AWARD NUMBER: W81XWH-15-1-0392

TITLE: Exogenic Human Heart in Gene Edited Animals

PRINCIPAL INVESTIGATOR: Daniel Garry, MD, PhD

CONTRACTING ORGANIZATION: Regents of the University of Minnesota
Minneapolis, MN 55455

REPORT DATE: September 2017

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel
Command Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
Exogenic Human Heart in Gene Edited Animals

Daniel J. Garry, MD, PhD
E-Mail: garry@umn.edu

Office of Sponsored Projects
Regents of the University of Minnesota
200 Oak ST SE
Minneapolis, MN 55455-2009

Congenital Heart Disease and heart failure are deadly. Cardiac transplantation is the only cure for heart failure and demand for hearts is significantly greater than the supply of donor hearts. The long range goal of this proposal is the production of a humanized heart using NKX2-5/HANDII/TBX5 knockout pigs as hosts for production. These animals may ultimately serve as an organ source for heart transplantation into humans. In addition to serving as a novel source of human hearts for the treatment of heart disease, the humanized pigs will also serve as a large animal model to study the regeneration of the human heart or response(s) to pharmacological agents or novel devices. During the second year of DOD funding we have established a productive cloning laboratory. We have examined multiple hiPSC lines regarding their ability to survive, proliferate and differentiate following delivery to the porcine parthenote blastocysts. Finally, we have initially examined the hiPSC-porcine parthenote chimeras following delivery to pseudopregnant gilts. This strategy has the capacity to have a profound impact on the development of emerging therapies for chronic cardiovascular diseases and transplantation that will benefit our military personnel, veterans and their families.
**Table of Contents**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Introduction</td>
<td>1</td>
</tr>
<tr>
<td>2. Keywords</td>
<td>1</td>
</tr>
<tr>
<td>3. Accomplishments</td>
<td>1</td>
</tr>
<tr>
<td>4. Impact</td>
<td>9</td>
</tr>
<tr>
<td>5. Changes/Problems</td>
<td>9</td>
</tr>
<tr>
<td>6. Products</td>
<td>9</td>
</tr>
<tr>
<td>7. Participants &amp; Other Collaborating Organizations</td>
<td>10</td>
</tr>
<tr>
<td>8. Special Reporting Requirements</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Introduction: Congenital Heart Disease and advanced heart failure are both common and deadly. Cardiac transplantation is the only cure for heart failure and demand for hearts is significantly greater than the supply of donor hearts. The long range goal and the clinical significance of this proposal is the production of a humanized heart using NKX2-5/HANDII/TBX5 knockout pigs as hosts for production. These animals may ultimately serve as an organ source for orthotopic heart transplantation into humans. In addition to serving as a novel source of human hearts for the treatment of congenital heart disease and end stage or advanced heart failure, the humanized pigs will also serve as a large animal model to study the regeneration of the human heart or response(s) to pharmacological agents or novel devices. During the second year of funding, we have made considerable progress towards the completion of our specific aims. We used CRISPR/Cas9 gene editing technology to engineer mutant pig models that lack a heart. We have engineered new hiPSC lines, we have engineered GFP-labeled porcine blastomeres that we cloned, we have established one of the premier cloning laboratories in the country and have metrics that exceed those of the industry. We have established new protocols that allow us to investigate improved efficiencies of chimerism in vitro. Collectively, these advances will provide an outstanding platform for us to achieve all our goals. We believe that this humanized large animal model will be an important resource for regenerative medicine and will serve as a platform for generating personalized humanized porcine models. This strategy has the capacity to have a profound impact on the development of emerging therapies for chronic cardiovascular diseases and transplantation that will benefit our military personnel, veterans and their families. Given the tremendous morbidity and mortality of cardiovascular disease in our society, the potential impact of this proposal is significant.

Keywords: cardiogenesis, humanized heart, gene editing, somatic cell nuclear transfer technology, pig,

Accomplishments:

1) Successful CRISPR/Cas9 gene editing of porcine embryonic fibroblasts (E35) to combinatorially mutate NKX2-5, HANDII and TBX5
2) Established a premier Cloning (SCNT) laboratory and cloned more than 10,000 embryos during the past one year period
3) Achieved outstanding fusion rates, maturation rates, blastocyst rates and pregnancy rates that exceed those published by the cloning industry
4) Established new hiPSC lines that are at distinct pluripotency states
5) Engineered high efficiency pig-pig chimeras in vitro
6) Established conditions/protocols to achieve the longest described culture periods of pig embryos in vitro
7) Achieved high efficiency human-porcine chimeras in vitro with established integration
8) Engineered new reporter cell lines for porcine experimentation
9) Confirmed that hiPSCs delivered into the developing pig embryo form mesodermal derivatives
10) Established that human stem cells (hiPSCs) are viable and differentiate following the delivery into the nonviable porcine parthenote embryos (in vitro and in vivo)

Major goals of the Project

Specific Aim #1: To define the functional role of NKX2-5/HANDII/TBX5 in the porcine model.

Specific Aim #2: To define the capacity of human stem cell populations for porcine blastocyst complementation.

Specific Aim #3: To generate a humanized heart in the NKX2-5/HANDII/TBX5 mutant porcine host.

The progress towards the completion of each specific aim is outlined as follows.
Specific Aim #1: To define the functional role of NKX2-5/HANDII/TBX5 in the porcine model. During the past one year period, we have made considerable progress towards the completion of this specific aim. Using CRISPR/Cas9 gene editing technologies, we engineered porcine fibroblasts using multiplex gene editing to lack NKX2-5/HANDII/TBX5.

**Gene editing technologies to engineer porcine fibroblasts.** We utilized gene editing technologies to combinatorially edit, in a biallelic fashion, the NKX2-5, HANDII and the TBX5 loci in porcine fibroblasts (see Figure 1). These mutant fibroblasts were then used for SCNT (i.e. cloning). Cloned, mutant pig embryos were transferred to synchronized gilts and sacrificed at E18. The embryos were genotyped (to assure that they lacked NKX2-5/HANDII/TBX5), immersion fixed in 4% paraformaldehyde, cryoprotected, frozen and 7 micron thick sections were obtained.

**Guide RNA (gRNA) design and production:** Candidate gRNA sequences for pig NKX2-5, HAND2 and TBX5 were designed using the online tool “http://crispr.mit.edu/”. Two gRNAs per gene to create a small deletion were cloned into a single plasmid following the BsaI-mediated Golden Gate Cloning method using multiplex CRISPR/Cas9 Assembly System kit (Addgene Kit #1000000055). The resulting all-in-one CRISPR/Cas9 vector system having total six gRNAs was sequence verified for correct gRNAs by Sanger sequencing (see Figure 1).

**Tissue Culture and Nucleofection:** Pig fibroblasts were maintained at 38.5°C at 5% CO2 in DMEM supplemented with 15% fetal bovine serum, 5 ng/ml basic fibroblast growth factor, and 10 mg/ml gentamicin. The Nucleofactor 2b device (Lonza) was used to deliver the all-in-one CRISPR/Cas9 plasmid using program U-012. Approximately 600,000 cells were nucleofected with 6 µg of plasmid using the Basic Nucleofector™ Kit for Primary Mammalian Fibroblasts (#VPI-1002). Nucleofected cells were cultured for 2 or 3 days at 38.5˚C, and then analyzed for gene editing efficiency and plated for colonies.

**Dilution cloning:** Two or three days post nucleofection, 50 cells were seeded onto 10 cm dishes and cultured for two weeks. Colonies were picked on Day 14 after transfection by applying 10 µm autoclaved cloning cylinders around each colony. Colonies were rinsed with PBS and harvested via trypsin; then resuspended in DMEM culture medium. Two thirds of the resuspended colony was transferred into a well of 24-well plate and the remaining one third was collected into a PCR tube. The cell pellets were resuspended in 10 µl of lysis buffer (40 mM Tris, pH 8.9, 0.9% Triton X-100, 0.4 mg/ml proteinase K [NEB]), incubated at 65˚C for 30 min for cell lysis, followed by 85˚C for 10min to inactivate the proteinase K. Expanded clones were collected and cryopreserved.

**Analysis of gene-edits:** 1 µl of the proteinase K digested cell lysate was used for PCR using primers flanking the intended sites. Clones having small deletions for all three genes were identified from the PCR amplicons by agarose gel electrophoresis. PCR products from clones showing biallelic small deletions were cloned into pCR2.1 TOPO (Life Technologies) vector and sequenced using Sanger sequencing method. Frame shift mutation and premature stop codons were confirmed from the sequence analysis (Figure 1).

![Figure 1. CRISPR/Cas9 gene editing to product triple knockout porcine embryos.](image-url)
Using these newly engineered porcine fibroblasts, we have validated the genotype and phenotype of the triple knockout embryos having established the first and only SCNT laboratory to engineer cloned pig models at the University of Minnesota. During the past year, our laboratory successfully cloned more than 10,000 porcine embryos and achieved outstanding metrics including a maturation rate that exceeds 80%, a lysis rate less than 5% and a blastocyst rate greater than 35%. These metrics have transformed our laboratory and have impacted our productivity (Figure 2).

**Figure 2. Establishment of the first porcine cloning facility at the University of Minnesota.** During the past one year, we have recruited outstanding investigators and have cloned more than 10,000 pig embryos by achieving outstanding procedural metrics. This new technology will amplify and accelerate all of our studies focused on engineering humanized organs in gene edited pigs.

**Specific Aim #2: To define the capacity of human stem cell populations for porcine blastocyst complementation.**

**Pig-pig complementation to define the highest efficiency chimerism in early (morula) developing porcine embryos.** As a baseline study to define the very best chimerism that we could achieve (with the interspecies chimeras), we examined pig-pig chimeras in vitro. Initially, we cloned GFP-labeled embryonic fibroblasts that we produced, dissociated the early pig embryos and isolated GFP-labeled pig blastomeres.
We also produced porcine parthenotes and allowed them to developmentally progress (in vitro) to the morula stage. We injected two blastomeres into each morula and assessed whether the injected cells proliferated and migrated to specific regions of the developing parthenotes (Figure 3). Importantly, we restricted our analysis only to developing parthenotes. Following injection (time=0), we found ~ 40% of the total injected parthenotes contained GFP+ cells at 48h (2 days). The numbers of GFP+ parthenotes decreased to 20% and 7% at 96h (4 days) and 144h (6 days) respectively, post-injection (Figure 3). Next, quantitative analysis of GFP+ cells revealed a 3-4 fold increase in the number of GFP-labeled cells as early as 48h, indicating the pig blastomeres were able to survive and proliferate using these cell culture conditions (Figure 3). These GFP-labeled porcine blastomeres and their derivatives migrated and localized to various regions of the developing parthenotes including the ICM and trophoectodermal layer.

Figure 3. Formation of pig-pig embryonic chimeras in vitro. A). Schematic showing the steps involved during the pig-pig chimera experiment. B) Representative brightfield (left) and green fluorescence (right) images of GFP-labeled blastomeres injected into pig parthenotes at 6, 8 and 10 days of development. C) Quantitative analysis of the GFP+ parthenotes at various time periods. Quantification involved data from 3 independent experiments (40 parthenotes were analyzed at each time point). (D) Quantitative analysis of GFP+ cells per parthenote at the indicated time points. Quantification of data from three independent experiments. (E) Immunohistochemical analysis revealed that the GFP+ blastomeres integrated and contributed to both Oct4+/CDX2+ (open arrowheads; trophectoderm) and Oct4+/CDX2- (filled arrowheads; inner cell mass) populations.
Successful in vivo pig-pig complementation using GFP-labeled wildtype blastomeres. We recognize the need to also engineer pigs with human vasculature in order to prevent hyperacute rejection of the humanized heart. We have previously discovered that Etv2 in the mouse is both necessary and sufficient for the specification and the development of the vascular and blood lineages. We have further established that Etv2 is a master regulator of these lineages. Therefore, using CRISPR/Cas9 gene editing technology, we engineered pig embryos that lack ETV2. These mutant embryos are nonviable by E16 and completely lack blood and vascular lineages. To establish the blastocyst complementation technique, we cloned the ETV2 null pig embryo and at the morula stage of development, we injected 2-4 GFP-labeled wildtype pig blastomeres and implanted the chimeric embryos into a pseudopregnant gilt and harvested them at E18. The Etv2 null embryo lacks vasculature, blood and is nonviable by E17. Using blastocyst complementation with wildtype GFP-labeled blastomeres the lethality and the absence of the vascular and blood lineages were completely rescued (Figure 4A-I). Moreover, while the GFP-labeled blastomeres can contribute to other lineages such as smooth muscle, neuronal, and intestinal lineages, they preferentially give rise to the vascular and blood lineages (Figure 4J-O). The use of the ETV2 null pig embryo will be an important advance in engineering humanized vasculature in combination with the humanized heart for therapeutic purposes.

Figure 4. Pig-Pig blastocyst complementation rescues entire lineages. The pig-pig complementation of the ETV2 null embryo with wildtype GFP-labeled pig blastomeres rescues the lethality and the absence of vascular and blood lineages. A) Whole mount image of the pig-pig complemented ETV2 null embryo at E18 showing normal structures and the presence of blood (red). B) Darkfield fluorescence of the pig-pig complemented embryo in panel A showing GFP-labeled vascular and blood cells at low magnification (B) and at high magnification (C). D-F) Histological sections
revealing that every Tie2 labeled endothelial cell is also labeled with GFP verifying that the vasculature of the *ETV2* null embryo is completely rescued by the GFP-labeled wildtype pig blastomeres. I) Quantification of the pig-pig complemented embryo compared to control dilutions. J-L) Immunofluorescence histological sections of the complemented pig-pig embryo reveals wildtype GFP-labeled blastomeres also contributed to smooth muscle (J-L), neural tube (M) and intestine (N) in the chimeric porcine embryo. O) Quantification of the chimerism demonstrates that 100% of the vasculature and the blood are contributed by the wildtype GFP-labeled blastomeres (and their derivatives) and other tissues with less than 40% chimerism.

**Engineering new hiPSC cell lines:** Over the past one year period, we have engineered additional hiPSC lines. This will allow us to have the necessary quality control to monitor drift and changes that may ultimately impact the efficiency related to interspecies chimerism. Human embryonic fibroblasts were infected with a reprogramming STEMCCA-OSKM Doxycycline-inducible viral cassette to generate induced pluripotent stem cells (iPSCs). Reprogramming fibroblasts were grown in doxycycline supplemented media for 30 days until embryonic stem cell like colonies appeared (Figure 5), then individual colonies were picked and grown in naïve vs. primed conditions. Once iPSCs were established, H2B-EGFP was knocked in to the AAVS1 locus by ZFN mediated targeting (Figure 5).

![Figure 5: Engineering new hiPSC lines for interspecies chimerism studies. a) Brightfield image of human embryonic fibroblasts. b) Brightfield image of naïve human induced pluripotent stem cell (iPSC) colonies derived from fibroblasts described in panel (a) after 30 days of OSKM induction. c) GFP positive hiPSC colonies following H2B-EGFP knockin to the AAVS1 locus (Scale bars 100µm).](image)

Using the hiPSC-EGFP lines we have established in vitro culture conditions to examine conditions and signaling pathways that could be interrogated and modified to increase the efficiency of human-porcine chimerism (Figure 6).
**Figure 6.** Engineering human-porcine chimeric embryos in vitro. A) Image showing single cell suspension of nuclear GFP-labeled hiPSCs. B) Brightfield image of the cell delivery process (hiPSCs) into the E4 pig morula. C) Green fluorescence image of the GFP-labeled hiPSCs in the capillary tube showing that two cells were injected into each parthenote.

**hiPSCs and pig blastomeres integrate and form a developmentally competent embryo in vitro.** To confirm that hiPSCs can integrate with the pig blastomeres, we performed dye transfer studies. hiPSCs were loaded with calcein and these cells were further labeled with DiI and injected into the pig parthenotes (Figure 7). We observed that the pig blastomeres adjacent to the hiPSCs had green fluorescence (Figure 7). These results indicated that the calcein dye from the hiPSCs was transferred (via gap junctions) to adjacent pig blastomeres.

**Figure 7.** Calcein transfer from injected hiPSCs to pig blastomeres through gap junctions, 24hrs after injection. (A) The injected parthenotes using brightfield microscopy. (B) The injected cells loaded with Calcein (green). (C) The injected cells labeled with DiI. (D) Calcein transfer from the injected hiPSCs to the pig blastomeres. Scale bars, 20μm.

To exclude the possibility that the GFP-labeling of the pig blastomeres was due to the leakage of calcein from the hiPSCs as a result of cell death/lysis, we injected variable concentrations of Calcein AM into the blastocele (Figure 8). As shown in Figure 8, we did not observe any green fluorescence in the pig blastomeres which further supports the notion that hiPSCs and pig blastomeres integrate and communicate via gap junctions.

**Figure 8.** Calcein AM injection into the parthenotes. Calcein AM with 1/2 or 1/4 of the concentration of the loaded hiPSCs was injected into the parthenote blastocele. Note that no Calcein was observed in the blastomeres. Scale bars, 20μm.
Pig-hiPSC complementation

We next utilized nuclear GFP-labeled hiPSC to monitor whether the hiPSC cells were able to survive, proliferate and migrate in the porcine embryo. We injected two primed hiPSCs into the decompacted pig morulae and followed the cells at multiple time points (Figure 9). Similar to the pig-pig complementation studies, we restricted our analysis only to healthy and developing parthenotes. As compared to pig-pig injection experiments, we found ~ 20% of the total injected parthenotes contained GFP* cells at 48h (2 days). The numbers of GFP+ parthenotes were subsequently decreased to 10% and 5% at 96h (4 days) and 144h (6 days) respectively, post-injection (Figure 9). Next, quantitative analysis of GFP+ cells revealed ~2-fold increase in the number of green cells 48h. The GFP+ cells were increased further by 3-4 fold at 96h and 144h time points, indicating the hiPSCs were able to survive and proliferate in the developing pig embryo (Figure 9). Analysis of GFP+ cell localization revealed that these cells migrated to the distinct region of the developing parthenotes such as the ICM and trophoectodermal layer. These studies are the first to describe long-term culture conditions of chimeric (hiPSC in the pig parthenotes) embryos (Figure 9).

Figure 9. GFP-labeled hiPSCs contribute to porcine embryonic chimeras in long-term cultures. A) Schematic showing the steps involved during the human-pig chimera experiment. B) Representative GFP-brightfield merged (left) and green fluorescence (right) images of the primed-hiPSCs injected into pig parthenotes at 4, 6, 8 and 10 days of their development. C) Quantitative analysis of the GFP+ parthenotes at various time periods. Quantification involved data from 4 independent experiments (30 parthenotes were analyzed at each time point). The white boxed area is magnified and shown in the right panel. (D) Quantitative analysis of GFP+ cells per parthenote at the indicated time points. Quantification includes data from four independent experiments.
Specific Aim #3: To generate a humanized heart in the NKX2-5/HANDII/TBX5 mutant porcine host.

Studies have been initiated to examine the chimerism of primed vs. naïve hiPSC in the developing mutant pig embryo. We plan to pursue these studies during the third year of funding.

Training activities: Training activities were provided for summer trainees (an undergraduate student who worked in the Garry laboratory), MSTP student, postdoctoral fellows and research associates. In addition, Dr. Garry delivered two talks on this subject to the University of Alabama (Biomedical Cardiovascular Engineering Meeting hosted by Dr. Jay Zhang). Dr. Mary Garry will be traveling to the University of Missouri to receive training on C-Section delivery of cloned pigs on September 25-27, 2017.

Plans for results disseminated to communities of interest: In the upcoming year, we will participate in the AHA and Keystone Molecular Cardiovascular Scientific Meetings. Next month we will be presenting our data at the Asilomar Conference. Dr. Garry has been invited to the 2018 AAAS meeting in Austin, TX to give an oral presentation focused on humanized organs using blastocyst complementation. We will also have two additional manuscripts that we are submitting for publication within the upcoming 3 month period.

Publications


Plans during the upcoming reporting period to accomplish the goals: We have essentially completed our goals for Specific Aims #1 and #2. During the upcoming year, we will initiate and accelerate our studies focused on the hiPSC complementation of the triple mutant (NKX2-5/HANDII/TBX5) pig embryo (Specific Aim #3).

Impact on the development of the principal discipline of the project?

Our studies further support the importance of achieving a naïve state for the hiPSCs compared to a primed state (see Specific Aim #2). Therefore, in addition to our planned studies, we will use single cell RNA-seq to define the molecular program associated with the pig blastomeres vs. the hiPSCs in order to enhance our understanding of the pathways that need to be modified to achieve increased efficiency of human-pig chimerism. These studies will impact the field of stem cell biology and regenerative medicine.

Impact on technology transfer: Nothing new to report.

Changes/Problems: We initially collaborated with Dr. Fahrenkrug at Recombinetics, Inc. pertaining to the chimeric human-porcine parthenote studies related to this project. Dr. Fahrenkrug has since left Recombinetics. Prior to his departure, Dr. Fahrenkrug elected to withhold gene edited cells (for which we paid in full) that were produced and required for the completion of our collaborative studies. As of today, we still have no access to these gene edited cells and there has been no interactions between our laboratory and Recombinetics (recall that Recombinetics was charged with providing the cloning services for the project along with the gene edited pig fibroblasts). In spite of this obstacle to our productivity, we established somatic cell nuclear transfer technology in the Garry laboratory and have we cloned more than 10,000 porcine embryos. Furthermore, we also utilized other gene editing strategies (CRISPR/Cas9 vs. TALENs) to engineer the pig mutant fibroblasts. Additionally, we have established farm operations, surgical facilities for embryo transfer and harvest and for neonatal delivery and care and abattoir services. These facilities are within 60 minutes of my laboratory. These new facilities and new technologies have accelerated our studies and we are on schedule toward completing our specific aims as outlined in our original grant application. The costs for reengineering
the porcine fibroblasts and the establishment of cloning initiatives within our laboratory have been necessary to continue this project but have also been a significant burden for our lab. While our overall goals have not changed, we have modified the SOW to decrease the number of interspecies chimeras.

**What was the impact on society beyond science and technology:** Nothing new to report.

**Changes/Problems:** We are currently assembling a manuscript focused on the ethics of organ allocation for transplantation which would highlight the democratization of organ availability with the engineering of humanized organs in a genetically modified pig.

**Participants and individuals working on this project:**

Daniel Garry, MD, PhD is the PI and has overseen all aspects of the project

Naoko Koyano-Nakagawa, PhD will reduce her effort on this project as Specific Aims #1 and #2 are largely completed. Geunho Maeng, PhD and Xiaoyan Pan, PhD will largely replace the effort provided by Naoko Koyano-Nakagawa, PhD as Drs. Maeng and Pan have expertise using hiPSCs, cloning (SCNT) pig embryos and morula/blastocyst complementation studies.

Mary Garry, PhD is a Co-I who has focused on the morphological characterization of the pig mutants and establishing the cloning facility within the Garry Laboratory.

**Has there been a change in active other support of the PI:** During the past one year period, Dr. Garry received funding as a co-investigator for the NHLBI supported Progenitor Cell Translational Consortium. There is no scientific overlap.

**Other new organizations involved as partners?** Nothing new to report

**Progress obtained and related to the Statement of Work:**

**Major Task 1:** Comprehensive characterization of the WT and NKX2-5/HANDII/TBX5 null porcine embryos.

**Response:** We have comprehensively characterized the triple knockout porcine embryos and have determined that they are lethal and lack a heart using lineage specific immunohistochemistry and morphological techniques. While we performed ultrasound analysis, these mutant porcine embryos lack a heart and therefore echocardiographic analysis could not be completed (this assessment was proposed in the event that our strategy to eliminate or delete the heart was unsuccessful).

**Major Task 2:** Pig-Pig blastocyst complementation.

**Response:** As outlined in the subtasks, we have isolated GFP-labeled pig embryonic fibroblasts, we have cloned the GFP-labeled pig fibroblasts and produced GFP-labeled blastomeres. We have performed pig-pig blastocyst complementation as outlined above (see Figure 3 above). We have undertaken in vivo pig-pig blastocyst complementation as outlined in Subtasks 2-4 and these studies are in progress.

**Major Task 3:** Examine human stem cell sources for production of chimeric preimplantation embryos.

**Response:** As outlined in Figures 5-9 we have examined more than 10,000 porcine parthenotes following the delivery of both naïve and primed hiPSC populations in vitro. While our analysis has included a much greater number of blastocysts (initially we only proposed to evaluate 700 blastocysts and to date we have evaluated more than 10X the proposed number), we have limited the hiPSCs to 4 human stem cell lines. We have also
examined different media compositions and the impact of temperature on the viability of the hiPSCs following delivery into the porcine parthenotes.

Figure for Major Task 3: Evaluation of culture conditions for hiPSC-injected parthenotes. (A) Number of porcine parthenotes containing live hiPSCs (shiPS9-1) over short-term culture. (B) Number of live hiPSC cells per parthenotes over short-term culture. (C,D) Blastocyst rate of parthenotes injected with hiPSCs (SH91) at d4 or d6 after activation, scored at 24h (C) and 48h (D). (D) Development rate of parthenotes to morula (compaction) and blastocyst stages in three porcine media. (F-K) Cell viability was assessed by TetraZ assay after culturing three hiPSC lines at indicated temperatures in NCSU-23 (F-H) or PZM-MU2 (I-K) media mixed with mTesR\textsuperscript{TM}1 medium.

Major Task 4: Examine human stem cell sources by implantation of parthenote chimeras.

Response: As outlined, we have undertaken hiPSC-porcine parthenote complementation and surgically transferred the chimeric embryos into pseudopregnant gilts and analyzed the chimeric embryos at E17/E18
using morphological and immunohistochemical analyses. In total we have examined approximately 1,100 chimeric human-pig parthenotes following implantation into psudeopregnant gilts.

**Figure for Major Task 4: Detection of hiPSCs in porcine host.** (A-F) hiPSCs were prelabeled with Dil (A-C) or EdU (D-G) and injected into parthenotes. Embryos were analyzed 48h later. Dil and HNA immunohistochemistry (A-C) or EdU and HNA detection identified the same cells (D-G). (H-K) Genomic in situ hybridization (H,I) and HNA immunohistochemistry (J,K) on human (H,J) and pig (I,K) sections reveal specificity. (L,M) Genomic qPCR using Alu and CytB primers. Scale bars, 50 μm.

**Major Task 5:** Examine longterm engraftment of human stem cell- *NKKX2-5/HANDII/TBX5* null porcine embryos in vivo.

**Response:** These studies will be challenging to complete due to the lack of funding (Subtask 1 – 6).