Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format. Questions? Contact Health Research Program staff at 717-783-2548.

1. **Grantee Institution:** The Trustees of the University of Pennsylvania

2. **Reporting Period (start and end date of grant award period):** 1/1/2009-12/31/2012

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** Gearline R. Robinson-Hall, BSF

4. **Grant Contact Person's Telephone Number:** 215-746-6821

5. **Grant SAP Number:** 4100047654

6. **Project Number and Title of Research Project:** 8 - Reprogramming Cells in Studies of Heart and Lung Development and Repair

7. **Start and End Date of Research Project:** 1/1/2009-6/30/2011

8. **Name of Principal Investigator for the Research Project:** John D. Gearhart, PhD

9. **Research Project Expenses**.

   9(A) Please provide the total amount of health research grant funds spent on this project for the entire duration of the grant, including indirect costs and any interest earned that was spent:

   $ 1,344,999.97

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name	Position Title	% of Effort on Project	Cost
Morrisey	Co-Investigator	14%	$25,377.37
Muthukumaraswamy	Research Specialist	68%	$22,315.38
Chokas	Postdoc Researcher	100%	$24,706.50
Cobb	Research Specialist A	100%	$28,268.40
Cui	Postdoc Researcher	100%	$31,940.00
Esteso	Research Fellow	27%	$22,708.38
Goings	Research Specialist B	21%	$27,232.19
Pacheco	Postdoc Researcher	35% Yr 1-2 15% Yr 2-3	$20,647.00; $9,529.52
Pinto	Research Specialist C	29%	$55,151.73
Stewart	Research Specialist D	23%	$20,245.45
Zubcevic	Postdoc Researcher	100%	$29,999.97

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name	Position Title	% of Effort on Project
Gearhart	Principal Investigator	10%
Gruber	Co-Investigator	5%

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

Type of Scientific Equipment	Value Derived	Cost
Confocal Microscope	Provides high-quality confocal microscopy; now shared with departmental imaging core.	$251,050.13
Olympus Microscope	Provides high-quality microscopy of both slides and live fluorescent cells	$104,756.09
Stand-alone HPLC-CED	Allowed for detection of dopamine by neurons produced via direct reprogramming.	$35,132.61

10. Co-funding of Research Project during Health Research Grant Award Period. Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes_____ No____X____

If yes, please indicate the source and amount of other funds:

11. Leveraging of Additional Funds

11(A) As a result of the health research funds provided for this research project, were you able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes_____ No____X____

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert "not funded" in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

A. Title of research project on grant application	B. Funding agency (check those that apply)	C. Month and Year Submitted	D. Amount of funds requested:	E. Amount of funds to be awarded:
None	☐NIH ☐ Other federal (specify:_____) ☐ Nonfederal source (specify:_)		$	$

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes____X____ No_____

If yes, please describe your plans:

We will be submitting grants to the NIH and to the AHA later this year.

12. Future of Research Project. What are the future plans for this research project?

Our results with this study have been exciting and important in considering a strategy for heart disease therapy. We are going to pursue this work vigorously.

13. **New Investigator Training and Development**. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes____X____ No_____

If yes, how many students? Please specify in the tables below:

	Undergraduate	Masters	Pre-doc	Post-doc
Male				
Female				1
Unknown				
Total				**1**

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic				
Non-Hispanic				1
Unknown				
Total				**1**

	Undergraduate	Masters	Pre-doc	Post-doc
White				1
Black				
Asian				
Other				
Unknown				
Total				**1**

14. **Recruitment of Out-of–State Researchers**. Did you bring researchers into Pennsylvania to carry out this research project?

Yes_____ No____X____

If yes, please list the name and degree of each researcher and his/her previous affiliation:

15. **Impact on Research Capacity and Quality**. Did the health research project enhance the quality and/or capacity of research at your institution?

Yes____X____ No_____

If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

With the reprogramming of cells to a cardiac fate we have opened a new and potentially transforming modality for treating heart disease.

16. Collaboration, business and community involvement.

16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

Yes_____ No____X____

If yes, please describe the collaborations:

16(B) Did the research project result in commercial development of any research products?

Yes_____ No___X____

If yes, please describe commercial development activities that resulted from the research project:

16(C) Did the research lead to new involvement with the community?

Yes_____ No____X____

If yes, please describe involvement with community groups that resulted from the research project:

17. Progress in Achieving Research Goals, Objectives and Aims.

List the project goals, objectives and specific aims (as contained in the grant agreement). Summarize the progress made in achieving these goals, objectives and aims for the period that the project was funded (i.e., from project start date through end date). Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.

Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance Review Report containing the comments of the expert review panel, and the grantee's written response to the Final Performance Review Report, will be posted on the CURE Web site.

There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not print as boxes (□) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.

Aim 1: To directly reprogram adult cardiac fibroblasts to cardiac progenitor cells.

1.1 Validation of the TroponinT-GCaMP Calcium Reporter

We constructed a GCaMP-reporter lentivirus that allows for real-time detection of calcium flux in live cells driven by the cardiomyocyte-specific Troponin T (*TNNT2*) promoter (Figure 1A). The effectiveness of the TroponinT-GCaMP5-Zeo reporter lentivirus was tested in cardiomyocytes isolated from mouse embryos at 14.5 dpc. As shown in Figure 1B, the reporter vector drives cardiomyocyte-specific expression of GCaMP5, a protein that exhibits bright green fluorescence in the presence of high intracellular Ca^{2+} levels and dim fluorescence with low Ca^{2+}. Thus, the rhythmic oscillation of intracellular Ca^{2+} that couples excitation and contraction in cardiomyocytes can be readily observed using a standard GFP filter on a fluorescent microscope.

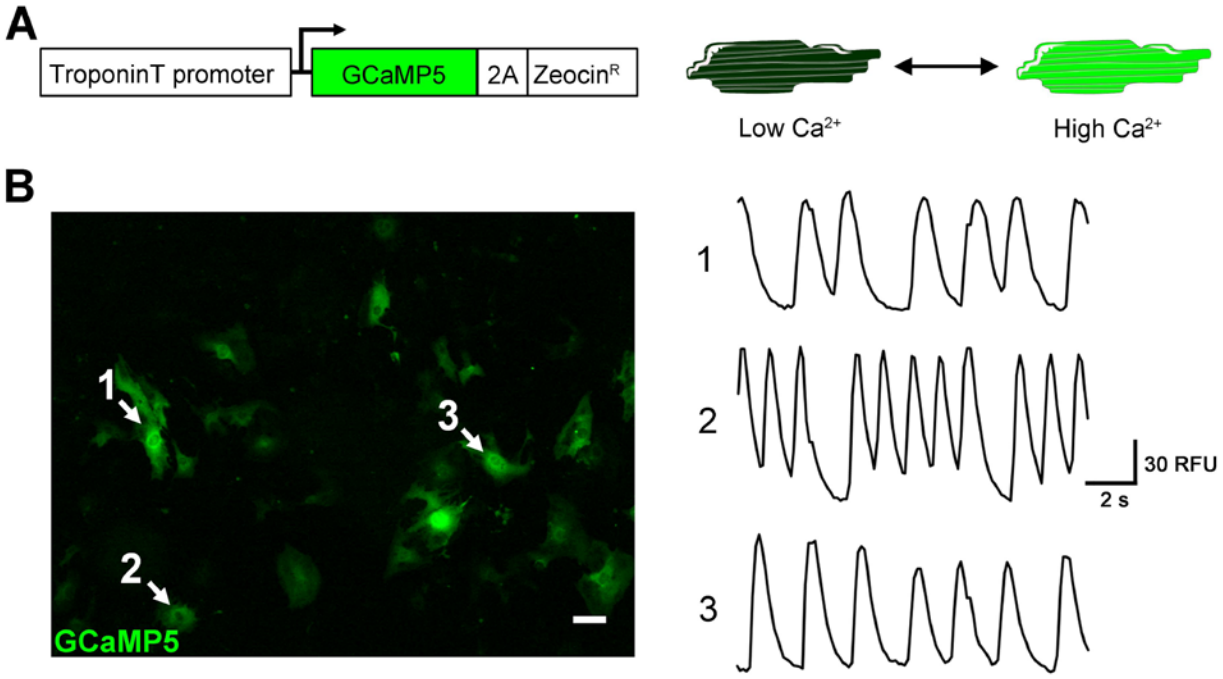

Figure 1. The TroponinT-GCaMP5 calcium reporter.
A) The Troponin T (*TNNT2*) gene promoter drives expression of the genetically-encoded calcium indicator, GCaMP5, and a zeocin resistance cassette. The GCaMP protein consists of a Ca^{2+}-sensitive calmodulin domain fused to GFP. When intracellular Ca^{2+} is low, the calmodulin domain blocks proper folding of GFP, resulting in a low level of GFP fluorescence. When intracellular Ca^{2+} is high, the calmodulin domain shifts to permit GFP folding and bright fluorescence.
B) Validation of the TroponinT-GCaMP5 reporter in embryonic cardiomyocytes. Cardiomyocytes were isolated from mouse embryos at 14.5 dpc and transduced with reporter lentivirus. GCaMP activity was recorded 48 hours post-transduction. White arrows indicate regions examined for oscillating fluorescence, shown in the right panel. RFU, Relative Fluorescence Units calculated using the "Intensity v Time Monitor" in ImageJ software.

1.2 Evaluation of Transcription Factor Combinations for their Capacity to Convert Fibroblasts into induced Cardiomyocytes

Mouse embryonic fibroblasts were transduced with doxycycline-inducible lentiviral vectors to drive expression of cardiac transcription factors. In combination with the reverse tetracycline transactivator (rtTA), these vectors allow for the activation and inactivation of transgene expression using Tet-on regulation. Each lentiviral vector contains a single transcription factor Open Reading Frame (listed in Table 1). Initial studies compared the previously published 3-factor combination of Gata4, Mef2c, and Tbx5 (GMT) with a 4-factor combination consisting of GMT plus Nkx2.5 (NGMT). NGMT proved more effective than GMT for the generation of putative iCMs, as indicated by immunostaining for α-myosin heavy chain, cardiac Troponin T, and sarcomeric α-actinin. NGMT also produced beating cardiomyocytes, with 0.7 ± 0.3% of cells exhibiting GCaMP activity at 14 days post-induction compared to 0.03 ± 0.02% for GMT (Figure 2C), a 22.5-fold increase over GMT. When we compared the more recently published combination of GMT plus Hand2 (HGMT), an increased number of functional iCMs (1.3 ±

0.1%) was detected. To determine whether the enhancing effects of Nkx2.5 and Hand2 were overlapping, we added both factors to GMT and found greater enhancement of functional iCM generation (HNGMT, 1.6 ± 0.3% GCaMP+), a 52-fold increase over GMT alone. In addition, we compared the recently published combination of Myocardin, Mef2c, and Tbx5 (DMT, 0.06 ± 0.01%) and DMT plus Nkx2.5 (DMNT, 0.4 ± 0.2%). Percentages of GCaMP+ cells at Day 14 post-induction are likely an underrepresentation of conversion efficiency given the continued proliferation of non-reprogrammed MEFs. Wells that began with 75,000 cells at initial plating contained an average of 2.04 x 10^6 cells on Day 14 (across all reprogramming conditions). Representative GCaMP+ iCMs generated with HNGMT are shown in Figure 2B, which is a single frame of a GCaMP movie overlaid with a red fluorescent image to show the PGK-H2B-mCherry vector that was used to quantify all cells in a given field of view. GCaMP activity was detected as early as 6 days post-induction, with robust calcium flux and spontaneous beating evident by Day 14. In MEFs that were not transduced with transcription factor lentiviruses, rare (fewer than 1 in 1000) cells exhibited steady green fluorescence, likely due to integration of the reporter vector in genomic regions of high transcriptional activity. However, no oscillations in GCaMP fluorescence were detected in MEFs that did not receive transcription factors, with greater than 2 x 10^7 cells analyzed.

Transcription Factor	Accession Number	Open Biosystems Clone ID
GATA4	BC105108.1	8144111
MEF2C	GQ129221	100068523
TBX5	EU446658	100069830
NKX2.5	DQ894104.2	100008564
Hand2	BC172639	100069447
Myocd	BC167199	100068099

Table 1. Transcription factor open reading frames cloned into FU-tetO-Gateway

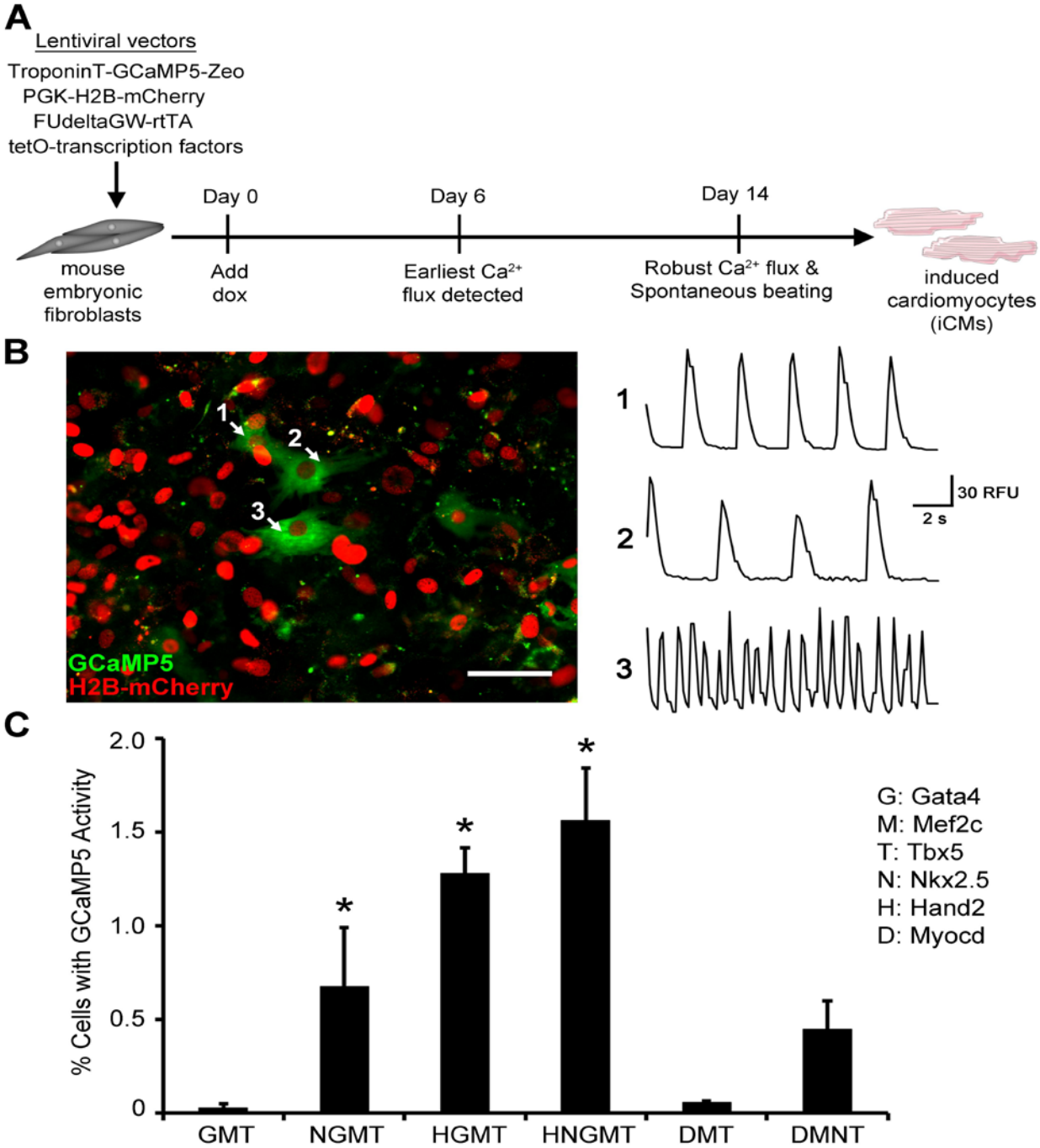

Figure 2. Functional quantification of direct fibroblast reprogramming to induced cardiomyocytes.

A) Schematic of direct fibroblast reprogramming to induced cardiomyocytes (iCMs).

B) GCaMP activity in beating iCMs, 14 days post-induction. The left panel corresponds to a single frame of a GCaMP movie, which shows Ca^{2+} flux in spontaneously beating iCMs generated by HNGMT treatment, overlaid with a red fluorescent image to show the PGK-H2B-mCherry vector that was used to quantify total cells. The right panel traces oscillations in GCaMP fluorescence, corresponding to oscillations in intracellular Ca^{2+}, at the regions marked by the white arrows. Scale bar 50μm. RFU, Relative Fluorescence Units calculated using the

"Intensity v Time Monitor" in ImageJ software.
C) Quantification of functional iCMs using GCaMP activity. Functional iCMs were identified on the basis of repeated GCaMP oscillation in 10s movies made at 14 days post-induction. Activity is expressed as the percentage of cells displaying repeated GCaMP oscillation in 10s movies divided by the total number of cells per field. *$p<0.05$ in comparison to GMT.

1.3 Immunocytochemical Analysis of induced Cardiomyocytes

Immunostaining for cardiomyocyte markers revealed expression of α-myosin heavy chain, cardiac Troponin T, and α-actinin, with sarcomere structure clearly visible (Figure 3). Staining for smooth muscle myosin heavy chain (Myh11), a marker that exclusively labels smooth muscle cells during mouse embryonic development, revealed that all combinations tested produced putative smooth muscle cells in addition to iCMs (Figure 3B). No expression of α-myosin heavy chain, cardiac Troponin T, α-actinin, or smooth muscle myosin heavy chain was detected in cells that were not transduced with transcription factor lentiviruses. Vascular endothelial markers (CD31 and von Willebrand factor) were not detected, suggesting that the reprogramming process did not involve a tripotent cardiac progenitor cell intermediate. In HNGMT-treated wells, rare cells were identified that stained double-positive for markers of smooth muscle and cardiomyocytes. Combinations DMT and DMNT induced widespread expression of smooth muscle myosin heavy chain, a finding that is consistent with the known role of Myocardin as a key regulator of smooth muscle development.

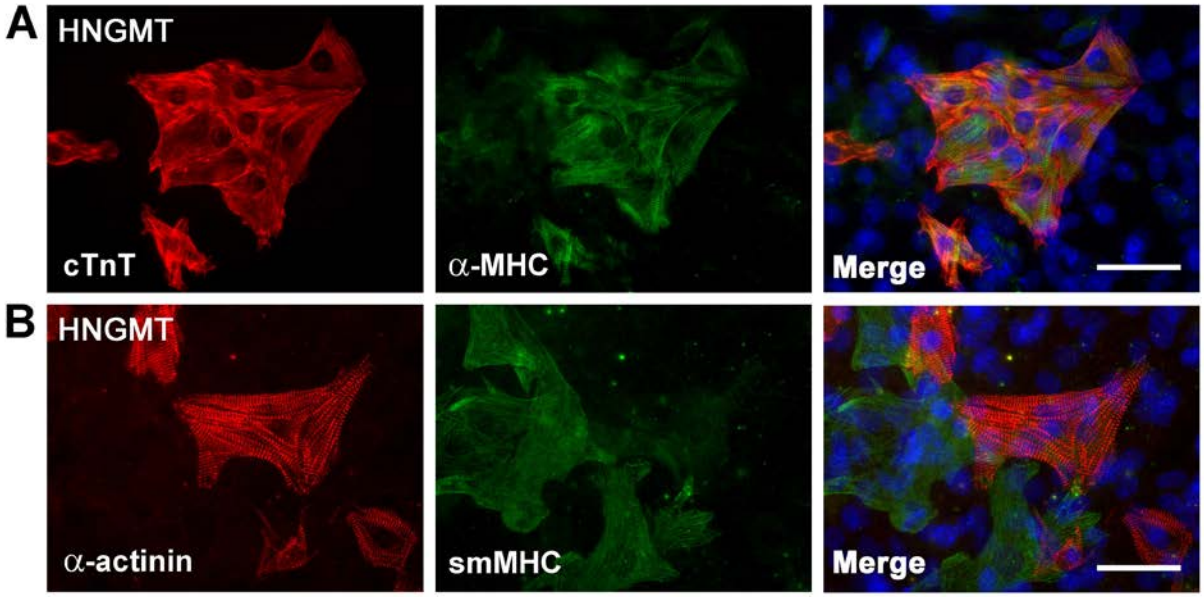

Figure 3. Immunocytochemical analysis of Day 14 iCMs. Cardiomyocyte markers cardiac Troponin T (cTnT), α-myosin heavy chain (α-MHC), and sarcomeric α-actinin are expressed in HNGMT-iCMs. Putative smooth muscle cells were also detected by staining for smooth muscle myosin heavy chain (smMHC, Myh11). DAPI stain (blue) marks nuclei. Scale bars 50μm.

1.4 Markers of Cardiac Progenitor Cells were not Detected in MEFs

Although MEFs were prepared from embryos following removal of the viscera, including the developing cardiovascular system, we considered the possibility that other embryonic tissues, such as the neural crest, might contribute migratory cardiac progenitors to the MEF population. We evaluated untreated MEFs for their expression of *Isl1*, a gene expressed by cardiac progenitor cells of both second heart field and neural crest origin, as well as *Mesp1*, a master regulator of cardiac cell fate. Neither marker was detected via either quantitative RT-PCR or immunocytochemistry.

1.5 Analysis of iCM Stability upon Inactivation of Reprogramming Factors

To determine the stability of the reprogrammed iCM phenotype as well as the minimum duration of factor expression required to drive reprogramming, we examined the effect of turning off exogenous HNGMT at various timepoints. We compared continuous factor expression (in the continued presence of doxycycline) to doxycycline removal after 5, 10, and 15 days (Figure 4A). Although induction for 5 days was sufficient to generate GCaMP+ cells (0.6 ± 0.2% at Day 14 post-induction), functional iCMs could not be detected at later timepoints. This suggests that short-term factor expression produces cells that are only partially reprogrammed. With 10 or more days of factor induction, however, functional iCMs persisted after the inactivation of exogenous transcription factor expression. This was further demonstrated by immunostaining for sarcomeric α-actinin 28 days post-induction (Figure 4B). No iCMs with sarcomeric structure were detected in the condition with 5 days of HNGMT expression. Although 10 days of factor induction was sufficient to generate iCMs, the percentage of GCaMP+ cells in this condition declined over time, a trend not observed in cells that received 15 days of factor induction. GCaMP+, beating iCMs could still be found 3 weeks following doxycycline removal (shown in Figure 4C).

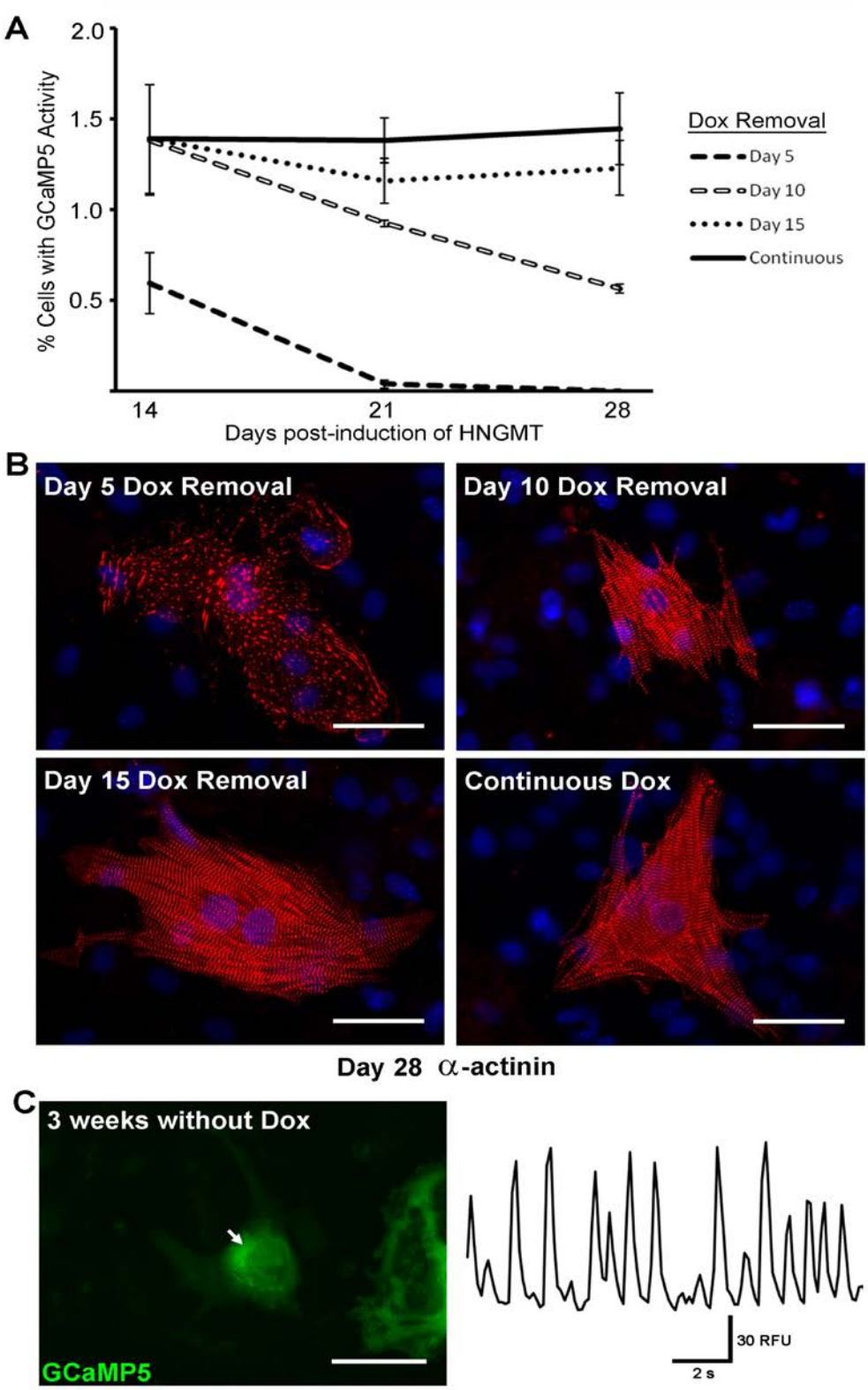

Figure 4. Stability of the reprogrammed iCM phenotype.
A) GCaMP activity was quantified at 14, 21, and 28 days post-induction, comparing HNGMT-treated cells with doxycycline applied continuously or withdrawn at 5, 10, or 15 days post-

induction. Beating, GCaMP+ iCMs could be detected on Day 28 for all conditions excepting Day 5 Dox removal.
B) Immunostaining for sarcomeric α-actinin in representative HNGMT-iCMs, 28 days post-induction. DAPI stain (blue) marks nuclei. Scale bars 50μm.
C) GCaMP activity recorded on Day 36 post-induction, with doxycycline removed on Day 15 (thus 3 weeks without exogenous transcription factor induction). Right panel shows oscillation in GCaMP fluorescence at the region indicated by the white arrow, left. Left panel is a single frame of a GCaMP movie, which shows both GCaMP activity and robust cell contraction. Scale bar 50μm.

1.6 Assessment of Genes that Encode the Excitation-Contraction Complex

To gain insight into the mechanism by which certain factor combinations produce greater numbers of functional iCMs, we examined the expression of genes encoding the complex set of proteins that mediate Ca^{2+} flux to couple excitation with contraction (Figure 5A). To generate an enriched population of iCMs, we made use of the zeocin resistance gene present in the TroponinT-GCaMP reporter by adding zeocin to the culture medium on Day 14 post-induction and collecting RNA for microarray analysis on Day 21. Gene upregulation was subsequently confirmed using quantitative RT-PCR. As shown in Figure 5B, key components of the excitation-contraction complex were upregulated in iCMs. In particular, calsequestrin, the calcium channel $Ca_V1.2$, and phospholamban were most highly upregulated in the transcription factor combinations that produced the largest percentages of functional iCMs.

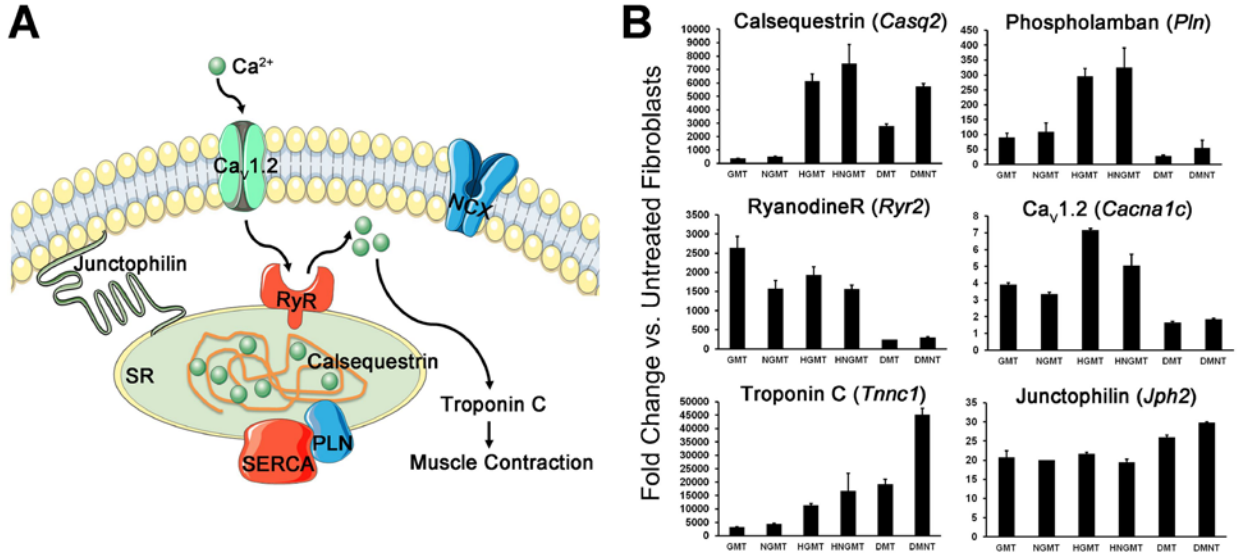

Figure 5. iCM expression of components of excitation-contraction coupling.
A) Key components of the excitation-contraction system: Action potential firing causes an inward Ca^{2+} current to enter the myocyte via $Ca_V1.2$ channels. The influx of Ca^{2+} ions stimulates Ryanodine receptors (RyR) to release additional Ca^{2+} into the cytosol from the calsequestrin stores of the sarcoplasmic reticulum (SR), causing the activation of Troponin C and the troponin-tropomyosin complex that mediates contraction. Cytosolic Ca^{2+} levels are then rapidly reduced by a combination of mechanisms including Na^+/Ca^{2+} exchange by NCX and pumping into the SR by the Phospoholamban (PLN)-regulated sarcoendoplasmic reticulum calcium ATPase (SERCA). Junctophilin helps to tether the SR to the sarcolemma. Diagrams made with Servier

Medical Art, with permission.
B) Expression of excitation-contraction genes in iCMs. Microarrays were performed on RNA collected from putative iCMs on Day 21 post-induction and upregulated genes were validated by quantitative RT-PCR. Calsequestrin, $Ca_V1.2$, and Phospholamban are most highly expressed in iCMs generated by HGMT and HNGMT, the factor combinations that produced the most GCaMP+ cells.

1.7. Discussion and Conclusions

Our work demonstrates a robust, convenient method for the quantification of fibroblast reprogramming to functional cardiomyocytes. The TroponinT-GCaMP reporter is scalable; it can be used to monitor calcium oscillations in a single cell or to evaluate the functionality of thousands of cells simultaneously. The present study focused on mouse embryonic fibroblasts; future work will determine whether the HNGMT combination is effective at direct *in situ* reprogramming of cardiac fibroblasts. The finding that iCMs remain stably reprogrammed long after the removal of exogenous reprogramming factors is encouraging. The minimum requirement of approximately ten days of factor expression to effect reprogramming is consistent with reports of transdifferentiation to other cell types. However, it is also evident that short-term exposure to reprogramming factors can produce unstable cells that are not fully converted. Furthermore, the beating iCMs generated in this study are likely immature, given that adult cardiomyocytes do not beat spontaneously in cell culture. These findings raise potential concerns for the safety of *in vivo* reprogramming strategies that will need to be evaluated. Future work will determine whether iCMs generated both *in vitro* and *in vivo* can undergo maturation to adult cardiomyocytes. It will also be important to identify the subtype of iCMs (i.e. atrial, ventricular, or nodal) and investigate whether different combinations of reprogramming factors produce iCMs of different subtypes.

Aim 2: Generate and characterize induced pluripotential stem (iPS) cells from congenital heart disease patients to determine the molecular cause of this important category of human congenital disease.

Our work has now expanded beyond the generation of iPS cells from children with congenital heart disease to understanding the mechanisms of CHD formation using iPS cells. Previously, our in vivo experiments using fresh source tissue from patients at the Children's Hospital of Philadelphia led to the successful production of iPS cells. We have now carried this work on considerably to completely characterize these cells and differentiate them into all developmental lineages.

1) Of the proposed library of CHD patient samples, we need just three more to finish our recruitment.
2) We have screened clones by IHC and demonstrated expression of the pluripotentiality markers NANOG, SOX2, OCT4, SSEA3, and SSEA4.
3) We have screened clones by RTqPCR and demonstrated expression of the pluripotentiality markers NANOG, SOX2, OCT4 is a quantitative manner. Importantly, we now

identify that the source fibroblasts (cardiac or dermal) appear to impart an expression signature upon the reprogrammed iPS cells.

4) We have differentiated these iPS cells in vivo (teratoma production) and in vitro (embryoid body production) proving the fidelity of these iPS cells to produce multiple lineages

5) We have directly differentiated human iPS cells in cardiac myocytes and are now working to further characterize these cells by qPCR and IHC.

Dr. Gruber left the University before the project end date.

Aim 3: Determine whether non-pulmonary cells can be directly reprogrammed into the pulmonary epithelial lineage using known and recently identified regulators of pulmonary epithelial differentiation.

Studies characterizing molecular pathways that promote lung repair and regeneration.

miR302-367 mediated lung regeneration. To test whether treatment of animals with miRNA mimics directed towards miR302-367 would improve lung regeneration, we injured mice using naphthalene which specifically depletes the secretory epithelium of the large airways. We dosed mice with miR302a, miR302c, and miR367 mimics at various time points after injury. We have found that treatment of mice from day2-day5 with miR302a, miR302c, and miR367 mimics leads to enhanced airway epithelial regeneration as assessed by increased secretory cell regeneration. This is coupled with an increase in secretory cell proliferation. These studies have led us to perform follow-up experiments to define the exact dose and timing necessary for improvement in lung regeneration. These studies should be completed in the next 6-12 months.

miR302-367 mediated increase in cardiomyocyte proliferation. Using our new mouse models of a miR302-367flox allele and a miR302-367 (R26R-CAG) cre-activatable gain of function allele, we have shown that miR302-367 is necessary for early cardiomyocyte proliferation during development. Moreover, over-expression of miR302/367 promotes cardiomyocyte proliferation even in postnatal animals. Remarkably, miR302-367 over-expression leads to cardiomyocyte proliferation up to 3 weeks after birth, far past the normal period for postnatal cardiomyocyte proliferation (which normally ceases by 7 days after birth). We have also shown that induction of miR302-367 in adult mouse hearts leads to increased cardiomyocyte proliferation, albeit in only very few cells. We are now testing whether in the setting of adult ischemic injury, miR302-367 can promote re-entry of cardiomyocytes into the cell cycle which may help improve cardiac regeneration in humans.

Using an assay to assess the targets of miR302-367, we have shown that expression of this miR cluster activates the Hippo signaling pathway which can regulate organ size through enhancing cell proliferation and inhibiting apoptosis. We have shown that YAP, an essential effector of Hippo signaling, is required for miR302-367 mediated cardiomyocyte proliferation. We are now determining whether reactivation of YAP in the adult heart can promote re-entry of postnatal cardiomyocytes into the cell cycle.

HDAC mediated regulation of lung development and regeneration. Foxp1/2/4 associate with the NuRD chromatin remodeling complex which contains the potent histone deacetylase enzymes HDAC1 and -2. We have now shown that the combined loss of HDAC1/2 in the developing lungs leads to a failure to develop Sox2+ endoderm progenitors and the subsequent failure to development the entire proximal compartment of the lung i.e. bronchi and bronchioles. HDAC1/2 regulate Bmp4 expression which is, in turn, dramatically up-regulated upon loss of HDAC1/2. This increase in Bmp4 expression, in part, leads to the loss of proximal airway development in the HDAC1/2 null lungs. We have also shown that HDAC1/2 directly regulate Rb1 which in turn regulates lung endoderm proliferation. Postnatal loss of HDAC1/2 does not affect airway epithelial homeostasis but does lead to a dramatic inhibition of airway epithelial regeneration after napthalene based injury. These studies indicate the importance of chromatin remodeling complexes in both lung development and regeneration and provide a mechanistic understanding of the previous findings that showed a decrease in HDAC activity and HDAC2 expression in COPD patients. A manuscript describing these studies has recently been accepted by Developmental Cell.

We are also exploring the role of HDAC3 in lung development. HDAC3 associates with a different chromatin remodeling complex, primarily the NCoR complex which is involved in lipid synthesis in the liver. Loss of HDAC3 leads to a partially penetrant (approx. 75%) lethal phenotype at birth. This phenotype appears to be associated with a loss of proper alveolarization. We are continuing to characterize these mutants with a focus on whether HDAC3 is required for proper lipid synthesis in alveolar type 2 epithelial cells, one of the most active producers of phospholipid in the body.

Combined function of Foxp1/2/4 in lung development and regeneration. As previously demonstrated, the lung expresses three Foxp factors, Foxp1/2/4. Given their high level of similarity, function, and expression pattern, we have deleted all three genes in the developing lung endoderm using the Shhcre system. This has been particularly difficult given that Foxp1 and Foxp2 are linked on mouse chromosome 6. Loss of Foxp1/2/4 in the developing lung endoderm results in a dramatic phenotype characterized by a loss in airway branching and decreased expression of most lung restricted genes analyzed including Nkx2.1 and SP-C. Transcriptome analysis of Foxp1/2/4 deficient lungs shows an overall decrease in lung specific gene expression with a concomitant increase in expression of genes expressed in different tissues, most notably the kidney and thyroid. Two clusters of genes, Hoxa9-13 and Pax2/8/9, are dramatically up-regulated upon loss of Foxp1/2/4 and the majority of additional changes in the transcriptome can be attributed to their increase expression and activity. Our current hypothesis is that Foxp1/2/4 are essential for promoting and maintaining lung endoderm identity through suppression of critical transcription factors normally expressed in other tissues such as Hoxa9-13 and Pax2/8/9. This suppression allows for proper expression of factors such as Nkx2.1 and others that are critical for promoting the lung endoderm program. We are currently testing whether this knowledge will help to promote lung differentiation from pluripotent stem cells.

Cooperative roles for Foxp1/4 in lung development and regeneration. This year we published our first paper on the cooperative roles that Foxp1/4 play in both lung development and postnatal regeneration in the journal Development. In this report, we showed that loss of Foxp1/4 leads to enhanced formation of the goblet cell lineage at the expense of the normal secretory or Clara cell

linage. We identified Agr2, a protein disulfide isomerase, as a direct target of Foxp1/4 and showed that its de-repression was responsible, at least in part, for the increase in goblet cell differentiation in these mutants. We also deleted Foxp1/4 specifically in postnatal secretory lineage and showed that loss of these two genes results in a failure to maintain the secretory linage phenotype. Foxp1/4 deficient airway epithelium transitions into a goblet like phenotype over the course of several weeks. This leads to the inability of this mutant epithelium to regenerate after naphthalene induced injury. Together, these studies highlight the importance of Foxp1/4 mechanisms in both promoting and maintain the secretory epithelial phenotype in the lung.

Papers relevant to this grant in the last year

Li, S, Wang, Y, Zhang, Y, Lu, MM, Demayo, FJ, Dekker, JD, Tucker, PW, Morrisey, EE. Foxp1/4 control epithelial cell fate during lung development and regeneration through regulation of anterior gradient 2. Development (2012) 139:2500-2509.

Wang Y, Tian Y, Morley MP, Lu MM, DeMayo FJ, Olson EN, and Morrisey EE. Development and regeneration of Sox2+ endoderm progenitors is regulated by a HDAC1/2-Bmp4/Rb1 regulatory pathway (in press-Developmental Cell)

Anokye-Danso, F, Snitow, M, Morrisey, EE. Promotion of pluripotent cellular reprogramming by microRNAs. Journal of Cell Science (2012) 125: 4179-4187.

Kadzik RS, and Morrisey EE. Directing lung endoderm differentiation in pluripotent stem cells. Cell Stem Cell. 2012 Apr 6;10(4):355-61

18. Extent of Clinical Activities Initiated and Completed. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
__X_ No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
__X__No

If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed. (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

_____Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

_____Number of subjects originally targeted to be included in the study
_____Number of subjects enrolled in the study

Note: Studies that fall dramatically short on recruitment are encouraged to provide the details of their recruitment efforts in Item 17, Progress in Achieving Research Goals, Objectives and Aims. For example, the number of eligible subjects approached, the number that refused to participate and the reasons for refusal. Without this information it is difficult to discern whether eligibility criteria were too restrictive or the study simply did not appeal to subjects.

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:
_____Males
_____Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
_____Not Latinos or Hispanics
_____Unknown

Race:
_____American Indian or Alaska Native
_____Asian
_____Blacks or African American
_____Native Hawaiian or Other Pacific Islander
_____White
_____Other, specify:_____
_____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.)

19. Human Embryonic Stem Cell Research. Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____Yes
__X_ _ No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
_____Yes
_____ No

19(C) Please describe how this project involved human embryonic stem cells:

20. Articles Submitted to Peer-Reviewed Publications.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication or paper submitted for publication, listed in the table, in a PDF version 5.0.5 (or greater) format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, the number of the publication and an abbreviated research project title. For example, if you submit two publications for PI Smith for the "Cognition and MRI in Older Adults" research project (Project 1), and two publications for PI Zhang for the "Lung Cancer" research project (Project 3), the filenames should be:

Project 1 – Smith – Publication 1 – Cognition and MRI
Project 1 – Smith – Publication 2 – Cognition and MRI
Project 3 – Zhang – Publication 1 – Lung Cancer
Project 3 – Zhang – Publication 2 – Lung Cancer

If the publication is not available electronically, provide 5 paper copies of the publication.

Note: The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

Title of Journal Article:	Authors:	Name of Peer-reviewed Publication:	Month and Year Submitted:	Publication Status (check appropriate box below):
1. Optimization of Direct Fibroblast Reprogramming to Cardiomyocytes Using Calcium Activity as a Functional Measure of Success	Russell C. Addis, Jamie L. Ifkovits, Filipa Pinto, Lori D. Kellam, Paul Esteso, Stacey Rentschler, Nicolas Christoforou, Jonathan A. Epstein, and John D. Gearhart	Journal of Molecular and Cellular Cardiology	January 2013	✓Submitted ☐Accepted ☐Published

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes____X____ No_____

If yes, please describe your plans:

An article is currently under review for publication.

21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project. Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis, or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None.

22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment. Describe major discoveries, new drugs, and new approaches for prevention, diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None.

23. Inventions, Patents and Commercial Development Opportunities.

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes_____ No____X____

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

a. Title of Invention:

b. Name of Inventor(s):

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____

If yes, indicate date patent was filed:

e. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____
If yes, indicate number of patent, title and date issued:
Patent number:
Title of patent:
Date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No____

If yes, how many licenses were granted?_____

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes___ No____

If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes_____ No____X____

If yes, please describe your plans:

24. Key Investigator Qualifications. Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages. *For Nonformula grants only – include information for only those key investigators whose biosketches were not included in the original grant application.*

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Gearhart, John David	Professor, Cell and Developmental Biology Professor, Animal Biology
eRA COMMONS USER NAME (credential, e.g., agency login) JGEARHA1	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Pennsylvania State University	B.Sc.	1964	Ag Biological Sciences
University of New Hampshire	M.Sc.	1966	Plant Genetics
Cornell University (Ithaca)	Ph.D.	1970	Genetics & Development

Research Support

Ongoing

Commonwealth of Pennsylvania SAP #4100047654 (Fluharty) 1/01/09-12/31/12
Molecular Mechanisms Involved in Reprogramming Cells
The goal of this project is to explain the process by which adult cells are converted to pluripotent cells and provide information that can be used to make generations of iPS more efficient and safe for clinical use.
Role: Co-Investigator

American Heart Association Myogenesis (Epstein) 4/1/09-3/31/13
Directing Cardiac Myogenesis in Development and Adult Progenitors: the Role on Wnt and Notch
The overall goal of this grant is to define the roles of Notch and Wnt signaling pathways during development and differentiation of cardiac progenitors during embryogenesis, and to determine whether parallel effects of these signaling pathways modulate adult cardiac progenitor behavior in mice and humans.
Role: Co-Investigator

National Institute of Health 1U01HL100405-01 (Morrisey/Epstein) 9/30/09-9/26/16
Expansion of cardiac and hematopoietic progenitors by Wnt and Notch
The Penn/UW Progenitro Consortium proposes to characterize the ability of Wnt and Notch signaling to expand progenitors in vivo as well as ex vivo.
Role: Co-Investigator

Commonwealth of Pennsylvania SAP# 4100050912 (Fluharty) 1/01/10-12/31/12
Generating Tissues from Stem Cells in the Pathways to Therapy
Studies will increase knowledge of stem cells that are involved in heart, lung, cartilage and tendon development and/or maintenance that will be utilized to direct, promote or enhance the repair of these organs and tissues.
Role: Co-Investigator

Completed

Commonwealth of Pennsylvania SAP #4100043362 (Gearhart) 6/01/08-5/31/12
Penn Center of Excellence for Regenerative Medicine
The major goal of this project is to support a Center of Excellence for Regenerative Medicine. Initiatives include a unique opportunity to reach out to collaborating institutions within the Commonwealth and address critical issues affecting the citizens of Pennsylvania.
Role: Principal Investigator

Commonwealth of Pennsylvania SAP #4100047654 (Fluharty) 1/01/09-6/30/11
Reprogramming Cells in Studies of Heart and Lung Development and Repair
The major goal of this project is to harness new technologies in regenerative biology and explore whether these techniques can be used to promote cardiac and pulmonary tissue regeneration.
Role: Co-Investigator

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Edward E. Morrisey	Professor of Medicine Scientific Director, University of Pennsylvania Institute for Regenerative Medicine
eRA COMMONS USER NAME (credential, e.g., agency login) MORRISEY	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY
University of Illinois, Urbana-Champaign, IL	B.S.	1982-1986	Microbiology
Northwestern University, Evanston, IL	Ph.D.	1990-1994	Molecular and cell biology
University of Chicago, Chicago, IL	Postdoc	1994-1998	Molecular cardiology

Research Support

Ongoing

R01-HL064632
NHLBI
Edward E. Morrisey (PI)
The Role of GATA6 in Lung Development 4/1/10-3/31/14
The major goals of this project are: 1) Define the functional interactions between GATA6 and Nkx2.1 on target promoters and during lung development, 2) Define the minimal peptide sequence in GATA6 that mediates GATA6 and Nkx2.1 interactions and determine its importance in vitro, and 3) Determine the temporal requirement of GATA6 in lung epithelium using a floxed GATA6 allele and temporally inducible cre mouse line. Overlap with current proposal: none

R01-HL071589
NHLBI
Edward E. Morrisey (PI)
Winged-helix repressors and lung development 12/01/11-11/30/15
The major goals of this proposal are to elucidate the role of Foxp1 and Foxp2 transcription factors during lung development with a specific emphasis on: 1) gene expression analysis during lung development, 2) structure/function analysis of Foxp1 and Foxp2 to determine important functional domains for repression, 3) characterization of interaction with CtBP-1, and 4) in vivo gene knock-outs of Foxp1 and Foxp2 to determine the importance of each factor during lung development.
Overlap with current proposal: none

R01-HL087825
NHLBI
Edward E. Morrisey (PI)

Regulation of airway morphogenesis and differentiation by Wnt signaling
12/01/07-11/30/12
The major goals of this proposal are to determine the role of canonical Wnt signaling in lung epithelial development, determine the role of non-canonical Wnt signaling in lung epithelial development, and define the mechanism by which Wnt signaling regulates lung epithelial differentiation and development.
Overlap with current proposal: none

American Heart Association (PI: Epstein)
Jon Holden DeHaan Foundation Myogenesis Center Grant
PI of Project 2: Wnt signaling in cardiovascular progenitors 04/01/09 – 03/31/13
The major goals of this project are to 1) Define the role of c-Kit positive cells in cardiac development and adult myocardial injury repair. This includes determining whether c-Kit+ cells generate cardiac myocytes in development or in the adult heart. 2) Determine how Wnt signaling affects c-Kit positive cells self-renewal and differentiation. This includes determining whether Wnt agonists can drive expansion of c-Kit+ cardiac progenitors.
Overlap with current proposal: none

U01-HL100405
NIH/NHLBI
Edward E. Morrisey (PI)
Expansion of cardiac and hematopoietic progenitors by Wnt and Notch
09/30/09-09/29/16
The purpose of this proposal is to determine how Wnt and Notch affect cardiac regeneration and repair will provide crucial insight into whether these pathways can be harnessed for therapeutic use with a focus on pluripotential stem cells (ES and iPS).
Overlap overlap with current proposal: none

U01-HG006398
NIH/NIGMS
Co-PI's: Daniel J. Rader, Edward E. Morrisey, and Stephen A. Duncan
iPS-derived hepatocytes to study lipid phenotypes associated with GWAS lipid loci 7/1/11-6/30/16
The purpose of this grant is to generate iPSCs from 300 patients with previously identified GWAS loci associated with lipid disorders and use novel techniques to enhance throughput and fidelity of hepatocyte generation from these iPSCs.
Overlap with current proposal: none

U01-HL110942
NIH/NHLBI
Edward E. Morrisey (PI)
Epigenetic regulation of lung repair and regeneration
12/1/11-11/30/16
Overlap with current proposal: none

Peter J. Gruber, M.D., Ph.D.

Dr. Gruber joined the University of Utah Health Care in 2011 as Pediatric Section chief, in the Division of Cardiothoracic Surgery, and is a diplomate of the American Boards of Surgery, Thoracic Surgery, and Congenital Cardiac Surgery Subspecialty.

He is co-Director of the Heart Center at Primary Children's Medical Center and devotes 25% of his time to research as an investigator in the Molecular Medicine program. He is Principal Investigator on a 5-year ($1,675,000) NIH National Heart, Lung and Blood Institute (NHLBI) grant titled: Histone deacetylase-mediated reduction of ischemia reperfusion injury.

He reviews proposals for the NIH, AHA, and the Children's Research Foundation and reviews for numerous journals including Circulation, Development, Journal of Clinical Investigation, Journal of Molecular and Cellular Biology, and The Annals of Thoracic Surgery.

He is a committee member on the American Board of Thoracic Surgery, American Society for Clinical Investigation, and is a Surgical Co-Chair for the Pediatric Critical Care Congress. He has published numerous manuscripts book chapters and lectures internationally.

He has published in the Journal of Clinical Investigation (1996, 2003), Development (1998), Nature (2005, 2010), FASEB J (2008), Journal of Thoracic and Cardiovascular Surgery (2006), Nature Medicine (2007), Journal of Biological Chemistry (2010), and Cell Stem Cell (2011).

Board Certification and Academic Information

Academic Departments	Surgery - Associate Professor, University of Utah Health Care
Academic Divisions	Cardiothoracic Surgery, University of Utah Health Care
Board Certification	American Board of Surgery

American Board of Thoracic Surgery
American Board of Thoracic Surgery (Sub: Congenital Cardiac Surgery)
National Board of Medical Examiners

Academic Bio

Dr. Gruber completed his undergraduate and medical training at the University of Pennsylvania with a degree in Biochemistry and Biophysics and followed by an M.D., Ph.D in the MSTP program with postgraduate training at Johns Hopkins Hospital, and the Children's Hospital of Philadelphia. He is currently the D. Rees and Eleanor T. Jensen Presidential Chair and Chief of Pediatric Cardiothoracic Surgery. Scientifically, he is best known for his work in human congenital heart disease, particularly investigations in the molecular basis of defects of cardiac morphogenesis and their relationship to cardiac progenitor cells. His clinical interests revolve around all aspects of complex congenital cardiac repairs and transplantation. Dr. Gruber is a member of AOA and the American Society for Clinical Investigation.

Education History

Type	School	Degree
Fellowship	The Children's Hospital of Philadelphia Pediatric Cardiothoracic Surgery	Fellow
Fellowship	Johns Hopkins Hospital Cardiothoracic Surgery	Fellow
Fellowship	Hôpital Necker-Enfants Malades	Visiting Fellow
Fellowship	Centre Chirurgical Marie-Lannelonge, Le Plessis	Visiting Fellow
Residency	Johns Hopkins Hospital General Surgery	Resident
Fellowship	University of California, San Diego, American Heart Association Bugher Foundation Center for Molecul Cardiac Development	Fellow
Residency	Johns Hopkins Hospital General Surgery	Resident
Internship	Johns Hopkins Hospital General Surgery	Intern
Doctoral Training	University of Pennsylvania Biochemistry and Biophysics	Ph.D.
Professional Medical	University of Pennsylvania School of Medicine NIH Medical Scientist Training Program	M.D.
Undergraduate	University of Pennsylvania Biochemistry	B.A.