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):** 1/1/2010-12/31/2013

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:** 4100050912

6. **Project Number and Title of Research Project:** 10 - Direct Reprogramming of Fibroblasts and Astrocytes to Dopaminergic Neurons

7. **Start and End Date of Research Project:** 11/23/2010-12/31/2013

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:

   $ 1,640,054.32

   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, First Name	Position Title	% of Effort on Project	Cost
Addis, Russell	Sr. Research Investigator	20% Yrs 1-2; 100% Yrs 3-4	$94,120.27
Clark, Jennifer	Postdoctoral Researcher	100% Yrs 2-3	$32,388.91
Esteso, Paul	Research Fellow	45% Yr 2	$ 3,625.02
Ifkovits, Jamie	Postdoctoral Researcher	100% Yrs 1-2	$34,595.00
Katz, Andrew	Lab Intern	100% Yrs 1-2	$12,851.65
Kellam, Lori	Postdoctoral Researcher	100% Yr 4	$49,834.08
Mercedes, Maria	Research Specialist	58%	$26,792.49
Pinto, Filipa	Research Specialist	33%	$78,357.17
Richardson, Dean	Co-Investigator	5%	$16,845.60

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, First Name	Position Title	% of Effort on Project
Gearhart, John	Principal Investigator	<1%

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
None		

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 plan to apply for additional funding from the Michael J. Fox Foundation and the NIH to continue this work.

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

We will continue to work with our collaborator, Gene Redmond at the St. Kitts Biomedical Research Foundation, to perform *in vivo* conversion of astrocytes to dopaminergic neurons in an African green monkey model of Parkinson's Disease.

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_____ No____X_____

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

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

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

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

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_____ No____X_____

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

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.

Specific Aims

AIM 1: Generate and characterize dopaminergic neurons via direct reprogramming of mouse embryonic fibroblasts. Hypothesis: ectopic expression of transcription factors normally expressed in midbrain dopaminergic neurons is sufficient to directly reprogram fibroblasts to dopaminergic neurons.
Result: This aim was succesfully completed. Details are provided below.

AIM 2: Determine the minimal set of transcription factors sufficient to reprogram mouse embryonic fibroblasts to dopaminergic neurons.
Result: This aim was succesfully completed. Details are provided below.

AIM 3: Reprogram primary mouse astrocytes to dopaminergic neurons. Hypothesis: the same set of transcription factors (or perhaps a subset thereof) that can reprogram fibroblasts can reprogram astrocytes to dopaminergic neurons.
Result: This aim was succesfully completed. Details are provided below.

To identify a combination of transcription factors that is sufficient to mediate reprogramming to dopaminergic neurons, twelve transcription factors known to play critical roles in midbrain dopaminergic neuron development and/or maintenance were cloned into the doxycycline-inducible lentiviral vector FU-tetO-Gateway (Figure 1A). Seventy-four unique combinations of these vectors were used to transduce mouse embryonic fibroblasts for the initial factor screen. RNA of transduced cells was harvested after 7 days of doxycycline-induced factor expression and assayed via RT-PCR for expression of tyrosine hydroxylase (Th) and DOPA decarboxylase (Ddc), the enzymes of dopamine synthesis. All experiments were performed in triplicate. The three-factor combination of ASCL1, LMX1B, and NURR1 resulted in the most robust expression of Th and Ddc (Figure 1A-B). This combination was selected for further analysis.

In order to increase the efficiency of cells receiving all three transcription factors, as well as to reduce variability resulting from different ratios of the factors reaching individual cells, we constructed a polycistronic vector. The open reading frames of ASCL1, LMX1B, and NURR1 were linked via recombinant PCR such that viral 2A peptide sequences separate the three genes, as shown in Figure 1C. A glycine-serine-glycine (GSG) linker was included upstream of each 2A sequence to facilitate protein cleavage. The ASCL1-P2A-LMX1B-T2A-NURR1 cassette (hereafter referred to as ALN) was inserted into FU-tetO-Gateway to produce the tetO-ALN vector. Cleavage at the 2A sites was validated by performing *in vitro* transcription and translation using the T7 promoter located at the start of the Gateway recombination site in the presence of biotinylated lysine. A streptavidin-HRP western blot confirmed complete cleavage at both the T2A and P2A sites (Figure 1D). The tetO-ALN lentiviral vector delivered ALN to both astrocytes and fibroblasts at >99% efficiency, as determined by immunocytochemistry for the C-terminal V5 tag on NURR1 (data not shown).

Characterization of gene and protein expression in induced dopaminergic neurons

Astrocytes were transduced with FUdeltaGW-rtTA (the reverse tetracycline transactivator) and tetO-ALN. The following day, doxycycline was added to astrocyte medium to induce ALN expression (Day 0). After four days in astrocyte medium with doxycycline, transduced cells were switched to NB27G neuronal medium. Doxycycline was removed on Day 10. After 14 days from the initial ALN induction, 35.1 ± 1.5% of cells expressed type III beta-tubulin (clone TUJ1), a neuronal marker. 50.9 ± 3.3% of TUJ1+ cells were also positive for tyrosine hydroxylase, yielding an overall conversion rate of 18.2 ± 1.5% (Figure 2A). Quantification was performed by counting a total of 3357 cells in three independent reprogramming experiments.

We constructed a lentiviral reporter vector in which the cell surface marker, CD4, is expressed under the control of the neuron-specific *MAP2* gene promoter. To further characterize the induced dopaminergic neurons, we sorted ALN-derived neurons on Day 14 using the *MAP2*-CD4 reporter vector and magnetic beads conjugated to anti-CD4. RNA was isolated from sorted cells and assayed via RT-PCR for expression of a panel genes shown in Figure 3. Gene expression in sorted neurons was compared to astrocytes that did not receive ALN as well as mouse embryonic stem cells that had been differentiated to dopaminergic neurons via co-culture with PA6 stromal cells. Sorted neurons displayed robust upregulation of genes expressed in midbrain dopaminergic neurons such as Pitx3, Lmx1a, Engrailed-1, aldehyde dehydrogenase, Foxa2, the vesicular monoamine transporter Vmat2, Msx1, and the dopamine transporter. Immunocytochemistry revealed expression of Girk2, a potassium channel widely expressed in A9/substantia nigra dopaminergic neurons, in virtually all (>99%) of the Th-immunoreactive cells (Figure 2B). Otx2, a protein expressed in mature A10/ventral tegmental area dopaminergic neurons, was not detected. Induced dopaminergic neurons exhibited robust synaptophysin expression (>99% of Th-immunoreactive cells, Figure 2C), suggesting the capacity to form synaptic connections.

Functional characterization of induced dopaminergic neurons

To evaluate the electrophysiological phenotype of induced dopaminergic neurons, patch clamping was performed on induced neurons between days 9 and 26 of initial ALN induction. Neurons were identified by screening for GFP driven by the *MAP2* promoter. Current clamp recordings in Day 9 cells showed an immature spiking pattern – generally a single action potential per step current injection. By Day 14-21, neurons demonstrated repetitive firing of action potentials in individual step current injections (Figure 4A). Of 30 patched cells, 24 fired action potentials (80%). Recorded cells had an average resting membrane potential of -55.4 mV. In voltage clamp, large sodium and potassium currents were seen (Figure 4B), with an average maximum I_{Na} of 1546 pA. Electrophysiological properties of induced dopaminergic neurons are summarized in Table 1. Recordings were made on neurons generated in four independent reprogramming experiments.

It has been well established that midbrain dopaminergic neurons are pacemaker neurons, spontaneously firing action potentials at a rate between 1 and 9 Hz with an average of 4.5 ± 1.7 Hz. We observed spontaneous firing of action potentials in 43% of recorded *MAP2*-GFP+ induced neurons, a fraction that is consistent with the 50.9 ± 3.3% of neurons that were immunoreactive for tyrosine hydroxylase (Figure 4C). The frequency of spontaneous firing ranged from 0.9 to 8.6 Hz, with an average of 5.6 ± 1.2 Hz. The pacemaker activity of dopaminergic neurons is accompanied by rhythmic fluctuations in intracellular calcium ion concentration. To determine whether induced dopaminergic neurons exhibit calcium

oscillations, we transduced astrocytes (prior to reprogramming) with a lentiviral vector containing the genetically-encoded calcium indicator GCaMP3 driven by the *MAP2* promoter. We detected robust calcium oscillations with an average frequency of 0.49 ± 0.11 Hz (Figure 4E).

To confirm that induced dopaminergic neurons produce and release dopamine, we stimulated cells with 56 mM KCl and measured dopamine release via HPLC. Cells assayed at 17 days post-transduction with ALN released dopamine in response to membrane depolarization while un-transduced cells (0F) did not, as shown in Figure 4D. No significant levels of epinephrine, norepinephrine, or serotonin were detected.

Direct reprogramming of fibroblasts to dopaminergic neurons

Given the widespread use of fibroblasts as a starting cell population in reprogramming, we tested whether our polycistronic vector was effective on mouse embryonic fibroblasts. The ALN vector was able to reprogram mouse embryonic fibroblasts to Th+/TUJ1+ neurons, as shown in Figure 5, with an overall efficiency of 9.1 ± 0.9%. Fibroblast-derived dopaminergic neurons exhibited large sodium currents and spontaneous action potential firing (Figure 5B-D). Dopaminergic neurons generated from fibroblasts using a nearly identical set of transcription factors (ASCL1, LMX1A, and NURR1) were recently described and characterized in detail.

Discussion

Our study is the first to demonstrate direct reprogramming of astrocytes to dopaminergic neurons. These neurons exhibit gene and protein expression patterns that are consistent with A9 midbrain dopaminergic neurons. Given the mounting evidence that cells retain some epigenetic memory when driven to pluripotency and subsequent differentiation, such memory is almost certain to be maintained in direct reprogramming. Global epigenetic profiling that compares authentic dopaminergic neurons to those derived from astrocytes and fibroblasts will be informative in determining the relative completeness of these reprogramming processes. It will also be important to compare the ability of astrocyte- and fibroblast-derived dopaminergic neurons to engraft and function in animal models of PD.

In addition to providing a novel source of dopaminergic neurons for use in cell-based therapies for PD, our use of astrocytes as the starting population allows an approach that may obviate the need for grafting altogether – direct *in vivo* reprogramming to replace lost neurons. Such an approach is facilitated by the development of a polycistronic vector, especially since one of the three factors, ASCL1, has been shown to reprogram astrocytes to non-dopaminergic neurons in the absence of additional factors. The fact that the polycistronic ALN vector reprograms astrocytes at such a high efficiency is also noteworthy, given the history of polycistronic vector use in generating iPS cells. A single vector delivering transcription factors that induce pluripotency was shown to be effective, but at a significantly lower rate than that which could be achieved when the factors were delivered individually. This was presumed to indicate that a particular ratio of reprogramming factors is ideal for inducing pluripotency. In the case of reprogramming to dopaminergic neurons, we can conclude that either the ratio of factors is not as important or, less likely, that our polycistronic vector fortuitously delivers the factors in the appropriate proportions. The present study takes a crucial step toward to ultimate goal of *in vivo* reprogramming of astrocytes to dopaminergic neurons. Future work will determine the feasibility of this approach in animal models of PD.

As described above, our group was the first to report the generation of dopaminergic neurons via transdifferentiation of astrocytes, a cell type that is a promising candidate for *in vivo* conversion strategies (Addis et al, PLoS ONE 2011). We identified a combination of 3 transcription factors – ASCL1, LMX1B, and NURR1 (ALN) – that is capable of efficiently converting both mouse fetal astrocytes and mouse embryonic fibroblasts to functional iDNs (induced dopaminergic neurons). When this reprogramming technique was applied to fetal human astrocytes, a key species difference was observed: while rodent astrocytes required all three of the transcription factors listed above to convert to dopaminergic neurons, human astrocytes required only two: ASCL1 and LMX1B. Surprisingly, the presence of NURR1 inhibited the reprogramming process in the human cells. Although the reason for this difference between rodent and primate cells remains undetermined, it suggests that a primate model may be more appropriate than rodent for studying *in vivo* reprogramming that may someday be applied to humans.

For several years, we have participated in a collaborative research effort with Dr. Eugene Redmond, MD, at Yale University. Dr. Redmond is the founder of the St. Kitts Biomedical Research Foundation (SKBRF), a world leader in primate research. Monkeys treated with the drug 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) exhibit parkinsonian symptoms as a result of the selective degeneration of midbrain dopaminergic neurons, making this model the gold standard of animal models for PD. In an effort to drive conversion of astrocytes to iDNs in vivo, we injected 4 adult asymptomatic MPTP-lesioned African green monkeys (*Chlorocebus sabaeus*) with Adeno-associated viral vectors (AAVs) expressing the transcription factors ASCL1 and LMX1B in a bicistronic expression cassette. The striatum in one hemisphere received the reprogramming factors while the striatum of the other hemisphere received a GFP control. In this manner, we are able to have both an experimental treatment and a control in the same animal. This allows us to gain valuable data from a minimal number of animals. Two months following the delivery of viral vectors to the striatum, the monkeys were sacrificed. Punch biopsies were taken of representative regions of the striatum to perform HPLC to evaluate dopamine levels. The brains were then fixed, sectioned, and immunostained for markers of astrocytes and dopaminergic neurons to evaluate the success of the reprogramming effort. Immunostaining for tyrosine hydroxylase, a key marker of dopaminergic neurons, indicated that no striatal astrocytes had been successfully converted to iDNs. This result was further validated by HPLC for dopamine, which showed no significant difference in the samples taken from reprogramming factor-treated versus GFP-treated tissue.

In the same set of experiments, we isolated astrocytes from adult African green monkeys. This valuable resource allowed us to perform our first *in vitro* experiments using adult monkey astrocytes. In preliminary studies, we found that our polycistronic vector expressing the ALN reprogramming factors was ineffective at converting the monkey cells to iDNs, as was the 2-factor combination of ASCL1 and LMX1B. No evidence of neuronal conversion could be detected whatsoever. We hypothesized that one or more of the reprogramming factors lacked necessary cofactors to mediate transdifferentiation. To overcome this hurdle, we engineered versions of the factors that were fused to the VP16 transactivation domain. VP16, originally isolated from herpes simplex virus, is a powerful activator of gene transcription. It has been widely used in molecular and cellular biology in applications such as two-hybrid screens when

potent transcriptional activation is desired. Recently, the VP16 transactivator was shown to significantly enhance the generation of induced pluripotent stem (iPS) cells when fused to the reprogramming factors Oct4, Sox2, and Nanog. To generate VP16 fusions, we engineered a drug-inducible lentiviral vector with a Gateway (Invitrogen) site that permits the cloning of an open reading frame (ORF) and places an N-terminal VP16 sequence on each gene. Using this vector, we generated transactivated fusions of ASCL1, LMX1B, and NURR1. By testing combinations of these VP16 fusions with the native forms of each factor, we have identified VP16-ASCL1 as being the key factor to mediate transdifferentiation of adult primate astrocytes into neurons. When VP16-ASCL1 is expressed with native LMX1B and NURR1, monkey dopaminergic neurons are readily produced. To our knowledge, this is the first report of any adult primate cell (human or non-human) being directly converted to dopaminergic neurons. We will further characterize these monkey iDNs using immunohistochemistry for additional key markers of dopaminergic neurons, electrophysiology to characterize action potentials, and HPLC to examine dopamine release. We will also confirm the stability of the reprogrammed phenotype by demonstrating that neurons remain functional upon inactivation of exogenous reprogramming factors.

In the last six months of 2013, we continued our studies of three transcription factors – ASCL1, LMX1B, and NURR1 – and evaluated their ability to directly convert adult monkey astrocytes into dopaminergic neurons. We constructed a lentiviral vector in which the astrocyte-specific Vimentin promoter (*Vim*) drives expression of a polycistronic cassette consisting of ASCL1, LMX1B, and NURR1 (abbreviated ALN). As a control vector, we also built *Vim*-GFP. This *Vim*-GFP vector was validated to drive robust transgene expression in adult African green monkey astrocytes *in vitro*. However, the *Vim* promoter did not drive expression of the ALN polycistronic cassette at a high enough level to permit effective conversion of astrocytes to dopaminergic neurons. Repeated experiments showed that although low levels of ALN could be detected, no dopaminergic neurons were generated. Accordingly, we have discontinued the use of the *Vim* promoter lentiviral vectors. In their place, we have returned to the adeno-associated viral (AAV) vectors that we used in the past. Due to size limitations of AAV versus lentiviral vectors, the polycistronic ALN cassette cannot be packaged into an AAV backbone. We therefore have generated AAV vectors expressing each transcription factor individually. While this adds an extra layer of complication that is not found when using a polycistronic vector, it permits us to use the clinically-applicable AAV system. Given our earlier results that AAV serotype 5 can effectively transduce African green monkey astrocytes *in vivo*, we are confident that this is the best method as we move forward.

As noted above, we engineered versions of the transcription factors ASCL1, LMX1B, and NURR1 that were fused to the VP16 transactivation domain. We identified VP16-ASCL1 as being the key factor to mediate transdifferentiation of adult primate astrocytes into neurons. However, subsequent experiments have called into question the notion that VP16 is required. We built a polycistronic lentiviral vector in which the VP16-ASCL1 fusion was expressed along with LMX1B and NURR1: VP16-ASCL1-LMX1B-NURR1. Surprisingly, this construct did not drive effective conversion of adult monkey astrocytes to dopaminergic neurons. Closer examination of the individual transgenes via Western blotting and immunocytochemistry revealed that VP16-ASCL1 was not being properly expressed. This may have been due to the 2A peptide sequence that remained on the C-terminus of ASCL1 following 2A cleavage. When

we switched from the polycistronic cassette to individually expressed transcription factors, we found that ordinary ASCL1 (i.e. without the VP16 fusion) was capable of driving conversion of astrocytes to dopaminergic neurons (see Figure 6, below). Previous failures of the original ALN cassette (published in PLoS One in 2011) in monkey cells may not have been due to a lack of VP16, as we hypothesized in our most recent annual report, but rather due to improper folding of ASCL1-2A. We have now engineered AAVs to express ASCL1 with and without VP16, along with separate AAVs for LMX1B, its homolog LMX1A, and NURR1. These were submitted to the Penn Vector Core for packaging in December of 2013. Although the experiments using these new vectors will be completed after the end of this grant funding period, we have made substantial progress toward understanding the requirements for *in vivo* conversion of adult monkey astrocytes into dopaminergic neurons. The results of these studies should prove invaluable toward the development of novel regenerative medicine-based therapies for Parkinson's Disease.

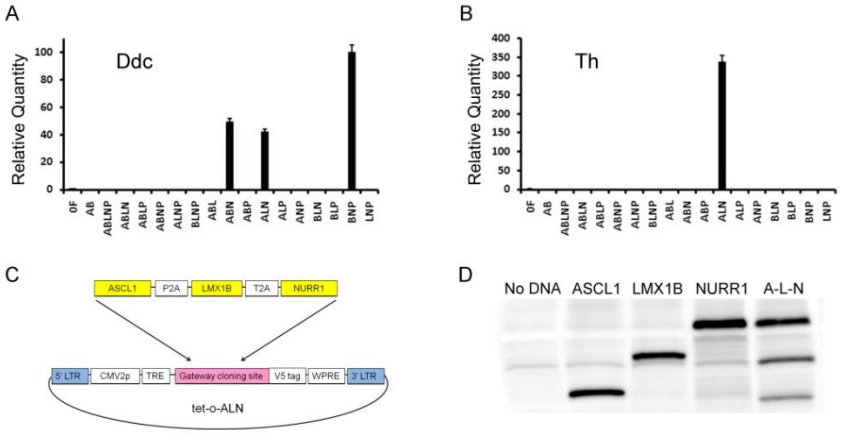

FIGURE 1. Transcription factor screen and polycistronic vector construction. (A-B) Evaluation of transcription factor combinations to induce reprogramming. RT-PCR results for a subset of the 74 transcription factor combinations tested for their ability to induce expression of DOPA decarboxylase (Ddc) and tyrosine hydroxylase (Th) in mouse embryonic fibroblasts after 7 days of factor expression. 0F: uninfected control; A: ASCL1; B: BRN2; L: LMX1B; N: NURR1; P: PITX3. The combination of ASCL1, LMX1B, and NURR1 (ALN) was the only combination to give robust expression of both Ddc and Th. (C) Polycistronic vector containing open reading frames of human ASCL1, LMX1B, and NURR1 linked by viral 2A sequences. (D) Complete cleavage at 2A peptide sites confirmed by *in vitro* transcription and translation (TnT) of lentiviral plasmids in the presence of biotinylated lysine. Streptavidin-HRP Western blot shows all newly synthesized protein. Lane 1: No DNA TnT control. Lanes 2-4: TnT performed on original single-factor plasmids. Lane 5: TnT for polycistronic ALN plasmid. Band intensity is proportional to the number of lysine residues present in each protein sequence.

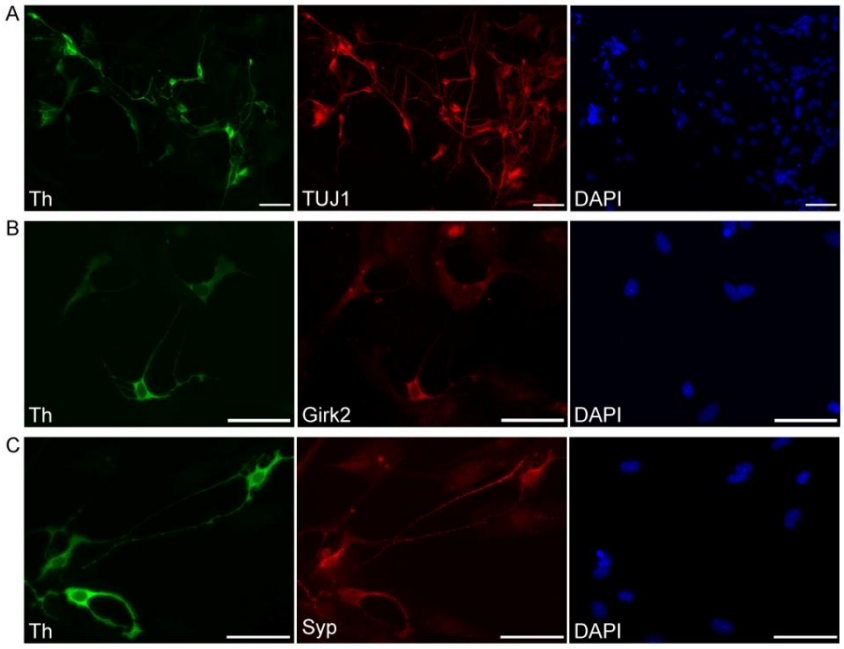

FIGURE 2. Immunocytochemical characterization of astrocyte-derived dopaminergic neurons. Astrocyte-derived dopaminergic neurons, 17 (A) to 19 (B,C) days post-induction. (A) Efficient conversion of astrocytes to dopaminergic neurons demonstrated by immunocytochemistry for the neuronal marker TUJ1 (type III beta tubulin) and the dopaminergic marker tyrosine hydroxylase (Th); the pan-nuclear marker DAPI is also shown. (B) Th positive neurons express the potassium channel Girk2, a marker of A9 (substantia nigra) dopaminergic neurons. (C) Punctate synaptophysin (Syp) expression in tyrosine hydroxylase (Th) positive neurons, indicating the potential to form synaptic connections. Scale bars 50 μm.

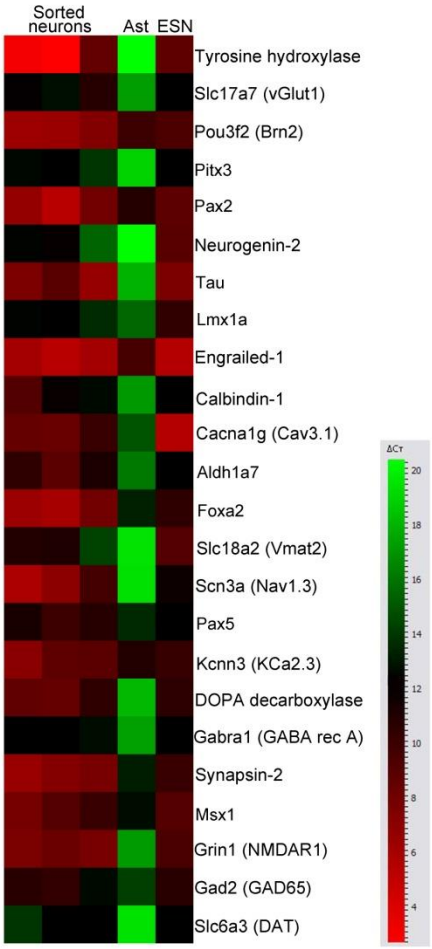

FIGURE 3. Transcriptional profile of astrocyte-derived dopaminergic neurons. Heat map of quantitative RT-PCR results comparing astrocyte-derived neurons magnetically sorted for a *MAP2*-CD4 reporter. Ast, uninfected astrocytes. ESN, mouse embryonic stem cell-derived neurons, generated via co-culture with PA6 stromal cells. Induced dopaminergic neurons express markers consistent with midbrain dopaminergic neurons. Color scale indicates change in Ct (threshold cycle) relative to the normalizing actin control. Higher delta Ct values correspond to lower relative gene expression, with every Ct decrease of 3.3 representing a ten-fold increase in relative expression.

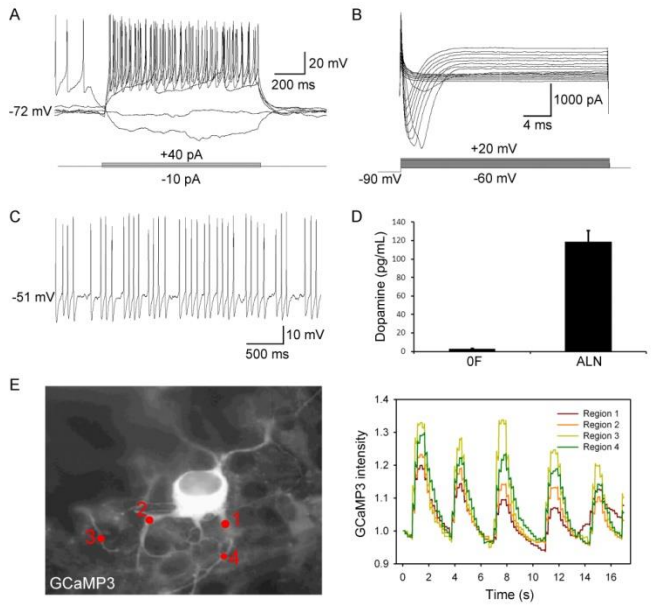

FIGURE 4. Functional characterization of astrocyte-derived dopaminergic neurons. (A) Action potential firing characteristics of induced dopaminergic neurons. Five overlapping traces are depicted derived from whole-cell current clamp recording of a representative induced dopaminergic neuron, elicited in response to hyperpolarizing and depolarizing current injection, increased from -10 to +40 pA in 10 pA increments. (B) Voltage-dependent sodium currents in induced dopaminergic neurons. Membrane potential was initially held at -90 mV and incrementally increased from -60 to + 20 mV in 5 mV depolarizing steps. (C) Spontaneous action potential firing, consistent with a dopaminergic neuron pacemaker phenotype. The recording was conducted at resting membrane potential (-51 mV). (D) Dopamine release quantified by HPLC. Membrane depolarization was induced with 56 mM KCl at 17 days post-infection. 0F: uninfected astrocytes; ALN: induced dopaminergic neurons. (E) *MAP2*-GCaMP3 reveals rhythmic oscillations of intracellular calcium. Left panel displays a single frame of a recording of oscillating levels of intracellular calcium, which is presented as a histogram of GCaMP3 fluorescence intensity in the adjacent panel.

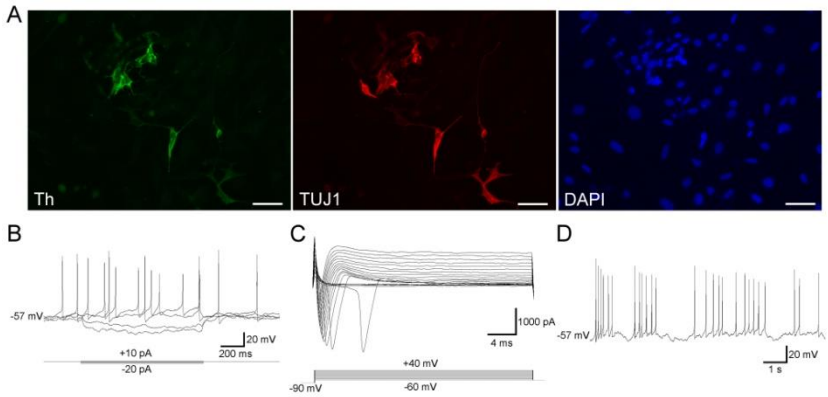

FIGURE 5. Fibroblast-derived dopaminergic neurons. (A) The ALN polycistronic vector is effective in converting fibroblasts to dopaminergic neurons, demonstrated by immunocytochemistry for the neuronal marker TUJ1 (14.9 ± 2.3% positive) and the dopaminergic marker tyrosine hydroxylase (9.1 ± 0.9% positive); the pan-nuclear marker DAPI is also shown. Scale bars 50 µm. (B) Action potential firing characteristics of fibroblast-derived dopaminergic neurons. Four overlapping traces are depicted derived from whole-cell current clamp recording of a representative induced dopaminergic neuron, elicited in response to hyperpolarizing and depolarizing current injection, increased from -20 to +10 pA in 10 pA increments. (C) Voltage-dependent sodium currents in fibroblast-derived dopaminergic neurons. Membrane potential was initially held at -90 mV and incrementally increased from -60 to + 40 mV in 5 mV depolarizing steps. (D) Spontaneous action potential firing, consistent with a dopaminergic neuron pacemaker phenotype. The recording was conducted at resting membrane potential (-57 mV).

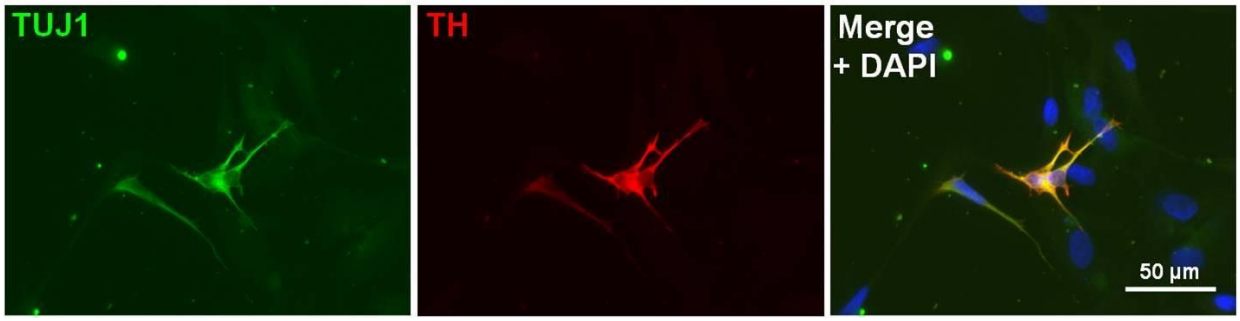

FIGURE 6. Dopaminergic neurons (iDNs) generated by direct reprogramming of adult monkey astrocytes. TUJ1: type III beta-tubulin, a pan-neuronal marker; TH: tyrosine hydroxylase. Immunocytochemistry was performed three days following induction of reprogramming factors ASCL1, LMX1B, and NURR1.

	Capacitance (pF)	RMP (mV)	Spontaneous firing (%)	AP freq (Hz)	Threshold (mV)	I_{Na}-max (pA)	I_K-max (pA)	IR (GΩ)
Mean	16.22	-55.39	43.00	5.60	-27.73	1546.00	1381.91	1.48
SEM	0.76	1.46	11.00	1.17	2.27	175.21	151.51	0.23
N	8	16	21	7	11	11	11	16

TABLE 1. Electrophysiological properties of astrocyte-derived dopaminergic neurons. RMP: resting membrane potential; AP freq: frequency of spontaneous action potentials; I_{Na} and I_K: Maximum sodium and potassium currents; IR: input resistance.

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, and an abbreviated title of the publication. For example, if you submit two publications for Smith (PI for Project 01), one publication for Zhang (PI for Project 03), and one publication for Bates (PI for Project 04), the filenames would be:

Project 01 – Smith – Three cases of isolated
Project 01 – Smith – Investigation of NEB1 deletions
Project 03 – Zhang – Molecular profiling of aromatase
Project 04 – Bates – Neonatal intensive care

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. Efficient Conversion of Astrocytes to Functional Midbrain Dopaminergic Neurons Using a Single Polycistronic Vector	Russell C. Addis, Fu-Chun Hsu, Rebecca L. Wright, Marc A. Dichter, Douglas A. Coulter, John D. Gearhart	PLoS One	Submitted August 2011; Published December 2011	☐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:

We plan to publish our results of *in vivo* conversion of astrocytes to dopaminergic neurons in an African green monkey model of Parkinson's Disease.

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.

This project led to the major discovery that astrocytes can be directly converted to dopaminergic neurons. This opens up a new avenue of regenerative medicine therapy for Parkinson's Disease, which is marked by the loss of dopaminergic neurons in the midbrain. By discovering the genes required for effecting these conversions in a dish, we have allowed for future research to test the feasibility of *in vivo* reprogramming approaches for Parkinson's Disease.

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.

John D. Gearhart, Ph.D., is the James W. Effron University Professor and is the Director of the Institute for Regenerative Medicine at the University of Pennsylvania. He is a developmental geneticist and his research over the past several decades has been directed at an understanding the molecular and cellular basis of human embryonic development. Dr. Gearhart is a leader in the development and use of human reproductive technologies, embryo and germ cell manipulations and in the genetic engineering of cells. In 1998, Dr. Gearhart and his research team at Johns Hopkins published the first report on the derivation of pluripotent stem cells from germ cells of the human embryo. These cells have the capacity to

form all cell types and tissues present in the human body and are considered a major starting point for the development of a wide variety of cell-based therapies in the new field of regenerative medicine. His research is focused on the basic science of stem cells, stem cell specialization, and the generation of cell-based therapies for a number of diseases and injuries.

Dr. Gearhart was a founding member of the International Society for Stem Cell Research and serves on a number of advisory boards and committees of foundations, institutes and professional societies involved in stem cell research and policy and science outreach and has served as a consultant or expert witness for many governmental agencies, in states, at the national level and to governments of foreign countries. He currently serves on the FDA Advisory Committee for Cellular, Tissue and Gene Therapies and the World Anti-Doping Authority, Gene Doping Expert Group.

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
Russell C. Addis	Senior Research Investigator
eRA COMMONS USER NAME (credential, e.g., agency login)	
RADDIS	

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 Pennsylvania	B.A.	05/02	Biochemistry & biology
Johns Hopkins University School of Medicine	Ph.D.	05/08	Human genetics & molecular biology

PROFESSIONAL POSITIONS

2002 – 2008 Johns Hopkins University School of Medicine
Graduate student in Human Genetics & Molecular Biology
Advisors: John D. Gearhart and Andrew S. McCallion

7/2008 – 6/2009 University of Pennsylvania Perelman School of Medicine
Postdoctoral researcher, Institute for Regenerative Medicine
Advisor: John D. Gearhart

7/2009 – present University of Pennsylvania Perelman School of Medicine
Senior Research Investigator, Institute for Regenerative Medicine
Department of Cell and Molecular Biology

HONORS AND AWARDS

2012 Award for best trainee presentation, Children's Hospital of Philadelphia / Penn Institute for Regenerative Medicine Stem Cell Retreat

2008 Scholarship to First International Symposium on Human Embryonic Stem Cell Research, Paris, France

PROFESSIONAL ORGANIZATIONS

American Heart Association
American Society for Cell Biology
International Society for Stem Cell Research

PEER-REVIEWED PUBLICATIONS

1. Jamie L. Ifkovits, **Russell C. Addis**, Jonathan A. Epstein, and John D. Gearhart. Inhibition of TGFβ Signaling Increases Direct Conversion of Fibroblasts to Induced Cardiomyocytes. Accepted by *PLoS One.* In press.

2. Kalliopi Stasi, DaVida Goings, Jiayan Huang, Lindsay Herman, Filipa Pinto, **Russell C. Addis**, Dahlia Klein, Mina Massaro-Giordano, and John D. Gearhart. Optimal isolation and xeno-free culture conditions for limbal stem cell function. *Investigative Ophthalmology and Visual Science*. 2013 Sep 12. [Epub ahead of print]

3. **Russell C. Addis** and Jonathan A. Epstein. Induced Regeneration – the progress and promise of direct reprogramming for heart repair. *Nature Medicine*. 2013 Jul;19:829-836.

4. **Russell C. Addis**, Jamie L. Ifkovits, Filipa Pinto, Lori D. Kellam, Paul Esteso, Stacey Rentschler, Nicolas Christoforou, Jonathan A. Epstein, and John D. Gearhart. Optimization of Direct Fibroblast Reprogramming to Cardiomyocytes Using Calcium Activity as a Functional Measure of Success. *Journal of Molecular and Cellular Cardiology*. 2013 Apr 13; 60C:97-106. doi: 10.1016/j.yjmcc.2013.04.004. [Epub ahead of print].

5. Nicolas Christoforou, Malathi Chellappan, Andrew F. Adler, Robert D. Kirkton, Tianyi Wu, **Russell C. Addis**, Nenad Bursac, and Kam Leong. Transcription Factors MYOCD, SRF, Mesp1 and SMARCD3 Enhance the Cardio-Inducing Effect of GATA4, TBX5, and MEF2C during Direct Cellular Reprogramming. *PLoS One.* 2013 May 21;8(5):e63577.

6. **Russell C. Addis**, Fu-Chun Hsu, Rebecca L. Wright, Marc A. Dichter, Douglas A. Coulter, and John D. Gearhart. Efficient conversion of astrocytes to functional midbrain dopaminergic neurons using a single polycistronic vector. *PLoS One*. 2011; 6(12)e28719. Epub 2011 Dec 9.

7. He N. Xu, **Russell C. Addis**, DaVida F. Goings, Shoko Nioka, Britton Chance, John D. Gearhart, and Lin Z. Li. Imaging Redox State Heterogeneity Within Individual Embryonic Stem Cell Colonies. *Journal of Innovative Optical Health Sciences*. 2011 Jul; 4(3):279-288.

8. **Russell C. Addis**, Megana K. Prasad, Robert L. Yochem, Xiangcan Zhan, Timothy P. Sheets, Joyce Axelman, Ethan S. Patterson, and Michael J. Shamblott. OCT3/4 regulates transcription of histone deacetylase 4 (Hdac4) in mouse embryonic stem cells. *Journal of Cellular Biochemistry*. 2010 Oct 1; 111(2):391-401.

9. Ethan S. Patterson, **Russell C. Addis**, Michael J. Shamblott, and John D. Gearhart. SOX17 directly activates Zfp202 transcription during in vitro endoderm differentiation. *Physiological Genomics*. 2008 Aug 15; 34(3):277-284.

PATENTS

"Direct conversion of astrocytes into dopaminergic neurons using a single polycistronic vector." Provisional patent disclosure #Y6288, May 2012.