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:** 1 - Molecular Mechanisms Involved in Reprogramming Cells

7. **Start and End Date of Research Project:** 1/1/2009-12/31/2012

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:

   $ 1,945,180.56

   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
Addis	Postdoc Researcher Sr. Research Investigator	11% Yr 1-2 71% Yr 2-4	$22,999.88 $155,453.70
Clark	Postdoc Researcher	10%	$4,621.22
Goings	Research Specialist B	42%	$53,476.92
Kellam	Postdoc Researcher	45% Yr 1-3 47% Yr 4	$77,760.45 $80,905.66
Mercedes	Research Specialist A	97%	$16,442.40
Pinto	Research Specialist C	43%	$81,594.22

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	< 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
Facscanto II Flow Cytometer IVD	For analyzing and quantifying cell populations	$144,358.56
Olympus Microscope	For fluorescent imaging and analysis of cells	$30,219.20
Bioanalyzer 2100 Electrophoresis System	For assessing quality and quantity of RNA prior to analysis	$23,267.70
5' Cell LG A2 115V Scope	Biohazard Laminar Flow cabinet for culturing cell lines	$13,608.87
TS100F LED Tissue Culture Microscope Nikon E800 Microscope Upgrade	For use in viewing and imaging live cells on tissue culture dishes	$34,923.44

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:

An RO1 grant will be prepared for submission to the NIH this Fall.

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

If the results obtained so far are supported by ongoing experiments, Dr. Kellam will have made a substantial contribution to the field of reproductive biology with major implications for clinical use. We will continue these studies with high priority.

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				2
Female				
Unknown				
Total				**2**

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic				1
Non-Hispanic				1
Unknown				
Total				**2**

	Undergraduate	Masters	Pre-doc	Post-doc
White				1
Black				
Asian				
Other				1
Unknown				
Total				**2**

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.

We hope to capitalize this work at Penn, both with our collaborators in the School of

Veterinary Medicine and extend this to Gyn/OB in the School of Medicine. It will also establish Penn's leadership in this exciting field.

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.

Two major reprogramming targets were funded through this grant: cells of the heart early embryonic cells. The reprogramming of fibroblasts to cardiomyocytes became a valuable tool for investigating a number of parameters associated with the introduction of exogenous transcription factors into cells, having them expressed conditionally and in assessing the degree to which cells were reprogrammed. All of this information has been important in not only succeeding with the heart cells but also we have successfully applied it to a variety of other cell types (not funded through CURE funds). This has enabled our group to become recognized 'experts' in this technology.

Specific Aim1: Determine the variables and extent of the reprogramming process at the single cell level.
Specific Aim 2: Determine whether there are limits to the reprogramming process.

<u>Reprogramming to heart cells</u>

Cardiovascular disease remains the number one killer of people worldwide. The World Health Organization (WHO) 2012 World Health Statistics outline that global cardiovascular/diabetes attributable mortality for people aged 30-70 years old in 2008 was approximately 32.1% while that from cancer accounted for about 19.6% of deaths. Of these myocardial infarction is the most prevalent, with a US incidence of approximately 1.5 million individuals per year [1]. Nearly half of these patients are under the age of 65. A secondary maladaptive physiologic response results in congestive heart failure in approximately 40% of these individuals. Notably there has been a decrease in cardiovascular mortality during the past decade (40.3% in 1999 to 27.1% in 2009) with a concomitant shift in presentation such that transmural myocardial infarction (ST-elevation MI or STEMI) declined from 133 to 50 per 100,000 person-years [2]. One implication of such epidemiologic changes is an increasing population of patients with chronic cardiovascular dysfunction who could potentially benefit from cellular interventions given a relatively preserved though

dysfunctional myocardium. Notably, a unifying feature of the majority of cardiovascular illnesses, weather congenital or acquired, is myocardial hypocellularity.

The potential of regenerative approaches to ameliorate cardiac morbidity and mortality have gripped the field of Cardiology, though an ideal cell type for transplantation or regeneration has not been identified. A number of potential cell sources have been considered and some even been evaluated in clinical trials. These trials have included skeletal myoblasts, mesenchymal stem cells, bone marrow derived mononuclear cells, etc., and demonstrated safety and feasibility, though not yet clinically effectiveness[3]. A pair of ongoing clinical trials are evaluating the use of patient-derived resident cardiac stem cells [4,5]. While not powered to demonstrate hard clinical outcomes the CADUCEUS trial demonstrated a smaller scar area in treated patients [4].

Another attractive source of cells would be that generated by transdifferentiation of the patient's own cells, allowing in-situ generation of contractile cardiomyocytes from the hypertrophic fibroblast-rich scar. However, fully differentiated cells have been historically thought to be locked into phenotypically stable states. This is often illustrated using Waddinton's epigenetic landscape imagery where differentiation is represented as a progressively restricted set of hills and valleys [6]. Progressive cell-fate determination is thought to lock cells into increasingly narrow phenotypes though the epigenetic closing of whole transcriptional programs. Nonetheless, several relevant examples of metaplasia seen clinically, such as the conversion of columnar to stratified squamous epithelium in the cervix in response to chronic irritation, challenge this notion. Furthermore, classic experiments in which Xenopus somatic nuclei were transferred into enucleated egg cells revealed the capacity for the egg cytoplasm or "ooplasm" to unlock the nuclear material, allowing these hybrid cells to undergo embryonic development [7].

For many years investigators have sought to define the materials that enable the reprogramming of somatic nuclei. This was finally achieved in 2006 when a Japanese group, led by Shinya Yamanaka, performed a now classical experiment. These investigators selected a list of candidate genes, based primarily on knowledge gleaned from embryonic stem (ES) cell work, which were systematically transduced into primary mouse embryonic fibroblasts (PMEF) using retroviral expression vectors. The result was formation of induced pleuripotent stem cells (iPS) capable of self-renewal and pleuripotent differentiation, as seen with ES cells [8]. This approach provides a means to obtain patient specific embryonic-like stems that can be used for potential transplantation, to study the mechanisms involved in human cardiogenesis or to analyze the potential determinants of congenital and acquired disease in-vitro with cells from particular patients with known clinical outcomes. This advance clearly adds a new dimension to the contribution that stem cell biology has provided to the understanding of cardiac biology.

The ability of transcription factors to modify cell fate has been clearly established. Classically, MyoD is termed a myogenic regulatory factor (MRF) for its broad capacity to induce myogenic fate when ectopically expressed in multiple differentiated cell types [9]. Similar single factors capable of inducing cardiogenic cell fate were sought without fortune. Interest in cellular reprogramming was revived when in 2006 two Japanese investigators,

Takahashi and Yamanaka, demonstrated that ectopic expression of 4 transcription factors could drive epigenetic and phenotypic reprogramming of fibroblasts to such extent that they behaved like embryonic stem cells in culture. These cells, termed induced pluripotent stem cells (iPS), could form teratomas, be incorporated into tissues from all embryonic germ layers in blastocyst injection experiments, including gonadal stem cells [8], and yield live, fertile mouse pups during tetraploid complementation [10]. In-vivo cellular reprogramming was first demonstrated by converting exocrine to endocrine pancreatic cells in mice [11]. By using endocrine cell-tropic adenoviruses the investigators were able to induce pancreatic beta cell transcriptional profile through the ectopic expression of three genes known to developmentally critical for these cells: Ngn3, Mafa and Pdx1. Furthermore, one week after virus injection into diabetic mice, investigators noted increased glycemic control that lasted through the 8 weeks of the study [11].

Transcription factor-induced transdifferentiation to cardiomyocytes was first described in the mouse embryo by Takeuchi and Bruneau[12]. Transfection of Gata4 and Tbx5 was sufficient to induce ectopic beating cardiac foci only in mesoderm when cotransfected with Baf60c, a cardiac-enriched subunit of the Swi-Snf-like chromatin-remodeling complex. No such effect was seen in endoderm and ectoderm was not reliably transfected [12]. Similarly David et al. (2008) showed that over expression of Mesp1 in the Xenopus embryo and embryonic stem cells led to a significant increase in cardiogenesis though this may occur through a broader increase in lateral plate mesoderm, where the cardiac crescent forms embryonically, as there was an increase of vascular structures seen in these transgenic ES cultures as well [13].

Developmentally the heart receives cellular contributions from a variety of sources, including the primary and secondary heart fields and cardiac neural crest (Reviewed in Vincent and Buckingham[14]). A new developmental source of cardiomyocytes has been recently described. Using lineage tracing experiments two independent groups have suggested the proepicardial organ, developmental source of cardiac fibroblasts, can give rise to a small number of cardiomyocytes. By permanently labeling cells during development that express Tbx18 [15] or Wt1 [16], embryonic epicardial markers, investigators have shown a contribution from these epicardial descendants to the myocardium. These results would suggest that the decision to form a cardiac fibroblast or a cardiomyocyte is made late in development and cardiac fibroblasts may, therefore, have a cardiomyocyte-permissive epigenetic background.

Additional efforts to increase cardiogenesis have sought to utilize small molecules. One such small molecule, Isoxazole, was identified by its capacity to increase expression of Nkx2-5 in a high throughput assay using an embryonal carcinoma cell line [17]. Furthermore, it is capable of increasing cardiac gene expression in Granulocyte colony-stimulating factor (GCSF)-mobilized human peripheral mononuclear cells and to enhance cardiac repair by these cells in a rat model of cryoinjury [17]. Isoxazole has also induced cardiac expression in cells derived from Notch-expressing stem cell-like epicardial cells while decreasing their pro-fibrotic profile. The effect was, however, insufficient to induce regeneration or to mitigate the fibrotic repair induced by a myocardial infarction in treated mice [18].

We sought to reprogram fibroblasts to cardiac progenitor cells or cardiomyocytes with a collection of ectopically expressed transcription factors and small molecules. Based on our

understanding of cardiac development, progenitor cell biology, pleuripotent reprogramming and novel cardiac gene discovery studies in our laboratory, using mES-derived CPCs, we sought to establish a list of candidate cardiac reprogramming factors. By combinatorial ectopic expression and supplementation with cardiogenic or epigenetically active small molecules we aim to determine a minimal set of factors capable and sufficient to induce cardiac cell fate efficiently. Furthermore we wanted to evaluate whether cardiac fibroblast were more amenable to this process than those from non-cardiac sources.

Materials and Methods

Lentiviral cloning, virus production and transduction

Cardiac transcription factor ORFs without stop codons were purchased from Open Biosystems in gateway vectors when available or PCR-cloned into pENTR/D-TOPO (Invitrogen, K240020) and gateway cloned into the FU-tetO-GW-V5 lentiviral backbone. PCR-cloned sequences were amplified using the Takara LA PCR kit Ver 2.1 (Takara, RR013A). Lentiviral plasmids were restriction digest and sequence confirmed and transformed onto One Shot Stbl3 chemically competent E. coli (Invitrogen) for amplification under antibiotic selection. Protein expression from lentiviral vectors was assessed by V5 tag western blotting following in-vitro transcription with the TNT-T7 coupled reticulocyte lysate system (Promega, L4610) as well as transduction into mouse and human cells. For virus production lentiviral expression and helper vectors were lipofected into LentiX-293T cells (Clontech, 632180). A solution containing 1.5 mL of OPTI-MEM (Gibco, 31985), a total of 24 ug of plasmid (FU 50%, psPAX2 32%, pMD2.G 18%) and 60 uL of Lipofectamine 2000 (Invitrogen, 11668) was used to lipofect one 10 cm plate of 90% confluent 293Ts. Fresh media was replaced after 12 hours and virus-containing supernatant was collected 24 and 48 hrs later. Viral production was evaluated with LentiX Go-Stick (Clontech, 631243). Supernatant was filtered with a 22 um Steriflip (Millipore). For transduction an aliquot of 500 uL of each virus was added to 10^5 cells in one well of a 6-well plate with polybrene (8 ng/uL, Sigma, H9268) and cultured overnight. Fu-rtTA alone was transduced at the time of plating. Additional transductions were carried out in consequent days such that no more than 3 different viruses were transduced on any given day.

Isolation of mouse cells

Mouse embryos were isolated at 12.5 dpc and used for primary mouse embryonic fibroblast isolation [8] and heart explant culture for cardiac fibroblast isolation [19]. For PMEF isolation the head, visceral organs and gonads were removed and remaining tissues were washed, triturated and treated with a solution of 0.15% trypsin/EDTA (Gibco) and collagenase IV (1 ug/mL, Sigma, C5138) for 10 minutes at 37^0C with shaking. After trypsin neutralization the solution was passed through a 100 um filter, pelleted and plated onto gelatin coated culture plates. For cardiac fibroblast isolation the hearts were removed from the same 12.5 dpc embryos. After grossly removing the atria explanted hearts were washed in ethanol, minced and plated one each on 35 mm gelatin coated plates. Fibroblasts were allowed to grow from cultured explants until confluent, approximately 1 week. Upon reaching confluency cultures were rinsed in Ca^{2+}/Mg^{2+} free DPBS (Gibco, 14190), trypsinized (0.05%, Gibco, 25300) for 5 minutes at 37^0C and neutralized with serum. The remaining heart chunks were removed by

filtering through a 70 um filter. After pelleting, cells were cultured on 10 cm gelatin coated plates.

Thy1 magnetic isolation

Thy1 is a relatively non-specific cell surface marker that in the heart is expressed in a subset of cardiac fibroblasts but not myocytes. For Thy1 isolation cells grown from heart explants were collected by a solution containing TrypLE Express (Invitrogen, 12604) and collagenase IV (10 ug/mL) at 37^0C for 3 min. After neutralizing with serum-containing media cells were pelleted and resuspended in PBSS (up to 10^7 cells in 500 uL). Cells were incubated on a shaker at 4^0C for 30 min with magnetic bead conjugated anti-mouse CD90.2 antibodies (50 uL per 10^7 cells, BD IMag, 551518) then pelleted and resuspended in 500 uL of PBSS. A MACS/Milteny magnetic isolation system was used as outlined by the manufacturer. MS columns (130-042-201) were placed on the magnet and equilibrated with PBSS by gravity. The cell solution was passed twice through the magnetic column (negative fraction) by gravity. The MS column containing the Thy1+ cell fraction was then removed from the magnet and 500 uL of fresh PBSS were allowed to flow through by gravity followed by 500 uL of PBSS passed through the column by using the plunger. The resulting Thy1+ cardiac fibroblasts were pelleted, resuspended in DMEM:M199 media and plated at 10^6 cells per 10 cm plate and transferred after 24-48 hrs to 6-well plates at 10^5 cells per well for transduction.

Cell culture

Mouse cells were grown on gelatin coated plates in DMEM (Gibco, 11965) with 10% FBS (Atlanta Biologicals, S11550), 1% Sodium Pyruvate 100 mM (Gibco, 11360), 1% GlutamaX (Gibco, 35050), 1% MEM NEAA (Gibco, 11140), 0.1% 2-mercaptoethanol (Gibco, 21985) and 0.05% Gentamycin (50 mg/mL, Gibco, 15750). After transduction cells were maintained in similar medium containing DMEM and M199 at 2:1 ratio (Gibco, 11150). Human dermal fibroblasts (CELLnTEC) were cultured on gelatin-coated plates with recommended CnT05 media (CELLnTEC) through lentiviral transduction and transferred to DMEM:M199 media from the time of doxycycline supplementation. Media was supplemented were indicated with doxycycline (10 ng/mL, Sigma, D9891), RG108 (10 ng/mL, CalBiochem, 260920), BMP4 (10 ng/uL, 314-BP), and changed every other day except where shorter incubations were indicated. Isoxazole was added to the media in DMSO as previously reported [17]. Briefly 2 uL of 100 uM solution in DMSO was added per 10 mL of media every other day beginning on day 2. All recombinant proteins were purchased from R&D Systems.

Immunocytochemistry

Cells were plated for 48 hrs on fibronectin-coated (20 ng/mL, 1hr at 37^0C) glass coverslips fixed and permeabilized in 4% PFA with 0.15% TritonX for 15 min and washed 3X5 min with PBS. Cells were blocked in PBST with 1% BSA (Sigma, B4287) and 10% donkey serum (+/- goat serum where appropriate) for 30 min and incubated with primary antibody for 1 hr in PBST with 1% serum and washed 3X5 min. Secondary antibodies were incubated for 1 hr in PBST. All incubations were done at room temperature. Coverslips were mounted on glass slides using ProLong Gold with DAPI mounting media (Invitrogen, P36935) and imaged using a Xenon fluorescent light source and an Olympus IX-81 microscope. The primary antibodies used at 1:200 were anti-alpha-actinin (Sigma, A7811), anti-Myosin Heavy Chain (MF20; eBioscience, 53-6503), anti-Tropinin T (Tnnt2; Thermo, MS-295-PO)

and those used at 1:500 were anti-Vimentin (Millipore, AB5733) and rabbit anti-V5 (Abcam, ab9116). Secondary antibodies were donkey anti-mouse 488 (1:500, A-21202), donkey anti-rabbit 594 (A-21207) and goat anti-chicken 594 (A-11042), both used at 1:1000. All secondary antibodies were purchased from Molecular Probes.

Western Blotting

Whole cell lysates were collected using protein loading buffer (Fermentas, R0891) and NuPAGE reducing agent (Invitrogen, NP0009) and boiled for 5 minutes at 99^0C. Lysates were run on 4-15% Tris-HCl ready gels (BioRad, 161-1104) and transferred to PVDF membranes. Blots were blocked using 5% milk in TBST for 30 min. Primary anti-V5 (1:5,000, Invitrogen, 46-0705) or anti-Actin (Millipore, MAB1501R) atibodies were incubated for 1 hr in TBST with milk. The blot was washed 3X5 min in TBST. HRP-conjugated secondary anti-mouse IgG antibody (Promega, W402B) was incubated for 1 hr and consequently washed 3 times in TBST. Chemiluminescent visualization was obtained after 5 min incubation with ECL Plus WB detection system (GE Healthcare, RNP2132). All incubations were done at room temperature.

RNA isolation and Quantitative-PCR

For RNA isolation cells were rinsed in DPBS, trypsinized (0.05%, Gibco), neutralized with TNS (Lonza, CC-5002) and passed 1:10 onto fibronectin-coated coverslips. The remaining cells were lysed for RNA collection using the ABI Gene Expression Cells-to-CT kit (4399002). 22.5uL of lysate were used for cDNA synthesis as recommended by the manufacturer. Q-PCR was done using both TaqMan primer/probes and customized microfluidic TaqMan array cards, which evaluate 32 genes of interest in triplicate per sample, and were run on an ABI 7900HT Real Time PCR system. Data was analyzed using the Delta-Delta-CT method as well as ABI's Data Assist analysis program and using a maximum CT of 37, which was included as zero-expression in the analysis.

Results

Cardiogenic Factor Selection

Candidate reprogramming genes were selected based on studies undertaken in our laboratory to discover novel cardiac genes using mES-derived CPCs as well as to define the critical transcriptome of these cells. In addition we reviewed the literature for genes necessary and/or critical to cardiac development. A list of 15 candidate genes was selected (Table 1) and cDNAs were gateway cloned into the Dox-inducible FU-tetO-GW-V5 lentiviral backbone. Two small molecules were selected for their reported capacity to improve cardiac repair and induce cardiac gene expression (Isoxazole), to diminish DNA methylation and improve the efficiency of iPS reprograming (RG108, a DNMT inhibitor). The potential contribution of the developmental mitogen BMP4, critical to cardiogenesis, was also assessed.

NKX2-5	**MEF2C**	**GATA4**	**TBX5**	**MESP1**
MYOCD	**HAND1**	**HAND1**	**BAF60C**	**BRACHYURY**
RBM24	**GATA5**	**GATA6**	**ISL1**	**HOPX**

Table 1. List of candidate cardiac reprogramming genes

Lentiviral Expression/Transduction

Mouse cardiac and embryonic fibroblasts were plated at 10^5 cells/well of a 6-well plate and transduced with Fu-rtTA at that time. Lentiviral expression vectors containing V5-tagged human cardiac transcription factors were transduced on the following day. Lentiviral expression of candidate reprogramming genes was assessed by western blotting with an anti-V5 antibody (Figure 1) and by quantitative RT-PCR, which could differentiate between endogenous mouse and ectopic human transcripts. On consequent days fibroblasts were transduced with up to 3 different lentiviruses daily to a maximum of 15 genes (Table 1).

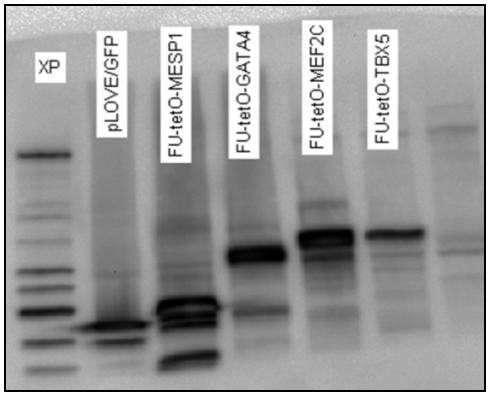

Figure 1. Characteristic western blot with anti-V5 antibody. Thy1+ cardiac fibroblasts treated with doxycycline for one week. pLOVE/GFP is a positive control. Lane 7 is a negative control.

Mef2c is Necessary for Induction of the Cardiac Transcriptional Program in Fibroblast

Initial experiments were undertaken by expressing 14 factors (Table 1 minus RBM24) in PMEFs. Gene expression was assessed by quantitative-PCR, beginning at day 5, and compared to the cells at day 0 (Day at which doxycycline was added) after lentiviral transduction. This revealed upregulation of cardiac transcription factors Nkx2-5, Gata4 and Tbx5, cardiac sarcomeric proteins alpha-Mhc and cTnT, and secreted protein Nppa. Conversely expression of fibroblast specific protein (Fsp1) was downregulated. These trends were maintained through day 20 (Figure 2A). Removal of Mef2c from the transduction cocktail resulted in loss of expression of all cardiac factors except Gata4 and loss of Fsp1 downregulation (Figure 2B).

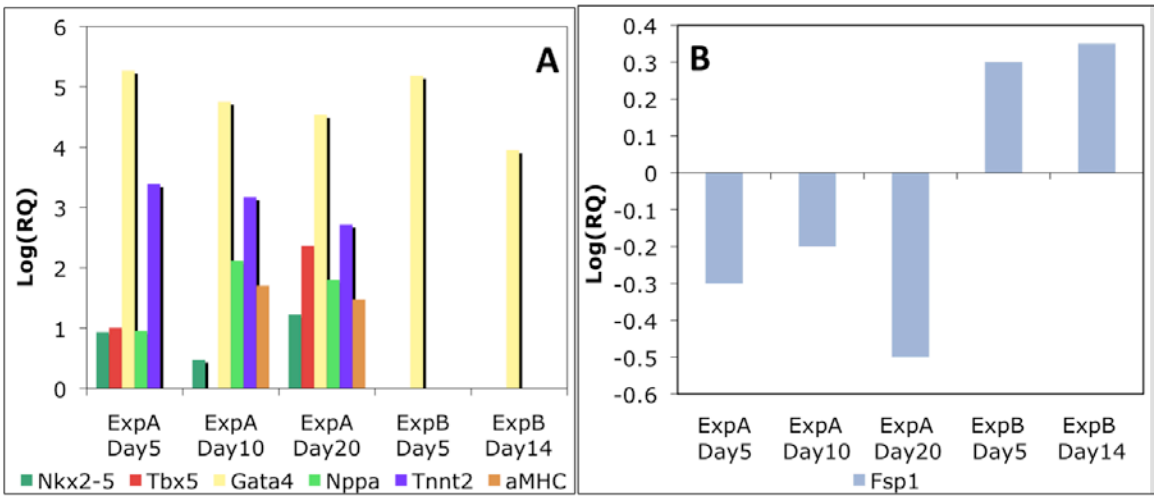

Figure 2. Effect of Mef2c removal from 14-factor reprogramming cocktail. Quantitative gene expression in primary embryonic fibroblasts treated with 14 factors (ExpA) or 13 factors, by removing Mef2c (ExpB). Assessment of cardiac gene expression (2A) or fibroblast specific expression (2B) in the presence (ExpA) or absence (ExpB) of Mef2c.

Reproducing Mef2c, Gata4, Tbx5 reported reprogramming to cardiomyocytes

During this portion of the experiment a manuscript was published stating that 3 cardiac factors (Mef2c, Gata4 and Tbx5 abbreviated MGT) were sufficient for cardiac reprogramming in fibroblasts. In response to this publication we aimed to recapitulate the published results. Expression of MGT with the originally published retroviral as well as with our own lentiviral expression vectors was attempted. Broad activation of a cardiac transcriptional program was evident. Gene expression assayed with TaqMan array cards showed broad cardiac transcriptional induction (Table 2). The sarcomeric protein cTnT was most rapidly and significantly upregulated (Figure 3). Induced expression in embryonic fibroblast clustered by treatment and time. Activation of the endogenous Mef2c, Gata4 and Tbx5 loci was seen after one month of doxycycline treatment (Figure 3). Notably removal of Doxycycline at 2 weeks resulted in continued reprogramming when expression was assessed at 1 month, though to a lesser extent that when Doxycycline treatment was continued (Figure 3).

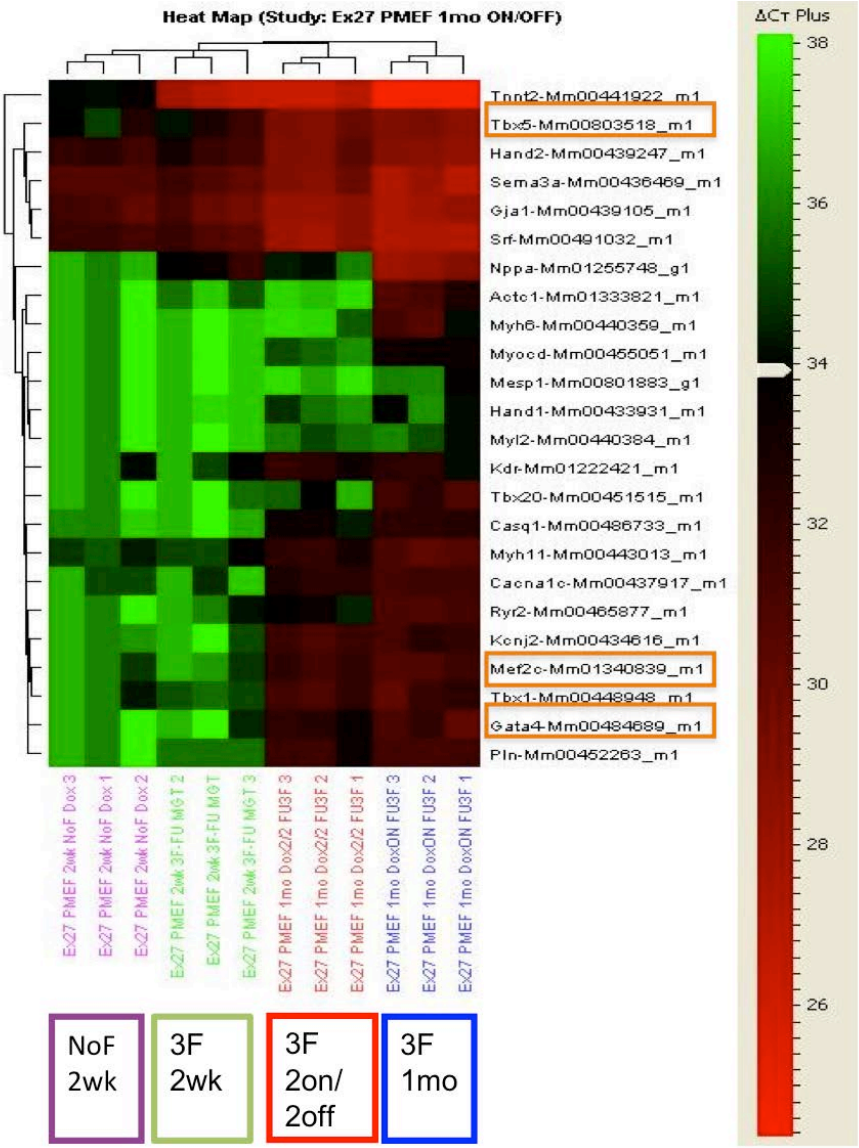

Figure 3. Cardiac expression of MGT transduced mouse fibroblasts. Q-PCR assessed expression of cardiac transcription. Expression clusters based on treatment and time. Doxycycline was maintained throughout the experiment except on 3F 2on/2off, where it was removed after 2 weeks.

1	Gapdh	Mm99999915_g1*	17	Vwf	Mm00550376_m1*
2	18S	Hs99999901_s1	18	Gja1	Mm00439105_m1*
3	Nkx2-5	Mm00657783_m1	19	Srf	Mm00491032_m1*
4	Gata4	Mm00484689_m1*	20	Ryr2	Mm00465877_m1*
5	Tbx5	Mm00803518_m1*	21	Csq	Mm01293333_m1*
6	Mef2c	Mm01340839_m1	22	Nppa	Mm01255748_g1*
7	Mesp1	Mm00801883_g1*	23	Kir2.1	Mm00434616_m1*
8	Myocd	Mm00455051_m1*	24	alpha-TM	Mm00600378_m1*
9	Isl1	Mm00627860_m1	25	Cacna1c	Mm00437917_m1*
10	Hand1	Mm00433931_m1*	26	Pln	Mm00452263_m1*
11	Hand2	Mm00439247_m1*	27	Kdr	Mm01222421_m1*
12	Myh6	Mm00440359_m1*	28	Sema3a	Mm00436469_m1*
13	Actc1	Mm01333821_m1*	29	Irx4	Mm01340286_m1*
14	Tnnt2	Mm00441922_m1	30	Tbx1	Mm00448948_m1*
15	Myl2	Mm00440384_m1*	31	Tbx20	Mm00451515_m1*
16	Myh11	Mm00443013_m1*	32	Hopx	Mm00558630_m1*

Table 2. Genes in Mouse TaqMan Array Card.

Effect of Isoxazole and Mesp1 addition on MGT Cardiac Reprogramming

The small cardiogenic molecule Isoxazole (ISO) was kindly provided by the laboratory of Dr. Schneider at UTSW. We began experiments to assess the additive effect of Isoxazole and Mesp1 (MGTP) expression on MGT reprograming of Thy1+ cardiac fibroblasts. Addition of 2 uL of 100uM Isoxazole in DMSO, or vehicle alone, was added per 10 mL of media beginning on day 2 of doxycycline and throughout the remainder of the experiment. Fresh media was replaced every 48 hrs as above. Gene expression was evaluated on day 0 and at 2 weeks by Q-PCR TaqMan array cards. Expression was then normalized to that in untreated cells and compared to that of mES-derived CPCs, isolated from embryoid bodies, by sorting for GFP expression that is driven by a cardiac specific Nkx2-5 enhancer, on day 6 of differentiation, as previously described [20]. Broad transcriptional upregulation of cardiac transcription factors (figure 4A), sarcomeric proteins (figure 4B) and ion channels (Figure 4C) was induced in at 2 weeks. There was no appreciable increase of cardiac expression by addition of Mesp1 above the MGT baseline either in the presence or absence of Isoxazole (figure 4). Addition of Isoxazole to either MGT or MGTP transduced cells resulted in an unexpected decrease in the extent of cardiac reprogramming, based on endogenous cardiac expression (figure 4). However, no evidence of histological organization of either alpha-Mhc or cTnT or of spontaneous beating was found with any of the above conditions (data not shown).

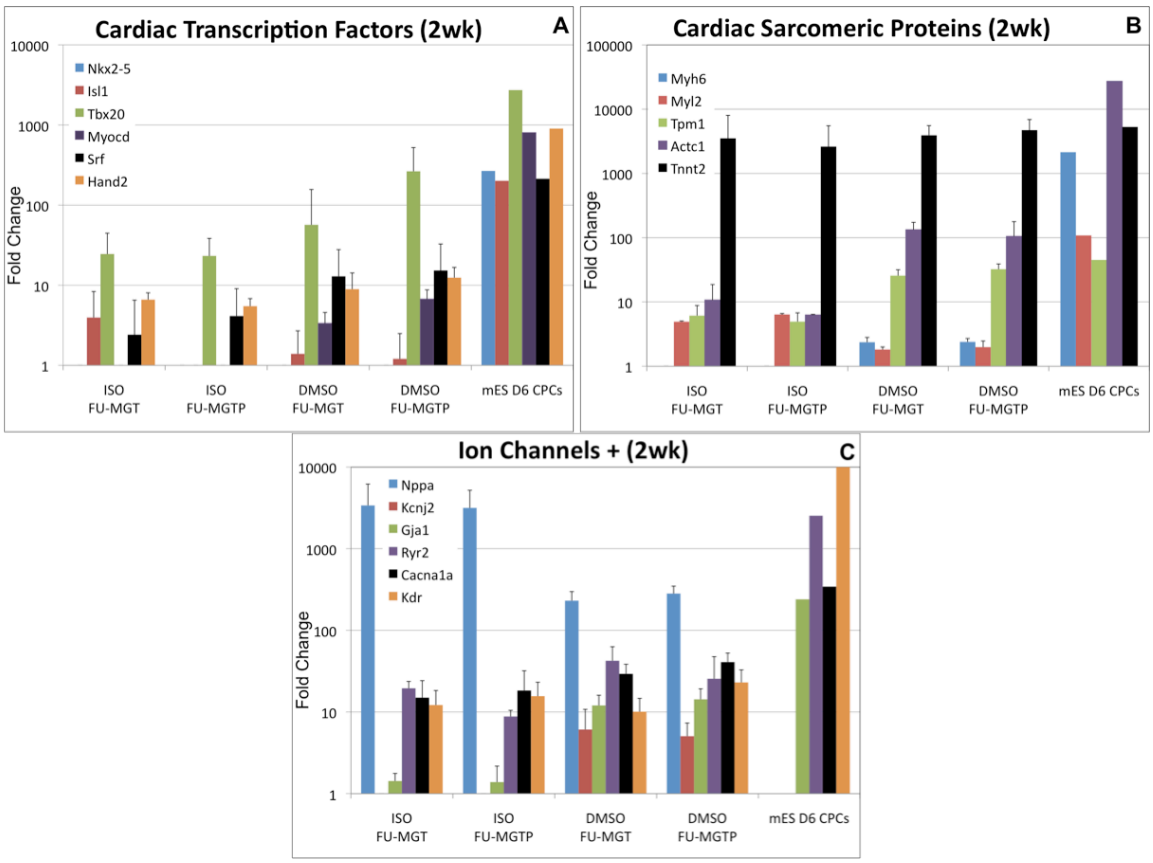

Figure 4. Effect of Mesp1 expression and Isoxazole supplementation on MGT reprogramming of Thy1+ cardiac fibroblasts. Expression assessed by Q-PCR after 2 weeks of doxycycline. Treatments include MGT and MGT+Mesp1(P) in the presence of Isoxazole (ISO) or vehicle (DMSO) and are compared to mES-derived CPCs isolated form embryoid bodies at day 6 or differentiation. Expression of cardiac transcription factors (A), Sarcomeric proteins (B) or Ion Channels and other characteristic cardiac proteins (C) is shown.

Effect of RG108, BMP4 and Gata5 addition on MGT Cardiac Reprogramming

Next we evaluated addition of RG108, BMP4 and Gata5 to MGT reprogramming. Mouse PMEFs and Thy1+ cardiac fibroblasts (CF) were transfected with MGT and additionally treated with RG108 +/- BMP4 or transfected with a Gata5 expressing lentivirus and compared to baseline MGT reprogramming. Assessment of gene expression was done at 3 weeks by Q-PCR using TaqMan array cards. Cardiac expression in PMEFs (figure 5A, 5C, 5E) and cardiac fibroblast (figure 5B, 5D, 5F) was enhanced by addition of RG108 +/- BMP4 or Gata5. Expression of the transcription factors HopX (5D) and Myocardin (5B) as well as the ion channel Casq1 (5F) was only seen in cardiac fibroblasts treated with additional factors.

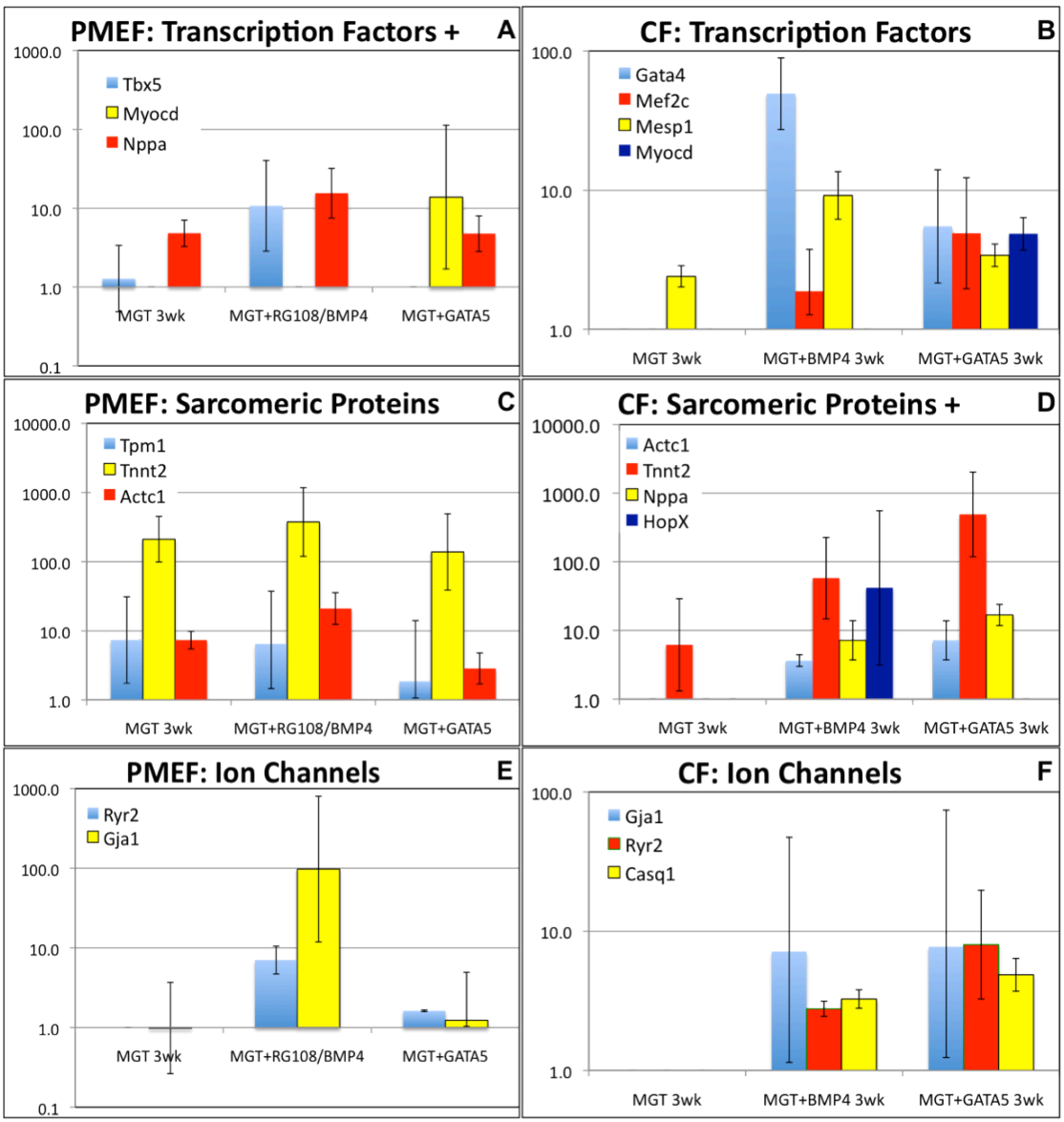

Figure 5. Effect of Gata5 sxpression and RG108, BMP4 supplementation on MGT reprogramming of PMEFs and Thy1+ cardiac fibroblasts. Expression assessed by Q-PCR after 3 weeks of doxycycline. Treatments include MGT and MGT+Gata5 or RG108 +/- BMP4. Expression of cardiac transcription factors (PMEF 5A, CF 5B), Sarcomeric proteins (PMEF 5C, CF 5D) or Ion Channels (PMEF 5E, CF 5F) is shown.

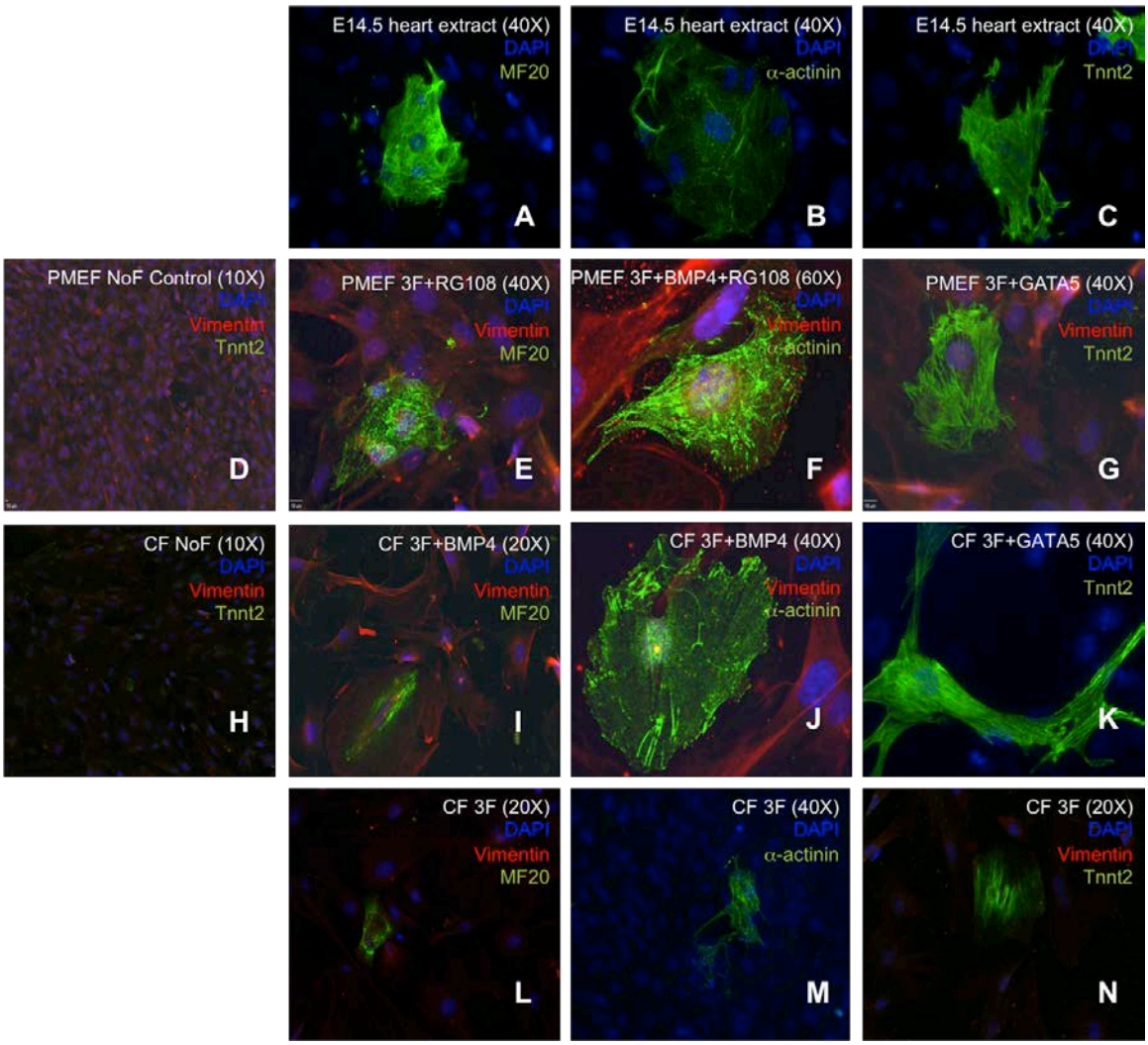

Figure 6. Histological assessment of cardiac sarcomeric proteins in mouse cells. Mouse Thy1+ cardiac fibroblasts and PMEFs were assessed after 3 weeks of doxycyxline treatment by staining with anti-cardiac troponin (Tnnt2), anti-alpha-myosin heavy chain (MF20), and anti-alpha-actinin (EA53) antibodies. Staining was compared to that present in explants of E14.5 mouse hearts (6A-C). Untreated control PMEF (6D) and cardiac fibroblasts (6H) are stained with Tnnt2 are representative. PMEF treated with MGT+ (6E-F), cardiac fibroblasts treated with MGT+ (6I-J), and cardiac fibroblasts treated with MGT alone (6L-N) represent best histologic images seen.

Histologic analysis of PMEF and Thy1+ cardiac fibroblasts transduced with MGT+ Gata5 or treated with RG108 +/- BMP4 display much improved histology with marked sarcomeric organization of alpha-actinin (figure 6F, 6J) and cardiac troponin (figure 6G, 6K) Organization of alpha-myosin heavy chain (figure 6E, 6I) was characterized by less organized, punctate agglomerations qualitatively different that those seen in explants from E14.5 mouse embryonic hearts (figure 6A). No positive staining for these sarcomeric proteins was found at any point when assessing untreated cardiac fibroblasts or PMEFs. Representative images of cTnT staining are included (figure 6D, 6H). MGT treatment alone resulted in at best cTnT fibril formation though this was markedly rare (figure 6N). In most cases positively staining MGT treated cells showed minimal puncta of expression with markedly immature structural organization (figure 6L) of any sarcomeric protein assessed.

MGT and MGTP Reprogramming of Human Dermal Fibroblasts

We evaluated the effect of MGT transduction on human dermal fibroblasts (hDF). Cells were fetal foreskin fibroblasts obtained from CELLnTECH and cultured with proprietary media as suggested by the manufacturer. As above, they were plated at 10^5 cells/well of a 6-well plate and transduced with FU-rtTA at the time of plating. MGT +/- Mesp1 (MGTP) transduction was carried out 24 hrs later in the presence of polybrene, with no more than 3 lentiviral vectors per day. Media was changed daily during transductions. Transduced cells were left in fresh media for one day after the final transduction before adding Dox to the media on day 0. Subsequently media was changed every 48 hrs.

Evaluation of cardiac gene expression was done by human-specific TaqMan array Q-PCR cards (Table 3). The same list of genes as those assayed for mouse cardiac reprogramming were assayed (Table 2). Cells were collected on day 0, 2 weeks and 1 month. Expression was normalized to that of cells on day 0. Heat-map analysis reveals that addition of MESP1 to the reprograming cocktail reduces the efficiency of cardiac transcriptional induction at 2 week (figure 7). This is unlike the effect seen in mouse where there is negligible difference between MGT and MGTP transduction (Figure 4). Of note, experiments with MGTP transduction in mice were carried out in the presence of Isoxazole or DMSO vehicle while those of human cells were not.

1	GAPDH	Hs99999905_m1	17	VWF	Hs00383230_g1*
2	18S	Hs03928990_g1*	18	GJA1	Hs00173810_m1*
3	NKX2-5	Hs00231763_m1*	19	SRF	Hs00911700_m1*
4	GATA4	Hs00171403_m1*	20	RYR2	Hs00411887_m1*
5	TBX5	Hs00361155_m1*	21	CSQ	Hs00166405_m1*
6	MEF2C	Hs00231149_m1*	22	NPPA	Hs01109515_m1*
7	MESP1	Hs00251489_m1*	23	KIR2.1	Hs00165960_m1*
8	MYOCD	Hs00538071_m1*	24	ALPHA-TM	Hs00165966_m1*
9	ISL1	Hs00158126_m1*	25	CACNA1C	Hs04194727_g1
10	HAND1	Hs00231848_m1*	26	PLN	Hs00892842_m1*
11	HAND2	Hs00232769_m1*	27	KDR	Hs00265315_m1
12	MYH6	Hs00271949_m1*	28	SEMA3A	Hs01579431_m1*
13	ACTC1	Hs00396596_m1*	29	IRX4	Hs00154281_m1*
14	TNNT2	Hs00212560_m1*	30	TBX1	Hs00160179_m1*
15	MYL2	Hs00261238_m1*	31	TBX20	Hs00224610_m1*
16	MYH11	Hs00182371_m1*	32	HOPX	Hs00169795_m1*

Table 3. Genes in Human TaqMan Array Card.

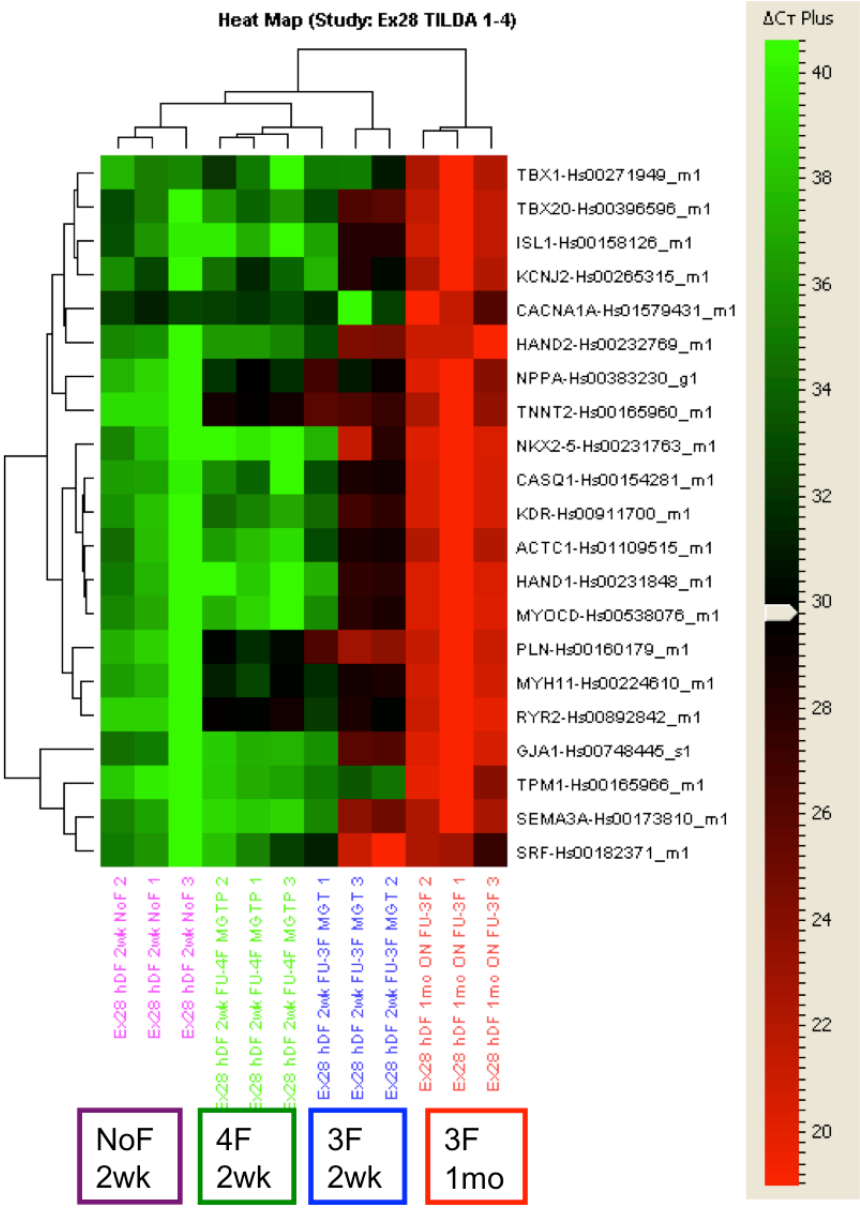

Figure 7. Cardiac expression of MGT and MGTP transduced human dermal fibroblasts. Q-PCR TaqMan array Card assessed expression of cardiac transcription. Expression clusters based on treatment and time.

When comparing MGT induced-cardiac gene expression in hDF (Figure 7) to that in mouse cells (Figure 3) it is evident that the human cardiac gene expression assayed is more globally induced, as indicated by the near global upregulation (bright red) in the 1 month MGT transduced hDFs. We must recall, however, that since we are using lentiviral vectors expressing the human cDNAs of MEF2C, GATA4, TBX5 and MESP1 endogenous expression of these four genes could not be evaluated by Q-PCR in the induced human dermal fibroblasts. High relative expression of a number of critical cardiac transcription factors (figure 8A), sarcomeric proteins (figure 8B) and ion channels (figure 8C) was present in MGT transduced hDFs. Expression was normalized to that of 2 week untreated hDFs.

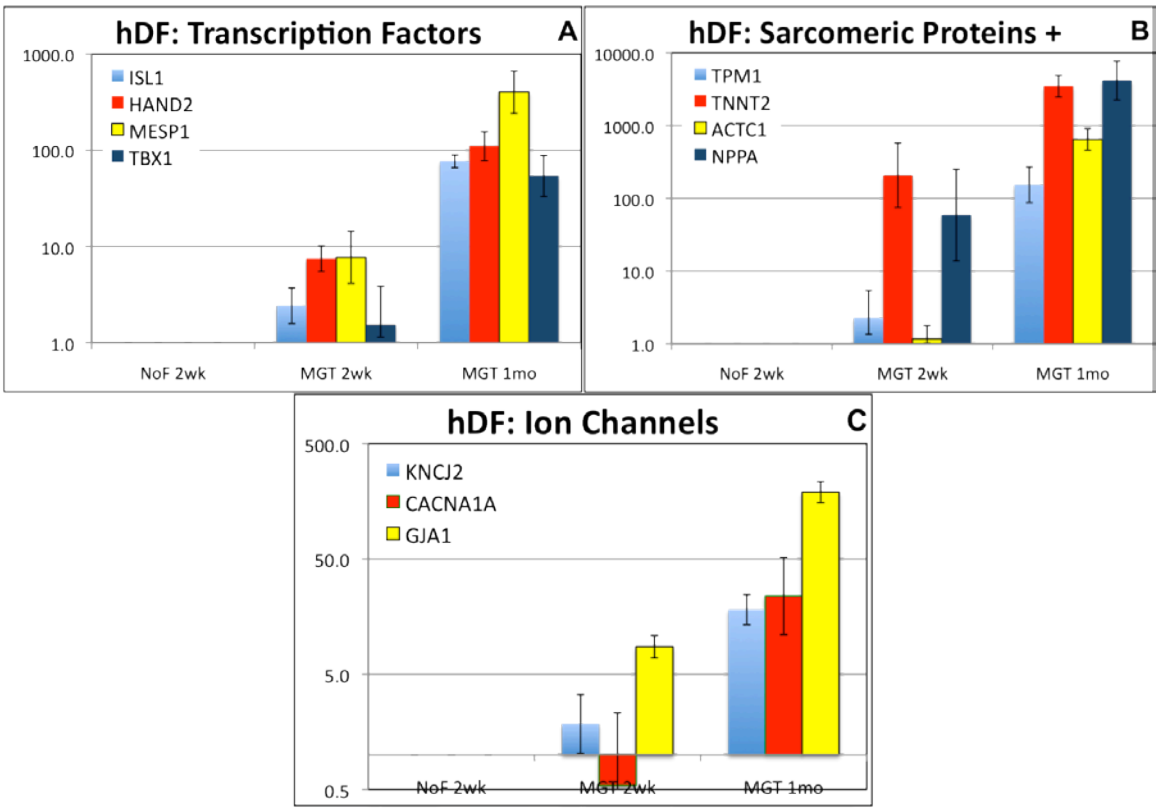

Figure 8. Effect of MGT transduction on hDF at 2 weeks and 1 month. Expression assay with TaqMan arrays and normalized to the untreated hDFs at 2 week. Y-axis displays relative fold expression.

Histologic analysis of hDF revealed cells that were immature and poorly organized, similar to those seen in MGT transduced mouse cells. Alpha-actinin expression can be seen in small number of cells to be organizing in arrayed puncta, as is present in developing sarcomeres (Figure 9). Thickened alpha-actinin+ fibrils are most evident on the short end of these elongated cells.

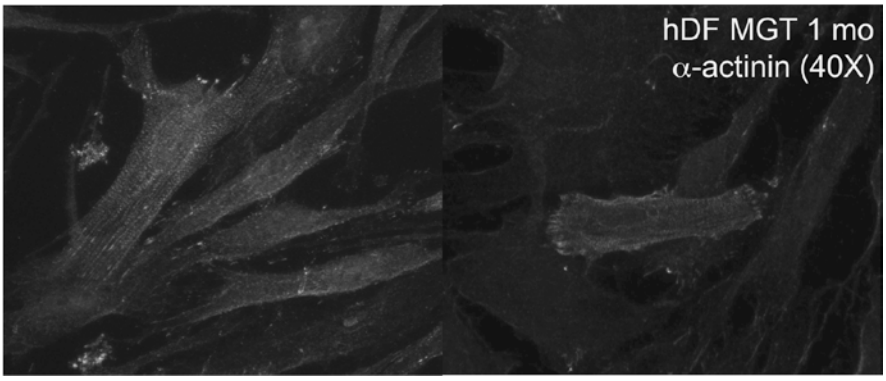

Figure 9. Histological assessment of alpha-actinin expression in MGT transduced hDFs at 1 month.

SUMMARY

The study of heart regeneration and cardiac reprogramming has shown remarkable progress in the last decade. A number of critical events have helped to energize and excite this field. The first was the demonstration of the regenerative capacity of zebrafish hearts. These teleost were able to respond to amputation of the ventricular apex with cardiac regeneration and functional recovery [21]. Analysis of this model has revealed a number of signaling pathways critical to cardiac regeneration including fibroblast growth factor [22], retinoic acid [23], hedgehog, insulin-like growth factor and transforming growth factor-β [24]. Mammalian cardiac regeneration has also been described in 1 day-old neonatal mice, though this capacity is lost by the time mice are 7 days old [25].

The second such event was the elegant confirmation of cardiomyocyte proliferation demonstrated by Bergman et al. based on the elevated levels of worldwide radiation during the period of nuclear testing, which ended sharply with the Limited Nuclear Test Ban Treaty of 1963 (2009). These investigators demonstrated unequivocally that cardiac myocyte proliferation occurred in an aged-dependent manner, such that a 20 year-old had cardiomyocyte yearly proliferation rate approaching 1% while the rate were closer to 0.4% in a 75 year-old [26]. Unfortunately, given the unique use of accumulated radiation as basis for their measurements no assessment of the effect of cardiac insult on proliferation could be obtained. Of note, data from mouse experiments suggests that myocardial injury can give rise to de-novo cardiomyocyte formation in the adult heart and that this process can be potentiated with thymosine-β4 supplementation [27].

The third such event was the description of pleuripotent reprogramming by ectopically express factors in mouse [8], and soon thereafter in human cells [28,29]. This protocol has been quickly adapted to cardiac reprogramming and resulted in a number of strategies, including ours, to generate cardiac myocytes directly from somatic cells. The first such publication describes the use of virally transduced Mef2c, Tbx5 and Gata4 to generate cardiomyocytes from fibroblasts [19]. Investigators admit that the efficiency of this protocol is approximately 1 beating cell per 1 X 10^6 cell transduced (personal communication). Thus while this is an effective way to induce the cardiac transcriptional program it yields poor functional outcomes. Furthermore this report has been the source of significant controversy [30]. More recently additional induction in-vivo with MGT+Hand2 (MGTH) has been shown to be able to ameliorate the extent of scarring following myocardial infarction and to improve functional outcomes in these mice, though in-vitro reprogramming remained markedly inefficient [31]. A similar capacity to ameliorate myocardial infarction in mice was also described by MGT alone and potentiated with the proangiogenic molecule T-β4 [32]. Transfection of a miRNA cocktail containing miR-1, miR-133, miR-208 and miR-499 mimics was also sufficient to induce cardiac reprogramming in-vitro and in-vivo following myocardial infarction in mice [33]. High-jacking the process of iPS formation [34] demonstrated efficient reprogramming to cardiomyocytes by limiting the induction time period of the reprogramming factors, abrogating leukemia inhibitory factor (LIF) supplementation and treating cells with low-serum defined media that included BMP4 supplementation and JAK-STAT inhibition. Resulting cells were up to 40% cTnT+ and showed characteristic beating and calcium transients [34]. Notably, JAK inhibition also markedly increased the reprogramming efficiency of miRNA-reprogramming [33].

Our experiments have demonstrated the capacity of MGT to efficiently induce cardiac transcription in mouse embryonic and cardiac fibroblasts. Furthermore we have evaluated supplementation by a number of small molecules and additional candidate genes. We have demonstrated that supplementation of MGT with BMP4, RG108 and Gata5 result in improved transcriptional induction and histological organization of sarcomeric proteins. A critical element lacking from the work in this field has been an efficient way to measure physiologic outcomes in reprogrammed cells. Ongoing work in our laboratory has demonstrated such capacity by labeling induced cells with a calcium-calmodulin/GFP fusion (GCaMP) reporter (Addis et al. Submitted), which responds to increasing intracellular calcium concentrations by fluorescing and vice-versa [35]. This is a significant advance. Consequent reprogramming efforts can now be undertaken using this physiological metric as a means to modulate reprogramming protocols. Finally we have demonstrated the capacity of human dermal fibroblasts to undergo limited cardiac induction with MGT ectopic expression. Consequent studies will focus on using the functional GCaMP reporter to optimize both mouse and human cardiac reprogramming.

These studies represent a significant contribution to the ultimate goal of clinical import of the current strategy: to be able to induce cardiac cellular and functional recovery by direct reprogramming of fibroblasts to cardiomyocytes. In this regard reprogramming to a multipotent, proliferative progenitor would be preferred, as it could potentiate regeneration and help restore local architecture with the necessary vasculature and supporting cells.

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Reprogramming to blastomeres

The goal of this research is to determine the capacity of known maternal-effect genes and oocyte-specific maternal genes encoding putative transcription factors to reprogram mouse embryonic stem cells to 2-cell blastomeres.

We identified a retrotransposon, Erv4/MuERV-L that is specifically expressed in mouse pre-implantation embryos and found that it is highly expressed in 2-cell (2C) embryos with reduced expression by the 8-cell stage. Quantitative RT-PCR analysis to determine the relative expression of Erv4 in various stages of preimplantation development as well as mouse embryonic stem cells (mESCs) revealed Erv4 is not detected in blastocyst staged embryos or in the mESC lines analyzed, but is highly expressed in 2-cell embryos, making Erv4 an ideal marker of the 2-cell stage embryo. We generated a lentiviral reporter construct with the Erv4 5'LTR and a portion of the Gag promoter driving expression of green fluorescent protein (GFP), to enable identification of cells with putative blastomere characteristics following treatment with lentiviral vectors containing the candidate reprogramming factors described below.

To induce blastomere-like cells, we transduced mESCs with combinations of mRNAs of the blastomere specific factors sub-cloned into the doxycycline inducible lentiviral vector FU-TetO-GW. These mRNAs are transcription factors specifically up-regulated in blastomeres compared to later pre-implantation stages and mESCs. This list includes, Ctcf, Hsf1, Bnc1, Tbx3, Tbx4, Obox1, Obox3 Obox5, Lhx8 and the zinc finger proteins (Zfp 1, 99, 108, and 386). At the time of factor transduction we also transduced the Erv4-GFP reporter lentivirus. We transduced the transcription factors listed above in a variety of combinations, however we have not observed either up-regulation of the Erv4-GFP reporter or any phenotypic or morphological changes indicative of a change towards the blastomere fate.

Evidence suggesting factors found in the egg cytoplasm shown to be key players in the reprogramming of nuclei during somatic cell nuclear transfer could be important in reprogramming mESCs towards a 2C-like state were also explored. Histone H1foo, responsible for the replacement of histones on donor nuclei during reprogramming, and histone H3.3, a variant of the core histone H3, specifically up-regulated in oocytes and related to transcriptional up-reguation of key pluripotency/totipotency genes, are two such

factors. We sub-cloned cDNAs representing histones H1foo and H3.3 into lentiviral vectors to force expression as described above. Addition of these factors to the 'reprogramming mix' did not up-regulate expression of the Erv4-GFP reporter or induce any morphological changes. Identification of a third histone, macroH2A, highly expressed in mESCs but not in 2C blastomeres led us to believe inhibition of this histone in mESCs may enable initialization of the reprogramming process. To that end, we used siRNAs to attempt to suppress macroH2A expression in mESCs however after repeated trials could not sufficiently inhibit expression of macroH2A in mESCs.

While the primary role of this research is to reprogram a mouse embryonic stem cell back to a 2-cell like blastomere, a process that is putatively controlled by factors in the maternal germ-line from the oocyte cytoplasm, we also attempted to reprogram somatic cells to the male germ lineage by using factors involved in maintenance and self-renewal of the spermatogonial stem cells (SSCs) located in testis. As in the experiments described above, we forced expression of specific SSC transcription factors using the doxycycline inducible lentiviral vector, FU-TetO-GW to covert somatic cells to spermatogonial stem cells. We chose factors previously defined as important for SSC establishment or maintenance, including Bcl6b, Etv5, Id4, Klf4, Myc, Ngn3, Oct6, Sohlh1, Sohlh2, and Sox2, to transduce to primary mouse embryonic fibroblasts (MEFs). MEFs were also transduced with a lentivirus bearing a GFP reporter under the control of the Dazl promoter, a gene that has been shown to be up-regulated specifically in the primary spermatogonia of the testis. Transduced and non-transduced MEFs were placed in media containing growth factors known to support germ cell development in vivo for 6 days, and then transitioned to media known to support long-term culture of SSCs. One specific combination of 4 factors is sufficient to induce morphological changes in MEFs consistent with SSCs in culture with cells growing as indicative 'clump-forming' cells that express the Dazl-GFP reporter (FIG 1). Further, analysis of transduced cells for germ cell specific proteins by immunocytochemistry, including DAZL, DDX4, and PLZF (FIG 2) and gene expression analysis by quantitative RT-PCR indicates these cells have up-regulated germ cell specific genes (FIG 3). Further analysis of these putatively reprogrammed cells revealed they maintain SSC-like morphology for over 20 passages, maintain expression of the proteins described above and continued expression of the Dazl-GFP reporter. Based on down-regulation of Dazl-GFP reporter, these cells are also dependent on glial-cell line derived neurotrophic factor (GDNF), a factor shown to be necessary for the maintenance and proliferation of SSCs both in vivo and in vitro.

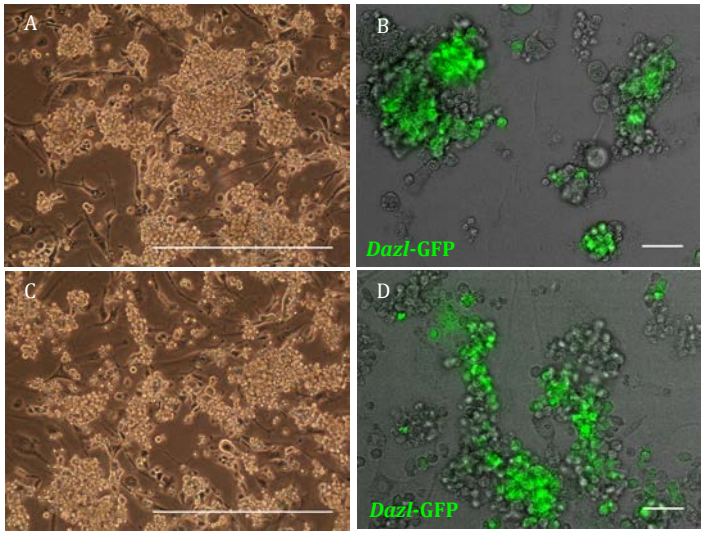
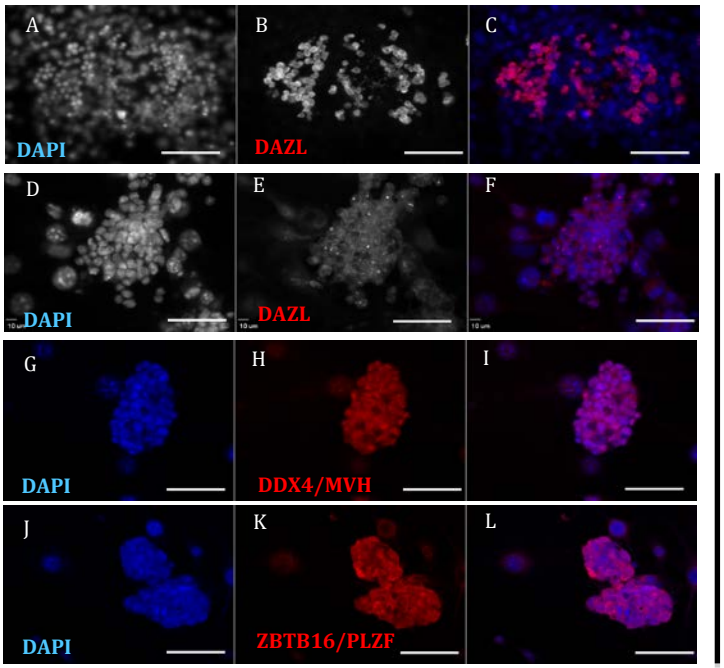


FIG 1. MEFs transduced with 4 factor combination exhibit clump-forming morphology at passage 14 (*A*) and express the *Dazl*-GFP reporter (*B*) and maintain this morphology and reporter expression out to passage 26 (*C*, *D*).



SSC

4Factor

FIG 2. SSC marker expression in 4 Factor reprogrammed cells. A. DAZL expression in cultured SSCs explanted from testis (*B)*. Expression of DAZL, DDX4, and PLZF in 4 factors transduced pMEFs reprogrammed to resemble SSCs morphologically (*E*, *H*, *K)*. DAPI labeling nuclei (*A*, *D*, *G*, *J)* and overlay of DAPI (*C*, *F*, *I*, *L*).

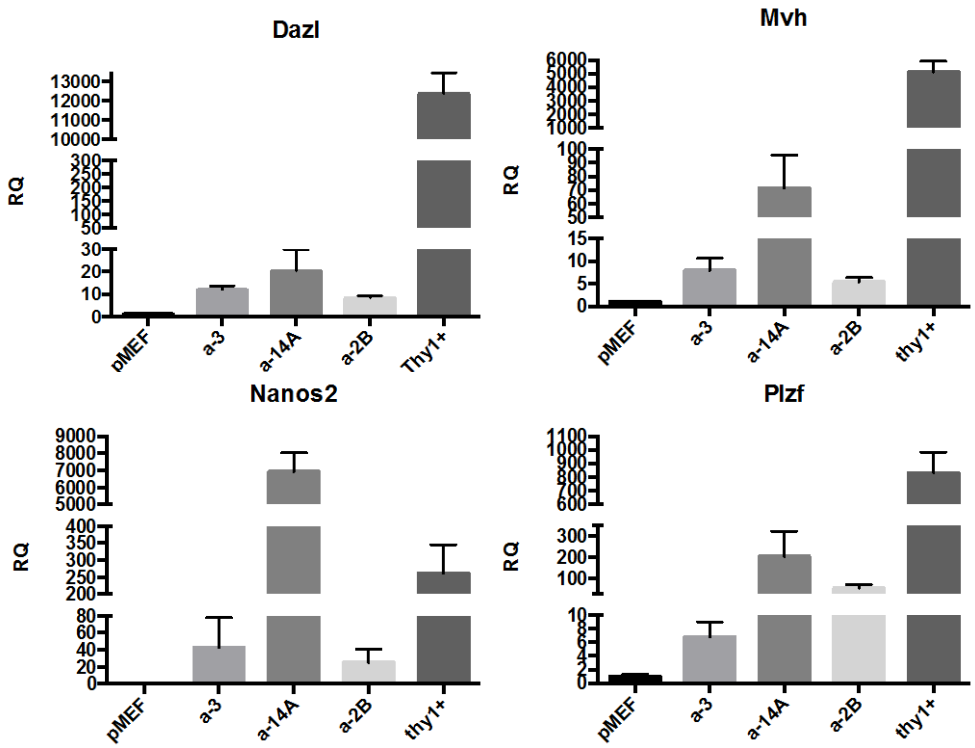


FIG 3. Quantitative RT-PCR for iSSC marker expression in 4 Factor reprogrammed cells. Fold change (RQ) for germ cell specific genes, *Dazl*, *Mvh* (*Ddx4*), and *Plzf* in 4 factors transduced pMEFs reprogrammed to iSSCs. Relative expression to starting pMEF population is shown for three representative clones (a-3, a-14A, a-2B) and Thy1 enriched testicular germ cells (SSCs).

Poster Presentation: Contribution of Specific Transcription Factors in the Reprogramming of Mouse Somatic Cells Towards Spermatogonial Stem Cells. LD Kellam and JD Gearhart. International Society for Stem Cell Research Annual Meeting 2011.

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. None				☐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:

This work will be published in a high visibility journal upon completion, which is expected this coming Fall. Anytime we have to demonstrate functional cells upon grafting into animal models, it takes time.

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.

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