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:** 3-Generating Tissues from Stem Cells in the Pathways to Therapy

7. **Start and End Date of Research Project:** 1/1/2010 – 12/31/2012

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

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:

   $ 682,557.54

   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
Chen, Christopher	Co-Investigator	5%	$ 9,486.00
Engleka, Kurt	Sr. Research Investigator	50%	$22,434.21
Epstein, Jonathan	Co-Investigator	3%	$21,647.61
Hwang, Ha Young	Postdoctoral Researcher	100% Yrs 1–2	$32,439.19
Irshad, Allana		100%	$ 1,036.75
Li, Deqiang	Postdoctoral Researcher	100% Yrs 2–3	$54,147.00
Li, Li	Research Specialist	35%	$25,724.95
Manderfield, Lauren	Postdoctoral Researcher	3%	$ 5,705.72
Margulies, Kenneth	Co-Investigator	5%	$22,128.66
Yang, Jifu	Research Specialist	35%	$42,860.32

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%
Morrisey, Edward	Co-Investigator	5%
Mu, Anbin	Research Specialist	10%
Boudou, Thomas	Postdoctoral Fellow	25%

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____X____ No_____

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

American Heart Association – Jon Holden DeHaan Research Centers Grant - $2,000,000

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___X____ No_____

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:
Epigenetic regulation of lung repair and regeneration	☒NIH ☐ Other federal (specify:_____) ☐ Nonfederal source (specify:_)	6/11 (awarded 12/11)	$2,500,000/ over 5 years	$2,500,000/ over 5 years
Cardiac Microtissue Force System for Preclinical Drug Screening and Toxicity Assessment	☒NIH ☐ Other federal (specify:_____) ☐ Nonfederal source (specify:_)	4/13	$196,409	$196,409

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 on pursuing NIH funding to continue the work done on each of the aims.

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

We will continue to develop and utilized the cardiac microtissue model for preclinical drug screening and mechanistic inquiries related to myocardial biology and myocardial

regeneration/myogenesis.

We will continue to assess the role of miR302-367 in lung regeneration and determine whether small molecular mimic versions can be used to promote regeneration in vivo.

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				
Non-Hispanic				2
Unknown				
Total				**2**

	Undergraduate	Masters	Pre-doc	Post-doc
White				1
Black				
Asian				1
Other				
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_____ 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____X____ No_____

If yes, please describe the collaborations:

The health research fund support helped initiate new collaborations between investigators from the School of Medicine and the School of Engineering at the University of Pennsylvania.

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

Yes____X____ No_____

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

A new startup company, Innolign Biomedical, LLC was initiated during the course of funding from the health research funds.

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

Yes_____ No_____X____

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

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

List the project goals, objectives and specific aims (as contained in the grant agreement). Summarize the progress made in achieving these goals, objectives and aims for the period that the project was funded (i.e., from project start date through end date). Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was

submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

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

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

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

Aim 1: Tissue fabrication for regenerated myocardium.

1A: Adapt existing techniques to enable manipulation of biomechanical stress in tissue arrays and evaluate the effects of static and cyclic stress on fabricated myocardial tissues.

Work on Aim 1A has been completed and published in the following manuscript: Boudou, T. et al. A microfabricated platform to measure and manipulate the mechanics of engineered cardiac microtissues. Tissue Eng Part A. 2012 May; 18(9-10):910-9. We inadvertently failed to acknowledge CURE funding in the manuscript when it was first published. We have contacted the publisher to correct this error. The online version of the article is being revised to include CURE Program as a funding source, and a correction page will be included in the May issue of Tissue Engineering, Part A. We will make sure that CURE funding is appropriately cited in all other future publications stemming from this research.

In this work we showed that engineered myocardial tissues can be used to elucidate fundamental features of myocardial biology. We used microelectromechanical systems technology to generate arrays of cardiac microtissues (CMTs) embedded within three-dimensional micropatterned matrices. Microcantilevers simultaneously constrain CMT contraction and report forces generated by the CMTs in real time. We were able to routinely

produce ~200 CMTs per million cardiac cells (<1 neonatal rat heart) whose spontaneous contraction frequency, duration, and forces could be tracked. Independently varying the mechanical stiffness of the cantilevers and collagen matrix revealed that both the dynamic force of cardiac contraction as well as the basal static tension within the CMT increased with boundary or matrix rigidity. Cell alignment is, however, reduced within a stiff collagen matrix; therefore, despite producing higher force, CMTs constructed from higher density collagen have a lower cross-sectional stress than those constructed from lower density collagen. We examined the effect of electrical stimulation on cell alignment and force generation within CMTs and found that the combination of electrical stimulation and auxotonic load strongly improves both the structure and the function of the CMTs. We provided proof-of-principle for the use of our technique for high-throughput monitoring of drug-induced changes in spontaneous frequency or contractility in CMTs as well as high-speed imaging of calcium dynamics using fluorescent dyes. Together, these results highlight the potential for this approach to quantitatively demonstrate the impact of physical parameters on the maturation, structure, and function of cardiac tissue and open the possibility to use high-throughput, low volume screening for studies on engineered myocardium. This work was performed using neonatal rat ventricular myocytes.

More recently, we have explored the possibility of deriving cardiac precursors for use in tissue fabrication from alternative sources. One potential source would be to reprogram fibroblasts (connective tissue cells) to adopt cardiac muscle precursor characteristics by expressing defined factors. In 2010, Ieda et al. published that 3 transcription factors could convert fibroblasts to myocardial cells in vitro (Cell, 142: 375-86, 2010). Therefore, the Epstein and Gearhart laboratories worked to optimize this process and to determine if 3D tissue constructs would promote more mature differentiation of directly reprogrammed cells. Our initial results were recently published: Addis RC, Ifkovits JL, et al. Optimization of direct fibroblast reprogramming to cardiomyocytes using calcium activity as a functional measure of success. J Mol Cell Cardiol. 2013 Jul;60:97-106.

1B: Determine whether co-culturing of human c-kit+ cardiac progenitors cells with neonatal rat cardiac myocytes in fabricated multicellular constructs promotes their maturation and integration.

Aim 1B focused on the use of human c-kit+ cardiac progenitors. Our attempts along these lines have not been successful to date since we have not observed adequate incorporation of c-kit+ progenitors into contracting cardiac microtissues and we have abandoned this approach.

1C: Determine the morphological and functional characteristics of fabricated multicellular constructs derived exclusively from iPS cells, fibroblasts and collagen.

Aim 1C. The goal of this aim was to evaluate the use of pluripotent stem cells (iPS) in combinations with mesenchymal stem cells, fibroblasts and collagen in the micro-tug experimental design.

As noted during last year's progress report, initial studies employed iPSC-derived cardiac

myocytes (iPS-CM), fibroblasts and mesenchymal stem cells (MSCs) in varying proportions to form functional cardiac micro-tissues (CMTs). In these trials, use of fibroblasts with iPS-CMs was associated with excessively high static stress and low tissue stability. CMTs from iPS-CMs and MSCs displayed very good stability with the routine production of >100 CMTs form 106 cells. Moreover, a mix of 75% iPS-CM/25% MSC had the highest active (systolic) tension development, though this was still far below the level of static (diastolic) tension. As with prior studies with CMTs derived from neonatal rat cardiac myocytes, we observed that physical parameters (stiffness of the cantilevers on which CMTs form) affected the active and static force generation, rate of maturation and structure of the human CMTs. Further phenotyping of functional responses demonstrated that there was a negative force-frequency response and no positive inotropic response to isoproterenol; and these findings suggest that the CMTs are still functionally immature at the 7-10 days after cell seeding.

Aim 2: Building cartilage and tendon from equine induced pluripotent stem cells (EqiPSCs). We generated and characterized Induced pluripotent stem cells from horse somatic cells (part 1). We also showed that the EqiPSCs could be successfully differentiated into cells of connective tissues such as bone and cartilage and potentially into tendon-like cells (part 2). We were able to show this capability within the time frame of the grant and we look forward to pursue collaborations with Dr. Robert Mauck and Dr. Kurt Hankenson, whom have expressed interest in using the EqiPSCs in translational applications for the horse, maintaining a fruitful collaboration between the Schools of Medicine, Veterinary and Bioengineering.

Below is the work done for Parts 1 and 2 of this aim.

Reprogramming of Horse somatic cells with a single vector

In order to generate pluripotent stem cells from Equine somatic cells we used a polycystronic lentiviral vector expressing the four transcription factors of mouse origin Oct4, Klf4, Sox2 and c-myc [Sommer et al., Stem Cells, 2009] (Figure 1A). The cDNAs are encoded under the constitutive promoter E1Fα and separated by the elements F2A, IRES and E2A, respectively. Dermal fibroblasts (DFs) from a 2 year-old male (Figure 1B) as well as mesenchymal stem cells (MSCs) from a 7 months old female embryo were used for reprogramming (not shown). One day after transduction with STEMCCA lentiviruses the cells were dissociated and transferred to a plate of irradiated mouse embryonic fibroblasts (MEFs) in media containing LIF (Figure 1A). Colonies resembling ES-like cells were visible within 5 days (Figure1C) and picked and dissociated by day 10 using trypsin supplemented with the Rock Inhibitor Y-27632. Established colonies showed a ESCs-like morphology similar to iPSCs and ESCs of other species, with tight cells and bright edges (Figure 1D). All the clones picked that expanded beyond passage 5 were tested for the expression of the transgenes. In Figure 1E we observe that both Oct4 and c-myc were present in all the lines run in parallel with the founder cells and the STEMCCA positive control. In addition we confirmed the correct number of chromosomes for several of these iPS cell lines by preparing chromosome spreads, which showed either 62XX or 62XY karyotypes depending on which cell source was used for reprogramming (Figure 1F and not shown). The EqiPS were passaged every 2-3 days and kept expanding for periods up to 6 months with no apparent loss of pluripotency maintaining

a stable karyotype and their morphological characteristics, demonstrating true self-renewal capacity *in vitro*.

Horse iPSCcells exhibit markers expression similar to pluripotent stem cells

We followed up on the characterization of the EqiPSCs by testing the expression of hallmark pluripotency genes by immunofluorescence. As we observe in Figure 2, the EqiPSCs express OCT4, NANOG, LIN28, SSEA1 and SSEA4 for the derived DF1 line (Figure 2A), using antibodies that recognize the horse proteins. While we cannot neglect the fact that the Oct4 antibody may be detecting the expression of the exogenous mouse Oct4, we do see upregulation of the OCT4 expression levels by RT-PCR when using primers specifically designed for detection of the horse sequences (Figure 2B). We used a mES line and confirmed that these primer sets were not able to detect expression of the mouse Oct4 (data not shown). Moreover, we also see increased expression of NANOG and LIN28, REX1 and SOX2 at the transcriptional level. Figure 2C shows the increased expression of OCT4 and SSEA1 as seen by FACS analysis for the line DF1, while the founder cells (DF) and feeders were negative.

Teratoma

To evaluate the pluripotency of EqiPSCs we tested the ability of the EqiPSCs to differentiate into cells from the three germ layers, both *in vivo* and *in vitro.* Approximately 3 million cells were injected subcutaneously in the flank of immune-deficient NSG (NOD SCID Gamma common chain null) mice. The injected mice developed tumors, which were ready for harvesting within 8 to 12 weeks, at which point they were dissected and frozen for tissue histology analysis. Represented in Figure 3A-E is a teratoma from the DF1-EqiPSC line. The tumors contained different types of cells as observed with H&E staining (Figure 3A-B) and include cells from mesoderm, positive for Desmin (Figure 3C); endoderm, positive for Sox17 (Figure 3D); and neuroectoderm, expressing TUBB3 (Figure 3E). We confirmed the horse origin of these tumors by using antibodies against CD44 and CD90 that recognize horse cells but not mouse (not shown). Immunodeficient mice injected with similar numbers of DFs and MSCs failed to form tumors.

***In vitro* differentiation: neuroectoderm**

In order to determine the *in vitro* pluripotency of the EqiPSCs, we directed these cells to differentiate into different lineages and specific cell types. To induce neurogenesis in the pluripotent stem cells, we used a protocol established by Kawasaki [Kawasaki et al., PNAS, 2002] in which culturing mouse embryonic stem cells on PA6 cells was shown to induce the differentiation into neural cells. We followed the same protocol and after 2 weeks in culture, the DF1-EqiPSC line expressed high levels of tubulin beta 3 (TUBB3), a marker of neuronal cells, as seen by immunofluorescence (Figure3F). Moreover we were able to identify neurite outgrowths (detail image) from these cells indicating a specialization of the differentiated EqiPSCs into neuronal cells.

***In vitro* differentiation: endoderm**

We also tested the ability of these cells to proceed through endodermal lineage by inducing their differentiation into hepatocytes. For that purpose we added HGF and FGF4 to the culture media [Reed and Johnson, J. Cell Physiol., 2007]. After 2 weeks in this culture

media, the cells were fixed and expression of endodermal specific markers was assessed. Figure 3G-I shows the expression CD34, SOX17 and FOXA2, respectively.

In vitro differentiation: mesoderm

We then tested the ability of these cells to differentiate into more specific cell types with potential use in horse therapies. For that purpose we used a protocol that has been optimized for the differentiation of human MSCs into adipocytes. The cells were fed every 2-3 days with 3 cycles of induction/maintenance adipogenic media, in which the induction media includes dexamethasome, indomethacin and 3-isobuty-l-methyl-xanthine. At the end of the induction, the cells were fixed and the formation of oil droplets was observed, as determined by Oil red O staining (Figure 3J). EqiPSCs were induced to osteogenic lineage by treatment with dexamethasone, ascorbate and β-glycerophosphate for three weeks. Mineral and calcium deposits visualized by Alizarin Red and Von Kossa stainings, respectively, confirmed ossification (Figure 3K-L). These areas of strong mineralization were not observed in non-osteogenic induced cultures (insets, Figure 3K-L). These results show that EqiPSCs can be successfully differentiated into fat and bone cells, as well as cells from the neuroectoderm and endoderm lineages *in vitro*.

Direct differentiation of EqiPSCs into Chondrocytes

Equine iPS cells could be an unlimited source of cells for cartilage repair, as large number of cells can be quickly obtained. MSCs are usually induced to chondrogenesis by aggregation, or micromass cultures, and exposed to transforming growth factor–β (TGF- β). Recent studies have shown that human embryonic stem cells could differentiate into the chondrogenic lineage either by a progressive differentiation protocol as well as by a direct method using micromasses [Gonget al., J. Cell Physiol. 2010; Oldershaw et al., Nat. Biotechnol., 2010]. We followed up on the direct differentiation of EqiPSCs by using high density micromass cultures treated with TGFβ3 to undergo direct differentiation into chondrogenic lineage. We applied this protocol to feeder-depleted cultures of the DF1-EqiPSC line and allowed cells to differentiate for 3 weeks. We included in this differentiation analysis primary chondrocytes (Chs) from horse cartilage and the parental DFs, to better evaluate the potential of the EqiPSCs to differentiate into cartilage cells. After 21 days, the micromasses were fixed, paraffin embedded and sectioned. Acidic polysaccharides, a major component in mature cartilage, are easily identified by a bright blue color in tissues stained for Alcian Blue (Figure 4A). The differentiated DF1 line shows equivalent expression of this matrix component to the Chondrocytes (Figure 4B), while DFs failed to yield significant levels of proteoglycans (Figure 4C), indicating a diminished capacity to differentiate into chondrocytes. Freshly cut sections from the DF1 and Chs micromasses were used to extract RNA, and several markers associated with chondrogenesis were evaluated by qRT-PCR (Figure 4D) and compared to the undifferentiated samples from day 0. SOX9, a transcription factor, which is highly expressed in early chondrogenesis was still upregulated after 3 weeks of differentiation. Expression of COL2A1, an extracellular matrix (ECM) protein specific to cartilage, and ACAN, a non-collagenous ECM protein present in more mature cartilage, also increase with differentiation. Col10A1 is associated with hypertrophy, indicating differentiation along an endochondral ossification. At the end of differentiation, the cells did not express the pluripotency markers Oct4 and Nanog (not shown).

Expression of tendon related genes in TGFb3 induced EqiPSCs

When a tendon injury occurs, there is formation of scar tissue, which increases the chance of recurring injuries. In current treatments, stem cells, mostly MSCs are injected directly into the site of injury [Paris and Stout, Theriogenology, 2010], with the anticipation that with the right cues and signaling these cells will differentiate into tendon cells *in situ*. Still, the risk of re-occurring injury is high, so, it is of paramount importance to be able to understand better the tendon differentiation so other strategies can be employed. MSCs and other adult stem cells, such as adipocyte stem cells and umbilical stem cells, have been shown to express markers characteristic of tendon fibroblasts, when BMP12, BMP14, or other members of the TGFβ family are added to the culture media. In rat, it has been shown that GDF5 (BMP14) improved the healing of damaged tendon [Rickert, Growth Factors, 2001]. More recently another study showed that TGFβ3 improved the expression of tendon associated markers in embryo-derived stem cells [Barsby and Guest, Tissue Eng. Part A, 2013]. In our study we used both TGFβ3 (20ng/µl) and GDF5 (100ng/µl) growth factors to determine if our EqiPSCs would differentiate into tenocytes along with control sample where no growth factor was added. Scleraxis, a transcription factor important during tendon development showed increased expression with time of differentiation, but this increase was not significantly different between the different conditions, as observed by RT-qPCR analysis (Figure 5A). Other tendon associated markers, such as the type I Collagens (Col1A1 and Col1A2), a collagen abundant in tendons, as well as Tenascin C (TNC), and Decorin (DCN), show increased expression when compared to the control samples, at the transcriptional level (Figure 5B). At day 7 the fold change is maximum for COL1A1/2 and TNC. Supporting this result, protein analysis by western blots (Figure 5C) also show increased expression of Tenascin C both at day 7 and day 14 (1.4 and 1.5 times, respectively), when TGFβ3 is added to the culture media, while Decorin expression is not significantly different across conditions. Immunofluorescence at day 7 show a qualitative increased expression of Col1A2 as seen in Figure 5D, for both TGFβ3 and GDF5 induced cells. We also observe expression of Decorin, Tenascin C and Tenomodulin (Tnmd, tendon specific marker), at day 7 of differentiation (Figure 5D). These results indicate that TGFb3 promotes the expression of tendon related genes when added to cultures of EqiPSCs.

Our results suggest that EqiPSCs can be used as a powerful alternative to MSCs to generate osteocytes, chondrocytes and tendon-like cells thus demonstrating the potential use of these cells for the treatment of damaged bones and cartilages in horses.

Figure 1 - Generation of Equine iPSCs

A. Time frame of the generation of EqiPSCs. Established colonies were then used in the subsequent studies. **B**. Morphology of dermal fibroblasts (DF) from a 2 year old thoroughbred gelding, used for the generation of EqiPSCs. **C.** Emerging colonies with ES-like morphology (arrows) at day 5. **D.** Morphology of an established EqiPSC clone (DF1-EqiPSC) after 5 passages. **E.** Expression of Oct4 and c-myc of mouse origin from the polycistronic transgene STEMCCA in established EqiPSCs clones DF1-DF5. Also shown the expression of the endogenous controls GAPDH (horse specific) and 18S (recognizes horse and mouse). **F.** Chromosome spreading of the clone DF1, stained with DAPI. The DF1-EqiPSC line shows a normal 62 XY karyotype. Inset: chromosome arrangement as described by Richer [Richer et al., Hereditas, 1990]. Scale bars represent 200µm (B-D) and 10µm (E).

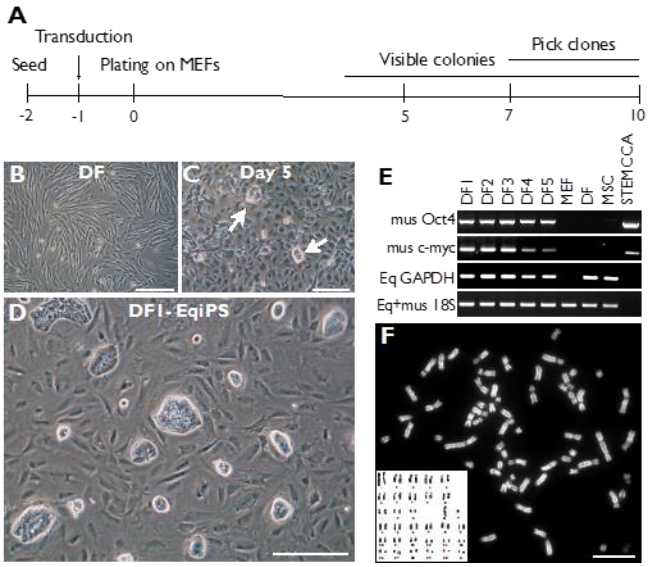


Figure 2 - Characterization of EqiPSCs

A. Immunofluorescence staining of the clone DF1 with pluripotent markers OCT4, NANOG, LIN28, SSEA1 and SSEA4, counterstained with DAPI. Scale bars represent 50µm. **B.** Fold increase by RT-qPCR of horse pluripotent genes OCT4, NANOG, SOX2, LIN28 and REX1 in the clone DF1 relative to parental DFs. Shown are mean ± SD. **C.** FACS analysis of OCT4 and SSEA1 expression for the clone DF1, the parental DF and feeders. In the upper right corner, staining with isotype control IgG (green).

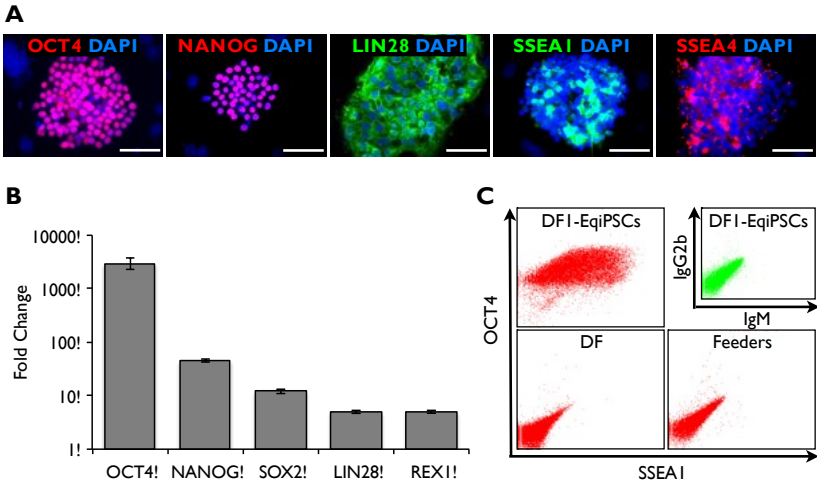


Figure 3 - EqiPSCs differentiation *in vivo* and *in vitro*

A-E. Teratoma formation for the clone DF1. Hematoxylin and eosin staining (**A**, **B**) and immunohistochemical staining (**C-E**). The teratoma contained cell types representing all three germ layers. Markers used were DESMIN as a mesodermal marker (**C**); SOX17 as an endodermal marker (**D**) and BIII TUBULIN as a neuroectodermal marker (E). Sections were counterstained with DAPI.

F-L. EqiPSCs were directed to differentiate into specific lineages *in vitro*. For neuronal induction, the differentiated cells were stained with a BIII TUBULIN antibody. For endodermal lineage induced cells were stained with CD34 (**G**), SOX17 (**H**) and FOXA2 (**I**)) antibodies. Cells were counterstained with DAPI. **J.** Adipogenic differentiation of DF1 clone. Oil Red O staining was used to visualize the formation of oil droplets. **K-L.** Differentiation of the clone DF1 into osteoblasts. Mineral and calcium deposits confirmed ossification by staining with Alizarin Red (**K**) and von Kossa (**L**) stainings, respectively. These areas of strong mineralization are not observed in non-osteogenic induced cultures (insets, lower left corners). Scale bars represent 400 µm (A, B) and 50 µm (C-E), 100µm (F-I, K) and 25µm (J,L).

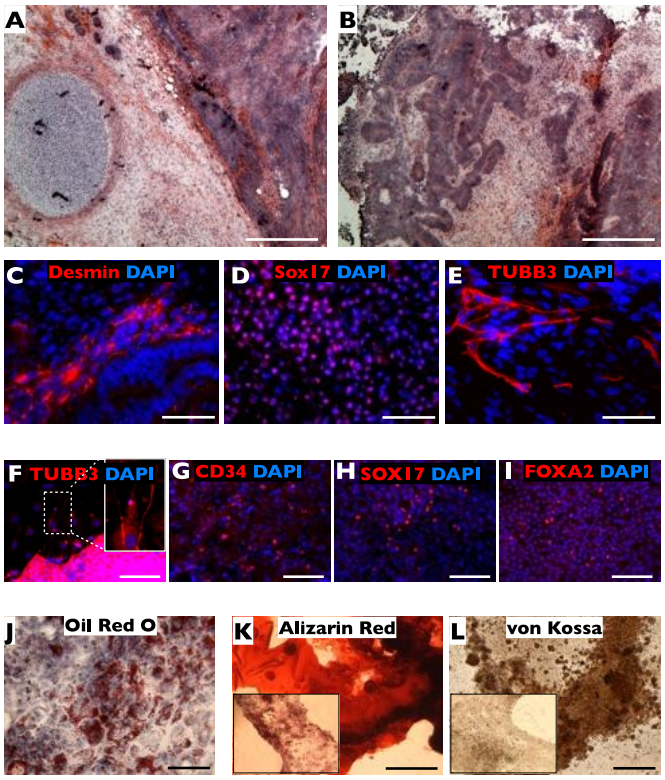


Figure 4 - Differentiation of EqiPSCs into Chondrogenic lineage

Chondrogenesis was confirmed by Alcian Blue staining for DF1 (**A**), chondrocytes (Ch, **B**) and parental dermal fibroblasts (DF, **C**). Cells were counterstained with Nuclear Fast Red. Scale bars represent 200 µm. **D**. Fold change by RT-qPCR for the expression of chondrogenic marker genes from the pluripotent state (day 0) to chondrocytes at day 21 of differentiation. Shown are Sox9, COL2A1, ACAN and COL10. Data shown are mean ± SD.

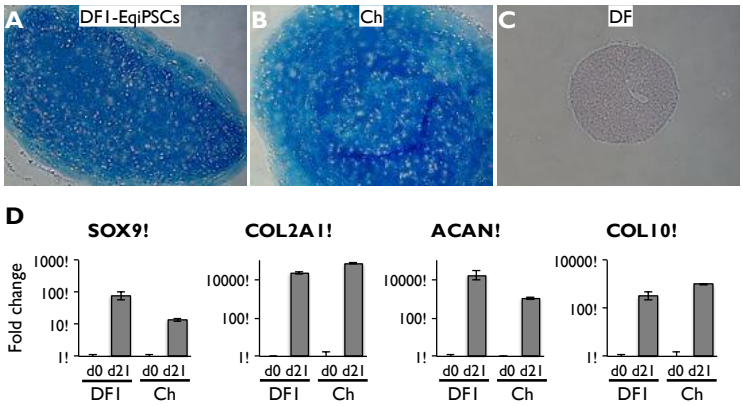
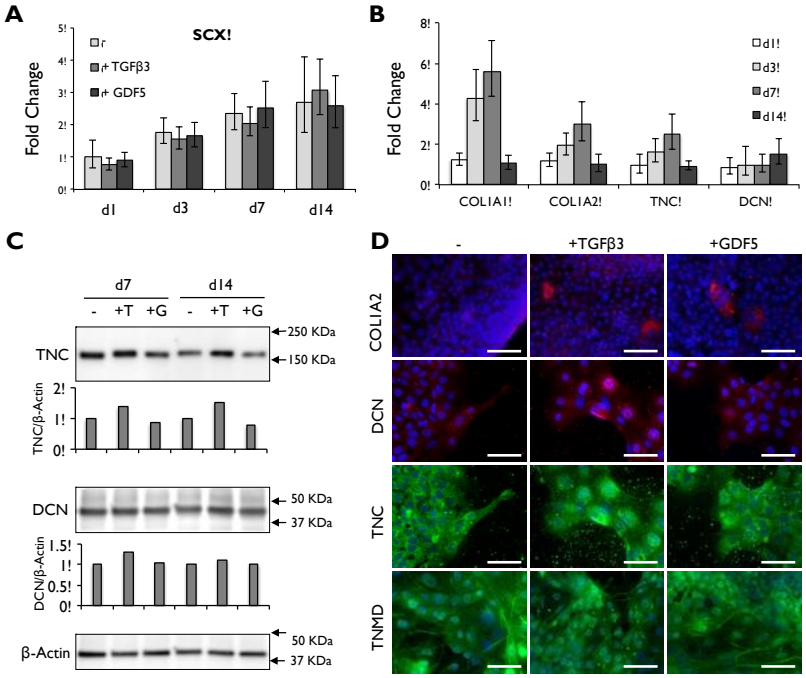


Figure 5 - Differentiation of EqiPSCs into Tenogenic lineage

A. Increased scleraxis expression by RT-qPCR for DF1 cells when induced with TGFβ3 and GDF5 or allowed to spontaneously differentiate (-). Control condition at day 1 was used as reference. **B**. Fold change by qRT-PCR for the expression of the tenogenic marker genes COL1A1, COL1A2, TNC and DCN with time for TGFβ3 induced differentiation, using control samples as reference. The data show the average of 3 experiments and error bars are mean ± SD. **C**. Immunoblots of whole cell lysates were probed with antibodies against TNC, DCN and β-actin at days 7 and 14 for control (-) and induced with TGFβ3 and GDF5 samples. Graphs show expression of TNC and DCN using β-actin as a reference. **D**. Immunofluorescence with COL1A2, DCN, TNC and TNMD at day 7 for differentiated DF1 cells. Scale bars represent 50µm.



Aim 3: Characterizing pulmonary epithelial cells that can affect repair in the mouse lung. The expression of specific genes, such as miR-302/367, will be tested to see if they can expand lung epithelial progenitors in vivo (normal tissue); whether they can expand lung epithelial progenitors after injury; and whether miR-302/367 and Gata6 are required for the effective engraftment of lung epithelial cells in vivo.

We have explored the role of miR302-367 in regulating lung epithelial regeneration. As part of this proposal we have generated two new mouse lines to characterize the role of this miRNA cluster: loss of function miR302-367flox/flox mice and R26R-miR302-367 gain of function mice. We have shown that loss of miR302-367 expression during lung development (using the Shhcre deletor) does not lead to a dramatic phenotype and these mice are viable. Combined loss

of miR302-367 along with the related miRNA cluster miR17-92 does lead to a partially penetrant phenotype which we are currently characterizing.

Conversely, over-expression of miR302-367 during lung development expands lung endoderm progenitors while inhibiting differentiation. We have now shown that in the adult lung, loss of miR302-367 inhibits the ability of airway epithelium to regenerate after naphthalene induced injury. Over-expression of miR302-367 in this injury model leads to a de-differentiated epithelium that cannot regenerate well indicating that persistent expression of these miRNAs is deleterious. To overcome this limitation, we have used miR302-367 mimics or small molecule replacements and shown that transient treatment of naphthalene injured mice accelerates regeneration, primarily through increased proliferation of the endogenous progenitor population in this region of the lung called variant Clara cells.

We have also discovered that miR302-367 is expressed in the early heart during development. Loss of miR302/367 leads to a partially penetrant perinatal lethality in mice. Interestingly, over-expression using our R26R-miR302/367 knock-in allele results in extension of cardiomyocyte proliferation beyond the normal first week after birth. This leads to a cardiomegaly phenotype due to a profound increase in cardiomyocyte proliferation. We have also over-expressed miR302/367 in the adult mouse heart and have shown that it can re-activate cardiomyocyte proliferation. Moreover, small molecular mimics for these miRNAs can be used to promote cardiac repair after injury in the adult heart.

We have also explored the underlying mechanism by which miR302-367 promotes lung and cardiac regeneration. We have identified that these miRNAs target important kinases within the Hippo pathway including Lats2, Mst2, and Mob1b. Inhibition of these kinases by miR302-367 expression leads to decreased Yap phosphorylation and inhibition of Hippo signaling which increases cell proliferation. Finally, knockdown of Yap in cardiomyocytes inhibits the ability of miR302-367 to promote proliferation in these cells. Taken together, miR302-367 is a potent inducer of cellular proliferation and may be leveraged to promote lung and cardiac regeneration after acute injury.

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. 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:

We are currently writing a manuscript on the role of miR302-367 in lung regeneration and another manuscript on the role of this cluster in cardiac regeneration. We expect both of these manuscripts to be submitted in the next 6 months.

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.

Jonathan Epstein, M.D., is the William Wikoff Smith Professor of Cardiovascular Research, Chairman of the Department of Cell and Developmental Biology, and Scientific Director of the Penn Cardiovascular Institute. Dr. Epstein graduated from Harvard College in 1983, Harvard Medical School in 1988, and completed his Residency and Fellowship in Medicine and Cardiology at the Brigham and Women's Hospital, where he also completed an HHMI Postdoctoral Fellowship in Genetics. In 1996, he accepted a position as Assistant Professor of Medicine in the Division of Cardiology at the University of Pennsylvania.

Dr. Epstein's research has focused on the molecular mechanisms of cardiovascular development and implications for understanding and treating human disease. His group has been at the forefront of utilizing mouse models of congenital heart disease to determine genetic and molecular pathways required for cardiac morphogenesis, including the role of neural crest in cardiovascular development. This line of investigation led to the identification of TBX1 as a candidate gene for DiGeorge syndrome, a common human condition associated with congenital heart disease, and to the implication of a series of other candidate genes for congenital cardiac disorders. The Epstein group has discovered novel signaling pathways regulating vascular patterning, angiogenesis and blood vessel growth. Genetic studies in mouse models have demonstrated a critical function for the gene responsible for von Recklinghausen Neurofibromatosis (NF1) in the vasculature. Using mouse and zebrafish models, novel members of the semaphorin family of guidance molecules have been implicated in the growth and patterning of both large and small vessels. Studies of cardiac development have been extended to implicate similar pathways in adult cardiac homeostasis and disease, with direct implications for the development of new therapeutic agents for heart failure and myocardial infarction. In addition to his research commitments, Dr. Epstein practices medicine at the Philadelphia Veteran Administration Hospital.

Christopher S. Chen, M.D., Ph.D., is the Skirkanich Professor of Innovation in the University of Pennsylvania's Department of Bioengineering. He is also a faculty member of the Cell Biology and Physiology Program as well as the Cell Growth and Cancer Program. He is the Director of the Tissue Microfabrication Laboratory and founding Director of the Center for Engineering Cells and Regeneration. Dr. Chen has been an instrumental figure in the development of engineered cellular microenvironments in order to engineer cell function. The goal of Dr. Chen's research is to identify the underlying mechanisms by which cells interact with materials and each other to build tissues, and to apply this knowledge in the biology of stem cells, tissue vascularization, and cancer.

Dr. Chen has received numerous honors, including the Presidential Early Career Award for Scientists and Engineers, the Angiogenesis Foundation Fellowship, the Office of Naval Research Young Investigator Award, the Mary Hulman George Award for Biomedical Research, and the Herbert W. Dickerman Award for Outstanding Contribution to Science. He serves as a member of the Faculty of 1000 Biology, the Board of Trustees for the Society for BioMEMS and Biomedical Nanotechnology, Editor for BioInterphases and Molecular and Cellular Biomechanics, and a member of the Defense Sciences Study Group. He received his A.B. in Biochemistry from Harvard, M.S. in Mechanical Engineering from M.I.T., and Ph.D. in Medical Engineering and Medical Physics from the Harvard-M.I.T. Health Sciences and Technology Program. He earned his M.D. from the Harvard Medical

School. He was Assistant Professor in Biomedical Engineering and in Oncology at Johns Hopkins University prior to being appointed Associate Professor at the University of Pennsylvania.

Dr. Kenneth Margulies, M. D., is a clinician-scientist with NIH-supported training in both integrative and cell physiology. His research over the past 20 years has focused primarily on the pathophysiology and treatment of heart failure employing a variety of in vitro and in vivo techniques in both animal models and patient-based research. Throughout this period, he has remained active in clinical heart failure and heart transplantation medicine at two of the largest programs in the U.S.

Recognizing the opportunities and perils of human heart tissue research, the Margulies lab initiated a human heart tissue research program 20 years ago and this work has been continuously funded by the NIH since 1997. Most of this work has involved studies examining regulation of contractility, myocardial remodeling and recovery, transcriptional regulation or endogenous myocardial repair in human hearts. Through a continuous commitment to carefully procuring human heart tissue, the Margulies Lab has established a human heart tissue bank that now includes over 18,000 human heart specimens from more than 850 hearts. This human heart tissue bank has supported extramural research at 33 academic laboratories in the U.S., including 27 outside of Penn, with at least 38 peer-reviewed manuscripts derived from human tissue studies to date.

Dr. Margulies' ongoing research focuses on mechanisms of cardiac remodeling and the regulation of contractility, metabolism and growth in failing human hearts. The Lab has been studying the role of endogenous cardiac repair mechanisms and the dynamics through which native progenitor cells are engrafted to the failing heart. For the past 4 years, Dr. Margulies and Dr. Chris Chen from Biongineering have been developing and optimizing an engineered cardiac microtissue (CMT) model that uniquely balances the goals of clinical relevance and experimental rigor for mechanistic inquiries, target validation and drug screening applications relevant to myocardial biology.

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.

Edward Morrisey, Ph. D., is Professor of Medicine and Cell and Developmental Biology. He is also the Scientific Director of the Institute for Regenerative Medicine at the University of Pennsylvania. The goal of his laboratory is to define the molecular mechanisms underlying lung and cardiac development and to determine how these mechanisms can be harnessed to improve repair and regeneration in these tissues. His lab has been a leader in the study of the development and repair/regeneration of the respiratory and cardiovascular systems. They were the first laboratory to demonstrate that Wnt signaling is required for specification of the pulmonary endoderm fate within the anterior foregut as well as described a role for Wnt signaling in lung vascular development. They have a current focus on how Wnt signaling, non-coding RNAs, forkhead transcription factors, and epigenetic mechanisms regulate cardiopulmonary endoderm/mesoderm development and repair and regeneration. Their ultimate goal is to better understand how signaling and epigenetic pathways regulate cardiopulmonary development and regeneration, which may lead to the development of novel therapeutic approaches for lung and cardiac diseases.