AWARD NUMBER: W81XWH-17-1-0659

TITLE: Scalability and Safety Studies in Clinical-Grade Pluripotent-Derived Myogenic Progenitors for Therapeutic Application in DMD

PRINCIPAL INVESTIGATOR: Rita Perlingeiro, PhD

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

REPORT DATE: October 2019

TYPE OF REPORT: Annual

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
October 2019	Annual	30 Sep 2018 - 29 Sep 2019

4. TITLE AND SUBTITLE

Scalability and Safety Studies in Clinical Grade Pluripotent- Derived Myogenic Progenitors for Therapeutic Application in DMD

5a. CONTRACT NUMBER

5b. GRANT NUMBER

W81XWH-17-1-0659

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Rita Perlingeiro, PhD

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Rm 450 McNamara Alumni Center
200 Oak St. SE
Minneapolis, MN 55455

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

SA Med Research ACQ Activity
820 Chandler St.
Fort Detrick, MD 21702

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

During the second year of this grant, we finalized optimization of the CD54+ MACS-purification protocol (in vitro and in vivo validation) as well as completed the growth curve studies as well as the in vivo experiments using the optimized media composition. Whole genome sequencing and mapping of integration sites has also been achieved.

15. SUBJECT TERMS

ACURO- Animal Care and Use Review Office; cGMP- Current Good Manufacturing Practice; FACS- Fluorescence-Activated Cell Sorting; FBS - Fetal Bovine Serum; HS - Horse Serum;
HRPO/ACURO- Human Research Protection; iPSC- Induced Pluripotent Stem Cell; ITS Insulin-Transferrin-Selenium; KOSR –

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified		19b. TELEPHONE NUMBER *(Include area code)*

Reset

Standard Form 298 (Rev. 8/98)
Prescribed by ANSI Std. Z39.18

Table of Contents

Page

1. Introduction…….1

2. Keywords…….1

3. Accomplishments……..……..1

4. Impact……...……4

5.Changes/Problems...….……4

6. Products……..……..…..……4

7.Participants & Other Collaborating Organizations……....5

8.Special Reporting Requirements……n/a

9. Appendices……quad chart

1. INTRODUCTION

This application builds on our successful experimental studies developing pluripotent stem cell-derived myogenic progenitors to promote long-term muscle regeneration for Duchene Muscular Dystrophy. The purpose of this project is to optimize manufacturing and purification of our myogenic cell product in compliance with current good manufacturing practice (cGMP). Once this is achieved and validated in preclinical studies, we will be in a strong position to begin IND filing, and a phase 1 safety trial for Duchene Muscular Dystrophy

2. KEYWORDS

ACURO - Animal Care and Use Review Office
cGMP - Current Good Manufacturing Practice
FACS - Fluorescence-Activated Cell Sorting
FBS - Fetal Bovine Serum
HS - Horse Serum
HRPO/ACURO – Human Research Protection
iPSC - induced Pluripotent Stem Cell
ITS - Insulin-Transferrin-Selenium
KOSR – Knockout Serum Replacement
LiPSC-ER2.2 - GMP-manufactured human induced pluripotent stem cell line used in this project
MACS - Magnetic Cell Sorting
PAX7 - Paired box protein 7, transcription factor, important for muscle development and adult muscle regeneration
pCCL - third generation self-inactivating lentiviral vector
rtTA - reverse tetracycline-controlled transactivator
TA - Tibialis Anterior muscle

3. ACCOMPLISHMENTS

3.a What were the major goals of the project?

Major Goal 1: Optimization of purification strategy using MACS	Proposed timeline (Achieved)
Milestone Achieved: HRPO/ACURO Approval	Month 4-6 (100%)
Milestone Achieved: *in vitro* validation purification protocol	Month 9 (100%)
Major Goal 2: Transplantation studies with MACS-purified myogenic progenitors	
Milestone Achieved: *in vivo* validation purification protocol	Month 21 (100%)
Major Goal 3: Scalability studies (growth curves)	Months
Milestone Achieved: Growth rates obtained for all conditions evaluated.	Month 18 (100%)

Major Goal 4: Scalability studies (*in vivo)*	
Milestone Achieved: *in vivo* validation scalability studies	Month 19 (100%)
Major Goal 5: Safety of iPAX7 myogenic progenitors	
Milestone Achieved: Whole genome sequencing and subsequent analysis as well as Cytogenetics	Month 15 (100%)
Major Goal 6: Verification of clinical-grade "compatible" purification and scale up of LiPSC-ER2.2 iPS cell-derived myogenic progenitors	
Milestone Achieved: Generation and *in vitro* validation of clinical grade "compatible" myogenic progenitors	Month 33 (0%)
Major Goal 7: *in vivo* validation of clinical grade "compatible" myogenic progenitors	Months
Milestone Achieved: *in vivo* validation of clinical grade "compatible" myogenic progenitors	Month 36 (0%)

3.b What was accomplished under these goals?

1) Major activities:
Our major activities during the last 12 months involved:
- Finalized *in vitro* and *in vivo* validation of MACS-based purification protocol for iPAX7 myogenic progenitors
- Finalized optimization of culture conditions for the scalability of iPAX7 myogenic progenitors (growth curves)
- Finalized *in vivo* validation of scalability studies
- Determined the safety of iPAX7 myogenic progenitors

2) Specific objectives:
- To generate clinical grade "compatible" myogenic progenitors
- To perform *in vitro* validation of clinical grade "compatible" myogenic progenitors
- To perform *in vivo* validation of clinical grade "compatible" myogenic progenitors

3) Significant findings/developments:
In the last 12 months, we completed the optimization of the protocol for the MACS-purification of $CD54^{+}$ myogenic progenitors. We also developed a GMP-compliant protocol for the expansion of human pluripotent stem cell-derived skeletal muscle progenitors to enable clinical application. In addition, a comprehensive risk assessment analysis of Pax7-induced pluripotent stem cell-derived myogenic progenitors was completed.

Aim 1 *- To define the optimal purification strategy for the clinical application of pluripotent-derived myogenic progenitors.*

This is a critical aspect when generating pluripotent stem cell-derived tissue specific progenitors for therapeutic application, not only for efficiency but especially for safety to avoid the presence of contaminating undifferentiated pluripotent stem cells. Previous results from our whole transcriptome sequencing studies followed by flow cytometry validation showed distinct up-regulation of 3 surface markers following PAX7 induction: the Intercellular Adhesion Molecule 1

(ICAM1 or CD54), Syndecan2 (SDC2 or CD362), and Alpha9 Integrin (ITGA9 or α9β1). We found the triple$^+$ fraction (CD54$^+$α9β1$^+$SDC2$^+$) to be virtually 100% GFP$^+$ (PAX7$^+$), and purification of this sub-fraction resulted in myogenic progenitors able to efficiently differentiate into myotubes *in vitro* and contribute to muscle regeneration *in vivo*. Both readouts were indistinguishable from cells sorted based on GFP expression. Nevertheless, because purification strategies compatible with clinical application preferably involve the use of magnetic beads, here we optimized a method to reliably and efficiently purify pluripotent stem cell-derived myogenic progenitors. Considering the very high levels of CD54 expression in PAX7-induced cells, and that CD54^{high} cells are also positive for both α9β1 and SDC2 (Magli et al, Cell Reports, 19:2867-2877, 2017), here we focused on investigating whether CD54 could be used as a single marker for the isolation of myogenic progenitors. This would be preferred in terms of cost and feasibility.

In Year 1, we confirmed the enrichment of the target myogenic progenitor cell population through CD54$^+$ cell isolation. We also performed pilot studies to determine the most efficient concentration of antibody in regard to cell number.

In the last 12 months (Year 2), we performed several experiments to determine the parameters for the efficient enrichment of the CD54$^+$ cell target population. After testing the purification of 2 x 10^6 cells using MS Miltenyi columns in the presence of biotin-conjugated anti-CD54 antibody (2.5ul or 5ul of antibody per 10^6 cells) and anti-biotin beads (0.5ul, 1ul, 2ul, 4ul, 8ul and 16ul of beads per 10^6 cells), we determined that 2.5ul antibody/2ul anti-biotin beads/10^6 cells represent the most efficient volume of these reagents for safer and scalable production of CD54$^+$ myogenic progenitors. Next, CD54$^+$ myogenic progenitors purified by MACS and FACS were compared to the FACS-purified triple$^+$ fraction (CD54$^+$α9β1$^+$SDC2$^+$) population in terms of *in vitro* myogenic differentiation and *in vivo* muscle regeneration. Similar outcome was observed among the 3 experimental groups (at least 10 recipient mice per group), providing validation for the use of MACS-purified CD54 myogenic progenitors for clinical application.

Aim 2 *- To develop a GMP-compliant protocol for the expansion of human pluripotent-derived skeletal muscle progenitors to enable clinical application.*

To enable basic research work towards clinical translation, it is required to adapt all culture methodologies to a GMP/clinically friendly protocol. The first step in this direction is to identify all components of each cell culture step that are from animal origin, and eliminate or replace them with a chemically defined substitute and/or acceptable cGMP reagent. The goal here is to develop a GMP protocol based on reagents that carry minimal risk to the eventual recipients of the cell product to enable the generation of cGMP pluripotent stem cell-derived myogenic progenitors. Therefore, the objective is to determine the conditions that provide optimal growth while retaining optimal *in vivo* regenerative potential.

Since pilot studies in which we totally removed FBS impaired dramatically cell expansion (data not shown), we opted for media compositions that maintained FBS. On the other hand, horse serum was eliminated from the culture medium – it was only maintained in the control condition (#1). We have tested 6 different media compositions (as outlined in Fig. 1B):

Condition #1: Control (15% FBS + 1% KOSR + 10% HS)
Condition #2: 15% FBS + 11% KOSR
Condition #4: 15% FBS + 11% KOSR + N2B7 supplement
Condition #6: 15% FBS + 11% KOSR + ITS
Condition #7: 4% FBS + 11% KOSR + ITS
Condition #11: 15% FBS + 11% KOSR + ITS + N2B7 supplement

We found that the combination #7, comprising of a low percentage of FBS (4%), combined with 11% of KOSR and 1% ITS, is sufficient to promote robust *in vitro* myogenic commitment, proliferation and *in vivo* engraftment (Fig. 1A-E). This media composition was selected based on optimal readout in terms of *in vitro* expansion, myotube differentiation, and *in vivo* regeneration upon transplantation into injured mice. In addition, this condition uses less than a third of the usual concentration of FBS, which is another advantage. Data shown in Fig. 1C-E made use of CellStack at the expansion stage, which allowed for the generation of very large numbers of myogenic progenitors. Based on this validation, we plan to use culture condition #7 combined with CellStack for the generation of PSC-derived myogenic progenitors for clinical application. Lastly, we also tested the effect of non-enzymatic and enzymatic reagents for the efficient recovery of myogenic progenitor before intramuscular transplantation and we found that the GMP-compatible TrypLE reagent does not affect myogenic progenitor engraftment potential (data not shown).

Aim 3 *- To complete a comprehensive risk assessment analysis of Pax7-induced pluripotent-derived myogenic progenitors*.

Our findings, to date, show that the use of lentiviral vectors is required for optimal inducible expression of Pax7, and subsequently expansion of Pax7^{+} myogenic progenitors in culture. Since there are lentiviral vectors, such as the pCCL, already approved for clinical trials (Aiuti et al., 2013), we have switched our inducible system to this lentiviral backbone. In Year 1, we showed that LiPSC-ER2.2 iPS cells that had been transduced with pCCL-PAX7 and pCCL-rtTA viruses displayed normal karyotype. During the last 12 months, we performed transplantation studies which confirmed that myogenic progenitors generated using this vector backbone are as efficient as the original counterparts (data not shown) in promoting *in vivo* muscle regeneration.

To assess the safety, we performed whole genome sequencing in iPAX7 LiPSC-ER2.2 pluripotent stem cells that had been modified with the pCCL backbone lentiviral vector. This approach allows the identification of viral integration sites, SNPs and CNVs, which might occur during the generation of the inducible iPS cell lines. Whole genome of iPax7 LiPSC-ER2.2 iPS cells was sequenced at 43x coverage using the Chromium platform from 10x Genomics (available at the University of Minnesota Genomic Center). For the bioinformatic analysis, we used the resources available at the Minnesota Supercomputing Institute (MSI). Raw reads were mapped using the canonical human genome (hg38) as reference to estimate coverage of the iPS cell genome. Next, we used the viral vector genome to identify chimeric reads containing both human and viral genome, which represent the viral integration sites. This approach identified 419 unique viral integrations. Following annotation of the viral integration sites relative to the position of the known human coding genes, we observed the viral integration sites are mostly located within genes (322/419). This is not surprising as lentiviral vectors integrates preferentially within active regions of chromatin, which are mainly associated with gene transcription (Molecular Therapy, 19:1273-86, 2011). Because this analysis was performed on the bulk cell population, we identified several integration sites. In any case, we expect that the number of integrations will be lower after clonal isolation (which we intend to do when working with the clinical grade iPS cells). Among others, viral integrations were also detected within potential tumor suppressor genes. This finding highlights the importance of clonal isolation and precise identification of integration sites to ensure safety of an iPS cell-based therapy.

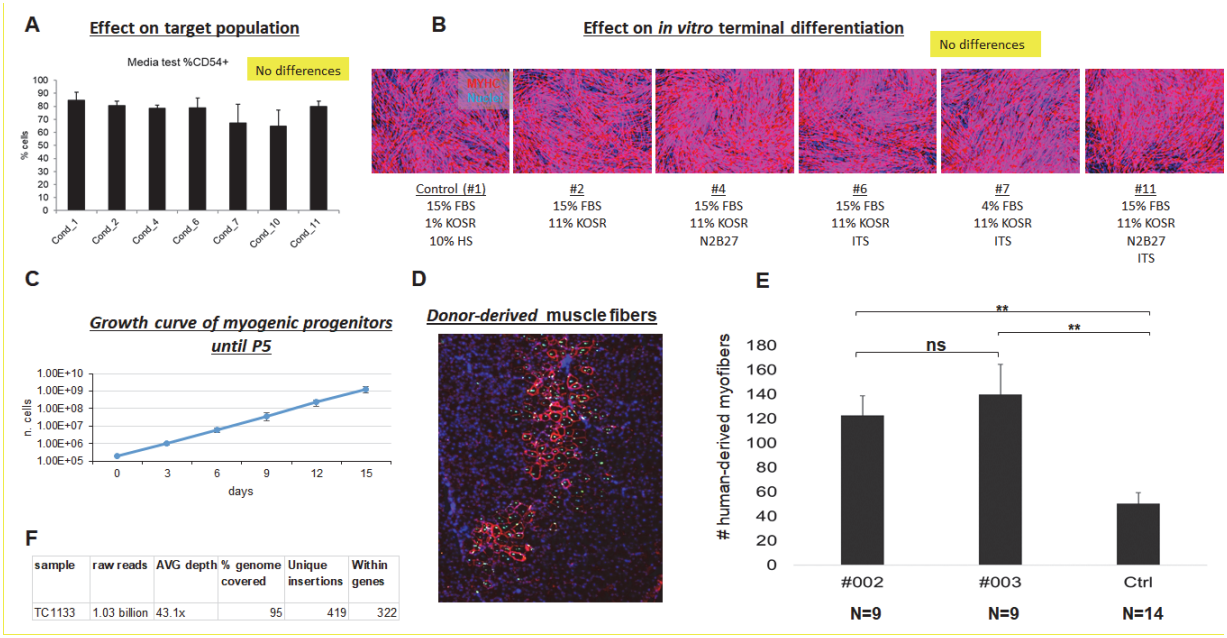

Figure 1. Significant developments. A-B) Comparison of different media compositions on the ability to promote myogenic commitment. Graph in panel A shows the frequency (mean ± standard error) of the CD54⁺ target population. No differences were found. CD54⁺ cells from these different media conditions were then purified and induced to terminally differentiate into myotubes. Panel B reports the results of immunostaining for myosin heavy-chain (MYHC; in red). DAPI stains nuclei (in blue). All conditions displayed a similar differentiation capability. C) Growth curve of myogenic progenitors (expanded in media #7) until passage 5. D-E) Transplantation results. Representative image shows engraftment of human iPSC-derived myogenic progenitors grown under condition #7 (D), as shown by staining for human specific DYSTROPHIN (in red) and human nuclei (Lamin A/C; in green) in transplanted TA muscles. DAPI (blue) was used to counterstain nuclei. Plot (E) shows quantification of human-derived myofibers in TA muscles injected with 2 independent preparations of iPAX7 LiPSC-ER2.2 cells expanded under condition #7 (#002 and #003) or control (ctrl #1) conditions. ns: not significant. ** *p*-value < 0.01. F) Whole genome sequencing results of iPAX7- LiPSC-ER2.2 iPS cells. Approximately 95% of the genome was sequenced at 43x coverage. 419 unique viral integration sites were identified, 322 of them located within genes.

3.c What opportunities for training and professional development has the project provided?

"Nothing to Report".

3.d How were the results disseminated to communities of interest?

"Nothing to Report".

3.e What do you plan to do during the next reporting period to accomplish the goals?

We will complete milestones associated with major goals 6 and 7, as described above.

4. IMPACT

4.a What was the impact on the development of the principal discipline(s) of the project?
"Nothing to Report".

4.b What was the impact on other disciplines?
"Nothing to Report".

4.c What was the impact on technology transfer?
"Nothing to Report".

4.d What was the impact on society beyond science and technology?
"Nothing to Report"

5. CHANGES/ PROBLEMS

5.a Changes in approach and reasons for change
"Nothing to Report".

5.b Actual or anticipated problems or delays and action or plans to resolve them
"Nothing to Report".

5.c Changes that had a significant expenditures
"Nothing to Report".

5.d Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
"Nothing to Report"

6. PRODUCTS

6.a Publications, conference papers, and presentations
"Nothing to Report".

6.b Website(s) or other Internet site(s)
"Nothing to Report".

6.c Technology or techniques
"Nothing to Report".

6.d Inventions, patent applications, and/or licenses
"Nothing to Report".

6.e Other products
"Nothing to Report".

7. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

7.a What individuals have worked on the project?

Name	Project Role	eRA Commons ID	Person Months	Contribution to Project
Rita Perlingeiro	PI	rperlingeiro	1.80	Overall oversight of the project.
Alessandro Magli	Assistant Professor	amagli	2.4	Dr. Magli assisted with experimental design and led whole genome sequencing studies and subsequent analysis.
James Kiley	Researcher 2	N/A	6.0	Mr. Kiley performed the studies involving the optimization of purification and scalability of pluripotent stem cell-derived myogenic progenitors
Tania Incitti	Postdoctoral Associate	tincitti	1.2	Dr. Incitti performed transplantation experiments and subsequent analysis.
David McKenna	Co-PI	dmckenna	0.6	Dr. McKenna provided expertise in the design and implementation of studies.

7.b Has there been a change in the active other support of the PD/PI(s) or senior/ key personnel since the last reporting period?

"Nothing to Report".

7.c What other organizations were involved as partners?

"Nothing to Report"

8. SPECIAL REPORTING REQUIREMENTS

N/A