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

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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 –

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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
CliniMACS - Magnetic Cell Sorting using a clinically compatible instrument produced by Miltenyi Biotec
DMD – Duchenne Muscular Dystrophy
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
NSG-mdx^{4cv} mice – Immunodeficient mouse model of Duchenne Muscular Dystrophy
NSG mice – Nod Scid Gamma-chain immunodeficient mice
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 (100%)
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 (100%)

3.b What was accomplished under these goals?

1) Major activities:
Our major activities during the last 12 months involved:
- Finalized generation of clinical grade "compatible" myogenic progenitors
- Finalized *in vitro* validation of clinical grade "compatible" myogenic progenitors
- Finalized *in vivo* validation of clinical grade "compatible" myogenic progenitors

2) Significant findings/developments:
In the last 12 months, we successfully tested the protocol for the cGMP-compliant purification of $CD54^+$ myogenic progenitors. We demonstrated that these human pluripotent stem cell-derived skeletal muscle progenitors are endowed with robust in vitro myogenic differentiation potential for over several passages, thus allowing scalability. Most importantly, we validated that CD54+ cGMP-compliant myogenic progenitors retain high *in vivo* muscle regenerative capability, both at 2 and 6 months following intramuscular injection.

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 ($CD54^+\alpha9\beta1^+SDC2^+$) 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 Year 2, we determined the most efficient ratio of cells:antibody:magnetic beads for safer and scalable production of $CD54^{+}$ myogenic progenitors using MACS. We also showed that both FACS- and MACS-purified CD54+ myogenic progenitors are characterized by a comparable muscle regeneration potential, thus validating the clinical potential of this strategy.

In the last 12 months (Year 3), we transitioned to a clinical grade-compatible system for the large-scale production of CD54+ myogenic progenitors. Using the CliniMACS apparatus available at the University of Minnesota Center for Translational Medicine, we performed large scale production of CD54+ myogenic progenitors derived from Pax7-inducible LiPSC-ER2.2 iPS cells. As shown in Fig. 1A, the CliniMACS system enables efficient purification of CD54+ cells from a mixed population. These purified progenitors maintain CD54+ expression for several passages (we tested up to passage 10; Fig. 1B). We also demonstrated that this clinically-compatible cell product expresses also other myogenic progenitor markers, such as Integrin α9β1 (Fig. 1C). Lastly, CliniMACS-purified CD54+ myogenic progenitors display robust ability to differentiate into myotubes *in vitro* for several passages, confirming their myogenic potential (Fig. 1D).

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.

In Year 2, we determined the conditions that retained *in vivo* regenerative potential while providing optimal growth. Following the test of different media compositions, we found that 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. 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, we also determined that 1) culture flasks do not require gelatin coating for *in vitro* expansion, and 2) the GMP-compatible TrypLE reagent does not affect myogenic progenitor engraftment potential.

In the last 12 months (Year 3), we applied these conditions to generate our target population using the clinical grade “compatible” CliniMACS (described in Aim 1). To ensure that expansion

potential is maintained also in the large-scale production, we expanded CD54+ cells using 3-layers CellStacks instead of T75 flasks. By plating 50 million cells at each passage, we observed an average 7-fold expansion until passage 5 (Fig. 1E). Importantly, we also tested the *in vivo* muscle regenerative potential of CliniMACS-purified CD54+ myogenic progenitors, which was compared to the small column MACS-purified counterpart. 8-week post-intramuscular injection in NSG mice, TA muscles were collected and examined for donor-derived DYSTROPHIN expression. As shown in Fig. 1F, multiple myofibers expressed both human DYSTROPHIN and human LAMIN A/C. Quantification of engraftment showed that CliniMACS-purified cells display enhanced muscle regenerative potential when compared to the small MACS column purified counterpart (Fig. 1G). We hypothesize the superior engraftment potential displayed by the CD54+ CliniMACS-purified myogenic progenitors is probably a consequence of 1) the superior purity of CD54-enriched population, 2) decreased cellular stress during the standardized CliniMACS purification, 3) other factors. We also tested whether delivery of these clinical grade “compatible” myogenic progenitors would provide a potential long lasting therapeutic effect, measured as expression of human DYSTROPHIN. We injected a large cohort of NSG animals (16 mice) with CliniMACS-purified CD54+ myogenic progenitors and evaluated skeletal muscle engraftment 2 and 6 months later. Quantification of donor-derived myofibers revealed no significant differences in engraftment levels between these two time points (Fig. 1H), thus suggesting that CliniMACS-purified CD54+ cells may provide long lasting therapeutic effect. Upcoming future studies will focus on later time points for long-term engraftment (9-12 months; this was not possible here due to Covid-19 reduced activities). We also tested the regenerative potential of these CliniMACS-purified CD54+ myogenic progenitors in a small cohort of *NSG-mdx^{4cv}* mice, which model DMD. We observed consistent engraftment in all transplanted recipients (Fig. 1I-J). Using isolated muscle force measurements in this small cohort of transplanted mice (n=6), we observed a positive trend in improvement of muscle strength in transplanted TA muscles when compared to PBS-injected controls (Fig. 1K), but this needs to be repeated with a larger cohort as required for functional studies.

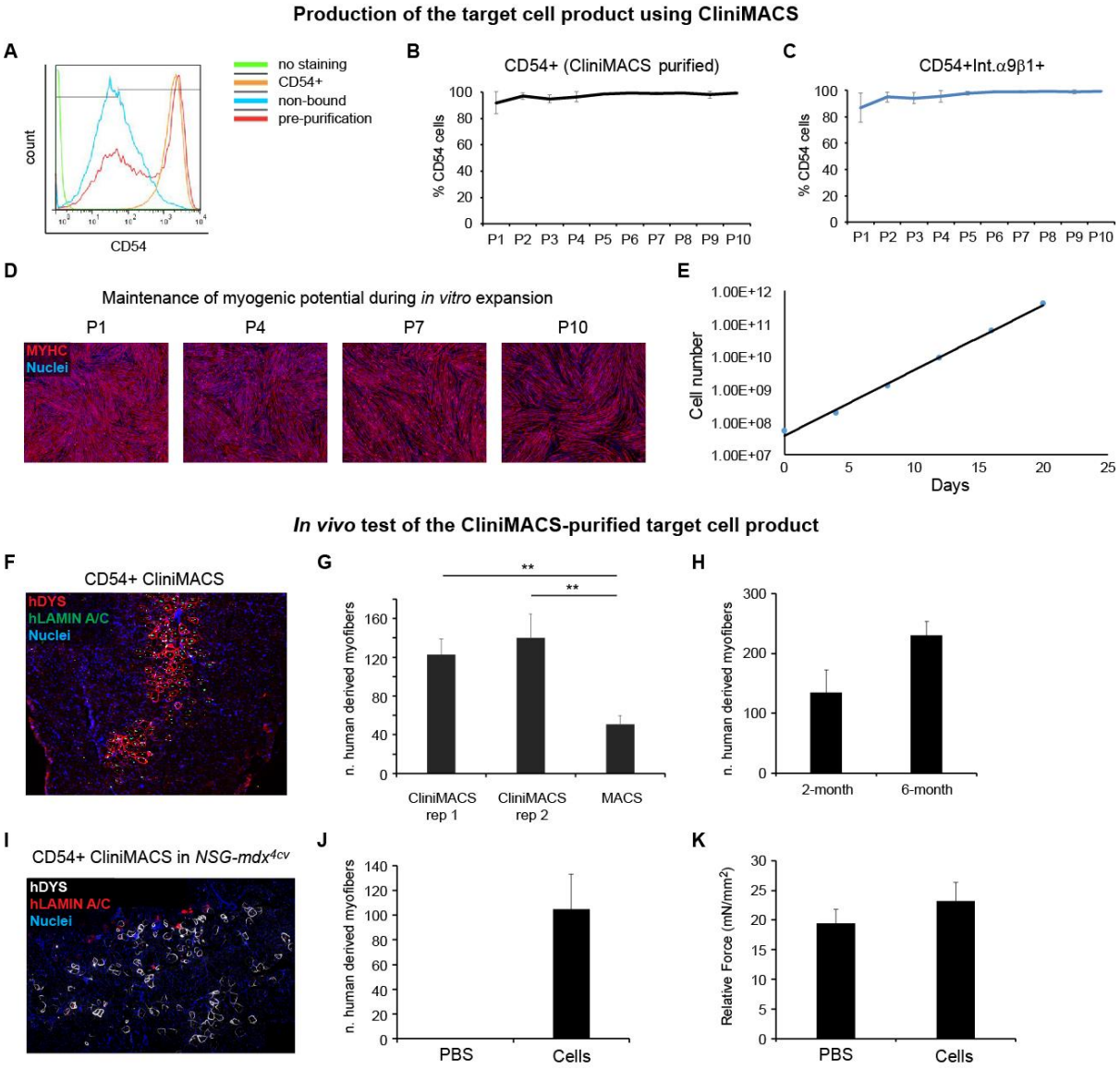

Figure 1. Significant developments. A) Efficient purification of CD54+ cells using CliniMACS. Plot reports the frequency of CD54+ cells before (pre-purification) and after (CD54+) CliniMACS-mediated purification. Non-bound fraction represents cells not retained by the CliniMACS column. Cells were cultured in media #7 and purified using anti-CD54-biotinylated antibody and anti-biotin beads. B) Frequency of CD54+ cells in the CliniMACS output. Cells were expanded for multiple passages using media #7. At each passage, an aliquot of the total sample was analyzed by flow cytometry using anti-CD54 and anti-Int.α9β1 antibodies. Graph reports Mean ± Standard deviation on n=3 independent experiments. C) Frequency of CD54+Int.α9β1+ cells in the CliniMACS output. Graph reports Mean ± Standard deviation on n=3 independent experiments. D) Expansion potential of CD54+ CliniMACS-purified myogenic progenitors in multilayer cell stacks. Over 5 passages, cell displayed a ~7000 fold expansion. E) Terminal differentiation capability of CliniMACS-purified myogenic progenitors at passages 1, 4, 7 and 10 (P1-P4-P7-P10). 200,000 cells were plated in 24-well plates and then switched to differentiation media (no dox in serum-free media). 5 days later, cells were fixed and processed for immunostaining using the MYHC antibody and DAPI. F) Representative pictures from Tibialis Anterior muscles following transplantation of CD54+ CliniMACS- and MACS-sorted cells cultured in media #7. Cryosections were processed for immunostaining using human-specific antibodies for DYSTROPHIN (red) and LAMIN A/C (green). DAPI was used for nuclear staining. G) Quantification of muscle fiber engraftment from panel F. Bars represent Mean + Standard Error. **p<0.01. H) Evaluation of long-term muscle regenerative potential. Muscles transplanted with CD54+ CliniMACS-purified myogenic cells were collected 2- and 6-month post-injection and subjected to immunostaining. Quantification of muscle fiber engraftment shows maintenance of donor cells over time. Bars represent Mean + Standard Error. I) Evaluation of engraftment upon intramuscular delivery of CD54+ myogenic progenitors in mdx-NSG mice. J) Quantification of average engraftment across 6 recipients. K) Force measurement of TA muscles from PBS- and CD54+ myogenic progenitor (Cells)-injected mdx-NSG mice. Bars represent Mean + Standard Error.

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?

● Every year we discuss our research progress with DMD patients and families during the Muscular Dystrophy Research Day, an event organized by the University of Minnesota's Paul and Sheila Wellstone Muscular Dystrophy Center.

● Webinar on “Muscle regeneration from iPS cells and therapeutic applications for muscular dystrophies”, FSHD Society, Zoom presentation, June 2020

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

N/A

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

● Presentations:

- "Optimization Studies to Enable a Phase 1 Clinical Trial of iPS Cell-Derived Myogenic Progenitors for DMD", Marzolf Symposium, University of Minnesota, Minneapolis, MN, October 21, 2019.
- “iPS Cell-Derived Myogenic Progenitors for the Treatment of Muscular Dystrophies: how far are we”? Cardiovascular Conference Series - UCSD, San Diego, CA, USA, May 2019.
- “From iPS cells to Skeletal Muscle Precursors: Potential for Gene Correction and Therapeutic Application”, The 44th Congress of FEBS, Krakow, Poland, July 2019.

● We are currently writing a manuscript on these results.

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