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ORIGINAL RESEARCH

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Optimizing transport methods to preserve function of self-innervating muscle cells for laryngeal injection

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Abstract

Objectives: Recently, our laboratory has discovered a self-innervating population of muscle cells, called motor endplate-expressing cells (MEEs). The cells innately release a wide variety of neurotrophic factors into the microenvironment promoting innervation when used as an injectable treatment. Unlike other stem cells, the therapeutic potential of MEEs is dependent on the cells' ability to maintain phenotypical cell surface proteins in particular motor endplates (MEPs). The goal of this study is to identify transport conditions that preserve MEE viability and self-innervating function.

Methods: Muscle progenitor cells (MPCs) of adult Yucatan pigs were cultured and induced to generate MEEs. Effects of short-term cryopreservation methods were studied on MPC and MEE stages. A minimally supplemented medium was investigated for suspension-mediated transport, and MEEs were loaded at a constant concentration (1×10^7 cells/mL) into plastic syringes. Samples were subjected to varying temperatures (4, 22, and 37°C) and durations (6, 18, 24, and 48 h), which was followed by statistical analysis of viability. Transport conditions maintaining viability acceptable for cellular therapy were examined for apoptosis rates and expression of desired myogenic, neurotrophic, neuromuscular junction, and angiogenic genes.

Results: Cryopreservation proved detrimental to our cell population. However, a minimally supplemented medium, theoretically safe for injection, was identified. Transport temperature and duration impacted cell viability, with warmer temperatures leading to faster death rates prior to the end of the study. Transport conditions did not appear to affect apoptotic rate. Any expression change of desirable genes fell within the acceptable range.

Conclusions: Transport state, medium, duration, and temperature must be considered during the transport of injectable muscle cells as they can affect cell viability and

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expression of integral genes. These described factors are integral in the planning of general cell transport and may prove equally important when the cell population utilized for laryngeal injection is derived from a patient's own initial muscle biopsy.

KEYWORDS
autologous muscle-derived cells, laryngeal injection, neuro-muscular junction, neurotrophin

1 | INTRODUCTION

Cellular therapy has been at the forefront of research in regenerative medicine for the past few decades. In recent years, numerous clinical trials have begun to pave the way for transition from bench-side research into mainstream clinical care.[1–4] Specifically, within the medical field of Otolaryngology, cellular therapy is being actively investigated for its potential to address complex pathologies within the head and neck region.[5,6] Autologous muscle-derived cells (AMDCs) are a particularly interesting cellular therapy because of unique ease of harvest (small muscle biopsy), incorporation into innate tissue, and potential to improve myogenic growth and function.[7] As an industrial leader in this field, Cook Myosite is actively researching AMDCs, concurrently developing good manufacturing practice (GMP) procedures for clinical trials.[7]

Our lab has studied AMDCs for laryngological applications including laryngeal reconstruction and vocal fold paralysis.[8–13] Our initial investigations used AMDCs alone, followed by genetically engineered AMDCs that release neurotrophic factors.[10–12] With the goal of making the model streamlined and safer, we then created a vector-free model of a self-innervating muscle cells.[13–15] In brief, AMDCs were differentiated into myotubes (multinucleated cells), which were cultured to induce the expression of aggregated cell surface proteins and motor endplates (MEPs). As MEPs are most stable in the form of innervated neuromuscular junctions (NMJs), the cells innately release various neurotrophic factors triggering axonal sprouting/regeneration. Thus, we refer to the cells as self-innervating or, more precisely, motor endplate-expressing cells (MEEs). To date, we have found that MEEs innately secrete neurotrophic factors, which encourage reinnervation in vivo, lay down the framework for NMJ formation, and still holding the myogenic benefits of AMDCs.[8,9,13,15] Notably, our group recently discovered that MEEs incorporate into native tissue and continue to have functional gene expression even 1 month after the initial injection.[16] Despite both AMDCs and MEEs being derived from skeletal muscle biopsies, widespread differences exist between AMDCs and MEEs, including retention of "stemness" and trophic factor expression, respectively. Thus, before MEE therapy can be brought into clinical trials, there is a need to develop MEE-specific GMP protocols.

Integral to GMP protocol development includes understanding cell reaction to transport. Depending on culture and administration locations, cells may be subjected to varying transport times. Environmental stressors are inherent, so understanding if MEE functionality changes during transport holds the utmost importance. Furthermore, an idealistic transport protocol would permit cells to be safely administered via injection directly upon arrival. Given the multiple meticulous processing steps of freezing, thawing, and removing toxic substances, cryofreezing already sits at a disadvantage.[17] Suspended state transport in commonly used cell culture media is also unacceptable considering toxic substances (phenol red) and other additives (antibiotic-antimycotic molecules, animal serum) are not safe for human injection. The former and latter, thus, reveal a need to identify efficient and safe transport that maintains MEE viability and functionality, to optimize clinical application.

The effects of transport conditions have been studied for blood products, whole organs, and a variety of stem cells.[18–20] However, unlike other stem cells, MEE transport conditions have to preserve both the cell viability and the ability to maintain complex surface proteins (MEPs), which result in their therapeutic benefit (the release of NFs). This is the first study of its kind to investigate the ideal transportation conditions for the MEE population. In this study, we investigate the effects of transport state, medium, time, and temperature on cell viability, apoptosis, and gene expression to better understand that the optimal manner injectable MEEs can be transported for clinical administration.

2 | METHODS

2.1 | Autologous muscle progenitor cell isolation and culture

A visual overview of the complete experiment is displayed in Figure 1. Muscle progenitor cells (MPCs) were isolated from muscle biopsies of Yucatan minipigs based on previously described methods.[16,21] Further detail available in Supporting Information S1.

2.2 | Differentiation and induction of MPCs into MEEs

MPCs were cultured toward differentiation and subsequent induction based on methods previously described by our group.[16,22] Further details are available in Supporting Information S1.

2.3 | Testing the effects of cryopreservation

In order to observe the effects of standard cryofreezing methods, fresh MPCs were subjected to long-term (4 months) cryofreezing via

standard methods, suspended in FBS with 10% DMSO (Sigma-Aldrich, St. Louis, MO) at −80°C in 1.2 mL screw-top cryogenic vials.[23] These frozen MPCs were thawed, cultured to confluency, and then subjected to our lab's MEE generation protocol. Relative gene expression was measured in frozen MPCs, frozen-derived MEEs, freshly harvested MPCs, and fresh-derived MEEs. To further assess the effects of such transport conditions on our cell population, MEEs created from freshly harvested MPCs were cryopreserved for 24- and 48-h, in order to see the effects on viability ($n \geq 3$).

2.4 | Selection of container for suspension-transport

Plastic 1 mL slip tip syringes (Beckton, Dickinson and Company, Franklin Lakes, NJ) were utilized rather than glass syringes or bottles due to previously reported lack of difference in effect on stored cells, lower cost, greater durability, and clinical ease of use.[19] Differing needle gauges were not tested, as our group has previously reported a lack of difference in MEE survival.[24]

2.5 | Selection of density utilized for suspension-transport

A consistent density of 1×10^7 cell/mL medium was utilized throughout this study, as this is the density our group continues to utilize during in vivo studies.[13,16]

2.6 | Selection of temperature utilized for suspension-transport

Temperatures of 4, 22, and 37°C were selected to stimulate the settings of standard refrigeration, room temperature, and temperature at which cultured cells are usually incubated.

2.7 | Selection of time utilized for suspension-transport

Time points of 6, 12, 18, 24, and 48 h were selected to stimulate various same-day, overnight, 1-day, and 2-day shipping.

2.8 | Selection of medium utilized in suspension-transport

To maximize clinical utility and efficiency, transport medium (TM) must be nontoxic to allow therapeutic injection upon arrival at a health care site. Based on the previously reported efficacy of high glucose Dulbecco's modified eagle medium (DMEM) in the transport of cellular therapeutics, a minimally supplemented medium of this base was utilized.[20] Ultimately, MEEs were suspended in either TM (high glucose DMEM with no phenol red, no HEPES buffer, no fetal bovine serum (FBS), no sodium pyruvate [DMEM-m, Fischer], with 10 nM L-glutamine [Fischer]) or 1× phosphate buffered saline (PBS). PBS was incorporated into the study to serve as a comparative control. Cell solutions were then taken up into 1-mL plastic syringes through 16-gauge needles. Loaded syringes were capped and placed in environments differing by temperature but free of light and supplemental CO_2. The pH was measured at 0, 24, 48, and 72 h to determine changes in respective media via electronic probe or blinded pH paper testing.

2.9 | Simulating MEE transport conditions

After determining that TM provided greater stability for physiologic pH than PBS, additional testing was performed. MEEs suspended in TM within syringes were placed in various temperatures (4, 22, and 37°C) and times (6, 12, 18, 24, and 48 h) free of light and supplemental CO_2 ($n \geq 3$).

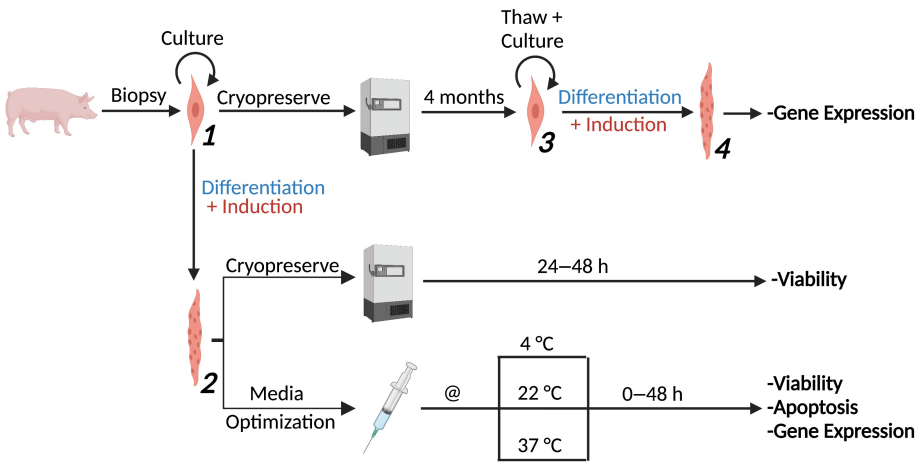

FIGURE 1 Illustrative overview of the experiment (created with BioRender.com). Cryopreservation of MPCs and MEEs was analyzed. Following the identification of a suitable transport medium, MEEs were subjected to conditions of varying temperature and time (1 = fresh MPCs, 2 = fresh derived MEEs, 3 = frozen MPCs, and 4 = frozen derived MEEs).

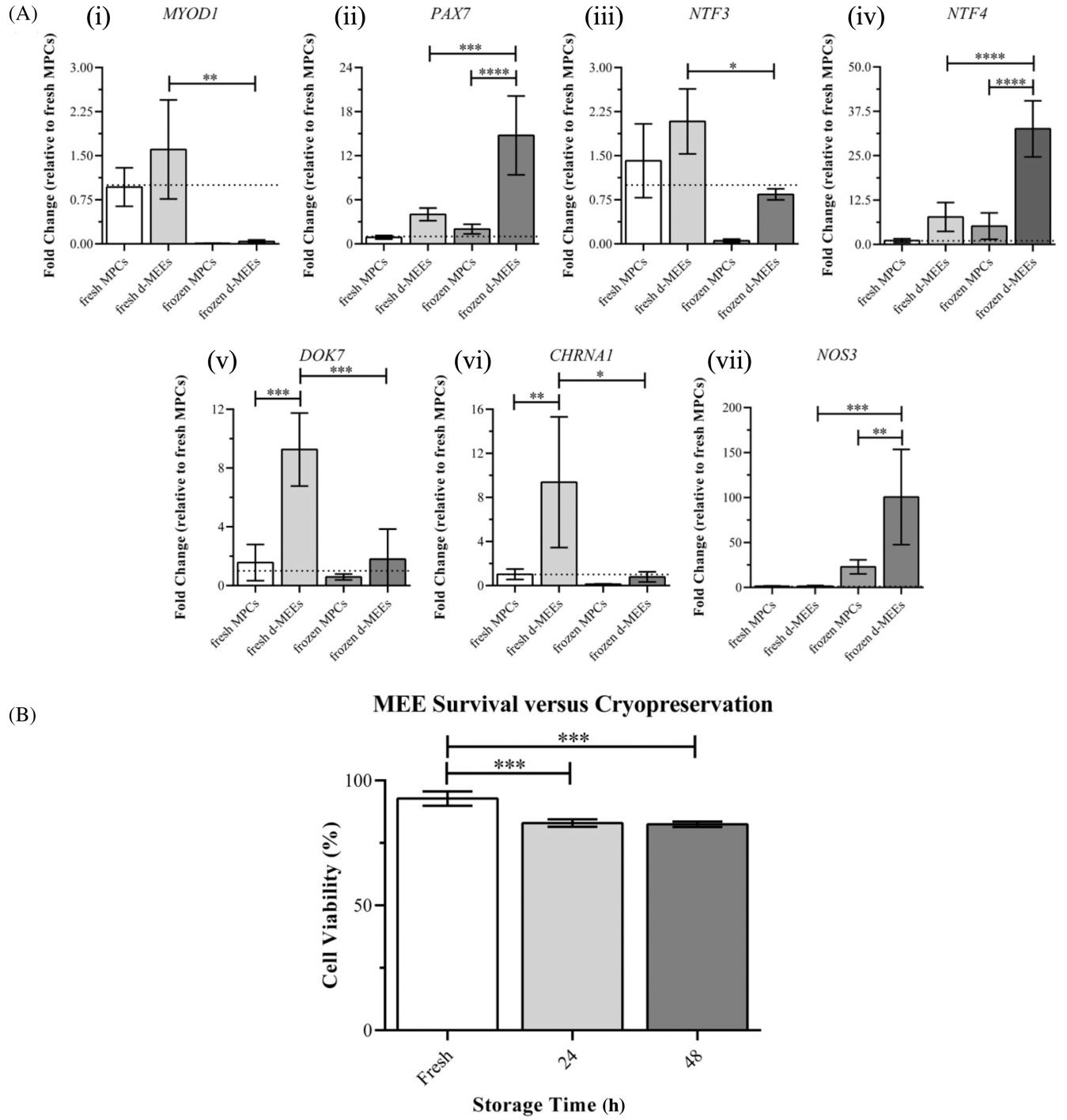

FIGURE 2 (A) Gene expression profiles of fresh MPCs, MEEs derived from fresh MPCs (fresh d-MEEs), frozen MPCs, and MEEs derived from frozen MPCs (frozen d-MEEs). Expression of (A-i) Myogenic Differentiation 1, (A-ii) Paired Box 7, (A-iii) Neurotrophin 3, (A-iv) Neurotrophin 4, (A-v) Docking Protein 7, (A-vi) Cholinergic Receptor Nicotinic Alpha 1 Subunit, and (A-vii) endothelial Nitric Oxide Synthase were measured ($n \geq 3$) (B) The percentage of MEEs (derived from fresh MPCs) alive following 24- and 48-h cryopreservation transport times, compared to the viability of freshly harvested MEEs ($n \geq 3$) (*$p \leq .05$, **$p \leq .01$, ***$p \leq .001$, ****$p \leq .0001$, dotted line denotes fold change of one).

2.10 | Flow cytometry (cell viability and apoptosis assessments)

Transported cells were initially characterized by assessing viability and apoptosis rate via methods previously described by our group.[24] Of note, only samples above our established viability threshold of 80% (10% above the Food and Drug Administration's recommendation) were further analyzed for apoptotic rate.[25] Further details are available in Supporting Information S1.

2.11 | Polymerase chain reaction (apoptotic and MEE-related gene expression)

Transport samples above the viability threshold were also tested for gene expression as previously described by our group.[16] Further details are available in Supporting Information S1, including polymerase chain reaction targets in Table S1.

2.12 | Statistical analysis

GraphPad Prism was utilized for data graphing and statistical analysis. $p < .05$ was utilized throughout for statistical significance. Tukey or Dunnett Multiple Comparison tests were utilized as appropriate for viability and gene expression analysis. Regression lines (setting y-intercepts at respective starting media pH's) were calculated when analyzing media pH changes.

3 | RESULTS

3.1 | Effect of cryopreservation on MEE behavior

When MPCs were subjected to standard cryopreservation methods, there proved to be detrimental effects on eventual gene expression. Frozen-derived MEEs had significantly altered expression of numerous myogenic, neurotrophic, NJM-formation, and angiogenic genes when compared with MEEs derived from fresh MPCs (Figure 2A i–vii). Additionally, cryopreservation proved significantly detrimental to the viability of MEEs derived from freshly harvested MPCs, with drops of 17.01% and 17.55% at short-term 24- and 48-h transport times, respectively (Figure 2B).

3.2 | Media pH test

The starting pH of TM and PBS were 8.32 and 7.30, respectively. With the exception of cells suspended in TM at 37°C, this medium had only modest changes in pH across the 48-h transport time when used for cell suspension (Figure 3). Given the ability to remain above average physiological pH (7.4) in the majority of transport temperatures with fresh derived MEEs, the previously reported advantage of a DMEM-based medium was confirmed, and TM was ultimately selected as the TM for our study.[19]

3.3 | Viability of fresh MEEs across transport conditions

The proportion of fresh-derived MEEs lacking propidium iodine (PI) positivity is reported as a percentage alive within a viability plot (Figure 4). The starting viability of all samples averaged just below 93%. A total of 4°C presented as the best transport temperature, maintaining 80% of cells alive through 24 h. A total of 22°C also proved to be a relatively effective transport temperature, with maintaining the viability through 18 h. MEEs stored at 37°C quickly dropped below 80% alive within the first 6 h. Significant differences in viability between freshly harvested MEEs (0 h) and those stored at 4, 22, and 37°C were reached by 48, 24, and 6 h (respectively).

3.4 | Effects of transport on MEE apoptosis

The effects of transport time and temperature were analyzed to understand the impact of these respective parts of the transport process. With 4°C transport maintaining the highest viability throughout the study, samples at this temperature were selected to examine the effect of differing transport times. Additionally, given the viability of MEEs was only close to the 80% threshold at the 6 h, samples at this timepoint were selected to examine the effect of differing transport temperatures. Neither transport time nor temperature led to significant changes in the expression of apoptotic marker p53 (Figure 5A). Such results were confirmed via flow cytometry, with only a small percentage of cells in each transport sample residing in early apoptosis (Figure 5B).

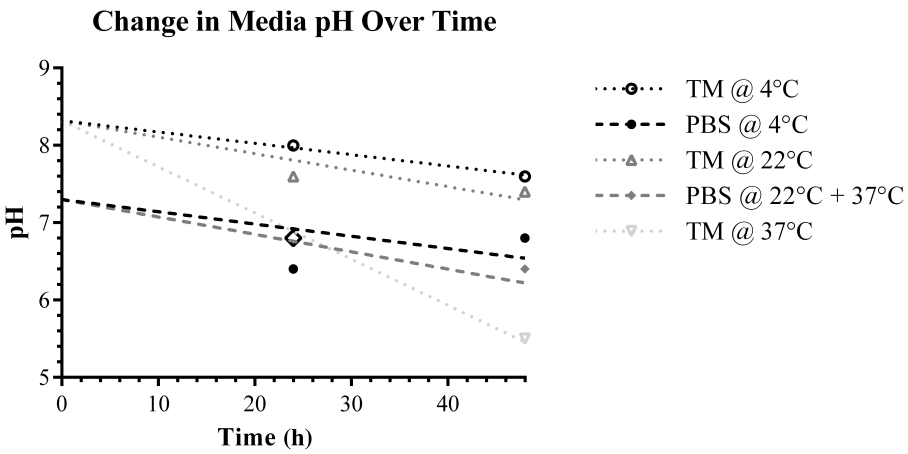

FIGURE 3 Media pH changes over time at differing temperatures for fresh derived MEEs suspended in DMEM-based transport medium (TM) and PBS, displayed as regression lines (◇ = overlap of multiple data points).

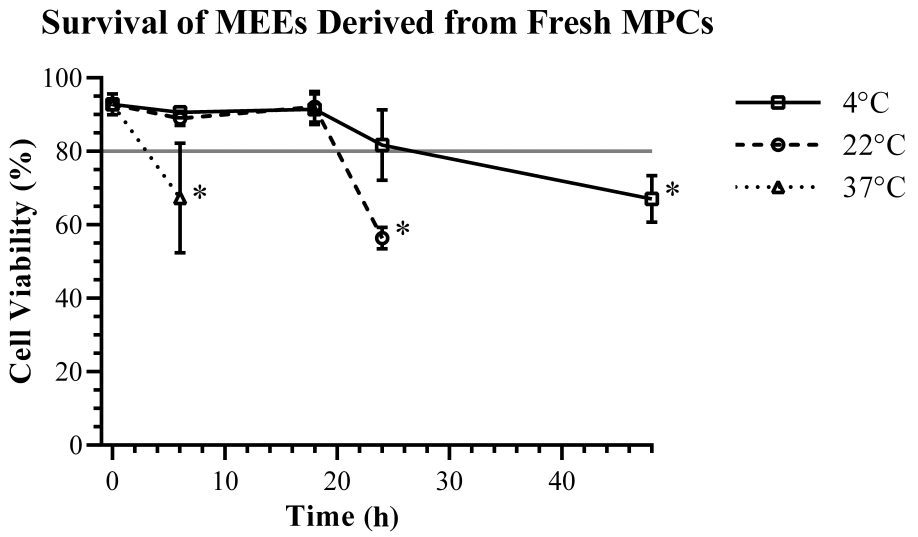

FIGURE 4 MEE (fresh derived) survival at differing transport temperatures and times reported as average percentage and error. The 80% viability cut-off was maintained over 24 h, was breached after 18 h, and was crossed within 6 h for the 4, 22, and 37°C temperature conditions, respectively ($n \geq 3$) (* = significant difference compared to freshly harvested MEE viability reached, testing discontinued). Interestingly, the viability of MEEs derived from frozen MPCs was consistently worse when compared with the fresh-derived cell MEEs (Figure S1).

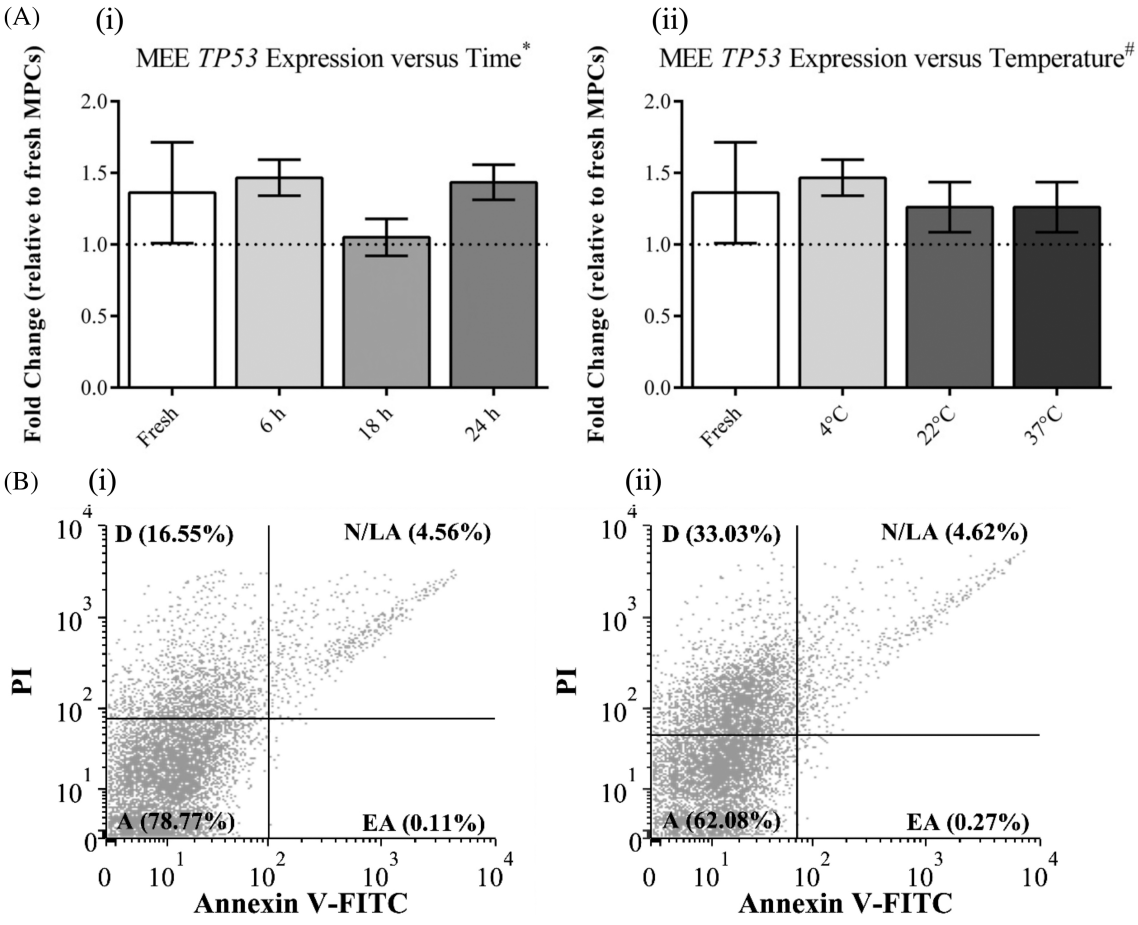

FIGURE 5 Apoptotic gene expression profiles of MEEs (derived from fresh MPCs) relative to freshly harvested MPCs that were freshly harvested and those subjected to differing transport (A-i) times and (A-ii) temperatures ($n \geq 3$). Representative dot plots of MEEs at (B-i) latest timepoint and at (B-ii) highest temperature (4°C 24 h and 37°C 6 h, respectively) displaying consistent relatively low percentages of cells in early apoptosis (A = alive, D = dead [PI+], EA = early apoptosis [Annexin V-FITC+], N/LA = necrosis/late apoptosis [PI+ and Annexin V-FITC+], * = constant temperature of 4°C, # = constant time of 6 h). Interestingly, the expression of p53 in MEEs derived from frozen MPCs was similar to that of freshly derived MEEs (Figure S2).

3.5 | Effects of suspension transport on MEE gene expression

Further analysis was conducted to examine the effect of transport on fresh-derived MEE functionality. Transport time and temperature appear to have a widespread effect on myogenic gene expression (Figure 6A,B). Regarding neurotrophic genes, transport time and temperature both had a widespread effect on *NTF3* expression (Figure 6C), but neither transport variable had a significant effect on *NTF4* expression (Figure 6D). Neither time nor temperature significantly impacted the expression of genes related to NMJs (Figure 6E,F). Temperature seemed to have a greater impact on angiogenic gene expression than time (Figure 6G).

4 | DISCUSSION

Critical to cellular therapeutic development is ensuring the cellular product profile is maintained from creation on-site to clinical administration off-site. MEEs present a special challenge given that self-innervating ability is entirely dependent on the capacity to maintain healthy but non-innervated MEPs. Thus, developing an effective transport protocol for autologous MEEs must maintain cells and surface MEPs in healthy states so they can actively release neurotrophic factors. Such a protocol will facilitate MEE utility in the clinical setting. Herein, we studied how MEE survival, health, and quality can be maintained through examining differences in transport state, medium, time, and temperature. Our study revealed that effective suspension-mediated transport is plausible in a medium free of toxic or xenographic additives when kept at colder temperatures, for up to 24 h.

4.1 | Effects of MPC cryopreservation on MEE behavior

Despite its utility for long-term storage of skeletal muscle cells, we found cryopreservation unsuitable for short-term transport.[26] Cryofreezing at the MPC stage deterred desirable gene expression when generating MEEs, as evident by statistical differences in all measured genes (Figure 2A). The cryopreservation process imparts inherent stress on cells through the freeze–thaw cycle leading to external structural and internal functional damage.[27] The upregulation of *NOS3* detected would be consistent with cellular strain, as *NOS3* has been shown to be related to oxidative and ischemic stress and is influential in angiogenesis.[28–30] Furthermore, our findings suggest cryopreservation-related stress may impact gene expression through multiple mechanisms: premature differentiation of MPCs or MPCs dedifferentiation (initial or sustained). The former is supported by the fact that *MyoD* expression returns to low levels near the end of the myoblast differentiation process.[31] The latter is supported by the upregulation of *PAX7*, potentially maintaining a pool of quiescent undifferentiated progenitor (satellite) cells that retain the ability for regenerative capacities, remodeling, and further proliferation.[31,32] Regardless of the exact mechanism, a shift away from intentional

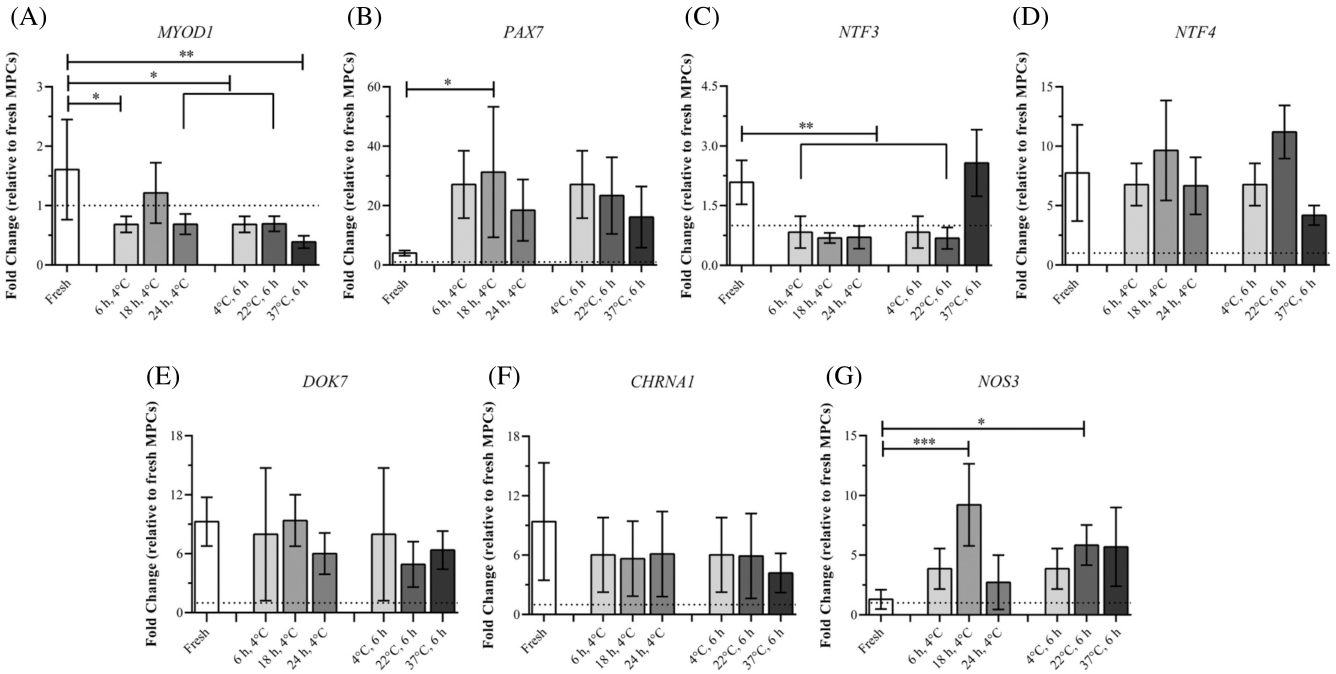

FIGURE 6 Gene expression profiles of freshly harvested MEEs (derived from fresh MPCs) in comparison with MEEs stored at various times and temperatures. Expression of (A) myogenic differentiation 1, (B) paired box 7, (C) neurotrophin 3, (D) neurotrophin 4, (E) docking protein 7, (F) cholinergic receptor nicotinic alpha 1 subunit, and (G) endothelial Nitric Oxide Synthase were measured and analyzed based on differences in transport time or temperature ($n \geq 3$) (*$p \leq .05$, **$p \leq .01$, ***$p \leq .001$, dotted line denotes fold change of 1).

differentiation during our MEE induction protocol was associated with decreased expression of NMJ-induction genes and *NTF3* (a factor in muscle spindle differentiation), feasibly explained by a protective physiologic shift toward premature differentiation or dedifferentiation.[33,34] Considering that *NTF3* and *NTF4* preferentially influence different receptor tyrosine kinase (Trk) pathways (TrkC and TrkB, respectively), the fact that *NTF3* expression declined while *NTF4* simultaneously increased is not surprising.[33] In contrast to *NTF3*, *NTF4* has been shown to be upregulated following injury, have protective effects, and assist in beneficial remodeling in aging muscle.[35–37] Thus, *NTF4* upregulation, similar to *NOS3*, likely plays a role in cellular stress response.

4.2 | Effects of MEE cryopreservation on MEE behavior

Cryopreservation at the MEE stage also revealed detrimental effects at a cellular level, observed by the statistically significant fall in cell viability at the studied timepoints (Figure 2B). Such falls in viability are likely due to the stress of the fresh-thaw cycle and less from transport time as similar end viabilities were observed at both 24 and 48 h in the cryopreserved groups.[27] Coupled with the fact that additional washes would be required to rid cell samples of toxic cryoprotectant additives to assure safety prior to injection, cryopreservation is not optimal for short-term transport to a clinical setting. However, further investigation is warranted to assess whether cryopreservation can significantly impact differentiated MEEs past the point of revival following long-term storage.

4.3 | Media pH test

With respect to its utility in suspension-mediated transport, a minimally supplemented DMEM-based medium proved effective per its buffering ability (Figure 3). DMEM contains alkaline bicarbonate, which holds a greater capacity to balance acidic metabolites produced by cells in anaerobic conditions when compared with PBS, which simply contains inorganic salts. By maintaining a medium pH above physiologic pH (7.4) across preferable temperature conditions for 48 h with cells in suspension, a cellular solution that is also free of toxic or xenographic additives would likely be safe to inject into humans after transport.

4.4 | Viability of fresh MEEs across transport conditions

When MEEs suspended in TM were formally subjected to differing transport conditions, the temperature had an inverse relationship with viability (Figure 4). This observation is consistent with the phenomenon that metabolic rate is related to environmental temperature.[38] Our TM contains essential amino acids and glucose. When temperature is low, plasma membranes are more rigid (limiting nutrients crossing) and activation energies of enzymatic reactions are not reached as easily.[38] This creates an environment promoting basal survival rather than proliferation. When temperatures are high, the opposite is true as illustrated by why many cells (including skeletal muscle) are cultured at 37°C. Along with increased metabolism and cell turnover are increases in the related internal metabolic stress and medium pH change (generating environmental stress). This explains why cell viability is maintained longest at the coldest 4°C temperature. An additional explanation for the apparent latent effect of transport time on viability in the 4 and 22°C samples, includes the fact that MEEs are being transferred from medium with serum (induction medium) to one without serum (TM) as they are harvested from culture flasks and prepared for transport. Such a change in preferred nutrient source, going from aerobic to anaerobic environments, and being held in cooler temperatures likely leads to changes in metabolism type occurring within MEEs. Transitioning from oxidative breakdown of lipids in serum to anaerobic glycolysis of glucose in serum-free medium takes time as a cell needs to sense the environmental change and then adjust internal mechanisms.

4.5 | Effects of transport on MEE apoptosis

Interestingly, apoptosis did not appear to be affected by transport conditions of time nor temperature as evidenced by gene expression and cell marker tagging (Figure 5). Despite this, there was an observed increase in *TP53* expression relative to MPCs. These observations appear consistent with the proposed theory that differentiated skeletal muscle cells have increased resistance to apoptosis secondary to increased expression of antiapoptotic factors, and as a result, preferentially undergo programmed autophagy.[39,40] This warrants attention, demonstrating potential added robustness of skeletal muscle for cellular therapy. This could also serve as direction for future study, as genes specifically upregulated in MEEs secondary to significant stress would provide insight into cell behavior.

4.6 | Effects of suspension transport on MEE gene expression

The FDA recommends that somatic cellular therapies should have at least 70% viability when submitting for approval.[25] We imposed a cutoff of 80% viability as suitable for MEE injection to further optimize the efficacy of this cellular therapeutic. In transport samples that maintained the viability cutoff, any change in gene expression (compared with freshly harvested MEEs) was within the relative acceptable range (Figure 6). *NTF4*, *DOK7*, and *CHRNA1* expressions did not differ significantly from freshly harvested MEEs in any of the analyzed samples. *NTF3* expression did decline over time in many samples. However, the fold change dropped minimally from 2 to 1 suggesting the drop may simply be a result of moving the MEEs out of the induction medium. With respect to *NOS3* expression, considering that at 4°C

expression increases at 18 h but decreases back to comparable levels at 24 h, such change is likely due to the slight inherent variability that is associated with testing gene expression. However, when analyzing *NOS3* expression in samples where temperature increases above 4°C, such observations are likely explained by the previously described relationship between temperature and metabolism (and related oxidative stress).[28–30] With the 18 h 4°C transport group being the only sample (paying close attention to 24 h 4°C) significantly different from fresh MEEs with respect to *PAX7* expression, this likely was also due to inherent variation of gene expression measurements. Despite widespread minimal decreases in *MyoD* fold change being similar to those seen in *NTF3*, it is worth mentioning that such an observation may be the response of the MEEs securing a sufficient satellite precursor cell pool as hypothesized in the cryopreserved samples.[31] This is noteworthy, as this reveals the ability of MEEs to maintain desirable gene expression and regenerative ability through the transport process.

5 | CONCLUSION

This study identifies a transport protocol for MEE cells to be shipped overnight within a physiologic culture medium, thereby standardizing bench-to-bedside processing. Our data reveal the robustness of MEEs through maintained cell viability, desirable gene expression, and regenerative capacity at cold temperatures (4°C) and various timepoints (up to 24 h), when suspended in a minimally supplemented DMEM-based medium. When clinically translated, the MEE cells in TM could be safely injected into the larynx upon arrival at any healthcare facility, thereby facilitating widespread distribution of this injectable therapy.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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