Gene Engineered Mesenchymal Stem Cells: Greater Transgene Expression and Efficacy With Minicircle Vs. Plasmid DNA Vectors in a Mouse Model of Acute Lung Injury

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Research

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Abstract

Background: Acute lung injury (ALI), and in its severe form the Acute respiratory distress syndrome (ARDS), results in increased pulmonary vascular inflammation and permeability, and is a major cause of mortality in many critically ill patients. Although cell-based therapies have shown promise in experimental ALI, strategies are needed to enhance potency of mesenchymal stem cells (MSC) to develop more effective treatments. Genetic modification of MSC has been demonstrated to significantly improve therapeutic benefits of these cells; however, the optimal vector for gene transfer is not clear. Given the acute nature of ARDS, transient transfection is desirable to avoid off target effects of long-term transgene expression, as well as the potential adverse consequences of genomic integration.

Methods: Here, we explored whether a minicircle DNA (MC) DNA vector containing human angiopoietin 1 (MC-ANGPT-1) can provide more effective platform for gene-enhanced MSC therapy of ALI/ARDS.

Results: At 24 hours after transfection, nuclear-targeted electroporation using a MC-ANGPT1 vector resulted in a 3.7 fold greater increase in human ANGPT1 protein in MSC conditioned media compared to the use of a plasmid ANGPT1 (pANGPT1) vector (2048±567pg/mL vs. 552.1±33.5pg/mL). In the lipopolysaccharide (LPS)-induced ALI model, administration of pANGPT1 transfected MSC significantly reduced bronchoalveolar lavage (BAL) neutrophil counts by 57%, while MC-ANGPT1 transfected MSC reduced it by 71% (p<0.001) by Holm-Sidak's multiple comparison test. Moreover, compared to pANGPT1, the MC-ANGPT1 transfected MSC significantly reduced pulmonary inflammation, as observed in decreased levels of proinflammatory cytokines tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-2 (MIP-2). pANGPT1 transfected MSC significantly reduced BAL albumin levels by 71% , while MC-ANGPT1 transfected MSC reduced it by 85%.

Conclusions: Overall, using a minicircle vector, we demonstrated an efficient and sustained expression of the ANGPT1 transgene in MSC and enhanced therapeutic effect on ALI model compared to plasmid. These results support the potential benefits of the MC-ANGPT1 gene enhancement of MSC therapy to treat ARDS.

Introduction

Acute respiratory distress syndrome (ARDS) was first described five decades ago in patients with tachypnea and refractory hypoxemia, as main symptoms complemented by diffuse opacities on chest radiographs (1). Common causes of ARDS include pneumonia, trauma, multiple transfusions, severe burns, and septic shock. The incidence of the disease ranges from 10 to 86 cases per 100,000 per year (2), with hospital mortality in those with severe ARDS at 46% (3). Many survivors have persistent pulmonary dysfunction, skeletal-muscle weakness and reduced health-related quality of life (4, 5). Lung-protective ventilation is employed to provide support for the failing respiratory system (6); however, patients diagnosed with ARDS still have limited treatment options and poor prognosis.
Mesenchymal stem cells (MSC) therapy is being considered as a promising intervention for treating ARDS due to the immunomodulatory and anti-inflammatory ability of these cells (7, 8). Previous studies have demonstrated that bone marrow-derived mesenchymal stem cells (BM-MSC) can reduce pulmonary inflammation and lung permeability (9–11). The immunomodulatory capacity of MSC is believed to be largely mediated through secretion of paracrine/endocrine factors and/or direct interaction with host immune cells (12, 13). There are ongoing translational efforts to study the safety and efficacy of MSC in patients with ARDS, including the recently completed START (14, 15) and MUST-ARDS trials (16). More recently, in rapid responses to COVID-19 pandemic, MSC are being deployed in human trials studying SARS-COV2-induced ARDS (17). For example, Leng et al. have reported improved pulmonary functions and symptoms of COVID-19 patients who received MSC therapy (18).

We have previously reported improved therapeutic efficacy of MSC in the LPS-induced acute lung injury (ALI) in mice can be enhanced by plasmid DNA angiopoietin-1 transfection (pANGPT1)(19). As a vascular protective factor, ANGPT1 reduces endothelial permeability and inhibits leukocyte-endothelium interactions by modifying endothelial cell adhesion molecules and cell junctions (20–23). However, plasmid bacterial sequences induce innate immune responses that limit the amount and duration of transgene expression and can exacerbate inflammation due to inherent bacterial CpG content (24). Minicircle DNA vectors consist of a circular expression cassette devoid of the bacterial plasmid DNA backbone, which results in less immunogenicity and more sustained transgene expression in quiescent cells/tissues (25, 26). Serra et al. demonstrated that MSC transfected with minicircles has higher VEGF expression compared to plasmid based transfection using an in vitro angiogenesis model (27). Additionally, Mun et al. showed minicircle delivery via microporation significantly improved transfection efficiency compared to cationic liposome system (28). Herein, we compared the minicircle DNA system to a conventional plasmid vector for the transfection of MSC, and examined the potential impact on therapeutic benefit in ALI murine model.

**Materials And Methods**

**Characterization, Culture, and Transfection of MSC**

A frozen vial of murine MSC (isolated from male C57Bl/6J mice; courtesy of Tulane Center for Gene Therapy, New Orleans, Louisiana, United States) was thawed and expanded as previously described (29). Differentiation of MSC was evaluated using a Mesenchymal Stem Cell Functional Identification Kit (R&D Systems,) per manufacture instruction and previous publication (19). The full-length coding sequence of human ANGPT1 (1115 bp) was cloned into the expression vector pVAX-CMV-1 (Sigma,) as previously described (30). For minicircle (MC)-Ang-1 cloning, the full-length coding sequence Templet Ang-1/pENTR223.1 was used. The ZYCY10P3S2T E.coli in the presence of Kanamycin 50 µg/ml was used for minicircle production. Competent bacteria have been inserted with parental CMV-Ang-1 to generate MC-Ang-1 by addition of 20% L-arabinose. Using Minicircle Production Strain approach, the bacterial backbone is excised and degraded (SBI technology) (Fig. 1A). The circular DNA fragments from the parental plasmid (Minicircle) was extracted using the EndoFree Plasmid Maxi Kit (Qiagen). MSC were
transfected by nuclear-targeting electroporation using nucleofection (Amaxa, Lonza). MSC were nucleofected with empty plasmid (pVAX), human ANGPT1 plasmid (pANGPT1), empty minicircle DNA (MC), and human ANGPT1 minicircle DNA (MC-ANGPT1). Cells receiving transfection reagent only were used as transfection control (mock). To evaluate ANGPT1 protein expression over time, media was changed daily and storage for further analysis by ELISA (R&D Systems), following manufacturers’ recommended protocols. Cell viabiliaty was evaluated by trypan blue exclusion.

**Murine Model of LPS-Induced ALI**

All animal procedures were approved in advance by the Animal Care Committee of University of Ottawa (Ottawa, Ontario, Canada, protocol number OGH/RI-42). C57Bl/6J Female mice (10–12 weeks) were anaesthetized with 120 mg/kg of ketamine with 6 mg/kg of xylazine. Acute lung injury was induced by intratracheal instillation of 5 mg/kg of LPS (100 µg/mouse, *E. coli* 055:B5; Sigma) dissolved in 30 µL of normal sterile saline. A suspension of $2.5 \times 10^5$ cells MSC in PBS (100 µl total volume) was slowly infused to each mouse via a jugular venous canula 30 min following LPS challenge.

After infusion, the canula was withdrawn, the vein ligated, and the incision sutured using silk suture. Mice were then sacrificed 3 days after LPS to evaluate the therapeutic efficacy by collecting tissues for analysis. Bronchoalveolar lavage (BAL) fluid were obtained by inserting a catheter into the trachea, and slowly injecting and aspirating 1 mL of saline three times. Cells and fluids obtained from BAL were used for analysis. Total cell counts were determined using a hemocytometer. BAL was then centrifuged at 800 $g$ for 10 min at 4 °C, and supernatant was collected and storage at -80 °C for further analysis; cell pellet was resuspended and cytopspun onto slides. Differential cell counts were determined on BAL cytospin slides stained with Hemacolor (EMD Chemicals). Number of neutrophils was calculated as the percentage of neutrophils multiplied by the total number of cells in the BAL fluid sample. All analyses were performed in a blinded fashion.

**Measurement of Albumin, IgM, Cytokines, and Chemokines**

Albumin and IgM levels in BAL fluid samples were measured using with a murine-specific albumin ELISA kit (ALPCO Diagnostics) and a murine-specific IgM ELISA kit (Bethyl Laboratories), following manufacturers’ recommended protocols respectively. Cytokine and chemokine levels (IFN-γ, TNF-α, IL-6, IL-1β, MIP-2 and MCP-1) in BAL fluid were measured multiplex Luminex immunoassays kit (Bio-Rad).

**Statistics and Softwares**

Data in figures are represented as individual data points in a scatter plot with bar to indicate the mean. Differences between the treated groups versus the injured group (LPS/PBS) were assessed using a one-way ANOVA (with post hoc comparisons using Bonferroni or Holm-Sidak’s test) with statistic software (GraphPad Prism version 8). A value of $p < 0.05$ was considered statistically significant. Illustration of generating minicircle was created using BioRender.com.
Results

Minicircle DNA Transfected MSC Released Significantly More ANGPT1 Proteins Compared to Plasmid Transfection

Significantly higher levels of ANGPT1 proteins were detected in conditioned media of MSC transfected with ANGPT1 coding sequence using minicircle (MC-ANGPT1) vs. plasmid (pANGPT1) vectors at 24 hours post transfection (2048 ± 567 pg/mL vs. 552.1 ± 33.5 pg/mL, respectively; Fig. 1B). ANGPT1 daily production was highest at day 1 and 2 after MC-ANGPT1 transfection, followed by reduction at the day 3 to 719.6 ± 54.3 pg/mL and continued to decrease by day 7 to 487.2 ± 22.5 pg/mL, with an empty MC vector at 361.4 ± 18.4 pg/mL, or with mock transfection at 384.6 ± 19.6 pg/mL (Fig. 1C). MSC transfected with MC-ANGPT1 and pANGPT1 showed similar morphology and viability at 24 h after transfection (90.2 ± 2.3 vs 89.8 ± 3.3; Fig. 1D). Overall, our data showed transfection in MSC using minicircle can result in higher level of ANGPT1 protein overexpression compared to using plasmid.

Overexpression of ANGPT1 with MC-DNA Vector Transfection Strategy Enhanced Therapeutic Efficacy of MSC in an Animal Model of Acute Lung Injury

Acute lung injury was induced by intratracheal LPS challenge in mice (Fig. 2A), followed by treatment with PBS or MSC (5 groups) at 30 minutes, and mice were then sacrificed at 3 days after LPS. Lung airspace inflammation, evaluated by number of neutrophils in bronchoalveolar lavage (BAL), was significantly reduced in all MSC-treated mice (MSC transfected with empty plasmid or MC, pANGPT1 or MC-ANGPT1 compared to LPS/PBS group), while mice receiving MC-ANGPT1 transfected MSC showed a strong trend towards reduced lung inflammation compared to pANGPT1 transfected MSC (p = 0.07) (Fig. 2B).

Overexpression of ANGPT1 using MC-DNA Vector Significantly Reduced Pulmonary Inflammation Compared to Plasmid-Based Strategy

We next assessed pulmonary inflammation caused by LPS-induced ALI by measuring the levels of inflammatory cytokines in BAL fluids. Proinflammatory cytokines, such as TNF-α, IFN-γ, IL-1β, IL-6, MCP-1 and chemokine MIP-2, were all significantly elevated in BAL fluid of mice in LPS/vehicle group in response to LPS exposure, compared to naïve animals receiving vehicle only (Fig. 3). Treatment with MSC (with mock transfection, empty plasmid or empty minicircle) decreased the level of proinflammatory cytokines, while transfected with MC-ANGPT1 decreased cytokine levels significantly to levels close to the baseline values observed in naïve mice (p < 0.0001, LPS/vehicle versus pANGPT1 or MC-ANGPT1 transfected MSC-treated groups). Furthermore, MC-ANGPT1 transfected MSC resulted in significantly greater reduction in pulmonary cytokine levels compared to pANGPT1 transfected MSC (p = 0.001 for TNF-α, p = 0.05 IFN-γ, p = 0.0001 IL-6, p = 0.0001 MCP-1 and p = 0.0001 MIP-2; Fig. 3).

Overexpression of ANGPT1 using a MC-DNA Vector was Significantly More Effective in Reducing Pulmonary Vascular Permeability
The LPS administration induced increase in pulmonary vascular permeability due to the loss of integrity of the alveolar-capillary membrane barrier, which can be detected by measuring levels of high molecular weight proteins such as albumin (Fig. 4A) or IgM (Fig. 4B) in BAL fluid. Compared to sham mice (sham/vehicle group), mice challenged with LPS (LPS/vehicle group) showed significant increases in albumin and IgM ($p < 0.0001$). Mice treated with non- or mock-transfected MSC showed modest reduction in pulmonary vascular permeability, while mice receiving MSC transfected with ANGPT1 (MSC with pANGPT1 or MC-ANGPT1 transfection) showed further reduction. However, MC-ANGPT1 transfected MSC showed the greater reductions of albumin and IgG in BAL fluids compared pANGPT1-transfected MSC ($p < 0.05$ for albumin; $p < 0.0001$ for IgM, Fig. 4).

**Discussion**

We have previously reported improved therapeutic efficacy of MSC engineered to overexpress ANGPT1 using plasmid DNA, in the LPS-induced ALI in mice (19). Here, we showed that overexpression of ANGPT1 using minicircle can further reduce alveolar neutrophil infiltration, pulmonary inflammatory mediators, and lung vascular permeability compared to treatment using the traditional plasmid system.

In recent years, various strategies have been reported to increase MSC therapeutic potential *in vitro* and *in vivo*. Pre-treatment of MSC using hypoxia, pharmacological agents, physical or chemical factors has been shown to improve MSC survival and even boost potency of MSC in angiogenesis and immunomodulation (31). However, the pre-treatment approach has the disadvantage of the effect being weaken or lost immediately after removal of the pre-treatment factor. In contract, genetic modification of MSC may offer a more persistent beneficial effect compared to pre-treatment strategy. The overexpression or knockdown of specific genes has been used to enhance MSC therapeutic potential by modulating survival, migration, adhesion and, regenerative and immunomodulatory effects (32). Promising therapeutic benefits were also obtained after injection of MSC in various preclinical models, such as heart and liver failure, blood production, neural injuries, sepsis and cancer (33).

Plasmids are circular or linear extrachromosomal DNA elements found in genomes of prokaryotes (34). Though it is an essential and ubiquitous tool applied in molecular biology, traditional plasmid itself has many limitations for being used in genetic modification of MSC for therapeutic purposes. The classical plasmid systems typically have low efficiency in transfecting primary cells with shorter duration of transgene expression. Additionally, traditional plasmid has been reported to lead to unwanted inflammatory response due to its unmethylated bacterial backbone, which in turn silences the transgenes and/or elimination of the plasmid from the cytoplasm (35).

To produce an enhance and sustained transgene expression, optimization of plasmid construction technology had been evolving. Modifications have been explored in regions of the promoter and enhancer elements, polyadenylation signals, as well as removal of CpG motifs and antibiotic resistance genes (36). The minicircle system described in the current manuscript is a plasmid-based, minimized DNA vectors (26). To obtain minicircles, recombination sequences are added between the transgene expression
cassette and the bacterial backbone. Using a minicircle producer *E. coli* strain, the addition of L-Arabinose turns on the recombination process and elimination of the plasmid backbone. This process results in a significant removal of CpG motifs and a reduced size which, when used for transfection, can lead to less immunogenic responses and higher transfection efficiency (25, 26).

Indeed, minicircle has been described to have enhanced and prolonged transgene expression in quiescent cells when applied *in vitro* or *in vivo*, such as in airway cells (37). Dad *et al.* used minicircle vector-mediated delivery of nucleases, such as Zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), for genome edition in HEK 293T cells. They demonstrated that this approach was more efficient, safe and less toxic than conventional plasmid (38). *In vivo* delivery (muscle injection) of short hairpin RNA interference targeting prolyl hydroxylase-2 (shPHD2) using minicircles vectors, compared to vehicle and plasmid-treated mice, showed improved postischemic neovascularization after hindlimb ischemia (39). Minicircles deployed with magnetofection were shown to achieve high, safe and sustained transgene expression (up to 4 weeks) transfection levels in primary neural stem cells (NSC) *in vitro* compared to the plasmid system. Furthermore, NSC daughter cells, such as neurons and oligodendrocytes, also showed transgene expression in minicircle engineered model, which was not observed when using the conventional plasmid transfection system. (40).

**Conclusion**

In the current study, we demonstrated for the first time ANGPT1 encoded in MC-transfected MSC, compared to plasmid, can achieve a more efficient and sustained expression of the ANGPT1 protein levels, leading to an enhanced therapeutic effect in rescuing animal model of ALI model. By overexpressing ANGPT1 in MSC, we demonstrated that genetic modification of MSC can significantly improve the therapeutic benefits of these cells; therefore, leading to a promising therapeutic approach to treat ARDS. The robust effects of MC-MSC on inflammatory mediators and permeability in the ALI model suggest this strategy represents an alternative strategy to the conventional non-viral plasmid vectors on genetic modification of MSC. Cellular and gene therapy is an innovative, disruptive field, and a lot of progress is being made. With advances in less immunogenic and more efficient transgene expression, genetically modified MSC will remark the next generation of MSC therapies in the near future.

**Abbreviations**

ALI  
Acute Lung Injury  
ANGPT-1  
Angiopoietin 1  
ARDS  
Acute Respiratory Distress Syndrome  
BAL  
Bronchoalveolar Lavage
IL-6
Interleukin-6
IFN-γ
Interferon Gamma
LPS
Lipopolysaccharide
MC
Minicircle
MCP-1
Monocyte Chemoattractant Protein-1
MIP-2
Macrophage inflammatory protein-2
MSC
Mesenchymal stem cells
pANGPT1
plasmid ANGPT1
shPHD2
short hairpin RNA
TALENs
transcription activator-like effector nucleases
TNF-α
Tumor Necrosis Factor Alpha
VEGF
Vascular Endothelial Growth Factor
ZFNs
Zinc Finger Nuclease

Declarations

Ethics approval and consent to participate: All animal procedures were approved in advance by the Animal Care Committee of University of Ottawa (Ottawa, Ontario, Canada, protocol number OGH/RI-42).

Consent for publication: Not applicable

Availability of data and materials: Not applicable

Competing interests: The funding institution had no role in the conception, design or conduct of the study, data collection or analysis, interpretation or presentation of the data, or preparation, review or approval of the manuscript. We also like to declare the following conflicts of interest: D.J.S. holds a patent for MSC therapy for the treatment of acute lung injury, and S.H.J.M. and Y.D. have received personal fees from
Northern Therapeutics that are outside of this submitted work. The remaining authors have disclosed that they do not have any conflicts of interest.

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**Authors’ contributions:** MF: manuscript preparation and interpretation of data. JW and YD: acquisition of data. LSM, DJS: manuscript preparation. SHJM: study design, interpretation of data and statistical analysis, manuscript preparation.

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