Received: 18 March 2020 | Accepted: 15 July 2020

DOI: 10.1002/sctm.20-0122

TISSUE ENGINEERING AND REGENERATIVE MEDICINE

STEM CELLS TRANSLATIONAL MEDICINE

Overexpression of alpha-1 antitrypsin in mesenchymal stromal cells improves their intrinsic biological properties and therapeutic effects in nonobese diabetic mice

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Funding information
Department of Veterans Affairs, Grant/Award Number: VA-ORD BLR&D Merit I01BX004536; National Institute of Diabetes and Digestive and Kidney Diseases, Grant/Award Numbers: DK118529, DK120394, 1R01DK105183

Abstract

Islet/β cell dysfunction and death caused by autoimmune-mediated injuries are major features of type 1 diabetes (T1D). Mesenchymal stromal cells (MSCs) have been used for the treatment of T1D in animal models and clinical trials. Based on the anti-inflammatory effects of alpha-1 antitrypsin (AAT), we generated human AAT engineered MSCs (hAAT-MSCs) by infecting human bone marrow-derived MSCs with the pHAGE CMV-a1aT-UBC-GFP-W lentiviral vector. We compared the colony forming, differentiation, and migration capacity of empty virus-treated MSCs (hMSC) and hAAT-MSCs and tested their protective effects in the prevention of onset of T1D in nonobese diabetic (NOD) mice. hAAT-MSCs showed increased self-renewal, better migration and multilineage differentiation abilities compared to hMSCs. In addition, polymerase chain reaction array for 84 MSC-related genes showed that 23 genes were upregulated, and 3 genes were downregulated in hAAT-MSCs compared to hMSCs. Upregulated genes include those critical for the stemness (ie, Wnt family member 3A [WNT3A], kinase insert domain receptor [KDR]), migration (intercellular adhesion molecule 1 [ICAM-1], vascular cell adhesion protein 1 [VICAM-1], matrix metalloproteinase-2 [MMP2]), and survival (insulin-like growth factor 1 [IGF-1]) of MSCs. Pathway analysis showed that changed genes were related to growth factor activity, positive regulation of cell migration, and positive regulation of transcription. In vivo, a single intravenous infusion of hAAT-MSCs significantly limited inflammatory infiltration into islets and delayed diabetes onset in the NOD mice compared with those receiving vehicle or hMSCs. Taken together, overexpression of hAAT in MSCs improved intrinsic biological properties of MSCs needed for cellular therapy for the treatment of T1D.

KEYWORDS

diabetes onset, human α-1 antitrypsin, mesenchymal stromal cells, stemness, type 1 diabetes

Charlie Strange and Hongjun Wang share senior authorship.

1 | INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease in which the immune system attacks insulin-secreting pancreatic β cells, leading eventually to β cell death and the deficiency of insulin. More than 1.25 million Americans are living with T1D, including 200 000 youths (<20 years) and more than 1 million adults (≥20 years). Therapies for T1D include a monitored diet, daily insulin to maintain blood glucose levels, immunotherapy, and pancreas and islet transplantations.[1] However, insulin therapy is not a cure, and years of research on pancreas or islet transplantation have shown only limited success.[2,3] Therefore, less than one-third of people with T1D are consistently achieving target blood glucose control levels. Given the enormity and cost of the problem, implementation of effective preventive or treatment strategies for T1D is vital.

Mesenchymal stromal cells (MSCs) are rare adult stem cells in the body that can migrate to sites of injury and exert immunomodulatory, tissue repair, and regenerative functions.[4] They can be isolated from the bone marrow, adipose, placental, umbilical cord, or other tissues and easily expanded to apply toward therapeutic doses ex vivo.[5] They are identified by plastic-adherence; expression of cell surface markers including CD29, CD90, CD44, and CD73; lacking expression of CD45, CD34, CD11b, CD19, and HLA-DR; and preserving differentiation potential into adipocytes, chondrocytes, and osteocytes.[6,7] MSCs exert protective effects through the release of pro-mitotic, antiapoptotic, anti-inflammatory, and immunomodulatory soluble factors while mitigating metabolomic and oxidative stress imbalance and restoring homeostasis. The immunosuppressive properties are based on their production of nitric oxide, indoleamine 2,3 dioxygenase, transforming growth factor β (TGF-β), and many other substances.[8-12] The immunomodulatory function of MSCs is observed in many disease models including encephalomyelitis,[13] T1D,[14] multiple sclerosis,[15,16] and graft vs host disease.[17]

Studies demonstrate that systemic infusion of MSCs in murine models of diabetes delay onset of diabetes, improve glycemic control, reduce pancreatic insulitis, prevent autoimmune destruction and regenerate pancreatic tissue.[18-21] Importantly, intravenously infused MSCs migrate to pancreatic islets or the injured pancreas in the T1D mouse models.[22,23] The safety and efficacy of MSC therapy has been tested in clinical trials for patients with new onset T1D[24,25] or chronic pancreatitis with autologous islet transplantation.[26]

Alpha-1 antitrypsin (AAT) is an acute phase reactant and serine protease inhibitor that inhibits various enzymes including neutrophil elastase, cathepsin G, and others.[27,28] AAT is synthesized primarily in the liver, but also is expressed in macrophages, monocytes and epithelial cells.[29] Human AAT (hAAT) is used as a drug for AAT deficiency.[30] AAT also possesses anti-inflammatory effects through suppressing cytokine production, complement activation, and immune cell infiltration,[31-34] inhibiting cell apoptosis,[35-40] and promoting cell growth and proliferation.[41,42] A single injection of AAT to nonobese diabetic (NOD) mice reduced the intensity of insulitis, increased β cell mass, promoted β cell regeneration, and prevented the onset of diabetes via modulating T regulatory cells.[39] AAT infusion improved islet survival and function after transplantation.[40,43] In this study, we tested whether overexpression of hAAT in MSCs enhances their intrinsic biological properties needed for MSC cell therapy. In addition, we also infused them into the NOD mice and evaluated the resulting effects on the prevention of diabetes onset.

Significance statement

The use of mesenchymal stromal/stem cells (MSCs) as a therapeutic tool represents a promising new intervention as increasing evidence demonstrates that MSC therapy can effectively target several injury pathways in a variety of autoimmune and inflammatory diseases. However, most human studies using MSCs alone have not been successful at sustained suppression of the autoimmune response. Based on the protective effects of alpha-1 antitrypsin (AAT), we generated AAT-overexpressing MSCs and demonstrated improved innate properties of MSCs with sustained efficacy in the prevention of onset of type 1 diabetes. Such a novel approach may help improve the efficacy of MSC therapy for diabetes.

2 | MATERIALS AND METHODS

2.1 | Mice

Female NOD mice at 6-8 weeks of age were purchased from the Jackson Laboratory (Bar Harbor, Maine) and used for the experiments. All mouse studies were approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina.

2.2 | Culture of MSCs

Bone marrow specimens from healthy donors were purchased from ATCC (Old Town Manassas, Virginia). Cells were isolated from bone marrow using gradient separation and cultured in complete medium (Dulbecco's modified Eagle's medium [DMEM], supplemented with 10% fetal bovine serum (FBS), 1% penicillin and streptomycin) in 5% CO_2 atmosphere at 37°C. Cells between passages 3 and 6 were used for the experiments.

2.3 | Lentivirus infection

Human MSCs were transduced with the pHAGE CMV-a1aT-UBC-GFP-W lentiviral vector encoding hAAT with the green fluorescent protein (GFP) reporter or with control lentiviruses as described previously.[44] In brief, MSCs at passages 2-3 were seeded at a density of 0.5×10^6 cells per well in 6-well plates and cultured overnight at

37°C with 5% CO_2. Cells were then incubated with viruses at multiplicity of infection of 20 for 16 hours before changing to fresh complete medium. Presence of GFP^+ cells were detected under a fluorescent microscope 96 hours after viral infection.

2.4 | Western blot

Cells were lysed in ice-cold protein lysis buffer. Proteins (30 μg) were separated by SDS-PAGE, transferred to PVDF membranes, and incubated with antibodies against hAAT (Cell signaling Technology, Danvers, Massachusetts), or glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and followed by incubation with the horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology). Signals were visualized using an ECL detection kit (Thermo Fisher Scientific, Waltham, Massachusetts).

2.5 | Enzyme-linked immunosorbent assay

Cells were cultured in six-well plates in complete medium with antibiotics in the absence of FBS for 96 hours. Cell culture supernatant was collected and hAAT levels in cell culture medium were measured using the human AAT enzyme-linked immunosorbent assay kit (Abcam, Cambridge, UK) according to the manufacturer's recommendations.

2.6 | Colony-forming unit-fibroblast assay

MSCs were seeded at a density of 150 single cells per well in a six-well plate. Cell culture medium was replaced every 4 days. Fourteen days after incubation, cells were washed twice, fixed with 100% methanol and stained with 0.5% crystal violet. Colonies were observed under a microscope and those containing at least 150 cells with fibroblast morphology were scored as a colony-forming unit-fibroblast (CFU-F) colony.

2.7 | MSCs differentiation assay

MSCs were seeded in 12-well plates and grew to 100% confluence for the differentiation assay. For adipocyte differentiation, cells were incubated in DMEM supplemented with 10% FBS, 50 μg/mL ascorbic acid, 0.5 mM 1-methyl-3-isobutylxanthine (IBMX), 10 nM dexamethasone and 10 μg/mL insulin. At 2 weeks after treatment, cells were washed and fixed with 10% neutral buffered formalin for 30 minutes, and then stained with Oil Red O for 50 minutes before observation. Osteogenic differentiation was induced by culturing MSCs in DMEM supplemented with 10% FBS, 50 μg/mL ascorbic acid, 10 mM β-glycerophosphate, and 10 nM dexamethasone for 3 weeks. The final product was fixed with 10% neutral buffered formalin for 30 minutes, and then stained with Alizarin Red (pH = 4) for 1 hour before observation. Chondrogenic differentiation was induced using a pellet culture system by culturing the cell pellets in 15-mL conical tubes containing DMEM supplemented with 5% FBS, 1% L-glutamine, 10% ITS and Premix tissue culture supplement (Invitrogen), 100 nM dexamethasone and 10 ng/mL transforming growth factor beta (TGF-β, R&D system) for 21 days. The aggregates were fixed with 10% neutral buffered formalin overnight, frozen in OCT, sectioned and stained with Alcian blue as described.[45] All reagents were from Sigma-Aldrich unless otherwise stated.

2.8 | Real-time quantitative polymerase chain reaction

RNA was extracted from cells using the RNeasy Micro kit (Qiagen) and reverse transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad). mRNA expression of the adipogenic marker peroxisome proliferator-activated receptor 2 (PPAR 2), osteogenic markers (Runx2 and osteocalcin), and the chondrogenic marker (type II collagen a1 chain) were measured in differentiated MSCs by quantitative polymerase chain reaction (qPCR). The reaction was performed in SYBR green Mastermix (Bio-Rad) on a CFX-96 Real-Time PCR system thermal cycler. Gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method, and relative gene expression levels were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.9 | RT2 profiler PCR array for MSCs

RNA was extracted from MSCs followed by cDNA synthesis according to the manufacturer's instructions (QIAGEN). Expression of 84 apoptosis-related genes was analyzed using RT^2 Profiler PCR Array Human Mesenchymal Stem Cells (Qiagen, 330 231 PAHS-082ZA) using the CFX96 Real-Time PCR Detection System from Bio-rad (Hercules, California).

2.10 | Cell wound healing assay

MSCs (0.5×10^6 per well) were seeded in six-well cell culture plates and cultured overnight to reach 90% confluence. A straight line in the cell monolayer was generated by scratching with a sterile 1-mL pipette tip across the center of the cell culture plate. Floating cells were removed, and cells were cultured in serum-free medium to avoid any effect of FBS on cell proliferation. Cells were observed after 24 hours. Percentage of scratch closure was measured and quantified by the ImageJ software.

2.11 | Cell migration assay

The directed migratory capacities of MSCs toward culture medium containing 30% FBS were evaluated using a modified Transwell

migration assay. MSCs (10 000 cells/cm^2) were placed in the top unit of an 8-mm pore filter Transwell chamber (BD Falcon) using the Corning FluorBlok Cell Culture Insert (Corning Inc. Corning, New York), and incubated in serum-free medium for 24 hours. The top units were then placed into a well that containing medium with 30% FBS. After overnight incubation, nonmigratory cells were removed from the upper side of the filter, and the filter was stained with the crystal violet to quantify cells migrated to the side containing 30% FBS as described.[46] Area covered by cells divided by total area was calculated.

2.12 | Cell proliferation assay

The proliferating rates of MSCs were measured using the Cell Proliferating kit II (Sigma-Adrich, St. Louis, Missouri) according to the manufacturer's instructions. Cells were dispersed into 100 μL of cell suspension in 96-well-plate (2000 cells/well) and cultured. Cell proliferation was measured using the Cell Counting Kit 8 Assay Kit (Abcam). In brief, cells were incubated with 10 μL of the Cell Counting Kit-8 solution for 1 hour and the absorbance at 450 nm was measured using a microplate reader. Cells were counted daily for 7 days.

2.13 | MSCs infusion into NOD mice

Female NOD mice at 8 weeks of age were given an intravenous infusion of hMSCs or hAAT-MSCs (0.5×10^6/mouse, in 0.2 mL of PBS). Mice receiving 0.2 mL of PBS were used as controls. Non-fasting blood glucose levels of mice were measured weekly starting at week 12 until 25 weeks of age using the Freestyle Lite blood glucometer (Abbott Inc., Abbott Park, Illinois). Mice with two non-fasting consecutive blood glucose levels of >300 mg/dL were considered diabetic.

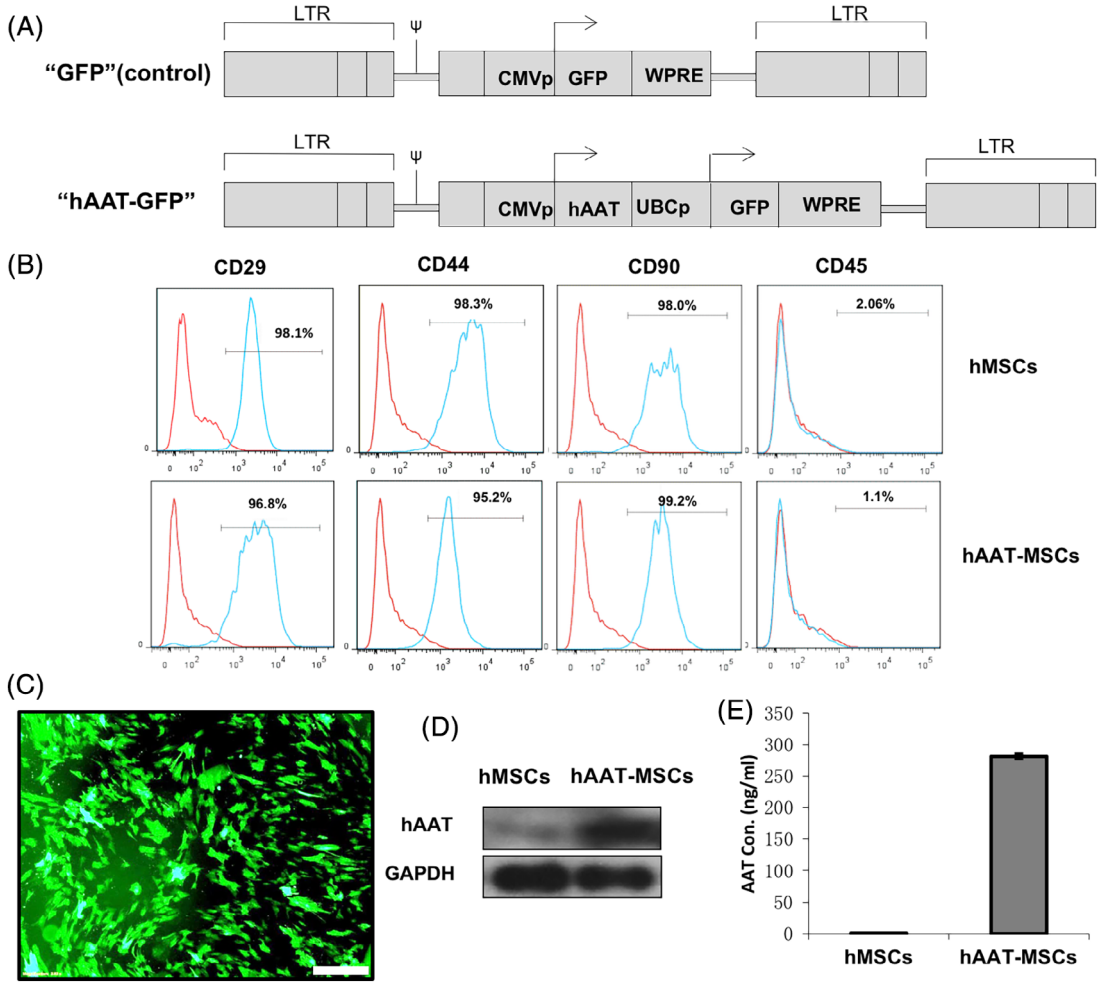

FIGURE 1 Generation and characterization of hAAT-MSCs. A, Schematic of the pHAGE CMV-hAAT-UBC-GFP-W and the control lentiviral constructs employed for MSC transduction. B, Representative FACS analysis of MSC surface makers CD44, CD29, CD90, and CD45 on hMSCs and hAAT-MSCs. C, GFP expression in hAAT-MSCs 96 hours after infection with the CMV-hAAT-GFP lentivirus, observed under a fluorescent microscope. Scale bar: 100 μm. D, hAAT protein expression in hAAT-MSCs or control MSCs at 14 days after viral infection measured by Western blot analysis. E, hAAT levels in cell culture medium of hMSCs or hAAT-MSCs measured by the human AAT ELISA kit (Abcam). hAAT-MSCs, human MSCs treated with hAAT-virus; hMSCs, human MSCs treated with control virus

2.14 | Hematoxylin and eosin staining of pancreas

The pancreases from female NOD mice treated with vehicle, hMSCs or hAAT-MSCs were collected 3 weeks after treatment (n = 6 in each group), fixed, and embedded in paraffin. At least three sections spanning every 100 μm of pancreas were selected from serial sectioned slides and stained for hematoxylin and eosin (H&E) using standard protocol.

2.15 | Statistical analysis

All in vitro experiments were repeated independently at least three times. Data were expressed as mean ± SEM. Differences between groups were compared for statistical significance by analysis of variance or Student's *t* tests. For the RT-PCR analysis, *P*-value was calculated using a parametric, unpaired, two-tailed distribution model with equal variance. The percentage of mice with hyperglycemia (onset of T1D) was plotted using Kaplan-Meier analysis using the Graphpad Prism 8.2.1 software, and area under the curve was calculated. Differences in the % of diabetic mice by week were not normally distributed and between group differences were calculated using the Log rank test. Serial non-fasting glucoses were normally distributed and were analyzed by analysis of variance with Tukey's multiple comparison test. $P < .05$ was considered significant.

3 | RESULTS

3.1 | hAAT-MSCs consistently secreted high levels of hAAT

Wilson and colleagues showed that pHAGE CMV-a1aT-UBC-GFP-W lentivirus-infected macrophages produced and secreted hAAT protein of identical size to normal hAAT protein that existed in human serum. The secreted biologically active hAAT was able to inactivate neutrophil elastase after 24 weeks of sustained gene expression, with no humoral or cellular immune responses to hAAT protein detected.[44,47] Based on these studies, we used the same viruses to infect MSCs (Figure 1A). We first analyzed the expression of MSC markers in transfected cells. More than 95% of hMSCs and hAAT-MSCs were positive for CD29, CD44, and CD90 and were negative for CD45. The majority of cultured cells were GFP positive after viral infection (Figure 1C). Increased hAAT expression was confirmed in hAAT-MSCs by Western blot (Figure 1D). Two weeks after transduction with viruses, the levels of hAAT secreted reached 280 ng/mL in hAAT-MSCs (Figure 1E). A high level of GFP expression and hAAT secretion were maintained throughout the study. Together, the above results demonstrate that we have generated MSCs that stably over-produce and secrete hAAT.

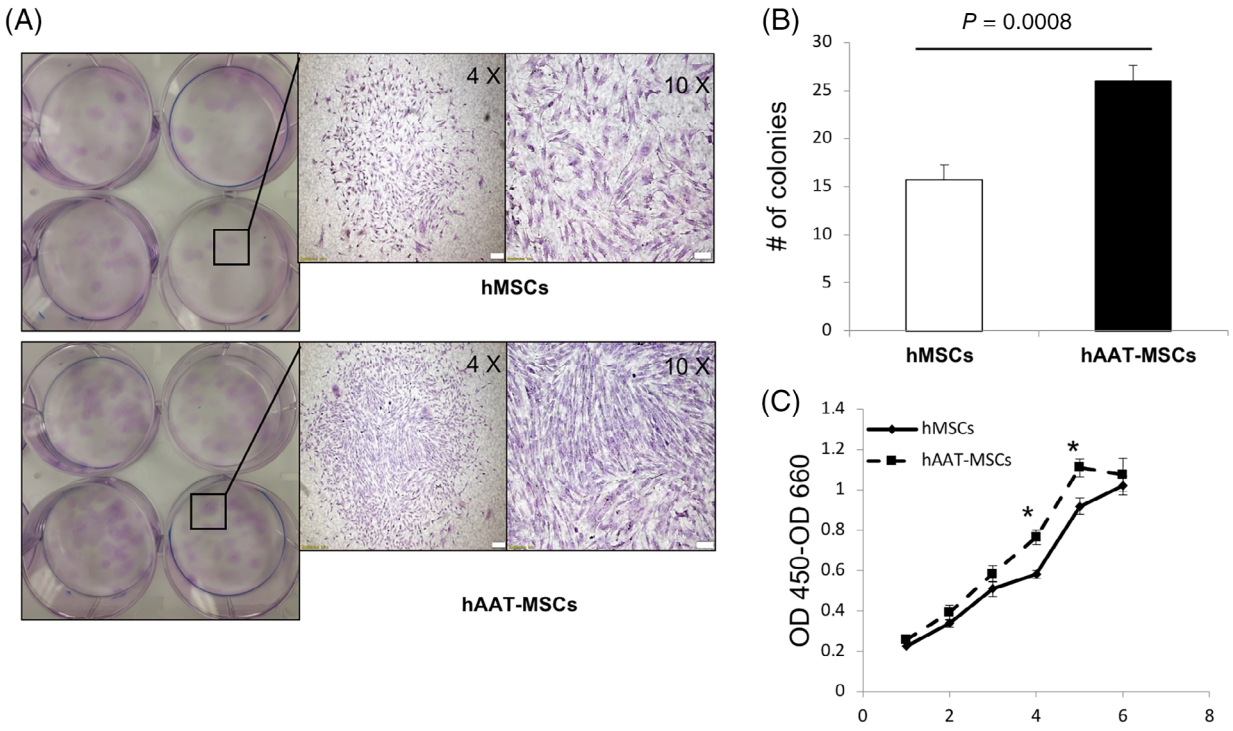

FIGURE 2 Colony forming capacity of hAAT-MSCs and hMSCs. A, Representative plates and total colony forming units colonies in hAAT-MSCs and hMSCs stained with crystal violet. Scale bars in b&e: 200 μm; Scale bars in c&f: 100 μm. B, Numbers of colonies at 14 days after culture in hAAT-MSCs and hMSCs. C, Cell proliferation rate measured at OD450-OC660 by the XTT assay. Bars represent mean ± SD. ***$P < .001$, Student's *t* test

3.2 | The CFU-F capacity of hAAT-MSCs in comparison to MSCs

Stem cells are expected to have robust clonal self-renewal ability. in vitro self-renewal capacity of hAAT-MSCs and MSCs were assessed by the CFU-F assay. The frequency of CFU-Fs was significantly higher in hAAT-MSCs compared with MSCs, with an average 26.50 ± 2.0 vs15.50 ± 1.5 colonies formed per 150 cells (Figure 2A,B). We further evaluated the proliferation rate of these cells. hAAT overexpression significantly increased cell proliferation compared with control MSCs, as shown by the Cell Proliferation Kit II (XTT) assay (Figure 2C), which suggested that the enhanced colony-forming capability of hAAT-MSCs might be caused by increased cell proliferation.

3.3 | The migration capacity of hAAT-MSCs compared with MSCs

The efficacy of MSCs in cell therapy largely depends on their homing or migrating ability to arrive at the site of injury.[48] Using a Transwell migration assay, the migratory capacities of hAAT-MSCs and MSCs were compared in vitro. Both hAAT-MSCs and control MSCs showed significant directional migration from serum-free medium toward medium containing 30% of FBS (Figure 3A). The mean migratory capacity as reflected by percentage of area covered by migrated cells of the hAAT-MSCs was 66.90% ± 2.64% vs 46.42 ± 1.81% for control MSCs (Figure 3A,B). The migration advantages of hAAT-MSCs were further confirmed by the scratch wound assay, in which hAAT-MSCs showed a significantly faster wound closure rate compared to control MSCs (Figure 3C,D). These data suggest that hAAT-MSCs had better migration capacity than control MSCs.

3.4 | Multilineage differentiation potential of hAAT-MSCs in comparison to control MSCs

MSCs have the potential to differentiate into multiple cell lineages that contribute to tissue repair. We compared the multilineage potential of hAAT-MSCs and control MSCs at P3 and P6. Cell type-specific staining showed that both hAAT-MSCs and MSCs

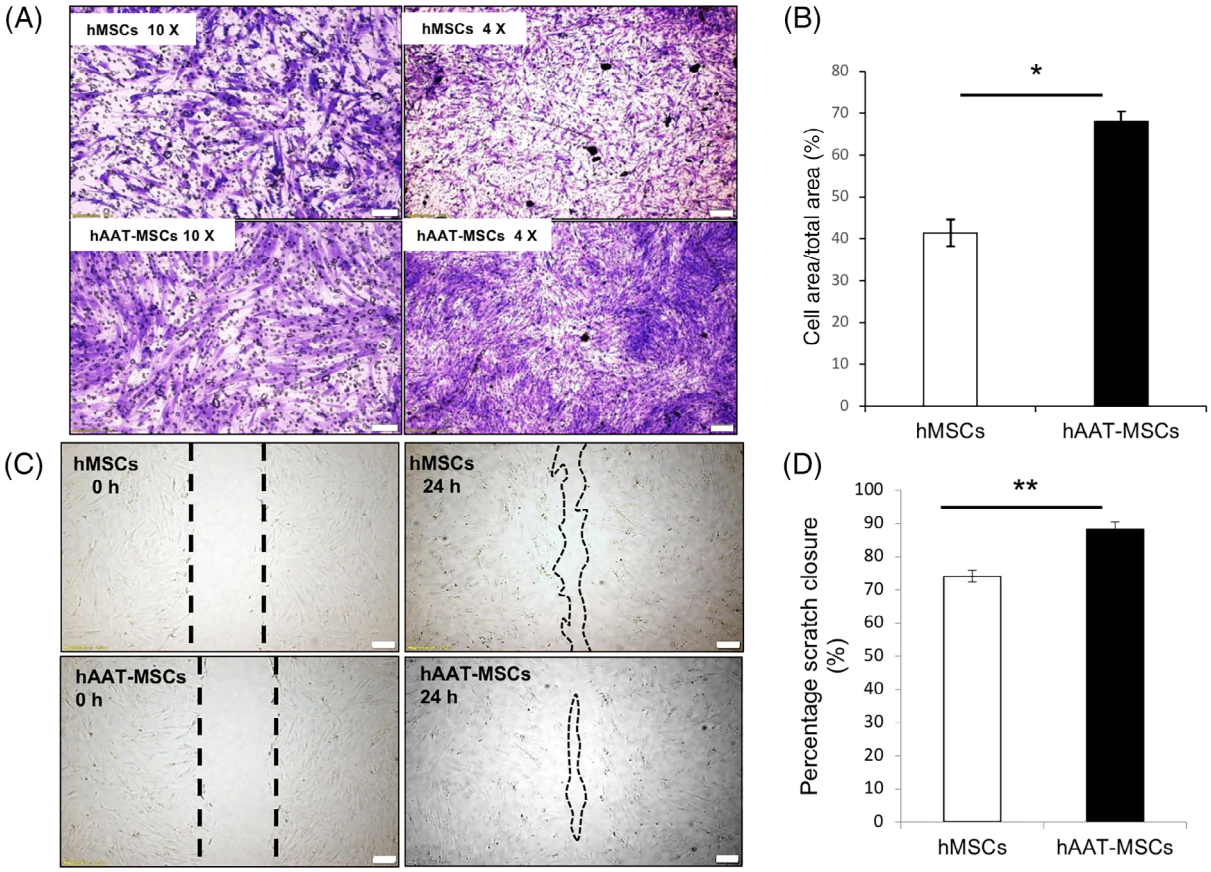

FIGURE 3 Migration capacity of hAAT-MSCs and MSCs. A, Cells migrated through the membrane to media containing 30% FBS were stained with crystal violet after overnight migration (magnification: ×4 or ×10). Scale bars in a&c: 100 μm; Scale bars in b&d: 100 μm. B, Crystal positive cell area divided by total cell area in hAAT-MSCs or MSCs. Columns shown are representative data (n = 3) from three individual experiments, mean ± SE, *P < .05, Student's t test. C, Representative images of a time-lapse sequence of scratch-wound assays after 24 hours in hAAT-MSCs and MSCs. Dotted lines define the wound area. Scale bar: 200 μm. D, Percentage of invasion in hMSCs and hAAT-MSCs calculated within 24 hours. Images were taken at 100× magnification. Data represent mean ± SE of three independent experiments. **P < .01, Student's t test

successfully differentiated into adipocytes, osteoblasts, and chondrocytes, when they were cultured in vitro in adipogenic, osteogenic, and chondrogenic differentiation media, and stained for different dyes specific for each cell types (Figure 4A). Quantitative PCR analysis detected up-regulation of the cell type-specific marker gene expression during differentiation. Compared with control MSCs, hAAT-MSCs derived adipocytes showed significantly increased expression of adipocyte-specific transcription factor, peroxisome proliferator-activated receptor gamma (PPARγ) and its downstream gene associated with adipogenesis, lipoprotein lipase (LPL), at 14 days postdifferentiation (Figure 4B). Similarly, expression of pro-osteogenic transcription factor Runx2, but not osteocalcin (OCN), was increased, at 14 days after differentiation (Figure 4C). Type II collagen a1 chain (Col2a1) expression was also increased, but the difference between hAAT-MSCs and controls was not significant at 21 days (Figure 4D). These results showed that both cell populations possessed trilineage differentiation potential.

3.5 | hAAT overexpression modified gene expression profiles in MSCs

To assess the impact of AAT-overexpression on gene expression of MSCs, we measured mRNA expression of 84 genes involved in maintaining the pluripotency and self-renewal status of MSCs using the RT^2 Profiler PCR Array for Human Mesenchymal Stem Cells. Our data showed that 23 genes were upregulated and 3 were downregulated at least 1.5-fold with $P < .05$ in hAAT-MSCs compared to control MSCs (Figure 5A,B). Upregulated genes include those critical for the stemness of MSCs (ie, WNT3A, KDR), migration (Notch homolog 1, NOTCH1, ICAM-1 and VCAM-1, MMP2), and cell survival (fibroblast growth factor 10, FGF10, IGF-1, VEGF). Gene set enrichment analysis showed that changed genes are mainly related to growth factor activity, positive regulation of cell migration, and positive regulation of transcription (Figure 5C). Again, change in gene expression profiles supported the improved migration, proliferation, and differentiation abilities in hAAT-MSCs.

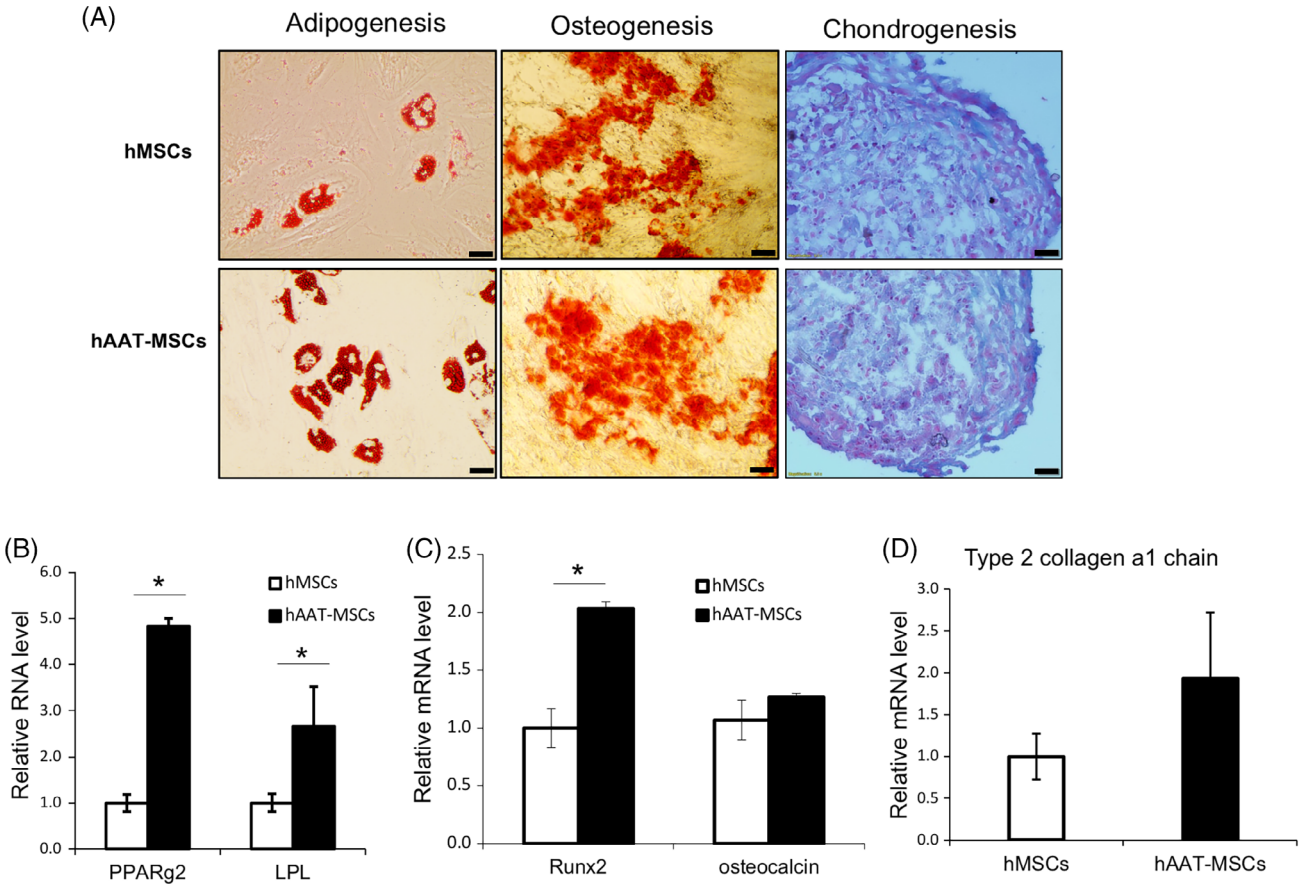

FIGURE 4 Multi-lineage differentiation and proliferation capacities of hAAT-MSCs and hMSCs. A, Adipogenic differentiation indicated by oil red staining; Osteogenic differentiation indicated by alizarin red staining; Chondrogenic differentiation indicated by alcian blue staining. B, Relative gene expression of PPARγ, LPL. C, Runx2, OCN and D, type 2 collagen a1 chain in cells derived from hAAT-MSCs or MSCs. $^{**}P < .01$, Student's *t* test. Scale bar: 100 μm

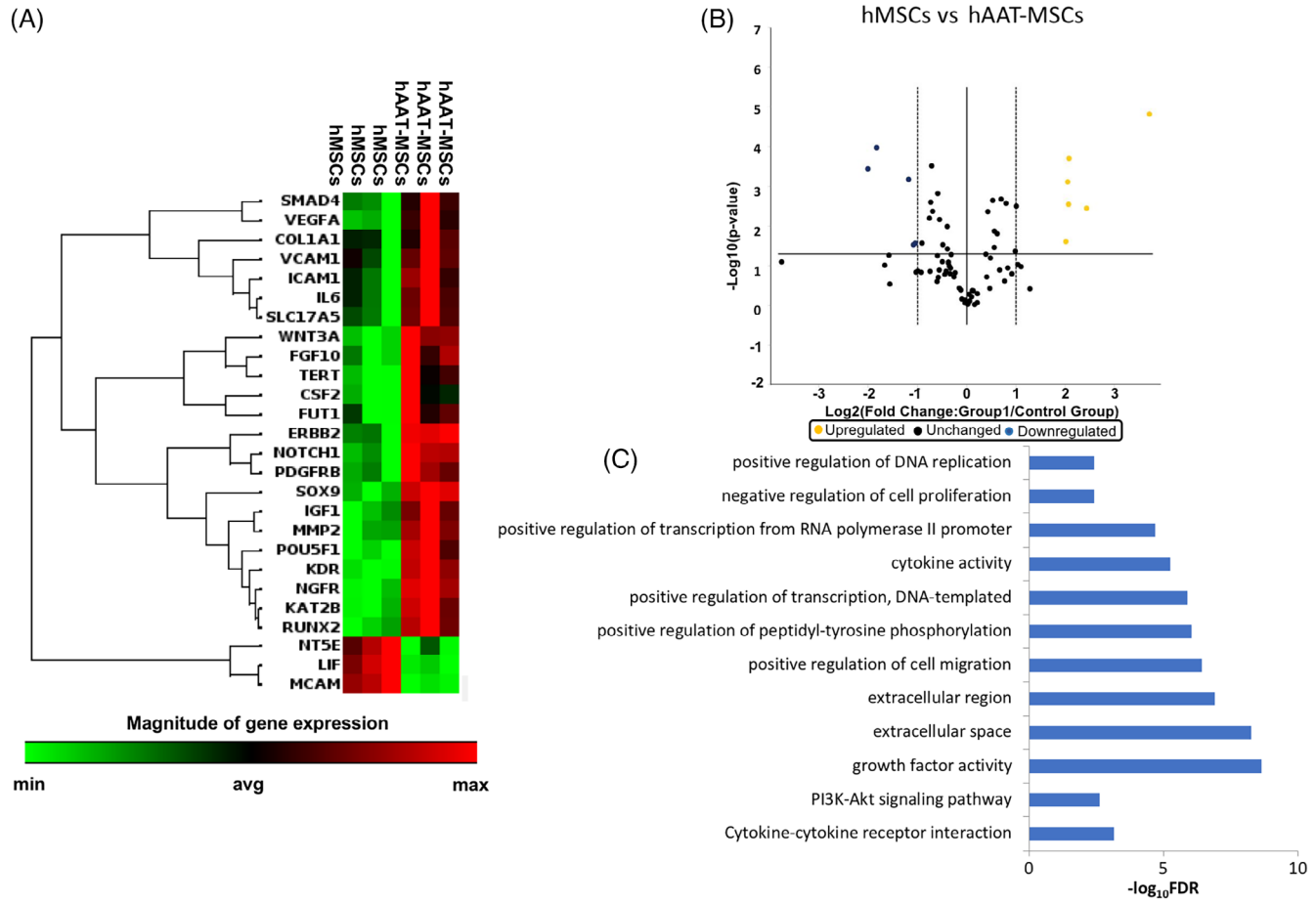

FIGURE 5 Transcriptional profile of mesenchymal stem cell related genes in hAAT-MSCs or MSCs. A, Heat map of top differentially expressed genes in MSCs and AAT-MSCs. Genes were identified using an >1.5-fold change and $P < .05$. Each column represents one cell sample. Gene expression shown by pseudocolor scale: red, upregulation of expression; green, downregulation of expression. B, Scatter plot report of the results from the RT2 Profiler PCR Array experiment, indicating positions of several noteworthy genes based on the fold-changes in expression between the control MSCs and the hAAT-MSC group. X-axis, normalized gene expression in control group; Y-axis, normalized gene expression in hAAT-SC group. Boundary used was fold-change ≥1.5. C, GSEA performed to identify the enrichment pathway by $P \leq .05$ and false discovery rate (FDR) ≤ 0.05 using the GSEA v2.2.1 software. Each bar represents an enriched pathway with significance determined using the −log FDR value (shown on X-axis). The *P*-values were calculated using Student's *t* test, with two-tail distribution and equal variances between two samples

3.6 | hAAT-MSCs delayed the onset of autoimmune diabetes

Systemic administration of MSCs delayed the onset of type 1 diabetes.[49] To test whether the beneficial effects of MSCs could be increased by hAAT overexpressed in MSCs in a preclinical T1D mouse model, we infused a single dose of hAAT-MSCs or control hMSCs (0.5×10^6 cells/mouse) to female NOD mice at the age of 8 weeks before diabetes onset. Blood glucose levels were monitored weekly from 12 weeks until 25 weeks after cell infusion. Infusion of hMSCs (n = 29) or hAAT-MSCs (n = 20) significantly delayed the onset of T1D, with hAAT-MSCs showing a more profound suppression of T1D onset until 18 weeks of age, compared with controls (n = 29). By 25 weeks of age, 76% of mice in the control group had hyperglycemia, while only 51.7% in the hMSCs group and 50% in the hAAT-MSCs group were hyperglycemic (Logrank test at week 25; CTR vs hMSCs: $P = .069$; CTR vs hAAT-MSCs: $P = .036$; MSC vs hAAT-MSC: $P = .74$), Figure 6A, The area under the curve of weekly % of diabetic mice is shown in Figure 6B. Mice receiving hMSCs or hAAT-MSCs trended with lower average non-fasting blood glucose levels compared to control NOD mice at most times measured (Figure 6C). The variability in glucose area under the curve through week 25 is shown in Figure 6D, $P > .05$.

We further performed H&E staining on the pancreatic tissue sections collected from control, hMSCs or hAAT-MSCs-treated mice at 3 weeks after cell infusion to identify immune cell infiltration into the islets (n = 6 per group, and 210-248 islets were analyzed in each group). Islets from pancreases of control mice showed typical immune cell infiltration, with 29.5 ± 11.6% of islets free of immune cell infiltration, compared to 35.9 ± 8.6% in the hMSCs and 51.0 ± 28.0% in the hAAT-MSC groups, respectively (Figure 6E). In summary, these data suggest that the administration of a single dose of hAAT-MSCs

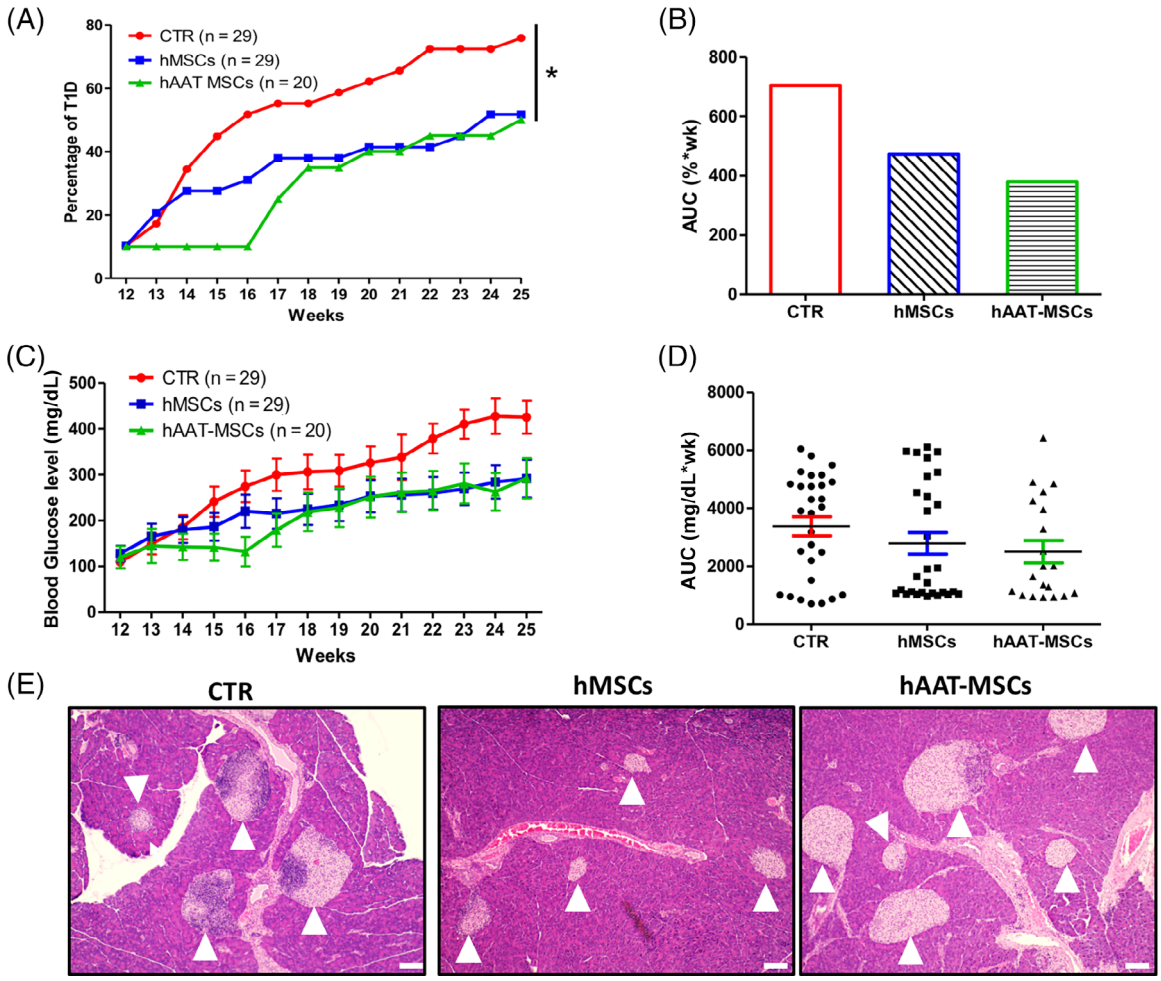

FIGURE 6 Infusion with MSCs delayed onset of T1D. A, NOD mice were either not treated (CTR, n = 29) or infused with one dose of hMSC (n = 29) or hAAT-MSC (n = 20) at 0.5×10^6/mouse. Mice blood glucose levels were measured weekly. Percentages of mice escaping normoglycemia was plotted. CTR vs AAT: $P = .069$ CTR vs hAAT-MSCs: $P = .036$, MSC vs hAAT-MSC: $P = .74$, by logrank test. B, Area under the curve of weekly percentage of T1D mice in each group. C, Average weekly non-fasting blood glucose levels in CTR, MSCs- or hAAT-MSC-treated NOD mice from week 12 until week 25. Error bars are SE. D, Area under the curve of blood glucose levels of each animal from week 12 to week 25. AUC: area under the curve. E, Representative H&E staining of pancreatic sections from control, hMSCs or hAAT-MSCs-treated NOD mice at 3 weeks after cell infusion. In each group, 210-248 islets from 6 mouse pancreases were counted. Scale bar: 100 μm. Arrows point to islets

increased early protection of the NOD mice from the onset of T1D compared to control MSCs, at least in part via suppression of immune cell infiltration into islets.

4 | DISCUSSION

In this study, we have demonstrated that engineered, bone marrow-derived MSCs overexpressing human AAT have improved proliferation, migration, and differentiation capabilities compared to control MSCs in vitro. This was supported by the change in expression of genes related to MSC stemness, migration, and differentiation. Furthermore, the enhanced capabilities of hAAT-MSCs translated in vivo in which infusion of hAAT-MSCs showed better early efficacy in the prevention of onset of T1D in the NOD mice compared with control MSCs. These data suggest that hAAT-engineered MSCs have improved capabilities needed for cell therapy.

Despite some unsolved problems and challenges, MSC cell therapy is widely used in animal studies and clinical trials for the treatment of a variety of diseases. in vivo cell tracking studies demonstrate that only a small percentage of cells arrive at the injured tissue after intravenous infusion, while most cells disappear after presentation to the lung and/or the liver.[50] Because of this problem, it would be benefical to improve the differentiation and migration functionality of MSCs in situations where there are numerical shortages of MSCs. Several approaches have been attempted to improve the natural properties of MSCs. For example, MSCs overexpressing TGF-β1 showed enhanced effects on proliferation, cell cycle turnover, and

prevention of cellular senescence and apoptosis.[51] MSCs cultured in hypoxic conditions increased their proliferation, self-renewing capacity and modulation of key genes and inflammation in vitro and in a murine hind limb ischemia model.[52,53] Licensing of adipose derived MSCs with INF-γ completely prevented graft vs host disease.[54] Treatment with PolyIC to activate Toll-like receptor 3 signaling improved the immunosuppressive effects of bone marrow and umbilical cord derived MSCs.[55]

Our strategy of overexpressing hAAT in MSCs contributes to an alternative new approach to enhance the natural properties of MSCs. Changes in gene expression in hAAT-MSCs further support the increased migration, differentiation, and proliferative capacities that may be need after intravenous infusion. VCAM1, ICAM1, and NOTCH1 play important roles in cell migration.[56] Matrix metalloproteinase-2 (MMP-2) and RUNX2 are potential chondrogenic markers during chondrogenic differentiation of MSCs.[57] Sox9 is reported as a modulator of MSCs survival and adipogenic differentiation.[58] Moreover, growth factors including VEGF, fibroblast growth factor 10 (FGF10), and insulin-like growth factor 1 (IGF-1) are involved in cell survival. Expression levels for a few genes including melanoma cell adhesion molecule (MCAM, or CD146) and leukemia inhibitor factor (LIF) were downregulated. CD146 and LIF are negatively related to cell proliferation, MSCs with low CD146 show higher proliferation.[59] Therefore, changes in these gene expressions support the improved innate properties of hAAT-MSCs.

In addition to their innate therapeutic effects, MSCs are one of the most promising stem cell populations for use in gene therapy studies and clinical trials, as they can be modified with a wide range of both viral and nonviral vector systems, and secrete therapeutic proteins such as hAAT, further enhancing their natural abilities to mediate repair within various tissues. AAT contributes to tissue repair by stimulating fibroblast proliferation and extracellular matrix production through the classical mitogen-activated signaling pathways.[41] AAT also play an essential role in cancer cell migration, invasion, and pericellular fibronectin assembly for facilitating lung colonization of lung adenocarcinoma cells.[60] We assume that the improved functionality of hAAT-MSCs were a combination effect of engineered MSCs and secreted AAT. This may be also true for the in vivo function of hAAT-MSCs in the NOD mice.

We found that treatment with hAAT-MSCs reduced immune cell infiltration into pancreatic islets. Since the pathogenesis of T1D is complex and patient specific, any proposed therapy must regenerate pancreatic β cell number as well as target the autoimmunity that initiated the disease. MSCs can be easily expanded to large numbers and have the potential to expand pancreatic β cell mass by halting islet death and by differentiating into insulin-secreting cells, which is a great advantage over other immune therapies such as low-dose IL-2 or Treg therapies.[61,62] In animal models, AAT has been shown to have a profound effect in the prevention of T1D. However, either treatment alone has not been successful at sustained suppression of the autoimmune response. Our data show in the new onset T1D model that a single infusion of hAAT-MSCs prevented the onset of T1D in the NOD mice until 18 weeks compared with hMSCs. Whether this additional protection was because of the enhanced migration, proliferation and differentiation capability of MSCs and/or the secreted AAT remains to be explored. We do not know if a more lasting effect might be obtained with serial infusions, since the durability of the therapeutic effects of MSCs is limited. Future studies to evaluate the methodology of serial infusions will be complicated and time consuming. Additionally, the mechanism by which the NOD mouse escaped the protection of hAAT-MSCs after 16 weeks is not known. Since the cells may have further differentiated by this time, any number of possibilities might be envisioned. Further experiments with exogenous AAT, re-infusion of MSCs at later times, or study of cell fate in the NOD mouse will be needed to explain this observation. Nevertheless, the strategy of using hAAT-MSCs may provide a more effective prevention for diabetes in susceptible individuals that use MSCs therapy.

5 | CONCLUSION

hAAT-MSCs showed improved stemness and differentiation ability compared to MSCs and were more potent in the prevention of T1D compared with MSCs in the NOD mouse. Such strategies may improve the treatment of type 1 diabetes and other diseases in the future.

ACKNOWLEDGMENTS

We thank Dr Andrew Wilson for providing the lentiviruses for this study. This study was supported by the National Institute of Diabetes and Digestive and Kidney Diseases grants # 1R01DK105183, DK120394, DK118529, and the Department of Veterans Affairs (VA-ORD BLR&D Merit I01BX004536) to H.W.

CONFLICT OF INTEREST

C.S. declared leadership position for AlphaNet providing disease management services to the Alpha-1 antitrypsin deficiency population, consultant role for Dicerna, CSL Behring, and Vertex on Alpha-1, researching from Adverum, Arrowhead, CSL Behring, Dicerna, Grifols, MatRx, Takeda, and Vertex on Alpha-1. All the other authors declared no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

L.S.: experimental design, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; W.G.: collection of data, data analysis, final approval of manuscript; J.W.: data analysis and interpretation, final approval of manuscript; W.H.: collection of data, final approval of manuscript; J.L.: collection of data, data analysis, manuscript writing and editing, final approval of manuscript; C.S.: conception and design, data analysis and interpretation, manuscript editing, final approval of manuscript; H.W.: conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: Song L, Gou W, Wang J, et al. Overexpression of alpha-1 antitrypsin in mesenchymal stromal cells improves their intrinsic biological properties and therapeutic effects in nonobese diabetic mice. *STEM CELLS Transl Med*. 2021;10:320–331. https://doi.org/10.1002/sctm.20-0122