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Recent advances on mesenchymal stromal cells applications for cancer therapy

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Recent advances on mesenchymal stromal cells applications for cancer therapy

Topic editors

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ORIGINAL RESEARCH
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Exosomes Derived From miR-212-5p Overexpressed Human Synovial Mesenchymal Stem Cells Suppress Chondrocyte Degeneration and Inflammation by Targeting ELF3

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Excessive chondrocyte degeneration and inflammation are the pathological features of osteoarthritis (OA), and altered miR-212-5p may contribute to meniscus and cartilage degeneration. Whether exosomes derived from miR-212-5p overexpressed synovial mesenchymal stem cells (SMSC-212-5p-Exos) could be utilized to treat degenerative chondrocytes is investigated in this study. Down-regulated miR-212-5p and up-regulated E74 Like ETS Transcription Factor 3 (ELF3) expression were detected in OA synovial tissues, which showed a negative correlation ($r = -0.55$, $p = 0.002$). miR-212-5p directly targeted ELF3 and regulated the relative expression of *ELF3* in SMSCs as indicated by luciferase reporter assay and RT-PCR. The relative expression of ELF3, chondrocyte degeneration-related molecules, matrix metalloproteinase, and inflammatory molecules were detected in chondrocytes stimulated with interleukin (IL)-1β or co-incubated with SMSC-212-5p-Exos or SMSCs-derived exosomes (SMSC-Exos). IL-1β induced up-regulation of ELF3, down-regulation of degeneration molecules (Collagen II, Aggrecan, and Sox9), up-regulation of matrix metalloproteinase (MMP-1, MMP-3, and MMP-13), and up-regulation of inflammatory molecules (IL-6, MCP-1, TNF-α, COX-2, and iNOS) could be inhibited by SMSC-212-5p-Exos or SMSC-Exos administration. When compared with the SMSC-Exos, SMSC-212-5p-Exos showed more treatment benefits. All of these indicate that SMSC-212-5p-Exos could suppress chondrocyte degeneration and inflammation by targeting ELF3, which can be considered as a disease-modifying strategy.

Keywords: miR-212-5p, synovial mesenchymal stem cells, ELF3, chondrocyte, SMSCs, osteoarthritis

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INTRODUCTION

Osteoarthritis (OA) is a disease that comprises progressive cartilage degenerative and synovial membrane inflammation, having an increased frequency of osteophyte formation and subchondral bone sclerosis in the aging population (Hunter and Bierma-Zeinstra, 2019; Katz et al., 2021; Sharma, 2021). No curative treatment has been applied in the clinic, and joint replacement remains the most commonly applied and effective therapy for the disability. Chondrocyte degeneration and

inflammation are the principal cause of OA, for chondrocytes are the only residents in the avascular articular cartilage to maintain the specialized structure. It is also reported that chondrocytes size could be potentially used to assess disease progression (Gratal et al., 2019). Therefore, the molecular mechanism underlying chondrocyte degeneration and inflammation is vital for OA treatment.

Exosomes are small size (30–100 nm), single-membrane organelles released by various cells to shunt loading bioactive molecules to target cells (Kalluri and LeBleu, 2020; O'Brien et al., 2020). Research has shown that exosomes derived from synovial mesenchymal stem cells (SMSC-Exos) have the potential to palliate the severity of interleukin (IL)-1β induced osteoarthritis (Wang et al., 2020; Qiu et al., 2021). Further research is urgent to confirm the effectiveness and feasibility of exosome-based therapy.

MicroRNAs (miRNAs) can post-transcriptionally regulate OA-associated genes in chondrocytes (Oliviero et al., 2019; Huang et al., 2021). Exosomal miRNAs differ significantly from those of the parent cell, and following uptake, exosomal miRNAs can mediate target gene repression and reprogramme the cellular response (Heidarzadeh et al., 2021; Moghadasi et al., 2021). In rheumatoid arthritis, miR-212-3p can reduce proliferation and promote apoptosis of fibroblast-like synoviocytes (Liu et al., 2018). On the other hand, altered miR-212-5p expression can lead to the meniscus and cartilage degenerative process in OA (Jiang et al., 2021). All of these indicate the treatment benefit of miR-212-5p delivery in OA and whether exosomes derived from miR-212-5p overexpressed SMSCs (SMSC-212-5p-Exos) could be utilized to treat OA is investigated in this study.

METHODS AND MATERIALS

Patient Enrollment

Based on the criteria of the American College of Rheumatology, thirty OA patients (18 females and 12 males; 50–74 years old; mean age of 62.3 ± 6.4 years) underwent total knee arthroplasty were enrolled. Meanwhile, the synovial tissues were collected from OA patients and 20 donors with accidental deaths (excluding those with OA-related diseases) or normal knee-joint synovium who were subjected to lower limb amputation after acute trauma or open reduction and internal fixation after fracturing of the tibial plateau (13 females and 7 males; 50–75 years old; mean age of 61.87 ± 7.44 years). The study was approved by the Xijing Hospital, and informed written consent was derived from each participant or close relatives.

Synovial Mesenchymal Stem Cells Differentiation and Transfection

Synovial membrane specimens were digested with 0.2% type I collagenase (Thermo Fisher) at 37°C overnight, which were further collected by centrifugation and seeded in a high-glucose DMEM medium (Thermo Fisher, Waltham, MA, United States) supplemented with 10% FBS for 4 days to allow cell attachment. The medium was refreshed every 3 days, and at day 14, SMSCs were obtained. After blocking with human BD Fc Block™, SMSCs were stained with the following antibodies (Becton Dickinson): anti-CD34, anti-CD44, anti-CD45, anti-Sca-1, and anti-CD105 antibodies to confirm the phenotype using Guava® easyCyte™ flow cytometer (Merck-Millipore, Billerica, MA, United States). At passage 3, SMSCs were switched to osteogenic differentiation medium (Sigma-Aldrich, St. Louis, MO, United States) for 2 weeks or StemPro Adipogenesis Differentiation Kit (Gibco) for 4 weeks. The miR-212-5p mimic or mimic-negative control (NC) (Sigma-Aldrich) were transfected with Lipofectamine® 3,000 (Thermo Fisher) at the concentration of 100 nM according to the manufacturer's instructions. The following sequences were used: miR-212-5p mimic (sense, 5′-ACCUUGGCUCUAGAC UGCUUACU-3'; and antisense, 5′-UAAGCAGUCUAGAGC CAAGGUUU-3′), mimic-negative control miRNA (sense, 5′-UUCUCCGAACGUGUCACGUTT-3'; and antisense, 5′-ACG UGACACGUUCGGAGAATT-3′).

Exosomes Isolation and Characterization

Conditioned medium of SMSCs or miR-212-5p overexpressing SMSCs was filtered with 0.22 μM filters (Merck-Millipore) and centrifuged (4,000×g) to concentrate the volume into approximately 200 μL, which was further ultracentrifuged (100,000×g, 1 h, 4°C) to obtain the exosomal particles. A transmission electron microscope (TEM) was utilized to observe the morphology of exosomes, and the size and distribution of exosomes were measured using dynamic light scattering (DLS) analysis.

Collection and Culture of Primary Chondrocyte

Primary chondrocytes were detached from cartilages dissected from the subchondral bone with 0.25 mg/ml collagenase P and 4 mg/ml protease, and the isolated chondrocytes (1×10^7) were seeded into the 6-well plates 1 day before treatment. When cell confluence reached 80%, 2 μg exosomes (EXO miR-NC and EXO-miR-212-5p) were introduced into the chondrocytes culture medium supplemented with 10 ng/ml IL-1β. Chondrocytes treated with PBS were regarded as the blank controls. After 48 h of treatment, cells were collected for subsequent use.

RNA Isolation and Quantitation

TRIzol (Invitrogen) was utilized to extract total RNA from chondrocytes or SMSCs. Total Exosome RNA & Protein Isolation Kit (Invitrogen, Waltham, MA, United States) was used to extract RNA and protein from exosomes for further analysis. TaqMan™ Advanced miRNA cDNA Synthesis Kit (Thermofisher) was utilized to reverse-transcript miRNA into cDNA, and RNU6B was utilized as an internal reference. Transcript® All-in-One-First-Strand cDNA synthesis supermix was utilized to reverse-transcript mRNA into cDNA,

and GAPDH was utilized as an internal reference. SYBR Green Master Mix (Roche, Penzberg, Upper Bavaria, Germany) was applied to detect the amplification. The primers were listed as following: ELF3, forward 5′- CATGACCTACGAGAAGCTGAG C-3′, reverse 5′- GACTCTGGAGAACCTCTTCCTC-3'; miR-212-5p, forward 5′- CAGTCTCCAGTCACGG-3′, reverse 5′- GAACATGTCTGCGTATCTC-3'; Collagen II (COL2A1), forward 5′- CCTGGCAAAGATGGTGAGACAG-3′, reverse 5′- CCTGGTTTTCCACCTTCACCTG-3'; Aggrecan, forward 5′-CAGGCTATGAGCAGTGTGATGC-3′, reverse 5′- GCT GCTGTCTTTGTCACCCACA-3'; Sox9, forward 5′- AGGAAG CTCGCGGACCAGTAC-3′, reverse 5′- GGTGGTCCTTCTTGT GCTGCAC-3'; MMP-1, forward 5′- ATGAAGCAGCCCAGA TGTGGAG -3′, 5′- TGGTCCACATCTGCTCTTGGCA -3'; MMP-3, forward 5′- CACTCACAGACCTGACTCGGTT-3′, reverse 5′- AAGCAGGATCACAGTTGGCTGG-3'; MMP-13, forward 5′- CCTTGATGCCATTACCAGTCTCC-3′, reverse 5′- AAACAGCTCCGCATCAACCTGC-3'; IL-6, forward 5′ AGACAGCCACTCACCTCTTCAG-3′, reverse 5′- TTCTGC CAGTGCCTCTTTGCTG-3; COX-2, forward 5′- CGGTGA AACTCTGGCTAGACAG-3′, reverse 5′- GCAAACCGTAGA TGCTCAGGGA-3'; iNOS, forward 5′- GCTCTACACCTC CAATGTGACC-3′, reverse 5′- CTGCCGAGATTTGAGCCT CATG-3'; GAPDH, forward 5′- CTGTGCCGTTGAATTTGC CG-3′, forward 5′- CGGGTTCCTATAAATACGGACTG-3'.

Luciferase Assay

The QuikChange Lightning Site-Directed Mutagenesis kit (Agilent, Santa Clara, CA, United States) was utilized to mutate the binding site of ELF3 with miR-212-5p. 3′-UTR fragment of ELF3 mRNA (mutant or wide type) was subcloned into pGL3 luciferase vector (Promega, Madison, WI, United States), which was further co-transfected with miR-212-5p mimic or mimic-NC into HEK-293T cells for 36 h with Lipofectamine 3,000. Subsequently, the luciferase activity was assayed with Luciferase Assay System (Promega).

Immunoblot Assay

The cellular lysates were quantified using the BCA protein concentration kit (20201ES76, Yeasen Company, Shanghai, China), and 20 μg lysates were separated with 10% SDS-PAGE electrophoresis and transferred to PVDF membranes, which were further blocked in Tris-buffered saline with Tween 20 (TBST) containing 0.1% Tween 20 and 5% skimmed milk powder and followed by incubation with the primary antibodies (Santa Cruz, Dallas, TX, United States) specific for CD63, CD9, Alix, ELF3, and GAPDH, and then incubated in peroxidase-conjugated secondary antibody (Sigma-Aldrich) at room temperature for 1 h. The dilution used for the primary antibodies against CD63, CD9, Alix, and ELF3 was 1:1,000, the dilution used for the primary antibody against GAPDH was 1:2000, and the dilution used for the second antibody was 1:2000. The signal was developed with an ECL system (GE Healthcare), and the relative intensity was normalized with GAPDH expression with NIH-Image J1.51p 22.

Elisa

According to the manufacturer's instructions, the concentrations of IL-6, MCP-1, MMP-3, MMP-13, and TNF-α were detected with commercial ELISA kits (eBiosciences, San Diego, CA, United States). Separated stock standard solutions and samples were measured with a SpectraMax M5 microplate reader at a wavelength of 450 nm.

Statistical Analysis

Statistical analysis were performed with Graph-Pad Prism 6.0. Data were presented as means ± SD. The Mann-Whitney test was performed to determine the statistical significance between any two groups, and Dunn's multiple comparisons test was used to compare multiple groups. p-value < 0.05 was considered statistically significant.

RESULTS

Down-Regulated miR-212-5p and Up-Regulated ELF3 Expression in OA Synovial Tissues

The relative miR-212-5p expression in OA synovial tissues was down-regulated compared with healthy control (**Figure 1A**, $p < 0.001$), and up-regulated *ELF3* mRNA expression (**Figure 1B**, $p < 0.001$) and increased protein expression of IL-1β (**Figure 1C**, $p < 0.001$) were also observed. The expression correlation analysis indicated that both the correlation between *ELF3* and miR-212-5p (**Figure 1D**, $r = -0.55$, $p = 0.002$) and the correlation between IL-1β and miR-212-5p (**Figure 1E**, $r = -0.47$, $p = 0.009$) were significantly negatively correlated. As expected, the correlation between *ELF3* and IL-1β was positively correlated (**Figure 1F**, $r = 0.41$, $p = 0.024$).

SMSC-Derived Exosomes Isolation and Confirmation

Spindle-like SMSCs were observed at passage 3 (P3) (**Figure 2A**), which could be induced into osteogenic cells confirmed by Alizarin Red S staining of calcium mineral deposits (**Figure 2B**) and induced into adipogenic cells confirmed by Oil Red O staining of small cytoplasmic lipid droplets (**Figure 2C**). Flow cytometry analysis confirmed the phenotype of SMSCs, which were positive for CD105, CD44, and SCA-1, and negative for CD34 and CD45 (**Figure 2D**). TEM showed the single membrane structure of SMSC-Exos (**Figure 2E**), and dynamic light scattering assayed that most exosomes ranged from 30 to 150 nm in size (**Figure 2F**). The relative protein expression of exosome markers (CD63, CD9, and Alix) was significantly up-regulated as detected by Western blot (**Figure 2G**). All these results confirmed the success of SMSC-Exos isolation.

miR-212-5p Directly Targets ELF3 in SMSCs

TargetScan (Agarwal et al., 2015) and miRBase (Ambros et al., 2003) database were utilized to predict the possible targets of

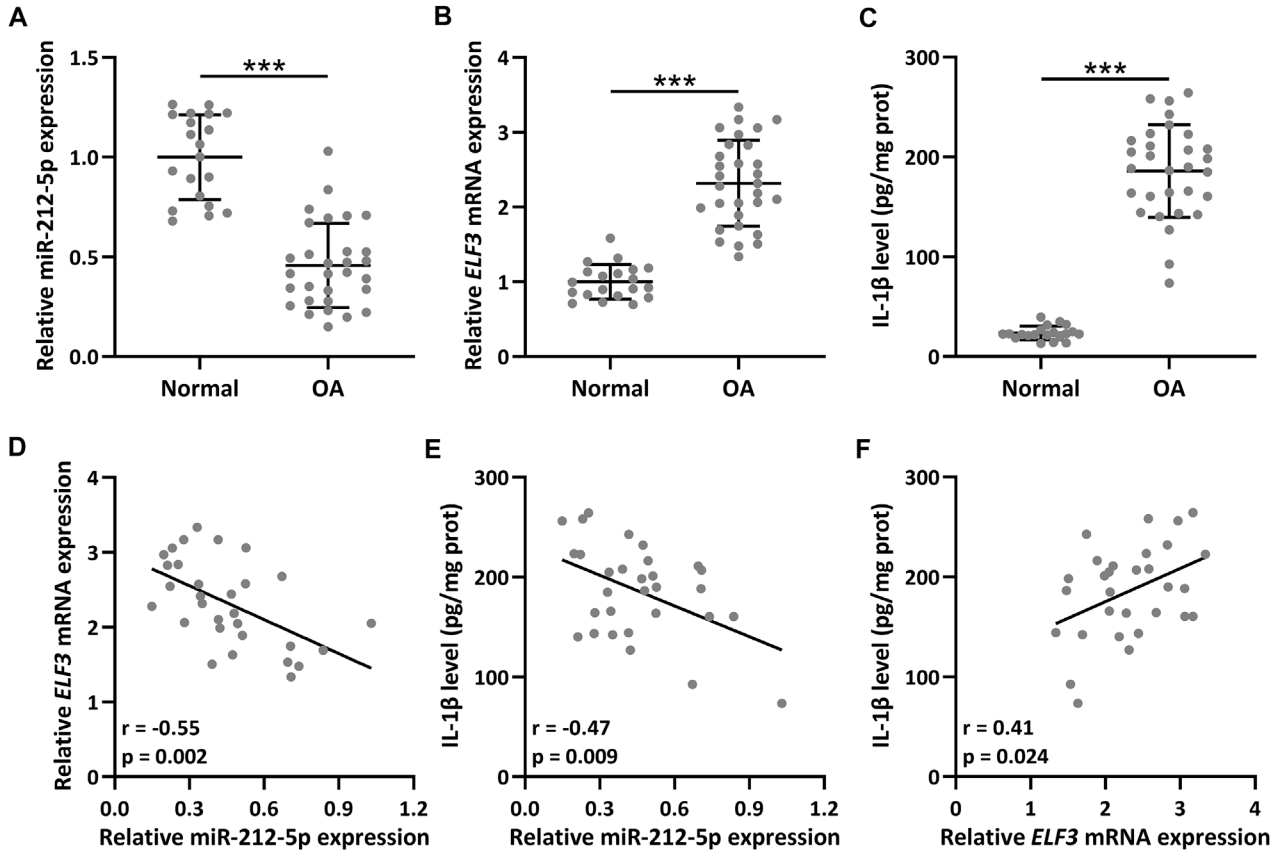

FIGURE 1 | The expression of ELF3 and miR-212-5p in OA ($n = 30$) and normal synovial tissues ($n = 20$). qRT-PCR was utilized to detect the relative expressions of miR-212-5p **(A)** and ELF3 **(B)**. ELISA was used to measure the IL-1β level **(C)**. Pearson correlation coefficient of miR-212-5p and ELF3 mRNA **(D)**, miR-212-5p and IL-1β level **(E)**, ELF3 mRNA and IL-1β level **(F)** in OA synovial tissues. $n = 30$. Mean ± SD. ***$p < 0.001$.

miR-212-5p. ELF3 was among the top 10 molecules that could be binding with miR-212-5p. 3′-UTR region of ELF3, containing the predicted miR-212-5p binding sequences (**Figure 3A**), was cloned into the luciferase vector. MiR-212-5p overexpression could significantly decrease the luciferase activity of ELF3 (**Figure 3B**). In addition, up-regulation of miR-212-5p was observed in miR-212-5p overexpressed SMSCs (**Figure 3C**), which could diminish the relative mRNA (**Figure 3D**) and protein (**Figures 3E**,F) expression of ELF3. These results indicated that miR-212-5p directly targeted ELF3 and regulated the expression of ELF3 in SMSCs.

Exosomes Derived From miR-212-5p Overexpressed SMSCs (SMSC-212-5p-Exos) Inhibit IL-1β Induced ELF3 Expression in Chondrocytes

Up-regulated miR-212-5p expression could be detected in SMSC-212-5p-Exos (**Figure 4A**), indicating that SMSCs exosomes can carry miR-212-5p to target cells. SMSC-212-5p-Exos showed no difference in size and membrane structure when compared with SMSC-Exos (data not shown). Different concentrations of IL-1β were used to stimulate chondrocytes, and it was found that decreased cell viability was observed in accordance with the increased IL-1β concentration (**Figure 4B**). SMSC-Exos could reverse the diminished cell viability induced by IL-1β in chondrocytes, and SMSC-212-5p-Exos showed more treatment benefit when compared with SMSC-Exos (**Figure 4C**). On the other hand, IL-1β incubated chondrocytes showed up-regulated ELF3 expression in both transcription (**Figure 4D**) and protein levels (**Figures 4E**,F), which can be reversed by the co-incubation with SMSC-Exos or SMSC-212-5p-Exos, while SMSC-212-5p-Exos showed the better treatment benefit. All of these results indicated that exosomal miR-212-5p inhibited up-regulated ELF3 expression in chondrocytes induced by IL-1β.

SMSC-212-5p-Exos Attenuate IL-1β Induced Chondrocyte Degeneration and Degradation

The relative mRNA expression of Collagen II (**Figure 5A**), Aggrecan (**Figure 5B**), and SOX9 (**Figure 5C**) in chondrocytes was decreased after the stimulation with IL-1β, while SMSC-

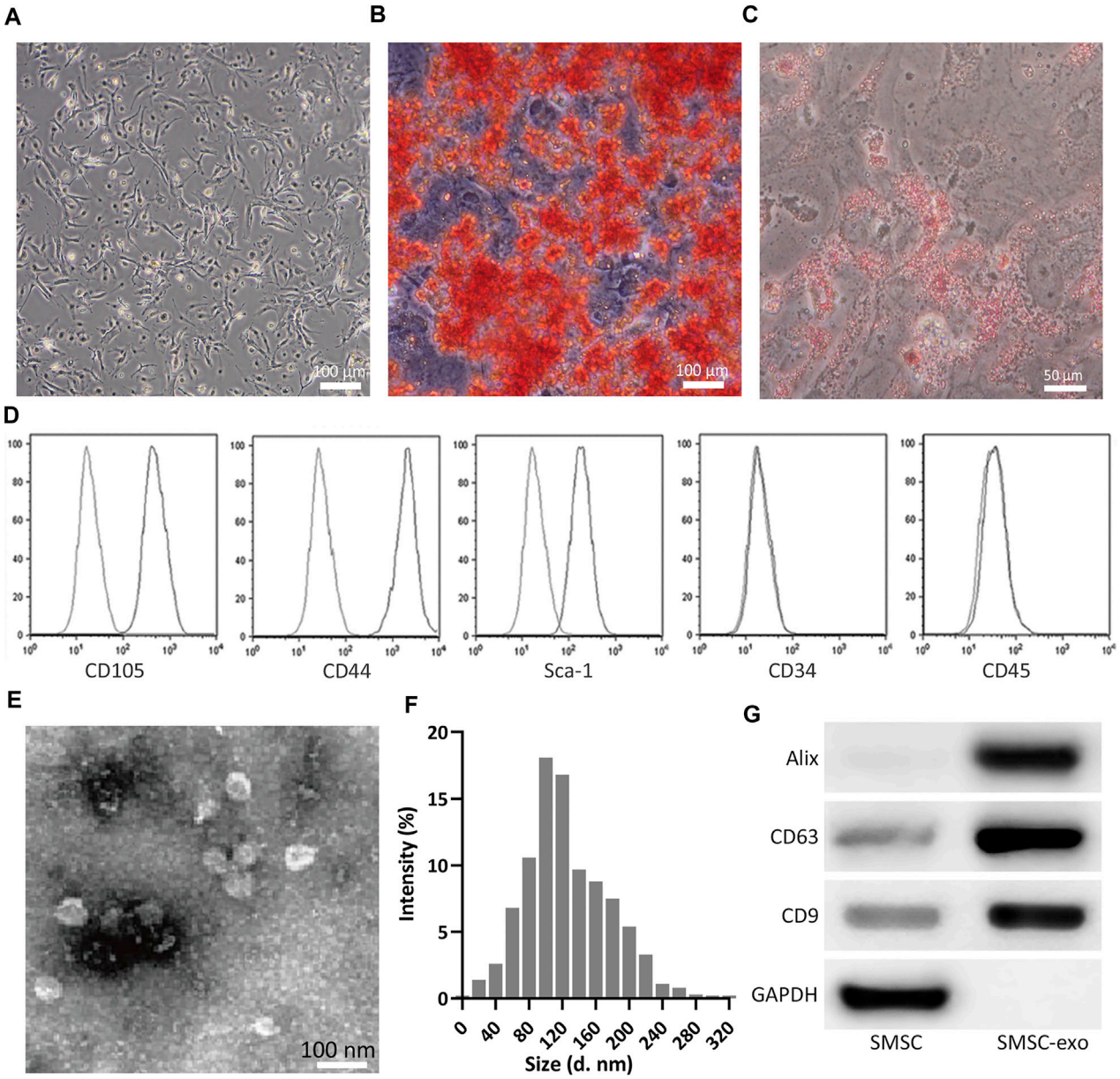

FIGURE 2 | Isolation of SMSCs and SMSC-derived exosomes (SMSC-Exos). **(A)** Observation of SMSCs morphology at passage 3. **(B)** After 14 days of osteogenic differentiation, matrix mineralization of SMSCs was stained with Alizarin Red S. **(C)** after 14 days of adipogenesis induction, SMSCs were stained with Oil Red O. **(D)** Fluorescence Activated Cell Sorting was performed on SMSCs for CD105, CD44, Sca-1, CD34, and CD45 at passage 3. **(E)** Identification of exosomes by TEM. **(F)** Nanoparticle Tracking Analysis of the isolated exosome. **(G)** Detection of CD9, CD63, and Alix expression by Western blot analysis between SMSCs and SMSC-derived exosomes (SMSC-Exos).

Exos, especially SMSC-212-5p-Exos, could reverse such reduced expression of chondrocyte degeneration related molecules. The relative content of MMP-1 (**Figure 6A**), MMP-3 (**Figure 6B**), and MMP-13 (**Figure 6C**) in the culture supernatant of chondrocytes was increased after the stimulation with IL-1β, while SMSC-Exos or SMSC-212-5p-Exos could inhibit the secretion of the relevant molecules. On the other hand, the relative expression of MMP-1 (**Figure 6D**), MMP-3 (**Figure 6E**), and MMP-13 (**Figure 6F**) in chondrocytes was increased after the treatment with IL-1β, while SMSC-Exos or SMSC-212-5p-Exos could reverse such increased expression. When compared with SMSC-Exos treatment, SMSC-212-5p-Exos treatment could show more treatment benefit. These results demonstrated that exosomal miR-212-5p could attenuate IL-1β induced chondrocyte degeneration and degradation.

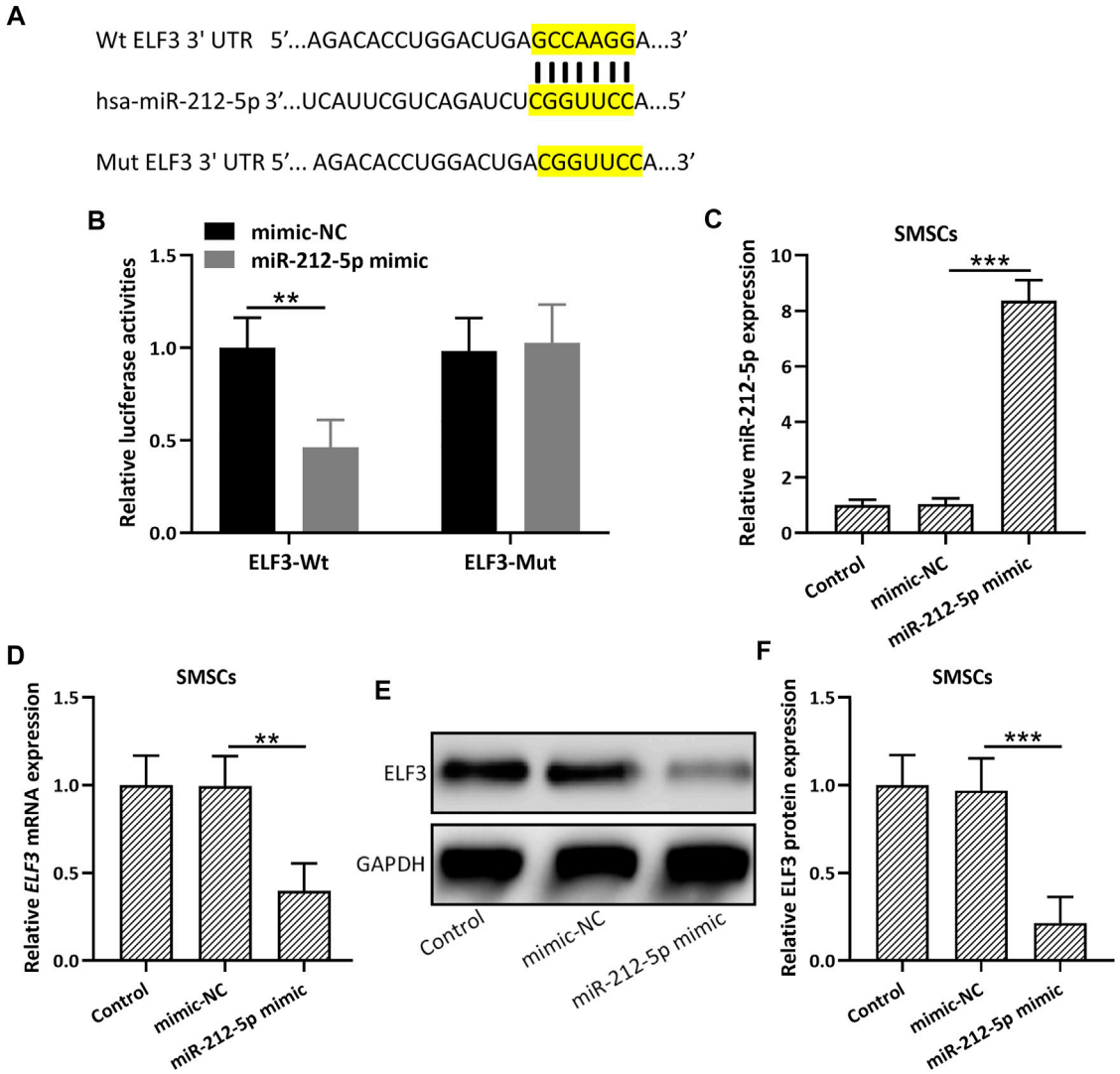

FIGURE 3 | miR-212-5p targeted ELF3 and regulated the expressions of ELF3 in SMSCs. **(A)** The predicted binding sequences of miR-212-5p with ELF3 mRNA were shown. **(B)** HEK-293T cells were co-transfected with luciferase reporters containing ELF3 3′-UTR (wide type or mutant) and miR-212-5p mimics or negative control. After 48 h of incubation, the relative luciferase activity was measured. $n = 3$. Mean ± SD. $^{**}p < 0.01$, Two-way ANOVA followed Turkey's multiple comparisons test. **(C)** qRT-PCR was used to assay the expressions of miR-212-5p in SMSCs and miR-212-5p overexpressed SMSCs. **(D–F)**, SMSCs were transfected with miR-212-5p mimics or negative control for 48 h qRT-PCR and Western blotting were used to analyze the mRNA and proteins expressions of ELF3. $N = 3$ for each group. Mean ± SD. $^{**}p < 0.01$, $^{***}p < 0.001$, One-way ANOVA followed Dunn's multiple comparisons test.

SMSC-212-5p-Exos Attenuate IL-1β Induced Inflammatory Responses in Chondrocytes

The relative content of IL-6 (**Figure 7A**), MCP1 (**Figure 7B**), and TNFα (**Figure 7C**) in the culture supernatant of chondrocytes was increased after the treatment with IL-1β, while SMSC-Exos or SMSC-212-5p-Exos could reverse such increased expression. On the other hand, the relative mRNA expression of IL-6 (**Figure 7D**), COX-2 (**Figure 7E**), and iNOS (**Figure 7F**) in chondrocytes was increased after the treatment with IL-1β, while SMSC-Exos or SMSC-212-5p-Exos could reverse such increased expression. It was worth noting that when compared with SMSC-Exos treatment, SMSC-212-5p-Exos treatment could decrease the levels of inflammatory molecules.

DISCUSSION

In addition to inflammation, dysregulation between the synthesis and degradation of extracellular matrix mainly mediated by type-II collagen, aggrecan, and matrix metalloproteinases contributes to the articular cartilage degradation and loss process (Martel-Pelletier et al., 2016). In this study, we utilize IL-1β induced chondrocytes to mimic the pathology of OA. We found that

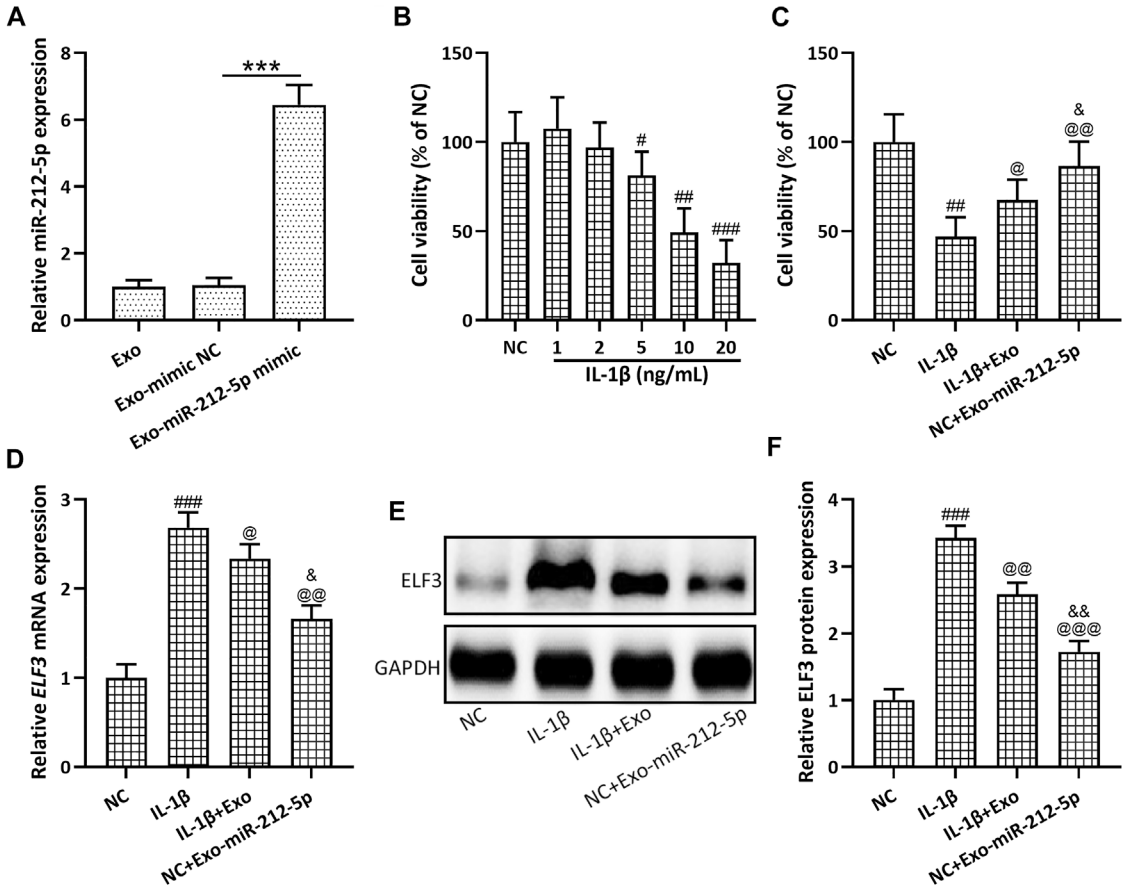

FIGURE 4 | Exosomes derived from miR-212-5p overexpressed SMSCs (SMSC-212-5p-Exos) inhibited up-regulated ELF3 expression in chondrocytes induced by IL-1β. **(A)** qRT-PCR was utilized to analyze the relative expressions of miR-212-5p from SMSC-Exos or SMSC-212-5p-Exos. **(B)** Cell viability of chondrocytes after individual treatment with different concentrations of IL-1β for 48 h. **(C)** Cell viability of chondrocytes after co-treatment with IL-1β and SMSC-Exos, or SMSC-212-5p-Exos for 48 h. **(D–F)** IL-1β induced chondrocytes were treated with SMSC-Exos or SMSC-212-5p-Exos for 48 h qRT-PCR and western blotting were used to analyze the mRNA and proteins expressions of ELF3. $n = 6$ for each group in cell viability analysis, $n = 3$ for each group in qRT-PCR and western blotting tests. #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ compared to NC. @$p < 0.05$, @@$p < 0.01$, @@@$p < 0.001$ compared to IL-1β. &$p < 0.05$, &&$p < 0.01$ compared to IL-1β+Exo. One-way ANOVA followed Dunn's multiple comparisons test.

SMSC-Exos showed the enrichment of miR-212-5p, which could target chondrocytes and further inhibit the ELF3 mediated chondrocyte degeneration, degradation, and inflammation process. This study highlights the significance and the need for further development of SMSC-212-5p-Exos on the clinical therapy of OA.

miR-132/212 clusters are maintained on down-regulation in mesenchymal stem cells at four different stages of transforming growth factor-β3-induced chondrogenesis differentiation (Yang et al., 2011). On the other hand, miR-132/212 clusters regulate hematopoietic stem cell maintenance and survival with age by buffering forkhead box O-3 (FoxO3) expression (Mehta et al., 2015). While no research has been performed to decipher the role of miR-212-5p in SMSCs. In our study, miR-212-5p is identified to contribute to the regulation of chondrocytes mediated by SMSC-Exos or SMSC-212-5p-Exos. All of these results indicate the universal utilization of miR-212-5p based treatment.

As transcription factors belonging to the ETS family, ELF3 participates in autoimmune and tumor neogenesis (Jedlicka and Gutierrez-Hartmann, 2008; Luk et al., 2018). The previous study demonstrates that ELF3 could modulate type II collagen transcription by prohibiting SOX9-CBP/p300-driven histone acetyltransferase activity in chondrocytes (Otero et al., 2017). On the other hand, ELF3 co-localizes with MMP-13 protein to regulate the transcription activity in human osteoarthritic cartilage (Otero et al., 2012). In accordance with the up-regulated SOX9 and MMP-13 expression testified in our study, all of these results define a pro-catabolic role for ELF3 to regulate MMP-13 and SOX9 mediated cartilage remodeling and degradation. Elevated ELF3 expression in OA cartilage tissues can act as a mediator of inflammatory and catabolism to destruct cartilage, which indicates the potential target of ELF3 to alleviate OA (Conde et al., 2018).

ELF3 is identified to direct target miR-212 to suppress nasopharyngeal carcinoma cells proliferation (Kang et al.,

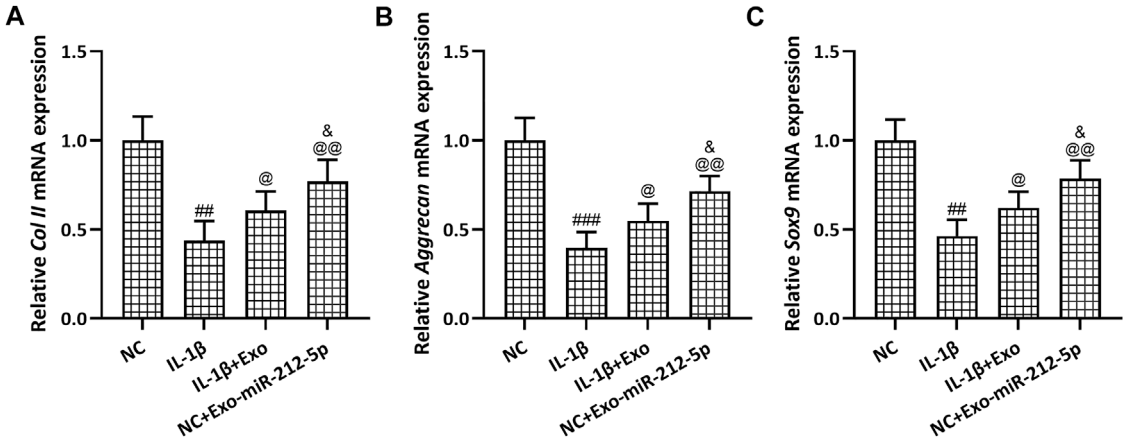

FIGURE 5 | SMSC-212-5p-Exos attenuated IL-1β induced chondrocyte degeneration. IL-1β induced chondrocytes were treated with SMSC-Exos or SMSC-212-5p-Exos for 48 h qRT-PCR was utilized to analyze the mRNA expressions of Collagen II **(A)**, Aggrecan **(B)** and Sox9 **(C)**. $n = 3$ for each group. ##$p < 0.01$, ###$p < 0.001$ compared to NC. @$p < 0.05$, @@$p < 0.01$ compared to IL-1β. &$p < 0.05$ compared to IL-1β+Exo. One-way ANOVA followed Dunn's multiple comparisons test.

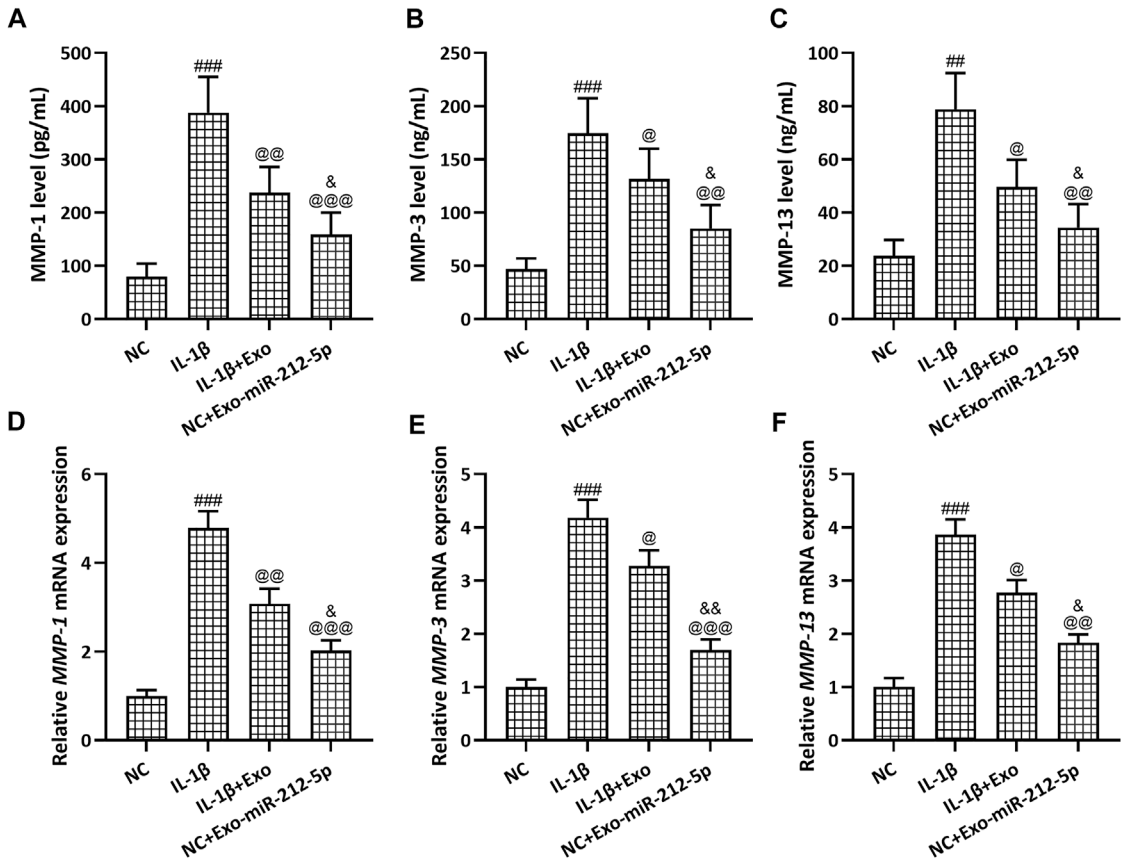

FIGURE 6 | SMSC-212-5p-Exos attenuated IL-1β induced MMP-1, MMP-3, and MMP-13 expression in chondrocytes. IL-1β induced chondrocytes were treated with SMSC-Exos or SMSC-212-5p-Exos for 48 h. MMP-1, MMP-3, and MMP-13 production in cellular supernatant were measured by ELISA **(A–C)**. mRNA expression of MMP-1, MMP-3, and MMP-13 from cell lysis were tested by qRT-PCR **(D–F)**. $n = 6$ for each group in ELISA analysis, $n = 3$ for each group in qRT-PCR test. ##$p < 0.01$, ###$p < 0.001$ compared to NC. @$p < 0.05$, @@$p < 0.01$, @@@$p < 0.001$ compared to IL-1β. &$p < 0.05$, &&$p < 0.01$ compared to IL-1β+Exo. One-way ANOVA followed Dunn's multiple comparisons test.

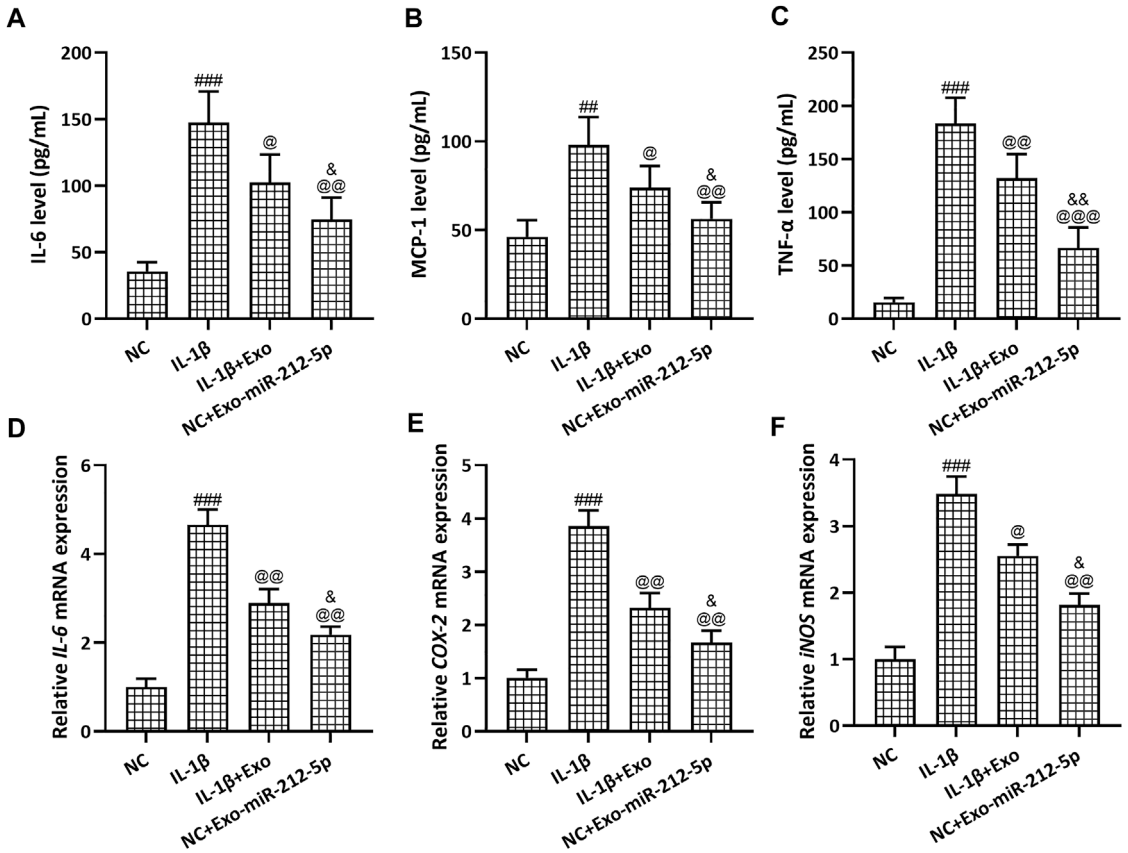

FIGURE 7 | SMSC-212-5p-Exos attenuated IL-1β induced inflammatory responses in chondrocytes. IL-1β induced chondrocytes were treated with SMSC-Exos or SMSC-212-5p-Exos for 48 h. IL-6, MCP-1, and TNF-α production in cellular supernatant were measured by ELISA **(A–C)**. mRNA levels of *IL-6*, *COX-2*, and *iNOS* from cell lysis were tested by qRT-PCR **(D–F)**. $n = 6$ for each group in ELISA analysis, $n = 3$ for each group in qRT-PCR test. ##$p < 0.01$, ###$p < 0.001$ compared to NC. @$p < 0.05$, @@$p < 0.01$, @@@$p < 0.001$ compared to IL-1β. &$p < 0.05$, &&$p < 0.01$ compared to IL-1β+Exo. One-way ANOVA followed Dunn's multiple comparisons test.

2020). In this research, miR-212-5p could target ELF3 to alleviate IL-1β induced chondrocytes degradation and inflammation. There are some limitations that should be indicated here. Microenvironment can alter the composition of exosomes (Skuratovskaia et al., 2020), and *in vivo* models should be constructed to demonstrate the therapeutic benefit of SMSC-212-5p-Exos. Whether other molecules, except miR-212-5p, could contribute to the treatment advantage in SMSC-212-5p-Exos compared with SMSC-Exos need further detailed analysis.

Accumulating investigations have indicated that exosomes will pave a new therapeutic paradigm for osteoarthritis treatment. Our study testifies that SMSC-212-5p-Exos could alleviate IL-1β induced chondrocytes degradation, degradation, and inflammation process involved in OA. It is worth noting that only *in vitro* model is utilized in the study, and the OA *in vivo* model should be constructed to demonstrate the treatment benefit of SMSC-212-5p-Exos. All in all, this study indicates that SMSC-212-5p-Exos could target ELF3-mediated OA pathology in chondrocytes, which can be considered as a future treatment option.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Xijing Hospital. The patients/participants provided their written informed consent to participate in this study. Written informed consent was not obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

Concept or design: TZ and ML. Acquisition of data: TZ, YL, XZ, JX, and ML. Analysis or interpretation of data: TZ, YL, and ML. Drafting of the manuscript: TZ, YL, XZ, JX, and ML. Critical revision of the manuscript for important intellectual content: All authors. All authors had full access to the data, contributed to the study, approved the final version for publication, and took responsibility for its accuracy and integrity.

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REVIEW
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Mesenchymal Stromal Cell-Derived Extracellular Vesicles as Biological Carriers for Drug Delivery in Cancer Therapy

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Cancer is the second leading cause of death worldwide, with 10.0 million cancer deaths in 2020. Despite advances in targeted therapies, some pharmacological drawbacks associated with anticancer chemo and immunotherapeutic agents include high toxicities, low bioavailability, and drug resistance. In recent years, extracellular vesicles emerged as a new promising platform for drug delivery, with the advantage of their inherent biocompatibility and specific targeting compared to artificial nanocarriers, such as liposomes. Particularly, mesenchymal stem/stromal cells were proposed as a source of extracellular vesicles for cancer therapy because of their intrinsic properties: high *in vitro* self-renewal and proliferation, regenerative and immunomodulatory capacities, and secretion of extracellular vesicles that mediate most of their paracrine functions. Moreover, extracellular vesicles are static and safer in comparison with mesenchymal stem/stromal cells, which can undergo genetic/epigenetic or phenotypic changes after their administration to patients. In this review, we summarize currently reported information regarding mesenchymal stem/stromal cell-derived extracellular vesicles, their proper isolation and purification techniques - from either naive or engineered mesenchymal stem/stromal cells - for their application in cancer therapy, as well as available downstream modification methods to improve their therapeutic properties. Additionally, we discuss the challenges associated with extracellular vesicles for cancer therapy, and we review some preclinical and clinical data available in the literature.

Keywords: mesenchymal stem/ stromal cells, extracellular vesicles, cancer therapy, drug delivery systems, cell-free therapy

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INTRODUCTION

Cancer is the second leading cause of death worldwide after cardiovascular diseases (Cortes et al., 2020; Lih et al., 2020). The GLOBOCAN cancer statistics estimated 19.3 million new cases and 10 million deaths worldwide in 2020 (Ferlay et al., 2021). Despite advances in targeted therapies and immunotherapies, tumors may not only develop drug resistance–in response to therapy or due to intrinsic intratumoral heterogeneity - and metastasize to distant organs, but also many patients do not benefit from the currently available therapies, or they suffer from off-target or immune-related

adverse effects (Martin et al., 2020; Saber et al., 2020). Moreover, some pharmacological difficulties are associated with anticancer chemotherapeutic agents, such as elevated toxicities, low bioavailability of free drugs, and drug resistance (Fang et al., 2020; Parodi et al., 2021). In consequence, nanodrug delivery systems–mainly liposomes - have been studied as an attractive option due to their several advantages, which include controlled drug release, protection from degradation in the circulation, reduced side effects, and increased drug solubility (Fang et al., 2020; van der Koog et al., 2021). However, artificial nanocarriers exert some limitations, such as rapid plasma clearance and accumulation in clearance organs, immunogenicity, unspecific targeting, and hypersensitivity reactions (De Jong et al., 2019; Attia and Mashal, 2021; van der Koog et al., 2021). In recent years, extracellular vesicles (EVs) have gained recognition as a new promising platform for drug delivery, with the advantage of their inherent biocompatibility because of their complex biological composition compared to artificial nanocarriers (van der Koog et al., 2021). Remarkably, mesenchymal stem/stromal cells (MSCs) emerged as a source of EVs for cancer therapy because of their inherent characteristics, with EVs either obtained from naïve or genetically engineered MSCs. In the next sections, we will discuss the properties that make MSCs suitable parental cells for the isolation of therapeutic EVs, as well as EVs manufacturing process and methods employed for their modification, with their respective benefits and limitations. Finally, we will review some pre-clinical and clinical data published in the last 5 years regarding EVs as drug delivery systems (DDSs) for cancer therapy.

GENERAL CHARACTERISTICS OF MESENCHYMAL STEM/STROMAL CELLS AND THEIR RELEVANCE IN CELL THERAPY

MSCs are non-hematopoietic multipotent precursor cells, first discovered by Friedenstein and colleagues in the bone marrow (BM) stroma, that contribute to the maintenance and regeneration of different connective tissues (such as bone, cartilage, adipose, and muscle tissues) (Gregory et al., 2005; Nombela-Arrieta et al., 2011). These cells can differentiate into multiple mesoderm-type cell lineages, like osteoblasts, chondrocytes, and endothelial cells, and non-mesoderm-type lineages, such as neuronal-like cells (Kassem et al., 2004). In addition, MSCs exhibit a high clonogenic nature, with rapid *in vitro* expansion and development of fibroblasts colony-forming units (Prockop and Oh, 2012), desirable properties when culturing parental cells for EVs isolation. In accordance with the criteria defined by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy, human MSCs are distinguished based on some minimal characteristics. These include their plastic-adherence, the expression of 95–99% levels of CD73, CD90, and CD105 surface markers, as well as the lack of expression of human leukocyte antigen (HLA)-DR, CD34, CD45, CD14 or CD11b, CD79a or CD19 markers, and also the ability to differentiate into adipogenic, chondrogenic and osteogenic lineages *in vitro* (Dominici et al., 2006; Brown et al., 2019). Because of their low expression of major histocompatibility complex (MHC) type I and lack of expression of MHC II molecules, MSCs are considered an immunoprivileged cell type, with a low risk of cellular rejection when used for cellular therapy (Lee and Hong, 2017). Apart from BM, MSCs can be isolated from other tissues, including adipose tissue (AT), dental pulp, umbilical cord (UC) blood and perivascular tissue, as well as from menstrual blood (Berebichez-Fridman and Montero-Olvera, 2018). While BM-MSCs are more difficult to obtain since they involve performing painful procedures to patients, AT-MSCs are relatively easy to isolate from AT available as a subproduct of cosmetic procedures (i.e., liposuction), while exhibiting similar phenotypic and functional properties to BM-MSCs (Berebichez-Fridman and Montero-Olvera, 2018). Regarding the self-renewal potential and proliferation capacity, MSCs may differ in these properties depending on their source of origin (Brown et al., 2019). Both human UC perivascular cells (HUCPVCs) and AT-MSCs have higher proliferation rates than BM-MSCs, being an attractive option to the latter for cell therapy (Kern et al., 2006; Yannarelli et al., 2013). Particularly, HUCPVCs overcome BM-MSCs limitation of reduced proliferation potential with increasing donor age and the increased heterogeneity between donors (Yannarelli et al., 2013).

Regarding their functional properties, MSCs are well known for their immunomodulatory effects both *in vitro* and *in vivo*. These cells secrete a variety of soluble factors and chemokines that mediate immunosuppression by inhibiting B and T cells, monocyte maturation, as well as the generation of regulatory T cells and M2 macrophages polarization (Nauta and Fibbe, 2007; Kim et al., 2013; de Witte et al., 2015; Jiang and Xu, 2020). Moreover, depending on the signals of the particular microenvironment, BM-MSCs can polarize into two different subtypes upon the stimulation of Toll-like receptors 3 or 4 (He et al., 2009). MSC1 or toll-like receptor 4-stimulated MSCs exhibit a pro-inflammatory phenotype, as they secrete the C-X-C Motif Chemokine Ligand (CXCL) 1, interleukin (IL)-6, IL-8 -among others-, whereas MSC2—or toll-like receptor 3-stimulated MSCs - secrete immunosuppressive factors, such as prostaglandin E2, indoleamine-2,3-dioxygenase, and IL-10 (Meisel et al., 2004; Tomchuck et al., 2008; de Witte et al., 2015). In addition to their immunomodulatory properties, BM-MSCs can mobilize, through peripheral circulation, to injured tissues during the healing process (Fu et al., 2019). Among the factors that regulate the migration of BM-MSCs, there are chemical factors, including CXCL12 chemokine, osteopontin, vascular endothelial growth factor, insulin growth factor 1, and transforming growth factor-beta, as well as mechanical factors such as mechanical strain, shear stress, matrix stiffness and microgravity (Fu et al., 2019). Once BM-MSCs reach the injury site, they can establish cell-to-cell contacts or secrete many bioactive factors that promote suppression of local immune responses, stimulation of fibrotic tissue formation, and modulation of angiogenesis and cell proliferation (Samsonraj et al., 2017).

During the last 20 years, MSCs have emerged as a promising source for cell therapy to treat several disorders because of their immunomodulatory and regenerative properties. While the first clinical trials with MSCs included osteogenesis imperfecta and graft vs. host disease (Horwitz et al., 1999; Lee et al., 2009), soon after, these cells began to be studied to treat immune-mediated and degenerative disorders (Saeedi et al., 2019). These pathologies include ischemia-reperfusion induced injury (Zhang et al., 2020), myocardial infarction (Ward et al., 2018), covid-19 pneumonia (Metcalfe, 2020), and cancer (Hmadcha et al., 2020). As Saeedi et al. reviewed in 2019, autoimmune diseases (25%), cardiovascular diseases (15%), and neuro degenerative diseases (12%) were the top-three diseases studied in clinical trials involving the administration of MSCs (Saeedi et al., 2019). Regarding MSCs use for treating cardiovascular diseases, these cells are known to have protective effects on the myocardium, by reducing inflammation and promoting angiogenesis and apoptosis resistance (Guo et al., 2020). However, their systemic administration caused embolism and inflammation, according to the clinical trials reports (Guo et al., 2020). Additionally, MSCs properties such as their homing capacity, immunomodulation, inhibition of inflammation and their ability to differentiate into neuron-like cells under specific *in vitro* conditions, have been studied for the treatment of neurodegenerative diseases (Yao et al., 2020). Recently, MSCs have been also tested in several clinical trials to treat the adverse effects caused by the SARS-CoV-2 infection, via their immunomodulatory and anti-inflammatory effects (Moradinasab et al., 2021).

With reference to the most suitable source of MSCs for cell-therapy, BM-MSCs are known to exhibit tropism for tumor sites and decreased immunosuppression, whereas AT-MSCs and UC-MSCs exert higher proliferation rates and are easier to isolate (Li et al., 2014). Additionally, both human UC-MSCs and AT-MSCs showed tropism toward cancer cells *in vitro* (Gondi et al., 2010; Chen et al., 2019). As Pulukuri et al. reported, the induction of urokinase plasminogen activator expression by histone deacetylase inhibitors could represent a strategy for enhancing the tumor tropism of MSCs (Pulukuri et al., 2010). Although nowadays it is still unclear which source of MSCs is better for therapeutic applications, we hypothesize that UC-MSCs could represent the best option for their use in cell-therapy, particularly in cancer, due to the mentioned properties.

Apart from using naïve MSCs for therapeutic applications, these cells can be modified by pre-conditioning with specific factors or employing genetic engineering techniques to enhance their therapeutic properties and/or reduce potential disadvantages (i.e., improvement of adhesion and survival or preventing premature senescence) (Wiredu Ocansey et al., 2020). For instance, human adult MSCs can be modified to deliver pro-apoptotic proteins or cytokines, such as tumor necrosis factor related apoptosis induced ligand (TRAIL), with positive anti-tumoral effects on colorectal cancer, or IL-12 overexpressing murine BM-MSCs that induced tumor cells apoptosis in melanoma and lung cancer murine models (Chulpanova et al., 2018). Additionally, an ongoing clinical trial (NCT03298763) is testing the administration of TRAIL-modified MSCs in combination with cisplatin/pemetrexed chemotherapy in metastatic non-small cell lung cancer patients. Moreover, it is easier to genetically modify MSCs than hematopoietic stem cells, while MSCs retain their *in vivo* activity after their modification (Attia and Mashal, 2021). Developing new and improved transfection methods –both chemical and physical - without associated risks –such as insertional mutations or adverse immune reactions –may facilitate those procedures (Attia and Mashal, 2021).

Despite those beneficial properties, the role of MSCs in cancer progression remains contradictory. On the one hand, a considerable amount of evidence supports the fact that BM-MSCs can migrate to primary tumors where the microenvironment educates them to become pro-tumoral, either as tumor-associated MSCs, or differentiated into tumor-associated fibroblasts as well (Hill et al., 2017; Giorello et al., 2021). In this way, BM-MSCs can promote tumor growth, immunosuppression, inflammation, drug resistance, angiogenesis, invasion and metastasis, and may even promote the establishment of pre-metastatic niches in distant organs (Gao et al., 2018; Pietrovito et al., 2018; Xu et al., 2018; Sanmartin et al., 2021). On the other hand, some studies showed that MSCs could exert inhibitory effects on tumor cells. For example, it was described that murine BM-MSCs inhibited tumor cell growth *in vitro* and *in vivo* in a cell number-dependent manner by inducing cell cycle arrest and apoptotic death in different cancer cell lines (Lu et al., 2008). Similarly, Bruno S. et al. also showed that human BM-MSC-derived EVs induced cell cycle arrest and inhibited tumor growth of hepatoma, Kaposi´s sarcoma, and ovarian cancer cell lines *in vivo* (Bruno et al., 2014). Li et al. published some contradictory results, showing that human BM-MSCs promoted the proliferation of hepatocellular carcinoma cells *in vitro*, and significantly inhibited invasiveness and metastasis through downregulation of transforming growth factor-beta (Li et al., 2010). Likewise, Bajetto et al. demonstrated *in vitro* that UC-MSCs anti-tumoral effects on glioblastoma cells were mediated by direct cell-to-cell contacts, while pro-tumoral effects involved releasing soluble factors (Bajetto et al., 2017). Moreover, Zhu et al. identified factor Dickkopf-1 as a key molecule - secreted byAT-MSCs - that may mediate the inhibitory effect of leukemia cells proliferation through the negative regulation of the WNT signaling pathway (Zhu et al., 2009). The evident discrepancies between studies may be due to the heterogeneity in MSCs sources and donors, as well as differences between types of cancer, cancer cell lines and *in vivo* models, and the lack of standardized culturing methods.

Apart from this controversy around the effects of MSCs on cancer cells, culture-expanded MSCs could potentially undergo malignant transformation and senescence-associated modifications at specific CpG sites (Wang et al., 2012a; 2012b). Senescent MSCs exert an altered phenotype, with decreased immunological properties and a higher production of pro-inflammatory cytokines, such as IL-6 and IL-8 (Drela et al., 2019). Nevertheless, it was subsequently demonstrated that MSCs exerting these abnormalities neither exhibited a growth advantage *in vitro* nor led to tumor formation in

immunocompromised mice (Rühle et al., 2019). According to the exclusion criteria proposed by the Cell Products Working Party and the Committee for Advanced Therapies, MSCs must be discarded upon identifying two identical abnormal metaphases on 20 metaphases in a karyotyping analysis (Rühle et al., 2019). However, even though no patients enrolled in clinical trials of MSC-therapies have been diagnosed with cancer (Lee and Hong, 2017), further studies need to be accomplished to assess this potential adverse effect in-depth. Furthermore, Di et al. demonstrated that *in vitro* aged human UC-MSCs (at passage 45) significantly promoted *in vitro* proliferation and migration of breast cancer cells and *in vivo* tumor progression compared with their 'young' counterparts (UC-MSCs at passage 5). They also identified IL-6 as the main mediator of these pro-tumoral effects (Di et al., 2014). It has also been shown that neonatal MSCs have higher proliferation potential in comparison to MSCs derived from adult sources (Drela et al., 2019). This evidence suggests that aged MSCs –or MSCs from aged donors - could represent a risk when used as anti-cancer cell therapies.

Another mechanism associated with MSC-based therapies is mitochondrial transfer. BM-MSCs are able to donate mitochondria to rescue cells from tissue damage through tunneling nanotubes (TNT), gap-junctions, or mitochondrial DNA transfer through EVs (Li et al., 2019a). In addition, MSCs from different sources may alter oxidative phosphorylation and reactive oxygen species generation through mitochondrial transfer, which leads to acute and chronic inflammation and apoptotic cell death (Stavely and Nurgali, 2020). However, mitochondrial transfer-based therapies could be disadvantageous in cancer patients since BM-MSCs that migrate to the primary tumor could transfer mitochondria to cancer cells, enhancing their chemo-resistance and proliferation rates (Pasquier et al., 2013). In this way, some investigators showed that the inhibition of TNT contacts –through Intercellular Adhesion Molecule 1 blocking - between UC-MSCs and human acute T cell leukemia Jurkat cells, lead to chemotherapy-induced tumor cell death (Paliwal et al., 2018), positioning TNT inhibition as a novel target therapy in some types of cancer. Finally, the therapeutic effect of MSCs in chemotherapy-induced tissue damage has also been studied *in vitro*. MSCs –independently of their tissue of origin- are radioresistant cells, as well as they appear to be unaffected by some chemotherapeutic agents commonly used for cancer treatment. This may be due to their high expression of anti-apoptotic proteins and their elevated antioxidant activity (Rühle et al., 2019). Further *in vitro* and *in vivo* studies are required to elucidate the mechanisms by which MSCs exert regeneration in the context of chemotherapy-induced injuries before the clinical translation of those results.

EXTRACELLULAR VESICLES AS A NEW PARADIGM FOR CELL-FREE THERAPY

Despite the fact that MSCs have been used in many clinical trials to treat several pathologies, the inoculation of viable MSCs into patients entails some hindrances. These include limited passaging before entering a senescent state –in particular with BM-MSCs -, cellular heterogeneity, the alteration of their differentiation potential under hypoxic conditions, the occurrence of epigenetic changes during culturing - in genes that regulate self-renewal -, excessive immunosuppression, and risk of lung embolism (Lee et al., 2009; Brown et al., 2019; Wang et al., 2020). Those are the main reasons why MSC-derived EVs have arisen as an interesting and potential option to MSCs for the treatment of several diseases, including cartilage defects, osteoarthritis, renal injury, hepatocellular injury, macular degeneration, and diabetes mellitus (Cai et al., 2020; Rostom et al., 2020). Since the discovery of EVs therapeutic properties, the number of published articles reporting their potential applications for the treatment of different pathologies raised from a few tens in 2015 to over 1,000 in 2021 (Racchetti and Meldolesi, 2021), thus reinforcing EVs potentiality when used as cell-free therapy. In addition, many groups previously described that MSCs mediate their biological and regenerative effects through the secretion of EVs (Phelps et al., 2018; Witwer et al., 2019), and that MSC-derived EVs seem to have the same effects as the parental cells while considered safer (Gratpain et al., 2021). Moreover, it was reported that MSC-derived EVs isolated from MSCs of different sources might be beneficial for treating specific diseases. For instance, BM-MSC-derived EVs exerted good properties for treating cartilage defects or osteoarthritis, due to their intrinsic bone tropism, while AT-MSC-derived EVs regulate inflammation in various disease models (Cai et al., 2020). Human UC-MSC-derived EVs were shown to promote neural restoration, heart repair, and protection of liver and kidney, through promoting angiogenesis and reducing apoptosis, as well as these cells are known to produce higher amounts of EVs when compared with MSCs from other sources (Cai et al., 2020). Particularly, using MSCs as parental cells for EVs isolation is advantageous since clinical-grade MSCs have been cultured for years for cell therapy applications, already meeting the regulatory requirements and following Good Manufacturing Practice (GMP) guidelines (Burnouf et al., 2019).

According to the International Society of Extracellular Vesicles, EVs are "nanosized lipid bilayer encapsulated membranes carrying proteins, lipids, nucleic acids and sugars that are shed by the majority of the cells into the extracellular milieu to mediate intercellular communication, by transferring molecules from parental/donor cells to target cells" (Soekmadji et al., 2020). Additionally, EVs can be classified based on their size, origin, and content (Battistelli and Falcieri, 2020). Exosomes are 30–200 nm vesicles originated from the endosomal compartment, secreted when multivesicular endosomes fuse with the cell membrane releasing their intraluminal vesicles in the extracellular space (Van Niel et al., 2018). Exosomes also have a complex membrane composition, consisting of a lipid bilayer with cholesterol and sphingomyelin present in lipid rafts. They can transport different molecular cargos –such as proteins, lipids, messenger RNAs (mRNAs) and microRNAs (miRNAs) - to target cells in both physiological and pathological contexts (Logozzi et al., 2019). Because of their endosomal origin, exosomes contain membrane transport and fusion proteins, tetraspanins (CD9, CD63, CD81), and proteins involved in the

multivesicular bodies biogenesis: programmed cell death 6 interacting protein (Alix) and Tumor susceptibility gene 101 (TSG101). These proteins are widely used as exosome positive markers, although their presence may vary depending on exosomes origin (Vlassov et al., 2012). Alternatively, microvesicles (MVs) are nanosized particles - 50–1,000 nm in diameter - generated through cell membrane budding after agonist activation, shear/physical stress, or oxidative stress, and may contain organelles proteins –such as those from the endoplasmic reticulum, Golgi, mitochondria and nucleus—(Boilard, 2018; Agrahari et al., 2019; Bodega et al., 2019). MVs also participate in intercellular communication, modulating several biological functions (Zi-Tong et al., 2022). It is important to highlight that since analytical techniques do not differentiate between exosomes and MVs due to their range overlapping, the International Society for Extracellular Vesicles recommends using the term small EVs (sEVs) instead (Witwer and Théry, 2019).

Finally, apoptotic bodies (ABs) are the largest membrane-bound extracellular vesicles –of 1–5 μm in diameter - generated during apoptotic cells disassemble (Jiang et al., 2017). ABs play a relevant role in the clearance of apoptotic cells by phagocytes and in cell-to-cell communication through their molecular cargos (Jiang et al., 2017). These large EVs may contain chromatin, glycosylated proteins, RNA, and even entire organelles - mainly mitochondria and nuclear fragments - (Irimie and Berindan-neagoe, 2020). While previously neglected, more attention has been recently focused on ABs. For instance, it was reported that ABs might be involved in cancer progression and metastasis (Gregory and Dransfield, 2018; Irimie and Berindan-neagoe, 2020). Liu et al. demonstrated - in a myocardial infarction model - that transplanted murine BM-MSCs that undergo apoptosis can promote angiogenesis through the release of ABs, by regulating autophagy in recipient endothelial cells (Liu et al., 2020). Furthermore, Li et al. used ABs purified from AT-MSCs to improve skin wound healing (Li J. et al., 2022). It was recently elucidated that ABs represent a heterogeneous population, including ABs with a size of less than 1 μm –defined as apoptotic MVs - which differ in their physiological and membrane integrity properties from larger ABs (Kakarla et al., 2020).

Regarding EVs therapeutic benefits for cancer therapy, they have intrinsic and target-specific homing capabilities when compared to free drugs or artificial nanocarriers, which may be related to the presence of specific surface proteins - such as integrins - and their ability to penetrate biological barriers –like the blood-brain barrier - (Berumen Sánchez et al., 2021). EVs also protect biological cargo –especially miRNAs and mRNAs–from degradation after their administration *in vivo* (Famta et al., 2022). Moreover, modern techniques allow EVs modification to increase tissue targeting efficiency (Antes et al., 2018), as we will discuss later. Particularly, MSC-derived EVs may exert similar tumor-homing properties to their parental cells, as exosomes from UC-MSCs were shown to concentrate mainly in osteosarcoma tumors *in vivo* (Quadri et al., 2022). In addition, it was demonstrated that EVs could passively reach the tumor microenvironment through enhanced permeability and retention effect (Li H. et al., 2022). MSC-derived EVs also have lower immunogenicity than MSCs (Li et al., 2021). However, as evidence suggests, MSCs from different tissue sources may release EVs that differ in their functional properties (Cai et al., 2020). This has to be considered when choosing the source of parental MSCs for EVs isolation, depending on the specific therapeutic goal. Moreover, recent innovations applied to the engineering of EVs have progressively circumvented the limitations of naive EVs (Ullah et al., 2021), as we will discuss later.

However, nothing is ever as simple as it seems. Despite several EVs-associated advantages, many groups reported some disadvantages. For instance, because of their similarities in size, EVs may be contaminated with viruses, many of which can incorporate their genetic material or proteins into EVs –i.e., Epstein-Barr virus and Kaposi's Sarcoma-Associated Herpesvirus - (McNamara and Dittmer, 2020). Moreover, the utilization of fetal bovine serum (FBS) for MSCs *in vitro* culturing not only represents a source of exogenous contaminating EVs, but also it was reported that FBS might alter MSCs phenotype, turning these cells immunogenic (Naskou et al., 2018; Lehrich et al., 2021). Additionally, it was shown that human BM-MSC-derived EVs might exert pleiotropic effects when administrated *in vivo* (Boulestreau et al., 2021). Finally, the cargo of BM-MSC-derived EVs can be highly dependent on cell culture conditions (Parfejevs et al., 2020). Heterogeneity in MSCs phenotype and secretome –including EVs - is also attributed to donor variability, variations in O_2 tension, genetic manipulation, senescence and oxidative stress (Ratushnyy et al., 2020; Costa et al., 2021). This evidence supports the requirement of additional efforts for *in vitro* clinical-grade MSCs culturing standardization, following current GMP guidelines.

Extracellular Vesicles Biodistribution and Tracking Assays

With reference to EVs biodistribution and pharmacokinetics upon their administration in animal models, evidence shows that they concentrate mainly in the liver, lungs, kidneys, and spleen (Kang et al., 2021). While most sEVs are located in the liver, larger EVs are concentrated mainly in the lungs (Kang et al., 2021). Differences between studies are attributed to the heterogeneity between EV sources, doses and routes of administration, animal models, chosen endpoints, as well as to mechanical factors –i.e., the more rigid the EVs are, the more efficient the uptake process is -, among others (Dang et al., 2020; Kang et al., 2021). Moreover, EVs clearance was reported to be rapid –between 1 and 6 h after administration - through renal and hepatic processing (Yin et al., 2020). This represents a limitation when using EVs as DDSs, since it may require the administration of multiple doses to achieve the desired therapeutic effect. Novel techniques are being developed in an attempt to improve EVs biodistribution and facilitate *in vivo* tracking. For example, Gangadaran et al. developed an *in vivo* bioluminescence imaging and tracking system for EVs based on *Renilla* luciferase (Gangadaran and Ahn, 2020). Similarly, Shimomura et al. designed novel fluorescent probes that bind EVs membranes - with no risk of EVs aggregation - for monitoring their uptake

(Shimomura et al., 2021). Additional methods for non-invasive *in vivo* tracking of EVs include near-infrared dyes –with the advantages of their intense signal, low autofluorescence of biological tissue in the spectral range used, and deep tissue penetration of near-infrared light-, as well as EVs modified with ultra-small superparamagnetic iron oxide for their analysis by magnetic resonance imaging (Gangadaran et al., 2018). In addition, EVs can be modified to delay their clearance from circulation or improve their biodistribution. Royo et al. showed that neuraminidase treatment –a glycosidase that alters EVs surface glycosylation profile - induced an accumulation in the lungs compared with untreated EVs (Royo et al., 2019). Moreover, since macrophages can take up EVs and clear them from the circulation, EVs can be engineered through the incorporation of CD47—a transmembrane protein that enables macrophages evasion -, which initiates a "don't eat me" signal (Imai et al., 2015; Kamerkar et al., 2017; Belhadj et al., 2020; Wei et al., 2021). Wei et al. reported that CD47-enriched EVs remained more time in circulation when compared with unmodified EVs (Wei et al., 2021). Similarly, the modification of EVs surfaces with polyethylene glycol (PEG) via PEGylation is another available strategy for improving EVs targeting (Clark et al., 2021).

Upon their arrival to the target tissue, EVs must be internalized by cells. There are several mechanisms by which EVs are up-taken by cells, depending on the specific type of EVs. These include clathrin-mediated endocytosis, caveolin-mediated endocytosis, macropinocytosis, phagocytosis, and direct fusion (Melling et al., 2019). Once incorporated into cells, EVs need to escape the endosomal compartment to prevent their degradation by lysosomes and cargo destruction. Some lysosomotropic molecules promote endosomal escape, such as chloroquine, amantadine, and ammonium chloride (Heath et al., 2019). Although the mechanisms underlying the endosomal escape of EVs are still not fully understood, Joshi et al. reported that EVs content release occurs from endosomes/lysosomes upon neutralizing endosomal pH and cholesterol accumulation to block EVs cargo exposure (Joshi et al., 2020). In addition, EVs can be engineered by expressing a pH-sensitive peptide, which allows EVs content release through the peptide fusion with the endosomal membrane upon endosomal acidification (Joshi et al., 2020). Nevertheless, additional efforts are needed to elucidate the mechanisms and the molecules involved in EVs processing after their incorporation by cells, in order to develop techniques that may improve cargo release.

Upstream and Downstream Processing

In the production of EVs for therapeutic applications, regulatory agencies request a complete characterization of the active drug and its mode of action (MoA). They also require making batch-to-batch comparisons through specific biochemical, biophysical, and functional/potency assays. In this way, the early characterization of the manufacturing process is crucial since it enables the design of untimely standard operation procedures and the identification of potential risks and bottlenecks in advance, guaranteeing consistency and reproducibility (Rohde et al., 2019). EVs manufacturing process can be divided into two big stages: upstream processing –which encompasses all the operations required to produce the conditioned media (CM) for EVs isolation -, and downstream processing –which includes EVs purification and concentration, as well as the final product formulation and characterization (Staubach et al., 2021).

According to the International Society of Cell Therapy recommendations, it is critical to evaluate the parental MSCs to determine their phenotype, genetic stability, and potential biological contamination. While bacteria can be eliminated through filtration, viruses are more difficult to remove due to their similarity in size and charge with sEVs (Burnouf et al., 2019). In consequence, both *in vitro* and *in vivo* testing are required. Despite several methods are available in the literature for viral inactivation –including solvents, detergents, irradiation, and nanofiltration-, their utilization may alter EVs structure and content (Burnouf et al., 2019). Regarding MSCs tissue source, as many studies suggest, MSCs isolated from cancer patients may exert some pathological alterations, so the obtaining of healthy volunteers-derived allogeneic MSCs for EVs isolation is highly recommended (Hofer et al., 2010; Fernandez Vallone et al., 2013; Rühle et al., 2019). Open/semi-open systems have been the most utilized for cell culturing, although they may be associated with variability between batches, risk of contamination, and lack of real-time process control (Roura et al., 2017). In counterpart, closed systems employment is related to reduced risk of contamination, higher yields and cell-mass expansion, GMP compatibility, reduced costs of production, and the incorporation of in-process controls (Roura et al., 2017). Furthermore, it was demonstrated that 3D spheroidal culturing of MSCs promotes their exosome secretion (Kim M. et al., 2018; Yan and Wu, 2020). Multilayered cell culture flasks, hollow fiber bioreactors, stirred-tank bioreactors, and spheroidal aggregates of MSCs are among the most popular 3D-culture closed systems (Maumus et al., 2020). In this regard, it is necessary to establish a robust production system when using these technologies –which can be time-consuming - not only to control environmental parameters that may affect MSCs phenotype, but also to generate lot-consistent populations of EVs (Whitford and Guterstam, 2019). As previously mentioned, FBS represents some disadvantages when employed for clinical-grade MSCs culturing. Chemically defined media, human platelet lysate, and platelet-poor plasma xeno-free supplements have emerged as an alternative to FBS, with some contradictory associated reports. While platelet-poor plasma-cultured AT-MSCs showed impaired proliferative potential and an altered phenotype, MSCs from different sources cultured with xeno-free supplement derived from human plasma were reported to maintain their genetic stability, phenotype, and homogeneity (Blázquez-Prunera et al., 2017; Mushahary et al., 2018; Silva-Carvalho et al., 2020). However, these supplements hold some associated drawbacks. Human platelet lysate may contain platelet-derived contaminating EVs and pose the risk of pathogen contamination due to pooling –which is performed to limit batch-to-batch variability between

donors - (Nyam-Erdene et al., 2021). In addition, the donor dependence for the obtainment of xeno-free supplements represents a limitation for industrial escalation of MSCs culturing. Moreover, since FBS removal may alter MSCs and MSC-derived EVs phenotype, EVs properties need to be corroborated after choosing a certain culturing media.

Concerning downstream processing, following CM harvesting, the first step includes multiple rounds of centrifugation or filtration to clarify the CM. Ultracentrifugation or density gradient centrifugation - used alone or in combination - are still considered the gold standards for sEVs purification and enrichment (Kain, 2005; Royo et al., 2020). Briefly, ultracentrifugation consists of a short centrifugation round at low speed to remove cells and cellular debris. Then, the supernatant is recovered and centrifuged 10,000–20,000xg for 20–30 min to pellet large ABs and MVs. sEVs remain in the supernatant and can be isolated by ultracentrifugation at high speed (100,000–120,000xg), with the time-lapse depending on the rotor size (Momen-Heravi, 2017). However, ultracentrifugation is associated with low yields, time-consuming centrifugation steps, mechanical damage to sEVs structure, and co-precipitation of contaminants - such as apolipoproteins A1/2 or B and albumin - (Busatto et al., 2018; Takov et al., 2019). Chromatographic techniques are commonly employed, including anion exchange chromatography, affinity chromatography, and size exclusion chromatography, which respective advantages and disadvantages are reviewed elsewhere (Staubach et al., 2021). Other novel approaches for sEVs purification encompass microchips, nanowires, and acoustic separation, as recently reviewed (Akbar et al., 2022). Similarly, new large-scale methods have been developed to improve EVs purification efficiency. For instance, tangential flow filtration is a novel technique that couples permeable membrane filtration and flow to obtain a concentrated sample of EVs from a colloidal matrix, and is more efficient, scalable, and involves less batch-to-batch variability than ultracentrifugation (Busatto et al., 2018). It requires a dead-end pre-filtration and a posterior step of membrane filtration (Gao et al., 2020). Zhang et al. also developed a purification strategy based on DNA aptamer-based magnetic isolation, mediated by CD63 binding (Zhang et al., 2019). However, as we previously mentioned, it is challenging to isolate highly pure populations of sEVs –this means the complete separation between exosomes and smaller MVs -. Using affinity chromatography with anti-CD63 antibodies may lead to the isolation of sEVs enriched in an exosome population, but the remaining antibodies or beads used for the purification process may interfere with further functional and potency assays by altering sEVs interaction with target cells (Tkach et al., 2018). When choosing a purification method, it is critical to compromise between EVs product purity and the method gentleness to maintain EVs functional properties. Another issue concerning EVs formulation is storage since it may alter EVs morphology, size, and particle concentration (Qin et al., 2020). After performing a comparative study, Wu et al. recommended storage at 4°C or −20°C for short-term preservation of sEVs and storage at −80°C for long-term preservation, in accordance with other studies (Herrmann et al., 2021; Wu et al., 2021). Since storage at −80°C implies high energy consumption and transportation issues, as well as repeatedly freeze and thaw cycles may alter EVs function, alternative storage conditions are being studied, such as lyophilization (Trenkenschuh et al., 2021).

After EVs purification, the final product needs to be characterized, as well as its safety, efficacy, and purity must be assessed through quality control procedures. EVs analysis is usually challenging due to the small size, biological complexity, and heterogeneity (Panagopoulou et al., 2020). As it happens with other biological and medical products, quality control procedures should evaluate identity, purity, potency, safety, and stability (Rohde et al., 2019). For instance, endotoxins from water, raw materials, equipment, and culture systems can contaminate EV formulations. Although affinity chromatography is the most used technique for endotoxin removal, novel and improved methods are being studied, including electronic biosensors and real-time monitoring systems (Schneier et al., 2020). EVs formulation purity is a standard normalization metric used to evaluate the product composition and to make comparisons between batches. It is essential for EVs dose determination and contaminant identification, and can be assessed through biophysical or biochemical methods. Regarding EVs characterization, nanoparticle tracking analysis - a method that tracks individual nanoparticles and derives their size and concentration in suspension– is used for EVs quantification and size determination, while electron-microscopy and cryo-electron microscopy are commonly used for EVs morphological characterization (Emelyanov et al., 2020; Comfort et al., 2021; Stam et al., 2021). Other methods used for EVs quantification include microfluidics, arrays, and polymerase chain reaction microfluidic systems (Panagopoulou et al., 2020). Multiplex bead-based assays are generally employed to evaluate and quantify EV surface markers, with EVs captured on antibody-coated beads coupled with flow cytometry read-outs (Wiklander et al., 2018). In this way, Kilic et al. recently developed a novel label-free multiplexed system to evaluate EVs surface protein profile through impedance spectroscopy (Kilic et al., 2022). Additionally, fingerprinting assays evaluate the presence or absence of a set of specific markers on EVs for quality control and determination of consistency between batches (Jeske et al., 2020). Finally, toxicity, safety, and efficacy require ultimate confirmation by the performance of qualified and well-standardized potency assays. These tests differ from functional assays since regulations define potency as "the specific ability or capacity of a product to effect a given result, as indicated by appropriate laboratory tests or by adequately controlled clinical data obtained through the administration of the product in the manner intended" (Nguyen et al., 2020). Although the US Food and Drug Administration recommends that potency assays should ideally represent the product MoA, this does not necessarily happen, because potency assays may not reveal information about EVs underlying mechanisms of action (Reiner et al., 2017; Gimona et al., 2021). Additionally, due to their biological complexity and, in consequence, their multifaceted MoA, the US Food and Drug Administration also

recommends the utilization of an array matrix consisting of several potency assays for EVs evaluation (Gimona et al., 2021). Moreover, since EVs may exert multiple MoA given a specific pathological context, potency assays should be specifically related to the disease model for which they are intended (Willis et al., 2017). In conclusion, the development and standardization of improved clinical-grade quantification and characterization techniques, as well as potency assays for evaluating therapeutic EVs formulations, are critical requisites for EVs successful translation into the clinic as DDSs.

Drug Loading

As previously demonstrated, MSC-derived EVs display high flexibility to modifications to improve their properties (Weng et al., 2021). This is a crucial feature for their usage as DDSs so that manufacturing processes frequently include endogenous or exogenous drug loading approaches. Regarding the endogenous approach, it involves the modification of the parental cell - through methods such as transfection/transduction or co-incubation of mainly small drugs with parental cells - followed by the purification of the modified MSC-derived EVs (Liao et al., 2019). The efficiency of the drug packaging into EVs depends mainly on its concentration inside cells (Susa et al., 2019). Transfection/transduction of parental MSCs leads to overexpression of mRNAs, proteins, or miRNAs. For instance, Li et al. cultured endometrial cancer cells with miR-302a-loaded EVs from modified UC-MSCs, showing a significant inhibition of the proliferation and migration of tumor cells through the blocking of AKT pathway (Li et al., 2019b). In the same way, Liu et al. demonstrated that BM-MSC-derived EVs containing let-7i miRNA –by modification of MSCs through a lentiviral vector transfection - inhibited lung cancer cells outgrowth both *in vitro* and *in vivo* (Liu J. et al., 2021). It is relevant to mention that viral testing - through high throughput techniques - has to be more exhaustive in those cases in which MSCs were genetically modified since the utilization of viral vectors may lead to insertional mutations and mutagenesis (Rohde et al., 2019). Some disadvantages are associated with endogenous drug loading, including low efficiency in RNA packaging into EVs and genetic instability in parental MSCs (Liao et al., 2019; Su et al., 2021). Thus, non-viral transfection methods began to be evaluated in MSCs, such as nanocarriers that interact with the plasma membrane, followed by cells uptake. These non-viral transfection methods are flexible and scalable, but showed reduced transfection efficiency as well as higher toxicities (Hamann et al., 2019). With reference to co-incubation methods, loading efficiency may be affected by drug properties, incubation periods, among others (Zhang X. et al., 2021), so co-incubation protocols should be optimized for each specific case.

On the other hand, through exogenous approaches, drugs are loaded into EVs after their purification by using different chemical and physical methods - which have to be analyzed in advance to evaluate potential alterations of EVs properties - (Herrmann et al., 2021). Among the most utilized techniques are co-incubation with drugs, electroporation, and sonication, while other methods such as cycles of freeze/thaw, EVs permeabilization with saponins, or extrusion, are less used (Zhao et al., 2020). Additionally, exogenous methods are classified into passive or active loading approaches. While the former includes co-incubation of EVs with hydrophobic drugs –whose loading efficiency depends on the drug gradient - the latter is frequently used for hydrophilic drugs that cannot spontaneously go across the EVs membrane (Herrmann et al., 2021). For example, Wei et al. reported the utilization of BM-MSC-derived EVs loaded with doxorubicin through passive co-incubation for osteosarcoma cells growth inhibition *in vitro* (Wei et al., 2019). Drug encapsulation efficiency –the mass of drug in EVs divided by the mass of drug added to the mix - is an important parameter that has to be determined when employing co-incubation methods since these techniques often result in low loading capacity (Villa et al., 2019; Li H. et al., 2022). In addition, it was reported that the lipid composition of the EVs may have an impact on the drug loading efficiency (Kooijmans et al., 2021). In counterpart, active loading methods can be classified into physical and chemical techniques. The former includes electroporation, sonication, and freeze/thaw cycles, while the latter involves using transfection reagents or saponins. Physical methods are characterized by a high loading efficiency compared with chemical methods, but may alter EVs membrane integrity and produce siRNA aggregation (Tang et al., 2019). Otherwise, chemical reagents may accomplish some toxicity levels (Tang et al., 2019).

Surface Engineering

In order to improve MSC-derived EVs targeting properties and reduce their systemic toxicity, various EVs surface engineering approaches can be employed. On the one hand, genetic engineering of parental cells to display specific proteins on EV surfaces can only be utilized for protein and peptide engineering (Richardson and Ejima, 2019). In this way, Dooley et al. developed a platform for the fusion of proteins of interest to full-length or truncated forms of Prostaglandin F2 receptor negative regulator or Brain acid soluble protein 1, which are two scaffold proteins present in EVs and selectively sorted into them, allowing surface display and luminal loading of a wide range of molecules (Dooley et al., 2021). Yim N. et al. also developed a tool for exosomes engineering, named 'exosomes for protein loading via optically reversible protein-protein interactions' (EXPLORs). This system integrates a reversible protein-protein interaction module controlled by blue light –based on photoreceptor cryptochrome 2 protein - with the endogenous process of exosome biogenesis –through using a truncated version of Calcium and integrin-binding protein 1 conjugated to CD9 -. This method enables selective cargo of the therapeutic protein into EVs and its delivery to target cells after removing the illumination source (Yim et al., 2016). Similarly, the SMART exos system is based on expressing distinct types of monoclonal antibodies on sEVs surfaces that bind specific surface proteins in target cells (Shi et al., 2020). The genetic engineering approach can also be used to generate fusion proteins between the green fluorescent protein and sEVs-specific proteins –such as tetraspanins– to label sEVs for biodistribution analysis (Levy et al., 2021). A limitation of this method is that only

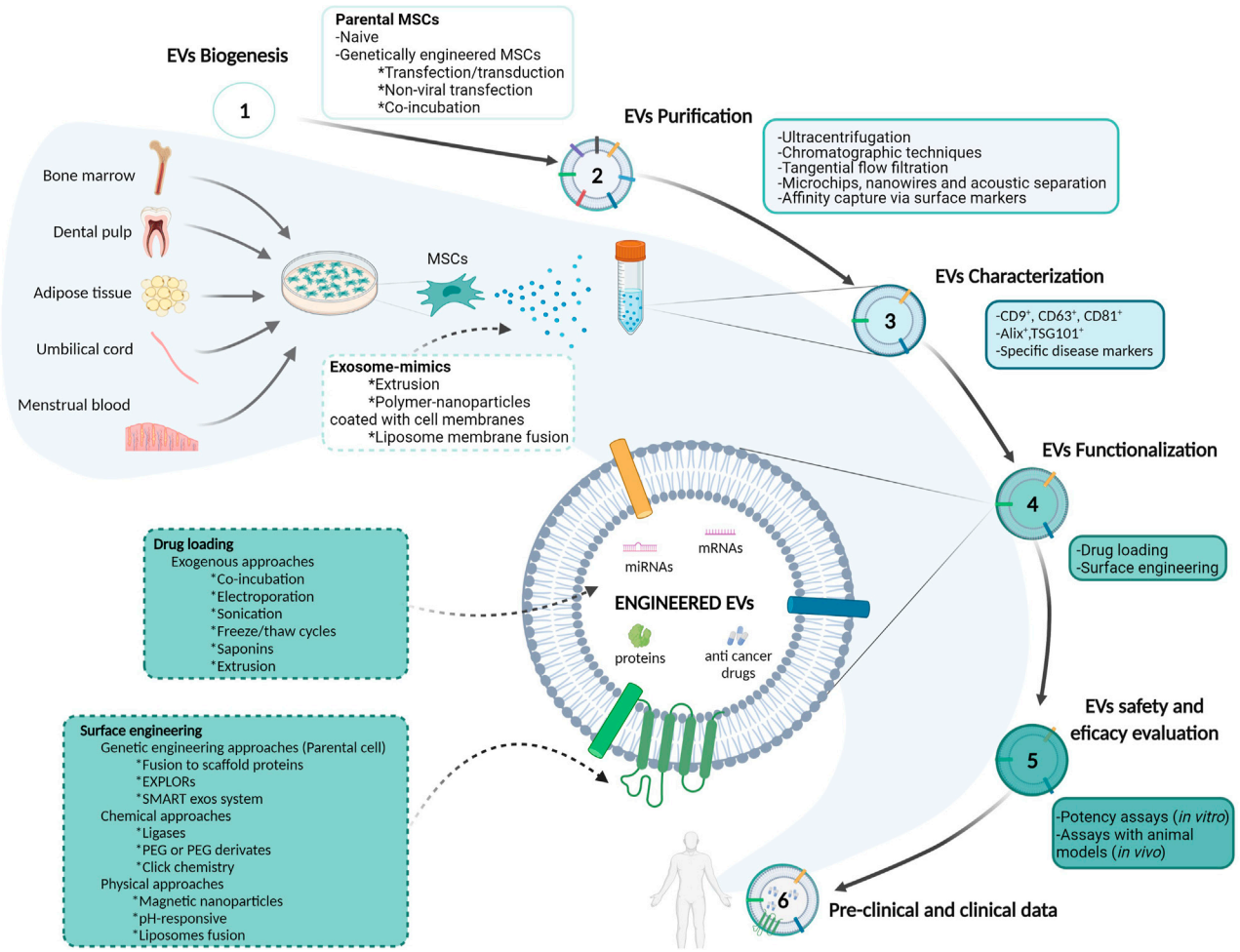

FIGURE 1 | Schematic representation of mesenchymal stem/stromal cells-derived extracellular vesicles isolation, purification, characterization and evaluation approaches for their utilization as drug delivery systems in cancer therapy. Abbreviations: Alix: apoptosis-linked gene 2–interacting protein X; EVs: extracellular vesicles; EXPLORs: exosomes for protein loading via optically reversible protein–protein interactions; miRNA: microRNA; mRNA: messenger ribonucleic acid; MSCs: mesenchymal stem/stromal cells; PEG: polyethylene glycol; TSG101: tumor susceptibility gene 101 protein.

a small population of sEVs become fluorescent, inducing variability in the signal intensity (Choi and Lee, 2016). Alternatively, specifically designed aptamers –single-stranded DNA/RNA oligonucleotides that can bind 3D structures and molecules with high affinity and specificity– can also be attached to EV surfaces through covalent linkage to improve their tumor tropism (Luo et al., 2019).

Regarding chemical approaches, EVs can be modified to anchor molecules or drugs on their surface through covalent linkage, PEG derivates, click chemistry techniques, and novel modification methods such as the utilization of ligases. Covalent linkage involves the employment of activated esters –i.e., hydroxysuccinimide esters - to link relatively simple molecules to the amino groups of EV surface proteins or anchor more complex biomolecules –such as antibodies– via biorthogonal reactions (Richter et al., 2021). In addition, nanobodies and other types of molecules such as lipid conjugates can be linked to EV superficial amino groups by using PEG derivates. These modified EVs showed an increased circulation time compared with unmodified ones (Kooijmans et al., 2016). Another popular approach is click chemistry –or azide-alkyne cycloaddition– which allows direct attachment of molecules to EV surfaces through covalent bonding. This method is simple with no impact on EVs structure, although it may alter EVs function by unspecific modification of proteins (Ramasubramanian et al., 2020). Pham et al. recently developed a covalent conjugation method of peptides or nanobodies to EV surfaces by employing protein ligases (Pham et al., 2021). They proved this method by anchoring an anti-epithelial growth factor receptor nanobody to EVs loaded with paclitaxel, which facilitated their tumor accumulation in a xenograft mouse model of epithelial growth factor receptor-positive lung cancer, and increased paclitaxel therapeutic efficacy (Pham et al., 2021). All these chemical methods require an additional purification step to prevent contamination of the final

formulation with the reagents used for EVs surface modification (Richardson and Ejima, 2019).

Furthermore, some physical approaches for EVs engineering have also received in-deep attention. For example, Mizuta et al. hybridized exosomes with magnetic nanoparticles to facilitate an efficient uptake by target cells after exposure to a magnetic field (Mizuta et al., 2019). Lee et al. also developed pH-responsive EVs containing hyaluronic acid grafted with 3-(diethylamino) propylamine and loaded with doxorubicin. These modified EVs responded to tumor pH (pH 6.5) and bounded to CD44 on HCT-116 human colorectal carcinoma cell line, inhibiting its growth *in vivo* (Lee et al., 2018). Finally, another emerging strategy is generating hybrid EVs by their fusion with liposomes. For instance, Piffoux et al. exploited the fusion of EVs –triggered by PEG - with functionalized liposomes, to enrich EVs with either lipophilic or hydrophilic drugs. These hybrid EVs showed no alteration of their structure and content and were more efficient in delivering chemotherapeutic compounds than their liposome precursor (Piffoux et al., 2018). All the information exposed in the previous sections is summarized in **Figure 1**.

Exosome Mimics

Due to the limitation related to the amount of EVs that can be purified from *in vitro* culturing of parental cells, more significant numbers of nanovesicles can be produced through the implementation of certain emerging techniques, such as cell extrusion or the generation of polymer-nanoparticles coated with cell membranes, to obtain the so-called exosome-mimics (Li S.-p. et al., 2018) (**Figure 1**). Exosome-mimics are similar to natural sEVs in their properties, drug loading capacity, and interaction with target cells, but obtained at higher amounts and considered safer than natural sEVs (Vázquez-Ríos et al., 2019). Extrusion involves the sequential squeezing of cells through a set of extrusion filters to obtain nanosized or microsized vesicles that generally share membranes with their parental cells (Wang et al., 2021). In counterpart, polymer-nanoparticles coated with cell membranes can be obtained by mixing purified exosomes or isolated cell membranes with artificial polymer-nanoparticles (Tian et al., 2020). Additionally, the use of cytochalasin B was shown to simplify the large-scale production of exosome-mimics from MSCs, as this compound causes actin filament dissociation and cell disintegration after shaking the treated cells, with the formation of multiple vesicles that are built from cell plasma membrane (Chulpanova et al., 2021). Exosome-mimics showed higher half-life in the blood, prolonged circulation retention than PEG-coated nanoparticles, and reduced clearance by macrophages (Li S.-p. et al., 2018). Additionally, "artificial exosomes" can be generated from conventional liposomes by adding specific molecules. For instance, Haraszti et al. incorporated one lipid (dilysocardiolipin) and three proteins (Ras-interacting protein Rab7, Desmoplakin, and Alpha-2-HS-glycoprotein) —which they found to be enriched in serum-deprived MSCs - into neutral liposomes to produce vesicles that mimic the tropism and cargo delivering of natural sEVs (Haraszti et al., 2019). In this way, the extensive knowledge obtained from the EVs field can be applied to artificial nanocarriers engineering to improve their biological and therapeutic properties.

PRE-CLINICAL AND CLINICAL DATA IN CANCER

The first known references MSC-derived EVs for cancer cell-free therapy were published in 2013 (Katakowski et al., 2013; Ohno et al., 2013). Since then, many pre-clinical studies have been published describing the anti-tumor effects of engineered MSC-derived EVs. Here, we review some pre-clinical studies published between 2018 and 2022 (**Table 1**). They widely differ in the source of MSCs, the tumor models, and the approaches employed for cargo loading or EV surface engineering. For instance, Wang et al. developed genetically engineered murine BM-MSCs to overexpress the miR-185, which is known to attenuate inflammation associated with oral leukoplakia (Wang et al., 2019). In this way, similar approaches for EVs modification could be used to prevent the malignant transformation of potentially malignant oral disorders. In addition, many works reported the study of engineered MSC-derived EVs to target the known as the *hallmarks of cancer*. As Hanahan and Weinberg described in their renowned article in 2011, tumor cells exert some distinctive and complementary capacities that allow them to grow, invade and disseminate to distant organs. These characteristics include sustained proliferative capacity, apoptosis evasion, genomic instability, angiogenesis, replicative immortality, inflammation, metabolism deregulation and immune system evasion (Hanahan and Weinberg, 2011). Dong et al. treated human UC-MSCs with the siRNA anti-ELFN1-AS1 –a long non-coding RNA highly expressed in colon adenocarcinoma cells - and found that the EVs purified from siRNA-ELFN1-AS1-treated UC-MSCs could inhibit colon adenocarcinoma cells proliferation and migration *in vitro*. With reference to immune evasion, Zhou et al. demonstrated that miR-424-5p delivery through AT-MSC-derived EVs partly exerted pro-inflammatory effects and enhanced anti-tumor cytotoxicity in human triple-negative breast cancer cells both *in vitro* and *in vivo*, through the downregulation of the PD-L1 pathway (Zhou et al., 2021). Chulpanova et al. genetically modified human AT-MSCs to overexpress human IL-2. The engineered MSC-derived EVs were able to activate human $CD8^+$ T cells, which in turn induced apoptosis in triple-negative breast cancer cells (Chulpanova et al., 2021). Furthermore, AT-MSC-derived EVs overexpressing miR-15a, which targets the demethylase KDM4 that is deregulated in colorectal cancer cells, were shown to diminish the immune evasion of tumor cells via the KDM4B/HOXC4/PD-L1 axis, both *in vitro* and *in vivo* (Liu L. et al., 2021). These results demonstrate that MSC-derived EVs may not only target the intrinsic tumor cells capacities, but also their ability to interact with the tumor microenvironment. Another study showed that the *in vitro* and *in vivo* delivery of miR-193a to colon cancer cells through BM-MSC-derived EVs inhibited their proliferation, migration and invasion through the Focal Adhesion Kinase targeting (Ying et al., 2020). This evidence supports the

TABLE 1 | Pre-clinical data from the 2018-2021 period, regarding extracellular vesicles evaluation as drug delivery systems for cancer therapy. Abbreviations: AT-MSCs: adipose-tissue mesenchymal stem cells; BM-MSCs: bone-marrow MSCs; DMBA: 7,12-dimethylbenzathracene; DOX: doxorubicin; EVs: extracellular vesicles; GRP78: glucose-regulated protein 78; HCC: hepatocellular carcinoma; LNA: locked nucleic acid; LPS: lipopolysaccharide; MUC1: mucin 1 cell surface-associated; NSCLC: non-small cell lung cancer; OPMD: oculopharyngeal muscular dystrophy; PCNA: proliferating cell nuclear antigen; PDAC: pancreatic ductal adenocarcinoma; TRAIL: tumor necrosis factor-related apoptosis-inducing ligand.

Type of Parental MSCs	Modification Method	Tumor/Malignant Disorder Model	Effects	Reported by
Murine AT-MSCs	Pre-conditioning with LPS, and loading of anti-oncogenic miRNA-16-5p through membrane fusion with liposomes	E0771 and 4T breast cancer cell lines, both *in vitro,* and *in vivo* through mouse subcutaneous models	Decreased tumor cell proliferation and migration, and enhanced tumor cell apoptosis *in vitro*	Li et al. (2020)
Murine BM-MSCs	Genetically engineered MSCs to overexpress the anti-oncogenic miR-185 in EVs	Oral leukoplakia (buccal lesions in a DMBA-induced OPMD mouse model *in vivo*)	Attenuated inflammation severity, significantly decreased incidence and the number of dysplasia in the OPMD tissue *in vivo*, through inhibition of AKT and PCNA pathways	Wang et al. (2019)
Human MSCs cell line (Lonza)	Lentivirus-transfected MSCs to overexpress the tumor suppressor miRNA-584	Human glioblastoma cell line (U87), and mouse xenografts	Reduced tumor cells proliferation, migration and invasion *in vitro*, and reduced tumor progression *in vivo*	Kim R. et al. (2018)
Human BM-MSCs	EVs loaded with paclitaxel	Human breast cancer cell line (MDA-MB-231) and subcutaneous mouse xenografts	Significantly decreased tumor cells viability *in vitro*, and inhibition of tumor growth *in vivo*, compared to naïve EVs	Kalimuthu et al. (2018)
Human UC-MSCs	EVs from pre-irradiated MSCs	Human malignant melanoma cell lines (A375 and G-361) and human breast cancer cell line (MCF-7), and their respective mouse xenografts	Decreased tumor growth *in vivo*, and significantly decreased number of metastatic foci *in vivo*	de Araujo Farias et al. (2018)
Murine BM-MSCs	Genetically engineered MSCs through a non-viral vector, to overexpress the anti-tumoral factor TRAIL	Subcutaneous mouse models of a mouse melanoma cell line (B-16-F0)	Reduced tumor size *in vivo*	Shamili et al. (2018)
Murine BM-MSCs	MSC-derived EVs loaded with DOX through electroporation. Surface engineering of EVs with carboxylic acid-end MUC1 aptamer	MUC1-positive murine colon carcinoma cell line (C26) and human breast cancer cell line (MCF-7), as well as C26 mouse xenografts	Higher cytotoxicity *in vitro*, and suppression of tumor growth *in vivo*	Bagheri et al. (2020)
Human AT-MSCs	Lentivirus-transduced MSCs to overexpress miR-199a	Human HCC cell lines (Huh7, SMMC-7721, and PLC/PRF/5), and a PLC/PRF/5 orthotopic mouse model with DOX treatment	Increased HCC cells chemo-sensitivity to DOX (by inhibiting mTOR pathway) *in vitro* and *in vivo*, compared to the free drug	Lou et al. (2020)
Human BM-MSCs	Transfection of MSCs with oligonucleotides of miR-1231 mimics	Human PDAC cell lines (BxPC-3 and MIA PaCa-2) and BxPC-3 subcutaneous mouse xenografts	Inhibition of PDAC cells proliferation, migration and invasion *in vitro*. Suppression of tumor growth *in vivo*	Shang, et al. (2019)
Human MSCs cell line (Lonza)	Lentivirus-transduced MSCs to overexpress tumor suppressor miR-124a	A panel of human glioma stem cell lines (GSC267, GSC20, GSC6-27, GSC8-11, and GSC2-14), and intracranial mouse xenografts	Significantly reduced viability and clonogenicity *in vitro*, and increased overall survival of animal models	Lang et al. (2018)
Human UC-MSCs	Lipotransfection of MSCs with a miR-375 mimic	Human esophageal squamous carcinoma cell lines (KYSE70, ECA109, and EC9706, and subcutaneous KYSE70 and EC9706 mouse xenografts	Inhibition of cell proliferation, invasion, migration, and tumorsphere formation *in vitro*. Promotion of apoptosis *in vitro*. Inhibition of tumor growth *in vivo*	He et al. (2020)
Human AT-MSCs, BM-MSCs and UCB-MSCs	MSCs engineered to express the yeast cytosine deaminase::uracil phosphoribosyl transferase suicide fusion gene, through MSCs transduction with a recombinant retrovirus	Human glioblastoma cells obtained from primary tumors	Tumor cell growth inhibition *in vitro*	Altanerova et al. (2019)
Murine BM-MSCs	MSC-derived EVs loaded with LNA modified antimiR-142-3p molecules via electroporation	Human breast cancer cell line (MCF-7) mammospheres	Reduced clonogenicity and tumorigenicity *in vitro*. Induction of apoptosis *in vitro*	Naseri et al. (2020)
BM-MSCs cell line (ScienCell)	MSC-derived EVs loaded with paclitaxel (through sonication) and gemcitabine (through reversible electroporation)	Human PDAC cell line (MiaPaca-2 cells, tumor spheroids), and a MiaPaca-2 orthotopic mouse model	Increased homing and penetrating abilities *in vivo*, compared to the free drugs. Anti-tumor efficacy *in vivo* and *in vitro*	Zhou et al. (2020)

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TABLE 1 | (*Continued*) Pre-clinical data from the 2018-2021 period, regarding extracellular vesicles evaluation as drug delivery systems for cancer therapy. Abbreviations: AT-MSCs: adipose-tissue mesenchymal stem cells; BM-MSCs: bone-marrow MSCs; DMBA: 7,12-dimethylbenzathracene; DOX: doxorubicin; EVs: extracellular vesicles; GRP78: glucose-regulated protein 78; HCC: hepatocellular carcinoma; LNA: locked nucleic acid; LPS: lipopolysaccharide; MUC1: mucin 1 cell surface-associated; NSCLC: non-small cell lung cancer; OPMD: oculopharyngeal muscular dystrophy; PCNA: proliferating cell nuclear antigen; PDAC: pancreatic ductal adenocarcinoma; TRAIL: tumor necrosis factor-related apoptosis-inducing ligand.

Type of Parental MSCs	Modification Method	Tumor/Malignant Disorder Model	Effects	Reported by
Human BM-MSCs	MSCs transfected with siRNA against GRP78	Human HCC cell lines (HepG2 and PLC), and HepG2 and PLC orthotopic subcutaneous and metastasis mouse models	Inhibition of Sorafenib-resistant HCCs growth and invasion *in vitro*. Inhibition of growth and metastasis *in vivo*	Li H. et al. (2018)
Human BM-MSCs	MSCs chemically transfected with a miR-199a mimic	Human glioma cell lines (U251, LN229, T98G, LN-18, SF-539 and A172) and U251 subcutaneous mouse xenografts	Inhibition of glioma cells proliferation, invasion and migration *in vitro*. Tumor growth inhibition *in vivo*	Yu et al. (2019)
Human BM-MSCs	MSCs chemically transfected with miR-144 mimic	Human NSCLC cell lines (A549, NCI-H1975, NCI-H1299), and NSCLC cell lines mouse xenografts	Inhibition of NSCLC cell proliferation and colony formation *in vitro*. Inhibition of tumor growth *in vivo*	Liang et al. (2020)
Human BM-MSCs	MSCs chemically transfected with a plasmid encoding miR-15a mimic	Human HCC cell lines (Hep3B and Huh7), and HCC mouse xenografts	Restriction of HCC cells proliferative, migrating, and invasive potentials *in vitro*. Promotion of HCC cells apoptosis *in vitro*. Reduced tumorigenicity and metastasis *in vivo*	Ma et al. (2021)
Human BM-MSCs	MSCs transfected with a lentivirus encoding miR-29a-3p mimics	Human glioma cell lines (U87MG and A172), and U87 mouse xenografts	Attenuated glioma cells migration and vasculogenic mimicry formation *in vitro*. Inhibition of tumor growth *in vivo*	Zhang Z. et al. (2021)

potential use of engineered MSC-derived EVs as a strategy for inhibiting the initial steps of the metastatic cascade. Finally, as we previously summarized in a previous review, there are some emerging pre-clinical strategies for targeting the establishment of the pre-metastatic niche (PMN) (Sanmartin et al., 2021). Although none of these mentioned therapeutic approaches consist of the utilization of MSC-derived EVs, these nanovesicles could be tested for the delivery of drugs that aim to target the PMN. For instance, some works described the use of antibodies to target soluble factors –such as the Dickkopf-related protein 1 and the C-C motif chemokine ligand 2– involved in bone/bone marrow PMN formation (Heath et al., 2009; Bonapace et al., 2014). Engineered MSC-derived EVs –particularly EVs isolated from BM-MSCs that exert tropism for bone/bone marrow PMN - carrying those antibodies in their surfaces could be developed as an improved strategy to alter the formation of the PMN and increase drug access to these niches.

Other engineered MSC-derived EVs include the combination of AT-MSCs pre-conditioning with LPS to downregulate the expression of CD90, with the loading of anti-oncogenic miRNA-16-5p into $CD90^{low}$-AT-MSC-derived EVs to enhance anti-tumoral effects (Li et al., 2020). Similarly, physical pre-conditioning techniques such as UC-MSCs irradiation were used to obtain MSC-derived EVs for melanoma treatment *in vivo*, showing promising results (de Araujo Farias et al., 2018). Furthermore, the usage of MSC-derived EVs as DDSs has been exploited to improve the biodistribution of chemotherapeutic drugs and reduce their severe systemic side effects. For example, paclitaxel has been previously associated with cardiotoxicity, myelosuppression, and neurotoxicity, so Kalimuthu et al. utilized MSC-derived EVs loaded with paclitaxel for the delivery of this chemotherapeutic agent to breast cancer cells while minimizing systemic adverse effects (Kalimuthu et al., 2018). Similarly, Shamili et al. isolated EVs derived from murine BM-MSCs overexpressing TRAIL and showed that the administration of the encapsulated form of TRAIL was better at reducing tumor size *in vivo* than the free drug (Shamili et al., 2018).

Regarding the clinical trials involving MSC-derived EVs for cancer therapy, only one phase I trial is registered at www.clinicaltrials.gov (NCT03608631). This clinical study is currently evaluating the best dose and associated side effects of MSC-derived exosomes loaded with KrasG12D siRNA (iExosomes) during the treatment of participants with metastatic pancreatic cancer bearing the KrasG12D mutation. Although gemcitabine has been the standard of care for this particular type of cancer, there is a poor response to this drug, leading to reduced overall survival (Long et al., 2011). Additionally, pancreatic cancer is a highly therapy-resistant tumor, even to available immunotherapies, due to its intrinsic low tumor mutational burden, immunosuppressive microenvironment, and acellular fibrous stroma that impairs drug access to the tumor (Bear et al., 2020; Stopa et al., 2020). In this way, MSC-derived EVs could be an exciting option to overcome drug access issues in pancreatic cancer patients and other cancer types with similar characteristics.

However, as Gupta et al. reviewed after performing a systematic analysis, there are many inconsistencies regarding

the doses of EVs used in pre-clinical studies (Gupta et al., 2021). Moreover, most of the analyzed articles lack of references to the rationale of the dose selection and treatment frequency. While EVs doses range from 0.001 to 100 mg of EV protein per kg. of body weight –and the same variability happens upon the use of particle number as EVs quantity determination -, the frequency of administration of EVs ranges from 1 to 6 times (Gupta et al., 2021). In this way, potency units as qualitative measures derived from potency assays could be used to homogenize the assessment of EVs doses, as Dal Collo et al. discussed after the development of an *in vitro* assay that measures T-reg induction by MSC-derived EVs (Dal Collo et al., 2020). Tieu et al. also reported that over two-thirds of the analyzed pre-clinical studies involved the administration of a single dose of MSC-derived EVs, and only a 28% of the studies reported the administration of multiple doses (Tieu et al., 2020). Furthermore, the EVs dose selection can depend on the particular disease, since it was reported that the EVs therapeutic dose required for neurological diseases is lower than the dose required for other systemic inflammatory diseases (Gupta et al., 2021). In the previously mentioned clinical study testing MSC-derived exosomes loaded with KrasG12D siRNA on pancreatic cancer patients (NCT03608631), the EVs dose used is not mentioned, while the treatment periodicity involves the administration of up to three infusions of EVs. For instance, when comparing with MSCs, clinical trials using MSCs to treat patients with Covid-19 pneumonia reported the infusion of one million of MSCs per kg. of body weight with up to three infusions of cells (NCT04444271, NCT04713878, NCT04429763). Additionally, Pacienza et al. showed that the use of HUCPVC-derived sEVs (released by 1×10^6 cells) in a rat lung preservation model had higher anti-inflammatory effects when compared with the administration of HUCPVCs (1×10^6 cells, via the pulmonary artery) (Pacienza et al., 2020). These results encourage the use of MSC-derived EVs as cell-free therapies in replacement of the administration of MSCs. Finally, additional efforts are needed to standardize EVs dose determination protocols.

DISCUSSION

Anti-cancer drug development is a challenging field because not only tumors can develop both intrinsic and extrinsic mechanisms of drug resistance, but also antineoplastic drugs may be associated with low bioavailability and off-target effects. Although MSC-based therapy has been investigated for many years for its application for cancer treatment due to their biological properties –mainly their low immunogenicity and tumor tropism - many groups reported contradictory results regarding the pro and anti-tumoral effects of MSCs. Although these discrepancies among studies have been attributed to the differences between tissue sources and donors and the lack of standardized protocols for MSCs isolation and *in vitro* culturing, researchers may tread carefully when using MSCs for cancer cell therapy. Consequently, the utilization of MSC-derived EVs –either naïve or modified to improve their delivery and targeting properties– as cell-free DDSs allowed to overcome some of the drawbacks associated with cell therapy since EVs are static and do not reproduce or represent a mutational risk when administered to patients. Moreover, because of their inherent biological complexity, EVs display better tropism and biocompatibility when compared with artificial nanocarriers. However, there are still some limitations associated with EVs as DDSs. To begin with, as any biological product, EV formulations must fulfill the requirements of regulatory agencies in terms of purity, safety, efficacy, and batch-to-batch homogeneity. The exhaustively controlled manufacturing processes and quality control protocols needed to accomplish GMP and regulatory standards also enormously increase the cost of EV production. Due to EVs biological complexity, it may be difficult to assess if the lipid bilayer, their content, or both are responsible for their therapeutic effect. Furthermore, the procedures employed for EVs engineering require additional purification steps to eliminate potential reagent contamination, and most of those procedures tend to increase even more the final product cost and may result in low yields as well. Additionally, it has to be kept in mind that, when using MSC-derived EVs, researchers need to keep investigating their not yet fully understood effects on tumor progression. Nevertheless, there has been an exponential development of new approaches to overcome those methodical and cost-associated limitations. For instance, the emergence of hybrid vesicles –by the fusion of sEVs and liposomes– and exosome-mimics brought together "the best of both worlds", shedding some light over the new emergent paradigm of biological nanovesicles as DDSs. In the last 4 years, many research groups have published pre-clinical data regarding the potential applications of engineered sEVs for cancer therapy, with some promising results. Since these reports are heterogeneous in terms of the engineering methods used, source of the parental MSCs, and the tumor models employed, new efforts are required to design and standardize protocols that will undoubtedly facilitate EVs translation to the clinic for theragnostic applications.

AUTHOR CONTRIBUTIONS

MS: investigation, writing - original draft, visualization. FB: writing - original draft. MG: writing - original draft. GY: review and editing, funding acquisition. NC: conceptualization, writing - review and editing, supervision, funding acquisition.

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Mesenchymal Stromal Cell-Derived Extracellular Vesicles Modulate Hematopoietic Stem and Progenitor Cell Viability and the Expression of Cell Cycle Regulators in an Age-dependent Manner

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Aging of the hematopoietic system is characterized by an expansion of hematopoietic stem and progenitor cells (HSPCs) with reduced capacity for engraftment, self-renewal, and lymphoid differentiation, resulting in myeloid-biased hematopoiesis. This process is mediated by both HSPC intrinsic and extrinsic factors, e.g., the stromal environment. A relevant cellular component of the bone marrow (BM) microenvironment are mesenchymal stromal cells (MSCs) which regulate fate and differentiation of HSPCs. The bi-directional communication with HSPCs is mediated either by direct cell-cell contacts or by extracellular vesicles (EVs) which carry bioactive substances such as small RNA, DNA, lipids and proteins. So far, the impact of MSC-derived EVs on human hematopoietic aging is poorly investigated. BM MSCs were isolated from young (n = 3, median age: 22 years) and aged (n = 3, median age: 70 years) donors and the EVs were isolated after culturing the confluent cell layer in serum-free medium for 48 h. $CD34^+$ HSPCs were purified from peripheral blood of healthy donors (n = 3, median age: 65 years) by magnetic sorting. Nanoparticle tracking analysis (NTA), transmission electron microscopy (TEM) and western blot detection of EV markers CD63, CD81 and Flotillin-1 revealed no significant differences between young and aged MSC-EVs. Interestingly, young MSCs secreted a significantly higher miRNA concentration than aged cells. However, the amount of distinct miRNAs such as miR-29a and miR-34a was significantly higher in aged MSC-EVs. HSPCs incubated with young EVs showed a significant increase in cell number and a higher viability. The expression of the tumor suppressors PTEN, a known target of mir-29a, and CDKN2A was increased in HSPCs incubated with young EVs. The clonogenic assay demonstrated a decreased colony number of CFU-GM after treatment with young EVs and an increased number of BFU-E/CFU-E after incubation with aged MSC-EVs. Xenogenic transplantation experiments showed no significant differences concerning the engraftment of lymphoid or myeloid cell compartments, but the overall human chimerism 8–16 weeks after transplantation was higher after EV treatment. In conclusion, our data suggest that HSPC

characteristics such as cell cycle activity and clonogenicity can be modulated by MSC-derived EVs. Further studies have to elucidate the potential therapeutic relevance of our findings.

Keywords: mesenchymal stromal cells, hematopoiesis, bone marrow, extracellular vesicles, aging, hematopoietic stem cells

INTRODUCTION

Hematopoietic cell transplantation (HCT) often represents the only possible treatment for hematological malignancies including leukemias as well as myelodysplastic syndromes. One major concern is to find a immunogenetically matched donor with age being the second-most relevant determinant of outcome after allogeneic HCT (Kollman et al., 2016; Dehn et al., 2019).

Aging is associated with changes in HSPC function and phenotype, including compromised regenerative potential, loss of quiescence, a deregulated cell cycle and increased metabolic activity, which is accompanied by a significant expansion of the stem cell pool and differentiation skewed towards the myeloid lineage (Akunuru and Geiger, 2016). Consequently, aged HSPCs (donors >50 years) have a reduced capacity to reconstitute hematopoiesis after allogeneic HCT, reducing the pool of HSPC donors (Kollman et al., 2001; Kollman et al., 2016).

Hematopoietic aging is determined by both HSPC intrinsic factors, such as accumulation of DNA damage, replicative stress, and decreased autophagy, as well as extrinsic factors conferred by the endothelial and stromal microenvironment (Ergen, Boles and Goodell, 2012; Kusumbe et al., 2016). However, the mechanisms and cellular components of the niche driving aging remain largely unknown.

A main cellular component of the bone marrow microenvironment (BMME) are mesenchymal stromal cells (MSCs). These multipotent cells can differentiate into adipocytes, chondrocytes and osteoblasts. Importantly, they regulate fate and differentiation of HSPCs and thereby their retention in the BM.

The communication with HSPCs is mediated either by direct cell-cell contacts or by extracellular vesicles (EV). These small membrane-surrounded particles carry different bioactive substances such as small DNA, RNA, lipids and proteins (Baglio et al., 2015; Haraszti et al., 2016). EVs are derived from the endosomal compartment or bud directly from the cell membrane. After incorporation in the recipient cell, they are able to modify the phenotype by transferring proteins or mRNA and changing the gene expression *via* small RNAs (De Luca et al., 2016; Preciado et al., 2019). Therefore, it is suggested that an age-related modification of EV cargo, such as miRNA levels, may have distinct impact on gene expression in the recipient cell.

It was shown that MSC-EV cargo in rats changes with age and that the gene and protein expression of HSPCs is influenced differently after incubation with EVs of young and old murine MSCs (Wang et al., 2015; Kulkarni et al., 2018). Unfortunately, we lack data considering the influence of aging on the EV content of human physiologically aged MSCs and their effects on human HSPCs.

In this study, we characterized EVs of young and aged MSCs and could demonstrate an age-dependent miRNA cargo. We focused on the analysis of miRNAs that are highly abundant in MSC-EVs and play an important role for hematopoiesis (Baglio et al., 2015; Morhayim et al., 2016; Kulkarni et al., 2018). Further, the different ability to support *in vitro* expansion of HSPCs and to influence surface marker expression was evaluated. Important target genes of investigated miRNA regulating differentiation and cell cycling of HSPCs were examined after incubation. Additionally, a potential increased *in vivo* engraftment of HSPCs was analysed by xenogeneic transplantation in NSG mice.

MATERIALS AND METHODS

Isolation and Culture of MSCs

BM MSCs were isolated from young (n = 3, median age: 22 years) and aged (n = 3, median age: 69 years) healthy donors (**Supplementary Table S1**) after obtaining informed written consent (Ethical Approval No. EK221102004, EK47022007) as described previously (Oswald et al., 2004; Hempel et al., 2016). The cells were expanded in Dulbecco's modified Eagle's medium-low glucose (DMEM, Gibco, Germany) with 10% fetal bovine serum (FBS, Gibco) and characterized according to the criteria of the International Society for Cellular Therapy (Dominici et al., 2006). MSCs were used in the second or third passage for all experiments.

Isolation of $CD34^+$ HSPCs

$CD34^+$ cells were isolated from leukapheresis of patients (n = 9, 47–67 years old, median age: 52 years) suffering from non-leukemic cancers (e.g., myeloma, lymphoma and sarcoma) after obtaining informed written consent. Stem cells were collected before high-dose chemotherapy in preparation of an autologous HCT. The cells were purified by immunomagnetic sorting using CD34 MicroBead Kit UltraPure (Miltenyi Biotec, Germany). The viability was determined by live-dead-discrimination using Countess II Automated Cell Counter (Thermo Fisher Scientific, Germany) and the purity of at least 98% was confirmed by flow cytometry.

MSC-EV and MSC-EV-miRNA Isolation

After reaching 80% confluency medium was changed to 100% DMEM. The medium was collected after 48 h and the cells were harvested and counted after staining with trypan blue for live-dead-discrimination. To deplete cell debris the medium was centrifuged at 380xg for 5 min and subsequently filtered through a 200 nm Millipore filter.

Afterwards the EVs were isolated with the exoEasy Maxi Kit (Qiagen, Germany) and the EV-miRNA was purified with the exoRNeasy Maxi Kit (Qiagen, Germany) according to the manufacturer's protocol. Briefly, the sample was mixed with the same volume of buffer XBP and given onto the column. After centrifugation at 500 *g* for 1 min at room temperature the column was washed with buffer XWP and centrifuged at 5.000xg for 10 min. To elute the EVs 400 µl of buffer XE was added onto the column before centrifugation at 500 *g* for 5 min. To increase the number of particles the column was eluted again and centrifuged at 5.000xg for 5 min.

To isolate the miRNA the EVs were bound on the columns analogously, but instead of eluting the particles were lysed in 700 µl Qiazol and the columns were spun down at 5.000xg for 5 min. The total RNA was isolated following the manufacturer's protocol. The samples were stored at -80°C.

Nanoparticle Tracking Analysis

The data concerning size distribution and the concentration of the particles were acquired on the ZetaVIEW S/N 243 (Particle Metrix GmbH, Germany). The samples were diluted 1:500 or 1: 1,000 in PBS. To process data ZetaView 8.05.05 SP2 was used.

Western Blot

Eluted EVs were lysed in 10X cell lysis buffer (Cell Signaling Technology, Germany) with protease inhibitor cocktail B (Santa Cruz, United States of America) and were mixed with Roti-Load 2 (Carl Roth, Germany). For reducing conditions DTT was added. 24 µl of the sample was loaded on a 10% or 12% SDS-polyacrylamide electrophoresis gel and transferred to a nitrocellulose membrane. Subsequently, it was blocked in 5% non-fat dry milk and incubated with anti-CD63 (Invitrogen, Germany) and anti-CD81 (Invitrogen, Germany) under non-reducing conditions, anti-Flotillin-1 (BD Biosciences, United States of America) and anti-Calnexin (Cell Signaling Technology, United States of America) under reducing conditions. Afterwards, the membrane was thoroughly washed and incubated with the secondary antibody coupled with a horseradish peroxidase. The chemiluminescence was detected using the ECL Plus Western Blotting Detection Reagent (Amersham, United States of America) on the Luminescent Image Analyzer LAS-3000 (FUJIFILM, Japan).

Transmission Electron Microscopy

To prepare the samples for Transmission Electron Microscopy (TEM) EV solutions were applied on a 300 mesh EM grid, airdried and fixed with 1% glutaraldehyde. The samples were washed twice with water and counterstained with uranyloxalate solution. Afterwards the EVs were examined under a transmission electron microscope Jeol JEM1400 Plus (Jeol, Germany) running at 80 kV acceleration voltage.

Quantitative miRNA-RT-PCR

The miRNA amount of the EV samples was calculated with the Qubit™ microRNA Assay Kit (Thermo Fisher Scientific, Germany). cDNA was synthesized using TaqMan® MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific, Germany). RT-PCR was performed using TaqMan® universal PCR Master Mix (Thermo Fisher Scientific, Germany) on a Quantstudio three cycler (Applied Biosystems, Germany). The following miRNAs were examined: let7a, miR-10a, miR-21, miR-23, miR-155, miR-221, miR-222 and miR-486, miR29a, miR34a. All primers were purchased from Applied Biosystems (**Supplementary Table S2**). The samples were run in duplicates. U18 was used as endogenous control according to Vriens et al. (Vriens et al., 2012). Amplicons were normalized to U18 applying the comparative CT (ΔCT) method.

In vitro Incubation of HSPCs With MSC-EVs

2.4×10^5 $CD34^+$ HSPCs were incubated with young or aged MSC-EVs collected from 1.2×10^5 cells or XE buffer as control in CellGenix SCGM (CellGenix, Germany) with 10% vesicle-depleted FBS supplemented with SCF, Flt3 ligand and IL-3 (10 ng/ml each, all from Miltenyi Biotec, Germany). The EV samples were pooled beforehand. For vesicle depletion, the FBS was centrifuged with an Amicon-Ultra 100 kDa filter (Millipore, Germany) for 55 min at 3.000xg. Each sample was run in duplicates.

After the incubation, the cells were washed and counted by Countess II Automated Cell Counter (Thermo Fisher Scientific, Germany). Subsequently, samples were pooled and further analyzed using flow cytometry, clonogenic assays and RT-PCR.

Flow Cytometry

HSPCs cultured with EVs were stained for surface markers using fluorescently labeled antibodies according to **Supplementary Table S3**. Corresponding human immunoglobulin G controls were used. Data was acquired on a BD FACS Calibur or LSRII. Data were analysed using FlowJo software (FlowJo, LLC, United States of America).

Fluorescent Labelling and Confocal Microscopy

Isolated EVs were labeled with fluorescent dye 10 µM DiI (Thermo Fisher Scientific). After incubation for 20 min at 37°C the dye was removed with Exosome Spin Columns (Thermo Fisher Scientific). 10^5 $CD34^+$ cells were incubated with DiI-EVs of $3*10^5$ MSCs or buffer as control for 24 h. Afterwards, a cytospin was used to concentrate the cells onto a microscope slide. After fixation in 4% paraformaldehyde, the nuclei were counterstained with DAPI. Images were acquired on a LSM 880 (ZEISS, Germany).

Clonogenic Assays

Colony-forming unit (CFU) assays were carried out using $CD34^+$ HSPCs harvested after 3 days of incubation with EVs. 500 cells were plated in Stem MACS HSC-CFU complete with Epo (Miltenyi Biotec, Germany). Colonies were counted after 2 weeks and classified with the StemVision system (Stem Cell Technologies, Germany). Each sample was run in duplicates.

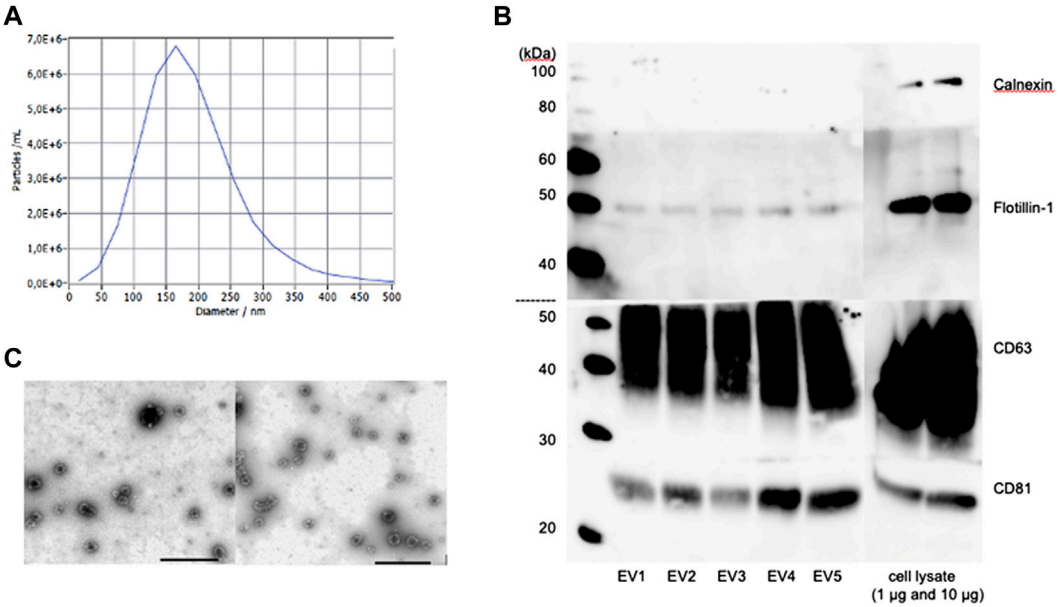

FIGURE 1 | Characterization of MSC-derived EVs of young and aged donors. **(A)** Nanoparticle Tracking Analysis of one representative sample. **(B)** Western Blot analysis of CD63, CD81, Flotillin-1 and Calnexin in different MSC-EV samples. **(C)** Representative TEM images of young MSC-EVs (left) and aged MSC-EVs (right), scale bar: 1,000 nm.

Quantitative RNA-RT-PCR

Total RNA were isolated from $CD34^+$ HSPCs after incubation with EVs using RNeasy Micro Kit (Qiagen, Germany) and reverse transcribed into cDNA using RevertAid cDNA synthesis kit (Thermo Fisher Scientific, Germany) with oligo-dT primers. Relative target quantity was determined using the comparative CT (ΔΔCT) method. RT-PCR was performed using SYBR Green/ROX PCR master mix (Thermo Fisher Scientific, Germany) and target specific primers for PTEN, CDKN2A and SIRT1 (**Supplementary Table S4**) on a Quantstudio three cycler (Applied Biosystems, Germany). Amplicons were normalized to endogenous GAPDH control. All samples were run in duplicates.

Xenogeneic Transplantation

NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NOD/SCID/IL2rc2/2, NSG) mice were purchased from The Jackson Laboratory (Jackson Laboratory, United States of America). They were kept in the animal facility at the Medical Theoretical Center of the University of Technology Dresden in accordance with German animal welfare legislation after approval of the Landesdirektion Sachsen (TVV 25/2015). Female mice (n = 3 per group) in the age of 8–9 weeks were used for the experiments. $CD34^+$ HSPCs were pre-incubated with EVs as described the *in vitro* studies. 1×10^5 cells were injected intravenously after whole body irradiation with 1 Gy. For the first 3 weeks after transplantation, water was supplemented with neomycin (1.17 g/l, Sigma-Aldrich, United States of America). Every 4 weeks peripheral blood was taken and after 16 weeks the animals were sacrificed and bone marrow was collected. All samples were stained with monoclonal antibodies (**Supplementary Table S3**) after lysis of red blood cells.

Statistical Analysis

Values were summarized as means, p values < 0.05 were considered significant. Statistical analyses including Student's t-Test and One-way ANOVA were calculated with GraphPad Prism 5.00 for Windows.

RESULTS

Characterization of MSC-Derived EVs From Young Vs. Aged Donors

After incubating a nearly confluent MSC layer in serum-free DMEM for 48 h, EVs were collected with a column-based isolation method. The MSC viability was proved to be over 95% in all samples. As a reference for EV concentration, the cell number was determined after trypsinization and was $2.4–3.3 \times 10^6$ MSCs (median 2.6×10^6).

NTA revealed no significant differences in concentration and size distribution between young and aged MSC-EVs. Particles larger than 400 nm could not be detected. The mean of the median diameter of the young and old MSC-EVs (n = 3 per group) was 170.7 ± 8.4 and 174.0 ± 11.0 nm, respectively (**Figure 1A**). The mean particle concentration was 2,83 ± 0,28 and 2,41 ± 0,49 $*10^3$ particles per MSC.

Next, the extracted EVs of young and old MSCs were characterized by Western Blot. The exosomal proteins CD63, CD81 and Flotillin-1 could be detected in all samples, whereas Calnexin, a typical cellular marker, was absent in all samples but present in the cell lysate (**Figure 1B**). The Transelectron Microscopy showed round vesicular structures with a hypodense center in isolates from both young and aged MSCs. The typical bilayer membrane

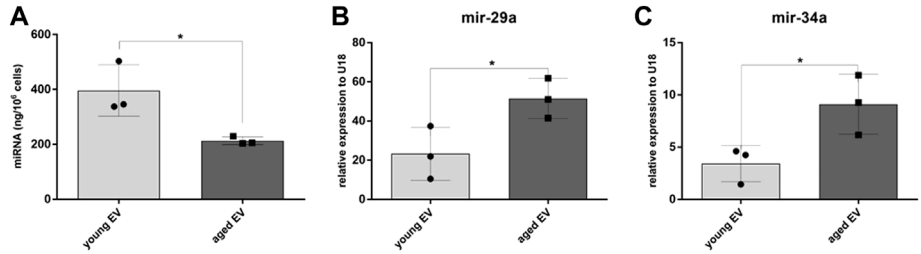

FIGURE 2 | Analysis of miRNA extracted from young and aged MSC-EVs. **(A)** The miRNA amount released per young and aged MSC-EVs was determined by Qubit fluorometry. **(B,C)** Expression of mir-29a and mir-34a in young and aged MSC-EVs was evaluated by quantitative real-time PCR. Data samples were normalized to U18, the bars represent the mean ± SEM from three different MSC donors in each group (n = 3), $*p < 0.05$.

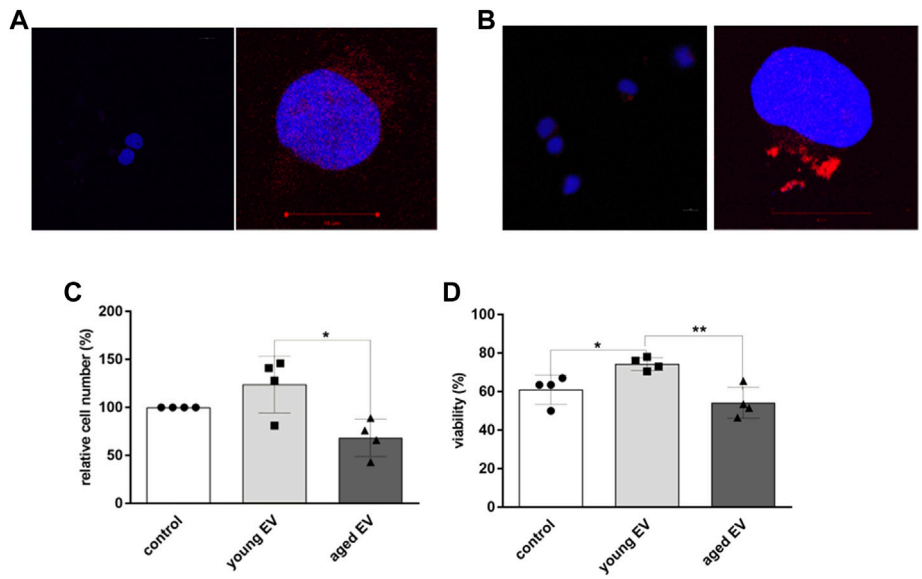

FIGURE 3 | Uptake of MSC-EVs and effects on HSPCs. HSPCs were either treated for 24 h with buffer **(A)** or with DiI-labeled EVs **(B)**. Representative images of confocal light microscopy are shown for lower (×20, left) and higher magnification (×63, right), nucleus of HSPCs is stained with DAPI (blue), EVs are labeled with DiI (red). Scale bar 10 µm. The cell number **(C)** and viability **(D)** was determined by trypane blue staining and counting of HSPCs after 72 h of incubation with young or aged MSC-EVs or buffer as control, respectively. Bars represent the mean ± SEM from four different HSPC donors in each group (n = 3), $*p < 0.05$, $**p < 0.01$.

could be observed. There were no obvious differences in the ultrastructure and the morphology between EVs from young and aged MSCs (**Figure 1C**).

Analysis of miRNAs in Young and Aged MSC-EVs

MSCEVs can transfer the cargoes to the recipient cells and thereby alter their activities.

Since small RNAs are highly abundant in EVs, the miRNA cargo was isolated and the concentration determined by Qubit analysis. Interestingly, the total amount of miRNA was significantly decreased in EVs derived from aged compared to young MSCs (**Figure 2A**). In a next step a panel of miRNAs relevant for hematopoiesis were quantified by PCR. U18 was used as endogenous control. The Ct value in the group of young MSC-EVs was 29.16 ± 0.96 and in the group of aged MSC-EVs 30.43 ± 0.86 confirming stable U18 expression in both sample cohorts. Whereas no relevant differences could be detected for let-7a, miR-10a, miR-21, miR-23, miR-155, miR-221, miR-222 and miR-486, the levels of miR-29a and miR-34a were significantly higher in EVs from aged MSCs (**Figures 2B**,C). This suggests an accumulation of specific miRNAs in aged EVs whereas the overall amount was decreased.

EV Transfer Into HSPCs

To investigate whether MSC-derived EVs can be incorporated by HSPCs, confocal microscopy was applied. After incubation of HSPCs with DiI labeled EVs for 24 h, an uptake could be detected independent on the age of the parental MSCs. Unstained EVs and buffer, respectively, were used as control (**Figures 3A,B**).

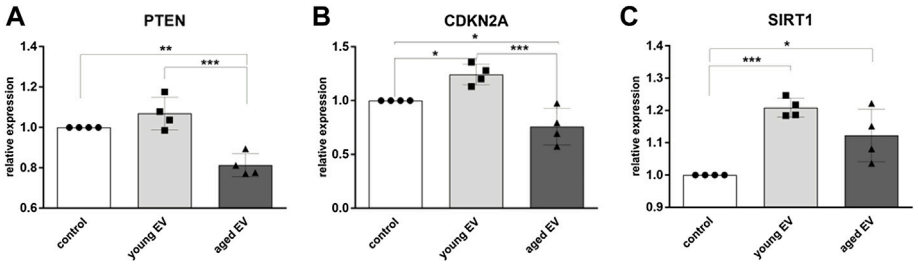

FIGURE 4 | Modulation of gene expression in HSPCs by MSC-EVs. The expression of PTEN **(A)**, CDKN2A **(B)** and SIRT1 **(C)** in HSPCs was evaluated after 72 h of incubation with young or aged MSC-EVs or buffer as control, respectively. Relative target quantity was determined using the comparative CT (ΔΔCT) method. Amplicons were normalized to endogenous GAPDH expression and the buffer control was set to 1. Cumulative data from four different HSPC donors are shown as mean ± SEM. Significance was assessed by one-way ANOVA with Tukey's multiple comparisons test. *$p < 0.05$, **$p < 0.01$ ***$p < 0.001$.

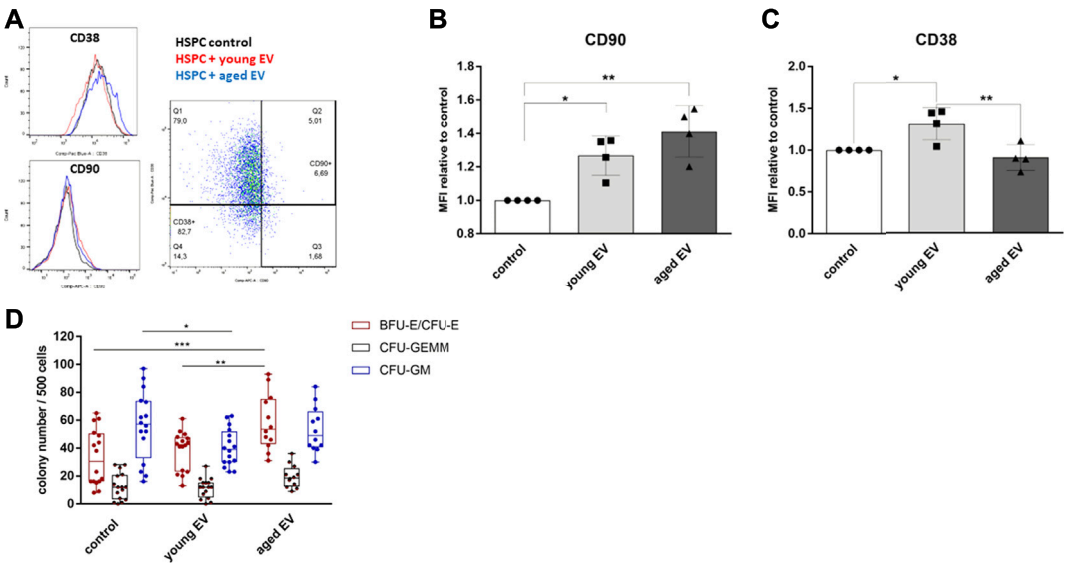

FIGURE 5 | Changes in HSPC surface molecule expression after incubation with MSC-EVs. The expression of CD90 and CD38 in $CD34^+$ HSPCs was analyzed by flow cytometry after 72 h of incubation with young or aged MSC-EVs or buffer as control, respectively. **(A)** Representative dot and histogram plots are shown for the detection of CD38 and CD90 expression. **(B)** Graphs show the CD90 and **(C)** CD38 MFI of $CD34^+$ cells in relation to their control. **(D)** A CFU-GEMM assay was performed for 14 days in methylcellulose medium and the colonies were classified by using the StemVision system. The bars represent the mean ± SEM from four different HSPC donors in each group, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by two-way ANOVA with Tukey's multiple comparisons test.

To analyze the impact of incorporated EVs on HSPC characteristics in more detail, the incubation time was increased for up to 72 h. Aged HSPCs (n = 4) were treated with EVs of young or aged MSCs, respectively, or with XE buffer only as control. EVs derived from young MSCs caused a significant increase in the cell number compared to cells treated with buffer (1.2-fold) or with old EVs (1.8-fold) (**Figure 3C**). Furthermore, the viability of HSPCs was significantly improved after incubation with young EVs (**Figure 3D**).

Gene Expression Analysis in HSPCs After EV Transfer

Incorporation of EVs and miRNA release can modulate gene expression of HSPCs. Therefore, the expression of the tumor suppressor gene PTEN, a known target of mir-29a, was decreased in HSPCs treated with aged EVs (**Figure 4A**). Interestingly, the expression of CDKN2A, encoding for the cell cycle inhibitors $p16^{INK4A}$ and p19, was significantly lower in HSPCs incubated with aged MSC-EVs. In contrast, the gene was upregulated in cells treated with young MSC-EVs (**Figure 4B**). SIRT1, encoding for the histone deacetylase Sirtuin-1 that regulates a balanced hematopoietic differentiation, is upregulated in both groups receiving EVs (**Figure 4C**).

Moreover, expression of p53, FOXO3, AKT1, Beclin-1 and ATG12 were investigated. However, no significant differences were detected in the expression levels of these genes.

Phenotypical Characterization of HSPCs After EV Transfer

Surface marker expression is an important indicator of differentiation and stemness of HSPCs and contributes to the definition of the functional properties, e.g., after transplantation. Therefore, the expression of the proteins CD38 and CD90 on $CD34^+$ population was investigated (**Figure 5A**). We could not detect significant changes in the absolute expression of these surface markers owing to relevant inter-individual differences. The mean fluorescence intensity (MFI) of the stemness marker CD90 normalized to the respective donor was increased after treatment with MSC-EVs with no respect to the donor's age (**Figure 5B**). However, the normalized MFI of CD38, a protein expressed on committed progenitors, was also significantly higher on HSPCs treated with young EVs too (**Figure 5C**).

Analysis of the Clonogenic Capacity of EV-Modulated HSPCs

To evaluate the clonogenic potential of HSPCs *in vitro* after EV treatment, colony-forming unit (CFU) assays were performed. The HSPCs of each group were able to differentiate into each type of colony. HSPCs treated with aged MSC-EVs showed a significant higher number of BFU-E/CFU-E colonies. Furthermore, the number of CFU-GM colonies was reduced in the group of HSPCs treated with young MSC-EVs compared to the control group (**Figure 5D**).

Evaluation of the *in Vivo* Engraftment Ability of EV-Treated HSPCs

To prove the beneficial effect of MSC-EVs on the engraftment potential of HSPCs *in vivo*, xenotransplantation assays in immunodeficient NSG mice were conducted. 1×10^5 pretreated HSPCs were transplanted. Long-term engraftment could be observed in all three groups assessed by the *de novo* generation of myeloid ($CD33^+$) and lymphoid ($CD3^+$ and $CD19^+$) cells (**Figure 6A**). There were no significant differences concerning the phenotype and the compartmental attribution of the blood cells neither in the spleen and the peripheral blood nor in the BM (**Figure 6B**).

The HSPCs treated with aged MSC-EVs showed a significantly higher week 12 peripheral blood chimerism (**Figure 6C**). The chimerism in bone marrow and spleen was also evaluated after 16 weeks. No significant

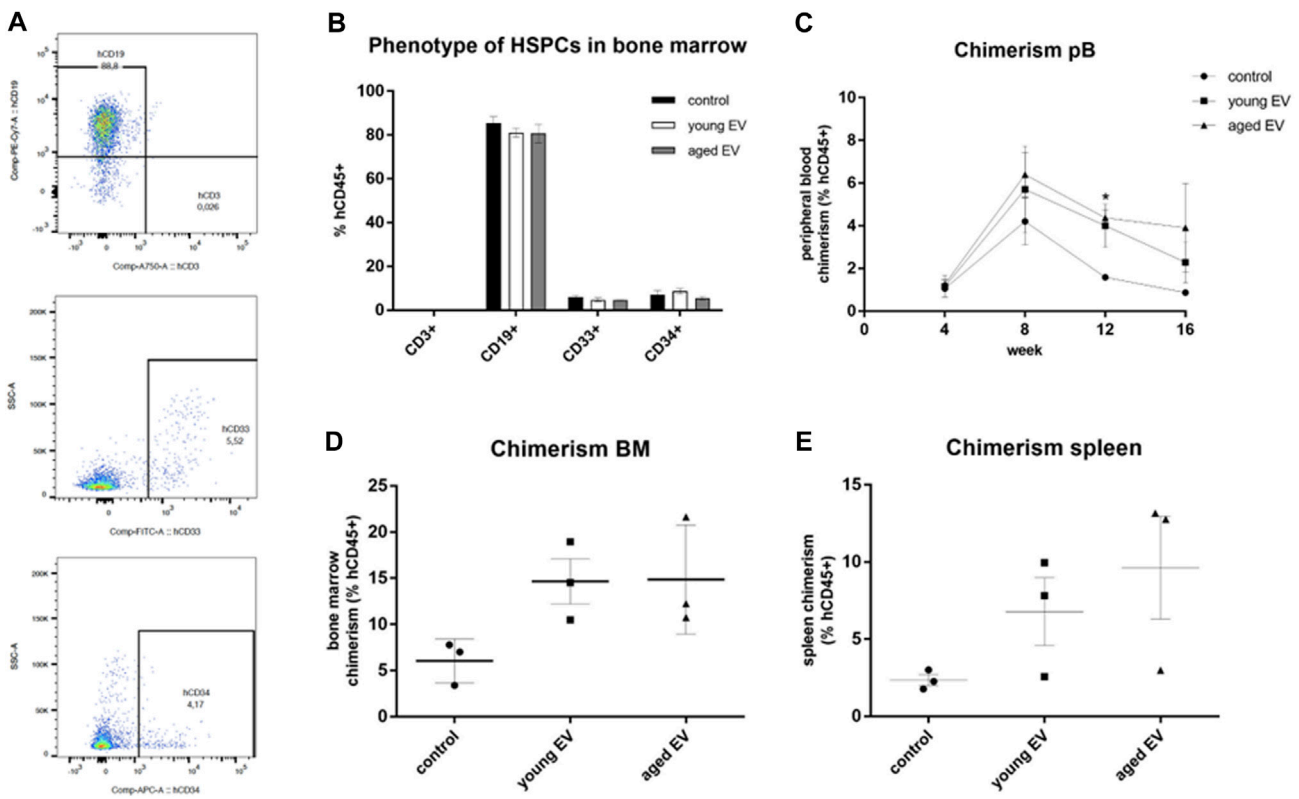

FIGURE 6 | Effects of MSC-EV treatment on HSPC engraftment *in vivo*. HSPCs were treated for 72 h with young or aged MSC-EVs or buffer as a control, respectively, and injected into NSG mice (n = 3 per group). Mice were kept for 16 weeks. Phenotype and chimerism was analyzed by flow cytometry after gating on human $CD45^+$ cells. **(A)** Representative dot plots for the detection of human cells expressing CD3, CD19, CD33 or CD34, respectively. **(B)** Phenotype of human $CD45^+$ cells in the BM. Lymphoid cells were detected by CD3 (T cells) and CD19 (B cells), myeloid cells by CD33 expression. **(C)** The chimerism defined as percentage of human $CD45^+$ cells amongst all $CD45^+$ cells was analyzed every 4 weeks in the peripheral blood. **(D,E)** The chimerism in the BM and in the spleen was analyzed 16 weeks after transplantation. The bars represent the mean ± SEM from three different mice in each group, $^*p < 0.05$.

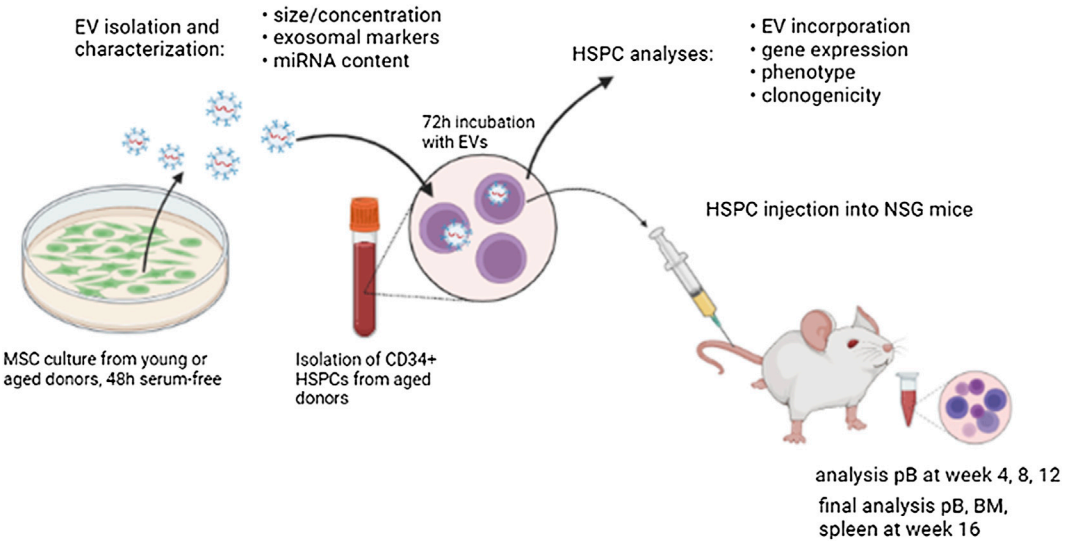

FIGURE 7 | Graphical abstract demonstrating the experimental setup.

differences could be observed, although the chimerism was always higher after EV treatment with no respect to the donor's age (**Figures 6D**,E).

DISCUSSION

Aging of HSPCs has a decisive impact on the outcome of HCT, is one of the reasons for decreased immune responses of elderly people and represents an important risk factor for the development of hematologic malignancies (Geiger, De Haan and Carolina Florian, 2013; Kollman et al., 2016). However, aging of HSPCs is not just a cell-intrinsic process, it is also influenced by an aging BMME (Walenda et al., 2010; Kusumbe et al., 2016). To this end, MSCs play a crucial role for the regulation of the HSPC fate. The communication between MSCs and HSPCs is mediated by direct cell-cell-interactions but also by the exchange of EVs. Recent studies have even shown an extension of health span in mice and highlight the meaning of these particles and their role in aging processes (Dorronsoro et al., 2021). However, the age of the MSCs as one factor influencing the potential of their EVs is often neglected in studies and examined just in few studies (Wang et al., 2015; Kulkarni et al., 2018).

We characterized young and aged MSC-EVs according to the recommendations of the International Society for Extracellular Vesicles (Théry et al., 2018). The EV morphology as analyzed by TEM, the protein cargo as examined by Western Blot and the size distribution calculated by NTA showed no difference between the two groups (**Figure 1**). However, the number of particles produced by each cell is significantly higher in aged MSCs. Fafián-Labora et al. also observed this increase in MSC-EVs of aged rats and hypothesized that it might be a mechanism to increase the disposal of intracellular proteins (Fafián-Labora et al., 2020).

miRNAs represent important mediators in the communication *via* EVs. MSC-EVs show a specific miRNA signature that is altered during aging (Baglio et al., 2015; Fafián-Labora et al., 2017). Interestingly, the total amount of miRNAs decreased in aged MSC-EVs samples what might represent one reason for an impaired ability to support HSPCs. The quantitative analysis revealed a different miRNA profile of young and aged MSC-EVs. The expression of miR-34a, a proapoptotic microRNA regulating the proliferation of cells (Rokavec et al., 2014), was increased in aged MSC-EVs (**Figure 2C**). Overexpression of miR-34a in HSPCs leads to a myeloid bias and is often detected in patients suffering from myelofibrosis (Bianchi et al., 2017). Moreover, miR-34a is typically upregulated in senescent cells and can be induced by cellular stress (Tazawa et al., 2007; Kulkarni et al., 2018). The expression of miR-29a known as an important smallRNA regulating cell cycle is also increased in aged MSC-EVs (**Figure 2B**). This miRNA supports the self-renewal of HSPCs, but can also lead to acute myeloid leukemia, when experimentally overexpressed in progenitor cells (Han et al., 2010).

In this study, we have examined for the first time effects caused by EVs of physiologically aged human MSCs on HSPCs of elderly people (**Figure 7**). Interestingly, young MSC-EVs can support the expansion of HSPCs *in vitro*, whereas aged MSC-EVs did not have beneficial effects on the cell survival and the proliferation (**Figures 3C**,D). This might be the consequence of a well-balanced regulation of cell growth and cell cycling in HSPCs treated with young EVs. Consistent with this assumption, we observed a downregulation of important genes in HSPCs after incubation with aged MSC-EVs. PTEN is a tumor suppressor which regulates cell growth in the AKT signaling pathway and that is important for the reconstitution after HCT (Yilmaz et al., 2006). It is also a target of miR-29a which is upregulated in old MSC-EVs (Morhayim et al., 2016). CDKN2A is another gene that is downregulated in HSPCs after treatment with old MSC-EVs. This gene encodes for proteins that regulate cell cycle especially in aged HSPCs (Furukawa et al., 2000; Janzen et al., 2006). In contrast, SIRT1 is upregulated in HSPCs treated with both young and old MSC-EVs. The encoded protein is a histone deacetylase regulating cell cycle and differentiation of HSPCs (Rimmelé et al., 2014). SIRT1 is a component in young and aged murine MSC-EVs

(Kulkarni et al., 2018). Thus, this mRNA is likely to be found in human MSC-EVs and may be transferred directly *via* EVs.

Consistent with this finding the MFI of CD90 increased after the treatment with MSC-EVs regardless of donor age. CD90 represents a marker indicating the stemness of HSPCs. However, it can also be found on MSCs and MSC-EVs (Ramos et al., 2016). An increased CD90 expression has been observed after culturing HSPCs on a MSC layer (Notta et al., 2011) which supports the hypothesis of a direct transfer of this protein *via* EVs.

The clonogenic assays revealed a restrictive effect of young MSC-EVs on the myeloid differentiation (**Figure 5D**). This supports the idea that especially the young BMME regulates the stem cell fate and avoid a myeloid bias observed in aged individuals (Dykstra et al., 2011; De Luca et al., 2016). Surprisingly, aged MSC-EVs seem to support the differentiation of HSPCs into erythroid colonies.

It is known that the engraftment of HSPCs is improved after co-culturing on a MSC layer (Huang et al., 2007; Bernardo, Cometa and Locatelli, 2012). However, the mechanism of support is not fully understood. Our results suggest EVs as one potential mediator to improve the engraftment indicated by a higher chimerism especially in the peripheral blood. In contrast to findings with murine cells, the effect does not seem to depend on the MSC donor's age (Kulkarni et al., 2018).

CONCLUSION

In conclusion, we provide evidence that human MSC-derived EVs underlie age-related changes in terms of their molecular cargo which might negatively impact recipient cells. Consequently, only young MSC-EVs supported the proliferation and viability of HSPCs *in vitro* and enhanced the expression of genes regulating cell growth and division. Additionally, MSC-EVs improved the engraftment of HSPCs *in vivo*. These data indicate a potential therapeutic relevance of MSC-EVs and may stimulate further translational studies to evaluate their role in age-related pathologies.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethikkommission an der TU Dresden. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Landesdirektion Sachsen, Dresden, Germany.

AUTHOR CONTRIBUTIONS

PF, MvB, MB, and MW contributed to conception and design of the study. PF, KM, RK, and MvB performed experiments and data analysis. PF and MW wrote the manuscript. MB, MvB, and MW edited the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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The Role of Hypoxia in Improving the Therapeutic Potential of Mesenchymal Stromal Cells. A Comparative Study From Healthy Lung and Congenital Pulmonary Airway Malformations in Infants

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Mesenchymal stromal cells (MSCs) play an important role in the field of regenerative medicine thanks to their immunomodulatory properties and their ability to secrete paracrine factors. The use of MSCs has also been tested in children with congenital lung diseases inducing fibrosis and a decrease in lung function. Congenital malformations of the pulmonary airways (CPAM) are the most frequently encountered lung lesion that results from defects in early development of airways. Despite the beneficial properties of MSCs, interventions aimed at improving the outcome of cell therapy are needed. Hypoxia may be an approach aimed to ameliorate the therapeutic potential of MSCs. In this regard, we evaluated the transcriptomic profile of MSCs collected from pediatric patients with CPAM, analyzing similarities and differences between healthy tissue (MSCs-lung) and cystic tissue (MSCs-CPAM) both in normoxia and in cells preconditioned with hypoxia (0.2%) for 24 h. Study results showed that hypoxia induces cell cycle activation, increasing in such a way the cell proliferation ability, and enhancing cell anaerobic metabolism in both MSCs-lung and MSCs-CPAM-lung. Additionally, hypoxia downregulated several pro-apoptotic genes preserving MSCs from apoptosis and, at the same time, improving their viability in both comparisons. Finally, data obtained indicates that hypoxia leads to a greater expression of genes involved in the regulation of the cytoskeleton in MSCs-lung than MSCs-CPAM.

Keywords: hypoxia, mesenchymal stromal cells, lung, congenital pulmonary airway malformations, transcriptomic analysis

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INTRODUCTION

Cell therapy is an important field that sees use in tissue engineering and regenerative medicine (Huang et al., 2009). Human mesenchymal stromal cells (MSCs) possess the ability to self-renewal and multilinear differentiation (Minguell et al., 2001; Pittenger and Martin, 2004) and have been isolated from different tissues such as fat, bones (Pittenger and Martin, 2004), bone marrow (Minguell et al., 2001), teeth (Huang et al., 2009), amniotic fluid (Kaviani et al., 2003), and urine (Chun et al., 2012). Thanks to their immunomodulatory properties and their ability to secrete several paracrine factors, they have been used for the treatment of cardiovascular diseases (Thakker and Yang, 2014), nerve injuries (Dadon-Nachum et al., 2011), bone regeneration (Fan et al., 2015) and respiratory disorders (Lou et al., 2021). Furthermore, the use of cell therapy with MSCs was also tested in children with congenital lung diseases (Pelizzo et al., 2020; Tong et al., 2021).

As reported, repeated administration of allogeneic MSCs improved respiratory conditions in children with interstitial lung disease. In particular, in the lung tissue, the regenerative processes are promoted by a pool of MSCs which would appear to be involved in the regeneration and architecture of tissues processes. In conditions such as congenital malformations of the pulmonary airways (CPAM), the therapeutic potential of MSCs ha also been proposed (Pelizzo et al., 2021).

CPAM are congenital pulmonary anomalies histologically characterized by the presence of multiple cysts located in the lung parenchyma. Although considered a rare disease, it is estimated to affect between one in 25,000 and 35,000 live births (Laberge et al., 2001; Duncombe et al., 2002). These lung lesions are the result of embryological damage in the early gestation period causing hyperproliferation and terminal bronchioles dilatation. The absence of normal alveoli induces pulmonary compression and hypoplasia and in some cases also a displacement of the mediastinum (Stocker et al., 1977; Stocker, 2009). CPAM are classified into 5 types according to the size and lesion location. Among these the most recurrent lesions are the type 1 lesion, which occurs in 60–70% of cases with intercommunicating cysts >2 cm, and CPAM types 2 in 15–20% of cases with cystic lesions <2 cm (MacSweeney et al., 2003). It can be asymptomatic or be responsible for recurrent infections or symptoms related to compression of the airways. To date, treatment in symptomatic patients includes surgical therapy with excision of the lesion (Leblanc et al., 2017). In the asymptomatic forms, indeed, the risk of developing infections and the potential development of tumors seem to support resection in childhood (Aslan et al., 2006; Adzick, 2009; Pelizzo et al., 2009; Peters et al., 2013; Gajewska-Knapik and Impey, 2015). MSCs have a crucial role in the microenvironment and regulation of tumor survival, growth, and progression (Pelizzo et al., 2017). However, MSCs could be useful for the lung regenerative process after surgical treatment of congenital pulmonary lesions.

For this reason, interventions aimed at improving the regenerative capacities of MSCs are needed. Several studies reported that the use of growth factors or exposure to hypoxic conditions improves the properties of MSCs (Khan et al., 2011; Fan et al., 2015). Oxygen (O_2) is a substrate needed by cells for energy production and cell metabolism (Wang et al., 2005). Cell proliferation and differentiation are biological processes regulated by O_2. Hypoxia preconditioning has been proposed as an approach to improve the therapeutic potential of MSCs; in fact, it has been seen that short-term exposure to hypoxia has an empowering effect leading to increased cell migration and improved proliferation, survival, differentiation and paracrine activities of MSCs (Annabi et al., 2003; Yang et al., 2022).

In this context, MSCs were harvested from infants after surgery for CPAM's lesions. Healthy lung tissue (MSCs-lung) and cystic tissue (MSCs-CPAM) were considered. In order to investigate the effect of O_2 concentration, MSCs-lung and MSCs-CPAM were exposed to *in vitro* normoxia or hypoxia (0.2% O_2) condition for 24 h. Subsequently, analogies and differences in transcriptomic profiles between MSCs-lung or MSCs-CPAM in normoxia against hypoxia were analyzed.

MATERIALS AND METHODS

Patients

Two male infants with diagnosed CPAM were admitted to the surgical ward for elective surgery including lobectomy. Microscopically, the lesion was diagnosed as CPAM type II by Stocker's classification. With parental consent, a portion of the lung tissue intended for histological analysis was used for the expansion of MSCs. Two samples were obtained respectively from the "healthy" tissue (called MSCs-lung) and from congenital lung tissue (called MSCs-CPAM). The study was performed according to the Declaration of Helsinki and with the approval of the Institutional Review Board of the "G. Di Cristina" (registration number 87 Civico 2017). Written informed consent was obtained by the parents and/or legal guardian of each infant after receiving information on the study.

Cell Isolation, Culture and Characterization

The MSCs were obtained from pulmonary tissue of two male infants diagnosed with CPAM and submitted to elective surgery, as previously reported (Pelizzo et al., 2017). The cells were expanded until passage 2. Subsequently, the cells were seeded at $2{,}5 \times 10^6$ cells in each Petri dish (150 × 25 mm) and kept in culture with MSCBM (Lonza, Basel, Switzerland) culture medium for 24 h inside an incubator at 37°C with a humidified atmosphere at 5% CO_2 in the air before hypoxic treatment.

The cells were characterized as previously reported for:

- proliferative capacity defined as calculated cell count (CCC),
- immunophenotype by flow-cytometry using monoclonal antibodies specific for CD73, CD34, CD90, CD14, CD45, CD31, CD105 class I and class II- HLA (Beckman coulter Milan, Italy),
- osteogenic and adipogenic differentation capacity was evaluated by *in vitro* adding specific stimuli. In particular,

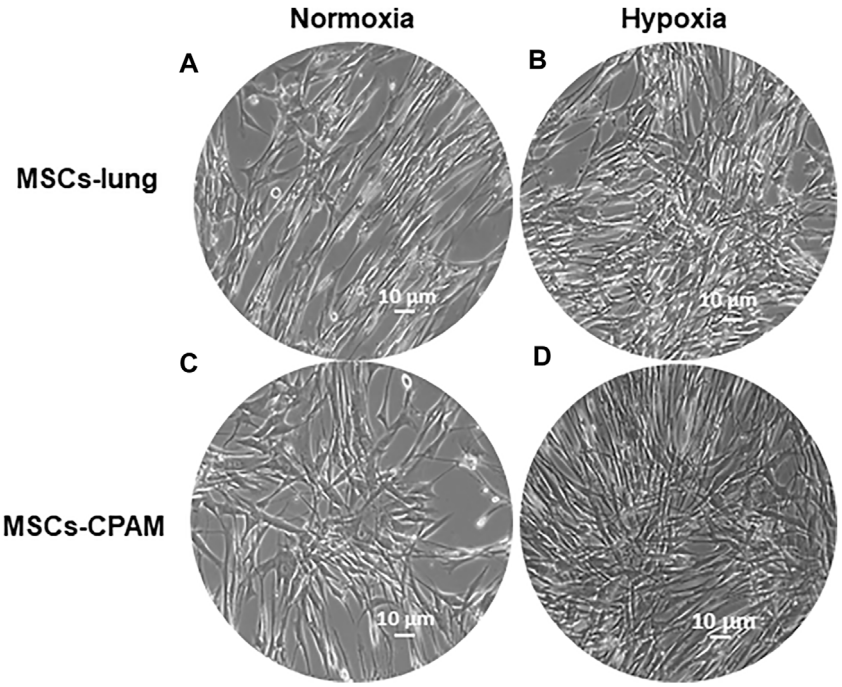

FIGURE 1 | MSCs-lung and MSCs-CPAM morphology observations under inverted light microscopy. **(A,C)** Normoxic controls. **(B,D)** Hypoxia samples. Scale bar = 10 µm.

for osteogenic differentation 10−7 M dexamethasone, 50 mg/mL L-ascorbic acid and 5 mM ß-glycerol phosphate were added to α-MEM 10% FBS (Euroclone, Milan, Italy), for adipogenic differentiation 100 mg/ml insulin, 50 mM isobutylmethylxanthine, 0.5 mM indomethacin were also added to culture medium. All reagents were from Sigma-Aldrich (Milan, Italy).

- The capability to enter senescence was evaluated maintaining cells in culture until the number of detached cells was ≤ to the number of plated cells.
- The immunomodulatory capacity was assesed at different ratio (1:2-1:20-1:200) on healthy donor peripheral blood mononuclerar cells (PBMC) activated with phytohemagglutinin (PHA).

Hypoxic Treatment

The cultured cells of both MSC-lung and MSCs-CPAM were seeded in a 6-multiwell plate with a density of 8×10^4 for each well. After seeding cells were maintained for 24 h in the standard medium MSCBM (Lonza) in incubator at 37°C with a humidified atmosphere at 5% CO_2. Then cultured cells of MSC-lung and MSCs-CPAM were subjected to hypoxic treatment by inserting the plates into the ProOx Model P110 (BioSpherix, New York, NY, United States) hypoxia chamber for 24 h at 0.2% of hypoxia. Cells were observed under inverted light microscopy (Leica Microsystem, Milan, Italy) to evaluate the morphological features in normoxic and hypoxic conditions. Cultured cells of MSC-lung and MSCs-CPAM were maintained in incubator with normoxic condition and used as control cells.

RNA Extraction

RNA isolation was performed using the Total Exosome RNA and Protein Isolation Kit (catalog # 4478545; Thermo Scientific, Rockford, IL, United States) following the manufacturer's instruction. The RNA quality and concentration were measured using Eppendorf BioSpectrometer fluorescence. TruSeq RNA Exome protocol (Illumina, San Diego, CA, United States) was used for library preparation. 3 batches were analyzed by RNA sequencing. Concisely, each sample RNA extracted was fragmented at 94 C for 8 min and then was synthesized the first strand of cDNA using the SuperScript II Reverse Transcriptase (Invitrogen, Milan, Italy). Subsequently, the second strand of cDNA was synthesized and purified using AMPure XP beads (Beckman Coulter, Brea, CA, United States). In the following step, in order to allow the adaptor ligation, the 3′ ends of the cDNA were adenylated and then was performed the indexing adapter ligation. The library was purified with AMPure XP beads. In order to enrich those fragments of DNA that have adaptors on both ends, and also to increase the quantity of library DNA a first PCR amplification step was carried out. The library has been validated through the Agilent Technologies 2,100 Bioanalyzer and then, 200 ng of each DNA library were combined for performing the first hybridization step. Magnetic beads coated with streptavidin were used to capture probes hybridized to the target regions, in order to eliminate nonspecific binding. After the enriched libraries were eluted from the beads, the second cycle of hybridization was performed, in order to obtain a wide

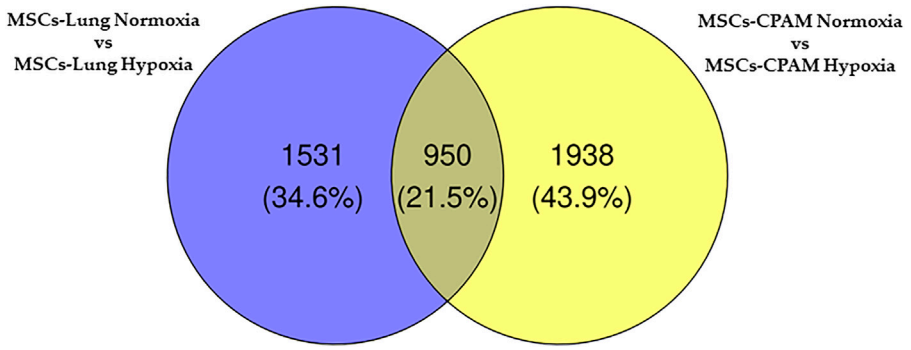

FIGURE 2 | Venn diagram of MSCs-lung and MSCs-CPAM in the different normoxia and hypoxia conditions. All the comparisons found 606 genes as different in statistical manner (the bottom intersection).

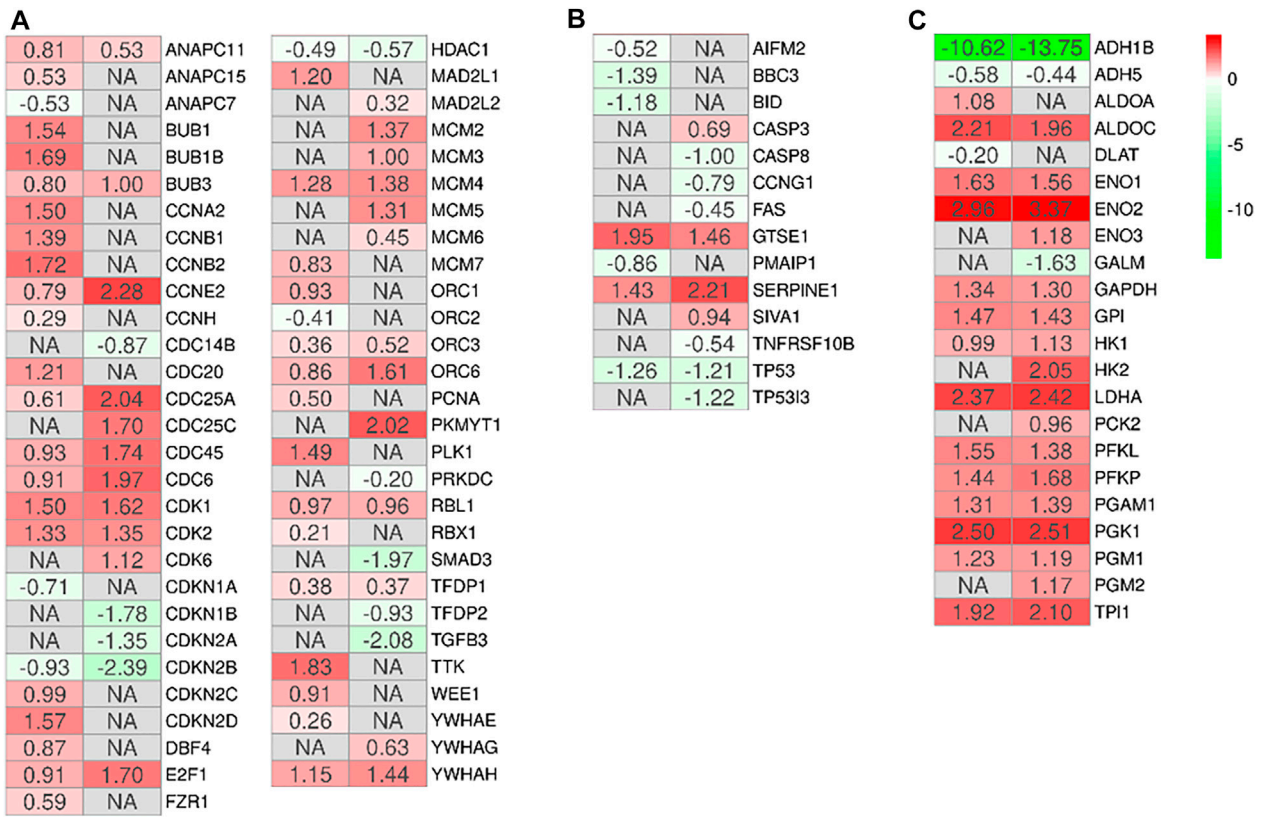

FIGURE 3 | Heatmap of genes involved in the activation of the cell cycle **(A)**, inhibition of the apoptosis **(B)** or activation of the cellular metabolism **(C)** in the comparison of MSCs-lung-Normoxia against MSCs-lung-Hypoxia or MSCs-CPAM-Normoxia (left column of each comparison) against MSCs-CPAM-Hypoxia (right column of each comparison). The green scale is related to downregulated genes whereas the red palette represents upregulated ones. NA value is put when the difference is not statistically relevant in the comparison. All fold changes are rounded to the second decimal digit.

specificity of regions of capture. Finally, the libraries were purified using the AMPure XP bead and then amplified. The final library was quantified and certified with the Agilent High Sensitivity Kit through a bioanalyzer. Subsequently, the library was normalized and was loaded for clustering on a MiSeq Flow Cell and then sequenced with a MiSeq Instrument (Illumina).

RNA-Seq Inspection

After the sequencing, the quality of the demultiplexed samples was verified using the software fastQC (version 0.11.5, Babraham Institute, Cambridge, United Kingdom) along with Trimmomatic (version 0.38, Usadel Lab, Aachen, Germany) (Bolger et al., 2014) to drop adapters or bad

bases. Each read was mapped to the relative gene of the human reference genome version GRCh38 through the spliced transcripts alignment to a reference (STAR)RNA-seq aligner (version 2.7.3a, New York, NY, United States) (Dobin et al., 2013). Then, the htseq-count (version 0.6.1p1, European Molecular Biology Laboratory (EMBL), Heidelberg, Germany) package (Anders et al., 2015) in python was used to count the transcripts and the differentially expressed genes (DEGs) for both the comparisons were analyzed in R (version 3.6.3, R Core Team) using the Bioconductor package DESeq2 (Love et al., 2014).

Western Blotting

Proteins extracted from all experimental groups were processed as previously described (Soundara Rajan et al., 2017). Briefly, 30 µg of proteins were resolved on SDS–PAGE gel and subsequently transferred to nitrocellulose sheets using a semidry blotting apparatus. Sheets were saturated for 120 min at room temperature in blocking buffer (1×TBS, 5% milk, 0.1% Tween- 20) and incubated overnight at 4°C in blocking buffer containing primary antibodies Bcl-2 (1:750; Santa Cruz Biotechnology, Dallas, TX, United States), Ki-67 (1:500; BioGenex, Fremont, CA, United States), Cleaved caspase-3 (1:1,000; Cell Signalling Technology, Milan, Italy), Cleaved caspase-8 (1: 400; Cell Signalling Technology), p53 (1:400; Merck Millipore, Milan, Italy) and ITGA4 (1:400; Abnova, DBA, Milan, Italy). β-Actin (1:750; Santa Cruz Biotechnology) was used as a loading control. After four washes in TBS containing 0.1% Tween-20, samples were incubated for 60 min at room temperature with peroxidase-conjugated secondary antibody diluted as 1:1,000 in 1 × TBS that contained 2.5% milk and 0.1% Tween-20. Bands were visualized and quantified by the ECL method with Alliance 2.7 (UVItec Limited, Cambridge, United Kingdom).

Statistical Analysis

To select the DEGs and reject the genes that did not differ statistically we used in DESeq2 the Welch's test applied to the negative binomial distribution. In detail, we corrected the *p*-value in q-value using the post-hoc Benjamini–Hochberg procedure and a threshold level of 0.05. Thus, only genes whose q-value was lower than 0.05 were defined as DEGs.

Furthermore, for both MSCs-lung and MSCs-CPAM comparisons we enriched the DEGs in both the comparison with the KEGG pathways using the clusterProfiler Bioconductor package (Yu et al., 2012). We used the default statistics test and we set 0.05 as the threshold. Even in this case, we used the false discovery rate post-hoc Benjamini–Hochberg to correct the *p*-value and drop the false positive pathways.

For protein expression the experiments were performed in triplicate. All data are expressed as mean ± standard deviation. Statistical analyses were performed using the Student's unpaired *t*-test for comparisons of two groups and one-way ANOVA followed by the Bonferroni method for comparisons of three or more groups. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Characterization of MSCs-Lung and MSCs-CPAM

MSCs-lung and MSCs-CPAM were successfully isolated and expanded from lung samples of both patients. As already reported they were plastic adherent, showed spindle shape morphology and high proliferative capacity, reaching ≥80% confluence in less than 5 days.

Cells were negative for CD34, CD14, CD45, and HLA-DR and positive for CD73, CD90, CD105, and HLA-I.

They did not differentiate into adipocytes or osteocytes, since lipid droplet formation or AP activity and calcium deposition was not observed.

MSCs-lung ceased to growth at P14 for both lines, while MSCs-CPAM at P19 and P16.

Moreover, they showed a dose dependent *in vitro* immunosuppressive activity on activated PBMC.

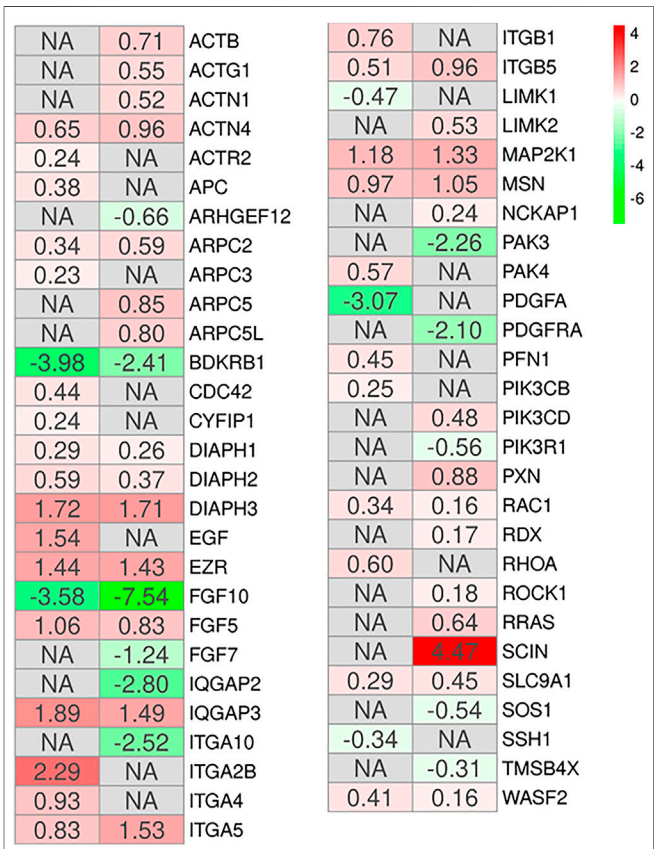

FIGURE 4 | Heatmap of genes involved in the gene ontology term "Epithelium development" and observed in the KEGG pathways "Regulation of actin cytoskeleton," "Focal adhesion" or "PI3K-Akt signaling pathway" in the comparison of MSCs-lung-Normoxia against MSCs-lung-Hypoxia (left column of each comparison) or MSCs-CPAM-Normoxia against MSCs-CPAM-Hypoxia (right column of each comparison). The green scale is related to downregulated genes whereas the red palette represents upregulated ones. NA value is put when the difference is not statistically relevant in the comparison. All fold changes are rounded to the second decimal digit.

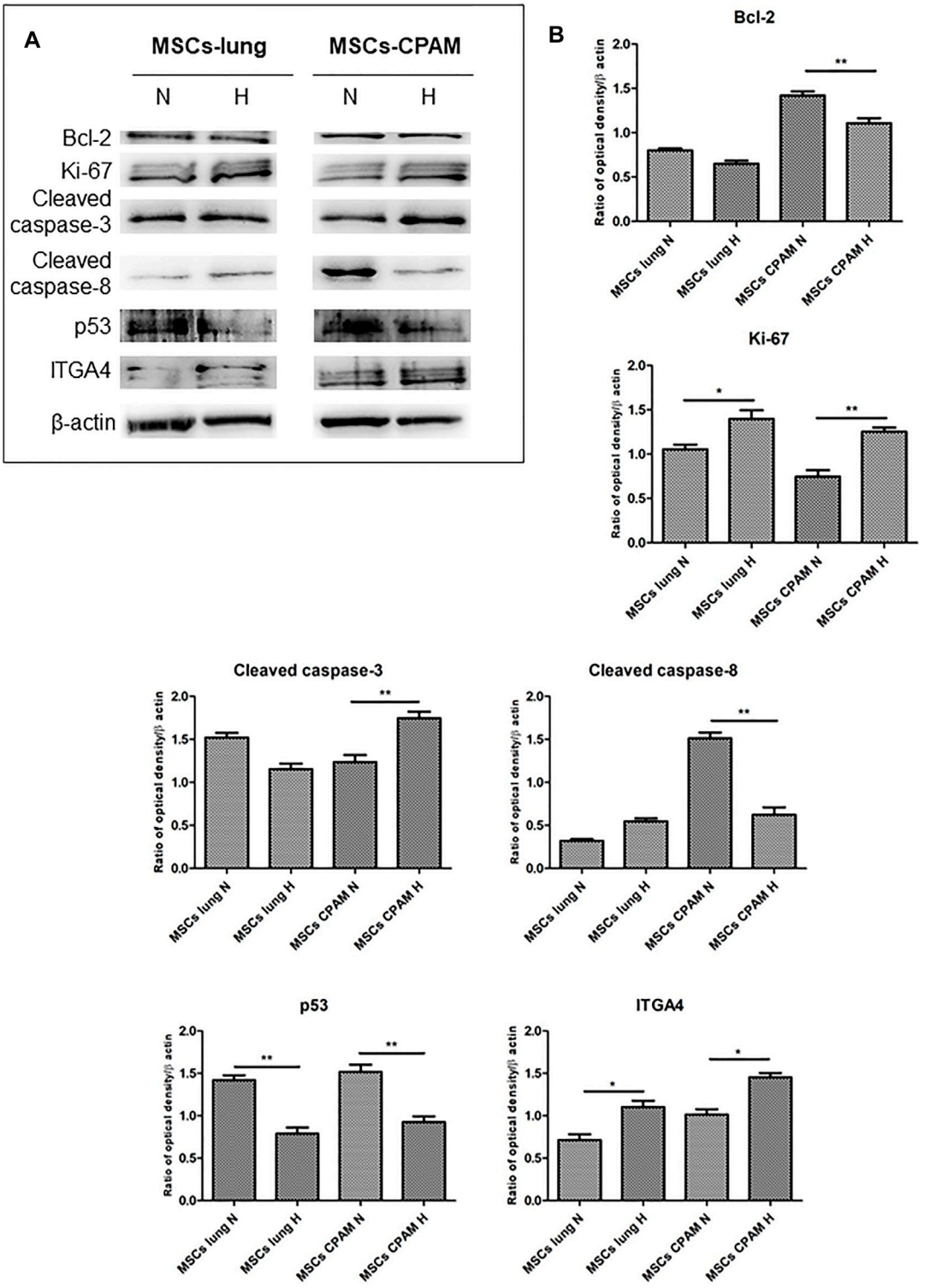

FIGURE 5 | MSCs-lung and MSCs-CPAM protein expression under Normoxic (N) and Hypoxic (H) conditions. **(A)** Specific band of investigated markers Bcl-2, Ki-67, Cleaved caspase-3, Cleaved caspase-8, p53 and ITGA4. Beta-actin was used as housekeeping protein. **(B)** Densitometric analyses of the studied markers. *$<p <$ 0.05; **$p < 0.01$.

Culture of MSCs-Lung and MSCs-CPAM

Cells were incubated under normoxic conditions and under hypoxic conditions. Cells morphology was recorded after hypoxic treatment using a light microscope. After hypoxic treatment, several spindle-shaped fibroblast-like cells were observed and their numbers evidently increased. In contrast to normoxic conditions, hypoxic conditions promoted the proliferation in MSCs-lung and MSCs-CPAM (**Figure 1**).

Transcriptomic Analysis

We focus our analysis on the DEGs observed in the two cell types (MSCs-lung or MSCs-CPAM) in the hypoxia condition compared to the normoxia condition. Thus, the analyzed comparisons are:

- MSCs-lung-Normoxia against MSCs-lung-Hypoxia
- MSCs-CPAM-Normoxia against MSCs-CPAM-Hypoxia.

The comparison of MSCs-lung-Normoxia against MSCs-lung-Hypoxia highlighted 2,481 DEGs (1,361 upregulated, 1,120 downregulated). On the other hand, the comparison of MSCs-CPAM- Normoxia against MSCs-CPAM-Hypoxia highlighted 2,888 DEGs (1,501 upregulated, 1,387 downregulated). The Venn diagram in **Figure 2** summarizes the similarity and differences in DEGs among MSCs-lung and MSCs-CPAM in normoxia or hypoxia conditions.

In order to observe the biological differences between MSCs-lung and MSCs-CPAM, we searched for the common enriched pathways. We focused our attention on the pathways "Cell cycle" (hsa04110), "p53 signaling pathway" (hsa04115), "Glycolysis/Gluconeogenesis" (hsa00010). The analysis of these pathways allowed us to observe 3 different processes involved in increasing the proliferative capacity of MSCs and which could support their role in tissue repair and regeneration. Indeed, treatment with hypoxia seems to increase their ability to proliferate through the activation of the cell cycle (**Figure 3A**) and inhibition of the apoptosis (**Figure 3B**), which is parallelly supported by the activation of the cellular metabolism (**Figure 3C**).

Additionally, literature shows that the MSCs have a potential role in tissue repair (Parekkadan and Milwid, 2010). The gene ontology biological process term "Epithelium development" collects all the genes involved in the progression and formation of the epithelium to the mature structure (Attrill et al., 2019). It is known that MSCs can graft as lung epithelium in order to carry out structural repair of the damaged lung (Weiss and Finck, 2010), and the GO "epithelium development" has been identified as involved in the processes of wound healing and epithelial regeneration in other pathological conditions (Pokrywczynska et al., 2019). Thus, we enriched the KEGG pathways all DEGs of the two comparisons MSCs-lung and MSCs-CPAM included in this biological term. The pathways enriched in both the two comparison are "Regulation of actin cytoskeleton (hsa04810)," "Focal adhesion (hsa04510)," and "PI3K-Akt signaling pathway (hsa04151)," were common pathways observed. The reorganization of the cytoskeleton, the cellular adhesion and the PI3Ks signaling are pathways involved in the migratory activity and in the tissue regeneration processes (**Figure 4**). This consideration is in line with the cell cycle activation since and cell proliferation. Interestingly, the MSCs-lung seems more inclined to trigger the activation of these processes than the MSCs-CPAM.

Protein Expression

Bcl-2, Ki-67, Cleaved caspase-3, Cleaved caspase-8, p53 and ITGA4 were investigated by Western blot analysis. The expression of Ki-67 and Cleaved caspase-3 was significantly increased in MSCs-CPAM cultured under Hypoxic conditions when compared to the MSCs-CPAM culture maintained under standard atmosphere parameters. On the other hand, Bcl-2 was down regulated in MSCs-CPAM maintained in hypoxic incubator in comparison with normoxic conditions. Cleaved caspase-8 and p53 were downregulated in MSCs-CPAM under hypoxic culture conditions when compared to the normoxic maintained culture. ITGA4 showed an increase expression in MSCs-lung and MSCs-CPAM under hypoxic conditions (**Figure 5**).

DISCUSSION

MSCs own the ability to differentiate into adipocytes, osteoblasts, chondrocytes and other tissues of mesodermal origin (Friedenstein et al., 1976; Colter et al., 2001) and possess specific immune regulatory properties (Krampera et al., 2003; Krampera et al., 2006). These properties make them interesting in cell therapy for the regeneration of damaged tissues of various origins, including epithelial tissues such as the lung. CPAM is a congenital disease characterized by cystic formations in the airways during fetal lung development and represents a model of study of congenital lung malformations which include the more complex form of pediatric interstitial lung disease. To date, surgery remains the only solution in symptomatic forms of CPAM (Leblanc et al., 2017). The regenerative process in the lung depends on a pool of lung MSCs which should allow lung regeneration with the maintenance of normal architecture during the regeneration phase (Pelizzo et al., 2017).

MSCs-based therapies represent a valid alternative for chronic lung diseases. However, it is known that MSCs, after transplantation, reduce their proliferative, survival, engraftment and paracrine properties. Several clinical studies have investigated cell therapy in chronic lung diseases (Kotton, 2012; Wecht and Rojas, 2016). The safety of MSCs therapy has only been demonstrated at an early stage and the relatively small number of recruited patients represents limitations yet to be overcome. The limited number of eligible lung donors and the potential risk of immunosuppression in patients associated with transplantation require further investigation (Weiss, 2014). Therefore, it is necessary to find strategies to improve the regenerative capacities of MSCs. The potential to enhance the benefits of MSCs has provided new opportunities that should be further explored. Indeed, the beneficial effects of hypoxic culture on proliferation and differentiation potentials of MSCs have been suggested as a strategy to improve MSCs' properties (Grayson

et al., 2007; Hung et al., 2012). As previously demonstrated (Pelizzo et al., 2017), results of the characterization of MSCs-lung and MSCs-CPAM demonstrated that the cells were adherent to the plastic and have the characteristic "spindle-shaped" morphology typical of MSCs. Moreover, hypoxic treatment did not induce morphological changes in MSCs demonstrating that it has no significant effects on the phenotype of MSCs, as already observed in other MSCs types (Valorani et al., 2012; Choi et al., 2014). Considering the involvement of hypoxia in increasing MSCs' properties, it was performed transcriptomic analysis focused on the comparison of MSCs-lung-Normoxia against MSCs-lung-Hypoxia and MSCs-CPAM-Normoxia against MSCs-CPAM-Hypoxia, in order to evaluate the impact of 24 h of hypoxia (0.2% O2) in MSCs-CPAM compared to MSCs-lung.

First, the effects of hypoxia on proliferation and cell cycle were evaluated. It is known that to ensure cell proliferation, the cell cycle must be controlled by a regulatory network and the transition from G1 to S phase must be highly controlled. In our analysis, data suggest the existence of O_2-dependent mechanisms that control cell proliferation. In the cell cycle, cyclins bind to cyclin-dependent kinases (CDK) in the G1 phase, then this will activate them and promote the phosphorylation of the retinoblastoma protein (Rb), with consequent release of E2F. E2F is a transcription factor that regulates the expression of proteins necessary for the transition from G1 to S (Bracken et al., 2004). Study results showed that the hypoxia conditions up-regulated *CCNE2* and *CDK2* genes in both MSCs-lung and MSCs-CPAM, *CCNA2* was up-regulated only in MSCs-lung and *CDK6* only in MSCs-CPAM. There are two classes of CDK inhibitory proteins (CDKI), p15, p16, p18, and p19, and the CDK protein/kinase inhibitory protein (CIP/KIP), composed of p21, p27, and p57 (Pavletich, 1999; Sherr and Roberts, 1999). Hypoxia reduced the expression of genes *CDKN1B*, *CDKN2A* and *CDKN2B* belonging to both classes in MSCs-CPAM; instead, it up-regulates two genes in the MSCs-lung *CDKN2C* and *CDKN2D*, and down-regulates two more *CDKN1A* and *CDKN2B*. In this way, it would appear that hypoxia facilitates the transition from G1 phase to the S phase better in MSCs-CPAM than in MSCs-lung. Indeed, *RB1*, the gene encoding Rb, was up-regulated by hypoxia only in the MSCs-CPAM comparison. The E2F1 gene, encoding the transcription factor E2F required for the transition from G1 to S were upregulated from hypoxia in both comparisons. However, the genes *TFDP1* and *TFDP2,* encoding the complex that controls the transcriptional activity of the numerous genes involved in the transition from G1 to S phase were upregulated by hypoxia only in the MSCs-lung comparison. As the G1/S transition is essential for cell cycle progression, these data demonstrate the maintenance of a high proliferative capacity in both MSCs-lung and MSCs-CPAM, as can be seen in **Figure 1**. In order to confirm cell proliferation, the Ki-67 Western blot investigation was performed, the results of which are presented in **Figure 5**. The cell proliferation antigen Ki-67 (Ki-67 or Ki67) is constitutively expressed in cells and is widely used as a marker of cell proliferation (Sobecki et al., 2016). Consistent with *in vitro* and RNA-seq investigations, Western blot analysis demonstrated that Ki-67 expression was significantly increased by hypoxia in both MSCs-CPAM and MSCs-lung (**Figure 5**), showing that hypoxia enhances the proliferation rate in MSCs. Moreover, it has been observed that cell cycle progression appears to be necessary for differentiation towards certain cell fates. Indeed, there appears to be a relationship between the cell cycle and cell differentiation (Jakoby and Schnittger, 2004). In particular, it has been observed that a cyclin of type D, proteins of the Rb family and CDK inhibitors, a subset of the phase transition proteins from G1 to S, are particularly involved in cell differentiation. Thus, some cell cycle proteins may regulate differentiation pathways, while simultaneously performing their cell cycle functions, as has been observed in terminal differentiation in the mammary epithelium (Caldon et al., 2010). In this context, recent evidence suggests that the tumor suppressor p53, in addition to the classical oncosuppressive activity, seems to have a role in the regulation of differentiation and development (Molchadsky et al., 2010). p53 regulates DNA repair, cell cycle, apoptosis, and senescence and cell proliferation (Liu et al., 2015b). Interestingly, in our analysis, the *TP53* gene that encodes for p53 was downregulated by hypoxia in both comparisons. Instead, the *CDKN1A* gene that encodes for p21, the main p53 target gene was downregulated only in MSCs-lung. Evidence of a significant decrease in p53 expression in both comparisons in hypoxic conditions was also confirmed at the protein level by Western analysis (**Figure 4**), suggesting that hypoxia ameliorates proliferation in both comparisons.

The results obtained show that hypoxia, in addition to stimulating the downstream genes to mediate the proliferation process, seems to deregulate the expression of the genes involved in the apoptosis process (**Figure 3B**). It is known that hypoxia preconditioning enhances the survival of MSCs, indeed it was observed that BMSCs in ischaemic tissues increased autophagy and decreased apoptosis, suggesting that hypoxia may result in a protective effect in MSCs. Hypoxia condition induces a similar effect also in BMSC survival *in vivo* (Liu et al., 2015a). Several findings demonstrated that 1–2% O_2 enhances the proliferation rate of human adipose-derived mesenchymal stem cells (Fotia et al., 2015; Wan Safwani et al., 2016). These evidences indicate that more deep investigations are necessary in order to improve stem cell function under stressed or pathological conditions. In this regard, our study results showed that hypoxia, in addition to reducing the pro-apoptotic *TP53* gene in both MSCs-lung and MSCs-CPAM, increases the levels of factors that negatively regulate the apoptosis processes, such as *SERPINE1* and *GTSE1,* in both comparisons. The overexpression of *SERPINE1* would appear to protect cells from apoptosis and increase cell migration while *GTSE1* acts as a negative p53 regulator (Monte et al., 2004; Pavón et al., 2015). In addition, in MSCs-lung are highlighted down-regulated genes *BBC3*, *BID* and *PMAIP1*, known for their pro-apoptotic activity. *BBC3* and *PMAIP1* belong to the pro-apoptotic class BH3 and are part of the apoptotic cascade mediated by p53, and *BID* is a mediator of mitochondrial damage induced by CASP8 and determines the release of cytochrome c (Westphal et al., 2011; Hikisz and Kiliańska, 2012). In compliance with the RNA-seq analysis, Western blot and statistical analyses also showed a significant

increase in the expression of cleaved-caspase 3 in MSCs-CPAM grown under hypoxic conditions more than in MSCs-CPAM maintained in standard atmospheric parameters. Contrarily, no significant difference was observed in the comparison of MSCs-lung under normoxic conditions compared to MSCs-lung under hypoxic conditions (**Figure 5**). This caspase is responsible for most of the proteolysis during apoptosis and the detection of cleaved caspase-3 is therefore considered a marker of apoptosis (Poreba et al., 2013). Therefore it would appear that hypoxia better protects MSCs-lungs from hypoxia than MSCs-CPAMs. Instead, in MSCs-CPAM, even though the *CASP3* gene was overexpressed by hypoxia, other apoptotic genes such as *CASP8*, *FAS* and *TNFRSF10B* were downregulated. *CASP8* encodes the Caspase 8 protein, a member of the cysteine-aspartic acid protease family, which is important in the execution phase of cellular apoptosis (Kruidering and Evan, 2000). In compliance with the transcriptomic analysis, also the Western analysis showed a significant reduction of the protein expression levels of cleaved-caspase-8 in MSCs-CPAM subjected to hypoxic conditions (**Figure 5**). *FAS* is a gene that codes for a member of the TNF receptor superfamily, involved in the regulation of programmed cell death. The interaction of this receptor induces the formation of a death-inducing signaling complex that includes Fas-associated death domain protein and caspase 8 (Waring and Mullbacher, 1999). *TNFRSF10B* encodes TNF Receptor Superfamily Member 10b, a receptor transduces an apoptosis signal (Zhao et al., 2014). Thus the downregulation of these pro-apoptotic genes and upregulation of those anti-apoptotic seems to protect more MSCs-lung than MSCs-CPAM from apoptosis. Further studies are needed to deepen the effect of hypoxia on the apoptotic process in MSCs-CPAM.

Stem cells constantly encounter hypoxic stress that hinders aerobic metabolism. Therefore, the upregulation of genes in the "Glycolysis/Gluconeogenesis" pathway under hypoxic compared to normoxic conditions, in both cell types, suggest that anaerobic metabolism was increased. In detail, *ALDOA* and *ALDOC* encode for two aldolase isoenzymes (A and C). They are two glycolytic enzymes that catalyze the reversible conversion of fructose-1,6-bisphosphate into glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. In this analysis, hypoxia upregulated both genes in MSCs-lung, while in MSCs-CPAM only the *ALDOC* gene was upregulated. Hypoxia also upregulated *ENO1*, *ENO2*, and *ENO3* in MSCs-CPAM, while in MSCs-lung it upregulates only *ENO1* and *ENO2*. In mammals, three genes encoding for three isoforms of the enzyme, α-enolase (ENOA), γ-enolase and β-enolase, respectively (Capello et al., 2011). In the second half of the glycolytic pathway, these enzymes promote the dehydration of 2-phospho-D-glycerate to phosphoenolpyruvate; conversely, in gluconeogenesis, these enzymes catalyze the hydration of phosphoenolpyruvate to 2-phospho-D-glycerate (Pancholi, 2001). *GP1*, *GAPDH* and *LDHA* are hypoxia-upregulated genes in both MSCs-lung and MSCs-CPAM. The *GP1* gene encodes the glucose-6-phosphate isomerase, responsible for the conversion of glucose-6-phosphate to fructose-6-phosphate, the second phase of glycolysis, and for the reverse reaction during gluconeogenesis (Kugler and Lakomek, 2000). Furthermore, in addition to its main role as a glycolytic enzyme, it can act as an angiogenic factor by promoting the motility of endothelial cells (Funasaka et al., 2001). *GAPDH* encodes glyceraldehyde-3-phosphate dehydrogenase, an enzyme important in glycolysis that promotes the conversion of D-glyceraldehyde 3-phosphate to 3-phospho-D-glyceroyl phosphate (Sirover, 2021). Furthermore, it has been observed that this enzyme also modulates the organization and assembly of the cytoskeleton (Schmitz and Bereiter-Hahn, 2002). *LDHA* encodes lactate dehydrogenase A, involved in the conversion of pyruvate into lactic acid during the last phase of glycolysis. Additionally, increased *LDHA* expression is required for glycolysis maintenance (Zheng et al., 2021). Therefore, the upregulation of the genes that code for glycolytic enzymes and the increase of the gene that codes for Lactate Dehydrogenase, make both MSCs-lung and MSCs-CPAM more dependent on anaerobic glycolysis for energy supply. These results would highlight the ability of hypoxia to facilitate the metabolic transition necessary to support the energy demands. Thus, the MSCs subjected to hypoxic challenges, increased genes encoding the glucose-6-phosphatase transporter, lactate dehydrogenase-A, glycolytic enzymes and glucose transporters to facilitate the glycolytic pathway. In this way, the modulation of glucose metabolism in MSCs, together with the down-regulation of anti-apoptotic factors represent a strategy to protect MSCs from apoptosis, improving their viability in hypoxic conditions (Hu et al., 2008).

Several studies have shown that MSCs in hypoxic conditions have a high regenerative potential. It has been seen that hypoxia increase cell proliferation, survival in damaged tissues after transplantation, and secretion of several bioactive factors (Noronha et al., 2019). Short-term hypoxic exposures induce functional changes in MSCs, such as alteration of glycolysis, reorganization of the cytoskeleton, and increased migratory activity (Zhidkova et al., 2021). In addition, mesenchymal-epithelial interaction plays an essential role in regeneration processes where it contributes to the maintenance of tissue homeostasis and its repair against damage (Demayo et al., 2002).

In this context, we inspected DEGs involved in epithelium development. Among all the pathways found in the two comparisons, "Regulation of actin cytoskeleton," "Focal adhesion," and "PI3K-Akt signaling pathway," were common pathways observed (**Figure 4**). This result is in compliance with the cell cycle activation given the involvement of the regulation of the cytoskeleton in cell proliferation (Bendris et al., 2015). The actin cytoskeleton is known to regulate cell adhesion and motility through its intricate participation in signal transduction and structural modifications. In both comparisons the hypoxic condition, upregulating many of the genes involved in this pathway, could reorganize the cytoskeleton thus influencing the processes necessary for the regulation of epithelia.

Among these genes, *RHOA* appears upregulated in MSCs-lung under hypoxic conditions compared to MSCs-lung in normoxic conditions. While *RAC1* appears upregulated in hypoxic conditions compared to normoxia in both MSCs-lung and MSC-CPAM, *CDC42* instead appears upregulated only in MSCs-lung in hypoxic conditions compared to MSCs-lung-Normoxia. These genes encode for members of the Rho family

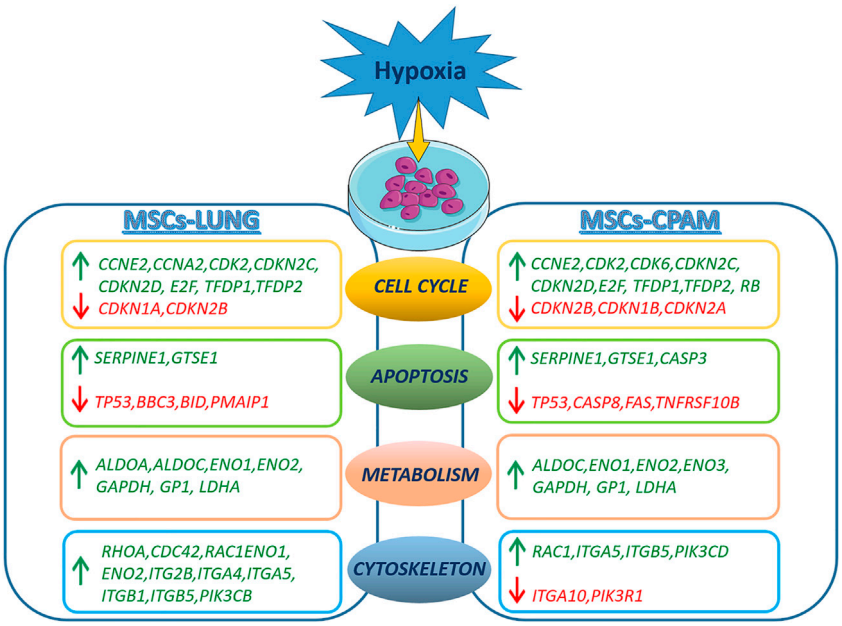

FIGURE 6 | Representation of DEGs observed in the MSCs-lung or MSCs-CPAM in hypoxia condition. The figure was made taking the images from Servier Medical Art (available at http://smart.servier.com/accessed on 20 January 2022), licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/accessed on 20 January 2022).

of small guanosine triphosphatases (GTPases), involved in the regulation of actin remodeling, adhesion site formation, and actomyosin contraction (Hall, 1998; Nobes and Hall, 1999). *RAC1* is involved in the formation of focal complexes and the formation of lamellipods through the polymerization of actin, while *CDC42* is mainly involved in cell polarity and phyllopod formation and transmits environmental signals to effector proteins, setting the orientation of the cell (Hanna and El-Sibai, 2013). The RHOA signaling cascade is believed to play an essential role in the migration of MSCs. Vertelov et al. found that in MSCs hypoxic conditions develop greater motility than normoxia in relation to *RHOA* activation hypoxia-induced, suggesting that elevated MSCs migration may occur through increased activation of *RHOA* (Vertelov et al., 2013). Therefore, hypoxia in MSCs-lung cells, through the upregulation of *RHOA* and *CDC42*, could increase the regulation of cell adhesion and migration processes.

RHOA activation occurs through a variety of factors such as growth factors, cytokines, adhesion molecules, integrins, G proteins and other biologically active substances (Loirand et al., 2006). During repair processes, components within the cellular matrix interact with integrins (Chen and Parks, 2009). Integrins are heterodimeric transmembrane glycoproteins receptors formed by non-covalently associated α and β subunits. It has been observed that hypoxic conditions, as well as promoting MSCs migration, self-renewal and delaying senescence, also induce an increase in subunits α1, α3, α5, α6, α11, αv, β1, and β3 (Saller et al., 2012). In our analysis, many integrin-coding genes were more up-regulated by hypoxia in MSCs-lung than in MSCs-CPAM. In detail, *ITGA5* and *ITGB5* are up-regulated in both comparisons, while *ITGA10* was up-regulated only in MSCs-CPAM. In MSCs-lung, instead, hypoxia upregulated *ITGA2B*, *ITGA4,* and *ITGB1*. In compliance with the RNA-seq analysis, Western blot and statistical analyzes showed a significant increase in the expression of ITGA4 in MSCs-lung. However, evidence of a significant enhancement in ITGA4 expression was also observed in MSCs-CPAM under hypoxia conditions compared to MSCs-CPAM in normoxic conditions, suggesting that hypoxia could improve MSCs properties. Indeed, it is known that the expression of ITGA4 in MSC mediates cellular adhesion and cell migration (Ocansey et al., 2020). In particular, the formation of heterodimers consisting of ITGB1 and ITGA4 increases the homing properties of MSCs (Kwon et al., 2018). Therefore, overexpression of integrins, especially in MSCs-lung, could promote the epithelial repair process.

Phosphatidylinositol 3-kinases (PI3Ks) are the well-known regulators of cell motility. In this study, hypoxia has induced the deregulation of the genes encoding for PI3Ks, in particular in MSCs-lung *PIK3CB* was up-regulated, while in MSCs-CPAM *PIK3CD* was up-regulated. However, in MSCs-CPAM the upregulation of this gene was counteracted by the downregulation of the *PIK3R1* gene. The PI3Ks are involved in cell proliferation, cell transformation, paracrine function and angiogenesis (Zhang et al., 2014). It is known that the PI3K/AKT pathway plays an important role in cell proliferation induced by hypoxia (Sheng et al., 2017). In compliance with this finding, hypoxia upregulated PI3K in MSCs-lung more than in MSCs-CPAM.

In conclusion, hypoxia treatment results in cell cycle activation with increased proliferative capacity and increased cell anaerobic

metabolism in both MSCs-lung and MSCs-CPAM. The analysis suggests a reduction in the apoptotic process in both comparisons, although two pro-apoptotic genes were observed up-regulated in MSCs-CPAM. Finally, data obtained indicate that hypoxia leads to a greater expression of more factors involved in cell motility, proliferation and cell migration in MSCs-lung than MSCs-CPAM (**Figure 6**). Therefore, the study data highlights that MSCs-CPAMs like MSCs-lungs can be isolated and expanded *in vitro* and exhibit morphology, phenotype and characteristics typical of MSCs. Noteworthy, these results suggest that exposing MSCs to hypoxia could be considered an innovative approach to lung repair and regeneration during disease progression and/or as post-surgical lung support after lung resection and encourages future studies to evaluate the importance of lung-derived MSCs in normal and pathological conditions.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA800623, https://www.ncbi.nlm.nih.gov/, PRJNA752960.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of the "G. Di Cristina "(registration number 87 Civico 2017). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

SS, FD and VZ wrote the manuscript. LC performed the computational and statistical analysis. GM, JP and FD performed cellular experiment. MA isolated MSCs. AV performed the library preparation. VC, GP and EM designed the study and revised the manuscript.

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REVIEW
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Chimeric Antigen Receptor T-Cells: An Overview of Concepts, Applications, Limitations, and Proposed Solutions

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Adaptive immunity, orchestrated by B-cells and T-cells, plays a crucial role in protecting the body from pathogenic invaders and can be used as tools to enhance the body's defense mechanisms against cancer by genetically engineering these immune cells. Several strategies have been identified for cancer treatment and evaluated for their efficacy against other diseases such as autoimmune and infectious diseases. One of the most advanced technologies is chimeric antigen receptor (CAR) T-cell therapy, a pioneering therapy in the oncology field. Successful clinical trials have resulted in the approval of six CAR-T cell products by the Food and Drug Administration for the treatment of hematological malignancies. However, there have been various obstacles that limit the use of CAR T-cell therapy as the first line of defense mechanism against cancer. Various innovative CAR-T cell therapeutic designs have been evaluated in preclinical and clinical trial settings and have demonstrated much potential for development. Such trials testing the suitability of CARs against solid tumors and HIV are showing promising results. In addition, new solutions have been proposed to overcome the limitations of this therapy. This review provides an overview of the current knowledge regarding this novel technology, including CAR T-cell structure, different applications, limitations, and proposed solutions.

Keywords: chimeric antigen receptor T-cell, adaptive immunity, autoimmune disorder, cancer immunotherapy, solid tumor, tumor infiltration

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1 INTRODUCTION

The global cancer burden, cancer incidence, and mortality estimations have increased rapidly. According to the International Agency for Research on Cancer, 19.3 million diagnosed cases and 10.0 million deaths worldwide in 2020 have been attributed to cancer (Sung et al., 2021). The relationship between cancer and the immune system was shown by Rudolf Virchow more than 150 years ago (Adams et al., 2015). Interest in immune system activation as a therapeutic approach for treating cancer began in the late 19th century when William Coley injected heat-inactivated bacteria into the tumor mass, resulting in its size reduction. Although the failure to achieve desirable clinical outcomes with early immunotherapies such as interferon-gamma (IFN-γ) and interleukin (IL)-2 treatments, novel immunotherapies launched in the 21st century have achieved robust clinical results,

establishing cancer immunotherapy as one of the foremost anchors of anticancer therapies (Lesterhuis et al., 2011; Jiang T. et al., 2016; Castro et al., 2018).

The effective eradication of cancer cells via the immune system involves several steps known as the cancer-immunity cycle, defined as a series of steps involving increased antitumor T-cell responses that are initiated upon recognition of the tumor-associated antigens (TAAs) captured from dying tumor cells by antigen-presenting cells (APCs) such as dendritic cells (DCs). Upon capturing TAA's, DCs get activated, express CCR7, mature, and 1) migrate to draining lymph nodes, 2) present the captured antigens to naïve $CD4^+$ and $CD8^+$ T-cells via the major histocompatibility complex (MHC) class I and II molecules, 3) express T-cell costimulatory molecules, for example, CD40, CD80, and CD86, 4) secrete critical cytokines to regulate T-cell responses, 5) activate naïve $CD8^+$ T-cells converting them into cytotoxic T-cells, which immigrate from lymphoid organs into the bloodstream and reach tissues and ultimately infiltrate the tumor. Activated cytotoxic T cells recognize the specific TAA (presented to them by DC's) found on MHC class I (MHC-I) molecules of tumor cells and kill the tumor cells via secreting perforins and granzymes that result in the release of additional TAAs, which trigger the initiation of another cycle of cancer immunity (Chen and Mellman, 2013).

Cancer eradication through cytotoxic immune responses is evident; however, cancers can grow progressively, suggesting their ability to mask and not be recognized by the immune system as seen in carcinogen-induced mouse models. This mechanism prompted Schreiber and others to hypothesize the immunoediting concept to explain the progressive growth of otherwise immunogenic cancers (Shankaran et al., 2001; Dunn et al., 2004; Schreiber et al., 2011; Matsushita et al., 2012). The immunoediting process of human cancers can be related to neoepitope presentation. Non-silent point mutations that lead to antigenic neoepitopes (T-cell recognition) are lost more frequently in cancers than in silent point mutations, thus preventing T-cells from recognizing and identifying cancer cells (Rooney et al., 2015). This concept suggests that the ability of cancers to progress and grow could be impaired by loss of immunogenicity; however, this perception alone contradicts another evidence that T-cells are adequately activated to enhance their cancer recognition by the administration of immune-activating cytokines or immune checkpoints releases such as programmed cell death-1 (PD-1) or cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) that leads to robust tumor responses in patients and mice (Chambers et al., 2001; Pardoll, 2012). T cells are central infiltrates of the heterogeneous tumor microenvironment (TME), and their population consists of naïve, effector, memory, and regulatory T cells (Hashimoto et al., 2018). The antigen stimulation of T cell receptors (TCRs) initiates an intrinsic program that guides the differentiation of T cells into cytotoxic effectors capable of eradicating the antigen; however, these cells start dying gradually except for a small number of surviving memory T cells that provide long-term protection against the antigen (Chang et al., 2014). Chronic exposure of T cells to the same antigen leads to remarkable alterations, thus affecting their activation and differentiation and eventually causing T-cell exhaustion (Wherry, 2011; Schietinger and Greenberg, 2014). T effector cell exhaustion is highlighted by the loss of effector functions such as proliferation, cytotoxicity, metabolic and transcriptional molecule alterations, and immune checkpoint upregulation (Guo et al., 2018; Li H. et al., 2019). Different factors have been identified that play several roles in T-cell exhaustion; the intrinsic factors relate to transcription, epigenetic, and metabolic factors, whereas the extrinsic factors include extracellular and cytokine interactions that create the TME and the immunosuppressive network (Maimela et al., 2019; Zhang et al., 2020). Therefore, the use of engineered T-cells targeting specific cell-surface antigens is considered a great approach to ensure specificity and overcome the shortcomings of other available immunotherapies.

In this review, we present a comprehensive prospect of the developmental and experimental progress in the field of chimeric antigen receptor (CAR) T-cell therapy while relating to some aspects of adaptive immunity as the rationale behind the evolution of this cutting-edge technology. The significance of this review is the broad inclusiveness of current therapeutic applications of CAR T-cells in hematological malignancies, solid tumors, and human immunodeficiency virus (HIV) infection while focusing on some recently published results of pre-clinical and clinical trials, pointing out some drawbacks, and suggesting some modifications.

2 ADOPTIVE IMMUNE THERAPY

Cancer immune therapy, which exploits the body's immune system to combat cancer cells, can be classified into three categories: adoptive cell therapies (ACTs), tumor vaccines, and immune checkpoint inhibitors (ICIs). These therapies have proven beneficial in patients with advanced tumors, and some have reached complete remission (Li D. et al., 2019). ACT is mainly based on the concept that the immune system can control a patient's cancer in the long-term and has been demonstrated by three independent approaches. The first approach involved tumor-infiltrating lymphocytes (TILs), which can be isolated from tumor lesions (e.g., melanoma) and expanded *in vitro*, followed by patient re-infusion, resulting in tumor regression and remission in a considerable number of patients. However, the downsides of the TILs approach included access limitations to the removable metastases or tumors, time-consuming preparation of T cells, and tumor-reactive T-cell clones were rarely found, which hindered the success of this strategy. The second approach involved T-cell receptor (TCR) engineering, where TCRs identified from TILs were virally transduced into peripheral blood T-cells, making them capable of inducing tumor regressions upon re-infusion into the patient. Unfortunately, this method was explicitly restricted because of its dependency on identifying MHC peptides expressed by tumors via their MHC complexes (Dudley et al., 2002; Zacharakis et al., 2018; Benmebarek et al., 2019). The third ACT approach is CAR-engineered T cells and is marked as the beginning of a new era in cancer therapy by providing a transformative approach to tumor

exclusion and gained attention over the other two as it offered a series of innovative modifications (Kershaw et al., 2006; Lamers et al., 2011; Mikkilineni and Kochenderfer, 2017). CARs are synthetic receptors that have the specificity of a monoclonal antibody and a signaling domain capable of inducing a cascade of events in the CAR-engineered immune cells (e.g., T-lymphocytes) upon target engagement. Engineering immune cells to express CARs is achieved by transferring protein-coding sequences using viral vectors (e.g., Lentiviral or Retroviral). CAR T-cells display immunological characteristics similar to activated T cells such as generating an immune response against target cells and expanding within the patient ensuring long-term protection (Porter et al., 2011; Grupp et al., 2013; Heiblig et al., 2015).

3 EVOLUTION OF CAR-T CELLS

Conventional T cells can distinguish between foreign peptide-MHCs (pMHCs) and the body's pMHCs via their TCRs, which can trigger a small number of agonist pMHCs compared with thousands self-pMHCs (Sykulev et al., 1996; Irvine et al., 2002; Huang et al., 2013). Genetic insertion of CARs, in immune cells, particularly T-cells, redirects them to target a preferred antigen (Jackson et al., 2016). CARs are bioengineered receptors which specifically target a desired antigen; almost 30 years ago, the first CARs were generated and undergone multiple modifications since they contributed to their development and evolution (Kobold et al., 2015; Lim and June, 2017). The flexibility of CARs arises from their ability to recognize antigens in the absence of MHC presentation, which is the opposite of innate TCRs (Lim and June, 2017). Additionally, CARs have advanced properties compared with conventional T-cells, as they combine the antigen-binding ability of monoclonal antibodies with T-cell self-renewal and lytic capacity (Ramos and Dotti, 2011; Curran et al., 2012; Maher, 2012). Also, TCRs can recognize short peptide sequences, whereas CAR T-cells can recognize several tumor antigens in different forms, such as proteins, glycolipids, and carbohydrates (Abbott et al., 2020). CAR T-cell recognition and destruction of tumor cells occur in an independent-manner of MHCs; this promotes enhanced cell recognition undisturbed by the tumor's ability to avoid MHC-restricted recognition of T-cells, such as the tumor's ability to encourage defective antigen processing by downregulating human leukocyte antigen (HLA) class I molecules (Dotti et al., 2014). It is considered an advantage where MHC expression is suppressed or lost due to the immunosuppressive cancer microenvironment (Garrido et al., 2016). CARs have been proven effective in treating cancers, especially hematological tumors. The specificity of CARs in targeting cancers makes them an appealing alternative to standard cancer treatments such as chemotherapy and radiation (Sadelain et al., 2013). CARs consist of three major domains: 1) extracellular domain (ectodomain), which can be further divided into an antigen-recognition domain, a single peptide on the cell surface cleaved from the mature CAR cell (Goulart et al., 2017). The antigen-recognition domain is a single-chain fragment variant (scFV) chiefly comprising of heavy and variable light chain regions composed of an antigen-specific immunoglobin separated by a flexible linker and attached to the transmembrane domain by a spacer (hinge) responsible for the transmission of receptor-binding signals (Zhang et al., 2017). 2) transmembrane domain is essential for receptor stability and surface expression; it is a hydrophobic alpha helix that extends in the cell membrane (Ramos and Dotti, 2011; Zhang et al., 2017). 3) intracellular domain (endo-domain), which upon stimulation, clusters and undergoes conformational changes, thus enabling the recruitment and phosphorylation of downstream signaling proteins (Cantrell, 2002; Su and Vale, 2018). The intracellular domain classifies CARs into five generations: first has a single activation domain, a cytoplasmic domain mostly CD3 zeta (CD3ζ), and some studies used the gamma chain (γ) of the Fc receptors, the second generation has CD3ζ plus one costimulatory domain, obtained from costimulatory molecules such as 4-1BB or CD28 connected to an activator domain (CD3ζ/γ chain of Fc receptor) to enhance both cell proliferative and cytotoxic competences of CAR T cells (Finney et al., 1998; Hombach et al., 2001; Acuto and Michel, 2003). The third generation is similar to the second generation but has multiple costimulatory domains with CD3ζ, such as 4-1BB and CD28, CD134, and CD137 (Sadelain et al., 2013; Zhang et al., 2017; Guedan et al., 2019). The fourth generation CARs, known as T cells redirected for universal cytokine-mediated killing (TRUCKs), were engineered to release transgenic cytokine-like interleukin 12 (IL-12) upon CAR signaling in the tumor tissue to overcome TME immunosuppression and endorse robust therapeutic outcomes (Chmielewski et al., 2014; Chmielewski and Abken, 2015, 2020). IL-12 is responsible for the induction of IFN-γ, perforin, and granzymes in T-cells, and inhibits Treg proliferation (Kubin et al., 1994; Cao et al., 2009). Other cytokines studied in the fourth generation are IL-15 and IL-18 (Hurton et al., 2016). IL-15 belongs to the γ-chain family and holds important properties for T cell expansion and survival (Klebanoff et al., 2004). Additionally, IL-18 CAR T-cells treatment of large pancreatic and lung tumors exhibited changes in the immune cell landscape related to the tumor; a significant increase in the macrophages (CD206– M1) and NKs (NKG2D+) was observed besides a decrease in Tregs such as M2 macrophages suppressive CD103+ DCs, suggesting the ability of "IL-18 TRUCKs" to sensitize large tumor lesions for efficient immune destruction (Chmielewski and Abken, 2017).The fifth generation of CARs is currently being explored; it is mainly designed based on the second generation. However, it contains a truncated cytoplasmic receptor (IL-12) and a β-chain domain (IL-2Rβ truncated intracellular interleukin 2β chain receptor) along with the transcription factor STAT3/5 binding motif (Tokarew et al., 2019) (**Figure 1**).

The structure and design of CARs contribute to their signaling mechanisms, effector functions, efficacy, and toxicity. The ligand recognition and signaling of CARs are affected by both the single-chain variable fragment (scFv) and cytoplasmic domains; however, the transmembrane and spacer domains (non-signaling) affect the function of CARs (Jayaraman et al., 2020). Generally, CAR T-cells can specifically recognize cancer cells and lyse them (Maggs et al., 2021).

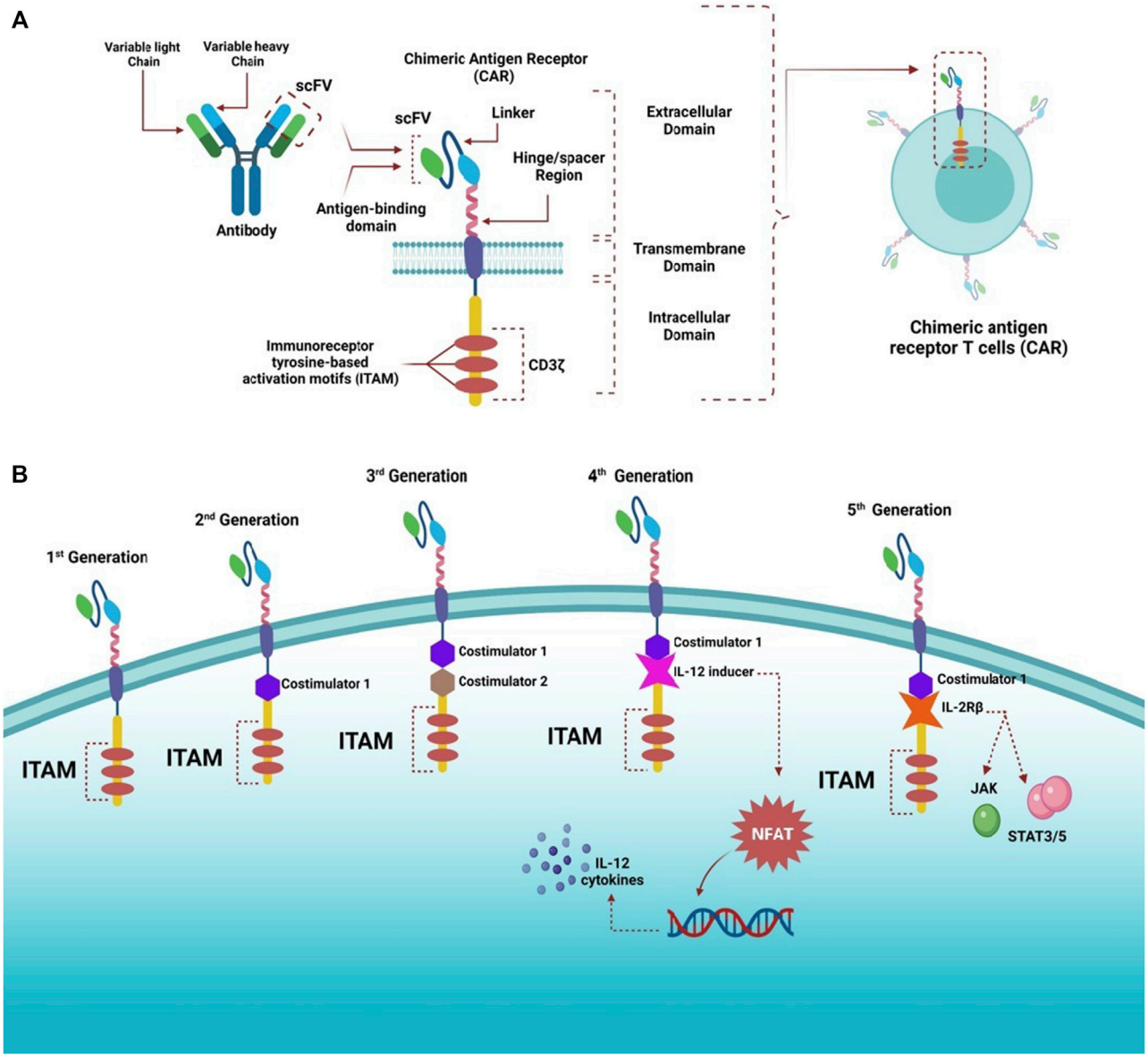

FIGURE 1 | Structure of CARs and different generations. **(A)** Highlights the general structure of CARs; they have an extracellular domain containing scFV derived from antibody variable heavy and light chains, linker, and a hinge/spacer region. All the extracellular structures provide flexibility and improve the binding affinity of the antigen. A transmembrane domain helps anchor molecules to the T cells, and an intracellular domain containing ITAM motifs, responsible for transmitting activating and costimulatory signals to T cells, is also present. **(B)** CARs have witnessed rapid advancement since the first generation, which contained only ITAM (CD3ζ) motifs as the T cell stimulatory molecule within the intracellular domain. The second generation had one costimulatory molecule, whereas the third generation had two costimulatory molecules to improve cytotoxicity and robustness of CAR-T cells. The fourth generation was designed based on the second generation but was paired with cytokine expressors (e.g., IL-12) under the control of NFAT transcription factor; therefore, this generation is referred to as T cell redirected for universal cytokine-mediated killing (TRUCKs). The fifth generation was also based on the second generation with additional intracellular domains of cytokine receptors (e.g., IL-2Rβ) to activate JAK and STAT3/5, stimulate cell proliferation, and enhance its persistence.

4 CLINICAL PREPARATION OF CAR-T CELLS

Despite various designs and tumor-specific scFVs, the manufacturing process of CAR-T cells remains constant (Wang and Rivière, 2016). In general, the personalized clinical production of CAR-T cells encompasses several steps followed by quality control testing through the entire process (Levine, 2015). The first step is collecting leukocytes from the patient (autologous) or the donor (allogeneic) from the peripheral blood via leukapheresis, in which only the leukocytes are extracted, and the rest of the blood products are returned to circulation (Brown and Adusumilli, 2016; Zhang et al., 2017). Second, T cells are augmented, separated, and washed with

leukapheresis buffer (Zhang et al., 2017; Gomes-Silva and Ramos, 2018). Third, at the CD4/CD8 composition level, the T-cell subsets are separated using specific antibody-coated bead conjugates or markers. The isolated cells are then cultured and activated by purified allogeneic or autologous APCs or by introducing beads coated with anti-CD3 or anti-CD28 monoclonal antibodies (or both along with feeder cells and interleukins) (Guedan et al., 2019). IL-2 is the most common growth factor used to induce the rapid growth of T cells (Wang and Rivière, 2016; Guedan et al., 2019). Recently, a study reported that a cytokine cocktail of IL-2, IL-7, and IL-15 induced better expansion of CD4 and CD8 CAR-T cells (Coppola et al., 2020).

Fourth, different methods have been considered to enable nucleic acid delivery to the obtained T cells. Usually, a foreign gene material (RNA or DNA) delivery into human cells can be accomplished using viral or non-viral vectors. Viral vectors are preferable for basic and clinical research because viruses have diverse expression characteristics, spend a fraction of time to reach clinically desired numbers of cultured T cells, and possess high transfer competency (Zhang et al., 2017; Gomes-Silva and Ramos, 2018). Viral vectors are used to encode CARs; with their reverse transcription potential, vectors convert RNA into permanently integrated DNA in the genome of the obtained T cells. These viral vectors include retroviruses, lentivirus, adenovirus, and adeno-associated virus. The most popular ones are genetically engineered retroviruses, more frequently used than gamma retroviral vectors. During the activation period, viral vectors are washed out of the culture by dilution and medium exchange (McGarrity et al., 2013; Zhang et al., 2017).

However, viral vectors present a possible safety hazard. The limitations of the viral vectors include tumorigenesis and toxicity caused by the insertion mutation used to generate immune reactions, and the limited carrier capacity and achieved titers are not sufficient (Wang et al., 2008). Therefore, to overcome the shortcomings of viral vectors, other methods such as mRNA transfection and non-viral vectors were used in the production of CAR-T cells. The most common were transposon-based non-viral vectors, facilitating safe and consistent DNA transfer into CAR T-cells. The sleeping beauty (SB) transposon system is the currently used substitute for viral-based vectors. It has been used to prepare $CD19^+$ CAR T-cells with antitumor properties *in vivo* and *in vitro* (Singh et al., 2015; Chicaybam et al., 2019). In 2014, an optimized protocol (GMP-compliant) was suggested to utilize the production of modified CAR T-cells by electroporation with CAR-encoding RNA, which helps in overcoming several drawbacks of classic viral transfection such as viral contamination, low time-efficiency, higher resource consumption, and off-target effects (Krug et al., 2014). In 2019, the optimized protocol was used in producing genetically modified CAR T-cells against melanomas; the CAR T-cells were electroporated and expanded with mRNA that encoded CAR targeting CSPG4, a surface protein highly expressed in most melanomas. The results showed that a high dosage of modified CAR T-cells could lyse 80% of melanoma cells after 20 h; the authors suggested a future expansion of their study to a full clinical trial (Wiesinger et al., 2019).

This approach has several advantages, such as improved integration of the transduced genetic material due to its low promoter activity (Yant et al., 2000), fewer epigenomic changes at the integration site, and reasonably low manufacturing costs (Izsvák et al., 2010). The only limitation in this approach is the low rate of transgenic material; however, it was considerably enhanced (Geurts et al., 2003). Nevertheless, the concerns remain; for instance, transient mRNA transfection requires several rounds of infusion, the possibility of mutagenesis, and SB transposon remobilization (Beatty et al., 2014). The fifth step is CAR-T cell expansion using bioreactors, which help cells divide and express CARs on the cell surface (Harrison et al., 2019). Finally, when the cells reach the clinically required volume, they are reinfused into the patient as a therapeutic agent. The infusion occurs 48–96 h after lymphodepletion chemotherapy to make room for the infused CAR-T cells (Turtle et al., 2016). The patient is then kept under observation for possible adverse effects within the first few days of infusion. The process lasts around 3 weeks, where cell preparation is the most time-consuming phase of treatment (Zhao and Cao, 2019) (**Figure 2**).

Interestingly, lymphodepletion chemotherapy is a crucial step before CAR T-cells infusion as it reduces endogenous lymphocyte numbers, thus increasing hemostatic cytokine availability promoting infused cells survival (Liang et al., 2020). Administration of T-cells to lymphodepleted patients has shown superior anti-tumor properties compared to lymphoreplete patients (Bechman and Maher, 2021). There have been several benefits to the lymphodepletion regimens, such as the non-myeloablative chemotherapeutic approach; this regimen results in the removal of endogenous lymphocytes that act as "cytokines sinks," which facilitate the accessibility of the infused T-cells to hemostatic cytokines like IL-15, IL-7, and IL-2, which stimulate JAK-STAT-mediated expansion (Gattinoni et al., 2005; Neelapu, 2019). In a lymphodepleted host, the memory cells proliferate in an antigen-dependent manner, unlike naïve T cells homeostatic expansion, which occurs in an MHC-dependent manner (Gattinoni et al., 2005; Klebanoff et al., 2005). It has been reported that lymphodepletion decreases immunosuppressive cells, such as myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs), while enhancing the APC cells' functionality and availability (Bechman and Maher, 2021). Immunosuppressive networks are negatively affected by lymphodepleting agents such as tryptophan metabolizing enzyme and indoleamine dioxygenase (IDO) (Hanafi et al., 2014; Ninomiya et al., 2015). Lymphodepletion also exerts certain positive effects on the microbiome. It enhances the translocation of microbes from the gastrointestinal tract, which lead to immunostimulatory impacts through Toll-like receptor ligation, resulting in an augmented release of IL-1β (Viaud et al., 2013; Lee et al., 2019). According to imaging studies, post lymphodepletion, the tumor-trafficking properties of adoptively infused cytotoxic T-cells were enhanced (Pittet et al., 2007). In a clinical trial (NCT03939026), which evaluates the safety and efficacy of certain lymphodepletion regimens, the phase I results suggest that fludarabine as a component in the lymphodepletion regimen is critical and

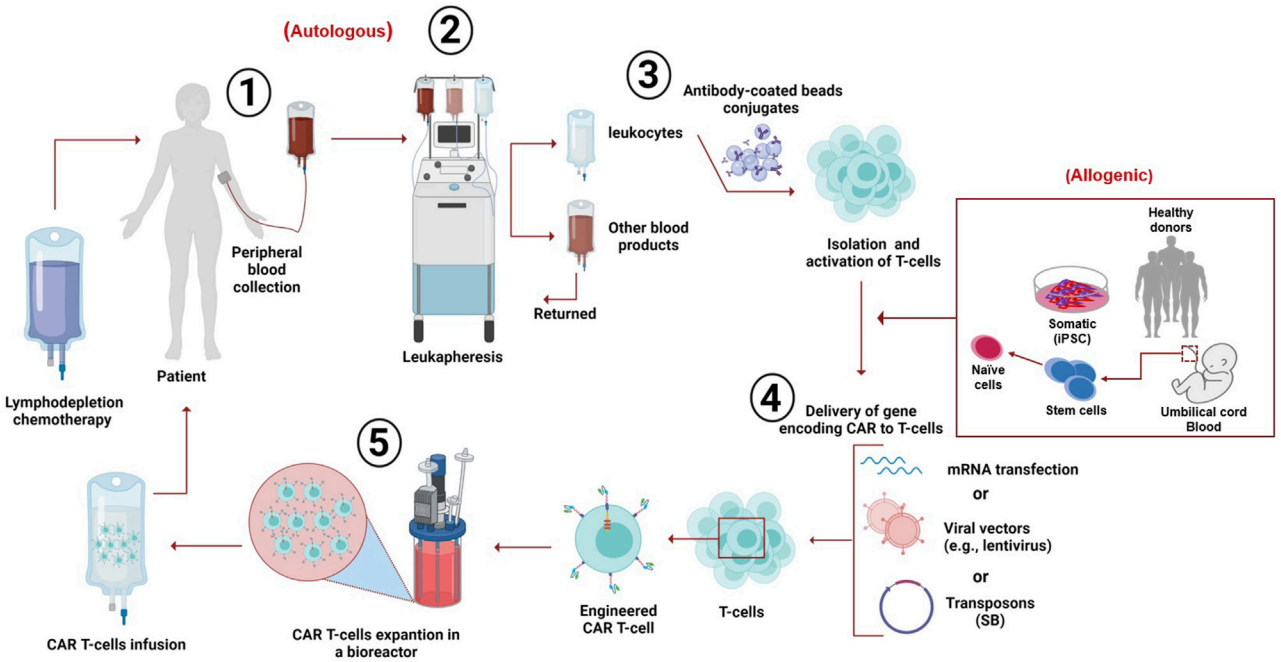

FIGURE 2 | Clinical production of CAR T-cells. The peripheral blood is withdrawn from the patient (autologous) or it can be obtained from the peripheral blood of a healthy donor (mononuclear cells), induced pluripotent stem cells (iPSC), or umbilical cord blood (allogeneic). The targeted T-cells are obtained by leukapheresis. Then, the T cells are separated and purified from other leukocytes using anti-CD3/CD28-coated beads; this process is followed by activation of the cells. Then, the genetic material encoding chimeric receptors is introduced into the T-cells via several known methods (such as mRNA transfection), viral vectors (e.g., lentivirus), or sleeping beauty (SB) transposons. The engineered T-cells expressing CARs are then expanded in a bioreactor. The patient receives chemotherapy for decreasing white cells blood count; after 48–96 h, the CAR T-cells are reinfused into the patient, followed by close monitoring for a few days to observe any adverse effects.

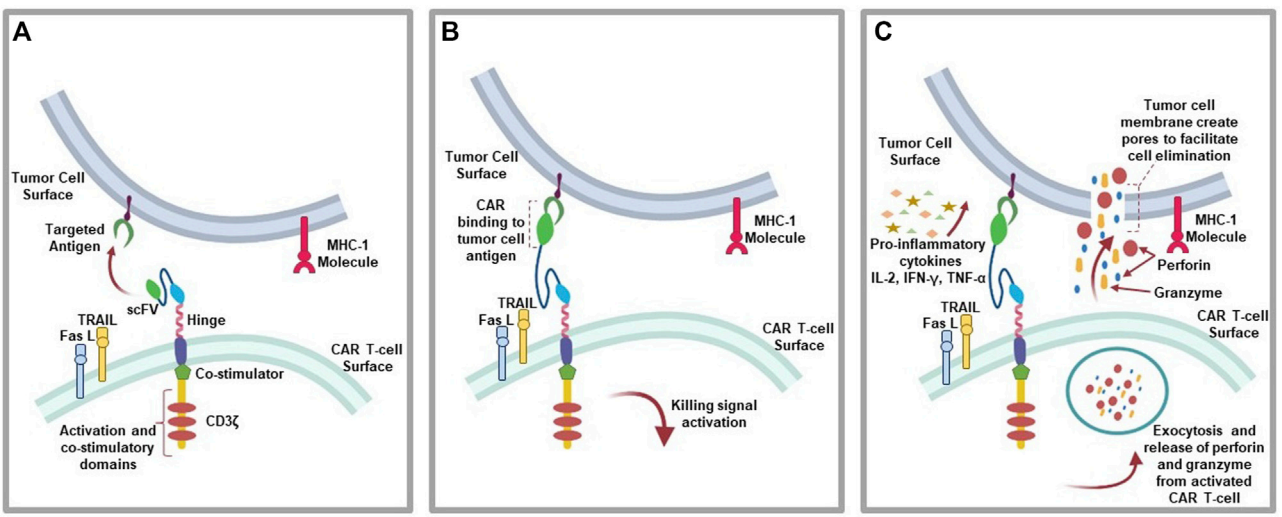

FIGURE 3 | CAR T-cell action: **(A)** CAR T-cells recognition of targeted antigen. **(B)** Chimeric antigen receptor binding to tumor-antigen. **(C)** Initiation of the antitumor (cytolytic) effects where the activated T-cells downstream the killing signaling by secreting granzymes and perforins, pro-inflammatory cytokines due to immune cell invasion, as well as initiating the expression of TRAIL and FasL pathways.

contributes to the efficacy of the procedure. Moreover, using a combination of fludarabine and cyclophosphamide (Flu/Cy) regimen is beneficial in multiple tumors; however, this combination is required for optimization in certain types of cancer and attenuation of the exerted toxicities of these agents. Although the benefits of lymphodepletion are undeniable, there

have been certain limitations, such as the short-lived span of lymphodepletion and the consequent immune restoration phase accompanied by a compensatory overshoot of both MDSCs and Tregs as indicated by preclinical and clinical studies (Bechman and Maher, 2021).

The mechanism of action of CAR T-cell involve the binding of CARs to a targeted antigen present on tumor cell surface via scFV recognition domain, which elicit anti-tumoral effects through the secretion of inflammatory cytokines (e.g., IL-2, IFN-γ, and TNF-α), cytolytic effector function via perforin and granzyme (Benmebarek et al., 2019), TNF-related apoptosis-inducing ligand (TRAIL), which binds to death receptors (e.g., DR4 and DR5) on tumor cells cell surface to activate graft-versus tumor effect (donor T-cells) (Watanabe et al., 2021). Also, tumor cell apoptosis can be initiated via the activation of caspase 8 and the formation of death-inducing signaling complex (DISC) leading to cell death mediated by mature caspase 3 subsequent cleavage of over 500 cellular substrate as a result of Fas and Fas ligand (FasL) pathway activation (Waring and Müllbacher, 1999; Nagata and Tanaka, 2017) (**Figure 3**).

5 CLINICAL APPLICATIONS OF CAR-T CELLS

5.1 Hematological Malignancies

5.1.1 Acute Lymphoblastic Leukemia

CAR-T cells are primarily used in hematological malignancies such as Acute lymphoblastic leukemia (ALL), characterized by a rapid proliferation of naïve cells in the bone marrow. CAR-T cells showed efficacy in treating ALL, especially the engineered T cells against CD19, as CD19 is a highly expressed biomarker of the B-cell lineage, responsible for B-cell malignancy of ALL. CD19 is a transmembrane glycoprotein involved in B-cell activation and is expressed throughout the developmental stages of B cells (Wang et al., 2012). Another potential target in B-ALL is the light chain of immunoglobulin CD20 (Gill et al., 2016; Jain and O'Brien, 2016). Conversely, T-cell malignancy of ALL (T-ALL) showed limited efficacy when the engineered CAR T-cells targeted CD19; therefore, another target (anti-CD5) showed effective elimination of a specific T-cell line expressing CD5 (Mamonkin et al., 2015). Anti-CD4 CAR-T cells are potential targets showing promising results against T-cell lymphoma (CD4 positive) models *in vivo* and *in vitro* (Pinz et al., 2016). Clinical trials evaluating multitargeted CAR-T cell therapy against ALL, such as single engineered CAR-T cells targeting both CD19 and CD22 and a combination of CAR-T cells with anti-CD19 and anti-CD20 to target each antigen independently, have yielded encouraging results (Huang et al., 2018). KYMRIAH ™ (tisagenlecleucel), which is a second generation CAR-T cell product (4-1BB costimulatory domain) directed against CD19 antigen, was approved by the Food and Drug Administration (FDA) for ALL in 2017 based on multicenter clinical studies, which established an overall remission rate of 81% in children and young adults with relapsed/refractory acute B-cell acute lymphoblastic leukemia (r/r B-cell ALL), and a best overall response rate of 52% in adults with relapsed/refractory diffuse large B-cell lymphoma (r/r DLBCL) (Thudium Mueller et al., 2021).

5.1.2 Acute Myeloid Leukemia

Acute myeloid leukemia (AML) results from genetic alterations in precursor cells that affect the growth and differentiation of hematopoietic cells, resulting in the accumulation of immature myeloid cells in the bone marrow and peripheral blood. These cells are incapable of turning into mature hematopoietic cells. CAR-T cell therapy in AML did not show the same success as seen in ALL, and the target of CAR T-cells in AML was CD123 and CD33; the latter was used in treating a patient and showed a significant reduction in tumor volume in the bone marrow; however, 9 weeks post-infusion, the patient experienced a relapse (Wang et al., 2015). Furthermore, the use of anti-CD123 CAR-T cells as a potential treatment of AML showed inadequate potency in "on-target-off-tumor" since CD123 is also expressed in normal tissues (e.g., endothelial tissue) and monocytes in relatively low levels compared with AML (Tettamanti et al., 2014). Therefore, other antigens have been investigated as new targets, including Lewis-Y (LeY) and CLEC12A antigens, and anti-LeY CART-cells were used in patients who eventually developed disease progression.

In contrast, the CD33was used as anti-CLEC12A-CD33 CAR T-cells, showed complete remission in a 44-year-old female patient with refractory AML (Ritchie et al., 2013; Morsink et al., 2019). In AML the application of CAR T cell therapy is limited by the absence of an AML-specific antigen. AML cells can express several cell surface antigens such as CD34, CD33, CD123, and many more. moreover, these antigens are also expressed by healthy Hematopoietic stem and progenitor cells (HSPCs) and their lymphoid and myeloid progenitors (Cummins and Gill, 2019). However, CAR-T cells are unable to distinguish between malignant and normal cells, unlike CD19 CAR T-cells, their complete elimination of the normal and malignant B cells resulting in B cell aplasia is considered clinically benign and manageable by intravenous immunoglobulin infusion; however, this is not the same in targeting myeloid antigens shared with normal myeloid progenitor as their elimination could be fatal due to bleeding complications and neutropenic infections (Mardiana and Gill, 2020). The aggressiveness of this disease and its ability to develop resistance against treatments requires substantial efforts to achieve remission. CAR T-cell therapy is a promising technology; however, the lack of leukemia-specific cell surface antigens could present a problem in designing CAR T-cells against AML (Mardiana and Gill, 2020). HSPCs frequently share antigens with AML. CAR T-cells expansion is threatened by AML blasts or prior exposure to chemotherapy that damages T cells. In addition, the AML's ability to evade the immune system by inducing various immunosuppressive mechanisms makes it challenging to achieve desired outcomes (Mardiana and Gill, 2020).

5.1.3 Chronic Lymphocytic Leukemia

Chronic lymphocytic leukemia (CLL) results in excessive mature lymphocytes in the blood, bone marrow, and lymphoid tissue (Kipps et al., 2017). The use of CD19 as a target in the case of CLL

by producing anti-CD19 CAR-T cells has shown remarkable results in patients with complete remission and minimal residual disease, and anti-CD19-CD28ζ CAR-T cells have shown promising results, according to the data from the National Cancer Institute (Porter et al., 2011, 2015; Kochenderfer et al., 2015). Pharmacokinetics plays an essential role in enhancing the outcomes and safety of CAR-T cell treatments, especially when it comes to the individual persistence of treatments, as it is considered the main goal in achieving the desired long-term antitumor effects (Norelli et al., 2016). In a recent study (NCT01747486), 42 patients (18 years and above) with CLL were treated with autologous CD19 CAR T-cells and 38 patients were infused with anti-CD19 CAR T-cells. Twenty-eight patients randomly received a low dose of (5×10^7) and high dose of (5×10^8), and 24 were evaluable for response assessment. After a short time, ten patients revived the high dose while eight were evaluable for response assessment. Follow-up ranged from 2 to 75 months; results showed that higher doses effectively induce a complete remission (CR) without excessive toxicities (Frey et al., 2020). One of the inevitable issues in CLL treatment is the antigen-negative relapse that has been threatening CAR T-cell therapy's success in CLL patients (Mancikova and Smida, 2021). The proportion of remissions in patients with CCL post CAR T-cell therapy remains the lowest compared to the spectrum of B-cell tumor patients. Current data are crucial for utilizing the clinical effects, and ibrutinib administration and partial reversing of the exhausted phenotype of CAR T-cells in CLL patient seem substantially promising. The genetic modification (insertion) of transgenic vector in the recipient T cells with systems such as CRISPR/Cas9 may contribute to treatment efficacy. The low quality of non-functional CAR T-cells derived from treated CLL patients could be improved using allogeneic CAR T-cells. The antigen-negative relapse could be alleviated using bispecific CARs targeting two antigens presented on the tumor cell surface. Suitable biomarkers must be identified and used as targets to design treatment and avoid infusion failure (Mancikova and Smida, 2021).

5.1.4 Non-Hodgkin's Lymphoma

Non-Hodgkin's lymphoma (NHL) consists of a group of neoplasms with various degrees of malignancy occurring in lymphocytes, lymphoid tissue, and histocytes at any stage of their development. These heterogeneous lymphoproliferative malignancies have a greater chance of dissemination to extranodal sites and are less predictable than Hodgkin's lymphomas (HL) (Singh et al., 2020). Anti-CD19 CAR-T cells have shown remarkable results in treating chemo-resistant lymphomas. Patients with refractory diffuse large B-cell lymphoma (DLBCL) had complete remission for more than 2 years (Neelapu et al., 2017a; Schuster et al., 2017; Tiberghien et al., 2017). CD22 is expressed in progenitor and differentiated B cells and is highly expressed in B-cell lymphomas and leukemia. Anti-CD22 showed promising results in four out of nine patients with a negative minimal residual disease and complete remission (Fry et al., 2015). Anti-CD20 and anti-CD23 CAR-T cells have also been used to treat NHL (Till et al., 2012; Zou et al., 2018). In 2017, the FDA approved YESCARTA (axicabtagene ciloleucel), which is a CD19 directed second generation CAR-T (CD28 co-stimulatory domain) cell product for the treatment of NHL.

5.1.5 Hodgkin's Lymphoma

Hodgkin's lymphoma (HL) is a common lymphoma derived from B cells. Hodgkin and Reed-Sternberg cells are rarely found in the tissues derived from mature B cells that lose their phenotype and co-express unusual hematopoietic cell markers (Küppers et al., 2012). HL cells highly express CD30; therefore, it was considered an ultimate target by engineered CAR-T cells, and clinical trials showed encouraging results where patients diagnosed with HL exhibited complete remission after anti-CD30 CAR-T cell therapy, wherein other patients either developed stable disease or relapse; however, the observations of anti-CD30 CAR-T cells did not show any toxicities or adverse events (Ramos et al., 2017; Wang et al., 2017).

5.1.6 Multiple Myeloma

Multiple myeloma (MM) is also a B-cell malignancy of long-lived plasma cells, which play a significant part in the immune defense system by producing antigen-specific immunoglobulins; in the case of malignancy, these cells excessively produce a specific immunoglobulin (containing two heavy chains and two light chains) and additional light chains, which can be detected in the blood. They are used to diagnose and monitor MM (Bird and Boyd, 2019). Disease management was compromised because of the unavailability of an ideal target. Syndecan 1 (CD138) was the target for the treatment of MM. This surface protein was expressed on both plasma cells and normal cells (epithelial), causing "on-target-off-tumor" toxicity. Nonetheless, Chinese clinical trials using CD138 as a target achieved stable disease in 4/5 patients (Heffner et al., 2012). Another target is the B-cell maturation antigen (BCMA), which is thought to be involved in all stages of B-cell differentiation and maturation and is highly expressed in myeloma cells; therefore, it is considered a better target in CAR-T cell therapy (Ali et al., 2016). A phase 1 clinical trial showed preliminary results regarding BCMA (anti-CD269) CAR-T cell therapy; one patient achieved complete remission for more than 3 months, whereas another patient showed an outstanding partial response to therapy. Additionally, a correlation was established between high treatment efficacy and higher doses. However, the higher the dosage, the more adverse events were seen, such as cytokine release syndrome, regardless of the use of BCMA or CD138 in therapy (Yang X. et al., 2019). The expression levels of CD19 in the plasma cells were low, but they were observed to be slightly higher in malignant cells and showed remission in a 43-year-old patient using CTL019 cells and CD19 as a target in MM. Cytokine release syndrome did not develop, and following several days of infusion, CTL019 cells were detected in the bone marrow and blood (Garfall et al., 2015). BCMA CAR T-cells were designed with signaling domain (CD3ζ) and CD28 (costimulatory domain) in a study (NCT02215967) conducted with 24 patients with MM; the cytotoxicity observed was minor post an infusion of a minimum dose ($0.3–3.0 \times 10^6$ cells/kg). The objective response rate (ORR) was 20%. The anti-tumor function with 81% ORR, while severe cytokine release syndrome (CRS) was reported in higher dosage

of CAR T-cells (9×10^6 cells/kg) (Brudno et al., 2018). Bispecific CAR T-cell (LCAR-B38M) was designed to target VHH1 and VHH2 epitopes of BCMA in a multicenter study (NCT03090659) on patients with MM. The findings included 88% ORR and 68% CR. The adverse events included leukopenia, thrombocytopenia, CRS, and pyrexia (Zhao W.-H. et al., 2018). In 2021, the FDA approved ABECMA (idecabtagene vicleucel) for MM. ABCEMA is a second generation CAR-T cell product directed against the BCMA tumor antigen.

5.2 Solid Tumors

5.2.1 Renal Cancer

Renal cancer (RCC) is one of the most diagnosed cancers in both men and women worldwide. RCC development is associated with several factors, including chronic kidney disease, smoking, hypertension, and obesity (Rossi et al., 2018; Capitanio et al., 2019). For many years, surgical intervention was the most effective treatment for RCC, known for its chemoresistance. Later, other treatments such as cytokine and tyrosine kinase inhibitors (TKIs) were approved, and when RCC showed possible immunological sensitivity, other immunotherapies were approved as well (Schepisi et al., 2020). CAR-T cell therapy of RCC targets carboxy-anhydrase-IX (CA-IX) as an antigen, which participates in the catalysis of carbon dioxide hydration (Bagley and O'Rourke, 2020; Bagley and O'Rourke, 2020) and is considered a critical antigen in RCC; however, it is also found in other normal tissues of gastric mucosa epithelium, small intestine epithelium, duodenum, and the biliary tree where it is expressed moderately (Yeku et al., 2017). The expression of CA-IX can be induced under hypoxic conditions in various tissues (Tafreshi et al., 2014). The first generation of CA-IX/CART-cells toward RCC was associated with high cytokine secretion due to cytotoxicity (Li et al., 2018).

5.2.2 Ovarian Cancer

Novel therapeutics are constantly required in Ovarian cancer (OC) as it is known for its high recurrence levels post-surgery and multi-agent chemotherapies. CAR-T cells are a novel therapy. In the context of ovarian cancer, they target tumor-associated glycoprotein 72 (TAG72); humanized TAG72-specific CAR-T cells exhibited cytokine production and cytotoxic activity in OC. In contrast, it also showed proliferation reduction and increased mouse viability in mouse models (Murad et al., 2018). Another target was mucin 16 (MUC16), which causes OC progression depletion after intraperitoneal and intravenous injection in mouse models, making it one of the potential targets, and an *in-vitro* study using Her-2 CAR-T cells on human OC cell line (SKOV3) expressing Her-2/neu reported growth suppression potential (Chekmasova et al., 2010). The antigen mesothelin was targeted by anti-Meso CAR-T cells, which inhibited proliferation and increased mouse viability. Additionally, 5T4-specific CAR-T cells and FRα-specific CAR-T cells exhibited inhibitory effects against OC cellular growth and progression (Zuo et al., 2017; Owens et al., 2018). In the dual design of CAR-T cells targeting both CD19 and mesothelin (MSLN-CAR NK-92) cells using lentivirus gene transfer, the MSLN-CAR molecules were highly expressed on the surface of NK-92, which led to the killing of $MSLN^+$ OC cells such as SKOV3 and OVCAR3 *in vitro* (Cao et al., 2020).

5.2.3 Lung Cancer

Lung cancer is one of the most diagnosed cancers worldwide and is considered one of the leading causes of death. Several antigens have been targeted to treat this cancer, including epidermal growth factor receptor (EGFR), which is highly expressed in the epithelium and epithelium-derived tissues compared with normal lung tissues. Because the receptor provides significant affinity for binding sites in lung carcinomas, it is one of the most therapeutic targets of CAR-T cells. Second-generation EGFR-CAR-T cells with CD137 co-stimulatory domain showed feasibility and safety in treating refractory/relapsed non-small cell lung cancer (Feng et al., 2016). Another candidate target was HER2, as it exhibited good therapeutic outcomes in refractory/recurrent $HER2^+$ sarcomas without any respiratory distress syndrome (RDS) signs. However, RDS was observed 15 min after cell infusion in one patient diagnosed with metastatic colon cancer to the lung and liver, plausibly because of an autoimmune reaction. Generally, the safety and efficacy of this anti-HER2 CAR-T cell in lung cancer depends on the levels of HER1 in patients and might be compromised because of RDS (Morgan et al., 2010).

Further antigens were considered, including MSLN, since it is expressed in 69% of lung adenocarcinoma (1/5 patients) and not in normal lung tissues and reduced tumor burden in mouse models (Carpenito et al., 2009; Kachala et al., 2014). The NSCLCs were found to overexpress transmembrane glycoprotein MUC1 and Prostate Stem Cell Antigen (PSCA), a glycosylphosphatidylinositol (GPI)-anchored cell surface antigen; therefore, they were preferred to be used in combination as potential targets for MUC1-CAR-T cells and anti-PSCA-CAR-T cells, which showed excellent efficacy compared with using a single antigen (Wei et al., 2017). Carcinoembryonic antigen (CEA) is overexpressed in nearly 70% of NSCLCs (Berinstein, 2002); however, patients who received anti-CEA CAR T-cell treatment had transient acute respiratory toxicity, possibly because of the expression of CEACAM5 on lung epithelial cells (Thistlethwaite et al., 2017). The tyrosine kinase-like orphan receptor 1 (ROR1) was used as a target; however, toxicity concerns are growing since it was also expressed in normal tissue. Therefore, to overcome this issue, selectivity of the target was improved by engineering CAR T-cells with synthetic Notch (synNotch) receptors specific for EpCAM or B7-Homolog 3 (B7-H3), a member of the B7 family of immune checkpoint molecules, which is expressed on ROR1+ tumor cells but not on $ROR1^+$ stromal cells, resulting in the regression of tumor cells without causing toxicity (Srivastava et al., 2019). The costimulatory role of CD80/CD86 makes it a suitable target for immune intervention, and upon binding to CTLA4 (CTLA4-CD80/CD86), T cells are downregulated via various mechanisms. In several NSCLC cells, the mRNA expression of CD80/CD86 was detected in normal tissues, risking autoimmunity reactions; hence, new strategies are

encouraged to overcome this risk by using CD80/CD86 CAR-T cells and enhancing its selectivity (Wroblewski et al., 2001; Egen et al., 2002).

5.2.4 Breast Cancer

Breast cancer (BC) is one of the leading causes of death in women, wherein 1.5 million women are diagnosed with BC worldwide each year. BC is diagnosed during routine screening or incidentally, and it could reach the lymph nodes and metastasize to other organs such as the brain (Sun et al., 2017; Seely and Alhassan, 2018). One of the most attractive targets for CAR T-cell therapy is triple-negative breast cancer (TNBC). This type of breast cancer lacks estrogen (ER), progesterone, and epidermal growth factor (EGFR) receptors (Harrer et al., 2019). The targeted receptors for CAR T-cell treatment include folate receptor alpha (FRα); as a result, the anti- FRα CAR T-cells killed *in vitro* TNBC cells. This antitumor activity correlates with the FRα antigen levels in the cells (Song et al., 2016). The MUC1 antigen is associated with different tumor invasiveness and metastatic behavior, including breast cancer, making it a potential treatment target (Zhou et al., 2019). Integrin αvβ3 is another tumor antigen expressed in different tumors, including BC tumors, and stimulates tumor cell survival and metastasis (Felding-Habermann et al., 2001). Tyrosine-protein kinase Met (c-Met) is a cell surface molecule expressed in almost 50% of breast tumors. After an intratumoral injection of c-Met CAR mRNA, the tumors were excised and analyzed via intratumoral injection immunohistochemistry, revealing inflammatory and necrotic responses (Tchou et al., 2012; Zhao et al., 2017). The ROR antigen was also used as a CAR T-cell target in BC, eliminating multiple layers of tumor cells deep in the tumor tissues above and beneath the basement membrane (Wallstabe et al., 2019). Recent clinical trials have targeted several antigens against BC, including HER2, MUCI, CEA, CD70, CD133, ROR1, and NKG2D ligands (Williams et al., 2017). The cell surface antigen mesothelin was found to be overexpressed in 67% of TNBC samples and is considered a potential target because of its involvement in the activation of intracellular pathways including MAPK, NFκB, and PI3K, resulting in tumor cell proliferation and resistance to apoptosis (Morello et al., 2016; Tchou et al., 2017). CSPG4 is a tumor glycoprotein found in 72.7% of TNBC lesions and believed to be associated with tumor cell survival and recurrence; it was primarily detected in TNBC stem cells responsible for resistance and relapse. Using anti-CSPG4 CAR T-cells in TNBC metastasis and progression can also be diminished; it can attack more than one target, including stromal cells, primary TNBC cells, and cancer-associated fibroblasts, which are considered to be crucial for maintaining the TME (Wang et al., 2010; Cooney et al., 2011; Harrer et al., 2019). Disialoganglioside GD2 is a BC stem cell antigen expressed in 35.5% of metastatic TNBC and is considered an immunotherapeutic target, and anti-GD2 CAR T-cells have been reported to show cytolytic activity in $GD2^+$ cell lines (Seitz et al., 2020; Xia et al., 2020). The TEM8 marker was found to be overexpressed in the vasculature of solid tumors. When anti-TEM8 CAR T-cells were used in the TNBC mouse model, explicit control of the tumor growth was observed without exhibiting any toxicity. On the other hand, in healthy mouse models, cytotoxic effects were observed, which might be due to the retroviral vectors used that might have affected the abundance of CAR T-cells (Risma and Jordan, 2012b; Chaudhary et al., 2012; Byrd et al., 2018). Another intriguing target is the human endogenous retrovirus family K (HERV-K) antigen, highly expressed in basal BC cells, similar to TNBC. Importantly, it is absent in nearly all normal human tissues. The anti-K CAR T-cells experimented with *in-vivo* BC mouse models showed slow tumor growth. The MDA-MB-231 cell line showed great lysis post-exposure to anti-K CAR T-cells prepared from cells obtained from patients with BC (Zhao et al., 2011; Wang-Johanning et al., 2012; Krishnamurthy et al., 2015; Zhou et al., 2015, 2016; Johanning et al., 2017).

5.2.5 Prostate Cancer

The second most frequently diagnosed malignancy in men is prostate cancer (PrC) and the fifth leading cause of death worldwide. According to GLOBOCAN 2018, the number of newly reported diagnoses in 2018 reached 1,276,106 cases worldwide, with a higher incidence in developed countries (Rawla, 2019). Prostate-specific membrane antigen (PSMA) has been used as a target by CAR T-cells in studies (*in vivo* and *in vitro*) and causes the proliferation and differentiation of $PSMA^+$ cells (Maher et al., 2002; Gade et al., 2005). In mouse models of metastatic PrC, diabetes, and severe combined immunodeficiency, the use of PSMA CAR T-cells eradicated metastatic PrC cells. The second generation CAR T-cells (containing co-stimulator CD28) offer a novel immune-targeted approach for metastatic PrC since it showed a better eradication effect than the previous generation (Ma et al., 2014; Zuccolotto et al., 2014). The anti-PSMA CAR T-cell dosage and protocols for metastatic PrC patients is being investigated in phase 1 clinical trials, in addition to the possible use of dual-targeted CAR T-cells targeting PSMA and transforming growth factor-β (TGFβ) and their safety in another phase 1 clinical trial (Slovin et al., 2013; Kloss et al., 2018). The prostate stem cell antigen (PSCA) is also an attractive target for CAR T-cell therapy; the first generation of CAR T-cells with the scFV of 7F5 antibodies exhibits antitumor effects in mice. In another study that used the 4-1BB co-stimulator, the activation of T cells was better than that by the CD28 co-stimulator (Hillerdal et al., 2014; Priceman et al., 2018). As a potential strategy, combined CAR T-cell therapy uses low-affinity PSCA CAR T-cells and high-affinity PSMA CAR T-cells to eliminate double-positive CAR T-cells in PrC (Feldmann et al., 2017). A different approach is to use diabodies (bispecific antibodies; BITEs) that simultaneously bind to specific T-cell receptor-associated molecules on the T-cell surface (e.g., CD3ε) and to a tumor-specific antigen expressed on the cancer cell surface (e.g., CD19; PSMA). The simultaneous engagement of BITEs with both CD3 and the specific antigen resulted in tumor cell lysis via the activation of cytotoxic T-cells. BITEs have also been reported to be overexpressed in tumor tissues compared to normal ones (Stone et al., 2012; Stieglmaier et al., 2015; Yang et al., 2016). These novel antibodies were evaluated in combating cells by targeting PSMA (Baum et al.,

2012; Friedrich et al., 2012; Feldmann et al., 2017). In animal models, these novel antibodies failed to block the proliferative activity of cancer; they only caused delayed tumor growth, which suggests that the use of diabodies as a single treatment would not achieve a sturdy cellular memory response (Hillerdal and Essand, 2015). However, in murine xenograft PrC models, the humanized bispecific antibody MOR209/ES414 caused tumor growth inhibition and improved survival. PSMA expression was reduced only in transferred and adaptive human T cells. In a recent study on xenograft models, BITE targets CD3 in T cells and PSMA in PrC cells. The results revealed their antitumor potential (Hernandez-Hoyos et al., 2016; Bailis et al., 2019). An additional target of PrC is the epithelial cell adhesion molecule (EpCAM; also known as CD326), a known stem cell antigen present in several tumors, including PrC (Gires et al., 2009; Ni et al., 2012). Recently in Europe, EpCAM-CD3 was approved for the treatment of malignant ascites. Using it as a TAA, it was developed to produce anti-EpCAM CAR T-cells capable of combating PC3M cells overexpressing EpCAM, thereby extending the survival of under-expressing EpCAM PC3 cells. However, further investigation of its efficacy in metastatic PrC is needed (Deng et al., 2015).

5.2.6 Liver Cancer

Liver cancer is a global health burden, with an estimated >1 million cases by 2025. The most frequently diagnosed type of liver cancer is hepatocellular cancer (HCC), contributing ~90% of all diagnosed cases. Many risk factors play a role in the progression of various diseases, such as hepatitis B and C infection, non-alcoholic steatohepatitis associated with diabetes mellitus, or metabolic syndrome (Llovet et al., 2021). The glypican-3 (GPC3) cell surface has been targeted in CAR T-cell therapy against the HCC xenograft mouse model and proved effective (Gao et al., 2014; Jiang Z. et al., 2016). Other targets are being investigated, including MUC 1, CEA, and epithelial cell adhesion molecules (Chen et al., 2018; Katz et al., 2019). A different target is the deletion-mutation form of EGFR (known as EGFRvIII), expressed in a wide range of cancer tissues, including HCC tissues. It was identified as a suitable target by CAR T-cells in an *in vivo* model (female BALB/cA-nude mice) and an *in vitro* SMMC7721 cell line (expressing high levels of EGFRvIII). The researchers used CAR T-cells by applying the transposon system (piggyBac), and the results showed antitumor effects in both *in vivo* and *in vitro* models (Ma et al., 2020).

5.2.7 Gastric Cancer

Gastric cancer (GC) is the fourth most commonly diagnosed type of cancer and the second cause of cancer-related death. Each year, the number of diagnosed patients is 990,000, of which 738,000 die (Machlowska et al., 2020). Different CAR T-cell targets against GC have been investigated, including folate receptor 1 (FOLR1) (Kim M. et al., 2018). HER2 is also a target in GC, and anti-HER2 CAR-T cells showed antitumor effects in MKN1 cells and mouse xenografts derived from a GC cell line with HER2 expression (Song et al., 2017). Several markers with diagnostic and functional importance have been studied as targets in GC, such as claudin 18.2 (CLDN 18.2), EpCAM, MUC1, CEA, EGFR2, natural-killer receptor group 2, member D (NKG2D), and MSLN. Other possible biomarkers that hold immense potential in GC include actin-related protein 2/3 (APR 2/3), desmocollin 2 (DSC2), B7H6 ligand, neuropilin-1 (NPR-1), cancer-related antigens CA-72-4 and CA-19-9, and anion exchanger 1 (AF1) (Zhang Q. et al., 2016). The use of anti-PSCA CAR T-cells on BGC-823, MKN-28, and KATO III GC cell lines and xenograft GC mouse models showed antitumor cytotoxicity post CAR T-cells peritoneal injection in mouse models resulted in tumor progression restriction (Wu et al., 2020).

5.2.8 Colorectal Cancer

Colorectal cancer (CRC) incidence has reached 1.85 million cases worldwide. The mortality rate has reached more than 850,000 deaths per year, making it the third most common cause of death among cancer-related deaths (Biller and Schrag, 2021). The targeted antigens in CRC are NKG2D, CEA, EGFR, MUC1, HER2, and CD133 (Li et al., 2021). The membrane-bound guanylyl cyclase2C (GUCY2C) has been used as a CAR T-cell target. It showed antitumor activity in both human and syngeneic xenograft CRC mouse models and is expressed in the intestinal apical surface, epithelial cells, and a proportion of the hypothalamic neurons (Magee et al., 2016, 2018). Anti-EpCAM CAR T-cells used against CRC cells and models exhibited cytotoxic lysis of the targeted cells that secreted cytotoxic cytokines, including IFN-γ and tumor necrosis factor-alpha (TNF-α), resulting in tumor growth and development in xenograft mouse models (Zhang et al., 2019). The tumor-associated glycoprotein 72 (TAG-72) was used as a CAR T-cell target in CRC. It was infused in patients via the hepatic artery and intravenously. The CAR T-cells were confirmed in the blood, and trafficking to the tumor tissue was confirmed by tumor biopsy. The results showed antitumor effects of the anti-TAG-72 CAR T-cells. However, the metastatic deposits were resistant to these cells and escaped the immune attack (Hege et al., 2017). Doublecortin-like kinase 1 (DCLK1), involved in the epithelial-mesenchymal transition (TME) and tumor progression, is a novel target for CRC immunotherapy and anti-DCLK1 CAR T-cells resulted in cytotoxicity and secretion of IFN-γ after incubation with CRC cells in two. Higher secretion levels were observed in three-dimensional cultures (Sureban et al., 2019).

5.2.9 Pancreatic Cancer

Pancreatic cancer (PaC) incidence has increased over the past few years, comprising 2% of all diagnosed malignancies and 5% of cancer-related deaths. Early diagnosis of PaC is challenging, and symptoms are not detectable at the early stages of the disease up to the advanced and metastatic settings. Most patients relapse, and the 5-year survival rate is 2% (Zhao and Liu, 2020). CXCR2-expressing CAR T-cells migrate more efficiently toward interleukin-8 (IL-8) and IL-8 containing TME, leading to a higher antitumor activity against αvβ6-expressing PaC xenografts (Whilding et al., 2019). B7-H3, also known as the CD276 antigen, was targeted by CAR T-cells in pancreatic adenocarcinoma *in vitro* and a metastatic xenograft mouse model, which proved efficacy (Du et al., 2019). Anti-CD133

CAR T-cells showed inhibitory activity against potential metastatic cells in HCC, colorectal carcinoma, and pancreatic carcinoma in phase I clinical trial (Wang et al., 2018). Other known antigens are being investigated for PaC CAR T-cell therapy, such as MUC-1 (Qu et al., 2004), fibroblast activation protein (FAP) (Tran et al., 2013), PSCA (Wu et al., 2020), CEA (Gansauge et al., 1996), mesothelin (Argani et al., 2001), CD24 (Jacob et al., 2004), and HER-2 (Komoto et al., 2009).

5.2.10 Brain Cancer

The burden of the brain and central nervous system cancers is high. However, they occur rarely and comprise approximately 1.5% of all diagnosed cancers, 80% of all adult primary brain cancers are gliomas, and the relative 5-year survival rate is 22% in brain cancer (Sandler et al., 2021). Various targets of CAR T-cells in brain cancer have been studied, including EGFRvIII, which has several limitations, including adverse events such as dyspnea and hypoxia in patients. Another potential end is that the heterogenic expression of this target in glioma tumors might lead to the accumulation of resistant variants able to escape CAR T-cell therapy (Goff et al., 2019; Rutkowska et al., 2019). In a human pilot study where IL-13Rα2 was used as a target for CAR-T cells in treating glioblastoma via multiple intracranial infusions, the treatment was well-tolerated and antitumor activity was observed in patients (Brown et al., 2015). A study on HER2 as a target showed that the third generation HER2-specific CAR-T cells with enhanced activity combined with PD-1 blockade successfully eliminated glioblastoma cells (Shen et al., 2019). Additionally, HER2-specific CAR T-cells were infused in 17 patients. The infusion was well-tolerated, no dose-limiting toxicities were observed, and CAR T-cell persistence was detected for up to 12 months after infusion. No disease progression was observed during 24–29 months of follow-up (Ahmed et al., 2017). B7-H3 was targeted against glioblastoma in mouse models, and anti-B7-H3 Car T-cells led to significant tumor regression and extended survival (Tang et al., 2019). B7-H3 mRNA exists in all normal tissues, but the microRNAs inhibit its translation; however, conditions such as inflammation might elicit B7-H3 expression in these tissues, making them a target of anti-B7-H3 CAR T-Cells (Xu et al., 2009). The inducer of extracellular matrix metalloproteinase, known as CD147, is responsible for the degradation of the extracellular matrix, allowing for tumor growth, invasion, and metastasis (Xiong et al., 2014). CD147 expression in glioma is significantly higher than that in normal tissues, and its expression is correlated with patient prognosis (Yang et al., 2013; Li et al., 2017a). A phase 1 clinical trial was performed to evaluate the anti-CD147 effect in recurrent glioblastoma patients; however, low levels of this antigen in several normal tissues despite high levels in malignant tissues sparked concern (Riethdorf et al., 2006; Liao et al., 2011; Tseng et al., 2020). GD2 is also expressed in glioblastoma patient samples and cell lines (Golinelli et al., 2018). Anti-GD2 CAR T-cells exhibited cytotoxic activity against neuroblastoma cell lines *in vitro* and subcutaneously grafted cell lines in mouse models and successfully eliminated orthotopic patient-derived diffuse midline glioma xenograft models (Prapa et al., 2015; Mount et al., 2018). Chlorotoxin (CLTX) is found in the death stalker scorpion venom [(DeBin et al., 1993). CLTX was found to selectively bind to primary tumor cells, while it is hardly detectable in different types of normal brain tissues (Lyons et al., 2002). CLTX directed-CAR T-cells were generated to target glioblastoma, which exhibited antitumor activity in orthotopic xenograft mouse models (Wang D. et al., 2020). NKG2D receptors are expressed in glioblastoma stem-like cells (Flüh et al., 2018; Yang D. et al., 2019). Chemotherapy or radiotherapy upregulates the expression of the NKG2D ligand in glioblastoma cells; therefore, the combination of radiotherapy and anti-NKG2D CAR T-cells led to the prolonged survival of immunocompetent mice grafted with intracranial glioma cells (Weiss et al., 2018). In human differentiated glioblastoma cells and cancer initiation cells, and subcutaneous tumor models showed cellular eradication after CAR T-cell therapy; however, NKG2D-ligands on normal tissues are expressed under distress, which may result in human toxicity (Yang D. et al., 2019). In preclinical studies, various targets, such as carbonic anhydrase (CAIX), CD70, chondroitin sulfate proteoglycan 4 (CSPG4), erythropoietin-producing hepatocellular carcinoma A2 (EphA2), and trophoblast cell surface antigen 2 (TROP2) (Maggs et al., 2021).

5.2.11 Malignant Pleural Mesothelioma

Malignant pleural mesothelioma (MPM) is an incurable, rare, and aggressive type of cancer that initiates at the serosal surfaces, including pleura, pericardium, peritoneum, and the vaginalis (in males), as a result of asbestos exposure, with an approximate survival of 8–14 months (Andujar et al., 2016; Carbone et al., 2019; Klampatsa and Albelda, 2020). In the United States, the incidence rate reached 3,200 diagnosed cases/year (Jane Henley et al., 2013), while in Europe, the cases are constant and are expected to have an increased trend between 2020 and 2025 (Carbone et al., 2019). MPM has three main histological mesothelioma subtypes: sarcomatoid, biphasic, and epithelioid (Yang et al., 2008). The disease is characterized by a significant therapeutic resistance and poor prognosis (Klampatsa and Albelda, 2020). Preclinical studies using mRNA electroporation exhibited potent anti-tumor effects (Zhao et al., 2010). In light of this, an initial study focusing (NCT01355695) on toxicity assessment was conducted using T-cells with transient expression of second-generation murine anti-mesothelin CAR containing CD3ζ and 41BB signaling domains (Maus et al., 2013; Beatty et al., 2014); in phase I safety trial none of the patients exhibited "on-target, off-tumor" toxicity post-infusion, and there was no evidence of clinical responses (Beatty et al., 2014; Klampatsa et al., 2017). However, an immediate anaphylactic reaction was observed in one of the patients post a delayed infusion of mesothelin CAR T cell, which was linked to the immunogenicity of the murine SS1 scFV used in the construction of CAR (Maus et al., 2013). After the safety confirmation of the transient CAR mesothelin expression, a second phase I clinical trial (NCT02159716) was conducted on 15 patients with mesothelioma, ovarian, and pancreatic cancer; the used CARs were expressing the same second-generation murine-based anti-mesothelin constructed using a lentiviral transduction vector (Haas et al., 2019). In this trial, two doses of T-cells were administered, and some

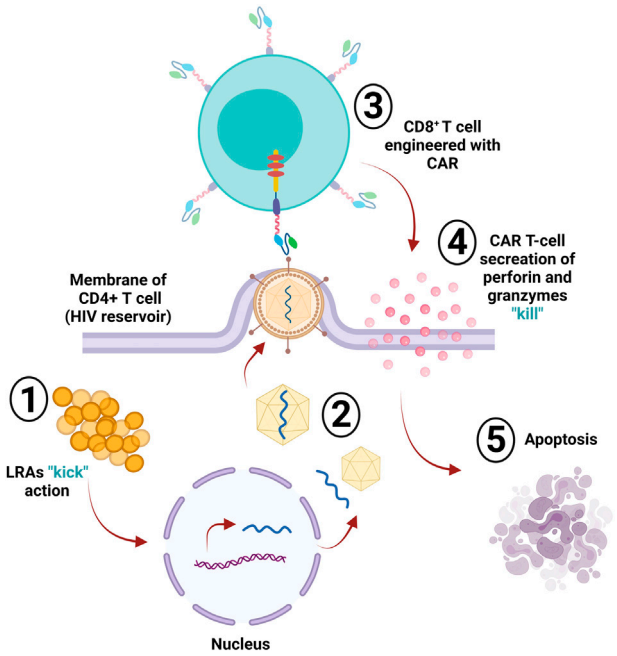

FIGURE 4 | HIV reservoir eradication. The "kick and kill" strategy is used to eliminate latently infected cells (reservoir); the "kick" part of this strategy depends on latency reversal agents (LRAs), which induce the virus via transcriptional reactivation of the incorporated provirus within the infected cell. The infected $CD4^+$ T-cell then starts producing and assembling the virus. Upon leaving the cell membrane, the engineered $CD8^+$ CAR T-cell will detect the expressed viral antigens; then, the "kill" action occurs via the secretion of perforin and granzymes, sending the cell into apoptosis.

cohorts used a lymphodepleting agent (cyclophosphamide). Although cyclophosphamide improved CART-meso expansion but did not enhance persistence beyond 28 days, the best overall response reported was stable disease in 11/15 patients (Haas et al., 2019). A third clinical trial (NCT03054298) was conducted using an active, fully human anti-mesothelin CAR and cyclophosphamide, administered via intravenous and intrapleural routes, respectively, to enhance the overall persistence and efficacy of CAR T-cells. In addition, researchers at Memorial Sloan Kettering Cancer Center are conducting a mesothelin-targeting CAR T cell trial to treat malignant pleural disease, including MPM (NCT02414269) based on preclinical studies of an orthotopic MPM mouse model. The study demonstrated that intrapleural administration of mesothelin CAR T-cell therapy was potent and had long-lasting antitumor efficacy (Adusumilli et al., 2014). The phase I/II clinical trial used CAR with human-derived anti-mesothelin scFV and CD3Z/CD28 signaling domain transduced by a retroviral vector; the CARs were administered via the intrapleural route in patients with primary and secondary pleural malignancies, with MPM patients being the main target population. A subset of the MPM patients had a subsequent administration of PD-1 checkpoint inhibitor (Pembrolizumab) to assess its efficacy in maintaining the prolonged activity of CAR T-cell therapy. Of the 27 patients who received cyclophosphamide, CAR T-cell therapy, and three doses of Pembrolizumab, 63% achieved either partial or complete response. Also, the CAR T-cells persisted and lasted for up to 42 weeks in the pleural fluid (Adusumilli et al., 2019).

5.3 HIV Infection

The human immunodeficiency virus (HIV) infects crucial cells in the human immune system, such as DCs, macrophages, and T helper cells ($CD4^+$ T cells) (Cunningham et al., 2010). The deterioration of $CD4^+$ T cells below critical levels renders the body susceptible to opportunistic infections (OIs) and the advancement of acquired immunodeficiency syndrome (AIDS) (Okoye and Picker, 2013). HIV-specific $CD8^+$ cytotoxic T lymphocytes (CTLs) play an essential role in recognizing viral antigens presented by HLA class I and killing the infected cells, resulting in limited viral replication *in vivo*; however, CTLs fail to provide sustainable HIV replication control without the use of a combination antiretroviral therapy (cART) (Jones and Walker, 2016). The CTL responses still fail to clear the virus from the body, even when using cART to delay disease progression and increase life expectancy. HIV remains an incurable disease, and one of the main reasons behind the failure of the immune system to clear out HIV infection is the reduction or absence of HIV viral antigen expression on infected yet latent $CD4^+$ T cells that act as viral reservoirs (Churchill et al., 2016). Viral reservoirs have been targeted by one strategy known as "kick and kill" or "shock and kill." This approach suggests the induction of the virus from the latent cells to promote HIV eradication via cell death or by immune surveillance, which clears the viral reservoir (Kim Y. et al., 2018). However, this approach has been investigated in clinical trials using latency reversal agents (LRAs), and the results are not promising (Rasmussen et al., 2014; Spivak et al., 2014; Søgaard et al., 2015). CAR T-cells are a promising approach for targeting and killing HIV-expressing cells (Kuhlmann et al., 2018) for multiple reasons: 1) long-term immune surveillance provided by CAR T-cells: the effector function of peripheral-derived CAR T-cells has been reported to be maintained for 6 months (Kalos et al., 2011; Kochenderfer et al., 2012; Maude et al., 2014). Moreover, hematopoietic stem cell (HSPC)-derived CAR T-cells persist longer and provide constant production of CAR T-cells as observed in HIV/AIDS animal models (Zhen et al., 2017). Additionally, HSC-based CAR T-cells were found in several lymphoid tissues in the gut, bone marrow, and several lymph nodes, all of which represent the main replication sites in non-human primate (NHP), infected models, with simian-human immunodeficiency virus (SHIV) (Zhen et al., 2017). Moreover, the long-lived immunological memory provided by CAR T-cells can be reprogrammed and differentiated into central memory or effector T cells (Kawalekar et al., 2016). 2) The trafficking capability of CAR T-cells to various types of tissues, including the central nervous system, is considered a potential harbor for latent HIV (Marban et al., 2016). Penetration of the blood-brain barrier has been a difficult task for drugs; however, evidence of anti-CD19 CAR T-cell trafficking to brain tissues and cancer cell elimination supports the concept that CAR-T cells may effectively target HIV reservoirs in the brain tissues (Grupp et al., 2013; Maude et al., 2014). Homing receptors can be added to CAR T-cells to increase their presence in the B cell follicle, which is another important HIV reservoir difficult for CTLs to

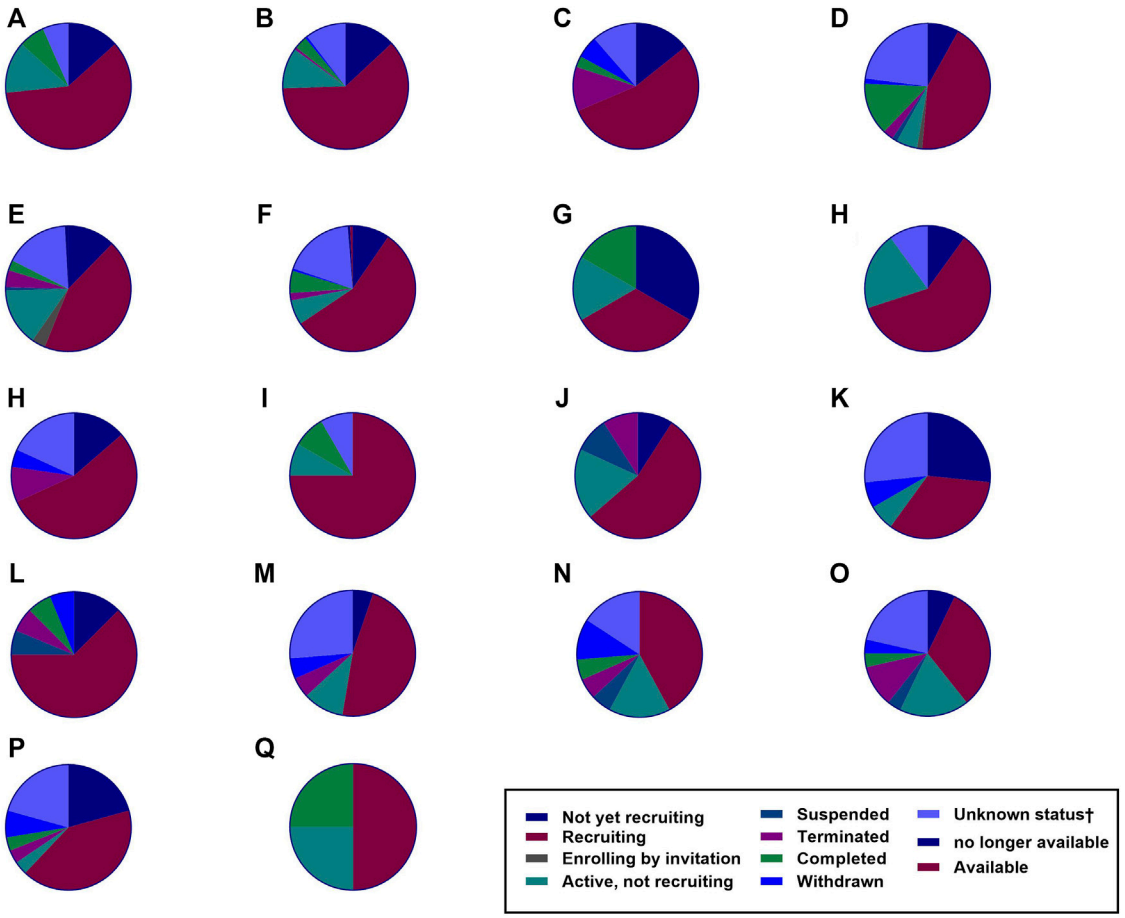

FIGURE 5 | The number of clinical trials. Several clinical trials have been investigating various malignancies as recorded by ClinicalTrials.gov. Based on the data up to January 2022, the number of these clinical trials is rising. The figure shows the number of CAR T-cell therapy clinical trials for hematological malignancies, solid tumors, and HIV infection (total = 789). **(A)** Hodgkin's lymphoma = 15 studies. **(B)** Acute myeloid leukemia = 35 studies. **(C)** Chronic lymphocytic leukemia = 74 studies. **(D)** Multiple myeloma = 114 studies. **(E)** Non-Hodgkin's lymphoma = 153 studies. **(F)** Acute lymphoblastic leukemia = 157 studies. **(G)** Human Immunodeficiency Virus = 6 studies. **(H)** Prostate Cancer = 10 studies. **(I)** Brain Cancer = 12 studies. **(J)** Renal Cancer = 12 studies. **(K)** Colorectal Cancer = 15 studies. **(L)** Ovarian Cancer = 16 studies. **(M)** Lung Cancer = 22 studies. **(N)** Gastric Cancer = 19 studies. **(O)** Breast Cancer = 19 studies. **(P)** Pancreatic Cancer = 28 studies. **(Q)** Liver Cancer = 29 studies. **(R)** Malignant pleural mesothelioma = 4 studies.

target (Haran et al., 2018). 3) The ability of CAR T-cells to target antigen in an MHC-independent manner helps in targeting HIV-infected cells and avoids viral downregulation of MHC-1 that leads to immune escape (Collins et al., 1998; Goulder and Walker, 1999; Wonderlich et al., 2011). The HIV CAR T-cell therapy targeted the primary HIV cellular receptor CD4, infused with CD3ζ signaling domain (CD4ζ) (Mitsuyasu et al., 2000; Walker et al., 2000; Deeks et al., 2002). The reason behind choosing CD4 as the reactive antigen in anti-HIV CAR T-cell design is its extensive targeting of all HIV isolates. Additionally, the binding sites of CD4 on the envelope protein are well preserved (Wang et al., 2019). The first generation CD4-based CAR-T cells have been tested in several clinical trials on HIV patients (Mitsuyasu et al., 2000; Walker et al., 2000; Deeks et al., 2002). The results showed a lack of durable control over viral replication; however, no treatment-associated toxicities were observed, and the persistence of modified cells continued for more than 10 years (Mitsuyasu et al., 2000). The first generation of CAR T-cells had certain impediments, such as limited *in vivo* expansion, susceptibility to apoptosis, and cytotoxicity (Heuser et al., 2003; Zhao et al., 2009). CAR T-cells were optimized into the second generation by adding costimulatory domains 4-1BB, resulting in 50-fold more compelling *in vitro* suppression of HIV replication than the previous generation (Leibman et al., 2017). *In vivo* studies showed that second generation CAR T-cells had superior expansion in response to the antigen, provided protection to $CD4^+$ T-cells against HIV infection, and CD4 reduction was decreased compared to the CARs without costimulatory molecules (Leibman et al., 2017). The costimulatory domain 4-1BB is superior in reducing viral rebound than the CD28 domain after antiretroviral therapy (ART) and 4-1BB-induced T-cell perseverance in the absence of the antigen (Zhang et al., 2007; Leibman et al., 2017). Developing third generation CARs with multiple costimulatory molecules enhanced effector function, survival, and proliferation. It also enhanced tumor targeting and killing (Savoldo et al., 2011).

TABLE 1 | CAR T-cell clinical trials with recorded results from ClinicalTrials.gov.

Condition	Enrollment	Status	Antigen	Phase	Results	NCT
B- cell lymphoma	43	Active, not recruiting	Anti-CD19 CAR T-cells	Phase I/ phase II	Complete remission (CR) of an assortment of the B-cell malignancies with durability for up to ≥3 years post 51% of anti-CD-19 CAR T-cell treatment with remission of 9 years and going. The adverse events were infrequent	(NCT00924326)
Metastatic melanoma and renal cancer	24	Terminated	Anti-VEGFR2- CAR T-cells	Phase I/ phase II	Adverse events registered Grade 3 of 4 toxicity with a presentation of hypoxia, nausea, vomiting, hyperbilirubinemia, elevation in aspartate transaminase, and alanine transaminase. The study was terminated due to the absence of observed impartial responses	(NCT01218867)
Metastatic cervical, pancreatic, lung, ovarian, and mesothelioma cancers	15	Terminated	Anti-mesothelin CAR T-cell	Phase I/ phase II	Adverse events were evident in this study, including anemia, constipation, thrombocytopenia, lymphocytopenia, and hypoxia. The study was terminated due to low and inadequate accrual	(NCT01583686)
Malignant gliomas	18	Completed	Anti-EGFRvIII CAR T-cells	Phase I/ phase II	The pilot clinical trial failed and led to severe adverse events such as hypoxia, dyspnea, and multi-organ failure. In addition, the CAR T-cell intervention had no significant impact on the glioblastoma and resulted in its progression	(NCT01454596)
Refractory B-cell malignancies in children and young adults	53	Completed	Anti-CD19 CAR T-cells	Phase I	The feasibility and safety of this treatment were evident. The anti-leukemic activity was remarked in chemoresistance patients. High responses rate was observed post-infusion in patients. Central nervous system (CNS) trafficking and clearance were detected in two cases. Minimum cytokine release syndrome was CAR T-cells expansion correlated. Toxicities were reversible	(NCT01593696)
Relapsed or refractory CD19 positive chronic lymphocytic leukemia (CLL) and small lymphocytic lymphoma (SLL)	42	Completed	Anti-CD19 CAR T-cells	Phase II	Anti-leukemic activity and long persistence of tranced cells were seen in patients. Upon further investigation, findings suggest that patients who achieved complete response showed an increased mass of the Anti-CD19 CAR T-cells mitochondria, which contributed to cells expansion and persistence	(NCT01747486)
Adult B-cell Acute Lymphoblastic Leukemia (B-ALL)	82	Terminated	JCAR015 Anti-CD19 CAR T-cells	Phase II	The clinical trial failed to achieve significant results as five patients suffered from cerebral edema as an adverse event, resulting in death, and the study was terminated for safety reasons	(NCT02535364)
B-cell Malignancies (B-Cell Lymphoma, Non-Hodgkin's Lymphoma)	27	Active, not recruiting	Anti-CD19 CAR T-cells. (Hu19-CD828Z)	Phase I	Patients had shown CR. This study suggested that enhancing the CAR T-cells design resulted in less neurotoxicity and CRS associated with low or mild cytokine production levels	(NCT02659943)

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TABLE 1 | (*Continued*) CAR T-cell clinical trials with recorded results from ClinicalTrials.gov.

Condition	Enrollment	Status	Antigen	Phase	Results	NCT
Multiple myeloma	6	Terminated	Anti-CD19 CAR T-cells. Post autologous stem cell transplantation (ASCT)	Phase II	No mortalities were reported. The serious adverse events were 1/6 patients suffered from CRS and upper respiratory tract infection (URI). The study was terminated due to administrative reasons	(NCT02794246)
B-cell Acute lymphoblastic leukemia in adults	1	Terminated	Anti-CD19 CAR T cells	Phase II	The patient died. The severe adverse events mentioned were paresthesia, encephalopathy, and gastric necrosis. The results were not discussed further, and the study was terminated due to admirative reasons	(NCT02935543)
Glioblastoma and gliosarcoma	3	Terminated	Anti- EGFRvIII CAR T-cells	Phase I	The mortalities were 3/3. The adverse events were confusion and generalized muscle weakness in 1/3. The study was terminated because the funding was not sufficient	(NCT02664363)
Multiple myeloma	12	Terminated	AUTO2 (APRIL CAR T-cells)	Phase I/phase II	The study mortalities were 6. Some patients have severe adverse events, including Acute myocardial infarction (AMI), pyrexia, lung infection, decreased neutrophil count, hypocalcaemia, metaplastic breast carcinoma, headache, and dyspnea. The study was terminated as the preliminary efficacy post-treatment was insufficient to guarantee further development	(NCT03287804)
B Cell Acute Lymphoblastic Leukemia (ALL)	23	Completed	AUTO3 (CD19/22 CAR T-cells)	Phase I/phase II	The mortality rate was 61.6% among patients who received high infusion doses; serious adverse events were anemia, febrile neutropenia, thrombocytopenia, pyrexia, cellulitis, encephalopathy, and seizure	(NCT03289455)
Relapsed/refractory B-cell malignancies	26	Active, not recruiting	Anti-CD20/19-CAR T-cells	Phase I	The results of this study suggest that the favorable infusion dosage is 2.5×10^6 cells/kg providing low toxicity and high efficacy in city profile and sustained efficacy at a dose of 2.5×10^6 cells per kg for relapsed, refractory B cell non-Hodgkin's lymphoma (NHL) and chronic lymphocytic leukemia (CLL) patients	(NCT03019055)
Relapsed/Refractory Multiple Myeloma	17	Active, not recruiting	KITE-585 CAR T-cells	Phase I	The overall mortality rate was 62.5%, and the adverse events were chest pain and hypoxia	(NCT03318861),
Advanced Lung Cancer	1	Terminated	Anti-PD-L1 CAR T-cells	Phase I	The patient developed severe CRS, which caused interstitial pneumonia disease. The study was terminated due to serious adverse events	(NCT03330834),
Acute Myeloid Leukemia (AML) Multiple Myeloma (MM)	8	Terminated	Anti-CD44v6 CAR T-cells	Phase I/phase II	The patients had adverse events of pyrexia, anemia, neutropenia. The study was terminated due to low patient recruitment and a lower-than-expected proportion of myeloma and leukemia expressing CD44v6. The study failed to be completed in a clinically relevant time frame	(NCT04097301)

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TABLE 1 | (*Continued*) CAR T-cell clinical trials with recorded results from ClinicalTrials.gov.

Condition	Enrollment	Status	Antigen	Phase	Results	NCT
$CD19^+$ Diffuse Large B-cell Lymphomas Follicular Lymphomas Mantle Cell Lymphomas	12	Completed	Anti-CD19 CAR T-cells	Phase I/ phase II	Serious adverse events included optic disorder, fever, hyperbilirubinemia, CRS, sepsis, hypercalcemia, delirium, acidosis, hypoxia, pleural effusion, non-cardiac related chest pain, and rash	(NCT02650999)
DLBCL Neurotoxicity Syndromes	25	Terminated	Evaluation of the Safety and Efficacy of Defibrotide in the Prevention of Chimeric Antigen Receptor-T-cell-associated Neurotoxicity	Phase II	Patients had febrile neutropenia, atrial fibrillation, myocardial infarction, asthenia, pyrexia, CRS, decreased appetite, neurotoxicity, tumor lysis syndrome, transient ischaemic attack, confusion state, pleural effusion, pulmonary embolism, and hypotension. The study was terminated because unplanned interim assessment on the first 20 efficacy evaluable patients was unlikely to meet the primary endpoint	(NCT03954106)
Relapsed or Refractory Neuroblastoma	17	Completed	Anti-GD2 CAR T-cells, (1RG – CART)	Phase I	Hypotension, capillary leak syndrome, neurological symptom, headache, hyponatremia, pyrexia, tachycardia, febrile neutropenia, and coagulopathy. Only 12 patients were subjected to therapy as two were withdrawn due to progressive disease, one died, and one withdrew the consent for the trial	(NCT02761915)
Myeloma-Multiple Myeloma, Plasma-Cell	13	Completed	Anti-SLAMF7 CAR T-cell	Phase I	Serious adverse events included CRS sinus tachycardia and fever	(NCT03958656)

Using the third generation CARs with CD3z-CD28-4-1BB as multiple domains, targeting the envelope glycoprotein GP120 (gp120) and anti-gp120 CAR T-cells in HIV infection showed increased effectiveness in lysing Env-expressing cells *in vitro* compared to CD4ζ CAR T-cells (Liu et al., 2016). Targeting HIV reservoirs by immune surveillance is difficult because of the ability of the virus to persist in various reservoirs and the lack of viral antigen expression in infected cells. The "kick and kill" strategy cause the transcription reactivation of the latently persistent provirus leading to viral antigen expression, making it detectable by the immune surveillance in ART-treated patients. The 'kick" strategy can be achieved by potent latency reversal agents (LRAs). Clinical studies in animals showed that LRA was well tolerated *in vivo* and induced HIV expression (Marsden et al., 2017). Although LRAs induce the virus killing by the immune system, it is insufficient, and reservoir eradication is inefficient (Thorlund et al., 2017). The CAR T-cells can exhibit the "kill" response in this strategy along with LRAs; this combination is necessary for effective reservoir eradication (Bashiri et al., 2018) (**Figure 4**). The kill action in the human system shows that CTLs, either $CD8^+$ or $CD4^+$, induce apoptosis by cytolytic perforin and granzyme (Yasukawa et al., 2000).

CAR T-cell therapy has been considered a potential treatment against other infectious diseases such as those caused by opportunistic fungi, hepatitis B virus (HBV), hepatitis C virus (HCV), and cytomegalovirus (CMV), and the data gathered from pre-clinical trials have shown promising results (Seif et al., 2019). The number of clinical trials of CAR-T cell therapies is increasing, and their observations are constantly changing, as it is a very attractive field of research with remarkable potential (**Figure 5**). However, according to ClinicalTrials.gov, only 21 studies had results in January 2022 (**Table 1**).

It is worth of mentioning that CAR T-cells potentials were recently applied against cardiac fibrosis (heart tissue stiffening and scarring). Rurik et al. were capable of designing an immunotherapy strategy to generate transient CAR T-cells able to identify fibrotic cells in the heart through injecting CD5-targeted lipid nanoparticles encompassing the needed mRNA to reprogram T lymphocytes, therapeutic CAR T-cells were successfully generated inside the body (*In vivo*). The heart disease in mouse model was analyzed and revealed that this approach has indeed succeeded in fibrosis reduction and cardiac function restoration (Rurik et al., 2022).

6 FDA APPROVED CAR T-CELLS THERAPIES

6.1 Axicabtagene Ciloleucel (YESCARTA™)

The first Food and Drug Administration (FDA) approved CAR T-cell therapy, axicabtagene ciloleucel (YESCARTA™) from Kite Pharma approved in 2017, comprises autologous genetically

modified T cells designed to produce CAR protein targeting CD19 expressing normal and malignant cells (Papadouli et al., 2020). It is used to treat adult large B-cell Lymphoma after two or more lines of systemic therapy, including DLBCL, high-grade B-cell lymphoma, primary mediastinal large B-cell lymphoma, and DLBCL arising from follicular lymphoma. The approval of this drug was based on a single-arm multicenter clinical trial (ZUMA-1; NCT02348216) conducted on 108 patients diagnosed with aggressive B-cell non-Hodgkin's lymphoma. The selection criteria were occurrence of refractory disease post a recent therapy or relapse post autologous hematopoietic stem cell transplantation within a year. The patients underwent lymphodepletion before receiving a single infusion of axicabtagene ciloleucel. The efficacy was evaluated in 101 patients as follows: ORR 72%, with a complete remission rate (CR) of 51%, the duration of response (DOR) was longer in patients with CR than in patients with partial remission (PR). The median DOR was not reached after 7.9-months (median follow-up). The estimated DOR was 2.1 months. Most common grade 3 (with incident ≥10%) adverse events occurred including fever, febrile neutropenia, encephalopathy, CRS, hypoxia, and hypotension; 25% exhibited severe adverse events, including neurotoxicity, CRS, serious infections, and prolonged cytopenia. In some patients, CRS and neurotoxicity were fatal. The FDA approved axicabtagene ciloleucel with recommendations of mitigation strategy and risk evaluation. The recommended dosage was 2×10^6 viable CAR-positive T cells/kg of body weight, following lymphodepletion chemotherapy by (Flu/Cy) (Neelapu et al., 2017a). In March 2021 (Yescarta, axi-cel), the FDA approved another directed CD19 T-cell therapy to treat adult r/r follicular lymphoma after two lines of therapy. The approval was based on collected data from a single-arm, open-label phase II clinical trial (ZUMA-5; NCT03105336). The clinical trial had 81 participants. The results were: ORR 91%, with CM of 60%, the median DOR was not reached within a year of CM rate of 76.2%, patients who underwent leukapheresis (n = 123) experienced a ORR of 89% with CM rate 62%. CRS (grade ≥ 3, 10%) occurred in 88%, and neurotoxicity occurred in 51% of all patients with non-Hodgkin's lymphoma (Colombo et al., 2021).

6.2 Tisagenlecleucel (KYMRIAH™)

The second FDA approved CAR T-cell therapy, tisagenlecleucel (KYMRIAH™) from Novartis pharmaceuticals approved in 2018, is a genetically modified autologous T-cell immunotherapy (CD19 directed) for adult patients with r/r large B-cell lymphoma post two or more lines of systemic therapy, including high-grade B-cell lymphoma DLBCL, and DLBCL arising from follicular lymphoma. The approval was based on phase II of a single-arm, open-label, multicenter clinical trial (JULIET; NCT02445248) conducted on adults with r/r DLBCL and DLBCL arising from follicular lymphoma (Schuster et al., 2019). The criteria included a condition that the subject must at least undergo two prior therapy lines with rituximab and anthracycline or have relapsed after autologous hematopoietic stem cell transplant. Patients had a single tisagenlecleucel infusion after the completion of lymphodepleting chemotherapy. The clinical trial had 68 eligible patients out of 115, and the outcomes were 50% ORR with a 32% CR rate. With a median follow-up time of 9.4 months, patients with the best overall response CR had longer DOR than that of patients with PR. Patients with CR estimated median DOR of (10.0 months) was not reached, while the estimated median DOR among PR patients was 3.4 months. The most common adverse events in 20% of the patients included CRS, pyrexia, nausea, infections-pathogens unspecified, fatigue, diarrhea, headache, edema, and hypotension. The recommended dose of tisagenlecleucel for adults with r/r DLBCL was $0.6–6.0 \times 10^8$ CAR-positive viable T-cells (Schuster et al., 2019).

6.3 Brexucabtagene Autoleucel (TECARTUS™)

Accelerated approval of brexucabtagene autoleucel (TECARTUS™) was granted by FDA in July 2020; this immunotherapy comprises autologous genetically modified T cells (CD19-directed) for the treatment of adult patients with r/r mantel cell lymphoma (MCL) (Wang M. et al., 2020). The clinical trial behind the approval was a multicenter, single-arm, and open-label (ZUMA-2; NCT02601313) trial. Seventy-four patients diagnosed with MCL were subjected to this study. These patients previously received anthracycline or bendamustine-containing chemotherapy, anti-CD20 antibody, and Bruton tyrosine kinase inhibitor. After completing lymphodepleting chemotherapy, patients received a single infusion of brexucabtagene autoleucel. Sixty out of 74 patients evaluated for efficacy in a minimum duration of 6 months follow-up showed 87% ORR, with a CR rate of 62%. The estimated DOR was not reached (0–29.2 months) after a median DOR of follow-up (8.6 months). Among all 74 patients who underwent leukapheresis, the ORR was 80%, and CR was 55%. The most common adverse reactions with grade 3 or higher (≥10%) included hypoxia, encephalopathy, leukopenia, anemia, neutropenia, thrombocytopenia, hypotension, hypophosphatemia, hypertension, hyponatremia, pyrexia, infection-pathogen unspecified, lymphopenia, hypocalcemia, and pneumonia. Due to the fatal or life-threatening neurotoxicity and CRS, the FDA approval came with risk evaluation and mitigation strategies. The recommended dose of brexucabtagene autoleucel was a single IV infusion of $(2 \times 10^6 – 2 \times 10^8)$CAR-positive viable T-cells/kg body weight post lymphodepleting chemotherapy of (Flu/Cy) (Wang M. et al., 2020).

In 2021 brexucabtagene autoleucel was approved to treat adult patients with r/r B-cell precursor ALL based on the data gathered from a phase II clinical trial (ZUMA-3; NCT02614066) (Shah et al., 2021). The study had 125 participants diagnosed with r/r B-cell precursor ALL. Patients received a single infusion of brexucabtagene autoleucel post completion of lymphodepleting chemotherapy. The outcomes included CR within 3 months post-infusion. Fifty-four patients were evaluable for efficacy, 28 achieved CR within 3 months with a median follow-up of 7.1 months, the CR median duration was not reached, and the CR duration for more than half of the patients was estimated to exceed 12 months. In 92% of patients, CRS occurred (≥grade 3, 26%); neurotoxicity occurred in 87% (≥Grade 3, 35%); most common adverse events were hypotension, CRS, encephalopathy,

fever, chills, headache, rash, edema, nausea, tachycardia, febrile neutropenia, musculoskeletal pain, hypoxia, diarrhea, tremor, constipation, infection with an unspecified pathogen, vomiting and decreased appetite. The recommended dose was a single IV infusion of brexucabtagene autoleucel (1×10^6–1×10^8) of CAR-positive viable T-cells/kg body weight preceded by (Flu/Cy) lymphodepleting chemotherapy (Shah et al., 2021).

6.4 Lisocabtagene Maraleucel (BREYANZI™)

In February 2021, lisocabtagene maraleucel (BREYANZI™) from Juno Therapeutics was approved by FDA for the treatment of adult patients with r/r large B-cell lymphoma after two or more lines of systemic therapy, including high-grade B-cell lymphoma, DLBCL, primary mediastinal large B-cell lymphoma, DLBCL arising from indolent lymphoma, and follicular lymphoma grade 3B (Abramson et al., 2020). Lisocabtagene maraleucel is a CD19- directed CAR T-cell immunotherapy comprised of autologous genetically modified T cells that produce CAR protein able to identify and eradicate CD19-expressing normal and malignant cells. The immunotherapy efficiency was evaluated in a single-arm, open-label, multicenter trial (TRANSCEND, NCT02631044); 192 patients underwent lymphodepleting chemotherapy before infusion. The outcomes included 73% ORR, with a CR rate of 54%, and the median time of first response was 1 month; 104/192 patients had CR, which lasted at least 6 months (65%), and some patients (62%) had a remission that lasted at least 9 months. The DOR was 16.7 months in patients who achieved CR; and the patients with PR had 1.4 DOR. Adverse events included CRS in 46% of the patients (grade 3 or higher, 4%); neurotoxicity occurred in 35% (grade 3 or higher, 12%). Three patients encountered fatal neurotoxicity. Other grade 3 or higher adverse events were prolonged cytopenia (31%) and infections (19%). Due to the fatal and life-threatening neurotoxicity and CRS, the FDA approval came with recommendations of risk evaluation and mitigation strategies. The recommended regimen was a single dose of $50–110 \times 10^6$ CAR-positive viable T-cells with a ratio of 1:1 of CD4 and CD8 components, intravenous (IV) infusion following (Flu/Cy) lymphodepletion (Abramson et al., 2020).

6.5 Idecabtagene Vicleucel (ABECMA™)

On March 2021, idecabtagene vicleucel (ABECMA™) from Bristol Myers Squibb was approved as the first cell-based immunotherapy for adult patients with r/r multiple myeloma after four or more preceded lines of therapy, including an anti-CD38 monoclonal antibody, an immunomodulator, and a proteasome inhibitor (Munshi et al., 2021). It is an autologous genetically modified B-cell maturation antigen (BCMA)-directed CAR T-cell therapy. In a multicenter study (NCT03361748), a total of 127 patients with r/r multiple myeloma were included to evaluate the safety and efficacy of the idecabtagene vicleucel; all patients received three (88% had received four or more) lines of antimyeloma therapies. In addition, 100 had received idecabtagene vicleucel with a dosage range of $300–460 \times 10^6$ of CAR-positive T-cells. The results showed a 72% ORR and a CR rate of 28%. Approximately 65% of patients had CR for at least 12 months. The most common adverse events included CRS, neurotoxicity, macrophage activation syndrome, prolonged cytopenia. Moreover, infection, fatigue, hypogammaglobulinemia, and musculoskeletal pain were designated as common side effects. Idecabtagene vicleucel was approved with recommendations of risk evaluation and mitigation strategies. The healthcare facility that houses this therapy must be specially certified to recognize and manage neurotoxicity and CRS. FDA called for a post-marketing observational study conducted by the manufacturer involving the patients treated with idecabtagene vicleucel (Munshi et al., 2021).

6.6 Ciltacabtagene Autoleucel (CARVYKTI ™)

The most recently FDA approved CAR T-cell therapy, in February 2022, is ciltacabtagene autoleucel (CARVYKTI™) from Janssen Biotech, Inc. This drug was approved for the treatment of r/r multiple myeloma post four or more prior lines of therapy including an anti-CD38 monoclonal antibody, an immunomodulatory agent (IMiD), and a proteosome inhibitor (PI). It is a genetically modified autologous CAR T-cell therapy directed by B-cell maturation antigen (BCMA). In a multicenter study CARTITUDE-1 (NCT03548207) ciltacabtagene autoleucel safety and efficacy of were evaluated in 97 patients with r/r multiple myeloma who presented disease progression post their last chemotherapy regimen; 82% of the patients had received four or more prior lines of antimyeloma therapy. The dosage of ciltacabtagene autoleucel given to patients was falling within the range of $0.5–1.0 \times 10^6$ viable CAR-positive T-cells/kg body weight. According to the International Myeloma Working Group Uniform Response Criteria for Multiple Myeloma, the efficacy was evaluated by an Independent Review committee based on the overall ORR and DOR response. The ORR 97.9%, and a median DOR of 21.8 and 12 months median duration of follow up. Most commonly observed adverse reactions of ciltacabtagene autoleucel were CRS, fatigue, hypogammaglobulinemia, pyrexia, musculoskeletal pain, nausea, infection, diarrhea, coagulopathy, encephalopathy, headache, vomiting, and constipation. Moreover, recommended dosage of (CARVYKTI™) ranges from $0.5–1.0 \times 10^6$ to a maximum dose of 1×10^8 viable CAR-positive T-cells/kg of body weight per single infusion. The approval of (CARVYKTI™) is restricted by a risk evaluation and mitigation strategy necessitating healthcare facilities that houses this therapy and their associated clinicians to be specially certified to recognize and manage neurotoxicity and CRS. FDA called for a post-marketing observational study conducted by the manufacturer involving the patients treated with ciltacabtagene autoleucel (Berdeja et al., 2021).

7 LIMITATIONS AND SOLUTIONS FOR CAR T-CELLS

The CAR T-cell technology has immense potential. Current clinically approved CAR T-cell therapies are KYMRIAH™ for ALL and DLBCL; YESCARTA™ for DLBCL and follicular lymphoma; TECARTUS™ for mantle cell lymphoma;

BREYANZI® for DLBCL and follicular lymphoma; and ABECMA® for MM. Unfortunately, all these approved CAR-T cell products exert serious but clinically manageable adverse effects such as cytokine release syndrome and neurotoxicity (Zhao Z. et al., 2018; Zheng et al., 2018). Notably, the delay in approving CAR T-cell therapies targeting other diseases has the following structural limitations.

7.1 Tumor Antigen Escape

Single antigen-targeting CAR-T cells might face tumor resistance after the initial high response rate. The decline in response and increase in resistance is due to partial or complete loss of target antigen expression. Tumor cells escape killing by encouraging mutations in the antigen-coding gene, leading to the downregulation of expression of alternative antigens that lack the antigen epitopes targeted by CAR T-cells (Majzner and Mackall, 2018; Sterner and Sterner, 2021). One strategy to overcome this hurdle is to design T cells equipped with two or more CARs to target multiple TAAs, suggesting that the escape mechanism would require mutation of several genes instead of one by engineering CARs with multi-specific targets such as bicistronic CAR T-cells, tandem CAR T-cells, co-administered CAR T-cells, or co-transduction CAR T-cells. However, finding more than one TAA in one tumor targeted by CAR T-cells may prove challenging in some malignancies, with respect to safety and effectiveness (Hegde et al., 2013; Jackson and Brentjens, 2015). In addition, the use of lymphodepleting agents before the adoptive T-cell transfer can enhance epitope spreading, leading to more specific antigen recognition (Cui et al., 2009). Additionally, combining CAR T-cell therapy with checkpoint inhibitors (Gargett et al., 2016; Li et al., 2017b; Heczey et al., 2017; Adusumilli et al., 2021), radiation (Weiss et al., 2018), vaccines (Slaney et al., 2017; Tanaka et al., 2017), other immune agonists (Khalil et al., 2016; Majzner et al., 2017) might also contribute to epitope spreading and immune escape restriction (Majzner and Mackall, 2018).

7.2 On-Target Off-Tumor

One of the most observed toxicities in CAR T-cell therapy is the "on-target-off-tumor," where the normal tissues express the same targeted antigen on the malignant tissues at variable levels, leading to a direct attack from CAR T-cells against the normal tissues and eventually resulting in toxic effects that can be detrimental (Sun et al., 2018). To overcome this roadblock, using affinity-tune CARs to recognize tumor cells that have increased density of surface antigens and preventing the involvement with normal tissues that express low-density surface antigens has been suggested (Zhao et al., 2009). This strategy can be executed by altering the binding region of scFV via mutagenesis or via the recombination of both heavy and light chains (Carter et al., 1992; Drent et al., 2017). Another potential avenue for solid tumors is to target tumor-restricted post-translational modifications, such as overexpression of truncated O-glycans such as Tn (GalNAca1-O-Ser/Thr) and sialyl-Tn (STn) (NeuAca2–6-GalNAca1-O-Ser/Thr) (Steentoft et al., 2018). Another suggested approach is CAR T-cell local administration to the disease site, which might contribute to the limitation of "on-target-off-tumor" toxicity as the on-target activity is focused on the malignant tissue, and the normal tissue interaction is disregarded (Sterner and Sterner, 2021). Inducible CAR-T cell products based on engineered synthetic Notch receptors are also being explored to mitigate the on-target off-tumor associated toxicities (Roybal et al., 2016).

7.3 Trafficking and Tumor Infiltration

One of the significant inadequacies in using CAR T-cell therapy in solid tumors is the ability of these cells to traffic and infiltrate the tumor because both immunosuppressive TME and physical barriers of tumor such as stroma restrain mobility and diffusion of CAR T-cells. The proposed approach uses the local administration as the delivery route, which disregards the need for the cells to traffic to the disease site (Sterner and Sterner, 2021). Another strategy developed to overcome the trafficking issue is the addition of chemokine receptor expression on CAR T-cells that match and respond to chemokines expressed by targeted tumors (Whilding et al., 2019). The physical barrier of the stroma mainly comprises an extracellular matrix with a primary component of heparin sulfate proteoglycan (HSPG). Upon its degradation, CAR T-cells can reach the tumor (Zhang B.-L. et al., 2016). Engineered CAR T-cells with heparinase expression have been shown to degrade HSPG, leading to enhanced tumor infiltration and elimination (Caruana et al., 2015). Likewise, fibroblast activation protein (FAP) was also targeted by CAR T-cells in animal models, which increased cytotoxic function by reducing the number of tumor fibroblasts (Wang et al., 2014).

7.4 Immunosuppressive Microenvironment

In the TME, several tumor-infiltrating cells contribute to immunosuppression, including MDSCs, regulatory T cells (Tregs), and tumor-associated macrophages (TAMs) (Quail and Joyce, 2013). These infiltrates and tumor cells contribute to the production of tumor-supporting growth factors, chemokines, and cytokines, and the antitumor immunity declines because of immune checkpoint proteins such as CTLA-4 or PD-1. Weak CAR T-cell responses could be regarded as a poor T-cell expansion and limited persistence period, indicating that the development of T-cell exhaustion is prompted by co-inhibitory pathways (Yin et al., 2018). Consequently, the combination of CAR-T cells with immunotherapy and checkpoint blockade is thought to be the next cutting-edge immunotherapy approach because it provides two major elements to secure strong immune responses: CAR T-cells provide tumor penetration and PD-1/PDL1 blockade to guarantee sustained and persistent T-cell function (June et al., 2018; Grosser et al., 2019). Recently, CAR-T cells have been engineered to be robustly resistant to TME immunosuppressive factors such as TFG-β-mediated inhibitory signals (Kloss et al., 2018). Furthermore, CAR T-cell engineering includes the addition of immunostimulatory signals such as stimulatory cytokines capable of increasing survival, proliferation, and antitumor activity while re-equalizing TME (Chmielewski et al., 2014). Various studies have been investigating numerous cytokines to create "armored CARs." The studies that focused on

proinflammatory cytokines apart from concentrating on inhibitory signals have depended on IL-12 secretion (Koneru et al., 2015), expression of IL-15 (Krenciute et al., 2017), and the redirection of immunosuppressive cytokine signaling (e.g., IL-4) towards proinflammatory cytokines (Mohammed et al., 2017).

7.5 CAR T-Cell-Associated Toxicities

T-cell therapy has been one of the most groundbreaking tools in cancer treatment; however, toxicities and associated fatalities have limited this approach's applications. To date, the characterization of the toxicities associated with CAR T-cell therapy has been broadly studied in patients receiving FDA-approved CAR T-cell therapy such as anti-CD19 CARs (Sterner and Sterner, 2021). Several factors determine the occurrence and intensity of (CRS), hemophagocytic lymphohistiocytosis (HLH), macrophage activation syndrome-like activation (MAS-L) (HLH/MASL), and immune effector cell-associated neurotoxicity syndrome (ICANS), including tumor type, specific target, and CAR design (Roex et al., 2020).

The most frequent acute toxicity associated with CAR T-cell therapy is the CRS; the cytokines involved are produced either by the infused CAR T-cells or by the CAR T-cell-responding immune cells such as macrophages. These cytokines include TNF-α, several interleukins such as IL-6, IL-2, -IL-2α, IL-8, IL-10, and IFN-γ, which were elevated in the patient's serum. Also, patients with severe CRS experience high-grade pyrexia, which can develop into an uncontrolled systemic inflammatory response with circulatory shock requiring vasopressors, vascular leakage, disseminated vascular clots, tachycardia, hypotension, hypoxia, and multi-organ system dysfunction. The severity of the CRS was correlated with the type of cytokines detected in the serum (Brudno and Kochenderfer, 2016; Shimabukuro-Vornhagen et al., 2018). Organ dysfunction can be reversed in most patients once CRS signs are recognized and managed early (Morris et al., 2021). Management of CRS using supportive care includes antipyretics, blood components transfusion, intravenous fluids, vasopressors, monoclonal antibodies (tocilizumab) used against the IL-6 receptor, and steroids in high-grade CRS. Both tocilizumab and steroids can control CRS in most cases. However, resistant CRS can also develop where the symptoms persist regardless of supportive treatments in a minority of patients, putting them at a high mortality risk (Yang X. et al., 2019).

ICANS is another common toxicity occurring after CAR T-cell infusion and is associated with treatment-related morbidity. However, the exact mechanism underlying the manifestation of neurologic toxicity remains indistinct. CAR T-cell facilitated inflammation-causing endothelial activation and disruption of the blood-brain barrier may play a central role (Holtzman et al., 2021). ICANS manifestation begins with toxic encephalopathy, aphasia, dysphasia, impaired motor function, and drowsiness. In severe cases, more severe symptoms occur, such as seizures, motor weakness, cerebral edema, and coma, most patients experiencing ICANS had earlier CRS that had subsided. Therefore, CRS could be considered an early sign of ICANS. Concurrence between ICANS and CRS occurs less frequently. ICANS is also reversible in patients who do not develop permanent neurological deficits (Morris et al., 2021). Management of ICANS aims to reduce the inflammatory response, which could be achieved by using Siltuximab (IL-6 antagonist), which prevents continuous IL-6 translocation across the blood-brain barrier (Gust et al., 2017). A high dose of corticosteroids shows sound central nervous system (CNS) penetration (Neelapu et al., 2017b). The use of levetiracetam or other antiepileptic agents can also be considered an option for treating severe neurological dysfunction as prophylaxis for seizures (Pehlivan et al., 2018). Additional studies are required to understand the mechanism underlying ICANS manifestation, associated risk factors, and optimal management required for CAR-T cell infusion.

HLH is a rare condition characterized by fever, hyperferritinema, splenomegaly, hypertriglyceridemia, coagulopathy, and cytopenia due to improper immune activation and cytokine release (Risma and Jordan, 2012a). In patients with low-grade CRS, HLH can occur; however, severe CRS might evolve into HLH. Thus, clinicians must pay attention to this condition to prevent fatal outcomes HLH/MAS post CAR T-cell therapy in association with CAR T-cell induced toxicities (CARTOX) score, which includes serum ferritin levels >10.000 ng/ml and one of the following: oliguria grade ≥3 or elevated serum creatinine grade ≥3, pulmonary edema, elevation in serum bilirubin, aspartate aminotransferase or alanine aminotransferase grade ≥3, and incidence of hemophagocytosis bone marrow (Mei et al., 2018). Management of HLH/MAS as mentioned in CRS and ICANS with anti-IL-6 agents and corticosteroids can be used. However, if the condition persists for almost 48 h, other interventions, such as intrathecal cytarabine and etoposide, especially in neurotoxicity-associated HLH (Neelapu et al., 2017b).

Several recommendations have been proposed to attenuate the toxicities resulting from CAR T-cells: 1) to ensure that the therapeutic efficacy is valid and no toxic overshooting of cytokines is occurring by monitoring the CAR T-cell activation threshold post-infusion. Activation of CAR T-cells is influenced by several factors, including tumor antigen expression levels on malignant cells, the affinity of the antigen-binding domain to target epitope, tumor burden, costimulatory elements of CARs (van der Stegen et al., 2015; Milone and Bhoj, 2018); 2) to achieve low affinity of the antigen-binding domain to ensure selectivity for tumors with high expression levels of targeted antigen; 3) hinge-region and transmembrane region modifications and optimization to control cytokine secretion levels and keep them within the therapeutic window as seen in anti-CD19 CAR T-cells where no CRS or ICANS were observed (Ying et al., 2019); 4) costimulatory domain can be customized based on tumor burden, tumor antigen binding domain engagement, antigen density, and toxicity concerns. Evidence suggests that 4-1BB costimulatory domains show lower toxicity risk, lower T-cell expansion levels, higher T-cell endurance. In contrast, CD28 costimulatory domains are associated with CAR T-cell onset rapid activation and consequent exhaustion. These properties make 4-1BB domains more preferable in cases of high disease burden or/and high tumor antigen density, and in cases of low surface antigen density or/and low-affinity antigen-binding domain CARs with CD28 costimulatory domains are more preferable (Salter et al., 2018); 5) CARs immunogenicity can be decreased by modifying

hinge region and/or transmembrane domain, which also contributes to CAR T-cell persistence improvement (Jonnalagadda et al., 2015; Sommermeyer et al., 2017); 6) neutralization of GM-CSF to overcome CRS and neurotoxicity, tyrosine hydroxylase inhibition by metyrosine or deletion of this enzyme in a myeloid cell-specific manner resulted in catecholamine and cytokine levels reduction (Staedtke et al., 2018), use of IL-1 antagonists to reduce neuroinflammation (Giavridis et al., 2018); 7) use of "off-switch" or suicide gene strategies to encourage selective elimination of CAR T-cells at the commencement of adverse events under a secondary agent control. However, the slow onset of antibody-mediated depletion limits the efficacy of this approach, especially in patients who require immediate reversal during acute and severe cytokine toxicities; therefore, faster switches such as inducible cas9 were developed and proved to deplete 90% of CAR T-cells within 30 min (Di Stasi et al., 2011; Jones et al., 2014). Engineering CAR T-cells with CD20 full-length expression or CD 20 mimotopes, which deplete CAR T-cells post rituximab treatment (Philip et al., 2014), use of switch off CARs (SWIFF-CARs) (Juillerat et al., 2019). The most significant limitation in utilizing the suicide gene strategy is the sudden cessation of therapy in rapidly progressing diseases, making this strategy a last resort. However, recently, the use of TKIs, which inhibit proximal TCR signaling kinases and suppress T cell activation (dasatinib), provide temporary inhibition of CAR T-cells. CAR T-cell activity would resume after toxicity has subsided (Sterner and Sterner, 2021). Additional studies are required to overcome all toxicities without affecting the activity and persistence of CAR-T cells.

7.6 Autologous Vs. Allogeneic

Although most of the clinical studies testing CAR T-cells depended on autologous T-cells, these therapies presented several limitations. The patient's cell generation is a cost-time-consuming process that holds a risk of manufacturing failure (Zhao J. et al., 2018). Additionally, it might result in a delayed availability of treatment, which could be problematic for patients with aggressive and highly proliferative diseases (Depil et al., 2020). The patients usually receive lymphodepleting chemotherapy, which might affect the quality and quantity of the starting autologous T cells (Ceppi et al., 2018); in contrast, allogeneic CAR-cells (derived from healthy donors) offer fully functional cells in high amounts allowing multiple generations of "off-the-shelf" CAR T cells products (Zhao J. et al., 2018; Depil et al., 2020). The heterogenic nature of tumor cell antigen expression and the immune evasion mechanisms developed by tumor cells require CAR T-cells with multiple antigen specificities (Walsh et al., 2019). This issue could be overcome by allogeneic T-cells capable of generating several CAR T-cells products with various antigen specifiers (multivalent), unlike autologous T-cells that are known to be capable of generating (monovalent) CAR T-cells (Martínez Bedoya et al., 2021). Allogeneic CAR T-cells can be obtained from several sources such as mononuclear cells from the peripheral blood of healthy donors that are capable of providing high numbers of fitter cells than the ones derived from the patients' blood as they have been subjected to radio- or chemotherapy (Depil et al., 2020). Umbilical cord blood is another source. Furthermore, adult somatic induced pluripotent stem cells (iPSC) can be produced by introducing specific transcription factors (Papapetrou, 2016) (**Figure 2**). Despite the advantages of allogeneic CAR T-cells, some limitations prevent their use in the CAR T-cells field. The first limitation is the graft-versus-host disease (GVHD) and the allo-rejection produced by the host immune cells, which would hinder the cells' anti-tumor activity (Martínez Bedoya et al., 2021). Changes within the design of the allogeneic CAR T-cells could overcome the GVHD; these changes include the employment of genetic engineering tools such as Zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN), and CRISPR/Cas9, which can be utilized in knocking-out T-cell receptor (TCR) and in attenuating the GVHD. Strategies to mitigate allorejection are being evaluated; chemo-resistant CAR T-cells are also being repeatedly tested through several rounds of administration to allow more profound or prolonged lymphopenia (Poirot et al., 2015; Valton et al., 2015). Overcoming the limitations of both autologous and allogeneic CAR T-cells is a great challenge but not impossible in such a fast-growing field.

8 CONCLUSION

The employment of adaptive immunity in treating chronic and malignant diseases has been the focus of many studies over the past few decades. The CAR T-cell revolution has changed the landscape of conventional therapies used in cancer and has provided new opportunities to test these technologies against other diseases. However, CAR T-cell therapy has few limitations, slowing its widespread clinical application as a routine treatment. To overcome these limitations, various *in vivo* and *in vitro* studies have suggested innovative strategies to enhance the efficacy of CARs against blood cancers and solid tumors. Several factors have been designated as necessary in CAR T-cell design, including tumor antigen expression levels on malignant cells, the affinity of the antigen-binding domain to the target epitope, tumor burden, and costimulatory elements of CARs. However, there is still a need to elucidate and resolve the issues associated with this intriguing technology. Therefore, further development of eccentric strategies to reduce CAR T-cell therapy limitations while maintaining antitumor efficacy, cellular persistence, and expansion will be necessary to magnify the clinical applications of this therapy. Notably, "off-the-shelf" CAR-T cell products with CRISPR-Cas9 genome-edited changes to manage toxicities and persistence will hold much promise. Additionally, the utilization of synthetic biology and cell engineering technologies might break the barriers impeding allogeneic CAR T-cells from being used as universal CAR T-cells, which could be pivotal in enhancing therapeutic outcomes and overall patient survival.

AUTHOR CONTRIBUTIONS

All authors listed had made a considerable, comprehensive, and perceptive contribution to the work and approved it for publication. AA, SA, and YA were involved in the study's design. The manuscript was drafted by AA, SA, YA, TA, SA, MA-S, and HA. SA and AA collaborated on the manuscript's review and editing. The final manuscript was read and approved by all authors.

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An Overview of Current Research on Mesenchymal Stem Cell-Derived Extracellular Vesicles: A Bibliometric Analysis From 2009 to 2021

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Background: Mesenchymal stem cell-derived extracellular vesicles (MSC-EVs) are important mediators of intercellular communication and participate in numerous physiological and pathological processes in the body. This study aims to introduce the research status, analyze the research hotspots, and predict the development trend through bibliometric analysis of MSC-EVs.

Methods: We searched all relevant literature on MSC-EVs from 2009 to 2021 in the Web of Science. R-bibliometrix, VOSviewer, and CiteSpace software were used to visualize the quantitative analysis of the published literature, including co-authorship, co-occurrence, citation, and co-citation, to provide objective presentation and predictions in the field.

Results: A total of 1595 articles and reviews on MSC-EVs published between 2009 and 2021 were identified. The annual publication outputs increased at an exponential rate, reaching as high as 555 publications in 2021. China contributed the most publications (n = 899, 56.36%) and had the most citations (n = 24,210). The United States had the strongest intensity of cooperation in this field. Shanghai Jiao Tong University had the maximum number of publications (n = 79). In terms of the number of publications and co-citations, the journal of Stem cell research & therapy ranked first. Camussi G was the most productive and most cited author. The top three themes in the research area were cell biology, research experimental medicine, and biochemistry molecular biology. Keyword co-occurrence and co-citation clustering analysis revealed that studies of MSC-EVs covered cellular origin (bone marrow mesenchymal stem cell, adipose-derived mesenchymal stem cell), injurious diseases (spinal cord injury, acute lung injury, ischemia/reperfusion injury, acute kidney injury, traumatic brain injury), tumor (breast cancer, tumor microenvironment), biological processes (drug delivery system, angiogenesis, inflammation, proliferation, differentiation, senescence), and molecular mechanisms (signaling pathway, signal transduction, oxidative stress, VEGF, TGF β).

Conclusions: Studies on MSC-EVs have shown a steep growth trend in recent years. Available studies mostly focused on the therapeutic effects and underlying mechanisms of

MSC-EVs in aplastic diseases. Multidisciplinary integration is a development trend in this field, and senescence-related topics might be the focus of future research on MSC-EVs.

Keywords: mesenchymal stem cell-derived extracellular vesicles, bibliometric analysis, CiteSpace, VOSviewer, therapeutic

1 INTRODUCTION

Extracellular vesicles (EVs) are nanoscale particles detached from the cell membrane or secreted by cells and carry the proteins, RNA, DNA, and lipids of the derived cells. EVs can be classified in different populations based on their biogenetic pathway, composition, and physical characteristics, such as size or density, giving rise to three major categories: apoptotic bodies (50–2000 nm), microvesicles (50–1500 nm), and exosomes (50–120 nm) (Simon et al., 2018). EVs, as mediators of material and signal communication between cells, participate in various physiological and pathological processes in the organism (Nawaz et al., 2016). From gonogenesis, embryogenesis to age-related cellular senescence, EVs are involved in almost the entire life cycle of human beings (Machtinger et al., 2016; Takasugi, 2018). Moreover, EVs play an important role in the occurrence and development of various diseases, such as cancer (Willms et al., 2018), neurodegenerative diseases (Hill, 2019), cardiovascular diseases (Gaceb et al., 2014), metabolic diseases (Akbar et al., 2019), and musculoskeletal diseases (Murphy et al., 2019). To date, the application potential of EVs is mainly in the diagnosis and treatment of diseases. Based on the analysis of EVs, cell-specific or disease-specific proteins can be identified, thereby contributing to disease diagnosis (Schou et al., 2020). The therapeutic potential of EVs, on the one hand, is as a vehicle for drug delivery (Kalluri and LeBleu, 2020), and on the other hand, the specific cell-derived EVs themselves have therapeutic effects.

In the 1970s, Friedenstein's research team extracted a type of spindle-shaped stromal cells from the bone marrow that can attach to plastic and differentiate into osteoblasts, chondrocytes, adipocytes, under corresponding *in vitro* conditions, and were subsequently defined as bone marrow-derived mesenchymal stem cells (BMSCs) (Väänänen, 2005). Mesenchymal stem cells (MSCs) are a class of adult stem cells originating from the mesoderm, with self-renewal and multi-directional differentiation potential (Li and Hua, 2017). MSCs can secrete a variety of cytokines and growth factors to regulate immunity, inhibit fibrosis and apoptosis, promote angiogenesis, activate endogenous stem/progenitor cells, and rebuild and maintain cellular microenvironmental niches (Andrzejewska et al., 2021). Furthermore, compared with embryonic stem cells, MSCs have the advantages of broad sources, easy access, low risk of tumorigenesis, and no ethical controversy (Li and Hua, 2017). Therefore, MSCs have become the main target of clinical translational research on stem cells.

In recent years, it has been demonstrated that the biological effects of MSCs largely depend on their secretome/EVs. After confirming that BMSCs and their conditioned medium can improve retinal ischemia/reperfusion injury, Mathew *et al.* further confirmed that EVs in the conditioned medium were the main components (Mathew et al., 2020). Zhang *et al.* compared the therapeutic effects of menstrual blood MSCs and small EVs derived from menstrual blood MSCs in the intrauterine adhesion (IUA) rat model, and no significant difference was detected between the two treatment methods. Specifically, both MSCs and small EVs could effectively repair endometrial damage, promote angiogenesis, and remodel fertility in IUA rats (Zhang S. et al., 2021). Mesenchymal stem cell-derived extracellular vesicles (MSC-EVs) can not only imitate the therapeutic effect of MSCs, but also have less risk, higher safety, and higher application value as a cell-free therapy. Moreover, MSC-EVs are not easily cleared by metabolism and can penetrate the blood-brain barrier due to their small size, which has advantages in the treatment of neurological diseases (Imafuku and Sjoqvist, 2021).

Bibliometric analysis is conducted to analyze the literature in the scientific field through qualitative and quantitative assessments, clarifies the development trends of scientific research objectively and visibly, and plays an important role in the current status of research and the prediction of the development direction (Brandt et al., 2019). In addition, it allows for the identification of collaborative relationships among authors, institutions, and countries, and the evaluation of the academic contribution of countries, institutions, journals, and authors in a specific field (Xia et al., 2022). Notably, the period of the included studies has a significant impact on the bibliometric results, therefore, timely updating is essential to grasp the research frontier. Recently, MSC-EVs have become a research hotspot, while the relevant bibliometric analysis studies are limited, mainly focusing on the EVs or exosomes without a specific source (Yiran et al., 2017; Wang et al., 2019), or the role of exosomes in a particular sort of disease, including cardiovascular disease, cancer (Ma et al., 2021; Shi et al., 2021; Teles et al., 2021). In this study, we performed a bibliometric analysis of literature on MSC-EVs published from 2009 to 2021, covering the number of publications, countries, institutions, authors, journals, and keywords, to summarise current thematic trends and hot topics and provide guidance for future research.

2 METHODS

2.1 Data Acquisition and Retrieval Strategy

Web of Science (WoS), a world-recognized database, was used to search for publications related to MSC-EVs. On December 31, 2021, we input the search terms "mesenchymal stem cell-derived extracellular vesicle", "mesenchymal stem cell-derived microvesicle", "mesenchymal stem cell-derived exosome", "mesenchymal stem cell-derived apoptotic body", "extracellular

vesicle derived from mesenchymal stem cell", "microvesicle derived from mesenchymal stem cell", "exosome derived from mesenchymal stem cell" and "apoptotic body derived from mesenchymal stem cell" in the WoS Core Collection including SCI-EXPANDED, with no restriction on language. The timespan was set from January 1, 2009, to December 31, 2021. A total of 2739 articles and reviews were obtained, and after excluding those on unrelated search topics (n = 1144), the remaining 1595 publications were used for subsequent analysis.

2.2 Bibliometric Analysis and Visualization

The analysis of annual production, the number of national, institutional, and journal publications, and the research areas were realized through the "Analyze Results" of WoS. The trend in publications over years was analyzed by the curve-fitting function of IBM SPSS Statistics 22.0 software (IBM Corp., Armonk, NY, United States). A two-tailed p-value < 0.05 was considered significant. Journal impact factors and category quartiles were acquired from the 2020 Journal Citation Reports (Clarivate Analytics, Philadelphia, United States). Bibliographic information for publications, including country distribution, year of publication, and citations, was automatically analyzed using the bibliometrix package in R 4.1.2. Statistical management of all data is carried out in Microsoft Excel 2016.

In this study, VOSviewer and CiteSpace are two key software for bibliometric analysis and visualization. WoS data were converted to txt format for further analysis. VOSviewer (1.6.17, Leiden University, the Netherlands) was employed to analyze citations of publications, co-authorship of countries/institutions/authors, co-citation of authors/journals/references, and co-occurrence of keywords. The VOSviewer parameter settings mainly included the counting method: full counting and item threshold, which was adjusted with different items. In the visual map, various nodes represent countries/institutions/journals/authors/references/keywords, the size of the nodes represents the number of publications/citations/frequency of occurrence, different colors represent clusters/the average appearing year, and the thickness of the line represents the strength of the link between the nodes. We used CiteSpace (5.8. R3) to construct a dual-map overlay for journals, cluster analysis of co-cited references and keywords, and detection of references and keywords with strong citation bursts (Chen, 2004). The parameters of CiteSpace were set as follows: link retaining factor (LRF = 3), look back years (LBY = 5), e for top N (e = 1), time span (2009–2021), years per slice (1), links (strength: cosine, scope: within slices), selection criteria (g-index: k = 25), and minimum duration (MD = 2 for keywords; MD = 5 for references).

3 RESULTS

3.1 Global Publication Trend

In total, 1595 publications related to MSC-EVs were identified in the WoS from 2009 to 2021. Of these, 1351 (84.70%) were indexed as "article" and 244 (15.30%) were indexed as "review". Between 2009 and 2021, the annual of publications grew rapidly from a few articles to over 500 articles (**Figure 1**). The annual growth trend is in line with the fitting curve $y = 0.510e^{0.5675x}$ (R^2 = 0.9731) (**Figure 1**). This indicated that MSC-EVs have gradually attracted the attention of scholars, and may become a long-standing research hotspot.

3.2 Distribution of Countries and Institutions

A total of 55 countries around the world contributed to the research on MSC-EVs (**Figure 2A**). Of these, 17 countries (30.9%) are in Europe, 10 countries (18.2%) in Asia, and two countries (3.6%) each in South America and North America. As shown in **Table 1** (Part A), China (899 publications, 56.36% of all articles) is the most productive country, followed by the United States (278, 17.43%), Italy (105, 6.58%), South Korea (80, 5.02%), and Iran (79, 4.95%). In terms of total citations, the top five countries are China (24,210 citations), the United States (14,615), Italy (6,485), Germany (4,652), and Japan (2,458) (**Figure 2B**). We analyzed the collaborative relationships between countries with more than 15 publications in the field, as shown in **Figure 3A**, where the size of the nodes represents the number of publications and the thickness of the connected lines represents the strength of the link. The United States has the highest total link strength (177 times) followed by China (132). The third to fifth places are Italy (64), Germany (59), and England (44).

A total of 1692 institutions are involved in this field. The top 10 institutions with the most publications are listed in **Table 1** (Part B). Shanghai Jiao Tong University (79 publications, 4.95% of all articles) contributed the maximum number of publications, followed by Nanjing Medical University (43, 2.70%), Zhejiang University (39, 2.45%), Central South University (37, 2.32%), and Jiangsu University (37, 2.32%). We analyzed the collaborations of 35 institutions with more than 15 publications. We excluded seven unrelated items and revealed the co-authorship of 28 institutions (**Figure 3B**). The five institutions with the highest total link strength were Shanghai Jiao Tong University (total link strength = 32 times), Second Military Medical University (23), Nanjing Medical University (21), Soochow University (18), and Tongji University (17). **Figure 3C** shows the average publication year of the 28 institutions above. The majority of institutions published papers after 2019, with greener or yellower colors. The institution with the earliest average year of publication is Jiangsu University (2018.25), and the institution with the latest average year of publication is Central South University (2020.64).

3.3 Analysis of Journals and Research Areas

Since 2009, a total of 1595 articles were published in 437 journals. The top 10 journals with the most publications are listed in **Table 2**. Stem cell research & therapy (147 publications, 9.22% of all articles) had the most publications, followed by International journal of molecular sciences (44, 2.76%), and Stem cells international (43, 2.70%). We analyzed a total of 120 journals that were co-cited more than 150 times (**Figure 4A**).

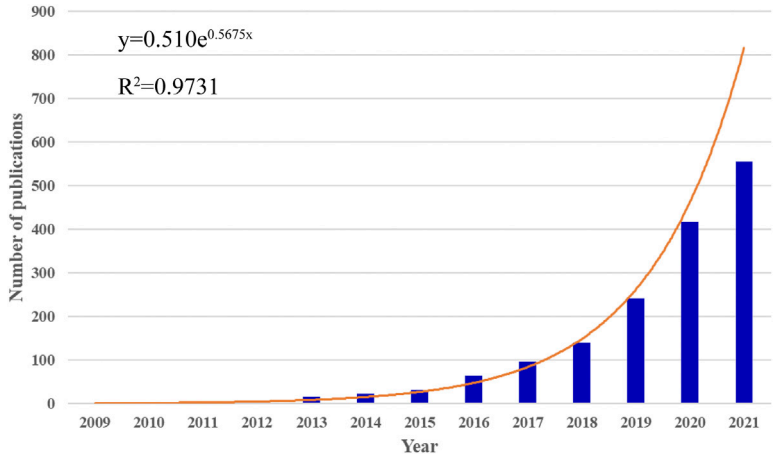

FIGURE 1 | Global trends in publications on MSC-EVs. The bar graph displays the trends in the growth of publications worldwide from 2009 to 2021. The curve shows the model fitting curves of growth trends in publications.

Table 2 shows the top 10 co-cited journals that published related articles. Top of the list is still Stem cell research & therapy (2707 citations), followed by Stem cells (2309), and Plos one (2274).

Overall, the identified publications are grouped into 63 research areas. As shown in **Table 3**, the most representative research area was Cell Biology (672 records, 42.13% of all articles), followed by Research Experimental Medicine (406, 25.46%), and Biochemistry Molecular Biology (185, 11.60%). In addition, a dual-map overlay of journals was used to analyze the association of subject categories between citing and cited journals. The spline wave from left to right describes the citation path, and this interaction illustrates the linkage of different research areas. Only one critical citation path marked in orange indicates that papers published in the journals in the area of Molecular/Biology/Immunology usually cited papers published in Molecular/Biology/Genetics journals (**Figure 4B**).

3.4 Analysis of Authors

The top 12 authors with the most publications and most citations are shown in **Table 4**. Camussi G, from the University of Torino, is the most productive author (21 articles), followed by Eirin A, from Mayo Clinica (19), and Lerman LO, from Mayo Clinica (16). As to citations in this field, Camussi G was ranked first (3379 citations) as well, followed by Bruno S, from the University of Torino (3,368), and Ciro Tetta, from Unicyte Srl (2,776). Notably, Camussi G had the highest number of publications and citations, indicating that he is probably at present the most active scholar and has made significant contributions to the development of the field.

We detected a total of 102 authors who co-authored more than five publications. Excluding the 64 items that were not connected, collaborations of 38 authors were shown (**Figure 5A**). The five authors with the highest total link strength were Qian H (total link strength = 74 times), Xu W (74), Yan Y (63), Zhang X (53), and Zhang B (49). **Figure 5B** showed the co-citation relationships for a total of 21 authors with more than 150 citations. In first place was Lai RC, with 601 citations, followed by Xin HQ (516), Bruno S (453), Théry C (390), and Zhang B (373).

3.5 Citation and Co-Citation Analyses

A total of 76 articles in this field have more than 150 citations (**Figure 6A**). The top ten most cited documents are shown in **Table 5**. There were 859 citations for "Mesenchymal stem cell-derived microvesicles protect against acute tubular injury", followed by "Mesenchymal stem cell-derived exosomes increase ATP levels, decrease oxidative stress and activate PI3K/Akt pathway to enhance myocardial viability and prevent adverse remodeling after myocardial ischemia/reperfusion injury", with 666 citations. The third-ranked article with the largest number of citations was "Concise Review: MSC-Derived Exosomes for Cell-Free Therapy", with 642 citations.

In total, 27 references, co-cited in more than 50 citations, were analyzed by VOSviewer (**Figure 6B**). **Table 6** lists the top ten references with the highest citations. The top five references with the largest number of citations were by Lim SK (2010; 301 citations), Lötvall JO (2007; 239 citations), Camussi G (2009; 199 citations), Dominici M, (2006; 181 citations), and Théry C (2018; 177 citations). The co-cited references were then clustered based on indexing terms. As shown in **Figure 6C**, the co-cited references were clustered into 16 major clusters: myocardial infarction, breast cancer, osteoarthritis, microvesicle, metabolic syndrome, spinal cord injury, senescence, bronchopulmonary dysplasia, vascular endothelial growth factor, tumor microenvironment, neurodegenerative disorders, acute lung injury, intervertebral disc degeneration, xenograft model, signal transduction, and reproductive toxicity.

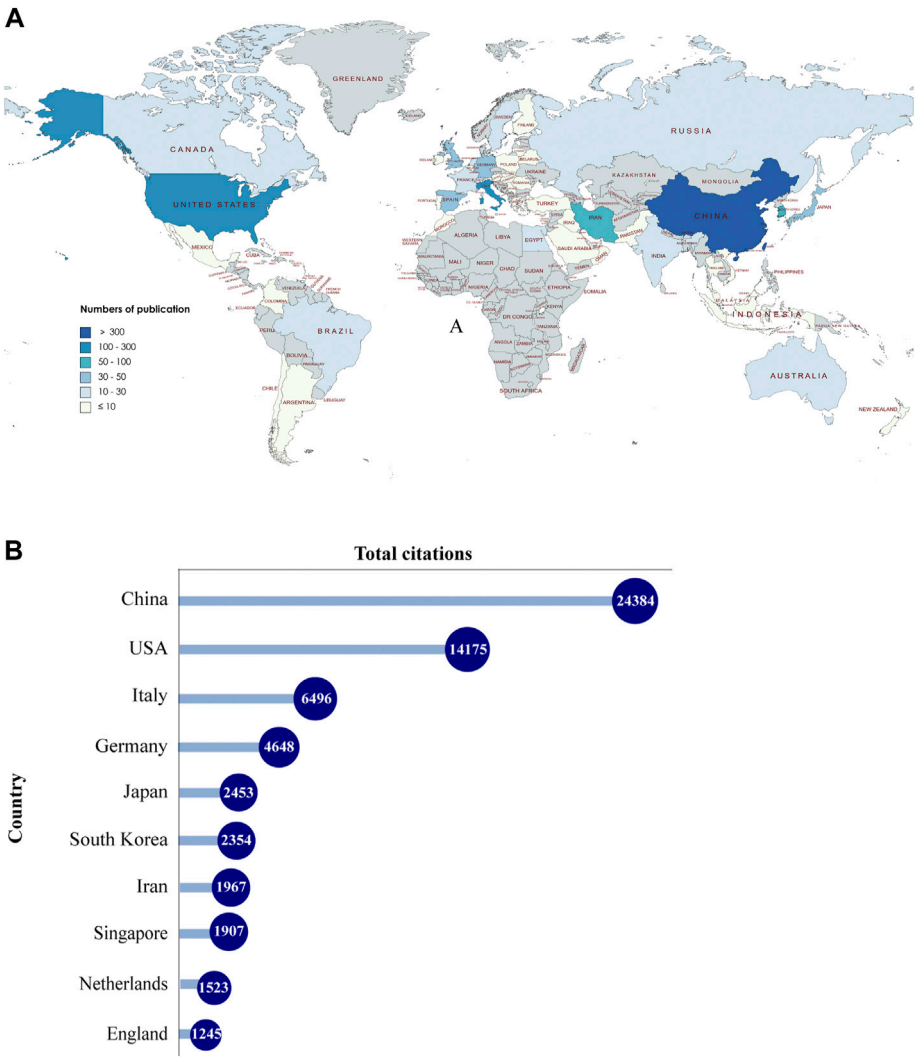

FIGURE 2 | Countries contributing to MSC-EVs. **(A)** World map showing the distribution of countries in this field. **(B)** Top 10 countries with the most total citations.

TABLE 1 | The top 10 countries and institutions with the most publications in the field of MSC-EVs.

Rank	Part A			Part B	
	Countries	Number of Publications	Number of Publications per 10 Million Population	Institutions	Number of Publications
1	China	899	6.37	Shanghai Jiao Tong University (China)	79
2	United States	278	8.39	Nanjing Medical University (China)	43
3	Italy	105	17.66	Zhejiang University (China)	39
4	South Korea	80	15.43	Central South University (China)	37
5	Iran	79	9.41	Jiangsu University (China)	37
6	Germany	50	6.01	University Of Turin (Italy)	36
7	Japan	45	3.58	Zhengzhou University (China)	36
8	Spain	41	8.66	Sun Yat Sen University (China)	35
9	England	35	5.21	University Of California System (United States)	34
10	France	30	4.45	Shandong University (China)	32

The top 25 references with the strongest citation bursts are presented in **Figure 6D**, among which, the article titled "Exosomes derived from human umbilical cord mesenchymal stem cells alleviate liver fibrosis", published in 2013, ranked first (strength = 26.1). Moreover, the citation bursts of articles published by Rani S, Baglio SR, and Zhang YL all lasted from 2016 to 2021.

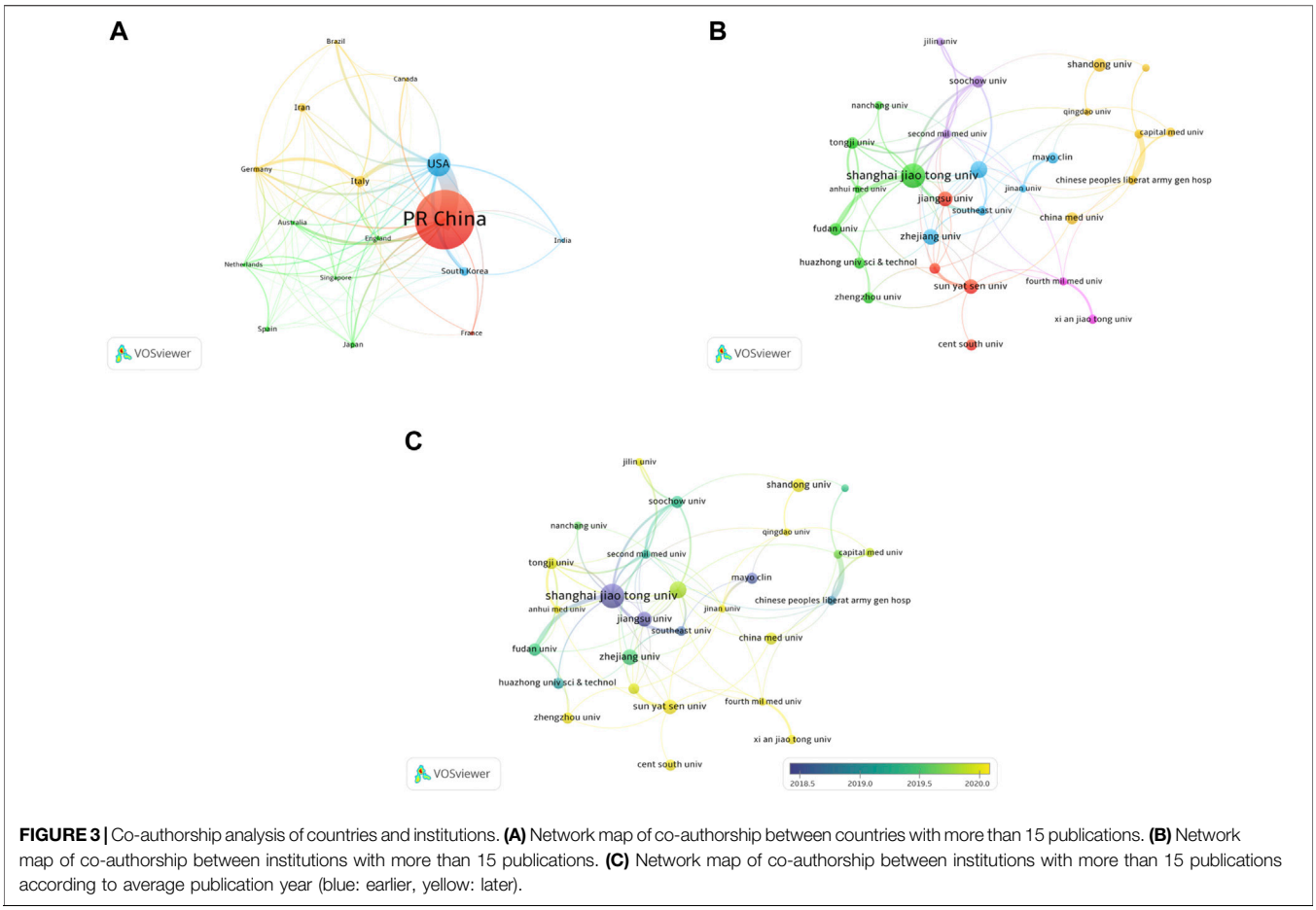

FIGURE 3 | Co-authorship analysis of countries and institutions. **(A)** Network map of co-authorship between countries with more than 15 publications. **(B)** Network map of co-authorship between institutions with more than 15 publications. **(C)** Network map of co-authorship between institutions with more than 15 publications according to average publication year (blue: earlier, yellow: later).

TABLE 2 | The top 10 most active journals and co-cited journals.

Rank	Top Journals	Records (n)	2020 IF	2020 JCR	Co-Cited Journals	Citations (n)	2020 IF	2020 JCR
1	Stem cell research & therapy	147	6.832	Q1	Stem cell research & therapy	2707	6.832	Q1
2	International journal of molecular sciences	44	4.101	Q2	Stem cells	2309	6.277	Q1
3	Stem cells international	43	5.443	Q2	Plos one	2274	3.24	Q2
4	Frontiers in cell and developmental biology	35	6.684	Q1	Scientific reports	1788	4.38	Q1
5	Scientific reports	34	4.38	Q1	Journal of extracellular vesicles	1655	25.841	Q1
6	Journal of cellular and molecular medicine	33	5.31	Q2	Stem cells and development	1320	3.272	Q2
7	Cells	29	6.6	Q2	Stem cells translational medicine	1217	6.94	Q1
8	Plos one	26	3.24	Q2	International journal of molecular sciences	1144	4.101	Q2
9	Aging-us	24	5.682	Q1	Stem cells international	1017	5.443	Q2
10	Life sciences	24	5.037	Q1	Blood	973	23.629	Q1

IF, impact factor; JCR, journal citation reports; Q, quartile in category.

3.6 Co-Occurrence Analysis of Keywords

VOSviewer was employed to analyze the keywords that occurred more than 25 times in all publications included. We obtained a total of 88 keywords, of which the five with the highest frequency were exosomes (787 times), extracellular vesicles (659), mesenchymal stem cells (374), stromal cells (370), and microvesicles 258) (**Figure 7A**). The average publication year of the identified keywords is indicated in the overlay visualization (**Figure 7B**). The chronological order is presented from dark blue to bright yellow. The majority of the keywords were published during 2019, while small extracellular vesicles, cartilage, chondrocytes, and senescence were relatively new keywords that emerged after 2020.

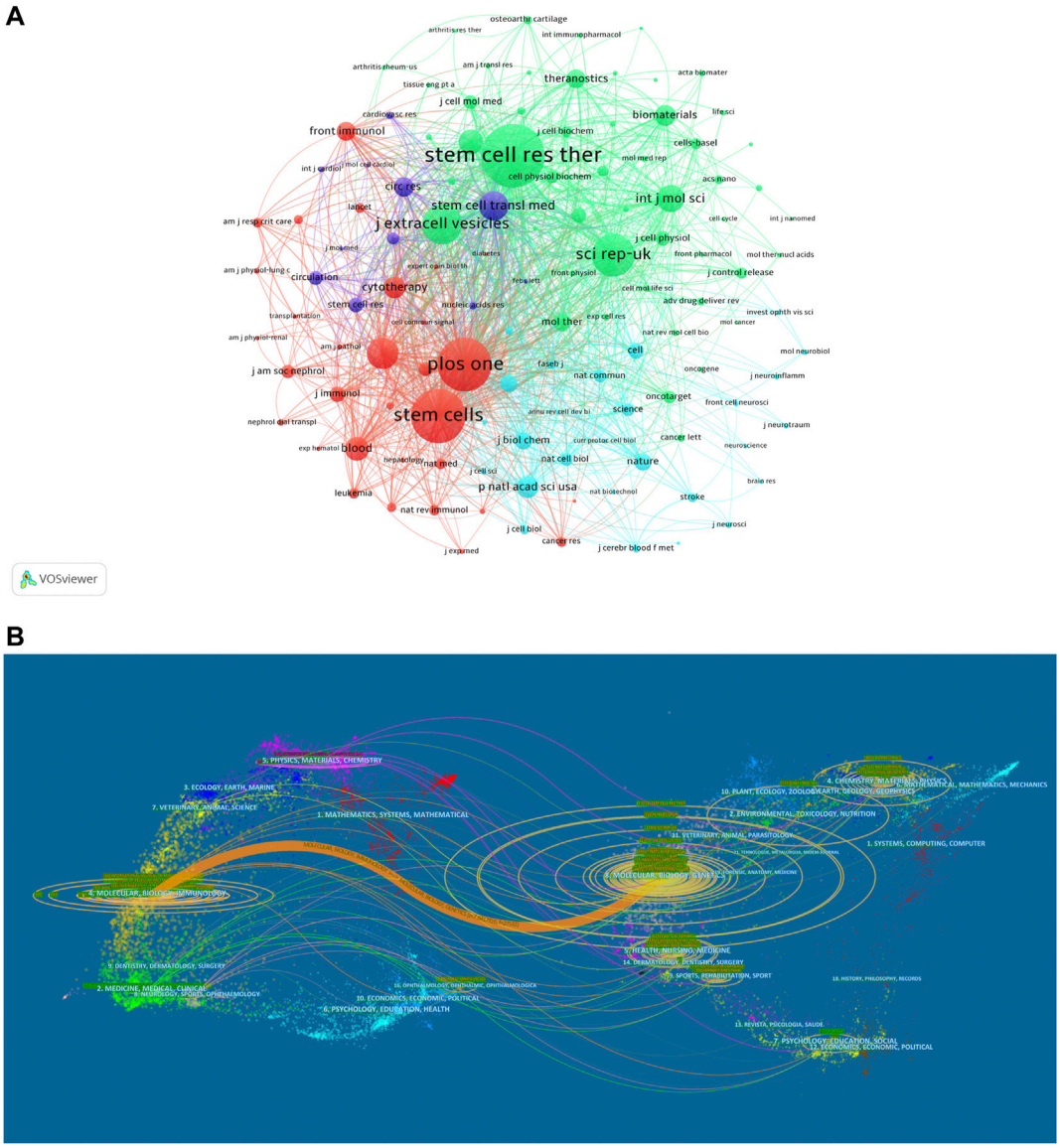

FIGURE 4 | Articles published in different journals on MSC-EVs. **(A)** Network map of journals that were co-cited in more than 150 publications. **(B)** The dual-map overlay of journals related to MSC-EVs.

TABLE 3 | The top 10 well-represented research areas.

Rank	Research Areas	Records (n)	% (of 1595)
1	Cell Biology	672	42.13
2	Research Experimental Medicine	406	25.46
3	Biochemistry Molecular Biology	185	11.60
4	Science Technology Other Topics	149	9.34
5	Pharmacology Pharmacy	131	8.21
6	Biotechnology Applied Microbiology	116	7.27
7	Oncology	109	6.83
8	Chemistry	87	5.46
9	Materials Science	83	5.20
10	Engineering	71	4.45

The time dynamic evolution of keywords clusters is presented in **Figure 7C**. In total, 19 clusters were identified, namely, spinal cord injury, mesenchymal stem cells, extracellular vesicles, renovascular disease, bone regeneration, ischemia/reperfusion injury, TGF β, rheumatoid arthritis, myocardial infarction, signaling pathway, osteoarthritis, drug delivery system, reperfusion injury, angiogenesis, bone marrow mesenchymal stem cells, white matter injury, Alzheimer's disease, acute myeloid leukemia, and adipose-derived mesenchymal stem cell. The average year of appearance for TGF-β was most recent, 2020, and the average year of appearance for osteoarthritis and acute myeloid leukemia was 2019.

TABLE 4 | The top 12 authors with the most publications and citations on MSC-EVs research.

Rank	Highly Published Authors	Country	Number of Publications	Highly Cited Authors	Country	Total Citations (n)
1	Camussi G	Italy	21	Camussi G	Italy	3379
2	Eirin A	United States	19	Bruno S	Italy	3368
3	Lerman LO	United States	16	Tetta C	Italy	2776
4	Qian H	China	15	Deregibus MC	Italy	2620
5	Bruno S	Italy	15	Qian H	China	2052
6	Liu W	China	15	Xu W	China	2051
7	Xu W	China	14	Collino F	Italy	1979
8	Giebel B	Germany	13	Chopp M	United States	1614
9	Orfei CP	Italy	11	Yan Y	China	1508
10	Ragni E	Italy	11	Zhu W	China	1497
11	De Girolamo L	Italy	11	Lai RC	Singapore	1486
12	Wang Z	China	11	Grange C	Italy	1448

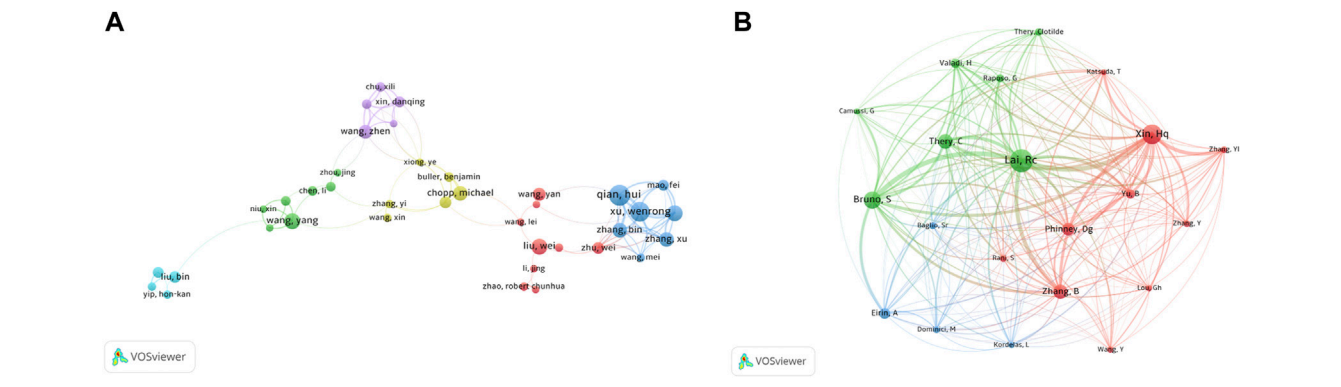

FIGURE 5 | Analysis of authors. **(A)** Network map of co-authorship between authors with more than five publications. **(B)** Network map of co-cited authors with more than 150 citations.

We detected the burst of keywords based on CiteSpace's algorithm of burst detection, where the minimum duration of the burst was set to 2 years. The discontinuous blue lines represent the timeline, specifically, each small blue rectangle represents 1 year, and the red part in the timeline represents the burst duration of the keyword. The top 20 keywords with the highest burst strength are shown in **Figure 8**. The most intense keyword was microvesicle (strength = 10.35), followed by horizontal transfer (8.98) and microparticle (7.33). The keyword with the longest burst time was messenger RNA, which lasted 9 years from 2009 to 2017. More meaningfully, the keyword "senescence" had outbreak citations most recently (2020-2021), which implied that the research on the linkage between MSC-EVs and aging might be research hotspots in the future.

4 DISCUSSION

In this study, we performed a bibliometric analysis of the literature published in the field of MSC-EVs from 2009 to 2021 based on visual management software. The temporal and spatial distribution of the literature, the contributions of countries, institutions, journals, authors, keywords, research areas, and future research hotspots were analyzed to clarify the evolutionary process and the changing in research focus and to guide researchers.

4.1 Overview of the Development of MSC-EVs

The earliest article that fits the theme of this study was published in 2009 by Bruno S et al. (Bruno et al., 2009). Since 2018, annual publications began to increase rapidly, with more than 500 publications in 2021, and articles in the past 3 years accounted for 76.11% of all identified articles. All these suggest that the research on MSC-EVs is developing rapidly as an emerging field. Furthermore, most of the top 10 most active journals and co-cited journals have impact factors above 5, indicating that the research in this field has relatively high academic value and the research findings are widely recognized. Stem cell research & therapy, which ranked first in both the number of publications and citations, reaching the top 1/4 (Q1) in the categories of Cell & Tissue Engineering, Cell Biology, and Medicine, Research & Experimental.

In terms of national contributions, China had the largest number of publications and citations in the world, indicating that China has become the most significant contributor to MSC-EVs research. We further analyzed the citations on average among countries with ≥50 publications and observed that Germany, Italy, United States were the top three countries with 92.96, 61.87, and 50.99 citations, respectively. Additionally, the United States played a leading role in this field, as it was far ahead of other countries in terms of centrality (0.52) and strength of collaboration (177). These findings suggested that the number of publications did not fully represent academic influence, and countries should encourage original creative discoveries and technological innovations beyond mere imitations and additions of previous breakthroughs. Ranking the academic achievements of research institutions, eight of the top 10 institutions came from China. The University of Turin in Italy had the most average citations (81.39) and an earlier publish year (2016.52).

4.2 Influential Authors and Studies in the MSC-EVs Research

Camussi G has published 21 papers on MSC-EVs, with a total of 3379 citations, and is the most productive and most cited author. From 2009 to 2021, Camussi G had publications every year except 2015 and could be considered a pioneer in the field. Camussi G mainly focused on the function of MSC-EVs in the repair of tissue damage and the key roles of various RNAs therein. In 2010, it was proposed that ribonucleoproteins involved in mRNAs and miRNAs transport, processing, and stabilization were present in MSC-EVs and that miRNAs highly expressed in extracellular vesicles might be involved in multi-organ development, cell

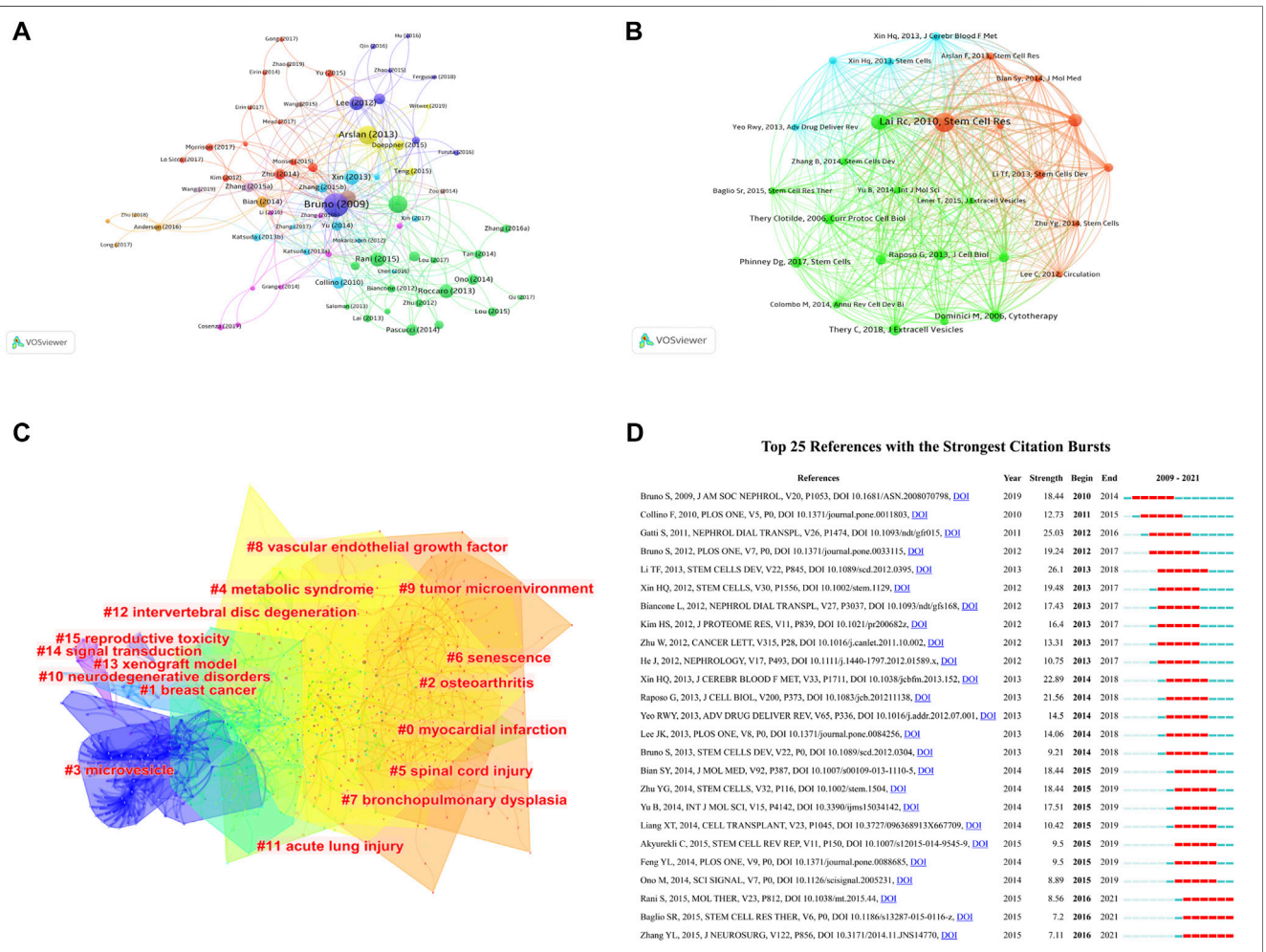

FIGURE 6 | Mapping of documents and references in studies on MSC-EVs. **(A)** Network map of citation analysis of documents with more than 150 citations. **(B)** Network map of co-citation analysis of references with more than 50 citations. **(C)** Clustering analysis of the co-citation network based on CiteSpace. **(D)** Top 25 references with strongest citation bursts based on CiteSpace.

TABLE 5 | The top 10 documents with the most citations in the field of MSC-EVs.

Rank	Title	Corresponding Author	Journal	2020 IF	Publication year	Total citations (n)
1	Mesenchymal stem cell-derived microvesicles protect against acute tubular injury	Camussi G	Journal of the american society of nephrology	10.121	2009	859
2	Mesenchymal stem cell-derived exosomes increase ATP levels, decrease oxidative stress and activate PI3K/Akt pathway to enhance myocardial viability and prevent adverse remodeling after myocardial ischemia/reperfusion injury	Arslan F	Stem cell research	2.02	2013	666
3	Concise Review: MSC-Derived Exosomes for Cell-Free Therapy	Phinney DG	Stem cells	6.277	2017	642
4	Microvesicles derived from human adult mesenchymal stem cells protect against ischaemia–reperfusion-induced acute and chronic kidney injury	Camussi G	Nephrology dialysis transplantation	5.992	2011	540
5	Systemic administration of exosomes released from mesenchymal stromal cells promote functional recovery and neurovascular plasticity after stroke in rats	Chopp M	Journal of cerebral blood flow and metabolism	6.2	2013	526
6	Mesenchymal Stem Cell-derived Extracellular Vesicles: Toward Cell-free Therapeutic Applications	Rani S	Molecular therapy	11.454	2015	519
7	BM mesenchymal stromal cell-derived exosomes facilitate multiple myeloma progression	Ghobrial IM	Journal of clinical investigation	14.808	2013	508
8	Exosomes mediate the cytoprotective action of mesenchymal stromal cells on hypoxia-induced pulmonary hypertension	Kourembanas S	Circulation	29.69	2012	503
9	Microvesicles derived from adult human bone marrow and tissue specific mesenchymal stem cells shuttle selected pattern of miRNAs	Collino F	Plos one	3.24	2010	437
10	Paclitaxel is incorporated by mesenchymal stromal cells and released in exosomes that inhibit *in vitro* tumor growth: a new approach for drug delivery	Pessina A	Journal of controlled release	9.776	2014	420

IF, impact factor.

TABLE 6 | The top ten co-citation analysis of cited reference on MSC-EVs.

Rank	Title	Corresponding Author	Journal	2020 IF	Publication year	Total citations (n)
1	Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury	Lim SK	Stem cell research	2.02	2010	301
2	Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells	Lötvall JO	Nature cell biology	28.824	2007	239
3	Mesenchymal stem cell-derived microvesicles protect against acute tubular injury	Camussi G	Journal of the american society of nephrology	10.121	2009	199
4	Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement	Dominici M	Cytotherapy	5.414	2006	181
5	Minimal information for studies of extracellular vesicles 2018 (MISEV 2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines	Théry C	Journal of extracellular vesicles	25.841	2018	177
6	Isolation and characterization of exosomes from cell culture supernatants and biological fluids	Théry C	Current protocols in cell biology[a]	NA	2006	171
7	Concise Review: MSC-Derived Exosomes for Cell-Free Therapy	Phinney DG	Stem cells	6.277	2017	170
8	Extracellular vesicles: exosomes, microvesicles, and friends	Raposo G	Journal of cell biology	10.539	2013	161
9	Mesenchymal Stem Cell-derived Extracellular Vesicles: Toward Cell-free Therapeutic Applications	Rani S	Molecular therapy	11.454	2015	150
10	MSC-derived exosomes: a novel tool to treat therapy-refractory graft-versus-host disease	Kordelas L	Leukemia	11.528	2014	145

IF, impact factor.

[a]Current protocols in cell biology *was not included in the 2020 Journal Citation Reports.*

survival, and differentiation (Collino et al., 2010). Camussi G was also devoted to the effect of MSC-EVs on renal injury, liver fibrosis, diabetes and its complications (wound ulcers), ischemia/reperfusion injury, brain injury, and tumors (Bruno et al., 2009; Bruno et al., 2014; Favaro et al., 2016; Lonati et al., 2019; Sisa et al., 2019; Chiabotto et al., 2021; Pomatto et al., 2021). Furthermore,

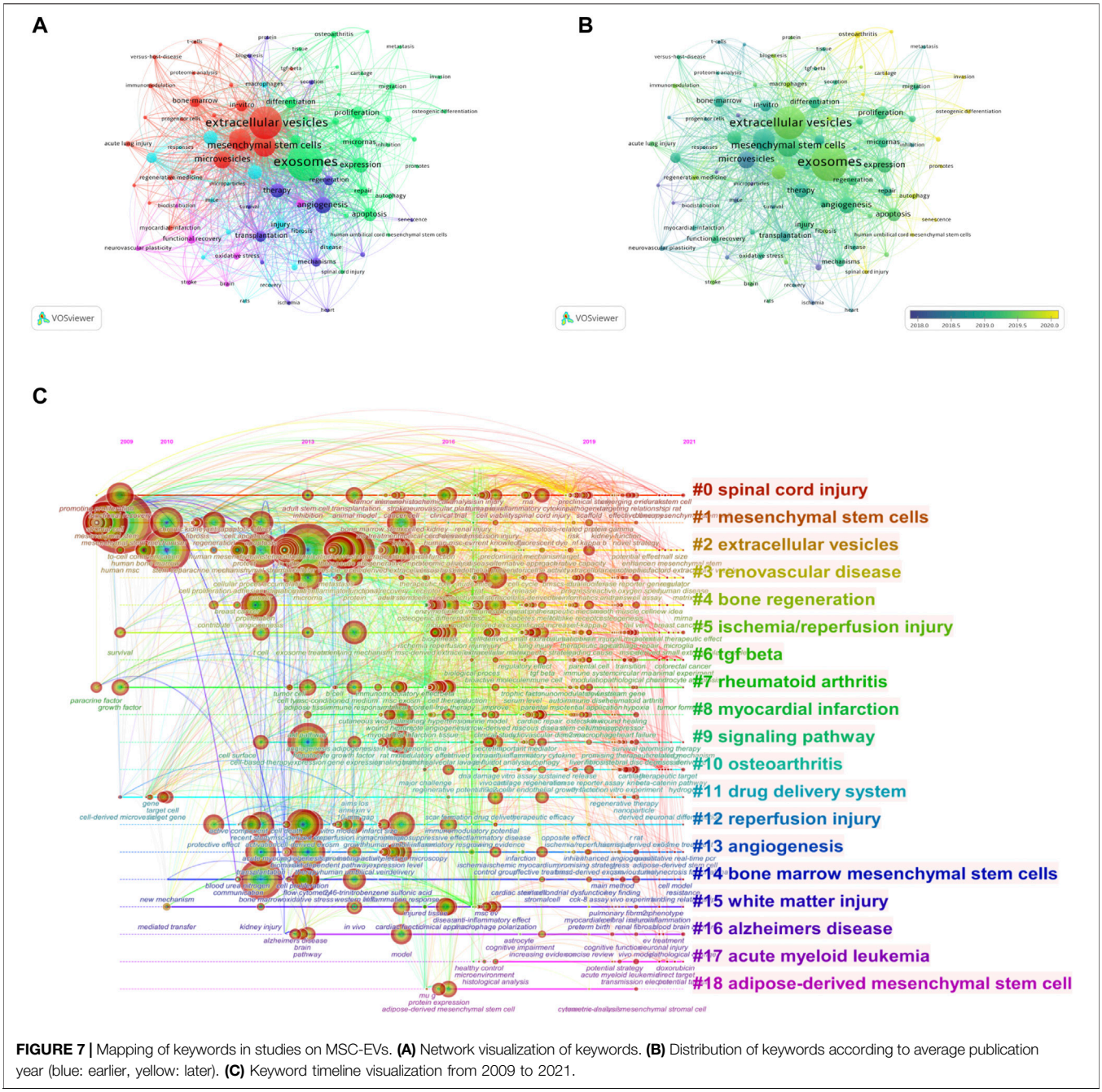

FIGURE 7 | Mapping of keywords in studies on MSC-EVs. **(A)** Network visualization of keywords. **(B)** Distribution of keywords according to average publication year (blue: earlier, yellow: later). **(C)** Keyword timeline visualization from 2009 to 2021.

Ratajczak MZ, Ratajczak J, Quesenberry PJ, Aliotta JM, and Lim SK *et al.* also laid a strong foundation for the pioneering field of MSC-EVs. In 2006, the Ratajczak laboratory from the University of Louisville demonstrated that embryonic stem cell (ES)-derived microvesicles can mediate the expansion and upregulation of pluripotency in hematopoietic progenitor cells through horizontal transfer of ES-derived mRNA (Ratajczak et al., 2006). The Rhode Island Hospital research team, to which Quesenberry PJ and Aliotta JM belonged, began to focus on the impact of niche on stem cell fate in 2007 (Aliotta et al., 2012) and proposed that microvesicles in the niche could affect the differentiation of marrow stem cells (Quesenberry and Aliotta, 2008; Quesenberry et al., 2015). And they had cooperated with Camussi G to conduct research on MSC-EVs since 2014 (Lindoso et al., 2014). Professor Lim SK from Nanyang Technological University analyzed the conditioned medium of ES-derived MSCs. The results suggested that MSCs could secrete abundant proteins and microparticles enriched for pre-miRNA, and these gene products were predicted to drive three major groups of biological processes: metabolism, defense response, and tissue differentiation (Sze et al., 2007; Chen et al., 2009). In brief, the early contributions of the above

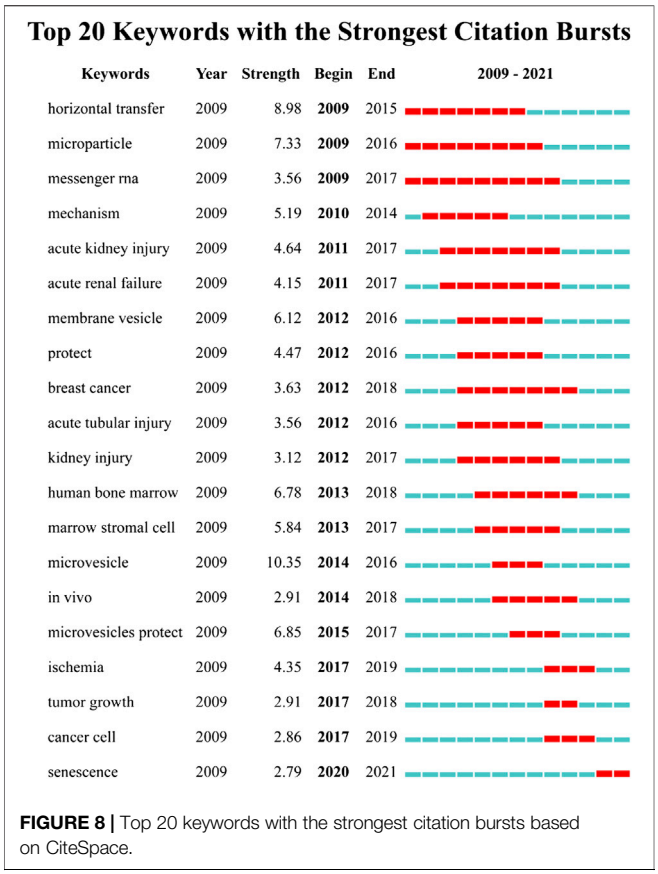

FIGURE 8 | Top 20 keywords with the strongest citation bursts based on CiteSpace.

teams all provided a solid foundation for the shift from paracrine to EVs in the study of MSCs.

Notably, collaboration among authors in this field is not close. Of the 102 authors with more than five publications, only 38 authors (approximately 1/3) could construct collaborative networks. Furthermore, in the near-linear cooperation map, almost all scholars are Chinese, and the cooperation is mostly confined within the research team. Scholars from different countries should strengthen cooperation, share advantageous platforms, exchange research progress, and form complementary advantages to achieve technological innovation and breakthroughs for the clinical translation of MSC-EVs.

The citation analysis of the publications and the co-citation analysis of references suggest the current research focuses on the therapeutic role of MSC-EVs in tissue repair and functional remodeling. In 2009, the study by Bruno S et al., with the most citations, reported that MSC-derived microvesicles initiate a proliferative program by transferring mRNA to residual renal tubular cells, thereby rescuing acute renal tubular injury (Bruno et al., 2009). The second most cited was the publication by Arslan F et al. which reported that infusion of MSC-derived exosomes into mice ischemic myocardium through the aorta could prevent ventricular dilatation and improve cardiac function (Arslan et al., 2013). Exosomes can improve energy imbalance, oxidative stress, and inflammatory responses induced by ischemia/reperfusion. Among the references with high co-citations, more attention lay on the extraction and identification criteria of EVs, in addition to the therapeutic potential of MSC-EVs in traumatic diseases. Théry C proposed a specific protocol for the isolation and characterization of exosomes from cell culture supernatants and biological fluids in 2006 (Dominici et al., 2006). In 2018, the International Extracellular Vesicle Society (ISEV) updated the Minimal Information for Studies of Extracellular Vesicles guidelines with the most important statement that exhaustive reporting is required when conferring specific functions on EVs, and when describing the delicate activities of EVs careful interpretation is necessary, given that the molecular mechanisms of its biogenesis and release remain to be explored (Théry et al., 2018). Notably, the guidelines or statements issued by the ISEV and the International Society for Cellular and Gene Therapies (ISCT) are recognized as the standard that MSC-EVs research should follow. The research group led by Rohde E, Lim SK, and Giebel B, respectively, contributed important knowledge to the field of MSC-EVs, and they participated in drafting several statements or reviews as members of ISEV and ICTG, including defining mesenchymal stromal cell (MSC)-derived small extracellular vesicles for therapeutic applications (Witwer et al., 2019), applying extracellular vesicles based therapeutics in clinical trials - an ISEV position paper (Lener et al., 2015), developing Best-Practice Models for the Therapeutic Use of Extracellular Vesicles (Reiner et al., 2017), and ISEV and ISCT statement on extracellular vesicles from mesenchymal stromal cells and other cells: considerations for potential therapeutic agents to suppress coronavirus disease-19 (Börger et al., 2020). Moreover, studies on RNAs wrapped in EVs were also the focus of high co-cited references and strong citation bursts in recent years. Valadi H et al. defined an exosomal shuttle RNA, which mainly includes mRNAs and miRNAs (Valadi et al., 2007). Most of the mRNAs were not present in the cytoplasm of the donor cell but could be delivered to the recipient cell and translated into new proteins to function. In an analysis of exosomes from BMSCs and adipose-derived MSCs (ADSCs), Baglio SR et al. demonstrated that stemness and multipotent of MSCs affected the composition of exosomes and that the RNA composition of exosomes was not the same as those of MSCs, with exosomes being able to selectively incorporate specific miRNAs (Baglio et al., 2015).

4.3 Research Hotspots and Future Trends

As shown in **Table 5**, MSC-EVs involved a wide range of research areas, forming a multidisciplinary convergence pattern dominated by cell biology. In particular, the emergence of materials science and engineering in the study of MSC-EVs strengthened the development of their therapeutic role. Different from MSCs, EVs lack the ability to self-renew and proliferate, hence the duration of activity maintenance is short. Yang et al. developed an injectable Diels–Alder crosslinked hyaluronic acid/PEG hydrogel, which achieved the sustained release of MSC-EVs in the joint cavity through degradation control, and hence, improved the therapeutic effect of osteoarthritis (Yang et al., 2021). Nanohydroxyapatite/poly-ε-caprolactone scaffolds with hyaluronic acid hydrogels mixed with MSC-derived exosomes as a sustained-release system could promote angiogenesis and osteogenesis (Zhang Y. et al., 2021).

Engineered EVs are obtained by genetic modification of donor cells or chemical modification of EVs for targeted delivery, increasing local concentrations at diseased sites, reducing toxicity and side effects, and maximizing therapeutic efficacy (Liang et al., 2021). Tsai HI et al. modified MSCs to obtain engineered EVs expressing FGL1 and PD-L1 on the membrane surface, which could target and bind to LAG-3 and PD-1 on the surface of T cells, thereby inhibiting T cell activation, and inducing immune tolerance in a heart allograft model (Tsai et al., 2022). The multidisciplinary combination helped to broaden the research horizon, overcome the research challenges, and lay the foundation for clinical translation in the field of MSC-EVs.

As shown in **Figure 7**, we can note that the therapeutic effects of MSC-EVs involved multi-system diseases, including neurological diseases, respiratory diseases, cardiovascular diseases, liver and kidney injuries, endocrine diseases, bone and joint diseases, reproductive system diseases, immune diseases, and cancer. The two diseases that appeared most frequently in the keywords were osteoarthritis and cancer. Osteoarthritis is a degenerative disease characterized by damage to cartilage, and cartilage degeneration is the most basic pathological change. MSC-derived exosomes could promote the proliferation of chondrocytes and inhibit their apoptosis to regenerate cartilage (Liu et al., 2018). Coordination the local inflammatory and regeneration microenvironment is crucial to the treatment of OA (Zhao et al., 2020). Zhang *et al.* demonstrated that MSC-derived exosomes attenuated inflammation and suppressed cartilage degeneration in the early stage, and subsequently restore matrix homeostasis for joint recovery and regeneration (Zhang et al., 2019). MSC-EVs may both promote tumor development and act as anti-tumor agents. MSCs in the tumor microenvironment could be reprogrammed by exosomes secreted by tumor cells, and MSC-derived exosomes then horizontally transferred information to neighboring cells, transforming the cellular milieu into one supportive of the tumor survival (Whiteside, 2017). Different MSC-EVs had diverse effects on specific tumors. For example, Guo *et al.* discovered that EVs derived from human umbilical cord-derived MSCs (hUCMSCs) promoted proliferation, migration, and invasion of lung cancer and accelerated tumor progression (Guo et al., 2021). Nevertheless, another study on the treatment of lung cancer with BMSCs-derived EVs suggested that EVs-derived let-7i could inhibit tumor proliferation and metastasis (Liu et al., 2021). Such differences might arise from the heterogeneity of MSCs, differences in EVs cargo, the diversity of malignancies' origin, and inconsistencies in experimental conditions (Weng et al., 2021). Using bioengineered EVs as delivery vehicles appears to be a more promising therapeutic approach in cancer, as they can transfer desired cargoes and confer enhanced targeting specificity (Walker et al., 2019).

From the co-occurrence of keywords, it can be observed that MSC-EVs come from a wide range of sources, among which BMSCs, ADSCs, and hUCMSCs are the main research candidates. Given the different types and abundance of proteins and RNAs in EVs derived from different MSCs, the selection of tissue-specific MSCs should be based on the pathological characteristics of diseases. According to available studies, ADSCs-derived EVs contained high levels of pro-angiogenic factors, BMSCs-derived EVs enriched with pro-differentiation and chemotactic proteins, and EVs secreted by hUCMSCs exhibited strong anti-inflammatory effects (Gorgun et al., 2021; Gupta et al., 2022). Therefore, further studies are warranted to elucidate the specific effects of different sources of MSC-EVs on various physiological processes and their strength, to guide application in disease therapy. Moreover, the safety of MSC-EVs in therapy should also be considered. The haemocompatibility of MSCs products is a key factor affecting patients' safety. One of the major risks of intravascular MSCs therapeutics is that the highly procoagulant tissue factor (TF) carried by MSCs may adversely trigger the instant blood-mediated inflammatory reaction. In turn, TF can be incorporated into EVs, introducing a potential risk to the MSC-EVs therapy (Moll et al., 2019; Ringdén et al., 2022). Compared with ADSCs and perinatal tissue-derived MSCs, BMSCs displayed lower levels of TF (Moll et al., 2019) and might be relatively safer. However, our understanding is not comprehensive enough, and further studies are needed to compare the safety of MSC-EVs from different sources and their possible risk factors.

Keyword burst detection indicated that senescence has been explosively cited since 2020 and is likely to be an emerging research hotspot. For one thing, the senescence of MSCs could affect the secretion, contents, and biological functions of EVs (Boulestreau et al., 2020). Increased secretion of EVs by senescent MSCs appears to remove unnecessary, toxic, and misfolded molecules, thereby the altered cargos of EVs may affect their biological function (Ahmadi and Rezaie, 2021). BMSCs-derived EVs from aged mice could mediate age-related insulin resistance by targeting adipocytes, myocytes, and hepatocytes via high expression of miR-29b-3p in EVs (Su et al., 2019). In contrast to EVs isolated from young MSCs, senescent MSC-EVs failed to protect against the LPS-induced acute lung injury model in mice (Huang et al., 2019). The reason for this difference may be that several miRNAs associated with macrophage polarization in aged MSC-EVs differ from those in young MSC-EVs, affecting the switch of macrophages to an anti-inflammatory phenotype. In addition, one of the characteristics of senescent MSCs is the senescence-associated secretory phenotype (SASP) including pro-inflammatory cytokines, chemokines, growth factors, and proteases (Turinetto et al., 2016). EVs may also represent a non-canonical part of SASP that contributes to cancer progression by inhibiting the immunomodulatory function of MSCs (Boulestreau et al., 2020). For another, MSC-EVs can also play a role in preventing physiological aging. BMSCs-derived EVs from young mice were able to reduce cellular senescence, improve stem cell function and extend the life span of mice (Dorronsoro et al., 2021). MSC-EVs also exhibited therapeutic potential in a range of age-related diseases, including myocardial infarction (Charles et al., 2020), breast cancer (Sandiford et al., 2021), osteoarthritis (Zhang et al., 2019), neurodegenerative disorders (Cone et al., 2021), and intervertebral disc degeneration (Xia et al., 2019), as seen in the co-citation clustering analysis. The

aging of the global population has led to a significant increase in the prevalence of age-related degenerative diseases, which not only negatively affects their quality of life but also imposes a significant burden on the healthcare system (Boulestreau et al., 2020). To solve this problem, an in-depth exploration of the role and mechanism of MSC-EVs in age-related diseases is urgently needed and necessary. Therefore, senescence-related topics might be the hotspot of future research on MSC-EVs.

4.4 Strengths and Limitations

We conducted a comprehensive bibliometric analysis of the research on MSC-EVs to introduce the research status, analyze the hotspots, and predict the research trend for the first time. In addition, a variety of tools, including R-bibliometrix, VOSviewer, and CiteSpace, were used to ensure the reliability and objectivity of the results. However, the present study still has several limitations. First, only the WoS database was used for relevant publication search, while the publications in other databases, such as PubMed, Embase, Scopus, and so on, might be omitted in this study, which may introduce the selection bias. Second, given that MSC-EVs is an emerging field of research, the latest studies published in high-quality journals might be overlooked in citation and co-citation analysis due to their low citations. Moreover, some publications related to MSC-EVs without a detailed definition of MSCs or EVs might be ignored. Finally, there is no uniform standard for parameter settings in Citespace, and hence, in the cluster and burst analysis, the outputs may vary slightly with different settings.

5 CONCLUSION

Using bibliometrics and visualization software, we summarized and analyzed the global research status, development trends, hotspots, and frontier themes of MSC-EVs. In recent years, the field of MSC-EVs has received great attention and grown rapidly. The keyword and co-citation clustering analysis indicated that current researches mainly focus on the therapeutic or supportive therapeutic efficacy of MSC-EVs in various diseases, such as cardiovascular diseases, neurodegenerative diseases, bone and cartilage diseases, and tumors. As well as the underlying mechanisms involved, including angiogenesis, improvement of the local microenvironment, and so on. Multidisciplinary collaboration is a trend in this field that could contribute to promoting the clinical application of MSC-EVs. The theme of linking senescence with MSC-EVs may be a frontier in the future.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

XZ was responsible for experiment conception and design, collection and assembly of data, data analysis and interpretation, and manuscript writing. YL and SW contributed to acquisition data and revised the manuscript. SZ and SL contributed to the revision of the manuscript. JT designed the work, provided technical guidance, and finally approved the manuscript. All authors read and approved the final manuscript.

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Mesenchymal stem cell-derived exosomes as new tools for delivery of miRNAs in the treatment of cancer

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Although ongoing medical research is working to find a cure for a variety of cancers, it continues to be one of the major causes of death worldwide. Chemotherapy and immunotherapy, as well as surgical intervention and radiation therapy, are critical components of cancer treatment. Most anti-cancer drugs are given systemically and distribute not just to tumor tissues but also to normal tissues, where they may cause side effects. Furthermore, because anti-cancer drugs have a low delivery efficiency, some tumors do not respond to them. As a result, tumor-targeted drug delivery is critical for improving the safety and efficacy of anti-cancer treatment. Exosomes are microscopic extracellular vesicles that cells produce to communicate with one another. MicroRNA (miRNA), long non-coding RNA (lncRNA), small interfering RNA (siRNA), DNA, protein, and lipids are among the therapeutic cargos found in exosomes. Recently, several studies have focused on miRNAs as a potential therapeutic element for the treatment of cancer. Mesenchymal stem cells (MSC) have been known to have angiogenic, anti-apoptotic, anti-inflammatory and immunomodulatory effects. Exosomes derived from MSCs are gaining popularity as a non-cellular alternative to MSC-based therapy, as this method avoids unwanted lineage differentiation. Therefore more research have focused on transferring miRNAs to mesenchymal stem cells (MSC) and targeting miRNA-loaded exosomes to cancer cells. Here, we initially gave an overview of the characteristics and potentials of MSC as well as the use of MSC-derived exosomes in cancer therapy. Finally, we emphasized the utilization of MSC-derived exosomes for miRNA delivery in the treatment of cancer.

KEYWORDS

mesenchymal stem cells, exosomes, micro RNA, cancer therapy, cell free therapy

Introduction

The human body comprises a variety of cell types that make up tissues and organs with distinct functions that contribute to long-term survival. Long ago, it was discovered that differentiated cells in several tissues, such as the skin, intestinal epithelium, and blood, have a short lifecycle and are unable to self-renew (Watt and Driskell, 2010). Stem

cells may self-renew and have the ability to differentiate into a variety of cell types in an organism. This discovery gave rise to the concept of stem cells, which are small unspecialized cells in the human body that lack a variety of phenotypic features observed in adult tissues and are used to maintain static and temporary cell types (Alvarez et al., 2012). Embryonic and non-embryonic stem cells (somatic stem cells) are the two basic types of stem cells. Embryonic stem cells are pluripotent, but somatic stem cells, mesenchymal stem cells (MSCs), for example, are multipotent stem cells (Singh et al., 2016). Because of their unique properties, such as self-renewal and the ability to differentiate into a variety of cell types, MSCs are among the most studied stem cells (Pittenger et al., 2019).

Friedenstein and colleagues were the first to isolate and define MSCs from bone marrow as adherent, highly replicative cells that can differentiate into mesodermal lineages such as osteoblasts, chondrocytes, adipocytes, and hematopoietic stroma (Friedenstein et al., 1966). In addition to bone marrow, MSCs can be isolated from variety of tissues (da Silva Meirelles et al., 2006). According to the International Society for Cellular Therapy (ISCT), MSCs must fulfill three minimal conditions; (1) adherence to plastic surface when cultured *in vitro*, (2) expression of the surface antigens CD73, CD90, and CD105, and absence of CD34, CD45, CD14 or CD11b, CD79α or CD19, and HLA-DR, (3) ability to form several mesodermal cell types, such as adipocytes, chondrocytes, and osteoblasts when cultured *in vitro* under appropriate conditions (Dominici et al., 2006).

MSCs are attractive therapeutic targets for a variety of disorders, including cancer treatment and tissue regeneration, because of their versatility and ability to self-renew. MSCs have undeniable medical potential; yet, their capacity to develop into tumor-associated fibroblasts (Mishra et al., 2008; Miyazaki et al., 2021), which promote tumor growth via their secretome (Liang W. et al., 2021), and resistance to apoptosis, makes them potentially dangerous (Bellagamba et al., 2016). MSCs have not been successfully used in anticancer therapy because of their contradictory involvement in cancer progression and regression. To effectively harness MSCs' therapeutic potential, it is critical to understand their underlying molecular pathways.

Exosomes are extracellular vesicles (EVs) produced by eukaryotic cells that serve as carriers for the transfer of membrane and cytosolic proteins, lipids, and RNA between cells, making them a key component of intercellular communication (Raposo and Stoorvogel, 2013). These membrane–bounded vesicles can be divided into three subtypes, exosomes (50–150 nm), microvesicles (100–1,1000 nm), and apoptotic bodies (500–5,5000 nm) (Doyle and Wang, 2019). Exosomes and other EVs have been found in a variety of tissues and biological fluids, including urine, blood, and cerebrospinal fluid. MicroRNAs (miRNAs) and proteins are mostly found in exosomes, which are enclosed by a lipid bilayer membrane (Zhang et al., 2018). Exosomes also contain other RNA types such as nucleolar RNA, long noncoding RNA, and ribosomal RNA, as well as DNA fragments (Sato-Kuwabara et al., 2015). Studies have shown that released exosomes can be guided to other cells *via* proteins found on cell surfaces (Neviani and Fabbri, 2015).

Exosomes derived from MSCs have been shown to possess potential benefits for the management of several pathological conditions, including cancer. MSC-derived exosomes have almost all of the properties of the original cells, in terms of paracrine effects and immunomodulatory functions. Recently, loading MSC-derived exosomes with defined cargos such as miRNAs has been suggested to be a promising strategy for the treatment of different diseases. Even more, genetically engineered miRNAs can be used in correcting the pathways disrupted in cancer. In the present review we discuss the function of exosomal miRNAs derived from MSCs in different type of cancers.

Biological functions of mesenchymal stem cells

MSCs share many properties with other stem cells, including robust self-renewal and multidirectional differentiation capacity. In previous studies, MSCs have been shown to be capable of differentiating into cells of the mesodermal, ectodermal, and endodermal lineages (Dominici et al., 2006; Paunescu et al., 2007). MSCs can regulate the immune system by interacting with immune cells and also have paracrine effects. Furthermore, because MSCs have a low immunogenicity, allograft matching requirements are less stringent, and immunological rejection is less likely. MSCs can thus be used as ideal seed cells for repairing tissue and organ damage caused by aging and pathological changes, and they also have broad clinical applications in the treatment of autoimmune diseases, inflammation-related diseases, and cancer (Farini et al., 2014).

MSCs exert their immunomodulatory activity through interacting with immune cells in both the native and acquired immune systems. First, MSCs decrease natural killer (NK) cell proliferation, cytotoxicity, and cytokine secretion by secreting prostaglandin E2 (PGE2), indoleamine 2,3-dioxygenase (IDO), and soluble human leukocyte antigen G5 (sHLA-G5) (Galland et al., 2017). MSCs can also influence the development of dendritic cells (DCs) by suppressing monocyte differentiation into DCs (Jung et al., 2007). MSCs can limit the expression of tumour necrosis factor (TNF) (Yan et al., 2018) and enhance the expression of interleukin 10 (IL-10) (Selleri et al., 2013) by DCs, which is likewise regulated by PGE2. MSCs also decrease the ability of naïve T cells to induce Th1 differentiation (Consentius et al., 2015), ultimately leading to immunosuppression.

In 2008, Le Blanc and Davies reported success in the treatment of graft versus host disease (GVHD) with allogeneic, semicompatible, and mismatched bone marrow-derived MSC transplantation, indicating that a strict match was not necessary in the treatment of GVHD

with MSCs (Le Blanc and Davies, 2015). The low immunogenicity of MSCs is crucial to the success of allogeneic MSC transplantation in preclinical and clinical settings. MSCs express major histocompatibility complex class I (MHC I) and lymphocyte function-associated antigen (LFA-3) on a constitutive manner, but only following stimulation with interferon-gamma they express MHC II and intercellular adhesion molecule (ICAM) (Tse, et al., 2003). Furthermore, MSCs do not stimulate the proliferation of peripheral blood mononuclear cells (PBMCs) showing low immunogenicity characteristics (Parys et al., 2017). Additionally, MSCs have the ability to significantly reduce the proliferation of activated T cells and interferon-gamma has a vital role in this process (Chinnadurai et al., 2014).

MSCs can migrate to the site of a lesion in a variety of illnesses, including inflammation, tissue damage, and tumors (Nitzsche et al., 2017). Several cell adhesion molecules and chemokine receptors expressed by MSCs influence their migration to the lesion site, and MSC-targeted migration to the lesion site is referred to as "homing" of MSCs (Naji et al., 2019) which is a multistep process that includes activation, adhesion, and migration (De Becker and Riet, 2016). First, inflammatory cytokines generated by inflamed or wounded tissues activate vascular cell adhesion molecule-1 (VCAM-1) on the surface of endothelial cells and α4β1 integrin (VLA-4) on the surface of MSCs, trapping MSCs on the endothelial cell surface (Uchibori et al., 2013). Following that, many growth factors generated by inflammation or damaged tissues might bind to MSC receptors and increase MSC adherence to endothelial cells (Lejmi et al., 2015). Finally, MSCs express matrix metalloproteinase 2 (MMP-2) and membrane type-1-MMP (MT-1-MMP), which activate proteasomes that breakdown the extracellular matrix and assist MSCs migrate across the basement membrane to the lesion site (Ries et al., 2007).

Mesenchymal stem cells in cancer therapy

Many mediators have been identified in the cross-talk between MSCs, the tumor microenvironment, and tumor cells. By triggering numerous signaling pathways, MSCs have different roles on the cells in the tumor microenvironment. MSCs can block Wnt signaling by regulating the Dickkopf-related protein 1 (DKK1) secreted by tumor cells, downregulating c-Myc and Cyclin D2 and upregulating the expression of P21CIP1 and P27KIP1, resulting to tumor cell suppression (Qiao et al., 2008; Zhu et al., 2009). By suppressing angiogenesis, naive MSCs can cause vascular endothelial cells to die (Otsu et al., 2009). On the contrary, MSCs have been shown to be linked to increased metastasis, tumorigenesis, and recurrence of tumors by producing cancer stem cells (CSCs) (Liu et al., 2011). MSCs also produce chemokines such as CXCR4 (Corcoran et al., 2008), CCL5 (Karnoub et al., 2007), ICAMs (Tsukamoto et al., 2012), and VCAMs (Hu et al., 2012). Breast cancer cells induce mesenchymal stem cells to secrete the chemokine CCL5, which subsequently acts in a paracrine manner on the cancer cells to promote motility, invasion, and metastasis (Karnoub et al., 2007). MSCs obtained from mouse lymphomas produce CCL2 and enhance cancer cell proliferation as well as the recruitment of immunosuppressive cells to lymphoid organs (Ren et al., 2012). MSCs originating from breast cancer tissues also produce some immunosuppressive mediators such as IL-4, TGF-β, and IL-10 (Razmkhah et al., 2011). Although the majority of studies aimed at using MSCs in cancer therapy have focused on their tumor-suppressing capabilities, these cells may potentially stimulate tumor progression by increasing metastasis, tumor angiogenesis, epithelial–mesenchymal transition, and disrupting immune surveillance (Hmadcha et al., 2020) (Figure 1). These unfavorable effects may appear depending on the number of MSCs injected, their source or origin, differentiation level, and tumor type, As a result, restrictions in MSC-based cancer therapy should be considered, and more research is needed to assess the safety and efficacy of such a therapeutic approach in the treatment of cancer.

Exosomes as drug carriers

Exosomes are more commonly used as drug delivery vehicles because of their transport capabilities in delivering functional content to specific cells. Some natural exosomes can be used as therapeutic agents because they contain endogenous anti-tumor biomolecules. Furthermore, bioengineered exosomes with extra required payloads and targeting specificity offer more promise in cancer treatment. In contrast to other regularly used drug delivery vehicles (e.g., liposomes), bioengineered exosomes have intrinsic targeting capabilities, low immunogenicity, high modification flexibility, and biological barrier permeability (Walker et al., 2019).

Different methods are currently being employed for the purification of exosomes, such as differential ultracentrifugation, density gradient ultracentrifugation, size exclusion chromatography, etc. (Wang et al., 2021). For isolation, the International Society for Extracellular Vesicles (ISEV) has established detailed guidelines. However, none of the methods were able to accomplish absolute purification, or total separation of exosomes from other biological products. Each approach has advantages and limitations, and combining them for optimum exosome enrichment may be recommended (Thery et al., 2018). It is necessary to characterize exosomes thoroughly according to ISEV's report for the validation of the isolation technique. Generally, Western Blot or ELISA are used for this purpose. The ISEV recommends identifying at least three

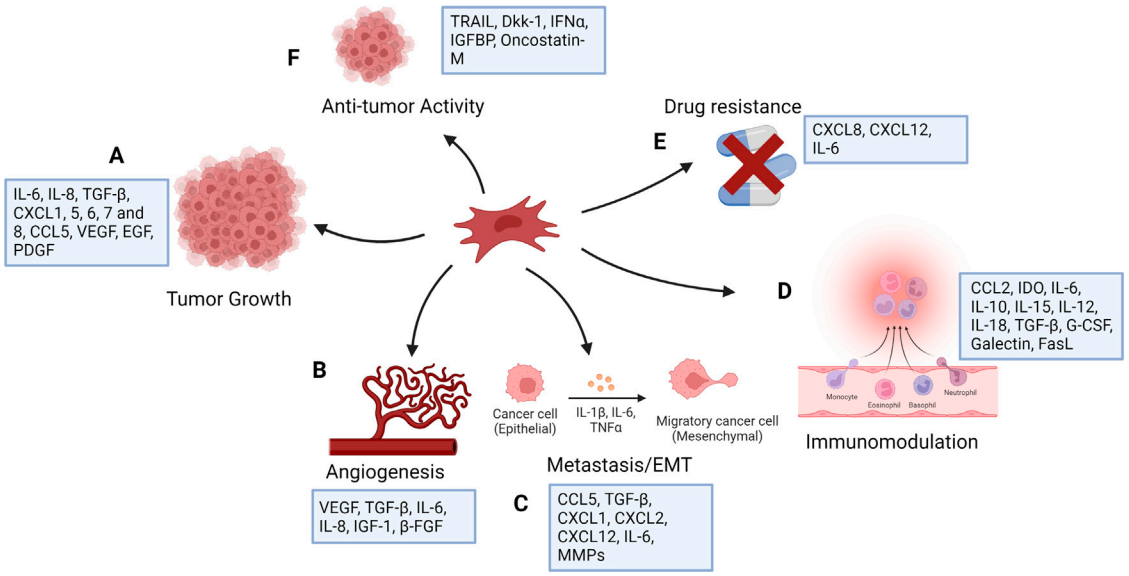

FIGURE 1
Functions of mesenchymal stem cells in cancer (created with BioRender). MSCs have number of effects on tumor cells, mostly increasing tumor growth as a result of their function in controlling inflammation and tissue repair. They affect tumor cell survival and stemness **(A)** and contribute to angiogenesis **(B)** by producing angiogenic factors. MSCs stimulate tumor cell motility, epithelial mesenchymal transition (EMT), and metastasis **(C)**, and secrete chemokines, including CXCL1, CXCL2, and CXCL12, and cytokines, including IL-6 and several matrix metalloproteinases (MMPs), which degrade the extracellular matrix to facilitate tumor cell migration. They show immunomodulatory function **(D)** and can induce drug resistance **(E)**. MSCs are generally pro-tumorigenic, however research has suggested that they may also have anti-tumor properties **(F)**.

positive and one negative protein markers. At least one transmembrane/lipid-bound protein (e.g., CD63, CD9, CD81) and one cytosolic protein (e.g., TSG101, ALIX) must be present as a positive protein marker. Single vesicle characterization requires imaging techniques (atomic force microscopy (AFM) and electron microscopy (EM)) and biophysical characterization (nanoparticle tracking analysis (NTA), tunable resistance pulse sensing (TRPS), dynamic light scattering (DLS), and flow cytometry (FC)) (Thery et al., 2018).

Bioengineered exosomes have greater therapeutic potential as delivery vehicles due their ability to transfer desired payloads and give better targeting specificity. To date, two key strategies for maximizing therapeutic efficacy of exosomes have been employed; (1) cargo engineering and, (2) surface engineering.

Cargo engineering

Different medicinal substances, such as drugs, proteins, and nucleic acids, can be encapsulated by exosomes. Pre-loading (before separation) and post-loading (after isolation) are the two main types of cargo loading techniques. In pre-loading, therapeutic molecules can be endogenously packed into exosomes during the biogenesis stage by modifying parental cells. This can be accomplished by manipulating the genetics of parental cells. Parental cells can overexpress therapeutic miRNAs, siRNAs, mRNAs, proteins, and peptides by transfection, which then be encapsulated into exosomes. Another method is to directly incubate drugs with parental cells, resulting in drug-containing exosomes (Herrmann et al., 2021). The post-loading occurs after exosomes are isolated. Exogenous payloads are passively or actively loaded into exosomes. After direct co-incubation, hydrophobic drugs can be mixed with the exosome lipid bilayer membrane and incorporated into the surface. The hydrophobic nature of the payloads and the concentration gradient of the molecules determine this passive loading method, which usually results in a poor loading capacity (Liang Y. et al., 2021). Different active loading strategies for hydrophilic molecules have been developed to temporarily permeabilize the hydrophobic lipid barrier, either physically or chemically, allowing the passage of the drug into exosomes. Electroporation, sonication, freeze-thaw cycles, and extrusion are examples of physical techniques that entail brief disruption of the exosome membrane by external forces (Walker et al., 2019). Electroporation is currently the most popular method, particularly for RNA encapsulation. Chemical techniques, on the other hand, use transfection reagents or permeabilizers like saponin to help payloads

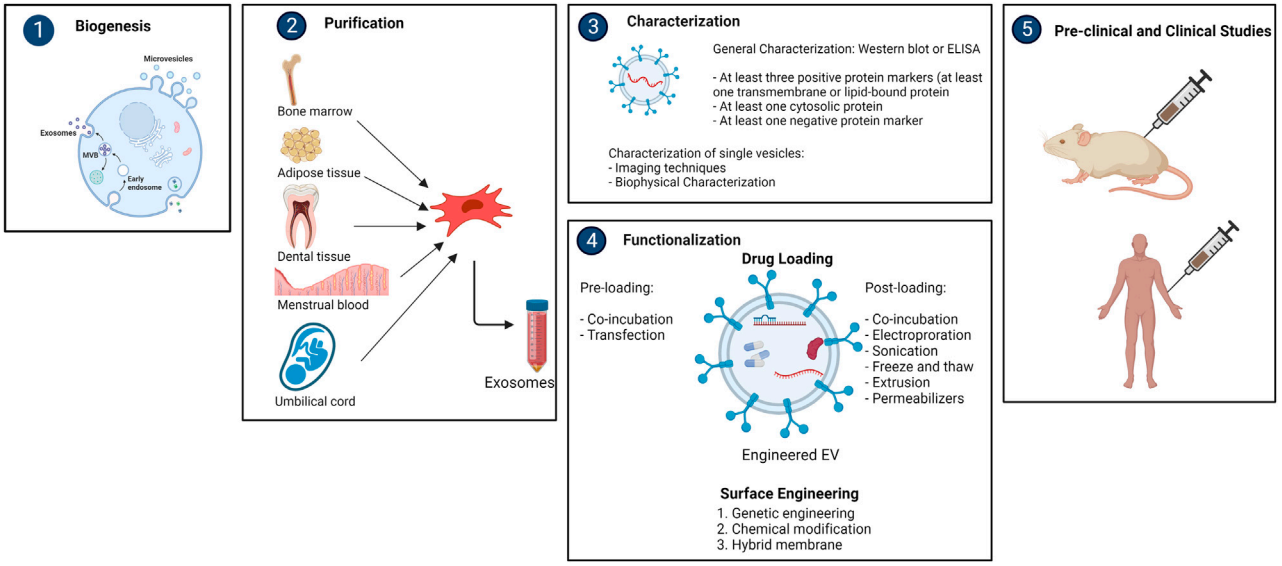

FIGURE 2
Overview of purification, characterization and functionalization of mesencyhmal stem cell-derived exosomes (created with BioRender).

enter the exosomes without disrupting its lipid bilayer structure (Haney et al., 2019).

Surface engineering

Exosomes isolated from distinct cell origins have different surface molecules, indicating that they are selective for specific recipient cells. The biodistribution and tropism of exosomes can be influenced by changing their surface, particularly their protein composition. The major purpose of surface engineering is to give exosomes more targeting specificity, raising the local concentration of exosomes at desirable localizations while lowering unwanted systemic toxicity. Genetic engineering, chemical modification, and hybrid membrane engineering are the three types of surface engineering technologies (Liang W et al., 2021) (Figure 2).

Mesenchymal stem cell-derived exosomes in cancer

Exosomes can be isolated from cell cultures or body fluids. The most common cell sources are MSCs, immune cells, and cancer cells. MSCs are the most abundant producer when compared to other cell sources, and they have a large expansion capacity for economically feasible exosome production (Kim et al., 2021). Additionally, MSCs can also be isolated from a variety of human tissues without having an ethical concern (Zhou et al., 2021). Numerous *in vivo* and *in vitro* studies demonstrate the immunoregulatory, pro-angiogenic, and tissue-regeneration properties of MSC-derived exosomes. For instance, MSC-derived exosomes alleviate the severity of myocardial injury (Ma J. et al., 2017); promote tissue damage repair (Zhang B. et al., 2015); and regulate the immune system (Ti et al., 2015). Other benefits include the prevention of acute tubular injury (Bruno et al., 2009), nerve injury (Drommelschmidt et al., 2017), and lung injury (Lee et al., 2012). Preclinical data have proven the safety of exosome therapy and scalability of their isolation methods from MSCs for clinical application. However, due to the lack of established cell culture conditions, suitable protocols for production, isolation, and storage of exosomes, optimal therapeutic dose and administration schedule, and reliable potency assays to assess the efficacy of exosome therapy, the use of MSC-derived exosomes in clinical settings is limited (Börger et al., 2017).

Recent studies have shown that MSC-derived exosomes play an important role in angiogenesis, tumor development, and tumor invasion. It is still unclear whether natural MSC-derived exosomes have beneficial or detrimental effects on tumors. Several studies have reported that natural MSC-derived exosomes enhanced tumor development. However, some studies suggested that these exosomes can prevent tumor progression. According to a prior study, the dual effect may be influenced by the origin of the MSC-derived exosomes, the dose and timing of the MSC injection, the kind of malignancy, and other parameters (Shojaei et al., 2019). Zhu et al. (2012) showed that exosomes released by MSCs could stimulate tumor growth *in vivo*. In xenograft mouse models of stomach and colon malignancies, exosomes generated from

human bone marrow mesenchymal stem cells (hBMSCs) promoted tumor growth. However, exosomes had no similar effects on tumor cells *in vitro*. Angiogenesis-related molecular signaling pathway activation was detected *in vivo* and *in vitro* with increased VEGF and CXCR4 mRNA levels, which corresponded to enhanced vascular density in tumor tissues *in vivo*. Finally, they showed that stimulation of the ERK1/2 and p38 MAPK pathways by hBMSC-derived exosomes increased VEGF and CXCR4 expression in tumor cells, resulting in increased angiogenesis and hence tumor growth *in vivo* (Zhu et al., 2012). In non-small cell lung cancer (NSCLC), MSC and MSC-derived exosomes promote malignancy by triggering epithelial mesenchymal transition, migration, autophagy, and also inhibiting apoptosis through the activation of the AMPK signaling pathway (Wang et al., 2022). In hepatocellular carcinoma (HCC) cells, MSC-derived exosomes increase proliferation, invasion, sphere formation ability and suppress apoptosis through TMBIM6. As a result of silencing TMBIM6, viability, sphere formation, invasion, epithelial mesenchymal transition and PI3K/AKT signaling pathway are suppressed, and apoptosis is triggered (Shang et al., 2022). Adipocyte-derived exosomes differentiated from MSC in breast cancer promote cell proliferation and migration, and also inhibit apoptosis via the Hippo signaling pathway. Suppression of the signaling pathway blocks the growth-promoting effect of adipocyte exosomes (Wang S. et al., 2019).

On the contrary, Wu et al. (2013) found that human umbilical cord Wharton's jelly mesenchymal stem cells (hWJMSCs)-derived exosomes could induce apoptosis and cell cycle arrest in T24, a bladder cancer cell line, by increasing the expression of caspase-3 and decreasing the phosphorylation of Akt. According to a study by Kalimuthu et al. (2016) treatment with MSC-derived extracellular vesicles led lung cancer cells to undergo apoptosis.

Functions of miRNA loaded mesenchymal stem cell-derived exosomes in cancer

miRNAs are a family of short single-stranded non-coding RNAs that regulate gene expression in target cells. They range in length from 20 to 25 nucleotides (Leavitt et al., 2019). miRNAs act at the 3′UTR of mRNAs to downregulate their translation or cause their degradation as part of the RNA-induced silencing complex (RISC) (Gu et al., 2009). miRNA expression can be altered due to many reasons such as germline and somatic mutations in miRNA genes, amplification or deletion of miRNA genes, epigenetic regulation in miRNA locus, changes in miRNA biogenesis mechanisms, editing and chemical modifications of miRNAs. These dysregulations result in up- or downregulation of miRNAs and predispose to the formation of many diseases, including cancer (Urbanek-Trzeciak et al., 2020). Under specific circumstances, miRNAs can act as tumor suppressors or oncogenes. It has been demonstrated that dysregulated miRNAs have an impact on the characteristics of cancer, including maintaining proliferative signaling, avoiding growth inhibitors, resisting cell death, triggering invasion and metastasis, and promoting angiogenesis (Table 1). miRNAs have been identified as possible biomarkers for the diagnosis and prognosis of human cancers and therapeutic targets (Peng and Croce, 2016).

Two types of miRNA-based approaches can be used to change the expression levels of target genes for therapeutic purposes: (a) miRNA suppression therapy when the target gene is downregulated and (b) miRNA replacement therapy when the target gene is upregulated. Usually, the reticuloendothelial system and the ribonucleases present in the blood rapidly degrade naked RNA. The stability of oligonucleotides can be improved by chemical modifications for *in vivo* delivery. Antisense oligonucleotide (ASO) technology was developed for studying miRNA, and the ASOs that are used to silence miRNA are called anti-miRNA oligonucleotides (AMOs) (Zhang and Farwell, 2008).

miRNA suppression therapy can remove miRNA suppression on the target mRNA, thus increasing the mRNA expression level. AMOs bind to the miRNA sense strand, block interactions between miRISC and its target mRNA, prevent the degradation of the mRNA, and thus allow the mRNA to be translated. In miRNA replacement therapy, miRNA mimics, synthetic double-stranded miRNA-like RNA molecules, can stimulate endogenous miRNAs and bind to mRNA of the target gene, resulting in posttranscriptional suppression (Fu et al., 2019). Since cancer is related with the deregulation of multiple genes and miRNAs, it is commonly accepted that focusing on just one target is insufficient for an effective treatment. Therefore high target specificity has been replaced with multi-specificity. In that regard, miRNA-based therapies are an advantage since they affect the regulatory sequence, commonly functioning on an entire pathway or even several pathways rather than just one gene (Baumann and Winkler, 2014).

Because of their negative charge and hydrophilic nature, miRNAs are difficult to cross the cell membrane. Additionally, they are destroyed after entering the body. Therefore, exosomes can serve as excellent carriers for miRNAs (Zhang et al., 2022). There are two methods for miRNA enrichment/loading in exosomes. The first strategy involves creating a cell line that overexpresses the desired therapeutic miRNA. The cell line then displays a high level of miRNA in their cytoplasm, followed by exosome secretion containing therapeutic miRNA. The second strategy involves separating exosomes from the source (cell lines or body fluids) and then loading them with selected miRNA by using chemical or physical approaches. Since it is widely known that increasing the quantity of miRNA in the cytosol may increase their passive loading in exosomes, it is possible to

TABLE 1 Examples of miRNAs and their roles in different cancers.

Cancer type	miRNA	Expression	Target	Pathway	Effect	References
Brain Cancer	miR-7	Downregulated	EGFR, PI3K- Akt EGFR, IRS1, IRS2	EGFR, PTEN-PI3K-Akt IGF-1R/Akt	Cell growth, cell cycle arrest Invasion, proliferation, cell cycle, survival/cell death	Liu Z. et al. (2014), Matos et al. (2018)
	miR-101	Downregulated	SOX9	Akt, Wnt, BMI1	Proliferation, migration, invasion	Liu et al. (2017)
	miR-29a/b/c	Downregulated	CDC42	CDC42-PAK	Migration, invasion	Shi et al. (2017)
	miR-146b-5p	Downregulated	TRAF6	TRAF6-TAK1	Cell proliferation, apoptosis resistance	Liu et al. (2015)
	miR-181c	Downregulated	NOTCH2	NOTCH	Tumor progression	Ayala-Ortega et al. (2016)
	miR-320a	Downregulated	SND1, β-catenin	TGFβ1	Cell proliferation, invasion, migration	Li et al. (2017)
	miR-21	Upregulated	EGFR, Akt, cyclin D, Bcl-2	EGFR, Akt	Apoptosis, TMZ resistance	Zhou et al. (2010), Wong et al. (2012)
	miR-221 miR-222	Upregulated	SOCS3	JAK/STAT	Invasion, migration, proliferation, angiogenesis	Xu C. H. et al. (2019)
	miR-10b	Upregulated	PTEN, p53, BIM E-cadherin, Apaf-1, PTEN/TGF-β1	TGF-β	Growth, invasion, apoptosis Proliferation, migration, EMT.	Sun et al. (2019), Ma C. et al. (2017)
	miR-181b	Upregulated	KPNA4	EMT	Growth, invasion, proliferation	Wang et al. (2015)
	miR-141	Upregulated	Jagged1	NOTCH	Growth	Gao et al. (2017)
Head and Neck Cancer	let-7c	Downregulated	IL-8		Radio-/chemoresistance	Peng C. Y. et al. (2018)
	miR-101	Downregulated	EZH2 CDK8	Wnt/β-catenin	Metastasis, EMT Tumorigenesis	Chen L. et al. (2019), Li et al. (2015)
	miR-124	Downregulated	STAT3	JAK/STAT	Tumor growth and metastasis	Xu et al. (2016)
	miR-let-7e	Downregulated	HMGB1	NF-κB	Migration, invasion	Ding C. et al. (2019)
	miR-206	Downregulated	MAP4K3	p38, JNK	Cell proliferation, apoptosis, multidrug resistance	Liu et al. (2019)
	miR-30a miR-379	Downregulated	DNMT3B	Retinoic acid pathway	Growth	Shiah et al. (2020)
	miR-125a	Upregulated	p53		Cell proliferation, migration, invasion	Chen J. et al. (2019)
	miR-134	Upregulated	PDCD7 WWOX		E-cadherin expression Suppressor inhibition	Peng S. Y. et al. (2018), Liu C. J. et al. (2014)
	miR-196b	Upregulated	PCDH-17		Cell proliferation, migration, and invasion	Luo M. et al. (2019)
	miR-144	Upregulated	mTOR	PI3K/Akt/mTOR	Cell proliferation, clonogenicity, migration, invasion, tumor formation	Shabani et al. (2018)
Breast Cancer	miR-126	Downregulated	VEGFA, PIK3R2	VEGF/PI3K/Akt	Angiogenesis	Zhu et al. (2011)
	miR-204	Downregulated	PI3K-α, c-SRC, VEGF, FAK, RAF1, MAPK	PI3K/AKT, RAF1/MAPK, VEGF, FAK/SRC	Angiogenesis	Salinas-Vera et al. (2018)
	miR-720	Downregulated	ADAM8	ERK	Metastasis	Das et al. (2016)
	miR-205	Downregulated	ZEB1, ZEB2, HER3, AMOT, erbB2/erbB3		Proliferation, invasion, metastasis	Wang et al. (2013), Zhang and Fan, (2015), Huo et al. (2016)
	miR-200 family	Downregulated	ZEB2, E-cadherin		Metastasis, invasion	Liu et al. (2018), Rogers et al. (2019)
	miR-203a-3p	Downregulated	ZEB2		Metastasis, invasion	Fahim et al. (2020)
	miR-1-3p	Downregulated	K-RAS, MALAT1		Proliferation, apoptosis	Chou et al. (2016), Jin et al. (2016), Fahim et al. (2020)
	miR-210	Upregulated	HRAS, PTK2, SHC1, HIF1a	Hypoxia VEGF signaling	Development of cancer, angiogenesis	Foekens et al. (2008)
	miR-182	Upregulated	FBXW7	HIF-1α- VEGF-A	Proliferation, angiogenesis	Chiang et al. (2016)

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TABLE 1 (*Continued*) Examples of miRNAs and their roles in different cancers.

Cancer type	miRNA	Expression	Target	Pathway	Effect	References
	miR-155	Upregulated	VHL	VHL/HIF-1α/VEGF	Angiogenesis	Kong et al. (2014)
	miR526b miR655	Upregulated	VEGFA, VEGFC, VEGFD, CD31, LYVE1	PI3K/Akt	Angiogenesis	Hunter et al. (2019)
	miR-20b	Upregulated	PTEN	PTEN-PI3K-Akt	Progression, angiogenesis	Zhou et al. (2014)
	miR-155 miR-203 miR-125a	Upregulated	SOCS1, SOCS3, STAT3, PIAS3, IL-6, IL-6R	JAK/STAT3		Lei et al. (2016), Ru et al. (2011), Park and Kim, (2019)
Gastrointestinal Cancer	miR-28-5p	Downregulated	AKT		Proliferation, migration	Xiao et al. (2018)
	miR-7	Downregulated	RelA/p65 Raf-1	NF-κB	Metastasis, tumor development, angiogenesis	Ye et al. (2019), Lin J. et al. (2020)
	miR-1299	Downregulated	ARF6		Proliferation, apoptosis, migration, invasion	Qiu et al. (2022)
	miR-223-3p	Downregulated				Zhou et al. (2017)
	miR-339-5p	Downregulated	Cdc25A			Luo A. et al. (2019)
	miR-148a-3p miR-181a-5p	Downregulated				Lin Z. et al. (2020)
	miR-497	Downregulated			Differentiation, lymph node metastasis	Zou G. et al. (2019)
	miR-100	Downregulated				Stroese et al. (2018)
	miR-181a	Upregulated	Caprin-1		Proliferation, apoptosis, invasion, metastasis	Lu et al. (2019)
	miR-653-5p	Upregulated	SOCS6-STAT3	JAK2/ STAT3 pathway	Proliferation, metastasis	Li Z. et al. (2021)
	miR-1301-3p	Upregulated	SIRT1		Proliferation, cell cycle, tumorigenesis	Luo et al. (2021)
	miR-106a miR-18a miR-20b miR-486-5p miR-584	Upregulated				Zhou et al. (2017)
	miR-34a-5p	Upregulated				Lin Z. et al. (2020)
	miR-199a-3p	Upregulated			Depth of invasion	Nonaka et al. (2014)
	miR-103 miR-720	Upregulated			Differentiation, lymph node metastasis	Nonaka et al. (2015)
	miR-19a-3p miR-19b-3p miR-25-3p miR-192-5p miR-223-3p	Upregulated				Zou X. et al. (2019)
Genitourinary Cancer	miRNA-199a-3p	Downregulated	Cyclin D1, c-Myc, mTOR EGFR		Proliferation, clonal expansion, regeneration	Liu et al. (2016)
	miRNA-203	Downregulated	IRS-1	ERK	Cell proliferation, cell cycle	Meng et al. (2020)
	miRNA-218	Downregulated	GAB2	PI3K/Akt/GSK-3β	Cell proliferation, migration	Tian et al. (2020)
	miRNA-1	Downregulated	c-Met	Akt/mTOR	Cell survival, proliferation	Gao et al. (2019)
	miRNA-31-5p	Downregulated	14–3-3 ε	PI3K/AKT/Bcl-2	Cell survival, proliferation	Zhao et al. (2020)
	miRNA-381	Downregulated	RELN	PI3K/Akt/mTOR	Autophagy, apoptosis	Liao and Zhang, (2020)
	miRNA-125b	Upregulated	p14ARF	p53	Cell proliferation	Amir et al. (2013)
	miRNA-486-5p	Upregulated		SMAD2/TGF- β PTEN/PI3K FoxO	Proliferation, development, pathogenesis	Yang et al. (2017)
	miRNA-4534	Upregulated		PTEN/PI3K/Akt	Migration, apoptosis	Nip et al. (2016)
Gynecologic Cancer	let-7d-5p	Downregulated	HMGA1	p53	Proliferation, chemosensitivity	Chen Y. N. et al. (2019)
	miR-101-5p	Downregulated	CXCL6		Colony formation, invasion, migration	Shen et al. (2019)
	miR-132	Downregulated	SMAD2		Lymph node metastasis	Zhao J. L. et al. (2015)
	miR-138-5p	Downregulated	SIRT1		Tumorigenesis, metastasis	Ou et al. (2018)

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TABLE 1 (*Continued*) Examples of miRNAs and their roles in different cancers.

Cancer type	miRNA	Expression	Target	Pathway	Effect	References
	miR-148b	Downregulated	CASP3		Cell proliferation, invasion, apoptosis	Mou et al. (2016)
	miR-508 miR-509–2 miR-526b	Downregulated	p53,SMAD4, NF-κB-1, MMP1, NOTCH1, SMAD4		Migration, invasion, lymph node metastasis, tumor progression	Chen et al. (2018)
	miR-16–1	Upregulated	CycE1		Controls the transition of cells from G1 to S phase	Zubillaga-Guerrero et al. (2015)
	miR-20a	Upregulated	TIMP2, ATG7		Lymph node metastasis, invasion	Zhao S. et al. (2015)
	miR-20b	Upregulated	TIMP2		Regulates the cytoskeleton and activates EMT, migration, invasion	Cheng et al. (2017)
	miR-27b	Upregulated	CDH11		Proliferation, cell cycle transition from G1 to S phase, migration, invasion	Yao et al. (2016)
	miR-106b-5p	Upregulated	GSK3B, VEGFA, PTK2	PI3K-Akt	Lymph node metastasis	Yi et al. (2018)
Hematologic Cancer	miR-3173	Downregulated	PTK2		Proliferation, migration, invasion	Tian et al. (2017)
	miR-181a	Downregulated	Smad7	TGF-β1	Proliferation, apoptosis, diagnostic sensitivity	Nabhan et al. (2017)
	miR-142-3p	Downregulated	MLL-AF4, HOXA7, HOXA9, HOXA10		Cell proliferation	Dou et al. (2013)
	hsa-miR-103a-3p hsa-miR486-3p	Downregulated	HOXA7, S100A10		Cell growth, motility, cell cycle progression, differentiation, Poor outcomes, chemoresistance	Huang et al. (2020)
	miR-21	Upregulated	PDCD4, PTEN, TPM1		Cell growth, invasion, angiogenesis, metastasis	Labib et al. (2017)
	miR-339-5p	Upregulated	BCL2L11, Bax, FGFR1		Cell cycle progression, apoptosis	Hu et al. (2018)
	miR-125b miR-17 miR-181b	Upregulated	PPP1CA, BTG2, PTEN		Proliferation, apoptosis	Vafadar et al. (2019)
	miR-187-5p	Upregulated	DKK2	Wnt/β-catenin	Proliferation, apoptosis	Lou et al. (2016)

transfect a designed miRNA into cells for exosomal therapy. Choosing the right cell type is one of the requirements for transfection. Although MSCs are the most commonly used "biofactories" for producing exosomes with loaded miRNA, there are some limitations in their utilization for therapeutic purposes. Initially, the cell system should be selected carefully according to the purpose of miRNA-loading. The disease being studied, the dynamics of communication between exosome-producing cells and the recipient cell, the rate of exosome secretion, and the capacity of exosomes to uptake exogenous therapeutic miRNAs should also be considered (Munir et al., 2020). Exosomes essentially have proteins on their surface, such as tetraspanins (CD-81, -82, -37, and CD-63), membrane trafficking proteins, cytoskeletal proteins, and two members of the Endosomal Sorting Complex Required for Transport (ESCRT) pathway, namely Alix and Tumor Susceptibility Gene 101 (TSG-101). The propensity of these proteins to target particular tissues is modest. Additionally, these proteins enable exosomes to accumulate in the liver, kidney, and spleen. They can be also eliminated through bile, renal filtration, and reticuloendothelial phagocytosis (Xitong and Xiaorong, 2016). Therefore, it is strongly advised to change the surface of exosomes in order to improve precise targeting and decrease the clearance rate. This can be accomplished by directly or genetically altering the exosome membrane proteins. Exosome surfaces can be directly altered using non-covalent or covalent techniques. In the non-covalent technique, exosomes and protein are combined. The covalent technique, on the other hand, involves the attachment of a peptide with covalent bonding. However, it remains to be unclear how effective these methods are for developing miRNA-enriched exosomes for targeted therapy. Both techniques have the potential for chemical contamination and have varying degrees of modification efficacy. Additionally, non-covalent attachment may dissociate under physiologic conditions (Hu et al., 2020). Genetic alteration involves producing a particular protein on the exosome surface which results in more homogenous population and sustained target specificity. It is more expensive than a direct approach.

TABLE 2 Effects of miRNAs delivered by mesencymal stem cell-derived exosomes in different cancer types.

Type of cancer	Source of MSC	miRNA	Target gene/Pathway	Effects	References
Brain Cancer					
Glioma		miR-584	CYP2J2, MMP-2, Bcl-2, Bax exp.	↓ proliferation, invasion, metastasis, ↑ apoptosis	Kim et al. (2018)
	Bone marrow (mice)	miR-133b	EZH2 exp. Wnt/β-catenin signaling pathway	↓ proliferation, invasion, migration	Xu H. et al. (2019)
	Bone marrow (human)	miR-34a	SIRT1 exp.	↑ cellular senescence	Li Q. et al. (2019)
	Bone marrow (human)	miR-199a	AGAP2 exp.	↓ proliferation, invasion, migration, ↓tumor growth (*in vivo*), ↑ chemosensitivity to temozolomide (*in vivo*)	Yu et al. (2019)
Glioblastoma multiforme	Wharton's jelly (human)	miR-124	CDK6 exp.	↓ migration, ↑ chemosensitivity to temozolomide	Sharif et al. (2018)
	Adipose tissue (human)	miR-4731		↓ proliferation stimulation of cell cycle arrest, apoptosis	Allahverdi et al. (2020)
	Bone marrow (human)	miR-512-5p	JAG1 exp. Notch signaling pathway	↓ proliferation stimulation of cell cycle arrest, ↓ tumor growth (*in vivo*) prolongation of survival (*in vivo*)	Yan et al. (2021)
	Bone marrow (human)	miR-30c	IL-6 exp	↓ migration, invasion, ↑ apoptosis	Mahjoor et al. (2021)
Neuroblastoma	Adipose tissue (human)	miR-124		↓ proliferation, ↑ apoptosis stimulate neuronal differentiation	Sharif et al. (2021)
Head and Neck Cancer					
Oral cancer	Bone marrow (human)	miR-101-3p	COL10A1 exp.	↓ proliferation, invasion, migration, ↓tumor growth (*in vivo*)	Xie et al. (2019)
Oral leukoplakia	Bone marrow (mice)	miR-185	Akt, caspase-3 and 9 exp.	↓severity of inflammation (*in vivo*), ↓number of dysplasia in the OPMD tissue (*in vivo*), ↑ apoptosis	Wang L. et al. (2019)
Thyroid cancer	Umbilical cord (human)	miR-30c-5p	PELI1, Ki-67, MMP-2 exp., PI3K-AKT signaling pathway	↓ proliferation, migration, ↓tumor growth (*in vivo*)	Zheng et al. (2022)
Breast Cancer					
	Bone marrow (mice)	LNA-antimiR-142-3p	miR-150, APC, P2X7R exp. Wnt signaling pathway	Penetration to the tumor site (*in vivo*), ↓ reduction of clone-formation, tumor-initiating abilitiy	Naseri et al. (2018), Naseri et al. (2020)
	Umbilical cord (human)	miR-148b-3p	TRIM59 exp.	suppressive effect on the progression, antitumor effect (*in vivo*)	Yuan et al. (2019)
	Adipose tissue (human)	miR-145	ROCK1, MMP9, ERBB2, TP53 exp.	↓ metastasis, ↑ apoptosis	Sheykhhasan et al. (2021)
	Umbilical cord (human)	miR-3182	mTOR, S6KB1 exp.	↓ proliferation, migration, ↑ apoptosis	Khazaei-Poul et al. (2021)
	Adipose tissue	miR-381	Wnt signaling pathway	↓ proliferation, migration, invasion, ↓ epithelial mesenchymal transition, ↑ apoptosis	Shojaei et al. (2021)
Gastrointestinal Cancer					
Esophageal squamous cell carcinoma	Umbilical cord (human)	miR-375	ENAH	↓ proliferation, migration, invasion, tumorsphere formation, ↑ apoptosis, ↓tumor growth (*in vivo*)	He et al. (2020)
Gastric cancer	Umbilical cord (human)	miR-6785-5p	INHBA exp	↓ angiogenesis, metastasis	Chen et al. (2021)
Pancreatic ductal adenocarcinoma	Umbilical cord (human)	miR-145-5p	Smad 3 exp	↓ proliferation, invasion, ↑ apoptosis, cell cycle arrest, ↓ tumor growth (*in vivo*)	Ding Y. et al. (2019)
Liver cancer	Adipose tissue (human)	miR-122	Genes involved in drug resistance or sensitivity	↑ susceptibility to chemotherapeutic drugs, ↑ anticancer activity of sorafenib (*in vivo*)	Lou et al. (2015)
	Adipose tissue (human)	miR-199a	mTOR signaling pathway	↑ sensitivity to doxorubicin	Lou et al. (2020)
Genitourinary Cancer					
Prostate cancer	Bone marrow (human)	miR-205	RHPN2 exp	↓ proliferation, invasion, metastasis, ↑ apoptosis	Jiang et al. (2019)

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TABLE 2 (*Continued*) Effects of miRNAs delivered by mesencymal stem cell-derived exosomes in different cancer types.

Type of cancer	Source of MSC	miRNA	Target gene/Pathway	Effects	References
Bladder cancer	Umbilical cord (human)	miR-139-5p	PRC1	↓ development of bladder cancer	Jia et al. (2021)
Gynecologic Cancer					
Endometrial cancer	Umbilical cord (human)	miR-302a	cyclin D1 exp. AKT signaling pathway	↓ proliferation, migration	Li X. et al. (2019)
		miR-499a-5p	VAV3 exp	↓ proliferation, endothelial cell tube formation, ↓ tumor growth and angiogenesis (*in vivo*)	Jing et al. (2020)
Cervical cancer	Bone marrow (human)	miR-144-3p	CEP55 exp	↓ proliferation, migration, invasion, ↑ apoptosis	Meng et al. (2021)
Ovarian cancer	Bone marrow (mice)	miR-424	MYB, VEGF, VEGFR exp.	↓ proliferation, migration, invasion of ovarian cancer cells, ↓ proliferation, migration, invasion, tube formation of human umbilical vein endothelial cells, ↓ tumorigenesis, angiogenesis (*in vivo*)	Li P. et al. (2021)
Hematologic Cancer					
Acute myeloid leukemia	Bone marrow (human)	miR-222-3p	IRF2 exp. IRF2/INPP4B signaling pathway	↓ proliferation, ↑ apoptosis	Zhang et al. (2020)
	Bone marrow (human)	miR-26a-5p	GSK3 exp. Wnt/β-catenin signaling pathway	promoting effect on AML progression	Ji et al. (2021)
Other cancer types					
Bone cancer	Bone marrow (human)	miR-143,		↓ proliferation, migration,	Shimbo et al. (2014),
	Bone marrow (mice)	miR-9-5p	REST, cytokine, MOR exp.	alleviation of bone cancer pain by modulating neuroinflammation in the central nervous system	Zhu et al. (2020)
Lung cancer	Bone marrow	miR-328-3p	NF2 exp. Hippo signaling pathway	promote formation and progression of cancer	Liu et al. (2021)
	Umbilical cord (human)	miR-320a	SOX4 exp. SOX4/Wnt/β-catenin axis	Antitumor effect	Xie and Wang, (2021)

Additionally, it raises safety issues, which makes it unsuitable for clinical uses (Ohno et al., 2013).

The effect of miRNAs carried by MSC-derived exosomes in tumor treatment is contradictory, with some research claiming that they can stimulate tumor growth and others claiming that they can repress tumor growth. In osteosarcoma, miR-208a in MSC-derived exosomes increased tumor growth by downregulating programmed cell death and activating the ERK1/2 pathway (Qin et al., 2020). Furthermore, MSC-exosome-derived miR-142-3p and miR-146a have been shown to stimulate tumor growth *via* many mechanisms (De Veirman et al., 2016; Li and Li, 2018). Similarly, miR-146a can enhance the progression of multiple myeloma, validating this concept (De Veirman et al., 2016).

On the other hand, anti-tumor effects of miRNA carrying MSC-derived exosomes have been shown by different groups (Kang et al., 2015; Renjie and Haiqian, 2015; Gopalan et al., 2018). In prostate cancer, human bone marrow MSC-derived exosomal miR-143 has been shown to inhibit cell proliferation, invasion, metastasis, and tumor growth (Che et al., 2019). miR-23b in MSC-derived exosomes can prevent tumor development, keep tumors dormant, improve patient's life quality, and lengthen survival time (Ono et al., 2014). In hepatocellular carcinoma, MSC-derived exosomes transfected with miR-122 can improve drug sensitivity (Lou et al., 2015). miR-34c in MSC has been proven to improve tumor sensitivity to radiotherapy in addition to enhancing chemical sensitivity (Wan et al., 2020). This shows that MSC-exosomes can be used in combination with conventional cancer treatments such as chemotherapy and radiotherapy.

miRNAs in MSC-derived exosomes have received a great deal of interest recently, and they are being studied largely for tumor inhibition. These studies differ from each other in terms of the cancer type of interest, selected MSC subtype, the way of miRNAs is transferred to MSCs, preferred miRNA and target genes according to the cancer type. The general approach in studies is to first detect and confirm miRNA and target genes that negatively regulate each other in bioinformatic studies or healthy/patient samples, and then detect alteration in the proliferation, apoptosis, migration and invasion capacities of

cancer cells after administration *in vitro* and *in vivo*. The general conclusion reached is that miRNA transfer with MSC-derived exosomes has positive effects. However, it is emphasized that such studies are at a preclinical stage, the data on the mechanism of action are still insufficient, and therefore studies should continue in order to reveal the mechanisms. We summarized the studies on the use of MSC-derived exosomes as vehicles for the delivery and restoration of miRNAs in Table 2, with the goal of developing an effective therapeutic strategy for various malignancies.

Conclusion

While significant progress has been made in the fight against cancer, it remains a leading cause of mortality in the twenty-first century, necessitating a greater understanding of the biology of cancer cells and their environment in order to create novel therapeutic options. Over the last three decades, researchers and clinicians have mostly concentrated on identifying cancer-specific targets and developing targeted medicines that can effectively destroy cancer cells while sparing their normal counterparts, decreasing undesired side effects. A variety of intriguing and very effective small compounds targeting cancer-specific mutations and/or altered signal transduction pathways that control cancer cell proliferation and survival have been developed as a result of this global endeavor.

MSC-derived exosomes have been identified as significant mediators in the therapeutic benefits of MSCs. MSC-derived exosomes can promote or inhibit tumor growth, but engineered MSC-derived exosomes are implicated in the suppression of cancer formation and progression by the delivery of numerous therapeutic compounds, including miRNAs. Dysregulation of miRNAs is thought to be involved in the initiation and progression of tumors. Furthermore, promising results show that restoring these regulatory miRNAs can be used as a therapeutic method in cancer treatment. Replacement of these molecules can contribute to the inhibition of cell proliferation, invasion, migration, and metastasis, along with increased sensitivity to chemotherapeutic drugs and activation of apoptosis through direct control of their target genes.

To summarize, recent findings have confirmed the capacity of MSC-derived exosomes to transport therapeutic miRNAs in a variety of malignancies, indicating that this approach is novel and extremely promising in the treatment of cancer. Despite the fact that MSCs have been shown to have anticancer properties, there have also been some conflicting claims about their roles in tumor growth. Hence, their potential in tumor progression should also be considered.

Author contributions

OD contributed to conception and design of the study. AD organized the literature. Both authors contributed to manuscript writing, revision, read, and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Genetic profiling of human bone marrow mesenchymal stromal cells after *in vitro* expansion in clinical grade human platelet lysate

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Mesenchymal stromal cells (MSCs) are non-hematopoietic cells that have a broad therapeutic potential. To obtain sufficient cells for clinical application, they must be expanded *ex vivo*. In the initial expansion protocols described, fetal calf serum (FCS) was used as the reference growth supplement, but more recently different groups started to replace FCS with platelet lysate (PL). We investigated in this study the impact of the culture supplement on gene expression of MSCs. Human bone marrow derived MSCs were expanded *in vitro* in FCS and PL supplemented medium. We found that MSCs expanded in PL-containing medium (PL-MSCs) express typical MSC immunomorphological features and can migrate, as their counterparts expanded in FCS-containing medium, through a layer of endothelial cells *in vitro*. Additionally, they show an increased proliferation rate compared to MSCs expanded in FCS medium (FCS-MSCs). RNA sequencing performed for MSCs cultured in both types of expansion medium revealed a large impact of the choice of growth supplement on gene expression: 1974 genes were at least twofold up- or downregulated. We focused on impact of genes involved in apoptosis and senescence. Our data showed that PL-MSCs express more anti-apoptotic genes and FCS-MSCs more pro-apoptotic genes. FCS-MSCs showed upregulation of senescence-related genes after four passages whereas this was rarer in PL-MSCs at the same timepoint. Since PL-MSCs show higher proliferation rates and anti-apoptotic gene expression, they might acquire features that predispose them to malignant transformation. We screened 10 MSC samples expanded in PL-based medium for the presence of tumor-associated genetic variants using a 165 gene panel and detected only 21 different genetic variants. According to our analysis, none of these were established pathogenic mutations. Our data show that differences in culture conditions such as growth supplement have a significant impact on the gene

expression profile of MSCs and favor the use of PL over FCS for expansion of MSCs.

KEYWORDS

mesenchymal stromal cell, expansion, platelet lysate, gene expression, transformation

Introduction

Mesenchymal stromal cells (MSCs) are non-hematopoietic cells residing in a wide range of tissues such as the bone marrow, adipose tissue and umbilical cord (Friedenstein et al., 1974; Maqsood et al., 2020). There is a broad interest in the therapeutical use of these cells due to their biological characteristics. MSCs can be expanded *in vitro* at levels sufficient for clinical use. They have immunomodulatory capacities and can escape immune recognition since they do not express MHC class II antigens. Moreover, MSCs have a multipotent differentiation potential and can migrate to sites of tissue injury and inflammation (Majumdar et al., 2000; Vater et al., 2011; Li et al., 2013; Stagg and Galipeau, 2013). Tissue repair applications with these cells cover different domains of medicine, including orthopedics (Bashir et al., 2014), cardiology and neurology (Chen et al., 2001; Fukuda and Fujita, 2005). Other important applications, currently under investigation in hematology, are treatment and/or prevention of acute and chronic graft-versus-host disease (Le Blanc and Ringden, 2005) and support of engraftment after hematopoietic stem cell transplantation (HSCT) (Pinho and Frenette, 2019). Very recently it was found that MSCs can also be used for immunosuppressive therapy in patients with COVID-19 (Sengupta et al., 2020; Shu et al., 2020; Rezakhani et al., 2021). For clinical use, systemic delivery of exogenous MSCs is the preferred route of injection but this approach requires that the expanded MSCs have sufficient capacity to extravasate and home to the target tissue (Sarkar et al., 2011).

At this moment there is also increasing interest to investigate whether MSC-derived extracellular vesicles (EVs) such as exosomes, could serve as a cell-free biologic in clinical practice. MSCs exert their function at least partially through paracrine mechanisms (Konala et al., 2016). It can be assumed that EVs possess characteristics similar to their parent cells and might have some advantages over MSCs such as a better safety profile, easier maintenance and a smaller size enabling them to circulate over longer distance without entrapment in the lungs, a well-known problem when *in vitro* expanded MSCs are infused through the blood circulation (Gao et al., 2001).

Because of the large discrepancy between the number of cells that can be harvested *in vivo* and the number needed for clinical use, MSCs must be expanded *in vitro* (Wechsler et al., 2021). Until now, fetal calf serum (FCS) is used as the reference culture medium supplement for expanding MSCs (Friedenstein et al., 1974). However this supplement has several disadvantages, such as the possibility of transmission of bovine pathogens (prions, viruses, mycoplasmas and bacteria), a batch-to-batch quality inconsistency that hampers reproduction of results and ethical concerns regarding methods of FBS collection (Cholewa et al., 2011; De Becker and Van Riet, 2015; Burnouf et al., 2016). Several studies suggested that human platelet lysate (PL) might be a good alternative for FCS, providing some important advantages. PL has a more favorable safety profile whereas it is a humanized culture medium supplement, it induces a higher cell proliferation rate with a shorter time to reach culture confluency and it is readily available. In addition to these advantages, FCS and PL are made up of different components as indicated in Table 1. It is important to note that the list of components in this table is not exhaustive as the scientific community agrees that both supplements are still not fully characterized and show–sometimes important - batch-to-batch variability (Price and Gregory, 1982; Horn et al., 2010; Crespo-Diaz et al., 2011; Kinzebach et al., 2013; Shih and Burnouf, 2015; Pilgrim et al., 2022). When considering all these factors, it can be assumed that the expansion culture protocol, including the growth medium can have major influence on the biological MSC characteristics. Previous studies already demonstrated that *in vitro* expansion conditions can affect the phenotype of MSCs and influence their multipotency, DNA repair and proliferation regulation (Kretlow et al., 2008; Wagner et al., 2008). Therefore, further research optimizing the culture medium composition and expansion methods of MSCs is mandatory.

Based on clinical studies using *in vitro* expanded MSCs, it can be assumed that the cells are relatively safe in terms of genetic stability. To date, spontaneous transformation of human bone-marrow derived MSCs hasn't been demonstrated yet (Barkholt et al., 2013; Budgude et al., 2020). Spontaneous transformation of MSCs has been observed so far only in expansion cultures using murine bone marrow cells. Aggressive sarcoma formation has been observed after transplantation of *in vitro* expanded murine MSCs in non-immunocompromised mice (Xu et al., 2012). These findings were supported by other studies (Tolar et al., 2007; Jeong et al., 2011; Xiao et al., 2013). The use of a medium supplement like PL that induces a higher proliferation rate, might be associated with a higher risk for genetic instability and malignant transformation.

TABLE 1 Overview of the different components of FCS and PL as culture medium growth supplement. This list is non-exhaustive and based on previously published data (Price and Gregory, 1982; Horn et al., 2010; Crespo-Diaz et al., 2011; Kinzebach et al., 2013; Shih and Burnouf, 2015; Pilgrim et al., 2022). (bFGF = basis fibroblast growth factor, EGF = epidermal growth factor, HGF = hepatocyte growth factor, VEGF = vascular endothelial growth factor, PDGF = platelet derived growth factor, IGF = insulin like growth factor, CCL = chemokine (C-C motif) ligand, CXCL = C-X-C motif chemokine ligand).

Fetal calf serum	Human platelet lysate
Proteins e.g. hemoglobin, albumin, low antibody concentration	Proteins e.g. albumin, immunoglobulins, fibrinogen
Major cations: Na^+, K^+, Ca^{++}	Coagulation factors
Major anions: Cl^-, PO_4^-	Adhesion molecules
Essential amino-acids	Protease inhibitors and proteoglycans
Trace elements and vitamins e.g. Selenium, vitamin A, vitamin E	Growth factors e.g. bFGF, EGF, HGF, VEGF, PDGF-AA, PDGF-AB, PDGF-BB, IGF-1
Growth factors	Chemokines e.g. CCL5, CXCL1, CXCL2, CXCL3
Cytokines	Cytokines
Carbohydrates	
Hormones e.g. insulin, cortisol, parathyroid hormone, progesterone, testosterone, growth hormone, thyroid hormones	
Glucose	
Cholesterol Non protein nitrogen	

In this study, we examined the impact of the culture medium supplement (PL *versus* FCS) on the genetic profile of *in vitro* expanded human mesenchymal stromal cells using genome wide RNA sequencing. In addition, we screened MSCs expanded in PL-based medium for the presence of tumor associated genetic variants by NGS using a targeted gene panel.

Material and methods

In vitro expansion of bone marrow-derived MSCs

Sternal bone marrow aspirates were obtained from healthy donors, following the informed consent. This study was approved by the Ethical Committee UZ Brussel (number 2001/23) For all donors MSCs were cultured in parallel in PL and FCS supplemented medium. The median age of the donors was 66 years (range 54–74 years) and 83% were male. Isolation of bone marrow mononucleated cells (BMMNC) was done by Lymphoprep density gradient centrifugation (Fresenius Kabi, Schelle, Belgium). BMMNC were plated in 10 ml DMEM +10% Hyclone FBS (Gibco, Fisher Scientific, Merelbeke, Belgium). According to the manufacturer, HyClone FBS is low in antibodies and high in growth factors to support robust growth and division of cells, including stem cells. In parallel, cells were cultured in α-MEM + 10% platelet lysate (Macopharma Benelux, Mouscron, Belgium) at a density of 60×10^6 cells per 75 cm^2 in Nunclon/Corning culture flasks (Nunc, VWR International, Leuven, Belgium) and incubated at 37°C with 5% humidified CO_2. Growth medium and non-adherent cells were discarded after 4 h. The adherent cell fraction was rinsed with DPBS (Gibco) and 15 ml DMEM +10% FBS or α-MEM + 10% platelet lysate was added to the culture flask. Cells were cultured for four passages. For some experiments, cells were harvested after ten passages. At each passage, cells were detached after a 5 min Incubation at 37°C with trypsin 0.25% (Gibco). After adding 10% fetal calf serum (FCS) (A&E Scientific, Enghien, Belgium) in RPMI, the cells were washed once in DPBS (Gibco). After each passage, the cells were harvested when the cultures reached subconfluent conditions (50%–80% coverage of the culture surface area). The number of population doublings during *in vitro* expansion was calculated as previously described (Jung et al., 2012).

Immunomorphological characterization of *in vitro* expanded MSCs

Approximately 10^5 MSCs were incubated with 10 µl monoclonal antibody conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE): CD105-FITC (Biolegend, ImTec, Antwerpen, Belgium), CD90-PE (Biolegend), CD73-PE (Biolegend) and CD166-PE (Biolegend). Unbound antibody was washed after 15 min with 3 ml PBS (Gibco). The cell pellet was resuspended in 0.5 ml PBS (Gibco). Samples were analyzed with the flow cytometer Macs Quant (Miltenyi Biotec, Bergisch Gladbach, Germany) with 10,000 events recorded for each condition. The morphology of cultured cells was evaluated using light microscopy.

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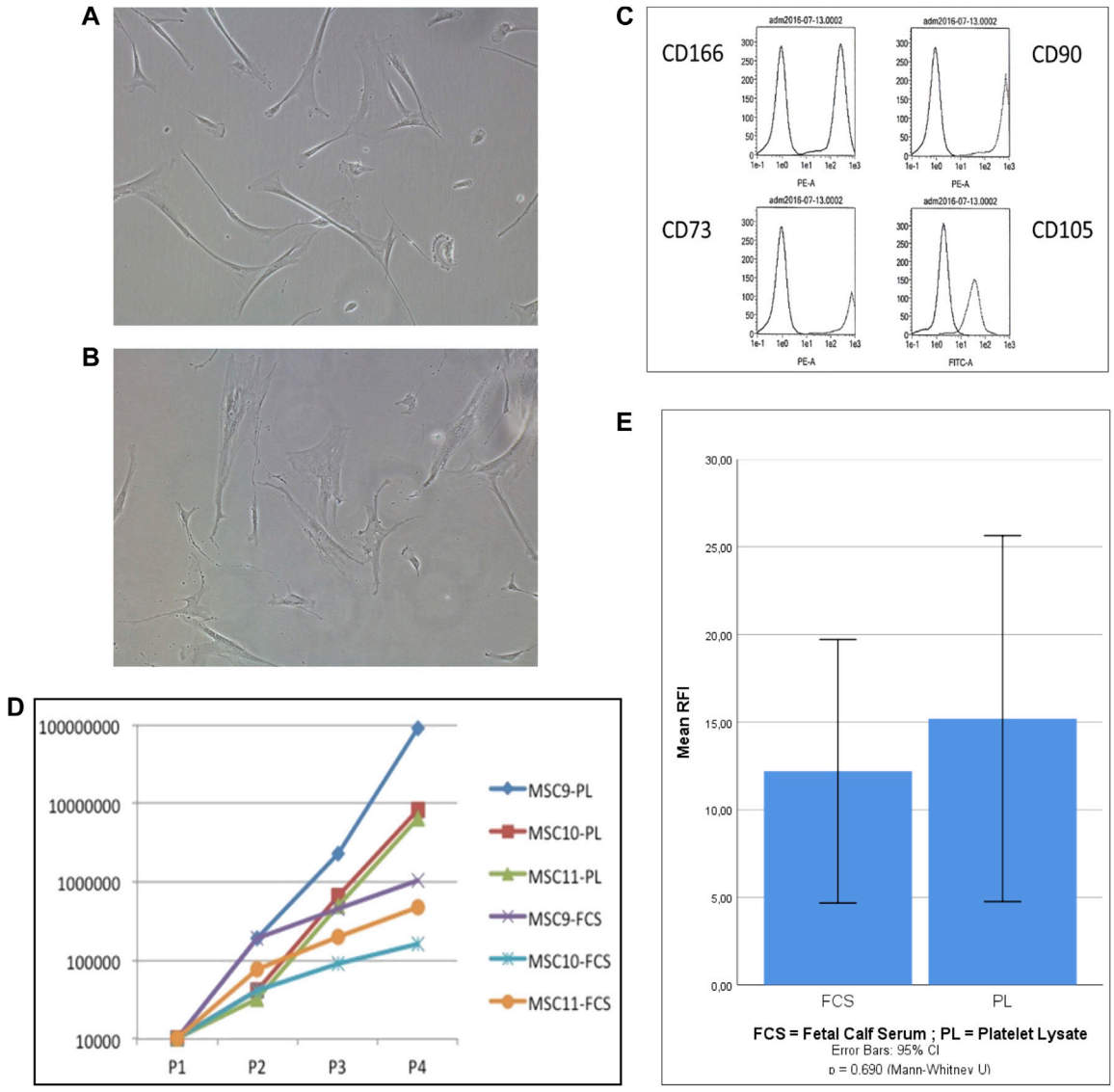

FIGURE 1
Characterisation of in vitro expanded MSCs. **(A,B)** Representative photographs of MSC's cultured in PL-based **(A)** and FCS-based **(B)** expansion medium (30×); **(C)**: Phenotype MSCs expanded in PL-based medium: the first peak represents the negative control antibody whereas the second peak represents the antigen-specific antibody; **(D)**: Proliferation capacity of MSCs expanded in PL-based medium versus FCS-based medium (n = 3), the number of cells is presented according to the number of passages; **(E)**: *In vitro* migration of MSCs cultured in FCS-based medium and PL-based medium (n = 5). Migration is expressed as mean relative fluorescence intensity (RFI). (p= 0.69, 95% CI, Mann Whitney).

In vitro migration of *in vitro* expanded MSCs

Prior to the migration assay, cells were labeled with the carbocyanine fluorochrome DiI (1,1′dioctadecyl-3,3,3′, 3′tetramethylindocarbocyanine perchlorate) (Molecular Probes, Fisher Scientific, Merelbeke, Belgium). DiI is a lipophilic molecule that incorporates in the cell membrane with specific spectral characteristics: absorption maximum at 549 nm and an emission maximum at 565 nm. MSC were incubated for 72 h with 10 µg/ml DiI at 37°C with 5% humidified CO_2. After incubation, the cells were harvested with trypsin. Since MSCs were labeled with a fluorescent dye, we used BD Falcon™ HTS FluoroBlok Inserts (BD Benelux, Erembodegem, Belgium). Their polyethylene terephthalate (PET) membrane blocks light transmission from 490 to 700 nm. As such, we can detect cells

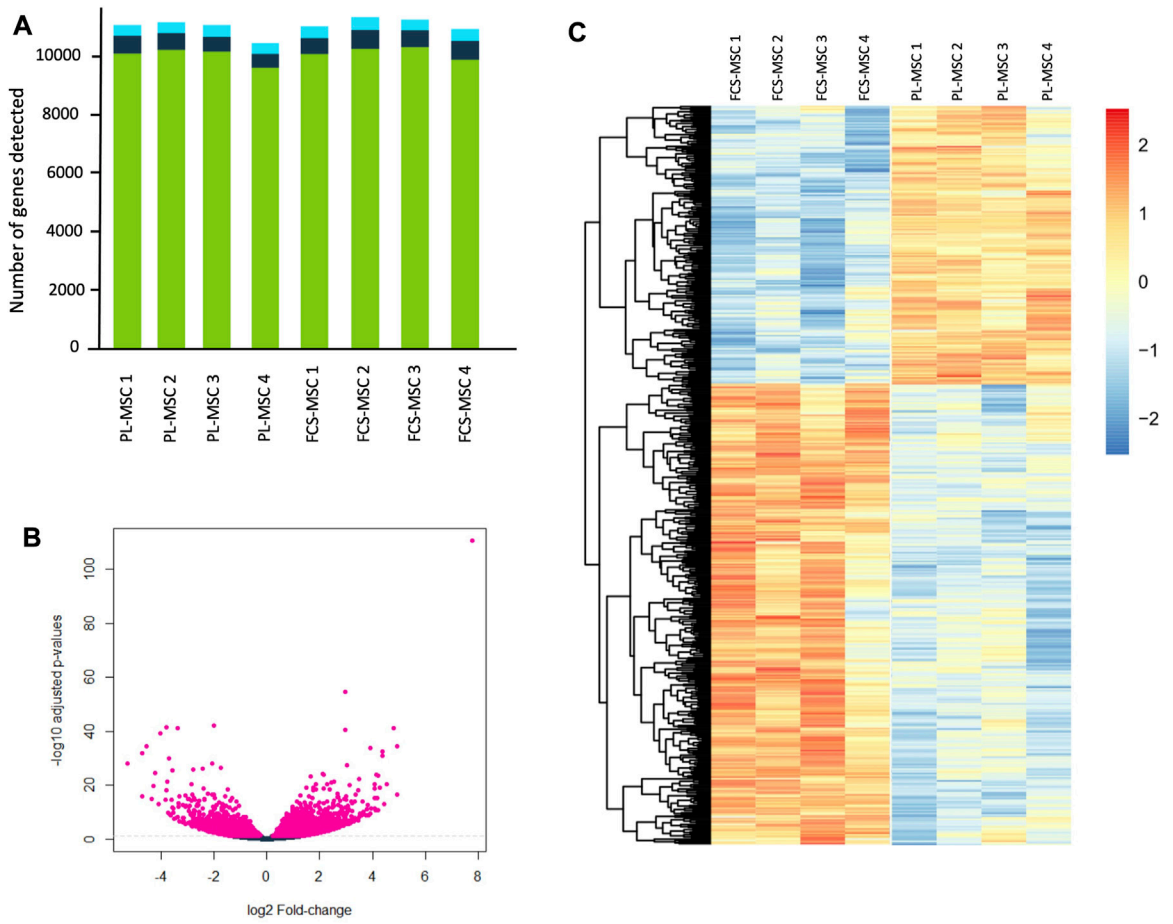

FIGURE 2
RNA sequencing of FCS-MSCs and PL-MSCs. **(A)**: Overview of the number of transcripts detected per sample for 4 different donors. The green bars represent coding genes, dark blue bars long non coding RNA and light blue bars other RNA species. **(B)**: Volcano plot showing -log10 adjusted *p*-values in function of the log2 fold change for FCS-MSCs *versus* PL-MSCs. Pink points indicate significantly differential expressed genes at FDR <0.05. FDR: false discovery rate. **(C)**: Heatmap showing the top 500 differentially expressed genes according to adjusted *p*-values at FDR <0.05 for FCS-MSCs *versus* PL-MSCs.

present in the lower compartment only. Once cells migrate through the pores of the membrane, they are no longer shielded from the light source. From this moment, they can be detected with a fluorescence plate reader. Data were assembled with the Fluoroskan Ascent plate reader and software (Thermo-Labsystems, VWR International, Leuven, Belgium). Migration assays were performed using filters coated with 50 µg Matrigel (BD Benelux) (gelled at 37°C for 1 h) and human bone marrow-derived endothelial cells (4LHBMEC-line) to analyze transendothelial migration. MSCs (5×10^4) in 100 µl RPMI were added to the upper compartment. The lower compartment contained 10% FCS in RPMI, which serves as a chemoattractant. Samples were incubated at 37°C overnight. The percentage migration was calculated as the ratio of signal intensity of migrated cells *versus* signal intensity of total input number of cells.

Poly A + RNA sequencing

Bone marrow samples from four different donors were used to culture MSCs in both culture medium supplements (FCS *versus* PL) for four passages. After *in vitro* expansion, MSCs were harvested and RNA was isolated using the miRNeasy Micro Kit (Qiagen, Venlo, Netherlands) with column DNase digestion, following the manufacturer instructions. The RNA concentration was determined using the Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Merelbeke, Belgium). RNA quality control was performed with the 2,100 Bioanalyzer microfluidic gel electrophoresis system (Agilent Technologies, Machelen, Belgium). The comparing RNA sequencing analyses was performed in collaboration with Biogazelle (Zwijnaarde, Belgium). Briefly, libraries for mRNA sequencing were prepared using the TruSeq stranded mRNA sample prep kit

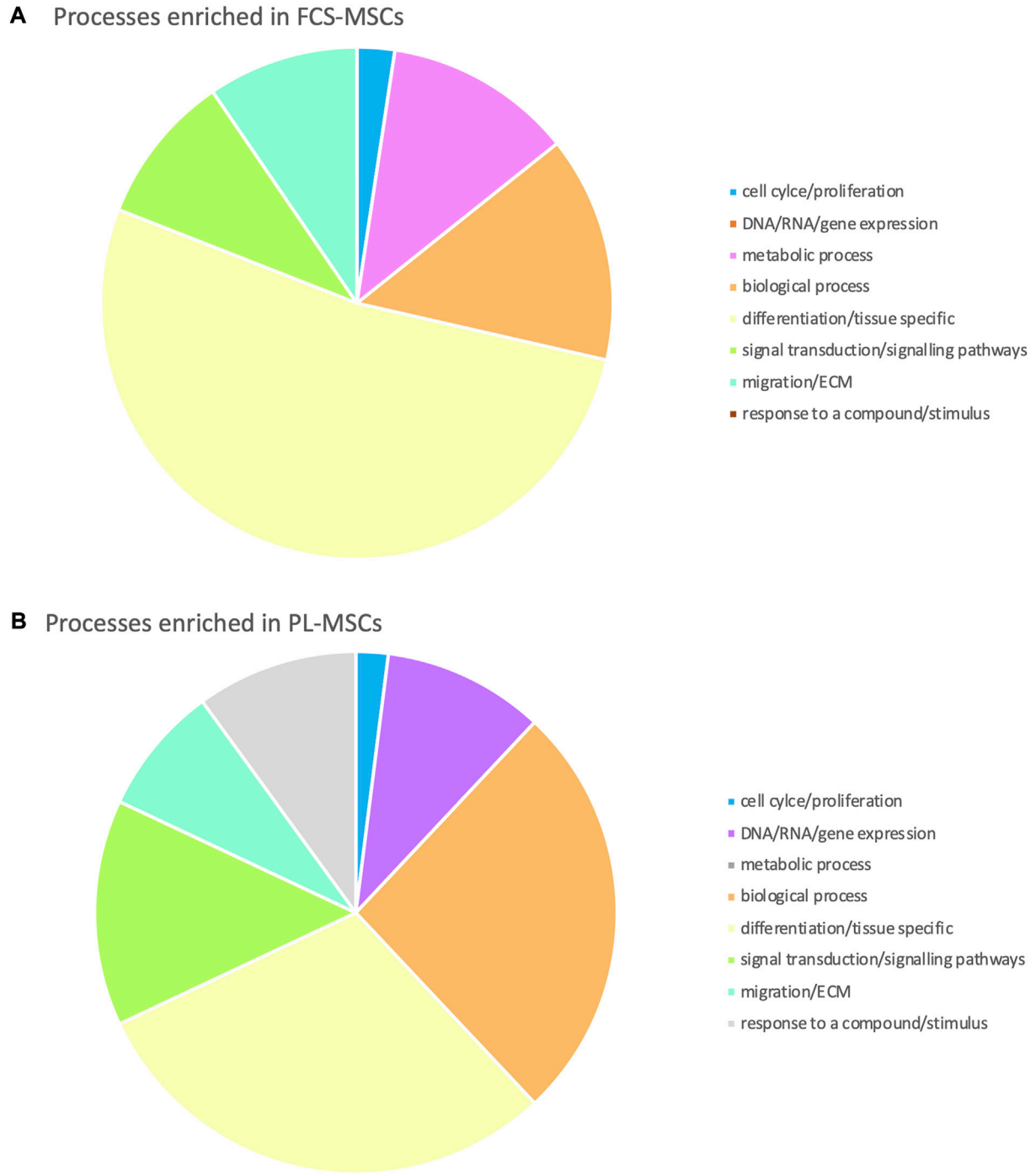

FIGURE 3
Gene Ontology analysis: processes enriched in **(A)** FCS-MSCs and **(B)** PL-MSCs ($q \leq 0,05$, false discovery rate adjusted p value $\leq 0,05$). Enriched processes are grouped in 8 major categories to get a better overview of processes impacted by differential expression due to use of a different growth supplement.

(Illumina Inc., San Diego, CA, United States). The starting 100 ng of RNA was mRNA enriched using the oligodT bead sytem (Illumina Inc.). Subsequently the isolated mRNA was enzymatically fragmented. Following to this, first and second strand synthesis were performed and the double stranded cDNA was purified with Agencourt AMPure XP (Beckman

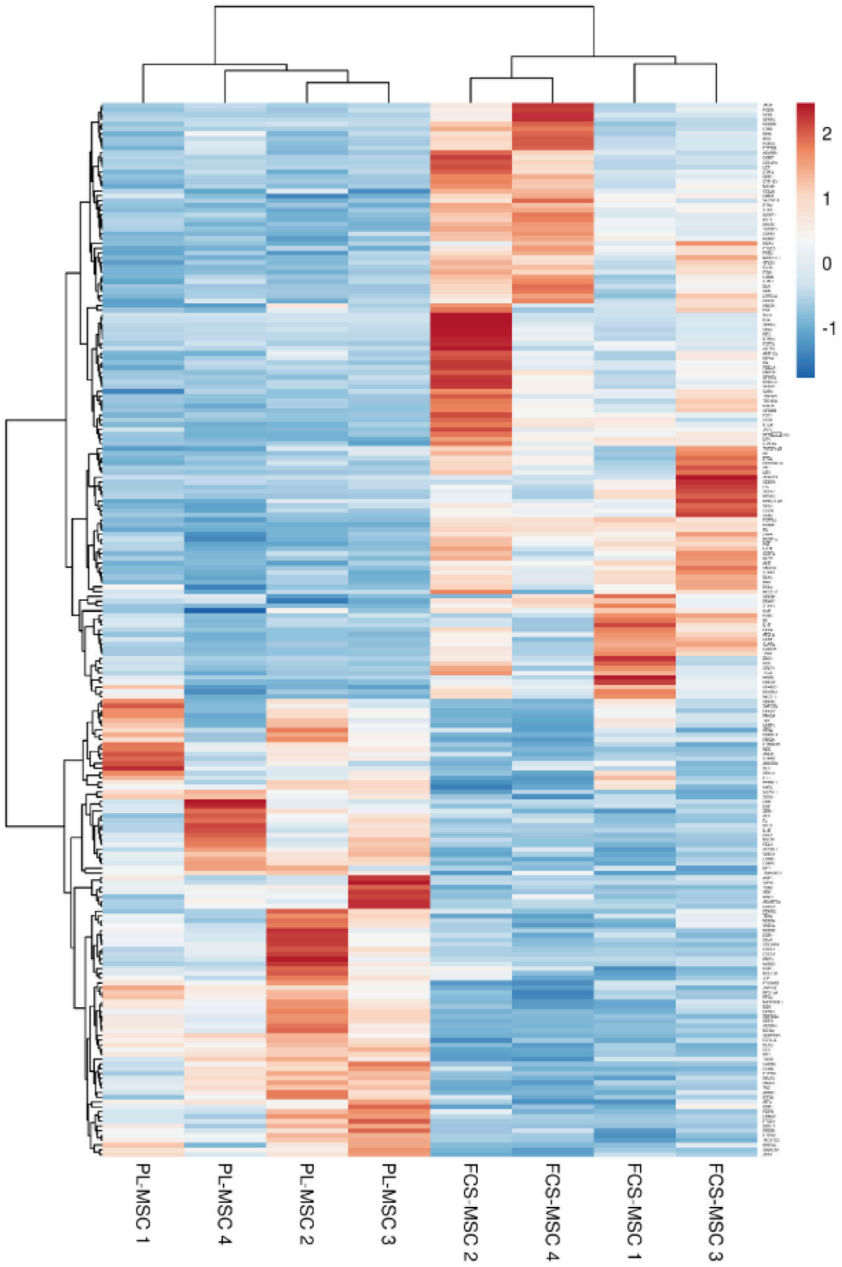

FIGURE 4
Heatmap generated based on FPKM of the 221 genes included in the Gene Ontology term 'regulation of cell population proliferation' (GO: 004212). Rows are centered and unit variance scaling is applied to the rows. Both rows and columns are clustered using correlation distance and average linkage.

Coulter, Brea, CA, United States). The cDNA was end repaired and the fragment ends were ligated by illumine sequencing adaptors. The library was purified with Agencourt AMPure XP (Beckman Coulter). The polyA + RNA stranded libraries were pre-amplified with PCR and purified with Agencourt AMPure XP (Beckman Coulter). The size distribution of the libraries was validated and the quality inspected on the 2,100 Bioanalyzer high sensitivity DNA chip (Agilent Technologies). According to the project specification (number of reads), high quality libraries were quantified using the Qubit 2.0 Fluorometer (Thermo Fisher Scientific). The concentration was normalized and the samples were pooled. Single-end sequencing was performed on the NextSeq500 according to the manufacturer instructions (Illumina Inc.). Transcriptome

data analysis based on eight samples with four in each group was based on the Tuxedo software package (Oracle Belgium, Vilvoorde, Belgium). The differentially expressed genes were finally organized in Gene Ontology categories using the open-source tool GOnet (Pomaznoy et al., 2018).

Senescence analysis of expanded MSCs by β -galactosidase staining

Senescent cells within the MSC cultures were identified with a β-galactosidase staining using the Senescence Cells Histochemical Staining Kit (Merck Life Science, Hoeilaart, Belgium) according to the manufacturer's instructions. MSCs of passage four were seeded in a 6-well plate at a density of 70.000 cells/well and FCS supplemented medium (Gibco) or PL supplemented medium (Macopharma Benelux) until reaching a sub confluent density. After addition of the staining mixture, the MSCs were incubated at 37°C without CO_2 overnight. Stained MSCs were evaluated with light microscopy using the EVOS M7000 (Thermo Fisher Scientific).

Detection of genetic variants by targeted gene sequencing

NGS analysis screening for genetic variants in 165 known tumor-associated genes was performed in collaboration with Brussels Interuniversity Genomics High Throughput core (BRIGHTcore), Universitair Ziekenhuis Brussel (UZ Brussel)/Vrije Universiteit Brussel (VUB), Brussels, Belgium. First, DNA was extracted from *in vitro* expanded MSCs using the QIAamp DNA Mini kit (Qiagen, Venlo, Netherlands), according to the instructions of the manufacturer. Eluted DNA was quantified on the Qubit 2.0 with the Qubit dsDNA HS Assay Kit (Life Technologies, CA, United States) after which DNA was stored at -20°C until library preparation. Next, DNA was requantified again using VICTOR Nivo™(PerkinElmer, Waltham, United States) prior to library preparation. 150 ng of input DNA was used to generate libraries with the KAPA HyperPlus kit (Roche Sequencing, CA, United States). Target enrichment was performed according to version 5.0 of the manufacturer's instructions with a homebrew Roche SeqCap EZ Choice probemix (Roche Sequencing, CA, United States) and homebrew IDT xGen Lockdown Probes for the final batch of sequenced samples (Integrated DNA Technologies, Inc. (IDT), Iowa, United States). A minimum of 14 million 2 × 100 bp reads were generated for each sample on the Illumina NovaSeq 6,000 system (Illumina Inc., CA, United States) using NovaSeq 6000 S2 Reagent Kit (200 cycles) kit. Illumina's bcl2fastq algorithm (version 2.19) was used to convert the raw basecall files into fastq files after which reads were aligned to the human reference genome (hg19) using BWA (version 0.7.10-r789) and picard (version 1.97) was used to mark the duplicate reads. Genome Analysis Toolkit (GATK) (version 3.3) was used to provide post-processing of the aligned reads which consisted of realignment around insertions/deletions (indels) and base quality score recalibration. The quality control on the post-processed aligned reads was performed by using samtools flagstat (version 0.1–19) and picard HsMetrics (version 1.97) which were also used to investigate the total number of reads, the percentage of duplicate reads, the mean coverage on target and the percentage of on-target, near-target and off-target bases. Variants were called with GATK Mutect2 (version 4) in tumor-only mode and final variant files were described using Alamut batch version 1.11 and Alamut database version alamut_db-1.5-2021.06.01.db.

Filtering and interpretation of variants was performed based on the recommendations of the ComPerMed guidelines (version 2 January 2019) (Froyen et al., 2019) using an in-house-designed script with the exclusion of the following variants: known artefacts in Alamut, synonymous variants, variants with a MAF (minor allele frequency) of >0.1% and variants with a VAF (variant allele frequency) of <3%. After data filtering, the remaining variants are subjected to manual inspection of the aligned reads in IGV version 2.6.3 (Integrative Genomics Viewer, © Broad Institute and the Regents of the University of California). Biological significance of the detected variants and classification as "pathogenic," "likely pathogenic" and "VUS" was assessed based on the recommendations described by Froyen et al. (Froyen et al., 2019).

Results

Culture and characterization of in vitro-expanded MSCs

Adherent cells were isolated by plastic adherence from mononuclear bone marrow cell suspensions and cultured in both types of expansion medium (PL-based *versus* FCS based) up to four passages (PL-MSCs and FCS-MSCs). For two donors, cells were cultured in PL-based medium up to 10 passages. FSC-MSCs showed a different shape and size compared to PL-MSCs (as shown in Figure 1). In PL-based medium (A) the cells showed in general a smaller cell size and had a more elongated (thinner) shape as compared to cells cultured in FCS-based medium (B). This morphology difference was inducible, as MSCs that were initially cultured in PL-based medium showed an increased cell size when they were passaged to FCS-based medium (data not shown). Flow cytometry analysis showed that the cells, expanded in both types of medium, had the same

TABLE 2 Differential expression of genes involved in apoptosis in MSCs expanded in FCS and PL supplemented medium. Genes are designated by their gene name and expression data were filtered based on fold change (at least twofold) and *p*-value ≤0.05. To provide additional information on relevance of gene expression mean FPKM values for cells expanded in both conditions are also provided. (FPKM = fragments per kilobase million). The direction of differential expression is shown in the last column. These genes are at least twofold upregulated in FCS or PL.

Gene	Fold change	*p*-value	Mean FPKM FCS	Mean FPKM PL	Differential expression
BCL2A1	6.7×	8.02×10^{-8}	0.25	2.28	Upregulated in PL
BCL3	2.2×	1.85×10^{-3}	3.09	6.91	Upregulated in PL
CASP1	2.2×	2.08×10^{-4}	0.75	1.58	Upregulated in PL
FAIM2	3.8×	1.68×10^{-4}	0.028	0.21	Upregulated in PL
BCL2	2.2×	7.65×10^{-3}	0.48	0.22	Upregulated in FCS
BMF	2.5×	2.09×10^{-2}	0.36	0.09	Upregulated in FCS
HRK	7.1×	9.72×10^{-6}	0.26	0.0086	Upregulated in FCS

phenotype and expressed four markers: CD90, CD105, CD73, and CD166, known to be expressed by true MSCs (representative phenotype is shown in Figure 1C). The expansion rate of MSCs in PL-based medium was higher than in FCS-based medium, as shown for 3 donors in Figure 1D. Cells expanded in PL–based and FCS-based medium showed during the 4 passages a mean population doubling of 10.7 (±2.1) *versus* 5.4 (±1.3), respectively. As shown in Figure 1E, the percentage of migrated cells was compared between PL-MSCs and FCS-MSCs. Comparing the migration through the mimicked endothelium, PL-MSCs did not show a significantly increased migration as compared to MSCs cultured in FCS-based medium ($p = 0.69$, 95% CI, Mann Whitney).

Differential gene expression of in vitro-expanded MSCs

The RNA expression profile of MSCs cultured in FCS was compared to the cells cultured in clinical grade human PL. The data are available in the ArrayExpress repository. In total, 11,000 genes were evaluated (Figure 2A) of which **2,433** were upregulated in FCS and **2,260** in PL. We further narrowed the pool of genes and retained only those that were at least twofold up- or downregulated. This resulted in **1974** differentially expressed genes, **1,178** were upregulated in FCS and **796** in PL. Fragments per kilobase million (FPKM) normalizes the reads for read depth and gene length. When applying this additional filter (FPKM ≥ 0,3) we still retain **1,666** differentially expressed genes. This means that even with these more stringent criteria, still almost 1 in 6 genes is differentially expressed when a different growth supplement is used. A volcano plot depicts the differentially expressed genes with the log2 fold change value in the X axis and the -log10 adjusted *p*-value in the Y axis. This gives a quick overview of the number of significantly differentially expressed genes (Figure 2B). The top 500 differentially expressed genes are represented in the heatmap shown in Figure 2C. In the RNA sequencing dataset, we found that the 4 surface markers used to characterize MSCs are expressed at high levels in both cell types: mean FPKM ranging from 56 to 118 in FCS-MSCs and from 28 to 140 in PL-MSC. Interestingly, CD166 is significantly upregulated in FCS-MSCs according to the criteria described above: 2.45-fold change, *p*-value 3.57×10^{-7} and FPKM 80 in FCS-MSCs vs. 28 in PL-MSCs. CD90 on the other hand also has a *p*-value below 0.05 and FPKM 56 *versus* 100 but falls just short of the fold change of 2 criterium at -1.87 and is upregulated in PL-MSCs. CD73 and CD105 are not differentially expressed in this analysis.

Using the online GOnet application enrichment for Gene Ontology (GO) terms was investigated. After selection of those GO terms that had a false discovery rate adjusted *p*-value of ≤0.05 we found that 92 GO terms were enriched: 42 for FCS-MSCs and 50 for PL-MSCs. We then organized these in 8 larger groups to reflect to which category of processes they belong (Figure 3). For both groups GO terms related to cell differentiation and biological processes made up more than half of enriched terms but differentiation and tissue specific GO terms were more prevalent in FCS-MSCs. Additionally, in PL-MSCs there is also enrichment of GO terms related to RNA/DNA/gene expression and responses to compounds or stimuli, these categories are not present in the GO analysis of FCS-MSCs.

Since PL-MSCs exhibit an increased proliferation rate we further analyzed the genes important for regulation of apoptosis and genes that play a role in cell senescence.

Apoptosis

Table 2 summarizes the genes involved in the regulation of apoptosis. Three important anti-apoptosis genes show higher expression in MSCs cultured in clinical grade human PL-based medium. We see an almost 7 times higher expression of *BCL2A1* (BCL2 related protein A1) compared to FCS-based

TABLE 3 overview of differential expression of genes reported to be upregulated in senescent MSC passages. Genes are designated by their gene name. Fold change, *p*-value and FPKM for each gene are detailed in the columns. FPKM will be highest in the condition where the gene is upregulated. The direction of differential expression is shown in the last column. Genes that are considered not to be significantly up- or downregulated ($p > 0.05$ and/or FPKM <0.3) are marked in italic. (Gene list from Wagner et al., 2008).

Gene	Fold change	*p*-value	Mean FPKM FCS	Mean FPKM PL	Differential expression
GPNMB	11.3	3×10^{-10}	93.8	4.16	Upregulated in FCS
MAN1C1	8.57	1.11×10^{-11}	1.20	0.0821	Upregulated in FCS
GCNT3	*3.73*	*0.0066*	*0.0584*	*0.00341*	*Upregulated in FCS*
PERP	1.58	0.03	18.3	11.0	Upregulated in FCS
MCOLN3	7.46	1.5×10^{-11}	1.09	0.0952	Upregulated in FCS
ENPP5	*1.39*	*0.27*	*1.88*	*1.296*	*Upregulated in FCS*
CTSK	3.63	0.0022	306	47.7	Upregulated in FCS
ATF3	−2.43	1.41×10^{-6}	4.75	10.7	Downregulated in FCS
DAB2	1.59	0.044	39.9	24.8	Upregulated in FCS
LY96	1.50	0.045	20.7	12.7	Upregulated in FCS
DNAJB4	−1.70	0.035	26.0	50.0	Downregulated in FCS
SLC16A6	*1.67*	*0.20*	*2.44*	*1.10*	Upregulated in FCS
SLC11A2	*1.49*	*3.18×10^{-6}*	*5.96*	*3.80*	Upregulated in FCS
STAT1	2.22	4.3×10^{-6}	100	41.5	Upregulated in FCS
CA11	*−1.46*	*0.44*	*0.0545*	*0.0654*	*Downregulated in FCS*
SEC14L4	*−1.24*	*0.72*	*8.82*	*6.56*	*Downregulated in FCS*
SCG2	*1.12*	*0.85*	*15.8*	*9.73*	*Upregulated in FCS*
GM2A	1.50	0.0090	1.28	1.30	Upregulated in FCS
IFIT1	*−1.06*	*0.80*	*110*	*101*	*Downregulated in FCS*
PRNP	*1.07*	*0.92*	*93.8*	*4.16*	*Upregulated in FCS*

medium. *FAIM-2* (Fas-apoptotic inhibitory molecule 2) is almost 4 times higher expressed while *BCL-3* (B-Cell Lymphoma 3) is approximately 2 times upregulated in PL-based medium. *CASP1* (Caspase 1) is a pro-apoptotic gene upregulated in PL-MSCs. Genes involved in apoptotic processes and upregulated in MSCs, cultured in FCS-based medium, are *BCL-2* (BCL2 apoptosis regulator), *BMF* (BCL2 modifying factor), (each approximately 2 times higher) and *HRK* (Harakiri) (7 times higher). BMF and HRK are pro-apoptotic genes whereas BCL-2 is an anti-apoptotic gene. When also factoring in a minimum FPKM value of 0.3, FAIM2 and HRK can no longer be retained as significantly differentially expressed genes.

The Gene Ontology term 'Regulation of cell population proliferation' (GO:004212) was enriched in our RNA sequencing analysis. We selected the 221 genes included in this term and generated a heatmap using the Clustvis online tool (Metsalu and Vilo, 2015). In this analysis MSC show similar expression profiles according to the growth supplement used as evidenced by their clustering according to expansion in FCS or PL (Figure 4).

Senescence of MSCs

In our hands FCS-MSCs show a blunted proliferation curve (Figure 1D) and cells become larger even at passage 4, PL-MSCs on the other hand do not appear to lose proliferation capacity at passage 4 and retain a small spindle shape morphology (Figures 1A,B). These might be signs of senescence in FCS-MSC. In 2008 a set of 33 genes that was differentially expressed in senescent MSCs was described. We cross-referenced this gene list with data obtained in our analysis (Tables 3, 4) (Wagner et al., 2008). We retrieved data on 20 genes reported upregulated in senescent MSCs. Of these 20 genes, 13 showed significant differential expression ($p \leq 0.05$ and/or FPKM>0.3). Eleven genes are upregulated in FCS-MSCs, 2 in PL-MSC (Table 3). A few genes were also reported to be downregulated in senescent MSCs. In our analysis, only CXCL6 was significantly differentially expressed and was highly downregulated (38x) in MSCs expanded in FCS-medium (Table 4). By β-galactosidase staining it could be confirmed that more MSCs were in senescence modus when the cells were cultured in FCS based expansion medium *versus* PL based expansion medium (Figure 5).

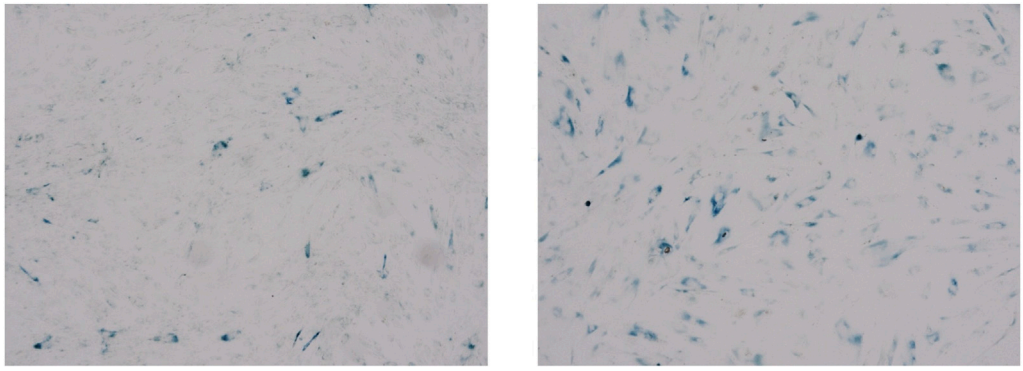

FIGURE 5
β-galactosidase staining of cultured MSCs. Cells from a representative donor were cultured in both media and stained after 4 passages. More β-galactosidase positive (blue colorored) cells were found in MSC cultures with FCS based expansion medium *versus* PL-based medium (4 × magnification) (P4 = passage 4).

TABLE 4 overview of differential expression of genes reported to be downregulated in senescent MSC passages. Genes are designated by their gene name in the first column. Fold change, *p*-value and FPKM for each gene are detailed in the columns. FPKM will be highest in the condition where the gene is upregulated. The direction of differential expression is shown in the last column. Genes considered not to be significantly up- or downregulated ($p > 0.05$ and/or FPKM <0.3) are marked in italic. (Gene list from Wagner et al., 2008).

Gene	Fold change	*p*-value	Mean FPKM FCS	Mean FPKM PL	Differential expression
CXCL6	−38.2	7.2×10^{-29}	0.0405	6.53	Upregulated in FCS
HAS1	*1.87*	*0.059*	*33.7*	*14.3*	*Upregulated in FCS*
RARRES1	*−1.56*	*0.43*	*0.567*	*1.33*	*Upregulated in FCS*
TNFSF11	*0.90*	*0.87*	*0.196*	*0.338*	*Downregulated in FCS*

Mutation profile of cultured MSCs

In the ten MSC DNA samples we investigated, 21 different genetic variants were detected as shown in Table 5, almost always with a high Variant Allele Frequency (VAF). Of these variants, only three are classified as "likely pathogenic" and these are located in *APC* (APC regulator of WNT signaling pathway), *NF1* (Neurofibromin 1) and *STAG2* (Stromal antigen 2). The remaining variants were either classified as VUS (17/21) or Ambiguous (1/21). The data are available in the ENA repository, accession number PRJEB55753.

Discussion

Initially, (FCS) was used as a growth supplement for culturing MSCs, and it has been considered a gold standard since then (Friedenstein et al., 1974). It is a rich source of many different growth factors and can act as a chelator for water insoluble nutrients, thus protecting the cells against shear damage (McGillicuddy et al., 2018). However, this medium has several disadvantages, such as xeno-immunization, pathogen transmission, batch-batch inconsistency but also ethical concerns (Cholewa et al., 2011; De Becker and Van Riet, 2015; Burnouf et al., 2016). Additionally bovine growth factors might not always be compatible with human cells, already in the early 80s Hornsby and colleagues found that for expansion of human adrenocortical cells, these responded better to horse than bovine serum (Hornsby et al., 1983). Around the same time Read et al. showed that bovine insulin-like growth factor-1 (IGF-1) has much lower affinity than human IGF-1 to the human receptor (Read et al., 1986). Human PL was proposed as a humanized alternative for FCS as a gold standard for the expansion of MSCs. This medium supplement has a more favorable safety profile since it is a humanized culture medium supplement and is readily available (Burnouf et al., 2016). In wound healing degranulation of platelets leads to secretion of cytokine and growth factors such as epidermal

TABLE 5 Overview of the genetic variants detected using a gene panel consisting of 165 tumor-associated genes. MSCs from donor 5 and 6 were harvested at passage 4 (P4) and 10 (P10) for sequencing, for all other donors cells were harvested at passage 4. VAF = variant allele frequency, VUS = variant of unknown significance.

Donor	Gene	cDNA	Protein change	VAF	Relevance of variant
1	*APC*	NM_000038.5:c.5427del	p. (Asp1810Ilefs*7)	4,1%	Likely pathogenic
	NOTCH1	NM_017617.4:c.4307C>T	p. (Ala1436Val)	49,4%	VUS
	DIS3	NM_014953.4:c.2646C>G	p. (Asn882Lys)	50%	VUS
	RET	NM_020975.4:c.364T>G	p. (Tyr122Asp)	57,8%	VUS
2	*RAD54L*	NM_003579.3:c.1784G>A	p. (Trp595*)	47,1%	Ambiguous
	ATR	NM_001184.4:c.3227A>G	p. (His1076Arg)	42,8%	VUS
	WT1	NM_024426.4:c.*10C>T	p. (?)	49,2%	VUS
	ATM	NM_000051.3:c.7114G>C	p. (Asp2372His)	51,5%	VUS
	NF1	NM_001042492.2:c.6904C>T	p. (Gln2302*)	36,8%	Likely pathogenic
3	*RAD54L*	NM_003579.3:c.883T>C	p. (Cys295Arg)	49,9%	VUS
	PTPN11	NM_002834.4:c.*13A>G	p. (?)	52,8%	VUS
4	*DICER1*	NM_030621.4:c.5738A>G	p. (Lys1913Arg)	48,2%	VUS
	STAG2	NM_001042749.2:c.3467 + 1_3467+3del	p. (?)	38%	Likely pathogenic
5 P4	*BRCA2*	NM_000059.3:c.3088T>G	p. (Phe1030Val)	50%	VUS
	ERBB2	NM_004448.3:c.1567C>T	p. (Pro523Ser)	49,5%	VUS
5 P10	*BRCA2*	NM_000059.3:c.3088T>G	p. (Phe1030Val)	49,6%	VUS
	ERBB2	NM_004448.3:c.1567C>T	p. (Pro523Ser)	48,6%	VUS
6 P4	*ERBB3*	NM_001982.3:c.2249G>A	p. (Arg750Gln)	48,9%	VUS
	DNMT3A	NM_175629.2:c.977G>A	p. (Arg326His)	3,6%	VUS
6 P10	*ERBB3*	NM_001982.3:c.2249G>A	p. (Arg750Gln)	52,9%	VUS
7	*ABL1*	NM_005157.5:c.2842C>T	p. (Pro948Ser)	52,5%	VUS
8	*ROS1*	NM_002944.2:c.2353G>A	p. (Val785Met)	50,5%	VUS
	BRCA1	NM_007294.3:c.4776C>G	p. (Asn1592Lys)	48,5%	VUS
	GNA11	NM_002067.5:c.911C>T	p. (Ala304Val)	47,7%	VUS

growth factor, PDGF, insuline like growth factor 1 and TGFβ1 and TGFβ2. These factors are important to start different aspects of wound healing (Martin P, 1997). In 2008 Ng and colleagues showed that platelet derived growth factor (PDGF), transforming growth factor β (TGFβ) and fibroblast growth factor (FGF) are important for proliferation and differentiation of MSCs (Ng et al., 2008). These growth factors appear to be present in abundance in PL, that is often generated by repeated cycles of freezing and thawing. Moreover, proteomics and cytokine array assays have confirmed the importance of platelet derived factors for *ex vivo* expansion of MSCs (Horn et al., 2010; Kinzebach et al., 2013). Additionally, it has also been shown that of all platelet derivatives that can be used for expansion of MSCs, only PL will affect the MSC proliferation rate (Bieback et al., 2009).

In this study we aimed to investigate the impact of culture conditions such as the choice of growth supplement on the genetic profile of MSCs.

Firstly, we characterized PL-MSCs expanded according to our protocol in a culture medium supplemented with clinical grade PL. These cells showed the typical spindle shaped morphology of MSCs and expressed CD73, CD90, CD105 and CD166 on the cell surface. Next MSCs from the same donor were cultured in parallel in both FCS and PL supplemented culture media. We could see that PL-MSCs have a higher proliferation rate than those cultured in FCS. *In vitro* transendothelial migration assays show a similar migration capacity *in vitro* for PL-MSCs and FCS-MSCs.

Changes in biological characteristics and gene expression profile in MSCs after alterations in the culture conditions have been reported previously (De Becker et al., 2007; Shi et al., 2007). Not just culture conditions but also the source used to obtain MSCs can have a significant impact on MSC gene expression profile and biological characteristics. Adipose tissue MSCs for example have less potential for bone formation and this is reflected by upregulation of genes that impact adipogenic but not osteocytic differentiation in these MSCs (Gluscevic et al., 2020). We hypothesized that the choice of growth supplement for *ex vivo* expansion of MSCs also might influence gene expression of these cells. Therefore, we performed whole genome polyA + RNA expression profiling on bone marrow derived MSCs from different

donors, cultured in parallel in FCS and PL supplemented medium. These analyses provided evidence that the gene expression profile changes considerably when a different growth supplement is used. Of 30,000 genes evaluated almost 4,700 were differentially expressed. Taking only those genes into account with at least a twofold change, p-value ≤0.05 and FPKM ≥0.3, still 1,666 genes were differentially expressed of which 973 were upregulated in FCS-MSCs and 693 in PL-MSCs. We found that of the 4 typical surface markers used to characterize MSCs, CD166 is significantly upregulated in FCS-MSCs and CD90 falls just short of the 2-fold change criterium but has a very low p-value and high FPKM suggesting that these data are robust. It has been shown previously that different conditions such as proliferative state, serum deprivation or cryopreservation can change the expression of surface antigens (Dudakovic et al., 2014; Camilleri et al., 2016; Krull et al., 2021). In future applications or design of quality control assays for release of MSC products, differential expression of some surface markers that reflect for example a post-proliferative state might be of interest.

To gain more insight in the function/role of all these genes, Gene Ontology analysis was performed using an online tool (Pomaznoy et al., 2018). This analysis shows that different processes are enriched in MSCs if cultured in FCS or PL. In FCS-MSCs 42 GO-terms are enriched, most of these are related to tissue specific processes and differentiation. In PL-MSCs 50 GO terms are enriched, in these cells differentiation/tissue specific and biological processes account for just over half of all GO terms enriched. In PL-MSCs terms related to RNA/DNA and gene expression and response to a compound/stimulus are also enriched which is not the case in FCS-MSCs. This type of analysis provides an enormous amount of information that should be interpreted carefully, for example in MSCs expanded with PL medium more genes could be upregulated in GO processes of migration, but this does not automatically translate into an improved migration capacity. The upregulated genes might also be inhibitory molecules. To clear this out the upregulated genes and their functions must be identified. In our study we are confronted with a very high number of differentially expressed genes and it is beyond the scope of this work to catalogue all. Since we–and others before us–observed an increased proliferation capacity of PL-MSCs we focused on differential expression of genes involved in apoptosis, proliferation and senescence as will be discussed further (Salvadè et al., 2010; Luzzani et al., 2015).

We identified 7 apoptosis related genes in our analysis. Of these 4 were upregulated in PL-MSCs and 3 in FCS-MSCs. Three of 4 genes upregulated in PL-MSCs are anti-apoptotic (BCL2A1, BCL-3, FAIM-2) whereas in FCS supplemented MSC 2 of 3 upregulated genes are pro-apoptotic (BMF, HRK). After more stringent filtering, including a FPKM threshold of 0.3 we find that FAIM2 in PL-MSCs and HRK in FCS-MSCs cannot be considered significantly upregulated in the respective culture conditions. This shows that we should interpret these data with caution, FPKM values are low in both conditions, signaling low gene expression. BCL2A1, a member of the BLC-2 protein family, expression is 6.7 times higher after expansion in PL containing medium. It forms hetero- or homodimers and acts as an anti-apoptotic regulator in a wide range of cellular processes. The protein encoded by this gene blocks the activation of caspases and reduces the release of pro-apoptotic cytochrome c from the mitochondria (Flores-Romero et al., 2019). BCL-3 is also upregulated after culture in PL, this molecule is involved in cell cycle regulation, plays a role in maintaining pluripotency of embryonic stem cells and is anti-apoptotic (Flores-Romero et al., 2019; Liu et al., 2022). The pro-apoptotic gene upregulated in PL-MSCs is CASP-1, a member of the caspase family which plays an important role in the execution of cell death (Valenti et al., 2021). BMF is upregulated in FCS-MSCs and is important for triggering apoptosis in response to intracellular damage (Piñon et al., 2009). BCL-2 is the anti-apoptotic gene upregulated in FCS-MSCs. This gene encodes a protein located on the outer mitochondrial membrane that blocks apoptosis of, among others, lymphocytes (Bruckheimer et al., 1998). These observations show differential expression of genes involved in apoptosis and perhaps suggest a different gene expression profile of proliferation associated genes. After all, we observed a number of population doublings in PL-MSC cultures that is approximately twice as high as in FCS-MSC cultures. Other groups have shown earlier that proliferation rate of MSCs in PL supplemented medium appears higher than in FCS supplemented medium (Horn et al., 2010; Crespo-Diaz et al., 2011; Kinzebach et al., 2013). As stated above RNA sequencing and subsequent GO analysis gives an abundance of information but in many processes large numbers of genes are involved. To get a visually informative overview of the differential expression of these genes a heatmap is a useful tool. Observing an increased proliferation potential and upregulation of 2 anti-apoptotic genes with higher FPKM in PL-MSCs and enrichment of the GO term 'Regulation of cell population proliferation' we created such a heatmap for these genes. This heatmap shows a clustering of MSCs according to growth supplement, confirming an effect of these supplements on expression of genes relevant for proliferation. A concern might be that differences in culture confluence levels influence expression of genes involved in proliferation as confluent cells will stop dividing. We harvested our cells at similar levels of subconfluency and thereby hope to minimize this possible bias in our analysis. Camilleri et al. showed that confluent and proliferating MSCs show different gene expression profiles. They identified CD168 as a gene exclusively expressed in proliferating cells and CD106 as a gene only expressed in confluent MSCs. In the samples we included in this analysis there was no difference in expression of both genes (data not shown).

Wagner and colleagues described the effects of senescence on MSCs. Prolonged passaging, up to 12 passages, resulted in morphological changes, the cells became larger and ultimately stopped to proliferate. At different passages they performed mRNA profiling. Based on these analyses they identified 29 genes that were significantly upregulated and 4 genes that were significantly downregulated in senescent passage MSCs (Wagner et al., 2008). Since we observed a higher proliferation rate in PL-MSCs but found the morphological aspect of senescence rather in FCS-MSCs (cells were larger), we evaluated expression of these genes in our RNA sequencing analysis comparing PL to FCS. In total 20 genes listed in the paper of Wagner were also included in our analysis, 65% (13/20) of genes that were upregulated in their analysis are also significantly differentially expressed in our analysis comparing FCS and PL as growth supplements. Of these 86% are upregulated in MSCs cultured in FCS-based medium ranging from 1.5–11 times. Wagner et al. also reported 4 genes that were downregulated (Wagner et al., 2008). In our analysis only 1 gene is statistically significant differentially expressed: CXCL6, it is highly downregulated (38 times) in FCS-MSCs. Based on these observations we can conclude that the gene expression profile of FCS-MSCs shows signs of senescence. Some genes were also upregulated in PL-MSCs but a much smaller number and to a smaller degree (1.7 and 2.4 fold). Beta galactosidase staining of cells is a well-established assay to screen for senescent cells. Using this technique we found more senescent cells in passage 4 FCS-MSCs than in passage 4 PL-MSCs, confirming our observations in the RNAseq analysis. Based on these results we favor the use of PL-MSCs for the *in vitro* expansion of MSCs. After all, senescent MSCs have diminished proliferative, differentiation and immunomodulatory capacities. They also appear to be less able to support hematopoiesis (Wagner et al., 2008; De Witte et al., 2017; Gnani et al., 2019).

To obtain a sufficient cell number for therapeutic applications MSCs are expanded *in vitro*. We have shown that especially in PL supplemented medium, MSCs can grow rapidly, and RNA profiling showed upregulation of anti-apoptotic genes. The last part of this study focuses therefore on the risk of malignant transformation MSCs expanded in PL *in vitro*. To screen for genetic changes that could lead to malignant transformation, we used a state-of-the art next generation sequencing approach to characterize the mutation profile of the coding DNA sequences of these MSCs in both low and high passages. Based on this analysis, only 21 different genetic variants in total were detected (after data filtering) in the 10 MSC DNA samples that have been sequenced. The majority (17/21, 81%) of these variants constituted of VUS (variants of uncertain significance). As the name suggests, these are all variants of which the biological significance is unclear, and where the currently available evidence is certainly insufficient to assume a potential pathogenic effect of these variants. Only three variants were detected that could be classified as "likely pathogenic" (in genes *APC*, *NF1,* and *STAG2*), meaning that there are reasons to assume a potential harmful effect of these variants but there is no consensus nor sufficient (functional) evidence to confirm this pathogenicity. Genetic variants in *APC* have been implicated in familial adenomatous polyposis (FAP) and colorectal cancer (Fodde R, 2002). The *APC* p.Asp1810Ilefs*7 variant had a low VAF (4,1%), indicating that not all cells carried the mutation and it does not appear to induce an immediate advantage for expansion *in vitro*. Similarly, although the *NF1* p.Gln2302* variant and the *STAG2* splice site variant we found were classified as likely pathogenic, no compelling evidence of a pathogenic functional effect of these particular variants could be retrieved in literature. Interestingly, based on a review of the clinical data from the donors, we found antecedents of colon polyps in the donor in whom the *APC* variant was found, although we did not detect the typical *APC* mutations seen in FAP (Fodde R, 2002). The donor in whom the *NF1* variant was detected did not show any familial or personal antecedents of neurofibromatosis but had antecedents of diabetes and psoriasis which implies a chronic state of inflammation and thus an increased risk of developing acquired mutations (Lonkar and Dedon 2011). The same observation was made in donor 4 who had a *STAG2* variant and suffered from COPD which is again a chronic state of inflammation. The cells in these donors have therefore had ample opportunity to acquire genetic variants *in vivo*. A baseline analysis of the BM sample at the start of the cell culture could give a definitive answer to the question whether these mutations discussed above were indeed pre-existing.

Importantly, we observed no major changes in the mutation signature of the donors between passage 4 and passage 10, as can be derived from Table 4. The fact that no additional (pathogenic) variants emerged in P10 provides further evidence and reassurance that no major genetic changes during *in vitro* expansion of MSCs occur that may lead to malignant transformation. When taking these observations into account together with the number of population doublings, these results indicate that at least the exonic (and clinically most important) DNA sequences remain largely stable during *in vitro* expansion of MSCs, supporting the safe use of PL for *in vitro* expansion of MSCs. In clinical trials with human MSCs there have been no reports of malignant transformation so far (De Becker and Van Riet 2015). However, murine MSCs have been shown to be prone to malignant transformation after prolonged *in vitro* expansion and Sole et al. were able to generate malignant cells *in vitro* from MSCs derived from an Ewing sarcoma patient (Xu et al., 2012; Sole et al., 2021). Kim et al. performed whole genome sequencing of MSCs at different passages and warned

for genomic instability of human MSCs in case of prolonged *ex vivo* expansion (Kim et al., 2017). Additionally, MSCs derived from patients with myeloid malignancies were reported to have cytogenic abnormalities or genetic variations (Kouvidi et al., 2016; Azuma et al., 2017). Our data suggest that human bone marrow derived MSCs expanded in PL supplemented medium are safe and unlikely to undergo malignant transformation after administration.

The data obtained in this study lead us to conclude that the selection of expansion medium can have a significant impact on the biological characteristics and gene expression profile of *in vitro* expanded human bone marrow derived MSCs. The option to replace the standard FCS-based medium by human platelet lysate–based medium for clinical grade MSC expansion seems to be advantageous: the cells retain MSC characteristics and exhibit a higher proliferation capacity without showing signs of senescence. FCS-MSCs on the other hand show signs of senescence already at passage 4 in our hands. Taking the higher *in vitro* proliferation rate of PL-MSCS into account, we could not provide any evidence for an increased risk of malignant transformation in the expanded MSCs. Future research should validate the biological effects of this human growth supplement as well as its potential to improve the clinical use of *in vitro* MSCs and their derived therapeutic products.

Data availability statement

The original contributions presented in the study are publicly available. NGS data set can be found in the ENA Repository https://www.ebi.ac.uk/ena/browser/ (accession number PRJEB55753). RNA seq data set can be found in ArrayExpress https://www.ebi.ac.uk/arrayexpress/ (accession number E-MTAB-12272).

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee UZ Brussel. The patients/participants provided their written informed consent to participate in this study.

Author contributions

AD: design and performance of the experiments, analysis of the data and writing of the manuscript RH: contribution to performance of the experiments and analysis of the data WD: contribution to performance of the experiments and analysis of the data KB: contribution to performance of the experiments and analysis of the data KM: analysis and interpretation of the NGS data IV: overall supervision, design of the experiments and the manuscript, review and revision of the manuscript draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The inhibitory effect of human umbilical cord mesenchymal stem cells expressing anti-HAAH scFv-sTRAIL fusion protein on glioma

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Glioma is the most common malignant intracranial tumor with low 5-year survival rate. In this study, we constructed a plasmid expressing anti-HAAH single-chain antibody and sTRAIL fusion protein (scFv-sTRAIL), and explored the effects of the double gene modified human umbilical cord mesenchyreal stem cells (hucMSCs) on the growth of glioma *in vitro* and *in vivo*. The isolated hucMSCs were identified by detecting the adipogenic differentiation ability and the osteogenic differentiation ability. The phenotypes of hucMSCs were determined by the flow cytometry. The hucMSCs were infected with lentivirus expression scFv-sTRAIL fusion protein. The expression of sTRAIL in hucMSCs were detected by immunofluorescence staining, western blot and ELISA. The tropism of hucMSCs toward U87G cells was assessed by transwell assay. The inhibitory effect of hucMSCs on U87G cells were explored by CCK8 and apoptosis assay. The xenograft tumor was established by subcutaneously injection of U87G cells into the back of mice. The hucMSCs were injected *via* tail veins. The inhibitory effect of hucMSCs on glioma *in vivo* was assessed by TUNEL assay. The hucMSCs migrated into the xenograft tumor were revealed by detecting the green fluorescent. The results showed that the scFv-sTRAIL expression did not affect the phenotypes of hucMSCs. The scFv-sTRAIL expression promoted the tropism of hucMSCs toward U87G cells, enhanced the inhibitory effect and tumor killing effect of hucMSCs on U87G cells. The *in vivo* study showed that hucMSCs expressing scFv-sTRAIL demonstrated significantly higher inhibitory effect and tumor killing effect than hucMSCs expressing sTRAIL. The green fluorescence intensity in the mice injected with hucMSCs expressing scFv-sTRAIL was significantly higher than that injected with hucMSCs expressing sTRAIL. These data suggested that the scFv conferred the targeting effect of hucMSCs tropism towards the xenograft tumor. In conclusion, the hucMSCs expressing scFv-sTRAIL fusion protein gained the capability to target and kill gliomas cells *in vitro* and *in vivo*. These findings shed light on a potential therapy for glioma treatment.

KEYWORDS

human umbilical cord mesenchyreal stem cells, single-chain antibody (scFv), human aspartyl-(asparaginyl)-β-hydroxylase, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), glioma

Introduction

Glioma is the most common primary intracranial tumor, and it is derived from neuroepithelium and accounts for 50%–60% of brain tumors (Grady et al., 2022; O'Connor et al., 2021). At present, the clinical treatment of glioma mainly includes neurosurgical resection, radiotherapy, chemotherapy, and immunotherapy (Grady et al., 2022). Nevertheless, patients have a mere median survival of 14 months, as well as a 5-year survival rate less than 10% (Birk et al., 2017). The recurrence rate of high-grade gliomas (WHO grade III-IV) is as high as 95% within 2 years after initial resection (Xiong et al., 2019). Drug or gene delivery vehicles commonly used to treat cancers, such as glioma include liposomes, nanoparticles, ionic polymers, microcapsules, and micropores (Ren et al., 2021). However, the clinical therapeutic value of these vectors is limited by factors, such as low drug or gene load, poor stability, and low targeting efficiency (Vikulina et al., 2019; Stephen et al., 2022). Therefore, cell-based targeted drug (gene) delivery systems have gradually attracted worldwide attention due to their unique advantages.

Cells that can be used as drug or gene delivery vehicles include red blood cells, immune cells, and stem cells (Wu et al., 2019). As a new type of drug or gene delivery cell carrier, human umbilical cord mesenchyreal stem cells (hucMSCs) present unique characteristics (Qu et al., 2020). The hucMSCs exhibit a faster renewal rate, stronger proliferation potential, and stronger immunosuppressive ability compared to adult mesenchymal stem cells derived from other mature tissues (Xu et al., 2018; Zha et al., 2021). Harvesting the umbilical cord cells is non-invasive and painless and has a wide range of sources without any ethical controversy (Coccini et al., 2022). Moreover, studies have reported that hucMSCs have the potential to cross the blood–brain barrier (BBB) (Sun et al., 2018).

Human aspartyl-(asparaginyl)-β-hydroxylase (HAAH), also known as spartate β-hydroxylase (ASPH), is a type II transmembrane protein and member of the alpha-ketoglutarate-dependent dioxygenase family (Lin et al., 2019). Its function is to hydroxylate aspartyl and asparagine residues in the epidermal growth factor-like domain of the synthesized protein (Greve et al., 2021). HAAH is not expressed in normal tissues, but highly expressed in a variety of tumor cells, including glioma (Yang et al., 2010; Chen et al., 2019; Lin et al., 2019), and is regarded as a broad-spectrum tumor-associated antigen (Zheng et al., 2020). It is a membrane protein expressed on the surface of tumor cells, and can be used for the accurate localization of cancer lesions (Babich et al., 2022).

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily of cytokines (Burgaletto et al., 2020). TRAIL can induce apoptosis of tumor cells but not normal cells (Yuan et al., 2018). TRAIL can specifically induce apoptosis by selectively binding to the TRAIL receptors on the surface of cancer cells, such as colon cancer cells, lung cancer cells, and glioma cells (Ion et al., 2019; Yoon et al., 2021). Under the MSCs homing effect, TRAIL-MSCs exhibit a tendency for homing to glioma lesions, significantly improving the local concentration of TRAIL in glioma lesions (Park et al., 2021). TRAIL can exist on the cell surface in the form of membrane protein (Wong et al., 2019). It can also be hydrolyzed by protease, shed from the cell membrane, and free in body fluids or blood in the form of soluble protein (sTRAIL) (Koliaki and Katsilambros, 2022). Studies have shown that the sTRAIL, like the membrane-bound form TRAIL, can induce apoptosis in a variety of tumor cell lines without affecting normal cells (Ma et al., 2005; de Bruyn et al., 2013).

A single-chain antibody (scFv) is a widely used recombinant antibody (Eskafi et al., 2021). It is a smaller unit with the ability to bind antigen. The advantages of scFv include lower molecular weight and easy modification (Eskafi et al., 2021). Thus, anti-HAAH scFv has potential application value in targeted therapy for glioma (Schaller et al., 2020). Currently, the use of anti-HAAH scFv for glioma-targeted therapy has not been reported yet. In this study, we constructed a plasmid expressing anti-HAAH single-chain antibody and sTRAIL fusion protein (scFv-sTRAIL), and explored the effects of the double gene modified hucMSCs on the growth of glioma *in vitro* and *in vivo*.

Materials and methods

Cell culture

Human glioblastoma cells U87G (kindly gifted by Prof. Hai Zhang of the Air Force Medical University) were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO, United States) with 10% fetal bovine serum (FBS, Sigma) and incubated at 37°C in a humidified atmosphere of 5% CO_2.

Human umbilical cord tissues were obtained from the Second Affiliated Hospital, Air Force Medical University. Informed consent was obtained from participants, and this study has been approved by the ethics committee of the Second Affiliated Hospital, Air Force Medical University (TDLL 2019-11-179). The hucMSCs were isolated from the Wharton's jelly of the human umbilical cord. The Wharton's

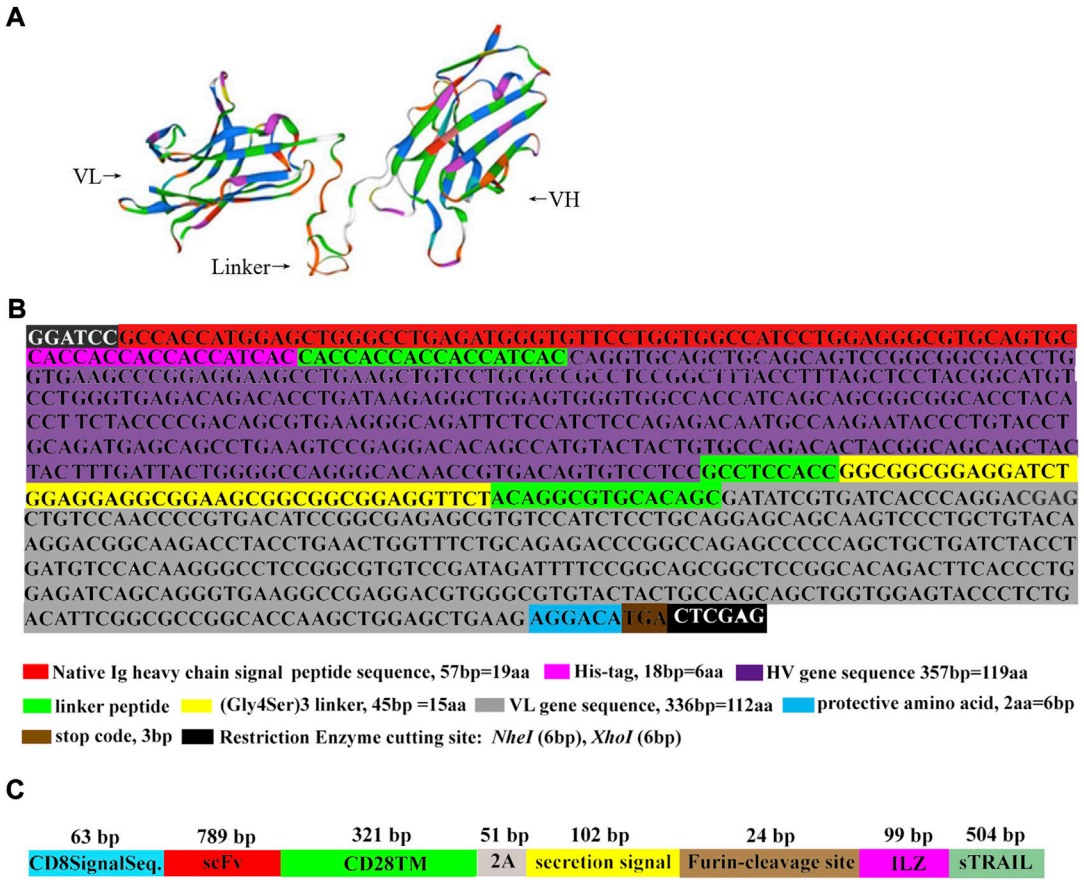

FIGURE 1
The schematic diagram of scFv and scFv-sTRAIL. **(A)** The mimic tertiary structure of scFv protein. **(B)** The results of scFv sequence determination. **(C)** The gene linear structural pattern diagram of the sequence encoding scFv-sTRAIL fusion protein.

jelly was separated in the laboratory ultra-clean workbench. After repeated washing with phosphate buffer saline (PBS), the Wharton's jelly was cut into 1 mm^3 tissue pieces, evenly spread in a 60 mm Petri dish, and maintained in DMEM supplemented with 10% FBS, 1% penicillin, and 1% streptomycin in an atmosphere of 37°C, 5% CO_2. After the cells were confluent, they were digested and passaged with 0.25% trypsin. For this experiment, the hucMSCs were limited to 4th ~ 5th generations (P4 ~ P5). The morphology of the hucMSCs was assessed using an inverted fluorescence microscope (Niko, Tokyo, Japan).

Identification of hucMSCs biomarkers by flow cytometry

The P4 hucMSCs were digested and PBS was added to prepare cell suspension. Cells were labeled using HLA-DR-PE, CD105-APC, CD90-PE, CD73-APC, CD19-APC, CD45-PE-Cy7, CD34-PE-Cy7 or CD14-PE antibodies (BioGems, Westlake Village, CA,United States) for 30 min at 4°C. Subsequently, cells were washed with PBS for three times. The above stained cells were analyzed using flow cytometry (BD Biosciences, San Jose, CA).

Identification of hucMSCs differentiation by alizarin red S staining and oil red O staining

The cell suspension of P4 hucMSCs (1× 10^8/L) was plated in six well plate with DMEM medium containing 10% FBS. When the cells confluency reached 70%, the osteoinductive complete culture medium or adipogenic differentiation complete culture medium (Guangzhou Cyagen biotechnology Co., Ltd., Guangzhou, China) was added, which was changed every 3 days. After 21 days, cells were stained using 0.1% alizarin red S (Sigma) or 2% oil red

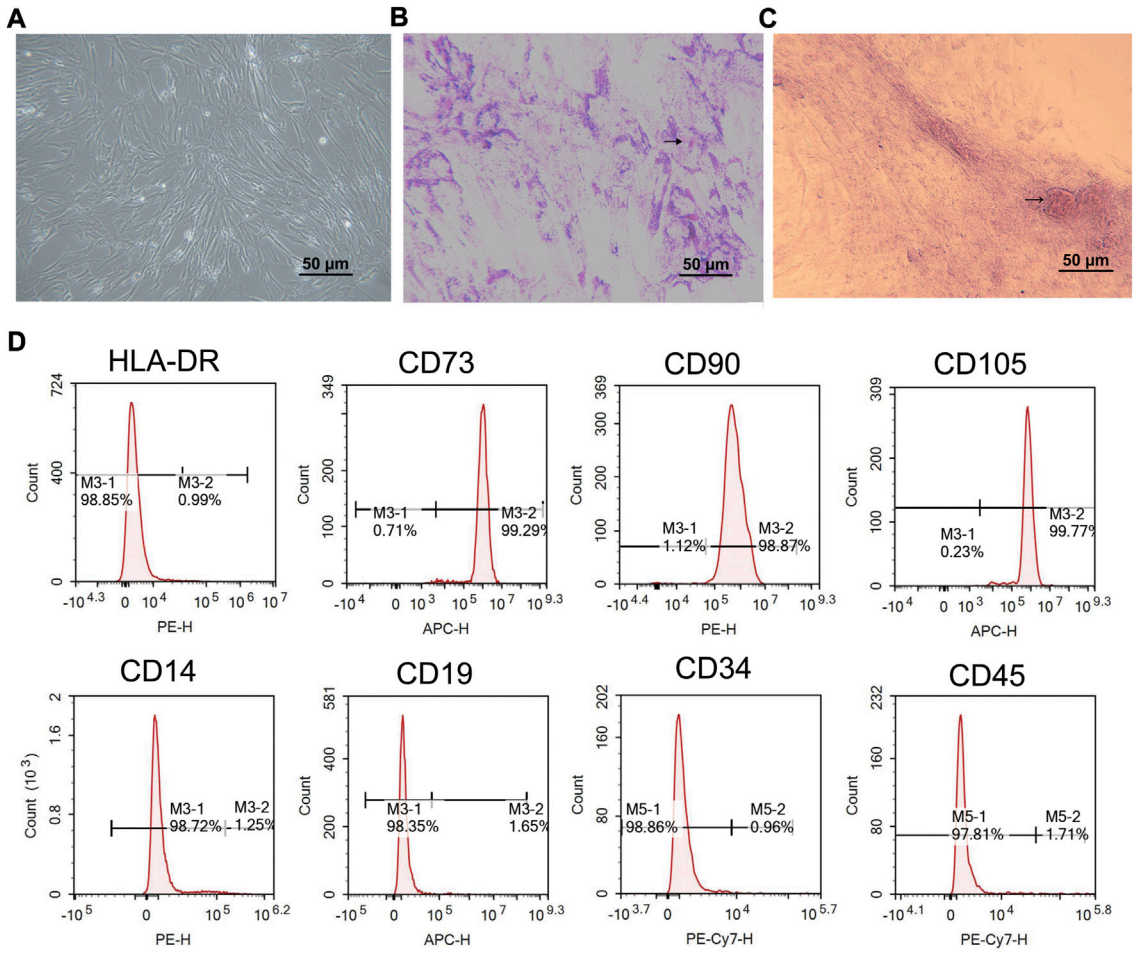

FIGURE 2
Characterization of hucMSCs. **(A)** Phase contrast picture of confluent huMSCs (x100). **(B)** Adipogenic differentiation observed with Oil Red O staining (x100). **(C)** Osteogenic differentiation observed with alizarin staining (x 100). **(D)** Flow cytometric analysis of hucMSCs specific biomarkers. Scale bar = 50 μm n = 3.

O (Cyagen biotechnology Co., Ltd.). The formation of calcified nodules or lipid droplet were observed under microscope.

Construction of plasmids expressing anti-HAAH scFv-sTRAIL fusion protein

The hybridoma cell line G3/F11 secreting anti-HAAH monoclonal antibody (McAb) was donated by Prof. Yingfeng Lei from the Microbiology Department of Air Force Military Medical University (Yang et al., 2011). The plasmids expressing anti-HAAH scFv were constructed according to previously described (Yang et al., 2011). Briefly, the total RNA of G3/F11 was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA) according to the instruction. The cDNA was synthesized *via* Transcriptor First Strand cDNA Synthesis Kit (Roche, Maryland, United States). Genes encoding the variable heavy and light chains (VH and VL) of the anti-HAAH antibodies were amplificated by polymerase chain reaction (PCR) using Taq DNA polymerase (Thermo Fisher Scientific, MA, United States) in a thermocycler (QIAGEN GmbH, Hilden, Germany). Based on the correctly sequenced VH and VL gene sequences, we designed the upstream and downstream restriction endonuclease sites (*BamHI* and *XhoI*), signal peptide, His-tag, Linker and other elements. The mimic tertiary structure of scFv protein was showed by SWISS-MODEL (Figure 1A). Based on the designed scFv gene sequence (Figure 1B) and the sTRAIL gene sequence at NCBI, the sequence encoding scFv-sTRAIL was designed. The gene linear structural pattern diagram encoding the scFv-sTRAIL fusion protein in this study was shown in Figure 1C. The sequences encoding scFv-sTRAIL fusion protein were chemically synthesized,

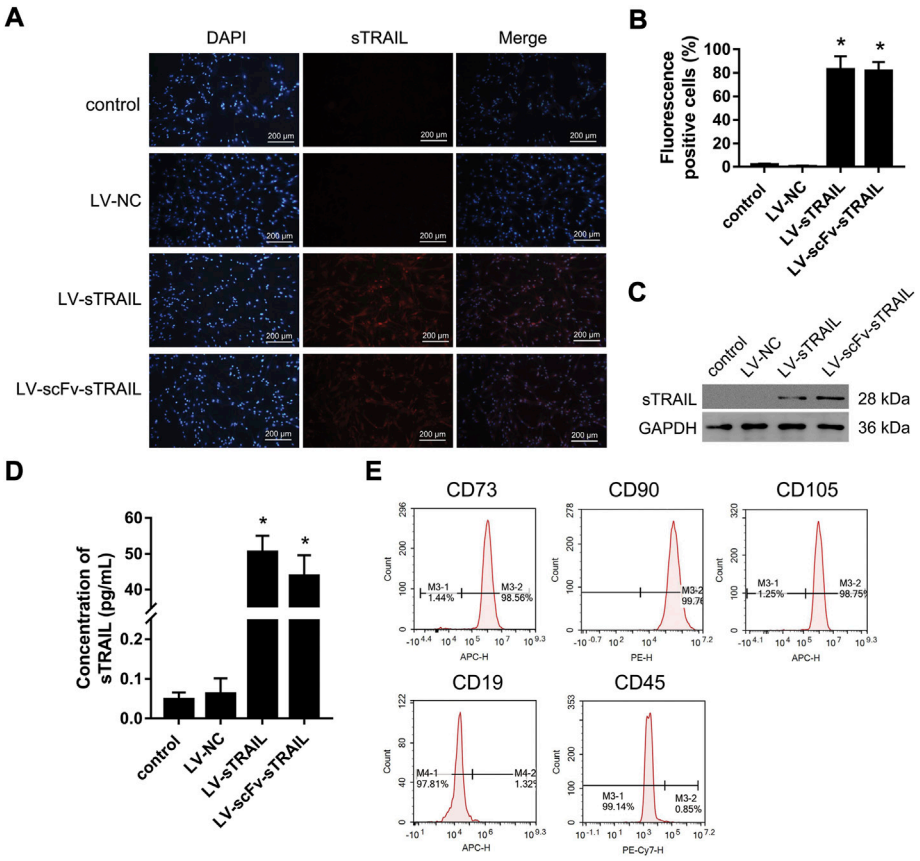

FIGURE 3
The effects of LV-scFv-sTRAIL infection on sTRAIL expression and biomarkers of hucMSCs. **(A)** The representative images of sTRAIL expression detected by immunofluorescence staining. **(B)** The quantitative data of sTRAIL positive cells. **(C)** Western blot detection of the expression of sTRAIL. **(D)** ELISA detection of the content of sTRAIL in the culture supernatant. **(E)** Flow cytometric analysis of hucMSCs specific biomarkers. Control group: normally cultured hucMSCs; LV-NC group: hucMSCs infected with empty plasmid lentivirus; LV-sTRAIL group: hucMSCs infected with lentivirus expressing sTRAIL; LV-scFv-sTRAIL group: hucMSCs infected with lentivirus expressing scFv-sTRAIL. Scale bar = 200 μm $n = 3$, $^*p < 0.01$ versus the LV-NC group.

cloned into pCDH-CMV-MSC-EF1-copGFP-T2A-Puro plasmids, and packaged into a lentivirus by Hanheng Biotech (Shanghai, China).

Infection of hucMSCs with lentivirus

The cell suspension of P4 hucMSCs was plated in six well plate. When cells were 70% confluent, hucMSCs were infected with lentivirus expressing empty plasmids (LV-NC group), lentivirus expressing sTRAIL (LV-sTRAIL group), or lentivirus expressing scFv-sTRAIL (LV-scFv-sTRAIL group). Medium containing puromycin (10 μg/ml; Invitrogen) was used to select stably infected cells. The hucMSCs normally cultured without infection were defined as control group.

Immunofluorescence staining

The hucMSCs were seeded into a 6-well dish containing cover slips and incubated for 48 h. The cells were taken out and were then washed twice using PBS, fixed for 15 min with 95% ethanol, permeabilized in 0.1% Triton X-100 and blocked for 1 h using 1% BSA in PBST. Cells were then incubated with mouse anti-TRAIL antibodies (ProteinTech Group, Wuhan, China) at 4°C overnight. After that, slides were washed three times with PBS. Then cells were incubation with Cy3-conjugated affinipure goat anti-mouse IgG (ProteinTech Group, Wuhan, China) and DAPI (1 μg/ml; Thermo Fisher Scientific, Inc.) in the dark for 1 h at room temperature. After washing with TBS and distilled water, fluorescent images were collected using Olympus FV3000RS confocal microscope, and fluorescent signal was quantified using ImageJ.

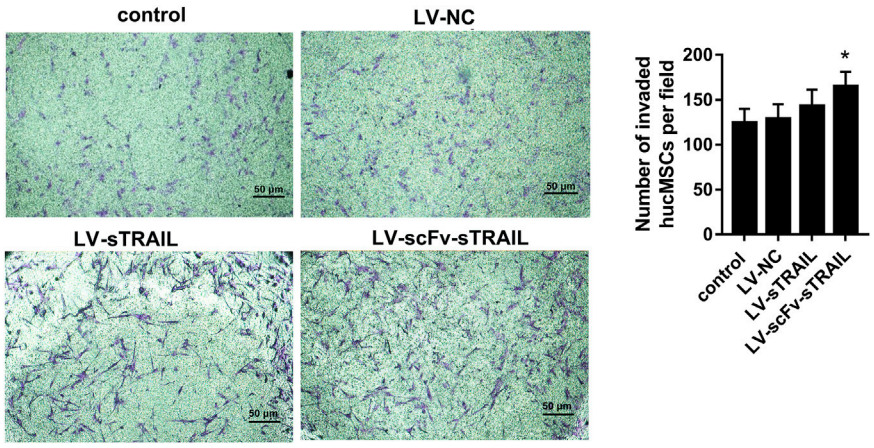

FIGURE 4
The effect of LV-scFv-sTRAIL infection on hucMSCs tropism toward U87G detected by the transwell assay (× 100). Scale bar = 50 μm $n = 3$, $*p < 0.05$ versus the LV-NC group.

Enzyme linked immunosorbent assay

ELISA was performed to detect the concentration of sTRAIL in culture supernatant of hucMSCs using the sTRAIL ELISA kit (Diaclone Research, Besancon, France). Absorbance was measured at 450 nM using a microplate reader (Bio-Rad Laboratories, Inc.). All samples were analyzed in triplicate.

Western blot

Western blot was used to verify sTRAIL protein expression in hucMSCs. Cells was lysed with RIPA buffer (Millipore, Bedford, MA. United States), and the protein concentration was tested by a Bradford protein assay kit (TIANGEN Co., Ltd., No.PH0325). Protein samples were separated by SDS-PAGE and transferred onto polyvinylidene fluoride membrane (Millipore). After blocking them with 3% nonfat milk, the membranes were incubated with rabbit anti-TRAIL antibody (Beyotime, Nantong, China) (1:200) and HRP-goat anti-rabbit IgG (CST) (1:5,000) successively. The proteins were detected by enhanced chemiluminescence. Finally, the bands were visualized using a Bio-Rad ChemiDoc apparatus (Bio-Rad, Hercules, CA, United States) and were analyzed using ImageJ software (ImageJ, Version 1.4). GAPDH was used as the internal reference.

Migration assay

Transwell migration assay was used to monitor the tropism of the hucMSCs towards glioma U87G cells. For this purpose, U87G cells (1×10^5/well) were plated in the lower chamber of the transwell plate (Thermo Fisher Scientific, Waltham, MA, United States). The hucMSCs (1×10^4/well) in each group were grown in the upper chamber. Cells in each group were incubated for 24 h at 37°C with 5% CO_2. The non-migrating cells in the upper chamber were gently wiped away with a cotton swab. The bottom of the chamber was stained with methanol and 0.1% crystal violet and then observed using a microscope. The number of invaded hucMSCs per field was hucMSCs number at an average of five-random non-overlapping fields.

Detection of U87G proliferation by CCK8 assay

U87G cells were seeded in 96-well plates (5×10^4/well) and pre-cultured for 24 h at 37°C with 5% CO_2. Subsequently, the culture supernatant concentrate (1:10, 100 μl/well) of hucMSCs in each group was added, followed by incubation for 24 h. A total of 10 μl/well of CCK8 reagent (Merck KGaA, Darmstadt, Germany) was added, followed by further incubation for 2–4 h. The absorbances were measured at OD450 nm using a microplate reader (Bio-Rad Laboratories, CA, United States).

Measurement of U87G apoptosis by flow cytometry

In this study, the effect of hucMSCs infected with LV-scFv-sTRAIL on U87G apoptosis was investigated using co-culture assay. For co-culture, U87G cells (1×10^5/well) were plated in the lower chamber of the transwell plate (Thermo Fisher Scientific) and incubated for 24 h. Then, hucMSCs (1×10^4/well) in each

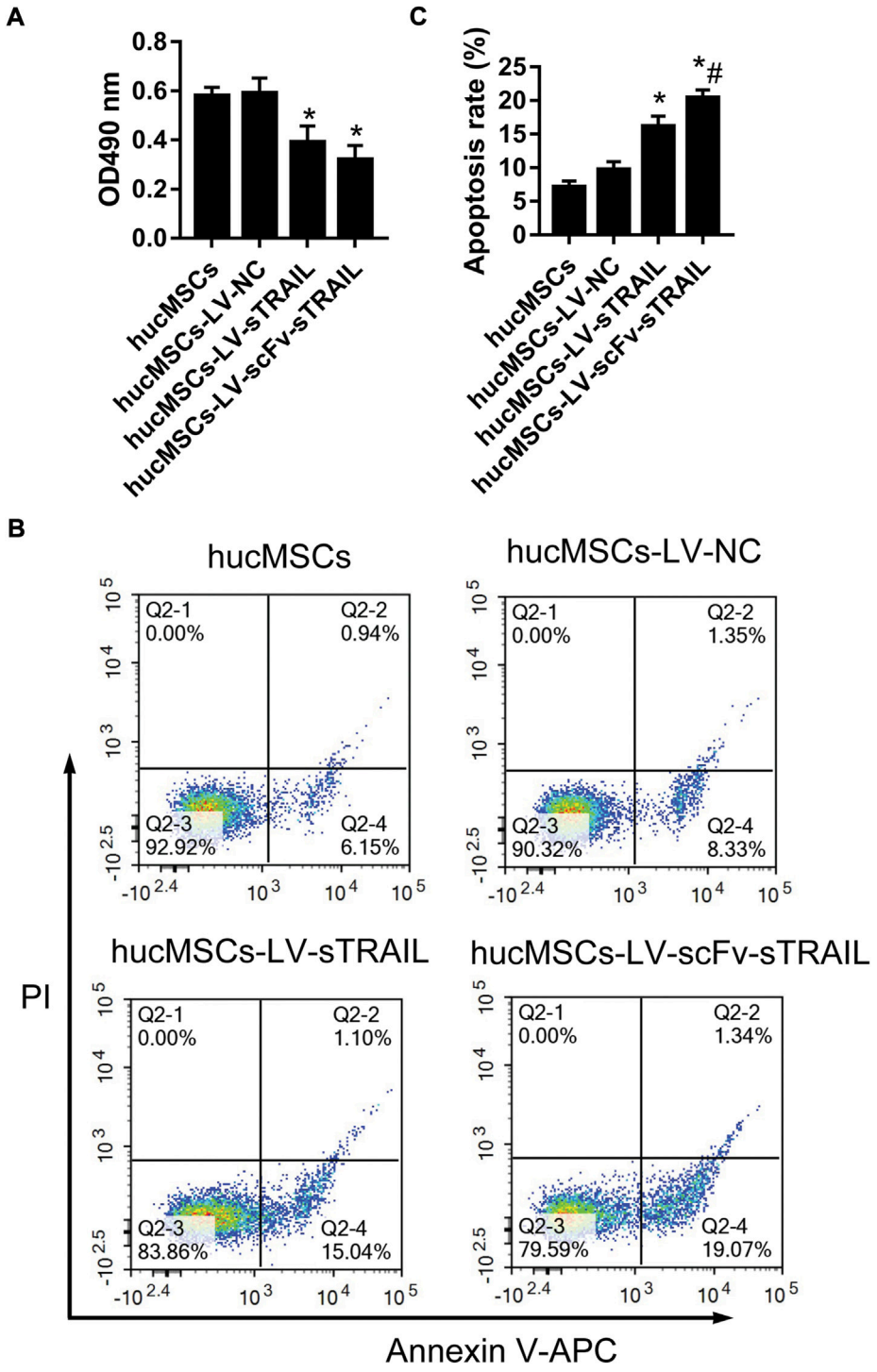

FIGURE 5
The effect of hucMSCs infected with LV-scFv-sTRAIL on proliferation and apoptosis of U87G. **(A)** The proliferation of U87G measured by CCK-8 assay. **(B)** The apoptosis of U87G measured by flow cytometric analysis. **(C)** The quantitative data of cell apoptosis. $n = 3$, $*p < 0.05$ versus the hucMSCs-LV-NC group; $\#p < 0.05$ versus the hucMSCs-LV-sTRAIL group.

group were grown in the upper chamber. After another 48 h, glioma cell apoptosis was detected using the Annexin V-APC/PI apoptosis detection kit (Invitrogen, Carlsbad, CA, United States). Briefly, the cells were resuspended in 300 μl binding buffer, followed by the addition of Annexin V-APC solution (5 μl). After 25 min of incubation at 4°C, the cells were resuspended for

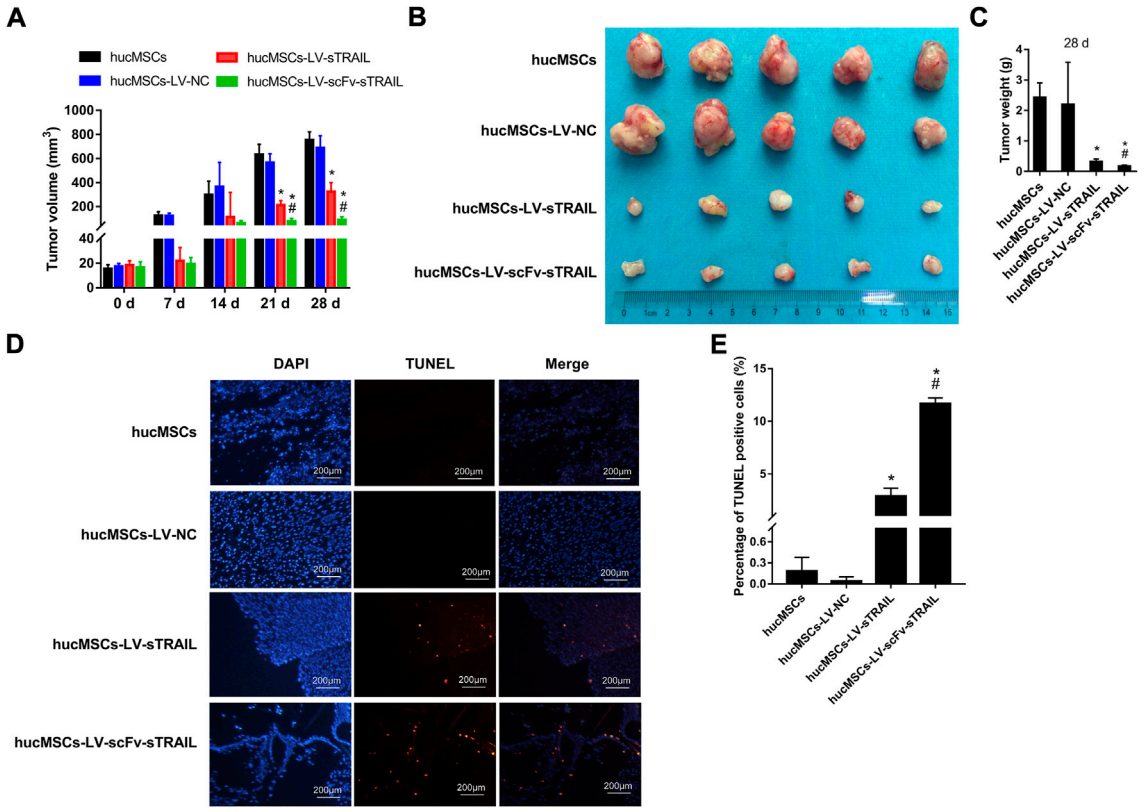

FIGURE 6
The effect of hucMSCs infected with LV-scFv-sTRAIL on glioma tumor growth. **(A)** Tumors volume. **(B)** The tumors isolated from mice. **(C)** Tumors weight on 28 days. **(D)** The representative images of apoptotic cells in U87G xenograft tumors detected by TUNEL assay. **(E)** The quantitative data of percentage of TUNEL positive cells. Scale bar = 200 μm $n = 5$, $^{*}p < 0.05$ versus the hucMSCs-LV-NC group; $\#p < 0.05$ versus the hucMSCs-LV-sTRAIL group.

10 min in a binding buffer with 5 μl of PI. Apoptotic cells were counted using a FACS analyzer (BD Biosciences).

Xenograft tumor assay

The immunodeficient BGR mice (SPF grade, 6–8 weeks old, 18–20 g) were purchased from the Laboratory Animal Center of Air Force Military Medical University. The experiments were carried out in strict accordance with the guidelines of the National Health and Medical Research Council for the Care and Use of Animals for Experimental Purposes in China, and were approved by the Institutional Animal Care and Use Committee of Chinese Academy of Sciences (No. 2020-A138). Glioma U87Gcells (2×10^6 cells) diluted in 200 μl of PBS were inoculated subcutaneously into the back of BGR mice. The tumor volume was calculated every 2 days by a vernier caliper: The volume was calculated as follows: volume = length × width^2 × 1/2. When the tumor volume reached 10–20 mm^3, mice were randomly divided into four groups (five mice/group) as follows: hucMSCs group (injected with control hucMSCs), hucMSCs-LV-NC group (injected with hucMSCs infected with pCDH empty plasmid lentivirus), hucMSCs-LV-sTRAIL group (injected with hucMSCs infected with LV-sTRAIL), and hucMSCs-LV-scFv-sTRAIL group (injected with hucMSCs infected with LV-scFv-sTRAIL). The mice in each group were injected with 1×10^7 cells (in 200 μl suspension) *via* their tail veins once a week for a total of four times. One week after the last inoculation, the mice were euthanized by subcutaneous injection with sodium pentobarbital (40 mg/kg). The tumors were isolated and the tumors weight were measured.

Because the lentiviral plasmids pCDH-CMV-MSC-EF1-copGFP-T2A-Puro used in this study could express GFP, hucMSCs migrated into the xenograft tumor could be reflected by detecting the green fluorescent. Briefly, tumor tissue were paraffin-embedded and 5 μm sections were made. Green fluorescent were visulized using Olympus FV3000RS confocal microscope, and fluorescent signal was quantified using ImageJ.

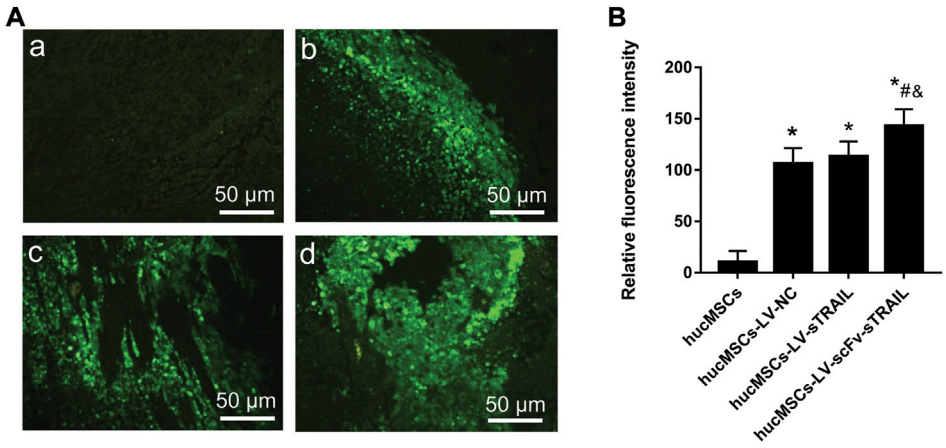

FIGURE 7
Fluorescence microscopic observation of paraffin embedded sections of tumor tissues. **(A)** Representative images of fluorescence microscope observations (magnification ×200). a: hucMSCs group; b: hucMSCs-LV-NC group; c: hucMSCs-LV-sTRAIL group; d: hucMSCs-LV-scFv-sTRAIL group. **(B)** The quantitative data of fluorescence intensity. Scale bar = 50 μm n = 5, *p < 0.05 versus the hucMSCs group; #p < 0.05 versus the hucMSCs-LV-NC group; &p < 0.05 versus the hucMSCs-LV-sTRAIL group.

TUNEL assay

The cell apoptosis in xenograft tumors were detected by the one-step TUNEL cell apoptosis detection kit (Beyotime, Nantong, China). Briefly, the slices were blocked with anti-fluorescence quenching blocking solution and then observed under a fluorescent microscope. The excitation wavelength of Cy3 is 550 nm, and the emission wavelength is 570 nm (red fluorescence). Fluorescent images were collected using Olympus FV3000RS confocal microscope, and fluorescent signal was quantified using ImageJ. The apoptosis rate = (the mean number of apoptotic cells in five random fields/total cell count in that five fields) ×100%.

Assessment of distribution of double gene modified hucMSCs *in vivo* by RT-PCR

The immunodeficient BGR mice (SPF grade, 6–8 weeks old, 18–20 g) were injected with LV-scFv-sTRAIL infected hucMSCs (5×10^7 cells in 200 μl suspension) *via* tail veins. At the 24, 48, 72 and 96 h, the mice were euthanized by subcutaneous injection with sodium pentobarbital (40 mg/kg). The brain, spleen, liver, kidney, heart and lung were collected for total RNA extraction. The cDNA was synthesized *via* Transcriptor First Strand cDNA Synthesis Kit (Roche). The presence of double gene modified hucMSCs were revealed by RT-PCR analysis for GFP (Caffario et al., 2017), and β-actin was used as loading control. The primer sequences used were as follows: GFP: 5′-AGGACAGCGTGATCTTCACC-3’ (sense) and 5′-CTTGAAGTGCATGTGGCTGT-3’ (antisense); and β-actin: 5′-ACAGAGCCTCGCCTTTGC-3’ (sense), 5′-GCGGCGATATCATCATCC-3’ (antisense).

Statistical analysis

Data were presented as means ± standard deviation (SD) of three independent experiments. For multiple-group comparisons, a significant one-factor analysis of variance (ANOVA) was used, followed by the Bonferroni test. $p < 0.05$ was considered to indicate a statistically significant difference. Analyses were performed using GraphPad Prism 5 (GraphPad Software Inc.; San Diego, CA, United States).

Results

Identification of the isolated hucMSCs

The hucMSCs from Wharton’s jelly of the human umbilical cord were incubated in DMEM with 10% FBS for 14 days. Spindle-forming fiber-like hucMSCs were densely arranged in a fingerprint or vortex shape (Figure 2A). After 3 weeks of adipogenic induction using the P4 cells, the formation of a large number of lipid droplets suggested that the cells have differentiated into adipocytes (Figure 2B). After osteogenic induction, the red-stained calcium nodules and scattered bone trabecular structures in the cells demonstrated the successful differentiation of cells toward osteocytes (Figure 2C). The flow cytometry results showed that CD73, CD90, and CD105 were presented as

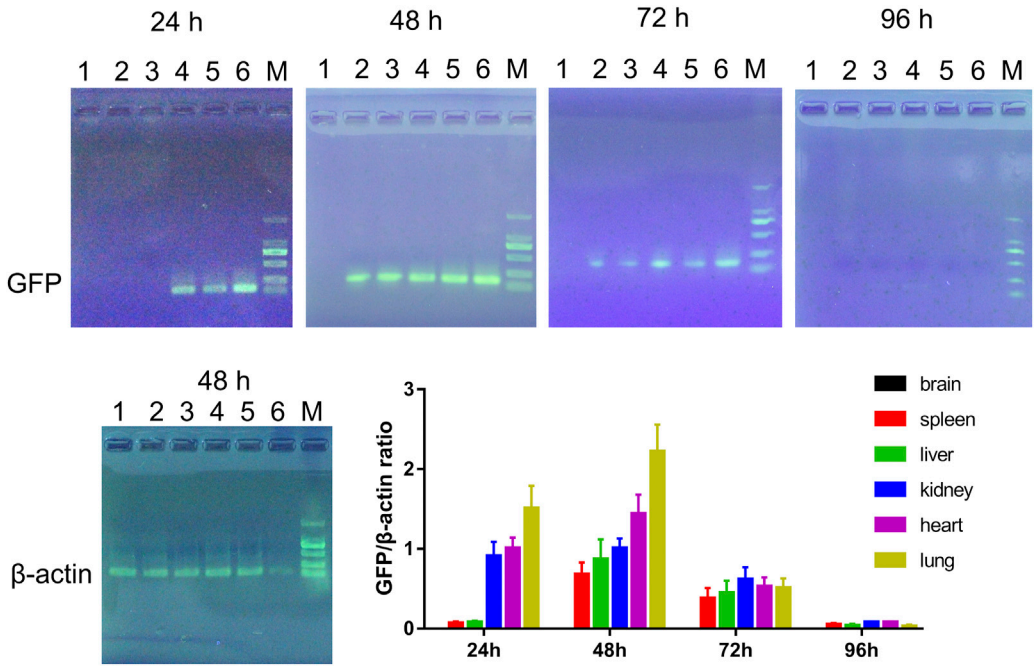

FIGURE 8
Representative electrophoresis images of RT-PCR products and the quantitative data. 1: Brain; 2: Spleen; 3: Liver; 4: Kidney; 5: Heart; 6: Lung; M: DL2000 marker. *n* = 3.

surface antigens, which are typical surface antigens of hucMSCs (Figure 2D). But the cells were CD14, CD19, CD34, CD45, and HLA-DR negative (Figure 2D). These data suggested the hucMSCs had been successfully obtained.

The LV-scFv-sTRAIL infection did not affect the phenotypes of hucMSCs

The hucMSCs infected with LV-sTRAIL and LV-scFv-sTRAIL both expressed sTRAIL successfully, as shown by immunofluorescence staining (Figures 3A,B) and western blot (Figure 3C). ELISA showed that the contents of sTRAIL in the culture supernatant of the LV-sTRAIL group and LV-scFv-sTRAIL group were both significantly higher than the LV-NC group (Figure 3D). There was no significant difference of the levels of sTRAIL between the LV-sTRAIL group and LV-scFv-sTRAIL group (Figures 3A–D).

Flow cytometry analysis demonstrated the hucMSCs infected with LV-scFv-sTRAIL were still CD73, CD90 and CD105 positive, and CD19 and CD45 negative (Figure 3E). This demonstrated that the LV-scFv-sTRAIL infection had no obvious effect on the phenotypes of hucMSCs.

The effect of LV-scFv-sTRAIL infection on hucMSCs tropism towards U87G

The tropism of the hucMSCs infected with LV-scFv-sTRAIL towards U87G *in vitro* was monitored by transwell migration assay. After crystalline violet staining, the number of cells that migrated across the submembrane was counted under the microscope. The results showed that the number of transmembrane cells in the LV-scFv-sTRAIL group was significantly higher than that in the LV-NC group (Figure 4).

The effect of hucMSCs infected with LV-scFv-sTRAIL on proliferation and apoptosis of U87G

The proliferation of U87G cultured with the culture supernatant of hucMSCs in each group were measured by CCK-8 assay. The data showed that proliferation of U87G in the hucMSCs-LV-sTRAIL group and hucMSCs-LV-scFv-sTRAIL group was significantly decreased contrast to that in the hucMSCs-LV-NC group (Figure 5A). After co-culture of hucMSCs and U87G, the effect of hucMSCs on U87G apoptosis was investigated. The data revealed that the apoptosis of U87G in the hucMSCs-LV-scFv-sTRAIL group increased significantly

compared to the hucMSCs-LV-NC group and hucMSCs-LV-sTRAIL group (Figures 5B,C).

The double gene modified hucMSCs inhibited glioma tumor growth *in vivo*

To evaluate the effect of hucMSCs infected with LV-scFv-sTRAIL on tumor growth *in vivo*, mice with xenograft tumor were established by subcutaneous inoculation with U87G cells. The hucMSCs infected with corresponding lentivirus plasmids were injected *via* the tail veins after the xenograft tumors were established. On the 21 days and 28 days after hucMSCs injection, the tumor volume in the hucMSCs-LV-sTRAIL group and hucMSCs-LV-scFv-sTRAIL group were both significantly smaller than that in the hucMSCs-LV-NC group ($p < 0.05$, Figure 6A). 28 days after hucMSCs injection, the tumors were isolated. The data showed that the tumor weight of the hucMSCs-LV-sTRAIL group and hucMSCs-LV-scFv-sTRAIL group were both significantly smaller than that of the hucMSCs-LV-NC group ($p < 0.05$, Figures 6B,C). TUNEL assay demonstrated that the cell apoptosis rates in the U87G xenograft tumor in the hucMSCs-LV-sTRAIL group and hucMSCs-LV-scFv-sTRAIL group were both significantly up-regulated than that in the hucMSCs-LV-NC group ($p < 0.05$, Figures 6D,E). As expected, hucMSCs infected with LV-scFv-sTRAIL showed a stronger inhibitory effect on tumor growth than hucMSCs infected with LV-sTRAIL ($p < 0.05$, Figures 6A–C). The cell apoptosis rate in the hucMSCs-LV-scFv-sTRAIL group was also significantly higher than that in the hucMSCs-LV-sTRAIL group ($p < 0.05$, Figures 6D,E).

The scFv conferred the targeting effect of hucMSCs to tumor tropism

To investigate whether the stronger inhibitory effect of hucMSCs infected with LV-scFv-sTRAIL on tumor growth was due to the targeting effect of scFv, we detected the hucMSCs migrated into the xenograft tumor. Because the lentiviral plasmids pCDH-CMV-MSC-EF1-copGFP-T2A-Puro used in this study could express green fluorescent protein (GFP), the presence of hucMSCs in the xenograft tumor could be revealed by detecting the green fluorescent. As shown in Figure 7, there was no significant difference between hucMSCs-LV-NC group and hucMSCs-LV-sTRAIL group ($p > 0.05$), suggesting the hucMSCs carrying foreign genes still maintained the tumor tropism ability *in vivo*. However, the fluorescence intensity in the hucMSCs-LV-scFv-sTRAIL group was significantly higher than that in the hucMSCs-LV-NC group and hucMSCs-LV-sTRAIL group ($p < 0.05$, Figure 7). These data suggested that the scFv conferred the targeting effect of hucMSCs tropism towards the xenograft tumor.

Distribution of double gene modified hucMSCs *in vivo*

To assess the distribution of double gene modified hucMSCs *in vivo*, the mice were injected with LV-scFv-sTRAIL infected hucMSCs. The presence of exogenous double gene modified hucMSCs in the brain, spleen, liver, kidney, heart and lung were revealed by RT-PCR analysis for GFP. As shown in Figure 8, 24 h after injection, hucMSCs were present in the kidney, heart and lung, but not brain, spleen and liver. 48 h after injection, hucMSCs could be detected in the spleen, liver, kidney, heart and lung, but not brain. After 72 h, the number of exogenous hucMSCs in each organ gradually decreased and almost could not be detected after 96 h.

Discussion

The success of targeted anti-tumor therapy largely depends on an effective targeted drug (gene) delivery system (Zhou et al., 2018). In this regard, hucMSCs exhibited significant tumor migration and could migrate or home to damaged tissues, lesions of the tumor, and metastases after systemic administration (Zhuang et al., 2021). Antibody-mediated targeted therapy is a novel molecular targeting therapy, which can greatly improve the targeting efficiency of genetically modified hucMSC (Ma et al., 2019). In this study, we showed that LV-scFv-sTRAIL elevated the tropism capacity of hucMSCs towards U87G. That indicated that the scFv increased the targeting of hucMSCs to U87G. Huyan (Huyan et al., 2017) found that in the presence of 10 μg/ml anti-HAAH-C mAb, the cytotoxicity of NK cells against hepatoma cell HepG2, cervical cancer cells HeLa, and breast cancer cells MCF-7 increased significantly. Furthermore, research has reported that anti-HAAH mAb (PAN-622) was used to prepare an imaging agent and radioimmunotherapy agent for the detection and treatment of metastatic breast cancer (Revskaya et al., 2017).

The scFv-sTRAIL fusion protein, which can be fused by tumor selective scFv and sTRAIL, can target and induce apoptosis of tumor cells, and it is expected to become a new candidate drug for tumor treatment (Muthu et al., 2020). Liu et al. (2021) demonstrated that sTRAIL gene-modified adipose-derived stem cells obtained tumor-restraining effect on hepatocellular carcinoma cells. The sTRAIL has tumor-

suppressive activity on circulating $CD44^+$ cells in patients with non-small cell lung cancer (Sun et al., 2020). The scFv425: sTRAIL fusion protein, comprises the EGFR blocking antibody fragment scFv425 and sTRAIL, could induce apoptosis of EGFR-positive tumor cells (Bremer et al., 2005). Nevertheless, the effect of the scFv-sTRAIL fusion protein on glioma development is still unclear. In this study, the U87G proliferation was significantly inhibited by hucMSCs infected with LV-scFv-sTRAIL. The apoptosis of U87G was induced by hucMSCs infected with LV-scFv-sTRAIL in co-culture method. In the *in-vivo* experiments, the weight and volume of glioma tumor were significantly inhibited by hucMSCs infected with LV-scFv-sTRAIL. The cell apoptosis of glioma tumor was obviously induced by hucMSCs infected with LV-scFv-sTRAIL. The double gene modification also enhanced the tropism ability of hucMSCs towards the xenograft tumor. Thus, the LV-scFv-sTRAIL infection enhanced the capacity of hucMSCs to target and kill gliomas cells *in vitro* and *in vivo*.

The BBB is a unique border separates brain parenchyma from the bloodstream. As reported, about 98% of prospective medications for brain disorders fail to penetrate BBB (Yarygin et al., 2021). Whether MSCs can cross the BBB is crucial for the clinical application of glioma treatment. Previous reports have shown that there was only 0.0005% MSCs cross the BBB in a traumatic brain injury rat model after intravenous injection (Harting et al., 2009). In another study, most of the injected hucMSCs were found to be distributed in the lung, heart, and liver, but were not detected in the brain at any of the time points after a single intravenous injection in a Alzheimer's disease mouse model (Park et al., 2016). In this study, we detected the distribution of double gene modified hucMSCs *in vivo* by RT-PCR according to the methods of a previous study (Cafforio et al., 2017). The mice were injected with LV-scFv-sTRAIL infected hucMSCs *via* the tail vein. The organs of mice were collected at different time points for RT-PCR detection. The results showed that LV-scFv-sTRAIL infected huMSCs could be detected in the spleen, liver, kidney, heart and lung, but not in brain. This distribution pattern was in line with a previous study (Park et al., 2016). Since the main purpose of this study was to investigate whether the double gene-modified hucMSCs could inhibit tumor growth *in vivo*, a mouse xenograft glioma model was used. The orthotopic glioma model was not used to investigate whether the double gene modified hucMSCs could cross the BBB. This is the main limitation of this study. It had been reported in the literature that the MSCs could migrated through the BBB for the treatment of brain diseases (Yarygin et al., 2021). But we did not detect the presence of exogenous hucMSCs in the present study, which is in line with expectations. The main reasons might be as follows: The subjects in the previous studies, in which the MSCs could cross the BBB, always had brain disease, such as glioma (Pacioni et al., 2017; Abdi et al., 2018), traumatic brain injury (Darkazalli et al., 2017), ischemic stroke (Do et al., 2021; Yarygin et al., 2021) or encephalitis (Bian et al., 2017). In these cases, the BBB was damaged, and lots of inflammatory mediators or chemokines released from inflammatory or tumor lesions in the brain became a decisive factor in promoting the recruitment and migration of therapeutic MSCs through BBB into infarct areas (Huang et al., 2018; Al-Kharboosh et al., 2020). However, in this study, the subjects we used to study the distribution of hucMSCs *in vivo* were normal healthy mice, which lacked the above-mentioned inflammatory mediators or chemokines to induce and recruit transfused exogenous hucMSCs through BBB. In our next research, we will construct an orthotopic glioma mouse model in the brain. Using the model, we will focus on whether these double gene-modified hucMSCs can cross the BBB. This is very important for the clinical application of this double-gene modified hucMSCs in the treatment of glioma.

In summary, the present study demonstrated that the hucMSCs expressing scFv-sTRAIL fusion protein exhibited significant tropism toward glioma, and inhibited the growth of glioma *in vitro* and *in vivo*. These findings shed light on a potential therapy for glioma treatment.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Informed consent was obtained from participants, and this study was approved by the ethics committee of the Second Affiliated Hospital, Air Force Medical University (TDLL2019-11-179). The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by The experiments were carried out in strict accordance with the guidelines of the National Health and Medical Research Council for the Care and Use of Animals for Experimental Purposes in China, and were approved by the Institutional Animal Care and Use Committee of Chinese Academy of Sciences (No. 2020-A138).

Author contributions

TX conceived and designed the study, performed the experiments, analysis of results and drafted the manuscript. XW collected umbilical cords, isolated and cultured MSCs. JR analyzed the data statistically, collection and arrangement of data, draw a chart. LZ performed the RT-PCR tests, gene cloning, and flow cytometry analysis. HY project

leader, directed the research, interpreted the results, review and editing. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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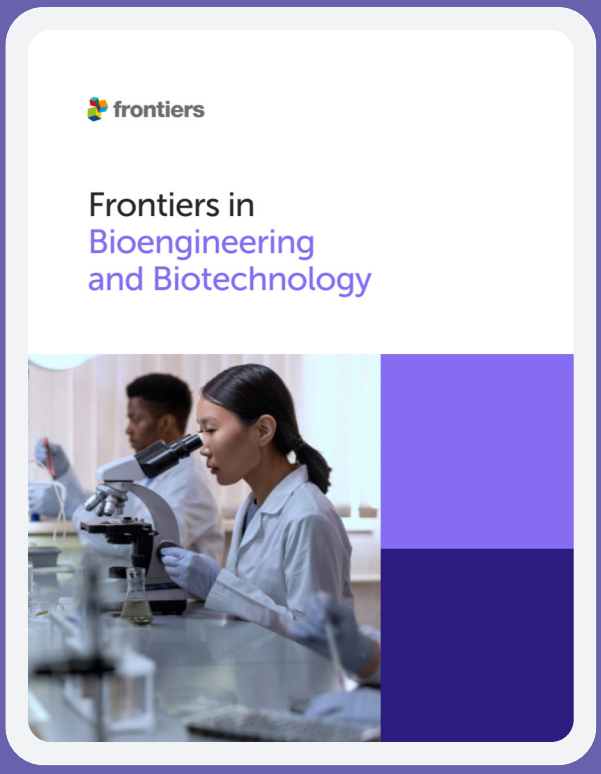

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