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Therapeutic applications of MSCs

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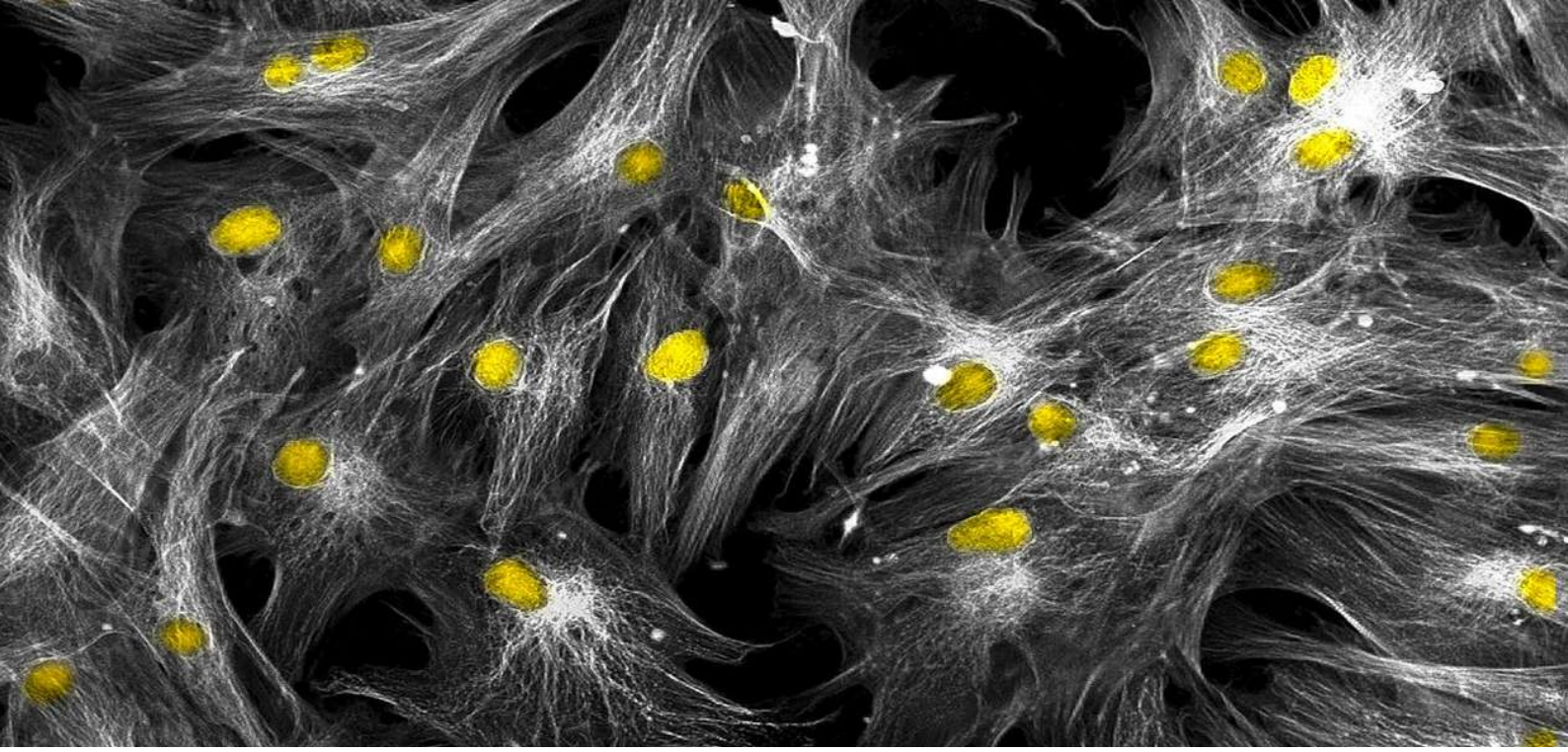

FOREWORD

Welcome to our eBook on the therapeutic applications of mesenchymal stromal cells (MSCs), which has been produced by RegMedNet in association with Biological Industries, part of the Sartorius group. This eBook aims to bring you the latest developments and leading opinions from key thought leaders in the field.

MSCs are multipotent cells with unique characteristics that make them ideal therapeutic candidates. The huge therapeutic potential of MSCs has led them to become incredibly desirable tools in regenerative medicine. Although there has been an increased demand for clinical-grade MSCs, poor quality control and inconsistent features of MSC-based products are remaining obstacles that need to be addressed.

In this eBook, we explore the translation of MSCs from the bench to the clinic and delve further into optimal culture conditions for hMSCs. We also take a look at the therapeutic potential of these cells for various diseases, including atherosclerosis.

We hope you enjoy reading about the latest MSC developments with us!

Annie Coulson
Editor, RegMedNet
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Translating MSCs from the bench to the bedside: an interview with Mira Genser-Nir

Mira Genser-Nir is a Senior Scientist in Advanced Therapies Division at Sartorius (Göttingen, Germany) with more than 10 years' experience at Biological Industries (Beit HaEmek, Israel) R&D, which is now part of the Sartorius group.

Mira has more than 15 years' experience in the biotech sector. She is experienced in developing genetic therapies using a liposomal drug delivery system at Omri biopharmaceuticals Ltd (Ness Ziona, Israel). Later, she joined Biogenics (Israel) and worked on the development of human skin biopump, an autologous platform for tissue-based protein drug delivery for gene therapy. Since 2009, Mira has led the development of serum-free, xeno-free and animal component-free culture platforms for the isolation, expansion, harvesting, differentiation and cryopreservation of hMSCs from various sources and T-Cells. Among the developed products is the MSC NutriStem® XF Medium, which has become the gold standard for isolation and expansion of clinical-grade hMSCs.

Mira Genser-Nir

1 What therapeutic potential do MSCs hold?

Human MSCs (hMSCs) are multipotent cells that exist in almost any niche of our body. They have the capability to renew themselves and differentiate into various cell types, mainly connective tissue lineages, such as adipocytes, osteoblasts and chondrocyte cells.

MSCs have multiple unique features, including their multipotency, their broad variety of tissue sources, their capability to home and engraft into injured tissues when injected intravenously, their potency as immunomodulators, their ability to secrete multiple bioactive molecules and being immuno-privileged, leading to lower immunogenicity. Moreover, these cells have high proliferation abilities (*in-vitro*) with minimal ethical issues.

These traits emphasize the huge therapeutic potential of hMSCs that led them to become one of the most cutting-edge areas in cell therapy today and a desirable tool in regenerative and transplantation medicine.

2 What challenges are associated with the translation of MSCs from the bench to the bedside?

One of the main challenges associated with MSC-based therapy is having off-the-shelf MSC based products. Currently MSCs are frequently used for autologous transplantation. Expanded usage of allogeneic MSCs may provide an easier source on a larger scale and an accessible option for patients' treatments, and it may also reduce costs.

In addition, any cell-based product is required to be accessible to the attending hospital or doctor. Currently MSC products are usually stored by cryopreservation and their use requires specially equipped facilities and skilled personnel, which also contributes to the expensive price of the product and the complexity of its use. Hence, the establishment of allogeneic and autologous cryo-banks of MSCs with the required conditions for handling, storing and shipping of cellular products to end users is needed to facilitate their usage.

Another challenge is having agreement on robust and relevant characterization criteria for clinical

The translation of MSCs from the bench to the bedside: an interview with Mira Genser-Nir

reliability and functionality of MSC-based products. In most clinical studies, the existing information regarding the MCSs is limited and relevant data such as the MSC origin and the culture process are not exposed even though they tend to vary and can have a decisive effect on MSC performance, dosage and administration. The poor quality control and inconsistent features of MSC-based products are main obstacles that have already led to the failure of MSC-based clinical applications and the disapproval of many products by the US FDA.

3 Why is culture media important in the manufacturing process?

In contrast to other bioprocesses that use a mammalian host to produce biologics (i.e., where the focus is the cell materials), such as CHO cells, cell-based therapy focuses on the cells themselves; they are the final target product and hence, the quality of the culture medium is crucial. Over the years, with the strength of regulatory demands, especially regarding clinical applications, the awareness of the importance of quality culture medium due to its influence on cells performance increased.

The culture platform, including the culture medium, auxiliary solutions (for the attachment, harvesting and freezing), as well as the cell culture manual, has a decisive effect on the quality of the cultured cells and may greatly impact on the cells proliferation, morphology, features and, eventually, on their therapeutic abilities. Hence, the culture medium (and all the culture platform) must be carefully selected and optimized specifically to the target cells.

4 Why are serum-free (SF) and xeno-free (XF) culture media preferred for the isolation and expansion of clinical grade hMSCs?

Serum contains more than 4000 components including proteins and growth factors that may impact on a cell's properties and features. Of course, this pool of components is not a matter of choice, hence it is not necessarily an optimal condition for MSCs. Moreover, some of these components may promote cell proliferation, whereas others may lead to cell differentiation or even toxicity and undesired interactions with human cells. Above all, with regards to MSCs, we found that *in vitro* proliferation rate under SF culture condition is higher, which typically correlates to a healthy cell morphology. That is in addition to being a safer culture platform without any ethical aspects and with reduced batch-to-batch variation.

From the regulatory aspect for clinical applications, there is an increased demand for production of MSCs under defined SF and XF culture conditions. Manufacturing of MSCs for clinical applications requires expansion of well-characterized cells produced under tightly controlled, consistent and reproducible culture conditions. This ensures consistent cells with uniform properties and predictable behaviours and reduces the risk of contamination with non-human pathogens (viruses, prions, mycoplasma) and endotoxins.

Therefore, MSC-based cell therapy applications need the elaboration of appropriate SF, XF culture media and defined culture conditions especially designed for MSCs, in order to minimize the theoretical health risk of using xenogeneic compounds. This will limit the immunological reactions once MSCs are transplanted and will

The translation of MSCs from the bench to the bedside: an interview with Mira Genser-Nir

promote high proliferation rate while maintaining MSCs unique features and authentication. It should be considered that optimized protocols for MSC isolation and *ex vivo* preparation for clinical use under SF, XF culture condition need to be well established and optimized as well.

5 With regards to MSCs, what have been the greatest advances in the past 5 years and what are your predictions for the next 5 years?

The greatest advances during the last 5 years, with the increasing demand for clinical application-based MSCs, are the shift from using autologous to allogeneic MSCs and the development of new MSC-based products such as cell-free exosomes and human pluripotent stem cell (hPSC)-derived MSCs. All of these may enable future off-the-shelf products. It should be noted that the COVID-19 global pandemic, has also led to significant progress in the research and development of MSC-based therapy for lung diseases with a dramatically increasing number of clinical studies and registered trials for the treatment of lung diseases (mainly COVID-19).

The shift from using autologous to allogeneic MSCs is an increasing trend and it enables more scalable, robust and accurate processes, although many clinical applications are still based on autologous MSCs. Regarding cell-free therapy approaches, the use of MSC exosomes opened new research directions and is becoming increasingly attractive since MSC exosomes were found to have the same therapeutic effects as MSCs – enabling tissue repair and regeneration. The use of MSC exosomes offers advantages over whole-cell therapy which is mainly due to their stability and very low immunogenicity, and there are currently several MSC exosome-based clinical applications. Finally, technological advances in induced PSC (iPSC)-derived cells led to the innovation of iPSC-derived MSCs. This approach reduces the variability associated with primary-isolated human MSCs and allows for an unlimited source of cells.

In the next 5 years, we are likely to see extensive data from the MSC-based clinical studies that are currently running, most of which are presently in Phase I–II. The results will ultimately impact on the fate of future MSC products.

At the same time, due to the huge progress and advances in MSC-based therapy approaches, it is anticipated that more off-the-shelf MSC products, including cells and cell-free treatments, will be available and accessible to a wider range of diseases. In parallel, there will likely be more developments and clinical trials utilizing MSC exosomes and iPSC-MSCs. In addition, it is anticipated that novel therapy approaches based on genetically engineered MSCs will also be available. Engineered MSCs may be directed to a more specific purpose and streamline the treatment with MSCs.

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Application Note

August 18, 2021

Keywords or phrases:
MSC Nutristem XF Medium, MSC isolation, MSC expansion, clinical applications, regenerative medicine, cell therapy, mesenchymal stromal cells, stem cell research, tissue regeneration, mesenchymal stem cells

MSC Nutristem® XF medium

Adipose Tissue Derived MSC Isolation Using Defined, Xeno-Free, Serum-Free Medium

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Abstract

Cell therapy is a very promising therapeutic approach. The impressive progress in the field of stem cell research, due to stem cells' infinite self-renewal ability and their potential to differentiate into other cell types, has laid the foundation for cell-based therapies for diseases which cannot be cured by conventional medicines.
Mesenchymal Stromal Cells (MSCs) can be found in a variety of tissues, including Adipose tissue, Bone Marrow, Dental Pulp, etc. and are capable of differentiating mainly into connective tissue cell types – Bone (osteocytes), cartilage (chondrocytes), muscle (myocytes) and fat (adipocytes).
In this study adipose tissue mesenchymal stromal cells (AT-MSCs) were derived from human adipose tissue, expanded and later used for a clinical application, using the defined MSC Nutristem® XF medium.
The isolation procedure and expansion protocol, including cell yield, phenotype and viability results are reviewed as followed.

Find out more: www.sartorius.com/MSC_NutriStem_XF_Medium

Introduction

Stem cell research has made impressive progress the last few years, laying the foundation for therapeutic approaches and very promising cell-based therapies, for the treatment of diseases which cannot be cured by existing conventional medicines.
Mesenchymal Stromal cells are multi potent cells that have the capability to differentiate into a variety of cell types, mainly adipose, bone, muscle and cartilage tissues. MSCs are found mostly in bone marrow, adipose tissue, cord blood, placenta and dental pulp and are known for their immunomodulatory affects. (1)
Because some sources of MSCs have relatively simple isolation techniques and MSCs in general have extensive differentiation potential and immune-modulation properties, these cells were introduced into the clinic for repairing tissue injuries, producing engineered tissues in-vitro for in-vivo transplantation, ameliorating immune-mediated diseases and other applications. (2)

Adipose tissue-derived MSCs (AT-MSCs) normally reside in the stromal vascular fraction of the adipose tissue. Subcutaneous adipose depots are abundant and easily accessible in large quantities with a minimally invasive procedure (liposuction aspiration). The AT-MSCs can easily be isolated by tissue digestion. (1)
In this experiment MSCs were extracted from adipose tissue, isolated and expanded, using MSC NutriStem® XF medium. The MSCs were then frozen and thawed, their parameters were assessed and they were used later for tissue regeneration applications.

Materials and Methods

Materials

- MSC NutriStem® XF Medium, Sartorius Cat. # 05-200-1, 05-201-1
- MSC Attachment Solution, Sartorius Cat. # 05-752-1
- DPBS no calcium, no magnesium, Sartorius Cat. #02-023-1
- NutriFreez® D10 Cryopreservation Medium, Sartorius Cat. # 05-713-1
- MSCgo™ Adipogenic Differentiation Medium, Sartorius Cat. # 05-330-1, 05-331-1-01, 05-332-1-15
- MSCgo™ Chondrogenic Differentiation Medium, Sartorius Cat. # 05-220-1, 05-221-1
- MSCgo™ Osteogenic Differentiation Medium, Sartorius Cat. # 05-440-1
- Recombinant Trypsin-EDTA solution, Sartorius Cat. # 03-079-1
- PBS, Biological Industries
- Collagenase (NB 4 standard grade), Serva Electrophoresis
- Male AB Serum, Access Biologicals
- Aanti-human CD13-PE, + isotype control PE, eBioscience
- Anti-human CD73-PE, + isotype control PE, eBioscience
- Anti-human CD90-PE-Cy7, + isotype control PE-Cy7, eBioscience
- Anti-human CD31-PE-Cy7, + isotype control PE-Cy7, eBioscience
- Anti-human CD45-Alexa Fluor 488, + isotype control Alexa Fluor 488, R&D Systems
- Oil Red O Stock, Science Cell
- Alcian Blue, Science Cell
- Alizarin Red S, Science Cell
- Crystal Violet, Sigma-Aldrich
- Chromosome Resolution Additive (CRA),Genial Genetics
- Acetic acid glacial 100%, Merck
- Methanol abs AR, Merck

Method

AT-MSC isolation:

AT-MSCs were isolated from adipose tissue, washed with sterile phosphate buffered saline (PBS; Biological Industries) to remove debris and red blood cells, following enzymatic digestion with collagenase. The processed tissue was centrifuged, and the cellular pellet was re-suspended in MSC NutriStem® XF medium.
Following isolation, the cells were seeded in tissue culture flasks, pre-coated with MSC attachment solution diluted 1:100 in DPBS no calcium, no magnesium, and then seeded in MSC NutriStem® XF medium supplemented with 2.5% Male AB Serum (Access Biologicals).

AT-MSCs passaging:

AT-MSCs flasks were washed with PBS (no calcium, no magnesium), and cells were harvested from the tissue culture flask using recombinant Trypsin-EDTA solution, centrifuged (7min, 300g), resuspended in MSC NutriStem® XF medium supplemented with Male AB serum, counted using NucleoCounter NC-200, and seeded in a new tissue culture flask at 2500-4500 cells/cm^2. Of note, for the AT-MSCs expansion period no attachment solution was applied, due to the addition of AB serum supplementing the MSC growth medium.

Cryopreservation of AT-MSCs:

P1 AT-MSCs were detached from the tissue culture flask using Recombinant Trypsin-EDTA solution, centrifuged, resuspended in MSC NutriStem® XF medium (supplemented with AB serum), and cell count and viability were assessed by NucleoCounter NC-200 (ChemoMetec). For cryopreservation, the cells were centrifuged, and the pellet was resuspended in the cold NutriFreez® D10 Cryopreservation Medium at a cell concentration of $5 \cdot 10^6$ cells/mL, and a total of $1 \cdot 10^6$ cells (200µl volume) was transferred into a cryogenic vial (1.8mL cryogenic tube, NuncTM, Thermo Scientific), immediately placed in 2-8°C pre-cooled Mr. Frosty, and inserted into a -80°C freezer. Following 12-24 hours at -80°C, the cryogenic vials were placed in liquid nitrogen for at least 14 days.

Thawing of cryopreserved AT-MSCs:

Following a minimum cryopreservation period of two weeks, the cryogenic vials were rapidly thawed (<1min) at 37°C water bath. The cells were slowly diluted with 5mL pre-warmed MSC MutriStem® XF medium supplemented with AB serum, centrifuged, the supernatant discarded, and the pellet resuspended with 1mL of MSC MutriStem® XF medium supplemented with AB serum.

AT-MSC count and assessment of cell viability:

Cell concentration and viability were measured by NucleoCounter NC-200 using vial-cassette, (ChemoMetec). The cryogenic vial volume was measured with a manual pipettor, and the total cells/vial was calculated by multiplying the NucleoCounter-measured cell concentration (cells/mL) with the vial volume (mL).

Evaluation of AT-MSCs adhesion capability:

1mL of thawed AT-MSCs, originating from one cryopreserved vial ($1 \cdot 10^6$ cells), was counted by NucleoCounter NC-200, and seeded in a T-25 tissue culture flask at a seeding concentration of 2500-4500 cells/cm^2 in MSC NutriStem® XF medium + AB serum, and incubated at 37°C (37°C, 5% CO_2 incubator) for 6-8 hours, after which the adherent cells were harvested with Recombinant Trypsin EDTA solution and counted by NucleoCounter NC-200.

AT-MSCs growth rate analysis:

AT-MSCs were thawed and seeded in a T-75 tissue culture flask, and expanded in MSC NutriStem® XF medium to passage P3 and P4. At passage P3 and P4, respectively, the adherent cells were harvested with Recombinant Trypsin EDTA solution, counted and their viability assessed by NucleoCounter NC-200. The growth rate (µ), doubling time (g), and population doubling level (PDL) were calculated for passage P3 and P4 AT-MSCs according to the following equations (A, B and C):

$$N_t = N_0 \cdot e\hat{}\mu t \quad \text{A}$$

$$g = (\ln(2))/\mu \quad \text{B}$$

$$PDL = 3.322 \cdot (\log(N_t) - \log(N_0)) \quad \text{C}$$

Where N_t is the number of cells at time t, N_0 is the number of cells at time 0, µ is the growth rate, and g the doubling time.

AT-MSCs identity analysis by flow cytometry:

The AT-MSCs stem/stromal cell identity was analyzed, at passages P3/P4, by flow cytometry, evaluating the presence of the surface markers CD13, CD73 and CD90 and the absence of the surface markers CD31 and CD45.

AT-MSCs differentiation assay into adipocytes, chondrocytes and osteocytes:
AT-MSCs were differentiated into the adipogenic, chondrogenic and osteogenic lineages using the respective differentiation medium; adipogenic (MSCgo™ Adipogenic Basal Medium, supplemented with MSCgo™ Adipogenic SF, XF Supplement Mix I, and Mix II, chondrogenic (MSCgo™ Chondrogenic Differentiation Medium, supplemented with MSCgo™ Chondrogenic Differentiation Supplement Mix, and osteogenic differentiation medium (MSCgo™ Osteogenic SF, XF, respectively. Following differentiation, the cells were evaluated by histological analysis and stained as follows: Adipocytes were stained with Oil Red O Stock to visualize lipids and fat deposits, in chondrocytes the sulfated proteoglycan present in cartilage tissue was stained with Alcian Blue, and in osteocytes the calcium deposits in the cells were stained with Alizarin Red S.

AT-MSCs fibroblast colony-forming unit (CFU-F) assay:
50 cells/well AT-MSCs (P4) were seeded in a 6-well plate (5 cells/cm2) using 2ml/well MSC NutriStem® XF medium on pre-coated plates (MSC attachment solution, diluted 1:100 in PBS) and were incubated for 10-14 days. Visible colonies are enumerated after fixation and staining of the plates with crystal violet dye. The % CFU-F of AT-MSCs was calculated by the following equation:

CFU-F (%)=(No.of colonies observed•100%)/(No.of seeded cells)

AT-MSCs karyotype analysis:
AT-MSCs were treated with CRA working solution (Chromosome Resolution Additive diluted 1:100 in Hank's Balance Solution and Colcemid solution. Following CRA and Colcemid incubations, the flasks were washed with PBS (without calcium and magnesium), harvested with recombinant Trypsin EDTA solution and centrifuged. The cell pellet was resuspended with hypotonic solution (Potassium chloride and Sodium citrate in water) and then washed three times with Fixer solution (1 part acetic acid with 3 parts of methanol). The karyotype analysis was performed at the Laboratory Services Division, Cytology Lab, Rambam Health Care Center, Israel.

Results

The AT-MSCs that were isolated from a donor's adipose tissue and their parameters were evaluated.

Cell viability of AT-MSCs:
The isolated AT-MSCs were cryopreserved at passage 1 (P1) at a cell concentration of $5 \cdot 10^6$ cells/mL, a total of $1 \cdot 10^6$ cells per cryogenic vial. The cryovials were immediately placed in 2-8°C pre-cooled Mr. Frosty and inserted into a -80°C freezer. The measured cell viability (by NucleoCounter NC-200) prior to cryopreservation was 96%.

Cell count and viability assessment of cryopreserved AT-MSCs following thawing:
Cryopreserved AT-MSCs were thawed (as described in the methods), counted and their cell viability assessed by NucleoCounter NC-200. Cell count was 1.0×10^6 (total cells/vial) and 94.4% of the cells were viable.

Evaluation of AT-MSCs adhesion potential:
AT-MSCs were counted and seeded in a T-25 tissue culture flask. Following 6-8 hours of incubation at 37°C, the adherent cells were harvested and counted by NucleoCounter NC-200. The total cell count was 1.0×10^6 and the Adherence (% adherent cells) was 100.0%.

AT-MSCs growth rate analysis:

AT-MSCs were counted and seeded in a T-75 tissue culture flask, at concentration of 2500-4500 cells/cm^2. At the end of passage P2, the adherent cells were harvested and their viability assessed by NucleoCounter NC-200. Cell viability was 98.0%.

The cells were seeded for passage P3 as well as for P4 at a concentration of 2500-4500 cells/cm^2. At the end of passage P3 and P4, the adherent cells were harvested, counted and their viability assessed, as well as the growth rate, doubling time, and population doubling level calculated. Results for P3 and P4 growth rate analysis are shown in table 1. In Figure 1, representative images are shown of the cryopreserved AT-MSCs following thawing (Figure 1A), at passage P3 (Figure 1B), and at passage P4 (Figure 1C).

Passage	% Viability	Growth rate - μ (1/h)	Doubling time - g (h)	PDL
P3	98.8	0.038	18.3	3.8
P4	98.1	0.0378	18.4	3.8

Table 1: AT-MSCs growth rate evaluation and viability assessment. The growth rate (μ), doubling time (g), and population doubling level (PDL) were calculated for passage P3 and P4 according to equations (A), (B), and (C), respectively (see Methods). Cell viability is presented as % viable cells; growth rate is presented as 1/h; doubling time is presented as hours.

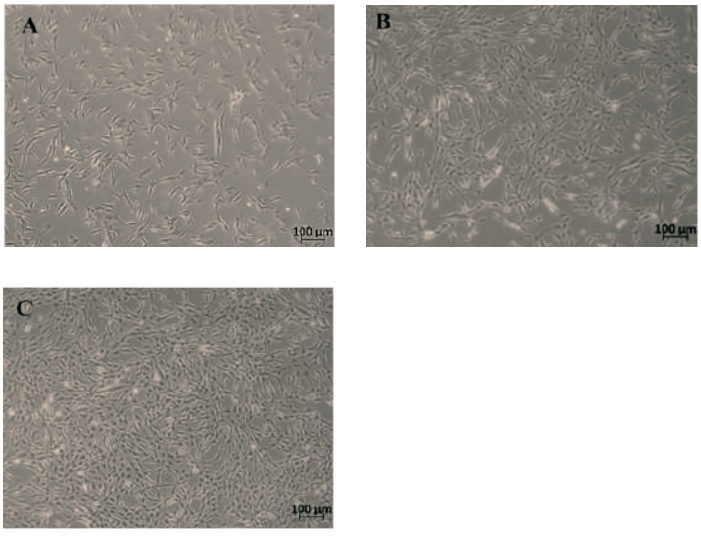


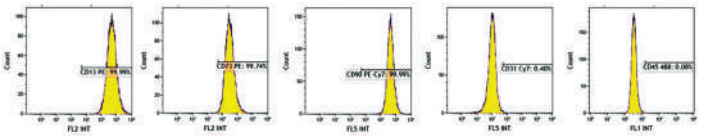
Figure 1: Representative images of AT-MSCs at passage P3 and P4. A) The cryopreserved cells (P1) were thawed and images were taken 6-8 hours after seeding (P2). For passage P3 as well as for P4 the cells were seeded at a concentration of 2500-4500 cells/cm^2 in T-75 tissue culture flasks. Representative images of AT-MSCs, taken prior to harvesting at the end of passage P3 (B), and P4 (C).

Identity analysis of the AT-MSCs:

The mesenchymal stromal cell identity of the AT-MSCs was evaluated at passage P3/P4 by flow cytometry analysis of the presence of the surface markers CD13, CD73, CD90, and the absence of the markers CD31, and CD45 (see Table 2 and Figure 2). The percentage of positive cells for the tested surface markers are presented in Table 2.

Tested surface markers (% positive cells)				
CD13	**CD73**	**CD90**	**CD31**	**CD45**
99.99	99.74	99.99	0.40	0.08

Table 2: AT-MSCs identity analysis. Data is presented as % positive cells for the respective surface marker. AT-MSCs expression level of the surface markers CD13, CD73, and CD90 should be >80% and the surface markers CD31 and CD45 <2%, according to Bourin et al., 2013.



CD13 CD73 CD90 CD31 CD45

Figure 2: AT-MSCs identity analysis. Flow cytometry identity analyses are presented for AT-MSCs. The percentage of surface marker positive cells is marked.

AT-MSCs differentiation potential:
AT-MSCs at passage P3/P4 seeded and grown in adipogenic, chondrogenic and osteogenic differentiation medium, in order to evaluate the cryopreserved AT-MSCs stemness. The cells were stained as follows: Adipocytes were stained on day 10 of adipogenic differentiation with Oil Red O Stock to visualize lipids and fat deposits (Figure 3A); In osteocytes the calcium deposits in the cells were stained with Alizarin Red S on day 18-21 of osteogenic differentiation (Figure 3B); in chondrocytes the sulfated proteoglycan present in cartilage tissue was stained on day 21 of chondrogenic differentiation with Alcian Blue (Figure 3C). Histological analysis of the AT-MSCs is presented in Figure 3.

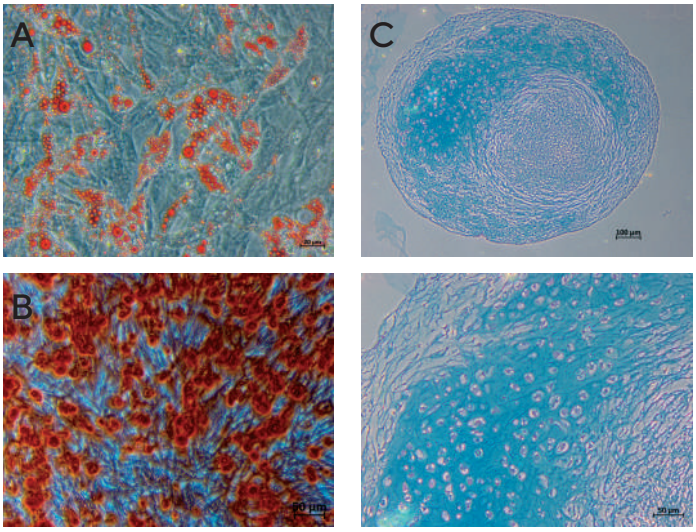


Figure 3: Differentiation potential of AT-MSCs into the adipogenic, chondrogenic and osteogenic lineages. Representative images of the differentiated AT-MSCs. The differentiated cells were stained as follows: A. Adipocytes were stained with Oil Red O Stock, B. Osteocytes were stained with Alizarin Red S, and in C. Chondrocytes were stained with Alcian Blue.

AT-MSC CFU-F assay
AT-MSCs on passage P4 were seeded at low density (5 cells/cm2) and incubated in MSC NutriStem® XF medium for 10-14 days. CFU-F results were 30%. The percentage of CFU-F was calculated according to the equation (see Materials and Methods). The image below shows a crystal violet-stained CFU-F assay well. (Figure 4).

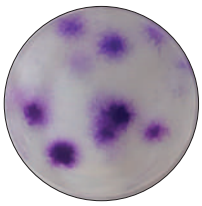


Figure 4: MSC Colonys stained with crystal violet. AT-MSC colony-forming unit (CFU-F) assay. CFU-F assay was performed. The image shows a crystal violet-stained CFU-F assay well.

Karyotype analysis
AT-MSCs were expanded to passage P6, and prepared for karyotyping, performed at Rambam Health Care Campus. The analysis results demonstrated the karyotype of the tested sample to be normal (Figure 5).

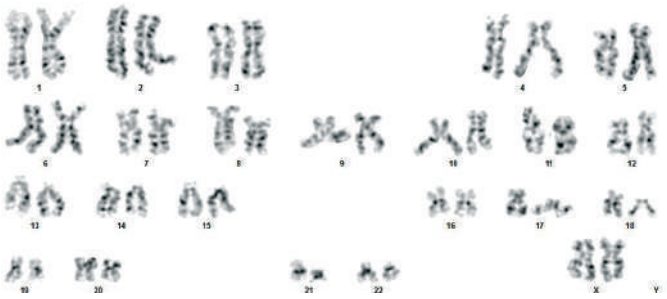


Figure 5: Karyotype analysis. Karyotype is presented for AT-MSCs.

Discussion

The study above illustrates the utility of the Sartorius MSC Nutristem® XF Medium when used to derive, isolate, and expand MSCs, as well as the MSCgo™ family of media to differentiate the aforementioned cells to the osteocyte, chondrocyte and adipocyte lineages.
MSCs are extremely sensitive cells and each stage of isolation and expansion must be adequate and sufficient. The smallest shift in cells environment and conditions might harm and influence their viability and potency.
Deriving cells for the purpose of cell therapy, regenerative medicine and other therapeutical applications has to be precise and consistent. Using the right media and creating the best conditions are crucial for achieving optimal outcomes.
MSC Nutristem® XF medium, a defined scientifically and regulatory supported product, was developed in order to ensure optimal MSC growth parameters such as high fold expansion rate, established differentiation potential, normal karyotype and typical MSC phenotype markers.

This application note describes the extraction of highly viable adherent AT-MSCs from human adipose tissue, their growth on tissue culture flasks, expansion, detachment, freezing and thawing – followed by evaluation of viability, differentiation analysis, karyotype analysis and many other parameters. MSC Nutristem® XF Medium was used for the isolation and expansion stages and between passages. The results demonstrate excellent cell counts after derivation and post thaw, very high viability, excellent differentiation potential, high percentage of cells presenting positive MSC markers for the identity analysis and overall support evidence for normal, viable and potent cells.

Conclusion

Using the right media and solutions is crucial and critical for achieving large numbers of high quality, viable, multipotent, normal phenotype and karyotype MSCs, suitable for regenerative medicine, cell therapy and other therapeutical applications.
This application note shows that MSC Nutristem® XF Medium supports desirable outcomes when isolating, expanding and maintaining MSCs, successfully ensuring the cells fine phenotype, viability and multipotency. These results demonstrate that MSC Nutristem® XF medium is a good reliable option for producing MSCs suitable for therapies and clinical applications.

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A Xeno-Free Culture System for hMSC From Various Sources Suitable for Initial Isolation and Expansion Toward Clinical Applications

Mira Genser-Nir, Sharon Daniliuc, Marina Tevrovsky, David Fiorentini.

Biological Industries, Beit Haemek, Israel.

Abstract

Human mesenchymal stem cells (hMSC) are multipotent adult stem cells present in a variety of tissue niches in the human body. hMSC have advantages over other stem cell types due to the broad variety of their tissue sources, since they are immuno-privileged, and for their ability to specifically migrate to tumors and wounds in vivo. Due to these traits hMSC have become desirable tools in tissue engineering and cell therapy. In most clinical applications hMSC are expanded in vitro before use. The quality of the culture medium and its performance are particularly crucial with regard to therapeutic applications, since hMSC properties can be significantly affected by medium components and culture conditions.

To date there is no efficient xeno-free (XF) medium for the initial isolation of hMSC from various tissues. In addition, most of the common culture media for growth and expansion of hMSC, as well as auxiliary solutions (for attachment, dissociation, and cryopreservation), are typically supplemented with serum or other xenogenic compounds. A defined serum-free (SF), XF culture system optimized for hMSC isolation and expansion would greatly facilitate the development of robust, clinically acceptable culture process for reproducibly generating quality-assured cells.

The present study evaluated a novel XF culture system, comprising MSC NutriStem® XF culture medium and all the required auxiliary solutions for the attachment, dissociation, and cryopreservation of the cells. The system was evaluated for initial isolation of hMSC from various sources, and for long-term culturing under SF, XF culture conditions suited for clinical applications.

Results show that the XF culture system for hMSC efficiently supports initial isolation and optimal expansion of hMSC from various sources, while maintaini MSC features: typical fibroblast-like cell morphology, phenotypic surface marker profile, differentiation capacity, self-renewal potential, and genetic stability.

Abbreviations

FBS	Foetal Bovine Serum
hMSC	Human Mesenchymal Stem Cells
hMSC-AT	Adipose Tissue derived hMSC
hMSC-BM	Bone Marrow derived hMSC
hMSC-PL	Placenta derived hMSC
hMSC-WJ	Wharton's Jelly derived hMSC
SF	Serum Free
XF	Xeno Free

Materials and Methods

Cells
hMSC (passage 0-5) from a variety of sources: AT WJ PL and BM were used in this study.

Initial isolation of hMSC
hMSC AT, WJ, and PL were isolated by enzymatic digestion followed by centrifugal separation to isolate the stromal | vascular cells. The pellet was re-suspended with MSC NutriStem® XF supplemented with 2% human AB+ serum (hMSC-AT and WJ) or w/o the addition of serum (hMSC-PL), and cultured on pre-coated plates with MSC Attachment Solution.
hMSC-BM mononuclear cells were collected from a bone marrow aspirate by density gradient centrifugation. The cells were washed and cultured on pre-coated plates (with MSC Attachment Solution) using MSC NutriStem® XF and serum-containing medium. The marrow stromal cells were selected by plastic adherence and were allowed to expand until reaching sub-confluence.

SF, XF culture system
hMSC were cultured in a SF, XF expansion medium (MSC NutriStem® XF, BI) on pre-coated dishes (MSC Attachment Solution, BI) or as indicated. Cells were seeded at concentrations of 5000-6000 viable cells/cm² and harvested using either MSC Dissociation Solution (BI) or Recombinant Trypsin Solution (BI).

Medium performance evaluation
Medium performance was evaluated by viable cell count, proliferation rate, cell morphology, multilineage differentiation potential into adipocytes, osteocytes, and chondrocytes, self-renewal potential, cell immunophenotype, and karyotype analysis.

Differentiation
hMSC expanded for 3-5 passages in MSC NutriStem® XF were tested for multilineage differentiation potential (into adipocytes, osteocytes, and chondrocytes) using differentiation media. Cells were fixed and stained with Oil Red O, Alizarin Red, and Alcian Blue, respectively.

CFU-F
hMSC were seeded at low densities (10, 50, and 100 cells/cm²) in MSC NutriStem® XF on pre-coated dishes (MSC Attachment Solution, BI) or as indicated, and cultured for 14 days following staining with 0.5% Crystal violet.

Flow Cytometry
hMSC were cultured for 2-5 passages in MSC NutriStem® XF followed by MSC identification by flow cytometry using positive and negative surface markers.

Karyotype
Genomic stability of hMSC was tested by G-banding karyotyping analysis.

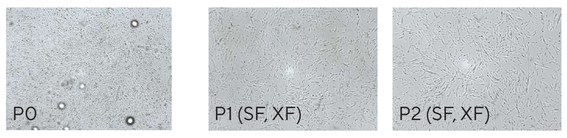
Results

I. Initial Isolation

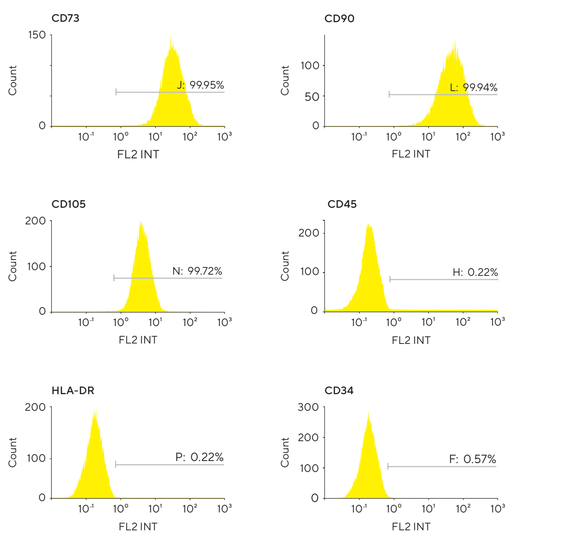
hMSC-AT

Figure 1: Evaluation of hMSC-AT isolation using MSC NutriStem® XF

A. MSC NutriStem® XF on pre-coated plates (MSC Attachment Solution)



B.

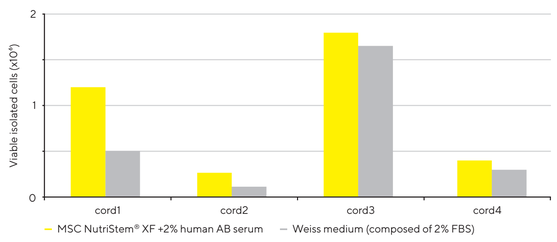


Adipose tissue-derived cells were seeded in MSC NutriStem® XF supplemented with 2% human AB+ serum on pre-coated plates with MSC Attachment Solution for the initial isolation and expansion of hMSC-AT (P0). The cells were cultured to 70-80% confluence before being sub-cultured. Further passages (P1-2) were done under SF, XF culture conditions, utilizing MSC NutriStem® XF culture medium on pre-coated dish. **A.** Representative images taken 4 days post initial seeding (P0) and 3 days post P1 and P2. **B.** Immunophenotyping results of hMSC-AT at passage 2 using FACS analysis. **Successful isolation of hMSC-AT that maintains a classical profile of MSC markers was achieved under XF conditions, utilizing MSC NutriStem® XF medium.**

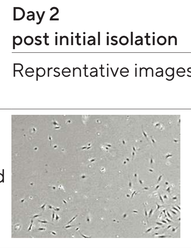
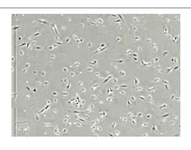
hMSC-WJ

Figure 2: Comparison of hMSC-WJ isolation utilizing MSC NutriStem® XF vs. FBS-containing medium

A.



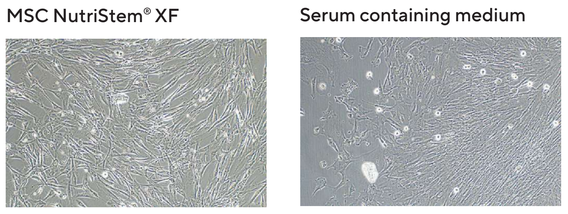
B.

	Day 2 post initial isolation	Day 7 post initial isolation	
	Reprsentative images	Total cells count Live	Dead
MSC NutriStem® XF (supple-mented with 2% human AB+ serum)		395,000	15,000
Weiss medium (composed of 2% FBS)		295,000	75,000

hMSC were initially isolated from 4 independent human cords utilizing XF medium (MSC NutriStem® XF supplemented with 2% human AB+ serum on pre-coated plates with MSC Attachment Solution) in comparison to serum-containing medium.
A. Comparing the amount of viable cells – passage 0. Cell count was measured by trypan blue exclusion assay. **B.** Representative images (×40) of cord 4 taken on Day 2 post initial isolation in each medium, and cell count results of Day 7 post initial isolation, respectively. **Initial isolation of hMSC–WJ under XF culture system is superior.**

hMSC-PL

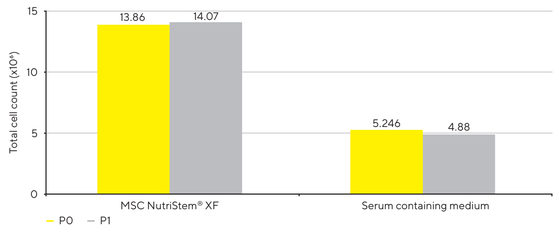
Figure 3: Isolation of hMSC-PL using SF, XF culture medium (MSC NutriStem® XF) and serum-containing medium



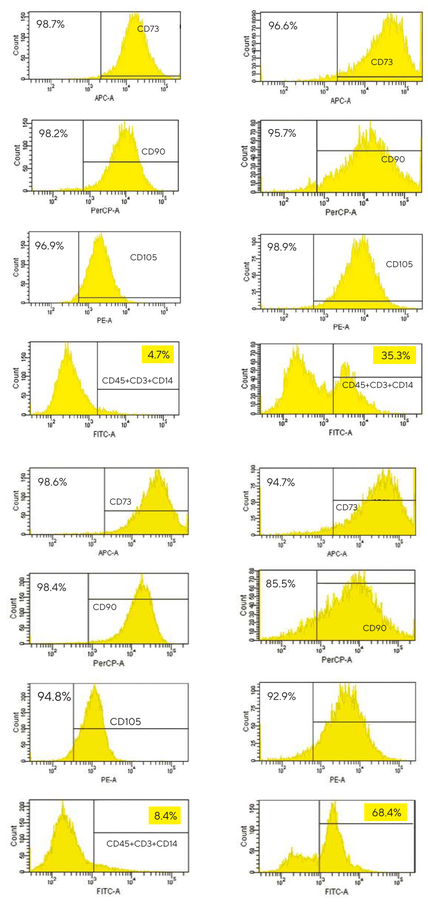
hMSC were isolated from frozen crude placenta under SF, XF culture conditions (MSC NutriStem® XF on pre-coated plates with MSC Attachment Solution, w/o supplementation of human AB+ serum) and in FBS containing medium. Representative images (×40) taken 11 days post initial isolation (P0). **Higher confluence is observed utilizing MSC NutriStem® XF w/o the requirement of human AB+ serum supplementation.**

Figure 4: Evaluation of hMSC-PL isolation using MSC NutriStem® XF vs. FBS containing medium

A.



B.



Comparison of hMSC-PL isolation from crude placenta 17 days post initial seeding (P0) and 7 days post P0 (P1) in each medium. **A.** Quantity of viable cells, measured by trypan blue exclusion assay. **B.** immunophenotype results using FACS analysis. **Initial isolation of hMSC–PL under SF, XF culture system using MSC NutriStem® XF (w/o supplementation of human AB+ serum) is superior: achieves a higher number of purer and viable cells that maintain a normal profile of MSC markers. Further expansion in MSC NutriStem® XF medium enhanced the advantages over serum-containing medium, leading to higher level of MSC purity (91.6% and 31.6%, respectively, emphasize in blue).**

SARTORIUS

Simplifying Progress

A Xeno-Free Culture System for hMSC From Various Sources Suitable for Initial Isolation and Expansion Toward Clinical Applications

Mira Genser-Nir, Sharon Daniliuc, Marina Tevrovsky, David Fiorentini.

Biological Industries, Beit Haemek, Israel.

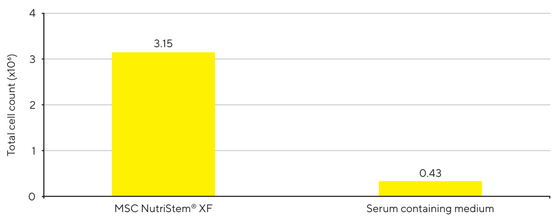
Results

I. Initial Isolation

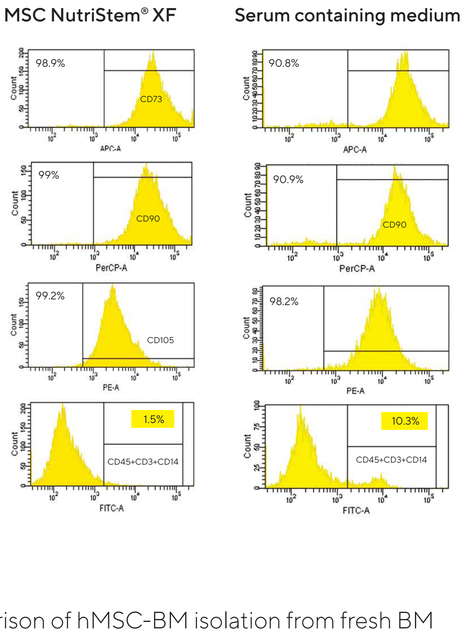
hMSC-BM

Figure 5: Evaluation of hMSC-BM isolation using MSC NutriStem® XF vs. FBS containing medium

A.



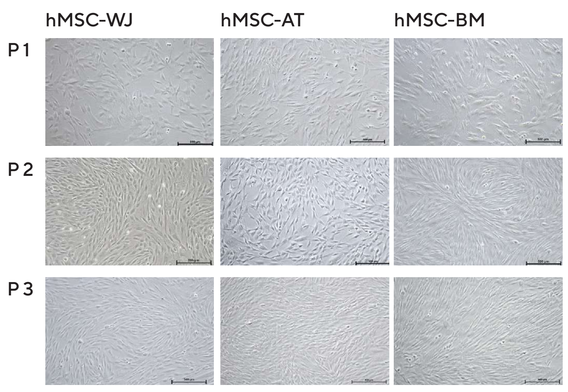
B.



Comparison of hMSC-BM isolation from fresh BM utilizing MSC NutriStem® XF and serum-containing medium (11-day assay). **A.** Cell count was measured by trypan blue exclusion assay. **B.** Immunophenotype using FACS analysis. **Initial isolation of hMSC-BM using MSC NutriStem® XF is superior: achieves MSC populations with a higher number of viable cells and higher levels of purity (98.5% and 89.7% respectively, emphasize in blue) that maintain a classical profile of MSC markers.**

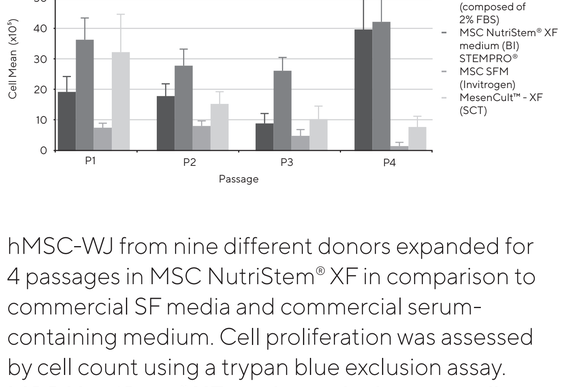
II. Expansion

Figure 6: Suitability for Various Sources of hMSC



hMSC derived from a variety of sources: WJ, AT, and BM cultured for 3 passages in the XF culture system (MSC NutriStem® XF, MSC Attachment Solution, MSC Dissociation Solution). Representative images taken on Day 3 of culture (×100). **MSC NutriStem® XF promotes proliferation of hMSC from a variety of sources while maintaining their fibroblast-like morphology.**

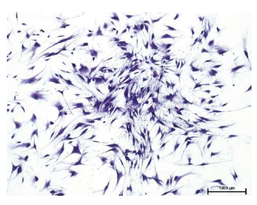
Figure 7: Proliferation



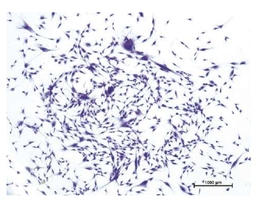
hMSC-WJ from nine different donors expanded for 4 passages in MSC NutriStem® XF in comparison to commercial SF media and commercial serum-containing medium. Cell proliferation was assessed by cell count using a trypan blue exclusion assay. **MSC NutriStem® XF produces the best overall expansion of hMSC-WJ.**

Figure 8: Self-renewal Potential

hMSC-BM

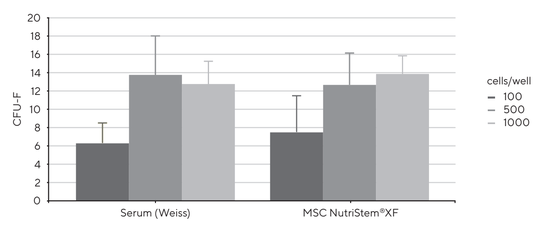


hMSC-AT



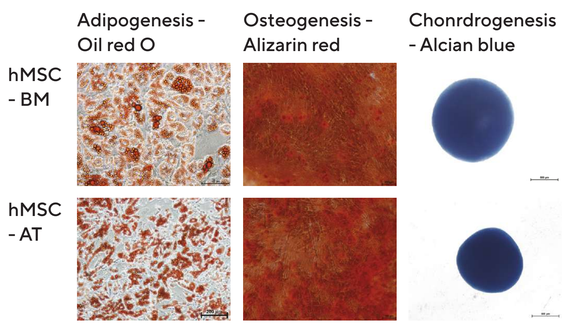
hMSC-BM and AT expanded in MSC NutriStem® XF for 3-5 passages prior to 14 days of CFU-F assay. Representative images of colonies stained with 0.5% crystal violet (×100). **hMSC cultured in MSC NutriStem® XF maintain their self-renewal potential.**

Figure 9: Self-renewal potential of hMSC-WJ



CFU-F assay of hMSC-WJ expanded for 5 passages in MSC NutriStem® XF and Weiss medium (2% FBS) in 3 different seeding concentrations. **hMSC cultured in MSC NutriStem® XF maintain their self-renewal potential.**

Figure 10: Trilineage differentiation potential



hMSC-BM and hMSC-AT expanded in MSC NutriStem® XF for 3-5 passages prior to trilineage differentiation using commercial differentiation formulations. Representative images of stained adipocytes (Oil Red O), osteocytes (Alizarin Red) and chondrocytes (Alcian Blue). **hMSC cultured in MSC NutriStem® XF maintain their multilineage differentiation potential.**

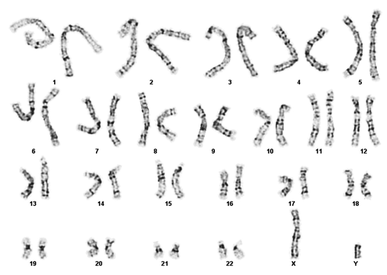
Figure 11: Immunophenotyping of hMSC-AT

	Seeding concentration [cells/cm²]							
	6000		5000		3000		1000	
	FBS containing medium	MSC NutriStem® XF	FBS containing medium	MSC NutriStem® XF	FBS containing medium	MSC NutriStem® XF	FBS containing medium	MSC NutriStem® XF
CD 34	0.31	0.91	0.16	0.3	0.52	1.32	0.17	0.48
CD 45	0.35	0.5	0.1	0.07	0.4	0.18	0.08	0.08
HLA-DR	1.54	0.3	0.91	0.11	7.16	0.16	2.27	0
CD 34+45+ HLA-DR	0.73	0.57	0.39	0.16	2.69	0.55	0.84	0.19
CD 73	99.96	99.97	99.96	99.98	99.52	99.76	99.94	99.96
CD 90	99.98	99.99	99.97	99.9	99.94	99.6	99.96	99.95
CD 105	99.99	100	100	99.98	100	100	100	99.96
CD 44	99.93	100	99.98	99.94	99.8	99.8	99.6	99.62

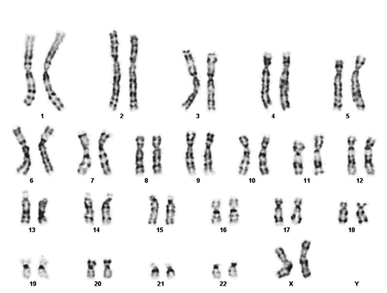
Comparison FACS analysis results of hMSC-AT cultured for 3 passages in different seeding concentrations using SF, XF culture medium (MSC NutriStem® XF) vs. FBS-containing medium. **hMSC cultured in MSC NutriStem® XF maintain a classical profile of MSC markers with a lower percentage of hematopoietic contamination in all tested seeding concentrations.**

Figure 12: Genomic stability of hMSC

hMSC-BM
46,XY



hMSC-AT
46,XX



G-banding karyotyping analysis of hMSC-BM and AT expanded for 4 passages in MSC NutriStem® XF. **hMSC cultured in MSC NutriStem® XF maintain genomic stability.**

Summary

- hMSC from various sources can be efficiently isolated using MSC NutriStem® XF supplemented with 2% human AB serum (AT, BM) or w/o (PL).
- A higher number of hMSC was obtained after isolation using MSC NutriStem® XF in comparison to FBS-containing medium.
- Using MSC NutriStem® XF for isolation of hMSC enhances purity of MSC population in earlier passages (decreasing hematopoietic contamination) compared to initial isolation using FBS-containing medium.
- The highest proliferation rate of hMSC from a variety of sources was achieved using MSC NutriStem® XF in comparison to other commercially available SF media.
- MSC NutriStem® XF supports long-term culture of hMSC from a variety of sources.
- hMSC cultured in MSC NutriStem® XF retain the essential MSC characteristics (fibroblast-like morphology, surface markers phenotype, multilineage differentiation, self-renewal potential, and genomic stability).

The developed XF culture system (MSC NutriStem® XF medium, MSC Attachment Solution, MSC Dissociation Solution, Recombinant Trypsin Solution, MSC Freezing Solution) supports the initial isolation and long-term expansion of hMSC from various sources, suitable for cell therapy and tissue enginnering.

Acknowledgements:

* We would like to thank Professor Mark L. Weiss, Kansas State University, Department of Anatomy and Physiology, Manhattan, KS, for his invaluable contribution to this study.

* We would like to thank Kasiak Research Pvt. Ltd., Maharashtra, India, for their cooperation in this study

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Short Communication

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Regenerative Medicine

Monocytes recruitment blocking synergizes with mesenchymal stem cell transplantation for treating myocardial infarction

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Background: Mesenchymal stem cell (MSC) transplantation is a promising therapeutic approach for acute myocardial infarction (AMI), however, research to date has demonstrated unsatisfactory results. **Materials & methods:** An AMI mouse model was established via left coronary artery ligation. AMI mice were treated with MSCs, anti-CCR2 or MSCs + anti-CCR2 and the effects of each treatment group were compared. Macrophage infiltration was analyzed by immunofluorescence staining and flow cytometry. **Results:** Implantation of MSCs + anti-CCR2 yielded a greater improvement in cardiac function and significantly reduced macrophage accumulation in the infarct site of AMI mice compared with the injection of MSCs or anti-CCR2 alone. Moreover, reduced macrophage infiltration was accompanied by reduced pro-inflammatory cytokine secretion in the injury sites and the low inflammatory response favored tissue regeneration. **Conclusion:** Treatment with MSCs and anti-CCR2 in combination may be a promising therapeutic strategy for AMI.

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Keywords: acute myocardial infarction • C-C chemokine receptor type 2 • inflammation • macrophages • mesenchymal stem cells

Acute myocardial infarction (AMI), a type of myocardial damage caused by acute and persistent ischemic hypoxia, leads to severe impairment of cardiac function [1]. Studies have found that myocardial infarction results in high levels of myocardial cell death in the infarcted area, which is eventually replaced by fibrous tissue [2]. Current interventions include drug interventions and surgical treatments, however, none of these strategies can restore heart scar tissues into normal myocardial tissues [3]. The most common and effective treatment for AMI is myocardial reperfusion, however, this can induce myocardial injury and cause further heart failure [4,5]. Thus, novel therapies for AMI are urgently needed.

The concept of using mesenchymal stem cells (MSCs) to treat AMI or myocardial infarction (MI) has gained increasing attention in recent years [6]. MSCs are multipotent progenitor cells with the capacity to differentiate into various types of cell, including macrophages and cardiomyocytes [7,8]. MSCs are reported to contribute to the remodeling of the microenvironment of infarct sites, which is beneficial for promoting the regeneration process and neovascularization [8]. Besides this, MSCs can be easily isolated from bone marrow (BM) or adipose tissues and further expanded *in vitro*. MSCs are immunologically tolerant and can be used for allogeneic cell therapy. Importantly, numerous preclinical studies have demonstrated that the transplantation of MSCs into the left ventricle of the heart facilitates cardiomyocyte repopulation at the infarct area, ameliorates heart injury and improves heart function [9]. However, the promising results from animal studies of MSC transplantation cannot be fully translated to human patients with MI [10], one of the reason being due to an excessive inflammation response. Studies have shown that after MI occurs, a large number of inflammatory cells, such as macrophages and monocytes, infiltrate the infarct sites, creating a severe local inflammatory environment, inducing cardiomyocyte cell death and extending ischemic injury. Migration of immune cells to the infarct site and reduction of inflammation can slow the deterioration of the infarction and promote the repair of damaged myocardial tissue [11].

Future Medicine

Targeting inflammatory response-associated proteins may be a good strategy in order to overcome these obstacles. Toll-like receptors (TLRs) play a key role in the regulation of cell death and pro-inflammatory cytokines expression. Genetic or pharmacological inhibition of TLRs, such as TLR2 or TLR4, strongly reduces MI size and alleviated adverse left ventricular remodeling consequences [12,13]. Similarly, direct targeting of IL-1β, a pro-inflammatory cytokine, can protect heart tissues from cardiac damage following AMI [14]. C-C chemokine receptor type 1 (CCR1), a G protein-coupled receptor, is engaged in immune cell homing. It should be noted that CCR1 is highly expressed on the injured myocardium but not on MSCs, and it has been demonstrated that the depletion of CCR1 reduces inflammatory recruitment and enhances tissue repair after MI [15]. CCR2 is an important mediator in a variety of cell recruitment mechanisms. In contrast to CCR1, CCR2 is expressed in both MSCs and heart tissues [16,17]. We hypothesized that the blockade of CCR2 may prevent excessive immune cell accumulation in the infarct sites, which may be beneficial for tissue healing. To the best of our knowledge, preclinical data on the effects of injecting a combination of MSCs and anti-CCR2 on acute MI is still lacking. In the current study, we aimed to address this question experimentally in a mouse model.

Materials & methods

Animal model construction

6- to 8-week-old male C57BL/6 mice were used in the experiments. Animal experimental protocols were approved by the ethics committee of Changhai Hospital, Naval Medical University and were performed in adherence to the National Institutes of Health Guidelines on the Use of Laboratory Animals. Mice were anesthetized with an intraperitoneal injection of ketamine (75 mg/kg) and xylazine (5 mg/kg). Ketamine hydrochloride (Cat. No. K-101) and xylazine hydrochloride (Cat. No. X-101) were purchased from Sigma-Aldrich (MO, USA). The mice were intubated with an endotracheal tube and ventilated with room air at a rate of 60 cycles/min, and tidal volume 1 ml per 100 g of body weight. A left thoracotomy was performed to expose the heart, and the proximal left anterior descending coronary artery was ligated. 60 min after the experiment, the MI mice were randomly divided into five groups: sham group; phosphate-buffered saline (PBS) control group: mice were injected with PBS; MSC group: mice were injected with MSCs (1×10^6 cells) at two sites near the border zone of infarction (medial and lateral zones) 1 h post MI accomplishment; anti-CCR2 group: mice were intravenously injected with anti-CCR2 antibody (2.5 mg/kg) every day for 3 days; combination group: mice were injected with MSCs and then received anti-CCR2 antibody administration. The anti-CCR2 antibody (Clone 8C3.1) was purchased from Merck Millipore (MA, USA). After the experiment, the mice were allowed to recover under appropriate care. Seven days postinjection, the infiltration macrophage population in the infarct area of the heart was determined by flow cytometry analysis of F4/80 and CD11b double-positive population.

Echocardiography & heart function assessment

A VisualSonics 770 echocardiography machine was applied to take the M-mode images of mice. The MI mice's hearts were observed in the short-axis between the two papillary muscles and each measurement was determined by the average data of three sequential heartbeats. The left ventricular ejection fraction (LVEF) was determined using the ultrasonic diagnostic equipment. To measure the left ventricular end-diastolic pressure (LVEDP) and rate of the ventricular pressure rise (+dp/dtmax), mice were anesthetized and a micro-catheter with a pressure transducer was inserted into the left ventricule through right carotid arteries. Then a multichannel physiological recorder was used to measure and record LVEDP and +dp/dtmax.

BM-MSC isolation & expansion

The isolation and expansion of bone marrow-derived MSCs (BM-MSCs) were performed as previously described [18]. The bone marrow was gently flushed out from the bone (tibias, femurs and humeri) cavity. The cells from bone marrow were washed twice and were filtered through a 70-um cell strainer (Corning, NY, USA). The mononuclear cells were cultured in Iscove's Modified Dulbecco's Medium suppled with 10% fetal bovine serum, penicillin and streptomycin (Gibco, NY, USA) at 37°C in a 5% CO_2 incubator. On day three, the cells appeared spindle-shaped and reached approximately 90% confluence. The cells were split at a 1–3 and cultured in fresh complete Iscove's Modified Dulbecco's Medium for further expansion. Cell passaging was performed every 4–6 days when the cell confluence reached 90%. Cells were ready for experiments after three passages. The BM-MSCs were subjected to cell characterization using flow cytometry analysis for biomarkers and microscopic analysis for cell morphology.

Immunofluorescence staining

Tissue samples were embedded in optimal cutting temperature (Tissue-Tek; Sakura Finetek, Torrance, CA, USA) and snap-frozen at -20°C. The tissue blocks were cut into 10-µm-thick sections. The tissue sections were washed, blocked using 5% bovine serum albumin, and incubated with anti-F4/80 antibody (Abcam, Cat. No. ab6640). After that, the tissue sections were washed and further incubated with a fluorescein (FITC)-conjugated secondary antibody (BioLegend, CA, USA). Nuclear DNA was stained using 4',6-diamidino-2-phenylindole (Thermo Fisher, MD, USA). The dilution of antibodies used for immunofluorescent staining was 1:400.

Flow cytometry assay

Cells were resuspended in 100 µl cold FACS buffer (PBS with 2% fetal bovine serum and 1 mM EDTA). Appropriate antibodies were added into the cell suspension for 30 min on ice. Cells were washed twice using cold FACS buffer and the cell population was analyzed using BD Accuri™ C6 Plus Cell Analyzer (BD Biosciences, CA, USA). All antibodies (allophycocyanin [APC]-conjugated anti-CD90, APC-conjugated anti-CD44, APC-conjugated anti-CD29, APC-conjugated anti-CD34, APC-conjugated anti-CD45, APC-conjugated anti-F4/80 and FITC-conjugated anti-CD11b) for flow cytometry were purchased from BioLegend. The dilution of antibodies used for flow cytometry analysis was 1:400.

Real-time quantitative PCR analyses

Total RNA was extracted with the use of TRIZOL reagent (Invitrogen, MA, USA) and treated with DNase I. The cDNA was generated from RNA using the SuperScript II Reverse Transcriptase Kit (Invitrogen). For gene expression analysis, the real-time quantitative PCR (RT-qPCR) were performed using SYBR Green PCR master mix (Applied Biosystems, MD, USA). The RT-qPCR was performed on an ABI 7500 instrument (Life Technology, CA, USA). The relative expression levels of target genes were normalized to *GAPDH*. The primer sequences are as following: *IL-6* sense 5'-CTGATGCTGGTGACAACCAC-3', *IL-6* antisense 5'-CAGACTTGCCATTGCACAAC-3'; *IL-1β* sense 5'-AAGCCTCGTGCTGTCGGAC-3', *IL-1β* antisense 5'-TGAGGCCCAAGGCCACAGGT-3'; *TNF-α* sense 5'-CATCTTCTCAAAATTCGAGTGACAA-3', *TNF-α* antisense 5'-CCAGCTGCTCCTCCACTTG-3'.

Statistical analysis

All data are presented as mean ± standard deviation. Comparisons between groups were assessed by one-way ANOVA with a Tukey's *post hoc* test where appropriate. $p < 0.05$ was considered statistically significant.

Results

Differentiation of BM-MSCs *in vitro*

In order to study the effect of BM-MSCs on AMI, they were first cultured and characterized. As illustrated in Figure 1A–E, the isolated cells were almost 100% positive for CD90, CD44 and CD29 (three MSC markers), while negative for CD34 (endothelial marker) and CD45 (hematopoietic marker). All the adherent cells exhibited uniform spindle-shaped and fibroblast-like morphology (Figure 1F).

Enhanced macrophages infiltration in the infarct area

Studies have shown that the infiltration of monocyte-derived macrophages into the infarct region is critical for cardiac-tissue repairing [9]. To confirm this phenotype, we evaluated the macrophage infiltration in the infarct area using immunofluorescence microscope analysis. Consistent with other observations, we also found that the population of macrophages, as demonstrated by F4/80 positive staining, were significantly elevated in the infarct region in murine models 1 day and 3 days postinfarction (Figure 2A & B).

Anti-CCR2 & MSC injection reduced macrophage accumulation in the infarct sites

It has been well-established that BM-MSC transplantation can improve the heart function in the mouse model of AMI [8]. To investigate whether the blockade of CCR2 can further improve the effect of MSCs on AMI, the mouse model of AMI was first established via left coronary artery ligation. After that, the AMI mice were injected with PBS, MSCs, anti-CCR2 antibody or a combination of MSCs and anti-CCR2 antibody (Figure 3A). The sham mice were used as a normal control group. The results showed that a dramatic increase of infiltrated macrophages was observed in the heart of AMI mice with PBS injection compared with normal healthy mice (Figure 3B & C). Noticeably, the injection of MSCs, anti-CCR2 antibody or BM-MSCs and anti-CCR2 antibody in combination

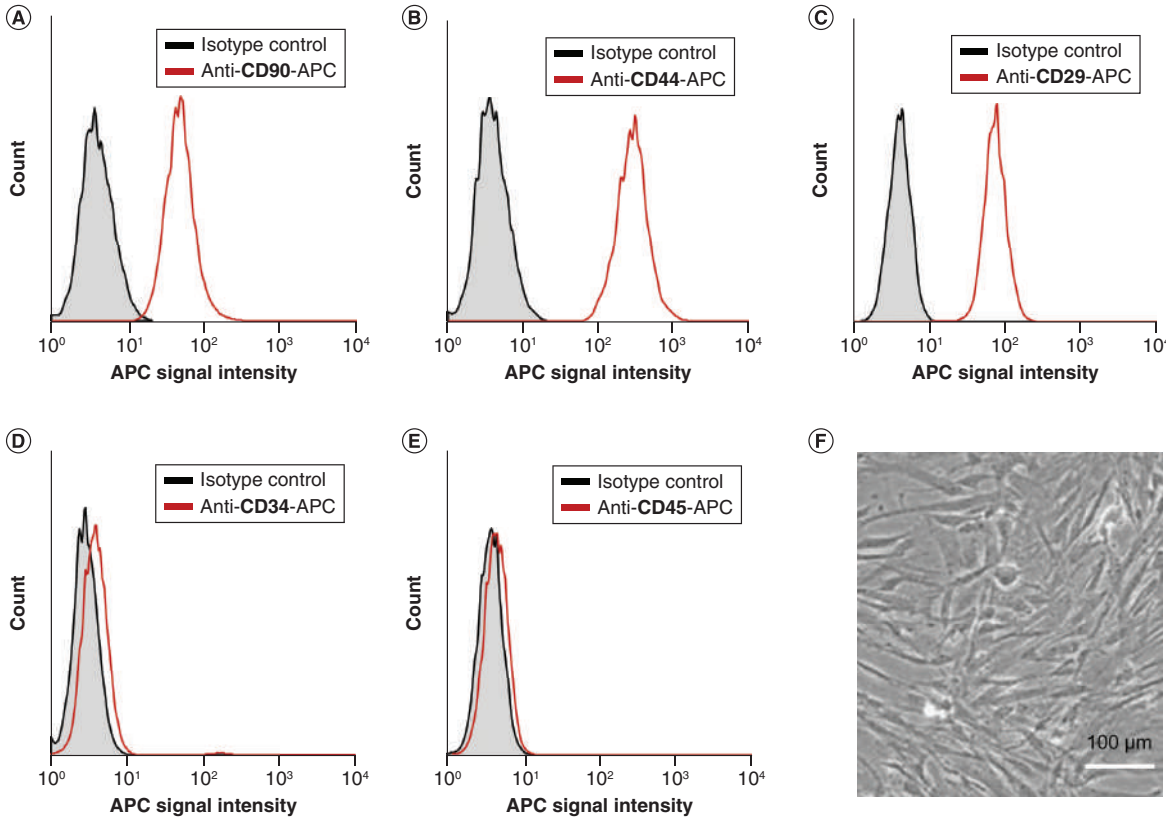

Figure 1. Characterization of bone marrow-derived mesenchymal stem cells. (A–E) Immunophenotypic profile of BM-MSCs. Flow cytometry histograms after three passages show the expression of selected surface molecules (including CD90, CD44, CD29, CD34 and CD45). Cells were stained with APC-labeled antibodies or isotype control antibody. The BM-MSCs were positive for CD90, CD44, and CD29 but negative for CD34 and CD45. **(F)** Morphology of murine BM-MSCs in culture. The majority of MSCs exhibit spindle or triangular shapes. Scale bar = 100 μm.
APC: Allophycocyanin; BM-MSC: Bone marrow-derived mesenchymal stem cell.

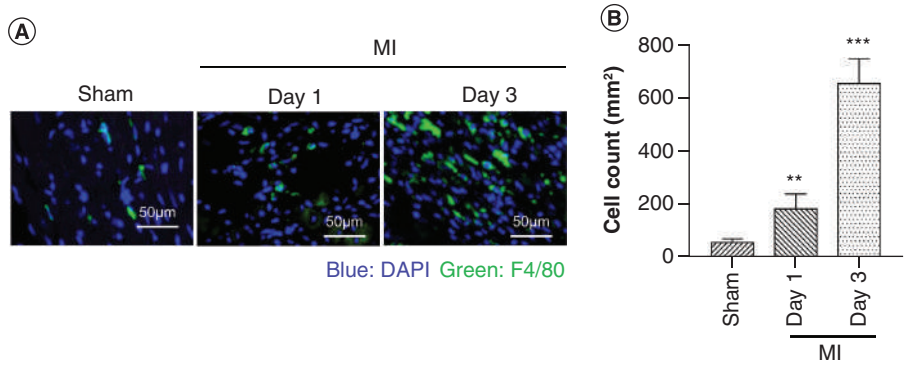

Figure 2. Macrophages were significantly increased in the infarct area in myocardial infarction mice. (A) Immunofluorescence revealed that the number of F4/80 macrophages was primarily increased in the infarct area on day 1 and day 3. Scale bars: 50 μm. **(B)** The numbers of F4/80$^+$ cells in infarct area were counted in the immunohistochemical samples described above.
**$p < 0.01$; ***$p < 0.001$, versus sham groups.
MI: Myocardial infarction.

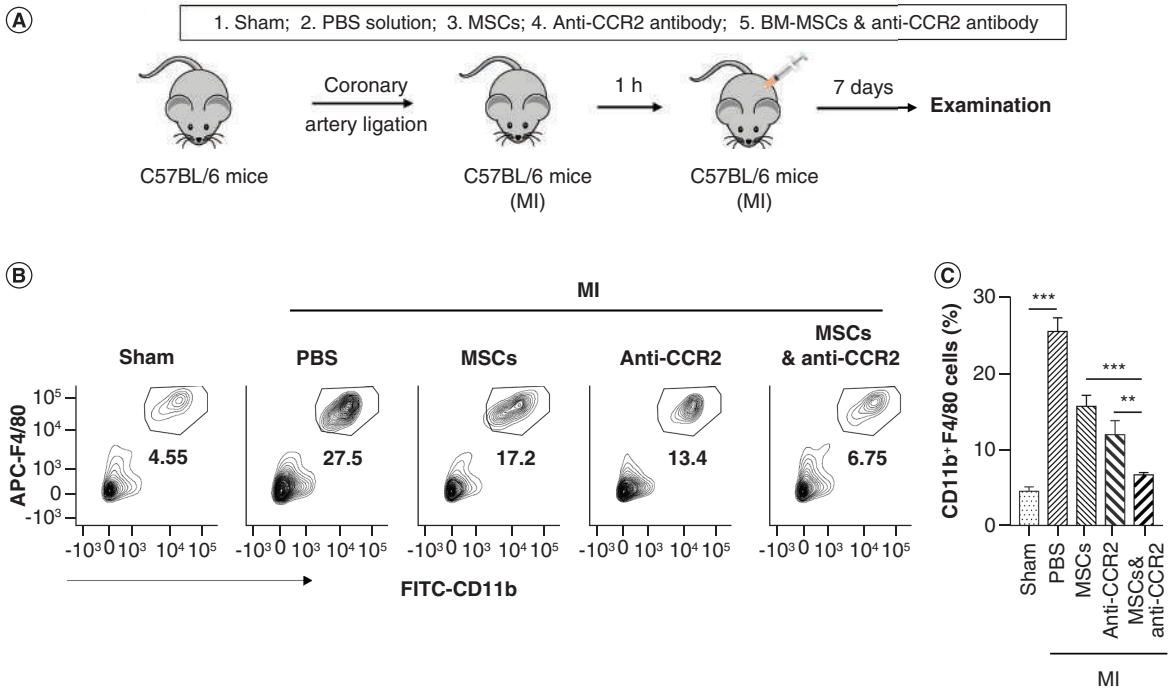

Figure 3. C-C chemokine receptor type 2 antibody suppresses infiltration of inflammatory cells in the myocardium of Mesenchymal stem cell transplanted mice. (A) The schematic depicts the experiment protocol. Mouse models of MI were established via coronary artery ligation. The MI mice were set into five groups: sham group; PBS group: mice were injected with PBS; MSCs group: mice were injected with MSCs at two sites near the border zone of infarction (medial and lateral zones) 1 h post MI accomplishment; anti-CCR2 group: mice were intravenously injected with anti-CCR2 antibody every day for 3 days; injection dose was 2.5 mg/kg. Combination group: mice were transferred with MSCs and then received anti-CCR2 antibody administration. **(B)** Flow cytometry analysis of infiltration of $CD11b^{+}F4/80^{+}$ cells peri-infarct area in the heart. Tissues from different groups were digested into single cells stained with FITC labeled CD11b and APC labeled F4/80 antibodies and then examined by flow cytometry. **(C)** Bar graph shows quantitative analysis of infiltrating F4/80+ cells. Data represent means ± SD.
$^{**}p < 0.01$; $^{***}p < 0.001$, n = 5.
APC: Allophycocyanin; BM-MSC: Bone marrow-derived mesenchymal stem cell; CCR2: Chemokine receptor type 2; FITC: fluorescein; MI: Myocardial infarction; MSC: Mesenchymal stem cell; PBS: Phosphate-buffered saline; SD: Standard deviation.

resulted in a significant decrease in the infiltration macrophage population in the myocardium of MI mice. The infiltration of macrophages was significantly reduced in the combination treatment group (MSCs and anti-CCR2 antibody) compared with single (MSCs or anti-CCR2 antibody) treatment groups.

Anti-CCR2 & MSC administration decreased pro-inflammatory cytokine expression in the injured sites

We further determined the expression levels of pro-inflammatory cytokines (IL-6, TNF-α and IL-1β) in the myocardium from the five groups of mice using the real-time PCR assay. Figure 4A–C showed that the expression levels of IL-6, TNF-α and IL-1β were markedly increased in AMI mice with PBS injection compared with healthy mice, respectively. The injection of either MSCs or anti-CCR2 antibody resulted in a remarkable decrease of IL-6, TNF-α and IL-1β expression. More importantly, the combination treatment (MSCs and anti-CCR2 antibody) yielded the most dramatic reduction of IL-6, TNF-α and IL-1β expression in the myocardium in AMI mice when compared with those in single (MSCs or anti-CCR2 antibody) injection AMI mice.

Anti-CCR2 & MSC injection improved cardiac function

The cardiac functions in the five groups of mice were measured using echocardiographic analysis (Figure 5A). The results demonstrated that the LVEF and +dp/dtmax were significantly decreased (Figure 5B & C), whereas, the LVEDP was markedly increased in AMI mice with PBS injection when compared with those in healthy mice (Figure 5D), suggesting that the cardiac function in AMI mice was significantly impaired. We found that MSCs or

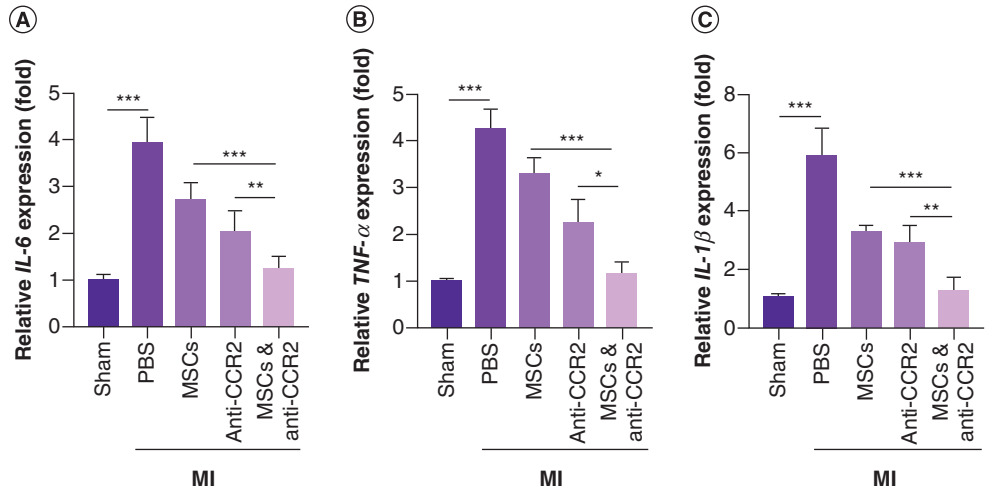

Figure 4. Chemokine receptor type 2 antibody suppresses the infiltration of expression of proinflammatory cytokines in the myocardium in mesenchymal stem cell transplanted mice. (A–C) Quantitative analysis of mRNA expression of proinflammatory cytokines and chemokines IL-1β, TNF-α and IL-6 in the border zone of infarct at 1 week post-MI. mRNA expression normalized to GAPDH expression. Data represent means ± SD.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, $n = 8$.
MI: Myocardial infarction; MSC: Mesenchymal stem cell; PBS: Phosphate-buffered saline; SD: Standard deviation.

anti-CCR2 antibody treatment can partially improve the cardiac function in AMI mice as seen through the partially restored values of LVEF, +dp/dtmax and LVEDP. Of note, the combination treatment (MSCs and anti-CCR2 antibody) surprisingly restored the values of LVEF, +dp/dtmax and LVEDP to nearly normal levels, which was a significant improvement compared with MSCs or anti-CCR2 antibody treatment alone.

Discussion

Tissue-resident macrophages exist in various tissues and organs throughout the human body and play a critical role in maintaining the normal function of the organs, such as Kupffer cells in the liver, microglia in the brain and cardiac macrophages in the heart [19]. These resident macrophages usually lose the ability of proliferation. The infiltrating macrophages, such as BM-MSCs, play a predominant role in tissue repair [8,9].

It has been reported that the cardiac resident macrophages can be divided into two distinct lineages based on the expression of CCR2. Studies have suggested that both cell populations (CCR2+ and CCR2- macrophages) orchestrate the cardiac tissue repair after MI [20]. The CCR2+ macrophages facilitate monocyte trafficking into the heart tissue and produce high levels of IL-6, TNF-α and IL-1β to induce inflammatory responses. Conversely, the CCR2- macrophages secrete high levels of growth factors, such as VEGF and IGF1 to promote tissue regeneration [16,21,22]. Thus, researchers have proposed that targeting CCR2-macrophages may be an ideal strategy to combat MI [23]. For example, early work using small molecular inhibitors to block the migration of CCR2+ macrophages has shown promising results *in vitro* but failed *in vivo* due to low potency in specific inhibition of CCR2 in the heart [24]. Later, Leuschner *et al.* reported that applying siRNA-mediated CCR2 silencing technology efficiently decreased CCR2 expression in monocytes and prevented macrophages accumulation in the inflammation sites [25]. The same group further reported another finding that targeting CCR2 using siRNA attenuated infarct inflammation and improved left-ventricular healing [26]. More importantly, systemic administration of anti-CCL2 neutralizing antibodies reduced macrophage mobilization and infiltration into the tumor tissues, leading to the suppression of tumor growth and metastasis [27]. Anti-CCL2 antibody therapy has also demonstrated beneficial effects in protecting the lung from injury in mice through the reduction of infiltrated monocyte/macrophages into the lung [28]. These data provide initial evidence that targeting CCR2 yielded promising results for MI treatment.

Although macrophages are heterogeneous populations, they can change their phenotype and function based on different stimuli. Macrophages can be broadly classified into M1-type or M2-type macrophages [29]. M1-type macrophages are associated with the pro-inflammatory response because they secrete a variety of pro-inflammatory cytokines, including IL-6, TNF-α, IL-12 and IL-1β. On the contrary, M2-type macrophages exhibit anti-inflammatory properties through the secretion of anti-inflammatory factors such as IL-10, VEGF and TGF-β [30].

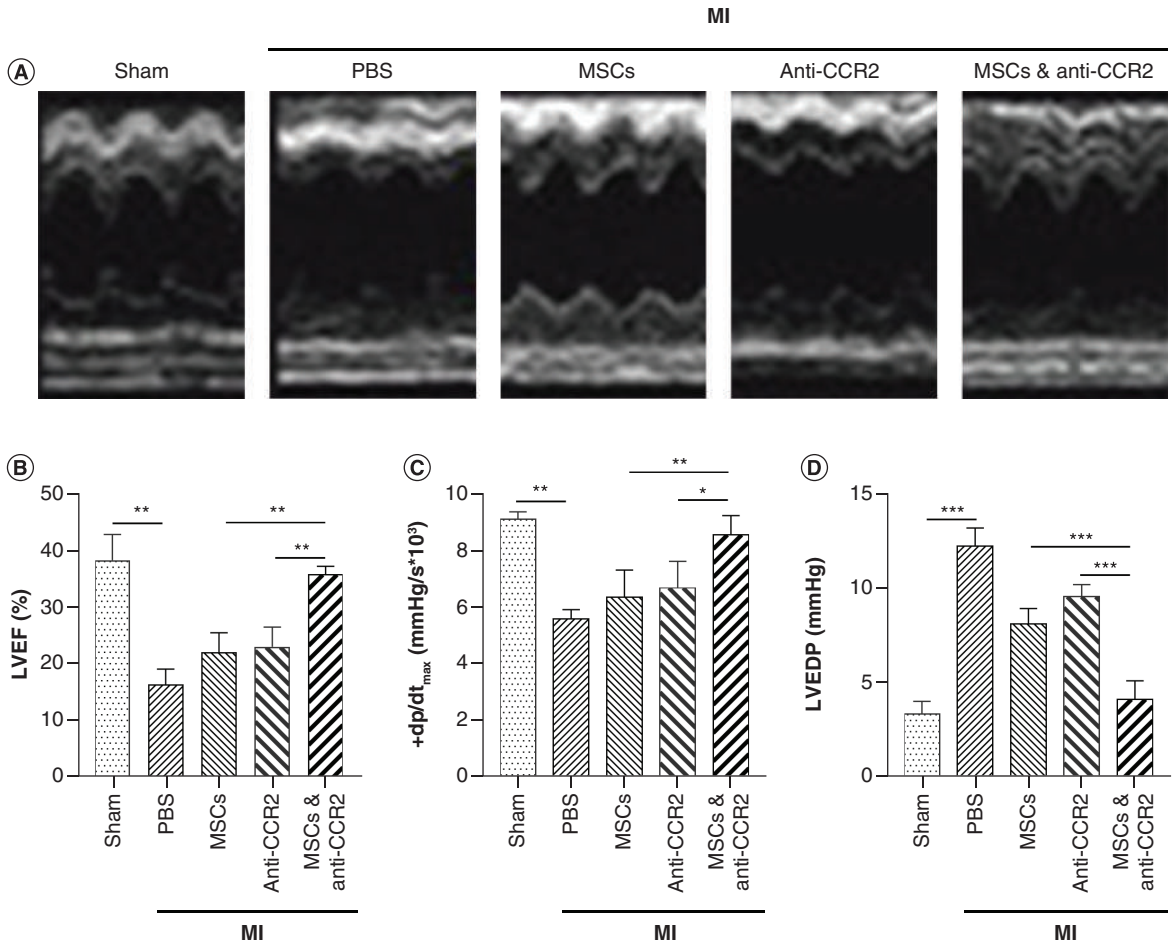

Figure 5. Monocyte recruitment blocking synergizes with mesenchymal stem cell transplantation for improving cardiac functional recovery in the mouse model of myocardial infarction. (A) Representative echocardiographic images 4 weeks after MI in different groups. **(B)** Left ventricular ejection fraction. **(C–D)** Hemodynamic analyses including $+dp/dt_{max}$ and left ventricular end diastolic pressure. Data represent means ± SD.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, $n = 5$.
MI: Myocardial infarction; MSC: Mesenchymal stem cell; PBS: Phosphate-buffered saline; SD: Standard deviation.

Upon the induction of AMI, the resident cardiac macrophages suffer from ischemia-induced cell damage. The monocyte-derived macrophages infiltrate into the ischemic region, further polarized into M1-type of macrophages, which play a major role in cleaning the debris and dead cells and producing pro-inflammatory cytokines [31]. However, studies have shown that the upregulated pro-inflammatory cytokine levels and overactivation of the inflammatory response in the heart tissue were associated with worsening outcome in MI patients. The excessive and consistent inflammatory response may impose stress on cardiac tissues, which may instigate cardiomyocyte apoptosis and disrupt cardiac tissue remodeling, ultimately leading to heart failure [32]. Indeed, inhibition of TNF-α or IL-1β resulted in improved cardiac function in animal models of heart failure [33,34].

Conclusion

In our study, we demonstrated that the injection of either MSCs or anti-CCR2 only partially, while injection of the combination of MSCs and anti-CCR2 substantially decreased macrophage infiltration and pro-inflammatory cytokine expression in the infarct area, as well as improved cardiac function. Our results were consistent with earlier findings that a persistent inflammation reaction contributes to worse outcomes following AMI.

Future perspective

The present study demonstrated that the MSCs + anti-CCR2 combination injection exerted beneficial effects on improving heart recovery and function through reducing the inflammatory reaction in infarct sites. Although the current results are encouraging, some critical questions still need to be addressed in future studies. For example, what is the underlying molecular mechanism of how the MSCs + anti-CCR2 combination treatment reduced the expression of IL-6, TNF-α and IL-1β expression? Can our animal experiment results be translated into human clinical trials? What is the safe dosage range of MSCs + anti-CCR2 combination injection for patients with MI? Successfully addressing these questions would profoundly contribute to the advancement of the treatment and management of patients with MI. Currently, we set out experiments to investigate the effect of MSCs and a serial dose of anti-CCR2 on the inflammatory responses and infarct size of the mouse with AMI. We also aim to explore the alternation of several transcriptional proteins in the heart tissue, such as NF-κB, and STAT1/3, which are being reported to play pivotal roles in the regulation of innate immune response and cell death (e.g., apoptosis and necroptosis).

Summary points

- There is an increase of infiltrating macrophages and inflammatory responses in cardiac infarct area.
- The transplantation of bone marrow-mesenchymal stem cells in supplement with antichemokine ligand 2 decreased macrophage accumulation and pro-inflammatory cytokine secretion.
- Injection of bone marrow-mesenchymal stem cells plus antichemokine ligand 2 mitigates infarct size and improves cardiac function.

Financial & competing interests disclosure

The study was supported by the National Natural Science Foundation of China (81570208). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

Animal experimental protocols were approved by the ethics committee of Changhai Hospital, Naval Medical University and were performed in adherence to the National Institutes of Health Guidelines on the Use of Laboratory Animals.

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Research Article

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Regenerative Medicine

In vitro potential of human mesenchymal stem cells for corneal epithelial regeneration

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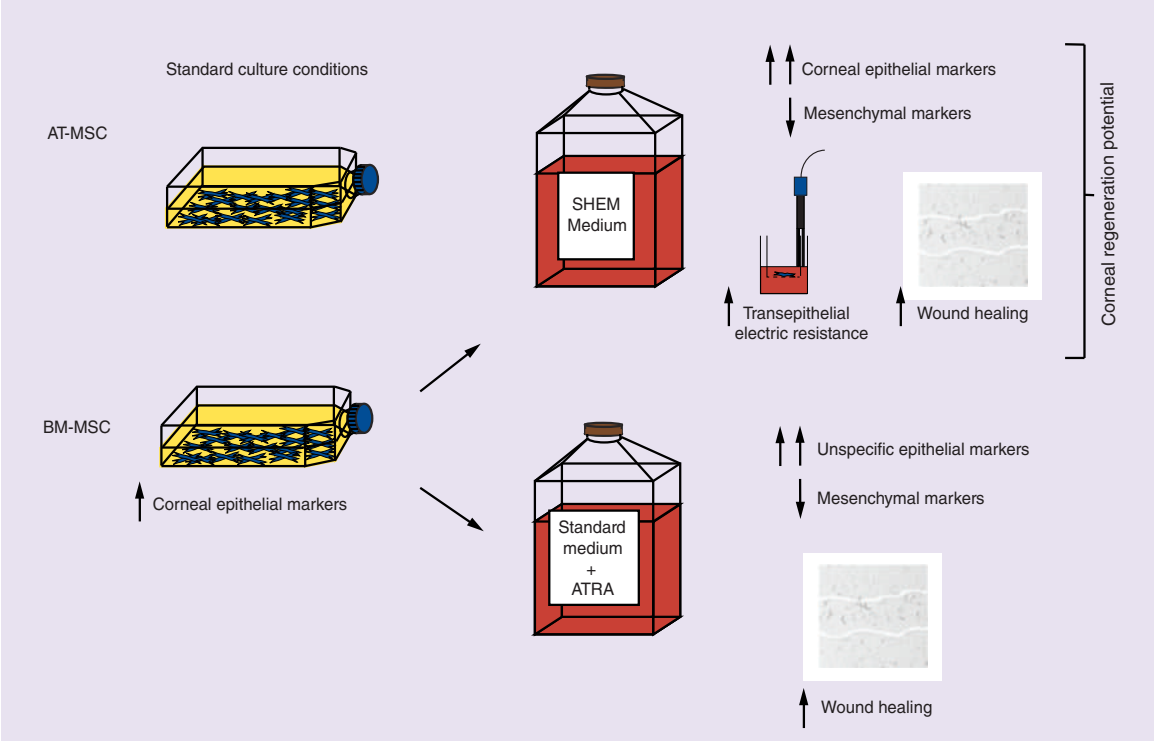
Aim: To determine the potential of mesenchymal stem cells (MSC) for corneal epithelial regeneration *in vitro*. **Materials & methods:** Bone marrow MSC (BM-MSC) and adipose tissue MSC were analyzed for corneal epithelial and mesenchymal markers, using limbal stem cells and corneal cells as controls. MSC with better potential were cultured with specific mediums for epithelial induction. Transepithelial electric resistance and wound healing assay with human corneal epithelial cells were performed. **Results:** BM-MSC showed better potential, increased corneal markers, and higher transepithelial electric resistance values when induced with limbal epithelial culture medium. Induced BM-MSC promoted better wound healing of human corneal epithelial cells by paracrine secretion. **Conclusion:** BM-MSC has potential for corneal epithelial induction in a protocol compatible with human application.

Graphical abstract:



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Future Medicine

Keywords: cell therapy • corneal epithelial differentiation • cytokeratins • limbal stem cell deficiency • limbal stem cells • mesenchymal stem cells • wound healing

The transparency and integrity of the cornea is maintained by tissue-specific stem cells called limbal stem cells (LSC). LSC are found in the basal epithelial layer of the limbic region (limbus), an anatomic boundary between the conjunctiva and the transparent cornea which comprises a circumferential area measuring 1.5 mm wide. LSC divide and generate differentiated progenies that renew the corneal epithelium [1]. During this differentiation process, the cells migrate from the limbic region to the central cornea and acquire new markers, such as cytokeratin (CK)12 and CK3, specific to differentiated corneal epithelium [2].

Partial or complete depletion of LSC – limbal stem cell deficiency (LSCD) – causes loss of transparency, chronic ulcerations, inflammation and conjunctivalization of the corneal surface because the corneal epithelium cannot be renewed. This impairs visual acuity and leads to blindness [3]. LSCD affects approximately 10 million people worldwide [4,5], and has several etiologies, including genetic and infectious factors, but the most common cause is environmental aggression, such as chemical burns [6]. In fact, ocular chemical burns represent 7.7–18% of all ocular traumas [7] and their prevalence has increased due to the use of corrosive household cleaners.

The current gold standard treatment for unilateral LSCD is cultivated limbal epithelial transplantation (CLET) with autologous LSC. LSC are collected from a minimally invasive limbal biopsy of the patient's healthy eye, expanded *in vitro*, and transplanted onto the damaged eye in a biocompatible carrier. This cell therapy approach has become well established since its first use in 1997 [3]. However, treatment of bilateral LSCD is a clear challenge because it depends on allogeneic transplantation and requires systemic immunosuppression to avoid immune rejection [8]. Since systemic immunosuppression can lead to adverse effects [9], it raises the need for alternative treatments.

In this scenario, mesenchymal stem cells (MSC) emerge as a promising source of stem cells because of their multiple properties. First, MSC differentiate toward various lineages *in vitro* [10]. They have the potential to express both corneal epithelial [11] and epithelial cell markers [12,13], implying an intrinsic potential for corneal epithelial-like or epithelial-like differentiation [14,15] and thus providing an alternative source of stem cells for LSCD transplantation. Second, MSC are used in regenerative medicine to treat conditions such chronic cutaneous ulcers [16,17], because they secrete paracrine trophic factors that accelerate the wound healing process through, enhancing the survival, proliferation and migration of resident cells, as well as suppressing the inflammation, among other actions [18]. Third, MSC do not express major histocompatibility complex DRII (HLA DRII) and are invisible to the immune system, preventing graft rejection [19,20]. Fourth, MSC from bone marrow or adipose tissue can be easily obtained because they are somatic stem cells, avoiding the ethical conflicts that arise with clinical application of embryonic stem cells. Last but not least, unlike induced pluripotent stem cells (IPSC), MSC are not linked to chromosomal instability or oncogenic transformation [21,22].

For these reasons, MSC have attracted attention in the treatment of LSCD, since they could serve as an alternative stem cell source for LSC and/or could help in wound repair and corneal regeneration in cases of bilateral LSCD. To date, MSC have been shown to be safe and effective in human application when compared with allogeneic LSC transplantation [23]. In view of these promising results, there is a need to study and optimize culture conditions suitable for clinical application, in order to increase the differentiation potential and regenerative capabilities of MSC.

In the present study, we explored the potential of human MSC for corneal epithelial regeneration by analyzing protocols to increase the expression of corneal epithelial markers and evaluating their wound repair potential. To this end, we compared and characterized adipose tissue MSC (AT-MSC) and bone marrow MSC (BM-MSC) for corneal, limbal, and epithelial cell markers, using human LSC and epithelial corneal cells as controls for expression. Then, we optimized a protocol compatible with human clinical application to induce corneal epithelial-like characteristics in MSC. We carried out an extensive analysis of epithelial corneal (CK12 and CK3), epithelial (CK19, pan-CK, CK15, ITGB1) and mesenchymal (α-smooth muscle actin [α-SMA], vimentin [Vim], snail) markers using several approaches, including real-time quantitative PCR (qPCR), western blot and immunofluorescence. We also evaluated the role of the Wnt/β-catenin signaling pathway, which is considered crucial during MSC differentiation into epithelial cells [24]. Moreover, we proved epithelial differentiation induction with a functional assay by transepithelial electrical resistance (TEER) measurements and also evaluated repair capabilities in an *in vitro* wound healing assay using human corneal epithelial cells (HCE).

Materials & methods

Cells

Informed consent for the use of human tissue for experimental purposes was obtained in accordance with the Declaration of Helsinki. Any active transmissible infections were excluded by serologic analyses.

BM-MSC were obtained from five healthy donors through iliac crest BM aspiration. Mononuclear fraction was selected using the Ficoll method and BM-MSC were isolated as described elsewhere [25]. AT-MSC from fresh human lipoaspirates were obtained by stromal vascular fraction isolation and cultured as previously described [26]. Human AT aspirates were collected from three healthy donors using plastic liposuction procedures, during planned lipoaspiration surgery.

MSC were cultured in MSC medium composed of α Minimum Essential Medium with Glutamax™ (αMEM-GT, Invitrogen, CA, USA) supplemented with 10% fetal calf serum (FCS, Invitrogen) and 1% antibiotics. MSC from each donor were characterized by flow cytometry analysis (data not shown) to assure MSC characterization guidelines accomplishment [27]. After the isolation passage, 5×10^5 MSC from each donor were pooled and used for characterization by flow cytometry, multilineage differentiation and experiments.

Adult human corneas and limbal tissue from different donors were obtained from the Ocular Tissue Bank of the Barcelona (BST-GenCat, Barcelona, Spain; www.bancsang.net/en_index/) and the Centro de Oftalmología Barraquer (Barcelona, Spain; www.barraquer.com/en/). LSC were isolated according to previous protocols [28] and cultured with supplemented hormonal epidermal medium (SHEM) consisting of: DMEM/Ham's F-12 (2:1 vol:vol) mixture (DMEM/F12; Invitrogen) supplemented with 2 mM L-Glutamine (Lonza, Verviers, Belgium), 5 μg/ml insulin (Sigma-Aldrich, Munich, Germany), 10 ng/ml EGF (hEGF, Sigma-Aldrich), 0.5% DMSO (Sigma-Aldrich), 0.4 μg/ml hydrocortisone (Sigma-Aldrich), 2 nM triiodothyronine (Sigma-Aldrich), 0.18 mM adenine (Sigma-Aldrich), 10% FCS and 1% antibiotics and cultured to sub-confluence. After the isolation passage, LSC from the three healthy donors were used for downstream experiments. Corneal epithelial cells (CO) were obtained by mechanical scrapping of the central corneal epithelium from four healthy donors, taking care to avoid the perilimbic region.

Flow cytometry characterization & sorting

MSC (2×10^5) were blocked with 10% FCS in 100 mM phosphate buffered saline for 10 min, and then incubated with conjugated antibodies for 30 min at room temperature (RT). Antibodies used in this study are detailed in Supplementary Table 1. Samples were analyzed by FACS (FACSCalibur™ Flow Cytometer, BD Biosciences, CA, USA) and data were evaluated by flow cytometry software (Summit, version 3.1; Cytomation, Fort Collins, CO, USA). Sorting experiments were carried out in a cell sorter (FACS Aria Fusion cell sorting cytometer, BD Biosciences). A mouse monoclonal IgG isotype was used as a negative control.

Clonogenic assay

For clonogenic experiments, 10 MSC/cm^2 were seeded in 35-mm diameter plates and cultured in MSC medium for 14 days. Then, MSC were fixed and stained with 0.5% crystal violet. Only colonies with diameters >2 mm were scored. The results are represented as percentages, applying a previously described formula [29].

Differentiation experiments

MSC (10^4 cells/cm^2) were plated and cultured in MSC medium for 24 h. Afterward the medium was changed to the respective induction medium and maintained for 3–4 weeks. The medium was changed every 48 h. The following experiments were performed:

Adipogenesis

Adipogenesis was performed with adipogenic induction medium [30] containing DMEM 1 g/l supplemented with 0.5 mM isobutyl methylxanthine (Sigma-Aldrich), 1 μM dexamethasone (Sigma-Aldrich) and 200 μM indomethacin (Sigma-Aldrich). Differentiation was confirmed at 28 days by Oil-Red-O staining.

Osteogenesis

Osteogenic induction was performed in DMEM 1 g/l medium with 1 μM dexamethasone, 50 μg/ml ascorbic acid (Sigma-Aldrich) and 10 mM glycerol 2-phosphate (Sigma-Aldrich) [30]. Experiments were analyzed at 28 days. Osteogenic differentiation was confirmed by Alizarin Red staining.

Chondrogenesis

MSC (2×10^5 cells/cm^2) were pelleted and chondrogenic induction was performed by adding DMEM 1 g/l supplemented with 0.4% ITS (Sigma-Aldrich), 350 μM L-proline (Sigma-Aldrich), 170 nM ascorbic acid, 1 mM sodium pyruvate (Lonza, Verviers, Belgium) and 10 ng/ml TGF-β1 (Sigma-Aldrich) [30]. Differentiation was allowed for 28 days. After that, pellets were fixed with 4% paraformaldehyde for 1 h at RT, stained with alcian blue and paraffin embedded before being sectioned at 5 μm.

Adhesion assay

For the adhesion assays, 96 well plates were coated with extracellular adhesion proteins (Sigma-Aldrich) collagen IV (COL-IV) at 50 μg/ml, laminin (LN) at 20 μg/ml or bovine serum albumin at 10 μg/ml as a control for substrate-binding experiments. BM-MSC (10^4 cells/cm^2) were seeded on the coated plates or directly on plastic in the MSC medium. After 30 min and 1 h, BM-MSC were washed, fixed and stained with 0.5% crystal violet. After washing, plates were air dried, and eluted with 33% acetic acid solution. Absorbance was measured at 590 nm (Tecan infinite® m200 pro, Tecan, Männedorf, Switzerland). Furthermore, to choose an adequate extracellular matrix protein that could promote stemness, with greater integrin-α6 (ITGA6) mRNA and protein expressions, we seeded 10^4 BM-MSC/cm^2 on coated plates with either LN at 20 μg/ml or COL-IV at 50 μg/ml and also directly on plastic (control), for 1 day in MSC medium.

Protocol for epithelial induction

BM-MSC (10^4 cells/cm^2) were seeded either on uncoated plates or on LN (20 μg/ml)-coated plates in MSC medium. The day after, the media were changed to control medium that comprised DMEM 1 g/l supplemented with 2% FCS, 2 mM L-glutamine and 1% antibiotics. Epithelial induction was performed by supplementing control medium with 1 μM of All-*trans*-retinoic acid (ATRA, Sigma-Aldrich) or using supplemented hormonal epidermal medium (SHEM) media (DMEM/Ham's F-12 [Invitrogen; 2:1 vol:vol] mixture, 2 mM L-Glutamine, 5 μg/ml insulin [Sigma-Aldrich], 10 ng/ml EGF [hEGF, Sigma-Aldrich], 0.5% DMSO [Sigma-Aldrich], 0.4 μg/ml hydrocortisone [Sigma-Aldrich], 2 nM triiodothyronine [Sigma-Aldrich], 0.18 mM adenine [Sigma-Aldrich], 2% FCS and 1% antibiotics). The medium was changed every other day and the differentiation was maintained for 7 days. BM-MSC were used at the third passage. To select the optimal concentration of ATRA that could favor epithelial induction attending mRNA expression, we first tested BM-MSC at ATRA concentrations of 1, 5, and 10 μM over 7 days.

Cytotoxicity assay

ATRA cytotoxicity was evaluated by measuring LDH. BM-MSC (10^4 cells/cm^2) were cultured with increasing concentrations of ATRA over 7 days (1, 5, 10, 45 and 100 μM) in MSC medium. The medium was changed every other day. Lactate Dehydrogenase Cytotoxicity Assay Kit (Thermo Scientific, MA, USA) was performed according to the manufacturer's instructions. Absorbance was measured at 490 nm (Tecan Infinite® m200 pro, Tecan, Männedorf, Switzerland).

Quantitative PCR analysis

Total mRNA was obtained (RNA PureLink® Mini Kit, Ambion, Invitrogen) and the concentration measured by absorbance (Tecan Infinite® m200 pro). Corneal epithelial cells scarified from the central corneas of human donors were used as positive controls for corneal epithelial markers, while cultured LSC were used as positive controls for limbal epithelial markers. RNA (1 μg) was reverse transcribed using Superscript III (Invitrogen), according to the manufacturer's instructions. The cDNA (1 μl) was used for quantitative PCR (qPCR) in a final volume of 18 μl with SYBR green reaction mix (Invitrogen) and a 0.2 μM primer concentration. We performed qPCR using Step one hardware and software (Applied Biosystems, Life Technologies, Glasgow, UK). The expression level of target genes was normalized to an internal 18 s control (RRN18S, TATAA Biocenter, Sweden) and represented as the relative expression, using $2^{-\Delta\Delta Ct}$. The sequences and annealing temperatures of PCR primers are listed in Supplementary Table 2.

Western blot

Total cell extracts were dissolved in an sodium dodecyl sulfate (SDS)-loading buffer. Corneal epithelial cells scarified from the central corneas of human donors were used as positive controls for corneal epithelial markers,

while cultured LSC were used as positive controls for limbal epithelial markers. The lysate (60–90 µg of protein) was electrophoresed on 12% SDS-PAGE. The separated proteins were transferred at 90 V for 90 min to nitrocellulose transfer membranes (BD Biosciences), and the membranes were then blocked for 1 h with 5% skimmed milk. Primary antibodies (Supplementary Table 1) were incubated overnight at 4°C. After washes, horseradish peroxidase-conjugated goat anti-mouse or swine anti-rabbit immunoglobulin (Dako Cytomation, Denmark) was added for 90 min at RT. Protein bands were revealed using an enhanced chemiluminescence substrate (Biological Industries, Reactiva, Barcelona, Spain) and were recorded on autoradiography film (Kodak Rochester, NY, USA). Western blots were analyzed by digitally scanning the blots and followed by densitometric analysis with ImageJ software [31]. Analyses were normalized to the loading control GADPH, except for the phosphorylated or dephosphorylated proteins, which were normalized to the respective total proteins.

Immunofluorescence

Cells grown on glass coverslips were fixed with 4% paraformaldehyde, permeabilized, and blocked. Coverslips were then incubated with primary antibodies for 60 min at 37°C in a humidified chamber. After several washes, the proper secondary antibody was added for 60 min at 37°C in a humidified chamber. The coverslips were mounted upside down with mounting medium (Vectashield, Vector laboratories, CA, USA) and the nucleus counterstained with 4',6-diamidino-2-phenylindole (DAPI). MSC were observed using an epifluorescence microscope (BX61; Olympus R-FTL-T; Olympus America, Inc., PA, USA) coupled with a digital image acquisition program (Olympus DP Controller). The antibodies used are listed in Supplementary Table 1.

Transepithelial electric resistance

BM-MSC (10^5 cells/cm^2) were seeded on 12-well transwell inserts (Corning, Inc., NY, USA) with 0.4-µm pore sizes in either control medium or the induction conditions. HCE cells, kindly gifted [32], were used as positive controls. Transepithelial electric resistance (TEER) was measured on days 1, 3, 5, and 7 with a Millicell ERS-2 Epithelial Volt-Ohm meter (Millipore, MA, USA) attached to an STX 100C Costar Probe (World Precision Instruments, FL, USA). The medium was changed after measurements. An empty insert and an LN-coated insert were used as background controls. The TEER values for the empty and LN-coated insert were 296 and 330 Ωcm^2, respectively.

In vitro wound healing assay

BM-MSC were seeded on transwell inserts (Corning), as commented, and cultured in control or induction mediums for 7 days. The inserts were then washed three-times with phosphate buffered saline to remove the medium completely, and then transferred to 12-well plates (Corning) where HCE monolayers were cultured to confluence and then wounded. Earlier, pictures of the wounds were taken under phase-contrast inverted microscopy (BX61; Olympus R-FTL-T) with a digital image acquisition program (Olympus DP Controller). Cells were then co-cultured for 6.5 h in control medium (DMEM 1 g/l, 2% FCS, 2 mM L-glutamine and 1% antibiotics). BM-MSC inserts were then removed and the wounds were relocated and photographed again. An empty insert and an LN-coated insert without cells were used as controls. The results were represented as the percentage of wound closure area by applying the following formula: 100 – [(wounded area at 6.5 h/wounded area at 0 h) × 100].

Statistical analysis

All experiments were performed at least in triplicate. One-way analysis of variance with Bonferroni post-test was used to compare the data for more than two groups, and two-tailed Student's t-tests were used to compare data between two groups; $p < 0.05$ were considered significant (PRISM, version 6.0 GraphPad Software, CA, USA). Results are presented as the mean ± standard error.

Results

CK12 expression was similar in BM-MSC & LSC, while BM-MSC & corneal epithelial cells shared similar levels of CK19

Both BM-MSC and AT-MSC were compared as they meet the criteria for MSC definition (Supplementary Figure 1) [27]. Using qPCR, western blot and immunofluorescence, we evaluated the expression levels of several corneal epithelial markers. We first analyzed the expression of the different markers in controls. The corneal differentiation markers E-cad, CK12, CK3 and PAX6 were highly expressed CO (Figure 1A & E). Cultured LSC expressed lower

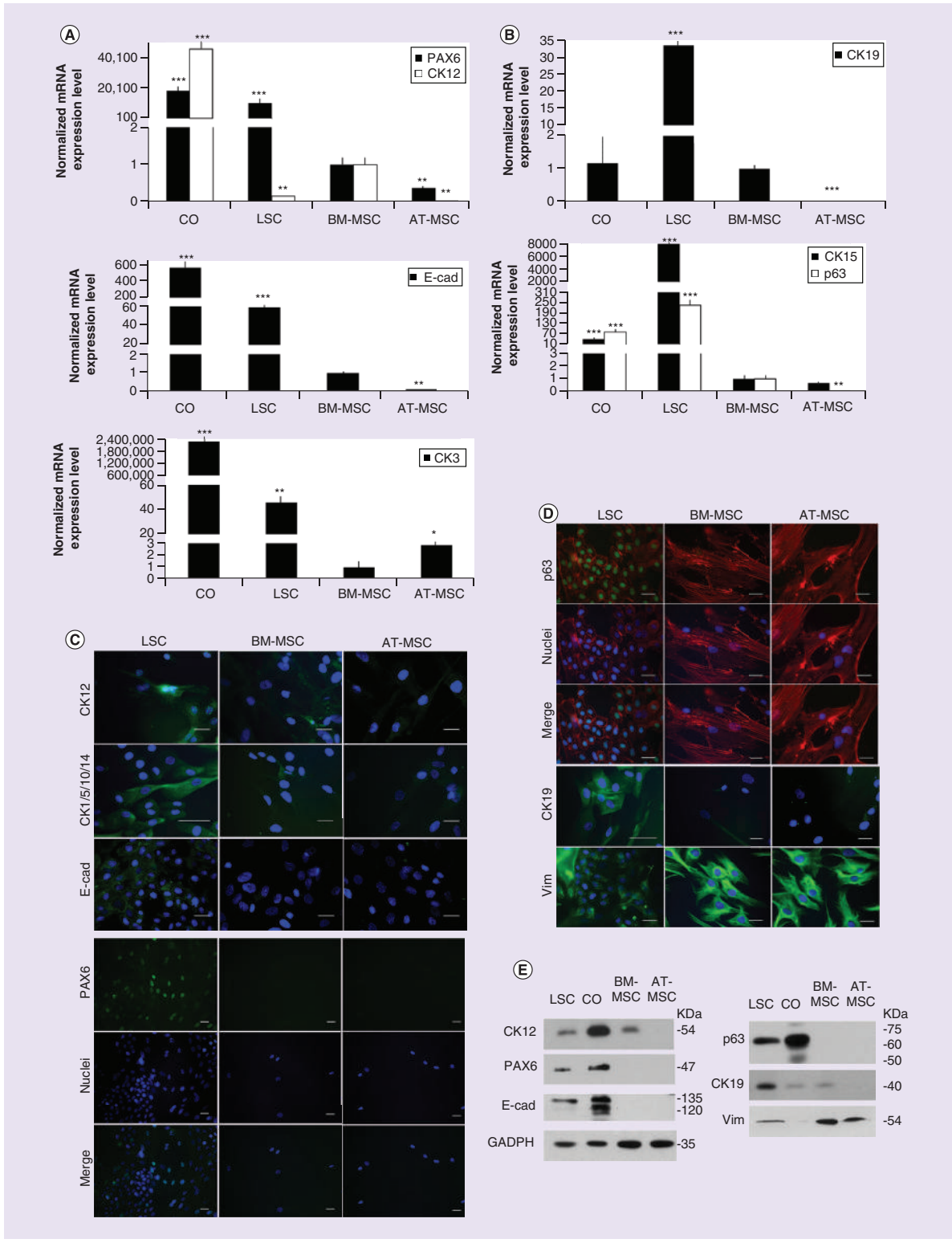

Figure 1. Expression of corneal, limbal and epithelial markers in BM-MSC and AT-MSC. (A) Normalized mRNA expression of corneal differentiation markers PAX6, E-cad, CK12 and CK3. **(B)** Normalized mRNA expression level analysis for progenitor epithelial markers CK15, CK19 and ΔNp63α. **(C)** Immunofluorescence for CK12, CK 1/5/10/14, E-cad and PAX6 in LSC, BM-MSC and AT-MSC. **(D)** Immunofluorescence for CK19, p63 and vimentin in limbal stem cells, CO, BM-MSC and AT-MSC. **(D)** Immunofluorescence for CK19, p63 and vimentin in limbal stem cells, CO, BM-MSC and AT-MSC. Red staining corresponds to phalloidin. Nuclei are counterstained with DAPI (blue). Epifluorescence microscopy. Scale bar = 15 μm. **(E)** Western blot analysis of CK12, PAX6, E-cad, p63, CK19 and vim. Statistical analysis performed using two-tailed Student's t-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Results are presented as means ± standard deviation of three independent experiments.

AT-MSC: Adipose tissue-derived mesenchymal stem cells; BM-MSC: Bone marrow mesenchymal stem cells; CO: Corneal epithelial cells; CK: Cytokeratin; DAPI: 4′,6-diamidino-2-phenylindole; E-cad: E-cadherin; LSC: Limbal stem cells; MSC: Mesenchymal stem cell; ns: No significant; Vim: Vimentin.

levels of CK12, PAX6 and E-cad (immature form) (Figure 1C & E) [33], but increased levels of CK15, CK19 and ΔNp63α compared with CO (Figure 1B). LSC were also positive for the mesenchymal marker Vim [34], while CO was not.

BM-MSC cultured with MSC medium expressed similar levels of CK12 compared with LSC, while AT-MSC cultured with MSC medium did not (Figure 1E). Moreover, levels of CK19 expression in BM-MSC were comparable to those in CO, while expression in AT-MSC remained negative (Figure 1E). LSC expressed pan-CK and detectable levels of CK1/5/10/14 were also seen in BM-MSC (Figure 1C). No PAX6, E-cad or ΔNp63 protein could be detected in either of the MSC lines, despite lower mRNA expressions (Figure 1C). In view of the mRNA and protein expression pattern, BM-MSC were chosen for corneal induction experiments.

LN substrate increases expression of the stemness marker ITGA6 compared with COL-IV

We tested whether COL-IV or LN could increase expression of ITGA6 mRNA and protein levels in BM-MSC, since it has been linked to stemness in MSC [35]. We also tested whether this protein could be related to increased clonogenic potential. LN significantly increased not only ITGA6 mRNA and protein expression but also integrin-β1 (ITGB1) mRNA expression compared with COL-IV (Figure 2A–D). Moreover, in an *in vitro* adhesion assay, LN exerted differential selection of cells because significantly decreased adhesion was observed when compared with COL-IV, bovine serum albumin and control, even at 1 h after cell seeding (Figure 2E & F). To evaluate changes in the clonogenicity of cells expressing ITGA6, we sorted ITGA6-positive (ITGA6+) and ITGA6-negative (ITGA6-) BM-MSC fractions. There was a significant increase in the clonogenic capability of ITGA6+ cells (14.83%) compared with ITGA6- cells (8.16%; Figure 2G–K). For these reasons, LN was chosen for further induction experiments.

SHEM medium increases corneal epithelial markers, ATRA 1 μM increases epithelial markers & both treatments decrease mesenchymal marker expression

Previous results led us to establish different epithelial induction treatments, such as 1 μM ATRA or SHEM. ATRA is commonly applied in epithelial differentiation [36], while SHEM is used to culture LSC [37]. We also used LN substrate (LN + ATRA, LN + SHEM) with both media. To evaluate the effects of the different treatments on corneal epithelial induction, we analyzed the expression of corneal epithelial and mesenchymal markers by qPCR, western blotting and immunofluorescence at day 7 of culture. Expressions of CK12, CK3, CK19, E-cad and ITGB1 were analyzed as epithelial markers, and expressions of Vim, snail and α-SMA were analyzed as mesenchymal markers. The concentration of ATRA for epithelial induction was selected based on previous experiments demonstrating that 1 μM ATRA exerted minimal cytotoxicity while increasing the expression of cytokeratins compared with 5 μM and 10 μM concentrations (Supplementary Figure 2).

CK12 protein levels increased in SHEM, LN and LN + SHEM (Figure 3A), although CK12 mRNA was increased in all the treatments (Figure 4A). CK3 protein levels only increased significantly in SHEM, but decreased in other treatments (Figure 3A). In contrast, CK19 protein levels were higher in ATRA treatments (ATRA and LN + ATRA) and significantly lower in SHEM treatments (SHEM and LN + SHEM), in agreement with qPCR results (Figure 3A & Figure 4A). The pan-CK proteins were only detected in ATRA treatments (ATRA and LN + ATRA) (Figure 3A). There were no differences in ITGB1 protein levels (Figure 3B), although an increased level of ITGB1 mRNA expression was detected in SHEM, LN + SHEM and LN + ATRA (Figure 4B).

Protein mesenchymal markers (Vim, snail and α-SMA) were lower in all the treatments, except in LN substrates (Figure 3B). Vim mRNA was also reduced in all the treatments except LN, and snail mRNA was reduced in SHEM and LN + SHEM (Figure 4B & C). N-cadherin protein expression was higher in ATRA treatments, but decreased with SHEM treatments (Figure 3B). We also carried out immunofluorescence experiments to evaluate the expression pattern of CK12, pan-CK, ITGB1, Vim, α-SMA and N-cad. These results corroborated the protein expression levels observed in the western blot experiments (Figure 5).

Both SHEM & ATRA treatments increase the wound healing capability of HCE cells

We explored the capability of treated BM-MSC to promote wound healing in HCE cells through paracrine secretion. This *in vitro* assay served as an approach to test the potential of the BM-MSC secretome to stimulate corneal epithelial wound healing by corneal epithelial cells *in vivo* [38]. The percentages of HCE wound closure increased with BM-MSC treated with either SHEM or ATRA (Figure 6). In addition, wound closure increased with BM-MSC seeded on LN substrate in control medium (MSC + LN), SHEM (SHEM + LN) and ATRA

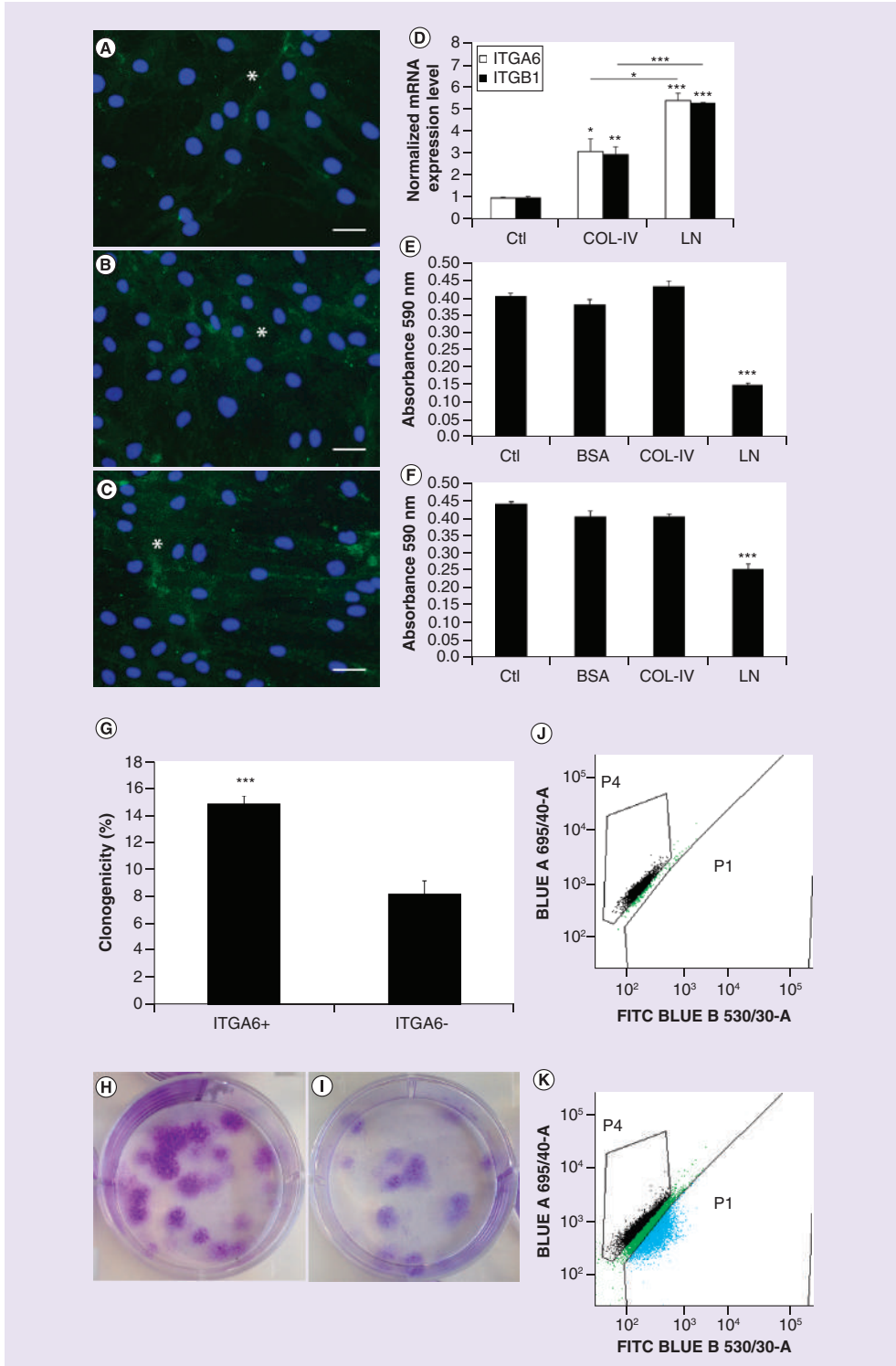

Figure 2. ITGA6 expression in bone marrow mesenchymal stem cells and differential adhesion on collagen IV and laminin substrates. (A–C) Immunofluorescence staining (asterisks) for ITGA6 in BM-MSC seeded without substrate **(A)**, on COL-IV **(B)** and LN **(C)** substrates; scale bar = 20 μm. **(D)** Normalized mRNA expression levels for ITGA6 and ITGB1. **(E & F)** Adhesion assay of BM-MSC on different substrates such as bovine serum albumin, control, collagen IV and laminin at 30 min **(E)** and 1 h **(F)**, evaluated by crystal violet assay. **(G)** Clonogenic assay. Results are presented as mean ± standard error of sixfold experiment. **(H)** Colony formation of the ITGA6+ fraction. **(I)** Colony formation of the ITGA6- fraction. **(J & K)** Sorting for ITGA6. **(J)** Negative isotype control; IgG conjugated with FITC. **(K)** P1 is the ITGA6+ fraction and P4 is the ITGA6- fraction. Results are presented as mean ± standard error of three independent experiments. Statistical analysis performed using two-tailed student's t-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). BSA: Bovine serum albumin; COL-IV: Collagen IV; Ctl: Control; FITC: Fluorescein isothiocyanate; ITGA6: Integrin α6; ITGB1: Integrin β1; LN: Laminin.

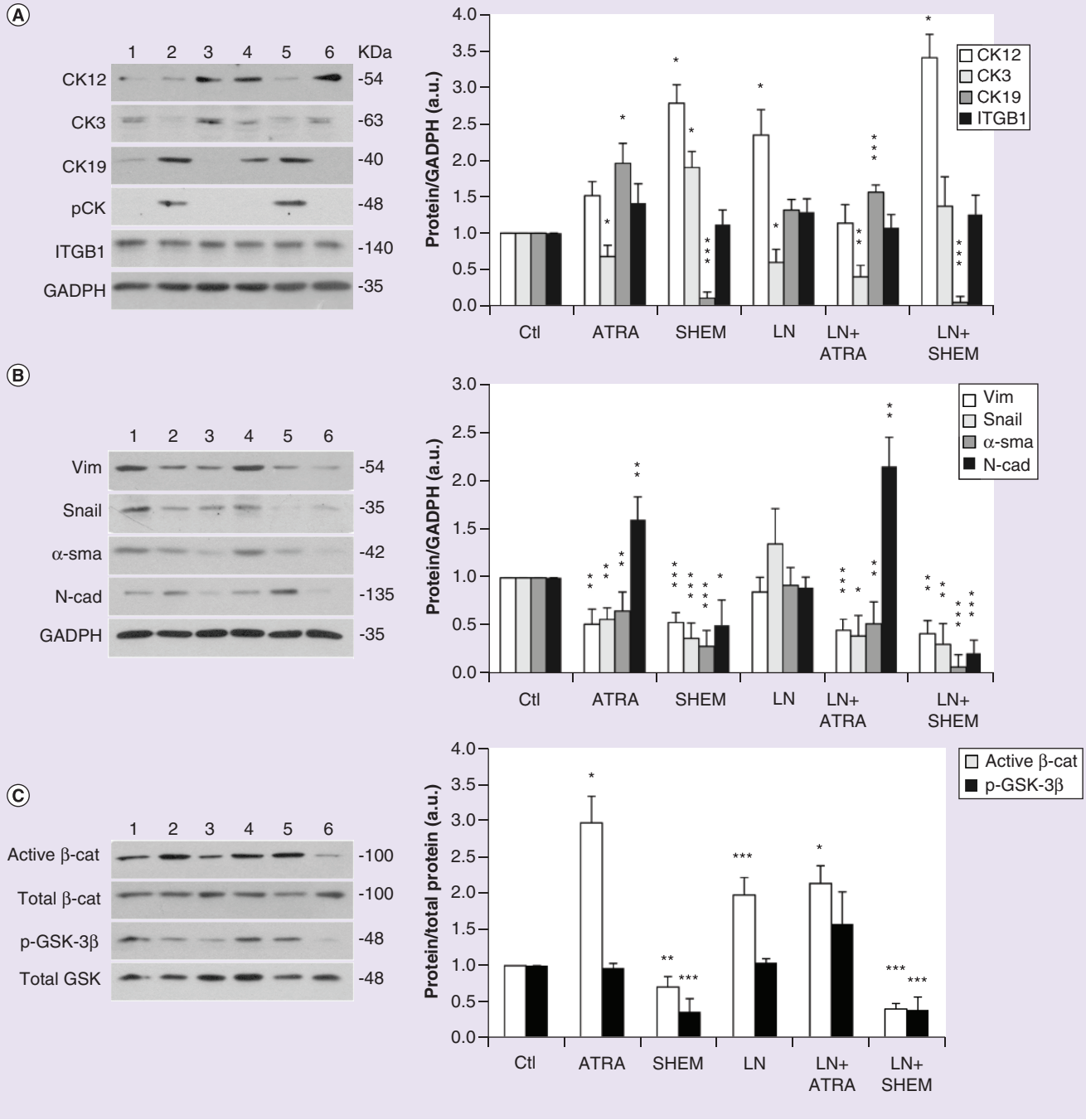

Figure 3. Western blot densitometric analysis of epithelial and mesenchymal cell markers and β-catenin signaling pathway in bone marrow mesenchymal stem cells. (A) CK12, CK3, CK19 and integrin β1 were used as epithelial cell markers and normalized to GAPDH expression. **(B)** Vimentin, snail, α-smooth actin and N-cadherin were used as mesenchymal cell markers and normalized to GAPDH. **(C)** Expressions of active β-catenin and phospho-glycogen synthase kinase 3β were normalized to the total cell protein concentration, total β-catenin and total glycogen synthase kinase, respectively. Lanes: 1. ctll; 2. ATRA; 3. SHEM; 4. LN; 5. LN + ATRA; 6. LN + SHEM. Results are presented as mean ± standard error of three independent experiments. Statistical analysis performed using two-tailed student's t-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).
ATRA: All-trans-retinoic acid; CK: Cytokeratin; Ctl: Control; LN: Laminin; SHEM: Supplemented hormonal epidermal medium.

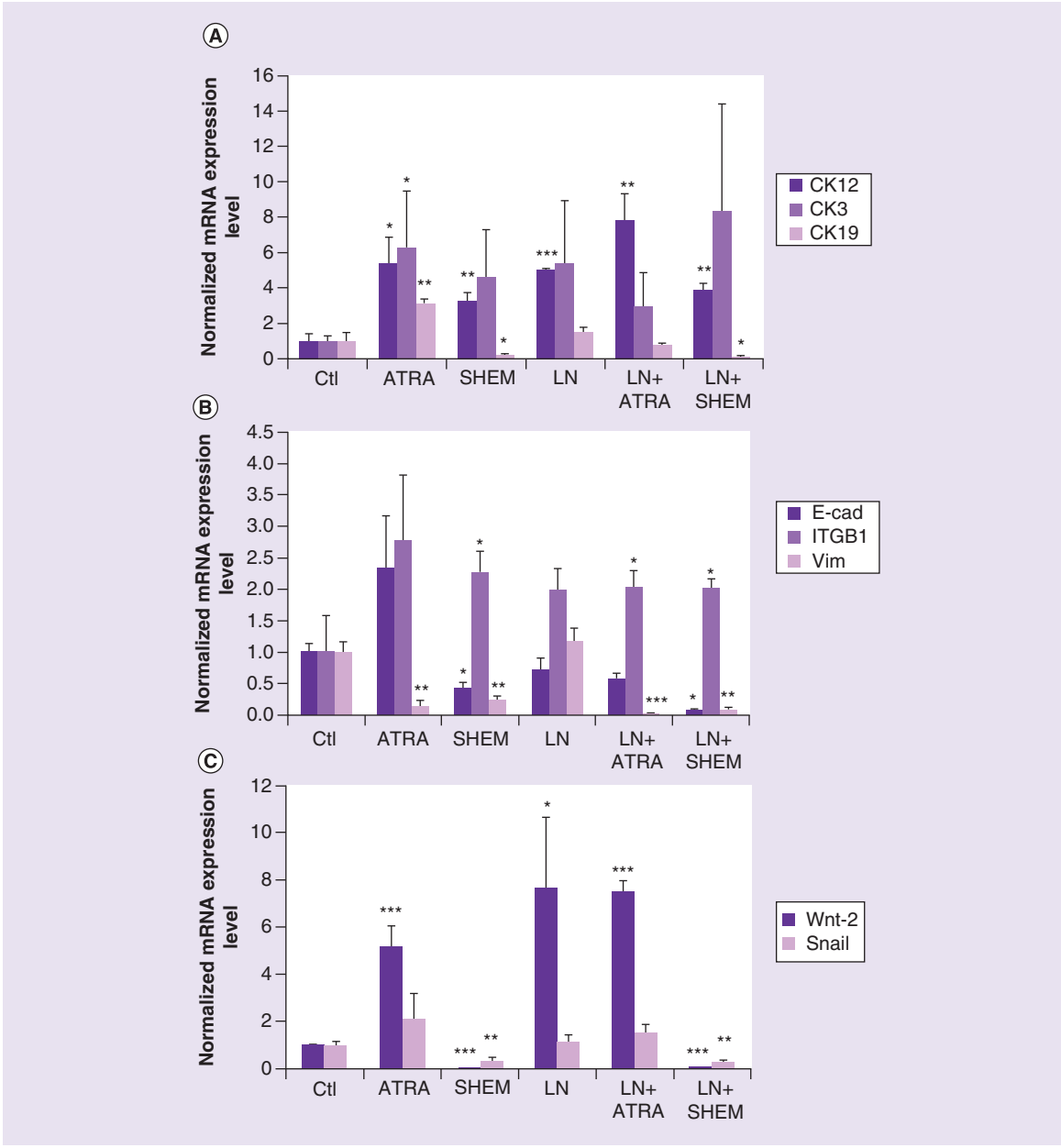

Figure 4. Normalized mRNA expression for corneal epithelial, epithelial and mesenchymal cell markers and for Wnt-signaling pathway in bone marrow mesenchymal stem cells. (A) CK12, CK3 and CK19 mRNA expression. **(B)** E-cad, integrin β1 and vim mRNA expression. **(C)** Wnt-2 and snail mRNA expression. Results are presented as mean ± standard error of three independent experiments. Statistical analysis performed using two-tailed student's t-tests (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).
ATRA: All-trans-retinoic acid; CK: Cytokeratin; Ctl: Control; E-cad: E-cadherin; LN: Laminin; SHEM: Supplemented hormonal epidermal medium; Vim: Vimentin.

(ATRA + LN) when compared with control conditions. Surprisingly, noninduced BM-MSC (MSC) seeded on an uncoated insert lacked the capability to increase wound closure rates.

SHEM medium increases TEER values

The TEER assay is a functional assay used to analyze the integrity of the epithelial barrier when determining corneal epithelial differentiation [39]. TEER values increased gradually with culture time, and were significantly higher in both SHEM treatments at days 3, 5 and 7 when compared with controls, without significant differences between

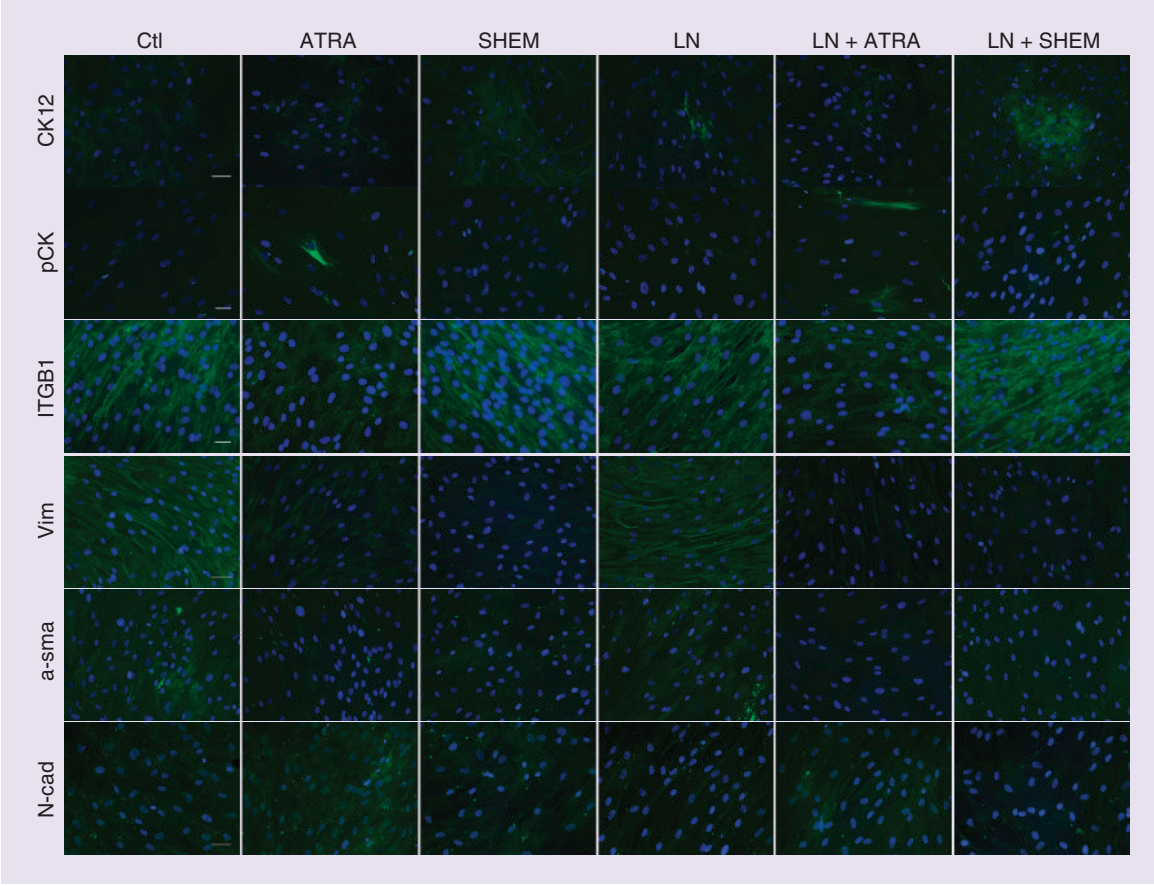

Figure 5. Indirect immunofluorescence for several markers. Note the increased expression of cytokeratin 12 in SHEM, LN and LN + SHEM compared with control, and the presence of pan-cytokeratin only in ATRA treatments (ATRA and LN + ATRA). There was also a decrease in the expression of the mesenchymal markers vimentin and α-smooth actin in the epithelial-induced bone marrow mesenchymal stem cells compared with control and LN, whereas N-cad expression was only reduced in the SHEM treatments and was augmented in the ATRA treatments. ATRA: All-trans-retinoic acid; CK: Cytokeratin; Ctl: Control; N-cad: N-cadherin; LN: Laminin; SHEM: Supplemented hormonal epidermal medium; Vim: Vimentin.

SHEM and LN + SHEM (Figure 7). The maximum TEER value for SHEM was achieved at day 5 of culture (93.83 Ωcm^2) and maintained until day 7 (83 Ωcm^2), while the maximum TEER value for LN + SHEM condition was achieved at day 7 (101 Ωcm^2). No differences were seen between control BM-MSC and ATRA, LN + ATRA or LN. The maximum values for control (65 Ωcm^2), ATRA (66.83 Ωcm^2) and LN + ATRA (66.33 Ωcm^2) were achieved at day 5, and for LN (67.33 Ωcm^2) at day 7 of culture. Positive control (HCE) showed higher TEER values from day 5 (137.66 Ωcm^2) to day 7 (148.33 Ωcm^2).

Wnt signaling protein expression decreased in SHEM medium but increased with ATRA

We also evaluated changes in Wnt/β-catenin expression, which is an important pathway for BM-MSC differentiation [40]. We studied Wnt-2 mRNA expression – an extracellular ligand that can activate the Wnt/β-catenin signaling pathway – and the protein levels of active β-catenin (active β-cat) and glycogen synthase kinase phosphorylated in serine 9 (p-GSK-3β), the inactive form of the enzyme which inhibits β-catenin [41]. We found that Wnt-2 mRNA expression was lower in both the SHEM treatments (SHEM and LN + SHEM), but increased in ATRA treatments (ATRA and LN + ATRA) and on LN substrate (Figure 3C). As expected, active β-cat protein levels decreased with SHEM treatment (SHEM and LN + SHEM) and increased with ATRA, LN and LN + ATRA (Figure 4C). The p-GSK-3β protein level was lower in SHEM treatments without significant changes in the other conditions (Figure 4C).

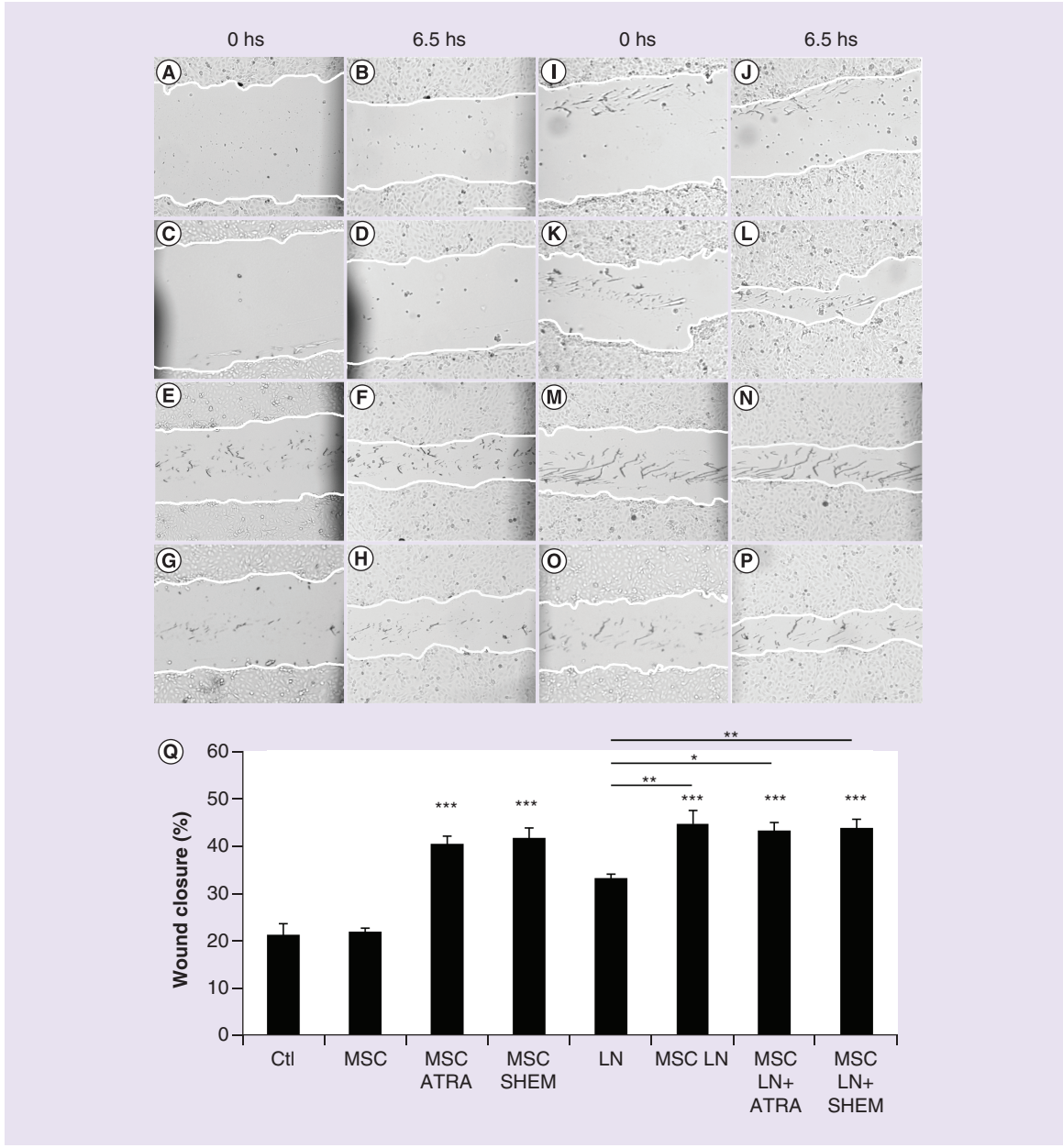

Figure 6. ***In vitro*** **wound healing experiment with human corneal epithelial cells.** BM-MSC were seeded on transwell inserts and cultured under epithelial-inducing conditions for 7 days. Confluent HCE cultures were wounded and phase-contrast micrographs taken. Cells were co-cultured for 6.5 h and wounds relocated and photographed again. **(A, C, E, G, I, K, M & O)** Wounded HCE monolayers at 0 h. **(B)** HCE wound after 6.5 h of co-culture with an empty insert that served as control. **(D)** HCE wound after 6.5 h with BM-MSC seeded without substrate in the control medium (MSC). **(F)** HCE wound after 6.5 h with BM-MSC cultured without substrate in medium supplemented with ATRA (1 μM; MSC ATRA). **(H)** HCE wound after 6.5 h with BM-MSC cultured without substrate in SHEM (MSC SHEM). **(J)** HCE wound after 6.5 h with laminin (20 μg/ml)-coated insert without MSC (LN). **(L)** HCE wound after 6.5 h with BM-MSC seeded on LN substrate in control medium (MSC LN). **(N)** HCE wound after 6.5 h with BM-MSC cultured on laminin substrate in medium supplemented with ATRA (MSC LN + ATRA). **(P)** HCE wound after 6.5 h with BM-MSC cultured on laminin substrate in supplemented hormonal epidermal medium (MSC LN + SHEM); scale bar = 300 μm. **(Q)** Graph showing the quantification of wound closure. Results are presented as mean ± standard error of 12 independent measures. Statistical analysis was by analysis of variance with Tukey's multiple comparison test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

ATRA: All-trans-retinoic acid; BM-MSC: Bone marrow mesenchymal stem cell; CK: Cytokeratin; Ctl: Control; HCE: Human corneal epithelial cell; LN: Laminin; MSC: Mesenchymal stem cell; SHEM: Supplemented hormonal epidermal medium.

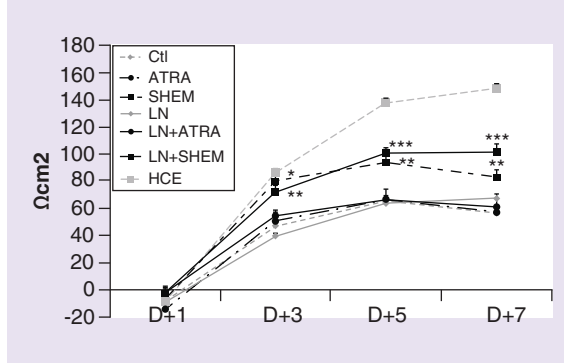

Figure 7. Bone marrow mesenchymal stem cells transepithelial electric resistance analysis after 7 days of epithelial induction. Cells were seeded into permeable transwell inserts: without substrate in a ctl medium; without substrate in a medium supplemented with 1 μM ATRA; without substrate in SHEM; on 20 μg/ml LN substrate-coated inserts in control medium (LN); on 20 μg/ml LN substrate-coated inserts in medium supplemented with 1 μM ATRA (LN+ATRA); and on 20 μg/ml LN substrate-coated inserts in supplemented hormonal epidermal medium (LN+SHEM). Bone marrow mesenchymal stem cells transepithelial electric resistance was carried out at different days, such as D+1, D+3, D+5 and D+7, during the epithelial induction conditions. Transepithelial electric resistance (Ωcm^2) results are presented as mean ± standard error of six independent measures. Statistical analysis performed using two-tailed student's t-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). ATRA: All-trans-retinoic acid; Ctl: Control; D: Day; HCE: Human corneal epithelial cell; LN: Laminin; SHEM: Supplemented hormonal epidermal medium.

Discussion

Nowadays, treatment for patients with corneal blindness due to bilateral LSCD is focused on the search for alternative sources of stem cells capable of differentiating to corneal epithelium or of promoting corneal wound repair, stimulating corneal epithelial cell migration, and decreasing inflammation through paracrine secretion. In this study, we optimized a protocol to increase the potential of MSC to acquire corneal epithelial characteristics, while promoting better wound healing. Our research is significant because MSC is a realistic alternative treatment for patients with bilateral corneal blindness, since MSC has the potential to regenerate corneal epithelium and is safe in humans [23].

We analyzed mesenchymal and corneal epithelial cell markers, which are used to evaluate the transdifferentiation of progenitor cells into epithelial [42,43] or corneal epithelial phenotypes [14,44–46]. ATRA induced the expression of non-specific cytokeratins for differentiated corneal epithelium (pan-CK and CK19), and served as control of unspecific epithelial lineage induction. Meanwhile, SHEM medium increased the expression of tissue-specific intermediate filament proteins of corneal epithelial cells [47,48], CK12 and CK3, indicating an induction of BM-MSC toward corneal epithelial characteristics. This is noteworthy, since some corneal epithelial derivation protocols with IPSC do not increase the expression of corneal markers such as CK12 or CK3 [49].

In addition, epithelial induction was also indicated by an overall decrease in the protein expression of mesenchymal markers. This supports previous reports demonstrating that ATRA induces epithelial differentiation in MSC [50]. Cells seeded on LN substrate and treated with SHEM medium showed the highest expression of CK12. In addition, LN alone enhanced the expression of CK12, indicating an effect of the substrate on BM-MSC induction. CK3 protein expression levels were highest in SHEM medium without the LN substrate, but failed to show any significant increase when seeded on LN + SHEM. This finding could be explained by an effect of LN, as LN alone reduced CK3 protein expression. It is possible that with longer culture times, we might have observed an increase in CK3 expression in both LN and LN + SHEM treatments because there is a delay in the increase in CK3 expression compared with the increase in CK12 in the corneal differentiation process of embryonic stem cells (ESC) [51].

The fact that induced BM-MSC guided better wound healing than noninduced BM-MSC pointed out an improved paracrine secretion profile with proregenerative effects boosted by the induction mediums. The greater HCE wound healing promoted by the BM-MSC seeded on LN without and additional induction treatment could also be explained by the modulatory effect of matrix composition on the MSC secretome [52,53]. It is well known that LN binds to EGFR, modifying cellular behavior [54]. Although further studies are needed to determine the soluble trophic factors involved in these positive results, our *in vitro* data agree with previous reports showing that *in vivo* corneal regeneration improves with epithelial-induced MSC when compared with noninduced MSC [55]. MSC cultured in standard conditions demonstrated their ability to improve total LSCD in rabbit models rather than partial LSCD [56]. Induction of MSC could be a reliable strategy to improve results in partial LSCD in animal models through enhanced paracrine effector functions. As reported elsewhere, MSC demonstrated efficacy in a clinical trial for LSCD treatment [23]. Since MSC were cultured in standard conditions for this trial, our results

highlight the trophic potential of induced BM-MSC to improve the outcomes of LSCD treatment with BM-MSC. Interestingly, our data indicate that SHEM conditions were associated with significantly increased TEER values in experiments with transwell inserts. The TEER values for SHEM-treated BM-MSC were higher than those found previously in BM-MSC induced toward corneal epithelial cells [14]. Taking all these factors into account, we can conclude that among our assayed conditions, SHEM treatment was the most appropriate medium for inducing BM-MSC to acquire corneal epithelium characteristics. The use of SHEM medium was approved by the Spanish Agency of Medicines and Health Products (AEMPS) for CLET application in a clinical trial for unilateral LSCD using autologous LSC (ClinicalTrial.gov identifier: NCT01470573). Thus, the use of this medium is suitable for the culture of BM-MSC for clinical application in humans. So, SHEM medium would be used to induce BM-MSC in further translational approaches for corneal surface regeneration.

The Wnt/β-catenin signaling pathway plays an important role during epithelial [57], chondrogenic, osteogenic and adipogenic differentiation [40] of BM-MSC. Furthermore, this pathway is involved in maintaining the undifferentiated state of LSC [58], and its repression is necessary to promote the differentiation of corneal epithelial progenitor cells into nonkeratinized corneal epithelium during development [59]. Inhibition of the Wnt/β-catenin signaling pathway enhances MSC differentiation toward epithelial cells [60] and its downregulation mediates the transdifferentiation of PAX6-transfected hair follicle stem cells into corneal epithelial cells [61]. The inhibition of this signaling pathway is also implied in the differentiation of IPSC toward corneal epithelium [44]. SHEM treatment inhibited the Wnt/β-catenin signaling pathway, thereby strengthening the induction of BM-MSC toward corneal epithelium.

BM-MSC expressed CK12 and CK19 while AT-MSC did not. These results demonstrate the cytoskeletal heterogeneity of MSC, as previously indicated [62]. Although CK expression was lower than in the corneal epithelium, our data indicate that cultured LSC and BM-MSC could share comparable CK12 protein expression. For this reason, BM-MSC were selected for corneal epithelial induction experiments. Although MSC were negative for the proteins p63, E-cad and PAX6, which are distinctive corneal phenotype markers [63], specific media or matrix compositions mimicking corneal micro-environmental cues [14,64], could be explored to increase the expression of corneal epithelial cytokeratins, as previously performed with MSC for simple epithelial differentiation [65]. Of note, we showed that isoform Δp63α was highly specific of LSC because isoforms Δp63β and Δp63γ were not expressed by these cells [66]. However, Δp63α, Δp63β and Δp63γ isoforms were expressed by the cornea, being the Δp63α isoform also the most expressed [67].

We considered the importance of niche cues for the proliferation, differentiation and stemness maintenance of corneal epithelial cells [2] and MSC [68]. We compared COL-IV and LN, as these are major components of the corneal epithelium basement membranes [69], and analyzed ITGA6 expression, because this has been demonstrated to increase MSC differentiation potential [35]. Consistent with previous reports [70], we found an increased clonogenic potential in the BM-MSC ITGA6+ subpopulation. We observed that BM-MSC seeded on the LN substrate showed increased levels of ITGA6 and decreased cellular adhesion, indicating selective attachment of BM-MSC.

Conclusion

In summary, our results indicate that BM-MSC can increase the expression of corneal epithelial cell markers when cultured in appropriate induction conditions. Furthermore, we provide details of some of the molecular mechanisms accompanying this process and present a protocol for corneal epithelial induction, compatible with human clinical application, which in turn can enhance the healing potential of MSC to regenerate a wounded monolayer of corneal epithelial cells. Our data will facilitate the development of an animal model, prior to human application.

Translational perspective

LSCD causes loss of corneal transparency, inflammation and conjunctivalization of the corneal surface leading to blindness. Unilateral LSCD is treated by cell therapy approaches with autologous cultured LSC transplantation (CLET) since 1997. Bilateral LSCD is a more challenging condition. Its treatment relies upon allogenic LSC transplantation along with systemic immunosuppression that entails adverse effects. One future perspective for the treatment of this pathology relies on cell therapy with MSC. In fact, MSC cultured in standard conditions have also demonstrated effectiveness in human clinical application in comparison with allogeneic LSC transplantation in cases of LSCD in a proof-of-concept clinical trial. In view of these promising results, there is a need to compare different sources of MSC and to study the culture conditions that can enhance their therapeutic potential. Our research demonstrated BM-MSC cultured with a specific LSC culture medium – SHEM medium – increased

the expression of corneal epithelial markers while improved their healing potential in *in vitro* approaches. Our research will pave the way for more translational research with MSC cultured with SHEM medium, since we also demonstrate that the paracrine effector functions of BM-MSC are central for the regenerative mechanisms of these cells *in vitro*. More studies would be needed in order to determine the exact mechanisms and pathways involved in these results.

Summary points

Comparison between bone marrow & adipose tissue mesenchymal stem cells

- Bone marrow mesenchymal stem cells (BM-MSC) expressed higher levels of corneal epithelial markers than adipose tissue mesenchymal stem cells (AT-MSC).
- BM-MSC express similar levels of cytokeratin (CK) 12 when compared with cultured limbal stem cells (LSC).
- BM-MSC express similar levels of CK19 when compared with corneal epithelial cells.

Effects of media in the expression of corneal epithelial, epithelial & mesenchymal markers in BM-MSC

- supplemented hormonal epithelial medium (SHEM), used to LSC culture, increased the expression of specific corneal epithelial markers in BM-MSC, such as CK12 and CK3, while decreased the expression of mesenchymal markers vimentin, snail, α-smooth muscle actin and N-cadherin.
- The use of all-*trans* retinoic acid (ATRA), increased nonspecific cytokeratins expression and decreased the expression of the mesenchymal markers.
- Transepithelial electrical resistance, a functional assay to evaluate epithelial differentiation, increased in BM-MSC when treated with SHEM.
- Wnt/β-catenin pathway was inhibited with SHEM while was activated with ATRA treatment.

Wound healing potential of BM-MSC

- SHEM and ATRA treated BM-MSC increased *in vitro* human corneal epithelial cells wound healing.

Author contributions

N Nieto-Nicolau contributed in conception and design of the study, administrative support, provision of study material, collection and assembly of data, data analysis and interpretation, manuscript writing and gave final approval of the manuscript. B Martín-Antonio contributed with administrative support, provision of study material, final approval of manuscript. C Müller-Sanchez did collection and assembly of data, data analysis and interpretation and gave final approval of manuscript. RP Casaroli-Marano contributed in conception and design, financial support, administrative support, provision of study material, collection and assembly of data, data analysis and interpretation, manuscript writing and gave final approval of manuscript.

Financial & competing interests disclosure

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Review

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Regenerative Medicine

Mechanisms underlying the therapeutic potential of mesenchymal stem cells in atherosclerosis

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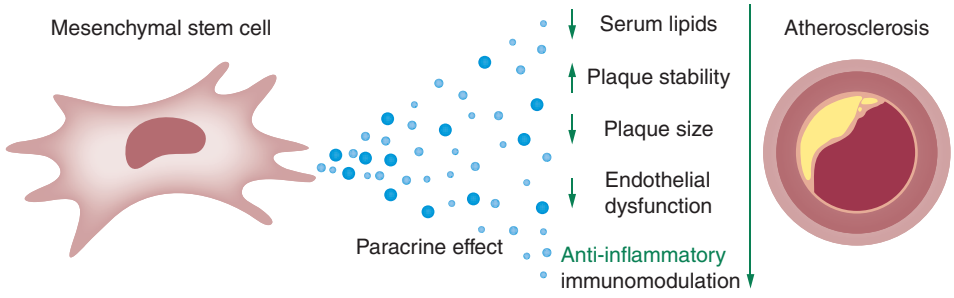
Atherosclerosis is a chronic inflammatory condition resulting in the formation of fibrofatty plaques within the intimal layer of arterial walls. The identification of resident stem cells in the vascular wall has led to significant investigation into their contributions to health and disease, as well as their therapeutic potential. Of these, mesenchymal stem cells (MSCs) are the most widely studied in human clinical trials, which have demonstrated a modulatory role in vascular physiology and disease. This review highlights the most recent knowledge surrounding the cell biology of MSCs, including their origin, identification markers and differentiation potential. The limitations concerning the implementation of MSC therapy are considered and novel solutions to overcome these are proposed.

Graphical abstract: Mesenchymal stem cells have been shown to have paracrine effects that affect the onset and progression of atherosclerotic disease. Notable protective effects include a reduction in serum lipids, plaque size and endothelial dysfunction, as well as an increase in plaque stability and overall anti-inflammatory immunomodulation. Created with BioRender.com.



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Keywords: atherosclerosis • immunomodulation • inflammation • mesenchymal stem cell • stem cell science • therapeutics

Despite current pharmacological and surgical management, atherosclerosis remains the leading cause of cardiovascular mortality worldwide [1]. Clinically, atherosclerosis most commonly manifests as coronary artery disease, acute myocardial infarction (AMI) and ischemic stroke. It is a chronic inflammatory condition driven by endothelial dysfunction, lipid accumulation and immune cell recruitment, resulting in the formation of plaques within the intimal layer of arterial walls [2]. Therefore, treatments aim to stabilize plaques, suppress inflammation and reduce hyperlipidemia [1]. Cell therapies present an innovative approach to the treatment of atherosclerosis [3].

Future Medicine

The identification of vascular resident stem and progenitor cell populations has provided a multitude of options upon which to base these novel cell therapies [4]. Studies have revealed several distinct stem cell niches, with varying potencies, in the vessel wall. These include multipotent and mesenchymal stem cells (MSCs), as well as unipotent endothelial, smooth muscle and adventitial macrophage progenitor cells [5]. Of these, MSCs are particularly popular as a potential therapy for various conditions, from osteoarthritis to AMI. According to clinicaltrial.gov, MSCs are currently involved in 1027 registered clinical trials to date.

An increasing body of evidence suggests that MSCs are a promising therapeutic avenue for atherosclerosis. The landmark CANTOS trial showed that targeting inflammation in atherosclerosis significantly reduced the rate of recurrent cardiovascular events and indeed, MSCs have demonstrated potent immunomodulatory effects [6]. Furthermore, there have been reports MSCs are lipid-lowering, support endothelial repair and may differentiate into functional vascular cells [7]. This provides MSCs with the potential to impair atherogenesis, reduce plaque size and even promote plaque stability.

Beyond their effects on atherogenesis, MSCs are readily available from a variety of sources, they exhibit potential for rapid expansion *in vitro* [6], demonstrate target specificity [8], and have a minimal risk profile [9] – all key aspects in determining the effectiveness of a clinical intervention. These functional and practical aspects provide MSCs with a therapeutic advantage over the other vascular resident stem cells. Thus, this review will detail the compelling evidence surrounding the therapeutic potential of MSCs in atherosclerosis, with the key studies outlined in Supplementary Table 1.

Mesenchymal stem cells

MSCs are a heterogeneous population of multipotent stromal cells that can differentiate into mesodermal lineage cells, including osteoblasts, chondrocytes and adipocytes [10]. Although originally identified in the bone marrow (BM), MSCs reside in most tissues [11], facilitating isolation from several human sources, including the umbilical cord, adipose tissue gingiva and skin [12].

The term 'MSC' was originally coined by Caplan and colleagues to describe the non-hematopoietic BM cells possessing broad differentiation potential [13]. Accordingly, *in vitro*, MSCs demonstrate self-renewal and commit to a mesodermal lineage, supporting their postulated *in vivo* role as a stem cell reservoir for tissue maintenance. Based on this model, MSCs were thought to support healthy vasculature through formation, repair and remodeling of arterial vessels.

However, Caplan has since suggested that MSCs be renamed 'medicinal signaling cells' when administered therapeutically [14]. This reflects a paradigm shift in response to substantial evidence showing that, when administered, MSCs do not differentiate to form new tissues *in vivo*, as the term 'stem cell' suggests. Rather, their primary function may be paracrine, by promoting tissue remodeling [15] and suppressing inflammation [16].

Identification of MSCs

Given their nonspecific fibroblast-like morphology [10], and the shared embryological origins of vascular stem cells, there is lack of consensus regarding key markers for MSC identification [5].

According to the published International Society for Cellular Therapy (ISCT) guidelines, the minimum criteria for MSC identification includes: cell adherence to a plastic surface, ability to differentiate into osteoblasts, adipocytes and chondrocytes *in vitro* and expression of surface markers, as seen in Figure 1B [17]. However, not all of these surface markers are unique to MSCs; CD73, for example, is also expressed by endothelial cells [18]. This has led some groups to adopt their own identification standards [19]. Thus, while the search for unique markers continues, caution must be taken when assessing studies involving MSCs. For this review, the studies referenced defined MSCs using the ISCT guidelines, unless stated otherwise.

MSCs in atherosclerosis

MSCs decrease endothelial dysfunction

The trigger for atherosclerotic plaque formation is endothelial dysfunction. Endothelial dysfunction involves a positive feedback loop wherein endothelial damage, triggered by pathological stimuli such as hypertension, allows oxidized LDL (ox-LDL) deposition, driving an immune response that supports atherogenesis [20].

A key signaling molecule following endothelial dysfunction is nitric oxide (NO). NO is produced by endothelial nitric oxide synthase (eNOS), and regulated by Akt-mediated phosphorylation [21]. In atherosclerosis, NO has demonstrated potent protective effects by inhibiting LDL oxidation, leukocyte adhesion, smooth muscle cell

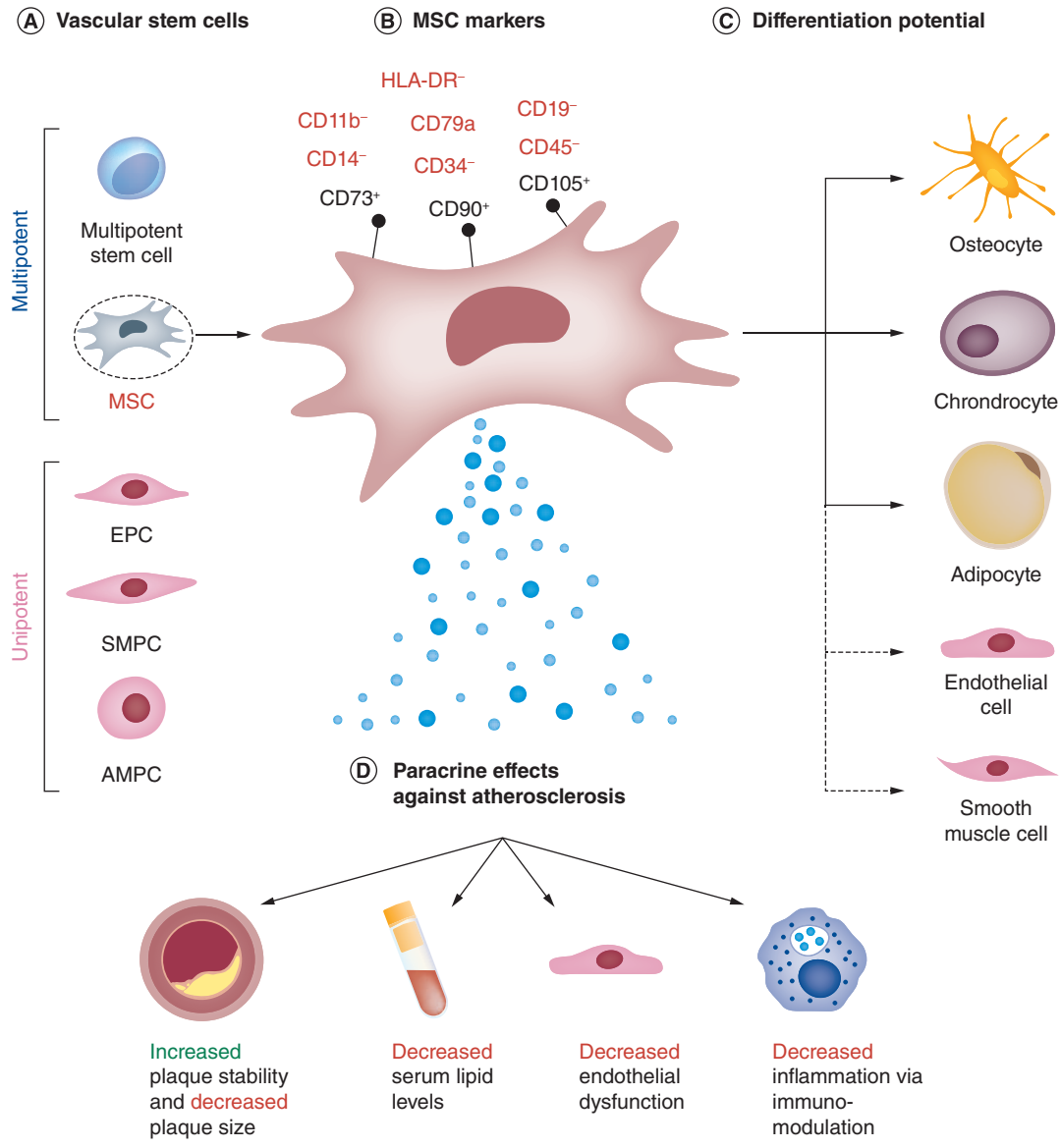

Figure 1. Schematic representation of the types of vascular stem cells, the markers of mesenchymal stem cells, the differentiation potential of mesenchymal stem cells and the paracrine effects of mesenchymal stem cells against atherosclerosis. (A) The types of multipotent and unipotent vascular resident stem cells. **(B)** The cell surface markers used to define MSCs according to the International Society for Cellular Therapy published guidelines. **(C)** MSC lineages. Solid arrows: confirmed lineage *in vitro* and *in vivo*; dashed arrows: potential lineage *in vivo*. **(D)** The key paracrine mechanisms of action by which MSCs may ameliorate atherosclerosis. Created with BioRender.com.
+: Expressed; −: Not expressed; AMPC: Adventitial macrophage progenitor cell; EPC: Endothelial progenitor cell; MSC: Mesenchymal stem cell; SMPC: Smooth muscle progenitor cell.

proliferation and platelet aggregation, while regulating vascular tone [22]. This is exemplified by the rapid progression of atherosclerosis noted in *eNOS*$^{-/-}$ mice [23]. However, Lin *et al.* found that ox-LDL inhibits NO production, via a reduction in Akt, and subsequently, eNOS phosphorylation, negating NO's protective effects and promoting endothelial dysfunction [24].

MSCs have been shown to successfully restore endothelial function, thus halting atherogenesis [25]. Culture medium sought from human skin-derived MSCs increased NO production in human aortic endothelial cells, demonstrating their paracrine potential [25]. Since, Lin *et al.* have revealed that human MSCs prevent ox-LDL-mediated inhibition of eNOS activity in human umbilical vein endothelial cells, through the phosphorylation and restoration of Akt/eNOS activity [24]. Importantly, intravenous delivery of allogeneic bone marrow-derived MSCs

(BM-MSCs) restored endothelium-dependent relaxation via an increase in phosphorylated Akt/eNOS, in a mouse model of atherosclerosis. Further investigations revealed that anti-IL-8 antibodies prevented eNOS stabilization, suggesting that the ability of MSCs to restore eNOS function is dependent on IL-8 secretion [24]. However, IL-8 is also an important pro-atherogenic cytokine involved in the early stages of plaque formation as it expedites leukocyte extravasation and endothelial cell adhesiveness [26]. Therefore, the timing of MSC delivery may be important to prevent an exacerbation of early plaque formation.

More recently, investigations of the Akt pathway revealed that MSCs also upregulate SIRT1 in endothelial cells [27]. SIRT1 is a key mediator of endothelial function as it protects against endothelial senescence and inflammation [28].

MSCs reduce hyperlipidemia

Hyperlipidemia is a well-established risk factor for atherosclerosis [2], and there is strong evidence showing that administration of MSCs reduces lipid levels in various hyperlipidemic animal models [29]. However, the mechanism by which this occurs remains unclear.

Frodermann and colleagues first observed that BM-MSCs significantly reduce serum cholesterol, particularly very-low-density-lipoproteins (VLDLs), in *LDLR*$^{-/-}$ mice, albeit 4 weeks post-administration [30]. A concomitant reduction in Kupffer cell LPL was observed, which aligns with previous literature [31]. Considering findings that TNF-α induces Kupffer cell LPL expression [32], Frodermann *et al.* suggested that the VLDL-lowering effect occurred via MSCs reducing TNF-α levels [30]. However, serum TNF-α was not reduced significantly in the experiment, casting doubt upon this proposed mechanism. Although subsequent studies demonstrated an MSC-mediated reduction in TNF-α [33], further investigation of this LPL mechanism must be conducted to support this mechanism.

Hong and colleagues demonstrated that gingival-MSC administration into *ApoE*$^{-/-}$ mice resulted in a reduction of total cholesterol and LDLs [29]. They also observed a reduction in SREBP-1c expression, a transcription factor involved in fatty acid biosynthesis, and an increase in PPAR-α expression, a transcription factor modulating fatty acid β-oxidation, as corroborated by other studies [34]. Combining these key observations suggests an alternative mechanism underlying MSC-mediated lipid-lowering. Indeed, Li *et al.* showed that administration of umbilical-cord-blood-MSCs to leptin-deficient mice resulted in a reduction in lipid levels [35]. They attributed this to an observed increase in PPAR-α and reduction in fatty acid synthase, an enzyme regulated by SREBP-1c, corroborating the findings of Hong and colleagues.

Overall, there is strong evidence that MSC administration lowers serum lipid levels, subsequently reducing lipid accumulation in plaques [36]. Although some studies found no change in lipid levels [24], their timescale was not long enough to observe an effect. While MSC-mediated anti-inflammatory signalling appears to underpin lipid reduction via altered lipid metabolism, further work is required to elucidate the exact mechanisms involved and to reproduce results in humans.

MSCs reduce inflammation

The classical risk factors of atherosclerosis, including: aging [37], hypertension [38], hypercholesterolemia [39], diabetes [40] and obesity [41] all promote inflammation. For instance, ox-LDL accumulation activates the NLRP3 inflammasome which drives the recruitment and migration of leukocytes into the plaque [42]. In the past decade, elucidation of MSCs anti-inflammatory properties [43], when primed in an inflammatory environment [44], highlights their therapeutic potential. The modulatory effects of MSCs on immune cells will be considered, with key studies summarized in Table 1.

Dendritic cells

Dendritic cells (DCs) are a type of antigen presenting cell implicated in atherogenesis via their role in priming and activating T cells [51]. *In vitro* studies have concluded that MSCs can inhibit dendritic differentiation and maturation, impair antigen uptake and decrease costimulatory molecule expression (CD80, CD86), thereby reducing T-cell activation and proliferation [52]. This was recently attributed to the secretion of extracellular vesicles containing miRNA-21-5p by MSCs. However, hyperlipidemia modifies DC function in inflammation [53], therefore, it is worth noting that these studies were not conducted in the presence of ox-LDL to simulate a hyperlipidaemic environment. Thus, future research needs to clarify the effect of MSCs on DCs in the presence of ox-LDL to recapitulate the lipid-rich environment of atherosclerotic plaques.

Table 1. Mesenchymal stem cells' effect in inflammation, particularly regarding T cells and macrophages in atherosclerosis.

Immune cell types	Reference	Source	Model	Techniques	Main findings	Ref.
T cells	Di Nicola Di *et al.*	Human BM-SC	*In vitro* human cell culture	Transwell migration assay	SCs suppress $CD4^+$/$CD8^+$ T cells; even without direct cell-to-cell contact	[45]
	Frodermann *et al.*	Mice BM-MSC	Pre-treatment with MSC in atherosclerosis mice model	Dilated supernatant	MSCs suppress $CD3^+$ T cells via cell-to-cell contact	[30]
Regulatory T cells	Frodermann *et al.*	Mice BM-MSC	Pre-treatment with MSC in atherosclerosis mice model	Dilated supernatant	Initial 51% increase in Tregs and progression to 10% decrease from baseline	[30]
	Wang *et al.*	Mice BM-MSC	MSC post-treatment in chronic atherosclerosis mice model	Histology of spleen, lymph node and aortic plaque Treg: effector T-cell ratio measured	MSCs promote an anti-inflammatory environment. MSCs increase number and activity of $CD4^+CD25^+FOXP3^+$ Treg subpopulation and decrease effector T-cell populations	[46]
	Cahill *et al.*	Mice BM-MSC	*In vitro* cell culture	Co-culture in six well plate	MSCs augment Treg induction via ligand Jagged-1 activation of Notch signaling	[47]
	Rashedi *et al.*	Human BM-MSC	*In vitro* human cell culture	Cell-to-cell contact in Transwell migration assay	MSCs augment Treg induction via TLR3 and TLR4 increasing Notch signaling	[48]
Macrophages	Adutler-Lieber *et al.*	A-MSC	*In vitro* human cell culture	Transwell migration assay. M2 markers: $CD206^+$, $CD163^+$ and $CD16^+$	A-MSCs can polarise macrophages into anti-inflammatory phenotype	[49]
	Li *et al.*	S-MSC	MSC post-treatment in chronic atherosclerosis mice model	Histology of aortic arch	S-MSCs decrease plaque size. S-MSCs promote a NF-κB dependant anti-inflammatory cytokine profile	[8]
	Zhang *et al.*	Human G-MSC	MSC post-treatment in chronic atherosclerosis mice model and in vitro human macrophage culture	M0, M1, M2 phenotype co-cultures. Histology – aortas, spleen cells, lymph node cells. G-MSC tracking *in vivo*	G-MSCs decreased plaque area and spleen/blood/lymph node macrophage numbers. G-MSCs can polarise macrophages into anti-inflammatory phenotype	[50]
	Wang *et al.*	Mice BM-MSC	*In vitro* macrophages cultured with ox-LDL	Histology: Oil-red-o staining Transwell membrane	Decrease in foam cell formation by decrease in scavenger receptors: CD36 and SRA	[46]

A-MSC: Adipocyte mesenchymal stem cell; BM-MSC: Bone marrow-derived mesenchymal stem cell; BM-SC: Bone marrow stromal cell; G-MSC: Gingival-derived mesenchymal stem cell; MSC: Mesenchymal stem cell; ox-LDL: Oxidized low-density lipoprotein; SC: Stem cell; S-MSC: Skin-derived mesenchymal stem cell.

T-lymphocytes

Nicola *et al.* showed that human BM-MSCs reduced DC-induced $CD2^+$ T-cell proliferation in a dose-dependent manner, via secretion of anti-inflammatory cytokines (TGF-β) [45]. Cell-to-cell contact was shown to augment this rate of inhibition [45]. Frodermann *et al.* [30] confirmed these findings in $CD3^+$ T cells, but controversially extrapolated that cell-to-cell contact is required to reduce their proliferation. Upon comparison of study methodologies (Table 1), contrastingly to Nicola *et al.*, Frodermann *et al.* diluted the MSC supernatant, which may explain the absent effect on T-cell proliferation.

More recently, focus has shifted toward exploring the atheroprotective effect of MSCs on Tregs [30]. With MSC therapy, Frodermann *et al.* observed a small initial increase in circulating Tregs, that progressively declined over time. However, mice were pre-treated with MSCs before atherosclerosis induction, limiting therapeutic relevance. Wang *et al.* [46] administered MSCs following the establishment of chronic atherosclerosis – a model with greater translational value (Table 1). Their results showed an increase in the number and activity of $CD4^+CD25^+FOXP3^+$ Tregs in the blood, spleen and most importantly, plaques. This may be driven by an MSC-mediated shift from a pro-inflammatory (IFN-γ, IL-6) to anti-inflammatory (IL-10 and TGF-β) environment, promoting Treg expression over effector T cells. MSCs are also thought to upregulate Tregs directly by activating the ligand Jagged-1 and TLR-3/TLR-4 on the MSC surface, both of which induce Notch signaling [47].

Monocytes & macrophages

In atherosclerosis, chemotaxis drives monocyte migration from the blood and adventitia into the intima, where they differentiate into macrophages. Mature macrophages express pattern recognition receptors, allowing them to engulf LDLs, becoming foam cells [54]. Multiple studies have shown MSC-mediated reductions in macrophage foam cell

formation *in vitro*, via modulation of scavenger receptor expression, including CD36, SRA1 and ATP-binding cassette transporter (Table 1) [46].

Macrophages also demonstrate high plasticity in different environments, being capable of expressing a spectrum of phenotypes ranging from pro- to anti-inflammatory. *In vitro*, MSCs were shown to reduce the expression of chemokine receptors on inflammatory monocytes and to promote phenotype switching to anti-inflammatory macrophages (Table 1) [50]. *In vivo*, MSC promotion of anti-inflammatory cytokine profiles and suppression of macrophage numbers systemically were used to explain the reduction in plaque size [8,50]. However, sparse *in vivo* data assessing MSCs' effects on macrophage density and phenotype switching specifically within the plaque, proves a major limitation. This may relate to difficulties in defining consistent macrophage surface markers [55]. Macrophage polarization is a dynamic process, in which environmental cues, such as lipid profiles, induce distinct transcriptional regulators that alter macrophage phenotype [56] and complicate the use of classical markers in atherosclerosis.

MSCs increase plaque stability

Several studies cite a reduction in plaque size as evidence for MSC efficacy in atherosclerosis [57,58]. However, plaque size is a poor sole indicator of disease severity, and the impact of plaque stability on risk of rupture must be considered [59].

Plaque rupture, and subsequent luminal thrombosis, is the predominant mechanism underlying AMI and ischemic stroke [60]. Established criteria can identify 'vulnerable plaques' and flag them for intervention. Reduced fibrous cap thickness (<65 μm) is the primary criterion, with additional indicators of vulnerability including the presence of a large necrotic core, significant macrophage and lymphocyte infiltrates, intraplaque hemorrhage and micro-calcifications [61].

The therapeutic application of MSCs in atherosclerosis appears to enhance plaque stability, targeting the above pathological changes observed in vulnerable plaques. Adoptive transfer of MSCs into rabbit and murine models resulted in significantly greater fibrous cap thickness, with increased vascular smooth muscle cell (VSMC) numbers and collagen content [62]. Although the mechanism is unclear, inhibition of VSMC, endothelial cell and macrophage apoptosis [62], and MMP expression (particularly MMP-1) [62] may contribute to the increase in fibrous cap thickness. However, given the complex relationship between MMPs and plaque stability, which is further obscured by contradictory findings surrounding the roles of MMP-2/9 in stabilization [63], considerable investigation must be undertaken to evaluate this speculation.

Intriguingly, MSCs have also demonstrated an ability to, both directly and indirectly, reduce foam cell formation by decreasing macrophage conversion to foam cells *in vitro* [46] and lowering circulating monocyte levels *in vivo* [30]. Consequently, this decrease in foam cell formation hinders progression toward a lipid-rich necrotic core.

The paracrine-mediated immunopotency of MSCs, both locally and systemically, is therapeutically relevant to plaque stability. Locally, MSCs induce macrophage phenotype switching from a pro- to anti-inflammatory profile [50,57]. While no effect on intra-plaque lymphocyte density has been observed [57], systemically, MSCs reduced effector ($CD62L^-$) $CD4^+$ and $CD8^+$ T-cell subpopulations, and decreased IFN-γ and IL-467, highlighting their capacity to repress cell-mediated inflammation [30]. These local and systemic immunomodulatory effects reduce inflammation and plaque infiltration, enhancing stability.

Increased fibrous cap thickness, inhibition of foam cell formation, and reduced leukocyte infiltration collectively ameliorate plaque vulnerability, demonstrating the value of MSC administration in promoting plaque stabilization.

MSCs & regeneration

The paracrine effects of MSCs in atherosclerosis have been outlined above. The controversies surrounding their differentiation potential and therapeutic benefits will now be discussed.

It is well defined in the literature that both BM-MSCs and adipose-derived MSCs (A-MSCs) can differentiate into endothelial-like cells (ELCs) *in vitro* [64]. Thus, current research has focused on optimisation, such as culturing with recombinant VEGF-A rather than VEGF [65]. Therapeutically, MSC-derived ELCs have been shown to promote angiogenesis and vascular repair *in vivo* [66], as illustrated by a double-blind randomized controlled trial on critical limb ischaemia that observed improved perfusion [67]. Although this trial does not focus on atherosclerosis directly, it does show a potential for MSCs in improving its downstream ischemic effects. Furthermore, following wire-induced vascular injury, Wang *et al.* found that BM-MSCs home to the site of injury, differentiate to ELCs and contribute to endothelial repair [68].

However, Wang *et al.* noted that while MSCs contributed to re-endothelialization, they also increased intimal hyperplasia and vascular stenosis. This contradicts reports that MSCs decrease atherosclerotic plaques, thus increasing their stability. This fuels the debate as to whether MSCs can contribute to atherogenesis [68]. MSCs have been shown to differentiate into VSMCs *in vitro* [69]. Kramann *et al.* [19] identified a well-defined population of MSCs expressing markers of VSMC differentiation ($Ptc1^+$, $Sca1^+$, PDGF receptor-β^+ and $CD34^+$). Use of inducible genetic fate-tracing experiments, which are more reliable than non-inducible techniques, confirmed that MSCs have the potential to differentiate into VSMCs *in vitro* [19]. Consequently, MSCs have been shown to contribute to up to 50% of intimal smooth muscle cells in atherosclerotic plaques, aggravating the disease [70]. However, as discussed by Caplan *et al.*, there is little evidence to show that MSC differentiation to VSMCs holds true *in vivo* [14]. Indeed, Iso *et al.* showed that MSCs did not differentiate into VSMCs [69]. While Liao *et al.* suggested that they may even differentiate into osteoblast-like cells in the intima, worsening plaque calcification, this only occurred only in the presence of an osteogenic inductor [71]. Interestingly, Caplice *et al.* found that BM transplantation contributed to VSMCs in existing atherosclerotic plaques [72]. However, this study could not confirm that the MSCs served as VSMC-progenitors. Furthermore, the plaque specimens were obtained from chemotherapy-induced immunocompromised patients who may have poorer systemic clearance of MSCs, explaining the excessive engraftment to existing plaques [73].

Although evidence suggests that MSCs cannot differentiate into functional VSMCs *in vivo*, caution still needs to be taken when using MSCs as therapy for atherosclerosis in humans, as its effect on plaque formation is still inconclusive [74]. Furthermore, MSCs differentiation potential *in vivo* to osteocytes, chondrocytes and adipocytes, is likely not replicated in the vasculature due to the unfavorable microenvironment and mechanical stimuli present.

Limitations of MSCs

When considering the application of MSC therapy in humans, safety is imperative. In rodent models, tumor formation was noted in early reports using MSCs. Particularly, BM-MSC transplantation was reported to induce gastric cancers and malignant sarcomas [75]. This has been attributed to a certain degree of chromosomal instability in BM-MSCs [76]. Nevertheless, standardized cell culture conditions and limiting the propagation duration can decrease the risk of chromosomal abnormalities [77]. Numerous clinical trials have reported no risk of tumorigenesis with MSCs and systematic reviews have demonstrated MSC safety in humans [78]. Another concern is MSCs may produce embolic events, when delivered systemically, due to their size. In animal models, MSCs often become entrapped in the lungs following administration, potentially limiting their therapeutic potential as they are unable to home to the site of injury. Furthermore, when delivered intra-arterially, cerebral infarcts have been reported, suggesting that safe routes of administration need to be developed [79]. Pulmonary entrapment has also been reported in human trials. However, this was noted to be a transient phenomenon which did not interrupt homing to myocardial injury [80]. This would suggest that MSCs therapeutic efficacy is not diminished via intravenous delivery, making it a safe and effective option. Lastly, MSCs have poor immunogenicity, therefore, MSCs obtained from allogeneic donors can be used in acute conditions for which autologous MSCs cannot be derived. However, some reports challenge this theory, noting that allogeneic MSCs are not intrinsically immunoprivileged and can be rejected by an immunocompetent host [81]. The possible differentiation of allogeneic MSCs *in vivo* may increase their immunogenicity [74].

In an attempt to overcome these limitations inherent to all cell therapies, there has been a recent surge of interest in cell-free based approcahes. A myriad of miRNA that modulate, either promote or inhibit, atherosclerosis plaque development have been identified along the years [82]. More recently, MSC-derived exosomes containing miRNAs have been proposed as a solution to overcome the cell-related MSC drawbacks described above, while delivering the therapeutic benefits of MSCs [83]. MSC-exosomes were first investigated in a myocardial/reperfusion injury mouse model in 2010 [84]. Its angiogenic and anti-apoptotic effects have been extensively described [85]. Sparse *in vitro* studies have demonstrated that MSC-exosomes are also associated with inhibition of inflammation, for instance by suppression of T-cell proliferation [86]. To this date, only one study has been conducted in atherosclerosis models, in which MSC-exosome injection into $ApoE^{-/-}$ mice was shown to reduce plaque area, potentially by reducing macrophage infiltration via the mir-let7/IGF2BP1/PTEN pathway and promoting macrophage polarisation into anti-inflammatory phenotypes via mir-let7/HMGA2/NF-κB pathway [87]. Despite the potential of cell-free based approaches to capitalise on the therapeutic benefits of MSCs while overcoming some of their limitations, the technique is not well established. Indeed, their therapeutic potenital in atherosclerosis is inconclusive and there is no literature on the safety and efficacy of exosome administration in clinical trials.

Considerations for therapeutic efficacy

This review demonstrates the compelling potential of MSCs as a therapy for atherosclerosis. However, translating pre-clinical data into clinical practice presents a challenge, as MSC use in atherosclerosis has yet to be investigated in clinical trials. Clinical trials using MSC therapy in other vascular diseases and heart failure have delivered promising results, attesting to the feasibility of MSC-based therapy for atherosclerosis [88]. Furthermore, there are many ongoing clinical trials which are aimed at demonstrating the safety and efficacy of MSCs in cardiovascular disease, as summarized by Guo *et al.* [89]. However, a consensus on how best to source, culture, pre-treat and administer MSCs has not been reached, resulting in variable therapeutic efficacy in clinical trials thus far.

Sourcing MSCs for therapeutic use is a controversial topic. BM-MSCs, first described in 1976, have been the most frequently used in vascular research [90]. However, BM aspiration is an invasive procedure that can cause serious, albeit infrequent, morbidity [91]. Furthermore, cell yield is low, at 1/100,000 cells, and decreases with age, as does proliferation potential [92]. In contrast, A-MSCs are easily isolated, provide a greater cell yield, and have a faster rate of expansion [93]. A-MSCs may also exhibit reduced osteogenic potential [94], thus, limiting their risk of contributing to plaque calcification. However, the properties of A-MSCs are dependent on anatomical location [93]. Thus, future investigations need to elucidate the location that offers the most effective MSC phenotype, to optimise and standardise subsequent research. Additionally, there is a lack of studies directly comparing the effectiveness of MSCs depending on their origin, therefore, the optimal source has yet to be established and requires further research.

As aforementioned, allogeneic MSCs could be considered due to their low immunogenicity and immunosuppressive properties [95]. Hare *et al.* demonstrated the superiority of allogeneic MSCs, since autologous MSCs are susceptible to age and co-morbidity-related decline in efficacy [96]. Indeed, the prevalence of atherosclerosis increases with age, and concomitantly, MSCs show an age-related decline in efficiency, with decreased proliferation and migration [97]. Oxidative stress has been implicated as a key factor involved in this decline. Pre-treating autologous MSCs could provide a potential solution to age-related senescence and negate the requirement for allogeneic MSCs which carry a risk of immune rejection. Zhang *et al.* investigated the use of MSCs in myocardial infarction and showed that pre-treating MSCs with asprosin, an adipokine that inhibits reactive oxygen species (ROS) generation, significantly improved MSC homing and proliferation while reducing apoptosis and scar size [98]. Lee *et al.* also demonstrated the effectiveness of pre-treatment with O-cyclic-phytosphingosine-1-phosphate which, through reversal of ROS damage, upregulated proliferation, migration and anti-apoptosis [99]. These studies propose innovative ways of rejuvenating autologous MSCs for the treatment of atherosclerosis. Additionally, stem cell manipulation prompts exciting possibilities of preconditioning for therapeutic enhancement of both autologous and allogeneic MSCs [100]. For example, priming MSCs with pro-inflammatory cytokines has been shown to upregulate their immunomodulatory effects [101]. Additionally, atorvastatin pre-treated MSCs displayed enhanced migratory ability and cardioprotective effects [102], while *N*-acetyl-L-cysteine upregulated their immunomodulatory effects [103]. Interestingly, induced pluripotent stem cell-derived MSCs acquire a rejuvenated gene signature, irrespective of donor age, providing an alternative method of overcoming MSCs age-related senescence [104].

The optimal route of MSC delivery is debated. Local administration is widely utilised in cardiac diseases [87]. Direct injection into the myocardium allows for precise delivery of MSCs, which may confer greater effectiveness [105]. However, this is an invasive procedure which may carry unnecessary risk. Intravenous infusion is easy to administer and has been preferentially used in the studies presented in this review. Furthermore, MSC homing to atherosclerotic regions is well-documented when given intravenously, counteracting the need for direct injection [24]. While this process is not fully elucidated, it is thought MSCs adhere to vascular endothelium via binding to P-selectin. *In vivo*, P-selectin is expressed on activated endothelium, particularly that of active atherosclerotic plaques [106,107]. Greater understanding of this mechanism could alter the administration of MSCs in order to further improve the proportion of MSCs that home to the site of vessel injury. Conversely, intra-arterial delivery may increase MSC delivery to target organs. However, as previously mentioned, it was associated with a risk of embolic events in animal models which was avoided when MSCs were administered intravenously [79]. Coronary artery injection, at the time of diagnostic angiography, may be a potential delivery method to investigate in future trials.

Current clinical trials using MSC therapy in AMI have used a dose of 1×10^6/kg (NCT01652209). However, it cannot be concluded definitively that this would be the optimal dosage in atherosclerosis. Additionally, pre-clinical studies demonstrate that the effects of MSCs are short-lived [24], and therefore, the possibility of multiple dosing should be explored.

A clearly defined target population is vital to any novel therapy. Based on the evidence presented in this review, administration of MSC therapy could reduce plaque size and risk of rupture, alleviating symptoms and decreasing the risk of future cardiovascular events. Thus, MSCs may be beneficial as an adjuvant therapy, aimed at reducing the dosage or duration of anticoagulation therapy following an acute coronary event or revascularisation procedure, particularly in patients with high bleed risk as assessed by the HAS-BLED score. Additionally, MSCs may provide a therapeutic alternative in patients who remain symptomatic despite optimal medical therapy, but for whom revascularization is inappropriate, including those with diffuse atherosclerosis. In turn, such patient populations should be the starting point for clinical trials involving MSC, though more appropriate targets may be identified as trial data is compiled.

Conclusion & future perspective

In conclusion, this review demonstrates the remarkable aptitude of MSCs as a treatment for atherosclerosis. Compelling evidence has been presented that MSCs can inhibit endothelial dysfunction, reduce hyperlipidaemia, decrease inflammation and stabilize existing atherosclerotic plaques. This combination of effects makes them suited to combat several pathological elements of atherosclerosis, thus rendering MSC therapy an excellent choice for future research.

To support their clinical translation, pre-clinical studies need to validate MSCs inability to differentiate into functional vascular cells *in vivo* to refute their possible contribution to atherogenesis. Additionally, tailoring the pre-conditioning of MSCs to optimise their atheroprotective effects presents an exciting avenue to pursue and, subsequently, apply clinically.

Clinical trials need to assess whether the preclinical effectiveness of MSCs in atherosclerosis can be translated to humans. This will clarify whether the aforementioned beneficial effects of MSCs affect hard end points, such as cardiovascular events and mortality. Additionally, practicalities such as the optimal dose and frequency of administration need to be established to maximize treatment efficacy. Subsequently, the process of MSC isolation, proliferation and pre-treatment should be standardized in order to improve clinical outcomes.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/rme-2021-0024

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Executive summary

- Atherosclerosis remains the leading cause of cardiovascular mortality worldwide.
- Mesenchymal stem cells (MSCs) provide a promising therapeutic option via attenuating risk factors involved in the pathogenesis of atherosclerosis, primarily through a paracrine mechanism.

MSCs decrease endothelial dysfunction

- The trigger for plaque formation is endothelial dysfunction. This is primarily regulated by LDL induced inhibition of nitric oxide (NO) production.
- MSCs prevent LDL-mediated inhibition of endothelial NO synthase, thus, increasing NO production and restoring endothelium-dependent relaxation.

MSCs reduce hyperlipidemia

- Hyperlipidemia is a well-established risk factor for atherosclerosis.
- MSCs significantly reduce circulating cholesterol and LDLs while increasing high-density lipoproteins.
- This process may be mediated via alterations in transcription factors involved in fatty acid biosynthesis and oxidation.

MSCs reduce inflammation

- Inflammation has recently been defined as a key pathological feature of plaque formation, progression and rupture.
- MSCs suppress both CD4 and CD8 T cells and increase the proportion of Treg cells.
- MSCs impair dendritic cell differentiation and maturation, thereby reducing T-cell activation.
- MSCs produce a phenotypic shift in macrophages from a pro-inflammatory to an anti-inflammatory state producing an anti-inflammatory cytokine profile with reduced foam cell formation.

MSCs increase plaque stability

- Plaque rupture, due to instability, is the predominant mechanism underlying myocardial infarction and ischemic stroke.
- MSCs decrease the size of atherosclerotic plaques which confers greater plaque stability.
- MSCs increase fibrous cap thickness, with increased smooth muscle cell and collagen content, creating a more stable plaque.
- As MSCs attenuate the inflammatory environment of an atherosclerotic plaque, this reduces plaque infiltration and enhances stability.

MSCs & regeneration

- There is controversy as to whether exogenous MSCs can differentiate into functional cells *in vivo*.
- MSCs have demonstrated the potential to differentiate into endothelial-like cells *in vivo*, improving vascular perfusion in models of hind limb ischemia.
- MSCs have also been shown to home to the site of vascular injury and contribute to re-endothelialization.
- However, MSCs may also contribute to intimal hyperplasia which can perpetuate vessel stenosis.

Limitations of MSCs

- In limited rodent models, MSCs led to tumor formation, however, the safety of MSCs has been persistently proven in human trials with no evidence of tumorigenesis.
- In animal models, MSCs have demonstrated a risk of vascular entrapment when delivered systemically, with intra-arterial delivery producing cerebral infarctions. Conversely, there been no ischaemic events in human trials.

Considerations for therapeutic efficacy

- While the safety and efficacy of MSCs in cardiovascular disease is being considered in clinical trials, a consensus of how best to source, culture, pre-treat and administer MSCs has not been reached.
- Bone marrow-derived MSCs have been the most frequently researched cell type, however, adipose-derived MSCs provide an appealing alternative given their simpler method of harvesting.
- Both intravenous and intra-coronary artery delivery of MSCs provide a promising mechanism for delivery due to the ability of MSCs to home to sites of atherosclerosis.

Conclusion

- MSCs have remarkable potential as a treatment for atherosclerosis.
- Clinical trials need to focus on the optimisation of sourcing, pre-treatment and delivery in order to maximise efficacy and standardize future research.

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