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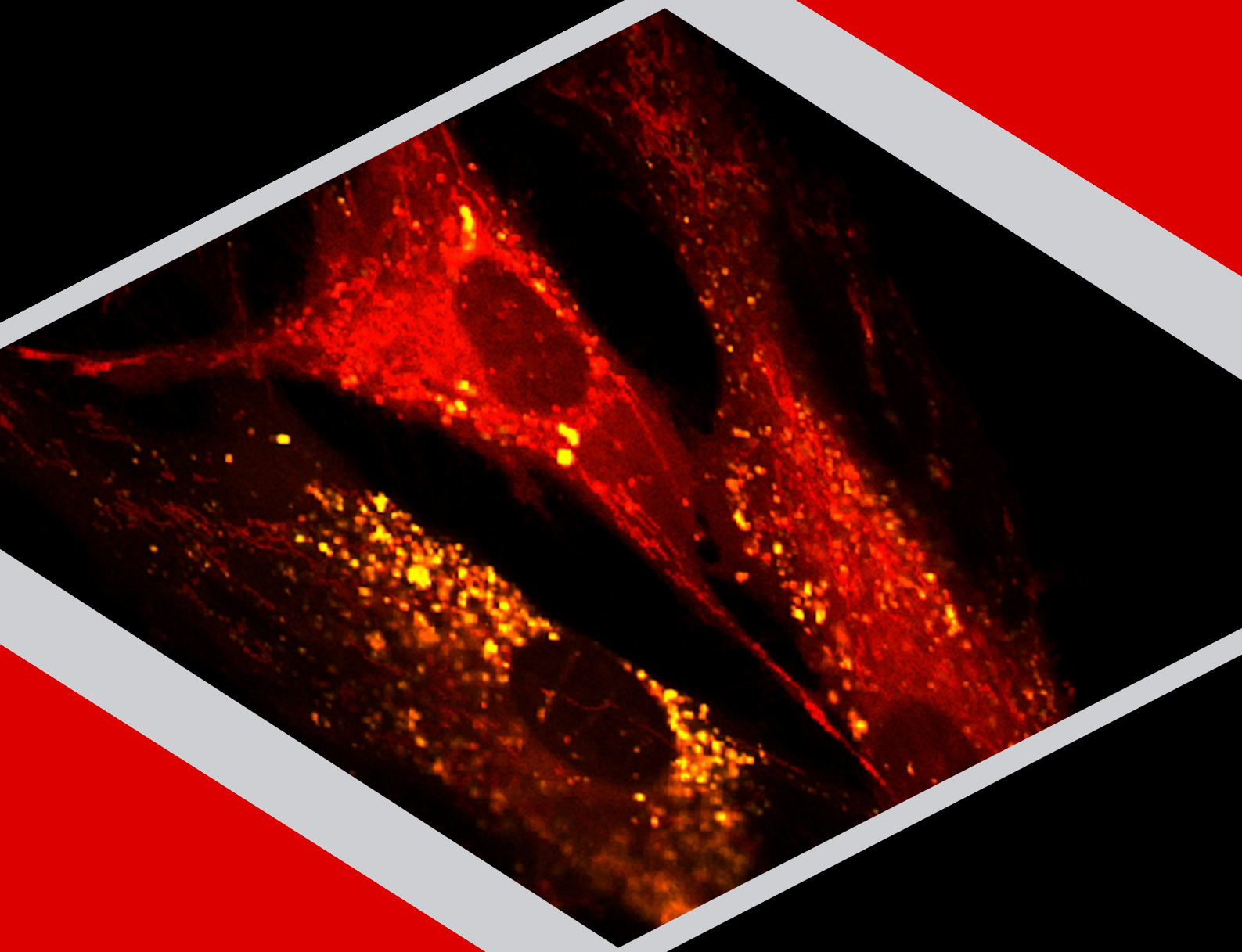

Mesenchymal stem cells: a cell culture handbook

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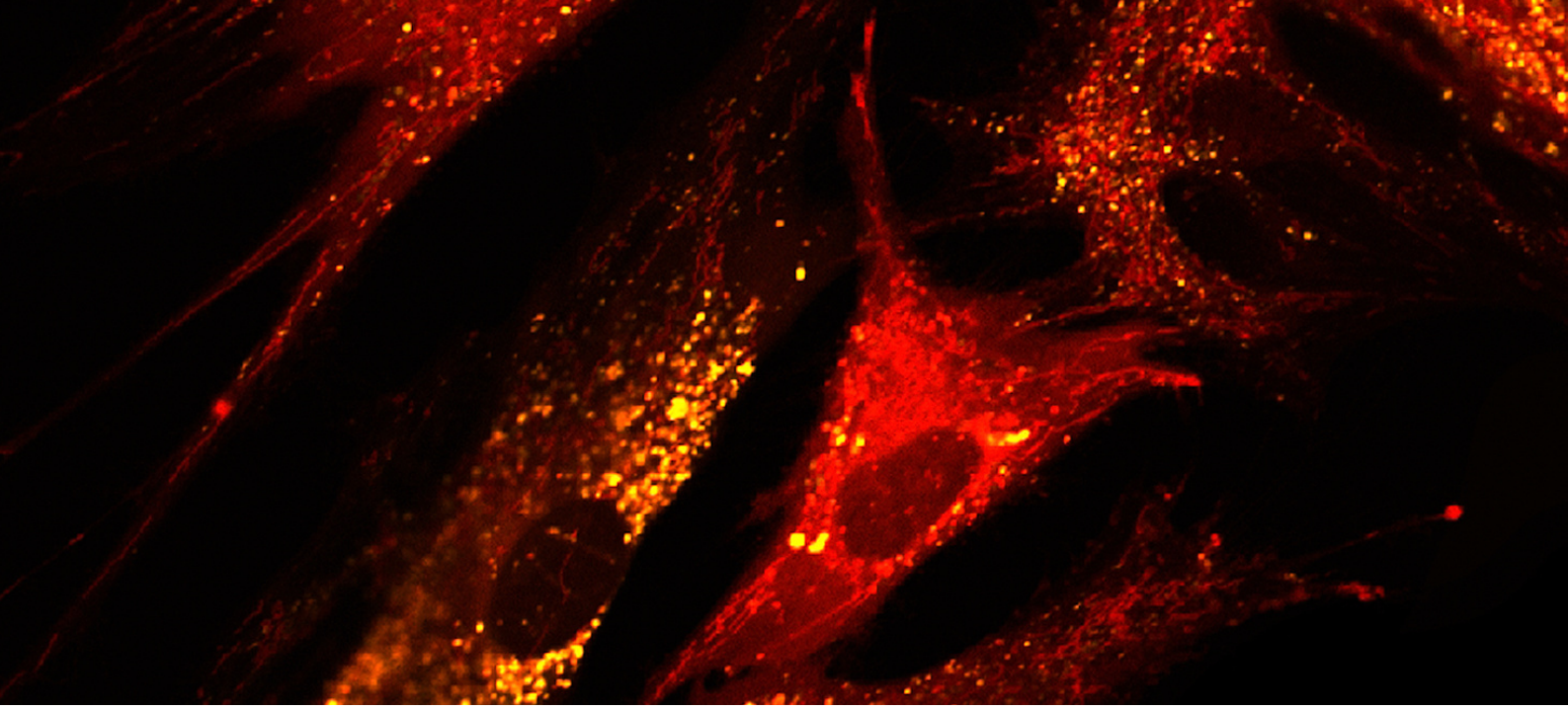

Foreword

We are pleased to present you with this eBook on mesenchymal stem cells: standardized cell culture media for cell therapy applications.

Mesenchymal stem cells (MSCs) have become extremely desirable tools in regenerative medicine due to their differentiation potential, immunomodulatory properties and their ability to promote tissue repair through the release of cytokines and growth factors.

When culturing MSCs for cell therapy applications, the cell culture medium is a critical factor. The medium used influences the growth, viability and differentiation of the cells and as a result affects the final therapeutic.

In this eBook, we delve further into the importance of having optimized cell culture media and protocols when working with MSCs for cell therapy applications, how different media types influence your cell culture, overcoming challenges when switching to xeno-free media and more!

We hope you enjoy reading these expert insights into standardized cell culture media for cell therapy applications with us.

Megan Giboney
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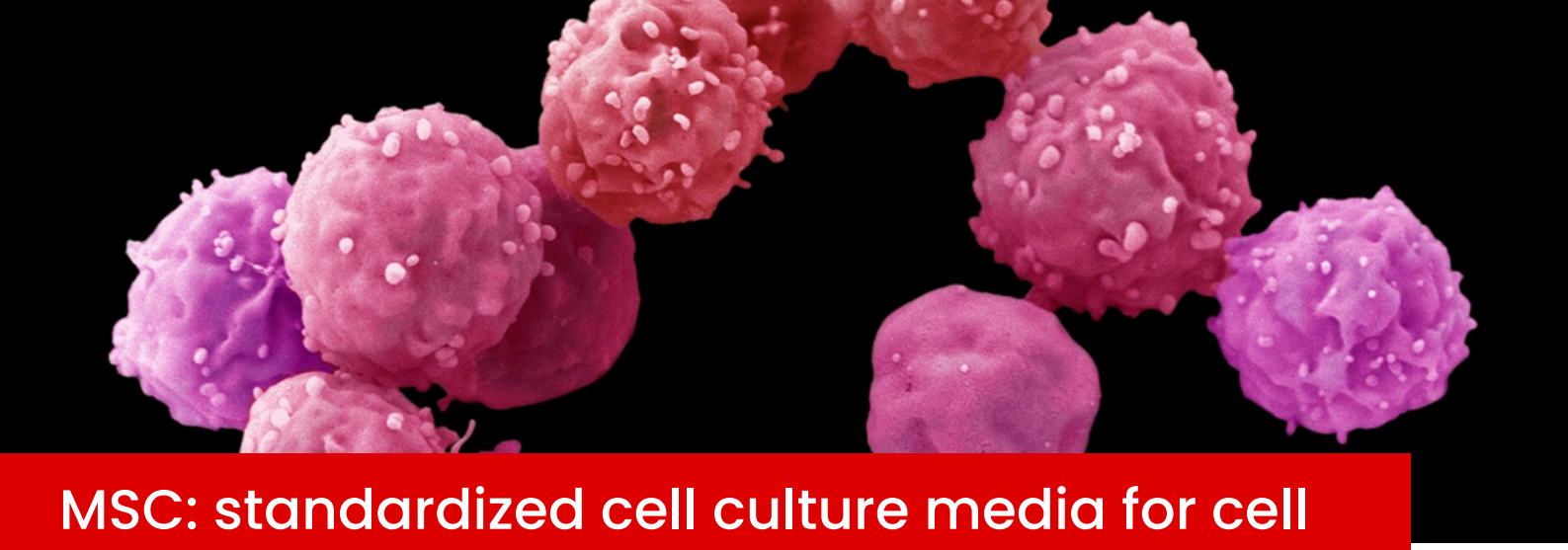

MSC: standardized cell culture media for cell therapy applications

Dr Hagen Wieland has been a member of PromoCell's R&D-team, located in Heidelberg, Germany, for more than 15 years. His main focus is on stem- and cancer-cell biology.

Hagen has gathered more than 20 years of cell culture experience in different research areas. Before joining PromoCell, he explored the pathogen defense mechanisms of monocytes and later gained a strong background in the isolation and culture of embryonic stem cells as well as various types of adult stem cells during his work in the scientific field of livestock biotechnology.

During his time at PromoCell Hagen led the development of several new products founding the company´s current stem-, blood- and cancer cell portfolio division. Examples are PromoCell's unique, fully functional cryopreserved macrophages along with the corresponding serum-free and xeno-free media or the defined and animal component-free Primary Cancer Culture System D-ACF. Another important product group are PromoCell's human mesenchymal stem cells (hMSC) of different tissue origins which are completed with a range of expansion and differentiation media including the serum-free and xeno-free MSC Growth Medium XF.

Dr Hagen Wieland
Research and development expert, PromoCell (Heidelberg, Germany)

1 Why is it important to refrain from using serum in cell culture?

Traditionally, cells have been propagated in vitro almost exclusively in standard culture media supplemented with varying amounts (2–20%) of fetal bovine serum (FBS). Like other undefined media supplements, FBS is not only beneficial to cell growth but also holds several unwanted fluctuating physiological, genetic and epigenetic cellular effects known to cause experimental variability.

As demonstrated for hormone-responsive cell lines, immune system-related cell types and stem cells undefined conventional culture media can interfere with cell properties and experimental readouts. For instance, it may falsify their responses to drugs, provoke unspecific bogus immune responses or cause unwanted differentiation of stem cells. Cells can also be significantly altered by culture conditions of this kind by the uptake and stable incorporation of potentially immunogenic animal-derived components e.g., non-human sialic acids. It follows that having the best possible controlled culture environment is key for obtaining more accurate results in cell culture and facilitates data analysis and interpretation.

In addition, an extensive body of national regulatory authorities stands by to enforce local safety regulations regarding the movement of serum-containing products from one country to another, as

well as the subsequent use of such products. Using media containing animal-derived serum in a regulated environment poses significant official challenges and is sometimes even impossible.

Apart from scientific and regulatory reasons, ethical concerns related to animal welfare represent another significant downside of using FBS in culture media, demanding at least for a reduction in the use of this type of serum to a necessary minimum. Using human serum instead of FBS could be a viable alternative but is, unfortunately, more expensive and does not perform as well for most types of cells as compared to FBS.

2 What makes it so difficult to simply swap out the serum in cell culture media?

Serum as a culture medium supplement exhibits a plethora of activities due to its highly complex composition. While some functionalities of serum in cell culture media have been identified decades ago, the current lack of rather defined and well-performing serum-free media for most types of human primary cells reflects the manifold undiscovered traits still submerged in this “magical liquid”. Not to mention the innumerable interactions potentially occurring between or with serum components e.g., between growth factor binding molecules and endogenous or exogenously added growth factors.

The provision of attachment factors, lipids, transferrin-bound iron and transport proteins are a few of the well-known functional mechanisms exerted by serum. Indeed the knowledge about many other of its functions is still patchy and frequently observational in nature with regard to one specific cell type.

As a result, the development of serum-free media is elaborate since all significant requirements of the cell, traditionally fulfilled by serum, need to be implemented in the serum-free formulation. Because of the sheer complexity of this task, serum-free media are mostly developed with a rather cell-type specific functional range limited to reproduction of the serum-mediated functions exclusively relevant for the respective cell type.

Frequently relevant serum functionalities are simply unknown and can therefore not be easily mimicked by more defined compounds. As a workaround serum-free media often contain serum fractions, human platelet lysate, hydrolysates of animal- or non-animal origin, or extracts e.g., from bovine brain, which are all nearly as ill-defined as serum itself.

Despite decades of research and thousands of publications on this topic to date, there is still no synthetic replacement precisely reflecting all major beneficial functions of serum as a universal medium supplement for various cell types.

3 Are there any differences in the end product?

Because they are also serum-free in most cases, one of the main challenges in xeno-free cell culture media products is to manage stability issues with sensitive media components e.g., cytokines and lipids.

Serum provides tremendous capabilities in stabilizing all kinds of delicate molecules in the medium and is responsible for keeping their integrity and bioactivity up for long periods of time. In contrast, more defined media lack these stabilizing effects rendering them prone to significant limitations in shelf-life and thermal stability. That´s why it is of utmost importance that the supplier instructions for the transport and storage of these products are strictly followed.

For these stability reasons and to achieve optimal performance, some xeno-free media formulations even require the addition of exogenous growth factors/cytokines or lipoprotein by the user just when reconstituting the complete medium.

4 Are all xeno-free media also serum-free and more defined when compared to FBS-supplemented media?

Not at all! There are two categories of xeno-free mesenchymal stem cell (MSC) media on the market. The first and most prevalent group is xeno-free media containing completely undefined supplements of human origin i.e., platelet lysate, fractionated or whole human serum. The latter, however, is used rarely since whole human serum tends to form extensive lipid precipitates leading to cloudiness of the media, which is unacceptable in most experimental settings. Due to this drawback of using complete human serum as a media supplement, at least most xeno-free will also be serum-free, which should, however, be explicitly stated by the media manufacturer in the product information of the referring product.

Hence, despite these media being xeno-free and mostly also serum-free their composition is far from being "defined" in any way – in fact, they are nearly as undefined as traditional media containing animal serum.

The second group of available xeno-free and serum-free MSC media refrain from using entirely undefined supplements in their formulations for the sake of a more defined and standardized product providing significant regulatory advantages in controlled environments, too. For example, the PromoCell Mesenchymal Growth Medium XF, which is also available in GMP grade, is such an exception since it contains neither human serum or fractions thereof nor platelet lysate. Instead, the only semi-defined omponent contained in this medium is highly purified albumin (HSA) derived from human plasma.

5 What challenges do you have to consider when switching to xeno-free cell culture? How do you overcome these?

Not all xeno-free media, which are mostly also serum-free, provide the same robustness of the culture process and functionality compared to their FBS-containing counterparts. Serum-free media are often slightly more cumbersome and expensive to use, while these media do, for example, not always support the efficient isolation and primary culture of human cells.

In order to minimize stability issues xeno- and serum-free media must be stored under the recommended conditions. Complete media bottles must never be pre-warmed or exposed for longer periods of time to ambient temperature and/or light. Instead, the medium should be picked from the refrigerator and the amount needed for the experiment should be dispensed before putting the media bottle back into appropriate storage conditions as quickly as possible.

In addition, frequent media changes keep the cells happy and improve the robustness of the serum-free culture process by replenishing spent nutrients and growth factors.

For subculturing cells grown in serum-free medium, trypsin activity must be quenched by a separate inhibitor after cell detachment since the trypsin inhibitors contained in serum are absent. Alternatively, non-enzymatic or self-inactivating cell detachment reagents can be used.

While undefined xeno-free MSC media containing platelet lysate or fractions of human serum may already contain cell attachment factors, more

defined xeno-free media usually must be used in combination with cultureware coated with an extracellular matrix e.g., fibronectin. Therefore, it is mandatory to make sure the used culture vessels are coated properly with an appropriate attachment substrate before use, where required. When switching to a xeno-free medium it is also advisable to check whether the cellular phenotype and biological functions of interest remain unchanged in the new culture setting. The researcher should also consider the possibility that in some experimental settings components contained in xeno- and serum-free media might interfere with downstream assays or applications established using a serum-containing culture system.

6 What would you consider the optimal culture medium for MSCs?

From a technical and regulatory perspective, the optimal MSC medium would be a culture medium of defined composition containing only non-animal/non-human sourced single chemical compounds and recombinantly expressed peptides/proteins of non-animal origin. The latter should be present at the minimum necessary number and concentration to reduce the cost and complexity of the formulation and minimize lot-to-lot variations, which may be potentially introduced even by recombinantly expressed proteins.

In addition, the optimal medium for MSC should be easy to handle and not require pre-coating of the culture surface. Indeed, anoptimal MSC medium should also support the primary isolation of MSCs and their extensive expansion without losing multipotency.

PromoCell®

GMP-compliant MSC culture media

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Xeno-free MSC culture: high yield, better viability and morphology

Application note

Background

Human mesenchymal stem cells (hMSCs) have potential for massively impacting the fields of cell-based therapy, tissue engineering and regenerative medicine due to their ready availability, stem cell properties and immunomodulatory activity. In order to create optimal growth conditions for human mesenchymal stromal cells *in vitro*, it is essential to mimic specific aspects of the *in vivo* microenvironment. Cell culture media are often supplemented with fetal bovine serum to provide nutrients and growth factors that promote the survival and growth of cells. However, serum use also suffers from a number of disadvantages such as unknown composition, batch-to-batch variation, the risk of infectious agents and contaminants, and animal welfare concerns. Due to the physiological variation of serum, it may also impede the reproducibility of research results or lead to their misinterpretation. Moreover, using serum-containing medium limits the impact of research results regarding clinical applications.

Serum-free culture medium is an excellent alternative that enables controlled and reproducible conditions. Being adherent cells, hMSCs require attachment molecules on the culture surface, some of which are provided by the serum and consequently missing under serum-free conditions. hMSCs therefore often exhibit altered adhesion, morphology, and proliferation when cultured in serum-free media. To avoid this, media suppliers often recommend precoating of the cell culture plasticware with extracellular matrix protein extracts such as fibronectin. Another recently developed strategy enables researchers working with serum-free media to use a chemically defined surface coating that comes in a ready-to-use form. This innovative new product, called myMATRIX MSC, creates optimal growth conditions for hMSCs by using biomimetic peptides and complex oligosaccharide molecules to mimic the human body's extracellular matrix. myMATRIX MSC is suitable for scientists who don't want to use products of animal origin. At the same time, it ensures consistently good quality without any fluctuations. myMATRIX MSC delivers fast results and is both easy to handle and cost-effective.

Coating	Medium	Passage 0 (6)		Passage 1 (7)		Passage 2 (8)	
		Cell count	Viability	Cell count	Viability	Cell count	Viability
myMatrix MSC	MSC Growth Medium XF	5.7	99	5.6	99	4.6	99
	Serum-free competitor medium	4.8	97	5.3	97	4.6	93
Fibronectin (human)	MSC Growth Medium XF	3.7	96	4.15	97	2.8	98
Tissue culture treated plastic	Serum-comtaining	2.2	96	1.25	94	1.25	94
	MSC Growth Medium XF	2.0	94	1.8	97	2.05	95
	Serum-free competitor medium	1.2	91	0.97	85	0.66	85

Tab. 1: Cell counts (in x10^5) and viability (in %) of MSCs in passages 6, 7 and 8 on different coatings and with different media compositions. *Cells grown on myMATRIX MSC in serum-free media (PromoCell MSC Growth Medium XF, serum-free competitor medium) show very high cell counts and viabilities. The best performance was observed using a combination of myMATRIX MSC and our MSC Growth Medium XF, yielding very high cell counts and 99% viability throughout the study. Cell expansion on fibronectin-coated plates and our xeno-free medium resulted in lower counts and viabilities compared to myMATRIX MSC and serum-free medium. Cell growth and viability was reduced on uncoated plasticware.*

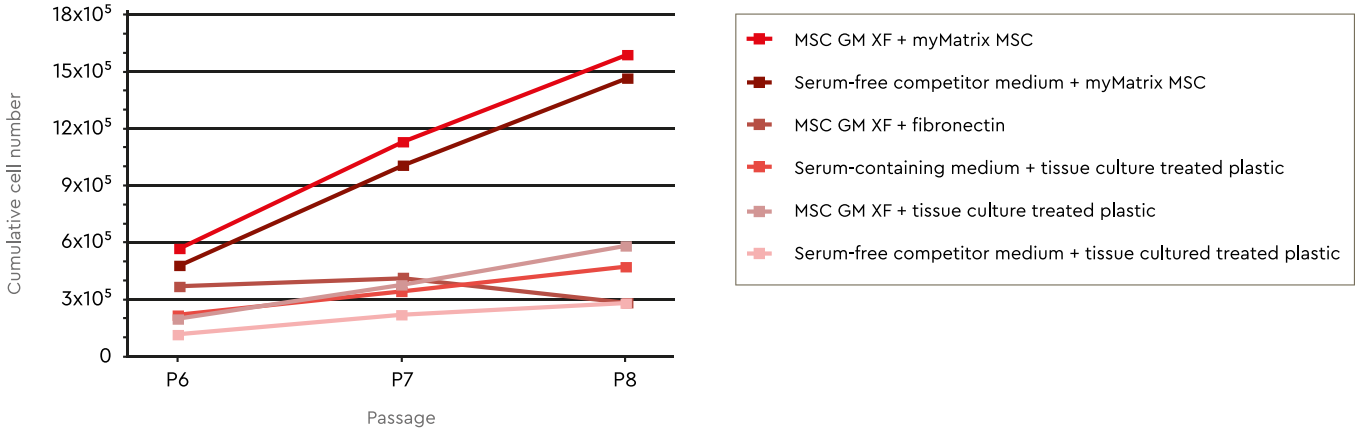

Fig. 1: Cumulative cell numbers of MSCs in passages 6, 7 and 8 on different coatings and with different media compositions. *The cumulative cell numbers demonstrate enhanced cell proliferation on myMATRIX MSC in serum-free medium, especially when using our MSC GM XF.*

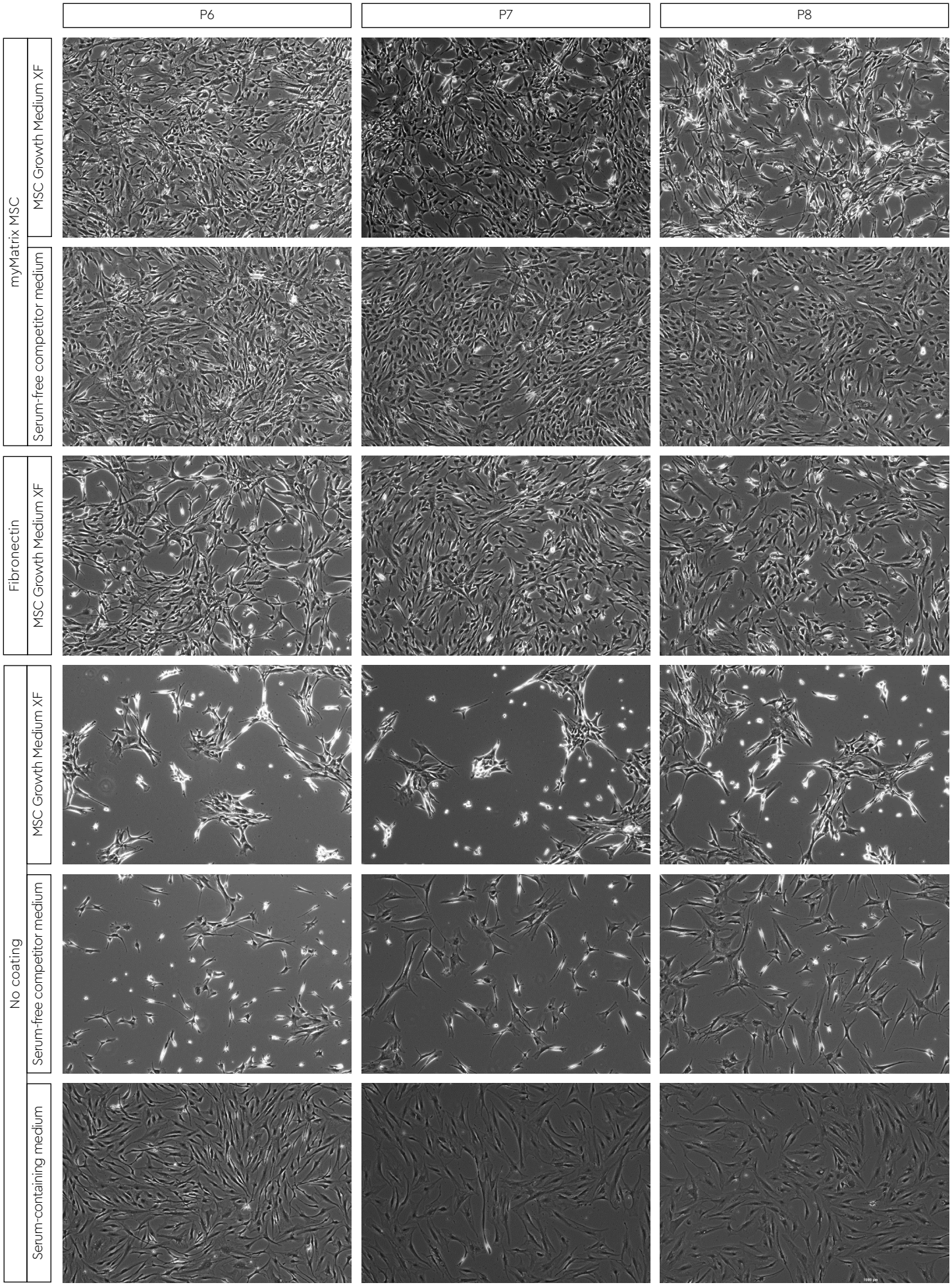

Fig. 2: Phase contrast images of MSCs in passages 6, 7 and 8 on different coatings and with different media compositions. *Cells grown in serum-free media (PromoCell MSC Growth Medium XF, serum-free competitor medium) on myMatrix MSC and on fibronectin-coated plates show a spindle-shaped, fibroblast-like morphology as well as smaller sizes than in serum-containing conditions. Tissue culture treated plasticware without coating was not sufficient to support MSC growth.*

Protocol for xeno-free expansion of MSCs

myMATRIX MSC are precoated plates that imitate the human extracellular matrix, thus providing optimal growth conditions for our human mesenchymal stem cells. Different media were used to compare cell culture conditions. MSCs were expanded until passage five in serum-containing medium on tissue culture treated plastic.

I. Serum- and xeno-free culture of MSCs on myMATRIX MSC

Materials

- Human bone marrow MSCs, passage 6 (C-12974)
- Serum-free medium: Mesenchymal Stem Cell Growth Medium XF
- (C-28019)
- Serum-free competitor medium
- Serum-containing medium: DMEM (low glucose, GlutaMAX™ Supplement, pyruvate, ThermoFisher Scientific, #21885025) + fetal bovine serum (10%, Sigma Aldrich, #F7524)
- Penicillin/Streptomycin (100 U/ml)
- Fibronectin from human plasma (Sigma Aldrich, #F2006)
- TrypLE Express (ThermoFisher Scientific, #12604013)
- Phosphate-buffered saline (PBS)
- Tissue culture treated cell culture vessels
- myMATRIX MSC (denovoMATRIX, #C0701)
- Trypan Blue stain

Use aseptic techniques and a laminar flow bench.

1

Fibronectin coating

Incubate the cell culture vessels with 5 µg/ml in PBS for two hours at 37°C.

2

Harvest and plate the cells

Harvest cells from existing culture at 70–80% confluence. Plate the cells at a density of 5.000 cells/cm² (5 ml per T25 flask) and incubate at 37°C and 5% CO_2. After four days take phase contrast images (4x).

3

Cell subculture

At 80% confluency, remove the medium from the flask, wash with PBS, and incubate the cells with TrypLE Express for three minutes at 37°C while tapping the flask. Then, stop trypsin activity by adding pre-warmed serum-containing medium and gently rinse the flask. Centrifuge the cell suspension for three minutes at 210 x g, remove supernatant, and resuspend the pellet in corresponding growth medium. Afterwards, count the cells and determine the viability with an automated cell counter by using an aliquot of resuspended cell pellet with Trypan Blue stain (1:1).

4

Expand the cells

After three passages, seed the cells on a 24-well plate and let them grow for three days for fluorescent staining.

II. Fluorescent staining of MSCs

Materials

- 4% paraformaldehyde (PFA)
- Phosphate-buffered saline (PBS)
- PBS with Ca^{2+}/Mg^{2+}
- 0.1% TritonX solution
- Blocking buffer/antibody solution (3% donkey serum in PBS)

Use aseptic techniques and a laminar flow bench.

1

Wash and fix the cells

Wash the cells gently and briefly in PBS with Ca^{2+}/Mg^{2+}. Then, fix the cells with 4% PFA for 10 minutes and rinse them twice with PBS.

2

Permeabilize the cells

Permeabilize the cells with 0.1% TritonX for 15 minutes and rinse them three times with PBS.

3

Incubate the cells with blocking buffer for one hour

4

Primary antibody

Incubate the cells with the primary antibody (Integrin β1/CD29, 1:500) solution for 1 hour. Then, wash the cells three times with PBS.

5

Secondary antibody

Incubate with the secondary antibody (AlexaFluor 488, 1:500) solution for 30–60 minutes in the dark. Wash the cells afterwards four times with PBS.

6

Incubate the cells with phalloidin-Atto633

7

Incubate the cells with Hoechst solution for 10 minutes in the dark

Afterwards, wash the cells three times with PBS and directly proceed with imaging using BioTek Lionheart FX microscope 4x objective.

Results

- Proliferation: best performance on our MSC Growth Medium XF (PC MSC GM XF) + myMatrix MSC
- Viability: highest on PC MSC GM XF + myMATRIX MSC (99%)
- Morphology: spindle-shaped, fibroblast-like, smaller than in serum-containing condition
- Actin/integrin β1: prominent stress fibers in serum-free competitor medium myMATRIX/TCT and PC MSC GM XF/TCT, discrete integrin spots (focal adhesions) especially in PC MSC GM XF FN/TCT, integrin staining of myMATRIX PC MSC GM XF and serum-free competitor medium comparable

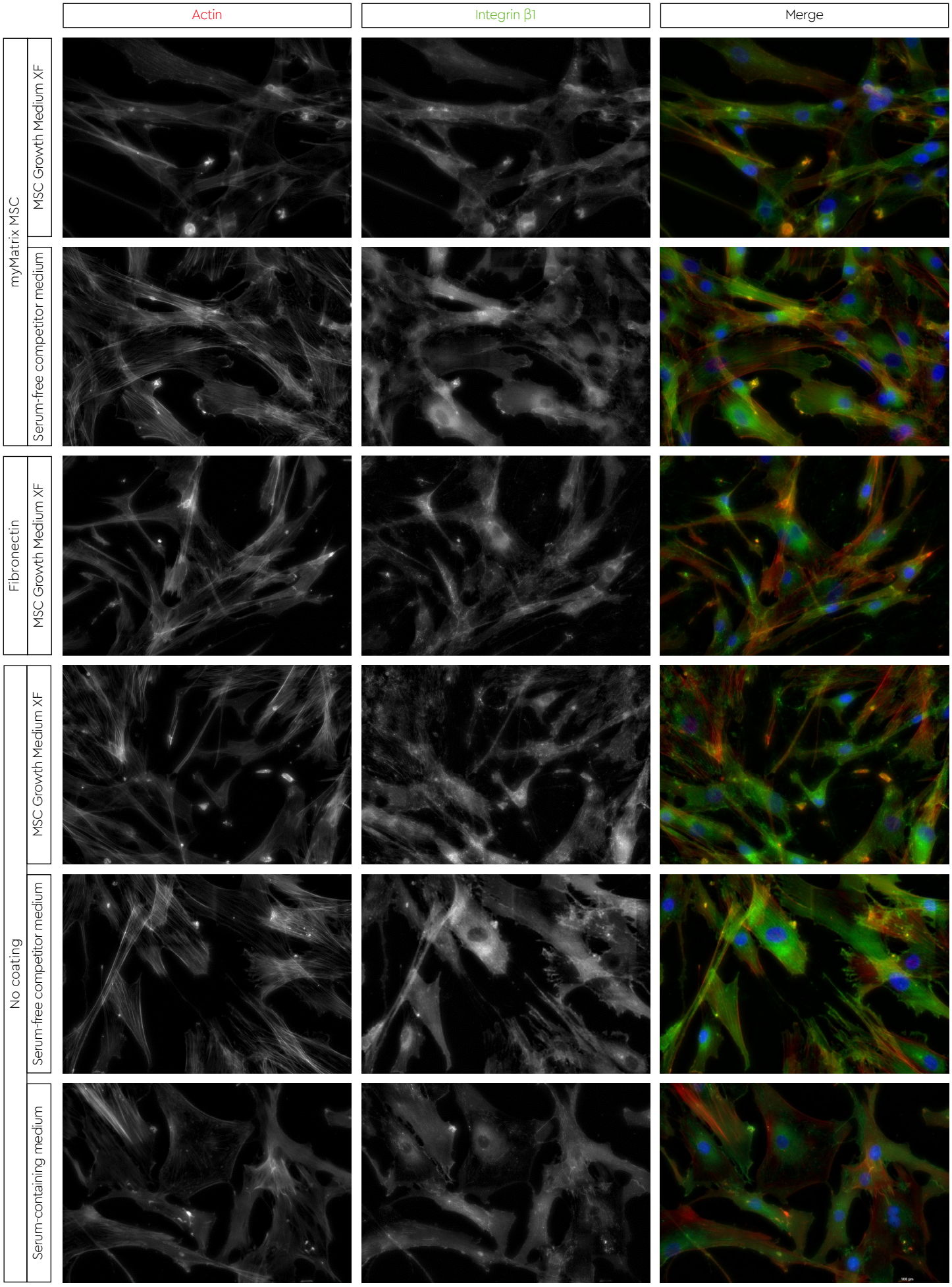

Fig. 3: Fluorescent stainings of MSCs in passages 6, 7 and 8. *Actin stress fibers are most prominent in cells cultured in PromoCell MSC Growth Medium XF and serum-free competitor medium without any coating. Discrete integrin spots (focal adhesions) are observed in all conditions, especially with our MSC Growth Medium XF on fibronectin-coated plasticware and without coating. The integrin pattern of cells grown on myMatrix MSC in serum-free media (PromoCell MSC GM XF and serum-free competitor) was comparable.*

Products

Product	Size	Catalog number
Human Mesenchymal Stem Cells from Bone Marrow (hMSC-BM)	500,000 cryopreserved cells 500,000 proliferating cells	C-12974 C-12975
Mesenchymal Stem Cell Growth Medium XF (ready-to-use)	500 ml	C-28019
screenMatrix www.denovomatrix.com/products/screenmatrix	96-well plate	S1001
myMATRIX MSC www.denovomatrix.com/products/mymatrix-msc	T75/T25 24-well plate/6-well plate	C0601/C0701 C0301/C0501

Related Products

Product	Size	Catalog number
Human Mesenchymal Stem Cells from Umbilical Cord Matrix (hMSC-UC)	500,000 cryopreserved cells 500,000 proliferating cells	C-12971 C-12972
Human Mesenchymal Stem Cells from Adipose Tissue (hMSC-AT)	500,000 cryopreserved cells 500,000 proliferating cells	C-12977 C-12978
Mesenchymal Stem Cell Growth Medium 2 (ready-to-use)	500 ml	C-28009
Mesenchymal Stem Cell Adipogenic Differentiation Medium 2 (ready-to-use)	100 ml	C-28016
Mesenchymal Stem Cell Chondrogenic Differentiation Medium (ready-to- use)	100 ml	C-28012
Mesenchymal Stem Cell Chondrogenic Differentiation Medium w/o Inducers (ready-to-use)	100 ml	C-28014
Mesenchymal Stem Cell Osteogenic Differentiation Medium (ready-to-use)	100 ml	C-28013
Mesenchymal Stem Cell Neurogenic Differentiation Medium (ready-to-use)	100 ml	C-28015
Accutase-Solution, primary human cell culture tested	100 ml	C-41310
Dulbecco's PBS, without Ca^{2+}/Mg^{2+}	500 ml	C-40232

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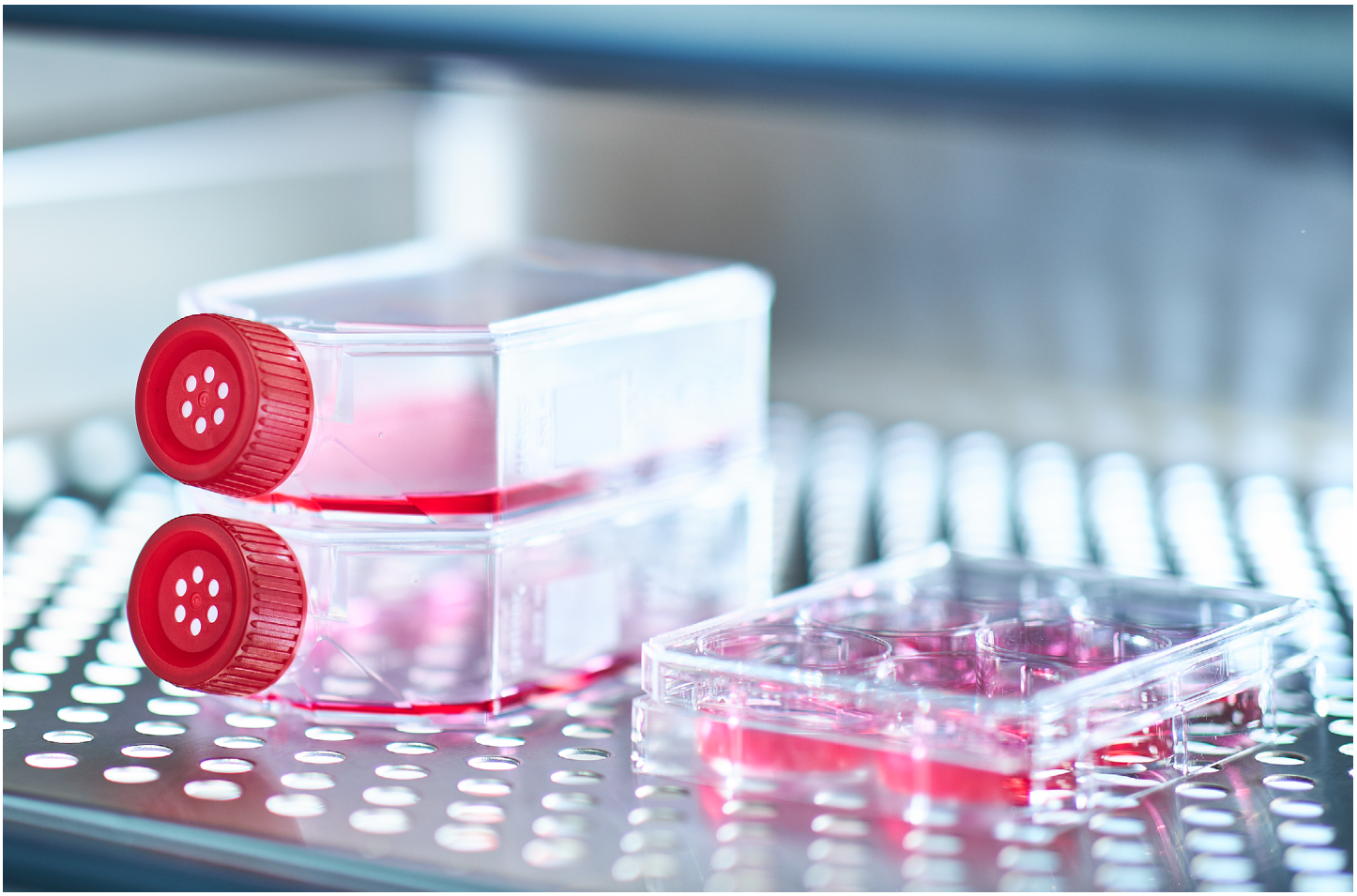
Mesenchymal stem cells: why optimizing manufacturing processes is key for a successful application

PromoCell®

Mesenchymal stem cells (MSCs) are the up-and-coming stars of cell therapy. They are able to differentiate, are very adaptable and can reduce inflammation, making them ideal for regenerative medicine approaches. But the demand for MSCs is far greater than supply. Pharmaceutical companies are racing to produce commercial-scale quantities of MSCs while guaranteeing quality standards.



In fact, in some ways, mesenchymal stem cells (MSCs) are not so different from high-tech computer chips. Even the tiniest component must function perfectly for the entire computer to work. Another parallel comes from the exponential growth of computing capacity in recent decades: Production and methods have had to sprint to keep up. Similarly, as the spectrum of potential MSC applications in regenerative medicine continues to increase, manufacturers must now shift to large-scale production. They need to provide a **high output of pharmaceutical-grade MSCs** made under stringent quality standards. The result: Patients will have sufficient access to these multitalented cells, which can restore normal functioning in the human body.

Stumbling blocks to large-scale MSC production

At the moment, producing standardized, well-characterized and high-quality MSCs in clinically sufficient quantities is an obstacle course. The first hurdle to be cleared is completing targeted preclinical research. This is mandatory, of course, so that authorities can validate processes and establish quality control criteria. But there is a further challenge: Because of the cellular heterogeneity of MSCs, scientists and industry have difficulty finding common ground. For example, how do they best identify, sort and track cells? And potential stumbling blocks of MSC expansion, freezing and thawing processes, along with final cell characterization, still need to be standardized. Yet the most troublesome aspect is coaxing these cells into performing optimally. After collection, MSCs need to be expanded *in vitro*, because **cellular therapies require far more cells than what scientist can extract from adult tissue.** In fact, MSCs make up just 0.01 percent of mononuclear cells in the bone marrow of adult donors. Their number in bone marrow aspirates is also low. For regenerative medicine to be effective, however, infusions must contain several million cells per kilogram of body weight (Mizukami and Swiech, 2018). This means that **the *in vitro* expansion phase is critical,** as it could negatively affect the function of MSCs and therefore their therapeutic potential. Donor-related factors such as age and gender, along with cell culture-related factors, can further influence how well MSCs reproduce (Fossett and Khan, 2012).

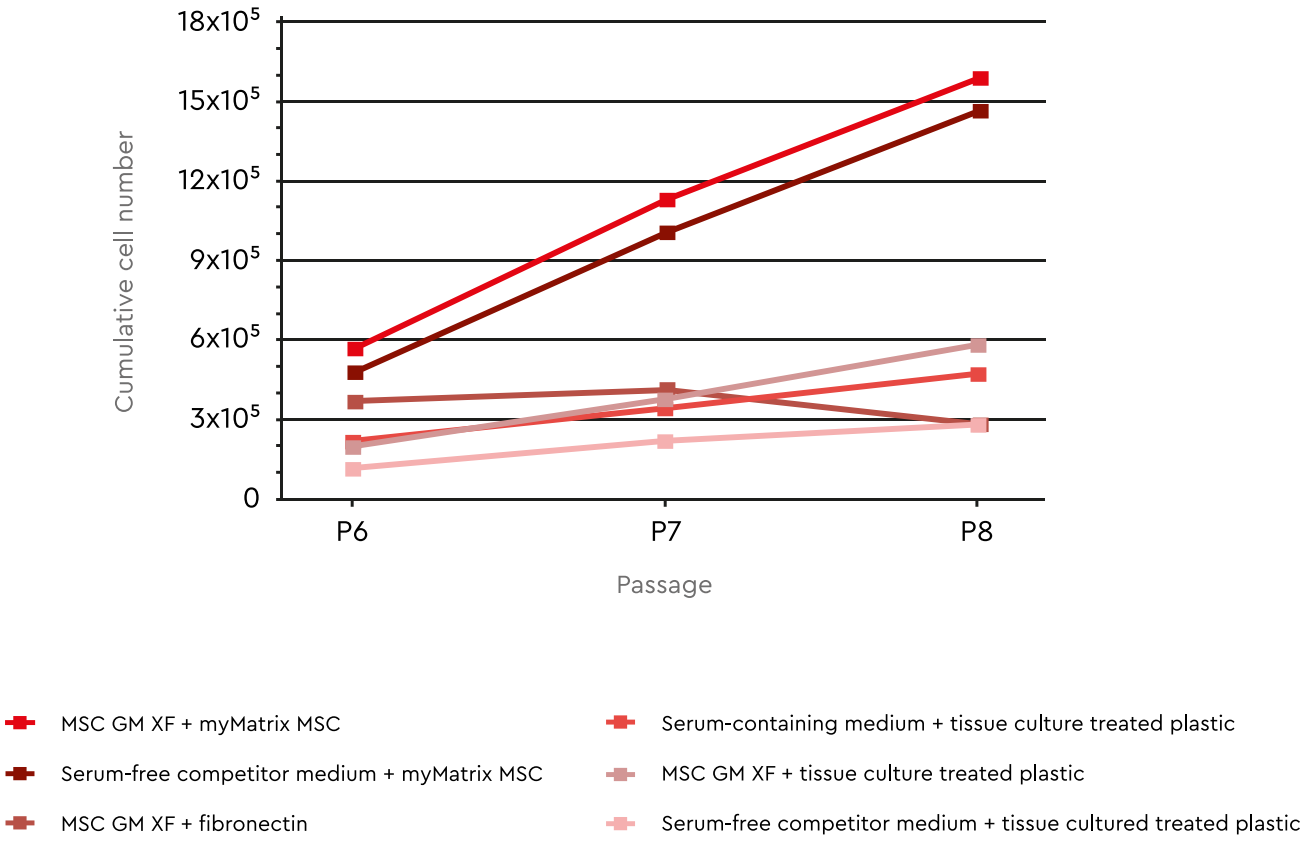


Figure 1: *Cumulative cell numbers of MSCs in passages 6, 7 and 8 on different coatings and with different media compositions. The cumulative cell numbers demonstrate enhanced cell proliferation in serum-free medium, especially when using our xeno-free and GMP-compliant MSC Growth Medium XF.*

Mesenchymal cells are also quite sensitive to their surroundings. When cultured in 2D monolayers, MSCs display signs of senescence. Their proliferation rate decreases, and their morphology changes from spindle shape to a flattened square shape as the number of passages increases (Hoch and Leach, 2014). Too much handling increases the risk of microbial contamination. And researchers do not yet agree on the optimal cell density for growth, with different studies suggesting different plating densities. (Sotiropoulou et al., 2006). Generally speaking, using lower seeding densities seem to support rapid expansion of cells. This makes cell culture procedure less time consuming and more cost-effective. At the same time, cell cultures are less likely to become contaminated or lose characteristic functions (Fossett and Khan, 2012).

Exploring various media, methods, and processes

Careful handling and alternative culture systems might motivate cell growth. With the aim to preserve key functional characteristics of MSCs and exploit their full therapeutic potential, researchers are testing various approaches:

1

3D culture

Using 3D culture systems instead of classical monolayers can help circumvent some limitations of 2D culture. A 3D system better resembles the *in vivo* situation and the physiological environment, both of which positively affect cellular functions (Egger et al, 2019).

2

Xeno-free environment

Using xeno-free standardized media can avoid xenogeneic responses. These include uncontrolled influences on MSC differentiation, caused by animal-derived culture supplements that are not well characterized. Regulators advise using xeno-free alternatives when defining safer and standardized protocols for producing therapeutic-grade cellular products (Cimino et al, 2017).

3

Bioreactors

Using bioreactors enables cell expansion in a fully closed, controllable and scalable culture system. This is crucial for high-scale production of MSCs in accordance to good manufacturing practices (GMP) and quality standards. Yet questions remain on using bioreactor systems – such as how to determine the best harvesting method, how to concentrate large volumes of cell solutions quickly without impairing cell viability and function, and when to replace traditional trypsin with newer enzymes that gently collect MSCs from microcarriers (Mizukami et Swiech, 2018).

4

Cryopreservation

Establishing new cryopreservation protocols for the use of nontoxic xeno-free supplements to store MSCs long term. Cryopreservation is the only method that can guarantee cellular products will be available quickly as well as in sufficient quantity to establish clinical-grade MSC banks for therapeutic applications (Rogulska et al., 2019, Lechanteur et al., 2016).

GMP-compliant MSC Culture Medium

Using high-quality cell culture media and reagents that comply with international regulatory guidelines is critical to ensure a seamless transition from bench to clinic. The EXCiPACT™ GMP standard for pharmaceutical excipients enables us to serve the growing demand of researchers working with our cell culture media and reagents in a regulated environment. Our product development pipeline is designed to ensure compliance with all relevant regulatory guidelines. By streamlining documentation of all raw materials, we reduce your regulatory burden, cost, and time. In addition, our manufacturing process enables us to offer customized products to meet your needs during product development and manufacturing.

Reach out to our experts to request your GMP service options

From bench to bedside: why consistent protocols are key for mesenchymal stem cell therapy

Every new therapy needs a set of ground rules and boundaries. This is why many ongoing preclinical research projects aim to establish regulatory guidelines. Such guidelines ensure that manufacturers produce MSCs according to standardized protocols, since medical experts need to have consistently high-quality cellular products to achieve positive treatment outcomes. In addition, when MSC therapeutics are reasonably priced, they will be used more often. Thus, more efficient manufacturing processes would lead to less expensive products, and lower costs would increase the availability of cell-based therapies for larger number of patients (Olsen et al., 2018). This **cost-efficiency is key** to unlocking access to the unique features of MSCs and to exploiting their therapeutic potential. As of July 2020, more than 700 recruiting, active or completed clinical studies in the US (clinicaltrials.gov) were investigating human MSCs in areas including heart conditions, neurological diseases, metabolic disorders and autoimmune diseases.

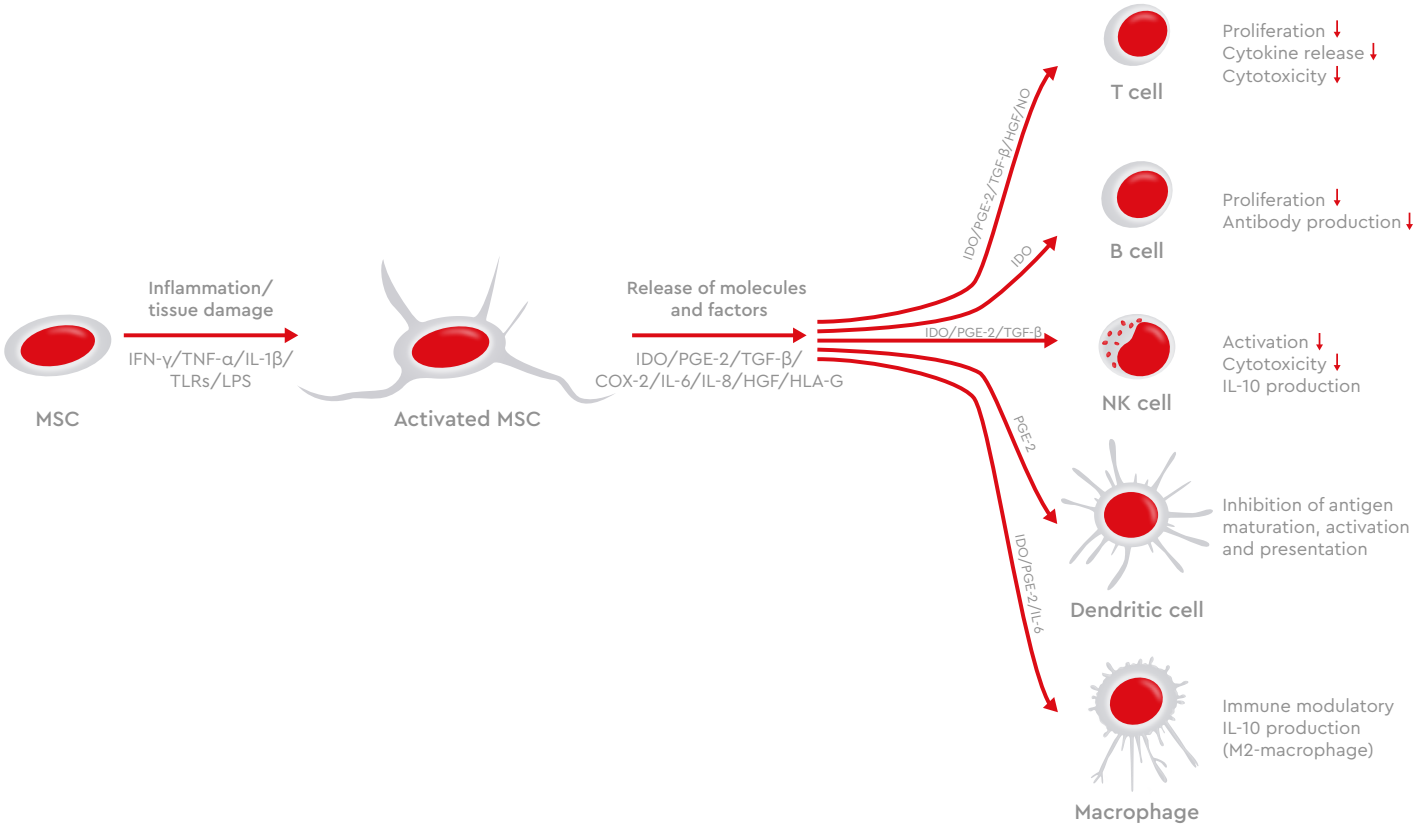


Figure 2: *MSCs modulate the innate and adaptive immune responses via the release of immunomodulatory molecules and factores. These immunomodulatory properties provide therapeutic benefits in the host body via paracrine effects and the stimulation of the host cells.*

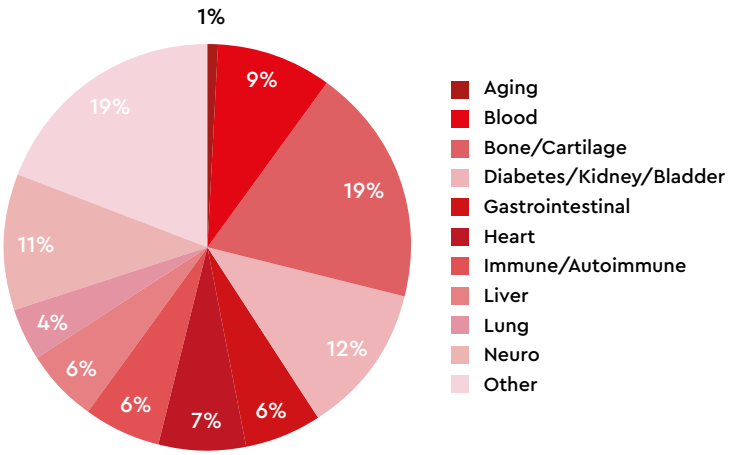
Human MSCs are also showing great promise in the treatment of graft-versus-host disease (GvHD), a common complication in patients receiving allogeneic hematopoietic stem cell transplants. In GvHD, donor immune cells attack recipient's tissues, such as in the skin, liver and gastrointestinal tract, where they can cause significant damage. Treatment is usually with systemic corticosteroids and immunosuppressants, but the condition often has a poor prognosis. However, the potential of MSCs to modulate the immune system benefit patients with GvHD (Zhao et al., 2019). Though most studies have shown that MSC therapy can improve overall survival for chronic GvHD, the results are sometimes conflicting. The evidence is not robust because of the small number of studies and the limited number of patients treated. Differences in protocols, means of MSC isolation, characterization and manipulation also make it difficult to compare MSC-based treatments. This example shows **how critical it is that production protocols are consistent and standardized** to deliver better clinical results (Godoy et al., 2019).

Ensuring quality, safety, and efficacy

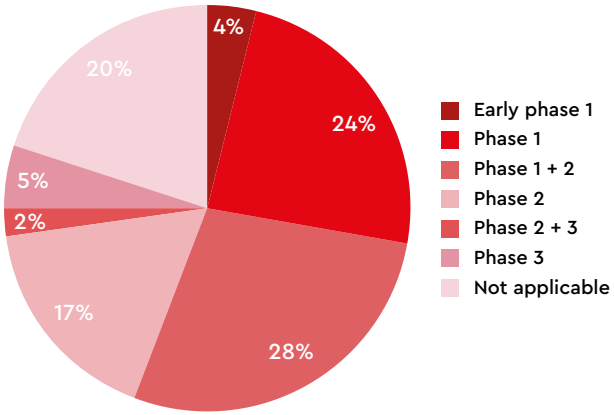
As with all pharmaceuticals, MSC-based therapies must be manufactured according to good manufacturing practices (GMP). However, there is no international consensus regarding quality control standards. At the national level, each research center agrees on production and application guidelines with the local authorities. A recent survey of US facilities that produce MSCs under GMP conditions offers an overview of the current best practices and shows how such practices affect product quality features such as cell yield, cell viability, purity, sterility, expression of surface markers and potency. This survey indicated that most facilities use bone marrow as MSC source, rely on plastic adherence to enrich those cells and use human platelet lysate as a media supplement. However, the study also highlighted a high variability across facilities in practices such as cell-plating densities, harvest times of the cellular product and methods used to assess product identity and potency (Phinney et al., 2019). Most production sites assess quality, safety and efficacy of MSCs based on their identity, morphology, growth characteristics, sterility, karyotype and efficacy *in vitro* and *in vivo*. For the International Society Cell and Gene Therapy (ISCT), the three criteria for assessing MSC identity are **plastic adherence, phenotypic expression of defined antigens and trilineage differentiation** (Dominici et al., 2006). With regard to cellular function, regulatory authorities in both the US and EU require a release assay related to the expected effect of the MSCs product *in vivo*. But due to the diverse therapeutic applications of MSCs, there is not just one specific test required to prove their efficacy. Nonetheless, the cell therapy community could benefit from a standard set of criteria allowing them to compare the results of clinical studies (Wilson et al., 2019).

The road to cell therapy remains long. Although numerous studies have shown that the clinical use of human MSCs is safe, feasible and effective for specific cases and conditions, regulatory agencies have approved only a few commercial products. The first commercial allogeneic product was approved in 2011 in Korea for treating traumatic and degenerative osteoarthritis. Since then, supported by basic research, other products have been approved for various indications (Mizukami and Swiech, 2018). To speed up the approval process, developers need to resolve scientific issues before they apply for market authorization. This would grant patients faster access to the benefits of new cellular therapies (McBlane et al., 2018). In the coming decade, the race for commercial MSC-based treatments will certainly accelerate. To match this increasingly rapid pace, the industry will have to **quickly establish consistent processes and standardize production.** Basic research will be essential to help the industry overcome today's hurdles and clear the path for a successful clinical application.

A) Therapeutic indications being addressed with MSCs



B) MSC clinical trials classified by clinical phase



Related Products

Product	Size	Catalog number
Mesenchymal Stem Cell Growth Medium XF (ready-to-use)	500 ml	C-28019
Human Mesenchymal Stem Cells from Bone Marrow (hMSC-BM)	500,000 cryopreserved cells 500,000 proliferating cells	C-12974 C-12975
Human Mesenchymal Stem Cells from Umbilical Cord Matrix (hMSC-UC)	500,000 cryopreserved cells 500,000 proliferating cells	C-12971 C-12972
Human Mesenchymal Stem Cells from Adipose Tissue (hMSC-AT)	500,000 cryopreserved cells 500,000 proliferating cells	C-12977 C-12978
Mesenchymal Stem Cell Growth Medium 2 (ready-to-use)	500 ml	C-28009
Mesenchymal Stem Cell Adipogenic Differentiation Medium 2 (ready-to-use)	100 ml	C-28016
Mesenchymal Stem Cell Chondrogenic Differentiation Medium (ready-to- use)	100 ml	C-28012
Mesenchymal Stem Cell Chondrogenic Differentiation Medium without Inducers (ready-to-use)	100 ml	C-28014
Mesenchymal Stem Cell Osteogenic Differentiation Medium (ready-to-use)	100 ml	C-28013
Mesenchymal Stem Cell Neurogenic Differentiation Medium (ready-to-use)	100 ml	C-28015
Accutase-Solution, primary human cell culture tested	100 ml	C-41310

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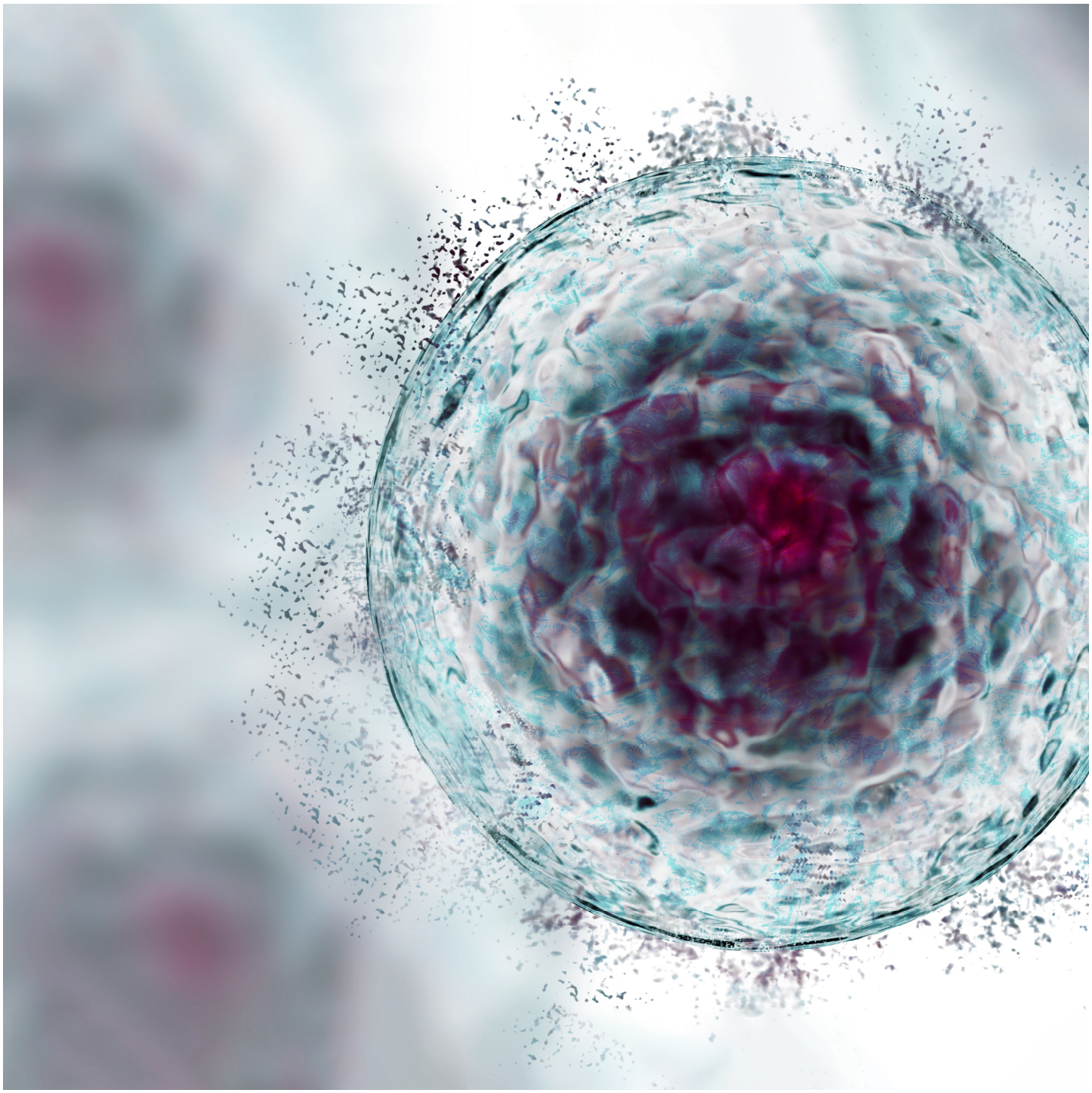
Tools for mesenchymal stem cell culture

PromoCell®

Solutions for world-class
stem cell research



Human Centered Science

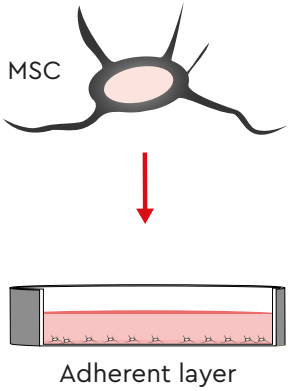
Human mesenchymal stem cells

Mesenchymal stem cells (MSCs) are multipotent cells that can self-renew and differentiate into various cell types. Under appropriate conditions (e.g., appropriate cell growth media and presence of growth factors and differentiation-inducing factors), MSCs can differentiate *in vitro* and *in vivo* into adipocytes, chondrocytes, osteoblasts, myocytes, neurons, hepatocytes, and pancreatic islet cells. In addition to their high differentiation potential, MSCs have immunomodulatory properties, which make them an attractive tool for cell and gene therapies. Compared with other types of stem cells, MSCs isolated from adult donors are relatively easy to culture and expand, given that an optimized culture medium is used. Therefore, MSCs are the most used cells in regenerative medicine. In addition to their use in research focused on understanding the mechanisms regulating organismal development, cell differentiation, and regeneration, MSCs have become a powerful tool for the development of cell-based therapies for various diseases, including cancer and degenerative conditions. To help you advance your stem cell research, we offer human MSCs (hMSCs) harvested from different tissues of donors and support the advanced development and manufacturing of cell and gene therapies. Our optimized cell culture media ensure that hMSCs have all the nutrients and factors they need to grow, self-renew, expand, and differentiate into adipocytes, chondrocytes, or osteoblasts.

MSC sources

Stem cells are often poorly characterized, and their use requires time-consuming and standardized protocols. To support the translation of cellular therapies, the International Society for Cell and Gene Therapy (ISCT) has described three key characteristics of MSCs:

1 Adherence to plastic



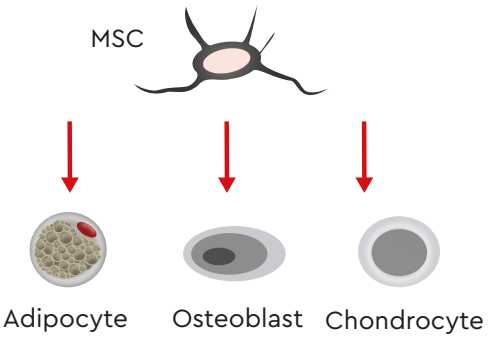
MSCs must adhere to plastic to grow under standard culture conditions

2 Marker expression

Positive ≥ 95% +	Negative ≤ 2% +
▪ $CD105^+$	▪ $CD45^-$
▪ $CD73^+$	▪ $CD34^-$
▪ $CD90^+$	▪ $CD14^-$ or $CD11b^-$
	▪ $CD79\alpha^-$ or $CD19^-$
	▪ $HLA\text{-}DR^-$

MSCs are characterized by the presence or absence of specific surface markers

3 Multipotent differentiation potential

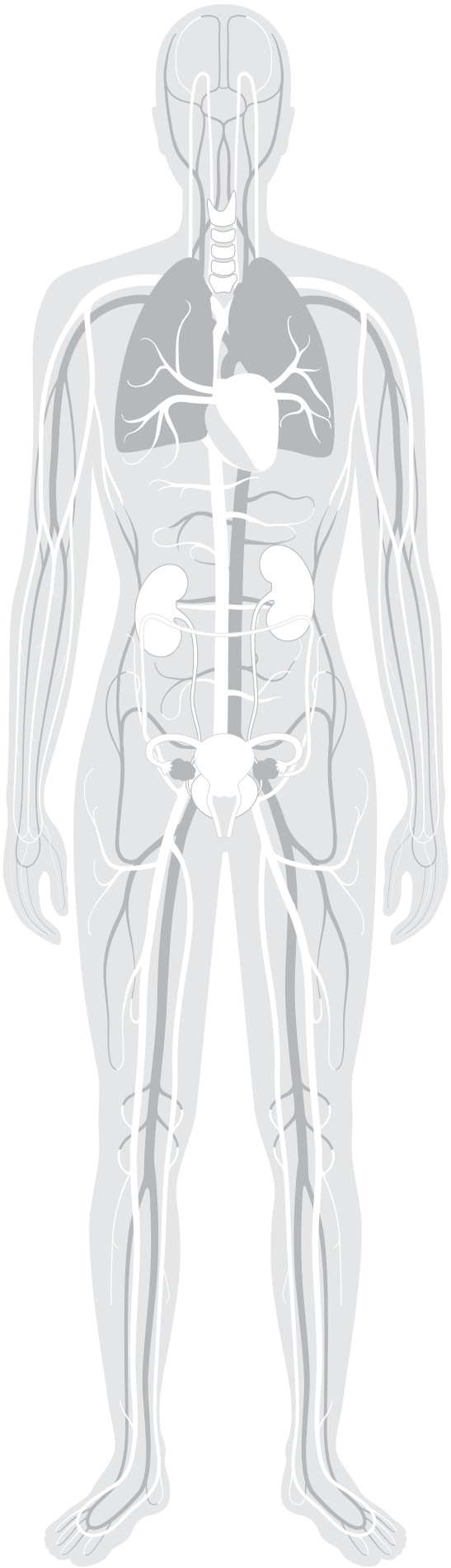


MSCs are multipotent and can differentiate into various cell lineages

We harvest our high-quality hMSCs from the adipose tissue, bone marrow, and umbilical cord matrix (Wharton's jelly) of healthy donors. Our hMSCs are tested for their ability to differentiate into adipocytes, chondrocytes, and osteoblasts *in vitro*. We help you save time and resources by providing well-characterized hMSCs with verified expression of MSC markers. Find out about all the key advantages of our MSC cultures down below:

Key advantages of our MSC cultures:

- MSCs with verified expression of surface markers that meet the ISCT criteria
- Standardized and well-characterized MSCs for reproducible research
- Possibility of large-scale production of various types of hMSCs isolated using ISCT standards
- Confirmed differentiation into osteoblasts, adipocytes, and chondrocytes
- Culture in our optimized MSC medium ensures expression of trilineage surface markers
- Custom MSC media according to your needs and regulatory requirements
- GMP-complient manufactoring of MSC media



Bone marrow

Human Mesenchymal Stem Cells from Bone Marrow (hMSC-BM)

Cat. No. C-12974
cryopreserved cells

Cat. No. C-12975
proliferating cells

Cat. No. C-14090
cell pellet

Umbilical cord

Human Mesenchymal Stem Cells from Umbilical Cord Matrix ((hMSC-UC)

Cat. No. C-12971
cryopreserved cells

Cat. No. C-12972
proliferating cells

Cat. No. C-14091
cell pellet

Adipose tissue

Human Mesenchymal Stem Cells from Adipose Tissue (hMSC-AT)

Cat. No. C-12977
cryopreserved cells

Cat. No. C-12978
proliferating cells

Cat. No. C-14092
cell pellet

Positive ≥95% +	Negative ≤2% +
▪ $CD105^+$ ▪ $CD73^+$ ▪ $CD90^+$	▪ $CD45^-$ ▪ $CD34^-$ ▪ $CD14^-$ or $CD11b^-$ ▪ $CD79\alpha^-$ or $CD19^-$ ▪ $HLA\text{-}DR^-$

MSC marker expression

According to the International Society for Cellular Therapies (ISCT) guidelines. Each lot is tested for its ability to differentiate *in vitro* into adipocytes, chondrocytes, and osteoblasts.

MSC expansion media

Our optimized growth media enable consistent growth and trilineage differentiation potential of hMSCs. Our media contain all the growth factors and supplements necessary for the robust expansion of hMSCs and generation of high-quality multipotent cells.

MSC Growth Medium 2

Our MSC Growth Medium 2 (C-28009) is an optimized low-serum medium for routine culture of hMSC.

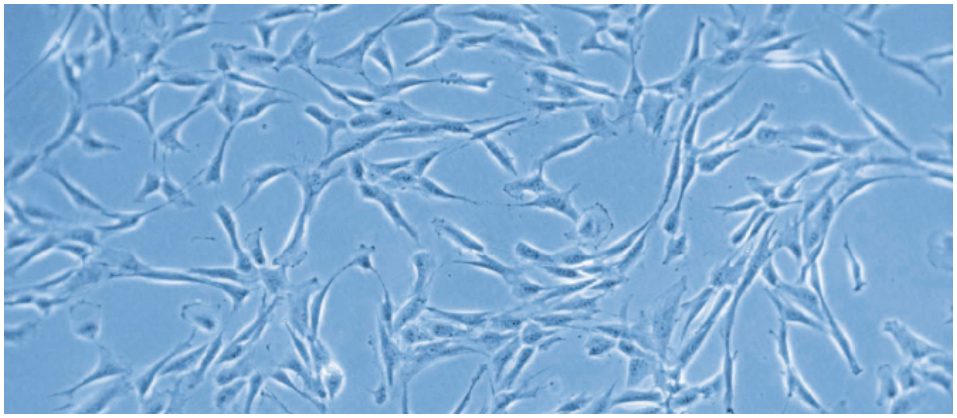
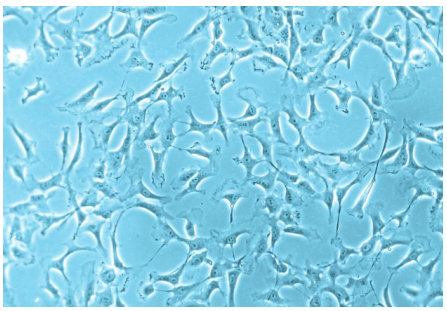


Fig. 1: Phase-contrast image of hMSCs isolated from bone marrow and cultured in MSC Growth Medium 2.

MSC Growth Medium XF

Our MSC Growth Medium XF (C-28019) is a serum-free and xeno-free culture medium. This medium is ideal for the standardized expansion and culture of hMSCs from the bone marrow, umbilical cord, and adipose tissue in fibronectin-coated plates.

A



B

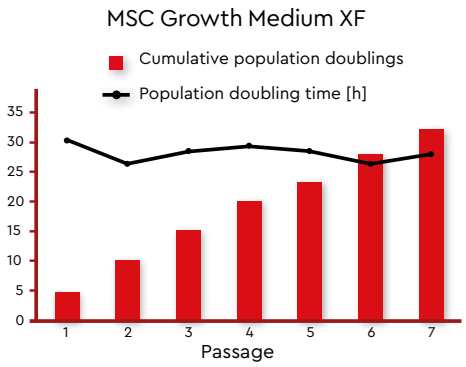


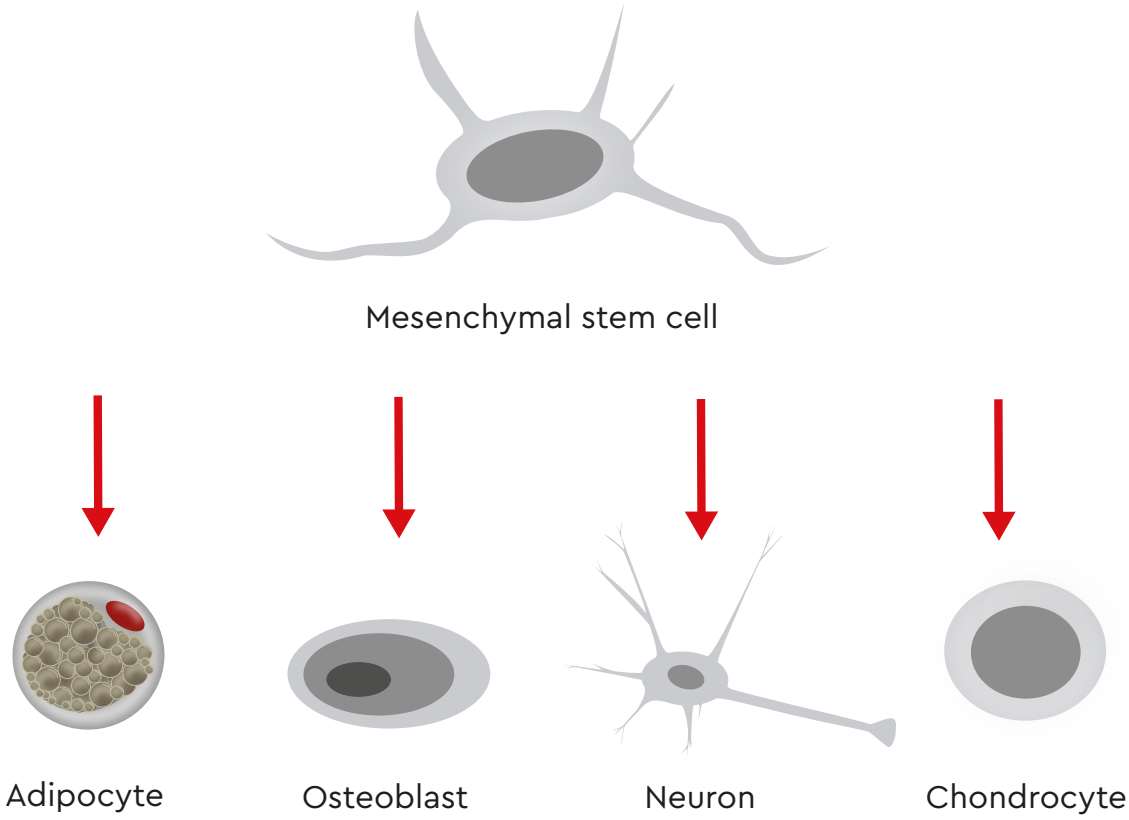
Fig. 2: Growth of bone marrow-derived hMSCs (hMSC-BM) in MSC Growth Medium XF. **A:** *Phase-contrast image of MSCs cultured in xeno-free MSC Growth Medium XF.* **B:** *Cumulative population doubling and doubling times of hMSCs over the course of 7 passages. Culturing in MSC Growth Medium XF enables a stable growth rate of less than 30 h/doubling even after prolonged in vitro culture (32 population doublings over the course of 7 passages).*

MSC Cryopreservation

Our Cryo-SFM is a defined, animal component-free, and protein-free cryopreservation medium. Our optimized and ready-to-use formulation is based on methylcellulose, DMSO, and other cryoprotectants recommended for the standardized cryopreservation of MSCs. Freezing primary human cells and stem cells in Cryo-SFM under standard freezing conditions ensures excellent viability, attachment, and growth performance after thawing.

MSC differentiation medium

We offer five MSC differentiation media that induce differentiation of MSCs into adipogenic, chondrogenic, osteogenic, or neurogenic lineages. The differentiation media have been designed for usage with our own cryopreserved Mesenchymal Stem Cells. In combination with our optimized growth media the differentiation media provide a complete and convenient workflow for the standardized induction of terminal differentiation.



Variations in cell culture conditions can lead to inefficient differentiation of hMSCs into adipocytes, chondrocytes, or osteocytes. Our optimized MSC Differentiation Media enables you to generate different types of differentiated human cells from hMSCs.

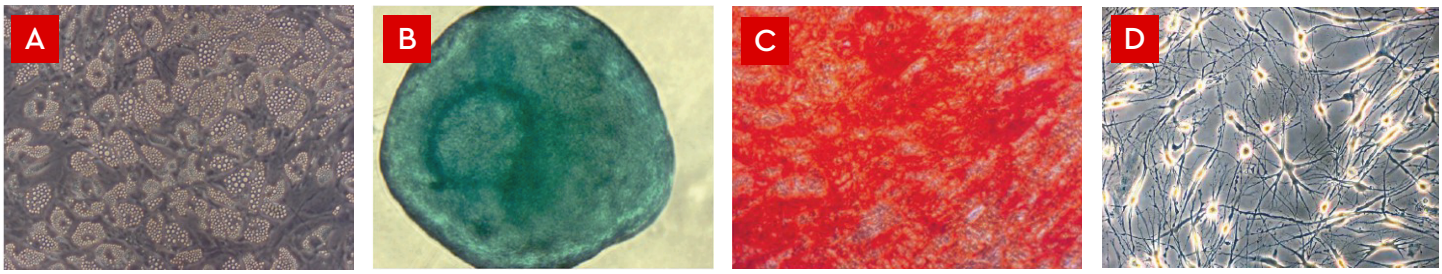


Fig. 3: In vitro differentiation of hMSCs into adipocytes, chondrocytes, osteoblasts, and neuronal cells. **A:** *Accumulation of lipid vesicles in adipocytes differentiated from bone marrow-derived hMSCs. The cells show typical characteristics of mature adipocytes, including intracellular lipid vacuoles.* **B**: *Alcian blue staining of cartilage spheroids after in vitro differentiation of hMSCs. Alcian blue stains cartilage extracellular matrix.* **C**: *Alizarin Red S staining of mature osteoblasts after in vitro differentiation of bone marrow-derived hMSCs. Alizarin Red S stains extracellular calcium deposits.* **D:** *Representative image of neurons after in vitro differentiation of bone marrow-derived hMSCs into neurogenic lineages.*

Visit our website to read our detailed step-by-step protocols together with instructions on how to detect successful differentiation:
promocell.com/scientific-resources/application-notes

MSC media for Cell & Gene Therapy

Because of the many advantages of MSC-based therapies compared with conventional pharmacotherapies, MSCs have become a powerful tool for the development of cell-based therapies for various diseases, including cancer and degenerative conditions. Regenerative medicine involves the use of therapeutics to repair tissue damage, restore degenerative tissues, promote wound healing, and replace dysfunctional tissues and organs. MSCs have high regenerative potential and have been used to regenerate bone, muscles, nerves, myocardium, liver, cornea, trachea, heart, and skin. A key challenge in cell therapies is that many cells is required to achieve a clinically significant therapeutic effect. MSC are easy to isolate from a wide variety of tissues, including the bone marrow, umbilical cord matrix, adipose tissue, tendons, lungs, and periosteum. MSCs can be expanded *in vitro*, given that an optimized culture medium is used; however, expanding MSCs in a standardized and well-characterized way can be challenging. MSCs are multipotent, versatile, and can differentiate into multiple cell types. Therefore, they can be used to replace various malfunctioning or degenerated tissues in the human body. Another advantage of MSCs as therapeutic products is that they are immune-privileged and are, therefore, able to escape immunological destruction. The immunomodulatory properties of MSCs make MSC-based therapies a promising treatment option for autoimmune, inflammatory, and hematologic diseases. MSCs can successfully migrate to tumors *in vivo*; therefore, they can be used to deliver anticancer drugs and tumor suppressor genes to tumor sites. The immunomodulatory properties of MSCs can be leveraged to modulate the tumor microenvironment and suppress tumor progression. MSCs can be engineered to activate immune cells, including macrophages, dendritic cells, and T cells. Collectively, these immune cells can eliminate cancer cells in primary tumors and metastatic sites. The ability of MSCs to migrate to tumor sites and activate immune cells provides an opportunity to combine them with CAR-T cells to enhance the function of engineered antitumor T cells. As with all therapeutic products developed for clinical applications, the safety of MSC therapies must be guaranteed. We manufacture our serum-free, xeno-free MSC culture medium using the highest-quality reagents and supplements, following all Good Manufacturing Practice (GMP) guidelines and international regulatory standards.

High-quality large-scale MSC lots

The commercial and clinical translation of cell-based therapies requires large-scale production of homogeneous and well-characterized MSC populations. Our expertise in cell isolation, media manufacturing, and quality control enables us to offer high-quality, large-scale MSC cultures to help you advance cell-based therapies. The use of large MSC lot sizes also minimizes donor- and source-dependent effects on cell characteristics.

What we offer:

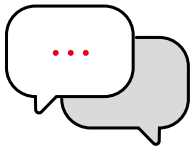
- **Customized large-scale industrial lots** of standardized MSCs from bone marrow and adipose tissue
- **Guaranteed MSC functionality and phenotypes** in accordance with ISCT guidelines
- **Optimized media** with supplements
- **Expert technical support**

Our solutions help you avoid time-consuming cell isolation and testing processes, as well as lot-to-lot variation problems.

Contact our experienced scientists to answer your questions and help you choose the right products: promocell.com/about-us/contact

GMP-compliant MSC culture

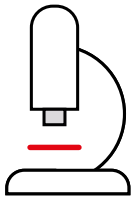
Using high-quality cell culture media and reagents that comply with international regulatory guidelines is critical to ensure a seamless transition from bench to clinic. The EXCiPACT™ GMP standard for pharmaceutical excipients enables us to serve the growing demand of researchers working with our cell culture media and reagents in a regulated environment. Our product development pipeline is designed to ensure compliance with all relevant regulatory guidelines. By streamlining documentation of all raw materials, we reduce your regulatory burden, cost, and time. In addition, our manufacturing process enables us to offer customized products to meet your needs during product development and manufacturing.



1

Contact us to discuss your questions & regulatory requirements

Personalized consultation to define the basic requirements of your project.



2

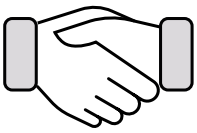
Evaluation of the best product for your project

During the first phase we support you in finding the best product tailored to your needs.

3

Customer Requirement Document Process (CRD Process)

Our customer requirement document process (CRD) supports you in identifying, determining and communicating your regulatory requirements. This process allows us to customize our products and documentation according to your needs, saving you significant time and effort.



4

Implementation of the product with our GMP-Service – what we offer:

- Documentation required for supporting your risk assessment
- Full traceability and raw material control
- Ensured lot-to-lot consistency using comprehensive quality control tests for composition, sterility, and biological activity
- Extended quality tests or documentation (e.g. endotoxin, osmolality) not covered in our standard quality control
- Custom media formulations and production processes to fulfil your raw material specifications and regulatory documentation requirements
- Upscaling and custom media bag production (e.g., 2L, 5L, 10L, 20L)
- Dedicated scientific and technical support

Our commitment to quality

Ethics and quality are at the heart of our business. We own the entire tissue collection and manufacturing process, which means that we can provide fast and direct ethical regulatory support. All our products comply with European biomedical conventions, ensuring that human rights and donor privacy are always protected. Our ISO certifications demonstrate our commitment to quality, and our EXCiPACT™ GMP certification enables us to produce our cell culture media and reagents according to GMP standards. Each year, 600 peer-reviewed publications feature PromoCell products. We operate in over 40 countries around the world, helping scientists with their research needs—we help your science move the world forward.

- Our Quality Management System is certified according the EXCIPACT™ GMP standard

- ISO 9001:2015 certification ensures that we "consistently provide products and services that meet customer and applicable statutory and regulatory requirements"

For more information access: www.promocell.com/compliance

Our tools for stem cell culture

Human stem cells and progenitor cells

Cell type	Description	Catalog no.	Marker	Recommended
Human Mesenchymal Stem Cells (hMSC)	Human Mesenchymal Stem Cells from Bone Marrow (hMSC-BM)	C-12974	$CD105^+/CD73^+/CD90^+$ and $CD45^-/CD34^-/CD14^-/CD19^-/HLA\text{-}DR^-$	C-28009 C-28019
	Human Mesenchymal Stem Cells from Umbilical Cord Matrix (hMSC-UC)	C-12971	$CD105^+/CD73^+/CD90^+$ and $CD45^-/CD34^-/CD14^-/CD19^-/HLA\text{-}DR^-$	C-28009 C-28019
	Human Mesenchymal Stem Cells from Adipose Tissue (hMSC-AT)	C-12977	$CD105^+/CD73^+/CD90^+$ and $CD45^-/CD34^-/CD14^-/CD19^-/HLA\text{-}DR^-$	C-28009 C-28019
hCD34+ -CB	Human CD34+ Progenitor Cells from Cord Blood	C-12921	$CD34^+$	C-28021 C-39891
hPC-PL	Human Pericytes from Placenta	C-12980	$CD105^+/CD146^+$ and $CD31^-/CD34^-$	C-28041

Cell culture media for expansion and differentiation

Cell type	Product	Size	Catalog Number
Human Mesenchymal Stem Cells (hMSC)	Mesenchymal Stem Cell Growth Medium 2 (Ready-to-use)	500 ml	C-28009
	Mesenchymal Stem Cell Growth Medium XF (Ready-to-use) 5	500 ml	C-28019
	Mesenchymal Stem Cell Adipogenic Differentiation Medium 2 (Ready-to-use)	100 ml	C-28016
	Mesenchymal Stem Cell Chondrogenic Differentiation Medium (Ready-to-use)	100 ml	C-28012
	Mesenchymal Stem Cell Osteogenic Differentiation Medium (Ready-to-use)	100 ml	C-28013
	Mesenchymal Stem Cell Chondrogenic Differentiation Medium without Inducers (Ready-to-use)	100 ml	C-28014
	Mesenchymal Stem Cell Neurogenic Differentiation Medium (Ready-to-use)	100 ml	C-28015
hCD34+-CB	Hematopoietic Progenitor Expansion Medium XF	500 ml	C-28021
	Cytokine Mix E for 100 ml HPC Expansion Medium XF	1 ml	C-39890
	Cytokine Mix E for 500 ml HPC Expansion Medium XF	5 ml	C-39891
hPC-PL	Pericyte Growth Medium 2 (Ready-to-use)	500 ml	C-28041

Reagents for cell dissociation and cryopreservation

Product	Size	Catalog Number
DetachKit	30 ml	C-41200
	125 ml	C-41210
	250 ml	C-41220
Accutase-Solution	100 ml	C-41310
Cryo-SFM	30 ml	C-29910
	125 ml	C-29912

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Reports

BioTechniques

Fast assay to predict multipotent mesenchymal stromal cell replicative senescence dynamics

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ABSTRACT

The major obstacle to the application of mesenchymal stromal cells (MSCs) in regenerative medicine is the expansion of the donor-derived cells *in vitro* to obtain high cell numbers in the shortest possible time. However, MSCs gradually undergo replicative senescence after a variable number of divisions that reduce their therapeutic efficacy, which needs to be determined before administration. The authors developed a fast and simple evaluation assay testing two senescence inducers, mitoxantrone (Mxt) and trichostatin A (TSA), to predict the onset of spontaneous replicative senescence of adipose-derived mesenchymal stromal cells (ASCs) and have confirmed the correlation between induced senescence and spontaneous replicative senescence in the assay using Mxt. This protocol facilitates the standardization of therapeutic ASCs and MSCs from other origins before application.

METHOD SUMMARY

Adipose mesenchymal stromal (stem) cells (ASCs) were isolated from the fat deposits of healthy donors at various anatomical locations, followed by long-term cultivation *in vitro* that differed in cumulative cell population doubling level and viability, as determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) assay and longevity (i.e., replicative senescence was determined after several passages using a β-galactosidase activity assay). This was compared in senescence induction assays, using mitoxantrone, a type II topoisomerase inhibitor, and trichostatin A, a histone deacetylase inhibitor. These fast and simple assays may replace the long-term replicative senescence potential assays that take several weeks for evaluation of results.

KEYWORDS:

adipose mesenchymal stromal (stem) cells (ASCs) ● cell longevity ● induced senescence ● mesenchymal stromal (stem) cells (MSCs) ● mitoxantrone ● replicative senescence ● trichostatin A

Mesenchymal stromal cells (MSCs), also called mesenchymal stem cells, are adult undifferentiated cells with self-renewal and clonogenic potential that are capable of differentiation into multiple cell lineages [1]. Due to these traits, MSCs are increasingly used in clinical practice for treatment in regenerative medicine, such as aesthetical surgery and in the treatment of many pathological conditions (e.g., tissue repair [2,3], autoimmune diseases [4], neurodegenerative diseases [5] and acute and chronic inflammation) [6]. Beneficial effects of MSCs on immune/inflammatory disorders of the lung were among the first to be reported [7], highlighting the potential of allogeneic use of MSCs in the treatment of COVID-19-related acute respiratory distress syndrome (ARDS) [8].

In addition to bone marrow, other sources of MSCs have been identified in a variety of fetal and adult tissues [1]. For any clinical application, the MSCs should fulfill the demand of the International Society for Cell Therapy for phenotypic and functional characterization [9]. MSCs have a limited lifespan due to their replicative senescence, which occurs after a cell type-specific number of population doublings in the *in vitro* culture conditions, thus representing a challenging task in the selection of the cell lines that will be most viable long-term. The onset of senescence in MSCs not only affects their longevity but also viability, self-renewal, differentiation capacity and secretome composition that, over time with cell aging, declines resulting in the impaired therapeutic potential of naive MSCs [10–15]. Unfortunately, the senescence status of cells cannot be predicted, unless the MSC's viability is followed *in vitro* through multiple passages and measurement of senescence cell fraction by the standard senescence marker, β-galactosidase (SA-bGal) activity [10,15].

Adipose-derived mesenchymal stromal cells (ASCs) are obtained from adipose tissue in rather high quantities and were therefore selected here for developing faster assays compared with routinely following *in vitro* spontaneous replication senescence to enable highly reliable standardization analyses, using senescence inducers. Here, the authors describe a method that would enable much more rapid characterization of MSC longevity and the immediate selection of good quality MSC cell lines after their isolation. The assays use mitoxantrone (Mxt), a type II topoisomerase inhibitor that disrupts DNA synthesis and repair via intercalation between DNA bases [16], and trichostatin A (TSA), a histone deacetylase inhibitor [17], both immediately inducing cell senescence. The data confirm a statistically

Table 1. Summary of donor characteristics and adipose-derived mesenchymal stromal cell yields.

Anatomical location	ASC code	Donor age (years)	Adipose tissue volume (ml)	Adipose tissue cells (n/ml)	Isolated cells (mean; n/ml)
Thighs	ASC-1	71	2.0	243,750	239,469†
	ASC-2	71	2.0	300,000	
	ASC-3	70	3.7	174,658	
Hips	ASC-4	35	1.5	91,666	74,305
	ASC-5	35	1.0	37,500	
	ASC-6	12	1.6	93,750	
Abdomen	ASC-7	35	1.0	75,000	41,667
	ASC-8	41	1.5	8333	

†The highest number of cells was isolated from the thighs compared with the hips and abdomen ($p < 0.05$).
ASC: Adipose-derived mesenchymal stromal cell.

significant correlation between chemically induced senescence in the Mxt assay and spontaneous replicative senescence of naive ASCs. This assay should be useful for the accelerated selection of high-quality ASCs for use in therapeutic applications. The assay is also applicable to other tissue sources of MSCs.

Materials & methods

Adipose tissue donors

Adipose tissue samples were collected from healthy donors who were undergoing esthetic surgery. The study was approved by the National Ethics Committee (Doc. no. 134/01/11). All the donors were female, with a mean age of 43 years, ranging from 12–71 years. Subcutaneous adipose tissue was taken from the thighs (n = 3), hips (n = 3) and abdomen (n = 2) from a total of seven donors (Table 1).

Isolation & culturing of adipose mesenchymal stromal cells

Isolation of ASCs from adipose tissue samples collected from healthy donors undergoing aesthetic surgery was performed according to the procedure described by Mahmoudifar *et al.* [18] with minor modifications. Briefly, the adipose tissue samples were washed with phosphate-buffered saline (PBS; PAA Lab, Pasching, Austria), cut into small pieces and dissociated with 0.1% collagenase I (Gibco, Life Tech. Corp., Paisley, UK) at 37°C with vigorous shaking. After 1 h, the collagenase was inactivated by adding heat-inactivated fetal bovine serum (FBS; PAA Lab), and the samples were centrifuged at 300 × g for 10 min. The pelleted cells were washed with erythrocyte lysis buffer (0.15 M NH4Cl, 0.1 mM EDTA, 0.01 M NaHCO3, pH 7.2–7.4; Sigma-Aldrich, Steinheim, Germany) and centrifuged at 300 × g for 5 min. The isolated cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) with 20% FBS, supplemented with 100 IU/ml penicillin, 0.1 mg/ml streptomycin and 2 mM L-glutamine (Gibco). The first ASC culture was denoted as passage 0. The medium was changed twice a week and the ASCs were replated at a density of 6000 cells/cm^2 after reaching 90% confluence.

Adipose-derived mesenchymal stromal cell characterization & differentiation

ASC identity was confirmed for all ASC lines at passage p7 by analysis of specific MSC cell surface marker expressions, such as the presence of CD13, CD29, CD44, CD73, CD90 and CD105 absence with less than 2% expression of the hematopoietic stem cell markers CD14, CD34, CD45 and HLA-DR. Briefly, one million ASCs were harvested, washed with PBS and labeled with antibodies or with appropriate isotype controls as instructed by the manufacturer (BD Biosciences, CA, USA). To exclude dead cells from the analysis, the cells were stained with propidium iodide (PI) and washed with PBS. The cells were analyzed by flow cytometry using a BD FACSCalibur™ and CellQuest Software (BD Biosciences). All ASC lines were tested for their selective differentiation into adipose, bone or cartilage-like cells as previously described [19].

Cumulative population doubling level

Growth profiling of the ASCs was performed by calculating their cumulative population doubling levels (CPDLs). The ASCs were counted at seeding and harvesting and their doubling levels were calculated according to the equation:

$$x = \{\log 10(NH) - \log 10(NI)\} \log 102$$

where NI is the initial ASC number at seeding and NH is the ASC number at harvesting [20]. The ASCs were passaged when they reached 90% confluence. The CPDL was obtained by adding the doubling level of the ASCs at each passage to that of the previous passage, with continuous cultivation from passage 1 until the cells ceased to proliferate (to at least passage 18).

Table 2. Adipose-derived mesenchymal stromal cells age-related cumulative population doubling levels at passages p6, p12 and p18.

Age group/(age average)	Cumulative population doubling level		
	p6	p12	p18
Older ASCs (70.7 years)	10.9 ± 5.8	26.7 ± 7.4	39.2 ± 8.8
Middle ASCs (36.5 years)	11.2 ± 2.2	26.0 ± 4.8	34.4 ± 4.0
Young ASCs (12 years)	9.5	19.1	24.7

Metabolic activity of adipose-derived mesenchymal stromal cells

The metabolic activity of the ASCs was determined using the MTT assay, as described by Mosmann *et al.* [21] with minor modifications. The concentration ranges for Mxt and TSA were selected based on data from Zhao *et al.* [22] and the preselection MTT test. Briefly, ASCs at passage p7 were seeded into 96-well plates (Corning, MA, USA) at a density of 5000 cells/well with 5 replicates for each condition and left to adhere for 24 h. Then 0.02–0.6 μM Mxt or 0.05–3.00 μM TSA was added. After 69 h, 0.5 mg/ml MTT (Sigma-Aldrich, Steinheim, Germany) was added to the ASCs, which were then incubated for 3 h at 37°C and 5% CO_2. The medium was removed and the formazan crystals were dissolved in dimethylsulphoxide (Sigma-Aldrich). The absorbance of the solution was measured at 570 nm (reference filter: 690 nm) using a Synergy™MX microplate reader (Bio-Tec Instruments Inc., VT, USA). The absorbance data are displayed as means ± standard deviation of 5 replicates from 3 independent experiments.

Replicative senescence & induced senescence of adipose-derived mesenchymal stromal cells

The spontaneous cell senescence that occurred under normal growth conditions *in vitro* was evaluated in ASCs at passages p6, p12 and p18. Briefly, the ASCs were plated on poly-lysine-coated coverslips at a density of 15,000 cells/coverslip in 24-well plates (Corning, MA, USA) and left to adhere overnight. The ASCs were fixed with 0.5% glutaraldehyde (Sigma-Aldrich, Steinheim, Germany) for 20 min at room temperature, and SA-β-gal activity at pH 6.0 was determined as previously described [20]. The fixed cells were washed twice with PBS and then incubated with 1 mg/ml 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside solution (X-gal substrate; Sigma-Aldrich). After 16 h, the ASCs were washed in distilled water and incubated with Hoechst 33342 (1:1000) to stain their nuclei. The percentage of senescent ASCs was determined by counting the senescent cells and nuclei per visual field under a light and fluorescent microscope, respectively. The results are presented as the ratio of the number of SA-β-gal-positive ASCs per the number of nuclei detected.

To induce senescence, the ASCs at passage 7 were plated onto poly-lysine-coated coverslips (15,000 cells/coverslip) and left to grow overnight. The next day, the ASCs were exposed to 0.02, 0.2, 0.4 or 0.6 μM Mxt (Sigma-Aldrich, Steinheim, Germany) or 0.05, 0.5, 1, 2 or 3 μM TSA (Sigma-Aldrich) for 72 h. The detection of SA-β-gal-positive ASCs was performed as previously described.

Statistical analysis

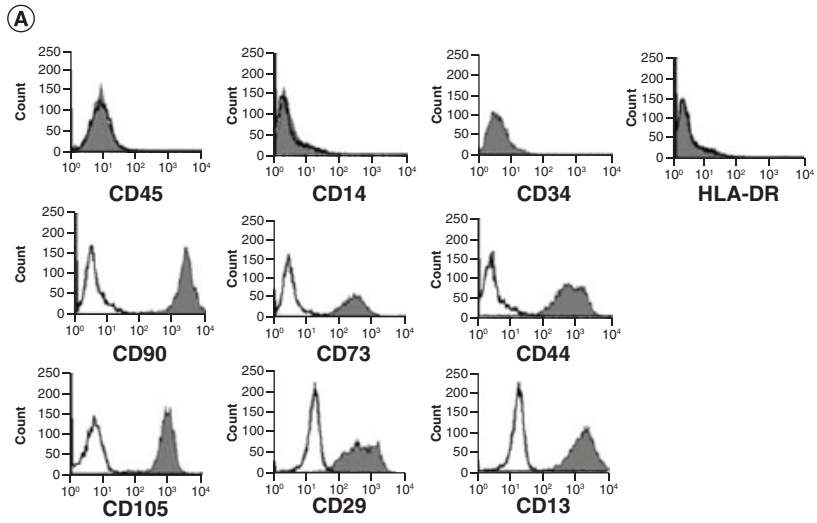
To test for spontaneous senescence progress in each cell line at passage p6, p12 and p18, determined by the number of SA-β-gal-positive cells and to test correlation of ASCs harvest site and CPDL, ANOVA with Tukey's posthoc tests was used. To test the effects of Mxt and TSA on ASC viability and senescence, treated ASCs were compared with untreated ASCs using ANOVA with Dunnett's posthoc tests. To test for correlations between ASCs replicative senescence at passage p12 and induced senescence in ASCs as a response to Mxt and TSA exposure, ANOVA with Fisher's LSD test was used. GraphPad Prism version 6 for Windows was used (GraphPad Software, CA, USA). Data are displayed as means ± standard deviation, with $p < 0.05$ considered statistically significant.

Results & discussion

Growth profiling & replicative senescence of adipose-derived mesenchymal stromal cells

After ASCs phenotype confirmation by selective MSC markers (Figure 1A & B) and specific differentiation profile into adipocytes, osteocytes and chondrocytes (Figure 1C) used to characterize MSCs as multipotent mesenchymal stromal cells, the proliferation capacity of each ASC line (ASC-1 to ASC-8) was determined. The cells were passaged until they ceased to proliferate. The growth curves determined in terms of CPDLs differed considerably among the ASC lines (Figure 2A). ASC-3 exhibited the highest proliferation rate, followed by ASC-5 and ASC-8. The remaining cell lines (ASC-1, ASC-2, ASC-4, ASC-6 and ASC-7) had similar proliferation profiles and formed a cluster of cells that showed nearly identical CPDLs after 100 days of cultivation. Comparison of the proliferation rates of ASC lines derived from three different anatomical sites showed that the proliferation of cells was not significantly different even after the highest number of passages, at p18 (Figure 2B). Similarly, when ASC lines were grouped according to the age of the donors (young, middle and old age), no significant differences were observed in terms of CPDL (Table 2). However, the number of ASC lines included in the study was too small to draw precise statistical conclusions.

Next, the presence of spontaneously senescent cells was evaluated by detection of SA-β-gal activity, the most frequently used standard for senescence detection. The proportions of SA-β-gal-positive cells varied among ASC lines at p6, p12 and p18, respectively (Figure 2C). However, the proliferation rates (CPDL) failed to reliably indicate the senescence status in ASCs at later passages (Figure 2). Thus, the CPDL assay should be replaced or only added to the more informative assay related inversely to the metabolic activity of cells.



B

Marker	Marker expression according to ASC source (%)							
	ASC-1	ASC-2	ASC-3	ASC-4	ASC-5	ASC-6	ASC-7	ASC-8
CD45	1.8	1.9	0.4	1.2	1.0	1.3	1.4	0.8
CD14	0.9	0.7	0.4	1.6	1.8	1.8	1.9	1.9
CD34	0.2	1.4	0.6	1.8	1.4	1.9	1.2	1.6
HLA-DR	0.9	0.5	1.1	1.4	0.7	0.9	1.6	0.7
CD90	99.3	98.4	99.9	98.7	98.6	96.8	98.5	99.4
CD73	98.4	99.3	98.6	95.3	98.9	97.2	99.9	97.6
CD44	99.8	99.1	99.3	98.8	95.2	95.9	98.7	96.1
CD105	99.5	95.6	99.9	99.2	95.9	95.6	96.8	99.4
CD29	99.5	98.6	98.7	96.9	99.2	98.9	97.1	95.7
CD13	99.4	99.4	98.7	98.9	96.3	96.8	97.0	99.0

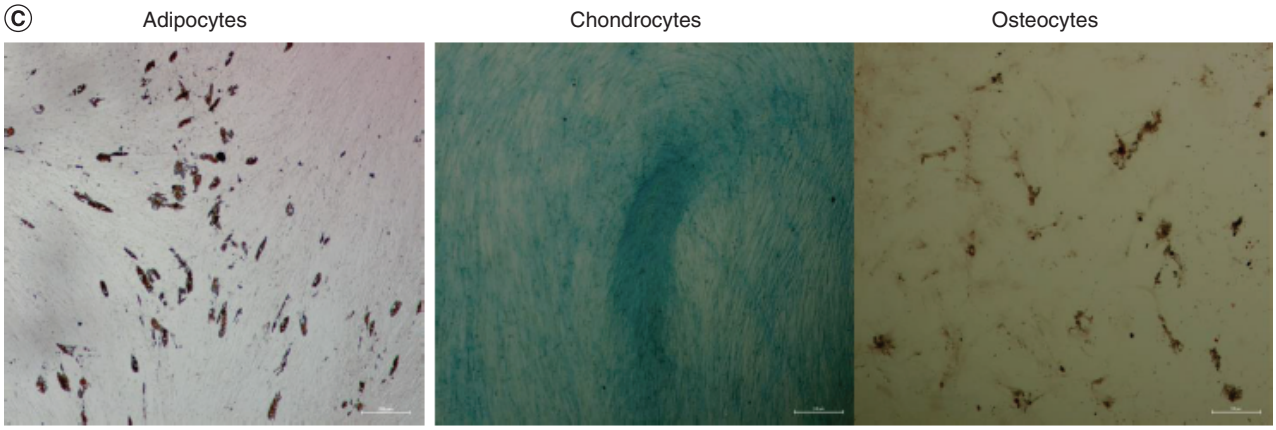


Figure 1. Characterization of adipose-derived mesenchymal stromal cells. (A) ASC surface marker expression, analyzed by flow cytometry for the characteristic MSC/ASC surface antigens CD45-FITC, CD14-FITC, CD34-FITC, HLA-DR-PE, CD90-PE, CD73-PE, CD44-PE, CD105-APC, CD29-APC, CD13-APC and the appropriate isotype (i.e., FITC-IgG1, FITC-IgG2a, PE-IgG1, PE-IgG2b and APC-IgG1), as described in the methods section. **(B)** Quantitative data for the MSC markers for all ASC lines used in the study, demonstrating their mesenchymal stromal cells origin. **(C)** Adipocytes, chondrocytes and osteocytes after differentiation of ASCs. Scale bars: 100 μm.
ASC: Adipose-derived mesenchymal stromal cell; MSC: Mesenchymal stromal cell.

Moreover, monitoring of senescence could be additionally assessed by new simple detection methods for phenotypic changes characteristic of cell senescence based on autofluorescence of cells using flow cytometry [23,24] or image-based assessment of change in morphology of senescent cells [25], which has also been shown to be a good indicator of cell senescence. To follow the mechanism of senescence in more detail, the above simple senescence detection techniques could be further supported by molecular, genomic, epigenomic and cytogenetic methods [15]. However, that was not the aim of this report.

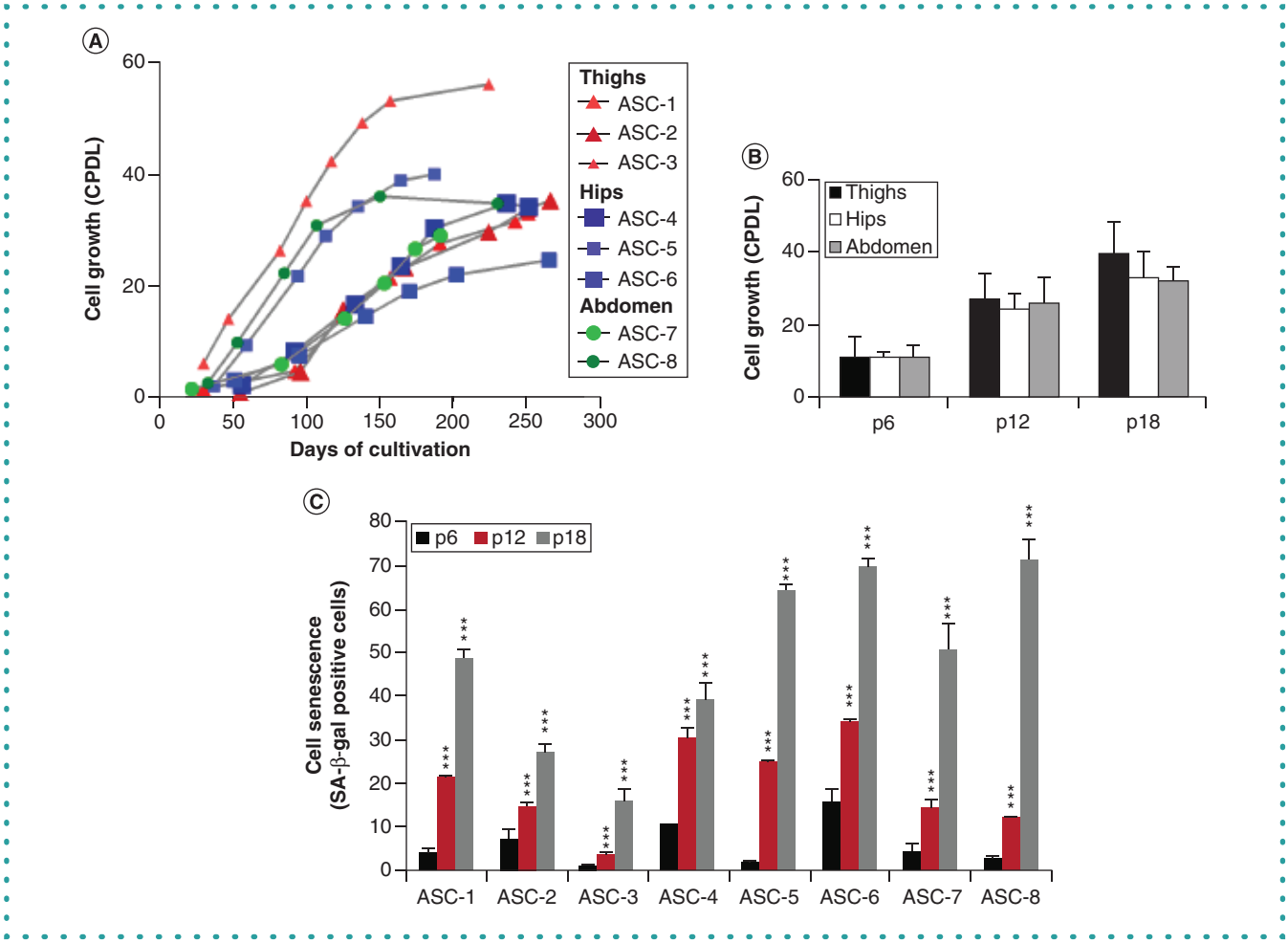


Figure 2. Growth curves and replicative senescence of adipose-derived mesenchymal stromal cells. (A) Long-term (250-day) growth curves of obtained ASC lines, quantified as described in the methods section. **(B)** CPDL of ASCs derived from different anatomical locations at the different passages, p6, p12 and p18. **(C)** SA-β-gal staining of the ASCs as a measure of spontaneous cell senescence during cell cultivation at early (p6) and late (p12 and p18) passage numbers. Quantification of senescent cells was performed by counting senescent cells and nuclei per visual field under a light and fluorescent microscope, respectively. Data represent the mean values ± SD of absorbance from three independent experiments.
***$p < 0.001$.
ASC: Adipose-derived mesenchymal stromal cell; CDPL: Cumulative population doubling levels.

Induced senescence of adipose-derived mesenchymal stromal cells

Since *in vitro* expansion is usually required to obtain sufficient numbers of cells for any particular application, the essential requirement for an ASC line at the time of application is that the cells retain genetic stability, viability and proliferation [26]. These properties strongly depend on cell-specific longevity (i.e., low or no senescence over several passages during ASC/MSC propagation *in vitro*) [14,23]. However, the changes are hardly noticeable at the initial stages of senescence before significant decreases in proliferation rate and altered morphology are observed [10,14,27]. At the molecular level, this is associated with gradual changes in the genetic and transcriptomic profile fingerprints of senescent cells. Cellular senescence-induced transcriptional profile changes in bone marrow–derived MSCs involve more than 5000 genes, including 31 miRNAs [12]. Differential miRNA expression in the aging versus young MSC-derived extracellular vesicles was observed, and these showed that aging MSC-derived extracellular vesicles were not suitable for the treatment of acute lung injury [28].

To avoid the prolonged expansion regimens needed to identify the most long-term, viable ASC/MSC lines, the current work established an evaluation assay that can be used to predict the onset of senescence. A rapid assay was developed using two senescence inducers, Mxt [16] and TSA [17,22], both of which affect cell cycle progression. The Mxt agent is an inhibitor of DNA topoisomerase II, which binds to DNA by intercalation, and the agent TSA is an inhibitor of histone deacetylase. As a consequence of the onset of senescence in ASCs after treatment with Mxt and TSA, their lower metabolic rate was detected.

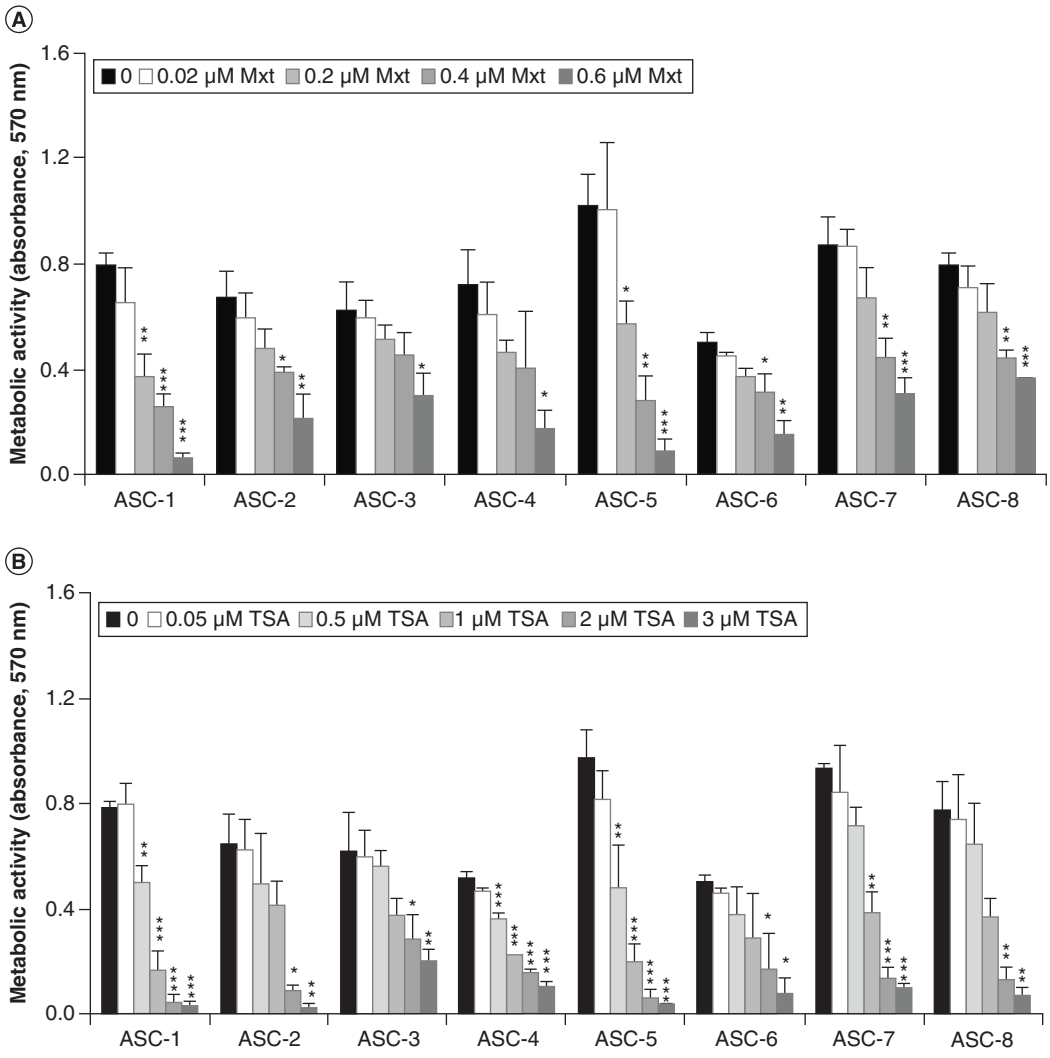


Figure 3. Metabolic activity of the adipose-derived mesenchymal stromal cells after exposure to the senescence inducers mitoxantrone and trichostatin A. Quantification of ASC metabolic activity after exposure to **(A)** mitoxantrone (Mxt) and **(B)** trichostatin A (TSA) using the MTT assay. Data represent the mean values ± SD of absorbance from three independent experiments.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.
ASC: Adipose-derived mesenchymal stromal cell.

After 72 h of treatment with Mxt (concentrations ranging 0.02–0.6 μM) and TSA (concentrations ranging 0.05 to 3.00 μM), the viability of ASCs in all ASC lines decreased drastically in a dose-dependent manner, although to different extents (Figure 3A & B). Notably, cell viability and senescence were used, two distinct inversely correlated processes, to demonstrate the effects of treatment with each of the senescence inducers.

Correlation of replicative & induced senescence

We anticipated that the response of ASCs to senescence inducers exposure would reflect the dynamics of their replicative senescence status. Variability in the onset of senescence was observed among ASC lines after Mxt- and TSA-induced senescence (Figure 4A & B). The results in Figure 4A compare the number of spontaneously senescent cells at passage 7 and passage 12 (last red-colored column in each graph). The numbers of senescent cells induced by exposure to 0.02–0.6 μM Mxt showed that the proportion of senescent cells gradually and significantly increased in all ASC lines, although to varying degrees.

However, when comparing the number of spontaneously senescent cells at high passage 12 and the number of senescent cells upon exposure to Mxt in individual ASC lines (Figure 4A), comparable numbers of SA-β-gal positive cells were found in lines ASC-1, ASC-2, ASC-3, ASC-6 and ASC-7 already upon exposure at 0.02 μM Mxt and at 0.2 μM Mxt in lines ASC-3, ASC-4, ASC-5 and ASC-6. Only in

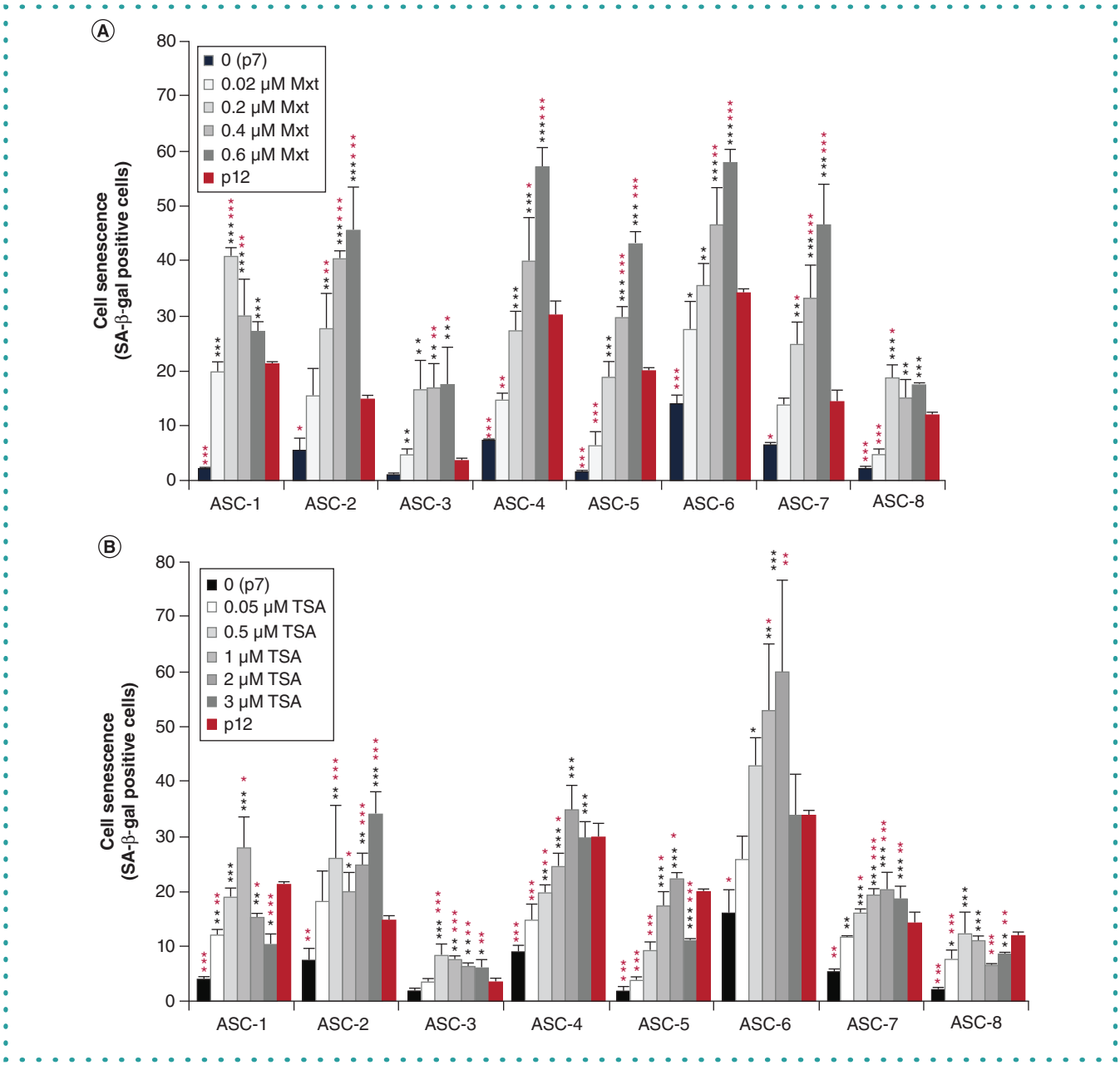


Figure 4. Senescence of the adipose-derived mesenchymal stromal cells during long-term cultivation and after exposure to senescence inducers.
SA-β-gal staining of the ASC's spontaneous cell senescence at late passage p12 and SA-β-gal staining of the ASCs after their exposure to **(A)** Mxt and **(B)** TSA at passage 7. Quantification of senescent cells was performed by counting senescent cells and nuclei per visual field under a light and fluorescent microscope, respectively. Comparison of senescent ASCs in control (black markings) and senescent ASCs at passage p12 (red markings) with respect to the ASCs exposed to Mxt and TSA, respectively. Data represent mean values ± SD from three independent experiments.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.
ASC: Adipose-derived mesenchymal stromal cell.

ASC-8, was a comparable proportion of SA-β-gal-positive cells found at passage p12 cells at higher concentrations of Mxt (0.4–0.6 μM). Although the tested ASC lines varied in terms of the proportion of senescent cells at the early passage, exposure to Mxt between 0.02 and 0.4 μM already reflected the extent of spontaneous replicative senescence that would be achieved in the late passage p12 in all ASC lines.

Exposure of passage 7 ASCs to concentrations range 0.05–3.00 μM TSA also resulted in an increase of senescent cells in all ASC lines, again to various extents (Figure 4B). Again, faster-responding ASC lines such as ASC-2, ASC-3, ASC-6 and ASC-7 already correspond to the extent of SA -β-gal-positive cells at passage 12 upon exposure to 0.05 μM TSA and slower responding cells such as ASC-1, ASC-6,

ASC-8 at 0.5 μM TSA (Figure 4B). In ASC-4, the comparable numbers of SA-β-gal-positive cells at passage p12 cells and SA-β-gal-positive cells were observed after exposure to 1 and 2 μM TSA. In the ASC-5 line, no correlation was found between the SA-β-gal-positive cells at passage p12 and at all tested concentrations of TSA. These data showed poor consistency in the proportional increase of responsiveness of ASCs to TSA.

Taken together, these results imply an association between the native, spontaneous replicative senescence of ASCs and induced senescence of ASCs triggered by the senescence inducer Mxt, but not by TSA. The limitation of the proposed protocol is that in this pilot study, its application of effects in a low number of ASCs and at passage 7 was demonstrated, whereas experiments at lower ASC passages would allow faster selection of quality cell lines, thus reducing workload and materials needed. To characterize each ASC line in terms of the senescence process in culture, we first examined the extent of replicative senescence in ASCs at passage 6 and at passage 7 and the extent of induced senescence in ASCs after exposure to various Mxt concentrations was determined, although the effects of senescence inducers should preferably be examined in identical and also lower ASC cell passages. In addition, the identified Mxt concentrations were not further validated on multiple ASC lines. Finally, the method has not yet been applied to bone marrow and other origins of tissues as the source of MSCs. The advantages of the method using this senescence inducer to determine the predisposition of a cell line toward early/late onset of senescence are that it is a technically simple and fast method in which ASCs and possibly MSCs of other tissue origins can be selected for clinical application in regenerative medicine and disease states.

Conclusion

Cell therapy represents an advanced medical tool that is increasingly used in experimental and clinical practice for the treatment of various diseases. MSCs are immunomodulatory cells used in the treatment of acute and chronic immune diseases, whereas autologous adipose tissue-derived MSCs (i.e., ASCs) are mainly used in regenerative medicine (e.g., aesthetic surgery).

Senescence is a progressive biological process that occurs both *in vivo* and *in vitro*. As the therapeutic potential of ASCs and MSCs diminishes with increased passages and progression to senescence, it is of utmost importance to use the primary cell cultures of the MSC lines that are the most senescence-resistant. However, to provide one to two million MSCs per kilogram of body weight for use in a single clinical setting [29] and to overcome the limitation of an extensive expansion of MSCs due to the onset of replicative senescence, cell lines should be subjected to a fast selection process that allows rapid identification of the most viable, long-lived cell lines. Therefore, senescence inducers could be used as a new analytical tool to preselect high-quality MSC lines for each application, avoiding lengthy and expensive *in vitro* expansion steps to validate replicative senescence.

This work is the first demonstration of an association between induced senescence and physiological longevity of ASCs isolated from adipose biopsies from different anatomical locations. The data showed that exposure of ASCs to the preferential senescence inducer Mxt, compared with TSA, can be used in a simple experimental method to identify the resistance of ASCs to the onset of replicative senescence. Although the tested ASC lines varied in their proliferation potential and in the proportion of senescent cells at early passage, exposure to Mxt reflected their tendency for faster progression of replicative senescence at late passage p12 in all ASC lines. Indeed, the results demonstrate a comparable number of SA-β-gal-positive ASCs due to replicative senescence at late passage 12 and SA-β-gal-positive ASCs after exposure to 0.02 μM, 0. 2 μM or 0.4 μM concentrations of Mxt. A limitation of the present study is that the status of replicative senescence of ASCs and the status of induced senescence of ASCs after Mxt exposure were not examined at the same passage and the identified Mxt concentrations were not validated in multiple ASC lines. Taken together, the advantages of this technically simple and fast method using senescence inducers lie in the prediction of the predisposition of a cell line to the onset of senescence of ASCs and possibly MSCs isolated from various tissue sources for allogenic or autologous application.

Future perspective

There is no doubt that advanced cell therapy is the most efficient approach to the treatment of tissue repair, certain autoimmune and neurodegenerative diseases and acute and chronic inflammation. First, Mxt should be tested in MSCs isolated from various tissue sources. Its broader application may also help tame ARDS in patients with COVID-19. Further, MSCs represent a pool of cells that can be used alone, attached to scaffolds or as delivery vectors that will be genetically engineered in the future to produce therapeutic proteins using CRISPR-Cas 9 technology. The longevity of sufficient amounts of these cells is still a bottleneck for wider application but can be overcome by Mxt assays to rapidly preselect the most viable MSC clones, ensuring their higher yield with superb clinical efficacy.

Author contributions

K Kološa conceptualized the study. A Leskovšek provided the tissue samples. T Rajar performed the experimental work. K Kološa analyzed the associated data and wrote the draft of this manuscript. T Lah provided grant support, input and contributed to the writing of the manuscript. All authors approved the final manuscript.

Financial & competing interests disclosure

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

Ethical conduct of research

The collection and use of adipose samples were approved by the National Ethics Committee, Slovenia (doc. no. 134/01/11).

Data sharing statement

All related data are available under request.

Open access

Executive summary

- Adipose tissue is a valuable and easily accessible source of MSCs (i.e., ASCs) used for cell therapy and in regenerative medicine. However, the quality of ASCs is highly variable, depending on the age and anatomical location of the donors.
- ASCs, similar to other MSCs, have a limited lifespan due to replicative senescence, which affects their viability and longevity and should be predetermined to ensure their successful expansion in the shortest possible time, which is particularly relevant when a larger number of cells are required for autologous application.
- Early passage of ASCs/MSCs exposed to a chemical inducer of senescence, mitoxantrone, correlates with endogenous dynamics of replicative senescence of ASCs. This simple assay allows early and efficient selection of clones for further expansion of the most viable cells and replaces long-term culturing to evaluate their spontaneous onset of senescence. The value of this innovative approach in cell biotechnology is in particular important due to the increasing use of cell therapy using genetically engineered allogeneic MSCs, similar to Car-T cells, for the treatment of various immune diseases, such as COVID-19.

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Short Communication

Regenerative Medicine

Novel hydrogel system eliminates subculturing and improves retention of nonsenescent mesenchymal stem cell populations

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Aim: To compare the physiological behavior of mesenchymal stem/stromal cells (MSCs) within an expandable tissue-mimetic 3D system relative to *in vitro* expansion in a traditional 2D system. **Methods:** Adipose-derived MSCs (ASCs) were continuously cultured for 6 weeks on either 2D culture plastic or in a 3D hydrogel system that eliminated subculturing. ASCs were assessed for senescence, 'stem-like' MSC markers, and ability for their secretome to augment a secondary cell population. **Results:** The 3D hydrogel system resulted in an enhanced retention of more regenerative, nonsenescent ASC populations that exhibited increased expression of 'stem-like' MSC surface markers. **Conclusion:** This study introduces a proof-of-concept design for a novel modular 3D system that can improve *in vitro* expansion of stem-like cell populations for future regenerative therapies.

Tweetable abstract: Novel tissue-mimetic 3D hydrogel system enhances the retention of nonsenescent ASC populations *in vitro* for up to 6 weeks in culture and eliminates the need to subculture, improving regenerative capacity of ASCs and their secreted biologics.

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Mesenchymal stem/stromal cells (MSCs) are a heterogeneous population of multipotent progenitor cells that are found in a variety of tissue sources within the human body, including bone marrow, dental pulp, umbilical cord and adipose tissue [1]. MSCs have earned a spotlight in the fields of tissue engineering and regenerative medicine for their intrinsically diverse regenerative and secretory properties [2]. The multipotent nature of MSCs originally garnered immense interest in the field of biomedical engineering due to the capacity for MSCs to differentiate into osteogenic, chondrogenic, adipogenic, nervous, skeletal and cardiac lineages to potentially form a neotissue or whole-organ replacement for diseased and damaged tissue within the body [1–3]. These types of therapies have often focused on the incorporation of MSCs with scaffolds and hydrogels of various complexities in order to control the differentiation of MSCs toward specific lineages.

Other MSC-based therapies that are currently being investigated include endovascular or direct injection of MSCs into locations of damaged tissue [2]. These therapies require further optimization due to cell death and radial diffusion of MSCs from the sites of injection, although ongoing research into delivery vehicles for MSC therapies such as scaffolds and hydrogel encapsulation have proven to be promising next steps in improving their efficiency and efficacy [4–9]. Similarly, another MSC-based therapy that has garnered interest involves the utilization of MSC-derived secretory products, called the secretome, as a form of acellular MSC therapy that circumvents some of the common limitations of cell-based therapies. MSCs' secreted byproducts include a heterogeneous variety of biomodulatory factors such as proteins, antioxidants, nucleic acids, exosomes and microvesicles, and have

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been shown to promote tissue regeneration and wound healing in a variety of applications [10–13]. Ultimately, for both cellular and acellular MSC-derived therapeutics, cells must be removed from a donor source and subsequently expanded *in vitro* to achieve sufficient cell numbers (often requiring anywhere from 10^7 to 10^9 MSCs at a minimum) to obtain a viable clinical product, which is associated with loss of a stem-like phenotype and often takes several weeks to achieve [14,15].

Traditionally, MSC *in vitro* expansion systems include the use of rigid, 2D plastic culture vessels to expand the cells as a monolayer and require multiple protease-dependent subculturing (passaging) events to achieve large enough cell quantities. Evidence suggests that traditional 2D culture systems are not ideal for stem cell expansion and can result in a loss of MSC multipotency, reduce viability, induce senescence and decrease secretion of regenerative factors [16–20]. Studies have demonstrated that stem cell differentiation and function are, in part, dependent on substrate mechanical properties [21,22], with more rigid substrates promoting osteogenic lineages and decreased viability, and softer substrates improving viability and the retention of 'stem-like' properties [21,23]. Thus, the decreased robustness of MSC populations seen in traditional 2D culture is likely due to a combination of the unphysiologically rigid substrate mechanics of 2D culture plastic, in addition to the overcrowding of cells in 2D monolayers and the need for continuous subculturing of cells. Ultimately, traditional 2D culture modalities likely result in less viable and more senescent cell populations over time, leading to impurities and/or an inconsistent secreted product that subsequently increases the variability between experimental assays and limits the potential downstream clinical benefits of MSC-derived therapies.

Early studies with 3D culture systems, including spheroid and organoid culture, have demonstrated their capacity to circumvent some of the limitations of 2D culture and improve the stem-like phenotype of MSC populations [24–26]. However, spheroid and organoid cultures are often limited in their size due to diffusional constraints and are prone to central regions of necrosis and cell death [27]. Additionally, spheroid/organoid culture can be labor-intensive and can require large and expensive bioreactor systems, such as stir-tank bioreactors, for long-term expansion applications that culminate in the need for artificial dissociation of the cellular clusters with proteases [24,28]. This process is especially true for industrial purposes with the goal of generating cellular or cellular-derived therapies. Moreover, a recent study investigating long-term culture of Wharton's jelly MSCs demonstrated an increased population of senescent cells after 3D spheroid culture [29]. Thus, the development of more efficient tissue-mimetic 3D culture systems that improve the long-term expansion of robust and stem-like cell populations are currently still under investigation. One promising approach is the utilization of 3D hydrogel cultures, due in part to the tailorability of the substrate composition and mechanics [30,31]. To date, many hydrogel systems have aimed to control differentiation and/or act as a delivery vehicle of stem cells, rather than to expand MSCs and maintain their stem-like properties [32–35]. Additionally, most current hydrogel formulations lack the porous microarchitecture that aids in cellular migration and nutrient diffusion.

In this study we set out to develop a proof of concept for a novel 3D hydrogel system that improves expansion outcomes of MSC populations, such as adipose-derived MSCs (ASCs). The customizable 3D hydrogel system is bioprinted and was formulated to be a bioinert substrate that closely mimicked native adipose tissue mechanics (Supplementary Figure 1) and ultimately acts like a tailored bioreactor for MSC expansion and collection of biologics. The 3D hydrogel system was constructed with a unique 'puzzle-piece' macrostructure design that enables easy addition of supplementary hydrogels. Additionally, the unique porous microarchitectural design permits mass transport and promotes cellular migration and proliferation, eliminating the need to subculture cells via cellular migration between hydrogels within the microchannels. The initial utilization of a bioinert substrate allowed for the investigation and observation of the potential role of the mechanical and dimensional properties of the 3D system on ASC senescence and stem-like phenotypic properties without introducing a bioactive substrate. We hypothesized that the softer, 3D hydrogel substrate would provide a more natural mechanical environment for the ASCs and result in the retention of nonsenescent stem-like ASC populations, relative to traditional 2D culture methodologies. Moreover, the unique architectural design would allow for continuous expansion of ASCs via cellular migration between the attached hydrogels, thus eliminating exposure of cells to negative 2D subculturing procedures and subsequent sequelae.

Materials & methods

Cell culture

Human ASCs (Lot #18TL212639, 23-year-old female, Black), human keratinocytes (KCs; Lot #18TL318559, 62-year-old male, Caucasian) obtained from Lonza (Basel, Switzerland) were utilized for this study. MSC-GM

Mesenchymal Stem Cell Growth Medium BulletKit™ was obtained from Lonza (#PT-3001) and used for ASCs. MesenCult™-ACF Plus Medium Kit (Stem Cell Technologies, BC, Canada; Cat. #05445) was used as the serum-free medium. DermaLife K Keratinocyte Medium Complete Kit was obtained from Lifeline Cell Technologies (MD, USA; #LL-0007) and used for KCs.

3D-printed hydrogel cell culture system

The bioinert 3D hydrogel system is approximately 1 × 1 × 1 cm and is a polyethylene glycol (PEG)-based system called a Tissue-Block (T-Block; Ronawk, KS, USA) which contains a unique microarchitectural design and was fabricated to resemble the mechanics of native adipose soft tissue (Supplementary Figure 1) and demonstrated no significant changes in mechanical properties over 3 months. The 3D hydrogels were placed into a glass six-well culture plate for culturing. Fibronectin is a commonly selected coating substrate for ASCs due to their natural secretion of fibronectin. Because the cells do not efficiently adhere/attach to the PEG-based polymer, both the 2D culture plastic/glass and 3D hydrogel were coated with fibronectin at a concentration of 5 μg/cm^2 to enhance the initial cell attachment. The concentration of fibronectin was standardized to surface area due to the inherent surface-area-to-volume differences between 2D and 3D systems. The approximate surface area of the 3D hydrogel was calculated from the 3D model used for bioprinting.

Expansion of ASCs & KCs

ASCs and KCs were seeded within a T-150 flask and cultured until ~80% confluence before subculturing (passaging). Subculturing of cells was performed by removing culture media, washing thrice with Hanks' balanced salt solution (HBSS, MA, USA; calcium-free, magnesium-free) and incubating with 0.05% Trypsin/EDTA (Lonza; Cat. #CC-3232) at 37°C for 5 min. Trypsin was neutralized with serum and the cells were centrifuged at 500 × g for 5 min, then pelleted and resuspended for reseeding on new 2D tissue culture plastic vessels. ASCs at passage 1 (P1) were reseeded onto 2D culture plastic or onto/within the bioprinted 3D hydrogel system via dropwise addition of a concentrated cell solution to the surface of the hydrogels. This process was repeated with the residual cell solution five times to ensure efficient cell seeding. This repetitive seeding process allowed for the cells to distribute throughout the microporous structure within the hydrogel system. Given that the increased surface area and attachment of additional hydrogels eliminated the need for subculturing for this study, a passage-equivalence time point was utilized to allow for analogous comparison with 2D culture. Thus, a passaging event typically occurred every 4–5 days in 2D culture for ASCs but not in 3D culture. After 4–5 days of 2D culture for P2 ASCs, the cells were then subcultured and considered to be P3 in 2D, and the 3D ASCs were then considered P3 passage-equivalent. Culture expansion was determined based on the known 2D and 3D surface areas, initial cell seeding density and average population doubling time of 2.25 days (experimentally determined in 2D; data not shown) for the ASCs in order to standardize cell numbers. ASCs were seeded at a standardized concentration of 5000 cells/cm^2 for assays. Media supplementation was standardized to 150 μl/cm^2 for ASC expansion to account for dilutional differences in surface-area-to-volume ratio between 2D and 3D culture.

ASC phenotype characterization

The MSC stem-like phenotype was evaluated for the ASCs at P1/2/6/10 via immunolabel characterization of three key MSC surface markers (CD73/90/105). ASCs were either continuously subcultured in a T-150 flask or allowed to expand within the 3D hydrogel system. At each respective passage time point, cells were seeded in 2D at a standardized density of ~5000 cells/cm^2 onto a 96-well glass culture plate (Cellvis, CA, USA; Cat. #P96-1.5H-N) for 2 days, fixed, then assessed for ASC phenotype via immunolabeling for surface CD markers. For ASCs within the 3D culture system, cells were fixed and stained *in situ*. Positive staining for CD73/90/105 and negative staining for CD34/45 was used to denote a stem-like MSC phenotype for this study. After fixation with 4% paraformaldehyde, cells were washed thrice with HBSS and placed in blocking buffer (1% donkey serum in HBSS) for 1 h. After blocking, primary antibodies for CD73 (Abcam, MA, USA; Cat. #133582; 1:100), CD90 (Abcam; Cat. #181469; 1:100), CD105 (Abcam; Cat. #231774; 1:100), CD34 (Abcam; Cat. #81289; 1:200) or CD45 (Abcam; Cat. #40763; 1:200) were added and the cells incubated overnight at 4°C. The next day cells were washed thrice and secondary antibodies were applied for 1 h, followed by three additional washes. Cells were counterstained with Hoechst 33342 (Invitrogen, MA, USA; Cat. #H3570; 1:1000) and Alexa Fluor® 647 Phalloidin (Invitrogen; Cat. #A22287; 1:1000). Immunofluorescence was assessed with a Revolve microscope using filters for 4′,6-diamidino-2-phenylindole (DAPI; EX-380/30, EM-450/50), fluorescein isothiocyanate (EX-470/40, EM-525/50); Texas Red

(EX-560/40, EM-630/75) and Cy5 (EX-630/40, EM-700/75) (Echo, CA, USA) and 20× objective (Olympus, Tokyo, Japan; UPlanSApo, 0.75NA). ASC stem-like phenotypic quantification was carried out in quadruplicate (n = 4), with images taken from a total of ten random fields of view per biological replicate (2D = per well; 3D = per hydrogel), to achieve up to 40 total measured values for each sample. Total nuclei were counted, and total positive cells were evaluated. Phalloidin counterstain was used to aid in localization of positive staining. ASCs characterized at P1 were used as a baseline for comparison (Supplementary Figure 2).

ASC senescence characterization

Similar to the ASC phenotyping methodology above, assessment of ASC senescence was performed. ASCs were continuously subcultured in a T-150 culture flask until each respective assay time point, when they were seeded onto a 96-well glass culture plate. ASCs in the 96-well plate were allowed to acclimate in serum-based media for 2 days, fixed, then assessed for senescent activity via immunofluorescent labeling of β-galactosidase activity with the CellEvent™ Senescence Green Detection Kit (Invitrogen; Cat. #C10850), per manufacturer's instructions. ASCs in the 3D system were assessed simultaneously at the P2/6/10 passage-equivalent time points. Similar to the method above, cells were counterstained with Hoechst and Phalloidin. Senescence characterization was carried out in quadruplicate (n = 4), with images taken from a total of ten random fields of view per biological replicate, to achieve up to 40 total measured values per sample. Total nuclei were counted, and total senescent positive and negative cells were determined.

Isolation of ASC-conditioned media

Media supplementation was standardized for ASC expansion to account for dilutional differences in surface-area-to-volume ratio between 2D and 3D cultures. Media were changed every 2 days. For ASC-conditioned medium (ASC-CM) collection, MSC-GM was removed and cells were washed with HBSS thrice, then cultured with serum-free MSC media for 48 h before collection (for both 2D and 3D cultures). Collected ASC-CM was then centrifuged at 1500 × g for 10 min to eliminate cell debris, Steriflip-filtered with a 0.22-μm filter and stored at -80°C until use.

KC activity after ASC-CM treatment

ASC-CM was collected at each respective time point per the protocol above. ASC-CM was then placed on KCs for up to 24 h to assess its capacity to modulate metabolic, proliferative or migratory activity to evaluate wound healing capabilities of the ASC-CM. For these studies, ASC-CM was added at a 1:1 ratio with KC growth medium. Experimental assays were performed per the manufacturer's instructions. PrestoBlue fluorescence was obtained at 560/590 nm (n = 4) and used for evaluation of metabolic activity after 24 h of ASC-CM treatment. Hoechst was added to PrestoBlue samples and values were displayed as average relative fluorescence unit values of PrestoBlue/Hoechst signal in order to control for potential differences in cell numbers and obtain approximate metabolic activity per cell. PicoGreen fluorescence was obtained at 485/535 nm (n = 4) and used for evaluation of cell number as a surrogate measurement of KC proliferation after 24 h of ASC-CM culture. Total cell numbers per 96-well plate were calculated based on an average DNA content of 7.7 pg/cell. KC scratch assays were performed to evaluate changes in wound size as a surrogate measurement of KC migration (n = 3). Migration images were taken using an ImageXpress Micro XLS Imaging System (Molecular Devices, CA, USA). The entire wound was imaged for each wound triplicate and three different wound regions per wound triplicate were used to calculate wound area. The three wound area values were averaged per triplicate and per time point for each group and displayed as percentage wound area recovered (n = 3).

Statistical analysis

All data are reported as means with standard error of the mean. Characterization analyses of ASC populations with immunolabeling for senescence and CD markers were evaluated with a two-way analysis of variance. KC metabolic, proliferative and migratory activities were evaluated with a two-way analysis of variance. Data were tested for normality via Shapiro–Wilk and Kolmogorov–Smirnov tests and plotted with a QQ plot. GraphPad Prism 9.0.2 software (GraphPad, CA, USA) was used for the analyses, and a p-value < 0.05 was considered significant.

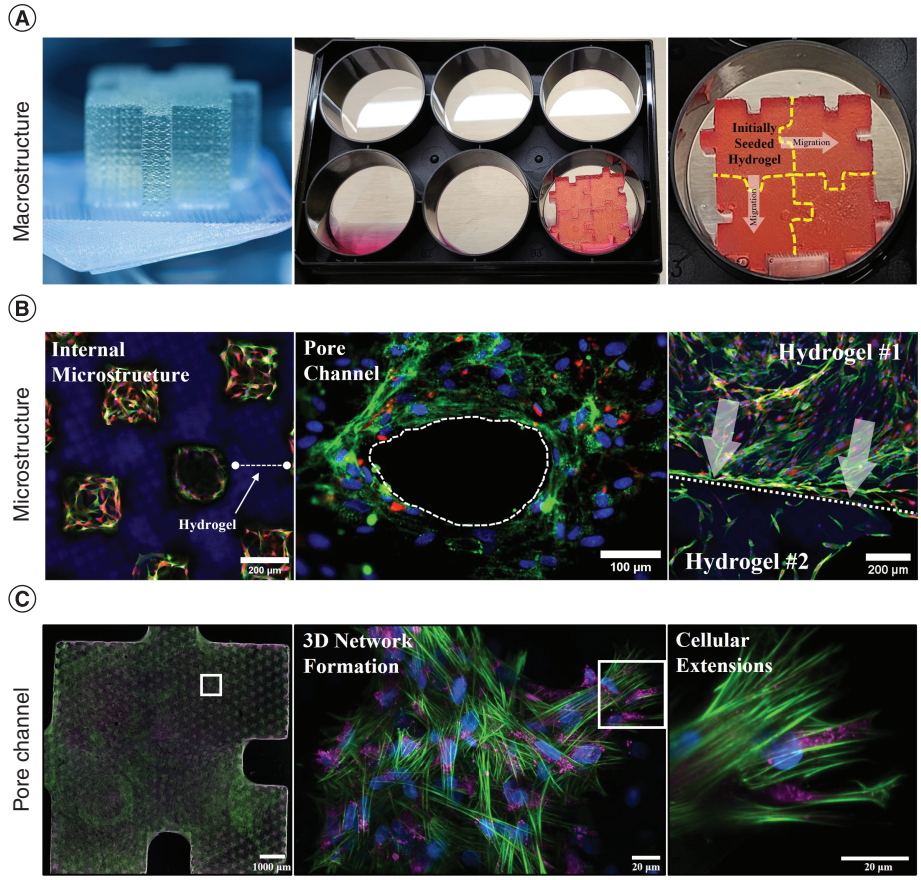

Figure 1. Unique 3D hydrogel design eliminates subculturing. (A) Depictions of the macrostructure of the hydrogel system. (Left) A single photographic image of the ~1-cm^3 3D hydrogel system. (Middle) A photographic image of four hydrogels connected to each other within a six-well plate. (Right) A photographic image of four hydrogels, connected with annotations depicting the migration of cells out/from an initially seeded hydrogel into/toward supplementally attached hydrogels without cells. **(B)** Depictions of the microstructure of the hydrogel system. (Left) Confocal microscopy image of a cross-section of the internal structure of the hydrogel depicting ASCs migrating and proliferating within the porous architecture. The small white arrow indicates polymeric struts of the hydrogel. Blue = Hoechst; green = phalloidin; red = Mitotracker. (Middle) Fluorescent image of ASCs lining an individual pore within the microstructure of the hydrogel system. Blue = Hoechst; green = wheat germ agglutinin; Red = Mitotracker. (Right) Confocal microscopy z-stack image of ASC migrating from an initially seeded hydrogel (Hydrogel #1) into a newly attached hydrogel (Hydrogel #2). Large white arrows indicate directionality of ASC migration; white dashed line indicates the junction of the two attached hydrogels. **(C)** Three images depicting a 3D z-stack of images within a single pore channel to highlight (middle) the 3D networks formed by ASCs and (right) cellular extension protruding from the cells. (Left) Low-magnification image of the entire stained hydrogel. Images in **(C)** were acquired by Nikon on their AXR Confocal Imaging System. Blue = Hoechst; Green = phalloidin; Pink = Mitotracker.
ASC: Adipose-derived mesenchymal stem/stromal cell.

Results

Unique 3D hydrogel design eliminates subculturing

The ~1-cm^3 3D-printed hydrogel system contains a unique 'puzzle-piece' macrostructure that allows the continuous addition of supplementary hydrogels (Figure 1A) and promotes the migration of cells from the primary seeded hydrogel into the newly attached hydrogels (Figure 1A & B). ASCs were cultured within/onto a single hydrogel system for 2 weeks and allowed to migrate throughout the hydrogel. Subsequently, an additional hydrogel was added for 5 days, and cells were allowed to migrate to the newly attached hydrogel. The cells were then stained and assessed for migration and proliferation between the two hydrogels (Figure 1B). ASCs were seen lining the porous channels beyond the superficial surface within the internal structure and can be seen forming networks within the hydrogel pores (Figure 1B & C). Cells were able to migrate both across the surface of attached hydrogels and within the microchannels, and can be seen with numerous cellular extensions and focal adhesions protruding from the

cells to aid in migration (Figure 1C). The interaction of ASCs in 3D and the formation of 3D networks within the hydrogel microarchitecture are further highlighted in Supplementary Videos 1 & 2. Notably, ASCs can be seen lining the surface of the hydrogel and migrating down into one of the pore channels, ultimately interacting in three dimensions with other cell populations within the pore.

Retention of stem-like MSC surface markers in 3D hydrogel over time

Evaluation of stem-like ASC populations over time was performed via immunolabeling quantification of the CD markers CD73/90/105 (Figure 2A). The prevalence of stem-like CD markers declined over time in both 2D and 3D systems; however, 2D culture had a significantly enhanced rate of loss of these markers relative to the 3D system (Figure 2B). Similarly, there was an apparent decrease in intensity of positively stained cells over time in both 2D and 3D systems. Culture within the 3D system resulted in a significant retention at all passage time points for all three positive stem-like markers, except for the initial P2 comparison of CD105. The significant loss of a stem-like phenotype in 2D culture can be seen clearly when comparing ASCs in 3D at P6 and P10 versus ASCs in 2D at P2 (Figure 2B). Over the course of five or nine passage equivalents in 3D (i.e., 3 or 6 weeks in the 3D system for P6 or P10, respectively) and only one passage event in 2D (seeded at P1), the ASCs had similar expression patterns for multiple CD markers, with only a significant difference between 3D expression of CD105 at P10 relative to 2D CD105 at P2 (Figure 2B). Both systems maintained a low population (<5%) of positive-staining cells for CD34 and CD45 markers.

Delayed induction of senescence in 3D hydrogel over time

The prevalence of senescent ASC populations over time was evaluated via fluorescent labeling of β-galactosidase activity, a commonly utilized surrogate measurement of cellular senescence (Figure 3A). The baseline expression of β-galactosidase activity was ~5% for both 2D and 3D ASCs at P2. Over the course of multiple passaging events in 2D, senescence was significantly increased in 2D, with 11.7 and 22.5% senescent cells at P6 and P10, respectively (Figure 3B). Conversely, in the 3D system there was no significant change in the prevalence of senescence over time in ASC populations for the time course of this study (6 weeks) (Figure 3B). Similarly, there were notable differences in cellular morphology in the 2D system versus the 3D system. More specifically, 2D ASCs appear to be more flattened, with a more heterogeneous morphological distribution and an apparent increase in cell size over time in culture, whereas the 3D ASCs maintained a more homogeneous morphology with no observable change in cell size or morphology.

Retention of ASC conditioned media wound healing capacity

Evaluation of the ASC-CM's functional capacity to modulate a secondary cell population was utilized to demonstrate the dynamic interrelationship between ASC population phenotype and ability to promote wound healing activity in KCs (Figure 4D). KCs were treated with either 2D or 3D ASC-CM from each respective time point for up to 24 h. KCs treated with ASC-CM from 2D cultures showed a significant drop in recovered wound area when treated with P6 and P10 ASC-CM, relative to P2. There was also a slight decrease noted in KC migratory activity observed with 3D ASC-CM from P10 relative to P2 (Figure 4A & B). Overall, 3D ASC-CM maintained a significantly higher ability to enhance KC migration and close their respective wounds, relative to their 2D ASC-CM counterparts. The metabolic activity of KCs demonstrated a decreasing trend when treated with ASC-CM from 2D-expanded cells, with a significant decrease noted in P10 relative to P2 ASC-CM (Figure 4C). No significant differences were observed with ASC-CM from 3D cultures over time. Similarly, the proliferative activity of KCs treated with ASC-CM from 2D cultures demonstrated a decreasing trend, with a significant difference between P10 and P2 ASC-CM, but no significant change noted when KCs were treated with ASC-CM from 3D cultures (Figure 4D). Moreover, the proliferative activity of KCs treated with 3D ASC-CM from P6 and P10 was significantly higher than that of their 2D ASC-CM counterparts.

Discussion

The inherent regenerative properties of MSCs have garnered immense interest for advancing the field of regenerative medicine and tissue engineering. However, MSCs typically must first be removed from a donor tissue source and cultured outside the body within an artificial environment not native to human tissue. To date, commercially available *in vitro* expansion systems are almost exclusively 2D in nature. Rigid 2D systems are unphysiological for the cells and rapidly result in the loss of MSC multipotent stem-like features, with subsequent loss of viability and

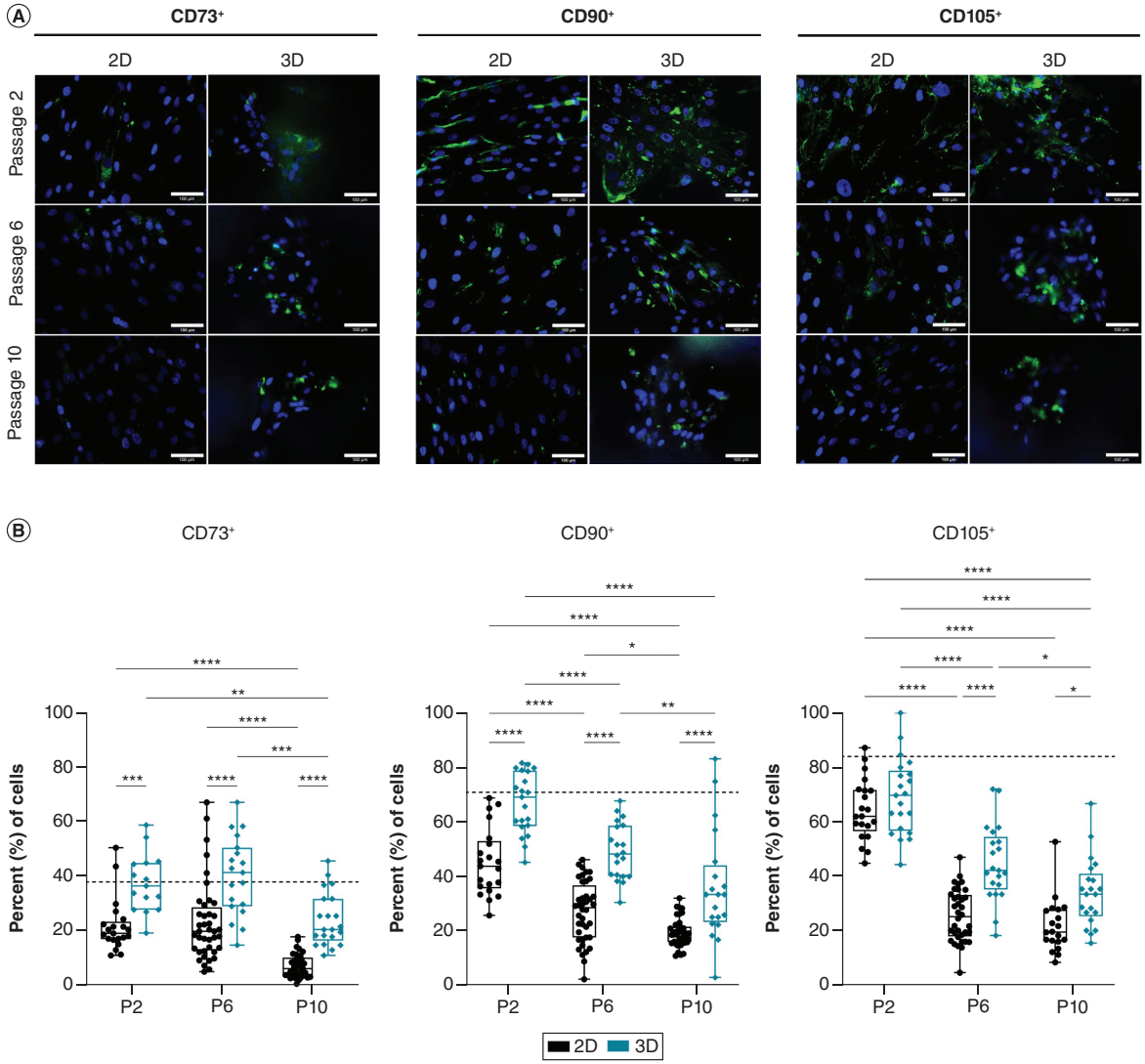

Figure 2. Retention of stem-like surface markers in 3D hydrogel over time. Adipose-derived mesenchymal stem/stromal cells (ASCs) were seeded at passage 1 (P1) within the 3D hydrogel system or traditional 2D culture. ASCs were continuously subcultured and assessed at P2, P6 and P10 for 2D culture. The ASCs in the 3D hydrogel system were compared with their respective 2D counterparts via passage-equivalent time points. Three additional hydrogels were added to each individual hydrogel at the 2-week mark and left for the remainder of the culture period to provide adequate surface area for continuous cell growth. **(A)** At each respective time point (P2, P6 and P10), representative images of 2D (leftmost columns) and 3D (right-most columns) cultured ASCs stained for either CD73 (left), CD90 (middle) or CD105 (right) are depicted. CD marker staining is denoted by green in the images. Samples were counterstained with Hoechst (blue) and phalloidin (not shown). **(B)** Quantification of imaging data was performed and total percentage of positive cells denoted by box-and-whiskers plots for each marker. Each individual point indicates quantification of a single image of ASCs in 2D (black circles) or 3D (teal diamonds) cultures using a 20× objective. Samples were analyzed in quadruplicate (n = 4). Scale bar = 100 μm. Error bars are standard error of the mean.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

induction of senescence [16,18,36]. These changes lead to MSC populations with significantly reduced regenerative capabilities, which is compounded by a lack of standardized cell culture conditions, creating a significant bottleneck in the growth and development of regenerative therapeutics. Thus, there is a critical need to develop culture systems for MSC expansion that are 3D and more tissue-mimetic in their mechanical, architectural and substrate composition properties and which can ultimately circumvent many of the limitations of traditional 2D culture, such as the continuous need for subculturing.

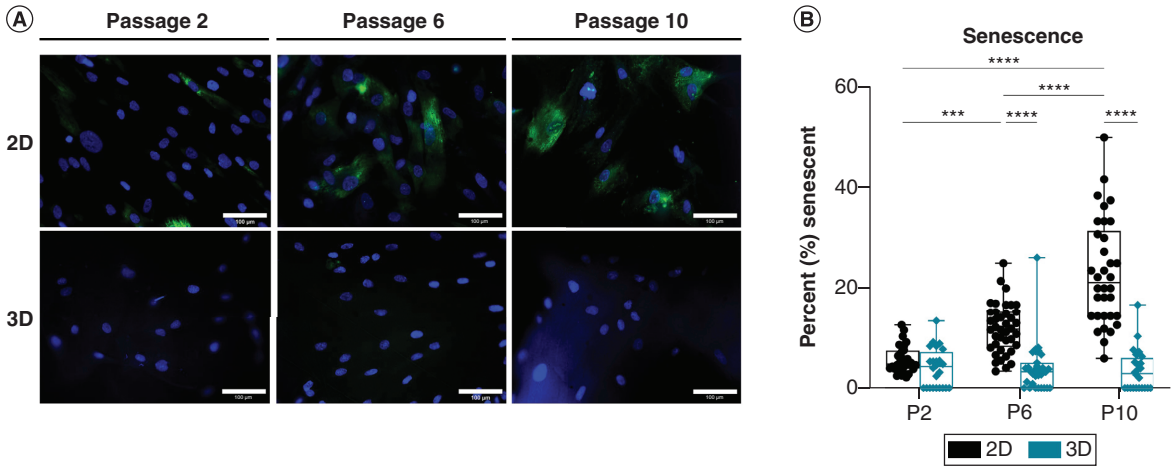

Figure 3. Delayed induction of senescence in 3D hydrogel over time. Adipose-derived mesenchymal stem/stromal cells (ASCs) were seeded at passage 1 (P1) within the 3D hydrogel system or traditional 2D culture. ASCs were continuously subcultured and assessed at P2, P6 and P10 for 2D culture. The ASCs in the 3D hydrogel system were compared with their respective 2D counterparts via passage-equivalent time points. Three additional hydrogels were added to each individual hydrogel at the 2-week mark and left for the remainder of the culture period to provide adequate surface area for continuous cell growth. **(A)** At each respective time point (P2, P6 and P10), representative images of 2D (top row) and 3D (bottom row) cultured ASCs stained for β-galactosidase (green) are depicted. Samples were counterstained with Hoechst (blue) and phalloidin (not shown). **(B)** Quantification of imaging data was performed and total percentage of positive cells denoted by box-and-whiskers plots for each marker. Each individual point indicates quantification of a single image of ASCs in 2D (black circles) or 3D (teal diamonds) using a 20× objective. Samples were analyzed in quadruplicate (n = 4). Scale bar = 100 μm. Error bars are standard error of the mean.
***$p < 0.001$; ****$p < 0.0001$.

Recent advancements in 3D systems have demonstrated progress toward producing MSC populations that are more stem-like. However, 3D systems such as spheroids, organoids, microspheres and many scaffold systems typically do not closely mimic the native mechanics of their cell/tissue source (e.g., adipose mechanics for ASCs) and often require large bioreactor systems and continuous subculturing to achieve large-scale cell numbers for clinical use. However, tissue-engineered hydrogel systems appear to be advantageous toward producing tailorable, tissue-mimetic systems for cell culture systems [30,31]. More specifically, the mechanotransductive response to the softer substrate of hydrogels is thought to aid in the retention of stem-like characteristics [23,37,38]. Unfortunately, most current hydrogel systems are manufactured to promote controlled differentiation of stem cells toward a specific tissue lineage and not to allow the cells to maintain a stem-like phenotype for long-term expansion [34,39–44]. Ultimately, an ideal MSC hydrogel expansion system would protect against senescence, while also improving the retention of a regenerative stem-like phenotype and permitting long-term expansion with minimal subculturing or user intervention.

Although MSC-based therapies have demonstrated promise, with over 1000 clinical trials to date listed with the US FDA, they have not advanced as quickly as previously thought. This is considered to be due, at least in part, to the detrimental impact senescent MSC populations may have on tissue regeneration [45,46]. Senescence is a progressive form of cell-cycle arrest, typically due to DNA and/or oxidative damage, which results in MSCs with impaired DNA-repair modalities that no longer proliferate and exhibit a loss of multipotency [47–49]. Moreover, senescent MSCs have been shown to secrete factors that negatively impact tissue regeneration and wound healing by impairing angiogenesis, increasing oxidative stress and exacerbating inflammation via the secretion of factors known as the senescence-associated secretory phenotype [50–52]. The composition of this phenotype can be heterogeneous and is dependent on the mechanism of senescence induction and environmental stimuli; therefore, this likely contributes to the heterogeneity in patient outcomes seen in clinical trials with both cell-based and acellular therapies [53–55]. Thus, developing an *in vitro* culture expansion system that limits/prevents the induction of senescence in healthy allogeneic or autologous MSC populations intended for patients would improve the efficacy and consistency of MSC-based clinical therapies.

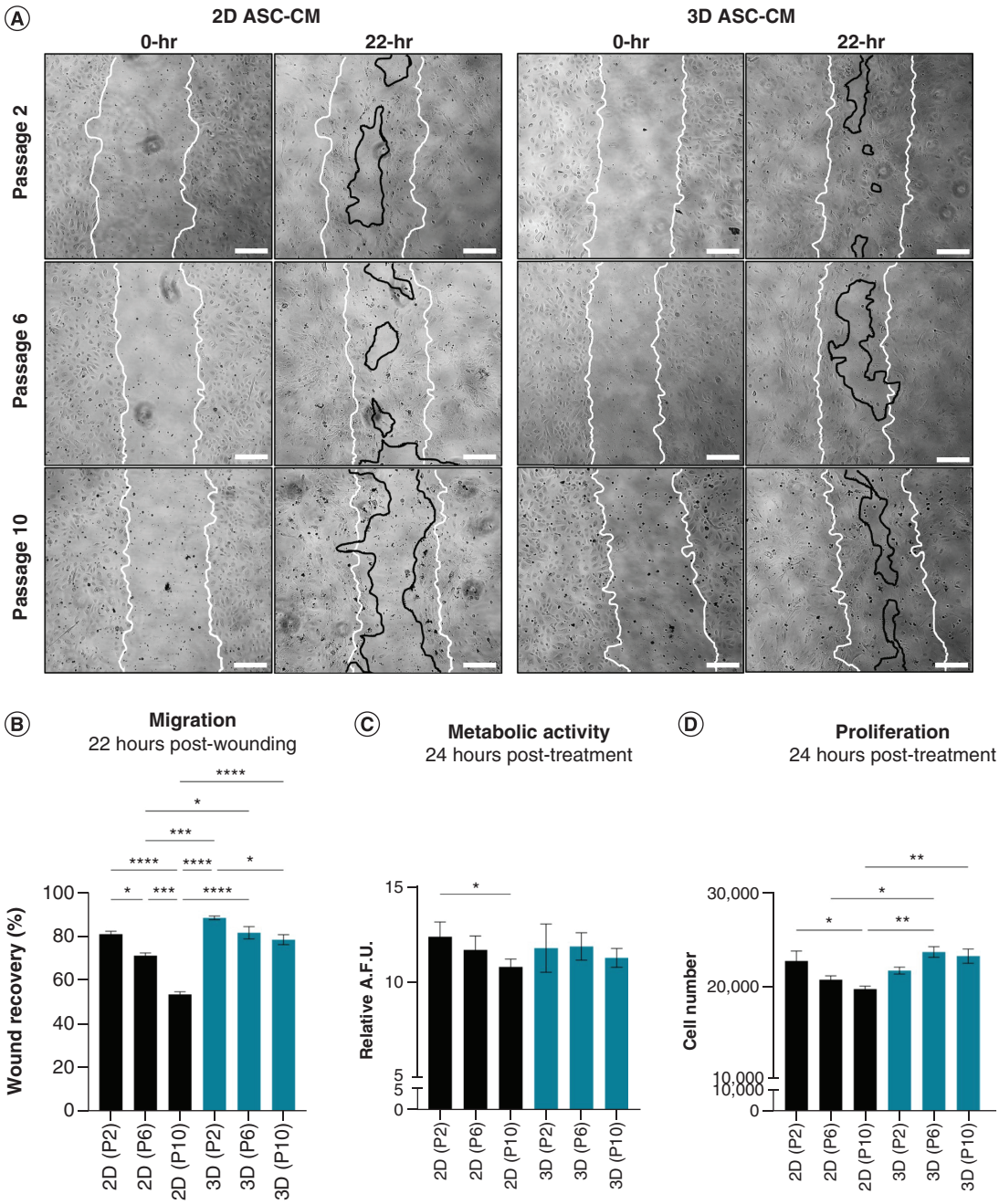

Figure 4. Retention of wound healing capacity in adipose-derived mesenchymal stem/stromal cell-conditioned media. ASC-CM from 2D and 3D from each respective time point was used to treat keratinocytes, which were then assessed for changes in their migratory, metabolic and proliferative activity. **(A & B)** Migratory activity was assessed via a scratch assay. **(A)** Whole-well image scans were acquired, and representative images of the wound images are provided. White solid lines denote original wound boundaries. Black solid lines outline the remaining wound area. **(B)** The average wound area after 22 h was determined and performed in triplicate. **(C)** Metabolic activity was quantified via PrestoBlue and is displayed as an average value of relative fluorescent units. **(D)** Proliferative activity was quantified via PicoGreen and the average cell number was determined. Keratinocytes treated with 2D ASC-CM are denoted with black bars; keratinocytes treated with 3D ASC-CM are denoted with teal bars. Scale bar = 500 μm. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. Error bars are standard error of the mean.
ASC: Adipose-derived mesenchymal stem/stromal cell.

In this proof-of-concept study, culture of ASCs within a traditional 2D culture system resulted in a significant increase in senescence, as previously established in literature. Conversely, the 3D hydrogel system resulted in no significant changes of senescence in ASC populations over the course of the 6-week study (i.e., 10 passage equivalents). Moreover, changes in cell morphology and size can be indicative of phenotypic changes; the apparent increase in cell size seen in the 2D ASCs may be associated with the induction of senescence and thus further supports the β-galactosidase imaging data and prior literature. Overall, these data support the previous hypothesis that 3D culture and substrate mechanics maintain a protective role against senescence. However, with our 3D hydrogel system the need for subculturing is eliminated, and secreted by-products are more readily accessible versus more traditional poured/molded hydrogels that lack a porous microarchitecture. Further studies will be needed to assess potential dynamic mechanisms at play (e.g., induction of telomerase, protection against oxidative damage or increased DNA repair mechanisms) to establish the protective role of 3D culture seen in this study. Moreover, evaluating what the inciting events are that drive the progression toward *in vitro* senescence, and whether they can be circumvented with appropriate culture systems, requires further investigation. This study offers insight into future design considerations for improving 3D cell culture modalities for MSC expansion.

Similarly, the stem-like phenotype of MSCs is critical to their regenerative potential and can rapidly change depending on the culture environment of the cells. MSC populations that differentiate and lose their stem-like characteristics result in variability of cellular phenotype and alterations in secretome composition, ultimately decreasing the consistency of regenerative MSC therapeutics, both cellular and acellular. Thus, MSC populations such as ASCs are often used within only a few passaging events in an attempt to circumvent the loss of regenerative potential. However, as we see in this study, even within one additional passaging event in 2D culture, ASCs significantly alter their expression of stem-like markers. Moreover, ASCs expanded in 3D culture for six or ten passaging equivalents (i.e., P6 or P10) over 6 weeks maintained similar expression levels of several markers relative to the baseline P2 ASCs, and a higher expression relative to their respective 2D counterparts, further highlighting the detrimental effects of 2D culture systems on MSC populations and the potential protective effects of 3D culture. The ability to improve the retention of stem-like properties within MSC populations for longer periods of time is desirable for a multitude of applications, including cell therapies, regenerative tissue engineering, immunotherapy and production of secreted biologics.

As previously mentioned, recent MSC therapies have expanded into investigating biologics as a potential regenerative therapy. MSC populations are highly adaptive in nature, and are known to sense their surrounding environmental stimuli and secrete factors accordingly. Thus, secreted bioactive compounds act to coordinate and bridge a variety of tissue reparative processes in an autocrine, paracrine or endocrine manner. In this study, the conditioned media from ASCs cultured in 2D versus the 3D system were collected over time and utilized as a therapeutic for a secondary cell population, KCs, as a means to functionally assess the phenotype of the ASC secretome toward promoting wound healing activity. Working under the hypothesis that the ASC phenotype deteriorates over time in 2D cultures but is sustained in the 3D system, we expected to see a gradual decline in regenerative capabilities of the ASC-CM from 2D cultures but minimal changes in ASC-CM from 3D cultures. This hypothesis was supported by the metabolic, proliferative and migratory data of KCs treated with ASC-CM. ASC-CM from 2D cultures demonstrated a steady decline in ability to augment KC activity and was consistently outperformed by its 3D counterpart over the course of the study. To our knowledge, this is the first direct comparison of ASC phenotypic changes over time in 2D and 3D cultures, and the first examination of how those changes potentially correlate to the regenerative wound healing capacity of the ASC secretome on KC functionality.

The limitations of this study include the formulation of the hydrogel system being intentionally bioinert in order to eliminate any contribution of a bioactive substrate, and thus it was not degradable. As a result, adequate removal of cells from this formulation was not feasible. Therefore, we utilized immunofluorescent labeling as an alternative to flow cytometry or RNA analysis to demonstrate senescence and phenotype of MSC populations. However, the ability to perform *in situ* visualization of an adherent population such as MSCs without the need to resuspend them is an advantage of immunolabeling over cytometry. Moreover, the relative comparison between 2D and 3D cultures, paired with the functional data of the ASC-CM, helps provide a supportive and holistic perspective that reinforces the immunolabeling data, although a more comprehensive analysis of the secretome is needed to assess both qualitative and quantitative changes. Future hydrogel formulations will aim to be biodegradable, such as insertion of enzyme-sensitive sequences or utilization of native matrix-derived compounds, while also incorporating the advantages and design of this proof-of-concept system, including the mechanics, modularity, architecture and

elimination of subculturing. Lastly, follow-up studies to directly compare the performance of this 3D hydrogel system versus other 3D systems will provide a better understanding of the relative benefits of this new system.

Conclusion

In this study we introduce the proof-of-concept of a novel 3D hydrogel system to demonstrate the benefits of culturing MSCs in a system that more closely resembles their native tissue mechanics. Our 3D system contains a unique architectural design that does not impede effective mass and fluid transport while also allowing the movement of cells within and between attached hydrogels, in effect providing a continuous 3D culture system that eliminates the need to subculture cells. The continuity is achieved by the addition of supplemental hydrogels to previously seeded hydrogels, much like attaching together two puzzle pieces. The porous microarchitecture creates a 'tunneling' system for the cells to interact in the x-, y- and z- planes and to migrate within and between hydrogels, in addition to surface migration at attachment points. This is the first demonstration of such a system, to our knowledge. The results of this study provide a framework for future studies; specifically, after establishing the beneficial effects of this bioinert 3D hydrogel system on ASC populations, future studies will aim to further tailor the 3D system's tissue-mimetic properties and control the regenerative activity of MSCs. Together, these data demonstrate the potential of a novel 3D culture system to enhance and retain the overall regenerative capacity of MSC populations over time, with the added benefit of further improving the regenerative and wound healing capabilities of MSC-derived biologics for a variety of tissue reparative applications.

Summary points

- Mesenchymal stem/stromal cell (MSC) populations are highly adaptable progenitor cell populations that contain inherent regenerative capabilities due to their multipotent and secretory nature.
- MSC-secreted by-products, also known as the secretome, contain a heterogeneous milieu of biomodulatory factors such as proteins, antioxidants, nucleic acids, exosomes and microvesicles. The secretome composition is dependent on MSCs' adaptive responses and environmental cues.
- The acellular MSC secretome has the ability to directly modulate tissue engineering and wound healing processes independent of MSC cellular feedback.
- In order to achieve enough MSCs for a clinical product, MSCs must be explanted from the body and expanded within an artificial environment not native to human tissue.
- To date, most commercially available *in vitro* expansion systems are 2D in nature, forcing MSCs to grow in a 2D monolayer. These rigid 2D systems are unphysiological and result in senescence, loss of MSC multipotency, and impaired secretory activity.
- Traditional 2D culture systems result in impurities and/or an inconsistent secretory and cellular product due to less consistent and viable MSC populations within 2D culture systems.
- Senescent MSCs have been shown to secrete factors that negatively impact tissue regeneration and wound healing.
- Recent advancements in 3D systems have demonstrated progress toward producing MSC populations that are able to maintain their stem-like characteristics for an extended period of time. However, most current 3D culture expansion systems still do not mimic native tissue mechanics for the MSC cell/tissue source.
- Tissue-engineered hydrogel systems offer many advantages for producing tailorable, tissue-mimetic systems for cell culture.
- Most current 3D hydrogel systems lack an inherent porous microarchitecture, are used as delivery vehicles and/or are manufactured to control and promote specific differentiation activity of MSC populations.
- Our novel 3D hydrogel system contains a unique architectural design that does not impede effective mass and fluid transport, permits the movement of cells within and between attached hydrogels and results in a continuous 3D culture system that eliminates the need for subculturing.
- Over the course of the 6-week study, our 3D hydrogel system resulted in no significant changes in senescence, whereas traditional 2D culture resulted in an approximately fourfold increase in senescent ASC populations.
- Traditional 2D culture resulted in a significant decline in expression of stem-like MSC markers within one passaging event, and this continued to decline over the course of the study. Loss of expression of stem-like MSC markers was significantly slower in adipose-derived MSCs (ASCs) cultured within the 3D hydrogel system.
- Deterioration of the ASC phenotype over time in 2D correlated with a gradual decline in the regenerative capabilities for the ASC-conditioned medium, whereas the regenerative capabilities for the ASC-conditioned medium from 3D culture were sustained.

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-2022-0140

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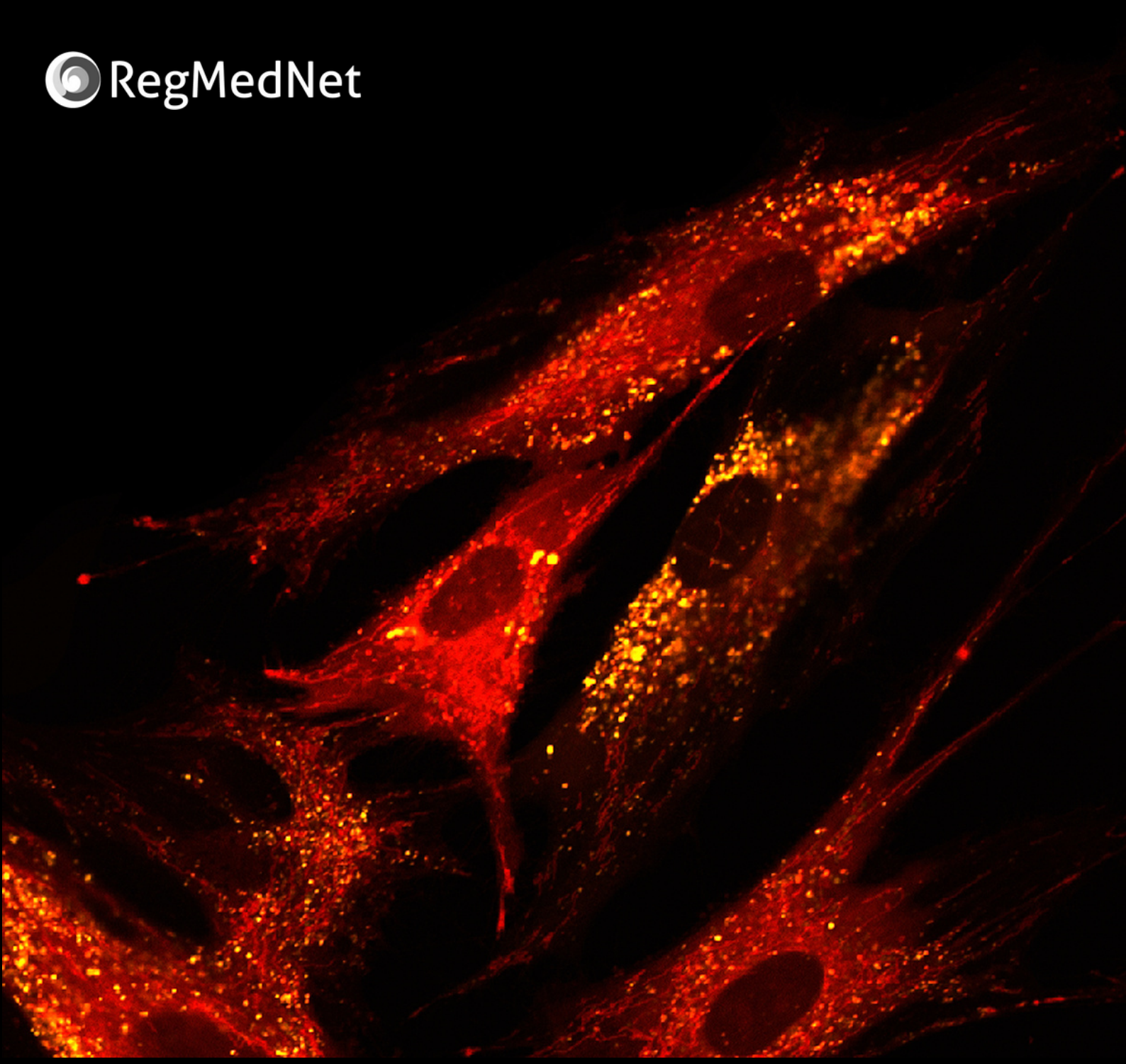

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