REVIEW ARTICLE

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Mesenchymal Stem Cell in 3D Culture: Diminishing Cell Senescence in Cryopreservation and Long-term Expansion

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Mesenchymal stem cells (MSCs) are widely recognized in cell treatment due to their capacity to secrete trophic factors, differentiate multipotent, and self-renew. Although there is growing evidence that MSCs have therapeutic benefits in various clinical settings, these cells eventually lose their ability to regenerate as they age, which increases cellular dysfunction. Several factors may affect MSCs aging, such as culture dimensions, cryopreservation process, and long-term expansion. Traditional two-dimensional (2D) culture conditions lack the complexities required to recreate MSCs in their natural environment. Meanwhile, three-dimensional (3D) culture mimics the niche, dynamic, and specialized microenvironments of the cells *in vivo*. The most used storage technique for MSCs, cryopreservation, requires a very low temperature reduction, which stresses cells and can cause the release of pro-inflammatory cytokines. For the utilization of MSCs in therapeutic applications, an *in vitro* expansion technique is required. Repeated expansion may reduce proliferative capacity, disrupts cellular shape, and impairs the somatic cell function of MSCs. Various processes and techniques may influence MSCs leading to cell aging. One of the culture methods, 3D culture, is shown to reduce the factors that will compromise the therapeutic effects of MSCs, especially cell senescence. The effect of culture dimensions, cryopreservation, and long-term expansion on cell senescence will be discussed in this review article.

Keywords: *cell aging, mesenchymal stem cell, 3D culture, cell senescence, cryopreservation, long-term expansion*

Introduction

Mesenchymal stem cells (MSCs) have been demonstrated in several studies to offer intriguing potential as regenerative therapeutics due to their capacity to self-renew, differentiate into various tissues, regenerate, and release immunomodulatory substances that can enhance the immune response (Figure 1).[1] One of the sources of MSCs, which has been utilized in investigations and treatments relatively frequently up to this point, is the umbilical cord. The effectiveness of umbilical cord MSCs as a treatment for heart failure[2], periapical periodontitis[3], rheumatoid arthritis[4], numerous studies of burn cases[5], and several studies to treat Coronavirus disease 2019 (COVID-19).[6] MSCs have

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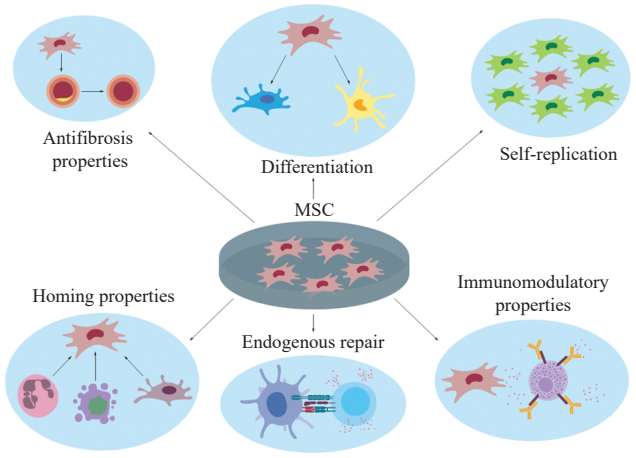

Figure 1. Properties of MSCs.(1) (Adapted with permission from Future Medicine).

to maintain its function and act as intended while treating degenerative disorders to be therapeutically effective.

Factors that can affect the capacity of MSCs to exert therapeutic benefits is its *in vitro* production process. The processes of culture, expansion, and cryopreservation are all included in the stages of MSCs manufacture. Two-dimensional (2D) is the most used *in vitro* culture techniques. As technology advances, the growing environment, namely the three-dimensional (3D) environment, is made feasible compared to its natural circumstances. As a result, the culture approach of MSCs should be considered, particularly for those that are more like their natural habitat since they can preserve their characteristics and functions.[7] Cell fatigue may result from the expansion process, which is an iterative one.[8] Storage at extremely low temperatures, in addition to the cryopreservation procedure, may also potentially reduce MSCs function.[9]

The development of age profiles (senescence) is one of the factors contributing to deteriorating the performance of MSCs.[10] Cells' ability to operate and provide therapeutic benefits can be further compromised by aging, which can cease the cell cycle. According to several studies, traditional 2D culture does not accurately reflect the natural growing environment of MSCs in the body, resulting in inferior functions and activities compared to 3D culture.[11,12] According to studies, the cryopreservation process decreases the immunomodulatory capacity of spinal cord MSCs and the ability of MSCs from the umbilical cord to adhere and proliferate.[9,13] Continuous cultivation can also lead to repeated cell fatigue, which accelerates aging.[8]

It is still challenging to keep MSCs functioning and having their therapeutic benefits, especially when the technology that produces MSCs is constantly evolving at the same time. In this review, information regarding MSCs culture in 3D environment and elaborate more on cryopreservation and long-term expansion effects in cell aging will be discussed, since studies with respect to this topic is still limited. Other than that, either diminishing or delaying cell aging on MSCs is in fact critical to achieve successful regenerative therapies using MSCs.

Cell aging

Cell aging displayes a constrained capacity for replication and stopped cell development.[14] When MSCs replication stops after growth with a certain level of repeatability, it enters a phase known as the Hayflick limit. The projected limit of MSCs is often observed at numbers between 30 and 40, the population doubling rate.[15] Senescence-associated-galactosidase (SA-gal) activity, the expression of cell cycle inhibitor proteins (p21, p16, and p53), changes in cell shape, and increased metabolic activity as evidenced by the activation of the glycogen synthase kinase (GSK3), adenosine monophosphate kinase (AMPK), and mamallian target of rapamycin (mTOR) pathways are just a few of the phenotypic characteristics of aging cells.[16]

Numerous variables can contribute to cellular aging, including DNA damage, oxidative stress, telomere shortening, mitochondrial malfunction, and abnormal oncoprotein activation. In addition to environmental variables, physiological mechanisms such as signaling cell development and repair processes can also cause aging.[17]

Cell aging is defined as a cell cycle process permanently interrupted and brought on by various reasons (Figure 2).[18] One of the leading causes of aging, which is brought on by physical, chemical, and stress stimuli damage, is DNA damage. Cells lose their equilibrium due to imbalances in synthesizing reactive oxygen species (ROS) and antioxidants, followed by aging.[19] Telomere shortening and oncogene activation are two additional aging factors that might trigger a DNA damage response (DDR). The p53/p32 and p16 signal routes significantly control aging, regardless of the received induction signal. The stimulus will activate DDR to trigger the transcription of the p53 protein, then activate p21, the cyclin-dependent kinase 1 (CDK1), which will prevent the phosphorylation of retinoblastoma tumor supressor protein (pRB) and the action of transcription

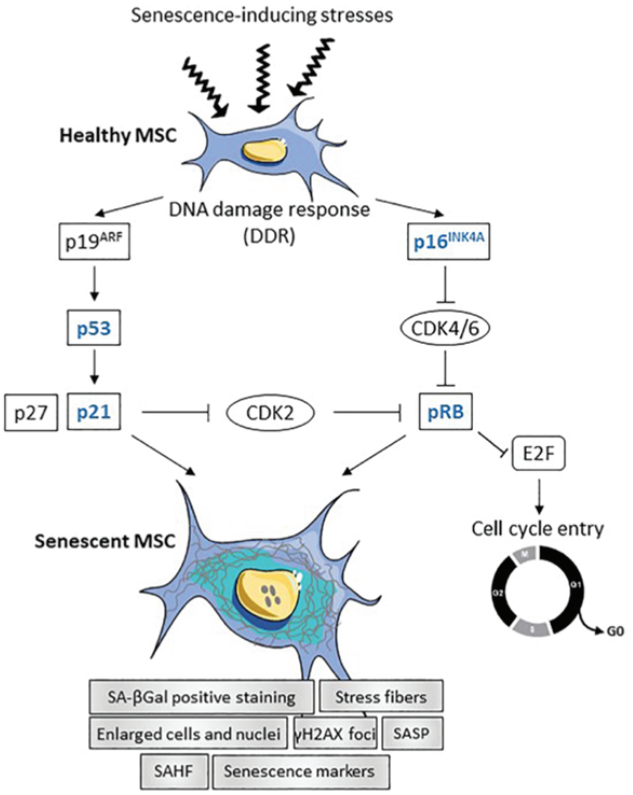

Figure 2. The mechanism of cell aging in MSCs.(18) SA-ß-gal: senescence-associated beta galactosidase, SASP: senescence-associated secretpry phenotype. (Adapted from Friontiers Media SA).

factor E2F. Activating these proteins will obstruct the cell cycle, causing cells to age.[19]

Senescence-associated-secretory-phenotype (SASP) cytokines are among the profiles that aging cells create, along with morphological alterations, increased SA-ß-galactosidase (SA-β-gal) activity, and SA-ß-gal expression. The shape of aged cells is often flatter and more extended than usual. The galactosidase beta 1 (GLB1) gene produces the lysosomal enzyme SA-gal, specifically secreted by aging cells. SA-β-gal measurements can be performed by staining and observed under a microscope and by the flow cytometry method. Aging cells synthesize and secrete a variety of cytokines and growth factors. Pro-inflammatory cytokines, including interleukin (IL)-6, IL-8, IL-1, and tumor necrosis factor (TNF)-α are often secreted cytokines. The flow cytometry technique may also be used to do SASP measurements.[20] Diverse circumstances cause the majority of signaling pathways to fuse on NF-kB lines. SASPs are cytokines that are highly expressed, most conserved, and intimately associated with NF-kB signaling pathways, especially IL-6 and IL-8.[21]

Conventional 2D culture

Most cell cultures and cell-based experiments are done on polymer or glass layers in 2D. Because 2D culture may be used to determine principles of cell biology, pharmacological activity, cell response to endogenous and external illnesses, mechanisms involved in cell development, and tissue morphogenesis, it plays a significant role *in vitro* cell investigations.[22] The bidimensional cell environment does not accurately reflect the natural environment of the cell, which is the 3D environment, even if 2D cell culture can help research the link between cell function and particular microenvironment components. Because it lacks most of the interactions that take place in the 3D environment, the 2D environment will lead cells to behave differently than they would in the natural 3D environment.[22] The main differences between 2D and 3D culture are described in Table 1.

MSCs are a specific kind of cell that adheres to a 2D dish culture's surface organically. Most of the culture containers for MSCs culture do not need to have a substrate or coating. The substrate is often present as serum in a growth media.[23] Even though 2D culture is a promising method for cultivating cells, several crucial MSCs features must be considered, including cell shape, physical contact between cells, and the degree of gene expression. Because a cell can only grow and extend in two dimensions, it has an elongated, flat form. The physical interaction of cells is becoming less representative of the physical interaction of innate cells. Due to the lack of physical contact between cells, the degree of gene expression is different from *in vivo* models, and communication between cells is similarly diminished.[24] As a result, continual 2D culture might lead MSCs to exhibit phenotypic alterations, one of which is a loss in differentiation capacity brought on by morphological changes in cells during cell subculture. Additionally, the proportion of surface marker proteins could decline.[11]

Several studies have shown cell aging results of stem cell cultured in 2D platform. Adipose derived-mesenchymal stem cells (AD-MSCs) in 2D culture express higher senescence-associated ß-galactosidase (SA-ß-galactosidase) than in 3D culture. It has been reported that SA-ß-galactosidase staining was more detectable at day 14 and 21. Another comparison of 2D and 3D culture shows a

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Table 1. Main differences of 2D and 3D culture.

Cellular Properties	2D	3D	Reference
Morphology	Fibroblast-like, spindle-shaped, aligned	Round-shaped adhering to 3D platforms	12
Doubling Time	Usually faster than 3D	Extended doubling time may occur	42
Cell Yield	Usually lower than 3D	High yield due to significant expanded space	39
Differentiation	Losing differentiation potential as expanded	Stronger differentiation capacity	39
Protein/Gene Expression	Lower due to lack of compexity	Higher expression levels, stemness, and pluripotent properties	51

significant increase of ß-galactosidase activity in adipose-derived mesenchymal stem cells (ASCs) in 2D culture.[23] The morphology of cells in 2D culture was seen more flattened and heterogenous with a clear increase in cell size within increasing passage.

Cryopreservation

Due to its infinite shelf life and ability to utilize small amounts of resources, cryopreservation has emerged as the industry standard for biological material stored in the cell therapy sector.[26] Indeed, cryostorage method has progressed from a small step in the cell therapy manufacturing process to a tool for expanding access to stem cell treatment and regenerative medicine. On the other hand, this cryopreservation process is growing slower than the cell therapy industry.

Cryopreservation of cells is associated with physical and molecular damage. There is ongoing debate over the usefulness of fresh cells vs. cryopreserved cells, as well as whether viability indicates functioning.[27] To date, using cryopreserved cells was suspected to be the root of failure in early MSC-based clinical studies.[27] Furthermore, the diversity in the result of MSC-based clinical trials has been presumed to be mostly attributable to functional changes in MSCs caused by the process of repeated thawing and freezing rather than the freezing method itself.[26]

As for the most used method, liquid nitrogen is used in cryopreservation. Cryopreservation keeps cells alive by reducing their metabolic activity at -196ºC. The most popular technique for lowering temperature is controlled rate freezing, which involves a temperature drop of 1º C per minute. A protective substance called a cryoprotectant must be added to the cell solution to preserve the cell from drastic temperature drop. Dimethylsulfoxide (DMSO) is a form of cryoprotectant that is often utilized. Cryoprotectants such as DMSO can infiltrate cells. DMSO acts by limiting cell dehydration and preventing the development of ice in the extracellular environment.[27]

Different cell conditions and sources may be affected differently by the cryopreservation method. Cryopreservation may lessen MSCs capacity for immunomodulation.[28] In contrast, hematopoietic stem cells can continue functioning and not significantly lose their potency even after being frozen for ten years.[29] Differences in the stages leading up to cryopreservation and the stage of cell resuscitation may be the source of the variety of effects of the technique.[30] Before cryopreservation, the settings for cell development can also impact how well cells perform, one of which is the culture dimension. Given the influence of 2D culture, which does not accurately reflect the natural environment and can alter the cell phenotype, 3D culture is anticipated to lessen the detrimental effects of cryopreservation on MSCs.

Studies regarding cryopreservation effect on 3D culture MSCs are still limited. The cryopreservation method of 3D-cultured MSCs may vary and may result in different outcome. There is a difference between preserving MSCs after 3D culture in cell suspension and preserving MSCs after 3D culture using its 3D platform. A study reported a significant difference between preserving MSCs in freely suspended colonies and preserving MSCs on microcarriers. MSCs preserved on microcarriers exhibit higher recovery post-thawing compared to freely suspended colonies of MSCs.[31] Another recent study conducting cryopreservation of MSCs in self-produced extracellular matrix (ECM) showed a retained 3D structure of ECM and did not exhibit a decline in viability.[32] These studies summarize that involving 3D platform in the MSCs cryopreservation process may improve cell recovery as well as maintain cell structure.

Long-term expansion of MSCs

The sources of MSCs are known to be relatively abundant, one of which is the umbilical cord.[33] However, tissue isolation often yields a small number of cells, whereas MSCs treatment calls for a large number. To achieve their therapeutic benefits, some illnesses and patients require up to hundreds of millions of cell counts.[34] Thus, an *in vitro* expansion procedure is necessary to use MSCs in therapeutic applications.[35]

The expansion process is an *in vitro* method to increase the number of cells. The passage number is a phrase used in this procedure to estimate cell age.[36] The passage rate begins at a low value once the cells are successfully isolated, then as the cell is subcultured, the passage rate increases. As a result, long-term growth will cause a rise in the passage rate, which indicates that cells are aging. Although the MSCs passage rate utilized for clinical applications varies, low passage rates, such as passage 3-5, are desirable. The consequences of replicative cell exhaustion, including reduced proliferation, morphological alterations, and the appearance of age markers, are brought on by long-term growth.[8]

The culture dimension in which MSCs are cultured also affect their function as well as performace. The expression of several genes involved in the fundamental function of MSC, such as vascular endothelial growth factor (VEGF), dimerized fibroblast growth factor (dFGF), bone morphogenetic protein 2 (BMP2) and C-X-C motif chemokine ligand 5 (CXCL5) decreased in 2D-cultured MSCs and increased with time. In contrast, the gene expression in 3D-cultured MSCs increased significantly. In other words, following prolonged extension, 3D culture can avoid a decline in the expression of genes involved in the fundamental function of MSCs.[11] Another study showed that adipose derived-mesenchymal stem cells (AD-MSCs) cultured in fibroblast-derived extracellular matrix downregulate senescence-related genes p16 and p21.[37] Up until now, there is still little evidence regarding the effect of senescence in long-term expansion of stem cell comparing 2D and 3D culture.

Mesenchymal stem cell in 3D culture

The 3D cell culture has been evolving and improving cell biology analysis starting from *in vitro* experiments, cancer studies, to drug delivery models.[38] This culture method resembles real environment where mechanisms of actions of cells take place in the living organism.[23] Therefore, various studies have shown that MSCs cultured in 3D environment exhibit higher stemness genes[39], sustain immune regulatory functions[20], promote cell yield[40], as well as supress cell senescence[11].

Regarding cell form, physical contact between cells, and the level of gene expression, 3D culture is more accurate in simulating the cell environment *in vivo*. Cells in a 3D environment can appear like cells in a natural 3D environment. In a 3D environment, there are cell-cell interactions which enhance cell-cell communication, furthermore increase the gene expression level in cells *in vivo* and in 3D-cultured cells.[24] Therefore, the performance and function of MSC may be maintained in a setting that more closely reflects the natural environment.[41] Using 3D culture is also beneficial in cell yield compared to 2D culture.[20] If large scale manufacturing of MSCs is intended, the 3D culture approach may be an alternative. Furthermore, in 3D cultures, many desired cellular properties are retained or even enhanced, enhancing their utility in fundamental and translational research.[42] The summary of several studies using 3D culture for MSCs and their outcome on cell aging are described in Table 2.

Various 3D platforms are now being developed for MSCs culture starting from hydrogels[25], extracellular matrix (ECM)[32], scaffolds[43], spheroid[44], to microcarriers[45] (Figure 3). There are static and dynamic 3D platform types, and for each type different culture technique are applied. ECM, scaffolds, and hydrogels can be applied for static 3D platforms, while spheroid and microcarriers can be applied for dynamic.[46] Compared to 2D culture, 3D culture technique has distinct and challenging cultivation process because of its three-dimensional environment. For example, MSCs cultured on microcarrier should be completely attached at the beginning and this requires procedure optimization to make sure all cells are attached.[45] Meanwhile, the 2D culture process is simpler because of its bidemensional environment. It is clear to see that 2D culture has an uncomplicated cultivation process but unfortunately, is inaccurate in mimicking MSCs natural environment.[23] Despite the challenges and other shortcomings, 3D culture is definitely an option to develop more specific and targeted MSCs for therapies seeing that the benefit is greater than 2D.

Alongside the ability to mimick real environment, 3D culture is known to enhance cell differentiation as well. The differentiation potential of MSCs cultured in a 3D platform is demonstrated by the strong expression of differentiation

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Table 2. Studies using 3D culture system in stem cells and its outcome on senescence.

Cell Type	3D Culture System	Cultureware Type	Outcome	Reference
AD-MSCs	Hydrogel	6 well plate	3D culture has the potential to improve senescence-related alterations.	12
hUC-MSCs	Bone Matrix-Mimicking Scaffold	Culture dish	The scaffold can preserve the stemness and youth of expanded hUC-MSCs	11
UC-MSCs	Honey Nanofibre Extracellular Matrix	Well plates	PVA:honey substrate can reduce ROS and senescence markers in UC-MSCs	52
ASCs	Spheroid	Ultralow culture flask	Upregulation of stemness and telomere maintenance	37
ASCs	Hydrogel	6 well plate	No significant increase in senescence over time of 3D culture ASCs	23
EMSCs	Spheroid	Culture plate	EMSCs in spheriod result in slower senescence than EMSCs in monolayer	49
hMSCs	Dissolvable microcarriers	Spinner flask	Low indications in senecence phenotype	53
Ad-MSCs	Fibroblast-derived extracellular matrix	Culture dish	Decreased expression of senescence-associated genes p16 and p21	35

AD-MSC: adipose derived-mesenchymal stem cell; hUC-MSC: human umbilical cord-mesenchymal stem cell; UC-MSC: umbilical cord-mesenchymal stem cell; ASC: adipose stem cells; EMSC: embryonic derived-mesenchymal stem cells; hMSC: human mesenchymal stem cells; Ad-MSC: adipose derived-mesenchymal stem cells.

genes such the osteocyte marker protein gene RUNX2, ALP, and Osterix/SP7. Additionally, chondrocyte cell transcription regulating proteins such as SOX9, SOX5, and SOX6 were expressed after MSCs growth utilizing microcarriers, together with greater levels of the chondrogenic extracellular matrix indicator COL2A1.[47] These proteins mainly indicate a chondrogenic differentiaion in MSCs. In contrast, MSCs grown with a microcarrier had decreased expression of the adipocyte cell marker protein gene, peroxisome proliferator-activated receptor 2 (PPAR2).[48] MSCs in 3D culture can still maintain the International Society of Cell and Gene Therapy (ISCT) requirements, particularly the expression of the marker proteins CD73, CD90, and CD105.[33] These are specific surface proteins for identification of MSCs where CD73 acts to identify multipotent potential of MSCs, CD90 involved in cell-cell and cell-matrix interaction, while CD105 is a protein marker for vascularization potential of MSCs.[49]

Due to different types of 3D models, different results in cell senescence have been found. Embryonic mesenchymal stem cells (EMSCs) grown in spheroids displayed a lower ß-galactosidase activity as much as 25% at passage 8, while EMSCs in 2D culture showed higher activity, approximately 37.5% at the same passage.[50] Another study using adipose mesenchymal stem cells (ASCs) cultured in hydrogel scaffold exhibited below 10% of ß-galactosidase activity at passage 10, while ASCs in 2D culture displayed higher up to 22.5% at the same passage.[25] Based on these studies, different senescence result may be obtained from different 3D models. Three-dimensional culture with scaffold may prevent mesenchymal stem cell senescence.

To summarize, most of the stem cells cultured in 3D platforms result in delayed senescence with increased stem cell properties. This culture method is very promising for stem cell industry to produce a better quality of stem cell products for clinical therapy. However, it still needs in

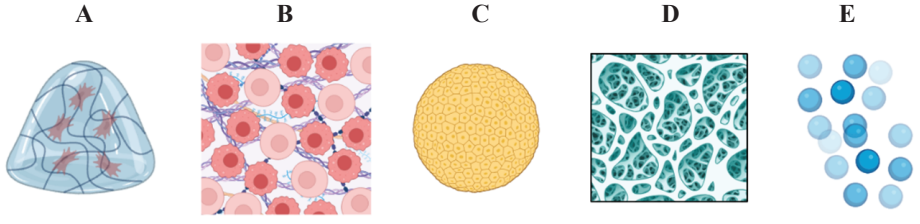

Figure 3. Examples of 3D cell culture. A: hydrogel, B: extracellular matrix (ECM), C: spheroid, D: scaffold, E: microcarriers.

depth study regarding the difference of 3D platforms since each type of the platforms may show different outcomes. Additionally, stem cell types from different sources may also contribute to variability of the results and therefore this needs a comprehensive study. In summary, there are several methods to influence cell senescence, such as moving from 2D to 3D culture, in order to get better outcomes in stem cell therapies.

Conclusion

The production process may have impact on MSCs. Several factors such as culture dimension, cryopreservation process, and long-term expansion of the cells may contribute to cell aging or senescence. Senescence can influence MSCs properties both *in vivo* and *in vitro*, which has substantial therapeutic and safety consequences. Numerous studies using the 3D culture approach have demonstrated improved results regarding MSCs features and age profiles. Therefore, 3D culture for stem cells may be a promising culture technique to produce sustained and healthy MSCs. This may lead to successful stem cell therapies as a result of utilizing non-senescent MSCs.

Authors Contribution

SF, RA, and CRS were involved in planning, drafted the manuscript, and designed the figures. All authors discussed the results and commented on the manuscript.

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