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Identification of senescent cells in multipotent mesenchymal stromal cell (MSC) cultures: current methods and future directions

Running title: Measuring senescent cells in MSC cultures

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Abstract

Regardless of their tissue of origin, multipotent mesenchymal stromal cells (MSCs) are commonly expanded in vitro for several population doublings, in order to achieve a sufficient number of cells for therapy. Prolonged MSC expansion has shown to result in phenotypical, morphological and gene expression changes in MSCs, which ultimately lead to the state of senescence. The presence of senescent cells in therapeutic MSC batches is undesirable, as it reduces their viability, differentiation potential and trophic capabilities. Additionally, senescent cells acquire senescence-activated secretory phenotype, which may not only induce apoptosis in the neighbouring host cells following MSC transplantation, but also trigger local inflammatory reactions. This review outlines the current and promising new methodologies for the identification of senescent cells in MSC cultures, with a particular emphasis on non-destructive and label-free methodologies. Technologies allowing identification of individual senescent cells, based on new surface markers, offer potential advantage for targeted senescent cell removal using new-generation senolytic agents, and subsequent production of therapeutic MSC batches fully devoid of senescent cells. Methods or a combination of methods that are non-destructive and label-free, for example involving cell size and spectroscopic measurements, could be the best way forward as they do not modify the cells of interest thus maximising the final output of therapeutic-grade MSC cultures. The further incorporation of machine learning methods has also recently shown promise in facilitating, automating and enhancing the analysis of these measured data.

Key Words: label-free, multipotent mesenchymal stromal cells, non-destructive, replicative aging, senescence

Abbreviations:

Bone Marrow Derived Mesenchymal Stromal Cells (BM-MSCs)

Colony-forming Units-fibroblast (CFU-f)

Critical Quality Attributes (CQAs)

Dental pulp Mesenchymal Stromal Cells (DP-MSCs)

International Society for Cellular Therapy (ISCT)

Histone Deacetylase (HDAC)

Human Mesenchymal Stromal Cells (hMSCs)

Mesenchymal Stromal Cells (MSCs)

Mean Telomere Restriction Fragment (mTRF)

Polymerase Chain Reaction (PCR)

Reactive oxygen species (ROS)

Senescence-associated β-galactosidase (SA- β-gal)

Senescence-associated Secretory Phenotype (SASP)

Single Telomere Length Analysis (STELA)

Vascular Endothelial Growth Factor (VEGF)

Introduction

Multipotent mesenchymal stromal cells (MSCs) have the ability to expand in culture, and with appropriate culture conditions, differentiate into at least three mesodermal cell lineages: osteocytes, chondrocytes and adipocytes [1]. In recent years, MSCs have attracted significant research and clinical interest due to their multipotent differentiation potential, trophic functions and applications in cell and gene therapy [2]. Due to their high proliferative capacity in vitro and the low risk of in vivo tumour formation in transplanted cells, MSCs have been employed in cell based therapy for various diseases including graft-versus-host disease, multiple sclerosis, myocardial infarction, liver failure and rejection after liver transplant [3, 4]. Moreover, cultured MSCs have been extensively used in tissue engineering applications to repair large articular cartilage and bone defects [5] or as a suitable vehicle for transplantation in both cell and gene therapy for the central nervous system [6]. However, the clinical outcomes of MSC transplantation remain variable, and this could be in part explained by difficulties in controlling both host responses and the fate of transplanted cells [7].

The variable outcomes may also be due to different MSC tissue sources used, such as bone marrow, adipose tissue, periosteum, synovium, umbilical cord, placenta, and skin [8, 9]. Furthermore, MSCs can be isolated and expanded through different procedures, with the use of different media, seeding densities and oxygen levels [10]. To minimise these inconsistencies, the International Society for Cellular Therapy (ISCT) issued a position statement in 2006 to propose the minimal criteria to define human MSCs [11]. While these criteria are useful for describing MSC purity in growing adherent cultures, additional potency assays are needed in order to predict clinically important factors such as MSC differentiation and immunomodulation capabilities, as well as growth factor production [10, 12].

It is well-documented that human MSCs (hMSCs) lose their differentiation potential after prolonged culture expansion in vitro [13, 14] and that the cells from late, pre-senescent passages may not be able to differentiate at all [2, 15, 16, 17]. Additionally, large numbers of senescent cells in MSC cultures negatively impact on cultures' immunomodulatory and homing abilities [18-20], which in turn may lead to lessened efficacy following implantation [16, 19]. Therefore, the assessment of MSC cultures for the presence of senescent cells may become one of the critical quality attributes (CQAs) [21] helping to ensure the best MSC product quality. This review article presents current methods for MSC senescence analysis,

and highlights promising new methods that are non-destructive and label-free with potential to become on-line [22] quality control tests [23] for therapeutic MSC products.

Replicative and stress-induced senescence in MSC cultures: main features

Cellular senescence is generally defined as the essentially irreversible arrest of cell proliferation [24]. Replicative senescence refers to irreversible growth arrest of human diploid cell strains after extensive serial passaging in culture [25]. MSC cultures are heterogeneous in relation to cell cycle progression of their individual cells [26], and as passage number increases, the relative proportions of fast-dividing highly-proliferative cells gradually decline, while slow dividing less-proliferative cells increase [2]. Ultimately, very late passage cultures are primarily composed of large, non-proliferative, senescent cells.

Besides replicative senescence [27], MSC senescence can also be stress induced, linked to the free radical theory of aging [28]. Free radicals/reactive oxygen species (ROS) are unstable molecules that have an unpaired electron, and their accumulation leads to oxidative stress, DNA damage, protein damage and mitochondrial dysfunction. Oxidative stress in normal cells is counter-balanced by anti-oxidants such as Superoxide Dismutase (SOD), catalase, peroxiredoxin, thioredoxin and glutathione systems. However, in senescent MSCs SOD activity is reduced, while ROS production is increased [2, 29, 30]. Persistent oxidative stress and DNA damage response can then initiate growth arrest and activation of p38MAPK, PI3K/AKT/mTOR/FOXO3 and p53 signalling pathways. Both p53 and p38MAPK pathways have been reported to be responsible for an irreversible cell cycle arrest in human MSCs exposed to exogenous ROS treatment, and the pharmacological inhibition of p38MAPK was sufficient to restore normal mitochondrial function and rescue MSC proliferation [31]. Therefore, endogenous ROS accumulation plays a key role in stress induced MSC senescence [2, 24, 29].

Mitochondrial dysfunction and continuous oxidative stress severely affect MSC metabolism. Unlike mild or transient oxidative stress, which enhances the proteasome activity, severe oxidative stress appears to inhibit it [32]. Suppression of proteasomes, which act to degrade damaged proteins (i.e. oxidized, misfolded and cross-linked), further exacerbates senescent cell phenotype [33]. With regards to lipid metabolism, senescent fibroblasts have been shown to possess decreased lipid synthesis and increased fatty acid oxidation, consistent with active mitochondrial oxidation in senescent cells [34]. Importantly, energy metabolism has recently emerged as an important determinant of the stem cell fate [35]. In their undifferentiated state, anaerobic glycolysis meets the stem cell need for proliferation, but they become more dependent on glucose oxidation during differentiation [36]. In one study, senescent MSCs did not appear to utilise anaerobic glycolysis and instead, produced ATP primarily via oxidative phosphorylation, a metabolic activity more consistent with differentiated cells [37]. However, a more recent study has shown that senescence-associated changes in MSCs included down-regulation of nicotinamide ribonucleotide and up-regulation of orotic acid, and reflected the metabolic switch from oxidative to glycolytic pathways, possibly to avoid further damage by ROS [38].

The exact molecular mechanism governing replicative MSC senescence is not clearly known. Telomeres have been associated with the molecular machinery for cell replicative lifespan, and their shortening is indicative of the cell aging process [39, 40]. Telomere shortening, and the loss of telomeric DNA through subsequent generations, is linked with replicative senescence and was previously reported in human fibroblasts, endothelial cells, myoblasts as well as MSCs [41-43]. However, modification to the telomeric structure is unlikely to be the only cause of MSC senescence following culture. As mentioned, DNA damage and oxidative stress are likely to be other contributory factors [29, 44] particularly as MSCs are commonly grown under supra-physiological oxygen levels [45].

Wagner et al. carried out comprehensive analysis of the changes in morphology, immunophenotype and differentiation capacity of MSCs serially-passaged in culture [15]. Having observed gradual changes in the global gene expression and miRNA expression throughout MSC passages, the authors concluded that senescence-like changes in MSCs were not only restricted to senescent passages, but were continuously acquired from the start of in vitro expansion [15]. Since senescence is a distinct cell state, while changes in gene and protein expression were observed gradually at the culture (cell population) levels, their data indicated a gradual accumulation of senescent cells from early- to late-passage MSC cultures. Therefore identifying senescent cells even in early MSC cultures may be of a significant scientific and therapeutic value.

In addition to growth arrest and widespread changes in chromatin organization and gene expression, senescent cells are characterised by a so-called senescence-associated secretory phenotype (SASP) [24]. The SASP secretome consist of growth factors and cytokines including interleukin-1 (IL-1), IL-3, IL-4, IL-6, IL-8, epidermal growth factor), hepatocyte growth factor, insulin growth factor, platelet derived growth factor, TGF-β and vascular endothelial growth factor (VEGF) [46] that amongst their other roles, regulate MSCs proliferation in culture and are crucial to MSC senescence as well [2]. Senescent MSC secretome can induce senescence and apoptosis in other cells [47], as well an inflammatory responses in neighbouring cells [16, 27]. Critically, transplantation of even small numbers of senescent cells can cause age-related disease phenotypes, such as osteoarthritis, as shown recently by Xu et al. [48].

In MSC mediated therapies, where large-scale MSC expansion in culture is often required prior to transplantation, the impact of senescent cells accumulation on MSC biological activities remains poorly understood. As mentioned above, enlarged and irregular cell shape and shortened telomere lengths of senescent cells in MSC cultures [15, 43] may affect their in vivo trafficking and proliferation. Senescent cell accumulation is likely to be responsible for a decrease in the overall multipotent differentiation ability of long term expanded MSCs [26, 49]. Finally, senescent cells SASP can accelerate ageing [48] or induce apoptosis [47] in the neighbouring cells. Thus, having a direct method for identification, quantification and ultimately, removal of senescent cells in MSC cultures would have a significant value for ensuring the quality of MSCs intended for clinical applications.

One of the earliest, indirect methods of assessing MSC senescence is the colony-forming units-fibroblast (CFU-f) assay [50, 51]. The measured clonogenic cell number indicates cells that remain viable and proliferative enough to form visible colonies, and therefore gives an indirect indication (by subtraction) as to the proportions of senescent (i.e. non-proliferative)

and poorly-proliferative cells. DiGirolamo et al. were first to use a CFU-f assay to study MSC cultures isolated from iliac crest bone marrow aspirates of normal volunteers. Their major findings were that the number of CFU-fs declined as MSC were expanded in culture, showing that the colony forming efficiency dropped to nearly 1% at passage 7 as compared to 25% at passage 2 [52]. As the cultures approached senescence, they retained the ability to differentiate into osteoblasts, but failed to differentiate into adipocytes. This has been confirmed in other independent studies [17, 43, 53]. Similarly, Schellenberg et al. showed more recently that in MSCs isolated from lipoaspirates, 18.4% of passage 1 cells generated CFU-f colonies [26]. This dropped significantly over subsequent passages, and after 10 passages, only 5% of the cells generated CFU-fs. Eventually, after 20 passages, colony formation was rarely observed [26]. These data not only indicated that MSCs were heterogeneous, but also confirmed the notion of replicative senescence occurring in both bone marrow and adipose derived MSCs.

Despite this assay being useful for the identification of senescent MSC passages (those containing no colony forming cells), it notably measures highly-proliferative, as opposed to senescent cells, and is time consuming and highly subjective. In a recent study by Zorin et al., the group confirmed that cells that tested positive for senescence were the highest in small diffuse colonies of human skin fibroblast cultures, and they originated from the cells with the least proliferative potential [54]. However, counting such small colonies as representative of pre-senescent cells remains inaccurate and very subjective. Thus, more accurate, quantitative and rapid characterisation techniques have been developed for better identifying individual senescent cells with a potential for their later removal in MSC cultures.

In the following sections, we discuss the current standards for characterizing senescent passages, or senescent cells in MSC cultures, as well as highlight promising new methods that employ specific markers in senescence measurements, summarized in Table 1. We propose that optimal methods of MSC senescence characterization should be quantitative, rapid, allow high-throughput, cost-effective and ideally, label-free and non-destructive.

Current Standards

Senescence-associated β-galactosidase activity (SA-β-gal) measurements

The detection of SA-β-gal activity is the most contemporary standard for the evaluation of cell senescence. The human acid β-D-galactosidase (β-Gal) is a eukaryotic hydrolase localized in the lysosome that catalyses the hydrolysis of terminal β-linked galactose residues in glycoproteins and glycolipids [55]. Under normal growth conditions, the enzymatic activity can be detected in cells at the optimal lysosomal pH 4.0. Senescence-associated beta-galactosidase (SA-β-Gal) is defined as beta-galactosidase activity detectable at the less favourable pH conditions (pH 6.0), most likely due to increase in lysosomal mass in senescent cells [56], as well as the accumulation of increased levels of GLB1 (gene encoding lysosomal β-D-galactosidase) mRNA and protein [57] in senescent cells. Currently, there are two types of SA-β-gal activity measuring methods: cytochemical/histochemical or fluorescence-based. The cytochemical method requires incubation of fixed cells with the chromogenic β-galactosidase substrate X-gal at pH 6.0. After staining, a blue colour develops in senescent cells within 2 hours, and the proportion of cells positive for SA-β-gal (i.e.

senescent cells) can be determined by simply counting the number of blue cells in the total population. If shielded from light, the blue staining is relatively stable and remains detectable in fixed specimens for several months [58].

The cytochemical method is time consuming, subjective and prone to operator errors. There may be false-positive results related to MSC state of confluency [58, 59]. Importantly, the degree of blue colour (representing the actual enzyme activity) is not standardized and can be biased when interpreted by different users. To overcome these drawbacks, more recent studies have proposed new programs and algorithms to implement automatic counting of senescent cells, which potentially offer unbiased and reproducible results when compared to manual counting [60].

For fluorescence detection of SA-β-gal activity, cells are incubated with C_{12}FDG, a β-galactosidase substrate that becomes fluorescent after cleavage by the enzyme. With this method, differences in SA-β-gal activity within and between cell populations could be more accurately evaluated [61]. In the study reported by Noppe et al. using cultured fibroblasts, the flow cytometric method provided measurements of SA-β-gal activity on a continuous fluorescence scale rather than strictly classifying positive or negative for SA-β-gal activity fractions. This was consistent with the suggestion made by Wagner et al., that senescent cell accumulation in MSC cultures is a continuous process rather than rapid event [15]. The flow cytometric method has the advantage of measuring SA-β-gal activity of living cells, whereas cytochemical methods are performed on fixed cells [61]. To maintain the internal pH of lysosomes in living cells at ~pH 6.0, bafilomycin A1 is commonly chosen rather than another more toxic lysosomal inhibitor chloroquine [58, 61]. Both agents promote lysosomal alkalization, acting as strong inhibitors of the vacuolar-type H(+)-ATPase that helps lysosomes maintain an acidic lumen [62].

As the fluorescence detection of SA-β-gal activity has been so far performed mostly on fibroblasts, method optimization is still required for characterizing senescent MSCs. This includes optimisation of C_{12}FDG concentration and staining time, MSC staining conditions (in a monolayer or in suspension), as well as 'fine-tuning' flow cytometry acquisition settings (for example, the number of acquired cells or the use of dead cell discrimination). As in the cytochemical method, results are highly sensitive to assay conditions and consequently it is possible that the fluorescent output is too weak for clear distinction between highly-proliferative and senescent cells in the MSC culture. Additionally, it remains to be a destructive method. However, as flow cytometry is a multiparameter technique, this can be combined with cell size measurements for clearer classification, as recently shown in Bertolo et al. [63] and elaborated further in the Promising New Methods section.

Telomere Length Measurements

Telomeres are repetitive nucleotide sequences at the end of the linear chromosomes that play a critical role in facilitating complete chromosome replication. In somatic cells, such as fibroblasts, telomeres shorten during each cell division till they reach a critical length at replicative senescence [64, 65]. As a result, mean telomere restriction fragment (mTRF) length analysis has been used to estimate the replicative history and predict senescence in MSC cultures [66]. Baxter et al. investigated the mTRFs of hMSCs from two donor groups

aged 0-18 and 59-75 years, and demonstrated a clear correlation between hMSC remaining proliferation potential and telomere length, both in culture and with donor age [43]. In this study, the telomere length indicative of the state of senescence was proposed to be 10kb [43]. Oja et al. similarly found that the mean telomere length was shortened at a constant rate during MSC culture, however, this was from 8.2±0.3 kbp at p1 to 6.08±0.6 kbp at senescence [67]. Thus, there is currently no consensus on the critical telomere length of MSCs at senescence, and whether it would be applicable to different types of MSCs, different culture conditions and different telomere measuring methods. This renders the telomere length measurement method not a definitive method for measuring MSC senescence.

Further problems of this method include that it cannot detect short telomeres that are present on a small number of chromosomes. As TRF reports an average of the lengths of telomeres, it does not provide information regarding single telomeres, this creates a significant limitation [66]. Polymerase chain reaction (PCR)-based telomere length analysis was developed to overcome the need for large quantities of DNA to evaluate telomere length [68], however, even when performed by experts, the PCR-based method results in variations due to differences in the method used for genomic DNA extraction, thus limiting its utility [66]. Notably, these methods are currently not suitable for measuring critical telomere length at senescence (i.e. identification of senescent cells) at the single-cell level.

To counter this, researchers have proposed assays that can detect the length of specific, individual telomeres to overcome the shortcomings of TRF and PCR-based assays that only provide measures of the average telomere length of the specimen. Baird et al. adapted the qPCR-based method to provide single telomere length analysis (STELA) [69]. Further adaptation of the STELA method is known as Universal STELA that allows for the detection of any critically short telomere, regardless of the chromosomal location [70, 71]. Although these methods provide insight regarding individual telomere lengths, they are technically challenging and are sensitive to the amount of template DNA. In conclusion, even with an eventual agreement on the critical telomere length of senescent MSCs, such methods remain destructive, labour-intensive and time-consuming (Table 1), and therefore are unlikely to be adopted as first-line quality-control assay for measuring senescent cells in MSC cultures.

Gene Expression Markers

MSCs isolated from different tissue sources, or the same tissue from different donors exhibit significantly different cellular behaviour [72]. Especially for MSCs derived from different tissue sources, the gene-expression profile and trilineage differentiation outcomes vary greatly [73]. Nevertheless, some common genes characteristic for cell cycle arrest at late passages may be applicable to all MSC tissue sources. Amongst those are cell cycle inhibitors $p16^{INK4A}$ and p21[74], and measuring these molecules at the transcript level is commonly utilised to characterise senescent passages of MSCs [2, 67, 75]. In the study carried out by Feng et al., MSCs derived from human dental pulp (DP-MSCs) showed characteristics of senescence as a function of donor age [76]. Their results indicated that DNA damage response and stress response may be the main contributors of DP-MSCs senescence induced by excessive activation of $p16^{INK4A}$ signalling, and that the dysfunction of DP-MSCs could be reversed by p16(INK4A) siRNA.

There are a number of other genes which could be indicative of MSC senescent passages. Wagner et al. have reported genes down-regulated during MSC replicative senescence including HSA1, ID1 and TNFSF11 [15]. In a more recent study by Churchman et al., the reduced expression of NANOG, TWIST1 and WIF1 in senescent MSC passages was found, as well as an increased expression of SPARC [17]. The decline in the NANOG, TWIST1 and WIF1 was proposed to indicate the loss of MSC multipotency during extended cultivation [77], and the steadily decline in the expression levels of CEBPA and FABP4 indicated the loss of their adipogenic potential [17].

In another study, Bellayr et al. evaluated predictive gene markers of aged human bone marrow-derived MSCs using a two-colour gene expression microarray. Overall, 81 genes were identified to be significantly different between passages 3 and 7 of the hMSCs cells studied. From their cell proliferation essay results, MSCs also demonstrated a greater potential for cell division at passage 3 than passage 7 [78]. Ten of the differentially expressed gene markers Bellayr et al. have identified (BST1, COL11A1, COL12A1, GALNT5, HAS1, KRT18, MEG3, PCM1, PENK, and SHB) were identical to those reported in the earlier gene marker study by Kulterer et al. [79]. Furthermore, two of the gene markers identified in Bellary's study, KRT18 and PRDX4, matched with those reported by Tanabe et al. [80] as well. Among these markers, Keratin KRT18 is the structural protein with a functional role in the stability of epithelial tissue [81], and for which significant gene expression changes were also reported in previous publications [80, 82].

From these results, there appears to be an emerging consensus on certain gene markers that can be used in combination to characterise senescent passages of cultured MSCs [78], however these markers are measured at the cell population rather than at the single-cell level. Although the gene marker method might be useful for rapid prediction of MSCs quality and cellular aging over consecutive passaging in culture, it is technically difficult and expensive, and therefore unlikely to be suitable for routine quantification of senescent cells in MSC cultures.

Gene Methylation and Epigenetic Markers

Epigenetic modifications play a central role in differentiation and development, and DNA methylation is the best characterized epigenetic modification [83]. In 2007, Shibata et al. proposed that the methylation state of the $p16^{INK4A}$ gene, a cyclin-dependent kinase inhibitor, could be monitored against the replicative senescence of MSCs during in vitro expansion [84].

Histone deacetylases (HDACs) are a class of enzymes that catalyse the removal of acetyl groups from ε-amino group of lysine residues in the histone tail [2]. During the progression of MSC senescence, HDAC inhibitors were found to downregulate polycomb group genes, and DNA methylation status of MSCs is proposed as another method for monitoring the gene expression of aging MSCs [2]. Gene expression can be regulated by DNA methylation through interference with transcription factors or methyl-CpG binding proteins, leading to silencing of respective promoter regions [85]. Koch et al. have studied the correlation between cellular senescence and DNA-methylation at specific CpG sites using human fibroblasts and MSCs from bone marrow [86]. Analysis of DNA-methylation level at six

CpG sites allowed reliable estimates of passage number in fibroblasts and MSCs, and the continuous changes in DNA-methylation in specific CpG sites were suggested as a marker to predict the state of cellular senescence of cultured MSCs [86]. Churchman et al. have also confirmed that the methylation of CpG islands had very strong associations with PD [17].

miRNA are non-coding RNAs of approximately 22 nucleotides in length that exert a post-transcriptional effect on gene expression, which are also epigenetic regulation markers [87]. Wagner et al. analysed the role of miRNA expression in determining MSC senescence on the molecular level and demonstrated that certain miRNAs (hsa-mir-371, hsa-mir-369-5P, hsa-mir-29c, hsa-mir-499 and hsa-let-7f) are up-regulated upon MSC replicative senescence [15]. They also showed that mir-29c directly targets DNA-methyl transferase 3A (DNMT3A) and 3B (DNMT3B) both of which are involved in cell proliferation and epigenetic regulation [15], and thus suggesting that senescence associated up-regulation of these miRNAs results in changes in the methylation pattern of proliferation inducing genes [15].

The primary drawback of measuring methylation as an indicator of senescence, similar to gene expression markers, is that it can only be performed at the cell culture level. It is important to note that gene specific methylation may be tissue-specific, and the genes of interest increase along with the number of tissues of relevance, which cause the method to be time-consuming and very costly [17, 88].

Promising New Methods

Although evaluating SA-β-gal activity to detect cell senescence is widely adopted across laboratories, the limitations of SA-β-gal method such as dependence on the state of cell confluence has been reported [89, 90] and alternative methods to measure MSC senescence, particularly at the singe-cell level have been developed [84]. Particularly for MSCs, it is important to consider from the outset how amendable these new methods would be for different types of scalable MSC manufacture approaches. For example, dynamic-culture bioreactors or planar-culture ‘cell factories’ [91-94], which may ultimately dictate the method employed. So far, the senescence status of MSCs grown on microcarriers in bioreactors continues to be assessed off-line [22] following MSC harvesting, plating in 2D and SA-β-gal activity staining in monolayer conditions [95]. Off-line here refers to monitoring by sampling a small volume of the cell culture for further analysis, either beside or away from the manufacturing line. Comparatively, on-line methods require no sampling and provide real-time information of the cells’ environment [22].

Prelamin A staining

Recent studies have revealed a correlation between accumulation of prelamin A and cellular aging [96, 97]. Progeria, a disease with symptoms of premature ageing and an average life span of 13 years, is resulted by heterozygous mutation in LMNA gene, and the affected protein cannot function correctly to mature lamin A [98]. Lamin A is a key structural component of the nuclear lamina, and is generated from a precursor protein, prelamin A [99]. Alteration to the nuclear lamina, a filamentous meshwork involved in regulation of the inner nuclear membrane and chromosomes, is recognised as a change associated with aging cells.

ZMPSTE24, a membrane zinc metalloproteinase [100], downregulated by in vitro aging in MSCs induces the upregulation of prelamin A, which leads to the induction of abnormal nuclear morphology and DNA damage [96]. Bellotti et al. have recently demonstrated that prolonged in vitro culture of human MSCs triggered replicative senescence and simultaneous accumulation of prelamin A, as measured by immunostaining for the full-length form of the lamin A precursor [101]. In another study utilising human skin fibroblasts, the oxidative stress- and replicative senescence was shown to affect prelamin A processing through the reduction of ZMPSTE24 expression [102]. Based on other previous reports showing that the accumulation of precursor of prelamin A induces molecular changes in MSCs such as multilineage differentiation [96], the group suggested that prelamin A accumulation could be used as a marker for detecting senescent MSCs in prolonged culture.

Cell surface proteins

Moravcikova et al. have recently used a high-throughput cell surface proteomic approach to analyse the changes in cell surface protein expression of CD45-/CD31-/CD34-/CD73+/CD105+ stromal cells in unpassaged BM MSC and subsequently determine the changes in cell surface markers with accompanying passage [103]. The group presented that the adhesion molecule CD106/VCAM1 is highly expressed in unpassaged MSC but decreases drastically with culture. This confirmed previous studies including ours [53, 104], however other studies showed increased CD106 expression [105], or random oscillation with passaging [106]. Similar controversy remains in relation to CD146 expression on in vitro aged MSCs [53, 104, 106].

Also for migration of MSCs, a metalloprotease associated with cell motility, CD10/neprilysin, has been shown to be rapidly upregulated in culture but decreased with early senescence [103]. Whereas no consensus yet exists in relation to a specific expression of classical MSC surface molecules on senescent MSCs, recent studies have identified new molecules such as cell surface protein DPP4 and membrane-bound vimentin to identify senescent cells [107, 108]. These studies were carried out using a combination of methods including mass spectrometry, flow cytometry and functional in vitro and in vivo assays. Having been confirmed by other independent studies, these new surface markers of senescent cells can be combined, in theory, with flow cytometry-based measurements of SA-β-gal activity and cell size, as well as be targeted for novel antibody-based senolytic approaches.

Cell surface lipids

Another recently proposed potentially flow cytometry-compatible method for measuring senescent cells in cultures and tissue samples is based on a histochemical staining of their lipofuscin content [109]. In senescent cells, aggregates of oxidized proteins, metals and lipids accumulate as lipofuscin. Although lipofuscins are auto-fluorescent, as further elaborated in the subsequent section, this fluorescence is very weak. Georgakopoulo et al. have utilized in their method a staining technique to enhance the detection of lipofuscin [109]. Of note, all the above methods still require a small sample of MSC culture to be sacrificed for the analysis, therefore remain destructive. In addition, their need for staining or fluorescent tags makes them non-label-free.

Another attractive option is cell plasma membrane lipid composition analysis that can be performed using tangential flow filtration [110-112]. It capitalises on the fact that plasma membranes of senescent cells become more rigid compared to their non-senescent analogues. While this biophysical method still requires some cells to be removed for the analysis, it being label-free leads to a reduction in the time required and cost of the assay.

Measurements of cell size

Cell enlargement is a key characteristic of senescent cells in culture [15, 67, 113], and as recently shown, possibly in vivo [75]. In the study by Biran et al., the authors utilized flow cytometry technology combined with high content image analysis to quantify DNA damage-induced senescent human fibroblasts in culture [75]. In their technique, they employed ImageStream X, an advance imaging flow cytometer capable of producing high resolution bright field and fluorescent images of individual cells. Prior to flow cytometry analysis they stained senescent fibroblasts for SA-β-gal activity and therefore, were able to correlate SA-β-galactosidase staining and the cell size, as well as other markers of cell proliferation and senescence such as Ki67 and HMGB1. Their results clearly showed a possibility of combining flow cytometry-based SA-β-gal measurements with the cell size analysis for the same sample of cells. The applicability of such methods for human MSCs has been recently shown in Bertolo et al. where SA-β-galactosidase activity in BM-MSCs was assessed using both chromogenic and fluorescent substrates and correlated with cell size and granularity [63].

Another recent study by Oja et al. analysing MSC cell size, has utilised live cell imaging analysis and reported that cell morphology and size could be a useful tool for evaluating the aging process and approaching senescence in cultured MSCs [67]. They suggested that cell area could be the most statistically significant and practical parameter for representing morphological changes that correlate with biochemical and gene expression markers of senescent cells. In their study, MSCs from passage 1 and 3 were uniform in size with around 1800 to 2500 μm^2 in cell area. Cells began to enlarge after passage 5 and resulted in a 4.8-fold increase in cell area at passage 6-9 as compared to passage 1. Other senescent markers such as the expression of $p16^{INK4a}$ and activity of β-galactosidase were reported to have a strong correlation with the increase in cell area [67]. With the cell size analysis methods, however, there is still a lack of a consensus on the critical cell size for senescent MSCs, and whether cell size is influenced by tissue source and culture conditions.

The way MSC cell size is measured – in growing cultures or on MSCs in suspension - needs to be evaluated and eventually, standardised. MSCs cultured in 2D conditions have the advantage of being non-destructively imaged for cell size analysis. However, such imaging modalities fail to perform for MSCs grown within microcarrier beads in bioreactors, as the beads interfere with imaging and also affect MSC spreading and shape depending on the beads being used. This make on-line cell size analysis challenging, therefore conducting off-line MSC cell size measurements post-harvesting is likely be more widely adopted in a near future. Measuring MSC size in suspension by flow cytometry, in combination with fluorescence-based assessment of SA-β-gal activity [63, 75] represents an attractive option.

Other label-free methods to identify and sort MSC subpopulations involve using microfluidics, where channels of micron-meter dimension are employed to sort cells in a high-throughput manner. For example, using a microfluidic method Yin et al. found that large cell populations (average cell size around 23-25 μm) expressed higher level of senescence associated β-galactosidase as compared to smaller cell populations (average cell size of 11-12 μm) [114].

Autofluorescence measurements

Another recently explored label-free and non-destructive method utilizes native biomarkers. Native biomarkers offer insights into the biochemistry of cells without the need for fluorescent labels or stains. Endogenous fluorophores are one such class of biomarkers and are responsible for autofluorescence in cells. Autofluorescence has found many applications in biomedical research and diagnosis [115], with lipofuscins being an indicator of cell senescence [116], as mentioned above. Fluorescence from lipofuscins have been demonstrated as feasible senescence indicators via conventional methods of fluorescence microscopy [117] and spectroscopy [118]. In these studies, autofluorescence intensities were observed to increase with increasing cell senescence in rat fibroblasts and neonatal rat cardiac myocytes respectively. Lipofuscin autofluorescence has similarly been probed in flow cytometry for HeLa cells [119]. Most recently, cell autofluorescence measured on a conventional flow cytometer was proposed to be 'a reliable in vitro marker of cellular senescence in human MSCs [63]'; although this interesting study is awaiting validation by other groups.

Despite the label-free and non-destructive advantage when using lipofusin fluorescence for cell senescence, there is still the challenge in acquiring autofluorescence due to its weak intensities. A potential workaround to this was recently explored in the method of cell lasing using endogenous fluorophores [120]. Through lasing, weak autofluorescence could potentially be amplified for easier detection.

Raman Scattering

Aside from autofluorescence, Raman scattering is another native signal from cells that does not require any labels. Raman spectroscopy has likewise been investigated for its ability in distinguishing between young and senescent cells. This was demonstrated in breast cancer cells [121], human umbilical cord MSCs [122] and in human dermal fibroblasts [123, 124]. In Raman spectroscopy, the Raman peaks provide biochemical and compositional information of the probed cells. Changes in intensities and spectral positions of these peaks thus correspond to changes in cells. However, like when using autofluorescence, Raman scattering has its drawbacks. In particular, changes in Raman peaks are typically too subtle for easy identification. In that regard, of note is the incorporation of machine learning methods in the more recent publications [123, 124] that largely facilitate the data analysis process.

Imaging and other label-free methods

More advanced optical techniques have also been reported to be capable of identifying senescent cells. These include light-sheet [125, 126] and digital holographic cytometry [127], methods which probe the biophysical aspects of cells to determine senescence. In particular, light-sheet cytometry uses the different scattering patterns of cells to determine their respective sizes and in turn senescence levels. On the other hand, digital holography cytometry essentially captures 3D images of cells and passes it through software for feature extraction. Apart from cell size and area, digital holography cytometry can generate information on cells' height, rates of division, motility and migration directionality. It is worth noting here that such acquisitions involve large amounts of data, typically incomprehensible by humans, therefore the involvement of machine learning and artificial neural nets has steadily gained popularity and importance in such technologies.

This is because the accuracy of cell counting based on images is critically dependent on image segmentation. Conventional computer vision methodologies achieve segmentation through classical image processing methods like filtering, intensity thresholding and feature detection. The typically reported challenges of applying such methods in live cell imaging pertain to segmentation accuracy; the consequent time required to separate accurately and inaccurately segmented images, as well as robustness across cell types and human operators [128]. Machine learning methods ease these challenges by automating segmentation and facilitating classification [129]. Deep learning, a category of machine learning, has recently shown exceptional performance in this area by offering the advantage of independently learning key features. It has been successfully applied in microscopy images of cells using various deep learning methods like deep convolution neural networks [130] and autoencoders [131].

Apart from segmentation for cell counting, machine learning methods have also been specifically applied in morphological profiling for identification of MSC subpopulations [132]. Finally, machine learning has also recently been explored for the discovery of CQAs that are to be assessed during the manufacture of stem cell-derived cell therapy products [133]. With tools like deep learning-based cell counting [134] being made freely available to the community, it is expected to see wide-spread applications in the MSC cell therapy area in the coming years.

The only example of non-destructive and label-free technology is a method recently reported by Tomita et al., where the authors detailed a non-invasive method for in vitro characterization of cell senescence in human fibroblasts based on their overall SASP profile of culture supernatants [135]. The authors suggested that their newly-developed polyion complex array allowed noninvasive tracking and early detection of replicative senescence progress even when a conventional marker such as SA-β-galactosidase indicates negative. This is awaiting an independent investigation but remains a very attractive possibility for on-line assessment of MSC senescence. Of note, such a method does reflect the state of MSC senescence at the population level, but still does not quantify individual senescent cells. Furthermore, the composition of the SASP appears to vary depending on the cell type from which senescent cells originated and how senescence was induced [136], this should be accounted for in future translational development of this assay.

Outlook and Future Directions

Based on the literature presented in this review, it can be proposed that methodologies allowing identification and quantification of senescent cells in therapeutic MSC batches would be advantageous for the MSC cell therapy field. When measured at cell culture level, a cut-off point can be developed, beyond which the cells could be considered unacceptably high in senescent cell burden, and hence therapeutically unsuitable. Such a test may represent one of the CQAs of manufactured MSC batches [21, 23].

Something even more advantageous would be having a method capable of identifying individual senescent cells particularly for autologous applications, where only small numbers of donor MSCs are available for expansion. In these situations, a cultures' senescent cells can be targeted for removal using current or new-generation senolytics [136]. Currently used senolytics, such as dasatinib and quercetin, target the Senescent Cell Anti-apoptotic Pathways based on the observation that senescent cells are resistant to apoptosis [136]. However classical senolytic agents may not always be very effective, as shown in a recent study [137]. Specific senescent cell surface markers as described in Kim et al. [108] may be more useful for not only to detect but also to selectively eliminate senescent cells using monoclonal antibodies conjugated to magnetic beads, or other cell separation technologies [138]. In large-scale MSC manufacturing processes, senescent cell removal can be performed during downstream processing stage [139], for example, as a part of cell harvesting, washing or cell concentration stages.

Amongst the standard methods, β-galactosidase based measurements are likely to stay for at least the next handful of years. As presented in this review, this method is capable of identifying individual senescent cells, and is already undergoing refinements in order to be combined with cell size measurements and high-throughput technologies such as flow cytometry or automated image analysis. Although the method remains destructive and not label-free, it currently serves as a positive control for other methods being developed, and as such is likely to continue to be in use for the near future.

The current consensus in the field of senescent cell biomarkers appears to be the use of multiple-marker signatures [111, 136]. As described above, biophysical-based cell characterisation methods offer the advantage of being label-free, while methods paired with flow cytometry allow for analysis of individual cells. Therefore, the best way forward may be a combination of methods involving cell size and label-free spectroscopic measurements since the combination has potential of being entirely non-destructive. Although such methods are not readily implementable for on-line monitoring at this point in time, they could still be particularly useful for rapid assessment of senescent cell numbers throughout the different stages of the manufacturing process. This is in contrast to conventional standards that take a long time and also irreversibly modify or even destroy the cells during examination, inevitably reducing the available number of MSCs for downstream processes. Furthermore, the combination of cell size and label-free spectroscopic measurements can be high throughput and thus time efficient with maximum output of the final therapeutic-grade MSC product. It should however be highlighted that spectroscopic measurements always require some form of excitation light. This could potentially pose the issue of photodamage of cells, which typically comes with prolonged exposure to light. Additionally, the limited reach of light into and out of non-homogenous mixtures like cell cultures, could restrict measurements

to just a single-layer. These factors would definitely have to be considered in the development of on-line methods for characterizing senescent cells following large-scale MSC manufacture in multi-layer culture vessels or on beads in bioreactors [140, 141].

Apart from characterising clinical-grade MSC batches for potential 'senescent cell burden', new culture conditions limiting MSC senescence should continue to be developed (Table 2). Historically, culturing MSCs in hypoxic conditions, without or with the addition of growth factors such as fibroblast growth factor, has prolonged the onset of senescence in MSCs. More recently, a better understanding of the effects of hypoxia, and the mechanisms of oxidative stress-induced MSC senescence has contributed to the development of new supplements to the culture media that improve MSC expansion with delaying senescence. For example, Antonioli et al. have recently reported that the addition of rapamycin (an inhibitor of the mTOR signaling pathway) to bone marrow MSC cultures delayed their senescence as evident by the diminished secretion of IL6 and elevated expression of NANOG gene, and this effect was associated with p16INK4A protein downregulation [142]. In another study, the supplementation of MSCs with nicotinamide (a precursor of NAD^+ that has been shown to reduce ROS generation) noticeably extended their replicative life span with a significant postponement in the onset of senescence [143]. Recent studies treating animal MSCs with ascorbic acid, or with an antioxidant cytokine C1q and tumor necrosis factor related protein 9 (CTRP9) [144], have also shown promise and need to be tested on human MSCs. In terms of selecting MSC tissue source for expansion, apart from tissue's propensity for certain lineage differentiation or trophic factor secretion, greater consideration should be given to MSC oxidative stress resistance [145] and desirably, longer onset of senescence. Even amongst perinatal cells, known to reach senescence later than adult tissue-MSCs [146], tissue specific differences exist, as shown recently in Kwon et al. study [147].

Finally, for cell therapy success, host tissue pathology and cell delivery considerations should be taken into account early in product manufacture ('beginning with the end in mind' or Quality by Design [23] concept). For example, for delivering MSCs into the joints and other hypoxic environments, their resistance to hypoxia may be a highly desirable feature to be tested and ultimately, quality-controlled. Overall, a holistic approach taking into consideration both cell therapy and host factors is currently needed to achieve a more rapid uptake of MSC cell therapies into the clinic. Predictive software algorithms and new machine learning technologies should be on the forefront of this endeavour.

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Method	Parameters Measured	Method Description	Single-cell or Cell population Level	Off-line only or Potential for on-line monitoring	Experiment Time	Destructive or Non-destructive	Labelled/Label-free	Type of Cell Tested
SA-β-gal measurements	β-gal activity	Cytochemical or fluorescent detection of SA-β-gal activity through staining or flow cytometry	Both	Off-line only	Less than 1 day	Destructive, requires a small sample of growing cells	Chromogenic or fluorescent substrate	Human diploid fibroblast-like cells[59] BM-MSC[15] Human fibroblast[58, 61]
Telomere length measurements	Critically short telomere length	Various: Mean telomere restriction fragment (mTRF); PCR-based telomere length analysis; Single telomere length analysis (STELA); Universal STELA	Method-dependent	Off-line only	Up to a week	Destructive, requires a sample of growing cells	End-labelling of telomeric oligonucleotide	BM-MSC[43, 67] Skin fibroblast cell[69, 70]
Gene expression markers	Gene expression	Candidate gene-expression measurements or profiling by microarray technology	Method-dependent	Off-line only	Up to a week for a small number of genes	Destructive, requires a sample of growing cells	cDNA labelling	BM-MSC [17, 73, 78-80] DP-MSCs[76] Human dermal and lung fibroblasts[77]

Gene methylation and other epigenetic markers		miRNAs, CpG sites with either linear increase or decrease in the methylation levels	Culture	Off-line only	Up to a week for a small number of candidates	Destructive, requires a sample of growing cells	DNA labelling	Human fibroblast cells[86] BM-MSC [2, 15, 17, 86] Adipose tissue mesenchymal stromal cells [86]
Prelamin A staining	Lamin A	Confocal imaging of prelamin A nuclear distribution following immunostaining	Both	Off-line only	Less than 1 day	Destructive, requires a sample of growing cells	Antibody labelling	BM-MSC[101]
Cell surface proteins	Various cell surface protein expression	Cell surface proteomic approach to analyse the changes in cell surface protein expression	Both	Off-line only	Less than 1 day	Destructive, requires a sample of growing cells	Antibody labelling	BM-MSC [53, 103, 104, 106] Human diploid fibroblast cells [107] Mouse lung fibroblasts [108]
Cell size measurements	Cell size	Cell images acquired by high-content screening microscopy or flow cytometry	Both	Potential for on-line monitoring	Less than 1 day	Currently destructive but can be modified for growing cells adherent to culture vessels	Histochemical staining but can be applied to holographic cytometry	BM-MSC[15, 67] Human fibroblasts[75]

Cell auto-fluorescence measurements	Lipofuscins	Conventional methods of microscopy and spectroscopy	Both	Potential for on-line monitoring	Few minutes to hours (depending on number of runs – each measurement takes <1min)	Non-destructive	Label-free	Rat fibroblasts[118] Neonatal rat cardiac myocytes[117] HeLa cells[119]
Cell Raman scattering measurements	Various biochemicals in cells (presented as Raman peaks)	Conventional Raman spectroscopy or microspectroscopy.	Both	Potential for on-line monitoring	Few minutes to hours (depending on number of runs – each measurement takes <1min)	Non-destructive	Label-free	Breast cancer cells[121] Human umbilical cord MSCs[122] Human dermal fibroblasts[123, 124]

Table 1. Current standards and new methods for characterizing senescent MSC passages or senescent cells in cultures.

Method category	The method	Mechanisms of action
Genetic modification - telomere based	Transduction by a retroviral vector containing the gene of human telomerase (hTERT) [148]	Increases MSC lifespan via telomere length preservation
Hypoxia	Culturing MSC in low oxygen conditions [149-151]	Reduces MSC oxidative stress and modifies cell metabolism; prevents the induction of p53 expression and mitochondrial ROS levels[152] Helps an induction of transcripts associated with HIF-1α
Hypoxia combined with growth factor supplementation or genetic manipulation	Hypoxia + fibroblast growth factor (FGF)	Increases MSC proliferation partially via ERK signalling pathway and HIF-1α expression enhancement [153]
	Hypoxia + Insulin-like growth factor-1 (IGF-1) knockdown	Increases autophagy of aged MSCs via decreased the activity of the Akt/mTOR signalling[154]
Genetic modification	Over-expression of Neuron-Derived Neurotrophic Factor (NDNF)	Enhances MSC proliferation and survival via increased Sirtuin 1 expression [155]
	Over-expression of Sirtuin 1	Regulates p53/p21 pathway [156]
Media supplements	Rapamycin	p16INK4A protein downregulation [157]
	Hybrid complexes of high and low molecular weight hyaluronan {Alessio, 2018 #176}	Unclear mechanism
	Nicotinamide (NAM)	Activation of autophagy through Sirtuin mobilization [143, 156, 158]
	Ascorbic acid	Reduction in ROS levels and inhibition of the AKT/mTOR signalling[159]
	C1q and tumour necrosis factor-related protein 9 (CTRP9)	Reduction in ROS levels and induction in superoxide dismutase (SOD) activity response[144]

Table 2: Methods to minimize MSC senescence during expansion