COMMENTARY
Detecting senescence: a new method for an old pigment

Hanna Salmonowicz and João F. Passos
Institute for Cell and Molecular Biosciences & Newcastle University Institute for Ageing, Newcastle upon Tyne NE4 5PL, UK

Summary
Cellular senescence is a state of irreversible cell cycle arrest induced by different types of cellular stressors. The field of senescence has made significant advances in the understanding of many of the mechanisms governing this phenomenon; however, a universal biomarker that unambiguously distinguishes senescent from proliferating cells has not been found. In this issue of Aging Cell, Evangelou and colleagues developed a sensitive method for identification of senescent cells in different types of biological material based on the detection of lipofuscin using an analogue of Sudan Black B (SBB) histochemical dye coupled with biotin, which they named GL13. The authors propose that this method is more sensitive and versatile than using SBB alone. Lipofuscin, a nondegradable oxidation product of lipids, proteins and metals, is found in senescent cells. Detection of lipofuscin using GL13 staining may be a more feasible method than others currently used for identification of senescent cells both in cell culture and tissues.

Key words: cellular senescence; lipofuscin; biomarker; aging.

Introduction
Cellular senescence has been defined as an irreversible cell cycle arrest which stops the propagation of damaged cells. It was first observed by Hayflick and Moorhead who demonstrated a limited replicative lifespan of human fibroblasts in culture (Hayflick & Moorhead, 1961).

Several stressors such as the shortening of telomeres, DNA lesions, oncogene activation, oxidative stress and others can induce cellular senescence (van Deursen, 2014). Depending on the trigger, senescence can be executed by several different effector pathways. The major ones comprise the p53-p21 and p16 pathways. Senescent cells experience dramatic changes at the level of gene expression, mitochondrial function (Correa-Melo et al., 2016) and epigenome (Cruijshanks et al., 2013). Furthermore, senescent cells have been shown to have a distinct secretome profile, known as senescence-associated secretory phenotype (SASP) (Coppé et al., 2008). SASP includes growth factors, extracellular matrix degrading proteins and pro-inflammatory cytokines. Through the SASP, senescent cells communicate with the immune system to orchestrate their own clearance and stimulate local progenitor cells to regenerate the tissue (van Deursen, 2014). However, the SASP is also capable of inducing senescence in adjacent, healthy cells, thereby contributing to tissue degeneration (Acosta et al., 2013). Impairment of clearance of senescent cells and chronic exposure to the SASP may result in the accumulation of senescent cells and paradoxically promote tumorigenesis.

The challenge of identifying a universal marker of senescence
Senescent cells have been recently shown to contribute causally to the aging process. Elimination of senescent cells by suicide gene-mediated ablation of p16

~42-expressing senescent cells in INK-ATTAC mice has led to improvements in healthspan and lifespan suggesting that senescent cells are drivers of aging (Baker et al., 2016). This has prompted the scientific community to identify new interventions to target senescence as a therapy against aging and age-related diseases (Zhu et al., 2015). However, despite remarkable advances, the detection of senescent cells, particularly in tissues, is still a major challenge.

There are several reasons, both of a biological and methodological nature, which have hindered the identification of specific markers able to determine whether a cell is senescent or not:

Firstly, while senescence is characterized by numerous changes in gene expression, very few of these differences are exclusive to senescent cells. Secondly, senescence is a kinetic, multifactorial process, with several phenotypic changes occurring at different time points following the initial cell cycle arrest. This could explain why aged tissues are highly heterogeneous, possibly containing cells at different stages of the senescent programme. Thirdly, senescent cells manifest the phenotype differently depending on the type of inducing stimuli or the cell type (van Deursen, 2014). Finally, recent data have highlighted that senescence may play different physiological roles in different contexts. For instance, an ‘acute’ type of senescence has been shown to play a beneficial role during processes such as development or tissue repair (Murioz-Espin et al., 2013; Demaria et al., 2014), while a ‘chronic’ type of senescence may contribute to aging and age-related disease. The recent realization that there may be different types of senescent cells in tissues has created an additional obstacle to the identification of a universal marker.

The detection of senescence-associated β-galactosidase (SA-β-Gal) activity at pH 6 is probably the most widely utilized method for identification of senescent cells (Dimri et al., 1995). Nevertheless, there are major limitations to this method. SA-β-Gal staining may occur in quiescent cells induced by confluency or serum starvation and in immortalized cells (Cristofalo, 2005). Furthermore, this method requires fresh, nonfixed material, which limits its applicability and the use of archived material. Its detection in tissues is technically challenging and has generated contradictory results. Given the growing realization that senescence is a multifactorial process, a multimarker approach is being favoured by many researchers in the field.

Examples of currently used markers are as follows: increased expression of cyclin kinase inhibitors p21 and p16 and absence of proliferation markers; telomere-associated DNA damage foci (Hewitt et al., 2012); senescence-associated heterochromatin foci (SAHF; Narita et al., 2003); loss of lamin B1 (Shimi et al., 2011); senescence-associated distension of satellites (SADS) (Swanson et al., 2013); and expression of components of the SASP (Coppé et al., 2008) amongst several others.

Correspondence
João F. Passos, Institute for Cell and Molecular Biosciences & Newcastle University Institute for Ageing, Newcastle upon Tyne NE4 5PL, UK. Tel.: +44 (0)1912081222; fax: +44(0)191 20 81101; e-mail: joao.passos@ncl.ac.uk
Accepted for publication 17 January 2017

© 2017 The Authors. Aging Cell published by the Anatomical Society and John Wiley & Sons Ltd. This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.
Nonetheless, there is also growing realization that many of these markers are not exclusive to all types of senescence and may only occur in specific cell types.

Detection of lipofuscin as a senescent marker

Lipofuscin is a nondegradable aggregate of oxidized lipids, covalently cross-linked proteins, oligosaccharides and transition metals which accumulate within lysosomes. It is a product of iron-catalysed oxidation and polymerization reactions of a variety of cellular structures and macromolecules (Terman & Brunk, 2004). Its discovery is credited to Hannover in 1842. Multiple studies indicate that lipofuscin accumulates in various tissues and species with age, particularly postmitotic tissues such as the brain and cardiac and skeletal muscle (Terman & Brunk, 2004). However, lipofuscin has also been shown to accumulate during replicative senescence of human fibroblasts (von Zglinicki et al., 1995). Lipofuscin is autofluorescent and can be visualized using fluorescent microscopy; however, several other histochemical methods have been described based on lipid detection, such as staining using Sudan Black B (SBB) amongst others.

In this article, Evangekou and colleagues designed and synthesized a structurally similar compound to SBB and coupled it to biotin. Commercially available SBB contain numerous impurities which impact on staining quality and justified the need to synthesize a new analogue. The chemical coupling with biotin allows its detection using antibiotic antibodies and thereby increases its detection sensitivity (Fig. 1). The authors show evidence for the versatility of this method: it can be detected in fresh, frozen cells and tissues, but also in fixed material. Furthermore, it can be identified in cells using both microscopy and flow cytometry. Their data indicate that GL13 staining can overcome some of the limitations of the standard SBB staining. SBB staining is less pronounced and requires a higher magnification.

The authors present multiple examples of positively stained cultured cells induced to senescence by replicative exhaustion, genotoxic stress, overexpression of p21 or oncogene activation. Furthermore, they reveal that mouse models of cellular senescence and human clinical samples of malignant lesions and irradiated tissue are characterized by elevated numbers of GL13-positive cells, when compared to controls.

Despite its obvious advantages, users should be aware of some possible limitations of the method. For instance, lysosomes may be loaded with degradable material, described as ‘temporary lipofuscin’, but also lipid droplets and glycogen which may be detected by methods such as SBB (Terman & Brunk, 2004). It is therefore plausible that ‘false positives’ may be detected using this method.

In addition, age-dependent accumulation of lipofuscin has been shown to occur in postmitotic tissues which in theory should not experience the phenotype originally described by Hayflick. This is at odds with the idea that lipofuscin accumulation is specific to senescent cells. One explanation is that similar pathways which drive senescence in mitotic cells can also be activated in postmitotic cells, as demonstrated in neurons (Jurk et al., 2012) and adipocytes (Minamino et al., 2009). Whether detection of lipofuscin overlaps with other senescent markers in postmitotic tissues warrants further investigation.

While the authors have convincingly demonstrated that lipofuscin accumulation correlates with senescent markers in cell culture and that lipofuscin increases in tissues with age, future work should investigate more thoroughly whether and to what extent the lipofuscin signal overlaps with other established senescent markers such as SA-β-Gal or p16 expression during aging in vivo. Finally, strategies such as the recently developed mouse models where p16-expressing senescent cells can be specifically eliminated (Demaria et al., 2014; Baker et al., 2016) could be applied to further ascertain the specificity of this method.

A separate question which arises from this work is whether lipofuscin accumulation is a mere consequence of the induction of the senescence programme or whether its accumulation contributes causally to the development of senescence. The latter hypothesis would be consistent with early findings which demonstrated that synthetic lipofuscin could induce senescence in human fibroblasts (von Zglinicki et al., 1995).

The last decade has seen incredible advances in the field of cellular senescence with the realization that senescent cells play important, albeit distinct, roles in vivo. However, one major challenge for the community is the identification of specific markers. The novel method proposed by Evangelou and colleagues offers a possible easy and versatile solution, but whether it works as a standalone marker or should be used in combination with several others remains to be determined.

Funding

Work in JP’s laboratory is funded by the Biotechnology and Biological Sciences Research Council BBSRC.

Conflict of interest

The authors declare no conflict of interests.
References

Acosta JC, Banito A, Wuestefeld T, Georgilis A, Janich P, Morton JP, Athineos D, Kang T-W, Lasitschka F, Andrusiak P, Pascual G, Morris K, Khan S, Jin H, Dharmalingam G, Snijders AP, Carroll T, Capper D, Pritchard C, Inman GJ, Longerich T, Sansom OJ, Benita SA, Zender L, J. (2013) A complex secretory program orchestrated by the inflammasome controls paracrine senescence. Nat. Cell Biol. 15, 978–990.

Baker DJ, Childs BG, Dunk M, Wijers ME, Sieben CI, Zhong JA, Saltness R, Jeganathan KB, Verzosa GC, Pezeshki A, Khazaei K, Miller JD, van Deursen JM (2016) Naturally occurring p16Ink4a-positive cells shorten healthy lifespan. Nature 530, 184–189.

Coppé JP, Patil CK, Rodier F, Sun Y, Muñoz DP, Goldstein J, Nelson PS, Desprez PY, Campisi J (2008) Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic Ras and the p53 tumor suppressor. PLoS Biol. 6, e201.

Correia-Melo C, Marques FDM, Anderson R, Hewitt G, Hewitt R, Cole J, Carroll BM, Miwa S, Birch J, Meza A, Rushton MD, Charles M, Jurk D, Tait SWG, Czapiewski R, Greaves L, Nelson G, Bohlolo-Y M, Rodriguez-Cuenca S, Vidal-Puig A, Mann D, Saretzki G, Quarato G, Green DR, Adams PD, von Zglinicki T, Korolchuk VI, Passos JF (2016) Mitochondria are required for pro-ageing features of the senescent phenotype. EMBO J. 35, 724–742.

Cristofalo VJ (2005) SA [beta] Gal staining: biomarker or delusion. Exp. Gerontol. 40, 836–838.

Crucickshanks HA, McBryan T, Nelson DM, VanderKraats ND, Shah PP, van Tuyn J, Singh Rai T, Brock C, Donahue G, Dunican DS, Drotar ME, Meehan RR, Edwards JL (2015) The Achilles’ heel of senescent cells: from transcriptome to senolytic drugs. Aging Cell 14, 644–658.

Hayflick L, Moorhead PS (1961) The serial cultivation of human diploid cell strains. Exp. Cell Res. 25, 585–621.

Hewitt G, Jurk D, Marques FDM, Correia-Melo C, Hardy T, Gackovska A, Anderson R, Taschuk M, Mann J, Passos JF (2012) Telomerases are favoured targets of a persistent DNA damage response in ageing and stress-induced senescence. Nat. Commun. 3, 708.

Jurk D, Wang C, Miwa S, Maddick M, Korolchuk V, Tsolou A, Gonos ES, Thrasivoulou C, Jill Saffrey M, Cameron K, von Zglinicki T (2012) Postmitotic neurons develop a p21-dependent senescence-like phenotype driven by a DNA damage response. Aging Cell 11, 996–1004.

Miramino T, Orimo M, Shimizu I, Kunieda T, Yokoyama M, Ito T, Nojima A, Nabetani A, Oike Y, Matsuura H, Ishikawa F, Komuro I (2009) A crucial role for adipose tissue p53 in the regulation of insulin resistance. Nat. Med. 15, 1082–1087.

Muñoz-Espin D, Cañamero M, Maraver A, Gómez-López G, Contreras J, Murillo-Cuesta S, Rodriguez-Baesa A, Varela-Nieto J, Ruberte J, Collado M, Serrano M (2013) Programmed cell senescence during mammalian embryonic development. Cell 155, 1104–1118.

Narita M, Núñez S, Heard E, Narita M, Lin AW, Hearn SA, Spector DL, Hannon GJ, Lowe SW (2003) Rb-mediated heterochromatin formation and silencing of EZF target genes during cellular senescence. Cell 113, 703–716.

Shimi T, Butin-Israeli V, Adami SA, Hanan RK, Biro RL, Shapira-Frommer R, Tischler AS, Mayerson J, Pardoll D, Hanahan D, Lowe SW (2006) The simultaneous loss of Lamin B1 and p16Ink4a cooperatively induces senescence. Nat. Cell Biol. 8, 499–508.

Terman A, Brunk UT (2004) Lipofuscin. Int. J. Biochem. Cell Biol. 36, 1400–1404.

van Deursen JM (2003) Programmed cell senescence during mammalian embryonic development. Cell 115, 1104–1118.

Narita M, Núñez S, Heard E, Narita M, Lin AW, Hearn SA, Spector DL, Hannon GJ, Lowe SW (2003) Rb-mediated heterochromatin formation and silencing of EZF target genes during cellular senescence. Cell 113, 703–716.

Shimi T, Butin-Israeli V, Adami SA, Hanan RK, Biro RL, Shapira-Frommer R, Tischler AS, Mayerson J, Pardoll D, Hanahan D, Lowe SW (2006) The simultaneous loss of Lamin B1 and p16Ink4a cooperatively induces senescence. Nat. Cell Biol. 8, 499–508.

Terman A, Brunk UT (2004) Lipofuscin. Int. J. Biochem. Cell Biol. 36, 1400–1404.

von Zglinicki T, Nilsson E, Dandekar T, Welle CK, Bhattacharyya M, Lucas CA, Pajuelo Ignacio, Rodriguez-Cuenca S, Vidal-Puig A, Mann D, Saretzki G, Quarato G, Green DR, Adams PD, von Zglinicki T, Korolchuk VI, Passos JF (2016) Mitochondria are required for pro-ageing features of the senescent phenotype. EMBO J. 35, 724–742.

Cristofalo VJ (2005) SA [beta] Gal staining: biomarker or delusion. Exp. Gerontol. 40, 836–838.