to be the product of an insertion of a large transposable element into the gene *cortex*, which demonstrates a (conditionally) beneficial role for what are considered to be genomic parasites. The timing of this mutation event, estimated by using the molecular footprint left by the selective sweep, also provides direct and independent evidence that the *carbonaria* morph arose in early 19th century Britain. Similar patterns of rise and fall in melanic forms of the peppered moth associated with coal pollution have been detected in eastern North America and continental Europe, and whilst the specific genetic identity and age of these polymorphisms remain to be resolved, *cortex* is likely to be involved. Intriguingly, this gene has also been shown to be involved in camouflage melanism in several species of moth and in the warning signal mimicry of *Heliconius* butterflies.

What is there left to learn from peppered moths? Even though the peppered moth has been and is the focus of much research, the extent to which larvae can change colour has only recently been documented. This raises the possibility that colour change may be more common amongst Lepidoptera larvae than is currently appreciated, and highlights the need for more research in this area. In addition, the larvae's ability to use extraocular photoreception makes the peppered moth a useful species in which to study the molecular, physiological and cognitive mechanisms that contribute to distributed sensing. This could inform the development of technologies that allow mobile objects to change colour to match their background. Moreover, the ability to manipulate larval colour allows us to produce living stimuli that can be used in experiments to address a range of questions about the evolution of many forms of adaptive colouration, and the co-evolution of prey appearance and behaviour. The co-existence of several adult morphs presents an ongoing opportunity for unravelling the interacting genetic and ecological factors involved in the maintenance of colour polymorphisms, and whether adaptation to major environmental change (e.g. pollution) relies more frequently on *de novo* mutations or pre-existing genetic variation. It also provides an excellent system in which to study the regulation and function of *cortex*, which acts as a primary developmental switch for colour pattern diversity in Lepidoptera.

Where can I find out more?

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[1]Max Planck Institute for Chemical Ecology, Hans Knöll Straße 8, Jena 07745, Germany. [2]Department of Evolution, Ecology and Behaviour, University of Liverpool, Liverpool L69 7ZB, UK. [3]Biosciences Institute, Faculty of Medical Sciences, Newcastle University, Henry Wellcome Building, Framlington Place, Newcastle upon Tyne NE2 4HH, UK.
*E-mail: John.Skelhorn@ncl.ac.uk

Primer

Cellular senescence

Marta Varela-Eirín[1,2] and Marco Demaria[1,*]

Cellular senescence defines a state of stable and generally irreversible proliferative arrest associated with various morphological, structural and functional changes (Figure 1), including enhanced expression and secretion of pro-inflammatory and tissue-remodelling mediators. This state is crucial in tissue physiology and pathology and arises as a response to potentially damaging stress signals. Whether the activation of a senescence state provides benefits or detriments for tissue function and homeostasis is strictly dependent on the context. Cell senescence acts as a potent tumour-suppressive mechanism limiting the proliferation of cells at risk of malignant transformation and supports the repair of acute tissue damage, but also represents a key driver of ageing and age-related diseases.

The concept of cell senescence was first described in 1961, in a pioneering study by Leonard Hayflick and Paul Moorhead. While expanding human primary fibroblasts at the Wistar Institute, Hayflick observed that the cells lose the ability to expand after a number of passages, despite remaining alive and metabolically active. This finding was in contrast to the concept that *ex vivo* cultured cells could be expanded indefinitely, initially introduced by Alexis Carris in 1912. Hayflick postulated that this cellular phenomenon of limited proliferative potential could be a reflection of organismal ageing. The observation that cells could only replicate and divide for a limited amount of times (between 40 and 60) was later coined as the Hayflick Limit by Frank Macfarlane Burnet in 1974. After some years of enduring criticisms, other scientists started to support Hayflick's theory and were able to reproduce his findings. Moreover, it became clear that one of the key mechanisms responsible for the limited replicative lifespan of primary cells was the shortening of telomeres. Telomeres are structures at chromosome ends that protect against end–end fusion, discovered by Elizabeth Blackburn and colleagues in 1988. More

recently, great progress has been made in demonstrating that cells can undergo senescence in response to different types of stress and significant efforts are being invested in characterising the phenotypic changes associated with a senescence state.

Here, we describe the mechanisms, markers and biological functions of cellular senescence. We primarily focus on the concept of heterogeneity and its consequences for the identification and therapeutic targeting of senescent cells.

Key features of senescence

When facing damage-inducing factors, cells activate repair pathways that can restore the functional integrity of the cell or, if the damage is irreversible, activate programmed death or entry into senescence. The senescence program provides pro-survival functions and resistance to extrinsic and intrinsic apoptosis. Once the senescence program is initiated, cells become unable to proliferate, even when mitogenic stimuli are present. This feature distinguishes senescence from another form of stable but reversible growth arrest — quiescence. The senescence-associated irreversible cell-cycle arrest is mainly characterised by the activation of two cyclin-dependent kinase inhibitors, $p21^{WAF1/Cip1}$ (p21) and $p16^{INK4a}$ (p16), which halt cell-cycle progression in G0/G1 phase. Although extensively used as a senescence biomarker, activation of a state of irreversible growth arrest is insufficient to unequivocally characterise cells as being senescent. Indeed, terminally differentiated and post-mitotic cells also indefinitely lose the ability to proliferate.

Senescent cells exhibit dramatic alterations of their morphology, with an enlarged, flattened and irregularly shaped body. Changes in morphology are easily measured in cell culture, but they can be difficult to detect *in vivo* because the enlargement of senescent cells can be restricted by tissue architecture. Morphological alterations mostly arise because of cytoskeleton rearrangements involving both vimentin intermediate filaments and microtubules.

Unresolved DNA damage, which occurs as a result of different intrinsic or extrinsic factors (e.g. oxidative stress, oncogene activation, ionising and non-ionising radiation or telomere erosion), activates a DNA-damage response involving sensor kinases that eventually leads to cell-cycle arrest via p53-mediated activation of p21. In addition, persistent DNA damage and partial disintegration of the nuclear envelope due to the reduced expression of the lamin B1 protein can lead to the accumulation of cytoplasmic chromatin fragments and activate a pro-inflammatory response via the cGAS–STING pathway.

Senescent cells remain metabolically active and acquire a hypersecretory behaviour. This hallmark is known as the senescence-associated secretory phenotype (SASP) and the secreted proteins represent a heterogeneous group of inflammatory cytokines, chemokines, matrix metalloproteinases and growth factors with context-dependent biological functions. The SASP reinforces and spreads senescence in an autocrine and paracrine manner, respectively, and also activates immune responses that can lead to the clearance of senescent cells. The inflammatory arm of the SASP is mainly triggered and regulated by the transcription

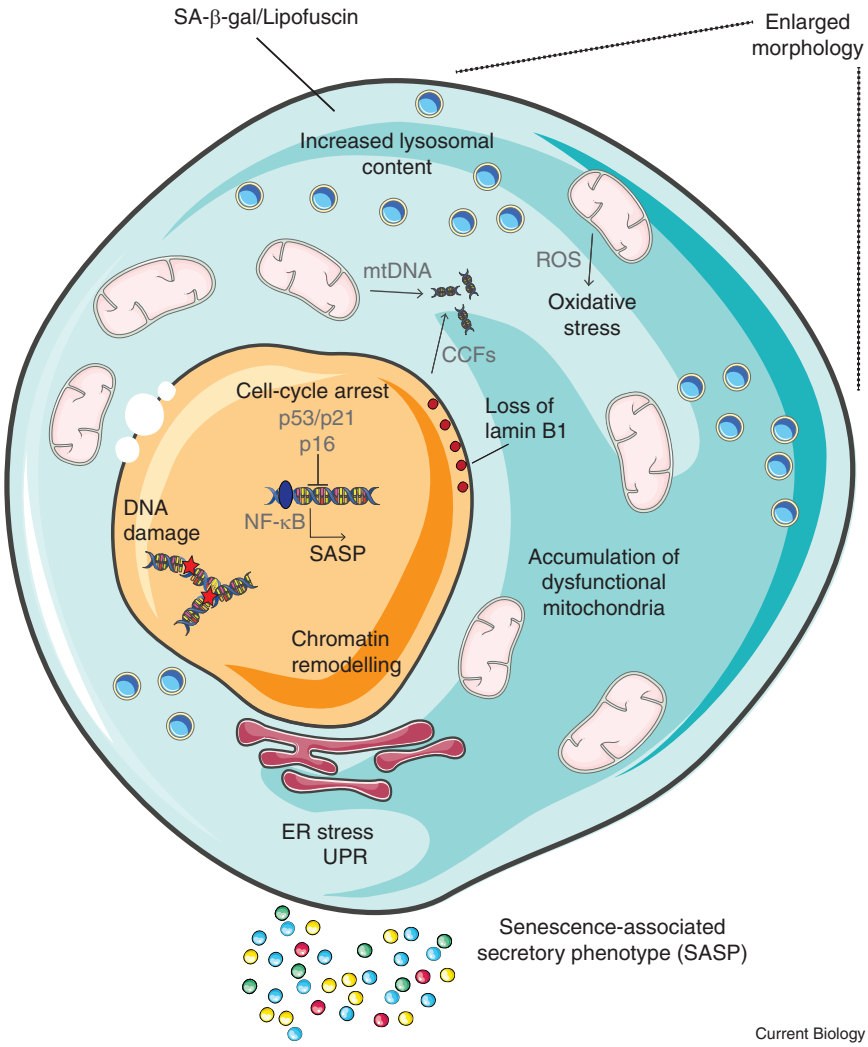

Figure 1. Hallmarks of the senescence phenotype.
General morphological, structural and functional alterations of senescent cells are shown. Cell-cycle-arrested senescent cells normally display enlarged and flattened morphology, accumulation of dysfunctional mitochondria and high lysosomal activity, as well as the presence of cytoplasmic mitochondrial DNA (mtDNA) and cytoplasmic chromatin fragments (CCFs). DNA damage and chromatin remodelling are usually also detected. Senescent cells exhibit overexpression of the cell-cycle inhibitors p21 and p16 and of several secreted factors. The nuclear lamina protein lamin B1 is consistently downregulated during senescence. ROS, reactive oxygen species; UPR, unfolded protein response.

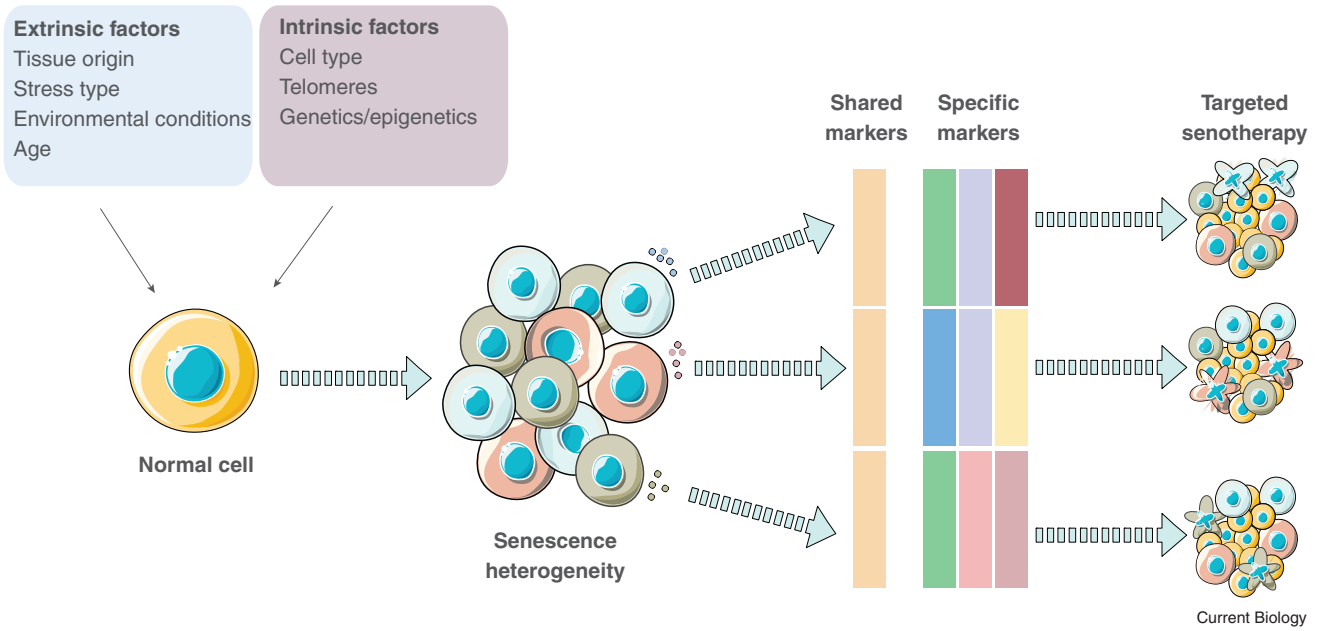

Figure 2. Senescence heterogeneity.
A combination of different extrinsic and intrinsic factors can promote senescence. However, specific senescence programs are highly dependent on the interaction between these various factors. As a result, senescent cells share common features, but each subpopulation exhibits subset-specific markers. Identification of specific markers can enable the selective senotherapeutic targeting of detrimental senescent populations.

factors NF-κB and C/EBPβ, which are activated as a consequence of the DNA-damage response, but also by other signalling factors that are normally interconnected (such as the transcription factor GATA4, mammalian target of rapamycin (mTOR), and the MAP kinase p38). Recent studies have demonstrated that SASP factors might be regulated in modules, each with a distinct origin, composition and biological activity. Catabolic processes are constantly activated in senescent cells as a mechanism to provide energy and materials for the secretion associated with the SASP. As a result, the number and size of lysosomes in senescent cells are increased, and the elevated lysosomal activity of the enzyme β-galactosidase (senescence-associated-β-galactosidase, SA-β-gal) is used as one of the main markers of cellular senescence. In addition to SA-β-gal analysis, increased lysosomal content can be evaluated by the detection of lipofuscin (deposits of crosslinked sugar and lipids with oxidized proteins) using Sudan Black B or its biotin-labelled analogue GL13. However, enhanced lysosomal content and activity are neither required nor unequivocal indicators of the senescent phenotype.

Senescent cells are also characterised by a deregulated metabolic profile. They accumulate dysfunctional and enlarged mitochondria, partly due to defects in mitophagy. Defective mitochondrial function arises as a consequence of decreased membrane potential, increased proton leak and reduced rates of fusion and fission. These features compromise the ability to generate ATP and contribute to the generation of reactive oxygen species. Oxidative stress is high in senescent cells and has a fundamental role in protein oxidation. As a result, proteins display an altered conformation, which leads to impaired function (such as changes to catalytic activity or protein–DNA interactions) and accumulation of insoluble protein aggregates, including those leading to lipofuscin formation. As a mechanism to cope with the accumulation of irreversibly misfolded proteins, cells enlarge the endoplasmic reticulum and initiate the unfolded protein response, reducing the synthesis of new proteins and promoting the export of the misfolded ones.

Senescence heterogeneity

To date, there is still no single universal marker that can identify senescent cells, and a combination of different biomarkers has been broadly exploited in an attempt to distinguish these cells from other normal non-proliferating cells (Figure 1). This combination includes markers of cell-cycle arrest (upregulation of p21 and p16, and a decrease in DNA synthesis, lamin B1 expression and levels of the proliferative marker Ki67), increased lysosomal activity (detection of SA-β-gal and lipofuscin) or activation of DNA damage response signalling (p53-binding protein 1 expression and γH2AX foci accumulation). In addition, RNA and protein expression of subsets of SASP components is also analysed (for example, the interleukins IL-6, IL-1α/β and IL-8, the chemokine CXCL1, the matrix metalloproteinase MMP1, and the cytokine GDF15). Nevertheless, even if some SASP factors are common to all senescent cells, the general composition of the SASP is highly variable. Moreover, recent evidence indicates that subtypes of senescent cells can re-enter the cell cycle under certain conditions. Hence, categorisation of senescence remains difficult due to the lack of individual and universal markers. Multi-parametric approaches — combining single-cell technologies based not only on transcriptomic and proteomic analysis

but also on analysis of circulating factors (such as small extracellular vesicles, long non-coding RNAs, and metabolites) — are proving to be useful in unveiling more specific senescence signatures (Figure 2), but these approaches remain too complex and expensive for routine work. Another issue is *in vivo* identification has proven difficult because senescent cells reside within tissues and their characteristics may differ from senescence-associated markers that are more prominent in cell culture.

Although the lack of universal markers has historically frustrated the senescence community, recent work has demonstrated that this might be less relevant than expected. Indeed, several studies have shown that distinct senescence subtypes coexist, and that senescence subset-specific phenotypes are determined by the combination of extrinsic and intrinsic factors to which these cells are exposed. Because different senescence subsets have distinct phenotypes and biological activities, the ability to identify such specific subpopulations, rather than focusing on the search for pan-senescence biomarkers, is becoming an emerging area of interest.

A stress stimulus will lead to different outcomes depending on the tissue origin of the cell, with divergent pathways being activated and distinct biomarkers being displayed. Interestingly, within a tissue subjected to the same stress, specific cell types will also respond differently, for example, by activating a different SASP. Recent single-cell RNA sequencing data also revealed that paracrine (secondary) senescent cells present a different SASP when compared with their primary senescent counterparts. In addition, the stress type that triggers senescence — whether it is extrinsic (such as irradiation or genotoxic drugs) or intrinsic (for example, telomere attrition or oncogenic activation) — is important. These different types of stress signals will lead to specific types of senescence that are named accordingly, such as telomere-dependent replicative senescence (RS), oncogene-induced senescence (OIS), stress-induced premature senescence (SIPS) or mitochondrial-dysfunction-associated senescence (MiDAS).

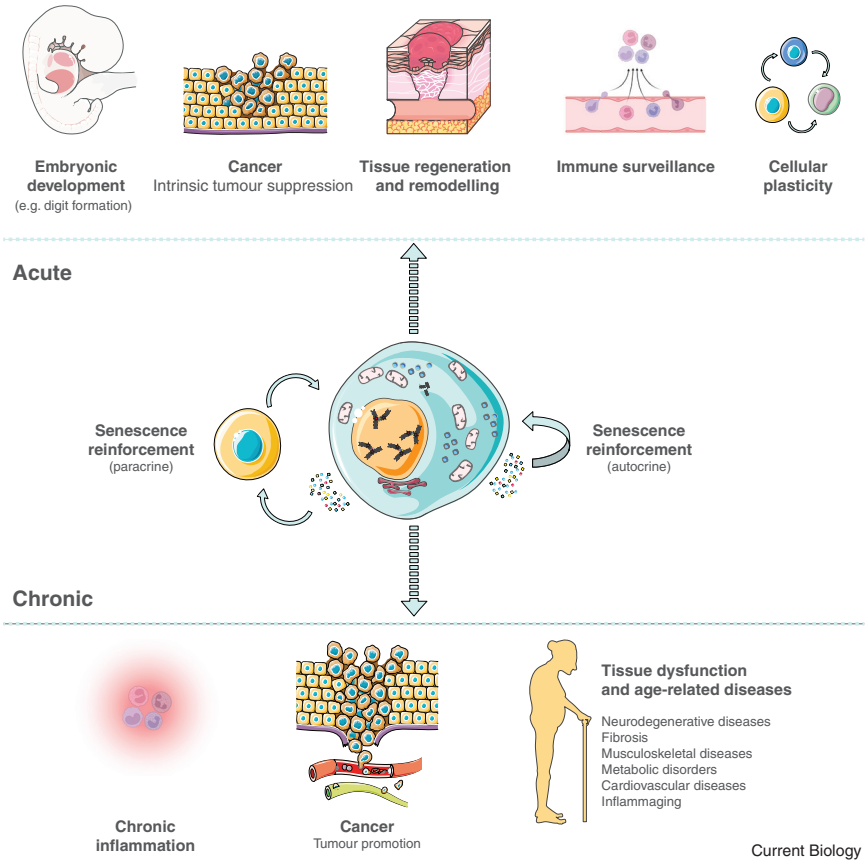

Figure 3. Biological functions of cellular senescence.
Senescent cells, mainly through the SASP, can have cell-autonomous and/or cell-non-autonomous functions that might depend on the age of the organism and the length of time that these cells reside in the tissue. Acute presence of senescent cells is normally associated with beneficial functions (top), while their chronic presence is associated with detrimental functions (bottom).

Senescence is also considered to be a dynamic multistep process, with the timing of the presence of these cells in a tissue being vitally important for their phenotype. This might also explain why senescence seems to be context dependent and has diverse outcomes and functions, as well as different clearance mechanisms.

Senescence in health and disease

Senescence has both beneficial and detrimental functions, depending on the tissue context and its persistence in the tissue (Figure 3). Unsurprisingly, senescence was originally reported as a tumour suppressor mechanism that limits the growth of potentially oncogenic cells. More recent work has shown that a programmed and transient senescence plays an important role during organogenesis in the embryo by facilitating elimination of short-lived structures. In addition, senescent cells can have beneficial biological functions via non-cell-autonomous functions. The transient presence of senescent cells in a tissue can promote de-differentiation and cellular plasticity of neighbouring cells in the context of a regenerative response. Furthermore, tissue remodelling (such as wound healing) is positively affected by short-term senescence, which also participates in the attenuation of tissue fibrosis by inhibiting the proliferation of hyper-proliferative myofibroblasts.

In contrast to the above-mentioned beneficial functions, the persistence of senescent cells is deeply associated with ageing, chronic inflammation and the development of age-related diseases, such as musculoskeletal dysfunctions (including osteoarthritis, osteoporosis and sarcopenia), fibrosis, neurodegeneration (for example, Alzheimer's and Parkinson's diseases), cancer and diabetes. After

injury, regeneration depends on both the proliferation of cells within the tissue, as well as the proliferation and differentiation of stem cells. However, the persistent accumulation of senescent cells in differentiated tissues, together with the loss of self-renewal and proliferation capacity in the stem cell pool, will compromise regenerative potential and tissue functionality. Furthermore, intrinsic mechanisms of apoptotic resistance of senescent cells may also protect cancer cells from dying before acquiring a fully senescent phenotype, bypassing growth arrest and resuming proliferation. The persistent accumulation of senescent cells can also halt tissue homeostasis through the progressive increase in SASP-associated secretion. In fact, the SASP contributes to the chronic low-grade inflammation that develops with age (inflammaging), together with the age-related decline in adaptive immunity (immunosenescence).

Because genetic elimination of senescent cells has been shown to improve age-related diseases and extend healthspan in animal models, different senescence-associated mechanisms are being exploited to develop new therapeutics. Significant effort is being invested in the identification of molecules that can specifically eliminate senescent cells (senolytics) or inhibit their SASP (senomorphics). Furthermore, emerging therapeutic opportunities are focused on enhancing immune-cell-mediated clearance of senescent cells. For development of senotherapeutics, cellular mechanisms that are altered in senescent cells are currently being targeted, for example via inhibition of pro-survival networks, modulation of the mTOR and NF-κB pathways, and promotion of immune-mediated clearance.

Outlook

Senescence is an intriguing phenotype with context-dependent pleiotropic roles in the organism. On one hand, certain senescent cells positively regulate important biological processes including tumour suppression, tissue remodelling and immune regulation. On the other hand, cellular senescence is associated with the onset and progression of disease and dysfunction and is considered a hallmark of ageing. Even considering that we still lack a deep understanding of the mechanisms and functions of senescent cells in each specific context, pro- and anti-senescent therapies are showing promising results in preclinical mouse models and in several ongoing human clinical trials.

However, our general understanding of the complex biological functions of senescence and the development of refined, more efficient and less toxic therapeutic interventions could benefit from a multi-parametric approach aiming to unveil subtype-specific biomarkers and signatures. In order to develop such an approach, we should take into account all of the potential intrinsic and extrinsic factors contributing to senescence heterogeneity. Moreover, it is imperative to properly characterise the particular context that is targeted, and to discover how to appropriately modulate the efficiency and kinetics of the clearance of senescent cells by the immune system. Different subsets of senescent cells coexist *in vivo* even within the same tissue, and the identification of the mechanisms that drive the formation of these particular subpopulations might help to develop senotherapies with significantly reduced toxicity. Anti-senescence therapies targeting specific detrimental subpopulations of senescent cells, while not affecting those senescent cells involved in beneficial processes or normal non-senescent cells, might promote healthy longevity with reduced toxicity. Similarly, pro-senescence interventions that promote beneficial senescence in certain cells and contexts, for example to facilitate optimal tissue repair, could be developed.

DECLARATION OF INTERESTS

M.D. is scientific advisor and shareholder of Cleara Biotech, and on the Scientific Advisory Board of Oisin Biotechnologies.

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[1]European Research Institute for the Biology of Ageing (ERIBA), University Medical Center Groningen (UMCG), University of Groningen (RUG), Groningen, The Netherlands. [2]CellCOM Research Group, Instituto de Investigación Biomédica de A Coruña (INIBIC), Servizo Galego de Saúde (SERGAS), Universidade da Coruña (UDC), A Coruña, Spain.
*E-mail: m.demaria@umcg.nl