Cellular Senescence in Acute and Chronic Wound Repair

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Abstract:

Cellular senescence, once thought an artefact of *in vitro* culture or passive outcome of ageing, has emerged as fundamental to tissue development and function. The senescence mechanism importantly halts cell cycle progression to protect against tumour formation, while transiently present senescent cells produce a complex secretome (or SASP) of inflammatory mediators, proteases and growth factors that guide developmental remodelling and tissue regeneration. Transiently present senescence is important for skin repair, where it accelerates extracellular matrix formation, limits fibrosis, promotes re-epithelialisation, and modulates inflammation. Unfortunately, advanced age and diabetes drive pathological accumulation of senescent cells in chronic wounds, which is perpetuated by a pro-inflammatory SASP, advanced glycation end-products and oxidative damage. Though the biology of wound senescence remains incompletely understood, drugs that selectively target senescent cells are showing promise in clinical trials for diverse pathological conditions. It may not be long before senescence-targeted therapies will be available for the management, or perhaps even prevention, of chronic wounds.

Introduction:

Hayflick and Moorhead (1961) first identified cellular senescence when they observed cultured primary human fibroblasts had a finite lifespan, undergoing irreversible cell cycle arrest following a limited number of population doublings. What was originally dismissed as a cell culture artefact is now widely acknowledged as a biological programme that globally regulates cell fate. In the years following Hayflick's discovery of replicative senescence, it emerged that cellular senescence could also be triggered by the activation of oncogenes (Serrano et al. 1997), highlighting the importance of senescence as a robust anti-tumour mechanism. More recently it has transpired that senescent cells are far from passive bystanders, instead displaying diverse phenotypes that contribute to tissue maintenance and dysfunction. In this Chapter, we discuss the diverse roles of cellular senescence in tissue repair

and pathology, highlighting exciting opportunities to develop senescence-targeted therapies to treat or prevent chronic wounds.

Cellular Senescence Pathways

Replicative and oncogenic stimuli are well documented drivers of senescence (Demaria et al. 2015), but senescence can be induced by various other intrinsic and extrinsic stressors including DNA breaks (di Fagagna 2008), oxidative and genotoxic stress (Nair et al. 2015; Erusalimsky 2020), epigenetic damage (Sidler et al. 2017), inflammation (Freund et al. 2010) and mitochondrial dysfunction (Chapman et al. 2019). Following insult, transient cell cycle arrest is induced via the p53/$p21^{WAF1/CIP1}$ axis. If the damage stimulus persists, the $p16^{INK4A}$/pRB tumour suppressor pathway can be engaged, causing irreversible senescence (He and Sharpless 2017). At the nexus of cell cycle regulation, p53 responds to telomere attrition (di Fagagna et al. 2003; Herbig et al. 2004) and broad damage-induced signals (Ou and Schumacher 2018). Cell fate following DNA damage and p53 activation is context-dependent with subsequently activated signalling pathways directing a cell towards quiescence, programmed cell death (apoptosis) or senescence. A plethora of extrinsic factors sway this response, including the cell type and stress severity, while at the molecular level, elaborate post-translational modifications affect p53 activity and function (Childs et al. 2014; Bourgeois and Madl 2018).

In most cases, p53 activation occurs in a DNA damage response (DDR)-dependent manner, whereby DDR sensors (e.g., ATM) phosphorylate p53 and its ubiquitin ligase, MDM2, leading to p53 stabilisation (Hu et al. 2012). p53 in turn transactivates p21, which governs the switch between proliferation, quiescence and senescence. p21 acts by modulating the expression of many p53 targets (Mijit et al. 2020) and can broadly prevent cyclin-dependent kinase (CDK) mediated inactivation of pRb (Bertoli et al. 2013). Importantly, p21 can induce transient growth arrest, because inactivation of the p53-p21 pathway in the absence of p16 reverses senescence (Beausejour et al. 2003). Thus, engagement of the pRB/p16 pathway is often required for irreversible senescence. p16 is one of three tumour suppressors encoded by the INK4A/ARF locus, whose main role is preventing phosphorylation of pRb via CDK4 and CDK6 (Takahashi et al. 2006). pRb binds to E2F family transcription factors, repressing the activation

of E2F target genes required for replication (Giacinti and Giordano 2006). Hence, failure to phosphorylate pRb prevents cell cycle progression. In addition to stimulating pRb-mediated inhibition of cell cycle progression, p53 promotes senescence by directly targeting E2F7, the only E2F family member upregulated during senescence (Aksoy et al. 2012), and a number of p53 responsive miRNAs (Xu et al. 2019). E2F7 and pRB then reinforce repression of E2F target genes by promoting heterochromatisation of E2F-responsive elements via recruitment of histone deacetylases and histone methyltransferases (Martínez-Zamudio et al. 2017).

While all forms of growth arrest are characterised by the presence of hypophosphorylated pRb family members (He and Sharpless 2017), there remain considerable differences between cells undergoing senescence versus those that become quiescent or terminally differentiated. Unlike cell quiescence, where cells enter cell cycle arrest in G0 and can re-enter at any time in response to mitogenic signals (Terzi et al. 2016), cellular senescence occurs at G1, G1/S and even G2 phases of the cell cycle (Blagosklonny 2011). Terminally differentiated cells develop specialised identities in response to developmental programming, whereas senescence is a fate shared by many cell types, often as a result of a sustained DDR (Chandler and Peters 2013). There are instances where the demarcations are less clear, because senescence is known to play a role in developmental processes (Muñoz-Espín et al. 2013; Storer et al. 2013), and terminally differentiated cells can undergo senescence (Jurk et al. 2012; Moreno-Blas et al. 2019), suggesting active inhibition of cell cycle is not always required for senescence.

In non-pathological states, senescent cells are typically removed from the body via apoptosis and immune clearance mechanisms. Following senescence induction, senescent cells undergo immunogenic conversion, producing a secretome of factors collectively known as the senescence associated secretory phenotype (SASP; Burton and Faragher 2015). The SASP can include a range of inflammatory chemokines that attract and activate various subsets of immune cells depending on their chemokine receptor repertoires (Acosta et al. 2013). This response thus crucially leads to elimination of senescent cells by virtue of the immune system. Indeed, various immune cell types, including Natural Killer (NK) cells, T cells, neutrophils and macrophages, are known to be involved in immune-mediated clearance of senescent cells (summarised in Sagiv and Krizhanovsky 2013).

Characterising Cellular Senescence - Detection

Reliable biomarkers for senescence detection are essential given its major role in aging and disease. However, detection of senescent cells remains challenging in the real-world setting for a number of reasons: 1) Senescence is context-dependent and cell type specific (Kirschner et al. 2020); 2) Senescence is highly dynamic with different markers associated with early and late stages (Mijit et al. 2020) and; 3) Several senescence markers are shared with other growth arrested states (Itahana et al. 2007). It is important to acknowledge that the individual senescence markers outlined in the following paragraphs will not be a feature of all senescent cells. Therefore, it is essential that more than one senescence marker is present for a cell to be identified as senescent.

Senescent cells possess a range of morphological, functional and molecular characteristics that can be influenced by intrinsic and extrinsic cues (**Figure 1**). Typically, senescent cells take on a flattened, elongated appearance with enlarged vacuoles and nuclei (Wang and Dreesen 2018; Neurohr et al. 2019). They exhibit heterochromatin at E2F promoters, termed senescence-associated heterochromatin foci (SAHF), which potentiate senescence by preventing E2F target gene transcription (Narita et al. 2003). SAHF are relatively easy to visualise as they stain notably with DAPI (Kosar et al. 2011) but are not useful for senescence detection in murine cells which are unable to produce robust SAHF (Aird and Zhang 2013). Histone loss and cytoplasmic chromatin fragments (positive for γH2AX and H3K9me3) have likewise been observed *in vitro* following replicative and oncogene-induced senescence (Ivanov et al. 2013). This observation suggests a compromised nuclear envelope, and is supported by loss of lamin B1, a major structural nuclear envelope protein, following senescence *in vitro* and *in vivo* (Shah et al. 2013; Wang et al. 2017; Saito et al. 2019). Given that persistent DNA damage is a primary cause of senescence, it is not surprising that senescent cells exhibit markers of DNA damage, such as γH2AX and ATM kinase, with reduced expression of DNA repair genes (Collin et al. 2018). However, DDR markers have been suggested to be of limited utility for *in vivo* senescence detection, where the majority of cells with a DDR are responding to reparable damage (Herranz and Gil 2018).

Senescence-associated beta galactosidase (SA-βGal) is the archetypical senescence biomarker, due to its expression across a broad range of senescent cell types and aged tissues (Dimri et al. 1995; Debacq-Chainiaux et al. 2009; Covarrubias et al. 2020). The lysosomal hydrolase detected by SA-βGal, B-D-galactosidase, is ordinarily detected in non-senescent cells at pH 4. However, senescence causes expansion of the lysosomal compartment, allowing detection of B-D-galactosidase at higher pH (Kuilman et al. 2010). Despite its wide use as a senescence biomarker, there remains controversy around the specificity of SA-βGal, with suggested non-specific staining of skin appendages *in vivo* and quiescent cells *in vitro* (Krishna et al. 1999; Cristofalo 2005; Lee et al. 2006). Prolonged incubation can also lead to nonspecific staining (González-Gualda et al. 2019), while the staining itself requires fresh tissue. Sudan Black B, which stains age-associated lipofuscin, has been recommended as an alternative to SA-βGal that can be used on archived tissues (Georgakopoulou et al. 2013). Nevertheless, SA-βGal staining remains the most extensively used method for detecting senescent cells, with commercial SA-βGal staining kits widely available. Note, in a recent study, Chia et al. (2021) failed to detect SA-βGal in young or aged human skin or acute wound tissue, despite observing upregulation of other senescence markers following injury.

Arguably the most specific marker of senescence *in vivo* is p16, accumulating with age in a variety of tissues (Jeyapalan et al. 2007; Hall et al. 2016; Hudgins et al. 2018). However, not all tissues show age-dependent accumulation of p16 (Idda et al. 2020), senescence can occur in a p16-independent manner (Prieur et al. 2011), and some cancer cells express p16 (Romagosa et al. 2011). In addition, p16 has been suggested as a characteristic of “normal” immunological phenotypes, such as macrophage polarisation (Hall et al. 2017) and T cell exhaustion (Sharpless and Sherr 2015). Other methods to detect senescence include the absence of proliferation (Biran et al. 2017), the presence of a SASP (Coppé et al. 2008), loss of lamin B1 (Freund et al. 2012), SAHF (Aird and Zhang 2013) and DNA damage markers (Wang et al. 2009; Hootan and Evans 2017). Given the variability in senescence phenotype/markers (Wang et al. 2009; Idda et al. 2020), combinatorial approaches to validate senescence in tissues are preferred, such as using SA-βGAL with proliferation markers (Itahana et al. 2013; Biran et al. 2017) or in conjunction with p16 and p21 staining (Ritschka et al. 2017).

Characterising Cellular Senescence – Outcomes

It is now widely accepted that senescent cells actively contribute to progressive tissue dysfunction. One major feature of senescent cells, important in the context of the tissue microenvironment, is their complex secretome, termed a SASP (Coppé et al. 2008). The SASP, like senescence, is a dynamic process regulated by factors such as Notch1 (Hoare et al. 2016) and established in a temporal and situation-dependent manner (Basisty et al. 2020). The SASP can include proinflammatory mediators, proteases, extracellular matrix (ECM) components and growth factors (Coppé et al. 2008; Freund et al. 2010; Elzi et al. 2012). Lipids and exosomal cargo are also important SASP components (Basisty et al. 2020; Wallis et al. 2020; Narzt et al. 2021). Interestingly, damage-associated molecular patterns, such as HMGB1 and specific toll-like receptors, are required for SASP induction (Davalos et al. 2013; Hari et al. 2019).

SASP factors not only reinforce cell cycle arrest in an autocrine manner (e.g., Acosta et al. 2008), but exacerbate inflammation, accelerate tissue breakdown and promote paracrine induction of senescence (Acosta et al. 2013; Davalos et al. 2013; Severino et al. 2013). Indeed, the pro-inflammatory SASP feature of senescent cells is often a DDR, controlled at the transcriptional level by NFkB (Rodier et al. 2009), C/EBP (Shao et al. 2016), mTOR (Herranz et al. 2015), p38MAPK (Freund et al. 2011) and Gata4 (Kang et al. 2015). Notably, the SASP can also be beneficial in particular situations, reinforcing senescence (Acosta et al. 2008), promoting senescent cell clearance (Eggert et al. 2016), preventing tumours (Lujambio et al. 2013) and aiding tissue repair (Demaria et al. 2014). While the SASP has clear implications for tissue homeostasis and pathology, understanding the context-dependent diversity of the SASP remains a key challenge.

Senescence from Embryogenesis to Tissue Ageing

It is well established that senescence is a dynamic stress response, evolved to prevent incipient neoplastic transformation (Campisi and d'Adda di Fagagna 2007). Tens of thousands of DNA alterations occur in an individual cell per day (Jackson and Bartek 2009). Therefore, along with other proofreading mechanisms, senescence is crucial to avert unrestrained proliferation of mutated cells. In young organisms, this process is highly efficient, with resulting senescent cells effectively cleared by the immune system. However, cellular

senescence is often considered a double-edged sword because as we age this process becomes perturbed, resulting in disease (Kowald et al. 2020).

Intriguingly, senescence has emerged as far more than an anti-cancer mechanism, or outcome of advanced cellular age. Indeed, it has now been shown to play diverse roles in the development and maintenance of tissues. Seminal publications documented senescent cell accumulation in the signalling hubs of murine and human embryos at restricted time windows (Muñoz-Espín et al. 2013; Storer et al. 2013). Detailed evaluation revealed the developmental importance of p21-dependent senescence, whereby senescent cells directed macrophage-mediated clearance and embryonic remodelling (Muñoz-Espín et al. 2013; Storer et al. 2013). Senescence and macrophage-mediated clearance has since been shown to be important for development of the inner ear in mice and chickens (Gibaja et al. 2019), and in the patterning of kidney, cement gland and brain of amphibians (Davaapil et al. 2017; Villiard et al. 2017). Senescence may be vital even earlier in development, as extravillous trophoblasts, required for placenta formation, lose their replicative potential and develop a SASP following invasion into the uterine lining (Velicky et al. 2018). Senescent cells are subsequently cleared from the endometrium by uterine NK cells (Brighton et al. 2017). These studies open questions around the origins of senescence, and whether its links to development (and tissue repair) precede its anti-tumorigenic role. However, the degree of conservation of senescence programming, and its functional requirements during development, remain largely unknown.

In contrast to ageing and pathology, where senescence is a stochastic damage response, senescence observed during development is instead a highly organised transient process with complimentary apoptotic and immune clearance mechanisms. At face value, it appears that transiently present and pathological senescence are dichotomous. A key factor governing the switch between beneficial and detrimental states appears to be effective immune-mediated clearance (Rhinn et al. 2019). During transiently present senescence observed during development (Storer et al. 2013), regeneration (Yun et al. 2015) and wound healing (Demaria et al. 2014), senescent cells are removed by macrophages, neutrophils, T lymphocytes and NK cells (Xue et al. 2007; Song et al. 2020). Immune cells are able to locate senescent cells by the factors they secrete (Iannello et al. 2013; Sagiv et al. 2013), controlled at the epigenetic level by BRD4 (Tasdemir et al. 2016). Senescent cells also express stimulatory ligands that bind the

NKG2D receptor and activate killing by T cells (Sagiv et al. 2016). In mouse tissues, developmental senescence appears to be solely modulated via p21, independent of p16 (Muñoz-Espín et al. 2013; Storer et al. 2013). However, similar to DDR linked senescence, developmental senescence and SASP requires TGFβ/SMAD signalling (Muñoz-Espín et al. 2013; Tominaga and Suzuki 2019).

The processes underpinning senescence accumulation in aged tissues are also not fully understood. Initial induction is likely mediated by replicative exhaustion, shortening of telomeres and activation of senescence pathways (Reaper et al. 2004). Subsequently, advanced age is associated with long-term exposure to intrinsic and extrinsic damage signals. At the molecular level, ageing perturbs the developmental machinery responsible for repressing senescence such that the INK/ARF locus loses repressive marks, hence increasing p16 sensitivity to induction (Martin et al. 2014). This combination of increased susceptibility and continuous damage signals heighten senescence onset, which is then reinforced in an intracrine manner by the production of a pro-inflammatory SASP (Acosta et al. 2008; Kuilman et al. 2008; Martien et al. 2013; Hsieh et al. 2017). Additionally, the SASP can potentiate senescence to the neighbouring microenvironment in a paracrine manner by activating a number of receptor pathways, including CCR2 (Acosta et al. 2013), TGFBR1 (Acosta et al. 2013; Bird et al. 2018; Ferreira-Gonzalez et al. 2018) and CXCR2 (Wilkinson et al. 2019a). The age-related SASP differs from developmental SASP, containing secreted factors known to drive widespread inflammation and tissue destruction. Ironically, the age-related SASP is also rich in potent mitogenic drivers, including proteases, growth factors and cytokines, which can enhance tumorigenesis (Coppé et al. 2008; Yoshimoto et al. 2013; Eggert et al. 2016). Consequently, a mechanism selected for its beneficial anti-cancer effects in the young can become maladaptive in later life. The diverse roles of senescence are summarised in **Figure 2**.

Another reason senescent cells accumulate during ageing is impairment in clearance mechanisms, such as redistribution of NK cell subtypes (Solana et al. 2014; Sagiv et al. 2016; Ovadya et al. 2018). It is widely acknowledged that ageing causes dysfunction to both the innate and adaptive immune systems, termed immunosenescence (Song et al. 2020). Some senescent cells evade NK- and CD8+ T cell-mediated clearance by expressing high levels of

HLA-E, which bind the inhibitory receptor NK2GA (Pereira et al. 2019). Others shed MICA and MICB to avoid detection by NKG2D (Muñoz et al. 2019). Moreover, age decreases expression of NKG2A in NK cells, thus reducing clearance mechanisms (Lutz et al. 2005). The SASP may also aid senescent cell evasion from immune clearance in certain contexts (Ruhland et al. 2016; Pereira et al. 2019).

The detrimental role of senescence during ageing is well established, where elevated numbers of senescent cells are associated with reduced tissue functionality in mice (Molofsky et al. 2006; Ovadya et al. 2018; Xu et al. 2018; Palmer et al. 2019; Cai et al. 2020) and humans (Justice et al. 2018; Gustafson et al. 2019), while the pro-inflammatory SASP contributes to many pathologies (Xu et al. 2015; Oubaha et al. 2016). Direct evidence comes from studies where transplantation of senescent cells to young mice induces disease states, such as osteoarthritis (Xu et al. 2017) and lower physical activity (Xu et al. 2018), likely via enhanced paracrine induction of senescence (da Silva et al. 2019). By contrast, selective removal of senescent cells is known to alleviate many age-related pathologies and extend lifespan in experimental models (Baker et al. 2011; Baker et al. 2016; Hashimoto et al. 2016; Roos et al. 2016; Ogrodnik et al. 2017; Xu et al. 2018; Yousefzadeh et al. 2018).

Senescence in Wound Repair and Regeneration

When a tissue repairs or regenerates, it re-uses processes associated with development and morphogenesis. This is true for senescence, where the SASP can enable reprogramming of cells to a stem cell-like fate following tissue injury (Mosteiro et al. 2016; Chiche et al. 2017) or during regeneration (Yun et al. 2015; Ritschka et al. 2017). The parallels between development and tissue regeneration are clear. Senescence is tightly regulated to aid limb regeneration in the salamander, with effective clearance mediated by macrophages, even following multiple rounds of amputation and regeneration (Yun et al. 2015). Senescence, mediated via Ccn1, is also required for regeneration of the embryonic murine heart (Feng et al. 2019), while ablation of senescent cells in zebrafish abrogates pectoral fin regeneration (Da Silva-Álvarez et al. 2020).

The sophisticated host response to injury can be described as four overlapping phases: haemostasis, inflammation, proliferation and remodelling (Eming et al., 2014; Gurtner et al., 2008). Each of these stages requires a temporal and dynamic interplay between various signalling cascades and cell types, where the role of senescence remains less well understood (Wilkinson and Hardman 2020a). The processes that occur during wound healing involve many mitogenic factors that enable partial epithelial-to-mesenchymal transition in keratinocytes to aid wound closure, and rapid proliferation of fibroblasts to restore the dermal matrix. Additionally, the complex population of immune cells provide an environment enriched for secreted factors that promote plasticity (Shaw and Martin 2016). Given the close links to cancer, developmental remodelling and regeneration, it is perhaps unsurprising that research is now uncovering vital roles for transient presence of senescence in tissue repair.

There is now strong evidence that induction of transiently present senescence is able to prevent excessive fibrosis following injury in multiple murine tissues (Krizhanovsky et al. 2008; Jun and Lau 2010; Meyer et al. 2016). Pivotal studies revealed that transiently present senescence occurs during murine skin wound repair, and that Ccn1 and Ccn2 are important drivers of this response (Jun and Lau 2010, 2017). Intriguingly, Ccn1 has also been reported as a pattern recognition receptor vital to prevent wound infection (Jun and Lau 2020). Demonstration that transiently present senescence is beneficial to healing was provided by Demaria et al. (2014), where specific ablation of p16- and p21-expressing cells significantly delayed cutaneous wound closure and reduced ECM deposition. Here, injury-induced senescent cells produced a PDGF-AA-rich SASP, crucial for stimulating myofibroblast differentiation and enabling effective healing. More recently, Hiebert et al. (2018) showed that Nrf2-triggered induction of senescence in fibroblasts accelerated both re-epithelialisation and ECM deposition. Transiently present senescence is not confined to skin wounds, with reported observations during corneal (Wang et al. 2019) and lung (Kobayashi et al. 2020) injury. In acute lung injury, p21 activation limits apoptosis and ameliorates tissue damage (Blazquez-Prieto et al. 2021).

The above investigations provide new insight into the importance of transiently present senescence during tissue repair. However, many questions remain unanswered. At what point do cells become susceptible to acute injury-induced senescence? Which cell types and why?

How are these cells effectively cleared to prevent the switch to a chronic state? It is likely that injury causes the release of a myriad of factors stimulating senescence induction, such as reactive oxygen species (ROS; Jun and Lau 2010; Passos et al. 2010), with others still to be identified. Effective clearance likely occurs by virtue of the diverse inflammatory profile of wounds (Wilkinson and Hardman 2020a), yet uncertainties remain around specificity and regulation. Finally, a major limitation of existing studies is that they are almost exclusively limited to *in vivo* models of wound repair, and we currently have limited understanding of how these observations will translate to human healing. In a recent proof-of-concept study, Chia et al. (2021) demonstrated that p21 and p53, but not p16, were induced in acute wound repair in young subjects, while neither p21, p53 or p16 were induced in older subjects.

Senescence in Chronic Wound Healing

Unlike internal organs, senescence in the skin is induced by a combination of intrinsic chronological ageing, and external factors, such as ultraviolet radiation exposure (Rittié and Fisher 2015). Indeed, the skin is characterised by a dense matrix of structural proteins, with degradation and remodelling of this ECM leading to loss of physiological and biomechanical integrity (Wilkinson and Hardman 2021a). Ultraviolet radiation is widely reported to induce skin senescence by increasing ROS levels (Herrling et al. 2006; Jenkins et al. 2011; Wang et al. 2017), with ageing epidermis and dermis both characterised by increased p16 and p21 positive cells (Ressler et al. 2006; Waaijer et al. 2012; Idda et al. 2020). Aged skin additionally exhibits loss of lamin b1 (Dreesen et al. 2013), shortened telomeres (particularly in the epidermis, Sugimoto et al. 2006), increased mutations in mitochondrial DNA (Berneburg et al. 1997) and an elevated SASP, including MMPs (Quan et al. 2009) and PAI-1 (Goldstein et al. 1994; Baker et al. 2008). Factors contributing to the age-related accumulation of SASP include higher levels of histone variant H2A.J in the epidermis, which promotes inflammation (Contrepois et al. 2017), and higher numbers of immunosuppressive cell types, reducing senescent cell clearance (Ruhland et al. 2016).

An important characteristic of skin is the high turnover of epidermal keratinocytes, essential to maintain the skin barrier (and repair epidermal damage). This high renewal capacity is aided by stem cell niches in the epidermal basal layer, hair follicles and sebaceous glands

(Pincelli and Marconi 2010; Donati and Watt 2015). Sebaceous gland function declines with age (Zouboulis et al. 2008), causing decreased production of enzymes required to synthesise long-chain fatty acids and cholesterol (Seyfarth et al. 2011). Age-associated loss of lipids and reduced keratinocyte renewal lead to an impaired skin barrier. Aged keratinocytes show altered cell cycle kinetics and lower proliferation rates (Giangreco et al. 2008; Charruyer et al. 2009), coinciding with accumulation of senescence (Zou et al. 2021). Intriguingly, hair follicle stem cell abundance is not altered with chronological age in mice (Giangreco et al. 2008), yet aged hair follicle stem cells possess lower chromatin accessibility, which is linked to decreased renewal capacity (Koester et al. 2021). The ability to regenerate tissues and repair injuries declines with age throughout the body (e.g., in the muscle; Jang et al. 2011; Sousa-Victor et al. 2014), while an age-related increase in p16 is associated with reduced stem cell capacity in the brain, kidney, haemopoietic system and other tissues (Janzen et al. 2006; Krishnamurthy et al. 2006; Molofsky et al. 2006).

Age-associated decline in skin barrier increases susceptibility to injury and infection. Thus, it is unsurprising that age is a major risk factor for the development of chronic, non-healing wounds, with high morbidity and mortality in patients (Guest et al. 2015; Han and Ceilley 2017). A second key risk factor for chronic wound development is diabetes. Diabetes is also closely linked to senescence as hyperglycaemia accelerates the formation of advanced glycation end-products, triggering oxidative damage and driving unrestrained inflammation (Stegenga et al. 2008; Fang et al. 2016; Moura et al. 2019; Wilkinson and Hardman 2021b). It has been known for more than twenty years that fibroblasts isolated from chronic wounds are predisposed to senescence (Mendez et al. 1998; Vande Berg et al. 1998; Agren et al. 1999; Stanley and Osler 2001). However, there has been little attempt to determine the molecular and cellular drivers of pathological wound senescence, nor to functionally demonstrate a link to poor healing outcome. We recently reported that diabetic macrophages are susceptible to senescence, showing that they delay healing in a non-aged murine model of diabetic wound repair (Wilkinson et al. 2019a). Interestingly, this process was modulated by CXCR2, an important senescence mediator (Acosta et al. 2008).

A hallmark of chronic wounds is prolonged and excessive local inflammation, where bacterial colonisation and impaired cell behaviours combine to drive extensive immune cell

recruitment and retention (Wilkinson and Hardman 2020b). This provides an optimum environment for rapid senescence induction. For example, neutrophils produce high levels of ROS which cause paracrine induction of senescence in neighbouring fibroblasts by telomere shortening (Lagnado et al. 2021). Chronic wounds also display elevated cytokines and chemokines, key SASP components that skew macrophages towards a pro-inflammatory state (Lujambio et al. 2013). A range of local factors, including pathogenic bacterial products (Muller et al., 2009; Elsayed et al. 2021) and tissue iron (Sindrilaru et al. 2011; Wilkinson et al. 2019b), likely reinforce chronic wound senescence by contributing to unresolved inflammation and perturbed immune cell function. Chronic wound milieu, which induces cellular perturbations associated with chronicity, such as epidermal hyperproliferation (Stojadinovic et al. 2008) and reduced angiogenesis (Lauer et al. 2000), has been shown to directly induce senescence in neonatal fibroblasts (Mendez et al. 1999).

What remains somewhat perplexing is that senescence and the SASP naturally induce pluripotency and regenerative capacity, yet during tissue ageing they contribute to inflammation and pathology (summarised in **Figure 3**). It could be this disparity reflects differences in the level and persistence of the response. This has been demonstrated recently, where short-term senescence induction in keratinocytes induced pluripotency and regeneration in skin grafts, while prolonged exposure to the SASP reduced pluripotency and increased numbers of p16+ve senescent cells (Ritschka et al. 2017). Conversely, the SASP itself could change over time in a context-dependent manner. For example, Hoare et al. (2016) showed that fibroblast SASP is characterised by a Notch-high early phase, and Notch-low late phase. The early phase was TGFβ-dependent and associated with tissue regeneration and immunosuppression, while the late phase was NFkB-dependent and linked to inflammation.

From a clinical perspective, extensive chronic wound recalcitrance highlights an urgent need to improve intervention strategies. While we are far from fully understanding the contribution of senescence to chronic wounds, studies into the role of senescence in other pathologies could provide a timely opportunity to re-purpose new and existing therapies. Indeed, the outcomes of senescent cell ablation using genetic models (Baker et al. 2011, 2016) have now been confirmed using drugs that selectively target cellular senescence, referred to as senolytics (Kirkland and Tchkonia 2020). Senolytics can act upon senescence machinery,

for example by targeting the pro-survival pathways (BCL-2 and others) that provide senescent cells with apoptosis resistance (Zhu et al. 2015; Chang et al. 2016; Hohmann et al. 2019). Elimination of senescent cells using senolytics in these models alleviated many age-related diseases and restored tissue function. Moreover, a BCL-2 family inhibitor reduced epidermal senescence and promoted hair follicle stem cell proliferation in mice with epidermal overexpression of $p14^{Arf}$ (Yosef et al. 2016). Another potential strategy is to target the SASP by blocking NFkB nuclear translocation (Moiseeva et al. 2013), inhibiting the JAK/STAT pathway (Xu et al. 2015; Farr et al. 2017) or suppressing BRD4 (Tasdemir et al. 2016).

To date, the majority of studies using senolytics to target chronic senescence have been pre-clinical. Therapeutic intervention for human disease is complex, particularly as most elderly patients suffer multimorbidity, presenting with two or more conditions (Guisado-Clavero et al. 2018). As chronic wounds primarily affect the elderly and/or diabetic, compatibility with other treatments must be considered to prevent contraindication or reduced efficacy. Despite these limitations, many senolytics are FDA-approved cancer drugs or natural products, making the clinical pathway for wound repurposing highly attractive. Indeed, a handful of recent clinical trials are starting to suggest benefit of senolytics in other chronic indications, such as patients with diabetic kidney disease (Hickson et al. 2019) or idiopathic pulmonary fibrosis (Justice et al. 2019), while others are underway (summarised in Robbins et al. 2021).

Concluding Remarks

Understanding of cellular senescence has progressed rapidly since the concept was first proposed in the 1960s. A process once thought to be an artefact of cell culture is now known to be essential for tumour suppression, developmental reprogramming, regeneration and wound repair. While the presence of transient senescence is beneficial for tissue maintenance, excessive senescence, as a result of age-related accumulation and defective immune clearance, contributes to many disease states. Although we are still a long way from unravelling the role of senescence in poor wound healing, preliminary *in vivo* studies have revealed the therapeutic potential of targeting senescence to promote wound repair. When coupled with emerging efficacy data from senolytic clinical trials in other chronic indications,

it is clear that a senolytic-based strategy for chronic wounds treatment could be a reality in the not so distant future.

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Figure Legends

Figure 1. Characteristics of senescent cells. Senescent cells undergo cell cycle arrest and feature DNA alterations such as senescence associated heterochromatin foci (SAHF), DNA-SCARS and markers of DNA damage. A disrupted nuclear envelope is accompanied by reduction in the structural nuclear envelope protein, lamin B1, and the release of chromatin into the cytoplasm. Senescent cells show mitochondrial dysfunction, with increased production of reactive oxygen species (ROS) and upregulation of pro-survival (anti-apoptotic) pathways. Morphologically, senescent cells appear flattened and elongated with enlargement of lysosomes, enabling detection by senescence-associated beta galactosidase (SA-βGAL) and lipofuscin. In addition, senescent cells feature a senescence associated secretory phenotype (SASP) containing proteases, cytokines, matrix metalloproteinases (MMPs) and extracellular vesicles (ECVs).

Figure 2. Diverse roles for senescence throughout life. Transiently present (short-term) senescence is required during development, tissue regeneration and wound repair. Here,

senescent cells produce a beneficial senescence associated secretory phenotype (SASP) that guides developmental patterning and tissue restoration following injury. Effective clearance of senescent cells during these processes prevents chronicity. By contrast, chronological ageing leads to accumulation of cellular stress which drives senescence. Chronic senescence is exacerbated by defective clearance mechanisms and unrestrained inflammation, leading to widespread tissue damage and increased risk of pathology.

Figure 3. Senescence in acute versus chronic wound healing. In acute wounds, transiently present senescent cells appear during late-stage healing, producing a senescence associated secretory phenotype (SASP) that aids extracellular matrix (ECM) deposition but prevents tissue fibrosis. Senescent cells are then cleared by the immune system, allowing full tissue resolution. During ageing and diabetes, advanced glycation end products (AGEs) and sterile inflammation promote the accumulation of senescent cells. Following injury, these resident senescent cells contribute to a pro-inflammatory environment that perpetuates senescence, causes tissue breakdown and prevents healing. Senescence (and inflammation) can also be exacerbated by chronic wound infection. AI Mϕs = anti-inflammatory macrophages. PI Mϕs = pro-inflammatory macrophages.