Investigating senescence in cellular plasticity and tissue regeneration

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To my family

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Abstract

Cellular senescence is a form of cell cycle arrest that is linked to tumor suppression and aging. It can be induced by replicative exhaustion, oncogenic signaling, DNA-damage and chemotherapeutic agents, acting to limit the proliferative capacity of damaged cells. Conversely however, it has been suggested that senescent cells also have pro-tumorigenic activity through the secretion of specific proteins, termed the senescence-associated secretory phenotype (SASP). Unexpectedly, we found that primary mouse keratinocytes undergoing oncogene-induced senescence exhibit an increase in the expression of skin stem cell-associated genes. Furthermore, we show that transient exposure to the SASP induces an increase in stem cell-associated genes in a paracrine manner, suggesting that the SASP can enhance tissue stemness. Importantly, by performing hair reconstitution assays with keratinocytes exposed to senescence-conditioned media, we demonstrate that these cells have functional stem cell capacity, suggesting that senescence can enhance regeneration *in vivo*. However, prolonged exposure to the SASP leads to paracrine senescence *in vitro* as a possible mechanism to counteract the aberrant regenerative stimulation. Furthermore, induction of senescence in single cells *in vivo* in the liver, induced expression of stem cell markers in the adjacent cells in a paracrine manner. Together this work suggests that the SASP is a regenerative mechanism that instructs stemness and plasticity, but if left unperturbed can have detrimental effects seen during aging and tumor formation.

Resumen

La senescencia celular es un tipo de arresto del ciclo celular ligado a la supresión tumoral y al envejecimiento que puede ser inducida por diversos mecanismos tales como el agotamiento replicativo, ciertos estímulos oncogénicos, daños en el ADN o ciertos agentes usados en quimioterapia. Su principal función es la de frenar la proliferación celular de células que han sido dañadas. Sin embargo, también se ha sugerido que la senescencia posee actividad tumorigénica a través de la secreción de ciertas proteínas extracelulares. Éste fenómeno de modulación de la respuesta a través de la secreción extracelular fue bautizado como "senescence-associated secretory phenotype" o SASP. De manera inesperada, nosotros hemos visto que la inducción de la senescencia mediante un oncogén en queratinocitos primarios de ratón conlleva un incremento en la expresión de genes específicos de células madre de la piel. Así mismo, hemos visto que el propio SASP tiene la capacidad de inducir de manera paracrina las propiedades de célula madre a células circundantes cuando éste es aplicado de forma transitoria. Además, a través de ensayos de reconstitución del pelo mediante el uso de queratinocitos murinos expuestos a medio condicionado de células senescentes, hemos podido funcionalmente demostrar que adquieren propiedades de células madre, lo que sugiere que la senescencia per se puede fomentar la regeneración *in vivo*. Sin embargo, también hemos visto que una exposición prolongada al mismo tipo de SASP induce senescencia *in vitro*, pudiendo actuar como un posible mecanismo de defensa frente a una regeneración aberrante. Así mismo, la inducción de la

senescencia en células aisladas de hígado *in vivo* promueve la aparición de marcadores de células madre en células adyacentes de manera paracrina. Globalmente, éste trabajo sugiere que el SASP aplicado de forma transitoria actúa como un mecanismo de regeneración celular que instruye propiedades de células madre y promueve la plasticidad celular. Sin embargo, cuando su efecto es prolongado en el tiempo desencadena efectos perjudiciales a nivel del organismo promoviendo el envejecimiento y la formación de tumores.

Preface

Cellular senescence is a form of proliferative arrest that occurs in response to stress such as aging, oncogenic stimulation, DNA damage and chemotherapy. Even though senescent cells are arrested in proliferation they remain metabolically active and can induce changes in gene expression and the microenvironment through the secretion of various factors termed the senescence-associated secretory phenotype (SASP). The role of the SASP is one of the main controversies in the field being able to both promote and suppress growth. Previous work in our laboratory had identified beneficial roles for senescence during tissue development in the embryo. Additionally, it noticed that during oncogene-induced senescence (OIS) there is an unexpected increase in stem cell gene expression. We thus decided to further investigate the significance and role of this senescence-associated stemness, and whether cellular senescence might play an instructive and enhancing role in tissue patterning and regeneration. Interestingly, this increase in stemness can also be induced in normal cells upon exposure to the SASP, suggesting that secreted factors by the senescent cells induce stem cell gene expression. Furthermore, we could demonstrate during *in vitro* and *in vivo* studies that cells exposed to the SASP for a short-term exhibit increased regenerative capacity, whereas long-term exposure to the SASP induces senescence arrest. These confounding effects, short-term stemness and long-term proliferative arrest during the course of senescence, may reconcile the regenerative and tumor suppressive activities of senescence and

thus resolve previous paradoxes surrounding senescence and the SASP.

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Introduction

1. Cellular Senescence

More than 50 years ago, Hayflick and Moorehead (1961) first described that cells in culture are not able to replicate indefinitely. Observations from culturing human embryonic fibroblasts and fibroblasts derived from adults showed that fibroblasts from adults ceased replicating before embryonic ones, demonstrating that cells had an intrinsically limited replication capacity. This permanent growth arrest was termed cellular senescence and was proposed to be related to organismal aging (Hayflick, 1965). Later, Harley et al. (Harley et al., 1990) discovered that DNA damage signals triggered by eroded telomeres that progressively shorten every time the cell divides, induced an irreversible cell cycle arrest. Further studies showed that senescence can be induced in normal as well as in cancer cells by a variety of stresses, including DNA damage and oncogenic signals (Jones et al., 2000; Serrano et al., 1997). Therefore, cellular senescence has been mainly implicated during aging as well as a tumor suppressive mechanism that functions as a protective barrier against intrinsic and extrinsic insults such as chromosomal instability and oncogenic stimulation altering the normal cellular functionality, thus leading to a potential malignant transformation. However, recent studies showed that senescence not only functions to simply cease growth, but has also been implicated in different biological roles including tissue repair, wound healing and during embryonic development, suggesting that senescence has additional unknown and complex functions.

1.1 Triggers of cellular senescence

Cellular senescence can be induced by a variety of intrinsic and extrinsic stimuli, including the following:

a) Telomere shortening and replicative senescence

Replicative senescence is the irreversible cell cycle arrest that somatic cells undergo after a finite number of replications at the end of their lifespan and is caused by telomere erosion. Telomeres are repetitive DNA sequences acting as protective caps at the end of chromosomes. Due to the unidirectional nature of the replication machinery, DNA polymerases cannot prime a new DNA strand, and therefore chromosomal ends are never completely copied, resulting in progressive shortening of telomeres (Harley et al., 1990). However, cells that express telomerase, which is an enzyme that can restore telomeric DNA sequences, do not undergo telomere erosion. While many cells in mice express telomerase, in humans it is only expressed in embryonic stem cells, cancer cells, certain adult stem cells and a few somatic cells like T cells (Campisi, 2013). Ultimately, when telomeres become critically short or dysfunctional they become irreparable. This causes suppression of DNA repair mechanisms with recognition of DNA double strand breaks leading to a persistent DNA damage response (DDR). The DDR machinery arrests the cell cycle mainly through phosphorylation and activation of tumor suppressor p53, which induces the cyclin-dependent kinase inhibitor p21 (Choudhury et al., 2007; d'Adda di Fagagna et al., 2003).

b) Oncogene-induced senescence

Senescence can also be induced by the aberrant expression of an oncogene, termed oncogene-induced senescence (OIS) and is of particular interest as it can function as a brake during the onset of tumorigenesis *in vivo*.

OIS was first described in primary human and mouse cells, where a constitutively active oncogenic $HRas^{V12}$ was expressed, causing cell cycle arrest, accumulation of p53 and $p16^{INK4a}$ and a morphological phenotype similar to the one seen in replicative senescent cells (Lin et al., 1998; Serrano et al., 1997). High levels of $H\text{-}Ras^{V12}$ induced a short hyperproliferation, leading to DNA replication errors and formation of double strand breaks, followed by activation of the DDR machinery, accumulation of p53 and the cyclin kinase inhibitor $p16^{INK4a}$ and subsequently cell cycle arrest. So far, up to 50 oncogenes have been discovered that can induce a senescence response (Gorgoulis and Halazonetis, 2010). Of note is the oncogene BRAF, which induces senescence in a DDR-independent manner, through a metabolic mechanism involving upregulation of mitochondrial pyruvate dehydrogenase (PDH) (Kaplon et al., 2013).

Together these findings suggest that OIS can act as a potent tumor-suppressive mechanism that prevents proliferation of cells prone to malignant transformation. Indeed, numerous *in vivo* studies discovered senescent cells in premalignant or benign tissues, but an absence in malignant tumors (Braig et al., 2005; Chen et al., 2005; Collado et al., 2005; Lazzerini Denchi et al., 2005; Michaloglou et al., 2005). For example, at the onset of lung and pancreatic

tumorigenesis, endogenous oncogenic Kras ($Kras^{G12V}$) was discovered to induce senescence (Collado et al., 2005). Furthermore, markers of cellular senescence were also found in E2F3-related hyperplasia of the pituitary gland (Lazzerini Denchi et al., 2005), in AKT-PTEN-associated prostatic intraepithelial neoplasia (Chen et al., 2005) and pre-malignant naevi (moles) that were associated with BRAF mutation (Michaloglou et al., 2005). The presence of senescent cells in premalignant tumors, but not in their correspondent malignant stages strongly indicates that OIS acts as an important tumor suppressive mechanism, which is discussed in more detail in a later section.

c) Loss of tumor suppressors

Additionally, loss of various tumor suppressors, like PTEN, RB (Retinoblastoma), NF1 (Neurofibronin) and VHL (von Hippel-Lindau disease tumor suppressor) trigger a senescence response. With the exception of RB inactivation, which induces senescence in a DDR-dependent manner, loss of PTEN, NF1 and VHL expression induces senescence without a DDR, but through activation of $p19^{ARF}$ and $p16^{INK4a}$ (van Deursen, 2014).

Evidence that loss of tumor suppressors induces senescence has been also shown *in vivo*. In the case of prostate intraepithelial neoplasia (PIN) resulting from the loss of PTEN, an increase of senescence markers has been discovered (Chen et al., 2005). Furthermore, neurofibromas, which are neoplastic lesions induced by hyperactivated Ras signaling due to an absence of NF1, were

associated with an increase of senescence as well (Courtois-Cox et al., 2006).

d) DNA damage and stress-induced senescence

As already mentioned above, replicative and oncogene-induced senescence results in a DDR and ultimately to senescence. Additionally, senescence can also be induced by other stressors that induce DNA damage including oxidative stress, ionizing radiation and chemotherapeutic drugs. For example, the drug etoposide, which inhibits topoisomerase II, induces DNA single-strand and double-strand breaks close to replication forks. Thereby, etoposide creates replication stress and DNA damage leading to cellular senescence (Marusyk et al., 2007; Robles et al., 1999).

Another form of stress-induced senescence is due to *in vitro* culture stress. This type of senescence induction is independent of telomere length. The senescence arrest is triggered by inadequate culture conditions such as suboptimal concentrations of growth factors and nutrients, oxidative stress due to culture in atmospheric O_2 as well as the absence of surrounding cells types and extracellular matrix components (Kuilman et al., 2010).

e) Tumor suppressor activation

Two major tumor suppressor pathways, the p53/ p21 and $p16^{INK4a}$/ pRB tumor suppressor networks, play crucial roles in the proliferation arrest and in the irreversibility of the senescent state (Narita et al., 2003; Serrano et al., 1997). Depending on the stimuli

that induce the senescence arrest either one or both of the p53/ p21 and $p16^{INK4a}$/ pRB pathways are involved in the establishment and/ or maintenance of the senescence response. Therefore, it is not surprising that overexpression or chronic activation of p53, p21, $p16^{INK4a}$ or pRB is enough to induce cellular senescence (Campisi, 2013). Furthermore, $p16^{INK4a}$ expression shows a gradual increase in cells and tissues of various organisms during aging (Herbig et al., 2006; Krishnamurthy et al., 2004; Liu et al., 2009; Ressler et al., 2006).

f) iPS reprogramming

During reprogramming of somatic cells into induced pluripotent stem cells (iPS) cellular senescence, mediated by p53 and p16 was also observed, which significantly limited the reprogramming efficiency (Banito et al., 2009). Upon overexpression of the reprogramming factors Oct4, Sox2, Klf4 and c-Myc, several studies noticed a DDR and senescence arrest, which might be caused by aberrant DNA replication and also by generation of reactive oxygen species (ROS) (Banito et al., 2009; Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009; Marión et al., 2009; Utikal et al., 2009). Multiple cells during this process resembled senescent cells as they displayed an enlarged cytoplasm and an increase of senescence-associated-β-Galactosidase activity (SA-β-Gal) and senescence-associated heterochromatin foci (SAHF), in addition to an upregulation of p53, p19, $p16^{INK4a}$ (Banito et al., 2009; Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009; Marión et al., 2009; Utikal et al., 2009). Indeed, inactivation of the cell-intrinsic

senescence tumor-suppressors led to increased reprogramming efficiency, although with greater genomic instability. Overall, these results are suggesting that cellular senescence is an intrinsic block to cellular reprogramming and is induced by reprogramming into iPS cells.

g) Epigenomic inducers

Epigenomic perturbations, in particular global relaxation of chromatin, can result in cellular senescence as well. Interestingly, these changes in chromatin organization can in some instances induce a senescence response without physical DNA damage. For example, histone deacetylase inhibitors induce chromatin relaxation, leading to reduced repression of $p16^{INK4a}$ and formation of senescence-associated heterochromatin (Munro et al., 2004). Furthermore, perturbations in chromatin organization can also be induced by suboptimal c-Myc or p300 histone acetyltransferase activity, which then also elicits an increase in $p16^{INK4a}$ expression (Bandyopadhyay et al., 2002; Campisi, 2013; Guney et al., 2006).

1.2 Characteristics of senescent cells

Even though different triggers can induce senescence, senescent cells have common characteristics that allow their identification. However, so far, no single marker for the senescent state has been described and not all senescent cells share the same characteristics. Therefore, senescent cells can only be identified by a combination of multiple characteristics, which include the following:

a) Growth arrest

Senescent cells exit from the cell cycle and unlike quiescent cells they are not able to respond to mitogenic signals such as serum or growth factors (Narita, 2007). Therefore, an absence of proliferation markers is used as one of the primary criteria to identify senescent cells. By definition also, the growth arrest is permanent, and has yet to be shown to be reversible in standard assays. However, some studies suggest genetic manipulation such as through depletion of p53 or p16 in senescent cells may affect this irreversibility (Beauséjour et al., 2003; Coppé et al., 2008; Dirac and Bernards, 2003; Kuilman et al., 2008).

b) Senescence-associated β-galactosidase (SA-β-Gal) activity

Senescent cells show an increase in lysosomal β-D-Galactosidase activity, which is thought to occur due to an increase in the lysosomal mass (Dimri et al., 1995). β-Galactosidase activity is detectable in senescent but not in proliferating, quiescent or terminally differentiated cells at the suboptimal pH 5.5-6.0 due to its overexpression (Lee et al., 2006). However, senescence can still occur in the absence of beta-galactosidase and SA-β-gal (Lee et al, 2006).

c) Senescence-associated secretory phenotype (SASP)

Even though senescent cells are arrested in proliferation, they stay metabolically active and secret a large number of chemokines,

cytokines, growth factors and extracellular matrix factors and remodelers (Coppé et al., 2008). Through the SASP, senescent cells are able to induce changes in gene expression and the tissue microenvironment, which can be either detrimental or beneficial. Furthermore, the SASP is a plastic phenotype and its composition differs among cell types as well depending on the stimulus that induced the senescent state. The SASP is discussed in more detail in the following section.

d) Senescence-associated heterochromatin foci (SAHF)

Senescent cells also undergo dramatic chromatin rearrangements, which are known as senescence-associated heterochromatin foci (SAHF). SAHFs are defined as facultative heterochromatin regions at several loci that contain certain pro-proliferative genes and presumably silence them. These foci are associated with trimethylation of lysine 9 of histone 3 (H3K9me3), heterochromatin protein 1 (HP1) and macroH2A, which are heterochromatin markers (Narita et al., 2006; Zhang et al., 2005). The reorganization of the chromatin in specific domains in senescent cells which leads to the formation of the SAHF is mediated by two chromatin regulators, namely histone repressor A (HIRA) and anti-silencing function 1a (ASF1a). Furthermore it has been shown that single chromosomes condense into a single SAHF (Zhang et al., 2005, 2007). However, SAHFs are not found in all types of senescence, and occur primarily in OIS, and not replicative or DNA-damage-induced types (Di Micco et al., 2011).

e) Expression of tumor suppressors and cell cycle inhibitors

Senescent cells also exhibit an increase in two crucial tumor suppressors, $p16^{INK4a}$ and $p19^{ARF}$ ($p14^{ARF}$ in human), which are encoded by the CDKN2A locus. Whereas $p16^{INK4a}$ is an inhibitor of CDK4 and CDK6 and prevents the inhibitory phosphorylation of RB, $p19^{ARF}$ functions by activating the transcription factor p53 (Lowe and Sherr, 2003). Furthermore, other downstream effectors are used for senescence detection, including p21 (Cdkn1a), p57 (Cdkn1c) and Promyelocytic Leukaemia (PML) (Lowe et al., 2004; Tsugu et al., 2000).

However, after SA-β-Gal expression, $p16^{INK4a}$ is so far, the second most commonly used marker for detection of senescent cells *in vitro* and especially *in vivo.* Whereas in most healthy cells and tissues of young samples, $p16^{INK4a}$ expression is very low or even undetectable, its expression is highly increased in cells and tissues upon certain stresses, such as tumorigenesis, wounding or aging. In fact, $p16^{INK4a}$ expression gradually increases upon aging of multiple vertebrate tissues (Sharpless and Sherr, 2015).

f) Enlarged nucleus and cellular size

Senescent cells also undergo morphological changes *in vitro*, such as a flat and enlarged phenotype. Additionally they become multinucleated and show intensive vacuolization (Hayflick, 1965; Serrano et al., 1997). However, *in vivo*, due to the restriction by

tissue architecture, senescent cells maintain normal morphology (Muñoz-Espín and Serrano, 2014).

g) DNA damage markers

As mentioned before, many senescence inducers cause genomic damage, resulting in persistent DNA damage foci and activation of the DDR machinery. These DNA damage foci can be identified by many markers of the DDR pathway, including γH2AX or p53-binding protein 1 (53BP1) (Bartkova et al., 2006; Di Micco et al., 2006; Sharpless and Sherr, 2015).

h) Lamin associated changes

Lamin proteins are components of the nuclear lamina, a fibrous network on the inside of the nucleoplasmic side of the nuclear membrane. Of these, Lamin B1, which connects the nuclear envelope to the chromatin, also plays a role during the senescence process. Lamin B1 levels are downregulated during senescence and therefore, an absence of lamin B1 serves as a good marker for senescence detection (Freund et al., 2012; Shimi et al., 2011). Additionally, lamin B1 levels contribute to global and local modifications in chromatin methylation, which are influencing gene expression and thereby strengthening the senescence response (Sadaie et al., 2013; Shah et al., 2013).

i) HMGB1 secretion

High mobility group box (HMGB) 1 is a non-histone protein that binds to DNA and changes its chromatin state to enable

transcription factors access to promoter regions (Grosschedl et al., 1994). Additionally, HMGB1 also functions as an alarmin which is a secreted factor released from cells upon damage or stress. Thus, HMGB1 is secreted upon stress to trigger an innate immune response. In particular, it has been shown that HMGB1 secretion precedes the induction of the SASP in cells undergoing senescence. Therefore, loss of HMGB1 serves as another marker for the detection of senescent cells (Davalos et al., 2013).

1.3 The senescence-associated secretory phenotype (SASP)

The fact that senescent cells continue to produce and secrete a rich cocktail of proteins into the environment has become one of the most interesting areas of senescence biology. Microarray analysis in 1999 of human skin fibroblasts undergoing replicative senescence showed that these cells exhibited a strong inflammatory response similar to that seen in wound healing (Shelton et al., 1999). Further studies demonstrated that senescence leads to a coordinated secretion of specific proteins, which occurs in different cell types following different senescence inducing stimuli (Coppé et al., 2006; Krtolica et al., 2001; Kuilman et al., 2008; Rodier et al., 2009), a feature that became known as the SASP. However, it has been shown that the SASP from Ras-induced senescent cells is markedly amplified compared to irradiation- or replication-induced senescent cells. Furthermore, the SASP from Ras-induced senescent cells already developed 2-4 days after oncogene expression, whereas

irradiation induced a SASP 4-7 days after the dose (Coppé et al., 2008).

Interestingly, many of the SASP aspects are conserved between human and mouse. The SASP consists of pro-inflammatory cytokines, including IL1-α, IL1-β, IL-6, IL-8 and TGF-β, inflammation-associated chemokines such as CXCL-1/-2/-3/-5/-7 and CCL2, but also growth factors like GRO-α, HGF and IGFBPs, cell surface molecules such as ICAMs (Coppé et al., 2008), as well as matrix remodeling factors (Coppé et al., 2010; Liu and Hornsby, 2007; Parrinello et al., 2005). Due to the great variety of the SASP components, cellular senescence is involved in a number of biological processes in a paracrine manner.

1.3.1 The roles of the SASP

In the past, cellular senescence has been mainly associated with tumor suppression and aging, mediated through cell-intrinsic cell cycle inhibition and arrest. However, through the secretion of the SASP, senescent cells can have multiple, sometimes paradoxical effects, promoting proliferation, invasion or paracrine senescence in neighboring cells, as well as instructing roles during wound healing, tissue repair and embryonic development.

It is thought that some functions of the SASP are to help prevent the proliferation of stressed or damaged cells, and to enable them to signal the neighboring cells about their compromised state and

prepare the surrounding tissue for repair. Simultaneously, another function of the SASP is to ensure that such stressed or damaged cells are cleared by the immune system. For instance, through secretion of SASP factors like WNT16B, IL-6, IL-8 and Gro-1, senescent cells reinforce their own growth arrest (Acosta et al., 2008; Kuilman et al., 2008) as well as cell cycle arrest in neighboring cells in a paracrine manner (Acosta et al., 2013). Furthermore, through secretion of the SASP, senescent cells can attract immune cells to ensure their own cell death and eventual clearance (Kang et al., 2011; Krizhanovsky et al., 2008; Xue et al., 2007). These features of the SASP help to prevent the continued proliferation and survival of damaged cells.

However, on the other hand, the SASP can stimulate proliferation in neighboring stromal or epithelial cells, induce angiogenesis and epithelial to mesenchymal transition (EMT), as well as disrupt the local tissue environment through the production of matrix-remodeling factors (Coppé et al., 2006, 2008; Krtolica et al., 2001; Parrinello et al., 2005) (Figure 1). These effects could on the one hand be beneficial and play a role in tissue homeostasis and repair. However, if in excess, they could become detrimental and contribute to a pro-tumorigenic environment. Therefore, recent studies suggested clear difference between acute and chronic senescence. While acute senescence is thought to be short-lived and beneficial, chronic senescence might result through an accumulation of senescent cells over time, which could create a pro-tumorigenic effect, through changes in the tissue and chronic inflammation. This

may play a role in the development of aging and age-associated diseases as well as during cancer development (van Deursen, 2014).

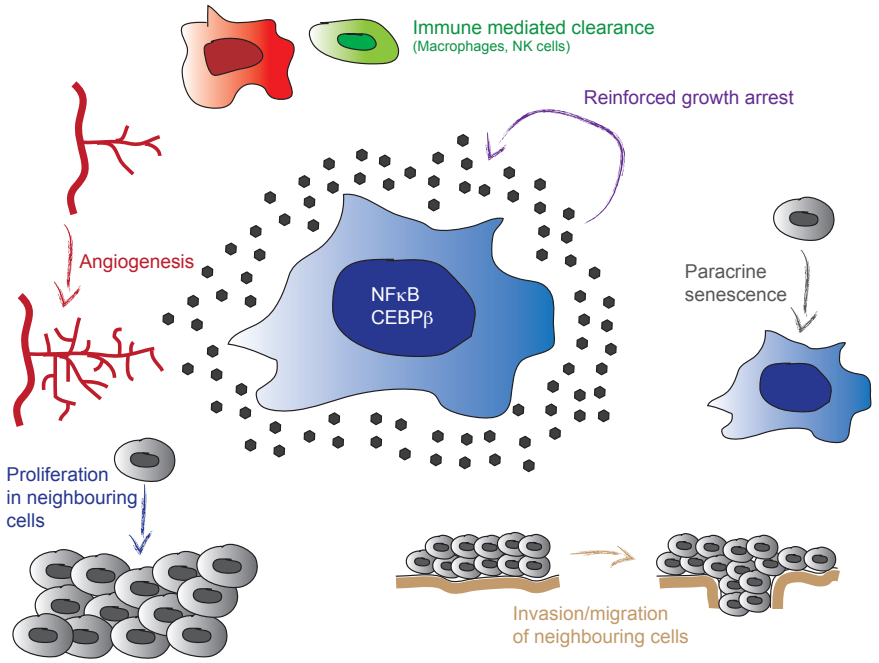

Figure 1. The multiple roles of the SASP (adapted from Freund et al., 2010).

1.3.2 SASP regulators

The majority of SASP components are positively regulated by the DDR proteins ATM, CHK2 and NBS1. These proteins are only able to induce a SASP after a persistent DNA damage response is established and thus a rapid activation of the DDR signaling after DNA damage is not sufficient to induce the SASP (Campisi, 2013). Additionally, it has been shown that the two transcription factors nuclear factor κB (NFκB) and CCAAT/ enhancer-binding protein-β

(CEBPβ) positively regulate the production of the SASP. Both transcription factors show increased expression in senescent cells and seem to be activated by genotoxic stress (Acosta et al., 2008; Kuilman et al., 2008). NFκB is one of the key regulators of the inflammatory response. Proteomic studies have shown that the NFκB subunit p65 accumulates on the chromatin of senescent cells and plays a major role in regulating the SASP by controlling the expression of numerous genes (Chien et al., 2011). Whereas depletion of NFκB in senescent cells substantially lowers the expression levels of GRO family members and several metalloproteinases, inhibition of CEBPβ significantly decreases two of the most upregulated SASP cytokines, namely IL-6 and IL-8 (Acosta et al., 2008; Chien et al., 2011; Coppé et al., 2011; Kuilman et al., 2008).
In contrast, the tumor suppressor p53 seems to restrain the SASP, as functional loss of the p53 protein causes a strong acceleration and amplification of the SASP (Coppé et al., 2008).

Recently, the transcription factor GATA4 has been identified to be required for SASP induction via direct activation of NFκB. In normal conditions, GATA4 is degraded by p62-mediated selective autophagy. However, upon senescence induction the DDR regulators ATM and ATR stabilize GATA4, inducing NFκB to initiate the SASP (Kang et al., 2015).
The mitogen-activated protein kinase p38 (p38MAPK) plays also a role in regulating the SASP, in a DDR-independent manner. Constitutive active p38MAPK is able to induce SASP mainly by

enhancing NFκB transcriptional activity, even without any senescence-inducing stimuli. Furthermore, inhibition of the p38MAPK reduces the secretion of some SASP components (Freund et al., 2011).

Recent studies also showed that the SASP can be regulated via epigenetic mechanisms. During senescence, the high mobility group box 2 (HMGB2) chromatin-binding protein re-localizes to SASP genes, promoting SASP gene expression, while knockdown of HMGB2 induces changes in heterochromatin marks at SASP gene loci, which resulted that these regions are incorporated into the SAHF and become silenced (Aird et al., 2016). Another study showed, that the expression of the oncoprotein and transcriptional activator MLL1 is associated with DDR activation during senescence. Depletion of MLL1 inhibited DDR-induced SASP activation by repressing pro-proliferative cell cycle regulators that are necessary for DDR activation (Capell et al., 2016). Additionally, the histone variant MacroH2A1 also undergoes re-localization on the chromatin during senescence, as it then is removed from SASP genes, which allows their expression (Chen et al., 2015b).

1.4 Senescence and its dual roles in cancer

Upon oncogenic mutation or non-reparable damage of a cell, mammalian cells can either undergo apoptosis or senescence. Whereas apoptosis leads to the fast removal of damaged or dysfunctional cells by death and phagocytes without inducing an

inflammation response, senescence induction not only prevents the dysfunctional cell from proliferating, but the senescent cell also influences the surrounding microenvironment, through the SASP. This could on the one hand be beneficial and tumor suppressive by inducing senescence in an autocrine/ paracrine manner, and through recruiting immune cells for its own clearance as well as the clearance of neighboring tumor cells. Conversely, however, the senescent cell can also create a pro-tumorigenic environment through secretion of the SASP. Therefore, cellular senescence can have roles in both tumor suppression and tumor promotion.

1.4.1 Suppressor

As already discussed before, senescence is mainly seen as a potent tumor suppressor mechanism that prevents proliferation of cells prone to neoplastic transformation (Figure 2). However, mutations that impair the senescence response, such as loss of p53 or $p16^{INK4a}$ could facilitate the transformation from pre-malignant to a malignant tumor (Narita and Lowe, 2005).

A study in which an oncogenic $Nras^{G12V}$ was stably induced into single hepatocytes in the mouse liver demonstrated that the senescence program is not only important for a cell intrinsic tumor suppression, but also for the induction of immune cell clearance of pre-cancerous cells (Kang et al., 2011). This study showed that senescent $Nras^{G12V}$ hepatocytes attract different immune cell

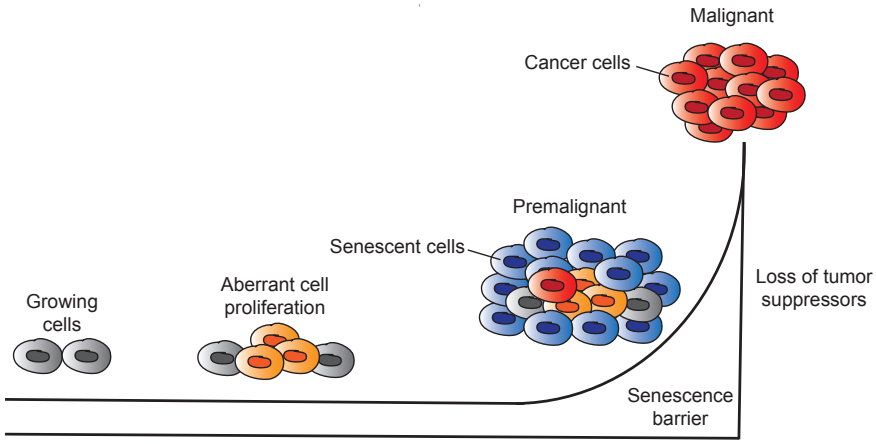

Figure 2. Senescence as a tumor suppressive mechanism. Abnormal activation of an oncogene can lead to aberrant cell proliferation, which eventually induces activation of a cellular senescence response. However, secondary mutations disabling cellular senescence are resulting in tumor progression (adapted from Narita and Lowe, 2005).

populations such as neutrophils, monocytes, natural killer cells, $CD4^+$ T cells and macrophages, which eliminated the senescent cells over time. Failure of this SASP-mediated immune clearance of pre-malignant senescent cells resulted in the development of liver carcinoma. Thus, in addition of suppressing tumorigenesis intrinsically by inducing cell cycle arrest in pre-cancerous cells, the senescence response is also essential to prevent cancer progression in a paracrine manner by stimulating immune cells to eliminate oncogene-expressing pre-malignant and malignant cells. This was further supported by a study in Scott Lowe's lab, where removal of senescence cells upon chronic liver damage resulted in increased liver fibrosis and cirrhosis accompanied with lower survival rate and higher incidence of hepatocellular carcinoma development. They could demonstrate that the SASP activates tumor-suppressive macrophages that are crucial to reinforce the cell cycle arrest and

removal of senescent cells, limiting transformation of normal cells and tumor formation (Lujambio et al., 2013).

Additionally, it was shown that tumor cells can be induced to senesce upon treatment with chemotherapeutic drugs or radiation, leading to tumor regression (Coppé et al., 2010; Roninson, 2003; Schmitt et al., 2002). Chemotherapeutic treatments can cause either apoptosis or senescence. In the case of chemotherapy-induced senescence, it was shown that mouse models of MYC-initiated lymphomas respond to the chemotherapeutic drug cyclophosphamide by inducing tumor cell senescence, which was mediated by $p16^{INK4a}$ and p53 (Schmitt et al., 2002). *In vitro* studies of human cancer cells showed that moderate doses of classic chemotherapy drugs induce senescence, while higher doses induce apoptosis (Roninson, 2003). Furthermore, analysis of biopsies from patients with lung, breast or prostate cancer after chemotherapy showed an increase in senescence markers, which was further associated with successful treatment, highlighting the tumor suppressive role of senescence *in vivo* (Coppé et al., 2008; te Poele et al., 2002; Roberson et al., 2005).

1.4.2 Promoter

Due to the paracrine effects of the SASP, senescent cells are also implicated during tumor development. For instance, xenograft studies showed that co-injection of senescent cells together with premalignant or fully malignant cells accelerated tumor development by stimulating tumor cell proliferation, invasion and

angiogenesis (Krtolica et al., 2001; Liu and Hornsby, 2007). Additionally, SASP components like IL-6 and IL-8 may also stimulate epithelial-to-mesenchymal transition in pre-malignant epithelial cells enabling invasion and migration through tissues, thus promoting metastatic properties of cancer (Coppé et al., 2008; Laberge et al., 2012; Parrinello et al., 2005).

Furthermore, it has been shown that cells induced to senesce in response to chemotherapeutic agents or radiation, secrete SASP factors that prevent surrounding tumor cells from being eliminated by the same chemotherapeutic treatment (Gilbert and Hemann, 2010; Sun et al., 2012). Additionally, chemotherapy-induced senescent cells may persist and become a source of chronic inflammation, leading to or enhancing many side effects of chemotherapy (Demaria et al., 2017; Ewald et al., 2010). Importantly, a recent study demonstrated that chemotherapy-induced senescence in non-tumor cells was also responsible for cancer relapse and metastasis. Elimination of these senescent non-tumor cells prevented or slowed down cancer relapse and the spread to distal tissue (Demaria et al., 2017).

1.5 Senescence and aging

From the beginning senescence had been associated with aging. This is already implied in the name, as the word senescence means “being old” or “the process of becoming old”. Aging is characterized by a gradual loss of function or degeneration. Several

studies showed that senescent cells not only accumulate with aging *in vitro*, but also in various tissues of aged human and aged mice (Baker et al., 2016; Dimri et al., 1995). Additionally, an increase of senescent cells has also been described at sites of multiple age-related diseases such as osteoporosis, neurodegenerative disorders, cardiovascular disease and arthritis (Campisi, 2013; Naylor et al., 2013). This accumulation of senescent cells may be due to an increase of DNA damage and/ or senescence-inducing stimuli. For instance, unrepaired genetic lesions in self-renewing cells are passed on to their progeny cells and can therefore accumulate over time. Mutations that result in growth or survival advantages, might lead to selection for these mutant stem cell clones and possible cancer formation. Thus, tumor suppressive mechanisms such as apoptosis or senescence prevent proliferation of damaged cells that have malignant potential. While on the one hand this could be beneficial in counteracting cancer development, on the other hand this tumor suppressive mechanism may play a role in aging by causing arrest or attrition of stem cells, or through the accumulation of non-proliferative cells in the aged tissue (Sharpless and DePinho, 2007). Therefore, senescence may be an example of antagonistic pleiotropy, in the meaning that it is beneficial early in life by preventing tumor formation, yet detrimental later in life by driving aging and age-related disorders. This theory is further supported by studies showing that loss of key senescence mediators like $p16^{INK4a}$ and p53 in mice can cause death from cancer at an early age (Sherr, 2000). In addition, studies in $p16^{INK4a}$ deficient and $p16^{INK4a}$ overexpressing hematopoietic stem cells, neural stem cells,

pancreatic islet cells and muscle stem cells demonstrated an association of gradual increasing $p16^{INK4a}$ levels and age-induced functional decline of these tissues (García-Prat et al., 2016; Janzen et al., 2006; Krishnamurthy et al., 2006; Sousa-Victor et al., 2014).

Interestingly, the reason senescent cells accumulate during aging is not known. It is also possible that the accumulation of senescent cells during aging is due to an age-related impairment in immune clearance (Burton, 2009; Hoenicke and Zender, 2012). The importance of clearance of senescent cells by immune cells has been demonstrated in a model of liver fibrosis and liver carcinoma and will be discussed later (Krizhanovsky et al., 2008; Xue et al., 2007). Even though senescent cells, through the SASP, still signal for their own removal, it is suggested that due to age-related immune impairment, senescent cells cannot be removed. Functional studies of hematopoietic stem cells, showed their functional decline with aging, resulting in defective lymphopoiesis and increased mylopoiesis, leading to impaired immune function (Wang et al., 2011).

Studies from the van Deursen lab further supported the beneficial effects of elimination of senescent cells during aging. They demonstrated that $p16^{INK4a}$-mediated senescence plays a key role as a driver of tissue and organismal aging, by using a unique mouse model in which they could eliminate p16-positive cells. Their mouse model consists of a construct that combines a $p16^{INK4a}$ promoter with an inducer of apoptosis, thus allowing the selective

elimination of $p16^{INK4a}$-positive cells in progeroid mice as well as during physiological aging. Clearance of $p16^{INK4a}$-positive cells increased the lifespan and health of the animals by delaying tumorigenesis and age-dependent functional decline in certain tissues such as heart and kidney (Baker et al., 2011, 2016). These studies suggest that $p16^{INK4a}$-mediated senescence acts as a block to regeneration during aging. Thus, it is not surprising that senescence is an intrinsic barrier to reprogramming. Various studies showed that knockdown of any of the main senescence effectors p53, p21 and $p16^{INK4a}$ significantly improved reprogramming rates and efficiency (Banito et al., 2009; Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009; Marión et al., 2009; Utikal et al., 2009). Interestingly, the decreased reprogramming efficiency from aged fibroblasts can be prevented by knockdown of $p16^{INK4a}$ (Li et al., 2009).

1.5.1 SASP and aging

So far it is not clear how senescence can drive age-related tissue dysfunction. It is possible that senescent cells contribute to the decline of the regenerative capacity that occurs during aging by changing the tissue environment through secretion of the SASP (van Deursen, 2014). In fact, by implanting cells from old mice into young mice, systemic factors from the young mice were able to reset the proliferation and regenerative capacities of aged muscle stem cells (Conboy and Rando, 2005). Also, previous work from our lab has shown that SASP-like inflammation from aged

keratinocytes can exert an inhibitory effect on tissue stem cells. Furthermore, treatment with drug inhibitors allowed stem cell activation (Doles et al., 2012) suggesting that the SASP can block stem cell function.

Additionally, the SASP consists of multiple potent inflammatory cytokines and chemokines, which may accumulate during aging or at sites of age-related disorders and thereby promote or accelerate chronic inflammation (Acosta et al., 2013; Nelson et al., 2012). Furthermore, via SASP secretion, senescent cells are also able to transmit the senescent phenotype in neighboring healthy cells (Acosta et al., 2013). Thus, paracrine senescence may intensify age-related tissue dysfunction.

1.6 Beneficial senescence

Cellular senescence is not only implicated to play a role during aging and as a tumor suppressor, but also partakes in other beneficial tasks during embryonic development, tissue repair and wound healing specifically due to the SASP (Demaria et al., 2014; Jun and Lau, 2010a; Krizhanovsky et al., 2008; Muñoz-Espín et al., 2013; Storer et al., 2013; Zhu et al., 2013).

1.6.1 Developmental senescence

The finding that senescence occurs under normal physiological conditions and plays an important role during embryonic

development was a surprising discovery that fueled several controversies in the field. This discovery is mainly based on studies in mouse embryos (Muñoz-Espín et al., 2013; Storer et al., 2013). However, developmental senescence has also been described in human (Muñoz-Espín et al., 2013), chicken (Storer et al., 2013), quail (Nacher et al., 2006) and amphibian (Davaapil et al., 2017; Villiard et al., 2017) embryos.

During mouse embryonic development, senescent cells were detected at multiple sites throughout the later stages of embryonic development such as in the endolymphatic sac of the inner ear, the embryonic kidney, the closing neural tube, the apical ectodermal ridge (AER) and the regressing interdigital webs of the limbs (Muñoz-Espín et al., 2013; Storer et al., 2013). These structures were identified as senescent as they were positive for SA-β-Gal, showed an increase in heterochromatin markers, absence of proliferation markers and an increase in the expression of the cell cycle inhibitors p21 and p15. However, developmental senescence seems to occur via a different mechanism, as there was an absence of DNA damage markers, telomere dysfunction and $p16^{INK4a}$, $p19^{ARF}$ and p53 expression. Thus, there might be a difference between developmental senescence, which is mainly dependent on p21, and senescence in non-embryonic tissues, which primarily depends on p53 and $p16^{INK4a}$. Interestingly though, analysis of tissues containing developmental senescent cells revealed a similar transcriptional profile to that of oncogene-induced senescence cells that contain multiple similar SASP factors (Storer et al., 2013). It is

thought that through secretion of the SASP, senescent cells influence proliferation of the adjacent cells and tissue patterning. Ultimately, the SASP signals enable recruitment of immune cells for senescent cell removal, highlighting that in a timely controlled manner, senescence may have a beneficial and instructive role (Muñoz-Espín et al., 2013; Storer et al., 2013).

1.6.2 Senescence during wound healing and tissue repair

Before the discovery that senescent cells play a role in instructing growth, patterning and tissue remodeling during embryonic development, senescence had been shown to be important during tissue repair and wound healing. Wound healing consists of four tightly regulated, but overlapping phases; namely inflammation, extracellular matrix (ECM) deposition, tissue formation and remodeling. Upon chronic injury and inflammation, excessive, non-functional ECM replaces parenchyma, leading to fibrosis, scarring and loss of tissue function (Jun and Lau, 2010b). For example, during liver damage, hepatic stellate cells (HSC) become activated, enter cell cycle and produce ECM proteins to stimulate hepatocyte proliferation and liver repair. Upon chronic or strong inflammation, HSC induce high amounts of ECM production, leading to fibrosis and eventually cirrhosis (Bataller and Brenner, 2005). One study induced liver fibrosis by chronic treatment with the liver damaging agent carbon tetrachloride (CCl4) leading to an induction of senescence in HSC (Krizhanovsky et al., 2008). The senescent HSC

decreased the proliferation rate of HSC and reduced ECM production, thus preventing the accumulation of fibrotic tissue. Additionally, the secreted SASP factors induced degradation of ECM components and eventually signaled natural killer cells to remove the senescent cells, thereby facilitating fibrosis resolution. Furthermore, mice that are deficient of senescence regulators such as p53 or $p16^{INK4a}$ showed both a strong increase in proliferating HSC and enhanced fibrosis (Krizhanovsky et al., 2008). During liver injury, damaged hepatocytes produce the ECM protein CCN1, which is a crucial mediator in inducing the senescence response in HSC through the accumulation of ROS (Kim et al., 2013). In human patient samples, an accumulation of SA-β-Gal positive cells can be detected in proximity to fibrotic scars (Wiemann et al., 2002). Also, CCN1 protein levels are highly elevated in human cirrhotic livers (Kim et al., 2013), suggesting that senescence through the SASP plays an important role during liver repair in humans.

During wound healing in the skin, a similar mechanism of fibrosis is necessary for proper tissue repair. CCN1 is also crucial to induce senescence in fibroblasts at later stages of wound healing in skin. The senescent cells then cease proliferation and increase expression of matrix remodeling factors to induce an anti-fibrotic effect (Jun and Lau, 2010a). Furthermore, the secretion of the early SASP factor platelet-derived factor A (PDGF-A) induces optimal wound closure through promotion of myofibroblast differentiation (Demaria et al., 2015). Altogether, these results suggest that

senescence plays an important role in reducing the level of fibrosis and accelerating wound closure during acute tissue damage.

2. The Skin

The skin, while being the largest organ found in mammals, is a dynamic organ with a complex structure consisting of the dermis, epidermis and associated appendages including hair follicles, nails, sebaceous glands and sweat glands. The different layers are composed of various cell types. The epidermis is the outermost layer of the skin and mainly consists of keratinocytes. Beneath the epidermis, lies the dermis, consisting of mesenchymal dermal fibroblasts dispersed with sensory nerves, immune cells and blood and lymphatic vessels. Underlying this is the subcutaneous tissue, which is made of fat and connective tissue (Blanpain and Fuchs, 2006).

The epidermis is also organized into different layers. The innermost layer of the epidermis consists of a single basal layer of mitotically active cells that can be identified by the expression of keratin 5 and keratin 14 (Fuchs and Green, 1980). These cells adhere to the underlying basement membrane that separates the epidermis from the dermis. Upon commitment of basal keratinocytes to undergo a process of terminal differentiation, basal cells detach from the basement membrane and move upwards towards the skin surface. During this terminal differentiation process, cells lose their proliferative capacity and progress through different layers

expressing specific differentiation markers. In the spinous layer, cells express the early differentiation markers keratin 1 and keratin 10. Upon progression of the differentiation process, spinous cells migrate first into the granular layer, expressing late stage differentiation markers such as prolifilaggrin and loricin and then into cornified layers where cells are positive for filaggrin. Altogether these layers are known as suprabasal layers. This process of increasing differentiation ends in the production of dead cornified cells building the outermost layer of the epidermal surface (Figure 3) (Fuchs and Green, 1980; Koster and Roop, 2007).

The different layers of the skin form a protective barrier against external stresses such as dehydration, pathogenic infections, traumatic insults, UV radiation and temperature stresses. In addition to wound repair after external injury, the skin undergoes continued rounds of self-renewal throughout adult life. Therefore, stem cells residing in the epidermis, hair follicle and sebaceous glands play a crucial role in maintaining tissue homeostasis, regenerating hair and repairing epidermis after injury (Blanpain and Fuchs, 2006).

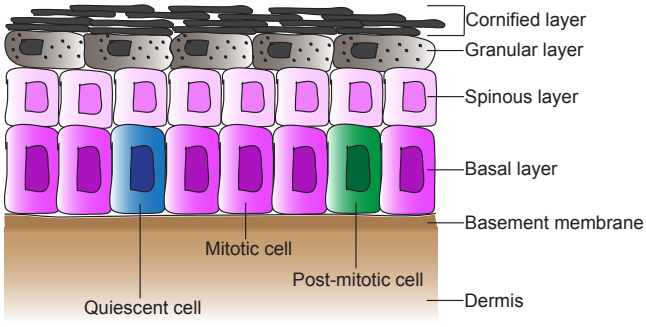

Figure 3. Structure of the mouse interfollicular epidermis (adapted from Solanas and Benitah, 2013).

2.1 Epidermal and hair follicle stem cells

The epidermis also consists of different regions and associated structures, including the interfollicular epidermis (IFE), hair follicles, sebaceous glands, lower isthmus and junctional zone. Each of these departments is maintained by different populations of epidermal stem cells (Figure 4).

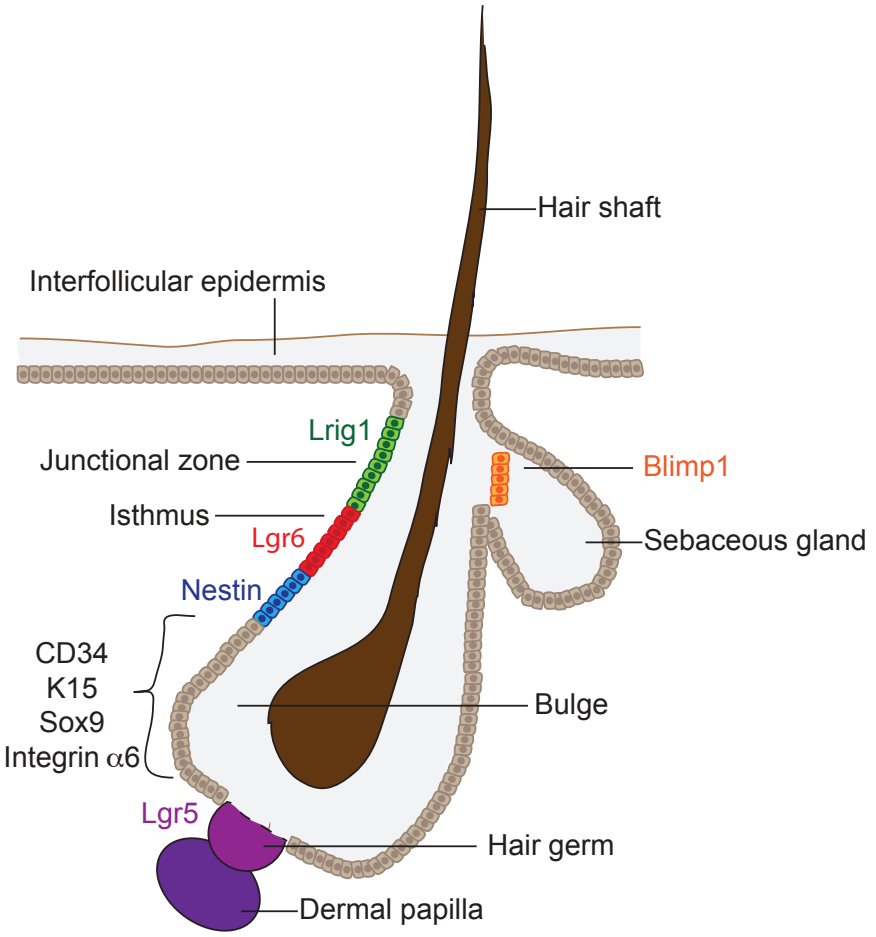

Figure 4. Schematic representation of the different stem cell pools in the mouse hair follicle (adapted from Solanas and Benitah, 2013).

However, so far, the best studied epidermal stem cell populations are found in the hair follicle, which contains a variety of spatially distributed hair follicle stem cell (HSFC) pools that can be identified by cell specific markers. Each of them contribute differently to hair follicle cycling. Perhaps the most studied HFSCs are found in the region of the hair follicle known as the bulge. Bulge stem cells can be identified by the expression of CD34, Keratin 15 (K15), Sox9 and Integrin α6 (Morris et al., 2004; Tumbar et al., 2004). Due to the non-homogenous characteristics of the bulge, the cells can be further divided into subpopulations of stem cells with different functions within the hair follicle. For instance, basal and suprabasal cells of the bulge are both positive for CD34, but differ in their Integrin α6 levels, which is high in basal cells and low in suprabasal cells (Blanpain et al., 2004). Bulge stem cells do not take part in normal tissue homeostasis of the epidermis, but are mainly involved in hair follicle growth.(Ito et al., 2005; Morris et al., 2004). However, upon wounding, these stem cells can contribute to additional structures, including the IFE, demonstrating that these cells possess a level of plasticity. Whereas CD34 expressing cells in the bulge are mainly quiescent, in the lower part of the bulge and hair germ reside more proliferative cells expressing Lgr5 and K15 (Jaks et al., 2008). Furthermore, a population of cells expressing Nestin, normally a marker for neural progenitor cells, is found in the upper part of the hair follicle bulge. Interestingly, this Nestin positive population is able to differentiate into multiple epidermal cell types as well as into neuronal, smooth muscle and adipose cell types (Li et al., 2003; Mignone et al.,

2007). Additionally, other stem cell populations and their specific markers have been characterized outside the bulge, including Blimp1 (Prdm1), which is expressed in the sebaceous gland (Horsley et al., 2006), Lgr6, which marks stem cells in the lower isthmus (Snippert et al., 2010) and Lrig1, marking stem cells in the junctional zone (Jensen et al., 2009) (Figure 4).

In order to characterize the stem-like properties of the various epidermal stem cells, different assays have been developed. Many epidermal stem cells are slow cycling cells and mainly quiescent. These slow cycling cells can be identified by DNA-labeled pulse chase experiments as they retain labeled nucleotides weeks after the first pulse (Kretzschmar and Watt, 2014). Additionally, clonogenic assays can be used to identify stem cells by their ability to form self-renewing colonies (Jensen et al., 2010). However, in order to assess the stem cells potential to give rise to all types of differentiated cells within a tissue, skin grafting assays are performed. In this assay, epidermal stem cells, along with dermal fibroblasts, are grafted into full-thickness wounds in the back of nude mice (Blanpain et al., 2004; Jensen et al., 2010). Depending on the population of epidermal stem cells, complete hair regeneration can be achieved. For example, CD34 positive hair follicle stem cells are able to regenerate epidermis with multiple hair follicles (Blanpain et al., 2004). Furthermore, primary mouse keratinocytes are used as a model system to study epidermal biology *in vitro*. By extracting cells from the epidermis of 1 to 2 day old mice, a population of keratinocytes derived from the proliferating basal

layer of the epidermis can be cultured. This culture contains then a mixture of stem and progenitor cells. These cells can also form hair follicles when grafted directly into full-thickness wounds. However, they have a limited proliferative lifespan due to culture stress, and lose the capacity to form hair follicles when cultured prior to grafting. Finally, primary keratinocytes can be used to model the molecular mechanisms of epithelial differentiation as high concentrations of $CaCl_2$ induce terminal differentiation and the cells then show patterns of differentiation marker expression similar to those seen in epidermal stratification *in vivo* (Lichti et al., 2008).

The different stem cell populations in the hair follicle are crucial in mediating the repetitive cycle of hair regeneration that the hair follicle undergoes during adult lifespan. The first hair follicle cycle occurs around 18 days after birth in mice. The hair follicle cycle consists of the following phases: degeneration (catagen), rest (telogen) and regeneration (anagen) (Blanpain and Fuchs, 2009). When hair growth stops, the follicle undergoes a destructive phase, where it degenerates the majority of the lower part of the follicle. The upper part, containing the bulge, stays intact with a pool of hair follicle stem cells that are necessary for the formation of the new hair follicle. After a short resting phase, where the bulge stem cells enter quiescence, they become activated again and induce matrix cell production and a new mature hair follicle is formed (Blanpain and Fuchs, 2009).

2.2 Papilloma

The importance of senescence as tumor suppressive mechanism can also be seen during papilloma formation, which is a benign tumor in the skin. The papilloma develops due to ectopic expression of oncogenic $KRas^{G12D}$ or $HRas^{V12}$ in skin stem cells, which induces aberrant proliferation of epithelial cells and ultimately triggering a senescence response. However, upon further mutations such as loss of p53, the benign papilloma progresses into invasive squamous cell carcinoma (SCC). Skin papillomas can be induced by treatment with 9,10-dimethyl-1,2-benzanthracene (DMBA) and 12-O-tetradecanoyl phorbol-13-acetate (TPA). Interestingly, in most DMBA/ TPA induced papillomas, a specific mutation of HRas was found, suggesting that the $HRas^{V12}$ mutation presents a selective advantage to epithelial cells (Quintanilla et al., 1986). In addition, these papillomas express several senescence markers and various studies showed that the SASP regulators CEBPβ, NFκB and p38MAPK, play an important role during papilloma formation. (Acosta et al., 2008; Budunova et al., 1999; Collado et al., 2005; Ewing et al., 2008; Kim and Pasparakis, 2014; Sun et al., 2007; Yamakoshi et al., 2009; Zhu et al., 2002).

2.2.1 DMBA/ TPA induced papilloma as an *in vivo* model for senescence

DMBA/ TPA induced papillomas are one of the most used *in vivo* skin models to study carcinogenesis in the mouse. First, the

mutagen DMBA is topically applied to the shaved dorsal skin of the mouse and initiates, at low doses, irreversible DNA damage with >90% of the cases generating a stable $HRas^{V12}$ mutation (Abel et al., 2009; Kemp, 2005). However, in order to then induce proliferation and tumor formation, the tumor promoter agent and inflammation inducing agent TPA is applied multiple times, which leads to clonal growths of the mutated cells and which manifest as outgrowth of the skin, known as papillomas. Interestingly, promotion of papillomas with TPA is possible up to one year after DMBA treatment, suggesting that the mutated cells are most likely cancer stem cells (Yuspa, 1998). It is thought that keratinocytes are the main cells carrying the mutation, which upon promotion increase their proliferative activity resulting in epidermal hyperplasia in the basal layer, skin thickening and ultimately benign tumor formation (Morris et al., 2004). Thus, a papilloma is described to have a stromal core surrounded by hyperplastic epidermis (Abel et al., 2009). In addition, multiple studies demonstrated that papilloma formation involves and is dependent on the aberrant accumulation of CD34 expressing stem cells demonstrated by the fact that DMBA/ TPA treatment in CD34 knockout mice does not lead to papilloma formation (Lapouge et al., 2012; Malanchi et al., 2008; Trempus et al., 2007).

Aim of the study

Aim of the study

Cellular senescence is implicated in having both beneficial and detrimental effects. On the one hand, it can act as a tumor suppressor mechanism, facilitate tissue repair and is important for fine-tuning embryogenesis. However, on the other hand, senescence can promote tumor formation, is implicated during organismal and tissue aging and can lead to tissue dysfunction. The aim of my thesis was to investigate whether cells undergoing oncogene-induced senescence also have beneficial effects on tissue growth and patterning as seen during embryonic development.

Results

The senescence-associated secretory phenotype induces cellular plasticity and tissue regeneration

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Discussion

3. Discussion

Cellular senescence has mainly been associated with tumor suppression and aging, mediated through both cell intrinsic cell-cycle inhibition and arrest, and cell-extrinsic communication through the SASP. However, through secretion of the SASP, senescent cells can also have paradoxical effects, promoting proliferation, invasion or paracrine senescence in neighboring cells. Additionally, emerging studies showed that cellular senescence is also implicated in complex biological processes such as embryonic development, tissue repair and wound healing. Our study helps to resolve previous paradoxes surrounding senescence and the SASP, demonstrating how senescence and the SASP is primarily a beneficial process that instructs stem cell function and regeneration, but when prolonged or in excess, can have detrimental effects resulting in paracrine senescence and decreased regenerative capacity.

3.1 Investigating oncogene-induced senescence in epithelial cells

As the majority of studies on cellular senescence have been performed on fibroblasts, we focused our investigation on oncogene-induced senescence in primary mouse keratinocytes. In this study, we discovered the paradoxical effect that cells that are undergoing senescence and losing their proliferative capacity increase the expression of genes that are associated with stem and cancer stem cells. Furthermore, we could show similar effects in

chemotherapy, irradiation and stress-induced senescence, suggesting that the increase of stem cell gene expression is not only linked to the oncogenic effects of Ras, but linked to the senescent state itself. However, the reasons why senescent cells might express stemness genes remained a question.

Depending on the degree and the kind of damage and the type of cell, a cell undergoes either apoptosis or senescence. In most cases, upon a stronger damage or overwhelming stress, a cell undergoes apoptosis, whereas senescence is caused by less severe damage (Childs et al., 2014). Thus, it is possible that the induced damage in our model is not high enough to induce apoptosis and at first seems reparable. Therefore, the cell might undergo a dedifferentiation process and increases its stemness in order to overcome the damage or stress. For example, in the hair follicle, stem cell markers like CD34, Lrig1, Nestin and Lgr6 identify separate populations of stem cells (Solanas and Benitah, 2013). However, we could show that the senescent induced CD34 positive cells also exhibited expression of Lrig1, Nestin and Lgr6. This result suggests that there is plasticity and an aberrant misexpression of stem cell markers in senescent cells. This would implicate that when a cell senses a damage it increases its stemness and becomes more plastic in order to then be able to respond to repair the damage like in a wound. However, the senescent program acts as a cell-intrinsic block in proliferation and likely then also prevents this highly plastic cell from proliferating further, as such a cell could be tumor initiating. Indeed, in various malignancies tumor cell plasticity can be observed (Varga et al.,

2014). Indeed, mutations in tumor suppressors and oncogenes can induce highly plastic phenotypes in cancer cells, which can dedifferentiate and acquire stem cell-like properties, promoting self-renewal and increasing the number of tumor-initiating cells (Varga et al., 2014). Our study further supports these findings, as we show that preventing cell cycle arrest in senescent cells by knockdown of p16 seems to not affect the increased stem cell gene expression in cells undergoing oncogene-induced senescence. As a result, these highly plastic cells have increased clonogenic capacities, implicating that cells bypassing senescence may be tumor-initiating cells. Indeed, another study demonstrated that cells that are escaping chemotherapy-induced senescence are highly clonogenic and exhibit an increase in cancer stem cell markers, highlighting the importance of senescence as a tumor suppressor (Achuthan et al., 2011).

3.2 Senescence induces plasticity in a paracrine manner

Our study further supports the growing work on the beneficial aspects of cellular senescence and in particular the SASP. Due to the instructive role of the SASP, it is likely favourable that upon damage or stress a cell enters senescence and does not undergo apoptosis. For example, apoptosis in the embryo is important to eliminate cells that are produced in abundance or that are no longer needed and is a cell intrinsic mechanism without any paracrine effects. A senescent cell on the other hand instructs the surrounding microenvironment through the secretion of the SASP. Thus, in the

case of developmental senescence or senescence during wound repair for instance, it is conceivable that the senescent cell does not get cleared away instantly, but that it stays viable and has an instructive paracrine role (Demaria et al., 2014; Jun and Lau, 2010a; Krizhanovsky et al., 2008; Muñoz-Espín et al., 2013; Storer et al., 2013). We could add to this data by showing that through the SASP, the senescent cell increases dedifferentiation and stem cell gene expression, stem cell functionality and regenerative capacity in a paracrine manner. Therefore, we think that when a cell exhibits a damage, the senescence response first prevents this damaged cell from proliferating and then through the SASP acts as a niche-like signaling center in order to maintain tissue homeostasis: It has been already shown that a senescent cell through secretion of ECM proteins and growth factors facilitates repair and growth of the surrounding tissue (Coppé et al., 2006; Demaria et al., 2014; Krizhanovsky et al., 2008). And, as we now describe, the SASP increases stemness and plasticity in the adjacent cells in order to replace the damaged cell and to promote tissue regeneration. Finally, the senescent cell then recruits immune cells for its own removal (Acosta et al., 2008; Kang et al., 2011; Kuilman et al., 2008; Xue et al., 2007).

The importance for a timed controlled clearance of senescent cells and therefore a limited SASP exposure has been demonstrated in various studies. First of all, senescence has been shown to promote tumor formation due to the promotion of cancer progression of pre-malignant cells by the SASP (Kang et al., 2011; Krtolica et al.,

2001; Liu and Hornsby, 2007; Rodier and Campisi, 2011; Yoshimoto et al., 2013). Second, during organismal and tissue aging, there is an increase of senescence as well as of SASP factors, such as IL-6 (Baker et al., 2011; Herbig et al., 2006; de Keizer, 2017; Krishnamurthy et al., 2004; Liu et al., 2009; Ressler et al., 2006). An accumulation of senescent cells, as seen during aging, can lead to a microenvironment that facilitates growth and progression of mutant cells which might then lead to tumor formation. Indeed, clearance of senescent cells during aging was shown to delay the onset of different types of cancer and to prolong the lifespan in mouse models (Baker et al., 2016). Based on the work presented here, it is tempting to speculate that the SASP from age-associated senescent cells could also promote tumor formation during aging by increasing cell plasticity.

Furthermore, it is thought that senescence plays a major role in inflammaging, which is the low, but chronic inflammation seen during aging, and which is implicated in the age-related functional decline as well as in various age-related pathologies such as atherosclerosis, diabetes and cancer (Franceschi and Campisi, 2014; Freund et al., 2010). It seems feasible that the accumulation of senescent cells with aging and at sites of age-associated disorders contributes to the observed tissue inflammation by the SASP, which consists of various pro-inflammatory cytokines, chemokines and growth factors (Campisi, 2013; van Deursen, 2014). Lastly, the SASP is able to induce senescence in neighbouring healthy cells and thereby increase the number of senescent cells in the

surrounding tissue (Acosta et al., 2013). Acosta et al. mainly focused their study on Ras-induced senescence in human fibroblasts and could show that in co-culture experiments OIS fibroblasts induced paracrine senescence in normal fibroblasts after around 7 days. Our data from conditioned media experiments in OIS keratinocytes confirm these findings, as there is a strong increase in senescence induction 6 days after SASP exposure. However, we are extending this previous published study by analysing SASP treated keratinocytes also at earlier time points. As described, we discovered that transient SASP treatment leads to an increase in dedifferentiation, stem cell gene expression and clonogenic capacity. Remarkably, those transient SASP treated cells were able to regenerate skin in hair regeneration assays. Notably, whereas freshly isolated newborn keratinocytes are able to fully regenerate skin, keratinocytes that have been placed in culture prior to transplantation, lose their regenerative capacity, resulting in formation of scar tissue with only a few hair follicles or pigment in the graft. However, transient exposure to the SASP seems to revert those cells back to a more plastic state as they are now able to form large thick patches of hair. Prolonged SASP treatment resulted then in a further increase of stem cell gene expression, but which also triggers a senescence response. Therefore, we propose that persistent SASP exposure leads to a cell intrinsic senescence arrest to block regenerative stimuli coming from the SASP in order to prevent an enhanced plastic cell from proliferation and possible tumor initiation.

Furthermore, recent studies using *in vivo* reprogrammable mice also demonstrated a link between senescence and cellular plasticity (Chiche et al., 2016; Mosteiro et al., 2016). Both studies used the same transgenic mouse model in which overexpression of the four transcription factors Oct4, Sox2, Klf4 and cMyc (OSKM) leads to cell dedifferentiation and reprogramming in multiple tissue types and ultimately to teratoma formation (Abad et al., 2013; Ohnishi et al., 2014). Interestingly, OSKM induction resulted in local induction of damage and senescence. Then, reprogrammed $Nanog^{+}$ cells were found in close proximity to areas of senescent cells (Mosteiro et al., 2016). As transient SASP exposure has already been shown to be beneficial during tissue repair and wound healing (Demaria et al., 2014; Jun and Lau, 2010a), it is not surprising that the SASP from injury-induced senescent cells can further enhance *in vivo* reprogramming (Chiche et al., 2016). Furthermore, OSKM induction in $p16^{Ink4a/Arf}$ null mice resulted in decreased levels of senescent and $Nanog^{+}$ cells suggesting a correlation between cellular senescence and cellular reprogramming. Interestingly, in this system, inhibition of NFκB also decreased the beneficial effects of the SASP on reprogramming, suggesting that common factors are involved between these studies and ours. Indeed, senescence through the SASP promotes *in vivo* reprogramming (Chiche et al., 2016; Mosteiro et al., 2016), correlating with our results that SASP can promote plasticity. Therefore, whereas senescence is a cell-intrinsic barrier to reprogramming (Banito et al., 2009; Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009; Marión et al., 2009; Utikal et al., 2009), it also enhances reprogramming efficiency in

neighbouring non-senescent cells through the secretion of the SASP (Chiche et al., 2016; Mosteiro et al., 2016).

3.3 NFκB and the SASP

Our data, in agreement with the literature, shows that inhibition of NFκB does not influence cell cycle arrest or the senescent phenotype. Instead, inhibiting the NFκB pathway resulted in reduced stem cell gene expression in senescent cells. In addition, our results demonstrated that knockdown of p16 in OIS cells does not influence stemness of senescent cells, but diminishes the proliferation block. Therefore, our study suggests that stem cell gene expression and cell cycle arrest are not linked together in senescent cells. Furthermore, these results implicate also a role for one of the main SASP mediators NFκB in controlling stem cell gene expression in senescent cells. Indeed, while knockdown of NFκB decreased stem cell gene expression in senescent cells, knockdown of other SASP regulators or factors, including CEBPβ, p38 or IL1α had no effect on the stem cell expression, further highlighting a potential specific contribution of NFκB. However, future studies are necessary to investigate whether the SASP from NFκB deficient senescent cells is no longer able to promote stemness and plasticity. A study in senescent human fibroblasts demonstrated that inhibition of NFκB prevented immune recognition by natural killer cells and therefore their own removal (Chien et al., 2011). This result suggests that senescent cells with suppressed NFκB activity might accumulate over time, as they

cannot signal for their own removal. If the SASP from those cells is still able to increase stemness and plasticity in the neighbouring cells, this would probably lead to aberrant regeneration and a pro-tumorigenic microenvironment. Therefore, especially regarding tumor development, it would be very important to analyse whether cells that are undergoing senescence, but have suppressed NFκB activity, still secrete an instructive and beneficial SASP. Furthermore, while NFκB is a potent mediator of age- and tumor-associated inflammation and dedifferentiation, it is also a major driver of hair follicle initiation and development (Schmidt-Ullrich et al., 2001; Zhang et al., 2009) and is involved during hair follicle stem cell regeneration upon skin wounding (Chen et al., 2015a). This further supports how NFκB-mediated processes regulating normal tissue development and regeneration can contribute to tumor formation and cancer stem cell fate when misregulated (Myant et al., 2013; Schwitalla et al., 2013).

3.4 Investigating senescence *in vivo*

A major problem in studying senescence is the lack of a single marker that can identify the senescent state. This problem becomes more evident *in vivo* due to the more heterogeneous and complex senescent phenotypes, highlighting the importance of using a compilation of multiple markers. Even though DMBA/ TPA papilloma has been widely used as an *in vivo* model of oncogene-induced senescence, there has been no detailed study published yet describing the distribution of senescent cells in the papilloma.

Surprisingly, our analysis showed that there are no SA-β-Gal positive cells in the epithelial layer of the papilloma, whereas in the dermis there is a high number of SA-β-Gal. SA-β-Gal assay is the most common used assay to identify senescent cells in tissues as well as in cells in culture, detecting cells that have enhanced lysosomal activity (Dimri et al., 1995; Young et al., 2009). However, SA-β-Gal activity can also be detected in non-senescent macrophages and osteoclasts (Bursuker et al., 1982; Kopp et al., 2007). Additionally, it has also been demonstrated that not all senescent cells are positive for SA-β-Gal (Lee et al., 2006). Thus, we analysed in detail the distribution of other senescence markers, including p16 and p21, demonstrating that there is also a population of senescent cells in the epithelial layer of the papilloma. The absence of positive SA-β-Gal staining in the epithelial layer, despite being positive for other senescence markers, could mean that there is a difference in the senescent state between epithelial and dermal cells in the papilloma. The DMBA/ TPA papilloma develops due to a combination of overexpression of $HRas^{V12}$ mutation in epithelial cells caused by the DMBA mutagen, and a chronic inflammatory signal from repeated treatment with TPA, which together, in an ongoing dynamic fashion, causes overproliferation of the mutated cells, a senescence response to limit the proliferation of these cells, which together manifests as a growing and expanding papilloma. Interestingly, this process depends on the accumulation of CD34 expressing skin stem cells (Abel et al., 2009; Lapouge et al., 2012; Malanchi et al., 2008; Morris, 2004). Based on our results, we are suggesting that first the $HRas^{V12}$ mutation in the keratinocytes leads

to dedifferentiation of keratinocytes to become a $CD34^+$ stem-like cell, and then to an aberrant proliferation of these $CD34^+$ cells. However, due to the aberrant proliferation and also increased stemness, these cells then become senescent as a tumor suppressive mechanism and start expressing senescence markers. Since this is a dynamic process, we can identify cells in the basal epithelial layer of the papilloma that express a proliferation marker as well as senescence markers. However, once the cell cycle arrest is established and these cells lose the proliferation marker, the cells detach from the basal layer and move upwards in the tissue. Thus, a papilloma shows a similar hierarchy as in the normal skin, but with aberrant expansion of each of the proliferation and dedifferentiation layers. At the same time, there is also an induction of senescence in the dermal fibroblasts. Whether this is also due to a mutation in the fibroblasts or whether the SASP coming from the keratinocytes induces paracrine senescence needs to be further investigated, but it is very likely that a SASP from the dermal fibroblasts contributes to the dedifferentiation and stem-like growth in the adjacent epithelial layers.

The additional analysis in another *in vivo* model of senescence, namely pancreatic intraepithelial neoplasia (PanIN), which is a pre-malignant lesion in the pancreas (Morton et al., 2010) further supports our described association between senescence and increased stemness. We could show that in those pre-malignant lesions, similar to the skin papilloma, there is an accumulation of senescent cells as well as an increased expression of the stem cell

markers CD44 and Nestin. This data suggests that the association of senescent cells and stem cell markers does not only occur in the skin, but also in other tissues. Furthermore, we extended our analysis in the liver, where we used a model of transposon-mediated delivery of $NRas^{G12V}$ to individual hepatocytes, which has been demonstrated to be an accurate inducer of transient senescence *in vivo* (Kang et al., 2011). Interestingly, there was an increased expression of the cancer and stem cell markers CD44 and Nestin adjacent to the senescent cell. However, since macrophages or other immune cells have been shown to express CD44 as well (Puré and Cuff, 2001), further analysis has to be done to exclude that the CD44 positive cells are not immune cells. Nevertheless, Nestin has been shown as an important factor for stemness and plasticity in the liver and the skin (Tschaharganeh et al., 2014). Therefore, this data suggests that the SASP is able to induce stemness and plasticity in the neighbouring cells. Interestingly in this case, the senescent cells themselves were not positive for any of the 2 stem cell markers examined, which could be due to a number of reasons. We analysed the tissue 6 days after senescence induction, a time point in which we did not see an increase in CD34 *protein* level in OIS keratinocytes *in vitro*. However, 6 days after SASP treatment alone we could identify CD34 positive cells. Another possible reason is that senescent hepatocytes do not show an increase in the 2 stem cell markers we analysed and we need to extend the analysis to other markers such as Epcam or CK19. However, it is also likely that our finding that senescent cells in culture express stem cell markers is a result of the tissue culture strategy used to study

senescence. In this way, every cell is infected with Ras, and each cell is likely instructed to dedifferentiate and turn on stem cell markers by the SASP from the surrounding cells. Therefore, the stemness may not be related to the endogenous Ras. This would also agree with the finding in the liver where the individual senescent cells are more isolated, and likely are not instructed by other senescent cells. Furthermore, as we demonstrate, knockdown of NFκB in cultures of senescent cells caused decreased stem cell marker expression, suggesting this feature was linked to the SASP.

3.5 Future directions

Altogether, however, our results help to resolve previous paradoxes surrounding senescence and the SASP, demonstrating how senescence and the SASP is primarily a beneficial process that instructs stem cell function and regeneration, but only when misregulated, can have detrimental effects seen during aging and cancer. However, the results presented in this thesis still leave various open questions.

1) How does the SASP induce plasticity and cell cycle arrest? In this study, we demonstrate that transient exposure to the SASP leads to increased stemness and plasticity, whereas longer SASP treatment induces a senescence response. However, the degree of their plasticity still remains a question and it would be interesting to investigate whether the transient SASP treated cells are able to dedifferentiate into other cell types upon stimulation or instruction.

Additionally, cellular reprogramming of somatic cells into induced pluripotent stem cells occurs through the global reprogramming of epigenetic marks (Buganim et al., 2012, 2013; Polo et al., 2012). Interestingly, there is a correlation between epigenetic marks that are remodelled during reprogramming, but which are also dysregulated during aging (Benayoun et al., 2015; Liu et al., 2013; Pollina and Brunet, 2011). Therefore, future studies comparing cellular reprogramming to pluripotency by expression of OSKM and transient and extended SASP treated cells could give important insights into the molecular mechanism how cellular senescence through the SASP is influencing plasticity and stemness.

2) Is SASP induced plasticity a part of normal tissue regeneration? The liver is a remarkable organ, being one of the few tissues that are able to regenerate after injury or tissue loss (Taub, 2004). The most widely used model to study liver regeneration is the 2/3 partial hepatectomy (PH) (Mitchell and Willenbring, 2008). Interestingly, shortly after PH there is an increase in p21 positive cells in the liver, followed by a later increase in proliferating hepatocytes (Liu et al., 2010; Tachibana et al., 2014). Furthermore, it has been shown that liver regeneration is impaired in IL-6 knockout mice (Cressman et al., 1996) and IL-6 levels are increased shortly after PH (Tachibana et al., 2014). Overall, since p21 is an important marker of senescence and IL-6 is a major component of the SASP (Coppé et al., 2010), these

results suggest a possible link between liver regeneration and senescence. Here we show that senescence in single cells in the liver *in vivo* induces tissue-specific expression of stem cell markers in a paracrine manner. Therefore, future investigations on whether the SASP instructs liver regeneration could give further insights in how cellular senescence is a beneficial regenerative mechanism.

3) What are the factors in the SASP that induce stemness and plasticity? The SASP consists of a variety of chemokines, cytokines, growth factors and extracellular matrix remodellers, is dynamic and varies between cell types (Coppé et al., 2008). It is obviously of high priority to identify which specific SASP factors contribute to the beneficial and instructive effects we could demonstrate. With regards to the link between the SASP and iPS cells, it was shown that IL-6 is a key SASP factor facilitating reprogramming (Chiche et al., 2016; Mosteiro et al., 2016). Interestingly however in our studies, IL-6 is not induced in primary mouse keratinocytes undergoing senescence, suggesting it is likely an additional factor. Indeed, we favour the idea that it is not necessarily a single factor, but more so that senescent cells exert a "niche-like" effect, though a combination of secreted factors. Identifying and isolating this possible cocktail of SASP factors might advance our understanding in regenerative medicine as well as in tumor-inhibition studies. Our identification of NFκB as an

important mediator in this process, should facilitate to identify these factors in future studies.

4) Is the SASP from senescent cells in aged tissues beneficial, or different? In our study, we mainly focus on the SASP at the onset of cells undergoing senescence, but we did not address whether the SASP changes during senescence. It might be possible that at the beginning of the senescence response, the SASP is very beneficial and instructive, however, it then changes its composition in order to recruit immune cells for its own removal as seen during developmental senescence (Storer et al., 2013). This is supported by a recent study demonstrating that Notch1 mediates a change in the SASP over time (Hoare et al., 2016). Another possibility might be that the change in SASP composition later on mainly induces paracrine senescence and tissue dysfunction. This might explain, why during aging an accumulation of senescent cells leads to tissue dysfunction and clearance of senescent cells delays functional decline in many tissues (Baker et al., 2011, 2016). Thus, in order to increase our understanding of the role of senescence during aging, it would be important to investigate whether and how the beneficial and instructive SASP seen during embryonic development (Muñoz-Espín et al., 2013; Storer et al., 2013), wound healing (Demaria et al., 2014; Jun and Lau, 2010a; Krizhanovsky et al., 2008) or in our study changes during organismal and tissue aging.

Conclusions

Conclusions

1) Primary mouse keratinocytes undergoing Ras induced senescence upregulate stem cell genes, while undergoing proliferative arrest and losing their clonogenic capacity.

2) Oncogene-induced senescence induces de novo specification of stem cell fate.

3) There is an association between senescence and increase in stem cell gene expression *in vivo*.

4) Keratinocytes that bypass senescence arrest through knockdown of p16 retain high stem cell gene expression, whereas inhibition of NFκB decreases stem cell signature.

5) Transient exposure to the SASP induces enhanced stemness and regeneration, whereas prolonged SASP treatment triggers paracrine senescence, likely as a tumor-suppressive response to aberrant regeneration.

6) Induction of senescence in single cells *in vivo* in the liver, induced expression of stem cell markers in the adjacent cells in a paracrine manner.

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