Senescence in RASopathies, a possible novel contributor to a complex pathophenotype

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ABSTRACT

Senescence is a biological process that induces a permanent cell cycle arrest and a specific gene expression program in response to various stressors. Following studies over the last few decades, the concept of senescence has evolved from an antiproliferative mechanism in cancer (oncogene-induced senescence) to a critical component of physiological processes associated with embryonic development, tissue regeneration, ageing and its associated diseases. In somatic cells, oncogenic mutations in RAS-MAPK pathway genes are associated with oncogene-induced senescence and cancer, while germline mutations in the same pathway are linked to a group of monogenic developmental disorders generally termed RASopathies. Here, we consider that in these disorders, senescence induction may result in opposing outcomes, a tumour protective effect and a possible contributor to a premature ageing phenotype identified in Costello syndrome, which belongs to the RASopathy group. In this review, we will highlight the role of senescence in organismal homeostasis and we will describe the current knowledge about senescence in RASopathies. Additionally, we provide a perspective on examples of experimentally characterised RASopathy mutations that, alone or in combination with various stressors, may also trigger an age-dependent chronic senescence, possibly contributing to the age-dependent worsening of RASopathy pathophenotype and the reduction of lifespan.

1. Introduction

Senescence is a permanent cell cycle arrest in response to various damaging external and internal stimuli and activated to limit the propagation of damaged cells. Senescence was initially observed in terminally replicative primary cells, which undergo a gradual shortening of telomeres with each cell division (Hayflick limit, Hayflick, 1965; Hayflick and Moorhead, 1961)). Replicative senescence is induced when telomeres reach a critical length and acts as a protective mechanism against genomic instability and tumour initiation by blocking the division of somatic cells (Fagagna et al., 2003; Kuilman et al., 2010). Telomere length is maintained by telomerase, an enzyme that is active in germ and certain stem cells, but undetectable in most somatic cells. Re-expression of telomerase in cells bypasses replicative senescence, a mechanism that is also found in numerous tumour cells (Bodnar et al., 1998; Ding et al., 2012; Perera et al., 2019). Further extensive studies identified that a novel type of senescence, generally termed “premature senescence”, may also be triggered by cellular damage induced by oncogene signalling, irradiation, oxidative stress and chemotherapy, among other stimuli (Kurz et al., 2004; Panganiban et al., 2013; Roninson, 2003; Serrano et al., 1997). Multiple studies have established the fundamental role of senescence as a major tumour suppressive mechanism (Lowe et al., 2004). Moreover, the accumulation of senescent cells was suggested to be an underlying cause of ageing and interventions aimed at the removal of senescent cells led to improved tissue homeostasis and delayed ageing (Baker et al., 2011). Recent

Abbreviations: CDK, cyclin-dependent kinase; PCNA, proliferating cell nuclear antigen; SA-β-GAL, senescence-associated β-galactosidase; DDR, DNA damage response; SAHF, senescence-associated heterochromatin foci; SDF, senescence DNA damage foci; TIF, telomere-dysfunction-induced foci; SADS, senescence-associated distension of peri- and centromeric satellites; SASP, senescence-associated secretory phenotype; OIS, oncogene-induced senescence; ROS, reactive oxygen species; AER, apical ectodermal ridge; TGF-β, transforming growth factor beta; SMAD, mothers against decapentaplegic homolog 1; PI3K, phosphoinositide 3-kinase; FOXO, forkhead box protein O1; CD4, cluster of differentiation 4; CD14, cluster of differentiation 14; CD16, cluster of differentiation 16; CD158d, cluster of differentiation 158d; MAPK, mitogen-activated protein kinase; CS, Costello Syndrome; CFCS, cardio-facio-cutaneous syndrome; LS, Legius syndrome; NS, Noonan syndrome; NSML, Noonan syndrome with multiple lentigines.

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studies expanded further the broad impact of senescence in organismal homeostasis by identifying a fundamental contribution to embryonic development and tissue repair (Adams, 2009; Da Silva-Alvarez et al., 2019; Rodier and Campisi, 2011).

In this review, we will highlight the hallmarks of senescence, including the positive and negative effects of senescence in organi
tinal homeostasis, and provide several examples of differentiated cells that undergo senescence or senescence-like phenotype. Finally, and most importantly, we will focus on implications of mutations in genes encoding for RAS-MAPK components in the induction of senescence in RASopathies. We believe that the induction of senescence in RASop
pathies could be an underestimated contributor to a complex clinical pathophenotype that often resembles premature ageing and its associ
ated ageing-related pathologies.

2. Senescence hallmarks

Senescent cells have several hallmarks that distinguish them from healthy neighbouring and quiescent cells (Gorgoulis et al., 2019). One major feature is known as senes
cence-induced cell cycle arrest, which is largely considered to be permanent (Blagosklonny, 2011; Campisi and d’Adda di Fagagna, 2007; Ogrodnik et al., 2019). The cell cycle arrest of senescent cells is mediated by an upregulation in the expression of cell cycle inhibitory genes in response to DNA damage, leading to the acti
vation of two major arms of cell cycle arrest, namely p53/p21WAF encoded by CDKN1A gene and p16INK4A (encoded by CDKN2A)/RB pathways. Senescence signals activate p53 and induce p21WAF expression, which in turn blocks cyclin-dependent kinase 2 (CDK2) complexes, thus blocking cell cycle progression or blocking DNA synthesis via the binding to proliferating cell nuclear antigen (PCNA) (Gartel and Radhakrishnan, 2005; Georgakilas et al., 2017; Waga et al., 1994; Xiong et al., 1993). Similar signals are also responsible for the expression of p16INK4A, which binds to and inhibits CDK4/6 complexes, protecting the RB tumour suppressor protein from inhibitory phosphorylation, which in turn blocks G1/S progression (Alcorta et al., 1996; Bruce et al., 2000; Serrano, 1997; Steven Zhang et al., 1999).

Senescent cells undergo morphological changes, adopting an enlarged flattened shape and have elevated levels of senescence
associated β-galactosidase (SA-β-Gal), thought to be a consequence of an enlargement of the lysosome (Lee et al., 2006). Additionally, the nuclei of senescent cells have an increased number of persistent foci that are associated with an accumulation of DNA damage response (DDR) proteins and epigenetic changes of chromatin (senescence-associated heterochromatin foci or SAHF) (Narita et al., 2003a). The DDR is a cluster of proteins coupled with either senescence DNA damage foci (SDF) or telomere-dysfunction-induced foci (TIP) and is created by an accumulation of p53-binding protein 1 (53BP1), histone H2AX (γH2AX) and ataxia telangiectasia mutated (ATM) proteins (Martínez-Zamudio et al., 2017). SAHF are chromatin structures that do not contain any active transcription sites and play a major role in blocking proliferative genes. These structures are identified by the presence of specific markers such as H1P1, histone H2A variant macroH2A and lysine-9 di- or tri-methylated histone H3 (H3K9Me2/3) (Aird and Zhang, 2013; Narita et al., 2003b; Zhang et al., 2007). In many senescence models, another epigenetic change which occurs in heterochromatin, is the senescence-associated distension of peri- and centromeric satellites (SADS) and this is common in progeria syndromes and normal physiological senescence (Parry and Narita, 2016; Swanson et al., 2013).

Furthermore, in distinct contrast to either quiescent or apoptotic cells, senescence cells are characterised by a specific secretome, called senescence-associated secretory phenotype (SASP). The SASP has a negative influence on the surrounding tissue through the release of inter
leukins (e.g. IL1α, β, IL6), chemokines (e.g. CXCL1), macrophage inflam
matory protein (MIP, Eotaxin) and proteases (e.g. MMP3, PAI1/2, TIMP1/2), among other factors (Coppé et al., 2010).

Last, but not least, senescence is associated with an impaired

3. The bright and the dark sides of senescence

3.1. Tumour suppression

In 1997, Serrano et al. described the premature senescence triggered by the expression of oncogenic RAS, which was termed oncogene
induced senescence (OIS) (Serrano et al., 1997), followed by similar observations in premalignant lesions (lung adenomas), but not in more advanced lesions (lung adenocarcinomas) (Collado et al., 2005). Similar phenomena were revealed in benign (nevi) and malignant (melanoma) melanocytic neoplasms induced by BRAF, NRAS and HRAS oncogenic mutations, where with the induction of senescence, nevi growth was blocked (Dankort et al., 2007; Di Micco et al., 2006; Roh et al., 2015; Serrano et al., 1997). The OIS response was also demonstrated for other oncogenes including RAF (Dankort et al., 2007), AKT (Chen et al., 2005; Miyagishi et al., 2004), E2F1 (Ogier et al., 2000) and cyclin E (Husova
et al., 2006). OIS is mainly mediated through the increased accumula

3.2. Embryonic development

In addition to a tumour suppressive role, senescent cells have addi

3.3. Adult tissue remodelling

The role of senescent cells in tissue remodelling processes has not only been demonstrated in developmental processes, but also in the maintenance of tissue homeostasis by influencing wound healing, tissue
repair and regeneration throughout the entire life span of an organism. Detailed insight into the mechanism through which senescent cells induce proper healing and tissue regeneration has been reported (Jun and II, Lau, 2010). Since they are metabolically active, senescent cells display a SASP, affecting the surrounding tissue via the enhancement of the motility and proliferation of adjacent cells and also recruit immune and phagocytic cells to eliminate senescent cells to restore the pre-damaged status of the tissue.

These effects were demonstrated in studies of activated hepatic stellate cells (HSCs) (Krizhanovsky et al., 2008), cardiac myofibroblasts (Meyer et al., 2016; Sumei et al., 2018), muscle regeneration (Le Roux et al., 2015), skin (Demaria et al., 2014) and the regeneration of hair follicles (Yosef et al., 2016). Upon damage, liver regeneration is mediat-ed by the stellate cells, which proliferate and produce extracellular matrix proteins that form the fibrotic scar. Ultimately, activated stellate cells become senescent cells are recognised and cleared by natural killer cells, thus enabling the resolution of fibrosis (Krizhanovsky et al., 2008). In the skin, a crucial repair event is myofibroblast activation, where cells drive wound healing and tissue repair. In vivo studies have demonstrated that senescent fibroblasts and endothelial cells located at the wound sites induce myofibroblast differentiation and accelerate wound closure by the secretion of platelet-derived growth factor AA (PDGF-AA) (Demaria et al., 2014). The involvement of senescence in the heart in myocardial fibrosis and postinfarction remodelling was tested in both rodent hearts and human ischemic tissues and indicated that the activation of senescence in myofibroblasts protects against increased perivascular and interstitial fibrosis and improves the postinfarct heart function (Meyer et al., 2016; Sumei et al., 2018).

Although human regeneration capacities are limited, it is worth mentioning that some organisms can regenerate their tissues throughout their lives. Interestingly, during limb regeneration in the salamander, there is a significant induction of senescence, which is rapidly cleared by macrophages (Yun et al., 2015).

3.4. Ageing

In contrast to the “bright side” functions of senescence, a “dark side” is associated with an age-dependent accumulation of senescent cells during normal ageing which has adverse consequences. Advanced age is a significant risk factor for most chronic diseases such as osteoporosis, cardiovascular disease, cancer, diabetes and neurodegenerative disorders (Laurent, 2012; Niccoli and Partridge, 2012). The causal link between senescence and ageing was first described in a mouse model of progeroid syndrome. Here, the selective clearance of senescent cells (p16INK4A-positive) resulted in an amelioration of ageing-related features (Baker et al., 2011; Sharpless, 2004). In aged organisms, the paracrine effects of senescent cells induced senescence in neighbouring cells and, when combined with an impaired immune clearance, led to an accumulation of senescent cells (McHugh, 2018). Senescence cells are not inert cells in a tissue because of their SASP, which contains metabolites, proteases, inflammatory cytokines and growth factors, and is undoubtedly associated with chronic inflammation and tissue remodelling, two hallmarks of ageing (Coppe et al., 2010). The accumulation of senescent cells is consistent with the idea that the elimination of these cells can restore tissue homeostasis after injury and delays ageing-associated disorders (Baker et al., 2011).

3.5. Senotherapies

Recently, more and more consideration has been given to target senescent cells by either genetic strategies or senotherapies (“senolytic” and “senomorphic” agents) or immune modulation as a potential therapy (Kim and Kim, 2019; Martel et al., 2020; Niedernhofer and Robbins, 2018).

Senolytic therapies have been described for use in a broad range of pathologies such as pancreatic cancer (Ruscetti et al., 2020) ageing-related pathologies disorders (Zhu et al., 2015) like neurodegeneration (Bussian et al., 2018), cardiovascular disease (Childs et al., 2018), bone loss (Farr et al., 2016) and, related to current viral pandemic developments, may prevent the transmission of the SARS-CoV-2 virus (Sargiacomo et al., 2020). The β-Gal dependent prodrug senescence-specific killing compound 1 (SSK1) provided an effective clearance of senescent cells and is an alternative to senolytic therapies. SSK1 conversion into gemcitabine by the lysosomal β-Gal selectively kills senescent cells through a p38MAPK-mediated apoptotic response. As a result of the treatment, there was a decreased senescence- and age-associated gene signature, attenuated low-grade local and systemic inflammation, and restored physical function in aged mice (Cai et al., 2020).

Senomorphic agents are compounds that are found in plants and dietary supplements, which regulate the SASP or pro-inflammatory secretome of senescence cells. In contrast to senolytics, they do not induce apoptosis in senescent cells. Candidate senomorphic molecules have been used in both in vitro and in vivo experiments and were shown to inhibit senescence in human umbilical vein endothelial cells and human diploid fibroblasts (Yang et al., 2015, 2014), delay ageing in progeric mouse models (Lee et al., 2016; Tilstra et al., 2012) and an improved skin healing in old mice (Kang et al., 2017). Contrary to senolytic agents, senomorphic molecules beneficial effects are achieved only by continuous long-term usage, constituting a significant drawback for their use.

During ageing, senescent cells accumulate in aged tissues and establish a specific environment that may help them to escape from the immune surveillance mediated by NK, CD4+ T cells and macrophages (Kang et al., 2011; Sagiv et al., 2016). Immunotherapies that accelerate immune-mediated clearance of senescent cells seem to be beneficial in reducing the accumulation of senescent cells (Soriani et al., 2009; Weiland et al., 2014).

4. Senescence in differentiated and terminally differentiated cells

Originally, senescence was associated with mitotic cycling cells that ultimately reach their proliferative limits and undergo the process known as classical replicative senescence (Hayflick, 1965; Hayflick and Moorhead, 1961). Adult stem cells are considered to be immortal, containing both the capacity to self-renew and also to differentiate into different cell types, thus maintaining organ and tissue homeostasis. However, stem cells can undergo senescence in response to intrinsic factors and/or niche ageing-induced stimuli (Janzen et al., 2006; Nishino et al., 2008; Schultz and Sinclair, 2016; Sousa-Victor et al., 2014), hence limiting their regenerative capacities which ultimately leads to ageing and ageing-related diseases.

Human and mouse studies have identified senescence or a senescence-like phenotype in differentiating and terminally differentiated cells in response to various intrinsic and extrinsic stressors, such as DNA damage, oxidative stress and inflammation (Herranz and Gil, 2018). In addition to stem cell senescence and ageing, the induction of senescence in differentiated cells and their accumulation within tissues leads to a dysfunctionality that may affect systemic physiological processes such as energy metabolism, bone remodelling, immunity and brain functions, to name only a few.

4.1. Liver

An accumulation of senescent cells in the liver during ageing is associated with an impaired homeostasis and underlies various patholo-gies. Senescence in hepatocytes was reported to correlate with fat accumulation and the underlying mechanism is related to an impaired mitochondrial utilisation of fatty acids (Aravintan et al., 2013; Ogrodnik et al., 2017; Wiemann et al., 2002). Moreover, a reduction of hepatosteatosis was seen by the removal of senescent cells by either
p16INK4A ablation or treatment with senolytic drugs (Ogrodnik et al., 2017). Furthermore, when undergoing a severe acute injury, liver hepatocytes activate a senescent program that blocks carcinogenesis via macrophage immunosurveillance, indicating that for liver cancer, the activation of senescence may have a therapeutic potential (Wang et al., 2018).

In addition to hepatocytes, senescence was also identified in human hepatic stellate cells (HSCs) in vivo and culture models, in response to either liver damage or microbial metabolites triggered by an obese state (Krizhanovsky et al., 2008; Odagiri et al., 2019; Yoshimoto et al., 2013). Interestingly, repetitive passaging triggers senescence whose SASP is strongly dependent on ERK1/2 MAPK activation (Odagiri et al., 2019). HSCs activation underlies liver fibrosis and its development to hepatocellular carcinoma. Induction of senescence in stellate cells and their clearance by natural killer cells reduces fibrosis, but a long-term induction of senescence contributes to an acute inflammation and hepatocyte transformation (Krizhanovsky et al., 2008; Yoshimoto et al., 2013).

4.2. White adipose tissue

Senescence-like phenotypes were also identified in other metabolic human diseases associated with ageing, like type 2 diabetes. An excessive caloric intake leads to obesity and an increased inflammation of adipose tissue which contributes to insulin resistance. Under these circumstances, enhanced oxidative stress and inflammation promotes p53-dependent senescence-like features in both the known major players of insulin resistance, mouse macrophages and visceral adipose cells (Minamino et al., 2009). Furthermore, in contrast to human non-diabetic controls, senescence features were identified with an increased fat inflammation in visceral fat biopsies collected from diabetic patients, suggesting that ageing of fat cells may have a sizeable impact in diabetes (Minamino et al., 2009).

4.3. Bone

A major ageing-related pathological feature is bone loss. Recently, the presence of senescence markers was reported in both human and mouse bone cells, suggesting that bone undergoes an accumulation of senescent cells with chronological ageing (Farr et al., 2016). Bone senescence seems to be an intricate process that results from cumulative effects that are triggered by senescent myeloid cells, T cells, B-cell, osteoblasts and osteocytes (Farr et al., 2016). The important role of senescence in ageing bone loss was demonstrated through rescue experiments using senolytic agents, which led to bone mass rescue and paves the way for using these molecules as a therapy in radiotherapy and ageing-related bone loss (Chandra et al., 2020).

4.4. Immune system

Senescence was also described in elderly people affected by chronic inflammation. A subset of CD14+CD16+ monocytes undergo senescence, which in turn leads to increased inflammation and proatherosclerotic activity in response to an increased adhesion to endothelial cells (Merino et al., 2011). Depending on their distinct polarization, monocytes become either the classically (inflammatory) or alternatively (non-inflammatory) activated macrophages. Activation by pro-inflammatory stimuli induce a p16INK4A-mediated senescence response and facilitates the polarization to the classically activated phenotype (Cadejko et al., 2011). For natural killer (NK) cells, senescence was reported as a response to the activation of an endosomal receptor (CD158d) during pregnancy. Functioning as a regulatory mechanism, this contributes to normal vascular adaptations, promoting vascular remodelling and angiogenesis, that supports foetal development (Rajagopalan and Long, 2012).

4.5. Brain

Senescence-like features were identified in brain cells and they may contribute to the development of neurodegenerative diseases (Baker and Petersen, 2018). Evidence of senescence markers were observed in neurons (Chernova et al., 2006; Forero et al., 2016a; Forero et al., 2016b; Jurk et al., 2012), microglia (Bachstetter et al., 2011; Sierra et al., 2007) and astrocytes (Bhat et al., 2012; Bitto et al., 2010), however, further studies are needed to prove the extent to which senescence affects brain cells and its physiological impact.

4.6. Heart

Senescence-like features were also identified in ex vivo grown cardiac progenitor cells (CPCs). In these cells, senescence was dependent on the activation of ERK1/2-MAPK (Choi et al., 2013). Murine cardiomyocytes collected from aged rats displayed premature senescence-like features that are induced by increased oxidative stress and are dependent on the PML-acetylated p53-p21WAF pathway (Maejima et al., 2008, 2006). Furthermore, human and mouse cardiac ageing, cardiomyopathy and heart failure are also related at least in part to the induction of senescence-like features in both cardiac stem cells and cardiomyocytes e.g. DNA damage, or increased cell cycle inhibitory genes expression (Daniele et al., 2004; Urbaneck et al., 2005). Moreover, senescence also has a negative influence on regeneration after post-myocardial infarction and a simultaneous inhibition of both RB1 and MEIS2 senescence-associated genes enhances cell cycle re-entry and repair (Alam et al., 2019).

Considering these literature reports, when mutations that trigger senescence occur in germlines (e.g. oncogenic mutations), their effects would be detectable throughout both embryonic development and the life span. However, a weaker senescence-like phenotype in differentiated cells may not be discernible before various intrinsic and extrinsic stimuli which would create a positive environment for an activation of senescence (e.g. ageing). Also, the positive and negative effects of senescence on tissue homeostasis strongly depend on whether senescence is either acute or chronic, the latter being generally associated with ageing and ageing-related disorders (Childs et al., 2015).

5. RAS-MAPK developmental disorders (RASopathies)

RAS proteins are fundamental molecular switches of many cellular signalling pathways and are indispensable for normal embryonic and postnatal development, controlling cell proliferation, differentiation, apoptosis and migration, to name only a few. HRAS, KRAS and NRAS are the founding members of the RAS superfamily of small GTPases and share significant sequence homology and largely overlapping functions (Mo et al., 2018). Somatic mutations in RAS and RAS-related genes are associated with several human cancers, KRAS (85 %) is the most frequently mutated, followed by NRAS (11 %) and HRAS (4 %) (Quinlan and Settleman, 2009). Oncogenic RAS somatic mutations lead to the constitutive activation of RAS protein that in turn hyperactivates its major downstream signalling arm in uncontrolled proliferation, the mitogen-activated protein kinase (MAPK) pathway (Santarpia et al., 2012). In addition to RAS, hyperactivation of MAPK is induced by somatic mutations in RAF kinases, which are direct effectors of RAS proteins that initiate the MAPK phosphorylation cascade. RAF kinases mutations, especially the BRAF mutations, were identified with a high incidence in malignant melanoma, and at a lower frequency in a wide range of other human cancers (thyroid, colorectal carcinomas, lung cancers, papillary craniopharyngioma, classical hairy cell leukaemia, and metanephric kidney adenoma) (Davies et al., 2002; Holderfield et al., 2014). It is reported that the BRAFV600E transversion, which encodes the constitutively active BRAFV600E oncoprotein, acts as the driver mutation in half of all melanomas. Mutations in BRAF occur frequently in the kinase domain, mediating the elevated kinase activity.
However, less common mutations that alleviate BRAF kinase activity are compensated for by activating CRAF, thereby still stimulating cellular signalling through the MAPK pathway (Garnett et al., 2005).

In contrast to somatic mutations, germline mutations in RAS-MAPK components lead to developmental disorders, collectively referred to as RAS-MAPK syndromes or RASopathies (Tidyman and Rauen, 2009). Germline mutations that occur in RAS genes and trigger RASopathies are gain-of-function mutations. RASopathy mutations do not only occur in RAS genes, but also affect many molecules involved in MAPK signalling: RAS abundance regulators (LZTR1), cycling regulators (GTPase-activating proteins and guanine-nucleotide exchange factors), RAF effectors (e.g. B- and C-RAF), phosphorylation cascade components (e.g. MEK1/2), MAPK positive (SHOC2) and negative (SPRED1) regulators, and ubiquitin ligases (Cbl). The large group of RASopathies includes defined syndromes: neurofibromatosis type 1 or NF1 (NF1 (Cawthorn et al., 1990; Viscochi et al., 1996; Wallace et al., 1990)), Noonan syndrome or NS (PTPN11 (Tartaglia et al., 2001), SOS1/2 (Roberts et al., 2007; Tartaglia et al., 2007), RASA2 (Chen et al., 2014), KRAS (Schubbert et al., 2006), NRAS (Cirstea et al., 2010), BRAS1-3, RIT1 (Aoki et al., 2013), BRAF (Sarkozy et al., 2005), CRF (Pandit et al., 2007), MEK1 (Nava et al., 2007), SHOC2 (Cereddus et al., 2009), CBL (Martinelli et al., 2010), LZTR1 (Yamamoto et al., 2015), Noonan syndrome with multiple lentigines or NS-ML (PTPN11 (Digilio et al., 2002; Legius et al., 2002), RAF1 (Pandit et al., 2007), capillary malformation–arteriovenous malformation syndrome or CM-AVMS (RASA1 (Eerola et al., 2003), Costello syndrome or CS (HRAS (Aoki et al., 2005)), cardio-facio-cutaneous syndrome or CFCS (BRAF (Nihori et al., 2006), MEK1/2 (Rodriguez-Viciana et al., 2006), KRAS (Nihori et al., 2006)), and Legius syndrome or LS (SPRED1 (Brems et al., 2007)). Although these disorders have distinct clinical characteristics, they have many overlapping features, such as cardiac malformations, craniofacial dysmorphism, skeletal abnormalities, learning difficulties and an increased risk of cancer.

6. Senescence in RASopathies (Costello syndrome)

Physiological ageing is associated with an increased accumulation of senescent cells that will ultimately have a negative influence on the regenerative capacity of tissues (Baker et al., 2013; Brack et al., 2007). Premature senescence was also identified in progeric syndromes that are associated with premature ageing (Fossel, 2003; Hasty et al., 2003). Interestingly, clinical observations have indicated that RASopathy patients are characterised by premature ageing-like features. Clear signs were observed with studies that thoroughly monitored the features of CS patients, where patients exhibit premature-aged skin and appearance, hair loss, reduced muscle tone, osteoporosis development and are affected by age-related cancers at a young age (e.g. bladder cancer) (Gripp and Lin, 2012; White et al., 2005). Unfortunately, no further publications exist that describe whether premature ageing is present, not only in CS, but also in other RASopathies. However, discussions have taken place with RASopathy patients support groups and clinicians who regularly monitor CFCS patients which describe that they may develop a premature ageing-like phenotype, but unfortunately there are no studies which regularly monitor CFCS patients which describe that they may develop a premature ageing-like phenotype, but unfortunately there are no studies that would support these observations (Martin Zenker, German Network for RASopathy Research-GeNeRARE, personal communication). Additionally, RAS-MAPK pathway activation is undoubtedly associated with senescence, either triggering (RAS and RAF) or transducing senescence signals (MEK1/2-ERK1/2) that modulate the senescence gene expression program. Given that RAS-MAPK is a crucial pathway in senescence and senescence is a major factor in tissue homeostasis and ageing, combined with the fact that either an inhibition of MEK1/2-ERK1/2 MAPK (Castillo-Quan et al., 2019; Slack et al., 2015) or senolytic therapies (Baker et al., 2011; van Deursen, 2019; Zhu et al., 2015) lead to an increased longevity and improved health in cellular and animal models, it is conceivable that senescence may trigger or contribute to pathologies and a putative premature ageing in RASopathies. Published mouse models of RASopathy show that their life span is reduced in organisms such as LTSPN11Y279C (Marin et al., 2011), CFCS BRAFV600E (Urosevic et al., 2011), NS KRASV12 (Hernandez-Porras et al., 2014), NS SOSIY546C (Chen et al., 2010), NS RAFI631V (Wu et al., 2011) and NS-CFCs BRAFV597V (Andreadi et al., 2012). It is interesting to note that the genetic background of the mouse may also influence the outcome of lifespan studies as described for NS KRASV12 (Hernandez-Porras et al., 2014) and reviewed in (Hernandez-Porras et al., 2015). This also seems to be true for the CS HRASG12V mouse model, where in a mixed background Bl6/129 has a similar lifespan as wild type controls (Schuhmacher et al., 2008), while our unpublished data indicates that in a pure Bl6 genetic background, the lifespan is reduced (Chennappan S et al., in preparation).

From all of the currently identified RAS-MAPK genes associated with both cancer and RASopathies, it has been demonstrated that RAS (G12, G13 and Q61 substitutions) and RAF kinases (BRAFV600E) oncogenic mutations are promoters of OIS (Mason et al., 2004; Michaloglou et al., 2005; Saretzki, 2010; Sarkisian et al., 2007; Serrano et al., 1997; Zhu et al., 1998). Senescence is also known to be induced through the MAPK kinase by RAF1 constitutively-activating mutations (regulatory residues Y340 and Y341) (Zhu et al., 1998) and gain of function mutations in MEK1 (MEK1Y568) in primary cells (Boucher et al., 2004; Lemieux et al., 2011; Lin et al., 1998).

To date, no studies have reported senescence in primary cells or biopsies collected from RASopathy patients. Despite the development of numerous cellular and animal models to study RASopathies (Jindal et al., 2015), senescence was largely unexamined, with a few exceptions for CS HRAS mutations. HRAS oncogenic mutations were identified only in CS, in which more than 80 % of mutations are caused by a G12S substitution, while stronger mutations such as G12 V, G12D and G12C are rarer and lead to an early lethality (Burkitt-Wright et al., 2012; Lo et al., 2008; Lorenz et al., 2012). In vivo and in vitro studies have also shown that senescence is triggered in response to HRAS mutations. In an in vitro model using human primary fibroblasts, viral transduction of CS mutants led to the induction of OIS, proven by SA-β-GAL staining, the activation of p53 and the induction of p16INK4A expression. As expected, various G12 and G13 mutations induced a strong downstream signalling, while, despite invoking a weaker downstream signalling, mutations that affected other regions of HRAS (K117R and V14T mutations), also led to senescence (Nihori et al., 2011).

Further support of HRASG12V mutation-induced senescence is provided by a CS-like transgenic zebrafish model (Santoriello et al., 2009). Despite an increased level of active HRAS, MAPK activation was not enhanced, but still resulted in reduced cellular proliferation and an increased presence of the senescence marker SA-β-GAL in brain, heart and gills of the adult fish. Surprisingly, an increased proliferation and gain in organ size was observed in kidney and liver, indicating that the activation of senescence in response to HRAS mutation is tissue/organ-dependent. Next, DDR marker analysis indicated that constitutive HRASG12V expression and cell cycle arrest was identified in different subpopulations of adult progenitor cells. These observations point towards a possible negative effect of HRAS activation and senescence of progenitor cells and may be an underlying cause for the age-worsening pathophenotype. Lastly, but not least, our unpublished data indicates that in a pure HRAS mouse model (Schuhmacher et al., 2008) indicates that CS cells at later passages have reduced proliferation, an enhanced ROS production, expression of p16INK4A and p21WAF cell cycle inhibitory genes and the induction of Hp1-positive SAHF (Chennappan S et al., 2019). Despite a lack of experimental data from RASopathy patients that would conclusively prove OIS, some clinical observations could be considered as prospective indicators of senescence. An important dermatological manifestation in CFCS, with a reduced incidence in CS patients, is the presence of melanocytic nevi (Siegel et al., 2012). These skin abnormalities are often regarded as precancerous lesions (Beyova et al., 2003) and despite an absence of morphological changes between nevus and adjacent cells, nevus melanocytes exhibit senescence-like
features, but cannot always be clearly distinguished from melanoma cells (Joselew et al., 2017; Tran et al., 2012). Nevertheless, nevus melanocytes arising from RAS and BRAF mutations are arrested and generally show an accumulation of markers such as SA-p16INK4A, p16INK4A and loss of lamin B1 (Gray-Schopper et al., 2006; Michaloglou et al., 2005; Wang and Dressen, 2018). Another major skin pathology that is associated with senescence is cutaneous papilloma, which in contrast to CFCS, arises with a high incidence in CS patients (Siegel et al., 2012). Papilloma are premalignant tumours lesions that contain senescence cells, which disappear upon tumour progression (Collado et al., 2005). Similarly to CS patients, a CS HRASG12V (CC/FR-HRASG12V) mouse model developed papilloma and an increased neoplastic potential due to a HRAS mutated gene imbalance was identified (Chen et al., 2009). In contrast to this CS mouse model, two other mouse models HRASG12V(P34L)-β-geo and HRASG12V(P61S)-β-geo were created and neither model developed papilloma (Oba et al., 2016; Schuhmacher et al., 2008). These discrepancies may be due to a reduced mutation strength (G12S) or the mouse background or genetic strategy that was used. Unfortunately, there are no reports as to whether senescence markers were identified in these mouse models.

7. Do RASopathy mutations have the intrinsic potential to induce senescence?

Unfortunately, barring the CS HRAS mutations in the above-mentioned studies, there are no further reports which investigated senescence in these RAS-MAPK gain-of-function syndromes or clearly state a premature ageing-like phenotype. Nonetheless, indirect evidence can be drawn from experimental data that investigated H/N/KRAS and BRAF mutations intrinsic properties and the activation of downstream signalling pathways. Data from biochemical and cell-based assays demonstrated that RAS and MAPK activation is enhanced in response to these germline mutations (Cirstea et al., 2013, 2010; Gremer et al., 2011, 2010; Pantaleoni et al., 2017; Rosenberger et al., 2009; Schubbert et al., 2006). The signalling strength of these RAS mutant proteins depends on the mutation location in structural motifs implicated in GDP/GTP cycling, and their interaction with regulators and effector proteins.

In contrast to CS HRAS oncogenic mutations that are compatible with embryonic survival, KRAS mutations at oncogenic residues that were not identified in NS and CFCS, clearly proved that KRASG12D mutation are lethal due to widespread embryonic abnormalities in mouse models (Tuveson et al., 2004). An answer to this difference may be provided by the high abundance and tissue wide KRAS expression in embryos, which indicates that a boost in KRAS mutant signalling would lead to lesions that are incompatible with embryonic survival (Newlaczyl et al., 2017). However, despite their enhanced signalling strength that leads to transformation, KRASG12D mutations also lead to OIS (Cisowski et al., 2016; Mohamed et al., 2018; Morton et al., 2010).

Numerous studies have identified the impaired intrinsic function of NS and CFCS KRAS mutants and their strength in activating MAPK (Cirstea et al., 2013, 2010; Gremer et al., 2011, 2010; Lorenz et al., 2013; Rosenberger et al., 2009). Biochemical studies and cell-based assays of NS KRAS mutants showed that all mutants induce the activation of RAS-MAPK and RAS-AKT pathways, though with different intensities. One important trait of oncogenic KRASG12D mutations is that the GAP-mediated GTP hydrolysis and KRAS inactivation are abolished and the protein is locked in an active state. It is conceivable that some mutations may trigger senescence. Experimental data indicates that similar to oncogenic G12 V mutant, KRASG12D, KRASG13D, KRASG59R, and NRASG61D are insensitive to GAP-mediated hydrolysis and accumulate in their active GTP-bound state, thus leading to a hyperactivation of MAPK. However, these effects may be dampened by a reduced affinity to the RAF kinase as measured by fluorescence polarization (Cirstea et al., 2010; Gremer et al., 2011). Furthermore, although the mutants’ biochemical properties were only partially tested in the original publication, NRASG12D/R/S/V and NRASG13D mutations ((De Filippi et al., 2009; Ekvall et al., 2015; Hakami et al., 2016) and https://cancer.sanger.ac.uk/cosmic/gene/analysis?ln=NRAS), and KRASG22R, KRASG24S, KRASG60A, respectively (https://cancer.sanger.ac.uk/cosmic/gene/analysis?ln=RAS_ENST00000311936, (Tate et al., 2018)).

BRAF mutations were identified in both NS and CFCS and molecular studies demonstrated that they have diverse potencies in activating downstream MAPK (Rodriguez-Viciana and Rauen, 2008; Sarkozy et al., 2009). Analyses of CFCS BRAF mutant kinase activities and the activation of its downstream MAPK target MEK1/2 kinase was compared to those from BRAF cancer mutations and indicated that some of the CFCS BRAF mutants (Q257R, S467A, L485 F, K499E and G534R) behaved similarly to the classical BRAFV600E oncogenic mutation and experimentally demonstrated as an OIS trigger ((Rodriguez-Viciana and Rauen, 2008) and https://cancer.sanger.ac.uk/cosmic/gene/analysis?ln=BRAF) and shows that this may lead to a senescence-like phenotype, but supporting studies are needed to convincingly prove this hypothesis.

Experimental data that has been reported for some RASopathy gene mutations may not yet provide enough evidence to link them to senescence. They lead to activation of MAPK (and probably other pathways as well) and one cannot exclude that intrinsic cellular stress in combination with ageing and its associated systemic inflammation and accumulation of cellular damage in various tissues, a “dark side” senescence-like phenotype may be induced. However, in the absence of senescence molecular studies in patient biopsies and cellular and animal models, this concept may remain only speculative.

RASopathy patients have a significant, but rather moderate risk of tumour development when compared to hereditary cancer syndromes (e.g. breast and cervical cancer) and develop diverse types of cancer. Tumour incidence depends on the mutated gene and mutation strength, which is related to the extent to which a mutation affects protein properties. Clinical reports have indicated that cancer incidence is 1.6 % in NS-ML, 3.5 % in CFCS, 3.9 % in NS, and 10.8 % in CS and that the collective occurrence of cancer was increased in RASopathy patients by the time they reach the age of 20 years to 4 % in NS and 15 % in CS (Kratz et al., 2015, 2011). The types of cancer observed are quite diverse and the median average age for some cancers is strongly lowered in RASopathies when compared to the normal population (average year NS/average year population): NS acute lymphoblastic leukaemia 2.3/13, glioma 9.5/43, rhabdomyosarcoma 4/49; CS rhabdomyosarcoma 2.3/49; bladder cancer 13.5/73; CFCS acute lymphoblastic leukaemia 5/13, Non-Hodgkin lymphoma 35.1/67; NS-ML acute myeloid leukaemia 9.5/67 (Kratz et al., 2015, 2011). These observations may indicate that cancers arising earlier in life may plausibly correlate with the premature ageing, particularly for CS patients. Taking into account the possibility that tumour incidence is reduced when compared to other inherited cancer syndromes, and corroborating it with implications of RAS-MAPK components as triggers or signalling transducers in senescence, and in particular in OIS, it is likely that the “bright side” anti-tumorigenic role of senescence may also be activated in these syndromes. Of major interest, as long as RASopathy patients are susceptible to cancer, it would be crucial to test whether senescence is activated in the early stages of tumour initiation and later be by-passed.
8. Conclusion and outlook

Extensive research aimed at understanding senescence and expanding its role beyond original replicative senescence and its view as an irreversible cell cycle arrest in response to various stressors that affect a wide range of cell types is needed. Currently, senescence is regarded as a biological process with deep implications in embryonic development, tissue repair and ageing by affecting both stem and differentiated cells. Senescence triggered by mutations in RAS and RAF oncogenes is a major mechanism against tumour initiation, but despite its positive effects, it may also contribute to various pathologies and premature ageing in RASopathies, a group of developmental disorders arising from germline mutations in RAS-MAPK components. The induction of either senescence or senescence-like features takes place at different paces and could be dependent on RASopathy gene mutations’ strength in dysregulating cellular signalling pathways and tissue homeostasis. In these syndromes, it is likely that senescence may have a ‘bright side’, as an anti-tumorigenic mechanism in response to RAS-MAPK hyperactivating mutations, but also a ‘dark side’ by impairing cellular processes that are implicated in tissue homeostasis and contributing to a premature-ageing phenotype. Firstly, senescence may contribute to impaired embryonic development that could lead not only to birth defects, but also early life pathologies. Secondly, the accumulation of molecular damage in various cells and detrimental systemic effects (e.g. inflammation) may lead to chronic senescence that will either initiate and enhance syndrome specific pathologies or promote premature ageing and ageing-related diseases in RASopathy patients.

Of further interest, in addition to the already defined premature ageing in CS patients that is triggered by HRAS mutations at oncogenic activity L597VBRAF mutant acts as an epistatic modifier of oncogenic RAS by enhancing signaling through the RAS/RAF/ERK pathway. Genes Dev. 26, 1945–1958. https://doi.org/10.1101/gad.193458.112.

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