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Senescence-associated secretory phenotype regulated by mTOR pathway in senescent cells

Sekreční vlastnosti senescentních buněk regulovaných mTOR dráhou

Bachelor's thesis

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Poděkování:

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Abstract

Senescence is a state of a cell characterized by permanent cell cycle arrest, growth of the cell, and other phenotypic changes, including proinflammatory secretome. Along with the beneficial aspects of senescence, it has an unfortunate outcome – it is a cause of age-related pathologies and tumor progression. Senecence-associated secretory phenotype (SASP) is responsible for that, as it alters the tissue microenvironment by the secretion of the inflammatory agents – chemokines, cytokines and other factors. Cellular senescence is linked to the activation of the mTOR (mechanistic target of rapamycin) nutrient- and mitogen-sensing pathway. Therefore, mTOR inhibition is a promising therapeutic strategy for the SASP suppression and of various types of cancer and age-related diseases. In this thesis I am going to summarize the current understanding of the mTOR's role in the mediation of SASP.

Key words: Senescent cells, senescence-associated secretory phenotype, mTOR pathway, MTORC1

Abstrakt

Senescence je stav buňky charakterizovaný trvalým zastavením buněčného cyklu, růstem buňky a dalšími fenotypickými změnami, včetně prozánětlivého sekretu. Vedle benefičních aspektů má senescence i neblahé důsledky - je příčinou patologických stavů souvisejících se stárnutím a progresí nádorů. Za to je zodpovědný sekreční fenotyp spojený se senescencí (SASP), který mění mikroprostředí tkání sekrecí zánětlivých látek - chemokinů, cytokinů a dalších faktorů. Buněčná senescence je spojena s aktivací dráhy mTOR (mechanistic target of rapamycin), která je zaměřena na živiny a mitogeny. Inhibice mTOR je proto slibnou terapeutickou strategií pro potlačení SASP a různých typů rakoviny a onemocnění souvisejících se stárnutím. V této práci mám v úmyslu shrnout současné poznatky o mTOR ve zprostředkování SASP.

Klíčová slova: Senescentní buňky, Sekreční vlastnosti senescentních buněk, mTOR dráha, MTORC1

Outline

List of Abbreviations

SASP	Senescence-associated secretory phenotype
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
mTOR	Mechanistic Target of Rapamycin
mTORC1	Mechanistic Target Of Rapamycin Complex 1
mTORC2	Mechanistic Target Of Rapamycin Complex 2
S6K	S6 Kinase
4E-BP1	Eukaryotic Translation Initiation Factor 4E-Binding Protein 1
ULK	Unc-51 Like Autophagy Activating Kinase
AKT	Protein Kinase B
SGK1	Serum/Glucocorticoid Regulated Kinase 1
PKC	Protein Kinase C
TSGs	tumor suppressor genes
CDK	cyklin-dependent kinase
ROS	reactive oxygen species
NK	natural killer cells
SAβGAL	senescence□associated β□galactosidase
AD	Alzheimer's Disease.
CVD	Cardiovascular Disease
T2DM	Type 2 Diabetes Mellitus
MCP	Monocyte Chemoattractant Protein
CCL	CC Chemokine Ligand
HCC	Heme Carrier Protein
MIP	Macrophage Inflammatory Protein
EGF	Epidermal Growth Factor
bFGF	Basic Fibroblast Growth Factor
HGF	Hepatocyte Growth Factor.

KGF	Keratinocyte Growth Factor (FGF7)
VEGF	Vascular Endothelial Growth Factor
SCF	Stem Cell Factor
SDF	Stromal Cell-Derived Factor
PIGF	Placenta Growth Factor
IGFBP	Insulin-like Growth Factor Binding Protein
MMP	Matrix Metalloproteinase
TIMP	Tissue Inhibitor of Metalloproteinases
PAI	Plasminogen Activator Inhibitor
tPA	Tissue Plasminogen Activator
uPA	Urokinase Plasminogen Activator
ICAM	Intercellular Adhesion Molecule
OPG	Osteoprotegerin
sTNFRI	Soluble TNF Receptor I
TRAIL-R3	TNF-Related Apoptosis-Inducing Ligand Receptor 3
uPAR	Urokinase Plasminogen Activator Surface Receptor
SGP130	Soluble Glycoprotein 130
EGF-R	Epidermal Growth Factor Receptor
PGE2	Prostaglandin E2
GM-CSE	Granulocyte-Macrophage Colony-Stimulating Factor
G-CSE	Granulocyte Colony-Stimulating Factor
IFN-γ	Interferon Gamma
BLC	B Lymphocyte Chemoattractant
MIF	Macrophage Migration Inhibitory Factor
CXCL	Chemokine (C-X-C motif) Ligan
IL	Interleukin
PI3K	Phosphoinositide 3-Kinase
Raf-1	Rapidly Accelerated Fibrosarcom
MEK	Mitogen-Activated Protein Kinase Kinase
ERK	Extracellular Signal-Regulated Kinase

eIF-4E	Eukaryotic Initiation Factor 4E
PIKK	Phosphatidylinositol Kinase-Related Kinase
PI4Ks	Phosphoinositide 4-Kinases
FKBP12	FK506 Binding Protein 12
RNAi	RNA Interference
FOXO	Forkhead Box
TOP	Terminal OligoPyrimidine
eEF2K	Eukaryotic Elongation Factor 2 Kinase

1. Introduction

Cellular aging is a process, which has been observed in all multicellular organisms. It is associated with a variety of cellular and physiological changes, such as the progressive decline in cell function and the accumulation of cellular damage, which is known to cause senescence. In culture, human diploid cells have a limited replicative lifespan of about 50 cell divisions before becoming senescent. This phenomenon, known as the "Hayflick limit", occurs as an outcome of progressive shortening of telomeres upon each cell division in the absence of telomerase expression (Sell 2007). Senescence is a biological process that occurs when cells cease dividing and enter a state of permanent arrest. Senescence can be either beneficial or injurious depending on the physiological context (He and Sharpless 2017). On one hand, senescence is implicated in the maintenance of tissue homeostasis, it occurs during normal embryonic development, where it contributes to the patterning of the embryo and it is considered to act as an important tumour suppression pathway. On the other hand, emerging evidence indicates that senescent cells may also promote deleterious effects including chronic inflammation and cancer promotion. Senescent cells are characterized by the secretion of a collection of factors that include cytokines and chemokines with pro-inflammatory properties, as well as various growth factors and proteases known as the senescence-associated secretory phenotype (SASP) (J.-P. Coppé et al. 2010). The molecular mechanisms governing SASP are not yet fully understood. However, there are a few pathways known to regulate SASP by influencing transcription factor NF-κB (Giroud et al. 2023). One of those ways is the mechanistic target of rapamycin (mTOR). The mTOR pathway is a key regulator in the control of cell growth and survival, therefore it is a major target of aging-associated diseases. It consists of two complexes, mTORC1 and mTORC2, which are activated by growth factors, nutrients, and hormones. For instance, mTOC1 phosphorylates proteins such as S6K, 4E-BP1, and ULK, which affect protein synthesis, autophagy, and metabolism. Meanwhile mTORC2 phosphorylates AKT, SGK1, and PKC proteins that play a role in cellular metabolism, survival, apoptosis, and motility (Et 2021). In recent years, the molecular mechanisms governing SASP have been the focus of intense research. This work aims to investigate the role of the mTOR pathway in the regulation of SASP in senescent cells.

2. Cellular senescence

2.1. The Role of Cellular Senescence in Aging and Cancer

Senescence is a cellular process characterised by a state of stable cell-cycle arrest and the acquisition of a distinct secretory phenotype in response to cellular stress (Domen et al. 2022). Senescent human cells arrest growth with a G1 DNA content, and the division cannot be stimulated by physiological mitogens (Beauséjour et al. 2003). With age, senescent cells progressively accumulate in tissues and might be the bridge connecting ageing to many age-related pathologies (Rachmian and Krizhanovsky 2023). The accumulation of cellular senescence is one of the main features of aged organisms (López-Otín et al. 2013). These cells are found primarily in renewable tissues and in tissues that experience prolonged inflammation. SASP promotes a state of systemic, chronic, low-grade inflammatory state, called "*inflammaging*," which is known to be one of the main risk factors for the development of such age-related diseases as Alzheimer's disease (AD), cardiovascular disease (CVD), type 2 diabetes mellitus (T2DM) and cancer (Balistreri et al. 2013).

Organisms with renewable tissues are prone to the development of cancer. Senescence is one of the mechanisms that prevent cancer development in cells at risk of neoplastic transformation (Campisi 2001). In contrast to normal cells, cancer cells are capable to escape senescence, thus gaining an unlimited replicative potential that leads to invasion, metastasis and additional features of malignancy (Hanahan and Weinberg 2011). Despite that, there are some factors capable of inducing senescence in cancer cells. These factors include oncogenic stress, triggered by the overexpression of certain oncogenes or loss of tumor suppressor genes (TSGs), DNA damage and metabolic changes (Courtois-Cox, Jones, and Cichowski 2008). This type of senescence response occurs immediately and, as well, independently of telomere shortening. This phenomenon is known as"premature" or oncogene-induced senescence (Kuilman and Peeper 2009).

2.2. Molecular mechanisms of senescence

DNA and other types of macromolecular damage ultimately lead to the termination of proliferation through activation of the p53/p21 ^{CIP1} and p16^{INK4a}/RB tumor suppressor pathways. Induction of p21 ^{CIP1} and p16 ^{INK4a} inhibits cyclin-dependent kinases CDK4, CDK6, (and CDK2 in the case of p21 ^{CIP1}) that are necessary to promote cell cycle progression (McHugh and Gil 2018). p16 is a crucial factor for ensuring the irreversibility of the cell cycle arrest, in the absence of p16 expression the senescence arrest can be reversed by inactivation of p53 (Beauséjour et al. 2003). In spite of the senescence-inducing role of p53, recent studies have shown that it can also cause reversible arrest without senescent morphology, leading to quiescence (Demidenko et al. 2010). Senescent cells can also promote cancer development by altering the cellular microenvironment. Normally senescence acts as a trigger of tissue remodeling during development and after injury. To achieve this, senescent cells arrest their proliferation, recruit phagocytic immune cells and promote tissue renewal (Muñoz-Espín and Serrano 2014). It is crucial to remark that not all cells that undergo the cell cycle arrest become senescent. Non-senescent arrest can be caused by withdrawal of serum growth factors and nutrients. Without growth factors, cells become quiescent (Blagosklonny MV., 2011).

2.3. Distinguishing Cellular Senescence and Quiescence

Senescence and quiescence are frequently used as interchangeable terms in the literature and despite the fact that common molecules play a role in the decision of cell cycle arrest, senescent and quiescent cells have distinctive phenotypes at both molecular and morphological levels (Terzi, Izmirli, and Gogebakan 2016). Cellular quiescence is, unlike senescence, a reversible non-proliferating state (Yao 2014). In the quiescence state, cells are halted in a stable position temporarily, i.e. G0 phase (Terzi et al. 2016). The choice between senescence and quiescence is determined by the activity of the mTOR pathway, where low mTOR activity results in quiescence and higher activity in senescence (Korotchkina et al. 2010).

2.4. Bystander Effects of Senescence and Intercellular Signalling

The tissue microenvironment is characterised by the phenotypes of the cells in a particular area and by the physical and chemical factors that surround them. There are several ways of

how senescent cells communicate with neighbouring cells. G. Nelson et. al. showed in their experiment that senescent cells induce a DNA damage response in surrounding cells through gap junction-mediated cell-to-cell contact and ROS-involving processes. Continuous exposure to senescent cells induced cell senescence in intact bystander fibroblasts. Therefore, senescent cells can induce a bystander effect, spreading senescence towards their neighboring cells *in vitro* and, possibly, *in vivo* (Nelson et al. 2012). Proteomic analysis and functional studies of the proteins transferred from senescent cells to NK and T cells revealed an alternative method of cell-to-cell communication. Based on the results of the research, the transfer is strictly dependent on cell-cell contact and CDC42-regulated actin polymerization and is mediated at least partially by cytoplasmic bridges (Biran et al. 2014). The other way of communication is via ROS-regulated cargo sorting into exosome-like small extracellular vesicles (sEVs), important mediators of the pro-tumorigenic function of senescent cells (Takasugi et al. 2017). Yet, the most pleiotropic effect on surrounding cells has senescence-associated secretory phenotype (SASP).

3. Biomarkers of senescence

Senescence, as a stress response, is a dynamic process involving multiple effector mechanisms combination of which determines the phenotype. Morphological changes observed in tissues that accompany senescence include the enlargement of the cell, it becomes flatter, more vacuolized, and in some cases multinucleated. However, in culture, senescent cells retain the normal morphology dictated by tissue architecture. There are several markers that, used in combination, are generally accepted to determine senescence both *in vitro* and *in vivo*. Biomarkers of senescence are specific molecules or characteristics that can be used to identify and quantify senescent cells. Senescence is typically detected through an assay that is done by using senescence□associated β□galactosidase (SAβGAL) activity at pH 6 and can be identified by flow cytometry using fluorescein di-D-galactopyranoside, a substrate that can be cleaved by galactosidase (Calcinotto and Alimonti 2017). This method relies on the aspect that senescent cells tend to have a higher content of lysosomes, which allows the detection of β□galactosidase (SAβGAL) at a lower pH level. This increase in lysosomal content is thought to be a result of enhanced autophagy in senescent cells and enlargement of the lysosomal compartment. Young A.R. identifies autophagy as an effector mechanism of senescence. It is activated during senescence and its activation is correlated with negative feedback in the PI3K–mammalian

target of rapamycin (mTOR) pathway (Young et al. 2009). As cellular senescence is identified as an irreversible state of cell cycle arrest, the absence of proliferative markers, such as Ki67 protein or 5□bromodeoxyuridine (BrdU) incorporation, is an essential condition to document senescence. Other canonical senescence markers comprise the most common mediators of senescence, including p16, ARF, p53, p21, p15, p27, hypophosphorylated RB, accumulation of lipofuscin, DNA damage foci, upregulation of microRNAs (miRNAs), as shown in Figure 1. Foci of heterochromatin are a feature of senescent cells and are known as senescence□associated heterochromatic foci (SAHF) (Narita et al. 2003). SAHF are preferentially formed during oncogene□induced senescence but not during replicative senescence or upon aging (Di Micco et al. 2011). Additionally to SAHF, senescent cells often exhibit remarkable rearrangement of chromatin structure, in the forms of PML (promyelocytic leukemia protein) nuclear bodies (Bernardi and Pandolfi 2007). Premature senescence induced by oncogenic RAS also decreases LB1 (nuclear lamin B1, a major component of the nucleus) expression through a retinoblastoma protein (pRb)-dependent mechanism (Shimi et al. 2011).

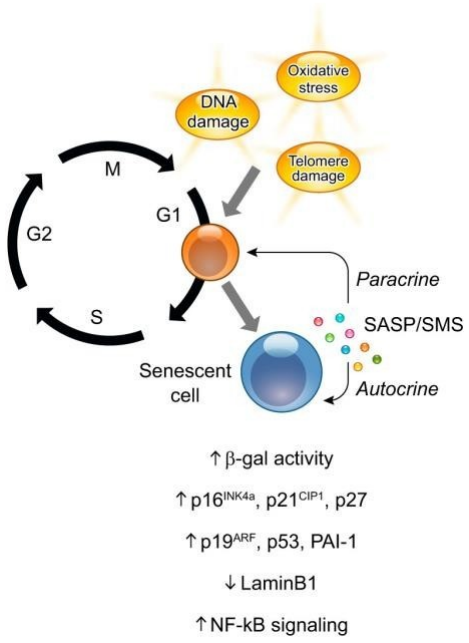

Figure 1. The diversity of biomolecules secreted by senescent cells includes growth factors, cytokines, chemokines, and proteases, is known as the senescence-associated secretory phenotype (SASP) or senescence-messaging secretome (SMS) (Calcinotto et al. 2019).

4. Senescence-associated secretory phenotype (SASP)

4.1 Secretory Profile of Senescent Cells

The senescence-associated secretory phenotype (SASP), also known as senescence messaging secretome (SMS), is a complex pro-inflammatory response, a characteristic feature of senescent cells, referring to the unique secretory profile of these cells (Acosta et al. 2013).
Among the inflammatory cues are molecules with pronounced tumor-controlling properties, both growth and invasion factors, and inhibitory factors, working directly or via recruited immune cells (Lasry and Ben-Neriah 2015).
When cells enter a state of senescence, they remain metabolically and transcriptionally active and undergo significant changes in their gene expression patterns, leading to the production and secretion of diverse variety of bioactive molecules, including pro-inflammatory cytokines, chemokines, growth factors, proteases, and extracellular matrix components (Lasry and Ben-Neriah 2015). Soluble signalling factors include interleukins IL-6, IL-7, IL-1a, -1b, IL-13, IL-15, chemokines IL-8, GRO-a,-b,-g [c], MCP-2, -4, MIP-1a and -3a, HCC-4, eotaxin-3, growth factors amphiregulin, epiregulin, heregulin, EGF, bFGF and HGF, KGF (FGF7), VEGF, angiogenin, SCF, SDF-1, PIGF, IGFBP-2, -3, -4, -6, -7, proteases and regulators MMP-1, -3, -10, -12, -13, -14, TIMP-1, TIMP-2, PAI-1, -2; tPA; uPA, cathepsin B and other inflammatory factors such as GM-CSE, G-CSE, IFN-γ, BLC and MIF. Figure 2 displays soluble factors sectreted by PRE and SEN cells. Soluble or shed receptors or ligands include ICAM-1, -3, OPG, sTNFRI, TRAIL-R3, Fas, sTNFRII, Fas, uPAR, SGP130, EGF-R. The non-protein soluble factors secreted within SASP are PGE2, ritric oxide, and reactive oxygen species. Lastly, insoluble factors of the extracellular matrix include fibronectin, collagens and laminin (J. P. Coppé et al. 2010).

These molecules are certain SASP factors such as IL-6, IL-8, GROα, and IGFBP-7, acting in an autocrine feedback loop to reinforce the senescence growth arrest via cooperation with the pRb and p53 tumor suppressor pathways in order to reduce the risk of malignant transformation in a cell-autonomous manner. Among CC ligand chemokine family members that are being upregulated in senescent cells are: MCP-2, -4, -1 (CCL-8, -13, -2), HCC-4 (CCL-16), eotaxin-3 (CCL-26), MIP-3α, and -1α (CCL-20,-3) (Bode-B&ouml et al. 2005)(Acosta et al. 2008).
Interestingly, despite p53 being one of the central mediators of the senescence growth arrest, is not required for the SASP. In fact, p53 inactivation in senescent cells enhances the expression and secretion of many SASP factors, although, the mechanism of that enhancement is yet to be discovered (Coppé et al. 2008).

One more feature of the SASP is its dynamic development over time (Coppé et al. 2008). It was stated, that in culture, cells develop a full SASP >5 days after senescence induction, whilst the SAGA develops within 24 h of damage. Not all SASP factors begin to be secreted at the same time. This gradual phenotypic transition is a feature conserved between cell types and senescence inducers. Genetic alterations, such as loss of p53 or gain of oncogenic RAS, lead to a more rapid acquisition of the SASP, suggesting that the SASP is a specific program triggered by genotoxic stress (J. P. Coppé et al. 2010).

In addition to the cell-autonomous tumor suppressor function of senescence, the paradoxical protumorigenic effects and contribution to age-related pathologies of the SASP have been noted (J. P. Coppé et al. 2010).

The majority of insoluble components of the ECM serve as enzymatic targets of secreted proteases. The accumulation of senescent cells could lessen the supportive role of the ECM due to changes in the senescent-associated proteolytic activity, therefore affecting the tissue structure, in particular tension and elasticity. Moreover, the more relaxed tissue structure coupled with elevated levels of matrix metalloproteinases (MMPs) could facilitate the movement and infiltration of tumor cells through the ECM, thereby promoting metastasis. The variety of proteases secreted by senescent cells extensively overlaps with those found in malignant tumors (J. P. Coppé et al. 2010).

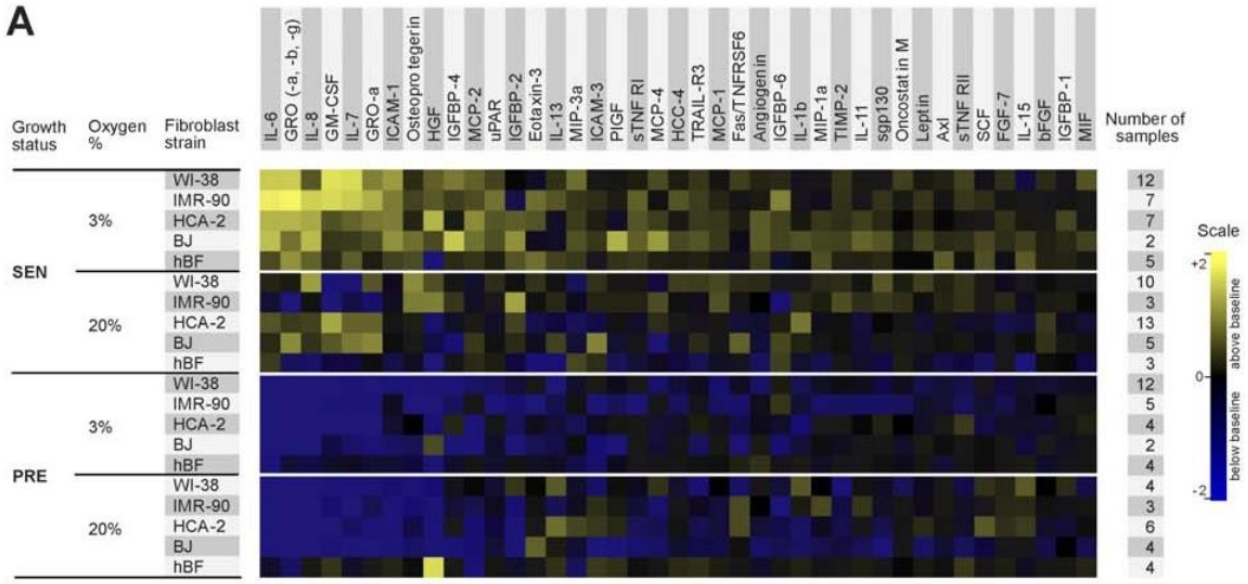

Figure 2. Soluble factors secreted by indicated cells. For each strain of cells, the PRE and SEN signals were averaged and used as the baseline. Signals above baseline are shown in yellow; signals below baseline are displayed in blue (Coppé et al. 2008).

4.2 Soluble Signaling Factors

Some of the most abundant soluble inflammatory signaling factors that are going to be discussed in this thesis include chemokines, which are a large family of small peptidic chemotactic cytokines that mediate communication between different cell types and are mainly involved in leukocyte chemoattraction (Mantovani, Bonecchi, and Locati 2006). Extracellular proteases have the ability to alter the microenvironment of the cells by cleaving membrane-bound proteins, degrading signaling molecules, and remodeling the extracellular matrix (Hornebeck and Maquart 2003).

Other soluble factors secreted by senescent cells include colony-stimulating factors (CSFs) like GM-CSF and G-CSF. Additionally, osteoprotegerin, which acts as a decoy receptor for tumor necrosis factor alpha, is found in abundant quantities in the extracellular environment of these cells. At senescence, other molecules such as prostaglandin E2 (PGE2) and Cox-2, the enzyme

that synthesizes PGE2 and other prostaglandins, are also significantly upregulated (J. P. Coppé et al. 2010).

Several key components of the SASP have been subjected to a more thorough investigation. Interleukin-6 (IL-6) is the most notable cytokine of the Senescence-Associated Secretory Phenotype (SASP). It's a multifunctional proinflammatory cytokine and it has been found to be linked with DNA damage and oncogenic stress-induced senescence in various cells such as keratinocytes, melanocytes, monocytes, fibroblasts, and epithelial cells of both mice and humans (Kuilman et al. 2008a). Moreover, IL-6 secretion seems to be directly regulated by continuous DNA-damage signaling (ATM and CHK2), independent of the p53 pathway (Rodier et al. 2009). Senescent cells can directly influence neighboring cells via the secretion of IL-6, particularly targeting cells that express the IL-6R (gp80) and gp130 signaling complex. That predominantly affects epithelial and endothelial cells of various functions and origins, thereby propagating the inflammatory responses characteristic of cellular senescence. Knockout of the janus kinase 2 (JAK2) (an upstream activator of the signal transducer and activator of transcription 3 (STAT3)) inhibition, has been found to modify the SASP composition without impacting the onset of senescence and the subsequent cell growth arrest (Toso et al. 2014). Particularly, targeting STAT3 diminishes the secretion of chemokines involved in myeloid-derived suppressor cell (MDSC) polarization, such as M-CSF, CXCL2, GM-CSF, IL-13, and IL-10, without influencing the levels of CCL2 (MCP-1) and CXCL10 (IP-10). Figure 3 displays the secretion of Il-6, Il-8 and p16, which is s is a cyclin-dependent kinase inhibitor in presenescent and senescent fibroblasts.

Interleukin 8 (IL-8), also termed chemokine C-X-C motif ligand 8 (CXCL8), is one of the most abundant factors produced by senescent cells. It is associated with inflammatory response as it serves as a chemoattractant (attracts neutrophils to sites of inflammation) and as a potential angiogenic factor (Brennan and Zheng 2007).

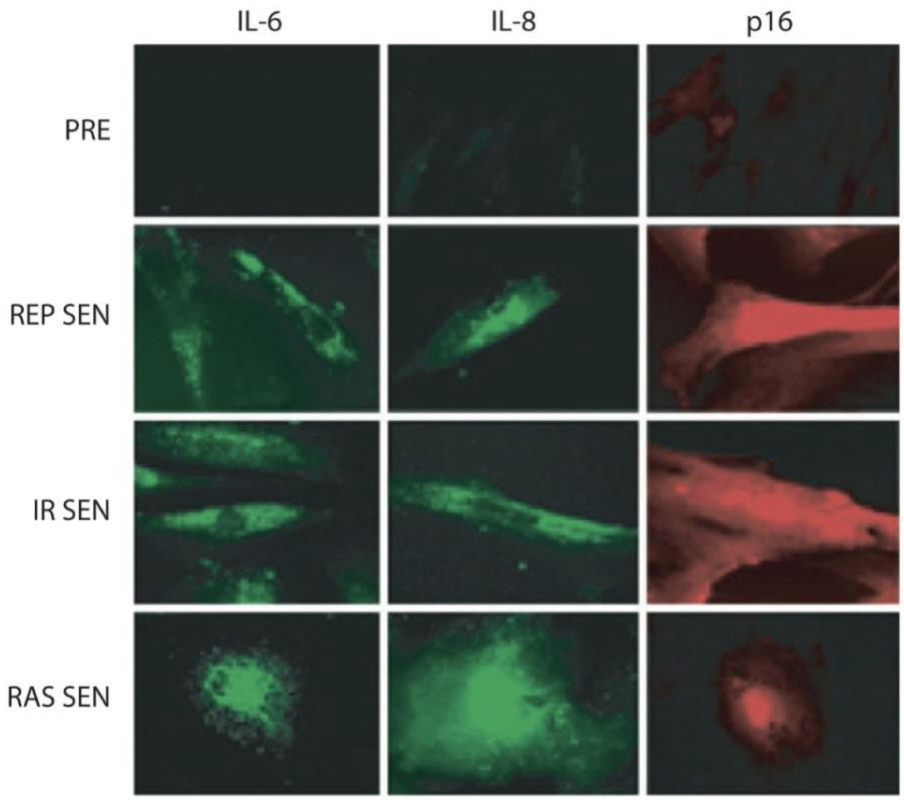

Figure 3. Inflammatory cytokines interleukin IL-6 and IL-8, along with the senescence marker p16 were detected in pre senescent (PRE) or senescent (SEN) human fibroblasts. The cells were induced into a senescent state through various methods, including replicative exhaustion (REP), exposure to ionizing radiation (IR), or the expression of oncogenic RAS (RAS) (J. P. Coppé et al. 2010).

One of the most studied interleukins among SASP is IL-1. The pathway of IL-1 is another one that has been empirically shown to be upregulated by senescent cells (J. P. Coppé et al. 2010). The cytokine interleukin-1 (IL-1) plays a crucial role in the inflammatory response. Many of the proinflammatory effects of IL-1, such as the up-regulation of cell adhesion molecules on vascular endothelia, occur at the level of transcriptional regulation. The transcriptional activation by IL-1 of cell adhesion molecules and other genes involved in the inflammatory response is largely mediated by NF-κB. In response to IL-1, the NF-κB inhibitory factor IKB is degraded, and NF-κB is released from its inactive cytoplasmic state to localize within the nucleus where it binds DNA and activates transcription, thus the key function of interleukin-1 (IL-1) is the activation of the transcription factor NF-κB (Croston, Cao, and Goeddel 1995).

Both IL-1α and IL-1β have the potential to influence adjacent cells via cell-surface receptors (IL-1 receptor/Toll-like receptor superfamily). The primary function of these receptors is to initiate the nuclear factor kappa B and activating protein 1 pathways (Mantovani et al. 2001). IL-1, particularly IL-1α, has been identified as a key positive regulator of IL-6 and IL-8 expression and secretion by senescent human fibroblasts through continuous stimulation of the IL-1R by cell surface-associated IL-1 Orjalo et al. 2009).

4.3 Extracellular Proteases

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that degrade various proteins in the extracellular matrix (ECM). The MMP family members that are consistently upregulated in human and mouse fibroblasts undergoing replicative or stress-induced senescence are stromelysin-1 and -2 (MMP-3 and -10, respectively) and collagenase-1 (MMP-1) (West et al. 1989). In some instances, the MMP-1 and -3 produced by senescent cells can also regulate the activity of the soluble factors present in the SASP. For example, these MMPs can cleave MCP-1, -2, and -4 and IL-8. A variety of other CXCL/CCL family members that constitute the SASP can also be cleaved by MMP-9, -2, or -7. These CXCL and CCL cytokines can originate from neighboring cells, such as leukocytes or tumor cells (Van Den Steen et al. 2003).

Further members of the proteases family involved in SASP-mediated tumor progression are serine proteases, along with the regulators of the plasminogen activation pathway. These factors include urokinase- or tissue-type plasminogen activators (uPA or tPA), the uPA receptor (uPAR), and inhibitors of these serine proteases, specifically PAI-1 and PAI-2 (Blasi and Carmeliet 2002).

4.4 Extracellular Insoluble Molecules and Nonprotein Secretions.

Fibronectin is one of the main glycoproteins of the extracellular matrix, it influences cell behaviour and tissue remodelling via the interaction with a wide range of macromolecules, including cell-surface receptors (integrins), components of the cytoskeleton, and other ECM molecules.
Senescent cells in culture and in vivo increase fibronectin expression (Kumazaki, Kobayashi, and Mitsui 1993).

4.5 Regulation of the Senescence-Associated Secretory Phenotype: Transcriptional and Post-Transcriptional Mechanisms

Various mechanisms have been implicated in the mediation of SASP, its variability and complexity. These mechanisms are yet to be studied, although, it is well recognized, that SASP is regulated on different levels, such as transcription, translation, mRNA stability, and secretion. The extensive scheme of the pathways leading to senescence is shown in Figure 4.

SASP is known to be associated with genotoxic stress (DNA damage response), DDR includes activation of sensor kinases (ATM/ATR, DNA□PK), formation of DNA damage foci that contain activated H2A.X histone protein (γH2A.X), and the induction of cell cycle arrest through activation of checkpoint proteins, specifically p53 (TP53) and the CDK inhibitor p21 (CDKN1A) (D'Adda Di Fagagna et al. 2003).

Secondly, most components of the SASP are upregulated at the level of mRNA abundance (Coppé et al. 2008). As it was mentioned earlier, the increase in mRNA levels of many factors depends on the transcription factors NF-κB and C/EBPβ, which have increased activity in senescent cells, are responsible for the increase in mRNA levels of many factors (Kuilman et al. 2008). Depletion of C/EBPβ transcription factor notably decreases the expression of both IL-6 and IL-8, which are among the most strongly upregulated SASP cytokines (Freund et al. 2010).

Several other transcription factors have been implicated in the upregulation of SASP, specifically p38 MAPK and GATA4 (Chien et al. 2011). p38MAPK is a novel DNA damage response-independent regulator of the SASP.
PTBP1 controls the alternative splicing of genes involved in intracellular trafficking such as EXOC7, thereby modulating the SASP (Georgilis et al. 2018).

The mTOR pathway has been implicated in the regulation of the SASP. It was found that mTOR accumulates at (auto)lysosomes and promotes the production of interleukin-6 (IL-6) and IL-8 in senescent cells (Salama et al. 2014).

Activation of the mTOR pathway promotes the translation of IL-1A, which activates transcription factors involved in SASP regulation. Cells that undergo oncogene-induced senescence secrete multiple IL2 (CXCR2)-binding chemokines in a program regulated by the NF-κB and C/EBPβ transcription factors and coordinately induce CXCR2 expression (Acosta et al. 2008).

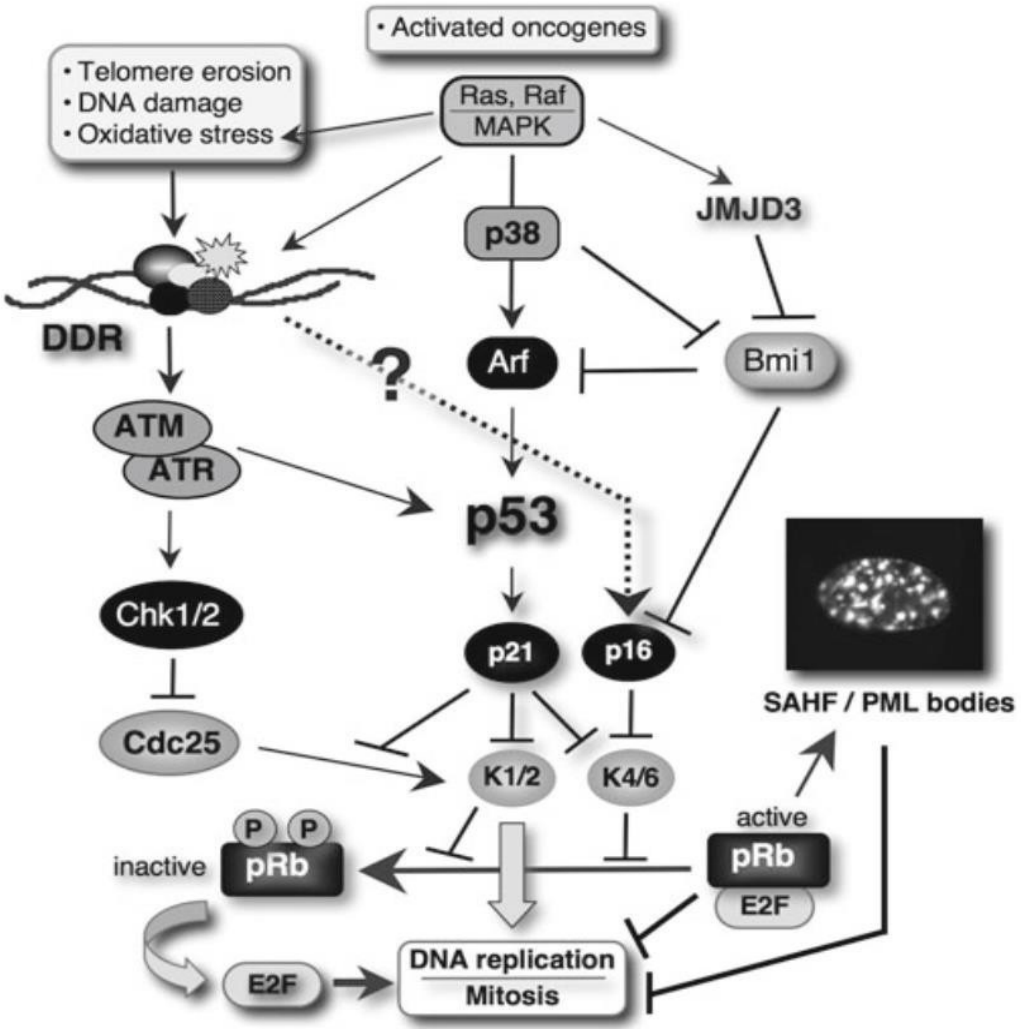

Figure 4. Molecular pathways leading to senescence-associated irreversible cell cycle arrest (Dulic 2013).

5. The mechanistic target of rapamycin (mTOR) pathway

5.1 Overview of mTOR Signaling

Senescence is induced by a variety of factors, normally classified as cytotoxic or mitogenic stimuli. These stimuli are known to initiate cell cycle arrest without blocking cellular growth (Blagosklonny 2006). In general, mitogens -- external stimuli that induce mitosis and cell division (growth factors like IGF-I and EGF). They trigger mitogen-activated pathways such as PI3-K, Ras, Raf-1, MEK, and Akt. Both PI-3K/Akt and Ras/Raf-1/ERK signaling pathways activate the target of rapamycin (mTOR) (Schmelzle and Hall 2000). Moreover, the initiation factor of translation (eIF-4E), which is a downstream effector of mTOR, has been shown to induce senescence (Ruggero et al. 2004). In this way, mTOR is one of the pathways responsible for one of the hallmarks of senescence, the enlargement of the cell, as it increases cell growth. Thus, to reverse senescence, it is critical to inhibit mitogen-activated pathways, especially mTOR.

Exploring the mechanistic Target of Rapamycin (mTOR) in greater detail, it resides at the nexus of an extensive signaling pathway that governs cell growth and metabolic processes across the eukaryotic domain. It is a conserved Ser/Thr kinase that orchestrates metabolic regulation and cell growth depending on environmental cues (Saxton and Sabatini 2017).

Genomic analyses reveal the ubiquity of the TOR gene across eukaryotes, with variations in gene count observed between species; for instance, while certain yeast species harbor two TOR genes, higher eukaryotes typically possess a singular TOR gene, indicating a conserved yet complex evolutionary trajectory of the TOR signaling pathway (Crespo and Hall 2002). Eukaryotic TORs, with a molecular weight of approximately 280 kDa proteins exhibit 40%–60% similarity in their primary sequence and belong to a group of kinases known as the phosphatidylinositol kinase-related kinase (PIKK) family. This family is characterized by a C-terminal Ser/Thr protein kinase domain that resembles the catalytic domain of phosphatidylinositol 3-kinases (PI3Ks) and PI4Ks. The amino-terminal to the kinase domain in TOR is the FKBP12-rapamycin binding domain (Wullschleger, Loewith, and Hall 2006). Notably, the FKBP12-rapamycin binding domain precedes the kinase domain, distinguishing TOR from its PIKK counterparts by lacking detectable lipid kinase activity, yet functioning as a Ser/Thr kinase.

(Tee and Blenis 2005)

5.2 The Central Role of mTOR in Cellular Regulation

In response to surrounding changes, such as variations in amino acid concentrations and energy availability, as well as stimulation by hormones and mitogens, cells efficiently alter their gene expression. A crucial player in this adaptation is the mechanistic target of rapamycin (mTOR), which regulates the translation of mRNA into proteins. This regulation is achieved by phosphorylating at least two essential translational regulators, enhancing protein synthesis. Particularly, hormones and growth factors activate G protein coupled receptors and receptor Tyr kinases, which trigger several key pathways that carry intracellular signals. Specifically, the phosphoinositide 3 kinase (PI3K)–AKT pathway and the Ras–ERK (extracellular signal regulated kinase) pathway activate mTORC1 signalling through inhibiting the negative regulator of mTORC1 —the tumour suppressor complex TSC1–TSC2 (Shaw and Cantley 2006). Inhibition of this complex is mediated mainly through the phosphorylation of TSC2 by several upstream kinases, including AKT, ERK, and ribosomal S6 kinase (RSK) (Ma and Blenis 2009).
The mTOR pathway intricately controls cellular size and G1 phase progression through its effector proteins, S6 kinases (S6Ks), and eukaryotic initiation factor 4E-binding proteins (4EBPs), illustrating the pathway's central role in integrating nutrient and growth factor signals (Wullschleger et al. 2006), (Fingar and Blenis 2004).

mTOR is named after a molecule that derives its name from rapamycin, an anti-fungal immunosuppressive macrolide produced by the bacterial species *Streptomyces hygroscopicus*, which was isolated from a soil sample from the Eastern Islands in the 1970s (Vezina, Kudelski, and Sehgal 2011). Rapamycin is known for its potent ability to inhibit cell growth and proliferation. mTOR kinase, the central component of the pathway, interacts with several proteins nucleating two distinct multi-protein complexes: MTORC1 and MTORC2 (Zoncu, Efeyan, and Sabatini 2010). Through these two complexes mTOR controls the translation.

The first link between aging and mTOR was found in S. cerevisiae. The deletion of the gene encoding the S6K orthologue in yeast – SCH9 resulted in the doubling of the lifespan (Fabrizio et al. 2001). The specific role of MTORC1 in longevity was identified through the RNA interference (RNAi) knockdown of mTOR (let-363) or the mTORC1 component raptor (daf-

15), which extended the lifespan in the nematode Caenorhabditis elegans (Jia, Chen, and Riddle 2004). In various genetic mouse models, a reduction in insulin/IGF-1-like signaling (IIS) (upstream of TOR) has been shown to increase lifespan. Reducing TOR activity is associated with increased lifespan not only in rodents, but in humans as well. It is observed that most, if not all, mutations that extend lifespan interact with the TOR pathway. Lifespan extension caused by reduced IIS is mediated by the FOXO family of transcription factors. Moreover, numerous studies have linked polymorphisms in FOXO3 with increased longevity in humans (Kenyon 2010).

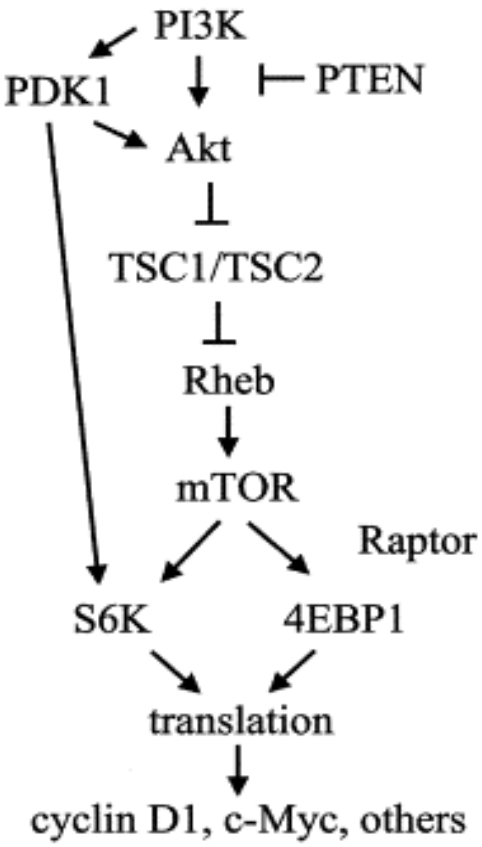

Figure 5. The illustration presents the current understanding of how mTOR is regulated via the PI3K/Akt pathway, as informed by biochemical and genetic research
(Sawyers 2003).

5.3 Regulation and Functions of mTORC1 and mTORC2

MTORC1 and MTORC2 regulate different branches of the mTOR network (Sarbassov et al. 2006). Regulating unique aspects of cell growth, these complexes have their own distinctive set of accessory proteins and differential sensitivity to rapamycin. Specifically, MTORC1 is the entity directly involved in the regulation of protein synthesis, activated in a GTP-dependent manner by the small GTPase protein Rheb (Ras homolog enriched in brain) (Long et al. 2005).

TSC2 serves as a crucial link connecting mTOR to the mitogen sensing PI3K signaling cascade, with Akt (upon activation by PI3K) phosphorylating and inactivating TSC2. Moreover, TSC2 controls TOR's ability to detect cellular energy levels. The AMP-activated and LKB1-dependent kinase, AMPK, phosphorylates and activates TSC2, leading to a reduction in TOR activity when cellular energy. In this way, AMPK is a conserved sensor of energy level which is activated in response to low ATP, thus, negatively regulating the mTORC1. Lastly, TOR has been associated with nutrient sensing via the Ras-like GTPase, Rheb, which is regulated by the GTPase-activating protein (GAP) activity of the TSC1/2 complex. The significance of mTOR and cell growth in tumor development is highlighted by its position downstream of the tumor suppressors TSC1, TSC2, LKB1, and PTEN, and by the growing effectiveness of the mTOR inhibitor rapamycin and its analogs against cancers caused by PI3K pathway misregulation (shown in Figure 5) (Richardson et al. 2004).

MTORC1 contains at its core mTOR, mLST8, FKBP12, DEPTOR, and the scaffold protein RAPTOR (regulatory associated protein of mTOR). MTORC1 integrates nutrient, growth-promoting, and stress-related signals and translates these inputs into adaptive responses that tune the balance between anabolism and catabolism. The Serine/Threonine kinase TOR, along with its associated proteins Raptor (regulatory associated protein of TOR), mLst8, PRAS40, and Deptor in mammals, forms a complex that oversees and integrates a wide range of intra- and extracellular parameters. This complex regulates cell size, proliferation, and lifespan through various downstream pathways (Guertin and Sabatini 2005). mTORC1 is known to regulate protein synthesis by phosphorylating key substrates involved in translation initiation and elongation (Thoreen et al. 2012).

mTORC1 communicates with two key players involved in cap-dependent translation initiation: 4E-binding protein 1 (4E-BP1), also known as EIF4EBP1 and 40S ribosomal protein S6 kinase (S6K) (Hao et al. 2020). One of the main ways mTORC1 regulates translation is by phosphorylating the eukaryotic initiation factor 4E-binding proteins (4E-BPs). mTORC1 phosphorylates 4E-BPs, leading to the release of eIF4E (eukaryotic translation initiation factor 4E). When 4E-BPs are hypophosphorylated, they bind to eIF4E and inhibit its interaction with the mRNA cap structure, thereby repressing translation initiation. Phosphorylation of 4E-BPs by mTORC1 results in their dissociation from eIF4E, allowing eIF4E to interact with eIF4G and form the eIF4F complex, which facilitates the recruitment of the ribosome to the mRNA

and enhances translation initiation, as shown in Figure 6 (Ma and Blenis 2009). The study by Carson et al found that the subset of mRNAs specifically regulated by mTORC1 consists almost entirely of transcripts with established 5′ terminal oligopyrimidine (TOP) motifs, or those with previously unrecognized TOP or related TOP-like motifs. This indicates a straightforward mechanism of mTORC1-dependent translation control focused on these specific mRNA features (Thoreen et al. 2012). mTORC1 also phosphorylates and activates S6K1, which in turn phosphorylates ribosomal protein S6 and other targets involved in translation initiation. Phosphorylation of S6 by S6K1 promotes ribosome biogenesis and protein synthesis. Additionally, S6K1 can regulate other cellular processes, such as cell growth, proliferation, and metabolism, through its downstream targets (Richardson et al. 2004).

Recent data point to the very specific role of mTORC1 in cell dynamics: its interaction with 4E-binding proteins (4E-BPs) is not able to directly affect cellular growth but modulates the proliferation by enabling the translation of key proteins in the cell cycle progression, such as cyclin D3 (Dowling et al. 2010).

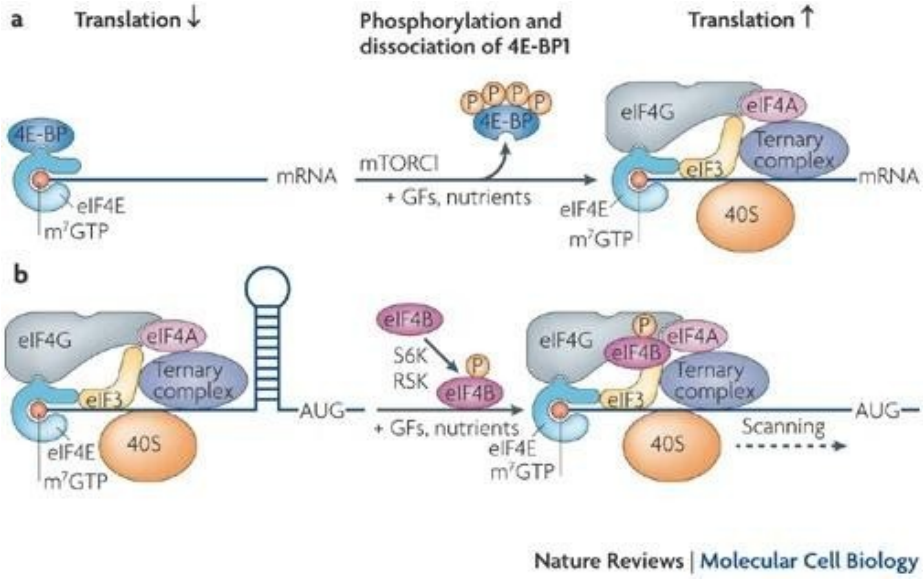

Figure 6. mTOR-dependent signaling through the 4E-BP1/eIF4E, S6K1 and eEF2K/eEF2 pathways (Tee and Blenis 2005).

mTORC2 complex is comprised of several components: mTOR, mSIN1 (mammalian stress□activated protein kinase□interacting protein 1), DEPTOR, and the scaffold protein RICTOR (rapamycin□independent companion of mTOR) (Laplante and Sabatini 2009). It has

been suggested that RICTOR and mSIN1 mutually reinforce each other, thereby forming the structural basis of mTORC2 (Polak and Hall 2006).

mTORC2 plays a pivotal role in several essential cellular processes. Evidence from RNAi-mediated knockdown of RICTOR in cultured cells has shown that mTORC2 orchestrates dynamic rearrangements of the cytoskeleton and the activation of pro-survival pathways in response to growth-promoting signals (Frias et al. 2006).

In contrast to mTORC1, mTORC2 is not believed to interact with FKBP12-rapamycin and is generally considered to be insensitive to rapamycin. mTORC2 is responsible for the phosphorylation and activation of Akt/PKB, which plays a crucial role in cell survival. It was indicated that rapamycin disrupts the formation of mTORC2 and that extended exposure to rapamycin in various cell types diminishes mTORC2 levels below the threshold required to sustain Akt/PKB signaling. The cells expressing a rapamycin resistant Akt/PKB mutant suppress the proapoptotic and antitumor effects of rapamycin (Sarbassov et al. 2006).

5.4 mTOR inhibitors in clinical use

In therapeutic approach mTOR inhibitors, such as rapamycin, are used to prevent organ transplant rejection and restenosis, and they are also considered promising therapies for cancer treatment (Meric-Bernstam and Gonzalez-Angulo 2009).
They are known to prevent the cell from the mTOR-driven proliferative response to nutrient availability and mitogenic stimuli. Even though mTOR inhibitors are used clinically as immunosuppressants, they are being investigated as anticancer drugs in recent years (Huang, Bjornsti, and Houghton 2003).

6. The role of mTOR in senescence and SASP

6.1 MTOR controls the pro-tumorigenic senescence-associated secretory phenotype by enhancing the translation of IL1A

Two recent studies have revealed that the mechanistic target of rapamycin (mTOR) kinase, a pivotal regulator of protein synthesis, also has a significant influence on the Senescence-

Associated Secretory Phenotype (SASP) by affecting mRNA translation, transcription, and stabilization.

In order to test the hypothesis that mTOR activity and SASP are linked Laberge et. Al. exposed senescent human fibroblasts to rapamycin, the selective MTORC1 complex inhibitor. Then they evaluated the secretion of pro-inflammatory cytokines, especially IL-6, one of the most abundant SASP components, which is displayed in figure 7 (Laberge et al. 2015).

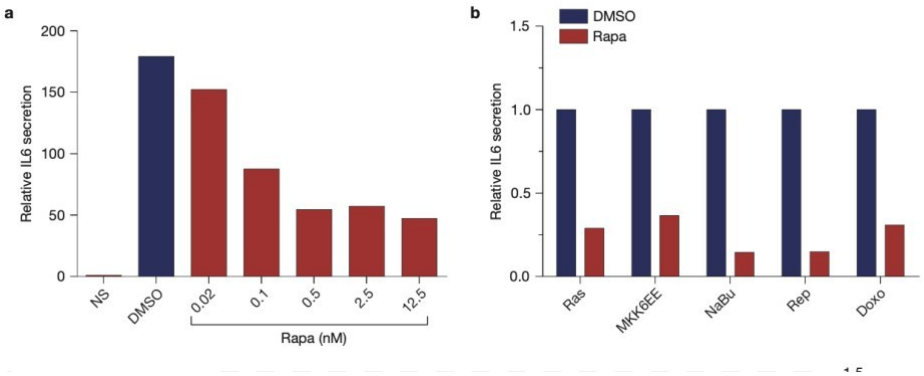

Figure 7 (a) displays that the secretion of IL-6 by rapamycin-treated fibroblasts is dose-dependent (Laberge et al. 2015).

(b) It is shown that rapamycin has been observed to inhibit the secretion of IL6 by human fibroblasts in which senescence was induced by various stimuli. In fibroblasts that were not senescent, rapamycin slightly reduced the minimal basal secretion of IL6. Moreover, rapamycin did not significantly alter the number of 53BP1 foci in senescent HCA2 or PSC27 cells, suggesting that it acts downstream of DNA damage response (DDR) signaling. Additionally, shRNA-mediated depletion of the mTORC1 component raptor or mTOR itself severely blunted IL6 secretion by senescent cells, further confirming the dependency of SASP production on mTOR activity (Laberge et al. 2015).

They observed that rapamycin significantly decreased IL6 secretion by three strains of normal human fibroblasts (HCA2 neonatal foreskin, WI-38 fetal lung, and PSC27 adult prostate), and two strains of immortal, but non-tumorigenic, human breast epithelial cell lines (MCF-10A and 184A1).

Rapamycin has been shown not only to reduce IL-6 secretion, but other SASP components as well, including several pro-inflammatory cytokines, chemokines and growth factors. Several SASP proteins were not affected, showing that rapamycin is a selective SASP modulator. Notably, all of the rapamycin-sensitive SASP factors were previously identified as targets of the NF-κB transcription factor (Freund, Patil, and Campisi 2011).

Interestingly, this dependency isn't just a result of the general decrease in protein synthesis seen with mTOR inhibition. Instead, the influence of rapamycin on these SASP components primarily occurs at the mRNA level, suggesting a specific regulatory role of mTOR on gene expression rather than a broad effect on protein production (Laberge et al. 2015).

While MTORC1 is mainly a translational regulator, the majority of SASP proteins are upregulated at the mRNA abundance level (Acosta et al. 2008). NF-κB stimulates the transcription of numerous SASP genes (Freund et al. 2011).

It has been previously identified that NF-κB and IL1A form a positive feedback loop that ultimately triggers the transcription of various genes encoding inflammatory cytokines. IL-1α, predominantly associated with the cell surface, serves as an early trigger for a pro-inflammatory network within the SASP by activating NF-κB, thus leading to the transcription of genes encoding inflammatory cytokines like IL-6 and IL-8 (Orjalo et al. 2009). IL1A is bound to the cell surface and is not secreted by the senescent cells. Although its presence on the surface of these cells significantly increases, as it plays a crucial role in the formation and maintenance of the SASP. The reduction of IL1A in senescent cells through shRNA led to a decrease in IL-6 secretion, a suppression that mirrors the effect caused by rapamycin. That suggests that the inhibition of mTORC1 could reduce the secretion of certain SASP components by interfering with the IL1A-NF-κB feedback loop (Laberge et al. 2015) (Orjalo et al. 2009). IL1A can bind to its cell surface receptor (IL1R1) through juxtacrine interaction, triggering a signaling cascade that ultimately leads to the degradation of IRAK1 (interleukin-1 receptor-associated kinase 1) and IκBα (also known as NFKBIA, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha), facilitating the nuclear translocation of NF-κB (Freund et al. 2010). Supporting this, Laberge et al. found that rapamycin treatment reduces the upregulation of IL-1α during senescence, differing from other SASP factors where the reduction is mostly at the transcriptional level. Instead, for IL-1α, the decrease is attributed to a drop in translation

efficiency, suggesting that mTOR specifically enhances the translation of IL1A mRNA, which encodes IL-1α, thereby stimulating NF-κB.

6.2 TOR – autophagy spatial coupling compartment (TASCC)

Cells undergoing oncogene-induced senescence (OIS) control their secretory characteristics by coordinating protein synthesis and autophagy within the TOR–autophagy spatial coupling compartment (TASCC). The TASCC is a distinct cellular compartment at the trans side of the Golgi apparatus, where (auto)lysosomes and mTOR are being accumulated during Ras-induced senescence (Narita et al. 2003).

The spatial coupling of mTOR and autophagy in the TASCC compartment during senescence indicates a possible mechanism for synchronizing protein synthesis and degradation, which may alter the production and secretion of SASP factors (Narita et al. 2011). Disruption of mTOR localization to the TASCC has been demonstrated to suppress interleukin-6/8 synthesis, suggesting a direct link between mTOR activity and SASP regulation.

6.3 mTOR controls the senescence-associated secretory phenotype by regulating the translation of MAPKAPK2

Herranz et. Al provides a distinct view on mTOR's regulation of the SASP. According to their findings, mTOR controls the translation of mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK2) through 4EBP1. MAPKAPK2 phosphorylates and directly suppresses the RNA-binding zinc finger protein 36L1 (ZFP36L1), involved in AU-rich element (ARE)-mediated decay that targets SASP components (Herranz, Gallage, and Gil 2015). Phosphorylation of ZFP36L1 by MAPKAPK2 at S54, S92, and S203 inhibits its binding to mRNAs thereby preventing the degradation of mRNA that encodes SASP components (Herranz, Gallage, Mellone, et al. 2015).

The team discovered that by inhibiting mTOR with the ATP-competitive mTOR kinase inhibitors, such as Torin1, the translation of MAPKAPK2 is significantly reduced, leading to less phosphorylation (and thus, activation) of ZFP36L1. As a result, ZFP36L1 is able to degrade mRNA transcripts encoding SASP factors, effectively suppressing the SASP. They identified

MAPKAPK2 (also referred to as MK2) as a particular target of mTOR-controlled translation during the process of senescence. MAPKAPK2, which is a downstream effector of p38 MAPK, has been demonstrated to phosphorylate the RNA-binding protein ZFP36L1, thereby inhibiting its activity in AU-rich element (ARE)-mediated mRNA decay (AMD). They put forth a model suggesting that mTOR specifically enhances the translation of MAPKAPK2 during senescence, which in turn inhibits the AMD activity of ZFP36L1, an mRNA-binding protein involved in ARE-mediated decay that targets certain SASP components (Herranz, Gallage, Mellone, et al. 2015).

7. Conclusion

The evidence from prior research shows that mTOR inhibitors blunted the protumorigenic SASP effect but did not reverse the proliferative arrest. This dual functionality is particularly important and beneficial due to the fact that the SASP has both tumor-promoting and tumor-suppressing roles depending on the context (Laplante and Sabatini 2012). However, it is critical to consider both the cell-autonomous and noncell-autonomous activities of senescence to maximize the potential benefits of senescence induction during cancer therapy. It has been shown that the SASP can also be tumor suppressive not only in preneoplastic tumors (represented by OIS) but also in full-blown cancer contexts, through both anti-proliferative effects.

The mechanism by which mTOR suppresses the SASP is by specifically reducing the translation of MAPAPK2. MAPKAPK2 phosphorylates and inhibits ZFP36L1, an mRNA-binding protein that is involved in ARE-mediated decay that targets SASP components. Consequently, mTOR inhibition leads to not only decreased translation but also reduced mRNA levels of the SASP (Herranz, Gallage, Mellone, et al. 2015).

Furthermore, it has been suggested that mTOR inhibition may shift cells from senescence to quiescence. Activation of mTOR initiates several negative feedback signals to the PI3K pathway, which can be further activated by inhibiting mTOR (Demidenko et al. 2009).

Understanding of the mechanism of SASP is crucial, as it could serve as a foundation for designing the strategy to reduce damaging effects of the process of aging. Careful consideration of mTOR inhibition of the SASP either by rapamycin analogs, ATP competitors, or mTOR/PI3K dual inhibitors might broaden its applications in cancer therapy.

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