Chapter 10
Potential Applications of Aptamers for Targeting Senescent Cells

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Abstract Cellular senescence is a stress response characterized by a permanent loss of proliferative ability. Different types of senescence reflect different forms of stress. Although cellular senescence plays a key role in maintaining tissue homeostasis and in suppressing tumorigenesis, it is widely appreciated that accumulation of senescent cells in tissue contributes to development of age-related dysfunctions and limits the efficacy of chemo- and radiotherapy. Consequently, novel therapeutic strategies targeting senescent cells, or their pro-inflammatory secretome, have been recently developed. The application of these novel therapeutic approaches requires biomarkers for in vivo detection of senescent cells for ageing and age-associated diseases, as well as for therapy-induced senescence in cancer patients. Aptamers are short oligonucleotide or peptide sequences able to bind with high affinity and specificity a wide range of cellular targets, including cell surface epitopes, thanks to their unique three-dimensional folding. Oligonucleotide aptamers have been widely applied for the discovery of biomarkers, since the early 1990s, and Affimers have recently emerged as valuable tools for biomarker discovery. In this chapter we briefly introduce applications of aptamer technologies for recognition of cell surface antigens and suggest that aptamers provide the perfect technology to identify novel senescence-specific biomarkers.

Keyword Senescence SASP · Senotherapy · Biomarkers · Aptamers · Affimers

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10.1   Cellular Senescence and the Growing Field
of Senotherapy

Cellular senescence is a stress response characterized by a permanent loss of pro-
liferative ability that can be activated by different damaging stimuli. As such, cellu-
lar senescence represents an evolutionarily conserved mechanism that prevents the
proliferation of damaged and potentially tumorigenic cells. Senescence also partici-
pates in mammalian embryonic development and critically contributes to embryonic
remodeling and patterning (Muñoz-Espín et al. 2013; Storer et al. 2013). The acti-
vation of p16^{INK4a}-pRb and p53-p21^{CIP1} signaling pathways sustain the senescence
cell cycle arrest. Cellular senescence is frequently associated with irreparable DNA
damage and a persistent DNA damage response (DDR) (Hewitt et al. 2012; Fuma-
galli et al. 2012; Sedelnikova et al. 2004), which is triggered as a result of telomere
shortening during replicative senescence (d'Adda di Fagagna et al. 2003), oncogene
activation in oncogene-induced senescence (OIS) (Bartkova et al. 2006; Di Micco
et al. 2006; Hills and Diffley 2014), and radiotherapy or chemotherapy in therapy-
induced senescence (TIS) (te Poele et al. 2002; Mirzayans et al. 2005). However,
senescence may also be induced in the absence of DNA damage. For instance, PTEN
inactivation induces senescence in the absence of DDR activation: PTEN-loss acti-
vates mTOR which enhances p53 translation and p21 expression, thus leading to per-
manent cell cycle arrest (Alimonti et al. 2010). Induction of cellular senescence with
drugs that modify chromatin structure, such as histone deacetylase inhibitors, occurs
in the absence of DNA damage (Pazolli et al. 2012). Sodium butyrate induces p21
expression and causes cellular senescence. Interestingly, sodium butyrate induced-
senescent cells display activation of DDR proteins, such as gamma-H2AX foci, with-
out detectable DNA damage (Pospelova et al. 2009). Curcumin treatment induces
senescence in vascular smooth muscle cells in a DNA damage-independent manner,
although activation of ATM is observed (Grabowska et al. 2015). Finally, develop-
mental senescence is not associated with DNA damage (Muñoz-Espín et al. 2013;
Storer et al. 2013).

Cellular organelles undergo senescence-specific alterations and critically con-
tribute to the development of the senescent phenotype. Enlargement of the lysoso-
mal compartment likely explain the increased activity of a well-established marker
of senescence, the so-called Senescence-Associated beta-galactosidase (SA-β-gal)
(Kurz et al. 2000). The DDR promotes PGC-1b-dependent mitochondrial biogenesis,
thereby increasing mitochondrial content in senescent cells. Mitochondria critically
contribute to cellular senescent-associated changes (Correia-Melo et al. 2016).

Senescent cells are transcriptionally and metabolically active, and develop a dis-
tinctive phenotype characterized by a flat and enlarged morphology. In addition,
senescent cells acquire an enhanced secretory ability, called senescence-associated
secretory phenotype (SASP) or senescence messaging secretome (SMS) (Coppé et al.
2008; Kuilman and Peeper 2009). The release of proinflammatory SASP factors is
critical for reinforcing the growth arrest, for triggering immune-mediated removal of
senescent cells and for activating wound healing (Salama et al. 2014; Serrano 2014).

Main transcriptional regulators of the SASP include NF-κB and C/EBPβ that cooperatively induce SASP expression (Acosta et al. 2008; Kuilman et al. 2008; Chien et al. 2011; Crescenzi et al. 2011). For instance, Acosta and colleagues demonstrated that NF-κB and C/EBPβ cooperatively induce the expression of CXCR2 ligands IL-8 and GROα in fibroblasts undergoing OIS (Acosta et al. 2008). Similarly, IL6 expression is induced by the transcription factor C/EBPβ upon exposure of cells to oncogenic stress (Kuilman et al. 2008). Both IL-8 and IL-6 play an important role in OIS reinforcing the senescence growth arrest. A critical role of RelA/p65 as a major transcription factor that accumulates on chromatin of senescent cells was demonstrated by Chien et al. (2011). Interestingly, suppression of RelA/p65 function not only abrogates the induction of proinflammatory cytokines IL-6, IL-8, CXCL1, but also reduces expression of intercellular adhesion molecules, such as ICAM-1, and several matrix metalloproteinases (Chien et al. 2011). RelA/p65 induces the expression of TNF-α and IFN-γ in drug-induced tumor senescent cells thereby sensitizing cells to Fas-mediated apoptosis (Crescenzi et al. 2011).

Activation of NF-κB during senescence depends on the cGAS-STING cytosolic DNA sensor pathway, in which cGAS detects endogenous senescence-specific cytoplasmic chromatin fragments (reviewed in Glück and Ablasser 2019). More recent data demonstrate that during OIS cGAS-STING controls the induction of TLR2 which acts as critical upstream mediator for the initiation of the SASP. TLR2 is activated by senescence-associated Damage-Associated Molecular Patterns (identified as acute-phase serum amyloids A1 and A2) and signals, downstream of STING, through NF-κB and p38[MAPK] to regulate the SASP and to reinforce the senescence growth arrest (Hari et al. 2019). Finally, a role for the JAK/STAT pathway in modulation of proinflammatory SASP components has been demonstrated (Novakova et al. 2010; Xu et al. 2015).

The SASP develops over several days in culture. Hence, expression of several SASP factors, notably the proinflammatory cytokines IL-6 and IL-8, requires up to 20d after DDR initiation before reaching maximal level (Coppé et al. 2008; Crescenzi et al. 2011). Furthermore, secretome composition is temporally modulated, changing with time from a TGF-β-driven to a C/EBPβ-driven profile (Hoare et al. 2016).

Interestingly, emerging evidences show that senescent cells manage to communicate with their neighbors not only through the SASP, but also through the release of small extracellular vesicles (EVs), namely exosomes and microvesicles (Takasugi 2018). Accordingly, various stresses known to induce cellular senescence increase the secretion of EVs (Takasugi 2018). EVs produced by senescent cells play a double role by removing harmful senescence-related cytoplasmic DNA fragments (Takahashi et al. 2017) and by delivering cargos of cell type-specific bioactive molecules (Takasugi et al. 2017; Sagini et al. 2018). Both normal and neoplastic senescent cells have been shown to modulate local tissue microenvironment through EVs. For instance, EVs secreted from senescent chondrocytes, isolated from osteoarthritis patients, induce paracrine senescence in nearby cells, thereby inhibiting cartilage extracellular matrix deposition. These effects are mediated by osteoarthritis-specific microRNA (miRNA) packaged in the EVs (Jeon et al. 2019). Similarly, primary human foreskin fibroblasts undergoing Ras-mediated senescence secrete exosomes

which mediate paracrine senescence. The interferon-induced transmembrane protein 3 (IFITM3), specifically accumulated within EVs, is responsible for transmitting senescence to normal neighboring cells (Borghesan et al. 2019). Interestingly, while EVs secreted from senescent cells elicit paracrine senescence, EVs isolated from human induced pluripotent stem cells reduce reactive oxygen species levels in senescent mesenchymal stem cells in culture, thereby reversing the senescent phenotype. Stem cells derived EVs also alleviate senescence in a progerin-induced premature aging cell model. High levels of EVs antioxidant enzymes are responsible for the anti-aging effects (Liu et al. 2019). In line with this, are the results of Zhang and colleagues that demonstrate that EVs isolated from hypothalamic neural stem cells ameliorate age-related cognitive declines in mice. Exosomal miRNA mediate these anti-aging effects (Zhang et al. 2017). Notably, senescent EVs can also promote protumorigenic effects: EVs enriched in EphA2 receptor bind to ephrin-A1 ligand expressed on cancer cells and promote their proliferation through reverse signaling (Takasugi et al. 2017). Notably, increased expression of EphA2 was detected in the stroma of human breast and ovarian cancer (Takasugi et al. 2017). Finally, there is also evidence that EVs deliver membrane-derived bioactive lipids that impact on immune system functions (Sagini et al. 2018).

An additional form of cell communication described in senescent cells is a direct intercellular protein transfer through cytoplasmic bridges. Although the functional consequences of direct intercellular transfer are not completely understood, is interesting to note that this kind of communication facilitates the clearance of senescent cells by NK cells (Biran et al. 2015).

It is now widely accepted that a prolonged permanence of senescent cells in tissue and their sustained secretory activity, result in chronic inflammation and critically contributes to both age-related dysfunctions and to a protumoral microenvironment (Sun et al. 2018; Wei and Ji 2018; Jackson et al. 2012). In the context of TIS, concerns have been raised regarding the ability of cancer cells to escape the senescence arrest, to resume proliferation and to drive tumor recurrence after radio- and chemotherapy (Roberson et al. 2005; Karimi-Busheri et al. 2010). Cancer cells that evade senescent growth arrest are endowed with stem cell properties, are more malignant, and resistant to drug therapies (Yang et al. 2017), representing a likely risk for tumor relapse. Notably, the SASP promotes cell plasticity and stemness also through paracrine actions (Mosteiro et al. 2016; Ritschka et al. 2017). In line with these observations, in vivo studies support the idea that accumulation of senescent cells results in detrimental tissue remodelling, both in normal and in neoplastic tissues. For instance, selective elimination of senescent cells by genetic approaches delays the onset and attenuates progression of age-related disorders, and extends lifespan (Jeon et al. 2017; Bussian et al. 2018; Baker et al. 2016; Baker et al. 2011). Similarly, specific ablation of TIS cells in the tumor microenvironment reduces both tumor relapse and the adverse effects of chemotherapy (Demaria et al. 2017). Notably, TIS induction was shown to be associated with adverse treatment outcome and decreased overall survival in cancer patients (Wu et al. 2012; Supiot et al. 2008; Sidi et al. 2011; Kim et al. 2016). Hence, available data suggest that senescent cells and the SASP contribute to organismal ageing and limit the efficacy of chemo- and radiotherapy (Muñoz-Espín

and Serrano 2014). Consequently, removal of senescent cells or the neutralization of their secretome, have become attractive therapeutic strategies to prevent senescence-associated dysfunctions, thus opening the new field of senotherapy research. Drugs that suppress or modulate the SASP without interfering with senescent growth arrest and senolytic agents that selectively induce apoptosis in senescent cells have been studied and are under development.

The possibility to pharmacologically reprogram the SASP towards an anti-tumorigenic rather than pro-tumorigenic profile was first highlighted by Toso and colleagues in the context of docetaxel-induced senescence in PTEN-null tumors, where pharmacological inhibition of the Jak2/Stat3 pathway resulted in activation of an antitumor immune response and enhanced chemotherapy efficacy (Toso et al. 2014). The neutralization of detrimental SASP components in the tumor microenvironment (TME), where senescent stromal cells promote proliferation and survival of nearby neoplastic cells, has also been studied focusing on senescent fibroblasts. In this setting, inhibition of p38^{MAPK} was found to inhibit SASP-mediated tumor growth driven by senescent fibroblasts (Alspach et al. 2014). Pharmacological inhibition of the chromatin regulator MLL1 in fibroblasts also abrogated the SASP preventing its pro-carcinogenic effects. Furthermore, similar results were obtained in etoposide-induced senescent MCF7 breast cancer cells, mimicking the clinical scenario of TIS in tumor cells (Capell et al. 2016). Simvastatin, an HMG-coA reductase inhibitor, decreased the SASP of senescent human fibroblasts, thereby reducing endocrine resistance in breast cancer (Liu et al. 2015). Similarly, the flavonoid apigenin suppressed the SASP and reduced detrimental paracrine effects of fibroblasts on breast cancer cells (Perrott et al. 2017). Rapamycin selectively suppressed the mTOR and NF-κB-dependent secretion of proinflammatory cytokines in senescent fibroblasts, thereby suppressing prostate tumor growth in mice (Laberge et al. 2015). Trabectedin, a DNA minor-groove binding anticancer drug, which causes a gene- and promoter-dependent modulation of transcription (Larsen et al. 2016), markedly decreased the expression of proinflammatory SASP cytokines in tumor cells and limited their pro-tumoral activities in vitro (Camorani et al. 2018). Importantly, Georgilis and colleagues identified 50 druggable, potential targets for therapeutic intervention, whose knockdown suppressed pro-tumorigenic effects of the senescent fibroblasts secretome on tumor growth (Georgilis et al. 2018).

However, expression profiles of different senescent cells are not uniform and the SASP appears to be stimulus-, time- and cell type-dependent (Hernandez-Segura et al. 2017; Wiley et al. 2017; Maciel-Barón et al. 2016) which makes the modulation of the SASP a complex task. Furthermore, in the context of TIS, the SASP has both tumor-suppressive and tumor-promoting effects: SASP cytokines are known to induce immune-mediated clearance of senescent tumor cells (Iannello et al. 2013; Tasdemir et al. 2016; Eggert et al. 2016) and interfering with these immunostimulatory signals might result in detrimental effects. For instance, in a murine model of lymphoma, the reduction of SASP proteins through NF-κB inhibition hampered immunosurveillance following TIS and led to drug resistance, early relapse and reduced survival (Chien et al. 2011). Together, these observations suggest that activation of cell-, time- and stimulus-specific senescence signaling pathways in tumor

cells results in different and even functionally-opposite SASPs (Burgess 2011; Llanos and Serrano 2016). Hence, therapeutic interference with key SASP regulators might require individual molecular profiles and monitoring of SASP signatures in cancer patients post-treatment.

As an alternative therapeutic strategy to prevent senescence-associated dysfunctions, senolysis, i.e. the selective removal of senescent cells, has been approached. Senolytic agents have been tested in pre-clinical models and have proved to be effective in alleviating age-related diseases and symptoms of progeria (Xu et al. 2018; Yousefzadeh et al. 2018; Farr et al. 2017; Roos et al. 2016). These studies support the clinical potential of senolysis for promoting healthy ageing. Comprehensive reviews have recently been published on this subject (Kirkland and Tchkonia 2017; Myrianthopoulos et al. 2019). In contrast, the effects of senolytic agents in TIS appear to be more multifaceted. For instance, the senolytic agent panobinostat effectively removed chemotherapy-induced senescent non-small cell lung cancer and head and neck squamous cell carcinoma cells, and proved more efficacious than multiple cycles of chemotherapy (Samaraweera et al. 2017). In contrast, the senolytic cocktail dasatinib plus quercetin, which effectively eliminated senescent fibroblasts (Schafer et al. 2017), not only showed no efficacy on TIS liver cancer cells but also displayed pro-tumorigenic effects in the absence of chemotherapy (Kovacovicova et al. 2018). These data again underline the complexity of senescent cancer cells and emphasize the need for further in vivo investigation of their features and their role(s) in the tumor microenvironment.

Future steps in senotherapy research would include the study of combination therapy with radiotherapy-chemotherapy plus senotherapy for cancer treatment, and advancing senotherapeutics from preclinical studies into the clinic for age-related diseases. Several clinical trials are ongoing for age-related diseases, aimed at evaluate the feasibility of senotherapy. For instance, a two-center, open-label study (ClinicalTrials.gov Identifier: NCT02874989) of senolytic treatment with Dasatinib and Quercetin was conducted in patients with idiopathic pulmonary fibrosis, supporting safety of the treatment and providing initial evidence of senolytic efficacy (Justice et al. 2019). In an open label Phase 1 pilot study (ClinicalTrials.gov Identifier: NCT02848131), the combination of Dasatinib plus Quercetin, administered to subjects with diabetic kidney disease, significantly decreased senescent cell burden (Hickson et al. 2019). A phase 1 clinical trial testing the safety and tolerability of a senolytic small molecule (UBX0101) for osteoarthritis treatment is ongoing (ClinicalTrials.gov Identifier: NCT03513016).

Application of senotherapies to cancer and age-related diseases will require specific and sensitive biomarkers for the detection of normal and neoplastic senescent cells in patients. In particular, assessment of senescence biomarkers in biological samples of cancer patients following neoadjuvant chemo-radiotherapy could help predict treatment responses and contribute to designing personalized therapies.

10.2 Markers for Senescence

The lack of specific markers for the in vivo detection of senescent cells hampers both the molecular characterization of cellular senescence in ageing, in ageing-related diseases and in cancer patients, and the development of senescence-specific molecular tools and probes for diagnostic, prognostic and therapeutic purposes. Assessment of SA-β-gal activity (Kurz et al. 2000) still represents the most commonly used method for identification of senescent cells, although this marker lacks appropriate specificity, being constantly expressed by senescent cells but also in several other conditions such as in confluent cells in culture (Severino et al. 2000; Yang and Hu 2005). Also, the use of fresh tissue, required for the detection of enzymatic activity of SA-β-gal, is incompatible with routine formalin-fixation and paraffin-embedding of clinical specimens. However, the high lysosomal β-galactosidase activity of senescent cells was successfully exploited for the delivery of galacto-oligosaccharide-encapsulated cytotoxic drugs to live senescent cells in vitro and in vivo, given that release of encapsulated material depended on digestion of the sugar-coat by the senescent-associated lysosomal β-galactosidase (Muñoz-Espín et al. 2018).

Combinations of markers indicative of cell cycle arrest, active DDR and distinctive morphological changes have been sometimes employed to detect senescent cells. For instance, co-staining of either SA-β-gal activity or histone H3K9 tri-methylation (H3K9me3, a repressive heterochromatin mark denoting cell cycle arrest) together with low Ki67 expression has allowed for detection of senescence in therapy-exposed cancer cells (Reimann et al. 2010). An irregular morphology and enlargement of nuclei is frequently observed in senescent cells, which has been related to alterations in components of the nuclear envelope and in specific downregulation of lamin B1 (Freund et al. 2012). Accordingly, the combination of SA-β-gal positivity and double Ki67-Lamin B1 negativity was used to detect senescent cells within untreated human invasive breast carcinomas (Cotarelo et al. 2016). However, although panels of biomarkers might improve our ability to identify senescent cells, there are technical challenges for the practical use of such panels in the clinic and in research.

While senescent cells differ from proliferating and quiescent cells in several aspects (Rodier and Campisi 2011), alterations in cell surface proteins are of greatest interest since surface proteins can be used both as senescence-specific biomarkers and as potential druggable targets. In addition, cell surface markers are easily detected using conventional methods on fresh samples and on fixed specimens. A screening for plasma membrane-associated proteins preferentially expressed in senescent cells led to the identification of DEP1, STX4 and B2MG proteins, whose co-expression marked senescent cells in culture and in tissue samples (Althubiti et al. 2014), although it was noted that STX4 was a better marker of $p21^{CIP1}$-induced senescence, whereas DEP1 was more specific for $p16^{INK4a}$-induced senescence. The combination of DEP1 and B2MG also allowed for flow cytometric identification of senescent cells. Mass spectrometry analysis of membrane- and cell surface-associated proteins from replicative senescent human diploid fibroblasts identified DPP4 (CD26) as a senescence surface marker. Interestingly, anti-DPP4 antibodies induced efficient

antibody-dependent cellular cytotoxicity against senescent fibroblasts, thus enabling their preferential elimination by natural killer cells (Kim et al. 2017). More recently, the plasma membrane-localized fatty acid translocase CD36 was found upregulated in various cell types during replicative senescence, OIS, and TIS (Chong et al. 2018).

Proteomic approaches unveiled not only specific quantitative changes but also qualitative modification of surface plasma membrane-associated proteins in senescent cells, such as protein glycation (or glycoxidation), carbonylation, and carbamylation (Itakura et al. 2016; Vanhooren et al. 2015). Indeed, specific non-enzymatic post-translational modifications of proteins and lipids have been proposed as hallmarks of ageing (Friguet 2002). For instance, the products of non-enzymatic glycation and oxidation (glycoxidation) of proteins and lipids, called advanced glycation end-products (AGEs), are known to accumulate in organisms in an age-dependent manner. It is also well known that reactive oxygen species induce progressive accumulation of oxidized and carbonylated proteins during aging (Baraibar et al. 2013). This age-related accumulation of oxidized proteins likely depends on a decreased efficacy of repair systems and/or failure of removal mechanisms (Chondrogianni et al. 2014). Among oxidized products, a non-degradable material referred to as lipofuscin, accumulates in the lysosomal compartment in aged tissues, and has been used as a senescence marker (von Zglinicki et al. 1995; Georgakopoulou et al. 2013). Recently, a novel sensitive technique for the detection of lipofuscin has been developed and applied for the detection of senescent cells in fresh, frozen and formalin-fixed biological specimens (Evangelou et al. 2017). Carbamylation, the spontaneous binding of isocyanic acid to proteins, also occurs continuously throughout life (Gorisse et al. 2016), and is easily detected on long-lifespan proteins such as extracellular matrix structural proteins. In order to characterize novel aging biomarkers, the key next step is to identify specific cell surface proteins targeted by these modifications in senescent cells. In line with this are the results of Frescas and colleagues that recently described the identification of a senescence-specific oxidative modification of vimentin (malondialdehyde (MDA)-modified vimentin), which is exposed on the surface of senescent fibroblasts and is secreted into the blood of senescence-prone SAMP8 mice (Frescas et al. 2017).

It is important to note that the acquisition of a senescent phenotype is associated to cell type- and stimulus-dependent variations in gene expression. For instance, by using differential display and DNA arrays, Toussaint and coworkers (Pascal et al. 2005) showed that, despite displaying a wide variety of common biomarkers of senescence at the morphological level, WI-38 foetal lung human diploid fibroblasts acquired different expression profiles when induced to undergo senescence by replicative exhaustion or by treatment with tert-butylhydroperoxide or ethanol. Similarly, using RNA-seq, Purcell compared expression profiles of replicative senescence with that of various types of stress-induced senescence in fibroblasts, and highlighted both similarities and differences in expression between different pro-senescence stimuli (Purcell et al. 2014). Interestingly, comparative bioinformatic analyses of high-throughput microarray data from nerve and muscle biopsies from young and aged healthy donors showed a limited number of common differentially expressed genes among the skeletal muscle and nervous tissues, thus confirming

the great heterogeneity that characterizes cellular senescence in vitro and in vivo (Voutetakis et al. 2015). Using numerous whole-transcriptome profiles of different fibroblast strains, Hernandez-Segura not only confirmed the stress- and cell-specific transcriptional response to senescence-inducing stimuli, but also showed that the gene expression profiles are temporally modulated (Hernandez-Segura et al. 2017). Recently, transcriptional heterogeneity of replicative senescent or stress-induced premature senescent fibroblasts was confirmed at single cell level using droplet-based single-cell mRNA sequencing (Tang et al. 2019). In this context, it is worth noting that gene expression analyses showed dramatically different profiles between senescent fibroblasts and senescent epithelial cells (Zhang et al. 2003). This observation, together with the stimulus-specific regulation of cellular senescence, suggests that the search for biomarkers of senescence for ageing and age-associated diseases, as well as for TIS in cancer patients, might require a large sampling effort, or that different biomarkers would eventually be used to identify different senescent phenotypes.

10.3 Potential Applications of Aptamers for Senescent Cells Biomarker Discovery and Targeting

The term aptamer (derived from the Latin aptus = to fit) was coined by Ellington and Szostak (http://ellingtonlab.org/blog/2014/12/1/on-aptamers) to describe molecules (oligonucleotides and, later, peptides) that bind to specific targets with high affinity and specificity, through structural recognition, thanks to their particular three-dimensional conformation, in the same way an antibody binds to an antigen (Ellington and Szostak 1990; Tuerk and Gold 1990; Ellington and Szostak 1992). Non-covalent interactions such as hydrophobic interactions, electrostatic interactions and van der Waals bonds mediate the binding of aptamers to their target molecules. Nucleic acid aptamers, usually ranging from 20 to 100 nt, are isolated from pools of random oligonucleotide sequences through an iterative process of selection and amplification, called SELEX (systemic evolution of ligands by exponential enrichment) (Tuerk and Gold 1990). Both RNA and single-strand DNA aptamers have been successfully selected using SELEX that can bind similar targets with similar affinity (McKeague and Derosa 2012; Lin and Patel 1997). However DNA aptamers are more stable than RNA aptamers (White et al. 2000), and the latter are usually chemically modified in order to enhance their stability (Kratschmer and Levy 2017; Germer et al. 2013).

Compared to antibodies, nucleic acid aptamers have a number of advantages. First, aptamers are chemically synthesized in the lab so their production does not require animals, is relatively easy and cheap, with low variability between batches. Aptamers can also be raised against non-immunogenic targets, such as small inorganic and organic molecules (https://www.aptagen.com/aptamer-index). Due to their small size, aptamers are minimally immunogenic (for instance, Zheng et al. 2017). Their small size also results in enhanced tissue penetration and reduced circulation time (Kaur et al. 2018; Keefe et al. 2010). Aptamers are amenable to chemical

modification in the sugar, base or phosphate backbone, which can improve both their resistance to nuclease degradation and their affinity for targets (Ni et al. 2017). Finally, aptamers can be easily coupled to different probes, nanoparticles or other biomolecules.

Focusing on the potential application of nucleic acid aptamer technology for the discovery of senescent cells biomarkers, it is important to note that SELEX can be carried out using as targets both purified proteins or complex targets, such as whole cells (cell-SELEX) and plasma (Zhuo et al. 2017; Shamah et al. 2008; Fitter and James 2005). Hence, SELEX could be employed to generate aptamers that specifically recognize (and possibly modulate the function(s)) of already known senescence biomarkers, such as DEP1 (Althubiti et al. 2014), DPP4 (Kim et al. 2017) and CD36 (Chong et al. 2018). On the other hand, cell-SELEX is a powerful strategy for discovery of unknown biomarkers and the cell-SELEX method could be used to identify aptamers that show high affinity for still unidentified membrane molecules on the surface of senescent cells (Berezovski et al. 2008; Sefah et al. 2010). The inclusion of a counter-selection step, with proliferating and/or quiescent cells as negative controls, would allow for enrichment of senescence-specific aptamers. It is important to note that aptamers generated through cell-SELEX recognize various kinds of biological molecules, for instance proteins, lipids and carbohydrates, in their native conformation (Li 2007). Aptamers are able to distinguish between closely related molecules. For instance, Chen and colleagues isolated an RNA aptamer able to distinguish the single amino acid mutant protein p53^{R175H} from the wild type p53 protein (Chen et al. 2015). Furthermore, aptamers are able to distinguish post-translational modifications (PTM) of target proteins (Díaz-Fernández et al. 2018; Gilbert et al. 1997). For instance, aptamers were produced that could recognize the hydrophobic farnesyl moiety in K-Ras (Gilbert et al. 1997). Highly specific DNA aptamers that specifically bound to histone H4 acetylated at lysine 16 (H4-K16Ac) were identified by Williams et al. (2009). DNA aptamers able to distinguish between peptides differing by a single glycosylation were developed, using N-glycosylated vascular endothelial growth factor (VEGF) (Rose et al. 2010). Notably, these aptamers were not specific for the sugar moiety itself, but rather the combination of the peptide sequence and the glycosylation. Since many types of PTM present in mammalian proteins are absent or different in bacteria (for instance ubiquitination and acetylation), it is important that recombinant proteins used as targets for SELEX are purified from mammalian cells if the aptamers are supposed to be used in mammalian cell systems, as demonstrated with the anti-cyclophilin B aptamer M9-5 (Ray et al. 2013). By binding to their target proteins, aptamers can modulate their functions, and aptamers working as both antagonists or agonists have been isolated. For example, an antagonistic DNA aptamer targeting PD-L1 efficiently blocked the binding between human PD-1 and PD-L1, thus restoring T cell functions and enhancing anti-tumor immune responses (Lai et al. 2016). An agonistic DNA aptamer, which bound to and stimulated the immune inhibitory receptor CD200R1, was shown to suppress inflammatory responses in vivo (Prodeus et al. 2018). Interestingly, this aptamer was capable of agonizing both murine and human CD200R1 (Prodeus et al. 2018).

Since their development in early 1990s nucleic acid aptamers have become important tools in a wide range of both clinical and research applications, such as ELISA and pull-down assays, flow cytometry, immunohistochemistry, drug delivery, functionalization of nanoparticles, and bioimaging (Zhu et al. 2015; Vandghanooni et al. 2018; He et al. 2018; Zhou and Rossi 2017). These observations strongly support the use of aptamers in the growing field of senotherapy. Although only one aptamer, Pegaptanib sodium, (Macugen; Eyetech Pharmaceuticals/Pfizer), an RNA aptamer directed against vascular endothelial growth factor (VEGF)-165, has been approved for therapeutic use in humans (Ng et al. 2006), other nucleic acid aptamers are at advanced stages of clinical trials (Kaur et al. 2018; Maier and Levy 2016).

Peptide aptamers were first described by Brent and coworkers (Colas et al. 1996), who based their approach on the concept that short peptides anchored at both amino and carboxy termini (i.e. conformationally constrained) are capable of high-affinity and specific interactions with other molecules. By using *Escherichia coli* Thioredoxin A protein as a rigid scaffold to display 20-residue random peptides, Brent and coworkers were able to select peptide aptamers that specifically bound human Cdk2, and, notably, inhibited its kinase activity. Since then, many other non-antibody protein scaffolds have been designed, which constrain variable peptide sequences for display, reviewed in Yu et al. 2017 and Simeon and Chen 2018. Here, we will focus on a recently developed, promising peptide aptamer technology known as Adhiron or Affimer system (Tiede et al. 2014, Tiede et al. 2017), developed by the BioScreening Technology Group (BSTG), from University of Leeds (UK) and their industrial partner Avacta (https://www.avacta.com/). Two different scaffolds were developed in this system: the first derived from human protease inhibitor Stefin A, the other derived from Cystatin A, a plant cysteine protease inhibitor. Each scaffold is capable of displaying one or two peptide variable regions of varying lengths (Tiede et al. 2014). Peptide aptamers derived from either of these two scaffolds are collectively called Affimers. Compared to antibodies, Affimers have a number of advantages. First, Affimers are easily expressed in *Escherichia coli* as well as in mammalian cells, and their production requires no animals (Dos Remedios and Peckham 2017). Both scaffolds are monomeric, highly stable (to both pH and temperature, with melting temperature between 70 and 100 °C) and small (~100 amino acids, 11 kDa, 3 nm in diameter). Their small size allows for better penetration into cellular and subcellular structures and tissue. Finally, the Affimer scaffolds show low immunogenicity (https://www.avacta.com/blogs/no-immunogenicity-issues-Affimer-technology), and can be subjected to chemical modification and conjugation.

Affimers recognizing specific targets are selected using a phage display strategy: a library of phage display Affimer reagents is screened against either purified target molecules or complex targets, such as whole cells, and a step of counter-screening may be used to increase specificity of isolated Affimers (Tang et al. 2017). Affimer libraries have been successfully screened against more than 500 target proteins and used as reagents in diagnostics, imaging and biomedical applications (recently reviewed in Kyle 2018).

Similar to nucleic acid aptamers, Affimers specifically recognize proteins with different types of post-translation modifications and are able to inhibit protein function

and/or protein-protein interactions by binding to their specific targets. For instance, Affimers directed against VEGFR2 inhibited VEGF-stimulated receptor phosphorylation and downstream signaling in HUVEC cells, eventually reducing VEGF-induced HUVEC tubulogenesis (Tiede et al. 2017). Affimers that specifically bound SUMO-1 or SUMO-2, or both, inhibited SUMO-mediated protein-protein interactions (Hughes et al. 2017). Inhibition of protein-protein interaction by Affimers was also described by Robinson and colleagues, who generated Affimers against the immunoglobulin receptor FcγRIIIa. These Affimers were able to selectively block IgG immune complex binding to FcγRIIIa (but not to FcγRIIa or FcγRIIIb), and abrogated FcγRIIIa-dependent effector functions in macrophage (Robinson et al. 2018). Affimers were also developed for in vivo imaging of tumor markers. For instance, high affinity Affimers recognizing Tenascin C, an extracellular matrix glycoprotein that is highly expressed in the tumor stroma of most epithelial malignancies (Yoshida et al. 2015), were isolated by Tiede et al. (2017). This Affimer, conjugated with biotin, allowed for the detection of Tenascin C in human colorectal cancer and glioblastoma xenograft tissue sections, with a specificity similar to antibodies. In addition, Rhodamine Red-labelled Affimer accumulated in Tenascin C expressing tumour in vivo. Interestingly, Affimers were quickly cleared from the circulatory system, compared to antibodies, allowing for rapid imaging of tumor (Tiede et al. 2017).

On the whole, these studies highlight the potentiality of Affimers as tools for the identification of novel senescence-specific cell surface biomarkers.

10.4 Conclusions

In this chapter we have highlighted the need for novel senescence biomarkers and the development of diagnostic, prognostic and therapeutic interventions for aging and cancer-related TIS. A wealth of data from high-throughput transcriptomics and proteomics studies indicate that the acquisition of a senescent phenotype, in normal as well as neoplastic cells, is associated with alterations of cell surface proteins and/or their modifications. These studies support the possibility of utilizing these quantitative and qualitative differences for developing senescence-specific markers.

Since the first reports in the 1990s, nucleic acid aptamers and the SELEX technology have been widely applied for the discovery of biomarkers, useful in research and in the clinic. More recently, the peptide aptamer (Affimers) technology has emerged as valuable tools for biomarker discovery. We propose that aptamers represent a promising strategy to succeed in identifying new senescence-specific markers.

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