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1 **Caloric restriction and cellular senescence**

2
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13

14

15 **Cellular senescence**

16 Cellular senescence was originally described as the limited proliferative capacity of cultured human
17 fibroblasts¹. This phenomenon, now termed replicative senescence, is caused by telomere erosion.
18 Currently, it is generally accepted that senescence can be prematurely induced by many different insults,
19 including oxidative stress, genotoxic stress, epigenetic changes, metabolic dysfunction, over activation of
20 oncogenes or loss of some tumor suppressor genes, and mitochondrial dysfunction².

21 The main defining characteristic of senescence is permanent growth arrest, and p53-p21 and p16^{INK4a} –
22 pRB are the two essential pathways responsible for this replicative arrest³. Interestingly, both p53 and
23 p16^{INK4a} are the most commonly mutated genes in cancer, suggesting that one of the evolutionary
24 advantages of the senescence response is to suppress the development of cancer⁴.

25 In addition to the growth arrest, which is a necessary but insufficient marker of senescent cells, many
26 features and molecular markers are used for identifying senescence. For instance, a commonly used
27 marker is the senescence-associated beta-galactosidase staining (SA-βgal)⁵. The increase in SA-βgal
28 activity is thought to be a consequence of increased lysosomal mass^{6,7}. Due the fact that there is no
29 universal marker, the combination of many senescence-associated hallmarks is currently used for the
30 unequivocal characterization and identification of senescent cells⁸.

31 Although p16^{INK4a} is expressed by many but not all senescent cells, it is now generally accepted to be one
32 of the most specific senescence markers *in vivo*^{9,10}. This led to the design of two distinct mouse models
33 where p16^{INK4a}-positive cells can be selectively eliminated^{11,12}. These models contributed much to our
34 understanding about the causal roles of senescent cells in many different processes that include aging,
35 age-related diseases, and wound healing¹¹⁻¹⁸.

36 Numerous senescence inducers can cause genomic damage that can remain unresolved. As a result, the
37 DNA damage response (DDR) is constantly active because of the chronic persistence of DNA damage
38 foci. Those foci are generally termed DNA-SCARS (DNA segments with chromatin alterations
39 reinforcing senescence)¹⁹. The persistent DDR signaling function as one of the main drivers of the
40 expression of the senescence associated secretory phenotype (SASP)²⁰.

41 The SASP is a complex mixture of secreted factors that can be divided in 3 main categories: 1) factors
42 binding to receptors (soluble signaling molecules, such as cytokines, chemokines, and growth factors), 2)
43 factors acting directly (matrix metalloproteases, serine proteases and small non-protein components, such
44 as reactive oxygen (ROS) and nitrogen species), and 3) regulatory factors (tissue inhibitors of
45 metalloproteases, the plasminogen activator inhibitor, and insulin-like growth factor binding proteins)²¹.

46 In addition to secreted factors, senescent cells can also influence their surrounding through juxtacrine
47 NOTCH/JAG1 signaling²² or via intercellular transfer of different molecules through cytoplasmic
48 bridges²³ or exosomes release²⁴. Importantly, some SASP components can reinforce senescence²⁵ and
49 induce senescence in neighboring cells (paracrine senescence)²⁶.

50 It is clear now that the composition of SASP factors is dependent on the type of cells and senescence
51 inducers²⁷. Moreover, the SASP can be regulated at different levels and it seems that most of the
52 pathways that are important for the establishment of this phenotype converge into activation of the NF- κ B
53 and the C/EBP β pathway²¹. Strikingly, the mechanistic target of rapamycin (mTOR) pathway, an
54 essential player in the aging process, is an important node in SASP regulation²⁸⁻³⁰.

55 Many age-related diseases, if not all, share a chronic low-grade inflammatory state referred to as
56 inflammaging³¹. Elimination of senescent cells reduces many proinflammatory factors such as IL-6, IL-1 α
57 and TNF- α ¹⁷, suggesting that the SASP is, at least in part, causing this increased inflammatory state in
58 old tissues. Given the fact that senescent cells are present in a small percentage in old tissues³², it is highly
59 plausible that the positive effect seen after senescent cells elimination is a consequence of SASP
60 suppression.

61 Growing body of evidence has demonstrated the implication of senescence in aging and age-related
62 pathologies. Hence, targeting senescent cells either by eliminating them using “senolytics” or inhibiting
63 the deleterious effect of SASP, might be a promising approach for enhancing healthy longevity³³. Several
64 literature reviews have covered the pharmacological elimination of senescent cells elsewhere³³⁻³⁵.

65 However, another plausible approach is to avoid the formation of senescent cells by preventing cellular
66 damage, and accumulating data in experimental animal models and humans suggest that caloric restriction
67 (CR) without malnutrition may be a promising intervention in this context.

68

69 **Calorie restriction**

70 Calorie restriction (CR) with adequate nutrient intake is the most powerful non-genetic intervention for
71 extending healthspan and lifespan in multiple animal models, including yeast, fruit flies, worms, and
72 rodents³⁶. In most strains of rats and mice a reduction of dietary calories by 20 up to 50% results in a
73 substantial extension of both average and maximal lifespan, even if mice with different genotypes
74 respond differently to the same degree of restriction³⁷. Not only these CR animals, supplied with the
75 appropriate amount of calories and nutrients, live significant longer, but many of the typical age-
76 associated chronic diseases are either prevented or delayed. For example, the incidence of cancer, the

77 leading cause of death in rodents, is drastically reduced in CR animals; similar reductions or slowing
78 down of disease progression have been observed for nephropathy, cardiomyopathy, diabetes, chronic lung
79 diseases, autoimmune diseases and neurodegenerative disease^{36,37}.

80 Accumulating data indicate that CR extends lifespan also in non-human primates³⁸. In Rhesus monkeys,
81 CR significantly improves metabolic health, prevents obesity, glucose intolerance/type 2 diabetes, and
82 postpones the onset of sarcopenia, hearing loss and atrophy of certain key subcortical regions of the brain,
83 including the caudate and putamen and the left insula (34). Moreover, CR in monkeys reduces morbidity
84 and mortality for cancer, cardiovascular disease and frailty^{39,40}. Indeed, in contrast to the scientifically
85 unsupported opinion that long-term CR promotes frailty⁴¹⁻⁴³, recent data from the Wisconsin CR Primate
86 study clearly show that the levels of weakness, poor endurance, slowness, low physical activity and frailty
87 were significantly lower in the CR than in the ad-libitum fed monkeys⁴⁴.

88 In humans, calorie restriction with adequate intake of vitamins and minerals causes many of the same
89 physiological, metabolic and molecular adaptations observed in CR animals. For example, moderate CR
90 leads to major improvements in all the classical cardiovascular risk factors, over and above those
91 conferred by weight loss, even when implemented in healthy young and middle-aged non-obese men and
92 women. Interestingly, these cardiometabolic adaptations are coupled with improvements in cardiac and
93 arterial function, including improvements in left ventricular diastolic function and heart rate variability⁴⁵.
94 Like in small mammals, CR in humans causes major modifications of several hormones that are
95 implicated in the pathogenesis of cancer and in the biology of aging⁴⁶. Serum concentrations of insulin,
96 testosterone, estradiol and several inflammatory markers were significantly lower, while IGFBP-1,
97 SHBG, adiponectin and cortisol concentration were higher in people practicing CR group than in controls
98 eating Western diets ad-libitum⁴⁵. Plasma triiodothyronine concentration, and as a consequence
99 average 24- hour, day- time and night- time core body temperature, were also significantly reduced,
100 supporting a strong CR-mediated inhibitory effect on metabolic rate and oxidative stress⁴⁷.

101

102 **CR-mediated molecular mechanisms promoting health and longevity**

103 The mechanisms mediating the health benefits of CR are not fully understood. Multiple systemic
104 metabolic, neuroendocrine and immunological adaptations coupled with cell-specific molecular
105 mechanisms are involved. For example, calorie restriction without malnutrition exerts a powerful effect in
106 improving insulin sensitivity and in reducing protein glycation, oxidative stress and free radical-induced
107 cellular damage⁴⁸⁻⁵⁰. The CR induced reduction of multiple anabolic hormones and growth factors causes
108 a down-regulation of the nutrient-sensing insulin/IGF signaling network and an activation of FOXO,

109 which modifies several “longevity genes”⁵¹, including endogenous antioxidant enzymes (e.g. SOD2,
110 catalase), DNA repair (e.g. DDB1) and autophagy (e.g. beclin-1, autophagin-1) genes⁵². Autophagy and
111 mitophagy are important for the removal of dysfunctional organelles, amyloid and other protein
112 aggregates that interfere with normal cell function⁵³. FOXO activation is also a powerful inhibitor of
113 cyclin D, a master regulator of cell cycle progression and cell proliferation. Another major adaptation to
114 CR is the reduction in plasma concentrations of inflammatory cytokines and a modest increase in
115 circulating cortisol that results in a reduction in systemic inflammation together with a protection against
116 aging-associated deterioration in immune function^{54,55}. Increased expression of protein chaperones such as
117 heat shock protein-70 is also important to improve proteostasis, the removal of damaged cellular proteins
118 and cellular stress resistance^{50,56}. As we will discuss later, other molecular effectors that have been shown
119 to mediate the health effects of CR include TOR⁵⁷, AMPK⁵⁸, sirtuins⁵⁹, and NRF2⁶⁰. Energy and amino
120 acids restriction cause an inhibition of mTORC1 activity, which in turn enhances autophagy, improves
121 proteostasis and stem cell function⁶¹. Overexpression of sirtuins (i.e. SIRT1, SIRT3 and SIRT6) improves
122 metabolic homeostasis through histone deacetylation, inhibits NF-κB signaling and increases genomic
123 stability⁵⁹. In addition, activation of AMPK and SIRT1 up-regulates PGC-1α, a key transcriptional factor
124 regulating mitochondrial function, antioxidant defenses, and fatty acid oxidation⁶².

125 **CR and cellular senescence**

126 Different studies have shown that CR reduce senescence markers in different mouse organs and human
127 colon mucosa^{9,63-66}. One of the main inducers of senescence is cellular damage. Therefore, it is highly
128 plausible that CR reduces the generation of senescent cells by preventing damage to occur. CR can
129 protect cellular deterioration in at least two major ways: interfering with the source of damage, for
130 example via decreasing oxidative stress and inflammation, or repairing/eliminating already present
131 damage, for example by increasing autophagy (Figure 1)^{49,50,54}.

132 An important source of cellular damage is oxidative stress, mainly caused by an accumulation of reactive
133 oxygen species (ROS). Historically, the beneficial effects of CR were thought to be the result of slow
134 metabolism resulting in reduced production of ROS⁶⁷, but it is now known that CR can actively regulate
135 many other defense mechanisms against oxidative stress^{52,68}. Numerous experiments have established that
136 sirtuins are critically required to reduce oxidative stress. In accordance, CR induces the expression of
137 SIRT3, which was shown to play an essential role in reducing oxidative damage and its related
138 pathologies^{69,70}. It is interesting to note that mitochondrial dysfunction caused by SIRT3 downregulation
139 can, in fact, induce senescence⁷¹. Another member of the sirtuin family, SIRT1, was reported to mediate
140 aspects of CR response⁷². Due to the fact that some studies have demonstrated the important role of
141 SIRT1 in regulating oxidative status within the cell⁷³, it is highly possible that SIRT1 mediates its effect

142 during CR by reducing levels of oxidative stress. These notions suggest that CR might prevent
143 senescence, in part, by upregulating the antioxidant defense program partly through the increase of
144 sirtuins function and in part through up-regulation of FOXO.

145 Autophagy, a major lysosomal degradation pathway that is activated by CR, might act as an anti-
146 senescence mechanism by clearance of damaged proteins and organelles including damaged
147 mitochondria^{50,74}. Intriguingly, selective elimination of dysfunctional mitochondria by mitophagy
148 alleviate many aspects of the senescence phenotype⁷⁵.

149 Another mechanism by which CR can prevent senescence is through enhancement of DNA repair
150 mechanisms. Interestingly, CR activates many pathways that can prevent and aid in resolving DNA
151 lesions. For instance, CR reduces age-dependent decline in non-homologous end joining (NHEJ)⁷⁶. In
152 addition, it can improve nucleotide excision repair (NER), increase the fidelity of polymerase alpha and
153 beta, and decrease their age dependent decline^{77,78}. Moreover, CR can also protect DNA by inducing the
154 base excision repair pathway (BER), both in young and aged animals⁷⁹.

155 It has been shown that CR can regulate longevity pathways that include the insulin/insulin growth factor I
156 signaling (IIS), both in rodents and human. Circulating IGF-1 levels were decreased during CR in rodents
157 ⁸⁰, whereas in human the effect was not direct. Long- term CR results in a persistent increase in serum
158 IGFBP- 1 leading to a decrease in IGF- 1:IGFBP- 1 ratio levels, which can probably inhibit IGF-1
159 signaling by decreasing free IGF- 1 in circulation⁸¹. Notably, prolonged exposure of cells to IGF-1 can
160 induce premature senescence through the regulation of SIRT1-p53 pathway⁸². Accordingly, the decrease
161 in IGF-1 activity, as a consequence of CR, might help in preventing premature senescence.

162 Finally, the mechanistic target of rapamycin (mTOR) is also negatively affected by CR. A large portion of
163 the senescence associated secretory phenotype components is regulated by mTOR²⁸, including factors that
164 can induce secondary senescence⁸³. In addition, mTOR can promote geroconversion, the conversion from
165 a proliferative arrest to irreversible senescence, and mitigation of its activity favors quiescence⁸⁴. For
166 these reasons, CR could also prevent the activation and spread of senescence by inhibiting mTOR.

167 **Conclusion**

168 CR is a well-established intervention for reducing age-associated chronic diseases and enhancing lifespan.
169 In this review, we have summarized some of the mechanisms by which CR exerts its beneficial effects,
170 highlighting their complexity and heterogeneity. However, reduction of cellular damage might well be
171 related, at least in part, to prevention of cellular senescence. In the next few years, it will be key to
172 monitor the effects of long-term CR on cell senescence in various tissues to determine whether there is

173 any organ-specificity for damage protection. It will be also of high interest to treat animals under CR with
174 senolytic drugs to prove any synergistic effect of lowering cellular damage and eliminating senescent
175 cells. Another important question is whether the absence of senescent cells during CR might trigger side
176 effects – for example promoting longer kinetic of wound healing, a known issue for animals under CR⁸⁵.
177 Finally, similar studies on the prevention of senescent cells should be directed towards alternative and less
178 invasive dietary interventions with anti-aging properties, such as intermittent fasting and/or protein
179 restriction⁸⁶⁻⁸⁸.

180

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187 The author apologizes for the omission of relevant work owing to space constraints.

188 Competing interests

189 M.D. is co-founder of Cleara Biotech, a company devoted to develop senolytic interventions. However,
190 he did not receive any compensation from the company related to this work.

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