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PERSPECTIVE

Rapamycin, proliferation and geroconversion to senescence

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ABSTRACT

Rapamycin inhibits cell proliferation, yet preserves (re)-proliferative potential (RPP). RPP is a potential of quiescent cells that is lost in senescent cells. mTOR drives conversion from quiescence to senescence (geroconversion). By suppressing geroconversion, rapamycin preserves RPP. Geroconversion is characterized by proliferation-like levels of phospho-S6K/S6/4E-BP1 in nonproliferating cells arrested by p16 and/or p21. mTOR-driven geroconversion is associated with cellular hyperfunction, which in turn leads to organismal aging manifested by age-related diseases.

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Introduction

In brief: proliferative potential is not actual proliferation

Rapamycin and other inhibitors of mTOR (mammalian Target of Rapamycin) maintain proliferative potential in non-proliferating cells [1,2]. This should not be misunderstood to mean stimulation of proliferation. In fact, rapamycin slows proliferation. The potential to proliferate is not actual proliferation; rather it is a hidden feature of quiescent cells that renders quiescence reversible. Rapamycin maintains the potential to proliferate in non-proliferating cells, enabling these cells can re-start proliferation when needed. We can use the term Re-Proliferative Potential (RPP) instead of proliferative potential to avoid confusion with actual proliferation.

In brief: is irreversible arrest reversible?

In senescence, cell cycle arrest cannot be reversed through growth stimulation using methods such as serum stimulation. However, this seemingly irreversible arrest can be reversed by switching off p16, p53/p21 and Rb [3–9]. Still, in practical terms, this arrest is irreversible because after re-entering the cell cycle from senescence, cells cannot proliferate (lost RPP) and eventually die in the attempt [9,10]. In Alzheimer's disease, for example, senescent neurons die after re-entering the cell cycle [11–13].

In brief: cell cycle arrest is not growth arrest

Growth of cellular mass and the cell cycle can be dissociated [14–20]. Normally, when a cell is arrested, it does not grow; it exists in a state known as quiescence, or G0 arrest [21–23]. During conversion from quiescence to senescence, or geroconversion, cellular size continues to increase exponentially until the cells acquire the senescent phenotype [17,24]. Geroconversion is thus a form of growth rather than of growth arrest [1,25], and it is driven by mTOR [22,23,26]. mTOR stimulates, rather than inhibits, cellular growth.

In brief: senescence is not just cell cycle arrest, and cell cycle arrest is not yet senescence

To become senescent, an arrested cell must undergo geroconversion [22–29]. As we will discuss, geroconversion is associated not only with loss of RPP, but also with a cellular hypertrophic (a large cell morphology), hypersecretory and hyperinflammatory phenotype (or senescence-associated secretory phenotype [SASP]) as well as lysosomal hyperactivation (β-Gal staining) and,

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most importantly, cell type-specific hyperfunction, which contributes to age-related diseases.

Two-step geroconversion model of cellular senescence

I*n vitro*, senescence involves two conflicting events: (i) cell cycle arrest and (ii) growth stimulation [15]. Growth stimulation drives conversion from arrest to senescence (geroconversion) [22,30]. Thus, a senescence program consists of arrest followed by geroconversion (Figure 1), which is driven in part by the growth-promoting mTOR pathway [1,22,25,30]. When the cell cycle is arrested but mTOR is still active, senescence develops [1].

Senescence cannot be completely understood in the realm of cell cycle arrest alone; mTOR-driven geroconversion must also be considered (Figure 2). In a two-dimensional model, markers of cell cycle arrest (p16 and p21) are accompanied by growth markers (phospho-S6 and cyclin D1). This two-dimensional view of senescence (cell cycle arrest plus geroconversion, Figure 2) enables us to not only to reconcile seemingly contradictory published findings, but also to manipulate and suppress cellular senescence. Rapalogs such as rapamycin and everolimus, as well as the pan-mTOR inhibitors, all suppress geroconversion and maintain quiescence [1,2,31–34].

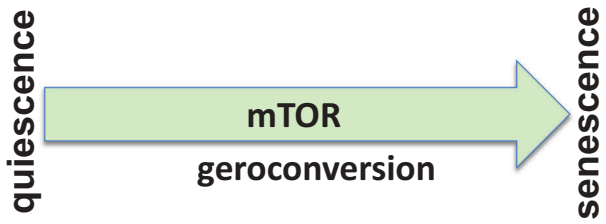

Figure 1. mTOR-driven geroconversion from quiescence to senescence.

Geroconversion and markers of senescence

During geroconversion, the mTOR and ERK/MAPK pathways are active, while cell cycling is blocked by p16/p21 (Figure 3). In a futile attempt to overcome the p16/p21-induced block, cyclin D1 is hyper-induced. At such high levels, cyclin D1 is a marker of senescence rather than of proliferation [10,35–38]. mTOR-driven geroconversion is associated with cellular hypertrophy [17,24,39] and hyperfunctions such as hypersecretion (or SASP) [40–44], ROS production [45], and lysosomal activation (β-Gal staining) [46–50]. These hyperfunctions in turn provoke compensatory reactions such as growth factor and insulin resistance [51–57],

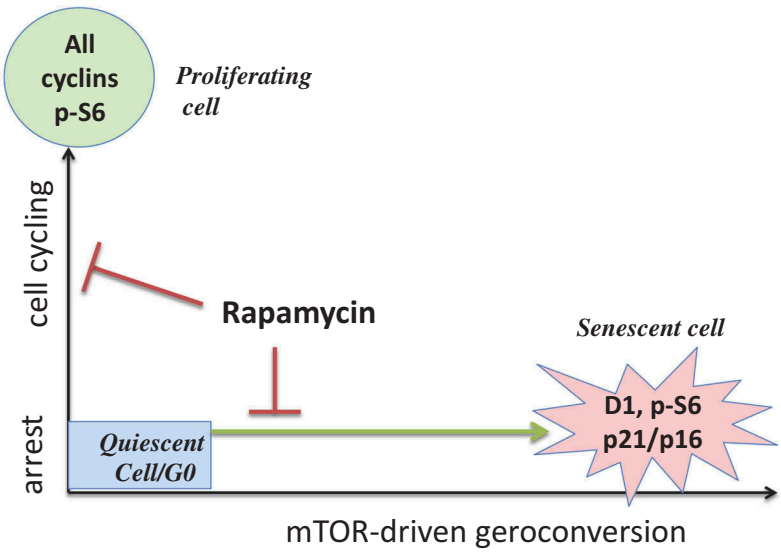

Figure 2. Two dimensional model: arrest vs mTOR activity.

There are two types of cell cycle arrest *in vitro*. The first type is quiescence (G0 arrest) caused by serum/nutrient starvation or by contact inhibition. Quiescence is associated with deactivated mTOR and ERK/MAPK pathways and low levels of all cyclins. Cells do not proliferate and do not grow in size. This type of arrest is easily reversible by re-addition of serum or by re-plating the cells at a low density. The second type is arrest caused by p21 or p16 in the presence of activated mTOR and ERK/MAPK, which drives geroconversion to senescence.

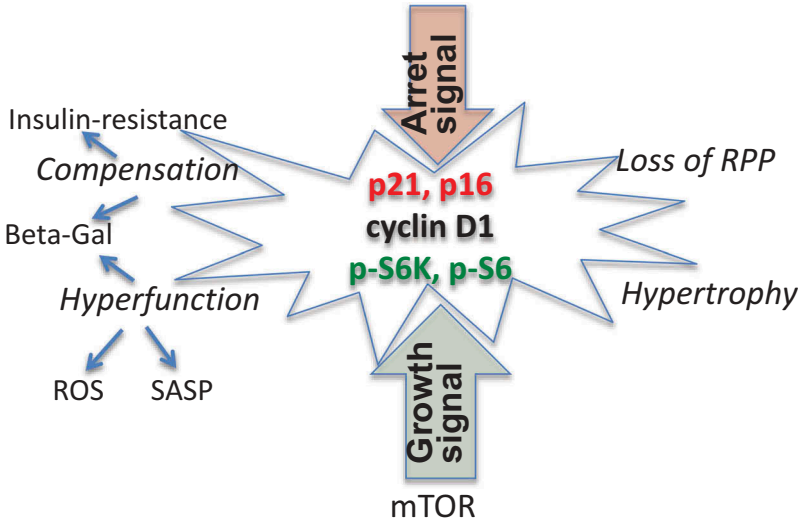

Figure 3. Senescent cells.

The conflicting signal model (cell cycle arrest plus mTOR-dependent growth stimulation) predicts the markers of senescence. Senescent cells are hyperfunctional, exhibiting a hypersecretory phenotype (or SASP), lysosomal hyperactivation (or β-Gal staining), high levels of cyclin D1, increased ROS production, pseudo-DNA-damage response, lipid accumulation, aerobic glycolysis and cellular hypertrophy (a large flat morphology). See text.

further lysosomal hyperactivation [49], and loss of RPP [10,38].

So-called golden marker of senescence and mTOR inhibitors

When the conflicting model of cellular senescence outlined above was published in 2003 [15], the golden marker of senescence was permanent arrest. We therefore tested whether rapamycin could prevent this "golden marker." Certainly, rapamycin decreases other markers of cellular senescence such as cellular hypertrophy [2,8,17,33,58,59] and the hypersecretory and hyperinflammatory phenotypes [8,40,60–63]. But these effects were anticipated, as rapamycin is a known antihypertrophic [25] and anti-inflammatory agent [64]. In contrast, the prediction that rapamycin would preserve proliferative potential (RPP) is counterintuitive. After all, rapamycin inhibits proliferation, which makes it crucial to confirm this prediction.

HT-p21 and HT-p16 cells respectively express IPTG-inducible p21 and p16 [1,9,65,66]. Cell cycle arrest can be switched off and on in these cells through the addition and removal of IPTG. If arrest was induced for only 1–2 d, proliferation re-started in most cells after removal of the IPTG. When arrest lasted longer than 3–4 d, however, cell proliferation did not re-start after IPTG removal [9]. When cells were treated with IPTG in the presence of rapamycin, cell proliferation re-started after the IPTG and rapamycin were washed out. Thus, rapamycin preserved RPP in p21/p16-arrested cells. Rapamycin similarly preserved RPP during cell cycle arrest caused by pharmacologic inhibitors of CDK4/6, DNA damaging drugs, HDACi and phorbol ester [29,58,65–67]. Suppression of senescence by rapamycin was further confirmed *in vitro* and *in vivo* [26,40,62–81]. In addition to rapamycin, everolimus and ridaforolimus (two rapalogs), pan-mTOR inhibitors, nutlin-3a (a p53-inducer), hypoxia and contact inhibition all inhibit mTOR and thus maintain RPP in arrested cells [2,32–34,58,82–86]. Suppression of senescence by pan-mTOR inhibitors is closely associated with dephosphorylation of 4E-BP1 at both rapamycin-sensitive and -insensitive sites [2,31–34].

Irreversible proliferative arrest due to loss of RPP

Although senescent cell cycle arrest is often said to be irreversible, it is technically reversible, if the correct method is used. It cannot be reversed using serum, nutrients, growth factors or other stimuli. Serum reverses quiescence caused by serum withdrawal,

but serum stimulation causes senescence when the cell cycle is blocked by p21 or p16 [1,58]. Similarly, quiescence caused by contact inhibition can be reversed by splitting cell cultures, but splitting senescent cultures only deepens senescence because mTOR is activated in sparse cell cultures [84,87,88]. It has therefore been suggested that the term "irreversible" be narrowed to "irreversible by mitogenic or oncogenic stimuli" [7].

Consider the mTOR-driven model of senescence. In quiescent cells, mTOR is deactivated (by serum/nutrient withdrawal, contact inhibition, hypoxia, etc.) and cyclin D1 is low; cells do not cycle and do not grow. Growth stimuli activate mTOR and induce cyclin D1, causing proliferation. However, strong growth stimuli can cause proliferation that is followed by arrest and geroconversion. For example, oncogenic Ras and Akt activate mTOR and induce cyclinD1, causing proliferation. But they can simultaneously induce p53, p21 and p16, thereby blocking the cell cycle [8,34]. This block cannot be reversed by growth stimulation, which only deepens the block and enhances mTOR-dependent geroconversion, but it can be reversed by inactivating p53, p21 and p16, for instance [3,15,89]. Once the cell cycle is unblocked, senescent cells re-enter the cell cycle but cannot undergo mitosis [9,10]). Moreover, these cells are hypermotile and literally tear themselves apart and eventually die (see micro-video in ref [10].). Thus, while cell cycle arrest is formally reversible, the loss of RPP renders it irreversible in practical terms. However, because rapamycin maintains RPP, cells in culture can regenerate once the cell cycle is unblocked.

Molecular definition of senescence

Although senescence can be defined as arrest that is irreversible by mitogenic or oncogenic (mTOR-activating) stimuli, this definition cannot be easily used in practice. Furthermore, RPP is a "potential" and is therefore difficult to test, especially *in vivo*. Defining senescence based on β-Gal staining is also problematic. β-Gal-staining is a marker of lysosomal hyperfunction [46–50]. Consequently, serum-starved and contact-inhibited cells are β-Gal-positive too [46,84], although these cells are not senescent (Figure 4). So can we distinguish β-Gal-positive quiescent and senescent cells? In β-Gal-positive *quiescent* cells, levels of phosphorylated S6, S6K and 4E-BP 1 are low or undetectable (Figure 4). In contrast, these proteins are highly phosphorylated in senescent cells (Figure 4). In β-Gal-positive quiescent cells, insulin

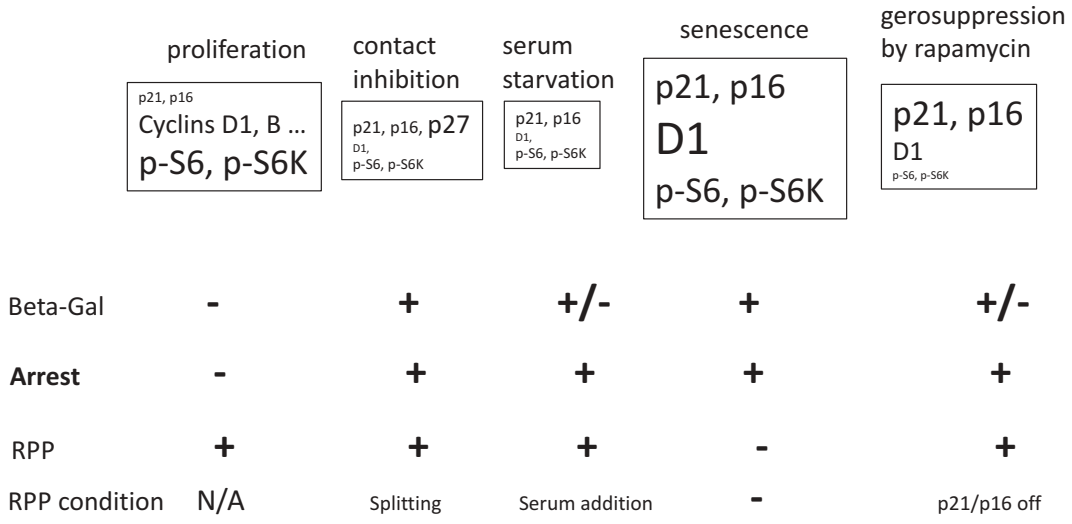

Figure 4. Characteristics of the main nonproliferative conditions.

Proliferation is shown for comparison. Cells are positive for cyclins and activated mTOR (phospho-S6/S6K/4EBP1). Four types of arrest are characterized by high (+) or moderate (±) β-Gal staining. Excluding senescence, the three other types of arrest are reversible (RPP+) under the indicated conditions. Contact inhibition (quiescence) is characterized by high p27 levels, small cell size, deactivated mTOR, and low cyclin levels; arrest is reversible by splitting cell cultures. Serum starvation (quiescence) is characterized by low levels of all molecular markers and small cell size. Senescence, in contrast, is characterized by super-induction of cyclin D1, high p21 or p16, activated mTOR pathway, large cells, and irreversibility. Rapamycin deactivates mTOR, decreasing cell size and rendering the condition reversible.

and other growth factors induce phospho-S6, whereas in senescent and proliferating cells, phospho-S6 is not further induced upon stimulation.

We can define senescence as practically irreversible arrest, a non-proliferative state, associated with proliferation-like mTOR activity (high levels of phospho-S6/S6K/4E-BP1). In addition, high levels of phospho-ERK and cyclin D1 coexist with p21 and/or p16 (Figure 4), and are associated with hypertrophy and hyperfunctions, including SASP, lysosomal hyperfunction (β-Gal staining), lipid synthesis (oil red O staining), ROS and lactate production. We suggest such cells can be identified using double-staining for phospho-S6 plus p16/p21, phospho-S6 plus β-Gal, or p16/p21 plus cyclin D1. A combination of all these markers may be the most valuable (Figure 4).

Cell culture and the organism

Rapamycin inhibits growth and slows geroconversion, which is a continuation of growth. In analogous fashion, organismal aging is a continuation of developmental growth [90–98]. Rapamycin (at high doses) slows cell proliferation within the organism, causing leucopenia, thrombocytopenia and mucositis and also decelerates organismal aging and its manifestations: age-related diseases [92].

In cultured cells, the senescence program consists of two steps: arrest plus geroconversion. Because most cells within organisms are quiescent, senescence consists of slow geroconversion. Why is it so slow? Contact inhibition and high cell density [84], hypoxia [83,99], and serum/nutrient starvation each deactivate mTOR. Within the organism, most cells are confluent or contact inhibited, and oxygen tension (1–3% O_2) as well as levels of nutrients/growth factors are low. These growth-limiting conditions may maintain quiescence for decades during a human lifespan.

In vitro, senescence is induced in sparse cell cultures in the presence of 21% oxygen and high levels of growth factors and nutrients. For example, glucose levels in DMEM are 5-fold higher than in normal blood, corresponding to levels associated with diabetic coma and causing complete insulin-resistance [55]. As a result, geroconversion is a fast event *in vitro*, especially in cancer cells. In fact, mTOR activity is much higher in cultured cells than in the organism and is inversely related to Akt activity [87].

Geroconversion and disease

mTOR-driven geroconversion is associated with enhanced tissue-specific functions (hyperfunctions), which drive age-related diseases. For example, vascular smooth muscle cell contraction, hypertrophy and hyperplasia all contribute to hypertension and atherosclerosis. Hyperfunction of adipocytes and hepatocytes increase blood cholesterol levels, contributing to atherosclerosis. Atherosclerosis, hypertension and thrombosis (due to platelet hyperfunction) can culminate in stroke and infarction and subsequent loss of organ function. Therefore, initial hyperfunction eventually leads to dysfunction and functional decline [90,100].

It was known by 2006 that rapamycin delays most age-related diseases [90]. As predicted [90], rapamycin prolongs the lifespan of mice [60,75,101–122]. Rapamycin and everolimus have been tested in healthy volunteers [123,124] and in the elderly [125–127]. A combination of rapamycin with several conventional life-extending drugs, known as the Koschei formula, is already being used for the elderly in the Alan Green Clinic in Little Neck, New York (https://rapamycintherapy.com).

"Repetitio est mater studiorum"

Rapamycin and other mTOR inhibitors suppress geroconversion. In the presence of rapamycin, time moves slower for cell growth, cycling and geroconversion [128]. Rapamycin does not reverse cell cycle arrest and does not stimulate proliferation. Like all gerosuppressants, rapamycin slows cell growth and geroconversion in arrested cells. Rapamycin also maintains RPP in quiescent/arrested cells. To observe this effect, the cell cycle block must be relieved – for example, by decreasing p16 and p21.

Two paradoxical effects of rapamycin

Notably, a two-dimensional model of aging predicts contradictory events.

1. Paradoxical prevention of cell cycle arrest by rapamycin

In replicative senescence, geroconversion starts before cell cycle arrest [30]. Therefore, to prevent

geroconversion, rapamycin should be added to proliferating cells. Although rapamycin may itself cause cell cycle arrest, transient treatment or low doses of rapamycin can trick cells, thereby preventing replicative senescence [34,59,129]. However, this phenomenon is not as it seems. Cells exhibit clonal proliferation after rapamycin as well as a change in the karyotype of chromosome 3 in the region containing the nucleolar organizer [129]. This proliferation is thus linked to chromosomal rearrangement, but a selective advantage is detected only in cells in which mTOR activity was inhibited. Similarly, rapamycin increases the efficiency of the cellular reprogramming of induced pluripotent stem cells (iPSCs) [130–132]. For example, brief treatment with nanomolar concentrations of rapamycin enhances cellular reprogramming, though more sustained treatment decreases reprogramming [132].

2. Paradoxical senescence-like state

In cell culture, high concentrations of rapamycin cause arrest in some cell types [133–136]. In the arrested cells, rapamycin slows geroconversion but does not block it completely [2,65]. This initially-reversible arrest may, in theory, slowly lead to geroconversion in the cultured cells. Thus, rapamycin does not cause senescence per se: it merely cannot suppress geroconversion completely. Rapamycin-induced senescence has been observed *in vitro* [135]. As an illuminating example, consider p53. Nutlin-3a, a p53-inducing drug, causes senescence in some cell types [137,138]. More precisely, nutlin-3a-induced p53 arrests the cell cycle, after which mTOR drives geroconversion in the arrested cells [82,138]. On the other hand, p53 (depending on its level and cell type) may slow geroconversion by inhibiting mTOR [42,138–140]. For example, a 3-day exposure to nutlin-3a causes reversible quiescence [141,142], whereas p21 and p16 induce senescence in the same cells [82]. By inhibiting geroconversion, nutlin-3a slows p21- and p16-induced senescence [82]. Suppression of senescence by p53 was observed in several models [60,143–145].

What is the difference between rapamycin, p53, and p21/p16? As illustrated in Figure 5, Rapamycin causes cell type-specific arrest but slows geroconversion universally. P53 readily and universally causes arrest but inhibits geroconversion under

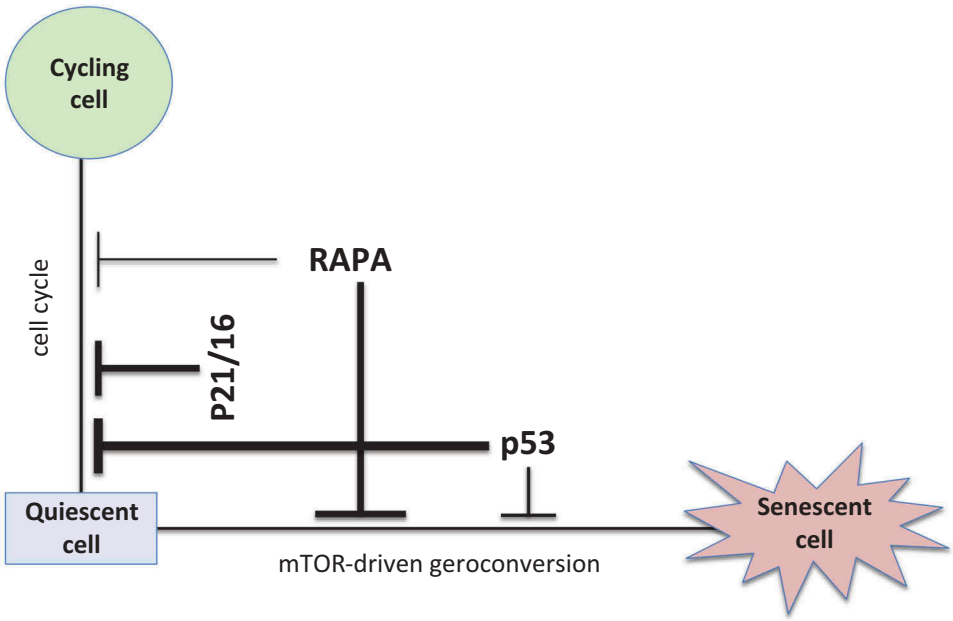

Figure 5. Paradoxical effects of p53 and rapamycin.

Senescence is cell cycle arrest plus mTOR-driven geroconversion. p21 and p16 cause cell cycle arrest without affecting mTOR, which then drives accelerated senescence. p53 causes cell cycle arrest and can moderately inhibit mTOR in a cell type-specific matter. By inhibiting mTOR, p53 suppresses geroconversion and maintains quiescence. When p53 does not inhibit mTOR, it causes senescence. mTOR inhibitors like rapamycin strongly inhibit mTOR and maintain quiescence in cells arrested by p21 or p16. However, rapamycin inhibits cell cycling in some cell types. If rapamycin-induced cell cycle arrest occurs, geroconversion to senescence may also occur (see text).

certain conditions [139]. P21 and p16 firmly and universally block the cell cycle without affecting geroconversion.

Disclosure statement

No potential conflict of interest was reported by the author.

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