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Accelerated Senescence of Cancer Stem Cells: A Failure to Thrive or a Route to Survival?

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<http://dx.doi.org/10.5772/intechopen.68582>

Abstract

Accelerated senescence of cancer stem cells (CSCs) represents an adaptive response allowing withstand cell death. TP53, the pivotal tumor suppressor plays an important role in this process by inducing a prolonged dual state with senescence and self-renewal as potential outcomes. Molecularly, this is achieved by activating both OCT4A (POU5F1) and p21CIP1. OCT4A suppresses the excessive activity of p21 preventing the immediate precipitation of apoptosis or terminal senescence. It persists as long as sufficient cellular energy remains; generated through autophagy, itself sequestering p16INK4A in the cytoplasm. As such, autophagic capacity is the bottleneck of these TP53-dependent senescence reversal processes, as well terminal senescence will follow if DNA damage is not ultimately repaired. In TP53 mutants the CSC-like state is boosted by stressed cells overcoming the tetraploidy barrier. These cells acquire additional DNA repair capacity through mitotic slippage and entrance to a sequence of ploidy cycles, allowing repair and sorting DNA damage, ultimately facilitating the genesis of mitotically competent daughter cells following final depolyploidisation. Again, autophagy is required to fuel this process. More detailed knowledge of these arcane processes anticipates the provision of anti-cancer drug targets, such as AURORA B kinase and Survivin, which ensure mitotic slippage and the continuity of ploidy cycles.

Keywords: accelerated senescence, cancer stem cells (CSCs), TP53, DNA damage, self-renewal OCT4A (POU5F1), p21CIP1, pluripotency, apoptosis, metastability, DNA repair, autophagy, p16INK4A, tetraploidy, ploidy cycles, AURORA B, Survivin, AMPK

1. Introduction

In 2001 Roninson and colleagues [1] published the now seminal article entitled “If not apoptosis, then what? Treatment-induced senescence and mitotic catastrophe in tumor cells”. After decades

of overwhelming attention toward apoptosis induction as the cure for cancer, this article gave birth to a new field in cancer research. They wrote: "Inhibition of the program of apoptosis has been reported to have little or no effect on clonogenic survival after treatment with drugs or radiation in several tumor cell lines. A decrease in apoptosis is compensated in such cell lines by an increase in the fractions of cells that undergo permanent growth arrest with phenotypic features of cell senescence. The senescent phenotype distinguishes tumor cells that survived drug exposure but lost the ability to form colonies from those that recover and proliferate after treatment. Although senescent cells do not proliferate, they are metabolically active and may produce secreted proteins with potential tumor-promoting activities."

Since that article, the induction of senescence was even claimed as the new goal of cancer treatment [2] and many researchers stepped on this path.

In this chapter, we describe our data over the last 2 decades, which along with other research, substantiates the exact opposite: that so-called accelerated cell senescence (ASC) (also called stress-induced premature senescence) and mitotic catastrophe (MC) are not desired goals of cancer treatment. Rather, we show that these processes can enable genotoxically treated cancer cells to escape cell death, not only by secretion of survival promoting components [3] but also by effective DNA repair. By stabilizing or recovering the innate stem properties of cancer stem cells (CSCs) senescence can be reversed by DNA damaged-induced ACS. To explore these concepts clearly it is first important to review and define the typical features of cell senescence and the biology of CSCs.

2. Biological features of cell senescence: what is clear and what is not?

Replicative senescence is usually dependent on *TP53/p21CIP1/pRb/E2F* pathway, whereas accelerated senescence can be mediated through *TP53/p21CIP1/pRb/E2F* or, *p16ink4a/pRb/E2F* pathway or both [4]. Cells acquire flat morphology, upregulate autophagy, and become positive for sa- β -galactosidase (pH 6.0) staining, indicative of high lysosomal activity. Erosion of telomeres and achievement of the Hayflick limit characterizes proliferative senescence, while in ACS the telomeres are not shortened [5]. However, the emergence of DNA strand breaks and the resulting DNA damage response (DDR) characterizes both proliferative and accelerated senescence [6]. Persistent irreparable DNA damage triggers the senescence-associated secretome [7], which is another feature of cell senescence. Emergence of endopolyploid cells, some capable of escaping senescence is also a typical feature of normal [8] and cancer cells [9, 10]. The reversibility of genotoxically induced senescence coupled to reversible polyploidy and its relation to stemness and population recovery are key phenomenon to understand if we wish to develop better cancer treatments [11–17]. The polyploidy component of senescence is associated with resistance to chemotherapy and involves mTOR activation; its inhibition causes senescence reversal, neo-expression of stem cell markers [18] and increased resistance of cancer cells to chemotherapy [19]. However, whether any senescing primary somatic cancer cell is capable of displaying the above features associated with senescence reversal or whether this only applies to cancer stem cells (CSCs) and how the recovery finally occurs is still largely unclear.

3. The common biological features of embryonic stem cells (ESCs) and CSC

It is now generally accepted that CSCs play a central role in cancer genesis and promotion. Firstly, they possess the developmental potential, being capable of sphere formation and the ability to differentiate into mesoderm, endoderm and ectoderm progenies. Many gene modules of ESC are found active in various cancers [20], in turn, aggressive tumors express the markers of ESC or germ cells [21–24]. Third, tumors also acquire epigenetic profiles of ESC under genotoxic [25–28] or hypoxic conditions [29], in association with overcoming the tetraploidy barrier. So, epigenetically, CSCs of highly de-differentiated tumors possess many features of ESC.

Epigenetic instability is another notable feature of ESC and likely also of CSC. ESCs possess 'poised', chromatin marking of key developmental genes. Such marking consists of large regions of H3 lysine 27 methylation harboring smaller regions of H3 lysine 4 methylation. These domains tend to coincide with genes of transposable elements (TE) expressed at low levels [30]. Some activity of TE may provide the transcriptional noise, which is necessary for the fate changes observed during early embryo development [31, 32]. Moreover, the enhancers in ESCs are enriched for the transposable elements and genetic variations associated with cancer [33].

An additional peculiarity of ESC (and likewise CSC) is the absence of the cell cycle G1/S restriction checkpoint: here OCT4 and NANOG activate cyclin D kinases cdk4 and cdk6 [34] to force ESC into S-phase. Therefore, damaged ESC cells typically accumulate in the G2 DNA damage checkpoint instead, whose relative weakness allows mitotic slippage [35, 36]. Moreover, stressed ESC and likely also CSC possess a peculiar intermediate post-slippage phase (not 4 N-G1) [37, 38] containing non-degraded cyclin B1, which is normally destroyed after mitosis. In irradiated lymphomas and HeLa cells this nondegraded cyclin B1 was found to be sustained by activated Mos kinase [39]. Some additional activators of meiotic prophase were also revealed [40, 41] indicating a possible trigger from the mitotic DNA damage checkpoint into a meiotic prophase-like state with its more effective recombination checkpoint. Potentially, this meiosis-like molecular setting allows CSCs to tolerate both DNA damage and tetraploidy and use this compartment for DNA repair by homologous recombination [14, 42]. All three facets of CSC biology; poised chromatin, an epigenetic shift to embryonicity, and the peculiar cell cycle checkpoints are apparently interrelated and involved in ACS, whose main hallmark is persistent DNA damage. The remainder of this chapter is an attempt to assess this notion within our experimental material.

4. Accelerated senescence of human fibroblasts induces senescing tetraploid cells with transient self-renewal potential

In one of our recent experimental systems, embryonal lung human fibroblasts (IMR90 cells) were grown in normoxia (20%) and 5% CO₂ and reached full senescence (proliferative arrest

with zero mitotic index) after 32–34 passages. Senescence was characterized by flat morphology of enlarged cells, nuclear positivity of p21CIP1 and cytoplasmic accumulation of p16INK4A; at the terminally stage nuclei were swelling and p16 entered the cell nuclei [43]. DNA cytometry revealed an accumulation of a portion of the prematurely senescing cells in the G2 compartment (**Figure 1A, B**) which were also overcoming the tetraploidy barrier with formation of a few (4–6%) tetraploid cells, which sometimes entered aberrant mitoses. These cells with large polyploid nuclei began to express both senescence markers (p21CIP1 and p16INK4A) with the self-renewal marker NANOG; these cells were also positive for DNA double strand breaks (DSB) (**Figure 1C–F**). The acquisition of bi-potentiality by a small proportion of normal

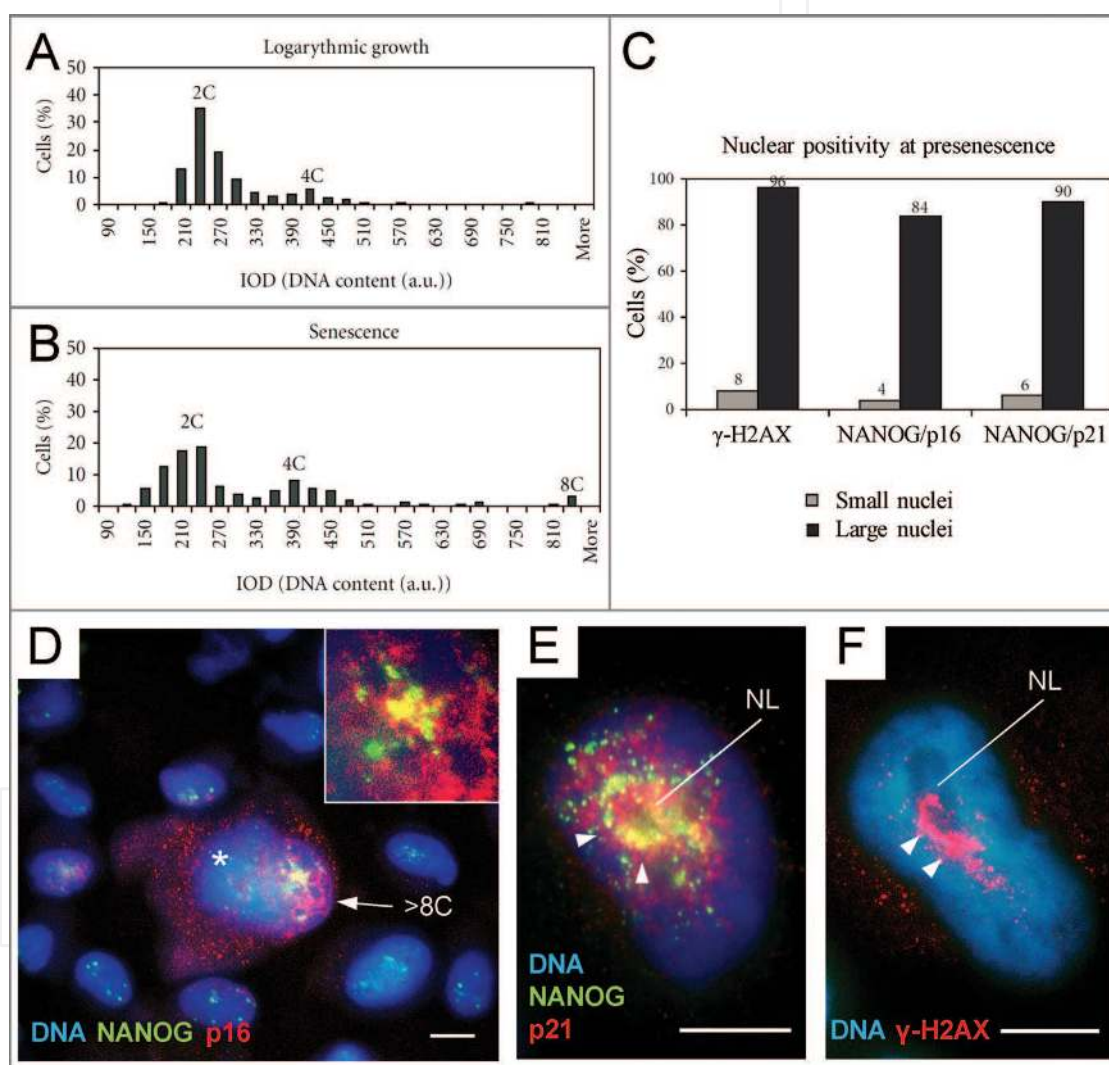


Figure 1. Characteristics of IMR90 human embryonal fibroblasts undergoing pre-senescence. Cells were cytopun, fixed and stained for DNA image cytometry. DNA content was determined for at least 200 cells in each condition and is represented as the percentage in a state of (A) logarithmic growth; (B) presenescence with some accumulation of cells in the G2M (4C) checkpoint and some increase of >4C DNA cell numbers. (C) shows the results from immunofluorescence studies staining for γ -H2AX, NANOG, P21CIP1 or p16INKA4A proteins with positive staining discriminated with respect to nuclei size; (D–F) – typical immunofluorescence patterns showing combinations of self-renewal (NANOG) and senescence (p21CIP1 and p16INK4A) markers in the same cells with large (>4C nuclei as measured by DAPI) nuclei, and positivity for DSBs γ -H2AX. Bars= 10 μ m. Republished from Ref. [43].

cells overcoming the G2M DNA damage checkpoint is reminiscent of stem cell activity; with the same lack of arrest in the G1/S checkpoint and weak G2M checkpoint. Nevertheless, these cells did not persist in the culture and NANOG expression was lost in later passages.

These observations heightened our interest in the role of senescence in the CSC model.

5. Ovarian germline cells challenged by genotoxic stress display the dual p53-dependent expression of p21CIP1 and OCT4A

As a model for CSC we chose the ovarian germline cancer cells PA1. These cells are wt *TP53*, possessing features of embryonal carcinoma [44]. They were treated with etoposide (ETO), the inhibitor of Topoisomerase II, to induce DSB through the impairment of S-phase [45]. Cells entered massive, prolonged, G2-arrest, acquired flat morphology, and signaled persistent DDR for about 4–5 days in nearly 100% of cell nuclei (positive for γ -H2AX and CHK2 foci) with some cells overcoming the tetraploid barrier (**Figure 2A, B**). The most surprising fact was that regulators of the opposing processes of senescence (p21CIP1) and self-renewal OCT4A (POU5F1) were highly induced in the same G2-arrested cells (**Figures 2C and 4A**). This was downstream of activated *TP53*, as both p21 and partly OCT4A became downregulated after *TP53* RNAi silencing (**Figure 2C**) [45]. Further study [46] revealed that p53-activated OCT4A down-regulated p21CIP1, moderating its expression and preventing cells from precipitating terminal senescence or apoptosis (**Figure 2D**), thus providing the opportunity for repair. Downregulation of p21 via OCT4 was previously shown in ESC [47].

Moreover, such stress-activated OCT4A was transiently disconnected from its self-renewal partners (SOX2 and NANOG), as those protein levels were low and/or not activated alongside Oct4A [46]. Therefore, the autoregulatory and feedforward loops seen in ESC [48] were not present, potentially due to the known down-regulation of the Nanog gene promoter by activated p53 [49] and/or the down-regulation of NANOG by high levels of OCT4A [50]. However, it is also possible that another, Cdk4-activating function of OCT4A [34] could be enhanced, while inhibiting action of p21 on Cyclin D/cdk4,6 checkpoint function reduced forcing escape of the damaged cells from G1/S checkpoint leading to their accumulation in G2-arrest. **Figure 3** presents these hypothetical relationships between the cell cycle and pluripotency functions of stress-activated OCT4A induced by DNA damage through activated p53.

The bizarre duality of OCT4A with p21CIP1 was subdued and cell cycle in the PA1 clones returned to normal, on days 7–14 (**Figure 2A**), while silencing of stress-activated OCT4A prevented recovery [46]. This transient undecided state in G2 arrest, between true senescence and true self-renewal, was thus lasting as long as wt*TP53* was activated by the DNA DSB.

In accordance with our findings in PA1 cells after ETO treatment, a recent high-throughput RNAi screen revealed the intrinsic roles of S and G2, functionally establishing that pluripotency control is hardwired to the cell-cycle machinery and that the ATM/ATR-CHEK2-p53 axis enhances the TGF- β pathway to prevent premature cell death [36]. As well, [3] showed that

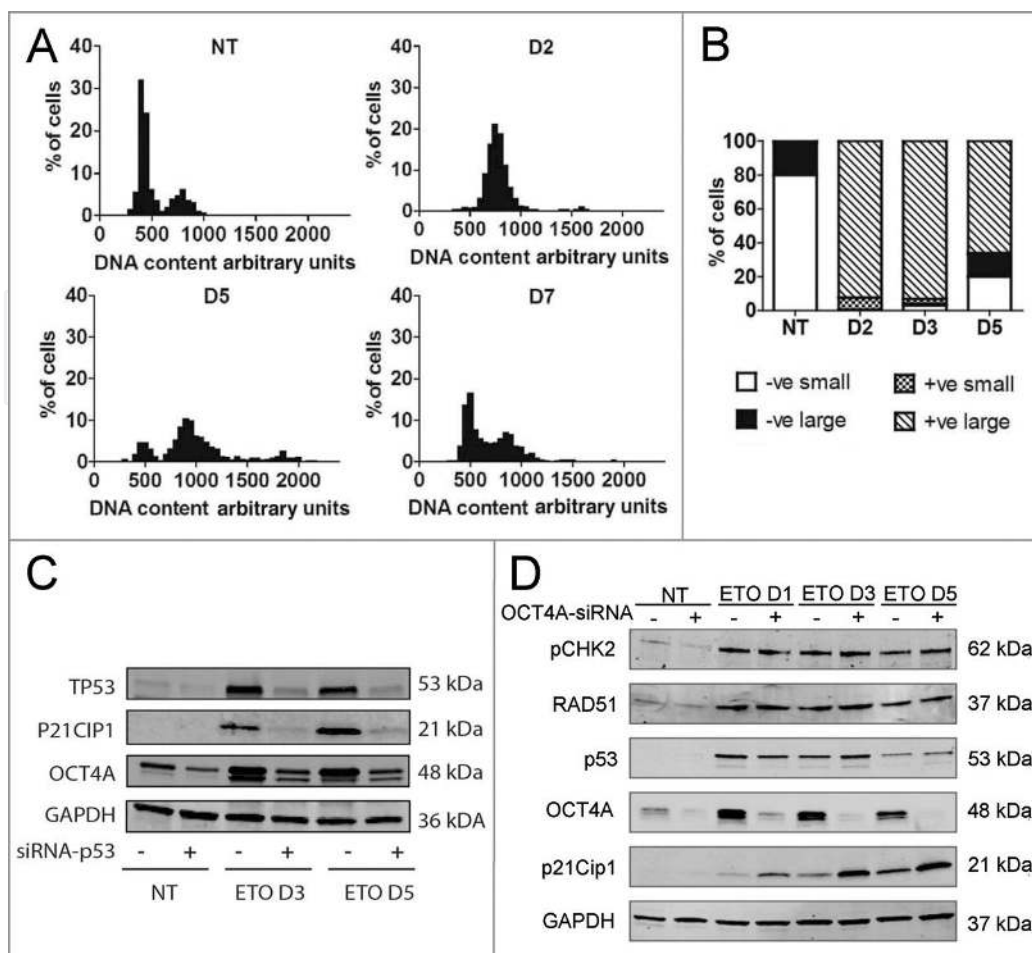


Figure 2. Response of PA -1 cells to ETO treatment. PA -1 cells were treated with 8 μ M ETO for 20 h, then washed and assessed at the indicated time point. **(A)** Cells were cytopun, fixed and stained for DNA image cytometry as previously described [46]. DNA content was determined for at least 200 cells in each condition and is represented as a percentage. Profound G2 arrest on day 2 was observed followed by the simultaneous emergence of a polyploid (>4C) and 4nG1 fraction on day 5 before the recovery of the normal cell cycle profile by day 7. **(B)** The proportion of pCHK2-positive cells was examined in the context of DNA content with cells sub-divided into small or large cells. In the NT control sample, all cells were pCHK2-negative with an expected nuclei size distribution (2C 80%; \geq 4C 20%). On day 2, all cells were pCHK2-positive and the vast majority of nuclei were large (\geq 4C). By day 5, cells with small nuclei appeared, all of which were pCHK2-negative. Data are representative of >three independent experiments; **(C)** Immunoblot analysis of TP53, OCT4A and P21CIP1 in PA-1 cells after ETO treatment. PA-1 cells were treated with non-target (ntg) siRNA (-) or siRNA-TP53 (+) for 24 h before treatment with 8 μ M ETO, washing after 20 h and cell lysates made and assessed by immunoblotting for p53, P21CIP1, OCT4A or GAPDH as a loading control at the indicated time-points (day 3 and 5). p53 was upregulated in response to ETO treatment and suppressed by siRNA-TP53. P21CIP1 and OCT4A were also upregulated by ETO treatment, and the upregulation was restricted by treatment with siRNA-TP53. Data are representative of three independent experiments. **(A–C)** republished from Ref. [45]. **(D)** OCT4A suppresses p21CIP1 and induces senescence. PA-1 cells were treated with ntg-siRNA or OCT4A-siRNA for 24 h before treatment with 8 μ M ETO for 20 h and replacement with fresh media. Protein expression was assessed at the indicated time points by immunoblotting. Cell lysates were made and assessed by immunoblotting for pCHK2, RAD51, p53, p21CIP1, OCT4A and GAPDH as a loading control. Republished from Ref. [46].

cellular senescence accompanying DNA damage or DNA damage as such favors cell reprogramming in vivo models. It should be noted however, that the frequency (chance) of survival in our PA1-ETO model was not high. It stresses the importance of another possible player in this “undecided” stage between senescence and self-renewal, of transcriptional noise.

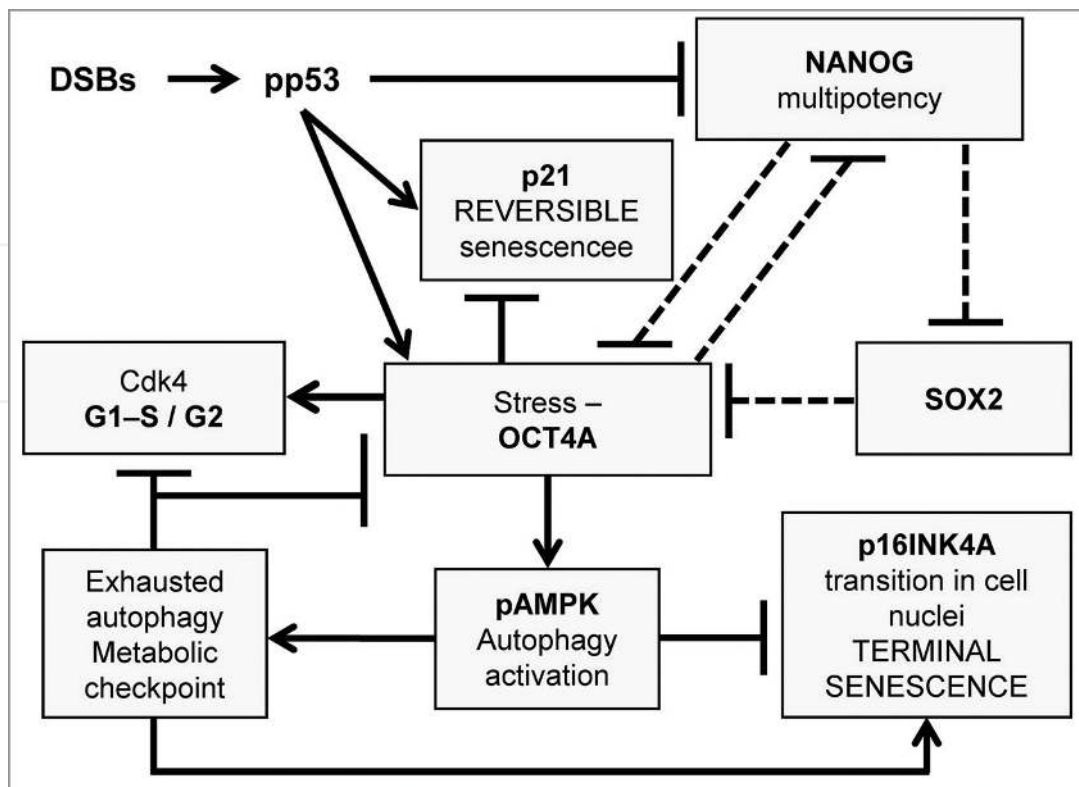


Figure 3. Schematic of the response of wt *TP53* cancer stem cells to genotoxic damage. P53 is activated and simultaneously induces p21CIP1 and OCT4A. OCT4A moderates the expression of p21CIP1 preventing apoptosis or terminal senescence [45, 46]. Activated p53 also downregulates the promoter of the Nanog gene [49]. Therefore, the multipotency circuit based on Nanog stimulation of OCT4A and SOX2 [48] (dashed lines) is interrupted. In addition, the overexpressed OCT4A also down-regulates NANOG [50]. Activated OCT4A also enhances the activity of cdk4 and favors cells exit from the G1/S restriction checkpoint [34] and accumulation in G2. Activated p53 also activates pAMPK, likely via OCT4A. pAMPK activates autophagy. Functional autophagy sequesters and digests p16INKA4 within the cytoplasm, preventing its transition into the cell nuclei and hence terminal senescence. With exhaustion of autophagic capacity (halting of autophagic flux) pAMPK activates a metabolic checkpoint [51], which precipitates cell death in the G1 and possibly also G2 cell compartments.

6. Transient bi-potentiality of CSC for senescence and self-renewal displays the population features of “noisy” expression and activated transposable elements

One of the interesting facets of this dual expression of self-renewal and senescence regulators in the PA1-ETO model was the high heterogeneity in response, with individual cells expressing wildly differing levels of OCT4 and p21 (**Figure 4A**). This explorative chaos continued for 4–6 days and culminated with massive cell death selecting a small proportion (<1%) of resistant survivors. Earlier studies on ESC observing the extensive heterogeneity and fluctuations of gene expression in individual stem cells led the authors to suggest that “noise” may be the central driving force behind multipotency [32, 50, 52].

Therefore, notably, a similar long ‘stochastic’ phase of choice between senescence and self-renewal (initiated by activating DNA repair and mesenchymal to epithelial transition), with

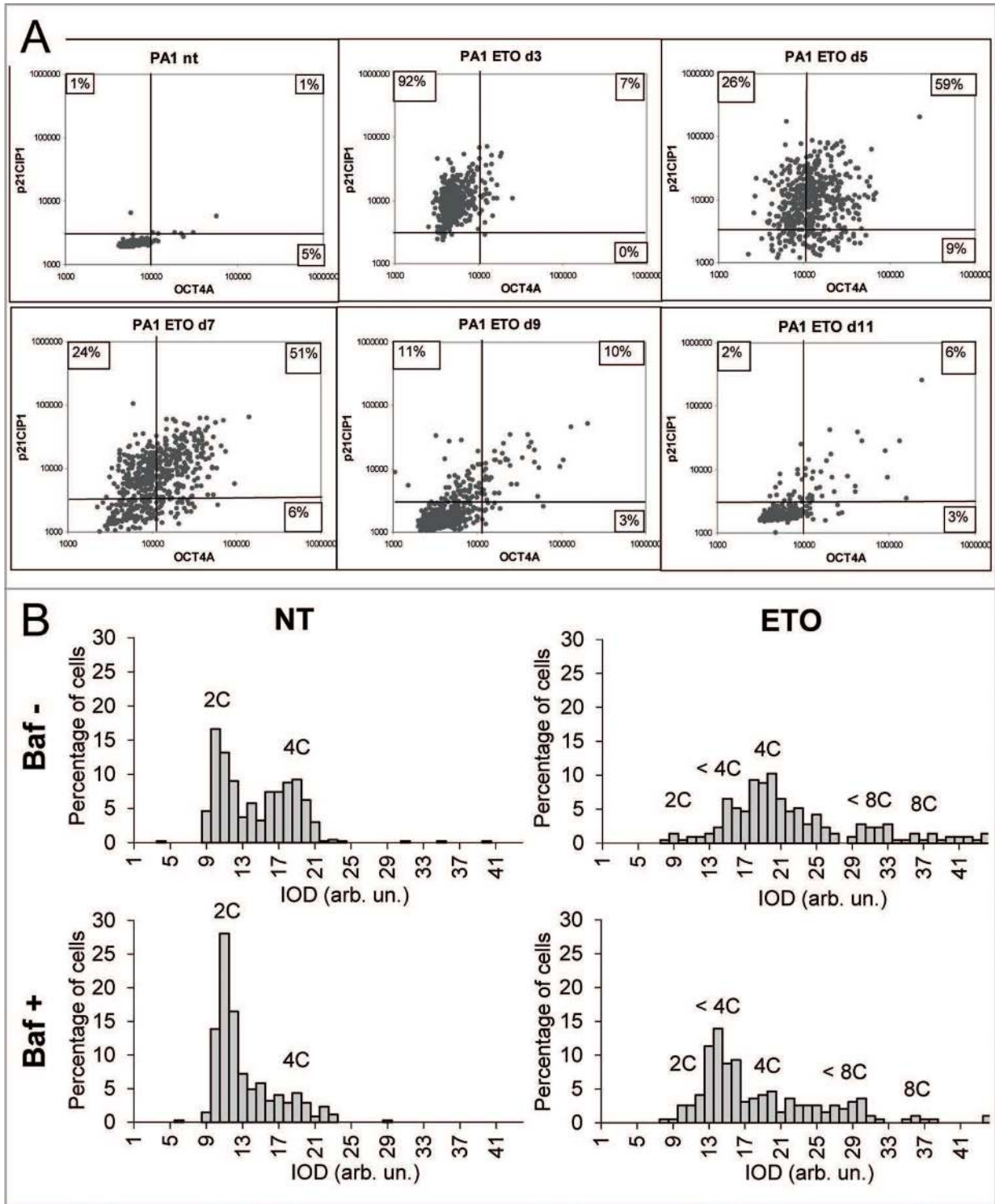


Figure 4. Population heterogeneity in expression of OCT4A and p21CIP1 in response of PA1 cells to ETO treatment. (A) Population heterogeneity in expression of OCT4A and p21CIP1 is extended for days 2–6 post ETO treatment, before recovery from day 7, when it reduces; (B) Population heterogeneity is accompanied by a degree of polyploidy as represented by DNA histograms on day 4, with underreplication of the cells in late S-phase of diploid and polyploid cell cohorts; this phenomenon is enhanced after impediment of autophagic flux. Republished from Ref. [46].

heterogenous activation of pluripotency genes, preceding the period of further determination of self-renewal circuitry has also been reported during the induction of pluripotent stem cells [53].

Our study of DNA histograms in ETO-treated PA1 cells revealed in addition to G2M arrest, a strong under-replication in late S-phase (**Figure 4B**), the time of constitutive heterochromatin replication [46]. Similarly, arrest in late S- and G2M phase was reported after Doxorubicin treatment in p53 mutant cancer cells [54]. This feature may therefore be equally required for any senescence causing release from silencing and subsequent activation of TE nested in constitutive heterochromatin as retrotransposition was found in replicative cell senescence [55, 56]. In particular, under-replication may cause de-repression of TE genes and result in the epigenetic activation of the developmental genes in the poised chromatin regions [30] enabling the reprogramming by senescence. Indeed, we observed the activation and clustering of *ALU* elements in the ETO treated PA1 cells [57]. The initial de-repression of transposons could provide the necessary “noisy” background of transcription allowing chaotic fluctuations of gene expression, which enable stochastic choice of the appropriate attractors for cell fate change and escape from terminal senescence.

7. The role of autophagy in preventing terminal senescence

Our further observations in the PA1 model showed the importance of autophagy in withstanding the proteotoxic stress following ETO treatment and its crucial role in maintaining viability; inhibition of autophagy culminated in chromatin fragmentation and nuclei disintegration [46, 57]. Stress-activated OCT4A mainly colocalized, and correlated in its nuclear concentration in individual cells, with activated AMP^{Thr172} kinase (**Figure 5A, B**). AMP-activated protein kinase (AMPK) serves as a general energy depletion sensor and activator of autophagy. The energy stress-response of AMPK is also tightly linked to the DDR of p53 [58, 59] and can induce a p53-dependent glucose-sensitive metabolic checkpoint [51] precipitating apoptotic cell death from the G1/S and likely autophagic death from G2M checkpoint [60].

We found, in addition, that active autophagy in PA1-ETO cells sequestered p16INK4A aggregates within the autophagic vacuoles (**Figure 5C i–ii**), while disability of autophagy enabled p16 diffuse distribution in the cell nuclei and caused terminal senescence with nuclear disintegration (**Figure 5 iii–iv**) preventing survival of ETO-treated cells [46]. The p53-dependent role of AMPK, its relationship with stress-activated OCT4A, and the role of autophagy in the prevention of terminal senescence for TP53 wild-type CSC cells is schematically presented in **Figure 3**.

However, in *TP53* mutants, the cells find an additional pathway for potential recovery – they undergo mitotic slippage and employ polyploidy for repair and sorting the damaged DNA.

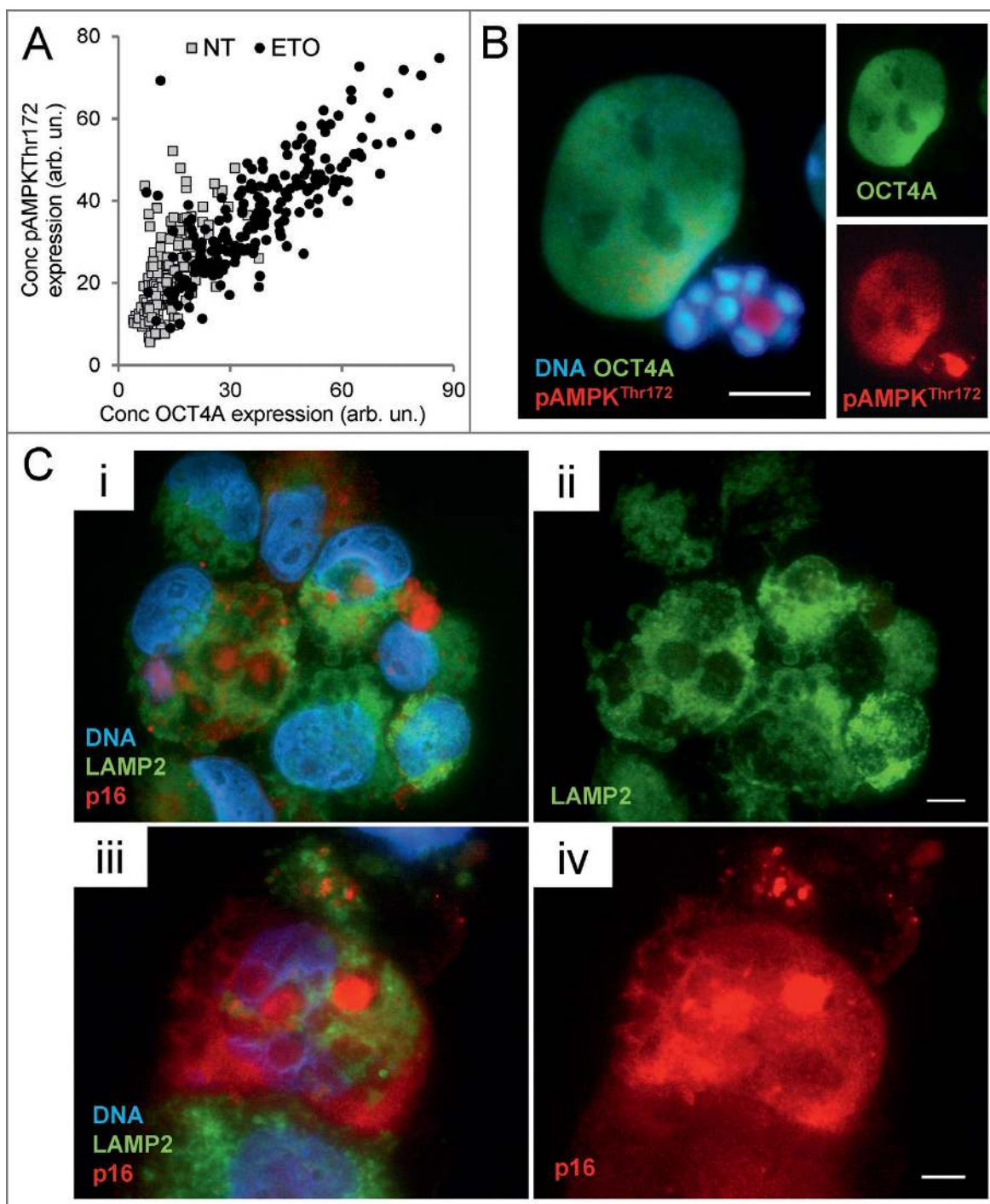


Figure 5. Autophagic response of PA1 cells to ETO-mediated DNA damage: (A) Scatterplot of image cytometry of OCT4A and pAMPK in individual cells assessed on day 4 after ETO treatment. There is a clear correlation between enhanced expression of OCT4A and pAMPK^{Thr172} of ETO-treated cells; (B) OCT4A and pAMPK^{Thr172} immunofluorescence in PA-1 cells 4 days after ETO-treatment. (C) The relationship between autophagy and senescence. PA1-ETO cells were treated without or with Bafilomycin A1, prior to media being removed and replaced with fresh media; cells were harvested 48 h later (day 4). (i) immunofluorescent staining for p16ink4a, LAMP2 or DAPI as shown via the BRG optical filter, (ii) only LAMP2 demonstrating high level of functional autophagy sequestering p16ink4a-containing aggresomes. (iii–iv) shows an example of autophagic failure, where sequestration of p16ink4a is partly lost, diffusing into the cytoplasm and nucleus, which is destroyed. Bars = 10 μ m. Republished from Ref. [46].

8. *TP53* mutants with persistent DNA damage undergo mitotic slippage, ploidy cycles, and are capable of reversing senescence alongside polyploidy

Some authors have reported that genotoxically treated cancer cells can paradoxically combine sa- β -gal-positivity (considered as a universal marker of senescence) with expression of Ki67, a hallmark signature of proliferation. This “swing phenotype” is apparently dependent on p21 and TERT [61]. Others have reported that sa- β -gal-positivity is also compatible with polyploid cells (induced by DNA chemotherapy) undergoing de-polyploidization and surviving [13, 16]. Overcoming the tetraploidy barrier in *TP53* mutants, boosting the self-renewal network [25, 26] – can likely convert tumor cells into CSCs or stabilize them. Moreover, paradoxically, genotoxically challenged *TP53* mutant tumor cells, which uncouple DNA replication from cell division and undergo mitotic slippage possessing both DNA DSBs and Ki67 expression (**Figure 6A**). As well the mitotic chromo-

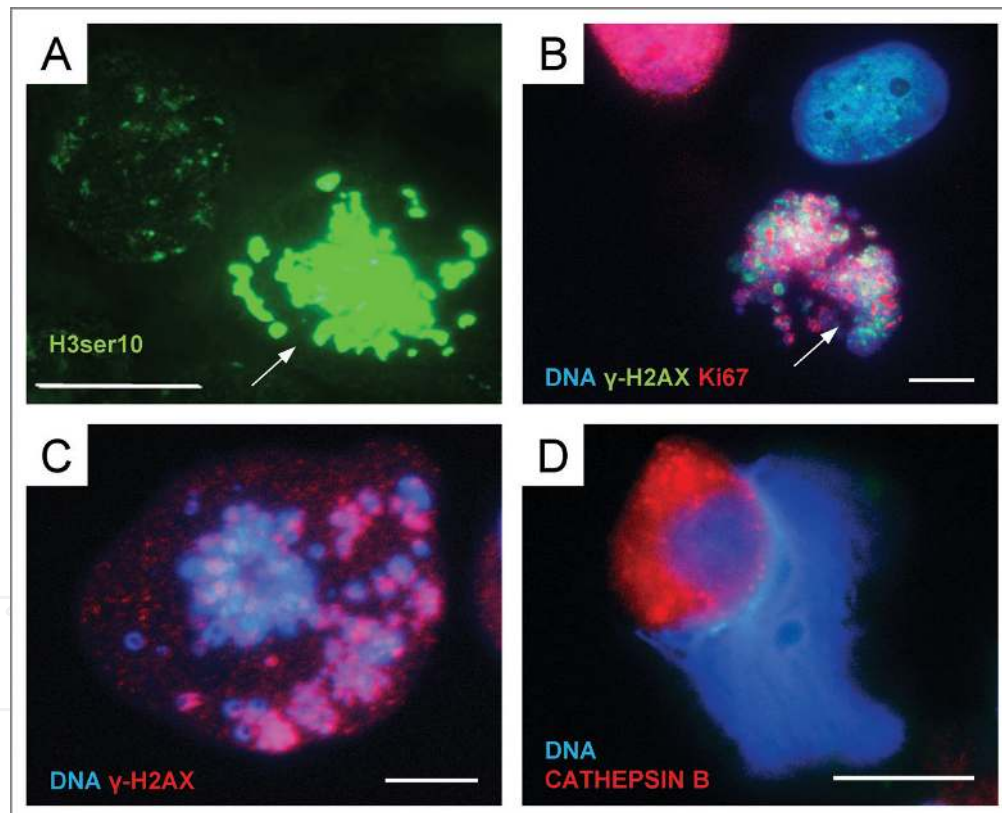


Figure 6. Mutant *TP53* tumors have additional options for repair and sorting of DNA damage in ploidy cycles. The genotoxic damage in mutant *TP53* cancer cell lines of various origins favors mitotic slippage with exaggerated: (A) H3ser10 activation by mitotic AURBK and (B) expression of proliferation marker Ki67 tolerating DNA damage detected by γ -H2AX foci. This is followed by (C) DNA damage sorting by micronuclei in the next mitosis of the polyploid cells and/or (D) by expelling and autophagic digestion of the whole subnuclei of multi-nucleated cells. (A) HeLa cells, 10 Gy irradiation, day 4; (B) MDA MB 231 breast cancer cells on day 4 after 100nM Doxorubicin treatment (in collaboration with A. Boiko); (C) SK-Mel-28 cell, 30Gy, day 2 (in collaboration with TR Jackson); (D) WI-L2-NS lymphoblastoma, 10Gy, day 6. Republished from Ref. [62]. Bars (A)=20 μ m; (B–D)=10 μ m.

some passengers, such as catalytically active Aurora B kinase (**Figure 6B**) and Survivin are expressed during mitotic slippage and in resulting polyploidy interphase [19, 63, 64]. Notably, activated AMPK, responsible for the metabolic aspect of senescence-associated autophagy, also possesses these same chromosome passenger features [65]. All of these observations indicate that stress-induced “senescent” cancer cells retain their proliferation potential through induced polyploidy coupled to active autophagy. During this process or/and in the next tetraploid/octaploid cell cycle they can additionally repair DNA [42] and also sort the un-repaired DNA damage in micronuclei (**Figure 6C**), as first reported by Haaf et al. [66]. The autophagic nature of this sorting found by Rello-Varona et al. [67]; has been reviewed previously in Ref. [68]. This sorting of the DNA damage through micronucleation was observed by us in several tumor cell line models after different kinds of genotoxic treatments as exemplified in **Figure 6 (A–C)**. The autophagic elimination of large DNA portions or whole sub-nuclei with damaged DNA was also observed (**Figure 6D**) [62, 68, 69] as another intriguing feature of the late post-damage events of genotoxically treated *TP53* mutants.

All this indicates that *TP53* mutants have a strong capacity for surviving genotoxic damage and reversing cell senescence by reversible endopolyploidy through a pathway involving boosted stemness. This pathway is in fact far away from the regulations of the typical mammalian cell cycle. More likely, these tumor cells exploit the life-cycle-like regulations of the unicellular organisms recapitulated from evolutionary ploidy cycles as we have postulated previously in Refs. [70, 71] and showed recently by bioinformatics study of polyploidy [72]. It only remains to add that in general tumor cells cannot bear wild type *TP53* and inactivate it, if not by mutations, then in many other ways [73]. Perhaps the increased ability to access additional routes to cell survival by overcoming senescence and repairing DNA damage as detailed above, also help explain this inactivation of *TP53* function in tumors.

Acknowledgements

Mr. Jekabs Krigerts is acknowledged for formatting the article and illustrations.

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