Polyploidy: The Link Between Senescence and Cancer

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Abstract: Polyploidy, a state of increased number of chromosomes, occurs often in plants and less frequently in animals. Polyploidization is recognized as a part of a developmental program and can be achieved *via* different mechanisms bypassing certain stages of the cell cycle. However, polyploidization also accompanies some pathological conditions as well as ageing. It is believed that tetraploid cells precede aneuploid ones in the early phases of tumor development. Division of tetraploid cells is restricted by the active tetraploid (4N G1) checkpoint. Tetraploid cells that are able to overcome this checkpoint give rise to increased genomic instability and tumor progression. Recently a cellular senescence program activated by oncogene expression was shown to act as a natural barrier against cancer development. Senescent cells were detected in many benign, but not malignant, human and animal tumors. Senescence can actually block cell transformation provided the 4N G1 checkpoint is active. Cancer cells that escaped the 4N G1 block are still able to undergo senescence upon anticancer treatment. Induction of cancer cell senescence is often correlated with high ploidy formation. Some polyploid cells can escape senescence and give progeny with numerical changes of chromosomes. Divisions of polyploid cancer cells on the road to senescence can be responsible for the ineffectiveness of anticancer therapy. Altogether, this implies polyploidy as a link between cellular senescence, cancer development and possible cancer renewal after treatment.

Keywords: Cancer, senescence, cell cycle, tetraploid G1, aneuploidy, polyploidy, endocycle.

1. INTRODUCTION

Eucaryotes contain diploid chromosome sets enabling sexual reproduction and genetic recombination. During division of somatic cells DNA replication must be restricted to only one event per cell cycle and followed by mitosis to yield two daughter cells, each with the same amount of DNA. The cell cycle of normal somatic cells is regulated with extremely high precision. This is achieved by a number of signal transduction pathways, known as checkpoints, which control cell cycle progression ensuring interdependency of the S-phase and mitosis, the integrity of the genome and proper chromosome segregation [1]. Despite this, polyploidy (or increased ploidy), the state of having a greater than diploid content of DNA, is widespread in plants and occurs also among animals [2]. Although the evolutionary consequences of polyploidy are questionable, polyploidization represents one of the most dramatic mutations known to occur. On the other hand, it seems to be well tolerated, at least as a part of the developmental program [3]. In mammals polyploidization occurs as a part of developmental program in trophoblasts [4] and megakaryocytes [5]. However, the number of polyploid cells also increases in certain tissues during ageing [6-8] and polyploidy accompanies cellular senescence [9,10].

A growing number of evidence indicates that polyploidy also arises during carcinogenesis and cancer development [11], thus the interest in the role of polyploidy and chromosomal instability is constantly growing among oncologists and gerontologists. In this review we will mainly focus on the role of polyploidy and chromosomal instability in cellular senescence of cancer cells and in carcinogenesis.

2. FROM MITOTIC CYCLE TO ENDOCYCLE

Polyploid cells can arise from different processes which generally can be divided into two categories: cell fusion (cell-cell fusion) and endocycling. Cell-cell fusion is fundamental to the development, physiology and pathology of a multicellular organism but it is beyond the scope of this review.

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Endocycle is a cell cycle repeated without cytokinesis. There are many mechanisms leading to polyploidy as the endocycle can bypass different stages of the S-phase and mitosis as observed during developmental processes [12] Fig. (**1**). As the endocycle is tightly connected to the cell cycle regulation and checkpoints we will briefly describe what is known about controlling cell division.

Quiescent somatic cells need to be stimulated with suitable growth factors to abandon the resting phase of the cell cycle (G0). Cycling cells enter the gap 1 (G1) phase followed by DNA synthesis (S) phase and gap 2 (G2) which precedes the mitotic (M) phase of cell cycle. Mitosis is finalized by cytokinesis giving two daughter cells containing the same quantity and quality of genetic material as the mother cell had.

The fidelity of the cell cycle is controlled by several checkpoints. During mitosis, a mitotic checkpoint (also known as a spindle assembly checkpoint) monitors the assembly of the spindle, preventing chromosome segregation until the correct bipolar attachment of chromosomes has been achieved. Three other cell cycle checkpoints operate in the interphase and are activated during DNA replication (S-phase) or if DNA is damaged by reactive oxygen species or other insults (G1 and G2). The signals of double-strand DNA breaks are transduced by the so called DNA damage response (DDR) pathway and determine cell fate as either of the three responses: transient cell cycle arrest (repair), stable cell cycle arrest (senescence) or cell death (apoptosis). DDR converges on key decision-making factors, such as cycle 25-CDC25 phosphatases, which arrest cell cycle for the time of repair, and p53 which either induces senescence or apoptosis by means of the cdk regulator p21 or the pro-apoptotic proteins belonging to the Bcl-2 protein family, respectively [13,14].

The cell cycle checkpoints are critical for protection against uncontrolled cell division which is the main feature of cancer development. There are a number of documented genetic lesions in the checkpoint genes, or in the cell cycle genes themselves, which result either directly in cancer or in a predisposition to certain cancer types. Moreover, many cancer cells are characterized by chromosomal instability (polyploidy/aneuploidy) [11,15].

Storchova and Pellman [11] introduced the term “abortive cell cycle”, to describe a process arising from a wide variety of defects concerning different aspects of cell division (DNA replication, mitotic spindle function and cytokinesis) and leading to tetraploidy.

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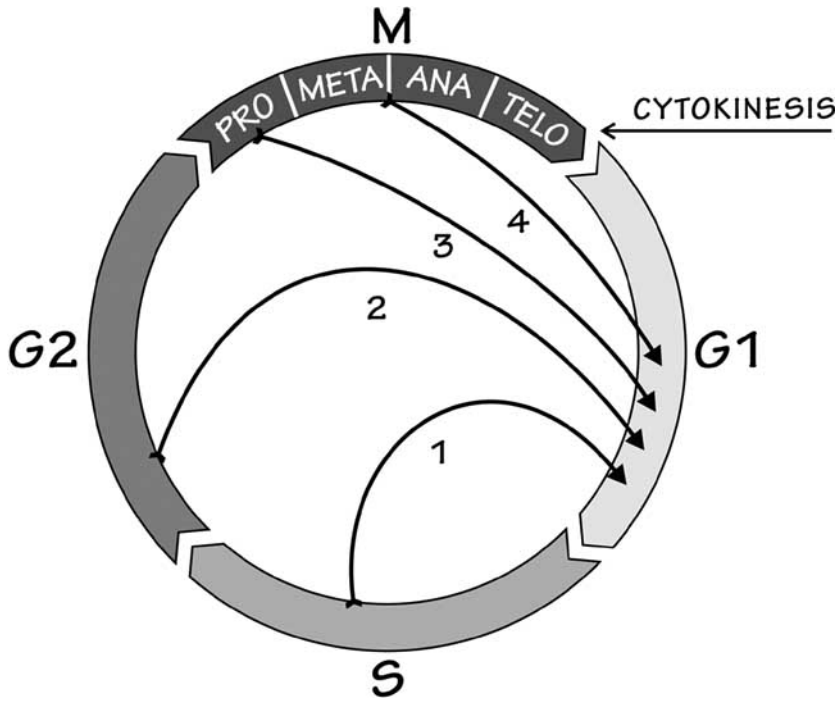

Fig. (1). Polyploidization is connected with bypassing different phases of cell cycle. Endoreduplication or endoreplication (1,2) - multiple S-phases without an intervening mitosis and cytokinesis; Endomitosis (3,4) - successive S-phases interrupted by a gap, during which the cell enters mitosis but does not complete it. Modified according to [12]

The reason is, that checkpoint activation often produces only transient delays in cell–cycle progression, and some cells can "slip" past the arrest.

In an adult organism there is a close correlation between the generation of polyploid cells and a variety of cellular stressors. The best example of this is liver undergoing oxidative damage or regeneration, heart muscle and vascular smooth-muscle cells which become polyploid in either rats or humans with hypertension, and fibroblasts during wound healing [11].

It seems that the aging process is a risk factor for polyploidy formation, at least in rat liver [8] and smooth muscle [6], as well as in human lymphocytes [16]. *In vitro* replicative senescence was shown to be associated with polyploidization both in endothelial cells [10] as well as in human [17] and chicken fibroblasts [18]. There is also one paper demonstrating *in vivo* that polyploid aortic vascular smooth muscle cells adopt a senescent phenotype [6]. Another spectacular example of changed ploidy is the brain of people suffering from Alzheimer disease [19]. It is hypothesized that the oxidative stress -induced DNA damage might either initiate cell cycle reentry in postmitotic neurons, or it may alter the components of a mitogenic signaling pathway. Moreover, the hyperdiploid cells can persist in the brain for a long time [20]

3. TETRAPLOIDY ON THE ROUTE TO CANCER DEVELOPMENT

The common feature of human cancer cells is aneuploidy, that is number of chromosomes different than 46. Epithelial tumors possess a nearby triploid DNA content, but most chromosomal aberrations observed in cancers are represented by a loss, gain or rearrangements of particular chromosomes rather than of the whole set of chromosomes. Interestingly, although cancer cells are known to be genetically unstable, many cancers that become clinically apparent have a relatively stable, though aneuploid, karyotype. Moreover both metastases and tumors that appear as a consequence of tumor relapse can have similar karyotype as the primary tumor. These observations indicate that loss of genetic stability is probably an early event in carcinogenesis and that chromosomal rearrangements give rise to clones that have certain growth advantages [21].

There are at least two mechanisms that could be responsible for aneuploidy generation at the early stage of cancer development, namely telomere attrition and polyploidization leading to intermediate unstable tetraploid cells.

a. Telomere Attrition

Telomeres protects the integrity of chromosomes by preventing chromosomal endings from homologuous recombination. They undergo gradual shortening after each cell division due to so called "end replication problem" as well as due to degradation caused by reactive oxygen species (ROS) that accumulate in the cell with the time of culture [22]. Since somatic cells do not express active telomerase, an enzyme that can restore telomere length, they finally irretrievably stop proliferation and become senescent when telomeres are critically short. However, due to mutations of tumor suppressor genes, such as p53 and p16, necessary for cell cycle arrest, cells with dysfunctional telomeres continue to proliferate, entering a period of slow growth called "crisis" that is characterized by genomic instability. During crisis, the unprotected telomere ends can be illegitimately fused through DNA repair mechanisms, leading to the non-reciprocal translocations. Due to lethal chromosomal aberrations the majority of cells undergo apoptosis, however rare cells that emerge from the crisis by activating mechanisms for telomere stabilization survive giving rise to cancer [14]. Although studies concerning the telomere-dependent process of induction of genomic instability were undertaken in cell culture models, there is a significant number of evidence that the same mechanism could operate *in vivo*. For example, genetically modified mice deficient for TERC (a telomerase RNA component) and p53 demonstrate increased incidence of epithelial cancers, which exhibit the complex cytogenetic profiles found in human epithelial cancers [23].

b. Tetraploid Cells in Early Stage of Cancer Development

It seems that tetraploidy might be a driving force in carcinogenesis as increased number of tetraploid cells was documented in

the early stage of tumor development [24]. Actually, tetraploidy and chromosomal instability were observed during the early stages of cervical carcinogenesis and predisposed cervical cells to aneuploidy [25]. Tetraploid cells were also documented in premalignant Barrett's esophagus biopsies and preceded the appearance of aneuploid cells in the later stages of cancer development [26]. Also development of pancreatic cancer in transgenic mice expressing the simian virus 40 tumor antigen was characterized by the sequential appearance of tetraploid and then multiple aneuploid cell populations [27].

The important issues are: how can tetraploid cells be generated from normal cells, and how can tetraploidy lead to chromosomal instability.

Concerning the first question it seems that several mechanisms can be involved in establishment of tetraploidy [28], such as aberrant DNA replication [29], endomitosis induced by defects in chromosome separation caused mainly by problems with mitotic spindle [30] or the lack of kinesin motor protein KiF4 necessary for cytokinesis [31]. It seems that Barrett's cancer cells undergo endoreduplication (the process in which a cell undergoes multiple S-phases without an intervening mitosis and cytokinesis) because despite elevated expression of genes necessary for G2/M transition, the condensed chromosomes were not present in isolated 4N cell fraction [32].

Answering the second question, that is how can tetraploidy lead to chromosomal instability, needs more explanation. Despite the fact that tetraploidy precedes the appearance of aneuploidy in solid tumors, it is generally believed that tetraploid cells are arrested in the 4N G1 phase of the cell cycle, *via* activation of the so called tetraploid checkpoint, which does not allow cells with duplicated chromosomes (namely those which undergo mitosis but not cytokinesis) to enter the next phase of the cell cycle. Indeed, Andreassen and colleagues [33], who have described this mechanism for the first time in non-transformed cells with failure in mitotic spindle checkpoint, documented that the tetraploid checkpoint was induced by active p53 targeting the cdks inhibitor, p21. Others documented that tetraploid cells could be arrested in the G1 due to an activated DNA damage checkpoint induced by DNA breaks acquired earlier during sustained mitosis and subsequent mitotic slippage [34]. DNA damage induces the so called DNA damage response pathway in which the key player is the p53 protein. Blagosklonny and colleagues [35] showed that p53 is crucial in arresting human arterial smooth muscle cells treated with the high concentration of mitotic poison, in the G1 and the 4N G1 phases of the cell cycle. Gannem and Pellman [28], although arguing the existence of a special 4N G1 "chromosomes counting" checkpoint admit that tetraploid cells are arrested, but due to stress-induced activation of so called "centrosome checkpoint". They concluded that abortive cell cycle leading to mononuclear or binuclear cells prevented their further proliferation. This was due to extra centrosome- and cytoskeleton abnormalities-driven p53 activation inducing cell arrest (senescence) or cell death (apoptosis), thus preventing cancer development [28].

Indeed, it seems that p53 keeps tetraploid cells arrested in the G1 as aneuploid cells generation was observed in cells lacking active p53. A good example supporting this idea are tetraploid p53-null mouse mammary epithelial cells giving rise to malignant mammary epithelial cancers when transplanted subcutaneously into nude mice [36]. Similarly, in Barrett's cancer cells the loss of p53 function correlated with increased fraction of tetraploids and progression to aneuploids [26].

Additional, however indirect and still not very strong, evidence proving instability of tetraploids was delivered by studies concerning cellular senescence. Namely, it is believed that cellular senescence is, like apoptosis, a barrier for cancer development as senescent cells do not proliferate, and they are detected in many benign, but not malignant, human and animal tumors [37]. Moreover, several oncogenes can induce cellular senescence as evidenced not only *in vitro*, but also *in vivo* [14]. Interestingly, human fibroblasts induced to senescence with H-ras-exhibited specific downregulation of genes involved in the G2/M checkpoint control and contained tetraploid cells (binucleated) that were arrested in the 4N G1 phase [38].

The role of p53 in the tetraploid G1 arrest has already been recognized, while the experiments performed by Borel and colleagues [39] on murine embryonic fibroblasts, indicated that the G1 tetraploid checkpoint is impaired in cells with compromised function of either p53 or the RB pocket proteins. This allowed for conclusion that RB pocket proteins, which function is suppressed in the great majority of tumors, play important role in tetraploidy control and mutations in those genes create the potential for aneuploidy augmentation in virtually all tumor cells. The authors also indicated the crucial role of centrosome multiplication in tetraploid cells escaping the G1 and undergoing aberrant mitoses. This is contrary to the already mentioned idea of an altered number of centrosomes as a main factor limiting the proliferation of polyploid cells [40]. However, it seems that the both scenarios are possible. According to Nigg and colleagues [41] the cells with a multiplied centrosomes due to severe loss of chromosomes, undergo apoptosis, however some of them could survive and give rise to increased genomic instability. Alternatively, supernumerary centrosomes can be forced to coalesce into two spindle poles, which allow the tetraploid tumor cells to expand through binary divisions. Very recently Ganem and colleagues [42] showed that cells with extra centrosomes routinely undergo bipolar cell divisions, but display a significantly increased frequency of lagging chromosomes during anaphase.

Thus, the answer to the second question of how tetraploid cells can give rise to aneuploid progeny, might be that, mutations compromising p53 could be responsible for the resumption of growth. However, as tetraploid cells are characterized by an increased number of centrosomes, they undergo aberrant mitoses leading either to apoptosis, or the survival of cells with normal or altered number of chromosomes.

4. INSTABILITY OF CANCER CELLS INDUCED BY TREATMENT

Effective treatment of cancer should result in cancer cells elimination. Hanahan and Weinberg [43] considered resistance to apoptosis as one of the seven fundamental features of cancer cells. On the other hand, many cancer cells are sensitive to treatment targeting different molecular pathways and leading to different types of cell death such as necrosis, apoptosis, autophagy, mitotic catastrophe and others [44]. Increasing knowledge concerning the mechanisms of cell cycle regulation and the crosstalk between DNA damage and mitotic checkpoints allowed to identifying new cancer cell responses to treatment, which do not necessarily end in cell death. Polyploidization falls into this category.

There is a growing body of evidence that cancer cells can undergo polyploidization upon treatment with both DNA-damaging agents and those targeting mitotic checkpoint [45]. Actually the list of agents inducing polyploidy in cancer cells is quite long and includes inhibitors of microtubule polymerization (*e.g.,* [46-49]), topoisomerase II inhibitors and poisons [50], deacetylase inhibitors [51], a natural agent curcumin [52] and others. Also, inhibiting activity of Aurora kinases which play a critical role in regulating mitosis and cell division, and are overexpressed in many cancers, might result in polyploidy [53-56].

Induction of polyploidy can be considered as a beneficial effect of the treatment. For instance presence of polyploid cells in drug-treated tumors was sometimes correlated with a positive response to the treatment as in the case of patients with colorectal carcinomas, who received chemotherapy [57]. Hyperploid cells, that were

observed within a population of Aurora A kinase inhibitor–treated cancer cells showed reduced capacity to undergo clonal growth, indicating that the recovery from hyperploidy was impaired [53]. Moreover, induction of polyploidy in non-small cell lung cancer cells, which was a result of combined treatment with an inhibitor of microtubule polymerization and γ-radiation, led to an increased apoptosis rate of cancer cells [58]. However, there are also reports showing that polyploidization of cancer cells can reflect the induction of protective mechanisms. Treatment of B16F10 melanoma cells with methotrexate results in cell death, but also in induction of significant polyploidy. The alteration of cell ploidy and proliferative capacity of melanoma cells reverted completely two weeks after drug treatment, indicating that polyploidyzation can be considered as a mechanism of drug resistance [59]. Similar conclusion was drawn from experiments on a taxol-resistant leukemic cell line, which underwent polyploidization upon taxol treatment, while in taxol-sensitive cell line no increase in ploidy was observed [60]. Reexpression of the T-box factor, Tbx2, in transformed human lung fibroblast cell line caused chromosomal aberrations and polyploidy and correlated with increased resistance to cisplatin treatment [61]. Thus polyploidization can help to survive the deleterious effect of treatment and allowed cancer to develop.

Indeed, polyploidization can lead to cancer renewal as polyploid cancer cells can give aneuploid progeny. Erenpreisa's group published a set of papers showing a sort of a cancer life cycle which consists of repeatable polyploidization and de-polyploidization processes [62,63]. They observed that X-ray irradiation of several cancer cell lines led to formation of multinuclear cells that were able to survive and underwent multipolar cell divisions giving rise to viable and proliferating cells with lower ploidy. The process of de-polyploidization was correlated with induction of some meiosis-specific genes and carried some morphological features of meiotic prophase I [64,65].

An alternative mechanism that enables polyploid/multinuclear cell to proliferate was proposed by the group of Rajaraman. According to their studies polyploid/multinuclear cells undergo a novel type of cell division, characterized by karyokinesis *via* nuclear budding, followed by asymmetric cytokinesis [66,67]. It has even been suggested that the capacity of a tumor to grow and relapse after treatment may rely on a small fraction of so called "cancer stem cells" (Raju cells) originating from giant cells [67]. However, it cannot be excluded that these "cancer stem cells" do not originate from polyploid cells, but represent a subpopulation of stem/progenitor cells residing in the population of cancer cells. The existence of cancer stem cells have already been documented in leukemia as well as in many solid tumors. In comparison with differentiated cells, cancer stem cells face a greater risk of accumulating mutations and are less prone to undergo senescence or apoptosis and might need fewer events to sustain uncontrolled growth [68].

5. POLYPLOIDIZATION OF CANCER CELLS INDUCED TO SENESCENCE

It is believed that cellular senescence (the state of permanent cell growth arrest), like apoptosis, is a barrier to tumorigenesis and cells with active SA-β-galactosidase (a common marker of cellular senescence) have been detected in early, but not late stages of carcinogenesis [37]. However, as emerges from seminal studies of Roninson's group, cancer cells still retain the ability to conduct the program of senescence [69,70]. Indeed, the induction of cancer cell senescence *in vitro* has been shown also by others, including our group [51,71-78], and the number of publications showing induction of cancer cell senescence upon treatment with many anticancer agents, including those with DNA-damaging activity, is constantly increasing. The presence of the SA-β-gal-positive cells has been reported also *in vivo* in specimens from breast cancer [79] and lung cancer patients who received chemotherapy [80] as well as in animal cancer model [81]. It is believed that the senescence of cancer cells is an important outcome in treatment of cancers - especially those resistant to apoptosis in response to many chemotherapy [82].

Moreover, senescence induced by DNA-damaging agents was associated with high polyploidy in several human cell lines [71, 72,78], however there are also other data showing no polyploidy generation. Studies performed by Jackson and Pereira-Smith revealed that senescent cancer cells can be stopped in the G1 or G2 phase of the cell cycle as it is in the case of doxorubicin-treated MCF-7 cells [83] or in the S-phase, as it was shown by us in the case of human colon cancer cells treated with methotrexate [84].

Senescence of normal cells is strictly connected with the activation of the p53/p21 pathway [85]. In human colon HCT116 cancer cells the level of p53 and p21 increased upon doxorubicin-treatment [72] but it seems that p21 is indispensable for cell survival, rather than for polyploidization/senescence (Mosieniak *et al.,* manuscript in preparation). The results of Chang and colleagues [71] indicate that p53 and p21 act as positive regulators of HCT116 cell senescence, but their function is neither sufficient nor absolutely required for response of tumor cells to treatment. Nonetheless Xu and colleagues [51] observed higher polyploidy/ senescence of HCT116 p21WAF1-/- or HCT116 p53-/- cells than of the wild-type ones. In hepatoma cells the p53/p21 pathway was not activated under polyploidization/senescence [78]. Altogether it seems that in cancer cells, which did not stop in the 4N G1 but continued endocycling, the role of p53/21 pathway is still awaiting elucidation. It can not be excluded that another tumor suppressor, namely pRb, is critical for blocking DNA replication and preventing endoreduplication of cells induced to polyploidization/ senescence [86,87]. This implies that the genetic background can influence molecular pathways leading to terminal growth arrest of cancer cells.

Regrettably, there are no many data elucidating the route to polyploidy of cancer cells induced to senescence. We showed that HCT116 cells induced to senescence did not enter mitosis but endoreduplicated, what was reflected by increase of DNA content (>16C) an impressive enlargement of nuclei and the lack of typical markers of mitosis [72]. Just recently our results were confirmed by others [88]. On the other hand, others showed that hepatoma cells induced to senescence became polyploidy by endomitosis which led to micronucleation [12].

Interestingly, we also observed micronucleation and very rare events of mitosis in HCT116 cells undergoing polyploidization. Micronuclei are the typical hallmark of cells which underwent mitotic catastrophe, that is cell death during prolonged M-phase, but we proved that those cells were viable [72]. According to Erenpreisa's group mitotic catastrophe is the phase in a cancer life preceding entry into the mitotic cycle and proper cell division [63].

6. INSTABILITY OF POLYPLOID/SENESCENT CANCER CELLS

As it was presented in the previous chapters, polyploid cells are potentially dangerous as they can undergo aberrant mitoses, giving rise to aneuploid progeny. One cannot exclude the possibility, that some polyploid cells can infrequently escape senescence and divide instead of ceasing proliferation. Indeed, a pulse dose of doxorubicin (24 h) resulted in appearance of SA-β-gal-positive HCT116 cells that did not stained for the proliferation marker, Ki-67, confirming their non-dividing status. However, the population of cells several days after treatment was very heterogeneous and some cells escaped senescence [72]. Others also documented the appearance of the population of small cells (among polyploid giant senescent ones), which displayed decreased sensitivity to drug treatment *in vitro* in comparison with the initial population [51]. Moreover, others showed that tumors generated by subcutaneous injection of cells that escaped senescence into rats were also resistant to the

dose of the drug that induced regression of the parental tumor [81]. In studies of Elmore *et al.* [89] MCF-7 breast cancer cells that escaped adriamycin-induced senescence were also shown to be intrinsically resistant to DNA-damage induced growth arrest, however evaluation of the DNA content and ploidy status was not included in those studies.

Chromosomal analysis of the population of cells which escaped senescence and were able to proliferate showed an increase in the amount of cells with an altered number of chromosomes comparing to the parental HCT116 cells [72]. Comparative genomic hybridization preformed by Puig *et al.* [81] on five distinct senescence- escaped cell lines and parental PROb prostate cancer cells demonstrated chromosomal aberrations in the former cells. These aberrations were not present in polyploid PROb cells, indicating that they were not related to a direct genotoxic effect of cisplatin used in these studies. Altogether, it seems that polyploidy formation that accompanies cancer cell senescence induced by anticancer treatment can lead to aneuploidy. Appearance of aneuploid cells that possess different genetic architecture can be potentially responsible for tumor relapse. Rajaraman *et al.* [67] suggested that small Raju cells observed by them originated from senescent cancer cells, however, they did not provide evidence that dividing giant cells were actually senescent ones.

Concluding, we are now well aware that despite many check-points in the cell cycle, controlling the quality and quantity of genetic materials, the system is leaky since polyploid cells are more frequent in diploid organisms that it could be expected. Moreover, cellular senescence, considered as a barrier for uncontrolled cell division, may be not perfect one. The terminal growth arrest can be preceded by appearance of unstable intermediate polyploidy which can escape senescence, divide and give rise to aneuploid progeny with preserved bipolar divisions. This seems to be true for sene-scence of both normal and cancer cells, although the phenomenon is still poorly recognized, and still not clearly documented in the case of normal cells. We predict that tetraploid G1 state can be more frequent than expected previously and that is often underestimated.

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ABBREVIATIONS

CDC25	=	Cell Division Cycle
Cdk	=	Cycle Dependent Kinase
DDR	=	DNA Damage Response
SA-β-gal	=	Senescence Associated- β-galactosidase
TERC	=	Telomerase RNA Component

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