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Cell Cycle News & Views

Ubiquitin and the DNA damage response: A new handle on histones

Comment on: Gatti M, et al. Cell Cycle 2012; 11:2538–44;
PMID:22713238; http://dx.doi.org/10.4161/cc.20919

Simon Bekker-Jensen and Niels Mailand*; Ubiquitin Signaling Group; Department of Disease Biology; Novo Nordisk Foundation Center for Protein Research; University of Copenhagen; Copenhagen, Denmark; *Email: niels.mailand@cpr.ku.dk; http://dx.doi.org/10.4161/cc.21718

The rapid accumulation of signaling and repair factors in the vicinity of DNA lesions is an integral part of the cellular DNA damage response (DDR) to DNA double-strand breaks (DSBs).[1,2] This is initiated by posttranslational modifications of core histones, to which various effector proteins bind. The priming modification is ATM-mediated phosphorylation of histone H2AX at Ser-139 (γ-H2AX), a mark that exclusively decorates nuclear sites of DNA damage.[2,3] Subsequently, accumulation of repair factors such as 53BP1 and the BRCA1 A complex impinges on the formation of K63-linked ubiquitin chains on histones H2A and H2AX, catalyzed in a sequential manner by the DNA damage-responsive E3 ubiquitin ligases RNF8 and RNF168. First, recruitment of RNF8 to DSB sites triggers initial Ubc13-dependent K63-linked ubiquitylation of H2A-type histones, generating landing pads for another E3 ligase, RNF168, which also acts in conjunction with Ubc13 to amplify and spread polyubiquitylation of H2A-type histones to levels sufficient to allow sustained retention of repair factors at the damaged chromatin.[1,4,5] These insights have given rise to the exciting concept of a DNA damage-specific "histone code," a series of sequential histone modifications that uniquely demarcate sites of DNA damage, distinguishing them from other chromatin landscapes. Such a concept is widely accepted for many transcriptional processes, where propagation of several inter-dependent histone marks are required to activate transcription at specific gene promoters.[6]

Despite impressive advances in our understanding of the molecular underpinnings of DSB-associated chromatin ubiquitylation, one fundamental aspect of the DSB-specific histone code that has remained elusive is the actual site(s) of RNF8/RNF168-mediated ubiquitylation on H2A-type histones. In a previous issue of *Cell Cycle*, Penengo and coworkers provide important new insight into this outstanding question.[7] By mass spectrometry analysis of ubiquitylation of epitope-tagged histones in cells overexpressing RNF168, the authors come to the surprising conclusion that the evolutionarily conserved lysine 13 and 15 residues are the chief acceptor sites of RNF8- and RNF168-catalyzed ubiquitylation on H2A-type histones. Lysines 118 and 119 have long been known as prominent sites of monoubiquitylation on these histones, and it is estimated that some 10–15% of all H2A molecules contain this modification.[8] A common assumption has been that RNF8 and RNF168 merely extend this very abundant mark into a K63-linked polyubiquitin chain. The new findings are attractive in suggesting that the local RNF8- and RNF168-generated ubiquitylation marks on H2A-type histones around DSBs differ distinctly in terms of both the chain topology and modification sites, lending further support to the concept of a DNA damage-specific histone code.

The exciting new findings by Gatti et al.[7] raise a number of important questions that will need to be addressed by future studies: first of all, it will be crucial to test by genetic means whether the newly identified ubiquitylation sites are in fact required to support recruitment of downstream factors to DSB-containing chromatin. Moreover, are both the K13 and K15 ubiquitylation sites in H2A important, and is K119/K120 monoubiquitylation dispensable for a productive DSB response? Issues like these are arguably difficult to resolve given the essential nature of histone H2A, its high expression level and the large number of genes that encode core histones. Nonetheless, experiments involving replacement of endogenous H2A-type histones with ubiquitylation-deficient variants, or at the very least functional interference with their normal ubiquitylation by overexpression of such mutants, will be instrumental for determining the relative importance of individual ubiquitylation sites in the DSB response.

Second, the identification of K13 and K15 as major acceptor sites for RNF8 and RNF168 should facilitate a better insight into the mechanistic basis of DSB-induced histone ubiquitylation and associated protein recruitment. Important questions are whether these modifications are confined to sites of DNA damage, if RNF8 and RNF168 are the major cellular K13/K15 ubiquitin ligases, or whether these ligases mostly extend K13/K15 monoubiquitylation primed by other E3s, as is often the case with Ubc13-mediated polyubiquitylations. Antibodies specifically recognizing K13/K15-ubiquitylated forms of H2A-type histones would be valuable tools for addressing these and related questions. Another key question is whether ubiquitylated K13 and K15 constitute direct binding platforms for RNF168 and RAP80. The identification of the sites of RNF8/RNF168 ubiquitylation in H2A sets the stage for a greater insight into the molecular interplay between readers and writers of DSB-associated histone ubiquitylation.

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Fenofibrate triggers apoptosis of glioblastoma cells in vitro: New insights for therapy

Comment on: Wilk A, et al. Cell Cycle 2012; 11:2660–71; PMID:22732497; http://dx.doi.org/10.4161/cc.21015

Antonio Giordano[1,2] and Marcella Macaluso[1,*]; [1]Sbarro Institute for Cancer Research and Molecular Medicine; College of Science and Technology; Temple University; Philadelphia, PA USA; [2]Department of Human Pathology and Oncology; University of Siena; Siena, Itlay; *Email: macaluso@temple.edu; http://dx.doi.org/10.4161/cc.21719

Fenofibrate, a peroxisome proliferator-activated receptor (PPARα) agonist, is a member of the fibrate class of anti-hyperlipidemic agents and has been widely used in the treatment of different forms of hyperlipidemia and hypercholesterolemia.[1] PPARα is a ligand-inducible transcription factor that belongs to the nuclear-hormone-receptor family and mediates peroxisome proliferation action. Interestingly, growing evidence has indicated that PPARα agonist fenofibrate exerts anticancer properties, probably because of its anti-proliferative and pro-apoptotic activities, and, importantly, exhibits low systemic toxicity.[2] However, it is not clear what role PPARα plays in the antiproliferative effect of fenofibrate, and the possibility of a PPARα-independent action is being considered. With respect to these mechanisms, in a recent issue of *Cell Cycle*, Wilk et al.[3] investigated the effect of fenofibrate treatment on the proliferative features of glioblastoma cells. Approximately, 50% of all primary brain tumors originate from glial cells, and glioblastoma multiforme (GBM) is by far the most common and most malignant of the glial tumors. Currently, the treatment of glioblastomas remains palliative and includes surgery, radiotherapy, chemotherapy and treatment with inhibitors and antibodies, which have been shown to prolong quality survival.[4]

In their study, Wilk et al.[3] show that fenofibrate can effectively induce growth arrest and trigger apoptosis of glial tumor cells in a dose- and time-dependent manner. Interestingly, the authors observed that fenofibrate-induced apoptosis was preceded by nuclear accumulation and serine phosphorylation of the transcription factor FoxO3A as well as by FoxO-dependent expression of the pro-apoptotic protein Bim. In addition, the authors observed that other agonists of PPARα were less effective in changing cell viability with respect to fenofibrate, and that PPARα silencing by siRNA only partially rescued glioblastoma cells from the treatment, suggesting that both PPARα-dependent and -independent mechanisms may play a role in the fenofibrate-induced activation of FoxO3A and Bim, which, in turn, triggers apoptosis.

Different studies have reported that deregulation of the $p16^{Ink4a}$-Cdk4-Rb cell cycle-controlling pathway is a common event in patients with GBM.[5,6] It would be very interesting to investigate the impact of fenofibrate treatment on p16-Cdk4/6-Rb axis and its relationship with apoptosis triggering and cytotoxicity effects in glioblastoma cells. Not all glioblastomas have the same biological abnormalities, and this may be the reason why different patients respond differently to the same treatment, and why different patients with the same tumor type have different outcomes. In this context, it would be interesting to disclose the pleiotropic effects of fenofibrate on cell growth and death signaling and to determine if the response to this drug could be critically determined by $p16^{Ink4a}$-Cdk4-Rb status in glioblastoma cells. Wilk et al.'s study provides evidence that fenofibrate treatment can effectively trigger apoptosis via FoxO3A in glioblastoma cells, and this information may represent the first step in elucidating the anticancer effect(s) of this drug. Further studies are necessary to evaluate the impact of fenofibrate treatment in modulating cellular growth and death signaling of glioblastoma cells and to shed light on how the use of this drug may improve current anticancer therapies.

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Blood cell fate changes without cell cycle transition

Comment on: Di Tullio A, et al. Cell Cycle 2012; 11:2739–46; PMID:22771961; http://dx.doi.org/10.4161/cc.21119

Matthias Stadtfeld; Skirball Institute; NYU School of Medicine; New York, NY USA; Email: matthias.stadtfeld@med.nyu.edu; http://dx.doi.org/10.4161/cc.21720

In recent years, a multitude of groundbreaking papers have demonstrated that cell fates can be altered almost at will by the enforced expression of defined sets of transcription factors. Such cell fate changes are referred to as reprogramming (if they involve the transition from a differentiated cell into a pluripotent stem cell)[1] or as transdifferentiation or direct lineage conversion (if they involve transitions between differentiated cell types).[2] Much of the attention that induced cell fate changes have received focuses on the possibility to generate patient-specific cells for potential tissue replacement applications or disease modeling. Nevertheless, the controlled tempering with cellular identity also represents a powerful tool to probe general mechanisms underlying development and

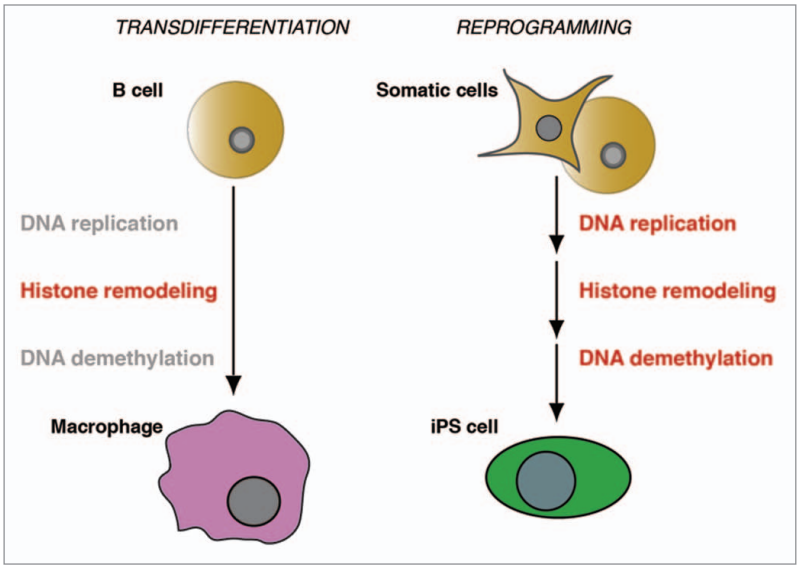

Figure 1. Scheme summarizing important differences between transdifferentiation and iPS cell reprogramming. Transdifferentiation events between somatic cells are rapid and can occur without cell division or apparent changes in promoter DNA methylation. Reprogramming somatic cells to pluripotency is a lengthy process with defined intermediate steps that requires cell division and DNA demethylation.

differentiation. In an elegant study published in a recent issue of *Cell Cycle*, di Tulio and Graf use a transdifferentiation system to investigate the role of the cell cycle during cellular commitment in the blood cell lineage.[3]

The relationship between DNA replication and proliferation, on one hand, and cell cycle arrest and terminal differentiation, on the other, has long intrigued cell biologists. It is generally accepted that terminal differentiation leads to cell cycle exit, and that this is an important regulatory mechanism during organ growth and regeneration. It is less clear how many, or if any, cell divisions are required for cells to change fate or to terminally differentiate. In fact, there is evidence that this may be dependent on cellular context. Thus, while fibroblasts or B cells undergoing reprogramming into induced pluripotent stem (iPS) cells often transit through the cell cycle dozens of times before entering the pluripotent state,[4] the conversion of fibroblasts[5] or hepatocytes[6] into neurons does not require cell division at all. Di Tulio and Graf studied the link between cell division and transdifferentation using a rapidly cycling pre-B cell line that expresses an inducible form of the myelomonocytic transcription factor C/EBPα.[7] These B cells can be triggered to differentiate into macrophage-like cells at essentially 100% efficiency in a matter of a few days. This makes them a unique tool to study transdifferentation and develop frameworks and hypotheses that can then be tested in less accessible experimental systems, such as animal models or primary cell cultures. The authors find that the majority of B cells undergo exactly one cell division before terminally exiting the cell cycle and adopting macrophage morphology, marker gene expression and behavior such as phagocytotic activity.[3] Preventing cell cycle transition significantly reduces the efficiency of transdifferentiation. However, a subset of cells adopts all macrophage characteristics tested, even in the presence of chemical inhibitors of DNA polymerase and without evidence for DNA replication. In fact, time-lapse imaging shows that cells that are not dividing transdifferentiate faster, and that the proportion of non-dividing cells increases with higher levels of C/EBPα. This demonstrates that cell division is not required to turn a B cell into a macrophage and provides further evidence that transdifferentiaton is mechanistically different from iPS cell reprogramming.

So, why can transdifferentiation succeed without cell cycle transition, while reprogramming cells to pluripotency apparently requires it? The answer to this might simply be that reprogramming involves large-scale epigenetic remodeling, while transdifferentiation does not. For example, since B cells and macrophages share a number of master blood cell regulators, C/EBPα partly operates by re-wiring a preexisting transcription factor network[8] by recruiting the transcription factor PU.1 to new target genes. During reprogramming, key components of the pluripotency network such as Nanog or Pou5f1 have to first be reactivated, as they are not expressed in somatic cells. This reactivation entails DNA demethylation, which during iPS cell formation takes more than a week to occur and might require DNA replication. In contrast, no detectable changes in promoter DNA methylation have been observed during B lineage cell into macrophage conversions using the C/EBPa overexpression system, while changes in histone tail modifications do occur[9] (**Fig. 1** summarizes differences between transdifferentiation and reprogramming). Many exciting questions remain unanswered. Exactly which molecular remodeling events during iPS cell formation require cell division, and how does this relate to physiological reprogramming events in the early embryo? Does transdifferentiation without cell division generate fully functional, mature cell types? Undoubtedly, further studies with sophisticated in vivo and in vitro cellular conversion models will point toward the answers.

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The yin and yang of proliferation and differentiation: Cyclin D1 inhibits differentiation factors ChREBP and HNF4α

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Frances M. Sladek; Department of Cell Biology and Neuroscience; University of California; Riverside, CA USA; Email: frances.sladek@ucr.edu;
http://dx.doi.org/10.4161/cc.21721

The yin-yang relationship between proliferation and differentiation is a fundamental tenet of development and plays a critical role in diseases such as cancer. Cyclins and cyclin-dependent kinases (CDK) are well-characterized drivers of the cell cycle; they have also been found to play a direct role in the regulation of genes not necessarily required for cell proliferation, and hence may be at the crux of this dichotomy. Cyclin D1, which increases in G_1 and remains elevated throughout S phase, is the best-studied of the cyclins and is amplified or overexpressed in many tumors. The canonical role of cyclin D1 is to activate CDK4/6, which, in turn, phosphorylate, and inactivate, the tumor suppressor Rb. Rb suppresses tumor growth by inhibiting E2F, a transcription factor (TF) that drives the expression of genes required for DNA synthesis. Less well-known activities of cyclin D1 that do not directly result in proliferation are CDK-independent interaction with TFs (e.g., nuclear receptors), effects on co-activator function (e.g., p300 and CBP) and widespread recruitment to gene promoters.

In a recent issue of *Cell Cycle*, Hanse, et al.[1] expand the list of cyclin D1-TF interactions with two important factors that regulate metabolism—carbohydrate response element binding protein (ChREBP) and hepatocyte nuclear factor 4 α (HNF4α). These interactions result in a decrease of expression of genes involved in glucose and lipid metabolism in hepatocytes: the net effect is a direct impact of cyclin D1 on metabolic homeostasis in the liver. This is of particular interest, since the liver not only is a major metabolic organ, but also has a remarkable proliferative capacity after partial hepatectomy or injury. The authors propose that after injury, the inhibition of expression of "luxury genes," such as those regulated by ChREBP and HNF4α, may be required so that cell resources can be re-directed to the more urgent task of repopulating the liver, and hence the need for cyclin D1 to repress their activity. Furthermore, cyclin D1 inhibits ChREBP and HNF4α by different mechanisms, suggesting an even broader role for this cyclin in transcription regulation. Cyclin D1 decreases ChREBP gene transcription and protein function in a CDK-dependent fashion. In contrast, cyclin D1 does not alter the level of HNF4α RNA or protein but does prevent its recruitment to and activity on target gene promoters, albeit in a CDK-independent fashion.

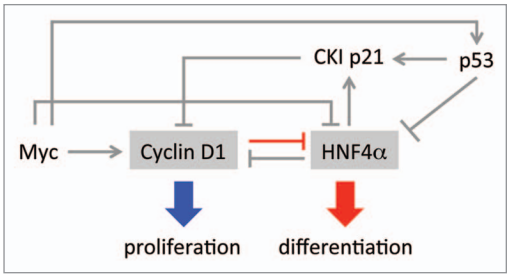

Figure 1. Cyclin D1 and HNF4α are at the center of a complex circuit that coordinately regulates cellular proliferation and differentiation. HNF4α and cyclin D1 negatively regulate each other and are in turn regulated by other modulators of the cell cycle, such as Myc, CKI p21 and p53. Red line is from Hanse et al.[1]

The action of cyclin D1 on HNF4α could have additional consequences. HNF4α, a member of the nuclear receptor superfamily, is one of the most abundant TFs in the adult liver and required for most liver-specific expression. It also acts as a tumor suppressor in the liver and directly inhibits cell proliferation.[2-4] Cyclin D1 now joins a cadre of other key players in proliferation that downregulate or antagonize HNF4α (**Fig. 1**). The oncogene c-Myc, which upregulates the expression of the cyclin D1 gene (*Ccnd1*), has been shown to compete with HNF4α for control of the cyclin-dependent kinase inhibitor p21 gene (*Cdkn1a*).[5] In contrast, HNF4α appears to downregulate the expression of *Ccnd1* as a liver-specific knockout of HNF4α results in a marked increase in cyclin D1 gene expression.[2] The tumor suppressor p53 has also been shown to inhibit HNF4α function and downregulate the expression of the *HNF4A* gene upon DNA damage;[6,7] decreased HNF4α may help set the stage for subsequent regrowth. While p53 is best known for its ability to inhibit the cell cycle (via upregulation of *Cdkn1a*), its expression is also increased by oncogenes such as Myc. Therefore, c-Myc can ostensibly inhibit HNF4α activity via cyclin D1, p53 or direct interaction with HNF4α.[5] Other pro-oncogenic factors that negatively affect HNF4α function include protein kinase C (PKC)[8] and Src tyrosine kinase.[9] While all of these factors (Myc, p53, PKC, Src) would be expected to cause a downregulation of HNF4α targets involved in differentiation, they will also result in an increase in proliferation by relieving the HNF4α-mediated repression of cyclin D1. Thus, cyclin D1 and HNF4α are at a nexus of a regulatory network that controls both proliferation and differentiation (**Fig. 1**). It will be of interest to determine whether ChREBP and other TFs inhibited by cyclin D1, especially those that drive differentiation, are part of this circuit.

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Tetraploid cancer cell precursors in ovarian carcinoma

Comment on: Lv L, et al. Cell Cycle 2012; 11: 2864–75; PMID:22801546; http://dx.doi.org/10.4161/cc.21196

Maria Castedo,[1,2,3] Laura Senovilla,[1,2,3] Ilio Vitale[1,2,3] and Guido Kroemer[1,4-7,*]; [1]INSERM; U848; Villejuif, France; [2]Institut Gustave Roussy; Villejuif, France; [3]Université Paris Sud-XI; Villejuif, France; [4]Metabolomics Platform; Institut Gustave Roussy; Villejuif, France; [5]Centre de Recherche des Cordeliers; Paris, France; [6]Pôle de Biologie; Hôpital Européen Georges Pompidou; AP-HP; Paris, France; [7]Université Paris Descartes, Paris 5; Paris, France; *Email: kroemer@orange.fr; http://dx.doi.org/10.4161/cc.21722

One of the initiating events of oncogenesis is tetraploidization, that is the generation of cells that contain twice as much DNA and chromosomes than their normal, diploid counterparts.[1] Tetraploidy may arise through illicit cell-to-cell fusion among somatic cells,[2] endomitosis[3] or endoreplication.[4] Tetraploidy has been observed in the early stages of cervical, colorectal, esophageal, ovary, mammary and other cancers.[5,6] Tetraploid cells exhibit an enhanced fitness in the context of DNA damage,[7] a property that may increase their survival during oncogenesis as well as after anticancer chemotherapies. In addition, tetraploid cells can undergo a subsequent depolyploidization cascade that ultimately results in rampant aneuploidy, due to multipolar divisions. Such multipolar division mostly leads to the death of daughter cells (due to lethal nullisomies or monosomies and/or desiquilibria in gene doses), yet occasionally yields cells that are fitter than their progenitors and can engage in progressive malignant transformation.[1] Altogether, these phenomena yield the histopathological aspect of anisokaryosis, which is a snapshot of transformation and tumor progression that morphologically reflects genetic heterogeneity.

The aforementioned tetraploidization/depolyploidization cascade is controlled at multiple levels, and a large panel of oncogenes and tumor suppressor genes can favor or inhibit the generation and survival of tetraploid cells as well as their depolyploidization.[3,8] For example, the inactivation of the tumor suppressor proteins Tp53 and Rb have been correlated with the incidence of tetraploidy in human cancers.[6] Moreover, the ectopic expression of the meiotic kinase Mos (which usually is only expressed during oocyte meiosis) in tetraploid tumor cells correlates with their propensity to undergo multipolar divisions.[8] Based on these observations, it can be expected that only a combination of genetic events that are compatible with multistep carcinogenesis can facilitate the process of tetraploidization/depolyploidization that marks the transition from normal diploidy/

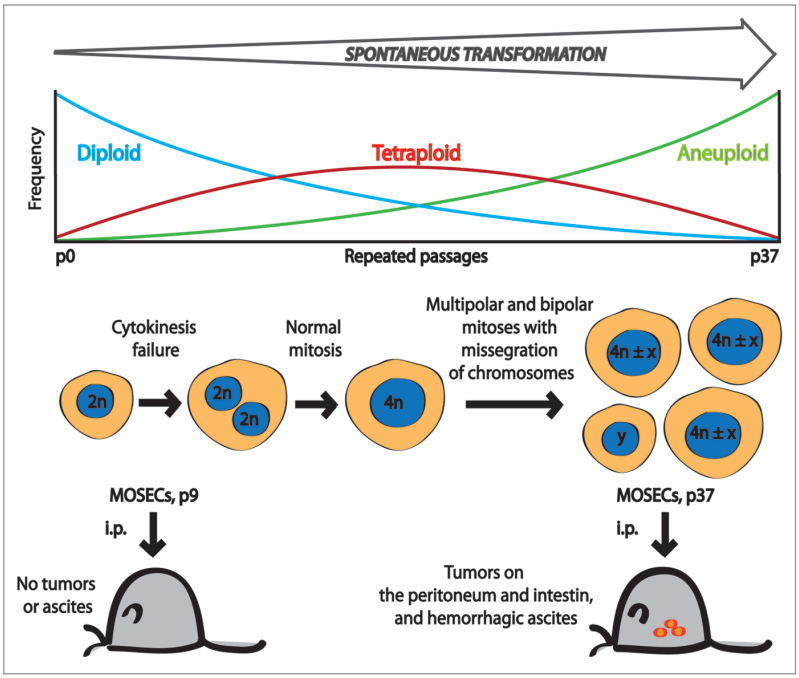

Figure 1. Tetraploidy in the malignant transformation of mouse ovarian surface epithelial cells (MOSECs). During the process of spontaneous transformation, the percentage of diploid (2 n) cells gradually decreased, while the fraction of tetraploid (4 n) cells increased, peaked at passage 19, then decreased with further passages in culture. At the same time, the frequency of aneuploid cells increased continuously until passage 37 (p37). Long-term live-cell imaging followed by FISH showed that cytokinesis failure is responsible for the generation of tetraploid cells and that most aneuploid cells derive from such tetraploids. When MOSECs from passage 9 were injected intraperitoneally into normal female C57BL/6 mice, no visible tumors or ascites were observed. In contrast, mice injected with late passage (p37) cells developed multiple tumors and had hemorrhagic ascites. (4 n ± x) and (y) represent near-tetraploid cells and other types of aneuploidy, respectively.

euploidy to metastable tetraploidy and subsequent aneuploidy.

In a recent issue of *Cell Cycle*, Lv L et al.[9] revealed the surprising finding that long-term in vitro culture of mouse ovarian surface epithelial cells (MOSECs) is sufficient to generate cancer cells, that is cells that generate tumors upon inoculation into syngenic control mice (**Fig. 1**). In the course of their experiments, Lv, et al. discover that cultured MOSECs initially are diploid, yet progressively become tetraploid after repeated passaging. Videomicroscopic studies indicate that such tetraploid MOSECs arise from cytokinesis failure of bipolar mitosis in initially diploid cells. Later, tetraploid cells gave rise to aneuploid offspring through chromosomal missegregation during either bipolar or multipolar mitoses, as documented by a combination of long-term videomicroscopy and fluorescence in situ hybridization (FISH) technology.[9] Altogether, these data reveal that aging normal MOSECs in vitro, in the absence of any chemical carcinogens, can lead to their malignant transformation.

The molecular events that are compatible with the spontaneous malignant transformation of aging MOSECs are elusive. Primary breast epithelial cells only can undergo tetraploidization and subsequent malignant transformation if they are derived from p53−/− mice, presumably because the inactivation of p53 is a prerequisite of the generation or survival of tetraploid cells.[3] Hence, it will be interesting to assess p53, as well as its upstream activators and downstream effectors in aging MOSECs. Aging primary fibroblasts undergo a "crisis" that results from progressive telomere shortening, followed by the reactivation of telomerase that is required for long-term proliferation. Since telomere attrition is a potent inducer of tetraploidization,[6] it will also be interesting to assess the expression of telomerase in MOSECs as they evolve from normal diploid to malignant aneuploidy via a transient state of tetraploidy.

Beyond these molecular details, it will be important to understand whether the in vitro culture of MOSECs constitutes a general model of aging. As a fascinating prospect, cell-intrinsic perturbations in cell cycle control and genomic maintenance might favor the accumulation of potential cancer cell precursors, hence explaining the ever-increasing incidence of malignancy in aging. It is also possible that accumulating tetraploid cells contribute to the progressive dysfunction of aging tissues. If this were the case, pharmacological interventions aiming at the selective elimination of tetraploid cells might constitute dual hits in so far as they would not only reduce the incidence of cancers, but that they would also mediate a general antiaging effect.

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