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A.A. STEPANENKO, V.M. KAVSAN
Institute of Molecular Biology and Genetics, NAS of Ukraine, Kyiv
E-mail: kavsan@imbg.org.ua

IMMORTALIZATION AND MALIGNANT TRANSFORMATION OF EUKARYOTIC CELLS

The process of cellular transformation has been amply studied in vitro using immortalized cell lines. Immortalized cells never have the normal diploid karyotype, nevertheless, they cannot grow over one another in cell culture (contact inhibition), do not form colonies in soft agar (anchorage-dependent growth) and do not form tumors when injected into immunodeficient rodents. All these characteristics can be obtained with additional chromosome changes. Multiple genetic rearrangements, including whole chromosome and gene copy number gains and losses, chromosome translocations, gene mutations are necessary for establishing the malignant cell phenotype. Most of the experiments detecting transforming ability of genes overexpressed and/or mutated in tumors (oncogenes) were performed using mouse embryonic fibroblasts (MEFs), NIH3T3 mouse fibroblast cell line, human embryonic kidney 293 cell line (HEK293), and human mammary epithelial cell lines (mainly HMECs and MCF10A). These cell lines have abnormal karyotypes and are prone to progress to malignantly transformed cells. This review is aimed at understanding the mechanisms of cell immortalization by different «immortalizing agents», oncogene-induced cell transformation of immortalized cells and moderate response of the advanced tumors to anticancer therapy in the light of tumor «oncogene and chromosome addiction», intra-/intertumor heterogeneity, and chromosome instability.

Introduction. Malignant transformation is the process by which cells acquire the properties of cancer. The first successful malignant transformation *in vitro* was achieved with the polyoma virus on Syrian hamster embryo cells, followed by transformation with chemical carcinogens in the mid-1960th (reviewed in [1]). Reports of human cell transformation using viruses and viral oncogenes appeared only in the late 1970th [1]. In early 80th it was shown that immortalized NIH3T3 mouse fibroblast cells introduced with total genomic DNA from human tumors were converted into cancer cells; later it was found that *H-RAS* gene harboring a point mutation induced transformation of NIH3T3 cells in culture and conferred on them the ability to induce tumors in nude mice (reviewed in [2]). These discoveries marked an advent of the intense searching for the abnormal genes influencing the development of human cancer that continues today [3].

Early works stated that *in vitro* transformation of human cells by a single carcinogenic agent in contrast to rodent cells was an extraordinarily rare event [1]. Moreover, a spontaneous immortalization following senescence was also an extremely rare event in human fibroblasts and epithelial cells, although it occurred commonly in rodent cells with varying frequencies depending on species from which the cells were derived [1, 4]. One of the explanations of intrinsic antineoplastic mechanisms of human cells was differences in telomere biology between human and murine cells. Mouse cells begin their replication *ex vivo* with extremely long telomeres: 3–10-fold longer than in identical human cells and the tendency for progressive telomere erosion might effectively be countered by the basal telomerase activity that is constitutively present in mouse cells [1, 4–8]. Additionally, the basal metabolic rate is about 7-fold higher in mice than in humans and this affects the levels of endogenous oxidants and other mutagens that are produced as by-products of normal oxidative metabolism resulting in 18-fold more breakdown products of DNA in mice [4]. Moreover, the rates of metabolic conversion of procarcinogens to carcinogens and the detoxification of many other potential mutagens can occur with greatly differing kinetics [4]. Furthermore, humans have more efficient DNA repair system, and the rate of 5-methylcytosine decline during cellular senescence is much slower in human cells than in mouse cells [1].

Nevertheless, telomerase-deficient primary mouse embryonic fibroblasts (MEFs) could be immortalized/transformed in culture, and generated tumors in nude mice following transformation [9]. It was concluded that telomerase is not required for establishment of immortalized cell lines, oncogenic transformation, or tumor formation in mice. Another research group transformed human primary fibroblasts and human primary mesodermal cells introducing simultaneously three oncogenes *E1A*, *MDM2*, and *H-RAS*^{V12}. These cells formed colonies in soft agar and tumors in mice, but they and the majority of the tumors derived from them lacked telomerase activity, and telomere erosion was observed [10]. Authors have deduced that telomere maintenance is not obligatory for tumorigenic conversion. To the point, human primary melanomas show telomere maintenance as a late event in tumor progression (metastatic melanoma), thus, telomere maintenance/immortalization is associated with progression rather than initiation of melanoma [11].

Furthermore, like primary human cells, primary MEFs require combination of two «hits» to acquire the capacity to form tumors [9, 12-19]. There are also cases of a conversion of normal primary rodent [20–23] and human [24–27] cells to fully transformed cells with a single oncogene under specific experimental (significant overexpression of oncogene) and culture conditions. Culture conditions significantly affect proliferative (before senescence) [5] and transformation potential of cells [8]. For example, wild type MEFs grown in serum-free medium supplemented with defined growth components (EGF, PDGF, insulin, high density lipoprotein, fibronectin, and transferrin) were refractory to transformation by oncogenic *RAS* + *E1A* [7]. Moreover, *RAS* + *E1A*-induced chromosome instability, colony formation and tumorigenesis of the *p53* −/− serum free-MEFs also could be attenuated by treating the cells with the free-radical scavenger N-acetylcysteine [7].

Finally, humans live, on average, 30–50 times longer than mice and undergo about 10^5 more cell divisions in a lifetime (10^{16} versus 10^{11} mitoses) [4]. Nevertheless, epidemiological studies have revealed that the life-time risk of developing cancer is comparable in both species. About 30 % of laboratory rodents have cancer by the end of their 2–3 year life-span and about 30 % of people have cancer by the end of their 70–80 year life-span [4].

Thus, it seems that *in vitro* (and likely *in vivo*) transformation process may be fundamentally similar in rodent and human cells and be significantly affected by non-physiological culture conditions *in vitro*.

Senescence. In contrast to germ cells and certain stem cells somatic cells have a limited lifespan, gradually slow in growth, and stop dividing, a process known as replicative senescence [28]. The finite replicative life span of normal cells in culture was first described approximately 50 years ago by Leonard Hayflick [29], and is often termed as the «Hayflick limit» [30]. The precise number of replicative doublings exhibited by cultured cells before they reach senescence depends on the species from which the cells are derived, the tissue of origin, and the age of the donor organism [31]. Cultured human primary fibroblastic cells generally display 50 to 80 population doublings (PD) [7, 32, 33], whereas explanted MEFs can divide just for 15–30 PD before undergoing senescence [5, 7]. Primary normal human astrocytes perform only about 20 PD before reaching senescence [34]. Human keratinocytes have an *in vitro* life span of 15–20 PD in serum-free chemically defined media, whereas keratinocytes grown on feeder fibroblasts proliferate for up to 50 PD [7, 32] and in F medium on feeder fibroblasts for up to 80 PD before senescencing [35]. Most published reports on cultured human epithelial cells have shown active growth for only 10 to 30 PD [32]. Significantly, simple changes in the culture conditions (defined growth factors instead of serum) could permit active growth of human mammary epithelial cells for up to 60 PD, whereas addition of oxytocin (endogenous antioxidant) gave about 20 PD of increased proliferation [32]. MEFs proliferate for more than 60 PD with no signs of replicative senescence under physiological oxygen levels (3 % versus 21 %) [7, 8]. Thus, primary cells undergo stress-associated senescence due to *in vitro* non-physiological standard culturing conditions, including disruption of cell-cell contacts, lack of heterotypic interactions between different cell types, the medium-to-cell ratio, persistent signaling pathways activation by mitogens, absence of appropriate survival factors, hyperoxia, and plating on plastic [5].

The process of senescence occurs both *in vitro* and *in vivo*. Cellular senescence *in vivo* is now recognized to play an active role as a tumor suppressor pathway [36, 37], in the loss of regenerative potential in aging tissues and in the pathogenesis of cardiovascular diseases [38]. Senescence *in vitro* is marked by the appearance of large, flattened vacuolated cells and characterized by the inability of cells to proliferate despite the presence of a steady supply of abundant nutrients, mitogens [39], ample room for expansion [33], and by the maintenance of cell viability/resistance to apoptosis and metabolic activity for months [37, 38, 40]. Once senescence is triggered, cells are not capable of re-entering the cell cycle or developing into tumors [36]. Moreover, senescent cells secrete a plethora of factors primarily involved in insulin-like growth factor and transforming growth factor β signaling, extracellular matrix remodeling, and inflammation. Altogether these secreted factors were referred to as the «Senescence-Messaging Secretome» or the «Senescence-Associated Secretory Phenotype» [33].

Senescent cells can be distinguished from presenescent, immortal, quiescent or terminally differentiated cells by histochemical detection of the biomarker senescence-associated β-galactosidase [41]. Senescence accompanies changes in nuclear morphology and formation of a distinct chromatin structure, called senescence-associated heterochromatic foci (SAHF). These foci are characterized by the accumulation of histone H3 trimethylated at lysine 9 and recruit heterochromatin proteins to the genes that are to be stably repressed during senescence [41]. Importantly, formation of SAHF and silencing of genes require an intact pRB pathway, since inhibition of $p16^{INK4A}$ prevents SAHF formation and leads to DNA replication [33, 41].

The onset of senescence is partly attributable to the shortening of telomeres by approximately 50–200 base pairs with each cell division to a threshold where it is recognized as DNA damage and thus initiates replicative senescence [31, 33, 38]. Critical telomere shortening and eventual dysfunction triggers a classical DNA damage response involving a number of cellular proteins, including ataxia telangiectasia mutated protein (ATM), check point kinase 1/2 (CHK1 and CHK2), $p16^{INK4A}$, p53, 53 binding protein 1 (53BP1), $p21^{CIP1}$, nijmegan breakage syndrome 1 protein (NBS1), plasminogen activator protein 1 (PAI1), and phosphorylated histone γ-H2A.X [41]. These cellular factors cooperate to initiate senescence, thereby preventing cellular proliferation in the presence of damaged chromosomes and hence limiting the acquisition of potential pathogenic mutations [33, 41].

However, telomere attrition is not the only stimulus for replicative senescence. Oxidative stress can induce or accelerate the onset, a phenomenon referred to as stress-induced replicative senescence. It occurs in several ways (reviewed in [38]): oxidative stress can activate critical cell cycle tumor suppressor proteins p53 and pRB by oxidative-stress-induced DNA damage such as double strand breaks. Oxidative stress can result in oxidative modifications of triple guanine repeats (TTAGGG) in sequences of telomeric ends making them more susceptible to breaks and enhancing the rate of telomere attrition. Oxidative-stress-induced premature senescence might be a function of a direct suppression of telomerase activity. *hTERT* gene (encodes catalytic subunit of human telomerase) expression is regulated by many transcription factors, including AP1, SP1 and NF-κB, all of which are redox regulated [42]. In any case, oxidative stress results in the loss of chromosomal integrity as manifested by chromosomal fusions, recombination and degradation, and contributes to DNA damage responses that eventually lead to the irreversible cell-cycle arrest/senescence or cell death through the activation of p53- and pRB-dependent functions [24, 43].

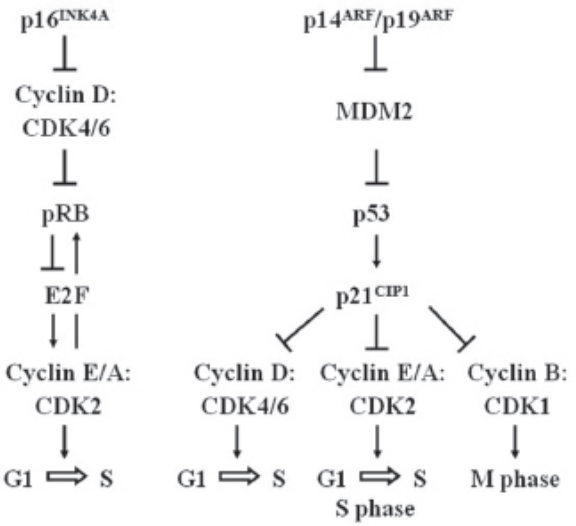
Immortalization. Senescence (telomere erosion-induced, oncogene-induced [33], or stress-induced [44]) forms a barrier against tumorigenesis. Overcoming of senescence and acquisition of immortality is an essential step in the process of malignant transformation [33, 45]. Cellular immortalization allows a cell to indefinitely proliferate while accumulating genetic abnormalities [27, 46]. Immortalized cells cannot grow over one another (contact inhibition) [47, 48], their proliferation ability is growth factor dependent [49] and their growth is anchorage-dependent (cells do not form colonies in soft agar) [50–52]. There are several mutually complementary mechanisms that contribute to

a cell being able to escape senescence and become immortal, including telomere length stabilization, epigenetic gene silencing by selective promoter methylation, oxidative DNA damage, inactivation of cell cycle regulatory genes such as *p16^{INK4A}*, *p53*, *pRB, p21^{CIP1}*, overexpression of a cellular oncogenic proteins such as c-MYC, BMI1 or through expression of viral oncogenes [41].

In vitro immortalization of various cell types was successfully performed by the introduction of viral genomes/oncogenes (Table 1), telomerase catalytic subunit (hTERT) (Table 2) as well as by enforced expression of transcription factors (e.g. c-MYC, BMI1, ZNF217, or β-catenin).

Genomes of viruses encode a number of regulatory and structural proteins but «immortalizating effect» can be attributed only to several of them. For SV40 viral oncoproteins responsible for immortalization correspond to the portion of the viral chromosome expressed early after infection, which encodes two proteins, the large T-antigen and the small t-antigen [92, 93]. For the adenoviruses viral oncoproteins are encoded by a subset of the early genes and termed the E1A proteins and the E1B proteins [93]. Experiments with the human papilloma viruses uncovered a similar set of early proteins called the E6 and E7 proteins [93, 94]. In the cell these oncoproteins bind to pRB and p53 causing their ubiquitin-dependent proteasomal degradation. It allows going through the cell cycle checkpoints in an uncontrolled manner. Role of pRB and p53 signaling pathways in cell cycle regulation is presented in Figure. Cells expressing these viral oncogenes continue proliferating beyond the population doubling level, at which their untreated counterparts become senescent, but they eventually cease proliferating in a state referred to as crisis [95–97]. A small number of cells within the population may acquire the ability to escape from crisis and form an immortalized cell line. In all such cell lines examined, escape from crisis has been shown to be associated with activation of a telomere maintenance mechanism.

Viral oncoproteins can bind to multiple other cellular proteins [92, 98], including several transcription factor complexes involved in *hTERT* transcription regulation [94]. For example, human papilloma virus E6 protein, *via* direct binding, increases c-MYC efficiency in activating the *hTERT* promoter and, on the other hand,



Schematic representation of pRB and p53 signaling pathways. p16^{INK4A} inhibits CDK4 and CDK6 preventing interaction with D-cyclins. CDK4 and CDK6 phosphorylate pRB leading to a partial loss of its ability to repress the E2F. When pRB-E2F suppressive interaction is relaxed, E2F transactivates genes involved in G1/S transition and in the initiation of DNA replication in S phase. CDK2-cyclin E complexes can further phosphorylate pRB resulting to complete its release from interacting with E2F and, thus, promoting S phase progression. P14^{ARF}/p19^{ARF} is an antagonist for MDM2 which, in turn, regulates p53 stability through its ubiquitin ligase activity. ARF sequesters MDM2 resulting in p53 activation and stabilization. p53 induces p21^{CIP1} expression. p21^{CIP1} associates with cyclin D-CDK4/6, E/A-CDK2, and cyclin B-CDK1 complexes, and has a universal inhibitory activity towards these CDKs thereby regulating G1/S transition, S and M phases of cell cycle

E6 is able, through its association with E6AP, to promote the degradation of the *hTERT* promoter transcriptional repressor NFX1-91 [94]. Usage of both viral oncogene and *hTERT* to induce immortalization has also been reported. For instance, pre-adipocytes, bone marrow stromal cells and ovarian surface epithelial cells were immortalized by introduction of HPV *E7* and *hTERT* [96]. Mechanistically, thus, process of immortalization induced by viruses corresponds to a process of cell cycle checkpoint proteins inactivation (pRB and/or p53) and restoration of telomerase activity resulting in telomere ends stabilization.

Ectopic expression of *hTERT* alone in presenescent or still dividing cells can effectively

immortalize them (Table 2). The native *hTERT* locus is embedded in a large nuclease-resistant chromatin domain in most normal human cells [97]. As a result, the *hTERT* promoter is stringently repressed in somatic primary cells. But in immortalized and tumor cell lines *hTERT* is often up-regulated, and these cells are capable to maintain stable telomere lengths by activation of a telomere maintenance mechanism; also, there is non-telomerase alternative mechanism of telomere maintenance [95–100]. Except cases with ectopic *hTERT* expression, in otherwise immortalized (and tumor) cells *hTERT* gene activation can occur in several ways probably mutually complementary to each other: through gene amplification; nonreciprocal translocation by chromosomal breakage at the *hTERT* locus and subsequent ligation to heterologous sequences by non-homologous end joining (NHEJ) mechanisms resulting in the chromosomal rearrangements upstream of its promoter; the activation of c-MYC and inhibition of histone deacetylases (HDACs) (reviewed in [97]). Hyperoxia or addition of exogenous H_2O_2 was shown to induce senescence of fibroblasts despite *hTERT* overexpression and exogenous H_2O_2 prevented hTERT-dependent immortalization of normal endothelial cells, whereas N-acetylcystein (antioxidant) permitted hTERT-dependent immortalization of endothelial cells [38]. Indeed, oxidative stress regulates hTERT at many levels, such as its gene expression, activity, and sub-cellular localization [42].

Immortalization of human and rodent cells was also achieved by different transcription factors. The c-MYC protein is a basic helix-loop-helix leucine zipper transcription factor that modulates

Table 1

Immortalization of human cells by viral genomes/oncogenes

Cell type	Immortalizing agent	Ref.
Astrocytes	SV40 T antigen	[53]
HS74BM diploid fetal bone marrow fibroblasts	SV40	[54]
IMR-90 diploid lung fibroblasts	SV40	[55, 56]
Ciliary epithelial cells	SV40	[57]
Fetal liver epithelial cell	SV40 T antigen	[58]
Mammary epithelial cells	SV40	[59]
Prostate epithelial cells	SV40	[60]
Tracheal epithelial cells	SV40	[61]
Uroepithelia cells	SV40	[62]
Cervical epithelial cells	HPV16/18	[63, 64]
Epidermal keratinocytes	HPV16	[65, 66]
Epidermal keratinocytes	HPV16 E6+E7	[67, 68]
Esophageal epithelial cells	HPV16 E6/E7	[69]
Foreskin keratinocytes	HPV16/18/ 31/ 33	[70, 71]
Gingival keratinocytes	HPV16 E6	[72]
HFE keratinocytes	HPV16 E7	[73]
Mammary epithelial cells	HPV16	[74]
Mammary epithelial cells	HPV16 E6+E7	[75]
Uroepithelial cells (from ureteral uroepithelium)	HPV16 E7	[76]
Urothelial cells (from ureteric or bladder tissue)	HPV16 E6+E7	[77]
WHE-7 fetal fibroblast	HPV16 E6	[72]
Embrionic kidney cells HEK293	Ad5	[78]
Bronchial epithelial cells	Ad12-SV40 fusion genome	[79]
Epidermal keratinocytes	Ad12-SV40 fusion genome	[80]

Indications. SV40 – simian polyomavirus; HPV16/18 – human papilloma virus types 16 and 18; Ad5 and 12 – adenovirus type 5 and 12.

Table 2

Molecular characteristics of immortalized human cell lines

Cells	Immort. agent	$p16^{INK4A}$	pRB	p53	$p21^{CIPI}$	hTERT*	Ref.
Adenoid epithelial cells and foreskin keratinocytes	hTERT	–	+ hyper phosphorylated	+	+	+	[81]
Ameloblastoma cells	hTERT	–	N/A	+	+	+	[82]
BJ fibroblasts, RPE-340 cells	hTERT	+/–	+	+	N/A	+	[83]
BJ fibroblasts	hTERT	N/A	+ unaffected	+	N/A	+	[84]
Cen3 fibroblasts	hTERT	↓/ –	N/A	+ mutated	↓	+	[24]
Esophageal epithelial cells	hTERT	↓	+ unaffected	+	↓	+	[69]
Foreskin fibroblasts	hTERT	↓ deletion	↑	+/↓ mutated	+/–	+	[85]
Gingival and periodontal ligament fibroblasts	hTERT	–	+ hyper phosphorylated	N/A	N/A	+	[72]
Dermal keratinocytes	hTERT	–	+ hyper phosphorylated	+	N/A	+	[86]
Mammary epithelial cells	hTERT	–	N/A E2F1 elevated	+	+	+	[87]
Epi gingival keratinocytes	HPV E6	–	+ hyper phosphorylated	N/A	↓	+	[72]
Esophageal epithelial cells	HPV16 E6/E7	↑	↓	↓	↑	+	[69]
Mammary epithelial cells	HPV16 E6 + E7	N/A	↓	↓	↓	N/A	[75]
WHE-7 fetal fibroblasts	HPV16 E6	–	+ hyper phosphorylated	N/A	↓	N/A	[72]
Foreskin fibroblasts	c-MYC	+	N/A	+	+	N/A	[88]
Prostate epithelial cells	c-MYC	–	N/A	+	+	+	[89]
Mammary epithelial cells	ZNF217	N/A	+ unaffected	+	N/A	+	[90]
Oral keratinocytes	Cyclin D1 + dominant-negative p53	N/A	N/A	+ mutated	N/A	ALT	[91]
Foreskin keratinocytes	Y-27632	+	N/A	+	N/A	+	[28]
KMST-6 fibroblasts	^{60}Co	–	+ hyper phosphorylated	N/A	↓	ALT	[72]
MDAH 087 skin fibroblasts	Aflatoxin B1/X-rays	–	+ hyper phosphorylated	+ mutated	↓	ALT	[72]
OUMS-24F fibroblasts	4-NQO	+	+ hyper phosphorylated	N/A	↑	ALT	[72]

Indications. RPE-340 – retinal pigment epithelial-340 cells; MDAH 087 – skin fibroblasts derived from a patient with Li-Fraumeni syndrome; Y-27632 – Rho kinase (ROCK) inhibitor; 4-NQO 4-nitroquinoline 1-oxide; ALT – alternative telomere maintenance mechanism; «↑» – overexpressed; «↓» – downregulated; «+» – unchanged level; * for hTERT – overexpressed; «–» – undetected; N/A – not analyzed.

expression of a cohort of genes, including those that function to promote cell growth and cell cycle entry [88, 101]. c-MYC up-regulates certain cyclin-dependent kinases (CDK4) and cyclins (A, B1, D1, D2, and E), and represses cyclin-dependent kinase inhibitors ($p15^{INK4B}$, $p21^{CIP1}$, and $p27^{KIP1}$) [48, 101, 102]. Mechanisms can be direct or indirect: for example, c-MYC directly binds to the cyclin B1 promoter, but optimal induction of expression occurs only when p53 is concurrently inactivated [102], whereas cyclin D1 expression is positively regulated through MYC/miR-378/TOB2/cyclin D1 functional module in human mammary epithelial cells [103]. $p21^{CIP1}$ expression is regulated both negatively and positively by c-MYC [104]. Induction of $p21^{CIP1}$ by *c-MYC* overexpression was p53-dependent in normal human and mouse fibroblasts and was associated with G2 arrest, whereas, inversely, c-MYC repressed $p21^{CIP1}$ transcription in p53-null mouse cells and in a human adenocarcinoma cell line [105]. Moreover, the *hTERT* promoter contains the MYC binding site (E-box) and is a direct transcriptional target of c-MYC [106]. *c-MYC* expression was reported to result in successful immortalization of rat kidney cells [22], mouse neural precursor cells [107], human neural stem cells (by v-MYC and c-MYC T58A mutant) [49], prostate epithelial cells [89], and foreskin fibroblast cells [88]. Interestingly, foreskin fibroblast cells had increased levels of $p16^{INK4A}$ and p53 and functional both $p16^{INK4A}$–pRB (pRB phosphorylation was reduced) and p53–$p21^{CIP1}$ parthways. Prostate epithelial cells preserved functional p53-$p21^{CIP1}$ pathway and had elevated $p16^{INK4A}$ but, nevertheless, pRB phosphorylation was maintained. Moreover, c-MYC allowed to tolerate ectopically overexpressed $p16^{INK4A}$ in prostate epithelial cells, whereas $p16^{INK4A}$ overexpression in foreskin fibroblast resulted in senescence. Foreskin fibroblast cells also showed epigenetically silenced *$p14^{ARF}$* (unfortunately, *$p14^{ARF}$* status was not analysed in prostate epithelial cells). Rodent cells immortalized by c-MYC characteristically inactivate the ARF–p53–$p21^{CIP1}$ pathway by loss of either functional p53 or 19^{ARF} [88]. $p14^{ARF}$/$p19^{ARF}$ is unique among c-MYC regulators. It selectively inactivates the hyperproliferative and transforming functions of c-MYC without affecting normal cell cycle progression or preventing c-MYC-mediated apoptosis [108]. Thus, p53 or $p14^{ARF}$/$p19^{ARF}$ inactivation is likely beneficial in cells immortalized by c-MYC.

Other transcription factor BMI1, a member of the Polycomb group of transcriptional repressors [109], was initially identified as an oncogene that cooperates with c-MYC in lymphomagenesis [110]. Moreover, BMI1 is positively regulated by c-MYC [37]. It was reported that overexpression of BMI1 down-regulated *$p16^{INK4A}$* and *$p19^{ARF}$* expression in mouse embryonic fibroblasts and resulted in their immortalization [96], immortalized primary human mammary epithelial cells (HMEC) [111] and nasopharyngeal cells [112]. In both latter cases immortalization was accompanied by telomerase activation. BMI1 caused the bypass of replicative senescence in normal human oral keratinocytes but did not immortalize them (no *hTERT* activation) [113]. *BMI1* introduction along with human papilloma virus *E6* gene but not with *E7* immortalized oral keratinocytes and it was associated with telomerase activation [113]. Introduction of *BMI1* as well as *$p16^{INK4A}$*-specific short hairpin RNA into human epithelial cells derived from skin, mammary gland and lung suppressed *$p16^{INK4A}$* expression and extended cells life span; subsequent introduction of *hTERT* in these cells resulted in their efficient immortalization with following maintenance of near normal diploidy [96]. The reason why some cell types become immortalized after *BMI1* introduction alone, whereas other cells do not is unclear. It was speculated that BMI1-induced immortalization mechanism may be tissue-dependent or because the cultured cells already underwent critical steps towards immortalization [113]. The mechanism whereby BMI1 promotes evasion of senescence involves, among other targets, transcriptional silencing of *CDKN2A* (cyclin-dependent kinase inhibitor 2A, which encodes both $p16^{INK4A}$ and $p14^{ARF}$) and *CDKN2B*, which encodes $p15^{INK4B}$ [42, 110, 114].

Transcription factor ZNF217 was able to immortalize human primary HMECs disturbing ARF-p53-$p21^{CIP1}$ pathway [90]. β-Catenin, a member of the Armadillo (ARM) repeat protein superfamily, activates transcription of target genes primarily by associating with the T cell factor/lymphoid enhancer-binding factor (TCF/LEF) family [115, 116]. Expression of β-catenin immortalized

primary mouse melanocytes directly repressing the expression of $p16^{INK4A}$ by binding to its promoter [117]. Other well known targets of β-catenin are c-MYC and cyclin D1 [115].

In fact, all mentioned targets of discussed transcription factors are only the «top of an iceberg». It was established that c-MYC regulates a total of 1469 target genes in HeLa cells and human primary fibroblasts [118], β-catenin in HCT116 colorectal carcinoma cells directly bound *in vivo* to more than 400 target genes [115]. ZNF217 was shown to target 103 genes in breast cancer cell line MCF7, 44 genes in colon cancer cell line SW480, and 101 genes in teratocarcinoma cell line Ntera2 [119]. Moreover, *NF-κB*, *STAT3*, *ERβ*, *JUN*, *ELK4*, *CEBP*, and *ETS1* were found among transcriptional regulator genes up-regulated by c-MYC in human B cell line P493 [120], and *EPAS1*, *ERF*, *FHL2*, *JUN*, *MNT*, *MYT1*, *RPO1-2*, *SOX4*, *TEAD4*, *TIEG1*, and *ZFP28* in pancreatic β-cells [121], which, in turn, regulate additional gene cohorts resulting eventually in global change in gene expression. C-MYC can regulate overall up to 10–15 % of all genes [120, 122], among which there are those regulating replication and reparation in S phase and chromosome separation during M phase [121–124].

Spontaneously immortalized cells emerge at an extremely low frequency (about 10^{-7}) during crisis *in vitro* [85, 97], but show the same general changes in cell cycle checkpoint pathways as all otherwise immortalized cells [125–127]. Thus, overwhelming majority of immortalized cells irrespective of «immortalizing agent» do not express $p16^{INK4A}$ cell cycle suppressor and this correlates with pRB hyperphosphorylation (inactivation) (Table 2). Indeed, it has been estimated that more than 70% of human immortalized and cancer cell lines lack functional $p16^{INK4A}$ due to promoter methylation, mutation, or homozygous deletion. In many instances the deletions affect both $p16^{INK4A}$ and $p14^{ARF}/p19^{ARF}$, but a substantial proportion of the missense mutations exclusively affect $p16^{INK4A}$, suggesting that $p16^{INK4A}$ itself plays significant and non-redundant role in tumor suppression [128]. Spontaneous reduction in $p16^{INK4A}$ expression due to promoter methylation (most often) or otherwise mechanisms during *in vitro* propagation of normal primary cells has been documented, for example, in HMEC [96, 129], fibroblasts derived from lung [96], human keratinocytes [96, 130], and human astrocytes [34]. Molecular mechanism of $p16^{INK4A}$ gene inactivation by epigenetic deregulating methylation during progression from primary cells to immortalized and pre-malignant cells is complex; it is under intensive investigation and may be different in mouse and human cells [128, 131–137].

Nevertheless, in contrast to frequent loss of $p16^{INK4A}$ expression *in vitro*, another well documented fact is that $p16^{INK4A}$ is overexpressed in certain samples of different cancer types. In tumors increased $p16^{INK4A}$ expression correlates statistically with *RB* loss of heterozygosity [138]. In spite of being tumor suppressor, overexpression of $p16^{INK4A}$, nevertheless, correlates with a poor prognosis and seems to be an unfavorable prognostic indicator [138].

ARF–p53–$p21^{CIP1}$ pathway is likely less critical for immortalization of human cells than $p16^{INK4A}$–pRB, because approximately in a half of analyzed works it was apparently functional (Table 2). Moreover, Odell et al. [139] examined more than a hundred spontaneously immortalized MEF cell lines and found that at least half of them had neither a p53 mutation nor loss of $p19^{ARF}$. Nevertheless, it is necessary to take into account that analysis of ARF-p53-$p21^{CIP1}$ pathway in most works (where it was shown functional) was performed once in certain population doubling (PD), but it could be inactivated later. For instance, hTERT immortalized cen3tel fibroblasts up to 108 PD had wild-type p53 sequence, whereas at late PDs (165 and 366 PDs) had a mutation in codon 161 [24].

Thus, it is possible to conclude that for successful immortalization cells must overcome senescence by inactivating $p16^{INK4A}$–pRB and /or ARF-p53-$p21^{CIP1}$ and crisis by maintaining their telomeres by activation of *hTERT* expression or by an alternative mechanism for lengthening telomeres (ALT) [99, 100]. However, it needs to keep in mind that every oncogene/«immortalizing agent» introduced into a cell has a great number of targets and multidirectional effects rather than being one-way agent. Thus, the process of immortalization is not simply a number of well defined events like inactivation of cell cycle negative regulators and activation of telomerase but, instead, is associated with karyotype/genome ab-

normalities (aneuploidy/gain or loss of additional chromosomes, translocations, deletions and amplifications) and, as a consequence, with global changes in gene expression [165]. All immortalized cells have abnormal karyotypes irrespectively of «immortalizing agent» (Table 3).

Also, significant changes in global gene expression can be reached through aberrant methylation of promoters. In HMECs during immortalization global aberrant DNA methylation changes occured in a stepwise fashion [129]. The first aberrant DNA methylation step coincided with overcoming stasis, and resulted in few to hundreds of changes, depending on how stasis was overcome (stress-inducing serum-free medium, benzo(a)pyrene or $p16^{INK4A}$ shRNA). A second step coincided with crisis/immortalization and resulted in hundreds of additional DNA methylation changes regardless of the immortalization pathway [129].

All together, it explains why across cell types and model systems genes in the cell cycle pathway, cytoskeletal genes, IFN pathway, IGF pathway, MAP kinase pathway, and oxidative stress pathway were identified as regulators of senescence/immortalization [41, 166]. It is worth noticing that virus oncoproteins induce more profound karyotype changes because of simultaneous ablation of pRB and p53 pathways, inactivation of which is directly linked with aneuploidy/polyploidy. In contrast, hTERT alone immortalized cells are suggested to be apparently genetically stable frequently showing near diploid karyotypes with lower abnormalities than otherwise immortalized cells [47, 142, 143, 147, 167–171]. It is clear that telomerase introduction into a cell can

Table 3

Karyotype abnormalities in immortalized cells

Immortalizing agent	Ref.
Cells immortalized by hTERT	
Human adenoid epithelial cells and foreskin keratinocytes	[81]
Human fibroblasts from two centenarian individuals	[140]
Human normal fibroblasts	[83, 84, 141, 142]
Sheep fibroblasts	[143]
Human bone marrow endothelial cells	[144]
Human small airway epithelial cells	[145]
Human mammary epithelial cells	[46]
Human myometrial and uterine leiomyoma cells	[146]
Swine umbilical vein endothelial cells	[47]
Human mesenchymal stem cells	[147]
Human fetal hepatocytes	[148]
Human meibomian gland epithelial cells	[149]
Cells immortalized by SV40 T large antigen	
Rabbit kidney epithelial cells	[150]
Human corneal epithelial cells	[151]
Human gingival keratinocytes	[152]
Human nasopharyngeal epithelial cells	[153]
Human bronchial epithelial cells	[154]
Human mammary epithelial cells	[155]
Cells immortalized by HPV16/18 E6/E7	
Human epidermal keratinocytes	[156]
Human smooth muscle cells	[157]
Human nasopharyngeal epithelial cells	[153]
Human extravillous cytotrophoblasts	[158]
Human bronchial epithelial cells	[159]
Spontaneously immortalized cells	
MEFs	[160]
Syrian hamster embryo cells	[161]
Human epidermal cells	[125]
Human keratinocytes	[162]
Murine neural crest-derived corneal progenitor cells	[126]
Immortalized by otherwise ways	
Mouse embryos cells by v-SIS or K51 oncogenes	[163]
Human mesenchymal stem cells by E6/E7 plus hTERT or BMI1, E6 plus hTERT	[147]
Human meibomian gland epithelial cells by SV40 LT antigen plus hTERT	[149]
Human bronchial epithelial cells by hTERT plus CDK4 or HPV16 E6/E7	[159]
Human bronchial epithelial cells by BMI1 plus hTERT	[164]
Human prostate epithelial cells by c-MYC	[89]
Oral keratinocytes by cyclin D1 plus mutant p53	[91]

stabilize telomeres preventing numerous chromosomal aberrations occuring *via* telomere dysfunction and the breakage-fusion-bridge mechanism [165]. Nevertheless, although BMI1 and hTERT immortalized human embryonic stem cells at passage 40 [172], as well as hTERT immortalized human neural progenitor cells isolated from the ventral telencephalons of first trimester embryos after more than 40 passages (80–120 PD) [170] had normal karyotypes, in contrast, hTERT immortalized bone marrow endothelial cell clones showed numerous abnormalities after 75 PD (more than 160 days) [144]. Interestingly, mass culture of these cells at 65 PD (120 days) had 47 chromosomes without any structural abnormalities and served this karyotype at 135 PD [144]. Also, prolonged cultivation of telomerase-immortalized human fibroblasts led to a premalignant phenotype, although hTERT-immortalized cells behaved similarly to primary cells during the first 150 PDs [141]. The possible pitfall of «normal» or near diploid karyotypes of hTERT immortalized cells can result from exploiting conventional cytogenetic techniques for karyotyping, which do not allow detecting the subchromosomal aberrations. In contrast, for example, SNP and CGH arrays revealed multiple genomic abnormalities in tumors with near diploid katyotypes.

How do immortalized and tumor cells become aneuploid? p53, a well known «genome safeguard», plays multiple roles in maintaining genomic stability in somatic cells. Loss of p53 functions promotes oncogenesis by inducing chromosomal instability and aneuploidy [173-176] and enabling efficient accumulation of genetic mutations [177]. Loss or mutational inactivation of p53 results in a high frequency of centrosome amplification in part *via* allowing the activation of CDK2-cyclin E (as well as CDK2-cyclin A), which is a critical factor for the initiation of centrosome duplication [178]. It also allows immature escaping from cell cycle G2 checkpoint arrest through inability of $p21^{CIP1}$ to inactivate CDK2, and this leads to reinforcement of CDK2-dependent NF-Y phosphorylation and NF-Y dependent transcription of the cell cycle G2-regulatory genes, including *CDK1*, *CDC25*, *cyclin A* and *B* [179].

There is also a link between pRB inactivation, cell aneuploidy and chromosome instability (CIN). Aneuploidy and CIN results from persistent defects in mitotic fidelity, and several mechanisms have been described that cause cells to missegregate whole chromosomes [176, 180, 181]. More than 50 proteins are able to trigger polyploidy/aneuploidy when are appropriately misregulated (mutation, depletion, knockdown or overexpression) [182]. If the dosage of any one of many proteins involved in ensuring chromosome segregation fidelity is disrupted by the missegregation of the chromosome carrying that gene, the resulting imbalance can further compromise chromosome segregation accuracy [176]. Importantly, pRB-E2Fs pathway directly regulates genes involved in bipolar spindle formation, chromosome-spindle association, chromosome cohesion, and the spindle assembly checkpoint (SAC) [183]. Acute pRB suppression in IMR90 cells [184], HCT116 cells [185], primary human fibroblasts [186], mouse embryonic fibroblasts [180], and mouse adult fibroblasts [187] caused misregulation of chechpoint genes [180, 185, 186] and, as a consequence, gave rise to centrosome amplification, multipolar spindles, anaphase bridges, lagging chromosomes, and micronuclei harbouring whole chromosomes resulting in polyploidy/aneuploidy and cancerogenesis [180, 184–188]. Moreover, pRB influences mitotic chromosome condensation in E2F-independent manner, and loss of pRB function can influence chromosome loss irrespectively of proliferation [189]. pRB can interact with the condensin II subunit CAP-D3, and this interaction is necessary for chromosome compaction in mitosis [189]. pRB depletion compromises centromeric localization of CAP-D3/condensin II and chromosome cohesion, leading to an increase in intercentromeric distance and deformation of centromeric structure [181]. These defects promotes merotelic attachment (occurs when one kinetochore is attached to both mitotic spindle poles), resulting in failure of chromosome congression and an increased propensity for lagging chromosomes following mitotic delay [181].

In contrast to pRB, p107 and p130 (also members of retinoblastoma family) are rarely found inactivated in human tumors [190], and this fact determined predominant research on pRB, but it hardly proves the unique importance of pRB in cell cycle regulation. Equally weighty roles of p107 and p130 may well be masked by a functional redundancy that they have with one

another. Such redundancy would drastically reduce the likelihood of their elimination from tumor cell genomes during tumor progression [191]. Indeed, these proteins are part of a «tumor-surveillance» mechanism and can suppress tumorigenesis [190, 192–194].

Another consequence of pRB and p53 pathways deregulation is compromised DNA damage surveillance and repair. More than 70 genes have been identified that have roles in DNA damage surveillance and repair [195], and many of them are regulated by pRB and p53 [183, 195].

pRB and/or p53 pathways deregulation and *hTERT* expression (or ALT) are markers and indispensable conditions of immortalization. Deregulated pRB and/or p53 pathways inevitably leads to numerical and structural chromosome aberrations. A cell attains immortality by a global change in gene expression, which accompanies karyotype changes. Although a few common chromosome aberrations might have been observed in different immortalized cell, karyotype changes, in general, have stochastic nature. Immortalized cells with aberrant karyotypes are prone to malignant transformation.

Transformation. Cancer cells display several hallmarks that can be distinguished from those of normal counterparts. These include immortalization (bypass of senescence), evasion of apoptosis, immune destruction and anti-growth signals, growth factor independence, reprogramming of energy metabolism (enhanced glycolysis), anchorage-independence, resistance to contact inhibition, migration, invasion/degradation of matrix components, angiogenesis, metastasis, inflammation, and genome instability, which generates the genetic diversity accelerating acquisition of all listed hallmarks [31, 196]. In addition to cancer cells, tumors exhibit another dimension of complexity: they contain a repertoire of recruited, ostensibly normal cells that contribute to the acquisition of hallmark traits by creating the «tumor microenvironment» [196].

Cancer genes are often classified according to whether they function in a dominant or recessive manner at the level of the cancer cell. Dominant cancer genes (also known as oncogenes) require only one of the two parental alleles present in a normal cell to be mutated, and the encoded protein is usually constitutively activated by the mutations. Recessive cancer genes (also known as tumor suppressor genes) require mutation of both parental alleles, and these usually result in inactivation of the encoded protein. More than 80 % of the currently known cancer genes are dominantly acting [3]. Census of cancer genes lists 467 genes (data on December 2011, www.sanger.ac.uk/genetics/CGP/Census), which are supposed to be causally implicated in cancer development when appropriately changed (point mutations, deletions, translocations or amplifications) [197]. However, studies in mice have suggested that more than 3000 genes, when appropriately altered, may have the potential to contribute to cancer development (see reference in [198]).

The process of cellular transformation has been intensively studied *in vitro* using cell-culture techniques. Most research works on this issue satisfy four criteria: the cells are immortalized, i.e., can grow indefinitely in culture; the cells can efficiently form colonies in soft agar; the cells can develop tumor in immunodeficient mice; the xenograft or orthotopic tumor in the mouse shows malignant histology to exclude a pseudo-tumor or a benign tumor [267]. Actually, there are a few interesting outliers. For example, cells obtained by stable overexpression of cyclooxygenase 1 in spontaneously immortalized human umbilical vein endothelial cells underwent contact inhibition, failed to grow under anchorage-independent conditions but grew aggressively as tumors in mice [202]. Human primary foreskin fibroblasts in which *E1A+H-RAS^{V12}+MDM2* were introduced, although able to form colonies in soft agar and tumors in nude mice, were not immortal and, if maintained in culture for an extended period of time (40–50 generations), underwent a crisis phase characterized by dramatically reduced proliferation and adoption of a senescent phenotype [10]. Cells were telomerase-negative, only few of them eventually survived this phase and these cells became telomerase-positive [10].

MAPK and PI3K-AKT signaling pathways in transformation: a double-edged sword. One prominent hallmark of transformed *in vitro* cells irrespectively of transforming agent is upregulation of RAS-dependent extracellular signal-regulated kinases 1 and 2 (ERK1/2) mitogen-activated protein kinase (MAPK) pathway, phosphoinositide-3-kinase (PI3K)-mammalian target of rapamycin (mTOR)-AKT pathway and overex-

pression of CDKs/cyclins. Actually, besides MAPK and PI3K/AKT signaling, other pathways can also be activated. Nevertheless, RAS-dependent extracellular signal-regulated kinase 1/2 (ERK1/2) mitogen-activated protein (MAP) kinase [268–270] and phosphoinositide-3-kinase (PI3K)-mammalian target of rapamycin (mTOR)-AKT cascades [271] are the key signal transduction pathways responsible for integrating the different environmental signals and relaying the information to the cell cycle control system. Both these pathways are hyperactivated frequently in transformed cells *in vitro* and tumors *in vivo*, and are involved in regulation of all aspects of normal and tumor cell biology (e.g., cell growth, proliferation, apoptosis, migration, invasion etc).

As it is reviewed in [268-270], ERK1/2 are required for cyclin D1 expression *via* regulation of FOS family members and c-MYC transcription factors, as well as inhibition of TOB1 and JUND, cyclin D1 expression negative regulators. The ERK pathway may assist in both the assembly and stabilization of cyclin D1-CDK4/6 complexes *via* HSC70. There are also a few reports implicating MAPK pathway in the regulation of cyclin D2 and cyclin D3 expression. ERK activity is required for proper nuclear translocation of CDK2, and in the nucleus ERK regulates phosphorylation of a CDK2 activating site. ERK can phosphorylate two of four phosphorylation sites of the cytoplasmic retention sequence of cyclin B1, which are necessary for nuclear localization of cyclin B1. ERK futher contributes to CDK1-cyclin B activation *via* RSK/MYT1/CDK1-cyclin B pathway. The RAS-ERK signaling pathway is involved in the mitogen-induced downregulation of *p27^{KIP1}*. The degradation of p27^{KIP1} at the G1/S transition depends on the accumulation of cyclin E and concomitant activation of CDK2, events that are conditional on earlier activation of cyclin D-CDK4/6 complexes by the ERK pathway.

Activation of ERK markedly enhances c-MYC protein stability, which can transcriptionally upregulate expression of certain cycline-dependent kinases (CDK4) and cyclins (A, B1, D1, D2 and E), and represses cyclin-dependent kinase inhibitors (p15^{INK4B}, p21^{CIP1} and p27^{KIP1}) [48, 101,102, 272]. ERK1/2 dislodge pRB from its interaction with lamin A, thereby facilitating its rapid phosphorylation and consequently promoting E2F activation and cell cycle entry [273]. Pyrimidine nucleotides serve as essential precursors for the synthesis of RNA and DNA, phospholipids, UDP-sugars and glycogen [268, 270]. The rate-limiting step in the pyrimidine pathway is catalysed by the carbamoyl-phosphate synthetase enzyme, which is part of the large multifunctional protein CAD [268, 270]. ERK2 directly phosphorylates CAD activating it. ERK may impact on global protein synthesis through direct regulation of ribosomal gene transcription [268].

Over a hundred putative AKT substrates have been reported. Targets among cell cycle regulating proteins are reviewed in [43, 274, 275]. GSK3-mediated phosphorylation of cyclin D and cyclin E and the transcripton factors c-JUN and c-MYC, which all play a central role in the G1-to-S phase cell-cycle transition, targets them for proteasomal degradation. Phosphorylation and inhibition of GSK3 by AKT enhances the stability of these proteins. AKT reduces p21^{CIP1} protein level through downregulation of p53-mediated transcription and activation of MDM2, and inhibits *p27^{KIP1}* expression *via* inactivation of FOXO family of transcription factors. AKT phosphorylates both p21^{CIP1} and the p27^{KIP1} cyclin-dependent kinase inhibitors leading to their cytosolic sequestration and phosphorylates and deactivates pRB leading to the activation of E2F. AKT induces p27^{KIP1} degradation *via* GSK3β/c-MYC/p27^{KIP1}.

Aberrant activation of mTORC1 is a common molecular event in a variety of cancers [276, 277]. Activation of the AKT and ERK pathways acts in a synergistic manner to promote mTORC1 signaling through phosphorylation of a tuberous sclerosis complex 2 (TSC2), GTPase activator protein (GAP), leading to the disruption of the TSC1-TSC2 complex as an inhibitor of RHEB, which in turn regulates mTORC1. AKT phosphorylates residues of TSC2 distinct from those phosphorylated by ERK [278]. Furthermore, the kinase RSK, a direct downstream substrate of ERK, can also phosphorylate TSC2 to inhibit the function of TSC1/TSC2 complex [270]. The S6K1 and 4E-BP1/eIF4E pathways represent critical mediators of mTORC1-dependent cell cycle control [279, 280] by promoting the cap-dependent

translation of many target mRNAs, including those encoding cyclins and c-MYC [274, 281]. mTORC2 also contributes to cell size and cell cycle regulation *via* AKT activation and, thus, contributing to TSC2 inactivation [275].

Nevetheless, it should be noted that the induction of cell cycle arrest by hyperactivation of the ERK1/2 pathway does occur in some cell lines and is frequently observed in non-immortalized primary cells. Expression of activated forms of RAS, RAF or MEK1 was shown to elicit cell cycle arrest in primary fibroblasts, Schwann cells, hepatocytes, T lymphocytes, keratinocytes, astrocytes, and epithelial intestinal cells (reviewed in [268]). Notably, the proliferation arrest observed in primary fibroblasts, astrocytes and epithelial intestinal cells is permanent and phenotypically related to cellular senescence [268]. This phenomenon is not restricted to MAPK pathway. AKT overexpression induced senescence of primary and immortalized esophageal epithelial cells [282], primary MEFs [283], primary human aortic endothelial cells, human dermal microvascular endothelial cells, and human umbilical vein endothelial cells [284, 285]. Moreover, senescence can be triggered in human cells by overexpression of *E2F1/3*, *CDC6*, *MOS* or deletion of *PTEN* and *NF1* (reviewed in [36]).

A robust and prolonged activation of ERK1/2 causes G1 arrest due to long-term $p21^{CIP1}$ induction [286, 287] and CDK2 inhibition and also induces the expression of p53 and the CDK inhibitors $p16^{INK4A}$ and $p15^{INK4B}$ in certain cell lines [36, 268]. Indeed, ERK pathway can induce $p21^{CIP1}$ transcription [287], translation [288], mRNA stabilization [289] and block proteasome-mediated $p21^{CIP1}$ degradation [287]. Constitutive activation of AKT promotes senescence-like arrest of cell growth *via* a p53/$p21^{CIP1}$-dependent pathway and this action is at least partly mediated by the forkhead transcription factor [284].

On the other hand, hyperactivated MAPK and PI3K-AKT pathways were documented in most, if not all, tumors and elevated $p21^{CIP1}$ expression was highlighted, for example, in rectal stromal tumors [290], lung adenocarcinomas [291], bladder tumors [292], colorectal carcinomas [293], ependymomas and astrocytomas [294], hepatocellular carcinomas [295], choroidal melanoma tumors [296], and rhabdomyosarcoma cells [288]. Importantly, in these cancers $p21^{CIP1}$ expression was associated with tumor malignancy and poor prognosis but not with long-term survival as it was expected.

Thus, oncogene-induced senescence occurs in primary cells, some immortalized and in benign but not in advanced tumors. It suggests that tumor cells gain resistance to $p21^{CIP1}$-mediated scenesence and inhibition of CDK/cyclin complexes. Oncogene-induced senescence can be bypassed by inactivating pRB and p53. Accordingly, if pRB and/or p53 are inactivated in a cell before an oncogenic event, senescence should be averted what is supported by numerous *in vivo* mouse modeling studies and by genetic analysis of human tumors [36]. Moreover, *RB* deficiency sharply increases the ability of RAS to bind guanine nucleotides, resulting in its activation [297].

Importantly, $p21^{CIP1}$ (and $p27^{KIP1}$ to a lesser degree) functions as an assembly- and activity-promoting factor for cyclin D-CDK4, cyclin E/A-CDK2, and cyclin B-CDK1 complexes when $p21^{CIP1}$ level is below a certain threshold, after which the presence of excess $p21^{CIP1}$ becomes inhibitory. Stoichiometry of $p21^{CIP1}$ is critical to allow or inhibit kinase activity [104, 286]. When one $p21^{CIP1}$ molecule is binding to cyclin-CDK, the complex is catalytically active, while binding of several $p21^{CIP1}$ subunits inhibits the complex. Thus, simultaneous overexpression of CDKs/cyclins with $p21^{CIP1}$ would create more active complexes fostering cell cycle progression and resistance to antimitotic stimuli.

Interestingly, in contrast, loss or decrease of cyclin dependent kinase inhibitor $p27^{KIP1}$ is commonly seen in many human cancers as lung, breast and prostate adenocarcinomas, gastrointestinal malignancies, brain tumors, and lymphoproliferative neoplasms [298]. Level of $p27^{KIP1}$ in epithelial cancers correlates with the pathologic tumor grade: high-grade, poorly differentiated tumors showing significantly lower $p27^{KIP1}$ protein than their well-differentiated counterparts [298]. Thus, selection of tumor cells against $p27^{KIP1}$ is likely beneficial, and $p27^{KIP1}$ has less profound role in CDK-cyclin complex assembly than $p21^{CIP1}$.

Karyotype evolution, selection and tumorigenecity. Accoding to the Duesberg's evolutionary chromosomal cancer theory [299–302], «activated oncogenes induce neoplastic transformation by

inducing random aneuploidy. Aneuploidy destabilizes the karyotype by unbalancing teams of proteins that segregate, synthesize and repair chromosomes in proportion to the degree of aneuploidy. Aneuploidy initiates and maintains karyotypic evolutions automatically because of the inherent instability of aneuploidy. Occasionally, rare cancer-causing karyotypes evolve stochastically. These cancer-causing karyotypes are then stabilized against the inherent instability of aneuploidy by selection for transforming function within narrow clonal limits of variation. Flexibility and heterogeneity of cancer karyotypes is the basis for the further, spontaneous evolutions that are known as tumor progression, such as metastasis and drug resistance». Most oncogenes deregulate DNA replication, centrosome amplification and chromosome segregation and lead to formation of DNA double strand breaks and chromosome instability. Indeed, oncogene and carcinogene induced chromosome instability is a driving force of cell immortalization and tumor evolution (Stepanenko and Kavsan, in preparation). For example, activated RAS induces DNA double strand breaks in NIH3T3 fibroblasts within a single cell cycle; other oncogenes, including MYC, cyclin E, MOS, CDC25A, E2F1 and sustained delivery of growth factors have similar effects in various cell types and in animal models (reviewed in [303]). Importantly, most of experiments detecting transforming ability of genes overexpressed and/or mutated in tumors (oncogenes) were performed using mouse and human cell lines (Table 4 and 5), which represent already immortalized cells with abnormal karyotypes (poly-/aneuploids with severe chromosome rearrangements) and are prone to progress to completely transformed cells under culture conditions.

Human embryonic kidney 293 cells (also often referred to as 293 cells, HEK 293, or less precisely HEK cells) is widely used human cell line both for basic molecular studies and as a vehicle for the production of recombinant proteines and viruses [304]. Originally named simply «293 cells» («293» designates a number of experiment), they were obtained by exposing human embryonic kidney cell culture to mechanically sheared fragments of adenovirus type 5 DNA (Ad5) [78]. After transformation the cells subcultured more than 100 times could be considered as an established/immortalized line and contained 4 to 5 fragments of Ad5 genome [78]. The transforming region of the human adenovirus is within the left 11 % of the viral genome encoding E1A and E1B proteins which are necessary and sufficient for mammalian cell transformation by Ads [305]. The integration site of the adenoviral DNA was mapped to chromosome region 19q13.2 [305].

Adenovirus-induced chromosome aberrations in human cells are well documented fact [306, 307]. Bylund et al. [304] performed cytogenetic studies on the 293 cells obtained from different sources. Karyotype analysis (G-banding and spectral karyotyping) showed that 293 cells (from ECACC, Salisbury, UK) cultured for less than ten days prior to harvesting was near triploid with 62–70 chromosomes/cell and had lots of chromosomal abnormalities. No additional chromosomal changes were found between 293 cells and 293^{aged} cells (in culture 6 months, more 100 PD). Thus, 293 cells exhibit the cytogenetic stability during culturing. Another work on 293 cell karyotype (cells were obtained from ATCC, Manassas, VA, USA) also showed triploidy of these cells [308] but with only partial overlap in chromosome gains/losses comparing with cells analysed by Bylund et al. [304]. Interestingly, original 293 cells obtained by Graham et al. [78] and tested at passage 8 were near-tetraploid and retained this ploidy at passage 38. On the other hand, it was revealed that 293 cell tumorigenic potential correlated with number of passages, that is, low-passage cells (less 52 passages) could not form tumors in mice in 8 weeks, whereas tumorigenicity reached 100% when the passage had exceeded 65 ($2 \cdot 10^7$ cells per injection; 10 of 10 mice had about 0.5 cm^3 in size tumors within 2 weeks, after 4 weeks tumor was as large as 2.0 $\cdot$ 1.5 $\cdot$ 1.3 cm^3) [309]. Nevertheless, there is no correlation between long-time culturing-induced tumorigenesis of parental 293 cells and karyotype instability [304, 309].

The possible reason why prolonged cultivation drives tumorigenic potential of parental cells might be deduced from investigations with NIH3T3 murine fibroblasts, which were used for transformation assays much more frequently than any other cells (Table 5). NIH3T3 cells were obtained in 1962 as spontaneously immortalized cells during long culturing using «3T3 protocol» [160]. Rubin documented [310–315] that spontaneous transformation of NIH3T3 (also of

Table 4

Transformation of human cells

Cell type	Immortalizing agent	Transforming agent	Ref.
Astrocytes	HPV E6 + E7 + hTERT	FoxM1B	[199]
Barrett's epithelial cells	hTERT	H-RAS^{G12V} + p53 knockdown	[200]
BJ fibroblasts	Primary normal cells	E1A + H-RAS^{G12V} + MDM2	[10]
cen3tel fibroblasts	hTERT	Culture propagation/spontaneous	[24]
Colorectal crypt cells	hTERT + SV40 large T antigen	MCLR (cyclic hepatotoxin peptide)	[201]
HUVEC	Spontaneous	COX1 (cyclooxygenase1)	[202]
Embryonic esophageal epithelial cells	HPV18 E6 + E7	Culture propagation/spontaneous	[203]
FHC fetal colon cells	Primary normal cells	MET wt or MET mutated	[25]
293 cells	Ad5	FAP (fibroblast activation protein)	[204]
293 cells	Ad5	PTTG1	[205]
293 cells	Ad5	RPMS1	[206]
293 cells	Ad5	ΔHER2 (Δ exon 16)	[207]
293 cells	Ad5	CnB (calcineurin B subunit)	[208]
293 cells	Ad5	VEGF111, 121, and 165	[209]
293 cells	Ad5	CCK2R mutated	[210]
293 cells	Ad5	ESM1	[211]
293 cells	Ad5	CD74	[212]
293 cells	Ad5	HCCR1	[213]
293 cells	Ad5	hBD3 (β-defensin 3)	[214]
293 cells	Ad5	PDX1	[215]
293 cells	Ad5	ROBO1	[216]
HMEC	hTERT	SV40 LT and st, p110α, RAS^{G12V}	[217]
HMEC	Primary normal cells	WNT1	[26]
HMEC	hTERT	c-MYC transcription factor	[48]
HMEC	Primary normal cells	MYC^{T58A}	[27]
MCF-10A	Spontaneous	EphA2	[218]
MCF-12A	Spontaneous	ESE1 transcription factor	[219]
MCF-10A, 12A	Spontaneous	αB-crystallin	[220]
MCF-10A	Spontaneous	CD8-IGF-IRβ chimera	[221]
MCF-10A	Spontaneous	hGH (human growth hormone)	[222]
MCF-10A	Spontaneous	EGFR + c-SRC	[109]
MCF-10A	Spontaneous	HOXA1 transcription factor	[223]
MCF-10A	Spontaneous	HER2^{V664E}	[137]
Oral epithelial cells	E6/E7	ErbB2	[224]
Ovarian epithelial cells	SV40 LT + hTERT	H-RAS mutated or ErbB2	[225]
Prostatic epithelial cells	SV40	FGF7 (fibroblast growth factor)	[226]
SV7tert cells	SV40 LT antigen + hTERT	PDGF (platelet derived growth factor)	[227]

Indications. HMEC – human mammary epithelial cells; SV7tert cells – derived from angiomyolipoma; FoxM1B – member of the Forkhead box transcription factor; RPMS1 – ORF of Epstein-Barr virus; PTTG1 – pituitary tumor-transforming 1; MutCCK2R – cholecystokinin-2 (CCK2)/gastrin receptor intron 4 retained; ESM1 – endocan, dermatan sulfate proteoglycan; CD74 – major histocompatibility complex, class II invariant chain; HCCR1 – human cervical cancer oncogene 1; PDX1 – pancreatic and duodenal homeobox-factor 1; ROBO1 – a member of round about family of transmembrane receptors.

Balb/c 3T3 mouse fibroblasts) cell line in monolayer culture is common event especially if cells were allowed to reach high density in routine passages (transformation of a diploid line of rat liver cells is also accelerated by the constraint of confluence). It also occurs in low density passages supplemented with low concentrations of serum. If however the cells are kept continuously and rapidly multiplying at low density in high serum concentration, not only do they remain nontransformed but they gradually lose the capacity for transformation under standard conditions [310–315]. Grown at different growth conditions (confluence, concentrations of serum) karyotypes of NIH3T3 cells analyzed at different passages (24, 253 and 385 passages) showed that although the chromosome complement of each of the passages was near triploid/hypotetraploid (76 ± 2.65, 74 ± 3.2 and 72 ± 2.3, respectively, instead of the normal 40 chromosomes in mice), there were more marker (i.e., abnormal) chromosomes in passage 385 cells than in the earlier passages [312]. Moreover, every one of the karyotyped cells of each passage was unique in the precise distribution of chromosomes. These results suggest that passaging and culture conditions can influence on aneuploid karyotype of NIH3T3 cells. It worth recalling that 293 cells also retained near triploid karyotype through more 100 doublings (6 months in culture) but modal number of chromosomes ranged from 62 to 70, that is, there are cell populations inside cell line that differ from each other [304]. In fact, genotypic and phenotypic variants constantly appear in the cell line populations. In addition to the passage number and the media, the selection of variants is also modulated by the temperature, humidity, and CO_2 concentration. Some cells can occur to be

Table 5

NIH3T3 cells transformation

Transforming agent	Ref.	Transforming agent	Ref.
mAChR	[228]	KIT (stem cell factor receptor)	[248]
AKT1myr	[229]	Lin28, Lin28B	[249]
AzI (antizyme inhibitor)	[230]	Matrigel	[250]
BCR-ABL + IL3R	[231]	Midkine	[251]
BI1 (Bax inhibitor 1)	[232]	Mina53	[252]
CDC42Hs (F28L)	[233]	MUC4 (mucin)	[253]
Cyclin T1	[234]	Nanog transcription factor	[254]
EEF1A2	[235]	Ornithine decarboxylase	[255, 256]
EGFR mutant	[236]	PAR	[257]
FGF (fibroblast growth factor)	[237–239]	PDGF	[258, 259]
F-LANa	[240]	Pleiotrophin	[260]
$G\alpha_q$ Q209L, wt$G\alpha_q$, $G\alpha_o$ Q205L	[241–243]	Polyamines	[261]
G6PD	[244]	RET mutants	[262]
HCCR1	[213]	c-SRC + nuclear oncogenes	[263]
HCCRBP1	[245]	v-SRC	[264]
HPV E7 truncated	[246]	v-SRC, STAT3-C	[265]
IMUP1 and 2	[247]	14-3-3γ	[266]

Indications. mAChR – muscarinic acetylcholine receptor; $CDC42Hs^{F28L}$ – full GTPase activity but spontaneous GTP-GDP exchange; EEF1A2 – protein elongation factor 1A2; EGFR – epidermal growth factor receptor; F-LANa – a member of Derlin family; $G\alpha_o$ Q205L and $G\alpha_q$ Q209L – lack of guanosine triphosphatase (GTPase) activity; G6PD – glucose-6-phosphate dehydrogenase; HCCR1 – human cervical cancer oncogene 1; HCCRBP1 – human cervical cancer oncogene binding protein 1; IMUP1 and 2 – immortalization-up-regulated protein 1 and 2; LIN28 and LIN28B – the RNA-binding proteins that block let-7 precursors from being processed to mature miRNAs and consequently derepress let-7 target genes; Matrigel – extract of basement-membrane proteins; Midkine – a heparin-binding growth factor; Mina53 – Myc-induced nuclear antigen; PAR - prostate androgen regulated; PDGF – platelet-derived growth factor; RET – receptor tyrosine kinase for members of the glial cell line-derived neurotrophic factor family; STAT3-C – constitutively dimerizable STAT3.

more physiologically advantaged to grow during culture monolayer constrains (e.g. confluence). Subsequent selection and clonal propagation of such cells can progressively replace the rest more growth restrained cells leading eventually to full replacement with the cell population with superior growth properties [316]. Selection explains why culturing 52 passages retained 293 cells growth restrained (cells did not form tumors in mice), whereas additional only 13 passages were enough to make cells fully transformed. To the point, authors stated that 293 cells were propagated as the cells grown to a 90 % monolayer [309]. Transformation can arise by the continuous fluctuation of growth states within cells, accompanied by the progressive selection of those states best suited to function under the selecting constraint [310–315]. The selection may foster cells carrying alterations that confer the capability to proliferate and survive more effectively than their neighbors [198]. Cell-cell contact interactions can conditionally determine suppression or selection of the neoplastic phenotype [315]. It is selection that plays a major role in the spontaneous neoplastic transformation of cells in culture [310–316].

Selection and evolution of cells *in vitro* and *in vivo* is universe phenomenon. Nielsen and Briand [317] demonstrated chromosome abnormalities and karyotypic evolution in a nontumorigenic (tested in nude mice) and noninvasive (tested *in vitro*), spontaneously immortalized cell line HMT-3522, derived from a fibrocystic breast lesion. During 205 passages, gain and loss of markers, loss of normal chromosomes, and duplication of the chromosome complement could be demonstrated. The variability increased during *in vitro* growth. This variability led to cells with different growth capacities from which sidelines might be selected and become stem lines. Selection in both directions (non-tumorigenic cells to tumorigenic and *vice versa*) was also described [154]. This work is of special interest and all observations documented by authors are presented here. The karyotypic changes were associated with the spontaneous acquisition of tumorigenicity in an immortalized human bronchial epithelial cell line NL20, which had been established by transfection of human bronchial epithelial cells with the SV40 T-antigen. When cells from passage 184 were inoculated into nude mice, a transplantable tumor was obtained. Subsequent passages of the NL20 cells *in vitro* did not yield further tumors by passage 205. Furthermore, the original tumorigenic NL20T cells lost the neoplastic phenotype after 25 passages *in vitro* and reverted to the nontumorigenic karyotype observed at passage 189. In contrast to the loss of the tumorigenic phenotype and karyotype, which occurred with *in vitro* passaging of the original tumor, when the NL20T cells were passaged in other nude mice, they continued to give rise to tumors; cells from the secondary tumors (NL20T-A cells) maintained a stable karyotype and remained tumorigenic even after 64 passages *in vitro*. A mixture of 10 % tumorigenic NL20T-A and 90 % nontumorigenic NL20 cells formed tumors in nude mice when cultured *in vitro* on fibronectin, but not on plastic; cytogenetic analysis demonstrated that the tumors and cell cultures were composed of tumorigenic NL20T-A cells, whereas cells cultured on plastic were identical to the nontumorigenic NL20 cells. Thus, neoplastic transformation in original cell line arose from *in vivo* selection of a small mutant clone, which had arisen in culture and was subsequently selected *in vivo* but was lost in *in vitro* culture [154]. The degree of karyotype heterogeneity determines selection rate and correlates with tumor latent period [318]. The karyotypes of tumors formed by spontaneously transformed Chinese hamster cells of high tumorigenic potential after a short latent period were similar to each other and to the injected cells. The karyotypes of tumors from cells of low tumorigenic potential and long latent periods were diverse, however. No chromosome aberration was common to every tumor. These results suggested that preneoplastic cells whose phenotypes were not directly capable of tumor formation could progress *in vivo* and that karyotype instability played an important role in providing cell variants for tumor progression [318].

NIH3T3 cells were fully transformed (showed both anchorage independent growth in soft agar and tumor formation in mice) by a number of transforming agents depicted in Table 5. Actually, as Rubin emphasized «the effectiveness of the NIH3T3 cell line as a target for demonstrating the transforming capacity of oncogenes depends on its partially transformed state, which needs only a nudge from an added oncogene to

progress to more advanced transformation» [315, 319]. Unfortunately, only several works traced karyotype changes accompanied by oncogene transformation. For example, karyotypic analysis of parent NIH3T3 cells and NIH3T3 containing an activated N-RAS oncogene showed that, although the modal chromosome number was comparable for both cell types, number of unstable chromosomes and forms of abnormalities were different [320]. Another work demonstrated that parental NIH3T3 cells contained 71 chromosomes (hypotetraploid), whereas EJ-NIH3T3 (NIH3T3 cell line carrying the transfected human activated H-RAS sequence of EJ human bladder carcinoma cells) contained 60 chromosomes (triploid) [321]. Moreover, when the latter cells were treated with mutagenes (ethyl methanesulfonate and 8-azaguanine) and mutant clones were selected, they were resistant to retransformation by Kirsten sarcoma virus, DNA from EJ-NIH 3T3 cells, H-RAS, v-SRC, v-MOS, simian virus 40 large T-antigen, or polyomavirus middle T antigen [321]. Karyotype analysis showed that resistant clones had hyperpentaploid karyotypes (103 ± 9.7 chromosomes) [321]. Transfection of vector containing the mitochondrial D-loop gene from colorectal cancer cell line SW480 into NIH3T3 cells resulted in that NIH3T3 cells had significantly greater percentage of multiploid and aberrant chromosomes than control NIH3T3, and this correlated with ability to form colonies in soft agar [322]. Also, other group of investigations suggests that for stable transformation profound changes in genome must occur [232, 238, 246, 323].

MCF10A cell line is spontaneously immortalized human mammary epithelial cells with near-diploid karyotype harboring a number of chromosome abnormalities. Besides being frequently used in *in vitro* transformation assays (Table 4), MCF10A cell line was used for comprehensive analysis of the MCF10A series of cell lines representing progression towards obvious malignancy [165, 324]. The MCF10A progression model consists of three directly derived cell lines: the spontaneously immortalized MCF10A cells (do not show any characteristics of invasiveness or tumor formation), MCF10AT1 cells (MCF10A cells transformed by *H-RAS*), and MCF10CA1a cells (obtained from tumor in immunodeficient mice after xenograft transplantation of MCF10AT1 cells) [165, 324]. 47 chromosomes were found in MCF10A (gained additional chromosome 8) and the MCF10AT1 cell lines (additional chromosome 8 was deleted, but chromosome 9 was gained), whereas the malignant MCF10CA1a cell line had 50 chromosomes [165]. Four marker chromosomes were identified in MCF10A and MCF10AT1 and nine in the malignant MCF-10CA1a cell line [165]. Spectral karyotyping analysis showed that the premalignant MCF10AT1 gained additional translocations to the MCF10A, whereas the malignant MCF10CA1a had more translocations extra to both MCF10A and MCF10AT1 [165]. Array comparative genome hybridization (aCGH) showed that MCF10A had a number of gains and losses of different chromosome regions and progression towards full malignancy was accompanied by much more severe genomic aberrations. Importantly, regions of genomic loss/gain overlapped only partially among these three cell lines [165]. Another investigation exploiting the same model (MCF10A series of cell lines) confirmed the stepwise genome changes accompanying progression to full malignancy [324]. Moreover, combining SNP array with Gene Array authors showed correlation between DNA copy number gains and increased expression levels for genes located in these regions [324].

Analysis of tumorigenic potential of established seven VERO cell line strains (African green monkey kidney cells, the normal chromosome number is 60), of which 1 strain was hypotetraploid and the rest strains were hypodiploid, and 3 strains of HeLa cell line (all strains were hyperdiploid) showed that the cell strains were comparatively stable in terms of their heritable characters [325]. There were only little significant changes between passages but the tumorigenicity of strains was different among different karyotypic cells (from 7 VERO strains 2 appeared tumorigenic: 73 ± 3, and 68 ± 3 or 65 ± 4, hyperdiploids and 5 were nontumorigenic: 54 ± 2, 55 ± 2, 54 ± 2, 54 ± 2, 54 ± 2, all hypodiploids), all HeLa strains were hyperdiploid and tumorigenic [325]. The chromosome number variation of strains had positive relationships with their carcinogenesis and the chromosome number variation of cell line could be significantly changed when it developed to tumor in nude mice [325]. These observations were confirmed in experiments

with other cell cultures. Meningioma cells with multiple chromosomal abnormalities grew rapidly *in vitro* and induced tumors in 49 of 50 animals, whereas with simple karyotypes (less or 1 chromosomal abnormality) grew slowly *in vitro* and gave small, nongrowing tumors in mice [326]. Also, the average number of chromosomes in 293N cells (a subline of just obtained 293 cells in 1977 year derived from a tumor developed in a nude mouse) was significantly lower than for the parental line [78].

Thus, progression of immortalized or pre-neoplastic cells towards obvious malignancy is always accompanied by karyotype changes. The degree and diversity of karyotype changes determine tumorigenic potential of cell culture and latency period of tumor formation necessary for creation and/or selection of the most competitive malignant cells.

Gene copy number, mRNA and protein levels relationships in tumor cells. Actually, primary tumor cells and cancer cell lines are always poliploid/aneuploid, and have karyotypes ranging from 40 to 60 but occasionally exceeding 70 or more chromosomes [327]. Moreover, numerical large-scale and focal chromosome aberrations (losses/ gains/deletions/ translocations) were found in all samples of each type and subtype of tumors analyzed up to now (Table 6). Roschke et al. [328] using spectral karyotyping provided a description of the chromosomal complement of the NCI-60 cell line panel developed by the National Cancer Institute (NCI) for *in vitro* anticancer drug screening and reflecting diverse cell lineages (lung, renal, colorectal, ovarian, breast, prostate, central nervous system, melanoma, and hematological malignancies). 23 cell lines were identified as near-diploid (a chromosome modal number between 35 and 57), 22 as near-triploid (the chromosome modal number between 58 and 80), 13 as near-tetraploid (a chromosome modal number between 81 and 103), and 1 as near-pentaploid (chromosome modal number between 104 and 126) on the basis of the International System for Chromosome Nomenclature. The range of numerical changes (clonal chromosome gains and losses) ranged from 1 to 28. Number of structurally rearranged chromosomes (contained translocations, deletions, duplications, insertions, inversions, or homogeneously staining regions) ranged from 1 to 45 (38 cell lines had 10 and more structurally rearranged chromosomes). In addition, in 24 of the 59 cell lines ploidy heterogeneity was found (i.e., if the majority of cells had a near-triploid karyotype there might be an additional small populations of cells with a near-pentaploid or near-hexaploid count, or, in few cases, with a near-diploid count). Chromosome numerical and structural heterogeneity between cells in the same cell line was also documented [328].

Later, this NCI-60 cell line panel was used to elucidate correlation between gene copy number and mRNA levels for the same gene. The data showed a generally positive correlation between a given gene's copy number and its expression at the mRNA level supporting the generalization that DNA copy number is one factor (among others) that can influence gene expression [329]. In another work authors performed a global analysis of both mRNA and protein levels based on sequence-based transcriptome analysis (RNA-seq) and SILAC-based mass spectrometry analysis [330]. The study was performed in three functionally different human cell lines (the glioblastoma cell line U251MG, the epidermoid carcinoma cell line A431 and the osteosarcoma cell line U2OS). The changes of mRNA and protein levels in the cell lines using SILAC and RNA ratios showed high correlations, even though the genome-wide dynamic range was substantially higher for the proteins as compared with the transcripts [330]. Also, there was a moderate but significant correlation between global mRNA (RNA-seq) and protein levels (SILAC) of 1710 genes affected by amplification or deletion (SNP and CGH arrays) in seven human metastatic melanoma cell lines [331]. Whether does it mean that the end point of gene expression, the level of protein, is affected proportionally to copy number of gene in tumor cells? Measurement of expression levels of 6735 proteins was directly compared to the gene copy number in MCF7 breast cancer cell line [332]. Authors found that in the majority of cases, there was no direct correspondence between the gene copy number change and the corresponding protein change. Nevertheless, proteins encoded by amplified oncogenes were often overexpressed, while adjacent amplified genes, which presumably did not promote growth and survival, were attenuated [332]. Furthermore, authors revealed that the proteins of such complexes as the proteasome, ribosome, spliceosome, and NADH dehydrogenase always maintained equal protein ratios, despite varia-

tion in the gene copy number of their subunits. This was strictly true for the core complexes components, but to a lesser degree for peripheral proteins, which could also be involved in other processes [332]. Interestingly, levels of protein expression in aneuploid yeast strains largely scale with chromosome copy numbers, following the same trend as that observed for the transcriptome [333]. Thus, eventually to be definitely concluded, relationships between gene copy number, mRNA level and protein level of individual genes across the whole cancer genome should be analyzed. It would give the comprehensive understanding to which degree regulation of gene expression on different levels operates in tumor cells and which groups of genes are predominantly imposed on such regulation.

Inter- and intratumor heterogeneity. It is supposed that common (clonal) chromosome changes are the «drivers» of neoplastic transformation whereas rare chromosome changes (non-clonal) are likely the «passengers» in this process, which may be either nonfunctional or functional but constitute secondary events [3, 374, 375]. Nevertheless, non-clonal aberrations reflect the significant tumor feature: genome/chromosome instability and, as a consequence, inter- and intratumor genome heterogeneity [376]. Importantly, the main determinant of the ability of a population to evolve is the extent of heritable variation within the population [377]. Numerous studies have proved that intra-tumor genetic heterogeneity/clonal diversity is a key force driving transformation and tumor evolution (Stepanenko and Kavsan, in preparation).

Nobusawa et al. [374] have analysed by aCGH separate tumor areas of 14 primary glioblastomas (total, 41 tumor areas). They revealed

Table 6

Tumors and cell lines with multiple chromosomal abnormalities

Cancer type	Method	Number of samples	Ref.
Acute myeloid leukemia	aCGH	17	[334]
Bladder cancer	aCGH	22	[335]
Bladder carcinomas	aCGH	109	[336, 337]
Breast cancer	aCGH, SNP array, sequencing,	1143	[338–342]
Cervical carcinomas	aCGH, SNP array	40	[343, 344]
Colorectal carcinomas and adenomas	aCGH, SNP array	129	[345–348]
Endometrial carcinomas and carcinosarcomas	aCGH and karyotyping	82	[349]
Ewing's cancer	aCGH, spectral karyotyping	7	[350]
Gastric cancer	aCGH	31	[351]
Germ cell cancer	aCGH	24	[352]
Glioma	aCGH, SNP array, karyotyping	248	[353–359]
Head and neck squamous cell carcinomas	aCGH	43	[360, 361]
Lung cancer	Sequencing, SNP array, karyotyping	80	[362, 363]
Myelodysplastic syndromes and related myeloid malignancies	SNP array, karyotyping	430	[364]
Oral carcinomas	aCGH	60	[365]
Oropharynx and hypopharynx squamous cell carcinomas	aCGH	20	[366]
Ovarian epithelial tumors	aCGH	47	[367]
Pancreatic carcinomas	aCGH, sequencing	37	[368, 369]
Prostate tumours	Sequencing	7	[370]
Thyroid carcinomas and adenomas	aCGH	28	[371]
29 different tissues	aCGH	598	[372]
26 different tissues	SNP array	3131	[373]

Indications. aCGH – array comparative genome hybridization; SNP array – single nucleotide polymorphism array; Sequencing – massively parallel paired-end sequencing; MLDPA – multiple ligation-dependent probe amplification.

that chromosomal imbalances significantly differed among glioblastomas. In addition, there were numerous tumor area-specific genomic imbalances. Analysis of disseminated single cells in minimal residual disease has shown that there is a high level of genomic heterogeneity within individual lesions as well as between primary tumors and metastatic cells [376]. Giving comments on reports of breast cancer genomes analyses with high-throughput genomics thechnics [339, 342], Swanton et al. [378] concluded that results from these studies had revealed «perplexing breast cancer genome complexity with very few aberrations occurring in common between breast cancers. In addition, such complexity is compounded by evidence of profound genomic heterogeneity within individual breast tumors (intratumoral heterogeneity), where multiple tumor subpopulations have been identified, each with distinct genomic profiles heterogeneity occurring within individual breast cancers». Moreover, recurrent tumors always show appearance of new chromosome imbalances and gene mutations distinct from those, which were observed in most cells of a primary tumor but could be harbored by a small group of cells within a primary tumor or acquired *de novo* [371, 377, 380–389].

Intratumor genomic heterogeneity is created and fostered by chromosome instability (CIN. Although defects in chromosome cohesion, kinetochore-microtubule misattachments, assembly of multipolar mitotic spindles [182, 390–396], translocations containing breakpoints within fragile sites [397], satellite repeats in heterochromatin [398], cell-in-cell formation by entosis (as a result, cytokinesis frequently fails, generating binucleate cells that produce aneuploid cell lineages) [399], random fragmentation of the entire chromosome (chromothripsis) in which chromosomes are broken into many pieces and then randomly stitched back together [400, 401] can contribute to CIN, in cancer cells mechanism of centrosome amplification and clustering is proposed to be the major contributor to CIN. Importantly, there is compelling evidence that diverse oncogenes or carcinogenes induce centrosome deregulation and CIN (Stepanenko and Kavsan, in preparation).

Inter- and intratumor heterogeneity of gene mutations was also revealed by sequencing of all protein coding genes in several solid tumors, including glioblastomas, colorectal, pancreatic and breast cancers. It was found that individual solid organ tumors harbor approximately 40–80 clonal mutations per tumor in the coding regions of different genes, and although a few of these genes are mutated in a high proportion of tumors, the prevalence at which the majority are mutated among different tumors of the same cancer type is low [376, 377, 402]. 2576 somatic mutations were identified across 1800 megabases of DNA representing 1507 coding genes from 441 tumors comprising breast, lung, ovarian and prostate cancer types and subtypes [403]. Authors found that mutation rates and the sets of mutated genes varied substantially across tumor types and subtypes. Results of sequencing COLO-829 cancer cell line derived, before treatment, from a metastasis of a malignant melanoma demonstrated a total of 292 somatic base substitutions in protein-coding sequences [404]. As Fox et al. stated «each tumor displays a unique and diverse profile of mutated genes, but no new prevalently mutated genes are identified... Within an individual neoplasm, a few mutations are present throughout the population, a greater number are present in minority subclones, and the majority is found in only one or a few cells» [376]. Actually, if to calculate all abnormalities of noncoding genome regions, there are usually between 1000 and 10000 somatic substitutions in the genomes of most adult tumors, including breast, ovary, colorectal, pancreas cancers, and glioma [3]. All these data imply that tumors are really «oncogene addicted», but it has *not* revealed a possible «Achilles' heel» within the cancer cell that can be exploited therapeutically [3, 405], because «instead of long-anticipated common mutations, a large number of stochastic gene mutations were detected for each individual with the same cancer type» [406].

Chromosome instability and drug resistance. Cancer cells rapidly acquire resistance against numerous cytotoxic drugs or are even intrinsically resistant [379]. The chromosomes of cancer cells are extremely unstable compared to those of normal cells: 1 in 100 highly aneuploid human cancer cells loses or gains or rearranges a chromosome per cell generation [407]. Genetic variation within a cancer cell population reflects dynamics of clonal evolution and, importantly, serves as a reservoir of genetic diversity from which therapy-resistant

clones may arise [376]. *In vitro* studies have confirmed that CIN cells acquire multidrug resistance at an accelerated rate compared with diploid cells resulting from the selection pressure influenced by drug exposure. Moreover, mouse CIN cells became multidrug resistant even after deletion of all known multidrug resistance genes [319, 379]. To identify distinct therapeutic approaches to specifically limit the growth of CIN tumors, Lee et al. [408] focused on a panel of colorectal cancer cell lines, previously classified as either chromosomally unstable, CIN (+), or diploid/near-diploid, CIN (−), and treated them individually with a library of kinase inhibitors targeting components of signal transduction, cell cycle, and transmembrane receptor signaling pathways. CIN (+) cell lines displayed significant intrinsic multidrug resistance compared with CIN (−) cancer cell lines, and this seemed to be independent of somatic mutation status and proliferation rate [408].

According to Duesberg et al., karyotype plays the central role in drug resistance [379]. «When cancer cells acquire resistance against drugs, they acquire new karyotypic alterations and/or they lose old ones». Indeed, gene expression profiles of drug resistant cells differ from those of parental drug sensitive cells in the over- or underexpression of hundreds to thousands genes [379]. Comparison of the structures of the puromycin resistance-specific chromosomal alterations in four different human colon cancer lines indicates, that most but not all of resistance-specific chromosomal alterations were unique for each cancer cell [379]. Drug resistance correlates with chromosomal alterations [319, 407]. It is generated *de novo* in cancer cells by chromosome re-assortments [379]. The resulting level of resistance is proportional to the numbers of resistance-specific chromosomal alterations or «tumor heterogeneity» [379]. In the presence of cytotoxic drugs resistance-specific alterations are selected from the resultining variants by classical Darwinian mechanisms [379]. Chromosome instability, intertumoral and intratumoral heterogeneity present a challenge to personalized therapeutic approaches [378].

Conclusion. The intense searching for the abnormal genes influencing the development of human cancer revealed about 200000 somatic mutations in cancer genomes (COSMIC database) since the first somatic mutation that was found in *H-RAS* the quarter of a century ago [198]. Hundreds of genes are being considered and dozens genes/proteins have been used already as potential drug targets in clinical trials. However, at present benefits from oncogene directed therapy are still moderate. Large-scale tumor genome sequencing have failed to reveal «universal» cancer genes and, instead, «large numbers of diverse mutations have been identified dominating the cancer genome landscape» [406]. «A future of multiple targeted therapies and patient stratification, based on a mutational signature of defined key genes for each cancer type, seems less hopeful than initially anticipated» [376].

Now it is clear that «cancer progression is a stochastic process both at the genome and gene level, and is not a stepwise process defined by sequential genetic aberrations. Stochastic process frequently occurs prior to the key stages of immortalization, transformation and metastasis and results in inability to detect type- and stage-specific recurrent aberrations in solid tumors» [406]**.** Numerous somatic rearrangements, including whole chromosome and copy number gains and losses, chromosome translocations, and gene mutations participate in establishing the malignant cell phenotype. «Multiple rounds of proliferation, often counter-balanced by cell death, are required to produce macroscopic tumors, and genomic instability, observed in most cancers, is expected to constantly produce new mutations, which serve as raw material on which tumor evolution can work» [409]. The selection may weed out cells that have acquired deleterious mutations or it may foster cells carrying alterations that confer the capability to proliferate and survive more effectively than their neighbors [198]. A single cell can acquire a set of sufficiently advantageous mutations that allows it to proliferate autonomously, invade tissues and metastasize [198]**.**

CIN is the most common genetic abnormality of cancer cells and tumorigenic cell lines. The frequent losses and gains of whole chromosomes during cell divisions in CIN cancer cells trigger rapid alterations in gene dosage [379]. *In vitro* studies have confirmed that CIN cells acquire multi-drug resistance at an accelerated rate compared with diploid cells [379]. Furthermore, although most cancers are of monoclonal origin, the expansion of

the population size, which occurs after malignant transformation, coupled with the constant acquisition of mutations promotes the diversion into subclones and a dramatic increase in genetic tumor heterogeneity [377]. High genetic heterogeneity of tumors means high probability of pre-existent clones that are resistant to therapeutic intervention and can be selected by therapy resulting in therapy failure [409]. Severe genome rearrangements and intratumoral heterogeneity challenge oncogene directed therapy, while chromosome instability and karyotype evolution make each tumor «a moving rather than frozen» target.

Thus, a main driver of evolutionary adaptation during drug treatment is the genetic heterogeneity, which is fostered by CIN. New tools are necessary to study heterogeneity and to analyze changes in heterogeneity and clonal composition during drug treatment [410–412]. This would allow new insights into these processes and provide the basis to improve therapeutic outcomes based on tumor evolution and the specific targeting of distinct genomic instability mechanisms [377, 413].

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А.А. Степаненко, В.М. Кавсан

ИММОРТАЛИЗАЦИЯ И ЗЛОКАЧЕСТВЕННАЯ ТРАНСФОРМАЦИЯ ЭУКАРИОТИЧЕСКИХ КЛЕТОК

Чтобы стать полностью трансформированной опухолевой клеткой, нормальная клетка должна преодолеть ряд внутренних клеточных барьеров и приобрести большое число хромосомных изменений. Первым и необходимым шагом в злокачественной трансформации является преодоление старения, или иммортализация клетки. Иммортализированные клетки могут бесконечно долго пролиферировать в присутствии ростовых факторов и питательных веществ. Иммортализированные клетки никогда не имеют нормального диплоидного кариотипа, хотя во время роста подвергаются контактному ингибированию, не формируют колоний в мягком агаре (т.е. зависимый от подложки рост) и не формируют опухолей при введении иммунодефицитным мышам. Все эти свойства могут быть приобретены с дополнительными хромосомными изменениями. Множественные генетические изменения, включая приобретение/потерю целых хромосом или отдельных участков/локусов, транслокацию хромосом и генные мутации, необходимы для установления трансформированного фенотипа. Процесс клеточной трансформации достаточно хорошо изучен наклеточных культурах *in vitro*. Большинство экспериментов, выявивших трансформирующую способность генов (онкогенов), надэкспрессироанных и/или мутированных в опухолях, было выполнено с использованием таких клеточных культур, как мышиные эмбриональные фибробласты (MEFs), мышиная клеточная линия фибробластов NIH3T3, клеточная линия человеческой эмбриональной почки 293 (293 клетки) и эпителиальные клеточные линии молочной железы человека (главным образом, HMECs и MCF10A), которые представляют собой иммортализированные клетки (кроме первичных мышиных фибробластов) с измененными геномами (поли-/анеуплоиды со значительными хромосомными перестройками) и склонные к полной злокачественной трансформации при культивирования. Недавно обновленный список онкогенов включает более 467 генов, которые, как полагают, вовлечены в развитие опухоли, когда соответственным образом изменены (точковые мутации, делеции, транслокации или амплификации). Однако исследования на мышах свидетельствуют, что более 3000 генов могут вносить вклад в развитие опухоли. Целью настоящего обзора является понять механизмы клеточной иммортализации различными «иммортализующими агентами», онкоген-индуцируемой клеточной трансформации иммортализированных клеток и умеренный ответ на терапию из-за «склонности» опухоли к приобретению многочисленных генных и хромосомных изменений, внутри- и межопухолевой гетерогенности.

О.А. Степаненко, В.М. Кавсан

ІМОРТАЛІЗАЦІЯ ТА ЗЛОЯКІСНА ТРАНСФОРМАЦІЯ ЕУКАРІОТИЧНИХ КЛІТИН

Щоб стати повністю трансформованою пухлинною клітиною, нормальна клітина повинна подолати низку внутрішніх клітинних бар'єрів і придбати велику кількість хромосомних змін. Першим необхідним кроком у злоякісній трансформації є подолання старіння, або імортализація клітини. Імортализовані клітини можуть нескінченно довго проліферувати в присутності ростових факторів і поживних речовин. Імортализовані клітини майже ніколи не мають нормального диплоїдного каріотипу, тим не менш вони під час росту піддаються контактному інгібуванню, не формують колоній в м'якому агарі (тобто залежне від підкладки зрос-

тання) і не формують пухлин при введенні імунодефіцитним мишам. Всі ці властивості стабільно можуть бути придбані з додатковими хромосомними змінами. Множинні генетичні зміни, включаючи набуття або втрату цілих хромосом або окремих ділянок/локусів, транслокація хромосом і генні мутації, є необхідними для встановлення трансформованого фенотипу. Процес клітинної трансформації досить добре вивчений на клітинних культурах *in vitro*. Більшість експериментів з виявлення трансформуючої здатності генів (онкогенів), надекспресованих та/або мутованих в пухлинах, було виконано з використанням таких клітинних культур, як мишачі ембріональні фібробласти (MEFs), клітинна лінія мишачих фібробластів NIH3T3, клітинна лінія людської ембріональної нирки 293 (293 клітини) і епітеліальні клітинні лінії молочної залози людини (головним чином, HMECs і MCF10A), які представляють собою іморталізовані клітини (крім первинних мишачих фібробластів) зі змінними каріотипами (полі-/анеуплоїди зі значними хромосомними перебудовами) і схильні до повної злоякісної трансформації при культивуванні. Нещодавно оновлений список онкогенів включає понад 467 генів, що залучені, як вважають, до розвитку пухлини, коли відповідним чином змінені (точкові мутації, делеції, транслокації або ампліфікації). Однак дослідження на мишах свідчать проте, що понад 3000 генів можуть робити внесок у розвиток пухлини. Мета даного огляду зрозуміти механізми клітинної іморталізації різними «іморталізуючими агентами», онкоген-індукованої клітинної трансформації іморталізованих клітин і помірну відповідь на терапію через «схильність» пухлини до придбання численних генних та хромосомних змін та гетерогенністю усередині і між пухлинами.

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