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Review Article

Genetic instability of modified stem cells – a first step towards malignant transformation?

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Abstract: Induced pluripotent stem cells (iPSC) are important tools in regenerative medicine. Yet, it is becoming increasingly clear that the reprogramming process, including retroviral transduction with potent oncogenes like c-Myc and long-term cultivation, may induce genetic instability. Genetically altered iPS cells can grow out and dominate the cell culture. This review intends to comprehensively summarize the current knowledge on genetic instability of embryonic and iPSCs, with an emphasis on cytogenetic alterations, and compares these data with what is known from tumorigenesis.

Keywords: Induced pluripotent stem cells, gene therapy, chromosomal instability, genetic integrity, tumor, leukemia, clonal evolution, chromosome, mutation, P53

Introduction

Induced pluripotent stem cells (iPSC) provide important tools to understand basic functions of stemness and reprogramming, to perform pharmacologic testing, to develop disease models and to perform gene correction before transfer into the patient [1, 2]. They also have great potential for regenerative medicine, providing cellular sources to replace defective cells, particularly in age-related diseases. However, a major concern in applying iPS cells in the clinic is whether they maintain their genetic integrity. There is increasing evidence that iPS cells may acquire genetic and epigenetic alterations during reprogramming and cultivation [3, 4]. Retroviral gene transfer to express reprogramming factors may itself result in insertional mutagenesis by activating cellular oncogenes. It may be associated with an increased genetic instability and finally lead to clonal and even malignant outgrowth [5].

Stem cells give their genetic information to millions of daughter cells. Therefore, it is of utmost importance that they protect this genetic information from deleterious damage. The genetic information is coded in about 20.000-25.000 genes that are systematically ordered in our 46 chromosomes and regulated by an epigenetic layer of information, e.g. by DNA methylation and chromatin modifications. Although epigenetic modifications control reprogramming and stemness, they are not further discussed in this review. Somatic mutations are estimated to occur at a rate of ~3.4×10^{-7} per cell division in normal humans [6]. Yet we have developed efficient repair systems and immune surveillance to eliminate cells carrying gene mutations or chromosome defects. Stem cells in particular have developed efficient mechanisms to maintain their genomic integrity (For review please refer to [7, 8]).

How to define genetic instability?

In principle, there are three different modes of genetic instability: acquisition of mutations, mismatch repair deficiency and chromosomal instability. Mismatch repair deficiency is caused by mutations in DNA repair genes like *MLH1* or *MSH2* that are responsible for resolving replicate errors within microsatellites. In the case of mismatch repair deficiency, short repetitive sequences may show an increased frequency of length mutations, especially in highly proliferating tissues. In contrast, chromosomal instability leads to gross alterations of chromosome

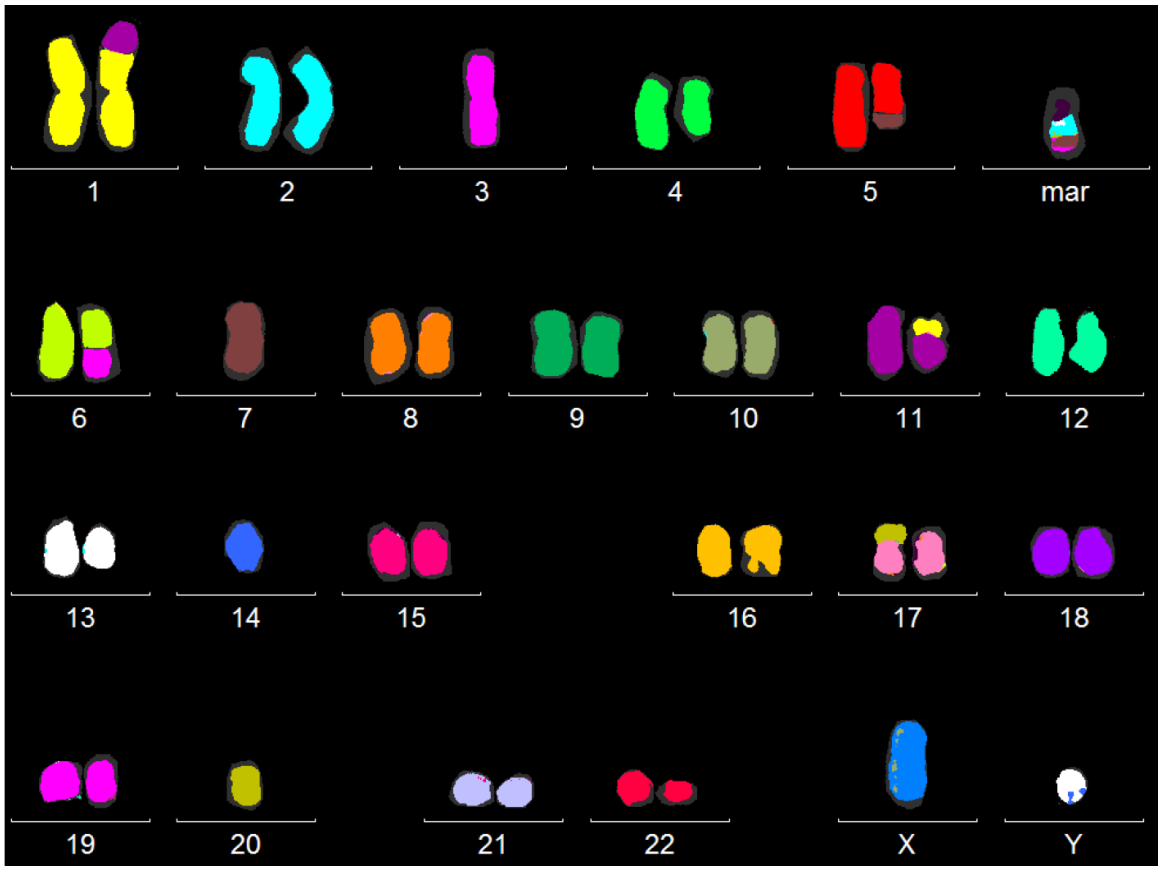

Figure 1. mFISH karyogram of a patient with myelodysplastic syndrome and a complex karyotype: by simultaneous hybridization of six fluorochromes onto metaphases, each chromosome is marked with a specific mixture of fluorochromes. A specialized software module (MetaSystems GmbH, Altlussheim, Germany) depicts the chromosomes in so-called pseudo-colors as shown here. In addition to classical banding analysis, this is a useful method to identify unknown chromosomal material or cryptic aberrations.

number or structure. Chromosomal instability refers to the cell-to-cell variability, i.e. chromosome aberrations that differ from cell to cell, that may ultimately lead to the outgrowth of chromosomally aberrant clones [9]. Frequently, chromosomal instability is used synonymously with the presence of chromosome aberrations, but this is the consequence rather than chromosomal instability *per se*.

Ways to decipher genetic instability

Karyotyping and fluorescence in situ hybridization (FISH)

Microscopic analyses of chromosome number and structure within single cells requires the preparation of metaphases and the application of chromosome banding techniques to construct a karyotype. This is still the gold standard to obtain an overview of the genetic integrity of reprogrammed cells. However, the resolution of this method is only about 5 to 10 Mb. Higher resolution of defined genetic regions can be achieved by FISH using fluorochrome-labeled DNA probes complementary to the regions of interest. After binding to the complementary regions, the probes produce distinct fluorescence signals within the metaphase or interphase cell that allow investigation of the number, structure and localization of the respective genetic regions with high resolution. However, only defined regions, complementary to the respective probes, can be investigated. In multicolorFISH (mFISH), a simultaneous hybridization of six fluorochromes onto metaphases, each chromosome is marked with a specific mixture of fluorochromes. With specialized software modules, each chromosome appears in a different so-called pseudo-color (**Figure 1**). This method is very helpful in clarifying the ori-

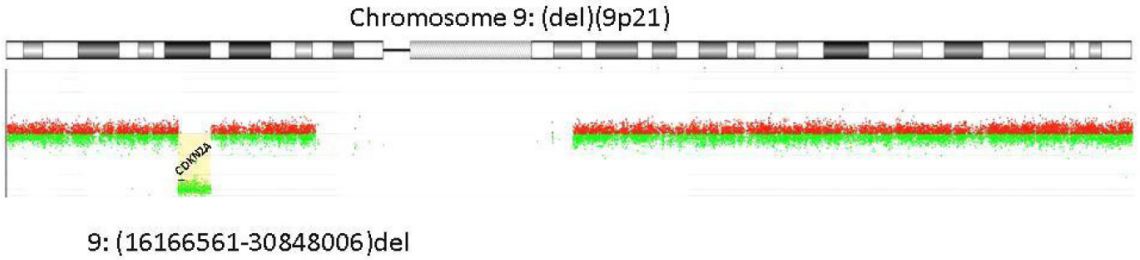

Figure 2. Array-CGH Result: Genomic profile of chromosome 9 as determined by Human Genome 400k Microarray (Agilent Technologies, Santa Clara, CA, USA). Below the ideogram of chromosome 9, its profile resulting from Genomic Workbench (Agilent) is given: log2 ratios of array probes are plotted against the chromosomal localization indicating a homozygous deletion of around 14.7 Mb affecting the region from 16.166561-30.848006 Mb.

gin of complex chromosomal rearrangements or marker chromosomes [10]. For example, it is useful for the identification of translocations that might not be detectable by classical banding analysis due to a similar banding pattern of the aberrant chromosomes. However, only inter-, not intrachromosomal rearrangements can be detected. A disadvantage of this method is the disability to detect deletions or inversions.

High-resolution array comparative genomic hybridization (aCGH)

High-resolution array comparative genomic hybridization (aCGH) or single nucleotide polymorphism (SNP) arrays are the appropriate techniques to detect small gains or losses up to single exons. The methods are based on the comparison of test genomes (e.g. cultured iPSC or initial fibroblast cells) to a reference genome. Fluorescently labeled probes are hybridized to oligonucleotide probes immobilized to a microarray (up to several million per array) slides. Probe design is very flexible in array-CGH, facilitating dense coverage of nonpolymorphic unique sequence probes of genomic regions, whereas SNP arrays depend on the genomic location of SNPs. Copy-number differences are calculated from the fluorescence intensities of the fluorochromes. Significant deviation from unity in the ratios of the fluorescence intensity values is indicative of a deletion or gain in the test compared to the reference DNA (**Figure 2**).

Nowadays, next generation sequencing is introduced to detect mutations in small cell populations with a very high sensitivity, given that coverage is at least 1000x. Meanwhile, whole-genome sequencing has identified more than one thousand heterozygous single-nucleotide variants (SNVs) in human iPS cell lines [11].

Genetic instability in reprogrammed cells

Recently, several reports brought attention to the presence of genetic alterations in embryonic stem (ES) and iPS cells [12-14]. Human iPS cell lines from different laboratories, independent of the reprogramming methods used, were found to contain an average of five protein-coding mutations. Some mutations preexisted in small subclones, the others occurred during the reprogramming process [12]. Significantly more copy number variants (CNV), i.e. gains or losses of genomic regions, are present in early-passage human iPS cells than intermediate-passage human iPS cells, fibroblasts or human ES cells [13]. Most CNVs are formed *de novo* and render the affected cells at a selective disadvantage. Therefore, genetically aberrant cells are rapidly counter-selected. Thus, it seems possible to generate iPS cell lines without copy-number changes, if appropriate conditions for reprogramming and non-integrating vectors are used [11]. However, copy-number variations frequently affect tumor suppressor genes and oncogenes [13, 15] and include homogeneously staining regions (HSR), cytogenetically detectable gene amplifications that are considered a hallmark of cancer cells [16]. Actually, many genes of ES cells deregulated due to copy-number variations are functionally linked to cancer [17].

Investigating different sources of mouse (induced) pluripotent stem cells, four hotspots of chromosomal aberrations were detected: full trisomy 11 (with a minimally recurrent gain in 11qE2), full trisomy 8, and deletions in chromo-

Table 1. Genomic alterations of embryonic stem (ES) cells

Cell type	Conditions	No of aberrant / analysed clones	Karyotype	Literature
mES cell lines	Culture in medium containing leukemia inhibitory factor	22 of 29 clones from 9 cell lines	Trisomy 8 in 21 clones; Trisomy 15 in 2 clones T(Y;12)(Tel;C1), Inv(3)	Liu X et al, Dev Dyn (1997) [19]
mES cell lines	Cell lines from different institutions	35 of 88 cell lines	Trisomy 8, loss of sex chromosome, trisomy 11	Sugawara A et al, Comp Med (2006) [56]
mES cell lines	Various conditions	49 of 129 cell lines with gene expression profiles obtained from the GEO database*	Trisomy 8, trisomy 11, deletion 10qB and 14C-14E	Ben-David U & Benvenisty N, Stem Cells (2012) [18]
hES cell lines	Prolonged culture	13 of 13 cell lines 18 of 18 cell lines	Gains of chromosomes 12, 17 and X; i(12)(p10); Smallest region of amplification: 17q11-q12, 17q25-ter; other aberrations	Baker DE et al, Nat Biotechnol (2007) [16] and review therein
hES cell lines (HS181, SHEF-3, SHEF-1)	Transfer to a feeder-free culture system	2 of 3 cell lines	47,XY,+14 47,XX,+12/48,idem,+der(20)	Catalina P et al, Mol Cancer (2008) [22]
hES cell lines (HUES1, HUES3, HUES4)	Prolonged culture	2 of 17 cell lines	Trisomy 12 der(2)	Cowan C et al, N Engl J Med (2004) [57]
hESC	Long-term culture	4 of 5 cell lines analysed by FISH and aCGH	Amplification of 20q11.21	Lefort N et al, Nat Biotechnol (2008) [25]
hES cell lines HS-181, HS293	Long-term culture on human feeder-layers	0 of 2 cell lines	Genetically stable	Catalina P et al, Leuk Res (2009) [58]
hESC	Cell lines from different institutions	12 of 38 cell lines with gene expression profiles obtained from the GEO database*	Trisomy 12 and 17	Mayshar Y et al, Cell Stem Cell (2010) [23]
hES cells	Cell lines from different institutions	17 of 17 cell lines analysed with SNP arrays	843 copy number variations (CNV), 50kb to 30Mb in size in karyotypically normal cell lines, Gain of 1p36, 2p11, 7q35, 14q32, 22q11, loss of 15q11	Närvä E et al, Nat Biotechnol (2010) [17]
hESC	Cell lines from different institutions	42 of 125 cell lines analysed with karyotyping and high-resolution SNP arrays	Extra copies of chromosomes 1, 12, 17, 2 or X, Minimal amplicons 17q25 and 20q11.2, Gain of 12p, Loss of 10p13-pter, 18q21-qter, 22q13-qter	The International Stem Cell Initiative, Nat Biotechnol (2011) [14]
hESC	Different time points during cultivation	69 cell lines analysed with high-resolution SNP arrays	Duplications of 12p, 20q and X chromosome, trisomy 15	Laurent LC et al, Cell Stem Cell (2011) [15]
hESC	Prolonged culture	1 of 3 cell lines analysed by G-banding, aCGH, FISH	ins(2;?)(p16;?) ish amp(1)(q21q32)	Dekel-Naftali M et al, Eur J Hum Genet (2012) [21]

*chromosome aberrations were detected by a methodology based on gene expression profiling (Ben-David et al., Cell Stem Cell 2011 [59]).

Table 2. Genomic alterations of induced pluripotent stem (iPS) cells

Cell type	Factors used for repro-gramming	No of aberrant / analysed clones	Karyotype	Literature
miPS cell lines	4 factors including c-Myc	6 of 9 cell lines	Trisomy or Robertsonian translocation of chromosome 14, trisomy 8	Chen Q et al, Chromosome Res (2011) [29]
miPS cell lines	Various conditions	27 of 127 cell lines with gene expression profiles obtained from the GEO database*	Trisomy 8, trisomy 11/ gains of 11D-11E, deletions of 14C-14E	Ben-David U & Benvenisty N, Stem Cells (2012) [18]
hiPSC	Non-Silencing of 4 factors	1 of 4 clones using karyotyping and mFISH	Translocation between chromosome 1 and 17, gain of 1q, trisomy 5	Ramos-Mejia V et al, Cell Res (2010) [26]
hiPSC	Cell lines from different institutions	13 of 66 cell lines with gene expression profiles obtained from the GEO database*	Trisomy 1, 9, 12 and 17, gain of 12p and 17q, Loss of 15q	Mayshar Y et al, Cell Stem Cell (2010) [23]
hiPS cell llines	No influence of reprogramming factors	22 hiPS cell lines analysed with SNP arrays	Median number of of copy number variations (CNV) 109; higher number in early than in late passages	Hussein SM et al, Nature (2011) [13]
hiPSC	Cell lines from different institutions	3 of 11 cell lines analysed with karyotyping and SNP arrays	Extra copies of chromosome 12, inversion of chromosome 5	The International Stem Cell Initiative, Nat Biotechnol (2011) [14]
hiPS	Different time points during cultivation and differentiation	37 cell lines analysed with high-resolution SNP arrays	Deletions of regions containing tumor suppressor genes, duplications of regions containing oncogenes	Laurent LC et al, Cell Stem Cell (2011) [15]
hiPSC	Reprogramming by - c-MYC - 3 factors - 4 factors	4 of 8, 2 of 6, 3 of 4 clones, resp, using array containing probes for chr. 5-13 and parts of chr. 4 and 14	Deletion of chromosome 9, 13, 14, duplication of chromosome 13, gain of chromosome 8, amplification of chromosome 7	Pasi CE et al, Cell Death Diff (2011) [28]
hiPSC	Prolonged culture	1 of 2 cell lines analysed by G-banding, aCGH, FISH	ish amp(1)(q21qter)	Dekel-Naftali et al, Eur J Hum Genet (2012) [21]

*chromosome aberrations were detected by a methodology based on gene expression profiling (Ben-David et al., Cell Stem Cell 2011 [59]).

somes 10qB and 14qC-14qE. The most recurrent aberration in mouse PSCs, gain 11qE2, turned out to be fully syntenic to the common aberration 17q25 in human PSCs, while other recurrent aberrations were found to be species-specific. Analysis of rhesus macaque PSCs revealed a gain in chromosome 16q, syntenic to the hotspot in human 17q [18]. Therefore, Ben-David and Benvenisty strongly emphasize that attention should be paid to the thorough cytogenetic characterization of ESC and iPSC. This is also recommended to exclude chromosome aberrations in cell lines used as control, which may lead to false conclusions. Chromosome aberrations may interfere with germline transmission of ESC, as described by Liu et al. for trisomy 8 [19]. During the last few years, comprehensive studies have been performed to genetically characterize large numbers of murine and human ES and iPS cell lines. The findings of these studies are summarized in **Tables 1** and **2**.

A 3.1Mb gain in 1q found in cultured human ES cells includes *JARID1B,* a polycomb-related histone demethylase [14] that is overexpressed in different human tumors like breast, prostate cancer or uveal melanoma. Moreover, a jumping translocation of chromosome 1q seems to inhibit senescence during neural differentiation of hESC into a neural stem cell population that could be propagated for more than 50 passages [20]. Jumping translocations of 1q are recurrent chromosome aberrations of hematologic neoplasms and pediatric brain tumors associated with a poor prognosis. Also, in another report, recurrent gain of chromosome 1q was described in one hESC and one hiPSC line, providing clonal advantage in culture [21].

Repeatedly, trisomy 12 or an isochromosome 12p were observed in human ES and iPS cell lines (**Tables 1** and **2**). Catalina et al. [22] reported clonal advantage of cells with trisomy 12 in hESC lines. Mayshar and colleagues [23] report that a substantial number of human iPSC and ES cell lines carry full and partial chromosomal aberrations - again, a high incidence of chromosome 12 duplications resulting in significant enrichment for cell-cycle-related genes like *NANOG*. Such aneuploidy may limit the differentiation capacity and increase the tumorigenicity of human iPSCs. This result was further substantiated by the data of Moon et al. [24], who found that undifferentiated CHA3-hESCs with trisomy 12 undergo abnormal cell division with multiple spindles and that these ESC derivates are able to form a tumor-like tissue after transplantation in mice. Notably, trisomy 12 is a recurrent chromosome aberration in different malignancies like chronic lymphocytic leukemia, and isochromosome 12p is the typical change of testicular germ cell tumors [http://cgap.nci.nih.gov/Chromosomes/Mitelman].

The International Stem Cell Initiative [14] analysed 125 human ES cell lines and 11 iPS cell lines, from 38 laboratories worldwide, for genetic changes occurring during culture. Most lines remained karyotypically normal, but there was a progressive tendency to acquire changes on prolonged culture, commonly affecting chromosomes 1, 12, 17 and 20. Notably, an amplicon in chromosome 20q11.21, including three genes expressed in human ES cells, *ID1, BCL2L1* and *HM13*, occurred in >20% of the lines. This amplification at 20q11.21 had been described previously in four cell lines of different origin and was proposed to provide a selective advantage to hES cells in culture [25]. Of the genes located within the amplified region, *BCL2L1* may be a strong candidate for driving culture adaptation of ES cells.

iPSC lines that do not silence the expression of the ectopic reprogramming factors may display enhanced propensity to genomic instability [26]. To this end, excisable vectors or transgene-free reprogramming, under serum-free and feeder-layer-free conditions, may significantly decrease the risk of genetic instability and malignant transformation [27]. Comparative genomic hybridization analysis of stem cells reprogrammed by expressing c-Myc revealed the presence of genomic deletions and amplifications, whose signature was suggestive of oncogene-induced DNA replication stress. The genomic aberrations were, to a significant degree, dependent on c-Myc expression and their presence could explain why p53 inactivation facilitates stem cell reprogramming [28]. This is in line with our observations in nine independent murine iPS (miPS) cell lines from three laboratories where we found recurrent trisomy and/or translocation of chromosome 14 to be associated with the use of c-MYC for reprogramming [29]. Other key factors regulating genetic instability are ATM and P16 [30].

Yet it seems possible to generate iPSCs lacking gene-disrupting mutations using current repro-

gramming methods. Using whole-genome paired-end DNA sequencing and a sensitive algorithm, only few (one or two) spontaneous sequence variants per line and no evidence for endogenous retro-element transposition were identified [31]. It is also possible to produce gene-corrected disease-specific iPSC lines that do not show any chromosomal alterations if investigated by spectral karyotyping and high-resolution array CGH analysis [2].

Key regulators of genetic instability in stem cells

Except for rare mismatch repair deficiency or chromosomal breakage syndromes caused by inherited mutations of components of different DNA repair pathways, the reasons for increased genetic instability are poorly understood. Clearly, mutations may occur under physiological conditions in normal cells, mostly due to replicative errors. Accordingly, normal cells can acquire numerical chromosome aberrations during mitosis. Metabolic processes generate oxidative species, potent mutagens. Thus, at a low frequency, genetic changes are an integral part of living cells.

DNA damage may also be induced by X-rays, radio- and chemotherapy and by a plethora of mutagenic substances. As long as mutated cells are recognized and eliminated, genetic changes will cause no harm. However, if replicative errors accumulate in highly proliferative cells, DNA repair systems and immune surveillance mechanisms may be overloaded and no longer able to protect genetic stability. With regard to genetic instability of stem cells, quiescence is a highly effective safeguard mechanism.

It is difficult to decipher the mechanisms that increase the rate of genetic instability and allow the outgrowth of genetically defective clones. Oncogenes, tumor suppressor genes, telomere attrition or a combination of several factors have been discussed. Very recently, RNase H2, an enzyme required to remove endogenous ribonucleotides from DNA was shown to be essential for genomic integrity in early embryogenesis [32].

There may be various factors inducing genetic instability during the process of reprogramming: insertional mutagenesis, transcription factors like c-MYC used for reprogramming, enhanced proliferation, cell cultivation over long periods of time, etc. Extensive passaging seems to be associated with an increased rate of genetic instability. Low rates of mosaicism observed in late, but not in early passages imply a direct correlation between number of passages and aneuploidy rate [21]. Chromosome aberrations may already be present in somatic cells, particularly from aged individuals, used for reprogramming. Recently, it has been shown that cellular reprogramming is capable of reversing aging-related features in somatic cells, despite the presence of genomic alterations [33].

c-MYC-induced genetic instability and telomere attrition

There is an obvious correlation between extent of chromosomal aberrations and transcriptional factors used for their reprogramming. Particularly the oncogene *c-MYC,* one of originally four factors to generate induced pluripotent stem (iPS) cells [34] has been demonstrated to induce genetic instability. As mentioned above, we have observed trisomy and translocations in miPS cell lines from three laboratories, which provided a growth advantage over the miPS cells with a normal karyotype. Remarkably, there was a significantly higher frequency of chromosome aberrations in the miPS cell lines induced with c-Myc than those without c-Myc [29]. Sabine May and colleagues elegantly showed that the overexpression of *c-MYC* leads to telomere aggregates that reflect the onset and propagation of breakage-bridge-fusion cycles initiated by end-to-end telomeric chromosome fusions. c-Myc-dependent telomere remodeling thus precedes the onset of genomic instability and subsequently leads to chromosomal rearrangements [35]. Investigating murine hematopoietic stem cells overexpressing c-myc, we could show that loss of the histone methyltransferase suv39h1 can prevent the induction of chromosomal instability. Suv39h1-deficient (c-myc-overexpressing) cells had short, yet less critically short telomeres than wild-type (c-myc-overexpressing) cells, indicating that alternative telomere lengthening may play a role to stabilize the telomeres [36]. Here we have to bear in mind that one single critically short telomere - and not the average telomere length - may induce a chromosome segregation defect or a chromosomal rearrangement.

Telomere erosion is physiologically associated with aging. Cells from the elderly have shortened telomeres and increased genetic instability, thus possibly explaining why tumor risks rise with age and why aged somatic cells can be reprogrammed less efficiently than cells from younger individuals. Several components of the telomerase complex have been implicated in telomere maintenance and long-term genomic stability in ES cells [37]. One of them is Zscan4 regulating telomere extension in ES cells. Knockdown of Zscan4 leads to telomere shortening and to increased rate of chromosome abnormalities [38].

TP53 - the guardian of the genome

TP53 is considered to be the most important gene to protect genetic stability and is also a key regulator of stem cell maintenance and pluripotency. After genetic damage occurs in a cell, *TP53* induces a halt in the cell cycle to allow repair, or alternatively induces senescence or apoptosis, in case the damage cannot be repaired properly. TP53 has a critical function in regulating stem cell quiescence in adult hematopoietic stem cells [39]. Moreover, high expression levels of TP53 promote differentiation of embryonic stem cells [40] and limit efficient reprogramming of somatic cells to iPSC [41, 42]. This may be due to the critical role of TP53 in preventing the reprogramming of cells carrying various types of DNA damage, including short telomeres, DNA repair deficiencies, or exogenously inflicted DNA damage [43]. P53 deletion impairs clearance of chromosomal-instable stem cells in aging telomere-dysfunctional mice [44]. TP53 is part of a huge network. Several regulators like necdin, a growth-suppressing protein [39], aurora kinase A [45] or miR-138, directly binding to the 3' untranslated region (UTR) of TP53, seem to control *TP53* expression [46] in adult and embryonal stem cells. Two downstream targets of P53, i.e. Puma and p21, represent cooperating checkpoints limiting self-renewal and chromosomal instability of somatic stem cells in response to telomere dysfunction [47].

Lessons to be learned from genetic defects in somatic stem cells

If we wish to understand the consequences defined genetic changes may have for genetically modified stem or iPS cells, it may be worth evaluating the harm they cause in somatic stem cells. From genetic analyses of leukemias and solid tumors, we have a broad knowledge of recurrent abnormalities associated with certain tumor subtypes and with clinical outcome. It is clear that the activation of oncogenes like *c-MYC* and the inactivation of tumor suppressor genes cooperate with telomere attrition and epigenetic changes to transform cells. There are different modes of activation of an oncogene, like point mutations, amplification or translocation next to enhancer elements. Also, there are different modes of inactivation of a tumor suppressor gene, like inactivating or dominant-negative mutations, deletions, uniparental disomy or loss of the whole chromosome.

Not surprisingly, *TP53* is the gene most frequently altered in human malignancies. In myeloid neoplasms, *TP53* mutations predict high relapse rates and a very poor prognosis [48, 49]. Notably, *TP53* mutations have been identified in the majority of myeloid neoplasms with a complex karyotype carrying a high number of chromosome aberrations, *per se* the most important factor to indicate an extremely poor prognosis. The influence of *TP53* on genetic stability is further highlighted by recent observations that *TP53* mutations are present in nearly all pediatric brain tumors exhibiting signs of chromothripsis, a kind of "shredding," and complex rearrangements within one or a few chromosomes [50]. *TP53* mutations seem to provide a profound survival advantage, since small clones with *TP53* mutations identified only by next generation sequencing can induce disease progression years later [49]. Yet, the recent advances in next generation sequencing have allowed the gain of deeper insights into clonal evolution and shown that there is a significant genetic diversity and highly dynamic growth and disappearance of genetically aberrant clones in human malignancies [51]. These studies will hopefully allow in the future key players like *TP53* that drive malignant transformation and progression to be distinguished from by-stander lesions. This knowledge will certainly be extremely helpful in judging the malignant potential of defined genetic lesions in reprogrammed cells.

Malignant transformation of genetically modified stem cells after gene therapy

There have been several reports that patients in different gene therapy trials have developed

leukemias. This allows us to better understand what genetic alterations provide a selective growth advantage to genetically modified hematopoietic stem cells. Unequivocally, vector integration into the host genome is the first step towards malignant transformation. As mouse transplantation models have clearly shown, even the integration of one vector upstream of a potent oncogene is sufficient for insertional mutagenesis [52]. There seems to be a limited number of oncogenes that are prone to insertional mutagenesis, particularly *EVI1* inducing myeloid malignancies like myelodysplastic syndromes and acute myeloid leukemias, and *LMO1* and *LMO2* inducing T lymphoblastic leukemias. Notably, in addition to insertional mutagenesis into *LMO2,* T cell leukemias from patients having undergone gene therapy of SCID-X1 frequently contained chromosome aberrations, for example deletions of 6q and 9p affecting the tumor suppressor gene CDKN2A or a SIL-TAL1 fusion [53, 54]. These changes are known as recurrently altered chromosome regions of sporadic T lymphoblastic leukemias, demonstrating that chromosome aberrations contribute to leukemogenesis of genetically modified stem cells. In a gene therapy trial for chronic granulomatous disease, two patients developed myelodysplasia (MDS) due to insertional mutagenesis into the *EVI1* locus. Both patients showed monosomy 7, a typical chromosome aberration of MDS that triggered the clonal expansion of the malignant clone [55].

Conclusions

From the knowledge we have today about genetic instability of genetically modified and reprogrammed cells, we can deduce that a better understanding of the factors that induce an increased genetic instability is warranted before extensive clinical use. There is evidence that enhanced cell culturing, prolonged expression of transcription factors like c-Myc and inactivation of TP53 may play a significant role. A better understanding will also include reliable criteria to classify genetic alterations according to risk for malignant transformation [27]. It will be of the utmost importance to compare genetic alterations in hES and iPS cells with those recurrently found in malignancies. As we show, there are surprising parallels, if we base the comparison on those genetic lesions that provide a selective growth advantage *in vitro.* Another challenge will be to develop appropriate animal models to investigate the malignant potential of defined genetic alterations *in vivo.* Integrating all this knowledge will enable us to set up highly qualified laboratories dedicated to rigorous genetic screening of reprogrammed cells with state-of-the-art technologies. Careful genetic analyses will help to reduce risks of clinical use when reprogrammed cells are transferred into patients.

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Conflict of interest

None of the authors has editorial or financial conflicts of interest (e.g., consultancy, stock ownership, equity interests, patent or licensing agreements).

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