

Cellular reprogramming and cancer development

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Cancer develops through the accumulation of genetic and epigenetic abnormalities. The role of genetic alterations in cancer development has been demonstrated by reverse genetic approaches. However, evidence indicating the functional significance of epigenetic abnormalities remains limited due to the lack of means to actively modify coordinated epigenetic regulations in the genome. Application of the reprogramming technology may help researchers to overcome this limitation and shed new light on cancer research. Reprogramming is accompanied by dynamic changes of epigenetic modifications and is therefore considered to be a useful tool to induce global epigenetic changes in cancer genomes. We herein discuss the similarities between reprogramming processes and carcinogenesis and propose the potential use of reprogramming technology to help understanding of the significance of epigenetic regulations in cancer cells. We, also discuss the application of induced pluripotent stem cell technology to cancer modeling based on the similar characteristics between pluripotent stem cells and cancer cells.

Epigenetic Modifications in Carcinogenesis

Cancer development is caused by a series of genetic mutations in cancer related genes, including oncogenes, tumor suppressor genes and genes related to genome stability. However, recent studies suggest that epigenetic modifications also play critical roles in tumorigenesis.¹ “Epigenetics” is defined as meiotically and mitotically inherited regulations of gene expression that are not accompanied by alteration of the DNA sequence. Two major epigenetic regulations observed in mammals are DNA methylation and post-translational modification of histone proteins. Accumulating evidence highlights the significance of epigenetic dysregulation in tumorigenesis and in the maintenance of cancer cells.^{2,3}

Abnormal patterns of genomic methylation in cancer cells are characterized by global losses of genomic methylation and increased methylation at specific loci, predominantly CpG islands that are often localized near transcription start sites. DNA methylation is a type of covalent modification in which a methyl group is added to a cytosine in the genome. In mammalian cells, DNA methyltransferases (DNMTs) are involved in the establishment and maintenance of DNA methylation. DNMT1 has high catalytic activity and shows a preference for hemimethylated DNA. It plays a role in maintaining the

genomic methylation levels during DNA replication. In contrast, DNMT3A and DNMT3B carry out *de novo* methylation at non-methylated CpG dinucleotides. Although DNMT3L does not exhibit enzymatic activity, it participates in DNA methylation by regulating other methyltransferase activities. Human tumors often display an aberrant expression of DNMTs. Higher levels of DNMT1 and DNMT3A/3B are frequently observed in a wide variety of cancers. Previous studies have suggested that such altered expressions of DNMTs could partly explain the abnormal patterns of genomic methylation observed in cancer cells.⁴

The functional significance of both global hypomethylation and site-specific hypermethylation in cancer has been suggested in previous studies.² Global genomic hypomethylations are frequently observed in both benign and malignant tumors.^{5,6} The clinical outcomes observed in several malignancies indicate that genomic hypomethylation is correlated with poor prognoses in cancer patients, suggesting that loss of DNA methylation can be a marker for tumor prognosis. It is noteworthy that global DNA hypomethylation is associated with abnormal chromosomal structures, as observed in patients with ICF (Immunodeficiency, Centromeric instability and Facial abnormalities) syndrome^{7,8} who harbor mutations of *DNMT3B*. In addition, embryonic stem (ES) cells lacking *Dnmt1* with decreased genomic methylation levels demonstrate increased mutation rates, implying the importance of maintaining the genomic methylation level for preserving genomic integrity.^{9,10} Indeed, DNA hypomethylated mice that harbor hypomorphic alleles of the *Dnmt1* consistently succumb to thymomas, indicating that global DNA hypomethylation promotes carcinogenesis possibly through the induction of chromosomal abnormalities.¹¹ This notion has been further supported by the results of experiments using mice with heterozygous mutations in the tumor suppressor gene *Nf1* in conjunction with *Dnmt1* hypomorphic alleles. In these mice, global DNA hypomethylation promotes the loss

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of heterozygosity in the mutated gene, leading to tumor formation.

Conversely, site-specific hypermethylation is associated with the silencing of tumor suppressor genes.⁴ For instance, treatment of cancer cells with 5-aza-2-deoxycytidine (5-Azac), which blocks DNMTs activity, causes increased expression of tumor suppressor genes and results in growth arrest, differentiation and/or apoptosis both *in vitro* and *in vivo*.¹² In addition, *Apc* mutant mice heterozygous for *Dnmt1* develop significantly decreased numbers of intestinal tumors.^{13–15} Although the opposing effects of the genomic methylation on tumor development seem to be contradictory, these effects were demonstrated in one experiment utilizing *Apc* mutant mice with global DNA hypomethylation. In this study, the forced reduction of genomic methylation resulted in the promotion of early lesions with losses of *Apc*; however, the overall development of large lesions was inhibited.^{16,17}

Imprinted genes display a characteristic parent-of-origin-specific DNA methylation pattern that results in the expression of only a single allele. DNA methylation that maintains the monoallelic expression of imprinted genes is established during gametogenesis and is important for fetal growth regulation and perinatal development.^{18,19} Loss of imprinting (LOI) (either biallelic expression or complete silencing of imprinted genes) has been implicated in the progression of several tumors.^{20,21} For instance, the aberrant biallelic expression of the insulin-like growth factor-2 (*IGF2*) gene is thought to promote tumorigenesis through inhibition of apoptosis or expansion of undifferentiated cells.²² Indeed, LOI is a significant risk factor for human colorectal carcinogenesis. In addition, LOI at *PEG3*, *P57* and *IGF2R* has been implicated in the development of oligodendrogliomas, breast cancer and hepatocellular carcinomas, respectively.^{23–25} Although there is reported evidence that LOI at the *IGF2* locus promotes tumorigenesis,²⁶ it remains unclear if the observed LOI is merely a consequence of altered epigenetic regulation in already transformed cells. Furthermore, the question whether LOI at other various imprinted genes also plays a causal role in cancer development is yet to be answered. Importantly, chimeric mice derived from imprinting-free ES cells develop multiple tumors,²⁷ suggesting a causal link between LOI and cancer development in mice.

Histone is a structural unit of nucleosomes that is important for packing of genomic DNA. Accumulating evidence suggests that post-translational modification of histones (e.g., acetylation, methylation, ubiquitylation, phosphorylation, sumoylation and ribosylation) controls the activity of transcription of surrounding DNA. Epigenetic silencing is associated with at least two distinct histone modifications: polycomb-based histone H3 lysine 27 trimethylation (H3K27me3) and H3K9 dimethylation (H3K9me2).²⁸ Polycomb group (PcG) proteins are components of polycomb repressor complex 1 (PRC1) and PRC2. Enhancer of zeste 2 (EZH2), a member of PRC2, exhibits histone methyltransferase activity with substrate specificity to H3K27.²⁹ H3K27me3 serves as a signal for specific binding of the chromodomain of the other PRC, PRC1, that includes BMI-1,

RING1, HPC and HPH.³⁰ The binding of PRC1 to the methylated histone hampers the recruitment of transcriptional activation factors such as SWI/SNF to surrounding genomic regions, resulting in the prevention of RNA polymerase II from transcription initiation.^{31,32} Conversely, H3K9me2 primarily plays a role in transcriptional control. G9a is a mammalian H3K9 methyltransferase that can repress gene transcription activity by inducing local H3K9me2 and H3K9me3 at target promoters.^{33,34}

Recent studies suggest that histone methyltransferases play roles in the promotion and progression of human cancers. For example, dysregulation of PcG proteins has been observed in a variety of cancers. EZH2 is upregulated in several types of cancer, including metastatic prostate cancer, lymphomas and aggressive breast cancer.³⁵ An increased expression of EZH2 is correlated with a poor prognosis in patients with prostate and breast cancer. In addition, frequent somatic mutations in *EZH2* have been reported in hematopoietic malignancies, consistent with the hypothesis that histone modifications are functionally involved in cancer development.³⁶ BMI-1, a member of PRC1, is frequently overexpressed in human medulloblastoma cell lines and primary tumors.³⁷ In addition to the findings that altered expressions of PcG proteins are closely related to tumorigenesis, the expression of G9a is also upregulated in cancers such as hepatocellular carcinomas.³⁸ It has been shown that G9a is associated with epigenetic silencing of tumor suppressor genes and with the maintenance of malignant phenotypes, providing additional evidence that the aberrant regulation of histone modifications contributes to cancer development.

As described above, emerging evidence indicates that cancer cells harbor altered epigenetic modifications correlated with altered expressions of cancer-related genes. It is important to note that DNA methylation, histone modification, chromatin remodeling and transcriptional regulation are closely interconnected in mammals.^{39,40} Several methyl-CpG-binding proteins (e.g., MeCP2 and MBD2) interact with histone deacetylase and recruit corepressor proteins. This evidence implies a mechanism linking DNA methylation and histone modification. Therefore, a comprehensive analysis of epigenetic modifications, including global analyses of DNA methylation, histone modifications and miRNA expression, in conjunction with a gene expression analysis is necessary to understand cancer epigenetics. Reverse genetic approaches have enabled us to elucidate the functional roles of specific “genetic” alterations in cancer cells. However, to elucidate the functional roles of “epigenetic” alterations, there have not yet been available experimental methods to efficiently control the coordinated epigenetic modifications. Active modification of epigenetic regulation would be a powerful tool to perform functional analyses of epigenetic regulation in cancer cells. In this context, it is noteworthy that technologies for cellular reprogramming enable us to induce dynamic changes in epigenetic modifications. Application of the reprogramming technology to cancer cells to actively alter their epigenetic status might eventually enhance our understanding of cancer epigenomes.

Epigenetic Regulations in Cellular Reprogramming

Global changes in epigenetic modifications can be observed during mammalian embryogenesis and are directly linked to the regulation of pluripotency and cellular differentiation. Although the developmental process in mammals is considered to be unidirectional, previous studies have demonstrated that differentiated cells can be converted into undifferentiated stem cells by artificial manipulation. For example, nuclear transplantation (NT) can reprogram a terminally differentiated cell into a pluripotent stem cell that can give rise to all types of cells in the body.^{41–43} Recent groundbreaking studies have demonstrated that the overexpression of four transcription factors, *Oct4*, *Sox2*, *Klf4* and *Myc*, reprograms differentiated cells into “induced pluripotent stem cells” (iPSCs).^{44,45} These studies have extended our understanding of the mechanisms underlying pluripotency acquisition, maintenance and differentiation.

Derivation of iPSCs from somatic differentiated cells is accomplished by erasing the epigenetic modifications associated with the maintenance of cellular identity and inducing epigenetic modifications similar to those found in early embryos while leaving genetic sequences unaltered. The earliest event in iPSC derivation has been suggested to be genome-wide changes in the histone modification H3K4me2 at more than a thousand loci, including large subsets of pluripotency-related or developmentally regulated gene promoters and enhancers. In contrast, the patterns of repressive H3K27me3 modification remain largely unchanged except for focused depletion occurring specifically at positions where H3K4 methylation is gained. Notably, these chromatin regulation events precede transcriptional changes within corresponding loci.⁴⁶ In addition, “partially” (or “unsuccessfully”) reprogrammed cell lines display incomplete repression of lineage-specifying transcription factors. These observations suggest that changes in epigenetic modifications play critical roles in the early stages of cellular reprogramming and that incomplete epigenetic repression of key genes may be a bottleneck in the transition to pluripotent states.⁴⁷ Several studies have demonstrated that treatment with DNMT inhibitors and histone modifiers such as VPA can improve the overall efficiency of the reprogramming process.^{48,49} This evidence emphasizes the importance of global changes in epigenetic modifications for successful reprogramming.

Similarities Between Cancer Cells and Pluripotent Stem Cells

The process of iPSC derivation shares similar characteristics with cancer development. During the reprogramming, somatic differentiated cells acquire unlimited proliferation properties and self-renewing activities.⁵⁰ This is also one of the most important events in carcinogenesis. It has also been suggested that iPSCs and cancer cells share similar characteristics of cell metabolism.⁵¹ Metabolites involved in transmethylation, cellular respiration and energy production functionally affect cellular reprogramming.⁵² Changes in metabolic status which occur during somatic cell reprogramming are largely similar to

those observed in cancer development.⁵³ This is in agreement with the results of previous studies demonstrating that similar pathways are associated with both oncogenesis and the induction of pluripotency.⁵⁴ Such similarities between pluripotent stem cells and cancer cells suggest that tumorigenesis and reprogramming processes may be partly promoted by overlapping mechanisms. Indeed, previous studies have suggested that reprogramming factors are involved in the development of various types of cancer.

Oct3/4 (also known as *Pou5f1*), a transcription factor that has been recognized as a fundamental factor, maintains pluripotency in ES cells and primordial germ cells.^{55,56} Although *Oct3/4*'s role during preimplantation development is to maintain embryonic cells in a pluripotent undifferentiated state, previous studies have suggested that *Oct3/4* expression may possibly play a role in cancer development. *OCT3/4* has been proposed to be a useful marker for germ cell tumors (GCTs) such as seminomas and embryonal carcinomas.⁵⁷ *OCT3/4* is functionally involved in the self-renewal of GCT cells possibly through the maintenance of undifferentiated states. Importantly, the forced induction of *Oct3/4* in adult somatic cells results in dysplastic growth in epithelial tissues through the inhibition of cellular differentiation in a manner similar to that in embryonic cells.⁵⁸ These findings suggest that *Oct3/4* expression affects epigenetic regulations and contributes to the maintenance of undifferentiated proliferating states in somatic cells. The notion may provide a possible link between transcription factor-mediated reprogramming and carcinogenesis.

A wide variety of cancers express increased levels of *MYC*. *MYC* oncogene is frequently translocated in multiple myeloma and is one of the most highly amplified oncogenes in many different human cancers. It should be noted that *Myc* is an important transcriptional regulator in ES cells and it significantly promotes the process of iPSC derivation. *Myc* targets in ES cells are predominantly involved in cellular metabolism, the cell cycle and protein synthesis pathways, whereas the targets of core reprogramming factors such as *Oct3/4*, *Nanog* and *Sox2* are frequently related to developmental processes. Interestingly, *Myc* preferentially binds to promoters of actively transcribed genes with the histone H3 lysine 4 trimethylation (H3K4me3) signature and enhance their transcriptions in both pluripotent stem cells and cancer cells.^{59,60} List of the genes activated under these promoters might explain the background mechanism of the unlimited proliferation of both pluripotent stem cells and cancer cells.

As described above, transcription factor-mediated reprogramming of somatic cells might be also involved in cancer development. Considering that epigenetic regulations are important during reprogramming, it is possible that epigenetic regulations modulated by the reprogramming factors also play a role in carcinogenesis. Therefore, uncovering the process of cellular reprogramming might eventually provide a better understanding of cancer epigenomes that are responsible for the unlimited growth properties of cancer cells.

Risk of Tumor Development in the Clinical Application of iPSCs to Cell Transplantation Therapy

Pluripotent stem cells are expected to be a promising source of cells for cell transplantation therapies. iPSCs have offered a solution to the ethical objections of ESC usage and to possible immune rejection against ESC-derived cells after transplantation into unmatched individuals. Therefore, iPSCs provide a novel and efficient approach for patient-specific regenerative therapy. One of the risks of using iPSCs in cell transplantation therapy is cancer development from iPSC-derived cells. As mentioned above, pluripotent stem cells share a number of cellular and molecular properties with cancer cells. Both divide rapidly with unlimited proliferative activity,⁶¹ both lack contact inhibition⁶² and both exhibit high telomerase activity.⁶³ Similarities between iPSCs and cancer cells have also been observed with respect to overall gene expression patterns^{64–66} and epigenetic status.⁶⁷ Indeed, one of the important characteristics of pluripotent stem cells is the ability to form teratomas in immunocompromised mice.

For the safe application of iPSCs in regenerative medicine, a number of possible mechanisms in which iPSC-derived cells become cancerous should be considered to avoid such cancer development. First, it is possible that contamination with undifferentiated pluripotent stem cells may result in teratoma formation when transplanted in patients. Given that an iPSC line may consist of a heterogeneous rather than a homogeneous cell population, it is also possible that contamination with unsuccessfully reprogrammed cells or mutated iPSCs might lead to cancer development. Second, since the derivation process of iPSCs requires a number of cell divisions, genetic mutations may be accumulated during the establishment of iPSCs *in vitro*, which may be a potential risk for cancer development. In addition, the reprogramming process is likely to participate in the activation of stress response pathways in the cells, which might also cause the accumulation of genetic mutations, regardless of the reprogramming method used.⁶⁸ Third, previous studies have demonstrated that epigenetic modifications play a role in genomic integrity. Therefore, dynamic changes in epigenetic modifications that occur during iPSC generation might induce genomic instability, leading to genetic alterations in cancer-related genes. Finally, based on the proposed concept of “epigenetic field defect” which is involved in the increased risk for cancer development,⁶⁹ it should be also considered that altered epigenetic modifications in iPSCs-derived cells might create cancer-susceptible populations after the integration of transplanted iPSC-derived cells.

Recent studies have described unique genetic and epigenetic properties of iPSCs distinct from those of ESCs, which might increase the risk of cancer development in iPSC-mediated transplantation therapy. These studies have examined copy number variations (CNVs)⁷⁰ and point mutations of protein-coding regions⁷¹ across the genome by utilizing next generation sequencers. Additionally, genome-wide DNA methylation patterns of ESCs and iPSCs have been analyzed at the single-base level using whole genome bisulfite sequencing.⁷² These studies,

along with other investigations, have proposed that reprogramming and the subsequent expansion of iPSCs *in vitro* may result in the accumulation of various genetic and epigenetic abnormalities at the chromosomal, subchromosomal and single-base levels.⁷³ Such chromosomal abnormalities appear soon after the establishment of iPSCs,⁷⁴ whereas chromosomal abnormalities are not generally observed in ESCs. The frequency of mutations in iPSCs has been estimated to be ten times higher than that in fibroblasts.⁷¹ In addition, there are greater number of subsequent CNVs in iPSCs, which are not found in the cells of origin or in human genomes of comparable background, than in ESCs.^{70,74} Similarly, epigenetic analyses of iPSCs feature retained epigenetic marks of the cells of origin. Another potential difference between ESCs and iPSCs is the status of genomic imprinting. Recent studies have found variety in the expression of imprinted genes among different lines of both murine and human iPSCs.^{75,76} Considering the critical role of genomic imprinting during the developmental process, aberrant silencing or the activation of imprinted genes during reprogramming might affect the differentiation capacity of pluripotent stem cells. Indeed, aberrant imprinting at a single imprinted gene cluster in murine iPSCs results in an impaired developmental potential, thus suggesting that epigenetic variations at imprinted loci can affect the biological behaviors of iPSCs. It is important to note that aberrant imprinting is evident in some types of human cancers and it is also associated with cancer development.^{77,78} Therefore, an aberrant expression of imprinted genes in iPSCs might also affect the tumorigenicity of iPSC-derived cells after cell transplantation therapy.

In contrast, a particularly sensitive analysis for genetic sequencing have subsequently demonstrated that a subset of point mutations found in iPSC lines already exist in a small minority of fibroblasts used for reprogramming, suggesting that the reprogramming process itself may not induce genetic instability.⁷⁰ In addition, large number of CNVs acquired during early passages of iPSCs disappear after subsequent growth *in vitro*, which implies that iPSCs with altered CNVs are negatively selected during the maintenance of iPSCs *in vitro*.⁷⁰ With regard to alterations in DNA methylation patterns, it is also noteworthy that other studies examining a large number of pluripotent stem cells have concluded that it is difficult to distinguish iPSCs from ESCs by DNA methylation patterns. Furthermore, previous studies suggested that the different induction and culture conditions in each laboratory can cause the variable expression patterns.

Taken together, it is still controversial whether the featured genetic and/or epigenetic alterations are inevitable risks of iPSCs for the future clinical therapies (Figure 1). As ESCs also show considerable variation in terms of both genetic and epigenetic contexts, the proposed alterations in iPSCs reported in previous studies may simply represent heterogeneity among different pluripotent stem cell lines. In addition, it has been demonstrated that iPSC lines, as well as ESC lines, show huge variations in differentiation propensity, which implies the presence of variation in tumorigenic risk

as well. Therefore, further studies using a large number of different iPSC/ESC lines are required before we conclude the stability of iPSCs in terms of genome/epigenome integrity. The development of proper strategies to determine and select better iPSCs will lead to the safe application of iPSCs in cell transplantation therapy.

Attempts to Generate Safe iPSCs for Clinical Application

As we described in the previous section, the potential obstacles to the efforts to develop secure iPSCs for clinical application are attributable to the lack of knowledge regarding the mechanisms of tumor development from iPSC-derived cells. In addition, it has not been determined if iPSC-derived cells are actually associated with an increased risk of cancer development. Yet several attempts have already been made to decrease the risk of cancer development from iPSC-derived cells. Based on the assumption that *c-Myc* plays a role in iPSC-associated cancer development, previous studies have been conducted to search for alternatives to replace *c-Myc* in the induction of iPSCs. *L-Myc* and *Glis1* were found to exhibit stronger and more specific activities in promoting iPSC generation in the absence of *c-Myc*.^{79,80} *L-Myc* has shorter amino acid sequences in the N-terminal region compared with the other two *Myc* members. *Glis1* is a Gli-like transcription factor that is enriched in unfertilized oocytes and embryos at one cell stage. Notably, although both factors markedly enhance the generation of iPSCs from both murine and human fibroblasts, no increases in the number of tumor formation events is observed in iPSC-derived chimeric mice, suggesting that these factors might have advantages in the establishment of secure iPSCs for clinical application.

iPSCs were initially derived by the transduction of reprogramming factors in fibroblasts with integrating viruses carrying transcription factors.⁴⁵ It should be noted that at least one of these reprogramming factors are frequently expressed in various types of cancer. It has also been suggested that reactivation of reprogramming factors predisposes iPSCs to genomic instability.⁸¹ Because an ectopic expression of either *Oct3/4* or *Klf4* can induce dysplasia *in vivo*,^{58,82} reactivation of integrated transgene might cause cancer development from iPSC-derived cells. Accordingly, various methods have been developed to eliminate such safety concerns by means of elimination of genome integrations. For example, transduction of purified recombinant reprogramming proteins,^{83,84} synthetic RNA introduction,⁸⁵ infection with nonintegrating Sendai virus,⁸⁶ *piggyBac* transposon^{87,88} and gene introduction using episomal vectors⁸⁹ have been used to generate iPSCs free from genomic integration of exogenous genes. However, the reprogramming efficiency of these approaches is often substantially lower than that of the methods using genome-integrating approaches, and the quality of resultant iPSCs remains to be scrutinized. Further studies will be required to determine the

safe reprogramming method that is applicable to the future cell-based regenerative medicine.

Application of iPSC Technology to Cancer Research Reprogramming of cancer cells

Because cellular reprogramming actively modifies the epigenetic state of a cellular genome without affecting its genetic constitution, reprogramming technologies can be used as tools to analyze the impact of epigenetic regulations on cancer cells. Historic experiments conducted in frogs have demonstrated that kidney carcinoma nuclei can be reprogrammed and that the resulting nuclei support the early development of frog embryos to the tadpole stage.⁹⁰ It has also been reported that by utilizing nuclear transfer into enucleated oocytes, the nuclei of leukemia, lymphoma and breast cancer cells are able to support normal preimplantation development to the blastocyst stage, although they fail to give rise to ES cells. However, interestingly, under the absence of key oncogene expression, the nuclei from cancer cells show the potential to give rise to ES cells. Namely, murine blastocysts cloned from melanoma cells with doxycycline-dependent *RAS*-inducible system give rise to ES cells in the absence of *RAS* expression with the potential to differentiate into multiple cell types, including melanocytes, lymphocytes and fibroblasts.⁹¹ Chimeric mice produced from these ES cells develop melanomas with higher penetrance, shorter latency and expanded tumor spectrums after retransduction of *RAS* in the melanocyte lineage in comparison to that observed in donor mouse models. Importantly, the chimeric mice produced from melanoma nuclear-transferred ES cells have nontumorigenic melanocytes, even after retransduction of *RAS*. This observation suggests that epigenetic reprogramming can revert melanoma cells to non-neoplastic melanocytes, highlighting the impact of epigenetic regulations in cancer cells.

Reprogramming by means of nuclear transfer requires special equipment and a high skill. In contrast, the discovery of iPSC generation technology provides a simple and reproducible method to reprogram differentiated cells into pluripotent stem cells.^{44,45} Taking advantage of the transcription factor-mediated reprogramming method, a number of studies have been conducted to reprogram cancer cells. However, these experiments have revealed that cancer cells are resistant to reprogramming. Recently, several groups have succeeded in reprogramming cancer cells into an iPSC-like state. iPSC-like cells have been generated from chronic myeloid leukemia cells,^{92,93} melanoma cells⁹⁴ and gastrointestinal cancer cells.⁹⁵ Although these studies have demonstrated that particular cancer cell genomes can be reprogrammed into iPSC-like states, reprogramming efficiency seems to be extremely low in cancer cells, suggesting that some properties of cancer cells strongly hamper cellular reprogramming. Considering that cellular reprogramming is a process in which dynamic epigenetic changes are required, the fact that cancer cells are resistant to cellular reprogramming raises the possibility that cancer cells have stable epigenetic regulations that compete with the exogenous reprogramming forces derived from

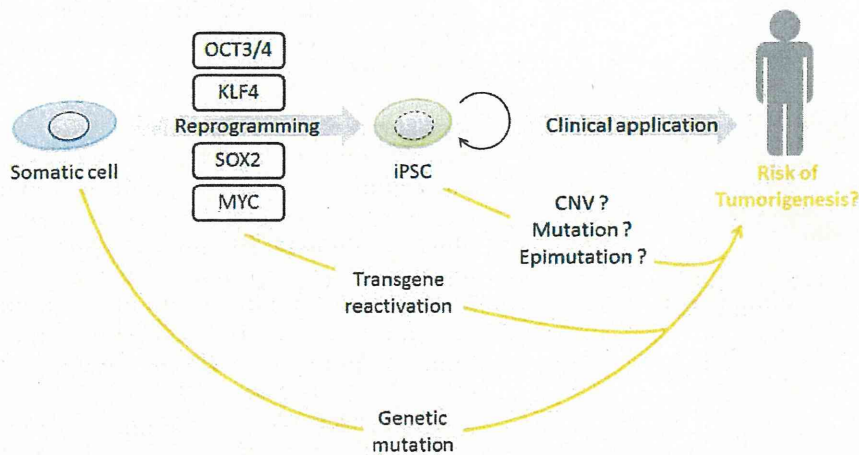


Figure 1. Potential risk of tumor development in the clinical application of iPSCs. Cancer development is a potential risk of using iPSCs in cell transplantation therapy. The genomic and epigenomic instability of iPSCs may cause tumor development from iPSC-derived cells, yet the instability has not been fully elucidated.

induced reprogramming factors. Uncovering the underlying mechanisms that explain the resistance of cancer cells to epigenetic reprogramming may be of great help in advancing understanding of the epigenetic stability of cancer cells, which may eventually contribute to the development of efficient therapeutic strategies targeting epigenetic modifications in cancer cells. In addition, understanding the molecular basis for the reprogramming resistance in cancer cells may further reveal a mechanism for the low efficiency of normal somatic cell reprogramming.

Concept of cell type-specific carcinogenesis

Tumors develop through the accumulation of multiple mutations in oncogenes and tumor suppressor genes. Oncogenic mutations are often observed in a tissue- or cell type-specific manner.⁹⁶ Similarly, the cancer mutation spectrum is different between cancers arising from different organs. In hereditary cancer syndromes, patients are predisposed to tumor development in specific tissues despite the presence of mutated cancer-related genes in all somatic cells. This suggests that the effects of cancer-relevant mutations are highly influenced by cell type-specific contexts in different environments. As cellular reprogramming enables us to erase the cellular identity of original cell types without altering the genomic sequences including genetic abnormalities in cancer cells, redifferentiation of cancer cells into cells of other tissue types should be useful method to directly demonstrate the concept of cell type-specific carcinogenesis. Actually, redifferentiation of reprogrammed cancer cells has been demonstrated in leukemia cell model. However, CML-derived iPSC cells failed to recapitulate cancer phenotypes, even after the differentiation into hematopoietic cell lineages.⁹³ Although the concept of cell-type specific carcinogenesis has not yet been proven, application of the iPSC technology to cancer cell reprogramming would be a useful tool for the future achievement of demonstrating this concept (Figure 2a).

Disease modeling using reprogramming technologies

Cancer-derived iPSCs are also expected to provide a novel experimental opportunity to establish disease models. It is interesting to note that iPSCs generated from a blood sample obtained from an imatinib-sensitive CML patient were found to be resistant to imatinib although the CML-derived iPSCs consistently expressed BCR-ABL oncoproteins.⁹³ Although imatinib treatment is a highly effective therapy for CML,⁹⁷ a minority of patients either fail to respond to imatinib. The altered response to imatinib in CML-iPSC suggests that cancer-derived iPSCs could be a novel platform to investigate the effect of the differentiation status on the response of cancer cells to external signals such as therapeutic agents, which could contribute to the development of effective therapeutic strategies. In this context, cancer cell reprogramming may be useful for understanding cancer cell behaviors that are related to distinct differentiation status.

Forced modifications of differentiation states in cancer cells may further lead to increasing understanding of the hierarchical control of differentiation in cancer cells. Such hierarchical control might be associated with the concept of cancer stem cells. Given that epigenetic regulations determine the heterogeneity underlying the concept of cancer stem cells, it might be possible that reprogramming technology could be applicable to modeling cancer stem cells through active modification of both epigenetic regulations and cell differentiation status.

iPSC technology might be also available to examine whether dedifferentiation process occurs during cancer development. Cancer cells frequently express progenitor-related genes that are exclusively expressed in the tissue-specific stem/progenitor cells of the originated tissue.⁹⁸ The hypothesis that cancer arises from tissue-specific stem/progenitor cells suggests that the undifferentiated properties of cancer cells may be consequence of the expansion of original undifferentiated cells. Therefore, it remains to be determined whether a dedifferentiation process is

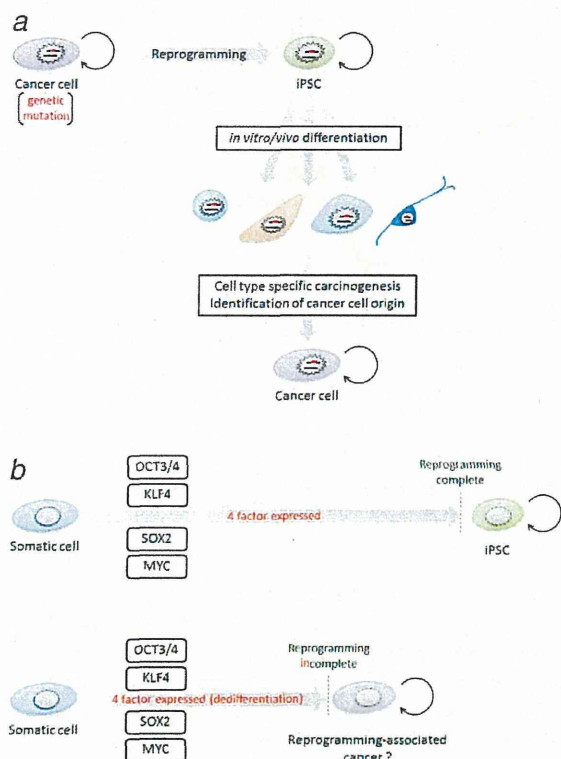


Figure 2. Application of iPSC technology for cancer research. Reprogramming technology is considered to be a useful tool to induce global epigenetic changes and to alter differentiation status of cancer cells. (a) Reprogramming of cancer cells and differentiation of the cancer cell-derived iPSCs. Cellular reprogramming actively modifies the epigenetic/differentiation state of cancer cells without affecting its genetic constitution. The reprogrammed cancer cells may provide a novel platform to demonstrate the concept of cell type-specific carcinogenesis and to identify the cancer cell origin. (b) Cancer modeling. Expression of Yamanaka 4 factors can reprogram somatic differentiated cells into undifferentiated stem cells with unlimited proliferation properties. Reprogramming technology might be available to model reprogramming-associated cancers, which is accompanied by dedifferentiation process with altered epigenetic regulations.

actually involved in the oncogenic process. Somatic cell reprogramming entails the erasure of gene expression programs characteristic of differentiated somatic cells and the reactivation of embryonic patterns of gene expression characteristic of the pluripotent state.^{99,100} Importantly, it has been shown that, not only stem/progenitor cells, but also somatic differentiated cells, can be reprogrammed into pluripotent stem cells, suggesting that differentiated cells can actually acquire self-renewing activities during the process.¹⁰¹ As an increased expression of pluripotency-related factors is frequently detectable in poorly differentiated cancers,⁶⁵ the dedifferentiation process may actively promote the development of certain types of cancers with transcriptional networks similar to those of pluripotent stem cells. Together with the fact that the factors that drive reprogramming are oncogenes or have been linked to cellular transforma-

tion, reprogramming technology might be useful to model reprogramming-associated cancers that are accompanied by dedifferentiation (Figure 2b).

Identification of cancer cell origin

In many types of cancers, the cell of origin remains unknown. The identification of the origin of cancer cells enables us to expand our knowledge of cancer development, which facilitates the discovery of more effective chemopreventive approaches. For instance, comparative studies of the cell of origin and the resultant cancer cells may reveal the key events that are directly involved in cancer development. Additionally, recent studies have suggested that the characteristics of the cell of origin often persist in the consequent tumor cells and may play critical roles in the propagation of tumor cells *in vivo*. Therefore, identification of the cell of origin should be beneficial to understand biological properties of cancer cells.

Based on the concept of cell type-specific carcinogenesis, it is expected that the reprogrammed cancer genome can exert their cancerous properties only when cancer genomes are matching with a particular cell type. In this context, reprogrammed cancer cells that harbor genetic mutations sufficient for cancer development but still retain multidifferentiation properties may be available for identifying cell types that give rise to cancer cells. In addition, recent studies have proposed that solid cancers arise from relatively undifferentiated cells such as tissue-specific stem cells. For instance, the conditional knock out of the *Apc* tumor suppressor gene in tissue-specific stem cells specifically results in the frequent development of intestinal tumors,¹⁰² while only a few tumors develop when *Apc* is depleted in progenitor/differentiated cells in crypts. The hypothesis of cancer stem cells also holds that cancer emerges from primitive tissue stem cells, yet it remains to be determined whether the concept can be applied for a wide variety of cancers. Cancer-derived iPSCs may also provide a platform for determining the particular differentiation status permissive for cancer development.

Conclusion

Technologies that induce pluripotent stem cells confer unlimited growth ability on somatic differentiated cells, a hallmark of cancer cells. It is important to note that the reprogramming process does not require changes in genomic sequences, thus indicating that changes in epigenetic modifications play a central role in this process. Application of reprogramming technologies for cancer cells might therefore be useful for uncovering the role of epigenetic regulations in cancer cells. Moreover, cancer development caused by the introduction of reprogramming factors might be applicable to cancer modeling that is predominantly dependent on epigenetic dysregulation with impaired gene expressions and altered differentiation status.

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Activation-Induced Cytidine Deaminase Alters the Subcellular Localization of Tet Family Proteins

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Abstract

Activation-induced cytidine deaminase (Aid), a unique enzyme that deaminates cytosine in DNA, shuttles between the nucleus and the cytoplasm. A recent study proposed a novel function of Aid in active DNA demethylation via deamination of 5-hydroxymethylcytosine, which is converted from 5-methylcytosine by the Ten-eleven translocation (Tet) family of enzymes. In this study, we examined the effect of simultaneous expression of Aid and Tet family proteins on the subcellular localization of each protein. We found that overexpressed Aid is mainly localized in the cytoplasm, whereas Tet1 and Tet2 are localized in the nucleus, and Tet3 is localized in both the cytoplasm and the nucleus. However, nuclear Tet proteins were gradually translocated to the cytoplasm when co-expressed with Aid. We also show that Aid-mediated translocation of Tet proteins is associated with Aid shuttling. Here we propose a possible role for Aid as a regulator of the subcellular localization of Tet family proteins.

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Introduction

DNA methylation is a stable epigenetic feature that is involved in gene silencing and the maintenance of long-lasting cell memories [1]. Dynamic regulation of the DNA methylation pattern is crucial for mammalian development, as well as differentiation and reprogramming [2,3]. In particular, the active loss of 5-methylcytosine (5mC) independent of cell division is considered to be a major initial event in the epigenetic reprogramming of early mammalian embryos [4]. It has been demonstrated that the loss of 5mC at the paternal pronucleus of a zygote is linked to the accumulation of 5-hydroxymethylcytosine (5hmC) [5–7]. The 5hmC is converted from 5mC by the ten-eleven translocation (Tet) family of proteins [8], and therefore 5hmC is considered to be an intermediate formed during the active DNA demethylation process in early embryos.

A recent study proposed a novel model for the removal of 5hmC, wherein activation-induced cytidine deaminase (Aid) induces the deamination of 5hmC, which is followed by base excision repair (BER), resulting in the conversion of 5hmC into unmethylated cytosine [9]. Based on this model for active DNA demethylation, coordinated actions of both the production and removal of 5hmC may regulate the conversion of 5mC into unmethylated cytosine. However, little is known how these proteins involved in the production and removal of 5hmC affect each other.

Aid is a well-known enzyme that converts cytosine into uracil in single-stranded DNA, causing somatic hypermutation and class switch recombination [10,11]. Aid is mainly localized in the cytoplasm under steady state conditions, but has the ability to

shuttle between the nucleus and the cytoplasm [12,13]. Previous studies suggested that the changeable localization of Aid, which is mediated by its shuttling, plays a role in controlling its activity as a DNA modifier [14,15]. Considering that 5hmC is localized at the nucleus, the shuttling of Aid may also contribute to the modulation of 5hmC removal. In addition, Tet family proteins show translocation into the nucleus from the cytoplasm during the early developmental stage, when the rapid generation of 5hmC is observed [16]. Therefore, it is possible that distinct subcellular localization of the Tet family and Aid controls the production and removal of 5hmC, leading to the regulation of active DNA demethylation. In the present study, we examined the relationship between the Tet family and Aid from the view of their subcellular localization. We herein demonstrate that Aid has an effect on the subcellular localization of the Tet family, and that this is associated with Aid shuttling.

Materials and Methods

DNA constructs

Mouse Tet1 (GU079948, DDBJ), Tet2 (GU079949, DDBJ), Aid (NM_009645.2, NCBI), Apobec1 (NM_031159.3, NCBI) and Apobec2 (NM_009694.3, NCBI) were cloned from mouse embryonic stem cells, and Tet3 (NM_183138.2, NCBI) was obtained from mouse embryonic fibroblasts by PCR amplification with KOD plus Neo (TOYOBO) using primers as described in Table S1. The Tet family fragments were subcloned into pcDNA4HisMax (Life Technologies), and the Aid, Apobec1 and Apobec2 fragments were subcloned into pcDNA4MycHis (Life Technologies). Plasmids encoding the Xpress-tagged catalytic