Review

The causal relationship between epigenetic abnormality and cancer development: in vivo reprogramming and its future application

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Abstract: There is increasing evidence that cancer cells acquire epigenetic abnormalities as well as genetic mutations during cancer initiation, maintenance, and progression. However, the role of epigenetic regulation in cancer development, especially at the organismal level, remains to be elucidated. Here, we describe the causative role of epigenetic abnormalities in cancer, referring to our in vivo studies using induced pluripotent stem cell technology. We first summarize epigenetic reorganization during cellular reprogramming and introduce our in vivo reprogramming system for investigating the impact of dedifferentiation-driven epigenetic disruption in cancer development. Accordingly, we propose that particular types of cancer, in which causative mutations are not often detectable, such as pediatric cancers like Wilms’ tumor, may develop mainly through alterations in epigenetic regulation triggered by dedifferentiation. Finally, we discuss issues that still remain to be resolved, and propose possible future applications of in vivo reprogramming to study cancer and other biological phenomena including organismal aging.

Keywords: cancer, epigenetics, iPS cell, in vivo reprogramming

Genetic mutations and epigenetic alterations in cancer development

It is generally accepted that genetic mutations cause cancer. Indeed, cancer cells harbor multiple genetic mutations. The well-known genetic mutations observed in human cancers include mutations at the TP53, KRAS and PIK3CA genes.1) A number of previous studies provide evidence that these mutations are indeed functionally significant in cancer development, and several proteins or pathways with genetic mutations, such as EGFR, HER2, ALK, etc., are now targets for molecular-targeted therapies and the targeted treatment sometimes exert remarkable benefits for patients.

In vivo studies using the forward/reverse genetics approach have been employed to investigate whether and how a certain genetic mutation is mechanistically involved in cancer development. For example, mutation of the APC gene is known to be the first genetic event in multistep carcinogenesis in the colon,2) and a causal relationship between APC gene mutations and colon cancer development in vivo has been established from the fact that the Apc mutant mouse, a model mouse for familial adenomatous polyposis (FAP), develops intestinal tumors. The Apc min/+ mouse, which contains a truncating mutation in the Apc gene, develops multiple intestinal adenomas, affirming that APC gene mutations are causative for intestinal neoplasms.3)

In contrast, accumulating evidence suggests that most cancer cells harbor altered epigenetic modifications in addition to aberrations in the genomic sequence when compared with adjacent tissues. Epigenetics is defined as the study of changes in gene function that are mitotically and/or meiotically
heritable and that do not entail a change in DNA sequence. DNA methylations and histone modifications at various amino acid residues are major epigenetic modifications. The reduction of global DNA methylation levels was the first epigenetic alteration to be described in cancer cells. Indeed, global DNA hypomethylation as well as site-specific DNA hypermethylation has been observed in the vast majority of cancers. To date, the role of altered DNA methylation patterns in cancer development has been extensively analyzed.

The Apc min/+ mouse model has provided important insights into the cooperative involvement of genetic mutations and epigenetic alterations in multistage cancer development in vivo. Particularly, the identification of microadenomatous lesions with the loss of Apc heterozygosity in the colon of the Apc min/+ mouse provides an opportunity to consider the involvement of epigenetic abnormalities in cancer, especially cancer progression in vivo. As described above, the Apc min/+ mouse develops intestinal adenomas as the result of a spontaneous loss of Apc heterozygosity. In this mouse model, however, the majority of intestinal polyps are located in the small intestine and are hardly detected in the colon, a distribution rather different from that in FAP patients. We have found that there exist many intramucosal small adenomatous lesions (microadenomas) in the colon (usually <300 μm), which exhibit a loss of Apc heterozygosity and the subsequent accumulation of β-catenin, and that only a subset of microadenomas progress into macroscopic tumors. These findings suggest that, in Apc min/+ mice, the loss of Apc function is responsible for the initiation of colon adenomas, but is not sufficient for full-blown tumor development, and that other factors play important roles in the transition of microadenomas into colon tumors.

It is interesting to note that well-known driver-mutations in human colon cancers are not detectable in colon tumors in this model (unpublished data), suggesting that the genetic mutations in the human adenoma-carcinoma sequence are not involved in the progression. Notably, subsequent studies using the same mouse model have revealed the significance of DNA methylation in colon tumor development. These studies demonstrated that the forced reduction of global DNA methylation levels causes chromosomal instability, promoting the loss of Apc heterozygosity, which leads to increased microadenoma formation, but also suppresses the transition of early microadenomas into macroscopic tumors. In contrast, the forced expression of de novo DNA methyltransferase Dnmt3b accelerates the transition of colonic microadenomas to macroscopic tumors, while the deletion of Dnmt3b suppresses this progression.

Fig. 1. Functional involvement of DNA methylations in murine colon tumor development and progression. Forced reduction of DNA methylation causes chromosomal instability and promotes the loss of Apc heterozygosity, which leads to increased microadenoma formation. The DNA hypomethylation suppresses the transition of early microadenomas into macroscopic tumors. In contrast, the forced expression of de novo DNA methyltransferase Dnmt3b accelerates the transition of colonic microadenomas to macroscopic tumors, while the deletion of Dnmt3b suppresses this progression.
**Causes of epigenetic alterations in cancer**

Although previous studies demonstrated that altered epigenetic modifications substantially affect cancer development in vivo, it remains to be fully understood how epigenetic abnormalities occur during cancer development. Large-scale cancer genome sequencing projects have identified a number of mutations at epigenetic regulator genes across a wide range of cancer types, and thus clearly demonstrated that some of the epigenetic abnormalities observed in cancers are attributable to the impaired function of epigenetic factors caused by genetic mutations.\(^{15}\)

This raises the possibility that epigenetic alterations are primarily caused by genetic aberrations and therefore that cancer is in essence a “genetic disease.”

Several studies have demonstrated that environmental factors, such as cancer-promoting inflammatory stimuli, diet, and smoking, induce changes in DNA methylation patterns,\(^{16}\) suggesting that external signals can induce epigenetic alterations in cancer cells presumably without affecting the genomic sequence. Chronic inflammation due to *Helicobacter pylori* (*H. pylori*) infection, which strongly promotes gastric cancer development, induces aberrant changes in DNA methylation in the stomach.\(^{17}\)

Notably, aberrant DNA methylations are detectable even in non-cancerous tissues in *H. pylori*-infected stomachs and are designated ‘epigenetic fields for cancerization’, which are proposed to mark preneoplastic lesions in the stomach.\(^{18}\)

Hypoxia, a common feature of solid cancers, can also promote the progression of cancer. Notably, hypoxic tumors often exhibit increased promoter hypermethylation, suggesting that the hypoxic condition may induce epigenetic alterations. Mechanistically, the increased promoter methylation is associated with a reduced expression of TET enzymes and decreased 5hmC at gene promoters and enhancers, supporting the notion that hypoxia inhibits TET-mediated DNA demethylation in tumors.\(^{19}\)

Taken together, these findings suggest a causal link between altered environmental factors and epigenetic aberrations that promotes cancer development.

Recent studies further indicated the impact of environmental factors on epigenetic regulation in stem cells. We have shown that mouse embryonic stem cells (ESCs) generated in the presence of 2i (inhibitors of Mek1/2 and Gsk3beta), which can induce ground state pluripotency in mouse ESCs, show a global loss of DNA methylation including methylation at imprinting control regions, resulting in impaired autonomous embryonic development.\(^{20},^{21}\)

These reports imply profound effects of environmental factors on epigenetic regulation and subsequent biological consequences for stem cells.

**Involvement of cellular dedifferentiation in cancer development**

Several studies have suggested that cancer cells arise from somatic stem cells.\(^{22},^{23}\)

In intestinal tumorigenesis, Barker et al. demonstrated that Lgr5-expressing intestinal stem/progenitor cells are prone to transformation.\(^{23}\)

In contrast, other studies have proposed that the dedifferentiation of mature cells triggers tumor development in the intestine. Schwitalla et al. showed that Wnt signaling together with elevated NF-kB signaling can induce the dedifferentiation of non-stem cells in the intestine, resulting in the acquisition of tumor-initiating capacity with stem cell properties.\(^{24}\)

In lymphoma-genesis, using a conditional knocked-out system for *Pax5*, the master regulator of B-cell lineage, Cobaleda et al. demonstrated that mature B-cells can be converted into aggressive lymphoma through dedifferentiation into the progenitor state.\(^{25}\)

Also, several studies that aimed to explore the origin of glioma cells have demonstrated that this tumor can arise in differentiated lineages through dedifferentiation.\(^{26},^{27}\)

Collectively, these results suggest that dedifferentiation is involved in the initiation of cancer development in diverse cancer types. Considering that epigenetic regulation plays a central role in cellular differentiation and maintenance, it is possible that the dedifferentiation process could be mediated by epigenetic regulation as well. Therefore, one important question is whether epigenetic regulation-mediated dedifferentiation has a causative role in cancer development. However, given that previous studies employed genetic manipulation to induce dedifferentiation, they did not directly demonstrate that epigenetics-driven dedifferentiation affects cancer development.

**Technology for cellular reprogramming and its applications to the cancer epigenome**

In mammals, classically, it was considered that once a cell differentiates into a particular cell type with a distinctive function, the cell permanently loses the potential for diverse functions and stably maintains its identity. This unidirectional differentiation process is often compared to a ball rolling down a hill into a landscape with peaks and valleys (Fig. 2). The pluripotent stem cell is located at the top of the hill, and each differentiated cell state lies at the bottom
of each distinct valley. Once the developmental stage proceeds with cellular differentiation, cells are unable to climb back to the top of the hill and become precursor cells again, nor can they cross the ridges that separate the individual valleys and transdifferentiate into other differentiated cell types.

However, accumulating evidence suggests that this dogma is not necessarily valid, and that cellular identity can be altered by artificial reprogramming technology. Pioneering studies employing the nuclear transfer technique showed that a somatic nucleus is reprogrammable into a pluripotent nucleus. In addition, the forced expression of the transcription factor MyoD induces myogenic differentiation in many cell types, suggesting that specifically differentiated somatic cells can also be reprogrammed into other types of differentiated cells. The decisive breakthrough in cell reprogramming came when Takahashi and Yamanaka succeeded in generating induced pluripotent stem cells (iPSCs) from fibroblasts, which can differentiate into any cell lineage of the body. In the context of previous studies, they hypothesized that the factors playing important roles in the maintenance of ESC identity may also play a critical role in the induction of pluripotency in somatic cells. As they expected, the forced induction of four transcription factors (Yamanaka factors), Oct3/4, Sox2, Klf4, and c-Myc, all of which are highly expressed and contribute to the maintenance of ESC identity, resulted in the generation of iPSCs (Fig. 2).

iPSC technology has also led to remarkable progress in the direct reprogramming of specialized cell types into others. As is the case for MyoD-induced transdifferentiation, direct reprogramming is basically achieved by inducing the transcription factors that govern the transcriptional network of the target cell lineage (Fig. 2). Collectively, cell identity is maintained, at least in part, by a stable transcription network unique to any individual cell type, but the forced expression of another set of cell type-specific transcription factors can activate the transcriptional network of the resulting cell type, which leads to cell fate conversion. The important implications of cellular reprogramming are that 1) during the reprogramming process, dynamic alterations in epigenetic modifications take place while the underlying DNA...
sequence remains unchanged, and 2) once a cell type conversion has occurred, this specific set of cellular properties is intrinsically maintained without expression of the exogenous transcription factors.\textsuperscript{31},\textsuperscript{32} Taken together, the transient activation of the cell type-specific key transcriptional network can reorganize the epigenetic regulation of somatic cells.

It is noteworthy that the reprogramming process into iPSCs has characteristics similar to those of cancer development. During iPSC derivation, somatic cells acquire unlimited proliferation properties and self-renewal activities, phenomena shared with cancer cells.\textsuperscript{30} It is also suggested that both iPSCs and cancer cells exhibit a similar metabolic status, namely activated glycolysis,\textsuperscript{37},\textsuperscript{38} and poorly differentiated aggressive cancers often have an ESC-like transcriptional signature.\textsuperscript{39} These similarities suggest that there may exist shared mechanisms governing tumor development and iPSC derivation. Indeed, a previous report showed that the ectopic expression of Oct3/4 causes dysplasia in epithelial tissues, indicating that pluripotency-related genes can functionally act as cancer initiators even in non-germ cell lineages.\textsuperscript{40} Also, the depletion of p53, a major tumor suppressor gene, significantly promotes the derivation of iPSCs,\textsuperscript{31},\textsuperscript{42} demonstrating that common signals could be a roadblock on the path toward both cancer and pluripotency.

Collectively, considering that the conversion of somatic cells to iPSCs is accompanied by dynamic changes in epigenetic regulation but not in the underlying genomic sequence raises the possibility that epigenetic regulation for the derivation of iPSCs might be associated with dedifferentiation-mediated cancer development. Together, these findings provide a rationale for exploring the causal relationship between dedifferentiation and cancer development using iPSC technology.

**Dissection of the cancer epigenome using in vivo reprogramming: a proof of concept for epigenetic cancer**

In view of the above considerations, we investigated the association between dedifferentiation-associated epigenetic regulation and cancer development using in vivo reprogrammable mice that have a transgenic system for the Yamanaka factors\textsuperscript{43} (Fig. 3). We employed chimeric transgenic mice that
were generated using ESCs in which the four factors can be induced under the control of doxycycline (Dox). Continuous expression of the factors resulted in the development of multiple teratomas in various organs (Fig. 4). It is noteworthy that in vitro culture of these teratoma cells resulted in the derivation of iPSCs capable of chimeric contribution in mice, thus confirming that somatic cells are reprogrammable in vivo.

Interestingly, when incomplete reprogramming was induced in these mice by the withdrawal of Dox treatment before teratoma formation, the mice developed cancers showing invasion of surrounding tissues. (B) Left panel: Continuous expression of the reprogramming factors induces teratomas, which consist of differentiated cells of the three germ layers, (a) neural tissue (ectoderm); (b) glandular epithelium (endoderm); (c) cartilage (mesoderm). Middle panel: The kidney tumor developed by incomplete reprogramming consists of epithelial (d), stromal (e), and blastema-like (f) compartments, which are representative histological features of Wilms’ tumors, a common pediatric kidney cancer. Right panel: A histology of human Wilms’ tumor containing epithelial, stromal, and blastema-like compartments.

Fig. 4. Premature termination of in vivo reprogramming causes kidney cancer development resembling human Wilms’ tumor. (A) Continuous expression of the reprogramming factors results in the development of multiple teratomas in various organs. However, when incomplete reprogramming is induced in these mice by the withdrawal of Dox treatment before teratoma formation, the mice develop cancers showing invasion of surrounding tissues. (B) Left panel: Continuous expression of the reprogramming factors induces teratomas, which consist of differentiated cells of the three germ layers, (a) neural tissue (ectoderm); (b) glandular epithelium (endoderm); (c) cartilage (mesoderm). Middle panel: The kidney tumor developed by incomplete reprogramming consists of epithelial (d), stromal (e), and blastema-like (f) compartments, which are representative histological features of Wilms’ tumors, a common pediatric kidney cancer. Right panel: A histology of human Wilms’ tumor containing epithelial, stromal, and blastema-like compartments.
gene expression profile, and an abnormal epigenetic regulation of \(Igf2\), a representative imprinting gene (Fig. 4).

Notably, no genetic mutations in cancer-related genes were detectable in Dox-withdrawn cancer cells. Given that mice have much shorter life span compared with humans, the lack of detectable mutations may be attributable to the short latency of cancer development. Interestingly, these cancer cells were readily reprogrammable into iPSCs with shorter latency and higher efficiency, which also reflects the partial reprogramming state. Moreover, these kidney tumor-derived iPSCs contributed to chimeric mice and differentiated into apparently normal non-neoplastic kidney cells, indicating that kidney cancer cells in this model have not undergone irreversible genetic transformation. We propose that particular types of cancer can develop mainly through the disruption of epigenetic regulation.

The fact that human Wilms’ tumors sometimes lack detectable recurrent mutations, including mutations at well-known genes that are associated with Wilms’ tumor development such as \(Wt\) and \(Ctnnb1\), supports this notion.\(^{47}\) The fact that children with Beckwith-Wiedemann syndrome (BWS) possess genetic abnormalities at imprinting genes including \(IGF2\) and develop Wilms’ tumor\(^{48}\) may also support the notion that an abnormal epigenetic regulation of \(Igf2\) might play an crucial role in kidney tumor development in our model.

Furthermore, our previous study demonstrated that partial reprogramming in vivo causes the development of hepatoblastoma-like liver cancers and pancreatoblastoma-like pancreatic cancers, as well as Wilms’-like kidney cancers, all of which are representative pediatric cancers. Therefore, childhood cancers, which appear not to accumulate genetic mutations with patient age, might be candidate epigenetics-driven cancers. Consistently, recent studies have demonstrated that childhood cancers such as medulloblastomas, neuroblastomas, and rhabdoid tumors have very few recurrently
mutated genes.\(^{49–51}\) Given that epigenetic regulation can be modulated by chemical compounds, the epigenetic abnormality that drives cancer development might be a promising therapeutic target.

Open questions to be resolved in human cancer epigenetics

It should be noted, however, that our experimental model utilizes an artificial expression system to induce the dedifferentiation, and the study does not provide direct evidence that dedifferentiation is actually involved in the development of human Wilms’ tumor. Further detailed studies using human samples are needed in order to clarify the role of dedifferentiation-related epigenetic regulation in human cancer development.

Our model suggests that partial reprogramming may drive particular types of cancer. However, it is unlikely that expression of the Yamanaka factors is directly responsible for the dedifferentiation that eventually causes the development of human cancers. It would be of great interest to identify a natural phenomenon that induces dedifferentiation. A previous study demonstrated that gene sets other than the Yamanaka factors are able to induce the complete reprogramming of somatic cells, albeit at very low efficiency.\(^{52}\) It is possible that incomplete reprogramming can be achieved by the expression of much more diverse gene sets than we expected, and that such a setting might be induced by abnormal environmental factors, such as inflammation and hypoxia.

One example of cellular reprogramming induced by environmental factors appears to be metaplasia. Metaplasia is a reversible change in which one differentiated cell type is replaced by another mature cell type. It is a common phenomenon and can be regarded as an adaptive replacement of cells that are sensitive to stress by cell types that tolerate the adverse environment. Of note, metaplasia has clinical significance because some metaplasia, such as Barrett’s esophagus and intestinal metaplasia of the stomach, are well known to have an increased risk of cancer development. Nevertheless, details of the mechanism of metaplasia are not fully elucidated. Whereas Wang et al. have proposed that residual embryonic cells can be precursors of Barrett’s-like metaplasia,\(^{53}\) Fujii et al. found that chronic infection with *H. pylori* converted gastric epithelial cells to intestinal epithelial cells via tissue-stem-like progenitor cells, where *H. pylori* infection induces the aberrant expression of the intestine-specific transcription factors *CDX1* and *CDX2*.\(^{54}\) Indeed, ectopic *CDX1* activates the stemness-associated reprogramming factors *SALL4* and *KLF5*, resulting in the reprogramming of gastric epithelial cells into tissue-stem-like progenitors, which eventually allow transdifferentiation into intestinal epithelial cells.\(^{54}\) These findings support the idea that an external stimulus such as infection by pathogenic organisms (e.g. *H. pylori*) and subsequent inflammation can induce the dedifferentiation of somatic cells. As mentioned above, it is noteworthy that inflammation-inducible NF-kB signaling, a common cytokine signal, accelerates the formation of intestinal tumors initiated by dedifferentiation.\(^{24}\) Dedifferentiated cells arising as a result of inflammation may easily acquire cancer-related properties. Given that aberrant de novo DNA methylation is observed at some loci upon aging\(^{55}\) and that DNA methylation at promoters and enhancers is generally associated with gene silencing, it is also possible that somatic cells in children and/or progenitor cells in fetuses may have higher plasticity than those of adults, which may render them more prone to dedifferentiation, thus leading to the development of childhood cancers. Together, the possibility of naturally occurring dedifferentiation is worth further exploration.

Whether the acquisition of epigenetic signature associated with cellular reprogramming is involved in cancer progression (not only initiation) in adults is also an open question. Poorly differentiated aggressive cancers often have an ESC-like transcriptional signature.\(^{39}\) For example, *SALL4*, one of the major pluripotency-related genes, can be expressed in cancers derived from several organs and its expression is often correlated with a worse prognosis.\(^{56–58}\) However, the mechanism for the acquisition of this phenotype has remained unclear. Currently, we are exploring the role of the epigenetics-mediated acquisition of the ESC-like signature on cancer progression by employing genetic cancer models with the *in vivo* reprogramming system.

We have also incorporated reprogramming technology into the *Apc* min/+ mouse model to dissect the cancer epigenome in multistage cancer development.\(^{59}\) Colon tumor cells with a loss of *Apc* function in this model were reprogrammed into iPS-like cells. Surprisingly, the majority of genes that were affected by *Apc* mutations in reprogrammed tumor cells (RTCs) did not overlap with genes affected in the intestine, suggesting cellular context-dependent gene regulation in response to oncogenic mutations. Notably, RTC-derived differentiated cells exhibited neoplastic growth exclusively in the
intestine, but not in other tissue types in vitro or in vivo. This phenotype suggests that cancer cell properties are largely dependent on cellular context, and that cell type-specific epigenetic control is important for the maintenance of cancer properties. Furthermore, the majority of intestinal lesions in RTC-derived mice remained as microadenomas. The results suggest that genetic mutations in tumor cells are not sufficient for full-blown tumor development, thus underscoring the significance of epigenetic regulation during multistage cancer development.

**Future directions of in vivo reprogramming**

Cellular reprogramming technology provides a unique tool for dissecting epigenetic regulation in cancer cells. However, this technology cannot control specific loci that are epigenetically altered. Epigenome editing, namely, locus-specific modulation of the epigenetic status, has become feasible and will contribute to a better understanding of epigenetic regulation in various biological phenomena.60),61) Future studies combining the technology of in vivo reprogramming with that of epigenome editing could clarify the locus responsible for the phenotypes observed in in vivo reprogrammable mice and uncover the detailed molecular mechanisms of pathological changes at the organismal level.

Aging is one of the major risk factors for various diseases including cancer.62) Notably, somatic cell reprogramming into iPSCs resets cellular damage-, stress- and senescence-associated epigenetic marks in vitro, suggesting that reprogramming technology can induce the rejuvenation of senescent cells. Several intriguing studies have employed in vivo reprogramming to investigate cellular senescence
and organismal aging. Mosteiro et al. have shown that reprogrammed cells in vivo appear in close proximity to clusters of senescent cells in reprogrammable mice. Interestingly, these senescent cells promote reprogramming through senescence-associated secretory phenotypes (SASP), especially by producing IL-6. These results, which have been supported by other studies, provide the functional cross-talk between cellular reprogramming and senescence. Although considerable progress in understanding cellular senescence has been made in vitro, it remains largely unknown how cellular senescence affects the cellular microenvironment (i.e., surrounding cells and the stroma), organ function, and organismal aging. Considering that the prevention and treatment of age-related diseases are important health issues, detailed studies of cellular senescence in relation to in vivo reprogramming will undoubtedly contribute to unveiling age-related functional impairment in the human body and eventually to the comprehensive understanding of age-related diseases, including not only cancer, but also non-malignant diseases.

Closing remarks

Although the rationale for dedifferentiation as a driver for cancer development has been unclear, previous studies have suggested the involvement of dedifferentiation in the development of diverse cancer types. Recent studies utilizing iPSC technology, which can initiate global changes in epigenetic modifications without affecting the underlying genome information, have provided strong evidence for a causative and primary role of dedifferentiation-associated epigenetic regulation in cancer development. Further studies aiming to identify external stimuli that induce a loss of somatic cell identity should unveil the role of dedifferentiation as a natural causative phenomenon in human cancer development. Investigations using in vivo reprogramming would be useful for developing novel strategies aimed at both the treatment and prevention of cancer and age-related diseases.

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Profile

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