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Review

Epigenetic regulation leading to induced pluripotency drives cancer development *in vivo*

Kotaro Ohnishi [a,b], Katsunori Semi [a,c], Yasuhiro Yamada [a,c,*]

[a] Center for iPS Cell Research and Application (CiRA), Kyoto University, Kyoto 606-8507, Japan
[b] Department of Medicine, Gifu University Graduate School of Medicine, Gifu 501-1194, Japan
[c] Institute for Integrated Cell-Material Sciences (WPI-iCeMS), Kyoto University, Kyoto 606-8507, Japan

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ABSTRACT

Somatic cells can be reprogrammed into induced pluripotent stem cells (iPSCs) by the transient expression of reprogramming factors. During the reprogramming process, somatic cells acquire the ability to undergo unlimited proliferation, which is also an important characteristic of cancer cells, while their underlying DNA sequence remains unchanged. Based on the characteristics shared between pluripotent stem cells and cancer cells, the potential involvement of the factors leading to reprogramming toward pluripotency in cancer development has been discussed. Recent *in vivo* reprogramming studies provided some clues to understanding the role of reprogramming-related epigenetic regulation in cancer development. It was shown that premature termination of the *in vivo* reprogramming result in the development of tumors that resemble pediatric cancers. Given that epigenetic modifications play a central role during reprogramming, failed reprogramming-associated cancer development may have provided a proof of concept for epigenetics-driven cancer development *in vivo*.

Contents

1. Introduction

During cellular reprogramming, multiple cellular processes act synergistically in a sequential manner [1–3]. However, the precise mechanism underlying the process of somatic cell reprogramming toward pluripotent stem cells remains unclear [4]. The dynamic reorganization of epigenetic modifications is considered to play a fundamental role in inducing pluripotency, leading to iPSC derivation [5]. Indeed, the functional importance of epigenetic regulation during the reprogramming process has been highlighted by recent studies showing that epigenetic modifications represented a bottleneck for iPSC derivation in both the early and late stages of cellular reprogramming [6,7]. The fact that the DNA methylation status at imprinted loci is associated with the quality of iPSCs supports the notion that proper remodeling of epigenetic modifications is essential to achieve successful reprogramming [8].

The efficiency of iPSC derivation is generally low, and only limited populations of intermediate cells give rise to iPSCs, which hampers the complete understanding of the reprogramming

* Corresponding author at: Center for iPS Cell Research and Application (CiRA), Kyoto University, 53 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan. Fax: +81 75 366 7093.
E-mail address: y-yamada@cira.kyoto-u.ac.jp (Y. Yamada).

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process [9]. It is known that non-iPSC like colonies often appear in culture dishes at the intermediate stage of cellular reprogramming *in vitro*. Although cells deviated from successful reprogramming contain metastable cells [10–12], the nature of such failed reprogramming state is unclear. Considering the fundamental role of epigenetic regulation in cell fate maintenance and conversion, it is expected that the failed reprogramming is attributable to the incomplete/unsuccessful reorganization of the epigenetic modifications.

It is noteworthy that the process of cell reprogramming toward a pluripotent state shares many characteristics with cancer development, although the process is not accompanied by the genetic alterations that are believed to be the causative abnormalities in most cancers. Somatic differentiated cells acquire self-renewing activities with unlimited proliferation properties during iPSC derivation, which is also a critical event during carcinogenesis. The metabolic switch to glycolysis that occurs during somatic cell reprogramming is similarly observed during cancer development [13,14]. Furthermore, Ben-Porath et al. demonstrated that a subgroup of transcriptional regulators that are highly expressed in embryonic stem cells (ESCs) are preferentially expressed in poorly differentiated aggressive tumors, suggesting that pluripotency-related transcriptional activity contributed to the development of particular types of cancer [15]. Such similarities suggest that the reprogramming process and cancer development may be at least partly promoted by overlapping mechanisms.

Indeed, previous studies demonstrated that a loss of tumor suppressor function is associated with the efficient induction of pluripotency [16,17]. Additionally, the currently used reprogramming factors generally include *Myc* and *Klf4*, both of which act as oncogenes in particular somatic cells. *Oct3/4*, a critical factor for somatic cell reprogramming, is overexpressed in germ cell tumors (GCTs) [18], and it is functionally involved in the self-renewal of GCT cells, possibly through the maintenance of an undifferentiated state [19]. Together, these findings may provide a link between transcription factor-mediated reprogramming and cancer development.

To investigate the relationship between cellular reprogramming and cancer development, it would be useful to analyze the reprogramming process in the *in vivo* environment. However, most of the previous studies investigating cellular reprogramming have been performed *in vitro*, and it has been difficult to adapt the *in vitro* phenotypes to uncover the association of reprogramming with cancer development *in vivo*.

2. *In vivo* expression of *Oct3/4* results in the expansion of dysplastic cells

Oct3/4 is highly expressed in pluripotent stem cells and is one of the essential transcription factors required for cellular reprogramming [1,2]. Hochedlinger et al. previously induced *Oct3/4* in various cell types in living mice *in vivo* [20]. Transgenic mice with a reverse tetracycline transactivator at *Rosa26*, together with a doxycycline (Dox)-responsive element, followed by the *Pou5f1* (*Oct3/4*) gene at *Collagen 1a1* were used in their study. The Dox-treated *Oct3/4*-induced mice developed dysplasia in many epithelial tissues, including the intestine, skin and forestomach [20]. Importantly, the dysplastic growth was dependent on transgenic *Oct3/4* expression, since withdrawal of Dox treatment resulted in a reversion of the dysplastic cells into normal-looking cells. These dysplastic cells revealed similar properties to tissue stem/progenitor cells, suggesting that the maintenance of the proliferating tissue stem/progenitor state caused the expansion of the dysplastic cells. The dysplastic cell expansion was accompanied by the upregulation of β-catenin, which is observed in a wide variety of cancers [21–23]. However, such dysplastic lesions did not show any evidence of metastasis, which is one of the hallmarks of cancer. Considering the critical role of *Oct3/4* in cellular reprogramming, this dysplasia model implied a possible association of reprogramming with cancer development. However, it should be noted that *Oct3/4* alone is not sufficient to reprogram most somatic cells. Therefore, the results did not provide direct evidence that somatic cells are reprogrammed toward a pluripotent state *in vivo*, nor that reprogramming itself is involved in cancer development.

3. *In vivo* reprogramming

In contrast to the results in *Oct3/4* single-induced mice, a later study by the Hochedlinger group using four reprogramming factors (*Oct3/4*, *Klf4*, *c-Myc*, and *Sox2*)-inducible mice suggested the possibility that somatic cells can be reprogrammed *in vivo* [24]. The reprogrammable mice frequently developed spontaneous teratomas, presumably because of leaky expression of the reprogramming factors. Given that pluripotent stem cells can form teratomas when inoculated into immunocompromised mice, the teratoma formation in the reprogrammable mice suggested that iPSCs arose *in vivo*, and these could give rise to teratomas. This notion has been further proven by recent studies with the controlled expression of reprogramming factors *in vivo* [25,26]. For example, Abad et al. showed that circulating cells in the four reprogramming factors-induced mice contain pluripotent cells capable of contributing to chimeric mice. We also demonstrated that iPSC-like cells could be established from teratomas in the four factors-induced mice, and such iPSC-like cells may indeed have pluripotency, with the ability to robustly contribute to chimeric mice. Collectively, these studies revealed that somatic cells can be reprogrammed by the transduction of four factors *in vivo*, and suggested that the *in vivo* reprogramming system could be a unique and powerful tool to study the *in vivo* behavior of failed reprogrammed cells, as well as fully reprogrammed cells. It is important that teratomas are benign tumors. Therefore, while these results demonstrated that *in vivo* reprogramming induces benign teratoma formation, they did not connect cellular reprogramming with malignant tumor development.

4. Premature termination of *in vivo* reprogramming causes malignant tumor development

Notably, a phenotype distinct from the teratoma formation was observed in mice with transient expression of the four reprogramming factors [26]. It was shown that the initial histological change after the induction of reprogramming factors *in vivo* is the expansion of dysplastic cells in many organs. Interestingly, the early dysplastic cells were able to revert to a non-dysplastic phenotype upon the withdrawal of reprogramming factor expression, which was similarly observed in the single *Oct3/4*-induced mice (Fig. 1). This is not surprising, because *Oct3/4* was one of the factors induced in these mice. However, in sharp contrast to the *Oct3/4*-induced dysplastic cells or the four factors-induced early dysplastic cells, the four factors-induced late dysplastic cells (Dox treatment for longer than 7 days) often did not revert to a non-dysplastic state, and many of these cells continued to grow even after the withdrawal of the transgene expression (Fig. 1). Tumor formation was also observed in some organs, such as the intestine, kidneys and pancreas. These tumors were able to form secondary tumors in the subcutaneous tissues of immunocompromised mice. Furthermore, the tumor cells revealed invasive growth into surrounding tissue and metastasis into lymph nodes, both of which are known to be a hallmarks of cancer [27]. Similar transgene-independent tumors were induced by three factors lacking the *Myc* transgene, but not by three factors without the *Oct3/4* transgene, a critical

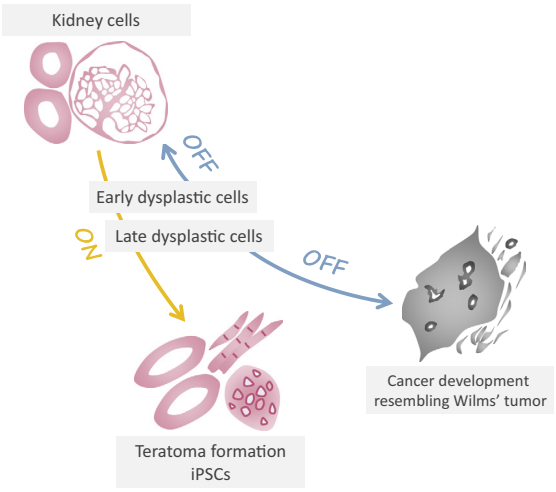

Fig. 1. *In vivo* induction of four reprogramming factors. Somatic cells can be reprogrammed into pluripotent stem cells *in vivo*, which is accompanied by teratoma formation. Dysplastic cell growth in somatic cells can be detectable in the intermediate stage of *in vivo* reprogramming. The early dysplastic cells revert to a non-dysplastic cells upon the withdrawal of the reprogramming factor expression. In contrast, the late dysplastic cells do not revert to a non-dysplastic state, and they continue to grow even after the withdrawal of the transgene expression, resulting in development of cancer, which resembles pediatric cancers, such as Wilms' tumors.

factor for reprogramming, demonstrating a tight connection between cancer development and reprogramming. Taken together, these findings indicated that premature termination of *in vivo* reprogramming results in cancer development, providing *in vivo* experimental evidence that failure in reprogramming into PSCs can drive cancer development.

5. Epigenetic regulation in failed reprogramming-associated cancer cells

The previous study dissected the ESC transcriptional program into three distinct modules; the Core, Myc, and Polycomb repressive complex (PRC) modules [28]. The ESC-Core module often includes genes related to the maintenance of pluripotency, while the ESC-Myc module contains a group of Myc target genes in ESCs. The ESC-PRC module is generally repressed in ESCs, and includes the genes that are bound by the Polycomb repressive complex in pluripotent stem cells. Of note, it was shown that the ESC-Core module and ESC-Myc module are similarly activated in both failed reprogramming-associated cancer cells and pluripotent stem cells when compared with those in normal kidney cells, indicating that there are shared characteristics between failed and successful reprogramming processes (Fig. 2). In contrast, many ESC-PRC module genes are aberrantly activated exclusively in failed reprogramming-associated cancer cells (Fig. 2). Since partially reprogrammed cells generated *in vitro* also reveal the failed repression of ESC-PRC module genes [12], the cancer cells in this model possess similar characteristics to the partially reprogrammed cells, suggesting that the partial reprogramming is associated with the failed reprogramming-associated cancer development.

However, aberrant activation of ESC-PRC target genes in cancer cells appears to contradict the current understanding of cancer epigenetics (Fig. 2). Previous studies found that ESC-PRC target genes are preferential targets for aberrant DNA hypermethylation in cancers [29–31]. Considering the repressive role of DNA hypermethylation at gene promoters, it would be expected that ESC-PRC targets are silenced in these cancers. Indeed, the previous study demonstrated that PRC2 target genes tend to be repressed in poorly differentiated cancers [15].

Altered DNA methylation patterns are the most extensively analyzed epigenetic abnormality in cancer cells [32]. Site-specific DNA hypermethylation and global DNA hypomethylation are shared alterations in the vast majority of cancers [33,34]. Indeed, in failed reprogramming-related cancers, a reduced representative bisulfite sequencing [35] analysis revealed global changes in the DNA methylation patterns (Fig. 2). However, in these failed reprogramming-related cancers, DNA hypermethylation at the proximal promoter regions was not evident, and the global DNA methylation level was comparable to that of normal cells, indicating a lack of both site-specific DNA hypermethylation and global DNA hypomethylation. Collectively, failed reprogramming-associated cancers showed distinguishable epigenetic properties from general cancers.

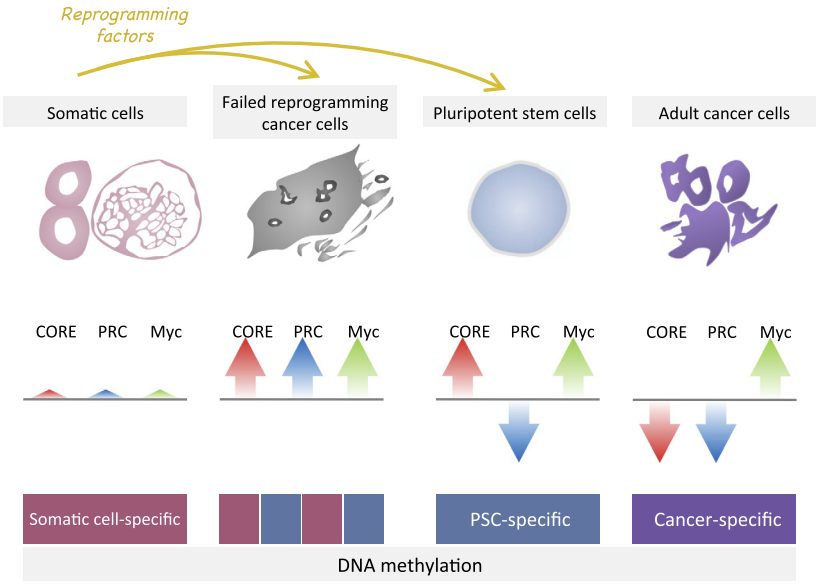

Fig. 2. Epigenetic regulation in failed reprogramming-associated cancer cells. The failed reprogramming-associated cancer cells reveal the different characteristics in epigenetic modifications from pluripotent stem cells or adult cancer cells. They show the activation of ESC-PRC module and the lack of cancer-specific DNA methylation alterations.

These discrepancies in epigenetic regulation raise an important concern regarding the application of this cancer model to investigate human cancer development. Given that failed reprogramming-associated cancers were induced through ectopic expression of four reprogramming factors that are not simultaneously expressed in somatic cells, these cancers are artificial, making the model less useful to uncover the mechanisms of human cancer development. However, it is noteworthy that failed reprogramming-associated cancers have a number of shared characteristics with pediatric cancers. Intriguingly, failed reprogramming-associated cancers in the kidney resembled Wilms' tumors, the most common pediatric kidney cancer. Additionally, the histology of liver tumors and pancreatic tumors showed similarities to those of hepatoblastomas and pancreatoblastomas, respectively. Of particular note, the ESC module activation in human Wilms' tumors shows a different pattern from that of adult cancers [26]. Wilms' tumors show the activation of all three modules (Core, Myc and PRC), recapitulating the findings in the mouse model. The failed reprogramming-associated cancer model could therefore be a model for pediatric cancers.

6. Genetic and epigenetic abnormalities in pediatric cancers

Cancer is considered to progress through multistep processes, which are associated with both genetic mutations and epigenetic abnormalities [27,32,36]. However, much attention has been paid to the role of epigenetic abnormalities in Wilms' tumors. Although some genetic abnormalities, such as *WT1*, *WTX* and *TP53* gene mutations [37,38] have been reported in Wilms' tumors, the incidence of these mutations is relatively low. In contrast, seminal studies identified that there are frequent epigenetic alterations in Wilms' tumors [39,40]. For example, the aberrant expression of *IGF2* with loss of imprinting, which is accompanied by increased DNA methylation at *IGF2*/*H19* locus, was identified in the majority of Wilms' tumors [40]. It is interesting to note that failed reprogramming-associated cancers in mice also showed abnormal DNA methylation at the *IGF2*/*H19* locus, with a number of shared molecular characteristics with Wilms' tumor. Similar types of epigenetic regulation between induced pluripotent cells and Wilms' tumor were further indicated by the previous findings that the chromatin profiles of Wilms' tumors resembled those of human ESCs [41]. Together, these findings indicated that Wilms' tumors harbor similar epigenetic changes as failed reprogramming-associated cancers induced by the transient expression of reprogramming factors. It is also noteworthy that some of epigenetics-related diseases such as imprinting disorders, Beckwith–Wiedemann syndrome and Angelman syndrome, are associated with pediatric blastomas [42,43]. Of note, the failed reprogramming-associated cancers in the liver and pancreas resembled hepatoblastomas and pancreatoblastomas, and the liver cancers were accompanied by the alteration of imprinting gene expressions. These findings provided a further connection between failed reprogramming-induced epigenetic regulations and pediatric blastoma development.

Recently, genome-wide sequencing studies have been performed in many types of cancers. These studies revealed that the rate of mutation is relatively low in most of the cancer-related genes [44]. A surprisingly low rate of genomic mutations was observed in pediatric cancers. Together with a previous finding regarding the close association between altered epigenetic modifications and the poor prognosis of pediatric cancers [45], the epigenetic changes associated with failed reprogramming might drive the development of particular types of pediatric cancers. Whole genome sequencing studies in ependymomas suggested that some ependymomas could occur without detectable driver mutations [46,47]. Notably, the rate of single nucleotide variants was very low in the ependymomas that occurred in the posterior fossa, while DNA hypermethylation was observed in progressive ependymomas, which support the notion that specific types of tumors might arise depending on the specific alterations in the epigenetic information.

7. Epigenetic reorganization toward pluripotency drives cancer development

Although altered epigenetic regulation can be detected in most cancer types, such epigenetic alterations may be attributable to the genetic mutations as a secondary effect. Consistent with this notion, somatic mutations at genes known to regulate the epigenetics have been often identified in a wide range of cancer types. For example, some glioblastomas harbor mutations in the H3.3-ATRX-DAXX pathway [48,49]. A mutation was detected in H3F3A, and was located at or just near the amino-terminal tail of the protein, which leads to post-transcriptional repression or activation.

It is of particular interest that failed reprogramming-associated cancer cells could be reprogrammed into iPSCs by the additional expression of reprogramming factors [26]. Notably, kidney cancer-derived iPSCs could contribute to adult chimeric mice that had normal-looking kidney tissues. Furthermore, tumor formation was not observed even in the elderly chimeric mice. Given that the DNA sequence remains unchanged during the reprogramming and re-differentiation of the reprogrammed cells, these findings indicated that the failed reprogramming-associated cancer genome is competent for normal development, and that genetic transformation is not a determinant of cancer development in this model. This further highlighted the primary and causative role of epigenetic regulations associated with induced pluripotency in the development of particular types of cancer.

However, it should be stressed that these findings do not provide evidence that the dedifferentiation process itself is involved in the development of human cancers, including Wilms' tumors. Further studies will be needed to identify the cancer types that are promoted by reprogramming-associated epigenetic changes. A better understanding would provide novel insights into effective cancer treatment targeting epigenetic regulation.

8. Conclusion

iPSC induction is accompanied by the acquisition of unlimited proliferative activity in somatic cells. Since epigenetic regulation plays a central role in the reprogramming process, it is suggested that epigenetic regulation leading to induced pluripotency confers self-renewing activity on somatic cells. Indeed, transient expression of reprogramming factors *in vivo* resulted in the development of cancer, independent of genetic transformation. A better understanding of the epigenetic regulation involved in induced pluripotency should be applicable to dissect the role of epigenetic regulation in cancer development, which eventually may contribute to the discovery of novel preventive or therapeutic strategies for cancers.

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