れ，線維肉腫では*Nf1*；*p53*のLOHが検出されたことから，DNA低メチル化によるゲノム不安定性を背景とする発癌促進作用が，個体レベルにおいても示されたものと考えられる．このように，同じDNA低メチル化モデルマウスでも，発癌モデルや対象臓器によってまったく反対の発癌修飾作用を示し，DNAメチル化修飾によるエピジェネティック制御は発癌に複雑に関与していることが予想されている．

Loss of imprintingと発癌

DNAメチル化異常が関与するエピジェネティック異常にloss of imprinting（LOI）があり，癌をはじめとするさまざまな疾患への関与が示唆されている．Holmら[10]は，一過性に*Dnmt1*の発現を停止させることで，インプリンティングに異常をもったimprinting-freeマウスES細胞（IF-ES細胞）の作製に成功した．IF-ES細胞由来のIF-マウス胎仔線維芽細胞（mouse embryonic fibroblast：MEF細胞）は，コントロールMEF細胞にくらべ増殖能が高く，免疫不全マウスでの造腫瘍能が確認された．IF-ES細胞から作製されたキメラマウスでは，自然発生腫瘍の多発がみられた[10]．癌細胞に観察されるLOIが，実際に生体で発癌促進的にはたらく結果が示され，エピジェネティック異常と発癌促進の関連をさらに深めた実験と考えられる．この結果は，Sakataniら[11]が報告したinsulin-like growth factor-2（*Igf2*）LOIモデルのMinマウスにおける腸管腫瘍形成促進を示した結果とともに，LOIの発癌促進作用を個体レベルで示したものと考えられる．

DNA低メチル化による相反する発癌修飾作用

*Dnmt1*低発現によるDNA低メチル化マウスを用いた実験から，DNA低メチル化は，臓器特異的に発癌修飾作用が異なることがわかった[8)12)~15)]．この一見矛盾する現象を理解するために，われわれはDNA低メチル化モデルのMinマウス腸管発癌への修飾を再検討した．その結果，DNA低メチル化は，過去の報告のようにMinマウスの大腸腫瘤の発生を抑制するものの，早期病変である粘膜内微小腺腫の発生を逆に促進することが明らかとなった[12]．増加した微小腺腫には高頻度に*Apc* LOHが検出され，DNAメチル化レベルの低下により*Apc* LOHの頻度が上昇していることが示唆された[12]．さらに，DNA低メチル化Minマウスでは肝腫瘍が多発し，DNA低メチル化による腫瘍促進作用がつぎつぎに観察された[12]．以上のように，強制的なDNAメチル化レベルの低下は，臓器特異性の修飾のみならず，発癌ステージ特異的な修飾作用を示すことがわかった．

*Dnmt3b*と大腸発癌修飾作用

DNAにメチル基を新たに付加する*Dnmt3b*のコンディショナルノックアウトマウスでは，粘膜内大腸微小腺腫から大腸腫瘤への進展が強く抑制される．一方，*Dnmt3b*の強制発現マウスでは，粘膜内大腸微小腺腫から大腸腫瘤への進展が促進されることが報告されている．*Dnmt3b*強制発現マウスに発生した腫瘍には，*H19* differentially methylated region（*H19* DMR），およびsecreted frizzled-related protein 2（*Sfrp2*），*Sfrp4*，*Sfrp5*の異常DNAメチル化が認められ，DNA高メチル化の積極的な発癌への関与を示す結果と考えられた．*Dnmt3b*の強制発現モデルでは，組織学的に正常にみえる大腸粘膜にもすでに大腸腫瘍に検出されたDNAメチル化異常が観察された[16]．異常DNAメチル化を示す正常様粘膜は，発癌母地としての重要性が示唆される．この現象は，epigenetic field of cancerizationの概念[17]にも応用でき，癌予防や発癌リスク評価などの観点からも興味深いと考えられる．

細胞初期化技術を用いた癌細胞のエピジェネティクス研究

癌細胞におけるエピジェネティック修飾の重要性を示した研究で，癌細胞の核移植実験がある[18]．核移植により作製された*RAS*発現メラノーマと同じ遺伝子配列異常を有するES細胞から作製されたキメラマウスに非腫瘍性のメラノサイトが形成された事実は，エピジェネティック修飾状態の変化は，遺伝子配列異常を有する癌細胞の異常増殖をも停止させうることを明確に示した．

一旦形成された遺伝子配列異常は不可逆的であると考えられるのに対し，エピジェネティック修飾は改変可能であることから，エピジェネティック修飾を標的とした癌予防，および癌治療の可能性が検討されている．一方でTakahashiら[19)20)]による人工多能性幹細胞（induced pluripotent stem cell：iPS細胞）作製技術は，細胞のエピジェネティック修飾状態を積極的に改変させうる技術ととらえることができる．この技術を癌細胞に応用し，癌細胞のエピジェネティック修飾状態を積極的に変化させることで，癌細胞の性質を大きく変えることができるかもしれない．細胞リプログラミングの技術の進歩および詳細なメカニズムの解明は，癌細胞のエピジェネティック異常解除の突破口となり，癌治療や発癌予防の開発へ発展できるかもしれない．

おわりに

マウスモデルを用いた癌エピジェネティクス研究によって，生体でのエピジェネティック修飾異常による発癌修飾作用が明らかとなってきた．その一方，改めてエピジェネティック修飾自体の複雑性も浮かび上がってきた．今後の研究発展には，正常細胞を含めたエピジェネティック制御メカニズムの解明が不可欠であると考えられる．本稿で注目したDNAメチル化以外にも，クロマチン構造変化にかかわる数々のエピジェネティクス制御因子を俯瞰的にとらえながら，癌細胞でのエピジェネティック異常を個体レベルで明らかにすることにより，より安全で効果的な治療法開発が可能となるだろう．細胞のエピジェネティック修飾状態を自由に，かつ領域特異的に改変しうる技術の確立が期待される．

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

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Abstract

Activation-induced cytidine deminase (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

domain (CD) of Tet1 (1367–2039 amino acids: aa), Tet2 (1044–1921aa) and Tet3 (697–1668aa) were generated by subcloning of the DNA fragments into BamHI and NotI sites for Tet1, EcoRI and XhoI sites for Tet2 or EcoRI and NotI sites for Tet3 of pcDNA4HisMax. Plasmids encoding Xpress-tagged mutants deficient in the catalytic domain (ΔCD) of Tet1 (1–1366aa), Tet2 (1–1043aa) and Tet3 (1–696aa) were also generated. The Aid mutants, AidΔNES (1–187aa) and AidΔN26 (27–198aa), were subcloned into BamHI/XhoI-digested pcDNA4MycHis. We used the KOD plus mutagenesis kit (TOYOBO) to generate a point mutant for Aid (F193A) and mutants for the Tet family catalytic domain, which include Tet1CDm (D1652Y, D1654A), Tet2CDm (H1304Y, D1306A) and Tet3CDm (H950Y, D952A).

Cell culture and cDNA transfection

Human embryonic kidney cells (HEK293FT) (Invitrogen) and human colon cancer cells (DLD-1) (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal bovine serum. Both of them were transiently transfected with plasmid DNA by FuGENE6 (Promega) according to the manufacturer's instructions, followed by immunofluorescence or co-immunoprecipitaion 48 h post-transfection, unless otherwise noted.

Immunofluorescence

The cells were fixed and permeabilized with cold 100% methanol for 10 min on ice. For 5hmC staining, permeabilized cells were treated with 4 N HCl for 10 min, followed by 1.5 M Tris-HCl (pH 8.8) treatment for 10 min, before being blocked with 1% BSA. The cells were incubated with primary antibodies; anti-Xpress mouse monoclonal antibody (mAb) (Life Technologies), anti-Myc rabbit polyclonal antibody (MBL), anti-5hmC rabbit polyclonal antibody (Active Motif) or anti-Aicda rabbit polyclonal antibody (Abcam) overnight at 4°C, followed by Alexa Fluor-conjugated secondary antibodies (Life Technologies) for 1 h, and DAPI staining for 5 min at room temperature. After washing with PBS containing with 0.05% Tween 20, the samples were mounted by using the Prolong Gold Antifade Reagent (Life Technologies), followed by curing on a flat surface in the dark overnight at 4°C. For four color staining, a Zenon Alexa Fluor labeling kit (Life Technologies) was used. The images were captured by a confocal laser microscope (OLYMPUS, FV1000). To score the subcellular localization in DLD-1 cells, we counted all of the fluorescence positive cells on 4-well chamber dishes (BD). When using HEK293FT cells, we counted cells in randomly acquired fields on the 4-well chamber dishes. In the case of co-transfection, co-expressed cells were counted and scored according to the Tet localization. Scoring of the subcellular localization was performed as indicated in Fig. S1.

Immunoprecipitation and immunoblotting

Transfected HEK293FT cells were lysed in EBC buffer (50 mM Tris-HCl pH 8.0, 120 mM NaCl and 0.5% NP40 for detecting Xpress-tagged protein, or 1.0% NP40 for detecting Myc-tagged protein) with a protease inhibitor cocktail (SIGMA). The cell lysates were incubated for 3 h at 4°C with Dynabeads M280 sheep anti-mouse IgG (VERITAS) which had been pre-treated with an anti-Xpress mAb or an anti-Myc mAb (Enzo life science) for 1 h. After washing the immunoprecipitates four times with EBC buffer, the beads were boiled with Laemmli SDS-sample buffer. This supernatant was separated by SDS-PAGE and transferred to a PVDF membrane (Millipore). For immunoblotting of the Xpress-tagged protein, after the membrane were blocked with 2% nonfat dry milk in PBS containing 0.05% Tween20, they were incubated with an anti-Xpress mAb followed by anti-mouse IgG antibody conjugated to HRP specific for naive IgG (Novagen). For Myc-tagged protein blotting, after being blocked, the membrane was incubated with anti-Myc antibody conjugated to HRP (MBL). Each antibody was diluted in Can Get Signal for immunoblotting (TOYOBO). Protein bands were visualized using the Pierce ECL plus Western Blotting Substrate (Thermo), and detected with a LAS4000 instrument (GE HealthCare).

Statistic analysis

The statistical significance of differences between two groups was determined by the Mann-Whitney U test. A value of $p<0.05$ was considered to be statistically significant. The numbers of samples are referred to as "n" in each graph.

Results

Aid alters the subcellular localization of Tet1 from the nucleus to the cytoplasm

A previous study showed that Tet family proteins generate 5hmC, whereas Aid facilitates the conversion of 5hmC into cytosine [9]. In this study, we investigated the effect of simultaneous expression of Aid and Tet on their subcellular localization. We transfected C-terminally Myc-tagged Aid or N-terminally Xpress-tagged Tet1 into HEK293FT or DLD-1 cells, and examined the subcellular localization of ectopically expressed proteins. Aid was observed mainly in the cytoplasm, whereas Tet1 was predominantly localized in the nucleus when the single proteins were overexpressed. When cells were co-transfected with expression plasmids for Aid and Tet1, the Tet1 localization was altered from the nucleus to the cytoplasm in the co-transfected cells, whereas Aid remained in the cytoplasm (Fig. 1).

To determine the domain responsible for the altered localization of Tet1, we performed a subcellular localization analysis using a series of deletion constructs for Tet1, as previously reported [17]; full length (FL) (1–2039 amino acids: aa) which was used in the experiment shown in Fig. 1, the catalytic domain (CD) (1367–2039aa), and the N-terminal domain (ΔCD) (1–1366aa), which lacks CD (Fig. 2A). The Tet1FL plasmid and these mutants were transfected individually with or without the plasmid for Aid. At 48 hrs after transfection, the subcellular localization of Tet1 and Aid was examined by confocal microscopy (Figs. 2B and S2). In the case of single transfection, all Tet1 mutants were predominantly localized in the nucleus, and Aid was mainly localized in the cytoplasm. However, when Tet1FL or Tet1CD was co-expressed with Aid, Tet1 was translocated to the cytoplasm (N: 0%, N+C: 9%, C: 91% for FL, and N: 18%, N+C: 35%, C: 47% for CD, respectively). In contrast, Tet1ΔCD remained in the nucleus even when co-expressed with Aid (N: 75%, N+C: 19%, C: 6%), suggesting that the catalytic domain of Tet1 plays a role in the altered localization of the protein (Figs. 2B and C). Since a previous study indicated that the subcellular localization of Aid is affected by the position of the tag [18], we also carried out co-transfection experiments using untagged Aid protein. It was confirmed that untagged Aid, as well as C-terminal-tagged Aid, also affected the localization of Tet1CD (Fig. S3), supporting the notion that Aid expression alters the subcellular localization of Tet1.

Next, to examine whether this effect is specific to Aid, we carried out the same experiments by using Apobec1 and Apobec2, instead of Aid, both of which show the similar enzymatic activity to Aid [19,20]. In particular, Apobec1 has been shown to shuttle between the nucleus and the cytoplasm [21]. We observed that overexpressed Apobec1 and Apobec2 were localized at both the

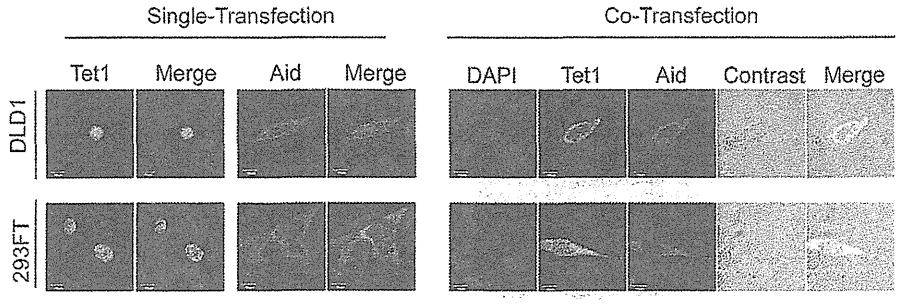

Figure 1. Overexpressed Aid alters the subcellular localization of Tet1. Images of cells transiently expressing N-terminally Xpress-tagged Tet1 or C-terminally Myc-tagged Aid. Tet1 was predominantly localized in the nucleus, whereas Aid was mainly localized in the cytoplasm 48 h after transfection in both DLD-1 and HEK293FT cells. When cells were co-transfected with a plasmid expressing Aid, the Tet1 subcellular localization was altered to the cytoplasm, where Aid was mainly localized. The scale bar is 10 μm.
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nucleus and the cytoplasm in DLD-1 cells regardless of the presence or absence of Tet1CD (Figs. S4). In contrast to the translocation of Tet1CD in the presence of Aid, Tet1CD always remained in the nucleus even when co-expressed with Apobec1 or Apobec2 (Figs. S4B and D, $p = 0.11$, with vs without Apobec1, $p = 0.38$, with vs without Apobec2). These results suggest that the altered subcellular localization of Tet1CD is not attributable to the artificial effects due to Aid overexpression.

Tet1 translocation is independent of its enzymatic activity

We observed that the subcellular localization of Tet1 was altered in the presence of Aid, but that Tet1ΔCD remained in the nucleus, implying that Tet1 enzymatic activity for the conversion of 5mC to 5hmC is associated with the translocation of Tet1. To test this, a Tet1CD mutant construct (CDm), which has mutations in the catalytic domain (D1652Y and D1654A) and lacks enzymatic activity, was generated (Fig. 3A) [17]. When Tet1CDm was solely transfected into DLD-1 cells, it was localized in the nucleus. However, the enzyme activity, which was detected by immunostaining for 5hmC, was not observed at all in Tet1CDm-expressing cells while it was evident in Tet1CD- and Tet1 FL-expressing cells (Fig. 3B). We also confirmed that Tet1ΔCD and Aid had no ability to produce 5hmC (Figs. 3B and S5A).

We next examined the subcellular localization of Tet1CDm when it was co-expressed with Aid in DLD-1 cells. Despite the lack of enzymatic activity in Tet1CDm, simultaneous expression of Aid and Tet1CDm caused the altered localization of Tet1CDm, and no significant difference in the localization of Tet1CD and Tet1CDm was observed when they were co-expressed with Aid ($p = 0.144$, CD vs CDm) (Figs. 2C and 3C). We obtained the similar observation using HEK293FT cells (Fig. S2). These results

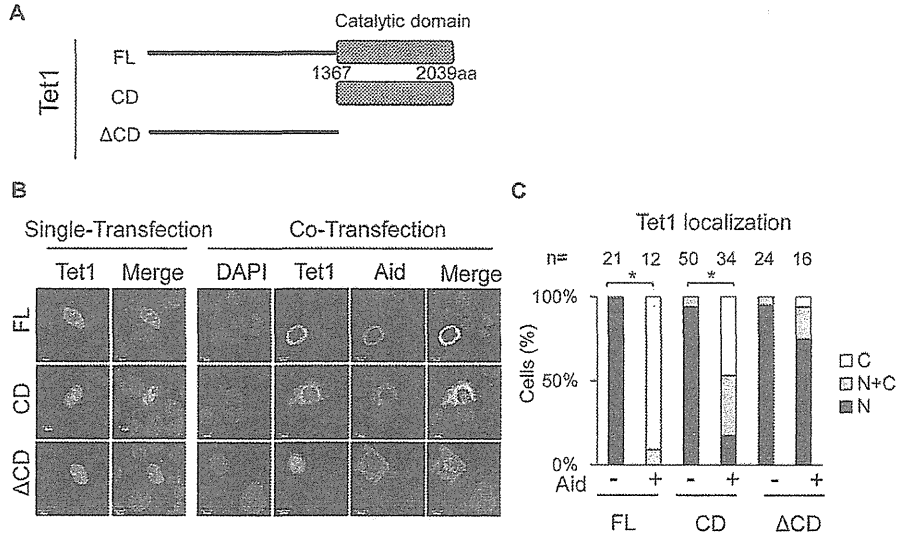

Figure 2. Tet1 translocation requires its catalytic domain. (A) A schematic representation of the Tet1 structure and its mutants used in this study. (aa = amino acid). (B) Confocal images of DLD-1 cells transiently expressing N-terminally Xpress-tagged Tet1 mutants with or without C-terminally Myc-tagged Aid. All Tet1 constructs (FL, CD and ΔCD) were localized in the nucleus when solely expressed in DLD-1 cells. When co-expressed with Aid, Tet1FL and Tet1CD were translocated to the cytoplasm, whereas Tet1ΔCD remained in the nucleus. (C) Each bar represents the proportion of cells with the different localizations of Tet1. The number (n) of cells indicated above each bar was scored according to their subcellular localization. N (black); nuclear localization, N+C (gray); both nuclear and cytoplasmic localization, C (white); cytoplasmic localization in multiple microscope fields. The scale bars in images are 10 μm. *$p<0.01$.
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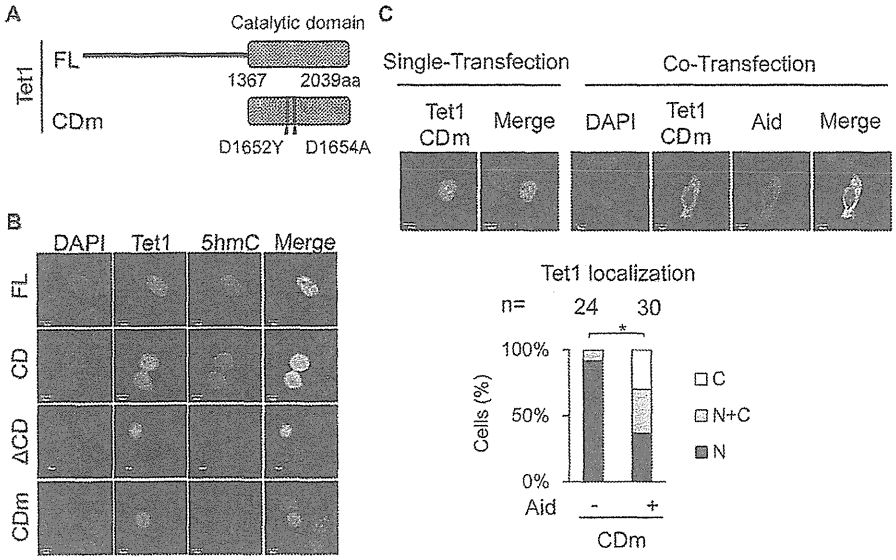

Figure 3. Tet1 translocation in the presence of Aid is independent of the Tet1 enzymatic activity. (A) A schematic representation of the Tet1CD mutant (CDm) used in this study. Tet1CDm were tagged with N-terminal Xpress. (B) Tet1FL and CD had enzyme activity and produced 5hmC, but Tet1ΔCD and CDm did not. (C) C-terminally Myc-tagged Aid expression altered the subcellular localization of Tet1CDm, which lacks the enzymatic activity. The upper panels are representative images of DLD-1 cells transiently expressing Tet1CDm with or without simultaneous expression of Aid. The lower graph shows the percentage score of the examined transfected cells (indicated as a number). The scale bars are 10 μm. *p<0.01. N (black); nuclear localization, N+C (gray); both nuclear and cytoplasmic localization, C (white); cytoplasmic localization in multiple microscope fields.
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indicate that, although the catalytic domain of Tet1 is important for the Aid-mediated translocation of Tet1, the translocation occurs independently of its enzymatic activity.

Co-expression of Aid has similar effects on the subcellular localization of other Tet family proteins

The Tet family proteins include Tet1, Tet2 and Tet3. We therefore examined whether the effects of Aid co-expression were also observed for other Tet family members. To perform these studies, FL, CD, ΔCD and CDm constructs for both Tet2 and Tet3 were generated (Fig. 4A) based on a previous report [17], and their subcellular localization in the presence or absence of Aid was examined. In both Tet2 and Tet3, the FL and CD proteins exhibited enzymatic activity, whereas the ΔCD and CDm mutants did not (Fig. S5B). Single expression of Tet2FL or its mutants led to the localization of the proteins primarily in the nucleus (Figs. 4B and C). However, in solely Tet3-expressing cells, Tet3ΔCD showed cytoplasmic localization, even in the single transfectants, although Tet3FL, Tet3CD and Tet3CDm were localized in both the nucleus and the cytoplasm (Figs. 4D and E), indicating that the catalytic domain of Tet3 is responsible for the nuclear localization of Tet3. Simultaneous expression of Aid and either of Tet FL, CD or CDm resulted in the altered subcellular localization of both Tet2 and Tet3 into the cytoplasm (Figs. 4B–E). These findings suggest that Aid alters the subcellular localization of all three Tet family proteins, and that this occurs independently of enzyme activity to produce 5hmC.

Translocation of Tet1 by Aid is associated with Aid shuttling

We next addressed how nuclear Tet1 is translocated into the cytoplasm by Aid. To understand the mechanism, we first examined the localization of both Tet1CD and Aid at different time points (10 h, 24 h, 48 h) after simultaneous transfection into HEK293FT cells. At 10 h after transfection, the subcellular localization of Tet1CD was mainly in the nucleus (N: 90%, N+C: 10%, C: 0%) while Aid was primarily expressed in the cytoplasm, showing the same localization pattern in the single transfected cells. At 24 h after transfection, the proportion of cells with cytoplasmic Tet1CD increased (N: 41%, N+C: 52%, C: 7%), and at 48 h after transfection, most of the Tet1CD were co-localized with Aid in the cytoplasm (N: 3%, N+C: 30%, C: 67%) (Figs. 5A and B). In contrast, Aid could be detected in the cytoplasm throughout this experiment.

It is worth noting that Aid is a shuttling protein that is translocating between the nucleus and the cytoplasm [12,13]. Since a gradual increase in the number of cells with cytoplasmic Tet1CD was observed, we evaluated whether Tet1 translocation is associated with Aid shuttling. We performed immunofluorescence experiment by using full length Aid (Aid FL) and its mutants which are impaired in nuclear-cytoplasmic shuttling; Aid lacking NES (AidΔNES_1-187aa), Aid having a single point mutation in the NES (Aid F193A) [22] [23] or Aid lacking the N terminus of Aid, which loses the important sequences for nuclear entry (AidΔN26_27-198aa) [12] (Fig. 6A). As expected, AidΔNES transfected cells revealed an increased number of cells with nuclear localization of Aid (ΔNES; N: 27%, N+C: 33%, C: 40%) when compared with Aid FL- transfected cells (N: 3%, N+C: 28%, C: 69%) (Figs. 6B and C). In addition, Aid F193A showed an increased localization of Aid at the nucleus (N: 23%, N+C: 43%, C: 34%) than Aid FL did. (Figs. 6B and D). Next, we co-transfected these Aid mutants with Tet1CD in DLD-1 cells and examined the effect of the expressions of Aid mutants on the Tet1CD subcellular localization (Figs. 6E and F). Co-expression with AidΔNES resulted in a decrease in the number of Tet1CD-translocated cells (N: 55%, N+C: 20%, C: 25%) compared with

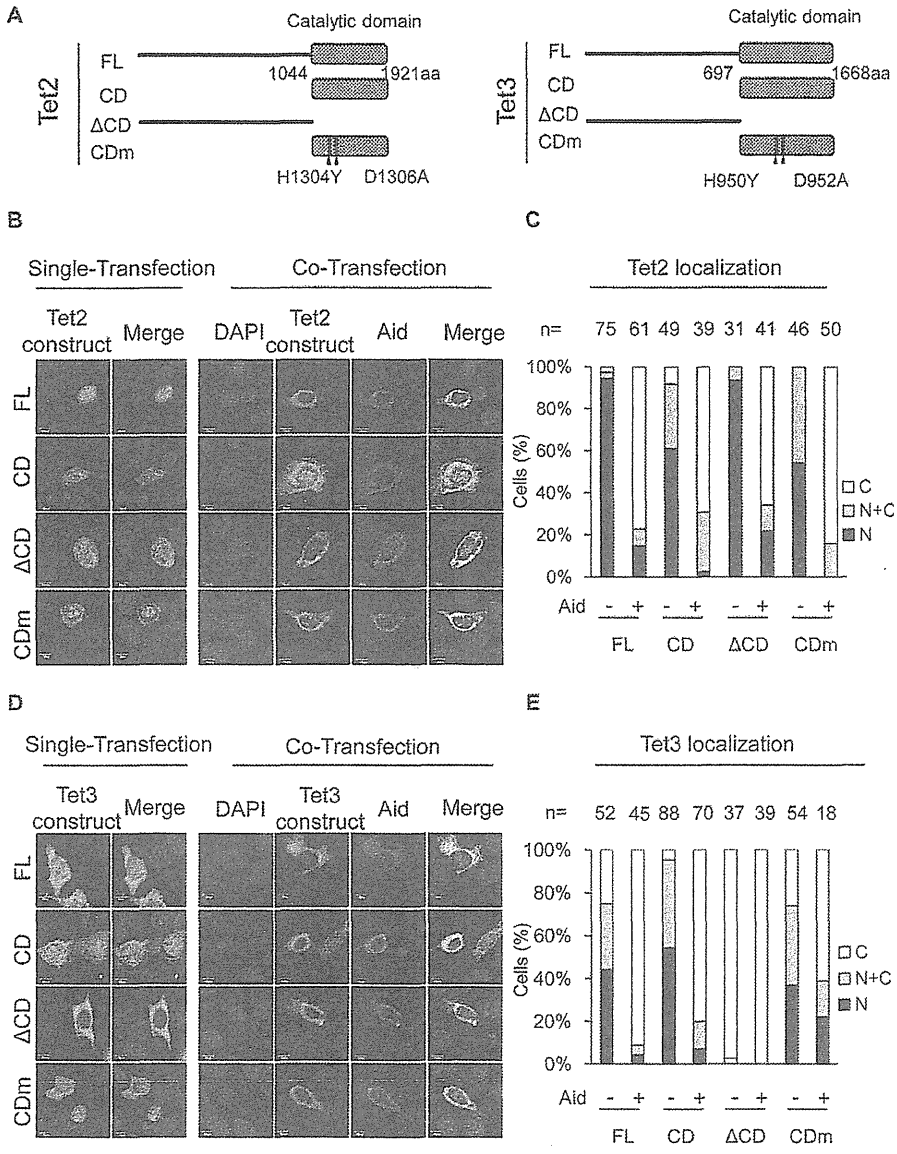

Figure 4. The subcellular localization of Tet2 and Tet3 is altered by Aid expression. (A) A schematic representation of the Tet2 and Tet3 structures and their mutants used in this study. (B) N-terminally Xpress-tagged Tet2 or its mutants with or without Aid tagged with C-terminal Myc were imaged by confocal microscopy in transiently transfected DLD-1 cells. (C) The number (n) of cells indicated above each bar was scored according to Tet2 subcellular localization. All Tet2 mutants were translocated to the cytoplasm in the presence of Aid ($p<0.01$, vs in the absence of Aid). (D, E) Simultaneous expression of N-terminally Xpress-tagged Tet3 and Aid-Myc. Tet3FL, CD and CDm were translocated to the cytoplasm when co-expressed with Aid ($p<0.01$, single-expression vs co-expression). Tet3ΔCD was localized in the cytoplasm regardless of the Aid expression. The scale bars are 10 μm. N (black); nuclear localization, N+C (gray); both nuclear and cytoplasmic localization, C (white); cytoplasmic localization in multiple microscope fields.
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that induced by with Aid FL (N: 18%, N+C: 35%, C: 47%) (Figs. 2C and 6E). Similarly, when co-expressed with a single point mutant AidF193A, the proportion of Tet1CD-translocated cells were decreased (N: 57%, N+C: 17%, C: 26%) in comparison to those when co-transfected with Aid FL (Figs. 2C and 6F). In addition, we performed the similar experiment using AidΔN26, which has defect in nuclear entry. AidΔN26 was predominantly localized at the cytoplasm in the case of single expression (N: 0%, N+C: 5%, C: 95%) (Fig. 6G). When co-expressed with AidΔN26, Tet1CD remained in the nucleus (N: 72%, N+C: 24%, C: 4%) (Figs. 2C and 6H). Taken together, these findings imply that Aid shuttling, which is mediated by the N-terminus and C-terminus domains of Aid, is associated with the Aid-mediated translocation of Tet1, and suggest that Tet1 translocation is dependent on the subcellular localization of Aid.

Interaction between Aid and Tet1

In this study, we found that nuclear Tet1 is translocated to the cytoplasm by Aid, and that the translocated Tet1 is co-localized with Aid. We next examined whether Aid interacts with Tet1

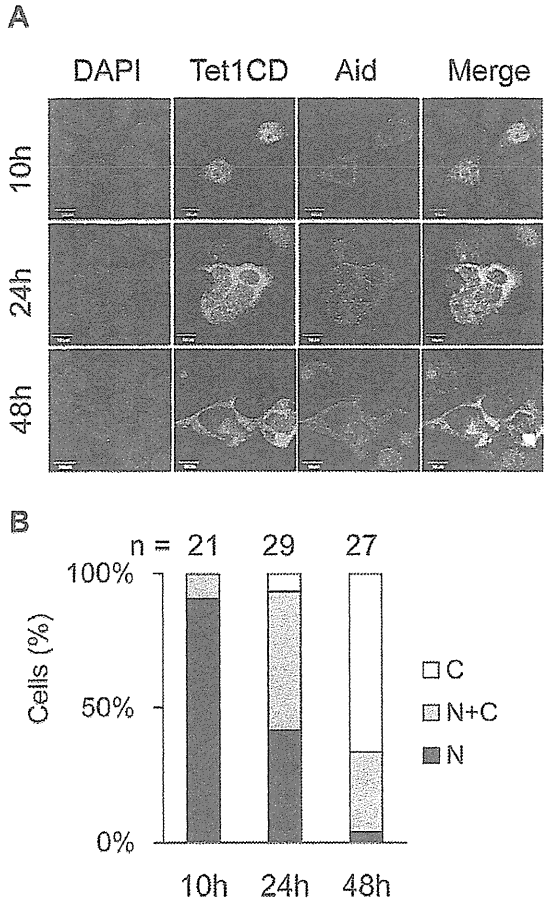

Figure 5. Nuclear Tet1 is gradually translocated into the cytoplasm by the simultaneous expression of Aid. (A) Confocal images of HEK293FT cells transiently co-expressing Tet1CD tagged with N-terminal Xpress and Aid tagged with C-terminal Myc at different time points (10 h, 24 h and 48 h) after co-transfection. The scale bars in images are 10 μm. (B) The number (n) of cells indicated above each bar was scored according to the Tet1CD subcellular localization. The number of cells with cytoplasmic Tet1CD gradually increased after co-transfection. N (black); nuclear localization, N+C (gray); both nuclear and cytoplasmic localization, C (white); cytoplasmic localization in multiple microscope fields.
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during this Aid-mediated translocation of Tet1. We carried out co-immunoprecipitation (co-IP) – immunoblotting (IB) using HEK293FT cells transfected with either or both the Xpress-Tet1CD and Aid FL-Myc vectors. Empty vectors were used as a negative control. Aid FL-Myc was co-precipitated with an anti-Xpress mAbs for Xpress-Tet1CD, and this association was confirmed by reciprocal IP with anti-Myc mAbs (Fig. 7A). Moreover, we observed a decreased interaction of AidΔNES with Tet1CD, although both AidΔNES and Tet1CD were localized in the nucleus (Fig. 7B). This result indicates that the NES domain of Aid is associated with the interaction between Aid and Tet1.

Discussion

Aid shuttles between the nucleus and the cytoplasm, interacting with several molecules, such as RNA polymerase II [24], CTNNBL-1 [25] and GANP [26], in order to target the IgV region and/or the S region DNA. Previous studies proposed that the shuttling of Aid plays a role in preventing excessive DNA mutation in the nucleus [18,27]. In the present study, we showed that the simultaneous expression of Aid and Tet family enzymes causes the altered subcellular localization of the Tet family proteins. Furthermore, the translocation of Tet was affected by Aid shuttling between the nucleus and the cytoplasm. These results suggest that Aid shuttling might have another function; altering the subcellular localization of Tet family members. However, it should be also noted that the level of Aid induced in this experiments seems to be substantially higher than that of physiological condition. Considering such artificial experimental system, further analyses for endogenous proteins are required to conclude the physiological function of Aid in the translocation of Tet family enzymes.

Although the physiological relevance of our findings remains to be established, it is important to note that the expression of both Tet family proteins and Aid is restricted to specific cell types. It was reported that Aid is highly expressed in oocytes [28], while Tet3 is expressed at high levels in oocytes and zygotes [6], thus indicating that both Tet3 and Aid are abundantly expressed in oocytes. Of note, Tet3 is localized in the cytoplasm in oocytes, but it translocates into the male pronucleus of zygotes shortly after fertilization [16]. Therefore, it seems that there is dynamic regulation of the subcellular localization of Tet family members during the early stage of development. Considering that simultaneous expression of Aid and Tet family members caused the translocalization of Tet proteins into the cytoplasm in this study, it is possible that endogenously expressed Aid contributes to the cytoplasmic localization of Tet3 in oocytes.

DNA methylation is critical for mammalian development and cellular differentiation [29]. In mammals, active genomic demethylation contributes to the genome-wide erasure of the DNA methylation observed in preimplantation embryos and primordial germ cells (PGCs) [30,31]. However, the mechanisms underlying active DNA demethylation in mammals have been highly controversial, although multiple mechanisms have been proposed [32–34]. Recently, an additional model was reported, wherein Aid facilitates the conversion of 5hmC into cytosine [9,35], and forms several complexes with thymine DNA glycosylase and GADD45a, which are involved in active DNA demethylation [36]. Our findings may also support the notion that Aid plays a role in DNA demethylation while interacting with several related factors.

To determine whether the altered subcellular localization of Tet contributes to the altered production of 5hmC, we performed immunodetection for 5hmC in dually-transfected cells (expressing both Tet1 and Aid), where Tet1 was translocated into the cytoplasm. Although Tet1 CD had already been translocated into the cytoplasm, 5hmC was still detectable in the nucleus (Fig. S6). Therefore, we could not conclude whether the translocation of Tet can affect the production of 5hmC in cells expressing both proteins. One of the possible explanations for our observation is that 5hmC is, to some extent, stable after its production, which made it difficult to detect an alteration in the 5hmC levels under our experimental conditions.

In summary, our present findings indicate that Aid regulates the subcellular localization of Tet family proteins, and that this is associated with Aid shuttling. The subcellular localization of proteins is crucial for their functional activity and is associated with their functional diversity [37,38]. Since both Aid and the Tet family proteins are involved in the modification of 5hmC, the coordinated action of these proteins might control epigenetic modifications by affecting the subcellular localization of Tet family

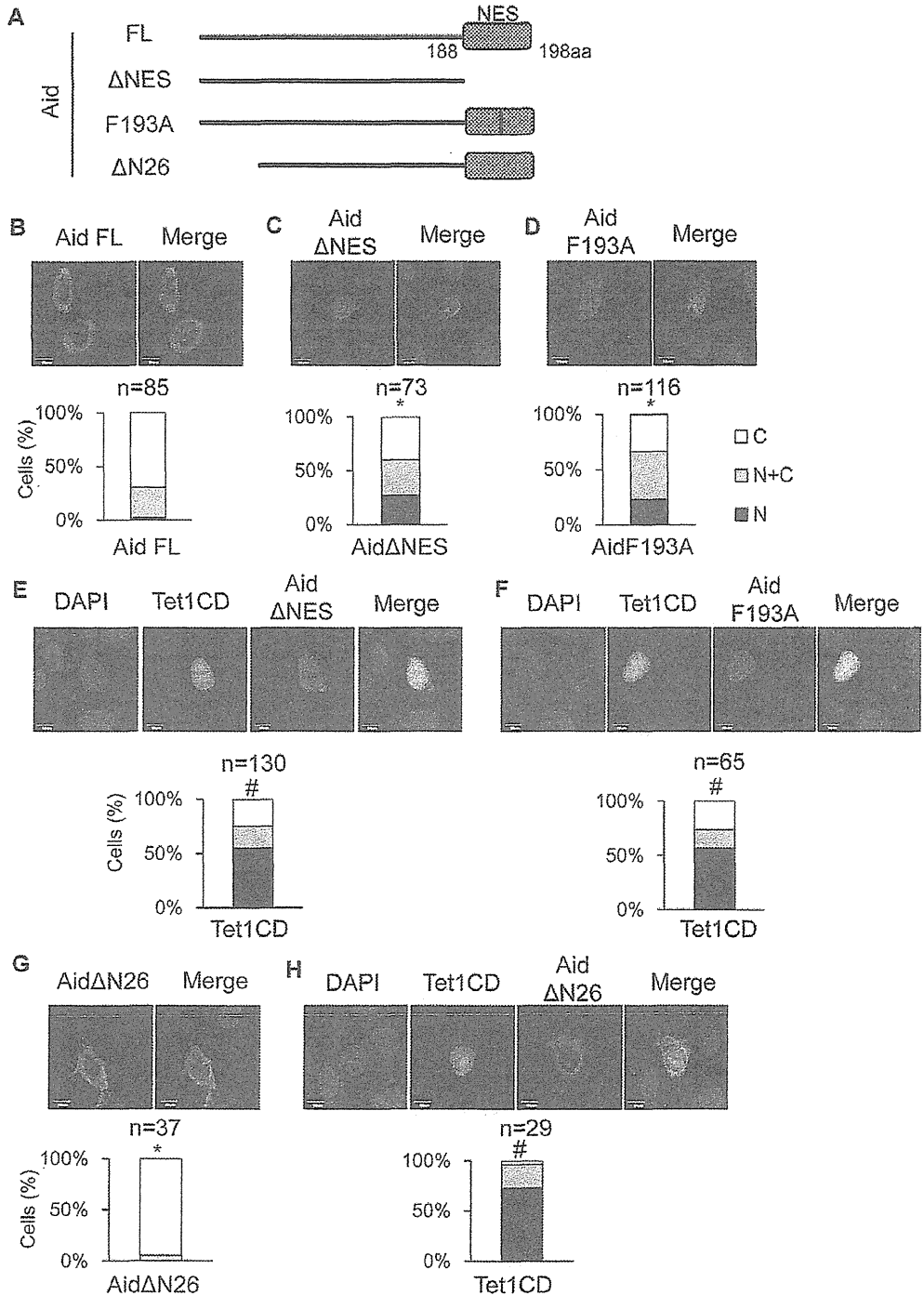

Figure 6. Aid shuttling is associated with Aid-mediating transcloation of Tet1. (A) A schematic representation of the Aid structure and its mutants used in this study. All Aid constructs were tagged with C-terminal Myc. (B–D) The upper figures are representative confocal images of DLD-1 cells transiently expressing only Aid FL (B), ΔNES (C) or F193A (D). The lower figure represents the proportion of cells with different subcellular localization of Aid. Aid mutants defect in NES showed the increased nuclear localization. *p<0.05 vs Aid FL. (E, F) The upper figures are representative confocal images of DLD-1 cells transiently co-expressing AidΔNES and Tet1CD (E), or AidF193A and Tet1CD (F). Tet1CD were tagged with N-terminal Xpress. The lower figure shows the proportion of cells with different localizations of Tet1 (E; AidΔNES and Tet1CD, F; AidF193A and Tet1CD). Aid mutants, which exhibit impaired shuttling between the nucleus and the cytoplasm, failed to alter the subcellular localization of Tet1. #p<0.05 vs with Aid FL. (G) The upper figures are representative confocal images of DLD-1 cells transiently expressing only AidΔN26. The lower figure represents the proportion of cells with different subcellular localization of AidΔN26. *p<0.05 vs Aid FL. (H) The upper figures are representative confocal images of DLD-1 cells transiently co-expressing AidΔN26 and Tet1CD. The lower figure shows the proportion of cells with different localizations of Tet1. #p<0.05 vs with AidFL. The scale bars are 10 μm. N (black); nuclear localization, N+C (gray); both nuclear and cytoplasmic localization, C (white); cytoplasmic localization in multiple microscope fields.
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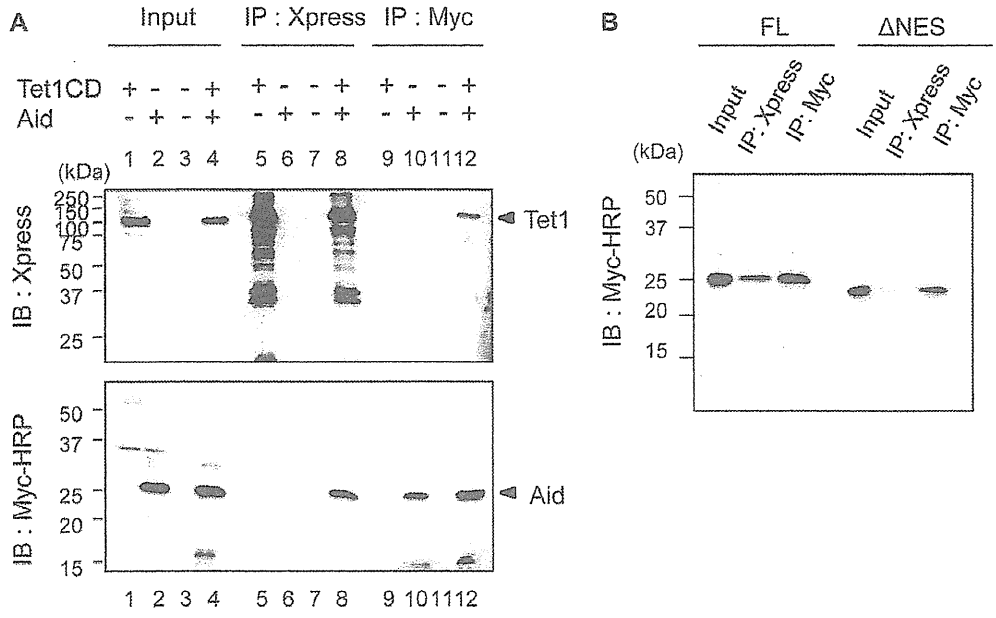

Figure 7. Aid interacts with Tet1CD. (A) Tet1CD was co-immunoprecipitated with Aid FL. Lysates from HEK293FT cells transfected with N-terminally Xpress-tagged Tet1CD, C-terminally Myc-tagged AidFL or both of them were immunoprecipitated (IP) by anti-Xpress mAbs or anti-Myc mAbs. Immunoblotting (IB) was performed by using an anti-Xpress Abs or anti-Myc-HRP antibody. Lane nos. 1, 5 and 9 were single transfections of Tet1CD. Lane nos. 2, 6 and 10 were single-transfections of Aid. Lane nos. 3, 7 and 11 were for mock transfection. Lane nos. 4, 8 and 12 shows the results for the co-transfection of Tet1CD and Aid. (B) The Co-IP experiment was performed by using lysates from HEK293FT cells co-transfected with N-terminally Xpress tagged-Tet1CD and C-terminally Myc-tagged Aid FL, or with N-terminally Xpress-tagged Tet1CD and C-terminally Myc-tagged AidΔNES. Despite the similar localization of AidΔNES and Tet1CD in the nucleus, AidΔNES revealed a decreased association with Tet1CD compared to Aid FL.
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proteins as we described in this study. Further studies are warranted to uncover the functional and physiological significance of the Aid-mediated translocation of Tet, which may eventually extend our understanding of the regulation of 5hmC production and active DNA demethylation.

Supporting Information

Figure S1 Representative images for each subcellular localization. Representative images of each subcellular localization are shown. Dominant immunofluorescent signals in the nucleus and cytoplasm were regarded as nuclear localization (N) and cytoplasmic localization (C), respectively. Similar signal intensity in both the nucleus and the cytoplasm was regarded as C+N. Scale bars are 10 μm.
(TIF)

Figure S2 Aid alters the subcellular localization of Tet1 in HEK293FT cells. HEK293FT cells expressing Aid also revealed translocation of Tet1CD and CDm. Consistent with the results in DLD-1 cells, Tet1ΔCD was retained in the nucleus even in the presence of Aid. The scale bars are 10 μm.
(TIF)

Figure S3 Untagged Aid expression results in the subcellular translocation of Tet1. Untagged Aid was detected by an anti-Aid polyclonal antibody. Untagged Aid was mainly localized in the cytoplasm, which was the same as Myc-tagged Aid. Simultaneous expression of untagged Aid and Tet1CD caused the altered localization of Tet1CD in the cytoplasm. The scale bars are 10 μm.
(TIF)

Figure S4 Apobec family has not an effect on the subcellular localization of Tet1. (A) The upper figures were confocal images of DLD-1 cells transiently expressing C-terminally Myc-tagged Apobec1. The lower graph represents the proportion of cells with different subcellular localization of Apobec1. (B) The upper figures were images of DLD-1 cells transiently co-expressing C-terminally Myc-tagged Apobec1 and N-terminally Xpress-tagged Tet1CD. The lower graph represents the percentage of the different subcellular localization of Tet1CD on the co-expressing cells. (C) The upper was images of DLD-1 cells transiently expressing C-terminally Myc-tagged Apobec2. The lower represents the proportion of cells with different subcellular localization of Apobec2. (D) The upper were images of DLD-1 cells transiently co-expressing C-terminally Myc-tagged Apobec2 and N-terminally Xpress-tagged Tet1CD. The lower showed the proportion of cells with different subcellular localization of Tet1CD on the co-expressing cells. The scale bars are 10 μm. N (black); nuclear localization, N+C (gray); both nuclear and cytoplasmic localization, C (white); cytoplasmic localization in multiple microscope fields.
(TIF)

Figure S5 Detection of 5hmC by immunostaining using in DLD-1 cells. (A) Aid alone could not produce 5hmC. (B) The FL and CD had enzymatic activity, whereas the ΔCD and CDm proteins did not in both Tet2 and Tet3. Aid was tagged with C-terminal Myc and Tets were with N-terminal Xpress. The scale bars are 10 μm.
(TIF)

Figure S6 5hmC remains in the nucleus even after Tet1CD transfer to the cytoplasm in HEK293FT cells. The 5hmC could be still detected in the nucleus, even though Tet1CD was translocated from the nucleus to the cytoplasm in the presence of Aid. Aid was tagged with C-terminal Myc and Tets were with N-terminal Xpress. The scale bars are 20 μm.
(TIF)

Table S1 Primer sets for cloning Tet family and Aid used in this study. F: forward primer, R: reverse primer
(DOC)

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Author Contributions

Conceived and designed the experiments: YA AW KS YY. Performed the experiments: YA. Analyzed the data: YA AW YY. Wrote the paper: YA AW YY.

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UICC global cancer control

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Cellular reprogramming and cancer development

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Special Section Paper

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-Azadc), 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 inhibition[62] 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 regions[71] 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 vectors[89] 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 cells[94] 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

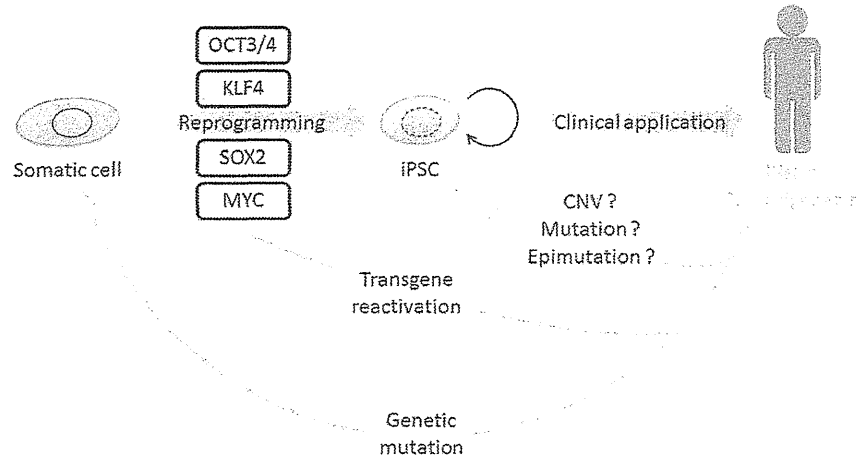

Figure 1. Potential risk of tumor development in the clinical application of iPSCs. Cancer development is a potential risk of using iPSCs in cell transplantation therapy. The genomic and epigenomic instability of iPSCs may cause tumor development from iPSC-derived cells, yet the instability has not been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

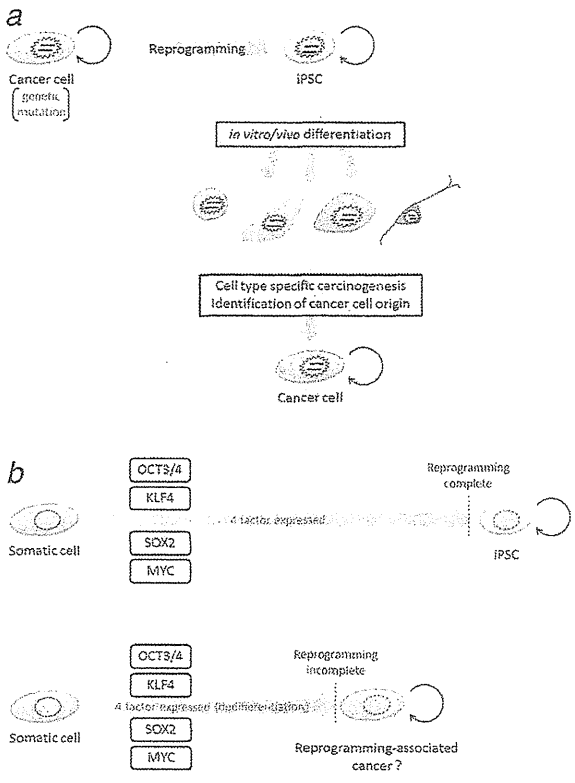

Figure 2. Application of iPSC technology for cancer research. Reprogramming technology is considered to be a useful tool to induce global epigenetic changes and to alter differentiation status of cancer cells. (*a*) Reprogramming of cancer cells and differentiation of the cancer cell-derived iPSCs. Cellular 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 transformation, 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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