AKT signaling is associated with epigenetic reprogramming via the upregulation of TET and its cofactor, alpha-ketoglutarate during iPSC generation

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Abstract

Background: Phosphoinositide-3 kinase (PI3K)/AKT signaling participates in cellular proliferation, survival and tumorigenesis. The activation of AKT signaling promotes the cellular reprogramming including generation of induced pluripotent stem cells (iPSCs) and dedifferentiation of primordial germ cells (PGCs). Previous studies suggested that AKT promotes reprogramming by activating proliferation and glycolysis. Here we report a line of evidence that supports the notion that AKT signaling is involved in TET-mediated DNA demethylation during iPSC induction.

Methods: AKT signaling was activated in mouse embryonic fibroblasts (MEFs) that were transduced with OCT4, SOX2 and KLF4. Multiomics analyses were conducted in this system to examine the effects of AKT activation on cells undergoing reprogramming.

Results: We revealed that cells undergoing reprogramming with artificially activated AKT exhibit enhanced anabolic glucose metabolism and accordingly increased level of cytosolic α-ketoglutarate (αKG), which is an essential cofactor for the enzymatic activity of the 5-methylcytosine (5mc) dioxygenase TET. Additionally, the level of TET is upregulated. Consistent with the upregulation of αKG production and TET, we observed a genome-wide increase in 5-hydroxymethylcytosine (5hmC), which is an intermediate in DNA demethylation. Moreover, the DNA methylation level of ES-cell super-enhancers of pluripotency-related genes is significantly decreased, leading to the upregulation of associated genes. Finally, the transduction of TET and the administration of cell-permeable αKG to somatic cells synergistically enhance cell reprogramming by Yamanaka factors.

Conclusion: These results suggest the possibility that the activation of AKT during somatic cell reprogramming promotes epigenetic reprogramming through the hyperactivation of TET at the transcriptional and catalytic levels.

Keywords: AKT signal, TET, αKG, DNA demethylation, Reprogramming, iPSC cells

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Background

PI3K/AKT signaling is activated downstream of extracellular stimuli such as growth factors and hormones via receptor tyrosine kinases and G-protein-coupled receptors [1]. Activated AKT phosphorylates serine and threonine residues of multiple target proteins, and...
cellular survival, proliferation, metabolism and growth are promoted as downstream effects of target phosphorylation. Research from our laboratory and others has shown that AKT activation enhances cellular reprogramming or dedifferentiation in vivo and in vitro [2]. Germ cell-specific Pten-deficient mice, which display constitutively activated AKT in germ cells, develop testicular teratomas that emerge from dedifferentiated primordial germ cells (PGCs) at the neonatal stage [3]. We have also shown that AKT activation in PGCs increases the efficiency of their dedifferentiation to pluripotent embryonic germ cells (EGCs) in vitro and that AKT can even replace bFGF, a cytokine required for EGC induction under the conventional protocol [4]. AKT activation also improves the efficiency of iPSC production [5].

In the process of iPSC induction, many functions of cellular physiology (e.g., metabolism, the epigenetic state associated with transcriptional networks, morphology and proliferation rates) dramatically change during the shift from a somatic cell state to a pluripotent stem cell state. Among these changes, metabolic remodeling occurs during the very early stage of reprogramming [6, 7]. Within 2 days following the transduction of Yamanaka factors (OCT4, SOX2 and KLF4; OKS), terminally differentiated cells with low glycolytic activity and an intermediate level of mitochondrial oxidative phosphorylation activity show a shift their metabolic state toward high glycolytic and oxidative phosphorylation activities, known as an OXPHOS burst. Recently, cellular metabolic remodeling has been recognized as an important mechanism controlling the pluripotency state by providing not only energy and cellular building blocks but also rate-limiting substrates for enzymes regulating epigenetic modifications, such as αKG and acetyl-CoA [8–11]. Epigenetic reprogramming occurs during the intermediate stage of iPSC generation. Genome-wide DNA hypomethylation activates pluripotency-associated genes, followed by the reestablishment of DNA methylation to repress cell type-specific genes [12]. TET family dioxygenases catalyze the sequential oxidation of 5mC to 5hmC, 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), ultimately leading to DNA demethylation. Thus, TETs play an essential role in epigenetic reprogramming during iPSC generation [13–15].

In this study, we observed molecular events in cells undergoing reprogramming with activated AKT in the context of cellular metabolism and DNA methylation and hydroxymethylation. Our results illustrate the association of epigenome reprogramming promoted by PI3K/AKT signaling with metabolic remodeling during iPSC generation.

Methods
Mice
C57BL/6 mice for maintaining the Oct4-EGFP transgenic mouse line [16, 17] and KSN/Slc nude mice for the teratoma formation assay (see below) were purchased from Japan SLC. All animal care and experimental procedures were carried out in accordance with the Guidelines for Animal Experiments of Kitasato University and were approved by the Institutional Animal Care and Use Committee of Kitasato University (Approval number: SAS2004). The study was carried out in compliance with the ARRIVE guidelines (https://arriveguidelines.org).

Mouse embryonic fibroblast derivation
MEFs were isolated from Oct4-EGFP transgenic mice [16, 17]. Multiple E13.5 male embryos were mixed and cultured in DMEM supplemented with high glucose (Nacalai Tesque), penicillin–streptomycin (Nacalai Tesque) and 10% FBS (Equitech-Bio).

Plasmid construction
To construct pMYs-AKT-Mer, the BamHI-Xhol fragment from the pCAGGS-myr-AKT-Mer-IRES-EGFP-pA plasmid [18] was cloned into the pMys retroviral vector, which was linearized, and subjected to IRES-GFP cassette removal by BamHI and SalI digestion (pMYs-IRES-GFP was kindly provided by Dr. Kitamura [3, 19]). To construct the pMYs-Idh1 retroviral vector, Idh1 was amplified from a cDNA library synthesized using in-house-cultured ES cells (c57bl/6 and DBA/2 mixed background) by RT-PCR (forward primer: 5′-AGT TAATTAAGGATCCATCCATGTCCAGAAAAATCCA AGG-3′; reverse primer: 5′-TTATTTTATCGTCGACTT AAAATGTGGCTTGAGCT-3′). Then, the RT-PCR amplicon was digested with BamHI and SalI, followed by ligation to the pMYs retroviral vector, linearization and removal of the IRES-GFP cassette by BamHI and SalI digestion. We performed site-directed mutagenesis to produce the IDH1-R132H mutant retroviral vector from pMYs-Idh1 using a mutagenesis primer set (forward primer: 5′-CCATCATCATGTCATGCCCCATACATGCA TATGGGAG-3′; reverse primer: 5′-GTCCCATGTA GCCATGTATGATCAG-3′). pMYs-Flag-TET2CD was kindly provided by Dr. Xu of the Chinese Academy of Sciences.

Retroviral infection and iPSC derivation
The retroviral transduction of the Yamanaka factors (OSK) along with AKT-Mer, IDH1 or IDH1(R132H) was performed following the original protocol with some modifications [20]. Briefly, 3 × 10⁶ plat-E cells were plated in a 60-mm dish 1 day before transfection with 4.5 μg of the pMXs-Oct4, pMXs-Sox2, pMXs-Klf4,
pMYs-AKT-Mer, pMYs-Idh1, pMYs-Idh1(R132H) or pMYs-Flag/TET2CD plasmid using X-tremeGENE 9 (Merck), following the manufacturer’s instructions. At 48 h posttransfection, the retroviruses in plate E medium were collected, mixed and filtered via PVDF filters with a 0.45 µm pore size (Merck, Cat# SLHVR33RS). Next, OE-MEFs that had been plated at 3 × 10^6 cells per 60-mm dish the day before were infected with retroviruses supplemented with 4 µg/mL polybrene (Sigma-Aldrich, Cat# H9268). At 3 dpi, the transfected cells were replated in a 6-well plate with LIF containing ES medium-DMEM (Nacalai Tesque Cat# 08459-64) supplemented with 10% FCS (Gibco, Cat# 10270, Lot# 42Q3056K), 0.1 mM non-essential amino acids (Nacalai Tesque Cat# 06344-56), 2 mM L-glutamine (Nacalai Tesque Cat# 16948-04), 0.1% 2-mercaptoethanol, 100 U/mL LIF (made in the laboratory) and streptomycin/penicillin (Nacalai Tesque Cat# 09367-34). At the same time, 4OHT (Sigma-Aldrich, Cat# H7904) (final concentration, 1 µM) was added to three wells to activate AKT, and ethanol (vehicle control) was added to another 3 wells for the AKT-nonactivated controls. AOA (Sigma-Aldrich, Cat# C13408) (final concentration, 1 mM) or DM-αKG (Sigma-Aldrich, Cat# H9268). At 3 dpi, the transfected cells were replated in a 6-well plate with LIF containing ES medium-DMEM (Nacalai Tesque Cat# 08459-64) supplemented with 10% FCS (Gibco, Cat# 10270, Lot# 42Q3056K), 0.1 mM non-essential amino acids (Nacalai Tesque Cat# 06344-56), 2 mM L-glutamine (Nacalai Tesque Cat# 16948-04), 0.1% 2-mercaptoethanol, 100 U/mL LIF (made in the laboratory) and streptomycin/penicillin (Nacalai Tesque Cat# 09367-34). At the same time, 4OHT (Sigma-Aldrich, Cat# H7904) (final concentration, 1 µM) was added to three wells to activate AKT, and ethanol (vehicle control) was added to another 3 wells for the AKT-nonactivated controls. AOA (Sigma-Aldrich, Cat# C13408) (final concentration, 1 mM) or DM-αKG (Sigma-Aldrich, Cat# H9268) (final concentration, 0.8 mM) was added to the culture medium beginning at either 3 dpi, to examine the levels of αKG and 5hmC (Fig. 4f, g) or 7 dpi, to determine reprogramming efficiency (Fig. 4h, i).

**Teratoma formation assay**

Established iPSCs (1 × 10^6) were subcutaneously injected into the dorsal flank of nude mice (Japan SLC). At 4–5 weeks after transplantation, the tumors were dissected from the mice. The teratomas were fixed with 4% paraformaldehyde in PBS and embedded in OCT compound (Sakura Finetek Japan). Sections (7 µm thick) were stained with hematoxylin and eosin.

**Cell cycle analysis**

At 7, 8, 9 and 10 dpi, 1 × 10^6 cells undergoing reprogramming were harvested in tubes, fixed with 1% PFA for 15 min at room temperature, washed with PBS and resuspended in 100 µL PBS. A total of 900 µL of 70% ethanol chilled at −30 °C was added, with mixing by tapping, followed by 50 µL RNase (50 µg/µL) and 200 µL propidium iodide (100 µg/µL). Next, the mixture was incubated for 30 min at room temperature. The cells were collected by centrifugation (960 × g for 5 min), resuspended in 1% FCS in PBS and passed through a mesh. Fluorescein was measured with a Gallios flow cytometer (Beckman Coulter) and analyzed with Kaluza software (Beckman Coulter).

**Proteomic analysis**

Three wells per experimental group were analyzed independently. Cells were harvested at 10 dpi by trypsinization and washed with PBS. The cell pellet was stored at −80 °C until protein extraction. The methods used for protein extraction and trypsin digestion have been described previously [21]. See Additional file 5 for further methodological details.

**Ion chromatography–tandem mass spectrometry for anionic metabolites**

Cells from each experimental group were plated in three wells of 6-well plates and analyzed independently as follows. The culture medium from cells undergoing reprogramming at 10 dpi was replaced with DMEM without glucose (Nacalai Tesque) supplemented with 4.5 g/L D-glucose-13C6 (Sigma-Aldrich), followed by incubation with 5% CO2 for 1 h at 37 °C. Then, the medium was aspirated, and the cells were washed in ice-cold PBS three times with chilling, followed by snap freezing in liquid nitrogen. The plates were stored at −80 °C until metabolite extraction. The methods used for metabolite extraction and cytosolic fractionation to remove mitochondria from cell lysates have been described previously [22, 23]. See Additional file 5 for further details.

**Microarray analysis**

Total RNA was extracted using the miRNeasy Mini Kit (QIAGEN) according to the manufacturer’s instructions. Cyanine-3 (Cy3)-labeled cRNA was prepared from 100 ng RNA using the Low Input Quick Amp Labeling Kit (Agilent) according to the manufacturer’s instructions, followed by RNeasy column purification (QIAGEN). Dye incorporation and the cRNA yield were checked with a NanoDrop ND-1000 spectrophotometer. Slides were scanned immediately after washing on an Agilent DNA Microarray Scanner (G2565CA) using the one-color scan setting for 8 × 60 k array slides (scan area 61 × 21.6 mm, scan resolution 3 µm, dye channel set to green, green PMT set to 100% and TIFF file dynamic range of 20 bits). The scanned images were analyzed with Feature Extraction Software 10.7.3.1 (Agilent) using the default parameters to obtain background-subtracted and spatially detrended processed signal intensities.

**Immunoblotting**

Proteins were extracted using RIPA buffer (50 mM HEPES pH 7.9, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS), followed by protein concentration measurement using a Pierce BCA Protein Assay Kit.
For SDS-PAGE, 20 µg protein was applied per lane. Proteins were detected with anti-TET2 (diluted to 1:1000) (Cell Signaling Technology) and anti-β-ACTIN (diluted to 1:1000) primary antibodies using ImageQuant LAS 4000 (GE Healthcare).

RNA sequencing
Total RNA was isolated from cells using the AllPrep DNA/RNA Mini Kit (QIAGEN) according to the manufacturer’s instructions. Standard polyA libraries were prepared using the KAPA Stranded mRNA-Seq Kit for Illumina (Kapa Biosystems) according to the manufacturer’s protocol. Libraries were subjected to 50-bp single-end sequencing on the Illumina HiSeq 1500 platform. The sequence data were trimmed using Trim Galore (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) and mapped against the mouse reference transcriptome using Salmon [24].

qRT-PCR
Total RNA was isolated from cells using the RNeasy Mini Kit (QIAGEN) according to the manufacturer’s instructions. Reverse transcription was performed by using a ThermoScript RT-PCR System (Invitrogen) and an oligo (dT) primer. Gene expression levels were measured with Stratagene MX3000P (Agilent Technologies) using FastStart Universal SYBR Green Master Mix (Rox) (Roche). See Additional file 5: Table S1 for the primers.

Bisulfite sequencing
Genomic DNA was isolated from cells using the Cica Genesus Total DNA Prep Kit (Kanto Chemical) according to the manufacturer’s instructions. The isolated DNA was subjected to the bisulfite reaction as described previously with some modifications [25, 26]. The automated shell scripts and the read me text are available in Additional files 2, 3 and 4 [27]. See Additional file 5 for further details.

Dot blot assay
Total DNA was extracted from cells by RNase and protease digestion, followed by phenol/chloroform purification. The DNA concentration was measured using a Qubit system (Thermo Fisher Scientific) and diluted to 100 ng/µL with distilled and deionized water. To denature the genomic DNA, 0.5 µL of 1 N NaOH was added to 4.5 µL of DNA solution, after which the mixture was heated to 95 °C for 10 min and neutralized by adding 0.75 µL of 5 M ammonium acetate. The genomic DNA (1.5 µL) was blotted onto a nylon membrane (Hybond-N+, GE Healthcare) (i.e., 117 ng genomic DNA per spot). The membrane was incubated at 80 °C for 30 min and UV cross-linked at 70 mJ. The blotting membrane was soaked in blocking reagent (BlockingOne, Nacalai Tesque) for 1 h at room temperature, washed briefly with TBS with 0.1% Tween-20 (TBST), incubated with a polyclonal anti-5hmC antibody (Activemotif, cat # 39069) diluted 1:5000 with Can Get Signal Solution 1 (ToyoBo Life Science) at 4 °C overnight, washed with TBST three times for 10 min each, incubated with an HRP-linked anti-rabbit IgG antibody (GE Healthcare, cat # NA934) for 1 h at room temperature and washed with TBST three times for 10 min each. The resulting signals were detected by ECL chemiluminescence (Nacalai Tesque) using ImageQuant LAS 4000 (GE Healthcare). The signal intensity of the dots was quantified using ImageJ software. To plot a standard curve of the dot signal intensity versus the amount of 5hmC, we used a synthesized oligonucleotide consisting of 57 nucleotides including seven 5hmCs (5′-AGAAT[X1]GGTTATAGG[X2]GGGAGACATAGAACTGCG[X3]G[X4]GTG[X5]GTG[X6]GTCAC[X7]GAAC-3′, [X]: 5hmC). Twofold dilutions of the oligonucleotide were prepared starting from the highest 5hmC concentration of 400 fmol/µL and treated in the same way as the genomic samples described above.

Statistical analysis
To evaluate reprogramming efficiency, iPSCs from each group were plated in three wells of 6-well plates. Then, the GFP-positive colonies in each well were counted. For dot blotting, transcriptomic, proteomic and metabolomic analyses, qRT-PCR, and bisulfite sequencing, cells from the three wells per experimental group were assessed independently. Statistical analyses of the iPSC generation assay, dot blot analysis, metabolomic analysis, qRT-PCR and methylation analysis data were performed using the unpaired Student's t-test. Statistical analyses of the RNA sequencing data were performed using the edgeR package [28], and p values were calculated after adjusting for the false discovery rate. Data are shown as the means, and error bars represent standard deviations.

Data and software availability
The accession numbers of the RNA-seq data and the microarray data reported in this paper are GEO: GSE161344 and GEO: GSE161399, respectively. The accession number of the proteomic analysis reported in this paper Japan ProteOme Standard Repository (JPOST; https://repository.jpostdb.org/) is PXD014856. The metabolomic analysis data reported in this paper are available in Additional file 1.
Results
Conditional activation of AKT enhances iPSC induction efficiency
To investigate the role of activated AKT during iPSC induction, we employed a fusion protein of myristoylated AKT and a modified estrogen receptor (AKT-MER) [4]. In this system, AKT can be conditionally activated by administering the MER ligand 4-hydroxytamoxifen (4OHT). For the induction of cell reprogramming, we used mouse embryonic fibroblasts (MEFs) carrying a transgene encoding enhanced green fluorescent protein gene (EGFP) under the control of the Oct4 promoter and enhancer (OE-MEFs) [16, 17]. The OE-MEFs enabled us to identify cells undergoing reprogramming by observing EGFP fluorescence. We transduced the OE-MEFs with AKT-MER in combination with OSK (OSKA) via retroviral vector infection (Day 0). At 3 days postinfection (dpi), we split the cells into two groups, which were cultured with (OSKA + 4OHT: AKT activation) or without 4OHT (OSKA − 4OHT: AKT nonactivation) (Fig. 1a). We assessed the effect of AKT activation on iPSC induction by counting the number of GFP-positive colonies at 7 and 10 dpi. At 7 dpi, the average number of GFP-positive colonies in the OSKA + 4OHT group was 450 ± 179.5 (i.e., the reprogramming efficiency was 0.15 ± 0.06% when the number of infected cells was $3 \times 10^3$), whereas no GFP-positive colonies were observed in the OSKA − 4OHT group ($n = 3, p = 0.045$, Student’s t-test) (Fig. 1b). At 10 dpi, there were 1094 ± 33.3 GFP-positive colonies (reprogramming efficiency: 0.36 ± 0.01%) in the OSKA + 4OHT group compared with 295 ± 65.0 (reprogramming efficiency: 0.10 ± 0.02%) in the OSKA − 4OHT group ($n = 3, p = 0.00033$, Student’s t-test) (Fig. 1b). The effects of 4OHT on the cells that were not transduced with AKT-MER were limited in terms of iPSC induction efficiency and gene expression (Figs. 1b and Additional file 5: Fig. S1). We established iPSC lines from GFP-positive colonies in the OSKA + 4OHT group and confirmed their pluripotent ability to differentiate into the three germ layers using a teratoma formation assay (Fig. 1c). The results indicated that the conditional activation of AKT greatly accelerates the timing and improves the efficiency of somatic cell reprogramming by Yamanaka factors.

We examined cell cycle progression with or without AKT activation from 7 to 10 dpi using fluorescence-activated cell sorting to measure DNA contents following propidium iodide (PI) staining. It has been reported that an accelerated cell cycle rate is important for somatic cell reprogramming [29, 30]. Additionally, one of the major effects of AKT on cell physiology is to promote proliferation by phosphorylating the cell cycle checkpoint proteins p21 and p27 [1]. The proportion of G1-phase cells was significantly lower at all assessed time points, whereas that of S-phase cells was significantly higher among AKT-activated cells (OSKA + 4OHT) than nonactivated cells (OSK with and without 4OHT: OSK − 4OHT and OSK + 4OHT, respectively, and OSKA − 4OHT) at 8 dpi (Fig. 1d).

These results indicate that activated AKT promotes the G1-to-S phase transition, thereby accelerating the cell cycle during iPSC induction.

Expanded glucose anabolism during iPSC induction with activated AKT
As AKT participates in glucose metabolism by regulating GSK and FOXO, we performed a metabolomic pathway tracing analysis by adding 13C-labeled glucose to the cell culture medium at 10 dpi. This experiment enabled us to understand how the carbon flows derived from the labeled glucose were modified in each metabolic pathway, where non-13C-labeled molecules corresponded to metabolites derived from intracellularly accumulated substrates or extracellular nonglucose substrates. The volcano plot showed an overall increasing trend of the anionic metabolites that we measured (Fig. 2a). Among 313 metabolites, 103 metabolites showed a statistically significant increase, whereas only 5 metabolites showed a statistically significant decrease in AKT-activated cells relative to AKT-nonactivated cells. The flow of glucose carbon into the glycolysis pathway and the pentose phosphate pathway was significantly increased in AKT-activated cells (Additional file 5: Fig. S2a,b). De novo nucleotide synthesis for genome replication, represented by inosine monophosphate (IMP) and deoxynucleotide triphosphates (dGTP, dATP, dCTP and TTP), was notably elevated (Additional file 5: Fig. S2c,d). The carbon flow into the TCA cycle was also significantly increased (Fig. 2b). The levels of intermediates of the TCA cycle, including citrate, cis-aconitate, isocitrate and αKG, significantly altered in AKT-activated cells. Because glucose-derived citrate is exclusively produced in mitochondria, this result suggests a higher level of mitochondrial activity. Importantly, these molecules are involved in fatty acid and amino acid synthesis.

Consistent with the metabolomic analysis, a proteomic analysis at 10 dpi followed by KEGG pathway analysis revealed that proteins involved in glycolysis and the pentose phosphate pathway as well as fatty acid metabolism, amino acid biosynthesis and carbon metabolism were significantly enriched among the proteins upregulated in AKT-activated cells (Additional file 5: Figs S2a,b and S3a,b). Overall, the metabolomic and proteomic analyses showed that iPSC induction with AKT activation shifts...
Fig. 1  Enhancement of iPSC induction by conditional AKT activation. 

a. A schematic representation of conditional AKT activation during iPSC induction. OE-MEFs were transduced with reprogramming factors (OSK) and AKT-Mer (A). Cells were plated in 6-well plates at 3 dpi. Next, 4OHT (the Mer ligand) was added to three wells to activate AKT, and vehicle (ethanol) was added to the remaining three wells as a control. GFP-positive colonies were counted at 7 and 10 dpi.

b. The numbers of GFP-positive colonies at 7 and 10 dpi. Cells without AKT-Mer transduction (OSK−4OHT and OSK+4OHT) were observed to record the intrinsic effects of 4OHT on reprogramming (n = 3).

c. Representative images of GFP-positive colonies and histological sections illustrating the three germ layers that differentiated from AKT-activated iPSCs transplanted into nude mice. The epidermis, muscle and mucosal gland correspond to the ectoderm, mesoderm and endoderm, respectively.

d. Cell cycle analysis by DNA content quantitation, as assessed by PI staining and flow cytometry between 7 and 10 dpi (n = 3). *p < 0.05; **p < 0.01; ***p < 0.001 by the unpaired Student’s t-test.
the glucose metabolic state toward anabolic processes supplying cellular building blocks (e.g., nucleotides and fatty acids), which could support an accelerated cell cycle rate (Fig. 1d).

Enhanced epigenetic reprogramming during iPSC induction with activated AKT
Next, we performed RNA sequencing at 10 dpi. A total of 559 genes were significantly upregulated, and 429 genes were significantly downregulated in the OSKA + 4OHT group relative to the OSKA − 4OHT group (fold change > 2, adjusted p value < 0.05, n = 3). KEGG pathway analysis revealed that genes involved in metabolism-related pathways, infection responses and cell cycle regulation were significantly enriched among the genes upregulated in AKT-activated cells (Fig. 3a). On the other hand, genes involved in cell adhesion, motility, the cytoskeleton, cancer and TGF-β signaling were enriched on the other hand, genes involved in cytoskeleton, cancer and TGF-β signaling were enriched, whereas no CpGs were significantly hypomethylated at a single-CpG resolution, whereas no CpGs were significantly hypermethylated in AKT-activated cells (Additional file 5: Fig. S5). Taken together with the TET-mediated genome-wide increase in 5hmC, these findings indicate that pluripotency-related genes are upregulated in AKT-activated cells relative to AKT-nonactivated cells by promoting DNA demethylation at cis-regulatory regions.

Involvement of increased αKG levels in epigenetic reprogramming during iPSC induction
To determine whether the elevated levels of 5hmC in AKT-activated cells were associated with TET activity
and pluripotency acquisition, we transduced mutant isocitrate dehydrogenase 1 (IDH1-R132H) together with OSKA to inhibit TET activity. IDH1 functions in the cytosol, and normal IDH1 (IDH1-WT) converts isocitrate into αKG, whereas IDH1-R132H converts αKG into 2-hydroxyglutarate (2HG), which inhibits TET activity [35] (Fig. 4a). We measured cellular αKG and 2HG and genome-wide 5hmC levels at 10 dpi. We also assessed the reprogramming efficiency at 10 and 14 dpi. The level of αKG was not affected by IDH1-WT but was significantly
activated cells (OSKA at 3 dpi) prevented the increase in the αKG level in AKT-cellular αKG levels [10]. AOA administration beginning which is an inhibitor of aminotransferases and decreases possibility, we evaluated aminooxyacetic acid (AOA), assessed by the number of GFP-positive colonies was significantly decreased by IDH1-WT at 10 dpi but had recovered by 14 dpi (Fig. 4e). The number of GFP-positive colonies was decreased by IDH1-WT at 10 dpi but had recovered by 14 dpi, suggesting that IDH1-WT altered the timing, rather than the efficiency, of somatic cell reprogramming (Fig. 4e). These data indicate that elevated levels of 5hmC depend on TET activity and are associated with an increased reprogramming efficiency. In addition, cell reprogramming with activated AKT increases cytosolic αKG during iPSC induction because IDH1-R132H, which functions in the cytosol but not in mitochondria, prevents the increase in the αKG level induced by AKT activation [36] (Fig. 4b). This was confirmed by measuring αKG in the cytosolic fraction without mitochondria (Fig. 2c).

Because TET2 is indispensable for iPSC generation and TET1 or TET2 overexpression during iPSC induction improves the reprogramming efficiency, the elevated expression of Tet1 and Tet2 in cells undergoing OSKA +4OHT-induced reprogramming is potentially involved in the enhanced reprogramming efficiency [13, 37, 38]. Additionally, it is highly possible that the elevated production of αKG is critical for enhanced reprogramming in the OSKA +4OHT group. To investigate this possibility, we evaluated aminoxyacetic acid (AOA), which is an inhibitor of aminotransferases and decreases cellular αKG levels [10]. AOA administration beginning at 3 dpi prevented the increase in the αKG level in AKT-activated cells (OSKA +4OHT) in a dose-dependent manner (Fig. 4f). We also found that the increase in 5hmC in the OSKA +4OHT group was suppressed by AOA administration in a dose-dependent manner (Fig. 4g). Taken together, these data show a strong correlation between αKG levels and genome-wide 5hmC levels. To assess the effect of AOA on the reprogramming efficiency, we administered AOA to cells at 7 dpi because its administration at 3 dpi interfered with cell proliferation. AOA administration significantly decreased the number of GFP-positive colonies at 10 dpi (Fig. 4h). This decrease was rescued by the simultaneous administration of dimethyl-αKG (DM-αKG), a cell membrane-permeable αKG derivative (Fig. 4h). However, DM-αKG administration in AKT-nonactivated cells (OSKA – 4OHT) did not improve the reprogramming efficiency (Fig. 4h), suggesting that an increase in the αKG level in conjunction with the upregulation of TET genes is critical for promoting reprogramming. Therefore, we investigated the effect of DM-αKG in cells undergoing reprogramming with TET2 catalytic domain (TET2CD) overexpression (OSKT2). The OSKT2 group showed a higher reprogramming efficiency than the OSK group at 14 dpi (Fig. 4i). Moreover, DM-αKG administration in the OSKT2 group beginning at 3 dpi significantly improved the reprogramming efficiency (Fig. 4i). Taken together, these results suggest that enhanced 5hmC production and the consequent promotion of somatic cell reprogramming are associated with the synergistic effect of TET overexpression and αKG overproduction in AKT-activated cells.

Discussion

In this study, we found that glycolysis was enhanced in AKT-activated cells undergoing reprogramming, leading to the accumulation of the downstream TCA cycle metabolite αKG during iPSC induction by OSK.
Fig. 3 (See legend on previous page.)
Additionally, while it has been reported that Tet1 and Tet2 expression is induced during iPSC induction, we observed higher expression of Tet1 and Tet2 in AKT-activated cells than in AKT-nonactivated cells [13, 39]. We speculate that the increases in aKg and TET at least partly account for the genome-wide elevation of 5hmC as well as DNA hypomethylation at ESC super-enhancers, for the following reasons. First, the levels of aKg were correlated with the genome-wide 5hmC levels, suggesting that aKg may play a role in DNA demethylation during somatic cell reprogramming. Second, the inhibition of aKg production abrogated iPSC induction. Furthermore, we showed that simultaneous TET2CD transduction and DM-aKg administration synergistically enhanced the reprogramming efficiency; thus, they partially reproduced the effect of AKT activation on reprogramming.

In addition, these results support the idea that AKT signaling is involved in the enhanced production of TET and aKg. The possible mechanisms of this effect include the following. First, TET overexpression may be induced by introducing Yamanaka factors, as Tet1 and Tet2 have super-enhancers with OCT4 binding sites [40], and it has been reported that the AKT-mediated phosphorylation of OCT4, SOX2 and KLF4 upregulates their activity [41, 42]. Second, aKg overproduction involves the AKT-FOXO1 axis [5]. AKT inactivates FOXO1 through direct phosphorylation, which activates glycolysis and mitochondrial function and may result in aKg overproduction [43]. Alternatively, because TET expression was found to be upregulated in cells undergoing reprogramming, these observations might reflect the consequences of an increase in reprogramming mediated by AKT. Further studies are required to reveal the direct and/or indirect linkages among AKT activation, TET expression and aKg production.

Which pathway is responsible for the overproduction of cytosolic aKg is another intriguing question (Fig. 2c). In this study, we used labeled glucose to show that the glucose-derived aKg level was elevated. Glucose-derived aKg accumulates in the cytosol via two pathways: (1) cytosolic citrate transferred from the mitochondria via citrate carrier proteins expressed on the mitochondrial membrane is metabolized to aKg by IDH1, and (2) aKg is produced as a TCA cycle intermediate in the mitochondrial matrix. Cellular aKg (b) and 2HG (c) levels in cells undergoing reprogramming induced by OSKA, OSKA + IDH1 and OSKA + IDH1 R132H were quantified by ion chromatography–tandem mass spectrometry at 10 dpi. Genome-wide 5hmC levels were quantified by dot blot analysis (d). GFP-positive colonies were quantified at 10 and 14 dpi (e). f and g: Correlation between aKg levels and genome-wide 5hmC levels at 10dpi. aOA administration at 3 dpi reduced aKg levels in AKT-activated cells in a dose-dependent manner, as quantified by ion chromatography–tandem mass spectrometry (f). Genotype-wide 5hmC levels of cells undergoing reprogramming with or without aOA administration at 3 dpi were quantified by dot blot analysis (g). h: Requirement for upregulated aKg production for an enhanced reprogramming efficiency in AKT-activated cells. GFP-positive colonies were counted at 10 dpi. aOA administration at 3 dpi reduced the reprogramming efficiency, but the simultaneous administration of DM-aKg rescued the reprogramming efficiency. i: Effects of DM-aKg on the reprogramming efficiency with or without TET2CD overexpression. GFP-positive colonies were counted at 14 dpi. The dot blot images shown in Figs. 3 and 4 are from the same membrane as shown in Additional file 5: Fig. S4. Therefore, the ‘total’ in Fig. 3b, ‘IDH1-’ in d and ‘AOA-’ in g are the same as ‘OSKA’ shown in Additional file 5: Fig. S4.
Fig. 4 (See legend on previous page.)
Moreover, AKT suppresses Chk1, p21 and p27, which are negative regulators of the cell cycle via phosphorylation, and phosphorylates Mdm2 to repress p53 [1]. Through RNA-seq followed by pathway analysis, we also found that MET is promoted in AKT-activated cells, which is associated with the improvement of the reprogramming efficiency by AKT [31]. Several other roles of AKT in cellular reprogramming and pluripotency, including apoptosis inhibition and the respiratory shift mediated by activated AKT in mitochondria, have also been proposed [45, 46]. Altogether, our results showing the role of AKT in epigenetic reprogramming associated with metabolic remodeling add new insight into the wide-ranging roles of AKT signaling in cellular reprogramming.

Conclusion
In summary, we performed a multimomics analysis to observe the effects of AKT activation on somatic cell reprogramming. These findings may provide novel insights into the dynamic link between AKT signaling and epigenetic regulation mediated by metabolic remodeling in pluripotency acquisition.

Abbreviations
PBK: Phosphoinositide-3 kinase; iPSCs: Induced pluripotent stem cells; PGCs: Primordial germ cells; MEFs: Mouse embryonic fibroblasts; EGFP: Enhanced green fluorescent protein; OE-MEFs: Mouse embryonic fibroblasts carrying Oct4-EGFP transgene; dpi: Days postinfection; FACS: Fluorescent activated cell sorting; oKg: α-Ketoglutarate; DM-oKg: Dimethyl-α-ketoglutarate; SMC: S-Methylcytosine, 5hmC: 5-Hydroxymethylcytosine, SIC: S-Formylcytosine, 5CaC: 5-Carboxylcytosine, 4OHt: 4-Hydroxymethylxoylen, OSK: OCT4, SOX2 and KLF4, AKT-MER: Fusion protein of myristoylated AKT and modified estrogen receptor; OSAK: OCT4, SOX2, KLF4 and AKT-MER, TET2: TET2-catalytic domain; OSAK-T2: OCT4, SOX2, KLF4 and TET2-catalytic domain; IDH1: Isocitrate dehydrogenase 1; 2HG: 2-Hydroxylglutarate; AOA: Aminoxyacetic acid.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s13287-021-02578-1.

Additional file 1: Data 1: Metabolome data for OSAK — 4OHT and OSAK + 4OHT at 10 dpi.

Additional file 2: Auto made shell script used for quantification of DNA methylation.

Additional file 3: Automated shell script used for quantification of DNA methylation for Linux.

Additional file 4: Read me for automated shell script used for quantification of DNA methylation.

Additional file 5: Supplementary Methods, References, Tables and Figures.

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Authors’ contributions
Yo.S. contributed to the design and performance of most of the experiments and wrote the manuscript. Yu.S. and E.S. performed and analyzed the metabolomic data. A.M. performed most of the experiments. Yu.K. performed the dot blot analysis and analyzed the results. R.K. performed the proteomic analysis. K.A. and M.S. performed the bisulfite sequencing analysis. T.I. performed the western blot analysis. T.Y. analyzed the transcriptomic data. E.K. performed the qRT-PCR. T.N., M.S. and F.I. held collaborative discussions on the data. Yo.K. performed the proteomic analysis and analyzed the data. Ta.K. performed the dot blot analysis and the RNA sequencing and analyzed the results. To.K. initiated, designed and led the study and wrote the manuscript, with all authors contributing to the final version.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from public databases (see Methods), supplementary information and the corresponding author on reasonable request.

Declarations
Ethic approval and consent to participate
This study does not report on or involve the use of human data or tissue. All animal care and experiments were carried out in accordance with Guidelines of Animal Experiments of Kitasato University and were approved by Institutional Animal Care and Use Committee of Kitasato University (Approval number: SAS2004). The study was carried out in compliance with the ARRIVE guidelines (https://arriveguidelines.org).

 Consent for publication
Not applicable.

Competing interests
The authors declare no competing financial and non-financial interests.

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