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α-Ketoglutarate Accelerates the Initial Differentiation of Primed Human Pluripotent Stem Cells

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SUMMARY

Pluripotent stem cells (PSCs) can self-renew or differentiate from naïve or more differentiated, primed, pluripotent states established by specific culture conditions. Increased intracellular α-ketoglutarate (αKG) was shown to favor self-renewal in naïve mouse embryonic stem cells

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AUTHOR CONTRIBUTIONS

T.T., A.C.C., S.L.E., and D.B. performed experiments. I.L. and K.H. provided key reagents and expert guidance. T.T., J.H., T.G.G., D.B., and M.A.T. participated at differing levels in designing the study and analyzing data. T.T. and M.A.T. wrote the paper with help from T.G.G. and D.B.

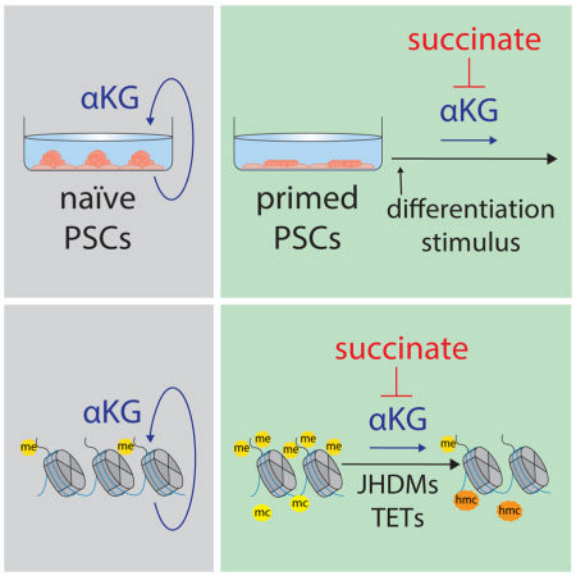
Conflict of Interest

The authors declare that they have no conflicts of interest.

(mESCs). The effect of αKG or αKG/succinate levels on differentiation from primed human PSCs (hPSCs) or mouse epiblast stem cells (EpiSCs) remains unknown. We examined primed hPSCs and EpiSCs and show that increased αKG or αKG/succinate ratios accelerate, and elevated succinate levels delay, primed PSC differentiation. αKG has been shown to inhibit the mitochondrial ATP synthase and to regulate epigenome-modifying dioxygenase enzymes. Mitochondrial uncoupling did not impede αKG-accelerated primed PSC differentiation. Instead, αKG induced, and succinate impaired, global histone and DNA demethylation in primed PSCs. The data support αKG promotion of self-renewal or differentiation depending on the pluripotent state.

eTOC blurb

α-ketoglutarate (αKG) is an important cofactor for demethylation reactions that helps to maintain naive pluripotent stem cells. TeSlaa et al. show that at later stages of pluripotency, αKG can promote early differentiation, highlighting that the cellular context and potentially the stage of cellular maturity can alter the effect of αKG.



INTRODUCTION

Human pluripotent stem cells (hPSCs) may self-renew or differentiate into all three germ layers (Takahashi et al., 2007; Thomson et al., 1998), but use in regenerative medicine is limited by generally inefficient differentiation strategies (Blanpain et al., 2012). During in vitro differentiation, hPSCs undergo a metabolic shift that increases respiration (OXPHOS) and reduces glycolysis, with inhibition of this transition impeding differentiation (Moussaieff et al., 2015; Zhang et al., 2011; Zhou et al., 2012). Despite the importance of this metabolic shift, differentiation protocols have focused on manipulating key signaling pathways and have overlooked metabolic contributions.

α-Ketoglutarate (αKG), a tricarboxylic acid (TCA) cycle metabolite, is a cofactor for αKG-dependent dioxygenase enzymes, which include JmjC-domain containing histone

demethylases (JHDMs) and Ten-eleven translocation (TET) enzymes (Kaelin Jr and McKnight, 2013). αKG can also bind and block the mitochondrial ATP synthase and inhibit mTOR signaling (Chin et al., 2014). Addition of cell permeable dimethyl-αKG (dm-αKG) to culture media enhances self-renewal and inhibits the differentiation of naïve state mouse embryonic stem cells (mESCs) likely by promoting histone and DNA demethylation (Carey et al., 2015). Interestingly, hPSCs grown in standard conditions are in a primed, or more developmentally mature pluripotent state, similar to post-implantation mouse epiblast stem cells (EpiSCs) (Greber et al., 2010; James et al., 2005; Tesar et al., 2007). A role for αKG in primed mouse or human PSCs has not been explored.

Naïve and primed PSCs show many molecular differences including self-renewing conditions, epigenetic states, and metabolism (Greber et al., 2010; Leitch et al., 2013; Marks et al., 2012; Ware et al., 2014; Zhou et al., 2012). A consensus naïve state for hPSCs, however, remains somewhat elusive. Culture conditions that establish naïve-like hPSCs each yield slightly different transcriptional profiles (Huang et al., 2014). Uncertainty about whether naïve hPSCs offer differentiation advantages over traditional primed hPSCs (Pera, 2014) emphasizes the remaining importance of primed hPSCs as an option for potential clinical applications.

Metabolites other than αKG have been shown to play a role in PSC self-renewal and differentiation. Removal of methionine, which provides methyl groups for DNA and histone methylation, potentiates PSC differentiation (Shiraki et al., 2014). Increased acetyl-CoA delays PSC differentiation, histone acetylation, and maintains expression of OCT4 (Moussaieff et al., 2015). Oxygen levels can both enhance reprogramming to pluripotency or the differentiation of hPSCs depending on environmental context (Mathieu et al., 2014; Xie et al., 2014). mESCs are dependent on threonine catabolism for histone and DNA methylation (Shyh-Chang et al., 2013; Wang et al., 2009). Here, we investigate the role for αKG during primed PSC differentiation.

RESULTS

TCA Cycle Metabolite Production in hPSCs

Respiration is reduced in hPSCs compared to their differentiated counterparts, suggesting that TCA cycle metabolite production could be low (Zhang et al., 2011; Zhou et al., 2012). To examine the TCA cycle, stable isotope labeling experiments were performed in media promoting self-renewal (E8 media) or encouraging differentiation (E6 media) (Figure S1A and S1B). Using the E8/E6 system, culture medias differ by only two factors which are excluded from the E6 medium, bFGF and TGF-β, ensuring differences in metabolism are due to different cell states. A shift in the oxygen consumption rate (OCR) to extracellular acidification rate (ECAR) ratio confirmed a shift toward OXPHOS with E6 media differentiation (Figure 1A). Furthermore, glutamine withdrawal reduced oxygen consumption, implicating glutamine as a TCA cycle fuel in hPSCs (Figure 1B).

Despite a low OCR/ECAR ratio, hPSCs showed a robust contribution of [U-^{13}C] glucose into TCA cycle metabolites (Figure 1C). Glutamine withdrawal increased the glucose contribution to TCA cycle metabolites αKG, succinate, and malate in E8, but not in E6,

culture conditions (Figure 1C). The mass isotopologue distribution (MID) of citrate indicates the contribution of [U-^{13}C] glucose to the TCA cycle, with m+2 and m+3 isotopologues indicating initial entry of glucose into the TCA cycle and m+4, m+5, and m+6 isotopologues indicating ^{13}C glucose carbons that have cycled through 1 or 2 turns (Figure 1D). In similar conditions no differences in the citrate MID in E8 and E6 cultures were detected (Figure 1E), but glutamine withdrawal for 18 h resulted in an increase in m+4 and m+6 citrate isotopolgoues in undifferentiated hESCs (E8) (Figure 1F). Thus, glucose-derived carbons are retained through 1 or 2 turns of the TCA cycle in the absence of glutamine in self-renewing hPSCs. Consistent with this result, glutamine withdrawal led to a decrease in unlabeled αKG (m+0) in E8 conditions (Figure S1C).

The MID of citrate with [U-^{13}C] glutamine indicates the amount of citrate derived from glutamine after one (m+4) or two turns of the TCA cycle (m+2) (Figure 1G). Increased m+4 citrate was detected in E8 compared to E6 conditions, suggesting a lower contribution of glutamine to the TCA cycle in E6 differentiated hPSCs (Figure 1H). However, 40% of glutamate, which is generated directly from glutamine, was unlabeled in E6 conditions (Figure S1D). Measurement of extracellular glutamate levels revealed net uptake of glutamate by cells cultured in E6 medium (Figure 1I). Detection of [U-^{13}C] glutamate uptake confirmed these results (Figure 1J), with increased conversion of glutamate into αKG occurring in differentiated cells (E6) only in the absence of glutamine (Figure 1K). The data suggest that glucose and glutamine are the major contributors to the TCA cycle in hPSCs, whereas other metabolites, such as glutamate, also fuel the TCA cycle in early differentiated hPSCs.

In proliferative cells, αKG producing transaminases (TAs), which transfer amine groups from glutamate to α-keto acids to form amino acids (Figure S1E), have high activity (Coloff et al., 2016). Glutamine inclusion in hPSC culture medium, which provides glutamate for TAs, increases αKG, alanine, and aspartate levels, products of these TAs (Figure S1F and S1G). To further study TA activity, [$^{15}N_2$] glutamine was used to quantify the transfer of ^{15}N from glutamate to amino acids (Figure S1E), and ^{15}N was detected in alanine, aspartate, isoleucine, serine, and methionine (Figure 1L). Expression of glutamate pyruvate transaminases (GPT) and glutamic oxaloacetic transaminases (GOT) was confirmed in hPSCs, verifying their contribution to αKG production (Figure S1H). Robust αKG production in primed hPSCs prompted studies into a role for αKG in PSC differentiation.

αKG Accelerates Multi-lineage Primed PSC Differentiation

Neuroectoderm (NE) differentiation was induced in primed H1, H9, UCLA1, and HIPS2 hPSCs by dual SMAD inhibition (Chambers et al., 2009). Dm-αKG significantly increased the percentage of PAX6, an essential transcription factor for NE specification in humans (Zhang et al., 2010), expressing cells by day 4 of differentiation (Figures 2A–2C). MAP2C and NESTIN positive cells modestly increased with dm-αKG treatment (Figure 2C and S2A). To determine whether this effect was lineage specific, endoderm differentiation was induced by high concentration Activin A exposure (D'Amour et al., 2005). On day 2, dm-αKG significantly increased the percentage of H9 cells expressing SOX17, a definitive

endoderm transcription factor (Figures S2B and S2C). Combined, the data support that αKG accelerates the early differentiation of multiple hPSC germ lineages.

NE differentiation was examined with added αKG, which unlike dm-αKG is dependent on membrane transporters for uptake, and resulted in an increase in PAX6 positive cells (Figure 2D and 2F). In contrast to dm-αKG, which shows a dose-dependent increase in cell differentiation, αKG levels beyond 4mM did not further stimulate NE differentiation (Figure 2D–2G). Added αKG and dm-αKG each increased intracellular αKG and TCA cycle metabolite levels, although only 12mM dm-αKG reached significance (Figure 2H and 2I).

Consistent with prior results showing that dm-αKG supports naïve mESC self-renewal (Carey et al., 2015), an increase in alkaline phosphatase staining was detected in naïve mESCs incubated with dm-αKG during 48h of LIF withdrawal (Figure S2D). Since dm-αKG accelerates primed hPSC differentiation, we examined the role for αKG in primed mouse PSCs, or EpiSCs. Addition of dm-αKG to EpiSCs induced to differentiate by withdrawal of bFGF and Activin A (Greber et al., 2010) accelerated the rate of Oct4 inactivation (Figures 2J, 2K, and S2E–S2G). Therefore, αKG can accelerate differentiation of both mouse and human primed PSCs.

αKG has recently been shown to bind and inhibit the mitochondrial ATP synthase subunit β leading to mTOR inhibition (Chin et al., 2014). To determine whether ATP synthase inhibition contributes to αKG-accelerated primed PSC differentiation, an OXPHOS uncoupling agent, carbonyl cyanide 4-(trifluoromethoxy)-phenylhydrazone (FCCP), was used during NE differentiation. FCCP inhibits ATP production as a mitochondrial inner membrane protonophore that dissipates the H^+ ion electrochemical gradient that runs the ATP synthase. dm-αKG accelerated NE differentiation in H9 hESCs incubated with FCCP (Figures S2H and S2I), suggesting at an alternative mechanism. Furthermore, hPSCs treated with an inhibitor of ATP synthase, oligomycin, during NE differentiation showed almost no PAX6 expression after 4 days despite addition of pyruvate and uridine to promote cell survival (Birsoy et al., 2015; Sullivan et al., 2015) (Figure S2J and S2K). Since ATP synthase inhibition delays or inhibits NE differentiation which opposes the accelerating effect of dm-αKG, we conclude that αKG does not accelerate differentiation of primed PSCs through inhibition of ATP synthase.

Succinate Accumulation Delays hPSC Differentiation

A second potential mechanism for αKG accelerated hPSC differentiation is stimulation of epigenome-modifying dioxygenases. In this event, an αKG-dependent dioxygenase competitive inhibitor, such as succinate (Xiao et al., 2012), would impair differentiation. Cell permeable succinate, dimethyl succinate (dms), resulted in a decreased percentage of PAX6 expressing hPSCs during NE differentiation compared to hPSCs incubated with dm-αKG alone (Figures S2L and S2M). Inhibition of succinate dehydrogenase A (SDHA), which converts succinate to fumarate, either with a chemical inhibitor, 3-nitropropionic acid (NPA), or with shRNA should also cause succinate accumulation (Figure 3A). NPA treatment decreased the αKG/succinate ratio 34-fold with a 14-fold mean increase in succinate and 2.4-fold mean decrease in αKG (Figures 3B and S3A). NPA delayed NE-specifying PAX6 expression (Figures 3C and S3B–S3D), and the loss of pluripotency

marker SSEA3 in hPSCs differentiated into NE (Figure S3E). Knockdown of SDHA, confirmed by immunoblot and reduced OCR (Figures S3F–S3H), also delayed PAX6 and MAP2B expression during NE differentiation, which was rescued by dm-αKG (Figures 3D, 3E and S3I).

We further assessed the role of succinate in differentiation by embryoid body (EB) formation, which contrasts with lineage-directed differentiation by removal of bFGF rather than by addition of supplements or inhibitors. OCT4 was almost eliminated in EBs expressing a scrambled shRNA, whereas shSDHA expression maintained elevated OCT4 expression (Figure S3J and S3K). Validating these results, inhibition of SDHA with NPA significantly impaired OCT4 repression in EB differentiation compared to control (Figures S3L).

TA Inhibition Delays hPSC Differentiation

To decrease αKG levels, chemical inhibitors of αKG producing TAs were used (Figure 3A). Aminooxyacetic acid (AOA), a pan-TA inhibitor, reduced ^{15}N transfer from [$^{15}N_2$] glutamine to alanine, aspartate, isoleucine, serine and methionine in hPSCs (Figure S3M). L-cycloserine (cyclo), a GPT inhibitor, decreased ^{15}N transfer to alanine in hPSCs treated with cyclo (Figure S3M). Both inhibitors caused a significant decrease in αKG levels and other TCA cycle metabolite levels, and had no effect on basal respiration (Figure 3F, S3N and S3O). AOA impaired PAX6 and MAP2B activation during NE differentiation (Figure 3G, 3H and S3P). A mixture of non-essential amino acids, including alanine and aspartate, had no effect on AOA treatment, whereas supplementation with dm-αKG rescued the block in differentiation caused by AOA (Figure 3G, 3H, and S3P). Cyclo caused a less dramatic, but significant, decrease in the percent MAP2B positive cells corresponding to its relatively smaller effect on αKG levels compared to AOA (Figure 3I and 3J). Supplementation with dm-αKG restored MAP2B levels in cyclo treated cells (Figure 3I and 3J). Therefore, decreased αKG levels cause a delay or inhibition of directed differentiation.

To determine whether TCA cycle flux impacts differentiation, dichloroacetate (DCA), an inhibitor of pyruvate dehydrogenase kinase (PDK), was utilized. PDK inhibits pyruvate dehydrogenase (PDH) activity. Therefore, DCA increases PDH activity and glucose flux into the TCA cycle, which elevates ETC activity (Figure S4A). Low levels of DCA increased PAX6 positive cells on day 4 of NE differentiation, whereas higher DCA levels had no effect (Figure S4B and S4C). dm-αKG further enhanced differentiation at 0 and 3mM DCA, but not at 1mM (Figure S4B and S4C). These results do not support changes in TCA cycle flux as a mechanism for αKG in accelerated hPSC differentiation. Rather, the data indicate that succinate delays and αKG promotes the initial differentiation of primed PSCs most likely through actions on αKG-dependent dioxygenases.

αKG/Succinate Regulates the Epigenome of Differentiating hPSCs

To evaluate the role of αKG-dependent dioxygenases during hPSC differentiation, an inhibitory αKG mimetic, dimethyloxalylglycine (dmog) was used. Exposure to dmog inhibited PAX6 expression at concentrations that did not affect cell number (Figure 4A, 4B, and S4D). To assess the effect of the αKG on TET enzymes, dot blots were performed and

levels of 5-hydroxymethylcytosine (5hmc) and 5-methylcytosine (5mc) in DNA were measured from H9 and UCLA1 hESCs after 4 days of NE differentiation. dm-αKG exposure caused a two-fold increase in the 5hmc/5mc ratio, whereas NPA caused a significant decrease in this ratio (Figure 4C, 4D, and S4E). The data suggest a significant role for TET enzymes in αKG accelerated differentiation of primed hPSCs. To evaluate αKG regulation of JHDMs, histone lysine 4 trimethylation (H3K4me3) and histone lysine 27 trimethylation (H3K27me3), were assessed by intracellular flow cytometry. In general, NPA treatment during NE differentiation in UCLA1 hESCs led to an increase in histone marks, whereas dm-αKG led to a decrease (Figures 4E–4H and S4F–S4J). Analysis of a broad array of histone post-translational modifications by immunoblot revealed an overall repressive effect of dm-αKG on global lysine trimethylation, but little effect on global monomethylation or acetylation marks (Figure 4I).

Overall, the data suggest that an increased αKG/succinate ratio accelerates, and a decreased αKG/succinate ratio retards, initial primed PSC differentiation by titered cofactor/inhibitor activities on epigenome remodeling enzymes, including TET enzymes and JHDMs. This interpretation is consistent with the proposed αKG/succinate mechanism for maintained naïve pluripotency in mESCs through epigenetic regulation (Carey et al., 2015), only with an inverse cell fate outcome in the primed pluripotent state with induced differentiation.

DISCUSSION

This study reveals an unanticipated differentiation-promoting role for αKG in primed PSCs. Recent results showed that αKG supports self-renewal of naïve mESCs potentially by promoting the demethylation of histones and DNA (Carey et al., 2015). Our data supports a similar mechanism with an opposite outcome in the context of primed PSCs induced to differentiate. Consistent with a context-specific role for αKG, TET enzymes and JHDMs also have dual roles in the self-renewal and differentiation of mESCs. TET1 promotes reprogramming to naïve pluripotency, whereas triple knockout of the TET enzymes impedes differentiation (Costa et al., 2013; Dawlaty et al., 2014). JHDMs are a large class of enzymes that have functions in both naive pluripotency and differentiation. For example, JMJD3 and UTX promote naïve pluripotency (Carey et al., 2015), whereas Jarid1b is involved in neural differentiation (Schmitz et al., 2011) The data suggest that αKG enhances the activity of a large family of enzymes that may both maintain low DNA and histone methylation levels favoring the naïve PSC state, and also promote epigenome remodeling during induced primed PSC differentiation. These opposing outcomes are consistent with the state-dependent effects of oxygen levels in maintaining pluripotency or promoting differentiation (Mathieu et al., 2014; Xie et al., 2014). Both DNA and histone methylation levels are lower in naïve compared to primed PSCs (Hackett and Surani, 2014; Leitch et al., 2013). These epigenome differences, along with metabolome differences between naïve and primed pluripotent states, could support a differential role for αKG identified in this study. Our results suggest a model for αKG promotion of induced differentiation by primed PSCs from demethylation reactions that help silence pluripotency genes and activate lineage specific genes to accelerate induced multi-lineage differentiation. A key goal of hPSC research is to develop mature and functional cells for regenerative medicine. Increased αKG could be useful for improving primed state PSC differentiation.

EXPERIMENTAL PROCEDURES

Standard procedures were followed for immunoblotting, confocal microscopy and shRNA knockdown, as described in the Supplemental Experimental Procedures.

Cell Culture

Primed hPSCs were passage onto feeder-free matrigel (Fisher) in mTeSR1 medium and passaged with Gentle Cell Dissociation Reagent (Stem Cell Technologies) for most experiments. For experiments performed in Essential 8 (E8) or Essential 6 (E6) medias (Stem Cell Technologies), hPSCs were switched from mTeSR1 to appropriate media at passage. EpiSCs were grown in feeder free conditions in medium containing bFGF and Activin A on fibronectin. Further details are provided in the Supplemental Experimental Procedures.

Seahorse Measurements

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) assays were performed as previously described (Zhang et al., 2012). hPSCs were plated onto an XF24 microplate (Seahorse Bioscience) at 10^5 cells/well or 10^6 cells/well with 10 μM Y-27632 (BioPioneer). The next day, 1h prior to the assay, the medium was changed to XF Media (Seahorse Bioscience) supplemented 17.5 mM glucose. Cell metabolic rates were measured using an XF24 Extracellular Flux Analyzer (Seahorse Bioscience). Basal respiration was determined by quantifying OCR prior to and after the addition of 1 μM rotenone (Sigma) and 1 μM antimycin A (Sigma).

Metabolite Extraction and Analysis

Cellular metabolites were extracted with 80% ice-cold methanol and UHPLC/MS measurements of metabolite levels was performed and analyzed as previously described (Thai et al., 2014). Details are provided in the Supplemental Experimental Procedures.

Glutamate Uptake

Levels of glutamate in culture media was measured using a BioProfile Basic Analyzer (Nova Biomedical). Details are provided in the Supplemental Experimental Procedures.

Neuroectoderm Differentiation

NE differentiation was performed as previously reported (Chambers et al., 2009; Shiraki et al., 2014). Details are provided in the Supplemental Experimental Procedures.

Flow Cytometry Analysis

Cells were collected with Gentle Cell Dissociation Reagent and processed using the Cytofix/Cytoperm Kit (BD Bioscience). Cells were analyzed with either an LSRII or LSRFortessa (BD Bioscience).

Dot Blot Analysis and Quantification

DNA was collected with the DNeasy Blood and Tissue Kit (Qiagen) and quantified using a NanoDrop 1000 Spectrophotometer (ThermoFisher). DNA was denatured at 99°C for 5 min, put on ice, and neutralized by adding ammonium acetate to a final concentration of 0.66M. 400ng of each sample was spotted on Amersham Hybond-N+ (Fisher) nylon membranes and baked at 80°C for 2h. Membranes were blocked with 5% skim milk for 3h and incubated with primary antibody overnight. The immunoblot procedure was followed by IRDye-conjugated seconary antibodies. Blots were imaged with an Odyssey Fc (Licor) and quantified with Image Studio Version 5.2.5 (Licor).

Statistical Analysis

Values are presented as the mean ± SD or mean ± SEM. Data were analyzed with Prism (GraphPad). Pairwise comparisons were analyzed using two-tailed Student's *t*-test. Other data were analyzed using one-way or two-way ANOVA with correction for multiple comparisons. In all cases, $p < 0.05$ was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Birsoy K, Wang T, Chen Walter W, Freinkman E, Abu-Remaileh M, Sabatini David M. An Essential Role of the Mitochondrial Electron Transport Chain in Cell Proliferation Is to Enable Aspartate Synthesis. Cell. 2015; 162:540–551. [PubMed: 26232224]

Blanpain C, Daley GQ, Hochedlinger K, Passegué E, Rossant J, Yamanaka S. Stem cells assessed. Nat Rev Mol Cell Biol. 2012; 13:471–476. [PubMed: 22678486]

Carey BW, Finley LWS, Cross JR, Allis CD, Thompson CB. Intracellular α-ketoglutarate maintains the pluripotency of embryonic stem cells. Nature. 2015; 518:413–416. [PubMed: 25487152]

Chambers SM, Fasano CA, Papapetrou EP, Tomishima M, Sadelain M, Studer L. Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. Nat Biotech. 2009; 27:275–280.

Chin RM, Fu X, Pai MY, Vergnes L, Hwang H, Deng G, Diep S, Lomenick B, Meli VS, Monsalve GC, et al. The metabolite [agr]-ketoglutarate extends lifespan by inhibiting ATP synthase and TOR. Nature. 2014; 510:397–401. [PubMed: 24828042]

Coloff, Jonathan L.; Murphy, JP.; Braun, Craig R.; Harris, Isaac S.; Shelton, Laura M.; Kami, K.; Gygi, Steven P.; Selfors, Laura M.; Brugge, Joan S. Differential Glutamate Metabolism in Proliferating and Quiescent Mammary Epithelial Cells. Cell Metabolism. 2016; 23:867–880. [PubMed: 27133130]

Costa Y, Ding J, Theunissen TW, Faiola F, Hore TA, Shliaha PV, Fidalgo M, Saunders A, Lawrence M, Dietmann S, et al. NANOG-dependent function of TET1 and TET2 in establishment of pluripotency. Nature. 2013; 495:370–374. [PubMed: 23395962]

D'Amour KA, Agulnick AD, Eliazer S, Kelly OG, Kroon E, Baetge EE. Efficient differentiation of human embryonic stem cells to definitive endoderm. Nat Biotech. 2005; 23:1534–1541.

Dawlaty, Meelad M.; Breiling, A.; Le, T.; Barrasa, MI.; Raddatz, G.; Gao, Q.; Powell, Benjamin E.; Cheng, Albert W.; Faull, Kym F.; Lyko, F., et al. Loss of Tet Enzymes Compromises Proper Differentiation of Embryonic Stem Cells. Developmental Cell. 2014; 29:102–111. [PubMed: 24735881]

Greber B, Wu G, Bernemann C, Joo JY, Han DW, Ko K, Tapia N, Sabour D, Sterneckert J, Tesar P, et al. Conserved and Divergent Roles of FGF Signaling in Mouse Epiblast Stem Cells and Human Embryonic Stem Cells. Cell Stem Cell. 2010; 6:215–226. [PubMed: 20207225]

Hackett, Jamie A.; Surani, MA. Regulatory Principles of Pluripotency: From the Ground State Up. Cell Stem Cell. 2014; 15:416–430. [PubMed: 25280218]

Huang K, Maruyama T, Fan G. The Naive State of Human Pluripotent Stem Cells: A Synthesis of Stem Cell and Preimplantation Embryo Transcriptome Analyses. Cell Stem Cell. 2014; 15:410–415. [PubMed: 25280217]

James D, Levine AJ, Besser D, Hemmati-Brivanlou A. TGFβ/activin/nodal signaling is necessary for the maintenance of pluripotency in human embryonic stem cells. Development. 2005; 132:1273–1282. [PubMed: 15703277]

Kaelin, William G., Jr; McKnight, Steven L. Influence of Metabolism on Epigenetics and Disease. Cell. 2013; 153:56–69. [PubMed: 23540690]

Leitch HG, McEwen KR, Turp A, Encheva V, Carroll T, Grabole N, Mansfield W, Nashun B, Knezovich JG, Smith A, et al. Naive pluripotency is associated with global DNA hypomethylation. Nat Struct Mol Biol. 2013; 20:311–316. [PubMed: 23416945]

Marks H, Kalkan T, Menafra R, Denissov S, Jones K, Hofemeister H, Nichols J, Kranz A, Francis Stewart A, Smith A, et al. The Transcriptional and Epigenomic Foundations of Ground State Pluripotency. Cell. 2012; 149:590–604. [PubMed: 22541430]

Mathieu J, Zhou W, Xing Y, Sperber H, Ferreccio A, Agoston Z, Kuppusamy Kavitha T, Moon Randall T, Ruohola-Baker H. Hypoxia-Inducible Factors Have Distinct and Stage-Specific Roles during Reprogramming of Human Cells to Pluripotency. Cell Stem Cell. 2014; 14:592–605. [PubMed: 24656769]

Moussaieff A, Rouleau M, Kitsberg D, Cohen M, Levy G, Barasch D, Nemirovski A, Shen-Orr S, Laevsky I, Amit M, et al. Glycolysis-Mediated Changes in Acetyl-CoA and Histone Acetylation Control the Early Differentiation of Embryonic Stem Cells. Cell Metabolism. 2015; 21:392–402. [PubMed: 25738455]

Pera, Martin F. In Search of Naivety. Cell Stem Cell. 2014; 15:543–545. [PubMed: 25517463]

Schmitz SU, Albert M, Malatesta M, Morey L, Johansen JV, Bak M, Tommerup N, Abarrategui I, Helin K. Jarid1b targets genes regulating development and is involved in neural differentiation. The EMBO Journal. 2011; 30:4586–4600. [PubMed: 22020125]

Shiraki N, Shiraki Y, Tsuyama T, Obata F, Miura M, Nagae G, Aburatani H, Kume K, Endo F, Kume S. Methionine Metabolism Regulates Maintenance and Differentiation of Human Pluripotent Stem Cells. Cell Metabolism. 2014; 19:780–794. [PubMed: 24746804]

Shyh-Chang N, Locasale JW, Lyssiotis CA, Zheng Y, Teo RY, Ratanasirintrawoot S, Zhang J, Onder T, Unternaehrer JJ, Zhu H, et al. Influence of Threonine Metabolism on S-Adenosylmethionine and Histone Methylation. Science. 2013; 339:222–226. [PubMed: 23118012]

Sullivan, Lucas B.; Gui, Dan Y.; Hosios, Aaron M.; Bush, Lauren N.; Freinkman, E.; Vander Heiden, Matthew G. Supporting Aspartate Biosynthesis Is an Essential Function of Respiration in Proliferating Cells. Cell. 2015; 162:552–563. [PubMed: 26232225]

Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 2007; 131:861–872. [PubMed: 18035408]

Tesar PJ, Chenoweth JG, Brook FA, Davies TJ, Evans EP, Mack DL, Gardner RL, McKay RDG. New cell lines from mouse epiblast share defining features with human embryonic stem cells. Nature. 2007; 448:196–199. [PubMed: 17597760]

Thai M, Graham Nicholas A, Braas D, Nehil M, Komisopoulou E, Kurdistani Siavash K, McCormick F, Graeber Thomas G, Christofk Heather R. Adenovirus E4ORF1-Induced MYC Activation Promotes Host Cell Anabolic Glucose Metabolism and Virus Replication. Cell Metabolism. 2014; 19:694–701. [PubMed: 24703700]

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic Stem Cell Lines Derived from Human Blastocysts. Science. 1998; 282:1145–1147. [PubMed: 9804556]

Wang J, Alexander P, Wu L, Hammer R, Cleaver O, McKnight SL. Dependence of Mouse Embryonic Stem Cells on Threonine Catabolism. Science. 2009; 325:435–439. [PubMed: 19589965]

Ware CB, Nelson AM, Mecham B, Hesson J, Zhou W, Jonlin EC, Jimenez-Caliani AJ, Deng X, Cavanaugh C, Cook S, et al. Derivation of naïve human embryonic stem cells. Proceedings of the National Academy of Sciences. 2014; 111:4484–4489.

Xiao M, Yang H, Xu W, Ma S, Lin H, Zhu H, Liu L, Liu Y, Yang C, Xu Y, et al. Inhibition of α-KG-dependent histone and DNA demethylases by fumarate and succinate that are accumulated in mutations of FH and SDH tumor suppressors. Genes & Development. 2012; 26:1326–1338. [PubMed: 22677546]

Xie Y, Zhang J, Lin Y, Gaeta X, Meng X, Wisidagama Dona RR, Cinkornpumin J, Koehler Carla M, Malone Cindy S, Teitell Michael A, et al. Defining the Role of Oxygen Tension in Human Neural Progenitor Fate. Stem Cell Reports. 2014; 3:743–757. [PubMed: 25418722]

Zhang J, Khvorostov I, Hong JS, Oktay Y, Vergnes L, Nuebel E, Wahjudi PN, Setoguchi K, Wang G, Do A, et al. UCP2 regulates energy metabolism and differentiation potential of human pluripotent stem cells. The EMBO Journal. 2011; 30:4860–4873. [PubMed: 22085932]

Zhang J, Nuebel E, Wisidagama DRR, Setoguchi K, Hong JS, Van Horn CM, Imam SS, Vergnes L, Malone CS, Koehler CM, et al. Measuring energy metabolism in cultured cells, including human pluripotent stem cells and differentiated cells. Nat Protocols. 2012; 7:1068–1085. [PubMed: 22576106]

Zhang X, Huang CT, Chen J, Pankratz MT, Xi J, Li J, Yang Y, LaVaute TM, Li XJ, Ayala M, et al. Pax6 Is a Human Neuroectoderm Cell Fate Determinant. Cell Stem Cell. 2010; 7:90–100. [PubMed: 20621053]

Zhou W, Choi M, Margineantu D, Margaretha L, Hesson J, Cavanaugh C, Blau CA, Horwitz MS, Hockenbery D, Ware C, et al. HIF1α induced switch from bivalent to exclusively glycolytic metabolism during ESC-to-EpiSC/hESC transition. The EMBO journal. 2012; 31:2103–2116. [PubMed: 22446391]

Highlights

- hPSCs produce TCA cycle metabolites despite low OXPHOS
- α-ketoglutarate (αKG) promotes early differentiation of hPSCs
- Accumulation of succinate or depletion of αKG delays differentiation of hPSCs
- αKG/succinate alters histone methylation corresponding to differentiation kinetics

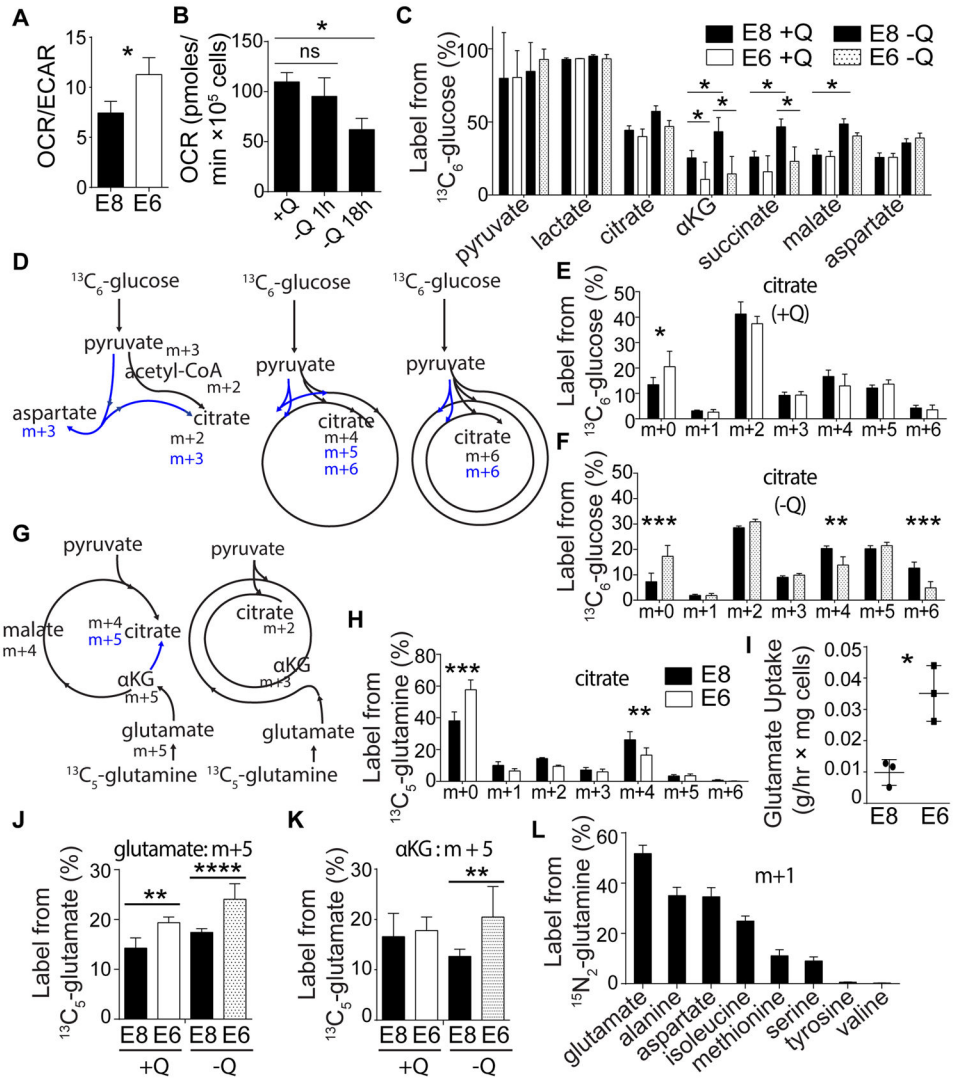

Figure 1. Production of TCA cycle metabolites in hPSCs

(A) Ratio of oxygen consumption rate (OCR) to extracellular acidification rate (ECAR) in H9 hESCs cultured in medium containing (E8) or lacking (E6) bFGF and TGFβ.

(B) OCR quantified in H9 hESCs grown in media containing glutamine or with glutamine removed for 1h or 18h.

(C) Fractional contribution of ^{13}C labeled metabolites from [U-^{13}C] glucose after 18h quantified by UHPLC-MS.

(D) Schematic illustrating how the mass isotopologue distribution (MID) of citrate from [U-^{13}C] glucose reveals the contribution of glucose-labeled metabolites through multiple turns of the TCA cycle.

(E, F) MID of citrate in H9 hESCs from [U-^{13}C] glucose in conditions containing (E) or lacking glutamine (F) are shown.

(G) Schematic of [U-^{13}C] glutamine labeling of the TCA cycle. The m+5 isotopologue of citrate can be produced by reductive carboxylation of glutamine (blue). The m+4 and m+2

isotopologues contain carbons derived from glutamine after one or two turns of the TCA cycle, respectively.

(H) MID of citrate from [U-^{13}C] glutamine in H9 hESCs cultured in E8 or E6 medium.

(I) Measurement of glutamate uptake from culture medium in H9 hESCs maintained in E8 medium or differentiated in E6 medium.

(J) M + 5 isotopologue of glutamate in H9 hESCs indicating increased uptake of [U-^{13}C] glutamate from the culture medium in differentiated cells (E6) grown with or without glutamine.

(K) M + 5 isotopologue of αKG in H9 hESCs grown in [U-^{13}C] glutamate.

(L) M + 1 isotopologue of listed amino acids in H9 hESCs grown with [$^{15}N_2$] glutamine reflecting the activities of multiple αKG-producing TAs.

Data represent mean ± SD of at least three biological replicates. *p≤0.05; **p≤0.01; ***p≤0.001; ****p≤0.0001. P values were determined by an unpaired two-tailed Student's *t*-test (A, I), by one-way (B) or by two-way analysis of variance (ANOVA) (C, E, F, H, J, K) with correction for multiple comparisons.

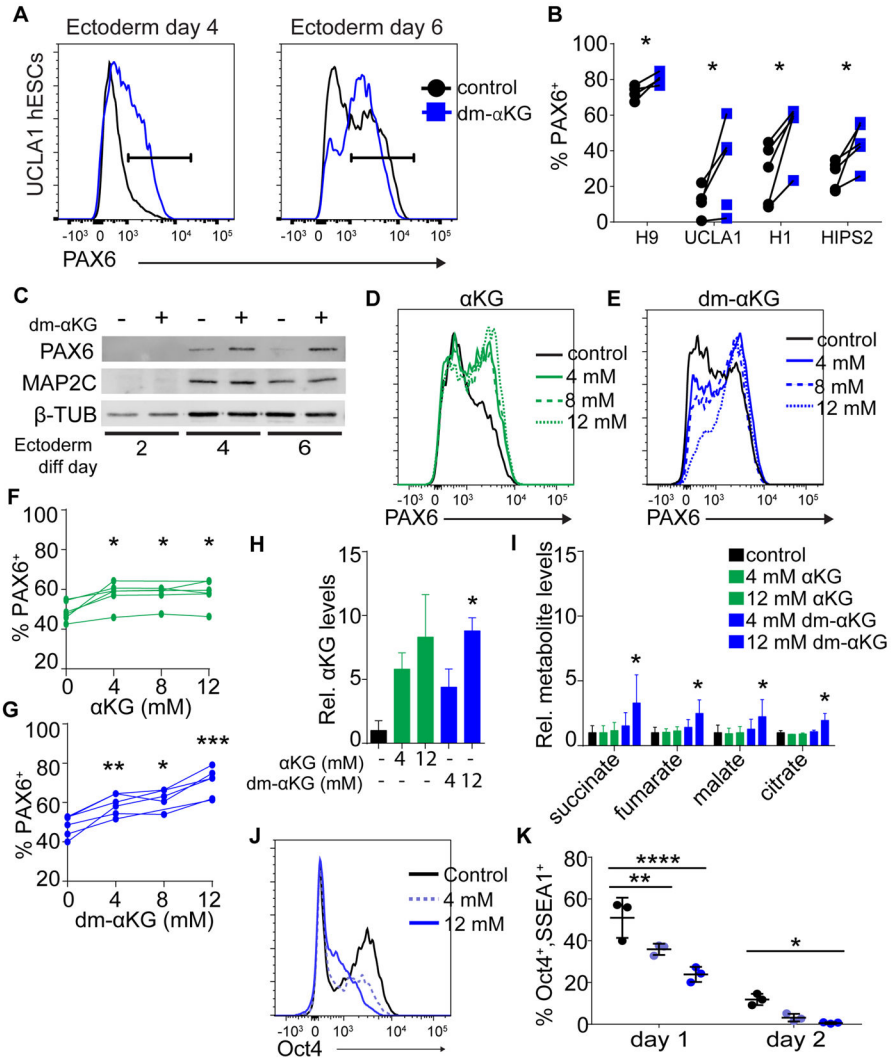

Figure 2. αKG accelerates differentiation of primed pluripotent stem cells

(A) Flow cytometry analysis of PAX6 transcription factor expression in UCLA1 hESCs encouraged to differentiate into NE at 4 or 6 days. 12mM dm-αKG incubated (blue) and control (black) traces are shown.

(B) Flow cytometry quantification of the percent PAX6 expressing cells at 4 days of NE differentiation for H9, UCLA1 and H1 hESCs and HIPS2 hiPSCs. H9, UCLA1 and H1 cells were incubated with (blue) or without (black) 12mM dm-αKG, and HIPS2 hPSCs were incubated with (blue) or without (black) 6mM dm-αKG. Lines connect pairs of independent biological replicates.

(C) Immunoblot of ectoderm markers PAX6 and MAP2C after 2, 4, and 6 days of NE differentiation of H9 hESCs.

(D–E) Flow cytometry of PAX6 expression in H9 hESCs on day 4 of NE differentiation with indicated amounts of (D) αKG or (E) dm-αKG added to the culture medium.

(F–G) Percent H9 hESC cells positive for PAX6 expression on day 4 of differentiation plotted against the concentration of added (F) αKG or (G) dm-αKG. Lines connect independent biological replicates.

(H–I) UHPLC-MS quantification of fold change of (H) αKG and (I) other TCA cycle metabolite levels in H9 hESCs incubated with the listed concentrations of αKG or dm-αKG. Error bars represent SEM of 3 biological replicates.

(J–K) Flow cytometry analysis (J) and quantification (K) of Oct4 expression in EpiSC-1 cells differentiated by growth factor withdrawal for 24h under control conditions, with 4mM dm-αKG, or with 12mM dm-αKG.

Data represent mean ± SD of three biological replicates unless otherwise noted. *p≤0.05; **p≤0.01, ***p≤0.001; ****p≤0.0001. P values were determined by a ratio paired two-tailed Student's *t*-test (B, F, G, H) or by two-way ANOVA with repeated measures and with correction for multiple comparisons (I, K).

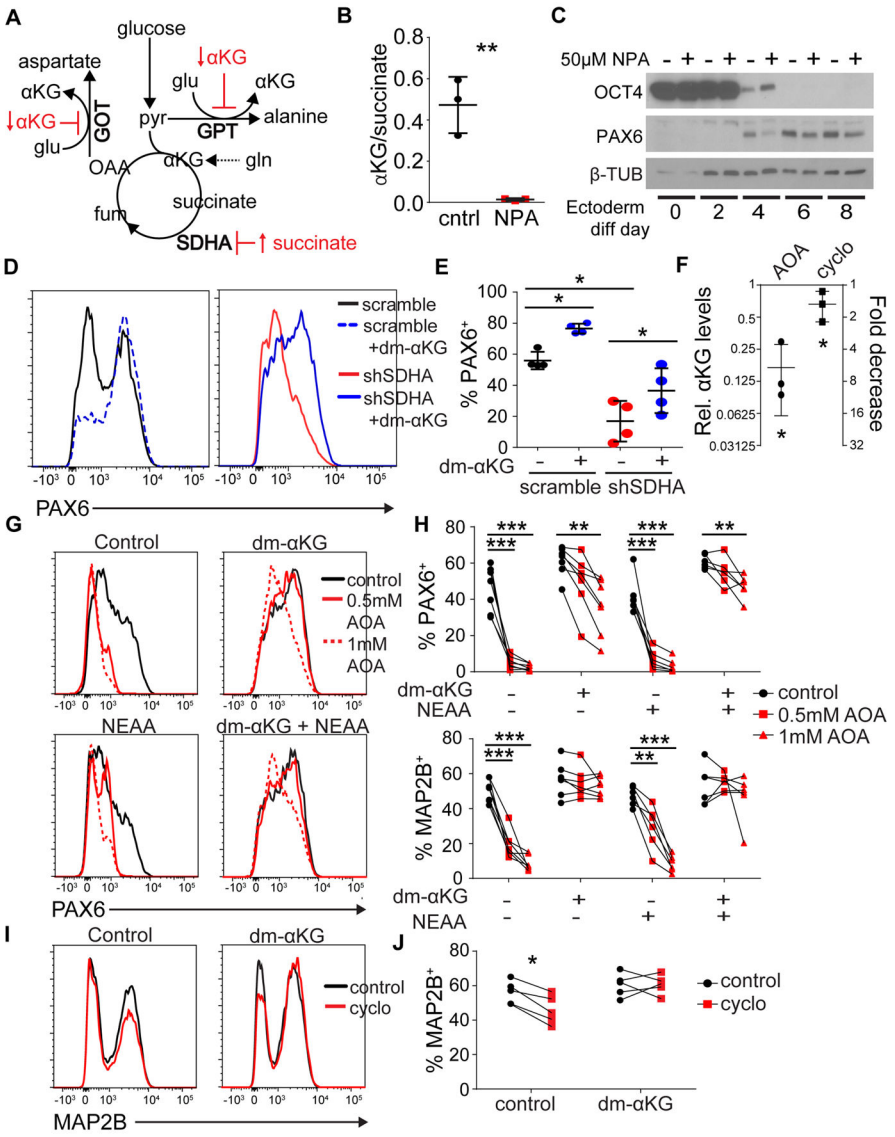

Figure 3. Decrease in the αKG/succinate ratio delays hPSC differentiation

(A) Inhibition or depletion of succinate dehydrogenase subunit A (SDHA) causes succinate accumulation. Inhibition of TAs, such as glutamate-pyruvate transaminase (GPT) or glutamic oxaloacetic transaminase (GOT), results in a decrease in αKG levels. Both of these manipulations cause a decrease in the αKG/succinate ratio which is predicted to inhibit αKG-dependent dioxygenases.

(B) αKG/succinate ratio in control (black) or 10μM NPA incubated (red) H9 hESCs for 18h quantified by UHPLC-MS.

(C) Immunoblot of OCT4 and PAX6 at indicated time points of NE differentiation with or without 50μM NPA for H9 hESCs. β-TUB, β-tubulin.

(D–E) Flow cytometry analysis of PAX6 expression in H9 hESCs expressing shRNA targeting SDHA or scramble control shRNA treated with dm-αKG where indicated at day 4 of NE differentiation.

(F) UHPLC-MS quantification of fold change in αKG levels with TA inhibitors aminooxyacetic acid (AOA) and cycloserine (cyclo) compared to drug carrier controls. (G–I) Flow cytometry analysis of PAX6 and MAP2B expression in H9 hESCs on day 4 of NE differentiation co-incubated with AOA (F, G) or 50μM cyclo (H, I) supplemented with 12mM dm-αKG, 1mM non-essential amino acids (NEAA), or both where indicated. Data represent mean ± SD of at least three biological replicates. *p≤0.05; **p≤0.01; ***p≤0.001. P values were calculated using an unpaired Student's *t*-test (B, F), or a one-way (E) or two-way ANOVA (H, J) with correction for multiple comparisons.

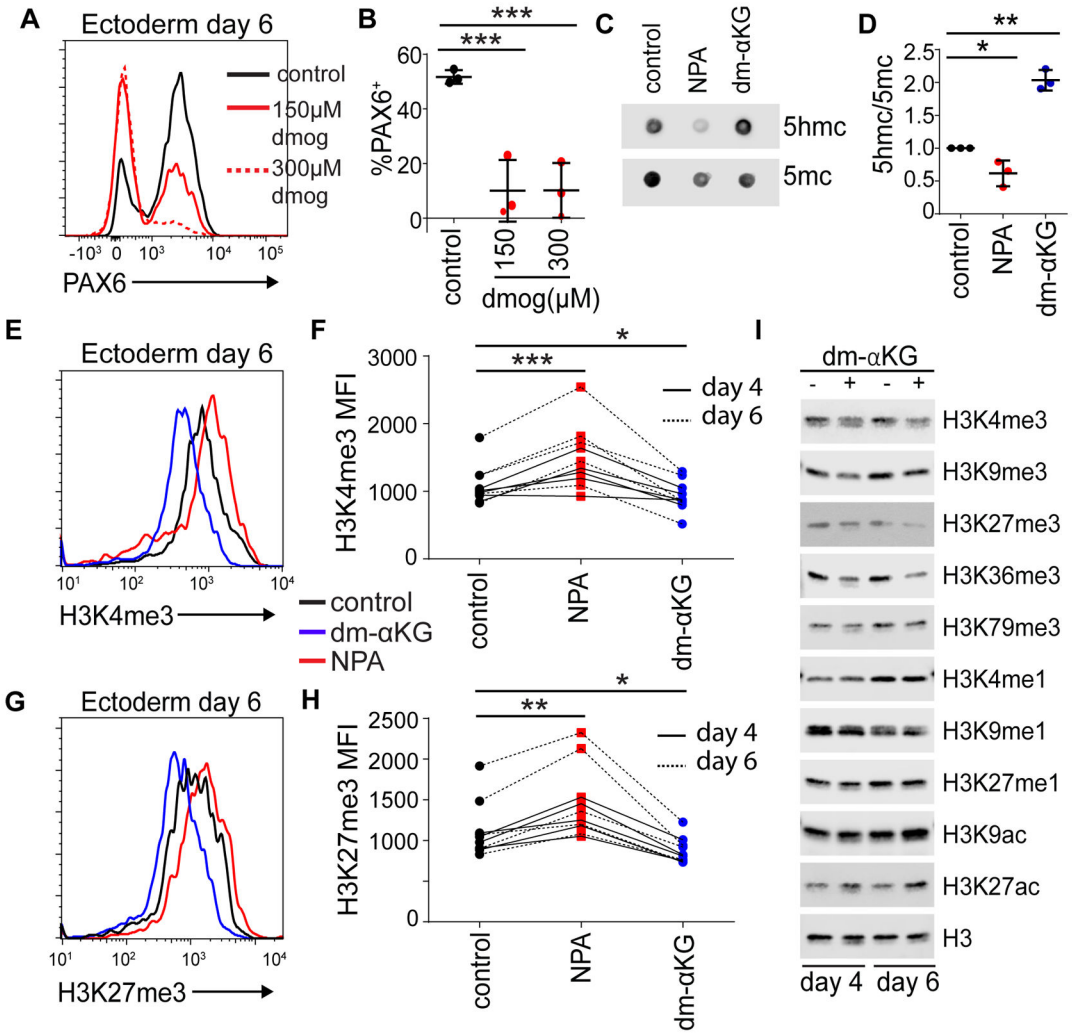

Figure 4. Changes in the α-KG/succinate ratio alter DNA hydroxymethylation and histone methylation

(A, B) Flow cytometry analysis of PAX6 expression in H9 hESCs after 6 days of NE differentiation with the indicated concentrations of αKG-dependent dioxygenase inhibitor, dimethyloxalylglycine (dmog).

(C, D) Dot blot quantification of 5-hydroxymethylcytosine (5hmc) and 5-methylcytosine (5mc) in DNA from H9 hESCs after 4 days of NE differentiation under control conditions or with 50μM NPA or12mM dm-αKG added.

(E, F) Flow cytometry analysis of H3K4me3 mark shown as mean fluorescent intensity (MFI) for UCLA1 hESCs at 4 (solid line) or 6 days (dotted line) of NE differentiation incubated without (black) or with12mM dm-αKG (blue) or 50μM NPA (red). Lines connect independent biological replicates.

(G, H) Flow cytometry analysis of H3K27me3 mark shown as MFI for UCLA1 hESCs at 4 (solid line) or 6 days (dotted line) of NE differentiation incubated without (black) or with 12mM dm-αKG (blue) or 50μM NPA (red). Lines connect independent biological replicates.

(I) Immunoblot of histone methylation and acetylation marks in UCLA1 hESCs differentiated into NE at 4 or 6 days with or without dm-αKG incubation.

Data represent mean ± SD of at least three biological replicates. *p≤0.05; **p≤0.01; ***p≤0.001. P-values were determined by one-way (B, C) or two-way analysis of variance (ANOVA) with repeated measures and correction for multiple comparisons (F, H).