Retinol and ascorbate drive erasure of epigenetic memory and enhance reprogramming to naïve pluripotency by complementary mechanisms

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Epigenetic memory, in particular DNA methylation, is established during development in differentiating cells and must be erased to create naïve (induced) pluripotent stem cells. The ten-eleven translocation (TET) enzymes can catalyze the oxidation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) and further oxidized derivatives, thereby actively removing this memory. Nevertheless, the mechanism by which the TET enzymes are regulated, and the extent to which they can be manipulated, are poorly understood. Here we report that retinoic acid (RA) or retinol (vitamin A) and ascorbate (vitamin C) act as modulators of TET levels and activity. RA or retinol enhances 5hmC production in naïve embryonic stem cells by activation of TET2 and TET3 transcription, whereas ascorbate potentiates TET activity and 5hmC production through enhanced Fe^{2+} recycling, and not as a cofactor as reported previously. We find that both ascorbate and RA or retinol promote the derivation of induced pluripotent stem cells synergistically and enhance the erasure of epigenetic memory. This mechanistic insight has significance for the development of cell treatments for regenenerative medicine, and enhances our understanding of how intrinsic and extrinsic signals shape the epigenome.

epigenetic memory | naive pluripotency | DNA methylation | vitamin A/C | TET

Epigenetic modification is a mechanism used to stably enforce and maintain gene expression patterns between different cell types. Cytosine methylation is perhaps the most intensively studied of these modifications. A low level of cytosine methylation (<30% of CpG dinucleotides) is one of the few features that distinguish the most basal stem cells of the body—naïve embryonic stem cells (nESCs)—from stem cells primed for differentiation (1–5) or committed to somatic lineages (70–85% CpG methylation) (6, 7). Importantly, inhibitors of the DNA methylation maintenance machinery can accelerate the reprogramming of differentiated cells into induced pluripotent stem cells (iPSCs) (8, 9). These features suggest that reduced DNA methylation, either globally or at specific genomic regions, is a fundamental property of naïve pluripotency (Fig. 1*A*).

A key pathway of active DNA demethylation involves the ten-eleven translocation (TET) protein family. These Fe^{2+}- and oxoglutarate-dependent enzymes remove methylated cytosine (5mC) by converting it to 5-hydroxymethylcytosine (5hmC) and further oxidized derivatives (10–13) (Fig. 1*B*). TET proteins can contribute to locus- specific demethylation in nESCs (1, 14), and their depletion reduces the expression of pluripotency genes and increases methylation at their promoters (12, 15). Furthermore, forced expression of TET1 and TET2 dramatically enhances iPSC reprogramming in a catalytically dependent manner (16–18). Nevertheless, the molecular signals that control TET activity in nESCs, and how they can be manipulated during reprogramming, are poorly characterized. For example, although ascorbate (vitamin C) is known to enhance 5hmC production in a TET-dependent manner (19–23), the mechanism by which this occurs is unclear (Fig. 1*B*).

Here we report that ascorbate enhances 5hmC production and potentiates TET catalysis, not as a cofactor as reported previously, but rather by reduction of Fe^{3+} to Fe^{2+}, making it available for participation in the TET enzyme catalytic center. Retinol, the most common form of vitamin A in the body, is chemically unrelated to ascorbate but similarly enhances the production of iPSCs (24). We discovered that it also increases 5hmC production and DNA demethylation in a TET-dependent manner. This is achieved not by an effect on enzymatic activity, but rather through increased TET2 and TET3 expression. We show that increased TET2 mRNA is dependent on an evolutionary conserved retinoic acid (RA) receptor element (RARE) in the first intron of its underlying gene. Finally, given the overlapping effects of retinol and ascorbate on 5hmC production and DNA demethylation, we tested their effects on the reprogramming of primed cells to naïve pluripotency. We found synergistic effects between these two vitamins in a manner

Significance

Naïve embryonic stem cells are characterized by genome-wide low levels of cytosine methylation, a property that may be intrinsic to their function. We found that retinol/retinoic acid (vitamin A) and ascorbate (vitamin C) synergistically diminish DNA methylation levels and in doing so enhance the generation of naïve pluripotent stem cells. This is achieved by two complementary mechanisms. Retinol increases cellular levels of TET proteins (which oxidize DNA methylation), whereas ascorbate affords them greater activity by reducing cellular Fe^{3+} to Fe^{2+}. This mechanistic insight is relevant for the production of induced pluripotent stem cells used in regenerative medicine, and contributes to our understanding of how the genome is connected to extrinsic and intrinsic signals.

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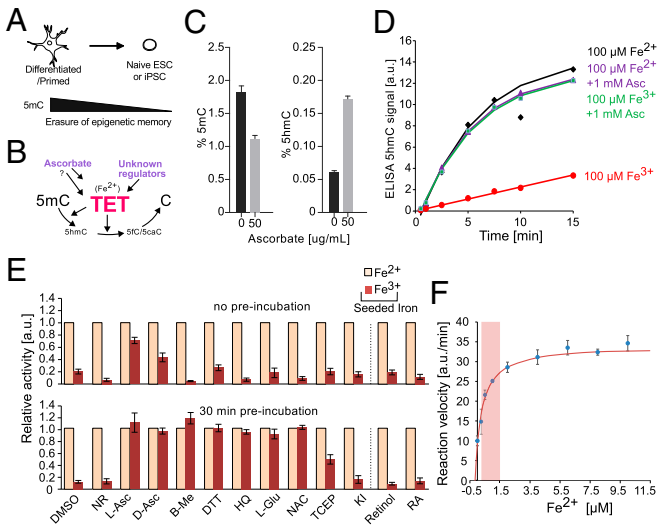

Fig. 1. TET protein catalytic activity in vitro is rescued by the addition of Fe^{2+}, ascorbate, or other antioxidants. (*A*) Major loss of DNA methylation, globally and at gene promoters, is associated with naïve pluripotent cells. (*B*) Demethylation during reprogramming is affected by the activity of the Fe^{2+}-dependent TET hydroxylases, which create 5hmC and other oxidized derivatives (5fC and 5caC). However, what regulates TET levels is largely unknown, and the mechanism by which factors such as ascorbate affect TET-mediated oxidation are unclear. (*C*) Global levels of 5mC and 5hmC (%) in nESCs following 72 h of supplementation with 50 μg/mL ascorbate (± SD; $n = 3$). (*D*) Kinetics of TET1-CD–mediated 5hmC production when supplemented with 100 μM iron (Fe^{2+} or Fe^{3+}) and 1 mM ascorbate (corresponds to 172.12 μg/mL). (*E*, *Upper*) Relative activity of TET1-CD when supplemented with 100 μM iron (Fe^{2+} or Fe^{3+}) and various antioxidants. (*E*, *Lower*) The same experiment repeated, with the antioxidant and iron mix preincubated for 30 min before the addition of TET1-CD (± SD; $n = 4$). NR represents reaction conditions without added reducing agents. (*F*) Relative activity of TET1-CD at various Fe^{2+} concentrations encompassing those seen in cellular contexts (0.2–1.5 μM, indicated by a red box). The mean apparent dissociation constant (K_d) for this reaction was determined to be 0.41 ± 0.05 μM ($n = 2$).

predicted by their interdependent effects on epigenetic memory erasure.

Results

Ascorbate Enhances 5hmC Production Not as a Cofactor of TET, but by Reduction of Fe^{3+} to Fe^{2+}. Ascorbate has been shown to drive DNA demethylation in cultured cells in a TET-dependent manner (19, 21, 22, 25); however, this effect was not measured in a genome-wide or fully quantitative fashion. We determined cytosine modification levels in nESCs using mass spectrometry and found that 50 μg/mL ascorbate supplementation over 72 h decreased 5mC by almost one half (1.7-fold reduction) and increased 5hmC by 2.8-fold (Fig. 1*C*). The absolute loss of 5mC (0.71 pp) was 6.5-fold greater than the increase in 5hmC (0.11 pp), implying that ascorbate drives a genuine loss of cytosine modification along with increasing 5hmC equilibrium levels.

Current biochemical studies report that ascorbate acts as a bound cofactor of the TET proteins (19, 21–23). This hypothesis is based largely on the observation that antioxidants other than ascorbate have no effect on TET activity in cultured cells (19, 21–23). Moreover, several other Fe^{2+}- and oxoglutarate-dependent enzymes are reliant on ascorbate as a bound cofactor (26). The classic example of this is collagen prolyl 4-hydroxylase (C-P4H), which in the absence of substrate undergoes a partial reaction involving decarboxylation of oxoglutarate and oxidation of the iron center to the inactive Fe^{3+} ion. Without ascorbate, this "uncoupled" reaction essentially destroys C-P4H activity in <1 min (27, 28) and is thought to be responsible for many of the symptoms associated with vitamin C deficiency.

To test whether ascorbate is required for TET function, we measured the in vitro activity of the recombinant murine TET1 catalytic domain (TET1-CD) using an ELISA plate assay. Under neutral conditions (pH 6.8) and 10 or 100 μM Fe^{2+} supplementation, no stimulation of hydroxymethylation activity was observed (Fig. 1*D* and Fig. S1*B*). This observation contrasts previous findings, including those of Yin et al. (23), who reported that 50–500 μM ascorbate was able to enhance TET function. One possible explanation for these discordant results could be related to the more basic reaction conditions used by Yin et al. (pH 8.0), in which Fe^{2+} is >100 times more likely to spontaneously oxidize to Fe^{3+} (29). We tested this hypothesis and found that at pH 8.0, nearly all Fe^{2+} ions became oxidized within 3 min (Fig. S1*A*), and ascorbate was able to rescue TET activity (Fig. S1 *B* and *C*). In contrast, at pH 6.8, only negligible oxidation of Fe^{2+} occurred after 15 min, and, accordingly, ascorbate was not able to enhance TET catalytic activity (Fig. S1 *A*–*C*). When TET1-CD was preincubated in the absence of methylated DNA substrate for 30 min (conditions that promote uncoupled reactions with other Fe^{2+}- and oxoglutarate-dependent enzymes), no loss of TET activity was seen (Fig. S1*D*). This demonstrates that unlike C-P4H (27, 28), there is no obligate requirement of ascorbate for TET activity.

To further test the hypothesis that ascorbate functions as a bound cofactor, we inspected the structure of the TET2 catalytic domain. In the presence of oxoglutarate and the target base, there is insufficient space for ascorbate to also bind in the catalytic pocket (Fig. S1*E*). Consequently, if ascorbate acts to reduce TET-bound iron, then at active concentrations it should compete with substrate for a position in the catalytic pocket. However, even with 2 mM L-ascorbate, TET1-CD activity was not stimulated and was only slightly inhibited (Fig. S1*F*). As such, we conclude that ascorbate does not act within the catalytic pocket to reduce bound iron.

An alternative hypothesis is that ascorbate supports TET function by converting Fe^{3+} (the most common form of iron in the cell) into Fe^{2+}, the catalytically relevant state for this class of enzymes. To test this hypothesis, we performed in vitro 5mC oxidation reactions of TET1-CD in the presence of Fe^{3+} (Fig. 1*E*). Consistent with the dependency of TET enzymes on Fe^{2+}, we observed only weak enzymatic function (~5–12% of previous activity); however, on addition of 1 mM ascorbate to the reaction mixture, the oxidative capacity was reinstated (Fig. 1*E*). To verify that this effect is not specific to TET1, we repeated our experiments using recombinant TET2 and TET3 catalytic domain proteins (TET2-CD and TET3-CD, respectively), and found similar results (Fig. S1*H*). Along with demonstrating stereoisotopic insensitivity (both D and L forms were effective), our results suggest that Fe^{3+} is weakly bound by TET and can be readily replaced by Fe^{2+}. A subsequent competition assay confirmed this point. A small amount of Fe^{2+} relative to Fe^{3+} (up to 100-fold less) was sufficient to retain full TET1-CD activity in vitro (Fig. S1*G*).

Prompted by the foregoing observations, we analyzed whether other common reducing agents can cause a similar effect as ascorbate in vitro. Following supplementation with DTT, tris(2-carboxyethyl)phosphine and reduced glutathione, 5mC oxidation activity under Fe^{3+} conditions recovered mildly (Fig. 1*E*). In contrast, hydroquinone and *N*-acetyl-L-cysteine showed little or no effect. Nevertheless, different reducing agents are known to show variable efficiencies for Fe^{3+} reduction (30). As such, we preincubated the reaction mixture containing Fe^{3+} ions for 30 min in the presence of a reducing agent and subsequently started the reactions by the addition of TET1-CD. This time, we observed robust recovery of TET1-CD activity for almost all reducing agents used (Fig. 1*E*). These results further emphasize the critical

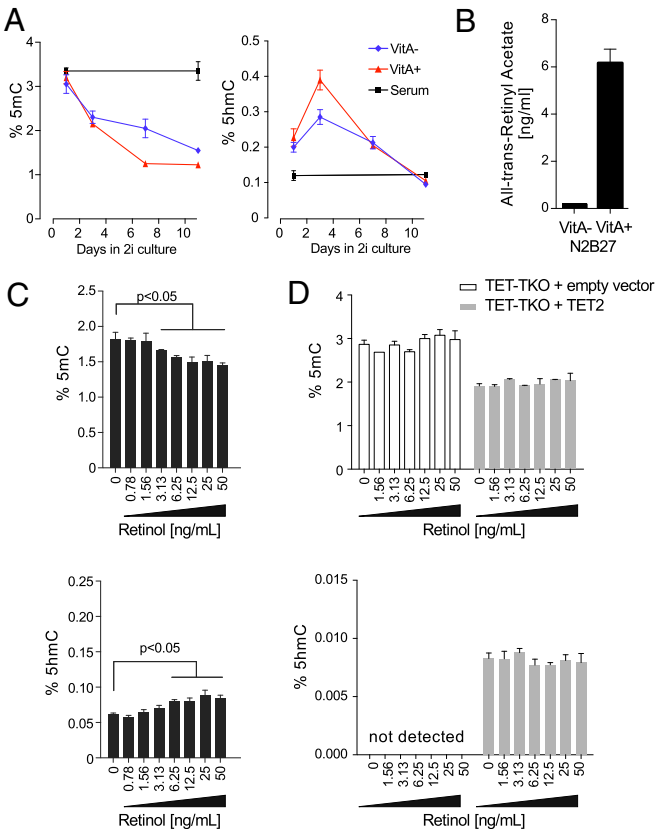

Fig. 2. Retinol increases 5hmC and reduces 5mC in nESCs. (*A*) Global levels of 5mC and 5hmC (%) in serum-grown ESCs following 11 d of reprogramming in 2i/LIF medium either with vitamin A (VitA+) or without vitamin A (VitA–) (± SD; $n = 3$). (*B*) Retinyl acetate levels in VitA+/– 2i/LIF media formulations (± SD; $n = 3$). (*C*) Global levels of 5mC (*Upper*) and 5hmC (*Lower*) in nESCs supplemented with increasing levels of retinol for 72 h (black bars) (± SD; $n = 3$). (*D*) Global levels of 5mC (*Upper*) and 5hmC (*Lower*) in 2i/LIF-conditioned TET-TKO ESCs that were partially rescued by forced expression of TET2 (dark gray) and were exposed to retinol for 72 h (± SD; $n = 3$).

role of appropriate iron levels and valency for TET activity, as opposed to the presence of ascorbate per se.

To support this proposition, we examined the rates of reactions performed at different Fe^{2+} concentrations (Fig. 1*F*), and were able to infer an apparent dissociation constant (0.41 ± 0.05 μM). Significantly, this value falls within the reported range of labile Fe^{2+} concentrations in resting erythroid and myeloid cultured cells (0.2–1.5 μM) (31), suggesting that cellular labile iron concentration can directly modulate TET function in cells.

Retinol Enhances 5hmC by Activating TET Expression. We and others have previously reported that reprogramming of serum-grown ESCs to naïve pluripotency causes a profound decrease in DNA methylation levels (1–4), an effect precipitated by the "2i" signaling inhibitors PD0325091 and CHIR99021. We also have shown that at 72 h of reprogramming, there is a simultaneous peak of 5hmC in the genome (1). To identify the molecular mechanism underlying this increase in 5hmC, we investigated the composition of the base medium used for growing the cells. There are two different formulations of the defined base medium used with 2i inhibition, the standard N2-B27 mixture originally designed for supporting the growth of neural stem cells (32) and another mixture in which the B27 supplement is without vitamin A, referred to herein as VitA+ and VitA–, respectively. When these two media types were used to convert serum-grown ESCs to naïve conditions, VitA+ treated cells were found to have greater loss of 5mC and increased 5hmC after 72 h (Fig. 2*A*).

Mass spectrometry analysis of these different formulations at working concentrations revealed that the VitA+ medium contains ∼6 ng/mL of all-trans retinyl acetate (Fig. 2*B*), a common form of vitamin A used in cell culture media. Retinyl acetate and retinol show similar biological effects at equal concentrations; however, retinol is the most common form of vitamin A in human blood. As such, we supplemented nESCs grown in VitA– media with increasing levels of retinol up to 50 ng/mL, and found that it resulted in a 27% increase in 5hmC and a 26% decrease in 5mC (Fig. 2*C*, black bars). Cosupplementation of ascorbate with increasing retinol concentrations additively decreased 5mC and increased 5hmC, except at high doses, where 5hmC levels became saturated (Fig. S2*A*, gray bars).

To understand how 5hmC is increased and 5mC is decreased by retinol treatment, we tested TET triple-knockout (TET-TKO) ESCs under the same conditions (Fig. 2*D*). No retinol-dependent decrease in 5mC was observed in these cells, indicating a requirement of the original retinol effect on TET proteins and 5hmC. When we partially rescued 5hmC production in the TET-TKO cells by forced expression of either TET1 or TET2 (the two most abundant TETs in ESCs), we found no increase in 5hmC upon retinol supplementation, and also no loss of 5mC (Fig. 2*D* and Fig. S3*A*). Thus, we reasoned that retinol does not affect TET protein stability or the efficiency of catalysis. Supporting this

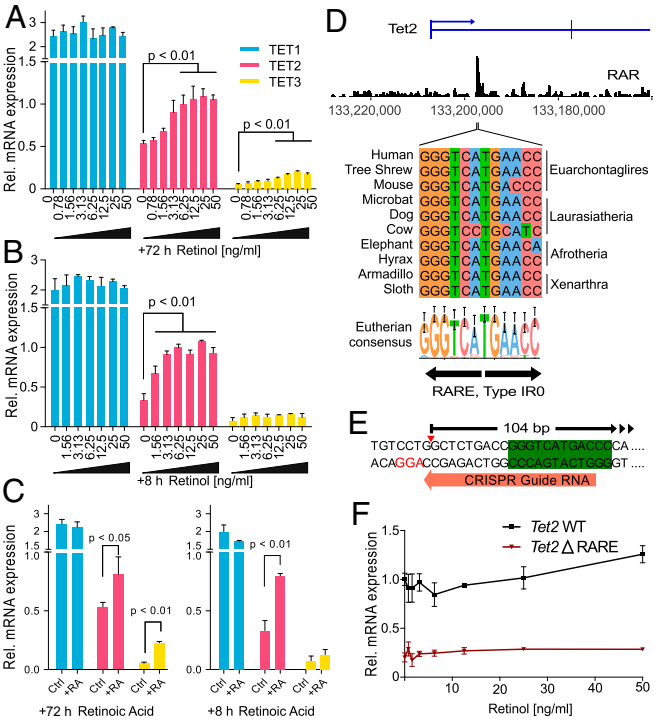

Fig. 3. Retinol enhances 5hmC in a TET2 and RA signaling-dependent manner. (*A*) Relative mRNA levels from TET1-3 in nESCs supplemented with retinol for 72 h (± SD; $n = 3$). (*B*) Relative mRNA levels from TET1-3 in nESCs supplemented with retinol for 8 h (± SD; $n = 3$). (*C*) Relative mRNA levels from TET1-3 in nESCs supplemented with RA (± SD; $n = 3$). (*D*) ChIP-seq of a pan-RAR antibody in ESCs (data analyzed from ref. 34). Underneath the major enrichment peak is an IR0-type RARE that is conserved throughout all mammalian superorders. (*E*) A 104-bp deletion was created encompassing the *Tet2* RARE (green box) using a CRISPR guide RNA (orange arrow) downstream of a protospacer adjacent motif (red text). The full deletion coordinates are NCBI37, chr3:133197151–133197253. (*F*) Relative TET2 mRNA levels in retinol-supplemented nESCs with the *Tet2* RARE deleted (*Tet2* ΔRARE). Wild-type (WT) control cells are included for comparison (± SD; $n = 3$).

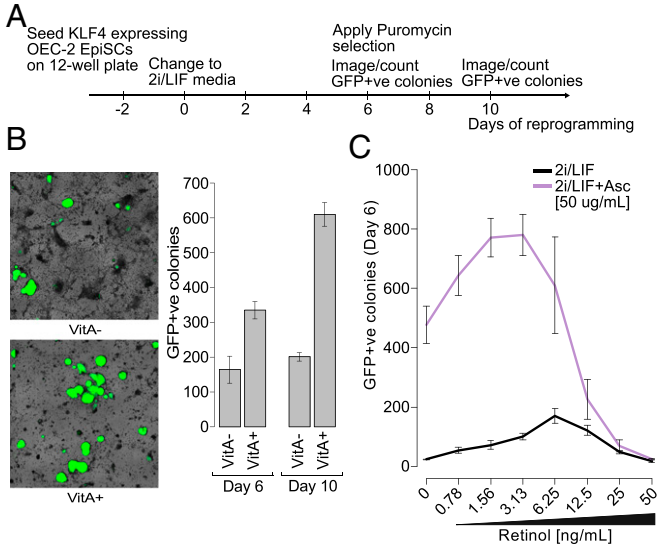

Fig. 4. Retinol and ascorbate enhance reprogramming of epiblast stem cells to naïve pluripotency. (*A*) Experimental setup for EpiSC reprogramming experiments. (*B*) *Oct4*:GFP$^+$ colonies following 6–10 d of reprogramming in VitA+/– 2i/LIF media. (*Left*) Representative microscopic field views at day 10 (4× magnification; the GFP channel is colored green and superimposed over a brightfield image of the same view). (*Right*) Frequency of reprogrammed colonies (± SD; $n = 3$). (*C*) Frequency of *Oct4*:GFP$^+$ colonies after 6 d of reprogramming in VitA– 2i/LIF media with supplemented retinol and ascorbate (± SD; $n = 3$).

proposition, even with 30 min of preincubation, neither retinol nor RA could rescue TET1-CD function in the presence of Fe^{3+} (Fig. 1*D*), nor could it enhance TET1-CD in the presence of optimal Fe^{2+} (Fig. S3*B*). As such, we reasoned that retinol might instead affect TET transcription.

To explore this idea further, we examined TET mRNA levels in nESCs over the same range of retinol concentrations as shown in Fig. 2. After 72 h, there was a clear dose-dependent increase in both TET2 and TET3 mRNA in response to retinol supplementation (up to a 1.5- and 4.3-fold increase, respectively) (Fig. 3*A* and Fig. S2*B*), indicating a direct transcriptional effect. Shorter stimulation of the cells with retinol (8 h) activated only TET2 (Fig. 3*B*). Retinol is a metabolic precursor of RA, which is a potent signaling molecule and morphogen that promotes the anteriorization of developing vertebrate embryos (33). Direct supplementation of cells with RA (1 mg/mL) for 72 h also increased TET2 and TET3 expression, with only TET2 activated after 8 h (Fig. 3*C*). Analysis of published chromatin immunoprecipitation and sequencing (ChIP-seq) data using a pan-RA receptor (RAR) antibody (34) revealed a strong peak of enrichment in ESCs 10.1 kb downstream of the *Tet2* promoter (Fig. 3*D* and Fig. S4). Underneath the apex of this peak, we discovered an inverted repeat consistent with IR0-type RAR elements (RAREs) (34, 35). This IR0 element is highly conserved throughout eutherian mammals, including all four major superorders (Fig. 3*D* and Table S1). In contrast, we found no RAR enrichment at *Tet1*, and although there was a small enrichment peak at *Tet3*, we could not detect any RAREs associated with this sequence (Fig. S4). To test whether the RARE is responsible for the effect of retinol on TET2 expression, we deleted it using CRISPR/Cas9 (*Tet2*ΔRARE; Fig. 3*E*). Stimulation of *Tet2*ΔRARE cells with retinol did not increase TET2 expression (Fig. 3*F*), indicating a direct role of the RARE in retinol-dependent regulation of TET2.

Retinol and Ascorbate Enhance Reprogramming of Epiblast Stem Cells to Naïve Pluripotency. To understand the relevance of our results from the perspective of DNA demethylation in a biological context, we asked how retinol and ascorbate affect iPSC production. Epiblast stem cells (EpiSCs) are derived from the embryo postimplantation (E6.5), have a highly methylated genome, and are unable to create chimeric mice on injection into a host blastocyst (36–38). They can be reprogrammed to naïve iPSCs in 2i/LIF media (albeit relatively inefficiently) by overexpression of at least one regulator of naïve pluripotency (36) and, in the case of the OEC-2 EpiSC line (39), show bright and homogenous expression of an *Oct4*:GFP reporter gene after 6–10 d of reprogramming (Fig. 4*A*).

We first compared VitA+ and VitA– reprogramming media (i.e., with and without retinyl acetate, respectively) on KLF4-overexpressing OEC-2 EpiSCs, and found that the latter resulted in less than one-half the number of *Oct4*:GFP$^+$ colonies after 6 d of reprogramming and almost one-third fewer after 10 d (Fig. 4*B*). When we tested this effect over the same range of retinol from Fig. 2, we found that the number of *Oct4*:GFP$^+$ colonies increased proportionally up to a concentration of 6.25 ng/mL (Fig. 4*C*, black line) but that beyond this point, further increases in retinol actually decreased the number of *Oct4*:GFP$^+$ colonies, such that at 50 ng/mL of retinol, almost no reprogrammed colonies were found. Combining ascorbate treatment with retinol enhanced reprogramming considerably, as previously reported in other systems (40, 41), but exhibited an interesting additional effect (Fig. 4*C*, purple line). The dose–response curve was clearly shifted to the left, meaning that the maximum number of *Oct4*:GFP$^+$ colonies produced resulted from a lower retinol concentration when cosupplemented with ascorbate (3.13 ng/mL). These results are consistent with the hypothesis that increased 5hmC and decreased 5mC due to retinol and ascorbate enhance the reprogramming of EpiSCs, and that their costimulation results in increased reprogramming synergistically.

Discussion

DNA methylation erasure in the germ line and during experimental reprogramming are closely linked to the acquisition of pluripotency. TET-mediated production of 5hmC can enhance DNA demethylation (reviewed in ref. 6); however, the mechanistic details involved with this process and what can be done to manipulate it are much less understood. Here we show that DNA demethylation and steady-state 5hmC levels can be enhanced in

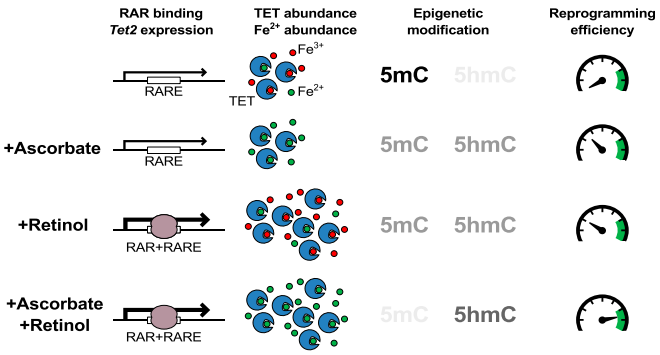

Fig. 5. Retinol and ascorbate enhance DNA demethylation, 5hmC production, and pluripotent stem cell reprogramming by synergistic mechanisms. The RARE in the first intron of *Tet2* allows increased expression of TET2 mRNA on stimulation of RA signaling (by retinol, retinyl acetate, or RA itself) and enhanced binding of the RAR (brown enzyme). In contrast, ascorbate increases the active iron (Fe^{2+}, green circles) required for the TET catalytic center by reduction from Fe^{3+} (red circles). Together, retinol and ascorbate additively enhance 5hmC production, resulting in greater removal of methylation from DNA. The enhancing effect of ascobate and retinol on naïve pluripotent stem cell reprogramming is greater than the sum of their individual effects.

nESCs by various retinoid forms and ascorbate through distinct mechanisms (Fig. 5).

We find that ascorbate supports TET activity not as an essential cofactor, but rather by reduction of nonenzyme-bound Fe^{3+} to Fe^{2+}. Unlike C-P4H, which is reliant on ascorbate for its activity, TET does not undergo uncoupled reactions that destroy its activity in the absence of substrate (Fig. S1*G*). Moreover, under conditions of sufficient Fe^{2+} and neutral pH, ascorbate does not enhance TET function (Fig. 1*D*). When faced with insufficient Fe^{2+} (and excess Fe^{3+}), ascorbate dramatically rescues TET activity, but other reducing agents can do this as well, provided that sufficient incubation time is provided (Fig. 1*E*). Although these results challenge the findings reported in the literature (19, 21–23), they are in complete agreement with the recent finding that redox-active quinones stimulate TET activity in cell culture through reduction of enzyme-free Fe^{3+} to Fe^{2+} (42). Taken together, these results imply that TET proteins are inherently sensitive to labile iron concentrations in the cell, an idea supported by the fact that the dissociation constant of Fe^{2+} and TET1-CD overlaps with the physiological range of labile iron in the cell (Fig. 1*F*).

In contrast to ascorbate, we found that retinol and RA do not have any effect on TET enzyme efficiency in cell culture (Fig. 2*D*) or in vitro (Fig. 1*E*), but instead enhance DNA demethylation and increase 5hmC by activating TET2 and TET3 expression (Fig. 3). We also found that human nESCs grown in media with vitamin A supplementation (i.e., retinyl acetate; Fig. 2*B*) have more genomic 5hmC than those grown without it (Fig. S5), supporting our original observations in mouse nESCs (Fig. 2*C*) and implying that this is a conserved regulatory effect. TET2 activation has been previously associated with RA treatment in embryonal carcinoma cells (43); however, the nature of this association is unknown. Given that TET2 responds to retinol and RA stimulation within 8 h in a manner dependent on a eutherian-conserved IR0-type RARE within its first intron (Fig. 3), we conclude that *Tet2* is a direct target of RA signaling.

When ascorbate and retinol are supplemented in combination, RA signaling will increase TET protein levels and ascorbate will potentiate its activity (Fig. 5). A prediction arising from this scenario is that the combined effect of retinol and ascorbate should be greater than the sum of their individual effects, owing to complementary effects on shared cellular components. Indeed, ascorbate treatment essentially "sensitizes" EpiSCs to lower levels of retinol (Fig. 4*C*), which suggests that the improvement in reprogramming by both small molecules is affecting the same pathway, the nexus of which is 5hmC.

Our results demonstrate that intermediate levels of RA signaling enhance the reprogramming of EpiSCs to naïve pluripotency (Fig. 4). Supplemented retinol above 6.25 ng/mL suppresses reprogramming in a dose-dependent manner, an effect that we speculate is due to the well-described ability of RA to stimulate differentiation. Interestingly, retinol levels in the serum of mice (44) and humans (45) is higher than the range that we tested; thus, adult levels of RA signaling may form one potential barrier by which somatic cells are protected from spontaneous de-differentation. A previous study showed that optimum levels of retinoid stimulation are critical for the reprogramming of EpiSCs to iPSCs, and that RA signaling strongly affects the reprogramming of mouse embryonic fibroblasts (24). That study further showed that small-molecule antagonists of RA signaling attenuate *β-catenin* and activate *Wnt* signaling in EpiSCs, thus providing a likely mechanism for how RA enhances reprogramming. We suggest that in addition to this effect, RA signaling enhances reprogramming by directly activating TET2 expression, a known reprogramming factor (16, 18).

In summary, our work provides mechanistic insight into how TET proteins remove epigenetic information during reprogramming to naïve pluripotency, and how this process can be manipulated through the use of retinol and ascorbate. Nevertheless, the significance of our work is not limited to the elucidation of these fundamental processes and their application to iPSC reprogramming. For example, our finding that TET activity is sensitive to physiological changes in iron suggests that TET may represent a conduit through which alterations in this ion are signaled to the genome. Moreover, the observation that RA signaling enhances TET2 expression could be relevant for the treatment of certain cancers. TET2 is a well-described tumor suppressor that is regularly mutated in a number of hematopoietic malignancies (46). Acute promyelocytic leukemia (APL) is a form of myeloid malignancy characterized by PML-RARα translocation and sensitivity to RA treatment, such that RA used in combination with arsenic trioxide can provide a 5-year event-free survival rate of >90% (47, 48), a dramatic improvement for what was once considered the deadliest form of acute leukemia. Nevertheless, a significant number of patients are resistant to RA treatment. A recent analysis found that 4.5% of patients with APL have mutations in TET2, and that a mutation in this and other epigenetic modifiers is a significant indicator of poor disease outcome (49). Our work provides a potential mechanistic explanation for RA insensitivity in patients with APL with TET2 mutations, and if proven in further experimentation, could affect the management of this disease.

Methods

Our methodology is described in detail in *SI Methods*. In brief, stem cell culture was performed according to standard techniques as reported previously (1, 16), with modifications as described in Tables S2 and S3. Mass spectrometry analysis of nucleosides was performed as described previously (50). An ELISA-based plate assay was used to quantify the 5hmC produced by a TET1 catalytic domain protein (TET1-CD) in vitro. EpiSC reprogramming was performed as described previously (39), with modifications as outlined in *SI Methods*.

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1. Ficz G, et al. (2013) FGF signaling inhibition in ESCs drives rapid genome-wide demethylation to the epigenetic ground state of pluripotency. *Cell Stem Cell* 13(3):351–359.
2. Habibi E, et al. (2013) Whole-genome bisulfite sequencing of two distinct interconvertible DNA methylomes of mouse embryonic stem cells. *Cell Stem Cell* 13(3):360–369.
3. Leitch HG, et al. (2013) Naive pluripotency is associated with global DNA hypomethylation. *Nat Struct Mol Biol* 20(3):311–316.
4. Yamaji M, et al. (2013) PRDM14 ensures naive pluripotency through dual regulation of signaling and epigenetic pathways in mouse embryonic stem cells. *Cell Stem Cell* 12(3):368–382.
5. Theunissen TW, et al. (2014) Systematic identification of culture conditions for induction and maintenance of naive human pluripotency. *Cell Stem Cell* 15(4):471–487.
6. Lee HJ, Hore TA, Reik W (2014) Reprogramming the methylome: Erasing memory and creating diversity. *Cell Stem Cell* 14(6):710–719.
7. Hon GC, et al. (2013) Epigenetic memory at embryonic enhancers identified in DNA methylation maps from adult mouse tissues. *Nat Genet* 45(10):1198–1206.
8. Mikkelsen TS, et al. (2008) Dissecting direct reprogramming through integrative genomic analysis. *Nature* 454(7200):49–55.
9. Theunissen TW, et al. (2011) Nanog overcomes reprogramming barriers and induces pluripotency in minimal conditions. *Curr Biol* 21(1):65–71.
10. Jurkowski TP, Jeltsch A (2011) Burning off DNA methylation: New evidence for oxygen-dependent DNA demethylation. *ChemBioChem* 12(17):2543–2545.
11. Tahiliani M, et al. (2009) Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science* 324(5929):930–935.
12. Ficz G, et al. (2011) Dynamic regulation of 5-hydroxymethylcytosine in mouse ES cells and during differentiation. *Nature* 473(7347):398–402.

13. Ito S, et al. (2011) Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. *Science* 333(6047):1300–1303.
14. von Meyenn F, et al. (2016) Impairment of DNA methylation maintenance is the main cause of global demethylation in naive embryonic stem cells. *Mol Cell* 62(6):848–861.
15. Williams K, et al. (2011) TET1 and hydroxymethylcytosine in transcription and DNA methylation fidelity. *Nature* 473(7347):343–348.
16. Costa Y, et al. (2013) NANOG-dependent function of TET1 and TET2 in establishment of pluripotency. *Nature* 495(7441):370–374.
17. Gao Y, et al. (2013) Replacement of Oct4 by Tet1 during iPSC induction reveals an important role of DNA methylation and hydroxymethylation in reprogramming. *Cell Stem Cell* 12(4):453–469.
18. Doege CA, et al. (2012) Early-stage epigenetic modification during somatic cell reprogramming by Parp1 and Tet2. *Nature* 488(7413):652–655.
19. Blaschke K, et al. (2013) Vitamin C induces Tet-dependent DNA demethylation and a blastocyst-like state in ES cells. *Nature* 500(7461):222–226.
20. Chen J, et al. (2013) Vitamin C modulates TET1 function during somatic cell reprogramming. *Nat Genet* 45(12):1504–1509.
21. Minor EA, Court BL, Young JI, Wang G (2013) Ascorbate induces ten-eleven translocation (Tet) methylcytosine dioxygenase-mediated generation of 5-hydroxymethylcytosine. *J Biol Chem* 288(19):13669–13674.
22. Dickson KM, Gustafson CB, Young JI, Züchner S, Wang G (2013) Ascorbate-induced generation of 5-hydroxymethylcytosine is unaffected by varying levels of iron and 2-oxoglutarate. *Biochem Biophys Res Commun* 439(4):522–527.
23. Yin R, et al. (2013) Ascorbic acid enhances Tet-mediated 5-methylcytosine oxidation and promotes DNA demethylation in mammals. *J Am Chem Soc* 135(28):10396–10403.
24. Yang J, et al. (2014) Signalling through retinoic acid receptors is required for reprogramming of both MEFs and EpiSCs to iPSCs. *Stem Cells* 33(5):1390–1404.
25. Chung T-L, et al. (2010) Vitamin C promotes widespread yet specific DNA demethylation of the epigenome in human embryonic stem cells. *Stem Cells* 28(10):1848–1855.
26. Kuiper C, Vissers MCM (2014) Ascorbate as a co-factor for Fe- and 2-oxoglutarate dependent dioxygenases: Physiological activity in tumor growth and progression. *Front Oncol* 4:359.
27. Myllylä R, Majamaa K, Günzler V, Hanauske-Abel HM, Kivirikko KI (1984) Ascorbate is consumed stoichiometrically in the uncoupled reactions catalyzed by prolyl 4-hydroxylase and lysyl hydroxylase. *J Biol Chem* 259(9):5403–5405.
28. De Jong L, Albracht SPJ, Kemp A (1982) Prolyl 4-hydroxylase activity in relation to the oxidation state of enzyme-bound iron: The role of ascorbate in peptidyl proline hydroxylation. *Biochim Biophys Acta* 704(2):326–332.
29. Morgan B, Lahav O (2007) The effect of pH on the kinetics of spontaneous Fe(II) oxidation by O2 in aqueous solution: Basic principles and a simple heuristic description. *Chemosphere* 68(11):2080–2084.
30. Petrat F, et al. (2003) Reduction of Fe(III) ions complexed to physiological ligands by lipoyl dehydrogenase and other flavoenzymes in vitro: Implications for an enzymatic reduction of Fe(III) ions of the labile iron pool. *J Biol Chem* 278(47):46403–46413.
31. Epsztejn S, Kakhlon O, Glickstein H, Breuer W, Cabantchik I (1997) Fluorescence analysis of the labile iron pool of mammalian cells. *Anal Biochem* 248(1):31–40.
32. Ying Q-L, Smith AG (2003) Defined conditions for neural commitment and differentiation. *Methods Enzymol* 365:327–341.
33. Rhinn M, Dollé P (2012) Retinoic acid signalling during development. *Development* 139(5):843–858.
34. Mahony S, et al. (2011) Ligand-dependent dynamics of retinoic acid receptor binding during early neurogenesis. *Genome Biol* 12(1):R2.
35. Moutier E, et al. (2012) Retinoic acid receptors recognize the mouse genome through binding elements with diverse spacing and topology. *J Biol Chem* 287(31): 26328–26341.
36. Martello G, Smith A (2014) The nature of embryonic stem cells. *Annu Rev Cell Dev Biol* 30(1):647–675.
37. Senner CE, Krueger F, Oxley D, Andrews S, Hemberger M (2012) DNA methylation profiles define stem cell identity and reveal a tight embryonic-extraembryonic lineage boundary. *Stem Cells* 30(12):2732–2745.
38. Hackett JA, et al. (2013) Synergistic mechanisms of DNA demethylation during transition to ground-state pluripotency. *Stem Cell Rep* 1(6):518–531.
39. Guo G, et al. (2009) Klf4 reverts developmentally programmed restriction of ground state pluripotency. *Development* 136(7):1063–1069.
40. Esteban MA, et al. (2010) Vitamin C enhances the generation of mouse and human induced pluripotent stem cells. *Cell Stem Cell* 6(1):71–79.
41. Schwarz BA, Bar-Nur O, Silva JC, Hochedlinger K (2014) Nanog is dispensable for the generation of induced pluripotent stem cells. *Curr Biol* 24(3):347–350.
42. Zhao B, et al. (2014) Redox-active quinones induces genome-wide DNA methylation changes by an iron-mediated and Tet-dependent mechanism. *Nucleic Acids Res* 42(3): 1593–1605.
43. Bocker MT, et al. (2012) Hydroxylation of 5-methylcytosine by TET2 maintains the active state of the mammalian HOXA cluster. *Nat Commun* 3(818):818.
44. Wei S, et al.; van Bennekum AM (2001) Biochemical basis for depressed serum retinol levels in transthyretin-deficient mice. *J Biol Chem* 276(2):1107–1113.
45. Thurnham DI, Mburu ASW, Mwaniki DL, De Wagt A (2005) Micronutrients in childhood and the influence of subclinical inflammation. *Proc Nutr Soc* 64(4):502–509.
46. Ko M, et al. (2015) TET proteins and 5-methylcytosine oxidation in hematological cancers. *Immunol Rev* 263(1):6–21.
47. Hu J, et al. (2009) Long-term efficacy and safety of all-trans retinoic acid/arsenic trioxide-based therapy in newly diagnosed acute promyelocytic leukemia. *Proc Natl Acad Sci USA* 106(9):3342–3347.
48. Lo-Coco F, et al.; Gruppo Italiano Malattie Ematologiche dell'Adulto; German-Austrian Acute Myeloid Leukemia Study Group; Study Alliance Leukemia (2013) Retinoic acid and arsenic trioxide for acute promyelocytic leukemia. *N Engl J Med* 369(2): 111–121.
49. Shen Y, et al. (2015) Mutations of epigenetic modifier genes as a poor prognostic factor in acute promyelocytic leukemia under treatment with all-trans retinoic acid and arsenic trioxide. *EBioMedicine* 2(6):563–571.
50. Bachman M, et al. (2014) 5-Hydroxymethylcytosine is a predominantly stable DNA modification. *Nat Chem* 6(12):1049–1055.
51. Ran FA, et al. (2013) Genome engineering using the CRISPR-Cas9 system. *Nat Protoc* 8(11):2281–2308.
52. Verschoor MJ, Molot LA (2013) A comparison of three colorimetric methods of ferrous and total reactive iron measurement in freshwaters. *Limnol Oceanogr Methods* 11(3): 113–125.
53. Hu L, et al. (2013) Crystal structure of TET2-DNA complex: Insight into TET-mediated 5mC oxidation. *Cell* 155(7):1545–1555.
54. Smith AG, Hooper ML (1987) Buffalo rat liver cells produce a diffusible activity which inhibits the differentiation of murine embryonal carcinoma and embryonic stem cells. *Dev Biol* 121(1):1–9.
55. Hu X, et al. (2014) Tet and TDG mediate DNA demethylation essential for mesenchymal-to-epithelial transition in somatic cell reprogramming. *Cell Stem Cell* 14(4): 512–522.