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Regulation of the Epigenome by Vitamin C

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Abstract

Emerging evidence suggests that ascorbate, the dominant form of vitamin C under physiological pH conditions, influences the genome activity via regulating epigenomic processes. Ascorbate serves as a cofactor for ten-eleven translocation (TET) dioxygenases that catalyze the oxidation of 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC), further to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), which are ultimately replaced by unmodified cytosine. The JmjC domain-containing histone demethylases also require ascorbate as a cofactor for histone demethylation. Thus, by primarily participating in the demethylation of both DNA and histones, ascorbate appears to be a mediator of the interface between the genome and environment. Furthermore, redox status has a profound impact on the bioavailability of ascorbate in the nucleus. In order to bridge the gap between redox biology and genomics, we suggest an interdisciplinary research field that can be termed “**Redox Genomics**” to study dynamic redox processes in health and diseases. This review examines the evidence and potential molecular mechanism of ascorbate in demethylation of the genome, while highlighting potential epigenetic roles of ascorbate in various diseases.

Keywords

Vitamin C; iron and 2-oxoglutarate-dependent dioxygenase; ten-eleven translocation dioxygenase; JmjC domain-containing histone demethylase; DNA demethylation; histone demethylation

INTRODUCTION

Vitamin C (L-ascorbic acid) is an essential, water-soluble micronutrient that exists predominantly as the ascorbate anion under physiological pH conditions. It is well established that ascorbate is an antioxidant and free radical scavenger and further, an essential cofactor in numerous enzymatic reactions.

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Most mammals, such as rodents, synthesize ascorbate *de novo* in the liver from glucose through a biosynthetic pathway. In contrast, humans as well as primates, guinea pigs and fruit bats no longer can synthesize ascorbate due to a mutant and nonfunctional enzyme, L-gulonolactone oxidase (**Gulo**), which catalyzes the last step of ascorbate biosynthesis (50). For these mammalian species, ascorbate is a vitamin that needs to be supplied through dietary sources and supplements.

Ascorbate, derived from either dietary sources or the liver, enters cells primarily through sodium-dependent vitamin C transporters (**SVCTs**). The high-capacity, low-affinity **SVCT1** is primarily responsible for ascorbate absorption and re-absorption in intestinal and renal epithelial cells. The high-affinity, low-capacity **SVCT2** distributes ascorbate to most tissues and is expressed more ubiquitously (91). The average concentration of ascorbate in the plasma of healthy humans or mice is ~50 μM . Currently, the recommended dietary allowances (**RDA**) by the Institute of Medicine (IOM) is 90 mg for adult males and 75 mg for adult females, although the tolerable upper intake level for adults is 2,000 mg per day. The daily adequate intakes (**AI**) for infants (0 – 12 months) is 40 ~ 50 mg. When the plasma ascorbate concentration drops to below 11.4 μM , there is a risk of developing scurvy, and is thus conventionally considered deficient. Once being transported across the plasma membrane, ascorbate accumulates within cells and its intracellular concentration can reach 1 ~ 10 mM (58). Thus, the majority of mammalian cells maintain highly elevated intracellular ascorbate concentrations compared to the extracellular milieu. For instance, neurons could have up to 10 mM of intracellular ascorbate, which is about 200 times higher than the extracellular ascorbate concentration (73).

Ascorbate is a relatively mild reducer and antioxidant. After a cascade of two-round oxidation and the loss of two electrons, the oxidized form of vitamin C, termed dehydroascorbic acid (**DHA**) is formed. Instead of utilizing **SVCTs**, **DHA** enters and leaves cells via facilitated glucose transporters (**GLUTs**). Once inside the cell, **DHA** can be rapidly reduced back to ascorbate. However, the reduced ascorbate is dominant and **DHA** is barely detectable in the plasma of healthy humans (43), suggesting that most cells take up and accumulate ascorbate primarily through **SVCTs**.

During evolution, primates and some other species have lost the ability to synthesize ascorbate due to accumulation of mutations in the *Gulo* gene. The antioxidant function of ascorbate generally appears to be compensated for by alternative reducing systems in these species (54). However, its role as a cofactor for iron and 2-oxoglutarate-dependent dioxygenases is irreplaceable, as these species develop scurvy, osteoporosis and other disease phenotypes unless provided with adequate dietary ascorbate. The iron and 2-oxoglutarate-dependent dioxygenases utilize Fe^{2+} as a cofactor and 2-oxoglutarate (**2OG**, also known as α -ketoglutarate) as a co-substrate; additionally, some of them require ascorbate as another cofactor for full catalytic activity (59). One classic member of this family is collagen prolyl 4-hydroxylase (**P4H**), which is well known for its involvement in scurvy. In the absence of ascorbate, the initial hydroxylation catalyzed by collagen **P4H** can proceed at a maximal rate. However, the catalytically inactive oxidized iron species (mostly Fe^{3+}) soon inactivates collagen **P4H**, which leads to an incomplete hydroxylation of proline residues in collagen and ultimately the characteristic signs of scurvy (23). When available,

ascorbate has the capacity to reduce oxidized iron species to catalytically active Fe^{2+} . Ascorbate thus assists collagen P4H to complete the hydroxylation and prevent scurvy. Overall, it has been well established that ascorbate is required to maintain a number of Fe^{2+} and 2OG-dependent dioxygenases in their fully active forms (59).

In recent years, tremendous progress has been made in regards to the identification of a number of novel Fe^{2+} and 2OG-dependent dioxygenases that catalyze the hydroxylation of methylated nucleic acids (DNA and RNA) and methylated histones. DNA and histone methylation are the major epigenetic hallmarks in the mammalian genome. It has also been shown that some of these nuclear dioxygenases require ascorbate as a cofactor to start and complete both DNA demethylation and histone demethylation processes. These unexpected findings uncover a previously unknown function of ascorbate in regulating the epigenome, which calls for a re-evaluation of the role of ascorbate in human health and diseases.

VITAMIN C AND DNA DEMETHYLATION

TET dioxygenases are Fe^{2+} and 2OG-dependent dioxygenases

The epigenome reflects the interface of a dynamic environment and the genome. Known epigenetic events include covalent modifications on nucleotides and histones, chromatin remodeling, and non-coding RNAs, which collectively constitute the epigenome. Methylation at the C⁵ position of cytosine (5-methylcytosine, **5mC**) is the major and best-characterized epigenetic mark of mammalian DNA. The transfer of a methyl group from the donor S-adenosylmethionine (**SAM**) to a cytosine is catalyzed by DNA methyltransferases (**DNMTs**), which are regarded as the **writers** of this epigenetic mark. After the methylation is completed, 5mC, especially in CpG dinucleotide context, can be recognized and then bound with a group of methyl-CpG binding proteins (**MBPs**). As **readers**, these MBPs then mediate multiple functions such as regulating transcription, initiating chromatin remodeling, maintaining genome stability and cellular identity. Although 5mC is a relatively stable epigenetic mark, it can be lost by passive dilution via a lack of maintenance by DNMT1 during DNA replication, which would result in passive demethylation. A few years ago, it remained largely unclear whether and how the methyl group in 5mC could be actively removed. The key question was whether or not there were DNA demethylases that existed as **erasers** to catalyze the removal of the methyl group from 5mC.

In 2009, the Heintz group reported the presence of an unusual DNA nucleotide, 5-hydroxymethylcytosine (**5hmC**), in the mouse brain (44). Although 5hmC constitutes less than 1% of total nucleotides, its percentage was higher in cerebellar Purkinje neurons than in granule cells. In contrast, there was no difference in 5mC content between these two distinct neuronal cell types. Interestingly, transcriptionally active euchromatin was enriched in Purkinje neurons while granule cells contained more of the transcriptionally inactive heterochromatin. Based on these results, Heintz et al suggested that 5hmC might play a role in the epigenetic regulation of neuronal functions (44). Interestingly, it appears that this is not the first time that 5hmC was identified in a genome. As early as 1952, 5hmC was detected in T-even bacteriophages (93). It was discovered that 5hmC could be incorporated into the viral genome to resist the attack of restriction enzymes from the bacteria. In 1972, 5hmC was also identified in mammalian DNA (70). However, 5hmC was generally regarded

as oxidatively damaged cytosine in the mammalian genome and might eventually be replaced by DNA-repair mechanism. Since then, not much attention has been given to 5hmC in terms of elucidating its possible physiological functions in the genome until the Heintz report spurred new interests.

The breakthrough in understanding the presence of 5hmC in the genome came from studies on a gene family that is known as Ten-eleven translocation (**TET**). *TET1* at chromosome 10q22 was originally named after the identification of this gene fused with the Mixed Lineage Leukemia (**MLL**) gene resulting from a chromosome translocation of 10q22 and 11q23 in acute myeloid leukemia (51). *TET1* appears to be a member of a well-conserved gene family that contains two other members (*TET2* at 4q24 and *TET3* at 2p12) in the human genome. Soon after, somatic mutations in *TET2* were identified in about 15% of patients affected by myeloid leukemia (12). However, the way in which the mutant TETs contributed to myeloid leukemia was unclear. As a matter of fact, the basic biological function of TETs was unknown at that time.

Through a series of elegant experiments, Rao's lab demonstrated for the first time that TET1 enzymatic activity involved the oxidation of 5mC to 5hmC (85), providing the first bona-fide active demethylation of DNA. Via bioinformatics approaches, TET dioxygenases were identified as mammalian homologs of the proteins JBP1 and JBP2 in trypanosome, a unicellular protozoan. These two proteins were known to oxidize the 5-methyl group of thymine. Upon forced expression of wild type (WT) and mutant TET1 in cultured cells, a dramatic reduction of 5mC signal was observed in WT, but not the mutant, TET1 overexpressing cells. *In vitro* enzymatic analysis further demonstrated that the recombinant TET1 catalytic domain catalyzed 5mC to 5hmC in methylated DNA fragments (85). Both JBP1 and JBP2 belong to the Fe²⁺ and 2OG-dependent dioxygenases family, suggesting that the enzymatic activity of TET dioxygenases may also depend on Fe²⁺ and 2OG. Indeed, withdrawing 2OG from the *in vitro* reaction almost completely blocked the conversion of 5mC to 5hmC. Furthermore, removing Fe²⁺ dramatically inhibited, but did not abolish it completely. The explanation for this result was that a certain amount of Fe²⁺ might be contained in the recombinant TET1 catalytic domain during protein purification process. Thus, TET1 was identified as a Fe²⁺ and 2OG-dependent enzyme that converts 5mC to 5hmC. Soon after, mouse TETs, including TET1, TET2 and TET3, were also identified as being able to oxidize 5mC to 5hmC (34).

Subsequent experiments further confirmed that TETs are Fe²⁺ and 2OG-dependent dioxygenases. First, the binding motif of Fe²⁺ in TETs include at least two histidine residues and one asparagine residue, which are conserved across JBP1, JBP2, human TETs and mouse TETs (78). Mutations at the Fe²⁺-binding sites in TET dioxygenases diminished 5hmC signal in the cultured cells (34). Secondly, 2OG is a critical intermediate metabolite of the Krebs cycle. The oxidative decarboxylation of isocitrate to 2OG is catalyzed by enzymes that are termed isocitrate dehydrogenases (IDH). Mutations in IDH1 and IDH2 have been associated with certain cancers, in which 5hmC is depleted. Instead of 2OG, the mutant IDH produces 2-hydroxyglutarate (2HG), which is also correlated with the reduction of 5hmC in cancers (94). In cultured cells, supplementation of 2HG suppressed the conversion of 5mC to 5hmC, suggesting that 2HG is an inhibitor of 2OG competing for the

binding site in TET dioxygenases (94). These lines of evidence have demonstrated that like P4H, the catalytic activity of TET dioxygenases is indeed dependent on Fe^{2+} and 2OG.

The Zhang group demonstrated that TETs further oxidize 5hmC to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (35). Both 5fC and 5caC could be excised by the DNA repair enzyme Thymine DNA glycosylase (TDG) to produce an abasic position, which is eventually replaced by an unmodified C as shown primarily by two groups (29, 53). Thus, 5hmC, 5fC and 5caC have been proposed as demethylation intermediates. So far, our knowledge of TETs-mediated DNA demethylation is the following: the Fe^{2+} and 2OG-dependent TETs consecutively oxidize 5mC to 5hmC, then to 5fC and 5caC which can eventually be removed from the genome and substituted by unmodified C, thus completing the process of DNA demethylation. Although it involves multiple steps, the TET-mediated oxidation, combined with base excision repair (BER), constitutes the most important and consistent pathway responsible for the active demethylation of DNA.

Vitamin C – an additional cofactor for TET dioxygenases

The requirement for ascorbate as an additional cofactor for P4H and other dioxygenases suggested a potential role for this reducing cofactor in TET-mediated DNA demethylation. Interestingly, studies have shown that ascorbate has the capacity to modify the status of DNA methylation in mammalian cells. For instance, ascorbate causes the widespread DNA demethylation of nearly 2,000 genes in embryonic stem cells (11). Ascorbate also enhances the generation of induced pluripotent stem cells (iPSC) from terminally differentiated cells, which is usually accompanied by genome-wide DNA demethylation (18, 84). These results indicate that ascorbate could be involved in the DNA demethylation process. However, it is unclear whether ascorbate participates directly in DNA demethylation and whether the facilitated DNA demethylation is mediated by the enhanced catalytic activity of TETs by ascorbate.

Initial *in vitro* enzymatic analysis suggested that ascorbate was not essential for TET-mediated hydroxylation of 5mC. It was reported that in the absence of ascorbate, recombinant TET1 converted 5mC to 5hmC at a similar efficiency as when ascorbate was included or excluded in the reaction (85). It is now understood that Fe^{2+} and 2OG could solely satisfy the need of TET dioxygenases in the short term to initiate the hydroxylation of 5mC, as they do for P4H. A possible premature exhaustion of TET enzymatic activity in the absence of ascorbate was not fully explored. It was not clear how long this reaction could last without ascorbate, especially when the *in vitro* stoichiometry of the reaction was not completely controlled. In some *in vitro* experimental settings, Fe^{2+} was obviously overloaded (molar ration of Fe^{2+} : TET1 > 20). In this case, Fe^{2+} might remain available for TET dioxygenases over short periods of time. Thus, under this condition, ascorbate is not required as a rejuvenator of TET dioxygenases via its reduction of the inactive oxidized iron species (Fe^{3+}) to the active Fe^{2+} state after TET dioxygenases have been inactivated by coupled or uncoupled decarboxylation of 2OG, as occurs in collagen P4H. In contrast, free Fe^{2+} is limited and stringently controlled within the cell. Therefore, these *in vitro* enzymatic analyses do not reflect the whole scenario of TET dioxygenases in catalyzing 5mC hydroxylation in the cell. The role of ascorbate as a potential cofactor for TET dioxygenases

to sustain and complete the hydroxylation of 5mC to 5hmC was therefore not excluded by these *in vitro* enzymatic analyses.

The Wang lab demonstrated for the first time that ascorbate enhanced 5hmC generation *in vivo* in cultured cells, most likely by acting as a cofactor for TET to hydroxylate 5mC (13, 62). This previously unknown function of ascorbate in DNA demethylation was subsequently validated in different cell types and modeled animals by other groups (2, 7, 96). 5hmC was originally thought to be detectable only in a few cell types such as stem cells and neurons, but not in other fully differentiated cells. For instance, 5hmC was previously reported as undetectable in cultured HEK-293 cells, and only detectable in the cells when TETs were forcibly overexpressed (35). Wang et al. found that mouse embryonic fibroblasts (MEF) expressed TETs at low but detectable levels as previously reported (14, 41), with TET3 at a higher level than TET1 and TET2. Thus, MEFs constituted an appropriate experimental system to analyze their enzymatic requirements in a cell-based experimental setting. Interestingly, standard cell culture media usually lack ascorbate in their formula. However, when ascorbate is available, it can effectively enter into cells via different transporters. The content of 5hmC was extremely low in MEFs cultured in ascorbate-free medium. Additions of ascorbate in a dose- and time-dependent manner enhanced the generation of 5hmC. The signal of 5hmC reached a plateau when MEFs were treated with 10 μ M ascorbate. Possibly due to the low expression of TETs in MEFs, ascorbate at a relatively low level could fulfill the requirement of TET dioxygenases to reach their maximal enzymatic activity. Surprisingly, the effect of ascorbate on 5hmC was quite rapid. The generation of 5hmC in MEFs could be induced by ascorbate in as little as 1 hr. The rapid effect suggested that no protein synthesis was required, rather activation of existing TET dioxygenases was enough to generate 5hmC (62). Indeed, ascorbate treatment did not change the expression of either TETs or IDHs.

Utilizing a variety of cell-based experiments, it was then tested whether ascorbate works as a cofactor for TET to enhance the conversion of 5mC to 5hmC. **(I)** Treatment with other reducers such as glutathione (GSH) did not change the level of 5hmC, suggesting that the effect of ascorbate on 5hmC could not be attributed to its role as a general reducer. **(II)** Knocking down the expression of TETs by short interference RNAs (siRNA) largely abolished the effect of ascorbate on 5hmC, indicating that it is TET dioxygenases that mediate the action of ascorbate on 5hmC generation. **(III)** Ascorbate transporter inhibitors such as phloretin or sulfinpyrazone decreased the effect of ascorbate on 5hmC generation, suggesting that intracellular accumulation of ascorbate is necessary for activating the catalytic activity of TET dioxygenases. **(IV)** The presence of ascorbate at physiological concentration has been shown to enhance the uptake of iron by cells, raising the possibility that the effect of ascorbate on 5hmC might be indirect and mediated by an ascorbate-induced increase in the cellular uptake of iron (46–48). However, removing iron from the culture medium did not affect the induction of 5hmC by ascorbate, which suggests that the effect of ascorbate on 5hmC is independent of cellular uptake of iron. **(V)** Cells cultured with different concentrations of glucose, a major precursor of 2OG, exhibited the similar level of 5hmC in response to ascorbate treatment. These results indicate that the effect of ascorbate on 5hmC is not dependent upon iron uptake, the expression of TET and IDH, or

the production of 2OG, which overall suggests that ascorbate may directly participate in the conversion of 5mC to 5hmC, most likely as a cofactor of TETs (13, 62). Embryonic stem (ES) cells are generally cultured in the medium without ascorbate. The Ramalho-Santos group observed that when added, ascorbate led to a rapid and global increase in 5hmC, which was followed by DNA demethylation of many gene promoters and upregulation of demethylated germline genes (2). Yin et al. reported that ascorbate directly enhanced the activity of purified C-terminal catalytic domain of TET2 to oxidize 5mC to 5hmC and 5fC, while other reducers such as spermidine, vitamin B1, vitamin E, glutathione, NADPH, and L-cysteine did not have this effect. After treatment with ascorbate, mouse ES cells displayed significant increases in 5mC oxidation products, particularly 5fC and 5caC. Furthermore, 5hmC was decreased in various tissues in Gulo knockout mice (96). These results suggest that ascorbate could be a cofactor for TET in the cascade oxidation of 5mC. Chen et al. reported that TET1, in an ascorbate-dependent manner, regulated 5hmC formation at loci critical for the reprogramming of iPSC (7).

From *in vitro* enzymatic activity analysis to cell and animal-based experiments, the available evidence strongly suggest that ascorbate is a cofactor for TET dioxygenases in the conversion of 5mC to 5hmC, and further 5fC and 5caC, thus modulating DNA demethylation (Fig. 1).

Vitamin C, DNA demethylation and epigenetic reprogramming

The amount of ascorbate needed for TETs apparently depends on the protein level of TET dioxygenases. The expression level of TETs is low in most terminally differentiated cells such as fibroblasts but is relatively high in neuronal cells such as Purkinje cells (26, 44). However, TETs are expressed at a much higher level in embryonic tissues, especially at early developmental stages (86). It is known that epigenetic reprogramming occurs during the mammalian embryonic development, which involves DNA demethylation and re-methylation. For a long time, how the DNA is demethylated in pre-implanted embryos has been unclear. It is now known that both TET-mediated oxidation and passive dilution participate in the demethylation process. Immediately after fertilization, 5mC in the paternal chromatin is rapidly replaced by 5hmC via TET3-mediated oxidation, which cannot be maintained during the rapid DNA replication in pre-implanted embryos leading to passive demethylation and the erasing of paternal 5mC patterns (33). In contrast, the maternal DNA is only passively demethylated by dilution due to the inhibition of DNMT1; further TETs-catalyzed oxidization of 5mC is not involved (42). However, one recent report showed that both the paternal and maternal chromatin went through active demethylation from zygote to four-cell embryos (89). A second round of epigenetic reprogramming happens in primordial germ cells (PGC), which also involves in TETs-mediated active demethylation (42).

Currently available evidence suggests that TETs-mediated active demethylation is one of the major pathways for DNA demethylation during early embryonic development. As a result, it could be possible that varying levels of ascorbate during embryonic development affect epigenetic reprogramming of both germ cells and the developing embryo, which could lead to distinct consequences. To date, studies on the effects of ascorbate contents on DNA methylation-demethylation dynamics during embryonic development are still lacking.

However, several studies suggest that deficiency of ascorbate is linked to certain types of developmental defects. For example, women at high risk for neural tube defect (NTD) recurrence tend to have lower leucocyte ascorbate levels compared with low-risk women (3, 77). Genetic variation in SVCT2, which might affect intracellular ascorbate levels, is also associated with the risk of preterm birth (16). However, due to the fact the ascorbate consumption from dietary sources and supplements is almost impossible to control quantitatively in human subjects, published results of ascorbate supplement on birth defects or preterm delivery are mixed. It is worth noting that ascorbate supplementation in pregnant women should be aimed at satisfying the needs of TETs and other Fe²⁺ and 2OG-dependent dioxygenases in both the mother and the embryo. In the case of sufficient ascorbate dietary intake, higher-doses of ascorbate may not necessarily exert additional benefits. Although ascorbate is most probably essential for TETs-mediated active demethylation in embryonic development, it is unclear how much ascorbate is needed from diet and/or supplements to completely satisfy the requirement of TETs in the prevention of embryonic defects caused by inappropriate DNA demethylation.

Vitamin C and the loss of 5hmC in cancer

In contrast to the relatively high level of 5hmC in embryos, especially in pre-implanted embryos and PGCs, cancer cells have very low or undetectable 5hmC. Studies have shown that loss of 5hmC is a novel epigenetic hallmark of most, if not all, types of human cancer (9, 36, 40, 45, 57, 65, 67, 71, 94, 95). The Rao lab was the first to provide convincing evidence that links the mutant TET1 and an impaired generation of 5hmC in leukemia (85). Since then, many groups have shown the loss of 5hmC in various cancers. In 2012, Shi et al published the inverse correlation of 5hmC with melanoma progression in humans, and a possible treatment in animal models. The content of 5hmC is relatively high in healthy melanocytes but is gradually lost during progression from benign nevi through advancing stages of primary and metastatic melanoma (49). The global loss of 5hmC disrupts the normal dynamics of DNA methylation-demethylation and affects genome-wide gene expression, which could eventually lead to malignant transformation. Besides mutations in TETs and IDHs (19), a decreased expression of TETs and IDHs is also attribute to the loss of 5hmC in cancer (49).

Genetic variation in SVCT1 and SVCT2 has been associated with the risk of certain types of cancer including advanced colorectal adenoma (17), muscle-invasive bladder cancer (25), gastric cancer (92) and non-Hodgkin lymphoma (82). It should be noted that these genetic associations have not yet been independently verified in other cohorts. Also worth important consideration are the potential functional consequences of these associated variants on ascorbate transportation, which remain largely unclear. A search of Cosmics, a cancer somatic mutation database (<http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/>), reveals that mutations in SVCTs have been identified in many types of cancer such as breast cancer, colorectal adenoma, brain tumors and others. Recurrent mutations in the splicing factor SF3B1 have been identified in chronic lymphocytic leukemia, uveal melanoma as well as other cancers (27, 72). In at least one of these types of cancers, chronic lymphocytic leukemia, the mutant SF3B1 causes a truncated, most likely nonfunctional, SVCT2 that can result in intracellular deficiency of ascorbate in cancer cells (72). Furthermore, the uptake

rate of ascorbate, the dominant form of ascorbate in the plasma, by a melanoma cell line is only ~50% of the uptake rate of the healthy melanocytes (83). These studies suggest that in addition to the genetic alterations in TETs and IDHs, variations in SVCTs might also contribute to the loss of 5hmC in cancer cells. Further studies are required to determine whether there is a local ascorbate deficiency in cancer cells.

In 1976, Linus Pauling proposed the treatment of cancer patients with intravenous ascorbate, followed by oral maintenance. This proposal was met with some skepticism, in part due to controversial experimental support, but also due to the lack of a complete picture of the effect of ascorbate on cancer cells; the newly recognized role of ascorbate in the epigenetic modulation of gene activity might shed a new light on this issue. It has been shown that overexpressing TET1 in breast cancer and overexpressing TET2 in melanoma could partially reestablish a normal 5hmC profile in the cancer cells and decreases malignancy especially invasiveness (32, 49). These findings suggest that the means of rebuilding the 5hmC content could offer a potential treatment for these cancers. However, it might not be feasible to clinically overexpress TET or IDH in patients. Our unpublished data show that treatment with a physiological concentration of ascorbate can decrease the malignant phenotype of melanoma cells *in vitro* by partially reestablishing the global content of 5hmC. As a cofactor for TETs, ascorbate enhances and possibly maximizes the catalytic activity of the existing TETs in cancer cells. The content of 5hmC induced by ascorbate in a melanoma cell line is comparable to the effect of overexpressing TET2 in the same cell line (49).

Ascorbate is a safe and well-tolerated dietary supplement that is readily available. Thus, it is feasible that ascorbate could be conveniently utilized in patient care. It is notable that ascorbate especially at high pharmacological concentrations has a long and widely discussed history in treating cancer. Further, possibly due to the fact that it is nearly impossible to quantitatively control dietary ascorbate consumption in human subjects, a solid epidemiological link between ascorbate deficiency and the incidence of cancer has not been established. However, a recent meta-analysis shows that higher intake of ascorbate might have a protective effect against the risk of lung cancer (52). Another meta-analysis supports the notion that post-diagnosis ascorbate supplementation may be associated with a reduced risk of mortality; dietary ascorbate intake is associated with a reduced risk of total mortality and breast cancer-specific mortality (28). It will not be surprising if some of the beneficial effects of ascorbate on cancer were found to be mediated by the induction of 5hmC generation in cancer cells.

Vitamin C and peripheral neuropathy

In the late 1960s, it was found that peripheral neuropathy developed in volunteers after ascorbate had been withdrawn from diet and dietary supplement for a longer period (~70 days). Reported complaints of the volunteers included numbness of the calves, hypoesthesia to light touches and pain (30, 31). These complaints, along with other signs of peripheral neuropathy gradually disappeared after ascorbate was resupplied to the volunteers. Since the potentially damaged peripheral nerves are nearly impossible to obtain from volunteers suffering ascorbate deficiency, a detailed histopathology of this induced peripheral neuropathy in humans is still unclear. Further, it also remains unknown how deficiency in

ascorbate damages the peripheral nerve. Recent *in vitro* studies and animal modeling have provided convincing evidence linking deficient ascorbate and peripheral neuropathy.

In peripheral nerves, Schwann cells form myelin sheaths, which encircles axons to provide metabolic support and allow for fast nerve conduction. In the 1980s, ascorbate was identified as an essential factor to initiate and promote myelin formation by Schwann cells *in vitro* (5). In the defined medium used for co-culture of neurons and Schwann cells, myelin is not formed if ascorbate is absent in the medium (66). In contrast, myelin formation by olfactory ensheathing cells is not dependent on ascorbate (1), suggesting the specificity of the requirement of ascorbate for the myelin formation by Schwann cells. Previous studies focused on the known regulatory role of ascorbate in the formation of collagen- and laminin-containing extracellular matrix (ECM) (60, 15). However, recent animal-based studies suggest that the effect of ascorbate on ECM such as collagen cross-linking may not play a key role in promoting axonal myelination.

It has been shown that ascorbate enters and accumulates in Schwann cells primarily via SVCT2 (20). Loss of one allele in the gene SVCT2 (SVCT2^{+/-}) causes a lower protein level of SVCT2, which would result in deficient ascorbate within Schwann cells, but not in extracellular milieu. Hypomyelination develops in peripheral nerves of SVCT2^{+/-} mice (21), suggesting that the intracellular ascorbate is the key to myelination. Thus, collagen cross-linking induced by ascorbate in the extracellular matrix might not play an important role in the myelination formed by Schwann cells as previously thought. G. Wang's unpublished observation indicates that ascorbate significantly increases 5hmC content and shifts the transcriptome pattern in cultured Schwann cells. It is now believed that ascorbate may have much broader impacts on the critical stages of myelination including Schwann cell activation, proliferation, differentiation and myelination by shifting their epigenome and transcriptome.

Charcot-Marie-Tooth disease (CMT) is a hereditary peripheral neuropathy. Type 1A of CMT (CMT1A), which is characterized by abnormalities in myelin, is mainly caused by duplication of a region on chromosome 17p12-p11.2 and mutations in the gene *peripheral myelin protein 22* (PMP22), which reside in the same region (74). Treatment with large doses of ascorbate ameliorates the neuropathy of a CMT1A mouse model, in which PMP22 is overexpressed (69). Unfortunately, subsequent clinical trials on CMT1A patients presented somewhat disappointing results – no significant benefits were observed after treatment with ascorbate (1.5 g/day) for 1~2 years (6, 68). The reasons for this disappointing clinical trial are still under debate. Perhaps, 1~2 years of treatment are still too short for ascorbate to exert a measurable clinical outcome improvements in this slowly progressive disease (22). Alternatively, the CMT1A mouse model may have certain fundamental differences in the phenotype manifestation. For example, the mouse model synthesizes endogenous ascorbate whereas patients do not have this biosynthetic pathway. Additionally, one study showed that ascorbate combined with vitamin B2 and a synthetic reducer idebenone accelerates the recovery of vision in patients affected by Leber hereditary optic neuropathy (LHON) (56). Overall, the mechanistic role of ascorbate in myelin formation requires further examinations, which will aid in the design of future clinical trials aiming at

the delay or prevention, rather than the cure, of peripheral neuropathy in patients carrying a CMT1A mutation.

VITAMIN C AND HISTONE DEMETHYLATION

Histone demethylation

The basic unit of eukaryotic chromatin is the nucleosome, which is composed of a short length of DNA wrapped around an octamer that consists of 2 copies of each histone (H2A, H2B, H3 and H4). Although not a component of the core histones, H1 binds the nucleosome “beads” and could be involved in the higher-order assembly of chromatin. Like other proteins, histones are also substrates for post-translational modifications (PTM). The known PTMs on histones include methylation, acetylation, phosphorylation, ubiquitination, sumoylation and others (75). These PTMs, which are catalyzed by different enzymes can target, yet sometimes compete for, amino acid residues in the histones. The dynamic PTMs in the histone modulate chromatin structure, genome stability and gene transcription.

Methylation at lysine and arginine residues is the major epigenetic modification in histones. Along with DNA methylation, histone methylation is a key component in the epigenome. The most extensively studied methylation occurs on histone H3 at lysine (K) 4 (H3K4), H3K9, H3K27, H3K36, H3K79 and H4K20 as well as on histone H3 at arginine (R) 2 (H3R2), H3R8, H3R17, H3R26 and H4R3. The methyl donor in histone methylation is S-adenosylmethionine (SAM), the same as the donor for DNA methylation. The addition of methyl groups to either lysine residues or arginine residues is catalyzed by histone methyltransferases (HMT) as writers of this epigenetic mark. There are many HMTs that belong to three families of enzymes; SET-domain-containing protein family, DOT1-like protein family methylate lysines and protein arginine N-methyltransferase (PRMT) family methylates arginines (24). In DNA methylation, only one methyl group is added to the cytosine. In histone methylation, there could be one, two or three methyl groups added to the single nitrogen of lysines resulting in monomethylated, dimethylated or trimethylated lysines respectively. Similarly, arginines could also be monomethylated and dimethylated (either symmetric at two nitrogens or asymmetric at a single nitrogen of the arginine), but not trimethylated. A large number of histone methylation binding proteins, as readers, bind methylated histones and further influence the recruitment of chromatin-modifying effectors, local chromatin structure and exert distinct impacts on genome functions depending on specific lysine or arginine residues. For example, methylation of H3K4 is associated with actively transcribed genes while methylation at H3K27 is a hallmark of silenced chromatin (37).

Histone methylation was once thought to be irreversible, thus, an everlasting PTM on histones. In 2004, the Shi group successfully identified the first lysine-specific histone demethylase 1 (LSD1 also known as KDM1A) (79) to remove methyl groups from monomethylated or dimethylated H3K4 and H3K9, thus resolving the debate of whether or not the histone methylation is reversible. LSD2, the other member of the LSD family, was later isolated to have similar enzymatic activity of histone demethylation like LSD1 (38). Via a flavin adenine dinucleotide (FAD)-dependent amine oxidase reaction, LSD family demethylases cleave the α -carbon bond of the methylated lysine to form an imine

intermediate, which is hydrolyzed to form formaldehyde, releasing one molecule of H₂O₂ and the demethylated lysine (80). It appears that the LSD family demethylases can only demethylate monomethylated and dimethylated lysines, but not the trimethylated (me₃) lysines in histone, suggesting the existence of other types of histone demethylase (64).

Vitamin C – an additional cofactor for JmjC domain-containing histone demethylases

In 2006, the Zhang group purified a JmjC domain-containing histone demethylase 1 (**JHDM1**), which specifically demethylates H3K36 in the presence of Fe²⁺ and 2OG. (88). Shortly after, the Zhang group also identified that the transcriptional repressor JHDM3A demethylates trimethylated H3K9 and H3K36 (39). So far, about 20 proteins that belong to the JmjC domain-containing histone demethylase family have been discovered to have the catalytic capacity to demethylate histones (63). It is now known that the JmjC domain-containing demethylases can demethylate mono-, di-, and trimethylated histone lysine residues. JmjC domain-containing histone demethylases, like TETs, also belong to the Fe²⁺ and 2OG dioxygenase superfamily. The Zhang group for the first time reported that ascorbate is required for optimal catalytic activity of JHDM1 and the demethylation mediated by JHDM3A is halted when ascorbate is withdrawn from the *in vitro* assay (39, 88). Although the role of ascorbate in histone demethylation was only examined in *in vitro* assays in these studies and the effect of ascorbate in other members in the JmjC domain-containing histone demethylases has not yet been reported, it is reasonable to deduce that ascorbate could be a cofactor for the JmjC domain-containing histone demethylase family, thus modulating histone demethylation in a similar way as it does on DNA demethylation (Fig. 2).

Vitamin C, histone demethylation and epigenetic reprogramming

Ascorbate appears to be important in the late phase of reprogramming from terminally differentiated cells (10). The key role of ascorbate in the transition from pre-iPSC phase to fully reprogrammed iPSCs was first uncovered by the Pei group (8). It appears that H3K9 methylation is a barrier during somatic cell reprogramming into iPSCs. Ascorbate helps histone demethylases switch the pre-iPSC fate on or off by regulating the H3K9 methylation status of the core pluripotency loci; in addition to modulating DNA demethylation (11, 18, 84). Furthermore, ascorbate stimulates proliferation of bone marrow mesenchymal stem cells (MSC) and improves the efficiency of iPSC generation from the MSC (97). The impact on DNA and histone demethylation may underlie the gene expression profiles altered by ascorbate treatment in MEFs during iPSC induction (90) (Table 1).

Ascorbate is also involved in cell differentiation. For example, ascorbate enhances T cell maturation, an effect that is intrinsic to lymphoid cells and independent of T cell receptor (TCR) rearrangement. Inhibition of DNA and histone methylation further promotes the effect of ascorbate on T-cell differentiation, which suggests a role of ascorbate participated active demethylation of DNA and histone in T cell maturation (55).

CELLULAR AVAILABILITY OF VITAMIN C AND REDOX GENOMICS

The available data provide evidence for the role of ascorbate as a critical mediator of the interface between the genome and environment, principally by participating in epigenomic demethylation. Thus, any genetic and environmental factors that influence the synthesis, absorption, transportation and metabolism of ascorbate could have significant consequences for genome integrity, development, pluripotency, and ultimately health and disease by modulating the epigenetic control of genome activity.

Insufficient dietary intake is the major cause for systemic ascorbate deficiency. Diet and lifestyle are known to dramatically influence the level of ascorbate in the human body. Even in a developed country like the USA, more than 7% of the population (>20 million individuals) is estimated to be deficient in ascorbate (concentrations <11.4 μM in plasma), according to a recent survey (76). Cigarette smokers, low-income families and individuals carrying certain genetic variants in SVCTs have higher risk of ascorbate deficiency (87). Further, the turnover rate of vitamin C appears to be quite rapid, suggesting that the number of people with short-term ascorbate deficiency could be even higher (81). Ascorbate deficiency also can happen locally in certain types of cells. For example, intracellular ascorbate concentration in hepatocytes is decreased in aged mice due to a lower expression of SVCT1, as compared to young controls (61). Certain environmental exposures such as arsenic compounds also can diminish ascorbate levels and alter the epigenome of peripheral blood mononuclear cells (4).

Both systemic and local deficiency affects the availability of ascorbate to the cell. Furthermore, the transport of ascorbate to the nucleus is the key for ascorbate to reach to the chromosomes, which then influences the epigenome by participating in the demethylation of DNA and histones. More importantly, ascorbate is merely one component of the redox network in the cell. The degradation and recycling of ascorbate is largely controlled by the redox status. For example, the status of redox couples including GSH/GSSG, NADH/NAD and FADH₂/FAD are capable of impacting the ascorbate/DHA redox couple, and thus, the availability of ascorbate to the DNA and histone demethylation machinery. Consequently, redox status in the cell, especially in the nucleus, will have a profound impact on the epigenome via ascorbate. It is well known that redox status changes along with development, aging, health and diseases. Bridging the gap between redox biology and genomics, we suggest an interdisciplinary research field that can be termed “**Redox Genomics**” to study the dynamic role of redox in health and diseases. Embryonic development, aging and many diseases such as chronic inflammation, cancer, diabetes, and neurodegenerative disorders, could be reassessed from the perspective of Redox Genomics.

Furthermore, many pre-clinical studies utilized cell- and animal (especially rodents) based models. It appears that ascorbate is not even contained in the formulation of most culture media. Thus, the critical regulatory role of ascorbate in the epigenome might have been overlooked in many cell-based studies. In addition, rodents can synthesize ascorbate *de novo* (unlike humans), therefore, these previous rodent studies were not equipped to detect how variation in ascorbate availability might affect the epigenome. We suggest that the availability of ascorbate should be addressed in future pre-clinical studies.

SUMMARY POINTS

- Ascorbate regulates DNA demethylation as an essential cofactor for TET dioxygenases.
- Ascorbate regulates histone demethylation as an essential cofactor for Jmjc domain-containing histone demethylases.
- Ascorbate is a key mediator of the interface between the genome and environment.
- Ascorbate is critical in maintaining the epigenome, especially at early embryonic stages.
- Failure to maintain the catalytic activity of TET dioxygenases and Jmjc domain-containing histone demethylases by deficient ascorbate contributes to different diseases.
- Ascorbate is an important micronutrient with functions far beyond scurvy prevention.

FUTURE ISSUES

Considering its cofactor role for the epigenetic enzymes, ascorbate, if deficient temporally and spatially, will impair the epigenome and contribute to certain phenotypic changes, and eventually lead to diseases. However, it is also understood that excessive ascorbate may not necessarily be beneficial for the epigenome. From an epigenomics perspective, the future goal should be to address ascorbate deficiency rather than elevating ascorbate to a very high level by large doses supplementation or injection. This novel epigenetic function of ascorbate needs to become known to the general public. Individuals with genetic variations affecting SVCTs should be alerted to the need for proper supplementation. RDA of ascorbate for women at early pregnancy may be reevaluated when the role of ascorbate in epigenetic reprogramming is taken into consideration. Deficient ascorbate may be involved in many diseases such as birth defects, cancer and diabetic complications. These need to be studied in patients as well as cell- and animal-based models. As previously noted, ascorbate is largely absent in the formulation of most media used for culturing cells. This suggests that the critical regulatory role of ascorbate in the epigenome has been completely overlooked in numerous cell-based studies. On the other hand, rodents have been overwhelmingly used in various disease modeling and drug screening. However, unlike humans, rodents synthesize ascorbate *de novo* in the liver. This implicates that the rodents-based studies have completely ignored the effect of variation in ascorbate availability (as in humans) on the epigenome. These issues need to be addressed sooner rather than later in the biomedical research field.

CONCLUSIONS

A novel function of ascorbate in epigenomic regulation has been uncovered by recently published studies. Ascorbate directly participates in epigenomic demethylation by serving as a cofactor for the demethylases. The bioavailability of ascorbate thus has a profound impact on the epigenome. Based on this knowledge, a novel interdisciplinary research field termed

Redox Genomics is emerging to meet the needs to study the epigenomic role of redox in health and diseases.

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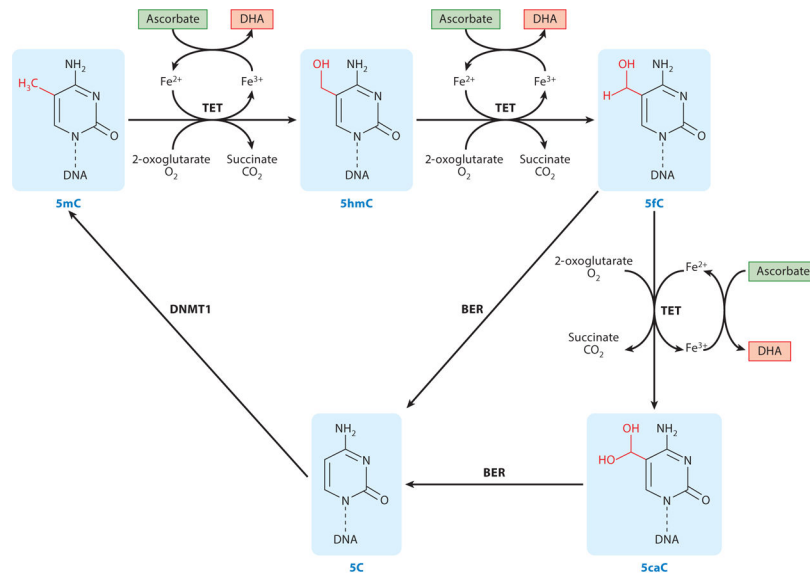


Figure 1. The role of ascorbate in DNA demethylation

As a cofactor, ascorbate participates in the cascade oxidation of 5mC, to 5hmC, to 5fC and to 5caC catalyzed by TET dioxygenases. 5fC and 5caC are then replaced by unmodified 5C by BER machinery. Additionally, unable to maintain 5hmC in the newly synthesized DNA also leads to passive demethylation. The methylation of an unmodified 5C could be reestablished by DNMT1, thus complete a cycle of DNA methylation–demethylation.

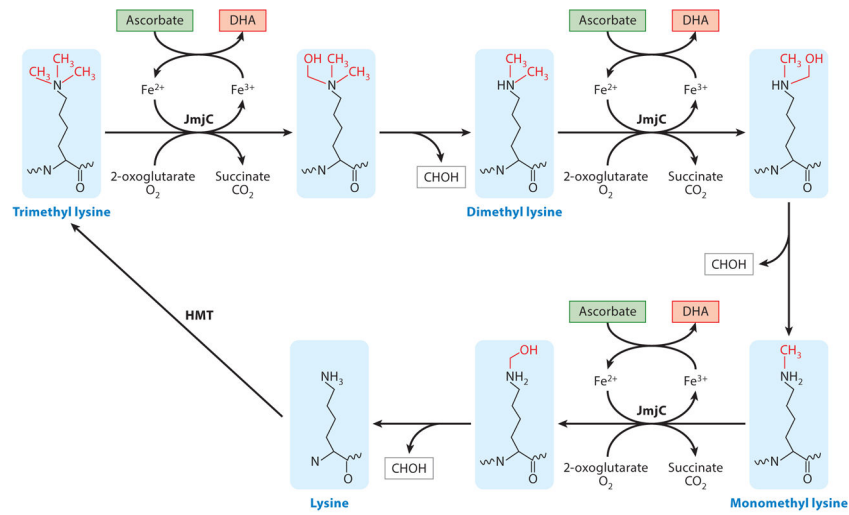


Figure 2. The role of ascorbate in histone demethylation

As a cofactor for JmjC domain-containing histone demethylases (JmjC), ascorbate participates in the oxidation of tri-methylated, di-methylated and mono-methylated lysine in histones, which is followed by a spontaneous removal of the hydroxymethyl group. The methylation of an unmodified lysine in histones could be reestablished by HMT, thus complete a cycle of histone methylation–demethylation.

Table 1

Gene expression changes induced by ascorbate administration in MEFs.

Chr	Gene	Gene description	Affymetrix ID	Effect
11	Ccl8	Chemokine (C-C motif) ligand 8	10379535	increased
19	As3mt	Arsenic (+3 oxidation state) methyltransferase	10463704	increased
19	Ifit1	Interferon-induced protein with tetratricopeptide repeats 1	10462623	increased
5	Oasl2	2'-5' oligoadenylate synthetase-like 2	10524621	increased
6	Usp18	Ubiquitin specific peptidase 18	10541307	increased
16	Rtp4	Receptor transporter protein 4	10434778	increased
8	Ddx60	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60	10571984	increased
10	Aldh112	Aldehyde dehydrogenase 1 family, member L2	10371332	decreased
11	Tgtp1	T cell specific GTPase 1	10385533	increased
11	Xaf1	XIAP associated factor 1	10378068	increased
3	Gbp3	Guanylate binding protein 3	10496580	increased
1	Csprs	Component of Sp100-rs	10347925	increased
13	Akr1c18	Aldo-keto reductase family 1, member C18	10407435	increased
6	Strip2	Striatin interacting protein 2	10536949	increased
7	Irf7	Interferon regulatory factor 7	10569102	increased
10	Ddit3	DNA-damage inducible transcript 3	10366881	decreased
2	Zbp1	Z-DNA binding protein 1	10490150	increased
2	Trib3	Tribbles homolog 3 (Drosophila)	10488608	decreased
16	Mx2	MX dynamin-like GTPase 2	10437224	increased
11	Dhx58	DEXH (Asp-Glu-X-His) box polypeptide 58	10391207	increased

Note: Listed here are the 20 genes which expression are significantly altered by the administration of ascorbate (50 µg/ml). The data were extracted from the GEO database that was deposited by Wang T, et al with the accession number GSE19377 (92).