Ascorbate is a multifunctional micronutrient whose synthesis is lacking in primates

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Ascorbate (vitamin C) is an essential micronutrient in primates, and exhibits multiple physiological functions. In addition to antioxidative action, ascorbate provides reducing power to α-ketoglutarate-dependent non-heme iron dioxygenases, such as prolyl hydroxylases. Demethylation of histones and DNA with the aid of ascorbate results in the reactivation of epigenetically silenced genes. Ascorbate and its oxidized form, dehydroascorbate, have attracted interest in terms of their roles in cancer therapy. The last step in the biosynthesis of ascorbate is catalyzed by L-gulono-γ-lactone oxidase whose gene *Gulo* is commonly mutated in all animals that do not synthesize ascorbate. One common explanation for this deficiency is based on the increased availability of ascorbate from foods. In fact, pathways for ascorbate synthesis and the detoxification of xenobiotics by glucuronate conjugation share the metabolic processes up to UDP-glucuronate, which prompts another hypothesis, namely, that ascorbate-incompetent animals might have developed stronger detoxification systems in return for their lack of ability to produce ascorbate, which would allow them to cope with their situation. Here, we overview recent advances in ascorbate research and propose that an enhanced glucuronate conjugation reaction may have applied positive selection pressure on ascorbate-incompetent animals, thus allowing them to dominate the animal kingdom.

Key Words: ascorbate, vitamin C, glucuronate conjugation, detoxification

Vitamins are nutrients that are, in the original meaning, essential micronutrients that cannot be synthesized by an organism. About a century ago, Szent-Györgyi[1] discovered that high levels of ascorbate (Asc) are stored in the adrenal gland, and this stimulated research interest in this compound. The inability to synthesize a vitamin is generally due to a lack genes responsible for their syntheses that developed during the evolutionary process, however, applying this scenario to Asc is not quite correct. In fact, most organisms, including mammals, are able to synthesize Asc from glucose as the primary carbohydrate. On the contrary, the ancestors of primates and some other species once had the ability to synthesize Asc but have lost this ability during their evolution.[2,3]

Humans need to ingest massive amounts of Asc compared to other vitamins for health maintenance; the recommended daily requirement for Asc is 75–110 mg/day for adult humans.[4] A high excretion rate from the body through the kidneys; a half-life being approximately one day, is the main contributor to this massive requirement. Regarding physiological roles, antioxidation and electron donation in the synthesis of biological compounds, such as collagen and catecholamines, are well established functions of Asc.[5,6] Recent advances in this area have revealed that the contribution of Asc can be extended to the oxygen-dependent regulation of gene expression via the hypoxia-inducible factor (HIF)-1α[7] and the epigenetic regulation of genes.[8,9] In the meantime, since the proposal by Linus Pauling on the use of Asc for cancer therapy, the benefits of high doses of Asc in the treatment of cancer has been a subject of considerable debate.[10] Accumulating evidence, based on basic and clinical studies, are now confirming that Asc can, in fact, be useful in cancer prevention and treatment.[11]

Phylogenetically, vertebrates higher than fish possess the *Gulo* encoding L-gulono-γ-lactone oxidase (Gulo), which catalyzes the last step in Asc synthesis.[12] A mutation in *Gulo* occurred about 63,000,000 years ago, which makes primates unable to synthesize Asc.[13] In fact, mutations have been observed in *Gulo* in all incompetent animal species, which include guinea pigs, bats, passeriformes birds, and teleost fish.[3,14,15] These animals can ingest sufficient amounts of Asc from foods in ordinary life, so that Asc production may not be essential for their health. However, the question remains about what has allowed these incompetent animals in which *Gulo* was mutated to dominate during their evolution.

In this review article, we briefly overview the multiple functions of Asc with specific emphasis on recent advances in our understanding of the beneficial roles of Asc in our health and propose a mechanism regarding selection pressure that may have allowed animals that are unable to produce Asc continue to dominate.

Anti-oxidative and Redox Reaction of Asc

The chemistry of ascrobic acid has been extensively overviewed,[16,17] and this is not the subject of this article. Accordingly, only minimal information concerning the structure and the chemical nature of Asc that are required for our understanding of its physiologica funcitons, are provided here. While ascorbic acid is present mainly in three states: the fully oxidized form (dehydroascorbate; DHA), the ascorbyl radical and the fully reduced monoanion form, Asc is the dominant form at physiological pH and is a potent reducing agent (Fig. 1). Asc readily undergoes a one-electron oxidation to form an ascorbyl radical and then DHA. In other words, due to its high electron-donating ability, Asc is involved in a variety of reactions that require reducing equivalents in both enzymatic and non-enzymatic reactions. The resulting oxidized form, DHA is either recycled by several processes that are described below or is converted into 2,3-diketo-L-gulonic acid, which is then further metabolized via L-xylose, L-threonic acid plus oxalic acid, or L-xylonic acid and L-lyxonic acid.[6] Asc concentrations in the plasma/extracellular fluid and cells/tissues are 0.04–0.08 mM and 0.3–10 mM, respectively.[10,18] A short half-life of

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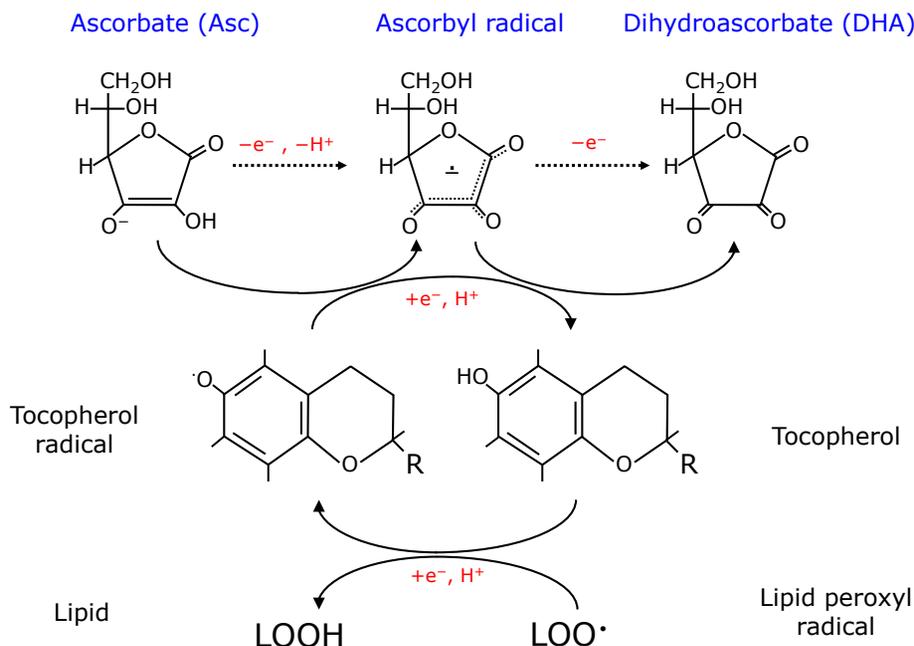
Fig. 1. Interconversion of Asc, ascorbyl radical and DHA and their roles in the vitamin E-mediated elimination of lipid radical. Three major forms of ascorbic acid and their redox-mediated interconversion are depicted on the top. Vitamin E (tocopherol) donates electron to lipid hydroxyl radical (LOO•) and is converted to tocopherol radical. Asc may donate an electron to a tocopherol radical for recycling and is converted to an ascorbyl radical, which is then oxidized to DHA. Asc collectively can protect lipids from oxidation reactions as well. Schemes show conceptual reactions and are not stoichiometric. R; alkyl side chain.

approximately one day in mammals is mainly due to the high excretion rate and insufficient reuptake in the kidney. Due to less abundance of DHA and high level of reductive recycling, the issue of how much of the DHA-derived metabolites are subsequently metabolized remain ambiguous.

Anti-oxidation is a major function of Asc and has been extensively reviewed.[17,19,20] Asc is a safe and relatively cheap antioxidative compound and is generally used as a food additive in commercial food products. The antioxidative capacity of Asc is largely attributed to its ability to donate an electron and be converted into a radical species, typically a hydroxyl radical, a vitamin E (α-tocopherol) radical and a thyl radical.[19] While Asc is a highly water-soluble molecule and can function to suppress hydrophilic radical species, vitamin E provides protection against lipid peroxidation by donating electrons to lipid peroxyl radicals (LOO•). Experimental data indicate that Asc is able to recycle vitamin E radicals back to vitamin E, and hence, also functions to protect lipids against peroxidation. Because of this, both Asc and vitamin E are often discussed together as nutritional antioxidants.[16,21] Because lipid peroxidation products trigger ferroptosis, a newly identified type of iron-dependent non-apoptotic cell death,[22,23] Asc may also be involved in the suppression of ferroptosis via the reductive recycling of vitamin E, a potent anti-ferroptotic compound. In the meantime, Asc is not a very effective scavenger of superoxide, a primary oxygen radical, according to the rate constant for this reaction $(2.7 \times 10^5 \text{ M}^{-1}\text{s}^{-1})$[24] which is about half that for the spontaneous dismutation of superoxides. However, because of the abundance of Asc in tissues, this allows the reaction between superoxide and Asc to proceed sufficiently rapidly to suppress superoxide to permissible levels *in vivo*.[25] Because superoxide is the oxygen radical that is first produced under a variety of reactions with oxygen consumption, its elimination would be of importance for suppressing subsequent radical chain reactions and hence provide relief from oxidative stress.

Asc is also closely associated with iron homeostasis and function. In cooperation with ferrireductase including duodenal cytochrome B, ferric iron (Fe^{3+}) is reduced to ferrous iron (Fe^{2+}) by Asc and is then taken up by apical membranes in the intestinal lumen via the divalent metal ion transporter DMT1.[26] Hence, an Asc insufficiency decreases the intestinal absorption of iron and causes iron-deficient anemia.[27] Asc stimulates the synthesis of the iron-storage protein ferritin, inhibits its lysosomal degradation, and decreases the efflux of iron from cells.[28] Moreover, Asc-dependent enzymatic reactions are dependent on iron in most cases and copper occasionally.[5] It is noteworthy, however, that Asc in pharmacologic concentrations is converted to excessive levels of ascorbyl radicals and is simultaneously involved in production of hydrogen peroxide in a free-iron dependent manner in extracellular fluid.[29] Thus, Asc may also act as a pro-oxidant and exert cytotoxicity, depending on the redox environment, which appears to be an underlying mechanism for the anti-cancer action of Asc.[30]

Outline of Asc Biosynthesis

Asc is synthesized dominantly in the liver from glucose as a primary carbohydrate via several processes,[31,32] and the pathway cross-talks with other glucose metabolic pathways (Fig. 2). Accordingly nutritional conditions can have a significant effect on Asc synthesis and can have an influence on the Asc levels in the body. Upon feeding, cells take up glucose from the blood via a glucose transporter (GLUT) and phosphorylate glucose to glucose-6-phosphate (glucose-6-P) via the action of hexokinase/glucokinase. Glucose-6-P is then either degraded by glycolysis, converted to other carbohydrates via the pentose phosphate pathway, or isomerized to glucose-1-phosphate (glucose-1-P) by phosphoglucomutase. Glucose-1-P uridylyltransferase, also referred to as UDP-glucose pyrophosphorylase, then converts glucose-1-P into UDP-glucose which serves as the communal
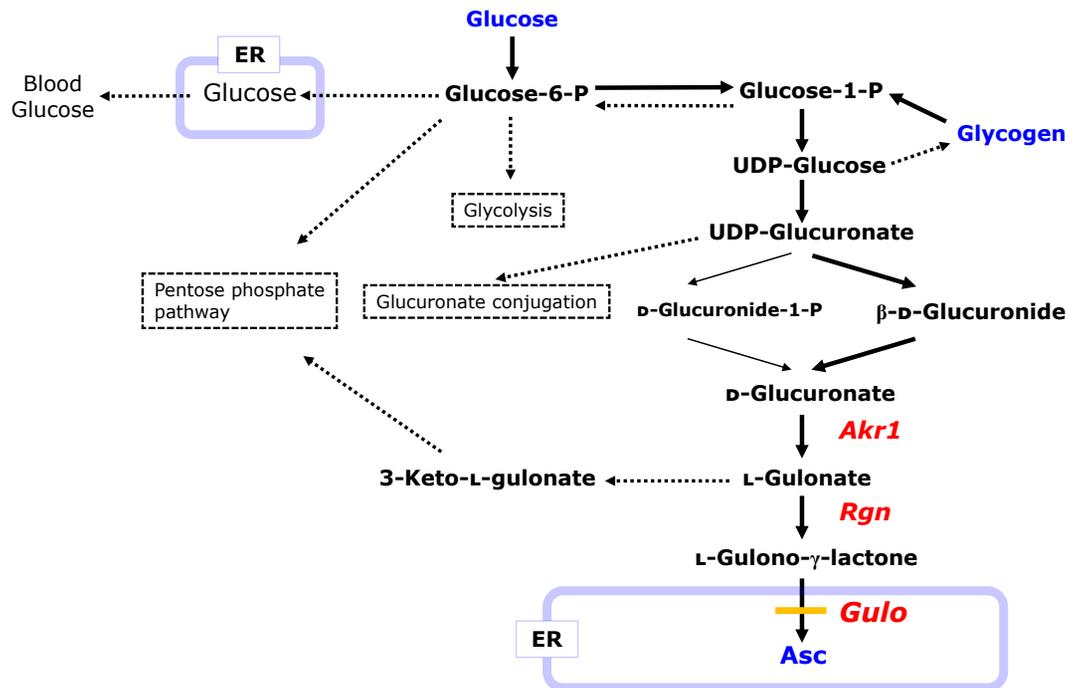
Fig. 2. Pathways for Asc synthesis and other related carbohydrate metabolism pathways from glucose. Solid lines indicate carbon flow to Asc synthesis. Dotted lines indicate flows that simultaneously or occasionally procced depending on the physiological conditions of the animals. Gulo in the ER is absent in incompetent animals of the Asc synthesis.

precursor for glycogen synthesis and Asc synthesis.[32]

UDP-glucose is oxidized to UDP-glucuronate, which then flows to the Asc synthesis or is used for glucuronate conjugation for purposes of detoxification.[33] In the biosynthesis of Asc, D-glucuronate is produced from UDP-glucuronate via either D-glucuronide-1-phosphate (D-glucuronide-1-P) or D-β-glucuronide. The resulting D-glucuronate is reduced by members of the aldo-keto reductase family (Akr)-1[34] to L-gulonate, which is then converted to either L-gulono-γ-lactone for Asc synthesis or 3-keto-L-gulonate leading to the pentose phosphate pathway. The conversion of L-gulonate to L-gulono-γ-lactone is catalyzed by gluconolactonase (Rgn). L-Gulono-γ-lactone is transported into the endoplasmic reticulum (ER) lumen and eventually oxidized to Asc by the catalytic action of Gulo.[32]

Glycogen is an energy storage form of carbohydrate, and glycogenolysis, upon stimulation with a hyperglycemic hormone such as glucagon, provides glucose to the body via the blood. In this response, glucose-1-P is released by glycogen phosphorylase, isomerized to glucose-6-P by phosphoglucomutase, and finally dephosphorylated to glucose by glucose 6-phosphatase (G6Pase) in the lumen of the ER (Fig. 3). Glucagon, the levels of which are increased in blood under hypoglycemic conditions and the principle hormone for maintaining blood glucose levels, stimulates glycogenolysis but does not activate Asc synthesis.[35] There is a large body of evidence to support the conclusion that Asc is also synthesized upon activation of glycogenolysis in competent animals such as rodents.[2,32] For example, Asc synthesis is reportedly stimulated under glutathione depletion by either inhibiting glutathione synthesis[36] or activating glutathione conjugation in rodents,[35,37] although blood glucose levels remain unchanged by these treatments.[37,38] For this purpose, glucose-1-P is converted into UDP-glucose only for Asc synthesis. Thus, there appears to be mechanisms that preferentially force glycogen-derived glucose-1-P to flow toward Asc production. These phenomena imply the existence of a line of communication between the glutathione system and the Asc system, but the molecular basis for this crosstalk is not clearly understood.[32] We will revisit this issue in a later section and discuss it from evolutional aspect of Asc synthesis.

It is also of note that oxygen consumption by Gulo produces hydrogen peroxide as a byproduct, which may be reduced to water during the oxidative folding of secretory proteins. Although an excessive production of hydrogen peroxide may trigger oxidative stress and eventually ER stress partly due to stimulating the misfolding of proteins,[39] a causal connection between the hydrogen peroxide released by the Gulo reaction and ER stress is obscure. Upon the dysfunction of Gulo in Asc-incompetent animals, upstream intermediary compounds of L-gulono-γ-lactone tend to flow toward other metabolic pathways and may be metabolized in different ways.

Asc Transport and the Reductive Recycling of DHA

The resulting Asc is released into the blood and delivered to organs. Asc is taken up by cells via a member of the Na⁺-dependent vitamin C transporter (SVCT) family that is driven by the Na⁺ gradient (Fig. 4).[40,41] On the other hand, DHA is taken up via facilitative glucose transporters [42] that include GLUT1, GLUT3, and GLUT4[43,44] as well as others.[45] Given presence of abundant levels of Na⁺ in the extracellular fluid and that Asc is the major form in the blood and extracellular milieu,[46] SVCTs dominantly contribute to the uptake of Asc by cells and generate large concentration gradients.[47] Consequently, Asc levels are maintained at high levels in cells, 0.5 to 10 mM compared to 50 to 80 µM in blood plasma.[48] The distribution of the two isoforms of the Asc transporters is discrete: SVCT1 (also referred to as solute carrier family 23 member 1; Slc23a1) in the intestine, kidney and liver cells, whereas it is SVCT2 (Slc23a2) in the brain, eye and other organs. SVCT1 which is expressed in the intestine and kidney, is involved in the absorption of Asc from nutritional sources and reabsorption from filtrate,
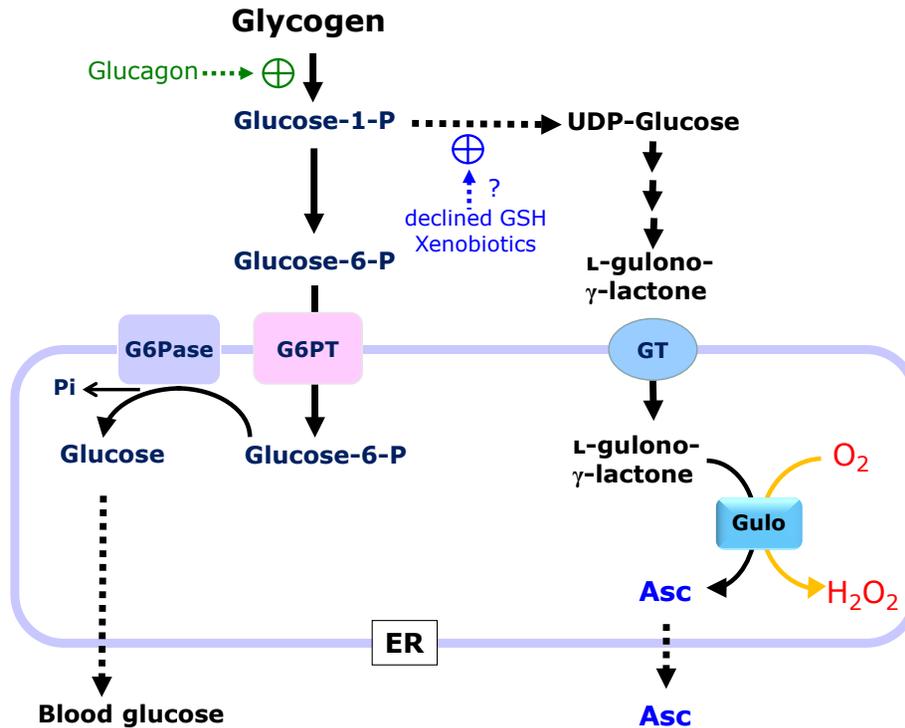
Fig. 3. Roles of ER in the production of glucose via glucose 6-P and Asc via UDP-glucose from glycogen in rodents. Glucose 1-P from glycogen is the common precursor for plasma glucose and Asc. Glucagon stimulates glycogenolysis but is not involved in UDP-glucose formation. In the meantime, xenobiotics and glutathione consumption stimulate the production of UDP-glucose and the consequent Asc synthesis, but not blood glucose. Dephosphorylation of glucose 6-P by glucose 6-phosphatase (G6Pase) releases glucose in the ER. Gulo oxidizes ʟ-gulono-γ-lactone to Asc by means of the oxidation of molecular oxygen, leading to the production of hydrogen peroxide.
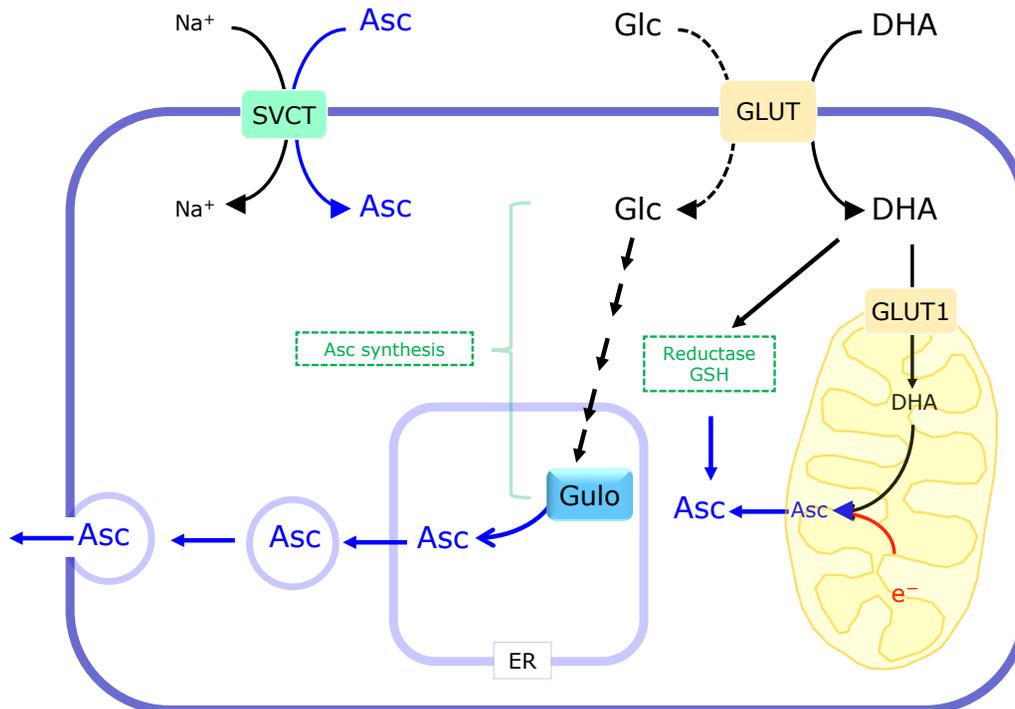
Fig. 4. Transport system for Asc and DHA and the reductive recycling of DHA. Asc is transported via the SVCT which is driven by a Na$^+$ electrochemical gradient. Some types of GLUT are responsible for DHA uptake as well as glucose (Glc) uptake. DHA is reduced to Asc by either enzymatic reactions or an electron donation from glutathione. DHA is partly transported into mitochondria via GLUT and reduced to Asc by electrons from the respiratory chain.

respectively. SVCT2 is expressed in the brain and accounts for the uptake of Asc from the cerebrospinal fluid,[49] and Asc has significant roles in neuronal functions, can be therapeutic target of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and Huntington's disease.[50]

The birth of a mouse with a deficiency in SVCT2 is accompanied by respiratory failure and intraparenchymal brain hemorrhage and dies within a few minute after birth.[45] An SVCT2 deficiency also impairs adrenal chromaffin cell function and decreases the tissue adrenalin and noradrenalin and plasma levels of corticosterone, although these appear to not be the cause for lethality.[51] Regarding cellular organelles, next to the ER, mitochondria are rich in Asc. SVCT2 is reportedly present in the mitochondrial membrane and appears to be responsible for the mitochondrial transport of Asc.[52]

Genetic ablation demonstrates the roles of SVCT1 in renal Asc reabsorption and perinatal survival.[53] Although SVCT1 is generally believed to be responsible for the intestinal absorption of Asc, its transport across the intestines is normal in SVCT1-knockout (KO) mice,[53] indicating the presence of an alternative transport mechanism. Functional analyses of the glucose transporters GLUT2 and GLUT5-12 that are expressed in Xenopus oocytes indicate that GLUT2 and GLUT8 are able to take up DHA.[54] Hence, DHA is taken up from intestinal fluids via GLUT2 and GLUT8, and after reduction to Asc, is recruited through the liver. Asc is abundantly present in the central nervous system. SVCT2 is highly expressed and appears to function in the cortex and cerebellum of the fetal rat brain.[55] However, the blood-brain barrier may interfere with the absorption of hydrophilic compounds, including Asc. GLUT1, which is expressed on endothelial cells at the blood-brain barrier, appears to transport DHA into the brain and is responsible for supplying Asc to neurons.[56] Asc has multiple roles in the brain, so please refer to recent review articles[18,50] concerning details regarding the regulation of Asc homeostasis and functions. Because the mouse can synthesize Asc, the ablation of these transporter genes under a genetic background with defected Asc synthesis may cause the production of different phenotypes.

DHA is partly reduced by glutathione,[20] and at a more accelerated rate, by that utilize an electron from glutathione or NADH/NADPH. NADH-cytochrome b5 reductase is one such enzyme that is responsible for the reduction of DHA,[57,58] but there appears to be more enzymes involved in its reductive recycling.[59,60] For example, protein disulfide isomerase (PDI) catalyzes the reduction of DHA in conjunction with disulfide bond formation in proteins within the ER, which, as viewed from the opposite side, implies that DHA plays a role in oxidative protein folding.[61] DHA is incorporated into mitochondria via GLUT1 which is localized on the inner mitochondrial membrane[62] and is then reduced to Asc by an electron from either glutathione[63] or the electron transport complex III.[64] Upon reduction, Asc may be transported out of mitochondria via SVCT2.

Asc as a Cofactor for Non-heme Iron Dioxygenases

In addition to antioxidation, another well-recognized action of Asc is to donate electrons for enzymatic processes that require reducing equivalents for the production of a variety of compounds, such as collagen, monoamines, and steroid hormones.[5,65] Asc is a cofactor for α-ketoglutarate (αKG)-dependent non-heme iron dioxygenases that include prolyl 4-hydroxylase for collagen synthesis and a unique prolyl hydroxylase for HIF-1α.[7,66] The prolyl hydroxylases utilize Asc, molecular oxygen and αKG as co-substrates for proline hydroxylation and result in the formation of DHA, carbon dioxide and succinate (Fig. 5A).

Collagens are the most abundant proteins in the body,

constituting ~30% of the total protein mass in the human body, and the proline residues are highly hydroxylated (~100 in each molecule).[67] Asc is a well-established electron donor for the prolyl 4-hydrolase reaction that is required for the maturation of collagen. Scurvy is a disease that is accompanied by aberrant collagen synthesis and is caused by a Asc insufficiency.[68] On the contrary, the excessive production of collagen, notably by myofibroblasts, may cause fibrosis under pathological conditions and result in tissue remodeling.[69] Vertebrates produce three types of collagen prolyl 4-hydroxylase that function in the ER where disulfide bond-mediated oxidative protein folding occurs (Fig. 5B). Prolyl 4-hydroxylases consist of a unique catalytic α-subunit, which is responsible for the actual proline hydroxylation, and a common β-subunit, which is identical to PDI.[66] PDI alone functions as an independent enzyme that plays roles in the oxidative folding of general secretory proteins in the ER as well as in the restoration of erroneous disulfide bonds.[70] Hence, prolyl 4-hydroxylases may be the self-contained enzyme that participates in the maturation of collagen. The requirement of PDI as a subunit for prolyl 4-hydroxylases may be associated with the lethality in *PDI*-KO mice due to the inability to synthesize collagen.[71] While Asc supports collagen synthesis by donating an electron for proline hydroxylation, DHA may be involved in the oxidative folding of collagen and other secretory proteins by accepting an electron via PDI.[61] However, because the rate of PDI-medicated reduction of DHA is not very rapid,[72] the extent of the contribution of DHA to oxidative protein folding is obscure.

Animal Models with Defected Asc Synthesis

About 60 years after the discovery of Asc by Szent-Györgyi,[1] genetic defects in the human gene encoding Gulo were characterized.[13] Among rodents, the guinea pig also carries an evolutionary defect in the *Gulo* gene[73] and has been utilized as a model animal for Asc function *in vivo*.[74] A natural mutant the Wistar rat strain, osteogenic disorder Shionogi (ODS), also carries a mutation in *Gulo* and develops scurvy,[75] despite it being less popular as a pathological model compared to the guinea pig. Since the mouse is the most popular laboratory animal, a huge amount of data on gene function and metabolism has accumulated. Thus, the mouse with a genetic ablation of the specific gene for Asc synthesis would be more advantageous as a pathological model animal.

The *Gulo*-KO mouse, which exhibits pathological conditions similar to scurvy in humans, was established by the homologous recombination of the gene[76] and has been employed successfully to study the physiological action of Asc.[77] The penultimate reaction in the Asc synthesis, the dehydration of L-gulonate to L-gulono-γ-lactone, is catalyzed by Rgn. The gene product of Rgn was found to be identical to the senescence marker protein 30.[78] The preceding reaction of Rgn is the NADPH-dependent reduction of D-glucuronic acid to L-gulonate.[79] This process is catalyzed by two closely related enzymes, namely, aldehyde reductase (Akr1a) and aldose reductase (Akr1b) in the mouse, which are members of the aldo-keto reductase superfamily.[34,80] The contribution of Akr1a and Akr1b in the mouse is 85–90% and 10–15%, respectively. Even in the presence of ~15% Asc for the wild-type (WT) mouse, the *Akr1a*-KO mouse starts dying after 20 weeks and does not survive beyond one year, whereas Asc supplementation extends their life-span to a considerable extent.[81] On the other hand, the *Akr1b*-KO mouse shows no such phenotypic abnormality.[34] These genetically modified mice have been extensively employed in studies of the roles of Asc from the standpoints of physiology and pathophysiology.[77,82]

While the oxidative conversion of L-gulono-γ-lactone to Asc appears to be the sole reaction catalyzed by Gulo, additional functions may reside in other gene products that are involved in
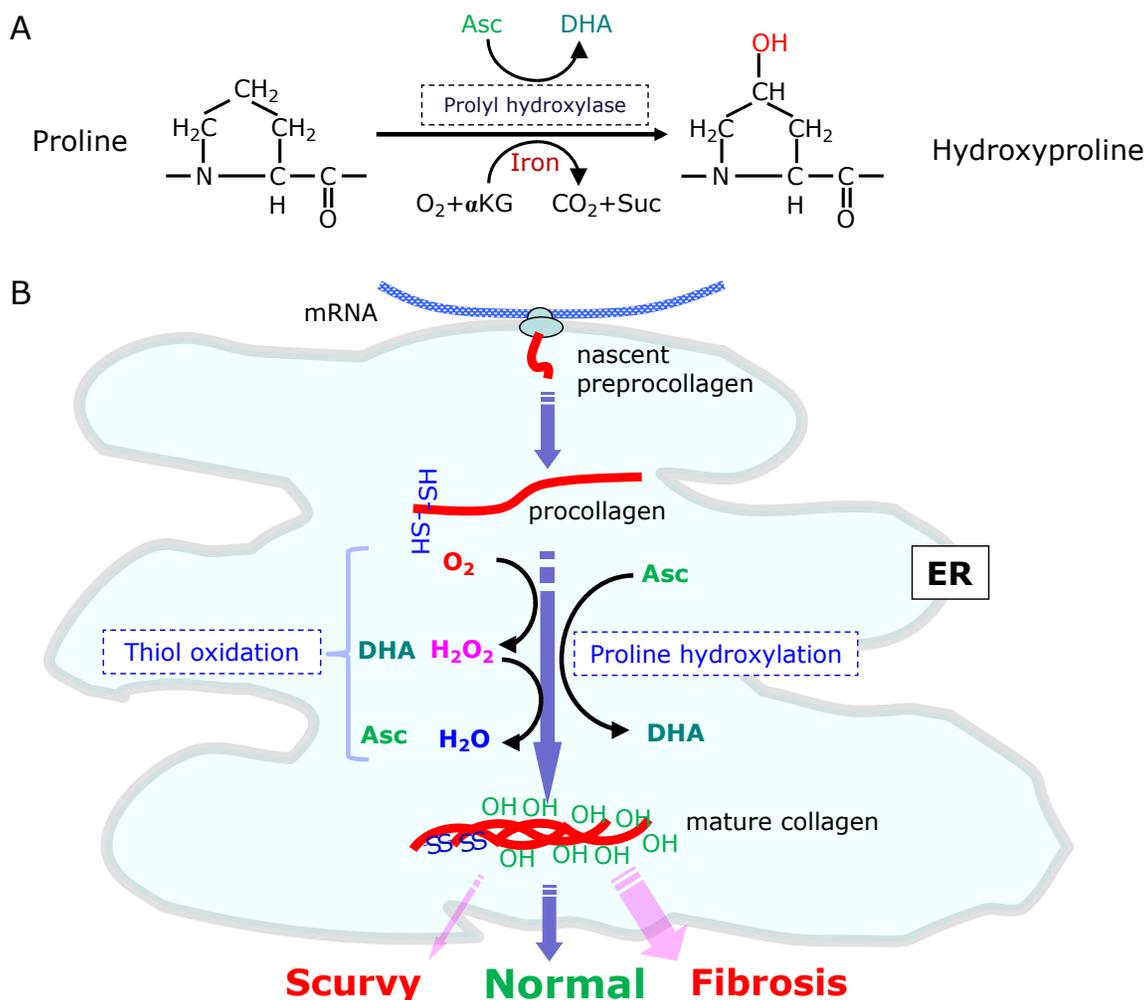
Fig. 5. Proline hydroxylation and the maturation of collagen in the ER. (A) Proly hydroxylase catalyzes the oxidative conversion of proline to hydroxyproline by employing Asc, molecular oxygen and α-ketoglutarate (αKG) and results in the production of DHA, carbon dioxide (CO_2) and succinate (Suc). (B) Oxidative folding and the prolyl hydroxylation of collagen proceed in the ER lumen. Procollagen and other secretory proteins undergo oxidative folding by means of ERO1 and other thiol oxidases. The resulting procollagen is hydroxylated at proline residues by prolyl hydroxylase coupled with Asc oxidation at multiple positions (~100 proline residues in a molecule) and then secreted out of the cell. DHA may also be involved in thiol oxidation. Both an Asc insufficiency and abundance may cause pathological conditions, such as scurvy and fibrosis, respectively.

the synthesis of Asc. Notably in the case of Akr1a/1b, multiple carbonyl compounds can serve as substrates for these enzymes,[83] and hence, an *Akr1a*-deficiency may result in the development of novel phenotypes beyond those expected from the Asc depletion, as has been observed in some reports.[84,85] Moreover, such a genetic deficiency may also change the upstream metabolic flow and result in the development of phenotypes that are not directly associated with Asc functions. To attribute the corresponding phenotype to an Asc deficiency, a simple but reliable criterion is based on observations of whether ot not Asc supplementation could rescue the animals. EDTA, a chelator of divalent cations is sometimes included in supplemental Asc solutions in order to suppress the spontaneous oxidation of Asc. However, because the inclusion of EDTA may cause deficits in metal absorption by metal chelation, notably iron, in the intestine and cause adverse effect in animals, researchers must take this into account in investigations of Asc-involved biological actions. Considering the sufficient absorption of DHA by intestinal cells via GLUT,[53,54] in our studies, we do not include a metal chelator in the Asc solution but, instead, change the Asc-containing water every 2–3 days to avoid the formation of such artifacts.

The genetically modified mice are particularly advantageous

in investigating the physiological consequences of an Asc deficiency. For example, when brain slices of the *Rgn*-KO mouse are subjected to the hypoxia-reperfusion treatment, Asc effectively suppresses superoxide production as judged by measuring the fluorescence of lucigenin.[86] Asc deprivation causes pulmonary emphysema with decreased collagen synthesis in the *Rgn*-KO mouse.[87] A double deficiency of *Rgn* and *SOD1*, which encodes the superoxide dismutase isoform which is abundantly present in cytosol and to a more limiter extent in mitochondria, shows premature death and aberrant lipid metabolism in the absence of Asc supplementation.[88] We recently found that a double deficiency of *Akr1a* and *SOD1* causes inevitable death in the mice within several weeks after the Asc supplementation is ceased.[25] Among the organs examined, oxidative lung injury appears to be the primary cause for the death of the double KO mouse. These observations strongly support the requisite role of Asc in eliminating superoxide from the body, notably in the lung that is continuously exposed to hyperoxygenic conditions compared to other organs.

To the contrary, an Asc deficiency due to genetic ablation does not always validate the conclusion that Asc is required for enzymatic reactions that have been demonstrated in *in vitro*

assays. For example, normal collagen status is observed in the *Gul*-KO mouse despite the complete absence of Asc and eventual death.[89] In the *Rgn*-KO mouse, the collagen content is in the skin is not affected, but the levels in the lung are decreased.[90] When the *Akr1a*-KO mouse is subjected to unilateral ureteral obstruction, a pathological model for kidney fibrosis, collagen production is elevated in both *Akr1a*-KO and WT mice, but no evident difference is observed between the two genotypic mice.[81] In other instances, while the levels of noradrenaline and adrenaline are low in adrenal glands of *Rgn*-KO mice,[91] these levels are not changed in the brain of the *Akr1a*-KO mouse.[92,93] Instead, the levels of adrenaline and noradrenaline are somewhat elevated in the *Gulo*-KO mice compared to WT mice in a water restraint stress model.[94] These observations imply that Asc is not the sole electron donor for these enzymatic systems, but that other redox-active compounds may compensate for the Asc-insufficient situation. Studies employing Asc-deficient animals, hence, demonstrate that Asc is actually involved in multiple reactions, but, in some instances, redox compounds other than Asc appears to be involved. Thiol compounds, more specifically, glutathione, are promising alternatives to Asc due to their abundance and electron donating ability.
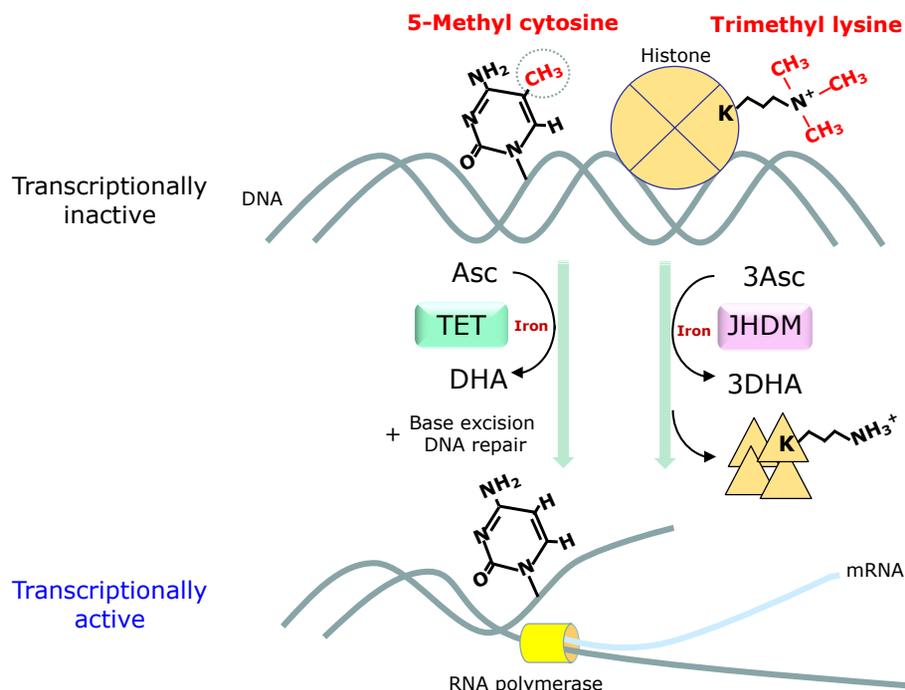
Epigenetic Action of Asc

Reactive oxygen species (ROS) cause base modifications in DNA and lead to genetic mutations, which may ultimately induce cancer development. 8-Oxo-7,8-dihydroguanine (8-oxoG), is the most frequently modified base, and consequently causes transversion of the GC pair to a TA pair. Hence, the DNA repair glycosylase OGG1 that excises 8-oxoG plays a pivotal role in the suppression of ROS-induced mutation and cancer.[95] While the protective effects of Asc against oxidative damage appear to largely dependent on its antioxidant ability,[96] recent studies have revealed that Asc regulates gene expression via a novel

mechanism. The methylation of DNA and histones contributes to the epigenetic regulation of gene expression. DNA methylation at the 5-position of cytosine (5mC) is catalyzed by DNA methyltransferases (DNMTs). While DNMT3 family enzymes are responsible for *de novo* methylation and newly establishing the 5mC in DNA, DNMT1 is involved in the maintenance of 5mC in the daughter strand during DNA replication.[97] In these cytosine methylation reactions, *S*-adenosylmethionine, which is produced in the process of methionine metabolism, serves as a methyl group donor. Cytosine methylation catalyzed by DNMTs tends to cause chromatin compaction, leading to heterochromatin formation and consequently the silencing of some genes.

In the meantime, histones undergo a variety of modifications, which includes methylation of lysine and arginine residues, acetylation, and ubiquitination, and also affect gene expression epigenetically.[98] Concerning Asc functions, the methylation status in lysine residues is a subject of interest. Histone H3-specific methyltransferases were initially found to methylate lysine 9.[99] Thereafter, several specific histone lysine methyltransferases and histone lysine demethylases were identified.[98] While lysine acetylation is largely correlated with the transcriptional activation of genes, the methylation of lysine results in either transcriptional activation or suppression, depending on the position and degree of methylation in the histones.

Because Asc modulates gene expression epigenetically, it has attracted the attention of researchers in the fields of stem cell and cancer research. For example, Asc in fact enhances the generation of induced pluripotent stem cells (iPSCs) from both humans and mice.[100] Asc is a cofactor for Fe^{2+}- and αKG-dependent dioxygenases that involve the demethylation of DNA and histones (Fig. 6).[9,101,102] The ten-eleven translocation (TET) methylcytosine dioxygenase is the enzyme that catalyzes the conversion of 5mC into 5-hydroxymethylcytosine[103] and then 5-carboxylcytosine, which

Fig. 6. Epigenetic action of Asc towards gene activation. Electron donation from Asc to TET reaction supports the conversion of 5-methyl cytosine to 5-hydroxyl cytosine and then 5-carboxylcytosine, which is consequently replaced to cytosine by the base excision DNA repair system. Jumonji C (JmjC)-domain-containing histone demethylases (JHDMs) removes methyl groups from lysines in histones. Demethylation of DNA and histones may be able to activate consequent gene expression. Only concept of the reactions are presented schematically.

is consequently replaced with an unmodified cytosine via a base excision DNA repair system.[104] Asc was first found to induce TET-dependent DNA demethylation in embryonic stem (ES) cells.[105] The TET-catalyzed reaction is accompanied by the conversion of Fe^{2+} to Fe^{3+}. The electron-donating nature of Asc supports the reductive recycling of Fe^{3+} to Fe^{2+}. Consequently, genes that have been epigenetically silenced by the methylation of cytosine undergo reactivation by Asc-supported TET reactions.[8]

The Jumonji C-domain-containing histone demethylase 1 (JHDM1) is the first identified histone demethylase that specifically demethylates histone H3 at lysine 36 (H3K36) and is conserved from yeast to humans.[106] JHDM1a/1b has been demonstrated to be the Asc-dependent H3K36 demethylase in mouse embryonic fibroblasts during reprogramming into iPSCs.[107] As the end of this reaction, trimethyl lysine is converted back to lysine in conjunction with the oxidation of three Asc molecules to DHA. Currently, the family contains approximately 20 proteins that catalytically remove methyl groups from methyl lysine with the aid of Asc.[108] Through the epigenetic remodeling of chromatin, an Asc deficiency would be expected to affect a variety of physiological processes, including development, differentiation, and aging and be consequently involved in the development of pathological conditions in animals.

Reactions of Asc in Prevention and Treatment of Cancer

An early trial with high-dose Asc in the treatment of caner patiens resulted in beneficial action,[109,110] but controversial results were reported in later studies.[111,112] Thus, the use of Asc in the treatment of tumor growth has been a subject of debate since the hypothesis was first announced by Linus Pauling.[11] Asc, in fact, exerts opposing effects, either carcinogenic or tumoricidal, in cancer, which largely depends on the redox status of the microenvironment. Iron can contribute to carcinogenic processes in several ways.[113] Fe^{3+} bound to ferritin is not toxic in the bound form, however, due to its electron-donating ability, Asc causes the free form of Fe^{3+} to be converted to Fe^{2+}. Fe^{2+} supplies electrons for the reductive conversion of hydrogen peroxide to hydroxyl radicals, which are highly reactive, oxidize a variety of molecules including DNA and results in the mutation of genes. Thus, Asc, in conjunction with free iron, may result in elevated ROS levels and exhibit carcinogenicity. By contrast, Asc eliminates ROS as an antioxidant and hence prevents their mutagenic action.

Recent studies have unveiled the anti-tumorigenic action of Asc in which serveral mechanisms are involved. The results of animal experiments indicate that Asc is beneficial in suppressing carcer development; the growth and metastasis of transplanted tumor cells observed in *Gulo*-KO mice were decreased by supplementation of Asc at physiological concentrations.[114–116] Asc appears to suppress chemical carcinogenesis as well. The administration of *N*-nitrosodiethylamine causes the complete death in *Rgn*-KO mice within 4 months and therefore provides no information regarding tumorigenesis.[117] Although *Akr1a*-KO mice are also mostly dead within a half-year, a few surviving mice developed extreme hepatocellular carcinomas, and Asc-supplementation markedly suppressed hepatocellular carcinoma development.[118] Because methylation of cytosine in DNA and lysine residues in histones epigenetically suppresses the expression of genes that include tumor suppressor genes, their Asc-mediated demethylation may lead to their reactivation.[9,98] Thus, the epigenetic actions of Asc would very likely contribute to the anticarcinogenic action.

Electron donation from Asc to a specific prolyl hydroxylase is responsible for the regulation of HIF-1α, which is a transcription factor and the master regulator of genes whose expression increases oxygen availability.[119] HIF-1α levels are increased in many solid tumors and reportedly support their development because the inner mass of solid tumor tends to be hypoxic. The constitutive activation of HIF-1α, hence, induces genes that increase the supply of oxygen and energy production, leading to the survival and proliferation of tumor cells. Asc is utilized for the hydroxylation of specific prolyl residues in HIF-1α, leading to the generation of a binding site for the von Hippel-Lidau (pVHL) tumor suppressor protein, which is made up of a ubiquitin ligase complex (Fig. 7). After polyubiquitination, HIF-1α undergoes degradation by proteasomes. As a result, Asc in the presence of oxygen may restrict the proliferation and survival of tumor cells.[7,120]

There is an emerging body of evidence to indicate that pharmacologically high-doses of Asc acts as a potential therapeutic agent for some types of cancers.[120–122] Because the plasma Asc level is strictly controlled; ≈80 μM,[123] the oral administration of a high dose of Asc does not reach effective levels. To administer a high dose of Asc for therapeutic purposes, intravenous administration is required; e.g. the intravenous administration of 10 g of Asc to cancer patients results in 1–5 mM concentrations in the plasma.[124,125] Upon Asc treatment, mitochondrial oxidative metabolism is stimulated and this increases the production of superoxide and hydrogen peroxide, which then disrupts iron metabolism in cancer cells.[126] The resulting labile iron together with hydrogen peroxide sensitizes cancer cells, notably non-small-cell lung cancer and glioblastoma, to pharmacological Asc doses in a redox-active manner. The synergistic action of Asc and iron coordinately trigger lipid peroxidation, which results in ferroptosis in cancer cells.[30,127]

In the meantime, colorectal cancer cells bearing mutations in *Kras* and *Braf* are selectively killed by the action of a high-dose of DHA.[128] The strategy for killing cancer cells by DHA is different from that for Asc. In this anti-tumor action, glutathione is oxidized in the process of the reduction of DHA to Asc, which results in an elevation in ROS levels. Oxidative DNA damage then stimulates the poly-(ADP-ribose) polymerase-mediated consumption of NAD^+, leading to energy depletion and the eventual death of cancer cells. In fact, clinical trials using cancer patients with a fasting-mimicking diet and vitamin C appears to be promising therapeutics for the treatment of colorectal cancer and possibly other tumors with *KRAS* mutations.[129] Thus, Asc at physiologic concentrations prevents tumorigenesis, whereas both Asc and DHA at pharmacological doses exert tumoricidal actions.

Evolutional Aspect of Asc Synthesis

Animals inherently do not possess some genes that are responsible for certain types of metabolism, which leads them to be incapable of synthesizing the corresponding compounds, e.g., essential amino acids and most vitamins. Contrary to these, Asc-incompetent animals had originally possessed functional *Gulo* but have lost its function due to mutations in the course of evolution.[13] Thus, scenario dealing with Asc synthesis is not comparable to those of essential amino acids or other vitamins. If sufficient Asc is not ingested, our bodies suffer disadvantages due to the inability to produce Asc. Nevertheless, Asc-incompetent animals, including primates, the guinea pig, the bat and passeriformes birds have actually survived.[3,14] In fact, these Asc-incompetent animals have even prospered. The teleost fish, which is the most abundant group in the modern fish family, also cannot synthesize Asc. Different from other Asc-incompetent animals, however, no relic of *Gulo* has been found in the teleost fish genome by searches of gene data bases.[15] Because the ancestral species can synthesize Asc, it is postulated that *Gulo* was completely eliminated during the
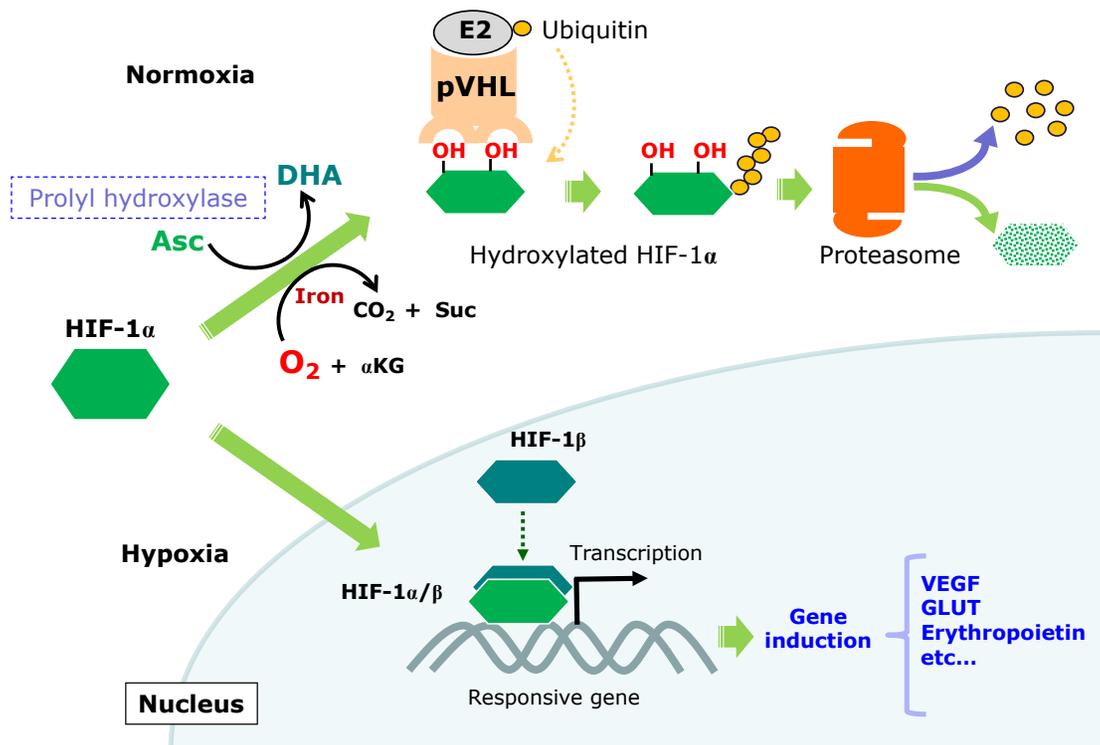
Fig. 7. Roles of Asc in controlling HIF-1α action. Under normoxic conditions, prolyl hydroxylase catalyzes the hydroxylation of specific proline residues in HIF-1α, which leads to polyubiquitination by the ubiquitin ligase activity of pVHL, followed by degradation via proteasomes. Under hypoxic conditions, the short oxygen supply suppresses proly hydroxylation, which results in the stabilization of HIF-1α. After translocation to the nucleus, HIF-1α dimerizes with HIF-1β, which is constitutively present largely in the nucleus, and together with other transcriptional factors, activates transcription of corresponding genes, such as erythropoietin, vascular epidermal growth factor (VEGF), and GLUT.

course of evolution of the teleost fish.[3] It is of note that Asc incompetence in these animals is caused solely by mutations in *Gulo*, which can be attributed to independent events during the evolutionary process.

The reason why Asc-incompetent animals are no longer able to synthesize Asc but remain prosperous is not understood and this constitutes an interesting issue from the standpoint of evolution and nutritional biochemistry as well. The most common explanation for the inability to synthesize Asc in primates is as follows; primates can climb trees and therefore gain access to sufficient amounts of Asc-rich foods, notably fruits, and hence do not need to synthesize Asc by themselves.[3] This notion may sound reasonable if the animals do not develop serious damage to their health caused by an Asc insufficiency. In fact, however, Asc exerts a variety of important roles in animal physiology, and an Asc insufficiency causes an unhealthy situation, and in severe cases, fatal conditions such as scurvy.[68] Most genetic mutations, as are found in hereditary diseases, are disadvantageous to the host animals, and hence, tend to be eliminated from the gene pool by death or failure in reproduction. When the mutation is neutral, meaning the mutation does not influence the survival of animals, both the original and mutated genes can be maintained in the gene pool of the animal species because there is no selection pressure on them. However, no active *Gulo* remains among primates, which suggests that either *Gulo* itself is disadvantageous or the mutated *Gulo* is more advantageous for these animals. The question therefore arises as to what mechanism has actually made the incompetent animals lose their ability to synthesize such an important nutrient by assuming the risk of a deficiency disorder?

Gulo is an ER-resident enzyme that converts L-gulono-γ-lactone to Asc by an oxidation reaction in conjunction with the reduction of molecular oxygen to hydrogen peroxide. Because even a very high dose of Asc is not toxic to animals in the general sense,[123] the presence of excessive levels of Asc does not appear to be harmful to animals. Hydrogen peroxide, a byproduct of the Gulo reaction, is released into the ER lumen, and once excessive levels are reached, it may exert unfavorable events due to its oxidizing power.[130] If Gulo-derived hydrogen peroxide is toxic enough, Asc-competent animals would be more vulnerable to the ER stress compared to the incompetent animals. In fact, the ER is the most hydrogen peroxide-rich organelle and this situation appears to be caused by the action of endoplasmic reticulum oxidoreductin 1 (Ero1), which is involved in oxidative protein folding in the ER and produces large amounts of hydrogen peroxide during the course of the enzymatic reaction.[131,132] Several peroxidases are localized in the ER and utilize hydrogen peroxide for thiol oxidation during oxidative protein folding in the ER.[70] Peroxiredoxin 4 (Prdx4) is one such thiol oxidase that oxidizes thiols in the PDI family or newly synthesized proteins to disulfides.[133] The genetic ablation of *Prdx4* alone shows no obvious changes in the mouse,[134] but a mouse with a double deficiency of *Prdx4* and *Ero1* develops atypical scurvy due to Asc consumption and insufficient collagen production.[135] Thus, an Asc insufficiency rather than excessive hydrogen peroxide appears to cause deleterious effects in the ER. This notion is also supported by the finding that an insufficient supply of oxygen decreases the production of hydrogen peroxide and the capacity for oxidative protein folding, which consequently leads to ER stress.[31] Moreover, no reports have yet shown that *Gulo*-derived hydrogen peroxide alone induces ER damage, although excessive ROS, which is caused by a double deficiency of *Prdx4* and *SOD1*, induces ER stress and hepatic damage in mice.[136] Thus far, there is no robust
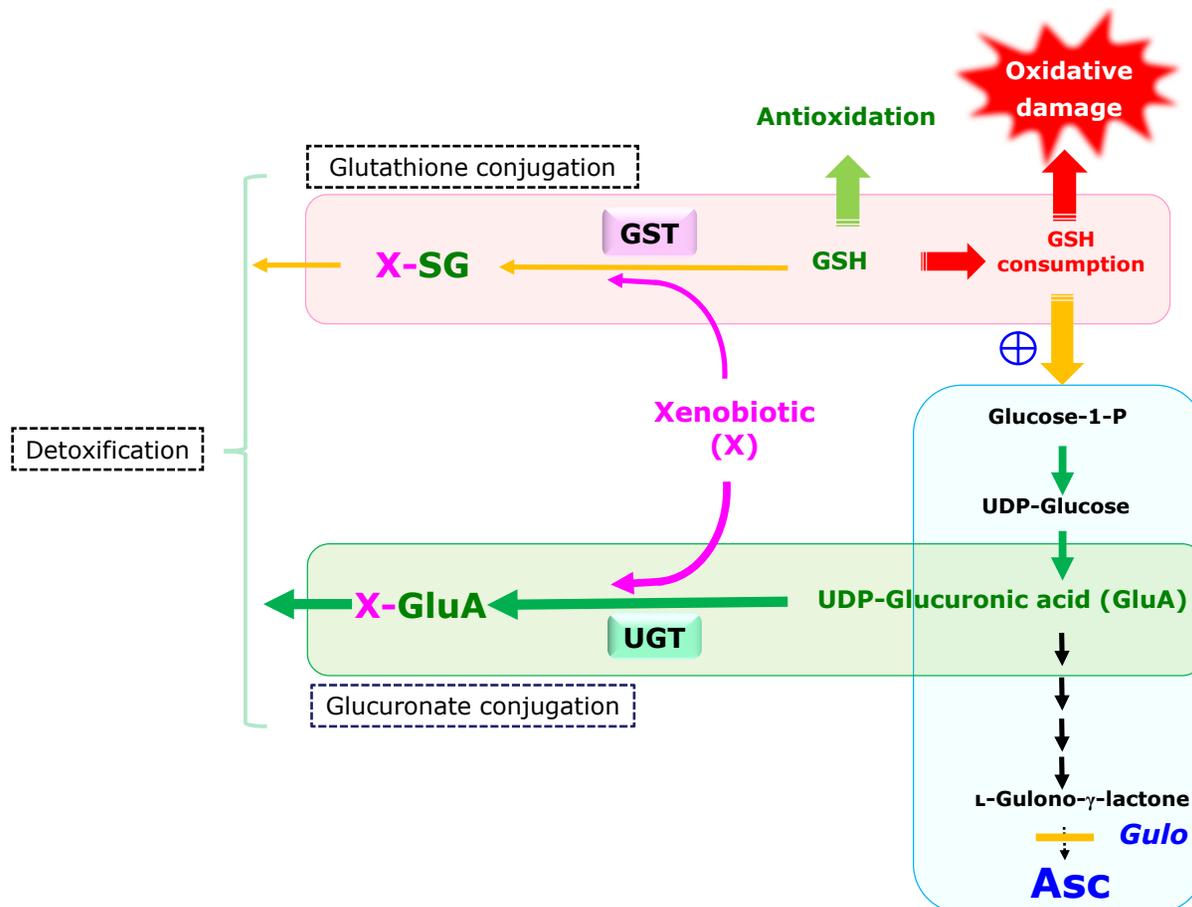
Fig. 8. Schematic representation of and crosstalk between two detoxification pathways, glutathione conjugation and glucuronate conjugation in the liver. While glutathione conjugation is catalyzed by glutathione *S*-transferases (GST), glucuronate conjugation is catalyzed by UGT. UGT may also catalyze the hydrolysis of UDP-glucuronate, resulting in the formation of D-glucuronate. Pathways for glucuronate conjugation and Asc synthesis share the metabolic processes up to UDP-glucuronate. Ablation of enzymes responsible for the downstream reaction causes the accumulation of intermediary compounds that include UDP-glucuronate. As a result, the glucuronate conjugation reaction is enhanced in animals that have a defect in Asc synthesis and preserve glutathione for antioxidation.

body of evidence to show that the Gulo-catalyzed reaction is a disadvantage in competent animals. Given that *Gulo*-deficient, Asc-incompetent animals are rather prosperous among close animal groups in the animal kingdom now, it is rational to conclude that the Asc-incompetent animals have gained an advantage in gaining prosperity in return for the loss for Asc synthesis.

Enhanced Detoxification via Glucuronate Conjugation Reactions May be Used to Rationalize the Loss of Asc Synthesis

The question still remains as to what function is gained in return for the loss of Asc synthesis and has allowed incompetent animals to become dominant during the course of evolution. Instead of focusing on the Gulo-catalyzed reaction itself, a re-examination of the upstream metabolic pathway of the Asc synthesis may provide a clue in this issue. The Asc-synthesizing pathway constitutes one of the glucose metabolism networks and the processes up to UDP-glucuronate is shared with that of glucuronate conjugation (Fig. 8). UDP-glucuronate either proceeds to form L-gulonate for Asc synthesis or becomes the substrate for conjugation reactions of xenobiotics, which is performed by UDP-glucuronosyltransferase (UGT).[33,137] Sharing the processes between the Asc synthesis

and glucuronate conjugation may explain why xenobiotics stimulate Asc synthesis. UGT itself or a closely related enzyme may be responsible for the following removal of UDP from UDP-glucuronate.[59] If UGT is identical to the enzyme, it can be regarded as the gate-keeping enzyme that dictates which pathways UDP-glucuronate takes, either glucuronate conjugation or Asc synthesis, depending on the presence or absence of xenobiotics. Upon the mutation in *Gulo*, however, UDP-glucuronate dominantly flows to the glucuronate conjugation reaction. Consequently, a loss of the Asc synthesis would result in enhanced detoxification by means of stimulated glucuronate conjugation.

While three major conjugation reactions, glucuronate conjugation, glutathione conjugation and sulfate conjugation, are involved in detoxification of xenobiotic compounds, which are mostly hydrophobic in nature, the inhibition of glutathione synthesis by buthionine sulfoximine[36] or glutathione consumption by stimulated glutathione conjugation[37] reportedly increases Asc-synthesis in rodents. In the case of the xenobiotic-mediated glutathione consumption, the accelerated conversion of UDP-glucuronate to glucuronate is a likely cause for the elevated Asc synthesis.[35] Hence, two detoxification pathways, glutathione conjugation and glucuronate conjugation, appear to be coordinately functioning from the standpoint of the detoxification of xenobiotics. Although, no metabolic pathways

are directly shared between the two conjugation reactions, these pathways are in fact under common genetic regulation. Especially Nrf2 is a redox-sensitive transcriptional regulatory factor that activates antioxidative genes, including those for glutathione synthesis, and also genes for detoxification, which includes UGT.[138] Because glutathione is also a pivotal electron donor for glutathione peroxidase reactions in antioxidation, it is conceivable that a preferential metabolic flow to glucuronate conjugation would save glutathione by providing relief for the glutathione conjugation reaction, which would lead to an augmented antioxidative capacity. Fruits are generally rich in Asc but also sometimes contain toxic compounds, notably in immature stages.[139] Considering that ancestral primates obtain food that could contain relatively high levels of toxic compounds, individuals that have acquired strong detoxification systems, by means of glucuronate conjugation, and an antioxidation system, by means of glutathione, in return for waiving Asc synthesis would have an advantage in terms of survival.

This hypothetical adaptation may rationalize prosperity of the Asc-incompetent animals. However, is there evidence for it? Our recent studies on drug-induced hepatic injuries caused by acetaminophen (APAP) may provide a clue to this issue.[140–142] APAP is a popular antifebrile and analgesic agent and is generally regarded as a safe medicine. Upon an overdose, however, APAP induces liver injury, which is a leading cause of drug-induced acute liver failure in several countries.[143] Numerous studies have been carried out and the rsults show that ingested APAP is mostly excreted via either glucuronate conjugation or sulfate conjugation[144,145] but is also partly oxidized to N-acetyl-p-benzoquinone imine (NAPQI) by cytochrome P450 (CYP) enzymes.[146] The resulting NAPQI then undergoes glutathione conjugation, which leads to the consumption of glutathione and consequent hepatic damage. The administration of N-acetyl cysteine, a precursor for cysteine and eventually glutathione, is the established treatment for an APAP overdose,[143] implying the importance of glutathione maintenance in liver homeostasis.

We employed Akrla-KO mice to examine the protective effects of Asc on APAP-induced hepatic injury. While the Akrla-KO mice are more vulnerable to high doses of APAP than the WT mice, Asc supplementation renders the Akrla-KO mouse to be markedly resistant to APAP hepatotoxicity.[140] These results alone can be simply explained by assuming that Asc exerted a hepatoprotective action via antioxidation against elevated ROS, which are elevated by the CYP reaction and the consumption of glutathione due to glutathione conjugation of NAPQI. Our most recent study, however, had some unexplainable results based on the simple antioxidant action of Asc.[142] Under conditions of an iron load, the hepatotoxicity of APAP was comparable between WT and the Akr1a-KO mice. To our surprise, however, Asc supplementation dramatically ameliorated the APAP-induced hepatic injury in the iron-loaded Akr1a-KO mice but had no effect on the iron-loaded WT mice. Because WT mice possesses both endogenously produced Asc and supplemented Asc, the Asc contents are actually higher in the WT mice than in the Akr1a-KO mouse, and hence, the results cannot be rationalized by Asc function alone. In the meantime, our metabolomic study on the Akr1a-KO mice indicated that the Akr1a-KO mice had an excessive accumulation of glucuronate (41-fold in the Akr1a-KO vs the WT mice) and its oxidized metabolite saccharate (102-fold in the Akr1a-KO vs the WT mice) in the liver.[80] These collective results imply that elevated oxidative stress due to an Asc deficiency in the Akr1a-KO mice makes them vulnerable to APAP, but the suppression of ROS production by Asc supplementation reinforces hepatic resistance, which is accomplished by efficient detoxification via enhanced glucuronate conjugation.

It should also be noted that the levels of glucuronate-conjugated APAP are actually the highest in the guinea pig among rodents that were administered APAP.[147] Guinea pig is consistently less sensitive to APAP hepatotoxicity. These circumstantial evidences support our hypothesis on the prosperity of Asc-incompetent animals; the restriction in the carbohydrate flow to Asc synthesis enhances the detoxification of APAP by stimulated glucuronate conjugation, and at the same time, preserves glutathione, which collectively leads to the KO mouse being more resistant to APAP. Thus, the preferential carbohydrate flow to glucuronate conjugation reaction due to the loss of Asc synthesis appears to be advantageous to Gulo-deficient animals. This hypothesis explains the phenomena observed in the Asc-incompetent animals and genetically modified mice but is largely based on circumstantial evidences at this moment. Thus, further experimentation will be required to verify this hypothetical mechanism for the loss of Asc synthesis.

Brief Summary

Asc is an essential micronutrient the synthesis of which primates have lost a long time ago. Because Asc exerts pivotal actions in a variety of metabolic processes and gene expression as well as functioning as an antioxidant in the body, a deficiency causes scurvy and is associated with cancer and many other diseases due to dysfunctions in physiological processes. The issue of why primates have lost synthesizing such an important molecule remains a mystery. Herein, we propose a hypothetical mechanism, which is different from the ordinary explanation; i.e., the loss of Asc synthesis due to the mutation in Gulo enhances detoxification reactions by glucuronate conjugation, which is accomplished by a preferential carbohydrate flow.

Acknowledgments

Abbreviations

Akr	aldo-keto reductase
Akr1a	aldehyde reductase
Akr1b	aldose reductase
APAP	acetaminophen
Asc	ascorbate
CYP	cytochrome P450
D-glucuronide-1-P	D-glucuronide-1-phosphate
DHA	dehydroascorbate
DNMT	DNA methyltransferase
ER	endoplasmic reticulum
Ero1	endoplasmic reticulum oxidoreductin 1
Fe^{3+}	ferric iron
Fe^{2+}	ferrous iron
Glc	glucose
glucose-6-P	glucose-6-phosphate
glucose-1-P	glucose-1-phosphate
GLUT	glucose transporter
G6Pase	glucose 6-phosphatase
Gulo	L-gulono-γ-lactone oxidase
HIF	hypoxia-inducible factor
H3K36	histone H3 at lysine 36
iPSC	induced pluripotent stem cell
JHDM	Jumonji C-domain-containing histone demethylase
αKG	α-ketoglutarate
KO	knockout

5mC	DNA methylation at the 5-position of cytosine
NAPQI	N-acetyl-p-benzoquinone imine
8-oxoG	8-oxo-7,8-dihydroguanine
PDI	protein disulfide isomerase
Prdx4	peroxiredoxin 4
Rgn	gluconolactonase
ROS	reactive oxygen species
SVCT	Na^+-dependent vitamin C transporter
TET	ten-eleven translocation
UGT	UDP-glucuronosyltransferase
WT	wild-type

Conflict of Interest

No potential conflicts of interest were disclosed.

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