

**RESTORATION OF VITAMIN C PRODUCTION IN *GULO*^{-/-} MICE
USING GENE THERAPY**

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Abstract

The effectiveness of vitamin C in treatment of cancer and heart disease is a matter of debate. While some studies show that vitamin C intake is correlated with improved clinical outcome in cancer patients and is associated with better cardiovascular health, others did not. In this thesis, we examine the biochemical and pharmacological properties of this vitamin in the hope that they will be conducive to resolving this controversy.

In Chapter 1 of this thesis, we present a compilation of three publications reviewing the current knowledge about this nutrient, including its chemical and biological properties, with focus placed on its therapeutic potentials. From these literatures, we arrived at the hypothesis that vitamin C, at pharmacological concentrations in the serum, may have mitigative effect on cancer and cardiovascular disease.

In Chapter 2 of this thesis, we examine the effectiveness of an alternative vitamin C delivery method using gene therapeutic vectors in a humanized transgenic mouse model. These mice have been rendered defective in endogenous vitamin C production by genetic knockout of gulonolactone oxidase (GULO)-encoding gene (*Gulo*), which is responsible for catalyzing ascorbic acid biosynthesis. **In an earlier study**, we constructed gene therapeutic helper-dependent adenoviral vectors (HDA_d) carrying the coding sequence for *Gulo* under either human phosphoenolpyruvate carboxykinase (PEPCK) promoter (HDA_d-PEPCK-*Gulo*) or murine cytomegalovirus(mCMV) immediate-early promoter (HDA_d-mCMV-*Gulo*). **In this study**, we sought to examine the ability of these vectors to mediate the expression of GULO and the production of ascorbic acid in human hepatocellular carcinoma (HEPG-2) and *Gulo*-knockout (*Gulo*^{-/-}) mice. We

found that HEPG-2 infected with HDAd-*mCMV-Gulo* expressed GULO, which can be readily detected in cells infected at a multiplicity of infection (MOI) of 10 viral particles per cell (vp/cell) using immuno-based blot. Immunoblot also showed that GULO expression occurred at 18 h post-infection in cells treated with HDAd-*mCMV-Gulo* at a MOI of 500 vp/cell. Vitamin C production was observed in HEPG-2 treated with HDAd-*mCMV-Gulo* as measured by HPLC-electrochemical detection (HPLC-ECD). We showed that vitamin C production is dependent on the substrate, gulonolactone, concentrations. *Gulo*-knockout mice treated with 2×10^{11} vp expressed GULO in the liver. Using HPLC-ECD, we showed that the serum vitamin C concentrations of these mice were elevated to levels comparable to those of the wild type mice (60 μ M) after 4 days of infection and were maintained at 30 μ M for the duration of the experiment (23 days and ongoing). Similar elevation was observed in urine and tissue vitamin C concentrations in vector-treated animals. In conclusion, we demonstrated here that gene therapeutic HDAd-*mCMV-Gulo* vectors are able to mediate the expression of GULO and endogenous production of vitamin C in human cells and in *Gulo*^{-/-} transgenic mice. Taken together, these findings support the feasibility of gene therapy as a novel vitamin C delivery method to achieve supra-physiological concentrations of vitamin C in the blood.

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Chapter 1: Background

1.1 Literature Review

In this chapter, I review current knowledge on vitamin C, including its molecular structure, chemistry, biological properties and its implications in health and disease. This chapter is presented as a compilation of three published works.

- Li, Y. and H.E. Schellhorn. 2007. New developments and novel therapeutic perspectives for vitamin C. *J Nutr.* 137:2171-84.
- Li, Y. and H.E. Schellhorn. 2008. *Vitamin C*. In *Nutrition and Exercise Concerns of Middle Age*. J.A. Driskell Ed., CRC Press, Taylor and Francis LLC
- Li Y, Schellhorn HE. 2007. Can ageing-related degenerative diseases be ameliorated through administration of vitamin C at pharmacological levels? *Med Hypotheses*. 68:1315–7.

1.1.1 Basic physiologic functions of vitamin C

“The medical profession itself took a very narrow and very wrong view. Lack of ascorbic acid caused scurvy, so if there was no scurvy there was no lack of ascorbic acid. Nothing could be clearer than this. The only trouble was that scurvy is not a first symptom of a lack but a final collapse, a premortal syndrome and there is a very wide gap between scurvy and full health.”

—Albert Szent-Gyorgyi Nobel Prize winner for his discovery of vitamin C

The above quotation, taken from Szent-Gyorgyi Nobel Prize acceptance speech, was remarkably prescient. Few nutritional issues have received as much attention or been as hotly debated as the human dietary requirement for vitamin C since the discovery of this vitamin in 1932. The recognition that vitamin C may be important in cancer and heart disease have spurred renewed interest in dietary vitamin C requirements with the view that amounts consumed should account for a potential therapeutic role in ameliorating chronic disease.

Our growing understanding of the mechanisms of vitamin C transport, newly-described physiological roles and potential involvement of vitamin C in cancer and heart disease have led to calls for re-appraisals of the dietary requirements for this vitamin (Carr and Frei, 1999; Levine *et al.*, 2001; Li *et al.*, 2006). In this review, I will examine the function and regulation of vitamin C transporters, and their implications in vitamin C treatment at both experimental and clinical stages. I will focus on recent evidence

supporting a potential role for vitamin C in degenerative disease, including cancer and cardiovascular disease, and will review the new developments in animal models that will be critical tools in resolving outstanding questions.

1.1.1.1 Chemical structure and purification/synthesis

Chemical structure

The most common and naturally-occurring form of vitamin C is L-ascorbic acid (**Figure 1**). It exists as a white crystal with molecular weight of 176.1 g/mol. L-ascorbic acid is freely soluble in water. Once dissolved in water, its two enolic hydroxyl groups can be sequentially deprotonated, giving rise to two pKa values, pKa1= 4.17 and pKa2= 11.57 (**Figure 1**). Vitamin C can also exist as dehydroascorbic acid (DHA) (**Figure 1**), which is the oxidized form of ascorbic acid. While structurally similar to ascorbic acid, DHA exhibits some unique physiological properties and is transported by a different mechanism, which will be discussed in detail in later sections. At physiological pH (pH 7.4), L-ascorbic acid is readily deprotonated at the first and more acidic hydroxyl group (pKa1= 4.17) to form ascorbate, a salt of ascorbic acid (**Figure 2**).

When encountering single-electron carrying compounds, including free radicals and reactive oxygen species, in solution, ascorbate can donate one electron to these compounds and itself becomes ascorbyl radical (**Figure 3**). Unlike other single-electron radicals, ascorbyl radical is extremely inert. This is attributable to its unique molecular structure in which the unpaired electron is delocalized in the pi molecular orbital of the ascorbyl ring and thus stabilized by a resonance structure (**Figure 3**). Therefore, this

simple redox reaction replaces reactive radicals with a relative unreactive compound, and as a consequence, offers antioxidant protection to the biological system.

Synthesis

In animals, vitamin C is synthesized from L-gulonolactone by the enzyme gulonolactone oxidase. Plants use a different pathway utilizing enzyme L-galactolactone dehydrogenase to synthesize vitamin C (Smirnoff, 2001).

L-ascorbic acid is synthesized from L-glucose in a series of biological reactions. In most mammals, it is produced in the liver using glucose extracted from the hepatic glycogen storage. The rate-limiting reaction of vitamin C biosynthesis is the oxidation of gulonolactone by gulonolactone oxidase (GULO).

Unlike most mammals, humans do not have functional GULO, and, as a result, cannot produce vitamin C. This nutritional defect is caused by inactivating mutations of the gene encoding GULO (*Gulo*). Because of this defect, humans are dependent on dietary vitamin C intake for metabolic needs.

1.1.1.2 Basis for biochemical deficiency in humans and other primates.

In most mammals, vitamin C is normally synthesized from glucose. In mammals that lack the terminal biosynthetic step, the precursor is decarboxylated to form xylulose that can be used in other synthetic pathways. Primates and guinea pigs independently lost the ability to synthesize vitamin C 40 million years ago (Hu and Willett, 2002). The gene has since become a so-called pseudogene (a highly-mutated gene that no longer codes for a functional protein). Reasons for the evolutionary loss of this function have been the

subject of considerable speculation (Nandi *et al.*, 1997). In an evolutionary sense, the loss likely had little effect on the reproductive success of primate populations since the amounts of vitamin C required for growth are very low and could be easily obtained in the diet. It has been estimated that most non-human primates easily obtain 1-2 grams per day in their diet (Milton, 1999). In contrast, humans generally consume significantly less because they consume fewer foods that are rich in vitamin C and also because cooking food inactivates vitamin C. As a result, even in North America, many individuals do not obtain even the relatively low levels (~60 mg per day) recommended by the Food and Drug Administration (FDA) (Ames, Gold, and Willett, 1995). It should be noted that the revised standard, the Dietary Reference Intake (DRI), is now 90 mg per day (Anon, 2000) although even this level could be considered inadequate for satisfying dietary antioxidant needs. Primates, particularly humans in the Western world, occupy a very different world than the one in which the inactivating mutation first arose. Rather than bacterial and virus-mediated mortality, the principal causes of death in Western society today are primarily heart disease (Hu and Willett, 2002) and cancer (Ames, Gold, and Willett, 1995), both of which are influenced by diet and, relevant to this review, are suspected to be aggravated by inadequate consumption of dietary oxidants such as vitamin C (Sato and Udenfriend, 1978). The RDA levels, based on the need to alleviate the symptoms of scurvy (Carr and Frei, 1999), are likely far below those required for antioxidant function (Ames, Gold, and Willett, 1995).

1.1.1.3 Physiological functions of vitamin C

Vitamin C plays important roles in a broad spectrum of physiological processes. The lack of vitamin C intake in humans results in a well-defined set of symptoms, including fatigue, poor wound healing, depression and tooth loss, which are collectively referred to as scurvy. If left untreated, the individual would die within months. In the following sections, I discuss the basic physiological functions of vitamin C.

Collagen biosynthesis

Collagen is the major component of the extracellular matrix. A longitudinal protein, collagen fibers aggregate to form a tough, rope-like structure that provides structural support for various tissues. It confers strength and elasticity to the skin. Aging-related degradation of collagen is a common cause for wrinkles and poor wound healing in aging populations. In addition, collagen is the major component of cartilage and bone, and is responsible for the fracture resistant property of these structures. Therefore, insufficient collagen synthesis may contribute to increased bone fracture and poor post-fracture recovery in aging humans.

Vitamin C is a cofactor for collagen biosynthesis (Murad *et al.*, 1981). It functions as an electron donor for proline hydroxylase, lysine hydroxylase and procollagen-proline 2-oxoglutarate 3-dioxygenase, which are responsible for post-translational modification and maturation of collagen. Inadequate intracellular vitamin C levels result in formation of defective collagen, leading to collagen deficiency in the body. This deficiency manifests itself in characteristic scurvy symptoms such as bleeding gum, skin deterioration, and tooth loss. In its early stages, these symptoms can be alleviated by

consumption of vitamin C-containing foods, such as lemons and oranges, a regimen commonly used during 18th century sea voyages.

L-carnitine biosynthesis

Carnitine is essential for fat metabolism. It carries long chain fatty acids from the cytosol into the mitochondrial matrix, where these fatty acids can be broken down to provide metabolic energy (Rebouche, 1991).

L-carnitine is produced from amino acids L-lysine and L-methionine. Vitamin C functions as a cofactor in carnitine biosynthesis in a similar manner as in collagen maturation. It is the electron donor of two carnitine synthetic enzymes, *N*-trimethyl-L-lysine hydroxylase and γ -butyrobetaine hydroxylase (Rebouche, 1991). Vitamin C deficiency results in decreased levels of carnitine in the body, and, in turn, impaired fatty acid metabolism, which is manifested as fatigue and lethargy (Ha, Otsuka, and Arakawa, 1991). These symptoms are commonly documented in cases of scurvy. Supplementation of vitamin C by dietary means has been shown to restore carnitine synthesis in animals (Ha, Otsuka, and Arakawa, 1991).

Iron absorption

Iron is the vital component of the hemoglobin of the red blood cell, which transports oxygen in the body for energy metabolism. Low levels of iron are the cause of iron deficiency anemia, characterized by diminished capacity of the red blood cell to carry oxygen into body tissues. Similar to hemoglobin, myoglobin, which is responsible for oxygen storage in muscle cells, also relies on iron to trap oxygen molecule. Therefore,

iron deficiency also leads to poor energy metabolism in muscles cells, resulting in fatigue and inability for prolonged exercise (Hallberg, Brune, and Rossander, 1986).

Although iron deficiency anemia is multifactorial, poor iron absorption is the most common cause for this ailment. Vitamin C has been shown to facilitate iron absorption from diet in a dose-dependent manner (Hallberg, Brune, and Rossander, 1986). An electron-donating agent, ascorbic acid reduces ferric iron (Fe^{3+}) to the more soluble ferrous form (Fe^{2+}), which is then absorbed in the small intestine (Levine *et al.*, 1999). In addition to acting on iron directly, vitamin C also potentiates the activity of ferric reductase, which catalyzes the conversion of ferric iron to the more absorbable ferrous iron (Herbert, Shaw, and Jayatilleke, 1996). Therefore, diets rich in vitamin C and iron may counteract the effect of iron deficiency anemia.

Conversion of dopamine to norepinephrine

Norepinephrine deficiency can cause depression and hypochondria, which are commonly observed in scurvy patients (Diliberto, Daniels, and Viveros, 1991). Ascorbic acid is a cofactor for dopamine hydroxylase and peptidylglycine amidating monooxygenase, which are enzymes involved in norepinephrine biosynthesis (Diliberto, Daniels, and Viveros, 1991). Therefore, adequate levels of vitamin C may be important for preventing these psychiatric disorders.

Vitamin C as a key biological antioxidant

Antioxidants are reducing compounds that protect the biological system from oxidative damage caused by ROS and nitrogen species (RNS). ROS and RNS are produced as the byproducts of normal mitochondria respiratory processes involving

oxygen as the terminal electron acceptor. Incomplete utilization of oxygen gives rise to free radicals, which are compounds carrying unpaired electrons and are toxic to various components of the biological system. Common biological ROS and RNS include superoxide, hydrogen peroxide and hydroxyl radical. In addition, some ROS are produced as a defense mechanism against invading pathogens. For example, superoxide is produced by NADPH-oxidase to destroy engulfed microorganisms in phagocytes.

ROS and RNS are detrimental to the biological system, as they can induce irreversible oxidative damage to various cellular structures and macromolecules, including DNA, lipids and proteins. Accumulating with age, ROS is believed to be one of the major causes for aging and age-related degenerative diseases, including cancer, heart disease and neurodegeneration.

Oxidative damage caused by ROS results in redox imbalance inside the cell, which, in turn, triggers redox-sensitive signaling pathways leading to tumorigenesis. Indeed, many key oncogenic pathways have been shown to be responsive to the reduction-oxidation state of the cell, and their alteration may lead to abnormalities in various cellular processes including uncontrollable proliferation (Catarzi *et al.*, 2002), growth of new blood vessels or angiogenesis (Hughes, Murphy, and Ledgerwood, 2005), and inhibition of programmed cell death or apoptosis (Souici *et al.*, 2000), all of which contribute to the formation of tumors. Moreover, ROS and RNS also induce genomic instability, as DNA, the major component of chromosomes, is extremely susceptible to oxidative damage by these compounds. Reactions between DNA and ROS, such as

hydroxyl radical, result in oxidative modification of the genetic material, leading to mutagenesis and carcinogenesis (Marnett, 2000; Cooke *et al.*, 2003).

Oxidative stress induced by ROS and RNS is also a cause for the initiation and progression of heart disease. For example, superoxide produced by vascular cells (Griendling, Sorescu, and Ushio-Fukai, 2000) oxidizes low-density lipoprotein (LDL), resulting in highly reactive oxidized LDL (oxLDL), which triggers a sequence of atherosclerotic events. First, oxLDL enters macrophages in the blood and transforms them into cholesterol lipid-laden foam cells (de Villiers and Smart, 1999; Li and Glass, 2002). These cholesterol-laden macrophages adhere to the blood vessel wall and start to accumulate in blood vessels (Parthasarathy *et al.*, 1989; Quinn *et al.*, 1987). These cells elicit multiple inflammatory responses in the circulatory system (Ross, 1999; Stoll and Bendszus, 2006) and induce programmed cell death of endothelial cells composing the vessel wall (Dimmeler *et al.*, 1997; Harada-Shiba *et al.*, 1998). These events mark the onset of atherosclerosis and cardiovascular dysfunction.

Endothelial NO is essential for modulating cardiovascular homeostasis and protecting the vascular system. It stimulates relaxation of the vascular smooth muscle, allowing vasodilatation and unhindered blood flow (Ignarro *et al.*, 1987). NO also prevents inflammation (Decaterina *et al.*, 1995; Cornelis *et al.*, 1994) and programmed cell death in endothelial cells (Khan *et al.*, 1996; Dimmeler *et al.*, 1997). These mechanisms counteract the effects of atherosclerosis, thus preventing the onset of cardiovascular disease.

NO synthesis is susceptible to ROS attack. For example, superoxide alters the NO synthetic pathway by oxidative modification. This has two effects: 1) reduction of endothelial NO, and, consequently, diminished protection offered by NO, and 2) further production of ROS and RNS by the hijacked NO pathway (Vasquez-Vivar *et al.*, 1998). Indeed, impaired endothelium-dependent vessel relaxation is commonly observed in individuals with endothelial dysfunction (Lapu-Bula and Ofili, 2007).

Oxidative modification of macromolecules by ROS and RNS has been proposed as a mechanistic basis for ageing-related neurodegeneration. The brain consumes disproportionately large quantities of oxygen and is composed of oxidation-prone fatty acids. Therefore, it is extremely sensitive to oxidative damage (Floyd, 1999). ROS in the brain triggers inflammatory responses and elicits damage to protein and lipid components, which contribute to the onset of neurodegenerative disease such as Alzheimer's disease.

As these age-related degenerative diseases, including cancer, heart disease and neurodegeneration, are attributable to accumulation of ROS and RNS over time, reduction of ROS and RNS levels in the body is essential for delaying and preventing the onset of these diseases, especially in aging populations.

Vitamin C is an ideal biological antioxidant. When encountering ROS and RNS, vitamin C donates a single electron to these molecules, hence reducing them to their unreactive state. Vitamin C itself becomes a single electron-carrying ascorbyl free radical. However, unlike other free radicals such as superoxide or hydroxyl radical, ascorbyl free radical is relatively inert, as the reactive electron is delocalized and stabilized by the unique molecular structure (**Figure 3**). Vitamin C is soluble in biological fluids such as

the blood, and, as such, can destroy blood borne ROS and RNS before they reach tissues and cause damage. In addition, vitamin C is non-toxic and can be used therapeutically to maintain the antioxidant/oxidant balance in the body. Indeed, a large number of studies have shown that ascorbic acid protects the biological system against oxidative damage induced by various ROS and RNS.

Vitamin C prevents oxidative damage to DNA (Lutsenko, Carcamo, and Golde, 2002; Noroozi, Angerson, and Lean, 1998; Pflaum *et al.*, 1998; Sweetman, Strain, and McKelveyMartin, 1997), lipids (Barja *et al.*, 1994; Kimura *et al.*, 1992) and protein (Barja *et al.*, 1994; Cadenas, Rojas, and Barja, 1998; Hoey and Butler, 1984). Consumption of vitamin C-rich diets is inversely related to the levels of oxidative DNA damage (Deng *et al.*, 1998; Fraga *et al.*, 1991; Rehman *et al.*, 1999; Thompson *et al.*, 1999). Not only does vitamin C offer structural protection to biological molecules, it also modulates redox-sensitive signal transduction pathways, leading to increased cell cycle arrest and programmed cell death in response to DNA damage (Reddy, Khanna, and Singh, 2001; Catani *et al.*, 2002), and attenuation of cell proliferation (Bowie and O'Neill, 2000; Carcamo *et al.*, 2002; Han *et al.*, 2004). These mechanisms together may give rise to antitumorigenic effects of ascorbic acid. These effects were reviewed in detail in later sections.

1.1.1.4 Vitamin C Transport

As a polar compound with a relatively large molecular weight, vitamin C cannot readily cross the cell membrane by simple diffusion. The flux of vitamin C in and out of the cell is controlled by specific mechanisms, including facilitated diffusion and active

transport, which are mediated by distinct classes of membrane proteins such as facilitative glucose transporters (GLUTs) and sodium vitamin C cotransporters (SVCTs), respectively.

Facilitated diffusion through GLUT transporters

Gradient-driven transport of the oxidized form of vitamin C, dehydroascorbic acid (DHA), is mediated by a class of facilitative glucose transporters (GLUTs), which has no detectable affinity for the reduced, biologically-active forms such as ascorbic acid and ascorbate. The reduced vitamin C, DHA, can be indirectly imported by a three-step mechanism involving 1) extracellular oxidization of ascorbate to DHA, 2) transport of DHA by the GLUT transporter and 3) intracellular reduction of DHA to ascorbate (Figure 4).

The GLUT transporters mediate the absorption of DHA in an energy-independent manner, and their kinetic properties can be robustly modelled by Michaelis-Menten kinetics (Rumsey *et al.*, 1997). Based on apparent transport affinities (K_m), GLUT1 and GLUT3 are the major transporters for DHA influx among GLUT isoforms, and have kinetic constants similar to those of glucose transport (Rumsey *et al.*, 1997). A third DHA transporter, GLUT4, was later identified (Rumsey *et al.*, 2000). GLUT1 and GLUT3 are predominantly located in osteoblast (Qutob, Dixon, and Wilson, 1998), muscle (Korcok *et al.*, 2003) and retinal cells (Hosoya *et al.*, 2004), and mediate the influx of DHA in these cells. GLUT1 is also expressed on the endothelial cells at the Blood Brain Barrier (BBB), and is responsible for accumulation of vitamin C in the brain (Agus *et al.*, 1997) (Figure 4).

Sharing the same transporters as glucose, GLUT-mediated transport of DHA is competitively inhibited by glucose (Agus *et al.*, 1997; Li *et al.*, 2006; Rumsey *et al.*, 1997; Rumsey *et al.*, 2000; Vera *et al.*, 1995). This raises the possibility that changes in serum glucose levels, especially those occurring during disease, may attenuate the bioavailability of vitamin C leading to secondary pathologies due to the concomitant depletion of circulating vitamin C. Indeed, this characteristic type of secondary pathology has been observed under hyperglycemic conditions caused by diabetes (Baynes, 1991; Chen *et al.*, 2005; Ng *et al.*, 1998), and may be treated, at least partially, by clinical administration of vitamin C.

In addition to glucose inhibition, the GLUT transporters are also subject to hormonal control. In the presence of both follicle-stimulating hormone and Insulin-like growth factor I, the expression of GLUT 1 is up-regulated in granulosa cells (Kodaman and Behrman, 1999). Similarly, GLUT4 expression in cells is stimulated by addition of insulin (Rumsey *et al.*, 2000).

The impact of serum glucose levels and endocrinal hormone status on vitamin C transport underscores the necessity of examining serum glucose concentrations in conjunction with vitamin C levels to understand how alterations in vitamin C status contribute to various diseases in humans.

The facilitated transport mechanism by GLUTs has been implicated in the protection against oxidative damage (Huang *et al.*, 2001). Administration of DHA has been shown to protect neural cells from experimentally-induced ischemic stroke by increasing antioxidant levels through GLUT-mediated vitamin C accumulation (Huang *et*

al., 2001). This may also protect against reactive oxygen species (ROS) generated from mitochondrial respiration, which is of particular interest in human nutrition, because oxidative respiration in mitochondria are the major source of biological ROS in the cell. As oxidative damage is a key contributor to age-related degenerative diseases, these findings support the therapeutic potential of intracellular vitamin C, and implicate DHA, in conjunction with the GLUT transport system, as potential targets in treatment of these diseases. Unlike the SVCT transporters, GLUT-mediated vitamin C transport is insensitive to feedback inhibition by ascorbate, and, as such, is more suitable for high-dose vitamin therapy than its counterpart.

Active transport by SVCT transporters

In addition to the facilitated mechanism, vitamin C is also transported by active Sodium-Vitamin C cotransporters (SVCTs), which transport ascorbate directly into the cell. Based on K_m values, SVCTs have higher affinity for ascorbate than do GLUTs for DHA, and thus are considered high-affinity vitamin C transporters (Tsukaguchi *et al.*, 1999). The SVCT system mediates ascorbate transport at the expense of the sodium electrochemical gradient across the cell membrane, which has to be maintained by active sodium-potassium-ATPase, and, as such, are classified as secondary active transporters (Wilson, 2005) (**Figure 4**). Unlike the GLUT transporters, SVCTs are substrate-saturable and sensitive to ascorbate feedback inhibition (MacDonald, Thumser, and Sharp, 2002), which limits the maximum effective vitamin C concentrations attainable by oral ingestion.

There are two isoforms of SVCT transporter: hSVCT1 (*slc23a2*) and hSVCT2 (*slc23a1*). However, the Human Genome Organization (HUGO) gene names for these two transporters have recently been reassigned: SVCT1 and SVCT2 are encoded by *SLC23A1* and *SLC23A2*, respectively (Takanaga, Mackenzie, and Hediger, 2004). Comparison of the two isoforms reveals that SVCT2 has a higher affinity (Liang, Johnson, and Jarvis, 2001), but lower transport capacity (Liang *et al.*, 2003) for ascorbate than SVCT1. The distribution and functions of the two SVCT isoforms are distinct (**Figure 4**). SVCT1 is predominantly expressed in epithelial cells, including those of the intestine, kidney and the liver, and can transport ascorbate exceeding the internal requirement of these cells (Tsukaguchi *et al.*, 1999). Hence it is often referred to as the “bulk” transporter of ascorbate. In contrast, SVCT2 is localized to metabolically-active and specialized cells, such as those of the brain, eye and placenta (Takanaga, Mackenzie, and Hediger, 2004; Tsukaguchi *et al.*, 1999), and has been implicated in the maintenance of intracellular vitamin C levels vital for neuronal function and the protection against oxidative stress (Qiu *et al.*, 2007).

Both isoforms of SVCT are subject to substrate feedback inhibition by ascorbate. The expression of SVCT1 is attenuated by high concentrations of ascorbic acid (4.5 mg/ml) in vitro (MacDonald, Thumser, and Sharp, 2002). As SVCT1 is the high-capacity, bulk transporter of vitamin C, its downregulation by ascorbate effectively limits the maximum achievable concentration of plasma vitamin C by oral ingestion (Wilson, 2005), and is considered a major obstacle in high-dose vitamin C therapy (Padayatty *et al.*, 2004). Similar to its isoform, SVCT2 is also sensitive to, and its activity varies

inversely with, the changes in intracellular ascorbate levels (Dixon and Wilson, 1992), which was suggested to play a regulatory role in maintaining ascorbate homeostasis inside the cell (Wilson, 2005). This feedback mechanism presents similar challenge to that of using SVCT1 to accumulate intracellular vitamin C, as pharmacologically increased intracellular ascorbate will likely induce a feedback inhibition, causing downregulation of SVCT2 and, in effect, restoration of intracellular ascorbate to its normal physiological levels (Dixon and Wilson, 1992). Nevertheless, the brain vitamin C levels can be effectively increased via the three-step GULT transport using DHA as substrate (Huang *et al.*, 2001).

In addition to substrate inhibition, an age-related decline in SVCT1 expression in rat liver cells has recently reported (Michels, Joisher, and Hagen, 2003). If this is true in humans, it may help explain the observation that elderly individuals require higher levels of dietary vitamin C to reach serum ascorbate concentrations comparable to those of younger individuals (Brubacher, Moser, and Jordan, 2000). As the effect of this decline can be compensated by increased vitamin C intake (Michels, Joisher, and Hagen, 2003), it has been suggested that clinical or nutritional treatment leading to moderately increased serum vitamin C levels might be beneficial for elderly individuals (Wilson, 2005). Unlike SVCT1, age-related decline is not observed in SVCT2 levels in the liver, perhaps as a result of low abundance of this transporter in the liver (Michels, Joisher, and Hagen, 2003). Future studies with tissues rich in SVCT2, such as brain and retina, may reveal any potential roles for ageing on this transporter, as well as consequent changes in vitamin C

accumulation and physiological abnormalities that might contribute to age-related diseases.

SVCT2 is also essential for perinatal survival of mice (Sotiriou *et al.*, 2002). It is required for vitamin C transport across the placenta as well as prenatal distribution of ascorbate into various tissues of the unborn mouse (Sotiriou *et al.*, 2002). Furthermore, newborn mice carrying null mutation of *SLC23A2* die of respiratory failure and brain hemorrhage shortly after birth, suggesting a vital but unknown role for vitamin C in lung and brain tissues during early development (Sotiriou *et al.*, 2002). The overt phenotypic difference between *SLC23A2*^{+/-} and wild type mice is reflective of the delicate correlation between SVCT2 activity and the intracellular ascorbate levels (Sotiriou *et al.*, 2002), which may be important for maintaining optimal intracellular vitamin C required for certain tissues. For example, overexpression of human SVCT2 transporter in mice leads to abnormal elevation of vitamin C levels in the retina, which results in damages to the eye (Fan *et al.*, 2006). A number of single-nucleotide polymorphisms (SNPs) at the *SLC23A2* locus have been identified among human populations (Eck *et al.*, 2004), and certain allelic variants are shown to associate with preterm birth in humans (Erichsen *et al.*, 2006), raising the possibility that vitamin C may be implicated in premature birth in humans.

In summary, the two major vitamin C transporters, GLUT and SVCT, regulates the tissue-specific vitamin C levels, and must be considered in treatments aiming to achieve high intracellular ascorbate levels. Indeed, a major difficulty in achieving high effective concentrations of vitamin C by oral administration is attributable to inhibition of

these transporters. Alternative administration methods, such as intravenous injection, that bypasses the renal system can temporarily raise serum vitamin C levels to pharmacological range (Padayatty *et al.*, 2004). Alternatively, treatments altering the activity of a specific vitamin C transporter may potentiate localized accumulation of vitamin C, and may be utilized when specific tissue is targeted for therapy. However, such interventions require better understanding of the physiological activities and tissue distributions of various vitamin C transporters *in vivo*. Transgenic animals harboring knockout mutations of SVCT2 (Sotiriou *et al.*, 2002) or overexpressing this transporter (Fan *et al.*, 2006) are excellent *in vivo* models for studying the function of this transporter. Mice defective in SVCT1 have not yet been made available and, given the fact that wild type mice do not rely on vitamin C absorption for survival, may not be suitable for modeling the nutritional requirement for this vitamin in humans. A double knockout mouse that carries SVCT1 null mutation and defective in vitamin C biosynthesis would be an invaluable tool and may yield insights into the function of SVCT1 in humans. An alternative strategy using a “chemical knock-out substrate” that is exclusively recognized and transported by one specific system has also been devised (Corpe *et al.*, 2005). The advantage of this substrate analog, 6-bromo-6-deoxy-L-ascorbic acid, is that it is specific for the SVCT system and, as such, allows the contributions of the GLUT and SVCT pathways in vitamin C transport to be assessed independently (Corpe *et al.*, 2005).

Vitamin C bioavailability

Bioavailability, or the effective concentration, of ascorbic acid is dependent on both intestinal absorption and renal excretion. Vitamin C, consumed either with diet or

dietary supplements, is absorbed by the epithelial cells of the small intestine by the SVCT1 transporter or, subsequently, diffuses into the surrounding capillaries and then the circulatory system. In the kidney, circulating ascorbic acid is filtered from the glomerulus capillary bed into the Bowman's capsule through a general filtration mechanism. Ascorbic acid, while passing through the proximal convoluted tubule, is reabsorbed into the capillary bed surrounding this portion of the renal tubule through renal epithelial cells by the SVCT1 transporter. The difference between the amount of ascorbic acid filtered and the amount reabsorbed constitutes renal excretion.

Together, intestinal absorption and renal excretion determine the serum level of vitamin C and hence its bioavailability. At low concentrations, most vitamin C is absorbed in the small intestine and reabsorbed from the renal tubule. However, at high concentrations, SVCT1 becomes saturated, which, combined with ascorbate-mediated SVCT1 downregulation, limits the amount of ascorbic acid absorbed from the intestine and reabsorbed from the kidney. This imposes a physiological restriction on the maximal effective serum vitamin C concentration (or its bioavailability) that is attainable by oral consumption (Padayatty *et al.*, 2004). This value has been determined to be ~200 $\mu\text{mol/L}$ (Padayatty *et al.*, 2004), although "normal" physiological serum concentrations of ascorbate in healthy humans range from 60 to 100 $\mu\text{mol/L}$.

1.1.1.5 Pharmacology of vitamin C

Recommended daily allowance

The current recommended daily allowance (RDA) for vitamin C proposed by the Food and Nutrition Board of the U.S. National Academy of Sciences is 120 mg per day

(Levine *et al.*, 1999). This value is derived from the estimated average requirement (EAR) for vitamin C, which is the amount of vitamin C required by half of the healthy population at a certain life stage. This calculation takes into account several key dietary and physiological factors including dietary availability, effective serum concentration as a function of dose, bioavailability and potential adverse effects (Levine *et al.*, 1999).

Another RDA based on adequate intake value (AI) has also been proposed. AI value is determined from a group of healthy individuals and is more suitable for determining nutrient intake of individuals (Levine *et al.*, 1999). Based on this value, it is recommended that 200 mg of vitamin C should be taken per day, which can be acquired from 5 servings of fruits and vegetables. At this level, tissue vitamin C saturation is reached (Levine *et al.*, 1999). Although vitamin C can be consumed from a variety of sources, including diet and dietary supplements, it is generally recommended that vitamin C intake come directly from dietary sources. Therefore, 5 servings of fruits and vegetables, which provide 200 mg vitamin C, are recommended to healthy individuals under normal conditions (Levine *et al.*, 1999).

Active and passive smokers

The requirement for vitamin C in individuals exposed to cigarette smoking is likely higher than in nonsmoking population. Cigarette smoke contains free radicals which can deplete circulating vitamin C in exposed individuals (Lykkesfeldt *et al.*, 2000). The reduction in serum vitamin C levels caused by smoking can reach up to 40% (Ross *et al.*, 1995). Nevertheless, moderate supplementation can efficiently replete serum vitamin C in smokers (Lykkesfeldt *et al.*, 2000). Therefore, to acquire similar levels of circulating

vitamin C to non-smokers, active and passive smokers require higher levels of dietary intake (Northrop-Clewes and Thurnham, 2007).

Individuals with hemochromatosis

Vitamin C prevents iron deficiency anemia by facilitating iron absorption. Some have argued that the same mechanism, if occurs in individuals with hemochromatosis, may exacerbate the risk of iron overload (Herbert, Shaw, and Jayatilleke, 1996). For example, vitamin C reduces ferric to ferrous iron through the Fenton reaction in vitro, resulting in the formation of reactive radicals which are detrimental to various cellular components (Herbert, Shaw, and Jayatilleke, 1996). Indeed, multiple cell and tissue damage has been reported in patients with iron overload when supplemented with ascorbic acid (Herbert, Shaw, and Jayatilleke, 1996; Nienhuis, 1981). Therefore, it may be advisable to reduce or avoid vitamin C supplementation in these individuals (Fraga and Oteiza, 2002). The frequency of hereditary hemochromatosis in Caucasian populations is 0.4—1% (Adams, 1999), and can be identified with a simple serum ferritin test or genetic testing. Physicians are encouraged to conduct such tests before recommending vitamin C supplementation in their patients, especially Caucasians.

Individuals prone to kidney stone

One of the metabolic end products of ascorbic acid is oxalate (HELLMAN and Burns, 1958), which can complex with calcium ion and potentially facilitate the formation of calcium oxalate kidney stone, and is, in itself, a determinant of kidney stone formation (Chalmers, Cowley, and Brown, 1986). A number of studies have been conducted in healthy subjects and stone-forming patients to examine the effect of vitamin C

supplementation on oxalate excretion in the urine. However, due to the difficulties in oxalate assay techniques, their findings are largely inconsistent (Baxmann, De, and Heilberg, 2003). Using improved assay methods, a study reports a increase of 61% and 41% in urinary oxalate levels after supplementation of 1 or 2 g ascorbic acid in calcium stone-forming patients (Baxmann, De, and Heilberg, 2003). This increase may exacerbate the crystallization of calcium oxalate, and, in effect, elevate the health risk in individuals with genetic predisposition toward kidney stone formation (Baxmann, De, and Heilberg, 2003). Therefore, these individuals should be cautioned regarding the potential danger of vitamin C supplementation. While vitamin C increase urinary excretion of oxalate, a direct association between kidney stone risk and vitamin C intake has not been established, and, in fact, findings from several large perspective studies contradict the practice of vitamin C restriction in kidney stone prevention (Curhan *et al.*, 1996; Curhan *et al.*, 1999).

Toxicity

Vitamin C is essentially non-toxic, which, in part, is attributable to the reduction in absorption and reabsorption when high levels of vitamin C is taken orally. Intravenous injection of ascorbic acid, which bypasses the intestinal absorption, can result in the blood vitamin C levels to about 13 mmol/L, which is 140-fold higher than the maximum oral levels, and no adverse effects at this level have been reported (Padayatty *et al.*, 2004).

Dietary sources

Fresh fruits and vegetables are the principle natural sources of vitamin C. Five servings of fruits and vegetables are generally recommended for healthy individuals

(Levine *et al.*, 1999). Some of the common dietary sources of vitamin C are listed in **Table 1**. It should be noted, however, that vitamin C content is also dependent on methods for food preparation and storage. For example, boiling vegetable may result in 50% to 80% loss and long term storage can also significantly reduce vitamin C content (Levine *et al.*, 1999).

At physiological concentrations, ascorbic acid protects LDL from oxidative modification by 1) quenching ROS and RNS in the blood and 2) enhancing the resistance of LDL to oxidation (Retsky, Freeman, and Frei, 1993), and, in effect, attenuates the levels of harmful oxLDL in the circulation (Retsky and Frei, 1995; Martin and Frei, 1997; Alul *et al.*, 2003). In addition, ascorbic acid also mitigates the effects of oxLDL on the biological system. For instance, it protects smooth muscle cells and macrophages from oxLDL-induced cell death (Siow *et al.*, 1998; Asmis and Wintergerst, 1998) and attenuates oxLDL-related atherogenic inflammation (Griffiths *et al.*, 2003; Mo *et al.*, 2003). Inside the cells, vitamin C protects normal NO synthesis (Marui *et al.*, 1993; Baker, Milstien, and Katusic, 2001). Adequate concentrations of vitamin C are also essential to maintain the structural integrity of the aorta (Maeda *et al.*, 2000; Nakata and Maeda, 2002).

As vitamin C potentiates the antioxidant capacity of the biological systems and offers protection for various cellular components, some have proposed that it may attenuate the process of ageing and progression of age-related degenerative diseases (Li and Schellhorn, 2007; Li and Schellhorn, 2007).

1.1.2 Novel therapeutic perspectives for vitamin C

1.1.2.1 Cardiovascular disease (CVD)

Cardiovascular disease (CVD) is multifactorial with many identifiable risk factors, including diet, tobacco smoking, diabetes and hypertension (Balady *et al.*, 2007). Diet, as a modifiable determinant, is important in the prevention of CVD. While some studies reported that consumption of vitamin C-rich foods, such as fruits and vegetables, is correlated with a reduced risk of CVD (Block *et al.*, 2001; Joshipura *et al.*, 2001; Liu and Tan, 2000), others have reported contradictory results (Muntwyler *et al.*, 2002). Apart from well-recognized confounding phenomena, the inconsistency is due, at least in part, to our limited understanding of the mechanisms of action of this vitamin on different pathophysiological variables contributing to cardiovascular complications, and, as such, calls for more focused mechanistic studies on the interaction of ascorbic acid with contributors of specific vascular pathology. In this section, both epidemiological and experimental evidence pertaining to the roles for vitamin C on the prevention and treatment of CVD is reviewed with focus on the mechanisms of action that may contribute to potential benefits of vitamin C.

Epidemiological evidence

High dietary content of vegetables and fruits reduces the risk of heart disease (Block *et al.*, 2001; Joshipura *et al.*, 2001; Liu and Tan, 2000). This association is partially attributable to antioxidants, such as vitamin C and vitamin E, present in these foods, which protect biological molecules from oxidative damage. This is supported by compelling evidence that oxidative damage due to free radicals is a major cause of CVD

(Taniyama and Griendling, 2003). Many epidemiological studies, including observational studies and randomized controlled trials, have examined the relationship between antioxidants and incidence of CVD. However the results and conclusions of these studies are not uniform. While some observational studies report a negative correlation between dietary intake of vitamin C, in itself or in combination with other antioxidant vitamins, and the risk of cardiovascular complications (Kaufmann *et al.*, 2000; Salonen *et al.*, 2000; Salonen *et al.*, 2003; Taniyama and Griendling, 2003), this association is not seen in randomized controlled trials (Stanner *et al.*, 2004; Willett and Stampfer, 2001). The findings of these epidemiologic studies have been systematically reviewed, and the causes of their discrepancy analyzed (Asplund, 2002; Morris and Carson, 2003).

Apart from reliance on subject self-report, susceptibility to measurement error and short intervention duration commonly associated with these studies, the inconsistency is also caused by confounding effects, as vitamin supplementation tends to correlate with other positive lifestyle factors, which may in themselves contribute to the reduction of cardiovascular risk (Asplund, 2002). In addition, epidemiological studies do not readily examine specific physiological conditions of the subjects, and as vitamin C may have opposing effects (antioxidant vs. prooxidant) under different physiological conditions (Balakrishnan *et al.*, 2003; Chen *et al.*, 2003), cancellation of positive and negative outcomes within a pooled sample population may result in the lack of treatment effect. This further underscores the importance of understanding of the mechanisms of action of this vitamin and its interaction with other physiological variables in the biological system. Indeed, research into the therapeutic effects of vitamin C on CVD has recently re-focused

on the elucidation of potential mechanisms of action that may contribute to its therapeutic potentials in cardiovascular disease.

Oxidative stress, vitamin C, and CVD

Oxidative stress induced by both reactive oxygen species (ROS) and reactive nitrogen species (RNS) play a major role in the initiation and progression of cardiovascular disease (Taniyama and Griendling, 2003). Among ROS and RNS, superoxide is the most biologically relevant radical in vasculature, as it is naturally produced by most vascular cells (Griendling, Sorescu, and Ushio-Fukai, 2000) and can mediate the generation of other ROS and RNS, leading to augmentation of oxidative damage (Taniyama and Griendling, 2003). For a detailed review on the effects of ROS on the vascular system, we recommend an article by Taniyama and Griendling (Taniyama and Griendling, 2003).

The effects of oxidative stress on the cardiovascular system are multifold and include 1) ROS-induced apoptosis of endothelial cells (Dimmeler *et al.*, 1999; Dimmeler and Zeiher, 2000), 2) induction of inflammation by oxidative modification of the expression of proinflammatory genes (De Keulenaer *et al.*, 2000) and cell adhesion (Marui *et al.*, 1993), 3) reduction of intracellular bioavailability of vasodilator NO (Forstermann and Munzel, 2006) and, 4) oxidative modification of low-density lipoproteins (LDL) (Stocker and Keaney, 2004). All of these contribute to clinical manifestations of CVD.

Antioxidants quench free radicals, and thus prevent oxidative damage to the cardiovascular system (Gey, 1990; Tardif, 2006). In the following section, the

mechanisms by which vitamin C can influence the cardiovascular health are reviewed with emphasis on interaction with key molecules/pathways of the vascular system, including LDL, vitamin E and the nitric oxide synthetic pathway.

Oxidative modification of LDL

Oxidative modification of LDLs by ROS, such as superoxide and hydroxyl radicals, generated by subendothelial cells transforms native LDLs into highly bioreactive oxidized-LDLs (oxLDLs), which initiates a sequence of atherogenic events in the subendothelial space marked by 1) increased intake of oxLDL by macrophages and the consequent formation of cholesterol lipid-laden foam cells (de Villiers and Smart, 1999; Li and Glass, 2002), 2) upregulation of intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM) receptors by activated macrophages, leading to influx of monocytes into subendothelial space and further accumulation of macrophages (Parthasarathy *et al.*, 1989; Quinn *et al.*, 1987), 3) subsequent elicitation of multiple inflammatory responses (Ross, 1999; Stoll and Bendszus, 2006), and 4) induction of apoptosis of endothelial cells (Dimmeler *et al.*, 1997; Harada-Shiba *et al.*, 1998). These events lead to the initiation and progression of atherosclerosis and other symptoms of heart disease. As oxidative modification of LDL occurs in the early stages of vascular dysfunction, and is instrumental to the progression of atherosclerosis, strategies to reduce LDL modification may help prevent the onset of atherosclerosis (Gotto and Farmer, 2006; Nissen *et al.*, 2005; Sacks, 2004).

As the origin of oxLDL formation can be traced to the elevation of ROS and RNS, a reduction in the concentration of these species and restoration of vascular redox balance

by water soluble antioxidants such as ascorbic acid may be effective in attenuating oxLDL-mediated endothelial dysfunction. For instance, physiological concentrations of ascorbic acid (50—100 μ M) in vitro attenuate oxidative modification of LDL induced by transition metals (Retsky and Frei, 1995; Retsky *et al.*, 1999), homocysteine (Alul *et al.*, 2003) and myeloperoxidase-derived HOCl (Carr *et al.*, 2000; Carr and Frei, 2002), as well as those naturally produced by human vascular endothelial cells (Martin and Frei, 1997). Two key mechanisms are responsible for these actions: 1) ascorbate quenches aqueous ROS and RNS, decreasing their bioavailability in the plasma, and 2) ascorbate reduces the affinity of LDL-bound ApoB protein for transition metal ions, and this, in effect, enhances the resistance of LDL to metal ion-dependent oxidation (Retsky, Freeman, and Frei, 1993). In addition to preventing oxLDL formation, vitamin C also counteracts the damaging effects of existing oxLDL on different vascular components. For example, vitamin C protects arterial smooth muscle (Siow *et al.*, 1999) and mature human macrophages (Asmis and Wintergerst, 1998) from oxLDL-induced apoptosis. It also attenuates the atherogenic inflammatory response by inhibiting oxLDL-related ICAM-I overexpression and monocyte adhesion (Griffiths *et al.*, 2003; Mo *et al.*, 2003; Rayment *et al.*, 2003; Son *et al.*, 2004); and spares intracellular glutathione from oxLDL-stimulated modulation (Siow *et al.*, 1998). This, in effect, further increases the antioxidant capacity of the cell (Siow *et al.*, 1998). Moreover, when applied with other antioxidants, vitamin C augments anti-atherogenic effect in a synergistic manner. For example, ascorbic acid can interact with estradiol in vitro, enhancing its ability to inhibit oxidation of LDL (Huang *et al.*, 1999; Hwang *et al.*, 2000). In combination with vitamin

E, vitamin C prevents oxLDL-induced overexpression of vascular endothelial growth factor (VEGF) and its receptor responsible for atherosclerotic plaque formation (Nespereira *et al.*, 2003; Rodriguez *et al.*, 2005), and decreases plasma VCAM-1 and ICAM-1 responsible for monocyte adhesion and inflammation (Tousoulis *et al.*, 2003). The synergism between vitamin C and vitamin E can, at least in part, be ascribed to the ability of ascorbic acid to regenerate vitamin E from α -tocopherol radical (Neuzil, Weber, and Kontush, 2001), therefore restoring and augmenting the intrinsic antioxidant property of vitamin E.

Vitamins C and E

Vitamin E, in the form of α -tocopherol, is a key lipophilic antioxidant in human circulation and the vasculature, and plays a role in many key processes contributing to the onset and progression of atherosclerosis (Kaliora, Dedoussis, and Schmidt, 2006). As a lipophilic antioxidant, vitamin E can interact with the lipid components in the vascular systems, notably LDL, and protects them from atherogenic oxidative modification (Burton and Ingold, 1986). Conversely, the lipid-bound α -tocopherols (α -TOH) can be oxidized by aqueous-phase radicals, and transformed into reactive tocopherol radicals, which, in turn, react with the unsaturated lipids of the lipoprotein, initiating lipid oxidation by a tocopherol-mediated peroxidation (TMP) reaction (Bowry and Stocker, 1993; Ingold *et al.*, 1993). Oxidized vitamin E can be reduced back to its antioxidant form by other aqueous-phase reductants (Ingold *et al.*, 1993).

Ascorbic acid reacts rapidly with the tocopherol radical, reducing it back to its native form (Nagaoka *et al.*, 2007). As such it may be conducive for vitamin E

regeneration in biological systems (Neuzil, Weber, and Kontush, 2001). In addition, ascorbate can sequester aqueous radicals in the plasma before they can oxidize vitamin E in the lipid phase, and, in effect, affords preemptive protection for lipid-bound tocopherols. In cigarette smokers, the rate of the blood vitamin E oxidation caused by increased oxidative stress is substantially attenuated by vitamin C supplementation (Bruno *et al.*, 2005; Bruno *et al.*, 2006), indicative of a vitamin E-recycling role for vitamin C, and a potential cooperative relationship between vitamins C and E. Indeed, this cooperativity against oxidation of lipoproteins has been shown both in vitro (Murugesan *et al.*, 2005) and in vivo (Krishnamoorthy *et al.*, 2007; Kumar *et al.*, 2004; Murugesan *et al.*, 2005; Ramanathan, Balakumar, and Panneerselvam, 2002). Furthermore, vitamins C and E can interact synergistically in protection against the development of cardiovascular disease (Carr, Zhu, and Frei, 2000). For example, when applied in combination they synergistically attenuate copper-mediated LDL oxidation in vitro (Abudu *et al.*, 2004), downregulate the expression of endothelial VEGF and its receptor VEGF-2 (Nespereira *et al.*, 2003; Rodriguez *et al.*, 2005), and decrease the activation of NADPH oxidase while increasing that of superoxide dismutase (SOD) leading to reduced levels of oxidative stress (Chen *et al.*, 2001). These cooperative interactions between these two vitamins have important clinical ramifications, as they provide a mechanistic basis for combined therapy (co-antioxidant therapy) in treatment of CDV. Indeed, the therapeutic value of multivitamin treatment has been supported by a growing body of clinical evidence (Antoniades *et al.*, 2003; Engler *et al.*, 2003; Fang *et al.*, 2002; Liu *et al.*, 2002; Tousoulis *et al.*, 2003).

Endothelial NO and vitamin C

Nitric oxide (NO) is produced from L-arginine by nitric oxide synthase (NOS) in the presence of NOS cofactor (6R)-5,6,7,8-tetrahydro-L-biopterin (BH₄). Endothelial NO produced by endothelial NOS (eNOS) modulates cardiovascular homeostasis and protects the vascular system by several mechanisms. As an endothelial signal molecule, NO stimulates vascular smooth muscle relaxation, allowing vasodilatation and unhindered blood flow (Ignarro *et al.*, 1987). It also participates in more complex regulatory pathways of vascular inflammation (Napoli *et al.*, 2006). For example, it prevents adhesion of leukocytes to the interior wall of blood vessels (Kubes, Suzuki, and Granger, 1991), activation of proinflammatory adhesion molecules and cytokines (Decaterina *et al.*, 1995; Khan and Sinha, 1996), and suppression of endothelial cell apoptosis (Dimmeler *et al.*, 1997; Khan and Sinha, 1996). These mechanisms contribute to a broad spectrum of physiological effects that inhibit atherosclerosis (Napoli *et al.*, 2006; Rubio and Morales-Segura, 2004).

However, the NO biosynthetic pathway is sensitive to oxidative modification by ROS, and its alteration has detrimental consequences for the vascular system. In the presence of superoxide anion, produced by NAD(P)H oxidase and uncoupled eNOS, NO is oxidized to peroxynitrite, which, in turn, oxidizes eNOS cofactor BH₄, leading to the inactivating uncoupling of eNOS from BH₄. The uncoupled eNOS exhibits altered enzymatic activity, reducing molecular oxygen to superoxide, which, in effect, leads to significant reduction of endothelial NO and accumulation of ROS and RNS in the vasculature (Vasquez-Vivar *et al.*, 1998). The former is manifest by impaired

endothelium-dependent vessel relaxation commonly observed in hypertensive animal models (Li *et al.*, 2006; Tai *et al.*, 2005) and patients with endothelial dysfunction (Lapubula and Ofili, 2007). The latter causes further NO oxidation and eNOS modification, leading to augmented oxidative stress to the vascular system. Indeed, aberrant NO metabolism is closely correlated with, and may be instrumental to, the development of cardiovascular disease.

Vitamin C protects normal NO synthesis by modulating the redox states of its components. It stabilizes endothelial BH4 level by regenerating BH4 from its oxidized form in a series of reduction reactions (Baker, Milstien, and Katusic, 2001; Heller *et al.*, 2001; Huang *et al.*, 2000; Marui *et al.*, 1993). As BH4 is an essential cofactor for eNOS and its oxidative inactivation is the major contributing factor to NO pathway aberration and consequent clinical manifestations (Hong *et al.*, 2001; Landmesser *et al.*, 2003; Shinozaki *et al.*, 1999), the stabilization and reactivation of the endothelial BH4 by ascorbic acid, and the consequent restoration of the normal biological activities of eNOS (d'Uscio *et al.*, 2003; Smith, Visioli, and Hagen, 2002) and endothelial NO accumulation, may represent a key mechanism by which vitamin C impacts overall endothelial health.

Vitamin C attenuates the activity of NAD(P)H oxidase and the production of superoxide in vitro (Wu *et al.*, 2007) and in vivo (Chen *et al.*, 2001; Papparella *et al.*, 2007). As NAD(P)H is the major source of endothelial superoxide (Mohazzab, Kaminski, and Wolin, 1994) responsible for the initial uncoupling of eNOS, its inactivation by vitamin C and E suggests that these two vitamins may prevent the onset of eNOS uncoupling-induced endothelial dysfunction by inhibiting the early events of this

process. As a free radical scavenger, ascorbic acid at high concentrations reacts directly with superoxide, decreasing its availability, and in effect inhibiting superoxide-mediated NO inactivation (Jackson *et al.*, 1998). Ascorbic acid may also preserve the normal enzymatic activity of eNOS by preventing the S-nitrosylation of the regulatory cysteine residues on eNOS (May, 2000).

In addition, ascorbate directly mediates the production of NO by reducing nitrite compounds, thereby maintaining high levels of local tissue NO concentrations independent of the NO synthesis (May, 2000). It potentiates the responsiveness to NO stimulation by increasing the sensitivity of guanylyl cyclase to NO signaling (Murphy, 1999). However the biological relevance of these *in vitro* phenomena is still unknown.

The protective role for vitamin C on NO and its biosynthetic pathway is supported by clinical evidence that administration of vitamin C improves endothelium-dependent vasodilatation in patients with endothelial dysfunction (de Sousa *et al.*, 2005; Plantinga *et al.*, 2007). As endothelial dysfunction generally marks the onset of atherosclerosis and CDV, vitamin C supplementation may be beneficial during early stages of CVD.

Vitamin C and collagen

Animal studies with *Gulo*^{-/-} mice unable to produce vitamin C show that ascorbic acid deficiency gives rise to structural abnormalities in the wall of the aorta, which is caused by defects in collagen and elastin synthesis (Maeda *et al.*, 2000). *Gulo*^{-/-} mice with concurrent *Apoe* null mutation (*Gulo*^{-/-}*Apoe*^{-/-}), which leads to arterial lesion and hyperglycemia, have lower collagen content in atherosclerotic plaques when fed on low vitamin C diet (Nakata and Maeda, 2002). Lower collagen content leads to instability of

plaques, hence facilitating rupture and making them high-risk (Nakata and Maeda, 2002). However, whether or not these collagen-deficiency associated abnormalities are applicable in humans is not yet clear.

1.1.2.2 Cancer

The idea of using vitamin C in treating and preventing cancer was first proposed in 1949 by Klenner, and later supported by Cameron and Pauling who, in a controversial study, showed that administration of high-dose ascorbic acid improved the survival of patients with terminal cancer (Cameron and Pauling, 1974; Cameron and Pauling, 1976; Cameron and Pauling, 1978). Their results led to the proposal of using mega doses of vitamin C to combat degenerative diseases, including cancer and cardiovascular disease.

One of the most important modifiable determinants of cancer risk is diet. Several research panels and committees have independently concluded that high fruit and vegetable intake decreases the risk of many types of cancer (Block, 1992; Steinmetz and Potter, 1996). As vitamin C is present in large quantities in these foods, it is conceivable that the reduction in cancer risk associated with the consumption of fruits and vegetables may be, at least in part, attributable to dietary vitamin C. This is supported by two large prospective studies, which showed that plasma vitamin C concentration is inversely related to cancer mortality in human subjects (Khaw *et al.*, 2001; Loria *et al.*, 2000). However, contradictory results have also been reported (Bjelakovic *et al.*, 2007; Blot *et al.*, 1993). The inconsistency in the vitamin C-cancer correlation and lack of sufficient mechanistic basis for its therapeutic action has critically undermined the scientific

plausibility of using vitamin C in clinical treatment or prevention of cancer (Padayatty *et al.*, 2006).

One of the most critical findings that have cast doubt over the effectiveness of vitamin C in treating cancer is the Moertel study, a randomized, placebo-controlled clinical study, in which high-dose was given orally to advanced cancer patients, but with no effect detected (Moertel *et al.*, 1985). It contradicted the findings of early studies conducted by Cameron *et al.* in which clear improvement in the health-status of terminal cancer patients were shown after high-dose intravenous vitamin C treatment (Cameron and Pauling, 1974; Cameron and Pauling, 1976; Cameron and Pauling, 1978). The discrepancy between these studies may be attributed to the difference in the plasma vitamin C concentrations achieved by different administration methods: the former administered vitamin C exclusively orally, whereas the latter both orally and intravenously. Maximum plasma vitamin C concentrations achievable by oral administration are limited by the renal systems, which eliminates excess ascorbic acid by decreasing the reabsorptive capacity of the proximal tubule. In contrast, as intravenous injection bypasses the renal absorptive system, it is able to temporarily elevated plasma concentrations to high levels (Padayatty *et al.*, 2004). This pharmacokinetic property of ascorbic acid was demonstrated recently in healthy subjects: intravenous administration resulted in substantially higher (~70-fold) plasma vitamin C levels than those attainable by oral dose (Padayatty *et al.*, 2004). In light of these results, it is likely that higher plasma concentrations were achieved in the Cameron study which used both IV and oral administrations, but not in the Moertel study, in which only oral administration was used.

The difference in effective vitamin C concentrations may have, in turn, contributed to the observed discrepancy in the therapeutic outcome between these two studies. Indeed, a recent case study examining the clinical history of three cancer patients and the treatment they received supports the notion that high-dose vitamin C administration through intravenous injection produces potential anti-tumor effects in certain types of cancer (Padayatty *et al.*, 2006).

Newly available pharmacokinetic data, improved understanding of its transporters and their regulations, and the growing evidence on its therapeutic efficacy have stimulated interest to reassess the scientific plausibility of using vitamin C in prevention and treatment of cancer. Though diverse in topic and methodology of investigation, most recent studies on vitamin C and cancer have been conducted around two central themes: 1) the effects of **high-dose** ascorbic acid on the development and progression of tumor and, 2) the **mechanisms of action** that may contribute to the anti-cancer effect of this vitamin.

High-dose i.v. vitamin C administration

Since achieving high levels ascorbic acid by intravenous injection was shown as feasible in vivo (Padayatty *et al.*, 2006), research has re-focused on the implications and applicability of high-dose intravenous vitamin C administration in cancer therapy. Pharmacologic concentrations of ascorbic acid (0.3—20 mM), which are comparable to those attained by IV administration, were shown to selectively target and kill tumor cells in vitro (Chen *et al.*, 2005). In contrast, physiological concentrations of ascorbic acid (0.1 mM) did not have any effect on either tumor or normal cells (Chen *et al.*, 2005). This

tumor-killing phenomenon was attributed to the pro-oxidant property of vitamin C, which, at high concentrations, mediates the production of toxic hydrogen peroxide (Chen *et al.*, 2005). This provides a potential mechanism of action for the anti-tumor effect of vitamin C and implicates it as a pro-drug in cancer treatment (Blot *et al.*, 1993; Chen *et al.*, 2005; Padayatty *et al.*, 2004). The manifestation of this effect in a real clinical setting has also been examined (Padayatty *et al.*, 2006). Conducted in accordance with the standard guidelines provided by the US National Cancer Institute, a case study examined the treatment effects of IV vitamin C administration on cancer progression in three patients with well-documented case histories (Padayatty *et al.*, 2006). In all three cases, high-dose intravenous vitamin C therapy effectively retarded the progression of malignant tumor, and improved the health status of these patients (Padayatty *et al.*, 2006). Unfortunately, the information on the plasma vitamin C concentrations of these patients is not available to establish a causal relationship between the route of administration, the resultant effective concentrations and the observed therapeutic effect. Nonetheless, this association can be reasonably assumed based on findings of a previous pharmacokinetic study (Padayatty *et al.*, 2004). However, it is difficult to ascertain the exact contribution of vitamin C in the clinical outcome, as all subjects under examination were receiving other forms of therapeutic treatments concurrent with high-dose vitamin C therapy (Assouline and Miller, 2006). Moreover, alternative explanations for this outcome cannot be readily ruled out. As point out by the authors (Padayatty *et al.*, 2004) and others (Assouline and Miller, 2006), the observed remission of cancer in these cases may be attributable to spontaneous remission or as the consequence of prior treatments

(Assouline and Miller, 2006) instead of ascorbic acid administration. Therefore, the therapeutic value of high-dose vitamin C administration in cancer progression or remission is not unequivocally supported by this study.

A recent clinical study, which prospectively examined the effects of IV vitamin C treatment on the health-related quality of life in terminal cancer patients, shows a direct positive correlation between vitamin C treatment and the health status of these patients (Yeom, Jung, and Song, 2007). After 1 week of high-dose therapy, the global health/quality of life, on both the functional, such as emotional and cognitive, and the symptom scale, such as fatigue and pain, were significantly improved in 39 terminal cancer patients (Yeom, Jung, and Song, 2007). Though not curative, vitamin C treatment in this case was shown to have successfully fulfilled an equally-important goal in combating cancer—the improvement in the quality of life, which is particularly critical in patients at the terminal stages of this disease, and, as such, vigorously pursued by clinicians. Although showing a direct relationship between vitamin C treatment and therapeutic benefits, the results of this study are not unequivocal. For example, like the aforementioned case study, the plasma ascorbic acid concentrations which resulted from the treatment were not measured. As in many cancer patients, especially those at the terminal stages, the absorption and excretion of certain drugs, including vitamin C, may be altered due to physiological abnormalities, which in turn may influence bioavailability. Thus the plasma vitamin C concentrations in these patients may not be comparable to those measured in healthy subjects in the early pharmacokinetic study (Padayatty *et al.*, 2004). For this reason, it is imperative to obtain direct information on plasma vitamin C

concentrations in future clinical studies, especially when cancer patients are employed as test subjects. Secondly, because of a lack of control groups, it is unclear whether the improved status in these patients is a directly result of vitamin C treatment. Nevertheless, the encouraging findings of these clinical (Yeom, Jung, and Song, 2007) and case studies (Padayatty *et al.*, 2006) have stimulated new interests for more systematic research. Recently phase I trial studies have been conducted to collect preliminary data on the efficacy, safety and pharmacokinetics of high-dose intravenous therapy, and systematically examine its potential application in cancer treatment (Assouline and Miller, 2006). The results of their study will certainly yield more insights into this type of therapeutic approach.

Mechanism of action

Parallel to clinical case/prospective studies examining the anti-cancer effects of high-dose vitamin C, experimental studies designed to investigate the mechanisms of action contributing to the therapeutic effect of vitamin C are concurrently being conducted, including its antioxidant or prooxidant function, its ability to modulate signal transduction and gene expression and its potential role in tumor metastasis.

Antioxidant and pro-oxidant. At physiological concentrations, vitamin C is a potent free radical scavenger in the plasma, protecting cells against oxidative damage caused by ROS (Carr and Frei, 1999). The antioxidant property of ascorbic acid is attributed to its ability to reduced potentially-damaging ROS, forming, instead, resonance stabilized and relatively stable ascorbate free radicals (Buettner and Sharma, 1993). This mechanism is manifest in a number of cyto-protective functions under physiological conditions,

including prevention of DNA mutation induced by oxidative stress (Lutsenko, Carcamo, and Golde, 2002; Noroozi, Angerson, and Lean, 1998; Pflaum *et al.*, 1998; Sweetman, Strain, and McKelveyMartin, 1997), protection of lipids against peroxidative damage (Barja and Hernanz, 1994; Kimura *et al.*, 1992), and repair of oxidized amino acids to maintain the integrity of proteins (Barja and Hernanz, 1994; Cadenas, Rojas, and Barja, 1998; Hoey and Butler, 1984). The effects of vitamin C on these three classes of biological molecules have been thoroughly reviewed by Carr and Frei (Carr and Frei, 1999). As DNA mutation is a major contributor to the age-related development of cancer (Deng *et al.*, 1998; Halliwell, 2000), attenuation of oxidation-induced mutations by ascorbic acid constitutes a potential anti-cancer mechanism. In vitro studies showed that intracellular vitamin C, at normal to high physiological concentrations (60—100 μM), decreases oxidative stress-induced DNA damage by neutralizing potentially mutagenic ROS (Lutsenko, Carcamo, and Golde, 2002; Noroozi, Angerson, and Lean, 1998; Pflaum *et al.*, 1998; Sweetman, Strain, and McKelveyMartin, 1997). In parallel with cell culture studies, several observational studies using 8-hydroxydeoxyguanosine (8-OHdG) as an indicator have found an inverse correlation between consumption of vitamin C-rich diets and the levels of oxidative DNA damage in vivo (Deng *et al.*, 1998; Fraga *et al.*, 1991; Rehman *et al.*, 1999; Thompson *et al.*, 1999). However, some have questioned the reliability of 8-OHdG as a biomarker of DNA damage (Halliwell, 2000). Moreover the view that DNA oxidation is directly involved in cancer initiation has not been substantiated by empirical evidence (Poulsen, Prieme, and Loft, 1998).

Conversely and paradoxically, ascorbic acid may function as a **pro-oxidant**, promoting oxidative damage to DNA (Stich *et al.*, 1976). This occurs in the presence of free transition metals, such as copper and iron, which are reduced by ascorbate, and, in turn, react with hydrogen peroxide, leading to the formation of highly reactive and damaging hydroxyl radicals (Stich *et al.*, 1976). However, the relevance of this in vitro phenomenon under normal physiological conditions in vivo has been questioned, as most transition metals required in this reaction exist in inactive, protein-bound form in vivo (Halliwell and Gutteridge, 1986). Interestingly, however, when used at pharmacological concentrations (0.3—20 mM), ascorbic acid displays transition metal-independent pro-oxidant activity, which is more profound in cancer cells and results in the death of these cells (Chen *et al.*, 2005). Further kinetic experiments showed that this tumor cell-killing response is dependent upon ascorbate incubation time and **extracellular** ascorbate concentration (Chen *et al.*, 2005). The findings of this study contradict a view that in vitro cancer-killing by vitamin C is a mere artifact due to the presence of free transition metals in the culture medium (Clement *et al.*, 2001; Golde, 2003). As demonstrated in this study, transition metal chelation had no effect on preventing cell death, indicative of a metal-independent mechanism in effect (Chen *et al.*, 2005). Another unexpected observation is that extracellular ascorbate was identified as the source of this anti-cancer effect; contrary to the traditionally-held view that intracellular vitamin C is the major contributor, though significance of this has yet to be determined. Although the mechanism of action for this cancer-killing effect has been identified, the reasons for the selectivity have not yet been confirmed. Nonetheless, the selective toxicity may be

attributed to several intrinsic properties of cancer cells, including reduced concentrations of antioxidant enzymes, such as catalase (Sun *et al.*, 1993; Yamaguchi, Sato, and Endo, 1992) and superoxide dismutase (Huang, He, and Domann, 1999; Sun, Colburn, and Oberley, 1993), increase in intracellular transitional metal availability (Gonzalez *et al.*, 2005), and better accumulation of DHA through GLUT transporter overexpression (Kawamura *et al.*, 2001; Kurata *et al.*, 1999), all contributing to the augmented intracellular hydrogen peroxide concentrations. Therefore, a nutritional regimen resulting in increased generation of hydrogen peroxide in vivo may be exploited as a means for inducing tumor-specific cyto-toxicity. Indeed, such regimen, in which high-dose vitamin C is administered in combination with other micronutrients and transitional metals, has recently been proposed (Gonzalez *et al.*, 2005).

The effective concentration of vitamin C required to mediate the cancer-killing mechanism can be easily achieved by intravenous injection (Chen *et al.*, 2005; Padayatty *et al.*, 2004), and maintained by repeated dosing in vivo, which provide plausibility for clinical application. Nonetheless more in vivo experiments are needed to gather and examine the pharmacologic information on IV vitamin C treatment.

Whether vitamin C functions as an antioxidant or pro-oxidant is dictated by at least three factors: 1) the redox potential of the cellular environment, 2) the presence/absence of transition metals, and 3) the local concentrations of ascorbate (Gonzalez *et al.*, 2005). The third factor is particularly relevant in treatments that depend on the antioxidant/prooxidant property of vitamin C, as it can be readily manipulated and controlled in vivo to achieve desired effects. For this reason, many pharmacokinetic

studies have been devoted to elucidate the mechanisms by which vitamin C is transported in the body.

Signal transduction, gene expression, and vitamin C. It is well known that the intracellular redox changes caused by oxidants and antioxidants can modulate the expression of genes involved in signal transduction pathways leading to cell cycle progression, cell differentiation and apoptosis (Allen and Tresini, 2000). Its ability to function as both an intracellular antioxidant and prooxidant provides a basis for a regulatory role for vitamin C in these mechanisms, which, with recent advances in cell culture systems and high-throughput screening techniques, are continuously being elucidated. For example, microarray studies showed that cells treated with ascorbic acid at low pharmacologic concentration (1 mM) increase expression of apoptotic genes induced by UV-irradiation and DNA damage, indicative of its role as a modulator of gene expression (Catani *et al.*, 2001). Ascorbate enhances the expression of both MLH1, a MutL homologue required for DNA mismatch repair machinery, and p73, a p53 homologue, increasing the cellular susceptibility to apoptosis, especially in the presence of DNA-damaging agents (Catani *et al.*, 2002). As the induction of MLH1 is a critical determinant in a cell's decision between pathways leading to either accumulation of mutation and subsequent tumorigenic progression or apoptosis (Catani *et al.*, 2002), these data support an anticancer role for intracellular vitamin C. The therapeutic relevance of vitamin C in cancer is further substantiated by its ability to activate the apoptotic program in DNA-damaged cells independent of p53 tumor suppressor through an alternative pathway mediated by p73, which, in contrast, is functional in most tumor types (Ikawa,

Nakagawara, and Ikawa, 1999). Ascorbate also stabilizes p53, and, in effect, augments the apoptotic response of Hela cells to chemotherapeutic agents (Reddy, Khanna, and Singh, 2001). At pharmacological concentration (1 mM), it decreases the Bcl-2/Bax ratio in the cytosol and mediates the mitochondrial release of cytochrome C, leading to the activation of the caspase cascade and apoptotic processes (Park *et al.*, 2004). This provides a mechanistic basis for combined therapy with vitamin C and chemotherapeutic drugs, as vitamin C potentiates the effectiveness of such drugs and, consequently, reduces the undesirable collateral damage to healthy cells (Catani *et al.*, 2002). However, the concurrent use of antioxidants such as ascorbic acid as chemotherapeutic agents is still controversial. A recent review article has been devoted to address this issue (Moss, 2006).

Vitamin C, at millimolar intracellular concentrations, inhibits the activation of NF κ B, a rapid response transcription factor, by preventing the tumor necrosis factor (TNF α)-mediated degradation of its inhibitor (I κ B) in different human cell lines as well as primary cells through independent mechanisms (Bowie and O'Neill, 2000; Carcamo *et al.*, 2002; Han *et al.*, 2004). As NF κ B induces transcription of genes involved in both inhibition of apoptosis and promotion of cell proliferation, its overexpression directly contributes to malignancy, and it has been implicated in a variety of cancers (Inoue *et al.*, 2007). Repression of constitutive activation of NF κ B by vitamin C can induce cell cycle arrest and apoptosis in these cells, and attenuate tumor progression in different types of cancer. Moreover, in vitro overexpression of the epidermal growth factor receptor family member Her-2/neu constitutively induces NF κ B activation, which likely contributes to the transformed phenotype in mammary tumor cells (Pianetti *et al.*, 2001). The recent

advances in transgenic animal models facilitate the examination of these phenomena in vivo. For example, the availability of Her-2/neu mice overexpressing this receptor (Andrechek and Muller, 2000) and *Gulo* knockout mice unable to produce vitamin C (Maeda *et al.*, 2000) provides possibility to create a strain of bi-transgenic knockout mice for examining the in vivo effects of high-dose vitamin C administration on breast cancer progression.

Ascorbate and its lipophilic derivatives attenuates cell proliferation, arrest cell cycle and induce apoptosis in human glioblastoma tumor and pancreatic cancer cells by downregulating the expression of insulin-like growth factor-I receptor (Naidu *et al.*, 2001; Naidu *et al.*, 2003). Cell cycle arrest induced by vitamin C can be also attributable to its ability to prevent the activation and nuclear accumulation of the mitosis-inducing phosphatase Cdc25C, hence providing a mechanism to restore cell cycle checkpoints in p53-deficient cells (Thomas *et al.*, 2005). The inhibitory effect is more potent in the lipophilic derivatives of ascorbate (Naidu *et al.*, 2001), which may have better intracellular accumulation. Therefore it is possible that synthetic vitamin C derivatives with increased lipophilicity exhibits higher bioavailability in vivo and thus improved therapeutic efficacy.

Can vitamin C attenuate metastasis? The spread of cancer, or metastasis, is initiated by disrupting the physical confinement imposed by the extracellular matrix (ECM) through the primary malignant cell-induced degradation of collagen structure (Gupta and Massague, 2006). As vitamin C is an essential factor for collagen maturation and stabilization, it is hypothesized that ascorbic acid may retard tumor spreading by

potentiating the stability of the ECM, especially in light of the observation that neoplastic invasion exhibits similar pathological manifestations as vitamin C deficiency (Gonzalez *et al.*, 2005). Unfortunately the effects of vitamin C on metastasis through collagen stabilization have not yet been examined *in vivo* due to the lack of appropriate animal models. Interestingly, in Gulo knockout mice, a vitamin C-independent pathway for collagen biosynthesis may exist in mice, as vitamin C restriction in Gulo knockout mice results in no detectable alteration in the levels of angiogenesis (Parsons *et al.*, 2006), a prerequisite for en masse tumor growth that requires sufficient collagen deposition. However whether the similar phenomenon occurs in humans and its possible implications are still unclear. In addition, conflicting results have been reported. For example, in the same mouse model, vitamin C depletion was shown to significantly attenuate tumor growth by impairing angiogenesis (Telang *et al.*, 2007), an observation that has cast some doubt over the anti-tumorigenic property of vitamin C. However, as pointed out by the authors, their findings are based on an implanted tumor that displays unusual dependence on angiogenesis (Telang *et al.*, 2007). Therefore, whether this mechanism is applicable for other clinical tumors in humans is largely uncertain. Moreover, tube formation of human endothelial cells, a process that mimics blood vessel formation, is attenuated by ascorbic acid at high physiological concentration (200 μM), but enhanced in a dose-dependent manner at normal physiological concentrations (< 100 μM) (Telang *et al.*, 2007), indicative of a dual-effect of vitamin C in blood vessel formation. However, the effects of supraphysiological (200 μM) or pharmacological levels (> 1 mM) of vitamin C

on angiogenesis in vivo, which are more relevant in clinical vitamin C therapy, were not investigated in this study.

Though not fully understood, there are two opposing views on the role of the collagen-stabilizing function of vitamin C on en masse tumor growth: 1) by stabilizing collagen, ascorbic acid fortifies the ECM and stromal structures, leading to better confinement of neoplastic cells to their primary sites and preventing tumor growth and metastasis (Gonzalez *et al.*, 2005), and 2) conversely, the same function may also facilitate the formation of new blood vessels, providing the prerequisite for malignant tumor growth (Telang *et al.*, 2007). The interplay of these effects in vivo, especially under pharmacological levels of vitamin C, is far from clear. However, with the availability of Gulo knockout mice and better understanding of collagen biosynthesis new research are being conducted to understand the mechanistic basis of these phenomena.

In addition to angiogenesis, cancer cells can also modify their energy metabolic pathways to adapt to the low oxygen microenvironment in the interior of a solid tumor (Leo, Giaccia, and Denko, 2004; Vaupel, 2004). This is achieved by activation of hypoxia-responsive gene expression networks controlled by hypoxia-inducible factor-1 α (HIF-1 α) (Harris, 2002). The activation of HIF-1 α by cancer cells is instrumental in both tumor growth and metastasis (Maxwell *et al.*, 1997; Vaupel, 2004). Ascorbate functions as a cofactor for hydroxylation of HIF-1 α (Knowles *et al.*, 2003). Proline hydroxylation targets HIF-1 α for ubiquitin-mediated degradation and thus decreases HIF-1 α levels in the cells. Furthermore, intracellular ascorbic acid can directly attenuate basal or hypoxia-induced expression of HIF-1 α in human primary and cancer cells (Vissers *et al.*, 2007).

The negative impact of ascorbate on HIF-1 α expression raises the question of whether intracellular vitamin C can inhibit the hypoxia-induced adaptation of solid tumor and thus restrict tumor growth and metastasis.

1.2 New developments

Since the publication of these articles, there have been several new discoveries on this vitamin, some of which are of significant relevance to this work. In the next section, I will review the findings of these studies.

1.2.1 Vitamin C and hypoxia-inducible factor (HIF)

The role for vitamin C in the inhibition of HIF has been reviewed in Section 1.1.2.2. To recapitulate, ascorbic acid can sequester reactive oxygen species (ROS) released by mitochondria upon oxygen deficiency, and, in so doing, attenuate ROS-mediated HIF stabilization. Likewise, ascorbic acid can function as a cofactor in proline hydroxylation, which targets HIF-1 α for ubiquitin-mediated degradation. Both processes lead to decreased HIF-1 α levels in the cells. As HIF-1 α is implicated in tumour angiogenesis and metastasis, inhibition of this transcription factor by ascorbic acid likely attenuates the progression of solid tumours. This effect has been shown primarily in cell cultures (Vissers *et al.*, 2007).

Recently, an *in vivo* study showed that immunodeficient mice engrafted with tumour cells exhibited decreased levels HIF-1 α in these cells when maintained on vitamin C water as compared to those deprived of dietary vitamin C (Gao *et al.*, 2007). In addition, a reduction in tumour volume was also observed in vitamin C-supplemented mice engrafted with cells carrying wild type HIF-1 α . In contrast, mice engrafted with tumour cells carrying proteasomal degradation-resistant HIF-1 α did not display comparable levels of tumour reduction, suggesting that ascorbic acid induces tumour reduction primarily through HIF-1 α attenuation (Gao *et al.*, 2007).

This study, however, did not provide any information on the serum vitamin C concentrations in these mice. As mice produce ascorbic acid endogenously, it is unclear whether dietary supplementation used in this study has resulted in any significant increase in effective vitamin C concentrations in supplemented mice as compared to deprived mice.

1.2.2 Pharmacologic levels of vitamin C generates hydrogen peroxide in vivo

As discussed in Section 1.1.2, vitamin C, at pharmacologic concentrations (0.3-20 mM), selectively targets and kills tumour cells *in vitro* by production of hydrogen peroxide (Chen *et al.*, 2005). This selective toxicity towards cancer cells provides a mechanistic basis for using high-dose ascorbate in cancer treatment. The possibility of achieving high level serum vitamin C has also been demonstrated. Intravenous injection of ascorbate raised serum vitamin C to supra-physiologic levels for at least 4 h (Padayatty *et al.*, 2004). However, this study did not report any physiologic or therapeutic effects of serum vitamin C at this level. Recently, intravenous ascorbate injection into rats has been shown to promote the formation of ascorbyl radicals and, in turn, hydrogen peroxide in extracellular fluid (Chen *et al.*, 2007). Levels of hydrogen peroxide formed *in vivo* as a result of high serum ascorbate were comparable to levels that lead to selective tumour killing, indicating that the selective toxicity observed in cell cultures may be replicable in animal models (Chen *et al.*, 2007). However, as the animal model used in this study was not a cancer model, a direct connection between high serum ascorbate and tumorigenesis cannot be established.

Chapter 2: Restoration of Vitamin C production by gene therapy

2.1 Introduction

Emerging evidence in *in vitro* experiments has led to the hypothesis that supra-physiological levels of ascorbic acid in the serum may be necessary for its anti-tumor effects (Li *et al.*, 2006; Li and Schellhorn, 2007). However, in humans, serum and tissue vitamin C levels are tightly controlled by intestinal absorption and renal excretion (Padayatty *et al.*, 2004). As a result, high-physiologic concentrations of serum vitamin C required for anti-cancer effects cannot be achieved by oral ingestion (Padayatty *et al.*, 2004).

Administration methods resulting in supra-physiologic levels of vitamin C have been investigated. Intravenous injection of ascorbate bypasses barrier in intestinal absorption and results in peak levels of vitamin C in the blood comparable to those required for tumor-killing in cell cultures (Chen *et al.*, 2005; Padayatty *et al.*, 2004).

As vitamin C deficiency is a genetic disorder, and animals with the wild type allele of the *Gulo* gene produce large quantities of ascorbic acid (Data not shown), I propose that complementation of this genetic defect in scurvy-prone animals by gene therapy can restore vitamin C production and result in sustained levels of ascorbic acid in the blood. In this study, I investigate the feasibility of using gene therapy as an alternative vitamin C delivery method. Here, I characterized previously constructed helper-dependent adenoviral vectors (HDAd) carrying the coding sequence for GULO (*Gulo*)

under the control of murine cytomegalovirus (mCMV) immediate-early promoter (HDAd-mCMV-*Gulo*) or human phosphoenolpyruvate carboxykinase (PEPCK) promoter ((HDAd-mCMV-*Gulo*). I tested the ability of these gene therapeutic vectors to mediate the expression of GULO in human hepatocellular carcinoma (HEPG-2) cells and in transgenic mice carrying genetic knockout of *Gulo*. Ultimately, serum and urine vitamin C levels of these mice treated with either HDAd-mCMV-*Gulo* vectors or maintained on dietary vitamin C were measured using HPLC with electrochemical detection (HPLC-cECD) and compared to wild type mice of the same strain background.

This is the first study that both recognizes vitamin C deficiency as a genetic disorder and attempts to correct it by genetic means. Similar to IV vitamin C administration, endogenous vitamin C production bypasses intestinal absorption, and, as such, may lead to supraphysiologic concentrations of vitamin C in the serum. In addition, it can mediate constitutive ascorbic acid production and thus sustained levels of vitamin C in the blood. By manipulating the regulatory elements for the *Gulo* transgene, this delivery model allows controlled production of vitamin C in targeted tissues. This provides the opportunity for target-delivery of toxic doses of ascorbic acid to cancer cells, hence offering additional selective toxicity to tumor cells.

2.1.1 Gulonolactone oxidase (GULO)

Gulonolactone oxidase (GULO) (50 kDa) is the key enzyme catalyzing the final, rate-limiting reaction of the ascorbic acid biosynthetic pathway (Nishikimi *et al.*, 1994). Unlike most mammals, primates lost the ability to manufacture vitamin C through an

inactivating mutation on the GULO encoding gene (*Gulo*) (Nishikimi *et al.*, 1988), and, as such, are dependent on dietary sources for survival.

Cross hybridization experiments have shown that the human genome contains sequences that exhibit certain homology to the wild type *Gulo* cDNA derived from rats (Nishikimi *et al.*, 1988), which suggests that humans have retained partial sequence of the GULO coding sequence. In subsequent studies using spot blot hybridization, human *Gulo* pseudogene was mapped onto chromosome 8 at region p21.1 (Nishikimi *et al.*, 1994). Further sequencing analysis on the 3'-half of the gene showed the complete deletions of exon VIII and XI, and occurrence of point mutations and partial deletions on exon X and XII. In addition, two *Alu* insertions were located between exon X and XII (Nishikimi *et al.*, 1994). Later computer-assisted study showed that human *Gulo* cDNA contains five exons, as compared to the 12 exons present in wild type rat. Consistent with early findings, several disruptive mutations were located in the existing exons (Inai, Ohta, and Nishikimi, 2003).

The reasons for these mutational events leading to this nutritional disorder still remain a topic of speculation. To date, several hypotheses have been proposed to address the question of why we lost the ability to produce vitamin C despite our absolute need for this nutrient (Benzie, 2003; Calabrese, 1982; Challem, 1997; Millar, 1992). One group suggested that the loss of endogenous vitamin C indirectly resulted in a selective mechanism against the ageing populations of primates, leaving more food available for the young and fertile (Millar, 1992). Yet another group proposed that, since vitamin C is a potent antioxidant, loss of its endogenous production likely led to accumulation of free

radicals, which might have increased the rate of mutation and, as such, propelled the evolution of primates (Challem, 1997). Despite differences in the mechanism of selection, it is generally agreed that, under the conditions around the time of this inactivation, selective advantages were conferred by losing *Gulo*.

The onset of GULO inactivation was estimated to have occurred around 30 million years ago (Nishikimi *et al.*, 1994). This is based on phylogenetic evidence and actual evolutionary events: a) as GULO mutation occurs in both New World and Old World monkeys, the mutation event must have taken place before their divergence, 35—45 million years ago; and b) as prosimians have functional GULO and simians do not, this mutation must have happened after the divergence of these two lineages, 50—65 million years ago (Nishikimi *et al.*, 1994).

Recently, with the advance of molecular techniques, large amounts of DNA sequence information have become readily available, providing the possibility of re-estimating the time of this inactivation event using DNA sequence data. A survey of the human genome showed that, out of the 12 exons constituting the functional *Gulo*, exons 1-6, 8 and 11 are missing from the human *Gulo* pseudogene (Ψ *Gulo*), whereas exons 7, 9, 10 and 12 remain structurally intact (Ha *et al.*, 2004). By comparing the existing exon sequences of human Ψ *Gulo* and wild type mouse *Gulo*, the time of this loss of function mutation in primates could be inferred. Similar type of estimation has been done for guinea pig Ψ *Gulo*, which, like that of human, is non-functional but with structurally intact exons (Nishikimi *et al.*, 1994).

2.1.2 Helper-dependent adenovirus vectors

First Generation Adenovirus vectors (FGAd) carrying the mCMV-*Gulo* have been created and shown effective in mediating GULO expression and vitamin C production in cell cultures (Ha *et al.*, 2004). However, it is unclear whether these vectors are able to mediate vitamin C production in animals.

The reason that I constructed HDAd vectors is that, despite their ability to mediate vitamin C production *in vitro*, FGAd vectors express adenoviral genes from the vector backbone (Morral *et al.*, 1997), which evokes host immunity and results in cytotoxicity. The consequences include immuno-targeting of viral proteins, elimination of infected cells, shortened transgene expression, and more seriously, acute and chronic host reactions (Dai *et al.*, 1995; Yang, Gao, and Ji, 1994).

In comparison, HDAd vectors lack viral genes, and, as such, do not evoke adaptive host immunity, which allows long-term transgene expression in transduced organs (Clemens *et al.*, 1996; Fisher *et al.*, 1996; Kochanek *et al.*, 1996; Mitani *et al.*, 1995; Parks *et al.*, 1996). However, despite their ability to evade adaptive host immunity, HDAd vectors elicit acute immune responses in the host, which is triggered by viral capsid proteins (Muruve *et al.*, 2004). Acute host immune response to internalized HDAd capsid is characterized by early induction of inflammatory cytokine and chemokine after infection but before any viral gene expression can occur (Muruve *et al.*, 1999). It is also observed in animals infected with transcriptionally inactive virus (Schnell *et al.*, 2001).

HDAd is advantageous over other transgene delivery vectors in long-term expression of the therapeutic transgene with minimal toxicity. The problems currently associated with this system include comparably complicated preparation, innate host

response and non-linear pharmacokinetics. In the context of this study, HDAd vectors are suitable because Ad vectors exhibit tropism towards the liver which is the site where endogenous vitamin C production in non-scurvy animals occurs.

2.1.3 Mouse model

The lack of appropriate animal models has been a major obstacle in studying the physiologic effects of ascorbic acid. Most animals, including laboratory rodents, such as mice and rats, carry the wild type allele of the *Gulo* gene, and, as such, are able to produce endogenous vitamin C, making controlled induction of vitamin C deficiency impossible. Guinea pigs have been used as a model for studying the nutritional effects of ascorbic acid, as they, like humans, are unable to produce vitamin C due to a *Gulo* genetic defect. Intraperitoneal injection of GULO has been shown to rescue vitamin C deficiency in guinea pigs provided that they are also supplemented with gulonolactone and other essential vitamins (Hadley and Sato, 1988). However, guinea pigs cannot be used to study the more advanced properties of vitamin C such as those involved in anti-tumorigenesis, because there have not been any established guinea pig cancer models.

Most cancer animal models are established in mice, making them an ideal background for studying the mechanisms of action of a candidate anti-cancer drug. To this end, transgenic mice carrying a null allele of the *Gulo* gene have been constructed (Maeda *et al.*, 2000). Targeted knockout was introduced into the coding sequence of the endogenous *Gulo* gene by deleting exons 3 and 4 on the C57BL/6 background (Maeda *et al.*, 2000). Mice homozygous of this allele (*Gulo*^{-/-}) have been shown to depend on dietary vitamin C for survival (Maeda *et al.*, 2000). Weight loss was observed in mature *Gulo*^{-/-}

mice maintained on vitamin C deficient diet for 5 weeks. Plasma vitamin C concentrations were measured to be 1.7 $\mu\text{g/ml}$ (9.7 μM) in *Gulo*^{-/-} mice and 11.1 $\mu\text{g/ml}$ (63.3 μM) in wild type mice. With dietary vitamin C supplementation through drinking water (0.3 g/L), plasma vitamin C levels in *Gulo*^{-/-} mice were increased to 5.7 $\mu\text{g/ml}$ (32.5 μM) (Maeda *et al.*, 2000).

The creation of *Gulo*^{-/-} mouse model opens the possibility for examining the effect of vitamin C on different biologic or pathologic processes. By crossing this model with other existing mouse models carrying a particular disease marker, one could generate a bi-transgenic mouse model, in which the effects of exogenous vitamin C on the disease marker can be studied. For example, crossing *Gulo*^{-/-} mice with BigBlue mice carrying an indicator for mutation frequency gives rise to doubly transgenic *Gulo*^{-/-} mice harboring a mutation marker, which allows for the examination of the effect of vitamin C on mutation frequency.

In the context of this study, these *Gulo*^{-/-} mice can be used to examine the levels of vitamin C production mediated by the HDAd vectors. As mice are significantly smaller in size than guinea pigs, their application as models can substantially reduce viral dose.

2.1.4 HPLC-electrochemical detection (HPLC-ECD)

High-performance liquid chromatography (HPLC) electrochemical detection (ECD) is an effective and sensitive way to separate and measure ascorbic acid in biological samples. In this section, I present a brief review on HPLC-ECD and its application in quantification of ascorbic acid.

A HPLC instrument usually consists of several components: a mobile phase reservoir, a pump, an autosampler/sample injector, a column and a detector (**Figure 5**).

The mobile phase reservoir contains large quantities of mobile/liquid solution (1-4 L), which is pumped continuously through the system and carries the injected sample through the column into the detector. A key feature of the mobile phase is that it stabilizes the molecule to be assayed. In ascorbic acid measurement, 0.2 M potassium phosphate buffer (pH3.0) supplemented with 0.2 mM EDTA is frequently used. The pump is an essential component of a HPLC system. It should be able to withstand high pressure and drive the mobile phase through the system at a specific flow rate. Sample injector is where the sample is injected into the system. It is often controlled by a computer to deliver a defined volume of the sample. Most injectors are integrated with an autosampler, which can be programmed to deliver a large number of samples from a multi-vial tray in an automated fashion, greatly reducing the labor of the assay. Once injected into the HPLC system, the sample is carried by the mobile phase into the column, where different substances are separated based on their interaction with the column. Like other chromatographies, the type of column selected for separation of a particular substance is based on specific properties of that compound and coexisting species in the sample. In the case of vitamin C separation, a hydrophobic Carbon-18 (C-18) column is used. C-18 column separates different compounds based on their hydrophobicity: hydrophobic species interact more strongly with the column and, in effect, are retained, whereas polar compounds such as ascorbic acid do not bind to the column and, as such, are eluted out at an earlier time. As a hydrophobic stationary phase (column) is used to

separate a polar species, this type of HPLC is commonly referred to as “reversed phase chromatography.” With a specific set of parameters, including the type of mobile phase, flow rate, and column type, a given species is eluted from the column at a defined time in relation to the solvent front, and this time is referred to as the “elution time” or “retention time.” In the case of ascorbic acid, it is eluted at a retention time of approximately 1.1 min, at a flow rate of 3 ml/min through a XDB-C18 analytical column (particle size 5 μ m, 4.6 x 250 mm). A typical chromatogram of vitamin C is shown in **Figure 6**.

After separation, different compounds in a sample are detected by a detector, resulting in a chromatographic representation of the elution sequence consisting of a series of “peaks.” These peaks assume a bell shape and the area covered by a peak is proportional to the concentration of a particular species. Therefore, elution time can be used to identify a species and the size of the chromatographic peak can be used to quantify that species. There are different types of detectors. In vitamin C detection, absorbance-based detector and electrochemical detector are frequently used. Absorbance-based detector detects a species based on its ability to absorb/transmit light at a specific wavelength, and the absorbance is, within certain range, proportional to the concentration of the species, hence allowing quantitative measurement. Ascorbic acid absorbs strongly at a wavelength of 245 nm, which has been used for its quantification. Electrochemical detector detects substances that are redox active (reducible or oxidizable) and requires a conducting mobile phase, and is suitable for reversed phase HPLC applications, such as ascorbic acid detection. There are two modes of electrochemical detections: coulometric and amperometric. In coulometric detection, reduction or oxidization of a substance at the

electrode surface reacts to completion, exhausting all reactants and resulting in a current signal. This signal is proportional the mass of the substance present in the sample. In ascorbic acid measurement, coulometric detection is more commonly used. Ascorbic acid has a reduction potential (E'_0) of -223mV at pH 3.0. With the settings in this study: Guard Cell at -200 mV, Analytical Cell Channel 1 at -150 mV, Channel 2 at 150 mV, ascorbic acid is readily detected with a response factor of 2×10^{-12} μg vitamin C per unit area.

2.2 Materials and methods

2.2.1 Helper-dependent adenovirus vectors

2.2.1.1 Construction of HDAd-*mCMV-Gulo* and HDAd-*PEPCK-Gulo* vectors

The *XbaI-SalI* fragment containing *Gulo* under the mCMV promoter was subcloned into the recipient vector pSC11PA at *XbaI-XhoI* sites, resulting in shuttle plasmid pYDL1 (pSC11MC-*Gulo*), which carries the mCMV-*Gulo*-PolyA construct flanked by *I-SceI* and *I-CeuI* restriction sites. The *I-SceI-I-CeuI* fragment from pYDL1 was then subcloned into an adenovirus precursor vector pSC15B (Shi, Graham, and Hitt, 2006) which contains viral *cis*-acting elements, hence resulting in the helper-dependent adenoviral (HDAd) vector pSC15BMC-*Gulo* (**Figure 7A**). Likewise, the *SmaI-SalI* fragments containing *Gulo* cDNA was subcloned from pMNH1 into plasmid pSC11PECKPS6801 at *ClaI-XhoI* site treated with T4 DNA polymerase, generating the shuttle vector pYDL2 (pSC11PECK-*Gulo*) with *Gulo* cDNA under the PEPCK promoter. The *I-SceI-I-CeuI* fragment from pYDL2, which contains PEPCK-*Gulo*-PolyA construct, was subsequently inserted into the stuffer plasmid pSC15B (Shi, Graham, and Hitt, 2006), and resulted in HDAd vector pSC15BPEPCK-*Gulo* (**Figure 7B**).

Viable HDAd vectors were produced by transfection of HEK293Cre4 cells with pSC15BmCMV-*Gulo* or pSC15BPEPCK-*Gulo* plasmid, followed by infection with Ad2 helper virus at a MOI of 5pfu/cell as described previously (Ng, Parks, and Graham, 2002). The HDAd vectors isolated from plaques were then propagated and amplified to large quantities in HEK293Cre4 cells with Ad2 helper virus according to standard protocols (Ng, Parks, and Graham, 2002).

2.2.1.2 Purification of HDAd-*mCMV-Gulo* and HDAd-*PEPCK-Gulo* vectors

Amplified HDAd-*mCMV-Gulo* and HDAd-*PEPCK-Gulo* vectors were purified by two rounds of CsCl ultracentrifugation as described (Ng, Parks, and Graham, 2002). Briefly, infected 293Cre4 cells exhibiting CPE were lysed with 0.5% sodium deoxycholate for 30 min, followed by 1 h DNaseI (50 µg) digestion at 37 °C. A volume of 5 ml supernatant from cell lysate was overlaid onto each CsCl step gradient consisting of 3 density steps: 0.5 mL of 1.5 g/mL, 3 mL of 1.35 g/mL and 3 mL of 1.25 mL, from bottom to top. The crude cell lysates containing virion were centrifuged at 35000 rpm for 1 h at 10 °C. The viral bands locating at the 1.25/1.35 interface were collected and subjected to a continuous gradient centrifugation in 1.35 g/mL CsCl at 4°C for 24-48 h. The resultant viral bands were collected and dialyzed with Slide-A-Lyzer® Dialysis Cassettes with MWCO of 10,000 at 4°C against three changes of 500 mL 10 mM Tris-HCl pH 8.0 for 36 h. HDAd titer was determined by spectrophotometric measurement of DNA concentration at 260 nm (Thermo Multiscan).

2.2.1.3 Characterization of HDAd-*mCMV-Gulo* and HDAd-*PEPCK-Gulo* vectors

Purified viral particles were lysed in pronase-SDS solution (0.5 mg/mL pronase, 0.5% SDS, 10 mM Tris-Cl, pH 7.4, 10 mM EDTA, pH 8.0) at 37 °C. Viral DNA was precipitated in 3M sodium acetate, pH 5.2, and 95% ethanol at -20 °C for 30 min. The DNA precipitate was washed with 70 % ethanol and resuspended in water. *Gulo* specific primers were used to probe for the *Gulo* gene in viral genome of both HDAd vectors in a standard PCR reaction.

2.2.2 Cell cultures

2.2.2.1 HEK293 cell culture

Human embryonic kidney 293 cells were grown at 37°C and 5% CO₂ in minimal essential media F-11 (MEM F-11) supplemented with 2 mM L-glutamine and 10% fetal bovine serum (FBS). Fungizone amphotericin B (2.5µg/ml) and penicillin/streptomycin (100U/ml) were added to the media to prevent microbial growth. G418 (200µg/ml) was added to 293Cre4 cell media to maintain stably transfected *cre4* gene. After infection with the HDAd vectors, cells were maintained in serum-poor media (2% FBS) under the same conditions.

2.2.2.2 HEP G-2 cell culture

Human hepatocellular carcinoma HEP G-2 cells were grown at 37°C and 5% CO₂ in D-minimal essential media (D-MEM) supplemented with 2 mM L-glutamine and 10% fetal bovine serum (FBS). Fungizone amphotericin B (2.5µg/ml) and penicillin/streptomycin (100U/ml) were added to the media to prevent microbial growth. After infection with the HDAd vectors, HEP G-2 cells were maintained in serum-poor media (2% FBS) under the same conditions. Cells were trypsinized and split 1:5 every 3 days or until confluence.

2.2.3 In vitro experiments

2.2.3.1 Protein extraction

To extract intracellular proteins from cell cultures (6-well plates), cells were first washed with PBS, and 250 µl CellLytic M lysis/extraction reagent (Sigma) was added to the cell surface (35 mm) followed by a 15 min incubation. Cells were then scraped off from the plate and centrifuged at 12,000 g for 20 min at 4°C. Cell free supernatants were

extracted and protein concentrations were determined by Bradford Assay using bovine serum albumin as standard.

To extract intracellular proteins from the liver, resected livers were thawed on ice and homogenized in CellLyticMT reagents (1 g tissue/2 ml reagent) (Sigma). Homogenized samples were centrifuged at 12,000 g for 20 min at 4°C. The protein containing supernatant was extracted and centrifuged again at 12,000 g for 20 min at 4°C to eliminate any residual cell debris. Protein concentrations were determined by Bradford Assay using bovine serum albumin as standard.

2.2.3.2 GULO Western blot

Cell lysates from cells cultures or tissues were boiled for 10 min in the presence of 2% SDS and 5% β -mercaptoethanol to denature cellular proteins. Protein samples (30 μ g) were subjected to SDS-polyacrylamide electrophoresis using a stacking gel with 4% upper gel and 10% running gel. After electrophoresis, resolved proteins were transferred to a PVDF membrane for 1 h at 90 V. Membrane blots were then blocked overnight in 5% milk dissolved in tris-buffered saline tween-20 (TBST) buffer. The blocked blots were then incubated for 1 h with primary antibody (rabbit) specific for the N-terminal domain of GULO (Kim *et al.*, 2006). After probed with the primary GULO antibody, blots were washed with TBST buffer and hybridized with secondary goat anti-rabbit antibody (Biorad) for 1 h. After a second round of washing with TBST, blots were incubated in ECL detection reagents (GE Healthcare) and exposed to ECL Chemiluminescence film (GE Healthcare), and developed.

2.2.3.3 HPLC-electrochemical detection of ascorbic acid

Intracellular and extracellular fluids were prepared and diluted in 5% metaphosphoric acid supplemented with 2mM EDTA (5% MPA-EDTA) prior to HPLC analysis. The HPLC system (Agilent 1200 Series) is consisted of a micro vacuum degasser, a binary pump SL, a high performance autosamplers and an Agilent XDB-C18 analytical column (particle size 5 μm , 4.6 x 250 mm).

Mobile phase was composed of 0.2 M KH_2PO_4 (pH 3.0) and 2mM EDTA and was pumped at a flow rate of 3 ml/min. The retention time of ascorbic acid with this setting is approximately 1.1 min. Eluted ascorbic acid was measured by an ESA CoulochemIII coulometric electrochemical detector. The ESA electrochemical detector was set as follows: Guard Cell at -200 mV, Analytical Cell Channel 1 at -150 mV, Channel 2 at 150 mV. Output signal from Channel 2 was used for vitamin C measurement. Calibration curve was established using ultrapure L-ascorbic acid (Sigma) prepared in 5% MPA-EDTA. System programming and chromatogram analysis were performed using EZChrom Elite software (Agilent). The response factor calculated from the calibration curve is 2×10^{-12} μg ascorbic acid per nC signal output.

2.2.3.4 Expression of GULO in HEP G-2 cells treated with *Gulo*-carrying vectors

To determine whether HDAd-mCMV-*Gulo* and HDAd-PEPCK-*Gulo* vectors can mediate GULO expression *in vitro*, human HEP G-2 cells were infected with HDAd vectors and the intracellular proteins were probed using Western blot for GULO expression. A 6-well plate of confluent HEP G-2 cells was used in this experiment. Serum rich media (10% FBS) was removed from the plate, HDAd vectors at a multiplicity of infection (MOI) of 500 viral particles (vp) per cell were added onto the cell surface.

Serum poor media (2% FBS) was then added to each well (3 ml/well). Infection was allowed for 48 h. At 48 h post-infection, intracellular proteins were extracted from the cells and Western blot was performed to probe for GULO expression as described previously.

2.2.3.5 Expression of GULO as a function of multiplicity of infection

To determine whether GULO expression varies with gene dosage, I infected HEP G-2 cells with increasing MOI of HDAd-mCMV-*Gulo*. Again serum-rich media were removed from 6-well plates carrying confluent HEP G-2 cells. HDAd-mCMV-*Gulo* at MOIs of 0, 1, 10, 50, 75, 100, 200, 400, 500, 750, and 1000 vp/cells was added into each well. Infection was allowed for 48 h, after which intracellular proteins were extracted and Western blot was conducted as described. In addition to GULO, β -actin levels in cells were also determined for the purpose of loading control.

2.2.3.6 Time-dependent expression of GULO in HDAd-infected cells

To establish a timeline for GULO expression after infection, I infected HEP G-2 cells with HDAd-mCMV-*Gulo* with a MOI of 500 vp/cell for different durations. After infection with HDAd-mCMV-*Gulo* as described previously, intracellular proteins were isolated from infected HEP G-2 cells at 0, 6, 18, 24, 36, 48 and 120 hours post-infection. GULO expression was probed using Western blot as described.

2.2.3.7 Production of ascorbic acid in HEP G-2 infected with HDAd-mCMV-*Gulo*

Confluent HEP G-2 cells (3.5 cm well) were infected with HDAd-mCMV-*Gulo* at a MOI of 500 vp/cell as described. Serum-poor media (3 ml) with or without L-gulononic- γ -lactone (10 mM) (Sigma) was added to the infected cells. Infection/incubation was

allowed for 48 h. Cells were trypsinized and washed twice with PBS. Cells were resuspended in 300 µl of 5% MPA-EDTA and lysed by freeze-thaw method. Cell lysates were centrifuged at 12,000 g for 20 min at 4°C. Cell-free supernatants were analyzed for ascorbic acid using HPLC-ECD as described previously.

2.2.3.8 Gulonolactone-dependent production of ascorbic acid

To examine the relationship between ascorbic acid production in cells infected with HDAd-mCMV-*Gulo* and the concentrations of L-gulonic-γ-lactone, I incubated HDAd-treated HEP G-2 cells with increasing concentrations of gulonolactone.

Confluent HEP G-2 cells were infected with HDAd-mCMV-*Gulo* at a MOI of 500 vp/cell as described previously. Serum-poor media supplemented with 0, 0.05, 0.1, 1, 2, 5 and 10 mM gulonolactone ($n=3$) was added to the infected cells. Cells were incubated for 48 h and intracellular fluids were obtained and analyzed for ascorbic acid production as described before.

2.2.4 Animal experiments

2.2.4.1 Mice

Adult male C57BL/6 and C57BL/6 *Gulo*^{-/-} mice were used for the experiments (Maeda *et al.*, 2000). All mice were housed in the Central Animal Facility at McMaster University under controlled conditions with free access to food and water. All experiments were performed according to Animal Utilization Protocols (Schellhorn 06-02-11). Mice were fed on vitamin C deficient diet and were given drinking water

containing 330 mg/L vitamin C to prevent scurvy (Maeda *et al.*, 2000). Vitamin C water was withdrawn 1 month prior to this study to eliminate vitamin C from the body.

2.2.4.2 Administration of HDAd-*mCMV-Gulo* vectors

Mice were anaesthetized with isoflurane. A volume of 0.3 ml of either 0.9% saline or HDAd vectors prepared in saline was injected into the tail vein of anaesthetized mice. After administration of treatments mice were housed three per cage according to the treatment received.

2.2.4.3 Collection of biological fluids

Blood samples were collected from the saphenous vein using heparin-coated blood collecting tubes (Kimble-Chase), and centrifuged at 1000 g for 5 min. Cell-free plasma (supernatant) were diluted (1:10) with 5% MPA-EDTA and centrifuged at 12,000 g for 30 min at 4°C to eliminate protein precipitate. Supernatant was extracted and stored at -80°C.

Urine samples were collected directly from the penis of the mouse and diluted with 5% MPA-EDTA and stored at -80°C until use.

2.2.4.4 Liver extraction

Mice were anaesthetized with Ketamine (150mg/kg) and Xylazine (10mg/kg) by intraperitoneal injection, and were perfused systemically with PBS through the left ventricle of the heart. After the liver has discolored, it was resected and frozen immediately in liquid nitrogen and stored at -80°C until use.

2.2.4.5 Expression of GULO in mice treated with HDAd-*mCMV-Gulo* vectors

Gulo^{-/-} mice were anaesthetized with isoflurane and infected with HDAd-*mCMV-Gulo* vectors by intravenous injection at doses of 0, 1x10¹¹ and 2x10¹² viral particles. At 48 h post-infection liver was extracted and GULO expression probed with Western blot as described.

2.2.4.6 Vitamin C production in mice treated with HDAd-*mCMV-Gulo* vectors

Wild type and *Gulo*^{-/-} mice were deprived of dietary vitamin C for 1 month, and were assigned into 4 groups according to treatments received (**Table 2**). Briefly, Group 1, consisting of 4 *Gulo*^{-/-} mice, was maintained on vitamin C deficient diet and water; Group 2, consisting of 4 *Gulo*^{-/-} mice, was treated with HDAd-*mCMV-Gulo* vectors at a MOI of 2x10¹² viral particles and maintained on vitamin C deficient diet and water; Group 3, consisting of 4 wild type mice, was maintained on vitamin C deficient diet and water; Group 4, consisting of 4 *Gulo*^{-/-} mice, was maintained on vitamin C water (330 mg/ml).

Blood samples were collected from these mice three times a week for 1 month. Urine samples were collected on day 4. Ascorbic acid concentrations in these samples were measured using HPLC-ECD as described previously.

2.2.5 Data analysis and processing

2.2.5.1 Data analysis

HPLC-ECD chromatographs were identified, integrated and exported using EZChrom Elite software (Agilent). All data were processed with Microsoft Office Excel 2003.

Graphics, including gel photographs and data charts were processed and edited with Microsoft Office PowerPoint 2003.

2.3 Results

2.3.1 Helper-dependent adenovirus vectors

2.3.1.1 HDAd-*mCMV-Gulo* and HDAd-*PEPCK-Gulo* vectors

HDAd-Gulo vectors carrying the mCMV promoter and the PEPCK promoter were constructed and characterized. A structural map of these vectors was shown in **Figure 8**. Diagnostic PCR revealed a 1.3 kb fragment corresponding to the size of the primer flanking region on *Gulo* cDNA in both vectors (**Figure 9**).

2.3.2 In vitro experiments

2.3.2.1 GULO is expressed in HEP G-2 cells infected with HDAd-mCMV-*Gulo*

To determine whether HDAd-mCMV-*Gulo* and HDAd-PEPCK-*Gulo* vectors are able to mediate GULO expression *in vitro*, human HEP G-2 cells were infected with the HDAd vectors at a multiplicity of infection (MOI) of 500 vp per cell for 48 h. GULO expression in these cells was determined by Western blotting with GULO specific antibody. GULO with molecular weight of 50kDa was detected in cells infected with HDAd-mCMV-*Gulo* (**Figure 10, Lane 2**) but not in those infected with HDAd-PEPCK-*Gulo* vectors (**Figure 10, Lane 1**). No expression was detected in mock infected cells with PBS (**Figure 10, Lane 3**), confirming that human cells do not express endogenous GULO.

2.3.2.2 GULO expression is dependent on the MOI of HDAd-mCMV-*Gulo* vectors

To determine whether GULO expression varies with gene dosage, I infected cells with increasing MOIs of HDAd-mCMV-*Gulo* vectors. We found that the expression of GULO was dependent on the MOI used within the test range (**Figure 11**). The minimal MOI that resulted in detectable expression was 50 vp/cell (**Figure 11, Lane 4**). Levels of intracellular GULO increased quantitatively with MOIs from 50 vp/cell to 1000 vp/cell. At 1000 vp/cell, however, cell detachment was observed. Intracellular β -actin levels remained constant in all cells independent of MOI of the HDAd vectors.

2.3.2.3 GULO expression exhibits time-dependence

To establish a timeline for GULO expression after infection, I infected HEP G-2 cells with HDAd-mCMV-*Gulo* at a MOI of 500 vp/cell for increasing durations. I isolated cell-free extracts at different time points for a period of 120 h. We found that levels of GULO expression increased with the duration of infection (**Figure 12**). Minimal detectable GULO was produced at 18 h post-infection (**Figure 12, Lane 3**). A gradual increase in signal strength, indicative of increase of intracellular GULO levels, was observed from 18 h to 120 h, with a drastic elevation from 24 h to 36 h (**Figure 12, Lane 4 and 5**). I observed cell death after 120 h of infection. As expected, cell lysate generated from uninfected cells at 48 h post-infection did not contain any detectable levels of GULO (**Figure 12, Lane 8**), consistent with previous observations.

2.3.2.4 Vitamin C is produced in HDAd-infected cells supplemented with gulonolactone

To examine whether cells treated with the HDAd vectors also produced ascorbic acid, I infected HEP G-2 cells at a MOI of 500 vp/cell for 48 h in serum-poor media with

or without gulonolactone—the substrate for ascorbic acid. I measured the intracellular concentrations of ascorbic acid in these cells using HPLC-ECD as described. With settings specified in this study, ascorbic acid was eluted at 1.1 min with an ECD response factor of 2×10^{-12} μg per unit area (nC) (**Figure 6 and Figure 9**). We found that HDAd-mCMV-*Gulo* vector-treated cells produced approximately 52 fmol/cell ascorbic acid when supplemented with 10 mM gulonolactone (**Figure 14**). Uninfected HEP G-2 cells maintained in media containing 10 mM gulonolactone produced 0.2 fmol/cell ascorbic acid (**Figure 14**), which is significantly lower than that produced by treated cells (1:300), confirming that human cells do not readily produce ascorbic acid as a result of GULO inactivation. Interestingly, HEP G-2 cells maintained in media devoid of gulonolactone did not produce any detectable levels of ascorbic acid, even when treated with HDAd-mCMV-*Gulo* at 500 vp/cell (**Figure 14**).

2.3.2.4 Vitamin C production is dependent on exogenous gulonolactone concentrations

To test whether gulonolactone levels affect ascorbic acid production in human cells treated with the HDAd vectors, I infected HEP G-2 cells with 500 vp/cell HDAd-mCMV-*Gulo* vectors and supplemented these cells with increasing concentrations of gulonolactone ($n=3$). I measured intracellular vitamin C levels after 48 h and found that vitamin C production increased as a function of exogenous gulonolactone concentrations from 0.05 mM to 5 mM. However, an increase of gulonolactone from 5 mM to 10 mM did not result in elevated vitamin C production (**Figure 15**).

2.3.3 Animal experiments

2.3.3.1 GULO was expressed in *Gulo*^{-/-} mice infected with HDAd-mCMV-*Gulo* vectors

Gulo^{-/-} mice were infected with HDAd-mCMV-*Gulo* vectors at low and high doses: 1×10^{10} and 2×10^{11} vp per animal, respectively. Viral vectors were delivered by intravenous injection. After 48 h, mice were perfused with PBS and GULO expression in the liver was measured by Western Blotting with GULO antibody. Mice infected at high dose (2×10^{11} vp) of the HDAd vector expressed detectable levels of GULO in the liver (Figure 16, Lane 3). However, mice infected at low dose (1×10^{10} vp) did not produce any detectable GULO (Figure 16, Lane 2). As expected, mice subjected to mock infection with PBS did not express any GULO (Figure 16, Lane 1), confirming that *Gulo*^{-/-} mice do not produce structurally-intact endogenous GULO as a result of the genetic knockout.

2.3.3.2 *Gulo*^{-/-} mice treated with HDAd-mCMV-*Gulo* vectors exhibited elevated serum vitamin C levels

To determine whether *Gulo*^{-/-} mice treated with HDAd-mCMV-*Gulo* produced ascorbic acid, blood samples were collected from mice infected with the HDAd vectors at a MOI of 2×10^{11} vp by IV injection ($n=3$). Blood samples from wild type mice ($n=3$) and untreated *Gulo*^{-/-} mice ($n=3$) maintained on vitamin C deficient diet were also analyzed for comparison. All mice were deprived of exogenous vitamin C for 1 month prior to the experiment. Ascorbic acid concentrations were measured in the blood samples of these mice on the day of infection and 4 days post-infection using HPLC-ECD.

Gulo^{-/-} mice treated with HDAd-mCMV-*Gulo* vectors ($n=3$) exhibited a 6-fold increased in blood vitamin C concentrations from Day 0 (10 ± 5 μ M) to Day 4 (62 ± 15

μM) (Figure 17). In contrast, no increase was observed in untreated *Gulo*^{-/-} mice ($n=3$), which had the lowest blood ascorbic acid levels on both days ($1.9 \pm 1 \mu\text{M}$ and $3.0 \pm 2.5 \mu\text{M}$) (Figure 17). Wild type mice did not exhibit any significant change in blood vitamin C concentrations between Day 0 ($71 \pm 20 \mu\text{M}$) and Day 4 ($62 \pm 8.5 \mu\text{M}$). However, they had the highest levels of serum vitamin C on both days (Figure 17).

2.3.3.3 *Gulo*^{-/-} mice treated with HDAd-mCMV-*Gulo* vectors exhibited elevated urine vitamin C levels

In addition to blood samples, urine samples were collected from these animals on Day 4 of treatment. Ascorbic acid in the urine was again measured using HPLC-ECD. Urine samples collected from *Gulo*^{-/-} mice treated with HDAd-mCMV-*Gulo* vectors contained $1.1 \pm 0.4 \text{ mM}$ ascorbic acid, which was 20-fold higher than those from untreated *Gulo*^{-/-} mice ($53 \pm 11 \mu\text{M}$) on the same day (Figure 18). Wild type mice again had the highest vitamin C concentrations in the urine ($7.4 \pm 0.5 \text{ mM}$) (Figure 18). Compared to blood vitamin C concentrations, urine vitamin C levels in all three groups were more than 5-fold higher (Figure 18).

2.3.3.4 *Gulo*^{-/-} mice treated with HDAd-mCMV-*Gulo* vectors had elevated tissue vitamin C levels

To examine the intracellular vitamin C status in tissues, the liver and the kidney were resected from *Gulo*^{-/-} mice treated with HDAd-mCMV-*Gulo* vectors ($n=1$), untreated *Gulo*^{-/-} mice ($n=1$) and wild type mice ($n=1$). Intracellular fluids were extracted from these tissues and analyzed using HPLC-ECD for vitamin C content.

Gulo^{-/-} mice treated with the HDAd-mCMV-*Gulo* vector exhibited elevation of both liver and kidney ascorbic acid levels (Liver: 47 µg/g tissue and Kidney: 51 µg/g tissue) as compared to the baseline values observed in untreated *Gulo*^{-/-} mice (Liver: 1.5 µg/g tissue and Kidney: 20 µg/g tissue, respectively) (**Figure 19**). An increase of 40-fold was observed in the liver and a 2-fold increase was detected in the kidney (**Figure 19**). Wild type mice had the highest levels of ascorbic acid in both tissue types (37 µg/g tissue and 137 µg/g tissue, respectively) (**Figure 19**). In addition, ascorbic acid levels in the kidney were significantly higher than those in the liver in untreated *Gulo*^{-/-} mice and wild type mice. This difference was not detected in *Gulo*^{-/-} mice infected with HDAd-mCMV-*Gulo* vectors.

2.3.3.5 Long-term vitamin C production in *Gulo*^{-/-} mice treated with HDAd-mCMV-*Gulo* vectors

To determine the duration of *in vivo* vitamin C production mediated by HDAd-mCMV-*Gulo*, I measured ascorbic acid concentrations in the serum of mice receiving different treatments (**Table 1**) for a period of 23 days. *Gulo*^{-/-} mice infected with HDAd-mCMV-*Gulo* vectors exhibited a 6-fold increase in blood vitamin C levels from Day 0 to Day 4 (**Figure 20, closed circle**). Compared to baseline levels observed in untreated *Gulo*^{-/-} animals, which fluctuated around 5 µM (**Figure 20, open circle**), these mice had, on average, 7-fold higher serum vitamin C concentrations (35 µM). This level was maintained for at least 23 days (**Figure 20**). Wild type animals had the highest levels of vitamin C concentration in the blood, which was on average 65 µM with some fluctuations (**Figure 20, closed triangle**). And *Gulo*^{-/-} mice maintained on dietary vitamin

C (330 mg/L) had intermediate levels of serum vitamin C (approx. 25 μ M) (**Figure 20, open triangle**). Large fluctuations have been observed in *Gulo*^{-/-} mice maintained on dietary vitamin C in drinking water. For example, on Day 0 these mice had 4.4 ± 1.0 μ M ascorbic acid in the blood, compared to 33 ± 12 μ M on Day 23.

2.4 Discussion

Ascorbic acid is an essential nutrient produced by most mammals. Endogenous vitamin C production occurs primarily in the liver using glucose as the precursor. Unlike most mammals, humans do not synthesize ascorbic acid endogenously and, as such, must consume vitamin C from diets for survival. This innate nutritional deficiency is caused by a genetic defect of gulonolactone oxidase encoding gene (*Gulo*) mapped to the short arm of chromosome 8 (Nishikimi *et al.*, 1988). This in turn renders the conversion of L-gulonolactone to L-ascorbic acid impossible, leading to endogenous vitamin C deficiency.

Because of this genetic defect, humans must consume vitamin C containing foods. The lack of dietary intake leads to rapid exhaustion of vitamin C in body reserves and subsequent onset of hypoascorbemia, marked by characteristic scurvy symptoms. Collagen malformation and the subsequent degradation of extracellular matrix and fibrous tissues are the main pathologic mechanisms contributing to the classical symptoms of scurvy. At early stages of hypoascorbemia, replenishment of vitamin C in the body through dietary means is in itself sufficient for alleviating this ailment. However, if not intervened, prolonged vitamin C deficiency is fatal.

While dietary intake results in blood vitamin C levels that are sufficient for preventing hypoascorbemia and related symptoms (~100 μ M), it is unable to achieve supraphysiologic or pharmacologic levels of ascorbic acid in the blood, which may be necessary for its therapeutic efficacy against tumor cells (Chen *et al.*, 2005; Padayatty *et al.*, 2004). This is due the tightly controlled vitamin C absorption/reabsorption mechanisms of the body. Intravenous injection has been shown to effectively elevate

serum vitamin C concentrations to pharmacologic levels, at which hydrogen peroxide toxic to tumor cells was produced (Chen *et al.*, 2005; Padayatty *et al.*, 2004). This is mainly due to the ability of intravenous injection to bypass intestinal absorption and deliver ascorbate directly into the blood. However, this high serum vitamin level is short-lived. After initial delivery, ascorbic acid is rapidly eliminated from the blood through renal excretion (Chen *et al.*, 2005; Padayatty *et al.*, 2004). It declines to normal physiologic levels (~100 μ M) within four hours (Chen *et al.*, 2005; Padayatty *et al.*, 2004).

As vitamin C deficiency in humans is a genetic disorder due to the loss-of-function mutations at the *Gulo* locus, complementation of this mutation from an extra-chromosomal vector expressing functional GULO likely results in restored ability for constitutive endogenous vitamin C production. To test this hypothesis, I cloned wild type mouse *Gulo* gene into helper-dependent adenoviral vectors under the control of either mCMV or PEPCK promoter, and examined the ability of these vectors to mediate the expression of wild type GULO and production of vitamin C in human cells and in transgenic *Gulo*^{-/-} mice.

2.4.1 GULO expression in HEP G-2 cells infected with the HDAd vectors

Infection of HEP G-2 cells with the HDAd vectors followed by Western blot for intracellular GULO showed that GULO was expressed in human liver cells treated with HDAd-mCMV-*Gulo* at a MOI of 500 vp/cell for 48 h, as indicated by a 50 kDa protein which hybridizes with GULO-specific antibody (**Figure 10, Lane 2**). Cells infected with HDAd-PEPCK-*Gulo* did not show detectable levels of GULO (**Figure 10, Lane 1**). This,

however, should not be interpreted simply as evidence for lack of GULO expression, as GULO might be produced but at a level that was undetectable by Western blot. Reverse-transcription PCR (RT-PCR) for *Gulo* mRNA detected the presence of *Gulo* transcript (mRNA) in HEK293 cells infected with HDAd-PEPCK-*Gulo* vector (data not shown), indicating that it has been expressed at least at the mRNA level. The PEPCK promoter is a relatively weak promoter and is active only in certain tissues (Beale, Clouthier, and Hammer, 1992). In contrast, mCMV promoter is a strong promoter. In the context of the promoter-*Gulo* construct, the mCMV promoter is able to mediate high-level expression of the downstream transgene in the cell whereas PEPCK promoter mediates comparably low-level *Gulo* expression. Therefore, the quantitative difference in GULO production is likely manifested at the mRNA level, which is supported by our previous observation that HEK293 cells infected with HDAd-mCMV-*Gulo* vector produced significantly more *Gulo* mRNA than those infected with HDAd-PEPCK-*Gulo* (data not shown). As expected, uninfected HEP G-2 did not express GULO (Figure 10, Lane 3), which is consistent with the fact that human cells do not produce structurally intact GULO. Due to the lack of detectable GULO expression, HDAd-PEPCK-*Gulo* vector was excluded from further study.

Having established that GULO is produced in human cells infected with HDAd-mCMV-*Gulo* vectors, I investigated the correlation between gene dose and levels of intracellular GULO expression as well as time-dependent expression of GULO. As the infectious titer (pfu/ml) of HDAd vectors cannot be readily quantified using conventional plaque assay, I relied on the physical titer (vp/ml) to estimate gene dose, which is

expressed as MOI (vp/cell). I showed that levels of intracellular GULO increases with increasing MOIs (gene dose) up to 1000 vp/cell (**Figure 11**). This suggests that the expression of GULO mediated by mCMV promoter is not subjected to negative feedback control, as intracellular GULO levels continued to increase as a function of MOIs.

Time-course experiments showed that GULO production increased with time to 120 h post-infection at which point cell death occurred. Therefore, it is very likely that the duration for GULO expression exceed 120 h, which was later confirmed in animal experiment (**Figure 20**). In addition, the minimum amount of time required for detectable GULO production is 18 h post-infection (**Figure 12, Lane 8**), though expression likely has occurred prior to this time, but the protein was not detectable.

2.4.2 Vitamin C production in HEP G-2 cells infected with HDAd-mCMV-*Gulo* vectors

Having shown that HDAd-mCMV-*Gulo* vectors are able to mediate GULO expression in human cell cultures, I investigated further to determine whether ascorbic acid has been produced in vector-infected cells. HPLC-ECD analysis was conducted on cell lysates to measure intracellular vitamin C concentrations. We found that HEP G-2 cells treated with HDAd-mCMV-*Gulo* vectors and supplemented with 10 mM L-gulonolactone had substantially higher levels of intracellular ascorbic acid than uninfected cells maintained in 10 mM of the substrate (**Figure 14**), suggesting that the intracellular GULO expressed from the extra-chromosomal mCMV-*Gulo* construct (**Figure 8A and Figure 10**) was able to catalyze the conversion of gulonolactone to ascorbic acid in cells. Lacking functional GULO, untreated human cells are defective for

this enzymatic reaction (**Figure 14**). Interestingly, HEP G-2 cells infected with HDAd-mCMV-*Gulo* vectors also had undetectable quantities of ascorbic acid if exogenous gulonolactone was not supplemented (**Figure 14, Column 3**), indicating that human cells require not only exogenous enzyme but also exogenous substrate for ascorbic acid production. This requirement for gulonolactone supplementation suggests that human cells are not able to produce endogenous gulonolactone at levels sufficient for vitamin C biosynthesis. As the inactivation of GULO occurred around 30 million years ago (Nishikimi *et al.*, 1988), other loss-of-function mutations may have occurred in other enzymes in the vitamin C biosynthetic pathway, leading to reduction of its intermediates especially the terminal intermediates such as gulonolactone. From the viewpoint of evolution, there was likely no selective pressure to keep the pathway intact if the final product has been removed. On the contrary, as gulonolactone has no known function other than being the substrate for ascorbic acid production and its synthesis consumes glucose, which is a vital energy source, the collapse of this pathway was likely favored by natural selection. Indeed, the same requirement for exogenous gulonolactone has been observed in guinea pigs, which, like humans, also lost GULO. In their study, GULO replacement was shown to rescue scurvy but only when gulonolactone was also administered to the animal (Hadley and Sato, 1988), indicative of a similar lack of endogenous gulonolactone production in guinea pigs.

As vitamin C production by infected human cells requires exogenous gulonolactone, I investigated whether vitamin C production varied quantitatively as a function of gulonolactone supplementation. Intracellular ascorbic acid levels increased

with increasing concentrations of exogenous gulonolactone from 0.05 mM to 5 mM (**Figure 15**). An increase from 5 mM to 10 mM did not result in elevated vitamin C production (**Figure 15**). These observations together suggest that ascorbic acid production inside the cell is directly related to the intracellular gulonolactone concentration, which is proportional to extracellular gulonolactone provided in the media. The equilibrium between intracellular and extracellular gulonolactone levels is likely re-established as those inside the cell were being consumed. However, from 5 mM to 10 mM, saturation of GULO by this substrate may have occurred, resulting in the lack of further increase in vitamin C synthesis (**Figure 15**). At high intracellular concentrations, vitamin C export by HEP G-2 may have also occurred, although ascorbic acid content in the media was not examined.

Taken together, our results suggest that HDAd-mCMV-*Gulo* vectors were able to mediate the expression of functional GULO in human cells, which, if supplemented with the substrate, also produced ascorbic acid.

2.4.3 GULO expression in mice infected with HDAd-mCMV-*Gulo* vectors

It was observed that HDAd-mCMV-*Gulo* vectors mediated the expression of GULO in human cells (**Figure 10—12**), I then examined whether infection with the HDAd vector also leads to GULO expression in animals. We found that *Gulo*^{-/-} mice infected with HDAd-mCMV-*Gulo* vectors at a dose of 2×10^{11} vp expressed GULO in the liver (**Figure 16 Lane 3**); whereas *Gulo*^{-/-} mice infected at 1×10^{10} vp did not produce any detectable levels of this protein (**Figure 16 Lane 2**).

The lack of detectable GULO production at low dose (1×10^{10} vp) may be attributable to either insufficient levels of GULO expression which is below the detection limit of Western blot or inability of the HDAd vector to transduce the liver at low dose and, consequently, complete lack of expression. Indeed, it is known that hepatic transduction by adenovirus exhibits a non-linear dose-response relationship (Tao *et al.*, 2001). It was shown previously in a similar study using IV injection of adenoviral vectors that, at low vector dose ($1\text{--}3 \times 10^{10}$ vp), the expression of transgene was largely undetectable, but at a higher dose (1×10^{11} vp), the transgene was expressed at disproportionately high levels (Tao *et al.*, 2001). Furthermore, co-infection of an unrelated adeno vector enhanced liver transduction by the transgene-carrying vector, resulting in higher transgene expression (Tao *et al.*, 2001). Based on these observations, the authors proposed a threshold effect of hepatic transduction by adenovirus, in that only vector doses above this threshold can lead to liver transduction (Tao *et al.*, 2001). The physical structure contributing to this threshold phenomenon was shown to be the Kupffer cells of the liver, which form a protective barrier for hepatocytes and, in the context of adenovirus infection, takes up viral particles, resulting in compromised vector availability for hepatocytes (Tao *et al.*, 2001). Therefore only when Kupffer cells have been saturated (threshold point), can adeno vectors effectively infect and transduce liver cells. Indeed, our observations are consistent with findings of this study in that we only detected GULO expression at a viral dose of 2×10^{11} vp IV, comparable to the high vector dose used in their study, and failed to detect any expression at low dose (1×10^{10} vp), which is likely attributable to the threshold effect.

Nonetheless, production of GULO by hepatocytes infected at high dose of HDAd-mCMV-*Gulo* confirmed that the HDAd vector not only mediated GULO expression *in vitro*, it also led to the production of GULO in *Gulo*^{-/-} animals. The lack of hepatic GULO in uninfected mice further confirms that *Gulo*^{-/-} mice do not produce this protein.

2.4.4 Ascorbic acid production in mice infected with HDAd-mCMV-*Gulo* vectors

To determine whether vitamin C was produced in *Gulo*^{-/-} mice treated the HDAd vector, I analyzed vitamin C content in biological samples derived from blood, urine and tissues of these mice using HPLC-ECD.

We found that after 4 days of infection, *Gulo*^{-/-} mice treated with HDAd-mCMV-*Gulo* vectors exhibited a 6-fold elevation in serum vitamin C ($62 \pm 15 \mu\text{M}$) compared to day 0 ($10 \pm 5 \mu\text{M}$) (**Figure 17**), strongly suggesting that ascorbic acid has been produced as a result of vector treatment. In comparison, untreated *Gulo*^{-/-} mice displayed consistently low serum vitamin C levels on both day 0 and day 4 ($1.9 \pm 1 \mu\text{M}$ and $3.0 \pm 2.5 \mu\text{M}$), confirming that these mice can not accumulate vitamin C in the body. Serum vitamin C concentrations in *Gulo*^{-/-} mice deprived of dietary vitamin C was previously reported to be $9.7 \mu\text{M}$ (Maeda *et al.*, 2000), which is comparable to vitamin C levels in treated mice on the day of treatment (Day 0), but higher than untreated mice ($1.9 \pm 1 \mu\text{M}$ and $3.0 \pm 2.5 \mu\text{M}$) (**Figure 17**). This discrepancy may be attributable to different assays used in our study and theirs. However, the discrepancy observed in our study between serum vitamin C concentrations in untreated mice and those obtained before treatment (3-fold difference) cannot be explained. Regardless, the treatment effect of HDAd-mCMV-*Gulo* vectors is unequivocal, as evidenced by the significant elevation in vitamin C

concentrations after treatment ($62 \pm 15 \mu\text{M}$), which can only be attributed to the expression of functional GULO and subsequent intracellular vitamin C production. Therefore, it is evident that the HDAd-mCMV-*Gulo* vector is sufficient to rescue vitamin C deficiency in *Gulo*^{-/-} mice. Unlike humans and guinea pigs, *Gulo*^{-/-} mice do not require exogenous gulonolactone for vitamin C synthesis. This independence of exogenous supplementation suggests that *Gulo*^{-/-} mice produce gulonolactone endogenously. These mice carry targeted knockout of *Gulo* by means of transgenics rather than natural selection, and the defective vitamin C biosynthetic pathway has not been subjected to any evolutionary processes, and therefore likely remains intact. In humans and guinea pigs, this pathway, since the inactivation of GULO 30 million years ago, has likely been subjected to mutational insults and eventually collapsed. As discussed previously, the complete collapse of inactivated vitamin C synthetic pathway may have been selected for to conserve glucose. As a result, humans and guinea pigs, unlike transgenic mice, do not produce endogenous gulonolactone.

In addition to *Gulo*^{-/-} mice, I also examined serum vitamin C concentrations in wild type mice for comparison. We found that wild type mice produced the highest levels of ascorbic acid on both day 0 and day 4, and the levels (71 and 62 μM) were similar to the reported values (63 μM) (Maeda *et al.*, 2000).

Next, I measured ascorbic acid concentrations in the urine samples of these animals 4 days after infection. We found that *Gulo*^{-/-} mice treated with HDAd-mCMV-*Gulo* vector displayed a significant elevation (20-fold) in urine vitamin C levels ($1.1 \pm 0.4 \text{ mM}$) from the baseline values observed in untreated *Gulo*^{-/-} mice ($53 \pm 11 \mu\text{M}$) (Figure

18). Vitamin C status in the urine reflects the amount excreted by the renal system to maintain homeostasis, and thus is proportional to circulating vitamin C in the blood. The 20-fold elevation in HDAd-treated *Gulo*^{-/-} mice suggests that these animals produced vitamin C endogenously, and the accumulation of ascorbic acid in the blood resulted in elevated levels of renal excretion. The highest levels of renal excretion were found in wild type mice (7.4 ± 0.5 mM) (**Figure 18**), suggesting these mice also had the highest levels of vitamin C production. In all three groups, urine vitamin C levels were significantly higher than blood vitamin C levels (**Figures 17 and 18**). This is particularly evident in treated *Gulo*^{-/-} mice (20-fold) and in wild type mice (100-fold), both of which were capable for endogenous vitamin C production, suggesting that the majority of ascorbic acid produced was eliminated. This is indicative of active vitamin C elimination by the renal system, which further underlines the difficulty to maintain high concentrations of ascorbic acid in the serum for therapeutic purposes. It is unclear as to why wild type mice, and perhaps other non-scurvy animals, produce large quantities of ascorbic acid only to “wash it off” in the urine. This dynamic balance seems to be particularly “wasteful,” considering that the precursor for this process is glucose. On the other hand, this dynamic balance is perhaps necessary for swift response to environmental insults, as large amounts of ascorbic acid can be rapidly accumulated in the body by reduction of renal excretion, which can be achieved by upregulating the sodium-vitamin C co-transporter 1 (SVCT1). As discussed previously, increase in SVCT1 levels leads to elevated tubular vitamin C re-absorption, which, in turn, reduces its excretion. Therefore, the pattern expression of SVCT1 in non-scurvy animals such as mice may be of interest

in understanding the physiologic significance of high concentrations of ascorbic acid in the body.

As in the blood and urine, vitamin C levels in tissues exhibited the same patterns (**Figure 19**). In the liver, *Gulo*^{-/-} mice treated with the HDAd vector accumulated comparable levels of ascorbic acid as in the wild type. Untreated *Gulo*^{-/-} mice, in contrast, had the lowest liver vitamin C content (30-fold less) (**Figure 19**). As the liver is the site for endogenous vitamin C synthesis, the intracellular ascorbic acid level in liver cells is likely representative of the degree of vitamin C biosynthesis. Vector-treated *Gulo*^{-/-} mice and wild type mice had significantly higher vitamin C content in the liver compared to baseline values (**Figure 19**), indicating active ascorbic acid biosynthesis in these mice. Untreated *Gulo*^{-/-} mice had the lowest level in the liver, which is consistent with the fact that they are unable for endogenous vitamin C synthesis, and this level (1.5 µg/g tissue) may represent the amount required for internal use by the liver. In the kidney, however, these mice had 15-fold higher vitamin C concentrations than in the liver (20 µg/g tissue), again indicative of vitamin C accumulation in the kidney for excretion. Kidney vitamin C levels were similar to liver levels in vector-treated mice, but were 3-fold higher than those in the liver of wild type animals, suggesting that these mice are actively excreting ascorbic acid through the kidney.

Taken together, these observations suggest that HDAd-mCMV-*Gulo* vectors mediated the production of ascorbic acid in the liver of *Gulo*^{-/-} mice independent of exogenous gulonolactone by expressing wild type GULO from the extra-chromosome transgene.

2.4.5 Long-term vitamin C production in mice treated with HDAd-mCMV-*Gulo* vectors

Having established that *Gulo*^{-/-} mice treated with HDAd-mCMV-*Gulo* vectors produced ascorbic acid, I then examined the duration of production as reflected by steady-state vitamin C levels in the blood. Steady state vitamin C concentrations in vector-treated *Gulo*^{-/-} mice were 7-fold higher than basal levels observed in untreated *Gulo*^{-/-} animals, and were maintained as such for at least 23 days (**Figure 20, closed circle**), which suggests that long-term vitamin C production is achievable in mice infected with the gene therapeutic vector HDAd-mCMV-*Gulo*. Untreated *Gulo*^{-/-} mice had consistently low vitamin C content in the blood with an average of approximately 5 µM (**Figure 20, open circle**) with small day-to-day fluctuations. Compared with previously reported serum vitamin C concentrations in these mice, which averaged around 10 µM (Maeda *et al.*, 2000), this level is significantly lower. This discrepancy may be caused by differences in sample preparation and assaying method as discussed previously. What is more striking between this study and previous studies on these mice is the discrepancy in the phenotypic manifestations of vitamin C deficiency. According to a previous study, *Gulo*^{-/-} mice deprived of dietary vitamin C exhibited rapid weight loss and died within 5 weeks (Maeda *et al.*, 2000). However, our study showed that *Gulo*^{-/-} mice maintained on vitamin C deficient diet survived for more than 3 months without any discernible scurvy symptoms. They are however smaller in size as compared to vitamin C supplemented mice. In humans and guinea pigs, deprivation of dietary vitamin C leads to scurvy and death within a few months. The reason why these mice can survive for more than 3

months is not clear. A recent study has reported a vitamin C-independent pathway for collagen biosynthesis in mice, suggesting that vascular tissue integrity would likely not be compromised in *Gulo*^{-/-} mice deprived of exogenous vitamin C (Parsons *et al.*, 2006). This provides the explanation as to why classical scurvy symptoms, marked by tissue degeneration, did not occur in these mice. Paradoxically, small quantities of ascorbic acid were detected in the blood of *Gulo*^{-/-} mice in our study (5 μ M) as well as in the previous study (10 μ M) (Maeda *et al.*, 2000) and the source of this vitamin C has not been identified. It is possible that these levels of vitamin C in the blood are sufficient for mice to thrive. Furthermore, untreated *Gulo*^{-/-} mice also excreted significant quantities of vitamin C (53 μ M) through the urine after 1 month vitamin C deprivation (**Figure 18**), indicating that they have either acquired vitamin C from unidentified sources or were able to produce small quantities of ascorbic acid independent of GULO, especially when gulonolactone is present. Indeed, we have previously observed small quantities of vitamin C (0.2 fmol/cell) in HEP G-2 cells maintained in 10 mM gulonolactone (**Figure 14**). Therefore, it is possible that the presence of high concentrations of gulonolactone (reactant) may be sufficient for shifting the vitamin C reaction equilibrium towards ascorbic acid (product) without the need for GULO. It was this slow reaction that resulted in low levels of ascorbic acid in *Gulo*^{-/-} mice and in human cells supplemented with gulonolactone. In comparison, humans and guinea pigs, due to the lack of both gulonolactone and GULO, are unable to produce even minute amounts of ascorbic acid.

Wild type mice and *Gulo*^{-/-} mice maintained on vitamin C water both had blood vitamin C levels comparable to those reported previously (Maeda *et al.*, 2000), although

they exhibited day-to-day fluctuations. *Gulo*^{-/-} mice on dietary vitamin C supplementation is of some clinical interest, as they mimic human nutritional conditions with respect to this vitamin. Plasma vitamin C levels in these mice are generally lower than those in humans, which may be attributable to physiologic differences in intestinal absorption and renal excretion between these two species.

In conclusion, we have shown that HDAd-mCMV-*Gulo* is sufficient in mediating expression of GULO and endogenous production of ascorbic acid in human cells and in *Gulo*^{-/-} mice. In comparison to intravenous injection, which results in peak but short-termed (4 h) elevation of vitamin C in the blood (Padayatty *et al.*, 2004), gene therapy using HDAd vectors leads to high steady concentrations of ascorbic acid for a longer duration (>23 days). It elevates the balance set by homeostasis by continuously producing ascorbic acid, which is simultaneously being eliminated by the renal system. This dynamics resembles vitamin C homeostasis in non-scurvy animals.

Our study also suggests that vitamin C excretion through the renal system is the major obstacle in achieving pharmacologic levels of ascorbic acid required for its therapeutic efficacy against cancer. As vitamin C renal excretion is inversely related to SVCT1 activities, overexpression of SVCT1 in renal tubular epithelial cells likely potentiates ascorbic acid re-absorption, which in turn leads to accumulation of vitamin C in the blood.

While demonstrating genetic complementation by gene therapeutic adenovectors led to *in vivo* vitamin C production, this study does not address whether vitamin C production has given rise to any therapeutic effects. This is due primarily to the lack of

scurvy symptoms in *Gulo*^{-/-} mice under vitamin C deprivation. To examine whether vitamin C gene therapy offers any therapeutic effects, a model with specific disease marker, such as cancer marker, is needed. This model can be constructed by crossing *Gulo*^{-/-} mice with an existing mouse model carrying such marker. Alternatively, the guinea pig, which is a standard scurvy model, can be used to determine whether gene therapy can alleviate the basic pathologies associated with vitamin C deficiency.

Figures and tables

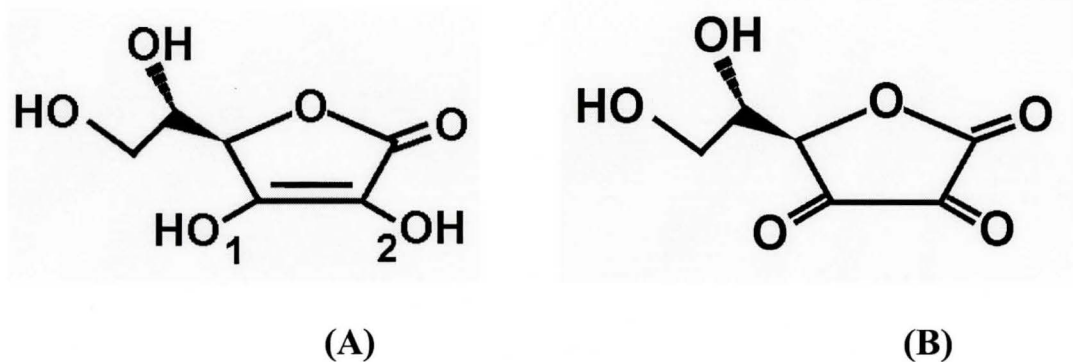


Figure 1 Molecular structure of L-ascorbic acid (A) and dehydroascorbic acid (B).

L-ascorbic acid has a molecular weight of 176.1 g/mol. It carries two acidic hydrogen atoms with pKD values of 4.17 and 11.57, respectively (A). Dehydroascorbic acid (DHA) is the oxidized form of ascorbic acid. It is another naturally-occurring structure of vitamin C (B).

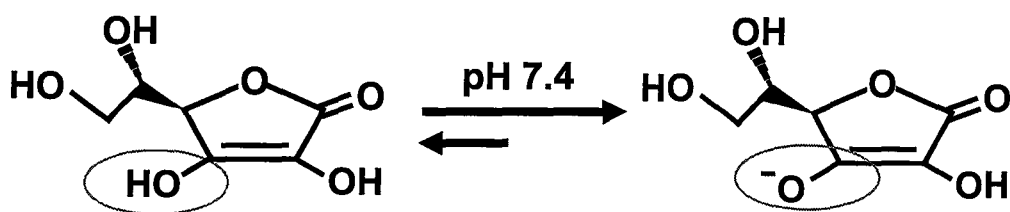


Figure 2 Formation of ascorbate from ascorbic acid in solution at physiological pH.

In solution L-ascorbic acid is readily deprotonated at physiological pH (pH 7.4) to form ascorbate.

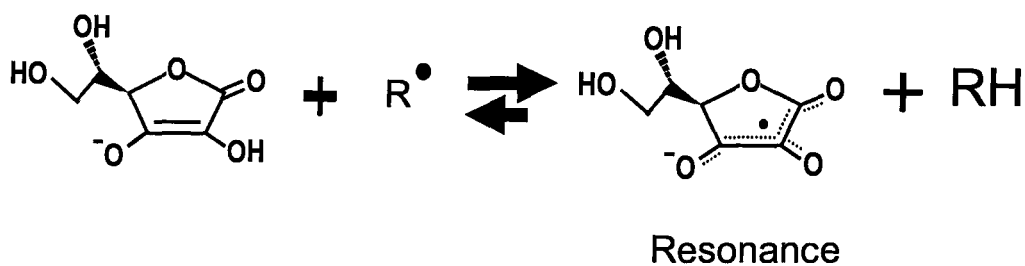


Figure 3 Neutralization of single-electron carrying radicals (R) by ascorbate.

Ascorbate donates a single electron to free radicals (R) in solution, resulting in reduced form of these radicals (RH). Ascorbate itself becomes singular electron-carrying ascorbyl radical, which, unlike other radicals, is unreactive, as the electron is stabilized by resonance.

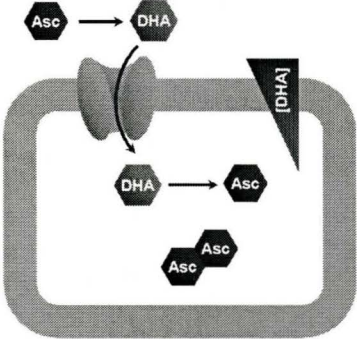
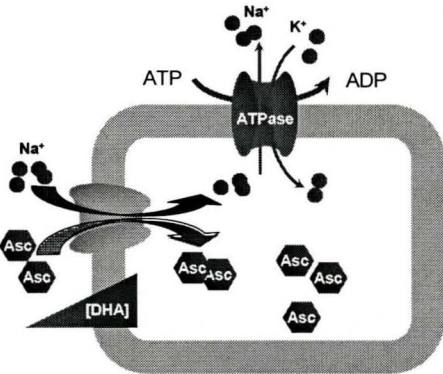
	Transporter	Mechanism	Distribution	Regulation
A	GLUT1 GLUT3 GLUT4		Osteoblast, astrocyte, muscle cells and retinal cells	Glucose: Competitive inhibition Insulin: Stimulates transport
	SVCT1 SVCT2		Intestinal, renal and liver epithelial cells Brain, retinal, and placental cells	Ascorbate: Substrate feedback inhibition of SVCT1 expression Ascorbate: Substrate feedback inhibition of SVCT2 expression

Figure 4 Major mechanisms for Vitamin C transport.

Vitamin C is transported by both Glucose-transporter (GLUT) in an Energy-independent, three-step mechanism (A) and by secondary active sodium vitamin C cotransporters (SVCT) in an ATP-dependent manner (B). Transport via GLUT requires extracellular oxidation of ascorbate to dehydroascorbate (DHA), which is imported by GLUT and reduced back to ascorbate in the cell. The concentration gradient of DHA is maintained (A). Ascorbate can be coupled to sodium ions and transported directly by SVCT. The excess intracellular sodium ion is actively exported in exchange for extracellular potassium ions through a Sodium-potassium ATPase. From (Li and Schellhorn, 2007b)

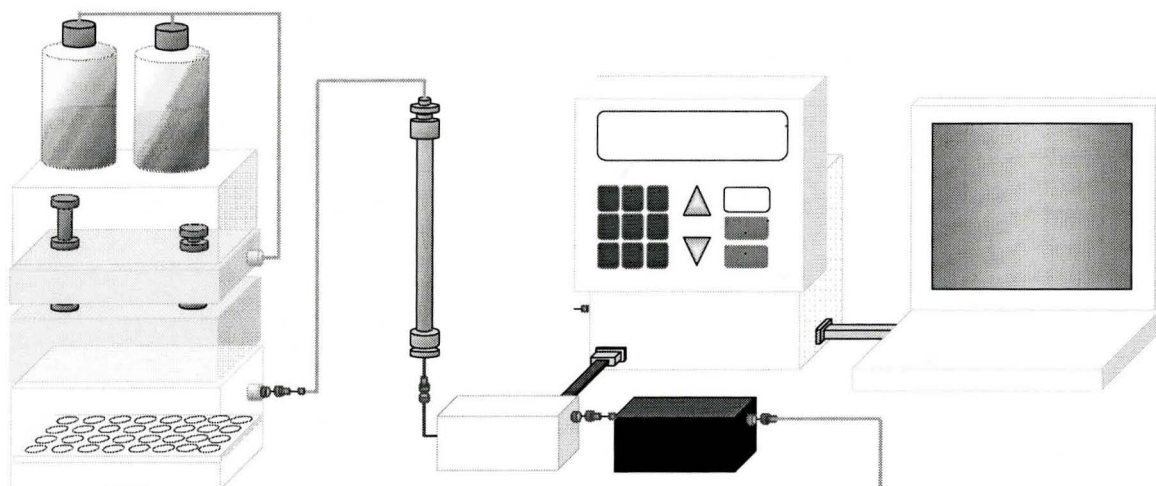
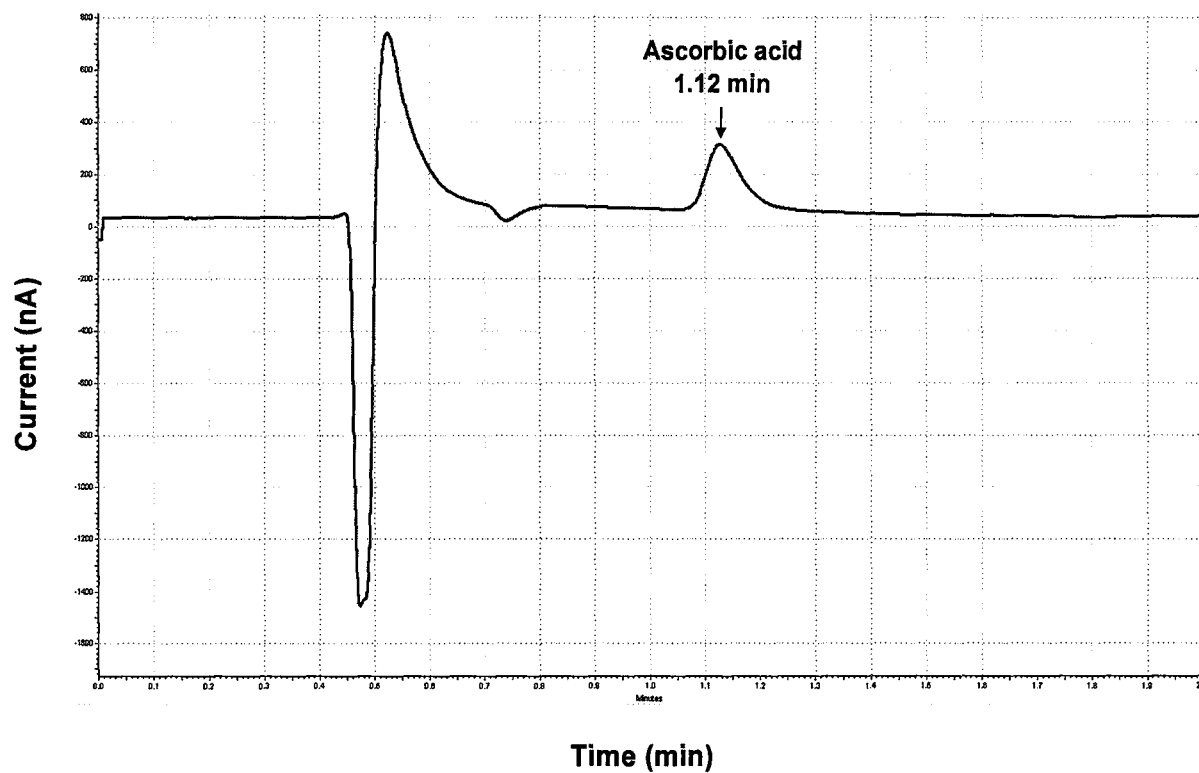


Figure 5 High-Performance Liquid Chromatography (HPLC)

Essentials components of a HPLC system are shown here: Mobile phase, binary pump, autosampler, column, dual-cell electrochemical detector and a computer.

(A)



(B)

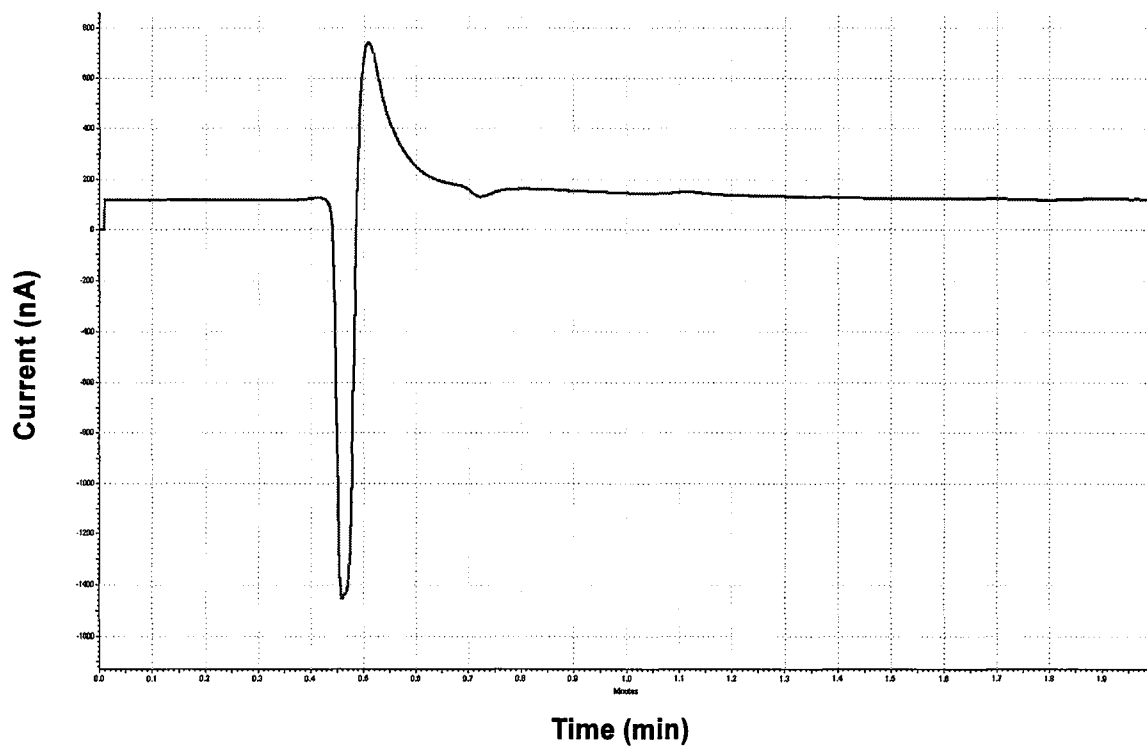
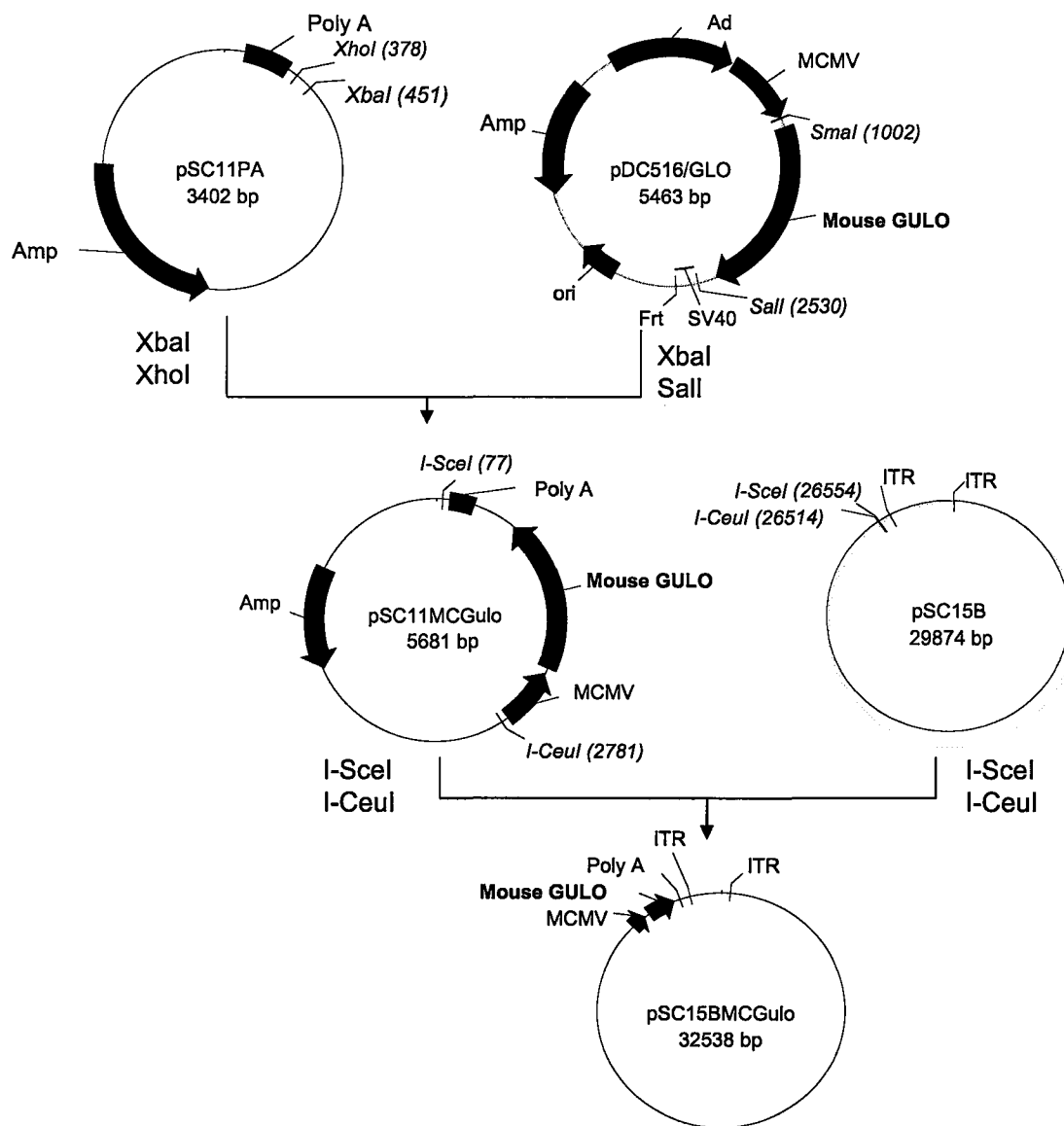


Figure 6 HPLC-ECD chromatograms of ascorbic acid.

A sample chromatogram of ascorbic acid elution is shown here. Ascorbic acid prepared in 5% *meta*-phosphoric acid is eluted at 1.12 min (min), resulting in a bell-shaped current signal (A). In comparison, the signal is absent in chromatogram of 5% *meta*-phosphoric acid devoid of ascorbic acid (B). The flow rate was set at 3 ml/min. A XDB-C18 analytical column (particle size 5 μ m, 4.6 x 250 mm) was used for separation.

(A)



(B)

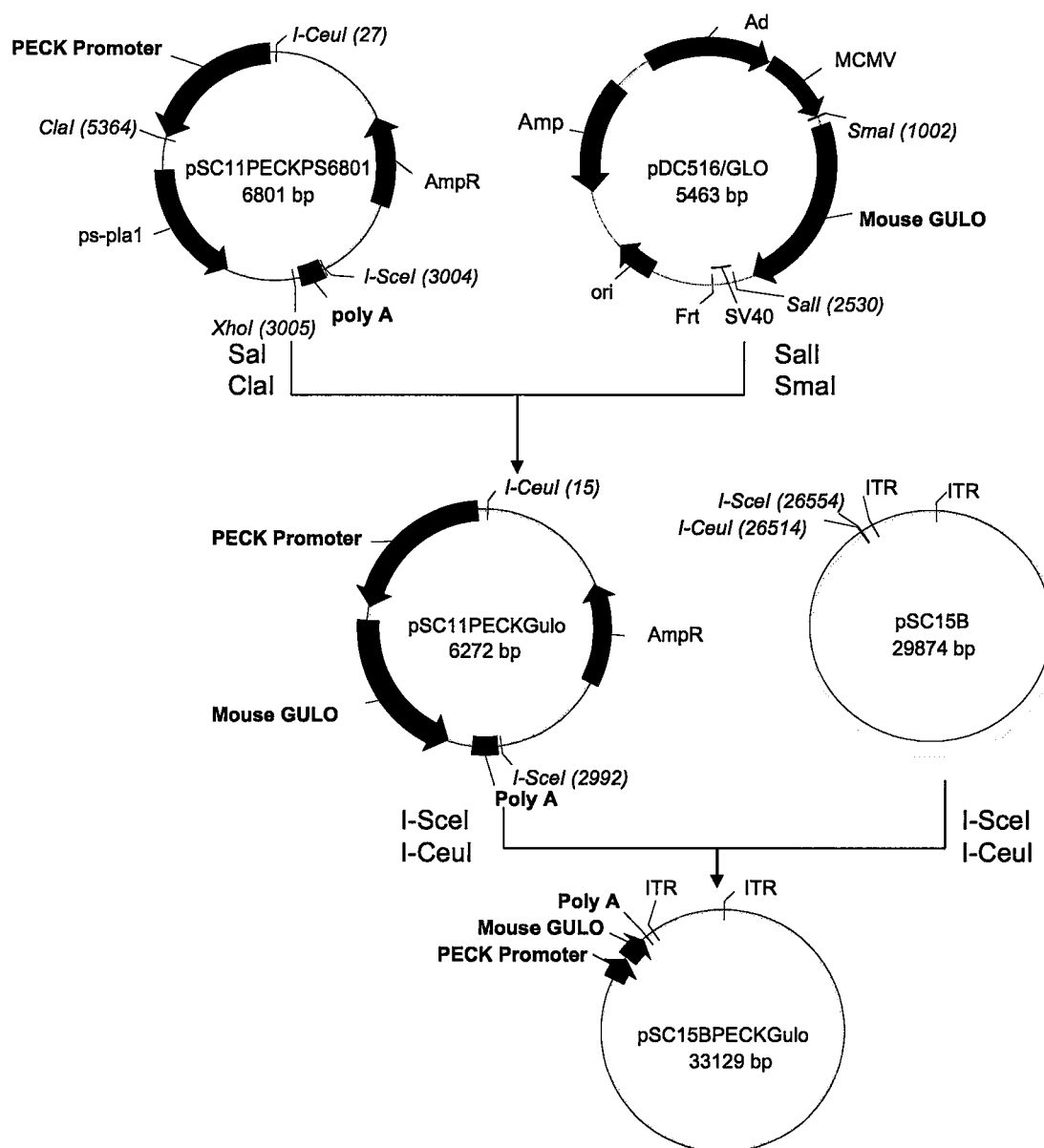
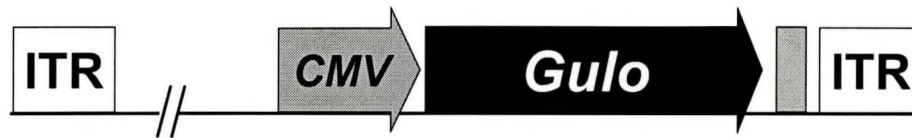


Figure 7 Construction of *Gulo* shuttle plasmids and HDAd precursor vectors.

The *XbaI*-*Sall* fragment containing mCMV promoter-*Gulo* construct from pMNH1 was ligated into the *XbaI*-*XhoI* site in vector pSC11PA (A). The *Sall*-*SmaI* fragment containing *Gulo* cDNA was inserted into the *Sall*-*Clal* site in vector pSC11PECKPS680 (B)

(A)



(B)

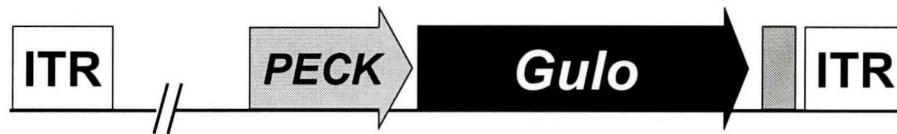


Figure 8 Structural maps of HDAd-mCMV-Gulo (A) and HDAd-PEPCK-Gulo (B) vectors.

HDAd-mCMV-Gulo vector has a 32 kb genome consisting of the mCMV-Gulo-polyA construct flanked by two inverted terminal repeats (ITR) (A). HDAd-PEPCK-Gulo vector has a 33 kb DNA consisting of the PEPCK-Gulo-polyA construct flanked by two ITRs (B). Neither of the two vector carries structural adenovirus genes.

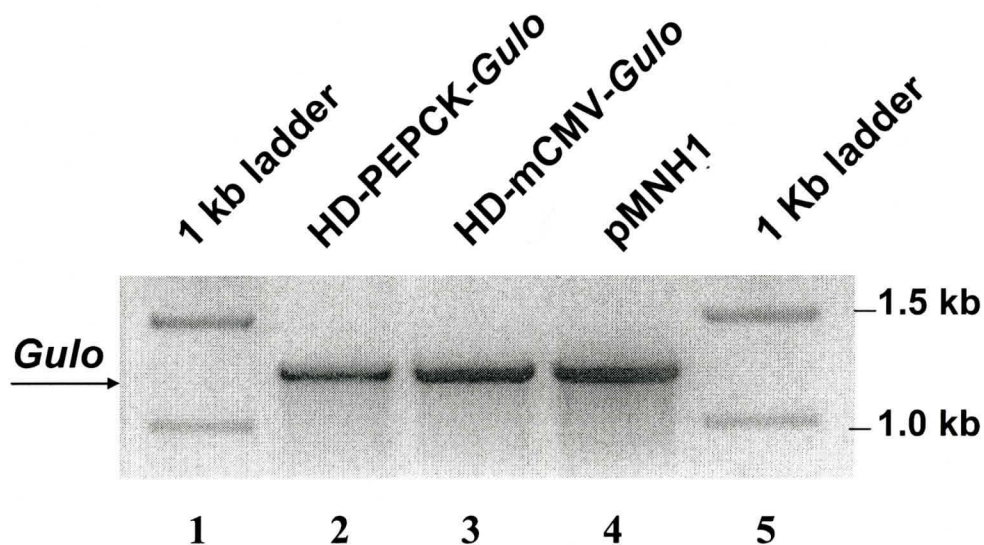


Figure 9 Diagnostic PCR detected a 1.3 kb signal.

PCR reaction using *Gulo* sequence specific primers detected a signal of 1.3 kb in length on template viral DNA isolated from purified HDAd-PEPCK-*Gulo* (Arrow, Lane 2) and HDAd-mCMV-*Gulo* (Arrow, Lane 3). The same signal was detected in PCR reaction using *Gulo* cDNA carrying plasmid pMNH1 (Arrow, Lane 8).

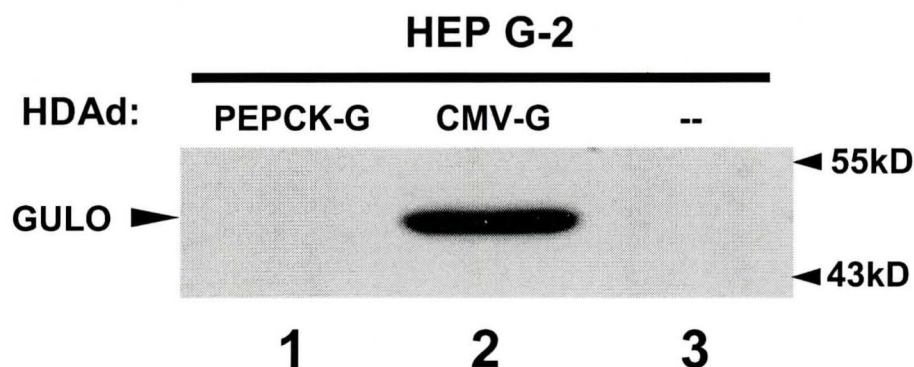


Figure 10 Expression of GULO in cells treated with HDAd-mCMV-Gulo vectors.

Western blot of GULO expression using GULO specific antibody showed that HEP G-2 infected with HDAd-mCMV-Gulo vectors at MOI of 500 vp/cell for 48 h expressed the 50 kDa GULO protein (Lane 2). Cells infected with HDAd-PEPCK-Gulo vector at the same MOI for the same duration did not produce detectable levels of GULO (Lane 1). No endogenous GULO expression in HEP G-2 cells was detected (Lane 3).

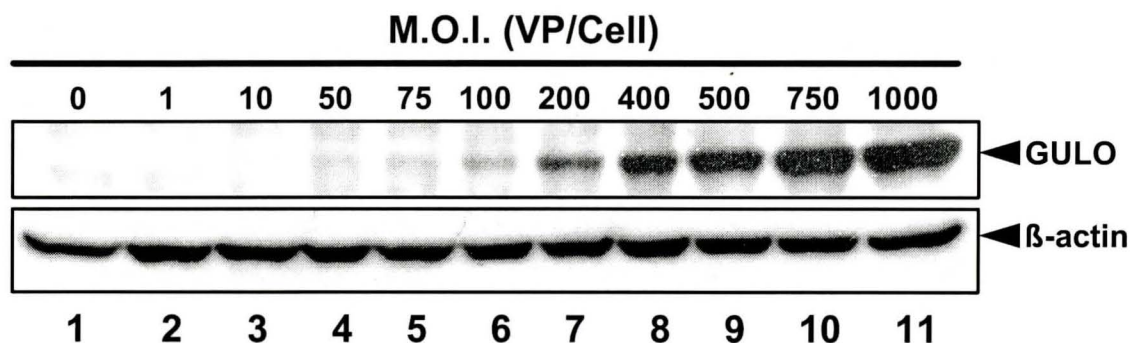


Figure 11 *GULO* expression was dependent on the MOI of HDAd-mCMV-Gulo vectors.

Western blot of GULO showed that GULO expression increased with increasing MOI of the HDAd vectors. The minimal MOI resulting in detectable expression was 50 vp/cell (Lane 4). At 1000 vp/cell, cell death was evident. Western blot of β -actin was used as loading control. Cells were infected for 48 h.

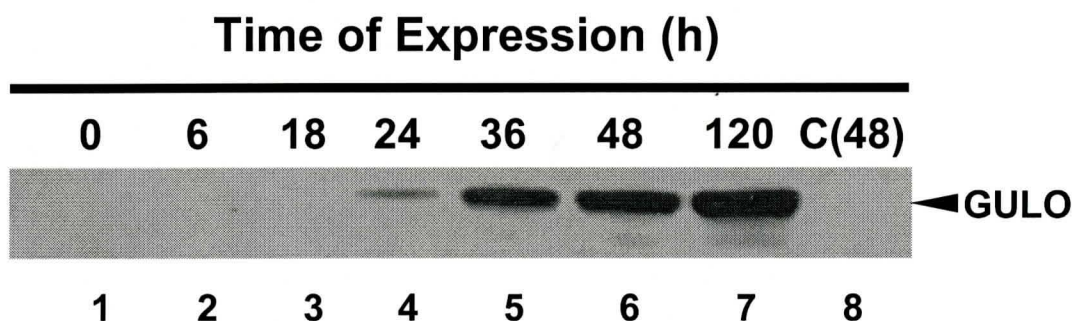


Figure 12 *GULO expression in infected cells increased with time.*

Western blot showed that HEP G-2 cells infected with HDAd-*mCMV-Gulo* vectors at a MOI of 500 vp/cell expressed GULO, and the level of express increases with the length of infection. GULO production was detected after 18 h of infection (Lane 4). Cell death occurred at 120 h.

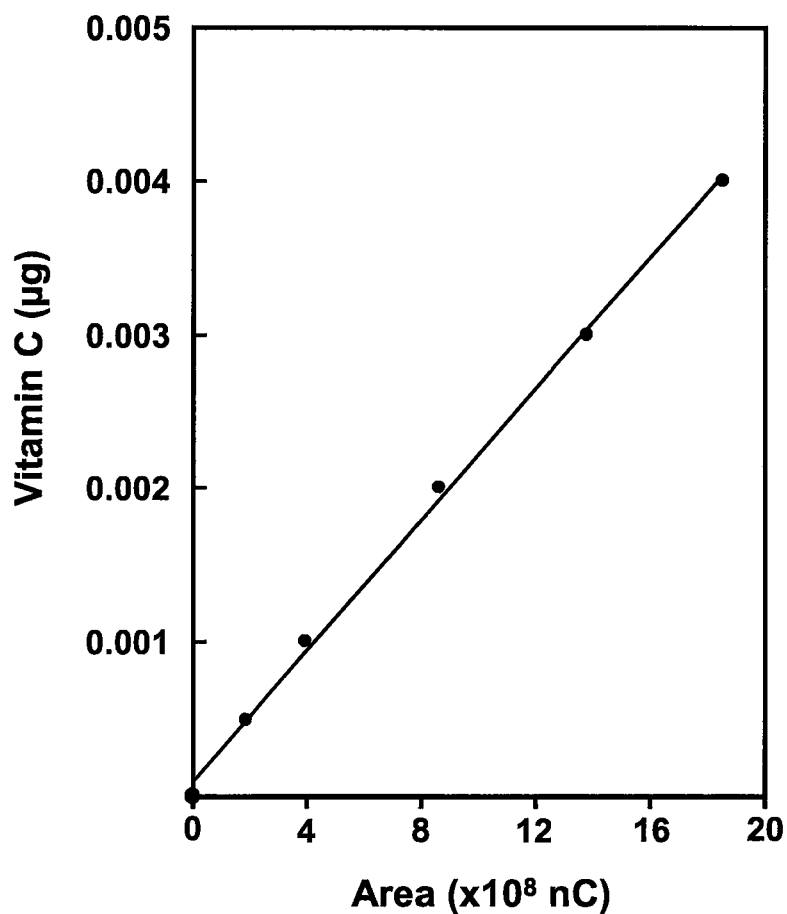


Figure 13 Response factor for ascorbic acid.

A standard curve of ascorbic acid versus electrochemical signals (area) was established using known concentrations of ascorbic acid and HPLC-ECD methods with the following parameters: Guard Cell at -200 mV, Analytical Cell Channel 1 at -150 mV, Channel 2 at 150 mV. The response factor was calculated to be 2×10^{-12} µg ascorbic acid per unit area (nC). R^2 value was calculated to be 0.998. ($n=1$)

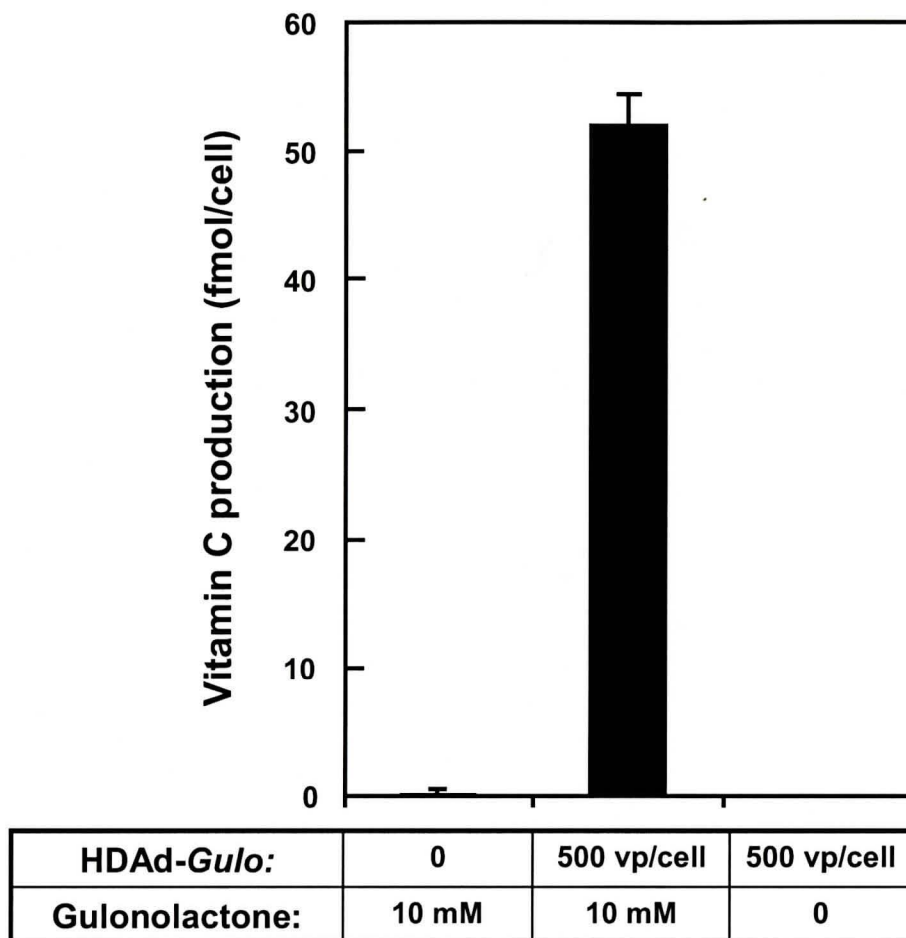


Figure 14 Ascorbic acid is produced in HDAd-mCMV-Gulo-treated HEP G-2 cells supplemented with gulonolactone.

HEP G-2 cells were infected with 500 vp/cell of the HDAd vector and maintained in the presence of 10 mM gulonolactone. Intracellular vitamin C concentrations were measured with HPLC-cECD 48 h after infection. HDAd-treated cells receiving gulonolactone produced approximately 50 fmol ascorbic acid per cell (Second column). In contrast, untreated cells maintained on equal amount of gulonolactone did not display significant vitamin C production (First column). In addition, HDAd-treated cells failed to produce ascorbic acid without gulonolactone (Third column). ($n=3$)

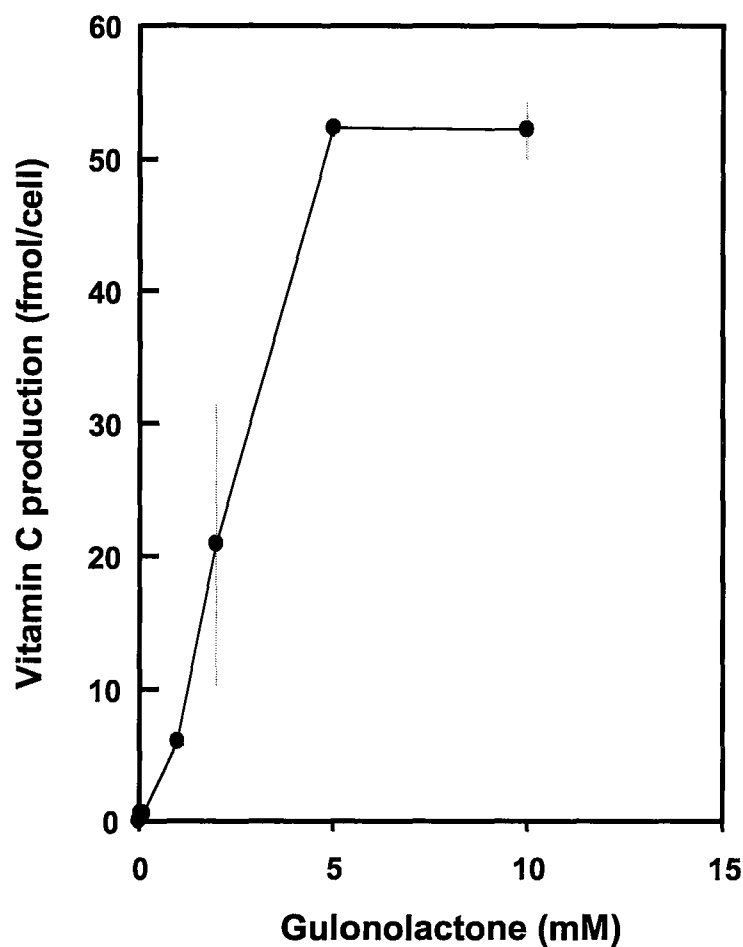


Figure 15 Ascorbic acid production is dependent on gulonolactone concentrations.

HDAd-*mCMV-Gulo* infected (500 vp/cell) HEP G-2 cells were incubated in media containing increasing concentrations of gulonolactone for 48 h. Intracellular ascorbic acid concentrations measured by HPLC-cECD increases with increasing levels of gulonolactone (0.05 mM to 5 mM). However, elevation of gulonolactone levels from 5 mM to 10 mM did not result in increased vitamin C production. ($n=3$)

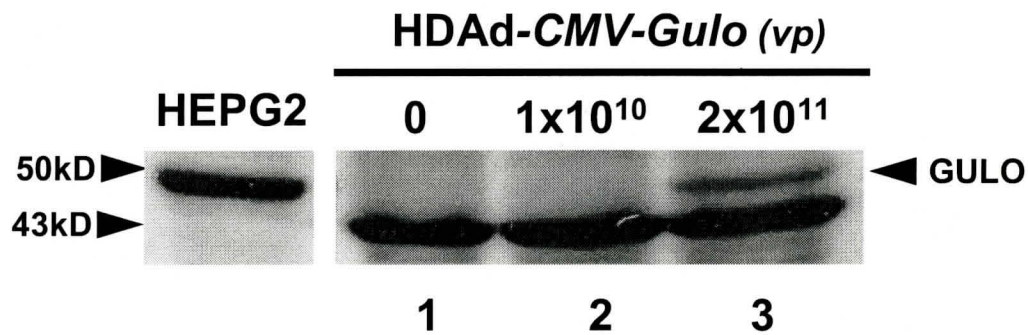


Figure 16 *Gulo*^{-/-} mice treated with HDAd-mCMV-Gulo vectors expressed GULO in the liver.

C57BL/6 *Gulo*^{-/-} mice were infected with the HDAd vectors, either at low-dose: 1×10^{10} or high-dose: 2×10^{11} viral particles, and tested for GULO expression using western blot 48 h after treatments. High-dose infection resulted in the expression of GULO in the liver (Lane 3), whereas low-dose infection did not lead to appreciable GULO expression (Lane 2). Uninfected mice did not expression GULO (Lane 1). Western blot of GULO in cell lysate from infected HEP G-2 was used as a reference (Left panel).

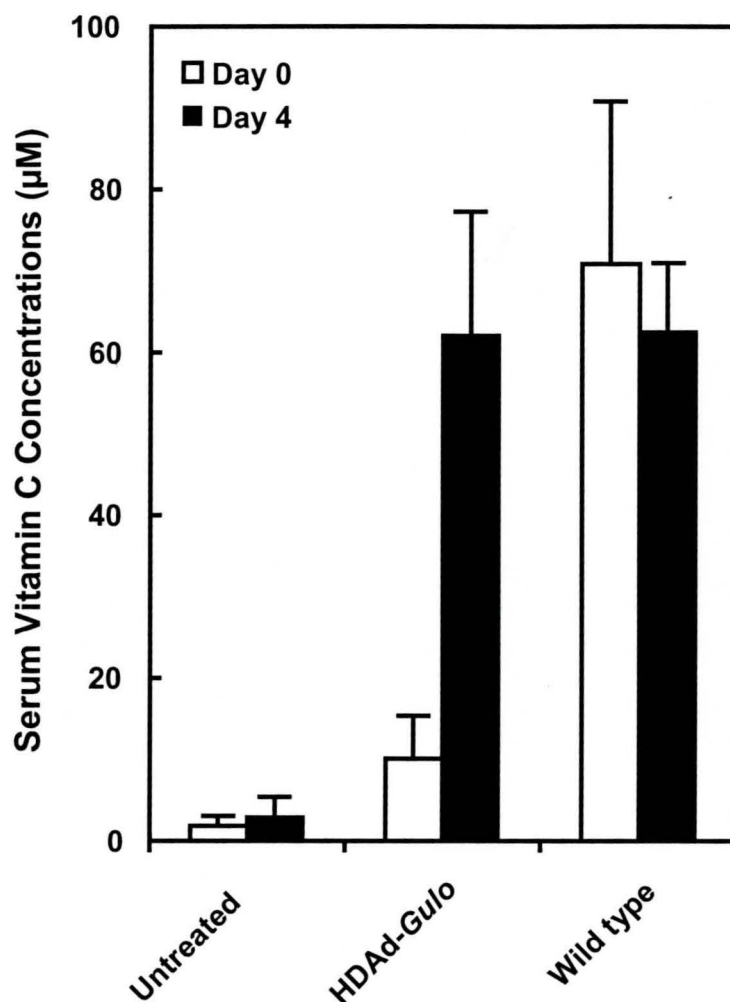


Figure 17 *Gulo*^{-/-} mice treated with HDAd-mCMV-*Gulo* vectors displayed elevated vitamin C levels in the serum.

Mice were infected with the HDAd vector (2×10^{11} vp) via IV injection. Blood samples were collected from the saphenous vein of mice on the day of treatment (Day 0) and four days after treatment (Day 4). Ascorbic acid concentrations were measured with HPLC-cECD. The HDAd vector-treated *Gulo*^{-/-} mice exhibited increased serum vitamin C levels on Day 4 as compared to Day 0 (HDAd-*Gulo*). In contrast, untreated mice did not display this elevation, and their serum vitamin C levels remained low on both days (Untreated). Wild type mice had the highest vitamin C concentrations on both days, and their serum ascorbic acid concentration did not fluctuate significantly. ($n=3$)

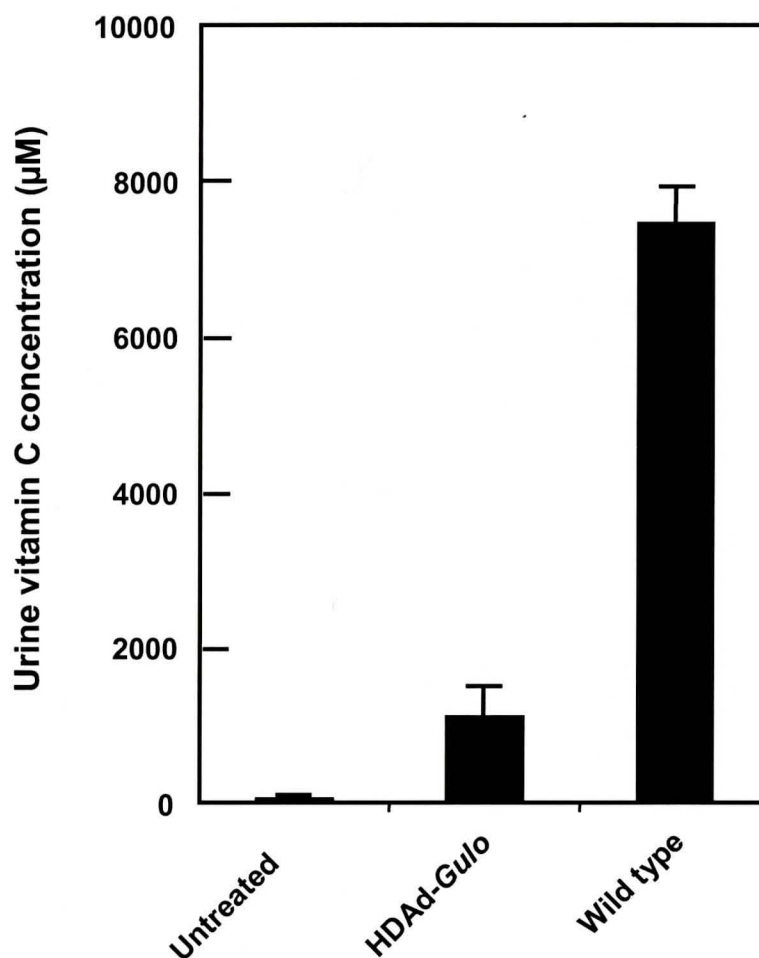


Figure 18 *Gulo*^{-/-} mice treated with HDAd-mCMV-Gulo vectors displayed elevated levels of vitamin C in the urine.

Urine samples were collected all three groups on Day 4 of treatment and analyzed with HPLC-cECD for ascorbic acid. Untreated *Gulo*^{-/-} mice had the lowest level of vitamin C in the urine (53 ± 11 μM). The HDAd-vector-treated *Gulo*^{-/-} mice displayed increased urine vitamin C levels (1.1 ± 0.4 mM) compared to untreated mice (53 ± 11 μM). Again wild type mice had the highest concentrations of ascorbic acid in the urine (7.4 ± 0.5 mM)

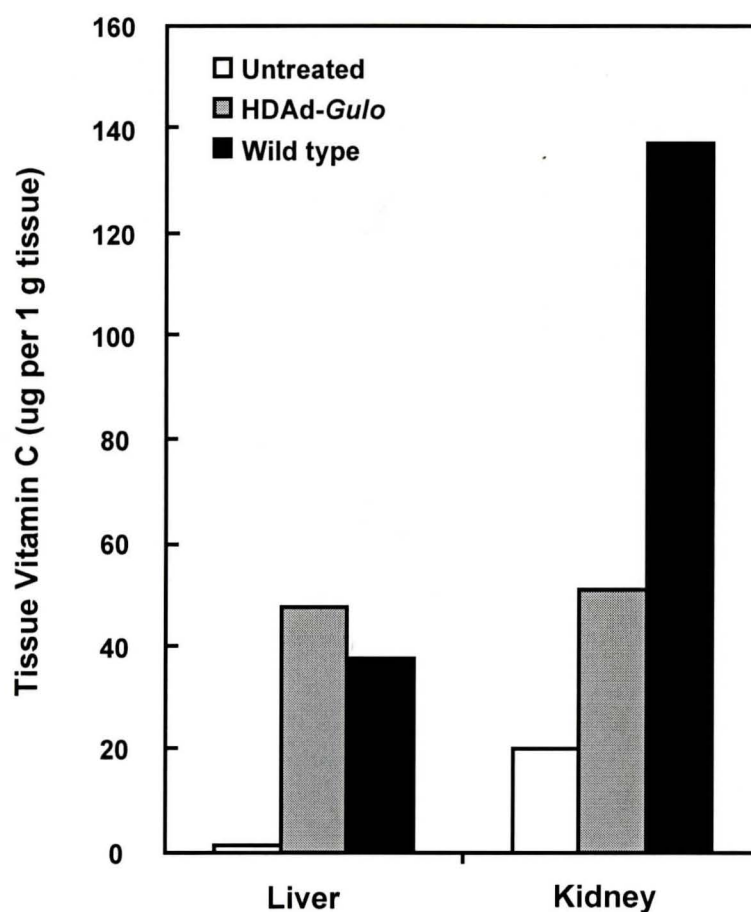


Figure 19 *Gulo*^{-/-} mice treated with HDAd-mCMV-Gulo vectors exhibited elevated levels of vitamin C in the liver and kidney.

Four days after treatment, intracellular ascorbic acid levels in the liver and kidney were measured using HPLC-ECD. Untreated *Gulo*^{-/-} mice had the lowest levels of intracellular vitamin C in both the liver (1.5 $\mu\text{g/g}$ tissue) and the kidney (20 $\mu\text{g/g}$ tissue). An increase in vitamin C level was detected in mice treated the HDAd vector in both the liver (46 $\mu\text{g/g}$ tissue) and the kidney (51 $\mu\text{g/g}$ tissue).

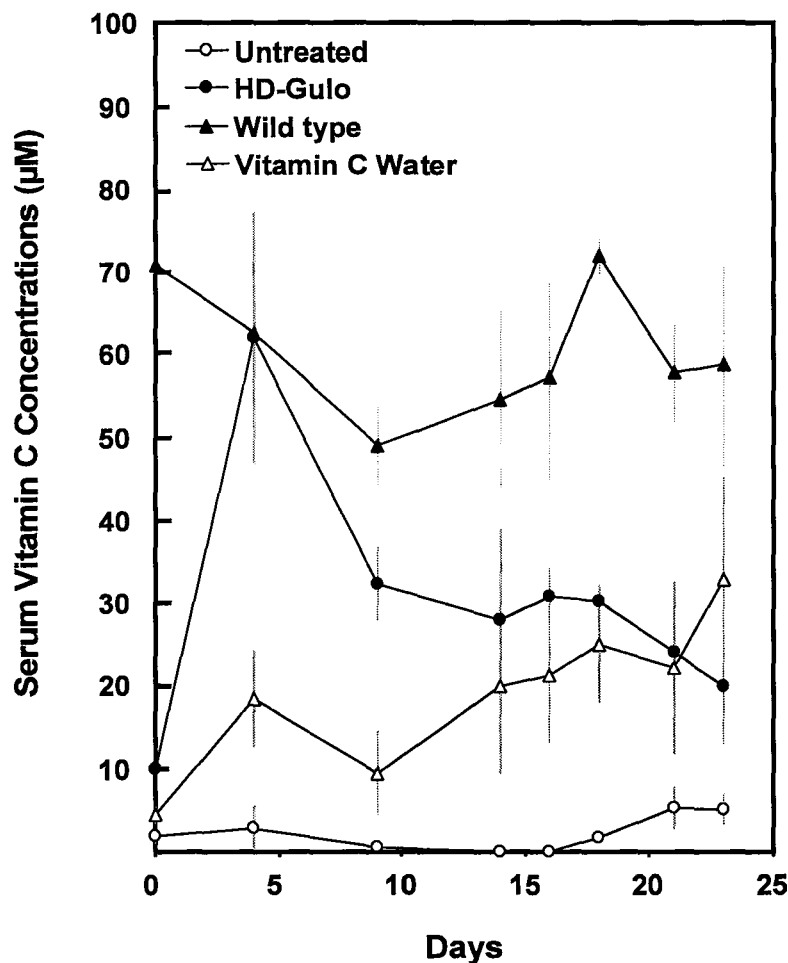


Figure 20 Long-term ascorbic acid production in *Gulo*^{-/-} mice was mediated by HDAd-mCMV-*Gulo*.

Serum vitamin C concentrations were measured for a period of 30 days using HPLC-ECD. *Gulo*^{-/-} mice infected with HDAd-mCMV-*Gulo* vectors exhibited a 6-fold increase in blood vitamin C levels from Day 0 (10 µM) to Day 4 (60 µM) (closed circle). Compared to the baseline levels observed in untreated *Gulo*^{-/-} animals (<5 µM) (open circle), these mice displayed, on average, 20-fold higher serum vitamin C concentrations (approx. 30 µM), which was sustained for at least 30 days. Wild type animals had the highest levels of vitamin C concentration (closed triangle). *Gulo*^{-/-} mice maintained on dietary vitamin C had intermediate levels of serum vitamin C (open triangle).

Table 1. Dietary sources of vitamin C

Source (Size)	Vitamin C, mg
Fruit	
Strawberries (1 Cup, sliced)	95
Kiwi fruit (1 Medium)	75
Orange (1 Medium)	70
Cantaloupe (1/4 Medium)	60
Mango (1 Cup, sliced)	45
Watermelon (1 Cup)	15
Juice	
Orange (1 Cup)	100
Grapefruit (1 Cup)	70
Fortified Juice	
Grape (1 Cup)	240
Apple (1 Cup)	100
Cranberry cocktail (1 Cup)	90
Vegetables	
Pepper, red or green	
Raw (1 Cup)	130
Cooked (1 Cup)	100
Broccoli, cooked (1 Cup)	120
Brussels sprouts, cooked (1 Cup)	100
Cabbage	
Red, raw (1 Cup)	40
White, raw (1 Cup)	20
Cauliflower (1 Cup)	50
Potato, baked (1 Medium)	25

Table 2 Treatments

Groups	Genotype	Treatment
1	<i>Gulo</i> ^{-/-}	Vitamin C deprivation
2	<i>Gulo</i> ^{-/-}	2x10 ¹¹ HDAd- <i>Gulo</i>
3	<i>Gulo</i> ^{-/-}	Vitamin C water
4	Wild type	No treatment

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Appendices

Appendix 1. Standard Operating Procedures (SOPs)

A.1.1 Cell cultures

A.1.1.1 HEK 293

Materials

Grow Medium

Minimal essential medium (MEM)-F11
10% fetal bovine serum (FBS)
2mM L-glutamine
2.5 µg/ml fungizone amphotericin B
100 U/ml penicillin/streptomycin

Growth Condition

5% CO₂ at 37 °C

1X Citric Saline

5 g KCl
2.2 g Na Citrate
Dissolve in ddH₂O and bring to 500 ml
Autoclave for 15 min at 15 lb./sq in pressure

Procedures

Culturing Frozen Cells

1. Warm Growth Media in a 37°C water bath for approximately 1hr.
2. Remove a vial of cell stock from liquid nitrogen and place it in a 37°C water bath until it completely thawed.
3. Transfer 20 ml of the pre-warmed Growth Media into a 150 mm dish.
4. Gently invert the thawed cell stock to ensure cells are well suspended.
5. Transfer the cells stock into the 150 mm dish and gently mix by mild agitation.

6. Incubate in a CO₂ incubator according to the Growth Conditions.
7. After 2 days, feed the cells with fresh Growth Media.

Feeding

1. Warm Growth Media in a 37°C water bath for approximately 1hr.
2. Remove the dish from the CO₂ incubator.
3. Decant the supernatant (old media).
4. Slowly transfer fresh pre-warmed Growth Media into the dish.
5. Return the dish to CO₂ incubator for 2~3 day until approx. 90% confluency is reached.
6. Split the 90% confluent culture 1 in 3.

Splitting Cell Culture

1. Warm Growth Media and 1X Citrate Saline in a 37°C water bath for approximately 1hr.
2. Remove the dish from incubator and decant the supernatant.
3. Rinse the dish with 5ml of 1X pre-warmed Citrate Saline for 10 s.
4. Carefully decant the Citrate Saline.
5. Repeat Steps 3 and 4 expect leaving a trace of Citrate Saline to cover the cell monolayer.
6. Leave the dish for 2 min and gently tap the dish sideways against a solid surface to detach cells from the bottom of the dish.
7. Re-suspend the cells in 6 ml warm Growth Media and distribute into 3 new 150 mm dishes containing 20 ml warm Growth Media.

8. Incubate in a CO₂ incubator according to the Growth Conditions
9. Feed the cells after 2~3 day incubation, and continue incubating until 90% confluency is reached.
10. Split 1:3 again.

Freezing Cells for Long Term Storage

1. Remove dishes containing 90% confluent cells from the incubator and decant the media.
2. Detach the cells as described previously.
3. Re-suspend cells in 2 ml 100% FBS.
4. Add 200 µl of DMSO and gently mix the suspension.
5. Transfer the suspension into 2 cryogenic vials.
6. Freeze the cells at -80°C for 2~3 days and transfer to liquid nitrogen bath for long term storage.

A. 1.1.2 Human hepatocellular carcinoma cells (HEP G-2)

Materials

Grow Medium

Dulbecco's Minimal essential medium (DMEM)

10% fetal bovine serum (FBS)

2mM L-glutamine

2.5 µg/ml fungizone amphotericin B

100 U/ml penicillin/streptomycin

Growth Condition

5% CO₂ at 37 °C

1X Trypsin-EDTA

1X Phosphate buffer saline (PBS)

Procedures

Culturing Frozen Cells

1. Warm Growth Media in a 37°C water bath for approximately 1hr.
2. Remove a vial of cell stock from liquid nitrogen and place it in a 37°C water bath until it completely thawed.
3. Transfer 20 ml of the pre-warmed Growth Media into a 150 mm dish.
4. Gently invert the thawed cell stock to ensure cells are well suspended.
5. Transfer the cells stock into the 150 mm dish and gently mix by mild agitation.
6. Incubate in a CO₂ incubator according to the Growth Conditions.
7. After 2 days, feed the cells with fresh Growth Media.

Feeding

1. Warm Growth Media in a 37°C water bath for approximately 1hr.
2. Remove the dish from the CO₂ incubator.

3. Decant the supernatant (old media).
4. Slowly transfer fresh pre-warmed Growth Media into the dish.
5. Return the dish to CO₂ incubator for 2~3 day until approx. 90% confluency is reached.
6. Split the 90% confluent culture 1 in 3.

Splitting Cell Culture

1. Warm Growth Media, 1XPBS and 1XTrypsin-EDTA in a 37°C water bath for approximately 1hr.
2. Remove the dish from incubator and decant the supernatant.
3. Rinse the dish with 10 ml of 1X PBS for 10 s.
4. Carefully decant the Citrate Saline.
5. Repeat Steps 3 and 4.
6. Add 0.5 ml 1X Trysin-EDTA
7. Leave the dish for 2 min and gently tap the dish sideways against a solid surface to detach cells from the bottom of the dish.
11. Re-suspend the cells in 6 ml warm Growth Media and distribute into 3 new 150 mm dishes containing 20 ml warm Growth Media.
12. Incubate in a CO₂ incubator according to the Growth Conditions
13. Feed the cells after 2~3 day incubation, and continue incubating until 90% confluency is reached.
14. Split 1:3 again.

Freezing Cells for Long Term Storage

8. Remove dishes containing 90% confluent cells from the incubator and decant the media.
9. Detach the cells as described previously.
10. Re-suspend cells in 2 ml 100% FBS.
11. Add 200 μ l of DMSO and gently mix the suspension.
12. Transfer the suspension into 2 cryogenic vials.
13. Freeze the cells at -80°C for 2~3 days and transfer to liquid nitrogen bath for long term storage.

A.1.2 Helper-dependent Adenovirus vectors

A.1.2.1 Determination of Physical Titer of Purified HDAd

Procedures:

1. A volume of 50 µl of HDAd stock is added into 0.5 ml TE buffer supplemented with SDS to 0.1%.
2. Blank is set up by adding 50 µl of 10 mM Tris-Cl, pH 8.0, 10% glycerol into 0.5 ml TE buffer with 0.1% SDS
3. Incubate for 10 min at 56°C
4. Vortex briefly
5. Add the blank into a 1 ml Quartz quvette and zero the spectrophotometer (SHIMADZU UV-VIS, Model UV-1201, serial no. 40083J) at 260nm wavelength
6. Transfer the blank back to the micro-tube and add the sample in to the same quvette and determine the absorbance of the sample at the same wavelength
7. Calculate the physical titer (PT) according to the following relationship:
$$\text{P.T. (particle per ml)} = (\text{OD}_{260} \times \text{dilution factor} \times 1.1 \times 10^{12}) \times 36 / (\text{size of vector in kb})$$

A.1.2.2 Infection of HEP G-2 cells with HDAD vectors

Materials

Grow Medium

Dulbecco's Minimal essential medium (DMEM)

2% fetal bovine serum (FBS)

2mM L-glutamine

2.5 µg/ml fungizone amphotericin B

100 U/ml penicillin/streptomycin

Growth Condition

5% CO₂ at 37 °C

1XPhosphate buffer saline (PBS)

Procedures

1. Warm Media and PBS in a 37°C water bath for approximately 1hr.
2. Remove the dish from the CO₂ incubator.
3. Remove media from the plate (6-well, 3cm well plate).
4. Wash cells gently with pre-warmed PBS twice to eliminate residual growth media
5. Add 0.2 ml pre-warmed PBS containing appropriate quantities of HDAd vectors
6. Allow absorption at 37°C for 1h.
7. Slowly transfer fresh pre-warmed Media into the dish.
8. Return the dish to CO₂ incubator for 2~3 day until approx. 90% confluency is reached.

A.1.3 Animals

A.1.3.1 Genotyping

Reagents:

BASE SOLUTION

1. 50X stock solution: Mix together 250 ml 5N NaOH, 20mL 0.5M EDTA, and sterile ddH₂O to 900mL total volume. Adjust pH to 12.0 with NaOH or HCl, and then adjust volume to 1000mL total with ddH₂O.
2. 1X working solution (25mM NaOH, 0.2mM EDTA, pH=12): Combine 1mL 50X stock with 49mL sterile ddH₂O.

NEUTRALIZATION SOLUTION

1. 50X stock solution (2M TRIS-HCl, pH=5.0): Dissolve 315.2g TRIS HCl in 900mL sterile ddH₂O, and adjust pH to 5.0 with NaOH or HCl. Adjust final volume to 1000mL.
2. 1X working solution (40mM Tris-HCl pH=5.0): Combine 1mL 50X stock with 49mL ddH₂O.

Procedures:

1. One toe is clipped from a mouse aged five days through adult.
2. Within one hour of cutting, the toes are placed into a 1.5 ml tube.
3. Add 75 μ L of 1X base solution to each tube, and place the tubes at 95° for 30 min.
4. After 30 min, cool the samples to room temperature or below.
5. Add 75 μ L 1X neutralization solution to the samples and vortex.

6. Use 4 μ L of this mixture in a 25 μ L PCR reaction.

PCR reaction

Reaction Mix

Reagents	Volume (μ L)
ddH ₂ O	12.5
10X PCR buffer w/t MgCl ₂ ^a	2.5
MgCl ₂ (25 mM) ^b	1.5
dNTP Mix (10 mM) ^c	0.5
P3 Forward Primer (25 μ M) ^d	1
P2 Reverse Primer (25 μ M) ^d	1
P4 Reverse primer (25 μ M) ^d	1
Template DNA	4
Taq Polymerase (1 U/ μ L) ^e	1

Note:

- Consists of 750mM Tris-HCl (pH 8.8 at 25°C), 200mM (NH₄)₂SO₄, 0.1% Tween 20. Produced by Fermentas.
- Produced by Fermentas.
- dNTP mix consists of dATP, dCTP, dGTP and dTTP with concentration of 10 mM, Produced by Fermentas, Stock No. R0191.
- Three diagnostic primers were designed as instructed by Maeda *et al.*, and synthesized by Mobix lab.
- Taq polymerase was produced by Fermentas, stock No. #EP0403.

Thermo Steps

PCR Step	Temperature	Duration	Number of Cycles
Initial denaturation	94	2 min	1
Cycled denaturation	94	20 s	30
Annealing	48.8	30 s	
Extension	72	30 s	
Terminal extension	72	7 min	1

Note: Eppendorf MasterCycler was used for PCR

7. 10~20 μ L PCR product is subjected to agarose gel electrophoresis as described previously

8. A product of 330 bp infers wild type GULO. 230 bp indicates mutated GULO.

A.1.3.2 Blood collection and blood sample preparation

Materials

- Equipments:
 - Restrainer
 - Blood Collecting Tubes (Heparinized)
 - Peanut clipper (shaver)
 - 25 G needle
- Reagents:
 - 5% MPA-EDTA
 - 5 g meta-phosphoric acid
 - 0.4 ml 0.5 M EDTA
 - ddH₂O to 100 ml

Procedures

1. Lure the mouse into the restrainer
2. Pull the hind leg out (left leg if right-handed)
3. Shave the exterior of the hind leg using peanut clipper
4. Locate the saphenous vein on the hind leg
5. Puncture the vein with 25 G needle
6. Collect the venous blood with blood collection tube driven by surface tension
7. Return the animal to its cage
8. Expel the blood into a microtube centrifuge immediately at 1000 g for 5 min
9. Transfer 20µl cell free plasma into 180µl 5% MPA-EDTA
10. Centrifuge for 30 min at 12,000 g at 4°C
11. Extract the supernatant and store at -80°C

A.1.4 Western blot

A.1.4.1 Purification of intracellular proteins

From cells (6-well plate):

1. Remove the growth medium from the cells
2. Rinse the cells twice with PBS
3. Add 300 μ l CelLytic M reagent (Sigma) to each well
4. Scrape cells the pippet tip
5. Incubate with rocking for 15 min
6. Collect cells and centrifuge for 30 min at 12,000 g at 4°C
7. Remove the protein-containing supernatant to a pre-chilled tube
8. Store at -70°C

From the liver:

1. Weigh out approx. 200 mg liver from frozen specimen in an microtube (1.5 ml).
2. Add 1 ml cold CelLytic MT reagent (Sigma) to the liver sample
3. Transfer into a tissue homogenizer (Mortar and pestle)
4. Homogenize liver sample until there is no more chunks of tissues
5. Transfer into a microtube (1.5 ml) and centrifuge for 30 min at 12,000 g at 4°C
6. Carefully transfer the supernatants to another pre-chill microtube
7. Store at -70°C

A.1.4.2 SDS-polyacrylamide gel electrophoresis

Protein quantification and preparation

1. Protein concentrations in the samples are quantified by Bradford assay using BSA as standard
2. Proteins are denatured by boiling for 10 min
3. 30 μ g of protein is loaded to each well

SDS-PAGE

1. SDS running gel (10%) is cast according to the following recipe

SDS Running Gel (10%)	Volume (ml)
ddH ₂ O	4
1.5M Tris-Cl pH 8.8	2.5
10% SDS	0.1
30% Ary/Bis	3.3
10% APS	0.1
TEMED	0.01
Total Volume	10

2. After the running gel has solidified (10 min) cast the stacking gel (4%)

SDS Running Gel (10%)	Volume (ml)
ddH ₂ O	3.6
1.5M Tris-Cl pH 8.8	0.63
10% SDS	0.05
30% Ary/Bis	0.66
10% APS	0.05
TEMED	0.005

3. After the upper gel has solidified (15 min) load denatured protein samples (30 μ g per well)
4. Electrophoresis at 100 V until dye fronts run out the gel

A.1.4.3 Protein transfer

Protein transfer

- Equipments:
 - Transfer chamber and sandwich insert
 - Three blot papers approx. 7x10cm
 - One PVDF membrane 7x10cm
- Reagents:
 - 1XTransfer buffer:
 - 57.6 g Glycine
 - 12.1 g Tris
 - 800 ml methanol
 - ddH₂O upto 4 L
- Procedure:
 1. Rinse the gel with ddH₂O to remove chunks of gel
 2. Soak the PVDF membrane with methanol
 3. Soak the PVDF membrane, blot papers and spacer in the transfer buffer
 4. Assemble the cassette and AVOID air bubbles
 5. Inert the cassette into the chamber
 6. Add the transfer buffer into the chamber and cassette
 7. Transfer in cold room for 1 h at 90 V or overnight at 20 V
 8. Try the membrane for at least 10 min

A.1.4.4 Immunoblot

- **Reagents:**

- 1XTBST:
 - 26.1 g NaCl
 - 30 ml 1M Tris-Cl pH 8.0
 - 1.5 ml Tween 20
 - ddH₂O upto 3L
- 5% milk in 1xTBST
- Primary antibody
 - Anti-Gulo developed in rabbits
 - Reconstitute in 200µl PBS stored at -20°C
 - 15 µl of the reconstituted antibody was added to 15 ml of milk
- Secondary antibody
 - Goat anti-rabbit (Bio-rad 170-6515)
 - 10 µl of secondary antibody was added to 30 ml of milk
- Detection reagents (Amersham biosciences)
 - Detection reagent 1 (RPN2106V1)
 - Detection reagent 2 (RPN2106V2)

- **Procedures**

1. Take the membrane out of the cassette and try for 10—60 min at room temperature
2. Block the PVDF membrane in ~20ml of 5% milk for 1h with 80 rpm agitation
3. Incubate with primary antibody in milk at 4°C overnight
4. Wash the PVDF membrane three times (10min each) with TBST buffer
5. Add 20ml previously prepared secondary antibody and incubate at room temperature for 1 h with 80 rpm agitation
6. Wash again as step 4.
7. Discard remaining TBST
8. Add 5 ml Detection reagent 1 and 5 ml Detection reagent 2 directly on the gel and shake for 1 min
9. Seal the membrane with cling film and AVOID any air bubbles

10. In the dark room expose the membrane with Hyperfilm (RPN2114K) for appropriate amount of time
11. Develop the film

A.1.5 HPLC-electrochemical analysis for ascorbic acid

Materials

- Equipments:
 - A HPLC system (Agilent 1200 series)
 - Agilent XDB-C18 analytical column (particle size 5 μm , 4.6 x 250 mm).
 - CoulochemIII electrochemical detector (ESA)
- Reagents:
 - 5% MPA-EDTA
 - Ultra pure ascorbic acid
 - Mobile phase
 - 27.2 g KH_2PO_4
 - 4 ml 0.5 M EDTA
 - ddH₂O to 1 L
 - Adjust pH with Phosphoric acid to pH 3.0

Procedures

1. Filter mobile phase through 0.2—0.4 μm filter
2. Place pump inlet A1 into the mobile phase reservoir
3. Turn on all components of the HPLC system and the CoulochemIII detector
4. Once the system has been stabilized, open the EZChrom software
5. Develop a new method with the following settings
 - Flow rate: 3ml/min
 - Autosampler: 96-well format
 - Column compartment: 1→6 or where the column is attached
 - Dioarray detector: wave length 240 nm and UV on for 2 min
 - Coulochem electrochemical detector:
 - i. Guard cell: -200 mV

- ii. Analytical cell: Channel 1 at -150 mV, Channel 2 at 150 mV
 - iii. Run time: 2 min
- 6. Save the method
 - 7. Go to Sequence Wizard and follow the instructions
 - 8. Start the sequence

Note:

- 1. Should run the mobile phase for at least 15 min to stabilize the system
- 2. According to these settings ascorbic acid should be eluted out at 1.1 min
- 3. A multi-peak complex (solvent front) will be eluted out before ascorbic acid
- 4. If appropriate, standard vitamin C with known concentrations should be used as quality controls
- 5. Autosampler light should be turned off to prevent decomposition of ascorbic acid

Appendix 2. Peer-Reviewed Publications 2006—2007

- Li,Y., Schellhorn,H.E. (2006) Can ageing-related degenerative diseases be ameliorated through administration of vitamin C at pharmacological levels? *Med Hypotheses*.
- Li,Y., Schellhorn,H.E. (2007) New developments and novel therapeutic perspectives for vitamin C *J.Nutr.* **137**: 2171-2184.
- Li,Y., Schellhorn,H.E. (2007) Rapid kinetic microassay for catalase activity *J.Biomol.Tech.* **18**: 185-187.