DEVELOPMENT OF ROBUST ANIMAL MODELS FOR VITAMIN C FUNCTION

BY

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Abstract

Vitamin C inhibits the oxidation of biologically important molecules and may have a potential protective role against cancer, cardiovascular diseases, and aging. Clinically relevant models of vitamin C function are essential for understanding the role of the antioxidant in the pathogenesis of these complex diseases, and its therapeutic potential. In this thesis, we examine ascorbic acid synthesis and deficiency in animal models, and develop these animal models into powerful tools to examine specific questions of vitamin C function. This thesis first presents a review on the existing animal models for antioxidant function in human nutrition, focusing on their clinical relevance in chronic diseases. We concluded that equivocal proof of beneficial effects of high dose antioxidant supplementation has not been established, and further investigations of animal models of antioxidant function are needed to resolve outstanding questions.

We then examined the feasibility and efficacy of an alternative vitamin C delivery method using gene therapeutic lentivirus vectors in a guinea pig model of vitamin C function. The guinea pig exhibits an inactivated gulonolactone oxidase gene (*Gulo*), which is required for endogenous ascorbic acid synthesis, and as such must acquire vitamin C from the diet. Using a lentivirus vector carrying the mouse *Gulo* under the murine cytomegalovirus (mCMV) promoter, which was previously developed as a part of my undergraduate thesis, we examined the ability of this gene therapeutic vector to mediate the expression of GULO and the production of ascorbic acid in guinea pigs. At a titre of 10^{10} viral particles per animal, the life of lentivirus-treated guinea pigs were prolonged by 35 days compared to the scorbutic control, which was given an ascorbic

acid-free diet. Ascorbic acid was produced in the liver of the treated guinea pigs, but the amount produced was not sufficient to elevate plasma concentrations or fully correct the metabolic deficiency. We conclude that lenti-mCMV-*Gulo* is able to mediate the expression of GULO and endogenous production of vitamin C in guinea pigs.

To test the role of vitamin C in cancer etiology and outcome, we are currently in the process of introgressing the *Gulo*$^{-/-}$ inactivation mutation, developed by Maeda *et al.* in 2000, from the C57BL/6 strain background into the FVB/N strain background. The FVB/N strain is also the background for several models of *erbB2/neu* overexpression in human breast cancer, associated with increased metastasis and low patient survival rates. Taken together, this thesis develops two animal models of vitamin C function, which can be employed in future applications.

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Chapter 1: Animal models for antioxidant function in human nutrition

1.1 Introduction

Increased levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) during stress are highly toxic to biological systems. Cellular antioxidants quench these, as well as other free radicals, to protect biologically important molecules – proteins, lipids, and DNA – from oxidative damage. However, with the recognition that reactive species can participate in signaling pathways and oxidative stress modulates gene expression, the nutritional importance of antioxidants and the complexity of antioxidant function are being re-evaluated.

Rodents have been used extensively to model diseases associated with oxidative stress and aging, such as cancer, cardiovascular diseases, and neurodegenerative diseases. These diseases are chronic and complex, with regulatory pathways that are incompletely understood. However, due to genetic and physiological difference from humans, these animals may not robustly model all aspects of disease symptoms and etiology (Table I). Nonetheless, there is increased interest in the use of animal models to provide new insights on the complex processes of oxidative damage-related diseases. As well, since key antioxidants are diet associated, use of animal models may provide guidance on recommended levels of dietary antioxidants, reviewed by the National Academy of Sciences. In this review, we examine animal models that mimic human antioxidant status, and evaluate the therapeutic implications of antioxidants in chronic diseases. Application of the models to current research goals is also discussed.

1.2 Vitamin C

Scurvy has been known since antiquity, but the recognition that fresh fruits and vegetables prevented scurvy came slowly. In 1753, James Lind showed that citrus fruits prevented scurvy in what is widely considered the first clinically controlled experiment. By 1795, limes and lemons became standard issue at sea in the British navy. Studies of the disease and the factor that prevents it then ceased for over a century. In 1907, before even the development of the "vitamin" concept, guinea pigs were serendipitously found to develop scurvy when fed a diet of grains and flour. The suspected antiscorbutic factor (referred to as "water-soluble C"), L-hexuronic acid, was isolated by Albert Szent-Gyorgyi and Joseph Svirbely in 1928-1932, first from animal adrenal glands and then from paprika peppers. L-hexuronic acid was proven to be the antiscorbutic factor by a team led by Charles King in 1932. Its chemical structure was identified in 1933 by Walter Haworth who, together with Szent-Gyorgyi, renamed the compound to ascorbic acid.

Today, the term "vitamin C" includes both ascorbic acid and dehydroascorbic acid. In addition to its antiscorbutic effects, ascorbic acid – but not dehydroascorbic acid – acts as a water-soluble antioxidant. When encountering single-electron carrying species, ascorbic acid donates a single electron to the reactive species, and is itself oxidized to semidehydroascorbate. Semidehydroascorbate is extremely inert as a single-electron radical, offering antioxidant protection to the biological system. Semidehydroascorbate can be further oxidized to dehydroascorbate (DHA) either chemically or enzymatically (Figure 1).

Whereas ascorbic acid is produced endogenously in most animals, higher primates of the suborder Haplorhini (which includes prosimians, Old World monkeys, apes, and New World monkeys) are among the few groups that lack this ability (Pollock and Mullin 1987). This is due to the loss of *Gulo*, which encodes for gulonolactone oxidase (GULO), responsible for the last step of ascorbate synthesis (Nishikimi *et al.* 1988). The guinea pig, the first animal known to develop scurvy, also lacks GULO (Burns *et al.* 1956). The Osteogenic Disorder Shionogi (ODS) rat was then isolated with GULO deficiency (Mizushima *et al.* 1984; Kawai *et al.* 1992), before the mouse became widely accepted as the vertebrate model organism. Thereafter, a number of mouse mutants unable to synthesize vitamin C were generated and isolated in the early 2000s. Other animals lacking *Gulo* include one pig mutant (Hasan *et al.* 2004), passerine birds (Chaudhuri and Chatterjee 1969), teleost fish (Moreau and Dabrowski 2000), and some species of bats (Cui *et al.* 2011a). Vitamin C uptake requires the expression of glucose transporters (GLUT) for facilitated diffusion of dehydroascorbic acid after extracellular oxidation of ascorbic acid, or sodium vitamin C co-transporters (SVCT) for active transport. Both isoforms of SVFTs, SVCT1 and SVCT2, co-transport a sodium and an ascorbate ion across the cellular membrane against a concentration gradient. The excess sodium in the cytoplasm is then pumped out through a sodium-potassium ATPase (Li and Schellhorn 2007).

1.2.1 Guinea pig

Similar to humans, guinea pigs cannot synthesize ascorbic acid due to the lack of GULO (Burns *et al.* 1956), and develops scurvy when vitamin C is absent from the diet.

The *Gulo* gene was later confirmed to be a pseudogene with many accumulated mutations (Kawai *et al.* 1992). Inactivating mutations in the *Gulo* gene is estimated to have occurred 13-15 million years ago in guinea pigs, and 61-74 million years ago in Haplorhine primates (Lachapelle and Drouin 2011).

Both guinea pigs and humans exhibit unique molecular characteristics that may be an evolutionary response to reduced ascorbate levels. Guinea pigs and humans exclusively express the Glut1 on the erythrocyte membrane, which preferentially transports DHA, while ascorbate-synthesizing animals express Glut4, which transports glucose (Montel-Hagen *et al.* 2008). As well, guinea pig ileum (Stevenson and Brush 1969) and human ileum (Stevenson 1974) both transport ascorbate against an electrochemical and concentration gradient, while rats do not have the same intestinal transport capacity (Horio *et al.* 1985), indicating that humans and guinea pigs have enhanced vitamin C transport capacity to compensate for the lack of ascorbic acid synthesis. Thus, the guinea pig may mimic the human physiology more closely than other rodents, and thus is a particularly useful animal to model ascorbate metabolism. Guinea pigs are also strikingly similar to humans in cholesterol metabolism. For example, unlike other rodents, both guinea pigs and humans carry cholesterol in LDL (Fernandez and McNamara 1989). The guinea pig is thus a unique model to study cholesterol metabolism and cardiovascular diseases.

Studies using the guinea pig primarily focused on the physiological manifestations of vitamin C deficiency and supplementation, defining the importance of vitamin C in maturation (Lykkesfeldt 2002; Lykkesfeldt and Moos 2005), including age-related

deterioration of sight (Hayes *et al.* 2011) and hearing (McFadden *et al.* 2005; Heinrich *et al.* 2008). Millimolar concentrations of vitamin C is selectively toxic to tumour cells (Casciari *et al.* 2005), as vitamin C generates H_2O_2 at high concentrations, and tumour cells often do not express catalase. Vitamin C supplementation moderately protects guinea pigs against cardiovascular risk factors (Bell *et al.* 2001; Wolkart *et al.* 2008) and smoke-induced pathologies (Panda *et al.* 2001; Banerjee *et al.* 2008; Ray *et al.* 2010). However, increased vitamin C and vitamin E intake have limited efficacy in mitigating syndromes caused by maternal alcohol consumption, and furthermore, produces deficits in infant behaviour when pregnant guinea pigs are not given alcohol (Nash *et al.* 2007). The mechanism for this potential adverse effect of vitamin C supplementation is unknown (Nash *et al.* 2007). Results obtained using guinea pigs provide evidence both for (Kapsokefalou and Miller 2001) and against (Chen *et al.* 2000) the apparent pro-oxidant properties of ascorbic acid, where ascorbic acid increases rather than decreases the cellular flux of reactive oxygen species (ROS).

The GULO enzyme, modified with glutaraldehyde (Sato and Walton 1983) and PEG (Hadley and Sato 1989), can rescue guinea pigs from scurvy in enzyme replacement therapies. Ascorbate synthesis occurs as long as L-gulonolactone, the substrate of GULO, is provided (Sato and Walton 1983; Hadley and Sato 1989), indicating that the endogenous pool of L-gulonolactone in the guinea pig is not enough to support GULO activity. Repeated intraperitoneal administrations of the modified enzyme and the substrate can prevent scurvy for a prolonged period of time (Hadley and Sato 1988), but intravenous injections of the enzyme remains highly immunogenic and causes death

(Hadley and Sato 1989). Interestingly, all animals showed an increase in plasma ascorbic acid before death (Hadley and Sato 1989).

1.2.2 ODS rat

The Osteogenic Disorder Shionogi (ODS) rat is a spontaneous mutant isolated with hind limb disorders, reduced weight gain, infertility, and premature death (Mizushima *et al.* 1984). These rats harbor a spontaneous mutation in a single autosomal gene *od*, later identified as a point mutation in *Gulo*, leading to a cysteine to tyrosine substitution at the 61st amino acid residue (Kawai *et al.* 1992). This mutation decreases detectable GULO concentration and activity by 90%, but does not affect the amount of mRNA present (Nishikimi *et al.* 1989), likely due to miscoding introduced by the mutation.

Results from several cancer studies indicate that vitamin C-deficient ODS rats, vitamin C-supplemented ODS rats, and wildtype controls capable of vitamin C synthesis, are equally susceptible to chemical-induced carcinogenesis (Mori *et al.* 1988; Mori *et al.* 1990; Mori *et al.* 1991; Yuann *et al.* 1999). Unsupplemented ODS rats exhibit increased metabolic activation of promutagens in the liver (Mori *et al.* 1993), but have a decreased risk of urinary bladder carcinogenesis (Mori *et al.* 1997). Similarly, the effect of vitamin C on cardiovascular diseases remains unresolved. Vitamin C deficiency increases lipid peroxidation (Kimura *et al.* 1992) and lowers serum apolipoproteins concentrations (Ikeda *et al.* 1996), indicating that adequate vitamin C is needed to prevent atherosclerotic diseases. On the other hand, vitamin C deficiency lowers blood pressure, antagonizes hypertension (Horio *et al.* 2001), and confers resistance to ischemic insults of the heart

(Vergely *et al.* 2001). Thus, it is still unclear whether vitamin C influences the prevention and treatment of cancer or cardiovascular diseases.

The efficiency of orally administered DHA and ascorbic acid was investigated using the ODS rat. Dietary DHA supplementation achieves only 10% of the tissue ascorbate concentration achieved by a molar equivalent of dietary ascorbic acid (Ogiri *et al.* 2002). This is not observed in humans (Tsujimura *et al.* 2008), but differences exist between the two studies, such as dose of DHA and ascorbic acid used, length of treatment, method of detection, and tissue/body fluid tested. Thus, the nutritional requirements of vitamin C in humans may need to be re-evaluated.

1.2.3 Gulo *targeted knockout mouse*

The *Gulo*$^{-/-}$ mouse was constructed by deleting exons 3 and 4 from *Gulo* in a C57BL/6 background (Maeda *et al.* 2000). This renders *Gulo* fully non-functional, and vitamin C supplementation is required to maintain viability. Interestingly, the plasma ascorbate level of *Gulo*$^{-/-}$ mice maintained on vitamin C-deficient diet does not reach zero upon dietary restriction, and is instead maintained at 15% of wildtype levels (Maeda *et al.* 2000), suggesting an uncharacterized pathway to generate a small amount of ascorbate. Consistently, vitamin C levels in unsupplemented *Gulo*$^{-/-}$ mice after 5 weeks of deficiency are still 25-30% of wildtype levels in major organ stores such as the adrenal gland, lungs, and the brain (Kim *et al.* 2012). Similar to observations made with guinea pigs, vitamin C levels increased before endpoint in several tissues, including the intestines, heart, adrenal gland, and spleen (Kim *et al.* 2012).

Vitamin C deficiency in the *Gulo*$^{-/-}$ mouse leads to aortic wall damage and increased plasma total cholesterol (Maeda *et al.* 2000), suggesting an increased risk for cardiovascular diseases in deficient animals. However, when the *Gulo*$^{-/-}$ genotype was crossed into the *Apoe*$^{-/-}$ mouse model for atherosclerosis, vitamin C deficiency does not influence either the development, distribution, or size of atherosclerotic plaques on the aortic sinus (Nakata and Maeda 2002). Similarly, hemorrhages and disrupted aortic vasculature in *Gulo*$^{-/-}$ animals are reported in some studies (Maeda *et al.* 2000; Telang *et al.* 2007), but not others (Parsons *et al.* 2006). The effect of vitamin C deficiency in cancer is also equivocal. In one study, implanted tumours were larger in vitamin C-deficient mice, and tumour growth slowed in response to oral vitamin C treatment (Telang *et al.* 2007). In another, the incidence, onset, and growth of induced mammary tumours were independent of plasma vitamin C levels (Parsons *et al.* 2006). In studies of neurofunction, vitamin C deficiency in the *Gulo*$^{-/-}$ mouse impairs motor skills, but not cognition (Harrison *et al.* 2008). Importantly, vitamin C supplements that are sufficient to maintain basic physiological conditions was not sufficient to maintain cognitive health in the AP/PSEN1/*Gulo*$^{-/-}$ mouse, predisposed to cognitive decline and age-dependent neurodegenerative disease (Harrison *et al.* 2008), indicating an increased need for vitamin C supplementation with age.

As the genomics and genetics of the mouse have been extensively studied, the *Gulo*$^{-/-}$ mouse has become the model of choice in studying the role of vitamin C in complex diseases. Vitamin C production has been successfully restored in the *Gulo*$^{-/-}$ mouse using a gene therapeutic adenovirus (Li *et al.* 2008), making it possible to robustly

manipulate physiological ascorbate levels. Unlike guinea pigs treated with modified GULO enzyme (Sato and Walton 1983; Hadley and Sato 1989), it is not necessary to provide exogenous L-gulonolactone to the virus-treated *Gulo*$^{-/-}$ mice (Li *et al.* 2008), indicating that L-gulonolactone is produced at sufficient levels in the *Gulo*$^{-/-}$ mouse for GULO activity.

1.2.4 sfx *mouse*

The spontaneous fracture (*sfx*) mouse was a mutant in the BALB/cBy background, isolated with a defining phenotype of spontaneous bone fractures shortly after weaning (Beamer *et al.* 2000). The mutation responsible was later mapped to *Gulo*, with a precise deletion of all 12 exons (Jiao *et al.* 2005; Mohan *et al.* 2005). Similar to the *Gulo*$^{-/-}$ mouse and in contrast to humans, plasma ascorbate concentration does not reach zero in the *sfx* mice (Mohan *et al.* 2005). Vitamin C supplementation in the drinking water corrects all abnormalities, including the spontaneous bone fracture, as well as lower body weight, infertility, and premature death (Mohan *et al.* 2005).

Use of the *sfx* mouse model has been focused on the osteoarticular manifestations of ascorbic acid deficiency (Xing *et al.* 2007; Kim *et al.* 2010). Transcription profiling of the *sfx* mouse confirmed the regulatory role of vitamin C on collagen genes and osteoblast differentiation, while suggesting possible involvement in hormone metabolism and immunity (Yan *et al.* 2007). However, it is noted that the analysis could not distinguish between deregulated gene expression due to *Gulo* deficiency, and expression changes in response to disease (Yan *et al.* 2007).

It is notable that the defining phenotype of the *sfx* spontaneous mutant – bone fragility – is not observed in the *Gulo*$^{-/-}$ mouse. It is likely that neither mouse models exactly mimic all aspects of vitamin C metabolism and function in humans, thus limiting any direct and simplified application of results obtained. These limitations notwithstanding, studies using the two *Gulo* mutants can further our understanding of the interactions of vitamin C with principal factors in complex diseases, through the development of double knockout/mutants.

1.2.5 SVCT1 and SVCT2 targeted knockout mice

Vitamin C is actively transported by two isoforms of sodium vitamin C transporters, SVCT1 and SVCT2. The SVCT1 and SVCT2 knockout mice are therefore thought to be capable of modeling severe vitamin C deficiency. SVCT1, responsible for vitamin C absorption in the intestine, kidney, and liver, was inactivated in the 129S/SvEvTac mouse (Corpe *et al.* 2010). Ascorbate excretion was increased 18-fold in the SVCT1$^{-/-}$ mouse, but as these mice expressed functional *Gulo*, it was easily compensated for by an increase in ascorbate synthesis. Interestingly, there is a 45% perinatal mortality associated with the genotype of the dams but not of the pups, indicating a relationship between vitamin C status during pregnancy and perinatal survival (Corpe *et al.* 2010).

In contrast to the "bulk" transport characteristics of SVCT1, expression of SVCT2 is localized to highly specialized neuronal, retinal, and placental cells, where vitamin C is accumulated to very high levels. The SVCT2$^{-/-}$ mouse, also in the 129S/SvEvTac background, exhibit marked reduction in ascorbate concentration in the central nervous

system (Sotiriou *et al.* 2002), and may be ideal to study the role of vitamin C in neuronal function. However, the SVCT2$^{-/-}$ mouse die within minutes of birth with brain hemorrhage and respiratory failure (Sotiriou *et al.* 2002). The precise mechanism leading to death is not understood (Bornstein *et al.* 2003; Harrison *et al.* 2010; Gess *et al.* 2011). As SVCT2 is required for placental transport of vitamin C, this vitamin may be essential for *in utero* development.

The SVCT2$^{-/-}$ mutation was later moved to the C57BL/6 background and crossed with the apolipoprotein E deficient (*Apoe*$^{-/-}$) mouse, an atherosclerosis model (Babaev *et al.* 2011). Contrary to expectations, restriction of vitamin C intake in the double mutant decreased atherosclerotic lesion size (Babaev *et al.* 2011). In contrast to results obtained from the SVCT2$^{-/-}$*Apoe*$^{-/-}$ mouse, severe vitamin C deficiency in the *Gulo*$^{-/-}$ SVCT2$^{+/-}$ mouse exacerbates atherosclerosis (Babaev *et al.* 2010). The role of vitamin C in cardiovascular diseases is thus unclear.

1.2.6 SMP30/GNL knockout mouse

The senescence marker protein-30 (SMP30) knock out mouse was originally constructed in the C57BL/6 background as a model of aging and premature death (Ishigami *et al.* 2002). SMP30 was subsequently identified to be gluconolactonase (GNL) in ascorbate synthesis (Kondo *et al.* 2006). Despite that an alternative pathway bypasses the need of GNL and produces a small flux of ascorbate, SMP30$^{-/-}$ mice fed with vitamin C-deficient diet still develop scurvy, characterized by bone fracture, rachitic rosary (overgrowth of the rib cartilage), and premature death (Kondo *et al.* 2006).This

alternative pathway only bypasses GNL and is still dependent on functional GULO for ascorbic acid production (Kondo *et al.* 2006).

Vitamin C deficiency caused by the SMP30$^{-/-}$ mutation accelerates many aspects of senescence and aging also observed in humans, including cataract formation (Ishikawa *et al.* 2012), decline of glucose/insulin tolerance (Hasegawa *et al.* 2010), decline of skin integrity (Arai *et al.* 2009), and hearing loss (Kashio *et al.* 2009). On the other hand, low vitamin C intake ameliorates CCl_4-induced liver fibrosis (Park *et al.* 2010a; Park *et al.* 2010b; Ki *et al.* 2011). The oxidant-antioxidant balance is disrupted in the SMP30$^{-/-}$ mouse, causing pulmonary emphysema (Koike *et al.* 2010), damage in the central nervous system (Kondo *et al.* 2008) and damage in the immune system (Chung *et al.* 2010). Mechanistic investigations are needed to critically examine the effects of vitamin C deficiency in the SMP30$^{-/-}$ mouse, and to establish the potential of vitamin C as an anti-aging therapeutic.

1.2.7 Other animal models

There is renewed interest in the ability of bats to synthesize vitamin C. An early survey of 50 species of bats has concluded that all bats lack *Gulo* and require dietary vitamin C (Birney *et al.* 1976). It was later determined that 2 additional species of bats do express functional GULO capable of producing vitamin C *in vitro*, albeit at lower levels than murine GULO (Cui *et al.* 2011a). There are differing degrees of gene attenuation through mutation among species of bats (Cui *et al.* 2011b), suggesting that the loss of *Gulo* began very recently – less than 3 million years ago. Gene reactivation events have also been identified (Cui *et al.* 2011a; Cui *et al.* 2011b). As such, bat *Gulo* may be

considered an intermediary between a functional, protein-encoding gene such as the murine *Gulo*, and a pseudogene such as the human *Gulo*. Bat models may serve as an important tool in investigating the functional role of vitamin C and the evolutionary significance of the loss of its synthesis.

A family of Danish pigs has recently acquired a spontaneous mutation in *Gulo* (Hasan *et al.* 2004). This is an isolated incidence in pigs, and despite the recognition that the biochemistry and physiology of pigs more closely resembles humans than rodents, it has not been examined further. Other animals naturally lacking *Gulo* include teleost fish (Cho *et al.* 2007) and passerine birds (Chaudhuri and Chatterjee 1969), none of which have been developed into experimental models, but have been frequently used in studies of genomics and bioinformatics. Finally, all Haplorhine primates lack functional *Gulo*. Studies using primates are restricted to reports of their daily vitamin C intake, which is several times that of humans (Milton and Jenness 1987).

1.3 Vitamin E

Vitamin E was first identified as an essential nutrient for infant development by Widenbauer in 1938, treating premature newborn infants suffering from growth failure with wheat germ oil. In 1948, Gyorge and Rose discovered the antihemolytic properties of tocopherol, and in 1949, α-tocopherol was used by Gerloczy to prevent and cure edema. By the 1960s, vitamin E deficiency is widely known to cause hemolytic anemia, and supplementation of the vitamin in infant formulas has since eradicated the disease caused by vitamin E deficiency.

More forms of the lipid-soluble vitamin have since been discovered, including four tocopherols and four tocotrienols. Vitamin E provides antioxidant protection to the cell membrane by scavenging peroxyl radicals – compared to polyunsaturated fatty acids, vitamin E reacts 1000 times faster with peroxyl radicals (Buettner 1993). The tocopheryl radical thus formed is then reduced back to its active form by other electron donors, such as ascorbic acid (Figure 1).

Of the eight molecules included in the term vitamin E, α-tocopherol is the most biologically active, preferentially accumulated (Vatassery *et al.* 1983), and by far the most studied. Rodents have been routinely used to study vitamin α-tocopherol deficiency and function in starvation and supplementation studies, as they (and all animals) require vitamin E in the diet. Genetic models of severe vitamin E deficiency include two independently developed mice lacking α-tocopherol transfer protein (α-TTP), which controls plasma and tissue α-tocopherol concentrations by exporting α-tocopherol from the liver.

1.3.1 Ttpa *knockout mouse*

The *Ttpa* gene encodes for α-tocopherol transporter protein, an vitamin E exporter expressed in liver cells. Two *Ttpa* knockout mice have been developed, both in the C57BL6 background. One group deleted all of exon 1 (Jishage *et al.* 2001), while another deleted the 5' UTL sequences of exon 1, resulting in the deletion of the transcription start codon (Terasawa *et al.* 2000). Both types of $Ttpa^{-/-}$ mice have very low or undetectable levels of α-tocopherol, and are infertile in the absence of vitamin E. Both mice exhibit

neuronal degeneration leading to progressive ataxia and age-related behavioural defects (Yokota *et al.* 2001; Gohil *et al.* 2004; Yoshida *et al.* 2010).

The *Ttpa*$^{-/-}$ mouse constructed by Jishage *et al.* (2001) can be employed as a model for delayed onset, slowly progressive neuronal degeneration caused by vitamin E deficiency-induced chronic oxidative stress (Yokota *et al.* 2001), and other aging-related pathologies (Tanito *et al.* 2007; Yoshida *et al.* 2010). Infertility of the *Ttpa*$^{-/-}$ mouse was investigated using this mouse construct, with the conclusion that α-tocopherol is important for placentation, but not necessary for fetal development (Jishage *et al.* 2001; Kaempf-Rotzoll *et al.* 2002; Jishage *et al.* 2005). The question of male fertility was not explored in these studies. This mouse is also used to explore participation of vitamin E in parasitic infections, showing that low vitamin E status confers resistance to infection, as a result of enhanced oxidative damage to the parasites (Herbas *et al.* 2009; Herbas *et al.* 2010; Shichiri *et al.* 2012).

Using the *Ttpa*$^{-/-}$ mouse developed by (Terasawa *et al.* 2000), α-tocopherol sensitive genes were identified in the brain (Gohil *et al.* 2003; Gohil *et al.* 2004), heart (Vasu *et al.* 2007), muscle (Vasu *et al.* 2009), and lungs under conditions known to induce oxidative stress (Gohil *et al.* 2007; Vasu *et al.* 2010). Adequate vitamin E levels are needed for proper inflammatory (Schock *et al.* 2004) and allergic (Lim *et al.* 2008) immune responses. Interestingly, vitamin E-deficient mice have improved glucose tolerance and sensitivity to insulin, indicating a role in the pathogenesis of type 2 diabetes mellitus (Birringer *et al.* 2007). Finally, results obtained from the *Ttpa*$^{-/-}$ *Apoe*$^{-/-}$ double mutant indicate that vitamin E deficiency does not cause atherosclerosis, but does

promote it though a mechanism independent of lipid oxidation (Terasawa *et al.* 2000; Suarna *et al.* 2006).

1.4 Uric acid

Uric acid is the metabolic end product of purine nucleoside catalysis. It is a major contributor in the total antioxidant defense in humans, and is in the highest concentration of all antioxidants in the human blood (Maxwell *et al.* 1997). Uric acid donates a single electron to reactive species, and is itself converted to the relatively stable urate radical, which then decays to allantoin (Figure 1). Uric acid cannot scavenge superoxide (Kuzkaya *et al.* 2005), and the presence of other water-soluble antioxidants such as vitamin C is absolutely required for complete antioxidant protection. Uric acid also exhibits a secondary antioxidant effect by chelating iron (Davies *et al.* 1986).

Uric acid accumulates in high concentrations in humans and other New World monkeys, due to a loss of the gene encoding urate oxidase (UO) (Wu *et al.* 1989). In other mammals, UO further oxidizes uric acid to the more soluble allantoin, which is excreted as the predominant nitrogenous waste in the urine. The loss of UO closely parallels the loss of GULO in higher primates, indicating a possible substitution of uric acid for ascorbic acid as the major antioxidant in these species. A UO knockout mouse has been constructed to model the effect of the loss of UO in humans and its resulting uric acid accumulation (Wu *et al.* 1994).

1.4.1 Urate oxidase (UO) knockout mouse

The UO knockout mouse was constructed by inserting a stop codon in exon 3 of the *uox* gene, resulting in complete loss of UO activity and a 10-fold increase in serum

uric acid level (Wu *et al.* 1994). While heterozygous animals are phenotypically normal, complete UO deletion is lethal unless allopurinol, an inhibitor of uric acid synthesis, is administered. This lethality prevented the UO knockout mouse to be widely used to model hyperuricemia and gout, since human sufferers of those conditions only have an increased risk of death due to increased risk of other renal and cardiovascular diseases. The UO knockout mouse has been used to test new therapies for the treatment of hyperuricemia, including a PEG-modified uricase (Kelly *et al.* 2001) and a self-regulating UO expression unit (Kemmer *et al.* 2010) that spontaneously shuts down when physiological urate levels are reached.

1.5 Synergistic effects of antioxidants

As vitamin C and vitamin E are the major dietary cellular and lipid antioxidants, respectively, sparing and/or synergistic effects between the two vitamins have been extensively studied. Deficiency in either antioxidant results in lower tissue concentrations of the other in the ODS rat (Tanaka *et al.* 1997), indicative of a sparing effect between the two vitamins. In the guinea pigs, combined but moderate deficiencies (>50% of control levels) in vitamin C and E causes death following a distinct clinical syndrome, with paralysis in the limbs and difficulty breathing (Hill *et al.* 2003), attributed to severe damage in the brainstem and spinal cord (Burk *et al.* 2006). Supplementation of both vitamin C and E prevents kidney failure (Durak *et al.* 2002), ameliorates asthma (Gu *et al.* 2003), and reinstated the cough reflex at high O_2 concentrations (Brozmanova *et al.* 2006). On the other hand, increased vitamin C and E intake does not prevent lipid oxidation in the liver when guinea pigs are fed oxidized fats (Keller *et al.* 2004), and have limited

efficacy in preventing neurobehavioural defects caused by maternal consumption of alcohol (Nash *et al.* 2007). Additionally, very high levels of vitamin C and E supplementation increases the incidence of lung lesions in guinea pigs exposed to cigarette smoke, indicative of a co-carcinogenic activity in cigarette smoke-induced lung cancer (Fiala *et al.* 2005).

Other antioxidants that have been included in studies of the antioxidant network include selenium (Se), a micronutrient essential for antioxidant enzymes, and glutathione (GSH), a tripeptide synthesized in all animals. Se and vitamin C double deficiency decreases vitamin E concentration in guinea pig tissues (Bertinato *et al.* 2007), and results in muscle cell death (Hill *et al.* 2009). Combined supplementation of Se and vitamin C protects against alcohol-induced oxidative stress (Sivaram *et al.* 2003) and hyperlipidemia (Asha and Indira 2004). Changes in GSH concentration inversely influence vitamin C levels in the guinea pig, indicative of a potential homeostatic regulation of these two antioxidants (Dalton *et al.* 2000; Shang *et al.* 2002).

1.6 Conclusion

The perceived adequacy of the dietary intake of antioxidants as an absence of disease has been challenged for nearly a century, but equivocal proof of beneficial effects of high dose supplementation has not yet been established. Variables such as different duration or dose of supplementation, the gender, age, and animal species, and different techniques used for scoring complex observations such as chronic oxidative damage, all contribute to the disagreement of whether supra-physiological levels of vitamin C is beneficial. Additionally, as antioxidants may influence both metabolic and cell signaling

networks, it is particularly important to investigate the effects of their deficiency and supplementation in the context of whole animal systems. Finally, oxidative stress and aging are chronic and complex, and there is not yet a consistent method of study. Further investigations of animal models of antioxidant function are needed to resolve these outstanding questions, and to achieve an understanding of the role of antioxidants in oxidative stress and age-related diseases.

Chapter 2: Restoration of endogenous vitamin C production in the guinea pig

2.1 Introduction

Although marginal vitamin C deficiency is not physically manifested, and severe vitamin C deficiency (scurvy) is now rarely reported in developed countries, low vitamin C status has been associated with a number of chronic diseases (Schleicher *et al.* 2009), such as cardiovascular diseases, respiratory diseases, cancer, and inflammatory diseases, which are some of the leading causes of morbidity and death in developed countries. Lower plasma vitamin C concentration is associated with cardiovascular risk factors such as hypercholesterolemia (Ginter *et al.* 1969; Ginter 1975) and high blood pressure (Myint *et al.* 2011). Decreased vitamin C levels are consistently observed in smokers, both active and passive (Institute of Medicine and Related Compounds 2000), and vitamin C therapy has been suggested to prevent lung cancer (Dey *et al.* 2011). Vitamin C is known to be accumulated at high levels in immune cells, and is rapidly depleted during inflammation (Watson *et al.* 2010). However, equivocal proof of the role of vitamin C deficiency in the symptoms and etiology of these diseases is lacking, and its mechanism of action is still unknown.

Although the amount ingested by most humans is sufficient to prevent scurvy, larger amounts may be required to offer protection against disease-causing oxidative damage. Compared to human consumption levels of vitamin C, for example, other mammals synthesize (Chatterjee 1973; Stone 1979) and ingest (Boure 1949; Pauling 1970) ascorbic acid in far greater excess, which is then excreted through the urinary system. However, supra-physiological levels of vitamin C cannot be easily achieved in humans

through ingestion due to saturation of absorption and low expression of vitamin C transporters (Levine *et al.* 1996). Intravenous injections of ascorbic acid can elevate its plasma concentration to up to 25fold higher than achievable by ingestion, which is comparative to the concentration of ascorbic acid required to selectively kill tumour cells by generating H_2O_2 (Chen *et al.* 2005). However, this elevation in vitamin C concentration by intravenous injection is subject to rapid renal clearing, and can only be maintained for a few hours (Padayatty *et al.* 2004). Thus, it is necessary to employ alternative means to achieve sustained high levels of vitamin C in the body.

In contrast to most other animals, humans and guinea pigs are among the few species that cannot synthesize ascorbate endogenously. This is due to independent loss-of-function mutations in the gene encoding for gulonolactone oxidase (GULO), which catalyzes the last step of ascorbate synthesis (Burns 1957). An enzyme replacement therapy method has previously been employed to rescue vitamin C production in the guinea pig. The guinea pigs were treated with chemically modified GULO (Sato and Walton 1983; Hadley and Sato 1989), and ascorbic acid was produced as long as both the modified enzyme and L-gulonolactone, the substrate of GULO, are repeatedly administered (Hadley and Sato 1988). However, repeated injection of the enzyme remains highly immunogenic and causes death (Hadley and Sato 1989), and thus is not a viable method to maintain high levels of vitamin C in the guinea pig as a model for vitamin C function.

Thereafter, gene therapy methods have been employed to restore GULO function in human cell lines, guinea pigs, and the constructed *Gulo*$^{-/-}$ mouse. A first generation

adenovirus carrying the mouse *Gulo* (AdGLO1) was first tested in human hepatocytes. The transfected cells expressed functional GULO and produced vitamin C when supplemented with L-gulonolactone (Ha et al. 2004). AdGLO1 was then tested on guinea pigs and found to reduce weight loss associated with vitamin C deficiency. However, at a titer of 10^9 viral particles per animal, *Gulo* mRNA could not be detected in the liver, and *de novo* vitamin C synthesis was not detected (DS and HES, unpublished data).

First generation adenoviruses, in which proteins necessary for the expression of other early and late genes are deleted, are known to have significant limitations in investigative and therapeutic potential due to transient transgene expression (a few weeks) and vector-associated inflammation (Danthinne and Imperiale 2000). In contrast, helper-dependent adenoviruses, lacking all viral genes and requires a "helper" virus for assembly, are easily prepared, relatively long-lasting (2-3 years), and mediate efficient transgene expression (Jiang *et al.* 2011). A helper-dependent adenovirus expressing mouse *Gulo* under the mouse CMV promoter (HDAd-mCMV-*Gulo*) was therefore constructed (Li *et al.* 2008) and tested on *Gulo*$^{-/-}$ mice, a transgenic mouse constructed to model GULO deficiency in humans (Maeda *et al.* 2000). At a titer of 2×10^{11} viral particles per animal, *Gulo-/-* mice infected with HDAd-mCMV-*Gulo* expressed GULO in the liver and produced ascorbic acid. Serum ascorbate concentration in virus-treated animals was elevated to 62 ± 15 μM, which is comparable to the wildtype concentration (Li *et al.* 2008).

Helper-dependent adenoviruses, in which all viral genes are deleted except those necessary for DNA replication and packaging (Danthinne and Imperiale 2000), elicit

pronounced immune response in immunocompetent animals and humans, and lacks persistent target gene delivery (Sugiyama *et al.* 2005). Lentiviruses, in contrast, are significantly less immunogenic, and can mediate prolonged target gene expression, as the transgene is integrated into the host cell genome (Sugiyama *et al.* 2005). Similar to adenoviruses, lentiviruses can infect both dividing and non-dividing cells (Sugiyama *et al.* 2005). These properties make lentiviruses an ideal vector for use in gene therapy. Two lentivirus carrying *Gulo* gene, one under the mCMV promoter (lenti-mCMV-*Gulo*) and one under the PEPCK promotor (lenti-PEPCK-*Gulo*) were thus constructed and tested in human hepatocytes as part of my undergraduate thesis. The mCMV promoter mediates very high expression levels of its downstream genes, enabling easy detection of their expression (Cheng *et al.* 2004), while the PEPCK promoter mediates long-term downstream gene expression at a more physiologically relevant level (Chakravarty *et al.* 2005). The lenti-mCMV-*Gulo* transfected cells expressed functional GULO and produced vitamin C at a concentration of 0.67 ± 0.10 fmol/cell (RY and HES, unpublished data). In cells transfected with lenti-PEPCK-*Gulo*, *Gulo* was successfully integrated into the cell genome, but neither GULO expression nor vitamin C synthesis were detectable (RY and HES, unpublished data).

The guinea pig has traditionally been used to model the effects of vitamin C deficiency and supplementation. There is extensive literature relating guinea pig vitamin C status to cardiovascular diseases, cancer, and aging. Recent developments in mouse model include completely sequenced genomes and well-characterized mutants of other genetic diseases. These advantages notwithstanding, guinea pigs remain a closer mimic of

vitamin C metabolism and function in humans. In contrast to rodent models, where loss of *Gulo* occurred recently in the evolutionary scale, guinea pigs lost *Gulo* 13-14 million years ago (Lachapelle and Drouin 2011). Under evolutionary pressures that both selected for the loss of *Gulo*, guinea pigs and humans may have developed similar adaptations that compensate for this loss. For example, both guinea pigs and humans use alternative pathways to metabolize gulonolactone, the immediate precursor of vitamin C. As well, the status of other antioxidants, such as glutathione and uric acid, are altered in humans and in the guinea pig (Lykkesfeldt 2002), possibly due to the evolutionary change in vitamin C status. Thus, in this project, guinea pigs have been chosen as a model for vitamin C metabolism and the restoration of vitamin C synthesis.

Similar to humans, the plasma vitamin C concentration of wildtype guinea pigs can be easily manipulated by supplementation in the diet, ranging from severely deficient to physiologically normal. To achieve supra-physiological levels of vitamin C, the guinea pigs were treated with gene therapeutic lentiviruses carrying the mouse *Gulo* under the mCMV promoter, constructed as a part of my undergraduate thesis. It is hypothesized that lentivirus-delivered *Gulo* can mediate GULO expression and ascorbic acid synthesis, thereby correcting the metabolic deficiency that renders guinea pigs dependent on dietary vitamin C. This is a necessary step towards the development of the guinea pig into a robust animal model for studies of vitamin C function, and its effect against oxidative diseases. Due to the large size of guinea pigs, however, nearly 10 times the amount of lentivirus is needed to effectively mediate transgene expression in a guinea pig compared to a mouse. Thus, replication was left out, as this project serves as a pilot study to test the

feasibility and efficacy of the restoration of endogenous vitamin C production in the guinea pig.

2.2 Experimental design

In Experiment 1, two guinea pigs were employed (#1 and #2) to test and compare the efficacy of helper dependent adenovirus-delivered and lentivirus-delivered GULO, establish proper endpoints and monitoring techniques, and determine the minimum effective titre. Both guinea pigs were maintained on a vitamin C-deficient diet for this experiment. Following a period of weight loss, vitamin C treatment was given through two doses of 3% vitamin C in saline by IP injection, and both guinea pigs were allowed to recover for 10 days. After the injection of 10^{10} viruses, GP #1 was also given weekly injections of L-gulonolactone, the substrate of GULO, to determine if it needs to be provided to the virus-treated guinea pigs for vitamin C production. The weights of the guinea pigs were monitored daily, and blood samples were collected from the saphenous vein twice per week to sample for ascorbic acid content.

From Experiment 1 it was determined that (1) the minimum effective titre is 10^{10} viral particles/animal, (2) weekly injections of gulonolactone is required for vitamin C production, and (3) scorbutic animals lose weight due to decreased food intake. With these parameters determined, Experiment 2 was performed with four animals (#3-#6). To reduce weight loss due to inanition, Jello treat was given to all animals two times per week. The ingredients of Jello include sugar, gelatin, adipic acid, fumaric acid, salt, artificial flavour, and colour. The weights of the guinea pigs were monitored daily, and

blood samples were collected from the saphenous vein twice per week to sample for ascorbic acid content.

2.3 Methods

2.3.1 HEK293ft cell culturing

Human embryonic kidney 293ft cells were grown at 37^{o}C and 5% CO_2 in D-minimal essential media (D-MEM) supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 0.1mM non-essential amino acids, 1mM sodium pyruvate, and 200μg/ml geneticin (G-418) (Li *et al.* 2008). Penicillin/streptomycin (100U/ml) was added to the media to prevent microbial growth. After infection with the lentiviral vector plasmids, cells were maintained in media under the same conditions. Cells were trypsinized and split 1:7 every 3 days or until confluence. Cells were stocked in 10% DMSO in complete media every 3 days or until confluence.

2.3.2 HEP G-2 cell culturing

Human hepatocellular carcinoma HEP G-2 cells were grown at 37^{o}C and 5% CO_2 in D-MEM supplemented with 10% (FBS), 2mM L-glutamine, 0.1mM non-essential amino acids, and 1mM sodium pyruvate. Penicillin/streptomycin (100U/ml) was added to the media to prevent microbial growth. Cells were trypsinized and split 1:5 every 5 days or until confluence. Cells were stocked in 10% DMSO in complete media every 3 days or until confluence. During lentivirus titration, HEP G-2 cells were maintained on complete media supplemented with 5μg/μl blasticidin (bsd) under the same conditions after transfection.

2.3.3 Construction of lentiviral vectors carrying Gulo

The pLenti6-mCMV-GULO plasmid vector contains *Gulo* under the mCMV promoter, and was available from previous work (MH and HES, unpublished). Plasmid vectors pLP-PLP1, pLP-PLP2, and pLP-VSVG encode lentiviral structural precursor genes gag/pol, rev, and VSV-G, respectively (Invitrogen Catalogue #V496-10). To obtain ultra-clean DNA suitable for transformation into mammalian cell lines, these pLP plasmids, as well as the pLenti6-mCMV-GULO, were extracted using QIAGEN HiSpeed Plasmid Midi Kit (Cat # 12643), and then further purified using the phenol-chloroform procedure. Viable lentiviral vectors were produced by co-transfecting HEK293ft cells with all four plasmids. Transformation of HEK293ft cells was done using a modified calcium phosphate method (Miller 1992). Briefly, 10μg of each plasmid was suspended in 200μl of 2.5M $CaCl_2$, and 200μl of 2x HEPES buffer was added to the solution and mixed by tapping gently. After 1 minute, the calcium phosphate-DNA suspension is immediately transferred into complete growth medium with freshly trypsinized cells. The plates were then rocked to mix and incubated overnight at 37^{o}C and 5% CO_2. The DNA-containing media was replaced with complete media the following day. Virus-containing media were collected on Days 2, 4, and 7, and stored at -80^{o}C until use.

Purification of lentiviral vectors was done by polyethylene glycol (PEG) precipitation. Briefly, collected media was centrifuged at 7000xg for 15 minutes to remove cells. The supernatant (~4ml) was collected and added to 1ml of 50% PEG 8000, 2.4% NaCl, pH 7.2. The mixture was refrigerated overnight at 4^{o}C and centrifuged at

2300xg for 45 minutes. The virus pellet was resuspended in 500µl of 1x PBS and stored at -80^{o}C until use.

2.3.4 Titration of lentivirus

Purified lentiviruses were serial diluted and added to 10 volumes of complete media in Falcon tubes and tapped gently to mix. The media of an overnight HEP G-2 culture was then replaced with the virus-containing media, and the culture was incubated overnight at 37^{o}C and 5% CO_2. The virus-containing media was removed the following day and replaced with complete media with 5µg/µl blasticidin (bsd), and incubated overnight at 37^{o}C and 5% CO_2. Cells were then washed 2 times with 1x PBS and trypsinized since HEP G-2 cells do not detach from the bottom of the plate. Dead cells were removed with the media on the following day, and transfected cells were subsequently maintained on 5µg/µl bsd. Blasticidin-resistant cell colonies and counted and multiplied with the dilution factor to obtain the lentivirus titre.

2.3.5 Guinea pigs

Adult male Hartley guinea pigs (Crl:HA) were used for these experiments. All guinea pigs were housed in the Central Animal Facility at McMaster University under controlled conditions, with free access food and water. All experiments were performed according to Animal Utilization Protocols and reviewed by the McMaster Bioethics Research Board. Guinea pigs were fed a vitamin C-deficient diet and were given drinking water containing ascorbic acid at 330 mg/L to prevent scurvy. Guinea pigs were deprived of dietary ascorbic acid 10 days before lentivirus injection to eliminate vitamin C carryover (DS and HES, unpublished results).

2.3.6 Administration of viral vectors, L-gulonolactone, and vitamin C

Guinea pigs were anaesthetized with isoflurane. A 3-ml volume of either HDAd-mCMV-*Gulo*, Lenti-mCMV-*Gulo*, or 0.9% saline was injected into the intraperitoneal cavity of anaesthetized guinea pigs. A 4 ml volume of 500mg L-gulonolactone or 0.9% saline was injected into the intraperitoneal cavity of anaesthetized guinea pigs weekly. During vitamin C treatment, a volume of 3% ascorbic acid equal to 10% of the animal weight was injected into the intraperitoneal cavity of anaesthetized guinea pigs. Guinea pigs were housed either according to the viral vector received at two animal per cage, or housed individually.

2.3.7 Blood collection

Blood samples were collected from the saphenous vein into heparin-coated blood collecting tubes (Kimble Chase, Vineland, NJ), and centrifuged at 1000 xg for 5 min. Cell-free plasma (supernatant) was diluted (1:10) with 5% MPA-EDTA and centrifuged at 13,000 xg for 30 min at 4^{o}C to eliminate protein precipitation. Supernatant was extracted and stored at -80^{o}C until use.

2.3.8 Extraction of protein from liver

Guinea pigs were anaesthetized with sodium pentobarbital (35 mg/kg) by intraperitoneal injection, and were perfused systemically with Lactated Ringer's Solution (LRS) through the left ventricle of the heart. The liver was then resected, frozen immediately in liquid nitrogen, and stored at -80^{o}C until use.

To extract intracellular proteins from the liver, 1 g of resected livers were thawed on ice and homogenized in 10 ml of liver buffer (50mM sodium citrate, 50mM potassium

phosphate buffer at pH 7.0). Homogenized samples were centrifuged at 13,000 xg for 20 min at 4^{o}C. The protein-containing supernatant was extracted and centrifuged again at 12,000 xg for 20 min at 4^{o}C to eliminate any residual cell debris. Protein concentrations were determined by Bradford assay, using bovine serum albumin as a standard.

2.3.9 Liver Gulo assay for ascorbic acid production

L-gulonolactone was dissolved in the liver buffer at 15 mM, and was mixed with the liver extract at a ratio of 2:1. The final concentration of L-gulonolactone in the reaction mixture was 10 mM. The reaction mixture was incubated at 37^{o}C for 0h, 2h, 4h, and overnight. To stop the reaction, 50 µl of 1.5% MPA was added to the reaction mixture. The resultant reaction mix was centrifuged at 13,000 xg for 5 minutes, and the ascorbic acid-containing supernatant was extracted.

2.3.10 HPLC-electrochemical detection of ascorbic acid

Prior to HPLC analysis, all samples were prepared and diluted in 5% MPA-EDTA. The HPLC system (Agilent 1200 Series) consists of a microvacuum degasser, a binary pump SL, a high performance autosampler and an Agilent XDB-C18 analytical column of 4.6 x 250mm, particle size 5µm.

The mobile phase for the detection of ascorbate on the HPLC is 0.2M KH_2PO_4 at pH 3.0. The flow rate is set at 2 ml/min with a maximum pressure of 400 bar. The pressure usually stabilizes at 350 bar. The retention time of ascorbic acid is approximately 1.9 min at a flow rate of 2 ml/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 and

Channel 2 at +150 mV. Output signal from Channel 2 was used for vitamin C measurement. Standard curves were established using ultrapure L-ascorbic acid (Sigma) dissolved in 5% MPA-EDTA, prepared fresh every run. System programming and chromatogram analysis were performed using EZChrom Elite Software (Agilent). Specifically, the electric charge signals (output area; nC) were obtained by integrating the current (signal peak; nA) with respect to elution duration (time; min) using the Agilent EZChrom Elite software (Agilent). The response factor calculated from the calibration curve is 5×10^{-11} μg of ascorbic acid per nC signal output.

2.3.11 Data analysis

HPLC-ECD chromatographs were identified, integrated and exported using EZChrom Elite software (Agilent). All data were processed with Microsoft Office Excel 2007. Graphics, including gel photographs and data charts were processed and edited with Microsoft Office PowerPoint 2007.

2.4 Results

2.4.1 A titre of 10^8 lentivirus/animal is insufficient to prevent scurvy

The body weight of guinea pig (GP) #1 and #2 are shown in Figure 2. Both animals continued gaining weight after vitamin C restriction. After the injection of 10^8 lentiviruses on Day 0, weight gain continued until Day 10, when the weight of GP #1 (lentivirus-injected) rapidly decreased, consistent with the development of scurvy. The weight of GP #2 (adenovirus-injected) also showed a slight trend of decrease. After given vitamin C treatment, the rapid weight loss was reverted (Figure 2).

The minimum effective titre to prolong life is 10^{10} lentivirus/animal

After a recovery period of 10 days after vitamin C treatment, GP #1 and #2 were injected with 10^{10} lentiviruses. Both animals then gained weight at a steady pace for 20 days. On Day 47, GP #1 began to again lose weight, and reached endpoint on Day 56. Weight gain in GP #2 was also attenuated on Day 47, and the animal began losing weight on Day 54. Endpoint was reached on Day 62 for GP #2 (Figure 2), and the animal was euthanized to prevent unnecessary suffering.

2.4.2 The plasma ascorbic acid concentration is not elevated by lentivirus treatment

The plasma ascorbic acid content of guinea pigs used in Experiment #1 is shown in Figure 3. The normal plasma ascorbic acid concentration in healthy guinea pigs is around 15ng/µl, which was rapidly reduced after restriction of vitamin C in the diet. Immediately before the first injection of viruses on Day 0, ascorbic acid concentration in the plasma temporarily increased in both animals, but declined again after the injection (Figure 3). On Day 27, when the second virus injection occurred, the plasma ascorbic acid contents in both guinea pigs were close to zero. It was transiently increased in both animals immediately before reaching endpoint.

2.4.3 The liver extract contains ascorbic acid at sacrifice and further produces ascorbic acid when L-gulonolactone is available

At 0h of reaction time, the reaction mixture containing the liver extract of GP #1 and 10 mM L-gulonolactone contained 1.3 ng vitamin C/mg protein, while the reaction mixture containing the liver extract of GP #2 and 10mM L-gulonolactone contained 1.6 ng vitamin C/mg protein (Figure 4). Incubation of 10mM L-gulonolactone alone did not produce any ascorbic acid. The ascorbic acid content was moderately increased when

both reaction mixtures were incubated for 2h and 4h. When the reaction mixtures were incubated overnight, the ascorbic acid content decreased (Figure 4).

2.4.4 Lentivirus treatment prolongs guinea pig survival after withdrawal of dietary vitamin C

In Experiment 2, the animal weights were recorded and shown in Figure 5. All four animals continued to gain weight after vitamin C restriction. GP #5, the negative control maintained on vitamin C-free water, stopped gaining weight on Day 12. Its weight was maintained for an additional 25 days, and endpoint was reached on Day 37. GP #3 and #4, the lentivirus-injected animals, continued gaining weight until Day 47. Both animals then lost weight and reached endpoint on Day 58 (Figure 5). GP #6, the positive control returned on vitamin C-supplemented water on Day 0, continued gaining weight at a steady pace of approximately 7.2g/day (Figure 5).

2.4.5 The plasma ascorbic acid concentration is not elevated by lentivirus treatment

The plasma ascorbic acid content of guinea pigs used in Experiment 2 is shown in Figure 6. The normal plasma ascorbic acid concentration in healthy guinea pigs is around 15ng/µl, which was reduced to zero after vitamin C restriction and prior to treatment. The plasma ascorbic acid concentration was undetectable until the end of the experiment in GP #5, the negative control maintained on vitamin C-free water. In GP #3 and #4, ascorbic acid concentration in the plasma temporarily increased after the injection of lentiviruses, but remained close to zero throughout the experimental period. Plasma ascorbic acid levels in both animals were transiently increased immediately before reaching endpoint (Figure 6). In GP #6, the positive control returned on vitamin C-

supplemented water on Day 0, the plasma ascorbic acid content increased and exceeded the normal levels for a period of 15 days, before decreasing to normal levels of around 15ng/µl (Figure 6).

Figure 7 shows some example traces of HPLC analyses of the plasma collected from lentivirus-treated animals (GP #3-4; Figure 7A), the negative control (GP #5; Figure 7B) and the positive control (GP #6; Figure 7C). Ascorbic acid is eluted at approximately 1.9 min and signal strength, corresponding to ascorbic acid concentration, is determined by integration from valley to valley.

2.5 Discussion

Ascorbic acid is accumulated against a concentration gradient in almost all cells and tissues (Levine *et al.* 2006). This internal ascorbic acid store causes a delay between the start of vitamin C deficiency and scorbutic collapse. As observed here and in previous studies, guinea pigs restricted of vitamin C intake continue to gain weight for around 20 days (Figure 2, Figure 5) (Shilotri 1977; Anthony *et al.* 1979). After around 20 days of weight gain, weight loss in guinea pigs restricted of vitamin C intake is abrupt and rapid (Figure 2). Treatment by daily injections of high dose (3%) vitamin C effectively terminated weight loss in 2 days and lead to a full recovery of weight in 10 days (Figure 2), indicating that neither guinea pig in Experiment 1 was producing ascorbic acid endogenously after injections of 10^8 viral particles.

Scorbutic collapse in guinea pigs has been attributed to the loss of collagen renewal (Shilotri 1977; Anthony *et al.* 1979). However, during Experiment 1, it was noticed that the rapid weight loss was closely associated with a decreased intake of both

food and water. It appears that loss of appetite and inanition plays a role in the rapid weight loss observed in the experimental guinea pigs. Thus, in Experiment 2, animals were given Jello two times per week, as it is appetizing and high in sugar. The Jello mix does not contain ascorbic acid, as confirmed by HPLC analysis. GP #5, the negative control maintained on vitamin C-free water, continued gaining weight 22 days after vitamin C restriction. Thereafter, instead of expiring in 7-10 days, GP #5 maintained its weight and persisted for an additional 25 days (Figure 5). Thus, while collagen is undoubtedly decreasing during vitamin C deficiency, loss of appetite and inanition makes a significant contribution to the rapid weight loss observed in vitamin C-deficient guinea pigs. This illustrates that the precise pathological mechanism of vitamin C deficiency is still poorly understood.

Previously, modified GULO (eg., with PEG or glutaraldehyde) has been administered as an enzyme replacement therapy to rescue guinea pigs from scurvy (Sato and Walton 1983; Hadley and Sato 1988; Hadley and Sato 1989). The activity of GULO was transient and the treatment elicited critical immune responses in many animals, which highlights the advantages of gene therapy methods. It was established in those studies that L-gulonolactone, the substrate of GULO, must be provided for ascorbate production (Hadley and Sato 1988; Hadley and Sato 1989). Thus, L-gulonolactone was supplemented by injection in lentivirus-treated guinea pigs after the injection of 10^{10} viruses in the second half of Experiment 1 as well as in Experiment 2. While the animals eventually succumbed to sickness and reached endpoint, there was a clear treatment effect

compared to the negative control, GP#5 (Figure 5), as lentivirus-treated animals (GP #3 and #4) survived an additional 21 days before reaching endpoint.

Despite that lentivirus-treated guinea pigs (GP #3 and #4) lived longer than the negative control (GP #5) (Figure 5), the plasma ascorbic acid levels for GP #3 and #4 were not greatly elevated. While GP #5 consistently contained undetectable levels of ascorbic acid in the plasma, the plasma ascorbic acid concentrations of GP #3 and #4 were only slightly higher, and this elevation was not consistent (Figure 6). This indicates that ascorbic acid was indeed synthesized in the livers of treated animals, but the amount produced was not sufficient to maintain health. This is supported by the fact that liver extracts of both lentivirus and adenovirus-treated animals in Experiment 1already contained ascorbic acid without *in vitro* incubation with gulonolactone (Figure 4, 0h reaction time). Furthermore, incubation of the liver extracts with external L-gulonolactone increased ascorbic acid content in the reaction mixtures (Figure 4, 2h and 4h reaction time), indicating that GULO was expressed and functional in the livers of the virus-treated guinea pigs. Overnight incubation of the reaction mixtures decreased ascorbic acid levels to lower than what was detected at 0h reaction time (Figure 4, ON reaction time). This indicates that ascorbic acid was indeed produced in the liver of the virus-treated animals, and was oxidized during the prolonged incubation, since ascorbic acid is unstable in solution at physiological pH.

Interestingly, ascorbic acid concentration in the plasma is elevated before endpoint (Figure 3, Figure 6). Similar observations have been made previously in the

guinea pig (DS and HES, unpublished data), as well as in the literature in the *Gulo*$^{-/-}$ mouse (Kim *et al.* 2012). The significance of this observation is not yet known.

2.6 Conclusion

In this project, we employed a gene therapy approach in the guinea pig, and achieved functional rescue of GULO and restoration of endogenous vitamin C production. Lentivirus-delivered *Gulo* was able to mediate GULO synthesis and expression. Though the metabolic deficiency was not completely corrected, there was a clear treatment effect as the lentivirus-treated guinea pigs lived longer than the negative control by 21 days. The lentivirus-treated guinea pigs were able to produce ascorbic acid endogenously, albeit at small amounts. With further experiments, it will be possible to develop the lentivirus-treated guinea pig into a robust animal model to study the relationship between vitamin C and oxidative diseases.

Chapter 3: Introgression of the *Gulo*$^{-/-}$ allele to the FVB/N mouse

3.1 Introduction

Mouse models have been instrumental in studies of complex human diseases, providing a powerful tool to analyse genetic differences that underlie disease risk factors, etiology, manifestation of symptoms, and clinical outcome. To ensure orthogonality in these genetic studies, it is necessary to work with inbred, or genetically identical, mice. Many such strains have been developed, with detailed characterizations of a wide array of biochemical and behavioural phenotypes. As each inbred strain has unique biochemical characteristics and susceptibility to diseases, it is sometimes necessary to introgress a specific gene mutation from one strain to another, creating congenic strains. This is done by repeated backcross to the recipient strain for a minimum of 10 backcross generations while selecting for the mutation of interest, reaching a mutant mouse with a 99.90% identity to the recipient genome (Morel *et al.* 1996; Yui *et al.* 1996).

The *Gulo*$^{-/-}$ mouse was constructed in the C57BL/6 background (Maeda *et al.* 2000), the most widely used inbred mouse commonly used to produce transgenic mice. This strain is ideal in research areas including cardiovascular biology and developmental biology, but has a low susceptibility to tumours (Paigen *et al.* 1990; Champy *et al.* 2008). Thus, although vitamin C has been implicated in the development and progression of cancer, the C57BL/6 strain is a poor choice of animal model for such studies. In contrast, the FVB/N strain is highly susceptible to chemically induced squamous cell carcinomas, with a high rate of malignant conversion from papilloma to carcinoma (Hennings *et al.* 1993), and is ideal to study human cancer development and metastasis.

The proto-oncogene *erbB2/neu* encodes for a receptor tyrosine kinase that is a part of the epidermal growth factor receptor (EGFR) family, but with no known ligand (Cho *et al.* 2003). Overexpression of *erbB2* is observed in approximately 30% of human breast cancers (Slamon *et al.* 1989). Furthermore, overexpression of ErbB2 in breast cancer patients leads to metastasis (Tan *et al.* 1997; Moody *et al.* 2002), increases chemoresistance (Colomer *et al.* 2000; Classen *et al.* 2002), and is inversely correlated with survival rates and disease-free intervals (Tan and Yu 2007). Clearly, ErbB2 plays an important role in the progression of human breast cancer. *erbb2/neu* has been modeled extensively by our collaborator (WM) in the FVB/N strain (Muller *et al.* 1988; Guy *et al.* 1992; Muller *et al.* 1996). Thus, to study the role of vitamin C in human breast cancer, it is necessary to introgress the $Gulo^{-/-}$ mutation into the FVB/N background.

3.2 Experimental design

A strain is considered congenic after a minimum of 10 backcross generations to the recipient strain has been made, counting the first hybrid (F1) generation as generation 1 (N1) (Markel *et al.* 1997). $Gulo^{+/-}$ mice are paired with FVB/N mice in monogamous breeding systems, which ensures accurate records and produces the maximum number of litters. The strain designation for the hybrid, and eventual congenic, mouse, which is in the FVB/N background and carries the *Gulo* inactivating mutation, is FVB.B6-$Gulo^{tm1Mae}$, following the Guidelines for Nomenclature of Mouse and Rat Strains (International Committee on Standardized Genetic Nomenclature for Mice 2011).

3.3 Methods

3.3.1 Mouse strains

C57BL/6 *Gulo*$^{+/-}$ mice were purchased from the Mutant Mouse Regional Resource Centers (MMRRC; Stock number 000015-UCD). FVB/N mice were purchased from Charles River (Strain code 207). All mice were housed in the Central Animal Facility at McMaster University under controlled conditions with free access to food and water. Animal breeding and weaning were performed according to Animal Utilization Protocols and reviewed by the McMaster Bioethics Research Board.

3.3.2 Mouse genotyping

Upon weaning, all animals were tagged and tailed according to Animal Utilization Protocol. Genomic DNA was extracted using the Norgen Genomic DNA Isolation Kit (Cat. #24700). The *Gulo* genotype of the pups weaned were determined using multiplex PCR primers P2 (5'-CGCGCCTTAATTAAGGATCC-3'), P3 (5'-GTCGTGACAGAATGTCTTGC-3'), and P4 (5'- GCATCCCAGTGACTAAGGAT-3'). A 230-bp fragment derived with P2 and P3 from the *Gulo*$^{-}$ locus and/or a 330-bp fragment derived with P3 and P4 from the *Gulo*$^{+}$ locus distinguish homozygous, heterozygous, and wildtype mice.

3.4 Significance and future work

The role of vitamin C in the development of diseases has been a matter of constant debate (Li and Schellhorn 2007). As well, in light of recent evidence of the antitumourigenesis properties of ascorbic acid (Chen *et al.* 2005), there has been increased interest in the possible therapeutic value of supraphysiological ascorbic acid in

cancer patients. Combined with a robust model of *erbB2*-associated breast cancer, the *Gulo*$^{-/-}$ genotype in the FVB/N mouse will provide a powerful tool to assay for the effect of vitamin C status on mammary tumourigenesis and progression. Additionally, with the possibility to produce supraphysiological amounts of ascorbic acid through genetic complementation, it will be possible to answer why most animals produce large quantities of ascorbic acid only to excrete it in the urine, and whether this supraphysiological production of ascorbic acid has pharmacologic applications.

Figures and Tables

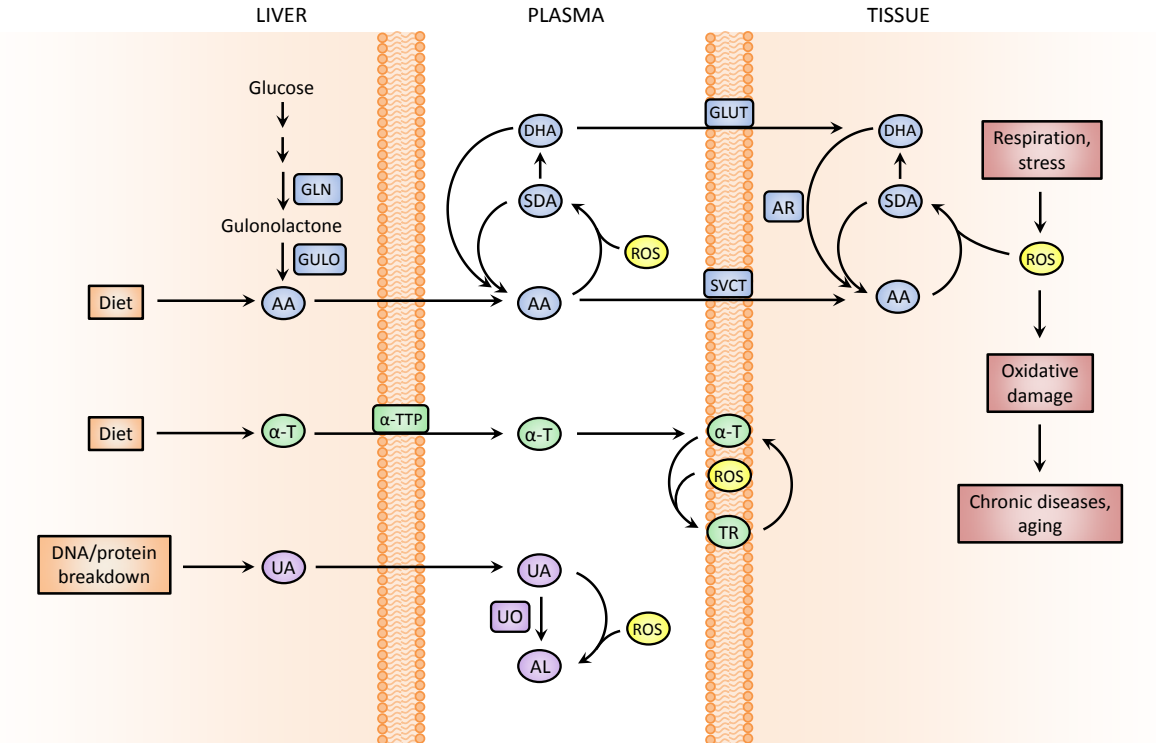


Figure 1. Mechanisms of action of human dietary antioxidants. Reactive oxygen species (ROS) can cause oxidative damage at high concentrations, which leads to chronic diseases and aging. ROS is produced in normal respiration due to superoxide leakage from the mitochondria. Production of ROS is elevated in conditions of stress. ROS can be quenched by antioxidants such as ascorbic acid (AA), α-tocopherol (α-T), and uric acid (UA). For descriptions of pathways, see text. AA, ascorbic acid; AL, allantoin; AR, ascorbate reductase; α-T, α-tocopherol; α-TTP, α-tocopherol transporter protein; DHA, dehydroascorbic acid; GLN, gluconolactonase; GLUT, glucose transporter; GULO, gulonolactone oxidase; ROS, reactive oxygen species; SDA, semidehydroascorbic acid; SVCT, sodium-dependent vitamin C transporter; TR, tocopherol radical; UA, uric acid; UO, urate oxidase.

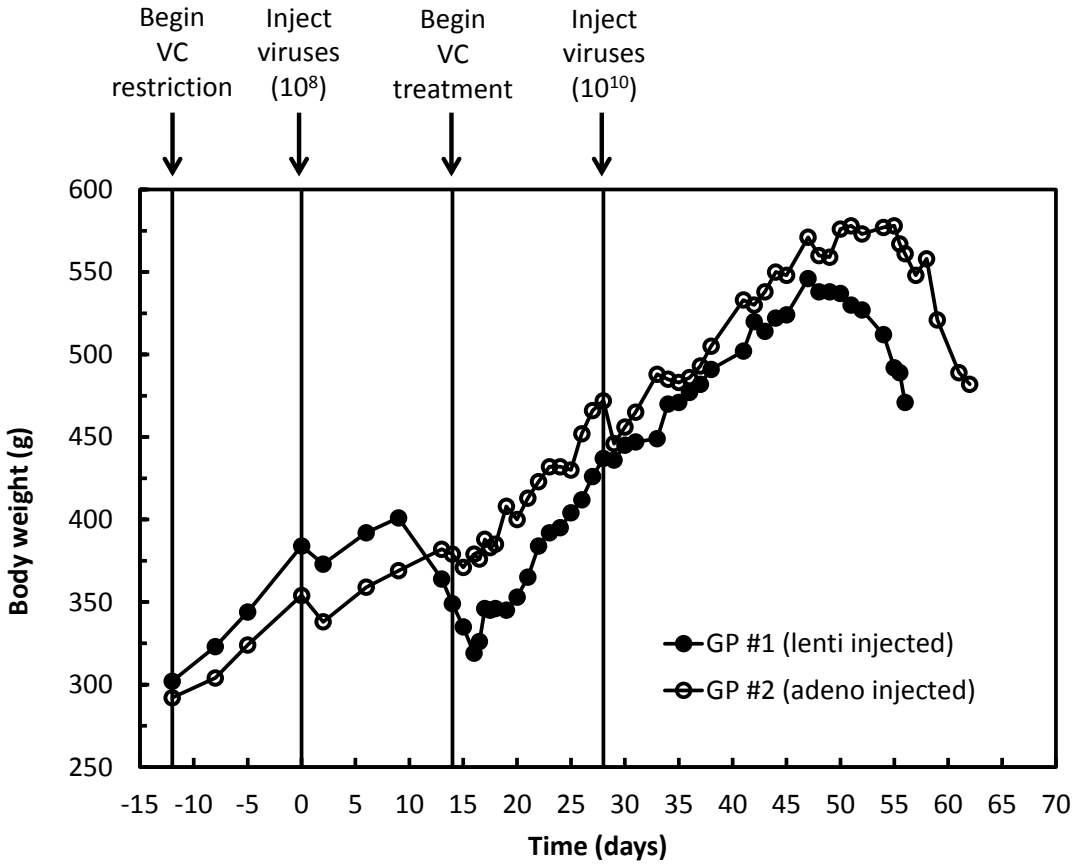


Figure 2. Guinea pig body weights of Experiment 1. Two guinea pigs, GP #1 and #2, were acclimatized for 1 week and fed vitamin C-deficient diet and vitamin C-free water for 10 days. On Day 0, GP #1 was injected with 10^8 particles of lenti-mCMV-*Gulo*, and GP #2 was injected with 10^8 particles of adeno-mCMV-*Gulo*. Their weights were monitored twice per week. Following a period of weight loss, both guinea pigs were injected with 10^{10} lenti- and adeno-mCMV-*Gulo*, respectively. GP #1 also received weekly injections of 500mg/4ml gulonolactone, the substrate of GULO, while GP #2 received 4ml of saline at the same time. Their weights were then monitored daily. VC, vitamin C.

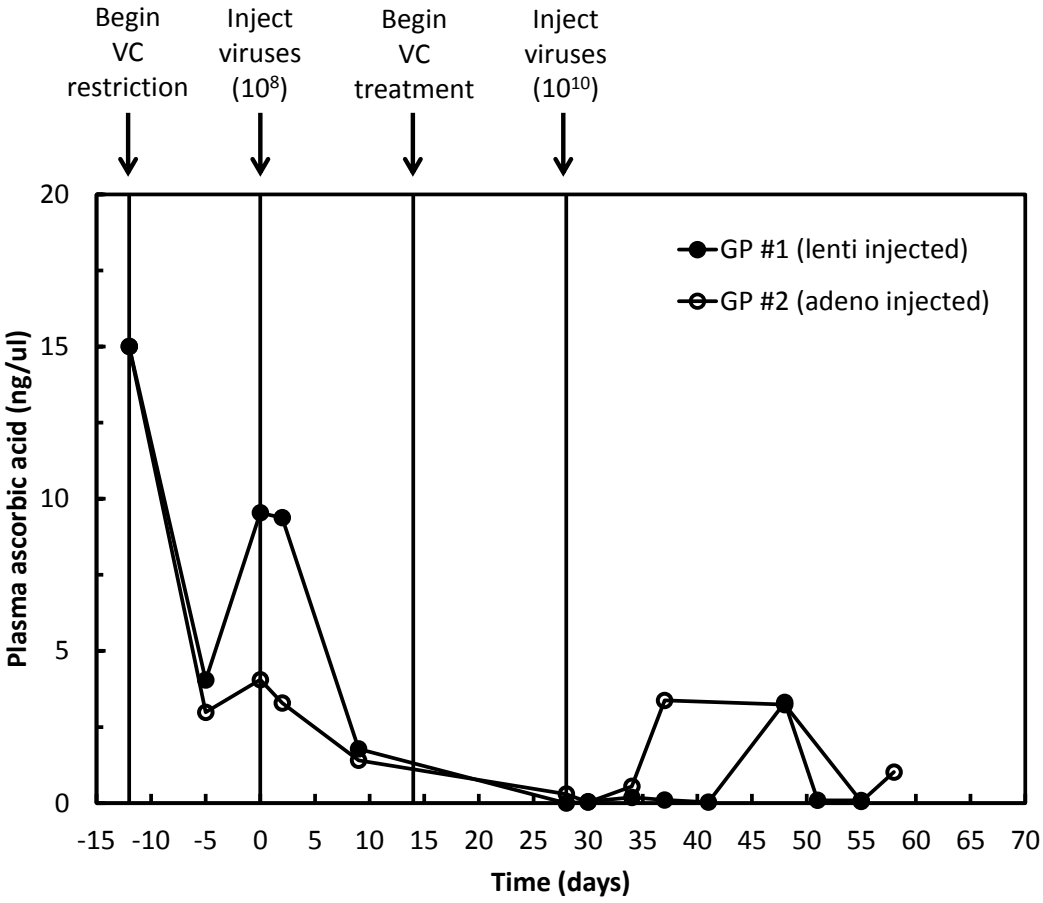


Figure 3. Guinea pig plasma ascorbic acid content in Experiment 1. Two guinea pigs, GP #1 and #2, were acclimatized for 1 week and fed vitamin C-deficient diet and vitamin C-free water for 10 days. On Day 0, GP #1 was injected with 10^8 particles of lenti-mCMV-*Gulo*, and GP #2 was injected with 10^8 particles of adeno-mCMV-*Gulo*. Following a period of weight loss, both guinea pigs were injected with 10^{10} lenti- and adeno-mCMV-*Gulo*, respectively. GP #1 also received weekly injections of 500mg/4ml gulonolactone, the substrate of GULO, while GP #2 received 4ml of saline at the same time. Blood was sampled from the saphenous vein twice per week. All blood samples were centrifuged to obtain the plasma, diluted 10x in 5% MPA, and analyzed using the HPLC for ascorbic acid detection and quantification. VC, vitamin C.

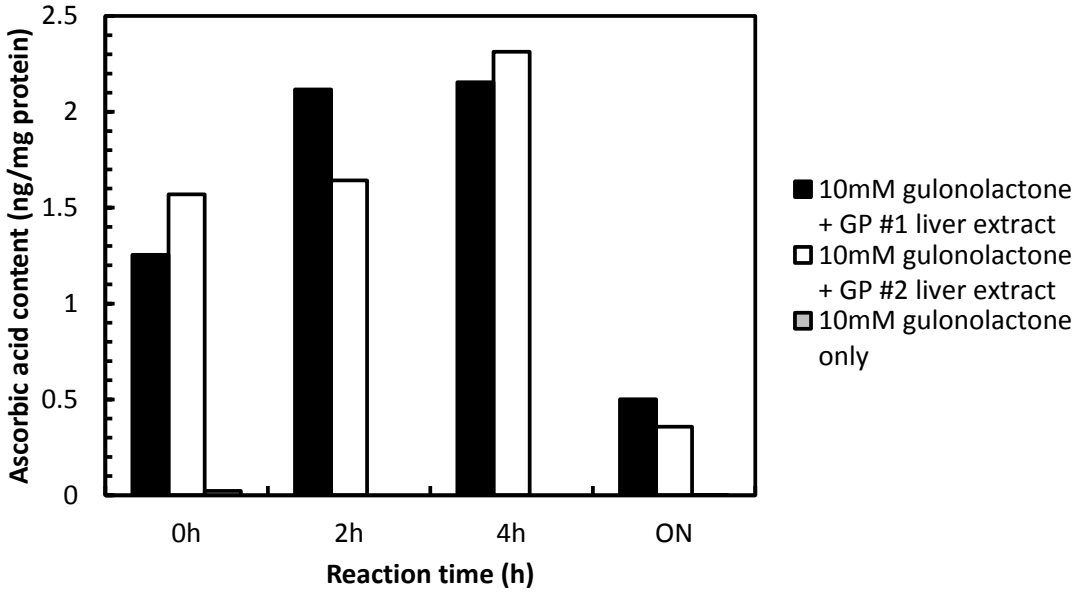


Figure 4. Enzyme assay using liver extracts from guinea pigs in Experiment 1. After reaching endpoint, GP #1 and #2 were euthanized and perfused with Lactated Ringer's Solution (LRS). The liver was collected from each animal, and total protein was extracted. The liver extracts were used in the enzyme assay with 10mM gulonolactone. Reaction mixtures were incubated in a 37^{o}C water bath for 0h, 2h, 4h, and overnight (ON). At the end of each reaction, 50µl of 1.5% MPA was added to stop the reaction. The reaction supernatants were extracted and analyzed for ascorbic acid using the HPLC. The ascorbic acid content was normalized to the total protein in the liver extract, determined by Bradford assay.

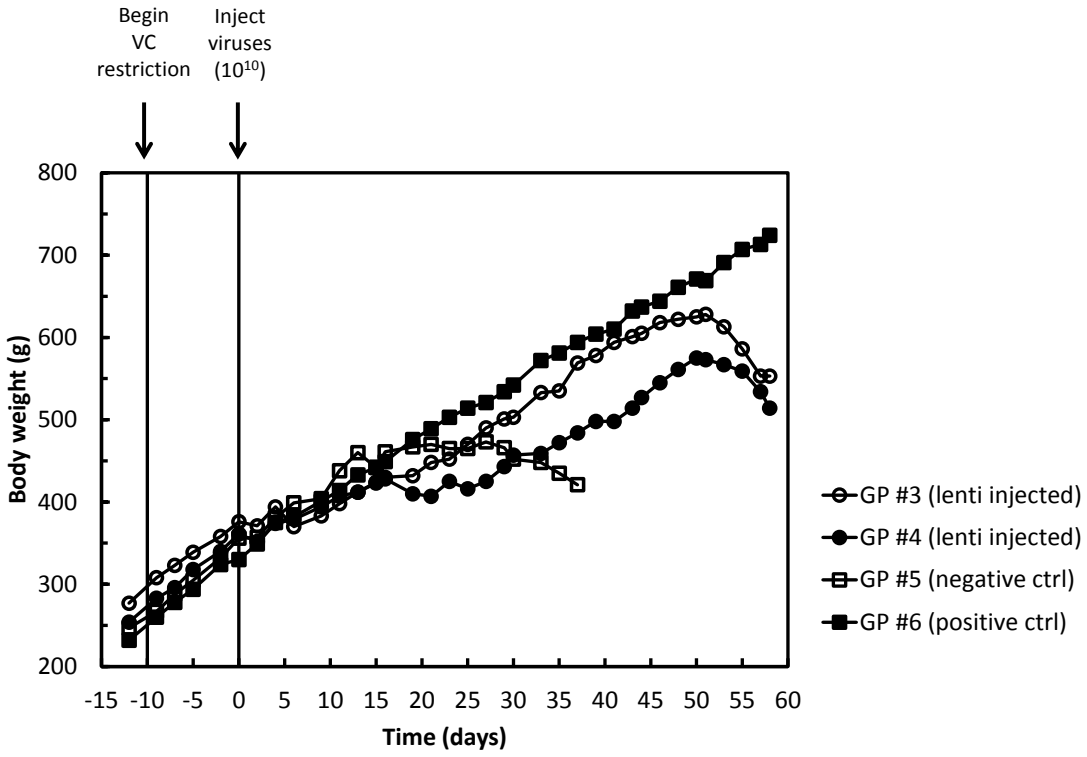


Figure 5. Guinea pig body weights of Experiment 2. Four guinea pigs, #3-6, were acclimitized for 1 week and fed vitamin C-deficient diet and vitamin C-free water for 10 days. On Day 0, GP #3 and #4 were injected with 10^{10} particles of lenti-mCMV-*Gulo*, and GP #5 and #6 were injected with saline of the same volume. Thereafter, GP #3-5 were maintained on vitamin C-free water, whereas GP #6 was returned on vitamin C-supplemented water. GP #3 and #4 also received weekly injections of 500mg/4ml gulonolactone, the substrate of GULO, while GP #5 and #6 received 4ml of saline at the same time. Their weights were then monitored daily. GP, guinea pig. VC, vitamin C.

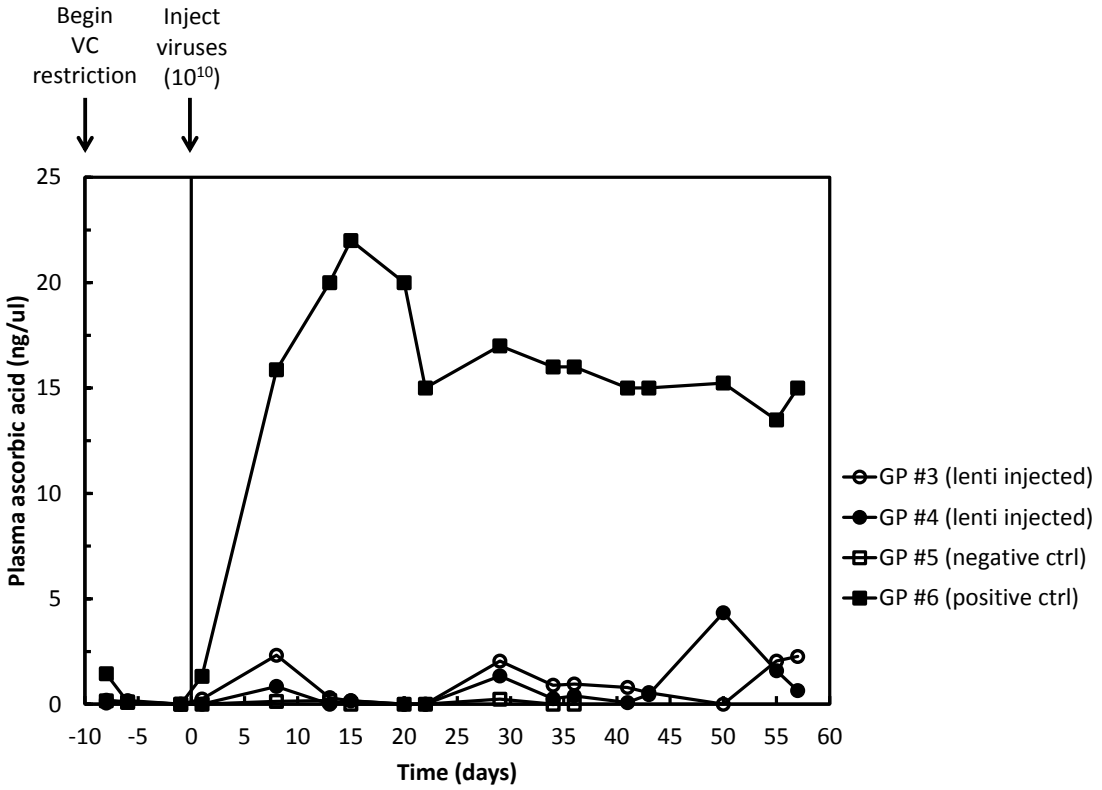


Figure 6. Guinea pig plasma ascorbic acid content in Experiment 2. Guinea pigs #3-6 were acclimatized for 1 week and fed vitamin C-deficient diet and vitamin C-free water for 10 days. On Day 0, GP #3 and #4 were injected with 10^{10} particles of lenti-mCMV-*Gulo*, while GP #5 and #6 were injected with saline of the same volume. Thereafter, GP #3-5 were maintained on vitamin C-free water, whereas GP #6 was returned on vitamin C-supplemented water. GP #3 and #4 also received weekly injections of 500mg/4ml gulonolactone, the substrate of GULO, while GP #5 and #6 received 4ml of saline at the same time. Blood was sampled from the saphenous vein twice per week. All blood samples were centrifuged to obtain the plasma, diluted 10x in 5% MPA, and analyzed using the HPLC for ascorbic acid detection and quantification. GP, guinea pig. VC, vitamin C.

(A)

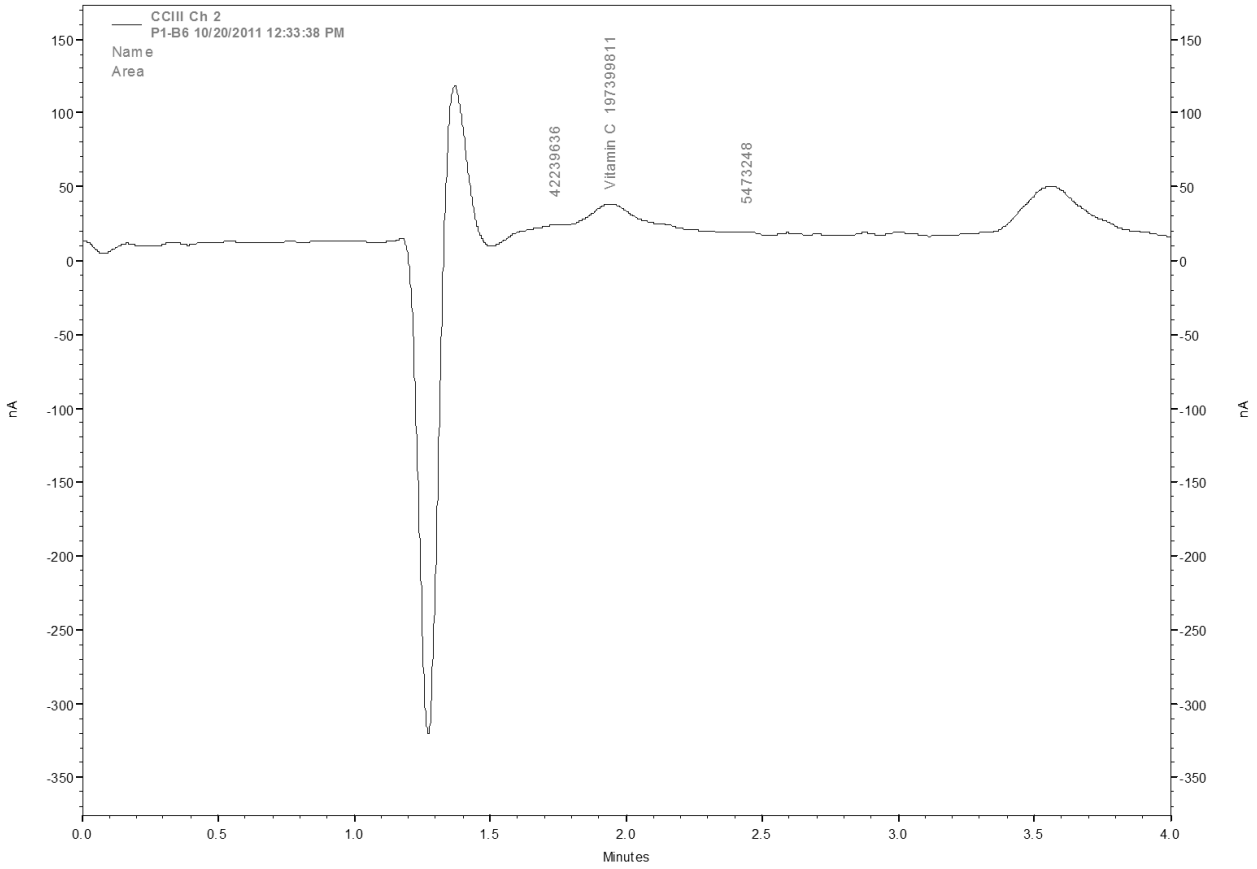
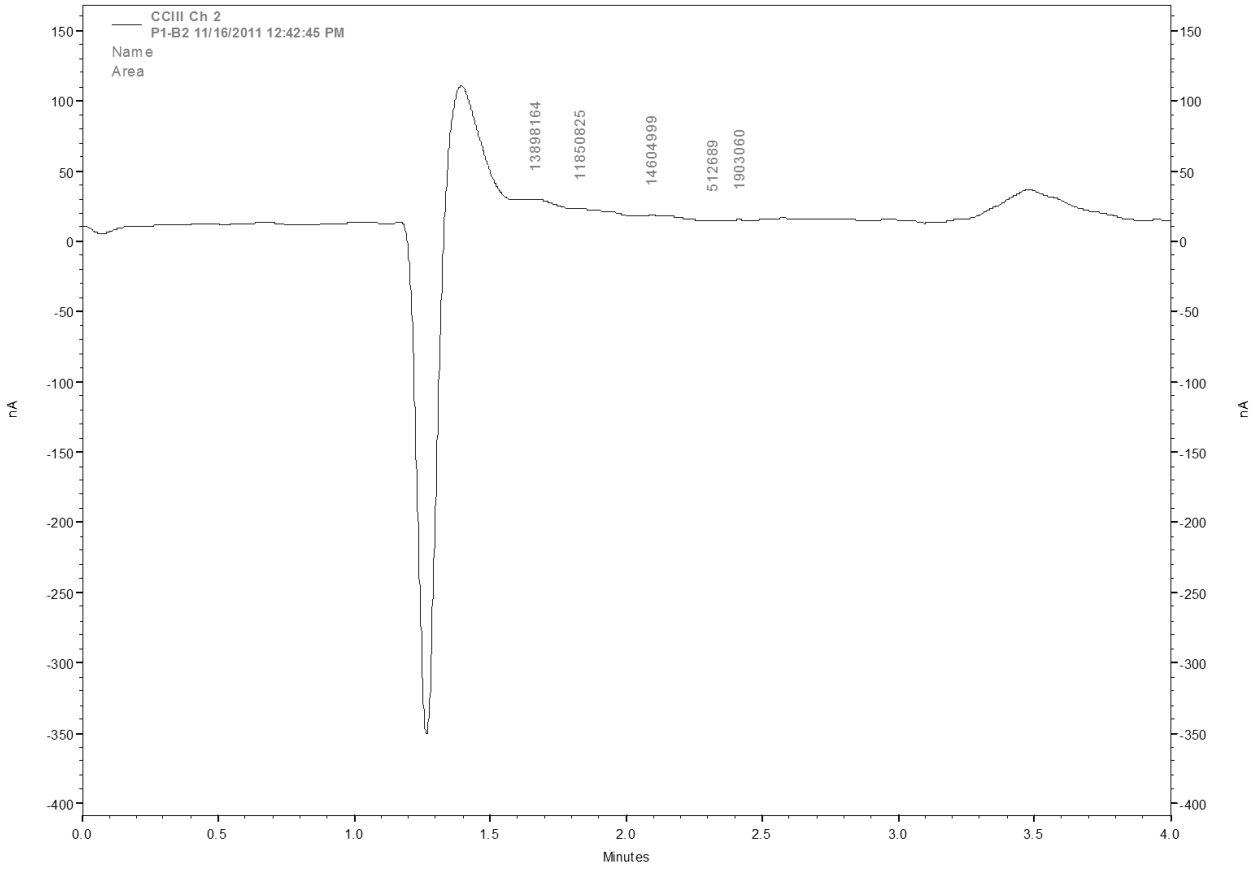


Figure 7. Example traces from HPLC analyses of guinea pig blood samples. (A), a plasma sample from GP #3, an experimental animal treated with lenti-mCMV-*Gulo* on Day 0 and containing a small concentration of ascorbic acid. (B), a plasma sample from GP #5, the negative control maintained on vitamin C-free water and containing no detectable ascorbic acid in the plasma. (C), a plasma sample from GP #6, the positive control returned on vitamin C-supplemented water on Day 0 and containing a high amount of ascorbic acid. Ascorbic acid is eluted at approximately 1.9 min and signal strength, corresponding to ascorbic acid concentration, is determined by integration from valley to valley.

(B)



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(C)

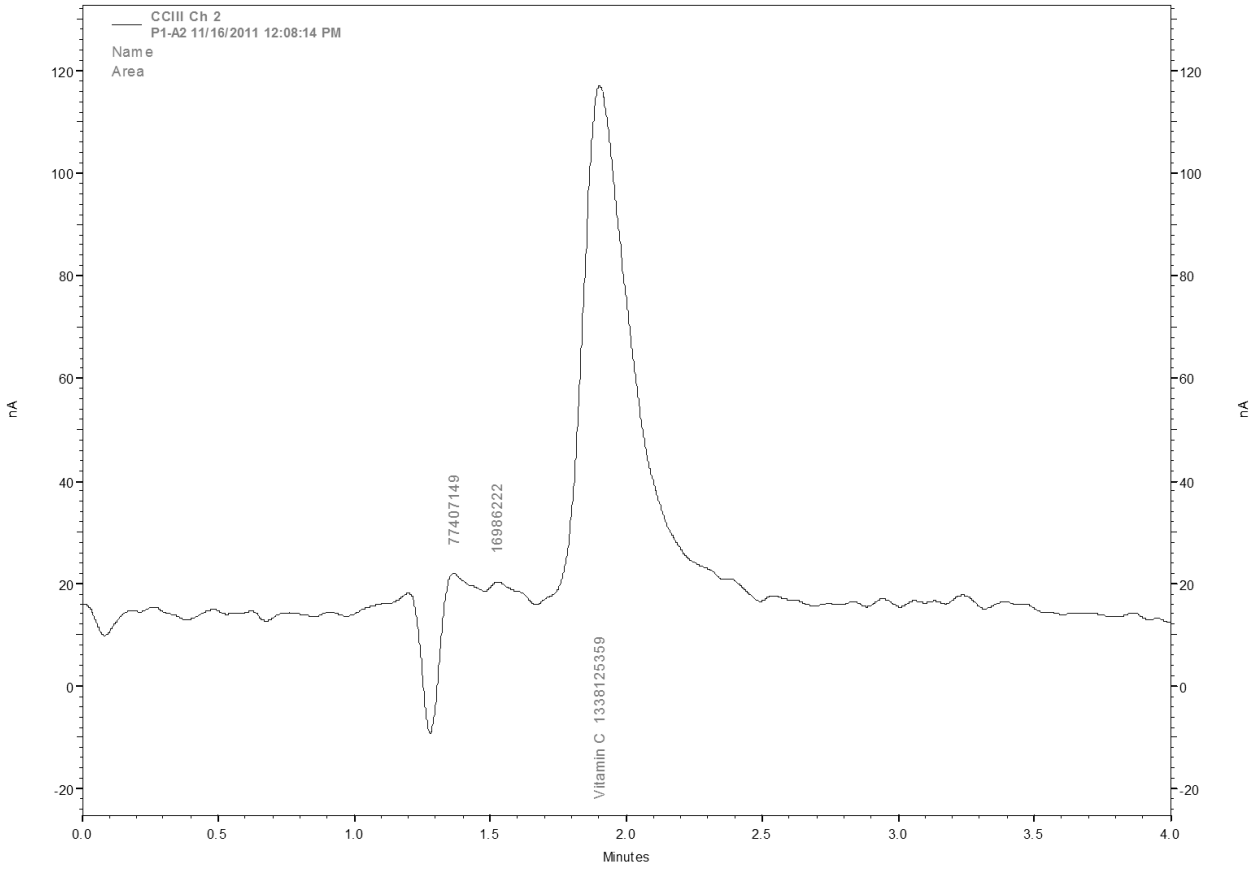

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Table I. Effects of antioxidant deficiency on factors of disease

Variables examined	**Animal model** *(Effect on antioxidant metabolism)*								
	Guinea pig *(no VC synthesis)*	**ODS rat** *(no VC synthesis)*	***Gulo*$^{-/-}$ mouse** *(no VC synthesis)*	***sfx* mouse** *(no VC synthesis)*	**SVCT1$^{-/-}$ mouse** *(impaired VC transport)*	**SVCT2$^{-/-}$ mouse** *(impaired VC transport)*	**SMP30$^{-/-}$ mouse** *(no VC synthesis)*	***Ttpa*$^{-/-}$ mouse** *(impaired VE transport)*	***uox*$^{-/-}$ mouse** *(no UA oxidation)*
Cardio-vascular disease factors									
Athero-sclerotic lesion/ plaque	-	-	NE (Nakata and Maeda 2002)	-	-	Inc (Babaev *et al.* 2010) Dec (Babaev *et al.* 2011)	-	Inc (Terasawa *et al.* 2000; Suarna *et al.* 2006)	-
Vasculature disruption	Inc (Bell *et al.* 2001)	-	Inc (Maeda *et al.* 2000; Telang *et al.* 2007) Dec (Parsons *et al.* 2006)	-	-	-	-	-	-
Blood pressure	Inc (Wolkart *et al.* 2008)	Dec (Horio *et al.* 2001)	-	-	-	-	-	-	-

Table I (cont). Effects of antioxidant deficiency on factors of disease

Variables examined	**Animal model** *(Effect on antioxidant metabolism)*								
	Guinea pig *(no VC synthesis)*	**ODS rat** *(no VC synthesis)*	***Gulo*$^{-/-}$ mouse** *(no VC synthesis)*	***sfx* mouse** *(no VC synthesis)*	**SVCT1$^{-/-}$ mouse** *(impaired VC transport)*	**SVCT2$^{-/-}$ mouse** *(impaired VC transport)*	**SMP30$^{-/-}$ mouse** *(no VC synthesis)*	***Ttpa*$^{-/-}$ mouse** *(impaired VE transport)*	***uox*$^{-/-}$ mouse** *(no UA oxidation)*
Hemorrhage	-	-	Inc (Maeda *et al.* 2000; Telang *et al.* 2007) Dec (Parsons *et al.* 2006)	-	-	-	-	-	-
Lipid peroxidation	NE (Keller *et al*. 2004)	Inc (Kimura *et al.* 1992)	-	-	-	-	-	Inc (Terasawa *et al*. 2000)	-
Serum apo-lipoprotein	-	Dec (Ikeda *et al*. 1996)	-	-	-	-	-	-	-
Total cholesterol	-	-	Inc (Maeda *et al.* 2000)	-	-	-	-	-	-

Table I (cont). Effects of antioxidant deficiency on factors of disease

Variables examined	**Animal model** *(Effect on antioxidant metabolism)*								
	Guinea pig *(no VC synthesis)*	**ODS rat** *(no VC synthesis)*	***Gulo*$^{-/-}$ mouse** *(no VC synthesis)*	***sfx* mouse** *(no VC synthesis)*	**SVCT1$^{-/-}$ mouse** *(impaired VC transport)*	**SVCT2$^{-/-}$ mouse** *(impaired VC transport)*	**SMP30$^{-/-}$ mouse** *(no VC synthesis)*	***Ttpa*$^{-/-}$ mouse** *(impaired VE transport)*	***uox*$^{-/-}$ mouse** *(no UA oxidation)*
Cancer factors									
Tumour size/growth	Inc (Casciari *et al.* 2005)	NE (Mori *et al.* 1988; Mori *et al.* 1990; Mori *et al.* 1991; Yuann *et al.* 1999)	Dec (Telang *et al.* 2007) NE (Parsons *et al.* 2006)	-	-	-	-	-	-
Angio-genesis	-	-	Inc (Telang *et al.* 2007) NE (Parsons *et al.* 2006)	-	-	-	-	-	-
Factors of aging									
Bone fragility	-	-	-	Inc (Beamer *et al.* 2000)	-	-	Inc (Kondo *et al.* 2006)	-	-

Table I (cont). Effects of antioxidant deficiency on factors of disease

Variables examined	**Animal model** *(Effect on antioxidant metabolism)*								
	Guinea pig *(no VC synthesis)*	**ODS rat** *(no VC synthesis)*	***Gulo*$^{-/-}$ mouse** *(no VC synthesis)*	***sfx* mouse** *(no VC synthesis)*	**SVCT1$^{-/-}$ mouse** *(impaired VC transport)*	**SVCT2$^{-/-}$ mouse** *(impaired VC transport)*	**SMP30$^{-/-}$ mouse** *(no VC synthesis)*	***Ttpa*$^{-/-}$ mouse** *(impaired VE transport)*	***uox*$^{-/-}$ mouse** *(no UA oxidation)*
Eyesight loss	Inc (Hayes *et al.* 2011)	-	-	-	-	-	Inc (Ishikawa *et al.* 2012)	Inc (Tanito *et al.* 2007)	-
Hearing loss	Inc (McFadden *et al.* 2005; Heinrich *et al.* 2008)	-	-	-	-	-	Inc (Kashio *et al.* 2009)	-	-
Sensori-motor skills	-	Dec (Hill *et al.* 2003)	Dec (Harrison *et al.* 2008)	-	-	-	-	Dec (Yokota *et al.* 2001; Gohil *et al.* 2004; Yoshida *et al.* 2010)	-

Table I (cont). Effects of antioxidant deficiency on factors of disease

Variables examined	**Animal model** *(Effect on antioxidant metabolism)*								
	Guinea pig *(no VC synthesis)*	**ODS rat** *(no VC synthesis)*	***Gulo*$^{-/-}$ mouse** *(no VC synthesis)*	***sfx* mouse** *(no VC synthesis)*	**SVCT1$^{-/-}$ mouse** *(impaired VC transport)*	**SVCT2$^{-/-}$ mouse** *(impaired VC transport)*	**SMP30$^{-/-}$ mouse** *(no VC synthesis)*	***Ttpa*$^{-/-}$ mouse** *(impaired VE transport)*	***uox*$^{-/-}$ mouse** *(no UA oxidation)*
Cognition	-	-	Dec when pre-disposed (Harrison *et al.* 2008)	-	-	-	-	Dec (Yokota *et al.* 2001; Gohil *et al.* 2004; Yoshida *et al.* 2010)	-
Glucose/ insulin tolerance	-	-	-	-	-	-	Dec (Hasegawa *et al.* 2010)	Inc (Birringer *et al.* 2007)	-
Liver fibrosis	-	-	-	-	-	-	Dec (Park *et al.* 2010a; Park *et al.* 2010b; Ki *et al.* 2011)	-	-

Table I (cont). Effects of antioxidant deficiency on factors of disease

Variables examined	**Animal model** *(Effect on antioxidant metabolism)*								
	Guinea pig *(no VC synthesis)*	**ODS rat** *(no VC synthesis)*	***Gulo*$^{-/-}$ mouse** *(no VC synthesis)*	***sfx* mouse** *(no VC synthesis)*	**SVCT1$^{-/-}$ mouse** *(impaired VC transport)*	**SVCT2$^{-/-}$ mouse** *(impaired VC transport)*	**SMP30$^{-/-}$ mouse** *(no VC synthesis)*	***Ttpa*$^{-/-}$ mouse** *(impaired VE transport)*	***uox*$^{-/-}$ mouse** *(no UA oxidation)*
Other									
Risk for smoking-related pathologies	Inc (Panda *et al*. 2001; Banerjee *et al.* 2008; Ray *et al.* 2010)	-	-	-	-	-	Inc (Koike *et al.* 2010)	-	-
Perinatal mortality	-	-	-	-	Inc (Corpe *et al.* 2010)	Inc (Sotiriou *et al*. 2002)	-	Inc (Jishage *et al*. 2001; Kaempf-Rotzoll *et al.* 2002; Jishage *et al.* 2005)	Inc (Wu *et al.* 1994)
Risk for hyper-uricemia	-	-	-	-	-	-	-	-	Inc (Wu *et al.* 1994)

VC, vitamin C; VE, vitamin E; UA, uric acid; inc, increased; dec, decreased; NE, no effect; -, information not available.

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Appendix A: Standard Operating Protocols

A.1 HEK 293ft cell culturing

A.1.1 Grow Medium

Dulbecco's Minimal essential medium (D-MEM)

10% fetal bovine serum (FBS)

2mM L-glutamine

0.1mM MEM Non-Essential Amino Acid (GIBCO 11140-050)

1mM MEM Sodium Pyruvate

200μg/ml geneticin (G-418)

100 U/ml penicillin/streptomycin (GIBCO 15140-122)

A.1.2 Growth Condition

5% CO2 at 37 °C

A.1.3 Other materials

0.05% Trypsin-EDTA (GIBCO 15140-054)

1x Phosphate Buffered Saline (PBS; Lonza 08211)

Dimethyl Sulfoxide (DMSO)

A.1.4 Culturing Frozen Cells

1. Turn on the biological safety hood; spray with 70% ethanol and wipe it down. Let the air flow in the hood for at least 30 minutes.
2. Warm Growth Media in a 37°C water bath for approximately 30 min.
3. Thaw a vial of cell stock from liquid nitrogen in a 37°C water bath.
4. To two 25cm^2 flasks, transfer 5 ml of the pre-warmed Growth Media each.
5. Gently invert the thawed cell stock to ensure cells are well suspended.
6. Transfer 0.5ml of the cells stock into each flask and gently mix by mild agitation.

7. Incubate in a CO_2 incubator according to the Growth Conditions overnight.
8. On day 2, feed with fresh Growth Media.

A.1.5 Feeding

1. Warm Growth Media in a 37°C water bath for approximately 30 min.
2. Remove cells from the CO_2 incubator.
3. Decant the supernatant (old media).
4. Slowly transfer 5ml of fresh pre-warmed Growth Media into the flask.
5. Return the dish to CO_2 incubator for 2-3 days until approx. 90% confluence.
6. Split the 90% confluent culture 1 in 7.

A.1.6 Splitting Cell Culture

1. Warm Growth Media, PBS, and 1x trypsin in a 37°C water bath for 30 min.
2. Remove the flask from incubator and decant the supernatant.
3. Rinse the flask with 5ml of 1x PBS, decant.
4. Add 0.5ml of 0.05% Trypsin-EDTA to the cells; incubate for 30-60 sec.
5. Gently tap the flask sideways against a solid surface to detach cells from the bottom of the dish.
6. Re-suspend the cells in 5 ml of warm Growth Media and distribute into 7 new flasks with 5 ml warm Growth Media.
7. Incubate in a CO_2 incubator according to the Growth Conditions
8. Feed cells after 2-3 day incubation, and continue incubating until 90% confluency is reached.
9. Split 1:7 again.

A.1.7 Freezing Cells for Long Term Storage

1. Remove flasks containing 90% confluent cells from the incubator and decant the media.
2. Rinse the flask with 5ml of 1x PBS, decant.

3. Add 0.5ml of 0.05% Trypsin-EDTA to the cells; incubate for 30-60 sec.
4. Resuspend cells in 5ml of complete media; transfer to 15ml conical tubes.
5. Centrifuge at 200xg room temperature for 5 minutes
6. Carefully aspire off the supernatant and resuspend cells in 1ml of complete media with 10% DMSO.
7. Transfer the suspension into 2 cryogenic vials.
8. Freeze cells at -80°C for 2~3 days and transfer to liquid nitrogen bath for long term storage.

A.2 HEP G2 cell culturing

A.2.1 Grow Medium

Dulbecco's Minimal essential medium (D-MEM)

10% fetal bovine serum (FBS)

2mM L-glutamine

0.1mM MEM Non-Essential Amino Acid (GIBCO 11140-050)

1mM MEM Sodium Pyruvate

100 U/ml penicillin/streptomycin (GIBCO 15140-122)

A.2.2 Growth Condition

5% CO2 at 37 °C

A.2.3 Other materials

0.05% Trypsin-EDTA (GIBCO 15140-054)

1x Phosphate Buffered Saline (PBS; Lonza 08211)

Dimethyl Sulfoxide (DMSO)

A.2.4 Culturing Frozen Cells

1. Turn on the biological safety hood; spray with 70% ethanol and wipe it down. Let the air flow in the hood for at least 30 minutes.
2. Warm Growth Media in a 37°C water bath for approximately 30 min.
3. Thaw a vial of cell stock from liquid nitrogen in a 37°C water bath.
4. To two 25cm^2 flasks, transfer 5 ml of the pre-warmed Growth Media each.
5. Gently invert the thawed cell stock to ensure cells are well suspended.
6. Transfer 0.5ml of the cells stock into each flask and gently mix by mild agitation.
7. Incubate in a CO_2 incubator according to the Growth Conditions overnight.
8. On day 2, feed with fresh Growth Media.

A.2.5 Feeding

1. Warm Growth Media in a 37°C water bath for approximately 30 min.
2. Remove cells from the CO_2 incubator.
3. Decant the supernatant (old media).
4. Slowly transfer 5ml of fresh pre-warmed Growth Media into the flask.
5. Return the dish to CO_2 incubator for 2-3 days until approx. 90% confluence.
6. Split the 90% confluent culture 1 in 5.

A.2.6 Splitting Cell Culture

1. Warm Growth Media, PBS, and 1x trypsin in a 37°C water bath for 30 min.
2. Remove the flask from incubator and decant the supernatant.
3. Rinse the flask with 5ml of 1x PBS, decant.
4. Add 0.5ml of 0.05% Trypsin-EDTA to the cells; incubate for 2-3 min.
5. Gently tap the flask sideways against a solid surface to detach cells from the bottom of the dish.
6. Re-suspend the cells in 5 ml of warm Growth Media and distribute into 5 new flasks with 5 ml warm Growth Media.
7. Incubate in a CO_2 incubator according to the Growth Conditions

8. Feed cells after 2-3 day incubation, and continue incubating until 90% confluency is reached.
9. Split 1:5 again.

A.2.7 Freezing Cells for Long Term Storage

1. Remove flasks containing 90% confluent cells from the incubator and decant the media.
2. Rinse the flask with 5ml of 1x PBS, decant.
3. Add 0.5ml of 0.05% Trypsin-EDTA to the cells; incubate for 2-3 min.
4. Resuspend cells in 5ml of complete media; transfer to 15ml conical tubes.
5. Centrifuge at 200xg room temperature for 5 minutes
6. Carefully aspire off the supernatant and resuspend cells in 1ml of complete media with 10% DMSO.
7. Transfer the suspension into 2 cryogenic vials.
8. Freeze cells at -80°C for 2~3 days and transfer to liquid nitrogen bath for long term storage.

A.3 Construction of lentiviral vectors carrying Gulo

A.3.1 Materials

2x HEPES-buffered saline: 140mM NaCl, 1.5mM Na_2HPO_4, 50mM HEPES

0.1x TE (pH 7.6): 1mM Tris-Cl (pH 7.6), 0.1mM EDTA (pH 7.6)

2.5M $CaCl_2$

Methanol

1x Phosphate Buffered Saline (PBS; Lonza 08211)

0.45μm filter (Millipore PVDF, ref SLHV033R5)

A.3.2 Transformation

1. Grow HEK 293ft cells to 90% confluency.

2. On day 0, trypsinize cells and collect cells into 15ml conical tubes.
3. Add 100µl of 2.5M $CaCl_2$ to a 1.5ml Eppendorf tube.
4. Add 25µl of pLP-PLP1, pLP-PLP2, pLP-VSVG, and either pLenti6-mCMV-GULO or pLenti6-PEPCK-GULO to the Eppendorf tube. The concentration of each plasmid is 0.5µg/µl. The total volume is 200µl.
5. Add 200µl of 2x HEPES-buffered saline; tap gently to mix.
6. Stand the tube for exactly 1 min. Transformation efficiency decreases dramatically if incubated longer than 1 min.
7. Add all 400µl of the DNA-$CaPO_4$ complex to the cells. The media should turn orange and turbid.
8. Transfer the mixture into a 25cm^2 flask.
9. Incubate flask at 37^oC 5% CO_2 overnight.
10. On day 1, replace DNA-containing media with complete growth media for HEP G2.
11. On day 2, 4, and 7, harvest supernatant and replace with fresh media. Filter virus-containing supernatant through 0.45µm filter to remove cells.
12. Centrifuge supernatant at 3000xg for 5 minute in room temperature.
13. Freeze in 1ml aliquots at -80^oC.

A.4 Concentration of lentiviruses

A.4.1 Materials

50% PEG6000

4M NaCl

1x Phosphate Buffered Saline (PBS; Lonza 08211)

50mM Tris-Cl pH 7.4

A.4.2 Concentration

1. To a 1 ml aliquot of virus, add 0.25ml 50% PEG6000, 0.1ml 4M NaCl, and 0.15ml PBS. Scale up if necessary.
2. Sit mixture at 4^{o}C for 1.5h, mixing every 20-30 min
3. Centrifuge at 7,000 xg at 4^{o}C for 10 min
4. Resuspend pellet in 1.0ml of 50mM Tris-Cl pH 7.4
5. Store in 0.5ml aliquots.

A.5 Titration of lentiviruses

A.5.1 Materials

Growth media for HEP G2

Blasticidin S HCl (Invitrogen A11139-03)

A.5.2 Infection

1. Grow HEP G2 cells to 90% confluency.
2. On day 0, Replace old media with fresh growth media; add serially diluted lentiviruses collected prior as described. Incubate cells overnight.
3. On day 1, remove virus-containing media and replace with media containing no blasticidin.
4. On day 2, remove media again and replace with media containing 5µg/ml blasticidin.
5. On day 4, trypsinize cells and replate in a fresh plate. Incubate overnight.
6. On day 5, replace media and dead cells by aspiration. Wash plate with 1x PBS and replenish the cells with fresh media containing 5µg/ml blasticidin.
7. Culture live cells as previously described as HEP G2 cells, except include 5µg/ml blasticidin in its growth media.

A.6 Tissue perfusion and collection on biohazard level 2 guinea pigs (LRS only)

Note: Perfusion with formalin is not covered in this SOP.

A.6.1 Materials

Class 2 biological safety cabinet (BSC) in biohazard level 2 room or PM room

Dissecting tray

Adult-sized diapers

Paper towels

Masking tape

Gaseous anaesthetic machine and isoflurane

Heparin (1000u/ml x .2ml)

Sodium pentobarbital (35mg/kg)

Scale

Syringes/needles

1ml/25G for heparin

1ml/23G for sodium pentobarbital

sandpaper-blunted 18Gneedle for puncturing the heart

Sandpaper

Surgical instruments (iris and mayo scissors, forceps)

Lactated Ringer's solution (LRS) (500ml/guinea pig)

Stand (for LRS)

Intravenous line

PPE

Collection tubes for tissue samples

Sealable container for level 2 material transport

70% ethanol in a spray bottle

A.6.2 Tissue perfusion

A.6.2.1 Set-up

1. Prep the biological safety cabinet (BSC) for use.

2. Set up a stand in the BSC. Hang a bag of Lactated Ringer's Solution (LRS) on the stand and connect an IV line to it. Use sandpaper to blunt the end of an 18G needle and attach it to the other end of the IV line.
3. Since perfusion with formalin is not required, only one IV line is enough. There is no need for a 3-way stopcock.
4. Put a layer of rabbit liner on to a dissecting tray and cover the whole tray.
5. Open up an adult-sized diaper and tape it down on to the dissecting tray.
6. Flush the IV line with LRS.
7. Weigh guinea pig, calculate the dose of sodium pentobarbital needed.

A.6.2.2 Dissection

1. Anaesthetize the guinea pig using isoflurane (See CAF SOP GEN727).
2. Inject sodium pentobarbital (35mg/kg), wait until guinea pig is deeply anaesthetized (10-20 minutes).
3. Tape animal to dissecting tray over the diaper. Make sure that the absorbent part of the diaper is directly below the upper body of the guinea pig.
4. Open skin over thorax and abdomen. Take the xiphoid process with forceps and lift the ribcage.
5. Cut through the ribs to expose the entire heart. The heart should still be beating and continue beating until the end of perfusion.

A.6.2.3 Perfusion

1. Inject heparin (1000u/ml x .2ml) into the left ventricle.
2. Wait approximately 10 heart beats.
3. Make sure LRS is flowing slowly through the IV line (with a blunted 18G needle attached to it). Insert the needle into the left ventricle. The heart should swell up with LRS.
4. Find the right auricle (flap above heart) and cut a small hole using a pair of iris scissors to allow blood to flow out.

5. Once perfusion begins, the IV line can be taped down and the flow of LRS can be increased. Monitor LRS dripping through the IV line as sometimes the needle can hit something in the heart and the flow could stop. Make sure blood is draining onto the diaper and being absorbed.
6. Allow blood to flow until it becomes clear (5-10 minutes). The liver should appear blanched.
7. Collect tissue samples and handle as level 2 biohazard material.

A.6.2.4 Clean-up

1. Fold the diaper closed with the absorbed blood and animal carcass inside. Detach rabbit liner from the tray and fold it closed with the diaper inside.
2. Gently slip the bundle into a biohazard bag. Dispose as level 2 waste.
3. Transport tissue collection tubes in a sealable container. Spray container with 70% ethanol before taking out of the BSC.
4. Treat all waste as level 2 biohazard waste. Animal carcasses are incinerated as per CAF SOP CAF056.
5. Check the LRS bag to make sure there's enough solution for the next perfusion.
6. When all animals are perfused, clean up BSC and work area following CAF SOP GEN127.

A.7 Protein extraction from the liver and enzyme assay

A.7.1 Materials

Liver buffer: 50mM sodium citrate, 50mM potassium phosphate buffer, pH7.0
Substrate solution: 50mM sodium citrate, 50mM potassium phosphate buffer, pH7.0, 3.75mM L-gulonolactone (final concentration 2.5mM) OR 15mM L-gulonolactone (final concentration 10mM)

A.7.2 Enzyme assay

1. Homogenize 1g of liver in 10ml of liver buffer
2. Centrifuge homogenate at 13,000 xg for 20 min
3. Extract supernatant; centrifuge again at 13,000 xg for 20 min
4. Extract supernatant; store at -20^{o}C
5. Perform Bradford assay to quantify total protein
6. Add 100µl of the liver extract to 200µl of substrate solution
7. Incubate in the 37^{o}C water bath for 0h, 2h, 4h and overnight
8. Add 50µl of 1.5% MPA to stop reaction
9. Centrifuge at 13,000xg for 5 min
10. Extract supernatant
11. Assay supernatant for ascorbic acid

A.8 Genotyping of Gulo knockout mouse

A.8.1 Materials

To book/request from CAF 24h in advance:

BSC in the CLN room (1U55)

Heating pad (optional)

Recovery cage (optional)

To autoclave in advance:

Tipbox(es)

Tubes

Surgical instruments – scissors & tweezers

To bring to CAF:

Ear notch

Silver nitrate sticks

Isoflurane (optional)

Liq N_2

Pen & paper

Cardkey

Other materials:

Norgen Genomic DNA isolation Kit (Cat #24700)

Primer P2: CGC GCC TTA ATT AAG GAT CC

Primer P3: GTC GTG ACA GAA TGT CTT GC

Primer P4: GCA TCC CAG TGA CTA AGG AT

A.8.2. Genotyping

A.8.2.1 Tailing

1. Prep BSC (GEN127.sop)
2. (Optional: anaesthetize mouse using isoflurane (GEN727.sop))
3. Notch ears, record ID
4. Clip mouse tail ~1cm
5. Wet silver nitrate sticks with water, apply to wound to stop bleeding
6. Flash freeze tail in labeled tubes
7. (Optional: recover mouse)
8. Re-cage
9. Store tail at -80C

A.8.2.2 Genomic DNA extraction

1. Use ~0.5cm of tail, store rest of tail at -80C again
2. Add 300µl digestion buffer, cut tissue up with small scissors
3. Add 10µl of RNase A (10mg/ml stock, ~10KUnitz)
4. Add 12µl of proteinase K, incubate at 55C for 1h
5. Add 300µl Binding Solution, vortex
6. Add 300µl 95% EtOH, vortex
7. Assemble column. Add 500µl lysate to column. Spin 3min at 5,200xg (8,000 RPM)

8. Repeat 7 with the rest of lysate
9. Add 500µl Wash Solution, spin 1min at 14,000xg
10. Repeat 9, spin 2min this time
11. Dry spin 2min, take column out & put in Eppi tubes
12. Add 200µl water, spin 1min at 3,000xg (6,000RPM) to hydrate DNA
13. Spin 2min at 14,000xg to elute
14. Can elute again (repeat 12-13) if needed
15. Sit on ice ~3h while running PCR. Dirty stuff precipitates. Collect 150µl into new tubes.

A.8.2.3 PCR

1. Mix PCR reactants:

ddH2O	12.5µl
10x PCR buffer w/o MgCl2	2.5µl
(Fermentas)	1.5µl
25mM MgCl2 (Fermentas)	0.5µl
10mM dNTP (Fermentas)	1µl
25µM primer P2	1µl
25µM primer P3	1µl
25µM primer P4	1µl
5u/µl Taq (NEB)	5µl
10x diluted genomic DNA	4µl
Total	25µl

2. PCR conditions:

95C	2min
95C	30s
52C	30s

72C	1:30
Repeat	30x
72C	7min
4C	hold

3. Run 3% agarose gel, use 100bp DNA ladder
    - Gulo+: band at 330bp (P3-P4)
    - Gulo-: band at 230bp (P2-P3)

A.9 HPLC-ECD analysis for ascorbic acid

A.9.1 Mobile phase

0.2M KH_2PO_4

Adjust pH to 2.9 using o-phosphoric acid

Filter through 0.2μm filter

A.9.2 HPLC equipment

A HPLC system (Agilent 1200 series)

Agilent XDB-C18 analytical column (particle size 5 μm, 4.6 x 250 mm).

CoulochemIII electrochemical detector (ESA)

A.9.3 Other Materials

5% MPA-EDTA (MPA Sigma-Aldrich 239275-100G)

Ultra-pure ascorbic acid (Sigma-Aldrich 255564-100G)

A.9.4 HPLC

1. Run mobile phase for at least 15min prior to any sampling to stabilize the system
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. a) 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 3 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: 3 min

    b) Alternatively, if the pump pressure is higher than 500 bars, develop the following method
    - Flow rate: 2ml/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 5 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: 5 min
6. Turn the autosampler light off to prevent decomposition of ascorbic acid.
7. Put at least 100µl of samples into a 96-welled plate before injection.
8. Autosampler can inject up to 100µl of liquid.
9. Samples may be run in a sequence using sequence wizard.