# Ascorbic-acid transporter Slc23a1 is essential for vitamin C transport into the brain and for perinatal survival

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**The only proven requirement for ascorbic acid (vitamin C) is in preventing scurvy[1,2], presumably because it is a cofactor for hydroxylases required for post-translational modifications that stabilize collagen[3]. We have created mice deficient in the mouse ortholog (solute carrier family 23 member 1 or Slc23a1) of a rat ascorbic-acid transporter, Svct2 (ref. 4). Cultured embryonic fibroblasts from homozygous *Slc23a1*$^{-/-}$ mice had less than 5% of normal ascorbic-acid uptake. Ascorbic-acid levels were undetectable or markedly reduced in the blood and tissues of *Slc23a1*$^{-/-}$ mice. Prenatal supplementation of pregnant females did not elevate blood ascorbic acid in *Slc23a1*$^{-/-}$ fetuses, suggesting Slc23a1 is important in placental ascorbic-acid transport. *Slc23a1*$^{-/-}$ mice died within a few minutes of birth with respiratory failure and intraparenchymal brain hemorrhage. Lungs showed no postnatal expansion but had normal surfactant protein B levels. Brain hemorrhage was unlikely to be simply a form of scurvy since *Slc23a1*$^{-/-}$ mice showed no hemorrhage in any other tissues and their skin had normal skin 4-hydroxyproline levels despite low ascorbic-acid content. We conclude that Slc23a1 is required for transport of ascorbic acid into many tissues and across the placenta. Deficiency of the transporter is lethal in newborn mice, thereby revealing a previously unrecognized requirement for ascorbic acid in the perinatal period.**

A deletion construct was prepared to create an early frame-shift mutation in the *Slc23a1* gene in embryonic stem (ES) cells[5] (Fig. 1*a*). Correctly targeted ES cell clones were injected into blastocysts to generate chimeras that gave germline transmission of the deletion allele. Genotypes from an F1 heterozygote intercross showed the expected fragment sizes by Southern-blot analysis (Fig. 1*b*). Reverse-transcriptase (RT)-PCR using primers that flanked the deleted segment of the *Slc23a1* transcript confirmed a deletion of the expected size in the transcript (Fig. 1*c*). To confirm that the mutant allele disrupted Slc23a1 function, we measured the uptake of $^{14}$C-labeled ascorbic acid by mouse embryonic fibroblasts in culture (Fig. 1*d*). Ascorbic-acid uptake was less than 5% of normal (0.12 versus 2.19 μmol/min).

We studied tissues from newborn mice heterozygous and homozygous for the mutant allele of *Slc23a1*. Ascorbic-acid levels were undetectable in the brain, pituitary, adrenals and pancreas and were markedly reduced in liver, kidney and muscle of *Slc23a1*$^{-/-}$ mice compared with *Slc23a1*$^{+/-}$ or wild-type littermates ($P << 1 \times 10^{-5}$, one-tailed Student's *t*-test) (Fig. 2). The concentration of ascorbic-acid in blood in *Slc23a1*$^{-/-}$ pups was also reduced compared with *Slc23a1*$^{+/-}$ pups ($P = 0.01$). Significant reductions of ascorbic-acid levels were also seen in most tissues of *Slc23a1*$^{+/-}$ newborns as compared with wild-type pups; reduced levels were also seen in the brain and blood, but not liver, of adult *Slc23a1*$^{+/-}$ mice (Fig. 2). Slc23a1 thus seems to be the predominant transporter of ascorbic acid in the fetus and is sensitive to gene dosage, resulting in a detectable biochemical phenotype in heterozygotes.

We supplemented pregnant female mice in *Slc23a1*$^{+/-}$ × *Slc23a1*$^{+/-}$ matings with oral ascorbic acid. Late-gestation pups (embryonic days (E)19.5–20.5) were killed following cesarean section and their plasma ascorbic-acid levels measured. Wild-type and *Slc23a1*$^{+/-}$ pups from mothers treated with oral ascorbic acid showed an increase from 76.5 to 133 μM and from 53.5 to 80.3 μM, respectively. Following oral ascorbic-acid supplementation of their mothers, *Slc23a1*$^{-/-}$ mice showed no change in plasma ascorbic-acid levels (35 μM without and 28.9 μM with supplementation). We therefore conclude that fetal Slc23a1 is a significant contributor to the maternal-to-fetal transport of ascorbic acid across the placenta.

Genotyping of newborn pups at birth revealed no fetal wastage: the mutant allele segregated in a mendelian fashion, generating wild-type, *Slc23a1*$^{+/-}$ and *Slc23a1*$^{-/-}$ genotypes at a ratio of 30:76:33 ($\chi^2 = 1.34$ with 2 degrees of freedom (d.f.), $P = 0.51$). All littermates weighed approximately the same and were grossly equivalent developmentally (data not shown). Immediately after birth, however, *Slc23a1*$^{-/-}$ mice developed respiratory distress and hemorrhaging over the head that was visible through the skin overlying the cranium (Fig. 3*a*). The hemorrhaging rapidly progressed from small petechiae to large ecchymoses. The newborns were unable to breathe and died within minutes. We performed cesarean sections to examine the *Slc23a1*$^{-/-}$ animals before birth. The phenotype of wild-type, *Slc23a1*$^{+/-}$ and *Slc23a1*$^{-/-}$ pups were initially indistinguishable *in utero*; however, *Slc23a1*$^{-/-}$ mice deliv-

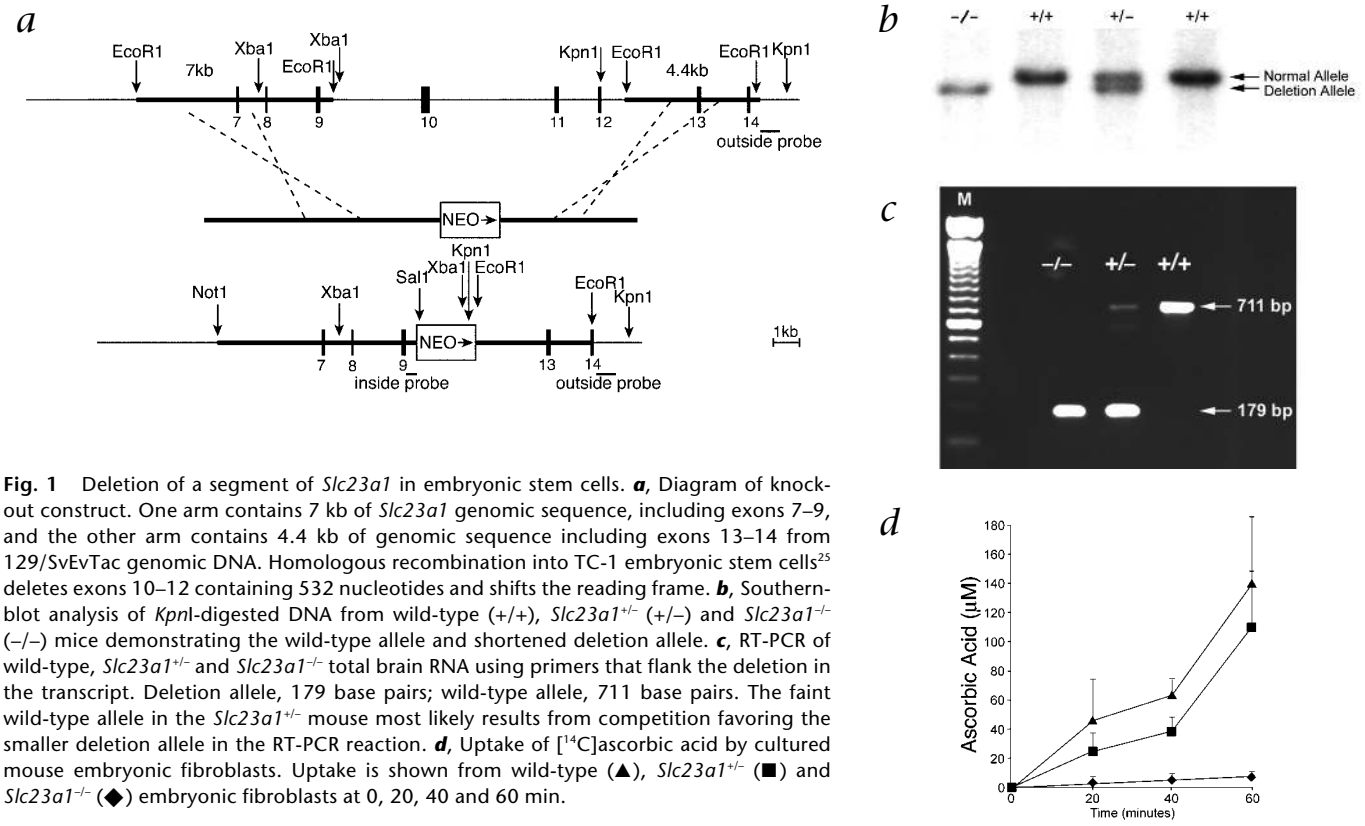


**Fig. 1** Deletion of a segment of *Slc23a1* in embryonic stem cells. ***a***, Diagram of knock-out construct. One arm contains 7 kb of *Slc23a1* genomic sequence, including exons 7–9, and the other arm contains 4.4 kb of genomic sequence including exons 13–14 from 129/SvEvTac genomic DNA. Homologous recombination into TC-1 embryonic stem cells[25] deletes exons 10–12 containing 532 nucleotides and shifts the reading frame. ***b***, Southern-blot analysis of *Kpn*I-digested DNA from wild-type (+/+), *Slc23a1*$^{+/-}$ (+/–) and *Slc23a1*$^{-/-}$ (–/–) mice demonstrating the wild-type allele and shortened deletion allele. ***c***, RT-PCR of wild-type, *Slc23a1*$^{+/-}$ and *Slc23a1*$^{-/-}$ total brain RNA using primers that flank the deletion in the transcript. Deletion allele, 179 base pairs; wild-type allele, 711 base pairs. The faint wild-type allele in the *Slc23a1*$^{+/-}$ mouse most likely results from competition favoring the smaller deletion allele in the RT-PCR reaction. ***d***, Uptake of [$^{14}$C]ascorbic acid by cultured mouse embryonic fibroblasts. Uptake is shown from wild-type (▲), *Slc23a1*$^{+/-}$ (■) and *Slc23a1*$^{-/-}$ (◆) embryonic fibroblasts at 0, 20, 40 and 60 min.

ered by cesarean section had the same rapid onset of respiratory distress and hemorrhaging as did full-term pups born vaginally.

At autopsy, the only abnormalities seen in *Slc23a1*$^{-/-}$ homozygotes were confined to the lungs and brain. The lungs of *Slc23a1*$^{-/-}$ mice did not float, indicating that the initial postnatal expansion did not occur. Microscopic examination of the lungs of *Slc23a1*$^{-/-}$ homozygous pups demonstrated no histological abnormalities other than failure of alveolar expansion. Levels of the precursor and mature forms of surfactant protein B (SP-B) as determined by western-blot analysis of lungs from *Slc23a1*$^{-/-}$ mice were not reduced compared with littermates (data not shown). There was extensive hemorrhage over the convex surface of the brain (Fig. 3*a*). Microscopic examination revealed widespread intraparenchymal hemorrhage (Fig. 3*b*). This distribution corresponded to the location of the most fragile blood vessels in the human fetal brain[6]. The *Slc23a1*$^{-/-}$ mice showed no bleeding in any other tissues such as lung, skin, muscle nor was there bleeding in the adrenal gland, another highly vascular tissue with a normally high ascorbic-acid content. Collagen modification, as evidenced by 4-hydroxyproline content, was indistinguishable in skin of animals with all three genotypes (data not shown).

We can draw three main conclusions from the work presented here. First, Slc23a1 is necessary for the prenatal transport of ascorbic acid into most tissues of the mouse, particularly in the central nervous system and the adrenal glands. Measurable reductions were seen even in mice heterozygous for the mutant allele, indicating a gene-dosage effect. Second, Slc23a1 has a major role in transport of the vitamin across the placenta. Finally, we uncovered an unanticipated and, as yet, incompletely understood phenotype of respiratory failure and intracerebral hemorrhage in the immediate newborn period in mice homozygous for a defective *Slc23a1* allele.

The mechanism of ascorbic-acid transport into the tissues of the whole animal has been studied over many years with somewhat conflicting results[7,8]. The marked reductions in tissue ascor-

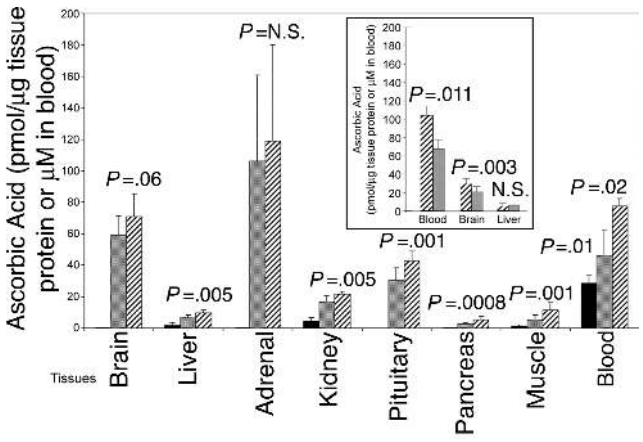


**Fig. 2** Ascorbic-acid levels in tissues (pmol/μg protein) and plasma (μM) from wild-type (▨), *Slc23a1*$^{+/-}$ (■) and *Slc23a1*$^{-/-}$ (■) littermates obtained by cesarean section. *P*-values (Student's *t*-test, one-tail, 17 d.f.) comparing ascorbic-acid levels in seven tissues from *Slc23a1*$^{+/-}$ and wild-type animals are as shown above the bars. *P*-values (Student's *t*-test, one tail, 13 d.f.) to test significance of differences in plasma ascorbic-acid concentrations are as shown between *Slc23a1*$^{+/-}$ and wild-type mice and between *Slc23a1*$^{+/-}$ and *Slc23a1*$^{-/-}$ mice. Inset, ascorbic-acid levels in tissues (pmol/μg protein) and plasma (μM) in adult wild-type and *Slc23a1*$^{+/-}$ mice aged 9–11 mo. *P*-values (Student's *t*-test, one tail, 7 d.f.) to test significance of differences in ascorbic-acid concentrations are as shown.

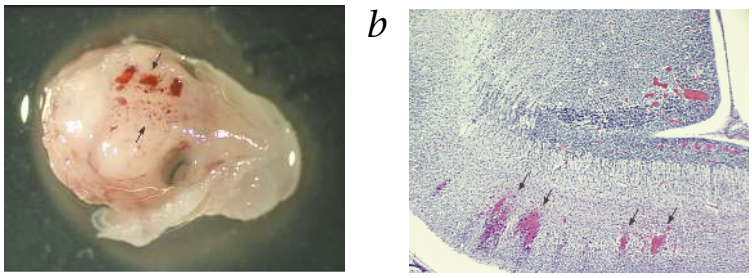

**Fig. 3** Cranium and cortex of newborn *Slc23a1*$^{-/-}$ mice. ***a***, Gross examination of surface of brain after removal of cranial bones showing petechiae and ecchymoses over the cerebral convexity (arrows). ***b***, Microscopic examination of areas of intraparenchymal hemorrhage in the cortex immediately adjacent to the subarachnoid space (arrows). Magnifications, ×2.5 (a) and ×450 (b).



bic-acid levels seen in *Slc23a1*$^{-/-}$ animals make it unlikely that the oxidized form of ascorbic acid, dehydroascorbic acid (DHA), and not ascorbic acid itself, is the predominant species transported from blood into most tissues[9,10] as Slc23a1 does not transport DHA (refs. 9,11,12). There is also a gene dosage effect in that significant reductions in ascorbic-acid levels were seen in newborn *Slc23a1*$^{+/-}$ mice that persists, at least in the brain, into adulthood. The marked reduction in ascorbic acid in brain and other tissues requires further investigation, as it is not known whether it is a loss of Slc23a1 function in neurons, ependyma, endothelial cells or some other cell type, singly or in combination, that has such a great impact on ascorbic-acid levels in these tissues.

In contrast to the brain and many other tissues, hepatic and blood ascorbic-acid levels were clearly measurable, although reduced, in late-gestation *Slc23a1*$^{-/-}$ mice. Ascorbic acid in late-gestation fetal liver most probably results from endogenous ascorbic-acid synthesis, which begins a few days before birth[13–15]. The reduced but measurable blood levels in *Slc23a1*$^{-/-}$ mice are also likely to reflect fetal hepatic synthesis and transport into the bloodstream. However, a role for Slc23a1 in the transport of endogenously synthesized ascorbic acid is suggested by the gene dosage effect seen in the reduced ascorbic-acid levels in the blood, but not liver, of adult *Slc23a1*$^{+/-}$ mice.

Placental transport of maternal ascorbic acid has been demonstrated[7,16] in the rat and guinea pig. However, there remains some uncertainty as to whether ascorbic acid or its oxidation product dehydroascorbic acid is the transported species and which transporter is responsible[7,8,11,12,17]. Our results with maternal ascorbic-acid supplementation suggest that Slc23a1 is required for placental transport of ascorbic acid. The involvement of Slc23a1 in placental transport is even more important in humans than in rodents because rodents, but not primates, synthesize ascorbic acid beginning late in gestation[13–15]. Human fetuses, in contrast, remain completely dependent on exogenous ascorbic acid throughout gestation and after birth.

The most striking observation from these studies is the syndrome of respiratory failure and intracerebral hemorrhage seen in *Slc23a1*$^{-/-}$ newborns immediately after birth. The abnormalities in lung function remain unexplained. *Slc23a1* is expressed in lung[5] and may allow ascorbic acid to play a previously unrecognized role in lung maturation. One obvious explanation for the respiratory failure—loss of surfactant production—was eliminated, although failure to deliver or maintain normal surfactant function in alveolar fluid itself cannot be excluded with current data. As for the intracerebral hemorrhage, it is unlikely to represent a bleeding diathesis due to generalized vascular fragility as is seen with scurvy. The level of 4-hydroxyproline in skin was normal in *Slc23a1*$^{-/-}$ newborns, suggesting that there was no generalized defect in post-translational processing of collagen as would be expected with ascorbic-acid deficiency and scurvy. As further evidence, the bleeding was confined to the brain and was not seen in many tissues with no detectable ascorbic acid such as pituitary, skin, pancreas or adrenal glands.

That mice with low levels of blood and tissue ascorbic acid due to a deficiency of Slc23a1 show some of the serious clinical complications of prematurity in human infants may have important implications for human health. Although postnatal administration of ascorbic acid to prevent the complications of premature birth remains controversial[18,19], our observation that mice deficient in Slc23a1 show some of the same complications associated with prematurity in humans suggests prenatal ascorbic-acid deficiency deserves a more thorough investigation for its role, if any, in the morbidity due to premature birth.

## Methods

**Creation of *Slc23a1*$^{-/-}$ mouse embryonic stem cells.** A mouse genomic bacterial artificial chromosome library constructed from strain 129S6/SvEvTac was screened for *Slc23a1* using mouse *Slc23a1* cDNA sequence[5]. A targeting vector was constructed to replace exons 10–12 with the aminoglycoside phosphotransferase gene (Neo$^R$) conferring neomycin resistance and removing 532 nucleotides of coding sequence to create a deletion and frameshift in *Slc23a1* mRNA (Fig. 1*a*). Embryonic stem (ES) cell colonies resistant to the aminoglycoside G418 were screened by Southern blotting to identify correctly targeted cell clones, 2 of which were then used for blastocyst injections to establish 2 lines of mice[20]. As both mouse lines were indistinguishable in their phenotype, all further experiments used mice derived from one of the ES-cell clones maintained on an inbred 129S6/SvEvTac background. Genomic DNA was isolated from tail biopsies by standard methods[21]. Animal experiments were conducted with the approval of the Animal Care and Use Committee of NHGRI under protocol GS98-13.

**RNA purification and reverse transcription.** Total RNA was prepared from brain of 3 littermates of E18.5, each of a different genotype with TRIZOL (Life Technologies, Carlsbad, California). Following DNAse treatment with deoxyribonuclease I, amplification grade (Life Technologies), first-strand cDNA was prepared using First-Strand cDNA synthesis kit (Pharmacia, Piscataway, New Jersey) and the primer 5′-CATAGCGGTGGTGAGAAGGAC-3′ which lies in exon 15 of *Slc23a1*. The amplification of cDNA was carried out by touchdown PCR (1 °C stepwise decrease starting from 75 °C, holding at 63 °C) using primers separated by 7 kilobytes (kb) in genomic DNA (ref. 5) and flanking the deleted segment, which stretches from exon 10a to exon 12 inclusive (Forward primer, 5′-GAGAGCCGGAAAGCACTGG-3′ located in exon 9; reverse primer, 5′-ACCGCCGTGATCATTCCAAAGAGT-3′ located between exons 13 and 14). No products were seen when reverse transcriptase was omitted.

**Fibroblast culture.** Embryonic fibroblasts were extracted from E13.5 mice embryos obtained by cesarean section. Following removal of extra-embryonic tissue and blood-containing embryonic organs, the embryos were rinsed with PBS and minced. Tissue clumps were removed by centrifugation, and fibroblast cultures were established in DMEM containing 15% v/v FBS (Gibco, Carlsbad, California), 1% v/v non-essential amino acids (Biofluids, Rockville, Maryland), 1% v/v penicillin-streptomycin (Biofluids) and 4 mM L-glutamine (Gibco). Cells were cultured at 37 °C in a 5% $CO_2$, 91% humidified atmosphere.

**Ascorbic-acid transport.** Transport assay was performed in triplicate on confluent cells grown on 60 × 15mm polystyrene plates[22]. One cell line of

each genotype (wild type, *Slc23a1*$^{+/-}$ and *Slc23a1*$^{-/-}$) was used. Before each assay, culture medium was removed and cells were washed twice and then incubated for 15 min at 37 °C in Hank's balanced salt solution (HBSS). At the end of the incubation, the HBSS was replaced with 500 µl HBSS containing 100 µM dithiothreitol and 100 µM [$^{14}$C]ascorbic acid (6 mCi/mmol, DuPont-NEN, Boston, Massachusetts). Incubation was carried out at 37 °C for 0, 20, 40 or 60 min. Following incubation, intracellular ascorbic acid was measured by scintillation spectrometry and converted to molar concentrations as described[22]. Data represent mean intracellular ascorbic acid in cell extracts, normalized for total protein. Tests for significance were made by Student's *t*-test on slopes determined by linear regression.

**Ascorbic-acid measurements in blood and tissues.** Blood was collected by suctioning the blood with a 0.9 × 25 mm needle on a 1.00-cc syringe from E18.5 mouse embryos killed by decapitation. Retro-orbital bleeds were performed on adult mice using heparinized capillaries. Blood was gently mixed in 7 U heparin (Elkin-Sinns, Cherry Hill, North Carolina) and kept on ice protected from light until further processing. Vitamin C measurements were made using HPLC with coulometric electrochemical detection, as described[23,24]. Tests for significance were made by Student's *t*-test. For maternal ascorbic-acid supplementation, pregnant females were treated with 2.5 mg/ml ascorbic acid in their drinking water throughout gestation.

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*Competing interests statement*

*The authors declare that they have no competing financial interests.*

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