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LETTER TO THE EDITOR

Loss of the sulfate transporter *Slc13a4* in placenta causes severe fetal abnormalities and death in mice

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Dear Editor,

Sulfate is an obligate nutrient for numerous cellular and metabolic processes important for fetal development [1]. Fetal tissues express sulfotransferases which conjugate sulfate (sulfonate) to molecules such as steroids and hormones, leading to their inactivation [2]. In addition, sulfonation of glycosaminoglycans is important for development of some tissues, as several growth factor gradients and ligand-receptor interactions are dependent on these sulfonated extracellular constituents [3]. Not surprisingly therefore, reduced sulfonation capacity has been linked to disorders of skeletal, eye, vascular and craniofacial development, demonstrating a critical requirement for sulfate in these tissues [4]. Despite a fundamental role for sulfate during development, the fetus appears to have a limited capacity to generate sulfate from sulfur-containing amino acids, and is thought to be reliant on maternal sulfate provision [5]. In fact, maternal serum sulfate levels increase two-fold during pregnancy, and provide a potential reservoir for fetal consumption [6, 7]. The elevated sulfate levels are due to increased sulfate reabsorption in the maternal kidneys, mediated by increased expression of the SLC13A1 sulfate transporter [7]. The importance of maintaining high maternal sulfate levels in pregnancy is highlighted by the hyposulfatemia seen in pregnant *Slc13a1*$^{-/-}$ mice, which causes fetal hyposulfatemia and late gestational fetal loss.

Despite its importance in fetal development, relatively little is known about how sulfate traverses the placenta. The sulfate transporter SLC13A4 is expressed predominantly in the placenta, where it is proposed to mediate sulfate supply to the fetus [8]. Previously we examined the spatial localization of all 10 sulfate transporters in human [9] and mouse placenta [8] and identified *Slc13a4* as the most abundant sulfate transporter localized to the transporting syncytiotrophoblasts. In the current study we describe the critical requirement of placental *Slc13a4* activity for normal fetal development in mice.

We generated *Slc13a4* knockout mice using ES cells sourced from the European Conditional Mouse Mutant Program (EUCOMM) to characterize the role of *Slc13a4* in placental sulfate transport during fetal development. The targeted *Slc13a4* allele (*Slc13a4*$^{tm1a(EUCOMM)Wtsi}$) contains a "knockout first" (KOF) targeting cassette (Supplementary information, Figure S1A), which splices to a *LacZ* trapping element after exon 2, generating a null allele (Figure 1H, Supplementary information, Figure S1A). Crosses between *Slc13a4*$^{+/KOF}$ mice yielded fewer pups per litter (n = 4) compared with litters from *Slc13a4*$^{+/KOF}$ x *Slc13a4*$^{+/+}$ matings (n = 8), and no *Slc13a4*$^{KOF/KOF}$ pups were found at birth, indicating loss of *Slc13a4* is embryonic lethal (Figure 1A). While loss of *Slc13a4* did not result in generalized growth restriction, *Slc13a4*$^{KOF/KOF}$ embryos displayed a variety of developmental abnormalities grossly detectable as early as embryonic day (E)12.5 (Supplementary information, Figure S2A), which became more severe at E14.5 (Supplementary information, Figure S2A) and E16.5 (Figures 1C and Supplementary information, Figure S2A), with fetal death occurring by E18.5. The most striking phenotypes at E16.5 were pale skin, subcutaneous oedema, craniofacial malformations, vascular hemorrhaging and skeletal defects (Figure 1C). We measured unidirectional maternal-fetal transfer of ^{35}S-sulfate as fetal accumulation of radioisotope at E12.5 after injection into the maternal circulation. At E12.5 ^{35}S-sulfate per gram of placenta was significantly lower in *Slc13a4*$^{KOF/KOF}$ embryos compared with *Slc13a4*$^{+/KOF}$ and *Slc13a4*$^{+/+}$ littermate controls (P < 0.001; Figure 1B, Supplementary information, Figure S3A). Additionally we measured elemental sulfur levels (Supplementary information, Figure S3C); *Slc13a4*$^{KOF/KOF}$ embryos exhibited an ~50% reduction in elemental sulfur compared with *Slc13a4*$^{+/KOF}$ and *Slc13a4*$^{+/+}$ littermate controls, in line with a reduced sulfate supply to *Slc13a4*$^{KOF/KOF}$ embryos.

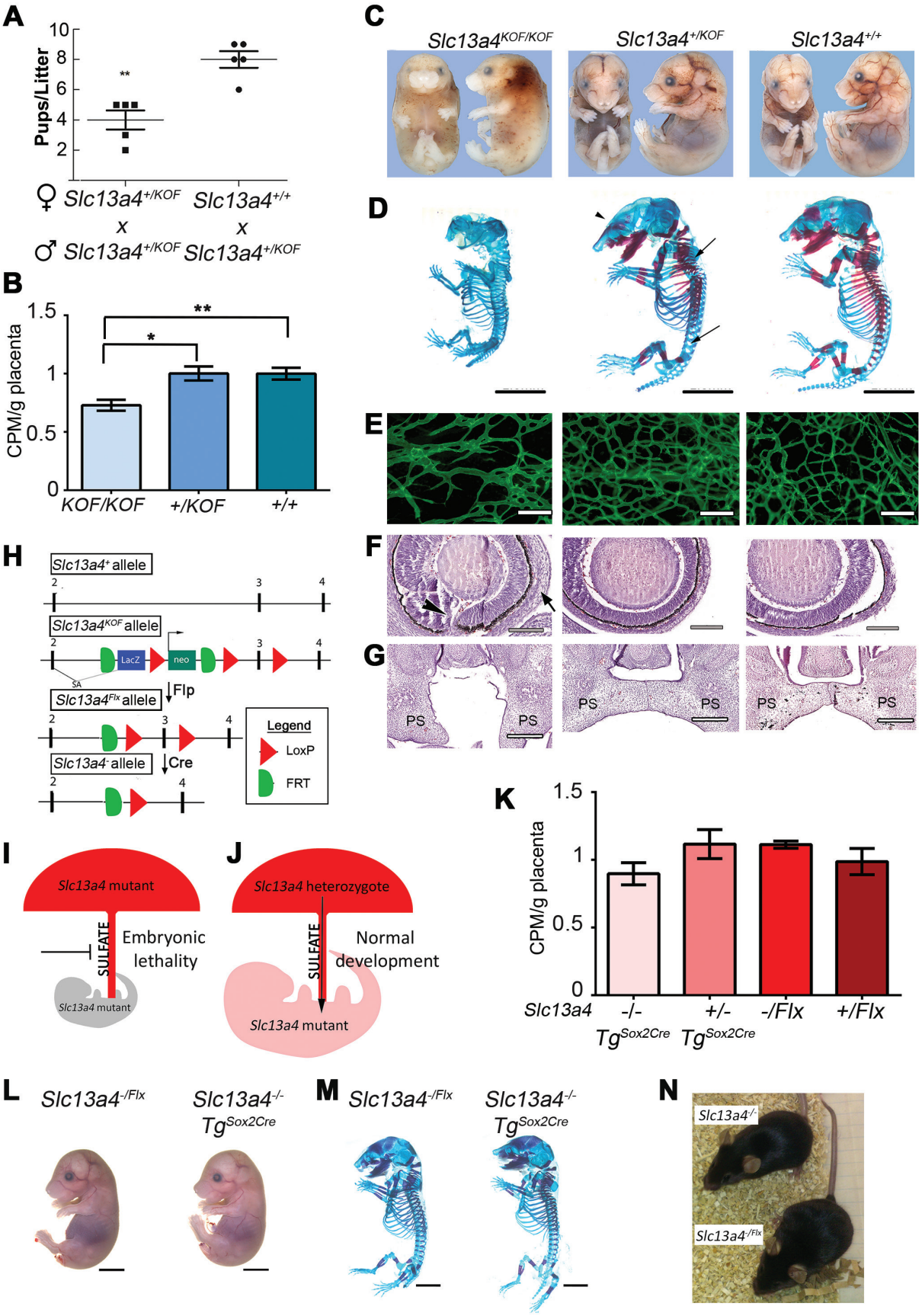
Mutations in genes required for intracellular sulfonation have been extensively studied and linked to developmental phenotypes in mice and humans [10]. For example, the sulfonation of ECM components such as heparin sulfate proteoglycans is critical for growth factor binding and signalling. Mutations within specific heparin

sulfotransferases cause defects in eye morphogenesis [11], skeletal and craniofacial development [11] or affect vascular patterning *in vivo* [12], depending on the organ or tissue the sulfotransferase is endogenously expressed. By contrast, the phenotypes present in *Slc13a4*$^{KOF/KOF}$ embryos affect multiple tissues, including the skeleton (Figure 1D), vasculature (Figure 1E), eye (iris coloboma and increased lens thickness) (Figure 1F) and palate (Figure 1G), all of which have been individually linked to sulfotransferases or sulfate transporter genes (Supplementary information, Table S1). As the phenotype of *Slc13a4*$^{KOF/KOF}$ embryos encompasses those of multiple sulfotransferase mutants, it is therefore likely the reflection of a global reduction in sulfate supply to the embryo via the placenta. Importantly, while the skeletons of *Slc13a4*$^{KOF/KOF}$ embryos displayed a total lack of calcification (alizarin red staining), *Slc13a4*$^{+/KOF}$ skeletons had an intermediate phenotype with reduced calcification compared with wildtype controls (Figure 1D, middle panel), suggesting that haploinsufficiency of *Slc13a4* is also sufficient to perturb or delay fetal skeletal maturation.

While the placenta expresses a multitude of transporters and channels to facilitate the supply of nutrients such as ions, amino acids, fatty acids, minerals, and glucose to the fetus, studies of gene knockout mice often overlook the contribution of placental supply when investigating the role of individual transporters in fetal development and adult disease. Importantly, the development of placentas from *Slc13a4* null embryos appeared normal throughout gestation (Supplementary information, Figure S4). This is an important observation as defects in placental development are known to cause fetal growth restriction [13]; we do not see a generalized growth restriction in *Slc13a4*$^{KOF/KOF}$ or *Slc13a4*$^{-/-}$ embryos. Normal development of *Slc13a4*$^{KOF/KOF}$ placentas therefore suggests that the tissue-specific embryonic phenotypes are the result of reduced placental SLC13A4 transport activity rather than generalized placental insufficiency and growth restriction as a result of aberrant placental development.

To confirm that the developmental phenotypes and late gestational lethality observed in *Slc13a4* null embryos are due to loss of placental *Slc13a4* activity, and not due to embryonal tissue-specific requirements for *Slc13a4*, we generated an *Slc13a4* conditional knockout allele by mating *Slc13a4*$^{+/KOF}$ mice with *FLPeR*-expressing mice to produce a conditional *Slc13a4* "floxed" allele (*Slc13a4*$^{+/Flx}$; Figure 1H, Supplementary information, Figure S1A). The conditional allele, when deleted globally through the maternal inheritance of the *Sox2*-Cre transgene (Tg) [14], produced the same phenotypes as seen with the *Slc13a4*$^{KOF/KOF}$. Furthermore, crosses of resultant

Figure 1 Loss of placental *Slc13a4* results in multiple developmental defects and embryonic lethality. **(A)** Litter sizes at P0.5 for *Slc13a4*$^{+/KOF}$ females mated with *Slc13a4*$^{+/KOF}$ males (n = 5 litters, 20 mice) were significantly smaller than for *Slc13a4*$^{+/+}$ females (n = 5 litters, 39 mice), $^{**}P < 0.01$; students t test **(B)** Placental transfer of ^{35}S-sulfate was significantly less in *Slc13a4*$^{KOF/KOF}$ embryos compared with *Slc13a4*$^{+/KOF}$ and *Slc13a4*$^{+/+}$ littermate controls (4 litters at E12.5: *KOF/KOF* n = 9; *+/KOF* n = 12; *+/+* n = 8. Error bars indicate ± SEM, * $P < 0.01$, $^{**}P < 0.001$; One-Way ANOVA with Tukey's test (*post hoc*) for multiple comparison. **(C)** Gross morphology of embryos at E16.5. Severe phenotypes were observed in *Slc13a4*$^{KOF/KOF}$ embryos, such as eye defects, oedema, pale appearance, and vascular hemorrhaging in the thoracic region. **(D)** E16.5 skeletons were stained with alcian blue for cartilage and alizarin red for mineralized bone. *Slc13a4*$^{KOF/KOF}$ embryos have multiple skeletal defects in the axial skeleton as well as the craniofacial region (left panel). *Slc13a4*$^{+/KOF}$ embryos (middle panel) exhibit an intermediate phenotype with reduced mineralization in areas of the axial skeleton (arrows) and regions of the skull (arrowhead) and have a disorganized rib cage. Scale bar, 1.0 mm, n = 4 for each genotype. **(E)** Wholemount ENDOMUCIN immunofluorescence on E14.5 embryo back skin revealed thickened vessels and a decrease in complexity of the vascular network in *Slc13a4*$^{KOF/KOF}$ embryos. Scale bar, 20 μm, n = 4 for each genotype. **(F)** *Slc13a4*$^{KOF/KOF}$ E14.5 embryos exhibit iris coloboma (left panel, arrowhead) and a thickened lens (arrow), scale bar, 10 μm, n = 4 for each genotype. **(G)** *Slc13a4*$^{KOF/KOF}$ embryos have craniofacial defects and cleft palate at E14.5, Scale bar, 100 μm. PS - palatal shelves, n = 4 for each genotype. **(H)** Schematic for generating the *Slc13a4*^{Flx} allele from the *Slc13a4*^{KOF} targeting allele (using *FLPeR* mice), and the subsequent generation of the *Slc13a4*$^{-}$ allele (using *Sox2-Cre* mice) **(I)** Loss of *Slc13a4* (either *Slc13a4*$^{KOF/KOF}$ or *Slc13a4*$^{-/-}$ alleles) in both placenta and embryo results in embryonic lethality. **(J)** Loss of *Slc13a4* in the embryo, but not the placenta (through epiblast-specific *Sox2*-Cre deletion of the *Slc13a4*^{Flx} allele) results in normal embryonic development (See also Supplementary information, Figure S6). **(K)** Fetal accumulation of ^{35}S-sulfate was not significantly different in *Slc13a4*$^{-/-}$*Tg*^{Sox2Cre} (n = 7) embryos compared with *Slc13a4*$^{-/+}$*Tg*^{Sox2Cre} (n = 3), *Slc13a4*$^{-/Flx}$ (n = 5) and *Slc13a4*$^{Flx/+}$ (n = 6) littermate controls. Total of three litters at E12.5, bars ± SEM, P = 0.8400, One-Way ANOVA with Tukey's test (posthoc) for multiple comparisons. See also Supplementary information, Figure S3. **(L)** Rescue of previously observed phenotypes in *Slc13a4*$^{-/-}$*Tg*^{Sox2Cre} embryos at E16.5 due to the retention of an *Slc13a4*$^{-/Flx}$ placenta (Scale bar, 1.0 mm) **(M)** Whole skeletal preparations of *Slc13a4*$^{-/-}$*Tg*^{Sox2Cre} embryos at E16.5 demonstrate the rescue of skeletal development in *Slc13a4*$^{-/-}$ embryos with an *Slc13a4*$^{-/Flx}$ placenta (Scale bar, 1.0 mm) **(N)** *Slc13a4*$^{-/-}$ mice from *Slc13a4*$^{Flx/Flx}$ X *Slc13a4*$^{-/+}$*Tg*^{Sox2Cre} crosses survive to adulthood and are indistinguishable from *Slc13a4*$^{-/Flx}$ littermate controls (See Supplementary information, Table S2 and Figure S1).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Slc13a4+ allele
Slc13a4KOF allele
LacZ
neo
SA
Flp
Slc13a4Flx allele
Cre
Slc13a4- allele
Legend
LoxP
FRT
I
Slc13a4 mutant
SULFATE
Embryonic lethality
Slc13a4 mutant
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Slc13a4 heterozygote
SULFATE
Normal development
Slc13a4 mutant
K
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1
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Slc13a4-/-
TgSox2Cre
M
Slc13a4-/Flx
Slc13a4-/-
TgSox2Cre
N
Slc13a4-/-
Slc13a4-/Flx

Slc13a4$^{+/-}$ mice also yielded *Slc13a4*$^{-/-}$ embryos with the same phenotypes as *Slc13a4*$^{KOF/KOF}$ embryos (Supplementary information, Figure S2B). We next mated homozygous floxed *Slc13a4* female mice with male *Sox2*-Cre deleter mice (*Slc13a4*$^{-/+}$*Tg*^{Sox2Cre} male X *Slc13a4*$^{Flx/Flx}$ female) in order to delete *Slc13a4* exon 3 within the embryo, while retaining placental *Slc13a4* expression; in *Slc13a4*$^{-/-}$*Tg*^{Sox2Cre} embryos (Figure 1I, J, Supplementary information, Figure S5 and S6) *Sox2*-driven Cre recombinase expression/activity is restricted to the embryonic epiblast, but is absent from extra-embryonic tissues such as the syncytiotrophoblast where *Slc13a4* is endogenously expressed. Litters from *Slc13a4*$^{-/+}$*Tg*^{Sox2Cre} x *Slc13a4*$^{Flx/Flx}$ crosses were analyzed on the day of birth (P0.5) and all the resultant pups were genotyped at postnatal day (P)8. *Slc13a4*$^{-/-}$*Tg*^{Sox2Cre} pups were present within each litter examined from these crosses (Supplementary information, Table S2). At E12.5 we measured unidirectional maternal-fetal transfer of ^{35}S-sulfate in litters from *Slc13a4*$^{-/+}$*Tg*^{Sox2Cre} x *Slc13a4*$^{Flx/Flx}$ crosses. There was no significant difference in fetal accumulation of ^{35}S-sulfate per gram of placenta between *Slc13a4*$^{-/-}$*Tg*^{Sox2Cre}, *Slc13a4*$^{-/+}$*Tg*^{Sox2Cre}, *Slc13a4*$^{-/Flx}$ and *Slc13a4*$^{+/Flx}$ littermate controls ($P = 0.840$; Figure 1K, Supplementary information, Figure S3B). *Slc13a4*$^{-/-}$*Tg*^{Sox2Cre} mice from these crosses were indistinguishable from heterozygous littermate controls and analysis of *Slc13a4*$^{-/-}$*Tg*^{Sox2Cre} embryos at E16.5 demonstrated the rescue of all previously observed phenotypes when *Slc13a4* expression was maintained in the placenta (Figure 1L, M). Importantly *Slc13a4*$^{-/-}$*Tg*^{Sox2Cre} mice survive through to adulthood (Figure 1N). The rescue of severe and lethal embryonic defects in *Slc13a4*$^{-/-}$*Tg*^{Sox2Cre} embryos when *Slc13a4* expression is retained in the placenta, but not the embryo itself, demonstrates the dependence of the fetus on access to circulating maternal sulfate stores via the placenta for normal development. These findings also confirm that the fetus cannot produce enough sulfate from the metabolism of sulfur-containing amino acids to sustain development. To date the role of placental sulfate supply in the development of fetal tissues with a high demand for sulfate metabolism and sulfonation reactions has not been widely considered. The current study highlights a placental contribution to sulfate-dependent fetal abnormalities, and warrants further investigation of placental sulfate transport in human gestation.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)