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Glutathione is essential for early embryogenesis – Analysis of a glutathione synthetase knockout mouse

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ABSTRACT

Glutathione (GSH) is present in all mammalian tissues and plays a crucial role in many cellular processes. The second and final step in the synthesis involves the formation of GSH from gamma-glutamylcysteine (γ-GC) and glycine and is catalyzed by glutathione synthetase (GS). GS deficiency is a rare autosomal recessive disorder, and is present in patients with a range of phenotypes, from mild hemolytic anemia and metabolic acidosis to severe neurologic disorders or even death in infancy. The substrate for GS, γ-GC, has been suggested as playing a protective role, by substituting for GSH as an antioxidant in GS deficient patients. To examine the role of GS and GSH metabolites in development, we generated mice deficient in GSH by targeted disruption of the GS gene (*Gss*). Homozygous mice died before embryonic day (E) 7.5, but heterozygous mice survived with no distinct phenotype. GS protein levels and enzyme activity, as well as GSH metabolites, were investigated in multiple tissues. Protein levels and enzyme activity of GS in heterozygous mice were diminished by 50%, while GSH levels remained intact. γ-GC could not be detected in any investigated tissue. These data demonstrate that GSH is essential for mammalian development, and GSH synthesis via GS is an indispensable pathway for survival.

1. Introduction

The tripeptide glutathione (γ-glutamyl-cysteinyl-glycine GSH) is present in all mammalian tissues and is an important antioxidant which participates in an array of crucial cellular processes [1–3]. Intracellular levels of GSH vary between 1–10 mM, with the highest levels present in the liver [4]. Decreased cellular levels of GSH have been observed in a number of diseases such as diabetes, cancer, and HIV infection in which increased oxidative stress has been implicated as the pathogenic mechanism [5].

GSH is metabolized by the six enzymes in the γ-glutamyl cycle [1]. The first of the two steps in the synthesis is rate-limiting and catalyzed by the enzyme glutamate-cysteine ligase (GCL; E.C. 6.3.2.2). In the second step, glutathione synthetase (GS; E.C. 6.3.2.3) adds a glycine residue to the dipeptide. GS is a dimer of two identical subunits with a subunit molecular weight of 52 kD, encoded by the unique glutathione synthetase gene localized on chromosome 20q11.2 [6] in humans (*GSS*) and on chromosome 2 H1 in mice (*Gss*) [7–9].

To date, 77 patients in 65 families with hereditary glutathione synthetase deficiency (GS deficiency) have been described clinically and genetically [1,10–13]. The disease is inherited in an autosomal recessive manner. Approximately 25% of the reported patients have died during the first year of life, whereas the others, during childhood, presented with clinical phenotypes spanning from isolated hemolytic anemia to additional metabolic acidosis and progressive dysfunction of the central nervous system [1]. Based on the clinical phenotype of the patients, the disease is classified as mild, moderate, or severe [14]. This considerable phenotypic variation is likely to reflect both genetic and environmental factors. We have previously reported genotype–phenotype correlation [12]. The GS activity in fibroblasts from different patients varies, and we have found children who are severely affected but survive in spite of undetectable GS enzyme levels by Western blot and less than 2% of control enzyme activity in fibroblasts [12,14,15]. Gamma-glutamylcysteine (γ-GC) protects against

Abbreviations: GCL, glutamate-cysteine ligase; GS, glutathione synthetase; γ-GC, gamma-glutamylcysteine; ES, embryonic stem cell; E, embryonic day; *Gss*, the mouse gene encoding the GS subunit; *GSS*, the human gene encoding the GS subunit; GGT, γ-glutamyltransferase; DTPA, diethylenetriaminepentaacetic acid; TBS, Tris-buffered saline.

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oxidative stress in yeast [16] and, since this dipeptide as well as cysteine accumulates in fibroblasts of patients with GS deficiency, we have proposed that γ-GC, to some extent, may substitute for GSH, thereby possibly explaining the survival of these patients [17]. This proposal is also supported by the fact that GCL deficiency is a rarer and more severe disease because these patients are deficient in both γ-GC and GSH.

Here we report the generation and analysis of a *Gss* null mutation in mice with respect to embryonic development, gene expression, and glutathione metabolism. Our study demonstrates that the null mutation causes early embryonic lethality, suggesting that the role of GSH in early embryogenesis is indispensable.

2. Materials and methods

The study protocol was approved by the Karolinska Institutet Ethic Board, and all experiments and animal handling were in accordance with the requirements of national authorities.

2.1. Generation of GS deficient mice

A clone containing exons 6 through 15 of Gss was isolated from a λ DNA library of the 129SvEv mouse strain (Stratagene). A pKO Scrambler NTKV-1903 targeting vector (Stratagene) was constructed to delete a 4.3 kb fragment of *Gss* encompassing exons 9–11. The vector contained a 2.6 kb 5′arm of homology, a 3.1 kb 3′arm of homology flanking a neo cassette (positive selection), and a TK gene (negative selection) (Fig. 1A). Sixty microgram of the vector were linearized with Sac I (New England Biolabs, NEB) and electroporated into the embryonic stem cell (ES) line RW-4wt derived from the 129/SvJ mouse strain at the Karolinska Centre for Transgene Technologies (KCTT), Karolinska Institutet, Stockholm, Sweden.

Following electroporation, clones were screened with G418 (positive selection) and gancyclovir (negative selection). The identified clones were digested with BamHI (NEB) and analyzed by Southern blot using a biotin labeled 5′ probe (BrightStarTM Psoralen-Biotin Nonisotopic Labeling Kit, Ambion) and SouthernMax and BrightStarTM Bio Detect Nonisotopic Detection Kit (Ambion) (Fig. 1A). In addition, Southern blot analysis with a neo probe was used to exclude multiple integration (data not shown). Clones positively tested for integration were injected into blastocysts from the C57BL/6 mouse strain which, in turn, were transferred into pseudo-pregnant females from the C57Bl/6 NCrl mouse strain. Resulting chimeras were identified by partial or complete agouti coat-color. A chimeric male was bred with C57BL/6 females, and agouti-colored offspring were screened by Southern blot analysis for germ line transmission of the targeted GS construct. Transgenic manipulation of the ES cells was performed at Karolinska Centre for Transgene Technologies, Karolinska Institutet, Stockholm, Sweden, and subsequent breeding at the animal facility, Karolinska University Hospital, Huddinge, Sweden.

2.2. Genotyping of pups and embryos by Southern blot and PCR

First generation (F1) offspring were screened by Southern blot, as described above, using 5 μg of tail DNA (Fig. 1B). PCR was used to screen offspring (F2–F5) and embryos at embryonic day (E) E7.5–E12.5 using three different primers (Fig. 1A): 5′-TTAAGATAGGGTCTCAGGTAAC-3′ (forward), 5′-CTGTCTAGCAGCTCGTTCTC-3′ (reverse), and 5′-AACTGTTCGCCAGGCTCAAG-3′ (reverse). The PCR conditions were 95 °C for 30 s for denaturation, 55 °C for 30 s for annealing, and 72 °C for 5 min for elongation, using DNA Polymerase (DyNAzyme EXT™, Finnzymes) in a Peltier Thermal Cycler (PTC-0200, MJ Research, Inc.).

Heterozygous mice were mated for timed pregnancies with noon of the day of the vaginal plug considered as E0.5. Females were killed by cervical dislocation, and embryos were genotyped by PCR at E7.5, E8.5, E9.5, E10.5, E11.5, and E12.5.

2.3. Production of polyclonal antibodies

Polyclonal antibodies towards human GS were produced by immunization of rabbits with human wild-type GS [15]. The antiserum consistently recognized a single band of expected size (52 kD) in Western blot analyses of lysates from human and mouse tissues as well as recombinant human GS [11].

2.4. Preparation of tissue samples

Mice were killed by carbon dioxide asphyxiation at 10 weeks of age. Organs were perfused with phosphate buffered saline (PBS) and immediately frozen in liquid nitrogen and stored at −80 °C until analysis. All of the following work was performed at 4 °C.

2.5. Western blot

Fifty to hundred milligram of tissue were homogenized with a polytron in 1 ml of lysis buffer containing Tris/HCl (20 mM pH 8.0), NaCl (137 mM), 10% glycerol, 1% Nonidet P-40, EDTA (2 mM), and a Complete Protease Inhibitor Cocktail tablet, according to the manufacturer's instructions (Roche). The homogenates were constantly agitated for 2 h and then centrifuged for 30 min at 16,000*g*. Protein concentration was determined by Bradford assay (Bio-Rad). *Samples containing 40 μg of cellular protein were analyzed by electrophoresis on a 9% polyacrylamide gel.* GS was detected using the specific polyclonal GS antiserum and visualized by Enhanced ChemiLumincence Western blotting reagents (Amersham Pharmacia Biotech). *Beta-actin (Abcam) was used as an internal loading control.*

2.6. Enzyme activity

GS activity in liver, kidney, spleen, and brain homogenates was analyzed as described [11].

2.7. Thiol content and indicators of oxidative stress in tissues

Tissues of kidney, liver, spleen, and brain were homogenized in serine borate buffer (100 mM Tris/HCl, 10 mM boric acid, 5 mM L-serine, and 1 mM DTPA (diethylenetriaminepentaacetic acid), pH 7.4). GSH, glutathione disulfide (GSSG), and cysteine (Cys) were measured as described [18].

2.8. Immunohistochemistry

Eight-week old mice were euthanized by carbon dioxide asphyxiation. Organs were placed in Accustain Bouin's solution (Sigma–Aldrich) overnight for fixation and routinely processed for paraffin embedding. Paraffin sections were deparaffinised in xylene and rehydrated through graded alcohol to H_2O. For antigen retrieval, sections were boiled in citric acid buffer (0.01 M, pH 6.0) for 10 min in a microwave oven. Endogenous peroxidase activity was quenched with 3% H_2O_2 in methanol for 10 min. To prevent non-specific binding, sections were incubated with 4% normal goat serum (DakoCytomation) in Tris-buffered saline (TBS), followed by the Vector avidin/biotin blocking kit (Vector Laboratories, Inc). Sections were incubated overnight at 4 °C in anti-GS antiserum. Control experiments were performed by pre-incubating the primary antibody with the recombinant GS protein. A biotinylated antibody derived from goat was used as a secondary antibody. Incubation in

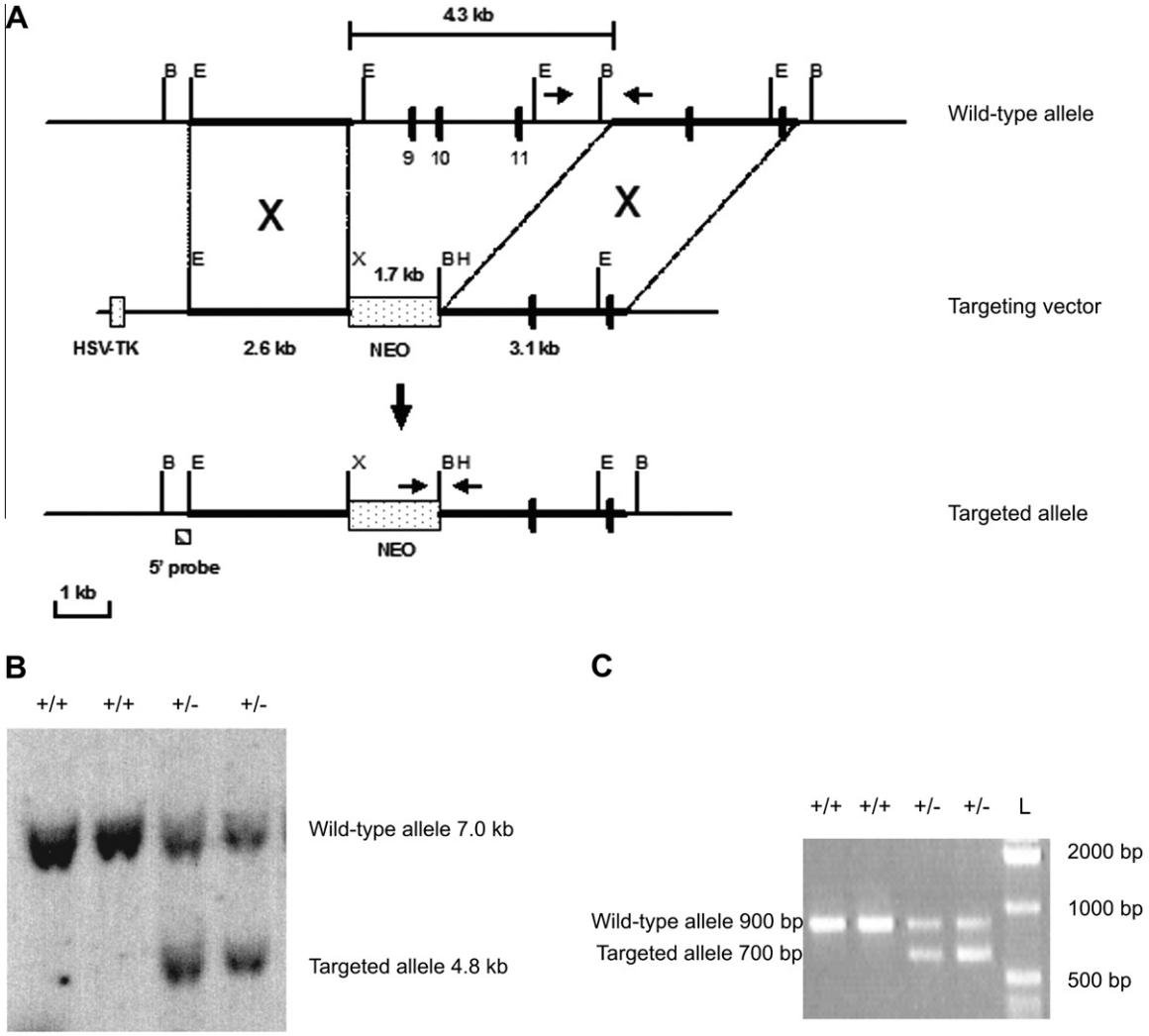

Fig. 1. Targeting scheme of the *Gss* locus and genotyping by Southern blot and PCR. (A) A 4.3 kb fragment, encompassing exon 9–11, was replaced by a neo cassette (positive selection marker) and a HSV-TK gene was used as a negative selection marker. A 5′external probe (slashed box) was used for Southern blot analysis of BamHI digested DNA extracted from ES cells or mouse-tail. The arrows show the three different primers which were used for PCR genotyping of embryos mouse-tail DNA. The forward primer is located inside the 3′arm of homology and the reverse primers are located inside the knock-out region and inside the neo cassette, respectively. Restriction sites: E, EcoRI; B, BamHI; H, HindIII; X, XhoI. (B) Southern blot analysis with a 5′external probe after BamHI digestion of mouse-tail DNA derived from heterozygote mating: +/−, heterozygotes; +/+, homozygotes for the wt allele. The wt allele gives rise to a 7.0 kb band and the targeted allele to a 4.8 kb band. (C) PCR genotyping of mouse-tail DNA and embryos: +/−, heterozygotes; +/+, homozygote for the wt allele; L, 1000 bp DNA ladder. The wt allele is represented by a 900 bp band and the targeted allele by a 700 bp fragment.

StreptABComplex/HRP (DakoCytomation) was followed by incubation in DAB Chromogen (DakoCytomation) to visualize antibody binding. Finally, sections were counterstained with Mayer Hematoxylin (Histolab Products AB), dehydrated through graded alcohol to xylene, and mounted in Pertex (Histolab Products AB).

2.9. Statistical methods

Data were analyzed employing the Statistical Package for Social Sciences, version 14 (SPSS, Chicago, USA).

3. Results

3.1. Generation of $Gss^{+/-}$ mice

A mouse genomic DNA clone, covering exons 6–15 of *Gss*, was isolated and a targeting vector was constructed to delete exons 9–11 that encode the substrate binding sites and catalytic site (Fig. 1A). After electroporation into mouse ES cells, clones were screened by positive and negative selection (Fig. 1A). Five of 260 clones had undergone homologous recombination, as analyzed by Southern blot. Multiple integration was excluded by the use of a neo probe (data not shown). Two of the five targeted ES cell clones were separately microinjected into C57BL/6 blastocysts and gave rise to twelve chimeras with a high contribution of ES cell derived agouti pigmentation to the coat-color. One of seven chimeric males was mated with C57BL/6 females, yielding 16 pups, 11 heterozygous, and five homozygous for the wild-type allele, as determined by Southern blot (Fig. 1B). Heterozygous $Gss^{+/-}$ offspring showed no differences in the morphological phenotype compared to wild-type $Gss^{+/+}$ mice.

3.2. Loss of both Gss alleles results in early embryonic lethality

The heterozygous $Gss^{+/-}$ offspring (F1–F5) were intercrossed giving rise to 203 mice. While 128 (63%) were heterozygous and 75 (37%) were wild-type, none was homozygous for the targeted allele indicating that homozygosity for the null mutation caused embryonic lethality.

Table 1
Genotype analysis of offspring from heterozygous intercross at different days of embryogenesis.

Day of embryogenesis	Total amount	Wild-type (+/+)	Heterozygous (+/−)	Homozygous (−/−)	Absorbed or too little material
E 7.5	48	5	25	0	18
E 8.5	30	9	13	0	8
E 9.5	9	1	7	0	1
E 10.5	17	4	5	0	8
E 11.5	9	0	6	0	3
E 12.5	13	1	4	0	8
	126	20	60	0	46

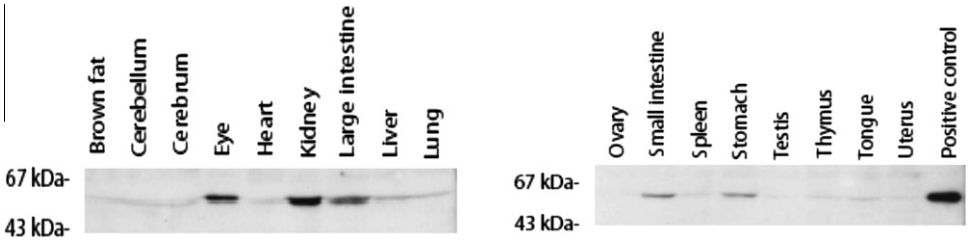

Fig. 2. Western blot analysis of GS protein levels in different tissues of wild-type $Gss^{+/+}$ mice. Western blot analysis was performed with 40 μg of GS protein. GS (52 kD) could be detected in different intensities with the strongest signals in kidney, large intestine and eye.

Embryos from heterozygous intercrossing were examined at different days of embryogenesis (E7.5–E12.5) (Table 1). Genotypes were determined by PCR (Fig. 1C). Ninety-one embryos appeared normal: 60 were heterozygous (+/−), and 20 were wild-type (+/+). Eleven embryos could not be genotyped because too little material was available. Thirty-five embryos were absorbed: 22 were heterozygous (+/−), two were wild-type (+/+), and 11 could not be genotyped. No homozygous $Gss^{-/-}$ embryo could be detected on any examined day of embryogenesis (Table 1). Thus, the data indicate that homozygous loss of GS results in failure to gastrulate before E7.5.

3.3. GS expression in tissues from wild-type and heterozygous mice

Immunohistochemistry analysis of different tissues from 10-week old heterozygous $Gss^{+/-}$ and wild-type $Gss^{+/+}$ mice showed strong reactivity to GS, mainly in epithelial cells of the large intestine, parts of the stomach mucosa, liver, bronchial epithel, and kidney. There was no obvious change in GS distribution between the different tissues of heterozygous and wild-type mice.

3.4. GS protein and GS activity in heterozygous $Gss^{+/-}$ mice

Using a polyclonal antiserum raised against human GS, western blot analysis of various tissues from wild-type $Gss^{+/+}$ mice showed highest expression of GS protein concentration in kidney, large intestine, and eye (Fig. 2). A decrease of approximately 50% in GS protein was revealed in liver and kidney of $Gss^{+/-}$ mice when compared with wild-type $Gss^{+/+}$ littermates (Fig. 3). In addition, GS activity was directly measured in liver, kidney, brain, and spleen of these animals; and the enzyme activity was diminished by approximately 50% in heterozygotes (Table 2).

3.5. GS enzyme activity, γ-glutamyl cycle metabolites, and indicators of oxidative stress

The consequences of targeted inactivation of one allele of the GS gene were investigated in tissue samples in terms of enzyme activity and levels of biochemical indicators of glutathione metabolism and oxidative stress. GS enzyme activity and GSH, GSSG, and Cys were measured in tissue samples from liver, brain, and spleen. In kidney, GSSG could not be detected. Both the enzyme activity and the biochemical indicators varied according to tissue type and genotype (Friedman, $P < 0.001$, Tables 2 and 3). Heterozygous $Gss^{+/-}$ mice exhibited approximately 50% decreased enzyme activity in all tissues (Mann–Whitney, $P < 0.002$, Table 2). In heterozygous $Gss^{+/-}$, GSSG was decreased in brain (T-test, $P = 0.04$) but not in liver and spleen (undetectable in kidney). In contrast, Cys and GSH did not vary according to *Gss* genotype in any tissue (Table 2). There were no variations in the enzyme activity and biochemical indicators according to sex. γ-GC could not be detected in any tissue (lower limit of detection of thiols: 60 fmol [18]).

4. Discussion

We have developed a transgenic mouse model for human GS deficiency in order to establish the pathophysiology of this disease and to gain a wider perspective of the complex functions of GSH. We decided to knock-out exons 9–11 of the mouse gene, since most of our GS deficient patients have mutations in the corresponding human exons (i.e., exons 6–8). These exons encode the substrate binding sites and catalytic site of the enzyme [11,19] and we, thus, expected to obtain a null *Gss* allele. Embryos, homozygous for the inactivated allele, failed to gastrulate before E7.5, while heterozygotes were born at a normal ratio to homozygous wild-type $Gss^{+/+}$ mice and with no obvious phenotype in an environment without any increased oxidative stress. This is partly at odds with our previous findings in humans where GS deficiency is a recessive disorder affecting patients postnatally, and some patients survive with mutations which were postulated to be null mutations [14]. The embryonic lethality argues against our previous hypothesis that increased levels of γ-GC in GS deficient patients may substitute for GSH, e.g. in defense against oxidative stress. A possible explanation for the survival of homozygous patients, in contrast to homozygous $Gss^{-/-}$ mice, could be that in humans there is residual activity of the GS enzyme, which is sufficient to permit some synthesis of GSH, while the null mutation in our experiment leads to a complete lack of GSH in homozygous $Gss^{-/-}$ mice.

The findings that heterozygous mice had an approximate 50% decrease in the amounts of GS protein (Fig. 3) and enzyme activity (Table 2) confirm that the *Gss* gene is unique. The ability or need

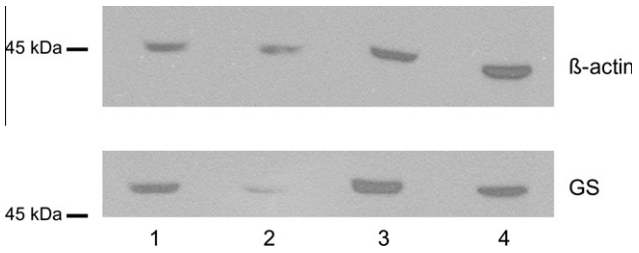

Fig. 3. Western blot analysis of GS protein levels in kidney and liver from heterozygous $Gss^{+/-}$ and wild-type $Gss^{+/+}$ mice. Western blot analysis was performed with 40 μg of GS protein. In $Gss^{+/-}$ mice a decrease of approximately 50% of GS protein in liver and kidney can be detected. (1) Liver, wild-type $Gss^{+/+}$; (2) liver, heterozygous $Gss^{+/-}$; (3) kidney, wild-type $Gss^{+/+}$; (4) kidney, heterozygous $Gss^{+/-}$.

for up-regulation of the expression of the remaining intact allele is limited, although the decrease in tissue levels of GSH is not significant in heterozygous $Gss^{+/-}$ mice.

Similar to the heterozygous mice, parents and siblings of patients are reported to be normal, although they have not been thoroughly investigated. The results in the present study were all obtained under environmental conditions without any additive oxidative stress, e.g., drugs and infections. The heterozygous $Gss^{+/-}$ mice appear healthy but may show symptoms when exposed to oxidative stress exceeding the normal range.

Interestingly, γ-GC was undetectable in all investigated tissues. Previously we found elevated levels of γ-GC compared to controls in fibroblasts of patients with homozygous GS deficiency [17]. The undetectable levels of γ-GC in the present study could be explained by the hypothesis that 50% of rest activity of the GS enzyme is sufficient to produce GSH without accumulating γ-GC. Vice versa, in homozygous patients with up to 2% of GS enzyme activity the blocked pathway leads to the previously described γ-GC accumulation in fibroblasts [17]. The relationship between the levels of GSH, GSSG, γ-GC, and Cys and the Gss gene state needs further investigation.

The GSH concentration in kidney was below the detection level of the present study. This may be explained by the observation that kidney has the highest capacity of all tissues to degrade GSH by γ-glutamyltransferase (GGT) [20], or post mortem degradation of the samples in spite of the precaution described in Section 2. This finding is in agreement with previously reported low GSH content in kidney [21]. In addition, the kidneys are unique in possessing the coexistence of carriers that can facilitate the uptake of intact GSH and pathways for GSH synthesis, degradation, and efflux [22].

In agreement with the present study, mouse embryos homozygous for interruption of the catalytic subunit of GCL (GCLC), i.e., the enzyme catalyzing the first step in the synthesis of GSH, die before E8.5 [23,24]. Similar to the $Gss^{+/-}$ mice, heterozygous GCLC mice appear fertile and healthy, and the small decrease in GSH levels did not result in a compensatory up-regulation of the expression of the GCLC gene.

Glutathione is a natural antioxidant in both male and female gametes and is crucial in the fertilization process and early embryo development [25]. Glutathione levels increase during maturation of oocytes and subsequently decrease by 90% during early embryo development in blastocysts, in comparison to concentrations in mitotic stage oocytes [25,26]. The mechanism for this may involve ATP-dependent *de novo* synthesis during oocyte maturation, which is switched off after fertilization [26,27]. During subsequent development, the embryo is exposed to increased oxidative stress that exceeds the antioxidative defense, resulting in a decrease in the GSH:GSSG ratio [28]. The expected initiation of GSH synthesis in

Table 2
GS activity in isolated tissue samples. The enzyme activity varied according to tissue (Friedman, $P < 0.001$) and genotype (Mann–Whitney, $P < 0.002$). The variation according to sex was inconsistent.

Genotype	*N*	Sex		Kidney GS pkatal/mg protein	Liver GS pkatal/mg protein	Brain GS pkatal/mg protein	Spleen GS pkatal/mg protein
+/+	4	♂	Median	418.0	70.8	35.1	19.5
			Range	365.5–490.7	62.8–81.0	25.3–40.6	16.8–20.5
+/+	4	♀	Median	290.0	98.1	37.1	15.3
			Range	259.3–318.8	70.7–107.3	29.3–40.5	12.8–17.8
+/−	4	♂	Median	192.8	30.1	25.0	6.9
			Range	151.7–198.6	21.1–40.9	20.5–28.1	6.2–10.5
+/−	4	♀	Median	149.2	32.3	20.5	9.9
			Range	138.3–178.2	31.1–32.5	17.7–22.1	7.3–11.5
Total	16	8/8					

Table 3
Biochemical indicators of glutathione metabolism in isolated tissue samples. GSH (glutathione), Cys (cysteine), GSSG (glutathione disulfide). Glutathione, Cys, and GSSG were measured as indicators of glutathione metabolism. All varied in a tissue specific manner (Friedman $P < 0.001$). Gamma-glutamylcysteine (γ-GC) was undetectable in all tissues. There was no significant variation according to sex. In heterozygotes, GSSG was decreased in brain (*T*-test, $P = 0.04$), but not in liver and spleen. The levels of Cys and GSH did not vary between the phenotypes.

Genotype	*N*	Sex		Liver			Brain			Spleen		
				GSH nmoles/mg protein	Cys nmoles/ mg protein	GSSG nmoles/mg protein	GSH nmoles/mg protein	Cys nmoles/mg protein	GSSG nmoles/mg protein	GSH nmoles/mg protein	Cys nmoles/mg protein	GSSG nmoles/mg protein
+/+	4	♂	Median	38.3	0.49	0.16	15.4	3.1	2.3	12.3	1.2	21.0
			Range	32.2–45.7	0.40–0.53	0.13–0.19	13.4–18.9	2.2–4.1	0.70–2.4	11.0–14.4	0.66–1.5	15.6–25.7
+/+	4	♀	Median	38.3	0.36	5.7	14.5	2.7	1.4	8.0	0.33	14.1
			Range	36.4–40.2	0.23–0.47	2.3–9.9	12.6–16.0	2.0–3.0	0.88–1.8	6.0–11.7	0.14–0.64	10.4–15.5
+/−	4	♂	Median	35.3	0.45	0.15	14.5	2.8	1.1	12.4	0.86	16.8
			Range	28.7–46.5	0.41–0.47	0.14–0.17	12.9–15.4	2.4–4.2	0.66–1.5	9.0–18.0	0.27–1.0	11.8–28.8
+/−	4	♀	Median	36.8	0.33	3.5	14.4	2.5	0.77	7.3	0.41	13.5
			Range	35.2–37.8	0.23–0.58	2.3–5.5	13.9–14.8	2.2–2.7	0.08–1.7	6.2–10.0	0.17–0.61	4.9–14.1
Total	16	8/8										

mice occurs late on day E3.0 [27]. Thus, the present data indicate that GSH is essential for early development, and that a block of GSH synthesis by GS deficiency is not compatible with embryonic survival in $Gss^{-/-}$ mice.

In conclusion, the present study confirms and extends the evidence that GSH is essential for early embryo development, and indicates that homozygous interruption of Gss cannot be compensated by, for example, an accumulation of γ-GC. Furthermore, heterozygous inactivation leading to decreased enzyme levels and activities is apparently not harmful during embryogenesis.

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