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Transgenic overexpression of glutathione *S*-transferase μ-type 1 reduces hypertension and oxidative stress in the stroke-prone spontaneously hypertensive rat

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Background: Combined congenic breeding and microarray gene expression profiling previously identified glutathione *S*-transferase μ-type 1 (*Gstm1*) as a positional and functional candidate gene for blood pressure (BP) regulation in the stroke-prone spontaneously hypertensive (SHRSP) rat. Renal *Gstm1* expression in SHRSP rats is significantly reduced when compared with normotensive Wistar Kyoto (WKY) rats. As *Gstm1* plays an important role in the secondary defence against oxidative stress, significantly lower expression levels may be functionally relevant in the development of hypertension. The aim of this study was to investigate the role of *Gstm1* in BP regulation and oxidative stress by transgenic overexpression of the *Gstm1* gene.

Method: Two independent *Gstm1* transgenic SHRSP lines were generated by microinjecting SHRSP embryos with a linear construct controlled by the EF-1α promoter encoding WKY *Gstm1* cDNA [SHRSP-Tg(Gstm1)1^{WKY} and SHRSP-Tg(Gstm1)2^{WKY}].

Results: Transgenic rats exhibit significantly reduced BP and pulse pressure when compared with SHRSP [systolic: SHRSP 205.2 ± 3.7 mmHg vs. SHRSP-Tg(Gstm1)1^{WKY} 175.5 ± 1.6 mmHg and SHRSP-Tg(Gstm1)2^{WKY} 172 ± 3.2 mmHg, $P < 0.001$; pulse pressure: SHRSP 58.4 ± 0.73 mmHg vs. SHRSP-Tg(Gstm1)1^{WKY} 52.7 ± 0.19 mmHg and SHRSP-Tg(Gstm1)2^{WKY} 40.7 ± 0.53 mmHg, $P < 0.001$]. Total renal and aortic *Gstm1* expression in transgenic animals was significantly increased compared with SHRSP [renal relative quantification (RQ): SHRSP-Tg(Gstm1)1^{WKY} 1.95 vs. SHRSP 1.0, $P < 0.01$; aorta RQ: SHRSP-Tg(Gstm1)1^{WKY} 2.8 vs. SHRSP 1.0, $P < 0.05$]. Renal lipid peroxidation (malondialdehyde: protein) and oxidized : reduced glutathione ratio levels were significantly reduced in both transgenic lines when compared with SHRSP [malondialdehyde: SHRSP 0.04 ± 0.009 μmol/l vs. SHRSP-Tg(Gstm1)1^{WKY} 0.024 ± 0.002 μmol/l and SHRSP-Tg(Gstm1)2^{WKY} 0.021 ± 0.002 μmol/l; (oxidized : reduced glutathione ratio): SHRSP 5.19 ± 2.26 μmol/l vs. SHRSP-Tg(Gstm1)1^{WKY} 0.17 ± 0.11 μmol/l and SHRSP-Tg(Gstm1)2^{WKY} 0.47 ± 0.22 μmol/l]. Transgenic SHRSP rats containing the WKY *Gstm1* gene demonstrate significantly lower BP, reduced oxidative stress and improved levels of renal *Gstm1* expression.

Conclusion: These data support the hypothesis that reduced renal *Gstm1* plays a role in the development of hypertension.

Keywords: glutathione *S*-transferase μ-type 1, hypertension, oxidative stress, stroke-prone spontaneously hypertensive rat, transgenic

Abbreviations: BP, blood pressure; GSSG: GSH, oxidized: reduced glutathione ratio; GSTM1, glutathione S-transferase μ-type 1; LVMI, left ventricular mass index/indices; PP, pulse pressure; QTL, quantitative trait locus/loci; RMI, renal mass index/indices; ROS, reactive oxygen species; SHRSP, stroke-prone spontaneously hypertensive rat; WKY, Wistar Kyoto

INTRODUCTION

Human essential hypertension is a complex polygenic disease with genetic heritability averaging approximately 40% and with strong influence of environmental factors and gene–environment interaction [1,2]. Heterogeneity in the general population and the polygenic complexities of the disease has meant that identification and functional validation of candidate genes has proved difficult in humans. A range of studies including genome wide association studies, meta-analysis and admixture mapping studies have successfully identified numerous loci associated with phenotypic variance for SBP and

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DBP [2–15]. However, only a small portion of the variance of blood pressure (BP) (i.e. ~5%) is explained by the loci discovered so far. To fully understand the functional roles of genetic loci on BP regulation it is essential to interrogate their impact in suitable rodent models. Rodent models are commonly used to discern and dissect genetic determinants of hypertension as they offer more favourable investigative opportunities because of reduced genetic heterogeneity, the capacity for controlled breeding and environmental conditions, and the ability to produce genetic crosses and analyse large numbers of progeny [16,17]. This translational approach will improve our knowledge and understanding of pathways, networks and gene environment interaction underlying essential hypertension.

The stroke-prone spontaneously hypertensive rat (SHRSP) is a well characterized experimental model for human essential hypertension, which develops a number of cardiovascular complications, including cardiac hypertrophy and stroke [18–20]. Similar to human disease, the genetic determination of BP variation in this model is complex and the result of multiple gene–gene and gene–environment interactions [21,22]. Genome-wide linkage studies have proved successful in the localization of large chromosomal regions containing quantitative trait loci (QTLs) for blood regulation in the SHRSP. In particular, previous work in our laboratory has identified at least two BP QTLs mapping to rat chromosome 2 [20]. These QTL were subsequently confirmed with the production and phenotypic analysis of chromosome 2 congenic strains, generated by introgressing regions of rat chromosome 2 from the normotensive Wistar Kyoto (WKY) strain into the SHRSP genetic background. This resulted in significant reduction of SBP and DBP when the congenic strains were compared with the SHRSP parental strain [23,24].

Combining this congenic strategy with genome-wide microarray expression profiling allowed the identification of the positional candidate gene, glutathione *S*-transferase μ-type 1 (*Gstm1*) [24,25]. This gene encodes a cytoplasmic glutathione *S*-transferase (GST) that belongs to the mu class of GST enzymes, which function in the detoxification of electrophilic compounds, including carcinogens, therapeutic drugs, environmental toxins and products of oxidative stress, by conjugation with glutathione [26]. GSTM1 protein is expressed in a wide range of tissues including the liver, endocrine tissues, brain, muscle tissues and kidney [27]. Our previous studies in the SHRSP rat demonstrate significantly downregulated expression of *Gstm1* in the kidney when compared with the WKY. As a result of this reduced *Gstm1* expression we hypothesise that the secondary defence against oxidative stress is compromised in SHRSP rats leading to oxidative stress-induced hypertension and end organ damage.

The aim of this study was to establish conclusive evidence that reduced *Gstm1* expression affects BP regulation and oxidative stress. Two independent SHRSP transgenic lines were created with the aim of reversing *Gstm1* deficiency by incorporation of the *Gstm1* gene from the normotensive WKY strain into the SHRSP genome.

MATERIAL AND METHODS

Animal strains

Inbred colonies of SHRSP and WKY have been maintained at the University of Glasgow since 1991, as described previously [20]. All animals were housed under controlled environmental conditions, fed standard rat chow (rat and mouse no. 1 maintenance diet, Special Diet Services) and water provided *ad libitum*. All animal procedures performed were approved by the Home Office according to regulations regarding experiments with animals in the United Kingdom.

Transgenic rat generation

Two independent transgenic lines of *Gstm1* SHRSP rats were created through incorporation of the *Gstm1* gene from the normotensive WKY strain into the SHRSP genome, which are designated SHRSP-Tg(Gstm1)1^{WKY} and SHRSP-Tg(Gstm1)2^{WKY}. Generation of these transgenic SHRSP rats involved microinjection of a 2.7-kb linear construct encoding wild-type (WKY) *Gstm1* under the control of the universal EF-1α promoter (Supplementary Fig. 1, http://links.lww.com/HJH/B27). They were generated using the same expression platform and microinjection fragment purification protocol employed as previously described [28]. See Supplementary file for extended methods.

Phenotypic measurements

The Dataquest IV telemetry system (Data Sciences International) was used for the direct measurement of SBP, DBP, pulse pressure (PP), activity and heart rate (HR) [23,29]. Male rats were implanted at 12 weeks of age with 1-week recovery, and haemodynamic parameters recorded until rats were 21 weeks of age. BP prior to 12 weeks of age was measured by tail cuff plethysmography [30]. Metabolic cages were used to collect 24-h urine samples from all animals before sacrifice. At sacrifice, weights for cardiac mass index, left ventricular (LV) mass index (LVMI) and renal mass index (RMI) were measured and corrected to tibia length. At sacrifice a range of tissues were harvested and either snap frozen in liquid nitrogen and stored at −80 °C or fixed in 4% buffered formaldehyde and paraffin embedded for histology or immunohistochemistry (IHC).

Urinary protein was measured using Thermo Pierce Protein Assay 660 (no. 22662; Thermo Fisher Scientific Inc, Waltham, Massachusetts, USA) with samples diluted one in five for assay. Urinary biochemistry analysis was carried out using a Beckman Coulter AU640 clinical chemistry analyser utilizing ion selective electrodes. Indirect glomerular filtration rates (GFR) were determined by a clinically validated automated analyser (c311, Roche Diagnostics, Burgess Hill, UK), using the manufacturer's calibrators and quality control material for isotope dilution-mass spectrometry. All measurements were normalized to kidney weight.

Echocardiography

Transthoracic echocardiography was used to assess cardiac geometry and contractility, as previously described [30,31]. Echocardiography was performed prior to sacrifice at 21 weeks of age. Animals were sedated and short axis

2-dimensional B-mode and M-mode images were taken through the left parasternal window at the papillary muscle levels using an ACUSON Sequoia C512 Echocardiograph (Siemens, Erlangen, Germany). Averaged data from six consecutive cardiac cycles from each M-mode tracing were used in the following equation for the calculation of LV mass [American society of echocardiography (ASE)-cube formula with Devereux correction factor] [LV mass = 0.8 (1.04 ((EDD + PWT + AWT) 3 − EDD3)) + 0.6], where PWT is the posterior wall thickness (mm), AWT is the anterior wall thickness (mm), EDD is the end diastolic dimension (mm). LV end-systolic volume (ESV) and LV end-diastolic volume (EDV) can be calculated from two-dimensional images according to a modified Simpson's rule. LV ejection fraction (LVEF) is then determined from EDV and ESV. Cardiac index is estimated as cardiac output (CO) adjusted for tibia length.

Renal histology

To assess evidence of renal disease, 3 μm sections from WKY, SHRSP and transgenic rat kidneys were stained with Harris haematoxylin and eosin and examined using an Olympus DP72 camera attached to Olympus BX51 microscope, and analysed using DP2-BSW software (Olympus, Hamburg, Germany).

Quantitative real-time PCR

Total RNA was extracted from kidney, aorta, heart and brain tissues from 5-week-old and 21-week-old rats using RNeasy kits (Thermo Fisher Scientific Inc.), treated with DNase Free (Thermo Fisher Scientific Inc.) and quantified using Ribogreen (Thermo Fisher Scientific Inc.). Normalization was confirmed by performing real-time PCR on TaqMan (Thermo Fisher Scientific Inc.) of β-actin with comparable threshold cycles. TaqMan probes for *Gstm1* (Rn00755117m1-labeled FAM) and β-actin (4352340E-labeled VIC) were multiplexed. Expression of *Gstm1* relative to β-actin in each sample was derived using the comparative (ΔΔCT) method [25].

For transgene or WKY (wild-type) gene expression, Exiqon custom locked-nucleic acid SYBR Green probes were designed to single nucleotide polymorphism (SNP) differences between WKY and SHRSP in exon 8 as previously described [25].

Western analysis of glutathione *S*-transferase μ-type 1 in rat kidney

Kidneys from 5-week-old and 21-week-old SHRSP, WKY and SHRSP-Tg(Gstm1)1^{WKY} and SHRSP-Tg(Gstm1)2^{WKY} rats were homogenized in protease inhibitor-containing buffer. Protein concentration was determined using a Bio-Rad BCA kit. Proteins were separated by PAGE and electroblotted onto a Hybond-P membrane (Thermo Fisher Scientific Inc,). Membranes were incubated with the appropriate primary antibody (anti-GSTM1 [25] 1 : 5000, antiβ-actin 1 : 1000) before repeated washing and application of a horseradish peroxidase-conjugated secondary antibody. Protein bands were detected by chemiluminescence (ECL kit; Thermo Fisher Scientific Inc), and visualized and quantified using a Bio-Rad Image Analyzer densitometry system.

Immunohistochemistry

Sections (6 μm) were obtained from kidneys and aorta of SHRSP, WKY, SHRSP-Tg(Gstm1)1^{WKY} and SHRSP-Tg(Gstm1)2^{WKY} male rats. Sections were blocked for 60 min with 2% serum in PBS, followed by overnight incubation at 26 °C with an antibody against GSTM1 [25]. A biotinylated secondary antibody, diluted in blocking reagent (ABC universal kit, two drops blocking serum + two drops supplied vectastain biotinylated antibody + 5 ml PBS) was added for 30 min at room temperature (RT). 3,3'-Diaminobenzidine (DAB) chromogen (DAB substrate kit) for universal and peroxidase secondary antibody was prepared following manufacturer's instructions. After treatment with antigen retrieval solution (Agilent, Stockport, Cheshire, UK), sections were blocked with 20% serum in PBS for 1 h. Washing with PBS was followed by incubation with a secondary antibody, at RT for 1 h. Sections were counterstained with Haematoxylin. Images shown were taken with Olympus DP72 attached to Olympus BX51 microscope, using DP2-BSW software (Olympus, Hamburg, Germany).

Oxidative stress measurements

Superoxide, hydrogen peroxide, nitric oxide, glutathione and lipid peroxidation measurements were performed in homogenized snap-frozen tissues from 5-week-old and 21-week-old animals. Superoxide was measured using lucigenin chemiluminescence in kidney tissue homogenized in lysis buffer [20 mmol/l of KH_2PO_4, 1 mmol/l of ethylene glycol-bis(B-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 1 μg/ml of aprotinin, 1 μg/ml of leupeptin, 1 μg/ml of pepstatin and 1 mmol/l of phenylmethylsulfonyl fluoride (PMSF)]. Fifty microliters of the sample were added to a suspension containing 175 μl of assay buffer (50 mmol/l of KH_2PO_4, 1 mmol/l of EGTA, and 150 mmol/l of sucrose) and lucigenin (5 μmol/l). NADPH (10^{-4} mol/l) was added to the suspension (300 μl) containing lucigenin. Luminescence was measured every 18 s for 3 min by a luminometer (AutoLumat LB 953; Berthold Technologies, Wildbad, Germany) before and after stimulation with NADPH. A buffer blank was subtracted from each reading. The results are expressed as counts per milligram of protein (percentage of control).

Glutathione levels were measured according to Cayman Glutathione Assay Kit (#703002) manufacturer's instructions. Hydrogen peroxide levels were measured using Invitrogen's AmplexR Red Hydrogen Peroxide/Peroxidase Assay Kit Amplex Red (Thermo Fisher Scientific Inc.) for tissues. Total nitrate and nitrite concentrations were measured using the Cayman Biochemical Nitrate/Nitrite Colorimetric Assay Kit.

Statistical analysis

Results are expressed as mean ± SEM. Haemodynamic parameters for WKY or transgenic strains were compared with SHRSP, using repeated measures analysis of variance (ANOVA) (general linear model). Other phenotypic comparisons between groups were performed by one way ANOVA with Tukey's multiple comparison test, unless stated otherwise. For cardiac parameter analysis statistical analysis was performed by a Student's *t* test.

RESULTS

Haemodynamic parameters

When measured by radiotelemetry, SHRSP-Tg(Gstm1)1^{WKY} and SHRSP-Tg(Gstm1)2^{WKY} rats demonstrated significantly reduced SBP (Fig. 1a), and PP (Fig. 1c) when compared with SHRSP ($P < 0.001$). DBP (Fig. 1b) and mean arterial pressure (Fig. 1d) were significantly reduced across the entire analysis period in SHRSP-Tg(Gstm1)1^{WKY} rats, whereas SHRSP-Tg(Gstm1)2^{WKY} rats demonstrated a significant reduction in diastolic and mean arterial pressure from approximately 18 weeks of age onwards ($P < 0.001$). There were no significant differences in HR or activity level across the measurement period between the four strains (Supplementary Fig. 2, http://links.lww.com/HJH/B27). Before 12 weeks of age, SBP measured by tail cuff plethysmography was significantly lower in WKY and the transgenic lines when compared with SHRSP ($P < 0.001$) (Supplementary Table 1, http://links.lww.com/HJH/B27).

Cardiac parameters

Cardiac parameters were assessed in rats at 21 weeks of age by transthoracic echocardiography immediately prior to sacrifice. LVMI was significantly reduced in WKY and SHRSP-Tg(Gstm1)2^{WKY} hearts compared with that of the SHRSP ($P < 0.05$). SHRSP-Tg(Gstm1)1^{WKY} rats displayed a trend towards a reduction in LVMI but did not reach statistical significance (Fig. 2a). Relative wall thickness in SHRSP-Tg(Gstm1)1^{WKY}, SHRSP-Tg(Gstm1)2^{WKY} and WKY rats was significantly lower compared with that of the SHRSP ($F = 7.9$, $P < 0.05$) (Fig. 2b). Stroke volume (SV) for SHRSP rats was significantly decreased when compared with WKY, SHRSP-Tg(Gstm1)1^{WKY} and SHRSP-Tg(Gstm1)2^{WKY} rats (Fig. 2c). CO in SHRSP rats was significantly decreased when compared with WKY, SHRSP-Tg(Gstm1)1^{WKY} and SHRSP-Tg(Gstm1)2^{WKY} rats (Fig. 2d). There were no significant differences in LV fractional shortening or ejection fraction between WKY, SHRSP, SHRSP-Tg(Gstm1)1^{WKY} and SHRSP-Tg(Gstm1)2^{WKY} (data not shown).

Renal parameters

Renal function data at 21 weeks of age are given in Table 1. Although there was a trend towards increased estimated GFR in SHRSP-Tg(Gstm1)1^{WKY} rats, there was no significance difference between all four strains ($P > 0.05$). There was also no significant difference between all four strains for urine sodium, potassium and chloride levels ($P > 0.05$). Levels of proteinuria in WKY, SHRSP-Tg(Gstm1)1^{WKY} and

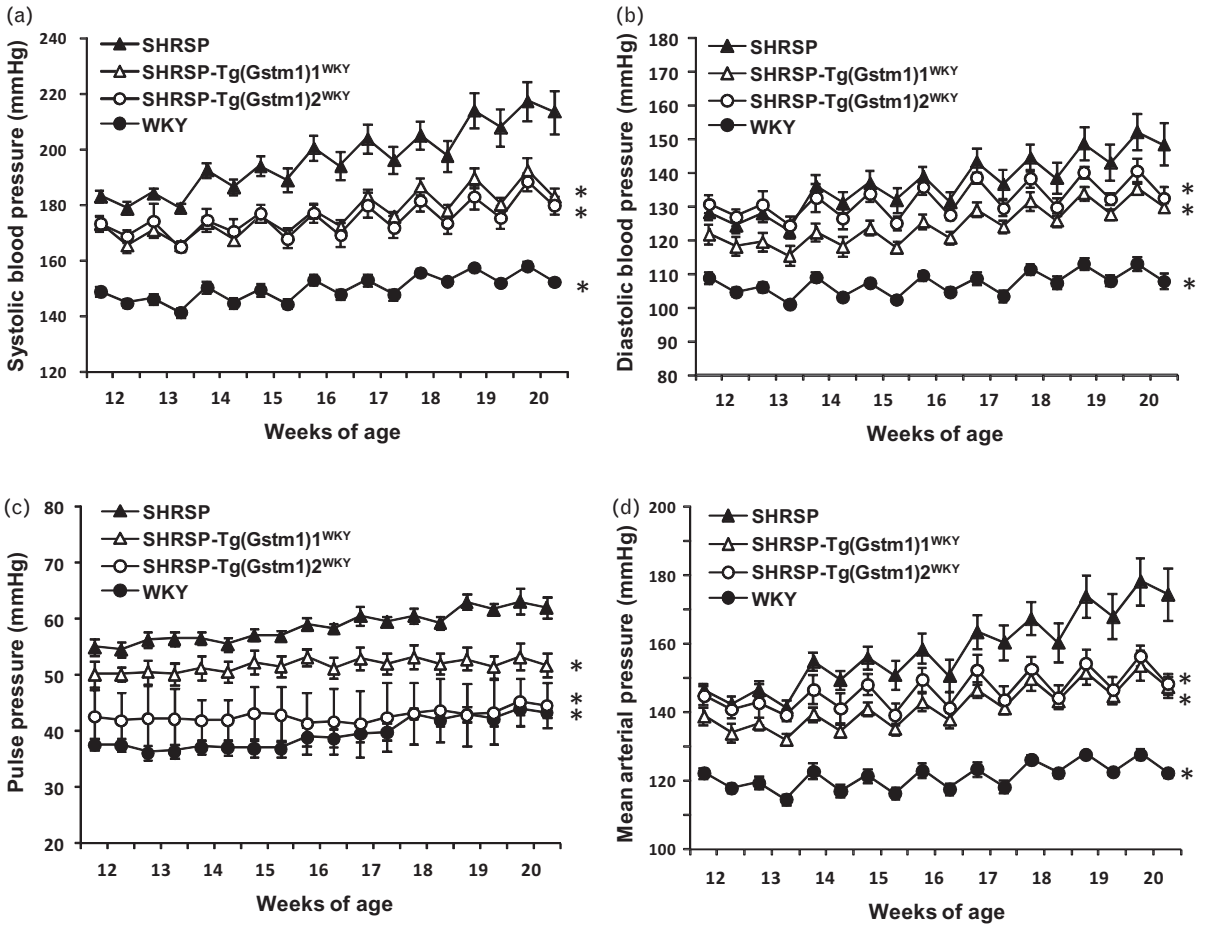

FIGURE 1 Haemodynamic parameters in SHRSP-Tg(Gstm1)1^{WKY}, SHRSP-Tg(Gstm1)2^{WKY} and parental strains. Significantly reduced (a) SBP, (b) DBP, (c) pulse pressure and (d) mean arterial pressure was observed in SHRSP-Gstm1)1^{WKY} ($n = 12$), SHRSP-Tg(Gstm1)2^{WKY} ($n = 5$) and Wistar Kyoto ($n = 15$) rats when compared with SHRSP ($n = 12$). Blood pressure was measured by radiotelemetry, and data illustrate weekly averaged night-time and daytime data points (*$P < 0.001$). Gstm1, glutathione-*S*-transferase-μ-type-1; SHRSP, stroke-prone spontaneously hypertensive.

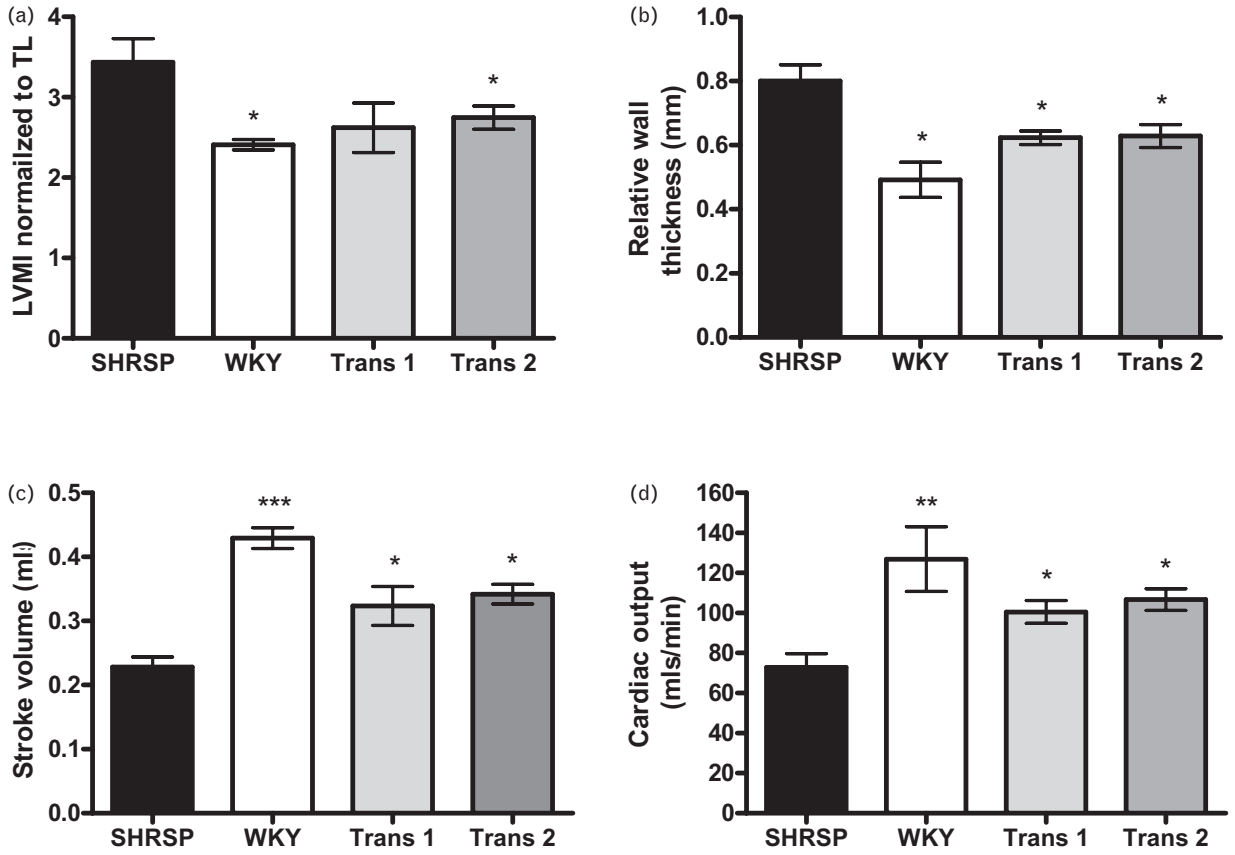

FIGURE 2 Cardiac parameters in SHRSP-Tg(Gstm1)1^{WKY}, SHRSP-Tg(Gstm1)2^{WKY} and parental strains. Significantly reduced (a) left ventricular mass index and (b) relative wall thickness, and significantly elevated (c) stroke volume, and (d) cardiac output were observed in SHRSP-Tg(Gstm1)1^{WKY} ($n=8$), SHRSP-Tg(Gstm1)2^{WKY} ($n=6$) and Wistar Kyoto rats ($n=8$) when compared with SHRSP ($n=8$). Cardiac parameters were measured by echocardiography at 21 weeks (*$P<0.05$, **$P<0.01$, ***$P<0.001$). TL, tibia length, Trans 1, SHRSP-Tg(Gstm1)1^{WKY}, Trans 2, SHRSP-Tg(Gstm1)2^{WKY}. Gstm1, glutathione-*S*-transferase-μ-type-1; SHRSP, stroke-prone spontaneously hypertensive.

SHRSP-Tg(Gstm1)2^{WKY} rats were significantly lower than that of the SHRSP ($P<0.05$). At 21 weeks of age, kidney mass, normalized to body weight, was significantly lower in WKY when compared with SHRSP ($P<0.0001$). RMI for SHRSP-Tg(Gstm1)1^{WKY} and SHRSP-Tg(Gstm1)2^{WKY} rats was not significantly different when compared with SHRSP.

Renal morphology, assessed by haemotoxylin and eosin staining at 21 weeks of age, showed normal arterioles in the WKY and transgenic animals, however renal arterioles in the SHRSP showed evidence of hyperplasia, a sign of accelerated hypertension (Fig. 3).

Renal glutathione *S*-transferase μ-type 1 mRNA and protein expression

Total *Gstm1* mRNA expression (Fig. 4a) and transgene specific (WKY variant of Gstm1) mRNA expression (Fig. 4b) in kidneys from rats at 5 weeks of age was significantly higher in SHRSP-Tg(Gstm1)1^{WKY}, SHRSP-Tg(Gstm1)2^{WKY} and WKY rats compared with SHRSP ($P<0.05$). This increase in renal *Gstm1* mRNA expression was paralleled by GSTM1 protein levels in SHRSP-Tg(Gstm1)1^{WKY} and SHRSP-Tg(Gstm1)2^{WKY} rats. Western blot analysis indicated significantly increased GSTM1 protein expression in SHRSP-Tg(Gstm1)2^{WKY} and WKY when

TABLE 1. Renal parameters in SHRSP, Wistar Kyoto, SHRSP-Tg(Gstm1)1^{WKY} and SHRSP-Tg(Gstm1)2^{WKY} rats at 21 weeks of age

	SHRSP	WKY	Trans 1	Trans 2
GFR (ml/min)	1.565 ± 0.206	1.642 ± 0.137	2.369 ± 0.309	1.605 ± 0.265
Na^{2+} (mg/ml)	83.98 ± 11.13	78.24 ± 8.433	59.29 ± 7.533	76.27 ± 20.70
K^{+} (mg/ml)	116.2 ± 14.02	149.1 ± 14.09	153.0 ± 23.60	115.1 ± 30.08
Cl^{-} (mg/ml)	100.1 ± 10.84	148.7 ± 30.58	99.93 ± 15.76	110.6 ± 30.99
RMI	4.242 ± 0.044	3.389 ± 0.049*	4.355 ± 0.366	4.115 ± 0.142
Proteinuria (mg/g)	29.98 ± 5.32	9.765 ± 4.776	8.464 ± 5.771	6.942 ± 2.810

GFR, glomerular filtration rate; $n=6$–8 per group; Gstm1, glutathione-*S*-transferase-μ-type-1; RMI, renal mass index (kidney/body weight ratio); SHRSP, SHRSP; Trans 1, SHRSP-Tg (Gstm1)1^{WKY}; Trans 2, SHRSP-Tg (Gstm1)2^{WKY}; WKY, Wistar Kyoto.
*$P<0.05$ vs. SHRSP.

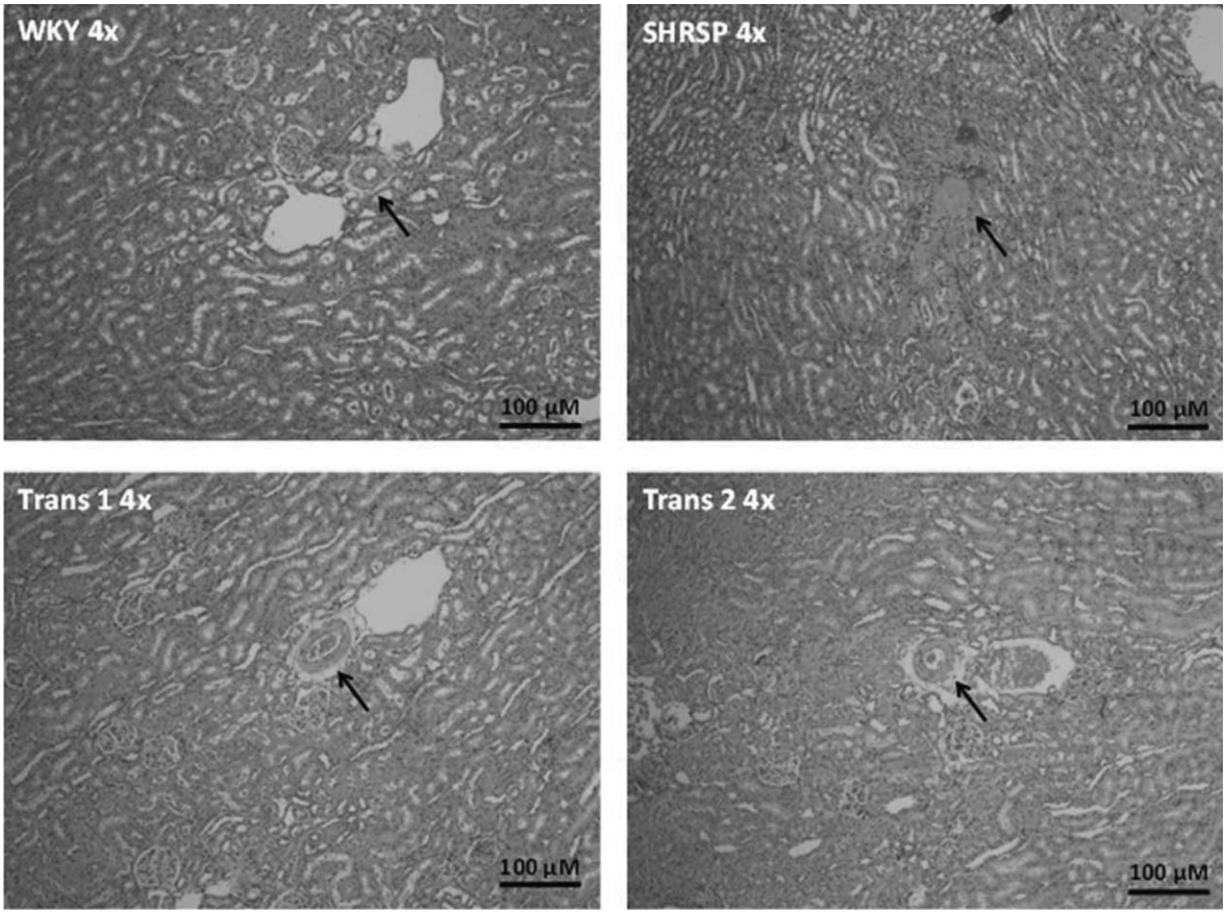

FIGURE 3 Representative renal histology. Kidney sections from rats at 21 weeks of age were stained with haematoxylin and eosin staining and showed no evidence of vascular pathology in Wistar Kyoto, SHRSP-Tg(Gstm1)1^{WKY}, SHRSP-Tg(Gstm1)2^{WKY} rats, but evidence of vascular hyperplasia was observed in SHRSP. Arrows indicate arcuate arteries. Bar = 100 μmol/l. Trans 1, SHRSP-Tg(Gstm1)1^{WKY}, Trans 2, SHRSP-Tg(Gstm1)2^{WKY}. Gstm1, glutathione-*S*-transferase-μ-type-1; SHRSP, stroke-prone spontaneously hypertensive.

compared with SHRSP, with a trend towards an increase in kidneys from SHRSP-Tg(Gstm1)1^{WKY} rats (Fig. 4c and d). IHC in kidneys from 5-week-old rats confirmed increased protein expression within the distal tubules of WKY and SHRSP-Tg(Gstm1)1^{WKY} rats when compared with SHRSP (Fig. 4e).

At 21 weeks of age, total renal *Gstm1* mRNA expression (Fig. 5a) and transgene specific mRNA expression (Fig. 5b) were significantly higher in WKY rats when compared with with SHRSP ($P < 0.05$). Transgene specific mRNA expression was also significantly increased in SHRSP-Tg(Gstm1)1^{WKY} rats, however SHRSP-Tg(Gstm1)2^{WKY} rats showed low transgene expression, which was not different from SHRSP (Fig. 5b). GSTM1 protein levels were significantly higher in WKY rats compared with that of the SHRSP ($P < 0.01$) but were not significantly different in SHRSP-Tg(Gstm1)1^{WKY} and SHRSP-Tg(Gstm1)2^{WKY} rats compared with SHRSP ($P > 0.05$) (Fig. 5c and d). IHC confirmed an increase in GSTM1 protein expression in distal tubules of WKY rats compared with SHRSP, with no increase in GSTM1 protein expression in the transgenic rats (Fig. 5e).

Vascular glutathione *S*-transferase μ-type 1 mRNA expression and immunohistochemistry

At 5 weeks of age vascular (aortic) total *Gstm1* expression showed a trend towards an increase in SHRSP-Tg(Gstm1)1^{WKY} and WKY rats, and was significantly increased in SHRSP-Tg(Gstm1)2^{WKY} rats when compared with the SHRSP ($P < 0.05$) (Supplementary Fig. 3A, http://links.lww.com/HJH/B27). At 21 weeks of age, *Gstm1* mRNA expression in aorta was significantly increased in SHRSP-Tg(Gstm1)1^{WKY} and WKY rats when compared with and SHRSP ($P < 0.005$) (Supplementary Fig. 3B, http://links.lww.com/HJH/B27). However, aortic *Gstm1* expression in SHRSP-Tg(Gstm1)2^{WKY} rats showed an increased trend but was not significantly different from WKY, SHRSP-Tg(Gstm1)1^{WKY} or SHRSP rats. IHC was performed on the aorta at 21 weeks of age to investigate GSTM1 protein expression. When quantified, percentage staining in the aorta demonstrated a significant increase in GSTM1 protein in WKY rats when compared with SHRSP ($P < 0.05$) (Supplementary Fig. 3C and D, http://links.lww.com/HJH/B27). There was a trend towards an increase in protein expression for aortas in SHRSP-Tg(Gstm1)1^{WKY} and

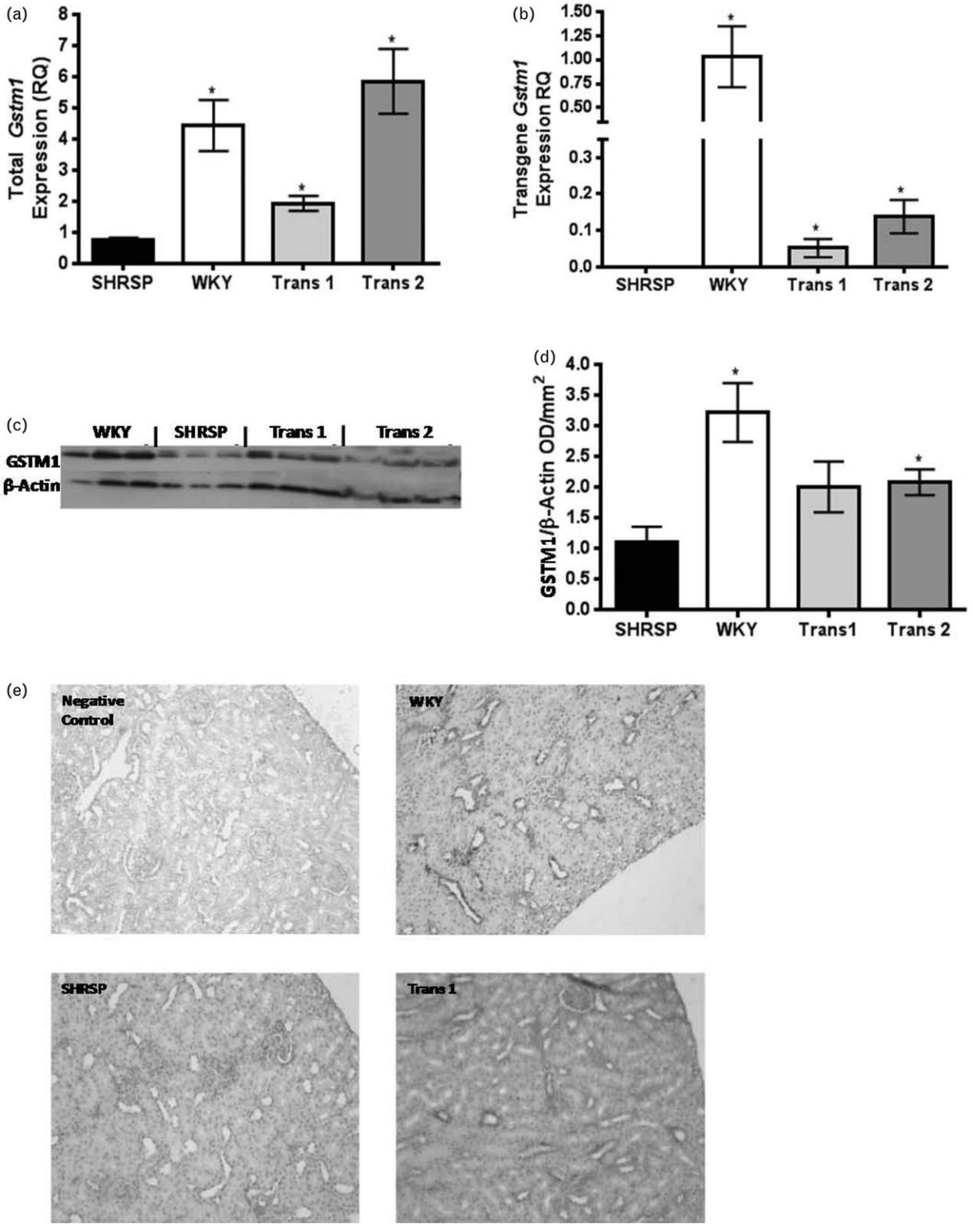

FIGURE 4 Renal glutathione *S*-transferase μ-type 1 expression in SHRSP-Tg(Gstm1)1^{WKY}, SHRSP-Tg(Gstm1)2^{WKY} and parental strains at 5 weeks of age. Glutathione *S*-transferase μ-type 1 expression in kidney at 5 weeks of age in SHRSP ($n=8$), Wistar Kyoto ($n=8$), SHRSP-Tg(Gstm1)1^{WKY} ($n=8$) and SHRSP-Tg(Gstm1)2^{WKY} ($n=3$) rats. (a) Total Gstm1 mRNA levels were significantly increased in Wistar Kyoto, SHRSP-Tg(Gstm1)1^{WKY}, SHRSP-Tg(Gstm1)2^{WKY} lines when compared with the SHRSP ($^*P<0.01$). (b) Wistar Kyoto specific glutathione *S*-transferase μ-type 1 mRNA levels were significantly higher in Wistar Kyoto, SHRSP-Tg(Gstm1)1^{WKY} and SHRSP-Tg(Gstm1)2^{WKY} rats when compared with SHRSP ($^*P<0.05$). (c) Representative western blot of whole kidney homogenates from parental and transgenic rats. (d) Increased glutathione *S*-transferase μ-type 1 protein expression was confirmed by densitometry with each band normalized to β-actin ($n=3$ for each strain) ($^*P<0.05$). (e) Immunohistochemistry of glutathione *S*-transferase μ-type 1 protein in whole kidney sections from Wistar Kyoto, SHRSP and SHRSP-Tg(Gstm1)1^{WKY} rats at 5 weeks of age (magnification = 10×). Trans 1, SHRSP-Tg(Gstm1)1^{WKY}, Trans 2, SHRSP-Tg(Gstm1)2^{WKY}. Gstm1, glutathione-*S*-transferase-μ-type-1; SHRSP, stroke-prone spontaneously hypertensive rat.

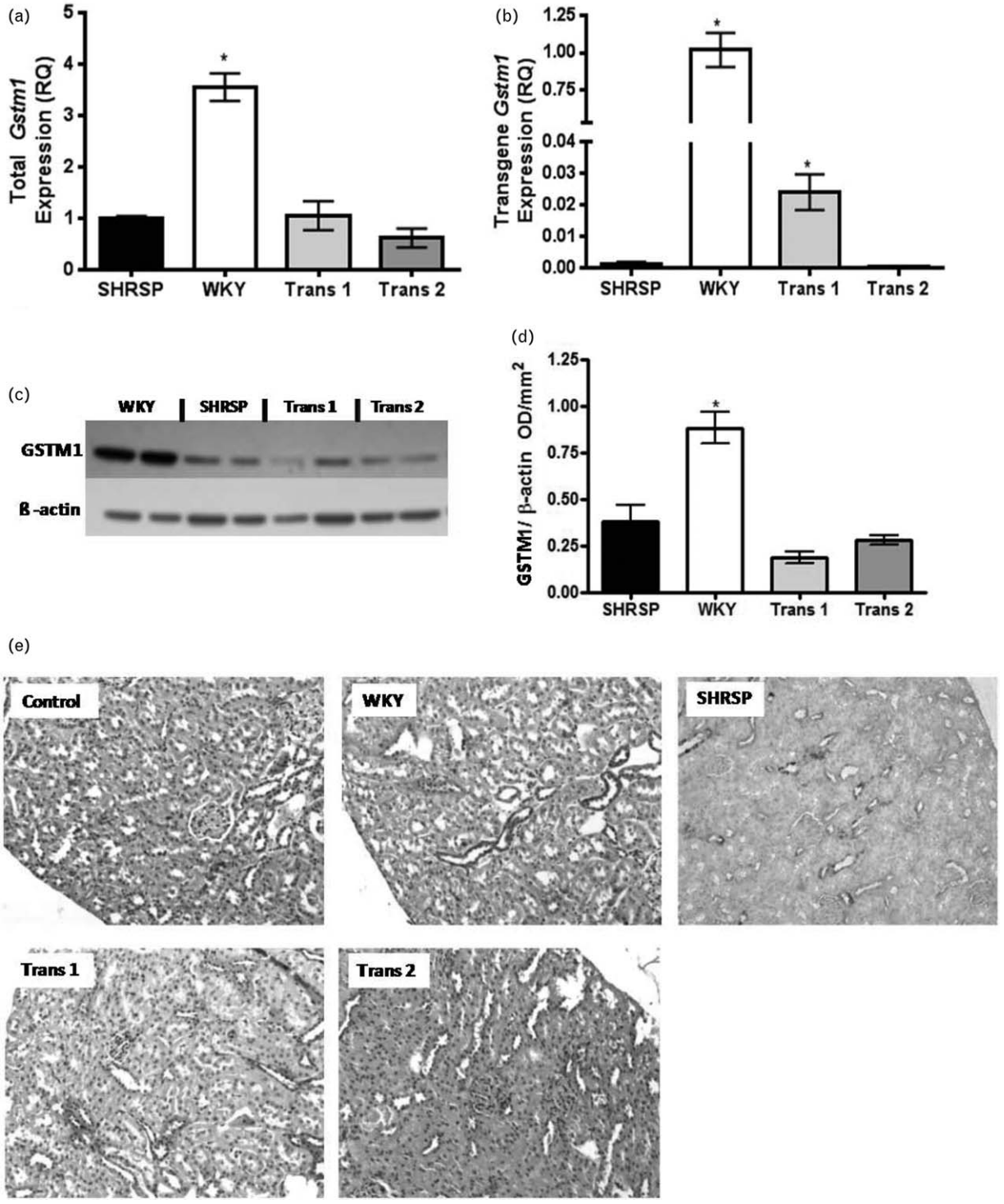

FIGURE 5 Renal glutathione *S*-transferase μ-type 1 expression between SHRSP-Tg(Gstm1)1^{WKY}, SHRSP-Tg(Gstm1)2^{WKY} and parental strains at 21 weeks of age. (a) Total glutathione *S*-transferase μ-type 1 expression in kidney at 21 weeks of age in SHRSP ($n = 8$), Wistar Kyoto ($n = 8$), SHRSP-Tg(Gstm1)1^{WKY} ($n = 8$) and SHRSP-Tg(Gstm1)2^{WKY} ($n = 6$) rats. Total glutathione *S*-transferase μ-type 1 levels were significantly increased in Wistar Kyoto rats when compared with SHRSP $^*P < 0.01$. (b) Wistar Kyoto specific glutathione *S*-transferase μ-type 1 mRNA levels were significantly higher in Wistar Kyoto and SHRSP-Tg(Gstm1)1^{WKY} rats when compared with SHRSP ($^*P < 0.05$). Representative western blot (c) illustrates increased glutathione *S*-transferase μ-type 1 expression in Wistar Kyoto when compared with SHRSP, which was confirmed by densitometry (d) with each band normalized to β-actin ($n = 3$ for each strain) ($^*P < 0.05$). (e) Immunohistochemistry of glutathione *S*-transferase μ-type 1 protein in whole kidney sections from Wistar Kyoto, SHRSP and SHRSP-Tg(Gstm1)1^{WKY} rats at 21 weeks of age (magnification = 10×). Trans 1, SHRSP-Tg(Gstm1)1^{WKY}, Trans 2, SHRSP-Tg(Gstm1)2^{WKY}. Gstm1, glutathione-*S*-transferase-μ-type-1; SHRSP, stroke-prone spontaneously hypertensive rat.

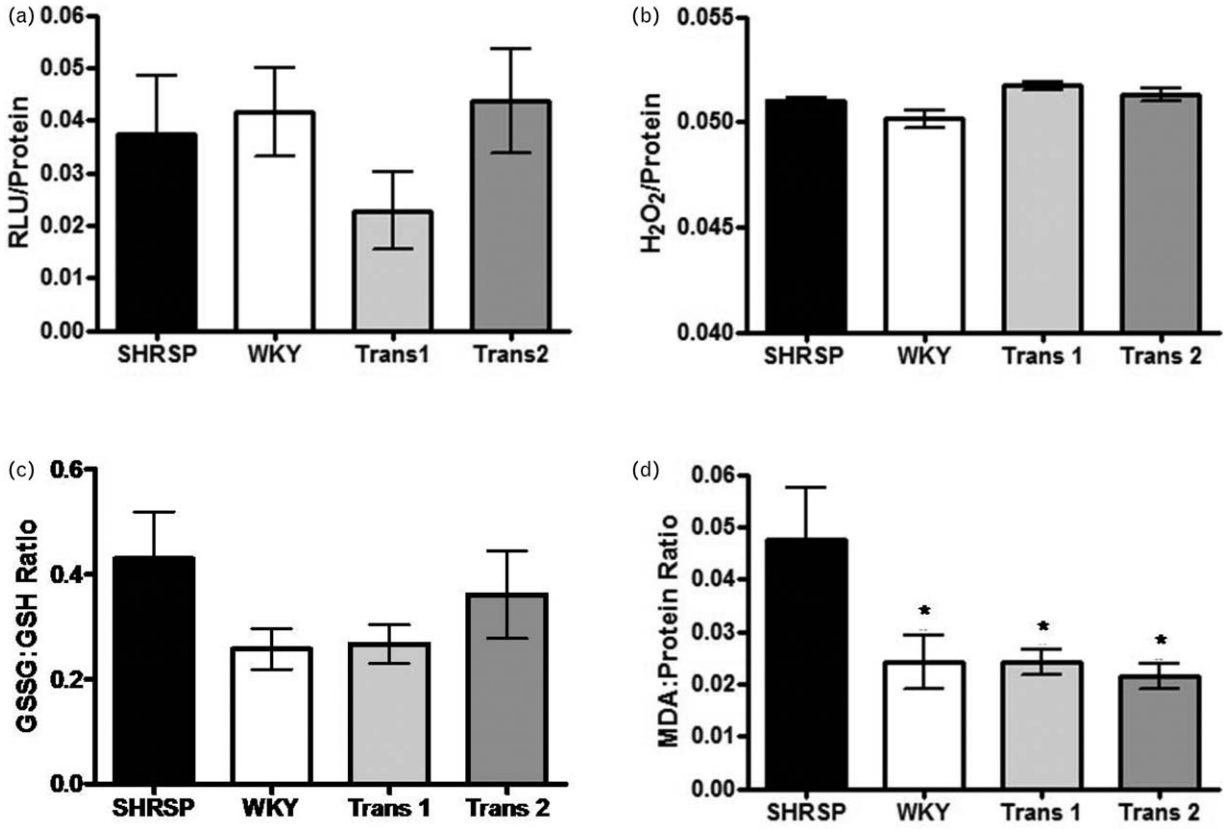

FIGURE 6 Renal oxidative stress measurements in SHRSP-Tg(Gstm1)1^{WKY}, SHRSP-Tg(Gstm1)2^{WKY} and parental strains at 21 weeks of age. (a) Renal superoxide levels measured by lucigenin chemiluminescence and (b) hydrogen peroxide levels measured by Amplex Red were not significantly different between SHRSP ($n = 8$), Wistar Kyoto ($n = 8$), SHRSP-Tg(Gstm1)1^{WKY} ($n = 8$) or SHRSP-Tg(Gstm1)2^{WKY} ($n = 6$) rats. Renal oxidative stress status determined by (c) oxidized : reduced glutathione ratio and (d) lipid peroxidation was lower in Wistar Kyoto, SHRSP-Tg(Gstm1)1^{WKY} ($n = 8$) and SHRSP-Tg(Gstm1)2^{WKY} when compared with SHRSP ($^*P < 0.05$). RLU, relative light unit; Trans 1, SHRSP-Tg(Gstm1)1^{WKY}, Trans 2, SHRSP-Tg(Gstm1)2^{WKY}. Gstm1, glutathione-*S*-transferase-μ-type-1; SHRSP, stroke-prone spontaneously hypertensive rat.

SHRSP-Tg(Gstm1)2^{WKY} rats which was not significantly different from either parental strain, indicating intermediate expression (Supplementary Fig. 3D, http://links.lww.com/HJH/B27).

Cardiac glutathione *S*-transferase μ-type 1 mRNA expression

At 21 weeks of age, cardiac total *Gstm1* expression was significantly increased in the SHRSP-Tg(Gstm1)1^{WKY} line when compared with WKY and SHRSP ($P < 0.05$). Cardiac *Gstm1* expression in SHRSP-Tg(Gstm1)2^{WKY} rats showed a trend towards increase, but was not significantly different from WKY, SHRSP-Tg(Gstm1)1^{WKY} or SHRSP rats (Supplementary Fig. 4A, http://links.lww.com/HJH/B27).

Effects of glutathione *S*-transferase μ-type 1 expression on renal and cardiac oxidative stress

At 21 weeks of age there was no significant difference between SHRSP, WKY or the transgenic rat lines for superoxide (O_2^-) levels in whole kidney homogenates (Fig. 6a), as measured by lucigenin chemiluminesence, or for hydrogen peroxide (Fig. 6b), as measured by Amplex Red Assay ($P > 0.05$). Oxidized : reduced glutathione (GSSG : GSH) ratios, showed a trend towards reduced levels in the WKY and both transgenic lines when compared with the SHRSP at 21 weeks of age (Fig. 6c). Lipid peroxidation measured using malondialdehyde (MDA) assay showed a significant reduction in renal MDA production in the WKY, SHRSP-Tg(Gstm1)1^{WKY} and SHRSP-Tg(Gstm1)2^{WKY} when compared with the SHRSP ($P < 0.05$), Fig. 6d. There were no significant differences in renal GSSG : GSH ratios between the four strains at 5 weeks of age (Supplementary Table 2, http://links.lww.com/HJH/B27), however renal GSSG : GSH ratios were significantly lower at 5 weeks of age when compared with 21 weeks of age (Supplementary Table 2, http://links.lww.com/HJH/B27).

DISCUSSION

In this study, the investigation of two independently generated transgenic rat lines, SHRSP-Tg(Gstm1)1^{WKY} and SHRSP-Tg(Gstm1)2^{WKY}, has demonstrated that increased expression of *Gstm1* improves BP regulation and reduces oxidative stress in the SHRSP rat. *Gstm1* deficiency was reversed by incorporation of the WKY *Gstm1* gene into the SHRSP genome leading to a significant reduction in SBP, DPB, MAP and PP. These haemodynamic improvements were paralleled by significant reductions in cardiac hypertrophy and improved cardiac function in both transgenic lines. Furthermore, renal oxidative stress, indicated by lipid peroxidation and GSSG : GSH ratio, was significantly

reduced in both transgenic lines when compared with SHRSP. These findings support the hypothesis that reduced *Gstm1* expression plays a causal role in the development of oxidative stress and BP elevation in the SHRSP rat.

The method of random transgene integration employed in this study may be viewed as a limitation when compared with the locus specific genome editing afforded by clustered regularly interspaced short palindromic repeats (CRISPR) technology [32]. However, this method for gene overexpression is still regularly used to generate genetically engineered rodent models, requires short development time and importantly is a method that we have previous success with in rats [33–37]. The generation of more than one transgenic line, using the same transgene and promoter, is critical to confirm that the phenotypic differences are the result of the transgene itself and not due to positional effects caused at the random insertion site. In independently generated lines it is highly unlikely that the transgene will be inserted into the identical genomic position. If significant phenotypic changes of similar magnitude and direction are observed for two independently generated lines then this provides corroborative evidence that the phenotype differences are due to a functional effect of the transgene.

Although both SHRSP-Tg(Gstm1)1^{WKY} and SHRSP-Tg(Gstm1)2^{WKY} transgenic rats demonstrate improved cardiovascular profiles, each line showed some unique molecular and phenotypic expression patterns. For example, while DBP for both SHRSP-Tg(Gstm1)1^{WKY} and SHRSP-Tg(Gstm1)2^{WKY} lines is significantly lower than that of SHRSP, the diastolic pressure profiles differ in the rate of increase over time (Fig. 1b). Similar phenotypic diversity between two independently generated lines has been demonstrated in the transgenic rescue of CD36 in the SHR rat, whereby, unique differences in insulin resistance and BP regulation were observed between two transgenic lines despite insertion of the same CD36 transgene and promoter [28]. It is clear that transgenes often do not behave as independent units, but are significantly and variably influenced by a number of factors leading to marked variations in expression patterns between different transgenic lines carrying the same construct [38]. For example, differences in mRNA and protein expression may occur due to the impact of enhancers that regulate neighbouring genes located in close proximity to the inserted transgene [39]. Although these enhancers normally regulate their respective associated gene, they can also affect the expression pattern of a transgene that is inserted near them. In addition, microinjection has the potential to insert multiple copies of a transgene into the recipient genome [28,40], which could lead to distinct copy number profiles within the two independently generated transgenic lines. Previous studies in other transgenic models have shown that copy number influences transgene expression resulting in differential phenotypic effects [41,42]. Another potential factor that could impact on the level of transgene expression is age-related increases in DNA-methylation [43]. For example, previous studies in rodent and pig transgenic models have revealed consistent age-related increases in DNA-methylation of ribosomal genes that correlated with inhibition of gene expression [41,44]. Future investigations will be necessary to determine if any of these factors play a role in the unique expression profiles between SHRSP-Tg(Gstm1)1^{WKY} and SHRSP-Tg(Gstm1)2^{WKY} rats.

Both transgenic lines demonstrated an increase in total and transgene specific *Gstm1* mRNA expression in kidneys at 5 weeks of age. However, this increased expression was not sustained at the later time point investigated (i.e. 21 weeks of age) in the SHRSP-Tg(Gstm1)2^{WKY} line. This return to SHRSP expression levels at 21 weeks is paralleled by renal protein expression in both SHRSP-Tg(Gstm1)1^{WKY} and SHRSP-Tg(Gstm1)2^{WKY} rats. An age-related decline in transgene expression is not unique to this investigation since previously published studies have shown that transgene expression levels can decline with time both *in vivo* and *in vitro* [45]. For example, enhanced green fluorescent protein (EGFP) expression, under the control of the EF-1α promoter, was found to be progressively limited during the later stages of development [45], and completely restricted in adult tissue of *Xenopus leavis* [46], medaka fish [47] and zebrafish [43]. Although these studies have reported a decrease in transgene expression levels during later stages of life, in line with the present findings, each of these studies demonstrate significant phenotypic effects as a result of early transgene expression [43,45–47]. Our data suggest that enhanced expression of *Gstm1* in the kidney prior to the onset of hypertension prevents the progression of hypertension in the transgenic SHRSP rat.

The kidney is highly vulnerable to the damage caused by reactive oxygen species (ROS), which can impact on its critical role in salt and water homeostasis, leading to altered renal vascular function and the development of hypertension [48,49]. In this study, we have demonstrated that the BP lowering effects of enhanced *Gstm1* expression are paralleled by reduced oxidative damage in the kidney. Specifically, *Gstm1* transgenic rats show significantly lower renal lipid peroxidation and reduced renal pathology as indicated by the absence of renal vessel hyperplasia and significantly reduced levels of proteinuria when compared with the SHRSP. The oxidative stress pathway is a complex cascade of events involving multiple types of ROS and antioxidant enzymes. We examined several components of this cascade, namely superoxide (O_2^-), hydrogen peroxide (H_2O_2), GSSG : GSH ratio and lipid peroxidation. Our data show that ROS generated early in the oxidative stress pathway (i.e. before point at which glutathione acts in the ROS cascade), are not significantly different in kidneys from SHRSP, WKY and transgenic rats. However, later components of the pathway (GSSG : GSH ratio and lipid peroxidation) are reduced in kidneys from the transgenic rats when compared with SHRSP. In contrast, previous studies in 20-week-old SHRSP rats demonstrated significantly increased basal and NADH stimulated O_2^- levels in renal cortex when compared with WKY rats [50]. Several factors may contribute to this difference in O_2^- levels between the two studies. For example, there are differences in the methods of O_2^- measurement between the studies, and whole kidney were used in the current study as opposed to renal cortex. However, similar to the current findings, there were no significant differences observed in renal H_2O_2 levels between SHRSP and WKY rats at 20 weeks of age. In addition to the 21-week time point we also examined

ROS levels in kidneys from rats at 5 weeks of age. Our data showed that there was no evidence of oxidative stress at this early time point in all four strains when compared with kidneys from 21-week-old rats. Therefore, improved *Gstm1* expression levels are evident in SHRSP-Tg(Gstm1)1^{WKY} and SHRSP-Tg(Gstm1)2^{WKY} rats before obvious differences in ROS levels or the onset of hypertension.

In parallel to the effect on BP, echocardiography measurements at 21 weeks of age demonstrate that cardiac hypertrophy (i.e. LVMI, relative wall thickness) and cardiac function parameters (i.e. SV, CO) were significantly improved in both transgenic lines compared with the SHRSP. In line with these cardiac changes, *Gstm1* mRNA expression levels were significantly increased in WKY hearts compared with SHRSP, with increased trends in expression in the transgenic rats. Currently it is not possible to determine whether the cardiac mass and function changes are due to direct effects of altered *Gstm1* expression in the heart or are a secondary effect of the significantly lowered BP in the transgenic animals and further investigation will be required to dissect these factors.

In conclusion, the production of two independently generated *Gstm1* transgenic lines on the SHRSP genetic background has provided a unique opportunity to investigate the impact of *Gstm1* deficiency on the development of hypertension and oxidative stress. This data supports the hypothesis that reduced renal *Gstm1* plays an important role in oxidative stress mechanisms underlying the development of hypertension and end organ damage. The clinical impact of the loss of *GSTM1* has recently been demonstrated in a subset of participants from the Atherosclerosis Risk in Communities Study [51]. In this community-based prospective cohort of black and white patients, zero or one copy of *GSTM1* was significantly and independently associated with higher risk of kidney failure and heart failure. These results suggest that *GSMT1* is a potential therapeutic target that warrants further detailed investigation, and our novel *Gstm1* transgenic SHRSP lines will provide an important experimental model for these future preclinical studies.

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Conflicts of interest

There are no conflicts of interest.

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