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Glutathione -S-Transferase μ 1 Regulates Vascular Smooth Muscle Cell Proliferation, Migration, and Oxidative Stress

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Abstract

Glutathione S-transferase μ-1, GSTM1, belongs to a superfamily of glutathione-S-transferases that metabolize a broad range of reactive oxygen species (ROS) and xenobiotics. Across species, genetic variants that result in decreased expression of the *Gstm1* gene are associated with increased susceptibility for vascular diseases, including atherosclerosis in humans. We previously identified *Gstm1* as a positional candidate in our gene mapping study for susceptibility to renal vascular injury characterized by medial hypertrophy and hyperplasia of the renal vessels. To determine the role of *Gstm1* in vascular smooth muscle cells (VSMCs), we isolated VSMCs from mouse aortas. We demonstrate that VSMCs from the susceptible C57BL/6 mice have reduced expression of *Gstm1* mRNA and its protein product compared to that of the resistant 129 mice. After serum stimulation, C57BL/6 VSMCs proliferate and migrate at a much faster rate than 129 VSMCs. Furthermore, C57BL/6 VSMCs have higher levels of ROS, and exhibit exaggerated p38 MAPK phosphorylation after exposure to H_2O_2. To establish causality, we show that knockdown of Gstm1 by siRNA results in increased proliferation of VSMCs in a dose dependent manner, as well as in increased ROS levels and VSM cell migration. Moreover, Gstm1 siRNA causes increased p38 MAPK phosphorylation, and attenuates the anti-proliferative effect of TEMPOL. Our data suggest that *Gstm1* is a novel regulator of VSMC proliferation and migration through its role in handling ROS. Genetic variants that cause a decremental change in expression of *Gstm1* may permit an environment of exaggerated oxidative stress, leading to susceptibility to vascular remodeling and atherosclerosis.

Keywords

Glutathione S-transferase μ-1; vascular smooth muscle cells; proliferation; migration; reactive oxygen species

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Disclosures
None.

Perspectives
Gene mapping and association studies have demonstrated that, across human rat and murine species, genetic variations of *Gstm1* gene are associated with cardiovascular diseases. The present study describes a novel function of *Gstm1* in the regulation of vascular smooth muscle cell proliferation and migration, perhaps through its role in handling oxidative stress. These findings implicate that genetic variants that cause even a modest decremental change in expression of *Gstm1* gene provide a permissive environment of exaggerated oxidative stress, leading to enhanced susceptibility to vascular remodeling and atherosclerosis. *Gstm1* may serve as a modifier of phenotype in disease states.

INTRODUCTION

Glutathione S-transferase μ-1, GSTM1, belongs to a superfamily of glutathione-S-transferases that metabolize a broad range of reactive oxygen species (ROS) and xenobiotics. There are 8 distinct classes of soluble GSTs that have been identified thus far according the substrate specificity, chemical affinity, structure and kinetic behavior of the enzyme (1,2). *GSTM1* belongs to the Mu (μ) class, and is one of the 5 μ class of *GST* genes in humans (3). In mice there are 7 *Gstm* genes, (4,5) and *Gstm1* is the most abundantly expressed among all *Gst* genes in the kidney (6).

In humans, a GSTM1 deficiency state exists in those carrying the null allele, *GSTM1(0)*, that arose from a recombination event during evolution between two highly homologous regions flanking this locus, resulting in deletion of a 20 kb segment (7,8). The prevalence of subjects carrying this allele in the homozygous state ranges from 30–50% in different human populations (9). The significance of this genetic variation in human was first recognized in cancer studies demonstrating that patients carrying the *GSTM1(0)* allele were at increased risks for colon and lung cancers (10),(11). In subsequent studies undertaken in cardiovascular disease, subjects homozygous for the *GSTM1(0)* allele were shown to have increased risks of hypertension (12) and atherosclerosis (13), and increased DNA alterations in atherosclerotic lesions of the abdominal aorta (14). Despite the circumstantial evidence for a role of the variant of the *GSTM1* gene in human disease, the exact contribution of this gene in the vasculature has not been well characterized.

In genetic studies of hypertension, genome-wide scans performed on several rat crosses identified QTLs involved in blood pressure regulation (15). Subsequently, congenic strains were derived to isolate one of these QTLs, and comparisons of microarray expression profiling of a congenic strain versus the original parental strains identified *Gstm1* as a positional and functional candidate gene (16). *Gstm1* mRNA expression was found to be reduced in the stroke-prone spontaneously hypertension rat (SHR) compared to the congenic and normotensive Wistar Kyoto (WKY) rats (17). The differences in mRNA expression levels were reflected at the protein levels in the kidney, and were inversely correlated with renal levels of ROS, suggesting that the pathophysiological role *Gstm1* in hypertension is likely to involve defense against oxidative stress (17).

In previous studies, we identified a single locus, *Msrvh1*, on chromosome 3 that was linked to renal vascular injury in a mouse model, the AT_{1A} receptor-deficient model, with vascular lesions characterized by medial hypertrophy and hyperplasia of the renal vasculature (18). As part of our screening process for possible positional candidate modifier gene(s), we first prioritized candidate genes as those that are differentially expressed between the susceptible (C57BL/6) and resistant (129S6) mouse strains. Within the critical interval of the *Msrvh1* region containing ~ 50 genes, only *Gstm1* exhibited robust and statistically significant differences in expression levels between the two strains. We found that the resistant 129 mice had twice the level of *Gstm1* expression compared to the susceptible C57BL/6.

Taken together, the evidence suggests that *Gstm1* plays a role in vascular homeostasis. The naturally occurring strain variation in *Gstm1* expression would provide a powerful model for testing the functional role of *Gstm1* in the vasculature. In this report, we determined strain differences in *Gstm1* expression in vascular smooth muscle cells (VSMCs), and assessed the role of *Gstm1* in VSMC proliferation, reactive oxygen species production, and migration.

MATERIALS AND METHODS

An expanded Materials and Methods section can be found online at http://hyper.ahajournals.org.

Primary vascular smooth muscle cell culture

VSMCs from aortas of 3–4 week old wild-type C57BL/6 (Jackson Laboratory, Bar Harbor, ME) and 129S6 (Taconic) were isolated by enzymatic digestion using collagenase, 1.5 mg/mL (Sigma), while suspended in Dulbecco's Modified Eagle Medium (DMEM) (GIBCO Laboratories) containing, L-glutamine, HEPES, penicillin and streptomycin. Cells were washed and grown in DMEM supplemented with 10% heat inactivated calf serum, penicillin (100 U/mL), streptomycin (100ug/mL) in 75 cm^2 Corning tissue culture flasks at 37°C in a humidified environment of 5% CO_2 and air.

Cell proliferation assays

Cell proliferation was measured using both MTS and BrdU assays.

Real-time RT-PCR

Total RNA was isolated from VSMCs or aorta by RNeasy Mini Kit (Invitrogen). One microgram of DNase I-treated RNA sample was reverse transcribed using SuperScript II First-Strand Synthesis System for RT-PCR (Gibco BRL) in a total reaction volume of 20 μL. Real-time RT-PCR was performed as described in the supplemental data.

DHE staining

VSMCs were seeded at 2×10^4/well in 24-well plate and allowed to grow overnight. Medium was then removed and cells were rinsed twice with HHBS, followed by addition of 1 mL HBSS to each well with 2.0 uM DHE. Cells were incubated at 37°C for 30 minutes in the dark. DHE was removed and cells were rinsed with HBSS twice, followed by addition of fresh HBSS. Fluorescence microscopy was performed after 30 min incubation.

Detection of H_2O_2 with DCF-DA

H_2O_2 in VSMC was measured using 2′7′dichlorofluorescein diacetate (DCFH-DA) (Sigma) probe. VSMCs at 5×10^3 per well were plated in a 96 wells plate, and allowed to grow overnight in DMEM medium. The medium was then removed and the cells were rinsed twice with HHBS. Cells were then incubated with 10 um DCFDA at 37°C with 5% CO2 for 30 min. The fluorescent signal was detected by a microplate reader (FLUOstar, OPTIMA, BMG Labtech, Germany) at 485 nm excitation and 535 nm emission.

Detection of Superoxide by lucigenin assay

Production of superoxide was measured by lucigenin-enhanced chemiluminescence response. Briefly, for cultured VSMCs, cell suspension was created by detachment with 0.25% trypsin and 0.02% EDTA. Cells were washed with modified Krebs buffer containingNaCl (130 mM), KCl (5 mM), $MgCl_2$ (1 mM), $CaCl_2$ (1.5 mM), K_2HPO_4 (1 mM), and HEPES (20 mM), pH 7.4, and were resuspended in Krebs buffer with 1 mg/mL BSA containing lucigenin (0.25 mmol/L). The cell concentration was then adjusted to 1×10^7/mL. To measure ROS production, the cell suspension was transferred into polypropylene tubes and assessed in a luminometer (OPTOCOMP I, GEM Biomedical Inc.). Counts were obtained at 10 minutes of incubation. Background counts determined in cell-free preparations were subtracted from total count.

RNA Interference and Cell Transfection

High-performance purity grade (>90% pure) small interfering RNAs (siRNAs) against Gstm1 (Gstm1-siRNA) was obtained from Ambion, Inc. siRNA, with a nonsilencing oligonucleotide sequence (nonsilencing siRNA) that does not recognize any knownhomology to mammalian genes, are used as a negative control (Control-siRNA). VSMCs were seeded at a density of 5×10^4 cells/well in 6-well plates and grown in DMEM containing 10% FCS. One day after

seeding, cells are transfected with 100 pmol of Control-siRNA, or 100 pmol of Gstm1-siRNA using lipofectomine NAiFect Transfection Reagent (Qiagen Inc) according to the manufacturer's instructions. Seventy-two hours post-transfection, the cells were then analyzed by Western blot, cell proliferation, DHE staining and Lucigenin, or migration assays.

Western Blot

The method used has been previously described (19). VSMC were lyzed in RIPA buffer with protease inhibitors. Rabbit anti-GSTM1 (generous gift of Dr. John Hayes (5)) was used at 1:2000 dilution.

Cell migration assay

Cell migration was assessed using 24-well plates with Transwell inserts (8.0 μm pore, Costar) as previously described (20).

Statistical analysis

Data are expressed as mean ± standard error (SE). For comparisons between strains, n = 3 aortas for each strain of 129 and C57BL/6 used for isolation of primary VSMCs. All experiments were performed in triplicates, and repeated 3–4 times. Student's t-test was used for all comparisons between two groups, and ANOVA was used for comparisons between three groups.

RESULTS

Strain differences in expression of *Gstm1* in VSMCs

Based on our previous gene mapping study demonstrating that *Gstm1* is a candidate gene for susceptibility to renal vascular pathology (18), we asked whether expression of *Gstm1* in vascular smooth muscle cells (VSMCs) is different between the susceptible C57BL/6 strain versus the resistant 129S6 (129) strain. We isolated VSMCs from the aorta from wild-type C57BL/6 and 129 mice and grew them in culture. By real-time RT-PCR and Western analysis, *Gstm1* mRNA and protein levels, respectively, were significantly decreased by 50% in C57BL/6 VSMCs compared to 129 VSMCs (Figure 1).

Strain differences in VSMC proliferation and reactive oxygen species production

While culturing primary VSMCs from the two different strains (n = 3 each), we noted that C57BL/6 VSMCs in culture proliferated and reached confluence at a much faster rate than 129 VSMCs. To better quantitate their growth rate, we measured VSMCs proliferation. By MTS assay (Figure 2A), starting at same cell number plating on day 0, with 10% serum, at 48 and 72 hours, C57BL/6 VSMCs proliferated two times faster than 129 VSMCs ($p \leq 0.03$). To confirm our observations, we also determined cell proliferation using the BrdU cell proliferation assay (Figure 2B). This assay also demonstrated a higher cell proliferation rate in the C57BL/6 VSMCs, and the difference was highly statistically significant by 24 hours.

Because a previous study showed that lower renal expression of *Gstm1* in stroke-prone SHR was associated with higher renal ROS levels compared to WKY rats (17), we examined ROS levels in our two different murine VSMC lines. As an indirect measure of superoxide levels, we performed dihydroethidium (DHE) staining of VSMCs in culture. At equal cell density by DAPI staining, DHE staining was significantly increased in C57BL/6 VSMCs compared to 129 VSMCs, suggesting increased superoxide levels (Figure 3A). We also assessed ROS (H_2O_2) generation in VSMCs using 2′7′dichlorofluorescein diacetate (DCFH-DA) probe. By detection of DCF fluorescence (Figure 3B) over a 50 minute interval, C57BL/6 VSMCs have much higher levels of ROS production compared to 129 VSMCs.

Reduction in Gstm1 causes increased cell proliferation and ROS production

To determine whether the difference in expression of *Gstm1* between the two different strains of VSMCs could directly cause differences in cell proliferation and ROS production, we used siRNA targeting *Gstm1* (Ambion, Inc). Using Gstm1-siRNA, we successfully knocked down *Gstm1* mRNA levels in both 129 and C57BL/6 VSMCs by 60–80% ($p < 0.05$) (Figure 4A). We next examined the effect of Gstm1 knockdown by siRNA on cell proliferation. As shown in Figure 4B, top panel, in both 129 and C57BL/6 VSMCs, compared to scrambled siRNA, Gstm1-siRNA resulted in significantly increased VSMC proliferation. Specifically, after 72 hours, 129 VSMC treated with Gstm1-siRNA had similar proliferation rate as C57BL/6 cells treated with scrambled-siRNA. Moreover, C57BL/6 cells treated with Gstm1-siRNA had significantly higher proliferation rate than cells treated with control-siRNA. Western blotting (Figure 4B, bottom panel) demonstrated that GSTM1 protein level in 129 Gstm1-siRNA treated cells were very similar to that of C57BL/6 control-siRNA condition. Furthermore, C57BL/6 cells treated with Gstm1-siRNA had barely detectable GSTM1 by Western analysis. Our data suggest that *Gstm1* exerts an anti-proliferative effect in a dose dependent manner.

We next determined whether knockdown of *Gstm1* expression in 129 VSMCs would affect ROS production. By DHE staining (Figure 5A), Gstm1-siRNA causes marked increase in superoxide production compared to control-siRNA. To better quantitate the differences, we performed an assay using lucigenin chemiluminescence. As shown in Figure 5B, in 129 VSMCs, Gstm1-siRNA caused a significant increase in superoxide production as measured by increased luminescence levels in VSMCs compared to control-siRNA.

Strain differences in p38 phosphorylation and effect of Gstm1 knockdown after exposure to hydrogen peroxide

ROS are thought to serve as second messengers that activate downstream kinases such as p38 MAP kinase (21). With two distinct VSMC lines with naturally occurring strain-dependent expression levels of *Gstm1*, we posited that the 129 VSMCs with higher expression levels of *Gstm1* would be more resistant than C57BL/6 VSMCs to p38 phosphorylation following H_2O_2 treatment. As expected, VSMCs from 129 demonstrated significantly less p38 phosphorylation compared to C57BL/6 after H_2O_2 exposure (Figure 6A). We next determined the effect of Gstm1 knockdown on p38 phosphorylation in 129 VSMCs. As shown in Figure 6B, Gstm1 siRNA resulted in a significant increase in p38 phosphorylation in 129 VSMCs after treatment with H_2O_2 compared to untransfected wild-type cells, or cells transfected with control-siRNA.

Reduction of Gstm1 attenuates anti-proliferative effects of TEMPOL

To determine whether the effect of Gstm1 on cell proliferation is mediated through its regulation of superoxide levels, we next assessed the effect of TEMPOL with and without Gstm1 knockdown. As shown in Figure 7A, Gstm1-siRNA blunted the inhibition of cell proliferation by TEMPOL by approximately 50% at both lower dose (2.5 mM) and higher dose (5 mM) of TEMPOL. Furthermore, with Gstm1-siRNA, the higher dose of TEMPOL was required to achieve similar degree of inhibition of cell proliferation as in the lower dose with control-siRNA. Conversely, TEMPOL attenuated the proliferative effect of Gstm1-siRNA (Figure 7B). Our data suggest that the effect of Gstm1 is mediated at least in part through its regulation of superoxide levels; loss of GSTM1 results in decreased clearance of superoxide, increased oxidative stress, and hence increased vascular smooth muscle cell proliferation.

Effects of *Gstm1* on vascular smooth muscle cell migration

It has been shown that ROS are key mediator for PDGF signal transduction, since blockade of H_2O_2 accumulation by catalase inhibits PDGF-induced migration of VSMCs (22). Since

reduction of *Gstm1* results in increased superoxide and H_2O_2 levels, we queried whether *Gstm1*, through its role in handling oxidative stress, regulates VSMC migration. We first examined migration rates between C57BL/6 and 129 VSMCs with low and high *Gstm1* expression, respectively. As shown in Figure 8A, the cell migration rates in untreated cells were similar between 129 and C57BL/6 cells. However, after stimulation with PDGF, cell migration rate was much faster in C57BL/6 VSMCs than 129 VSMCs. To determine if this difference might be contributed by differences in *Gstm1* expression, we used Gstm1-siRNA in 129 VSMCs to demonstrate causality. After Gstm1 knockdown with siRNA, 129 VSMCs migrated at a much faster rate compared to control-siRNA and untransfected conditions (Figure 8B).

DISCUSSION

Our studies demonstrate that genetic variation in expression of *Gstm1* is associated with differences in VSMC proliferation, ROS production, and cell migration. Furthermore, we establish a functional role of *Gstm1*, as we find that reduction of *Gstm1* in VSMC using siRNA directly causes increased cell proliferation, oxidative stress, and migration. Our findings of differences in proliferation between the 129 and C57BL/6 murine VSMCs are very similar to the earlier studies reporting that VSMCs isolated from thoracic aorta from the SHR strain proliferate faster than those from the WKY rats (23–27). More recently, *Gstm1* was identified as a positional and functional candidate gene for hypertension (15,16) and was found to be reduced in kidney tissues from stroke-prone SHR strain compared to the congenic and normotensive WKY rats (17). We suggest that the differences in cell proliferation between these two rat strains are also due, at least in part, to differences in *Gstm1* expression.

One important consideration is whether the effect we observe is specific to *Gstm1* or is due to combined effects of other differentially expressed *Gstm* genes. In this regard, we find that, in the mouse aorta, along with *Gstm1*, *Gstm4*, *Gstm5* are also more significantly highly expressed in 129 than C57BL/6 mice. However, similar to previous studies describing the relative expression of all *Gst* genes in the kidney (6), we also find that, in the aorta in both strains, the expression of *Gstm1* gene, relative to GAPDH, is significantly and several times higher than any other *Gstm* genes (Supplemental Figure S1), suggesting that, in the vasculature, *Gstm1* plays the most dominant role within its class, and perhaps among all the *Gst* classes, as GSTM1 is the most predominant glutathione s-transferase in both the mouse kidney (6) and lung (28), accounting for 45% and 60% of the total GST content, respectively. Moreover, we found no change in expression of the other 5 *Gstm* genes (*Gstm2 – Gstm6*) in VSMCs after reduction of Gstm1 by siRNA knockdown (data not shown). This suggests that the effect we observe *in vitro* is specifically due to knockdown of *Gstm1*, and that there is no loss or compensatory changes from other *Gstm* genes that could account for our observations. It is worth noting here that, while *Gstm7* has been reported, we have not been able to confirm expression of this gene using previously reported primers (29). We have re-designed primers for *Gstm7*, but have not successfully identified its expression in either mouse aortic tissues or isolated VSMCs.

Compared to the 129 strain, the C57BL/6 strain is more susceptible to atherosclerosis (30) and ocular neovascularization (31). In our genetic model of AT_{1A} receptor deficiency, we found that the C57BL/6 strain is more susceptible to renal vascular injury (18). Taken together, we speculate that the lower expression of *Gstm1* in the vasculature in the C57BL/6 strain contributes to their generalized increased susceptibility to vascular injury and remodeling.

Approximately 30–50% of individuals in most human populations completely lack the activity of the detoxifying enzyme gluthathione S-transferase M1. This results from homozygous inheritance of the *GSTM1* null allele, *GSTM1(0)*, resulting in a 20 kb segment deletion of the gene (7,8). The three genotypes, homozygously active *GSTM1/GSTM1*, heterozygously

deficient *GSTM1/GSTM1(0)*, and homozygously deficient *GSTM1(0)/GSTM1(0)*, are associated with a trimodal distribution of glutathione-conjugator activity, with high, intermediate and non conjugators, respectively (7,32). Examination of public databases reveals that *GSTM1* lies within a cluster with 4 other GSTM genes on human chromosome 1, and this region is a hot spot for gene copy number variation. Thus, it is likely that other *GSTM1* polymorphisms may also contribute to expression differences of *GSTM1* in humans. The exact polymorphism causing *Gstm1* expression differences in stroke prone SHR and WKY rats is unknown (17). We have sequenced the putative promoter region of the murine *Gstm1* gene, as well as all the exons, introns, and 3′ UTR region, and have not detected any sequence variation between the 129 and C57BL/6 mouse strains. Based on our gene mapping study (18), it is likely that the regulating variant affecting *Gstm1* expression, and that of *Gstm4* and *Gstm5*, is cis-acting and lies in a yet to be discovered regulatory region of the gene cluster.

In disease states such as atherosclerosis and arterial injury-induced neointimal hyperplasia, it is generally thought that a key component involves medial smooth muscle cell proliferation and migration into the arterial intima. We demonstrate here that VSMCs with lower *Gstm1* expression, from either naturally occurring genetic variation or si-RNA knockdown, have increased cell proliferation and migration rates. Our studies provide a possible mechanistic link for increased susceptibility to atherosclerosis in those carrying the *GSTM1(0)* allele.

The exact mechanism(s) by which *Gstm1* influences cell proliferation, ROS production and cell migration is unknown. We find that reduction of *Gstm1* expression results in increased production of ROS, which are thought to function as second messengers that activate the phosphorylation of downstream kinases such as p38 MAP kinase (21). We show that, with serum stimulation, *Gstm1* directly and dose-dependently affects vascular smooth muscle cell growth; the lower the *Gstm1* expression level, the higher the cell proliferation rate. Suh et al. previously showed that serum-induced proliferation of VSMCs is mediated by Nox1, a member of the NAD(P)H oxidase (33). NADPH oxidases are generally recognized to be the major contributor to ROS production in the vasculature (34). The pro-growth effect on VSMCs by the hormones angiotensin II, platelet derived-growth factor (PDGF), and thrombin, and the cytokine tumor necrosis factor alpha (TNFα) is thought to be via the common activation of NADPH oxidase (21). The result is an increased level of NADPH oxidase-derived $O_2^{\bullet -}$ that increases the activity of downstream kinases such as p38 MAP kinase and Akt (21). We find that C57BL/6 VSMCs are more susceptible to p38 phosphorylation compared to 129 when exposed to H_2O_2. Furthermore, p38 phosphorylation is virtually undetectable in 129 VSCMs at baseline, or even after exposure to H_2O_2, but is significantly enhanced after exposure to H_2O_2 in the presence of *Gstm1* siRNA. Taken together, we postulate that *Gstm1* modulates the NADPH oxidase-induced signaling pathway, perhaps through its role in regulating ROS levels.

Whether GSTM1 has superoxide dismutase and/or catalase-like activity remains to be determined. In our studies using low doses of TEMPOL, the addition of Gstm1 siRNA resulted in reduced effectiveness of TEMPOL to suppress cell proliferation. Moreover, TEMPOL attenuates the proliferative effect of Gstm1 siRNA. One alternative explanation is that GSTM1 reduces the effectiveness of TEMPOL simply through its metabolism of the compound. However, in 129 VSMCs, Gstm1-siRNA caused significant p38 phosphorylation after exposure to H_2O_2. These observations suggest that GSTM1 directly regulates ROS levels. It is possible that GSTM1 regulates intracellular ROS through its well-known catalytic activity in intracellular glutathione conjugation and metabolism, thereby regulating the redox state. In addition to its active catalytic site, GSTM1 also has a functional non-catalytic domain that inhibits activation of the ASK1-p38 signaling pathway. Under normal conditions, GSTM1, via its non-catalytic site, binds to the apoptosis signaling-regulating kinase-1 (ASK1), thereby inhibiting ASK1 activation (35). In conditions of stress such as heat shock or exposure to

H_2O_2, GSTM1 is thought to be released from ASK1, leading to activation of ASK1 and downstream kinases (36). Our finding that *Gstm1* expression regulates p38 phosphorylation is consistent with these earlier observations. Another possibility is that *Gstm1* modulates VSMC proliferation and migration through its role in protection against accumulation of lipid peroxidation products that are now recognized to influence cell proliferation and migration (37). It has been demonstrated that human subjects homozygous for *GSTM1(0)* have higher plasma levels of malondialdehyde (MDA), a product of lipid peroxidation (38).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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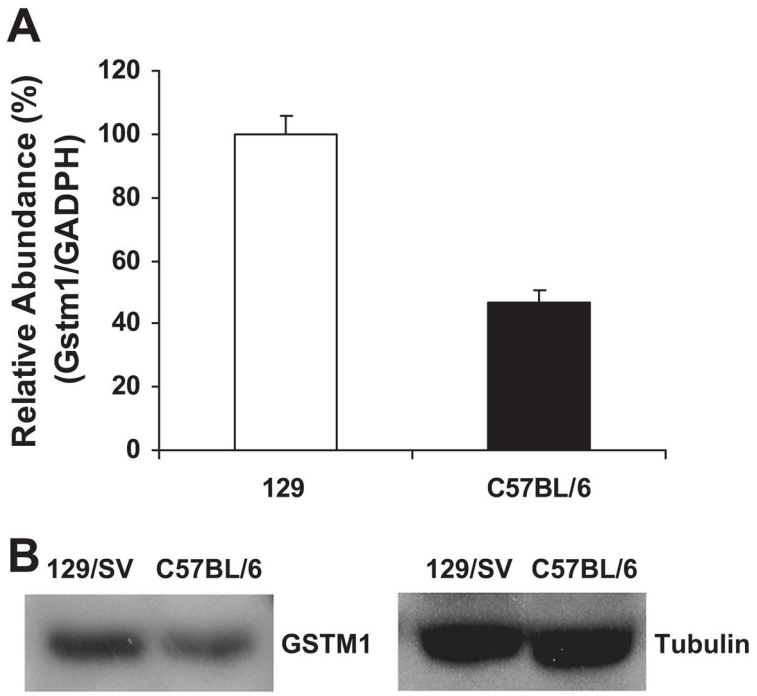

Figure 1. Strain differences in expression of *Gstm1* in vascular smooth muscle cells

A. Real time RT-PCR, expression of *Gstm1* is compared to GAPDH housekeeping gene. Values are expressed as relative abundance, with 129 value used as reference value, $p = 0.005$ between 129 vs. C57BL/6, $n = 3$ each, performed in triplicates. **B**. Western blot, showing abundance of GSTM1 enzyme is lower in C57BL/6 compared to 129 VSMCs.

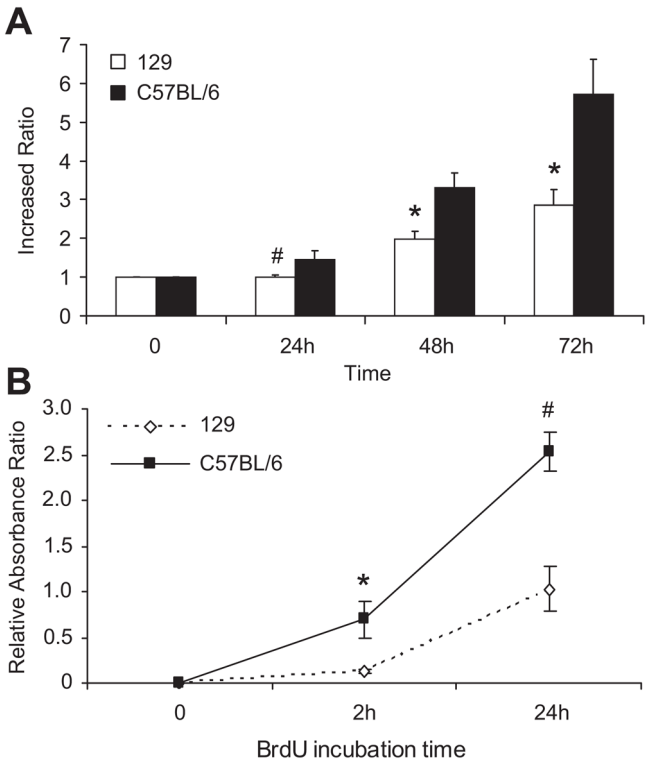

Figure 2. Strain differences in VSMC proliferation

A. By MTS assay, C57BL/6 VSMCs have higher proliferation rates compared to 129 VSMCs, # $p = 0.09$ at 24hr, * $p \leq 0.03$ at 48 and 72 hr. **B.** By BrdU assay, differences in cell proliferation between strains were detected as early as 2 hr, * $p = 0.01$, # $p = 0.001$. VSMCs were obtained from aortas from 3 separate mice from each strain. Cell proliferation assays were done using serum, performed in triplicates, and repeated 4 times in separate experiments. Cell passages between 1–3 were used and matched between strains.

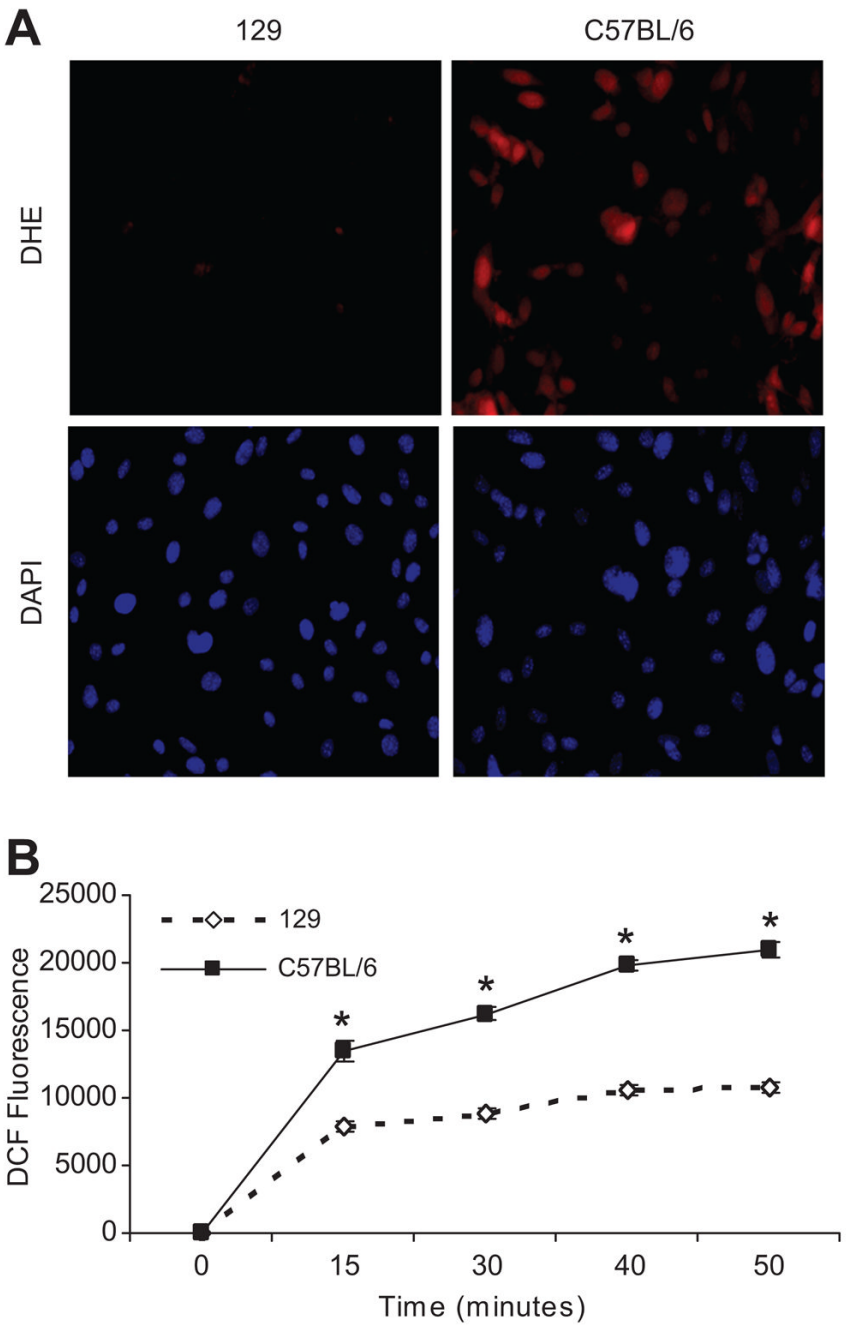

Figure 3. Strain differences in ROS production

A. DHE staining At relatively equal cell density determined by DAPI nuclear staining (lower left and right panels), there is very low level of DHE staining in the 129 cells (top left panel). However, in C57BL/6 VSMCs, there is dramatically higher level of DHE staining (top right panel), suggesting higher levels of superoxide. **B. DCF-DA assay.** The DCF fluorescent signal, a measure of H_2O_2 levels, is significantly higher in C57BL/6 VSMCs compared to 129, * $p \leq 0.001$; n = 3 each, performed in triplicates in 3 experiments.

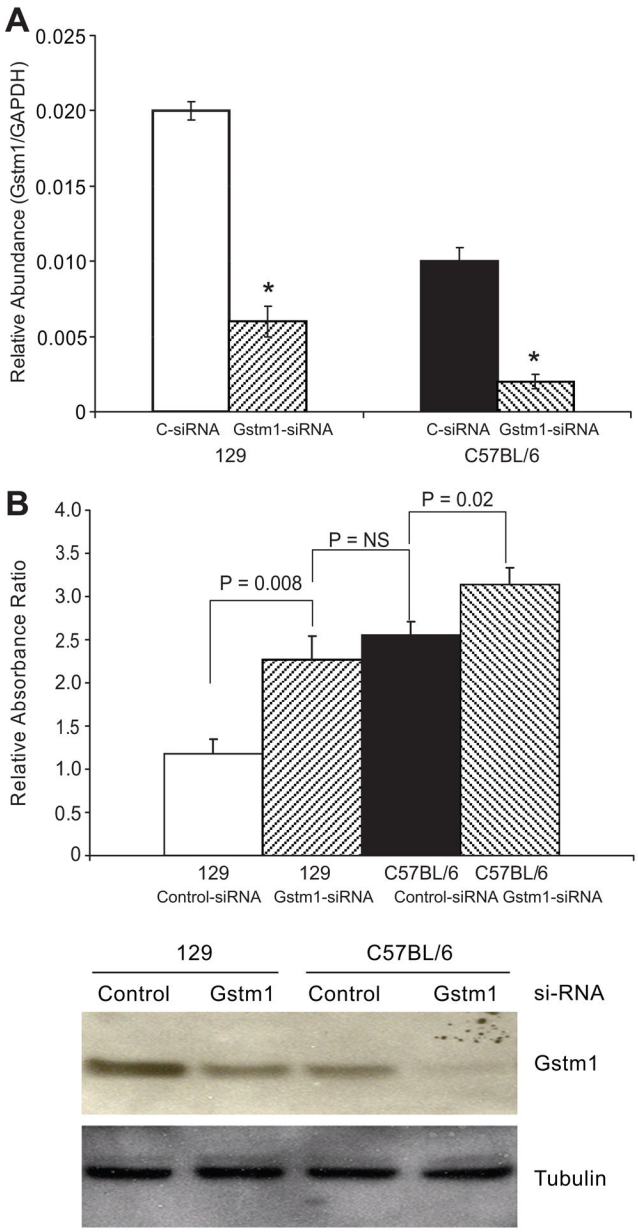

Figure 4. Effect of knockdown of *Gstm1* by si-RNA

A. Gstm1-siRNA successfully decreased *Gstm1* expression in both 129 and C57BL/6 VSMCs by 60–80%, * $p < 0.0005$ compared to control (C-siRNA); n = 3 each, performed in triplicates. **B. Upper panel**: Gstm1-siRNA resulted in significantly increased VSMC proliferation. 72 hours after si-RNA transfection, 129 VSMC treated with Gstm1-siRNA had significantly higher proliferation rate as 129 cells treated with control-siRNA ($p = 0.008$ 129 Control-siRNA vs 129 Gstm1-siRNA), but similar proliferation rate as C57BL/6 cells treated with Control-siRNA ($p = 0.40$ 129 Gstm1-siRNA vs C57BL/6 Control-siRNA). C57BL/6 cells treated with Gstm1-siRNA had an even higher proliferation rate than cells treated with control-siRNA ($p = 0.02$ C57BL/6 Control-siRNA vs C57BL/6 Gstm1-siRNA). N = 3 for each condition, performed in triplicates, in 3 separate experiments. **Lower panel**: Western analysis demonstrates successful knockdown of the enzyme in both cell lines compared to control-siRNA. Of note, Gstm1-siRNA reduced GSTM1 protein expression of 129 VSMCs to similar

level as seen in C57BL/6 cells treated with control-siRNA. C57BL/6 cells treated with Gstm1-siRNA had barely detectable GSTM1 protein levels.

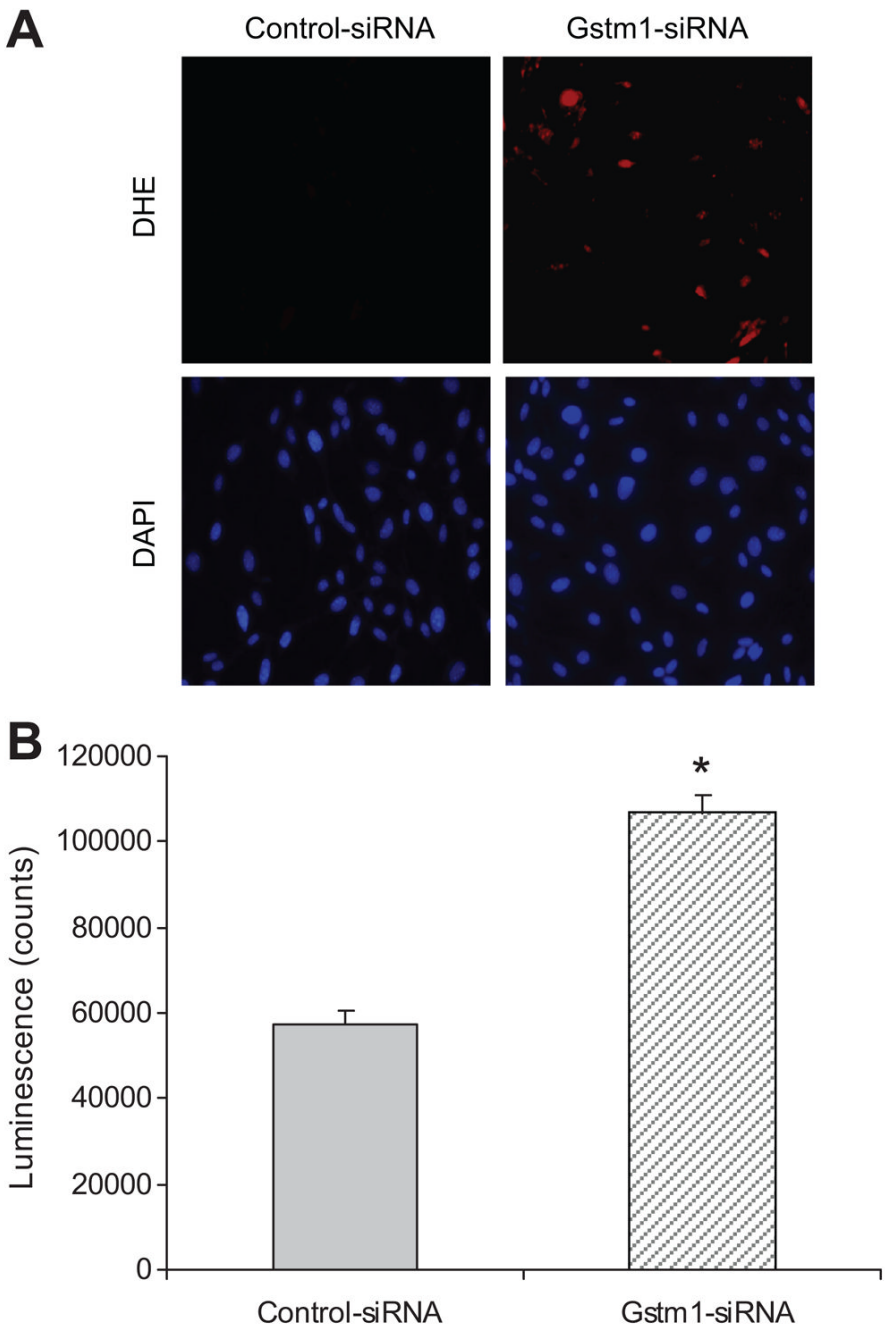

Figure 5. Effect of Gstm1 knockdown on superoxide levels in 129 VSMCs

A. DHE staining Compared to control-siRNA, Gstm1-siRNA resulted in significantly enhanced DHE staining (top panels) in 129 VSMCs. The bottom panels represent DAPI staining, showing relatively equal cell density. **B. Lucigen-enhanced chemiluminescence assay.** After transfection with Gstm1-siRNA, 129 VSMCs display significantly higher lucigenenin luminescence counts than 129 VSMCs transfected with control-siRNA, * $p < 0.005$, $n = 3$ each condition, performed in triplicates in 3 separate experiments.

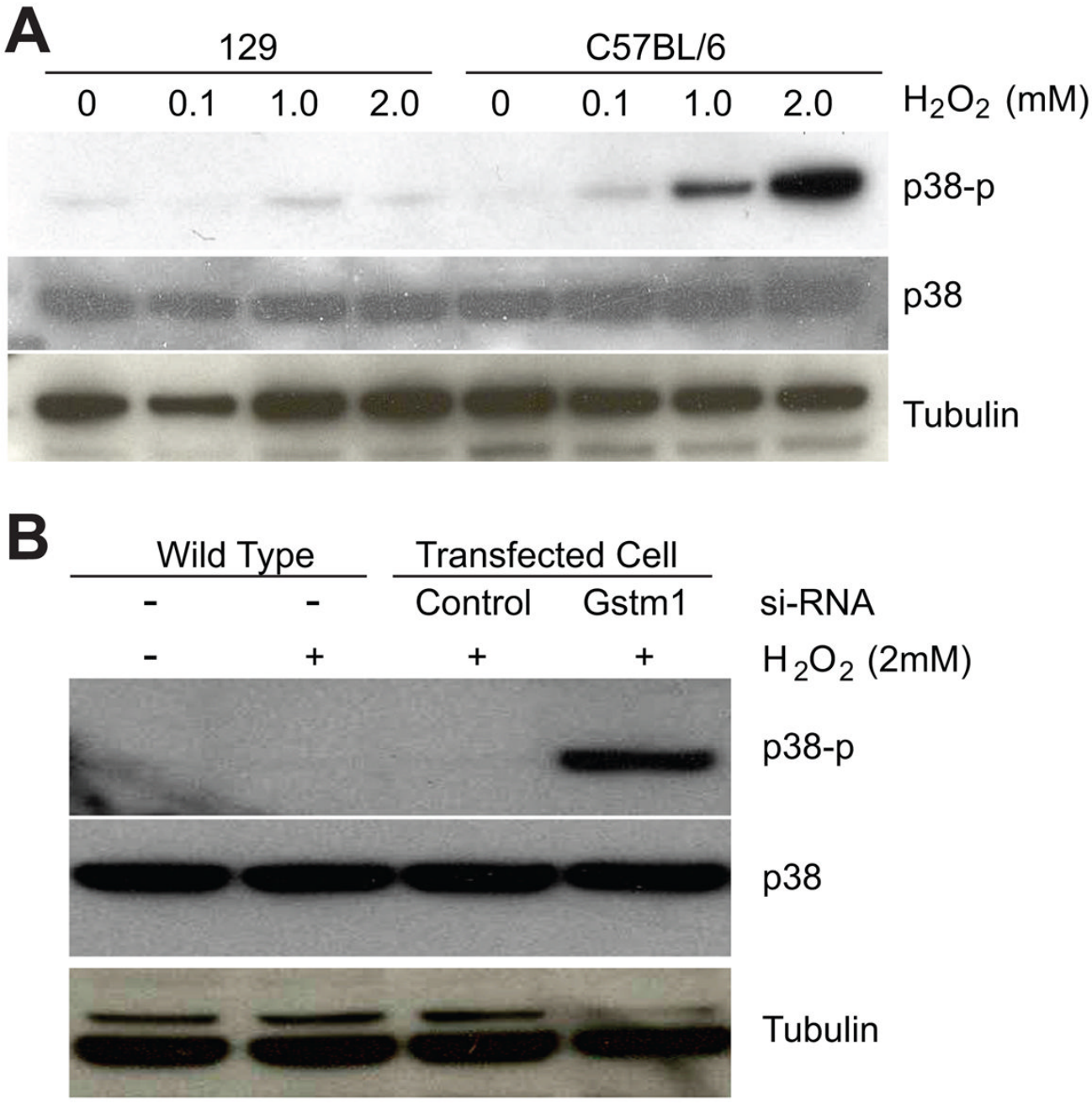

Figure 6. Activation of p38 kinase in response to H_2O_2. A. Strain differences in p38 MAP kinase phosphorylation

129 VSMCs, with higher levels of *Gstm*1, demonstrated attenuated p38 phosphorylation after treatment with H_2O_2 compared to C57BL/6 VSMCs. **B. Effect of Gstm1 knockdown on p38 phosphorylation in 129 VSMCs.** Gstm1-siRNA transfection resulted in significant increase in p38 phosphorylation compared to control-siRNA or un-transfected conditions in 129 VSMCs exposed to H_2O_2.

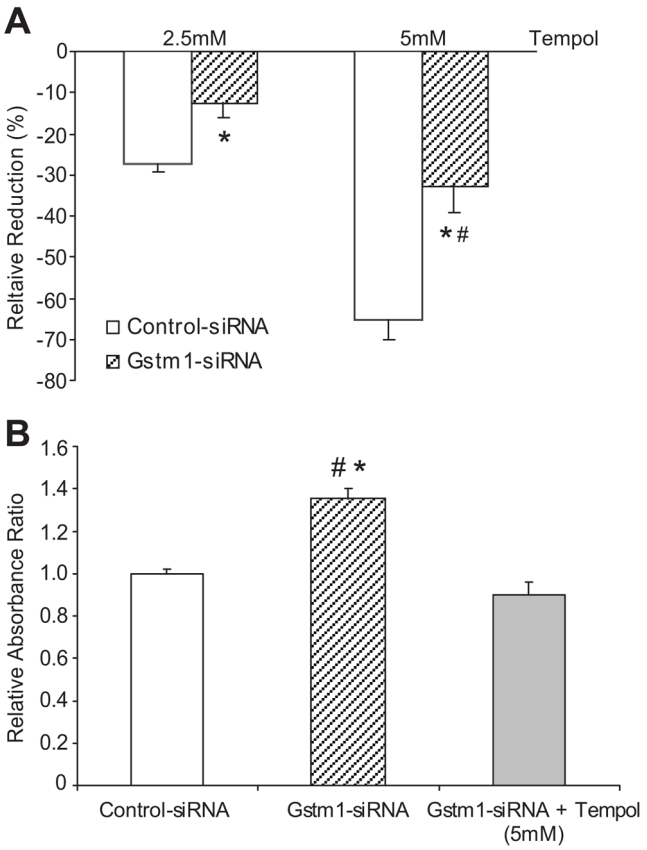

Figure 7. A. Effect of Gstm1 knockdown on inhibition of VSMCs proliferation by TEMPOL

Gstm1-siRNA attenuates the anti-proliferative effect of TEMPOL by ~ 50%, * $p \leq 0.008$ vs. control-siRNA, # p = NS for Gstm1-siRNA at 5 mM TEMPOL vs. control-siRNA at 2.5 mM TEMPOL. **B: Effect of TEMPOL on proliferative effect of Gstm1 knockdown.** TEMPOL blunts the proliferative effect of Gstm1-siRNA, * p = 0.006 versus control-siRNA, # p = 0.001 versus Gstm1-siRNA + TEMPOL (5 mM), p = NS for control-siRNA vs. Gstm1-siRNA + TEMPOL (5 mM). N = 3 for each condition, performed in triplicates in 3 separate experiments.

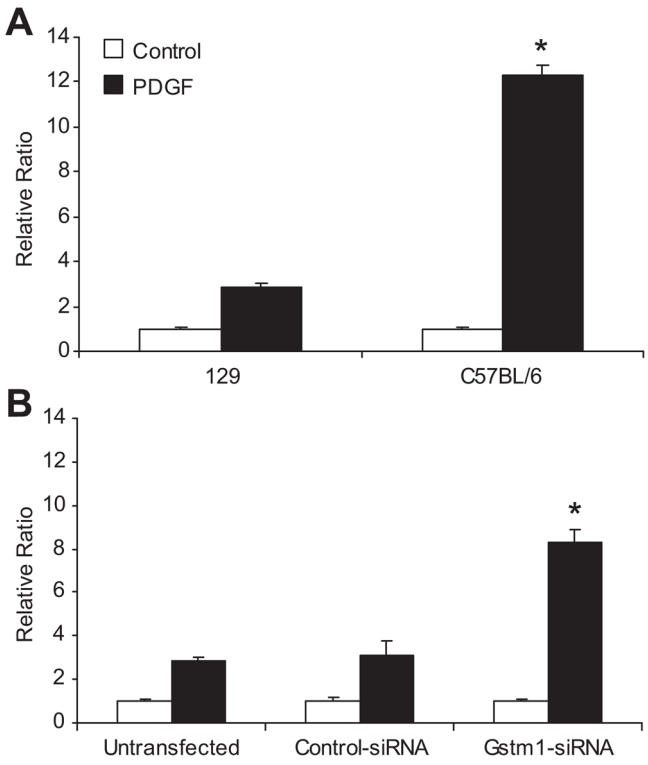

Figure 8. A. Strain differences in vascular smooth muscle cell migration

Basal VSMC migration was equivalent in 129 and C57BL/6 cell lines. However, after PDGF-BB stimulation, C57BL/6 VSMCs migrated at a much faster rate than 129 VSMCs, * $p = 0.002$, $n = 3$ each condition performed in triplicates. **B. Effect of Gstm1 knockdown on migration of 129 VSMCs.** Basal VSMC migration was similar between untransfected 129 cells and cells transfected with control- or Gstm1-siRNA. However, after stimulation with PDGF-BB, 129 VSMCs transfected with Gstm1-siRNA migrated at a much faster rate compared to untransfected or control-siRNA transfected cells, * $p = 0.009$, ANOVA, $n = 3$ each condition performed in triplicates in 3 separate experiments.