Deep sequencing of transcriptome profiling of GSTM2 knock-down in swine testis cells

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Glutathione S-transferases (GSTs), as members of a supergene family of multifunctional enzymes, play an important role in the defense to a wide array of toxic and carcinogenic substances by catalyzing the conjugation of glutathione (GSH) with a broad range of electrophilic compounds. Up to now, eight distinct classes of GSTs have been identified, whereas, different class of GST enzymes have different functions. Mu class (GSTMs) are mainly involved in eliminations of free radicals, peroxides, electrophilic reagents, heavy metals, also, they mediates and regulates cells protection and growth. Among the members of GSTMs, GSTM2 is a potential candidate involved in reproductive regulation due to high expression level in spermaduct, epididymis, testis, ovary, and oviduct, which was mentioned by a study for mammalian reproduction. It is reported that ova resists the endogenous and exogenous toxic substances by GSTM2 in ovary, which characterizes GSTM2 as a protector for germ cells. GSTM2 participates in the generation of prostaglandin E2 (PGE2) that is essential for testis maturation and embryo implantation. GSTM2 is up-regulated forcefully in luminal epithelium of uterine at the day 3 and day 4 after pregnancy, and additionally, progesterone is probably involved in up-regulation of GSTM2, which shows the necessity of GSTM2 in the preparation of uterus in blastocyst implantation process. Interestingly, the high expression of GSTM2 in progression of embryonic reactivation suggests the potential effect on embryo development.

In a previous study of our lab, it has been identified that a premature translation termination codon (PTC) caused by a nonsense mutation (CGA → TGA) resulting from a C27T substitution in the fifth exon of GSTM2. Nonsense-mediated mRNA decay (NMD) could degrade the mutated porcine GSTM2 mRNA because of the specific identification and degradation of aberrant transcripts harboring a premature termination codon (PTC). Interestingly, the homozygous genotype TT was not found in 164 individuals from Large White, Landrace, Meishan and Qingping pigs. The embryo with a GSTM2 TT genotype would be most likely to die or abort. To give insight into the role of GSTM2 in embryo development, RNA-seq was performed from ST cells treated with siRNAs targeting GSTM2.

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Results

Small interfering RNA treatment repressed GSTM2 in swine testis cells. Three pairs of siRNAs named si1, si2, and si3, were designed to suppress expression of GSTM2 in ST cells. The mRNA and protein level of GSTM2 was decreased significantly ($P < 0.01$) at 24 h after transfection (Supplementary Fig. S1a), and furthermore, si2 worked best for the suppression (Supplementary Fig. S1b).

Sequence quality and saturation analysis. RNA integrity was assessed by BioAnalyzer in the present study. The RIN value of all samples were over 7. The raw data which contained adaptor sequences were transformed into clean tags. Preparation and experiments of sample RNA were thought to be convincing if the tags contains N were less than 10% of total raw data, and copy number less than 2 tags was no more than 20%. The data showed that all the samples conformed to sequencing requirement and the experiments were successful (Supplementary Fig. S2a). In addition, the repeatability of RNA-seq was tested (Supplementary Fig. S2b). R values both from Spearman and Pearson analyses over 8 showed a high repeatability between two samples. The saturation analysis could be performed to check whether the number of detected genes keep increasing when sequencing amount (total tag number) increases. The data showed that when sequencing amount reaches 2 M or higher, the number of detected genes almost ceases to increase (Supplementary Fig. S2c). Thereby, the result indicated that the high-throughput Illumina sequencing data were exhaustive.

Data analysis of RNA-seq. In the present study, six cDNA libraries (TR1, TR2, TR3, CK1, CK2 and CK3) were established by reverse transcription of total RNA from three wells of treated ST cells (with siRNA2) and three wells of un-treated ST cells respectively. The standard analyses for quality control$^{14}$ was conducted to ensure the quality of RNA met the requirement for sequencing (Supplementary Fig. S3). A total of 11,927,452 (98.78%), 11,865,576 (99.01%), 11,870,090 (98.74%) clean reads were obtained from three treatment groups respectively. 11,790,005 (98.79%), 12,129,113 (98.75%), and 12,192,917 (98.85%) clean reads were obtained from three control groups respectively (Supplementary Table S2a). A total of 22,966,999 reads of treatment groups and 24,559,030 reads of control groups were mapped to reference gene. A total of 26,394,293 reads of treatment groups and 27,449,663 reads of control groups were mapped to reference genome respectively (Supplementary Table S2b). The raw data was obtained from transformed sequence data by Base Calling, and then the raw reads that contained adaptor sequence data and N were removed. Furthermore sequence data were filtered for low-quality reads at high level of stringency. After these steps, approximately 99% reads of the raw data were clean reads, whereas only 1% of the data came from adaptor sequences attached to empty vectors. The clean reads were mapped to the reference gene and the reference genome of pig (Supplementary Table S2b and Supplementary Table S3). The clean reads were mapped to the reference genome of pig (Supplementary Table S2b and Supplementary Table S3). The data showed that all the samples conformed to sequencing requirement and the experiments were successful (Supplementary Fig. S2a). In addition, the repeatability of RNA-seq was tested (Supplementary Fig. S2b). R values both from Spearman and Pearson analyses over 8 showed a high repeatability between two samples. The saturation analysis could be performed to check whether the number of detected genes keep increasing when sequencing amount (total tag number) increases. The data showed that when sequencing amount reaches 2 M or higher, the number of detected genes almost ceases to increase (Supplementary Fig. S2c). Thereby, the result indicated that the high-throughput Illumina sequencing data were exhaustive.

Differentially expressed genes (DEGs) between treatment groups and control groups. Total of 242 different transcripts within 131 up-regulated DEGs and 111 down-regulated DEGs were identified through the EdgeR analysis ($| \log_{2} \text{Ratio} | \geq 1$, FDR $\leq 0.001$) in treatment groups compared to control group (Fig. 1 and Supplementary Table S4).

Verification of DEGs with quantitative real-Time PCR (qRT-PCR). Several DEGs related to embryo development from RNA-seq were screened for validation by qRT-PCR (Fig. 1C). The results showed that the expression of SPP1, ADAMTS1, ITGA9, MUC4, and SRC was down-regulated, whereas STAT1, CYR61, DUSP1, TIMP3 and MMP19 was up-regulated, which were consistent with the expression profile of RNA-seq.

Gene ontology and KEGG pathway enrichment analysis for DEGs. GO classification of the DEGs revealed that 110 genes are involved in metabolic processes, 37 in stimuli, 38 in immune system processes, and 13 in biological adhesion, respectively (Fig. 2A). These 242 DEGs mainly participated in over 30 pathways according to PANTER, including inflammation mediated by chemokine and cytokine signaling pathways, integrin signaling pathways, Parkinson’s disease, angiogenesis, gonadotropin releasing hormone receptor pathway, interleukin signaling pathway, angiogenesis, gonadotropin releasing hormone receptor pathway, interleukin signaling pathway, EGF receptor signaling pathway, FGF signaling pathway etc. (Fig. 2B). Detail information of GO and pathway analysis was showed in Supplementary Table S5 and Supplementary Table S6, respectively.

Genes involved in the Maternal-Placental Interface and embryonic development. The knock-down of GSTM2 in ST cells alerts the expression of some genes involved in maternal-placental interface and embryonic development. Three SLC family genes (SLC5A10, SLC3A1, and SLC37A4) were down-regulated in GSTM2 knock-down ST cells. Another down-regulated DEGs is FGFFR4, which is a member of the fibroblast growth factor receptor (FGFR) family (Supplementary Fig. S4A). Furthermore, six genes related to the process of cell adhesion were down-regulated in the GSTM2 knock-down ST cells, including tetraspanin 3 (TSPAN3), disintegrin and metalloproteinase with thrombospondin motifs 1 (ADAMTS1), integrin, alpha V (ITGA9), GTP-binding RAS-like 3 (DRA53), immunoglobulin superfamily 11 (IGSF11) and secreted phosphoprotein 1 (SPP1, also known as osteopontin (OPN)) (Supplementary Fig. S4B). More than 30 IFN-stimulated genes were up-regulated, including JSG family, IFIT, IRF, MX etc. (Supplementary Fig. S4C). In addition, IFN-stimulated genes, some cytokines and chemokines in the endometrium related to implantation, including CCL2, CCL4, CCL20, CCL5, and CXCL8 were also up-regulated. Another up-regulated gene, CSF1, is a specific hemopoietic growth factor regulating survival, proliferation and differentiation of mononuclear phagocytes.

Suppression of STAT1 phosphorylation by overexpression of GSTM2. In order to provide more insight into relationship between GSTM2 and STAT1, we overexpressed GSTM2 in ST cells. Protein
level of STAT1 was decreased (Supplementary Fig. S5A,C) when GSTM2 was overexpressed. In addition, the mRNA expression level of downstream targets of STAT1 including ITGA5, SRC, and OPN were up-regulated (Supplementary Fig. S5B). Overexpression of GSTM2 suppressed the phosphorylation of STAT1 (Fig. 3A). Furthermore, co-immunoprecipitation assay indicated that GSTM2 could bind to STAT1 (Fig. 3B).

**Overexpression and interference assay of STAT1.**  STAT1 was knocked down in ST cells by RNAi (Supplementary Figure S6 A–D). The mRNA expression of MUC4, ADAMTS1, OPN, and ITGA5, which belong to downstream targets of STAT1, were decreased (P < 0.05, Fig. 4A,B), but did not have a significant change (P > 0.05) when STAT1 was overexpressed (Supplementary Figure S6 E,F). IFIT1, IFIT3, MX1, OAS1, OAS2, and immune-related genes were up-regulated (P < 0.05, Fig. 4C) in the presence of overexpression of STAT1.
Expression profile of GSTM2 and STAT1 in porcine endometrium. The expression level of GSTM2, STAT1 and some other DEGs was detected in porcine endometrium with different developmental stages, including day 0, day 12, day 15, day 18 and day 32 of gestation stages. The expression level of GSTM2 was increased at day 18 and day 32 compared to day 0 and day 12, whereas the STAT1 was increased at day 12, day 15, and day 18 gradually compared to 0d. Both GSTM2 and STAT1 had a highest expression level at day 18, whereas, the expression sharply decreased at day 32 (Fig. 5A). The expression level of downstream targets of STAT1 including immune-related genes IFIT1, IFIT3, ISG15, B2M, and adhesion process-related genes SPP1, MUC4, ITGAV and MX1, was also detected. Interestingly, all these genes expressed abundantly at day 15 and day 18 compared to day 0 and day 12 except for ITGAV, whereas, expression of SPP1, MUC4 and ITGAV began to increase at day 12. Furthermore, the expression of ISG15 and SPP1 was still high at day 32, whereas the others were resumed to the expression level of day 0. The expression pattern of MUC4, B2M and MX1 was similar to that of STAT1 (Fig. 5B).
Discussion

As one kind of important phase II antioxidant enzymes in vivo, GSTs were a wide array of toxic and carcinogenic substances. In this superfamily, mu class of GST could eliminate free radicals, peroxides, electrophilic reagents, heavy metals, and mediate cell protection and the regulation of cell growth. GSTM2, a member of GST mu class, was widely and highly expressed in various tissues including embryos, and testis \(^{11}\). In gestation stage of mice, GSTM2 expressed at a low level in luminal epithelium at day 3, but expressed highly at day 4 during early pregnancy \(^8\). In a previous study from our lab, the NMD-induced degradation of GSTM2 was most likely to cause embryonic death or abortion. Furthermore, this genotype that could be degraded by NMD was not found in 164 adults from Large White, Landrace, Meishan and Qingping pigs \(^{11}\). Here we knocked down GSTM2 by small interference RNAs in vitro to simulate the degradation of GSTM2 in vivo. High-throughput sequencing with the GSTM2-knockdown ST cells would be helpful to deeply understand the function of GSTM2.

The transcriptome data obtained from the present study were useful for the elucidation of function of GSTM2. Usually more than 18M reads from RNA-seq analyses are required for each sample to attain a saturated state for novel gene discovery and expressional analysis \(^{14,20}\). The quality control of our data revealed that the RNA-seq data were well qualified (Supplementary Table S2).

Bioinformatics analyses of DEGs discovered DEGs between the GSTM2-knockdown group and control group. After filtering with the standard, 242 DEGs were obtained from there six samples (Fig. 1B). Some down-regulated genes, including TSPAN3, ADAMTS1, ITGA5, DIRAS3, IGSF11 and SPP1, were involved in embryo implantation. Among them, TSPAN3 as a member of transmembrane 4 superfamily (TM4SF) involved in the adhesion, migration, proliferation, differentiation and signal transduction of cells \(^{21}\) has a high expression at the blastocyst of *Xenopus Laevis*, and furthermore played an important role in the communication between blastocyst and endometrium in mammals \(^{22-24}\) especially in cow \(^{25}\).

![Figure 4. Knockdown and overexpression experiment of STAT1 in ST cells.](image_url)

(A) Knockdown STAT1 in ST cells using siRNA and detect downstream genes at 24 h using q-PCR. (B) Knockdown STAT1 in ST cells using siRNA and detect downstream genes at 48 h using q-PCR. (C) Overexpress STAT1 in ST cells and detect downstream genes at 24 h using q-PCR. *P < 0.05.
According to our data, three SLC family genes (SLC5A10, SLC3A1, and SLC37A4), one FGFR family gene (FGFR4), and six cell adhesion-related genes (TSPAN3, ADAMTS1, ITGAV, DIRAS3, IGSF11, and OPN) were down-regulated in GSTM2-knockdown ST cells. Whereas, a lot of IFN-stimulated genes including ISG family, IFIT, IRF, MX, and some cytokines/chemokines including CCL2, CCL4, CCL20, CCL5, and CXCL8 were up-regulated. Among these genes, OPN was a powerful gene involved in embryo implantation. The expression of OPN was modulated by estrogen in pregnancy. OPN is also one component of the extracellular matrix (ECM) serving as an integrin ligand. Additionally, it was expressed in a small number of stroma cells at day 9 of pregnancy, the uterine LE adjacent to conceptus tissue beginning at day 12 and throughout the LE surface by day 20. Although OPN was clustered to estrogen-responsible gene, the concrete regulation of estrogen on expression of OPN was indirect through its interactions with ERα. The SRC family members enhanced the transcriptional activity of a variety of nuclear receptors, including ERα, ERβ, and PR. The expression of GSTM2, SRC and OPN was down-regulated significantly at the interface. Therefore, we suspected that GSTM2 regulated the expression of SRC that affected the activity of ERα and resulted in embryo implantation by suppressing OPN expression. OPN expressed both in endometrial epithelial cells and in trophoblast cells due to the interaction between OPN and trophoblast expression of integrin. This interaction achieved embryo adhesion and early communication to mother directly. OPN binds to the receptors including CD44 and integrins on the cell surface, and then initiated a variety of kinase cascades, including focal adhesion kinase (FAK) and phosphatidylinositol 3-kinase (PI3K)/apoptosis signal-regulating kinase 1 (AKT) signaling. The increase of adhesion complex assembly in OPN treated blastocysts was mediated through FAK- and PI3K-dependent signaling pathways. GSTM2 was a downstream gene of PI3K. Thus, knockdown GSTM2 may affect embryo adhesion by modulating interactions between OPN and integrins. OPN as a kind of ECM protein was degraded by MMPs. According to the data of RNA-seq, expression of MMP19 was raised in the GSTM2 knockdown group, which was likely to lower expression of OPN. However, the protease activity of MMPs was correlated with TIMPs, whereas, both of these proteins maintained the stability of the extracellular matrix. The expression of TIMP3 gene was increased in the GSTM2 knockdown group, whereas expression of MMP19 was elevated as well. Only if the expression of TIMPs/MMPs was balanced, can embryo implantation be carried out. The knock-down of GSTM2 may break the balance. Therefore we assumed that GSTM2 repressed the activity of ER via the regulation of SRC, then the expression level of OPN changed.

IFN-stimulated genes and some cytokines/chemokines were identified from DEGs, but there was no evidence to prove that GSTM2 could regulate these genes. STAT1 is involved in JAK-STAT pathway, which could be stimulated by some IFNs and cytokines, and regulates its downstream targets including some IFN-induced genes (IFNGs) and immune-related genes. Interestingly, previous study indicated that the interaction between GSTP1 and STAT3 regulated the phosphorylation of STAT3. For this reason, we assumed that GSTM2 would regulate

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**Figure 5. Temporal expression profiles of interesting genes.** Expression level of GSTM2, STAT1, SPP1, ITGAV, MUC4, IFIT3, ISG15, B2M, IFIT1, and MX1 in porcine endometrium. Day 0, 12, 15, 18, and 32 were selected for the detection. *P < 0.05, **P < 0.01.
phosphorylation of STAT1. The result of co-IP experiment indicated that GSTM2 could bind to STAT1 (Fig. 3B), the phosphorylation level, furthermore, was reduced by the overexpression of GSTM2 (Fig. 3A). It provided a better explanation for expression changes of downstream targets of STAT1. We performed the overexpression and interference assay of STAT1 to confirm whether these IFN-stimulated genes and some cytokines/chemokines were the downstream targets of STAT1. When STAT1 was knocked down, the expression of MUC4, ADAMTS1, OPN, and ITGAV were decreased (P < 0.05, Fig. 4A,B), whereas, IFIT1, IFIT3, MX1, OAS1 and OAS2, immune-related genes were up-regulated (P < 0.05, Fig. 4C) in the presence of overexpression of STAT1. Hence the activation of STAT1 would induce the up-regulation of IFNGs. IFNGs could enhance the uterus acceptance, however, the high expression of IFNGs would induce the blastocyst delay. Maternal immune recognition of embryos occurs through two major pathways. One is that the immune system can detect the presence of alloantigens or receptor ligands on the conceptus. The other is that the immune system could be activated by chemokines and cytokines produced by the conceptus. There were two classes of major histocompatibility complex (MHC) antigens, whereas, only MHC class I antigens were expressed on the surface of the conceptus. MHC class I molecules or transcripts could be detected throughout development to the blastocyst stage in cow. At this period, the period of placental attachment, little MHC antigens were expressed. Available evidence has indicated the down-regulation of MHC antigen class I in porcine trophoblast from days 14 to 25. Some chemokines and cytokines, including interferons and CCL, activated the immune system, started the pregnancy recognition and protected against some bacteria during the peri-attachment period. Some of MHC genes were up-regulated with the knock-down of GSTM2, including MHC class I antigens SLA7, SLA11 and SUSC-MIC1, and MHC class II antigens SLA-DQA1. Porcine conceptus trophoderm cells induced the expression of SLA class I and β2m genes through secretion of IFN-β or IFN-γ in uterine stromal, but this expression was silenced in LE in order to prevent immune rejection at the uterine-placental interface. SLA-DQA1 was expressed responding to IFN-γ from the conceptus. Additionally, it likely regulated immune responses at the maternal-fetal interface in order to support the maintenance of pregnancy in pigs. Many IFN-stimulated genes were up-regulated, including ISG family, IFIT, IRF, MX etc. (Supplementary Fig. S4C), whereas the effects of expression of Mx were different in pregnant and non-pregnant pigs. Changes of related genes suggested that GSTM2 was associated to immunological recognition of the conceptus. STAT1 participated in type I and type II interferon signaling pathways. In the type I interferon activating pathways, GSTM2 may be involved in IFNs signaling pathways by regulating STAT1.

We also detected the expression of related genes in vivo. The expression level of GSTM2 was increased at day 18 and day 32 compared to day 0 and day 12, whereas the STAT1 was increased at day 12, 15, and 18 gradually compared to day 0. The high level of STAT1 at day 12 and day 15 did not induce the increase of expression of GSTM2. Both GSTM2 and STAT1 expressed highest at 18d, whereas, the expression sharply decreased at day 32 (Fig. 5A). Day 18 is essential time point for pig embryo implantation. Maybe more GSTM2 was needed to suppress the effect of STAT1. The expression level of downstream targets of STAT1 containing immune-related genes IFIT1, IFIT3, ISG15, B2M, and adhesion process-related genes SPP1, MUC4, MX1 expressed highly at day 15 and day 18 compared to day 0 and day 12, whereas, expression of SPP1, MUC4 and ITGAV began to increase at day 12. Furthermore, the expression of ISG15 and SPP1 was still high at day 32, whereas the others were resumed to the expression level of day 0. The expression pattern of MUC4, B2M and MX1 was similar to that of STAT1 (Fig. 5B). A previous study about implantation showed that GSTM2 was highly expressed during the early stage of implantation, and this condition just occurred in a very short period. In addition, IFN was able to increase the capacity of the endometrium to adapt new conceptus, whereas overexpression of IFNG may induce delay of conceptus sometimes by 15–23 days. In order to protect conceptus from the delay induced by IFNG, expression of GSTM2 was up-regulated in the early stage of implantation. Porcine embryo implantation started at day 13 and finished at day 24 after pregnancy. In general, we assumed that GSTM2-STAT1 promoted embryo implantation. Blastocyst attachment to the LE was only achieved by the transitional labilization and the remodeling of uterine epithelium polarity after the synchronous exchange of signals between the conceptus and endometrial cells. Vascular permeability increases within 13 days of pig pregnancy, whereas, by day 15 of pregnancy, IFNs up-regulated a large array of IFN induced genes in the underlying stroma and glandular epithelium, including ISG15, IRF1, STAT1, SLA5 and B2M, which were likely to play roles in uterine remodeling to support placentalization. Another gene is F3, a tissue factor (TF) that exists in endometrial stromal cells and may play an important role during the period of embryo. Matrix metalloproteinases (MMPs) are a family of zinc-dependent neutral endopeptidases that regulated tissue remodeling during embryonic development, angiogenesis and wound healing. Among these, MMP19 was expressed throughout the menstrual cycle, thereafter, affected cell proliferation and angiogenesis, which were crucial for endometrial receptivity. Tissue inhibitor of matrix metalloproteinases (TIMPs) is an MMP inhibitor, and an abnormal balance between MMPs and TIMPs has been related to tumor invasion and metastasis in various human cancers, including endometrial cancers. In general, we constructed a potential network of GSTM2-STAT1 at the maternal-fetal interface in pigs (Fig. 6).

Methods

Samples Collection and Animal Care Protocol. All animals were raised under the same conditions. We obtained endometrium of day 0, 12, 15, 18, and 32 clinically healthy gestation sow from the Fine Farm of HuaZong Agricultural University. Tissue samples from endometrium were frozen in liquid nitrogen immediately after collection and stored at −80 °C prior to RNA extraction. The methods were performed in accordance with the approved guidelines from Huazhong Agricultural University, and scientific, ethical and legal principles of the Hubei Regulations for the Administration of Affairs Concerning Experimental Animals. All experimental protocols were approved by the Ethics Committee of Huazhong Agricultural University.
Cell culture. Swine testis (ST) cells were purchased from CCTCC (China Center for Type Culture Collection, Wuhan, China) were cultured in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% (v/v) bovine calf serum (Gibico, USA) in a culture flask at 37 °C under a humidified atmosphere of 5% CO2. At 60–70% confluence, cells were trypsin-digested for further sub-culturing or seeded into 6-well plates (2 ml/well) at a concentration of 1–2 × 10^5 cells per ml for siRNA transfection.

RNA interference. GSTM2 was widely expressed in various tissues including embryos, and testis with relative high expression. Hence, GSTM2 could be expressed by normal ST cells. Three pairs of GSTM2-specific siRNAs were designed targeting corresponding regions of porcine GSTM2 mRNA (Ambion). In addition, scrambled siRNA (Ambion) was used as a negative control. Briefly, the RNA interference transfection was performed as follows. Cell suspensions were reverse transfected in triplicate with siRNA via LipofectamineTM 2000 (Invitrogen, Carlsbad, CA, USA) and Optimem-I reduced serum media (OPTI-MEM I, Invitrogen) with the siRNA pooled at equal amounts to a final concentration of 50 nM. Six hours after transfection (Supplementary Fig. S1), the medium was refreshed, and the cells were further incubated for another 24 h. Triplicate wells of non-transfected cells were also included. To evaluate the effects of GSTM2 knock-down on cells and transfection efficiency, we assessed morphology and cell numbers using fluorescence microscopy. The other two siRNAs, negative control and mock control followed the same treatments. Three independent experiments were performed.

RNA isolation and real-time PCR. Total RNA isolated from transfected and control cells were assessed for integrity using a BioAnalyzer 2100 (Agilent, Santa Clara, USA) and for concentration and purity by the NanoDropTM 1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). After washes with Phosphate Buffered Saline (PBS), cells of each well were homogenized with 1 ml TRIzol reagent (Invitrogen) according to the manufacturer’s protocol followed by modifications after re-dissolving the RNA. The RNA was dissolved in 100μL RNase-free water by mixing up and down with a pipette and stored at −80 °C freezer.

Real-time PCR was performed using a Roche LightCycler 480 detection system (Roche, Switzerland) and the following PCR program: denaturation at 95 °C for 30 sec, amplification for 40 cycles at 95 °C for 5 s, annealing and extension at 58 °C for 30 s and 72 °C for 10 s. Primer sequences and expected product sizes are shown in Supplementary Table S1. Specific amplification for certain PCRs was assessed by melting curve. One negative control reaction in which cDNA template was replaced by water was performed to avoid potential contamination. The sample from each well was repeated three times, and the comparative Ct (△△Ct) value method was used for relative quantification. Four genes, eukaryotic translation elongation factor 1 alpha 1 (EEF1A1), heat shock protein 90 kDa alpha (cytosolic), class B member 1 (HSPCB), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and beta actin (ACTB), were chosen as potential housekeeping genes based on their uniformly high expression.
levels across groups from the sequencing data. Expression levels of these genes were assessed and used to normalize target genes via geNorm software\(^1\). Expression levels were considered undetectable when the Ct value of the targeted gene exceeded 35.

**Western blotting.** Western blotting was used to further validate the effect of RNAi on GSTM2 before RNA-seq. Transfected cells were homogenized in 1 ml of 25 mM Tris/1 mM ethylenediaminetetraacetic acid pH buffer, pH = 7.5. Homogenates were separated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (SDS-PAGE) and transferred to a PVDF membrane (Millipore, Bedford, MA) using a semidy electric electrophoretic apparatus. The blocked membranes (5% BSA in TBS buffer containing 0.1% Tween 20) were incubated with anti-GSTM2 (1:1,000; AbCam, Cambridge, MA) and anti-beta-actin antibodies (1:1,000; AbCam, Cambridge, MA) overnight at 4°C. The blots were extensively washed three times with TBST buffer for 10 min and incubated under gentle agitation with the secondary antibodies for immunodetection. The antigen-antibody reaction was incubated for 1 hour, and the cross-reacting proteins were detected. Prestained molecular weight markers 10–170 kD in weight (Fermentas, Canada) were used as standards.

**RNA-seq.** Deep sequencing was carried out at BGI (Shenzhen, China). Briefly, the total RNA samples were first treated with DNase I to degrade any possible contaminating DNA. Then, the mRNA was enriched using oligo (dT) magnetic beads (for eukaryotes) or by removing rRNAs from the total RNA (for prokaryotes). Mixed with the fragmentation buffer, the mRNA was fragmented into short fragments (approximately 200 bp). Then, the first strand of cDNA was synthesized using random hexamer primers. Buffer, dNTPs, RNase H and DNA polymerase I were added to synthesize the second strand. The double-stranded cDNA was purified with magnetic beads. End reparation and 3'-end single nucleotide A (adenine) addition was then performed. Finally, sequencing adaptors were ligated to the fragments, which were then enriched by PCR amplification. During the QC step, the Agilent 2100 Bioanalyzer and ABI StepOnePlus Real-Time PCR System were used to qualify and quantify the sample library. Total six cDNA libraries were constructed including three GSTM2-knock down groups and three negative control groups. The library products were sequenced using an Illumina HiSeqTM 2000. The RNA used in sequencing was the same with the samples for q-PCR analysis.

**Differential gene expression analysis.** Values of RPKM were used to evaluate the total number of genes expression in each well of ST cells sample and the DEGs among each comparison\(^2\). The DEGs were analyzed based on an algorithm as previously described\(^3\). The P-value corresponds to a differential gene expression test in which False Discovery Rate (FDR) was used to determine the threshold of the P-value in multiple tests. The Cluster 3.0\(^4\) was used to the clustering analysis. The R heatmap package\(^4\) was applied to the analysis of Pearson and Spearman clustering. The functional classification of genes was performed using KEGG\(^5\) pathway analysis.

**Bioinformatics and data analysis.** Identification of both pathway network and gene ontology (GO) categories was performed using IPA and the online tool PANTHER. All of the probe sequences from differential and positively expressed probes were first re-annotated with pig RefSeq RNA database from the porcine genome (Sscrofa10.2) from NCBI (Index of ftp://ftp.ncbi.nih.gov/genomes/Sus_scrofa/RNA/, last updated on October 2011). The unique gene symbol list from differential and positively detected probes was then uploaded into PANTHER (http://www.pantherdb.org/). The genes, transcripts, and proteins related to the Gene Ontology (GO) terms were identified. Then biological processes and pathways was obtained by these GO terms. The Sus scrofa genome was used as the reference gene list, which allowed for the identification of statistically significant biological processes and pathways from GO terms, which are represented in the over- and under-expressed between gene lists. More details related to the expected value and P-value calculation can be obtained online under Binomial Statistic Help from PANTHER.

**Co-immunoprecipitation assay.** ST cells were plated in 100 mm dishes the day before transfection. Then cells were transfected with GFP-pcDNA3.1 containing the cDNA encoding GSTM2 and incubated at 37°C in a humidified atmosphere of 5% CO\(_2\) for 24 h for transient expression. The GFP was used to confirm the equal amount of plasmid transfected each dish. The cells were washed with cold PBS three times and lysed in 1000 mL cell lysis buffer (Sangon Biotech, Shanghai). The lysate was centrifuged at 12,000 rpm for 5 min at 4°C. The supernatant was divided into two equal parts, one added with 2 μg anti-GSTM2, the other added with 2 μg IgG as control, whereafter incubated at 4°C for 5 h. Each aliquot was added with 50 μL Protein A/G beads (Millipore, USA) and incubated for 30 min. The beads were collected and rinsed with 1 mL PBS (containing 0.05% Tween 20) for 5 times (5 min one time). The beads were added with 20 μL water and 10 μL 5 × SDS PAGE buffer, boiled at 98°C for 10 min and laid on ice for 5 min. The supernatant was saved for western blotting analysis.

**Overexpression and interference experiments.** Complete CDS sequences of GSTM2 and STAT1 were obtained from NCBI. The Primers with restriction sites for full-length amplification were designed by Primer 5 software. Primer pairs were as follows: forward primer for GSTM2 (5'-GGGGTACCCCG CGAGGACGTCAAGGAGAA-3', underlined sequences recognized by Kpn1), reverse primer for GSTM2 (5'-GCTCTAGAAGCCATCTCCTGCTGCAAAGGCA-3', underlined sequences recognized by XbaI), forward primer for STAT1 (5'-GGGGATCCCATGTCGTCCAGGAG-3', underlined sequences recognized by BamHI), reverse primer for STAT1 (5'-GCTCTAGAGCCTTAGTCAGGCAAGGAG-3', underlined sequences recognized by XbaI). The amplification products were obtained by PCR with reaction mixture including 2 × Taq Master Mix 25 μL, cDNA library of ST cells 1 μL, forward primer 1 μL, reverse primer 1 μL, and dd water added to 50 μL. Then the PCR product was recovered by E.Z.N.A Gel Extraction Kit according to instructions (OMEGA), and additionally, double enzyme digestion reaction was performed with both PCR product and pcDNA3.1. The expression vectors of GSTM2 and STAT1 were acquired after connectivity,
transfected into ST cells followed by the same method as RNA interference experiment which was described previous, expect for the dose of expression vector for transfection was 1 μg (Supplementary Figs S5,S6).

**Statistical analyses.** All experiments were repeated three times. Data were given as mean ± SD. Student’s t-test was used for statistical comparisons. P value < 0.05 was considered to be statistically significant.

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The authors declare no competing financial interests.

**Author Contributions**

Conceived and designed the experiments: Z.R., Y.L. Performed the experiments: Y.L., Y.Z. Analyzed the data: Y.L., Y.J., Y.Z., J.J. Contributed reagents/materials/analysis tools: Y.L., Z.M., Y.J. Wrote the paper: Y.J., Y.L., Z.R.

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**Additional Information**

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