Trophoblast H_2S maintains early pregnancy via regulating maternal-fetal interface immune hemostasis

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Running Title: H_2S maintains early pregnancy

Precise: Trophoblast CBS-H_2S signaling is dysregulated in unexplained recurrent spontaneous abortion and reduced CBS decreases trophoblast function and results in embryonic resorption in association with perturbed Th1/Th2 imbalance at the maternal-interface. These findings support a role of CBS-H_2S signaling in the maintenance of early pregnancy.

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Supplemental Table 1

1.The target sequence of lentivirus CBS (LV-CBS)

TAGACGAAGCTTGGGCTGCAGGTCGACTCTAGAGGATCCCCGGGTACCGG
TCGCCACCATGCCTTCTGAGACCCCCCAGGCAGAAGTGGGGCCCACAGGC
TGCCCCCACCGCTCAGGGCCACACTCGGCGAAGGGGAGCCTGGAGAAGG
GGTCCCCAGAGGATAAGGAAGCCAAGGAGCCCCTGTGGATCCGGCCCGAT
GCTCCGAGCAGGTGCACCTGGCAGCTGGGCCGGCCTGCCTCCGAGTCCCC
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GAAGAAAATCGGGGACACCCCTATGGTCAGAATCAACAAGATTGGGAAGA
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GGAACACCGGGATCGGGCTGGCCCTGGCTGCGGCAGTGAGGGGCTATCGC
TGCATCATCGTGATGCCAGAGAAGATGAGCTCCGAGAAGGTGGACGTGCT
GCGGGCACTGGGGGCTGAGATTGTGAGGACGCCCACCAATGCCAGGTTCG
ACTCCCCGGAGTCACACGTGGGGGTGGCCTGGCGGCTGAAGAACGAAATC
CCCAATTCTCACATCCTAGACCAGTACCGCAACGCCAGCAACCCCCTGGCT
CACTACGACACCACCGCTGATGAGATCCTGCAGCAGTGTGATGGGAAGCT
GGACATGCTGGTGGCTTCAGTGGGCACGGGCGGCACCATCACGGGCATTG
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CCCGAAGGGTCCATCCTCGCAGAGCCGGAGGAGCTGAACCAGACGGAGC
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GCTCTCGTCCCTGCTTGCCGGGAAGGTGCAGCCGTCAGACCAAGTTGGCA
AAGTCATCTACAAGCAGTTCAAACAGATCCGCCTCACGGACACGCTGGGC
AGGCTCTCGCACATCCTGGAGATGGACCACTTCGCCCTGGTGGTGCACGA
GCAGATCCAGTACCACAGCACCGGGAAGTCCAGTCAGCGGCAGATGGTGT
TCGGGGTGGTCACCGCCATTGACTTGCTGAACTTCGTGGCCGCCCAGGAG
CGGGACCAGAAGGGTATGGACTACAAGGATGACGATGAC

2. The target sequence of lentivirus CBSRNAi (LV-CBS-RNAi)

ATTAAAAAACAAATTACAAAAATTCAAAATTTTCGGGTTTATTACAGGGA
CAGCAGAGATCCAGTTTGGTTAATTAATCGAGCGGCCGCCCCCTTCACCG
AGGGCCTATTTCCCATGATTCCTTCATATTTGCATATACGATACAAGGCTG
TTAGAGAGATAATTGGAATTAATTTGACTGTAAACACAAAGATATTAGTA
CAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCAGTTTTA
AAATTATGTTTTAAAATGGACTATCATATGCTTACCGTAACTTGAAAGTAT
TTCGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGGACAC
GATTATCGAGCCGACATCTCGAGATGTCGGCTCGATAATCGTGTTTTTTGA
ATTCTCGACCTCGAGACAAATGGCAGTATTCATCCACGGATCCTAACCCG
TGTCGGCTCCAGATCTGGCCTCCGCGCCGGGTTTTGGCGCCTCCCGCGGGC
GCCCCCCTCCTCACGGCGAGCGCTGCCACGTCAGACGAAGGGCGCAGCGA
GCGTCCTGATCCTTCCGCCCGGACGCTCAGGACAGCGGCCCGCTGCTCAT
AAGACTCGGCCTTAGAACCCCAGTATCAGCAGAAGGACATTTTAGGACGG
GACTTGGGTGACTCTAGGGCACTGGTTTTCTTTCCAGAGAGCGGAACAGG
CGAGGAAAAGTAGTCCCTTCTCGGCGATTCTGCGGAGGGATCTCCGTGGG
GCGGTGAACGCCGATGATTATATAAGGACGCGCCGGGTGTGGCACAGCTA
GTTCCGTCGCAGCCGGGATTTGGGTCGCGTTCTTGTTTGTGGATCGCTGTG
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TCGCTCGTGGGACGAGCGTGTGAGAGACGCAGGCTGTAGTCTGGGTCGCG
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GATGAGACGCTTGGAGGCGCTGAGT

psc16211-1
CcggacACGATTATCGAGCCGACATCTCGAGATGTCGGCTCGATAATCGTgtT
TTTT

Supplemental Table 2

Primer pairs used for qRT-PCR

Gene	Species	Forward primer	Reverse primer
GAPDH	Mouse	ATACGGCTACAGCAACAGGG	GCCTCTCTTGCTCAGTGTCC
TGF-β	Mouse	CTCCGTGGCTTCTAGTGC	GCCTTAGTTTGGACAGGATCTG
IL-1R	Human	TGTTGGGGAAGGGTCTACCT	GACCTCAACAATGTCTTCCCCA
COX2	Human	TCCCTTGGGTGTCAAAGGTAAA	TGGCCCTCGCTTATGATCTG

Supplemental Table 3 The effect of AOAA and PAG on embryos absorption

Group	NO.	Total number of embryos	Number of absorbed fetuses	Number of mice with embryo loss
Control	1	10	0	
	2	8	0	
	3	2	0	
	4	8	1	
	5	10	0	
	6	9	0	
	7	10	0	
Total	N=7	57	1	1/7
AOAA	1	10	3	
	2	8	0	
	3	9	0	
	4	7	1	
	5	13	3	
	6	8	6	
	7	12	4	
	8	11	0	
	9	10	2	
	10	9	3	
	11	8	1	
	12	6	3	
	13	10	1	
	14	10	3	
Total	N=14	131	30	11/14
PAG	1	8	1	
	2	4	0	
	3	9	2	
	4	8	0	
	5	10	1	
	6	10	0	
	7	11	2	
	8	9	2	
	9	8	3	
	10	3	1	
	11	7	1	
	12	10	1	
	13	8	2	
Total	N=13	105	16	10/13

Supplemental figure

Fig. S1 **Effect of NaHS and L-cysteine on JEG3 cell invasion**

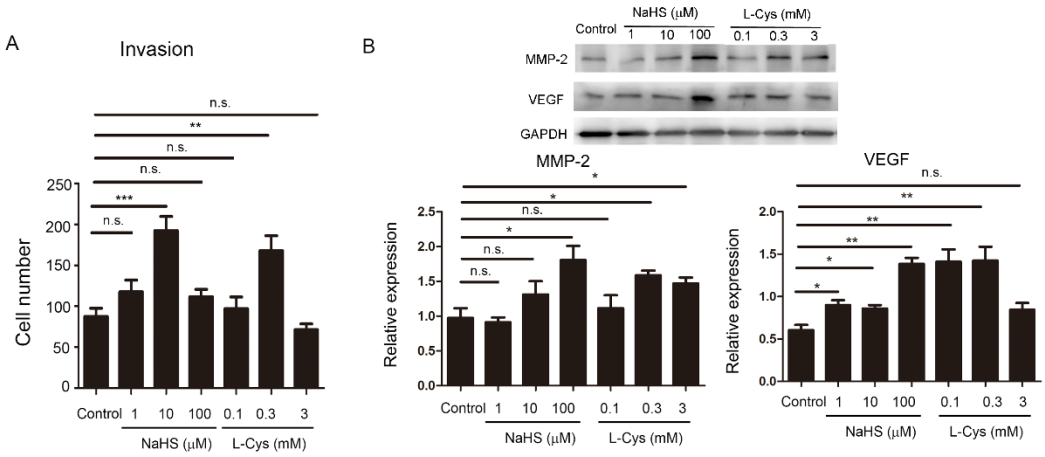
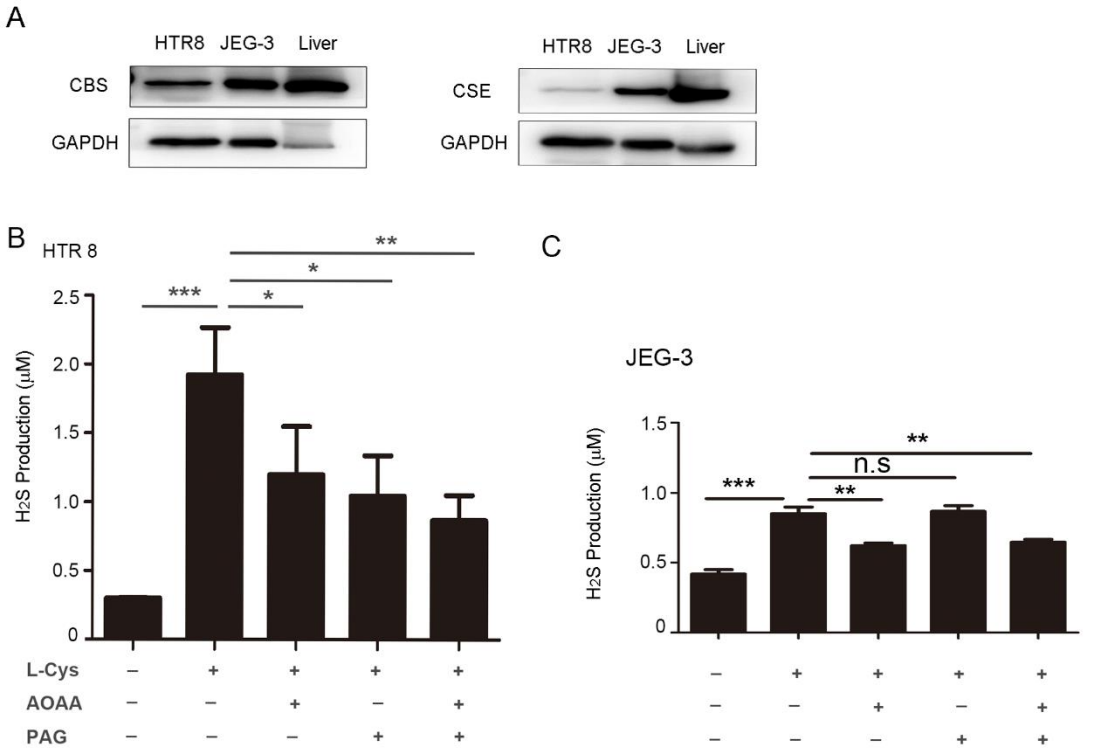


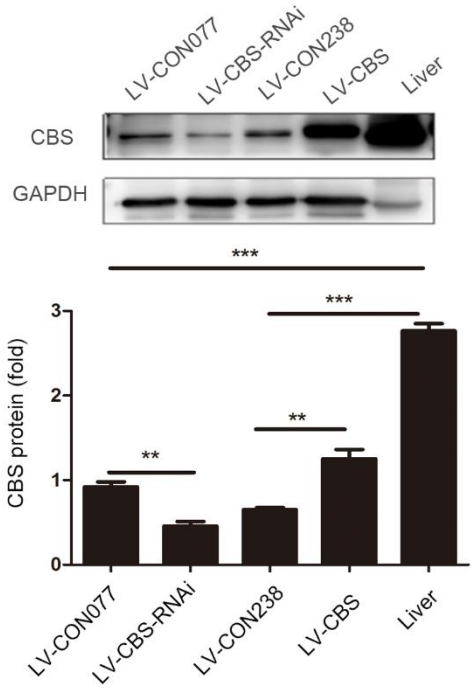
Fig.S1. JEG3 cells were treated with increasing concentrations of L-cysteine (L-Cys) or NaHS for invasion assay by using a trans-well system (A) or determining MMP-2 and VEGF protein expressions at 24 h by Western Blot with GAPDH as a control (B). Data were presented as means ± SD from at least 3 independent experiments. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; n.s., not significant.

Fig. S2. CBS and CSE protein expression in human trophoblast cell lines



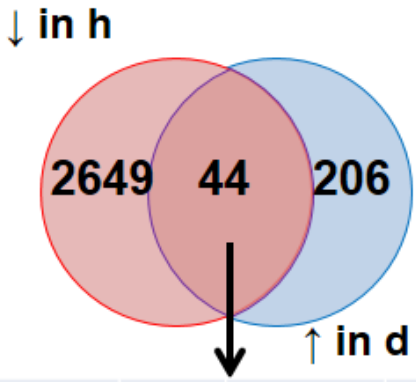
A: CBS and CSE proteins in human trophoblastic cell lines JEG-3 and HTR8/SVneo were analyzed by Western Blot with specific antibodies and GAPDH as a loading control and mouse liver was used as a positive control. B: The H_2S synthesizing ability of HTR8/SVneo (B) and JEG3 (C) cells in the presence of L-Cys were determined by the methylene blue assay. Data were presented as means ± SD from at least 3 independent experiments. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; n.s., not significant.

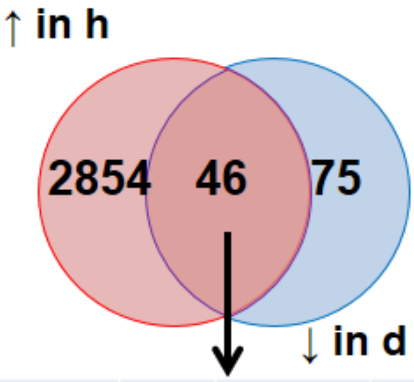
Fig.S3. CBS knockdown or overexpression in HTR8/SVneo cells



HTR-8/SVneo cells were transfected with lentiviruses carrying CBS siRNA (LV-CBSRNAi) or control siRNA (LV-CON077), or CBS cDNA (LV-CBS) or its control (LV-CON238). Proteins were analyzed by Western Blot with specific antibodies; GAPDH was measured as a loading control and mouse liver sample was used as a positive control. Data were calculated as means ± SD from at least 3 independent experiments. **, $p<0.01$; ***, $p<0.001$.

Fig. S4. The overlapped genes in CBS overexpression and knockdown HTR8/SVneo cells revealed by RNA-Seq analysis.



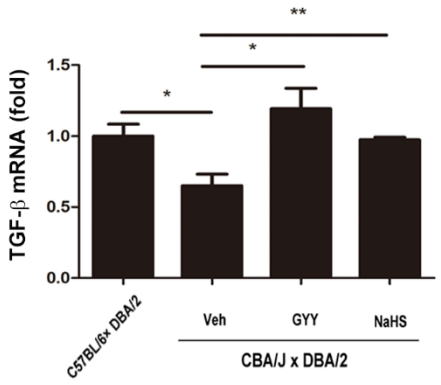


Genes	Log2(FC)	Genes	Log2(FC)
SNORA75	-2.72	PTGS2	-1.07
DPYSL3	-2.88	VWA5A	-2.31
BHLHE40	-1.57	DCN	-3.62
PLK2	-1.33	GBP4	-4.71
OPLAH	-4.75	EGR3	-2.42
PPP2R2C	-2.32	VCAM1	-1.93
KIRREL2	-1.69	RP11-54O7.18	-1.34
SERPING1	-2.44	RP4-724E16.2	-1.28
DIRAS3	-5.10	LUCAT1	-1.25
BMP6	-3.93	SNORA46	-5.11
LXN	-6.15	Metazoa_SRP	-1.55
DOC2B	-2.51	GS1-600G8.5	-1.95
PQLC2L	-4.98	KCNH2	-1.81
GRIN1	-2.31	IL1R1	-1.34
HSPA6	-3.14	BST2	-1.20
EFEMP1	-2.20	REEP1	-1.55
MEG9	-4.06	ACSM3	-1.19
CTB-133P21.1	-4.14	KYNU	-1.52
IFITM1	-1.17	HMX3	-2.42
TMOD1	-1.92	KLF7-IT1	-1.38
GBP2	-4.46	GALNT18	-1.92
XKR7	-3.45	FABP6	-1.36

Genes	Log2(FC)	Genes	Log2(FC)
TMEM200A	3.64	ANKRD20A5P	1.58
PBX1	3.27	RP11-1E6.1	2.99
TIMP3	2.41	RAB27B	1.31
ZFPM2	2.62	SLC16A6	1.47
ASPH	1.39	GPAT3	1.16
GALNT3	2.86	Y_RNA	1.04
INPP4B	1.66	AGTR1	1.28
AMIGO2	2.13	ABCG2	1.89
SYT1	1.90	ALX1	1.27
C6orf141	2.01	TLR6	1.80
FST	1.51	HOXA7	1.67
KCNT2	2.21	CDH10	1.26
ITGA1	1.70	EPHA3	2.25
TNFSF18	2.64	ACRBP	1.24
SRPX2	1.96	SELENOP	1.13
ZFPM2-AS1	2.33	DOK3	1.12
RBMS3	1.31	RNA5-8S5	1.98
IP6K3	2.26	RGS5	1.22
CYP1A1	2.11	CPA4	1.38
COL4A6	1.51	LINC00324	1.50
RYR2	2.36	SLC27A2	1.31
ADGRL2	1.04	MUC2	1.46
PDGFD	1.70	AMDHD1	1.87

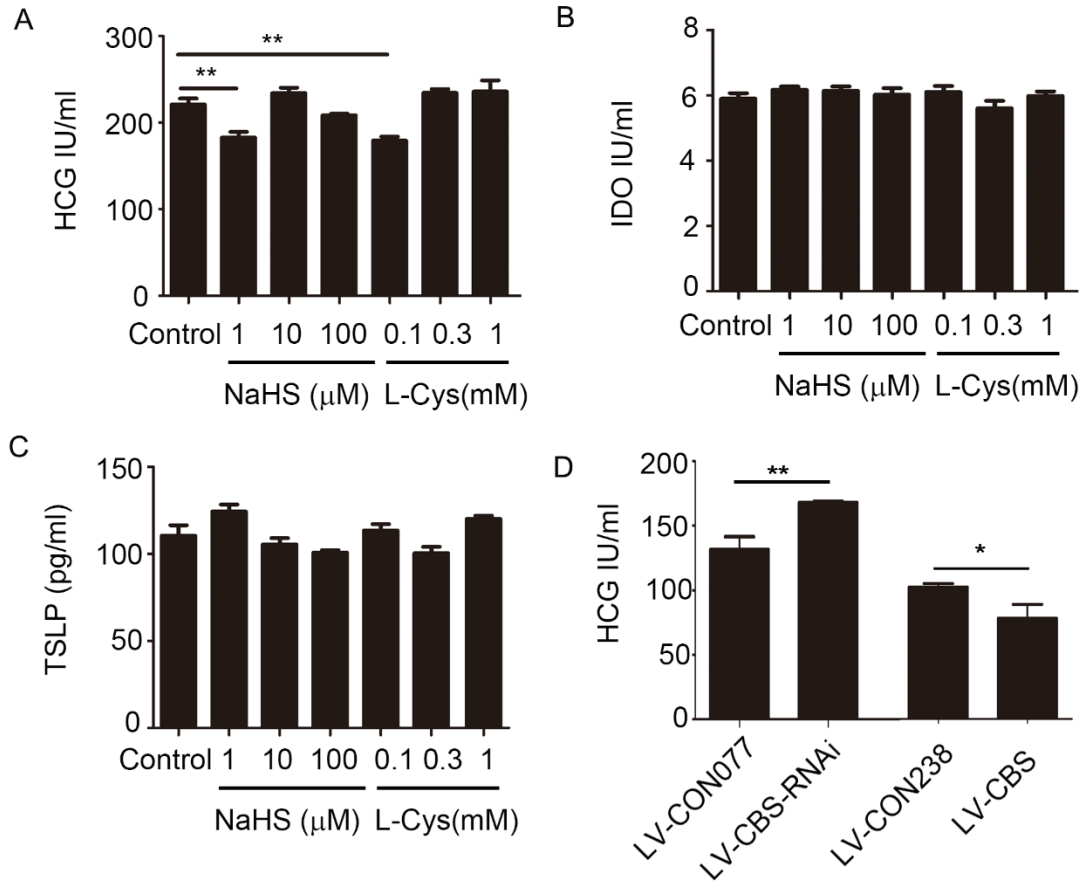
h: overexpression of CBS; d: knockdown of CBS; Up arrow: upregulated genes; Down arrow: downregulated genes. IL1R1 and PTGS2: Genes associated with inflammatory response related biological process; TIMP3,ITGA1 and PDGF: Genes associated with focal adhesion.

Fig. S5. **Effect of H_2S donors on decidual TGF-β mRNA in dams with resorbed fetuses in the spontaneous abortion mouse model.**



CBA/J x DBA/2 dams were treated without or with daily GYY (10 mg/kg) or NaHS (0.5 mg/kg) stating on GD 6.5 and then killed on GD12.5. C57BL/6 x DBA/2 dams were used as controls. Decidual RNA was extracted for determining TGF-β mRNA by RT-qPCR. Data were presented as means ± SD. n=4, *, $p<0.05$; **, $p<0.01$.

Fig. S6. Effect of H_2S on human chorionic gonadotropin (hCG), indoleamine 2,3-dioxygenase (IDO), and thymic stromal lymphopoietin (TSLP) production in HTR8/SVneo cells



Cells were treated with increasing concentrations of L-cysteine (L-Cys) or NaHS for 24 hours. The culture medium was collected for measuring levels of hCG (A), IDO (B), and TSLP(C) by ELISA. Levels of hCG secretion was also measured in the medium collected from HTR-8/SVneo cells transfected with CBS RNAi (LV-CBS-RNAi; LV-CON077 was the control) or CBS cDNA (LV-CBS; LV-CON238 was used as the control) (D). Data were calculated as means ± SD. n=4; *; $p<0.05$; **, $p<0.01$.