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Hyperhomocysteinemia is detrimental to pregnancy in mice and is associated with preterm birth

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ABSTRACT

Elevated levels of homocysteine produce detrimental effects in humans but its role in preterm birth is not known. Here we used a mouse model of hyperhomocysteinemia to examine the relevance of homocysteine to preterm birth. The mouse carries a heterozygous deletion of cystathionine β-synthase (*Cbs*$^{+/-}$). Gestational period was monitored in wild type and *Cbs*$^{+/-}$ female mice. Mouse uterine and placental tissues, human primary trophoblast cells, and human myometrial and placental cell lines were used to determine the influence of homocysteine on expression of specific genes *in vitro*. The activity of BK_{Ca} channel in the myometrial cell line was monitored using the patch-clamp technique. We found that hyperhomocysteinemia had detrimental effects on pregnancy and induced preterm birth in mice. Homocysteine increased the expression of oxytocin receptor and Cox-2 as well as PGE_2 production in uterus and placenta, and initiated premature uterine contraction. A Cox-2 inhibitor reversed these effects. Gpr109a, a receptor for niacin, induced Cox-2 in uterus. Homocysteine upregulated GPR109A and suppressed BK_{Ca} channel activity in human myometrial cells. Deletion of *Gpr109a* in *Cbs*$^{+/-}$ mice reversed premature birth. We conclude that hyperhomocysteinemia causes preterm birth in mice through upregulation of the Gpr109a/Cox-2/PGE_2 axis and that pharmacological blockade of Gpr109a may have potential in prevention of preterm birth.

1. Introduction

Developmental maturity in human fetuses is completed between 34 and 37 weeks of gestation. Prematurely born babies lack this process, which increases the risk of perinatal mortality and morbidity [1]. Though multiple factors are believed to be responsible, the exact mechanisms involved in preterm birth are poorly understood [2]. Recent epidemiological studies have suggested a potential role for increased maternal plasma homocysteine levels in premature birth [3]. Homocysteine is an intermediate in methionine metabolism. It is either remethylated to methionine by a folate/vitamin B_{12}-dependent enzyme (methionine synthase) or converted to cysteine by a vitamin B_6-dependent trans-sulfuration pathway with cystathionine as an intermediate. Deficiency in folate, B_{12} or B_6, can result in accumulation of homocysteine. Elevated levels of homocysteine are also seen in patients with mutations in N^5, N^{10}-methylenetetrahydrofolate reductase (MTHFR) and cystathionine β-synthase (CBS) [4,5]. MTHFR generates N^5-methyltetrahydrofolate, the coenzyme for methionine synthase, while CBS is an enzyme in the trans-sulfuration pathway. Elevated levels of homocysteine in circulation are causally related to increased risk of coronary, cerebral, and peripheral arterial diseases [5,6]. It is also a risk factor for preeclampsia, spontaneous abortion and placental abruption [7,8].

Cbs knockout mouse has been generated as an animal model of hyperhomocysteinemia to investigate the consequences of elevated levels of homocysteine [9]. The circulating levels of homocysteine are 204 ± 65 μM in homozygous mice (*Cbs*$^{-/-}$), 14 ± 3 μM in heterozygous mice (*Cbs*$^{+/-}$), and 6 ± 1 μM in wild type mice [9]. When the heterozygotes are bred, the ratio of wild type mice to heterozygous mice is close to the expected ratio based on the Mendelian inheritance, and the *Cbs*$^{+/-}$ mice have a lifespan comparable to that of wild type mice [9]. However, the number of homozygous mice is lower than expected, and the *Cbs*$^{-/-}$ mice that are born have markedly shortened lifespan, indicating that elevated levels of homocysteine not only have detrimental effects on pregnancy outcome but also on postnatal development and growth [9]. Here we report an important, hitherto unrecognized,

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biological feature of *Cbs*$^{+/-}$ female mice. We found that the heterozygous mice with significantly elevated levels of homocysteine in circulation always delivered pups prematurely, thus unraveling a causative role of hyperhomocysteinemia in preterm birth. Mechanistic studies show that preterm birth associated with elevated levels of homocysteine is due to premature uterine contraction caused by the upregulation of Cox-2 and oxytocin receptor, increased production of PGE2, and suppression of BK_{Ca} channel activity. The niacin receptor Gpr109a appears to mediate these effects of hyperhomocysteinemia because deletion of *Gpr109a* in *Cbs*$^{+/-}$ mice reverses the preterm birth.

2. Materials and methods

2.1. Generation of *Cbs*$^{+/-}$and *Cbs*$^{+/-}$/*Gpr109a* $^{-/-}$ mice and timed pregnancy

All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the Georgia Health Sciences University. Generation of *Cbs*$^{-/-}$ mice has been described previously [9]. We obtained a breeding pair of *Cbs*$^{+/-}$ mice (C57Bl/6 background) from Jackson Laboratories (Bar Harbor, ME, U. S. A.) for our studies. The mice were maintained in clear plastic cages with 12-h alternating light/dark cycles. Mice were fed a rodent diet (7004 Teklad S-2335 Mouse Breeder Sterilizable Diet, Harlan Laboratories, Indianapolis, IN, U. S. A.). Wild type and *Cbs*$^{+/-}$ mice were set up for mating at the end of the day, and females were examined on the morning of the following day for vaginal plug. If the plug was observed, the pregnancy was timed as 0.5 d of gestation, and the littering day was recorded for each mouse. The pups were genotyped, and the number of wild type, heterozygous, and homozygous pups in each litter was recorded. For collection of placenta and uterus, a longitudinal incision was made in the lower abdomen on 16th day of gestation, the uterine tract was taken out and cleared of perivascular tissue, and the fetus, placenta, and the adjoining uterine tissue were separated with scissors.

Gpr109a$^{-/-}$ mice were obtained from Dr. Stefan Offermanns (Max-Planck-Institute for Heart and Lung Research, Bad Nauheim, Germany). Generation of these mice has been described previously [10]. Female *Cbs*$^{+/-}$ mice were first crossed with *Gpr109a*$^{-/-}$ males to generate *Cbs*$^{+/-}$/*Gpr109a*$^{+/-}$ mice. The males and females of the *Cbs*$^{+/-}$/*Gpr109a*$^{+/-}$ genotype were then crossed to generate *Cbs*$^{+/-}$/*Gpr109a*$^{-/-}$ mice. Genotyping was accomplished by polymerase chain reaction using gene-specific primers. *Cbs*$^{+/-}$/*Gpr109a*$^{-/-}$ female mice were mated with *Cbs*$^{+/-}$/*Gpr109a*$^{-/-}$ males to examine the influence of *Gpr109a* deletion on gestational period in *Cbs*$^{+/-}$ female mice. This experiment was repeated with 5 different females, and the data are presented as means ± S. E. Statistical significance was first determined by ANOVA and then by Student's '*t*' test, and $p < 0.05$ was considered significant.

2.2. RT-qPCR

Placenta and uterine tissues were isolated from wild type, *Cbs*$^{+/-}$, and *Cbs*$^{-/-}$ mice; RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, U. S. A.). Real-time PCR was performed using SYBR Green fluorescein mix from ABgene (Absolute QPCR; Thermo Scientific, Surrey, UK) and a real-time PCR detection system (I-Cycler; Bio-Rad, Hercules, CA, U. S. A.). The same method was used to monitor the expression of specific genes *in vitro* in mouse uterine and placental tissues, human primary trophoblast cells, and the human placental (BeWo) and uterine myometrial (ULTR) cell lines, which were treated with or without homocysteine (500 μM; 72 h). The treatment was carried out inside a tissue culture hood under sterile conditions. Uterine and placental tissues were prepared from wild type pregnant mice on 16th day of gestation. The tissue fragments were first washed thoroughly in sterilized phosphate-buffered saline to remove blood, and then treated with homocysteine. Primary trophoblast cells were prepared from term human placentas as described previously [11]. BeWo and ULTR cells were cultured to 80–90% confluency and then treated with homocysteine. Total RNA was prepared from control and treated tissues/cells and used for RT-qPCR.

2.3. Western blot

Lysates from placental and uterine tissues were used for western blot. Membranes were probed with commercially available antibodies, followed by appropriate horseradish peroxidase-conjugated secondary antibodies (BIO-RAD, Hercules, CA, U. S. A.).

2.4. Immunohistochemistry

This was done on frozen sections using specific antibodies. After treatment with primary antibodies, tissue sections were incubated with appropriate AlexaFluor 555-conjugated IgG secondary antibodies (Invitrogen, Carlsbad, CA, U. S. A.). Hoechst was used as the nuclear stain. Slides were viewed with an epifluorescence microscope (Carl Zeiss Meditec, Oberkochen, Germany).

2.5. Measurement of homocysteine in mouse tissues

HPLC was used to measure the levels of homocysteine in mouse placentas of different genotype. Wild type mice were set up for breeding to obtain placentas of wild type genotype. Heterozygous mice were used for breeding to obtain *Cbs*$^{-/-}$ placentas. In both cases, pregnant females were killed on 16th of gestation and placental tissues were collected and genotyped. Tissues were homogenized in phosphate-buffered saline. The HPLC instrument included a controller (LCNet II), a solvent mixing module (MX-2080–31), an autosampler (AS-2055), and a fluorescence detector (FP-1520 (Jasco, Inc., Easton, MD, U. S. A.). Two HPLC columns were used: Supelco C18 and Supelguard C18 (Ascentis, Bellefonte, PA, U. S. A.). Homocysteine levels were measured by a fluorometric method using the fluorescent probe 7-fluorobenzofurazan-4-sulfonic acid that tags thiol groups. The values were normalized to the internal standard *N*-acetyl cysteine. Values were normalized to protein content of the tissue samples. Protein in tissue lysates was determined by Lowry's method. Three individual samples were prepared for each test group and measurements were repeated in duplicate.

The same technique was used also for the determination of plasma levels of homocysteine in wild type and *Gpr109a*$^{-/-}$ mice. Blood was collected from the retroorbital sinus in the presence of heparin as an anticoagulant, and plasma was isolated by centrifugation. Protein in plasma was precipitated with trichloroacetic acid, and the supernatant was used for estimation of homocysteine. Three mice were used for each genotype.

2.6. Analysis of uterine muscle contraction

Isometric tension analysis was performed with a Radnoti digital force transducer organ bath system (Model 159920) using mouse uterine ring preparations as previously described [12]. Briefly, on 16th day of gestation, uterine tissues from wild type and *Cbs*$^{+/-}$ pregnant female mice were dissected and cleaned of excess fat and connective tissue. Two to four 5-mm rings were obtained from each uterine tissue and prepared for isometric contractile force recordings. The uterine rings were then mounted on two triangular tissue supports, with one support fixed to stationary glass rod, and the other attached to a force–displacement transducer. Isometric contractions or relaxations were recorded in a computer using PowerChart software from AD Instruments (Colorado Springs, CO, U. S. A.). The tissue-bathing solution was the modified Krebs–Henseleit buffer: 122 mM NaCl, 4.7 mM KCl, 15.5 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgCl_2$, 1.8 mM $CaCl_2$, 11.5 mM glucose, pH 7.2. The solution

was oxygenated continuously (95% O_2/5% CO_2) and maintained at 37 °C. Uterine ring preparations were equilibrated for 90 min under a resting tension of 1 g, and fresh bath solution was added to the tissue chamber every 30 min to prevent accumulation of metabolic end products. After the initial equilibration, the tissue preparations were exposed to maximally effective concentrations of oxytocin (10 IU) to induce contractile response. After recording the response for about 15 min, homocysteine (100 μM) was added to the bath chamber. Again the response was recorded. To confirm the effect of homocysteine on oxytocin-induced contractions, the bath solution was replaced with fresh Kreb's buffer, and the order of the addition of oxytocin and homocysteine was reversed. Homocysteine (100 μM) was added to the bath solution first, the tissue response was recorded, and then oxytocin (10 IU) was added and the tissue response was recorded. The experiment was repeated with 3 wild type uterine tissues and 3 *Cbs*$^{+/-}$ uterine tissues, and the data were analyzed using PowerLab Chart5.1 Software. Results are given as means ± S. E.

2.7. Patch clamp

Patch clamp experiments were performed using the human uterine myometrial cell line ULTR. The bath solution consisted of 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 20 mM HEPES, and 20 mM glucose, pH 7.4. The patch pipettes with a resistance of 3 MΩ or less were made from capillaries of Corning glass 7052. To measure potassium currents, the tips of the patch pipettes were filled with a solution containing 90 mM KCH_3SO_3 (potassium methanesulfonate), 40 mM KCl, 5 mM $MgCl_2$, and 20 mM HEPES, pH 7.4, to approximate normal cellular [K^+] and [Cl^-]. The remainder of the pipette was back-filled with a similar solution containing 200 mg/ml amphotericin B. The experiments with cells were continued only if the voltage drop across the series resistance was reduced to ≤ 5 mV within 10–20 min after forming a gigaohm seal. To measure the resting membrane potential, the voltage reading was recorded while the current-clamp mode was switched on. Voltage clamp and voltage pulse generation were controlled with an Axopatch 200A patch clamp amplifier (Axon Instruments, Inc., Foster City, CA, U. S. A.) and data were analyzed with pCLAMP 6.0.3 software (Axon Instruments, Inc.), which is a comprehensive software package for acquisition and analysis of both whole-cell and single-channel currents. Voltage-activated currents were filtered at 2 kHz and digitized at 10 kHz, and capacitative and leakage currents were subtracted digitally. All drugs were diluted with fresh bath solution and perfused into the recording chamber.

2.8. PGE_2 assay

To measure the levels of prostaglandin E_2, we treated the uterine myometrial cells (ULTR) and placental syncytiotrophoblast cells (BeWo) with homocysteine chronically (cells exposed to homocysteine for 5 passages) and collected the culture medium. PGE_2 levels were measured by ELISA using a commercially available kit (Cayman Chemical Company, Ann Arbor, MI, U. S. A.). This assay was based on the competition between PGE_2 and a PGE_2-acetylcholinesterase (AChE) conjugate (PGE_2 Tracer) for a fixed amount of PGE_2 monoclonal antibody. When the concentration of PGE_2 in tissue samples varies, the amount of PGE_2 Tracer that is able to bind to the PGE_2 monoclonal antibody will be inversely proportional to the concentration of PGE_2 in the tissue sample. This antibody–PGE_2 complex binds to goat polyclonal anti-mouse IgG that had been coated in the well. The wells were washed to remove any unbound reagents, and then Ellman's Reagent, which contains the substrate to AChE, was added to the wells. The product of this enzymatic reaction was monitored by measurement of absorbance at 412 nm. The intensity of this color is proportional to the amount of PGE_2 Tracer bound to the well, which is inversely proportional to the amount of free PGE_2 present in the tissue sample.

2.9. Microarray

Wild type × wild type matings and *Cbs*$^{+/-}$ × *Cbs*$^{+/-}$ matings were set up, and placentas were collected on 16th day of gestation. The placentas from the heterozygote mating were genotyped to identify *Cbs*$^{-/-}$ placentas. Even though wild type placentas were obtained from the heterozygote matings, these were not used for microarray because these placentas were exposed to elevated levels of homocysteine during pregnancy in *Cbs*$^{+/-}$ female mice. Therefore, the wild type placentas used in microarray analysis were from wild type × wild type matings. Three wild type placentas and three *Cbs*$^{-/-}$ placentas were used for isolation of total RNA using the Trizol method. Double-stranded cDNA was synthesized from 80 to 100 ng total RNA through a random-primed reverse transcription using a dNTP mix containing dUTP. After purification, the cDNA was fragmented by incubation with a mixture of UDG (uracil-DNA glycosylase) and APE1 restriction endonucleases and end-labeled via a terminal transferase reaction incorporating a biotinylated dideoxynucleotide. The fragmented, biotinylated cDNA (5.5 pg) was added to a hybridization cocktail, loaded on a Mouse Gene 1.0 ST Gene Chip and hybridized for 18 h at 45 °C. Following hybridization, the array was washed and stained according to the Affymetrix protocols. The stained arrays were scanned at 532 nm using an Affymetrix GeneChip Scanner 3000, generating CEL files for each array.

Gene expression alterations were determined using PARTEK Genomics Suite. The data were then subjected to Principal Component Analysis. A gene summarization was then performed on the data, which estimates the intensity of individual genes by averaging the intensities of all the probe sets comprising the gene. The summarization is followed by an n-way analysis of variance (ANOVA) using a mixed model and methods of moment to equate ANOVA mean sum of squares to their expected values. The data was then analyzed using a two sample *t*-test for significance at $p < 0.05$ and a fold change cut off of 2.

3. Results

3.1. Hyperhomocysteinemia causes premature delivery in mice

Heterozygote matings (*Cbs*$^{+/-}$ × *Cbs*$^{+/-}$) used in the present study yielded a total of 63 wild type mice (*Cbs*$^{+/+}$), 115 heterozygous mice (*Cbs*$^{+/-}$), and 49 homozygous mice (*Cbs*$^{-/-}$) with a corresponding ratio of 1:1.8:0.8. The ratio of wild type and heterozygous mice in the litters was close to what was expected of Mendelian inheritance. The proportion of *Cbs*$^{-/-}$ pups was however less than expected, suggestive of significant embryonic lethality of the *Cbs*$^{-/-}$ genotype. *Cbs*$^{+/-}$ mice grew normal and there was no apparent phenotype associated with this genotype except for the significantly elevated homocysteine levels in plasma. *Cbs*$^{+/-}$ females were fertile, but interestingly pregnant *Cbs*$^{+/-}$ mice littered prematurely compared to wild type mice (Fig. 1A; gestation period in wild type mice, 20.0 ± 0.2 days; gestation period in *Cbs*$^{+/-}$ mice, 16.6 ± 0.1 days; $p < 0.001$). We also found that the placental weight and body weight of *Cbs*$^{-/-}$ fetuses were significantly lower compared to wild type and *Cbs*$^{+/-}$ littermates (Fig. 1B, C). The primary site of *Cbs* expression is liver, but we found that *Cbs* was also expressed in uterus and placenta (Fig. 1D, E). Homocysteine levels were higher in *Cbs*$^{-/-}$ placentas (0.17 ± 0.02 pmol/μg protein) than in wild type placentas (0.09 ± 0.01 pmol/μg protein; $p < 0.01$) (Fig. 1F).

3.2. Role of Cox-2 in premature delivery in *Cbs*$^{+/-}$ mice

We analyzed the differential gene expression in wild type and *Cbs*$^{-/-}$ placentas by microarray (Supplemental Table 1). One of the

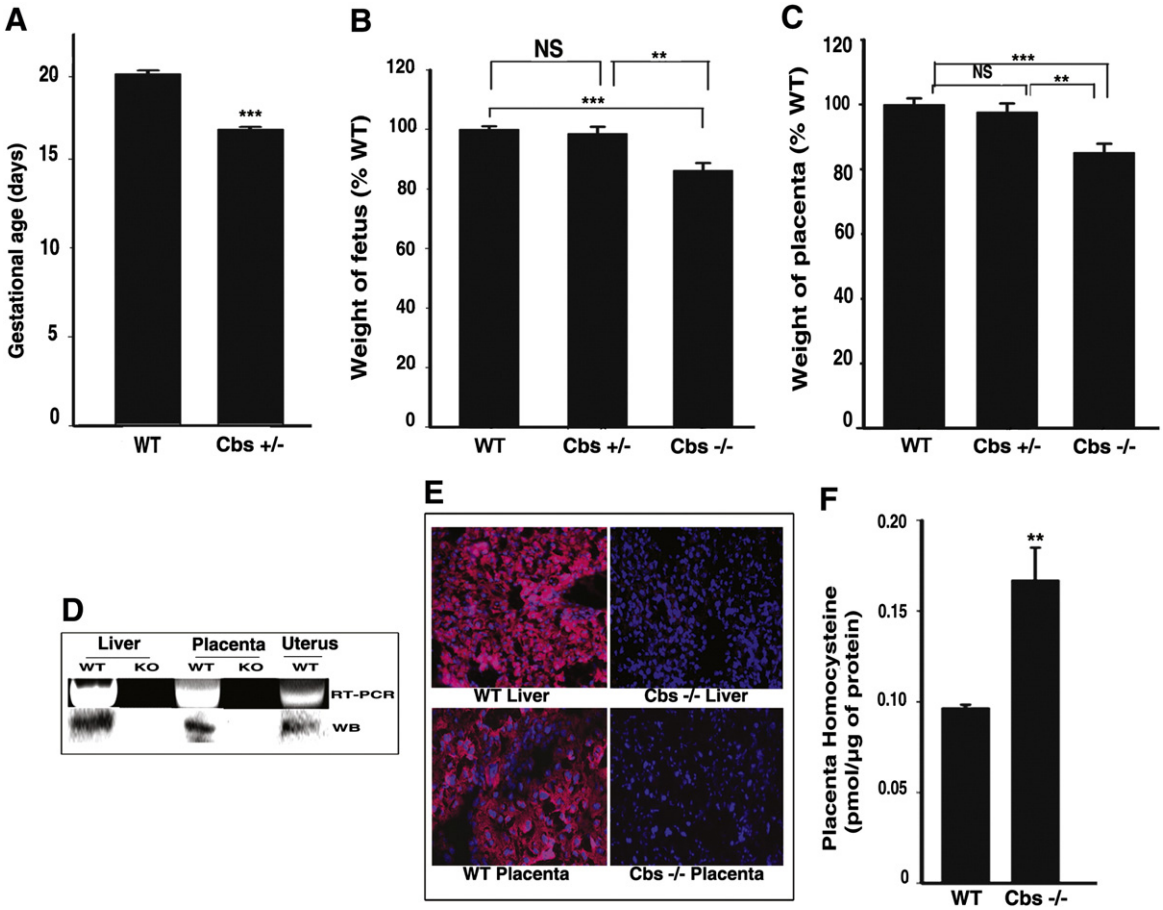

Fig. 1. Hyperhomocysteinemia causes premature delivery in mice. (A) Gestation period in wild type (WT) and *Cbs*$^{+/-}$ mice (Mean ± S. E., ***$p < 0.001$). (B) Fetal weight in wild type (WT) and *Cbs*$^{+/-}$ mice on 16th day of pregnancy (Mean ± S. E., NS, not significant; **$p < 0.01$; ***$p < 0.001$). (**C**) Placental weight in wild type (WT) and *Cbs*$^{+/-}$ mice on 16th day of pregnancy (Mean ± S. E., NS, not significant; **$p < 0.01$; ***$p < 0.001$). (D) RT-PCR and western blot analysis of cystathionine β-synthase (*Cbs*) expression in wild type (WT) and *Cbs*$^{-/-}$ (KO) liver, placenta and uterus. (E) Immunofluorescence analysis of liver and placenta for *Cbs* protein in wild type (WT) and *Cbs*$^{-/-}$ mice. (F) Homocysteine (Hcy) levels in wild type (WT) and *Cbs*$^{-/-}$ placentas on 16th day of pregnancy (**$p < 0.01$).

genes upregulated in *Cbs*$^{-/-}$ placentas was *Ptgs2* (*Cox-2*). We confirmed its upregulation by RT-qPCR, western blot and immunohistochemistry (Fig. 2A–C). The upregulation was also evident in *Cbs*$^{+/-}$ uterus (similar data could not be obtained for *Cbs*$^{-/-}$ uterus because *Cbs*$^{-/-}$ females do not survive long enough to reach the reproductive age). RT-qPCR showed a ~11 fold increase in Cox-2 expression in *Cbs*$^{-/-}$ placenta and ~5 fold increase in *Cbs*$^{+/-}$ uterus on 16th day of gestation. This raised the possibility that the preterm birth observed in *Cbs*$^{+/-}$ female mice may be due to homocysteine-induced increase in PGE_2 production through Cox-2. To confirm that Cox-2 was responsible for the premature birth in *Cbs*$^{+/-}$ mice, we administered Celecoxib, a selective Cox-2 inhibitor, by oral gavage to *Cbs*$^{+/-}$ pregnant mice once a day on 15th and 16th day of gestation (25 mg/kg body weight). This treatment increased the gestation period significantly (vehicle versus Celecoxib, $p < 0.01$) (Fig. 2D). In contrast, *Cbs*$^{+/-}$ pregnant mice treated with vehicle littered prematurely. We examined the influence of homocysteine on PGE_2 production in a human uterine myometrial cell line (ULTR) and a human placental trophoblast cell line (BeWo). Chronic treatment of these cells with homocysteine (cells exposed to homocysteine for 5 passages) increased PGE_2 secretion (Fig. 2E; $p < 0.01$ for BeWo; $p < 0.001$ for ULTR). We also examined the tissue levels of PGE_2 in wild type placentas and in placentas of different *Cbs* genotypes (Fig. 2F). The PGE_2 levels were higher in *Cbs*$^{-/-}$ placentas than in wild type or *Cbs*$^{+/-}$ placentas ($p < 0.05$). We also examined PGE_2 levels in wild type uterine tissue from wild type females mated with wild type males and in *Cbs*$^{+/-}$ uterine tissues attached to placentas of different *Cbs* genotypes. The latter were obtained from *Cbs*$^{+/-}$ females mated with *Cbs*$^{+/-}$ males. We found elevated levels of PGE_2 in *Cbs*$^{+/-}$ uterus compared to wild type uterus ($p < 0.01$) (Fig. 2F). Among the *Cbs*$^{+/-}$ uterine tissues attached to placentas of different *Cbs* genetic backgrounds, PGE_2 levels were higher in tissues attached to *Cbs*$^{-/-}$ placentas than in tissues attached to wild type or *Cbs*$^{+/-}$ placentas ($p < 0.05$).

3.3. Effect of homocysteine on uterine muscle contraction

We then monitored the contractile features of uterine muscle from wild type and *Cbs*$^{+/-}$ pregnant mice. Oxytocin induced the contraction of the uterine tissue from wild type as well as *Cbs*$^{+/-}$ mice; there was a trend of increased contraction in *Cbs*$^{+/-}$ uterine tissue, but the difference was not statistically significant (Fig. 3A, B). Homocysteine had a significant augmenting effect on oxytocin-induced contraction in wild type uterus, but this effect was significantly greater in *Cbs*$^{+/-}$ uterus ($p < 0.05$). Homocysteine itself caused uterine contraction, but the effect was much greater in *Cbs*$^{+/-}$ uterus than in wild type uterus ($p < 0.01$). Even though the effects of oxytocin by itself on uterine contraction were not different between wild type and *Cbs*$^{+/-}$ uterine muscle, in the presence of homocysteine, oxytocin elicited a greater effect on uterine contraction in *Cbs*$^{+/-}$ uterus than in wild type uterus ($p < 0.05$).

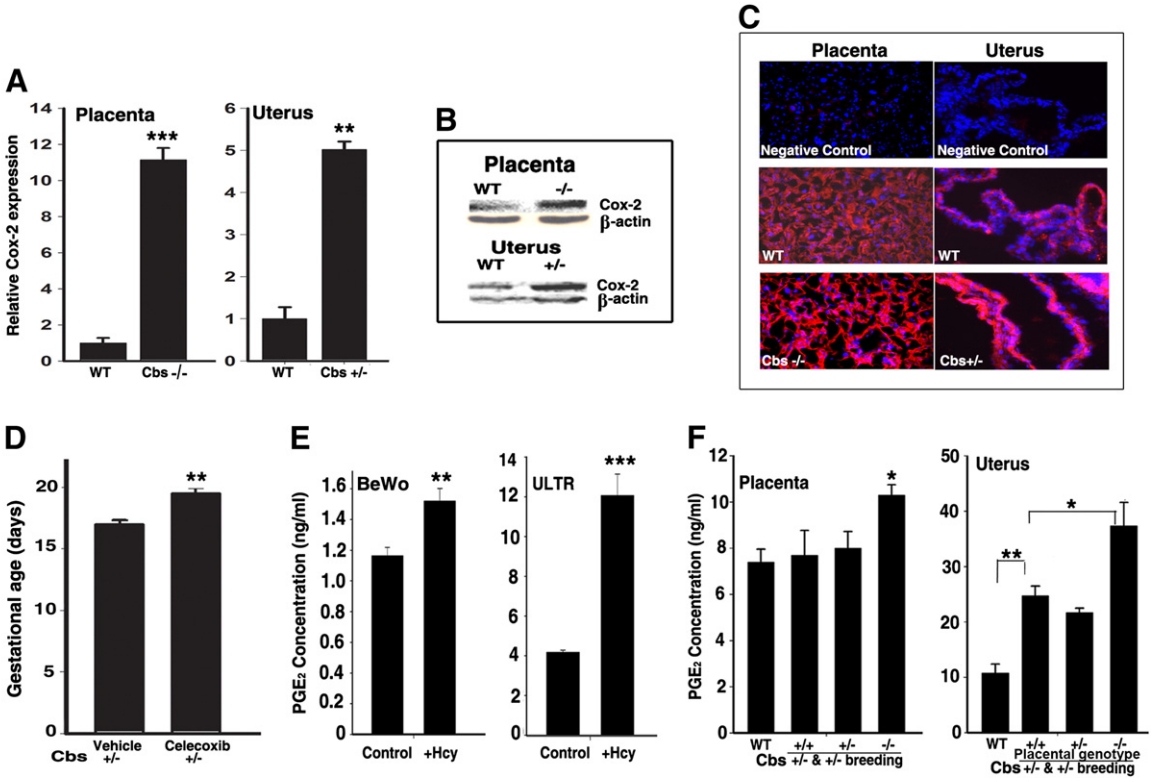

Fig. 2. Role of Cox-2 in premature delivery in *Cbs*$^{+/-}$ mice. (A) RT-qPCR analysis of Cox-2 mRNA in wild type (WT) placenta and uterus and in *Cbs*$^{-/-}$ placenta and *Cbs*$^{+/-}$ uterus on day 16 of pregnancy (**$p < 0.01$; ***$p < 0.001$). (B) Western blot of Cox-2 in wild type (WT) placenta and uterus and in *Cbs*$^{-/-}$ placenta and *Cbs*$^{+/-}$ uterus on day 16 of pregnancy. (C) Immunohistochemical analysis of Cox-2 in wild type placenta and uterus and in *Cbs*$^{-/-}$ placenta and *Cbs*$^{+/-}$ uterus on day 16 of pregnancy. (D) Significant reversal of preterm birth in *Cbs*$^{+/-}$ mice by Cox-2 inhibitor Celecoxib. The drug was administered at a dose of 25 mg/kg body weight by oral gavage once a day on day 15 and 16 of pregnancy (Mean ± S. E., **$p < 0.01$). (E) PGE_2 levels secreted by control and homocysteine (Hcy)-treated BeWo cells and ULTR cells (Hcy treatment: 0.5 mM, four passages; **$p < 0.01$; ***$p < 0.001$). (F) PGE_2 levels in placenta and uterus with different *Cbs* genetic backgrounds. Wild type (WT) placentas and uterine tissues were obtained on 16th day of gestation from matings wild type females with wild type males. In a separate experiment, matings of heterozygous mice were set up, and on 16th day of gestation, placentas and the regions of the uterine tissues attached to each of the placentas were separated. The placental tissues were genotyped. Mean ± S. E., *$p < 0.05$; **$p < 0.01$.

3.4. Effect of homocysteine on BK_{Ca} channel expression and activity in the uterine myometrial cell line ULTR

We examined the influence of homocysteine on expression of the two subunits of the BK_{Ca} channel in the human uterine myometrial cell line ULTR. This was prompted by published reports that BK_{Ca} channels play a critical role in uterine contraction [13–16]. In cells that were cultured in the presence of homocysteine for 5 passages, the α-subunit was upregulated whereas the regulatory β-subunit was downregulated (Fig. 3C). We then monitored the BK_{Ca} channel activity in these cells by measuring large outward K^+ currents using the whole-cell patch-clamp technique. Resting membrane potential was significantly less in homocysteine-treated cells than in control cells (Fig. 3D; $p < 0.05$). These findings were suggestive of depressed K^+ channel function. We then measured macroscopic currents generated by incremental 10 mV depolarizing steps (from −60 to +60 mV). Whole-cell recordings demonstrated a robust outward current whose kinetics suggested both a rapid and a more long-lasting component (Fig. 3E, left panel). Interestingly, both the peak and steady-state outward currents were reduced dramatically in homocysteine-treated cells. In the presence of 1 mM 4-aminopyridine, an inhibitor of voltage-dependent K^+ channels (Kv), the fast activation component was abolished leaving a prominent, slow-activating, non-inactivating outward current that was highly sensitive to blockade by 1 mM tetraethyl ammonium, which at this concentration exhibits selectivity for BK_{Ca} channels. These studies suggest that homocysteine exerts a powerful inhibitory influence upon outward potassium currents in uterine myometrial cells, and that a major component of these currents is indeed due to activity of BK_{Ca} channels.

3.5. Effect of homocysteine on the expression of oxytocin and progesterone receptors

We also compared the expression of several other genes that are related to uterine activity between wild type and *Cbs*$^{+/-}$ uterine tissues collected on 16th day of gestation. The expression of oxytocin receptor was increased several-fold in *Cbs*$^{+/-}$ uterus compared to wild type uterus (Fig. 4A, B). In heterozygote matings (*Cbs*$^{+/-}$ × *Cbs*$^{+/-}$), the expression level varied in different regions of the uterine horn depending on the genotype of the placenta that was attached to the given region. The expression was the lowest in the region attached to wild type placenta and highest in the region attached to *Cbs*$^{-/-}$ placenta. In contrast to oxytocin receptor, the expression of progesterone receptor was less in *Cbs*$^{+/-}$ uterus than in wild type uterus (Fig. 4C).

3.6. Effect of homocysteine on the expression of Cox-2 and the receptors for oxytocin and prostaglandins in placenta and uterus

Even though we have demonstrated that exogenous homocysteine induces Cox-2 expression in the human uterine myometrial cell line ULTR and the human placental trophoblast cell line BeWo, concerns may arise with these findings because of the transformed nature of these cell lines. Therefore, to validate our findings further, we used organ cultures of mouse pregnant uterine tissue and placental tissue. On gestation day 16, wild type pregnant mice were euthanized and uterine and placental tissues were collected. The tissues were chopped into fine pieces, washed thoroughly to remove blood, and then used for organ cultures under sterile conditions with or without homocysteine

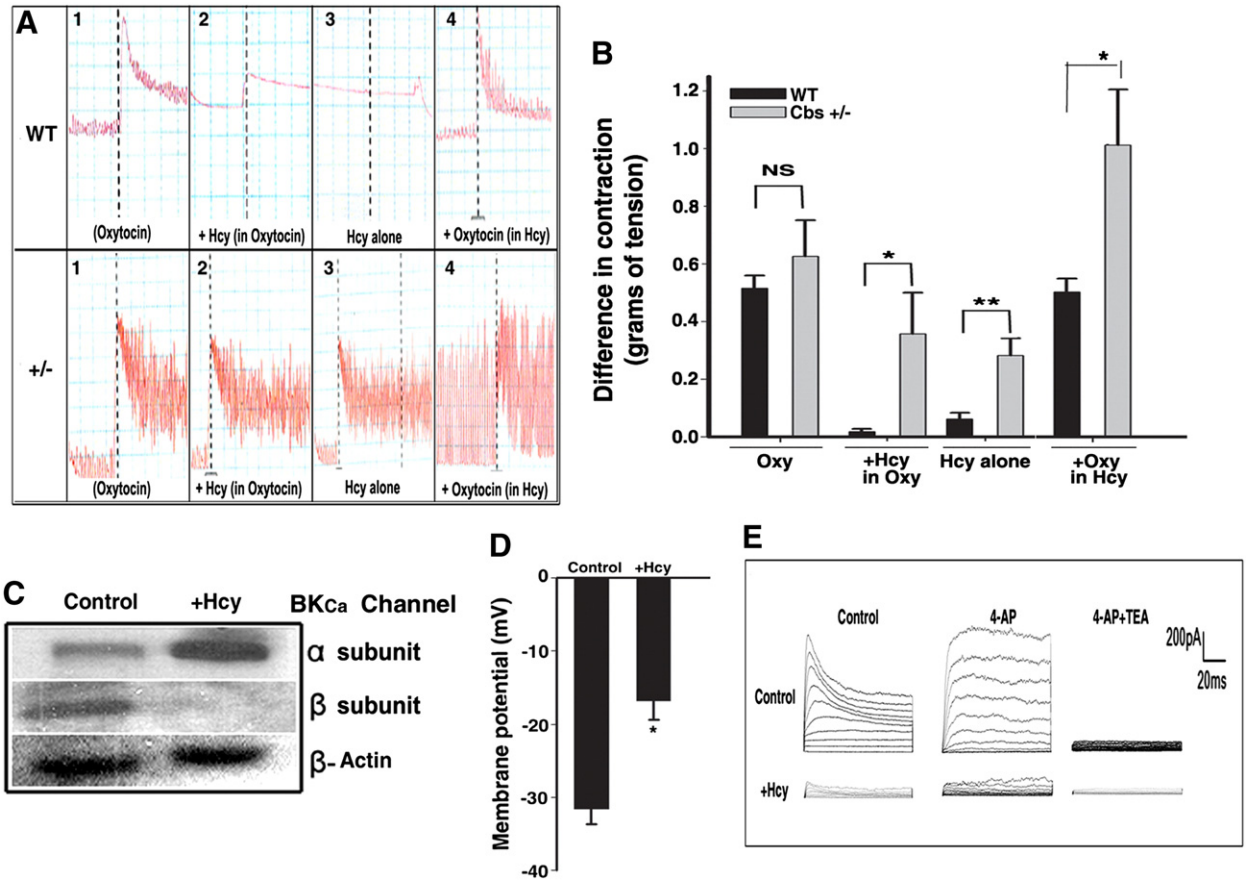

Fig. 3. Effects of homocysteine (Hcy) on uterine muscle preparations from 16-day pregnant wild type and *Cbs*$^{+/-}$ mice. (A) Muscle contraction tracings with uterine rings from wild type and *Cbs*$^{+/-}$ mice (oxytocin, 10 IU; Hcy, 100 μM). (B) Contractions from 3 different animals were quantified (Mean ± S. E., NS, not significant; $^{*}p < 0.05$; $^{**}p < 0.01$). (C) Western blot of the α- and β-subunits of the BK_{Ca} channel in control and Hcy-treated (0.5 mM; 4 passages) ULTR cells. (D) Resting membrane potential in control and Hcy-treated (0.5 mM; 4 passages) ULTR cells (Mean ± S. E.; $^{*}p < 0.05$). (E) Whole-cell patch-clamp recordings of outward currents in control and Hcy-treated (0.5 mM; 4 passages) ULTR cells in the absence and presence of 1 mM 4-aminopyridine (4-AP), an inhibitor of voltage-dependent (Kv) channels, with or without 1 mM tetraethylammonium (TEA), an inhibitor of BK_{Ca} channel.

(500 μM) for 72 h. Following the treatment, total RNA was isolated for analysis of gene expression. Similar experiments were also carried out with primary placental trophoblast cells prepared from normal term human placentas. We found that homocysteine treatment increased the expression of Cox-2 as well as that of the oxytocin receptor in all three preparations (Fig. 5). The prostaglandin receptor EP3 was also

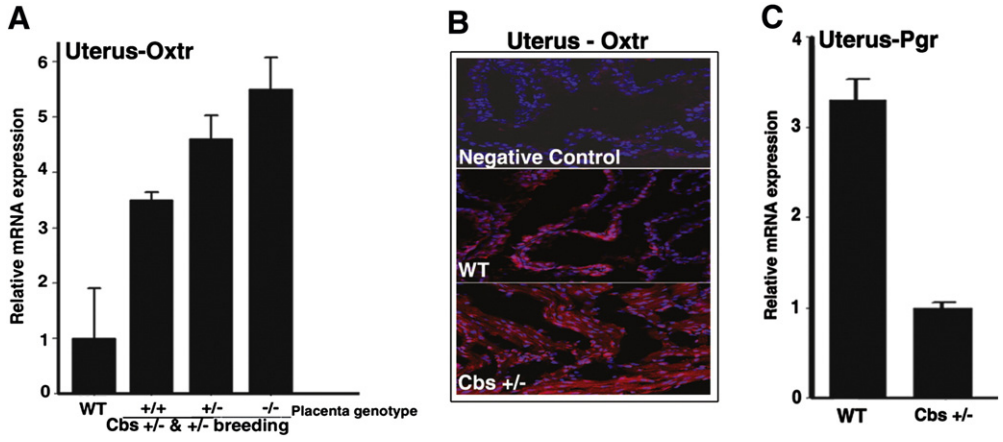

Fig. 4. Expression of oxytocin receptor and progesterone receptor in Cbs$^{+/-}$ mouse uterus. (A) RT-qPCR showing upregulation of oxytocin receptor (*Oxtr*) in *Cbs*$^{+/-}$ uterus on day 16 of pregnancy. Wild type (WT) uterine tissues were obtained on 16th day of gestation from matings of wild type females with wild type males. In a separate experiment, matings of heterozygous mice were set up, and on 16th day of gestation, placentas and the regions of the uterine tissues attached to each of the placentas were separated. The placental tissues were genotyped. The expression of oxytocin receptor was then analyzed using RNA isolated from the regions of the uterine tissue that were attached to placentas of different genotype. The genotype of the uterine tissue in all these regions is *Cbs*$^{+/-}$. (B) Immunohistochemical analysis of *Oxtr* in wild type and *Cbs*$^{+/-}$ uterine tissues on day 16 of pregnancy. (C) RT-qPCR showing downregulation of progesterone receptor (*Pgr*) in *Cbs*$^{+/-}$ uterus on day 16 of pregnancy.

upregulated markedly by homocysteine in human primary trophoblast cells, but this was not seen with organ cultures of mouse uterine and placental tissues.

3.7. Reversal of preterm birth in *Cbs*$^{+/-}$ mice by deletion of *Gpr109a*

Based on the published reports that GPR109A, a G-protein-coupled receptor for niacin, induces COX-2 and increases production of prostaglandins in some cell types [17,18], we wanted to know if homocysteine-induced preterm birth in mice was mediated through this receptor. We first examined if there are differences in circulating levels of homocysteine between wild type mice and *Gpr109a*$^{-/-}$ mice. The plasma concentration of homocysteine in wild type mice was 4.8 ± 0.1 μM, and the corresponding value in *Gpr109a*$^{-/-}$ mice was 3.2 ± 0.3 μM. This difference was significant ($p < 0.05$). We then generated *Cbs*$^{+/-}$ mice on *Gpr109a*$^{+/+}$ and *Gpr109a*$^{-/-}$ backgrounds and compared the gestational period between the two mouse lines. While *Cbs*$^{+/-}$ females on *Gpr109a*$^{+/+}$ background littered prematurely, *Cbs*$^{+/-}$ females on *Gpr109a*$^{-/-}$ background had normal gestational period (Fig. 6A). This reversal of preterm birth in *Cbs*$^{+/-}$ females upon deletion of *Gpr109a* was accompanied by reversal of Cox-2 expression in placenta and uterus (Fig. 6B). We also found that the expression of *Gpr109a* was increased in both *Cbs*$^{-/-}$ placenta and *Cbs*$^{+/-}$ uterus in response to hyperhomocysteinemia (Fig. 6C). The influence of homocysteine on *GPR109A* expression was also seen in BeWo and ULTR cells (Fig. 6D). Placenta and uterus from wild type mice express Gpr109a constitutively, and the expression is increased in mice with hyperhomocysteinemia (Fig. 6E).

4. Discussion

The findings of the present study can be summarized as follows. Elevated levels of homocyteine have detrimental effects in mice during pregnancy, which include significant embryonic lethality, decreased placental weight, decreased birth weight, and preterm delivery. Homocysteine is known for its profound effects on vasculature [19,20] and has been implicated in the pathogenesis of preeclampsia [7,8]. However, there have been no reports in the literature on the role of this amino acid in premature birth. Our present studies show for the first time that elevated levels of homocysteine in mice induce preterm birth. The underlying molecular mechanism involves homocysteine-induced upregulation of Cox-2 and consequent generation of the uterotonic molecule prostaglandin E_2 as well as upregulation of the oxytocin receptor. Cox-2 induction and increased production of PGE_2 are observed in the uterine and placental tissues obtained from the hyperhomocysteinemic pregnant mice. Exogenous homocysteine induces contraction of uterine muscle by itself and also potentiates oxytocin-mediated contraction *in vitro*. Administration of Celecoxib, a pharmacological blocker of Cox-2, to hyperhomocysteinemic pregnant mice provides significant protection against preterm birth. The ability of homocysteine to upregulate Cox-2 and oxytocin receptor is seen not only in human uterine myometrial and placental cell lines but also in organ cultures of mouse uterine and placental tissues and in primary cultures of human placental trophoblasts. Therefore, it appears that Cox-2 and its product PGE_2 in both uterus and placenta are likely to contribute to the premature uterine contraction seen in hyperhomocysteinemic female mice. Even though our studies have unequivocally demonstrated the induction of Cox-2

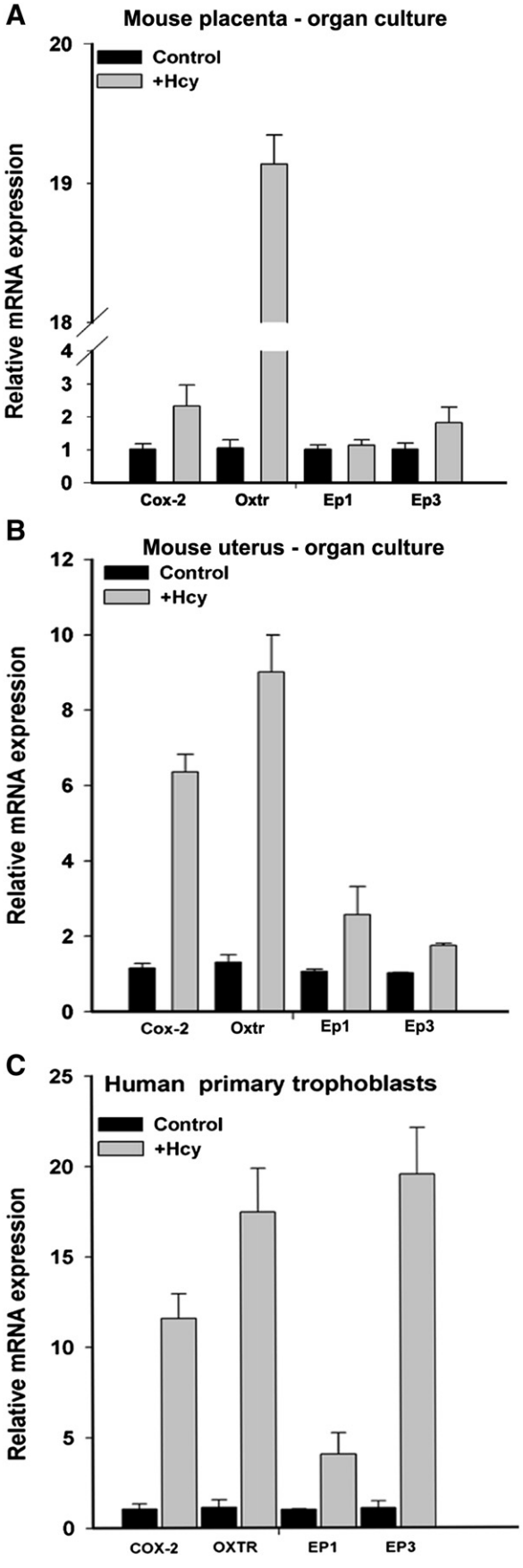

Fig. 5. Induction of Cox-2 and oxytocin receptor by homocysteine in organ cultures of mouse uterine and placental tissues and in human primary placental trophoblast cells. (A & B) Uterine and placental tissues were collected from wild type pregnant mice on day 16 of gestation. The tissues were cut into small pieces and washed thoroughly with sterile phosphate-buffered saline to remove blood. The tissue fragments were then cultured under sterile conditions for 72 h in the presence or absence of homocysteine (500 μM). Total RNA was then isolated and used for RT-qPCR. (C) Similar experiments were carried with primary trophoblast cells isolated from normal human term placentas.

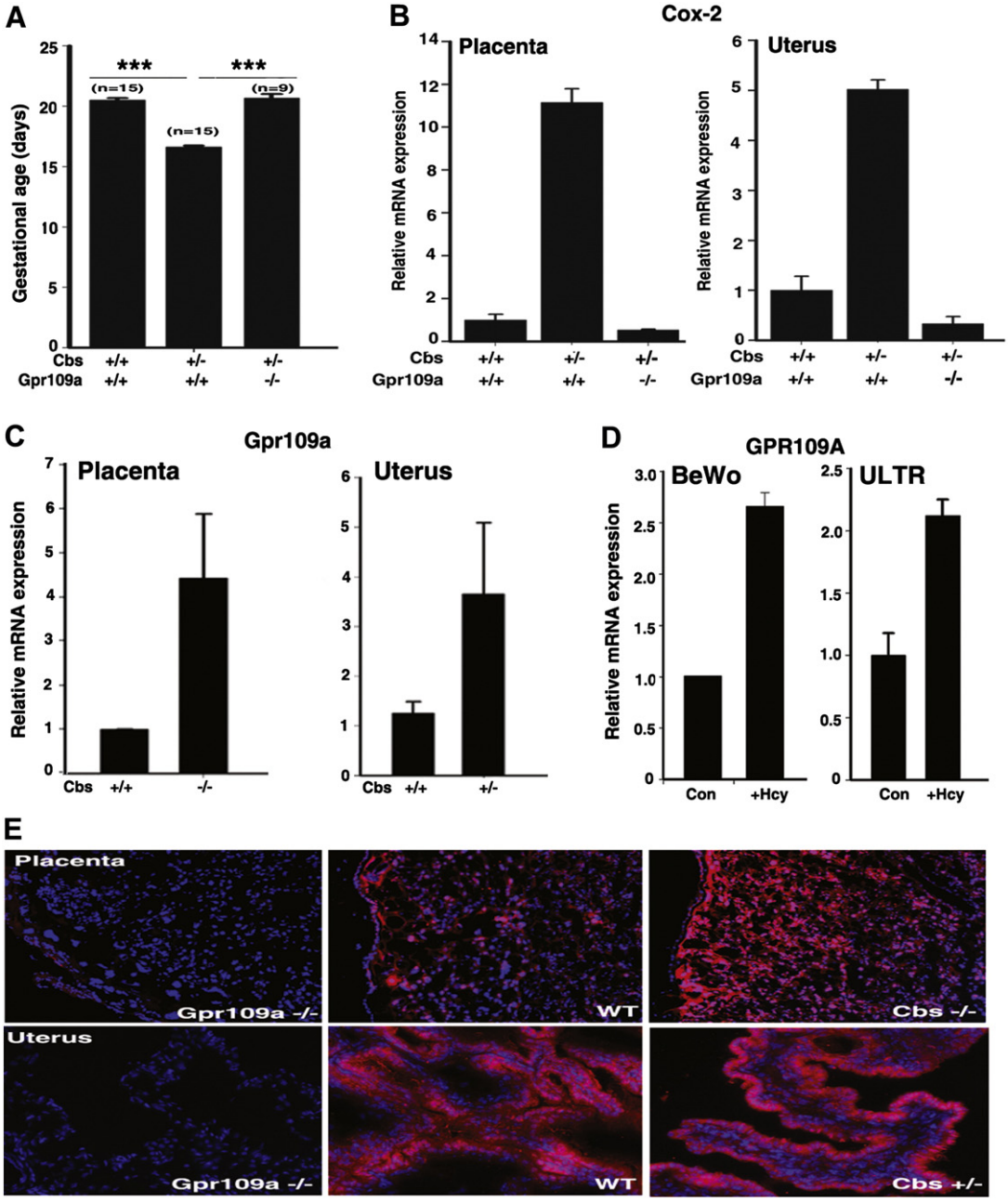

Fig. 6. Reversal of preterm birth in *Cbs*$^{+/-}$ mice by deletion of *Gpr109a*. (A) Gestational period in wild type mice and in *Cbs*$^{+/-}$ mice on *Gpr109a*$^{+/+}$ and *Gpr109a*$^{-/-}$ backgrounds (Mean ± S. E.; ***$p < 0.001$). (B) RT-qPCR of Cox-2 mRNA levels in placentas and uteri from wild type and *Cbs*$^{+/-}$ mice on *Gpr109a*$^{+/+}$ and *Gpr109a*$^{-/-}$ backgrounds on 16th day of pregnancy (Mean ± S. E.). (C) RT-qPCR of Gpr109a mRNA levels in wild type and *Cbs*$^{-/-}$ placentas and in wild type and *Cbs*$^{+/-}$ uterus; (D) RT-qPCR of GPR109A mRNA levels in control and Hcy-treated (0.5 mM; 4 passages) BeWo and ULTR cells. (E) Immunohistochemical analysis of Gpr109a expression in wild type placenta and uterus, *Cbs*$^{-/-}$ placenta and *Cbs*$^{+/-}$ uterus.

by homocysteine both in vivo and *in vitro*, the signaling events associated with the process remain to be investigated.

Normal pregnancy is associated with increased oxidative stress, and this is aggravated even further in pre-eclampsia [21]. Homocysteine is known to cause oxidative stress [5,6], and elevated levels of homocysteine constitute a risk factor for pre-eclampsia [7,8]. Since Cbs is an enzyme in the trans-sulfuration pathway that is essential for endogenous synthesis of cysteine, a limiting amino acid for glutathione biosynthesis, it is likely that disruption of the *Cbs* gene results in decreased glutathione and consequently increased oxidative stress. There are reports implicating oxidative stress in the induction of COX-2 [22]. Therefore, if elevated levels of homocysteine induce oxidative stress, this could serve as a signal in the homocysteine-induced upregulation of Cox-2.

It is not known at present whether preterm birth observed in *Cbs*$^{+/-}$ mice is evident in other mouse models of hyperhomocysteinemia. Such models have been described in the literature [23]. For example, nutritional manipulations (e.g., methionine loading via modified diet, limiting the dietary content of the vitamins folic acid, B_{12}, or B_6) can elevate the circulating levels of homocysteine in mice. However, there are limitations to the use of dietary models of hyperhomocysteinemia because dietary manipulations not only cause an increase in homocysteine but also lead to alterations in other metabolites and biochemical pathways. Therefore, even if any of these mouse models is associated with preterm birth, it may be difficult to ascertain unequivocally the role of homocysteine as the factor responsible for this phenotype. There are two other genetic models of hyperhomocysteinemia in

mice: *Mthfr*-deficient mouse [24] and methionine synthase-deficient mouse [25]. *Mthfr*$^{-/-}$ mice have elevated levels of homocysteine and suffer from decreased survival, and motor and gait abnormalities. Homozygous deletion of methionine synthase is embryonically lethal whereas heterozygous deletion of the gene results in no significant changes in circulating levels of homocysteine. Therefore, *Mthfr*$^{-/-}$ mice may provide an additional animal model suitable to investigate the relevance of hyperhomocysteinemia to preterm birth. Apparently, the gestational period in this mouse model has not yet been examined because we could not find any information in the literature on this issue. This was also the case with *Cbs*$^{+/-}$ mouse; this mouse model was first reported almost 20 years ago [9], but the gestational age was not investigated until the present study. Therefore, it would be of interest to investigate the gestational period in *Mthfr*$^{-/-}$ mice in the future to complement the present findings in *Cbs*$^{+/-}$ mice.

Homocysteine contains a reactive thiol group and is known to covalently modify proteins via a process called homocysteinylation [26] This process also interferes with collagen crosslinking by breaking down disulfide bonds through formation of mixed disulfides with cysteine residues in collagen [26]. In addition, collagen synthesis and cross-linking are also affected by mercaptopropionaldehyde, a metabolite of homocysteine [27]. The effects on vasculature and collagen are believed to be the primary reasons for the clinical symptoms associated with hyperhomocysteinemia, which include thrombosis, osteoporosis, and dislocation of lens [28]. Here we provide evidence for the first time that uterus is also a prime target for homocysteine. Elevated levels of homocysteine have profound effects on this tissue. The effects include changes in the expression of various genes that are closely associated with uterine function. Exposure of uterus to homocysteine increases the expression of oxytocin receptor but decreases the expression of progesterone receptor, thus simultaneously enhancing the contractile response of the tissue to oxytocin and decreasing the ability of progesterone to relax the tissue. In addition, induction of Cox-2 with consequent generation of prostaglandins also facilitates homocysteine-induced uterine contraction. The enhanced contractile response of uterine tissues from pregnant hyperhomocysteinemic mice to oxytocin provides supporting evidence for homocysteine-induced promotion of uterine contraction. Even though the present studies focused on the Cox-2/PGE_2 pathway as the underlying mechanism for the premature contraction associated with hyperhomocysteinemia, other pathways may also be potentially relevant. Homocysteine has been shown to increase the production of pro-inflammatory cytokines [29,30] and metalloproteinases [31,32], both of which have been implicated in promotion of uterine contraction and cervical ripening.

Even though there are significant differences between mice and humans in terms of biological processes associated with parturition, the present findings implicating elevated levels of homocysteine as a risk factor for preterm birth in mice are likely to be relevant to humans. There is evidence from epidemiological studies that folic acid deficiency is linked to preterm delivery in humans [33–38]. One of the biochemical hallmarks of folic acid deficiency is hyperhomocysteinemia, thus implicating homocysteine as a potential causative factor in preterm birth observed in humans with folic acid deficiency. Elevated levels of homocysteine have also been shown to be a pathogenic factor in preeclampsia, a condition associated with preterm delivery [7,8]. These observations suggest that plasma levels of homocysteine during pregnancy may have potential as a biomarker to predict the risk of premature birth. Various nutritional and genetic factors contribute to the plasma levels of homocysteine in humans. Since homocysteine is either converted to methionine by the folate/B_{12}-dependent enzyme methionine synthase or converted to cysteine by vitamin B_6-dependent trans-sulfuration pathway, deficiency in folate, B_{12} or B_6 can result in accumulation of homocysteine. Elevated levels of homocysteine are also seen in patients with mutations in methionine synthase and cystathionine β-synthase. However, the frequency of mutations in these two enzymes is rare in the general population. Another enzyme whose activity is related to circulating levels of homocysteine is N^5, N^{10}-methylenetetrahydrofolate reductase (MTHFR). This enzyme is obligatory in the synthesis of N^5-methyltetrahydrofolate necessary for the remethylation of homocysteine by methionine synthase. Mutations in MTHFR are quite prevalent in general population [39,40], and these mutations result in an increase in circulating levels of homocysteine. The most frequent genetic alterations in MTHFR are Ala222Val (C→T mutation) and Glu429Ala (A→C mutation). The variations in plasma levels of homocysteine in the general population are primarily due to MTHFR mutations coupled with dietary folic acid intake status. It would be interesting and important to determine if there is any significant correlation between circulating levels of homocysteine during pregnancy and the length of gestation in humans.

The involvement of the niacin receptor Gpr109a in homocysteine effects on uterus is an unexpected surprise finding. When we initiated this work, it was not even known that this receptor is expressed in the utero-placental unit. Present studies show that it is not only expressed abundantly in placenta and uterus but also plays a role in homocysteine-induced facilitation of uterine contraction. Exposure of both tissues to homocysteine enhances the expression of the receptor even further. The obligatory nature of the involvement of the receptor in preterm birth associated with hyperhomocysteinemia is supported by the findings that deletion of this receptor in hyperhomocysteinemic mice prevents preterm birth.

The findings of the present studies have immense clinical relevance considering the fact that preterm birth causes a significant increase in morbidity and mortality in babies. These studies demonstrate that elevated levels of homocysteine in blood in pregnant women could serve as a biomarker to predict the risk of premature delivery. Strategies to reduce the levels of homocysteine, such as nutritional supplementation with folic acid, B_{12}, and B_6, could be used as a prophylaxis to decrease the risk of premature delivery. MTHFR mutations decrease the affinity of the enzyme for N^5, N^{10}-methylenetetrahydrofolate; therefore, folic acid supplementation would be able to correct the defect in the enzyme activity by increasing the concentrations of the coenzyme available for the mutant enzyme. Pharmacologically, 17α-hydroxyprogesterone remains as the only drug used at present in pregnancy for prevention of preterm birth [41,42]. Our present studies open up a new avenue for development of novel drugs for prevention of preterm birth by focusing on antagonists that interfere with GPR109A signaling in the utero-placental unit.

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Disclosures

None.

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