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Research Article

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Combinatory approaches prevent preterm birth profoundly exacerbated by gene-environment interactions

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There are currently more than 15 million preterm births each year. We propose that gene-environment interaction is a major contributor to preterm birth. To address this experimentally, we generated a mouse model with uterine deletion of *Trp53*, which exhibits approximately 50% incidence of spontaneous preterm birth due to premature decidual senescence with increased mTORC1 activity and COX2 signaling. Here we provide evidence that this predisposition provoked preterm birth in 100% of females exposed to a mild inflammatory insult with LPS, revealing the high significance of gene-environment interactions in preterm birth. More intriguingly, preterm birth was rescued in LPS-treated *Trp53*-deficient mice when they were treated with a combination of rapamycin (mTORC1 inhibitor) and progesterone (P_4), without adverse effects on maternal or fetal health. These results provide evidence for the cooperative contributions of two sites of action (decidua and ovary) toward preterm birth. Moreover, a similar signature of decidual senescence with increased mTORC1 and COX2 signaling was observed in women undergoing preterm birth. Collectively, our findings show that superimposition of inflammation on genetic predisposition results in high incidence of preterm birth and suggest that combined treatment with low doses of rapamycin and P_4 may help reduce the incidence of preterm birth in high-risk women.

Introduction

Preterm birth in humans, defined as parturition occurring prior to 37 weeks of gestation, is still a global problem. One in 10 babies is born preterm worldwide, accounting annually for more than 15 million premature births (1–4). With more than 1 million deaths attributed to complications arising from prematurity each year, preterm birth is a leading cause of neonatal death (1, 5, 6). In addition, premature babies who survive are at increased risk for a number of health challenges, including respiratory distress, underdeveloped organ systems, and cerebral palsy with learning and developmental disabilities (1, 2, 7). Several risk factors, including genetic predisposition, infection/inflammation, oxidative stress, short cervix, progesterone (P_4) resistance, increased maternal age, and stretch signaling originating from multiple pregnancy, contribute to this multifactorial disorder (1, 8). Due to its complex nature, mechanisms underpinning preterm birth are not clearly understood.

While more than 60% of preterm births occur in developing countries in Africa and South Asia, a recent report from the WHO identified the United States as being among the ten countries with the highest numbers of preterm birth (1), underscoring the global nature of this problem. Etiologies behind high preterm birth rates in developing and developed countries may be disparate – infection/inflammation affects developing countries more, while assisted reproductive technology (ART) compounded by increased maternal age at conception as well as increased prevalence of diabetes and high

blood pressure are additional risk factors in developed countries (1). These observations suggest that preterm birth is the end result of many different causative factors. Therefore, multiple approaches and model systems are warranted in addressing this problem.

Mechanistic preclinical studies utilizing mouse models of preterm birth should help in addressing this problem. Inflammatory mediators such as endotoxin (LPS) and inflammatory cytokines (such as IL-1 β , IL-6, and TNF- α) are known to induce preterm labor coincident with ovarian luteolysis with a decrease in serum P_4 levels (9). Along the same lines, administration of RU-486 (mifepristone), a progesterone receptor (PR) antagonist, leads to similar effects (8, 10). However, these mouse models may not adequately define the mechanism of parturition timing, since human preterm birth is considered to occur without a drop in serum P_4 levels (11), although further studies are required to assess P_4 levels accounting for disparate etiologies of preterm birth.

Mouse and human studies have shown that aberrations in early pregnancy can be propagated during the subsequent course of pregnancy and lead to compromised pregnancy outcomes, including preterm birth (12). We have generated a mouse model harboring a conditional uterine deletion of *Trp53*, encoding tumor suppressor protein p53 (*Trp53^{loxP/loxP}Pgy^{Cre/+}*). These mice exhibit premature decidual senescence associated with heightened mTORC1 signaling early in pregnancy. Strikingly, they show genetic predisposition to preterm birth; approximately 50% incidence of spontaneous preterm delivery with fetal death and dystocia is observed in these females without a drop in serum P_4 levels (13). Progressive decidual senescence was coincident with aberrantly higher levels of COX2 and prostaglandin F synthase (PGFS), reflected in increased uterine levels of

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Table 1
p53^{del/d} mice are more susceptible to inflammation-induced preterm birth

Treatment	Genotype	Time of delivery	No. of dams	Rate of preterm birth	% Dead pups and resorptions/ total number of pups
75 µg LPS	<i>p53^{fl/fl}</i>	Day 17 0800h–1800h	5	83% (5)	100% (32/32)
		Day 19 1800h to day 20 0800h	1 ^A	0% (0)	67% (6/9)
		Total	6	83% (5)	93% (38/41)
50 µg LPS	<i>p53^{fl/fl}</i>	Day 17 0800h–1800h	7	71% (5)	67% (45/67)
		Day 19 1800h to day 20 0800h	5	0% (0)	0% (0/44)
37 µg LPS	<i>p53^{fl/fl}</i>	Day 17 1200h–1800h	1	25% (1)	100% (8/8)
		Day 17 1800h to day 18 1800h	3	75% (3)	100% (25/25)
	Total	4	100% (4)	100% (33/33)	
	<i>p53^{del/d}</i>	Day 19 0800h to day 21 0800h	12	0% (0)	1% (1/99)
Day 16 1700h to day 17 0800h		3	15% (3)	100% (16/16)	
Day 17 0800h–1800h		13	65% (13)	95% (81/85)	
10 µg LPS	<i>p53^{del/d}</i>	Day 17 1800h to day 18 1800h	4	20% (4)	100% (19/19)
		Total	20	100% (20)	94% (113/117)

Littermate *p53^{fl/fl}* (*Trp53^{loxP/loxP}Pgr^{Cre/+}*) and *p53^{del/d}* (*Trp53^{loxP/loxP}Pgr^{Cre/+}*) dams were used. Time of delivery is defined as the day of pregnancy when the dam first started delivering pups. Preterm birth is defined as delivery occurring before day 19 of pregnancy. LPS was administered i.p. on day 16 of pregnancy at 1200h. The rate of preterm birth was calculated as the number of females exhibiting preterm birth over the total number of females examined within a treatment group, while numbers in parentheses indicate the number of females. The percentage of dead pups and resorptions was calculated over the total number of pups delivered, while numbers in parentheses indicate their absolute numbers. ^AOne dam lost weight daily from days 16 through 19 of pregnancy, indicative of resorption, with 3 live pups; 9 placental scars were observed when the uterus was exposed post mortem.

PGF_{2α}, triggering myometrial contractility in a paracrine manner to induce preterm birth (13). These results led us to speculate that targeting COX2 and/or mTORC1 signaling could prevent preterm birth in this model. Indeed, we were successful in preventing preterm birth in *Trp53^{loxP/loxP}Pgr^{Cre/+}* females by oral gavage of celecoxib, a selective inhibitor of COX2, on day 16 of pregnancy or by inhibition of mTORC1 signaling by a low dose of rapamycin, which attenuated the progression of premature decidual senescence (13, 14).

Preterm birth is the result of many contributing factors, including genetic predisposition and environmental insults. Although gene-environment interactions are thought to contribute to the disease process, this concept has not been experimentally tested in a predisposed model. Our current studies provide evidence-based proof of principle that when genetic predisposition is superimposed with inflammation, the rate of preterm birth is profoundly enhanced. We show that *Trp53^{loxP/loxP}Pgr^{Cre/+}* females exposed to even a mild inflammatory insult are exquisitely susceptible to preterm birth, with a decrease in P₄ levels. Intriguingly, combination therapy with rapamycin and P₄ targeting both the decidual senescence imposed by *Trp53* deletion and the inflammatory insult can effectively rescue preterm birth. These findings point to gene-environment interactions in preterm birth through cooperative contributions of at least two sites of action (decidua and ovary) and an effective preventative strategy.

These studies in mice prompted us to assess whether similar signaling pathways are operative in human preterm delivery. We present evidence that a signature of decidual senescence with increased mTORC1 signaling and COX2 expression is active in human preterm birth. Superficial decidual cells adherent to the term placenta of women were isolated for culture and showed increased levels of the inflammatory mediators IL-6 and IL-8 in the presence of TLR4-specific LPS, but their levels were reduced by addition of P₄ and/or rapamycin. Collectively, the results suggest a close relationship between genetic predisposition and environmental insults in exacerbating preterm delivery.

Results

Trp53^{loxP/loxP}Pgr^{Cre/+} mice show increased sensitivity to inflammation-induced preterm birth. To generate mice with uterine-specific deletion of *Trp53*, we mated *Trp53^{loxP/loxP}* females with males expressing Cre recombinase driven by the *Pgr* promoter (*Pgr^{Cre/+}*), as previously described (13, 14). *Trp53^{loxP/loxP}Pgr^{Cre/+}* females show approximately 50% incidence of spontaneous preterm delivery with dystocia and fetal death compared with floxed littermates showing normal pregnancy outcome; preterm delivery is defined as birth occurring before day 19 of pregnancy (14). To compare the sensitivity of these females to inflammation with respect to preterm birth, we injected TLR4-specific LPS i.p. into *Trp53^{loxP/loxP}Pgr^{Cre/+}* and *Trp53^{loxP/loxP}Pgr^{Cre/+}* females. Ultrapure LPS was used to avoid contamination by other TLR agonists normally found in commercial preparations that are often used in preterm birth studies. *Trp53^{loxP/loxP}Pgr^{Cre/+}* females showed 100% incidence of preterm birth and/or fetal resorption and death when injected with 75 µg TLR4-specific LPS on day 16 of pregnancy. While a dose of even 50 µg was quite effective in inducing preterm birth (71%), lower doses of LPS (10 or 37 µg) were ineffective in inducing preterm birth in floxed females (Table 1). Remarkably, an injection of 10 µg LPS on day 16 induced preterm birth with stillbirth in all *Trp53^{loxP/loxP}Pgr^{Cre/+}* littermates examined (*n* = 20). These results clearly demonstrate that these females are exquisitely sensitive to preterm delivery.

Trp53^{loxP/loxP}Pgr^{Cre/+} females show exaggerated uterine prostaglandin production. Prostaglandins (PGs) are normally generated by the COX system, which exists in two isoforms, COX1 and COX2. While constitutive COX1 is considered to maintain basal levels of PGs, COX2-induced PGs are normally generated by inflammatory stimuli and are known to participate in parturition (13, 15, 16). Among various PGs, PGF_{2α} is implicated in parturition timing by synchronizing myometrial contractility. We have previously shown that levels of uterine COX1 remains unaltered in *Trp53^{loxP/loxP}Pgr^{Cre/+}* and *Trp53^{loxP/loxP}Pgr^{Cre/+}* females, while uterine COX2 levels are upregulated in *Trp53^{loxP/loxP}Pgr^{Cre/+}* females, with increased levels of PGFS and PGF_{2α} (13). We also found that

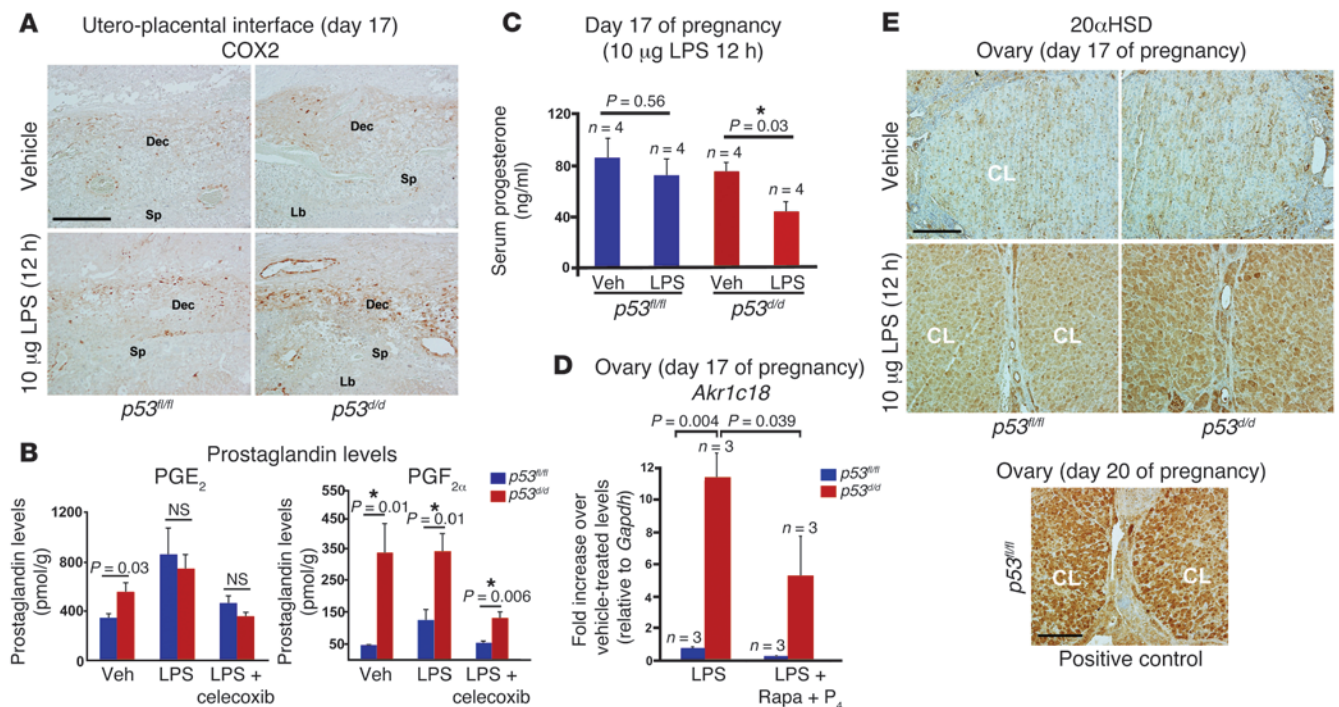


Figure 1

Mild inflammatory insult in $p53^{d/d}$ female mice upregulates decidual COX2 signaling and renders ovaries more sensitive to luteolysis. (A) Immunohistochemistry showed upregulated COX2 expression in the decidua of $Trp53^{loxP/loxP}Pgr^{Cre/+}$ ($p53^{d/d}$) mice 12 hours after an injection of 10 μ g LPS at 1900h on day 16 of pregnancy. Dec, decidua; Sp, spongiotrophoblast; Lb, labyrinth. Scale bar: 250 μ m. (B) Mass spectrometric analysis showed that uterine levels of PGF_{2 α} , but not PGE₂, are significantly upregulated in LPS-treated $p53^{d/d}$ females as compared with $Trp53^{loxP/loxP}Pgr^{+/+}$ ($p53^{fl/fl}$) littermates. This upregulation was suppressed by celecoxib treatment. Three to 6 independent samples isolated from each mouse were analyzed ($n = 3-5$ mice/treatment group; mean \pm SEM; * $P < 0.05$). (C) Serum P₄ levels were measured 12 hours after LPS or vehicle injection. $p53^{d/d}$ females showed significant decreases in serum P₄ levels as compared with $p53^{fl/fl}$ littermates, which did not show any significant differences (mean \pm SEM; * $P < 0.05$). (D) qPCR results showed significant upregulation of *Akr1c18* in ovaries of $p53^{d/d}$ females after LPS injection compared with those in $p53^{fl/fl}$ littermates. This upregulation was attenuated by rapamycin (Rapa) and P₄ treatment (mean \pm SEM). (E) Immunohistochemistry for 20 α HSD in CL of vehicle-treated $p53^{fl/fl}$ and $p53^{d/d}$ females showed similar signal levels, as compared with increased signal levels in CL of $p53^{d/d}$ females 12 hours after LPS injection, albeit with some increases in $p53^{fl/fl}$ CL. As expected, sections of ovaries from $p53^{fl/fl}$ females on day 20 of pregnancy prior to parturition showed higher expression of 20 α HSD (positive control). Scale bars: 100 μ m.

upregulation of COX2 primarily occurs in the decidua, suggesting that decidua-derived PGF_{2 α} causes myometrial contractions in a paracrine manner.

In the present study, we found that COX2 immunostaining was more intense in deciduae and endothelial cells of decidual vasculature of $Trp53^{loxP/loxP}Pgr^{Cre/+}$ females as compared with $Trp53^{loxP/loxP}Pgr^{+/+}$ females after 12-hour treatment with a low dose of LPS (10 μ g) given on day 16 of pregnancy (Figure 1A). Increases in decidual COX2 levels in $Trp53^{loxP/loxP}Pgr^{Cre/+}$ females after exposure to LPS were also reflected in higher levels of PGF_{2 α} without alterations in PGE₂ levels, as determined by mass spectrometric analysis (Figure 1B). Notably, these increased levels were similar to those in untreated $Trp53^{loxP/loxP}Pgr^{Cre/+}$ females, but much higher than in $Trp53^{loxP/loxP}Pgr^{+/+}$ mice treated with LPS. Increased levels of PGF_{2 α} after LPS administration were attenuated by oral administration of celecoxib. These results raised the question of how even a small insult results in such profound effects on preterm birth. Notably, acute exposure to LPS did not increase the intensity of senescence-associated β -gal staining (SA- β -gal), an established marker of cellular senescence, which was already higher in $Trp53^{loxP/loxP}Pgr^{Cre/+}$ deciduae (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI70098DS1).

Trp53^{loxP/loxP}Pgr^{Cre/+} females show ovarian insufficiency in response to a mild inflammatory stimulus. P₄ is an absolute requirement for pregnancy success, and withdrawal of P₄ signaling is critical for parturition (12). Therefore, we examined whether the ovarian output of P₄ is altered in the $Trp53^{loxP/loxP}Pgr^{Cre/+}$ females receiving 10 μ g LPS. Large doses of LPS ranging from 50 to 250 μ g have been shown to induce ovarian luteolysis (9), the functional and structural disintegration of corpora lutea (CL); this process triggers a rapid decrease in serum P₄ levels, leading to preterm birth or pregnancy termination in rodents (9, 17), although other systemic responses cannot be ruled out. Normally, $Trp53^{loxP/loxP}Pgr^{Cre/+}$ females do not show ovarian luteolysis or a decrease in serum P₄ levels but still exhibit approximately 50% spontaneous preterm birth (13). In the present investigation, we found that an injection of even 10 μ g LPS in $Trp53^{loxP/loxP}Pgr^{Cre/+}$ females on day 16 of pregnancy triggered a drop in serum P₄ levels 12 hours after injection (Figure 1C). This was reflected in higher expression of ovarian *Akr1c18*, encoding mouse 20 α -hydroxysteroid dehydrogenase (20 α HSD), which metabolizes P₄ to an inactive form (Figure 1, D and E). No significant drop in P₄ levels nor increased 20 α HSD expression at the mRNA or protein levels was noted in $Trp53^{loxP/loxP}Pgr^{+/+}$ females after such treatment. As previously shown by oth-

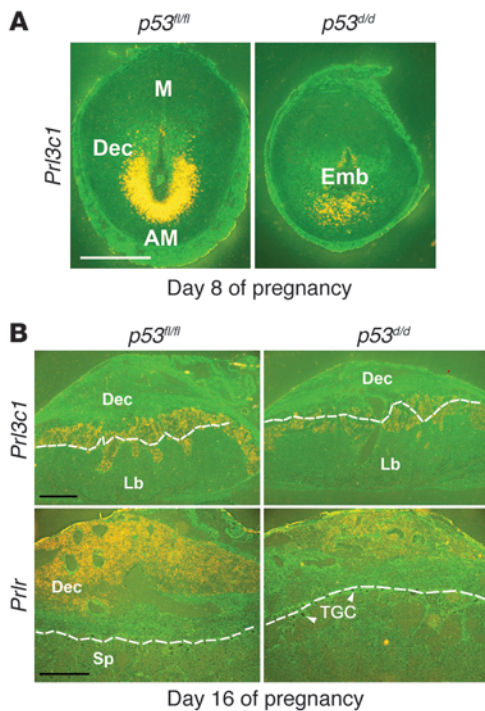


Figure 2

Expression of *Prl3c1* and *Prlr* is downregulated in decidua and at the decidual-placental interface in *p53^{did}* females. **(A)** In situ hybridization of *Prl3c1* in *p53^{fl/fl}* and *p53^{did}* deciduae on day 8 of pregnancy showing significant downregulation in *p53^{did}* decidua. Scale bar: 1 mm. **(B)** In situ hybridization of *Prl3c1* and *Prlr* in *p53^{did}* and *p53^{fl/fl}* deciduae on day 16 of pregnancy, again showing downregulation in *p53^{did}* females. Dotted lines demarcate the location of trophoblast giant cells (TGC) at the decidual-placental interface. Scale bar for *Prl3c1*: 1 mm; for *Prlr*: 500 μ m. Emb, embryo; AM, antimesometrial pole; M, mesometrial pole; Lb, labyrinth.

ers (18, 19), ovarian insufficiency in LPS-treated *Trp53^{loxP/loxP} Pgy^{Cre/+}* females was also reflected in upregulation of *Socs1* and *Socs3* expression (Supplemental Figure 2, A and B). These results suggest that ovaries in *Trp53^{loxP/loxP} Pgy^{Cre/+}* mice are more sensitive to luteolysis even with a mild environmental insult, raising the question regarding the mechanism for the heightened sensitivity of *Trp53^{loxP/loxP} Pgy^{Cre/+}* females to ovarian luteolysis with inflammatory stimulus, leading to preterm birth.

Trp53^{loxP/loxP} Pgy^{Cre/+} females express reduced levels of decidual prolactin-like protein J and prolactin receptor. It is known that decidua-derived factors serve as luteotrophins to extend the lifespan of the CL and maintain luteal P_4 secretion in rodents (20–22). In fact, experimentally induced decidualization in pseudopregnant rats extends the CL lifespan with continued P_4 secretion, as opposed to shortened CL lifespan in pseudopregnant rats without decidualization (23). Our recent proteomics analysis found downregulation of a decidua-enriched prolactin-like hormone, prolactin-like protein J (PLP-J), in *Trp53^{loxP/loxP} Pgy^{Cre/+}* deciduae (24). PLP-J was identified in rat decidua and is implicated in maintaining decidual cell survival (25–28). Our in situ hybridization results show that *Prl3c1*, encoding PLP-J, was highly expressed in the decidua surrounding the developing embryo on day 8 in *Trp53^{loxP/loxP} Pgy^{Cre/+}* females but was markedly downregulated in *Trp53^{loxP/loxP} Pgy^{Cre/+}* deciduae, consistent with our previous proteomics results (Figure 2A). We also found that expression of this gene was downregulated at the decidual-placental interface in *Trp53^{loxP/loxP} Pgy^{Cre/+}* females on day 16 (Figure 2B). Immunolocalization of CDX2 demarcated trophoblast cell invasion into the decidual bed on this day of pregnancy (Supplemental Figure 3). Prolactin receptor (*Prlr*), which is also expressed in the decidua (29, 30), was downregulated in this tissue on day 16 (Figure 2B). These results suggest that decidual health is inferior in *Trp53^{loxP/loxP} Pgy^{Cre/+}* females, which compromises its luteotrophic task, making the CL more vulnerable to even a small insult.

Rapamycin and P_4 with or without celecoxib rescue preterm birth in $Trp53^{loxP/loxP} Pgy^{Cre/+}$ females exposed to a low dose of LPS. As reported previously (13, 14), administration of either celecoxib or rapamycin rescues spontaneous preterm birth in *Trp53^{loxP/loxP} Pgy^{Cre/+}* females without any apparent adverse effects on the dam or fetuses. These results led us to test whether this treatment would effectively reverse inflammation-exaggerated preterm birth in *Trp53^{loxP/loxP} Pgy^{Cre/+}* females. First, we used rapamycin or celecoxib singly and found them to be insufficient in preventing LPS-induced preterm birth (Supplemental Figure 4 and Supplemental Table 1). We also found that although P_4 (2 mg) supplementation prior to and after LPS injection extended the parturition timing in *Trp53^{loxP/loxP} Pgy^{Cre/+}* females, higher rates of fetal death and/or stillbirth were frequently encountered under this condition (Supplemental Table 1). Moreover, a combination treatment of celecoxib plus rapamycin or of celecoxib and P_4 did not rescue preterm birth in *Trp53^{loxP/loxP} Pgy^{Cre/+}* females exposed to LPS (Supplemental Table 1). We next asked whether combinatory treatments with celecoxib, P_4 , and/or rapamycin would rescue preterm birth with neonatal survival. Both floxed and deleted mice received an oral gavage of rapamycin (0.25 mg/kg BW) on days 8, 12, and 16 of pregnancy, followed by an oral gavage of celecoxib (10 mg/kg BW) twice on day 16, once 3 hours prior to and 4 hours after LPS (10 μ g) injection. In addition, P_4 was given twice on day 16 at around the same time points as celecoxib. This combination treatment rescued preterm birth in *Trp53^{loxP/loxP} Pgy^{Cre/+}* females exposed to LPS, with survival of a full complement of pups (Figure 3, A–C, and Supplemental Table 2); maternal weight gain due to fetal growth from day 16 to delivery and neonatal pup growth over a period of 10 days were comparable to those of untreated *Trp53^{loxP/loxP} Pgy^{Cre/+}* females with term delivery (Supplemental Figure 5, A and B). However, this treatment schedule adversely affected fetal viability, with high incidence of resorption in littermate *Trp53^{loxP/loxP} Pgy^{Cre/+}* females (Figure 3C). These results were surprising and led us to reevaluate our approach to treating LPS-induced preterm birth in *Trp53^{loxP/loxP} Pgy^{Cre/+}* females without incurring adverse effects on fetal survival in control floxed littermates. We found that a combination of rapamycin and P_4 was not only sufficient to rescue preterm birth in *Trp53^{loxP/loxP} Pgy^{Cre/+}* females, but also did not significantly alter pregnancy outcome in *Trp53^{loxP/loxP} Pgy^{Cre/+}* females (Figure 3, A–C, and Supplemental Table 2). Again, this treatment did not interfere with maternal weight gain due to fetal growth during pregnancy or neonatal growth over a period of 10 days in either group (Supplemental Figure 5, A and B). An alternative schedule of rapamycin treatment on days 8, 10, and 12 of pregnancy with P_4 on day 16 was also effective in rescuing preterm birth in *Trp53^{loxP/loxP} Pgy^{Cre/+}* females and did not result in adverse pregnancy outcome in *Trp53^{loxP/loxP} Pgy^{Cre/+}* females (Supplemental Table 3). The combined treatment of rapamycin and P_4

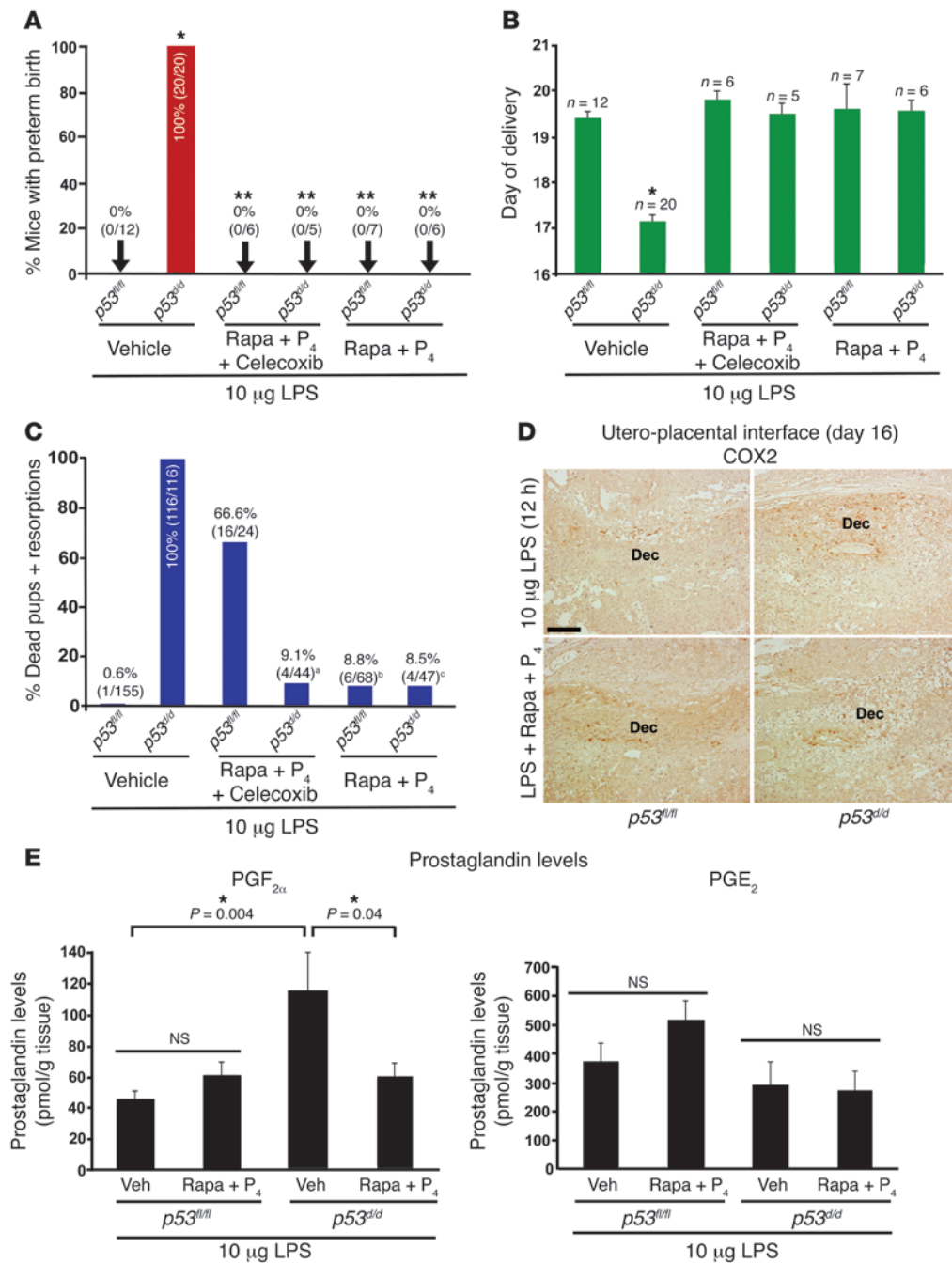


Figure 3 Preterm birth in *p53^{dd}* females was effectively rescued with combined treatment of rapamycin and P₄, without adverse effects on pregnancy outcome. (A) All *p53^{dd}* females examined under mild inflammation (10 μg LPS) showed preterm birth, which was rescued by a combination of rapamycin (Rapa) and P₄ treatment with or without celecoxib (mean ± SEM; *P < 0.05 compared with vehicle-treated control females; **P < 0.05 compared with LPS-treated females). (B) *p53^{dd}* females with these treatment schedules showed rescue of preterm birth as assessed by the day of delivery (mean ± SEM; *P < 0.05). (C) Combined treatment with rapamycin, P₄, and celecoxib adversely affects fetal health in *p53^{fl/fl}* females, but treatment with P₄ and rapamycin does not. a, b, and c denote dead pups/resorption sites in one dam in each group (see Supplemental Table 2). (D) Immunohistochemistry for COX2 in deciduae of LPS-treated *p53^{dd}* females showed decreased signals after treatment with rapamycin and P₄. Scale bar: 200 μm. (E) Mass spectrometric analysis of PGs shows that treatment with rapamycin and P₄ significantly lowered PGF_{2α} levels in *p53^{dd}* uteri challenged with LPS; uterine PGE₂ levels were not significantly different in similarly treated *p53^{fl/fl}* and *p53^{dd}* females. Three to six independent samples isolated per animal were analyzed (n = 3–5 mice/treatment group; mean ± SEM; *P < 0.05). Veh, vehicle.

prevented an increase in decidual COX2 (Figure 3D and Supplemental Figure 6), and this was reflected in reduced levels of PGF_{2α} compared with those in *Trp53^{loxP/loxP}Pgr^{Cre/+}* females treated with LPS alone (Figure 3E). Rapamycin and P₄ treatment also reduced the expression level of ovarian *Akr1c18* in LPS-treated *Trp53^{loxP/loxP}Pgr^{Cre/+}* females (Figure 1D), along with downregulation of ovarian *Socs1* and *Socs3* expression (Supplemental Figure 2). Collectively, these results point toward a potential therapeutic application for this combination treatment in preterm birth.

Higher mTORC1 signaling is correlated with increased senescence and upregulation of COX2 expression in human preterm deciduae. The next objective was to see whether our findings in mice were applica-

ble in the case of human preterm birth. Placentae to which the decidua basalis remained adherent were collected from women following vaginal delivery at term (37–41 weeks of gestation) and preterm (25–36 weeks); the etiology of preterm birth ranged from unknown to diagnosed infection (Supplemental Table 4). When placental-decidual sections were processed for SA-β-gal staining, we found clear evidence of positive β-gal staining in preterm deciduae, with little or no staining in term deciduae (Figure 4A, Supplemental Figure 7A, and Supplemental Figure 8). We also found increased intensity of nuclear immunolocalization of γH2AX, another marker of senescence associated with DNA damage response, in preterm deciduae (Figure 4B, Supple-

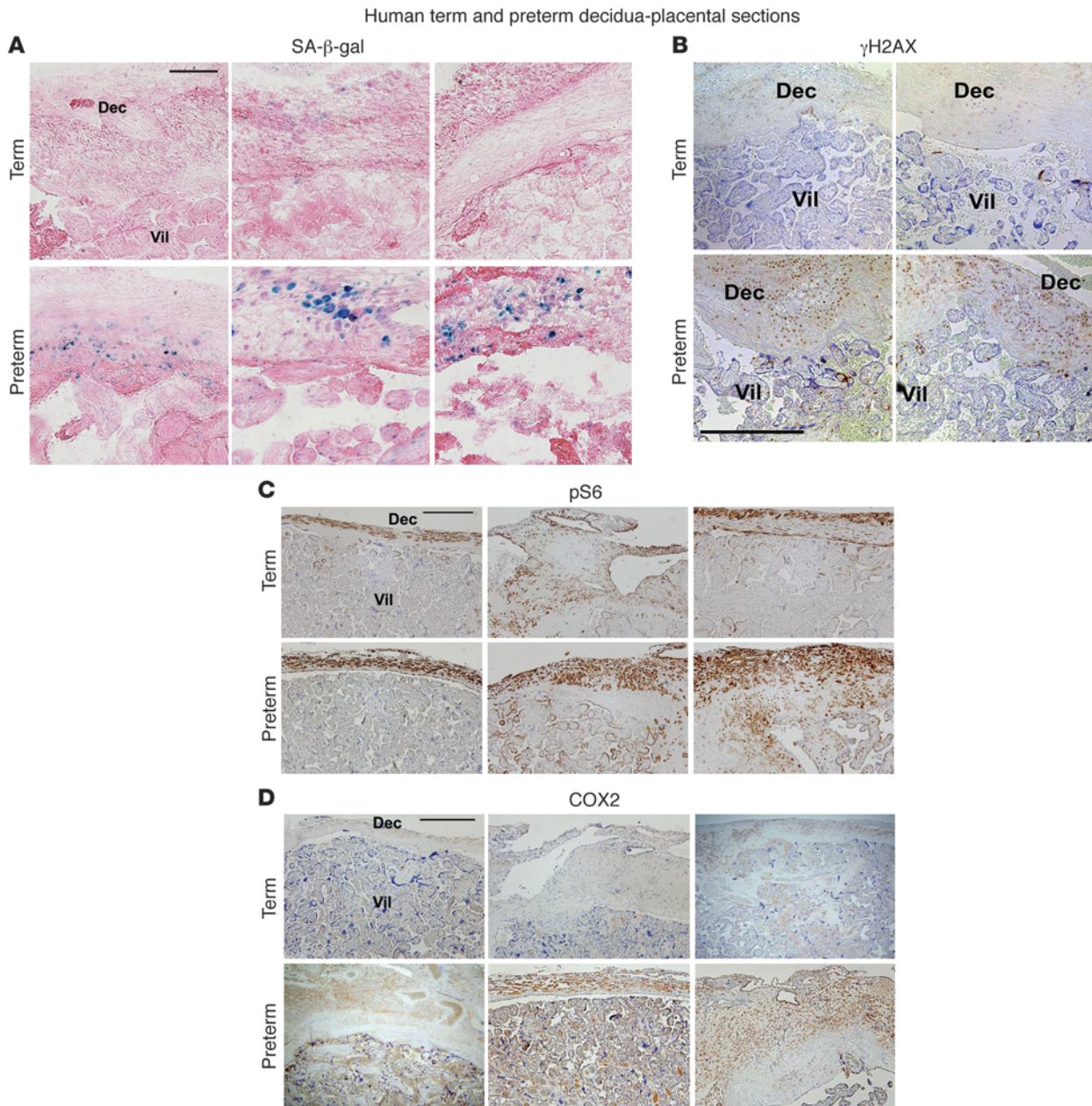


Figure 4
 Heightened decidual senescence, mTORC1 signaling, and COX2 levels are evident in human preterm birth. **(A)** Three representative images of increased SA-β-gal staining (blue) in sections of preterm decidua-placental samples compared with those from term delivery. **(B)** Two representative images showing increased signals for γH2AX, a senescence and DNA damage response marker, in preterm decidua-placental sections compared with those from term delivery. Immunostaining for pS6 **(C)** and COX2 **(D)** in sections of preterm decidua-placental samples compared with those from term delivery. The same 9 samples were processed as paraffin sections for COX2, pS6, and γH2AX immunostaining, while 9 additional samples were processed as frozen sections for SA-β-gal staining. Dec, decidua; Vil, villous trophoblast. Scale bars: 200 μm.

mental Figure 7B, Supplemental Figure 8, and ref. 31). Increased expression of these senescence markers correlated with increased immunostaining of COX2 and phosphorylated ribosomal protein S6 (pS6), a signature of heightened mTORC1 signaling (Figure 4, C and D, Supplemental Figure 7, C and D, and Supplemental Figure 8). To determine whether this decidual signature is specific to preterm delivery, we performed similar analyses in decidua-placental sections from women undergoing non-laboring cesar-

ean deliveries at 31–36 weeks gestation due to various pathologies, including preeclampsia, placenta previa, placental abruption, and fetal anomalies/distress. The signature was not observed in these deciduae (Supplemental Figure 9 and Supplemental Table 5). Collectively, the results provide evidence that decidual senescence is associated with higher mTORC1 and COX2 signaling in deciduae from preterm deliveries, corroborating the results in *Trp53^{loxP/loxP} Pgr^{Cre/+}* mice (13, 14).

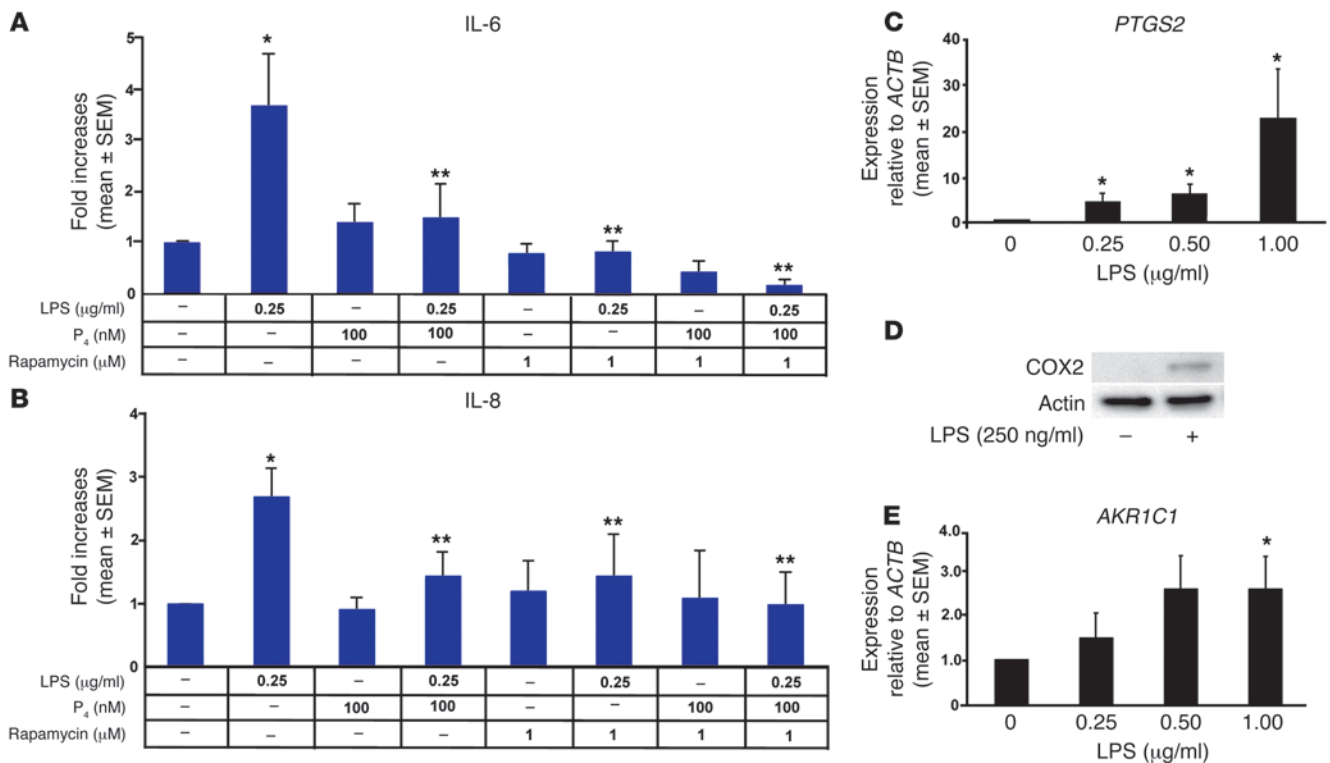


Figure 5

Levels of IL-6 and IL-8 and expression of *PTGS2* and *AKR1C1* in cultured human term decidual cells following exposure to LPS. (A and B) Decidual cells isolated from human term placentae showed increased secretion of IL-6 and IL-8 into culture media when exposed to LPS for 24 hours in culture; the levels were attenuated by treatment with rapamycin and/or P₄ (mean ± SEM; *P < 0.05 compared with vehicle-treated control; **P < 0.05 compared with LPS-treated cells). ELISA for IL-6 (n = 8) and IL-8 (n = 7) were repeated using independent samples. (C) qPCR results showed dose-dependent increases in decidual *PTGS2* expression levels 6 hours after LPS exposure. Experiments were repeated in 3 independent samples (mean ± SEM; *P < 0.05 compared with control). (D) Western blotting showed increases in decidual COX2 levels. One representative blot from 3 independent experiments is shown. (E) qPCR showed dose-dependent increases in decidual *AKR1C1* expression levels 6 hours after LPS exposure. Experiments were repeated in 3 independent samples (mean ± SEM; *P < 0.05 compared with control).

P₄ and/or rapamycin attenuate LPS-induced IL-6 and IL-8 levels in cultured human decidual cells. With these results in hand, we questioned whether an inflammatory insult will provoke inflammatory cytokine production in decidual cells. Human decidual cells adherent to term placentae were isolated and cultured as previously described (32). Vimentin and cytokeratin immunostaining (markers of stroma-derived decidual cells and of ectoderm-derived trophoblast and amnion cells, respectively) confirmed that isolated decidual cells were approximately 99% pure and negative for CD45 (pan-immune cell marker) (Supplemental Figure 10A). Notably, TLR4 was expressed in decidual cells free of immune cells (Supplemental Figure 10B). Decidual cells were cultured in the presence or absence of TLR4-specific LPS. We found that decidual cells exposed to LPS secreted higher levels of IL-6 and IL-8 (Figure 5, A and B), with increased decidual COX2 at the protein and mRNA levels after LPS exposure (Figure 5, C and D). Intriguingly, higher expression levels of decidual *AKR1C1*, encoding human 20αHSD, were also observed (Figure 5E), suggesting that the decidua can be a site for P₄ metabolism.

Since P₄ administration reduces the incidence of preterm birth in a specific population with higher risk of preterm birth and since P₄ is considered immunosuppressive and antiinflammatory (33), LPS-treated cells were cultured in the presence or absence of P₄ (100 nM). We found that P₄ reduced the levels of IL-6 and IL-8 in the spent

media (Figure 5, A and B). We then asked whether mTORC1 inhibitor would also suppress the levels of these cytokines in the presence of LPS. Indeed, rapamycin (1 μM) reduced the levels of IL-6 and IL-8 in the spent media (Figure 5, A and B), with further reduction by a combination of P₄ and rapamycin (Figure 5, A and B). While higher levels of decidual *PTGS2* after LPS exposure were suppressed by rapamycin, but not P₄, higher levels of decidual *AKR1C1* after LPS exposure were suppressed by rapamycin or P₄ treatment (Supplemental Figure 11, A and B). Collectively, these results suggest that LPS can increase the release of inflammatory cytokines/mediators and the primary P₄-metabolizing enzyme in decidual cells without the participation of immune cells, and that P₄ and/or rapamycin can dampen these responses.

Discussion

The highlights of the present study are that: (a) genetically predisposed females with uterine deletion of *Trp53* are more susceptible to preterm birth if exposed to a mild inflammatory stimulus; (b) under these conditions, preterm birth appears to involve both premature decidual senescence and ovarian luteolysis with a drop in P₄ levels; (c) targeting premature decidual senescence by inhibiting mTORC1 signaling and compensating the drop in P₄ levels by exogenous supplementation rescue preterm birth; (d) decidual senescence with increased mTORC1 and COX2 signaling is also

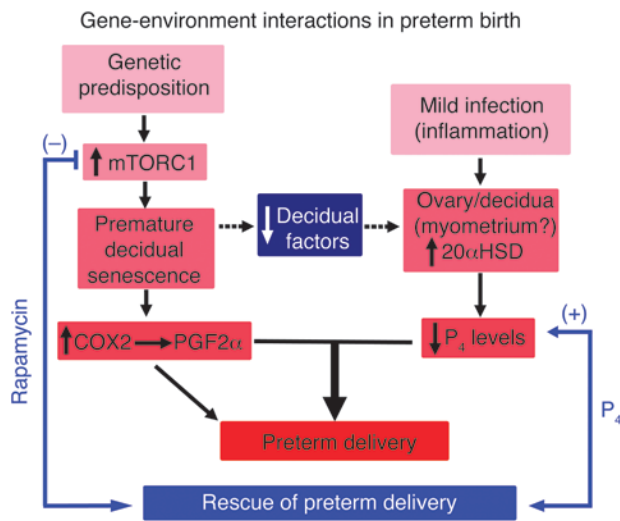


Figure 6 Proposed scheme of gene-environment interactions in preterm birth. In mice with uterine deletion of *Trp53*, premature decidual senescence arising from heightened decidual mTORC1 and COX2 signaling confers genetic predisposition to preterm birth. This genetic predisposition is remarkably aggravated by a mild inflammatory insult through a decrease in ovarian P₄ levels due to increased expression of 20αHSD, a P₄ metabolizing enzyme. Decidua-derived factors normally serve as luteotrophins to extend the CL lifespan; decidual health is presumably compromised in *Trp53^{loxP/loxP}Pggr^{Cre/+}* females due to premature senescence and reduced levels of decidual factors, conferring ovarian insufficiency and increased susceptibility to inflammation-mediated preterm birth.

evident in women undergoing preterm birth (Figure 6); and (e) cultured human term decidual cells respond to LPS, P₄, and rapamycin, as predicted from mouse experiments.

Paradoxically, the role of p53 in aging is dependent upon its cellular and physiological context (34–37). It has been shown that inactivation of p53 in mouse embryonic fibroblasts results in heightened cellular senescence with increased mTORC1 signaling, which is attenuated by rapamycin (38). This is consistent with our present and previous *in vivo* findings (14). The finding that spontaneous preterm birth occurred in approximately 50% of *Trp53^{loxP/loxP}Pggr^{Cre/+}* females with increased decidual senescence motivated us to ask whether another site of action could be targeted to reproducibly increase the incidence of preterm birth to 100% in *Trp53^{loxP/loxP}Pggr^{Cre/+}* females. Indeed, our present results showing preterm birth in all *Trp53^{loxP/loxP}Pggr^{Cre/+}* females treated with a low dose of LPS as opposed to none in floxed littermate females suggest that superimposition of even a mild inflammation on genetic predisposition can profoundly aggravate this phenotype. This raises the question as to the site of action of this second insult. While it is possible that *Trp53^{loxP/loxP}Pggr^{Cre/+}* deciduae are more responsive to inflammation than floxed deciduae, our results showing signs of ovarian luteolysis with a drop in serum P₄ levels after an exposure to 10 μg LPS in *Trp53^{loxP/loxP}Pggr^{Cre/+}* females, but not in *Trp53^{loxP/loxP}Pggr^{+/+}* dams, suggest that the ovary is also a potential target of this inflammatory insult in *Trp53^{loxP/loxP}Pggr^{Cre/+}* females. Although other systemic effects of LPS cannot be ruled out, a previous study showed antigen-induced inflammation during early pregnancy induces luteolysis leading to pregnancy failure, which could be rescued by P₄ supplementation (19); this is

consistent with our present findings. There is evidence that higher doses of LPS (50–250 μg) can trigger preterm birth in rodents with ovarian luteolysis and a decrease in P₄ levels (9, 39).

Our results showing reduced expression of *Prl3c1* and *Prlr* in deciduae of pregnant *Trp53^{loxP/loxP}Pggr^{Cre/+}* mice suggest that increased sensitivity to ovarian luteolysis under mild inflammation could be due to decidual insufficiency. There is physiological and molecular evidence that decidual factors affect CL lifespan (20–23). Gibori’s group has also shown that decidual “luteotrophins” regulate ovarian adenylyl cyclase activity, luteinizing hormone receptor, and steroidogenesis (40). In addition, we and others have previously shown that implantation failure in *Prlr* mutant females is rescued by P₄ administration, suggesting its effect at the ovarian level (29, 30). However, *Prlr* is also expressed in the decidua, and P₄-treated *Prlr* mutant mice fail to give a full complement of pups and show an increased number of resorptions even with continued P₄ administration, suggesting the significance of decidual *Prlr* in supporting the later course of pregnancy. The importance of decidual health is also reflected in our present findings of rescue of preterm birth timing in LPS-treated *Trp53^{loxP/loxP}Pggr^{Cre/+}* females with P₄ alone, but with a large number of resorptions and fetal deaths (~40%) (Supplemental Table 1). In contrast, in LPS-treated *Trp53^{loxP/loxP}Pggr^{Cre/+}* females, treatment with rapamycin and P₄ not only rescued preterm birth but maintained fetal survival (91% survival), which was comparable to that in floxed females under similar treatment conditions (Supplemental Table 2). It would be interesting to determine whether the decidua undergoing senescence influences CL function or whether both decidua and ovary are affected by inflammation in genetically predisposed females, such as *Trp53^{loxP/loxP}Pggr^{Cre/+}* mice. Nonetheless, our results provide evidence that *Trp53^{loxP/loxP}Pggr^{Cre/+}* deciduae and/or ovaries are more sensitive to exacerbation of preterm birth by inflammatory stimuli.

Our findings of decreased expression of *AKRIC18* by rapamycin and P₄ are consistent with a previous observation of attenuation of *AKRIC18* promoter activity in mouse luteal cells by rapamycin (21). We have previously shown that rapamycin inhibits decidual COX2 levels in *Trp53^{loxP/loxP}Pggr^{Cre/+}* females and in a cell line with increased mTORC1 activity (14). Moreover, our observations that attenuation of premature decidual senescence by rapamycin together with P₄ supplementation prevents preterm birth in *Trp53^{loxP/loxP}Pggr^{Cre/+}* females exposed to a small dose of LPS – without any observable effects on fetal viability and growth – suggest that targeting decidual senescence and ovarian luteolysis is a potential therapy for preventing preterm birth in the context of genetic predisposition and infection/inflammation.

The therapeutic approach using rapamycin and P₄ seems more desirable, since floxed mice given this therapy along with LPS did not appreciably show adverse effects on pregnancy outcome. A combinatory treatment with rapamycin and P₄ with celecoxib also rescued preterm birth in *Trp53^{loxP/loxP}Pggr^{Cre/+}* females, but this regimen produced adverse effects on pregnancy outcome in floxed littermates. The reasons for the rescue of preterm birth with normal fetal viability and health in *Trp53^{loxP/loxP}Pggr^{Cre/+}* mice versus widespread fetal death and resorption in floxed mice is not clearly understood at this time, although it is possible that under normal pregnancy conditions, drug-drug interactions between celecoxib and rapamycin may adversely affect fetal survival. In fact, co-administration of rapamycin and NSAIDs is not clinically recommended (41). It is also possible that other PGs inhibited by this combination may be harm-



ful to the overall health of normal pregnancy. Notably, celecoxib or rapamycin given alone in *Trp53^{loxP/loxP}Pggr^{Cre/+}* females completely rescued spontaneous preterm birth and had no apparent effects on fetal viability or growth in deleted and floxed females (13, 14).

With regard to the doses and schedule of rapamycin and P₄ treatments, rapamycin is normally given at a loading dose of 6 mg followed by a daily oral maintenance dose of 2 mg in transplant patients (42). However, the dosage depends on the response of the patient, and the daily maintenance dose can be up to the recommended limit of 40 mg (43). In our mouse studies, we used only 3 intermittent doses of 0.25 mg/kg BW rapamycin; however, it is difficult to directly compare the doses in mice and in humans due to differential metabolic and clearance rates of drugs.

Regarding the dose of P₄, the use of 2 mg P₄ in mouse studies is common and widespread in implantation and pregnancy maintenance (12, 44, 45). Besides, we used only 2 doses of P₄ on day 16 of pregnancy, which did not result in any adverse effects in floxed wild-type mice, delivering a full complement of healthy pups. In humans, there is a large range of P₄ doses given via different routes and for variable lengths of treatment, and the American College of Obstetrics and Gynecology has not yet identified an appropriate dose, route, or formulation for P₄ supplementation for the prevention of preterm birth (46). Two major clinical studies have shown that P₄ supplementation can reduce the incidence of preterm delivery in select patient populations. One study used 250 mg 17 α -hydroxyprogesterone caproate injections (i.m.) weekly from 16 to 20 weeks gestation until 37 weeks or delivery (47), while another study used 200 mg vaginal P₄ each night from 24 to 34 weeks gestation until 34 weeks (48). These long-term studies apparently did not show adverse effects on the mother and babies. Again, the doses used in these studies cannot directly be compared with mouse studies due to differential metabolic and clearance rates between the two species. Nonetheless, we believe that the dose of P₄ we have used is an appropriate treatment for our present mouse studies.

In this mouse model, we address two known effectors of preterm birth and its rescue. However, the multifactorial aspects of human preterm delivery must be recognized and further studied. Extension of parturition timing but with poor neonatal outcome in LPS-treated *Trp53^{loxP/loxP}Pggr^{Cre/+}* females by P₄ treatment alone suggests that it can address the ovarian insufficiency of P₄ secretion but cannot overcome the adverse effects of premature decidual senescence. Indeed, there is evidence that P₄ supplementation in humans can prevent preterm delivery only in certain patient populations with specific risk factors (47–51).

The role of p53 in pregnancy maintenance in relation to P₄ levels and its responsiveness remain to be ascertained. There is evidence that certain *TRP53* polymorphisms in women correlate with recurrent pregnancy failure (52); however, this issue remains unsettled (53). *TRP53* polymorphisms have also been associated with aging and lifespan in humans (36, 54). Our recent proteomics study showed that deciduae in *Trp53^{loxP/loxP}Pggr^{Cre/+}* females manifest an increased signature for oxidative stress, with downregulation of many antioxidant enzymes, including PRDX6 (24). PRDX6 plays a role during pregnancy in mice with deletion of *Fkbp52*, an immunophilin co-chaperone for nuclear PR, which show reduced uterine P₄ responsiveness (55–57). Therefore, it is possible that oxidative stress makes *Trp53^{loxP/loxP}Pggr^{Cre/+}* females more sensitive to preterm birth, since it is considered a contributing factor (1–3, 8). In-depth studies will be required to assess the definitive role of p53 at various stages of pregnancy.

Our results are clinically relevant because some aspects of the molecular signature observed in mouse studies are consistent with those observed in deciduae of patients undergoing preterm birth. As presented here, it is remarkable that decidual senescence indicated by SA- β -gal and γ H2AX staining (58, 59), along with higher mTORC1 and COX2 signaling, are also characteristics of human preterm deciduae. Interestingly, this signature was observed in deciduae irrespective of the etiology of preterm birth, ranging from unknown to diagnosed infection (e.g., chorioamnionitis). These results suggest that disparate signaling pathways converge toward mTORC1-induced decidual senescence and COX2 signaling. However, these studies must be repeated with a larger cohort of patients undergoing preterm birth. Nonetheless, the finding that P₄ and/or rapamycin inhibited the inflammatory cytokine release from cultured human term decidual cells in response to LPS suggests that maintaining decidual health will help to prevent preterm birth. It is interesting that TLR4 is expressed in human decidual cells free of leukocytes, suggesting a direct effect of TLR4-mediated effects in the decidua in addition to the effects exerted by immune cells. Whether results from cultured decidual cells correctly reflect the effects of inflammation/infection *in vivo* remains to be determined.

The placenta is a major source of P₄ in human pregnancy after 10 weeks of gestation, as opposed to the situation in rodents, in which ovaries are the major source of P₄ throughout the course of pregnancy (60). Although a decrease in P₄ levels in rodent models of preterm birth is well established, peripheral P₄ levels in women undergoing term and preterm delivery needs to be carefully assessed. A recent report from the United Kingdom shows decreases in salivary P₄ levels in women undergoing preterm birth before 34 weeks of gestation; this study suggested that P₄ levels are different in early preterm and late preterm birth (61). However, an earlier, U.S. study failed to observe such decline in salivary P₄ levels (62). Therefore, P₄ levels during human pregnancy in the context of the etiology of preterm birth and parturition timing remain unsettled. A recent report shows that microRNA-200a via STAT5b increases local metabolism of P₄ by increasing the expression of *AKR1C1* in immortalized human myometrial cells in culture (63). Another report shows *AKR1C1* expression in human deciduae (64). Our results showing increased *AKR1C1* expression levels in human term decidual cells in culture exposed to LPS, which could be attenuated by rapamycin or P₄ treatment, suggest that decidua is also a site for P₄ metabolism. It is interesting that the decidual *PTGS2* levels are downregulated by rapamycin, which is consistent with our previous and present findings (14). Collectively, human studies showing different aspects of P₄ signaling in parturition timing and multiple sites regulating P₄ levels indicate that further investigation is warranted.

P₄ executes its functions via two PR isoforms, PR-A and PR-B (65, 66). Analysis of promoter activity in cell culture systems suggests that while PR-A functions as a repressor, PR-B serves to increase P₄ signaling (67). Notably, the placenta does not express PR. Therefore, P₄ should exert its effects via decidual or myometrial PR; which site of P₄ signaling is more important in parturition remains to be ascertained. Functional withdrawal of P₄ signaling in the myometrium has been proposed to trigger labor in humans (67). There could be several reasons for withdrawal: reduced P₄ levels, local metabolism of P₄ in the myometrium and/or decidua, an altered ratio of PR isoforms (PR-A/PR-B), or reduced transactivation or heightened transrepression due to recruitment of coactiva-



tors or corepressors (68). There is also evidence that inflammation via NF- κ B can reduce P₄ effectiveness and PGF_{2 α} increases PR-A expression without affecting PR-B expression (69, 70). In addition, several studies reported that human labor is associated with reduced decidual expression of PR (71–73). Taken together, the evidence indicates that P₄ signaling in the context of myometrial contractility in human parturition requires further investigation.

Chronological aging is a contributing factor to cellular senescence (74). Therefore, it is possible that uterine senescence due to maternal aging compounded by environmental stressors, such as infection/inflammation, can increase the risk of preterm birth. Epidemiologic evidence suggests that advanced maternal age is associated with human preterm birth (75–77). Furthermore, women of advanced maternal age undergoing ART procedures show higher incidence of preterm birth, even when receiving oocytes from young donors (78, 79), suggesting that uterine factors can contribute to this disorder.

Although gene-environment interactions are assumed to be major contributors to preterm birth, this concept has not been experimentally interrogated. Our studies in mice provide evidence that when a genetic predisposition is superimposed by mild inflammation, the rate of preterm birth is profoundly exaggerated. More importantly, success in reversing preterm birth in our mouse model by rapamycin and P₄ provides clues to the cooperative contributions of at least two sites of action (decidua and ovary) toward preterm birth. Our present findings in mouse and human studies point toward decidual senescence as a contributor to preterm birth, a concept not previously entertained. These findings provide new insights and should encourage further investigation in the field. Future studies integrating findings from multiple models of preterm delivery will help to define the mechanism behind parturition timing and allow for the design of strategies to prevent preterm birth.

Methods

Mice. *Trp53^{loxP/loxP}Pgr^{Cre/+}* mice were generated as described previously (13). Briefly, *Trp53^{loxP/loxP}* mice (FVB/129) were crossed with *Pgr^{Cre/+}* mice (C57BL6/129) to generate mice with uterine deletion of *Trp53*. *Trp53^{loxP/loxP}* mice were obtained from the Mouse Models of Human Cancers Consortium, while *Pgr^{Cre/+}* mice were initially provided by J.B. Lydon and F.J. DeMayo (Baylor College of Medicine, Houston, Texas, USA). For experiments, littermate *Trp53^{loxP/loxP}Pgr^{+/+}* and *Trp53^{loxP/loxP}Pgr^{Cre/+}* mice were used. Mice were provided with autoclaved rodent LabDiet 5010 (Purina) and UV light-sterilized RO/DI constant circulation water ad libitum and were housed under a constant 12-hour light/12-hour dark cycle.

Analysis of parturition. Parturition events were monitored from day 16 through day 21 by observing mice daily, morning (0600h–0700h), noon (1200h), and evening (1800h–2000h). Birth timing was defined by the observation of the first born pup. Preterm birth was defined as birth occurring earlier than day 19 of pregnancy, with the day the vaginal plug was found designated day 1 of pregnancy. Dystocia was defined as difficult delivery lasting more than 12 hours. Resorption sites and placental scars were identified in dams showing preterm or difficult deliveries by examining the uterus after delivery. The number of pups/masses delivered were compared with the number resorption sites and placental scars identified.

Drug and LPS administration. Ultrapure TLR4-specific LPS (10, 37, 50, or 75 μ g/mouse, i.p.; Invivogen) was administered on day 16 of pregnancy at 1200h. The selective COX2 inhibitor celecoxib was suspended in 5% PEG400 and 5% Tween-80 dissolved in water by constant stirring and was given by oral gavage as indicated (10 mg/kg BW/dose). The mTORC1 inhibitor rapamycin (0.25 mg/kg BW/d) was suspended in the same vehicle

and given as a single oral gavage as indicated. The control group received vehicle alone. Progesterone was dissolved in sesame oil and administered subcutaneously (2 mg/0.1 ml/dose). Treatment schedules of various combinations of drugs are given in Supplemental Figure 4.

Measurement of PG profiles. Implantation sites from which fetuses and placentae had been removed were collected on day 16 of pregnancy. These tissues were flash frozen and stored at -80°C until used for extractions. Methanolic extracts of tissues were partially purified using C18 solid-phase extraction columns (Agilent), and PGs were quantified by HPLC–tandem mass spectrometry as previously described (13).

In situ hybridization. In situ hybridization was performed as described (13). Whole implantation sites were collected and flash frozen. Frozen tissue sections (12 μ m) were mounted onto baked poly-L-lysine-coated slides, fixed in cold 4% paraformaldehyde, acetylated, and hybridized at 45°C for 4 hours in formamide hybridization buffer containing ^{35}S -labeled *Plr3c1* (a gift from Michael Soares, University of Kansas Medical Center, Kansas City, Kansas, USA) and *Prlr* (long isoform) cRNA probes. RNase A-resistant hybrids were detected by autoradiography after 3- to 10-day exposure by using Kodak NTB-2 liquid emulsion. To compare mRNA localization in *Trp53^{loxP/loxP}Pgr^{+/+}* and *Trp53^{loxP/loxP}Pgr^{Cre/+}* tissues, we placed sections of tissues of both genotypes under similar experimental conditions onto the same slide and processed them for hybridization.

Immunohistochemistry. Immunostaining was performed in formalin-fixed, paraffin-embedded sections using specific antibodies to 20 α HSD (a gift from Geula Gibori, University of Illinois at Chicago, Chicago, Illinois, USA), COX2 (mouse, laboratory-generated; human, Santa Cruz Biotechnology Inc.), pS6 (Cell Signaling Technology), CDX2 (BioGenex), γ H2AX (Millipore), vimentin (Dako), pan cytokeratin (Dako), and CD45 (Dako) as described previously (13, 14). Tissue sections from *Trp53^{loxP/loxP}Pgr^{+/+}* and *Trp53^{loxP/loxP}Pgr^{Cre/+}* females on each day of pregnancy were processed onto the same slide.

SA- β -gal staining. Staining of SA- β -gal activity was performed as described previously (13, 14). In brief, frozen sections were fixed in 0.5% glutaraldehyde in PBS and stained for 6 hours in PBS (human tissues, pH 6.0; mouse tissues, pH 5.5) containing 1 mM MgCl₂, 1 mg/ml X-gal, and 5 mM each of potassium ferricyanide and potassium ferrocyanide. Sections were counterstained with eosin.

Densitometry of staining. The images of SA- β -gal staining and immunostaining were analyzed using inForm Image analysis software (PerkinElmer), which can detect the average signal intensity in the scanned area.

RNA isolation and quantitative PCR. RNA was prepared from homogenized tissues using TRIzol reagent (Invitrogen). RNA extraction was performed as described previously (13, 14). Quantitative PCR (qPCR) was performed using StepOnePlus Real-Time PCR System (Applied Biosciences). PCR was performed using the following primers: 5'-CTCTGAAGC-CAGGGAATGAG-3' and 5'-ATGGCATTCTACCTGGTTGC-3' for mouse *Akr1c18* (encoding 20 α HSD) (product size, 221 bp); 5'-TGTGCCGCGC-CATTAAGTG-3' and 5'-GGCATCTCACCCTCCACAAC-3' for mouse *Socs1* (product size, 125 bp); 5'-TGCTGGCCAAAGAAATAACCA-3' and 5'-GGTCACCCCTTGCCACTCT-3' for mouse *Socs3* (product size, 88 bp); 5'-TCCATGACAACCTTGGCATTG-3' and 5'-CAGTCTTCTGGGTG-GCAGTGA-3' for mouse *Gapdh* (product size, 72 bp); 5'-GATTGCC-CGACTCCCTTGG-3' and 5'-GTCTAGCCAGATTTCACCGT-3' for human *PTGS2* (encoding COX2) (product size, 250 bp); 5'-TGTGC-GATATTTGACCCTTGA-3' and 5'-TGCTGTAGCTTGCTGAAATCAC-3' for human *AKR1C1* (product size, 204 bp); 5'-CAACAAGGTGGGAAT-GCTT-3' and 5'-TGCCATTGAAAGCAACTCTG-3' for human *TLR4* (product size, 317 bp); and 5'-CACACTGTGCCATCTACGA-3' and 5'-CTCCTTAATGTCACGCACGA-3' for human *ACTB* (product size, 162 bp). *Gapdh* and *ACTB* served as housekeeping genes for mouse tissues and human cells, respectively.



Measurement of P_4 levels. Mouse blood samples were collected on day 16 of pregnancy at the prescribed time after treatments. Serum levels of P_4 were measured by EIA kits (Cayman Chemical).

Human samples. Term and preterm placentae were obtained from women with singleton vaginal term or preterm delivery. Placental samples from patients with hydramnios or newborns with any birth or chromosomal abnormalities were not included in the preterm study, although placental samples from patients with chorioamnionitis or preterm premature rupture of the membranes (PPROM) were included in the study. Detailed patient information for immunohistochemistry and SA- β -gal staining is shown in Supplemental Table 4. Placental samples from non-laboring patients undergoing cesarean section for preeclampsia, fetal distress/anomaly, placenta abruption, or previa were included as controls for the study (Supplemental Table 5). For decidual cell culture, term placentae were obtained from women undergoing elective cesarean section. Endometria for endometrial stromal cell culture were obtained from women undergoing hysterectomy due to benign gynecological diseases; none had hormone treatment 3 months prior to surgery. None of the women undergoing term vaginal delivery or term cesarean section showed any clinical or pathological signs of preterm delivery, infection, or other maternal or placental diseases. All women with singleton preterm vaginal delivery did not show clinical or pathological signs of other maternal or placental diseases apart from preterm delivery. Newborns did not have any apparent birth or chromosomal abnormalities.

Isolation and culture of human decidual cells. Human term decidual cells were isolated and cultured according to previously described protocols with minor modifications (32, 80). Briefly, term deciduae from women with cesarean section were scraped from the maternal surface of the chorion, minced, and digested in Ham's F-10 media containing 25 mg/ml collagenase and 6.25 U/ml DNase in a shaking water bath at 37°C for 30 minutes. Digested samples were passed through a 23-gauge needle to dissociate remaining cell clusters, centrifuged at 250 g for 5 minutes, and washed in the culture media. The cell pellet was resuspended in 20% Percoll, layered on a discontinuous (60%:50%:40%) Percoll gradient, and centrifuged at 540 g for 20 minutes. The upper cell layer was collected, washed, centrifuged, resuspended in 40% Percoll, layered on a discontinuous (55%:50%:45%) Percoll gradient, and centrifuged at 540 rpm for 20 min. The upper cell layer was washed and resuspended in DMEM/F-12 media containing 5% fetal bovine serum, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μ g/ml amphotericin B and plated onto 100-mm dishes. Cells were cultured at 37°C in a humidified 5% CO₂ chamber. When the cells attained confluence, they were dissociated with 0.25% trypsin-EDTA, harvested by centrifugation at 250 g for 5 minutes, and replated onto 100-mm dishes. Cells were passaged at least 3 times and plated in 12-well plates at 2×10^5 cells/well for experiments. The complete media were then removed and replaced with serum-free media containing antibiotics, and cells were cultured for an additional 12–24 hours before stimulation. Purity of the decidual cell population was determined by immunocytochemical staining of vimentin, pan-cytokeratin, and CD45, which served as markers for stromal cells, epithelial cells, and leukocytes, respectively. The purity of the passaged decidual cells was greater than 99%, as judged by positive staining for vimentin and negative staining for cytokeratin and CD45 (Supplemental Figure 10A).

Treatment of human term decidual cells. To evaluate the effects of LPS on expression of *PTGS2* and *AKR1C1* in term decidual cells, wells were replenished with serum-free media with or without TLR4-specific LPS and cells were incubated for 6 hours (qPCR) or 24 hours (Western blotting). After termination of cultures, total RNA or protein was isolated and used for qPCR or Western blotting, respectively. To evaluate the effects of P_4 and rapamycin on LPS-induced levels of *PTGS2* and *AKR1C1*,

cells were preincubated with P_4 or rapamycin for 24 hours before addition of LPS and cultured for an additional 24 hours. To evaluate the effects of P_4 and rapamycin on LPS-induced levels of IL-6 and IL-8, conditioned media were collected after termination of cultures, centrifuged, and stored at -80°C until assay. Concentrations of IL-6 and IL-8 were measured using specific ELISA kits according to the manufacturer's protocol (R&D Systems). Absorbance was read at 450 nm with a DigiScan Microplate Reader.

Western blotting. Protein extraction and Western blotting were performed as previously described (13, 14). Antibodies to COX2 and actin (Santa Cruz Biotechnology Inc.) were used. Bands were visualized by using an ECL Prime Western blotting detection system (GE Healthcare). Actin served as a loading control.

Statistics. Statistical analyses were performed using 2-tailed Student's *t* test. *P* values less than 0.05 were considered statistically significant.

Study approval. All mice used in this investigation were housed in the Cincinnati Children's Hospital Medical Center Animal Care Facility according to NIH and institutional guidelines for the use of laboratory animals. All protocols of the present study were reviewed and approved by the Cincinnati Children's Hospital Research Foundation Institutional Animal Care and Use Committee. Collection and processing of human samples were approved by the respective ethics committees at University of Tokyo and Yaizu City Hospital in Tokyo under the approved IRB protocol no. 3456, and all patients provided written informed consent. Tissue sample collections were based on the operating procedures of the University of Tokyo Tissue Procurement Resource, which strips samples of all patient identifiers before procurement (de-identified) and replaces these with new sample identifiers. This study was limited to female subjects because of the nature of the disease studied. Children were not included due to the rarity of preterm birth in the pediatric population.

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