Heightened uterine mammalian target of rapamycin complex 1 (mTORC1) signaling provokes preterm birth in mice

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Although preterm delivery is a major global health issue, its causes and underlying mechanism remain elusive. Using mutant mice, mimicking aspects of human preterm birth, we show here that uterine decidual senescence early in pregnancy via heightened mammalian target of rapamycin complex 1 (mTORC1) signaling is a significant contributor of preterm birth and fetal death, and that these adverse phenotypes are rescued by a low dose of rapamycin, an inhibitor of mTORC1 signaling. This role of mTORC1 signaling in determining the timing of birth in mice may help us better understand the mechanism of the timing of birth in humans and develop new and improved strategies to combat the global problem of preterm birth.

uterus | phospho-S6 | p21 | prostaglandins | parturition

Preterm birth has been a global health concern for far too long. Preterm birth results in the underdevelopment of organs and organ systems, particularly the respiratory tree, and carries an increased risk for cerebral palsy and learning and developmental disabilities (1, 2). Nearly 13 million preterm births and more than 3 million stillbirths occur each year, with prematurity being a direct cause of more than 1 million neonatal deaths per year (2). Prematurity, with its wide gamut of effects, costs nearly $26 billion annually in the US alone, in the form of infant healthcare, maternal care, early intervention services, special education for learning disabilities, and lost household productivity (2).

Potential risk factors for preterm birth include infection/inflammation, stress, genetic factors, and environmental insults, among others (2). Although the etiology is multifactorial, it is possible that many of these risk factors converge on an effector pathway to influence the timing of birth. These risk factors are known to contribute to the cellular senescence process (3–7). In the present study, using conditionally and ubiquitously deleted mouse models, we show that mammalian target of rapamycin complex 1 (mTORC1) signaling plays a key role in uterine senescence and the timing of birth.

Mammalian target of rapamycin, a serine/threonine kinase, plays important roles in cell proliferation and survival and is a catalytic subunit of two distinct complexes that drive separate signaling pathways: mTORC1 and mTORC2 (8). mTORC1 activation is suppressed by a complex consisting of tuberous sclerosis complex 1 (TSC1, or hamartin) and tuberous sclerosis complex 1 (TSC2, or tuberin), two distinct tumor suppressors (8). Phosphorylated Akt (pAkt) inhibits TSC activity by phosphorylating TSC2, thereby releasing the brake on mTORC1 signaling. There is evidence indicating that increased mTORC1 signaling accelerates the aging process, and that rapamycin (an inhibitor of mTORC1 signaling) or deletion of *Rps6k1* (a critical downstream target in mTORC1 signaling) delays this process, thereby extending the life span in various species, including mice (6, 9). There is also evidence suggesting that mTORC1 signaling plays a role in cellular senescence (8). Our findings in the present study suggest that increased mTORC1 signaling influencing decidual senescence early in pregnancy leads to preterm birth.

Results

Increased mTORC1 Signaling Promotes Preterm Birth. We previously reported that 50–60% of dams conditionally deleted of uterine *p53* ($p53^{d/d}$ = $Trp53^{loxP/loxP}Pgr^{Cre/+}$), generated by crossing *p53* floxed mice with those expressing Cre recombinase under the progesterone receptor (*Pgr*) promoter (10), experience spontaneous preterm birth with dystocia, stillbirth, and perinatal death. $p53^{d/d}$ females display senescence-associated growth restriction of uterine decidual cells with increased levels of pAkt and p21 (10). Although elevated pAkt and p21 levels were coincident with decidual senescence and preterm birth, the underlying mechanism linking pAkt and p21 with these changes has remained unknown.

Among its many roles, pAkt can stimulate mTORC1 signaling (11). Because activation of the PI3K-Akt pathway enhances mTORC1 signaling, and because increased mTORC1 signaling is implicated in advancing physiological aging (12), we speculated that increased mTORC1 signaling is a cause of exacerbating decidual senescence early in pregnancy, leading to preterm delivery in $p53^{d/d}$ mice. Indeed, we found higher levels of phosphorylated S6 (pS6), a downstream effector and a hallmark of mTORC1 activation, in decidua of $p53^{d/d}$ females compared with floxed littermates ($p53^{f/f}$ = $Trp53^{loxP/loxP}Pgr^{+/+}$) on days 8 and 16 of pregnancy (Fig. 1 *A–D*). These results prompted us to examine whether inhibition of mTORC1 signaling would influence the incidence of preterm birth in $p53^{d/d}$ mice. Pregnant $p53^{d/d}$ and $p53^{f/f}$ mice mated with WT fertile males were given an oral gavage of a single low dose of the mTORC1 inhibitor rapamycin (0.25 mg/kg body weight) or vehicle on days 8, 10, and 12 of pregnancy and were monitored for parturition phenotypes. Although none of the $p53^{f/f}$ females treated with either rapamycin or the vehicle experienced preterm delivery, more than 50% of the $p53^{d/d}$ dams treated with the vehicle experienced preterm birth with neonatal death. Surprisingly, preterm birth in $p53^{d/d}$ mothers was rescued by rapamycin treatment, and so was the incidence of neonatal death (Fig. 2 *A–C* and Table S1).

To examine whether rapamycin treatment had any adverse effects on fetal viability, we recorded the number of viable fetuses on day 16 under the same experimental conditions, and found comparable numbers in the $p53^{f/f}$ and $p53^{d/d}$ mice (Fig.

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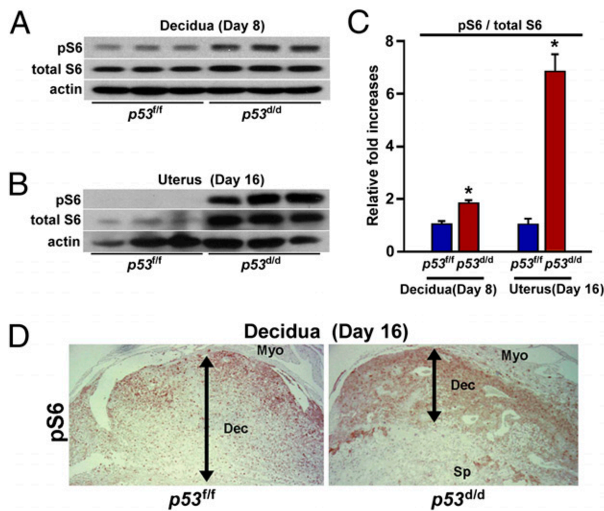

Fig. 1. $p53^{d/d}$ uteri exhibit higher pS6 levels on days 8 and 16 of pregnancy. (*A–C*) Deficiency of uterine *p53* up-regulates the levels of phosphorylated S6 (pS6) in day 8 decidua (*A*) and day 16 uteri (*B*). As assessed by Western blot analysis (*A* and *B*), relative levels of pS6 were normalized against total S6 (*C*). Three independent samples from different mice were examined in each group. Values are mean ± SEM. $^*P < 0.05$. Day 8 decidual tissues were isolated after dissecting out embryos. Day 16 uterine samples from which placentas and fetuses had been removed were used. (*D*) Uterine loss of *p53* enhances intensity of pS6 immunostaining in decidual cells. Brown deposits indicate sites of positive immunostaining. Decidual boundaries are demarcated by lines with arrows. Myo, myometrium; Dec, decidua; Sp, spongiotrophoblast.

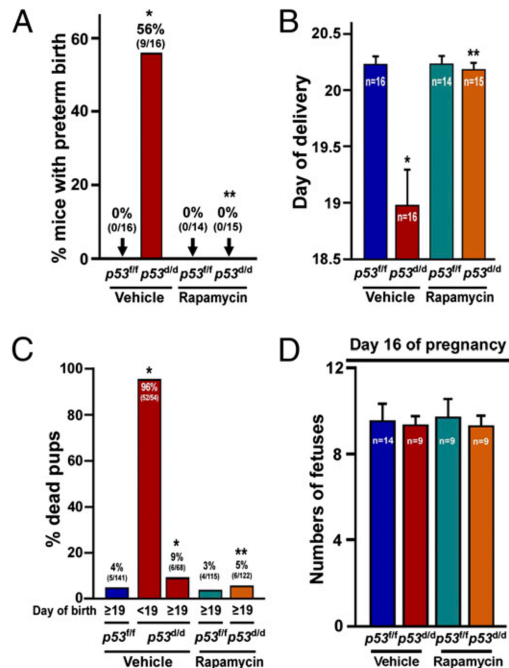

Fig. 2. Rapamycin rescues preterm birth in mice deleted of uterine *p53* ($p53^{d/d}$). (*A–C*) Preterm birth (deliveries earlier than day 19) and neonatal deaths in $p53^{d/d}$ mice are rescued by an oral gavage of an mTORC1 inhibitor rapamycin (0.25 mg/kg body weight/day) on days 8, 10, and 12 of pregnancy. In *A*, numbers in parentheses denote the ratio of dams with preterm birth/total dams. In *B*, day of deliveries are shown with numbers of animals examined indicated within the bars. In *C*, numbers in parentheses denote the ratio of dead pups/total pups. Values are mean ± SEM. $^*P < 0.05$ vs. vehicle-treated $p53^{f/f}$; $^{**}P < 0.05$ vs. vehicle-treated $p53^{d/d}$. (*D*) Number of viable fetuses in $p53^{f/f}$ or $p53^{d/d}$ females treated with vehicle or rapamycin. Values are mean ± SEM. $P > 0.05$.

2*D*). This rescue of preterm delivery prompted us to explore whether a single administration of rapamycin would rescue this phenotype. Indeed, even a single oral gavage of rapamycin at the same dose on either day 8 or day 12 rescued preterm deliveries in all mice examined, with a full complement of pups born to $p53^{d/d}$ dams ($n = 10$). The body weights of pups born to $p53^{f/f}$ and $p53^{d/d}$ dams were also comparable [mean ± SEM: $p53^{f/f}$, 1.4 ± 0.03 g/pup ($n = 138$ pups) vs. $p53^{d/d}$ plus rapamycin, 1.5 ± 0.06 g/pup ($n = 79$ pups)]. These results provide evidence that targeting mTORC1 signaling during the window of vulnerability early in pregnancy can be effective in preventing preterm birth with little adverse effects on fetal viability. Collectively, our findings suggest that attenuation of mTORC1 signaling can rescue preterm birth in $p53^{d/d}$ females.

Because increased mTORC1 signaling is known to advance the senescence process, we examined whether inhibition of mTORC1 signaling attenuates uterine senescence in $p53^{d/d}$ mice. In fact, decidua in $p53^{d/d}$ females, which exhibit higher levels of senescence-associated β-galactosidase (SA-β-gal) staining (10), were attenuated by rapamycin treatment (Fig. 3*A*). This observation is supported by a previous in vitro study showing that loss of *p53* increased mTORC1 signaling and cellular senescence in a human fibrosarcoma cell line (13). Taken together, these results suggest that rapamycin rescued preterm birth by attenuating the senescence process. We next asked whether decidual senescence also occurs in floxed littermate females at around the time of parturition. In $p53^{f/f}$ females, SA-β-gal staining was present primarily in decidual cells and not in the placenta (10), and in fact a gradual increase in SA-β-gal staining was seen on days 16, 18, and 19 of pregnancy before parturition (Fig. 3*B*). These findings suggest that decidual cells undergoing natural senescence during pregnancy becomes more intense as parturition approaches, underscoring the significance of decidual senescence in determining the timing of parturition.

Interestingly, WT mice given an injection of LPS (25 μg/mouse i.p.), an inducer of inflammation and preterm labor in mice that works by activating Toll-like receptor 4 (TLR4) and, to a lesser degree, Toll-like receptor 2 (TLR2) (14), increased the levels of pS6 and COX2 without changes in SA-β-gal staining (Fig. S1 *A* and *B*).

Deletion of p21 Rescues Preterm Birth in $p53^{d/d}$ Females. We next asked whether p21 (encoded by *Cdkn1a*), an inducer of cell cycle arrest and senescence (15), influences the timing of birth. We found that its levels are up-regulated in $p53^{d/d}$ uteri and this up-regulation is suppressed by rapamycin treatment (Fig. 3 *C* and *D*). Reduced pS6 levels confirmed that the rapamycin treatment regimen was effective in inhibiting mTORC1 signaling (Fig. 3 *C* and *D*). These results suggested that *p21* deficiency would also rescue premature delivery in mice deleted of uterine *p53*. To test this hypothesis, we generated mice with conditional deletion of uterine *p53* superimposed with a constitutive deletion of *Cdkn1a* ($Cdkn1a^{-/-}Trp53^{loxP/loxP}Pgr^{Cre/+} = p21^{d/d}p53^{d/d}$) by crossing $p53^{d/d}$ mice with $p21^{d/d}$ mice. These double-mutant mice were mated with WT fertile males. The number of viable fetuses on day 16 of pregnancy was comparable in $p53^{f/f}$, $p53^{d/d}$, $p21^{d/d}p53^{f/f}$, and $p21^{d/d}p53^{d/d}$ mice (Fig. 4*A*). Intriguingly, deletion of *p21* superimposed on uterine deletion of *p53* prevented preterm birth and neonatal death (Fig. 4 *B–D* and Table S2). As reported previously (16), *p21* deletion alone did not exhibit any adverse female reproductive phenotype. We also found that superimposition of *p21* deletion on *p53* deficiency attenuates decidual senescence, as detected by SA-β-gal staining (Fig. 5*A*). With these data in hand, we then asked whether the effects of rapa-

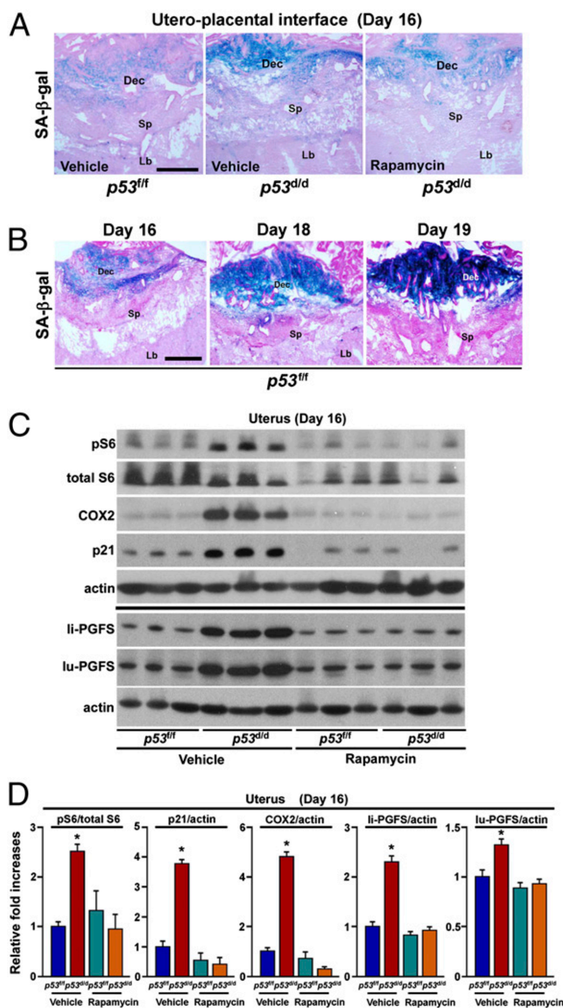

Fig. 3. Rapamycin attenuates senescence, mTORC1 signaling, and levels of p21 and COX2 in *p53*$^{d/d}$ females, whereas floxed *p53* females show progressive senescence in decidua approaching term pregnancy. (*A*) Uterine SA-β-gal staining in vehicle-treated *p53*$^{f/f}$ and vehicle- or rapamycin-treated *p53*$^{d/d}$ mice on day 16 of pregnancy. (*B*) SA-β-gal staining in uteri on days 16, 18, and 19 of pregnancy in floxed *p53* mice. (Scale bar: 500 μm.) At least three independent samples were examined in each group. Dec, decidua; Sp, spongiotrophoblast; Lb, labyrinth. (*C* and *D*) Up-regulated levels of pS6, COX2, p21, and PGFS in *p53*$^{d/d}$ uteri are suppressed by rapamycin treatment examined on day 16. Uterine samples from which placentas and fetuses had been removed were used. In *D*, whereas levels of pS6 were normalized against total S6, those of COX2, p21, and liver- and lung-type PGFS (li-PGFS and lu-PGFS) were normalized against actin. Values are mean ± SEM. $^*P <$ 0.05 vs. vehicle-treated *p53*$^{f/f}$. In each group, three independent samples from different mice were examined.

mycin or *p21* deficiency in rescuing preterm birth in *p53*$^{d/d}$ mice are mediated by cyclooxygenase-2 (COX2).

Deficient mTORC1 or p21 Signaling Down-Regulates Uterine COX2 Levels in *p53*$^{d/d}$ Females. Cyclooxygenase-derived prostaglandins (PGs) are known to participate in the parturition process. We previously showed that elevated uterine levels of prostaglandin F (PGF) 2α ($PGF_{2\alpha}$), resulting from increased levels of COX2 and PGF synthase (PGFS), were associated with preterm birth in *p53*$^{d/d}$ mice, and this phenotype was rescued by oral administration of celecoxib, a selective COX2 inhibitor (10). We surmised that mTORC1 and p21 are upstream regulators that influence the machinery for PGF synthesis. In fact, we found that rapamycin treatment or *p21* deficiency abrogates the rise in

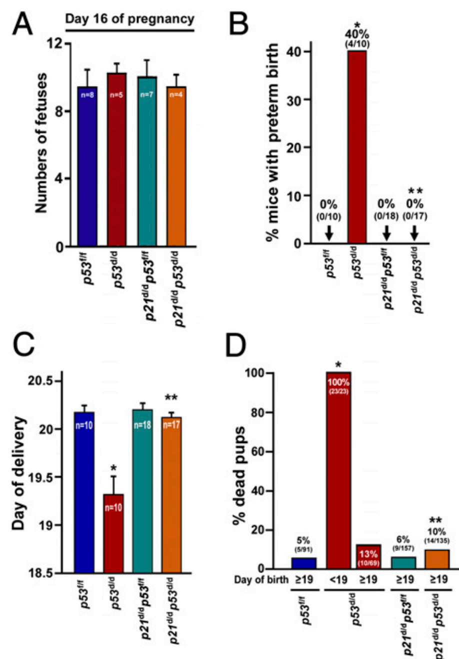

Fig. 4. Superimposition of *p21* deletion on uterine deficiency on *p53* rescues preterm birth and neonatal deaths. (*A*) Number of fetuses in *p53*$^{f/f}$, *p53*$^{d/d}$, *p21*$^{d/d}$*p53*$^{f/f}$, and *p21*$^{d/d}$*p53*$^{d/d}$ females on day 16 of pregnancy. Values are mean ± SEM. $P >$ 0.05. Numbers inside the bars indicate the number of mice examined. (*B*–*D*) Preterm birth (delivery earlier than day 19) was not observed in *p21*$^{d/d}$ *p53*$^{d/d}$ females. In *B*, numbers in parentheses denote the ratio of dams with preterm birth/total dams. In *C*, days of delivery are shown with numbers of animals examined indicated within the bars. In *D*, numbers in parentheses denote the ratio of dead pups/total pups. Values are mean ± SEM. $^*P <$ 0.05 vs. *p53*$^{f/f}$; $^{**}P <$ 0.05 vs. *p53*$^{d/d}$.

COX2 and PGF synthase that is seen in *p53*$^{d/d}$ uteri (Figs. 3 *C* and *D* and 5 *B* and *C*). However, serum levels of estradiol-17β and P_4 (Fig. S2 *A* and *B*), expression of 20α-hydroxysteroid dehydrogenase (20α-HSD; an enzyme that converts P_4 to an inactive metabolite during luteolysis), and ovarian morphology were not affected under these conditions (Fig. S3 *A*–*D*). These results illustrate that rapamycin or lack of *p21* rescues preterm birth in *p53*$^{d/d}$ mothers by suppressing uterine generation of $PGF_{2\alpha}$ by COX2 and PGFS, but does not affect ovarian function. There is evidence that elevated levels of COX2 are associated with senescence in fibroblasts in vitro, leading to higher levels of COX2-derived prostaglandins (17). Interestingly, uterine pS6 levels were not down-regulated in *p21*$^{d/d}$*p53*$^{d/d}$ uteri (Fig. 5 *C* and *D*), providing evidence that mTORC1 signaling is an upstream modulator of p21 and COX2 signaling that impacts preterm delivery. The underlying mechanism of elevated COX2 under increased mTORC1 signaling is not clearly understood, however.

It is possible that the up-regulation of COX2 in decidua is due to recruitment of immune cells. However, we found a comparable population of decidual natural killer cells, detected by periodic acid-Schiff (PAS) staining, in *p53*$^{f/f}$ and *p53*$^{d/d}$ mice on day 12 of pregnancy (Fig. S4), although this finding does not exclude the possibility of differential activation of this cell population in the two genotypes. Whether COX2 up-regulation contributing to inflammatory response leading to preterm birth stems from increased migration of other immune cells into the deciduum remains an open question. Our previous observation of highly localized COX2 expression in decidual cells, but not in a scattered pattern characteristic of immune cells, in *p53*$^{d/d}$ uteri on days 8 and 16 of pregnancy (10) support our premise that COX2 elevation is an effect specific to decidual cells under our exper-

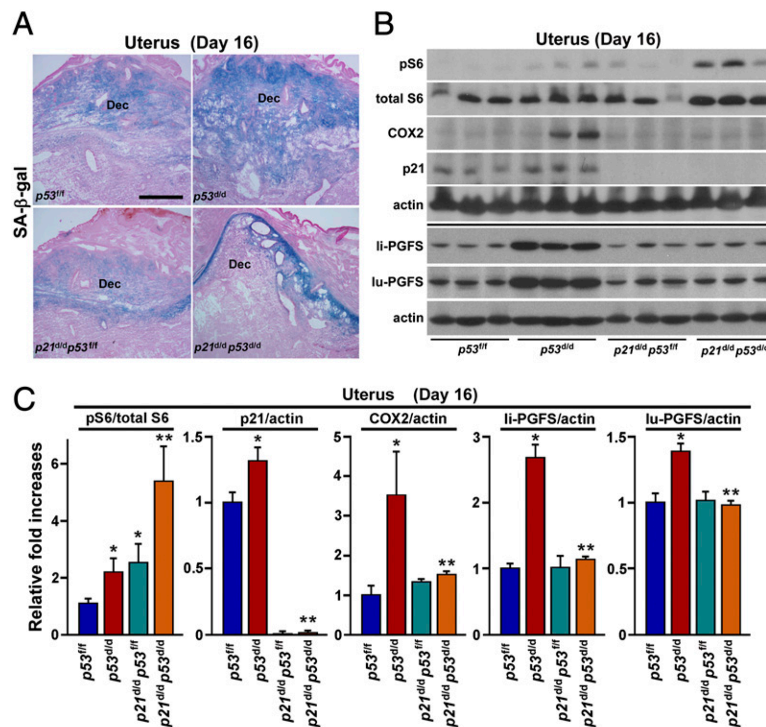

Fig. 5. Superimposition of *p21* deletion attenuates senescence and levels of COX2 and PGFS with no changes in pS6 in *p53*$^{d/d}$ females. (*A*) Uterine SA-β-gal activity in *p53*$^{f/f}$, *p53*$^{d/d}$, *p21*$^{d/d}$*p53*$^{f/f}$, and *p21*$^{d/d}$*p53*$^{d/d}$ mice on day 16 of pregnancy. Dec, deciduas. (Scale bar: 500 μm.) (*B* and *C*) Up-regulated levels of COX2 and PGFS in uteri with *p53* deletion are reversed by superimposition of *p21* deficiency on day 16; pS6 levels are not altered. Uterine samples from which placentas and fetuses had been removed were used for Western blot analysis. In *C*, although levels of pS6 were normalized against total S6, those of COX2, p21, and liver- and lung-type PGFS (li-PGFS and lu-PGFS) were against actin. In each group, three independent samples from different mice were examined. Values are mean ± SEM. $*P < 0.05$ vs. *p53*$^{f/f}$; $**P < 0.05$ vs. *p53*$^{d/d}$.

imental conditions. As discussed previously (10), we believe that prostaglandins derived from decidual COX2 function as paracrine effectors on the myometrium.

To investigate whether mTORC1 signaling can directly affect p21 and COX2 levels in cells with intrinsically high mTORC1 activity, we used *Tsc1*$^{+/+}$ and *Tsc1*$^{-/-}$ mouse embryonic fibroblasts (MEFs) in culture (18). As expected, both total S6 and pS6 levels were high in cultured *Tsc1*$^{-/-}$ MEFs, indicating heightened intrinsic mTORC1 activity. Moreover, p21 and COX2 levels were also higher in *Tsc1*$^{-/-}$ MEFs, and rapamycin attenuated the levels of pS6, COX2, and p21 in a time- and dose-dependent manner in *Tsc1*$^{-/-}$ MEFs (Fig. S5 *A* and *B*). These results demonstrate that inhibition of mTORC1 signaling negatively regulates the status of p21 and COX2. This finding is supported by evidence that increased mTORC1 signaling up-regulates p21 levels and that an mTORC1 inhibitor can abrogate this up-regulation in a lung carcinoma cell line (19).

Discussion

This work has revealed an unanticipated role of mTORC1 signaling in preterm birth upstream of p21 and COX2. We have shown that the mTORC1-p21-COX2 signaling axis is a critical component in the timing of birth, and that an intervention aimed at any of these three targets is capable of rescuing preterm delivery in mice, which has not been reported previously (Fig. 6).

Lye et al. (20) previously observed activation of PI3K-mTORC1 signaling in the proliferating myometrium in ovariectomized rats mimicking the hormonal milieu of early pregnancy. Our finding of increased mTORC1 signaling in decidua leading to preterm birth suggests that this pathway is active in a stage- and tissue-specific manner. This is evident from the localization of pS6 and COX2 in conjunction with SA-β-gal staining in decidual cells in *p53*$^{d/d}$ mice on day 16 of pregnancy.

Because chronological aging is a cause of cellular senescence (21), and decidual senescence is associated with premature delivery (10), it is possible that uterine senescence imposed by maternal aging or other stressors, including infection/inflammation, carries an increased risk for problematic parturition. Indeed, epidemiologic evidence indicates that increased maternal age is correlated with preterm delivery in humans (22, 23), and aging mice demonstrate declining p53 function (24). Our preclinical study mimicking aspects of human parturition certainly opens this important question to further investigation.

Although ovarian functions, such as ovulation and hormone secretion, are assumed to be altered with aging (25), there has been less investigative focus on uterine aging and its consequences for fertility. Epidemiologic evidence suggests that increased maternal age is a risk factor for pregnancy-associated complications, including preterm birth and preeclampsia (23, 25). In addition, women of higher maternal age who use assisted reproductive technologies to achieve pregnancy experience a higher incidence of preterm birth even when receiving oocytes from young donors (26, 27). This finding suggests that uterine senescence may be a potential contributor to these difficulties and warrants further investigation. Although causes of advanced maternal age-related perinatal complications remain elusive, our study provides a proof of concept that premature uterine senescence seeded early in pregnancy also may contribute to perinatal complications.

Rapamycin has been reported to cause remission of respiratory distress syndrome resulting from heightened mTORC1 signaling in the premature lung epithelium induced by preterm delivery of pups on day 17.5 (28), suggesting beneficial aspects of rapamycin

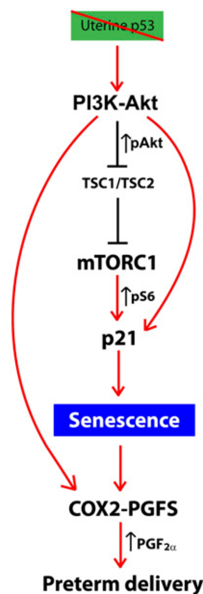

Fig. 6. The proposed signaling pathway to preterm birth. Uterine deletion of p53 in mice results in increased PI3K-Akt signaling, which suppresses TSC activity by phosphorylating TSC2. Thus, TSC's inhibition on mTORC1 signaling is released, reflecting higher levels of pS6, as well as p21 levels. These changes give rise to heightened cellular senescence associated with increased $PGF_{2\alpha}$ generation via the COX2-PGFS pathway, driving preterm birth.

during pregnancy. Rapamycin also reverses congenital hypertrophic cardiomyopathy in juvenile mice manifesting LEOPARD syndrome (29). Furthermore, TLR4- or TLR2-induced neutrophil activation and lung injury resulting from increased mTORC1 signaling is attenuated by rapamycin treatment (30). It is interesting to note that inflammatory signals resulting from TLR4 or TRL2 activation is a potential cause of preterm birth (31).

Rodent models of preterm birth induced by local or systemic application of LPS, mediated primarily by TLR4/TLR2 signaling, are widely used and are coincident with ovarian luteolysis and a drop in serum P_4 levels, which normally does not occur in human parturition (31, 32). However, each model has unique features, and their contributions to the understanding of preterm labor are complementary to studies using genetic models (10, 33). These models could be used in conjunction to explore gene–environment aspects of preterm birth and prematurity.

Our results showing increased mTORC1 activation and COX2 levels after LPS injection suggest that an acute, systemic inflammatory stimulus can induce preterm labor with activation of mTORC1 and COX2 signaling, but is incapable of altering the senescence status within this short period (~24 h). However, LPS has been reported to induce decidual and placental apoptosis (34); thus, it is possible that preterm birth resulting from increased mTORC1 signaling in our model or from an acute inflammatory stimulus is mediated by different cellular processes but ultimately converge on an mTORC1-COX2 pathway. Further investigation is warranted to unravel the intricacies of these pathways.

LPS can act both locally and systemically, and discerning the contribution of each type of action to preterm birth is difficult. Although there is evidence that local administration of LPS can induce preterm delivery (31, 32), possible systemic effects from these local applications cannot be excluded. Irrespective of local or systemic effects, inflammatory rodent models of preterm birth differ from the $p53^{d/d}$ mouse model, in which gradual accumulation of senescence factors from early stages of pregnancy are thought to predispose these mice to spontaneous preterm birth. We conjecture that this genetic model will respond more robustly to environmental stressors (i.e., inflammation/infection), providing an opportunity to study the gene–environment relationship in preterm birth. In this respect, genetic and environmental factors may initially participate in different pathways but converge to a final pathway involving COX2-derived PGs to induce preterm birth. Whether this has relevance to human preterm birth awaits further investigation.

Our findings demonstrating rescue of preterm birth in $p53^{d/d}$ dams from low-dose rapamycin administration are remarkable. Given that a significant percentage of preterm birth is thought to result from infection, inflammation, and immune responses, it remains to be ascertained whether low doses of rapamycin (antiaging and immunosuppressive) plus celecoxib (anti-inflammatory) could produce better therapeutic effects with minimal side effects. In addition, the downstream targets of mTORC1 or COX2 might be amenable to pharmacologic interventions to circumvent the side effects of rapamycin and COX2 inhibitors, and may prove beneficial in preventing preterm birth as well. Future studies will establish whether mTORC1 and TLR signaling independently or cooperatively converge on a final pathway amenable to targeting by combinatory therapeutic approaches.

Genetic components and environmental stressors have been implicated in preterm delivery but have not been experimentally tested. Notably, the signaling pathways triggering preterm delivery might not be equated with those occurring in normal parturition; preterm delivery represents an aberrant state of pregnancy. Thus, models exhibiting different aspects of preterm birth will provide a better understanding of the mechanism of preterm birth in humans.

Methods

Mice. Mice with uterine deletion of *Trp53* ($Trp53^{loxP/loxP}Pgr^{Cre/+} = p53^{d/d}$) were generated as described previously (10). $Cdkn1a^{-/-}Trp53^{loxP/loxP}Pgr^{Cre/+}$ ($p21^{d/d}p53^{d/d}$) mice were generated by mating $p53^{d/d}$ mice with $Cdkn1a^{-/-}$ ($p21^{d/d}$) mice. For experiments, littermate $p53^{f/f}$, $p53^{d/d}$, $p21^{d/d}p53^{f/f}$, and $p21^{d/d}p53^{d/d}$ females on the mixed background were mated with WT fertile males to induce pregnancy. All mice used in this investigation were housed in barrier facilities in the Cincinnati Children's Hospital Medical Center's Animal Care Facility according to National Institutes of Health and institutional guidelines for the use of laboratory animals. All protocols for the present study were reviewed and approved by the Cincinnati Children's Research Foundation's Institutional Animal Care and Use Committee. Mice were provided with autoclaved rodent LabDiet 5010 (Purina) and UV light-sterilized RO/DI constant circulation water ad libitum.

Analysis of Parturition. Parturition events were monitored from day 17 through day 21 by observing mice in the morning, at noon, and in the evening as described previously (10). Preterm birth was defined as birth occurring earlier than day 19 of pregnancy. The mTORC1 inhibitor rapamycin (0.25 mg/kg body weight/day) was suspended in 5% PEG400 and 5% Tween-80 dissolved in water by constant stirring and was given as a single oral gavage on days 8, 10, and/or 12 of pregnancy. The control group received vehicle alone. LPS (25 μg/mouse i.p.; Sigma-Aldrich) was administered on day 16 of pregnancy.

Western Blot Analysis. Protein extraction and Western blot analysis were performed as described previously (10). Immunoblots were performed using extracts of uteri from which placenta and fetuses, including fetal membranes, were removed. Protein lysates were run on 10% SDS/PAGE gels. Antibodies for total S6 (1:1,000; Cell Signaling) and phospho-S6 (serine 235/236, 1:1,000; Cell Signaling), COX2 (1:5,000; Cayman), p21 (1:1,000; Santa Cruz Biotechnology), liver- and lung-type prostaglandin F synthase (PGFS; a gift from Kikuko Watanabe, University of East Asia, Shimonoseki, Japan), actin (1:1,000; Santa Cruz Biotechnology), and 20α-HSD (a gift from Geula Gibori, University of Illinois, Chicago, IL) were used. The same blots were used for quantitative analyses of each protein. Bands were visualized using an ECL kit (GE Healthcare). Actin served as a loading control.

Immunohistochemistry. Immunostaining of pS6 was performed in formalin-fixed, paraffin-embedded sections using antibodies to pS6 (Cell Signaling).

PAS staining was used to identify dNK cells (35). To compare intensity of immunostaining between *p53*$^{f/f}$ and *p53*$^{d/d}$ tissues, tissue sections from both genotypes were processed onto the same slide.

SA-β-gal Staining. Staining of SA-β-gal activity was performed as described previously (10). SA-β-gal staining, a well-established marker of cellular senescence, is performed at pH 5.5–6.0. To compare the intensity of SA-β-gal staining, sections from different genotypes and on different days of pregnancy were processed on the same slide.

Statistics. Statistical analyses were performed using the two-tailed Student *t* test and Fisher's exact probability test, as appropriate. $P < 0.05$ was considered statistically significant.

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