Rethinking progesterone regulation of female reproductive cyclicity

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The progesterone receptor (PGR) is a ligand-activated transcription factor with key roles in the regulation of female fertility. Much has been learned of the actions of PGR signaling through the use of pharmacologic inhibitors and genetic manipulation, using mouse mutagenesis. Characterization of rats with a null mutation at the *Pgr* locus has forced a reexamination of the role of progesterone in the regulation of the female reproductive cycle. We generated two *Pgr* mutant rat models, using genome editing. In both cases, deletions yielded a null mutation resulting from a nonsense frame-shift and the emergence of a stop codon. Similar to *Pgr* null mice, *Pgr* null rats were infertile because of deficits in sexual behavior, ovulation, and uterine endometrial differentiation. However, in contrast to the reported phenotype of female mice with disruptions in *Pgr* signaling, *Pgr* null female rats exhibit robust estrous cycles. Cyclic changes in vaginal cytology, uterine histology, serum hormone levels, and wheel running activity were evident in *Pgr* null female rats, similar to wild-type controls. Furthermore, exogenous progesterone treatment inhibited estrous cycles in wild-type female rats but not in *Pgr*-null female rats. As previously reported, pharmacologic antagonism supports a role for PGR signaling in the regulation of the ovulatory gonadotropin surge, a result at variance with experimentation using genetic ablation of PGR signaling. To conclude, our findings in the *Pgr* null rat challenge current assumptions and prompt a reevaluation of the hormonal control of reproductive cyclicity.

progesterone | rat | reproductive cycles | PGR

The fundamental elements regulating the female reproductive cycle have been universally accepted for decades and include a hierarchy of control involving the hypothalamic/anterior pituitary/ovarian axis (1,2). The hypothalamus, through its secretion of gonadotropin-releasing hormone, drives anterior pituitary production of gonadotropins [luteinizing hormone (LH) and follicle-stimulating hormone (FSH)], which act on the ovaries to promote follicle development, ovulation, formation of the corpus luteum, and secretion of sex steroid hormones estrogen and progesterone. These two sex steroid hormones possess well-established actions on the female reproductive tract. At the core of the female reproductive cycle is a balance of sex steroid hormone negative and positive feedback regulation of gonadotropin secretion. Both estrogen and progesterone signaling pathways have been implicated in feedback control of gonadotropins and regulation of the female reproductive cycle (3–5). These concepts have been reinforced through phenotypic examination of mice possessing null mutations at either *Esr1* or *Pgr* loci (6–8), and through the use of pharmacologic inhibitors of estrogen and progesterone signaling. *Esr1* and *Pgr* encode the estrogen receptor 1 (also referred to as ER alpha) and progesterone receptor, respectively. These two nuclear receptors mediate many of the actions of estrogen and progesterone on the female reproductive system (9–11).

Historically, the rat represented the model organism for investigations on mammalian reproduction, including the regulation of female reproductive cyclicity (12–15). The advent of gene manipulation strategies in the mouse largely supplanted the rat, and over the last few decades, our understanding of mammalian reproduction has been greatly influenced by mouse mutagenesis experimentation (16). Development of genome editing strategies has decreased the dependence on the mouse and has created opportunities for investigating the regulation of mammalian reproduction in other animal model systems, including the rat. Analysis of rats with an ESR1 deficiency has further strengthened the importance of estrogen and ESR1 in regulating female reproductive cyclicity (17).

Here, using genome-editing strategies to produce *Pgr* null rats, we show that, as expected, progesterone signaling through the progesterone receptor (PGR) mediates progesterone action on the reproductive axis, including negative feedback regulation; however, PGR and progesterone signaling are not essential for female reproductive cyclicity. These results challenge a basic tenet of mammalian reproductive biology and force a reevaluation of the role of progesterone in the regulation of the female reproductive cycle.

Results

Targeted Disruption of the Rat *Pgr* Gene. Zinc finger nuclease mediated disruption of the *Pgr* locus in the rat (136-bp deletion within exon 1, $Pgr^{\Delta 136E1}$; Fig. S1) yielded a nucleotide frameshift and resulted in a premature stop codon and absence of detectable PGR protein in homozygotes, resulting in a null mutation and leading to confirmation of many of the hallmarks of progesterone action, including female infertility (Fig. 1 and Figs. S1–S3). $Pgr^{\Delta 136E1}$ null female rats exhibited failures in ovulation; ovulatory responses to

Significance

Progesterone possesses an essential role in regulating female fertility, with prominent actions throughout the female reproductive axis. The neuroendocrine actions of progesterone have been viewed as critical for the control of the female reproductive cycle. This basic principle has been reinforced by in vivo experimental paradigms, using hormone replacement as well as pharmacologic and genetic disruption of the progesterone receptor (PGR). Phenotypic characterization of *Pgr* null rats strengthens roles for progesterone in the regulation of female fertility, but not roles for progesterone as an essential determinant of female reproductive cyclicity, challenging an elemental principle of mammalian reproductive biology. Such findings demonstrate the benefits of genome editing in expanding available animal models for physiologic investigation.

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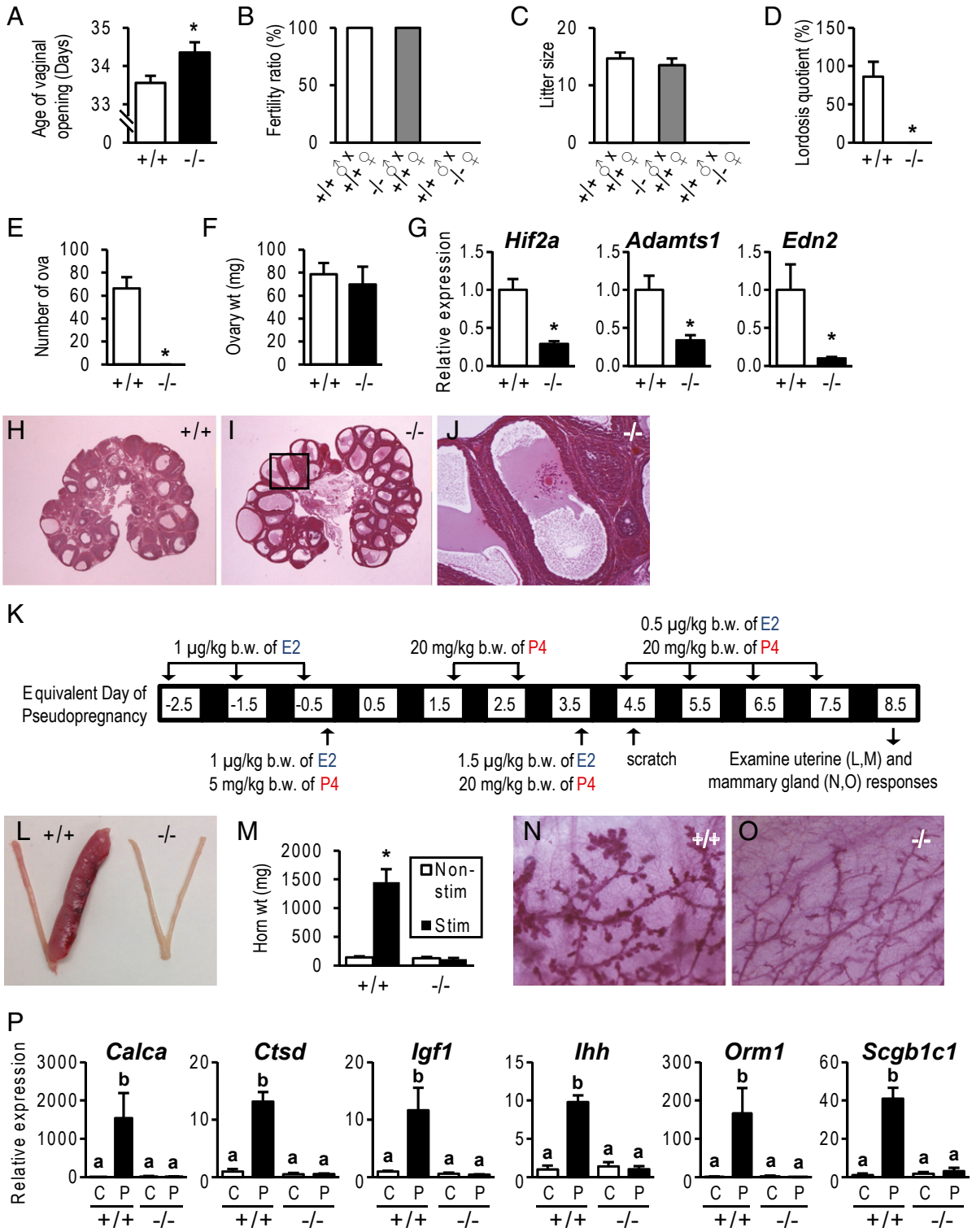

Fig. 1. Phenotypic characterization of $Pgr^{\Delta 136E1}$ null female rats. (*A*) Temporal assessment of vaginal opening in wild-type (+/+) and $Pgr^{\Delta 136E1}$ null (–/–) female rats ($n = 50$/genotype). (*B* and *C*) Fertility tests and litter sizes from wild-type males mated to wild-type and $Pgr^{\Delta 136E1}$ null female rats and $Pgr^{\Delta 136E1}$ null males mated to wild-type females. ($n = 6$/mating scheme). (*D*) Sexual behavior in wild-type and $Pgr^{\Delta 136E1}$ null female rats. The ratio of female lordosis behavior to male mounting was quantified ($n = 6$/genotype; Movie S1). (*E–J*) Effects of exogenous gonadotropins on ovulation (*E*), ovarian weight (*F*), gene expression (*G*), and hematoxylin and eosin-stained paraffin-embedded ovarian tissue sections (*H–J*) in wild-type and $Pgr^{\Delta 136E1}$ null female rats ($n = 6$/genotype). (*J*) Trapped oocyte within an unruptured follicle. (*K–M*) Examination of artificial decidualization in wild-type and $Pgr^{\Delta 136E1}$ null female rats. (*K*) Schematic presentation of hormone treatments (E2, estradiol; P4, progesterone). (*L*) Gross responses of uterine tissue to a deciduogenic stimulus. (*M*) Quantification of uterine horn weights from nonstimulated (Nonstim) and stimulated (Stim) uterine horns ($n = 6$/genotype). (*N* and *O*) Mammary gland development in hormonally treated wild-type and $Pgr^{\Delta 136E1}$ null female rats. (*P*) Examination of acute uterine responses to progesterone in wild-type and $Pgr^{\Delta 136E1}$ null rats. Progesterone responsive transcripts were monitored by quantitative RT-PCR (qRT-PCR) ($n = 6$/group; C, vehicle; P, progesterone). Results are presented as mean ± SEM. Asterisks or different letters above bars signify differences between means ($P < 0.05$).

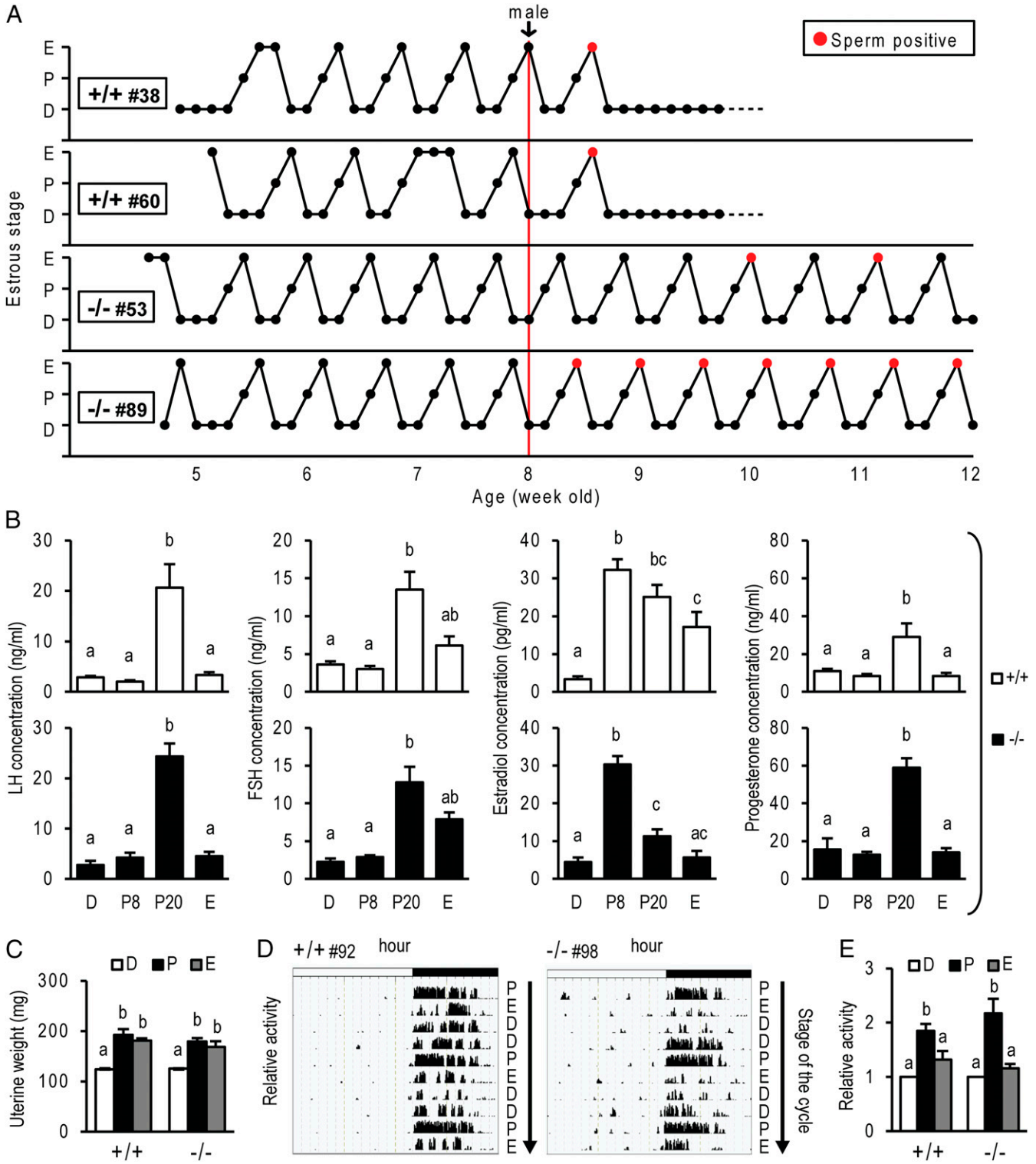

Fig. 2. Reproductive cyclicity in wild-type and $Pgr^{\Delta 136E1}$ null female rats. (*A*) Representative estrous cycle profiles of wild-type (+/+) and $Pgr^{\Delta 136E1}$ null (–/–) female rats. Estrous cycles were monitored for 7 wk by daily inspection of vaginal cytology (D, diestrus; P, proestrus; E, estrus). The graphs also indicate when males were introduced. Red points indicate the presence of sperm in the vaginal lavage. (*B*) Cyclic changes in hormone concentrations in wild-type (*Upper*) and $Pgr^{\Delta 136E1}$ null (*Lower*) female rats. Blood was collected from 8–10-wk-old females at 0800 h on the first day of diestrus (D; $n = 6$/genotype), proestrus (P8; $n = 6$/genotype), and estrus (E; $n = 6$/genotype), and also at 2000 h on proestrus (P20; $n = 14$/genotype). Serum LH, FSH, estradiol, and progesterone were measured. (*C*) Cyclic changes in uterine weight in wild-type and $Pgr^{\Delta 136E1}$ null rats. Uteri were collected from 8-wk-old female rats, weighed ($n = 6$/group), and analyzed histologically (Fig. S4). (*D* and *E*) Cyclic changes in activity patterns in wild-type and $Pgr^{\Delta 136E1}$ null rats. Representative activity patterns during estrous cycles (*D*) and quantification of relative activity during each stage of the estrous cycle (*E*; $n = 9$/genotype) Results are presented as mean ± SEM. Different letters above bars signify differences between means ($P < 0.05$).

exogenous gonadotropins; uterine responses to progesterone, including decidualization and induction of progesterone-dependent gene expression; hormone-dependent sexual behavior; and mammary gland branching morphogenesis (Fig. 1, Fig. S3, and Movie S1). A distinctive feature of ovaries from $Pgr^{\Delta 136E1}$ null females was the presence of oocytes trapped in unruptured follicles (Fig. 1*J* and Fig. S3). $Pgr^{\Delta 136E1}$ null male rats were fertile. These facets of the $Pgr^{\Delta 136E1}$ null female and male rat reproductive phenotypes are consistent with previous observations in the mouse (7, 18).

Reproductive Cyclicity in the Female $Pgr^{\Delta 136E1}$ Null Rat. Further phenotypic characterization of the $Pgr^{\Delta 136E1}$ null rat revealed a fundamental distinction from the *Pgr* null mouse described in earlier reports (19, 20). $Pgr^{\Delta 136E1}$ null female rats display highly regular and well-defined reproductive cycles (Fig. 2), in contrast to the acyclic *Pgr* null female mouse (19). Cyclic changes in vaginal cytology, wheel running activity patterns, hormone levels, and uterine weights were observed in both wild-type and $Pgr^{\Delta 136E1}$ null female rats (Fig. 2 and Fig. S4). Although cyclicity was observed in both genotypes, some differences between wild-type and $Pgr^{\Delta 136E1}$ null female rats were noted, including cyclic changes in serum levels of sex steroid hormones (Fig. 2) and estrous cycle length (Fig. S4). Another unexpected observation was the detection of sperm in vaginal lavages from $Pgr^{\Delta 136E1}$ null female rats cohabiting cages with males (Fig. 2). In some instances, the presence of sperm in the vaginal lavage exhibited a cyclic pattern coinciding with the estrus stage of the estrous cycle. Although $Pgr^{\Delta 136E1}$ null female rats showed clear deficits in hormone-elicited sexual behavior (Fig. 1), in the gonadally intact state, some females allowed males to mount, exhibiting enough elements of sexual receptivity to permit vaginal deposition of sperm by the male (Movie S2). Such observations are consistent with previous reports showing estrogen alone can facilitate sexual behavior in the rat (21, 22). The mating never yielded a pregnancy or pseudopregnancy; instead, $Pgr^{\Delta 136E1}$ null female rats continued to cycle. These observations prompted further analysis of progesterone signaling on female cyclicity.

Progesterone Regulation of Reproductive Cycles. Evidence exists for progesterone acting independent of its nuclear receptor, PGR (23, 24). As a consequence, we examined the actions of exogenous progesterone treatment on cyclicity. Adult wild-type and $Pgr^{\Delta 136E1}$ null female rats were s.c. implanted with progesterone pellets (2 × 200 mg/rat), and vaginal cytology was monitored daily for 2 wk. As expected, wild-type females ceased cycling, whereas $Pgr^{\Delta 136E1}$ null females continued to display uninterrupted cycles (Fig. 3). In addition, progesterone administration could shift a 4-d to a 5-d estrous cycle in wild-type females, but not in $Pgr^{\Delta 136E1}$ null females (Fig. S4). These data demonstrate that PGR is involved in the negative feedback regulation of the female reproductive axis; however, PGR and progesterone signaling are dispensable for female reproductive cyclicity in the rat.

Generation and Phenotypic Characterization of a Mutation Targeting Exon 3 of the Rat *Pgr* Gene. Because the *Pgr* mutation generated in the rat targeting exon 1 ($Pgr^{\Delta 136E1}$) differed from the previously characterized *Pgr* mutant mouse model (7), we sought to determine whether our phenotypic observations were biased by differences in the respective genomic manipulations. Clustered regularly-interspaced short palindromic repeat (*CRISPR*)/*Cas9* genome editing was used to produce a rat model possessing a complete deletion of exon 3 (encoding the DNA binding domain) within the rat *Pgr* gene ($Pgr^{\Delta E3}$; Fig. S5). The $Pgr^{\Delta E3}$ mutation resulted in a null mutation and phenocopied the $Pgr^{\Delta 136E1}$ mutant rat, including the manifestation of reproductive cycles (Fig. 4). Thus, phenotypic characterization of two distinct genetic models, both possessing deficits in progesterone signaling, indicate that in the rat, female reproductive cyclicity is independent of PGR.

Actions of Mifepristone on the LH Surge. Administration of progesterone receptor antagonists such as mifepristone (also known as RU486) have been consistently shown to disrupt the LH surge and interfere with cyclicity in the rat, monkey, and human (25–28), implicating progesterone signaling in the control of reproductive cyclicity. However, these findings are at odds with our observations with *Pgr* null rats. As a consequence, we examined the effects of mifepristone on the LH surge of wild-type and $Pgr^{\Delta 136E1}$ null rats. Mifepristone effectively inhibited the LH surge in wild-type rats, but not in $Pgr^{\Delta 136E1}$ null rats (Fig. 5). These results replicate earlier observations indicating that a progesterone receptor antagonist such as mifepristone disrupts the LH surge (25–28) and effectively demonstrates that these mifepristone actions are dependent on the PGR, but remain at odds with results with *Pgr* null rats. Thus, the downstream events after mifepristone engagement of PGR are fundamentally different from a biological response associated with the absence of PGR.

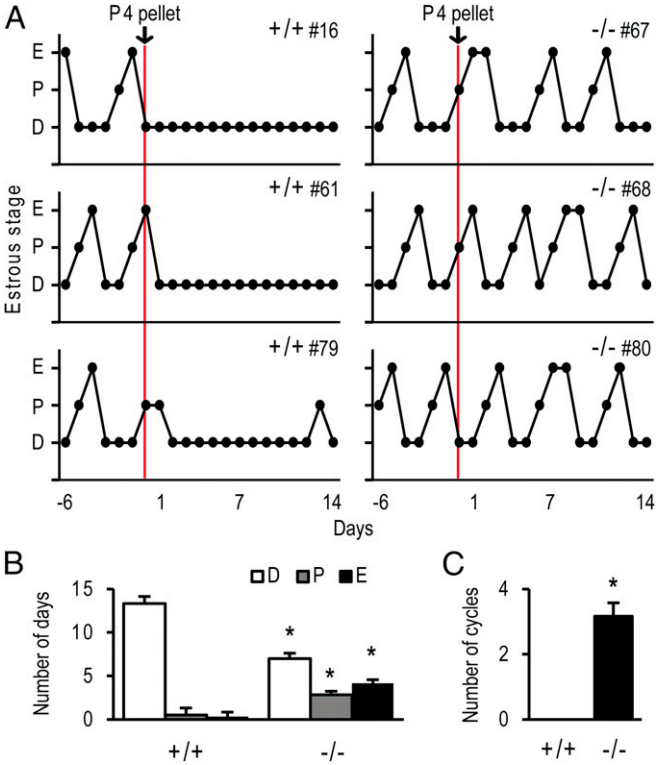

Fig. 3. Cyclicity in progesterone treated wild-type and $Pgr^{\Delta 136E1}$ null female rats. Progesterone (P4) pellets (400 mg/rat) were implanted s.c. into cyclic wild-type and $Pgr^{\Delta 136E1}$ null female rats. Cyclicity was monitored by daily inspection of vaginal cytology for 14 d. (*A*) Vaginal cytology profiles for three representative wild-type and three representative $Pgr^{\Delta 136E1}$ null female rats are presented (D, diestrus; P, proestrus; E, estrus). (*B* and *C*) Quantification of the number of days in each stage of the estrous cycle and the number of cycles during the 14-d test period is presented (n = 6/genotype). Results are presented as the mean ± SEM. Asterisks indicate significant differences between wild-type and $Pgr^{\Delta 136E1}$ null female rats ($P < 0.05$).

Discussion

Progesterone signaling through the PGR is essential for female fertility. Such insights were first demonstrated through mouse mutagenesis (7) and are now also evident in rats with null mutations at the *Pgr* locus. The absence of a functional PGR is associated with deficits all along the reproductive axis (e.g., hypothalamus/pituitary, ovary, uterus, and mammary gland) (7, 19, 20; 29, 30). Remarkably, disruption of PGR signaling in the rat does not interfere with cyclic changes in vaginal cytology, physical activity, circulating hormone levels, or uterine structure. Although PGR is important in the negative feedback actions of progesterone on the reproductive axis (31), the integrity of this activity is not required for cyclicity. Such observations are provocative. They contrast with the reported phenotype of *Pgr* mutant mice (19, 20, 29, 30), insights gained from hormone replacement experiments (13, 32), and the reported neuroendocrine

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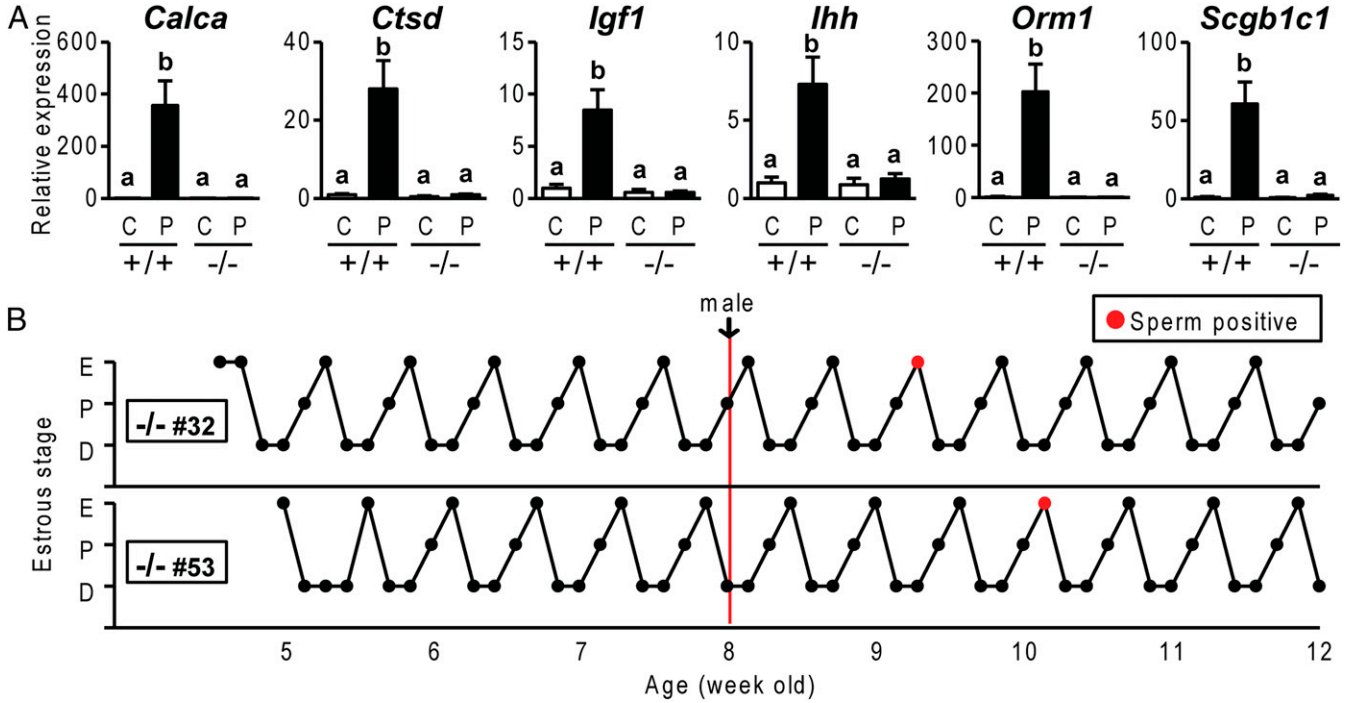

Fig. 4. Phenotypic characterization of $Pgr^{\Delta E3}$ null female rats. (*A*) Examination of acute uterine responses to progesterone in wild-type (+/+) and $Pgr^{\Delta E3}$ null (–/–) rats. Progesterone-responsive transcripts were monitored by qRT-PCR (n = 6/group). Results are presented as the mean ± SEM. Different letters above bars signify differences between means ($P < 0.05$). (*B*) Representative estrous cycle profiles of $Pgr^{\Delta E3}$ null females. Estrous cycles were monitored for 7 wk by daily inspection of vaginal cytology (D, diestrus; P, proestrus; E, estrus). The graphs also indicate when males were introduced. Red points indicate the presence of sperm in the vaginal lavage.

actions of progesterone receptor antagonists (25–28) and demand a rethinking of the hormonal control of reproductive cyclicity.

Cyclicity phenotypes of mice and rats with *Pgr* null mutations exhibit sharp differences (19, 20). Two strains of *Pgr* mutant rats, each possessing a distinct null mutation, exhibit cyclicity, whereas mice lacking a functional PGR do not cycle. Such a fundamental difference between these two murid species is unexpected. Species differences and experiential factors may affect substrates for progesterone action regulating the reproductive cycle. Mice are known to possess irregularities in their estrous cycle, as assessed by vaginal cytology, and are susceptible to interference by environmental and social factors, posing technical challenges not evident in the rat (33). Some of these potential factors affecting mouse reproductive cyclicity are transmitted through the olfactory system (34), a known substrate for progesterone action (35). Variability in the mouse estrous cycle is often addressed by experimental simulations of specific estrous cycle events, including pheromone-activated LH surges and hormone-activated LH surges in ovariectomized mice. *Pgr* mutant mice show deficits in both pheromone- and hormone-activated LH surges (19, 20, 29, 30). Because *Pgr* null rats exhibit robust and well-defined reproductive cycles, it is not necessary to artificially simulate events within the estrous cycle, making direct comparisons between mouse and rat *Pgr* mutants problematic. Observations with the *Pgr* null rat bring into question the involvement of progesterone and PGR signaling as an essential regulator of the female reproductive cycle. Alternatively, a mouse-centric interpretation may preserve the importance of PGR signaling in cyclicity, and instead focus on the emergence of a compensatory pathway controlling cyclicity in the *Pgr* null rat. Nevertheless, our observations highlight a practical benefit of genome editing, which enables the experimental implementation of the most appropriate animal models for physiologic investigation.

In addition to the acyclic phenotype of the *Pgr* null mouse, the actions of progesterone receptor antagonists have been used to support a role for progesterone signaling in the regulation of the female reproductive cycle. Our findings in *Pgr* null rats indicate that the actions of a PGR antagonist, such as mifepristone, in the presence of PGR is fundamentally different from the biology associated with the absence of PGR. In the present study, mifepristone treatment inhibited the LH surge in a PGR-dependent manner, whereas the LH surge occurred uninterrupted in *Pgr* null rats. This may be surprising to some, but not to those familiar with the molecular actions of mifepristone. Mifepristone physically interacts with PGR, facilitating the assembly of protein complexes distinct from those activated by PGR agonists (36, 37); differentially regulates chromatin remodeling (38); and guides ligand receptor complexes to unique regions within the genome (39). In addition, transcriptional targets of PGR within the mouse uterus identified through the use of mifepristone as an antagonist of endogenous ligand action (40) versus progesterone actions in wild-type and *Pgr* null tissues yield different profiles (41). Thus, caution is required in interpreting pharmacologic manipulations versus genetic

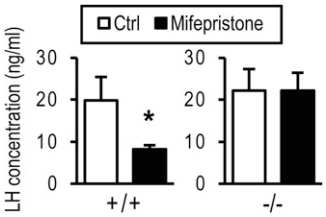

Fig. 5. The effects of mifepristone on the LH surge. Wild-type (+/+) and $Pgr^{\Delta 136E1}$ null (–/–) female rats were monitored for cyclicity by daily inspection of vaginal cytology. At 1330 h on proestrus, animals were treated with vehicle (sesame oil) or mifepristone (6 mg/kg). Animals were killed at 2000 h on proestrus and blood collected for measurement of serum LH concentrations. Sample sizes: wild type-vehicle, n = 12; wild type-mifepristone, n = 14; $Pgr^{\Delta 136E1}$ null-vehicle, n = 8; $Pgr^{\Delta 136E1}$ null-mifepristone, n = 8. Results are presented as the mean ± SEM. The asterisk indicates a significant difference between the vehicle and mifepristone treatments ($P < 0.05$).

interventions. Mifepristone does not simply block progesterone action through its interactions with the PGR but also creates epifunctions not normally ascribed to the PGR.

The phenotypic description of *Pgr* null rats resembles aspects of those previously described for a patient with progesterone resistance and normal menstrual cycles (42, 43). Collectively, these observations suggest that female reproductive cycles are driven by cyclic changes in circulating estradiol and are independent of progesterone signaling. Although the administration of compounds with progestagenic activities can interfere with female reproductive cycles, forming the basis of their use as oral contraceptives (44), endogenous progesterone signaling is not required for cyclicity in the rat, a model organism for mammalian reproduction, opening the question of its involvement in regulating female reproductive cyclicity in other species.

Methods

The University of Kansas Medical Center Animal Care and Use Committee approved all protocols performed. Animals, generation of targeted *Pgr* mutations, phenotypic characterization of wild-type and *Pgr* null rats, hormone measurements, Western blotting, immunohistochemistry, whole-mount mammary gland preparations, and statistical analyses are provided in the *SI Methods*.

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