Hurp Deficiency in Mice Leads to Female Infertility Caused by an Implantation Defect*[S]

Ching-Yen Tsai[‡], **Chen-Kung Chou**[§], **Chu-Wen Yang**[¶],
Yi-Chen Lai[‡], **Chih-Chia Liang**[‡], **Chun-Ming Chen**[‡],
and Ting-Fen Tsai[‡‖1]

From the [‡]*Department of Life Sciences and Institute of Genome Sciences, National Yang-Ming University, Taipei 112, Taiwan, the* [§]*Department of Life Science, Chang Gung University, Tao-Yuan 333, Taiwan, the* [¶]*Department of Microbiology, Soochow University, Taipei 111, Taiwan, and the* [‖]*Division of Molecular and Genomic Medicine, National Health Research Institutes, Zhunan 350, Taiwan*

First revealed in cancer studies, HURP (<u>h</u>epatoma <u>u</u>p-<u>r</u>egulated <u>p</u>rotein) is a cell cycle-associated gene with elevated expression in the G_2/M phase. Cell culture studies have revealed that HURP is an essential factor required for spindle formation and chromosome congression during mitosis. However, the function of HURP in an *in vivo* context has not been explored. We generated a *Hurp* knock-out (*Hurp*$^{-/-}$) mouse to investigate the role of HURP in development under normal physiological conditions. *Hurp*$^{-/-}$ mice develop normally and are indistinguishable from their wild-type littermates. Interestingly, breeding experiments revealed that *Hurp*$^{-/-}$ females are completely infertile, whereas the males reproduce normally. Ovulation, fertilization, and pre-implantation embryo development are normal; however, the *Hurp*$^{-/-}$ females are unable to form implantation sites due to an inability to undergo the decidual reaction. This is caused by a defect in endometrial stromal proliferation that leads to implantation failure. Additionally, HURP expression in the uterus coincides with the implantation stage and can be induced by estrogen treatment. Our results demonstrate for the first time that HURP affects endometrial stromal proliferation during implantation but is dispensable during normal development in mice; specifically, HURP has an essential function in uterine biology.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental "Methods," Figs. S1–S5, Tables 1 and 2, and additional references.

[1] To whom correspondence should be addressed: National Yang-Ming University, 155 Li-Nong St., Sec. 2, Peitou, Taipei 112, Taiwan. Tel.: 886-2-2826-7293; Fax: 886-2-2828-0872; E-mail: tftsai@ym.edu.tw.

First revealed in cancer studies, HURP (<u>h</u>epatoma <u>u</u>p-<u>r</u>egulated <u>p</u>rotein) is a cell cycle-associated gene with elevated expression in the G_2/M phase (1–3). HURP is a microtubule-associated protein and an essential component of the Ran-dependent complex involved in bipolar spindle assembly (4–6). HURP binds to microtubules through its N-terminal domain, and the phosphorylation of HURP by Aurora A regulates the activity and accessibility of HURP to bind to microtubules (7). Specifically, HURP plays a crucial role during chromosome congression, which is the process whereby microtubules attach to the kinetochore of chromosomes and move the chromosomes to the metaphase plate during mitosis; depletion of HURP in cells results in chromosome misalignment at the metaphase plate (8, 9). Although cell culture studies have revealed that HURP is an essential factor required for mitosis, the role of HURP in development under normal physiological conditions has not been explored. In this study, we applied a mouse genetics approach and discovered, serendipitously, that HURP deficiency leads to female infertility caused by implantation failure. Implantation of embryos into the uterus is a sophisticated and important process for viviparous animals. In mice, decidualization, which is characterized by endometrial stromal proliferation and differentiation, is crucial for the establishment of a fetal-maternal interface during implantation (10–12). However, the molecular mechanisms by which cell cycle events govern decidualization are poorly understood. Here, we demonstrated that HURP is an essential cell cycle regulator that is needed for successful stromal proliferation during the process of decidualization to transform the uterine endometrium into a receptive state for blastocyst implantation in mice. Most important, this is a newly discovered molecular influence of HURP on implantation and represents an example whereby a deficiency in a cell cycle-associated gene exhibits phenotypic effects specifically on certain tissues, in this case, the endometrial stroma, but seems to be dispensable in normal cell proliferation during mouse development.

EXPERIMENTAL PROCEDURES

Generation of Hurp Knock-out Mice—An insertion targeting vector was used to disrupt the *Hurp* gene in embryonic stem cells as described under supplemental "Methods" and in Fig. S2*A*.

Estrogen Treatment and Induction of Artificial Decidualization—Ovariectomized females were injected with estrogen (E_2)[2] or an oil (vehicle) control. Mice were killed at 2, 24, and 72 h after injection. For artificial induction of decidual reaction, ovariectomized females were treated with three daily injections of E_2. After 2 days of rest, mice were treated with daily injections of progesterone (P_4) and E_2. Six hours after the P_4 and E_2 injection on day 3, one uterine horn was infused with 30 μl of sesame oil to induce decidualization. The daily injections of P_4

[2] The abbreviations used are: E_2, estrogen; P_4, progesterone; BrdUrd, bromodeoxyuridine; pH3, phosphohistone H3; IHC, immunohistochemistry; RT, reverse transcription.

and E_2 were continued until the day of sampling. Uterine weights of the oil-infused and control horns were measured on day 5 after oil infusion.

Mating, Embryo Collection, and Implantation Sites—Female mice were bred with wild-type fertile males for uterus sampling at different pregnancy stages. Virginal plugs were checked in the morning before 10:00. Sampling of the pregnant uteri was carried out between 11:00 and 13:00. On day 4 of pregnancy (day 1 = vaginal plug), uteri were flushed with M2 medium to recover the pre-implantation blastocysts (13). The collected blastocysts were examined under a dissecting microscope (Leica). Implantation sites on day 6 of pregnancy were visualized by intravenous injection of Chicago Blue B dye (1% in phosphate-buffered saline; 0.1 ml/mouse) through tail veins (14). The number of implantation sites as demarcated by distinct blue bands was then recorded.

Immunohistochemistry, DNA Synthesis, and Mitotic Index—Antibodies against bromodeoxyuridine (BrdUrd; Dako M0744) and phosphohistone H3 (pH3; Upstate 06-570) were used for DNA synthesis and mitotic index analyses. Rabbit anti-mouse HURP polyclonal antibody was generated and used for HURP immunohistochemistry (IHC).

Detailed experimental procedures for RNA analysis, artificial decidualization, and IHC are described in the supplemental "Methods."

RESULTS AND DISCUSSION

Hurp Expression Coincides with Cell Proliferation in Wild-type Mice—Multiple tissues of Northern blot analysis showed that a higher level of *Hurp* mRNA was expressed in the testis, spleen, and thymus. Lower expression of *Hurp* mRNA was detected in the colon, ovary, and small intestine (supplemental Fig. S1A). To study the relationship between *Hurp* expression and cell proliferation in an *in vivo* context, we examine the temporal expression pattern of *Hurp* in the embryonic and postnatal tissues and used the different developmental stages of livers as the example. In the fetal liver, a high expression level of *Hurp* mRNA was sustained until embryonic day 15.5. The signal declined gradually thereafter in the embryonic liver and in the postnatal newborn until expression was barely detectable in the adult liver (supplemental Fig. S1B). Interestingly, the temporal expression pattern of *Hurp* coincided with the expression of the proliferation marker *Pcna* (supplemental Fig. S1B), implying that *Hurp* expression correlates with the ongoing cell proliferation of hepatocytes in mouse liver. Although most hepatocytes are arrested at the quiescent stage in the adult liver, quiescent hepatocytes are able to re-enter the cell cycle during liver regeneration, which is inducible by partial hepatectomy (15, 16). Our data indeed demonstrate that the expression of *Hurp* mRNA and protein can be transiently induced at 2 days post-hepatectomy, which coincides with the G_2/M phase during hepatocyte cell cycle progression, and then the level rapidly decreases to the basal level after 3 days post-hepatectomy (supplemental Fig. S1, C and D).

Normal Development without Overt Phenotype in $Hurp^{-/-}$ Mice—To study the role of *Hurp* involved in development under physiological conditions, we generated *Hurp* knock-out mice using gene targeting technology in embryonic stem cells

(supplemental Fig. S2A). Southern blot and Northern blot analyses demonstrated that the *Hurp* gene was disrupted with undetectable mRNA expression in the homozygous knock-out ($Hurp^{-/-}$) mice (supplemental Fig. S2, B and C). $Hurp^{-/-}$ mice, derived from a $Hurp^{+/-}$ heterozygous cross, were born at the expected Mendelian ratio (supplemental Table 1). Both male and female $Hurp^{-/-}$ mice are phenotypically normal, with no observable changes in morphology, growth curve, and behavior. Previous studies have identified HURP as a component of the mitotic apparatus involved in spindle formation and chromosome congression using different approaches, including proteomics study of HeLa cells (4), biochemical fractionation of microtubule-associated proteins from *Xenopus* eggs (5), and functional genomic screen of mitotic regulators in human tumor cells (6). However, our gene knock-out experiment revealed that HURP is dispensable in normal mouse development from the embryonic stage to adulthood. This could be due to redundancy whereby another yet-to-be identified gene product compensates for the loss of HURP in the knock-out mice.

Hurp Deficiency Leads to Female Infertility in Mice—To detect possible reproductive phenotypes, we performed a 6-month breeding study of $Hurp^{-/-}$ males and females mated with fertile $Hurp^{+/-}$ females and males, respectively. Interestingly, we found that the $Hurp^{-/-}$ females were completely infertile, with no pups at all being born over the 6 months of the breeding period, whereas the $Hurp^{-/-}$ males were fertile, showing no significant difference compared with the fertile $Hurp^{+/-}$ males (supplemental Table 1). To explore the underlying cause of this female infertility and to delineate whether HURP plays a critical role in female reproduction including ovarian and uterine functions, we first analyzed the early pregnancy events. The pre-implantation blastocysts recovered from the wild-type and $Hurp^{-/-}$ females mated with wild-type fertile males were examined. We found that the number of morphologically normal blastocysts recovered from the $Hurp^{-/-}$ females was comparable with that of wild-type females when examined on day 4 of pregnancy (Fig. 1A and supplemental Table 2); this indicated that ovulation, fertilization, and the pre-implantation development of blastocysts were all normal in the $Hurp^{-/-}$ female. In addition, hematoxylin and eosin staining of tissue sections revealed a normal histology of $Hurp^{-/-}$ ovary, which contained different stages of oocytes and corpus luteum similar to those in the normal female mice (supplemental Fig. S3). Together, these results indicate that the complete infertility observed in $Hurp^{-/-}$ females was not due to disruption of ovarian function or an absence of fertilization, but was due to either defective implantation and/or pregnancy failure following implantation. In mice, blastocyst attachment to the uterine luminal epithelium initiates the implantation process and is accompanied by increased endometrial vascular permeability at the implantation sites, which can be visualized by the Chicago Blue B method (14). Using this staining method, we found that, although wild-type females (n = nine mice) had an expected number of implantation sites when examined on day 6 of pregnancy, the uteri of $Hurp^{-/-}$ females (n = 12 mice) showed no sign of implantation (Fig. 1B). This result clearly indicates that the $Hurp^{-/-}$ uterus is
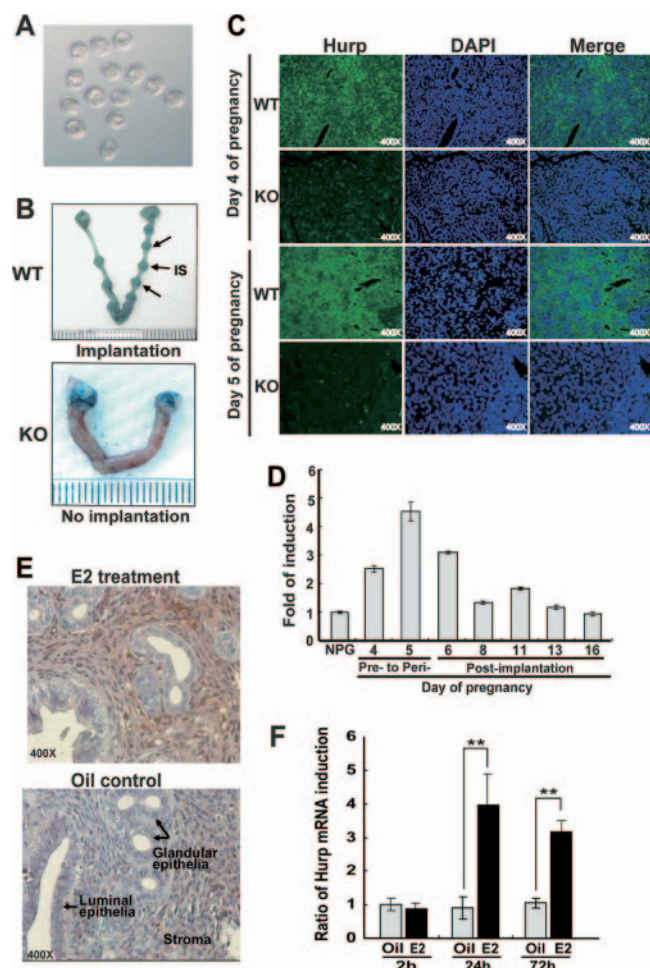
FIGURE 1. *Hurp*$^{-/-}$ **females are completely infertile and unable to form implantation sites (*A* and *B*) and expression of *Hurp* in the pregnant uteri of wild-type mice and ovariectomized mice injected with E$_2$ (*C–F*).** *A*, representative photograph of the morphologically normal blastocysts (embryonic day 3.5) recovered from two *Hurp*$^{-/-}$ female mice on day 4 of pregnancy. *B*, representative photographs of wild-type uteri with implantation sites (*IS*; indicated by *arrows*) and *Hurp*$^{-/-}$ uteri without implantation sites on day 6 of pregnancy (day 1 = vaginal plug). Implantation sites were visualized by intravenous injection of Chicago Blue B dye. The wild-type and *Hurp*$^{-/-}$ females were plugged by fertile wild-type male mice (wild-type females, $n = 9$; and *Hurp*$^{-/-}$ females, $n = 12$). *C*, IHC staining of HURP protein in the uterine sections prepared from wild-type (*WT*) and *Hurp*$^{-/-}$ (knock-out (*KO*)) females on days 4 and 5 of pregnancy. Nuclei were counterstained blue with 4′,6-diamidino-2-phenylindole (*DAPI*). Original magnification × 400. *D*, elevated levels of *Hurp* mRNA detected in the pregnant uteri at the pre- to peri-implantation stages of pregnancy using real-time RT-PCR analyses. There was a peak induction of *Hurp* mRNA expression on day 5 of pregnancy, which decreased thereafter to a basal level at a late pregnant stage. *NPG*, non-pregnant. *E*, representative photomicrographs of IHC staining of HURP protein at 72 h. This was induced in the endometrial stromal cells of the ovariectomized females that had received an intraperitoneal injection of E$_2$. *F*, quantification of *Hurp* mRNA expression in the uteri of the ovariectomized mice that had received intraperitoneal injections of E$_2$ or oil (vehicle) by real-time quantitative RT-PCR analyses. The analyses were carried out at 2, 24, and 72 h after injection. Wild-type C57BL/6 mice were used for the E$_2$ treatment experiments. The amount of total input cDNA was normalized using hypoxanthine-guanine phosphoribosyltransferase as an internal control. Three mice per group were used for these experiments. **, $p < 0.005$.

defective with respect to blastocyst implantation and implies an essential role for the HURP protein specifically in uterine receptivity.

Hurp Expression in the Endometrial Stroma Coincides with the Implantation Stage and Can Be Induced by Estrogen

Treatment—The crucial role of HURP in implantation was reinforced by studying the expression pattern of HURP in the pregnant uteri of normal mice. This expression pattern coincides with an increased stromal cell proliferation on days 4 and 5 of pregnancy (described below in Fig. 2), and this also coincides with the pre- to peri-implantation stages initiated by blastocyst attachment to the uterine luminal epithelium. In addition, IHC staining of HURP protein using a rabbit polyclonal antibody revealed the presence of strong expression of HURP in the stromal cells of the uterine endometrium (Fig. 1*C*). To study the temporal expression of *Hurp* mRNA, we performed real-time reverse transcription (RT)-PCR analyses using total RNA isolated from non-pregnant and pregnant uteri of wild-type mice at various stages of pregnancy. Our results showed that *Hurp* mRNA was induced on day 4 of pregnancy, and peak induction of *Hurp* mRNA expression was detected on day 5 of pregnancy; it decreased thereafter to a basal level during the late pregnant stage (Fig. 1*D*). The specificity of the antibodies against mouse HURP protein was confirmed by Western blotting using cellular extracts of regenerating livers (supplemental Fig. S1*D*). Moreover, our IHC staining of the uterine sections demonstrated the absence of HURP protein in the *Hurp*$^{-/-}$ uteri (Fig. 1*C*). To elucidate whether HURP can be induced by ovarian steroids such as E$_2$, which is a mitogen for the uterine endometrium during implantation, we performed ovariectomy and E$_2$ treatment experiments on wild-type adult mice. Uterine tissues were obtained from the ovariectomized mice that had received intraperitoneal injections of E$_2$ or oil (vehicle) for 2, 24, and 72 h. We found that at 24 and 72 h following the injection of E$_2$ into these mice, both protein and mRNA expression of *Hurp* was significantly induced in the endometrial stroma as detected by IHC staining and real-time RT-PCR, respectively (Fig. 1, *E* and *F*). Quantification revealed that there were 4- and 3-fold increases in the *Hurp* mRNA levels at 24 and 72 h after E$_2$ treatment, respectively (Fig. 1*F*). This suggests that *Hurp* is an E$_2$-responsive gene, the induction of which is regulated by the E$_2$ signaling pathway in the stromal cells. Alternatively, this induction could be due to the mitogenic action of E$_2$ on stromal proliferation, with up-regulation of *Hurp* expression being a consequence of cell cycle progression.

Defective Decidualization of the Hurp$^{-/-}$ *Uterus during Implantation*—Decidualization is a process of stromal transformation that is characterized by endometrial stromal proliferation and differentiation to form morphologically and functionally distinct cells. This process is critical for the establishment of successful pregnancy (10). To determine whether the infertility of *Hurp*$^{-/-}$ females is caused by impaired decidualization of the uterine stroma, we performed artificial induction of the decidual reaction (17). Ovariectomized wild-type and *Hurp*$^{-/-}$ females were first treated with E$_2$ and P$_4$. A decidualization reaction was then induced in one of the two uterine horns by an intraluminal infusion of oil, and the other horn was left unstimulated as a control. Uterine weight and gross morphology of the stimulated and control uterine horns were examined on day 5 after oil infusion. As shown in Fig. 2*A*, a robust reaction of decidualization was observed in the wild-type female. In contrast, the *Hurp*$^{-/-}$ uteri failed to show any significant induction of decidualization upon identical
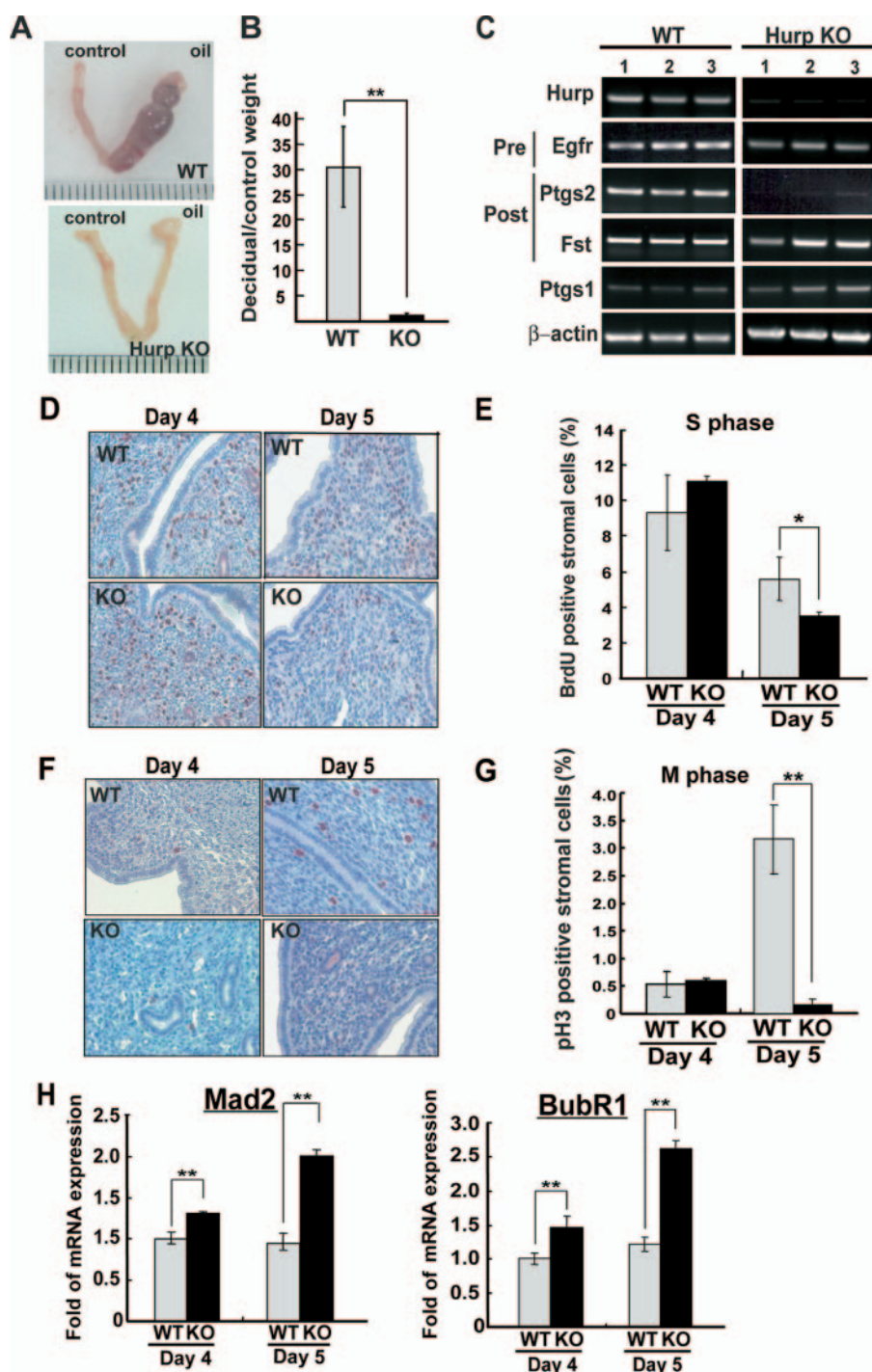
FIGURE 2. **Defective decidualization of the *Hurp*$^{-/-}$ uteri due to impaired cell proliferation of the endometrial stroma during implantation.** *A*, representative photographs of gross morphology of the decidual response in the wild-type (*WT*) and Hurp$^{-/-}$ (knock-out (*KO*)) uteri 5 days after the decidual stimulus. *B*, ratios of the weights of the decidual horn to the control horn measured on day 5 after the decidual stimulus (wild-type females, $n = 4$; *Hurp*$^{-/-}$ females, $n = 6$). **, $p < 0.005$. *C*, RT-PCR analyses of genes associated with pre- and post-implantation stages of pregnancy. Total RNA samples isolated from uteri 5 days after the decidual stimulus were used for RT-PCR analyses. *D*, representative photomicrographs of the S phase cells as monitored by BrdUrd incorporation into the replicating DNA molecules. The uterine sections were prepared from wild-type and *Hurp*$^{-/-}$ females on days 4 and 5 of pregnancy. Original magnification × 400. *E*, quantification of BrdUrd (*BrdU*)-positive cells. About 1000 stromal cells in random fields of BrdUrd-staining slides were examined for the presence of BrdUrd-positive cells for each mouse. *F*, representative photomicrographs of the mitotic cells as monitored by pH3 staining of the pregnant uteri prepared from wild-type and *Hurp*$^{-/-}$ mice on days 4 and 5 of pregnancy. Original magnification × 400. *G*, quantification of pH3-positive mitotic cells. About 1000 stromal cells in random fields of uterine sections were examined for the presence of pH3-positive cells for each mouse. The mean for each time point is expressed as a percentage of total stromal cells counted and is shown as the mean ± S.D. *H*, quantification of the mRNA levels of *Mad2* and *BubR1* using real-time quantitative RT-PCR. The amount of total input cDNA was normalized using hypoxanthine-guanine phosphoribosyltransferase as an internal reference. Three to five mice per group were used for these experiments. *, $p < 0.05$; **, $p < 0.005$.

stimulation. The decidual reaction was quantified by measuring the ratio of the weight of the stimulated uterine horn to the control horn. Our results clearly indicate that the stimulated horn of the *Hurp*$^{-/-}$ uteri failed to increase in size compared with wild-type uteri (Fig. 2, *A* and *B*). To elucidate the decidualization defect in the *Hurp*$^{-/-}$ females, we studied the differential expression of genes crucial for the pre-implantation and post-implantation stages (10, 18). Total RNA samples isolated from the stimulated uterine horns of wild-type and *Hurp*$^{-/-}$ females were subjected to RT-PCR analysis. This RT-PCR experiment revealed that gene expression related to the pre-implantation stage of uterine function, such as the *Egfr* gene, showed no obvious differences. However, for the *Hurp*$^{-/-}$ uteri, there was a significant decrease in gene expression crucial to the peri- and post-implantation stages, such as the *Ptgs2* gene (Fig. 2*C*). To further investigate the defective gene induction during decidualization, we performed an Affymetrix microarray analysis. Three independent microarray experiments were carried out using three RNA samples isolated from three individual mice of each group to obtain reproducible data. This analysis revealed significant differences between the wild-type and *Hurp*$^{-/-}$ uteri. In terms of cell cycle-associated gene expression, in the *Hurp*$^{-/-}$ uteri, a dramatic decrease in various G_2/M markers such as *Ccnb1* (cyclin B_1) and *Ccnb2* (cyclin B_2) was noted, but this did not occur with G_1 and S phase markers (supplemental Fig. S4*A*). This suggests cell cycle progress through the G_1 and S phases but the presence of an impairment at the G_2/M phase. In addition, gene products important to implantation and decidualization, such as the prostaglandin biosynthesis enzymes (19, 20) and decidual prolactins (21, 22), were significantly decreased or absent in the *Hurp*$^{-/-}$ uteri (supplemental Fig. S4, *B* and *C*). Together, the results of the artificial

decidualization experiments indicate that $Hurp^{-/-}$ females are unable to form implantation sites due to an inability to undergo the decidual reaction, and the later is likely to be due to a defect associated with cell proliferation, which leads to blockage of the further differentiation and development of the endometrium that supports embryo implantation.

Impaired Stromal Proliferation Leads to Failure of the Decidual Reaction in the $Hurp^{-/-}$ Uteri—Because *Hurp* was originally identified as a cell cycle-regulated gene, the expression of which is up-regulated during the G_2/M phase, we speculated that endometrial stromal proliferation may be affected in the $Hurp^{-/-}$ uteri and that this leads to a failure of the decidualization. To further illustrate the defect in stromal proliferation, we monitored the S and M phase cells by BrdUrd incorporation into the replicating DNA and IHC staining of pH3, respectively, using uterine samples prepared from wild-type and $Hurp^{-/-}$ females naturally mated with wild-type males. On day 4 of pregnancy, there was a comparably high percentage of S phase cells detected in the stromata of the $Hurp^{-/-}$ and wild-type uteri; only a few M phase cells were present in these uterine samples (Fig. 2, *D–G*). On day 5 of pregnancy, an elevated percentage of M phase cells was detected in the wild-type stroma. However, there was a significantly decreased percentage or near absence of M phase stromal cells detected in the $Hurp^{-/-}$ uteri (Fig. 2, *D–G*), which indicates that there was an impairment of cell cycle progression and that this would seem to caused by a block before the mitotic stage. This blockage further impaired cell progression into the next cell cycle as illustrated by the decreased S phase cells in the $Hurp^{-/-}$ stroma compared with the wild-type control on day 5 of pregnancy (Fig. 2, *E* and *G*). Thus, the *Hurp* deficiency causes a pre-mitotic block leading to impaired cell cycle progression of the endometrial stroma in the pregnant uteri. Previous cell culture studies revealed that HURP depletion leads to chromosome misalignment at the metaphase plate (4–6). In our mouse study, we have never seen stromal cells with visible condensed chromosomes in the uterine samples of *Hurp* knock-out mice; this may be attributable to cell cycle arrest at the pre-mitotic phase. However, we did find that HURP protein co-localized with condensed chromosomes in the wild-type uterine samples (supplemental Fig. S5). This observation is consistent with the role of HURP being involved in stabilizing and targeting K-fibers to the kinetochore of chromosomes (8, 9).

To further determine whether the mitotic spindle checkpoint is activated in the $Hurp^{-/-}$ stroma rendering the cell cycle arrest, we examined the expression levels of *Mad2* and *BubR1*, which are key components of the mitotic checkpoint complex (23, 24). As expected, significantly higher levels of *Mad2* and *BubR1* were detected in the $Hurp^{-/-}$ uteri compared with wild-type controls; this was revealed as 2.1- and 2.2-fold increases in the mRNA levels of *Mad2* and *BubR1*, respectively, on day 5 of pregnancy (Fig. 2*H*). These results indicate that, in the absence of HURP, checkpoint signaling is activated possibly because of lack of a functional mitotic apparatus in the stromal cells and that this causes an arrest of cell cycle progression; this activation of the mitotic checkpoint phenom-enon is similar to that observed previously in HURP-depleted HeLa cells (6).

In this study, we demonstrated that *Hurp* deficiency in mice leads to female infertility caused by impaired cell cycle progression of the endometrial stroma, which is arrested at the pre-mitotic phase. The lack of completion of stromal cell proliferation at an early stage of decidualization thus abrogates the latter events of stromal differentiation that follow the proliferation. This knock-out mouse model thus provides a useful system that allows the study of the *in vivo* effects of HURP on uterine physiology and the genetic pathways of HURP-mediated regulation of endometrial proliferation. This could be relevant to humans. We anticipate that a more thorough understanding of the molecular mechanisms that regulate uterine receptivity and implantation is of clinical relevance to the improvement of infertility-related medical treatments and may lead to the development of a novel contraceptive method.

REFERENCES

1. Bassal, S., Nomura, N., Venter, D., Brand, K., McKay, M. J., and van der Spek, P. J. (2001) *Genomics* **77,** 5–7
2. Tsou, A. P., Yang, C.-W., Huang, C. Y., Yu, R. C., Lee, Y. C., Chang, C. W., Chen, B. R., Chung, Y. F., Fann, M. J., Chi, C. W., Chin, J. H., and Chou, C. K. (2003) *Oncogene* **22,** 298–307
3. Yu, C. T., Hsu, J. M., Lee, Y. C., Tsou, A. P., Chou, C.-K., and Huang, C. Y. (2005) *Mol. Cell. Biol.* **25,** 5789–5800
4. Silljé, H. H., Nagel, S., Körner, R., and Nigg, E. A. (2006) *Curr. Biol.* **16,** 731–742
5. Koffa, M. D., Casanova, C. M., Santarella, R., Köcher, T., Wilm, M., and Mattaj, I. W. (2006) *Curr. Biol.* **16,** 743–775
6. Wong, J., and Fang, G. (2006) *J. Cell Biol.* **173,** 879–891
7. Wong, J., Lerrigo, R., Jang, C.-Y., and Fang, G. (2008) *Mol. Biol. Cell* **19,** 2083–2091
8. Sanderson, H. S., and Clarke, P. R. (2006) *Curr. Biol.* **16,** R466–R468
9. Wilde, A. (2006) *J. Cell Biol.* **173,** 829–831
10. Dey, S. K., Lim, H., Das, S. K., Reese, J., Paria, B. C., Daikoku, T., and Wang, H. (2004) *Endocr. Rev.* **25,** 341–373
11. Wang, H., and Dey, S. K. (2006) *Nat. Rev. Genet.* **7,** 185–199
12. Lee, K. Y., Jeong, J. W., Tsai, S. Y., Lydon, J. P., and DeMayo, F. J. (2007) *Trends Endocrinol. Metab.* **18,** 234–239
13. Hogan, B., Beddington, R., Costantini, F., and Lacy, E. (1994) *Manipulating the Mouse Embryo: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
14. Tranguch, S., Cheung-Flynn, J., Daikoku, T., Prapapanich, V., Cox, M. B., Xie, H., Wang, H., Das, S. K., Smith, D. F., and Dey, S. K. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 14326–14331
15. Greene, A. K., and Puder, M. (2003) *J. Investig. Surg.* **16,** 99–102
16. Fausto, N., Campbell, J. S., and Riehle, K. J. (2006) *Hepatology* **43,** S45–S53
17. Lee, K. Y., Jeong, J. W., Wang, J., Ma, L., Martin, J. F., Tsai, S. Y., Lydon, J. P., and DeMayo, F. J. (2007) *Mol. Cell. Biol.* **27,** 5468–5478
18. Hewitt, S. C., Deroo, B. J., Hansen, K., Collins, J., Grissom, S., Afshari, C. A., and Korach, K. S. (2003) *Mol. Endocrinol.* **17,** 2070–2083
19. Lim, H., Paria, B. C., Das, S. K., Dinchuk, J. E., Langenbach, R., Trzaskos, J. M., and Dey, S. K. (1997) *Cell* **91,** 197–208
20. Matsumoto, H., Ma, W. G., Daikoku, T., Zhao, X., Paria, B. C., Das, S. K., Trzaskos, J. M., and Dey, S. K. (2002) *J. Biol. Chem.* **277,** 29260–29267
21. Bole-Feysot, C., Goffin, V., Edery, M., Binart, N., and Kelly, P. A. (1998) *Endocr. Rev.* **19,** 225–268
22. Bao, L., Tessier, C., Prigent-Tessier, A., Li, F., Buzzio, O. L., Callegari, E. A., Horseman, N. D., and Gibori, G. (2007) *Endocrinology* **148,** 2326–2334
23. Yu, H. (2006) *J. Cell Biol.* **173,** 153–157
24. Chen, R. H. (2007) *J. Biomed. Sci.* **14,** 475–479