Hepatoma up-regulated protein, HURP, first revealed in cancer studies, is a cell-cycle associated gene with elevated expression in the G2/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 knockout (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 while 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 stroma 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 stroma proliferation during implantation but is dispensable during normal development in mice; specifically Hurp has an essential function in uterine biology.

Hepatoma up-regulated protein, HURP, first revealed in cancer studies, is a cell-cycle associated gene with elevated expression in the G2/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 stroma 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 stroma proliferation during the process of decidualization in order to transform the uterine endometrium into a receptive state for blastocyst implantation in mice. Most importantly, 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 to normal cell proliferation during mouse development.

EXPERIMENTAL PROCEDURES

Generation of Hurp knockout mice—An insertion targeting vector was used to disrupt the Hurp gene in ES cells as described in supplemental Fig. S2A and methods.

Estrogen (E2) treatment and induction of artificial decidualization—Ovariectomized females were injected with E2 or an oil (vehicle) control. Mice were sacrificed at 2h, 24h and 72h after injection. For artificial induction of decidual reaction, ovariectomized females were treated with 3 daily injections of E2. After two days of rest, mice were treated with daily injections of progesterone (P4) and E2. Six hours after the P4 and E2 injection on the third day, one uterine horn was infused with 30μl of sesame oil to induce decidualization. The daily injections of P4 and E2 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 11:00-13:00. 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 an i.v. injection of Chicago Blue B dye (1% in PBS; 0.1 ml per mouse) through tail vein (14). The number of implantation sites as demarcated by distinct blue bands was then recorded.

**Immunohistochemistry (IHC), DNA synthesis and mitotic index**—Antibodies against BrdU (DAKO M0744) and phospho-histone 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 IHC.

Detail experimental procedures of RNA analysis, artificial decidualization and IHC are described in the supplemental information.

**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 (E15.5). 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 the Hurp coincided with the expression of the proliferation marker Pena (supplemental Fig. S1B), implying that Hurp expression was correlated 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 demonstrated that the expression of Hurp mRNA and protein can be transiently induced at 2 days post-hepatectomy, which coincides with the G2/M phase during hepatocyte cell cycle progression, and then the level rapidly decreased to basal level after 3-day post-hepatectomy (supplemental Fig. S1C, D).

**Normal development without overt phenotype in the Hurp+/− mice**—To study the role of Hurp involved in development under physiological conditions, we generated Hurp knockout mice using gene targeting technology in ES cells (supplemental Fig. S2A). Southern blot and Northern blot analyses demonstrated that the Hurp gene was disrupted with undetectable mRNA expression in the homozygous knockout (Hurp−/−) mice (supplemental Fig. S2B, C). Hurp−/− mice, derived from a Hurp+/− heterozygous cross, were born at the expected Mendelian ratio (supplemental Table 1). Both males and females of the 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 MAPs from Xenopus eggs (5), and functional genomic screen of mitotic regulators in human tumor cells (6). However, our gene knockout experiment revealed that Hurp is dispensable to 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 knockout 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; while 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 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 to 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, H&E staining of tissue section revealed a normal histology of Hurp–/– ovary, which contained different stages of oocytes and corpus luteum similar to that 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 Chicago Blue B method (14). Using this staining method we found that, although wild-type females (n = 9 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 indicated that the Hurp–/– uterus is defective with respect to blastocyst implantation and implying an essential role for the Hurp protein specifically in uterine receptivity.

Hurst 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 uterus of normal mice. This expression pattern coincides with an increased stromal cell proliferation on days 4 and 5 of pregnancy (described later 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, immuno-histochemistry (IHC) staining of Hurp protein using a rabbit polyclonal antibody revealed the presence of strong expression of Hurp in the stromal cells of the uterus endometrium (Fig. 1C). To study the temporal expression of Hurp mRNA, we performed real-time 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 late pregnant stage (Fig. 1D). The specificity of the antibodies against mouse Hurp protein was confirmed by Western blotting using cellular extracts of regenerating livers (supplemental Fig. S1D). Moreover, our IHC staining of the uterine sections demonstrated the absence of Hurp protein in the Hurp–/– uteri (Fig. 1C). To elucidate whether Hurp can be induced by ovarian steroids such as estrogen (E2), which is a mitogen for uterine endometrium during implantation, we performed ovariectomy and E2 treatment experiments on wild-type adult mice. Uterine tissues were obtained from the ovariectomized mice that had received i.p. injection of E2 or oil (vehicle) for 2h, 24h and 72h. We found that at 24h and 72h following the injection of E2 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. 1E, F). Quantification revealed that there was a 4-fold and 3-fold increase in the Hurp mRNA level at 24h and 72h after E2 treatment, respectively (Fig. 1F). This suggests that Hurp is an estrogen responsive gene, the induction of which is regulated by the estrogen signaling pathway in the stroma cells. Alternatively, this induction could be due to the mitogenic action of estrogen on stroma 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 stroma 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 uterine stroma, we performed artificial induction of the decidual reaction (17). Ovariectomized wild-type and Hurp–/– females were first treated with estrogen (E2) and progesterone (P4). Then a decidualization reaction was induced in one of the two uterine horns by an intraluminal infusion of oil; while the other horn was left un-stimulated 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. 2A, a robust reaction of decidua lization was observed in the wild-type female. In contrast, the Hurp–/– uteri failed to show any significant induction of decidualization under identical stimulation. Quantification of the decidual reaction was by measuring the ratio of the weight of the stimulated uterine horn to the control horn. Our results clearly indicated that the stimulated horn of the Hurp–/– uteri failed to increase in size compared with wild-type uteri (Fig. 2A, 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. 2C). 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 in order 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 G2/M markers such as Ccnb1 (cyclin B1) and Ccnb2 (cyclin B2) was noted, but this did not occur with G1 and S phase markers (supplemental Fig. S4A). This suggests that cell cycle progress through G1 and S phases but the presence of an impairment at G2/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. S4B, C). Together, the results of the artificial decidualization experiments indicated 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 stroma proliferation leads to failure of the decidual reaction in the Hurp−/− uteri**—Since Hurp was originally identified as a cell cycle regulated gene, the expression of which is up-regulated during G2/M phase, we speculated that endometrial stroma proliferation may be affected in the Hurp−/− uteri and that this leads to a failure of the decidualization. To further illustrate the defect in stroma proliferation, we monitored the S-phase and M-phase cells by BrdU 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 were a comparably high percentage of S-phase cells detected in the stroma of the Hurp−/− and wild-type uteri; only a few M-phase cells were present in these uterine samples (Fig. 2D-G). On day 5 of pregnancy, an elevated percentage of M-phase cells were detected in the wild-type stroma. However, there was a significantly decreased percentage or near absent of M-phase stromal cells detected in the Hurp−/− uteri (Fig. 2D-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 wild-type control on day 5 of pregnancy (Fig. 2E, G). Thus, the Hurp deficiency causes a pre-mitotic block leading to impaired cell cycle progression of 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 knockout mice; this may be attributable to cell cycle arrest at the pre-mitotic phase. However, we did find that Hurp protein co-localizes 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 the Mad2 and BubR1 were detected in the Hurp−/− uteri compared to wild-type controls; this was revealed as a 2.1-fold and 2.2-fold increase in the mRNA levels of Mad2 and BubR1, respectively, on day 5 of pregnancy (Fig. 2H). 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 this causes an arrest of cell cycle progression; this activation of the mitotic checkpoint phenomenon is similar to that observed previously in the 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 stroma differentiation that follow the proliferation. This knockout 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 novel contraceptive method.
FOOTNOTES

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REFERENCES


FIGURE LEGENDS

Fig 1. Hurp^{−/−} females are completely infertile and unable to form implantation sites (A, B); expression of Hurp in the pregnant uteri of wild-type mice, and ovarioectomized mice injected with E2 (C-F). A, Representative photograph of the morphologically normal blastocysts (embryonic stage E3.5) 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 IS on day 6 of pregnancy (day 1 = vaginal plug). Implantation sites were visualized by an i.v. 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; Hurp^{−/−} females, n = 12. C, IHC staining of Hurp protein in the uterine sections prepared from wild-type (WT) and Hurp^{−/−}
(KO) females on day 4 and day 5 of pregnancy. Nuclei were counter stained blue with DAPI. Original magnification, 400X. D, Elevated levels of Hurp mRNA were detected in the pregnant uteri at the pre- to peri-implantation stages of pregnancy using real-time RT-PCR analyses. There is a peak induction of Hurp mRNA expression on day 5 of pregnancy and decreased thereafter to a basal level at late pregnant stage. NPG, non-pregnant E, Representative photomicrographs of IHC staining of Hurp protein at 72h; this was induced in the endometrial stromal cells of the ovariectomized females who had received an i.p. injection of E2. F, Quantification of Hurp mRNA expression in the uteri of the ovariectomized mice who had received i.p. injections of E2 or oil (vehicle) by real-time quantitative RT-PCR analyses. The analyses were carried out at 2h, 24h and 72 h after injection. Wild-type C57BL/6 mice were used for the E2 treatment experiments. The amount of total input cDNA was normalized using Hprt as an internal control. Three mice per group were used for these experiments. **p<0.005.

Fig 2. Defective decidualization of the Hurp<sup>−/−</sup> 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<sup>−/−</sup> (KO) uteri 5 days after the decidual stimulus. B, Ratios of weights of the decidual horn to the control horn measured on day 5 after the decidual stimulus. Wild-type females, n = 4; Hurp<sup>−/−</sup> 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 BrdU incorporation into the replicating DNA molecules. The uterine sections were prepared from wild-type and Hurp<sup>−/−</sup> females on day 4 and day 5 of pregnancy. Original magnification, 400X. E, Quantification of BrdU positive cells. About 1000 stromal cells in random fields of BrdU staining slides were examined for the presence of BrdU positive cells for each mouse. F, Representative photomicrographs of the mitotic cells as monitored by phospho-histone H3 (pH3) staining of the pregnant uteri prepared from wild-type and Hurp<sup>−/−</sup> mice on day 4 and day 5 of pregnancy. Original magnification, 400X. 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 are 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 Hprt as an internal reference. Three to five mice per group were used for these experiments. *p<0.05; **p<0.005.
HUPR deficiency in mice leads to female infertility caused by an implantation defect
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