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*Original Article*

# Energy supplementation rescues growth restriction and female infertility of mice with hepatic HRD1 ablation

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**Abstract:** Severe dietary restriction, catabolic states and even short-term caloric deprivation impair fertility in mammals including human, which is often reversible by restoration of the energy supplementation. The dysregulated crosstalk among multiple organs is possibly involved in this process. However, ideal experimental animal models are needed to illuminate functional crosstalk among distal organs during the starvation pathogenesis. We have recently discovered that conditional hepatic HRD1 gene deletion results in elevated energy expenditure and consequently leads to growth retardation and female fertility. Herein, we discovered that both growth retardation and female infertility of liver-specific HRD1 knockout mice could be fully rescued by additional energy supplementation upon HFD feeding. Hepatic HRD1 deletion appears to impair by the pituitary gland functions in secreting critical hormones in growth and female fertility including growth hormone (GH), follicle-stimulating hormone (FSH) and luteinizinghormone (LH) because a dramatic reduction in the sera levels of all three hormones were detected in liver HRD1 KO mice, which consequently shortened their tibia lengths and impaired the ovary functions in females. HFD feeding for six weeks largely restored all three hormones in liver HRD1 KO mice back to levels comparable with those in WT mice. In addition, the growth hormone induced activation of JAK-STAT5 pathway was inhibited by HRD1 deletion, and additional energy supplementation upon HFD feeding restored STAT5 transcriptional activation. Our studies establish a unique mouse model to study liver crosstalk with distal organs in regulating energy balance in growth and female fertility.

**Keywords:** HRD1, energy balance, growth retardation, female fertility & HFD

## Introduction

Energy balance makes an important role in all aspects in life including growth, body temperature and reproduction. When energy is scarce, partition energy favors the processes to ensure the survival of the individuals over those promoting growth and reproduction. In any situations when energy expenditure exceeding energy intake, energy prior spent on life-sustaining requirements to maintain body temperature. Facing survival difficult position, growth and reproduction inevitably becomes to a subordinate consideration of energy allocation [1, 2]. On the other hand, in addition to its association with type II diabetes, coronary heart diseases and cancer, obseity clearly increases women's risk of fertility impairment [3-5]. In females, the downregulation of the hypothalamic-pituitary-ovarian (HPO) axis, which consequently affects gonadotropin production, menstrual abnormalities and ovulation dysfunction, has been considered as the major reason for the negative energy balance induced infertility [6, 7]. While being extensively studied, the cellular and molecular mechanisms underlying how the imbalanced energy expenditure retards body growth and impair reproduction remain partially understood.

HMG-CoA reductase degradation protein 1 (HRD1), which is a class of E3 ubiquitin ligase, regulates rate-limiting enzyme HMGCR (HMG-CoA reductase) turnover in yeast [8]. Initially studies demonstrated that HRD1 plays a critical role in endoplasmic reticulum associated degradation (ERAD) system, which is required to destruct the misfolded or unfolded proteins

to rescue cells from ER stress response [9-11]. We have recently demonstrated that HRD1-ERAD serves as a key member to control the metabolic balance and energy expenditures through direct binding and catalyzing the ubiquitination of several metabolic enzymes in liver, a crucial organ for regulating energy balance in the body [12-14].

In this study, we demonstrated that the liver specific HRD1 gene deletion impairs the critical functions of the distal organ pituitary gland functions in secreting critical hormones GH, FSH and LH, and consequently leads to in growth retardation and female infertility. These defect of HRD1 LKO mice are likely due to the elevated energy expenditure, which presumably starves pituitary gland, because energy supplementation by feeding the mice with HFD fully rescued them from growth retardation and infertility.

## Materials and methods

### *Mice*

The liver specific HRD1 gene knockout (HRD1 LKO, $HRD1^{flox/flox}$-ALB-$Cre^{+}$) mice were used as previously described [15], the $HRD1^{flox/flox}$ littermates were used as WT controls. All mice were at the C57/BL6 genetic background and bred in a pathogen-free facility. All animal experiments were approved by the Institutional Animal Care and Use Committees (IACUC) at Northwestern University. Measurements of food intake, activity, and water intake were taken using a Comprehensive Lab Animal Monitoring System from the Northwestern University. Animals were maintained on a standard chow diet under 12-h light and dark cycles beginning at 5:00 a.m. and 5:00 p.m., respectively. The high-fat diet (45% kcal fat) was purchased from the Research Diet Inc (New Brunswick, NJ).

### *Real-time qPCR*

WT and HRD1 LKO mice were fasted overnight. WT and HRD1 LKO mice were fed with normal chew or with HFD for 6 weeks from the age of 6 weeks, RNA was isolated from their ovarian tissues collected from with TRIzol reagent cDNA was synthesized using the qScript cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD). iQ5 and SYBRGreen Detection system (Bio-Rad, Hercules, CA) were used for quantitative PCR (qPCR). qPCR was run on a Bio-Rad IQ2 PCR machine, and each PCR mixture contained 40 ng of cDNA template and 10 nM primers in 15 μl of SYBR Green reaction mix (Bio-Rad). The Expression values were normalized to those that were obtained with the control *actb* (encoding beta-actin). All the R2 (coefficient of determination) of the reaction with specific primers are > 0.99 and the efficiency of the PCR reaction in the range 100% ± 10% is as shown in the protocol from Thermo Fisher Scientific. Changes in gene expression levels were calculated by the 2-ΔΔCt method.

The primers were, for genotype of HRD1 knockout mice 5'-CTTCTGTGCAGCTGGTATTTGG-3' and 5'-ACACGTCTCCTGGGTGATCTAC-3'; for *Actin* 5'-ACGCAGCTCAGTAACAGTCC-3' and 5'-AGATCAAGATCATTGCTCCTCCT-3'; for *Hsd3b5* 5'-CACAGCAGCTGAGTCACAACAG-3' and 5'-AGTCCTAAGCACTTGCCCAGTAAT-3', *Cyp2d9* 5'-AGTCTCTGGCTTAATTCCTGAGGTT-3' and 5'-CGCAAGAGTATCGGGAATGC-3', *Cyp7b1* 5'-TGAGGTTCTGAGGCTGTGCTC-3' and 5'-TCCTGCACTTCTCGGATGATG-3' *and Cyp4a12* 5'-GCACAATCTCTTTTTTCTCCGTGTG-3' and 5'-GCAGGCACTGTTGGCCAA-3'; for *Bax* 5'-AAACTGGTGCTCAAGGCCC-3' and 5'-CTTGGATCCAGACAAGCAGC-3'; for *Bcl2* 5'-CACCCCTGGTGGACAACATC-3' and 5'-ATAGTTCCACAAAGGCATCCCAG-3'; for *Fas* 5'-TGCCAACCTGAAAACTAGGCT-3' and 5'-CCACCCCCTTCTCCCAATTC-3'. *Mup1* 5'-GAC TTT TTC TGG AGC AAA TCC ATG, *Mup1* 3'-GAG CAC TCT TCA TCT CTT ACA G. *ELOVL3* 5'-GGA CAG AGG CAC ACA CAA ACA, *ELOVL3* 5'-GCG CCT ACC AGG CCT AGA AT.

### *Ovarian histology*

The ovaries were cleaned by removing fat tissues after castrate, fixed in 10% formalin overnight and embedded in paraffin. Ovary tissues from mice were cut into sections and stained with hematoxylin and eosin (H&E) [16]. Corpus luteum (CL), is the follicle after ovulation which represent fertilizing capacity.

### *Estrous cycle analysis*

Estrus stages were determined by vaginal cytology. Mice vaginas were flushed with PBS and cells were transferred to slide, and stained with 0.1% crystal violet for 1 min after air dry. The slides were then washed with water for 1 min and dried.

The estrus cycle includes four stages, proestrus, estrus, metestrus and diestrus. Proestrus stage contains nucleated cells and leukocytes. The estrus stage is characterized by cornified cell alone. The predominant feature of metestrus stage is lightly stained cornified cells, with some leukocytes and nucleated cells. Diestrus is characterized by leukocytes, along with some nucleated and cornified cells [17].

*Hormone determinations*

Blood samples were obtained on days of diestrus at 5:00 p.m. and stored at -80°C until use. GH, IGF-1, FSH and LH were analyzed with ELISA kits (MyBioSource and Abcam, USA).

*Statistical analyses*

Statistical analysis was performed using the two-way, unpaired Student's *t* test, and $P < 0.05$ was considered significant. Data are expressed as mean ± standard derivation (SD).

## Results

*HFD reverses the growth retardation of hepatic HRD1 deficient mice*

While the body sizes were indistinguishable after birth, we have consistently observed that HRD1 deletion in liver leads to growth retardation from the age of 6 weeks [14]. As indicated in **Figure 1**, the body size of HRD1 LKO mice was markedly smaller than their wild-type littermate controls when fed with normal chew at the age of 12 weeks. The average body height and body weight of L-HRD KO was significantly decreased compared with that of WT mice (10.15 ± 1.62 cm *vs* 8.13 ± 0.79 cm; 19.94 ± 1.87 g *vs* 16.5 ± 2.69 g; $P < 0.05$) at the age of 12 weeks fed a chow diet (**Figure 1A-C**). Notably, high-fat-diet (HFD) largely reversed this growth retardation phenotype induced by hepatic HRD1 deletion as the body heights of HRD1 LKO mice were comparable 6 weeks after HFD compare to that of WT mice (**Figure 1A**, **1B**). While the body weight of HRD1 LKO mice on HFD are still lower than that of HFD fed WT control mice (**Figure 1C**), their body hights were comparable with WT mice under chew feeding (**Figure 1B**). Consistent with our observation that HFD largely rescue HRD1 LKO body heights, the HRD1 LKO mice had a similar average tibia length compared with the WT controls either with chew or with HFD feeding (**Figure 1D**, **1E**). These results indicate that conditional HRD1 knockout specifically in liver causes growth restriction in mice, which can be rescued, at least partially by energy supplements via HFD.

*HFD restores growth hormonal dysregulation in HRD1-deficient mice*

The growth retardation is often due to the reduction in the levels of growth hormone (GH), a proteohormone secreted by the pituitary, which is essential for longitudinal and long bones growth [18]. To elucidate the mechanisms underlying how the liver specific HRD1 gene deletion causes mouse growth retardation, we first analyzed their serum GH levels. Indeed, a dramatic reduction in the serum GH levels in HRD1 LKO mice compared to those in WT littermates fed with chew diet (CWT 35.73 ± 2.64 pg/ml; CKO 13.26 ± 3.07 pg/ml; $P < 0.01$), indicating that the liver specific HRD1 suppression impairs the pituitary functions at least for GH production and this GH reduction is likely responsible for the growth retardation of HRD1 LKO mice. To support our initial observation that HFD largely rescued HRD1 LKO mice from growth retardation, HFD feeding resumed GH production in HRD1 LKO mice (HWT 36.5 ± 2.26 *vs* HKO 31.36 ± 5.31 pg/ml) (**Figure 2A**). It has been well established that GH promotes mammal growth predominantly through its downstream mediator Insulinlike growth-factor 1 (IGF-1), which is produced by hepatocytes and consequently stimulates osteoblast and chondrocyte activity to promote bone growth [19, 20]. Of note, a more than 80% reduction in the serum IGF-1 levels in hepatic HRD1 KO mice was detected in comparison with WT on a chow diet (CWT 13.62 ± 0.64; CKO 1.61 ± 0.75 ng/ml; $p < 0.01$). Similar to GH, HFD feeding dramatically recovered IGF-1 production in HRD1 LKO mice (HWT 12.92 ± 0.56 *vs* HKO 12.4 ± 3.05 ng/ml) (**Figure 2B**).

The growth hormone achieves its physiological functions through activating the signal transducer and activator of transcription 5 (STAT5) for the transcription of genes including the induction of IGF-I [21, 22]. In addition to IGF-1, GH regulates the expression of several metabolic enzymes including *Hsd3b5, Cyp2d9, Cyp7b1, Cyp4a12, Mup1 and Elovl3*. As expected, the expression of *Hsd3b5, Cyp2d9, Cyp7b1, Cyp4a12, Mup1 and Elovl3* were dramatically

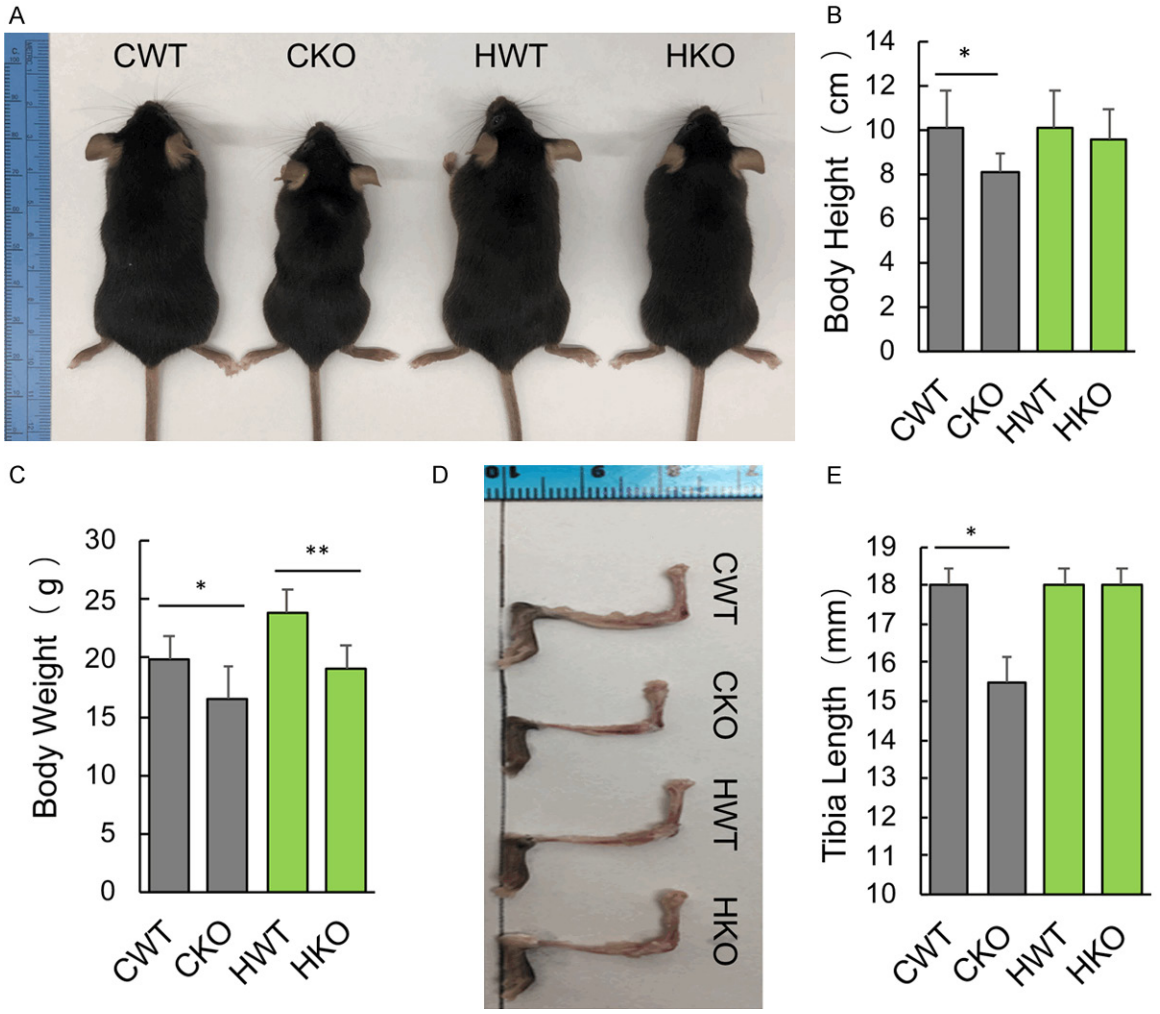


**Figure 1.** The effects of HFD on growth restriction of hepatic HRD1 KO mice. 6-week old HRD1 LKO and their littermate control mice were either on chew (CKO, CWT) or with HFD (HKO, HWT) for 6 weeks and euthanized. (A-C) Representative pictures (A), the average body length (B) and body weight (C) at the age of 12 weeks (n=5 for each group) are shown. (D, E) Representative tibia images of each group (D) and the average tibia length from 5 mice (E) are shown (mean ± s.d.). *$P < 0.05$. **$P < 0.01$ by unpaired Student's t test.

decreased in the liver by HRD1 deletion, which were also partially rescued by HFD (**Figure 2C**). Collectively, these results indicate that liver HRD1 is critical to regulate growth hormone production by pituitary possibly through controlling the energy expenditure. As a consequence, liver specific HRD1 deletion resulted in a small size of mice, and energy supplementation by HFD fully rescues GH production and body growth.

*The impaired fertility of female HRD1 LKO mice can be rescued by HFD*

We have observed that female HRD1 LKO mice are infertile [13]. Since the imbalanced energy expenditure often impairs female fertility, we reasoned whether energy supplementation by HFD feeding could rescue HRD1 LKO mice from infertility. The average estrous cycle length is 4-5 days in WT mice as characterized by the cornified cells [17]. HRD1 KO mice remained in diestrus phase, but rarely entered regular estrus cycle. Surprisingly, we observed a recovery of abnormal estrous cycles in KO mice even after 1 week of HFD feeding (**Figure 3A**), clearly indicating that the energy insufficiency is solely responsible for infertility of female HRD1 LKO mice. Further vaginal cytology analysis confirmed the periodic presence of proestrus stage with nucleated cells and leukocytes, cornified cells in estrus stage followed metestrus

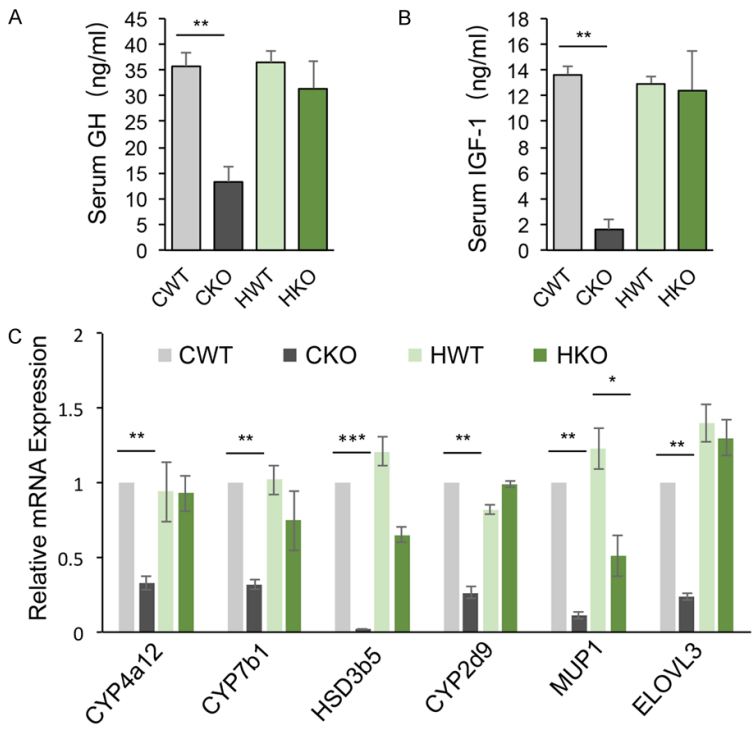


**Figure 2.** Analysis of growth hormones in HRD1 LKO and control WT mice. 6-week old HRD1 LKO and their littermate control mice were either on chew (CKO, CWT) or with HFD (HKO, HWT) for 6 weeks. (A, B) Their serum Growth hormone (A) and IGF-1 (B) were determined by ELISA; (C) Mice were euthanized at the age of 12 weeks, the levels of *Cyp4a12*, *Cyp7b1*, *Cyp2d9*, *Hsd3b5*, *Mup1* and *Elovl3* in liver tissues were determined by real-time PCR. Error bars represent data from 5 of mice each group. Statistical significance was evaluated by unpaired Student's t test; *$P < 0.05$; **$P < 0.01$.

stained all three types of cells and diestrus with leukocytes in WT mice, which are totally diminished in female HRD1 LKO mice fed with chew diet. A week after HFD feeding, a similar vaginal cytology in HRD1 LKO and control WT females was detected (**Figure 3B**). Breeding of HRD1 KO females with WT males under chew feeding for a period of 3 months demonstrated severe subfertility, but with a complete recovery to mate with males fed on HFD during this mating period. More importantly all HFD fed HRD1 LKO females became pregnant 3-4 weeks after HFD (**Figure 3C**). Therefore, the energy insufficiency in HRD1 LKO females, possibly due to the elevated energy expenditure as we recently reported, is responsible for their infertility, and can be fully rescued by additional energy supplementation.

Female fertility requires optimal levels of gonadotropins, both luteinizing hormone (LH) and follicle-stimulating hormone (FSH) [23]. To determine whether HRD1 LKO female mice fail to maintain LH and FSH levels under chew diet, we measured their levels in serum. Indeed, there was a more than 8 reduction in serum LH levels in HRD1 LKO mice under chew diet (CWT 10.85 ± 0.53; CKO 2.33 ± 0.24 ng/ml; $P < 0.01$), which level is clinically insufficient to maintain female fertility. 6 weeks after HFD feeding, the levels of LH in the sera of HDR1 LKO mice were fully recovered to a comparable level 10.12 ± 0.24 ng/ml as that in WT control mice (10.85 ± 0.53) (**Figure 3D**). Similar to that of LH, the serum levels of FSH was declined in HRD1 LKO mice under chew diet, which were fully returned to normal level 6 weeks after HFD fed (**Figure 3D**). Therefore, the impaired pituitary gland functions in HRD1 LKO mice is likely due to energy insufficiency, which can be fully recovered by additional energy supplementation.

*HFD rescues HRD1 LKO mice from follicular dysfunction*

LH plays a critical role in to promoting ovulation, which is essential for female fertility, while FSH inhibits the degradation of follicular atresia and promotes follicles development to the pre-ovulatory stage [24]. Since the hepatic HRD1 deletion resulted in the reduced production of both LH and FSH, we then analyzed the ovarian tissue sections by HE staining as reported [25]. As expected, WT mice with develop mature corpus luteum (CL), an area after the release of the egg follicle, which represent fertilizing capacity [25]. However, no mature follicles in chow-fed HRD1 KO mice were observed. After 6 weeks of HFD feeding, HRD1 LKO mice developed a comparable level of postovulatory corpus luteum as that in WT mice either with chew or with HFD (**Figure 4A**).

Elevated cell apoptosis has been detected in follicular atresia in mammals upon starvation [26], raising a possibility that the impaired female fertility of liver HRD1 mice is due to the

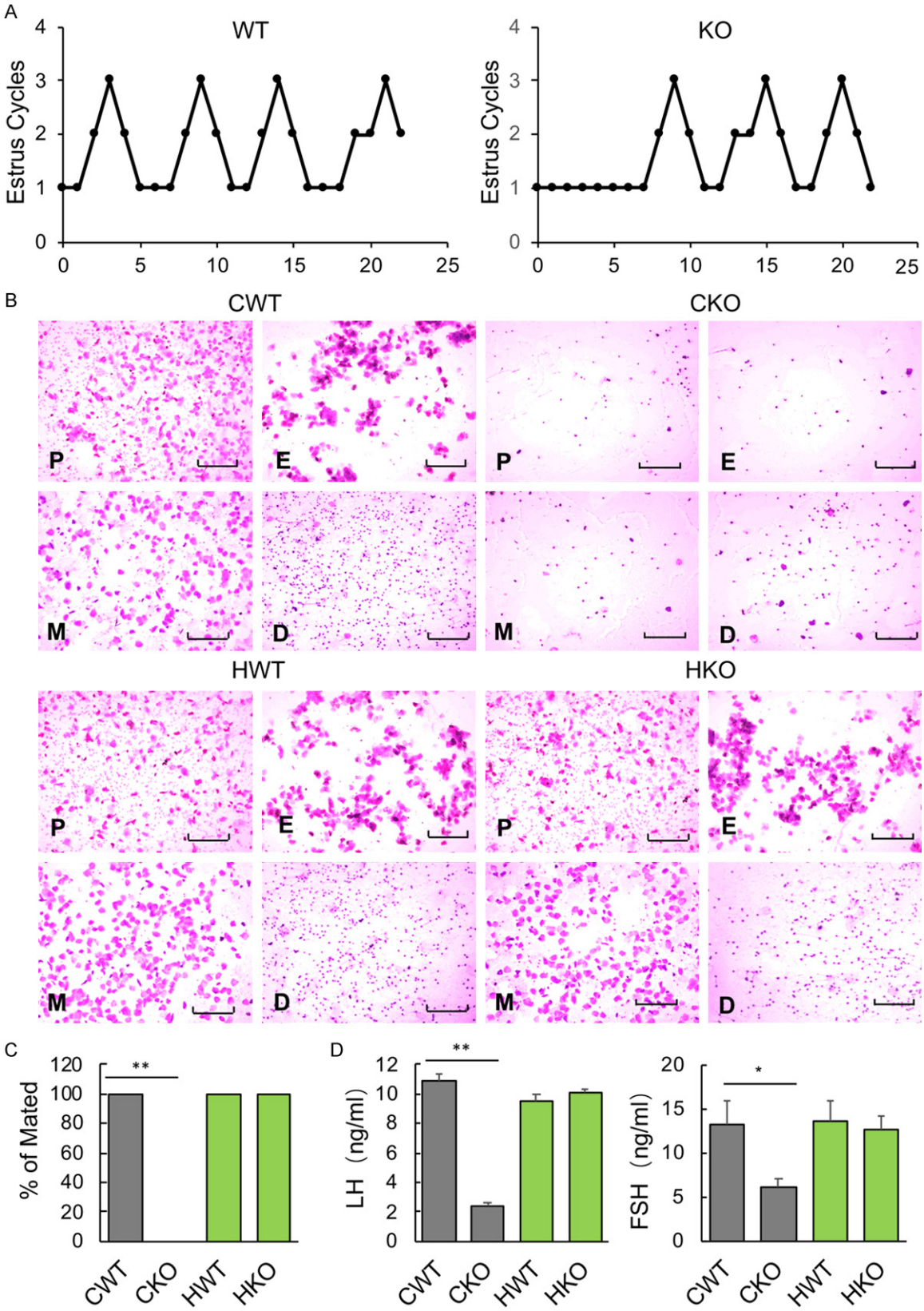


**Figure 3.** Fertility analysis in hepatic HRD1 LKO female mice under chew or HFD. HRD1 LKO and control WT females were fed with HFD from the age of 6 weeks. Mice under chew diet were used as controls. A. The estrus cycle was

determined from the day of starting with HFD (P: proestrus, E: estrus, M: metestrus and D: diestrus). B. Representative images from vaginal cytology analysis are provided. C. The mate rate from 5 mice in each group; D. Serum LH and FSH during diestrus phase were determine by ELISA (n=5 for each group). Statistical significance was evaluated by Student's t test; *$P < 0.05$; **$P < 0.01$.

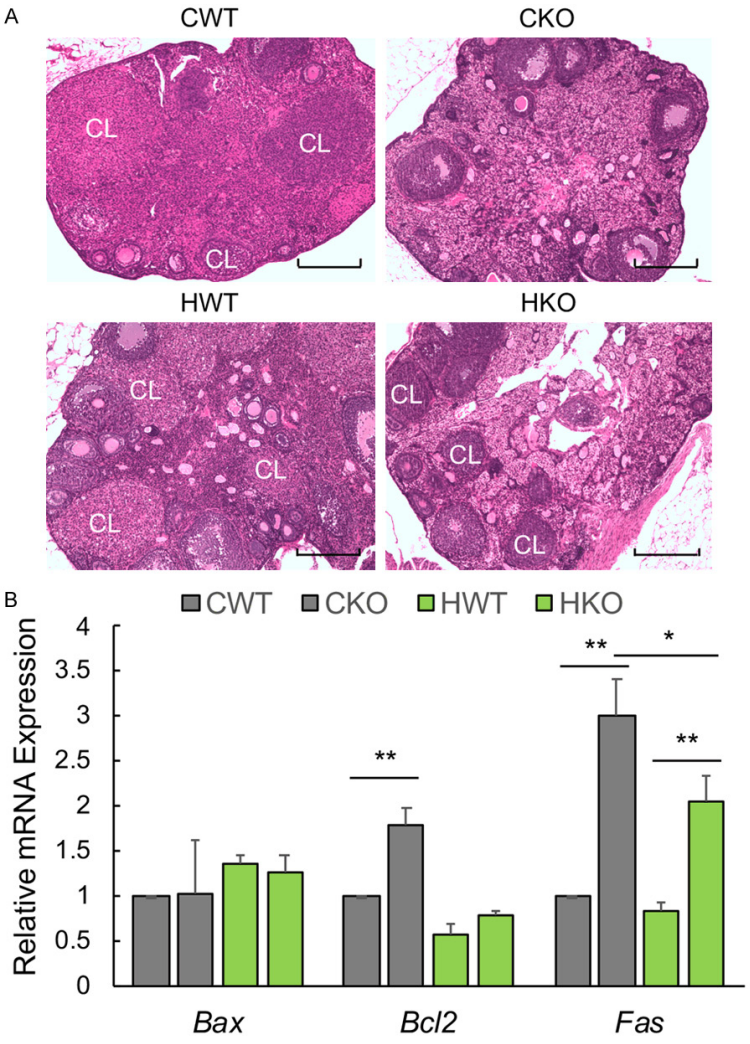


**Figure 4.** Analysis of ovary pathogenesis in HRD1 LKO mice. A. Ovarian histology from WT and HRD1 LKO mice fed with either chow diet or HFD. CL, corpus luteum; Scales, 250 μm; B. The expression of apoptosis genes including *Bax*, *Bcl2* and *Fas* in ovarian tissues were measured. Error bars represent data from 5 mice each group; Statistical significance was evaluated by Student's t test; *$P < 0.05$; **$P < 0.01$.

increased apoptosis. To support this notion, real-time PCR analysis detected significant increases in the expression of both *Bcl2* and *Fas,* but not *Bax,* in ovarian tissues from chow-fed HRD1 KO mice comparing to those in WT controls [27, 28]. Consistent with our observation that the follicular maturation was rescued in HDR1 LKO females by HFD, the expression of *Bcl2* returned back to a similar level to that in WT control. While not reduced back to a comparable level with that of WT, HFD still dramatically reduced the Fas expression levels by HFD in HRD1 LKO mice (**Figure 4B**). We did not detect any changes in the expression of the anti-apoptotic factor *Bax* by HRD1 deletion in mice regardless under chew or HFD feeding (**Figure 4B**). Therefore, the increase in follicular atresia apoptosis is involved in the infertility of female HRD1 LKO mice.

## Discussion

Severe dietary restriction, catabolic states and even short-term caloric deprivation impair fertility in mammals including human, which is often reversible by restoration of the energy supplementation. The dysregulated crosstalk among multiple organs is possibly involved in this process. However, ideal experimental animal models are needed to illuminate functional crosstalk among distal organs during the starvation pathogenesis. We have recently discovered that conditional hepatic HRD1 gene deletion results in elevated energy expenditure and consequently leads to growth retardation and female fertility. The current studies demonstrated that liver specific HRD1 gene ablation in mice caused their growth retardation and female infertility possibly due to the impaired pituitary function in production of both growth hormones and gonadotropins [19, 23, 29, 30]. Energy supplementation by HFD feeding can fully rescue the HRD1 LKO mice by restoring pituitary functions. Those studies define a crosstalk between liver HRD1 functions

with the distal organs including pituitary, bone and ovary in mice.

It has been well established that severe dietary restriction, catabolic states and even short-term caloric deprivation impair fertility in mammals including human, which is often reversible by restoration of the energy supplementation. Several genetically modified animal models have been reported to resemble energy deficiency in human [5, 31, 32]. Mice with systemic deletion of either leptin or insulin receptor substrate-2 (IRS-2) are significantly smaller and infertile [33]. Similar to that in HRD1 LKO mice, both leptin and IRS-2 KO mice have reduced growth hormones and gonadotropins, hormones produced by pituitary critical for body growth and female fertility [34]. All these strains of mice, with HRD1 LKO as the first liver specific but not systemic genetic modification, are useful model for the study of energy imbalance in growth and fertility.

We have recently demonstrated that the liver specific HRD1 deletion increases mouse energy expenditure, which appears to be largely responsible for the impaired growth and female fertility because additional energy supplementation largely restores the body growth and reproduction. Similar to IRS-2 KO mice or the starved human being, the pituitary hormone production in HRD1 LKO mice was dramatically reduced, including the growth hormone and LH. As a consequence of the reduced serum GH levels, a critical hormone controls bone growth, the tibia length of HRD1 LKO mice are substantially shorter. In liver tissue, GH controls liver metabolic response through its mediator IGF-1, which is also reduced by liver HRD1 deletion. To further support our observation of reduced GH levels, the downstream target genes of the transcription factor STAT5 were also reduced. Similar to that in IRS-2 deficient mice, the impaired pituitary function in production of GH was fully restored by HFD. Therefore, the energy deficiency in HRD1 LKO mice, possibly due to the elevated energy expenditure, impaired the pituitary production of GH and consequently leading to growth retardation.

In addition to GH, HRD1 LKO mice were unable to produce the optimal levels of the gonadotropins including LH and FSH, in females. LH is a pituitary hormone that is essential for sexual development and reproduction in both men and women. LH stimulates ovulation and production of progesterone by the corpus luteum that is necessary for the maturation of the uterine endometrium for implantation of the fertilized egg. FSH is also a pituitary hormone that regulates growth, sexual development and reproduction, including menstruation, follicular development and ovulation. Similar to that in human upon starvation [24, 25], both LH and FSH levels were reduced in HRD1 LKO mice. The mechanisms that control energy balance are in line with reproduction. Studies showed women with low body weight are at risk for reproductive neuroendocrine disorders, and experienced a decreased gonadotropins hormones LH and FSH, which is similar to our study [35-37]. Furthermore, energy restriction effects on cell apoptosis and follicular atresia [38]. Our findings of increased *Bcl2* and *Fas* imply a possibility that the elevated follicular atresia apoptosis, due to either reduced FSH and LH levels, or direct starvation of ovary, or both, causes the female infertility of HRD1 LKO mice.

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## Disclosure of conflict of interest

None.

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