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RESEARCH ARTICLE

High-fat, sucrose and salt-rich diet during rat spermatogenesis lead to the development of chronic kidney disease in the female offspring of the F2 generation

Xiaoli Zhang[1,2,3] | **Ahmed A. Hasan**[2,3] | **Hongwei Wu**[3,4] | **Mohamed M. S. Gaballa**[3,5] | **Suimin Zeng**[6] | **Liping Liu**[1] | **Li Xie**[1] | **Tobias Jung**[7] | **Tilman Grune**[7,8] | **Bernhard K. Krämer**[3] | **Burkhard Kleuser**[2] | **Jian Li**[1] | **Berthold Hocher**[1,3,9,10]

[1]Key Laboratory of Study and Discovery of Small Targeted Molecules of Hunan Province, School of Medicine, Hunan Normal University, Changsha, China

[2]Institute of Pharmacy, Freie Universität Berlin, Berlin, Germany

[3]Fifth Department of Medicine (Nephrology/Endocrinology/Rheumatology), University Medical Centre Mannheim, University of Heidelberg, Heidelberg, Germany

[4]Department of Nephrology, the First Affiliated Hospital of Jinan University, Guangzhou, China

[5]Faculty of Veterinary Medicine, Benha University, Toukh, Egypt

[6]The First Hospital of Traditional Chinese Medicine, Yiyang, China

[7]Department of Molecular Toxicology, German Institute of Human Nutrition Potsdam-Rehbruecke (DIfE), Nuthetal, Germany

[8]Department of Physiological Chemistry, Faculty of Chemistry, University of Vienna, Vienna, Austria

[9]Reproductive and Genetic Hospital of CITIC-Xiangya, Changsha, China

[10]Institute of Medical Diagnostics, IMD Berlin, Berlin, Germany

Correspondence
Jian Li and Berthold Hocher, Key Laboratory of Study and Discovery of Small Targeted Molecules of Hunan Province, School of Medicine, Hunan Normal University, Changsha 410013, China.
Email: angela13656355@qq.com and berthold.hocher@medma.uni-heidelberg.de

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Abstract

Effects of feeding male rats during spermatogenesis a high-fat, high-sucrose and high-salt diet (HFSSD) over two generations (F0 and F1) on renal outcomes are unknown. Male F0 and F1 rats were fed either control diet (F0CD+F1CD) or HFSSD (F0HD+F1HD). The outcomes were glomerular filtration rate and urinary albumin excretion in F1 and F2 offspring. If both outcomes were altered a morphological and molecular assessment was done. F2 offspring of both sexes had a decreased GFR. However, increased urinary albumin excretion was only observed in female F2 F0HD+F1HD offspring compared with controls. F0HD+F1HD female F2 offspring developed glomerulosclerosis (+31%; $p < .01$) and increased renal interstitial fibrosis (+52%; $p < .05$). RNA sequencing

Abbreviations: ACTR3B, Actin Related Protein 3B gene; CD, control diet; CD300LF, CD300 Molecule Like Family Member F gene; CKD, chronic kidney disease; ENPP6, Ectonucleotide Pyrophosphatase/Phosphodiesterase 6 gene; HFSSD, high-fat, high-sucrose and high-salt diet; TMEM144, Transmembrane Protein 144 gene.

Xiaoli Zhang and Ahmed A. Hasan contributed equally to this work.

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followed by qRT-PCR validation showed that four genes (Enpp6, Tmem144, Cd300lf, and Actr3b) were differentially regulated in the kidneys of female F2 offspring. lncRNA XR-146683.1 expression decreased in female F0HD+F1HD F2 offspring and its expression was ($r = 0.44$, $p = .027$) correlated with the expression of Tmem144. Methylation of CpG islands in the promoter region of the Cd300lf gene was increased ($p = .001$) in female F2 F0HD+F1HD offspring compared to controls. Promoter CpG island methylation rate of Cd300lf was inversely correlated with Cd300lf mRNA expression in F2 female offspring ($r = -0.483$, $p = .012$). Cd300lf mRNA expression was inversely correlated with the urinary albumin-to-creatinine ratio in female F2 offspring ($r = -0.588$, $p = .005$). Paternal pre-conceptional unhealthy diet given for two generations predispose female F2 offspring to chronic kidney disease due to epigenetic alterations of renal gene expression. Particularly, Cd300lf gene promotor methylation was inversely associated with Cd300lf mRNA expression and Cd300lf mRNA expression itself was inversely associated with urinary albumin excretion in F2 female offspring whose fathers and grandfathers got a pre-conceptional unhealthy diet.

KEYWORDS

epigenetics, high-fat-sucrose-salt diet, kidney function, paternal programming

1 | INTRODUCTION

The 'fetal programming' hypothesis proposes that environmental factors before birth can alter the offspring phenotype through a range of mechanisms.[1,2] These factors are most well defined in mothers for example, maternal undernutrition during pregnancy, maternal high protein diet during pregnancy, and glucocorticoid exposure of the fetus. While alterations in epigenome and phenotype of offspring by maternal factors are widely reported, there are also reports in the past years suggesting that fetal programming can also be paternally initiated.[3–6] For example, paternal smoking, age, and occupational chemical exposure are associated with an increased risk of cancer and mental health disorders in the offspring.

Fetal programming of kidney diseases was likewise established. Independent research teams worldwide showed that maternal undernutrition, caloric restriction, protein restriction or maternal dietary fat excess all have a negative impact on nephrogenesis and kidney structure, contributing to offspring kidney disease in later life.[7–9] Moreover, there is also evidence from human studies that comparable maternal factors increase the risk of kidney disease in the offspring.[10] While there were several studies investigating the impact of maternal factors on the offspring renal phenotype, the literature on the later-life effects of paternal factors for example, pre-conceptional unhealthy diet on the renal phenotype of the offspring is limited. *Chowdhury* et al. reported that paternal obesity can lead to increased triglyceride content in offspring kidneys with signs of tubular damage, such as cell sloughing, absence of brush border which may be indicative of early signs of kidney damage.[11]

Previously, we fed a diet resembling an unhealthy diet (high-fat, high-sucrose and high-salt diet [HFSSD], often consumed by young men) to male rats prior to mating and analyzed the effect on healthy outcome in offspring. It was shown that paternal pre-conception unhealthy diet had detrimental effects on F1 female offspring's glucose metabolism, which can be reversed by folate treatment of pregnant dams.[12] Furthermore, we found that a paternal pre-conceptional unhealthy diet predisposed the F2 offspring to mild liver functional alterations and alterations of gut microbiota in later life.[13]

In the current study, we analyzed the effect of feeding male rats a high-fat, high-sucrose and high-salt diet (HFSSD) over two generations (F0 and F1) on their offspring's (F2) kidney function. We defined abnormal kidney function by two parameters: decreased glomerular filtration rate (GFR) combined with increased urinary-albumin-to-creatinine ratio (UACR). In case of developing significantly declined GFR and significantly elevated UACR after exposure of the fathers and/or grandfathers to an unhealthy diet, we analyzed the renal morphology as well as the underlying molecular mechanisms causing paternal programmed of chronic kidney disease in the offspring.

2 | MATERIAL AND METHODS

2.1 | Animals

The study was conducted in Sprague-Dawley rats, including F0 generation (30 male rats, 45 female rats), F1 and F2 generation animals. The F0 generation rats were purchased from Hunan SJA Laboratory Animal Co., Ltd (Changsha, China) and were delivered at the age of 4 weeks. All the animals were allowed free access to water and food. The experimental protocols were approved by the Experimental Animal Center of the Hunan Normal University (Changsha, Hunan, China).

2.2 | Study design

F0 male rats were randomly divided into two study groups: (1) control diet (CD, $n = 15$); (2) high-fat, high-sucrose and high-salt diet (HFSSD, $n = 15$). A detailed description of the diet compositions is indicated in our previous study[13] (Table S1).

15-week-old F0 founder male rats were fed either a CD or HFSSD for 9 weeks. Then they were mated with F0 normal-weight, naturally cycling CD-fed dams to produce F1 offspring. The presence of a vaginal plug was designated as gestational day 1. The rats were mated for 4 days. After mating and throughout the gestational period, all the F1 dams were fed CD. The number of F1 offspring per litter was 13.89 ± 0.3 (SE) and was not differ significantly between the groups. We sampled randomly 173 F1 offspring (86 males and 87 females) out of 540 total offspring. Thus, the sample size was around one-third of the entire offspring. The F1 male offspring was allocated into two study groups based on F0 paternal diet before mating: (1) paternal control diet group (PatCD group): male F1 offspring of CD-fed F0 founders received CD from birth until the 24th week of age and were mated with CD-fed dams and their F2 offspring represented the F0CD+F1CD group; (2) paternal high-fat, high-sucrose and high-salt diet group (PatHFSSD group): male F1 offspring of HFSSD-fed F0 founders received CD from birth until the 15th week of age followed by HFSSD until the 24th week of age and were mated with CD-fed dams and their offspring represented F0HD+F1HD group. The average litter size of F2 offspring was 13 and was not differ significantly between the groups. We sampled randomly 65 F2 offspring (33 males and 32 females) out of 150 total offspring. In summary, based on F0 paternal diet and F1 paternal diet before mating, the female F2 offspring can be divided into two study groups (Figure 1): (a) F0CD+F1CD group: female F2 offspring of F0 and F1 male founders fed a CD; (b) F0HD+F1HD group: female F2 offspring of F0 and F1 male founders fed a HFSSD. All F2 offspring (F0CD+F1CD and F0HD+F1HD) were fed CD from birth until the 24th week of age. Before sacrifice, F2 offspring were fasted for 8 h, then rats were placed in a metabolic cage and 24 h-urine was collected. At the 24th week, they were sacrificed under deep anesthesia induced by intraperitoneal injection of 3% (wt/vol.) sodium pentobarbital solution. Afterwards, blood samples were collected and organs were harvested.

Blood pressure measurements, metabolic analysis, analysis of kidney morphology, lncRNA-mRNA expression profiling, real-time quantitative PCR, correlation analysis of lncRNAs and mRNAs and functional prediction, gene-specific DNA methylation were described in detail in the electronic supplementary materials (ESM).

2.3 | Statistical analysis

Normality of data distribution was checked using Shaprio–Wilk test. Student's *t*-test (unpaired, two-tailed) and the Mann–Whitney test were used to compare parametric and non-parametric data, respectively, between groups. Pearson's correlation analysis was used to assess correlations between mRNAs and lncRNAs. Unless otherwise indicated, results are expressed as the mean ± SE. A p value of <.05 was considered statistically significant. The data were analyzed using SPSS version 20.0 (SPSS, Chicago, IL, USA) and GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA). Heat maps were created using R version 3.5.3 (https://www.r-project.org/) and Cytoscape version 3.7.1 (https://cytoscape.org/) was used to construct the relational network.

3 | RESULTS

The primary outcome of the study in the F1 and F2 generations was a composite endpoint consisting of decreased GFR combined with increased urinary albumin to creatine ratio. Only if this endpoint was met, a detailed biochemical and morphological analysis was done.

3.1 | F0 founders

Detailed data on body weight gain, blood pressure and serum metabolite levels in the F0 male founders were presented in Table S2. The number of pups was similar in all groups; no effect of HFSSD on the number of pups, male/female ratio and birth body weight were detectable.

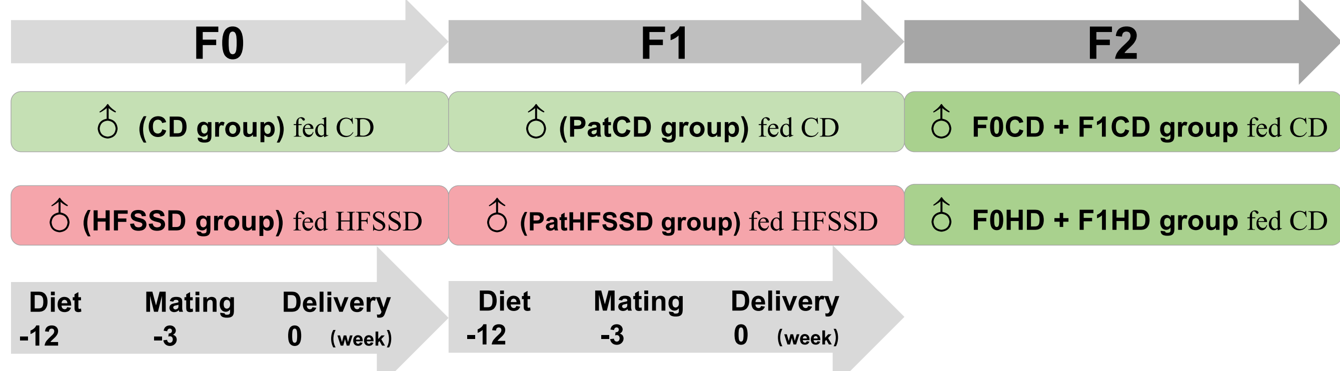

FIGURE 1 Study design for the F0, F1 and F2 generations. F0 founder male rats fed either a control diet (CD) or high-fat, high-sucrose and high-salt diet (HFSSD) for 9 weeks were mated with CD-fed dams to produce F1 offspring. The F1 male offspring was allocated into two study groups based on F0 paternal diet before mating: (1) Paternal control diet group (PatCD group): male F1 offspring of CD-fed F0 founders received CD from birth until the 24th week of age; (2) Paternal high-fat, high-sucrose and high-salt diet group (PatHFSSD group): male F1 offspring of HFSSD-fed F0 founders received CD from birth until the 15th week of age followed by HFSSD until the 24th week of age. Male PatCD group rats and male PatHFSSD group rats in 24th week of age were mated with female PatCD group rats to produce F2 offspring. Based on F0 paternal diet and F1 paternal diet before mating, the F2 offspring can be divided into two study groups: (1) F0CD+F1CD group: F2 offspring of F0 and F1 male founders fed a CD; (2) F0HD+F1HD group: F2 offspring of F0 and F1 male founders fed a HFSSD. All F2 offspring (F0CD+F1CD and F0HD+F1HD) were fed a normal diet (CD) for 24 weeks

TABLE 1 Effect of paternal HFSSD on kidney weights and kidney biomarkers in F1 offspring

Parameters	Female		Male	
	PatCD ($n = 7$)	PatHFSSD ($n = 11$)	PatCD ($n = 14$)	PatHFSSD ($n = 17$)
Body weight (100 days)	252.69 ± 4.47	266.96 ± 6.47	416.47 ± 8.50	425.20 ± 9.89
Relative left kidney weight (% to body weight)	0.32 ± 0.01	0.31 ± 0.01	0.32 ± 0.01	0.33 ± 0.01
Relative right kidney weight (% to body weight)	0.33 ± 0.01	0.32 ± 0.01	0.33 ± 0.01	0.33 ± 0.01
BUN (mmol L^{-1})	6.79 ± 0.33	6 ± 0.33	6.42 ± 1.03	5.27 ± 0.24
Plasma Cr (mmol L^{-1})	27.2 ± 1.03	24.05 ± 2.14	24.13 ± 3.72	24.3 ± 1.34
Plasma cystatin C (μg ml^{-1})	1.46 ± 0.11	1.53 ± 0.13	1.54 ± 0.18	1.75 ± 0.12
Urine volume (ml 24 h^{-1})	28.19 ± 3.69	6.23 ± 0.53**	45.46 ± 8.13	34.29 ± 6.26
Urinary microalbumin (μg 24 h^{-1})	82.56 ± 34.78	73.05 ± 14.01	65.99 ± 11.42	112.18 ± 33.61
Urine Cr (μmol 24 h^{-1})	65.49 ± 6.82	53.11 ± 4.51	95.66 ± 8.35	100.70 ± 9.47
Urinary albumin-to-creatinine ratio (μg $μmol^{-1}$)	1.25 ± 0.43	1.37 ± 0.24	0.74 ± 0.14	1.13 ± 0.27
GFR/body weight (ml 24 h^{-1} g^{-1})	10.15 ± 1.07	7.77 ± 0.91	10.87 ± 1.52	9.29 ± 1.16

Note: Values are given as mean ± SE.

Abbreviations: BUN, blood urea nitrogen; Cr, creatinine; GFR, glomerular filtration rate.

**$p < .01$ vs. PatCD.

3.2 | Kidney function in F1 offspring

The urinary albumin-to-creatinine ratio was numerically higher in F1 offspring of the PatHFSSD group of both sexes. However, the differences did not reach statistical difference. The GFR corrected by body weight was numerically lower in F1 offspring of the PatHFSSD group of both sexes. However, the differences again did not reach statistical difference (Table 1). Therefore, we further observed the effect of HFSSD on kidney function in F2 offspring.

3.3 | Body weight and kidney function in F2 offspring

Compared with the F0CD+F1CD group, the body weight of the female F0HD+F1HD group increased significantly at the 9th, 15th, 18th, 22nd and 24th week (Figure S1A). However, no significant differences in body weight were observed between the F0CD+F1CD and F0HD+F1HD in F2 male offspring (Figure S1B). The urinary albumin to creatine ratio in female F2 offspring of F0HD+F1HD group was significantly increased compared with the F0CD+F1CD group ($p < .01$, Figure 2A). Furthermore, the ratio of GFR/body weight ratio at study end was significantly lower in female F0HD+F1HD group than that in F0CD+F1CD group ($p < .05$, Figure 2B). However, no significant differences related to renal function were observed between the F0CD+F1CD and F0HD+F1HD in male F2 offspring (Table 2). Since only female F2 offspring met the composite study endpoint, a detailed morphological and molecular assessment was next done in just female F2 offspring.

3.4 | Kidney morphology

The number and size of glomeruli was similar among the study groups of female F2 offspring (Figure 2C,D). In female F2 offspring born to grandfather and father exposed to the HFSSD diet prior to mating glomerulosclerosis score was increased by 31% and renal interstitial fibrosis area was increased by 52% ($p < .01$, $p < .05$ vs. control female counterparts, respectively, see Figure 2E–H).

3.5 | Expression of mRNA and lncRNA in the kidney

Microarray profiling in a subset of animals (see Section 2) was done to identify candidate genes being potentially differentially regulated. The results of microarray profiling showed that 285 mRNAs were differentially regulated. (156 up-regulated mRNA and 129 down-regulated mRNA) when comparing the F0CD+F1CD and the F0HD+F1HD group in female F2 offspring ($p < .05$ and $|\text{fold change}| \geqq 1.5$ was set as the threshold for significant differential expression) (Figures 3A and S2A).

Using the same criteria, we identified 129 lncRNAs (94 up-regulated lncRNA and 35 down-regulated graduated lncRNA) (Figure S2B). Protein-protein interaction analysis of these DEGs highlighted the key module of RT1 family (a family of MHC class Ib genes) in the relational network (Figure S2C). Functional enrichment and KEGG analyses revealed that antigen processing and presentation, immune response, and antiviral function in F0HD+F1HD group differed from that in the F0CD+F1CD group (Figure S3A,B), suggesting that renal dysfunction in F2 female offspring might be related to altered immunological properties of the kidneys.

From the 285 DEGs, we chose the most promising 18 candidate mRNAs based on a statistical criterion ($p < .01$, $|\text{fold change}| \geqq 2$) in the arrays for confirmation in the entire study population by qRT-PCR (Table S4). Expression of Enpp6, Tmem144 and Cd300lf was decreased whereas Actr3b gene expression was increased in female offspring born to grandfather and father exposed to the HFSSD (Figure 3B).

3.6 | Correlation analysis of lncRNA and mRNA

To analyze the relationship between lncRNA and the 4 confirmed differentially expressed mRNAs (Enpp6, Actr3b, Tmem144 and Cd300lf), we performed Pearson correlation analysis using the microarray data. Based on Pearson correlation coefficient >0.4 or <−0.4 and p value <.05, we found that 20 lncRNAs had significant correlations with Enpp6, Tmem144, Cd300lf and Actr3b. (Figure 4A). Subsequently, we conducted qRT-PCR to verify these correlations in the entire study. We found that lncRNA XR_146683.1 and uc.239+ were significantly down-regulated in female offspring born to grandfather and father exposed to the HFSSD (Figure 4B, Table S5). Further correlation analysis showed that expression of lncRNA XR_146683.1 had a significant positive correlation with that of Tmem144 ($r = 0.44$, $p = .027$) (Figure 4C).

3.7 | DNA methylation of specific target genes in the kidney

The methylation rate of CpG islands in the promoter region of Enpp6, Tmem144, Actr3b and Cd300lf genes was analyzed. Violin plot of differential methylation fragments showed that the methylation rate of CpG islands in the promoter region of Cd300lf was significantly higher (Figure 5A, $p = .001$) in the female F0HD+F1HD group as compared to the F0CD+F1CD group. Cd300lf gene promotor methylation was inversely correlated with Cd300lf mRNA expression ($r = -0.483$, $p = .012$) (Figure 5B). Cd300lf mRNA expression itself was inversely correlated with the urinary albumin-to-creatinine ratio in female F2 offspring (see Table S7 and Figure 5C, $r = -0.588$, $p = .005$).

We next analyzed the Cd300lf gene promotor methylation in more detail. We analyzed the CpG site specific

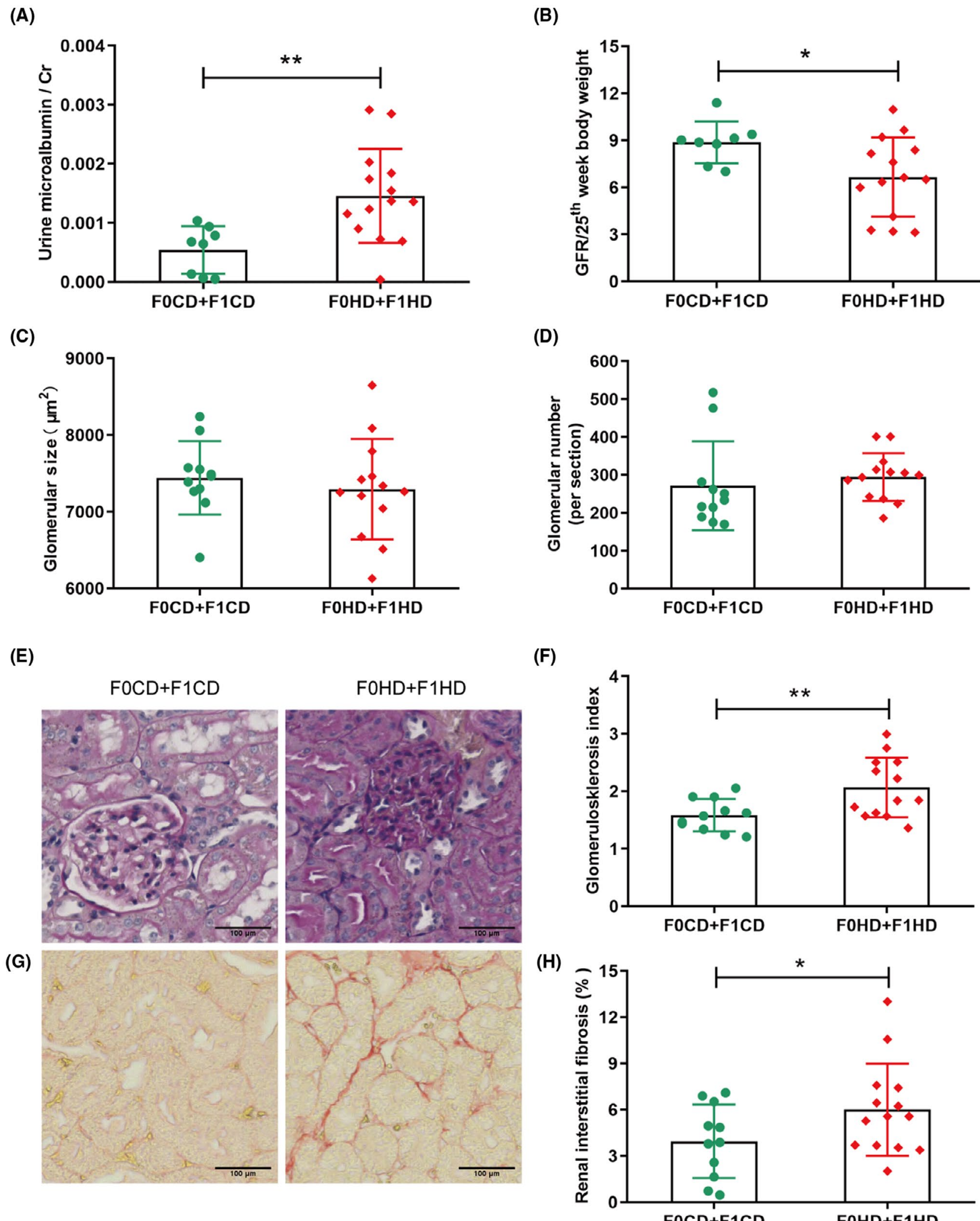

FIGURE 2 Effect of paternal HFSSD on urine indicators related to renal function and morphology of F2 female offspring. ratio of urine microalbumin and Cr (A); ratio of GFR and body weight of the 25th week (B); size of glomerulus (C); total number of glomerulus (D); representative photomicrographs of PAS-stained renal sections (magnification:×200) (E); glomerulosclerosis score (F); representative photomicrographs of picrosirius red stained renal sections (magnification:×200) (G); renal interstitial fibrosis area (H). F0CD+F1CD, F2 offspring of F0 and F1 male founders fed a CD; F0HD+F1HD, F2 offspring of F0 and F1 male founders fed a HFSSD. *$p < .05$ vs. F0CD+F1CD, **$p < .01$ vs. F0CD+F1CD

TABLE 2 Effect of paternal HFSSD on blood pressure, kidney weights and kidney biomarkers in F2 offspring

Parameters	Female		Male	
	F0CD+F1CD ($n = 11$)	F0HD+F1HD ($n = 15$)	F0CD+F1CD ($n = 18$)	F0HD+F1HD ($n = 19$)
Body weight at the 24th week	308.1 ± 5.55	322.64 ± 3.71*	585.78 ± 7.98	604.77 ± 8.50
Heart rate (bpm)	442.2 ± 6.96	411.55 ± 9.5*	414.68 ± 8.34	406.95 ± 11.25
Systolic blood pressure (mm Hg)	135.58 ± 3.33	133.82 ± 2.28	137.61 ± 2.43	135.19 ± 2.96
Relative left kidney weight (% to body weight)	0.29 ± 0.01	0.29 ± 0.01	0.28 ± 0.01	0.27 ± 0.01
Relative right kidney weight (% to body weight)	0.29 ± 0.01	0.29 ± 0.01	0.28 ± 0.01	0.26 ± 0.01
BUN (mmol L^{-1})	8.43 ± 0.24	7.89 ± 0.21	7.22 ± 0.18	7.35 ± 0.17
Plasma Cr (mmol L^{-1})	27.82 ± 1.05	29.4 ± 0.88	26.44 ± 0.81	27.68 ± 0.87
Plasma cystatin C (μg ml^{-1})	1.14 ± 0.05	1.17 ± 0.05	1.32 ± 0.03	1.43 ± 0.08
Urine volume (ml 24 h^{-1})	44.48 ± 8.79	14.33 ± 3.38**	45.16 ± 6.09	26.07 ± 4.38*
Urinary microalbumin (μg 24 h^{-1})	43.9 ± 27.11	90.9 ± 43.73*	109.26 ± 85.82	85.01 ± 60.74
Urine Cr (μmol 24 h^{-1})	72.47 ± 4.37	62.59 ± 5.77	131.24 ± 4.39	105.19 ± 9.87*
Urinary albumin-to-creatinine ratio (μg μmol^{-1})	0.55 ± 0.14	1.69 ± 0.31**	0.80 ± 0.14	1.08 ± 0.21
GFR/body weight (ml 24 h^{-1} g^{-1})	8.86 ± 0.47	6.66 ± 0.67*	8.42 ± 0.40	6.41 ± 0.64*

Note: Values are given as mean ± SE.

Abbreviations: BUN, blood urea nitrogen; Cr, creatinine; F0CD+F1CD: F2 offspring of F0 and F1 male founders fed a CD; F0HD+F1HD, F2 offspring of F0 and F1 male founders fed a HFSSD; GFR, glomerular filtration rate.

*$p < .05$ vs. F0CD+F1CD; **$p < .01$ vs. F0CD+F1CD.

methylation in the Cd300lf gene promotor region and could demonstrate that all CpG sites were differently methylated (different methylation rate) in all 7 promoter CpG sites (Figure 5D, Table S6). Pearson correlation analysis indicated that 5 CpG sites of Cd300lf showed negative correlation with the expression of Cd300lf (Figure 6).

We likewise analyzed whether the methylation rate of a given CpG site is correlated with other CpG sites methylation within the promoter region, Pearson correlation matrices were calculated and plotted as heat maps for each group. The resulting group-specific correlation patterns were clearly different. Regarding the correlation matrices of the methylation rate of CpG islands within the Actr3b and Cd300lf promoter, positive correlations, indicated by red, were more predominant in the offspring born to grandfathers and fathers on an unhealthy diet when compared with the offspring born to grandfathers and fathers on a normal diet (Figure 7A,B).

4 | DISCUSSION

The present study was designed to investigate the hypotheses that an unhealthy diet during spermatogenesis might have adverse effects on kidney function and morphology in the offspring. Several studies have already investigated and established the concept of maternal programming of cardio-metabolic and renal diseases,[1,2] while the evidence for paternal programming of kidney diseases is still limited.[11,14]

We fed male rats an unhealthy diet during spermatogenesis over two generations (F0 and F1) and studied the effects on kidney function in the offspring (F1 and F2). Impairment of both GFR and urinary albumin excretion was predefined as the endpoint of the initial phenotypic screening. If this composite endpoint was met, we performed a detailed morphological and biochemical analysis of the kidneys. F1 offspring demonstrated nonsignificant reduction of GFR and non-significant increase of urinary albumin excretion, while F2 offspring showed a significantly decreased GFR and a significantly increased urinary albumin excretion in females only. Thus, we investigated the female F2 offspring in more detail.

Studies on renal outcomes caused by paternal factors are limited and preliminary so far. In a rat model, paternal high fat diet resulted in increased triglyceride content in the kidneys of the offspring and tubular damage such as loss of brush border and cell sloughing, while there were no signs of glomerular damage.[11] Another study[14] showed that paternal exposure to synthetic glucocorticoids alter the expression and DNA methylation of the mineralocorticoid receptor-, estrogen alpha receptor-, and

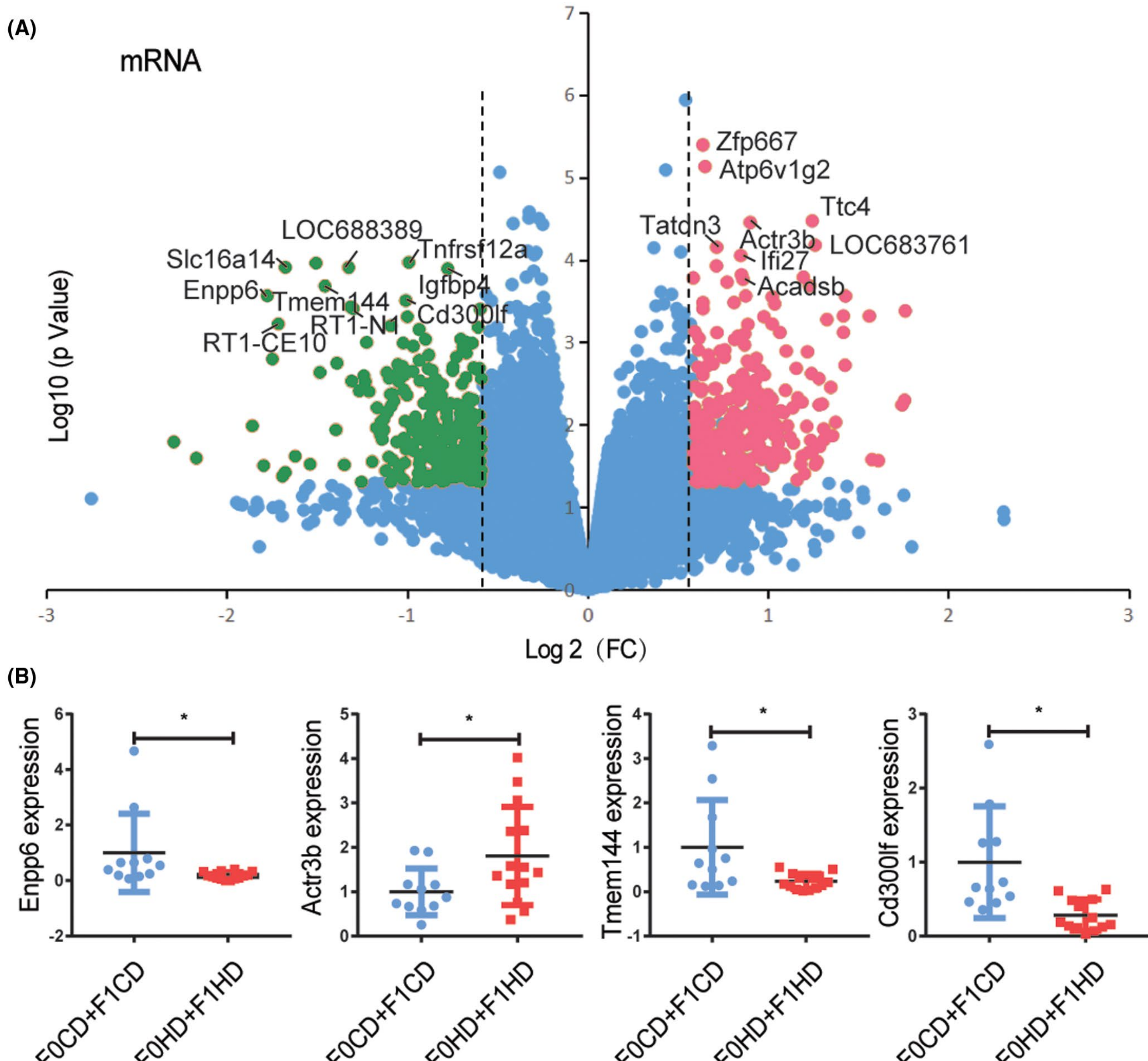

FIGURE 3 Analysis and verification of differentially expressed mRNAs. Volcano plot of differentially expressed mRNAs (A) based on |Fold change| ≥ 1.5 and $p < .05$ between the female offspring of F0CD+F1CD group and the F0HD+F1HD group. Blue dots indicate mRNA with no significant difference. Green dots indicate significantly down-regulated mRNAs, and red dots indicate significantly up-regulated mRNAs. The expression of *Enpp6*, *Cd300lf*, *Teme144* and *Actr3b* mRNA in kidney tissues of female F2 offspring was verified by qRT-PCR as only these four mRNAs showed significant differential regulation between the two groups (B). F0CD+F1CD, F2 offspring of F0 and F1 male founders fed a CD; F0HD+F1HD, F2 offspring of F0 and F1 male founders fed a HFSSD. *$p < .05$ vs. F0CD+F1CD

glucocorticoid receptor gene in the hippocampus and kidney of offspring.

The molecular mechanisms of paternal programming of renal outcomes in the offspring are not well studied. However, several studies investigated paternal programming of other organs such as liver,[15–17] cardiovascular function,[18] pancreas,[19] adipose tissue,[20] and reproductive system.[21] There is now convincing evidence that the underlying molecular mechanisms are mediated via epigenetic alterations of the sperm such as sperm DNA methylation, histone modifications, noncoding RNAs in the tip of the sperm.[22–25] These epigenetic marks of the sperm cause after fertilization specific epigenetic alterations in target organs such as the endocrine pancreas and finally phenotypic alterations in the offspring. In other words, early epigenetic marks in the sperm induced by environmental factors as listed above cause specific epigenetic alterations in offspring target organs leading to a specific phenotype

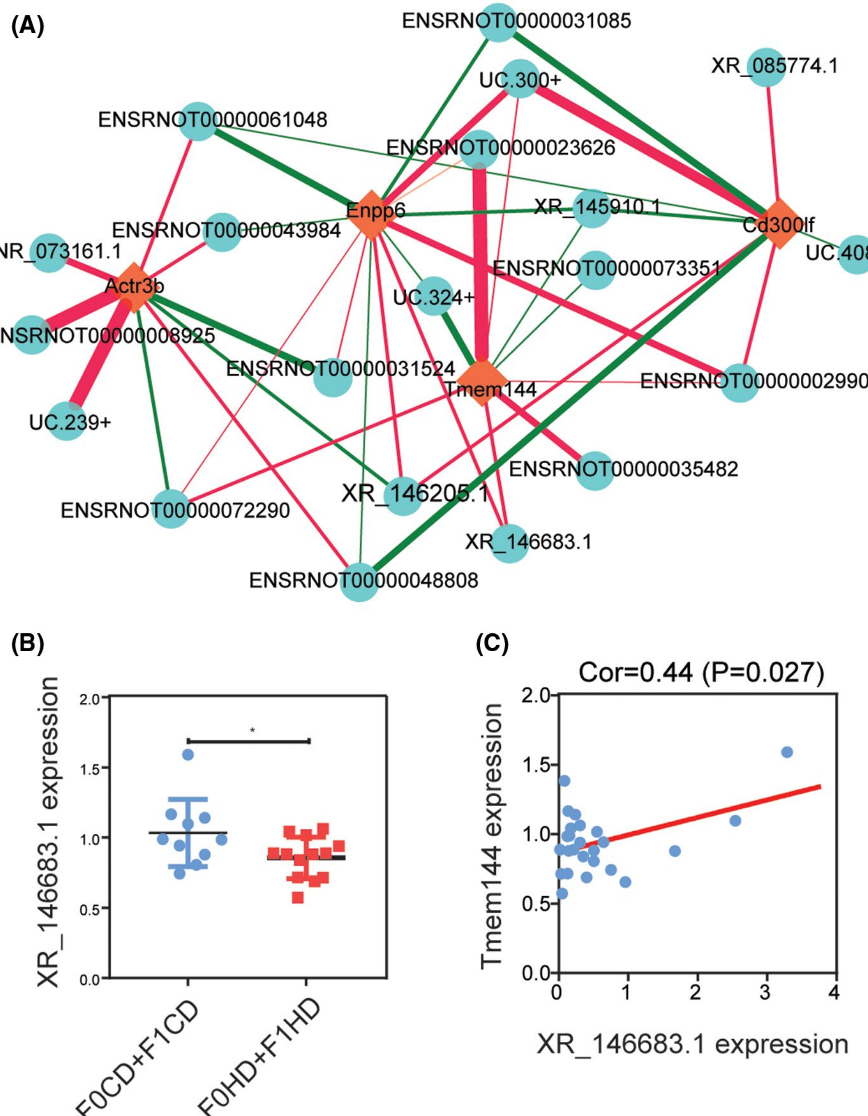

FIGURE 4 Correlation analysis of lncRNAs and mRNAs and verification of differentially expressed lncRNAs. (A) Correlation analysis of lncRNAs detected by the microarray approach and qPCR confirmed mRNAs. The circles represent lncRNAs and the diamonds represent mRNA. The red lines indicate positive correlation, and the green lines indicate negative correlation. The thicker the line, the higher the correlation coefficient. Pearson correlation coefficient >0.4 or <−0.4 and p value <.05 were set as screening criteria. (B) Renal lncRNA *XR_146683.1* expression in female F2 offspring. (C) Correlation analysis of renal lncRNAs *XR_146683.1 expression* and renal *Tmem144 mRNA expression*. F0CD+F1CD, F2 offspring of F0 and F1 male founders fed a CD; F0HD+F1HD, F2 offspring of F0 and F1 male founders fed a HFSSD. *p < .05 vs. F0CD+F1CD

in the offspring later in life. This was best shown so far for metabolic conditions such as glucose metabolism/insulin resistance.

We did microarray profiling of mRNAs and lncRNAs in order to characterize the target organ specific epigenetic alterations in the offspring kidneys in those offspring who developed a renal phenotype (decreased GFR and increased urinary albumin excretion) in order to better understand the molecular mechanisms associated with the phenotype in the female F2 offspring of the fathers and grandfathers on an unhealthy diet during spermatogenesis. We identified 285 mRNAs and 129 lncRNAs differentially regulated by RNA sequencing. The statistically best candidates in terms of P-value and fold change were validated using qRT-PCR and hence four candidate genes (ENPP6: Ectonucleotide Pyrophosphatase/Phosphodiesterase 6 gene, TMEM144: Transmembrane Protein 144 gene, ACTR3B: Actin Related Protein 3B gene, and CD300LF: CD300 Molecule Like Family Member F gene) were finally proven to be differentially regulated. Moreover, the renal expression of lncRNA XR-146683.1 was significantly down-regulated in female F2 offspring of the F0HD+F1HD group compared with control female counterparts and its expression was correlated with the

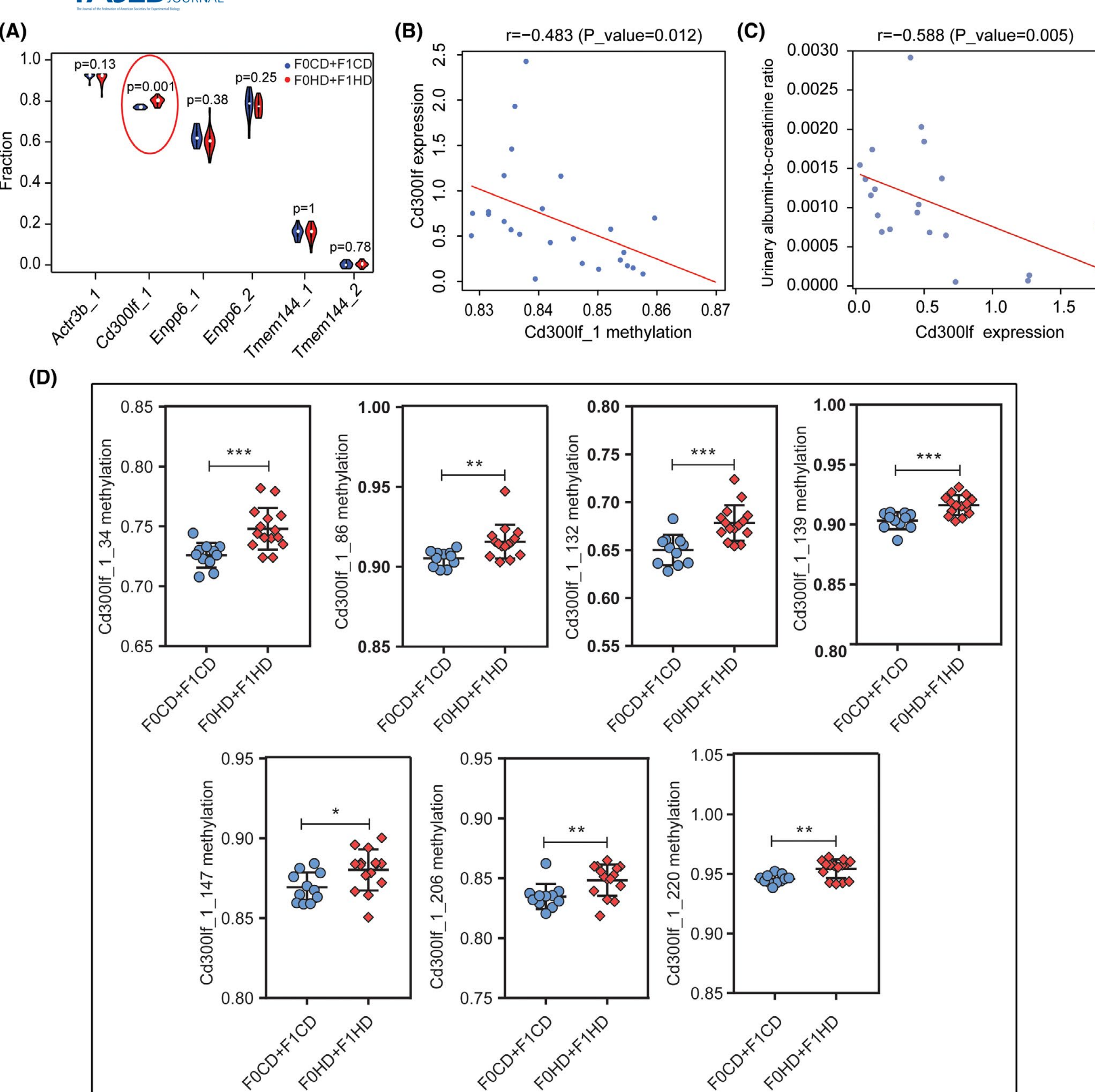

FIGURE 5 Relationship between *Cd300lf* DNA methylation—*Cd300lf* gene expression and urinary albumin excretion. (A) Comparison of overall *DNA methylation* in differently expressed genes between the F0CD+F1CD group and the F0HD+F1HD group in F2 female offspring. Fraction = methylated reads/Tatol reads detected in this fragment. (B) Correlation between methylation fragment of *Cd300lf* and the expression of *Cd300lf*. Differential CpG sites of *Cd300lf* between F0CD+F1CD group and F0HD+F1HD group in F2 female offspring kidney (C). (C) Correlation of *Cd300lf* gene expression and urinary albumin-to-creatinine ratio. (D) Comparison of methylation in kidneys of specific *Cd300lf* gene CpG sites between the F0CD+F1CD group and the F0HD+F1HD group of F2 female offspring. F0CD+F1CD, F2 offspring of F0 and F1 male founders fed a CD; F0HD+F1HD, F2 offspring of F0 and F1 male founders fed a HFSSD. *p < .05 vs. F0CD+F1CD, **p < .01 vs. F0CD+F1CD

expression of Tmem144. The methylation rate of CpG islands in the promoter region was significantly affected in the Cd300lf gene, but not in other differently regulated genes in the F2 female offspring of the F0HD+F1HD group compared to control female counterparts. The methylation rate of CpG islands in the promoter region of Cd300lf was inversely correlated with Cd300lf mRNA expression in F2 female offspring. Moreover, Cd300lf mRNA expression was correlated to urinary albumin excretion suggesting that an unhealths died during spermatogenesis in the F0 and F1 generation affects methylation of the of CpG islands in the promoter region of Cd300lf in female

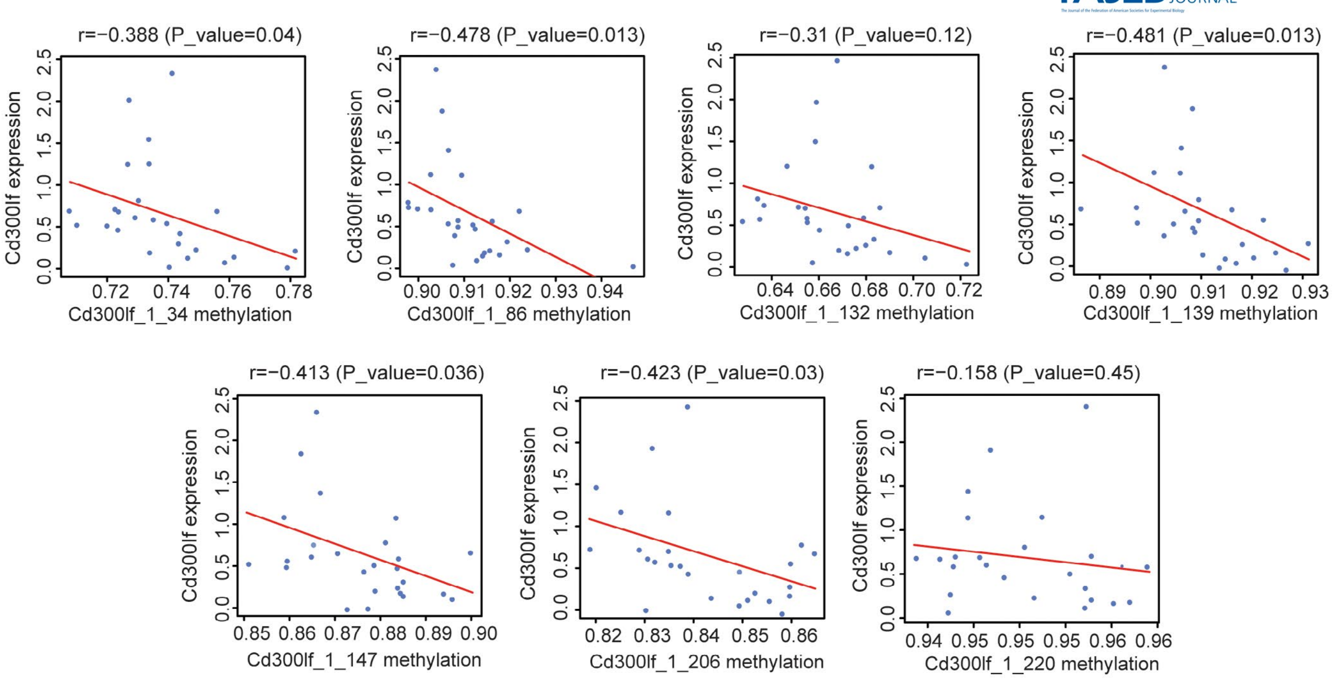

FIGURE 6 Correlation analysis between the degree of specific CpG site methylation and the expression of the corresponding *Cd300lf* mRNAs in F2 female offspring. A *p* value < .05 was considered statistically significant

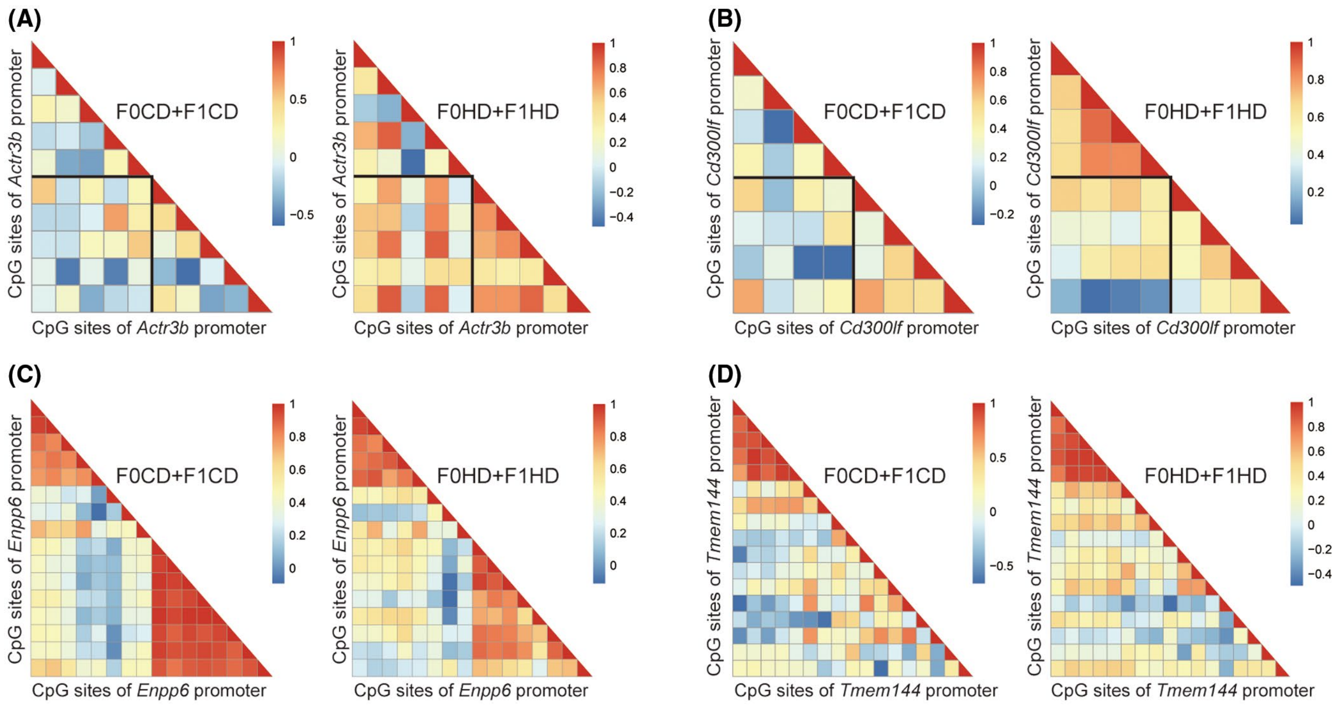

FIGURE 7 Heat maps of group-specific inter-CpG site correlation coefficients of DNA methylation of *Actr3b* (A), *Cd300lf* (B) *Enpp6* (C) and *Tmem144* (D) in female F2 offspring

offspring leading to alterations of Cd300lf gene expression and hence urinary albumin excretion. In addition, the methylation rate of two CpG sites (*Cd300lf*_1_132 and *Cd300lf*_1_147) in the promoter region of *Cd300lf* were positively correlated with the degree of glomerulosclerosis in F2 female offspring. Beside the degree of gene promoter methylation also the pattern of methylated CpG sites in a promoter of a gene might modulate its activity,[26] thus we analyzed treatment group-specific inter-CpG site correlation of DNA methylation and plotted the resulting correlation coefficients as heatmaps. Interestingly, different patterns were observed when comparing treatment

groups. Different correlation patterns between the degree of DNA methylation of one CpG site to another could result in a different net effect on gene expression by altering the attachment of transcription factors to gene promotors.

Cd300lf is expressed in monocytes and peripheral blood mononuclear cells.[27] *Cd300lf* acts as an inhibitory receptor for myeloid cells and mast cells.[28] It positively regulates the phagocytosis of apoptotic cells (efferocytosis) via phosphatidylserine (PS) recognition. It also plays an important role in the maintenance of immune homeostasis.[29] Phenotypes associated with *Cd300lf* include nervous system, homeostasis/metabolism and immune system phenotype,[30,31] but so far, few studies reported that this gene was involved in kidney diseases. Our findings are in agreement with studies suggesting that epigenetic modifications leading to glomerular and interstitial fibrosis through transcriptional regulation.[32,33] However, the specific molecular mechanism involved causing epigenetic changes of *Cd300lf* and subsequently alter kidney function and morphology in F2 female offspring need to be further analyzed.

The results of functional enrichment analysis of mRNAs and lncRNAs of our study showed that both differentially expressed mRNAs and lncRNAs were mainly enriched in antigen processing and presentation pathways. Similarly, a study by Jackson et al. found that maternal HFD can cause glomerulosclerosis and tubulointerstitial fibrosis in male offspring kidney by activating pro-inflammatory pathways.[34] A study conducted in a sheep model showed that maternal nutrient restriction during early fetal kidney development attenuates the effects of early onset obesity-related nephropathy, in part, through the downregulation of the innate inflammatory response.[35]

Our data suggest that paternal HFSSD over two generations resulted in functional alterations in the kidneys of female F2 offspring associated with increased glomerulosclerosis and tubulointerstitial fibrosis. The functional alterations observed in the F1 generation went into the same direction but did not reach statistical significance. For paternal programming induced by an unhealthy diet during spermatogenesis obviously two hits (high-fat, high-sucrose and high-salt diet during spermatogenesis in two subsequent paternal generations) are necessary. The effects were sex-dependent. Female F2 offspring are affected most. RNA sequencing followed by confirmatory qRT-PCR showed four differently regulated genes (Enpp6, Tmem144, Cd300lf, and Actr3b) in the kidneys of female F2 offspring. Methylation rate and pattern of CpG islands in the promoter region of Cd300lf was significantly invers correlated with Cd300lf mRNA expression in F2 female offspring ($r = -0.483$, $p = .0123$). Methylation pattern of the Cd300lf promotor was also altered, both effects might cause the observed effects on Cd300lf mRNA. Cd300lf mRNA expression itself was inversely correlated with the urinary albumin-to-creatinine ratio in female F2 offspring. Furthermore, the expression of lncRNA XR-146683.1 was significantly down-regulated in the F2 female offspring of fathers and grandfathers exposed to an unhealthy diet during spermatogenesis. Although we identified an epigenetic pathway linking Cd300lf methylation with urinary albumin excretion, it is more likely that complex alterations of gene expression are responsible for the observed renal phenotype in female F2 offspring of fathers and grandfathers on high-fat, high-sucrose and high-salt diet during spermatogenesis. In good agreement with this hypothesis, we saw that multiple renal genes (ENPP6, TMEM144, ACTR3B, and CD300LF) and also the non-coding RNA lncRNA XR-146683 are associated with the development of the renal phenotype of female F2 offspring (decreased GFR, increased urinary albumin excretion, glomerulosclerosis and renal interstitial fibrosis). Alteration of just a single pathway or even single genes as a result of fetal programming events during spermatogenesis and fetal development are rather uncommon, the environmental stimuli - high-fat, high-sucrose and high-salt diet during spermatogenesis in two subsequent paternal generations in our case—rather induce complex epigenetic marks[4,8,36–39] inducing the observed renal phenotype. How the interaction of these different epigenetic induced alterations in various gene expression patterns in the kidney finally causes the observed renal phenotype in female F2 offspring is yet unknown. Epigenetic alterations of paternal programming consist of two distinct epigenetic phenomena: the initial environmental factors—in our case the unhealthy pre-conceptional paternal diet—induced epigenetic alterations of the sperm (most important seem to be non-coding RNAs). This was first discovered in male rats exposed to a high fat diet. These initial alterations in the sperm causes later in life alterations in target organs such as the endocrine pancreas and fat tissues. DNA methylation of gene promotors of affected genes seems to be the main epigenetic alteration in this later stage of epigenetic changes.[19,40,41] We focused in our study on the late epigenetic effects in the kidney, see above. It is clearly a study limitation that we did not analyses epigenetic alterations in the sperm of the F0 and F1 generation and that we did not analyses epigenetic alterations in the less affected kidneys of the F1 generation.

Sex-dependency of fetal programming[37] and also paternal programming[19,40,41] is well known. A high paternal fat diet prior mating causes an impairment of glucose metabolism in particular in female offspring.[19] Potential underlying molecular pathways were reviewed recently.[37]

Taken together, our data suggest that a paternal pre-conceptional unhealthy diet might predispose in

particular female F2 offspring to chronic kidney disease. The mechanisms underlying this renal phenotype could involve differential regulation of the expression of mRNAs (Enpp6, Tmem144, Cd300lf, and Actr3b) and the lncRNA XR-146683.1 as well as alterations in the methylation pattern of CpG islands in the promoter region of the Cd300lf gene. This gene was reported to be involved in biological processes such as immune homeostasis and phagocytosis of apoptotic cells.

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DISCLOSURES

All authors declared no competing interests.

AUTHOR CONTRIBUTIONS

Berthold Hocher designed the study. Xiaoli Zhang, Ahmed A. Hasan, Suimin Zeng, Liping Liu and Li Xie performed the animal experiments and statistical analysis. Hongwei Wu performed bioinformatics analysis. Xiaoli Zhang, Ahmed A. Hasan, Jian Li and Berthold Hocher checked quality of the data. Xiaoli Zhang and Ahmed A. Hasan drafted the manuscript. Mohamed M. S. Gaballa, Jung Tobias, Grune Tilman, Bernhard K Krämer, Burkhard Kleuser, Jian Li and Berthold Hocher contributed to the revisions of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the methods and supplementary material of this article.

ORCID

Xiaoli Zhang https://orcid.org/0000-0003-0076-0854
Berthold Hocher https://orcid.org/0000-0001-8143-0579

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SUPPORTING INFORMATION

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