

·基础研究·

## 脂质合成异常增多加重糖尿病肾病和叶酸诱导的肾损伤

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**摘要:**【目的】探讨叶酸诱导的肾损伤模型和糖尿病肾病(DN)模型中脂肪酸代谢的改变及其影响。【方法】通过腹腔注射叶酸构建叶酸诱导的肾损伤模型。将9只成年C57BL/6J小鼠随机分为2组:生理盐水对照组(Saline,  $n=5$ )和叶酸干预组(FA,  $n=4$ )。FA组小鼠腹腔单次注射叶酸(250 mg/kg), Saline组则注射相应体积的生理盐水。采用高脂饮食联合链脲佐菌素注射构建DN模型。取11只成年C57BL/6J小鼠, 随机分为2组:对照组(ND,  $n=6$ )和模型组(DN,  $n=5$ )。ND组全程饲喂正常饲料; DN组饲喂高脂饲料8周后单次腹腔注射链脲佐菌素(100 mg/kg), 并继续饲喂高脂饲料至第16周。(1)采用HE和天狼星红染色观察4组小鼠肾脏的病理改变。(2)小鼠的肾脏组织进行RNA-Seq分析, 筛选差异表达基因(DEGs)并进行GO、KEGG和GSEA分析。采用RT-qPCR检测各组小鼠的脂肪酸合成基因和纤维化相关基因的mRNA水平; 制备肾脏冰冻切片进行油红O染色检测脂滴蓄积情况。(3)经10 ng/mL重组人TGF- $\beta$ 1处理或高糖条件培养的人肾皮质近曲小管上皮细胞(HK-2)通过RT-qPCR分析脂肪酸合成基因、纤维化相关基因的mRNA水平; 利用Bodipy 493/503染色检测HK-2细胞和同样处理的原代肾小管上皮细胞(PTECs)的脂滴蓄积情况。(4)经100  $\mu$ mol/L棕榈酸(PA)处理的HK-2细胞通过Bodipy 493/503染色评估脂滴蓄积情况。(5)利用SA- $\beta$ -gal染色分别检测4组小鼠肾脏组织的衰老情况。利用SA- $\beta$ -gal染色、Western blot和RT-qPCR评估PA处理的HK-2细胞衰老及衰老相关分泌表型因子的表达情况; 利用免疫荧光分析PA处理的PTECs对肾脏成纤维细胞活化的影响。【结果】(1)FA和DN组小鼠肾脏组织出现肾小管结构损伤和胶原纤维沉积的病理学改变。(2)FA和DN组的差异表达基因显著富集于代谢相关通路, 尤其是脂代谢相关通路。与Saline对照组相比, FA组脂肪酸合成基因*Srebp1*和*Fasn*的表达显著上调(*Srebp1*:  $t=2.445$ ,  $P=0.044$  4; *Fasn*:  $t=2.571$ ,  $P=0.037$  0); 与ND组相比, DN组*Srebp1*、*Acc1*和*Fasn*基因表达上调(*Srebp1*:  $t=3.354$ ,  $P=0.010$  0; *Acc1*:  $t=2.602$ ,  $P=0.031$  5; *Fasn*:  $t=2.358$ ,  $P=0.046$  1)。FA和DN组的纤维化相关基因*Col1a1*均呈现表达上调(FA:  $t=2.628$ ,  $P=0.034$  0; DN:  $t=3.602$ ,  $P=0.007$  0), 并伴有脂质蓄积的病理表现。(3)TGF- $\beta$ 1或高糖处理的HK-2细胞中*SREBP1*( $F=15.41$ ,  $P=0.004$  3; TGF $\beta$ 组:  $P=0.002$  9; HG组:  $P=0.045$  2)、*ACCI*( $F=30.30$ ,  $P=0.000$  7; HG组:  $P=0.001$  4)和*FASN*( $F=18.76$ ,  $P=0.002$  6; TGF $\beta$ 组:  $P=0.001$  7; HG组:  $P=0.031$  0)的mRNA水平显著上调, 且两种处理均诱导HK-2细胞和PTECs脂质蓄积。(4)FA和DN组肾脏中衰老细胞增多, 并且PA处理可诱导肾小管上皮细胞脂质蓄积并加快细胞衰老和成纤维细胞活化。【结论】叶酸性肾病和糖尿病肾病模型的肾脏中脂代谢相关基因的转录均发生异常, 脂肪酸合成过度则会引发脂质蓄积, 诱导PTECs衰老, 最终促进纤维化发生发展。

**关键词:**叶酸性肾病; 糖尿病肾病; 脂肪酸代谢; 脂质蓄积; 细胞衰老; 纤维化

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## Aberrant Increase in Lipid Synthesis Exacerbates Diabetic Nephropathy and Folic Acid-induced Kidney Injury.

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**Abstract:** **[Objective]** To investigate the changes and effects of fatty acid metabolism in folic acid (FA)-induced kidney injury and diabetic nephropathy (DN) mouse models. **[Methods]** The FA-induced kidney injury model was established via intraperitoneal injection of FA. Nine adult C57BL/6J mice were randomly allocated to two groups: saline control group (Saline,  $n=5$ ) and FA intervention group ( $n=4$ ). Mice in the FA group received a single intraperitoneal injection of FA (250 mg/kg), whereas those in the Saline group were administered an equivalent volume of normal saline. The DN model was constructed using a high-fat diet combined with streptozotocin (STZ) injection. Eleven adult C57BL/6J mice were randomly divided into two groups: normal diet control group (ND,  $n=6$ ) and DN model group (DN,  $n=5$ ). The ND group was fed a normal diet throughout the experiment; the DN group was maintained on a high-fat diet for 8 weeks, followed by a single intraperitoneal injection of STZ (100 mg/kg), and then continued on the high-fat diet until week 16. (1) Hematoxylin and eosin (HE) and sirius red staining were used to observe pathological changes in the kidneys of the four groups of mice. (2) RNA-Seq was performed on mouse kidney tissues to screen for differentially expressed genes (DEGs), followed by Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Gene Set Enrichment Analysis (GSEA). RT-qPCR was used to detect the mRNA levels of fatty acid synthesis and fibrosis-related genes. Lipid droplet accumulation was assessed by Oil Red O staining on frozen kidney sections. (3) Human renal cortical proximal tubular epithelial cells (HK-2) and proximal tubular epithelial cells (PTECs) were treated with 10 ng/mL recombinant human TGF- $\beta$ 1 or high glucose. RT-qPCR was used to measure the mRNA levels of fatty acid synthesis and fibrosis-related genes in HK-2 cells. Lipid droplet accumulation in HK-2 cells and PTECs was detected using Bodipy 493/503 staining. (4) HK-2 cells were treated with 100  $\mu$ mol/L palmitic acid (PA). Bodipy 493/503 staining was used to evaluate lipid droplet accumulation. (5) SA- $\beta$ -gal staining was used to assess senescence in kidney tissues from all four mouse groups. Cellular senescence and the expression of senescence-associated secretory phenotype (SASP) factors in PA-treated HK-2 cells were assessed using SA- $\beta$ -gal staining, Western blot, and RT-qPCR. The effect of PA-treated PTECs on renal fibroblast activation was assessed by immunofluorescence. **[Results]** (1) Both FA and DN groups showed pathological changes in kidney tissue, including renal tubular structural damage and collagen fiber deposition. (2) DEGs in both FA and DN groups were significantly enriched in metabolism-related pathways, especially lipid metabolism pathways. Compared to the Saline control, the expression of fatty acid synthesis genes *Srebp1* and *Fasn* was significantly upregulated in the FA group (*Srebp1*:  $t=2.445$ ,  $P=0.044$  4; *Fasn*:  $t=2.571$ ,  $P=0.037$  0). Compared to the ND group, *Srebp1*, *Acc1*, and *Fasn* gene expression was upregulated in the DN group (*Srebp1*:  $t=3.354$ ,  $P=0.010$  0; *Acc1*:  $t=2.602$ ,  $P=0.031$  5; *Fasn*:  $t=2.358$ ,  $P=0.046$  1). The fibrosis-related gene *Col1a1* was also upregulated in both FA and DN groups (FA:  $t=2.628$ ,  $P=0.034$  0; DN:  $t=3.602$ ,  $P=0.007$  0), accompanied by pathological signs of lipid accumulation. (3) In HK-2 cells treated with TGF- $\beta$ 1 or high glucose, the mRNA expression levels of *SREBP1* ( $F=15.41$ ,  $P=0.004$  3; TGF $\beta$ :  $P=0.002$  9; HG:  $P=0.045$  2), *ACCI* ( $F=30.30$ ,  $P=0.000$  7; HG:  $P=0.001$  4), and *FASN* ( $F=18.76$ ,  $P=0.002$  6; TGF $\beta$ :  $P=0.001$  7; HG:  $P=0.031$  0) were significantly upregulated. Both treatments also induced lipid accumulation in HK-2 cells and PTECs. (4) There was an increase in senescent cells in the kidneys of both the FA and DN groups. PA treatment induced lipid accumulation, accelerated senescence, and activated fibroblasts in renal tubular epithelial cells. **[Conclusions]** Transcriptional abnormalities of lipid metabolism-related genes were observed in the kidneys of both FA-induced kidney injury and diabetic nephropathy models. Excessive fatty acid synthesis leads to lipid accumulation, which induces senescence in PTECs and ultimately promotes the development and progression of fibrosis.

**Key words:** folic acid-induced kidney injury; diabetic nephropathy; fatty acid metabolism; lipid accumulation; cellular senescence; fibrosis

慢性肾脏疾病(chronic kidney disease, CKD)是一种以肾小球滤过率逐渐下降和肾脏结构功能异常(持续 $\geq 3$ 个月)为特征的临床综合征。若不及时干预,CKD可能进展为终末期肾病,对患者生命健康构成严重威胁。流行病学数据显示,CKD的全球患病率逐年上升,影响10%左右的全球人口(约8亿人),其中我国的患病率约为11%,相关死亡率为1.9%<sup>[1-2]</sup>。CKD的病理机制复杂,由糖尿病、高血压、肥胖及药物性肾损伤等多种因素驱动,最终导致肾纤维化。糖尿病肾病是CKD的主要类型之一,也是终末期肾病的重要病因<sup>[3]</sup>。此外,急性肾损伤(acute kidney injury, AKI),如由药物过量蓄积引发,可进一步演变为CKD,并诱发肾间质纤维化<sup>[4]</sup>。叶酸(folic acid, FA)诱导的AKI-CKD进展模型常用于研究肾毒性小管损伤及肾纤维化的发生机制。尽管CKD的发病率和死亡率持续攀升,但当前临床治疗手段的疗效有限<sup>[5]</sup>。因此,通过经典动物模型深入研究不同类型CKD的共同病理特征,并探索有效的干预靶点,对提升患者预后具有重要的科学意义和临床应用价值。脂代谢紊乱与肾脏纤维化的发生密切相关,肾脏内脂质异常蓄积及其引发的脂毒性是肾损伤和纤维化进程的关键驱动因素,干预脂代谢紊乱可有效延缓肾脏纤维化的发展<sup>[6]</sup>。脂质作为细胞功能和生物学过程的重要分子,在能量代谢中发挥核心作用<sup>[7-8]</sup>。脂代谢失衡可加速多种疾病的进展,包括肥胖、代谢综合征及2型糖尿病<sup>[9]</sup>。尽管肾纤维化的具体发病机制尚未完全阐明,但已有证据表明,肾脏内脂质异常蓄积可诱导炎症反应并促进纤维化进程<sup>[10]</sup>。衰老是肾纤维化的重要危险因素,衰老细胞通过分泌炎症因子和促纤维化因子等衰老相关分泌表型,进一步加速纤维化进程<sup>[11]</sup>。最近研究表明,脂代谢紊乱不仅与年龄相关疾病有关,还调控细胞衰老过程<sup>[7]</sup>。作为脂代谢的核心环节,脂肪酸代谢紊乱在多种疾病及器官纤维化进展中起到重要作用<sup>[12]</sup>。本研究旨在通过构建FA诱导的AKI向CKD进展模型及DN模型,系统比较脂肪酸代谢紊乱在不同病因驱动的肾纤维化过程中的作用,并深入探讨其潜在的共性分子机制。

## 1 材料与方法

### 1.1 试剂与仪器

高脂饲料(D12492)购自 Research Diets; 叶酸(F7876)、链脲佐菌素(18 883-66-4)、II型胶原酶(C6885)、表皮生长因子(E4127)、棕榈酸钠(P9767)、油红O粉末(00625)和SA- $\beta$ -gal(CS0030)购自Sigma公司;重组人TGF- $\beta$ 1(240-B-010)购自安迪(R&D);40  $\mu$ m过滤器(352340)购自Falcon公司;苏木素-伊红染色试剂盒(C0105S)、山羊血清(C0265)、PMSF(ST2573)、蛋白酶抑制剂(P1005)、RIPA裂解液(P0013B)和HRP标记的山羊抗兔/鼠二抗(A0208/A0216)购自碧云天;PVDF膜(IPVH00010)和ECL化学发光试剂盒(WBKLS0500)购自Millipore;牛血清白蛋白(bovine serum albumin, BSA, A8020)购自北京索莱宝科技有限公司;天狼星红(BP-DL029)购自南京森贝伽生物科技有限公司;反转试剂盒(RR036A)购自Takara;SYBR Green Master Mix(11202E508)购自翌圣;Bodipy 493/503(D3922)购自Invitrogen;Fibronectin抗体(66042-1-Ig)购自Proteintech公司; $\beta$ -actin抗体(4970S)、P53抗体(2524S)和DAPI(4083S)购自CST;P16抗体(sc-1661)购自Santa Cruz Biotechnology公司;胎牛血清(fetal bovine serum, FBS, 10091148)、青霉素-链霉素-谷氨酰胺(penicillin-streptomycin-glutamine, PSG, 10378016)和DMEM/F12(8122437)购自Gibco;DMEM/High glucose(SH30243.01)、RPMI(SH30809.01)和蛋白印迹成像系统(Amersham ImageQuant 800)购自Cytiva;倒置相差显微镜购自OLYMPUS;正置光学显微镜(Leica ICC50W)和共聚焦显微镜(Leica SP8)购自Leica;BCA试剂盒(23227)和实时荧光定量PCR仪(ABI QuantStudio 5)购自Thermo Fisher。

### 1.2 实验动物

20只7周龄SPF级的健康雄性C57BL/6J小鼠(体质量约20~22 g)购自北京斯贝福生物技术有限公司(生产许可证号SCXK(京)2019-0010)。小鼠未检测到细菌、病毒感染和寄生虫,合格证编号:110324221107787815。小鼠饲养于恒温(22  $^{\circ}$ C

$\pm 2$  °C)、12 h光照/12 h黑暗循环周期的SPF环境中,可自由进食和饮水。所有动物实验操作及管理均经广东医科大学动物福利与伦理委员会批准(批准号:GDY2004002)。

### 1.3 模型建立

1.3.1 叶酸性肾病模型 小鼠适应环境1周后随机分为两组:生理盐水对照组(Saline)和叶酸(FA)干预组。FA组小鼠单次腹腔注射叶酸(250 mg/kg),Saline组则注射相同体积的生理盐水,造模28 d后收集肾脏组织进行分析。小鼠禁食过夜,收集肾脏组织进行基因表达和表型分析。

1.3.2 糖尿病肾病模型 小鼠适应环境1周后随机分为对照组和模型组。对照组饲喂正常饲料,模型组饲喂高脂饲料。模型组饲喂高脂饲料8周后,单次腹腔注射链脲佐菌素(streptozotocin, STZ, 100 mg/kg),之后持续喂养高脂饲料至造模第16周。小鼠禁食过夜,收集肾脏组织进行基因表达和表型分析。

### 1.4 细胞培养及处理

人肾皮质近曲小管上皮细胞(HK-2)购自中国科学院细胞中心(上海,中国)。细胞在含100 mL/L的FBS和10 mL/L PSG的DMEM/F12培养基中培养。TGF- $\beta$ 1组加入终浓度为10 ng/mL的重组人TGF- $\beta$ 1进行处理;棕榈酸(palmitic acid, PA)组加入终浓度为100  $\mu$ mol/L的PA处理细胞;高糖(HG)组则更换为高糖培养基(DMEM/High Glucose, 25 mmol/L D-Glucose)培养细胞。原代肾小管上皮细胞(proximal tubular epithelial cell, PTECs)和原代肾脏成纤维细胞来源于同一只小鼠的肾脏。分离小鼠肾脏皮质,剪为1~3 mm<sup>2</sup>大小的碎块,加入含有II型胶原酶(100 ng/mL)的培养基,置于37 °C水平摇床进行消化。40  $\mu$ m细胞滤器过滤消化后的上清,滤液中的组分在含100 mL/L的FBS和20 mL/L PSG的DMEM/High Glucose培养基中进行培养以获得成肾纤维细胞。滤网上保留的肾小管在含100 mL/L的FBS、20 mL/L PSG和25 ng/mL表皮生长因子的DMEM/F12培养基中培养7 d分化为肾小管上皮细胞。肾原代成纤维细胞与PTECs共培养的实验步骤如下:首先,用PA处理PTECs 24 h后,收集其培养上清(即条件培养基),用于培养原代成纤维

细胞。随后,继续用PA处理PTECs 12 h,收集第二次条件培养基,并将其转移至原代成纤维细胞进行培养。接受两次条件培养基的原代成纤维细胞继续培养24 h。所有细胞均置于37 °C、体积分数5% CO<sub>2</sub>的培养箱中培养。

### 1.5 肾脏组织学染色

将小鼠肾脏组织置于40 g/L的多聚甲醛(paraformaldehyde, PFA)中固定。固定的组织依次置于浓度递增的乙醇(500 mL/L、700 mL/L、800 mL/L、900 mL/L、1 000 mL/L)中梯度脱水,二甲苯透明后进行石蜡包埋。石蜡包埋块切成5  $\mu$ m厚切片。切片于62 °C进行烘烤,经二甲苯脱蜡及梯度乙醇水化(1 000 mL/L、900 mL/L、700 mL/L)后,使用苏木素伊红(HE)染色试剂盒进行染色。苏木素染8 min后蒸馏水洗涤,随后伊红染50 s。切片经梯度乙醇(950 mL/L、1 000 mL/L)脱水、二甲苯透明,最后以中性树胶封片。使用光学显微镜随机采集图像,观察肾脏组织结构。

### 1.6 肾脏组织天狼星红染色

肾脏石蜡切片经烤片、二甲苯脱蜡及梯度乙醇水化后,使用商品化天狼星红染色试剂盒进行染色。切片置于染液中浸染,随后依次经梯度乙醇脱水及二甲苯透明,最后以中性树胶封片。使用光学显微镜随机选取多个视野采集图像,并用ImageJ软件进行胶原沉积面积百分比的定量分析。

### 1.7 肾脏组织油红O染色

经40 g/L PFA固定的肾脏组织经蔗糖梯度脱水进行OCT包埋,切成8  $\mu$ m厚的切片。称取0.5 g油红O粉末溶于100 mL的异丙醇中,用超纯水配置成600 mL/L的油红O染液,并使用0.45  $\mu$ m过滤器过滤。冰冻切片于室温复温,用600 mL/L的异丙醇浸泡1 min。随后切片置于油红染液中避光浸染10 min,超纯水洗涤2次。切片置于苏木素染液中浸染2 min,蒸馏水洗涤3 min后用超纯水洗涤1次,最后以甘油明胶封片。使用显微镜镜下随机选取视野采集图像,Image J软件进行阳性面积统计分析。

### 1.8 RNA测序与基因富集分析

本研究委托广州基迪奥生物科技有限公司对肾脏组织样本进行转录组测序(RNA-seq)。在

Illumina平台进行高通量测序后对测序数据进行生物信息学分析,包括序列比对、基因表达量计算、差异表达基因筛选,以及对转录水平上的差异表达基因进行功能注释和富集分析,涵盖基因本体论(gene ontology, GO)、京都基因与基因组百科全书(kyoto encyclopedia of genes and genome, KEGG)、基因集富集分析(gene set enrichment analysis, GSEA)分析等。利用DESeq2进行差异表达分析,筛选差异表达基因(differentially expressed genes, DEGs),设定 $\log_2(\text{fold change}) \geq 1.5$ 且校正后 $P\text{-value} \leq 0.05$ 为标准,定义为上调或者下调的差异表达基因。

### 1.9 RNA提取和实时定量PCR分析

采用Trizol法提取肾脏组织RNA。取1  $\mu\text{g}$  RNA,使用PrimeScript RT试剂盒(含gDNA消除剂)进行基因组去除和反转录合成cDNA。SYBR Green进行实时荧光定量PCR(qPCR),以 $\beta\text{-actin}$ 或者Hprt作为内参,通过 $2^{-\Delta\Delta CT}$ 方法计算目标基因的相对表达量。引物合成由广州艾基生物技术有限公司完成。

### 1.10 细胞脂滴Bodipy 493/503染色

将HK-2细胞或PTECs接种于含无菌爬片的24孔板中。细胞培养完成后PBS洗3次,加入40 g/L的PFA室温固定细胞15 min, PBS洗3次。使用Bodipy 493/503荧光染料(用PBS按1:1 000稀释)室温避光孵育10 min, DAPI(用PBS按1:1 000稀释)染核5 min,随后用PBS洗3次。滴加抗荧光猝灭剂并进行封片。使用徕卡共聚焦显微镜采集图像,用ImageJ软件对平均荧光强度进行统计分析。

### 1.11 SA- $\beta$ -gal染色

用衰老染色固定液固定肾脏冰冻切片或HK-2细胞,室温固定10 min后弃去,PBS洗3次。每孔加入SA- $\beta$ -gal(senescence-associated  $\beta$ -galactosidase)染液覆盖细胞,于37  $^{\circ}\text{C}$ 无 $\text{CO}_2$ 的培养箱中孵育,观察显微镜下的染色程度,用PBS终止染色。染色结束后,使用光学倒置显微镜随机选取视野采集图像,ImageJ软件统计阳性面积百分比。

### 1.12 免疫荧光染色

肾脏冰冻切片恢复至室温后,使用PBS洗去OCT包埋剂。使用100 mL/L山羊血清于37  $^{\circ}\text{C}$ 封闭

30 min。将Fibronectin抗体以1:600的比例稀释,并于4  $^{\circ}\text{C}$ 孵育过夜。用PBST洗涤切片,加入抗小鼠的荧光二抗,室温避光孵育1.5 h。DAPI室温避光染核5 min。再次PBST洗涤后,滴加抗荧光猝灭剂并进行封片。使用Leica共聚焦显微镜采集图像,用Image J软件对平均荧光强度进行统计分析。

### 1.13 蛋白免疫印迹实验

收集HK-2细胞,用PBS洗3次,并加入预冷的含PMSF和蛋白酶抑制剂的RIPA裂解液在冰上裂解30 min。裂解后的细胞组分在4  $^{\circ}\text{C}$ 条件下12 000 $\times g$ 离心10 min。收集上清,使用BCA试剂盒检测蛋白浓度。取25  $\mu\text{g}$ 蛋白于十二烷基硫酸钠-聚丙烯酰胺凝胶(SDS-PAGE)电泳分离,并采用湿转法将蛋白转移至PVDF膜。PVDF膜用50 g/L脱脂奶粉封闭90 min,根据彩色Marker大小与相应的一抗P16(1:600)、P53(1:1 000)或 $\beta\text{-actin}$ (1:1 000)4  $^{\circ}\text{C}$ 孵育过夜。洗涤后的PVDF膜加入1:2 000稀释的HRP标记的二抗,室温孵育90 min后再次洗涤,并加入ECL化学发光底物进行曝光。根据Image J软件分析的条带的灰度值,计算P16和P53蛋白相对与 $\beta\text{-actin}$ 的表达量。

### 1.14 统计学处理

运用GraphPad Prism 9.0软件进行实验数据的统计分析,所有数据均采用均数 $\pm$ 标准差(mean $\pm$ SD)表示。两组数据的比较采用 $t$ 检验;三组数据的比较采用单因素方差分析(ANOVA),并用Bonferroni比较方法进行两两比较(与对照组比较)。 $P < 0.05$ 代表有统计学差异。

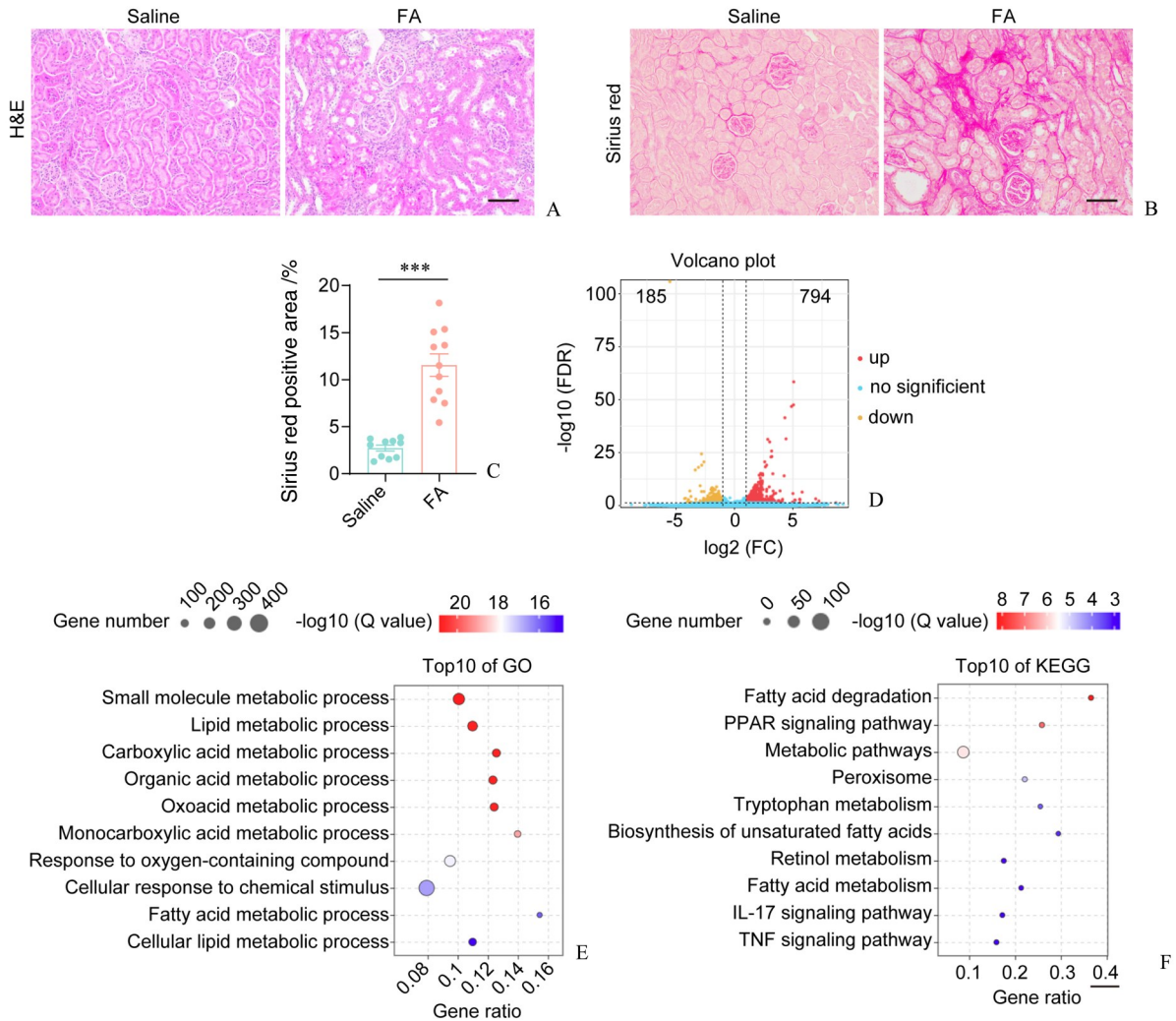
## 2 结果

### 2.1 叶酸处理导致肾脏代谢基因表达异常

肾脏切片的HE染色结果显示,叶酸FA组小鼠出现肾脏组织受损、肾小管扩张、炎症细胞浸润等病理现象(图1A)。与生理盐水对照组相比,FA组胶原沉积也显著升高( $t=6.876$ ,  $P < 0.000 1$ ),见图1B、C。以上病理染色结果显示单次大剂量腹腔注射FA成功建立了肾纤维化模型。为解析其分子水平的变化,我们对两组小鼠的肾脏组织进行了转录组测序,并筛选到794个FA组上调的基因和185个

下调的基因,见图1D差异表达基因的火山图。通过对这些差异基因进行GO和KEGG分析,发现代谢相关通路显著富集,如小分子代谢过程、脂代谢

过程、过氧化物酶体增殖物激活受体( Peroxisome proliferator-activated receptor, PPAR)信号通路等(图1E、F)。



A: HE staining of kidney sections (scale bar=100  $\mu$ m). B and C: Representative Sirius Red staining of kidney sections (scale bar=100  $\mu$ m) and quantitative analysis of the positive staining area. D and E: GO and KEGG pathway enrichment analyses of differentially expressed genes in the kidneys of saline-treated versus FA-treated mice,  $\log_2$  (fold change) $\geq$ 1.5. Mean  $\pm$  SD. Saline group ( $n=10$ ). FA group ( $n=11$ ). \*\*\* $P$ <0.0001 vs Saline group.

图1 叶酸处理导致肾脏中的代谢基因表达异常

Fig. 1 Dysregulation of metabolic genes in the kidneys of mice with folic acid-induced renal injury

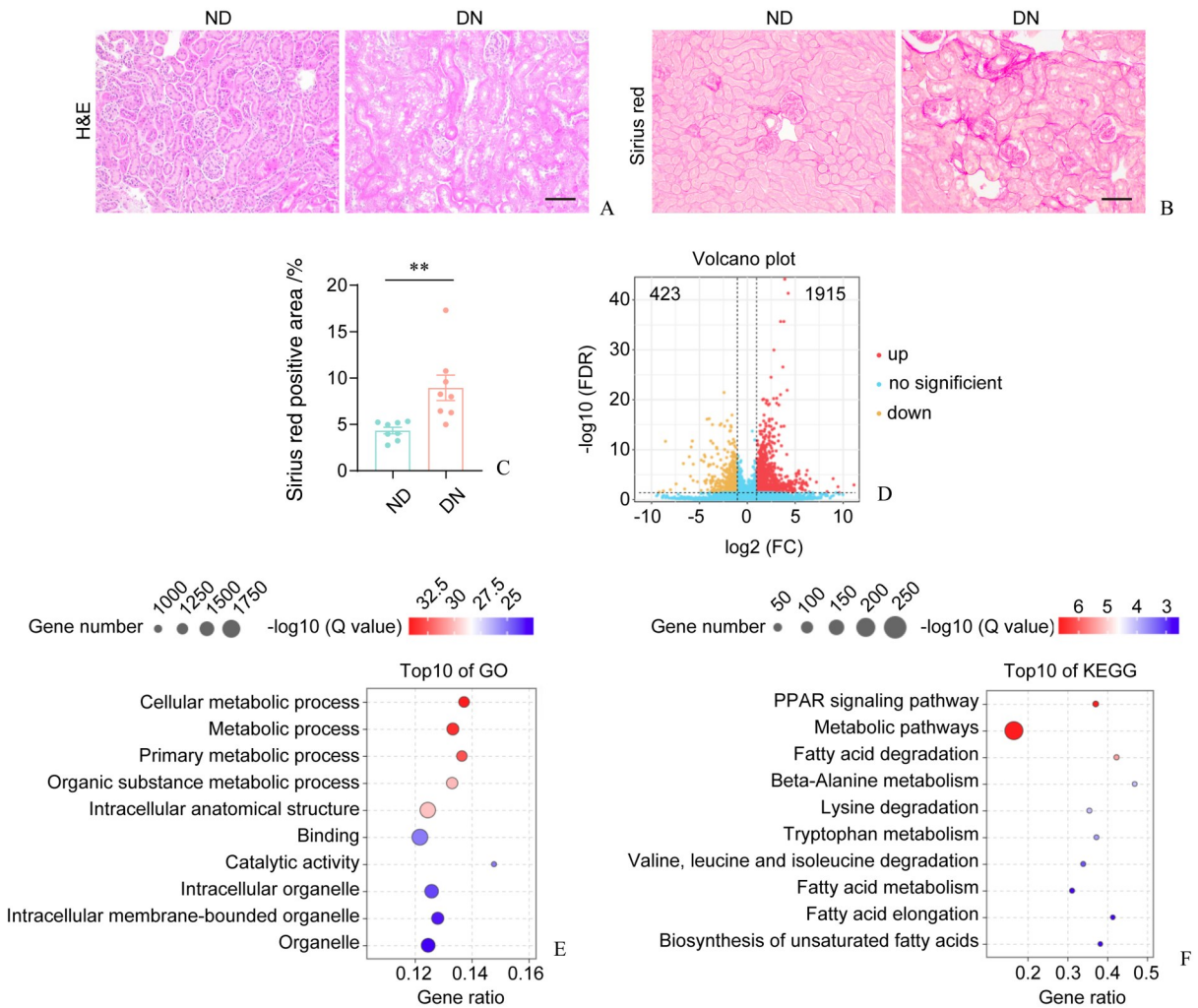
## 2.2 糖尿病肾病模型的肾脏中代谢基因表达失调

腹腔注射STZ和饲喂高脂饲料建立的糖尿病肾病模型的肾脏切片HE染色结果显示,糖尿病肾病组肾脏组织结构受损严重、肾小管上皮细胞空泡变性(图2A)。与正常饮食对照组(normal diet, ND)相比, DN组出现明显的胶原纤维沉积( $t=3.280$ ,  $P=0.0055$ ),见图2B、C。两组小鼠的肾脏组织的转录组分析筛选到1915个DN组上调的基因

和423个下调的基因(图2D)。对差异表达基因进行GO和KEGG分析,同样富集到多个代谢相关通路,如细胞代谢过程、PPAR信号通路、脂肪酸降解等(图2E、F)。

## 2.3 叶酸诱导的肾损伤模型和糖尿病肾病模型的肾脏中脂肪酸代谢基因表达异常

韦恩图分析显示,叶酸性肾病模型(Saline vs FA)和糖尿病肾病模型(ND vs DN)肾脏的差异表



A: HE staining of kidney sections (scale bar=100  $\mu\text{m}$ ). B and C: Representative Sirius Red staining of kidney sections (scale bar=100  $\mu\text{m}$ ) and quantitative analysis of the positive staining area ( $n=8$ ). \*\* $P < 0.01$  vs ND group. D and E: GO and KEGG pathway enrichment analyses of differentially expressed genes in the kidneys of non-diabetic (ND) versus DN mice,  $|\log_2(\text{fold change})| \geq 1.5$ . Mean  $\pm$  SD.

图2 糖尿病肾病模型的肾脏中代谢基因表达失调

Fig. 2 Dysregulation of metabolic genes in the kidneys of mice with diabetic nephropathy

达基因存在交集,共有550个核心差异表达基因(图3A)。针对这些差异表达基因的GO分析显示,脂质代谢过程、脂肪酸分解代谢等生物过程显著富集;KEGG分析则显示其在脂肪酸代谢、PPAR信号通路、脂肪酸降解等通路显著富集(图3B、C)。GSEA分析进一步支持两个模型中脂肪酸代谢通路的显著失调(图3D、E)。

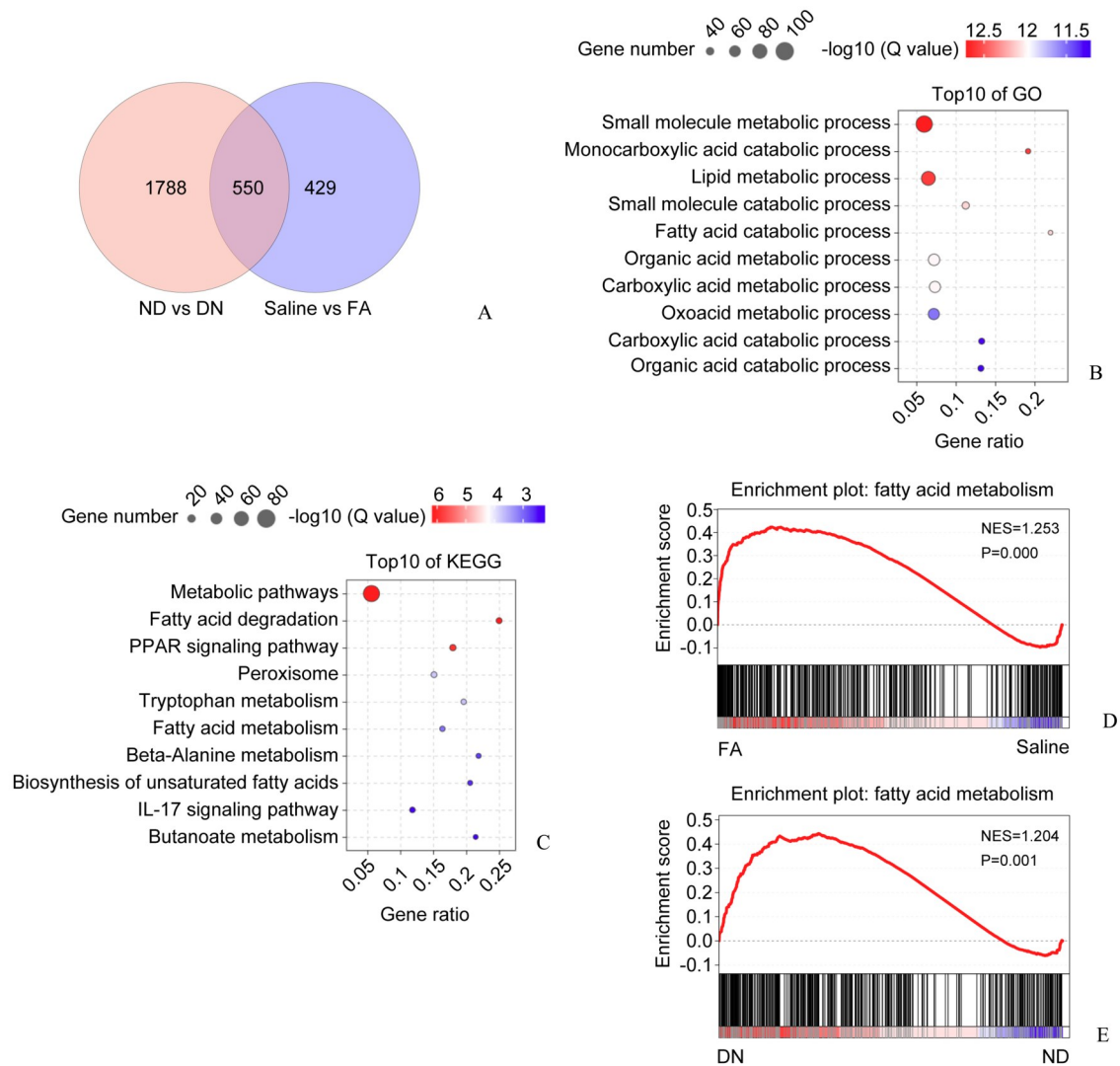
#### 2.4 叶酸诱导的肾损伤模型和糖尿病肾病模型的肾脏中脂肪酸合成基因上调导致脂质累积

与Saline组相比,FA组小鼠肾脏中的纤维化相关基因I型胶原*Colla1*、脂质合成转录因子*Srebp1*以及脂肪酸合酶*Fasn*的mRNA表达水平平均升高(*Colla1*:  $t=2.628$ ,  $P=0.0340$ ; *Srebp1*:  $t=2.445$ ,  $P=$

$0.0444$ ; *Fasn*:  $t=2.571$ ,  $P=0.0370$ ),见图4A、B。相较于ND组, DN组小鼠肾脏同样呈现*Colla1*、*Srebp1*、乙酰辅酶A羧化酶*Acc1*、*Fasn*的表达上调(*Colla1*:  $t=3.602$ ,  $P=0.0070$ ; *Srebp1*:  $t=3.354$ ,  $P=0.01$ ; *Acc1*:  $t=2.602$ ,  $P=0.0315$ ; *Fasn*:  $t=2.358$ ,  $P=0.0461$ ),见图4C、D。油红O染色结果进一步显示,相较于对照组,FA组和DN组小鼠均出现明显的脂质蓄积现象(FA:  $t=12.73$ ,  $P<0.0001$ ; DN:  $t=7.239$ ,  $P<0.0001$ ),见图4E-H。

#### 2.5 TGF- $\beta$ 1或高糖处理的肾小管上皮细胞中脂肪酸合成基因上调导致脂质累积

我们接下来用TGF- $\beta$ 1处理HK-2细胞模拟体内叶酸诱导的肾损伤模型, HG处理HK-2细胞模



A: Venn diagram depicting the overlap of DEGs between FA-induced and DN mouse models. B and C: GO and KEGG pathway enrichment analyses of the intersecting DEGs in FA-induced and DN mouse models. D: GSEA analysis of DEGs in FA-induced and DN mouse models.

图3 叶酸诱导的肾损伤模型和糖尿病肾病模型的肾脏中脂肪酸代谢基因表达失调

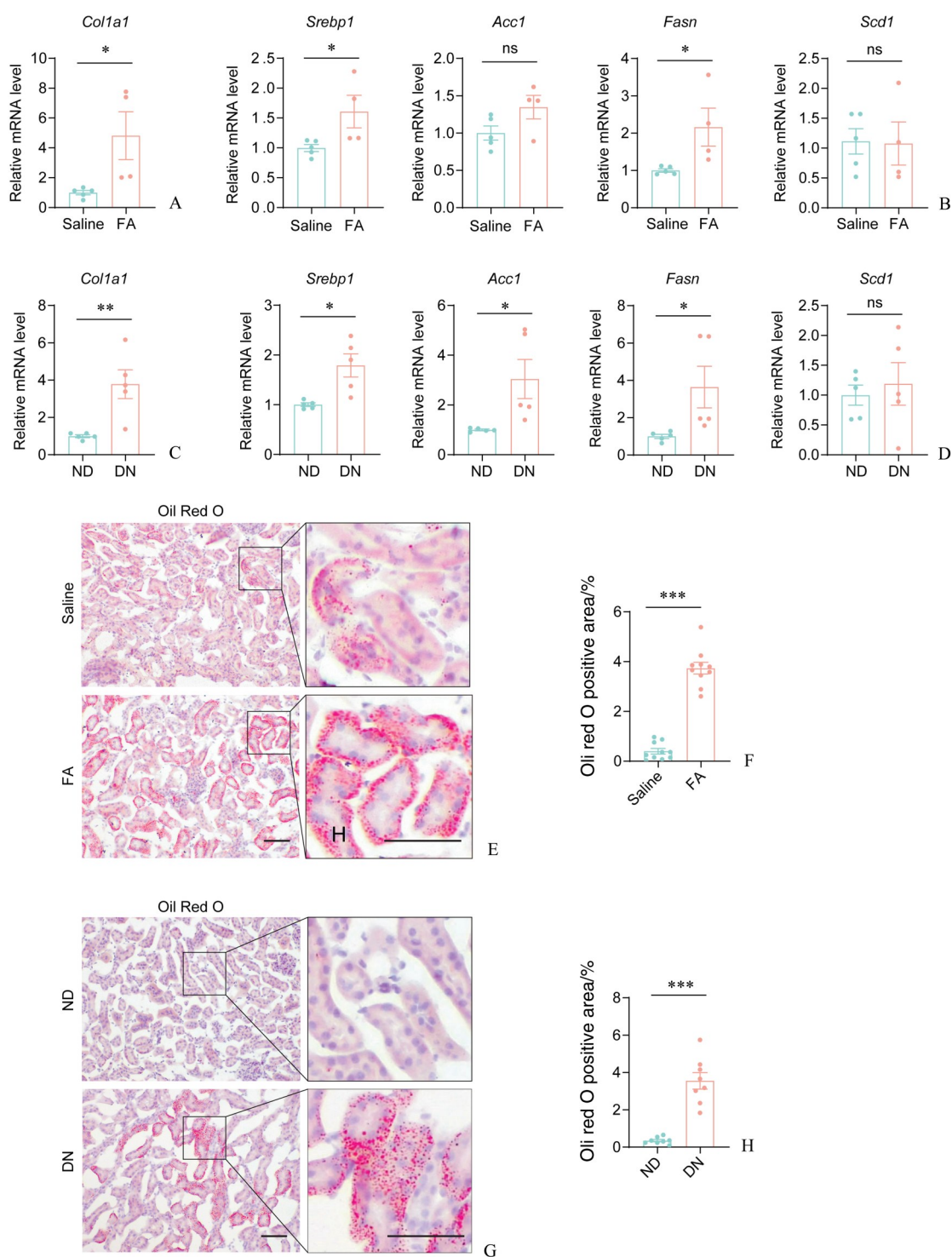
Fig. 3 Dysregulated renal fatty acid metabolism in folic acid-induced kidney injury and diabetic nephropathy in mice

拟糖尿病肾病模型。RT-qPCR结果显示,两种处理均显著上调HK-2细胞中纤维化相关因子 *ACTA2* 的表达 ( $F=28.07$ ,  $P=0.0009$ ; TGF $\beta$ 组:  $P=0.0006$ ; HG组:  $P=0.0064$ ; 图5A)。如见图5B所示,与对照组相比,脂肪酸合成相关基因 *SREBP1* 在两种处理条件下均表达上调 ( $F=15.41$ ,  $P=0.0043$ ; TGF $\beta$ 组:  $P=0.0029$ ; HG组:  $P=0.0452$ )。尽管TGF- $\beta$ 1处理组无差异, *ACCI* 基因在HG组表达上调 ( $P=0.0014$ )。脂肪酸合成酶 *FASN* 在两种处理条件下均表达上调 ( $F=18.76$ ,  $P=0.0026$ ; TGF $\beta$ 组:  $P=0.0017$ ; HG组:  $P=0.0310$ ), 而单不饱和脂肪酸 *SCD1* 在两种处理条件下表达无差异。基于叶

酸性肾病和糖尿病肾病模型中脂滴主要蓄积在肾小管上皮细胞,接下来我们通过Bodipy 493/503染色检测了两种不同处理条件下HK-2细胞和PTECs的脂质蓄积情况。与对照组相比, TGF- $\beta$ 1或高糖处理促使HK-2细胞和PTECs中的脂质蓄积(TGF $\beta$ 处理HK-2组:  $t=9.409$ ,  $P<0.0001$ ; HG处理HK-2组:  $t=7.114$ ,  $P<0.0001$ ; TGF $\beta$ 处理PTECs组:  $t=2.572$ ,  $P=0.0205$ ; HG处理PTECs组:  $t=5.241$ ,  $P<0.0001$ ; 图5C-J)。

## 2.6 脂质蓄积诱导的肾小管上皮细胞衰老通过旁分泌作用促进成纤维细胞活化

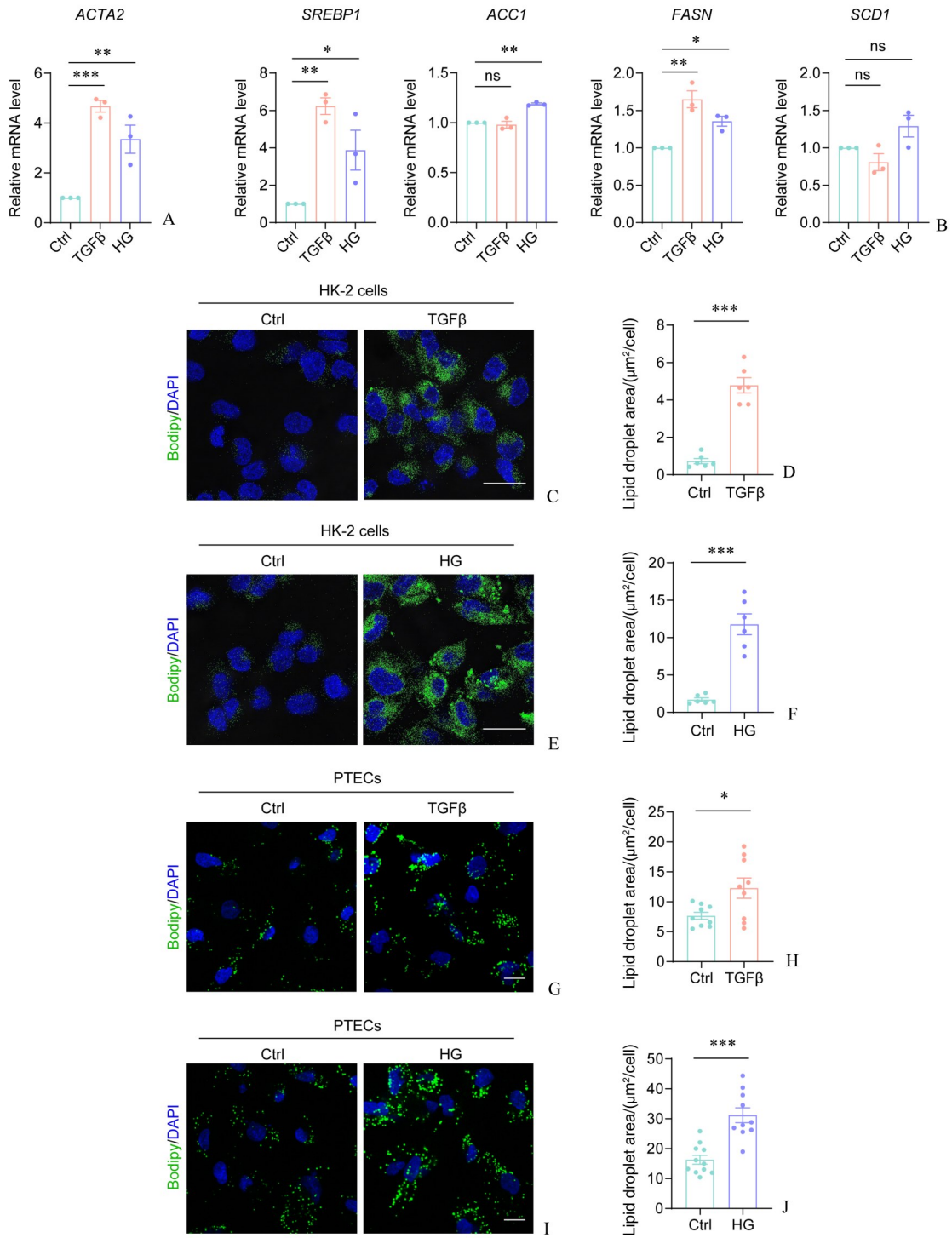
基于肾脏组织转录组的GSEA分析发现,细胞



A and B: mRNA expression levels of *Col1a1* and fatty acid synthesis genes in kidneys of saline-treated versus FA-treated mice. Saline group ( $n=5$ ). FA group ( $n=4$ ). C and D: mRNA expression levels of *Col1a1* and fatty acid synthesis genes in kidneys of non-diabetic (ND) versus DN mice.  $n=5$ . E-H: Representative Oil Red O staining of kidney sections (scale bar=100  $\mu\text{m}$ ) and quantitative analysis of the positive staining area. Saline group ( $n=10$ ). FA group ( $n=10$ ). ND group ( $n=8$ ). DN group ( $n=8$ ). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  and ns=no significant.

图4 叶酸诱导的肾损伤模型和糖尿病肾病模型的肾脏中脂质累积

**Fig. 4 Upregulation of fatty acid synthesis gene expression in the kidneys promotes lipid accumulation in FA-induced renal injury and DN mouse models**



A and B: mRNA expression levels of *ACTA2* and fatty acid synthesis genes in HK-2 cells treated with TGF- $\beta$ 1 or HG compared to controls.  $n=3$ . C-F: Representative Bodipy 493/503 staining of lipid droplets in HK-2 cells and quantitative analysis of lipid droplet area.  $n=6$ . G-H: Representative Bodipy 493/503 staining of lipid droplets in PTECs treated with TGF- $\beta$ 1 and quantitative analysis of lipid droplet area.  $n=9$ . I-J: Representative Bodipy 493/503 staining of lipid droplets in PTECs treated with HG and quantitative analysis of lipid droplet area. Ctrl group ( $n=11$ ). HG group ( $n=10$ ). Scale bar=10  $\mu\text{m}$ . \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.0001$  vs Ctrl group.

图5 TGF- $\beta$ 1或高糖处理的肾小管上皮细胞脂质累积

Fig. 5 Upregulation of fatty acid synthesis gene expression promotes lipid accumulation in HK-2 cells treated with TGF- $\beta$ 1 or high glucose (HG)

衰老相关通路在FA组显著富集(图6A),同时SA- $\beta$ -gal染色结果也显示FA组衰老水平显著升高( $t=6.088$ ,  $P<0.0001$ ),见图6B、C。同样,相较于ND组, DN组的GSEA分析和SA- $\beta$ -gal染色结果均显示肾脏衰老( $t=6.826$ ,  $P<0.0001$ ,图6D-F)。为了进一步明确脂质蓄积与细胞衰老的联系,我们用棕榈酸PA处理HK-2细胞诱导脂质蓄积。相较于对照组, Bodipy 493/503染色结果显示PA处理组出现明显的脂质蓄积( $t=6.40$ ,  $P<0.0001$ ;图6G、H), SA- $\beta$ -gal染色显示PA组细胞衰老加重( $t=7.652$ ,  $P<0.0001$ ;图6I、J)。Western blot和RT-qPCR结果显示,PA组衰老标志蛋白(P16和P53)以及SASP的促纤维化因子( $TGF\beta$ 、 $MMP2$ 和 $SPPI$ )显著高于对照组(P16:  $t=2.915$ ,  $P=0.0268$ ; P53:  $t=4.473$ ,  $P=0.0042$ ;  $TGF\beta$ :  $t=5.054$ ,  $P=0.0010$ ;  $MMP2$ :  $t=4.825$ ,  $P=0.0013$ ;  $SPPI$ :  $t=8.020$ ,  $P<0.0001$ ;图6K、L)。我们利用PA处理的PTECs的上清(条件培养基)培养成纤维细胞,并分析了成纤维细胞中纤维连接蛋白(fibronectin)的表达水平。免疫荧光结果显示,相较于BSA对照组,PA组Fibronectin表达水平显著增高( $t=10.75$ ,  $P<0.0001$ ),见图6M、N。以上结果提示PA诱导的脂质蓄积可能通过衰老肾小管上皮细胞的旁分泌作用促进邻近成纤维细胞的活化。

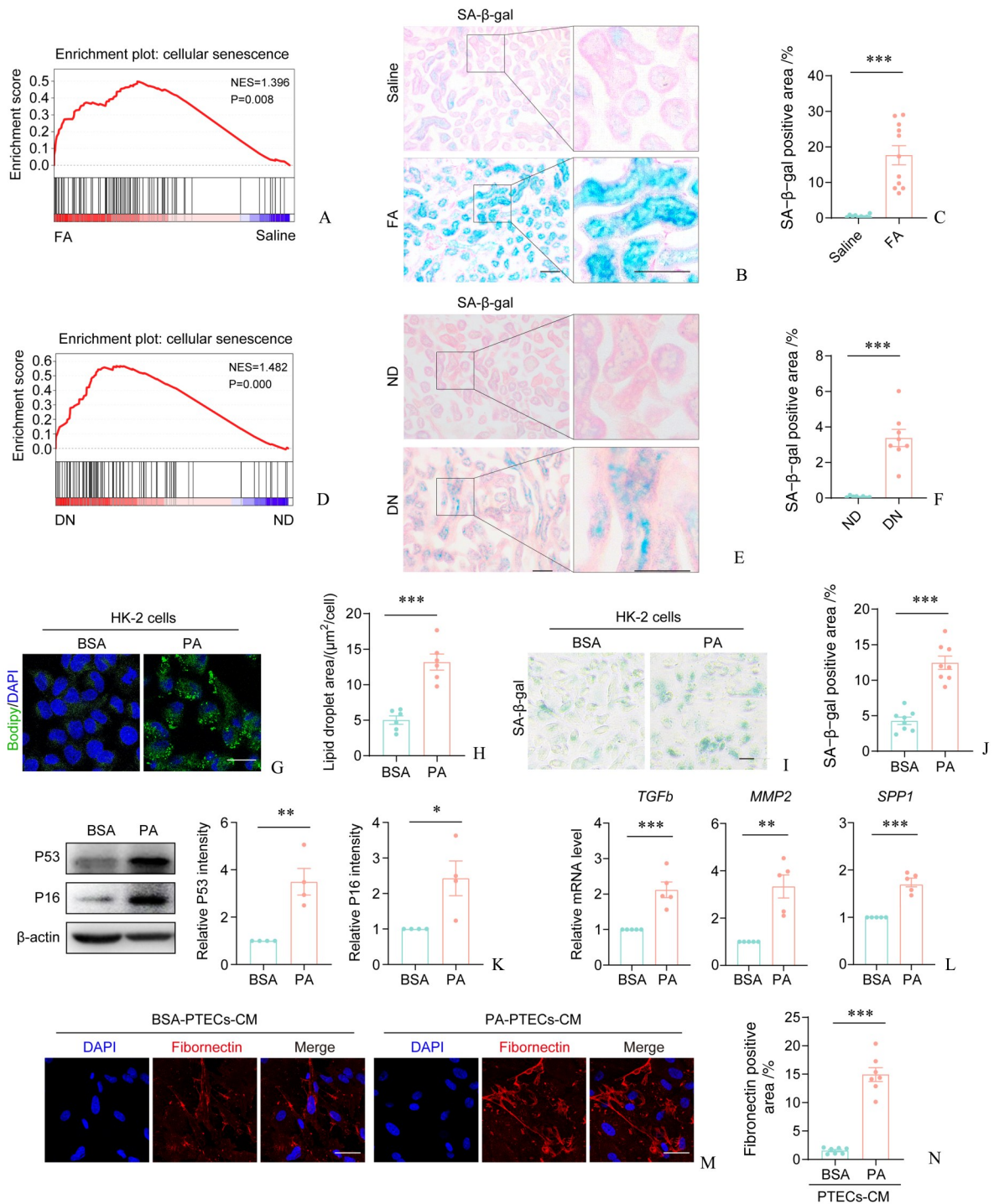
### 3 讨论

CKD是全球公共卫生领域的重要挑战,具有高发病率、预后不良的特点。CKD发展到后期其主要治疗手段,如血液透析和肾移植,不仅给患者带来显著的身心负担,还对社会医疗资源造成沉重压力。CKD的发病机制复杂,涉及TGF- $\beta$ 1/Smad信号通路、Wnt/ $\beta$ -catenin信号通路、细胞死亡途径以及线粒体功能障碍等多个关键因素<sup>[13]</sup>。由于目前临床上缺乏有效的防治策略,深入研究CKD的发病机制成为亟待解决的科学问题,而经典动物模型在揭示这些机制方面具有不可替代的作用。研究表明,尽管低剂量叶酸(维生素B9)具有抗氧化应激的保护作用,而高剂量叶酸则表现出肾毒性,可诱导AKI及CKD的发生<sup>[14]</sup>。本研究采用单次大剂

量腹腔注射FA建立肾毒性肾损伤模型。组织学分析结果显示,与对照组相比,叶酸组小鼠肾脏呈现显著的病理学改变,包括肾小管扩张、肾脏结构破坏、炎性细胞浸润以及肾间质胶原纤维沉积等。因此,深入探究DN的分子机制,寻找减缓疾病进展的新型治疗策略具有重要意义。既往研究表明,叶酸诱导的肾损伤机制涉及氧化应激、铁死亡以及线粒体功能障碍等多个方面<sup>[14-16]</sup>,提示可能叶酸引起了代谢相关的变化。本研究通过对两组小鼠肾组织的转录组分析,发现差异表达基因显著富集于代谢相关通路,包括小分子代谢过程、脂质代谢过程以及PPAR信号通路等。这些发现提示,即使在“非代谢”叶酸肾损伤模型中,代谢重编程仍然是病理进程中的关键因素。

DN是由糖尿病引起的慢性肾脏疾病,是糖尿病患者终末期肾病的主要原因<sup>[17-18]</sup>。尽管目前临床上已应用肾素-血管紧张素系统抑制剂、钠-葡萄糖协同转运蛋白2抑制剂以及胰高血糖素样肽-1受体激动剂等药物治疗DN,但这些疗法仍无法完全阻止疾病进展,患者仍面临较高的疾病进展风险<sup>[19]</sup>。近年来越来越多的研究强调脂质稳态失衡在DN病理进程中的关键作用<sup>[20-21]</sup>。例如,促进脂肪酸氧化的PPAR $\delta$ 分子的激动剂Seladelpar能减轻糖尿病肾病进程中的足细胞损伤、改善肾脏损伤<sup>[22]</sup>。糖尿病肾病患者中介导脂肪酸和胆固醇受体的转运蛋白或受体( $CD36$ 、 $SR-AI$ 、 $LOX-1$ )表达上调及脂肪酸氧化关键基因( $ACOI$ 、 $CPTI$ )表达下调会导致肾组织出现明显的脂质沉积<sup>[23]</sup>。构建2型糖尿病肾病实验动物模型的经典方法是采用高脂饲料喂养联合低剂量STZ腹腔注射,同样强调了代谢异常在糖尿病肾病中的作用。我们对2型糖尿病肾病小鼠的肾脏组织进行转录组分析,发现DEGs显著富集于脂质代谢相关通路。此外,通过比较叶酸诱导的肾损伤模型与DN模型,我们发现两模型共有的DEGs同样显著富集于脂质代谢相关通路,尤其是脂肪酸代谢过程。这一发现表明,脂代谢紊乱可能是多种慢性肾脏病发生与发展的共性病理机制。

近年研究表明,脂肪酸合成异常在肾脏疾病发



A: GSEA of cellular senescence related genes in kidney from FA mouse model. B and C: Representative images and quantification of senescent cells in kidney sections of FA mice, identified by SA-β-gal staining (scale bar=100 μm). Saline group (n=10). FA group (n=11). D: GSEA of cellular senescence related genes in kidney of DN mice. E and F: Representative images and quantification of senescent cells in kidney sections of DN mice, identified by SA-β-gal staining (scale bar=100 μm). n=8. G and H: Bodipy staining showing lipid droplets in HK-2 cells treated with palmitic acid (PA). Scale bar=10 μm. n=6. I and J: Representative images and quantification of senescent cells in HK-2 cells treated with PA, as determined by SA-β-gal staining. Scale bar=10 μm. n=8. K: Western blot images and quantification of P16 and P53 protein in HK-2 cells treated with PA or bovine serum albumin (BSA). n=4. L: RT-qPCR analysis of TGFβ, MMP2, and SPP1 mRNA expression in HK-2 cells treated with PA relative to BSA control. n=5. M and N: Immunofluorescence images and quantification of Fibronectin in fibroblasts cultured with conditioned medium from PA-treated PTECs. n=7. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.0001 vs Ctrl group.

图6 肾小管上皮细胞的脂质蓄积导致细胞衰老加重促进成纤维细胞活化

Fig. 6 Lipid accumulation in renal tubular epithelial cells accelerates cellular senescence and fibroblast activation

生发展中发挥重要作用。例如,降糖药利拉普肽通过下调 *Srebp1* 和 *Fasn*、上调 *ATGL* 和 *HSL*, 显著减轻糖尿病肾病大鼠肾小管脂质沉积,并改善棕榈酸诱导的肾小管上皮细胞脂质蓄积<sup>[24]</sup>;尽管脂代谢在DN中的作用已广受关注,但在叶酸诱导的肾损伤模型中的研究较少。本研究分析比较FA诱导的肾病和DN模型发现,脂肪酸合成基因(如 *Srebp1*、*Acc1*、*Fasn*)表达上调,且肾小管上皮细胞出现明显脂质蓄积。在体外研究中,我们同样发现转化生长因子 $\beta$ 1和高糖处理均显著上调HK-2细胞脂质合成关键因子 *SREBP1* 及其下游酶(*ACCI*、*FASN*)的mRNA水平。此外,两种处理均能诱导HK-2细胞和PTECs出现脂质蓄积。这些体内外结果一致表明,肾小管上皮细胞中脂肪酸合成通路异常激活引起了肾脏脂质蓄积。

衰老细胞的增多被认为是推动肾脏纤维化发展的关键因素。在小鼠缺血-再灌注肾损伤、单侧输尿管结扎模型以及CKD患者肾组织中,检测到衰老相关标志物(P16、P21、P53)的显著表达和衰老细胞的增加<sup>[25-26]</sup>。此外,研究表明,减少肾小管上皮细胞衰老可有效缓解肾纤维化进程<sup>[27]</sup>。本研究对叶酸诱导的肾病和糖尿病肾病模型的肾组织进行了分析。GSEA和SA- $\beta$ -gal染色结果显示,与

对照组相比,FA组和DN组均表现出明显的细胞衰老特征。为进一步验证脂质蓄积是否驱动细胞衰老,我们采用PA处理HK-2细胞以诱导脂质蓄积。PA处理显著加重HK-2细胞的衰老程度,提示脂质蓄积是细胞衰老的重要驱动因素之一。此外,我们发现PA处理的原代肾小管上皮细胞培养上清可促进成纤维细胞的活化。这些结果表明,脂质蓄积不仅诱导肾小管上皮细胞衰老,还可能通过衰老细胞的旁分泌作用激活成纤维细胞,从而加剧肾纤维化进程。

综上所述,本研究采用高剂量FA和高脂饮食联合STZ诱导的DN模型,发现FA组和DN组均出现肾小管扩张、炎性细胞浸润及纤维化等病理特征。转录组分析显示,DEGs显著富集于脂质代谢通路,尤其是脂肪酸代谢,提示代谢重编程是CKD和DN共性病理机制。体外实验进一步验证,TGF- $\beta$ 1和高糖处理HK-2细胞后,脂肪酸合成基因(*Srebp1*、*Acc1*、*Fasn*)上调,伴随脂质蓄积。此外,研究发现脂质蓄积诱导肾小管上皮细胞衰老,衰老细胞通过旁分泌作用促进成纤维细胞活化,加剧纤维化进程。这些结果表明,脂肪酸合成异常及脂质蓄积是多种肾脏疾病的核心机制,为开发靶向代谢的抗纤维化疗法提供依据。

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