

·基础研究·

光感受器 *Lss* 缺失通过线粒体功能障碍与氧化应激导致 年龄相关性视网膜退行性病变

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摘 要:【目的】探讨光感受器特异性敲除羊毛甾醇合成酶(*Lss*)是否引发年龄相关性视网膜退行性变及其作用机制。【方法】构建光感受器特异性 *Lss* 敲除小鼠(*Lss*^{flax/flax}; *Rho-Cre*, CKO)模型, 分别在 1、4、8 月龄时采用眼底照相、光学相干断层扫描、暗适应视网膜电图(ERG)、HE 染色等技术评估视网膜结构与功能; 以小鼠视网膜感光细胞系 661W 为体外模型, 通过 siRNA 敲低 *Lss*, 结合 Seahorse XF 线粒体压力测试、JC-1 染色、MitoSOX Red、DCFH-DA 探针检测线粒体功能及氧化应激, 糖酵解速率测试检测糖酵解能力, RT-qPCR 检测炎症因子, γ H2AX 免疫荧光检测双链 DNA 损伤; 转录组测序分析敲低 *Lss* 后的差异表达基因及富集通路。【结果】与对照组相比, 1 月龄 CKO 小鼠未见异常; 4 月龄出现轻微眼底病变; 至 8 月龄 CKO 小鼠视网膜外核层显著变薄、内/外节结构紊乱, 暗适应 ERG a 波、b 波振幅明显降低($P<0.05$); 转录组分析显示, 敲低 *Lss* 后共鉴定出 1 842 个差异表达基因(上调 826 个, 下调 1 016 个), 其中 *Lss* 为最显著下调基因, 显著富集于甾醇生物合成、氧化磷酸化、有氧呼吸链及线粒体 ATP 合成等通路($P<0.05$)。在 661W 细胞敲低 *Lss* 后, 线粒体基础呼吸($P=0.006$)、ATP 生成($P=0.015$)、最大呼吸($P<0.001$)及储备能力($P<0.001$)均显著下降, 基础糖酵解($P=0.005$)、代偿性糖酵解水平升高($P=0.028$), 同时伴随线粒体膜电位去极化($P<0.001$), 线粒体活性氧(ROS)($P<0.001$)及总 ROS 水平升高($P=0.013$), 炎症因子 *Ccl2*($P=0.004$)、*Ccl5*($P=0.012$)、*Cxcl1*($P<0.001$)、*Cxcl5*($P<0.0001$)、*Tgfb*($P=0.008$)、*Hgf*($P<0.0001$)、*Mmp3*($P=0.003$) mRNA 表达显著上调, γ H2AX 荧光强度增加($P=0.002$)。【结论】光感受器 *Lss* 缺失通过导致线粒体功能障碍、氧化应激及炎症反应, 导致年龄相关性视网膜退行性变, 为遗传性及年龄相关性视网膜退行性疾病提供了新的发病机制和潜在干预靶点。

关键词: 羊毛甾醇合成酶; 光感受器; 视网膜退行性疾病; 线粒体功能障碍; 氧化应激

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Lanosterol Synthase Deficiency in Photoreceptor Leads to Age-related Retinal Degeneration via Mitochondrial Dysfunction and Oxidative Stress

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Abstract: 【Objective】To investigate whether photoreceptor-specific knockout of lanosterol synthase (*Lss*) induces age-related retinal degeneration and to elucidate the underlying molecular mechanisms. 【Methods】Photoreceptor-specific *Lss* knockout mice (*Lss*^{flax/flax}; *Rho-Cre*, CKO) models were established. Retinal structure and function were assessed at 1,

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4, and 8 months of age using fundus photography, optical coherence tomography (OCT), scotopic electroretinogram (ERG) and hematoxylin and eosin (HE) staining. The mouse photoreceptor cell line 661W served as an in vitro model. *Lss* was knocked down via siRNA, followed by analyses including Seahorse XF mitochondrial stress test, JC-1, MitoSOX Red and DCFH-DA probes to evaluate mitochondrial function and oxidative stress, glycolytic rate assay for glycolysis ability, RT-qPCR for inflammatory factors, γ H2AX immunofluorescence for double-strand DNA damage detection, and transcriptome sequencing to identify differentially expressed genes and enriched pathways. 【Results】 Compared with controls, 1-month-old CKO mice showed no abnormalities. Mild fundus lesions appeared at 4 months. By 8 months, CKO mice exhibited significant thinning of the outer nuclear layer, disorganization of the inner/outer segment structure, and markedly reduced amplitudes of the dark-adapted ERG a- and b-waves ($P<0.05$). Transcriptome analysis revealed 1 842 differentially expressed genes (826 up-regulated, 1 016 down-regulated), with *Lss* being the most significantly downregulated gene; these genes significantly enriched in sterol biosynthesis, oxidative phosphorylation, aerobic electron transport chain, and ATP synthesis ($P<0.05$). In 661W cells, knockdown of *Lss* significantly reduced mitochondrial basal respiration ($P=0.006$), ATP production ($P=0.015$), maximal respiration ($P<0.001$), and spare respiratory capacity ($P<0.001$), while basal glycolysis ($P=0.005$) and compensatory glycolysis ($P=0.028$) were increased. These changes were accompanied by mitochondrial membrane potential depolarization ($P<0.001$), elevated mitochondrial reactive oxygen species (ROS) levels ($P<0.001$), and increased total ROS levels ($P=0.013$). Additionally, the mRNA expression of inflammatory factors, including *Ccl2* ($P=0.004$), *Ccl5* ($P=0.012$), *Cxcl1* ($P<0.001$), *Cxcl5* ($P<0.000 1$), *Tgfb* ($P=0.008$), *Hgf* ($P<0.000 1$) and *Mmp3* ($P=0.003$) was significantly upregulated. Moreover, γ H2AX fluorescence intensity was increased ($P=0.002$). 【Conclusions】 Photoreceptor-specific *Lss* deficiency induces mitochondrial dysfunction, oxidative stress and cellular inflammation, ultimately leading to age-dependent retinal degeneration. This study provides novel mechanistic insights and potential therapeutic targets for hereditary and age-related retinal degenerative diseases.

Key words: lanosterol synthase; photoreceptor; retinal degeneration; mitochondrial dysfunction; oxidative stress

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年龄相关性黄斑变性 (age-related macular degeneration, AMD) 是一种以黄斑区光感受器、视网膜色素上皮 (retinal pigment epithelium, RPE)、Bruch膜及脉络膜毛细血管复合体进行性退变为特征的致盲性眼病,且患病率持续攀升^[1]。AMD临床分为干性 (dry AMD) 与湿性 (wet AMD) 2类,其中干性AMD约占病例总数的85%~90%,其终末期表现为边界清晰的地图样萎缩 (geographic atrophy, GA),即RPE细胞及其支持的光感受器细胞发生不可逆性丢失^[2]。

视网膜光感受器细胞作为高度特化的神经元,具备极高的代谢活性与能量需求,对内环境稳态的失衡敏感^[3-4]。光感受器细胞内富含线粒体,依赖氧化磷酸化产生ATP,其正常功能维持需要极高的能量供给及充足的代谢底物从而实现光感受器外节 (photoreceptor outer segments, POS) 的不断更新。尽管基因突变、氧化应激和炎症反应已被证实参与了退变过程,但视网膜甾醇代谢稳态在感光细胞生存中的具体调控机制尚未完全阐明^[5]。光感受器

退行性病变,尤其是POS的丢失,是多种视网膜退行性疾病共同病理基础,且随年龄增长而逐渐加重,在AMD等疾病中尤为显著^[6]。然而,驱动光感受器随年龄发生退变的内在代谢机制尚未完全阐明。

近年来,视网膜代谢功能障碍被认为是驱动光感受器退行性病变的重要潜在机制。全基因组关联研究 (genome-wide association studies, GWAS) 显示,多种脂质代谢相关基因的多态性与AMD发病风险密切相关^[7-9]。这些研究提示脂质代谢稳态尤其是甾醇稳态在AMD发病机制中具有重要作用。尽管干性AMD发病占比较高,但当前临床治疗手段的疗效有限^[10]。因此,通过动物模型深入研究GA的病理特征,并探索有效的干预靶点,对改善患者预后具有重要的科学意义与临床价值。

羊毛甾醇作为胆固醇生物合成途径中的关键前体分子,其合成依赖于羊毛甾醇合成酶 (lanosterol synthase, *Lss*),在维持晶状体蛋白稳态和透明度方面具有重要生物学功能^[11-12]。值得注

意的是,Grassmann等^[13]对935例GA患者进行的GWAS发现,*Lss*基因位点(rs2839127)是仅有的2个与GA病灶进展相关的全基因组显著性位点之一,提示羊毛甾醇合成障碍可能与AMD相关的光感受器退行性病变存在潜在因果联系。然而,目前尚不清楚感光细胞如何依赖内源性羊毛甾醇维持其结构与功能稳态,亦缺乏直接的体内遗传学证据以证明羊毛甾醇缺失在光感受器中对年龄相关性视网膜退行性病变的致病作用。

基于上述背景,本研究通过构建光感受器特异性*Lss*条件性敲除小鼠模型,评估羊毛甾醇缺失对光感受器结构、功能及其随年龄变化的影响,并探索其驱动视网膜代谢重塑及退行性病变的机制,为理解年龄相关性光感受器变性的代谢基础及视网膜退行性疾病的防治提供新的理论依据。

1 材料与方法

1.1 实验动物

本实验所采用的4周至8月龄转基因小鼠均在中山眼科中心动物房中饲养(SPF级)[实验动物使用许可证号:SYXK(粤)2013-0189]。饲养间环境湿度60%左右、温度20℃~24℃,遵循12 h光照/黑暗交替周期,给予经过高压灭菌的标准啮齿类动物饲料和纯化水,所有小鼠均自由摄食、饮水。*Lss*^{flax/+}小鼠(C57BL/6J背景)均由江苏集萃药康生物科技股份有限公司通过CRISPR/Cas9基因编辑技术构建。*Rhodopsin-Cre*工具鼠如前所述^[14]。通过将*Lss*^{flax/flax}小鼠与*Rhodopsin-Cre*工具鼠交配,培育出光感受器细胞特异性敲除小鼠CKO(*Lss*^{flax/flax}; *Rhodopsin-Cre*)。实验对照组(Ctrl)为同窝出生的*Lss*^{flax/flax}或*Lss*^{flax/+}小鼠。本实验中所有动物操作均严格遵守国际眼科与视觉研究协会关于视觉和眼科研究中动物使用的声明,并获得中山眼科中心实验动物福利与伦理委员会批准与监督(批准号:Z2025079)。

1.2 实验细胞

661W小鼠视网膜光感受器细胞系由中山大学中山眼科中心刘春巧教授惠赠,培养于含100 mL/L胎牛血清和10 mL/L青霉素-链霉素的DMEM完全培养基。细胞每2 d~3 d换液一次,生长至80%~90%融合度时进行传代或实验处理。

1.3 细胞转染

待661W细胞生长至60%~80%汇合度时进行转染。本研究采用Lipofectamine RNAiMAX转染试剂(13778150, ThermoFisher)进行转染。靶向*Lss*的siRNA序列由吉玛基因合成,序列见附表1。



附表
Appendix table

1.4 眼底照相与视网膜光学相干成像

首先使用10 g/L戊巴比妥钠(生理盐水配制)腹腔注射麻醉动物,托吡卡胺-去氧肾上腺素滴眼液散瞳后采用小动物视网膜成像显微镜(Phoenix, Micron IV)行眼底照相及光学相干断层扫描(optical coherence tomography, OCT)检测。扫描时以视盘为中心,水平扫描获取视网膜图像。

1.5 电生理

采用Diagnosys Celeris ERG系统记录小鼠双眼视网膜电图(electroretinogram, ERG)。暗适应12 h后,充分麻醉散瞳,于小鼠角膜表面滴加羟丙甲基纤维素以保湿,随后安放角膜电极。在避光条件下进行暗适应ERG检测,依次以0.01、0.1、1和10 cd·s/m²这4个强度的白光闪烁刺激记录a波与b波。

1.6 石蜡切片苏木素-伊红染色

小鼠麻醉后,摘取眼球固定脱水后行石蜡包埋,作5 μm经视神经的切片。行苏木素-伊红(HE)染色后,采用TissueFAXs全景组织切片扫描系统采集图像。使用Image J软件测量全视网膜厚度、视网膜外核层(outer nuclear layer, ONL)厚度。

1.7 免疫荧光染色

细胞样本经40 g/L多聚甲醛固定15 min, PBST洗涤3次后,于含5 mL/L Triton X-100及50 g/L BSA的PBS溶液中通透并封闭1 h。随后加入兔源抗γH2AX(Ser139)-抗(2577, CST), 4℃孵育过夜; PBST洗涤3次后,以Alexa Fluor 555标记的二抗室温避光孵育1 h。再次洗涤后, DAPI复染细胞核。另取细胞, PBS洗涤3次后, 分别加入JC-1(C2006, Beyotime)或MitoSOX Red染料(M36007, invitrogen)进行染色, PBS洗涤后以Hoechst复染细胞核。所有荧光图像均采用LSM980共聚焦显微镜采集。

1.8 细胞线粒体压力测试、糖酵解速率测试

采用Seahorse XF24分析仪连续监测细胞的耗氧率(oxygen consumption rate, OCR)、糖酵解质子流出速率(glycolytic proton efflux rate, GlycoPER)。实验前一日,以每孔3×10⁴个细胞的密度接种于

XF24细胞培养板培养过夜。每组实验设置5个生物学重复。分别使用Seahorse XF细胞线粒体压力测试试剂盒(103015-100, Agilent)、Seahorse XF糖酵解速率测试试剂盒(103344-400, Agilent)进行测定。药物注射顺序及最终工作浓度:①线粒体压力测试(OCR测定):按顺序依次向探针加药孔A、B、C中注射最终工作浓度为1.5 $\mu\text{mol/L}$ 的寡霉素、1 $\mu\text{mol/L}$ 的解偶联剂FCCP,以及0.5 $\mu\text{mol/L}$ 的鱼藤酮与抗霉素A混合液(rotenone/antimycin A)。②糖酵解速率测试(GlycoPER测定):按顺序依次向探针加药孔A、B中注射最终工作浓度为0.5 $\mu\text{mol/L}$ 的rotenone/antimycin A,以及50 mmol/L的2-脱氧葡萄糖。测定结束后,根据各孔细胞总蛋白浓度对OCR及GlycoPER值进行归一化处理及数据分析。

1.9 蛋白免疫印迹实验

向分离的视网膜组织中加入含蛋白酶及磷酸酶抑制剂的RIPA裂解液提取蛋白。使用12% SDS-PAGE凝胶电泳分离后转印至PVDF膜。室温下50 g/L BSA中封闭2 h,4 $^{\circ}\text{C}$ 孵育兔抗 *Lss* 抗体(13715-1-AP, Proteintech)及小鼠抗 β -Actin抗体(AC004, ABclonal)过夜。之后,将膜与相应二抗在室温下孵育1 h,ECL化学发光试剂进行显影,Image Lab成像系统采集图像。

1.10 RNA提取与RT-qPCR

采用TRIzol试剂提取组织总RNA,并使用ReverTra Ace qPCR RT试剂盒将其反转录为cDNA。后续实时荧光定量PCR在LightCycler[®] 96系统上完成,使用 $2^{-\Delta\Delta\text{Ct}}$ 法分析基因的相对表达量,引物序列见附表2。

1.11 流式细胞术

处理好的细胞用0.25%胰酶消化2 min,加入含血清培养基终止消化,室温200 $\times g$ 离心5 min收集沉淀。PBS洗涤后分别用Mitox Red、JC-1和DCFH-DA试剂盒进行细胞染色,PBS洗涤细胞后上机检测。

1.12 转录组

转录组测序RNA-seq测序由北京贝瑞基因科技有限公司完成,具体方法参照文献^[15]。提取对照组与敲低 *Lss* 组的总RNA,每个样本取1 μg 用于建库。使用NEB Next[®] Ultra[™] RNA Library Prep Kit for Illumina[®]构建测序文库,并在Illumina HiSeq 2000平台上进行测序后进行生物信息学分析,包括京都基因与基因组百科全书(kyoto encyclopedia

of genes and genome, KEGG)基因本体论(gene ontology, GO)。将校正后 $P<0.05$ 的基因鉴定为差异表达基因。

1.13 统计学方法

实验数据以平均值 $\pm$ 标准误(Mean $\pm$ SEM)形式表示,每个实验至少重复3次。采用GraphPad Prism软件(version 8.0)制作统计图,采用双尾非配对 *t* 检验对两组间差异进行分析,方差齐性通过 *F* 检验评估,若方差不齐则采用Welch校正。 $P<0.05$ 为差异有统计学意义。

2 结果

2.1 光感受器中羊毛甾醇合成酶缺失导致年龄相关性视网膜变性

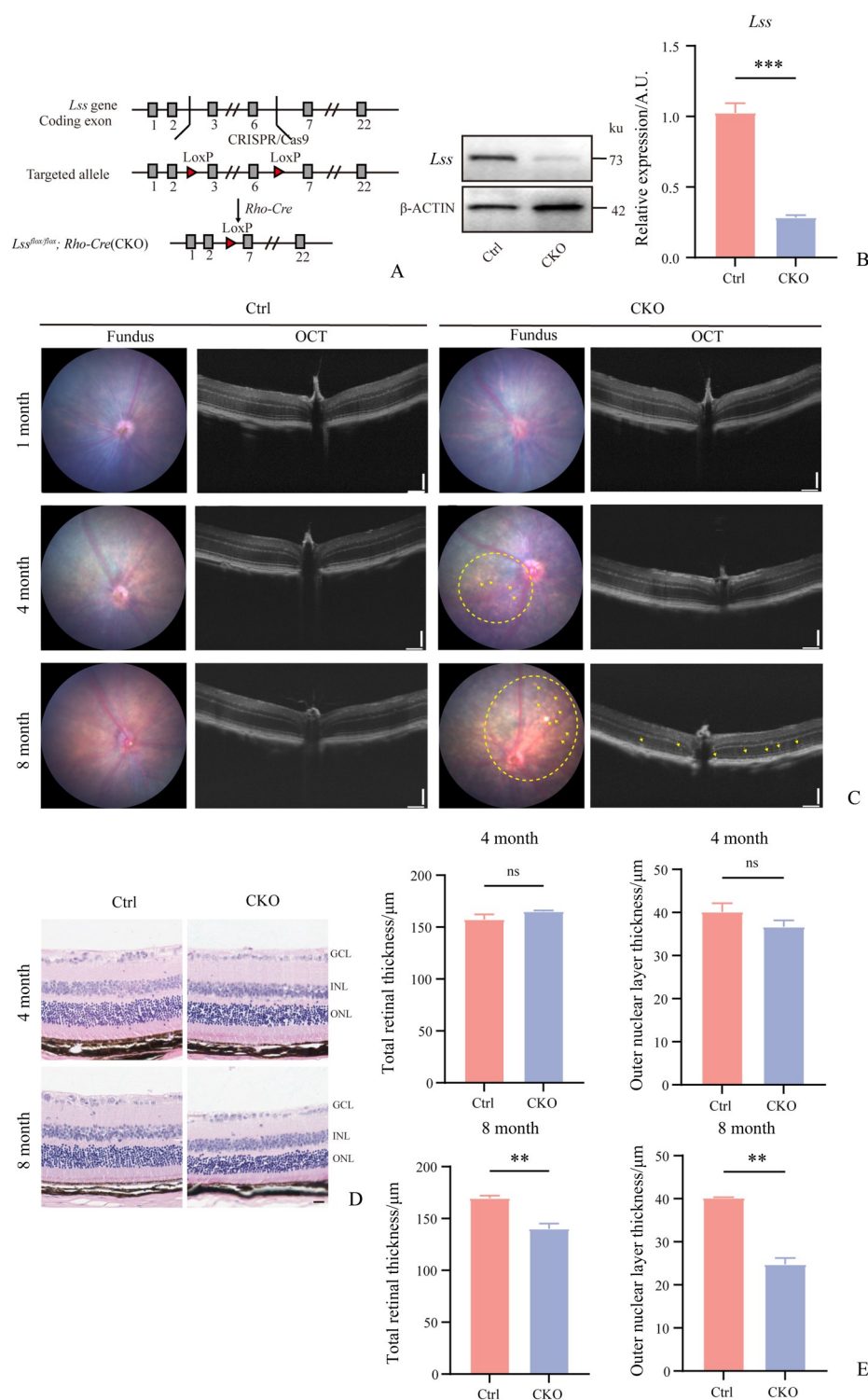
2.1.1 光感受器条件性敲除 *Lss* 小鼠的构建方案、鉴定与验证 通过CRISPR/Cas9方法在 *Lss* 基因的第3号外显子至第6号外显子两端插入 *LoxP* 位点,获得 *Lss*^{flax/+}小鼠,通过配繁获得纯合 *Lss*^{flax/flax}(Ctrl)小鼠,再与Rhodopsin-Cre工具鼠交配得到 *Lss*^{flax/flax}; *Rho-Cre*(CKO)小鼠,实现光感受器特异性敲除 *Lss* 基因(图1A)。Western blot结果显示,CKO组视网膜 *Lss* 蛋白显著低于对照组($t=10.410$, $P<0.001$;图1B)。

2.1.2 光感受器条件敲除 *Lss* 小鼠随年龄出现视网膜退行性疾病表型 眼底照相及OCT结果显示,CKO小鼠视网膜表现出随年龄增长而进展的退行性改变。1月龄时,CKO组与Ctrl组眼底及OCT表现均未见明显异常,视网膜各层结构完整。4月龄时,CKO组开始出现轻微眼底异常,总视网膜厚度、ONL厚度与对照组相比无显著差异(total retinal thickness: $t=1.544$, $P=0.213$; ONL thickness: $t=1.371$, $P=0.220$;图1C-E)。至8月龄时,CKO组眼底可见边界清晰的多发黄白色病灶,OCT图像可见明显的高反射灶,HE染色显示总视网膜厚度、ONL厚度显著变薄;而Ctrl组在各时间点均维持正常结构(total retinal thickness: $t=5.216$, $P=0.002$; ONL thickness: $t=10.670$, $P=0.002$;图1C-E)。

上述结果表明,光感受器细胞 *Lss* 缺失会引起进行性视网膜结构退变。

2.2 光感受器条件敲除 *Lss* 小鼠视网膜功能随年龄增长呈进行性下降

通过暗适应ERG的a波评估光感受器(主要是杆状细胞)的功能,b波评估双极细胞和水平细胞



A: Schematic diagram of CRISPR/Cas9-mediated conditional knockout strategy for *Lss* in photoreceptors driven by Rhodopsin-Cre. B: Quantitative analysis of relative *Lss* expression in retina of Ctrl and CKO ($n=3$ mice per group). C: Representative fundus photographs and OCT images of Ctrl and CKO mice at 1, 4 and 8 months of age showing yellow-white lesions (yellow arrows/dashed yellow ovals). D: Representative hematoxylin and eosin (H&E) staining of retinal sections from Ctrl and CKO mice at 4 and 8 months of age. Scale bar: 20 μm . E: Quantitative analysis of total retinal thickness and ONL thickness at 4 months and 8 months ($n=4$ mice per group). Welch's t -test was used for ONL thickness at 8 months. Data are presented as Mean $\pm$ SEM, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. Ctrl group.

图1 感光细胞特异性敲除 *Lss* 基因导致 *Lss* 表达降低及年龄相关性的视网膜结构改变

Fig. 1 photoreceptor-specific *Lss* knockout leads to reduced *Lss* expression and age-dependent retinal structural alterations

的功能。结果显示,在1月龄时,CKO组与Ctrl组小鼠的a波及b波振幅在各刺激强度下均无显著差异(a波:0.001 cd·s/m²时, $t=0.341$, $P=0.738$; 0.01 cd·s/m²时, $t=1.046$, $P=0.313$; 0.1 cd·s/m²时, $t=1.376$, $P=0.190$; 1 cd·s/m²时, $t=0.893$, $P=0.387$; 10 cd·s/m²时, $t=0.579$, $P=0.572$; b波:0.001 cd·s/m²时, $t=-1.279$, $P=0.221$; 0.01 cd·s/m²时, $t=-1.149$, $P=0.269$; 0.1 cd·s/m²时, $t=0.071$, $P=0.944$; 1 cd·s/m²时, $t=0.067$, $P=0.947$; 10 cd·s/m²时, $t=-0.235$, $P=0.817$;图2A-C)。然而,随着月龄增加,CKO组视网膜功能呈现进行性损伤。4月龄时,CKO组a波振幅在1、10 cd·s/m²刺激强度下显著低于对照组,b波振幅在0.01、0.1、1、10 cd·s/m²刺激强度下显著低于对照组(a波:0.001 cd·s/m²时, $t=-1.922$, $P=0.075$; 0.01 cd·s/m²时, $t=-0.239$, $P=0.814$; 0.1 cd·s/m²时, $t=-1.441$, $P=0.171$; 1 cd·s/m²时, $t=-3.375$, $P=0.005$; 10 cd·s/m²时, $t=-2.259$, $P=0.040$; b波:0.001 cd·s/m²时, $t=1.633$, $P=0.131$; 0.01 cd·s/m²时, $t=2.327$, $P=0.035$; 0.1 cd·s/m²时, $t=2.847$, $P=0.013$; 1 cd·s/m²时, $t=3.272$, $P=0.006$; 10 cd·s/m²时, $t=3.614$, $P=0.003$;图2D-F)。至8月龄时,视网膜功能损伤进一步加剧,CKO组小鼠的a波与b波振幅相较于均较Ctrl组发生显著下降。其中,a波振幅在0.1、1、10 cd·s/m²刺激强度下显著降低,b波振幅在除0.001 cd·s/m²各刺激强度下均显著降低(a波:0.001 cd·s/m²时, $t=-0.254$, $P=0.803$; 0.01 cd·s/m²时, $t=-2.022$, $P=0.062$; 0.1 cd·s/m²时, $t=-2.194$, $P=0.046$; 1 cd·s/m²时, $t=-2.852$, $P=0.013$; 10 cd·s/m²时, $t=-3.179$, $P=0.007$; b波:0.001 cd·s/m²时, $t=1.375$, $P=0.191$; 0.01 cd·s/m²时, $t=2.587$, $P=0.021$; 0.1 cd·s/m²时, $t=2.344$, $P=0.034$; 1 cd·s/m²时, $t=2.488$, $P=0.026$; 10 cd·s/m²时, $t=2.400$, $P=0.031$;图2G-I)表现为波形明显低平。以上结果表明,光感受器条件性敲除 *Lss* 基因可导致小鼠视网膜暗适应功能呈年龄依赖性进行性退变。

2.3 敲低 *Lss* 导致光感受器细胞能量代谢相关基因表达谱显著改变

为进一步阐明 *Lss* 缺失导致上述表型的分子机制,我们对661W细胞敲低 *Lss* 后进行了转录组测序分析。火山图分析显示,与对照组相比,共筛选出上调基因826个,下调基因1 016个(图3A)。针对这些差异表达基因的KEGG富集分析显示,差异表达基因显著富集于氧化磷酸化及谷胱甘肽代谢等

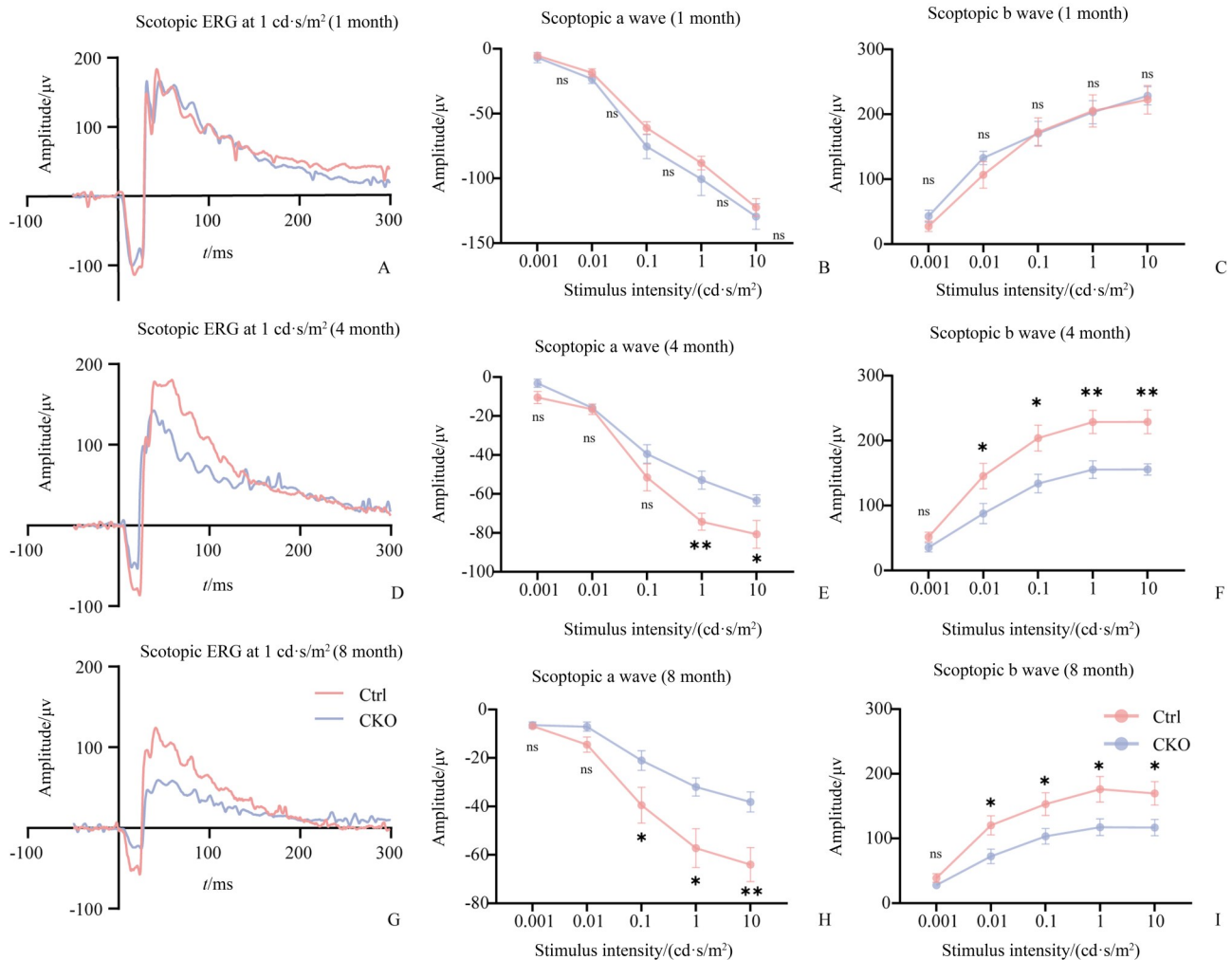
生物学通路(图3B)。GO通路分析进一步表明,在生物过程差异基因显著富集于有氧电子传递链、线粒体ATP合成偶联电子传递、细胞呼吸及固醇代谢等过程;细胞组分主要富集于线粒体、线粒体膜及线粒体蛋白复合物等区域;分子功能则显著富集于ATP依赖的活性、ATP结合及电子转运体活性等功能类别(图3C)。本研究进一步分析了线粒体氧化磷酸化复合体相关关键基因的表达特征。热图分析显示,与对照组相比,呼吸链复合体I-V的核心亚基基因在siLss组中下调。这些基因包括复合体I的 *Ndubf9*、*Ndubf8*、*Ndubf4*、*Ndufs5*、*Ndufs7*,复合体II的 *Sdhb*,复合体III的 *Uqcrl0*、*Cyc1*、*Uqcrlq*,复合体IV的 *Cox5a*、*Cox6a1*、*Cox7c* 以及ATP合成酶复合体V的核心元件 *Atp5g1*、*Atp5b*、*Atp5d*(图3D)。上述结果提示, *Lss* 敲除主要通过扰乱线粒体能量代谢、氧化磷酸化和固醇合成相关基因表达,进而驱动光感受器退行性病变。

2.4 敲低 *Lss* 抑制光感受器线粒体呼吸并促进糖酵解代偿

通过siRNA敲低 *Lss* 以探究其对感光细胞功能的影响。在661W细胞中,与Scramble组相比,siLss组 *Lss* mRNA水平较对照组降低约73% ($t=9.501$, $P=0.011$;图4A)。为进一步探讨 *Lss* 对光感受器能量代谢的调节作用,利用Seahorse XF分析仪检测了在661W细胞通过siRNA敲低 *Lss* 后OCR的变化(图4B)。线粒体功能检测显示,与Scramble组相比,siLss组细胞的基础呼吸水平显著降低($t=3.740$, $P=0.006$;图4C)。同时,siLss组用于ATP产生的偶联氧耗率也较对照组明显减少($t=3.750$, $P=0.015$;图4F)。

此外,在加入FCCP后,siLss组细胞表现出较低的代偿能力,其最大呼吸速率显著低于Scramble组($t=6.024$, $P<0.001$;图4E);相应的储备呼吸能力亦发生显著下降($t=6.810$, $P<0.001$;图4D)。糖酵解方面,siLss组基础糖酵解水平显著升高($t=3.590$, $P=0.005$;图4H),代偿性糖酵解能力亦显著升高($t=2.577$, $P=0.028$;图4I)。糖酵解实时曲线(图4G)显示,在Rotenone/Antimycin A阻断线粒体呼吸后siLss组仍能维持更高的glycoPER。

以上结果表明,敲低 *Lss* 基因会显著损害光感受器细胞的线粒体呼吸功能及能量储备,同时,细胞通过上调糖酵解途径实现代偿性代谢重编程,以维持基本能量供应。



A, D, G: Representative scotopic ERG waveforms elicited by a single flash of $1 \text{ cd} \cdot \text{s}/\text{m}^2$ in Ctrl (red) and CKO (blue) mice at 1 month (A), 4 months (D), and 8 months (G) of age. B, C: Scotopic a-wave (B) and b-wave (C) amplitudes as a function of stimulus intensity at 1 month of age ($n=8$ per group). E, F: Scotopic a-wave (E) and b-wave (F) amplitudes as a function of stimulus intensity at 4 months of age ($n=8$ per group). H, I: Scotopic a-wave (H) and b-wave (I) amplitudes as a function of stimulus intensity at 8 months of age ($n=8$ per group). Data are presented as Mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$ vs. Ctrl at the corresponding stimulus intensity.

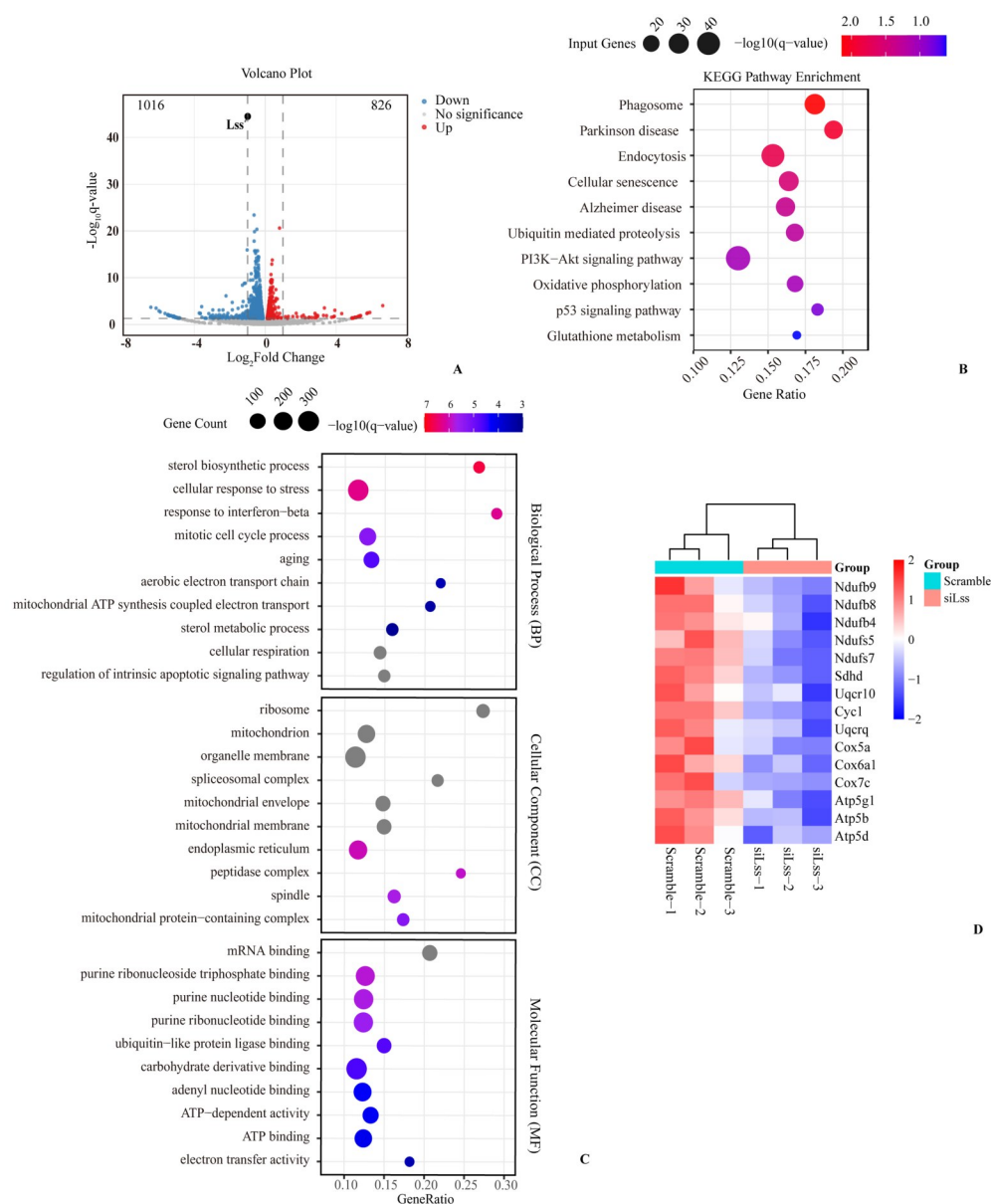
图2 光感受器条件性敲除 *Lss* 小鼠视网膜功能呈年龄依赖性进行性下降

Fig. 2 Age-dependent decline of retina function in photoreceptor-specific *Lss* knockout mice

2.5 敲低 *Lss* 导致光感受器细胞线粒体膜电位下降并诱发氧化应激

鉴于敲低 *Lss* 显著抑制了细胞的线粒体呼吸功能,本研究进一步通过 JC-1 染色和荧光探针检测了线粒体膜电位 ($\Delta\Psi_m$) 及活性氧 (reactive oxygen species, ROS) 水平的变化。流式细胞术 (图 5A-B) 及免疫荧光成像 (图 5C-D) 结果显示,与 Scramble 组相比,si*Lss* 组细胞的 JC-1 红/绿荧光比值显著降低 ($t=11.460$, $P < 0.001$) 且相对荧光强度减弱 ($t=4.419$, $P=0.005$),提示 *Lss* 缺失引起明显的线粒体膜电位去极化。

与此同时,为评估线粒体内的氧化应激状态,我们利用 MitoSOX Red 探针检测了线粒体超氧化物的产生。结果显示,si*Lss* 组细胞的线粒体超氧化物水平显著升高。流式细胞术定量分析 ($t=12.650$, $P < 0.001$; 图 5E-F) 与荧光显微镜观察 ($t=6.308$, $P < 0.001$; 图 5G-H) 均证实了这一结果。此外,DCFH-DA 染色结果显示,si*Lss* 组细胞内的总 ROS 水平亦显著升高 ($t=4.244$, $P=0.013$; 图 5I-J)。上述结果表明,敲低 *Lss* 不仅抑制线粒体呼吸链功能,还诱发线粒体膜电位丧失及严重的氧化应激损伤,这可能是导致光感受器细胞功能障碍的重要机制。



A: Volcano plot of RNA-seq data from 661W cells transfected with Scramble *vs.* siLss ($n=3$ per group). Red dots indicate significantly up-regulated genes (826 genes, $q < 0.05$); blue dots indicate significantly down-regulated genes (1016 genes, $q < 0.05$); gray dots indicate non-significant genes. *Lss* is marked as the most significantly down-regulated gene. B: Enriched KEGG pathways of differentially expressed genes. Bubble size represents gene count; color represents -log₁₀(q-value). C: Enriched GO terms in Biological Process (BP), Cellular Component (CC), and Molecular Function (MF). Bubble size represents gene count; color represents -log₁₀(q-value). D: Heatmap of differentially expressed genes (DEGs) involved in the mitochondrial oxidative phosphorylation (OXPHOS) complexes. The color scale indicates the Z-score of gene expression levels, where red represents up-regulation and blue represents down-regulation. The listed genes cover core subunits of all five mitochondrial respiratory complexes (Complex I - V).

图3 *Lss* 敲除后转录组差异表达基因及功能富集分析

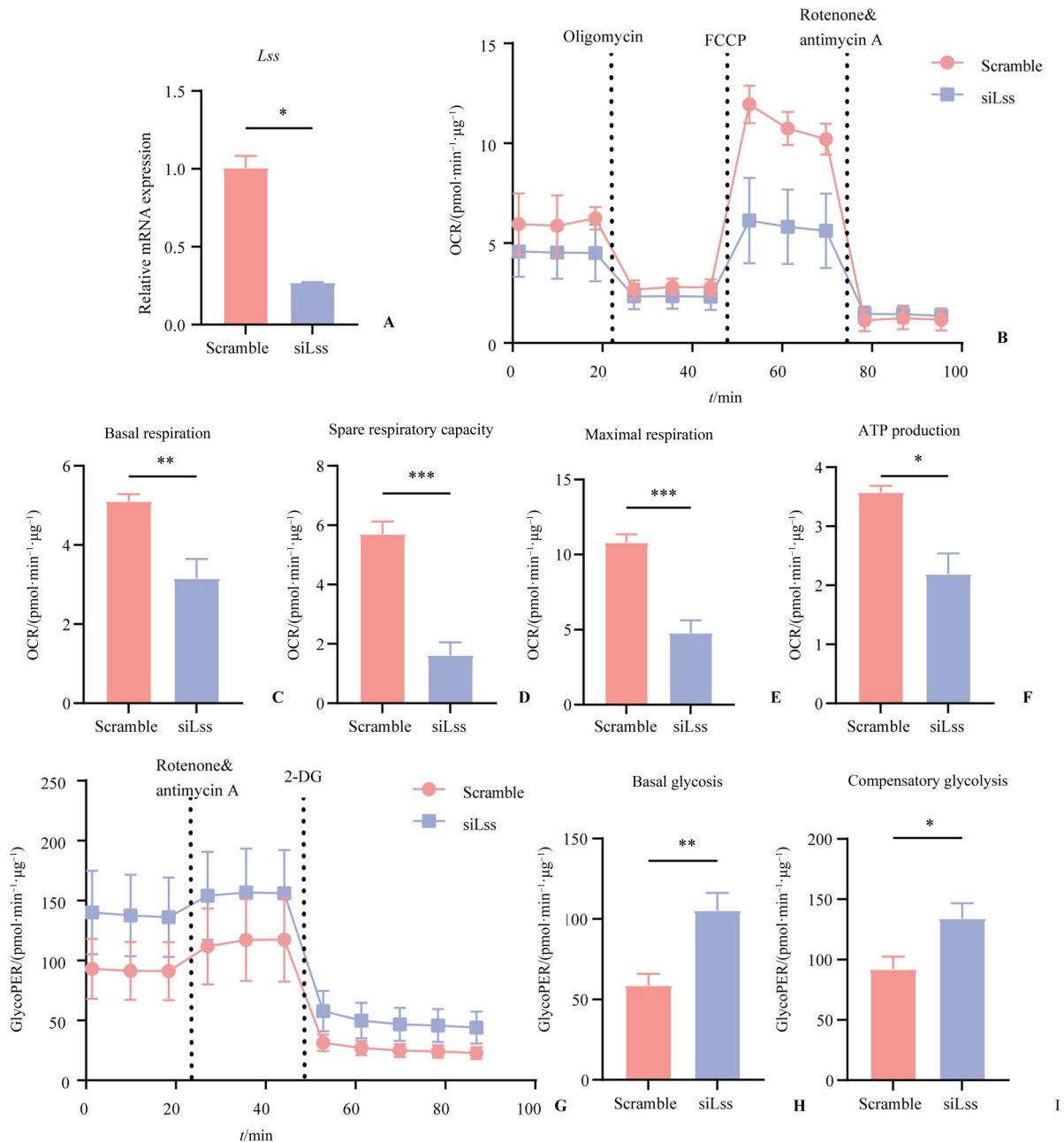
Fig. 3 Transcriptomic profiling and functional enrichment analysis of differentially expressed genes following *Lss* knockdown

2.6 敲低 *Lss* 上调感光细胞炎症因子表达并导致细胞DNA双链断裂损伤

线粒体功能障碍和ROS的过度累积通常会触发炎症反应,进而导致DNA损伤。为探究 *Lss* 缺失所导致的线粒体功能障碍和氧化应激是否

进一步引发炎症及DNA损伤,本研究检测了促炎因子 mRNA 表达及DNA双链断裂标志物 γ H2AX。

RT-qPCR结果显示,siLss组多个炎症相关基因的mRNA显著上调,包括趋化因子 *Ccl2*、*Ccl5*、



A: Relative *Lss* mRNA expression in 661W cells 48 h after transfection with scramble siRNA or siLss ($n=3$). Welch's t -test was used. B: Real-time OCR traces measured by Seahorse XF extracellular flux analyzer in scramble and siLss-transfected 661W cells during mitochondrial stress test. Oligomycin, FCCP, and rotenone/antimycin A were sequentially injected at the indicated time points ($n=5$). C-F: Quantification of basal respiration (C), spare respiratory capacity (D), maximal respiration (E), and ATP-linked respiration (F) derived from the OCR traces ($n=5$). G: Real-time glycoPER traces in Scramble- and siLss-transfected 661W cells during glycolytic rate assay. Rotenone/antimycin A and 2-deoxy-D-glucose were sequentially injected at the indicated time points ($n=5$). H, I: Basal glycolysis and compensatory glycolysis levels quantified from the glycoPER traces ($n=5$). Data are presented as Mean $\pm$ SEM. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs. Scramble group.

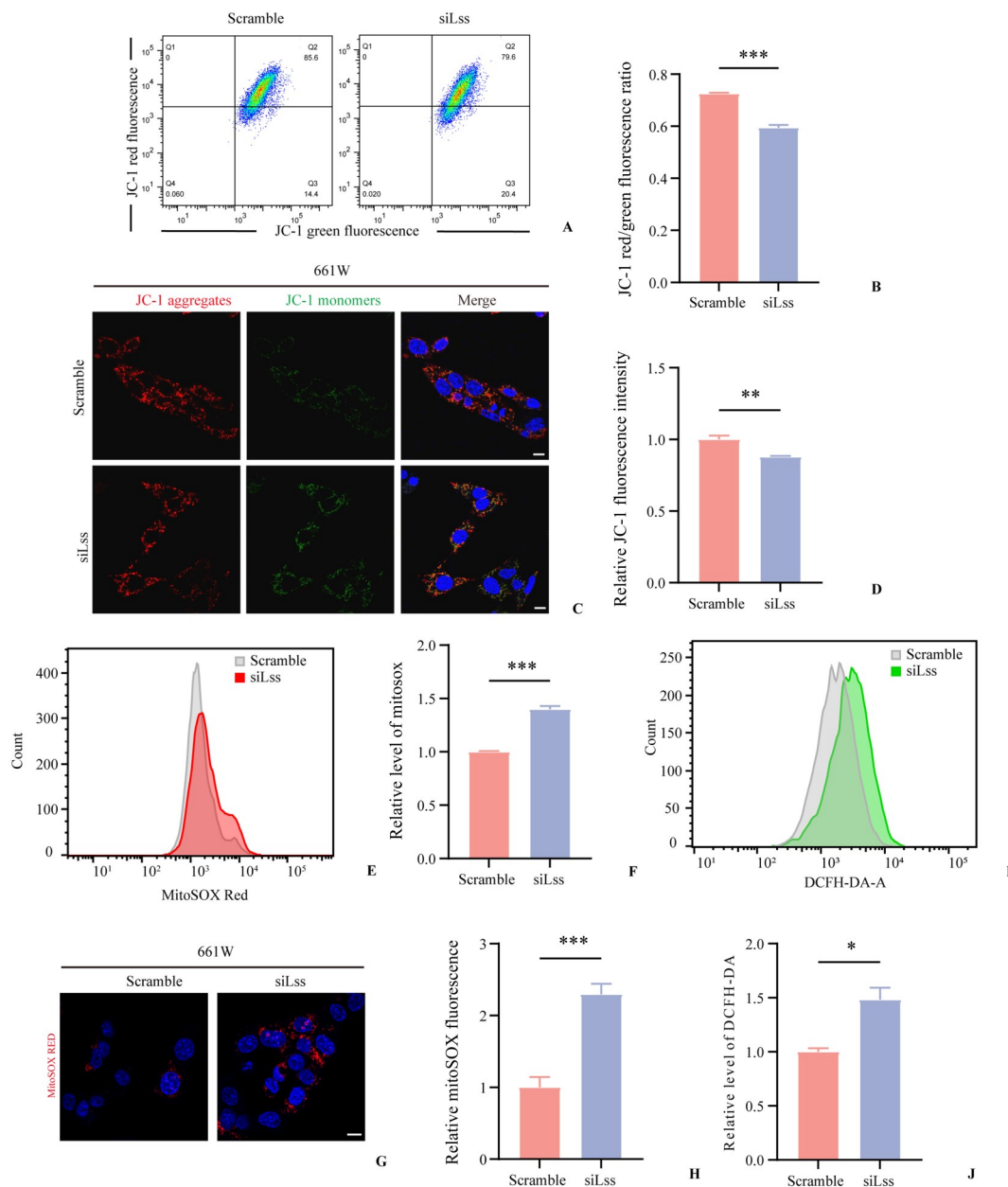
图4 敲低 *Lss* 抑制 661W 光感受器细胞线粒体呼吸并促进糖酵解

Fig. 4 *Lss* knockdown impairs mitochondrial respiration but enhances glycolysis in 661W photoreceptor cells

Cxcl1、*Cxcl5*、生长因子 *Tgfb*、*Hgf* 及基质金属蛋白酶 *Mmp3* 的 mRNA 水平均显著上调 (*Ccl2*: $t=6.030$, $P=0.004$; *Ccl5*: $t=8.999$, $P=0.012$; *Cxcl1*: $t=9.463$, $P<0.001$; *Cxcl5*: $t=23.080$, $P<0.0001$; *Tgfb*: $t=$

4.998, $P=0.008$; *Hgf*: $t=17.510$, $P<0.0001$; *Mmp3*: $t=17.600$, $P=0.003$; 图 6A)。

免疫荧光染色结果显示, siLss 组细胞核内 γ H2AX 阳性焦点显著增多(图 6B), 相对荧光强度



A: Representative flow cytometry plots of JC-1 staining (red aggregates vs. green monomers). B: Quantitative analysis of JC-1 red/green fluorescence ratio($n=3$). C: Representative immunofluorescence images of JC-1 aggregates (red), monomers (green), and merged signals. Scale bar: 10 μ m. D: Quantitative analysis of relative JC-1 fluorescence intensity($n=6$). E: Representative flow cytometry histograms of MitoSOX Red staining. F: Quantitative analysis of mitochondrial superoxide (MitoSOX Red) level($n=3$). G: Representative immunofluorescence images of MitoSOX Red staining. Scale bar: 10 μ m. H: Quantitative analysis of relative MitoSOX Red fluorescence intensity($n=5$). I: Representative flow cytometry histograms of DCFH-DA staining. J: Quantitative analysis of total cellular ROS (DCFH-DA) level ($n=3$). Data are presented as Mean $\pm$ SEM. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs. Scramble group.

图5 敲低 *Lss* 导致光感受器细胞线粒体膜电位下降并诱发氧化应激

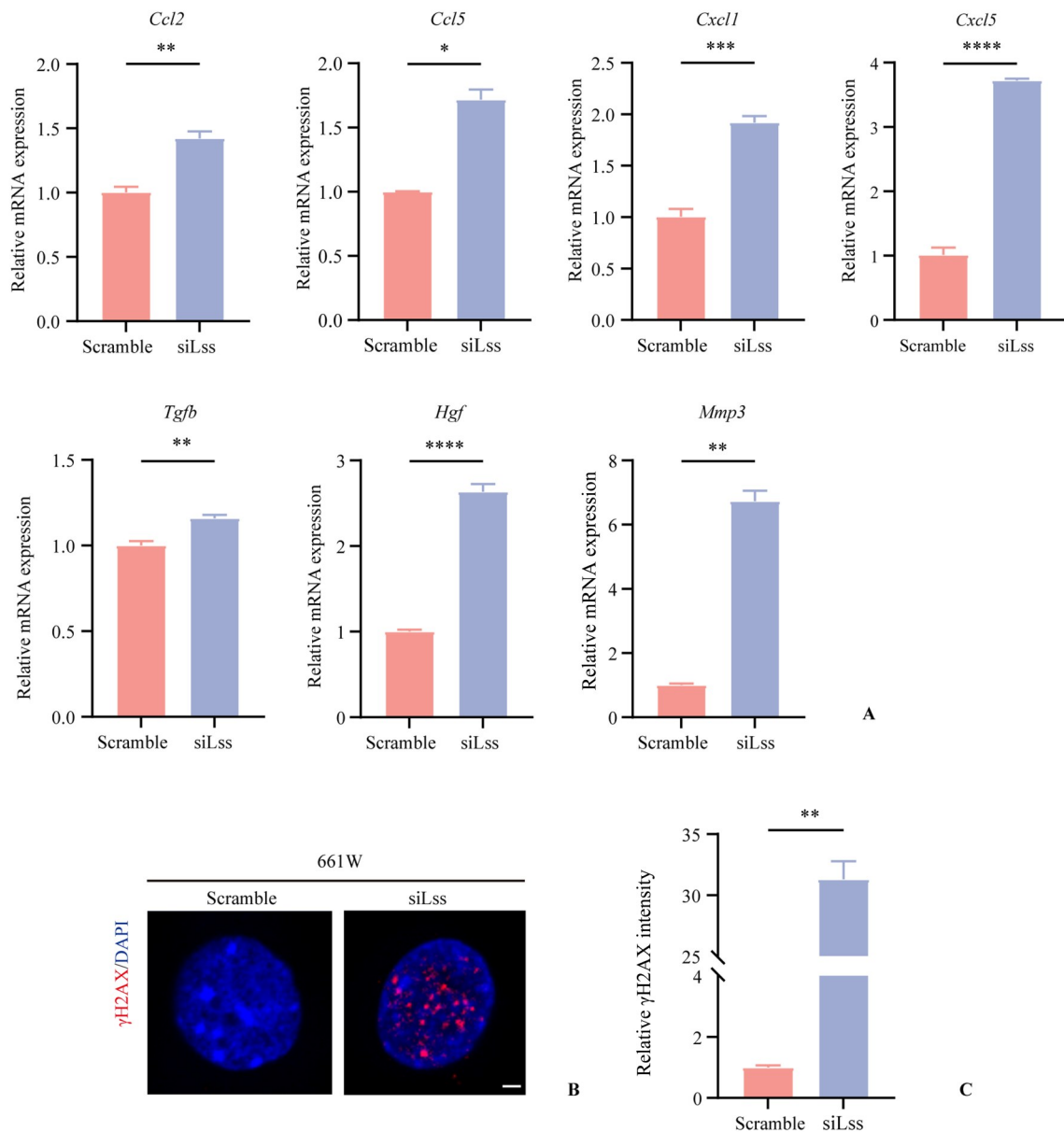
Fig. 5 *Lss* knockdown induces mitochondrial membrane potential depolarization and increased reactive oxygen species production in 661W photoreceptor cells

较Scramble组显著增加($t=20.510$, $P=0.002$;图6C)。

上述结果表明,敲低 *Lss* 所诱发的线粒体功能障碍与氧化应激可激活炎症因子表达,并最终导致DNA双链断裂损伤。

3 讨论

视网膜退行性疾病如AMD是导致中老年人群不可逆性视力丧失的主要原因,其核心病理为感光



A: Relative mRNA expression of inflammatory (*Ccl2*, *Ccl5*, *Cxcl1*, *Cxcl5*, *Tgfb*, *Hgf*, *Mmp3*) detected by RT-qPCR ($n=3$). Welch's t -test was used for *Ccl5* and *Mmp3*. B: Representative immunofluorescence images of γ H2AX (red) with DAPI nuclear counterstain (blue). Scale bar: 2 μ m. C: Quantitative analysis of relative γ H2AX fluorescence intensity ($n=3$). Welch's t -test was applied. Data are presented as Mean $\pm$ SEM. ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$ vs. control group.

图6 敲低 *Lss* 诱导 661W 细胞炎症因子 mRNA 显著上调及 DNA 损伤

Fig. 6 *Lss* knockdown induces up-regulation of inflammatory factor mRNA and DNA damage in 661W cells

细胞进行性丢失与视网膜功能衰退。尽管基因突变、氧化应激和炎症已被广泛研究^[4, 16],但现有研究多集中于 RPE 或全视网膜水平的脂质异常,感光细胞内甾醇代谢的证据仍空白,限制了对视网膜退行性疾病代谢基础的深入理解。基于此,构建并分析合适的动物模型对于阐明相关发病机制具有重要意义。本研究围绕“感光细胞内源性羊毛甾醇是否参与维持感光细胞稳态,及其在年龄相关视网膜

变性中的作用”这一科学问题,首次通过遗传学手段在体内构建了感光细胞特异性 *Lss* 条件敲除模型,揭示了羊毛甾醇缺失在感光细胞退行性病变及视网膜代谢重塑中的关键作用。本研究结果表明,感光细胞内源性羊毛甾醇缺失可引起随年龄进展的视网膜结构破坏和功能衰退,其机制可能与线粒体能量代谢障碍、氧化应激增强密切相关。

视网膜是机体中代谢最活跃的组织之一,其感

光细胞外节膜盘的不断更新对脂质和甾醇的周转有着极高的需求^[17-18]。羊毛甾醇作为胆固醇生物合成途径中的第一个环状前体分子,在成熟视网膜中的生物学功能尚未得到充分认识^[19]。本研究发现,感光细胞特异性羊毛甾醇合成受损的CKO小鼠在1月龄时视网膜结构基本正常,但自4月龄起逐渐出现视功能减退,并在8月龄时表现出显著的ONL变薄和萎缩。这一特征与临床上多种视网膜退行性疾病,尤其是AMD及部分视网膜色素变性中“早期代偿、晚期失代偿”的病程一致。

感光细胞是全身代谢最活跃的神经元之一,视杆细胞椭圆体带中线粒体占比高达54%~66%,其外节盘膜的持续更新及光信号传导过程高度依赖线粒体氧化磷酸化功能^[20]。有研究报道,羊毛甾醇可以通过诱导线粒体解偶联作用以及促进线粒体自噬保护多巴胺能神经元^[21]。此外,作为羊毛甾醇下游的重要中间体,链甾醇在巨噬细胞中充当连接甾醇稳态与炎症调控的关键枢纽。其缺失可引起线粒体呼吸链功能障碍、mtROS爆发性升高,进而激活NLRP3炎症小体,促进炎症并加速动脉粥样硬化进展^[22]。本研究中,敲低*Lss*后细胞的基础呼吸、最大呼吸能力、ATP产生及储备呼吸能力均显著下降,羊毛甾醇缺失会严重损害光感受器细胞的线粒体功能。结合转录组分析结果,*Lss*敲低后差异基因显著富集于氧化磷酸化、线粒体ATP合成、电子传递链及细胞呼吸相关通路。在光感受器细胞中,*Lss*的敲低导致了线粒体呼吸链复合体I至V的核心亚基基因表达下降,如复合体I的*Ndufb9*和*Ndufs7*、复合体II的*Sdhb*、复合体III的*Uqcrl10*、复合体IV的*Cox5a*以及复合体V的*Atp5g1*等。有研究指出线粒体复合物I的下调通过氧化应激导致光感受器外节损伤^[23]。因此,线粒体呼吸链复合体关键基因的表达下降,可能是*Lss*敲低组线粒体基础呼吸受损及ATP生成减少的直接原因。与成熟中枢神经元主要依赖线粒体氧化磷酸化不同,光感受器细胞约80%~90%的能量产生来自糖酵解^[24]。本研究中,尽管*Lss*敲低后导致线粒体呼吸严重受损,但细胞通过显著上调基础糖酵解和代偿性糖酵解维持能量稳态,这一适应性重编程可能是光感受器在面对线粒体损伤时的生存策略。然而,长期过度依赖糖酵解可能导致乳酸堆积、胞内酸化及氧化应激加剧,仍可能造成光感受器的损伤和视网膜退行性病变^[25-26]。

ROS的过量产生是线粒体功能障碍的直接后果^[27]。哺乳动物的视网膜极易受到氧化应激的影响,光感受器膜盘含有多不饱和脂肪酸,易发生氧化损伤^[28]。研究显示,羊毛甾醇对紫外线照射诱导的ROS产生具有一定保护作用^[29]。TLR4激活可诱导巨噬细胞内羊毛甾醇积累,从而增强膜流动性并促进ROS生成,最终提高吞噬与杀菌能力,提示羊毛甾醇在免疫相关氧化应激调控中具有重要作用^[30]。本研究通过JC-1、MitoSOX及DCFH-DA等多种手段证实,*Lss*缺失会导致线粒体膜电位下降及线粒体和细胞内ROS显著升高。这一结果提示,羊毛甾醇在感光细胞中可能具有维持线粒体氧化还原稳态的重要作用。持续的氧化应激是视网膜退行性疾病的致病因素之一,过量ROS可直接损伤脂质、蛋白和DNA。本研究进一步发现,敲低*Lss*后 γ H2AX显著升高,提示DNA双链断裂明显增加,说明羊毛甾醇缺失引发的氧化应激已超过细胞修复能力,导致基因组稳定性受损^[31]。这一发现为甾醇代谢异常与感光细胞遗传稳定性破坏之间建立了直接联系。

以上代谢紊乱最终引发了炎症反应和双链DNA损伤。视网膜作为中枢神经系统的延伸,炎症和DNA损伤被认为是年龄相关视网膜退行性疾病的重要驱动因素之一^[32]。CCL2-CCR2轴通过驱动慢性炎症并促进光感受器退变,在AMD发病机制中发挥重要作用^[33]。多种促炎因子显著上调,预示着受损的感光细胞不仅自身功能丧失,还可在局部微环境中放大炎症反应、干扰细胞间通讯并加速组织退变^[34-35]。双链DNA损伤是连接代谢障碍与细胞损伤的关键节点^[31, 36]。在本研究中,*Lss*敲低后同样观察到*Ccl2*、*Cxcl1*、*Tgfb*等促炎因子mRNA显著上调,同时 γ H2AX焦点数量明显增加,证实羊毛甾醇合成障碍所致的代谢重编程可直接触发炎症级联反应和DNA损伤应答,最终加速光感受器细胞的损伤与退变。

值得注意的是,甾醇代谢在视网膜稳态维持中的重要性已被多项研究证实。光感受器特异性敲除胆固醇外流转运体*Abca1/Abcg1*导致胆固醇积累,可直接促进光感受器退行性病变^[37-38]。同样,胆固醇代谢紊乱,如Cyp27a1/Cyp46a1双敲除、高脂饮食诱导已被证实可驱动年龄相关光感受器神经退行性病变^[39-40]。这些证据揭示了甾醇代谢失衡与光感受细胞退变之间的紧密联系,提示该通路中

的关键调控节点可能是潜在的干预靶点。本研究首次通过光感受器细胞特异性遗传模型,在体内直接证明羊毛甾醇在感光细胞长期稳态中的关键作用,并提出甾醇代谢异常可作为连接年龄、代谢失衡与感光细胞退变的重要枢纽。在转录富集结果中,同时出现了神经退行性疾病相关通路[如帕金森病(Parkinson's disease, PD)和阿尔茨海默病通路(Alzheimer's disease, AD)],提示线粒体功能障碍是中枢神经退行性疾病和视网膜退行性病变的共同病理机制之一。最新研究表明,提高线粒体递送效率可有效干预PD^[41]。虽然有研究报道,*Lss*合成产物羊毛甾醇可促进线粒体自噬保护神经细胞,羊毛甾醇水平下调与AD、PD的发生密切相关^[21, 42],但是否通过线粒体功能障碍导致AD和PD有待进一步探究。

本研究通过构建光感受器*Lss*缺失动物模型阐明了*Lss*基因在维持光感受器正常功能中的重要作用,但仍存在一定的局限性。GWAS研究表明,*Lss*基因与GA进展密切相关。干性AMD终末期以光感受器细胞的进行性丢失为核心病理特征,虽然光感受器特异性敲除*Lss*小鼠模型能部分模拟该过程,但小鼠视网膜没有黄斑结构,后续还需要在患者视网膜组织进一步研究。此外,基于*Lss*缺失在视网膜退行性病变中的重要作用,高表达*Lss*或补充羊毛甾醇是否可作为潜在的治疗手段有待于验证。

综上,本研究为理解年龄相关视网膜退行性疾病的发病机制提供了新角度。未来,靶向甾醇代谢、改善线粒体功能或抑制氧化应激相关通路,如局部补充羊毛甾醇、线粒体保护剂或抗炎药物均存在延缓感光细胞退变的干预价值。

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