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[DOI]10.12016/j.issn.2096-1456.202660123

· 基础研究 ·

调节性T细胞在口腔鳞状细胞癌低氧应激环境下抵抗铁死亡的机制研究

郭芮瑜, 章芳瑜, 徐骏, 陈万涛, 严明

上海交通大学医学院附属第九人民医院口腔颌面头颈肿瘤科, 上海交通大学口腔医学院, 国家口腔医学中心, 口腔疾病国家临床医学研究中心, 上海市口腔医学重点实验室, 上海市口腔医学研究所, 上海(200011)

【摘要】 目的 探讨低氧应激微环境下调节性T细胞(Tregs)抵抗铁死亡的代谢分子机制, 为寻找改善口腔鳞状细胞癌(OSCC)免疫微环境的潜在干预靶点提供科学依据。方法 本研究获单位医学伦理委员会审批, 通过多色免疫荧光检测OSCC患者临床病理标本中Tregs分布及低氧区域定位。动物实验获得单位实验动物伦理委员会审批。建立C57BL/6小鼠荷瘤模型, 收集颌下、肩胛下、腹股沟及腭窝淋巴结与脾脏, 研磨制备单细胞悬液。体外诱导C57BL/6小鼠初始CD4⁺T细胞分化为Tregs, 构建1%低氧培养模型, 设置常氧对照组与低氧组, 流式细胞术检测低氧环境下Tregs细胞活力。运用转录组学、代谢组学分析差异表达基因、代谢通路及胆固醇代谢产物变化。采用Western blot检测帕米结合蛋白(EBP)、谷胱甘肽过氧化物酶4(GPX4)、铁死亡抑制蛋白1(FSP1)、长链脂酰辅酶A合成酶4(ACSL4)表达。采用CCK-8法确定EBP抑制剂TASIN-30的安全浓度, 在此基础上, 设置常氧对照组、低氧组、低氧+TASIN-30组、低氧+TASIN-30+7-脱氢胆固醇(7-DHC)组, 流式细胞术检测各组Tregs活力; 细胞免疫荧光及流式细胞术检测胞内Fe²⁺含量、脂质过氧化水平及活性氧(ROS)累积量; 酶标仪检测还原型/氧化型谷胱甘肽(GSH/GSSG)比值; 透射电镜观察线粒体形态。通过C3H/HeJ小鼠体内抑瘤实验验证抑制EBP的体内效应及基于TCGA-HNSC队列的临床预后生存分析评估Treg铁死亡抵抗的临床预后价值。结果 OSCC肿瘤组织中Tregs大量富集且优先聚集于低氧区域, 且Tregs可在低氧状态下维持细胞存活; 低氧组胆固醇代谢通路上调, EBP表达升高($P < 0.001$), 7-DHC积累; 与低氧组相比, 低氧+TASIN-30组GPX4、FSP1表达降低($P < 0.001$, $P = 0.003$), ACSL4表达升高($P < 0.001$), 活细胞比例下降($P < 0.001$), Fe²⁺、脂质过氧化、ROS水平升高($P < 0.001$), GSH/GSSG比值降低($P < 0.001$), 线粒体皱缩; 外源性补充7-DHC则可逆转抑制EBP后的效应; 小鼠体内抑制EBP后可显著抑制肿瘤生长($P < 0.001$)。结论 Tregs在低氧微环境中上调EBP, 促进7-DHC积聚, 进而抑制Tregs铁死亡, 维持其存活及免疫抑制功能, 抑制EBP可削弱Tregs在低氧状态下抵抗铁死亡的作用。

【关键词】 口腔鳞状细胞癌; 肿瘤免疫; 低氧微环境; 调节性T细胞; 胆固醇代谢; 铁死亡; 依莫帕米结合蛋白; 7-脱氢胆固醇

【中图分类号】 R78 **【文献标志码】** A **【文章编号】** 2096-1456(2026)10-0953-17

【引用著录格式】 郭芮瑜, 章芳瑜, 徐骏, 等. 调节性T细胞在口腔鳞状细胞癌低氧应激环境下抵抗铁死亡的机制研究[J]. 口腔疾病防治, 2026, 34(10): 953-969. doi:10.12016/j.issn.2096-1456.202660123.

Mechanistic study of regulatory T cells resisting ferroptosis under hypoxic stress in oral squamous cell carcinoma GUO Ruiyu, ZHANG Fangyu, XU Qin, CHEN Wantao, YAN Ming. Department of Oral and Maxillofacial Head and Neck Oncology, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, College of Stomatology, Shanghai Jiao Tong University, National Center for Stomatology, National Clinical Research Center for Oral Disease, Shanghai Key Laboratory of Stomatology & Shanghai Research Institute of Stomatology, Shanghai 200011, China



微信公众号

【收稿日期】 2026-03-27; **【修回日期】** 2026-04-28

【基金项目】 国家自然科学基金面上项目(82173217)

【作者简介】 郭芮瑜, 医师, 硕士研究生在读, Email: ggry0601@163.com

【通信作者】 严明, 副研究员, 博士, Email: yanmingsjtu@163.com

Corresponding author: YAN Ming, Email: yanmingsjtu@163.com

【Abstract】 Objective To explore the metabolic molecular mechanism of regulatory T cells (Tregs) resisting ferroptosis under a hypoxic stress microenvironment, and to provide scientific support for finding intervention targets to improve the immune microenvironment of oral squamous cell carcinoma (OSCC). **Methods** This study was approved by the Institutional Medical Ethics Committee. Multiplex immunofluorescence was used to detect the distribution of Tregs and the localization of hypoxic regions in clinical pathological specimens from patients with OSCC tissues. Animal experiments were approved by the Laboratory Animal Ethics Committee. A tumor-bearing model was established in C57BL/6 mice. The submandibular, subscapular, inguinal, and popliteal lymph nodes were collected along with the spleen, and were homogenized to prepare single cell suspensions. C57BL/6 mouse naive CD4⁺ T cells were induced to differentiate into Tregs *in vitro*, and a 1% hypoxic culture model was established. Normoxia and hypoxia groups were established, and Treg viability under hypoxic conditions was assessed by flow cytometry. Transcriptomics and metabolomics were applied to analyze differentially expressed genes, metabolic pathways, and changes in cholesterol metabolites. Western blot was performed to detect the expression of emopamil-binding protein (EBP), glutathione peroxidase 4 (GPX4), ferroptosis suppressor protein 1 (FSP1), and acyl-CoA synthetase long-chain family member 4 (ACSL4). The safe concentration of the EBP inhibitor TASIN-30 was determined by the CCK-8 assay. On this basis, four groups were established: normoxia control, hypoxia, hypoxia+TASIN-30, and hypoxia+TASIN-30+7-dehydrocholesterol (7-DHC). Treg viability in each group was measured by flow cytometry; intracellular Fe²⁺ content, lipid peroxidation level, and reactive oxygen species (ROS) accumulation were detected by cellular immunofluorescence and flow cytometry; the reduced/oxidized glutathione (GSH/GSSG) ratio was measured using a microplate reader; and mitochondrial morphology was observed by transmission electron microscopy. *In vivo* tumor-inhibition experiments in C3H/HeJ mice were performed to validate the *in vivo* effects of EBP inhibition, and clinical prognostic survival analysis based on the TCGA-HNSC cohort was performed to evaluate the clinical prognostic value of ferroptosis resistance in Tregs. **Results** Tregs were highly enriched in OSCC tissue, preferentially accumulated in hypoxic areas, and could maintain cell survival under hypoxia. The cholesterol metabolism pathway was upregulated in the hypoxic group, with increased EBP mRNA expression ($P < 0.001$) and 7-DHC accumulation. Compared with the hypoxic group alone, the hypoxic + TASIN-30 group showed decreased GPX4 and FSP1 expression ($P < 0.001$, $P = 0.003$); increased ACSL4 expression ($P < 0.001$); a decreased viable cell ratio ($P < 0.001$); increased Fe²⁺, lipid peroxidation, and ROS levels ($P < 0.001$); a decreased GSH/GSSG ratio ($P < 0.001$); and mitochondrial shrinkage. Exogenous supplementation of 7-DHC reversed the effects of EBP inhibition. *In vivo* inhibition of EBP significantly inhibited tumor growth ($P < 0.001$). **Conclusion** Tregs upregulate EBP and promote 7-DHC accumulation in hypoxic microenvironments, thereby inhibiting Treg ferroptosis and maintaining their survival and immunosuppressive function. Inhibition of EBP can weaken the resistance of Tregs to ferroptosis under hypoxia.

【Key words】 oral squamous cell carcinoma; tumor immunity; hypoxic microenvironment; regulatory T cells; cholesterol metabolism; ferroptosis; emopamil-binding protein; 7-dehydrocholesterol

J Prev Treat Stomatol Dis, 2026, 34(10): 953-969.

口腔鳞状细胞癌(oral squamous cell carcinoma, OSCC)是一种常见的头颈恶性肿瘤,具备侵袭性强、易复发转移的特点,其病灶内部因肿瘤细胞异常增殖、血管分布紊乱且功能异常,形成典型的低氧微环境^[1-3]。这一微环境不仅推动肿瘤细胞恶性增殖、侵袭转移,还会重塑局部免疫微环境,介导免疫逃逸,是OSCC临床治疗耐受、预后不佳的重要诱因^[4-5]。调节性T细胞(regulatory T cells, Tregs)是机体维持免疫稳态的核心免疫抑制细胞,在OSCC低氧微环境中大量富集,成为肿瘤免疫抑制网络的关键组成部分^[6-8]。Tregs可通过直接抑

制效应T细胞的活化与增殖、分泌免疫抑制因子、阻断抗原呈递过程等多重途径,抑制机体抗肿瘤免疫应答,从而促进肿瘤细胞逃避免疫监视。而Tregs在低氧微环境中的存活能力与免疫抑制功能高度依赖细胞内代谢重编程的精准调控^[9-11]。

铁死亡是一种铁离子依赖性、以脂质过氧化过度累积为核心特征的程序性细胞死亡方式,区别于传统凋亡、坏死等死亡形式,已成为调控免疫细胞存活、重塑肿瘤免疫微环境的重要靶点^[12-13]。7-脱氢胆固醇(7-dehydrocholesterol, 7-DHC)作为重要的固醇代谢物,被证实可通过抑制脂质过氧化

发挥铁死亡拮抗作用,而依莫帕米结合蛋白(emopamil-binding protein, EBP)、7-脱氢胆固醇还原酶(7-dehydrocholesterol reductase, DHCR7)作为胆固醇代谢通路的关键调控分子,与7-DHC的合成代谢密切相关^[14-15]。目前,低氧微环境下Tregs的铁死亡调控机制尚未完全阐明,EBP/7-DHC在其中的作用更是鲜有报道。

本研究依托转录组学、代谢组学联合测序技术,结合分子干预与功能验证实验,系统性探究低氧状态下EBP、7-DHC的表达变化,及其对Tregs铁死亡的调控效应,旨在明确低氧微环境介导Tregs环境适应性的代谢机制,为阐明OSCC进展的代谢免疫调控网络提供新的实验依据。

1 材料和方法

1.1 主要试剂与仪器

流式细胞仪(NovoCyte Peatean, Agilent, 美国);激光共聚焦显微镜(LSM900, ZEISS, 德国);胎牛血清(A5669701, Gibco, 澳洲);RPMI-1640培养基(L210KJ, 源培生物, 中国);1×PBS(C20012500BT, Gibco, 澳洲);胰蛋白酶细胞消化液(C100C1, 新赛美, 中国);100×青霉素-链霉素(C100C5, 新赛美, 中国);多色免疫荧光染色试剂盒(AFIHC027, 艾方, 中国);Hypoxypore-Red549 Kits (HPB-HP7-100Kit, Hypoxypore, 美国);流式试剂(fixable viability kit, 423106; PerCP/Cyanine5.5 anti-mouse CD4, 100540; Alexa Fluor® 647 anti-mouse/rat/human FOXP3, 320014; Biogend, 美国);CD4⁺ T细胞分选试剂盒(480070, Biogend, 美国);白细胞介素-2(interleukin-2, IL-2) (200-02-100UG, Peprotech, 美国);转化生长因子-β(transforming growth factor-β, TGF-β) (100-21-100UG, Peprotech, 美国);抗CD3抗体(16-0031-86, ThermoFisher, 美国);抗CD28抗体(16-0281-86, ThermoFisher, 美国);一抗:兔抗人CD4抗体(ab288724, Abcam, 美国);鼠抗人叉头盒蛋白3(Forkhead Box Protein 3, FOXP3)抗体(14-4777-82, Thermo, 美国);鼠抗鼠EBP抗体(sc-374267, Santa, 美国);兔抗鼠谷胱甘肽过氧化物酶4(glutathione peroxidase 4, GPX4)抗体(52455, Cell Signaling Technology, 美国);兔抗鼠铁死亡抑制蛋白1(ferroptosis suppressor protein 1, FSP1)抗体(24972, Cell Signaling Technology, 美国);兔抗鼠长链脂酰辅酶A合成酶4(acyl-CoA synthetase long-chain family member 4, ACSL4)抗体(38493, Cell

Signaling Technology, 美国);兔抗鼠β-肌动蛋白抗体(4967, Cell Signaling Technology, 美国);辣根过氧化物酶标记的山羊抗兔IgG(HRP-conjugated goat anti-rabbit IgG)(SA00001-2, Proteintech, 美国);小分子化合物及检测试剂:TASIN-30(HY-164561, MedChemExpress, 美国);7-脱氢胆固醇(7-dehydrocholesterol, 7-DHC)(HY-113279, MedChemExpress, 美国);铁离子荧光探针(Rhonox-1)(HY-D1533, MedChemExpress, 美国);活性氧荧光探针(DCFH-DA)(HY-D0940, MedChemExpress, 美国);还原型/氧化型谷胱甘肽检测试剂盒(reduced glutathione/oxidized glutathione GSH/GSSG Assay Kit)(HY-K0311, MedChemExpress, 美国);脂质过氧化检测试剂盒(S0043, 碧云天, 中国)。

1.2 实验方法

1.2.1 临床标本多色免疫荧光检测 回顾性收集本院5例OSCC患者的肿瘤石蜡标本及对应的癌旁正常组织石蜡标本(男性3例、女性2例, TNM分期Ⅱ期3例、Ⅲ期2例),所有患者经病理确诊,未接受术前放化疗及免疫治疗。本实验遵循《赫尔辛基宣言》的原则,并经上海交通大学医学院附属第九人民医院医学伦理委员会审批(批件号:SH9H-2026-T218-1),鉴于研究仅使用既往诊疗中留存的匿名化石蜡标本,且不涉及患者隐私及干预措施,该委员会批准豁免患者知情同意。染色步骤:65℃烤片2h,二甲苯Ⅰ、Ⅱ脱蜡15min,乙醇梯度复水;柠檬酸钠-EDTA抗原热修复,冷却后过氧化物酶封闭液室温孵育15min,封闭液室温封闭1h。滴加一抗(CD4、FOXP3, 1:200),4℃湿盒避光孵育过夜,通用二抗孵育1h, TSA荧光染料避光显色5~10min;重复抗原修复及染色完成多标,含DAPI抗淬灭剂封片,共聚焦显微镜观察成像,每例样本随机选取3个视野进行统计。

1.2.2 动物实验 本研究所有动物实验遵循国家《实验动物管理条例》及《实验动物福利伦理审查指南》的相关规定,并经上海交通大学医学院附属第九人民医院实验动物伦理委员会审批(批件号:SH9H-2025-A1613-1),动物实验方案按伦理委员会批准的方案执行(未进行注册)。本研究遵循ARRIVE指南2.0。实验动物选用6~8周龄20~22g SPF级健康雄性C57BL/6、C3H/HeJ小鼠,购自赛业(苏州)生物科技有限公司, C57BL/6小鼠的合格证号:320981260100399942, C3H/HeJ小鼠的合格证号:320981260100055538,饲养于SPF级动物房内

(温度 22~25 °C, 相对湿度 50%~60%, 12 h 光/暗循环, 自由摄食和饮水, 每日监测动物体质量、活动状态), 随机笼位, 以单只小鼠作为独立实验单元。C57BL/6 小鼠用于构建肿瘤内低氧动物模型, C3H/HeJ 小鼠用于后续体内抑瘤实验。随机数字表法选取 6 只 C57BL/6 小鼠(根据预实验结果确定样本量)构建荷瘤模型组, 未设置对照组, 仅探究肿瘤内部低氧微环境特征。对数生长期小鼠 OSCC 细胞系 MOC1 细胞消化成单细胞悬液(浓度 1×10^7 个/mL), 每只 C57BL/6 小鼠右后足垫部位注射 1×10^6 个细胞。待足垫肿瘤体积生长至 100 ~ 150 mm³ 时, 纳入后续实验。纳入标准: 体表无破损、无感染、实验周期内体质量波动不超过初始体质量 10% 的小鼠; 排除标准: 接种后足垫未成瘤或肿瘤体积未达设定范围者; 接种部位出现破溃、出血、感染或坏死, 且经局部消毒处理 48 h 后无好转者; 实验期间出现严重恶病质(如弓背、竖毛、自发活动显著减少)者。本实验最终共纳入 6 只符合标准的 C57BL/6 小鼠, 无动物在实验终点前被剔除或终止。处死小鼠前 90 min 腹腔注射低氧示踪剂哌莫硝唑(60 mg/kg), 随后置于预先充入 5% 异氟烷 氧气混合气体(氧气流速 1 L/min) 的麻醉诱导盒中进行麻醉 2 min, 确认达到深度麻醉状态后颈椎脱臼法处死小鼠, 剥离肿瘤组织包埋切片后 -80 °C 保存^[16]。冰冻切片室温复温, 4% 多聚甲醛固定 15 min, 0.5% Triton X-100 室温孵育 10 min, 过氧化物酶封闭液室温孵育 15 min, 封闭液室温封闭 1 h 后滴加 FOXP3 一抗(1:200) 4 °C 孵育过夜。通用二抗室温孵育 1 h, 荧光素室温避光显色 5~10 min。抗体洗脱液 37 °C 处理 30 min, 封闭, 低氧染料(1:100) 4 °C 孵育过夜; DAPI 封片, 共聚焦显微镜采集图像。

1.2.3 Tregs 诱导分化、低氧模型构建及活性检测 随机数字表法取 1 只 6~8 周龄 SPF 级雄性 C57BL/6 小鼠, 于处死前 90 min 腹腔注射低氧示踪剂哌莫硝唑(60 mg/kg), 随后以颈椎脱臼法处死小鼠, 收集颌下、肩胛下、腹股沟及腘窝淋巴结与脾脏, 研磨制备单细胞悬液。1 mL 红细胞裂解液室温裂解 2 min, PBS 终止后离心, 细胞分选液 200 μ L 重悬细胞。20 μ L Fc 受体封闭抗体室温静置 10 min, 10 μ L CD4 偶联磁珠冰上孵育 15 min; 磁块分选 3 次获得纯化 CD4⁺ T 细胞。以含 TGF- β (10 ng/ μ L)、IL-2(10 ng/ μ L) 的 T 细胞完全培养基重悬细胞, 接种于 CD3、CD28 抗体包被的 24 孔板, 常规培养 72 h, 诱导高纯度 Tregs。将 Tregs 分为常氧对照组(20%

O₂) 和低氧组(1% O₂), 培养 24 h。采用 Fixable Viability 染料(1:1 000, 每管 100 μ L) 冰上避光染色 15 min, 流式细胞术检测细胞活力。

1.2.4 转录组学检测差异表达基因 取常氧组和低氧组 Tregs, Trizol 法提取总 RNA, 检测纯度和完整性。合格 RNA 样本进行转录组测序(Illumina NovaSeq 6000)。测序数据过滤后, 进行差异表达分析(|FC| > 1.5, $P < 0.05$), 转录因子富集分析(Metascape)、KEGG 通路富集分析(clusterProfiler)、GSEA 富集分析(GSEA 4.2.0), 热图绘制(pheatmap)。

1.2.5 代谢组学检测胆固醇代谢产物变化 收集常氧组和低氧组 Tregs, 液氮速冻, -80 °C 保存。干冰运输后, 预冷甲醇-乙腈提取代谢物, 高速离心、浓缩、复溶后用超高效液相色谱-串联质谱进行代谢组学测序(Thermo Hypersil GOLD C18 柱, 柱温 30 °C)。筛选差异胆固醇代谢产物($FC \geq 1.1$, $P < 0.05$)。

1.2.6 Western blot 检测蛋白水平变化 收集低氧组及常氧对照组 Tregs, RIPA 裂解液(含蛋白酶及磷酸酶抑制剂)冰浴裂解 30 min, 12 000 rpm、4 °C 离心 15 min, 取上清。BCA 法蛋白定量, 加入 5 $\times$ 上样缓冲液, 95 °C 变性 15 min。SDS-PAGE 电泳后转 PVDF 膜, 5% 脱脂牛奶封闭 2 h, 一抗 4 °C 孵育过夜, HRP 二抗室温孵育 1 h, ECL 化学发光显影。检测指标: EBP、GPX4、FSP1、ACSL4, 以 β -actin 为内参。

1.2.7 抑制 EBP 与挽救实验 将 Tregs 接种于 96 孔板(1×10^4 个/孔), 加入不同浓度的 EBP 抑制剂 TASIN-30(0、2.5、5、10、20、40、80 μ mol/L), 每个浓度 3 个复孔, 培养 48 h 后加 CCK-8 试剂孵育 2 h, 测 450 nm 吸光度, 计算 IC₅₀。后续实验选用 10 μ mol/L TASIN-30 作为 EBP 抑制剂, 并根据预实验结果, 以 25 μ mol/L 7-DHC 进行挽救实验。分组: 常氧对照组、低氧组、低氧+TASIN-30 组(10 μ mol/L TASIN-30 处理 24 h)、低氧+TASIN-30+7-DHC 组(10 μ mol/L TASIN-30+25 μ mol/L 7-DHC 处理 24 h)。

1.2.8 细胞免疫荧光和流式细胞术检测铁离子含量 将 10 mmol/L Rhonox-1 储存液用无血清培养基配制 5 μ mol/L 工作液; 将不同分组(组别与 1.2.7 相同) Tregs 调整密度至 1×10^6 个/mL, 离心后加入 1 mL 5 μ mol/L Rhonox-1 工作液, 37 °C 孵育 30 min, 无血清培养基重悬后用于共聚焦成像与流式细胞仪检测(Ex/Em=540/575 nm), 分析平均荧光强度。

1.2.9 细胞免疫荧光和流式细胞术检测脂质过氧

化 各组细胞(组别与1.2.7相同)加入5 $\mu\text{mol/L}$ BODIPY C11探针,37 $^{\circ}\text{C}$ 避光孵育30 min。共聚焦显微镜(氧化态Ex/Em=488/510 nm,还原态Ex/Em=581/591 nm)和流式细胞仪检测,计算氧化/还原荧光比值。

1.2.10 细胞免疫荧光和流式细胞术检测ROS 各组细胞(组别与1.2.7相同)加入10 $\mu\text{mol/L}$ DCFH-DA探针,37 $^{\circ}\text{C}$ 避光孵育30 min。共聚焦显微镜和流式细胞仪检测(Ex/Em=488/525 nm)平均荧光强度。

1.2.11 酶标仪检测GSH/GSSG GSH标准品配制成100 $\mu\text{mol/L}$ 储存液,逐步稀释为0、2.5、5、10、20、40 $\mu\text{mol/L}$ 标准工作液,配置GSH、GSSG检测液;收集各组细胞(组别与1.2.7相同),密度 1×10^6 个/mL,冰上裂解30 min,12 000 $\times g$ 离心10 min,取上清。按GSH/GSSG检测试剂盒说明书操作,酶标仪412 nm测OD值,计算GSH/GSSG比值。

1.2.12 透射电镜观察线粒体形态 收集各组Tregs(组别与1.2.7相同),调整细胞密度至 1×10^6 个/mL,1 mL 2.5%戊二醛固定液4 $^{\circ}\text{C}$ 避光固定2 h,1 mL 1%锇酸固定液4 $^{\circ}\text{C}$ 避光固定1 h,梯度乙醇脱水、包埋聚合,超薄切片(50~70 nm),醋酸铀和柠檬酸铅染色,透射电镜(80 kV)采集线粒体形态图像。

1.2.13 小鼠体内抑瘤实验 取6~8周龄SPF级雄性C3H/HeJ小鼠,动物适应性生长后皮下注射SCC7细胞(1×10^6 个/只)。成瘤后随机数字表法分为2组(每组5只,样本量依据预实验确定):对照组(腹腔注射PBS)和TASIN-30组(腹腔注射40 mg/kg TASIN-30,每日1次,连续10 d),随机笼位。每2 d测量肿瘤长径(L)和短径(W),计算体积 $V=(L\times W^2)/2$,分组及肿瘤测量、称重操作均采用单盲法评估。动物纳入标准为小鼠状态良好、无外伤感染、体重平稳;排除标准为造模后未成瘤、肿瘤生长异常及实验期间小鼠死亡,本实验无动物剔除,最终有效实验样本共10只。10 d后处死小鼠,于处死前90 min腹腔注射低氧示踪剂哌莫硝唑(60 mg/kg),随后置于预先充入5%异氟烷氧气混合气体(氧气流速1 L/min)的麻醉诱导盒中进行麻醉2 min,确认达到深度麻醉状态后以颈椎脱臼法处死小鼠,剥离肿瘤称重。

1.2.14 临床预后生存分析 基于TCGA-HNSC队列筛选OSCC样本,单细胞转录组数据(GSE140042)中提取Tregs亚群,结合FerrDb V3数

据库筛选Tregs中表达率>10%的铁死亡抑制基因;UCeI算法计算铁死亡抑制评分,将Tregs分为铁死亡抵抗型(Tregs_FerroR)与敏感型(Tregs_FerroS)亚群,筛选前100个显著上调基因构建Tregs_FerroR特征基因集,应用于癌症基因组图谱-口腔鳞状细胞癌(The Cancer Genome Atlas - Oral Squamous Cell Carcinoma, TCGA-OSCC)队列,计算患者特征得分;基于最大选择秩统计量确定最佳截断值,划分高分组与低分组;采用Kaplan-Meier法绘制总体生存曲线,并通过Log-rank检验评估组间生存差异。

1.3 统计学分析

所有细胞实验独立重复3次, n 值代表独立实验次数或每组单个小鼠数量。采用GraphPad Prism 9.0软件进行统计学分析,计量资料以均数 $\pm$ 标准差($\bar{x}\pm s$)表示,两组间比较采用独立样本 t 检验,多组间比较采用单因素方差分析(ANOVA), $P<0.05$ 为差异具有统计学意义。

2 结果

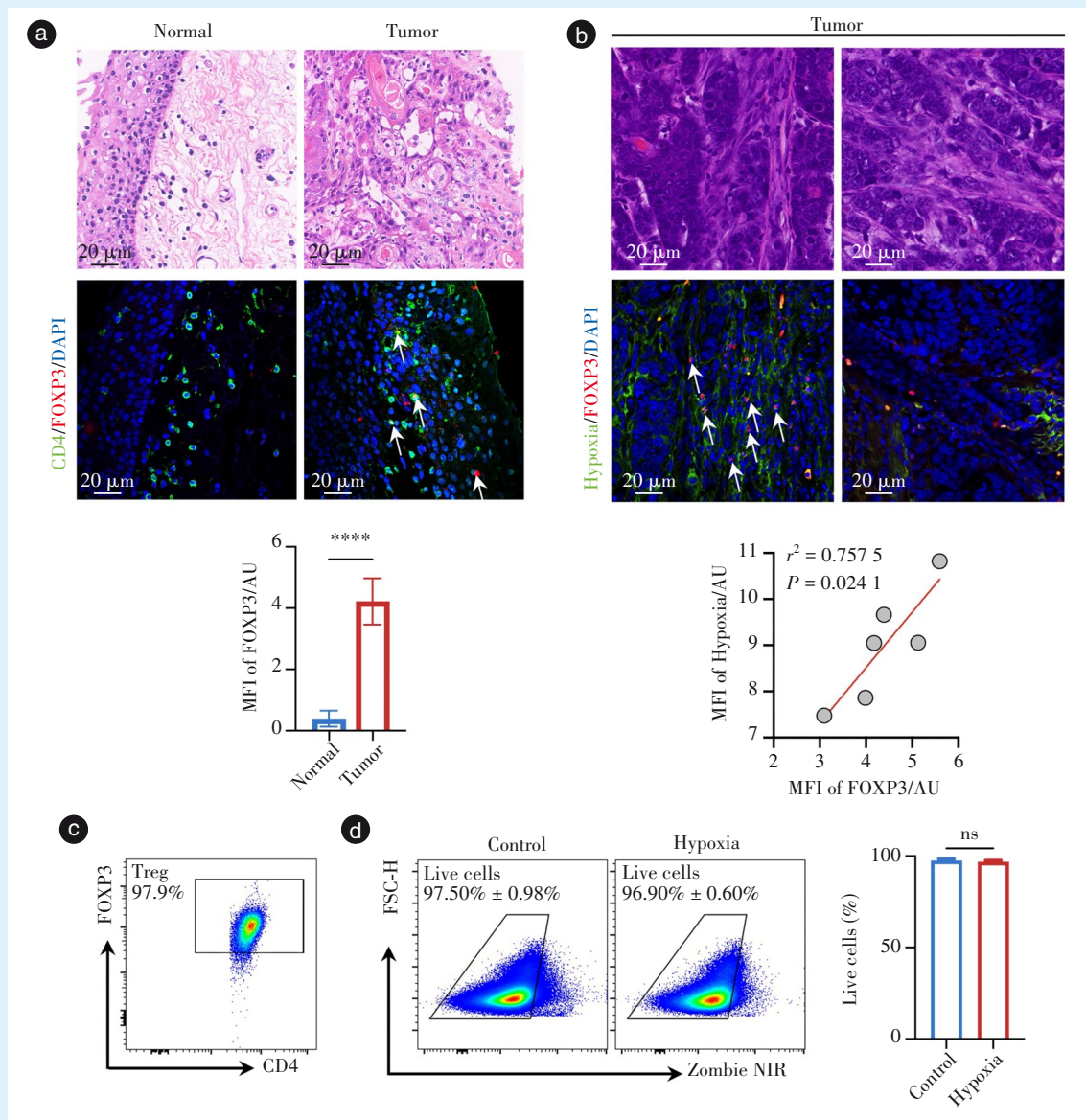
2.1 OSCC肿瘤组织中Tregs优先聚集于低氧区域

OSCC患者的原发灶及癌旁正常组织的免疫荧光染色显示,肿瘤组织中FOXP3⁺ Tregs数量显著高于癌旁组织($P<0.001$)(图1a)。小鼠荷瘤低氧模型表明,Tregs主要聚集于低氧区域,且低氧荧光强度与FOXP3荧光强度呈正相关(图1b)。流式结果显示,诱导Tregs纯度为97.9%,与常氧环境相比,低氧培养对Tregs细胞活力无明显影响($P=0.419$),表明Tregs可以在低氧环境中稳定存活(图1c、1d)。

2.2 低氧状态下Tregs能够抵抗铁死亡

对常氧与低氧培养的Tregs进行转录组测序,火山图显示低氧状态下存在差异表达基因($|\text{FC}|>1.5$, $P<0.05$)(图2a)。转录因子富集分析表明特异性蛋白1(specificity protein 1, Sp1)、核因子 κB 亚基1(nuclear factor kappa B subunit 1, Nfkb1)等转录因子在低氧组显著富集(图2b)。转录因子富集弦图显示,Sp1调控多种与胆固醇代谢密切相关的基因(图2c)。KEGG富集分析证实胆固醇代谢通路在低氧组显著上调,说明胆固醇代谢重编程是低氧诱导Tregs代谢改变的核心通路(图3a)。

结合转录因子分析,对转录组结果进行GSEA富集分析发现,低氧状态下Tregs的凋亡通路富集程度显著降低,表明Tregs可抵御低氧微环境诱发的细胞损伤,维持自身存活,而铁死亡相关通路富集程度显著升高(图3b、3c)。进一步拆解通路基



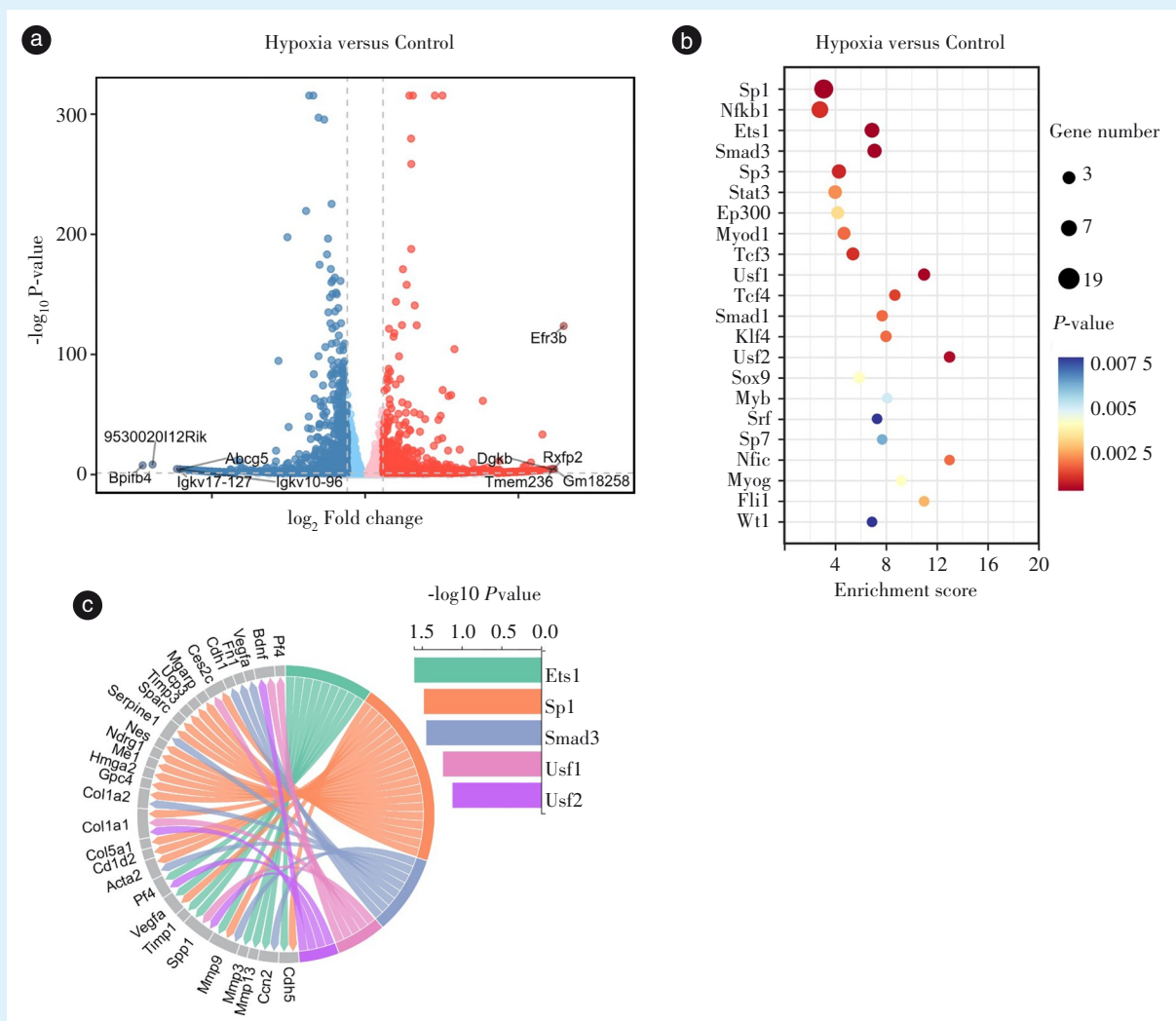
a: representative HE staining images of OSCC tumor tissue and adjacent normal tissue from patients with OSCC, and multicolor immunofluorescence staining of Tregs. Fluorescence labeling: DAPI (blue, nuclei), CD4 (green, T cell marker), and FOXP3 (red, Treg-specific marker). The white arrows indicate Tregs. The number of Tregs in OSCC tumor tissue was significantly higher than that in adjacent normal tissue ($n=5$ biologically independent replicates). b: representative HE staining images of OSCC tumor tissue from MOC1 tumor-bearing mice, and multicolor immunofluorescence staining of hypoxic regions and Tregs. Fluorescence labeling: DAPI (blue, nuclei), hypoxia probe (green, tumor hypoxic regions), and FOXP3 (red, Tregs). The white arrows indicate Tregs. Tregs were mainly accumulated in hypoxic regions of the tumor and rarely distributed in non-hypoxic regions. The fluorescence intensity of hypoxic regions was positively correlated with that of FOXP3. $n=6$ mice (6 biologically independent replicates). c: Treg purity was determined by flow cytometry. The purity of Tregs was 97.9%. d: the viability of Tregs cultured under normoxia and hypoxia was detected by flow cytometry. The number of viable Treg cells showed no significant decrease in the hypoxia group compared with the normoxia group ($n=3$ biologically independent replicates). Data were expressed as mean $\pm$ SD. Comparisons between two groups were performed using independent samples t test. ns: no significance. OSCC: oral squamous cell carcinoma. Tregs: regulatory T cells; FOXP3: forkhead box protein 3; MFI: mean fluorescence intensity; DAPI: 4',6-diamidino-2-phenylindole

Figure 1 Regional accumulation and hypoxia stress tolerance of Tregs in the hypoxic microenvironment of OSCC

图1 OSCC低氧微环境中Tregs的区域聚集特征及低氧应激耐受能力

因集后发现,低氧环境下Tregs中铁死亡抑制相关基因表达上调,铁死亡促进相关基因呈下调趋势,提示低氧可能重塑代谢从而抵抗低氧应激下的铁

死亡。基因热图显示,低氧状态下Tregs免疫抑制功能性基因T细胞免疫抑制受体(T-cell immunoreceptor with Ig and ITIM domains, Tigit)、C-X-C基序



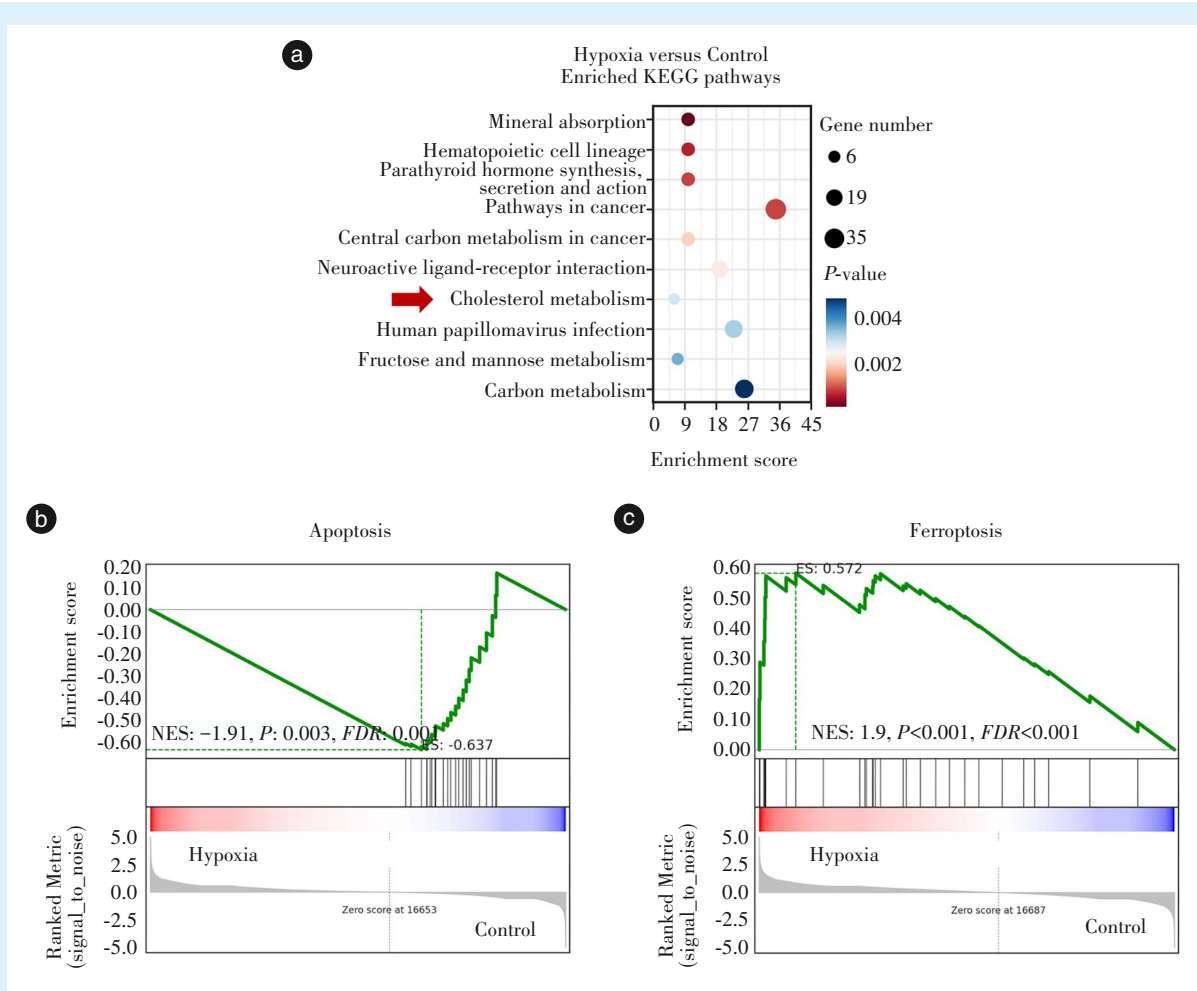
a: volcano plot of the transcriptome sequencing of Tregs in the hypoxia and control groups. The red dots represent significantly upregulated genes, the blue dots represent significantly down-regulated genes, and the grey dots represent genes with no significant difference. b: transcription factor enrichment analysis of hypoxia-upregulated genes. The transcription factors Sp1 and Nfkb1 were upregulated. c: chord diagram of transcription factor-regulated genes. Tregs: regulatory T cells; Bpifb4: BPI fold containing family B member 4; 9530020I12Rik: RIKEN cDNA 9530020I12 gene; Abcg5: ATP binding cassette subfamily G member 5; Igkv17-127: immunoglobulin kappa variable 17-127; Igkv10-96: immunoglobulin kappa variable 10-96; Efr3b: eighty-five requiring 3 homolog B; Dgkb: diacylglycerol kinase beta; Rxfp2: relaxin family peptide receptor 2; Tmem236: transmembrane protein 236; Gm18258: predicted gene 18258; Sp1: specificity protein 1; Nfkb1: nuclear factor kappa B subunit 1; Ets1: ETS proto-oncogene 1, transcription factor; Smad3: SMAD family member 3; Sp3: specificity protein 3; Stat3: signal transducer and activator of transcription 3; Ep300: E1A binding protein p300; Myod1: myogenic differentiation 1; Tef3: transcription factor 3; Usf1: upstream transcription factor 1; Tef4: transcription factor 4; Smad1: SMAD family member 1; Klf4: Krüppel-like factor 4; Usf2: upstream transcription factor 2; Sox9: SRY-box transcription factor 9; Myb: MYB proto-oncogene, transcription factor; Srf: serum response factor; Sp7: Sp7 transcription factor; Nfic: nuclear factor I C; Myog: myogenin; Fli1: friend leukemia virus integration 1 transcription factor; Wt1: Wilms tumor 1; P14: platelet factor 4; Bdnf: brain derived neurotrophic factor; Vegfa: vascular endothelial growth factor A; Fn1: fibronectin 1; Cdh1: cadherin 1; C1s2c: complement component 1, s subcomponent 2; Mgarp: mitochondria-localized glutamic acid-rich protein; Ucp3: uncoupling protein 3; Timp3: tissue inhibitor of metalloproteinase 3; Sparc: secreted protein acidic and cysteine rich; Serpine1: serpin family E member 1; Nes: nestin; Ndr1: N-myc downstream regulated gene 1; Me1: malic enzyme 1; Hmga2: high mobility group AT-hook 2; Gpc4: glypican 4; Col1a2: collagen type I alpha 2 chain; Col1a1: collagen type I alpha 1 chain; Col5a1: collagen type V alpha 1 chain; Cd1d2: CD1d2 antigen; Acta2: actin alpha 2, smooth muscle; Timp1: tissue inhibitor of metalloproteinase 1; Spp1: secreted phosphoprotein 1; Mmp9: matrix metalloproteinase 9; Mmp3: matrix metalloproteinase 3; Mmp13: matrix metalloproteinase 13; Ccn2: cellular communication network factor 2; Cdh5: cadherin 5

Figure 2 Hypoxia activates metabolic reprogramming of Tregs differential gene expression and transcription factor regulation

图2 低氧激活 Tregs 代谢重编程的差异基因表达与转录因子调控

趋化因子受体6(C-X-C motif chemokine receptor 6, Cxcr6)表达显著升高(图4),印证低氧状态下Tregs

不仅能存活,其免疫抑制功能也有增强。



a: Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis diagram of hypoxia-upregulated genes. The cholesterol metabolism pathway was significantly up-regulated. b-c: Gene Set Enrichment Analysis (GSEA) enrichment analysis diagram of hypoxia-upregulated genes. Under hypoxic conditions, the apoptosis pathway was downregulated and the ferroptosis pathway was upregulated. Tregs: regulatory T cells

Figure 3 Pathway enrichment analysis of hypoxia-activated metabolic reprogramming in Tregs

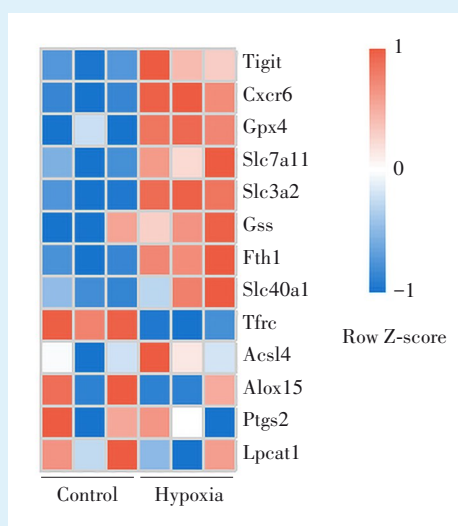
图3 低氧激活Tregs代谢重编程的通路富集分析

2.3 Tregs在低氧状态下胆固醇代谢水平升高

转录组火山图表明,低氧状态下大量胆固醇代谢相关基因上调($|\text{FC}|>1.1, P<0.05$)(图5a)。结合胆固醇合成通路示意图,推测低氧通过调控胆固醇合成通路重塑Tregs细胞代谢(图5b)。分析关键基因显示,胆固醇合成中关键分子Ebp在低氧组显著升高($P=0.002$)(图5c),甾醇C5-去饱和酶(sterol-C5-desaturase, Sc5d)同样显著升高($P=0.004$)(图5d),而Dhcr7表达无显著变化($P=0.217$)(图5e)。蛋白水平进一步证实,低氧环境可诱导EBP蛋白表达升高($P=0.001$)(图5f)。

2.4 低氧状态下Tregs中胆固醇合成中间产物增加

代谢组学结果显示,低氧培养的Tregs中多个胆固醇合成中间产物与衍生物表达升高($|\text{FC}|>1.1, P<0.05$)(图6a)。结合胆固醇合成末端通路示意图分析发现关键中间产物Zymoterol、7-DHC在低氧组呈现升高趋势,而总胆固醇含量反而呈下降趋势(图6b)。研究发现7-DHC具有抗铁死亡功能,能够通过抑制脂质过氧化来保护细胞免于铁死亡^[17]。这提示低氧可能通过Tregs内7-DHC的积累来抑制细胞铁死亡,从而调控Tregs的功能。



heatmap of differential gene expression of Tregs between the hypoxia and control groups. Ferroptosis resistance-related genes were downregulated under hypoxic conditions. Tigit: T-cell immunoreceptor with Ig and ITIM domains; Cxcr6: C-X-C motif chemokine receptor 6; Gpx4: glutathione peroxidase 4; Slc7a11: solute carrier family 7 member 11; Slc3a2: solute carrier family 3 member 2; Gss: glutathione synthetase; Fth1: ferritin heavy chain 1; Slc40a1: solute carrier family 40 member 1; Tfrc: transferrin receptor; Acsl4: acyl-CoA synthetase long chain family member 4; Alox15: arachidonate 15-lipoxygenase; Ptgs2: prostaglandin-endoperoxide synthase 2; Lpcat1: lysophosphatidylcholine acyltransferase 1. Tregs: regulatory T cells

Figure 4 Heatmap of hypoxia-regulated differential gene expression in Tregs

图4 低氧调控Tregs差异基因表达热图

2.5 抑制胆固醇合成关键靶点EBP可促进低氧状态下Tregs铁死亡

为探究胆固醇代谢在低氧诱导Tregs铁死亡中的作用,采用EBP抑制剂TASIN-30处理低氧培养的Tregs(图7a)。CCK-8法检测细胞活力确定TASIN-30安全处理浓度为22.94 $\mu\text{mol/L}$ 以下(图7b)。WB结果显示,相比于常氧,低氧状态下铁死亡抑制分子GPX4、FSP1表达升高($P<0.001$, $P=0.009$),铁死亡促进分子ACSL4表达降低($P<0.001$);而加入TASIN-30抑制EBP后,GPX4、FSP1表达显著下降($P<0.001$, $P=0.003$),ACSL4表达回升($P<0.001$)(图7c)。流式细胞术分析表明,低氧环境下Treg活细胞比例无明显减少,而加入TASIN-30后活细胞比例显著降低($P<0.001$),外源性加入7-DHC后,活细胞比例回升($P<0.001$)(图7d)。以上结果提示抑制EBP,减少7-DHC合成会削弱低氧状态Tregs抵抗铁死亡的作用。

2.6 阻断EBP可促进Treg中 Fe^{2+} 、脂质过氧化及活性氧累积,还原型谷胱甘肽减少,线粒体形态改变

Fe^{2+} 免疫荧光与流式结果显示,低氧状态下Tregs内 Fe^{2+} 水平较低,TASIN-30抑制EBP后 Fe^{2+} 水平显著升高,补充7-DHC可逆转该效应($P<0.001$)(图8a、8b)。BODIPY C11检测表明,低氧状态下Tregs内绿色氧化态荧光较弱,抑制EBP后氧化信号显著增强,7-DHC可逆转该趋势($P<0.001$)(图8c、8d)。DCFH-DA结果显示,低氧组Tregs ROS水平较低,抑制EBP后ROS显著升高($P=0.004$),7-DHC可拮抗该作用($P=0.008$)(图8e、8f)。低氧状态下Tregs内GSH/GSSG比值较高,抑制EBP后该比值显著降低,7-DHC可恢复其水平($P<0.001$),提示抑制EBP可降低Tregs的抗氧化能力,补充7-DHC可恢复其抗氧化水平(图8g)。透射电镜观察结果显示,低氧状态下Tregs线粒体形态正常,结构完整;抑制EBP后,线粒体出现典型铁死亡特征,表现为线粒体皱缩、形态异常;补充7-DHC后,线粒体形态基本恢复正常,表明抑制EBP可诱导Tregs线粒体发生铁死亡相关形态改变,而7-DHC可逆转该变化(图8h)。综上,抑制胆固醇合成关键靶点EBP可减少7-DHC的生成,削弱Treg对铁死亡的抵抗能力。

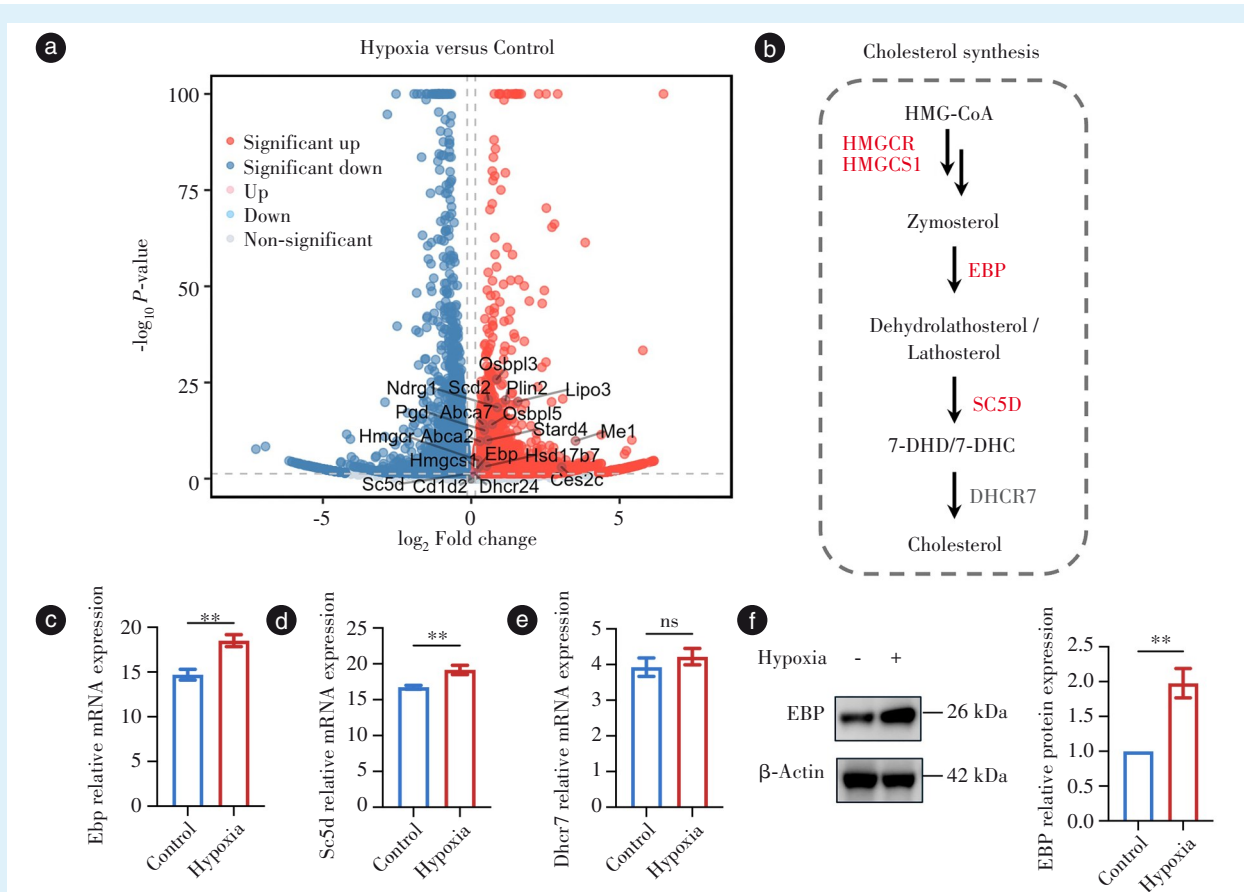
2.7 抑制EBP的体内抑瘤效应及Treg铁死亡抵抗的临床预后价值

与对照组相比实验组小鼠肿瘤体积显著缩小($P<0.001$)、肿瘤重量明显降低($P<0.001$),表明抑制EBP可有效抑制肿瘤生长(图9)。基于单细胞转录组定义的抗铁死亡Treg特征基因集分析TCGA-OSCC队列显示,抗铁死亡基因集评分较高患者总生存期显著更差($P=0.002$),提示其浸润与不良预后相关(图10)。

3 讨论

本研究聚焦围绕OSCC低氧微环境、Tregs、胆固醇代谢及铁死亡的关联展开探究,发现Treg能够适应低氧微环境,且低氧可介导Tregs胆固醇代谢重编程,促进细胞抵抗铁死亡,而阻断胆固醇合成关键分子EBP可逆转这一过程。Tregs在低氧微环境中的存活与功能稳态,高度依赖细胞内代谢重编程,而铁死亡作为新型程序性死亡方式,为靶向清除肿瘤浸润Treg、逆转免疫抑制提供了全新思路^[18-19]。

低氧是肿瘤微环境的重要特征,与免疫抑制



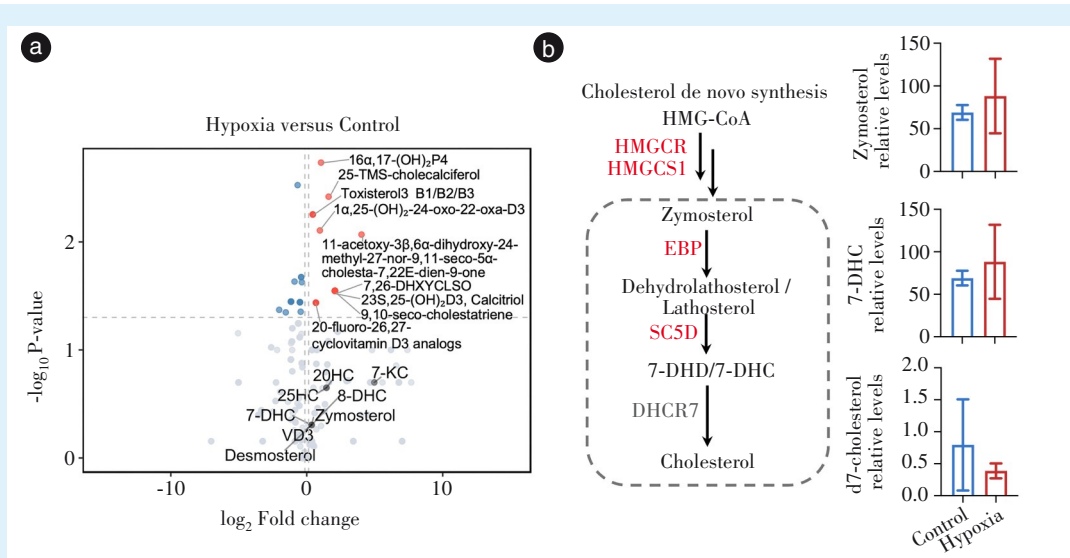
a: volcano plot of transcriptome sequencing of Tregs in the hypoxia and control groups. The red dots represent significantly upregulated genes, the blue dots represent significantly downregulated genes, and the gray dots represent genes with no significant difference. Cholesterol metabolism-related genes are highlighted, and most of them were upregulated. b: schematic diagram of cholesterol synthesis pathway. c-e: transcriptome sequencing results of Tregs in the hypoxia and control groups showed that Ebp and Sc5d expression was upregulated under hypoxic conditions, while Dhcr7 expression was not significantly upregulated ($n=3$ biologically independent replicates). f: the expression of the EBP protein under hypoxic conditions was detected by western blot assay, and EBP protein expression was upregulated under hypoxic conditions ($n=3$ biologically independent replicates). Data were expressed as mean $\pm$ SD. Comparisons between two groups were performed using independent samples t test. ns: no significance, ** $P < 0.01$. Tregs: regulatory T cells; Ndr1: N-myc downstream regulated 1; Pgd: phosphogluconate dehydrogenase; Hmgcr: 3-hydroxy-3-methylglutaryl-CoA reductase; Hmgcs1: 3-hydroxy-3-methylglutaryl-CoA synthase 1; Scd2: stearoyl-CoA desaturase 2; Abca7: ATP binding cassette subfamily A member 7; Osbp13: oxysterol binding protein like 3; Plin2: perilipin 2; Lipo3: lipase member O3; Osbp5: oxysterol binding protein like 5; Stard4: StAR related lipid transfer domain containing 4; Me1: malic enzyme 1; Abca2: ATP binding cassette subfamily A member 2; Ebp: emopamil binding protein; Hsd17b7: hydroxysteroid 17-beta dehydrogenase 7; Sc5d: sterol-C5-desaturase; Cd1d2: CD1d2 antigen; Dhcr24: 24-dehydrocholesterol reductase; Ces2c: carboxylesterase 2C; HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A; HMGCR: 3-hydroxy-3-methylglutaryl-CoA reductase; HMGCS1: 3-hydroxy-3-methylglutaryl-CoA synthase 1; 7-DHD/7-DHC: 7-dehydrocholesterol; DHCR7: 7-dehydrocholesterol reductase

Figure 5 Hypoxia regulates cholesterol metabolic reprogramming in Tregs

图5 低氧调控Tregs胆固醇代谢重编程

性Tregs浸润密切相关,共同介导肿瘤免疫耐受^[9, 20]。本研究证实OSCC肿瘤组织中Tregs浸润数量较多且富集于低氧区域,体外低氧培养Tregs并不会造成活细胞数量明显下降。这一发现提示Treg能够适应肿瘤低氧微环境,维持存活稳态,在肿瘤组织内定植富集。既往研究证实Tregs大量浸润是肿瘤免疫逃逸、病情进展的重要原因^[21-22]。实

体肿瘤普遍存在的低氧特征正是塑造免疫抑制微环境的核心因素之一^[2, 23]。低氧不仅驱动肿瘤细胞增殖、侵袭及放化疗耐受,更会重塑免疫微环境,促使Tregs大量浸润并维持免疫抑制表型,进而介导肿瘤免疫逃逸,这也是OSCC临床免疫治疗效果不佳的核心原因之一^[24-25]。多数经典研究聚焦Tregs的免疫抑制功能,并未探索其在恶性肿瘤环



a: volcano plot of the expression levels of intracellular cholesterol intermediates in Tregs under hypoxia by metabolomics analysis. The red dots represent significantly upregulated metabolites, the blue dots represent significantly downregulated metabolites, and the gray dots represent metabolites with no significant difference. Cholesterol synthesis intermediates and their derivatives are highlighted. b: schematic diagram of the terminal cholesterol synthesis pathway and box plots of the changes in expression levels of representative metabolites. Zymosterol and 7-DHC had an upward trend, while total cholesterol had a downward trend ($n=3$ biologically independent replicates). Data were expressed as mean $\pm$ SD. Comparisons between two groups were performed using independent samples t test. Tregs: regulatory T cells; 20HC: 20-hydroxycholesterol; 25HC: 25-hydroxycholesterol; 7-KC: 7-ketocholesterol; 8-DHC: 8-dehydrocholesterol; VD3: vitamin D3; HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A; HMGCR: 3-hydroxy-3-methylglutaryl-CoA reductase; HMGS1: 3-hydroxy-3-methylglutaryl-CoA synthase 1; EBP: emopamil binding protein (sterol isomerase); SC5D: sterol-C5-desaturase; 7-DHD/7-DHC: 7-dehydrocholesterol; DHCR7: 7-dehydrocholesterol reductase

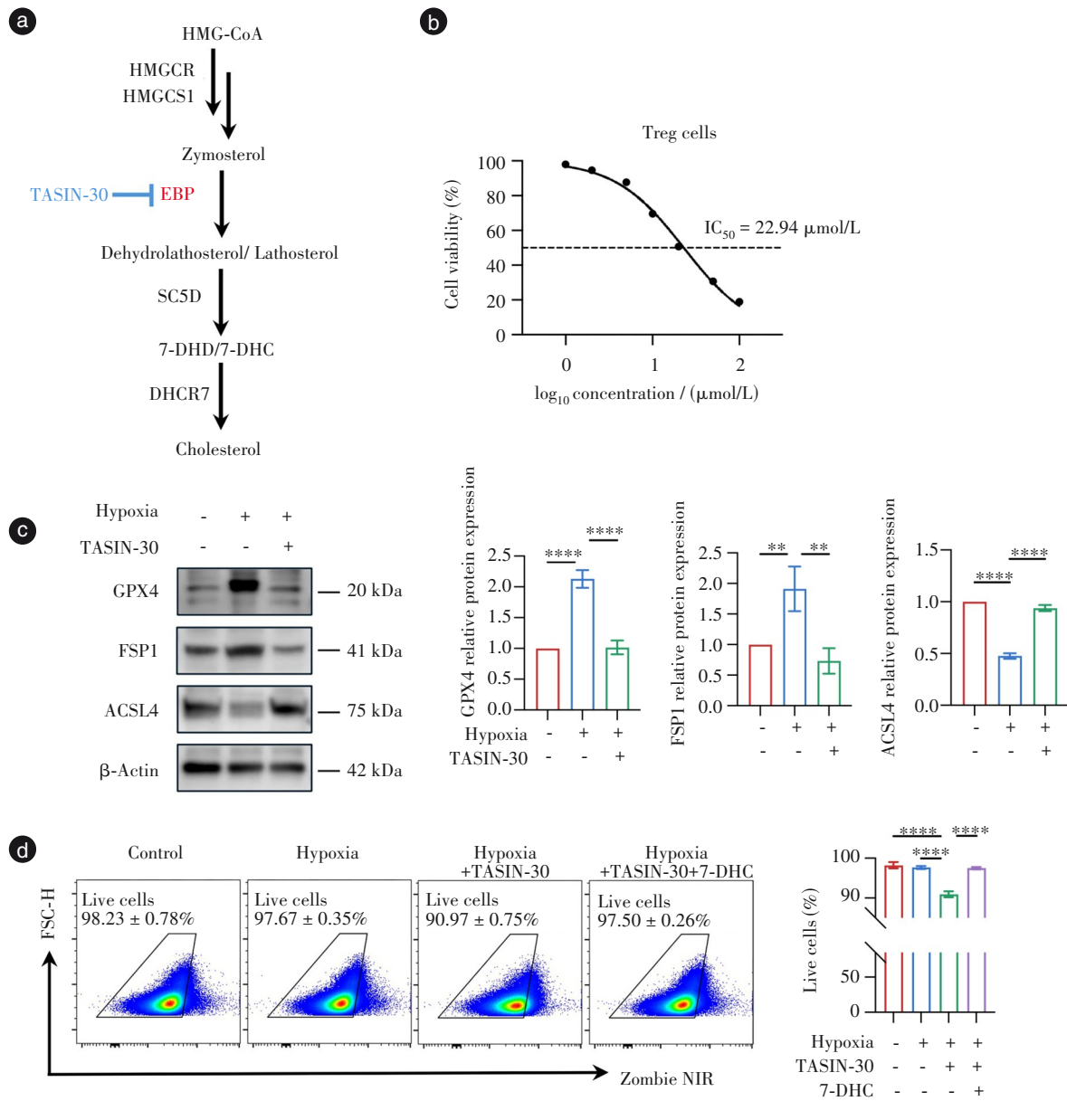
Figure 6 Increase in cholesterol synthesis intermediates in Tregs exposed to hypoxia

图6 低氧状态下Tregs中胆固醇合成中间产物增加

境中的存活适应机制——即Tregs如何突破环境应激、维持自身存活,这也是既往研究的局限^[26-27]。本研究聚焦于Treg自身的适应能力,明确Tregs在应激环境下通过代谢重编程抵抗铁死亡,应对低氧应激,维持自身存活。

代谢重编程是免疫细胞命运调控的核心机制^[28-29]。本研究通过转录组学分析发现,低氧可诱导Tregs发生胆固醇代谢重编程,这与既往研究中低氧调控免疫细胞代谢的结论一致,进一步证实胆固醇代谢在低氧免疫调控中的重要作用^[30-31]。SP1、NFKB1作为核心低氧应答转录因子明显富集,提示这两类转录因子正是低氧启动Tregs代谢重塑的关键媒介^[32-34]。研究表明Sp1可响应低氧应激调控下游基因转录,Nfkb1为经典炎症与低氧应答转录因子,二者均参与免疫细胞糖脂代谢、能量代谢的转录调控^[35-37]。Sp1可与固醇调节元件结合转录因子2(Sterol Regulatory Element Binding Transcription Factor 2, SREBF2)协同调控胆固醇合成通路的转录激活,是介导细胞胆固醇代谢重编程的关键转录因子^[38-39]。转录因子Nfkb1可通过

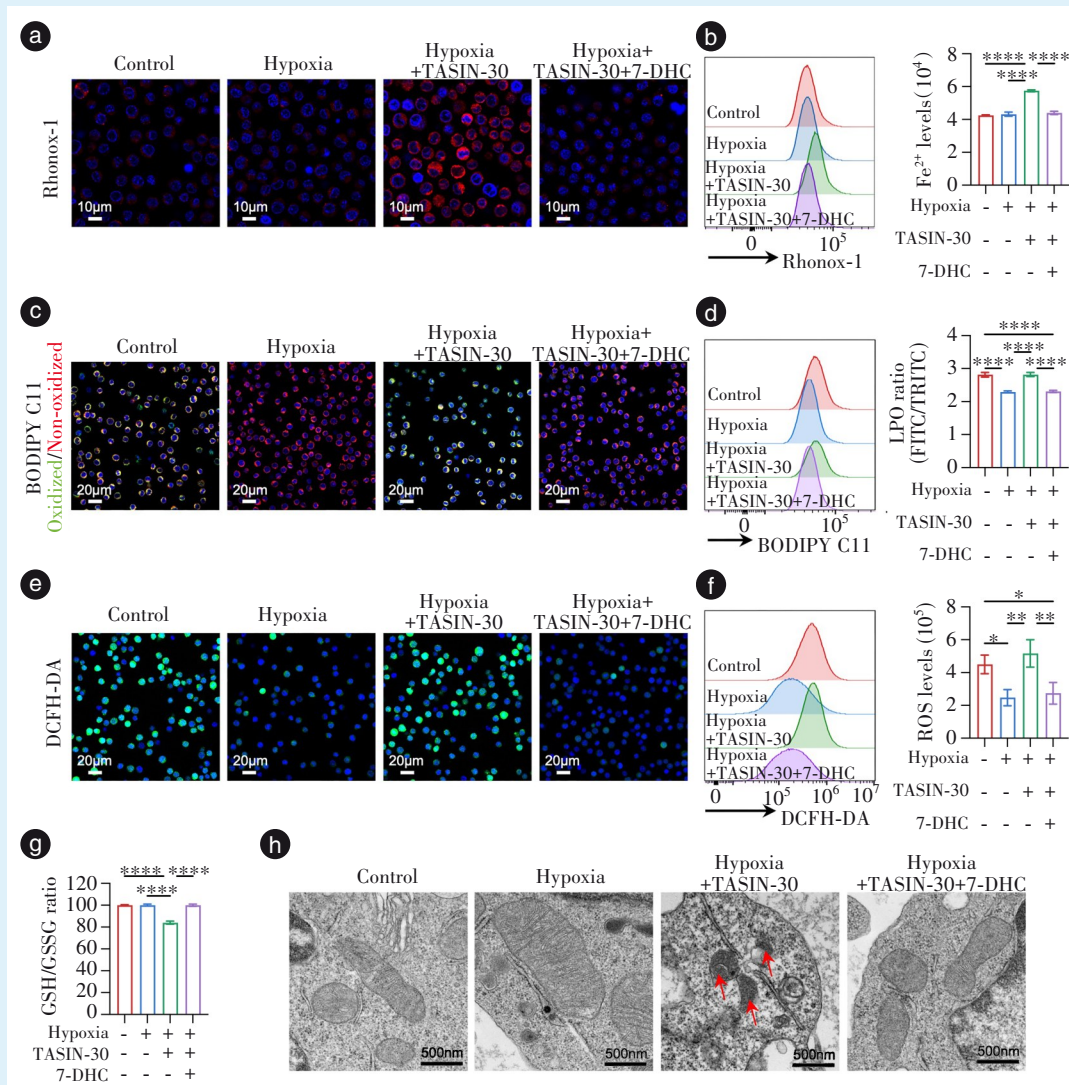
促进B细胞淋巴瘤-2(B-cell lymphoma 2, Bcl-2)、B细胞淋巴瘤特大分子(B-cell lymphoma-extra large, Bcl-xL)等不同抗凋亡基因的表达来抑制细胞凋亡,从而增强肿瘤细胞存活与增殖能力^[40]。不同于既往研究聚焦于低氧对Tregs功能或凋亡的单一调控,本研究发现低氧对Tregs死亡方式的调控具有特异性:凋亡通路富集程度显著降低,铁死亡抑制基因上调、促铁死亡基因表达下调,这说明Tregs在低氧环境中具有铁死亡抵抗能力,同时低氧上调Tigit、Cxcr6等免疫抑制功能基因,说明Tregs不仅能够存活,并且抑制功能增强,这也是Tregs能在恶劣的肿瘤低氧微环境中发挥免疫抑制作用的重要原因。既往对Tregs代谢的研究多集中于糖代谢、脂肪酸代谢,认为Tregs主要通过糖酵解维持基础存活,而对胆固醇代谢的关注多局限于总胆固醇水平的变化,未深入探讨胆固醇代谢与Tregs应激存活的关联,更未揭示胆固醇中间代谢产物的调控作用^[33, 41]。本研究对代谢通路研究发现,低氧可上调胆固醇合成关键分子EBP的表达,驱动Tregs内7-DHC积累,这正是介导Tregs铁死亡抵抗



a: schematic diagram of the cholesterol synthesis pathway and TASIN30-mediated EBP inhibition. b: CCK8 assay was used to detect the drug safety of TASIN-30 on Tregs, and the half-maximal inhibitory concentration (IC_{50}) was calculated as $22.94 \mu\text{mol/L}$ ($n=3$ biologically independent replicates). c: a western blot assay was used to detect the levels of GPX4, FSP1 and ACSL4 protein expression in Tregs in different groups, with β -Actin as the internal reference. The groups included the normoxic control group, hypoxia group, and hypoxia + TASIN-30 group. Compared with the normoxic control group, the levels of GPX4 and FSP1 expression in the hypoxia group increased, while the level of ACSL4 expression decreased. The GPX4 and FSP1 proteins were downregulated in the hypoxia + TASIN-30 group, while ACSL4 was upregulated ($n=3$ biologically independent replicates). d: flow cytometry was used to detect the viability of Tregs in different groups, including the normoxic control, hypoxia, hypoxia + TASIN-30, and hypoxia + TASIN-30 + 7-DHC groups. The proportion of viable cells was higher in the normoxic control and hypoxia groups, significantly decreased in the hypoxia + TASIN-30 group, and recovered after 7-DHC supplementation ($n=3$ biologically independent replicates). Data were expressed as mean $\pm$ SD. One-way analysis of variance (ANOVA) was used for comparisons among multiple groups. ** $P < 0.01$, **** $P < 0.0001$. Tregs: regulatory T cells; HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A; HMGCR: 3-hydroxy-3-methylglutaryl-CoA reductase; HMGC1: 3-hydroxy-3-methylglutaryl-CoA synthase 1; EBP: emopamil binding protein (sterol isomerase); SC5D: sterol-C5-desaturase; 7-DHD/7-DHC: 7-dehydrocholesterol; DHCR7: 7-dehydrocholesterol reductase; IC_{50} : half maximal inhibitory concentration; GPX4: glutathione peroxidase 4; FSP1: ferroptosis suppressor protein 1; ACSL4: acyl-CoA synthetase long-chain family member 4

Figure 7 Effect of EBP inhibition on hypoxic Tregs viability

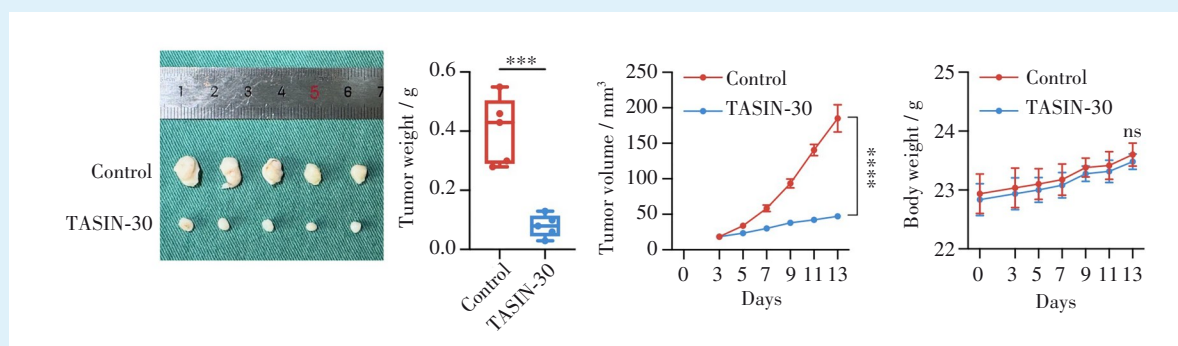
图7 抑制EBP对低氧状态下Tregs活力的影响



a: representative immunofluorescence images of ferrous ions in cells detected by Rhonox-1 staining. Fluorescence labeling: Rhonox-1 (red, ferrous ions), DAPI (blue, cell nuclei). The level of ferrous ions was low in the hypoxia group, increased in the hypoxia + TASIN-30 group, and decreased after 7-DHC supplementation. b: flow cytometry analysis of ferrous ion levels in cells. The trend of the results was consistent with that of immunofluorescence ($n=3$ biologically independent replicates). c: representative immunofluorescence images of cell lipid oxidation level detected by BODIPY C11 staining. Fluorescence labeling: BODIPY C11 (green, lipid oxidation products), DAPI (blue, cell nuclei). The lipid oxidation level was low in the hypoxia group, increased in the hypoxia + TASIN-30 group, and recovered to a level close to the hypoxia group after 7-DHC supplementation. d: flow cytometry analysis of cell lipid oxidation level. The results were consistent with immunofluorescence ($n=3$ biologically independent replicates). e: representative immunofluorescence images of intracellular ROS content detected by DCFH-DA staining. Fluorescence labeling: DCFH-DA (green, ROS), DAPI (blue, cell nuclei). The results showed that the ROS content was low in the hypoxia group, increased in the hypoxia + TASIN-30 group, and decreased after 7-DHC supplementation. f: flow cytometry analysis of intracellular ROS content. The results were consistent with immunofluorescence ($n=3$ biologically independent replicates). g: detection results of intracellular GSH, expressed as the GSH/GSSG ratio. The GSH/GSSG ratio was high in the hypoxia group, significantly decreased in the hypoxia + TASIN-30 group, and increased after 7-DHC supplementation ($n=3$ biologically independent replicates). h: transmission electron microscopy (TEM) images of cells to observe mitochondrial morphology. Representative TEM images of cells in each group are shown. The red arrows indicate shrunken mitochondria. The mitochondrial morphology in the hypoxia group was essentially normal, while the mitochondria in the hypoxia + TASIN-30 group showed shrinkage. The mitochondrial damage was improved after 7-DHC supplementation. The above representative images are from three biologically independent replicates. Data were expressed as mean $\pm$ SD. One-way analysis of variance (ANOVA) was used for comparisons among multiple groups. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$. Tregs: regulatory T cells; 7-DHC: 7-dehydrocholesterol; LPO ratio: lipid - peroxidation ratio; ROS: reactive oxygen species; GSH: reduced glutathione; GSSG: oxidized glutathione

Figure 8 Inhibition of EBP can weaken the ferroptosis resistance of Tregs under hypoxic conditions

图8 抑制EBP可削弱低氧状态下Tregs抗铁死亡作用

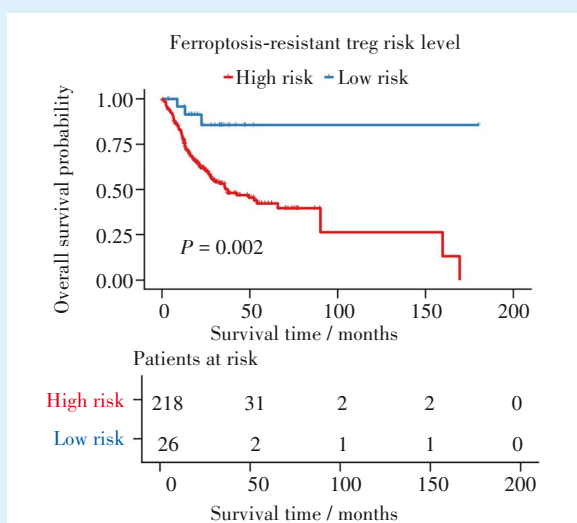


in vivo experiments confirmed that inhibition of EBP by TASIN-30 can significantly inhibit OSCC tumor growth. $n=5$ mice (5 biologically independent replicates). Data were expressed as mean $\pm$ SD. One-way analysis of variance (ANOVA) was used for comparisons among multiple groups.

*** $P < 0.001$, **** $P < 0.0001$. Tregs: regulatory T cells; OSCC: oral squamous cell carcinoma; EBP: emopamil-binding protein

Figure 9 Effects of Tregs ferroptosis-related regulation (EBP inhibition) on OSCC tumor growth in mice

图9 Tregs铁死亡相关调控(抑制EBP)对小鼠OSCC肿瘤生长的影响



clinical data analysis showed that patients with OSCC and higher Tregs ferroptosis resistance scores had shorter overall survival and worse prognosis (cutoff = 0.84, log-rank $\chi^2 = 3.29$). The patient whole-transcriptome and clinical follow-up cohort data used for survival analysis were obtained from the TCGA-HNSC project (<https://portal.gdc.cancer.gov/projects/TCGA-HNSC>). The single-cell transcriptome data utilized to calculate the feature scores were retrieved from the GEO database with accession number GSE140042 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE140042>). Ferroptosis-related gene sets were downloaded from the FerrDb V3 database (<http://www.zhounan.org/ferrdb/>). Tregs: regulatory T cells; OSCC: oral squamous cell carcinoma; EBP: emopamil-binding protein

Figure 10 Effects of Tregs ferroptosis-related regulation (EBP inhibition) on patient prognosis

图10 Tregs铁死亡相关调控(抑制EBP)对患者预后的影响

的核心机制。需要指出的是,本研究存在一定局限性:仅检测了Tregs免疫抑制相关功能基因(如 *Tigit*、*Cxcr6*)的表达水平,未开展体外功能验证实验(如Tregs对效应T细胞的抑制实验),无法直接证实胆固醇代谢重编程调控Tregs存活适应能力后,其免疫抑制功能是否同步受到影响,这也为本研究的后续完善指明了方向。

Fe^{2+} 、脂质过氧化与活性氧(reactive oxygen species, ROS)积累和还原型谷胱甘肽(reduced glutathione, GSH)减少是铁死亡的核心特征^[13, 42]。目前鲜有研究报道胆固醇中间代谢产物参与调控Tregs的铁死亡进程,本研究以此为切入点,通过抑制实验证实,阻断EBP可下调铁死亡负调控分子GPX4、FSP1的表达,上调促铁死亡蛋白ACSL4的水平,同时加剧细胞内 Fe^{2+} 、ROS堆积与脂质过氧

化,GSH降低以及线粒体形态改变,最终削弱低氧状态下Tregs铁死亡的抵抗作用,而外源性添加7-DHC则能够恢复Tregs抵抗铁死亡的作用。这一发现明确低氧通过上调EBP使得7-DHC积聚从而使得Treg在低氧状态下抵抗铁死亡这一全新机制,揭示了代谢重编程调控Tregs的新机制,是对既往低氧免疫调控、脂代谢与细胞死亡相关研究的补充与延伸。

体内实验结果表明,抑制EBP能有效抑制肿瘤生长,临床分析进一步揭示抗铁死亡Tregs高评分与OSCC患者的不良预后显著相关。TASIN-30的体内抑癌效应是多种因素共同作用的综合结果,因此EBP通过调控Tregs抗铁死亡能力增强其生存适应性可能是促进肿瘤进展的重要机制之一,但不能排除其对肿瘤细胞或其他免疫细胞影

响。这一机制为靶向EBP或Treg铁死亡通路提供了理论依据,后续本研究将采用Tregs特异性Ebp敲除小鼠重复荷瘤实验,验证EBP调控Treg抗铁死亡表型的特异性功能,同时评估联合免疫治疗的协同效果,进一步明确EBP在OSCC进展中的作用及临床应用潜力。

本研究的创新点在于揭示低氧微环境通过EBP调控胆固醇代谢促进Tregs抵抗铁死亡的机制,为OSCC治疗提供新的思路。TASIN-30作为EBP抑制剂是本研究机制验证的核心工具药,其在肿瘤代谢靶向治疗中的临床转化价值值得关注^[43]。该药虽然尚未进入临床试验阶段,但结合本研究结果,TASIN-30可通过促进Treg铁死亡改善OSCC免疫抑制微环境,未来可作为OSCC免疫治疗的增敏剂,与免疫检查点抑制剂、放化疗联合应用,但仍需开展大量临床前研究。

综上,Tregs在低氧微环境中通过上调EBP促进7-DHC积聚,抑制其铁死亡,EBP抑制剂TASIN-30可促进其铁死亡,抑制肿瘤生长。本研究为OSCC代谢干预提供了新的靶点,未来可依托本研究结果,补充Tregs体外功能验证实验,开展临床样本验证,优化TASIN-30的药物研发路径,探索其联合免疫治疗在OSCC中的应用价值,为改善OSCC患者预后提供全新策略。

【Author contributions】 Guo RY performed the experiments and wrote the article. Zhang FY, XU Q assisted the data analysis. Chen WT assisted the data analysis and participated in supervising the implementation of the project. Yan M designed the study and revised the article. All authors read and approved the final manuscript as submitted.

【Ethics Statement】 This study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the Medical Ethics Committee of Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine (Approval No. SH9H-2026-T218-1). This study also complied with the national Regulations for the Administration of Laboratory Animals and the Guidelines for Ethical Review of Laboratory Animal Welfare, and was approved by the Medical Ethics Committee of Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine (Approval No. SH9H-2025-A1613-1).

【Funding】 This study was supported by the National Natural Science Foundation of China (No. 82173217). The funder had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

【Competing interests】 All authors have completed the ICMJE uniform disclosure form at www.icmje.org/disclosure-of-interest/ and declare: support from the National Natural Science Foundation of China (No. 82173217) for the submitted work; no financial relationships with any organisations that might have an interest in the submitted work in

the previous three years; no other relationships or activities that could appear to have influenced the submitted work.

【Data availability statement】 The patient whole-transcriptome and clinical follow-up cohort data used for survival analysis were obtained from the TCGA-HNSC project (<https://portal.gdc.cancer.gov/projects/TCGA-HNSC>). The single-cell transcriptome data utilized to calculate the feature scores were retrieved from the GEO database with accession number GSE140042 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE140042>). Ferroptosis-related gene sets were downloaded from the FerrDb V3 database (<http://www.zhounan.org/ferrdb/>). The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author upon reasonable request.

【Generative AI statement】 The authors declared that generative AI was not used in the creation of this manuscript.

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(编辑 罗燕鸿)



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《口腔疾病防治》被美国化学文摘社(CAS)数据库收录

2025年6月,《口腔疾病防治》杂志通过美国化学文摘社(Cheical Abstracts Service, CAS)数据库的严格评审,正式被其收录。

CAS是美国化学会(American Chemical Society, ACS)的分支机构,是世界上最权威的化学信息机构,是全球唯一一家致力于发现、收集、整理所有已披露的化学物质信息的机构。

目前本刊已被美国Ulrichsweb/EBSCO/CAS数据库、瑞典DOAJ数据库、荷兰Scopus/Embase数据库、俄罗斯《文摘杂志》(AJ of VINITI)、日本JST数据库、瑞士HINARI数据库、WHO西太平洋地区医学索引(WPRIM)、波兰哥白尼索引(IC)等国际著名数据库收录。这标志着本刊在学术质量、开放获取和伦理规范等方面获得国际学术界的广泛认可。

《口腔疾病防治》将以建设世界一流科技期刊为目标,竭诚为广大读者、作者做好服务,促进中外学术交流和人类的口腔健康。

《口腔疾病防治》编辑部