论著·“骨关节病病理机制与靶向干预”重点号

糖皮质激素通过诱导血管内皮细胞旁分泌促纤维化因子加速激素性股骨头坏死纤维化

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[**摘要**] **目的**　探究激素性股骨头坏死(glucocorticoid-induced osteonecrosis of the femoral head,GONFH)模型中,损伤的血管内皮细胞对股骨头坏死区域纤维化进程的调控作用,并深入解析其潜在分子机制,为GONFH的纤维化干预及临床治疗提供理论依据。**方法**　①临床样本组织学检测:随机选取2023年12月至2025年12月期间大坪医院骨科收治的5例确诊为GONFH患者的股骨头样本作为试验组,同时选取5例因股骨颈骨折(femoral neck fracture,FNF)行手术治疗患者的股骨头样本作为正常对照组。对坏死反应带进行石蜡切片,HE染色、天狼星红染色、α-SMA/FN的Western blotting及CD31免疫荧光染色,评估组织形态、胶原沉积、纤维化相关蛋白表达及血管内皮细胞分布。②体外细胞实验:明确地塞米松(dexamethasone,DEX)处理后人脐静脉血管内皮细胞(human umbilical vein endothelial cell,HUVEC)分泌的因子对肌成纤维细胞活化的影响。应用转化生长因子-β(transforming growth factor-beta,TGF-β)诱导人骨髓间充质干细胞(human bone marrow mesenchymal stem cell,hBMSC)分化为肌成纤维细胞,成功后将HUVEC分为DEX处理组(作用48 h)和PBS对照组,收集2组细胞的条件培养基(conditioned medium,CM),将CM-DEX组和CM-PBS组分别与肌成纤维细胞共孵育48 h,Western blot检测肌成纤维细胞中α-SMA、Ⅰ型胶原蛋白(collagen type,Col Ⅰ)及FN的蛋白表达水平。③分子机制探究:提取CM-DEX组与CM-PBS组HUVEC总RNA,RNA转录组测序(RNA-seq),筛选2组间差异表达基因(DEGs),行GO功能富集和KEGG通路富集分析。收集2组HUVEC分泌蛋白行蛋白组学测序,筛选差异分泌蛋白,结合生物信息学分析其通路及与肌成纤维细胞活化的关联。**结果**　①临床样本检测结果:与FNF对照组相比,GONFH组股骨头反应带区域:HE染色示组织排列紊乱、纤维组织增生;天狼星红染色示胶原沉积显著增加($P<0.000\ 1$);α-SMA($P<0.01$)及FN($P<0.000\ 1$)蛋白表达升高。$CD31^+$血管内皮细胞数量增多($P<0.000\ 1$)且形成管状结构;α-SMA肌成纤维细胞数量增加($P<0.000\ 1$),且与CD31细胞空间分布邻近。②体外细胞实验结果:与CM-PBS组比较,CM-DEX组肌成纤维细胞中α-SMA($P<0.01$)及FN($P<0.01$)蛋白表达升高,Col Ⅰ表达无显著差异($P=0.131\ 0$)。③分子测序:RNA-seq显示,CM-DEX组HUVEC上调620个基因,筛选出30个促纤维化/炎症相关基因,前5位为*PTPRN2*、*TSC22D3*、*FKBP5*、*MAFB*、*SYP*。GO富集于蛋白结合、转录调控、蛋白转运等;KEGG富集于TGF-β、JAK-STAT、MAPK、SNARE囊泡运输及外泌体通路。蛋白组学显示,CM-DEX组HUVEC分泌20个促纤维化相关蛋白(如TGFBR1、SMAD2、RAB11B、DCN等)。GO富集于细胞外基质、胶原相关功能;KEGG富集于补体与凝血级联反应、细胞外基质(extracellular matrix,ECM)-受体互作、黏着斑等通路。**结论**　糖皮质激素可诱导血管内皮细胞异常活化,通过TGF-β、PI3K-Akt及囊泡运输/外泌体等通路,促进DCN、

[**基金项目**] 重庆市杰出青年科学基金(CSTB2024NSCQ-JQX0019);陆军军医大学科技创新能力提升专项(2023XQN44);陆军军医大学科技创新能力提升专项(2023XJS38)

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引用格式:靳润泽,朱卫文,李冰飞,等. 糖皮质激素通过诱导血管内皮细胞旁分泌促纤维化因子加速激素性股骨头坏死纤维化[J]. 陆军军医大学学报,2026,48(12):1667-1678. DOI:10.16016/j.2097-0927.202602073.

Citation:JIN R Z,ZHU W W,LI B F,*et al*. Glucocorticoids accelerate fibrosis in steroid-induced osteonecrosis of the femoral head by inducing paracrine pro-fibrotic factors from vascular endothelial cells[J]. J Army Med Univ,2026,48(12):1667-1678. DOI:10.16016/j.2097-0927.202602073.

RAB11B、TGFBR1等促纤维化因子高表达;这些因子旁分泌作用于成纤维细胞,促使其活化并过度合成ECM(Col Ⅰ、FN)。GONFH患者股骨头反应带中血管内皮细胞增多且与肌成纤维细胞空间邻近,进一步加剧纤维化进程。

[关键词] 股骨头坏死;糖皮质激素;血管内皮细胞;肌成纤维细胞;纤维化
[中图法分类号] R347.7;R363.21;R681.8 [文献标志码] A

Glucocorticoids accelerate fibrosis in steroid-induced osteonecrosis of the femoral head by inducing paracrine pro-fibrotic factors from vascular endothelial cells

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[**Abstract**] Objective To investigate the regulatory role of injured vascular endothelial cells in the fibrosis progression in the necrotic region of the femoral head in a glucocorticoid-induced osteonecrosis of the femoral head (GONFH), and further elucidate the underlying molecular mechanisms, so as to provide a theoretical basis for anti-fibrotic intervention and clinical treatment of GONFH. Methods ① Clinical sample histological examination: Femoral head samples were collected from 5 patients diagnosed with GONFH admitted to Department of Orthopedics of Daping Hospital between December 2023 and December 2025 and assigned to the experimental group. Meanwhile, femoral head specimens from 5 patients undergoing surgical treatment for femoral neck fracture (FNF) were selected as the normal control group. Paraffin sections from the reactive zone of necrotic tissues were prepared. HE staining, Sirius red staining, Western blotting for α-smooth muscle actin (α-SMA) and fibronectin (FN), and CD31 immunofluorescence staining were performed to evaluate tissue morphology, collagen deposition, expression of fibrosis-related proteins and distribution of vascular endothelial cells. ② *In vitro* experiments: The effect of factors secreted by dexamethasone (DEX)-treated human umbilical vein endothelial cells (HUVEC) on myofibroblast activation was determined. Human bone marrow mesenchymal stem cell (hBMSC) was induced to differentiate into myofibroblasts using transforming growth factor-β (TGF-β). HUVEC were then divided into a DEX treatment group (48 h) and a PBS control group, and conditioned medium (CM) was collected from both groups. CM-DEX and CM-PBS were co-incubated with myofibroblasts for 48 h, and Western blotting was applied to detect the protein expression levels of α-SMA, collagen type I (Col I), and FN in myofibroblasts. ③ Exploration of molecular mechanisms: Total RNA was extracted from DEX-treated and control HUVEC for RNA sequencing (RNA-seq) to screen differentially expressed genes (DEGs) between the 2 groups. Gene Ontology (GO) functional enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were conducted. Secreted proteins from both groups of HUVEC were collected for proteomic sequencing, and differentially secreted proteins were screened. Bioinformatics analysis was used to analyze their pathways and association with myofibroblast activation. Results ① Clinical sample findings: Compared with the FNF control group, the reactive zone of the femoral head in the GONFH group showed: disorganized tissue arrangement and fibrous hyperplasia by HE staining; markedly increased collagen deposition by Sirius red staining ($P<0.000\ 1$); and elevated protein expression of α-SMA ($P<0.01$) and FN ($P<0.000\ 1$). The number of CD31 vascular endothelial cells was increased ($P<0.000\ 1$) with evident formation of tubular structures, and

the count of α-SMA myofibroblasts was also elevated (P<0.000 1). In addition, α-SMA myofibroblasts were spatially adjacent to $CD31^+$ endothelial cells. ② *In vitro* experiment results: Compared with the CM-PBS group, the expression levels of α-SMA and FN were significantly increased in myofibroblasts of the CM-DEX group (both P<0.01), while no significant difference was observed in Col Ⅰ expression (P=0.131 0). ③ Molecular sequencing: RNA-seq results revealed that 620 genes were upregulated in DEX-treated HUVECs, and 30 of them were identified as pro-fibrotic/inflammation-related. The top 5 genes were *PTPRN2*, *TSC22D3*, *FKBP5*, *MAFB* and *SYP*. GO enrichment analysis indicated that these genes were mainly involved in protein binding, transcriptional regulation and protein transport. KEGG enrichment analysis revealed enrichment in TGF-β, JAK-STAT, MAPK, SNARE vesicular transport and exosome pathways. Proteomic analysis identified 20 pro-fibrotic proteins secreted by DEX-treated HUVEC, including TGFBR1, SMAD2, RAB11B and DCN. GO terms were predominantly enriched in extracellular matrix (ECM) and collagen-related functions. KEGG pathways were mainly enriched in the complement and coagulation cascades, ECM-receptor interaction and focal adhesion pathways. **Conclusion** Glucocorticoids induce abnormal activation of vascular endothelial cells, which promotes high expression of pro-fibrotic factors such as DCN, RAB11B, and TGFBR1 through the TGF-β, PI3K-Akt, and vesicle transport/exosome pathways. These factors act on fibroblasts in a paracrine fashion, promote fibroblast activation and excessive synthesis of ECM components including Col Ⅰ and FN. In the reactive zone of the femoral head in GONFH patients, the increased number of vascular endothelial cells and their spatial proximity to myofibroblasts further exacerbate the fibrosis process.

[**Key words**] osteonecrosis of the femoral head; glucocorticoids; vascular endothelial cells; myofibroblasts; fibrosis

Supported by the Science Fund for Distinguished Young Scholars of Chongqing (CSTB2024NSCQ-JQX0019), and the Special Project of Science and Technology Innovation Capacity Promotion of Army Medical University (2023XQN44, 2023XJS38). Corresponding authors: XIONG Yan, E-mail: xiongyandoctor@tmmu.edu.cn; ZHOU Lan, E-mail: zhou930@163.com

激素性股骨头坏死(glucocorticoid-induced osteonecrosis of the femoral head,GONFH)是因长期或过量使用糖皮质激素引发的股骨头缺血性坏死,好发于中青年人群,在长期接受激素治疗的患者中发病率显著升高[1]。近年来,由于临床糖皮质激素使用不当增多,GONFH发病率逐年上升。该疾病主要表现为髋关节疼痛、活动受限和关节功能障碍等,严重者可出现股骨头软骨下骨塌陷,必须行关节置换术[2]。目前保守治疗和手术治疗均存在局限性,迄今尚未达成GONFH治疗的有效共识。患者的预后多取决于塌陷前的早期干预,因此,早期诊断和治疗对改善预后十分重要[3]。研究显示,GONFH病理机制复杂,包括血流受损、凝血和内皮功能障碍、脂质代谢紊乱、骨内高压和骨质疏松症等[4-5],这些因素共同作用导致骨组织损伤和功能障碍,其中血管损伤、炎症反应及微环境改变被认为是GONFH发生发展的关键因素[6-8]。

纤维化是指组织在损伤或炎症刺激下,细胞外基质(extracellular matrix,ECM)过度沉积,最终取代正常组织结构并伴有功能性细胞减少的不可逆性病理过程[9]。其核心机制为肌成纤维细胞的异常活化与增殖,即在TGF-β、血小板衍生生长因子(PDGF)及白介素等关键因子作用下,成纤维细胞转化为肌成纤维细胞,活化的肌成纤维细胞大量合成ECM蛋白,并通过表达α-SMA介导组织挛缩。此过程还涉及基质金属蛋白酶失衡、持续氧化应激等多条信号通路的相互作用[10]。多种细胞因子失调和组织稳态失衡可引发广泛组织纤维化,使其具有疾病"普遍性"的特征,与心、肾、肝等多数器官功能障碍密切相关[9,11]。组织纤维化同样是股骨头坏死区域的重要病理改变[12-13]。在肌骨损伤研究中,纤维化及胶原沉积增加,可伴随关节炎症反应加重和骨结构破坏程度加深。但针对骨坏死区域纤维化病理及机制的研究仍较为缺乏。

组织损伤后的修复过程中,血管的新生与长入至关重要。新生血管不仅负责运输氧气和营养物质,还可分泌多种生长因子和细胞因子,从而调节周围组织细胞的增殖与分化[14]。血管内皮细胞作为血管的重要组成结构,直接参与局部血运重建和组织再生。在炎症、缺氧等刺激下,内皮细胞一方面可发生转化并形成肌成纤维细胞,直接参与胶原合成[15-16]。另一方面与基质细胞通过旁分泌信号密切调控互作[14,17],诱

导周围平滑肌细胞、成纤维细胞增殖与迁移,共同促进损伤修复[15]。血管内皮细胞损伤是GONFH的重要病理机制[18],激素可刺激内皮细胞分泌内皮素-1(ET-1)等血管收缩因子,以及纤维蛋白原诱导因子(PAI-1)和促血小板聚集因子等促凝血物质,进而引发血管收缩和血栓形成[18]。ET-1在硬皮病患者血清中表达升高,除收缩血管外,还可直接促进成纤维细胞向肌成纤维细胞转化,增加胶原合成[19]。此外,血管损伤会启动凝血级联反应,形成的纤维蛋白支架有助于细胞外基质沉积[20]。研究表明,活化的内皮细胞常引发或加重炎症反应,促使肌成纤维细胞过量合成ECM,加速肺纤维化等病理过程[21-22]。在新冠相关GONFH中,股骨头组织以广泛纤维化、大量新生血管及血栓形成为主要形态学表现[13]。值得注意的是,在股骨头坏死进程中,血管损伤通常早于骨内其他结构的明显改变[23]。坏死区与正常骨组织交界处的反应带内,血管血栓、炎性浸润及纤维化表现较为突出,但其具体机制尚不明确[24-25]。血管内皮细胞损伤与股骨头坏死反应带纤维化病变的关联及内在分子机制,仍有待深入研究阐明。糖皮质激素作为GONFH的核心诱因,其是否会通过调控血管内皮细胞功能状态,间接影响肌成纤维细胞活化。基于此,本研究以地塞米松(dexame thasone, DEX)处理人脐静脉血管内皮细胞(human umbilical vein endothelial cell,HUVEC)为体外模型,探究其分泌因子对肌成纤维细胞活化的影响,结合RNA转录组及蛋白组学技术,初步解析血管内皮细胞促纤维化的潜在通路,为GONFH的早期干预及靶向治疗提供新的研究思路与理论依据。

1 材料与方法

1.1 主要试剂

本研究中RPMI 1640培养基(12633012)、胎牛血清(10091148)均采购于赛默飞世尔科技公司,骨髓间充质干细胞(HUXMA-01001)及完全培养基(HUXMA)购自赛业(广州)生物科技有限公司。DAPI染色液(C1006)、抗荧光淬灭封片液(P0126)、RIPA蛋白裂解液(P0013K)、蛋白酶抑制剂混合物(P1005)以及0.25%胰酶(C0201)均采购于上海碧云天生物技术有限公司。Western blot实验与免疫荧光的关键一抗:α-SMA(14395-1-AP)、Collange Ⅰ(14695-1-AP)、β-actin(66009-1-Ig)、FN(66042-1-Ig)均采购于武汉三鹰生物技术有限公司。另外地塞米松购自石药集团公司,槲皮素(Q111274)和达沙替尼(D125110)采购于上海阿拉丁生化科技有限公司。兔二抗(AS014)、鼠二抗(AS003)、荧光二抗(A24924, AS039)、TGF-β1(RP01458)、ECL显色液(BRK0094)购自武汉爱博泰克公司。HE染色试剂盒和天狼星红染色试剂盒均购自上海碧云天生物技术有限公司。

1.2 主要方法

1.2.1 临床样本采集 GONFH组及股骨颈骨折(femoral neck fracture, FNF)组股骨头标本各5例,分别采集于2023年12月至2025年12月大坪医院骨科收治的接受全髋关节置换术的GONFH患者及FNF患者(均为住院病人)。2组纳入标准如下:①GONFH诊断依据《中国成人股骨头坏死临床诊疗指南(2020版)》确诊,FNF诊断通过X线确认;②GONFH患者有明确糖皮质激素使用史,且所有患者无髋部创伤、慢性酒精滥用、严重器官功能障碍、精神障碍等证据;③年龄18~65岁,平均年龄分别为(GONFH:45.1岁)和(FNF:44.6岁)性别分布随机均衡,每组n=5,男女各≥2人;④无其他骨代谢疾病、妊娠或哺乳、恶性肿瘤、器官移植、HIV感染或其他传染病、使用免疫抑制剂或抗凝剂;⑤GONFH组与FNF组标本的收集均获得了患者的知情同意。本研究经过大坪医院伦理委员会批准(2024-138)。GONFH组骨组织与FNF组对应的样本经骨刀切割成0.5 cm×0.5 cm×0.5 cm大小,而后立即转移至冰箱,长期保存于-80 ℃。

1.2.2 细胞培养及处理 本试验中使用的HUVEC和人骨髓间充质干细胞(human bone marrow mesenchymal stem cell, hBMSC)购自康宁公司,HUVEC在10%胎牛血清和1%青霉素-链霉素的RPMI 1640培养基的细胞培养皿中培养,hBMSC在含成人骨髓间充质干细胞完全培养基的T25细胞培养瓶中培养,以上细胞均置于5%二氧化碳的恒温培养箱(37 ℃)中培养,每隔2~3 d更换新鲜培养基。待细胞生长至90%汇合度时,以1∶3的比例进行传代培养。将HUVEC接种于6孔板内,分为PBS对照组与DEX处理组。药物干预48 h后,弃去原有培养基,更换新鲜完全培养基,继续常规培养48 h,备用。另取hBMSC接种于6孔板,加入TGF-β蛋白干预48 h,诱导其向肌成纤维细胞分化。诱导结束后,弃去诱导培养基,分别更换为上述收集的PBS处理、DEX处理的HUVEC条件培养基进行孵育培养。培养结束后,提取细胞总蛋白用于后续实验,或通过免疫荧光染色进行相关指标检测。

1.2.3 Western blotting检测 将股骨头组织完全匀浆,以及处理后的细胞经洗涤刮取后与含有1%蛋白酶抑制剂的RIPA裂解液混合,冰上裂解及离心后取上清液。收集的蛋白加入SDS-PAGE蛋白上样缓冲

液,经过95 ℃变性10 min,电泳2 h后转印至PVDF膜。随后,在5%脱脂奶粉中常温封闭1 h。加入一抗(稀释比例均为1∶2 000)并在4 ℃下孵育过夜,回收一抗后,PBST洗膜3次,每次10 min,加入二抗混合液(稀释比例均为1∶5 000)并在室温内孵育1 h。而后PBST洗膜3~5次,每次5~10 min。最后,结合ECL显色液进行显影和拍照。

1.2.4 天狼星红染色 所有股骨头组织在取材和固定后,先进行EDTA脱钙,制作石蜡切片。而后选取部分石蜡切片,经过常规脱蜡、水洗,使用天狼星红-苦味酸染色液浸染30~60 min,而后经过快速洗涤、二甲苯透明和中性树胶封片等步骤。分别在普通光学显微镜和偏振光显微镜下观察,评估骨组织内胶原纤维的分型、含量及排列情况。

1.2.5 HE染色 选取股骨头组织石蜡切片,经二甲苯脱蜡以及乙醇到蒸馏水的梯度水化后,浸入苏木精染液室温染色10 min,而后进行自来水冲洗、酒精分化以及返蓝后冲洗,接着用伊红染液室温染色3 min,经过自来水冲洗乙醇梯度脱水,滴加中性树胶封片,风干后镜检。

1.2.6 免疫荧光染色 骨组织石蜡切片经过脱蜡、抗原修复后,利用山羊血清在室温条件下封闭非特异性结合位点,弃去封闭液后加入一抗工作液(1∶100),4 ℃条件下孵育过夜,次日复温后用PBS洗涤3次,再滴加荧光二抗工作液(1∶200)室温避光孵育,孵育结束后洗涤3次,最后使用DAPI在避光条件下染核3 min,PBS洗去多余染液,封片后在荧光显微镜下观察和采集图像。

1.2.7 蛋白组学检测 HUVEC细胞分为2组(PBS和DEX),诱导48 h,更换新鲜无血清培养基培养48 h后收集上清,经过4 ℃ 300×*g*离心10 min,吸取上清后再4 ℃ 3 000×*g*离心10 min,确保将细胞或细胞碎片去除干净,将2组上清分别分装至预冷的冻存管,液氮速冻后通过干冰运输至杭州联川生物公司检测。利用蛋白质谱检测的原始数据进行蛋白质鉴定和数据库的匹配;对鉴定到的蛋白进行GO、KEGG数据库和亚细胞定位等常见功能数据库注释;最后展开蛋白质的定量分析、样本相关性分析和蛋白差异分析,对于差异表达蛋白展开差异蛋白功能分析,包括GO功能富集、KEGG功能富集和蛋白相关性分析等。

1.2.8 RNA-seq HUVEC细胞分为2组(PBS和DEX),诱导48 h,确定生长状态良好后弃培养基,PBS洗涤细胞2次后加入适量细胞裂解液并吹打混匀,转移至细胞冻存管后,干冰运输至杭州联川生物公司进行检测。获得原始数据后,首先过滤下机数据,保证测序数据的质量(Clean Data),然后将数据比对参考基因组,进行基因表达定量、GSEA、基因差异及富集等分析。

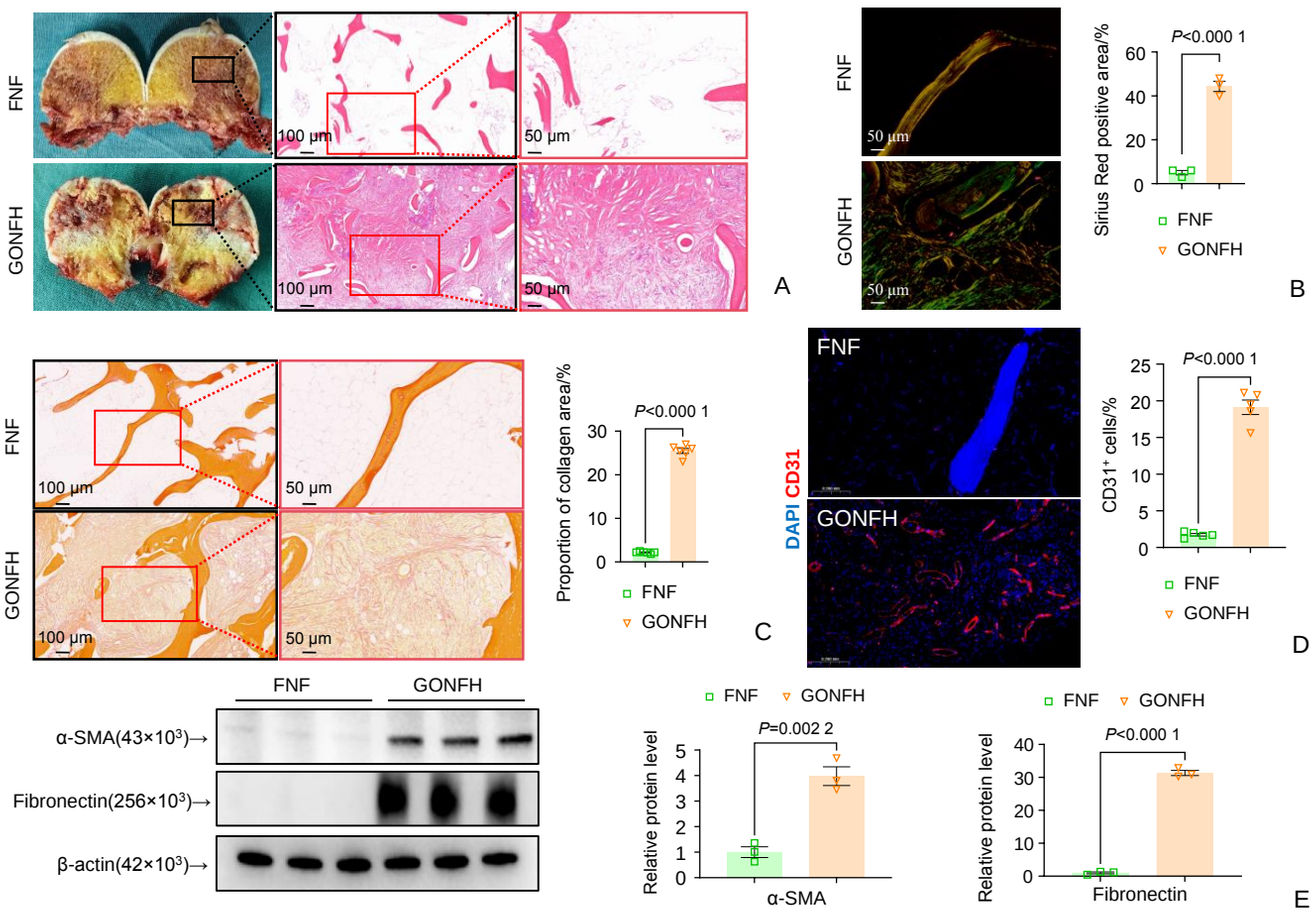
1.3 统计学分析

采用GraphPad Prism 10.4.1软件进行数据分析。所有试验保证至少3次重复,利用独立样本*t*检验比较两组的数据,多组间比较采用单因素方差分析。$P<0.05$代表差异具有统计学意义。

2 结果

2.1 GONFH股骨头坏死反应区微血管增多,且存在显著纤维化

本研究收集了GONFH患者及FNF患者的新鲜股骨头各5例,其大体形态显示(图1A):GONFH患者股骨头存在典型的缺血性病理分区,临床常将其分为“坏死区、反应带、正常区”3个区域,反应带为坏死区与正常区的交界部位[24]。对反应带组织切片进行HE染色,结果显示:GONFH组中可见大量淡红色无细胞致密纤维化区域,正常骨髓结构破坏,脂肪空泡减少,骨小梁间纤维组织弥漫性增生,呈淡红色均质条索状结构,纤维束粗细不等,交织成网,部分区域呈致密束状排列,纤维化区域显著增多(图1A)。对2组样本坏死反应带切片进行天狼星红染色,结果显示:GONFH组可见大量红色胶原纤维沉积,分布紊乱且交织成网状,局部胶原纤维呈团簇状聚集;视野内可见散在分布的微血管结构,血管壁周围紧贴呈环形环绕的胶原纤维,排列致密且规整,紧密包绕血管基底膜,远离血管壁的胶原纤维呈放射状向外周延伸($P<0.000\,1$,图1C)。由于天狼星红染色对胶原具有高特异性与灵敏度,在偏振光下,不同类型的胶原纤维呈特征性显色:Ⅰ型胶原为强双折射橙红色/黄色,Ⅲ型胶原为弱双折射绿色。偏振光显微镜下可见,GONFH反应带纤维化区域可见骨髓间质内Ⅰ型胶原与Ⅲ型胶原交织分布,提示基质细胞分泌活跃($P<0.000\,1$,图1B)。对反应带组织行CD31免疫荧光染色,可见广泛CD31阳性荧光信号,以及大量环状、短线状血管样结构,部分区域血管内皮细胞密度较低且排列紊乱($P<0.000\,1$,图1D)。提取2组反应带组织蛋白进行Western blotting检测,结果显示:与对照组相比,GONFH组肌成纤维细胞活化指标α-SMA($P=0.002\,2$)及FN($P<0.000\,1$)表达水平均显著升高(图1E)。上述结果表明,GONFH反应带内微血管呈反应性增生,数量较对照组骨组织显著增多,同时伴随肌成纤维细胞活化与大量胶原纤维沉积。



A: Representative gross and HE-stained images showing the corresponding reactive zones of the femoral head in the GONFH and FNF groups; B: Representative images of Sirius Red staining in the corresponding reactive zones of the femoral head in the GONFH and FNF groups under polarized light (n=3); C: Representative images of Sirius Red staining in the corresponding reactive zones of the femoral head in the GONFH and FNF groups (n=6); D: Representative images of immunofluorescence staining showing CD31 (red) in human femoral head of GONFH and control groups (n=5); E: Protein expression levels of fibronectin (FNF) and α-SMA in the corresponding reactive zones of the femoral head in the GONFH and FNF groups by Western blotting (n=3)

图1 GONFH股骨头坏死反应区微血管增多且存在显著纤维化

Figure 1 The necrotic reactive zone of patients with GONFH exhibits increased microvessels and significant fibrosis

2.2 地塞米松处理的血管内皮细胞中促纤维化相关通路显著富集

我们前期采用衰老相关β-半乳糖苷酶(SA-β-gal)染色试剂盒,对不同浓度DEX处理的HUVEC进行染色,发现2 μmol/L DEX处理即可诱导HUVEC发生明显衰老损伤。随后以此浓度DEX处理HUVEC 48 h,设置处理组(DEX组)与对照组(PBS组),收集细胞后送至公司完成RNA-seq检测分析(图2A)。对DEX组620个上调基因进行功能注释与筛选,共获得30个促纤维化相关差异基因并绘制热图(图2B),其中表达上调最显著的前5位基因为*PTPRN2*、*TSC22D3*、*FKBP5*、*MAFB*和*SYP*。GO富集散点图结果显示,DEX处理后的差异基因显著富集于蛋白结合(protein binding)、RNA聚合酶Ⅱ转录调控(regulation of transcription by RNA polymerase Ⅱ)、蛋白转运(protein transport)、内质网膜(endoplasmic reticulum membrane)、胞质囊泡(cytoplasmic vesicle)等条目(图2D)。这些条目主要涉及蛋白互作、基因转录调控、蛋白胞内转运及囊泡运输等生物学过程,其中蛋白转运及囊泡运输与细胞分泌功能直接相关,提示DEX可能通过激活分泌相关转运通路,介导促纤维化因子的合成与释放。KEGG通路富集散点图显示,JAK-STAT信号通路、TGF-β信号通路、MAPK信号通路及SNARE介导的囊泡运输通路等富集程度显著(图2C)。TGF-β通路为经典促纤维化调控通路,JAK-STAT与MAPK等通路参与细胞功能活化调控,而SNARE囊泡运输通路直接介导细胞分

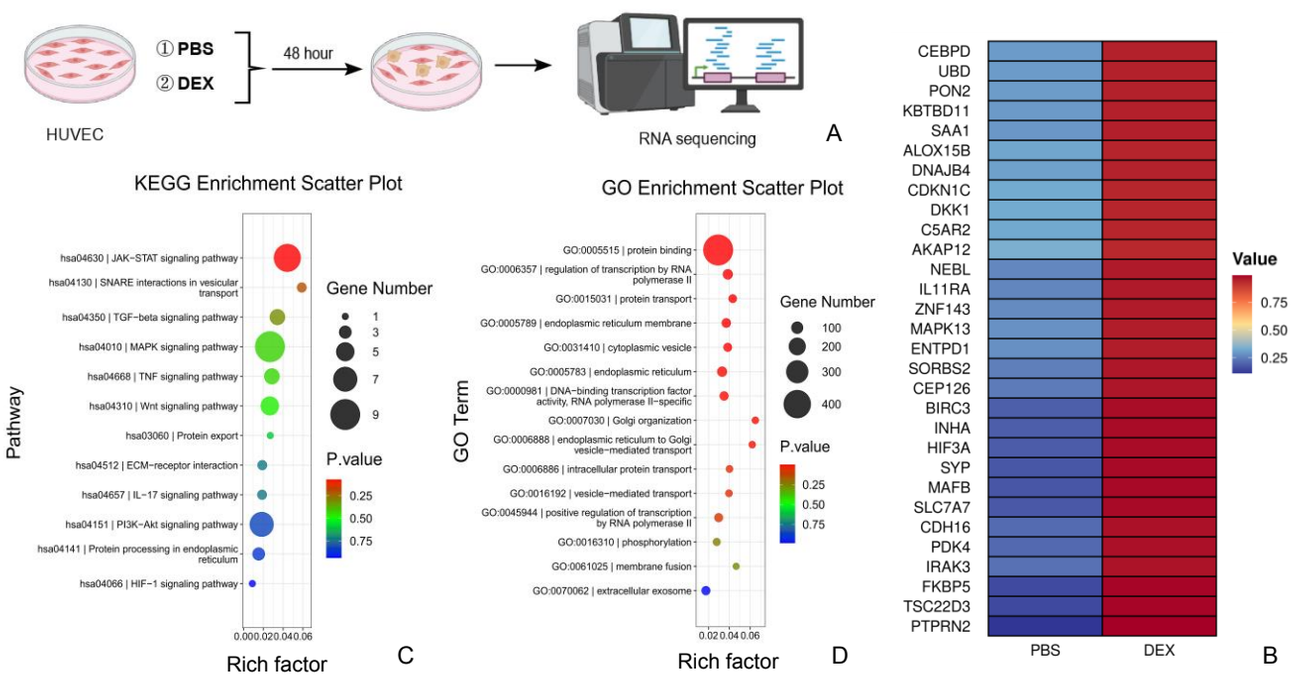
泌过程。据此推测,DEX处理后HUVEC可能通过外泌体等囊泡途径释放TGF-β、IL-11等促纤维化因子,进而影响周围成纤维细胞生物学行为。综上,转录组测序结果提示,DEX可促进血管内皮细胞中促纤维化相关基因表达及功能通路活化。

2.3 糖皮质激素处理的血管内皮细胞来源的上清促进了肌成纤维细胞的活化

为进一步验证糖皮质激素处理的血管内皮细胞是否促进肌成纤维细胞活化,我们设计了体外实验(图3A)。实验流程如下:首先以DEX及PBS分别处理HUVEC 48 h,更换新鲜培养基后继续培养48 h,收集上清;同时采用TGF-β诱导人骨髓间充质干细胞分化为肌成纤维细胞,将收集的HUVEC上清加入肌成纤维细胞中孵育48 h,最终检测α-SMA、Ⅰ型胶原及FN的蛋白表达水平。Western blotting显示,DEX组上清可显著诱导肌成纤维细胞活化标志物α-SMA(P=0.005 4)和纤连蛋白FN(P=0.009 6)的表达升高;DEX组Ⅰ型胶原蛋白表达较PBS组虽有升高趋势,但差异无统计学意义(P=0.131 0,图3B)。免疫荧光染色结果显示,DEX组上清可显著促进肌成纤维细胞α-SMA(P=0.002 4)和Ⅰ型胶原(P<0.000 1)的表达(图3C、D),提示肌成纤维细胞活化增强且胶原分泌增加。为明确GONFH中血管内皮细胞和纤维化是否相关,对GONFH组织切片进行CD31和α-SMA免疫荧光共染,结果显示,GONFH坏死区域微血管显著增生,且血管周围可见α-SMA阳性表达的肌成纤维细胞分布(P<0.000 1,图3E)。上述结果提示,血管内皮细胞可能通过分泌相关因子,促进肌成纤维细胞活化。

2.4 地塞米松通过多条信号通路诱导HUVEC分泌促纤维化蛋白

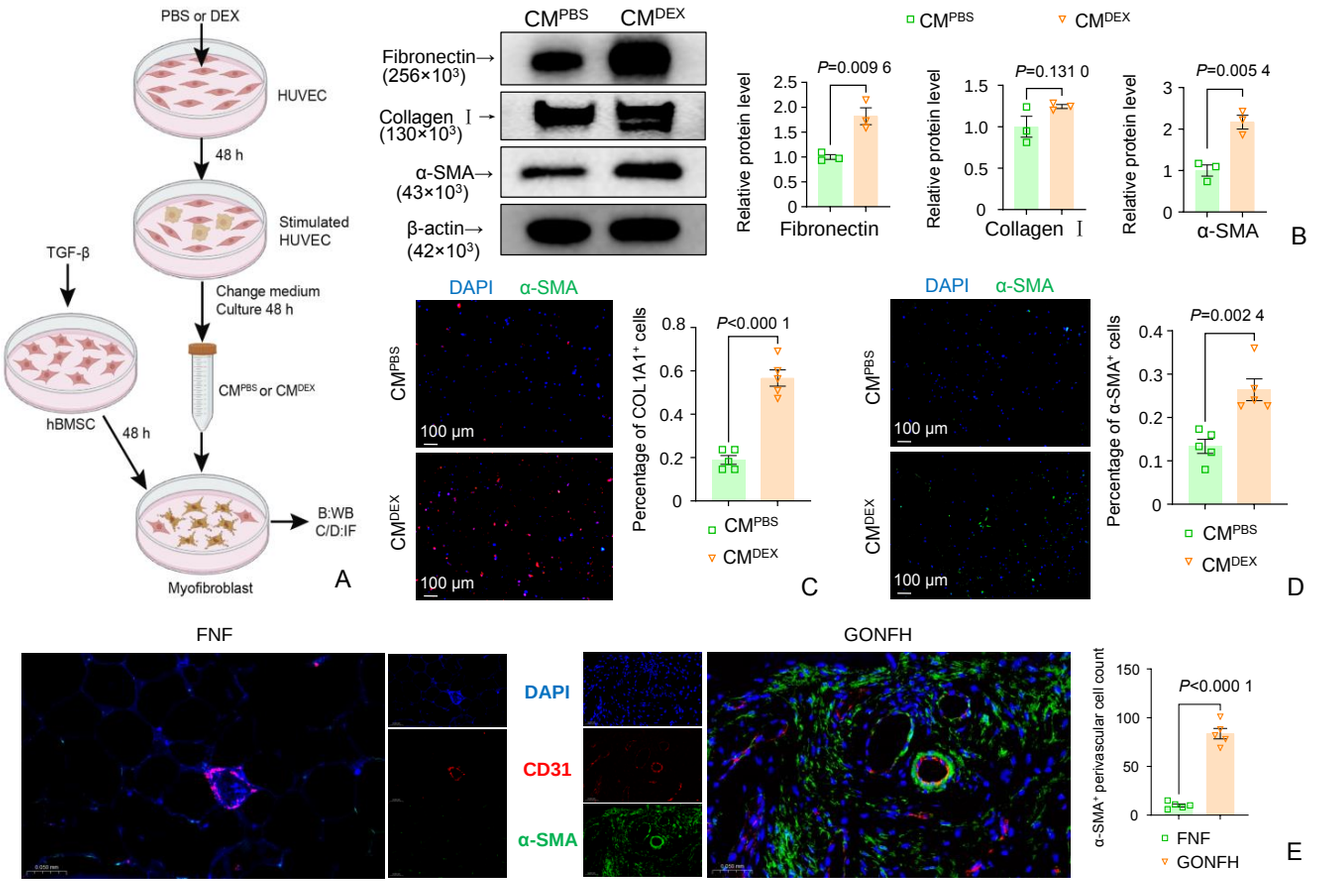
对DEX处理后HUVEC的条件培养基进行分泌蛋白组学分析,筛选出DEX组中20个纤维化通路相关的上调蛋白,热图结果显示(图4A):与纤维化及细胞分泌功能密切相关的核心蛋白均显著升高,其中包括TGF-β通路关键分子TGFBR1、SMAD2,囊泡运输相关蛋白RAB11B和ECM相关蛋白DCN等;而SMAD4、PTK2在2组中的表达差异相对较小。这些差异表达蛋白涵盖了促纤维化核心通路(TGF-β信号通路)、细胞分泌转运(囊泡运输)以及ECM重构等功能。KEGG通路富集柱状图中显示(图4B),肌肉细胞细胞骨架、黏着斑、PI3K-Akt信号通路及ECM-受体互作通路中的差异蛋白数量较多。其中黏着斑、ECM-受体互作是



A: Schematic diagram of the workflow procedures; B: Heatmap showing the 30 upregulated pro-fibrotic-related genes in the DEX group; C: KEGG pathway enrichment scatter plot from RNA-seq analysis; D: GO enrichment scatter plot showing differentially expressed genes between DEX-treated cells and the control group. HUVEC: human umbilical vein endothelial cell

图2 地塞米松处理的血管内皮细胞中促纤维化相关通路显著富集

Figure 2 Pro-fibrotic related pathways are significantly enriched in vascular endothelial cells after DEX treatment



A：Schematic diagram of the cell experimental workflow；B：Protein expression levels of α-SMA，Collagen Ⅰ and fibronectin in myofibroblasts after 48 h of conditioned medium（CM）incubation by Western blotting（n=3）；C：Representative images of immunofluorescence staining showing COL1A1（red）in myofibroblasts incubated with CM-PBS and CM-DEX（n=5）；D：Representative images of immunofluorescence staining showing α-SMA（green）in myofibroblasts incubated with CM-PBS and CM-DEX（n=5）；E：Representative images of immunofluorescence staining showing CD31（red）and α-SMA（green）in human femoral head of GONFH and control groups（n=5）

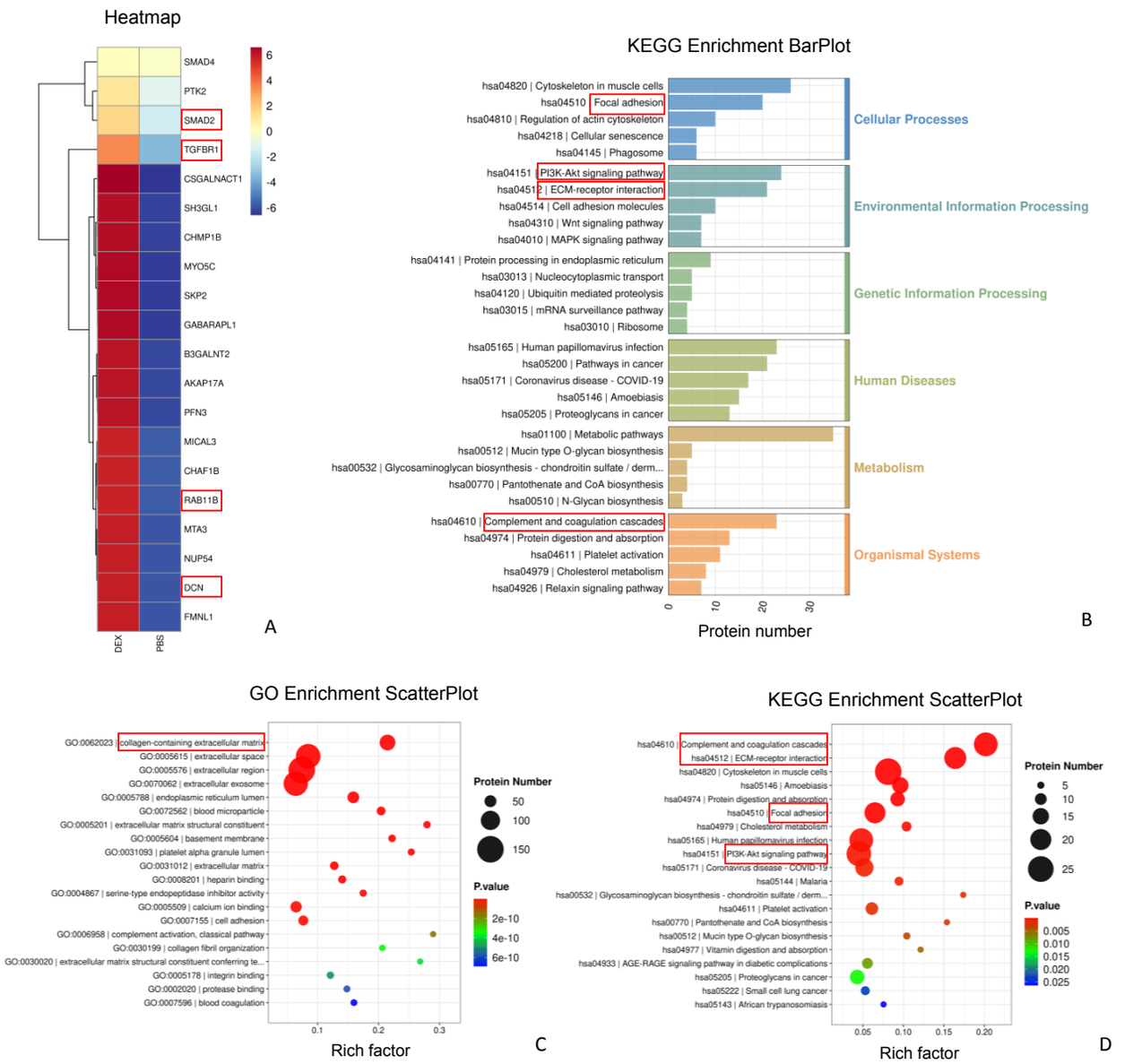
图3　糖皮质激素处理的血管内皮细胞来源的上清促进了肌成纤维细胞的活化

Figure 3　Supernatant derived from glucocorticoid-treated vascular endothelial cells promotes the activation of myofibroblasts

调控细胞外基质重构及细胞黏附的核心通路，PI3K-Akt信号通路参与细胞活化与功能调控，上述通路均为肌成纤维细胞活化及组织纤维化的关键调控通路。GO富集散点图显示（图4C），差异蛋白在“含胶原的细胞外基质”“细胞外空间”“细胞外区域”等条目上呈现高显著性富集，同时“细胞外基质结构组成”“胶原纤维组织”等与纤维化直接相关的功能条目也被富集。这提示DEX处理后，HUVEC的分泌蛋白组分高度聚焦于细胞外基质重构、胶原代谢等纤维化关键过程。KEGG通路富集散点图进一步证实（图4D），差异蛋白在补体和凝血级联反应、ECM-受体互作、肌肉细胞骨架、黏着斑等通路中呈现高显著性、高富集特征。此外，DEX处理组上清中TGFBR1、SMAD2及SMAD4表达显著升高，提示经典促纤维化TGF-β通路的激活；PI3K-Akt通路的富集反映细胞机械和黏附表型的转变；ECM相关组分DCN的上调则间接提示细胞外基质的重塑和胶原沉积，同时DEX组HUVEC的囊泡分泌能力显著增强。综上，这些通路的显著富集表明，DEX处理组的上清蛋白组分可能通过ECM互作、黏着斑、囊泡运输以及炎症调控等多种途径，在纤维化进程中发挥重要作用。

3　讨论

本研究核心在于解析了GONFH中血管内皮细胞损伤对于组织纤维化病理的关键作用。初步发现糖皮质激素可通过改变血管内皮细胞的分泌功能，激活多种与纤维化相关的信号通路。结合临床组织病理染色、体外细胞互作实验，以及RNA-seq和分泌蛋白组学测序分析，初步建立了血管内皮细胞与GONFH患者股骨头纤维化之间的关联。



A: Heatmap of secreted proteins in cells treated with PBS and DEX conditioned medium showing the 20 upregulated proteins associated with fibrotic pathways in the DEX group; B: KEGG pathway enrichment bar chart showing the highly significantly enriched pathways of differentially expressed proteins between the DEX group and the PBS group; C: GO enrichment scatter plot showing the enriched functional terms of differentially expressed proteins; D: KEGG pathway enrichment scatter plot showing the highly significantly enriched pathways of differentially expressed proteins between the DEX group and the PBS group

图4 地塞米松通过多条信号通路诱导HUVEC分泌促纤维化蛋白

Figure 4 DEX induces secretion of profibrotic proteins in HUVEC *via* multiple signaling pathways

3.1 衰老内皮细胞可通过SASP促进多种疾病的纤维化进程

衰老细胞是指处于细胞周期停滞的细胞,其核心特征为大量分泌衰老相关分泌表型(SASP),包括多种生物活性介质、生长因子和细胞因子(如TGF-β、TNF-α、IL-6等)、趋化因子、细胞外基质蛋白及酶类。本研究中RNA-seq结果显示,血管内皮细胞中存在细胞衰老相关信号通路的富集,包括p53/p21细胞周期抑制通路、NF-κB通路以及DNA损伤响应通路。越来越多的研究表明,衰老内皮细胞可通过局部慢性炎症促进多种纤维化疾病的进程。例如,在年龄相关性肾纤维化中,衰老的肾小球内皮细胞周围可形成炎症微环境,加速肾纤维化进展[26];在慢性心脏压力过载的小鼠模型中,抑制内皮细胞p53特异性衰老可以改善纤维化

程度[27];在特发性肺纤维化病理进程中,衰老内皮细胞通过SASP促进肺成纤维细胞向肌成纤维细胞转化[28]。此外,内皮细胞衰老还可分泌半乳糖凝集素3(Gal3),Gal3通过与肺成纤维细胞膜上的TGFBR1和巨噬细胞上的TLR4直接结合,促进成纤维细胞-肌成纤维细胞转化及NLRP3炎性小体激活,进而加速矽肺进展[29]。体外实验也证实,免疫复合物可触发内皮细胞活化,合成IL-6和TGF-β,介导皮肤成纤维细胞获得促纤维化表型[30]。因此,在不同的疾病中靶向衰老内皮细胞可能是未来改善疾病纤维化进程的重要策略。

3.2 外泌体可能是血管内皮细胞调控肌成纤维细胞的重要介质

RNA-seq结果提示,SNARE运输通路显著富集,结合囊泡运输相关蛋白RAB11B在DEX组显著上调,提示衰老或活化的血管内皮细胞分泌的上清在细胞因子的直接作用外,还可能借助细胞外囊泡(如外泌体)发挥调控作用。已有研究表明,内皮细胞来源的外泌体可能含有促纤维化因子,这些因子作用于不同靶细胞,可诱导其转化为活化的肌成纤维细胞[31]。例如,在系统性硬化症中,从患者血清中分离的外泌体可影响正常真皮成纤维细胞的基因表达模式,诱导其获得纤维化表型[32]。本研究观察到内皮细胞来源的囊泡运输及外泌体相关信号通路激活,提示囊泡运输与外泌体可能在血管内皮细胞与成纤维细胞的交互作用中发挥重要调控功能,其具体机制仍有待进一步深入研究。

3.3 TGF-β途径是调控组织纤维化的核心信号通路

TGF-β家族已被证明在广泛的病理过程中发挥重要作用,包括衰老、免疫调节、动脉粥样硬化和组织纤维化。作为组织纤维化的重要调节因子,TGF-β结合TGFβR2,招募TGFβR1并磷酸化SMAD2/3,与SMAD4形成复合物入核,调控纤维化相关基因的表达[33-34]。研究表明,在BCR-ABL1阴性骨髓增殖性肿瘤(myeloproliferative neoplasm, MPN)中,恶性JAK信号通过释放炎症因子驱动巨核细胞产生过量TGF-β,使MSC大量分化为肌成纤维细胞并合成胶原等纤维组织[35]。在GONFH中,BMSC来源的circ_0058792通过TGF-β/Smad7途径,调节成骨分化[36]。GONFH组织缺氧会激活细胞中的炎症小体,产生活性氧、TGF-β和一系列炎症因子。持续性的炎症会进一步刺激TGF-β等细胞因子和介质的分泌。同时,损伤或衰老细胞的异常积累也可产生促纤维化SASP——TGF-β[37]。本研究显示TGF-β通路存在显著富集,结合TGF-β相关蛋白在疾病进展中介导促纤维化效应,表明靶向TGF-β通路及其下游分子可能是未来GONFH抗纤维化药物研发的重点。

3.4 研究局限性与未来研究方向

本研究初步证实了血管内皮细胞在GONFH纤维化进程中的调控作用,但仍存在一定局限性:第一,临床样本量较小,研究结论需通过更大样本量的临床研究进一步验证;第二,体外细胞实验仅采用HUVEC和hBMSC,HUVEC主要来源于脐静脉,不能完全复制骨髓内血管内皮细胞的特征和功能,更可靠的方法是利用原代骨微血管内皮细胞进行进一步的研究;第三,外泌体及SNARE通路介导内皮细胞与肌成纤维细胞通讯的具体分子机制,尚需通过功能实验和动物模型进一步验证。

综上所述,本研究初步提示血管内皮细胞对肌成纤维细胞存在活化作用,这可能是GONFH纤维化进程中的关键节点,该发现为理解GONFH纤维化的病理机制提供了新视角,也为开发行之有效的股骨头坏死区域纤维化防治策略提供了理论依据。

利益冲突 本文所有作者声明不存在利益冲突

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(**收稿**:2026-02-13;**修回**:2026-04-01)

(编辑 栾 嘉)