·基础研究·

靶向抑制生长分化因子3减轻内皮细胞炎症反应并抑制脉络膜新生血管

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摘 要：【目的】揭示靶向抑制生长分化因子3（GDF3）对脉络膜新生血管（CNV）形成及内皮炎症反应的影响。【方法】构建激光诱导的小鼠CNV模型，通过RNA测序（RNA-seq）筛选差异基因。采用实时荧光定量PCR（RT-qPCR）和Western blot验证GDF3表达水平。通过siRNA及中和抗体拮抗内皮细胞GDF3，采用CCK8实验、划痕实验及成管实验评估内皮细胞增殖、迁移和血管生成能力。检测炎症/黏附分子表达变化，分析免疫细胞黏附和跨内皮迁移实验变化。在CNV模型中，通过玻璃体腔注射shRNA靶向抑制GDF3，结合免疫荧光染色观察CNV面积及免疫细胞浸润情况。【结果】GDF3在CNV组织中表达显著上调（$P<0.001$）。体外干预GDF3显著抑制内皮细胞增殖（$P<0.001$）、迁移（$P<0.001$）及血管生成能力（$P<0.01$），同时下调炎症/黏附分子表达（$P<0.001$），并显著减少免疫细胞黏附（$P<0.001$）和跨内皮迁移（$P<0.001$）。体内实验证实靶向抑制GDF3可显著抑制CNV的形成（$P<0.001$）并降低免疫细胞浸润（$P<0.001$）。【结论】靶向抑制GDF3可协同抑制内皮炎症反应和病理性血管生成，其作用可能与调控炎症微环境相关，为湿性年龄相关性黄斑变性（wAMD）治疗提供新思路。

关键词：年龄相关性黄斑变性；脉络膜新生血管；靶向抑制生长分化因子3；血管内皮细胞；炎症
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Targeted Inhibition of Growth Differentiation Factor 3 Attenuates Endothelial Cell Inflammatory Response and Suppresses Choroidal Neovascularization

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Abstract: 【Objective】 To investigate the effects of targeted inhibition of growth differentiation factor 3 (GDF3) on choroidal neovascularization (CNV) and endothelial inflammatory response. 【Methods】 A laser-induced CNV mouse model was established, and differentially expressed genes were screened by RNA sequencing (RNA-seq). GDF3 expression was validated by real-time fluorescence quantitative PCR (RT-qPCR) and Western blot. GDF3 in endothelial cells was antagonized by siRNA and neutralizing antibodies. Functional assays including CCK8 assay, scratch assay, and tube formation assay were performed to assess endothelial cell proliferation, migration, and angiogenic capacity. The expression changes of inflammatory/adhesion molecules were measured, and immune cell adhesion and transendothelial migration were analyzed. In the CNV model, shRNA was intravitreally injected to suppress GDF3 expression, with CNV

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lesions and immune cell infiltration quantified by immunofluorescence staining.【Results】GDF3 expression was significantly upregulated in CNV tissues ($P<0.001$). In vitro GDF3 intervention markedly suppressed endothelial cell proliferation ($P<0.001$), migration ($P<0.001$), and angiogenesis ($P<0.01$), downregulated the expression of inflammatory/adhesion molecules ($P<0.001$), and significantly reduced immune cell adhesion ($P<0.001$) and transendothelial migration ($P<0.001$). In vivo experiments confirmed that targeted inhibition of GDF3 significantly attenuated CNV formation ($P<0.001$) and decreased immune cell infiltration ($P<0.001$).【Conclusion】Targeted inhibition of GDF3 concurrently attenuates endothelial inflammatory response and pathological angiogenesis, potentially through modulating the inflammatory microenvironment. These findings provide novel insights for the treatment of wet age-related macular degeneration (wAMD).

Key words: age-related macular degeneration; choroidal neovascularization; growth differentiation factor 3; vascular endothelial cell; inflammation

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年龄相关性黄斑变性(age-related macular degeneration,AMD)是严重的致盲性眼病,是老年人失明的主要原因[1-2]。流行病学数据显示,2020年全球的AMD患者总数约为1.96亿人,预计2040年将增至2.88亿[3-4]。我国AMD患者基数庞大,2020年已达3 000万,伴随人口老龄化加剧,2040年预计突破5 000万,对社会经济和公共卫生体系构成严峻挑战[5-6]。AMD可分为干性(dry AMD)和湿性(wet AMD,wAMD)两种亚型,其中wAMD约占病例总数的10%~15%,却导致90%以上的AMD相关严重视力丧失[7]。wAMD核心病理特征为脉络膜新生血管(choroidal neovascularization,CNV)异常增生,并且这些新生血管结构紊乱、屏障功能缺陷,易引发视网膜下出血、视网膜脱离及纤维瘢痕形成,最终导致光感受器细胞不可逆损伤[8-9]。目前wAMD的临床治疗主要依赖玻璃体腔注射抗血管内皮生长因子(vascular endothelial growth factor,VEGF)药物(如雷珠单抗、阿柏西普),通过抑制血管内皮生长因子信号延缓CNV进展[10]。尽管此类疗法可部分改善血管渗漏并稳定视力,但仍存在显著局限,比如无法彻底抑制新生血管和渗漏,需频繁注射,容易引起眼内炎、晶状体损伤、视网膜损害等眼内并发症[11]。更重要的是,抗VEGF治疗存在耐药,对伴随的炎症微环境调控不足,无法阻断疾病复发[12]。因此,亟需找到更有效的治疗wAMD的新靶点。

炎症反应不仅是wAMD发病的核心机制之一,也是抗VEGF疗效减弱的重要诱因[13-15]。临床证据显示,wAMD患者房水中白细胞介素(interleukin,IL)-17、IL-12等促炎因子水平显著升高(67%,n=60),近半数患者γ-干扰素(interferon-γ,IFN-γ)和IL-4浓度高于健康对照[13],且抗VEGF治疗后76%患者(n=21)出现TNF-α等炎症因子水平升高[16]。此外,有研究发现紧密连接蛋白5(claudin-5)在炎症因子IL-6、TNF-α、CCL2等刺激下表达上调,并通过激活Rho相关含卷曲螺旋蛋白激酶2导致抗VEGF治疗耐药[14],而且靶向炎症因子IL-17可以有效缓解抗VEGF治疗抵抗[15]。这些研究提示炎症在wAMD新生血管增生和抗VEGF治疗耐药性中发挥十分重要的作用。因此,揭示CNV进程中调控炎症的关键分子靶点,开发新的干预策略,已经成为wAMD防治研究的热点和前沿方向。

生长分化因子3(growth differentiation factor 3,GDF3)作为转化生长因子-β(transforming growth factor-β,TGF-β)超家族成员,通过激活SMAD信号通路参与细胞增殖、分化及免疫调控[17-19]。研究显示GDF3是抗VEGF耐药相关基因[20],不过目前尚无研究报道GDF3是否介导VEGF耐药。更重要的是,GDF3可以促进巨噬细胞向M2型极化,并抑制M1型极化[21],而此前的研究表明M2型巨噬细胞促进CNV形成和炎症,而M1型巨噬细胞则可抑制CNV形成和炎症[22],这提示GDF3可能通过调控巨噬细胞表型转换影响CNV进程。但其在wAMD中的作用机制尚未明确。基于此,本研究旨在明确GDF3在wAMD新生血管和炎症中的作用,探索靶向GDF3治疗wAMD的应用潜能。

1　材料与方法

1.1　实验动物

本实验所使用的6~8周龄雄性C57BL/6J小鼠均从江苏集萃药康生物科技有限公司佛山分公司购买，动物许可证号：SCXK（粤）2020-0054。小鼠饲养于中山大学中山眼科中心动物房（SPF级）[实验动物使用许可证号：SYXK（粤）2018-0189]。动物房温度控制在（22±2）℃，湿度控制在60%左右。所有动物均定期7 d更换垫料，并提供充足的饮食和纯净水。动物房采用12 h明暗交替的照明周期。本实验的所有动物实验和操作均已获得中山大学中山眼科中心伦理委员会的批准（批准号：Z2021002），并遵循国家科技部及中山眼科中心的相关管理规定执行。

1.2　实验所用细胞体外培养

人视网膜内皮细胞（human retinal endothelial cells，HREC）细胞购买自美国ScienCell公司，培养于体积分数5%胎牛血清（fetal bovine serum，FBS）+体积分数1%内皮细胞生长补充剂+10 mL/L青霉素/链霉素双抗（Penicillin/Streptomycin，P/S）的内皮细胞完全培养基中。细胞每48 h进行一次换液，细胞长至80%~90%进行传代或者进行后续实验处理。HREC细胞代数在4~7之间用于实验。人单核细胞白血病细胞（human monocytic leukemia cell line 1，THP-1）细胞购买自美国ATCC公司，培养于体积分数10% FBS和10 mL/L P/S的RPMI1640完全培养基中，每3 d进行1次换液，每次换液吸取1/3原培养基，加入1/3新培养基。

1.3　siRNA转染内皮细胞

特异性靶向敲低*GDF3*的siRNA（序列为：5'-GGUUAUUCCUGGAGAUACU-3'；5'-GAGACUUAUGCUACGUAAA-3'）和阴性对照siRNA由擎科生物公司合成。将siRNA与转染试剂Escort IV（Sigma，L3287）混合，逐滴加入细胞培养皿内，轻轻混匀。将培养皿放入培养箱中3 h后，换成正常培养基，培养24~48 h后用于后续实验。

1.4　CCK8实验

siRNA转染24 h的内皮细胞经PBS清洗两次后，用胰酶消化，将细胞悬液以10^4个细胞/孔种于96孔板中，培养箱培养过夜。次日，去除培养基，将培养基与CCK8试剂以9∶1的比例，共100 μL加入上述细胞中，放回培养箱继续培养4 h后，用酶标仪测量450 nm吸光值。

1.5　内皮细胞划痕实验

siRNA转染24 h的内皮细胞以5×10^4个细胞/孔种于24孔板中，培养箱培养至细胞长满。用200 μL的枪头垂直于细胞进行水平划痕，用无血清的内皮培养基洗去划掉的细胞，在0 h和12 h拍照，并用Image J统计迁移率。

1.6　内皮细胞成管实验

将基质胶（Corning，356231）置于4 ℃冰箱进行溶解，以5 000×*g*，4 ℃离心3 min，将50 μL基质胶加入预冷的96孔板中，并放入培养箱中孵育30 min。siRNA转染24 h的内皮细胞以10^4个细胞/孔加到基质胶上，轻轻摇匀，6 h后拍照，并用Image J统计成管数量和长度。

1.7　免疫细胞黏附实验

siRNA转染24 h的内皮细胞以5×10^4个细胞/孔种于24孔板中，培养箱培养至细胞长满，形成单层细胞。1 μg/mL脂多糖（LPS，Sigma，L2630）或10 ng/mL TNF-α（MCE，HY-P7058-10）处理4 h。2.5 μmol/L的钙黄绿素（Beyotime，C2012）标记THP-1细胞30 min，PBS洗涤内皮细胞3次后每孔加入2.5×10^5个THP-1细胞共培养2 h。用PBS洗涤共培养的细胞3次，以洗去未黏附的THP-1细胞。随机选取12个视野/孔进行拍照，用Image J统计每个孔黏附的THP-1细胞的平均数量。

1.8　免疫细胞穿内皮细胞迁移实验

siRNA转染24 h的内皮细胞以5×10^4个细胞/孔种于transwell（EMD Millipore，ECM557）的上室中，培养24 h，形成单层细胞。加入5×10^5个钙黄绿素标记的THP-1细胞到内皮细胞中。加入体积分数10% FBS，50 ng/mL CCL2的1640培养基到下室中，继续培养12 h。将迁移到下室的THP-1细胞用150 ng/mL佛波酯（PMA，MCE，HY-18739）处理12 h，使THP-1贴壁，并随机选取12个视野/孔进行拍照，用Image J进行分析，统计每个孔跨内皮细胞迁移的THP-1细胞的平均数量。

1.9　RNA提取及RT-qPCR

利用TRIzol提取细胞或动物组织样品RNA，并进行RT-qPCR检测基因的表达。以β-ACTIN作为内参，使用$2^{-\Delta\Delta Ct}$法分析基因的相对表达量，并以对照作为1（表1）。

表1 RT-qPCR引物序列

Table 1 Primer sequence of RT-qPCR

Target gene	Primer sequence(5′-3′)	
hu-*GDF3*	F: GGTGGTGACTCTCAACCCTG	R: GGCAATGATCCACTTGTGCC
ms-*Gdf3*	F: ATGCAGCCTTATCAACGGCTT	R: AGGCGCTTTCTCTAATCCCAG
hu-*TNF-A*	F: ACACCATGAGCACTGAAAGC	R: CGATCAGGAAGGAGAAGAGG
hu-*CXCL1*	F: GCTTGCCTCAATCCTGCATC	R: AGTTGGATTTGTCACTGTTC
hu-*CXCL2*	F: GAAAGCTTGTCTCAACCCCG	R: GTTGGATTTGCCATTTTTCAGCA
hu-*CXCL3*	F: GTGAATGTAAGGTCCCCCGGA	R: TCAGTTGGTGCTCCCCTTGT
hu-*CCL2*	F: GAAAGTCTCTGCCGCCCTT	R: GGTGACTGGGGCATTGATTG
hu-*CCL5*	F: TCCTCATTGCTACTGCCCTC	R: TCGGGTGACAAAGACGACTG
hu-*IL-1A*	F: AGATGCCTGAGATACCCAAAACC	R: CCAAGCACACCCAGTAGTCT
hu-*IL-1B*	F: AATCTGTACCTGTCCTGCGTGTT	R: ATCGCTTTTCCATCTTCTTCTTTG
hu-*IL-6*	F: CAAGCGCCTTCGGTCCAGT	R: GGTTGGGTCAGGGGTGGTTA
hu-*IL-8*	F: TTTTGCCAAGGAGTGCTAAAGA	R: AACCCTCTGCACCCAGTTTTC
hu-*SELE*	F: CAGCAAAGGTACACACACCTG	R: CAGACCCACACATTGTTGACTT
hu-*ICAM1*	F: ATGCCCAGACATCTGTGTCC	R: GGGGTCTCTATGCCCAACAA
hu-*VCAM1*	F: GCTATGAGGATGGAAGACTCTGG	R: ACTTGTGCAGCCACCTGAGATC
hrm-ACTIN	F: GCCAACACAGTGCTGTCTGG	R: GGAGCAATGATCTTGATCTTC

F: forward primer; R: reverse primer.

1.10 Western blot

细胞或组织样品用RIPA裂解液进行裂解提取蛋白，按照试剂商的指示用二喹啉甲酸(bicinchoninic acid，BCA)法测量蛋白浓度。根据浓度吸取上样总量为20 μg蛋白所需的体积，加入5× SDS loading buffer，混匀，置于95 ℃煮样25 min。将蛋白液加入SDS-PAGE凝胶上110 V恒压跑胶2 h，然后转移到PVDF膜上，250 mA恒流转膜2 h。在室温下用50 g/L脱脂牛奶封闭1 h后，孵育一抗，4 ℃过夜。次日，室温孵育二抗1 h后进行显影，并用Image J对结果进行灰度统计。

1.11 构建靶向敲低Gdf3的shRNA质粒

靶向敲低*Gdf3*的shRNA利用BLOCK-iT™ RNAi Designer (Thermofisher)进行序列设计，并由擎科生物合成后用T4 DNA连接酶(Thermofisher，15224041)连接到载体Plko.1上。构建的shRNA质粒进行DNA测序确定靶序列已经连接到Plko.1载体，并利用DH5α (TIANGEN，CB101-01)感受态细胞进行转化和质粒提取试剂盒(TIANGEN，DP117-TA)提取转化的质粒。本实验所用的shRNA序列如表2所示。

表2 shRNA序列

Table 2 shRNA sequence

Target	Sequence (5′-3′)
sh*Ctrl*	CCGGCCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGGTTTTTG
sh*Gdf3*	CCGGGCTTAAGGATTGGAGCAGCAACTCGAGTTGCTGCTCCAATCCTTAAGCTTTTTG

1.12 激光诱导的小鼠脉络膜新生血管模型

小鼠脉络膜新生血管模型是经典的wAMD研究模型[23]。6~8周大小C57BL/6J小鼠随机分组进行麻醉和散瞳，在距离视盘2～3个视盘直径的12，

3，6，9点位置进行激光光凝（光斑大小75 μm，功率90 MW，持续时间75 ms，Oculight 红外激光系统810 nm，Iridex）。激光损伤后3天处死小鼠取眼球分离脉络膜进行RNA-seq和检测*Gdf3*的表达情况。取激光诱导后7 d的小鼠脉络膜进行免疫荧光染色，确定血管新生和炎症情况，其中未受激光诱导损伤的小鼠作为对照组（control，CTRL），经激光损伤的小鼠为处理组（choroidal neovascularization，CNV）。

1.13　玻璃体腔注射

取激光损伤后的小鼠，碘伏消毒眼表，用无菌注射针在小鼠眼角膜缘附近避开血管扎出小孔，轻轻按压眼球使房水流出。沿小孔注射1 μg/μL sh*Gdf3*（处理组）或sh*Ctrl*（对照组）质粒-PEI复合物或注射1 μg/μL GDF3中和抗体（处理组，anti-GDF3）或IgG（对照组），1 μL/眼。注射后3 d取小鼠脉络膜检测Gdf3的敲低情况。为检测CNV面积和免疫细胞浸润，在第一次注射后第3 d进行二次注射，剂量同上，取注射7 d后的小鼠脉络膜进行免疫染色分析。

1.14　免疫荧光染色

分离小鼠眼球，用40 g/L多聚甲醛室温固定1 h，剥去角膜、晶体、晶状体和玻璃体。分离脉络膜/RPE复合物，PBS冲洗。在体积分数为5%驴血清、5%BSA和0.3%Triton X-100的抗体稀释液中稀释抗体，包括离子钙结合适配器分子1（ionized calcium binding adaptor molecule 1，IBA1；Wako，019-19741），小鼠含生长因子样模体粘液样激素样受体（mouse EGF-like module-containing mucin-like hormone receptor-like 1，EMR1，又称F4/80）（eBioscience，11-4801-82）和同工凝集素B4（isolectin b4，IB4；Life Technologies，I21411）。其中IB4是血管内皮细胞标记物，用于指示CNV面积[24]；IBA1和F4/80是巨噬细胞/小胶质细胞标记物，用于指示炎症细胞浸润的面积[25-26]。将脉络膜/RPE复合物置于溶液中4 ℃孵育过夜。PBS洗涤脉络膜/RPE复合物3次，将RPE面朝上封片与载玻片。使用荧光显微镜（Zeiss，Germany）拍照，并使用Image J分析CNV染色定量CNV面积和炎症细胞浸润的面积。

1.15　统计学方法

应用GraphPad Prism 8软件进行柱状图作图和显著性分析，符合正态分布及方差齐性检验的计量资料以均数（mean）及标准差（SD）表示，两组比较使用双尾t检验。$P<0.05$视为有统计学意义。所有实验都独立重复3次及以上。

2　结 果

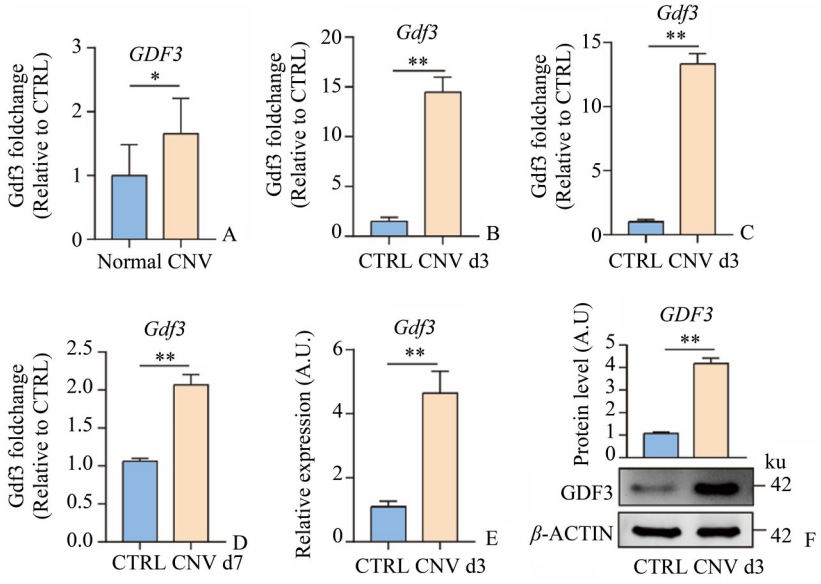
2.1　GDF3在脉络膜新生血管中表达上调

目前GDF3在wAMD病理性新生血管中的作用尚未明确，对AMD患者的RNA-seq数据集（GSE29801）进行分析显示，相较于同龄正常对照组（Normal），*GDF3*的mRNA表达水平在湿性AMD患者（CNV）的脉络膜/RPE样本中显著上升（t=2.275，P=0.039，图1A）。为了进一步探究GDF3在wAMD相关新生血管形成中的作用，本研究构建激光诱导的小鼠CNV模型，未受激光损伤的正常小鼠作为对照组（CTRL），并于激光损伤后第3天分离RPE/脉络膜复合体进行RNA-seq（GSE277800）。RNA-seq的结果显示相较于未受激光损伤的CTRL组小鼠，*Gdf3*在CNV中表达显著上调（t=14.380，P<0.001；图1B）。进一步对公共数据库的RNA-seq数据（GSE160011；GSE207171）进行分析也发现相较于CTRL组小鼠，*Gdf3*在CNV组显著上调（t=26.030，P<0.001，图1C；t=12.220，P<0.001；图1D）。RT-qPCR（t=10.140，P<0.001）和Western blot（t=25.060，P<0.001）实验进一步验证了相较于CTRL组小鼠，GDF3在CNV中显著上调（图1E，F）。这些结果提示了GDF3可能在CNV发生发展中发挥重要调控作用。

2.2　GDF3调控内皮细胞新生血管功能

2.2.1　敲低GDF3抑制内皮细胞体外新生血管功能

通过siRNA敲低GDF3表达以探究其对内皮细胞功能的影响。在HREC细胞中，与转染对照组（si*CTRL*）相比，siRNA处理可显著降低GDF3的mRNA（t=22.650，P<0.001；图2A）及蛋白表达水平（t=16.590，P<0.001；图2B）。通过CCK8实验检测细胞增殖能力，结果显示GDF3敲低组吸光度（OD450）较对照组下降约30%（t=22.260，P<0.001；图2C）。划痕实验进一步表明，GDF3敲低导致HREC迁移率较对照组降低约50%（t=5.511，P=0.003；图2D，E）。为了评估GDF3对血管生成能力的影响，采用基质胶成管实验分析显示，GDF3敲低显著减少管状结构分支节点数（t=6.600，P=0.003）和总长度（t=13.040，P<0.001；图2F-H）。



A: RNA-seq reveals that the mRNA level of *GDF3* in macular RPE-complexes is significantly upregulated in wAMD patients (CNV, n=4) compared to normal controls (Normal, n=12). B: RNA-Seq reveals that *Gdf3* is significantly upregulated in CNV lesions at day 3 post-laser injury. n=3 mice per group. C, D: Validation using publicly available RNA-seq datasets (GSE160011; GSE207171) confirms *Gdf3* upregulation in CNV tissues at days 3 and 7 post-laser injury. n=3 mice per group. E, F: RT-qPCR and Western blot analyses further validate increased GDF3 expression at both mRNA and protein levels in CNV lesions. n=3 independent experiments. Data are mean ± SD. Student's t-test was used. *P < 0.05, **P < 0.001. CNV: choroidal neovascularization.

图1 GDF3在激光诱导的小鼠CNV模型中表达上调

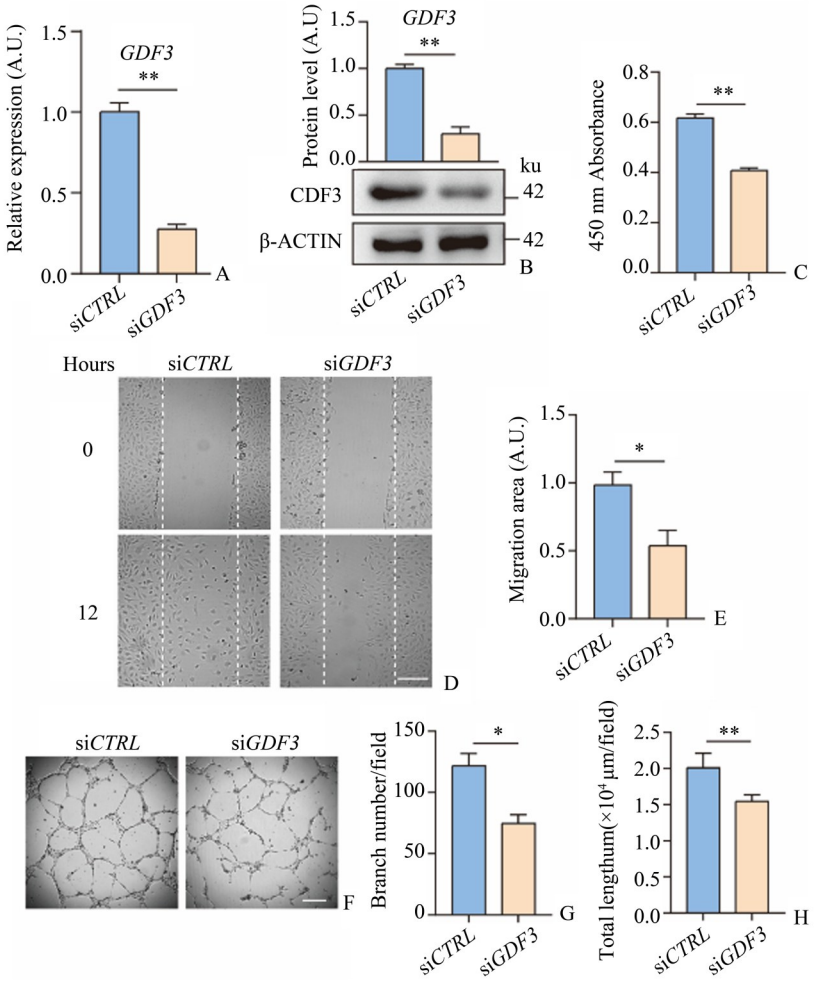
Fig. 1 GDF3 expression is upregulated in laser-induced CNV mouse model

2.2.2 拮抗GDF3抑制内皮细胞体外新生血管功能 为进一步验证GDF3的功能，采用1 μg/mL GDF3中和抗体(处理组，anti-GDF3)或相同浓度的IgG(对照组)处理HREC细胞24 h，并评估对其增殖、迁移和体外成管能力的影响。结果显示，相较于IgG处理，GDF3中和抗体可显著减少了内皮细胞的增殖(t=21.680，P<0.001；图3A)、迁移(t=7.120，P<0.001；图3B，C)和成管(t=8.034，P<0.01，图3D，E；t=3.741，P=0.020，图3D，F)，其抑制效果与siRNA敲低一致。这些结果说明GDF3调控内皮细胞的体外新生血管功能。

2.3 GDF3调控内皮细胞炎症

2.3.1 敲低GDF3抑制内皮细胞炎症 通过敲低GDF3进一步探究其对内皮细胞炎症通路的影响。结果显示，与转染对照组(si*CTRL*)相比，HREC细胞敲低GDF3显著下调*IL-8*(t=8.459，P<0.001)、*CXCL1*(t=9.377，P<0.001)、*CXCL3*(t=8.659，P<0.001)、*IL-1B*(t=8.898，P<0.001)、*TNF-A*(t=8.180，P<0.001)、*SELE*(t=7.362，P<0.001)、*CCL2*(t=7.262，P<0.001)、*IL-1A*(t=7.222，P<0.001)和*CCL5*(t=6.863，P<0.001)等炎症因子的表达(图4A)，同时抑制黏附分子ICAM-1(t=11.110，P<0.001，图4A；t=9.684，P<0.001；图4B，C)与VCAM-1(t=8.646，P<0.001，图4A；t=11.180，P<0.001；图4B，C)的表达。THP-1细胞黏附实验和跨内皮细胞迁移实验结果进一步证实相较于转染对照组(si*CTRL*)，敲低GDF3显著减少了黏附到内皮细胞的THP-1数量(t=17.760，P<0.001；图4D，E)，并降低了THP-1跨内皮细胞迁移的能力(t=11.060，P<0.001；图4F，G)。这些结果说明敲低GDF3抑制了内皮细胞炎症。

2.3.2 拮抗GDF3抑制内皮细胞炎症 与基因敲低结果一致，相较于对照组的IgG处理，GDF3中和抗体处理可显著降低炎症因子，如*IL-1B*(t=9.451，P<0.001)、*CCL2*(t=16.210，P<0.001)、*CXCL2*(t=7.909，P<0.001)、*SELE*(t=7.661，P<0.001)、*CXCL1*(t=15.650，P<0.001)、*IL-8*(t=14.070，P<0.001)、*TNF-A*(t=4.045，P=0.006)、*IL-1A*(t=7.686，P<0.001)、*CCL5*(t=9.740，P<0.001)和*IL-6*(t=9.023，P<0.001)，以及黏附分子如ICAM-1(t=9.708，P<0.001)和VCAM-1(t=4.474，P=0.004)的表达水平(图5A)，并抑制THP-1细胞粘附(t=



A, B: RT-qPCR (A) and Western blot (B) reveal that siRNA-mediated *GDF3* knockdown significantly reduces GDF3 expression in HREC. n=3 independent experiments. C: CCK8 assay demonstrates decreased HREC proliferation after *GDF3* knockdown. n=4 independent experiments. D, E: Scratch wound healing assay reveals reduced migration capacity of HREC upon *GDF3* knockdown. The quantification of migrated areas is shown in (E). Scale bar: 50 μm, n=3 independent experiments, 12 images for each group. F-H: Matrigel tube formation assay shows decreased branch points and total length under *GDF3* knockdown. The quantification of branch numbers per field and total length were shown in (G, H). Scale bar: 50 μm, n=3 independent experiments, 3 images for each group. Data are mean ± SD. Student's t-test was used. *P < 0.01, **P < 0.001.

图2　敲低GDF3抑制内皮细胞体外新生血管功能

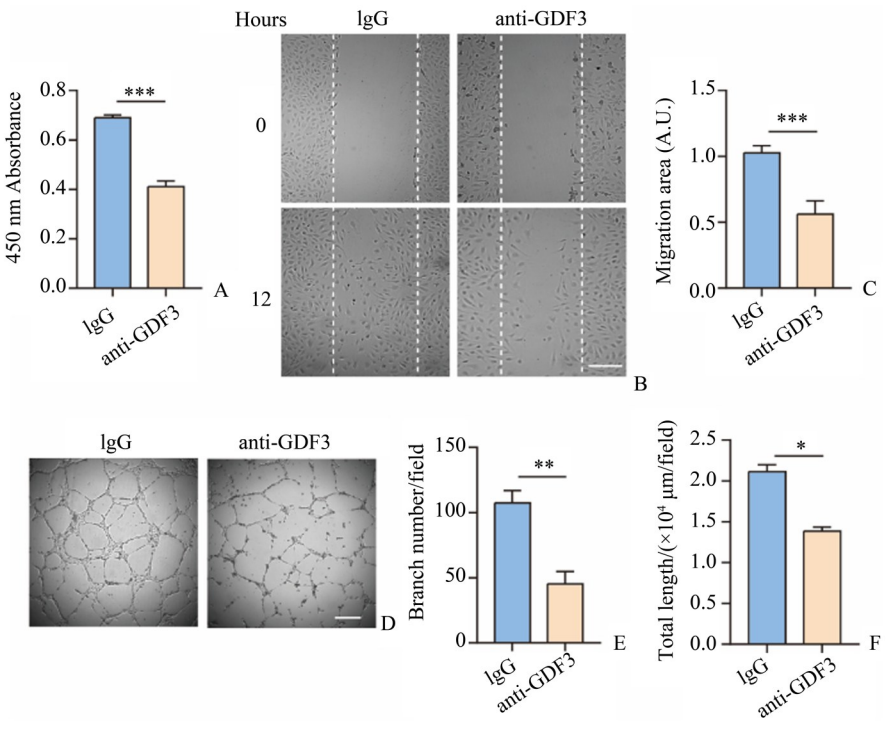
Fig. 2　GDF3 knockdown inhibits endothelial cell angiogenesis function in vitro

19.900，P<0.001；图5B，C）与跨内皮细胞迁移（t=11.710，P<0.001；图5D，E）的能力。上述结果提示，GDF3通过调控内皮炎症微环境参与CNV进程。

2.4　靶向GDF3抑制脉络膜新生血管形成和炎症

基于GDF3在内皮细胞炎症和体外血管生成中的重要作用，进一步探究靶向GDF3是否具有抑制CNV形成和炎症的作用。通过玻璃体腔注射shRNA靶向抑制小鼠脉络膜GDF3表达，RT-qPCR（t=15.860，P<0.001）和Western blot（t=9.092，P<0.001）的结果显示，与阴性对照sh*CTRL*相比，靶向GDF3的shRNA减少了GDF3在小鼠脉络膜中的表达约50%（图6A，B）。为评估GDF3抑制对CNV形成的调控效果，采用IB4染色定量CNV面积。结果显示，GDF3敲低组CNV面积较对照组减少约30%（t=7.422，P<0.001；图6C，D）。同时，通过F4/80和IBA1免疫荧光染色分析炎症细胞浸润的情况，发现GDF3敲低组脉络膜内F4/80阳性细胞浸润减少约35%（t=6.059，P<0.001；图6C，E），IBA1阳性细胞浸润减少约30%（t=2.509，P=0.025；图6F，G）。

上述结果表明，靶向抑制GDF3可能通过协同阻断病理性血管生成与炎症反应发挥治疗作用，为克服当前抗VEGF治疗的局限性提供了新策略。



A: CCK8 assay indicates attenuated HREC proliferation after GDF3 neutralization. n=4 independent experiments. B, C: Scratch wound healing assay demonstrates reduced migration capacity of HREC under GDF3 neutralization. The quantification of migrated areas is shown in (C). Scale bar: 50 μm, n=3 independent experiments, 12 images for each group. D–F: Tube formation assay confirms diminished branch points and total length by GDF3 neutralization. The quantification of branch numbers per field and total length were shown in (E, F). Scale bar: 50 μm, n=3 independent experiments, 3 images for each group. Data are mean ± SD. Student's t-test was used. *P < 0.05, **P < 0.01, ***P < 0.001.

图3 拮抗GDF3抑制内皮细胞体外新生血管功能

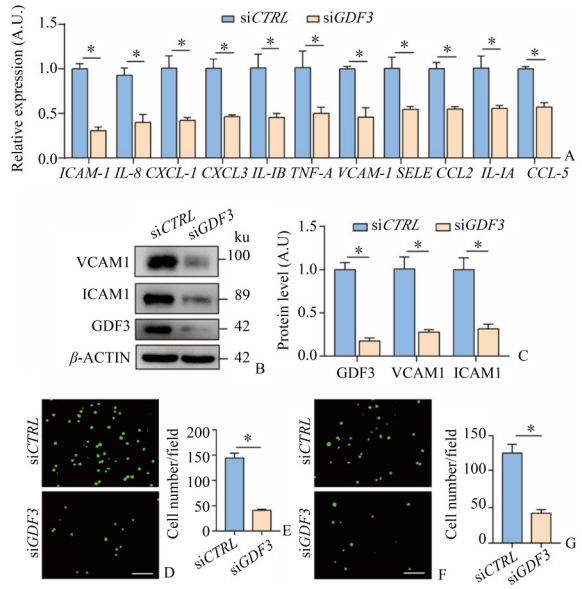
Fig. 3 GDF3 neutralization suppresses endothelial cell angiogenesis in vitro

3 讨 论

炎症反应不仅是CNV形成的驱动因素,其引发的血管渗漏还会进一步加剧局部炎症反应,这一病理特征使得炎症调控成为治疗wAMD的重点和难点[27]。越来越多的证据表明,内皮细胞炎症在CNV的进展中发挥着十分重要的作用[28-30],但是内皮细胞炎症调控CNV的机制尚不清楚。尽管抗VEGF药物通过抑制病理性新生血管可部分改善患者视力,但难以解决炎症问题[31-33],凸显开发兼具抗血管生成与抗炎双重功效疗法的迫切性。本研究聚焦wAMD潜在新靶点GDF3,首次揭示GDF3在CNV形成和炎症中的双重调控作用,为wAMD治疗提供新思路。

通过分析公共数据(GSE29801)中wAMD患者的GDF3表达水平,以及对前期研究中激光诱导CNV模型的RNA测序数据及公共数据(GSE160011、GSE207171)的分析,发现GDF3在病变组织中的显著上调(图1A-D),且其表达水平与炎症通路激活呈正相关,提示GDF3可能作为连接血管生成与炎症的关键节点分子。进一步的功能实验表明,GDF3可能通过双重机制促进CNV进展:①直接增强内皮细胞增殖、迁移及成管能力(图2-3);②诱导内皮细胞表达TNF-α等炎症因子并上调ICAM-1、VCAM-1等黏附分子的表达,进而招募单核/巨噬细胞浸润(图4-5)。值得注意的是,靶向抑制GDF3可同时抑制病理性新生血管和炎症细胞浸润(图6C-G),这一双重效应为突破现有单靶点疗法的局限性提供了可能。

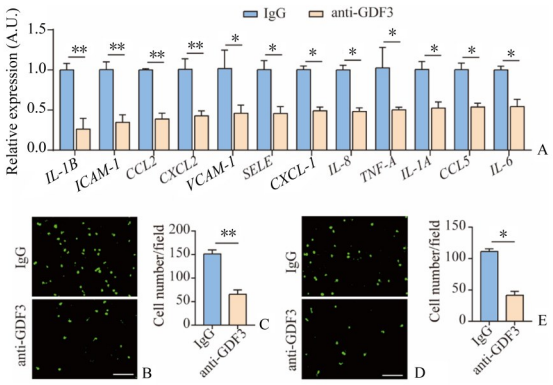
既往研究报道GDF3通过激活Smad2/Smad3信号通路调控炎症反应[21]。本研究进一步证实了GDF3在内皮细胞炎症中的调控作用。然而,GDF3调控内皮细胞炎症的分子机制仍待解析,后续可通过转录组测序联合磷酸化蛋白质组学技术系统阐明相关分子机制。值得注意的是,GDF3与抗VEGF治疗耐药性的临床关联为



A: RT-qPCR reveals the reduced expression of pro-inflammatory genes and adhesion molecules in *GDF3* knockdown HREC. n=4 independent experiments. B, C: Western blot shows reduced ICAM1 and VCAM1 expression after *GDF3* knockdown. n=3 independent experiments. D, E: THP-1 adhesion assay reveals decreased immune cell adhered to HREC under *GDF3* knockdown. The quantification of adhered THP-1 cells was shown in (E). Scale bar: 100 μm, n=4 independent experiments. F, G: Transendothelial migration assay demonstrates reduced THP-1 transmigration upon *GDF3* knockdown in HRECs. The quantification of migrated THP-1 cells was shown in (G). Scale bar: 100 μm, n=4 independent experiments. Data are mean ± SD. Student's t-test was used. *P < 0.001.

图4　敲低GDF3抑制内皮细胞炎症反应

Fig. 4　GDF3 knockdown inhibits endothelial inflammatory responses



A: RT-qPCR reveals the reduced expression of inflammatory genes and adhesion molecules in HREC after GDF3 neutralization. n=4 independent experiments. B, C: GDF3 neutralization in HRECs inhibited THP-1 cell adhesion. The quantification of adhered THP-1 cells was shown in (C). Scale bar: 100 μm, n=3 independent experiments, 12 images for each group. D, E: GDF3 neutralization in HRECs repressed the transendothelial cell migration of THP-1 cells. The quantification of migrated THP-1 cells was shown in (E). Scale bar: 100 μm, n=3 independent experiments, 12 images for each group. Data are mean ± SD. Student's t-test was used. *P < 0.01, **P < 0.001.

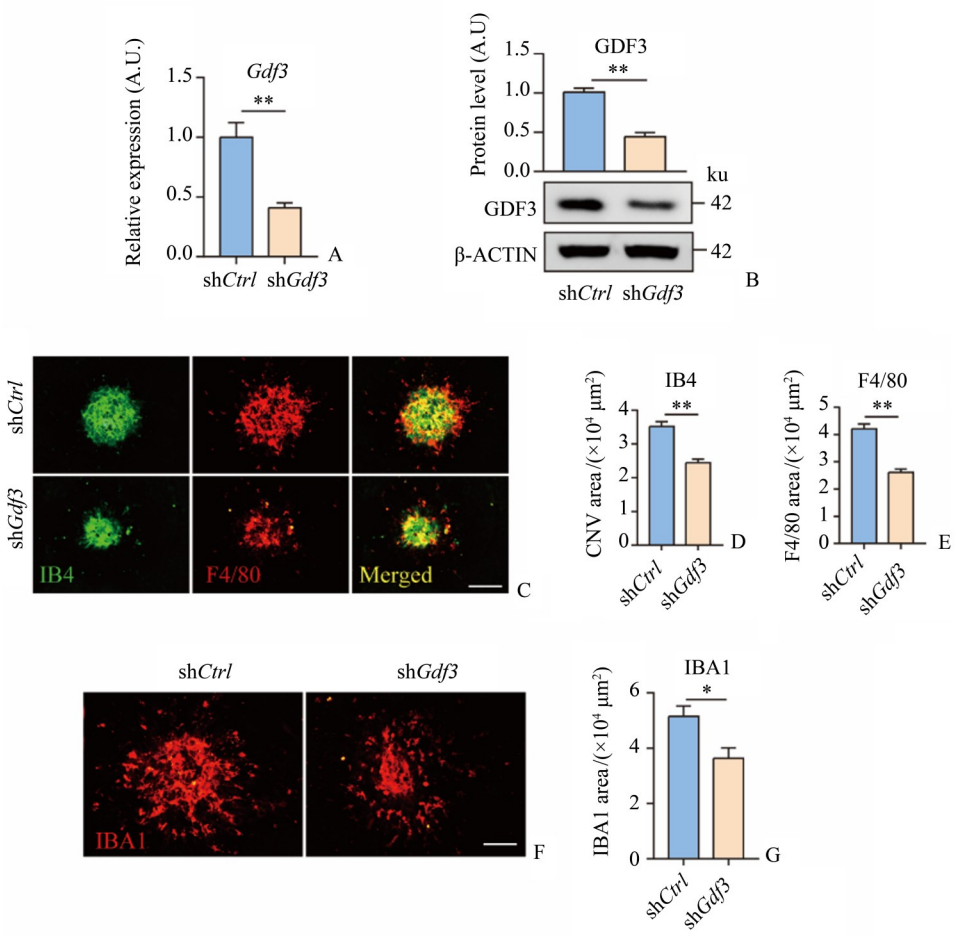
图5　拮抗GDF3抑制内皮细胞炎症

Fig. 5　GDF3 neutralization mitigates endothelial inflammation

GDF3促进血管新生的机制研究提供了新视角：作为wAMD的一线疗法，抗VEGF药物虽能抑制异常血管生长，但单药治疗易产生耐药性[34-35]，而近期研究发现GDF3是抗VEGF治疗耐药相关基因[20]。此外，GDF3的经典下游信号Smad2/3的激活可上调VEGF，促进血管新生[36-37]，而GDF3受体ALK4的抑制会降低VEGF水平并抑制血管新生[38-39]，提示GDF3可能通过下游信号调节VEGF表达参与CNV的调控。另外，研究显示在脂肪组织巨噬细胞中，GDF3通过PPARγ依赖性途径抑制脂肪细胞的脂解作用[40]，而巨噬细胞内的脂质积累被发现是抗VEGF治疗中产生耐药性的关键因素[41]。因此，GDF3除了可能通过调控巨噬细胞表型转换参与CNV进展[21-22]，还可能通过调节视网膜巨噬细胞的脂代谢状态，参与抗VEGF耐药性的产生。

本研究的治疗策略亦存在优化空间：shRNA靶向敲低GDF3的表达，虽然能抑制CNV形成和炎症，但是未能完全抑制炎症。这一方面受限于shRNA的敲低效率（约50%）（图6A-C），另一方面shRNA的效应时间较短，无法长期抑制GDF3的表达。近期研究显示，腺相关病毒（AAV）载体可实现眼底细胞中长期基因沉默[42-43]，而纳米颗粒缓释系统能提高抗体类药物眼内滞留时间[44-45]，这些技术有望提升GDF3靶向治疗的临床转化潜力，更高效和长期抑制GDF3。因此开发GDF3小分子抑制剂或中和抗体，并优化眼内药物递送系统，有望突破现有的治疗瓶颈，为wAMD患者提供更安全、长效的治疗方案。

综上，本研究明确了GDF3在CNV和内皮细胞炎症中的作用，确立其作为wAMD治疗新靶点的潜力。未来研究可从受体信号解析、临床转化验证及联合治疗策略这3方面深入探索，推动wAMD治疗从单一通路抑制向多靶点调控的转变。



A: RT-qPCR showing reduced *Gdf3* level in choroid/RPE complexes after *Gdf3* knockdown by shRNA. n=4 independent experiments. B: Western blot validates reduced GDF3 levels in choroid/RPE complexes after *Gdf3* knockdown by shRNA. n=3 independent experiments. C-G: Immunostaining of choroid/RPE wholemount shows reduced IB4 (green), F4/80 (red) and IBA1 (red) positive areas after *Gdf3* knockdown in mice with CNV. The quantification of the indicated areas was shown in (D, E, G). Scale bar: 100 μm. 3 independent experiments were performed (n=3 mice/group), and data of 8 mice were used for statistical analysis. Data are mean ± SD. Student's t-test was used. *P < 0.05, **P < 0.001.

图6 靶向GDF3抑制CNV形成和炎症

Fig. 6 Targeting GDF3 inhibits CNV progression and inflammation in vivo

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