



Fibulin-3调控组织金属蛋白酶抑制因子-3影响 椎间盘髓核细胞衰老的机制研究*

王许可¹, 张 杨², 罗 刚², 孔军珂³, 曹向阳¹, 王庆丰^{1△}

1. 河南省洛阳正骨医院(河南省骨科医院)(洛阳 471002); 2. 陆军军医大学新桥医院 骨科(重庆 400037);
3. 河南中医药大学(郑州 450046)

【摘要】目的 探讨fibulin-3通过调控组织金属蛋白酶抑制因子-3(tissue inhibitor of metalloproteinase 3, TIMP-3)表达而影响椎间盘髓核细胞衰老的分子机制。**方法** ①收集37例椎间盘术后患者的髓核组织及影像学资料,按照Pfirrmann分级评估椎间盘的退变程度,衰老相关 β -半乳糖苷酶(senescence-associated β -galactosidase, SA- β -gal)染色法确定髓核细胞的衰老程度,Western blot和ELISA检测fibulin-3蛋白水平,探讨fibulin-3与椎间盘退变和髓核细胞衰老之间的关系;②体外培养人椎间盘髓核细胞(nucleus pulposus cells, NPCs),观察连续传代过程中NPCs的增殖(CCK8法)和衰老(SA- β -gal染色法)情况,检测fibulin-3的表达水平以及促炎症因子和基质蛋白酶的表达情况。通过添加外源性fibulin-3验证其对NPCs增殖和衰老的影响;③通过基因过表达技术验证fibulin-3对NPCs凋亡和增殖的作用,结合凋亡抑制剂进行双向验证;④利用生物信息学分析fibulin-3与TIMP家族的关系,通过过表达fibulin-3并沉默TIMP-3基因的实验,验证其在NPCs衰老中的作用。**结果** ①在37例患者的椎间盘退变样本中,按Pfirrmann分级,退变等级越高, fibulin-3的表达越低。Spearman相关性分析显示椎间盘分级与NPCs的衰老等级负相关($r=-0.87$, $P<0.001$),与fibulin-3的表达量负相关($r=-0.79$, $P<0.001$);②随着NPCs传代次数增加, fibulin-3的表达逐渐降低,细胞增殖能力减弱,促炎症因子和基质蛋白酶的表达增高。添加外源性fibulin-3后,细胞形态和生长状态得到保持,细胞衰老显著抑制,促炎症因子和基质蛋白酶的表达明显降低;③基因过表达实验显示, fibulin-3减少了NPCs的凋亡,促进了细胞增殖,从而抑制了NPCs的衰老;④生物信息学分析显示fibulin-3与TIMP家族的TIMP-3存在显著关联。进一步实验验证发现,过表达fibulin-3能够增强TIMP-3的表达,而沉默TIMP-3基因后, fibulin-3对NPCs衰老的抑制作用显著减弱。这表明fibulin-3通过调控TIMP-3来抑制基质金属蛋白酶活性,影响细胞外基质的合成和降解,最终抑制髓核细胞的衰老。**结论** 本研究表明, fibulin-3通过调控TIMP-3在椎间盘髓核细胞衰老过程中发挥重要作用。具体机制包括: fibulin-3上调TIMP-3的表达,抑制基质金属蛋白酶活性,减少细胞外基质的降解,从而促进细胞外基质的合成;此外, fibulin-3通过减少细胞凋亡和促进细胞增殖,进一步抑制髓核细胞的衰老。因此, fibulin-3和TIMP-3在维持椎间盘健康和延缓退变过程中具有潜在的治疗意义。

【关键词】 Fibulin-3 髓核细胞 椎间盘退变 组织金属蛋白酶抑制因子-3

Fibulin-3 Regulates Tissue Inhibitor of Metalloproteinases 3 to Inhibit Senescence in Intervertebral Disc Nucleus Pulposus Cells WANG Xuke¹, ZHANG Yang², LUO Gang², KONG Junke³, CAO Xiangyang¹, WANG Qingfeng^{1△}.

1. Luoyang Orthopedic-Traumatological Hospital of Henan Province (Henan Provincial Orthopedic Hospital), Luoyang 471002, China; 2. Department of Orthopedics, Xinqiao Hospital, Army Medical University, Chongqing 400037, China; 3. Henan University of Chinese Medicine, Zhengzhou 450046, China

△ Corresponding author, E-mail: wangqf600@163.com

【Abstract】 Objective To investigate the effect of fibulin-3 on the senescence of intervertebral disc nucleus pulposus cells (NPCs) through the regulation of tissue inhibitor of metalloproteinases 3 (TIMP-3) expression and to elucidate the molecular mechanisms involved. **Methods** 1). The nucleus pulposus tissues and imaging data of 37 patients who had undergone intervertebral disc surgery were collected. The degree of degeneration of the intervertebral discs were classified according to the Pfirrmann grading system. The senescence degree of NPCs was determined using senescence-associated β -galactosidase (SA- β -gal) staining. Fibulin-3 expression levels were determined using Western blot and ELISA. The relationship between fibulin-3 and disc degeneration and NPCs senescence was investigated. 2). Human intervertebral disc NPCs were cultured *in vitro*. The proliferation and senescence of NPC across continuous passage were observed via CCK-8 assay and SA- β -gal staining, respectively. Fibulin-3 expression levels and the expression of inflammatory cytokines and matrix metalloproteinases were assessed. Exogenous fibulin-3 was added to verify its effect on the proliferation and senescence of NPCs. 3). The effect of fibulin-3 on the apoptosis and proliferation of NPCs was verified through gene overexpression, which was used in combination with an apoptosis inhibitor for bidirectional

* 河南省中医药科学研究专项课题(No. 2019ZY1032)和2023洛阳市社会发展类公益专项(No. 2302020A)资助

△ 通信作者, E-mail: wangqf600@163.com

出版日期: 2024-09-20

verification. 4). Bioinformatics analysis was performed to explore the relationship between fibulin-3 and the TIMP family. Experiments overexpressing fibulin-3 and silencing the *TIMP-3* gene were performed to verify their role in NPCs senescence. **Results** 1). The intervertebral disc degeneration samples from 37 patients were classified according to the Pfirrmann grading system. The higher the degeneration grade, the lower fibulin-3 expression. Spearman correlation analysis showed that the disc grade was negatively correlated with the NPC senescence grade ($r=-0.87$, $P<0.001$) and fibulin-3 expression ($r=-0.79$, $P<0.001$). 2). As the passage number of NPCs increased, fibulin-3 expression gradually decreased, cell proliferation ability weakened, and the expression of inflammatory cytokines and matrix metalloproteinases increased. After exogenous fibulin-3 was added, cell morphology and growth status were maintained, cell senescence was significantly inhibited, and the expression of inflammatory cytokines and matrix metalloproteinases was markedly reduced. 3). Gene overexpression experiments showed that fibulin-3 reduced NPC apoptosis and promoted cell proliferation, thereby inhibiting NPC senescence. 4). Bioinformatics analysis revealed a significant association between fibulin-3 and TIMP-3 of the TIMP family. Further experiments confirmed that overexpressing fibulin-3 enhanced *TIMP-3* expression, while silencing the *TIMP-3* gene significantly weakened the inhibitory effect of fibulin-3 on NPCs senescence. This indicates that, through regulating TIMP-3, fibulin-3 inhibits the activity of matrix metalloproteinases, affects the synthesis and degradation of the extracellular matrix, and ultimately inhibits NPCs senescence. **Conclusion** This study demonstrates that fibulin-3 plays a crucial role in inhibiting the senescence of intervertebral disc NPCs by regulating TIMP-3. The specific mechanisms involved are as follows, fibulin-3 upregulates *TIMP-3* expression, inhibits matrix metalloproteinase activity, and reduces extracellular matrix degradation, thereby promoting extracellular matrix synthesis. Additionally, fibulin-3 inhibits NPCs senescence by reducing apoptosis and promoting cell proliferation. Therefore, fibulin-3 and TIMP-3 have potential therapeutic significance in maintaining intervertebral disc health and delaying degeneration.

【Key words】 Fibulin-3 Nucleus pulposus cells Intervertebral disc degeneration Tissue inhibitor of metalloproteinases 3

椎间盘是脊柱中相邻椎骨之间的软组织结构,由纤维环和髓核组成,对脊柱的正常生理活动具有重要作用^[1]。髓核细胞的衰老是随着年龄增长逐渐发生的生理变化,表现为髓核的变性、脱水和萎缩,导致椎间盘的厚度和弹性降低^[2-3]。这种变化不仅影响脊柱的稳定性和运动功能,还可能导致一系列椎间盘相关疾病的发生。髓核细胞的衰老过程涉及细胞凋亡、细胞分化和细胞代谢等多个方面,是一系列复杂的生物学变化。近年来的研究表明,一些细胞外基质蛋白和相关分子在这一过程中发挥重要作用,其中fibulin-3和组织金属蛋白酶抑制因子-3(tissue inhibitor of metalloproteinase 3, TIMP-3)尤为值得关注^[4-5]。Fibulin-3是一种存在于细胞外基质中的结构蛋白,在细胞黏附、细胞外基质形成和细胞信号传导中发挥关键作用^[6]。研究发现, fibulin-3在椎间盘细胞衰老过程中通过抑制细胞凋亡和细胞老化来维持椎间盘的正常生理功能^[7-8]。此外, fibulin-3的表达水平与椎间盘退变程度呈负相关,提示其在椎间盘健康中具有重要作用^[4]。TIMP-3是一种组织抑制金属蛋白酶,在细胞外基质中的作用类似于调节因子,能够影响细胞外基质的合成、降解和修复^[9]。研究显示, TIMP-3在椎间盘细胞衰老过程中起到关键调节作用,通过抑制细胞凋亡和促进细胞代谢,延缓椎间盘的衰老^[10-12]。尽管已有研究表明fibulin-3和TIMP-3在椎间盘细胞衰老中扮演重要角色,但其具体分

子机制仍不完全清楚。本研究旨在探讨fibulin-3调控TIMP-3在人椎间盘髓核细胞(nucleus pulposus cells, NPCs)衰老中的作用机制。这不仅有助于进一步理解椎间盘衰老的分子机制,也为预防和治疗椎间盘相关疾病提供新的思路和方法。

1 材料与方法

1.1 人退变椎间盘组织的获取和NPCs的原代培养和扩增

本研究经河南省洛阳正骨医院(河南省骨科医院)伦理委员会批准。依托河南省洛阳正骨医院脊柱研究中心平台,获取手术后退变椎间盘组织标本。获取的标本来自于接受椎间盘手术的患者,共计37例,分别采集其椎间盘髓核组织。37例人髓核组织样本按照Pfirrmann分级 I 级5例, II 级8例, III 级8例, IV 级8例, V 级8例。因而分为轻度退变组(I 级)、中度退变(II - III 级)组和重度退变(IV - V 级)组。每个患者的髓核组织单独处理,未进行混合处理。在无菌条件下,使用解剖显微镜从椎间盘标本中分离出髓核组织。

通过酶消化和贴壁法分离和纯化出髓核细胞,并建立连续传代的体外培养体系。具体如下:将分离出的髓核组织用0.2%的 II 型胶原酶在37 ℃静置消化40 min,然后以1 000 r/min离心5 min,去除上清液。使用含糖的DMEM/F12培养液吹匀细胞,再次离心,重复此步骤

3次。用细胞计数板对离心后的细胞进行计数,并按 $1 \times 10^6 \text{ mL}^{-1}$ 的细胞浓度接种于底面积为 25 cm^2 的培养瓶中。每个培养瓶中加入5 mL含有100 U/mL青霉素、100 U/mL链霉素及20%胎牛血清的DMEM/F12培养液。在 37°C 、体积分数5% CO_2 的培养箱中培养细胞,每3 d更换1次培养液。当细胞融合率达到90%时,用0.25%的胰酶消化细胞,并按1:2的比例进行传代培养。每次传代的细胞密度和生长状态通过显微镜观察和计数板进行评估。NPCs通常可以传代5~6次,具体传代次数视细胞的生长状态和实验需求而定。在每次传代过程中,记录细胞的形态变化、增殖能力和凋亡情况,以确保实验结果的可靠性。

1.2 细胞实验分组

本实验中将细胞分为3组,分别为对照组、过表达fibulin-3组和TIMP-3基因敲低组(LV-shTIMP-3)。对照组:细胞不进行任何特定基因干预处理,仅使用普通培养基进行培养。过表达fibulin-3组:在细胞中添加外源性fibulin-3蛋白以促进fibulin-3的过表达。TIMP-3基因敲低组(LV-shTIMP-3):通过慢病毒介导的RNA干扰技术(shRNA)敲低TIMP-3基因的表达。

用完全培养基制备密度为 $5 \times 10^5 \text{ mL}^{-1}$ 的细胞悬液,接种到6孔板中, 37°C 培养12 h,细胞汇合度为50%~60%。每孔加入1 μL 质量浓度为100 $\mu\text{g/mL}$ 的fibulin-3(MCE, HY-P77921)重组蛋白溶液,处理72 h后,开展下一步实验。

用完全培养基制备密度为 $5 \times 10^4 \text{ mL}^{-1}$ 的细胞悬液,接种到6孔板中, 37°C 培养12 h,细胞汇合度为40%~50%。每孔加入2 μL LV-shTIMP-3病毒(上海吉凯基因科技有限公司)及20 μL HiTransG A感染液(上海吉凯基因科技有限公司), 37°C 培养16 h,更换为完全培养基,继续培养。感染约72 h后,荧光显微镜(Olympus IX73,日本)观察感染效率。待感染效率到达80%~90%后,开展下一步实验。

在上述处理基础上,进一步将每组细胞分为两组:Vehicle组和Z-VAD-FMK组。Vehicle组加入等体积的溶剂作为对照,Z-VAD-FMK组每孔加入30 $\mu\text{mol/L}$ 的Z-VAD-FMK(MCE, HY-16658B),处理24 h后,开展下一步实验。

1.3 Real-time PCR检测相关基因的表达

通过Trizol reagent(TaKaRa, Japan)提取NPCs中总的RNA,使用RNase-free DNase(TaKaRa, Japan)处理提取的总RNA。使用核酸蛋白测定仪(Eppendorf, Germany)检测并计算RNA浓度。采用PrimeScriptTM RT Master Mix逆转录(RT)-PCR体系(TaKaRa, Japan)逆转录合成cDNA。采用SYBR Green Master Mix reagent(TaKaRa, Japan)进行PCR扩增,总反应容量为20 μL 。Real-time PCR检测

GAPDH、MMP1、MMP2、MMP3、MMP8、MMP9、MMP13、p16、p21、p53、TIMP-1、TIMP-3、TNF- α 、IL-1 β 、IL-8以及fibulin-3的表达。读取Ct值,通过 $2^{-\Delta\Delta\text{Ct}}$ 公式计算出相对表达量。

1.4 Western blot

通过Western blot检测中度退变组和重度退变组髓核组织中fibulin-3蛋白的表达水平。向细胞沉淀中加入含有蛋白酶抑制剂PMSF的RIPA裂解液(Beyotime),裂解细胞后,测定蛋白质浓度,并向样品中加入5 $\times$ SDS-PAGE Loading Buffer(Beyotime)。处理后取上清30 μL 电泳。SDS-PAGE电泳:分离胶8%,积层胶6%,胶厚1.5 mm。电泳:在积层胶中以80 V电压电泳,分离胶中以120 V电压电泳。湿式转移电泳,300 mA转移1.5 h。膜封闭后加于需要检测蛋白的相关抗体(fibulin-3、TIMP-3、p16、p21、p53), 4°C 振荡过夜。洗膜,再先后加入生物素化羊抗兔IgG和碱性磷酸酶标记的亲合素,用NBT/BCIP底物显色。利用ImageJ软件分析目标蛋白灰度值与内参蛋白灰度值的比值,计算相对表达量。

1.5 ELISA检测NPCs中的fibulin-3表达情况

每组细胞沉淀中加入含有蛋白酶抑制剂PMSF的RIPA裂解液(Beyotime),裂解细胞后,12 000 r/min离心10 min,吸取上清液待测。在酶标包被板上设标准品孔8孔,质量浓度分别为240、120、60、30、15、7.5、3.75、0 ng/L。分别设空白孔(空白对照孔不加样品及酶标试剂,其余各步操作相同)、待测样品孔。在酶标板待测样品孔中先加样品稀释液40 μL ,然后再加待测样品10 μL (样品最终稀释度为5倍)。用封板膜封板后置 37°C 温育30 min,小心揭掉封板膜,弃去液体,甩干,每孔加满洗涤液,静置30 s后弃去,重复5次。每孔加入酶标试剂50 μL ,用封板膜封板后置 37°C 温育30 min,小心揭掉封板膜,弃去液体,甩干,每孔加满洗涤液,静置30 s后弃去,重复5次。每孔先加入显色剂A 50 μL ,再加入显色剂B 50 μL ,轻轻震荡混匀, 37°C 避光显色15 min。每孔加终止液50 μL ,终止反应(此时蓝色立转黄色)。依序测量各孔的450 nm波长吸光度值(A值)。以标准物的质量浓度为横坐标,A值为纵坐标绘出标准曲线,用标准物的质量浓度与A值计算出标准曲线的直线回归方程式,将样品的A值代入方程式,计算出样品质量浓度,再乘以稀释倍数,即为样品的实际质量浓度。

1.6 SA- β -gal实验检测NPCs衰老

通过观察SA- β -gal染色的阳性表达率,检测人髓核细胞的衰老程度。将NPCs接种于12孔板上,培养24 h后,使用细胞衰老相关的 β -半乳糖苷酶(senescence-associated

β -galactosidase, SA- β -gal)染色试剂盒(Cell Signaling Technology, USA)进行染色,用体积分数4%多聚甲醛固定细胞15 min,用PBS洗涤固定的细胞3次,每次5 min。将SA- β -gal染色液加入12孔板,将12孔板用封口膜密封,于37 °C孵箱保存过夜。将染色液去除,用PBS洗涤细胞2次,衰老阳性细胞呈现蓝色。采用相差显微镜观察(Olympus IX73, 日本),每孔随机选取6个区域照相,计数阳性染色细胞数以及染色细胞总数,计算阳性染色细胞数占染色细胞总数的百分比。

1.7 CCK8检测NPCs复制增殖能力

在96孔板中接种NPCs悬液(1000细胞/孔),将培养板放在培养箱中预培养(体积分数5%CO₂、37 °C)过夜。将Cell Counting Kit (CCK-8)(DOJINDO LABORATORIES, Japan)与含有10%血清的DMEM/F12完全培养基按1:10预混,将96孔板中的原培养基吸出弃掉,每孔加入100 μ L含有CCK8的培养基,培养箱内孵育2 h,酶标仪测定波长450 nm的吸光度。

1.8 流式细胞术检测细胞凋亡率

细胞处理结束后,收集并用冷PBS洗涤两次,然后重悬于1 $\times$ Annexin V结合缓冲液中,细胞浓度为1 $\times$ 10⁶ mL⁻¹。加入5 μ L FITC标记的Annexin V和5 μ L碘化丙啶(PI),在黑暗中室温孵育15 min。并通过软件分析,计算各组的凋亡率。

1.9 NPCs的衰老相关分泌表型检测

收集NPCs的培养基上清,采用Merck-Millipore公司ELISA液相芯片技术,检测不同培养代数NPCs分泌促炎症因子、趋化因子和基质金属蛋白酶(MMP1、MMP2、MMP3、MMP8、MMP9、MMP13、ADAMTS4、ADAMTS5、TNF- α 、IL-1 β 、IL-8、IL-6、IL-17、MCP-1)的情况,确定其衰老相关分泌表型(senescence-associated secretory phenotype, SASP)。

1.10 TIMP-3蛋白交互作用预测

本研究从公共数据库中获取TIMP3的蛋白质相互作用数据。使用STRING数据库(Search Tool for the Retrieval of Interacting Genes/Proteins)来预测TIMP-3的蛋白质相互作用网络。在STRING数据库中,本研究设定了高置信度的阈值(通常为0.7或更高),以确保所预测的蛋白质相互作用具有较高的可靠性。生成的TIMP-3蛋白交互图显示了TIMP-3与多种蛋白质之间的相互作用,包括基质金属蛋白酶(MMPs)和其他细胞外基质相关蛋白。

1.11 统计学方法

对于描述性数据,所有数据均以 $\bar{x} \pm s$ 形式表示。采

用Shapiro-Wilk检验对数据的正态性进行检验。当数据不符合正态分布时,使用非参数统计方法进行分析。两组间比较:对于符合正态分布且方差齐性的两组数据,采用独立样本 t 检验。对于不符合正态分布或方差不齐的两组数据,采用Mann-Whitney U 检验。对于符合正态分布且方差齐性的多组数据,采用单因素方差分析(ANOVA),并使用LSD或Tukey's HSD进行事后检验。对于不符合正态分布或方差不齐的多组数据,采用Kruskal-Wallis H 检验,并使用Dunn's检验进行事后分析。对于符合正态分布的变量,采用Pearson相关系数分析变量间的相关性。对于不符合正态分布的变量,采用Spearman秩相关系数分析。 $\alpha=0.05$ 。

2 结果

2.1 fibulin-3与患者髓核细胞衰老和椎间盘退变之间的关系

通过Western blot检测了中度退变(Ⅱ-Ⅲ级)和重度退变(Ⅳ-Ⅴ级)髓核组织中fibulin-3蛋白的表达水平,可以看出,随着椎间盘退变程度的增加(从Ⅱ-Ⅲ到Ⅳ-Ⅴ),fibulin-3蛋白的表达显著降低(图1A)。使用ELISA检测患者血清中fibulin-3的表达水平(图1B)。结果表明,fibulin-3的表达水平在椎间盘退变的过程中逐渐降低,而且这种降低趋势与椎间盘退变的程度呈负相关(图1C)。随着髓核细胞的SA- β -gal阳性细胞百分比(衰老标志物)的增加,fibulin-3的表达逐渐降低。fibulin-3的减少可能与髓核细胞的衰老加剧密切相关(图1D);椎间盘退变程度(Pfirrmann分级)与髓核细胞的SA- β -gal阳性细胞百分比(衰老标志物)呈显著正相关,表明随着退变加重,髓核细胞衰老程度增加(图1E)。

2.2 NPCs体外培养体系证实fibulin-3抑制连续传代对髓核细胞衰老的影响

本研究检测了不同传代的髓核细胞中fibulin-3的表达水平,并观察了fibulin-3对髓核细胞衰老的影响。实验结果表明,随着髓核细胞的传代次数增加,fibulin-3的表达水平逐渐降低(图2A),第2代细胞(p2)中fibulin-3的表达水平最高,而第10、18代细胞(p10、p18)中表达水平显著降低,差异有统计学意义($P<0.01$)。细胞增殖能力随着细胞代数的增加而减弱,通过CCK8检测显示,第18代细胞的增殖能力比第2代细胞降低了约50%(图2B, $P<0.01$)。髓核细胞在连续传代过程中经历了明显的衰老和凋亡现象,SA- β -gal染色显示,第18代细胞的衰老阳性率高于第2代细胞(图2C, $P<0.01$),流式细胞术检测结果表明,第18代细胞的凋亡率明显升高(图2D, $P<0.01$)。

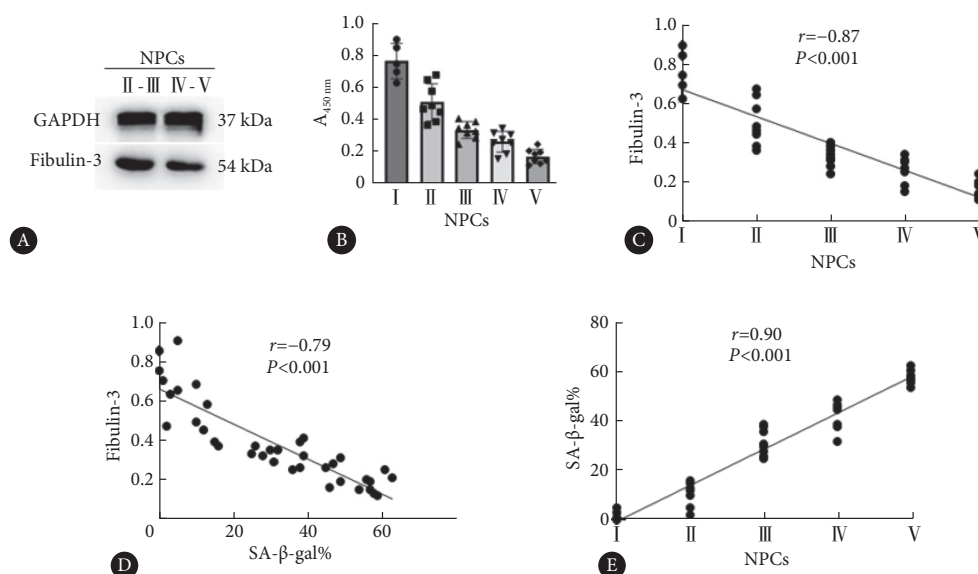


图 1 人髓核组织中fibulin-3与髓核细胞衰老和椎间盘退变有关

Fig 1 Fibulin-3 in human nucleus pulposus tissue is associated with nucleus pulposus cells (NPCs) senescence and intervertebral disc degeneration

A, Western blot analysis of fibulin-3 protein levels in moderately degenerated (II - III) and severely degenerated (IV - V) nucleus pulposus tissues. B, ELISA was performed to determine the fibulin-3 expression in NPCs from discs of different grades, measured at 450 nm absorbance (I : n=5, II : n=8, III : n=8, IV : n=8, V : n=8). C, Spearman correlation analysis between different grades and fibulin-3 expression, showing a negative correlation ($r=-0.87$, $P<0.001$). D, Spearman correlation analysis between different grades and NPCs from various grades, showing a negative correlation ($r=-0.79$, $P<0.001$). E, Spearman correlation analysis between the degree of senescence and NPCs from various grades, showing a positive correlation ($r=0.9$, $P<0.001$).

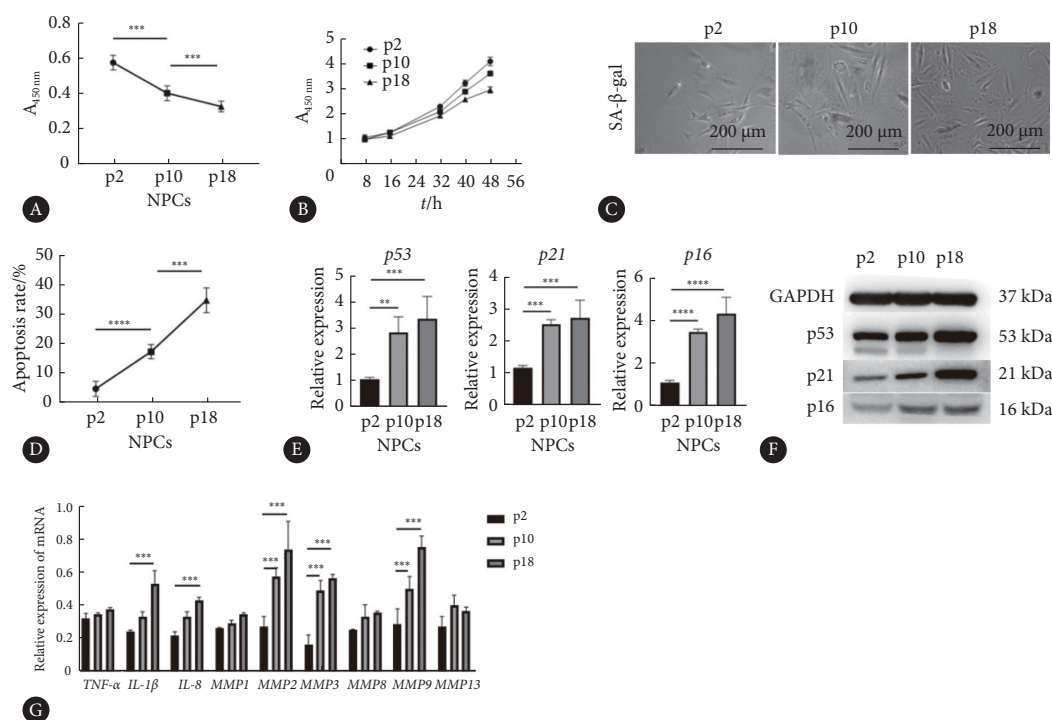


图 2 连续传代NPCs衰老程度与fibulin-3蛋白水平负相关

Fig 2 Negative correlation between the senescence level of serially passed nucleus pulposus cells (NPCs) and fibulin-3 protein level

A, ELISA detection of fibulin-3 expression in serially passed NPCs at 450 nm absorbance. B, CCK8 assay determination of proliferation in serially passed NPCs at 450 nm absorbance. C, SA-β-gal staining to assess the degree of senescence in NPCs, with representative images showing significant differences in senescence staining of serially passed NPCs. D, Statistical analysis of apoptosis rate in NPCs of different passages. E, Quantitative PCR analysis showing increased mRNA levels of senescence pathway proteins *p53*, *p21*, and *p16* in NPCs with increasing passages. F, Western blot analysis of *p53*, *p21*, and *p16* protein levels in NPCs showing an increase with successive passages. G, Quantitative PCR analysis showing increased expression of inflammatory cytokines and matrix metalloproteinases, indicative of the senescence-associated secretory phenotype (SASP), in NPCs with increasing passages. $n=3$. ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$.

此外,随着髓核细胞传代次数的增加(p2到p18),p53、p21、p16的基因和蛋白表达水平显著上调,表明衰老标志物的表达随细胞传代次数增加而升高(图2E、2F, $P < 0.01$)。此外,促炎症因子(如IL-1 β 和TNF- α)和基质蛋白酶(如MMP3和MMP13)的表达也随着传代次数增加而显著升高(图2G, $P < 0.01$),总之,与对照组相比, fibulin-3显著上调了凋亡相关因子p53、p21、p16,以及炎症因子IL-1 β 、IL-8的表达,同时也显著增加了基质金属蛋白酶MMP2、MMP3和MMP9的表达。这些结果表明, fibulin-3在促进细胞凋亡、增强炎症反应和加速细胞外基质降解中可能发挥了重要作用。

外源性fibulin-3提升了fibulin-3的表达水平,显著增强了细胞的增殖能力(图3A、3B),图3A展示了对照组和fibulin-3处理组在450nm处的吸光度测量结果,反映了细胞增殖或活力。结果显示, fibulin-3处理组的吸光度显著高于对照组,这表明fibulin-3处理显著增强了细胞的增殖能力。统计分析结果显示,处理组与对照组之间的差异有统计学意义($P < 0.0001$)。图3B展示了对照组和fibulin-3处理组中fibulin-3相对表达水平的测量结果。统计分析结果表明, fibulin-3处理组的表达水平高于对照组,差异

有统计学意义($P < 0.0001$)。实验组保持了较好的细胞形态和生长状态(图3C),显著抑制了髓核细胞的衰老(图3D、3E, $P < 0.01$),流式细胞术检测显示实验组细胞凋亡率显著降低($P < 0.01$),促炎症因子和MMPs的基因表达也降低,过表达fibulin-3显著降低了p53、p21和p16这三个关键衰老标记基因的表达水平,进一步证明了fibulin-3抑制了NPCs的衰老(图3F, $P < 0.01$)。这些结果表明,外源性fibulin-3能够有效抑制髓核细胞的衰老和凋亡,并降低促炎症因子和基质蛋白酶的表达。

2.3 fibulin-3通过减少凋亡和促进增殖抑制NPCs的衰老

本研究使用体外培养的人NPCs,评估了fibulin-3对细胞增殖、凋亡的影响。实验结果表明,在Vehicle组中,添加外源性fibulin-3能够显著降低细胞的凋亡并促进NPCs的增殖(图4A),CCK-8实验显示处理组的细胞增殖率高于对照组($P < 0.01$)(图4B)。此外,衰老标记物p53、p21和p16的表达也显著减少(图4C, 4D)。为了验证fibulin-3的作用机制,进一步引入凋亡抑制剂Z-VAD-FMK,发现Z-VAD-FMK组中, fibulin-3对细胞凋亡的抑制作用不再显著,细胞增殖与对照组无明显差异,且衰老标记物的表达未见明显变化(图4A ~ 4D)。这些结果表明,

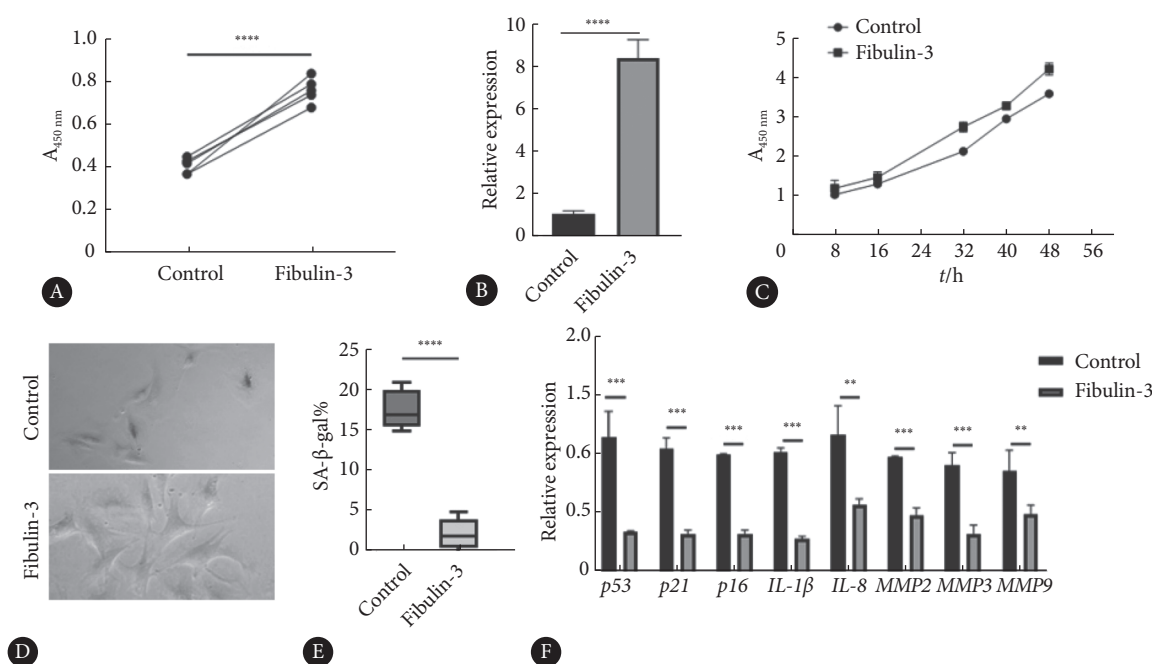


图3 fibulin-3通过抑制基质金属蛋白酶反应和促炎症因子分泌延缓连续传代NPCs的衰老

Fig 3 Fibulin-3 protein delays senescence of serially passaged nucleus pulposus cells (NPCs) by inhibiting matrix metalloproteinase activity and secreting pro-inflammatory cytokines

A, ELISA detection of fibulin-3 expression in the treatment group (fibulin-3) and control group (PBS) at 450 nm absorbance. B, Quantitative PCR validation of mRNA levels of fibulin-3 expression in the treatment and control groups. C, CCK8 assay detection of proliferation in NPCs from the treatment and control groups at 450 nm absorbance. D, SA- β -gal staining to assess the degree of senescence in NPCs, with representative images of senescence staining from the treatment and control groups ($\times 200$). E, Statistical analysis of senescence staining in NPCs from the treatment and control groups. F, Quantitative PCR analysis showing the secretion of inflammatory cytokines and matrix metalloproteinases in NPCs, indicating that the treatment group inhibited the senescence-associated secretory phenotype (SASP). $n=3$.

** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

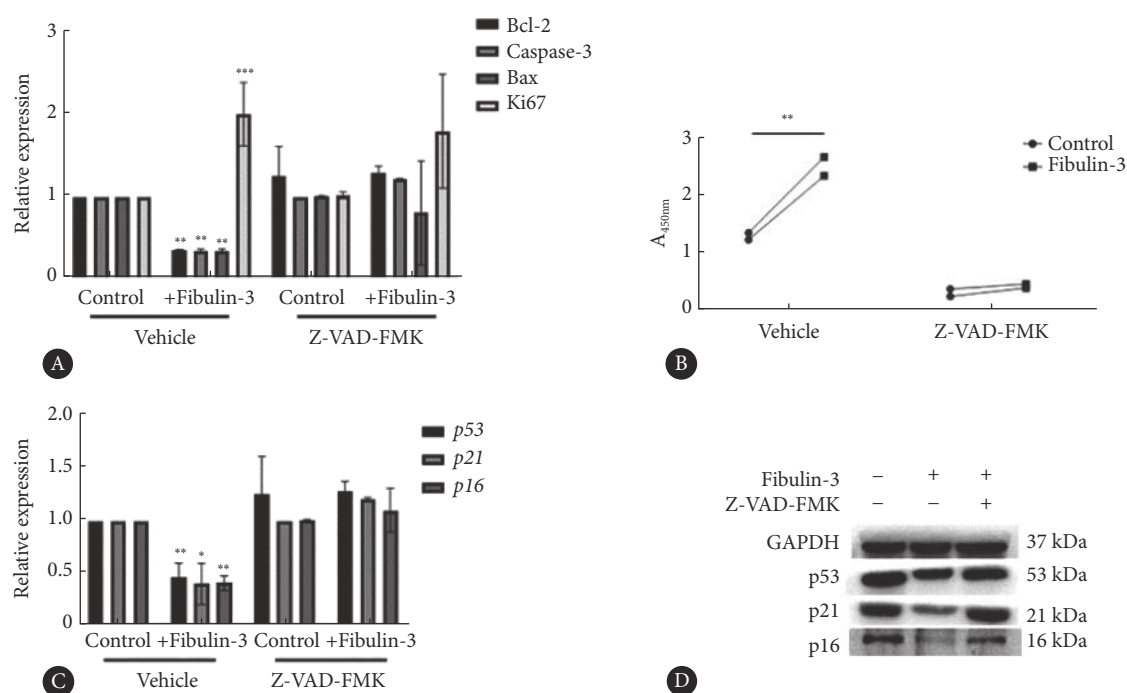


图4 fibulin-3通过抑制细胞凋亡和增强细胞活力延缓连续传代NPCs的衰老

Fig 4 Fibulin-3 protein delays senescence of serially passaged nucleus pulposus cells (NPCs) by inhibiting apoptosis and enhancing cell viability

A, Overexpression of the fibulin-3 gene in NPCs reduces the expression of apoptotic genes and increases the expression of proliferation markers. This effect is confirmed by using the apoptosis inhibitor Z-VAD-FMK, which abolishes the reduction in apoptosis caused by fibulin-3 overexpression. ** $P < 0.01$, *** $P < 0.001$, vs. control of vehicle. B, The effect of fibulin-3 overexpression on enhancing cell viability is abolished by using the apoptosis inhibitor Z-VAD-FMK (** $P < 0.01$). C, Overexpression of fibulin-3 in nucleus pulposus cells reduces the expression of senescence genes. This effect is confirmed by using the apoptosis inhibitor Z-VAD-FMK, which abolishes the reduction in senescence gene expression caused by fibulin-3. * $P < 0.05$, ** $P < 0.01$, vs. control of vehicle. D, Western blot analysis of the expression levels of p53, p21, and p16 proteins in NPCs from different groups. $n = 3$.

fibulin-3通过减少凋亡和促进增殖抑制了NPCs的衰老,而Z-VAD-FMK则部分抑制了fibulin-3的作用。

2.4 fibulin-3通过增强TIMP-3介导MMP调控NPCs的衰老

生物信息学分析预测TIMP-3的相互作用蛋白表明, TIMP-3与多种蛋白质,包括MMPs及其他细胞外基质相关蛋白之间存在相互作用(图5A)。本研究通过Real-time PCR、Western blot和ELISA检测了人NPCs在不同处理条件下的TIMP-3表达水平和MMPs活性。结果显示,在来源于37例人髓核组织提取的髓核细胞样本中,处理组TIMP-3的表达水平高于对照组,统计分析显示 $P < 0.01$ (图5B)。处理组中的MMP3和MMP13的活性显著降低,表明TIMP-3有效抑制了MMPs的活性(图5B~5C)。此外,通过LV-shTIMP-3处理后, TIMP-3的表达水平显著降低,进一步导致p16、p21和p53的基因和蛋白表达水平显著上调(图5D、5E、5G)。这些结果表明, TIMP-3的下调促进了与细胞衰老相关的基因表达(图5F、5G)。与此同时,基质金属蛋白酶(MMPs)的活性显著增加,特别是MMP2、MMP3和MMP9的浓度在LV-shTIMP-3组中显著升高(图5H),这些结果支持了TIMP-3的抑制通过影响

MMPs的活性调节了细胞外基质的降解过程。这些结果进一步证实了TIMP-3通过调控MMPs的活性,影响NPCs的衰老和细胞外基质的代谢。

3 讨论

fibulin-3和TIMP-3在NPCs衰老过程中发挥着重要作用^[13-14]。尽管已有研究表明这些分子在细胞外基质调控和细胞凋亡抑制方面的功能,但其在椎间盘细胞衰老过程中的具体机制尚未完全阐明^[15]。因此,本研究旨在通过体外实验模型,探讨fibulin-3和TIMP-3在NPCs衰老中的作用,为进一步理解其在椎间盘退变中的功能提供依据。

为了探讨fibulin-3和TIMP-3在NPCs衰老中的作用,本研究采用了体外培养的人NPCs模型。通过连续传代培养NPCs,模拟细胞衰老过程,并使用Western blot和ELISA技术检测fibulin-3和TIMP-3的表达变化。同时,添加外源性fibulin-3和TIMP-3以观察其对NPCs增殖和凋亡的影响。实验设计旨在通过多方面的检测手段,全面评估fibulin-3和TIMP-3在NPCs衰老过程中的作用。

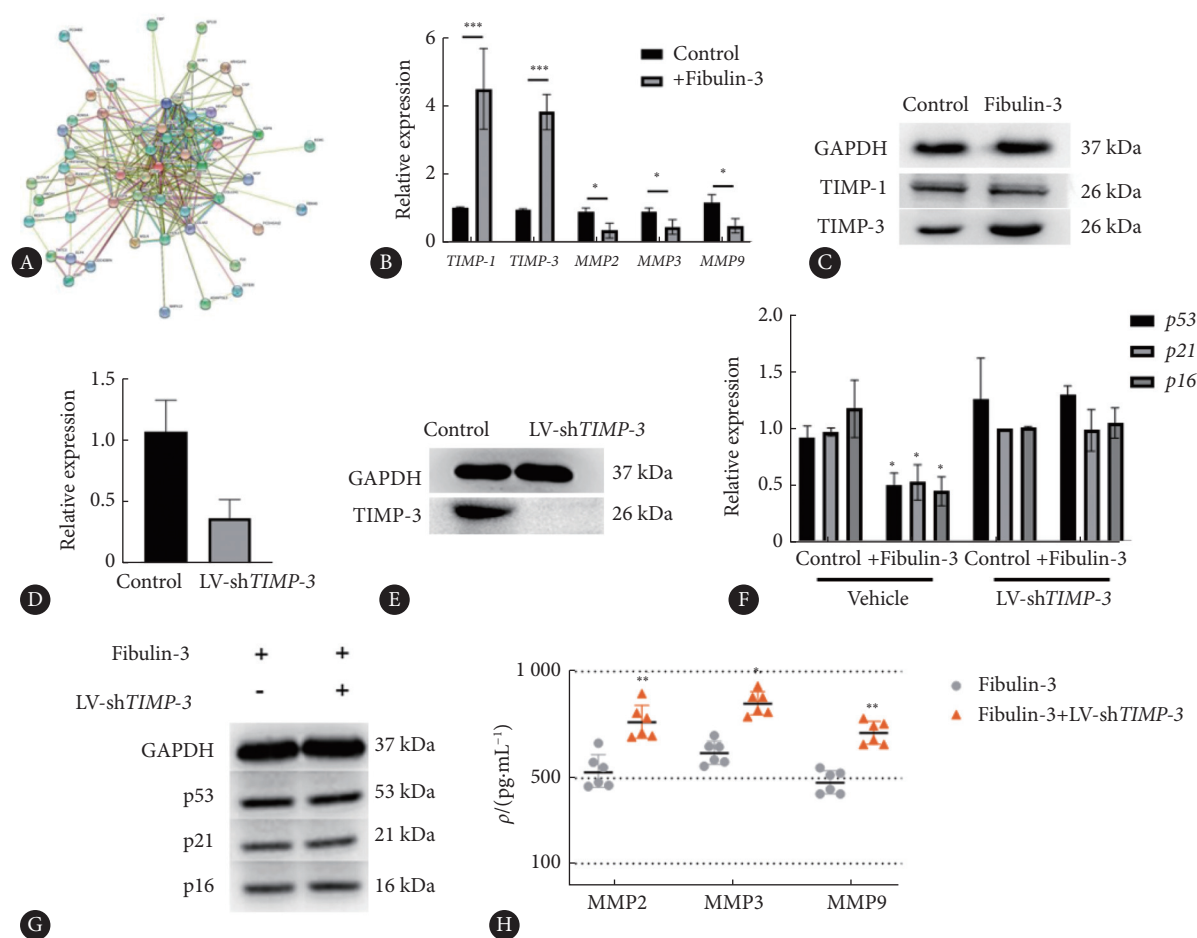


图 5 fibulin-3抑制基质金属蛋白酶反应延缓髓核细胞衰老与其能调控下游TIMP-3分子密切相关

Fig 5 Fibulin-3 protein inhibits matrix metalloproteinase activity to delay nucleus pulposus cell senescence, which is closely related to its regulation of downstream TIMP-3 molecule

A, Network diagram obtained from database searches showing proteins that interact with human fibulin-3, highlighting a close relationship with the tissue inhibitors of metalloproteinases (TIMPs) family. B, Quantitative PCR showing upregulation of TIMP-1 and TIMP-3 expression in NPCs overexpressing fibulin-3, with concurrent inhibition of matrix metalloproteinases (MMP2, MMP3, and MMP9). * $P < 0.05$, *** $P < 0.001$. C, Western blot analysis demonstrating increased protein levels of TIMP-3 in NPCs overexpressing fibulin-3, with no significant change in TIMP-1. D-E, Quantitative PCR validation of reduced TIMP-3 mRNA and protein levels in the TIMP-3 knockdown group (LV-shTIMP-3). F-G, Overexpression of the fibulin-3 gene in NPCs inhibits the expression of senescence genes, and this inhibitory effect is abolished by suppressing TIMP-3 expression. * $P < 0.05$, vs. control of vehicle. H, Multiplex cytokine detection showing the secretion levels of matrix metalloproteinases (pg/mL) in both groups. $n = 3$. * $P < 0.05$, ** $P < 0.01$, vs. fibulin-3.

实验结果显示,随着NPCs连续传代次数的增加, fibulin-3和TIMP-3的表达水平显著下降。进一步研究发现,添加外源性fibulin-3和TIMP-3可以显著抑制NPCs的衰老过程,这一效果主要通过减少细胞凋亡和促进细胞增殖来实现。这些发现表明, fibulin-3和TIMP-3在维持NPCs活力和功能方面具有重要作用,并通过不同的机制协同作用以延缓细胞衰老。

本研究结果支持了fibulin-3和TIMP-3在NPCs衰老中的关键作用^[16-17],并增进了对其具体机制的理解。然而,研究也存在一定局限性,例如体外模型是否完全反映体内环境,还有待进一步验证。此外, fibulin-3和TIMP-3在椎间盘退变中的具体信号通路及其相互作用机制仍需深

入研究。这些局限性提示未来的研究应结合体内实验和更复杂的细胞模型,以更全面地理解这些分子的作用。

这些发现提示, fibulin-3和TIMP-3可能成为椎间盘相关疾病的潜在治疗靶点^[18-19]。通过调节这两个分子的表达或功能,可能延缓或阻止椎间盘退变,从而减少腰痛的发生。未来研究可以尝试使用基因治疗技术增强 fibulin-3和TIMP-3的表达或功能,以促进髓核细胞的增殖并减少细胞凋亡,进而延缓椎间盘退变。然而,这些潜在的治疗策略仍需大量研究和验证。

综上所述, fibulin-3和TIMP-3在髓核细胞衰老和椎间盘相关疾病的发病机制中发挥着重要作用。它们通过抑制细胞凋亡和减轻MMPs反应,促进细胞外基质合成

和细胞增殖相关信号通路的激活,从而调节NPCs的衰老过程。未来研究应利用生物信息学技术,全面系统地研究fibulin-3和TIMP-3相关的信号通路,以揭示它们在椎间盘细胞衰老和相关疾病中的作用机制,为椎间盘相关疾病的治疗和预防提供科学依据和技术支持。

* * *

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Author Contribution WANG Xuke is responsible for conceptualization, data curation, methodology, project administration, resources, validation, writing--original draft, and writing--review and editing. ZHANG Yang is responsible for formal analysis, investigation, and methodology. LUO Gang is responsible for data curation, investigation, and methodology. KONG Junke is responsible for investigation and methodology. CAO Xiangyang is responsible for data curation, methodology, project administration, and supervision. WANG Qingfeng is responsible for conceptualization, data curation, formal analysis, funding acquisition, project administration, resources, supervision, and writing--review and editing. All authors consented to the submission of the article to the Journal. All authors approved the final version to be published and agreed to take responsibility for all aspects of the work.

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Declaration of Conflicting Interests All authors declare no competing interests.

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(2024-05-31收稿, 2024-07-15修回)

编辑 刘 华



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