

[DOI] 10.12016/j.issn.2096-1456.2022.11.002

• 基础研究 •

炎症环境下敲低 SHP2 对人牙周膜干细胞增殖和成骨分化的影响

张远, 赵庆, 吕浩东, 王天丛, 窦昭婧, 金玉琴, 季骏

南京大学医学院附属口腔医院·南京市口腔医院正畸科, 江苏 南京(210008)

【摘要】 目的 探讨炎症环境下含有 Src 同源结构域 2 的蛋白酪氨酸磷酸酶 2 (Src homology-2 domain containing protein tyrosine phosphatase-2, SHP2) 对人牙周膜干细胞 (human periodontal ligament stem cells, hPDLSCs) 增殖及成骨分化的调节作用和相关机制, 为牙周炎治疗提供新的靶点。方法 使用慢病毒感染构建敲低 SHP2 基因的 hPDLSCs 细胞系, 通过 RT-qPCR、Western blot 验证 SHP2 的敲低水平; 使用肿瘤坏死因子- α (tumor necrosis factor- α , TNF- α) 和白细胞介素-1 β (interleukin-1 β , IL-1 β) 在体外模拟炎症环境; CCK-8 检测正常及炎症条件下敲低 SHP2 对 hPDLSCs 增殖的影响; 碱性磷酸酶 (alkaline phosphatase, ALP) 染色、ALP 活性检测、ARS 染色、RT-qPCR 以及 Western blot 检测成骨指标; Western blot 检测敲低 SHP2 在炎症条件下, 对丝裂原活化蛋白激酶/核因子 κ B (mitogen-activated protein kinase/nuclear factor- κ B, MAPK/NF- κ B) 通路蛋白的影响。结果 慢病毒感染细胞 72 h 后在荧光显微镜下显示出明显的绿色荧光, 测得 SHP2 shRNA 重组慢病毒的滴度为 2.9×10^8 TU/mL; Western blot 及 RT-qPCR 检测发现, SHP2 敲低组的 SHP2 蛋白及基因的表达显著降低 ($P < 0.001$); 在正常条件下, 敲低 SHP2 在一定程度上抑制了 hPDLSCs 的增殖; 敲低 SHP2 后, hPDLSCs 的早期成骨指标增高, 包括 ALP 活性显著增高, ALP 和 COL-1 的表达增加 ($P < 0.05$), 然而敲低 SHP2 对 hPDLSCs 最终产生钙结节的量无明显影响。在 TNF- α 和 IL-1 β 诱导的炎症环境下, 敲低 SHP2 对 hPDLSCs 的增殖无明显影响 ($P > 0.05$), 敲低 SHP2 后, hPDLSCs 成骨相关指标均增加 ($P < 0.05$), 钙结节形成的量增多 ($P < 0.05$); 炎症环境下, 敲低 SHP2 后, hPDLSCs 中 p65 的磷酸化和 I κ B- α 的降解降低, 并且 NF- κ B 上游 MAPK 通路蛋白 p-p38 和 p-JNK 的表达降低 ($P < 0.05$)。结论 炎症环境下, 敲低 SHP2 对 hPDLSCs 的增殖无明显影响, 但可通过抑制 MAPK/NF- κ B 通路促进 hPDLSCs 成骨分化。

【关键词】 牙周膜干细胞; 增殖; 牙周炎; 肿瘤坏死因子- α ; 白细胞介素-1 β ; 炎症环境; Src 同源结构域 2 的蛋白质酪氨酸磷酸酶 2; 基因沉默; 成骨分化; 碱性磷酸酶; COL-1; MAPK/NF- κ B 信号通路



微信公众号

【中图分类号】 R78 **【文献标志码】** A **【文章编号】** 2096-1456(2022)11-0769-10

【引用著录格式】 张远, 赵庆, 吕浩东, 等. 炎症环境下敲低 SHP2 对人牙周膜干细胞增殖和成骨分化的影响[J]. 口腔疾病防治, 2022, 30(11): 769-778. doi:10.12016/j.issn.2096-1456.2022.11.002.

Effect of SHP2 knockdown on the proliferation and osteogenic differentiation of human periodontal ligament stem cells under inflammatory environment ZHANG Yuan, ZHAO Qing, LV Haodong, WANG Tiancong, DOU Zhaojing, JIN Yuqin, JI Jun.

Department of Orthodontics, Nanjing Stomatological Hospital, Medical School of Nanjing University, Nanjing 210008, China

Corresponding authors: JI Jun, Email: drjijun@126.com, Tel: 86-25-83620283; JIN Yuqin, Email: yuqinjinf@163.com, Tel: 86-25-83620283

【收稿日期】 2022-04-23; **【修回日期】** 2022-05-27

【基金项目】 国家自然科学基金项目 (81700939); 江苏省自然科学基金项目 (BK20200150); 江苏省医学青年人才基金项目 (QN-RC2016116); 南京市医学科技发展基金资助项目 (YKK16163, YKK16160); 江苏省六大人才高峰项目 (YY-100)

【作者简介】 张远, 医师, 硕士, Email: 2118647727@qq.com

【通信作者】 季骏, 主任医师, 博士, Email: drjijun@126.com, Tel: 86-25-83620283; 金玉琴, 主治医师, 博士, Email: yuqinjinf@163.com, Tel: 86-25-83620283

【Abstract】 Objective The purpose of this study was to clarify the regulatory effect and mechanism of Src homology-2 domain containing protein tyrosine phosphatase-2 (SHP2) on human periodontal ligament stem cell (hPDLSC) proliferation and osteogenic differentiation under inflammatory environment and to provide a new target for the treatment of periodontitis. **Methods** SHP2 was knocked down in hPDLSCs, and the transfection efficiency of SHP2 was detected by RT-qPCR and Western blot. An *in vitro* inflammatory environment was created using tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β). The effect of SHP2 knockdown on hPDLSC viability under normal and inflammatory conditions was detected by CCK-8, and the osteogenic capacity of hPDLSCs under normal and inflammatory conditions was detected by ALP staining, ALP activity, ARS staining, RT-qPCR and Western blot. The mechanism by which SHP2 knockdown affected the MAPK pathway and its downstream NF- κ B pathway under inflammatory conditions was assessed by Western blot. **Results** Green fluorescence was observed after transfection for 72 h, and the titer of SHP2 shRNA recombinant lentivirus was 2.9×10^8 TU/mL. SHP2 expression was significantly downregulated in lentivirus-transfected cells, as demonstrated by Western blot and RT-qPCR ($P < 0.001$). SHP2 knockdown inhibited hPDLSC proliferation to a certain extent and increased the expression of early osteogenic markers under normal conditions, including increased ALP activity and increased ALP and COL-1 expression ($P < 0.05$). However, SHP2 knockdown exerted no effect on mineralized nodule formation. In the TNF- α - and IL-1 β -induced inflammatory environment, SHP2 knockdown exerted no effect on hPDLSC proliferation ($P > 0.05$). Osteogenic markers were upregulated ($P < 0.05$), and mineralized nodules were significantly increased ($P < 0.05$) after SHP2 knockdown. Western blot analysis showed that p65 phosphorylation and I κ B- α degradation were reduced in SHP2-knockdown hPDLSCs in the inflammatory environment. Moreover, SHP2 knockdown significantly inhibited the expression of p-p38 and p-JNK MAPK, which represent pathways upstream of the NF- κ B pathway ($P < 0.05$). **Conclusion** SHP2 knockdown did not affect cell viability but promoted the osteogenic potential of hPDLSCs by inhibiting the MAPK/NF- κ B-mediated signaling pathway under inflammatory environment.

【Key words】 periodontal ligament stem cell; proliferation; periodontitis; tumor necrosis factor- α ; interleukin-1 β ; inflammatory environment; Src homology-2 domain containing protein tyrosine phosphatase-2(SHP2); gene silencing; osteogenesis; alkaline phosphatase; COL-1; MAPK/NF- κ B signaling pathway

J Prev Treat Stomatol Dis, 2022, 30(11): 769-778.

【Competing interests】 The authors declare no competing interests.

This study was supported by National Natural Science Foundation of China (No.81700939); Natural Science Foundation of Jiangsu Province (No. BK20200150); Jiangsu Provincial Medical Youth Talent (No. QNRC2016116); the Medical Science and Technology Development Foundation, Nanjing Department of Health (No. YKK16163, No. YKK16160); Six Talent Peaks Project in Jiangsu Province (No. YY-100).

牙周炎是细菌感染引起的慢性炎症性疾病,可激活宿主的免疫反应,破坏牙齿的支持组织,最终导致牙齿脱落^[1]。牙周炎的现有治疗方法旨在去除菌斑和控制局部炎症,然而恢复已经丧失的牙周组织依旧是一个挑战。基于干细胞的组织工程策略为牙周再生带来新的希望,人牙周膜干细胞(human periodontal ligament stem cells, hPDLSCs)是形成牙槽骨的理想细胞来源,但局部炎症微环境抑制了hPDLSCs成骨分化能力,因此明确hPDLSCs的成骨分化机制,尤其是在炎症环境下的成骨分化,对牙周炎治疗至关重要。含有Src同源结构域2的蛋白酪氨酸磷酸酶2(Src homology-2 domain containing protein tyrosine phosphatase-2, SHP2)是一种由非受体型酪氨酸蛋白磷酸酶11(tyrosine-

protein phosphatase non-receptor type 11, PTPN11)基因编码,并广泛表达的非受体蛋白酪氨酸磷酸酶(protein tyrosine phosphatase, PTP)。越来越多的证据表明,SHP2在骨骼发育和形成中起着至关重要的作用,同时SHP2与骨髓间充质干细胞(bone mesenchymal stem cells, BMSCs)的体外成骨和体内骨形成密切相关^[2-3]。但目前SHP2对干细胞的骨形成作用尚有争议,SHP2在不同环境以及不同的细胞可能对成骨有着不同作用^[4-5]。SHP2与炎症反应同样关系密切。SHP2与干扰素- β 的产生^[6]以及促炎细胞因子和趋化因子(包括IL-1 β)的产生密切相关^[7]。SHP2也被证实可能在不同的炎症通路中表现出正或负调节功能。由于SHP2同时参与成骨和炎症过程,笔者猜测SHP2可能参与调节

hPDLSCs的成骨分化。SHP2目前在炎症条件下是否调节hPDLSCs的成骨分化以及通过何种方式调节,目前尚无报道。本研究通过慢病毒感染敲低hPDLSCs中的SHP2基因,旨在探讨敲低SHP2对炎症环境下hPDLSCs增殖和成骨分化的影响,并研究相关机制。

1 材料和方法

1.1 主要材料及仪器

hPDLSCs (Sciencell, 美国); SHP2 shRNA 慢病毒表达载体 [pSLenti-U6-shRNA (PTPN11)-CMV-EGFP-F2A-Puro-WPRE, 和元生物, 中国]; 胎牛血清 (Gibco, 美国); 青链霉素 (Hyclone, 美国); 高糖DMEM培养基 (Gibco, 美国); PBS (Gibco, 美国); 甲醇 (CNW, 德国); 蛋白酶抑制剂 (碧云天, 中国); RIPA裂解液 (碧云天, 中国); 转膜缓冲液 (苏州新赛美生物科技有限公司, 中国); 电泳缓冲液 (南京金斯瑞生物科技有限公司, 中国); TBS缓冲液 (上海生物工程有限公司, 中国); 蛋白酶抑制剂 (碧云天, 中国); CCK-8试剂盒 (Dojindo, 日本); 碱性磷酸酶染色试剂盒 (碧云天, 中国); 茜素红S (Sigma, 美国); 氯化十六烷基吡啶 (Sigma, 美国); COL-1抗体 (1:1 000, Abcam, 美国); RUNX2抗体 (1:1 000, Abcam, 美国); ALP抗体 (1:1 000, proteintech, 美国); GAPDH抗体 (1:5 000, Proteintech, 美国); 核因子 κ B (nuclear factor kappa-B, NF- κ B)抗体 (p-p65、p65和I κ B α , 1:1 000, Affinity Biosciences, 美国); MAPK抗体 (p-ERK、ERK、p-p38、p-38、p-JNK和JNK, 1:1 000, Cell Signaling Technology, 美国); 兔源及鼠源二抗 (1:5 000, Proteintech, 英国); ECL试剂盒 (苏州新赛美生物科技有限公司, 中国); 总RNA提取试剂盒 (诺唯赞生物, 中国); HiScript III RT SuperMix for qPCR (+gDNA wiper) (诺唯赞生物, 中国); ChamQ Universal SYBR qPCR Master Mix (诺唯赞生物, 中国); 多聚甲醛 (博士德生物, 中国); 地塞米松 (Sigma, 美国); β -甘油磷酸钠 (Sigma, 美国); 抗败血酸 (Sigma, 美国)。

1.2 实验方法

1.2.1 细胞培养 按照低糖DMEM培养基+10%胎牛血清+1%双抗 (青霉素/链霉素) 配置完全培养基用于PDLSCs的培养。成骨诱导液为完全培养基额外添加成骨诱导成分 (50 μ M抗坏血酸, 10 mM β -磷酸甘油和100 nM地塞米松)。根据预实验及既往研究^[8-9], 使用10 ng/mL肿瘤坏死因子- α (tumor necrosis factor- α , TNF- α) 和10 ng/mL白细胞介素-

1 β (interleukin-1 β , IL-1 β) 刺激hPDLSCs创建炎症微环境。

1.2.2 SHP2 shRNA 慢病毒载体的构建 SHP2 shRNA慢病毒载体由和元生物公司构建, 根据Human PTPN11基因的转录本设计siRNA靶点, 安排引物合成。将单链的引物退火成双链oligo序列, 连接入双酶切线性化的RNA干扰载体, 替换原有ccdB毒性基因。菌落PCR筛选转化子, 筛选的阳性克隆进行测序验证。测序验证正确的克隆, 进行高纯度质粒抽提。

1.2.3 重组质粒的包装和滴度测定 将构建的SHP2 shRNA慢病毒载体和包装质粒共转染进入肾上皮细胞系HEK 293T细胞, 换液、培养后, 收集细胞上清液, 进一步离心获得病毒浓缩液。使用有限稀释法稀释病毒浓缩液, 用不同浓度梯度感染HEK 293T细胞, 荧光显微镜下观察各孔荧光细胞数量, 结合稀释倍数计算病毒滴度。

1.2.4 慢病毒转染 以 5×10^4 个/mL的密度将hPDLSCs接种于6孔培养板中, 待细胞融合度达到60%~80%之间时加入慢病毒感染 (MOI=20)。培养箱中培养4 h后观察细胞状态, 将无血清培养基更换为完全培养基, 继续培养。细胞培养72 h后在荧光显微镜下观察转染效率。加入嘌呤霉素培养筛选, 经增殖传代后, 通过RT-qPCR、Western blot验证敲低效率, 用于后续实验。SHP2低表达慢病毒 (shSHP2) 的靶序列为5'-ACACTGGTGATTAC-TATGA-3'。敲低SHP2的hPDLSCs组用shSHP2表示, 空载病毒组用shSHP2-NC表示。

1.2.5 CCK-8检测细胞增殖 在96孔板中加入100 μ L不同处理后的细胞悬液 (5×10^4 个/孔), 置于培养箱 (37 $^{\circ}$ C, 5% CO₂) 中孵育24 h, 使细胞贴壁。在培养箱中孵育适当的时间 (1、3、5、7 d) 后每孔加入10 μ L CCK-8溶液, 孵育1 h后酶标仪测量450 nm处的吸光度并绘制生长曲线。

1.2.6 碱性磷酸酶 (alkaline phosphatase, ALP) 染色及ALP活性检测 hPDLSCs成骨诱导3、7 d后, 去除孔板内的培养基, 用PBS洗涤细胞样品5次, 每次5 min。加入适量染色工作液, 将样品置于室温下, 避光孵育30 min。去除染色工作液, 蒸馏水洗涤2次, 终止显色反应。

hPDLSCs成骨诱导3、7 d后, 去除孔板内的培养基, 用PBS洗涤细胞样品3次, 每次5 min。使用细胞裂解液裂解细胞。每毫克蛋白样品每分钟生产的对硝基苯酚毫摩尔数作为ALP活性进行统计分析。

1.2.7 茜素红染色(alizarin red staining, ARS)及钙离子浓度分析 hPDLSCs成骨培养21 d后,用4%多聚甲醛固定15 min并用茜素红S染色液在室温下染色30 min,显微镜下观察并拍照。每孔加入1 mL 10%氯化十六烷基吡啶,在室温下充分溶解。使用酶标仪测量562 nm处的吸光度作为钙结节的半定量分析结果。

1.2.8 RT-qPCR 使用Vazyme RNA extraction kit提取总RNA,用HiScript III RT SuperMix for qPCR (+gDNA wiper)反转录试剂盒对RNA进行逆转录,获得cDNA,以cDNA为模板,用实时荧光定量PCR仪扩增。引物序列见表1。采用 $2^{-\Delta\Delta C_t}$ 相对定量法分析目标基因的相对表达水平。

表1 PCR 引物序列

Table 1 Sequences of PCR primers

Gene (human)	Primer sequence
IL-6	Forward 5'-GGTGTTCCTGCTGCCTTCC-3'
	Reverse 5'-GTTCTGAAGAGGTGAGTGGCTGTC-3'
IL-1 β	Forward 5'-TGATGGCTTATTACAGTGGC-3'
	Reverse 5'-TGTAAGTGGTGGTGGAGATT-3'
IL-8	Forward 5'-ACTCCAAACCTTTCCACC-3'
	Reverse 5'-CTTCTCCACAACCTCTG-3'
TNF- α	Forward 5'-CGTGGAGCTGGCCGAGGAG-3'
	Reverse 5'-AGGAAGGAGAAGAGGCTGAGGAAC-3'
SHP2	Forward 5'-AGTTACATTGCCACTCAAGGCTGC-3'
	Reverse 5'-TTGACGTTCTTAACACGCATGACC-3'
ALP	Forward 5'-GACCCTTGACCCCAACAAT-3'
	Reverse 5'-GCTCGTACTGCATGTCCCCT-3'
COL-1	Forward 5'-AGAACAGCGTGGCCT-3'
	Reverse 5'-TCCGGTGTGACTCGT-3'
RUNX-2	Forward 5'-GGAGTGGACGAGGCAAGAGTTT-3'
	Reverse 5'-AGCTTCTGTCTGTGCCTTCTGG-3'
OCN	Forward 5'-GGCAGCGAGGTAGTGAAGAG-3'
	Reverse 5'-GATGTGCTCAGCCAACTCGT-3'
GAPDH	Forward 5'-CGTCTCTCTCCTCCTCTGTT-3'
	Reverse 5'-CCATGGTGTCTGAGCGATGT-3'

IL-6: interleukin-6; IL-1 β : interleukin-1 β ; IL-8: interleukin-8; TNF- α : tumor necrosis factor- α ; SHP2: Src homology-2 domain containing protein tyrosine phosphatase-2; ALP: alkaline phosphatase; COL-1: type-I collagen; RUNX-2: runt-related transcription factor 2; OCN: osteocalcin; GAPDH: glyceraldehyde-3-phosphate dehydrogenase

1.2.9 蛋白提取以及 Western blot 实验 提取总蛋白并按BCA蛋白浓度测定试剂盒说明书测定蛋白浓度。根据BCA所测得蛋白浓度,上样不同体积的蛋白。蛋白分离后转移至PVDF膜,并置入封闭液内,室温下摇床封闭10 min。随后4℃过夜孵育一抗。TBST摇床洗涤3次,10 min/次。按照一抗

的源性选择不同的兔源或鼠源二抗,室温孵育1 h,通过化学成像发光仪曝光。用Image J软件进行灰度值分析。

1.3 统计学分析

采用GraphPad Prism 9.0软件进行统计学分析,采用Shapiro-Wilk法进行正态性检验,数据用均数 $\pm$ 标准差表示,两样本比较采用 t 检验。 $P < 0.05$ 为差异有统计学意义。

2 结果

2.1 SHP2 shRNA 慢病毒载体的测序结果

经过比对,重组克隆中插入的片段序列与选用的oligo序列完全一致(图1),表明SHP2 shRNA慢病毒载体构建成功。

2.2 慢病毒原液滴度测定结果

慢病毒感染HEK 293T细胞72 h后通过倒置荧光显微镜观察到绿色荧光(图2),测得SHP2 shRNA重组慢病毒的滴度为 2.9×10^8 TU/mL,表明转染成功。

2.3 建立体外炎症模型

使用10 ng/mL TNF- α 、IL-1 β 刺激hPDLSCs 24 h后,通过RT-qPCR检测炎症因子IL-6、IL-1 β 、IL-8和TNF- α 基因转录水平表达。如图3a所示,TNF- α 和IL-1 β 刺激hPDLSCs的炎症组IL-6($t = 14.84, P < 0.001$)、IL-1 β ($t = 130, P < 0.001$)、IL-8($t = 26.751, P < 0.001$)和TNF- α ($t = 6.775, P = 0.003$)基因表达水平均明显提高。诱导7 d后,RT-qPCR显示炎症刺激组成骨相关基因COL-1($t = 68.42, P < 0.001$)、RUNX2($t = 5.359, P = 0.006$)的表达降低(图3b)。成骨诱导7 d后,炎症组hPDLSCs的ALP染色浅于对照组,ALP活性也更低($t = 22.65, P = 0.002$)(图3c、3d)。成骨诱导21 d后,ARS染色同样显示相似结果,即炎症状态下hPDLSCs的钙结节矿化形成远少于对照组($t = 23.42, P < 0.001$)(图3e)。上述结果均证明炎症微环境会损伤hPDLSCs成骨分化潜力。

2.4 SHP2 敲低效率的检测

SHP2敲低组和空载病毒组细胞在荧光显微镜下均显示出明显的绿色荧光(图3f),提示病毒转染成功。通过Western blot及RT-qPCR检测发现,SHP2敲低组的SHP2蛋白及基因的表达显著降低($t = 9.739, P < 0.001; t = 8.474, P < 0.001$),并且与空载病毒组相比,SHP2表达量降低达70%以上(图3g、3h)。

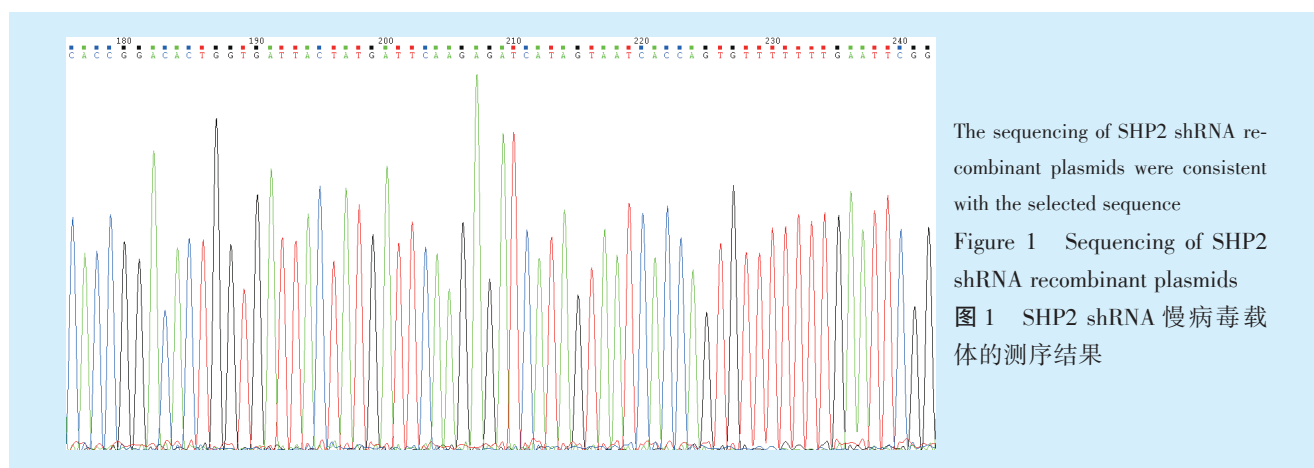


Figure 1 Sequencing of SHP2 shRNA recombinant plasmids

图1 SHP2 shRNA慢病毒载体的测序结果

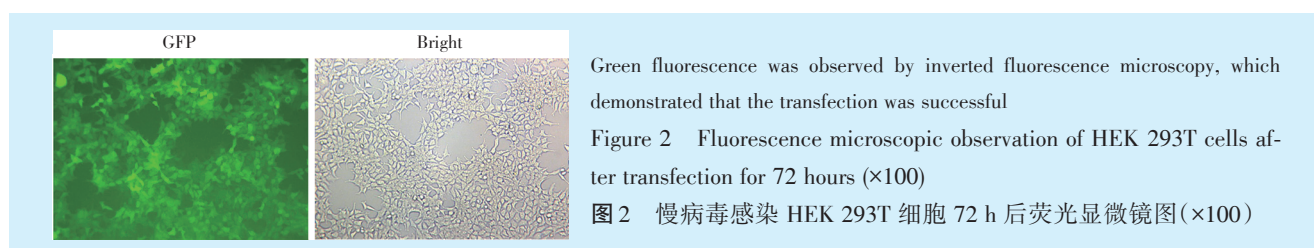


Figure 2 Fluorescence microscopic observation of HEK 293T cells after transfection for 72 hours ($\times 100$)

图2 慢病毒感染 HEK 293T 细胞 72 h 后荧光显微镜图($\times 100$)

2.5 敲低SHP2对hPDLSCs增殖活力的影响

在培养1 d后细胞增殖暂无明显改变,而3、5、7 d后,SHP2敲低组细胞活力低于对照组($P < 0.05$,图3i)。提示SHP2对hPDLSCs的增殖有一定的潜在作用。炎症条件下,SHP2敲低对hPDLSCs的增殖活力并无明显影响($P > 0.05$)。

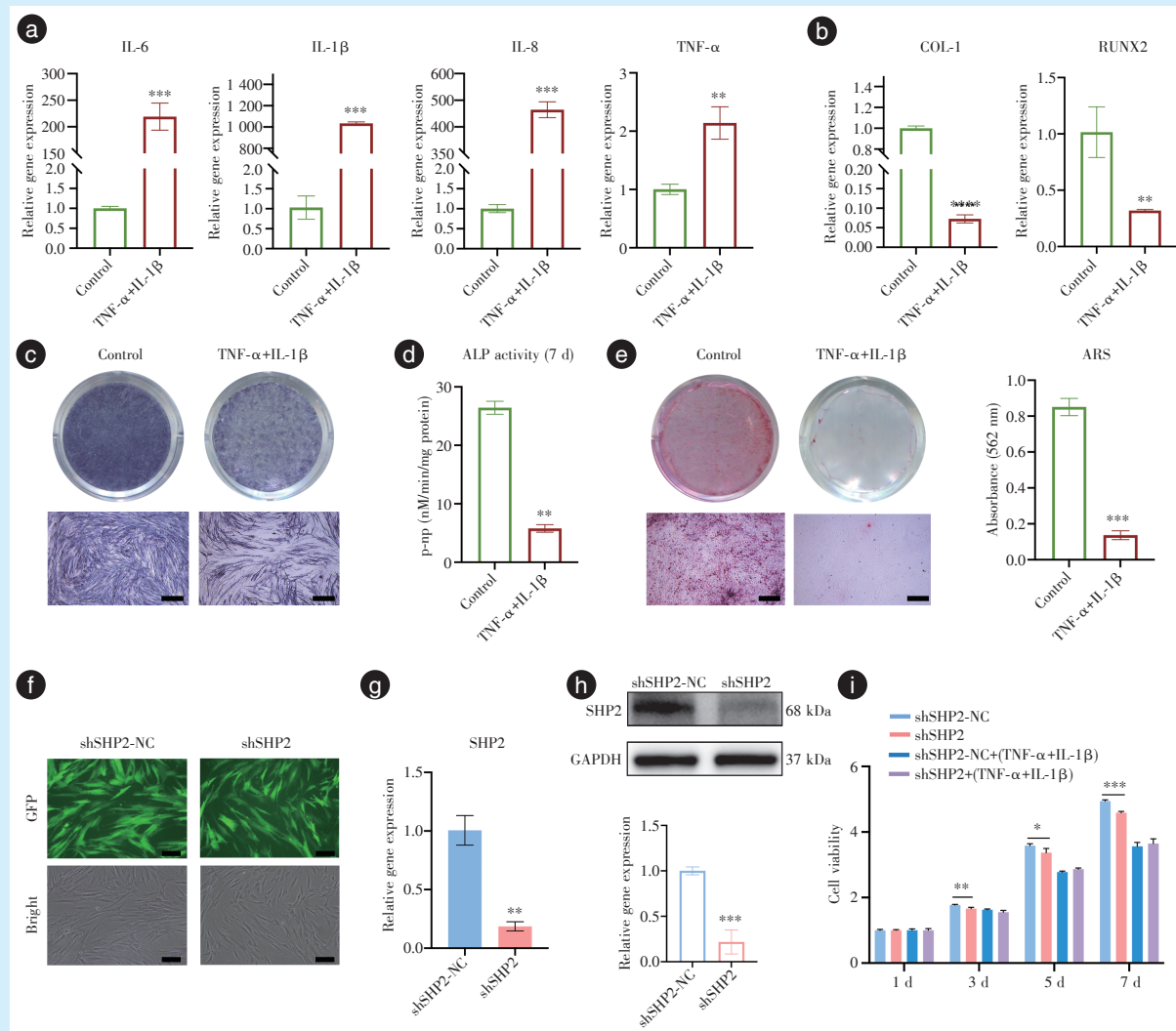
2.6 正常环境下敲低SHP2对hPDLSCs成骨分化的影响

成骨诱导3 d时,与对照组相比,SHP2敲低组ALP、COL-1基因表达均有增高($t = 3.628, P = 0.022; t = 11.010, P < 0.001$),而RUNX2和OCN基因表达无明显差异(图4a);在成骨培养3 d后SHP2敲低组ALP活性高于对照组($t = 5.423, P = 0.032$)(图4b)。在成骨诱导7 d时,SHP2敲低后相较对照组,只有COL-1基因表达增高($t = 4.402, P = 0.022$),其他基因均无明显变化(图4c),而成骨诱导7 d后的ALP活性检测显示,SHP2敲低后ALP活性提高($t = 6.947, P = 0.020$)(图4d)。在成骨诱导14 d后,SHP2敲低组的COL-1表达增高($t = 13.32, P < 0.001$),但RUNX2,ALP的表达无明显变化(图4e、4f)。ALP染色结果显示,与诱导3 d相比,诱导7 d的对照组及SHP2敲低组染色均加深,说明随着时间增加ALP在细胞质中进一步积累。此外,在成骨培养3 d后SHP2敲低组ALP染色深于对照组,但成骨诱导7 d后两组ALP的着色深度无明显

差异(图4g、4h)。经ARS染色实验证明SHP2敲低组的着色及钙结节的形成量与对照组无明显差异(图4i)。

2.7 炎症环境下敲低SHP2对hPDLSCs成骨分化的影响

成骨诱导3 d时,相较于对照组,SHP2敲低组的ALP($t = 6.823, P = 0.006$)、COL-1($t = 8.249, P = 0.004$)、RUNX2($t = 4.589, P = 0.019$)基因表达增高,OCN作为晚期成骨指标在3 d时并未表现出明显差异(图5a);SHP2敲低后,hPDLSCs的ALP活性升高($t = 5.064, P = 0.037$)(图5b)。成骨诱导7 d后,与对照组相比,SHP2敲低后ALP($t = 6.987, P < 0.001$)、COL-1($t = 8.992, P < 0.001$)、RUNX($t = 3.751, P = 0.033$)、OCN($t = 4.737, P = 0.018$)的表达增高(图5c)。成骨诱导7 d的ALP活性升高,与3 d的结果相同($t = 4.760, P = 0.041$)(图5d)。在成骨诱导14 d后,与对照组相比,SHP2敲低组的COL-1($t = 8.767, P < 0.001$)、ALP($t = 3.418, P = 0.027$)、RUNX2($t = 8.224, P = 0.004$)的蛋白表达均增高,这与RT-qPCR结果保持一致(图5e、5f)。从ALP染色结果上可见,成骨诱导3 d和7 d的SHP2敲低组着色均深于对照组(图5g、5h)。SHP2敲低组经ARS染色后,着色深度及钙结节的形成量高于对照组($t = 87.88, P < 0.001$)(图5i)。



a: the relative mRNA expression of inflammatory cytokines after culturing for 24 hours; b: the relative mRNA expression of osteogenic-related genes on Day 7; c: ALP staining on Day 7 after osteogenic induction; d: ALP activity assays on Day 7 after osteogenic induction; e: ARS and calcium deposition assays of hPDLSCs after 21 d of osteogenic induction; f: fluorescence microscopy was used to observe the intracellular fluorescence intensity of GFP; g: SHP2 mRNA expression was significantly decreased in the Lv-SHP2 group; h: SHP2 protein levels were significantly decreased in the Lv-SHP2 group, GAPDH served as an internal control; i: cell viability was examined using the CCK-8 assay. Scale bar: 400 μm . *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$. hPDLSCs: human periodontal ligament stem cells; IL-6: interleukin-6; IL-1 β : interleukin-1 β ; IL-8: interleukin-8; TNF- α : tumor necrosis factor- α ; COL-1: type-I collagen; RUNX-2: runt-related transcription factor 2; ALP: alkaline phosphatase; ARS: Alizarin red staining; SHP2: Src homology-2 domain containing protein tyrosine phosphatase-2; shSHP2-NC: negative control group; shSHP2: SHP2 knockdown group; shSHP2-NC + (TNF- α +IL-1 β): negative control group cultured in an environment containing TNF- α and IL-1 β ; shSHP2 + (TNF- α +IL-1 β): SHP2 knockdown group cultured in an environment containing TNF- α and IL-1 β

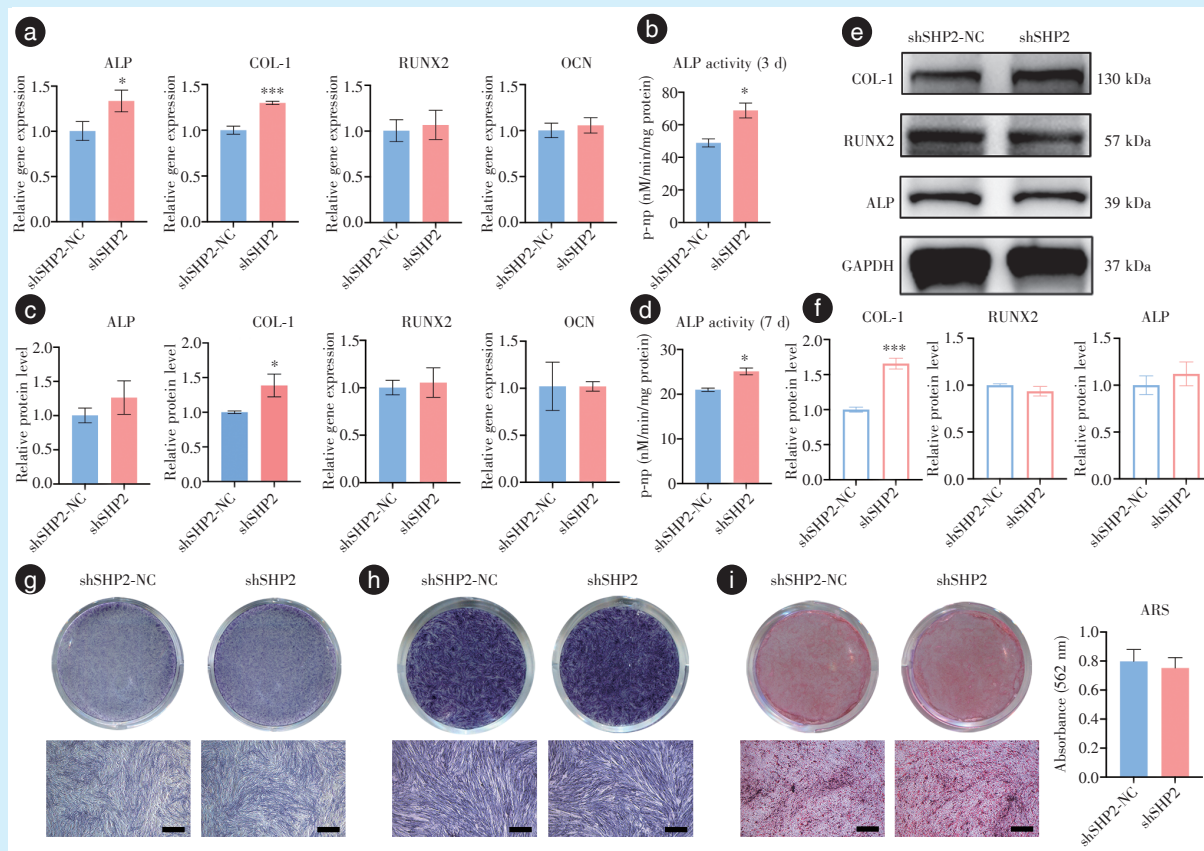
Figure 3 Establishment of an inflammatory environment for hPDLSCs with TNF- α and IL-1 β and SHP2 knockdown efficiency of hPDLSCs

图3 TNF- α 和IL-1 β 刺激hPDLSCs建立体外炎症环境及SHP2敲低效率检测

2.8 炎症环境下敲低SHP2通过抑制MAPK/NF- κ B通路促进hPDLSCs成骨分化

与非炎症组相比,炎症组p-p65的水平增高($t = 36.34, P < 0.001$),I κ B α 水平降低($t = 12.79, P < 0.001$),说明炎症因子(TNF- α 和IL-1 β)刺激30 min时已能够激活NF- κ B通路;而炎症环境下,敲低

SHP2可下调p-p65($t = 12.17, P < 0.001$),并且提高I κ B α 的表达水平($t = 8.979, P < 0.001$)(图6a)。如图6b所示,与非炎症组相比,炎症组p-p38和p-JNK的表达增高($t = 67.30, P < 0.001$; $t = 9.712, P < 0.001$);而炎症环境下,敲低SHP2基因可抑制p-p38和p-JNK的表达($t = 25.99, P < 0.001$; $t = 12.79, P = 0.002$)。



a: relative mRNA levels of osteogenic genes after 3 days of osteogenic induction; b: ALP activity of hPDLSCs was determined by ALP staining after osteogenic induction for 3 days; c: relative mRNA levels of osteogenic genes after 7 days of osteogenic induction; d: ALP activity of hPDLSCs after 7 days of osteogenic induction; e: COL-1, Runx2 and ALP protein accumulation determined by Western blot after 14 d of osteogenic induction; f: quantitative data represent the levels of analyzed proteins in e; g-h: ALP staining on Day 3 (g) and Day 7 (h); i: ARS staining on Day 21 and its semiquantitative results. Scale bar: 400 μ m. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. hPDLSCs: human periodontal ligament stem cells; ALP: alkaline phosphatase; COL-1: type-I collagen; RUNX-2: runt-related transcription factor 2; OCN: osteocalcin; ARS: alizarin red staining; shSHP2-NC: negative control group; shSHP2: SHP2 knockdown group

Figure 4 Effects of SHP2 knockdown on osteogenic differentiation of hPDLSCs under normal environment

图4 正常环境下敲低SHP2对hPDLSCs成骨分化的影响

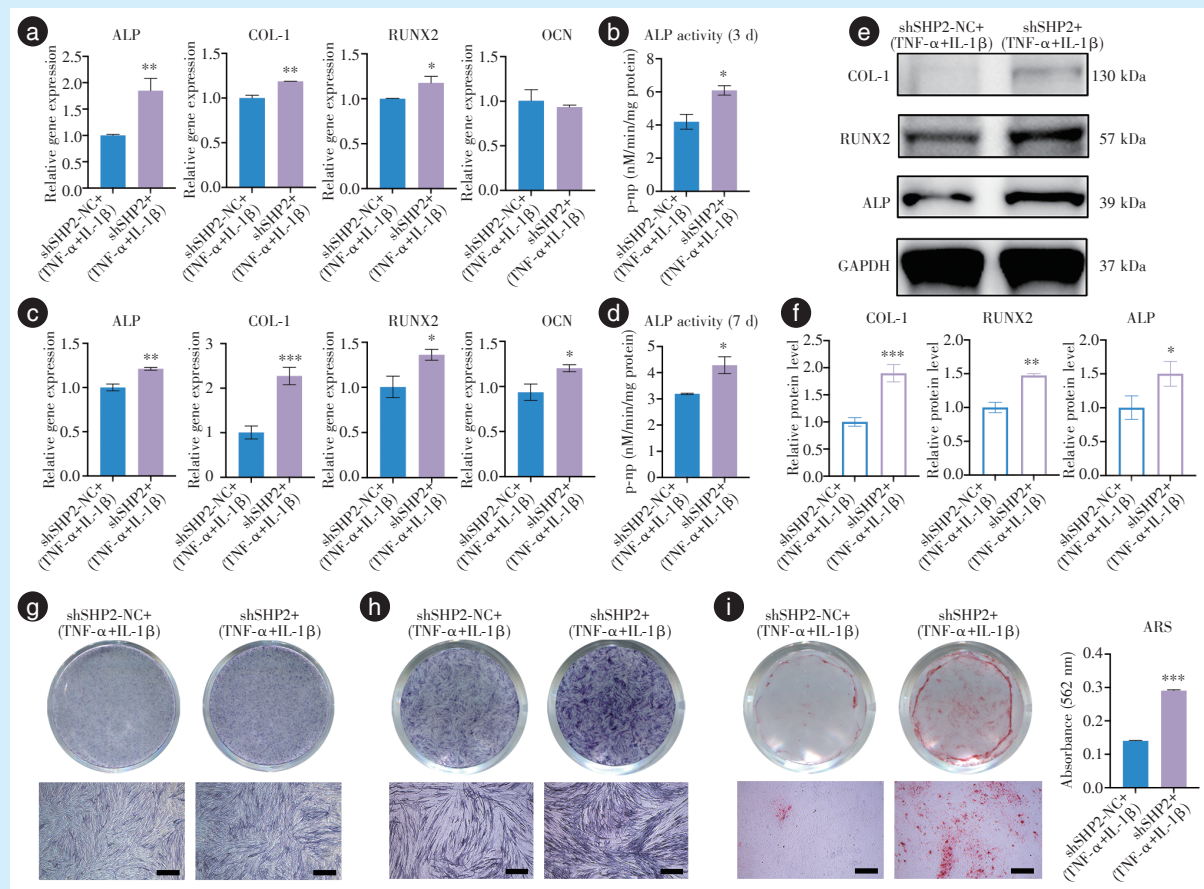
3 讨论

牙周炎是一种常见的破坏牙周支持组织的慢性炎症性疾病,在牙周炎期间,促炎细胞因子在牙周组织中大量分泌^[10-12],抑制PDLSCs的成骨能力,进一步造成牙周硬组织再生的困难。由于临床上大多数牙周组织的愈合阶段都发生在牙周炎症微环境下,因此研究炎症细胞因子对牙周病和干细胞介导的愈合过程的影响,对理解牙周再生机制及临床上进一步指导和优化牙周再生方法至关重要。

ALP在钙化过程中作为第一批功能基因,在骨骼发育早期表达。在骨矿化过程中,ALP增加局部无机磷的浓度,同时可水解抑制矿化结晶的焦磷酸盐,最终促进羟基磷灰石的形成。COL-1是骨骼

中有机细胞外基质中最丰富的成分,其主要功能是和胶原蛋白一同充当骨细胞的支架,并起着支撑作用。因此ALP、COL-1在早期成骨中意义重大,也是经典的早期成骨指标^[13]。本实验证明敲低SHP2可促进ALP、COL-1的表达,表明SHP2参与了上述早期矿化过程。

然而,牙周炎修复过程中的骨再生与正常状况下的不一样,牙周炎局部具有特殊的炎症环境影响了hPDLSCs再生牙周组织的能力,在炎症状态下hPDLSCs成骨能力减弱,显然不利于牙周硬组织的修复。研究表明,来源牙周炎患者的hPDLSCs成骨能力受损,表现为成骨细胞基因表达降低以及钙化结节沉积物和ALP活性减少^[14-15]。本实验也证明相比正常环境,在TNF- α 和IL-1 β 诱导



a: relative mRNA levels of osteogenic genes after 3 days of osteogenic induction; b: ALP activity of hPDLSCs was determined by ALP staining after osteogenic induction for 3 days; c: relative mRNA levels of osteogenic genes after 7 days of osteogenic induction; d: ALP activity of hPDLSCs after 7 days of osteogenic induction; e: COL-1, Runx2 and ALP protein accumulation determined by Western blot after 14 d of osteogenic induction; f: quantitative data represent the levels of analyzed proteins in e; g-h: ALP staining on Day 3 (g) and Day 7 (h); i: ARS staining on Day 21 and its semiquantitative results. Scale bar: 400 μ m. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. hPDLSCs: human periodontal ligament stem cells; ALP: alkaline phosphatase; COL-1: type-I collagen; RUNX-2: runt-related transcription factor 2; OCN: osteocalcin; ARS: alizarin red staining; shSHP2-NC + (TNF- α +IL-1 β): negative control group cultured in an environment containing TNF- α and IL-1 β ; shSHP2 + (TNF- α +IL-1 β): SHP2 knockdown group cultured in an environment containing TNF- α and IL-1 β

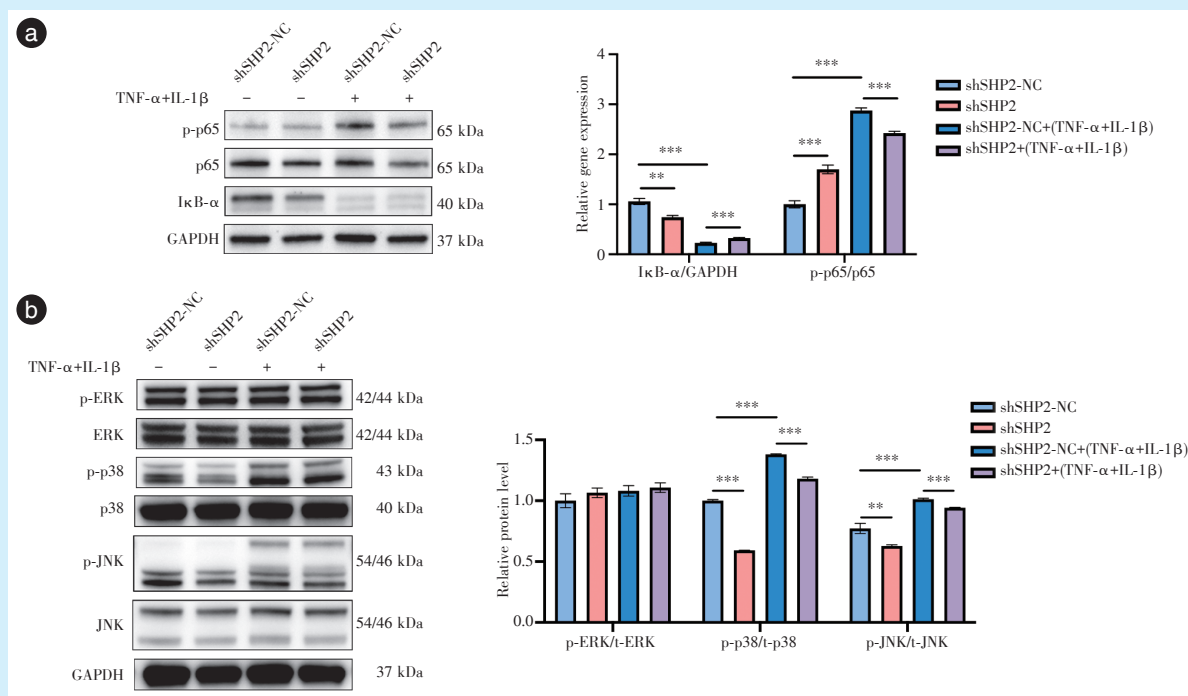
Figure 5 SHP2 knockdown enhanced the osteogenic differentiation of hPDLSCs in an inflammatory environment

图5 炎症环境下敲低SHP2可促进hPDLSCs早期成骨分化

炎症环境下, hPDLSCs成骨能力明显下降。因此, 如何提高hPDLSCs在牙周炎环境中的成骨分化能力, 使其受损的成骨能力得以恢复, 是干细胞治疗获得满意治疗效果的重要因素。于是笔者研究了SHP2敲低在炎症条件下对hPDLSCs增殖及成骨分化的影响, 发现SHP2敲低在炎症条件下增加了成骨标志物(ALP、COL-1和RUNX2)的表达, 证实了SHP2敲低在炎症环境下可以促进早期成骨。而在成骨诱导21 d后, 通过ARS评估钙沉积水平, 以及在14 d时通过Western Blot评估成骨相关蛋白水平, 证明了SHP2敲低对晚期成骨指标也有促进作用。相较于正常环境, SHP2敲低在炎症环境下对

hPDLSCs成骨分化的促进作用更为显著, 这意味着SHP2对于炎症下hPDLSCs的成骨分化起着重要作用。

NF- κ B是一种转录因子, 在炎症发作中起重要作用^[16]。在炎症状况下, NF- κ B通路下游产生炎症因子(如TNF- α)可抑制骨形成^[17]。在牙周炎疾病背景下, 炎症环境也被证实可通过NF- κ B通路抑制hPDLSCs的成骨修复效果。据现有研究表明, SHP2与NF- κ B信号通路关系密切^[18]。因此, 笔者认为NF- κ B信号通路可能参与炎症条件下SHP2介导的hPDLSCs成骨分化。本实验发现, 炎症刺激能够激活NF- κ B通路, 而SHP2敲低能够抑



Protein levels of key molecules of the NF-κB and MAPK signaling pathways after treatment for 30 minutes were determined by Western blotting. a: TNF-α and IL-1β increased p65 phosphorylation and IκB-α degradation, and SHP2 knockdown alleviated these effects; b: TNF-α and IL-1β significantly increased the phosphorylation of p38 and JNK, whereas SHP2 knockdown decreased the phosphorylation of p38 and JNK. hPDLSCs: human periodontal ligament stem cells; shSHP2-NC: negative control group; shSHP2: SHP2 knockdown group; shSHP2-NC + (TNF-α+IL-1β): negative control group cultured in an environment containing TNF-α and IL-1β; shSHP2 + (TNF-α+IL-1β): SHP2 knockdown group cultured in an environment containing TNF-α and IL-1β

Figure 6 Possible mechanism by which SHP2 knockdown promotes the osteogenic differentiation of hPDLSCs under inflammatory environment

图6 SHP2敲低促进炎症环境下hPDLSCs成骨分化的机制

制炎症状态下激活的NF-κB通路。MAPK通路位于NF-κB上游,有3条主要的分支路线即ERK、JNK、p38^[19]。多项研究证实了在炎症环境下,MAPK通路中的JNK、p38由炎症反应激活,而通过各种生物分子或药物抑制JNK/p38激活可有效下调促炎细胞因子、抑制炎症反应,实现多种炎症疾病的治疗^[20-22]。本实验同样发现SHP2可以调控MAPK通路中的p38、JNK磷酸化。

基于本实验,笔者发现敲低SHP2基因后,炎症条件下MAPK-NF-κB通路部分受到抑制,hPDLSCs的成骨分化似乎得到恢复。已有研究证明,p38、JNK及NF-κB通路在炎症状态下激活,这些通路被抑制后能够减轻炎症反应。此外,较低的炎症水平可促进干细胞的成骨分化,因此笔者推断高水平炎症环境下,敲低SHP2后,p38、JNK及NF-κB通路被抑制,减轻了炎症反应,从而恢复干细胞的成骨分化能力。有趣的是,与SHP2对大多

数细胞中NF-κB通路的影响相反,在正常条件下,SHP2敲低导致hPDLSCs中NF-κB通路激活。未来的研究需要进一步探索这种差异的潜在机制。

综上,本研究证明了在炎症条件下,敲低SHP2可能通过MAPK/NF-κB介导的信号传导,促进炎症环境中hPDLSCs的成骨分化。SHP2可能是治疗炎症相关骨骼疾病(如牙周炎)的潜在靶标,并为临床上牙周再生的干细胞治疗提供新的思路。

【Author contributions】 Zhang Y, Jin YQ and Ji J wrote the article and designed the study. Zhao Q, Lv HD, Wang TC and Dou ZJ collected, processed and analyzed the data. All authors read and approved the final manuscript.

参考文献

- [1] Zhang X, Gu H, Xie S, et al. Periodontitis in patients with psoriasis: a systematic review and meta-analysis[J]. Oral Dis, 2022, 28 (1): 33-43. doi: 10.1111/odi.13617.
- [2] Fan D, Liu S, Jiang S, et al. The use of SHP-2 gene transduced

- bone marrow mesenchymal stem cells to promote osteogenic differentiation and bone defect repair in rat[J]. J Biomed Mater Res A, 2016, 104(8): 1871-1881. doi: 10.1002/jbm.a.35718.
- [3] Zhang Y, Lu W, Zhao Q, et al. The role of the protein tyrosine phosphatase SHP2 in ossification[J]. Dev Dyn, 2022, 251(5): 748-758. doi: 10.1002/dvdy.449.
- [4] Kaneshiro S, Ebina K, Shi K, et al. IL-6 negatively regulates osteoblast differentiation through the SHP2/MEK2 and SHP2/Akt2 pathways *in vitro*[J]. J Bone Miner Metab, 2014, 32(4): 378-392. doi: 10.1007/s00774-013-0514-1.
- [5] Holm CK, Engman S, Sulniute R, et al. Lack of SIRP α phosphorylation and concomitantly reduced SHP-2-PI3K-Akt2 signaling decrease osteoblast differentiation[J]. Biochem Biophys Res Commun, 2016, 478(1): 268-273. doi: 10.1016/j.bbrc.2016.07.048.
- [6] An H, Zhao W, Hou J, et al. SHP-2 phosphatase negatively regulates the TRIF adaptor protein-dependent type I interferon and proinflammatory cytokine production[J]. Immunity, 2006, 25(6): 919-928. doi: 10.1016/j.immuni.2006.10.014.
- [7] Deng Z, Ma S, Zhou H, et al. Tyrosine phosphatase SHP-2 mediates C-type lectin receptor-induced activation of the kinase Syk and anti-fungal TH17 responses[J]. Nat Immunol, 2015, 16(6): 642-652. doi: 10.1038/ni.3155.
- [8] Albuquerque-Souza E, Schulte F, Chen T, et al. Maresin-1 and resolvin E1 promote regenerative properties of periodontal ligament stem cells under inflammatory conditions[J]. Front Immunol, 2020, 11: 585530. doi: 10.3389/fimmu.2020.585530.
- [9] Sun C, Liu F, Cen S, et al. Tensile strength suppresses the osteogenesis of periodontal ligament cells in inflammatory microenvironments[J]. Mol Med Rep, 2017, 16(1): 666-672. doi: 10.3892/mmr.2017.6644.
- [10] Cardoso EM, Reis C, Manzanares-Céspedes MC. Chronic periodontitis, inflammatory cytokines, and interrelationship with other chronic diseases[J]. Postgrad Med, 2018, 130(1): 98-104. doi: 10.1080/00325481.2018.1396876.
- [11] Damgaard C, Kantarci A, Holmstrup P, et al. Porphyromonas gingivalis-induced production of reactive oxygen species, tumor necrosis factor- α , interleukin-6, CXCL8 and CCL2 by neutrophils from localized aggressive periodontitis and healthy donors: modulating actions of red blood cells and resolvin E1[J]. J Periodontal Res, 2017, 52(2): 246-254. doi: 10.1111/jre.12388.
- [12] Bartold PM, Cantley MD, Haynes DR. Mechanisms and control of pathologic bone loss in periodontitis[J]. Periodontol 2000, 2010, 53(53): 55-69. doi: 10.1111/j.1600-0757.2010.00347.x.
- [13] Corn DJ, Kim Y, Krebs MD, et al. Imaging early stage osteogenic differentiation of mesenchymal stem cells [J]. J Orthop Res, 2013, 31(6): 871-879. doi: 10.1002/jor.22328.
- [14] Ballini A, Boccaccio A, Saini R, et al. Dental-derived stem cells and their secretome and interactions with bioscaffolds/biomaterials in regenerative medicine: from the *in vitro* research to translational applications[J]. Stem Cells Int, 2017: 6975251. doi: 10.1155/2017/6975251.
- [15] Chen X, Hu C, Wang G, et al. Nuclear factor- κ B modulates osteogenesis of periodontal ligament stem cells through competition with β -catenin signaling in inflammatory microenvironments[J]. Cell Death Dis, 2013, 4(2): e510. doi: 10.1038/cddis.2013.14.
- [16] Tak PP, Firestein GS. NF- κ B: a key role in inflammatory diseases[J]. J Clin Invest, 2001, 107(1): 7-11. doi: 10.1172/JCI11830.
- [17] Yamazaki M, Fukushima H, Shin M, et al. Tumor necrosis factor α represses bone morphogenetic protein (BMP) signaling by interfering with the DNA binding of Smads through the activation of NF- κ B[J]. J Biol Chem, 2009, 284(51): 35987-35995. doi: 10.1074/jbc.M109.070540.
- [18] Ouyang W, Liu C, Pan Y, et al. SHP2 deficiency promotes Staphylococcus aureus pneumonia following influenza infection[J]. Cell Prolif, 2020, 53(1): e12721. doi: 10.1111/cpr.12721.
- [19] Schulze-Osthoff K, Ferrari D, Riehemann K, et al. Regulation of NF- κ B activation by MAP kinase cascades[J]. Immunobiology, 1997, 198(1): 35-49. doi: 10.1016/s0171-2985(97)80025-3.
- [20] Wu CR, Yang QY, Chen QW, et al. Ghrelin attenuate cerebral microvascular leakage by regulating inflammation and apoptosis potentially via a p38 MAPK-JNK dependent pathway[J]. Biochem Biophys Res Commun, 2021 (552): 37-43. doi:10.1016/j.bbrc.2021.03.032.
- [21] Zhong Z, Liu Z, Zheng R, et al. miR-132-3p modulates DUSP9-dependent p38/JNK signaling pathways to enhance inflammation in the amnion leading to labor[J]. Int J Mol Sci, 2022, 23(3): 1864. doi: 10.3390/ijms23031864.
- [22] Wang JL, Ren CH, Feng J, et al. Oleonic acid inhibits mouse spinal cord injury through suppressing inflammation and apoptosis via the blockage of p38 and JNK MAPKs[J]. Biomed Pharmacother, 2020, 123: 109752. doi: 10.1016/j.biopha.2019.109752.

(编辑 罗燕鸿,张雪洋)



官网