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· 基础研究 ·

## miR-21 对人牙周膜干细胞增殖及成骨分化的影响

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**【摘要】目的** 探讨 miR-21 对人牙周膜干细胞(human periodontal ligament stem cells, hPDLSCs)增殖及成骨的影响,旨在为干细胞治疗牙周炎提供实验基础。**方法** 酶解组织块法分离培养 hPDLSCs,通过流式细胞仪检测表面分子 CD34、CD45、CD90、CD105 对 hPDLSCs 进行鉴定,采用 Lipofectamine 2000 将 miR-21 的 mimics (pre-miR-21)、inhibitor(anti-miR-21)以及各自的阴性对照转染至 hPDLSCs。实验分组:过表达组(mimics组)、过表达阴性对照组(mimics-NC组);抑制阴性对照组(inhibitor-NC组)、抑制组(inhibitor组)。实时荧光定量 PCR 检测 miR-21 转染效率;CCK-8、流式细胞术检测 hPDLSCs 的增殖;茜素红染色检测 hPDLSCs 的成骨能力和 Western blot 检测成骨相关基因 Runx2 的蛋白表达。**结果** miR-21 的 mRNA 表达量 mimics 组较 mimics-NC 组明显升高, inhibitor 组较 inhibitor-NC 组明显降低( $P < 0.05$ )。hPDLSCs 增殖及 S 期细胞比例 mimics 组较 mimics-NC 组升高, inhibitor 组较 inhibitor-NC 组减少,差异有统计学意义( $P < 0.05$ )。经茜素红染色后, mimics 组矿化结节多于 mimics-NC 组; inhibitor 组矿化结节少于 inhibitor-NC 组; Western blot 检测 mimics 组 Runx2 蛋白表达高于 mimics-NC 组, inhibitor 组 Runx2 蛋白表达量较 inhibitor-NC 组减少,差异有统计学意义( $P < 0.05$ )。**结论** miR-21 调控 hPDLSCs 的增殖和成骨向分化。

**【关键词】** 微小 RNA; miR-21; 牙周再生; 人牙周膜干细胞; 增殖; 成骨分化; 矿化结节; 成骨相关转录因子 2

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### Effect of miR-21 on the proliferation and osteogenic differentiation of human periodontal ligament stem cells

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**【Abstract】 Objective** To explore the effect of miR-21 on human periodontal ligament stem cells (PDLSCs) proliferation and osteogenesis and to provide a theoretical basis for the stem cell treatment of periodontitis. **Methods** hPDLSCs were isolated and cultured with the enzymatic tissue block method, and surface molecules (CD34, CD45, CD90 and CD105) were detected by flow cytometry. An miR-21 mimics (pre-miR-21) and inhibitor (anti-miR-21) were transfected into hPDLSCs by Lipofectamine 2000. The experiment groups: mimics-NC group, mimics group, inhibitor group, and inhibitor-NC group. The transfection efficiency of miR-21 was determined by qRT-PCR. Proliferation was detected by CCK-8 and flow cytometry. The osteogenic differentiation ability of hPDLSCs was determined by alizarin red staining. Western blot was used to detect the protein expression of osteogenic related genes: Runx2. **Results** The mRNA expression of miR-21 in the mimics group was significantly higher than that in the mimics-NC group; additionally, the expression in the inhibitor group was significantly weaker than that in the inhibitor-NC group ( $P < 0.05$ ). hPDLSCs proliferation and the S phase cell ratio in the mimics group were stronger than those in the mimics-NC group ( $P < 0.05$ ); those

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in the inhibitor group were weaker than those in the inhibitor-NC group ( $P < 0.05$ ). After alizarin red staining, the mimics group was found to have more mineralized modules than mimics-NC group, and the inhibitor group had fewer than that in the inhibitor-NC group. Runx2 protein expression in the mimics group was higher than that in the mimics-NC group ( $P < 0.05$ ), and expression was lower in the inhibitor group than in the inhibitor-NC group ( $P < 0.05$ ). **Conclusion** miR-21 can promote the proliferation and osteogenic differentiation of hPDLSCs.

**【Key words】** microRNA; miR-21; periodontal tissue regeneration; human periodontal stem cells; proliferation; osteogenesis differentiation; mineralized module; runt-related transcription factor-2

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人牙周膜干细胞(human periodontal ligament stem cells, hPDLSCs)是牙周组织中修复和再生重要的细胞群,因其具有免疫调节特性,且可以分化成为不同的细胞类型,如脂肪细胞、成骨细胞等,而被认为是牙周炎治疗的最有潜力的种子细胞。微小RNA(microRNA, miRNA)是18~25个核苷酸组成的非编码、高度保守的RNA,因其在细胞分化、凋亡、增殖及迁移等多种过程中的作用而受到广泛关注<sup>[1]</sup>。有报道miRNA在牙周炎的发生发展和宿主免疫发挥重要作用<sup>[2-3]</sup>。研究发现在牙周炎患者的血清miRNAs表达谱中,miR-21表达量异常,提示miR-21作为关键miRNA参与牙周炎的发生发展<sup>[4]</sup>。此外,有学者发现<sup>[5]</sup>,miR-21可以作为牙周炎控制的干预靶点,抑制牙周炎症。但miR-21在牙周骨再生方面的研究较少,因此,本实验通过研究miR-21对牙周膜干细胞增殖和分化的影响,旨在为牙周炎的治疗提供实验基础。

## 1 材料和方法

### 1.1 主要试剂和仪器

$\alpha$ -MEM培养基(Hyclone,美国),胎牛血清(四季青,中国),PBS(Hyclone,美国),青霉素、链霉素(Gibco,美国),0.25%胰蛋白酶(Gibco,美国),Lipofectamine 2000(Invitrogen,美国),细胞周期检测试剂盒(Sysmex,日本),CCK-8试剂盒(诺唯赞,中国),CD34(PE标记)、CD45(PE标记)、CD90(FITC标记)、CD105抗体(FITC标记),兔抗人Runx2抗体(Abcam,美国),鼠抗兔荧光二抗(Proteintech,美国),成人骨髓间充质干细胞成骨诱导分化培养基试剂盒(Cyagen,美国),CO<sub>2</sub>培养箱(Thermo,美国),倒置相差显微镜以及照相系统(Olympus,日本),流式细胞仪(Partec,德国)、pre-miR-21及其阴性对照pre-miR-21-NC,anti-miR-21及其阴性对照anti-miR-21(锐博,中国),实时荧光定量PCR仪(安

捷伦,美国)。

### 1.2 hPDLSCs的分离培养、鉴定

经患者知情同意,昆明医科大学附属口腔医院伦理委员会批准同意,收集因正畸治疗需要拔除的健康前磨牙,患者年龄为12~16岁。酶解组织块法分离培养hPDLSCs,原代培养至细胞汇合达70%时,进行传代培养。取P3代hPDLSCs,含2%胎牛血清的PBS洗涤两次,调整细胞浓度为 $1 \times 10^5$ 个/mL,加入PE标记的CD34、CD45和FITC标记的CD90、CD105的抗体,避光4℃孵育30 min,含2%胎牛血清的PBS洗涤两次,加入1 mL的含2%胎牛血清的PBS重悬,流式细胞仪上机检测进行鉴定。

### 1.3 细胞转染

待细胞融合至70%后,更换为不含青链双抗的 $\alpha$ -MEM培养基过夜培养,次日使用Lipofectamine 2000进行pre-miR-21及anti-miR-21转染。pre-miR-21为miR-21模拟物,化学合成的成熟miRNA双链,模拟天然存在的miRNA;anti-miR-21是miR-21拮抗物,为化学修饰的成熟miRNA互补单链,构建时,与成熟体序列的互补,参与抑制内源性miRNA的表达。实验分组为过表达组(mimics组)、过表达阴性对照组(mimics-NC组)、抑制组(inhibitor组)、抑制阴性对照组(inhibitor-NC组)。转染后24 h,实时荧光定量PCR检测转染效率,引物序列参见表1。

表1 引物序列

Table 1 Primer sequences

Primer	Sequence
miR-21-F	5'-GTGCAGGGTCCGAGGT-3'
miR-21-R	5'-GCCGCTAGCTTATCAGACTGATGT-3'
pre-miR-21-NC-F	5'-UUUGUACUACACAAAAGUACUG-3'
pre-miR-21-NC-R	5'-AAACAUGAUGUGUUUCAUGAC-3'
anti-miR-21-NC	5'-CAGUACUUUUGUGUAGUACAAA-3'
U6-F	5'-CTCGCTTCGGCAGCAC-3'
U6-R	5'-AACGCTTCACGAATTTGCGT-3'

#### 1.4 hPDLSCs 细胞增殖检测

1.4.1 CCK-8 检测 0.25%胰酶消化 P3 代 hPDLSCs, 将细胞以每孔  $4 \times 10^3$  个接种于 96 孔板中, 进行 miR-21 及 anti-miR-21 的转染, 实验分组: 过表达阴性对照组 (mimics-NC 组)、过表达组 (mimics 组)、抑制阴性对照组 (inhibitor-NC 组)、抑制组 (inhibitor 组)。分别在转染后 1、2、3、4 d, 酶标仪测定在 450 nm 波长处的吸光度。每组设 5 个复孔, 实验重复 3 次。具体操作参照 CCK-8 试剂盒说明书。

1.4.2 细胞周期检测 转染 48 h 后, 0.25%胰酶分别消化各组细胞, 离心弃上清, 加入 1 mL 的细胞周期检测 Buffer (试剂盒为一步法检测细胞周期), 流式细胞仪上机检测。

#### 1.5 hPDLSCs 成骨向分化检测

1.5.1 茜素红染色 转染后 24 h, 开始加入成骨诱导液 (含地塞米松、 $\beta$ -甘油磷酸钠、抗坏血酸、谷氨酰胺的成骨诱导分化基础培养基), 每隔 3 d 换液 1 次,

镜下观察到矿化结节后改为半定量换液, 至 21 d 时, PBS 洗 2 次, 4%多聚甲醛固定 30 min, 加入茜素红染色 3 min, PBS 洗 2 次, 镜下观察矿化结节。

1.5.2 Western blot 检测成骨相关蛋白 Runx2 在成骨诱导 7 d 后, 提取蛋白并测定浓度。行 SDS-PAGE 电泳, 半干法转膜, 10%脱脂奶粉封闭 2 h, 加一抗, 4 °C 孵育过夜, 加二抗孵育, TBST 洗膜, 条带显影及凝胶图像分析。

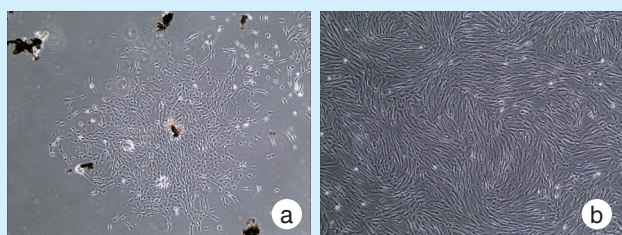
#### 1.6 统计学方法

使用 SPSS 18.0 统计学软件分析, 计量资料以  $\bar{x} \pm s$  表示, 采用单因素方差分析和两样本独立 *t* 检验,  $P < 0.05$  为差异有统计学意义。

## 2 结果

### 2.1 hPDLSCs 的原代培养和鉴定

原代细胞在培养 7 d 左右爬出, 细胞形态为长梭形。传代后的 hPDLSCs 生长状态良好, 呈典型的旋涡状生长 (图 1)。

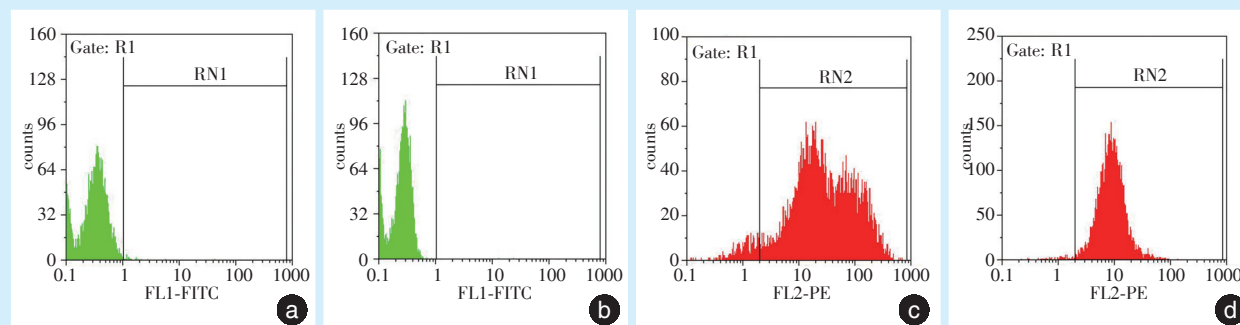


a: on the 6<sup>th</sup> day, primary hPDLSCs were seen around the tissue block ( $\times 100$ ); b: after passage, hPDLSCs showed typical vortex-like growth ( $\times 100$ ); hPDLSCs: human periodontal ligament stem cells  
Figure 1 Primary culture of hPDLSCs

图 1 hPDLSCs 原代培养

经流式细胞仪检测表面分子发现, 所培养的 hPDLSCs 低表达造血干细胞表面标志物 CD34

(1.51%)、CD45 (1.01%), 高表达间充质表面标志物 CD90 (94.16%)、CD105 (98.33%) (图 2)。



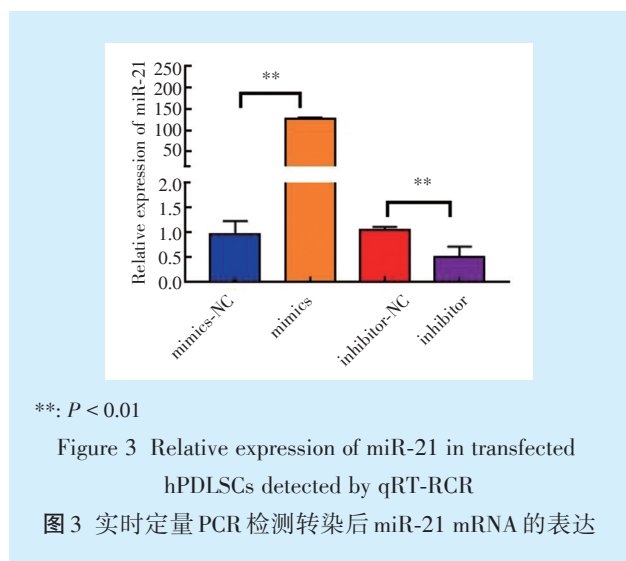
a: hPDLSCs showed low expression of hematopoietic stem cell surface markers: CD34 (1.51%); b: hPDLSCs showed low expression of hematopoietic stem cell surface markers: CD45 (1.01%); c: hPDLSCs showed high expression of mesenchymal surface markers: CD90 (94.16%); d: hPDLSCs showed high expression of mesenchymal surface markers: CD105 (98.33%); hPDLSCs: human periodontal ligament stem cells

Figure 2 Surface molecules of hPDLSCs were measured by flow cytometry

图 2 流式细胞仪检测 hPDLSCs 表面标志物

## 2.2 转染后 miR-21 mRNA 的表达

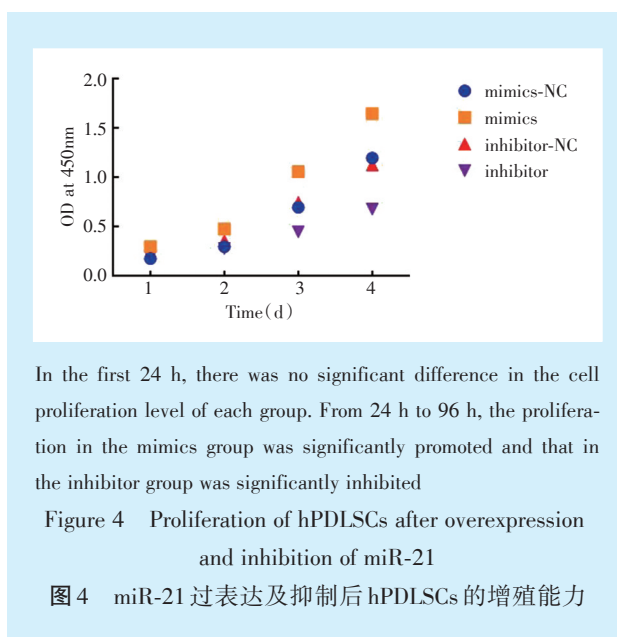
转染后 24 h, 通过 qRT-PCR 检测 hPDLSCs 中 miR-21 的 mRNA 表达水平, mimics 组明显高于 mimics-NC 组, 而 inhibitor 组的表达明显低于 inhibitor-NC 组, 差异均具有统计学意义 ( $P < 0.01$ ) (图 3)。



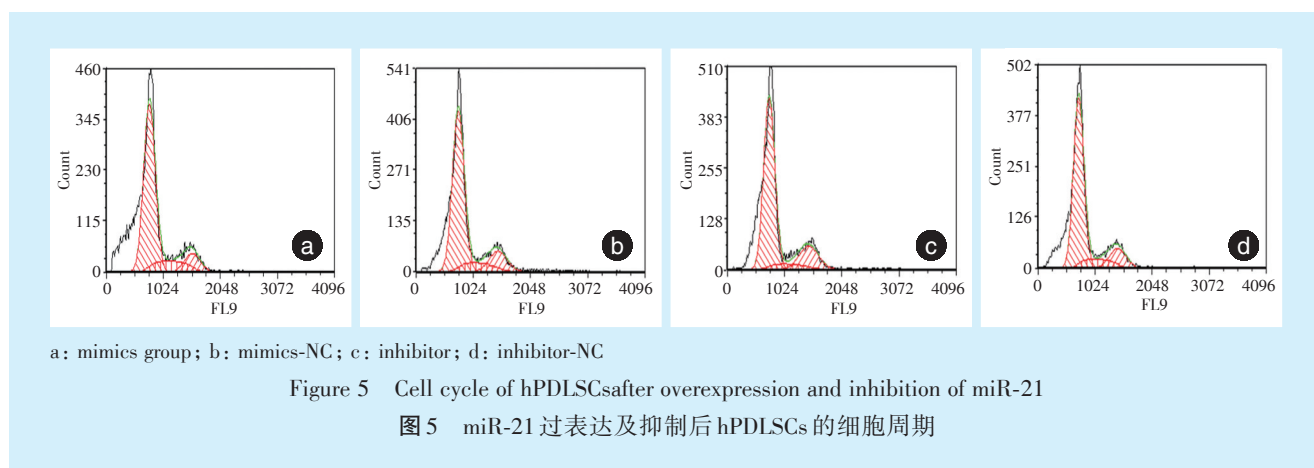
## 2.3 hPDLSCs 增殖检测

2.3.1 CCK-8 检测 hPDLSCs 的增殖经酶标仪检测 OD 值发现, 48、72、96 h 时, 与 mimics-NC 组相比, mimics 组细胞增殖明显增加 ( $P < 0.01$ ), 与 inhibitor-NC 组相比, inhibitor 组细胞增殖明显减少

( $P < 0.05$ ) (图 4)。



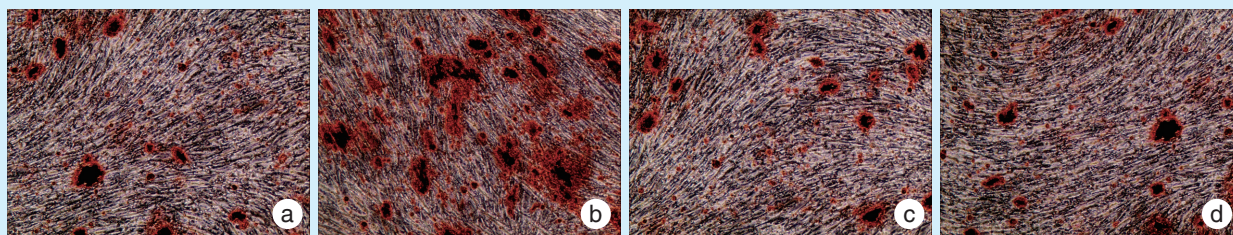
2.3.2 hPDLSCs 细胞周期检测 经流式细胞仪检测发现 (图 5), mimics 组 S 期细胞比例为 ( $14.67 \pm 0.14$ )%, 较 mimics-NC 组 ( $10.84 \pm 0.64$ )% 明显增加 ( $t = 10.127, P < 0.01$ ), 与 inhibitor-NC 组 S 期细胞比例 ( $11.72 \pm 0.57$ )% 相比, inhibitor 组 S 期细胞比例 ( $7.35 \pm 0.36$ )% 明显减少 ( $t = -11.334, P < 0.05$ )。



## 2.4 hPDLSCs 成骨向分化的检测

2.4.1 茜素红染色 茜素红染色后, 孔板照肉眼观, mimics 组的显色度明显高于 mimics-NC 组, inhibitor 组显色明显低于 inhibitor-NC 组; 在倒置显微镜下观察, mimics 组的矿化结节明显多于 mimics-NC 组, inhibitor 组矿化结节明显少于 inhibitor-NC 组 (图 6)。

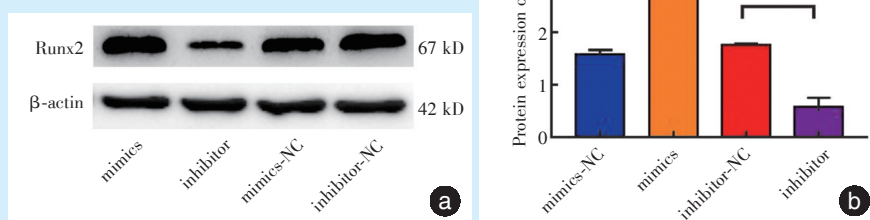
2.4.2 Western blot 检测成骨相关蛋白 Runx2 成骨诱导 7 d 后, 通过 Western blot 检测成骨相关蛋白 Runx2 蛋白表达水平, mimics 组明显高于 mimics-NC 组 ( $t = 12.798$ ), 而 inhibitor 组的表达明显低于 inhibitor-NC 组 ( $t = -12.098$ ), 差异具有统计学意义 ( $P < 0.05$ ) (图 7)。



a: mimics-NC; b: mimics; c: inhibitor-NC; d: inhibitor (× 100)

Figure 6 Images of mineralized nodules by alizarin red staining after overexpression or inhibition of miR-21 in the 4 groups

图6 miR-21 过表达或抑制后4组矿化结节茜素红染色图片



The expression level of the osteogenic related protein Runx2 in the mimics group was significantly higher than that in the mimics-NC group, and the expression in the inhibitor group was lower than that in the inhibitor-NC group. mimics vs. mimics-NC\*,  $P < 0.05$ . inhibitor vs. inhibitor-NC\*,  $P < 0.05$

Figure 7 Protein expression of Runx2 after overexpression or inhibition of miR-21

图7 miR-21 过表达或抑制后 Runx2 的蛋白表达情况

### 3 讨论

牙周炎是发生在牙齿支持组织、与免疫相关的一种慢性炎症破坏性疾病。目前对于牙周炎的治疗包括药物治疗<sup>[6]</sup>、手术治疗以及牙周基础治疗,但均只能控制炎症,不能对于已经缺损的牙周组织进行修复和再生<sup>[7]</sup>。因此,控制已有炎症恢复缺损的牙周组织成为牙周炎治疗的研究难点。牙周膜干细胞是牙周组织来源的间充质干细胞,具有自我更新、多向分化和免疫调节的特性,且在维持牙周内环境稳定方面具有重要作用。研究发现<sup>[8]</sup>,在小型猪牙周炎模型中,使用小型猪自体PDLSCs治疗牙周缺损,PDLSCs能够成骨向分化,再生牙周组织。因此干细胞治疗作为一种新的、可行的临床治疗手段,为炎症破坏性疾病提供新的治疗思路<sup>[9]</sup>。

miRNAs是一类在哺乳动物中高度保守的、非编码蛋白质的RNA,其能通过与靶基因的3'非编码区(3'UTR)碱基配对,引起mRNA的直接降解或翻译受到抑制,从而参与细胞的基本生物学过程,

如分化、增殖与凋亡<sup>[10]</sup>。此外,每一个miRNA可能同时调控多个基因的表达。miR-21位于17号染色体,其全长22nt,由于其在多个肿瘤细胞中高表达,又被称为oncomiR<sup>[11-12]</sup>。近年研究发现,miR-21能通过多种信号途径参与调控干细胞的自我更新和分化<sup>[13-14]</sup>。Chen等<sup>[15]</sup>发现阻断小鼠肉芽组织中皮肤干细胞miR-21的表达,可以抑制细胞迁移和分化,但仍有部分细胞通过ROS依赖途径促进增殖和自我更新;Trohatou等<sup>[16]</sup>证实miR-21通过调节Sox2而参与间充质干细胞(mesenchymal stem cells, MSCs)的增殖和分化;Zhang等<sup>[17]</sup>发现miR-21促进人羊膜间充质干细胞(human amniotic membranemesenchymal stem cells, hAM-MSCs)的增殖;在去卵巢小鼠的骨质疏松模型中<sup>[18]</sup>,miR-21的表达、骨钙素浓度和碱性磷酸酶活性下降;杨楠<sup>[19]</sup>发现miR-21靶向Sprouty 1参与骨髓间充质干细胞的成骨分化。以上提示,miR-21是参与细胞增殖和分化的关键miRNA。Runx2是骨特异性转录因子,在促进成骨分化中发挥重要作用,其表达量可反映向成

骨细胞转化的趋势。此外,Runx2的蛋白水平又受miRNA调控<sup>[20]</sup>,miR-21作为一种潜在的促成骨调节因子,参与调节Runx2的转录活性<sup>[21]</sup>。本研究结果显示,miR-21对Runx-2有正向调控作用。Xing等<sup>[22]</sup>发现脂质体转染法对细胞损伤小、无免疫反应、操作简单、重复性高。因此,本实验采用脂质体转染法,使用Lipo 2000转染pre-miR-21及anti-miR-21至hPDLSCs,调高/降低hPDLSCs中miR-21的表达。通过qRT-PCR检测miR-21的转染效率,排除转染试剂等对实验的干扰。通过CCK-8、流式细胞术检测细胞增殖、周期,发现miR-21能够促进hPDLSCs的增殖,与Zhang等<sup>[17]</sup>研究一致;通过茜素红和Western blot检测成骨分化能力,发现miR-21正向调控hPDLSCs的成骨向分化,这与杨楠<sup>[19]</sup>发现miR-21参与MSCs的成骨分化趋势一致。综上,本研究发现miR-21能够显著调控hPDLSCs的增殖和成骨向分化,但对于其作用的重要靶点及可能通路仍不明确,后续实验将进一步深入研究。

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