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MiR-21 敲低抑制异位内膜来源的子宫内膜干细胞增殖、迁移和侵袭

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[摘　要]　**目的：**探讨miR-21敲低对子宫内膜异位症异位内膜来源的子宫内膜干细胞(endometrial stem cells，EnSCs)增殖、迁移和侵袭的影响，并分析其潜在的机制。**方法：**收集子宫内膜异位症异位内膜组织和正常内膜组织，并分别分离EnSCs。通过慢病毒感染法敲减异位内膜来源的EnSCs中miR-21表达，通过EdU法和Transwell小室试验分别评估EnSCs增殖、侵袭和迁移。将慢病毒感染的异位内膜来源的EnSCs分别移植到BALB/c裸鼠皮下，通过近红外荧光成像检测囊肿的荧光强度，并检测囊肿组织的体积。通过蛋白质印迹法比较异位内膜和正常内膜来源的EnSCs中转化生长因子-β(transforming growth factor-β，TGF-β)相对蛋白质表达水平差异。并检测慢病毒感染的异位内膜来源的EnSCs中TGF-β相对蛋白质表达。**结果：**异位内膜来源的EnSCs中miR-21和TGF-β的相对表达水平明显高于正常内膜来源的EnSCs。敲减miR-21后，异位内膜来源的EnSCs的增殖、迁移和侵袭明显减弱，同时TGF-β降低。与阴性对照(negative control, NC)组比较，敲低miR-21的EnSCs形成的囊肿荧光强度明显降低，囊肿体积明显降低。**结论：**miR-21敲减抑制异位内膜来源的EnSCs增殖、迁移和侵袭，其机制可能与下调TGF-β表达相关。

[关键词]　miR-21；子宫内膜异位；转化生长因子-β；子宫内膜干细胞

MiR-21 knockdown inhibits the proliferation, migration and invasion of endometrial stem cells derived from ectopic endometrium

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Abstract　**Objective:** To investigate the effect of miR-21 knockdown on the proliferation, migration and invasion of endometrial stem cells (EnSCs) derived from ectopic endometrium, and explore the underlying mechanism. **Methods:** Ectopic endometrial tissues and normal endometrial tissues of endometriosis were collected and

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EnSCs were isolated respectively. MiR-21 knockdown in EnSCs derived from ectopic endometrium by lentivirus infection. Then, the proliferation, invasion and migration were evaluated by EdU assay and Transwell assay, respectively. EnSCs from ectopic endometrium infected by lentivirus were subcutaneously transplanted into BALB/c nude mice, respectively. The fluorescence intensity of allogeneic cysts were detected by near-infrared fluorescence imaging, then the cysts were excised and the volumes of cysts were measured. The relative protein expression levels of transforming growth factor-β (TGF-β) in EnSCs from ectopic endometrium and normal endometrium were compared by Western blot. The relative protein expression of TGF-β in EnSCs derived from ectopic endometrium infected with lentivirus was detected. **Results:** The relative expression levels of miR-21 and TGF-β in EnSCs from ectopic endometrium were significantly higher than those in EnSCs from normal endometrium. After miR-21 knockdown, the proliferation, migration and invasion of EnSCs derived from ectopic endometrium were significantly reduced, while TGF-β was decreased. Compared with the negative control group (NC), the fluorescence intensity and volume of cysts formed by EnSCs knockdown of miR-21 were significantly reduced. **Conclusion:** MiR-21 knockdown inhibits the proliferation, migration and invasion of EnSCs derived from ectopic endometrium, and the mechanism may be related to down-regulating TGF-β expression.

Keywords miR-21; endometriosis; transforming growth factor-β; endometrial stem cells

子宫内膜异位症是一种常见的多因素妇科疾病，影响10%~15%的育龄妇女[1]。该疾病的发生是子宫内膜组织在子宫腔外生长，会引起不同程度的疼痛症状和不孕[2-3]。并且，子宫内膜异位症存在癌变风险[4]。子宫内膜异位症的产生机制有几种假设，包括淋巴和血管转移、医源性直接植入、体腔组织化生、胚胎休息和间充质细胞诱导等[5]。子宫内膜干细胞(endometrial stem cells，EnSCs)异常被认为是子宫内膜异位症发病机制之一。有研究报道存在子宫内膜组织基质中的间充质干细胞(mesenchymal stem cells，MSCs)导致子宫内膜异位的发生[6-7]。Esfandiari等[8-9]的研究发现：子宫内膜干细胞在体外3D培养模型中参与了子宫内膜血管生成，这与早期子宫内膜异位症发生非常相似。Taylor[10]研究发现子宫内膜的骨髓间充质干细胞参与了子宫内膜异位症的发生和发展。

miRNA是由20~25个核苷酸组成的小RNA分子，其显著影响基因表达和调节转录后生物学效应。研究[11]表明miRNA的失调在子宫内膜异位症的发生和发展中起重要作用。为研究miR-21对EnSCs的作用，本研究分离异位内膜和正常内膜的EnSCs，进一步比较俩种来源的EnSCs中miR-21表达差异，然后对异位内膜来源的EnSCs通过慢病毒感染法来敲减miR-21表达，在体外观察增殖、侵袭与迁移的变化，在体内观察生长情况；并进一步分析miR-21与转化生长因子-β(transforming growth factor-β，TGF-β)的表达关系。

1 材料与方法

1.1 实验材料

1.1.1 子宫内膜异位症患者组织获取

收集8例行子宫全切术的宫颈上皮内瘤变3级(cervical intraepithelial neoplasia grade 3，CIN3)病变的子宫内膜异位症患者的异位内膜组织和正常内膜组织。对于本研究的目的和用途，每位患者及其家属均知情同意，该研究经本院伦理审查委员会批准。所有患者在3个月内接受过类固醇激素药物治疗。

1.1.2 实验仪器及试剂

细胞裂解缓冲液来自美国Bio-Rad公司。DMEM/F12培养基、胎牛血清(fetal bovine serum，FBS)、青霉素和链霉素、胶原酶和胰蛋白酶来源于美国Gibco公司。TRIzol试剂、ECL化学发光试剂盒来自美国Life Science公司。Ficoll分离液来自美国Sigma公司。GFP标记慢病毒载体构建的(Lenti-miR-21 shRNA-GFP)和对照(Lenti-miR NC-GFP)由上海吉玛制药技术有限公司合成。反转录试剂盒和荧光定量RT-PCR试剂盒来自日本Takara公司；FITC标记的CD140b抗体、FITC标记的CD34抗体、FITC标记的CD31抗体和TGF-β抗体购自美国Abcam公司。FITC标记的CD146抗体来自美国eBioScience公司。EDU细胞增殖检测试剂盒、β-Actin抗体和HRP标记的二抗来自江苏碧云天生物研究所。

1.2 方法

1.2.1 子宫内膜干细胞的分离

将收集的子宫内膜异位组织和正常子宫内膜组织分别在PBS中洗涤、切碎。37 ℃下，在胶原酶(1 mg/mL)中搅拌消化30~45 min。然后，将得到的细胞溶液过滤并离心，并通过Ficoll分离液分离单核细胞，并在PBS中洗涤。将分离的细胞在含有10%FBS和1%青霉素/链霉素抗生素的DMEM/F-12培养基中置于37 ℃，5%CO_2培养箱培养。当细胞大约80%汇合时，将它们用胰蛋白酶消化并减半，通过流式细胞术分选和扩增进行表征，最终分别得到来源于子宫内膜异位内膜组织的EnSCs和正常内膜组织的EnSCs。

1.2.2 细胞流式分析

通过流式细胞术对细胞表面标志物进行EnSCs的表征。将细胞与各种制造商推荐的浓度的子宫内膜干细胞标志物(FITC标记的CD146抗体、FITC标记的CD140b抗体)、血液标志物(FITC标记的CD34抗体)和内皮标志物(FITC标记的CD31抗体)一起孵育1 h，并通过流式细胞术分析。

1.2.3 细胞培养

来源于异位内膜的EnSCs和正常内膜的EnSCs培养于含10%胎牛血清的DMEM/F12培养基中培养。取生长期EnSCs用于RT-qPCR分析miR-21表达。

1.2.4 病毒感染

将浓度为2×10^5个来源于异位内膜的EnSCs接种至24孔板中，待细胞贴壁后，加入2 mL含有5 μg/mL聚凝胺，并分别加入20 MOI Lenti-miR-21 shRNA-GFP和Lenti-miR NC-GFP病毒液，继续培养24 h。经RT-qPCR检测感染效率后，取稳定感染的EnSCs用于后续实验。

1.2.5 细胞分组

来源于异位内膜的EnSCs分为NC组、miR-21 shRNA组、NC+Vehicle组、miR-21 shRNA+Vehicle组和miR-21 shRNA+TGF-β组。其中NC组为稳定感染Lenti-miR NC-GFP的EnSCs在继续培养24 h；miR-21 shRNA组为稳定感染Lenti-miR-21 shRNA-GFP的EnSCs在继续培养24 h；miR-21 shRNA+TGF-β组为10 ng/mL TGF-β刺激稳定感染Lenti-miR-21 shRNA-GFP的EnSCs 24 h；miR-21 shRNA+Vehicle组为稳定感染Lenti-miR-21 shRNA-GFP的EnSCs加入等体积的DMSO后再培养24 h；NC+Vehicle组为稳定感染Lenti-miR NC-GFP的EnSCs加入等体积的DMSO后再培养24 h。

1.2.6 EDU 掺入比色测定法

使用EDU比色测定法测量细胞增殖。在EdU掺入测定之前，将各组的EnSCs在无血清培养基中饥饿12 h。然后分别将EnSCs然后根据EDU比色检测试剂盒说明书步骤，加入10 nmol/L EDU试剂孵育1 h后，去除培养液加入4%多聚甲醛固定30 min；去除固定液，加入0.3% TRIzol试剂通透10 min；去除通透试剂，加入内源性过氧化物酶封闭20 min；去除封闭液，加入Click反应液避光孵育30 min；去除Click反应液，加入Streptavidin-HRP工作液孵育15 min；最后加入TMB显色液孵育30 min后加入终止液，使用酶标仪测定波长490 nm的OD值。

1.2.7 Transwell 小室试验检测细胞侵袭与迁移

将各组的EnSCs在无血清培养基中饥饿12 h，然后收集细胞，用含0.5%BSA的无血清的DMEM培养基制备无血清细胞悬液，调整细胞浓度为5×10^4个/mL，分别取100 μL细胞悬液加入8%基底胶涂覆的Transwell板(8 μm孔径，24孔室，美国Corning公司)或直接加入Transwell板上室上膜面。取600 μL含有10%FBS的DMEM/F12培养基加入下室。细胞培养24 h后，用棉签除去残留在上表面上的细胞。将膜插入物下表面上的细胞固定在75%甲醇中，然后用结晶紫染色。在光学显微镜下计数侵入基质胶并到达过滤器下表面的细胞。

1.2.8 RNA 萃取和 RT-qPCR

用TRIzol试剂萃取来源于异位内膜的EnSCs、正常内膜的EnSCs和各组的EnSCs的RNA。RNA经紫外分光仪定量后，每样本各取2 μg RNA，采用反转录试剂盒反转为cDNA。取各样本cDNA和引物，引物序列miR-21正向引物5'-GCGGCGGTAGCTTATCAGACTG-3'，反向引物5'-ATCCAGTGCAGGGTCCGAGG-3'；U6正向引物5'-CGCTTCGGCAGCCACATATACTA-3'，反向引物5'-CGCTTCACGAATTTGCGTGTCA-3'。按照荧光定量RT-PCR试剂盒构建RT-qPCR反应系统并利用ABI 7500荧光定量PCR仪进行RT-qPCR。PCR设置条件：94 ℃ 30 s，35个循环94 ℃ 2 s，56 ℃ 30 s，60 ℃ 1 min。

选用U6作为miR-21的内参，以$2^{-\Delta\Delta Ct}$法计算miR-21相对表达结果。

1.2.9 蛋白质印迹法分析

用细胞裂解液萃取来源于异位内膜的EnSCs、正常内膜的EnSCs和各组的EnSCs总蛋白质。蛋白质经定量后，每样本各取30 μg蛋白质进行10% SDS-PAGE分离。将每样本分离的蛋白质样品转

移到硝酸纤维素膜上，用5%脱脂奶粉在室温下封膜30 min。将膜与TBST中的TGF-β抗体(1∶500稀释度)在室温下孵育1 h，用TBST洗膜后，将膜与HRP标记的二抗在室温下孵育40 min。使用ECL试剂进行化学发光显影。通过Smart Chemi-TM灯分析系统扫描膜，并根据β-Actin的条带密度值，采用Quantity One软件对TGF-β的表达进行定量。

1.2.10 *动物实验*

10只SPF级6周龄雌性BALB/c裸鼠(19~21 g)购于北京维通利华实验动物技术有限公司上海分公司[生产许可证：SCXK(沪)2017-0011]，饲养于江苏科标医学检测有限公司动物公共实验中心SPF级动物房[使用许可证：SYXK(苏)2017-0010]。进行适应饲养3 d后，裸鼠分为NC组和miR-21 shRNA组，每组5只。将1×10^7个已感染Lenti-miR-21 shRNA-GFP和Lenti-miR NC-GFP的EnSCs分别对应接种于NC组和miR-21 shRNA组裸鼠腹股沟腹膜面。18 d后，囊肿进行体内荧光成像，并麻醉裸鼠，分离囊肿组织并使用公式$V=1/2(长\times宽^2)$计算囊肿的体积。

1.3 统计学处理

利用SPSS 17.0软件进行统计分析。采用均数±标准差($\bar{x}\pm s$)表示，对于两组定量资料比较采用双侧t检验分析方法。当$P<0.05$时差异为有统计学意义。

2 结果

2.1 通过流式分析来鉴定 EnSCs 细胞

流式细胞术分析对分离出来的人EnSCs进行鉴定。分离的细胞对CD140b和CD146呈阳性，对CD31和CD34呈阴性，CD140b和CD146是EnSCs标志物，CD34是造血标志物，CD31是内皮标志物(图1)。

2.2 异位内膜组织来源的 EnSCs 中 miR-21 高表达水平

来自异位内膜的EnSCs中miR-21的表达水平明显高于正常内膜来源的EnSCs(图2A，$P<0.01$)。对异位内膜来源的EnSCs感染Lenti-miR-21 shRNA-GFP和Lenti-miR NC-GFP，与阴性对照(NC)组相比，感染miR-21 shRNA-GFP后细胞中miR-21的表达降低(图2B，$P<0.01$)。

2.3 MiR-21 敲低在体外实验抑制 EnSCs 的增殖、迁移和侵袭能力

与NC组相比，Lenti-miR-21 shRNA-GFP感染的EnSCs细胞中EDU掺入OD值明显降低(图3A，$P<0.01$)。在Transwell实验中，与NC组相比，Lenti-miR-21 shRNA-GFP感染的EnSCs细胞，每个视野下的侵袭细胞(图3B，3C，$P<0.001$)和迁移细胞(图3D，3E，$P<0.001$)的数量明显减少。

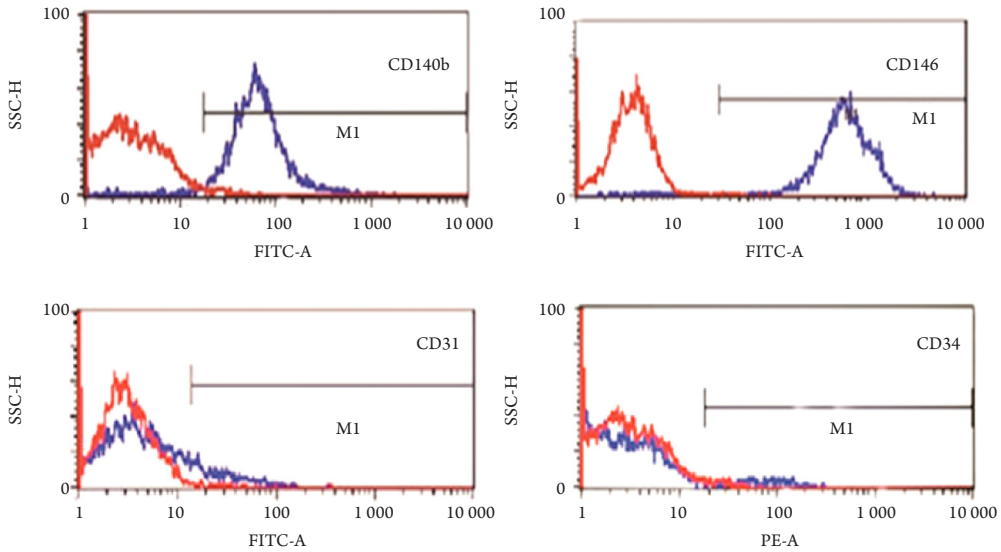

图1 通过流式细胞术对分离的EnSCs标志物(CD140 b和 CD146)、造血标志物(CD34)和内皮细胞标志物(CD31)进行流式细胞分析

Figure 1 Flow cytometric analysis of isolated EnSCs for EnSCs markers (CD140b, CD146), haemopoietic marker (CD34) and endothelial marker (CD31) respectively

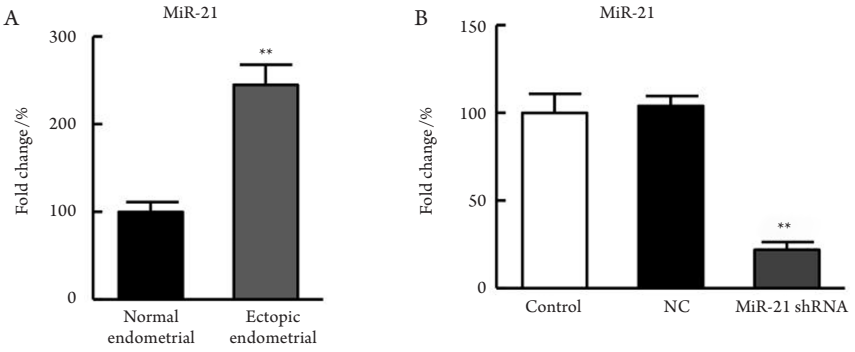

图2 异位内膜组织来源的EnSCs中miR-21的表达与miR-21 shRNA转染

Figure 2 Expression of miR-21 and transfection of miR-21 shRNA in EnSCs derived from ectopic endometrial tissues

(A)异位子宫内膜组织来源的EnSCs中miR-21的表达水平显著高于正常子宫内膜组织来源的EnSCs。n=8，与异位子宫内膜组织来源的EnSCs比较，$^{**}P$<0.01。(B)转染Lenti-miR-21 shRNA-GFP后，异位子宫内膜组织来源的EnSCs中miR-21的表达显著降低。n=4, 与阴性对照组比较，$^{**}P$<0.01。

(A) The expression level of miR-21 was significantly higher in EnSCs derived from ectopic endometrial tissues than in EnSCs from normal endometrial tissues. n=8, $^{**}P$<0.01 vs EnSCs derived from normal endometrial tissues. (B) Significant reduction of miR-21 expression in EnSCs from ectopic endometrial tissues after transfected with Lenti-miR-21 shRNA-GFP. n=4, $^{**}P$<0.01 vs NC group.

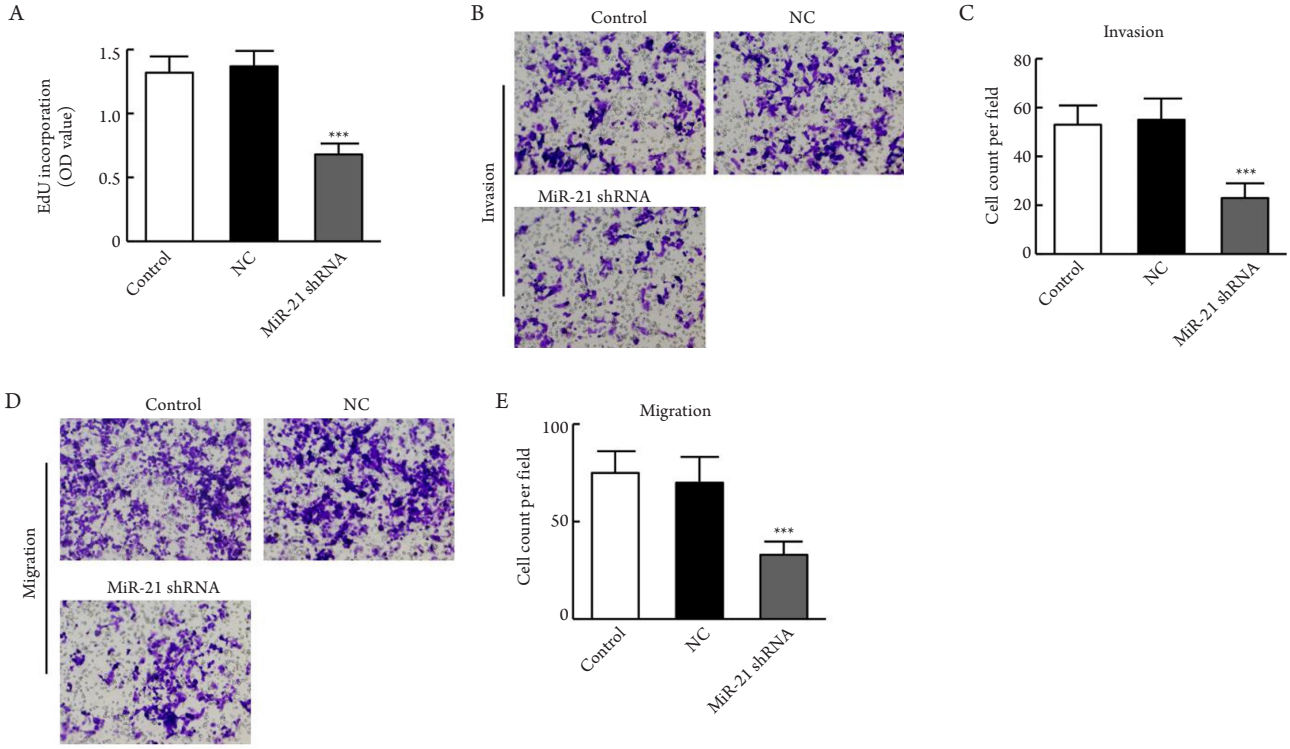

图3 MiR-21敲低在体外实验抑制异位子宫内膜组织来源的EnSCs的增殖、迁移和侵袭能力

Figure 3 MiR-21 knockdown reduces the proliferation, migration and invasion of EnSCs derived from ectopic endometrial tissues in vitro

(A)用EdU法评价转染Lenti-miR-21 shRNA-GFP的EnSCs的增殖情况。(B，C)Transwell法检测转染Lenti-miR-21 shRNA-GFP的EnSCs的侵袭(×200)。(D，E)Transwell法检测转染Lenti-miR-21 shRNA-GFP的EnSCs的迁移(×200)。n=4，$^{***}P$<0.001 vs NC group。

(A) Proliferation of EnSCs transfected with Lenti-miR-21 shRNA-GFP evaluated by EdU assay. (B, C) Invasion of EnSCs transfected with Lenti-miR-21 shRNA-GFP was detected by Transwell assay (×200). (D, E) The migration of EnSCs transfected with Lenti-miR-21 shRNA-GFP was detected by Transwell assay (×200). n=4, $^{***}P$<0.001 vs NC group.

2.4 EnSCs 异体囊肿检测

18 d后对囊肿进行近红外荧光成像分析。与NC组相比，Lenti-miR-21 shRNA-GFP感染的EnSCs细胞形成的囊肿总荧光强度明显降低(图4A，4B，$P<0.001$)。分离囊肿，与NC组相比，Lenti-miR-21 shRNA-GFP感染的EnSCs细胞形成的囊肿体积明显减小(图4C，4D，$P<0.001$)。实验结果表明miR-21敲低能在体内实验中抑制EnSCs生长。

2.5 MiR-21 shRNA 通过下调 TGF-β 表达抑制 EnSCs

蛋白质印迹法结果表明异位内膜来源的EnSCs中TGF-β蛋白质水平明显高于正常内膜来源的EnSCs(图5A，$P<0.001$)。与NC组相比，Lenti-miR-21 shRNA-GFP感染的EnSCs细胞中TGF-β蛋白质表达明显降低(图5B，$P<0.001$)。与miR-21 shRNA+vehicle组相比，miR-21 shRNA+TGF-β组EDU掺入OD值(图5C，$P<0.01$)、细胞迁移数和侵袭数均明显增加(图5D~5F，$P<0.001$)。

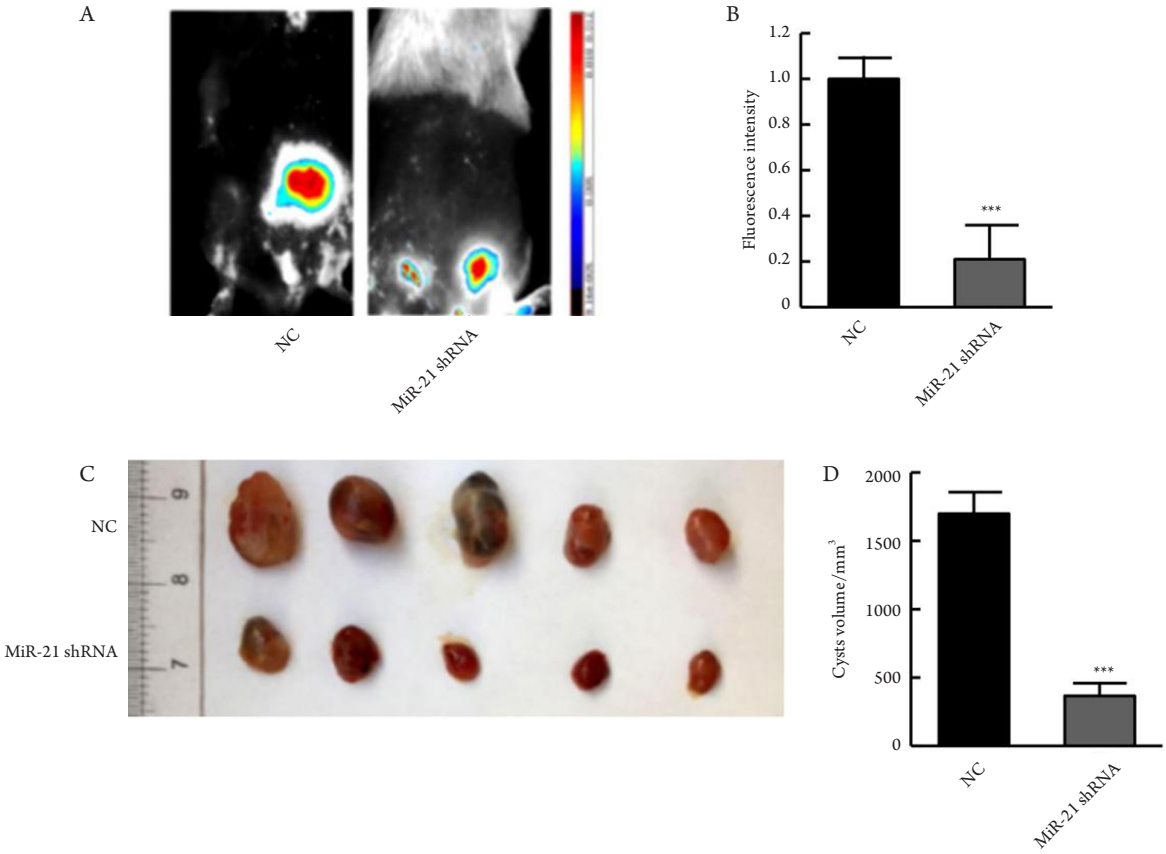

图4 MiR-21敲低在体内实验抑制异位子宫内膜组织来源的EnSCs的生长

Figure 4 MiR-21 knockdown inhibits the growth of EnSCs derived from ectopic endometrial tissues in vivo

(A)皮下移植转染的EnSCs 18 d后，囊肿的近红外荧光成像代表性图像。(B)皮下移植miR-21转染的EnSCs后，总荧光强度明显降低。(C，D)囊肿的宏观图像(C)和体积(D)。n=5，***$P<0.001$ vs NC group.

(A) Representative image of near-infrared fluorescence imaging of cysts after18 days of subcutaneous transplantate transfected EnSCs. (B) Total fluorescence intensity significantly reduced in subcutaneous transplantate miR-21 transfected EnSCs. (C,D) Macroscopic images (C) and volume of cysts (D). n=5, ***$P<0.001$ vs NC group.

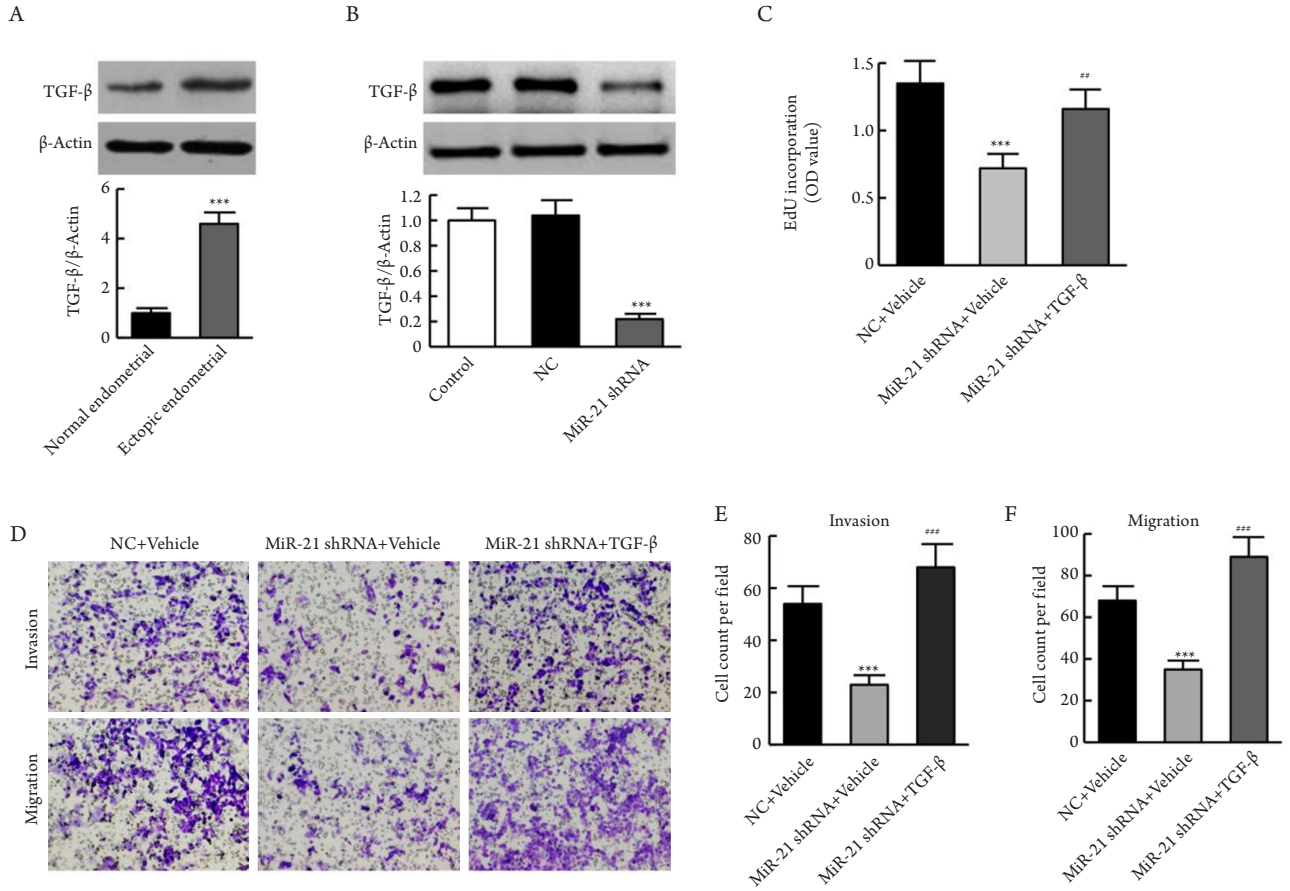

图5 miR-21敲低抑制EnSCs增殖、迁移与侵袭的作用可能与下调TGF-β表达相关

Figure 5 MiR-21 knockdown inhibits the proliferation, migration and invasion of EnSCs may via downregulation of TGF-β expression

(A)异位子宫内膜组织来源的EnSCs的TGF-β相对蛋白质表达水平。n=8，与异位子宫内膜组织来源的EnSCs比较，$^{***}P$<0.001。(B) Lenti-miR-21 shRNA-GFP 转染的EnSCs的TGF-β相对蛋白质表达水平。(C)NC+vehicle，miR-21 shRNA+vehicle和miR-21 shRNA+TGF-β组的EnSCs的增殖。(D~F)NC+vehicle、miR-21 shRNA+vehicle和miR-21 shRNA+TGF-β组的EnSCs的侵袭(D，E)与迁移(D，F)(×200)。n=4，与NC组或NC+vehicle组比较，$^{***}P$<0.001；与miR-21 shRNA+vehicle组比较，$^{##}P$<0.01。

(A) Relative protein expression levels of TGF-β in EnSCs derived from ectopic endometrial tissues, n=8, $^{***}P$<0.001 vs EnSCs derived from normal endometrial tissues. (B) Relative protein expression levels of TGF-β in Lenti-miR-21 shRNA-GFP transfected EnSCs. (C) Proliferation of EnSCs in NC+vehicle, miR-21 shRNA+vehicle and miR-21 shRNA+TGF- β groups. (D, E, F) Invasion(D, E) and migration (D, F) ability of EnSCs in NC+vehicle, miR-21 shRNA+vehicle and miR-21 shRNA+TGF-β groups (×200). N=4, $^{***}P$<0.001 vs NC group or NC+vehicle group. $^{##}P$<0.01, $^{###}P$<0.001 vs miR-21 shRNA+vehicle group.

3 讨论

子宫内膜异位症是育龄妇女的常见疾病，该疾病影响10%~15%的育龄妇女，并且患有子宫内膜异位症相关的不育和/或疼痛的妇女患病率高达35%~50%[1-3]。EnSCs异常是子宫内膜异位症的重要发病机制之一[5,12]。研究子宫内膜异位症发生发展过程中EnSCs的变化情况，对研究子宫内膜异位症有重要的参考意义。在本次实验通过异位内膜和正常内膜的EnSCs，检测发现miR-21在异位内膜来源的EnSCs中异常高表达。并且miR-21敲低后，体外实验发现异位内膜来源的EnSCs的增殖、迁移和侵袭受到抑制；体内实验发现EnSCs生长也发现受到抑制。

有研究[13-14]发现：TGF-β刺激能通过激活TGF-β/Smads和TGF-β/受体信号分子等信号通

路促进细胞增殖、迁移、侵袭与上皮间质转化(epithelial-mesenchymal transition，EMT)，而抑制TGF-β表达后，细胞的增殖、迁移、侵袭与EMT过程受到抑制。本研究同样采用了TGF-β刺激异位内膜来源的EnSCs，发现TGF-β刺激同样能增加EnSCs增殖、迁移和侵袭。本研究为分析miR-21敲低抑制EnSCs生长、迁移与侵袭的机制，进一步检测异位内膜和正常内膜来源的EnSCs，发现异位内膜和正常内膜来源的EnSCs中TGF-β表达异常高表达。同时，在miR-21敲低的异位内膜来源的EnSCs中发现TGF-β表达受到抑制。提示miR-21敲低对异位内膜来源的EnSCs增殖、迁移与侵袭的抑制作用可能与降低TGF-β表达有关。

本实验不足的地方在于没有直接干预TGF-β表达来论证TGF-β通路是miR-21促进EnSCs生长的必须通路，另外miR-21与TGF-β表达是直接关系还是间接关系也需要更多的实验论证。总之，本研究的目前结果显示：miR-21在异位内膜来源的EnSCs中高表达，而敲低miR-21表达，能抑制异位内膜来源的EnSCs增殖、迁移与侵袭。

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