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MiR-133b通过抑制SOX4调节膀胱癌细胞的增殖、克隆形成和侵袭能力

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[摘 要] **目的：**膀胱癌是泌尿系统最常见的恶性肿瘤之一，但其发病机制仍不十分清楚。虽然研究表明miR-133b可调控膀胱癌的发生，但其机制尚未明确。性别决定基因-区域转录因子4(sex determining region Y-box transcriptim factor 4，SOX4)在肿瘤的发生中起至关重要的作用，目前尚不清楚在膀胱癌中miR-133b与SOX4是否存在相互作用。本研究旨在探究膀胱癌组织和细胞中miR-133b和SOX4的表达情况及其对膀胱癌细胞增殖、克隆形成和侵袭能力的调控作用，并探讨在调节膀胱癌细胞生物特性过程中miR-133b与SOX4的相互关系。**方法：**获取2015年1至6月在中南大学湘雅二医院行手术切除的10例患者的膀胱癌及邻近癌旁组织标本，分别采用real-time PCR和蛋白质印迹法检测膀胱癌组织、癌旁组织、膀胱癌细胞系及正常膀胱上皮细胞中miR-133b的mRNA表达水平和SOX4的蛋白质表达水平。利用数据库TargetScan预测SOX4与miR-133b的相关性。利用脂质体Lipofectamine 2000为载体，将miR-133b模拟物、抑制物或SOX4的短发夹RNA(short hairpin RNA，shRNA)转染至T24细胞。应用双荧光素酶报告基因检测系统检测高表达miR-133b对T24细胞表达SOX4的影响。体外培养T24细胞14 d，计数培养克隆数并计算克隆形成率。使用CCK-8试剂盒检测T24细胞在不同时间点(0、12、24、48、72、96 h)的增殖情况。应用Transwell检测T24细胞的侵袭能力。**结果：**与癌旁组织和正常膀胱上皮细胞比较，膀胱癌组织和膀胱癌细胞系均低表达miR-133b、高表达SOX4，并且*miR-133b* mRNA与SOX4蛋白质在膀胱癌组织中的表达水平呈负相关(r=−0.84)。TargetScan数据库预测SOX4存在miR-133b结合位点，miR-133b模拟物转染可抑制膀胱癌T24细胞内SOX4的表达。MiR-133b模拟物或shRNA-SOX4转染均能抑制膀胱癌T24细胞的体外增殖、克隆形成和侵袭能力。相对于转染miR-133b抑制物，miR-133b抑制物和shRNA-SOX4共转染能够显著抑制膀胱癌T24细胞的增殖、克隆形成和侵袭能力。**结论：**MiR-133b和SOX4均能参与调控膀胱癌细胞的增殖、克隆形成和侵袭能力，并且miR-133b可以通过抑制SOX4的表达从而抑制膀胱癌细胞的增殖、克隆形成和侵袭能力。

[关键词] 膀胱癌；miR-133b；性别决定基因-区域转录因子4

MiR-133b regulates the proliferation, colony formation, and invasion of bladder cancer cells via inhibiting SOX4

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ABSTRACT

Objective: Bladder cancer is one of the most common urothelial tumors with high incidence and mortality rates. Although it has been reported that microRNA (miR)-133b can regulate tumorigenesis of bladder cancer, the mechanism remains unclear. Sex-determining region Y-box transcription factor 4 (SOX4) exhibits an important role in tumorigenesis, but it is unclear whether SOX4 and miR-133b are associated with regulation of pathogenesis of bladder cancer. This study aims to determine the expressions of SOX4 and miR-133b in bladder cancer tissues and cells, investigate their effects on the proliferation, colony formation, and invasion of bladder cancer cells, and to explore the association between miR-133b and SOX4 in regulating biological featurss of bladder cancer cells.

Methods: The bladder cancer and adjacent tissue samples of 10 patients who underwent surgical resection in the Second Xiangya Hospital of Central South Universty from Januray to June 2015 were obtained. The levels of miR-133b were tested by real-time PCR, and the protein levels of SOX4 were evaluated using Western blotting in bladder cancer tissues, matched adjacent tissues, and cell lines. The correlation between miR-133b expression and SOX4 expression in bladder cancer tissues was analyzed. Using the online database TargetScan, the relationship between SOX4 and miR-133b was predicted. MiR-133b mimics, miR-133b inhibitor, and short hairpin RNA (shRNA)-SOX4 were transfected into T24 cells by Lipofectamine 2000. The relationship between miR-133b and SOX4 was also verified by a dual-luciferase reporter assay. The proliferation of T24 cells cultured for 0, 12, 48, 72, and 96 h was evaluated by cell counting kit-8 (CCK-8) assay. The colony formation capacity of bladder cancer cells was tested after 14-day culture, and cell invasion capacity was evaluated with Transwell invasion assay.

Results: Bladder cancer tissue and bladder cancer cells had low level of miR-133b but high level of SOX4, compared with matched adjacent tissues and normal bladder epithelial cells. A negative correlation between *miR-133b* mRNA and SOX4 protein levels in bladder cancer tissues was also found (r=−0.84). The results of online database TargetScan showed that miR-133b targets at SOX4, and overexpression of miR-133b significantly attenuated the expression of SOX4 in T24 cells. Both overexpression of miR-133b and knockdown of SOX4 significantly inhibited the proliferation, colony formation, and invasion capacity of bladder cancer cells in vitro. SOX4 down-regulation restored the effects of miR-133b inhibitor on the proliferation, colony formation, and invasion capacity of T24 cells.

Conclusion: The up-regulation of SOX4 contributes to the progression of bladder cancer, and miR-133b can regulate the proliferation, colony formation, and invasion of bladder cancer cells via inhibiting SOX4.

KEY WORDS bladder cancer; miR-133b; sex determining region Y-box transcription factor 4

膀胱癌是世界范围内发病率和病死率均较高的恶性肿瘤之一[1-2]。2012年，全世界新增膀胱癌患者43万例，同年死于膀胱癌的患者16.5万例[2]。然而，目前对于膀胱癌发生发展机制的了解仍然十分有限。微RNA(microRNA，miRNA，miR)是一组短的非编码RNA，其能调节细胞的多种生物学活动，包括发

育、生长、分化和凋亡等[3-4]。此外，miRNA还能够通过抑制靶基因调节恶性肿瘤的发生发展[3, 5]。MiR-133b参与肿瘤的发生和转移，大多数恶性肿瘤中miR-133b的表达下调，如乳腺癌[6]、非小细胞肺癌[7]、结直肠癌[8]、食管鳞状细胞癌[9]和胶质瘤[10]。在膀胱癌中，miR-133b的表达水平也呈下调状态[11-14]。关于miR-133b与膀胱癌进展之间的关系，既往的研究结论并不一致。Pignot等[11]发现miR-133b的下调与膀胱癌的病理分期并无关联；但有研究[12, 15]发现miR-133b的异常下调与膀胱癌的侵袭性病理特征和不良预后显著相关。在作用机制方面，研究[13]发现miR-133b通过抑制在膀胱癌组织和细胞系中高度表达的B细胞淋巴因子-W(B cell lymphocyte factor W，Bcl-W)和丝氨酸/苏氨酸蛋白激酶1(AKT serine/threonine kinase 1，Akt1)从而调节膀胱癌细胞的增殖和凋亡。然而，目前关于miR-133b调节膀胱癌细胞生物学行为的机制尚未明确。

性别决定基因-区域转录因子4(sex determining region Y-box transcription factor 4，SOX4)是SOX转录因子C亚家族成员。SOX4在肿瘤的发生中起重要的作用[16]。过表达SOX4能够促进癌细胞向上皮间充质细胞转化及其侵袭和转移能力[17]。膀胱癌细胞中高表达的SOX4与癌症分期、患者的低生存率及病灶的侵袭性相关[18-19]。越来越多的证据[20-23]表明，在多种癌细胞中SOX4是miRNA的潜在调控靶点。但目前尚不清楚在膀胱癌中miR-133b与SOX4是否存在相互作用。本研究旨在探究膀胱癌组织和细胞中miR-133b和SOX4的表达情况及其对膀胱癌细胞增殖、克隆形成和侵袭能力的调控作用，并进一步探讨miR-133b在调节膀胱癌细胞生物特性过程中与SOX4的相互关系。

1 材料与方法

1.1 标本来源

标本来自2015年1月至6月在中南大学湘雅二医院行手术切除的10例膀胱癌患者的癌组织及邻近癌旁组织，冻存于−80 ℃冰箱。

1.2 试剂

膀胱癌细胞系5637、T24和SW780以及正常膀胱上皮细胞系SV-HUC-1购自中国科学院细胞库；miRNeasy Mini试剂盒购自德国Qiagen公司；miRNA Q-PCR检测试剂盒购自美国GeneCopoeia公司；Lipofectamine 2000试剂购自美国Invitrogen公司；双荧光素酶报告基因检测系统(DLR™，E1960)购自美国Promega公司；胎牛血清购自美国Gibco公司；SOX4一抗、羊抗兔和羊抗鼠二抗购自英国Abcam公司；GAPDH一抗购自美国SANTA公司；Transwell 6孔板购自美国Corning公司；Matrigel基底膜购自美国BD公司；CCK-8试剂盒购自上海碧云天生物技术有限公司；总蛋白质提取试剂盒购自美国ProMab公司。本研究获得了中南大学湘雅二医院伦理委员会批准(审批号：研179号)。所有患者签署知情同意书。

1.3 方法

1.3.1 细胞培养和转染

将细胞用含10%胎牛血清的DMEM培养基，在37 ℃、5% CO_2条件下进行培养。由上海GenePharma公司合成miRNA-133b模拟物、抑制物、阴性对照(NC)和SOX4短发夹RNA[(short hairpin RNA，shRNA)-SOX4]。使用Lipofectamine 2000™试剂将miRNA-133b模拟物、抑制剂、反义RNA(RNAi)和shRNA-SOX4转染至T24细胞。相应序列如下：MiRNA-133b模拟物，5'-UUUGGUCCCCUUCAACCAGCUA-3'；miRNA-133b抑制物，5'-UAGCUGGUGAGGCAAA-3'；RNAi，5'-UUCUCCGAACGUGUCACGUTT-3'；shRNA-SOX4，5'-GACAGCGAGATCCCTTC-3'。

1.3.2 Real-time PCR检测*miR-133b*基因表达量

根据miRNeasy Mini试剂盒说明从膀胱癌组织、癌旁组织及细胞(膀胱癌细胞系5637、T24和SW780及正常膀胱上皮细胞系SV-HUC-1)中提取总RNA。然后用miRNA Q-PCR检测试剂盒进行反转录及PCR定量检测。*MiR-133b*引物，正向5'-GTTTTGCCCCTTCAAC-3'，反向5'-TTTGGCACTA-GCACATT-3'；*U6*引物，正向5'-CTCGCTTCGGC-AGCA-3'，反向5'-AACGCTTCACGAATTTGCGT-3'。应用$2^{-\Delta\Delta Ct}$法计算*miR-133b*基因的相对表达量。

1.3.3 体外培养检测膀胱癌T24细胞的增殖能力

转染后，将T24细胞按1×10^5/孔的数量接种于96孔板中。按照说明书，用CCK-8试剂盒检测T24细胞在不同时间点(0、12、24、48、72、96 h)的增殖情况。

1.3.4 克隆形成实验检测膀胱癌T24细胞的克隆形成能力

转染后48 h，将T24细胞接种到直径6 cm的培养皿中，每皿1×10^4个细胞。培养14 d后，用戊二醛固定细胞，在室温下用0.5%结晶紫染色10 min。计数克隆形成数并计算克隆形成率：克隆形成率=(菌落数/种子细胞数)×100%。

1.3.5 Transwell侵袭实验检测膀胱癌T24细胞的侵袭能力

将T24细胞密度调整至5×10⁴/mL并接种到Transwell上室，下室充满培养基。24 h后，用95%乙醇固定细胞15 min，以苏木精染色10 min，于200倍倒置显微镜下计数染色细胞。

1.3.6 双荧光素酶报告基因检测系统分析miR-133b和SOX4的调节关系

利用在线数据库TargetScan预测SOX4是否存在miR-133b结合位点。双荧光素酶报告分析用于检测miR-133b是否直接靶向SOX4。构建报告基因*SOX4*：5'-AAAAAAACAAUUCAGGACCAAA-3'。转染24 h前将T24细胞以5×10⁴/孔的密度接种于48孔板中。转染时根据Lipofectamine 2000™试剂盒说明书将500 ng *SOX4*报告基因、0.75 μL *miR-133b*模拟物和NC进行转染。转染后48 h，收集细胞裂解物，并使用双荧光素酶报告基因检测系统检测萤火虫荧光素酶和Renilla荧光素酶的荧光活性值，计算萤火虫荧光素酶/Renilla荧光素酶的相对荧光值。

1.3.7 蛋白质印迹法检测膀胱癌组织和细胞系中SOX4蛋白质表达量

使用总蛋白质提取试剂盒从膀胱癌组织或细胞系中提取总蛋白质，经SDS-PAGE电泳后，使用Trans-Blot® Turbo™全能型蛋白质转印系统(美国BIO-RAD公司)在100 V下转膜70 min。在室温下，用5%脱脂奶粉封闭2 h后，用TBST缓冲液(含10 mmol/L Tris，pH值8.0，150 mmol/L NaCl，0.5%吐温20)洗膜3次，并在4 ℃摇瓶中与SOX4一抗(1∶1 000)或GAPDH一抗(1∶800)孵育过夜。用TBST洗涤3次后，将膜与羊抗兔抗体(1∶4 000)或羊抗鼠二抗(1∶8 000)在室温下孵育1 h。用ImageQuant LAS4000化学发光成像分析仪(美国GE公司)检测蛋白质条带。利用Image J软件计算SOX4蛋白质的相对表达量。

1.4 统计学处理

使用Graphpad Prism 6.01软件进行统计分析及制作统计图。计量资料以均数±标准差($\bar{x}\pm s$)表示，2组比较采用t检验。MiR-133b与SOX4表达量的相关性分析采用Spearman秩相关。P<0.05为差异具有统计学意义。

2 结 果

2.1 *MiR-133b*在膀胱癌组织和细胞系中表达下调

在膀胱癌组织中*miR-133b* mRNA水平低于癌旁组织(1.55±0.42 vs 2.85±0.80，P<0.01；图1A)。与SV-HUC-1细胞(3.28±0.32)比较，5637细胞(2.19±0.49)、T24细胞(1.01±0.10)和SW780细胞(1.81±0.26)中的*miR-133b* mRNA水平均下降(均P<0.05)，且以T24细胞下降最多(图1B)。

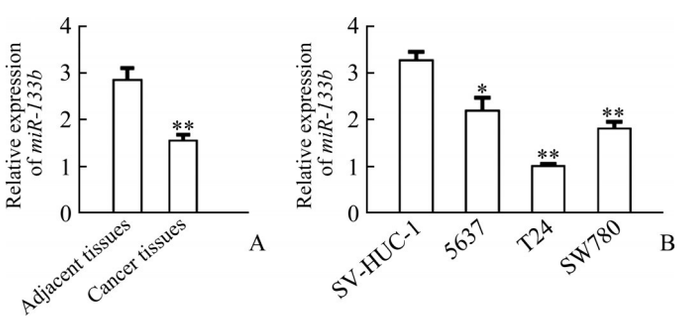

图1 *MiR-133b*在膀胱癌组织和膀胱癌细胞系中的表达水平降低

Figure 1 Down-regulation of *miR-133b* in bladder cancer tissues and cell lines

A: Comparison of the level of *miR-133b* in bladder cancer tissues and adjacent tissues; B: Comparison of the level of *miR-133b* in bladder cancer cell lines 5637, T24, SW780 and normal bladder epithelial cell line SV-HUC-1. *P<0.05, **P<0.01 vs adjacent tissues or SV-HUC-1 cells.

2.2 MiR-133b对膀胱癌T24细胞增殖、集落形成和侵袭能力的影响

与NC组相比，转染miR-133b模拟物能够显著抑制体外培养12 h(0.48±0.01 vs 0.51±0.02，P<0.05)、24 h(0.50±0.01 vs 0.57±0.02，P<0.01)、48 h(0.53±0.01 vs 0.64±0.02，P<0.01)、72 h(0.57±0.02 vs 0.79±0.01，P<0.01)和96 h(10.55±0.02 vs 0.92±0.01，P<0.01)时T24细胞的增殖(图2A)、克隆形成率[(4.43±

0.11)% vs (6.55±0.07)%，P<0.01；图2B]及侵袭能力[(35.67±2.08)个 vs (44.33±4.04)个，P<0.05；图2C]。

2.3 SOX4在膀胱癌组织和细胞系中的表达上调

与癌旁组织(0.15±0.09)相比，膀胱癌组织中SOX4的蛋白质表达水平显著上调(0.35±0.08，P<0.01；图3A、3B)。与SV-HUC-1细胞(0.16±0.34)相比，T24细胞中SOX4的表达水平也上调(0.65±0.12，P<0.01；图3C、3D)。

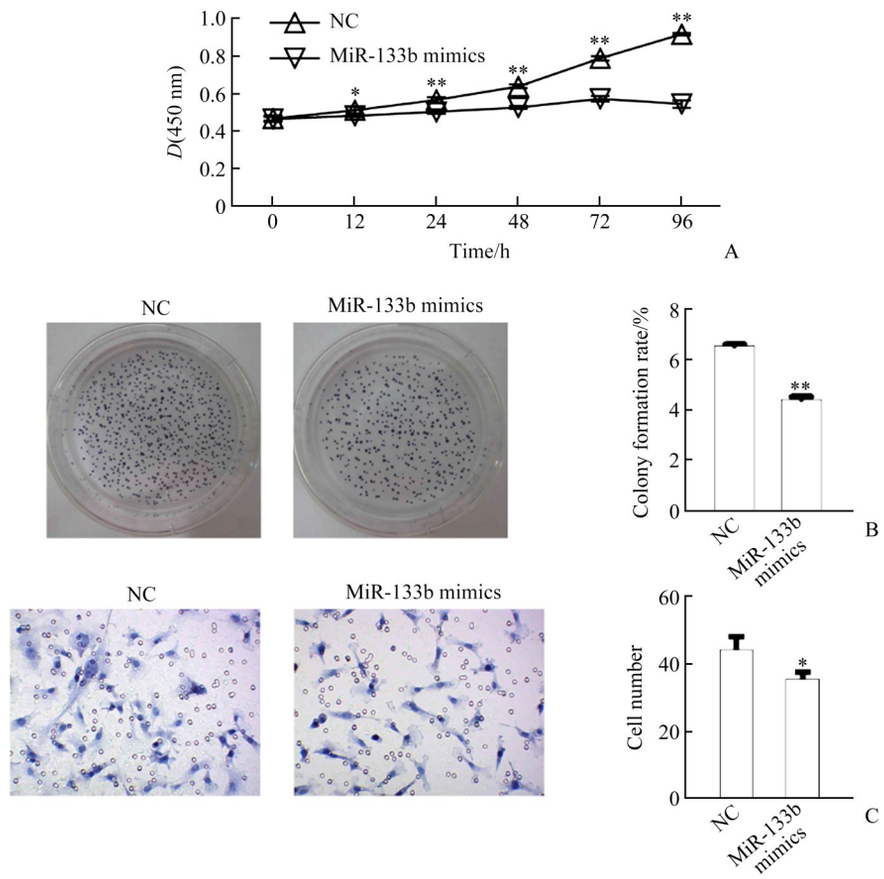

图2　过表达miR-133b抑制膀胱癌T24细胞的增殖、克隆形成和侵袭能力

Figure 2　Overexpression of miR-133b inhibits the proliferation, colony formation, and invasion

A: Proliferation rate of T24 cells treated with NC or miR-133b mimics transfection at different time of cell culture; B: Colony formation rate of T24 cells treated with NC or miR-133b mimics transfection; C: Invasion capacity of T24 cells treated with NC or miR-133b mimics transfection. *P<0.05, ** P<0.01 vs NC group.

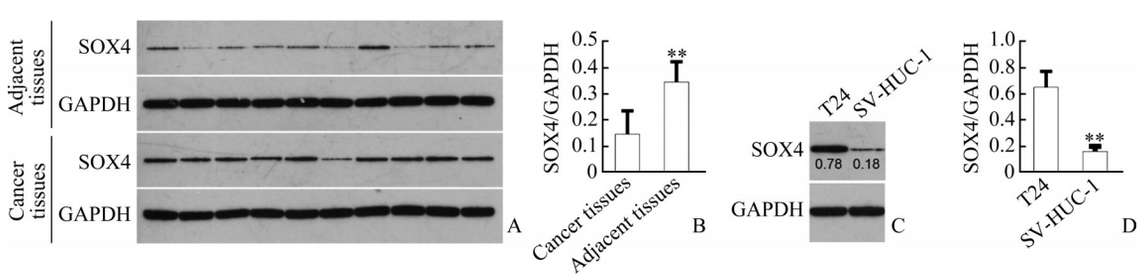

图3　膀胱癌组织和细胞系高表达SOX4

Figure 3　Up-regulated SOX4 expression in bladder cancer tissues and cells

A: Western blotting results of SOX4 protein in bladder cancer tissues and adjacent tissues; B: Relative expression of SOX4 protein in bladder cancer tissues and adjacent tissues; C: Western blotting results of SOX4 in SV-HUC-1 cells and T24 cells; D: Relative expression of SOX4 in SV-HUC-1 cells and T24 cells. **P<0.01 vs adjacent tissues or T24 cells.

2.4 SOX4促进膀胱癌细胞增殖、克隆形成和侵袭

与阴性对照(NC)相比，shRNA-SOX4转染显著抑制T24细胞的生物学特性，包括：体外培养12 h(0.44±0.01 vs 0.47±0.01，P<0.01)、24 h(0.46±0.01 vs 0.57±0.01，P<0.01)、48 h(0.50±0.01 vs 0.64±0.01，P<0.01)、72 h(0.52±0.01 vs 0.79±0.01，P<0.01)和96 h(0.50±0.01 vs 0.92±0.01，P<0.01)时的细胞增殖(图4A)、克隆形成率[(6.48±0.06)% vs (3.69±0.39)%，P<0.01；图4B)]及侵袭能力[(45.33±3.86)个 vs (17.00±1.63)个，P<0.01；图4C)。

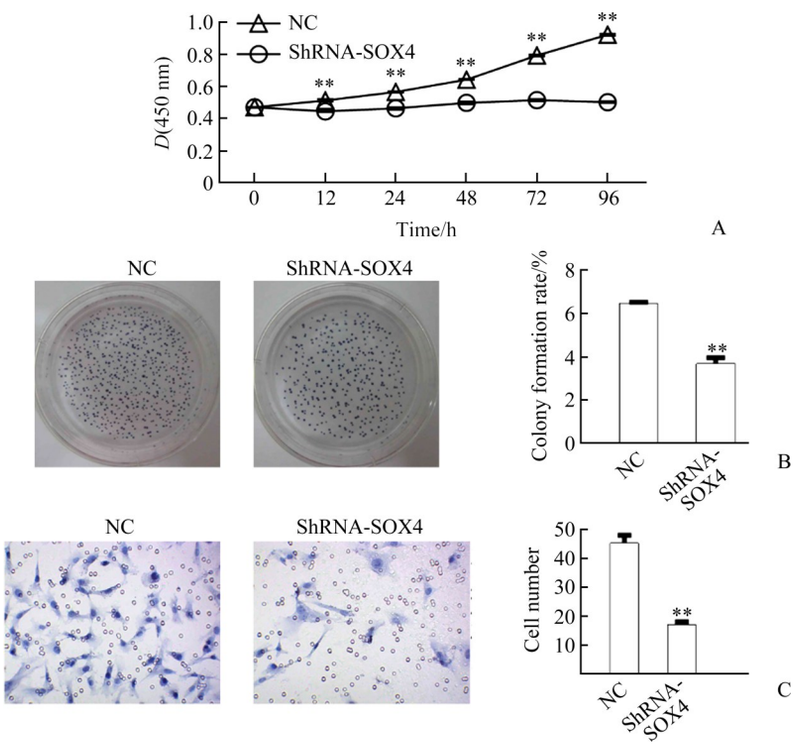

图4 SOX4促进膀胱癌细胞的增殖、克隆形成和侵袭能力

Figure 4 SOX4 promotes the proliferation, colony formation, and invasion of bladder cancer cells

A: Proliferation of T24 cells transfected with negative control (NC) or shRNA-SOX4 at different time of cell culture; B: Colony formation rate of T24 cells after negative control (NC) or shRNA-SOX4 transfection; C: Invasion capacity of T24 cells after negative control (NC) or shRNA-SOX4 transfection. **P<0.01 vs NC group.

2.5 *MiR-133b*抑制膀胱癌细胞中SOX4的表达

在膀胱癌组织中*miR-133b* mRNA相对表达量与SOX4蛋白相对表达量呈负相关(r=−0.84，P<0.01；图5A)。在线数据库TargetScan预测到SOX4存在*miR-133b*结合位点(图5B)。双荧光素酶报告系统发现*miR-133b*模拟物显著抑制荧光素酶活性(1.00±0.10 vs 0.16±0.01，P<0.01；图5C)。与NC相比，miR-133b模拟物转染显著抑制T24细胞中的SOX4蛋白质水平(图5D)。

2.6 MiR-133b通过抑制SOX4调节膀胱癌细胞的增殖、克隆形成和侵袭能力

与单转染miR-133b抑制物相比，miR-133b抑制物和shRNA-SOX4共转染显著抑制T24细胞在培养12 h(0.45±0.01 vs 0.53±0.01，P<0.01)、24 h(0.47±0.01 vs 0.59±0.01，P<0.01)、48 h(0.50±0.01 vs 0.69±0.01，P<0.01)、72 h(0.53±0.01 vs 0.88±0.01，P<0.01)和96 h(0.51±0.01 vs 0.83±0.01，P<0.01)的增殖(图6A)、克隆形成率[(3.50±0.10)% vs (8.32±0.08)%，P<0.01]和侵袭能力[(15.33±2.87)个 vs (70.33±3.69)个，P<0.01；图6B、6C]。

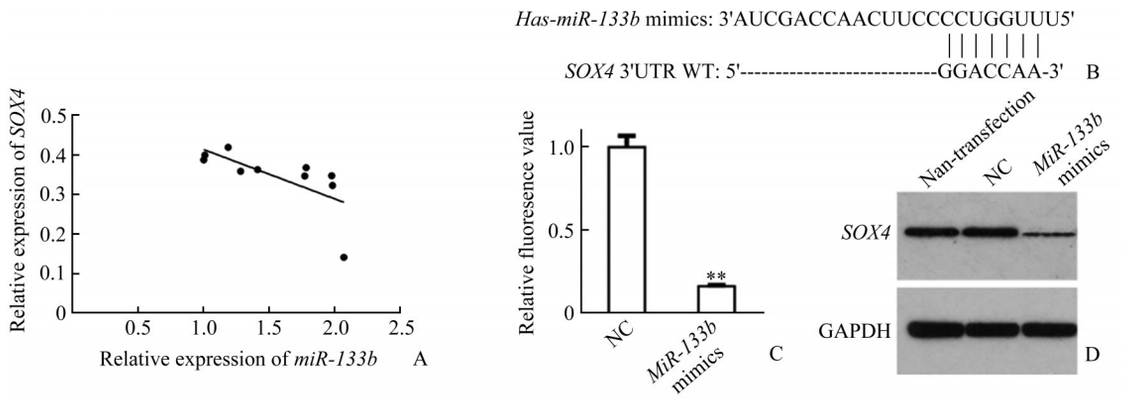

图5　*MiR-133b*抑制膀胱癌细胞中*SOX4*的表达

Figure 5　*MiR-133b* inhibits *SOX4* expression in bladder cancer cells

A: *MiR-133b* levels are negatively correlated with the protein level of *SOX4* in bladder cancer tissues. B: Binding target was predicted using the online database TargetScan. C: Renilla and firefly luciferase activities in T24 cells were measured after transfected with *miR-133b* mimics or negative control (NC). ***P*<0.01 vs the NC group. D: Levels of *SOX4* in T24 cells after transfected with *miR-133b* mimics or negative control (NC) were detected with Western blotting.

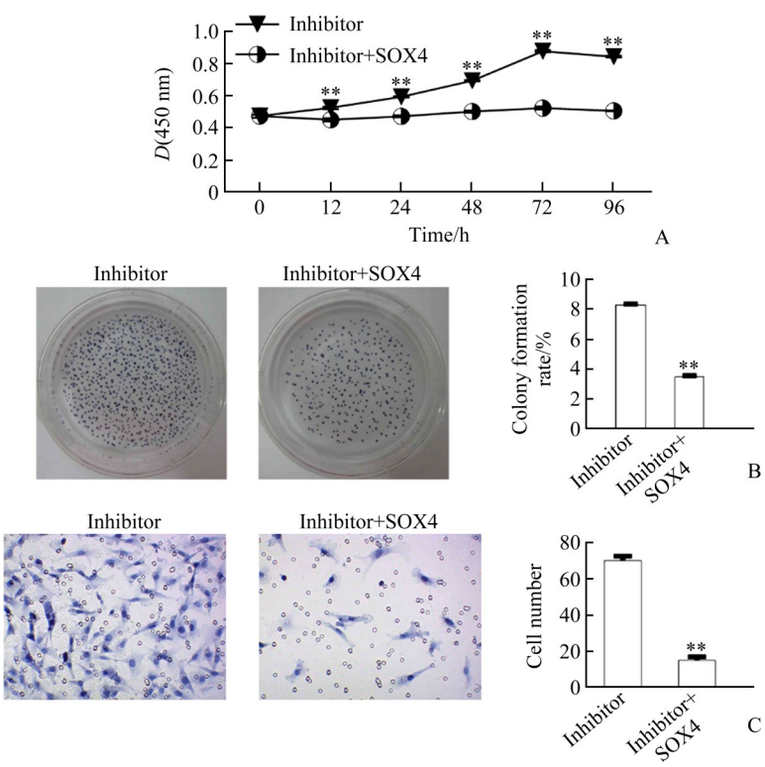

图6　MiR-133b通过SOX4调节膀胱癌细胞的增殖、克隆形成和侵袭能力

Figure 6　MiR-133b regulates the proliferation, colony formation, and invasion capacity of bladder cancer cells via SOX4

A: Proliferation of T24 cells treated with miR-133b-inhibitor (inhibitor) or miR-133b-inhibitor together with shRNA-SOX4 (inhibitor+SOX4) transfection at different time of cell culture; B: Colony formation rate of T24 cells after miR-133b-inhibitor (inhibitor) or miR-133b-inhibitor together with shRNA-SOX4 (inhibitor+SOX4) transfection; C: Invasion capacity of T24 cells after miR-133b-inhibitor (inhibitor) or miR-133b-inhibitor together with shRNA-SOX4 (inhibitor+SOX4) transfection. ***P*<0.01 vs miR-133b-inhibitor.

3 讨 论

深入探索癌细胞中异常表达的miRNA对膀胱癌发生发展的调节作用十分重要。Yoshino等[24]总结了多种在膀胱癌组织和细胞中异常表达的miRNA，包括miR-133b、miR-145-5p、miR-125b、miR-133a、miR-182。也有研究[12-15, 25]发现低水平表达的miR-133b对膀胱癌的发生发展具有重要的调节作用。本研究证实在膀胱癌细胞中低表达的miR-133b参与调节癌细胞的克隆形成、侵袭和增殖能力；miR-133b可能通过抑制SOX4调节膀胱癌细胞的生物学特性。

MiR-133b的低表达与肿瘤转移、侵袭和总生存率降低等不良临床预后相关[12-13, 15]。本研究证实膀胱癌组织及细胞系中均低表达miR-133b，而过表达miR-133b能够显著抑制膀胱癌细胞的克隆形成、侵袭和增殖能力，与此前的研究[11-14]结果一致。迄今为止，在膀胱癌细胞中已经发现了一些miR-133b的靶基因，如表皮生长因子受体、细胞外信号调节激酶、基质金属蛋白酶-2、Akt、Bcl-W和转凝胶蛋白2[13-14, 25]。但目前对于miR-133b调节膀胱癌细胞生物学特性机制的了解仍十分有限。

本研究证实在膀胱癌组织和细胞系中SOX4呈高表达，与以往研究[18-19, 26]观点一致。在肿瘤中，SOX4能够调节涉及转录、信号通路、细胞生长和凋亡的功能基因，也是许多miRNA的作用靶点[26]。MiR-212/132簇和miR-335可以通过靶向SOX4从而抑制乳腺癌的转移和迁移[20-21]。抑制miR-129-2能够增强子宫内膜癌细胞中SOX4的表达[22]。Dyrskjϕt等[27]发现miR-129可以通过增强SOX4的表达从而促进膀胱癌的生长和癌细胞的存活。本研究结果显示，干扰SOX4能够显著抑制膀胱癌细胞的增殖、克隆形成和侵袭能力。

MiR-133b和SOX4在膀胱癌细胞中的表达及作用特点提示二者存在调控关系。本研究利用在线数据库TargetScan预测到二者存在联系，也发现二者在膀胱癌组织中的表达呈负相关，并且进一步证实在膀胱癌细胞中过表达miR-133b能够抑制SOX4的表达。在功能调控方面，相对于转染miR-133b抑制物，miR-133b抑制物和shRNA-SOX4共转染能够显著抑制膀胱癌细胞的增殖、克隆形成和侵袭能力，证实miR-133b可以通过SOX4调节膀胱癌细胞。

综上所述，膀胱癌细胞低表达miR-133b，但高表达SOX4，这两种改变在膀胱癌细胞的增殖、克隆形成和侵袭中都具有重要作用；miR-133b可以通过抑制SOX4从而抑制膀胱癌细胞的增殖、克隆形成和侵袭能力。

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