

doi: 10.13241/j.cnki.pmb.2019.06.008

siRNA 干扰 TCAB1 对人主动脉平滑肌增殖的影响及机制*

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摘要 目的:探究 TCAB1 沉默对人主动脉平滑肌细胞(HASMC)增殖的影响及可能机制。**方法:**采用 RNAi 技术设计并合成靶向沉默 TCAB1 基因表达的三对特异性 siRNA 序列 (siTCAB1-331、siTCAB1-619、siTCAB1-749) 和一对阴性对照序列 (NC), 使用 lipo2000 将 siTCAB1、NC 转染 HASMC, 分为 3 个组: 干扰组 (siTCAB1)、空白对照组 (BC)、阴性对照组 (NC), 转染 24 小时倒置荧光显微镜观察细胞转染情况; 通过 RT-qPCR 和 Western blot 从 3 个干扰靶点中筛选效果最好的干扰靶点。进一步转染 siTCAB1-749 后, MTS 检测 HASMC 24、48、72 h 的增殖能力, 48 小时用 RT-qPCR 和 Western blot 检测 CyclinD1 表达量变化, 流式细胞术检测 HASMC 的细胞周期变化。**结果:**RT-qPCR 和 WB 结果显示 siTCAB1-749 为最好的干扰靶点; 转染 24、48、72 h 后, siTCAB1-749 组增殖水平明显低于 NC 组、BC 组 ($P<0.05$)。流式结果显示: siTCAB1-749 组处于 G1 期细胞比率有所增加, 处于 S 期细胞比率减少 ($P<0.05$), 且 siTCAB1-749 组细胞周期蛋白 cyclinD1 表达也下降 ($P<0.05$)。**结论:**沉默 TCAB1 能抑制 HASMC 的增殖, 其机制可能与阻碍细胞周期蛋白 cyclinD1 有关。

关键词:平滑肌细胞; 端粒酶; 卡扎尔体蛋白 1; 细胞增殖; 细胞周期

中图分类号: R-33; R329.2; R543.5 **文献标识码:** A **文章编号:** 1673-6273(2019)06-1039-05

The Effect and Mechanism of siRNA-targeting TCAB1
on the Proliferation of HASMC*ZHENG Xue-zhong¹, GAO Jing², CHEN Yu-hao³, LI Qing-qing⁴, WANG En-yang⁴, WANG Yi-xuan⁴, WANG-Hong^{4△}

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ABSTRACT Objective: To investigate the effect and potential mechanism of silencing TCAB1 on the proliferation of human aortic smooth muscle cells (HASMC). **Methods:** Three siRNA sequences (siTCAB1-331, siTCAB1-619, siTCAB1-749) targeting TCAB1 and one negative sequence (NC) were designed, synthesized and then transfected into HASMC with Lipofectamine-TM2000. HASMC were divided three groups: interference group, blank control group, negative control group. Cell transfection was observed under fluorescence microscope. The mRNA and protein expression levels of TCAB1 were detected by RT-qPCR and Western blotting, the best target was selected from the three interfering targets. The effective siRNA (siTCAB1-749) was further transfected into HASMC with Lipofectamine-TM2000. The changes of cell cycle and CyclinD1 expression of HASMC cells were assessed using flow cytometry, RT-qPCR and Western blotting, respectively. The proliferation of HASMC was assessed using MTS assay after transfecting siTCAB1-749 at 24 h, 48 h, 72 h. **Results:** RT-qPCR and Western blot indicated that the siTCAB1-749 was the best target in the three interfering targets. MTS revealed that the number of cell growth in siTCAB1-749 group was significantly lower than BC group and NC group at 24 h, 48 h, 72 h ($P<0.05$); the percentages of S phases in the cell cycle of siTCAB1-749 group was decreased and G1 phases was increased at 48 h ($P<0.05$). In addition, the expression of cyclinD1 was decreased in siTCAB1-749 group ($P<0.05$). **Conclusion:** Silencing TCAB1 can inhibit the proliferation of HASMC, and its mechanism may be related to the inhibition of Cell cycle protein D1.

Key words: Smooth muscle cells; Telomerase; TCAB1; Cell proliferation; Cell cycle

Chinese Library Classification (CLC): R-33; R329.2; R543.5 **Document code:** A

Article ID: 1673-6273(2019)06-1039-05

* 基金项目: 国家自然科学基金项目 (81270224)

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(收稿日期: 2018-06-18 接受日期: 2018-07-13)

前言

端粒是异染色质末端一段特殊线性结构,而细胞端粒酶是一种特殊核糖核蛋白酶,通过识别并结合于富含G的端粒末端,逆转录合成端粒,可维持端粒长度的稳定。一般认为端粒酶的组成,包括端粒酶逆转录酶(TERT)、端粒酶RNA和端粒酶相关蛋白等三部分组成^[1]。此外,与端粒酶复合体相关的蛋白还有Potin/reptin和端粒酶Cajal body蛋白1(TCAB1)^[2]。卡哈尔体(Cajal body, CB)是细胞核内核糖核蛋白加工的场所,TCAB1是端粒酶全酶的一个亚单位,在卡哈尔体含量丰富,TCAB1对端粒酶从卡哈尔体到端粒的运输是必需的^[3]。

端粒酶也表达于平滑肌细胞,研究表明端粒酶活性增加促进平滑肌细胞增殖,抑制端粒酶活性降低了血管平滑肌的生长^[4]。此外, Jacob等人报道高糖、高同型半胱氨酸能激活人主动脉平滑肌细胞端粒酶活性并促进其增殖^[5]。既往研究报道抑制TCAB1的表达能显著抑制肺癌、头颈部癌的增殖^[6,7],然而其在平滑肌中的调控机制是未知的。而抑制血管平滑肌细胞的增殖一直被认为是防治动脉粥样硬化(AS)形成及PCI术后狭窄的关键。因此,本研究主要探究了沉默TCAB1对HASMC增殖影响以及可能的分子机制,以期AS及PCI术后再狭窄的治疗提供新的思路。

1 材料与方法

1.1 主要材料

HASMC购自上海赛齐生物,胎牛血清购自sciencell公司,WRAP53兔多抗、cyclinD1鼠多抗、羊抗鼠IgG(H+L)二抗购于proteintech公司;GAPDH(兔多抗)及羊抗兔IgG(H+L)二抗购于碧云天生物技术公司;Lipo2000购于Invitrogen公司;Trizol、RT-PCR试剂盒、cellTiter96AQ(MTS)细胞增殖检测试剂购自美国Promega公司,FAM记的三对干扰序列(siTCAB1-331, siTCAB1-749, siTCAB1-749)及阴性对照序列、GAPDH引物序列购于上海吉码公司。

1.2 细胞培养和siRNA转染

用含10%胎牛血清的DMEM培养HASMC,置于37℃、5%CO₂浓度细胞培养箱中孵育,以0.25%胰酶消化细胞传代。取6-8代VSMC对数生长期的细胞接种于6孔板(约7×10⁴个/孔),不含抗生素10%血清DMEM培养,根据Lipo2000说明书,将siTCAB1-331, siTCAB1-749, siTCAB1-749及NC序列以90 nmol/L浓度转染HASMC,每组3个复孔。在37℃、5%CO₂条件下培养5 h后,换含10%FBS的DMEM继续培养^[8],转染24 h后,倒置荧光显微镜观察细胞,并拍照^[9]。

1.3 RT-qPCR检测TCAB1、cyclinD1 mRNA的表达

转染48 h后Trizol法常规提取RNA并定量,按照Promega反转试剂盒说明书进行反转录,反转录反应条件:42℃ 15 min, 70℃ 15 min, 4℃,得到的cDNA稀释4倍备用。通过查阅文献和pubmed-blast验证后确定TCAB1^[7]、CyclinD1^[10]引物并交由上海生工生物工程有限公司合成。TCAB1上游引物5'-CATATCTGGGACGCATTCACT-3',下游引物5'-GTTGAAGCCACAGAAGAGCTG-3';CyclinD1上游引物5'-CGTGGCCTCTAAGATGAAGGA-3',下游引物5'-CGGTG-

TAGATGCACAGCTTCTC-3';GAPDH上游引物5'-GTCATCAATGGAAATCCCATCA-3',下游引物5'-CCAGTGGACTCCACGACGTAC-3'。参照Promega荧光定量试剂盒说明书进行扩增,用CFX96实时荧光定量PCR仪完成,反应条件为10 min 95℃, 15 sec 95℃, 1 min 59℃共50个循环,基因的相对表达量用2^{-ΔΔCt}计算^[11]。

1.4 Western blot检测TCAB1、cyclinD1蛋白的表达

转染后48 h,提取细胞总蛋白并定量,各组上样量30 μg,经SDS-PAGE电泳后转膜,TBST漂洗3次,5%脱脂奶粉室温封闭1 h。封闭完成后,加入用TBST稀释的TCAB1(1:1000)、cyclinD1(1:2000)、GAPDH(1:5000)一抗,4℃孵育过夜,一抗孵育完成后,TBST漂洗3次,而后加入TBST稀释的相应二抗羊抗兔(1:1000)以及羊抗鼠IgG(H+L)(1:5000),室温下孵育1 h,二抗孵育完成后,TBST漂洗3次,加入ECL显色试剂,凝胶成像系统拍照。

1.5 MTS法检测HASMC的增殖

以3000个/孔细胞密度接种96孔板,每组设置6个平行孔。次日细胞生长融合至30%-50%时,以90 nmol/L浓度转染siRNA,转染方法同前。转染前(0 h)及转染后24、48、72 h,换上新鲜培养基100 μL,每孔加入MTS 20 μL培养箱孵育2 h后,在酶标仪上测490 nm吸光度值^[12]。

1.6 细胞周期检测

HASMC转染siRNA48 h后,收细胞,离心后70%的预冷乙醇固定过夜,离心、PBS洗涤后用含有PI、无DNA酶的RNA酶的预混液重悬细胞,置于暗室中37℃染色30分钟,上流式检测。细胞周期用ModFit LT软件分析^[13]。

1.7 统计学处理

统计学处理在SPSS 21.0软件包上进行。不同时间点测量细胞增殖采用重复测量资料^[14]。其它数据采用单因素方差分析,方差齐时选用LSD法进行组间多重比较,方差不齐时则选Tamhane's法进行比较^[15], $P<0.05$ 为有统计学差异。

2 结果

2.1 HASMC的培养和转染

第四代HASMC培养后第2天、第3天镜下图像(图a,b),倒置显微镜下细胞形态不一(多角形、梭形或不规则形),部分区域高低不等呈“峰、谷”状生长,符合平滑肌细胞的形态学特征。细胞转染siTCAB1和NC24 h后,用倒置荧光显微镜观察细胞可见大量绿色荧光(图d,e),表明siTCAB1转染成功。

2.2 TCAB1-siRNA干扰效果的筛选

转染48 h后,通过RT-qPCR检测各组TCAB1 mRNA的表达,三个干扰组中siTCAB1-331组、siTCAB1-749组mRNA表达水平下调($P<0.05$,图2a)。选取各组相对基因表达量均数和P值较小的siTCAB1-749组与BC组和NC组进行蛋白分析,Western blotting检测结果显示:转染TCAB1-siRNA-749组TCAB1表达水平明显低于NC组、BC组(图2b)。

2.3 沉默TCAB1表达对VSMC细胞的增殖影响

倒置显微镜下观察各组在转染后24 h、48 h细胞密度,结果显示:siTCAB1-749组24 h、48 h细胞密度和NC组、BC组比较显著减少(图3a);MTS检测法显示:不同时间点(24、48、

72 h), siTCAB1-749 组、NC 组、BC 组 HASMC 的 OD_{490} 值均随时间延长而增高 ($P<0.05$), 而转染 24、48、72 h 后, siTCAB1-749 组 OD_{490} 值明显低于 NC 组、BC 组 ($P<0.05$, 图 3b)。

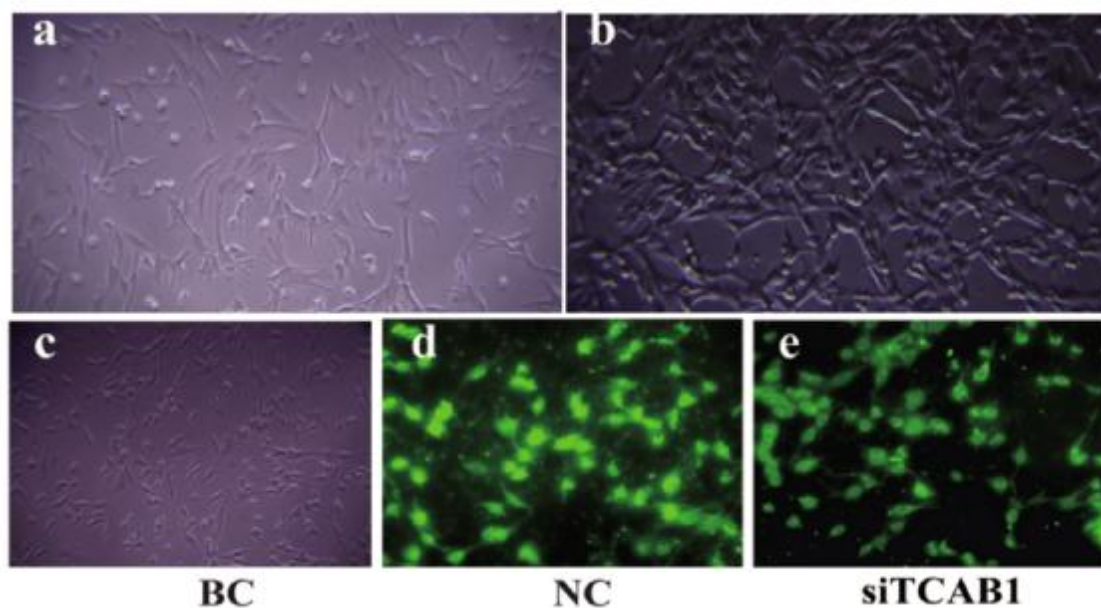


图 1 HASMC 的形态以及 siTCAB1、NC 序列在 HASMC 中的转染情况 (100 ×)

Fig. 1 The morphology of HASMC and the transfection of siTCAB1, NC in HASMC (100 ×)

注: 图 a, b 为第四代 HASMC 的形态 (100 ×); 图 d, e 为荧光显微镜下 siTCAB1、NC 序列的转染情况。

Note: The morphology of HASMC in four passages (100×) (Fig. a, b);

The transfection among the BC, NC and siRNA groups using a fluorescence microscope (Fig. d, e).

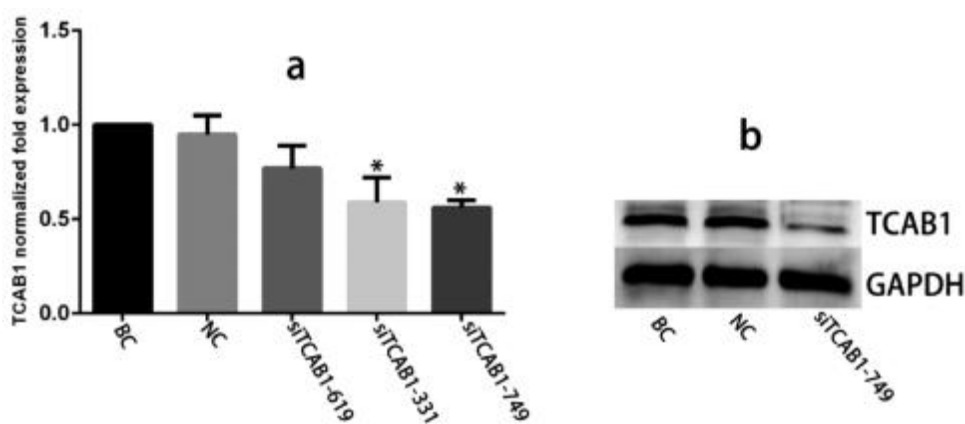


图 2 TCAB1 干扰靶点的筛选

Fig. 2 Screening of interference target of TCAB1-siRNA

注: 图 2a 为 HASMC 转染 siTCAB1 后各组 mRNA 表达变化, 与 NC 组和 BC 组比较, $*P<0.05$;

图 2b 为 Western blot 检测转染 siTCAB1-749 后 HASMC 中 TCAB1 的表达水平。

Note: the Relative expression of TCAB1 mRNA in HASMC after transfecting different interference sequence for 48 h in different groups, $*P<0.05$ (n=5) vs. BC and NC (Fig. 2a); Expression level of TCAB1 after transfecting siTCAB1-749 in HASMC by western blot assay (Fig. 2b).

2.4 沉默 TCAB1 表达对 HASMC 细胞周期及 CyclinD1 表达的影响

转染 siTCAB1-749 48h 后, siTCAB1-749 组 CyclinD1 mRNA 明显低于 NC 组、BC 组 ($P<0.05$, 图 4b)。Western blotting 结果显示 CyclinD1 蛋白表达量明显低于 BC 和 NC 组 (图 4c)。流式细胞术对 BC、NC、siTCAB1-749 组细胞周期进行检测, 各组 G1 期的细胞比率分别为 (27.4 ± 2.3) %、(28.1 ± 2.5) %、(31.4 ± 1.4) %; S 期细胞的比率分别为 (59.8 ± 0.2) %、(59.0 ± 1.9) %、

(52.2 ± 2.2) %, siTCAB1-749 组处于 G1 期细胞比率有所增加, 处于 S 期细胞比率减少 ($P<0.05$, 图 4a)。

3 讨论

Endorf EB 等研究表明动脉粥样硬化斑块中高表达 hTERT, 过表达 hTERT 可以促进动脉粥样硬化斑块内膜增生和血管平滑肌细胞增殖^[6]。反之, 通过反义寡核苷酸沉默端粒酶基因抑制了平滑肌的增殖^[7]。TCAB1 是端粒酶全酶的一个亚单位, 在

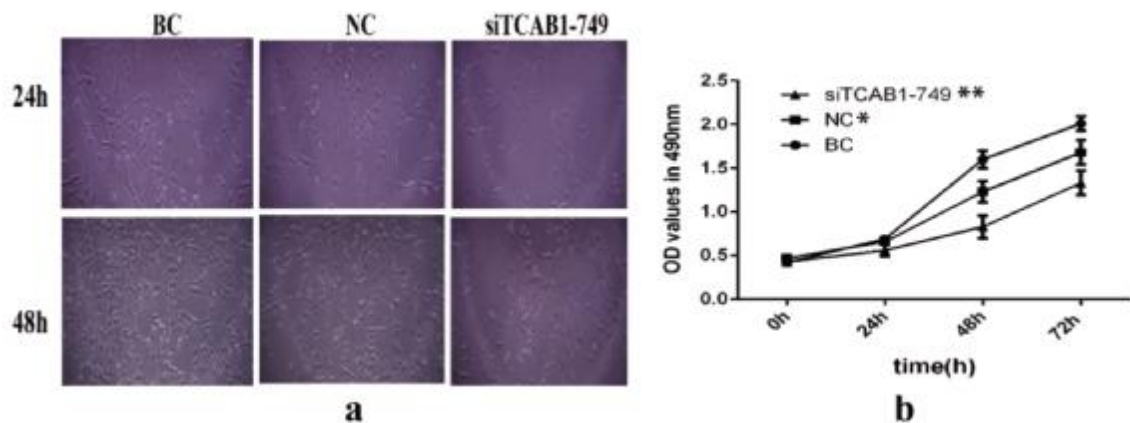


图3 沉默 TCAB1 表达对 HASMC 增殖的影响

Fig.3 Effect of siTCAB1-749 on the proliferation of HASMC

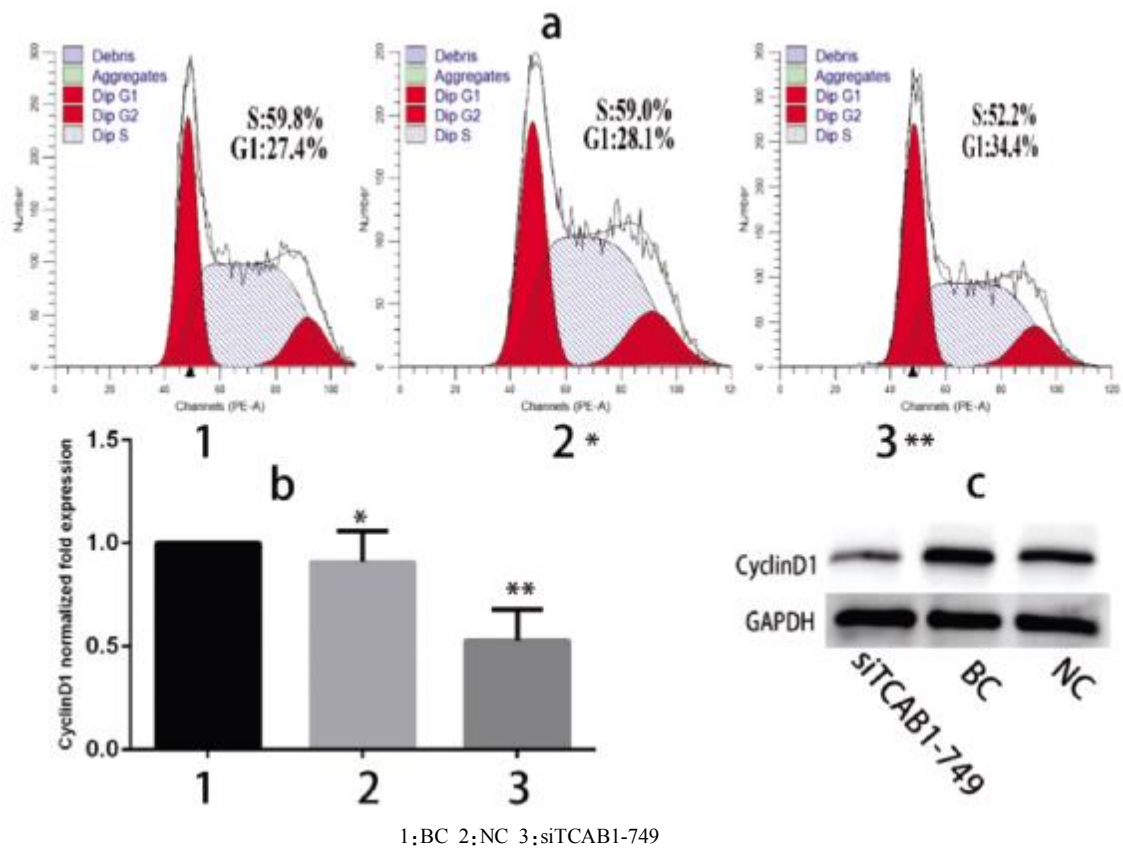
注:图 3a 为 TCAB1 干扰后显微镜下随机视野内细胞密度($100\times$);图 3b 为 MTS 比色法检测干扰 TCAB1 基因后 HASMC 细胞生长状况,** 与 NC 组和 BC 组比较 $P<0.05$, * 和 BC 组比较 $P<0.05$.Note: Visual fields of cells were captured separately at 24, 48 h, cell density were observed under inverted microscope($100\times$,Fig.3a);Cell growth and proliferation capacity were measured by MTS assay (Fig.3b, ** $P<0.05$ vs.BC and NC, * $P<0.05$ vs.BC n=6)

图4 沉默 TCAB1 对 HASMC 周期和 CyclinD1 表达量的影响

Fig.4 Effect of TCAB1 silencing on cell cycle and CyclinD1 expression of HASMC

注:图 4a 为沉默 TCAB1 48h 流式细胞仪检测细胞周期;图 4b 为 HASMC 转染 siTCAB1-749 后各组 CyclinD1mRNA 表达变化;图 4c 为 Western blot 检测转染 siTCAB1-749 后 HASMC 中 CyclinD1 的表达水平. ** 组与 NC 组和 BC 组比较, $P<0.05$.Note: Cell cycle were detected after transfecting siTCAB1-749 for 48h by FCM(Fig.4a); the Relative expression of CyclinD1 mRNA in HASMC after transfecting different interference sequence for 48h in different groups(Fig.4b); Expression level of CyclinD1 after transfecting siTCAB1-749 in HASMC by western blot assay (Fig.4c). (** $P<0.05$ vs.BC and NC)

卡哈尔体含量丰富,TCAB1 对端粒酶从卡哈尔体到端粒的运输是必需的,TCAB1 的缺失可阻止细胞 S 期端粒酶复合物运送至端粒末端^[3,6,18]。本实验合成三对 TCAB1siRNA 序列和 NC

序列,以 90 nmol/L 浓度将 siRNA 和 NC 序列成功转入 HASMC((图 d,e)),最终筛选出干扰效果最好的 siTCAB1-749 进行后续实验(图 2)。HASMC 转染 siTCAB1-749 24、48h 后,随

机视野细胞密度显著减少,细胞增殖水平明显降低,而 NC 组细胞增殖水平也降低(图 3b),这与 NC 组 CyclinD1 蛋白表达量轻微低于 BC 组相一致,可能是 lipo2000 对细胞的慢性毒性或者是由于阴性序列对细胞有一定的毒性作用所致,但是这种毒性作用并不是阴性序列沉默 TCAB1 基因所致,因为 qPCR 结果中 NC 组并未显示 TCAB1mRNA 的降低。

细胞周期蛋白在细胞周期的不同阶段发挥调控作用。在这些细胞周期蛋白中,细胞周期蛋白 D 非常重要。在细胞周期调控过程中,CyclinD1 参与细胞周期从 G1 期到 S 期的转换^[19,20]。因此,我们转染 siTCAB1-749 后 48 h,对 BC、NC、siTCAB1-749 组细胞周期进行检测,发现 siTCAB1-749 组处于 G1 期细胞比率与阴性对照组和空白组比较有所增加,处于 S 期细胞比率减少。Western blotting 结果显示 siTCAB1-749 组 CyclinD1 蛋白表达量明显低于 BC 和 NC 组,与细胞周期结果符合(图 4)。然而,沉默 TCAB1 抑制平滑肌的增殖是通过调节端粒酶的活性还是端粒的长度,其机制还需要进一步研究。综上所述,沉默 TCAB1 能抑制 HAVSMC 增殖,其机制与阻碍细胞周期蛋白 CyclinD1 有关。

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