TRPM7调控PI3K/AKT通路对人脑血管外膜成纤维细胞增殖和凋亡的影响

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摘要 人脑血管外膜成纤维细胞(HBVAFs)的异常增殖参与了血管增殖性疾病的发生发展。该研究探讨TRPM7(transient receptor potential melastatin 7)能否通过调控PI3K/AKT信号通路影响HBVAFs增殖和凋亡。体外培养HBVAFs细胞, 分为如下几组: parental(正常培养HBVAFs细胞)、si-NC、si-TRPM7、si-NC+IGF-1(PI3K/AKT信号通路激活剂)、si-TRPM7+IGF-1。通过qRT-PCR法检测*TRPM7* mRNA表达; CCK-8法检测细胞增殖能力; 流式细胞术检测细胞周期及凋亡情况; Western blot法检测目的蛋白水平。结果显示, si-TRPM7转染可显著降低HBVAFs细胞中TRPM7 mRNA和蛋白表达水平(P<0.001); 与si-NC组比较, si-TRPM7组细胞增殖活力下降(P<0.001), 细胞G_0~G_1期细胞比率上升(P<0.001), S期细胞比率下降(P<0.001), 周期调控蛋白CCND1、CDK2、CDK4水平均明显下降(P<0.001), 凋亡百分比增加(P<0.001), Bcl-2、p-PI3K及p-AKT蛋白表达下降(P<0.001), Bax、cleaved caspase-3及Cytochrome c蛋白表达增加(P<0.001); 而PI3K/AKT信号通路激活剂IGF-1处理可有效逆转si-TRPM7介导的上述改变(P<0.001)。这些结果提示, 干扰TRPM7表达可通过抑制PI3K/AKT信号通路激活发挥抑制HBVAFs细胞增殖并诱导凋亡的作用, 为TRPM7作为血管增殖性疾病的治疗靶点提供理论依据。

关键词 TRPM7; HBVAFs; 增殖; 凋亡; PI3K/AKT信号通路

TRPM7 Affects Proliferation and Apoptosis of Human Cerebral Vascular Adventitial Fibroblasts via PI3K/AKT Pathway

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Abstract The abnormal proliferation of HBVAFs (human cerebral vascular adventitial fibroblasts) contributes to the development of vascular proliferative diseases. This study aimed to investigate whether TRPM7 (transient receptor potential melastatin 7) could affect the proliferation and apoptosis of HBVAFs through PI3K/AKT signaling pathway. The cultured HBVAFs were randomly divided into the following groups: parental (normal cultured HBVAFs), si-NC, si-TRPM7, si-NC+IGF-1 (an activator of PI3K/AKT pathway), si-TRPM7+IGF-1. qRT-PCR was used for determining *TRPM7* mRNA expression. The cell proliferation was detected by CCK-8. Flow cytometry was performed to assess cell cycle and apoptosis. The levels of target proteins were evaluated by Western blot. The results showed that si-TRPM7 transfection significantly reduced the mRNA and protein expression of TRPM7

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in HBVAFs (P<0.001). Compared with si-NC group, si-TRPM7 transfection reduced cell viability (P<0.001), increased percentage in G_0-G_1 phase (P<0.001), decreased percentage in S phase (P<0.001), enhanced protein levels of CCND1, CDK2, CDK4 (P<0.001), raised apoptotic rate (P<0.001), reduced protein levels of Bcl-2, p-PI3K, and p-AKT (P<0.001), and elevated protein levels of Bax, cleaved caspase-3 and Cytochrome c (P<0.001). However, these si-TRPM7-mediated changes could be effectively reversed by IGF-1 treatment (P<0.001). These findings indicated that interference of TRPM7 could inhibit the proliferation and induce apoptosis of HBVAFs via inactivation of PI3K/AKT signaling pathway, which provided theoretical basis for TRPM7 serving as a potential therapeutic target for vascular proliferative disease.

Keywords TRPM7; HBVAFs; proliferation; apoptosis; PI3K/AKT signaling pathway

传统观念认为, 血管外膜只起到支持和滋养血管的作用。现在证实, 无论是血管炎症、早期内膜增厚还是血管损伤引起的血管壁晚期纤维化, 作为构成血管外膜最重要的成员之一血管外膜成纤维细胞(vascular adventital fibroblasts, VAFs)在血管重构中都发挥了核心作用[1]。大量证据表明, VAFs介导的血管重构直接参与动脉粥样硬化、高血压、肺动脉高压和血管再狭窄等疾病进程[2-3]。M型顺时受体电位通道7(transient receptor potential channel 7, TRPM7)是一种广泛表达的, 能够与细胞内囊泡和质膜中的C末端α-激酶结构域融合的通道蛋白, 在血管调节、高血压、肿瘤发展和免疫激活中发挥重要作用[4-5]。前期研究发现, TRPM7在主动脉狭窄大鼠模型中对血管炎症、高血压介导的血管外膜胶原堆积和细胞分型转化起调控作用[6]。TRPM7可促进机械拉伸应力引起的VAFs表型转化与炎症反应[7]。研究还发现, TRPM7在细胞增殖和凋亡调控中起关键作用[8]。但TRPM7对VAFs细胞增殖和凋亡的影响尚未可知。本研究旨在探讨TRPM7对人脑血管外膜成纤维细胞(human cerebral vascular adventitial fibroblasts, HBVAFs)增殖和凋亡的影响及相关分子机制, 为血管增殖性疾病的治疗提供新的潜在靶点。

1 材料与方法

1.1 材料与试剂

HBVAFs细胞、FM成纤维细胞培养基购自美国ScienCell公司; 胎牛血清购自美国Gibco公司; Lipofectamine® 2000、Trizol试剂购自美国Thermo Fisher公司; si-NC、si-TRPM7购自上海吉凯基因医学科技股份有限公司; 反转录与荧光定量PCR试剂盒购自北京百泰克生物技术有限公司; 细胞凋亡检测试剂盒购自翌圣生物科技(上海)有限公司; PI试剂购自上海江莱生物科技有限公司; PI3K/AKT通路激活剂IGF-1购自北京义翘神州科技股份有限公司; 兔源TRPM7抗体、pro-caspase-3抗体、HRP标记的山羊抗兔二抗购自英国Abcam公司; CCK-8试剂盒、兔源CCND1抗体、CDK2抗体、CDK4抗体、Bax抗体、Bcl-2抗体、Cytochrome c抗体、cleaved caspase-3抗体、p-PI3K抗体、PI3K抗体、p-AKT抗体、AKT抗体、GAPDH抗体购自北京博奥森生物技术有限公司。

1.2 方法

1.2.1 实验分组 HBVAFs细胞培养于含有10%胎牛血清的FM培养基中, 置于37 °C、含5% CO_2培养箱中培养。将对数生长期HBVAFs随机分为parental组(正常空细胞对照组)、si-NC组(si-NC转染组)、si-TRPM7组(si-TRPM7转染组)、si-NC+IGF-1组(转染si-NC换液后加入2 nmol/L IGF-1)、si-TRPM7+IGF-1组(转染si-TRPM7换液后加入2 nmol/L IGF-1)。*TRPM7* siRNA序列: 正向5′-GUC UUG CCA UGA AAU ACU CUU-3′, 反向5′-GAG UAU UUC AUG GCA AGA CUU-3′ (siTRPM7 #1); 正向 5′-AGG AGA AGA UGC AAU UAA ATT-3′, 反向 5′-UUU AAU UGC AUC UUC UCC UAG-3′ (siTRPM7 #2)。si-NC序列: 正向 5′- UUC UCC GAA CGU GUC ACG UTT-3′; 反向 5′-ACG UGA CAC GUU CGG AGA ATT-3′。转染6 h换液后, HBVAFs细胞在含有10%胎牛血清的培养基中继续培养24、48和72 h。

1.2.2 qRT-PCR检测*TRPM7*的mRNA水平 采用Trizol试剂从各组HBVAFs细胞中提取总RNA, 随后使用反转录试剂盒获得cDNA, 以cDNA为模板使用荧光定量PCR试剂盒进行PCR扩增。其中*TRPM7*正向引物序列为5′-CAC TTG GAA ACT GGA ACC-3′, 反向引物序列为5′-CGG TAG ATG GCC TTC TAC TG-

3′; *Bax*正向引物序列为5′-GAC GAA CTG GAC AGT AAC ATG GA-3′, 反向引物序列为5′-GCA AAG TAA AAG GGC GAC A-3′; *Bcl-2*正向引物序列为5′-CCC GTT GCT TTT CCT CTG G-3′, 反向引物序列为5′-ATC CCA CTC GTA GCC CCT CT-3′; *Cytochrome c*正向引物序列为5′-CTT TGG GCG GAA GAC AGG TC-3', 反向引物序列为5′-TTA TTG GCG GCT GTG TAA GAG-3′; *GAPDH*正向引物序列为5′-GCA CCG TCA AGC TGA GAA C-3′, 反向引物序列为5′- TGG TGA AGA CGC CAG TGG A-3′。以*GAPDH*为内参, 采用$2^{-\Delta\Delta Ct}$法计算*TRPM7* mRNA的相对表达水平。

1.2.3 CCK-8检测细胞增殖 不同处理组HBVAFs细胞接种于96孔板中(1×10^3个/孔), 分别培养24、48和72 h后, 每孔加入10 μL CCK-8溶液。细胞继续在37 °C培养箱培养2 h后, 使用酶标仪检测各孔450 nm处吸光度(*D*)值。

1.2.4 流式细胞术检测细胞周期 不同组别HBVAFs细胞接种于6孔板中, 继续培养48 h后, 收集细胞, 并使用70%冰乙醇固定过夜。预冷的PBS清洗细胞后加入500 μL染色液(含25 μL PI溶液和10 μL RNase A), 避光孵育30 min。随后, 流式细胞仪检测各组细胞周期分布。

1.2.5 流式细胞术检测细胞凋亡 HBVAFs细胞转染后继续培养48 h, 收集各组细胞, 冰冷PBS清洗细胞两次, 然后使用500 μL结合液重悬细胞。随后分别加入5 μL Annexin V-FITC和5 μL PI染液。避光孵育15 min后, 立即采用流式细胞仪检测各组细胞凋亡百分比。

1.2.6 Western blot法检测蛋白水平 收集各处理组HBVAFs细胞, 分别加入蛋白裂解液获取总蛋白。蛋白浓度定量后, 采用等量蛋白样品进行SDS-PAGE电泳, 随后转移到PVDF膜上。5%脱脂牛奶室温封闭1 h后, PVDF膜分别与不同的一抗稀释液(1:1 000) 4 °C孵育过夜。TBST洗膜三次后, 加入二抗稀释液(1:5 000)室温孵育1 h后, 采用ECL液显色, 获得可视化蛋白条带。使用ImageJ软件定量分析条带灰度值。

1.3 统计学处理

使用GraphPad 8.0软件进行统计分析。所用数据以均数±标准差($\bar{x}\pm s$)表示。采用单因素方差分析及LSD事后检验进行组间数据比较。$P<0.05$表示差异有统计学意义。

2 结果

2.1 干扰TRPM7对HBVAFs细胞增殖的影响

与si-NC转染组比较, si-TRPM7组HBVAFs细胞中TRPM7的mRNA和蛋白水平显著下降($P<0.001$), 提示siRNA成功转染进细胞并发挥干扰基因表达的作用。CCK-8结果显示, 干扰TRPM7可明显抑制HBVAFs细胞增殖($P<0.05$)(图1)。

2.2 干扰TRPM7对HBVAFs细胞周期进程的影响

进一步采用流式细胞术检测TRPM7对细胞周期的影响情况。与si-NC组比较, si-TRPM7转染可引起G_0~G_1期细胞比例增加, 而S期细胞比例下降($P<0.001$)。随后采用Western blot检测周期相关蛋白的表达变化情况。与si-NC组相比, si-TRPM7组细胞中CCND1、CDK2、CDK4蛋白水平均明显下降($P<0.001$)(图2)。

2.3 干扰TRPM7对HBVAFs细胞凋亡的影响

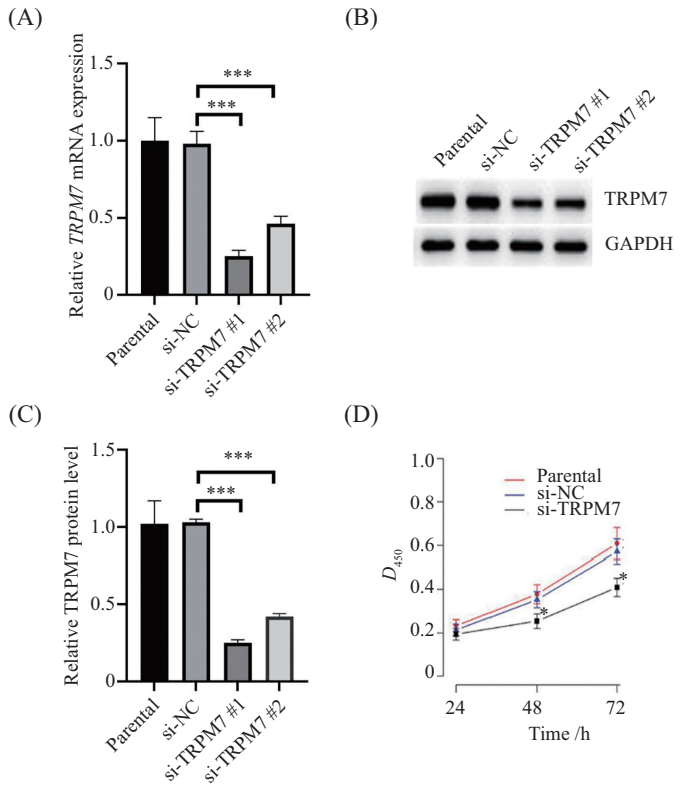
与si-NC组比较, si-TRPM7组细胞凋亡百分比显著上升($P<0.001$)。此外, 干扰TRPM7表达可引起HBVAFs细胞中Bax、Cytochrome c、cleaved caspase-3蛋白表达增加, 而Bcl-2和pro-caspase-3蛋白表达减少($P<0.001$)。与之一致的是, si-TRPM7组细胞中*Bax*和*Cytochrome c* mRNA 水平上升, 而*Bcl-2* mRNA水平降低(图3)。

2.4 干扰TRPM7对PI3K/AKT通路激活的影响

为了进一步探讨TRPM7对HBVAFs细胞增殖和凋亡调控的分子机制, 采用Western blot实验检测PI3K/AKT通路蛋白的表达水平。与si-NC组比较, si-TRPM7组p-PI3K和p-AKT蛋白表达均显著降低($P<0.001$)(图4)。

2.5 干扰TRPM7通过调控PI3K/AKT通路影响HBVAFs细胞增殖和凋亡

HBVAFs细胞进一步给予PI3K/AKT通路激活剂IGF-1处理, 从而阐释TRPM7是否通过调控PI3K/AKT通路调节HBVAFs细胞增殖和凋亡。与si-NC组比较, si-NC+IGF-1组细胞活力增强, 凋亡百分比减少, Bax、Cytochrome c、cleaved caspase-3蛋白表达下降, 而p-PI3K、p-AKT、Bcl-2和pro-caspase-3蛋白表达增加($P<0.05$, $P<0.001$)。与si-TRPM7组比较, si-TRPM7+IGF-1组胞活力增高, 凋亡百分比减少, Bax、Cytochrome c、cleaved caspase-3蛋白表达下降, 而p-PI3K、p-AKT、Bcl-2和pro-caspase-3蛋白表达上升($P<0.05$, $P<0.001$)(图5)。

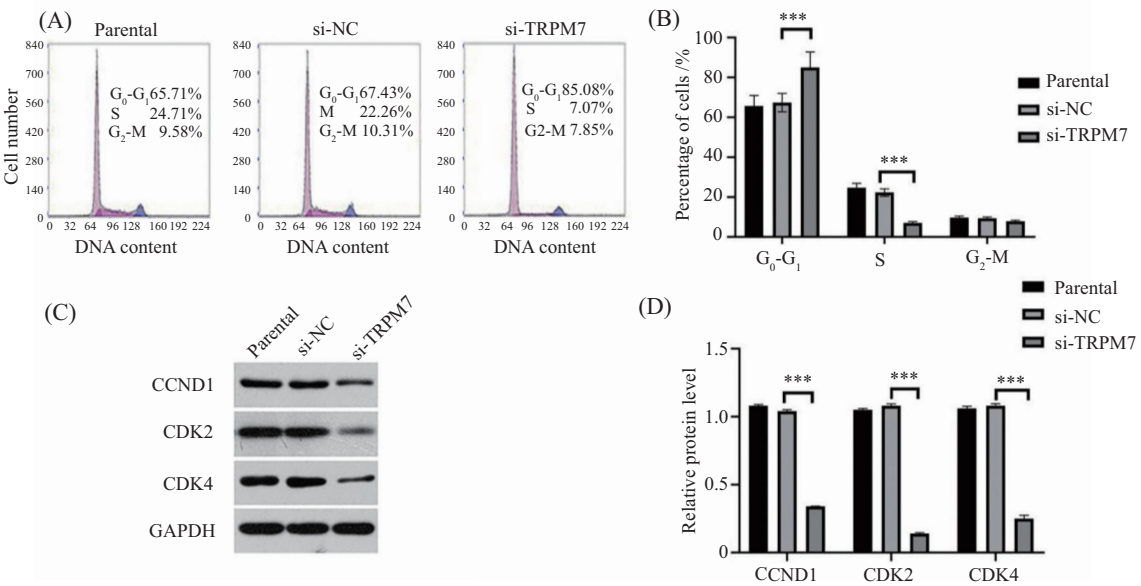


A: qRT-PCR实验检测*TRPM7*的mRNA表达水平; B: 采用Western blot法检测TRPM7的蛋白表达水平; C: TRPM7蛋白相对表达量的定量分析; D: 采用CCK-8实验检测HBVAFs细胞增殖情况。*P<0.05, ***P<0.001, 与si-NC组比较。

A: qRT-PCR for evaluating the mRNA expression of *TRPM7*; B: Western blot was performed to detect the protein level of TRPM7; C: the relative protein expression of TRPM7 was quantified; D: the proliferation of HBVAFs was detected by CCK-8. *P<0.05, ***P<0.001 compared with the si-NC group.

图1 干扰TRPM7表达对HBVAFs细胞增殖的影响

Fig.1 Effect of TRPM7 interference on the proliferation of HBVAFs

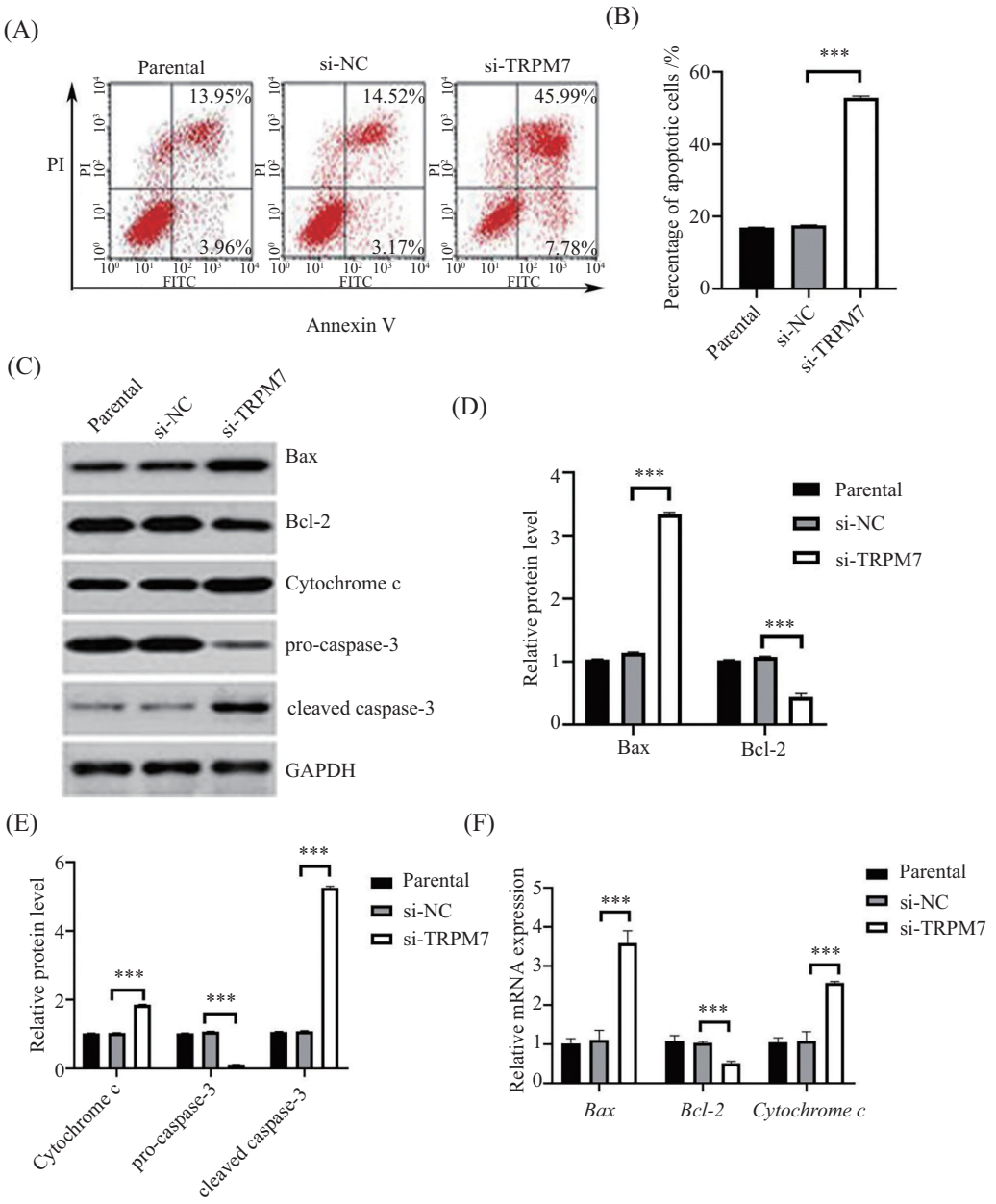


A: 流式细胞术检测各组细胞周期变化; B: G_0~G_1、S和 G_2~M细胞周期的百分比; C: Western blot实验检测细胞周期相关蛋白表达情况; D: 各周期蛋白相对表达量的定量分析。***P<0.001, 与si-NC组比较。

A: cell cycle progression was assessed by flow cytometry; B: the percentage of cells in G_0-G_1, S, and G_2-M phases; C: the expression of cell cycle-related proteins was detected by Western blot; D: the relative cell cycle-related protein expression was quantified. ***P<0.001 compared with the si-NC group.

图2 干扰TRPM7表达对HBVAFs细胞周期的影响

Fig.2 Effect of TRPM7 interference on cell cycle progression of HBVAFs



A: 流式细胞术检测各组细胞凋亡情况; B: 细胞凋亡百分比的定量分析; C: Western blot实验检测凋亡相关蛋白表达情况; D、E: 各凋亡相关蛋白相对表达量的定量分析; F: qRT-PCR实验检测凋亡相关蛋白的mRNA表达水平。***P<0.001, 与si-NC组比较。

A: apoptosis of cells in each group was determined by flow cytometry; B: the percentage of apoptotic cells was shown; C: the expression of apoptosis-related proteins was measured by Western blot; D,E: the relative protein expression was quantified; F: qRT-PCR for assessing the mRNA expression of apoptosis-related proteins. ***P<0.001 compared with the si-NC group.

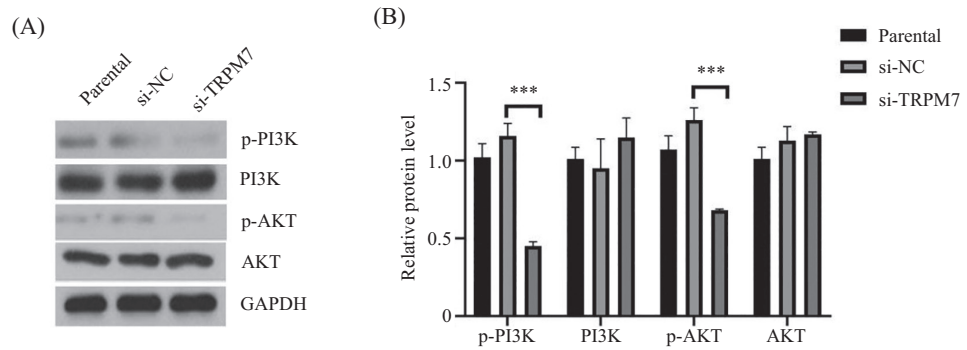
图3 干扰TRPM7表达对HBVAFs细胞凋亡的影响

Fig.3 Effect of TRPM7 interference on apoptosis of HBVAFs

3 讨论

近年来, VAFs在血管重构中的作用引起了越来越多的关注。VAFs向肌成纤维细胞的分型转化在动脉受损时新生内膜形成中发挥重要作用[9]。深入的研究表明, VAFs的激活、异常增殖和凋亡抑制是血管增殖性疾病的重要病理机制[10]。因此, 有效地抑制VAFs过度增殖并诱导其凋亡将在控制和治疗上述疾病中发挥关键作用。本研究主要探讨TRPM7对HBVAFs细胞增殖和凋亡的调控作用及相关机制。结果显示, TRPM7干扰可有效抑制HBVAFs细胞增殖并诱导周期阻滞和凋亡。

TRPM7被证明在血管平滑肌细胞功能障碍中发挥重要调控作用[11]。据报道, TRPM7在血管平滑肌细胞中可被高糖激活, 从而证明TRPM7可在高糖

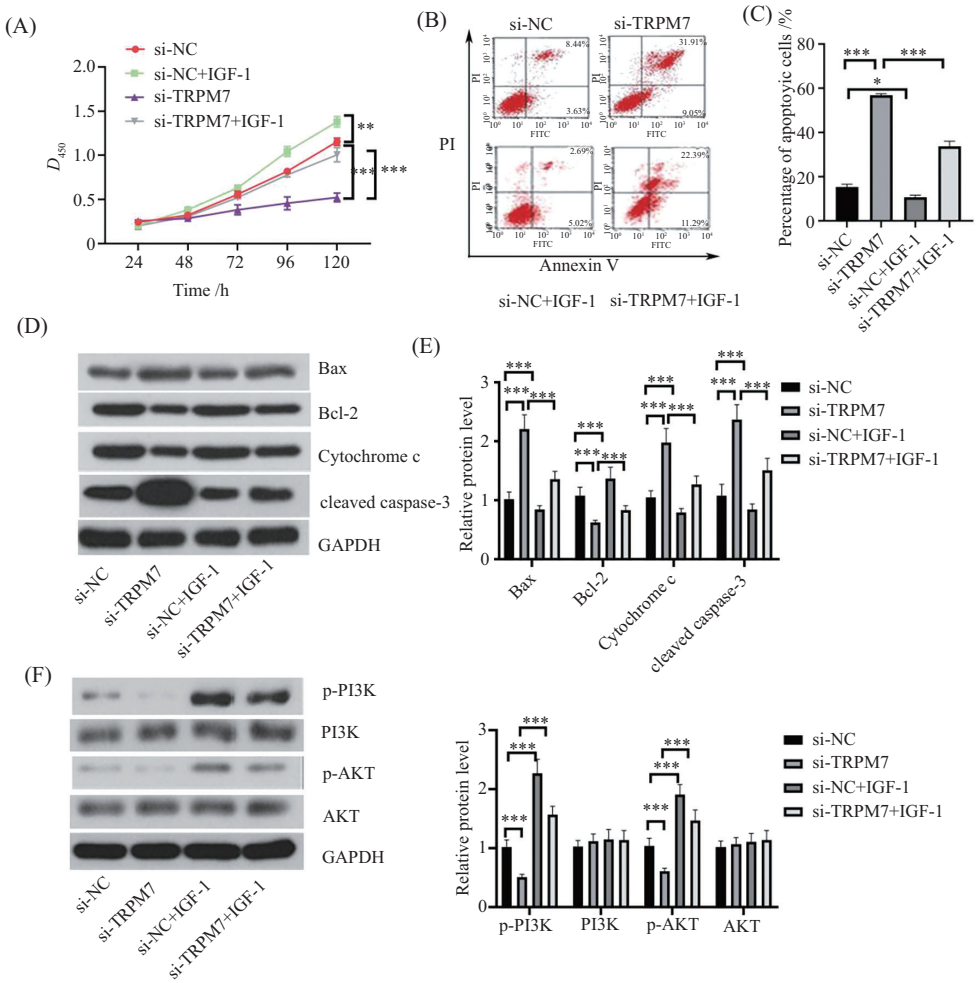


A: Western blot实验检测PI3K/AKT通路蛋白表达情况。 B: PI3K/AKT通路蛋白相对表达量的定量分析。***P<0.001, 与si-NC组比较。

A: the expression of PI3K/AKT pathway proteins was detected by Western blot. B: the relative protein expression of PI3K/AKT pathway was quantified. ***P<0.001 compared with the si-NC group.

图4 干扰TRPM7表达对PI3K/AKT通路激活的影响

Fig.4 Effect of TRPM7 interference on the activation of PI3K/AKT pathway



A: CCK-8实验检测各组细胞增殖; B: 流式细胞术检测各组细胞凋亡情况; C: 凋亡细胞百分比的定量分析; D: Western blot实验检测凋亡相关蛋白表达情况; E: 各凋亡相关蛋白相对表达量的定量分析; F: Western blot实验检测PI3K/AKT通路蛋白表达情况; G: PI3K/AKT通路蛋白相对表达量的定量分析。*P<0.05, **P<0.01, ***P<0.001, 与si-NC或si-TRPM7组比较。

A: CCK-8 was used to determine the proliferation of cells from different groups; B: apoptosis of HBVAFs was evaluated by flow cytometry; C: the percentage of apoptotic HBVAFs was calculated; D: Western blot for assessing the protein expression of apoptosis-related proteins; E: the relative protein expression was quantified; F: Western blot for determining the PI3K/AKT pathway protein expression; G: the relative protein expression of PI3K/AKT pathway was quantified. *P<0.05, **P<0.01, ***P<0.001 compared with the si-NC or si-TRPM7 group.

图5 干扰TRPM7通过PI3K/AKT通路调控HBVAFs细胞增殖凋亡

Fig.5 TRPM7 interference affects the proliferation and apophasis of HBVAFs via PI3K/AKT pathway

引起的血管损伤中发挥作用[12]。进一步的研究显示, TRPM7在主动脉狭窄大鼠VAFs中表达增高并促进血管外膜重构和炎症反应, 而抑制TRPM7表达可有效逆转上述病理改变[6], 这提示, TRPM7与血管增殖性疾病具有紧密相关性。然而, TRPM7对VAFs增殖和凋亡的作用还不明确。有研究发现, TRPM7表达抑制可有效抑制结直肠癌细胞增殖并诱导细胞周期阻滞[13]。亦有研究证明, 在膀胱癌细胞中降低TRPM7表达, 可诱导ERK通路激活, 从而抑制膀胱癌细胞活性并诱导凋亡[14]。本研究结果显示, 干扰TRPM7表达后HBVAFs细胞增殖能力下降并诱导G_1期细胞阻滞和凋亡; 并且si-TRPM7处理后, 细胞周期调节蛋白CCND1和CDK2/4水平均显著下降。此外, si-TRPM7可引起导致线粒体凋亡的Bax/Bcl-2表达失衡[15]及cleaved caspase-3和Cytochrome c蛋白表达增加[16], 提示干扰TRPM7表达可引起线粒体依赖的细胞凋亡。

为了深入探究TRPM7调控HBVAFs细胞增殖和凋亡生物功能的潜在分子机制, 本研究检测TRPM7对PI3K/AKT通路激活的影响。PI3K/AKT信号通路是控制细胞存活、代谢、生长、分化的重要调控通路[17]。PI3K/AKT通路的失调可导致其下游效应因子的异常改变, 从而引起细胞增殖和凋亡失控[18]。抑制PI3K/AKT通路激活可减轻血管内膜增生[19], 提示PI3K/AKT激活可促进血管增殖性疾病进展。PI3K/AKT通路与TRPM7具有紧密的联系。例如, 研究显示TRPM7可通过激活PI3K/AKT通路促进卵巢癌细胞上皮间质转化[20]。抑制TRPM7表达可通过调节PI3K/AKT通路在可生物降解镁合金抗炎中发挥重要作用[21]。本研究结果表明, 干扰TRPM7表达后HBVAFs细胞中p-PI3K、p-AKT蛋白水平明显下降, 提示干扰TRPM7表达可能通过抑制PI3K/AKT通路激活从而调控HBVAFs细胞增殖和凋亡。为阐明TRPM7是否通过调控PI3K/AKT通路发挥作用, 本研究进一步在转染si-TRPM7的HBVAFs细胞中加入PI3K/AKT通路激活剂IGF-1, 结果发现单独给予IGF-1处理后可激活PI3K/AKT通路从而增强HBVAFs细胞增殖能力并减少凋亡比率; 而si-TRPM7与IGF-1共处理可有效中和干扰TRPM7对HBVAFs细胞增殖和凋亡的调控作用。这些结果表明, 干扰TRPM7表达可抑制HBVAFs细增殖并诱导凋亡, 其作用机制可能与抑制PI3K/AKT信号通路激活有关。

综上所述, 干扰TRPM7表达可降低HBVAFs细胞增殖活力, 并引起G_1期细胞周期阻滞和凋亡, 而抑制PI3K/AKT通路激活是其潜在的调控分子机制。TRPM7可能作为血管增殖性疾病治疗的潜在靶点, 但其治疗作用的分子机制有待进一步深入探索。

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