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• 基础研究 •

TRPM3 通过 Wnt/ β -catenin 信号通路诱导上皮性卵巢癌细胞
上皮间质转化的作用研究 *陈利侠^{1,2} 张 汝¹ 苏敏君² 席晓薇¹ 孙云燕^{1Δ}

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摘要 目的:探讨瞬时受体电位离子通道 3(Transient receptor potential melastatin 3, TRPM3)对卵巢癌侵袭转移和上皮细胞间质转化(Epithelial mesenchymal transition, EMT)的影响及其分子作用机制。**方法:**采用小干扰 RNA 沉默上皮性卵巢癌细胞株中 HEY 及 SKOV3 中 TRPM3 的表达,通过 Transwell 实验和划痕实验检测上皮性卵巢癌细胞的侵袭和迁移能力的变化,Western Blot 检测 EMT 相关蛋白、Wnt/ β -catenin 通路相关蛋白的表达情况。**结果:**与对照组细胞相比,干扰组的上皮性卵巢癌细胞迁移和侵袭能力均明显减弱,EMT 相关蛋白的上皮细胞标志分子 E-cadherin 的表达上调,间质细胞标志分子 N-cadherin 和 EMT 相关转录调控因子 Snail 的表达下调,Wnt/ β -catenin 通路相关蛋白 CyclinD1、 β -catenin 的表达下调。**结论:**TRPM3 可能通过激活 Wnt/ β -catenin 通路促进卵巢癌细胞的上皮间质转化过程,进而增强其侵袭转移的能力。

关键词:卵巢癌;上皮间质转化;瞬时受体电位离子通道 3

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TRPM3 induces Epithelial-mesenchymal Transition via the Wnt/ β -catenin
Signaling Pathway in Epithelial Ovarian Cancer*CHEN Li-xia^{1,2}, ZHANG Ru¹, SU Min-jun², XI Xiao-wei¹, SUN Yun-yan^{1Δ}

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ABSTRACT Objective: The purpose of this study was to explore the role of TRPM3 (transient receptor potential melastatin 3, TRPM3) in the invasion and metastasis of ovarian cancer, its role in EMT (epithelial mesenchymal transition, EMT), and the molecular mechanism in epithelial ovarian cancer. **Methods:** We knocked down expression of TRPM3 in HEY and SKOV3 cells, using a small interference RNA. Using Transwell experiment and scratch migration assays, we tried to detect the invasion and migration of epithelial ovarian cancer cells. In addition, the expression of related proteins in EMT and Wnt/ β -catenin pathway was detected by Western Blot. **Results:** TRPM3 silencing increased the expression of E-cadherin and decreased the expression of N-cadherin, β -catenin and Snail. **Conclusion:** These results suggest that TRPM3 may enhance the ability of the invasion and metastasis of ovarian cancer cells by activating the Wnt/ β -catenin pathway.

Key words: Ovarian cancer; Epithelial mesenchymal transition; Transient receptor potential melastatin 3

Chinese Library Classification(CLC): R-33; R737.31 **Document code:** A

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前言

卵巢癌(ovarian cancer, OC)是女性生殖系统常见恶性肿瘤之一,恶性程度较高,患者确诊时多为晚期,五年生存率较低^[1]。临床上治疗晚期卵巢癌主要采用手术以及术后铂类、紫杉醇类辅助化疗^[2]。探索卵巢癌侵袭转移分子机制可为针对性的靶向治疗提供参考依据^[3-5]。

瞬时受体电位离子通道 3(transient receptor potential melas-

tatin 3, TRPM3)可介导钙离子、镁离子、锌离子等进入细胞,并且主要以通透钙离子为主^[6]。研究表明 TRPM3 参与机体的各种病理生理过程,但 TRPM3 在卵巢癌中的作用尚不明确。上皮细胞间质转化(epithelial mesenchymal transition, EMT)受多种信号传导通路调控,Wnt/ β -catenin 通路在 EMT 中是重要的信号通路,并且在细胞分裂、分化、粘附、凋亡中扮演重要角色^[7]。本研究拟在初步探讨 TRPM3、EMT 及 Wnt/ β -catenin 信号通路在上皮性卵巢癌中的生物学作用及其作用机制。

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1 材料与方

1.1 材料

1.1.1 细胞株 实验 HEY、SKOV3 细胞(人类上皮性卵巢癌细胞)由上海交通大学附属第一人民医院,临床转化研究院妇产科实验室提供。

1.1.2 主要试剂 青霉素-链霉素溶液、胎牛血清、DMEM/F12 培养基、胰蛋白酶(0.25%),购于 Hyclone 公司。细胞裂解液、Western Blot 配胶试剂盒、PMSF 和 SDS-PAGE 蛋白电泳上样缓冲液(5×)购于上海碧云天生物技术有限公司。转染试剂 Lipofectamine™ 2000 购于 Invitrogen 公司。蛋白浓度测定试剂盒 BCA Protein Assay Kit 购于 Thermo Scientific 公司。兔抗人 TRPM3 单克隆抗体购于美国 Abcam 公司。BD 基底膜基质胶购于 BD 公司。Transwell 小室(24 孔,孔径 8.0 μm)购于美国 Corning Incorporated 公司。

1.2 方法

1.2.1 细胞培养 HEY 和 SKOV3 细胞用含 10%胎牛血清、青霉素、链霉素 DMEM/F12 培养基,在细胞培养箱 (37 °C、5% CO₂)培养。用生长状态良好、融合度约 80%的细胞,胰酶消化传代,用于后续实验。

1.2.2 合成构建小干扰 RNA 实验所用小干扰 RNA(siRNA)由广州锐博生物技术有限公司设计合成,产品为常温干粉,用 ddH₂O 溶解成液体后冻存,并于 -80 °C 冰箱长期保存。siRNA 正义链 5' -CCUCUAAGUCUCGAGGAAAdTdT-3', 反义链 3' -UUUCCUCGAGACUUAGAGdTdT-5'。

1.2.3 细胞转染 以 HEY 细胞、5×10⁴ 个/孔,SKOV3 细胞、10×10⁴ 个/孔细胞数量接种在六孔板上,每孔加 3 mL 不含抗生素的 DMEM/F-12 细胞培养基,摇匀后细胞培养箱培养。当细胞密度达到 50%-70%,用无血清无抗的 DMEM/F-12 培养液(静止液),培养 12 h。配制溶液 A:5 μL siRNA 培养液+250 μL 静止液,混匀、室温静置 5 min。配制溶液 B:5 μL lipo2000+250 μL 静止液,混匀室温静置 5 min。A 液+B 液混匀室温孵育 20 min。混合液加入六孔板,细胞培养箱培养 6 h。6 h 后更换为含 10%胎牛血清的 DMEM F-12 培养液,在 37 °C、5%CO₂ 的细胞培养箱中培养,约 48 h 后提取蛋白。转染效果检测:培养 48 h 后,提取细胞蛋白质,Western Blot 检测目的蛋白表达。

1.2.4 Transwell 侵袭实验 用无血清无抗 DMEM/F-12 培养基与 BD 胶以 8:1 比例混匀,将 80 μL 混合液滴在 Transwell

小室底部膜的上室面中央,勿使气泡产生。放入细胞培养箱 5 h,待基底膜凝固。Transwell 小室上室加入含 10 万个细胞的无血清培养基细胞悬液 200 μL,下室加入 10%胎牛血清的培养基 600 μL,放入细胞培养箱,培养 16-24 h(HEY 细胞需培养 16 h,SKOV3 细胞需培养 24 h)。擦去小室膜顶端的细胞,4%多聚甲醛固定,0.1%结晶紫染色。倒置显微镜下观察拍照,随机选取 5 个低倍视野(×100)进行细胞计数,计算平均值。

1.2.5 Transwell 迁移实验 无基底膜包被,上室中的细胞悬液需培养 12-20 h (HEY 细胞需培养 12 h,SKOV3 细胞需培养 20 h),余同 Transwell 侵袭实验。

1.2.6 划痕实验 检测 TRPM3 对卵巢癌细胞迁移能力的影响。选择生长状态良好的 HEY、SKOV3 细胞(对照组 Control 组和转染组 si-RNA 组),PBS 清洗 2-3 遍后,胰酶消化,培养液终止反应,混匀种植至六孔板,尽量使每个孔细胞数目、密度相同。细胞箱中培养。六孔板底部用 200 μL 的枪头制造细胞划痕,每个划痕的宽度基本相同。PBS 洗涤 2-3 次,洗去细胞碎片,无血清培养基培养细胞。HEY 细胞分别于 0、12、24 h 在显微镜下观察细胞的生长情况,SKOV3 细胞分别于 0、24、48 h 在显微镜下观察细胞的生长情况,拍照前洗去漂浮的细胞,记录细胞不同时间段迁移情况。

1.2.7 Western Blot 检测 按上述方法转染细胞,Control 组和 si-RNA 组,48 h 后提取细胞蛋白,BCA 法测蛋白浓度,β-actin 为内参,配胶,60 V 恒压电泳,300 mA 转膜。5%的脱脂牛奶封闭 1 h,1×TBST 洗涤后孵一抗(鼠抗内参 β-actin 1:1000,兔抗人 TRPM3 1:10000; 兔抗人 E-cadherin、N-cadherin、Snail 1:1000)4 °C 过夜,第二天洗涤后孵二抗(兔抗 1:10000,兔抗 1:10000)1 h,1×TBST 洗涤,ECL 显影。Western Blot 结果采用 ImageJ 图像分析系统进行分析,得出各条带相对灰度值。

1.3 统计学分析

采用 Social Sciences (SPSS) 20.0 系统对相对灰度值进行统计学分析。采用 GraphPad Prism 软件进行统计分析,结果用 $\bar{x} \pm s$ 表示,组间两两比较采用 t 检验,以 $P < 0.05$ 为差异有统计学意义。

2 结果

2.1 TRPM3 在上皮性卵巢癌细胞 HEY 和 SKOV3 中有效沉默

如图 1 所示,转染特异性 si-RNA 的上皮性卵巢癌细胞 HEY 和 SKOV3 中 TRPM3 蛋白表达明显下降 ($P < 0.05$),表明

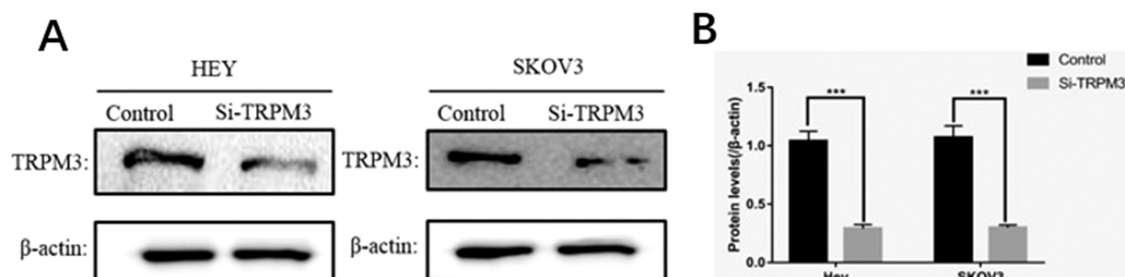


图 1 TRPM3 在上皮性卵巢癌细胞中有效沉默

Fig. 1 Effective silence of TRPM3 in the epithelial ovarian cancer cells

Note: A. Silencing TRPM3 expression in HEY and SKOV3 cell lines, decreased expression TRPM3; B. Grey intensity analysis of TRPM3 protein expression by ImageJ. β-actin served as a loading control. (***) $P < 0.001$

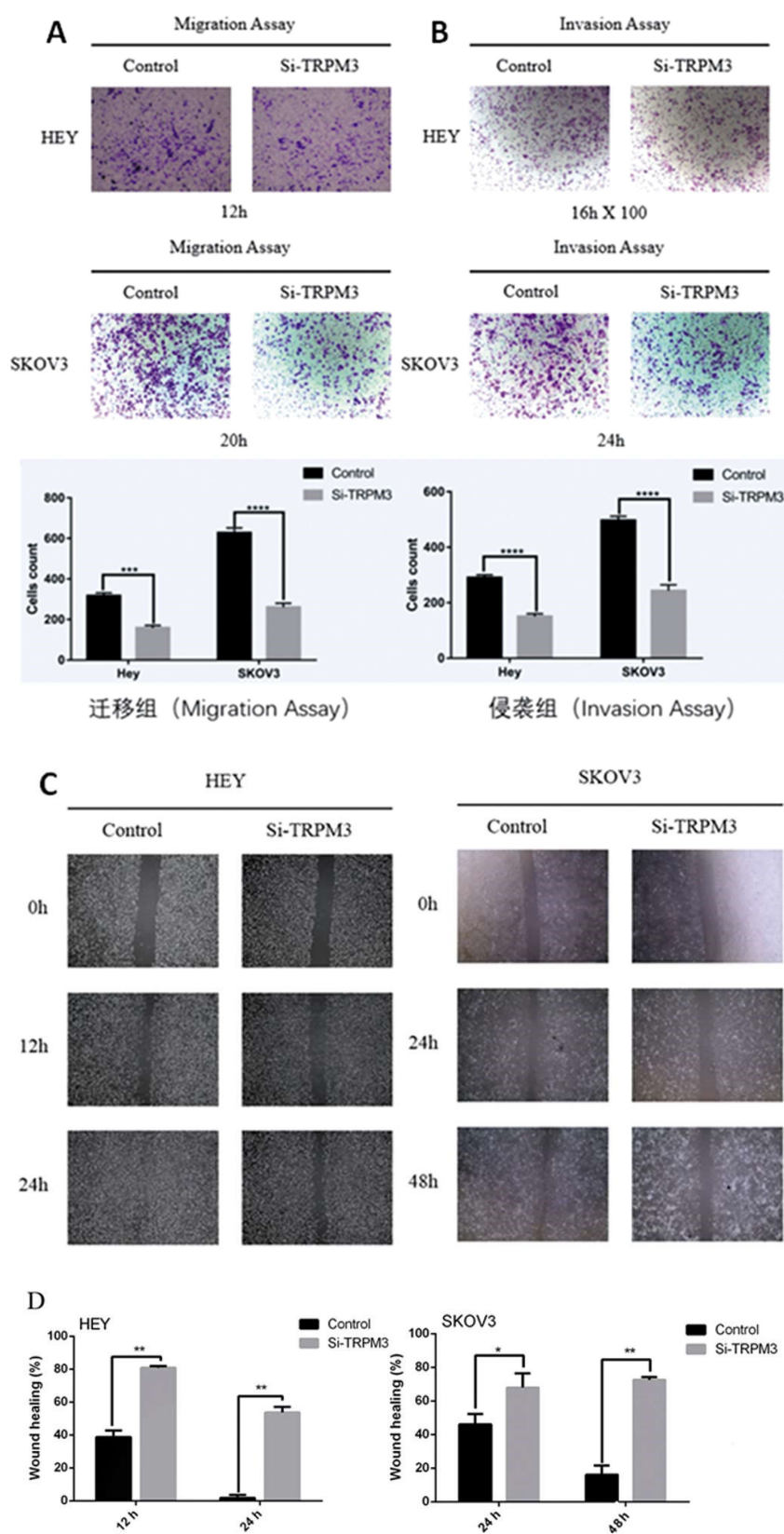


图 2 下调 TRPM3 的表达抑制上皮性卵巢癌细胞的侵袭迁移

Fig. 2 Downregulation of TRPM3 expression inhibited the invasion and migration of epithelial ovarian cancer cells

Note: A. The results showed that the number of migration cells after TRPM3 knockout was significantly smaller than that of the control group, and there was statistical difference between the si-RNA group and the control group; B. The results showed that the number of invasion cells after TRPM3 knockout was significantly smaller than that of the control group, and there was statistical difference between the si-RNA group and the control group; C. The results of scratch test showed that the wound healing time of HEY and SKOV3 cells was prolonged after knockout the TRPM3; D. The results of scratch test showed that the wound healing distance of HEY and SKOV3 cells was shorted after knockout the TRPM3. The magnification of the microscope is $200\times$ ($***P < 0.001$, $****P < 0.0001$)

TRPM3 在蛋白水平得到有效沉默。

2.2 下调 TRPM3 的表达抑制上皮性卵巢癌细胞的侵袭迁移

Transwell 实验结果显示:使用小干扰 RNA 沉默 TRPM3 表达后,HEY 和 SKOV3 细胞的迁移和侵袭能力减弱,对照组和转染组迁移侵袭细胞数之间有统计学差异($P<0.05$)。

2.3 下调 TRPM3 的表达影响上皮性卵巢癌细胞 EMT 相关蛋白的表达

白的表达

在 HEY 和 SKOV3 细胞中,敲除 TRPM3 后,上皮性细胞标志蛋白 E-cadherin 表达增加,而间质性细胞标志蛋白 N-cadherin 及转录调节因子 Snail 表达下降,表明 TRPM3 可以促进上皮性卵巢癌细胞发生 EMT(如图 3 所示)。

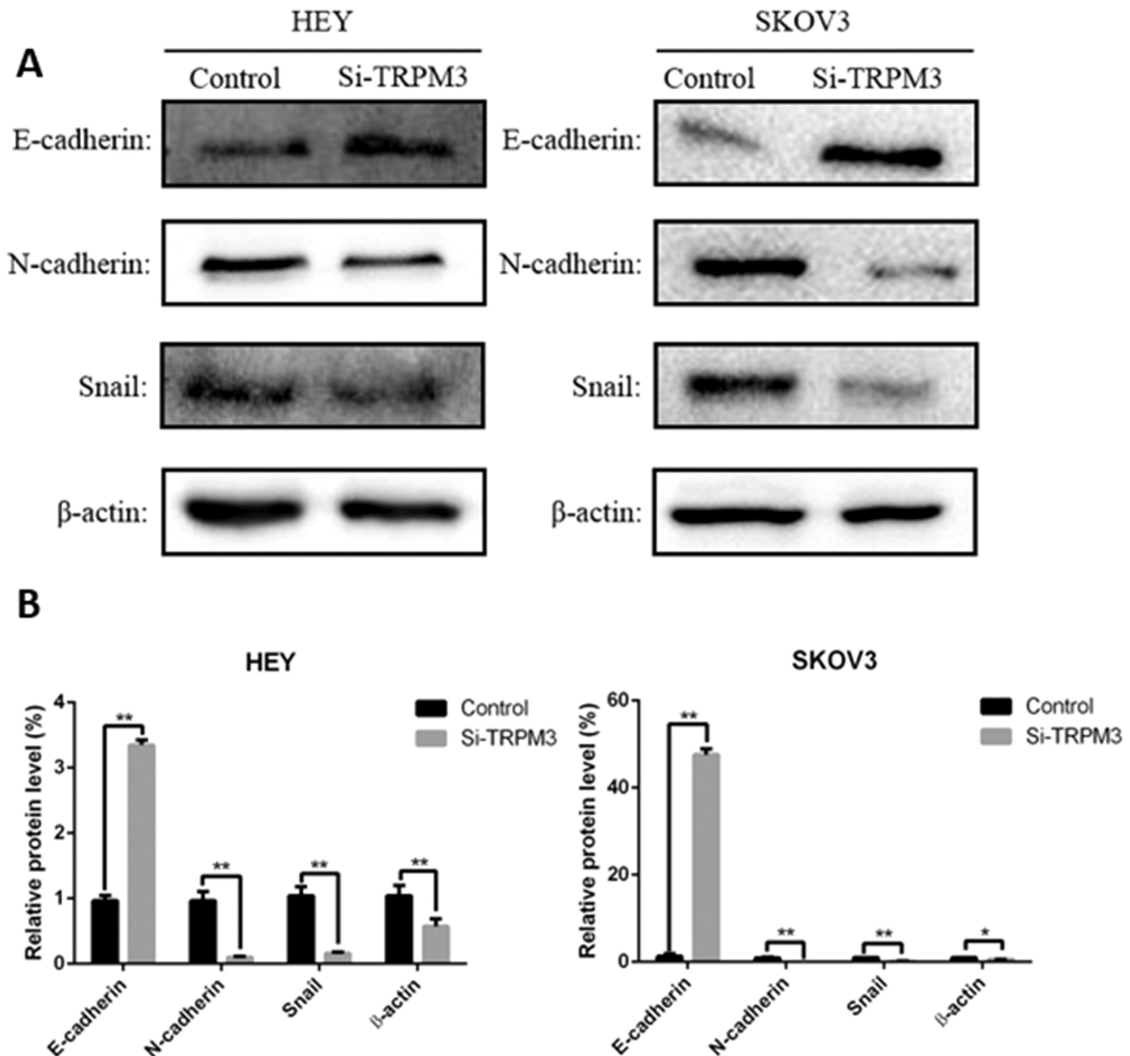


图 3 下调 TRPM3 的表达影响上皮性卵巢癌细胞 EMT 相关蛋白的表达

Fig. 3 Downregulation of TRPM3 expression mediated the expressions of EMT-related protein in the epithelial ovarian cancer cells

Note: A. Silencing the expression of TRPM3 in HEY and SKOV3 cells, the expression of E-cadherin was up-regulated, and the expression of N-cadherin and Snail was down-regulated. β-actin served as a loading control; B. Grey intensity analysis of E-cadherin, N-cadherin, and Snail protein expression by ImageJ. β-actin served as a loading control. (** $P<0.001$)

2.4 下调 TRPM3 的表达抑制 Wnt/β-catenin 信号通路的激活

Western Blot 结果显示:与对照组相比,在干扰 TRPM3 的 HEY 和 SKOV3 细胞中, Wnt/β-catenin 信号通路相关蛋白 β-catenin、Cyclin D1 的表达明显下降,表明 TRPM3 可以激活 Wnt/β-catenin 信号通路(如图 4 所示)。

3 讨论

EMT 存在多种上皮来源的恶性肿瘤中,是指在特定的条件下,上皮细胞向间质细胞表型转化,使得肿瘤细胞具有干细胞的生物学特征,并且促进产生肿瘤干细胞^[8-10],同时伴有上

皮型生物标志物 E-cadherin 表达的下调^[11,12],间质细胞标志蛋白 N-cadherin 的表达上调,及转录调控因子 Snail 表达增加。且多项研究证实恶性肿瘤侵袭转移过程中,EMT 发挥关键作用,其中包括上皮性卵巢癌^[13,14]。EMT 受多种信号传导通路调控,如肝细胞生长因子(Hepatocyte growth factor, HGF)、表皮生长因子(Epidermal growth factor, EGF)、转化生长因子(Transforming growth factor-β, TGF-β)、Notch 以及 Wnt/β-catenin 通路。

Wnt 信号通路作为 EMT 的重要调节因子在上皮性卵巢癌发生发展中起着重要作用^[15,16]。且大量研究表明 Wnt/β-catenin 信号通路在多种恶性肿瘤中是调节 EMT 过程的重要信号通路

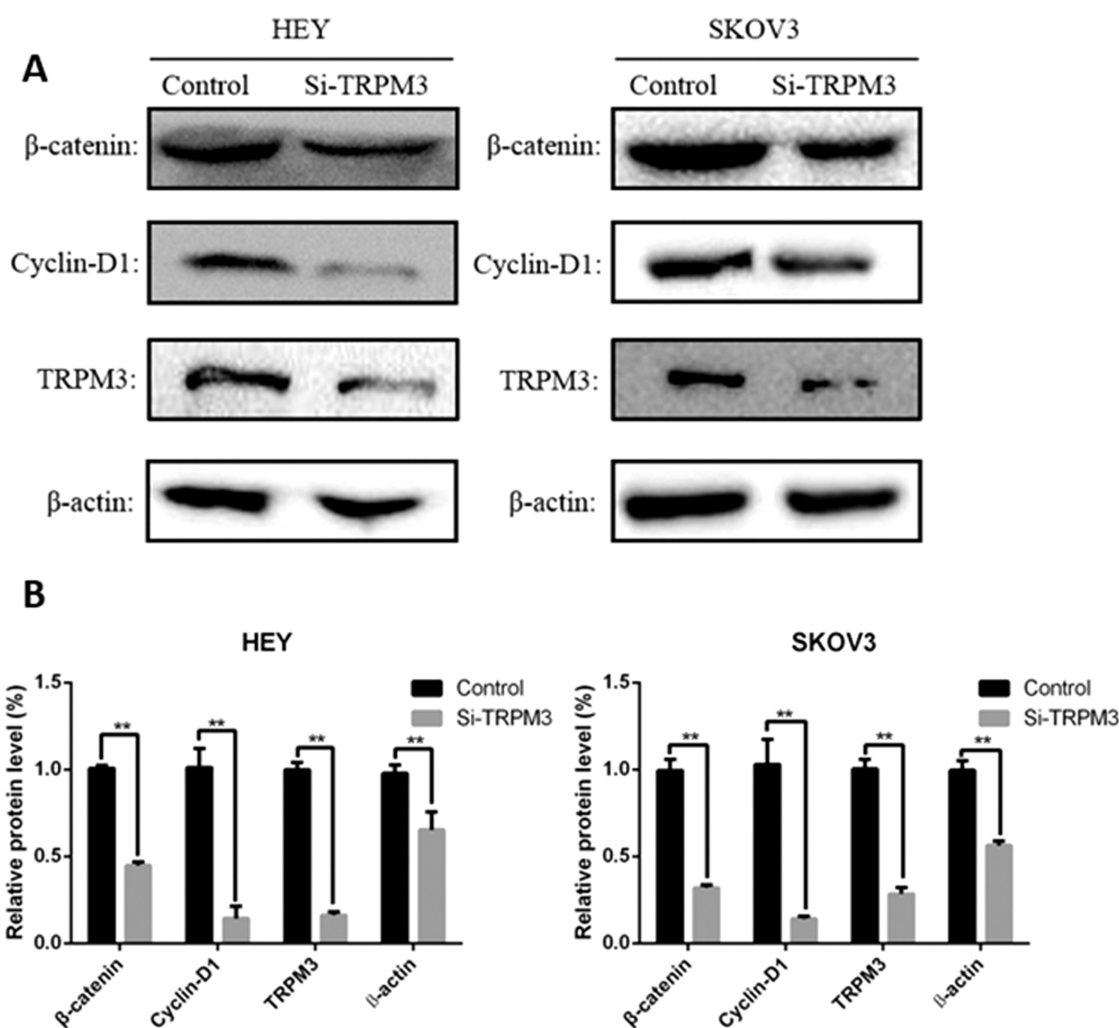


图 4 下调 TRPM3 的表达抑制 Wnt/β-catenin 信号通路的激活

Fig. 4 Downregulation of TRPM3 expression inhibited the activation of Wnt/β-catenin pathway in the epithelial ovarian cancer cells

Note: A. Silencing the expression of TRPM3 in HEY and SKOV3 cells, the expression of β-catenin was up-regulated, the expression of β-catenin, and Cyclin D1 were down-regulated. β-actin served as a loading control; B. Grey intensity analysis of β-catenin, and Cyclin D1 protein expression by ImageJ. β-actin served as a loading control. (***) $P < 0.001$

[17,18],并且在细胞分裂、分化、粘附、凋亡中扮演重要角色^[7]。Wnt/β-catenin 信号通路为经典 Wnt 通路,该通路激活后 β-catenin 磷酸化受到抑制,使得细胞内 β-catenin 浓度增高,与核内转录因子结合后,从而进一步激活 c-Myc、Cyclin D1 等靶基因的表达式^[19]。β-catenin 的磷酸化状态决定了下游靶基因转录的调控^[20]。同时,为了维持 E-cadherin 的粘附功能,胞质内的 β-catenin 与 E 钙黏蛋白 (E-cadherin) 结合,细胞核内的 β-catenin 可以激活进一步 Snail。E-cadherin 和 Snail 都是 EMT 过程中的相关蛋白,因此 Wnt/β-catenin 信号通路与 EMT 密切相关。

瞬时受体电位离子通道 3 (TRPM3) 是一类细胞膜上的重要阳离子通道,是 TRP 阳离子通道家族中的一员。人 TRPM3 基因包括 24 个外显子,位于人染色体 9q-21.12,其在多种组织和细胞(包括背根神经节、心肌细胞和胰腺 β-细胞)中广泛表达。TRPM3 在脂肪细胞、胰腺细胞和肾脏、眼、大脑表达,尤其在眼中高度表达,包括虹膜、视网膜色素上皮和视网膜,在其他组织中如卵巢、胰腺、脊索和睾丸也有一定的表达^[21]。研究表明 TRPM3 参与机体的各种病理生理过程,TRPM3 在背根神经节

神经元中可参与调控机体的避害行为^[22,23],在寡突胶质细胞的分化和中枢神经系统髓鞘的形成中也起到一定的作用^[24]。TRPM3 能够介导胰岛素的分泌^[25],还可以抑制血管平滑肌中炎症因子 IL-6 的表达。此外,还有研究显示肾透明细胞癌中 TRPM3 表达增加,TRPM3 能够促进肾透明细胞癌的生长,并通过诱导钙离子内流增加并刺激 MAP1LC3A (microtubule-associated protein light chain3A, LC3A) 和 MAP1LC3B (microtubule-associated protein light chain3B, LC3B) 自噬^[26]。最新的研究指出高血糖可诱导 TRPM3 高表达,TRPM3 可能参与了糖尿病肾病的发生发展^[27]。有研究指出,储存消耗^[13]、被 D-赤式鞘氨醇活化及细胞低渗可以激活 TRPM3。尽管其天然配体目前尚不清楚,但 TRPM3 可被细胞外神经甾体类固醇孕烯醇酮硫酸盐激活,并与 β 细胞中的胰岛素分泌正向结合^[28]。TRPM3 激活后诱导细胞内信号级联,涉及细胞内钙离子的升高,蛋白激酶 Raf、ERK 和 JNK 的激活以及反应性转录因子 AP-1、CREB、Egr-1 和 Elk-1 的活化。TRPM3 激活的抑制剂包括胆固醇、孕酮、甲氧胺酸、罗格列酮,对 TRPM3 诱导的基因活化有一定的影响,但效果不明显,这些化合物不能作为 TRPM3 特异性抑制

剂。TM3E3 是特异性 TRPM3 的抑制剂^[29], 可以部分性抑制 TRPM3 介导的离子电流和钙离子进入细胞。在功能上, TRPM3 的功能是一个相对有效的 Ca^{2+} 通道^[30], 介导 Ca^{2+} 流入细胞, 使得细胞内 Ca^{2+} 浓度升高。TRPM3 的刺激与体感神经元的热感, 胰腺 β 细胞的胰岛素分泌, 神经递质释放的调节, 虹膜收缩和肿瘤促进有关。

本研究中, Transwell 和划痕实验结果表明敲除 TRPM3 基因后, 上皮性卵巢癌细胞的侵袭迁移能力明显下降, 表明 TRPM3 具有促进上皮性卵巢癌细胞侵袭转移的作用。干扰 TRPM3 基因的表达后, 卵巢癌细胞中上皮性细胞标志蛋白 E-cadherin 下调, 而间质性细胞标志蛋白 N-cadherin 及转录调节因子 Snail 表达上调, 表明 TRPM3 可以促进上皮性卵巢癌细胞发生 EMT 进而促使其发生侵袭转移; 而同时 Wnt/ β -catenin 相关蛋白 β -catenin、CyclinD1 的表达下调。我们由此推测 TRPM3 很可能是通过激活 Wnt/ β -catenin 信号通路促进上皮性卵巢癌细胞发生 EMT。

然而, 目前仍然缺乏高特异性的 TRPM3 抑制剂, 未来工作中可将其作为研究目标。随着人们对 TRPM3 的作用和调控机制的深入了解, TRPM3 的其他组织特异性作用也将被发现, TRPM3 可能作为上皮性卵巢癌新的治疗靶标。

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