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·基础研究·

DKC1影响黏膜型黑色素瘤细胞的增殖、凋亡和细胞周期且与MEK-ERK通路相关

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[摘 要] **目的:**研究角化不良素假尿苷合成酶1(dyskerin pseudouridine synthase 1,DKC1)对黏膜型黑色素瘤细胞的增殖、细胞周期和凋亡的影响及其可能的机制。**方法:**qPCR检测DKC1 mRNA在黏膜型黑色素瘤细胞系HMV II、GAK和正常皮肤细胞株BJ中的表达水平;用DKC1 siRNA(si-DKC1组)和对照siRNA(si-Ctrl组)转染HMV II和GAK细胞,干扰48 h后用qPCR和Western blotting验证敲减效率,CCK-8法检测敲减DKC1对黏膜型黑色素瘤细胞增殖的影响,流式细胞技术检测对细胞凋亡和周期的影响,Western blotting和qPCR检测MEK-ERK通路相关分子表达变化。**结果:**HMV II、GAK细胞的DKC1 mRNA和蛋白表达水平均显著高于BJ细胞(均P<0.01)。转染siRNA 48 h后,与si-Ctrl组相比,si-DKC1组HMV II和GAK细胞中DKC1 mRNA和蛋白水平均显著降低(均P<0.01),细胞的增殖水平显著下降(P<0.05或P<0.01),细胞的凋亡率显著升高(均P<0.01)且促凋亡分子caspase 9、BAK和PUMA mRNA的表达显著升高(P<0.05或P<0.01),发生细胞周期阻滞(P<0.05或P<0.01),MEK和ERK1/2的磷酸化水平显著下调(P<0.05)。**结论:**敲减DKC1可抑制黏膜型黑色素瘤细胞的增殖,促进细胞周期阻滞并诱导细胞凋亡,其机制可能与MEK/ERK信号通路有关。

[关键词] 角化不良素假尿苷合成酶1;黏膜型黑色素瘤;增殖;凋亡;细胞周期
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DKC1 affects proliferation, apoptosis and cell cycle of mucosal melanoma cells relating to MEK-ERK pathway

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[Abstract] Objective: To investigate the effects of dyskerin pseudouridine synthase 1 (DKC1) on the proliferation, cell cycle and apoptosis of mucosal melanoma cells and its potential mechanisms. **Methods:** qPCR was used to detect the mRNA expression of DKC1 in mucosal melanoma cell lines HMV II, GAK and normal skin cell line BJ. HMV II and GAK cells were interfered with DKC1 siRNA (si-DKC1 group) and control siRNA(si-Ctrl group) respectively; 48 h later, qPCR and Western blotting were used to verify the interference efficiency. CCK-8 assay was used to detect the effect of DKC1 knockdown on the proliferation of mucousal melanoma cells. Flow cytometry was used to detect the apoptosis and cell cycles. Western blotting and qPCR were used to detect the molecule expressions of related pathways. **Results:** The mRNA and protein expression levels of DKC1 in HMV II and GAK cells were significantly higher than those in BJ cells (all P<0.01). After 48 h of siRNA transfection, compared with the si-Ctrl group, the mRNA and protein levels of DKC1 in HMV II and GAK cells of the si-DKC1 group significantly reduced (all P<0.01), the cell proliferation level significantly reduced (P<0.05 or P<0.01), and the apoptosis rate of cells significantly increased (all P<0.01); in addition, the mRNA expressions of pro-apoptotic molecules caspase 9, BAK and PUMA increased significantly (P<0.05 or P<0.01) and the cell cycle was blocked (P<0.05 or P<0.01); moreover, the phosphorylation levels of MEK and ERK1/2 were significantly reduced (P<0.05). **Conclusion:** Knockdown of DKC1 can inhibit the proliferation of mucousal melanoma cells, promote cell cycle arrest and induce apoptosis, and its mechanism may be related to MEK/ERK signal pathway.

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[Key words] dyskerin pseudouridine synthase 1 (DKC1); mucosal melanoma; proliferation; apoptosis; cell cycle
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黑色素瘤是一种病死率较高的恶性肿瘤，发病率每年递增3%~5%[1]。据2015年流行病学统计，中国黑色素瘤年均新发病例已超过2万人[2]。黏膜型黑色素瘤在欧美人群中发病率仅占总黑色素瘤的1%~2%，但在亚洲人群中这一比例却高达22.6%[3]。目前国际上没有针对黏膜型黑色素瘤的标准治疗指南可遵循，此外，由于黏膜型黑色素瘤具有诊断时分期晚、预后差、易复发和转移等特点，给临床治疗带来极大挑战[4-6]。因此，探索黏膜型黑色素瘤发生发展的机制，寻找治疗黏膜型黑色素瘤的潜在干预靶点，成为提高患者生存率的关键。

角化不良素假尿苷合成酶1(dyskerin pseudouridine synthase 1，DKC1)基因定位于人X染色体q28位点，在进化上高度保守。其编码的角化不良素蛋白(dyskerin)是一种假尿苷合成酶，可以将rRNA上的尿苷残基转化为假尿苷，从而使核糖体维持正常功能，还可以作为端粒酶组成部分调节端粒酶活性[7-9]。DKC1突变会引起X连锁性先天性角化不全(X-linked dyskeratosis congenita，X-DC)，其表现为核糖体功能障碍、组织增殖能力减弱和肿瘤易感性增加[10-13]。此外，DKC1还被报道与多种肿瘤相关：在成神经细胞瘤中DKC1的mRNA水平较正常细胞升高，且在临床队列研究中发现，DKC1高表达与患者预后密切相关[14]；在乳腺癌和肾透明细胞癌中发现DKC1低表达组的患者预后好于高表达组[15-16]，但相关机制尚不明确。关于DKC1在黑色素瘤发生发展中的作用及其机制尚不清楚，本研究拟探讨DKC1在黏膜型黑色素瘤中的功能及作用机制。

1 材料与方法

1.1 主要材料与试剂

黏膜型黑色素瘤细胞株HMV II、GAK和正常皮肤细胞株BJ购自美国ATCC。F10、F12培养基、胰蛋白酶、胎牛血清、双抗、Opti-MEM培养基购自Gibco公司，RNA提取试剂盒购自Thermo公司，逆转录试剂盒购自TaKaRa公司，qPCR试剂购自SYBR公司，凋亡检测试剂盒、CCK-8细胞增殖与毒性检测试剂盒均购自DojinDo公司，细胞周期检测试剂盒购自BD公司，引物合成由天一辉远公司、擎科公司完成，DKC1 siRNA和NC siRNA由吉玛公司合成，Western blotting(WB)所用抗体均购自Abcam公司，WB Loadding Buffer购自TaKaRa公司，Lipofectamine™ 2000试剂盒购自Invitrogen公司，流式细胞仪购自Beckman Coulter公司，Fast-7500 qPCR仪购自Thermo公司。

1.2 细胞培养及转染

将HMV II、GAK、BJ细胞分别置于F10、F12、DMEM培养基(含20%胎牛血清及1%双抗)中，放在37 ℃、5% CO_2孵箱培养至对数期。将HMV II、GAK接种于6孔板，24 h后，更换新鲜培养基，将细胞随机分为2组：敲减组(转染si-DKC1)和对照组(转染si-Ctrl)，使用Lipofectamine™ 2000试剂盒按说明书操作，转染siRNA干扰48 h后收集各组细胞进行后续实验(细胞RNA和蛋白的提取、周期和凋亡的检测)。

1.3 CCK-8法检测敲减DKC1对黑色素瘤细胞增殖的影响

待转染后的细胞长至汇合度80%左右时，重新消化、清洗，按3 000个/孔、共100 μl接种于96孔板，按CCK-8检测试剂盒说明书操作，第24、48、72、96 h时用酶标仪检测各孔在450 nm波长处的光密度(D)值，以D值代表细胞的增殖能力，绘制增殖曲线。

1.4 WB检测敲减DKC1对黑色素瘤细胞中相关蛋白表达的影响

转染48 h后收集各组细胞，提取总蛋白，采用BCA法检测蛋白浓度，参照文献[17]报道的方法行WB检测。采用Amersham Imager 600仪器分析蛋白条带灰度值，蛋白的相对表达水平用目的条带与内参(GAPDH)条带的灰度值比值表示。

1.5 qPCR检测细胞中基因的表达水平

提取细胞RNA，按逆转录试剂盒说明书完成逆转录后进行qPCR实验。qPCR具体步骤参见文献[17]的方法。应用Fast 7500 PCR仪、Fast 7500软件分析，以β-actin为对照，目的基因mRNA的相对表达量用$2^{-\triangle\triangle CT}$法计算。用Graphpad prism7软件行单因素分析并作图。

1.6 流式细胞术检测敲减DKC1对黑色素瘤细胞周期的影响

转染48 h后将各组细胞消化、PBS清洗、离心弃上清，每组细胞用1 ml预冷的PBS重悬，缓慢加入预冷的无水乙醇3 ml(乙醇终浓度为75%)，4 ℃静置过夜(18~24 h)后重新离心，预冷的PBS洗2遍，加入100 μl PBS重悬，转移至1.5 ml EP管中，每1×10^6个细胞加入0.5 ml PI染色液，24 h内上流式细胞仪检测细胞周期情况。

1.7 流式细胞术检测敲减DKC1对黑色素瘤细胞凋亡的影响

转染48 h后收集各组细胞，使用锥虫蓝染色法计数。每组样品加入1×Annexin V Binding Buffer，制

成终浓度为10^6个/ml的细胞悬液（不少于500 μl），取100 μl至1.5 ml EP管中，加入5 μl Annexin V-FITC结合物，再加入5 μl PI Solution，室温避光染色15 min，加入400 μl 1×Annexin V Binding Buffer，1 h内上流式细胞仪检测细胞凋亡情况。

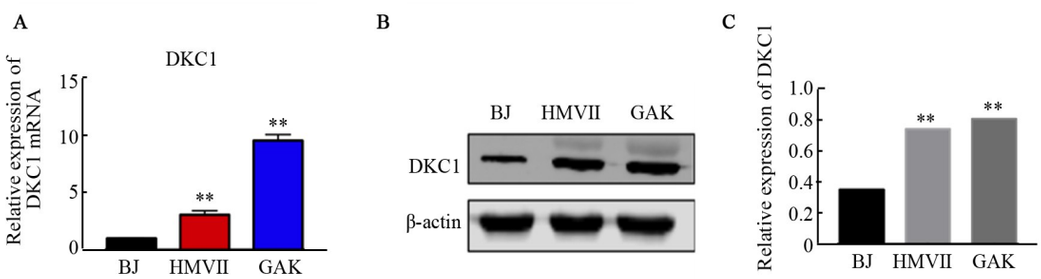
1.8　统计学处理

采用SPSS 16.0软件进行分析，所有实验独立重复3次，呈正态分布的计量数据采用$\bar{x}\pm s$表示，组间比较采用独立样本t检验，以P<0.05或P<0.01表示差异具有统计学意义。

2　结　果

2.1 DKC1在黏膜型黑色素瘤细胞中呈高表达

qPCR和WB检测结果显示，黏膜型黑色素瘤细胞HMV II、GAK的DKC1 mRNA表达水平(图1A)和蛋白水平（图1B、C）均显著高于正常的皮肤细胞BJ（均P<0.01）。



**P<0.01 *vs* BJ cells

A: The mRNA expression of DKC1 in BJ, HMV II and GAK cells detected by qPCR;

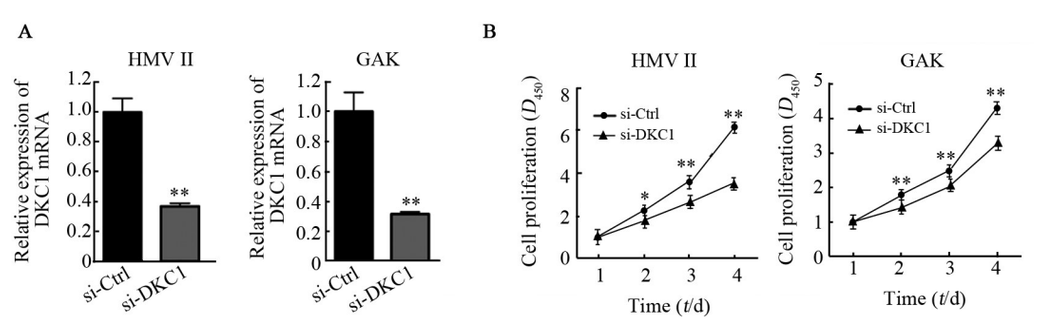
B, C: The protein expression of DKC1 in BJ, HMV II and GAK cells detected by WB

图1　DKC1在黏膜型黑色素瘤细胞和正常皮肤细胞中的表达

Fig.1　Expression of DKC1 in mucosal melanoma cells and normal skin cells

2.2　敲减DKC1抑制黏膜型黑色素瘤细胞的增殖

转染siRNA 48 h后，qPCR检测结果（图2A）显示，si-DKC1组HMV II和GAK细胞中DKC1 mRNA水平显著低于对照组（均P<0.01），说明DKC1得到有效敲减。CCK-8法检测结果（图2B）显示，转染siRNA 48 h起，si-DKC1组HMV II和GAK细胞的增殖水平显著低于对照组(P<0.05或P<0.01）。



*P<0.05, **P<0.01 *vs* si-Ctrl group

A: DKC1 knockdown efficiency was detected by qPCR; B: Knockdown of DKC1 inhibited the proliferation of mucosal melanoma cells

图2　敲减DKC1对黏膜型黑色素瘤细胞增殖的影响

Fig.2　Knockdown of DKC1 affected the proliferation of mucosal melanoma cells

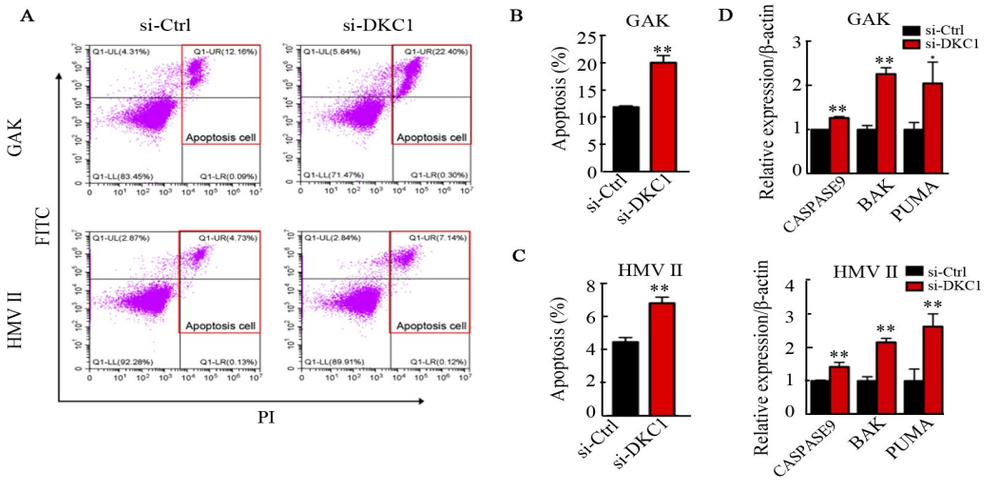
2.3　敲减DKC1促进黏膜型黑色素瘤细胞凋亡且影响相关分子表达

流式细胞术检测结果（图3A、B、C）显示，与si-Ctrl组相比，si-DKC1组HMV II和GAK细胞的凋亡率显著升高（P<0.01）；qPCR检测结果（图3D）显示，si-DKC1组HMV II和GAK细胞中促凋亡分子caspase 9、BAK和PUMA mRNA的表达较si-Ctrl组显著升高（P<0.05或P<0.01）。

2.4　DKC1敲减诱导黏膜型黑色素瘤细胞周期阻滞

流式细胞术检测结果（图4）显示，对于GAK细胞，相较于si-Ctrl组，si-DKC1组G0/G1期的细胞比例增多，而S期细胞比例减少（均P<0.05）；对于

HMV II细胞，相较于si-Ctrl组，si-DKC1组S期的细胞比例显著升高（P<0.01），而G0/G1期（P<0.01）和G2/M期（P<0.05）的细胞比例显著减少。

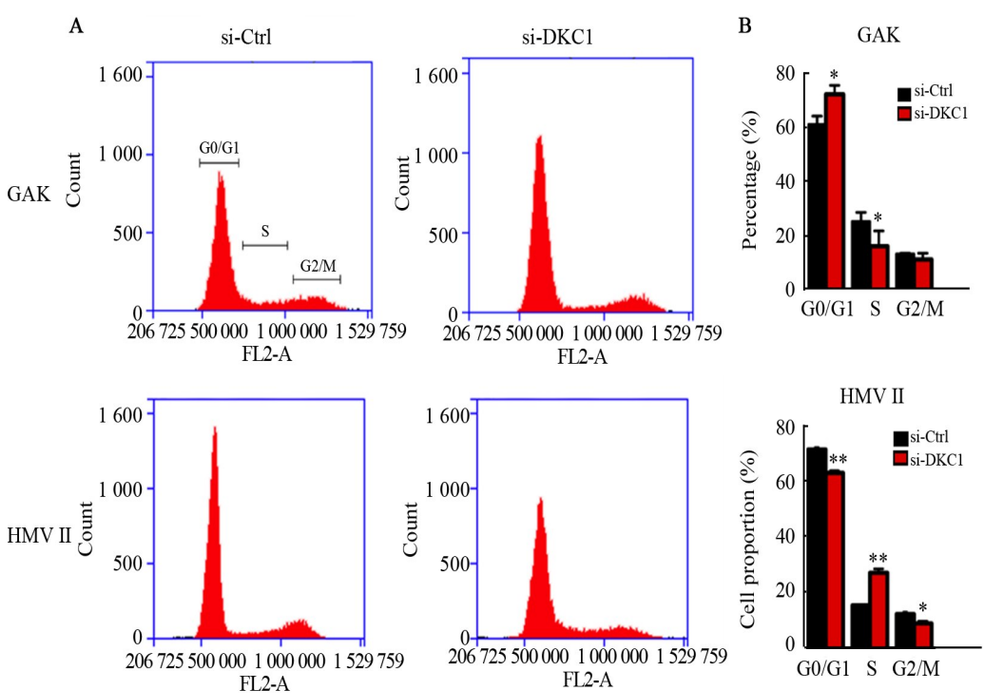


*P<0.05, **P<0.01 *vs* si-Ctrl group

A: Effect of DKC1 knockdown on apoptosis of GAK and HMV II cells was detected by Flow cytometry; B and C: Apoptosis rates of GAK and HMV II cells; D: Expression of apoptosis-related biomarkers was detected by qPCR

图3 敲减DKC1对黏膜型黑色素瘤细胞凋亡的影响

Fig.3 Knockdown of DKC1 affected the apoptosis rates of mucosal melanoma cells



*P<0.05, **P<0.01 *vs* si-Ctrl group

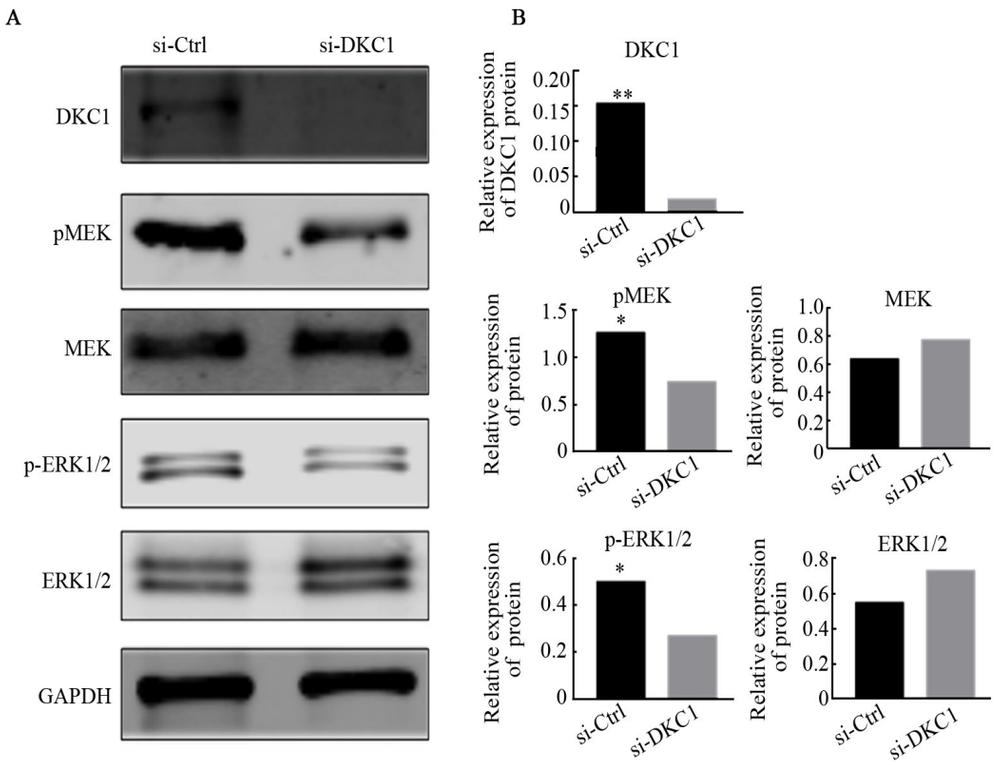
A: Cell cycle detected by Flow cytometry; B: Proportion of cells in different cycles

图4 敲减DKC1对黏膜型黑色素瘤细胞周期的影响

Fig.4 Effects of DKC1 knockdown on cell cycles of mucosal melanoma cells

2.5 DKC1敲减影响黏膜型黑色素瘤细胞MEK/ERK通路相关蛋白的表达

WB检测结果（图5）显示，与si-Ctrl组相比，si-DKC1组细胞中DKC1蛋白表达显著降低（P<0.01），且MEK和ERK1/2的磷酸化水平显著降低（P<0.05），而MEK、ERK1/2的总蛋白量无明显变化（P>0.05）。



*P<0.05, **P<0.01 *vs* si-Ctrl group

A: Effect of DKC1 down-regulation on the expressions of MEK, ERK1/2, phosphorylated MEK and ERK1/2 assessed by WB assay;

B: The quantification of MEK, ERK1/2, phosphorylated MEK and ERK1/2 compared with GAPDH as the control group

图5 敲减DKC1对MEK/ERK通路相关蛋白的影响

Fig.5 Knockdown of DKC1 affected the expressions of proteins related by MEK/ERK pathway

3 讨 论

DKC1与多种肿瘤的发生发展、恶性程度及预后有着密切联系，但其促进肿瘤发生发展的机制尚未阐明。有研究[9, 18]认为，DKC1不是通过端粒酶途径影响肿瘤发生发展，而是通过其编码的角蛋白作用于核糖体的翻译过程影响肿瘤进程。DKC1参与含有内部核糖体进入位点（internal ribosomal entry site，IRES）的mRNA的翻译过程，其中包括抑癌基因p53和p27、抗凋亡因子Bcl-xL和XIAP等，这导致DKC1参与的翻译过程发生错误会导致肿瘤易感性增加[19-22]。另外，DKC1可以对rRNA前体进行加工，并与单链选择性单功能尿嘧啶DNA糖基化酶1（single-strand-selective monofunctional uracil-DNA glycosylase 1，SMUG1）协同调控成熟rRNA的质量[23]，但关于DKC1如何调控抑癌基因的翻译尚不清楚。

RAS-RAF-MEK-ERK通路是一条在多种肿瘤中能被广泛激活的通路，与细胞增殖、耐药、周期阻滞和凋亡等肿瘤细胞生物学行为均具有密切关系[24-33]。本研究结果显示，敲减DKC1能抑制黏膜型黑色素瘤细胞增殖、诱导周期阻滞和凋亡，同时伴随MEK和ERK1/2蛋白磷酸化水平下调，推测DKC1可能通过MEK-ERK通路调控细胞周期阻滞和凋亡，从而调节黏膜型黑色素瘤细胞的增殖，影响黏膜型黑色素瘤进展。此外，有研究[34]发现DKC1的转录直接受癌基因MYC的调节。在成神经细胞瘤中，抑制DKC1表达可以使肿瘤细胞发生p53依赖的G1期阻滞[14]；在神经胶质瘤中，敲减DKC1能抑制细胞增殖、侵袭和迁移，且将细胞周期阻滞于G1期，并伴随着神经钙黏素、HIF-1α、MMP2、CyclinE2、CDK2和p27表达下调[35]；在肾透明细胞癌中，敲减DKC1可通过NF-κB/MMP-2信号通路抑制癌细胞增殖、侵袭和迁移[16]。本研究结果与上述报道一致，提示DKC1表达异常可以诱导细胞周期阻滞进而抑制细胞增殖是一种普遍的现象。值得注意的是，研究[36]发现，DKC1在前列腺癌中普遍上调，且发挥着维持蛋白质合成的重要功能，但DKC1的功能受损或是过表达均能促进肿瘤发展。同样，在垂体瘤小鼠模型中发现，DKC1的突变能促进体细胞癌的发生，且通过调控p27的翻译在肿瘤抑制中扮演关键角色[13]，提示DKC1可能在不同的肿瘤中发挥不同功能。

综上所述，本研究阐释了DKC1在黏膜型黑色素

瘤中的作用并对其相关通路进行了初步探索，证明DKC1可影响黏膜型黑色素瘤细胞的增殖、细胞周期和凋亡，且可能与MEK/ERK信号通路有关，该结果为筛选黏膜型黑色素瘤的潜在干预靶点提供了有意义的资料。

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