S1P/S1PR1通路调节LAMB3的表达对食管鳞癌细胞迁移、黏附和转移的影响

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摘要　　该文主要探讨1-磷酸鞘氨醇(sphingosine 1-phosphate, S1P)/1型1-磷酸鞘氨醇受体(sphingosine 1-phosphate receptor 1, S1PR1)通路调节层黏连蛋白亚基β3(laminin subunit beta 3, LAMB3)的表达对食管鳞状细胞癌(esophageal squamous cell carcinoma, ESCC)细胞增殖、迁移、黏附及转移的影响。使用GEO数据库分析*LAMB3*在ESCC癌组织与癌旁正常组织中的表达差异情况; 用Western blot检测人食管上皮细胞HEEC及人食管鳞癌Eca109、TE-1、KYSE-150细胞中LAMB3的表达量; 用S1P处理TE-1细胞, 或在Eca109细胞中敲低或过表达S1PR1, 采用Western blot检测LAMB3的表达情况; *S1PR1*-EGFP过表达载体和*LAMB3*小干扰RNA(siRNA)共转染Eca109细胞, 采用划痕实验检测迁移能力; 采用*LAMB3* siRNA或*LAMB3*短发夹RNA(shRNA)下调ESCC细胞LAMB3的表达, 过表达LAMB3的慢病毒上调ESCC细胞LAMB3的表达, 通过CCK8法、划痕实验、细胞–基质黏附实验分别检测ESCC细胞的增殖、迁移和黏附能力, Western blot检测上皮–间质转化(epithelial-mesenchymal transition, EMT)相关蛋白及基质金属蛋白酶9(matrix metalloproteinase 9, MMP9)的表达情况; 裸鼠尾静脉注射*LAMB3* shRNA敲低的Eca109细胞, 观察其转移能力及生存期。结果显示, LAMB3在ESCC癌组织及ESCC细胞中的表达均高于癌旁正常组织及正常食管上皮细胞; S1P作用后的TE-1细胞LAMB3表达水平增高; 在Eca109细胞中敲低S1PR1, LAMB3表达下调; 在Eca109细胞中过表达S1PR1, LAMB3表达上调, 促进细胞迁移, 并且干扰LAMB3表达可抑制上述作用; 敲低LAMB3的表达, Eca109、TE-1细胞增殖无显著变化, 而迁移、黏附受到明显抑制, 过表达LAMB3后, Eca109细胞增殖无显著变化, 而迁移、黏附增强; *LAMB3* shRNA敲低的Eca109细胞EMT过程被抑制, MMP9表达降低, 过表达LAMB3则出现相反的效应; 与NC shRNA组相比, 注射*LAMB3* shRNA敲低的Eca109细胞的裸鼠生存期明显延长, 肿瘤体内转移灶减少。该研究得出, LAMB3在ESCC细胞及组织中表达上调, S1P/S1PR1通路能诱导LAMB3的表达, 促进ESCC细胞的体外迁移、黏附和体内转移。

关键词　　1-磷酸鞘氨醇; 1型1-磷酸鞘氨醇受体; 层黏连蛋白亚基β-3; 食管鳞癌; 上皮–间质转化; 迁移; 黏附

Effects of S1P/S1PR1 Pathway Regulating LAMB3 Expression on Migration, Adhesion and Metastasis of Esophageal Squamous Cell Carcinoma Cells

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Abstract　　This study aimed to investigate the effects of S1P (sphingosine 1-phosphate)/S1PR1 (sphingosine 1-phosphate receptor 1) pathway regulating the expression of LAMB3 (laminin subunit beta 3) on the proliferation, migration, adhesion and metastasis of ESCC (esophageal squamous cell carcinoma) cells. GEO database was used to analyze the difference of LAMB3 expression between ESCC tissues and normal tissues. Western blot was used to detect the expression of LAMB3 in human esophageal epithelial cell HEEC cells and human esophageal squamous cell carcinoma Eca109, TE-1 and KYSE-150 cells. TE-1 cells were treated with S1P, or knockdowned or overexpressed S1PR1 in Eca109 cells, the expression of LAMB3 was detected by Western blot. *S1PR1*-EGFP overexpression vector and *LAMB3* siRNA (small interfering RNA) were co-transfected into Eca109 cells, and the migration ability was detected by scratch assay. LAMB3 siRNA or LAMB3 shRNA (short hairpin RNA) was used to down-regulate the expression of LAMB3 in ESCC cells, lentivirus with LAMB3 overexpression was used to up-regulate the expression of LAMB3 in ESCC cells. The proliferation, migration and adhesion ability of ESCC cells were detected by CCK8 assay, scratch assay, and cell-matrix adhesion assay, respectively, and the expression of EMT (epithelial-mesenchymal transition) -related proteins and MMP9 (matrix metalloproteinase 9) were detected by Western blot. Eca109 cells with *LAMB3* shRNA knockdown were injected into the tail vein of nude mice, and their metastatic ability and survival rate were evaluated. The results showed that the expression of LAMB3 gene and protein in ESCC cancer tissues and ESCC cells were higher than that in normal tissues and human esophageal epithelial cells. LAMB3 expression was increased in TE-1 cells treated with S1P. LAMB3 expression was down-regulated in S1PR1 knockdown Eca109 cells, and conversely, overexpression of S1PR1 in Eca109 cells up-regulated LAMB3 expression, promoted cell migration, and knockdown of LAMB3 inhibited the effect. LAMB3 knockdown by siRNA or shRNA had no significant effect on the proliferation of Eca109 cells and TE-1 cells. However, the migration and adhesion of Eca109 cells and TE-1 cells were considerably reduced by LAMB3 knockdown. Overexpression of LAMB3 had no significant effect on the proliferation of Eca109 cells. However, the migration and adhesion of Eca109 cells were considerably enhanced by LAMB3 overexpression. EMT process was inhibited and MMP9 was significantly decreased in LAMB3 stable knockdown Eca109 cells, and the opposite effect were showed in LAMB3-overexpressing Eca109 cells. In the nude mice model of metastasis, there were higher survival rate and fewer metastases in LAMB3 knockdown group than that in the negative control group. In this study, it concludes that LAMB3 is up-regulated in ESCC cells and tissues, and S1P/S1PR1 pathway inducing the expression of LAMB3 promotes ESCC cells migration and adhesion *in vitro* and metastasis *in vivo*.

Keywords　　sphingosine 1-phosphate; sphingosine 1-phosphate receptor 1; laminin subunit beta-3; esophageal squamous cell carcinoma; epithelial-mesenchymal transition; migration; adhesion

在常见的恶性肿瘤中, 食管癌的全球发病率居第7位, 总死亡率居第6位, 最常见的组织学亚型包括食管鳞状细胞癌 (esophageal squamous cell carcinoma, ESCC, 简称食管鳞癌)和食管腺癌 (esophageal adenocarcinoma, EAC)[1]。我国是世界上食管癌高发地区之一, 组织学亚型多为ESCC[2]。患者被诊断为食管癌时多处于中晚期, 且病程进展快, 患者生存率低[3]。

层黏连蛋白亚基β3(laminin subunit beta 3, *LAMB3*)基因编码层黏连蛋白332(laminin 332, LM-332)的β3链。LM-332是层黏连蛋白家族的主要成员, 由α3链、β3链、γ2链构成, α3、γ2链分别由基因层黏连蛋白亚基α3(laminin subunit alpha 3, *LAMA3*)、层黏连蛋白亚基γ2(laminin subunit gamma 2, *LAMC2*)编码[4]。研究发现, LAMB3在几种肿瘤组织与细胞中表达上调[5-9], 可促进肿瘤的侵袭和转移等。

本课题组在前期研究中发现, ESCC的进展与1-磷酸鞘氨醇(sphingosine 1-phosphate, S1P)/1型1-磷酸鞘氨醇受体(sphingosine 1-phosphate receptor 1, S1PR1)通路密切相关[10], 并且采用有参转录组高通量测序技术, 发现在过表达S1PR1的食管鳞癌Eca109细胞中, *LAMB3* mRNA的表达水平增加[11]。本研究将进一步探究在ESCC中, S1P/S1PR1通路对LAMB3的调节, 以及LAMB3对ESCC细胞增殖、迁移、黏附和体内转移能力的影响, 旨在为ESCC的发生发展机制、诊断、治疗、预后提供科学的依据。

1　材料与方法

1.1　材料

1.1.1　细胞株　　人食管上皮细胞HEEC由川北医学院病理学教研室何欣蓉教授惠赠, 人食管鳞癌Eca109细胞、TE-1细胞、KYSE-150细胞由中国科学院细胞库提供。

1.1.2　主要试剂　　RPMI 1640培养液及胰蛋白酶(0.25% Trypsin-EDTA)购自Gibco公司。胎牛血清(fetal bovine serum, FBS)购自浙江天杭生物科技股份有限公司。无脂肪酸牛血清白蛋白(fatty acid-free bovine serum albumin, FAF-BSA)购自Sigma公司。Lipofectamine™ 2000购自Invitrogen公司。S1P购自Cayman Chemical公司。*S1PR1*-EGFP(人*S1PR1*与EGFP融合表达的载体)及对照质粒Control-EGFP由美国麻省总医院吴美雄教授惠赠。CCK8试剂、Western blot试剂盒、Western blot所用二抗及β-actin一抗购自上海碧云天生物技术有限公司。S1PR1、LAMB3、E-钙黏蛋白(E-cadherin)、基质金属蛋白酶9(matrix metalloproteinases 9, MMP9)和波形蛋白(Vimentin)的一抗购自Proteintech公司。嘌呤霉素及纤连蛋白购自Solarbio公司。引物合成于生工生物工程(上海)股份有限公司。小干扰RNA(small interfering RNA, siRNA)由上海吉玛制药技术有限公司合成。

短发夹RNA(short hairpin RNA, shRNA)慢病毒载体的构建及病毒包装由上海吉凯基因科技有限公司完成。

1.2　方法

1.2.1　*LAMB3*在ESCC癌组织与癌旁正常组织中的表达情况　　在GEO数据库选取并下载包含ESCC基因表达的数据集GSE44021(含113对ESCC癌组织和癌旁正常组织样本)、GSE161533(含28对ESCC癌组织和癌旁正常组织样本), 提取*LAMB3*表达信息, 分析*LAMB3*在ESCC癌组织和癌旁正常组织中的表达差异。

1.2.2　细胞培养　　向人食管上皮细胞HEEC、人食管鳞癌细胞(Eca109、TE-1及KYSE-150)中加入RPMI 1640培养液(含10% FBS、100 U/mL青霉素、100 μg/mL链霉素), 并将其置于37 ℃、5% CO₂细胞培养箱中培养。

1.2.3　Western blot分析　　使用碧云天RIPA裂解液提取细胞总蛋白, 用BCA法检测总蛋白的浓度。细胞总蛋白(25 μg)经SDS-PAGE凝胶电泳(8%)分离后, 转至PVDF膜上, 室温下用5%脱脂奶粉封闭2 h。加入稀释后的一抗[S1PR1(1:500)、LAMB3(1:2 500)、E-cadherin(1:2 000)、MMP9(1:1 500)、Vimentin(1:2 000)、和β-actin(1:2 000)], 摇床4 ℃孵育过夜。TBST洗膜3次, 加入稀释后的二抗(1:1 000), 室温孵育70 min; TBST洗涤, 使用ECL发光液, 用Bio-Rad ChemiDoc™ XRS+化学发光凝胶成像仪曝光。

1.2.4　S1P处理TE-1细胞　　用含10% FBS的RPMI 1640培养液培养细胞, 然后将TE-1细胞接种于6孔板, 培养24 h, 换液为含0.1% FAF-BSA的RPMI 1640培养液(2 mL), 血清饥饿14 h后, 分别用含0、10、100 nmol/L S1P的0.1% FAF-BSA的RPMI 1640培养液(2 mL), 继续培养24 h, 收集细胞进行后续实验。

1.2.5　质粒转染Eca109细胞　　用含10% FBS的RPMI 1640培养液培养细胞, 然后将Eca109细胞接种于6孔板, 培养24 h, 换液为含0.1% FAF-BSA的RPMI 1640培养液, 用Lipofectamine™ 2000分别转染*S1PR1*-EGFP与Control-EGFP(即: S1PR1过表达组与对照组)。转染后6~8 h换液为含0.1% FAF-BSA的RPMI 1640培养液, 转染后48 h及72 h分别收集细胞提取总RNA及总蛋白。

1.2.6　siRNA转染Eca109、TE-1细胞　　用含10% FBS的RPMI 1640培养液培养细胞, 然后将细胞接

种于6孔板、12孔板或96孔板,培养24 h,按照说明书,用 Lipofectamine™ 2000将NC siRNA、*S1PR1* siRNA1、*S1PR1* siRNA2或NC siRNA、*LAMB3* siRNA1、*LAMB3* siRNA2分别转染细胞,在转染后的不同时间点进行相应检测。*S1PR1* siRNA1正义链序列为5′-CUG ACC UCG GUG GUG UUC AdT-dT-3′,反义链序列为5′-UGA ACA CCA CCG AGG UCA GdTdT-3′;*S1PR1* siRNA2正义链序列为5′-CGG UCU CUG ACU ACG UCA AdTdT-3′,反义链序列为5′-UUG ACG UAG UCA GAG ACC GAG;*LAMB3* siRNA1正义链序列为5′-GAU GGA GGA AGA UGU CAG AdTdT-3′,反义链序列为5′-UCU GAC AUC UUC CUC CAU CdTdT-3′;*LAMB3* siRNA2正义链序列为5′-CCA AAG GUG CGA CUG CAA UdTdT-3′,反义链序列为5′-AUU GCA GUC GCA CCU UUG GdTdT-3′;阴性对照NC siRNA正义链序列为5′-UUC UCC GAA CGU GUC ACG UdTdT-3′,反义链序列为5′-ACG UGA CAC GUU CGG AGA AdTdT-3′。

1.2.7 *S1PR1*-EGFP质粒和 *LAMB3* siRNA共转染 Eca109细胞 　用含10% FBS的RPMI 1640培养液将Eca109细胞接种于12孔板,培养24 h后换液为含0.1% FAF-BSA的RPMI 1640培养液,按照说明书用Lipofectamine™ 2000共转染质粒、siRNA,共转染分为4组(*S1PR1*-EGFP+NC siRNA、Control-EGFP+NC siRNA、*S1PR1*-EGFP+*LAMB3* siRNA1、*S1PR1*-EGFP+*LAMB3* siRNA2)。转染后6~8 h将其换液为含0.1%FAF-BSA的RPMI 1640培养液,继续培养并进行后续实验。

1.2.8 慢病毒感染及稳定细胞株的筛选 　根据慢病毒使用说明书进行细胞慢病毒感染及后续嘌呤霉素筛选稳定细胞株。敲低及过表达LAMB3的Eca109细胞分别分为3组及2组:对照组(命名为NC shRNA),LAMB3敲低组1(命名为*LAMB3* shRNA1),LAMB3敲低组2(命名为*LAMB3* shRNA2);对照组(命名为Control),LAMB3过表达组(命名为*LAMB3* OE)。取对数生长期的Eca109细胞,用含10% FBS的RPMI 1640培养液制备成浓度为$1×10^5$个/mL的细胞悬液,接种于24孔板中(500 μL/孔)。培养24 h(细胞汇合度约30%),加入病毒颗粒,置于细胞培养箱中培养14 h后,更换为含10% FBS的RPMI 1640培养液,继续培养72 h,用嘌呤霉素筛选阳性感染细胞后进行后续实验。慢病毒插入靶向*LAMB3*的序列分别

为CCA AGA GGG ATT TGA GAG AAT(*LAMB3* shR-NA1)及GAA GCT TCA ATG GTC TCC TTA(*LAMB3* shRNA2),对照载体插入序列为:TTC TCC GAA CGT GTC ACG T。

1.2.9 CCK8法检测细胞增殖 　用含10% FBS的RPMI 1640培养液将Eca109细胞或TE-1细胞接种于96孔板,24 h后转染相应siRNA,转染后第0、1、2、3天,按照说明书每孔加入10 μL CCK8试剂,酶标仪450 nm处检测吸光度(D)值。Eca109稳定细胞株接种于96孔板,接种细胞后的第0、1、2、3天,每孔加入10 μL CCK8试剂,检测D值。

1.2.10 划痕实验检测细胞迁移 　预先用碳素笔于12孔板底部画三条水平线,用含10% FBS的RPMI 1640培养液将Eca109细胞或TE-1细胞接种于12孔板中,接种后24 h(细胞汇合度约90%),用100 μL枪头于孔内正中划一道与孔底部三条水平线垂直交叉的竖痕,PBS洗涤,更换细胞培养液,拍照,按照说明书在各孔转染相应的siRNA,转染后不同时间点于同一视野拍照。Eca109稳定细胞株接种于12孔板,24 h后(细胞汇合度约90%),划痕并换液。含有划痕的图片用ImageJ软件分析。

1.2.11 细胞–基质黏附实验 　10 μg/mL的纤连蛋白包被96孔板,4 °C过夜后,用1%牛血清白蛋白(bovine serum albumin, BSA)于37 °C封闭1 h,将$1×10^4$个细胞接种于孔内,37 °C孵育1 h。PBS清洗,除去未贴壁细胞,每孔加入0.1%结晶紫染色后PBS洗涤3次,加入10%乙酸,静置5~10 min,在酶标仪上测定波长在595 nm处的D值,代表各组细胞的黏附能力。

1.2.12 裸鼠食管鳞癌转移模型 　雌性4~5周龄BALB/c裸鼠购自斯贝福(北京)生物技术有限公司,裸鼠体质量14~17 g。将对数生长期生长的Eca109稳定细胞株(NC shRNA、*LAMB3* shRNA1)浓度调整至$1×10^7$个细胞/mL,裸鼠随机分为2组(每组$n=5$),经侧尾静脉分别注射NC shRNA、*LAMB3* shRNA1细胞悬液0.1 mL。每日观察裸鼠活动、饮食及精神状态。每3天测量体重。裸鼠自然死亡后解剖肺组织及胸腹腔,观察有无转移灶。中性甲醛固定组织标本,苏木精和伊红(H&E)染色,并进行肿瘤体内转移的组织学评估。上述动物实验均遵照《中华人民共和国实验动物管理条例》的相关规定,并已通过川北医学院动物伦理委员会审查批准[NSMC伦理动物审〔2021〕80号]。

1.2.13 统计学处理 采用软件SPSS 25.0及Graph-Pad Prism 9分析实验数据, 计量资料以均数±标准差表示, 两组间比较采用t检验, 多组间比较采用方差分析, 当$P<0.05$时表示差异有统计学意义。

2 结果

2.1 *LAMB3*在ESCC癌组织、癌旁正常组织中的表达情况

GEO数据库的数据集GSE44021(图1A)、GSE161533(图1B)分析结果表明, *LAMB3*在ESCC癌组织中的表达均显著高于癌旁正常组织($P<0.001$)。

2.2 HEEC、Eca109、TE-1、KYSE-150细胞中LAMB3的表达情况

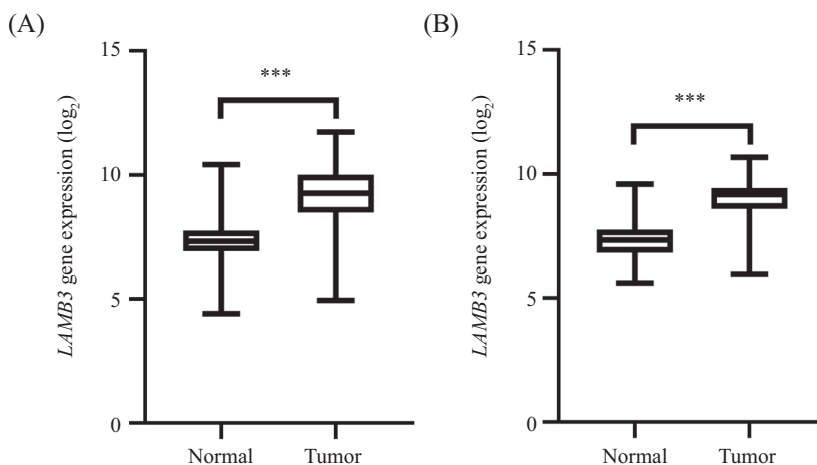采用Western blot方法检测人食管上皮细胞HEEC及食管鳞癌细胞株中LAMB3蛋白表达水平, 结果如图2所示, LAMB3蛋白在三种ESCC细胞中均显著高于人食管上皮细胞($P<0.001$)。

2.3 S1P对TE-1细胞LAMB3表达的影响

课题组前期发现, TE-1细胞高表达S1PR1[11]。因此, 我们用S1P刺激TE-1细胞, 检测LAMB3的表达情况。S1P作用TE-1细胞24 h后, 收集细胞总蛋白进行Western blot实验, 结果如图3所示。与未加S1P的对照组相比, S1P作用TE-1细胞24 h后, LAMB3蛋白表达水平升高($P<0.001$), 且呈浓度依赖性。
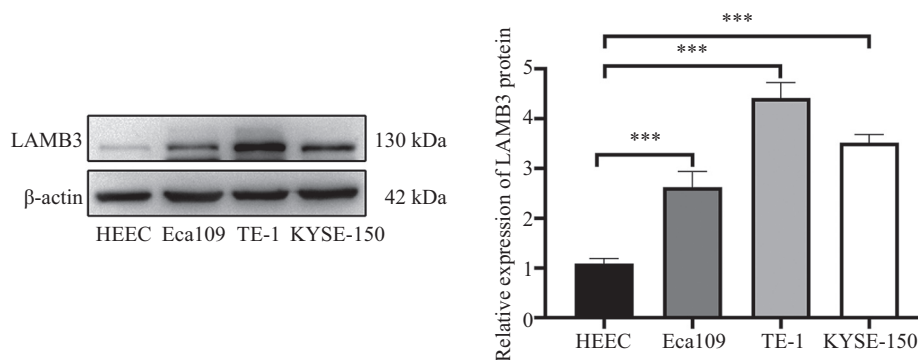
2.4 敲低或过表达S1PR1对Eca109细胞LAMB3表达的影响

课题组前期研究证实, 过表达S1PR1后, Eca109细胞*LAMB3* mRNA的表达水平增加[11], 因此, 我们

A: *LAMB3*在数据集GSE44021中癌组织与癌旁正常组织的表达情况; B: *LAMB3*在数据集GSE161533中癌组织与癌旁正常组织的表达情况。***$P<0.001$。

A: the expression of *LAMB3* in cancer and normal tissue in dataset GSE44021; B: the expression of *LAMB3* in cancer and normal tissue in dataset GSE161533. ***$P<0.001$.

图1 *LAMB3*在ESCC癌组织与癌旁正常组织中的表达

Fig.1 Expression of *LAMB3* in ESCC tissue and normal tissue
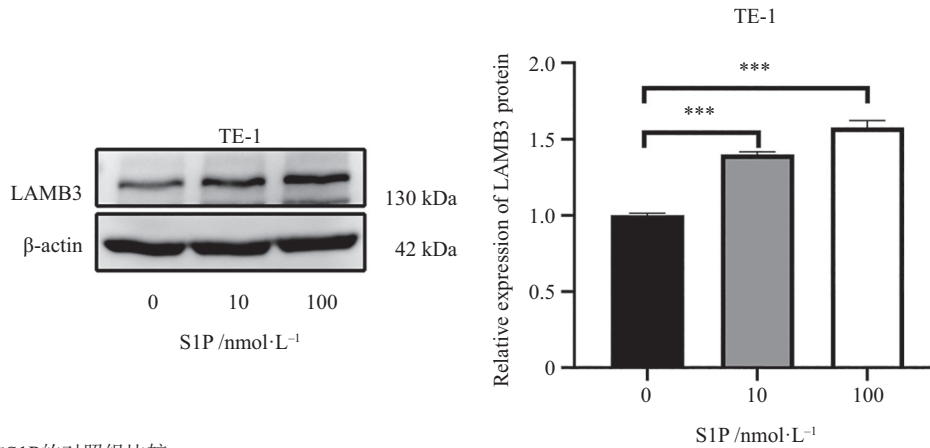

***$P<0.001$.

图2 HEEC、Eca109、TE-1、KYSE-150细胞中LAMB3蛋白表达水平

Fig.2 Expression of LAMB3 protein in HEEC, Eca109, TE-1 and KYSE-150 cells

在Eca109细胞中敲低或过表达S1PR1并检测细胞内LAMB3的表达, 相比于阴性对照组, S1PR1敲低组的S1PR1蛋白表达水平显著降低($P<0.001$, 图4A), LAMB3蛋白表达水平显著下降($P<0.01$, 图4A), 反之, S1PR1过表达组(转染$S1PR1$-EGFP)的LAMB3蛋

白表达水平显著升高($P<0.001$, 图4B)。

2.5 Eca109、TE-1细胞中敲低或过表达LAMB3的效率验证

Eca109、TE-1细胞转染 NC siRNA、$LAMB3$ siRNA1、$LAMB3$ siRNA2后, 72 h收集细胞总蛋白
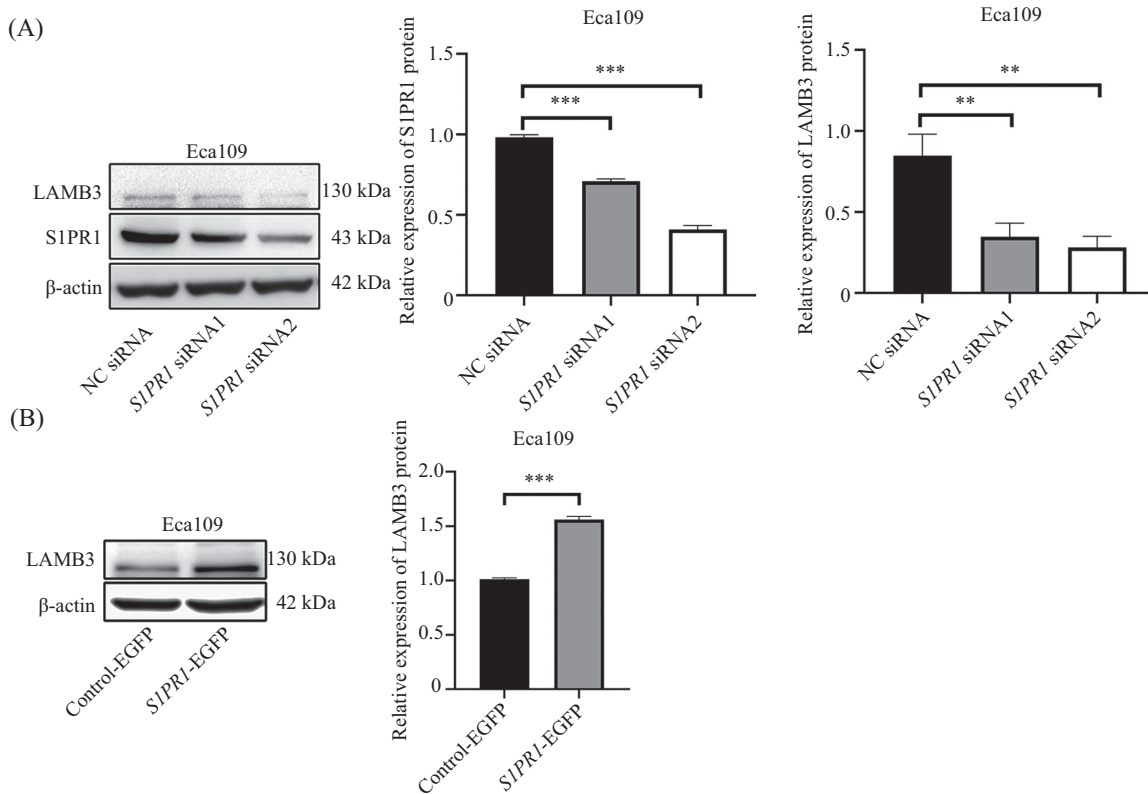

***$P<0.001$, 与未加S1P的对照组比较。
***$P<0.001$ compared with the control group without S1P.

图3 不同浓度S1P作用TE-1细胞后LAMB3蛋白表达水平
Fig.3 Expression of LAMB3 protein in TE-1 cells treated with different concentrations of S1P

A: 敲低$S1PR1$对Eca109细胞LAMB3表达的影响; B: 过表达S1PR1对Eca109细胞LAMB3表达的影响。**$P<0.01$, ***$P<0.001$。
A: effect of knockdown of $S1PR1$ on LAMB3 expression in Eca109 cells; B: effect of overexpression of S1PR1 on LAMB3 expression in Eca109 cells. **$P<0.01$, ***$P<0.001$.

图4 S1PR1敲低或过表达的Eca109细胞中LAMB3蛋白表达情况
Fig.4 LAMB3 protein expression in Eca109 cells with S1PR1 knockdown or overexpression

进行Western blot实验, 结果如图5A和图5D所示。相比于NC siRNA组, 转染了*LAMB3* siRNA1、*LAMB3* siRNA2的Eca109细胞和TE-1细胞的LAMB3蛋白表达水平显著降低($P<0.001$)。敲低或过表达LAMB3的Eca109稳定细胞株, 通过Western blot比较*LAMB3* shRNA1、LAMB3 shRNA2组与NC shRNA组或比较Control组与*LAMB3* OE组细胞的LAMB3表达量, 结果如图5B和图5C所示, Eca109细胞的LAMB3蛋白表达水平显著降低或增高($P<0.001$)。

2.6 *S1PR1*-EGFP质粒与*LAMB3* siRNA共转染对Eca109细胞迁移的影响

经分析得出, 如图6所示, 共转染后72 h, 4组细胞的迁移率如下: *S1PR1*-EGFP+NC siRNA组大于Control-EGFP+NC siRNA组($P<0.05$), 而*S1PR1*-EGFP+*LAMB3* siRNA1组和*S1PR1*-EGFP+*LAMB3* siRNA2组均小于*S1PR1*-EGFP+NC siRNA组($P<0.001$)。

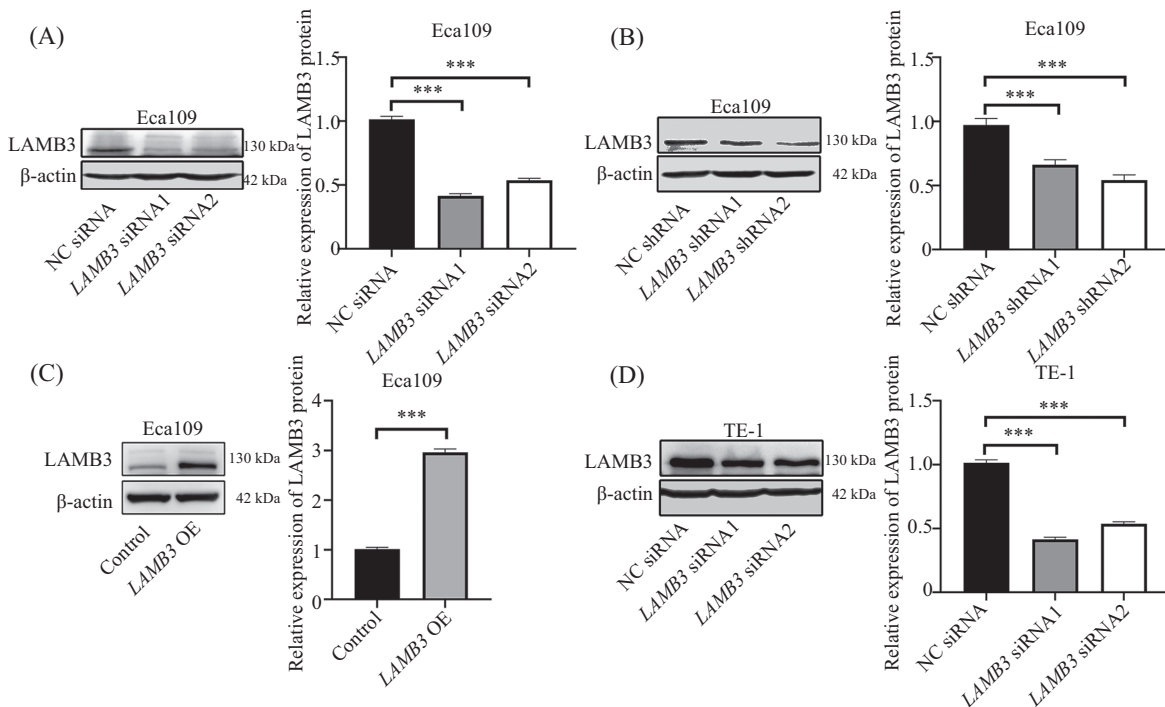## 2.7 敲低或过表达LAMB3对Eca109、TE-1细胞增殖的影响

CCK8结果如图7所示, 转染*LAMB3* siRNA1、

LAMB3 siRNA2的Eca109、TE-1细胞, 与阴性对照相比, 增殖能力均无明显变化(图7A和图7D); 敲低或过表达LAMB3的Eca109稳定细胞株的增殖能力也无显著变化(图7B和图7C)。

2.8 敲低或过表达LAMB3对Eca109、TE-1细胞迁移的影响

通过ImageJ分析划痕图片, 可以得出: 转染了*LAMB3* siRNA1或*LAMB3* siRNA2的Eca109细胞(图8A)、TE-1细胞(图8D)迁移率均小于阴性对照组细胞($P<0.05$, $P<0.001$)。敲低 LAMB3的Eca109稳定细胞株, 相较于对照组, 敲低组抑制了细胞的迁移($P<0.001$, 图8B), 反之, 过表达LAMB3促进了细胞的迁移($P<0.001$, 图8C)。

2.9 敲低或过表达LAMB3对Eca109细胞黏附的影响

在酶标仪上测定波长在595nm处的D值, 相较于对照组, LAMB3敲低组细胞对细胞外基质的黏附能力显著降低($P<0.05$, $P<0.01$, 图9A), 反之, 过表达LAMB3的Eca109细胞的黏附能力增强($P<0.001$, 图9B)。

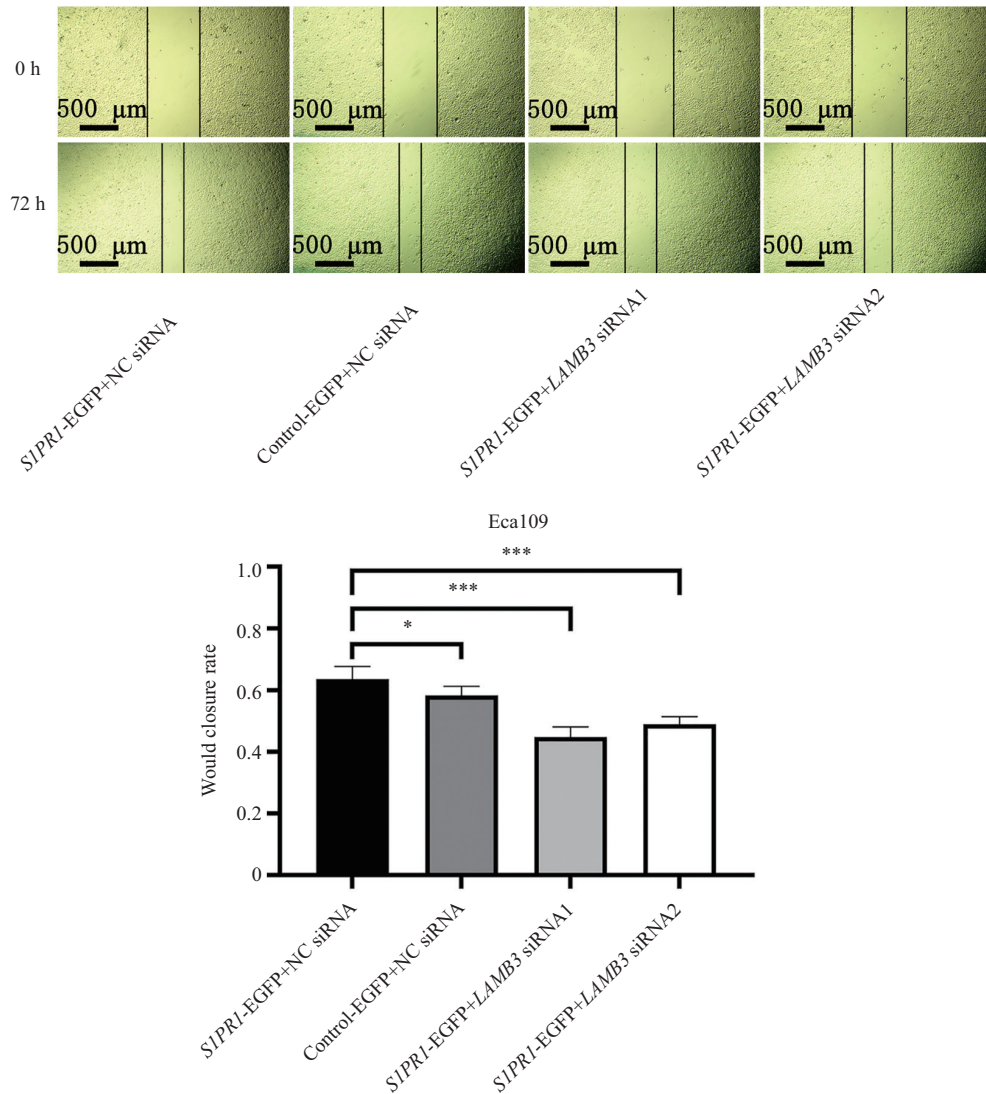A: 用siRNA敲低Eca109细胞*LAMB3*的表达; B: 用shRNA敲低Eca109细胞*LAMB3*的表达; C: Eca109细胞中过表达*LAMB3*; D: 用siRNA敲低TE-1细胞*LAMB3*的表达。***$P<0.001$。

A: knockdown of *LAMB3* expression in Eca109 cells with siRNA; B: knockdown of *LAMB3* expression in Eca109 cells with shRNA; C: overexpression of *LAMB3* in Eca109 cells; D: knockdown of *LAMB3* expression in TE-1 cells with siRNA. ***$P<0.001$.

图5 Eca109、TE-1细胞中敲低或过表达LAMB3的效率验证

Fig.5 Validation of the efficiency of knockdown or overexpression of LAMB3 in Eca109, TE-1 cells

Eca109

*P<0.05, ***P<0.001。
*P<0.05, ***P<0.001.

图6 ***S1PR1*-EGFP和*LAMB3* siRNA共转染对Eca109细胞迁移的影响**
Fig.6 Effect of *S1PR1*-EGFP and *LAMB3* siRNA co-transfection on migration of Eca109 cells

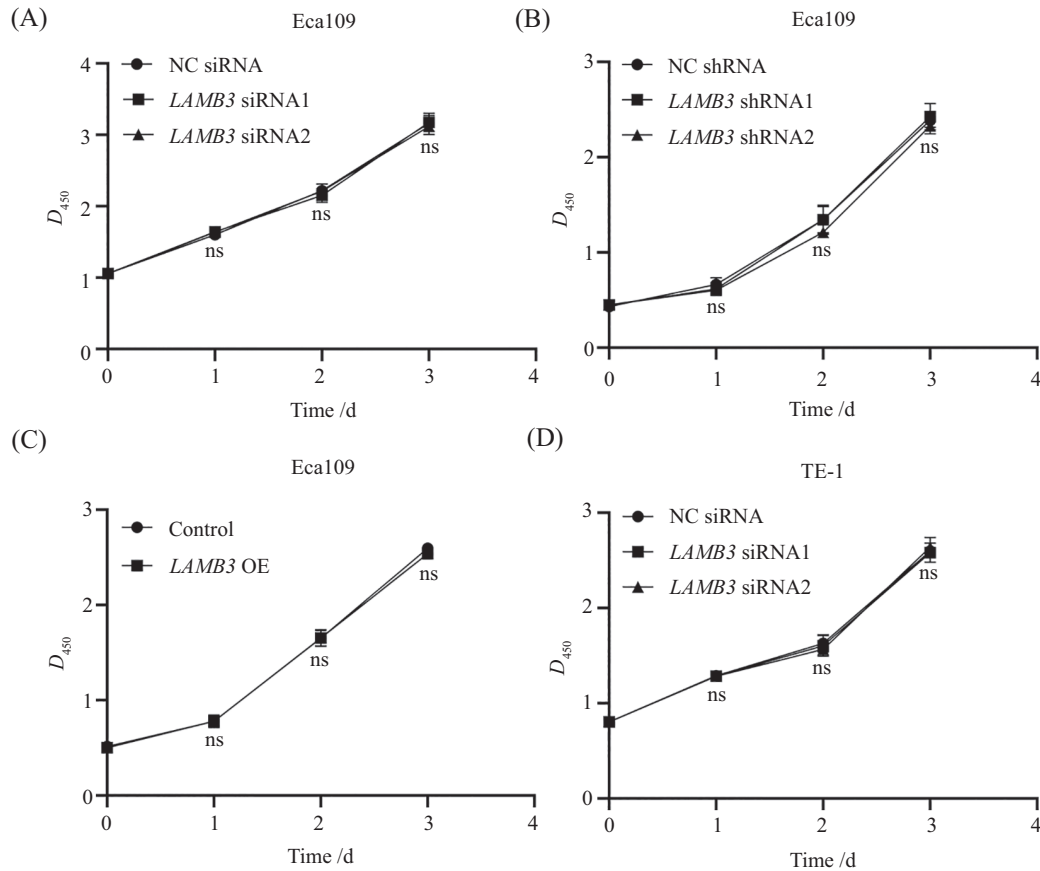## 2.10 敲低或过表达LAMB3对上皮–间质转化(epithelial-mesenchymal transition, EMT)相关蛋白及MMP9表达的影响

敲低LAMB3的Eca109稳定株相较于对照组细胞, EMT过程被抑制(E-cadherin表达量升高, Vimentin表达量降低), MMP9表达量降低($P<0.05$, $P<0.01$, $P<0.001$, 图10A), 反之, 过表达LAMB3的Eca109稳定株相较于对照组细胞, EMT过程被促进(E-cadherin表达量降低, Vimentin表达量升高), MMP9表达量升高($P<0.001$, 图10B)。

2.11 敲低LAMB3对Eca109细胞转移的影响

食管鳞癌Eca109细胞(NC shRNA、*LAMB3* shRNA1)经尾静脉注射构建裸鼠食管鳞癌转移模型。生存曲线显示: 注射后第37天, NC shRNA组裸鼠死亡1只, 注射后第39、40天, NC shRNA组各死亡2只, 而敲低组5只裸鼠全部存活; 与对照组相比, LAMB3敲低延长了总生存期($P<0.01$, 图11A)。解剖发现: NC shRNA组中有1只裸鼠胸膜可见一肿瘤样结节, 另1只腹膜可见一肿瘤样结节; 而敲低组胸腹腔未见异常。所有裸鼠的肺组织及上述疑似胸腹膜肿瘤结节均连续切片、H&E染色(图11B), 镜下发现: 敲低组有2只裸鼠的肺组织可见癌细胞; NC shRNA组裸鼠中腹腔结节、伴有胸膜结节裸鼠的肺组织及胸膜结节均可见大量癌细胞。与注射对照组

A~D: 敲低或过表达LAMB3的Eca109及TE-1细胞的增殖能力。 $^{ns}P>0.05$, 与阴性对照组比较。
A-D: proliferation ability of Eca109 and TE-1 cells knocked down or overexpressing LAMB3. $^{ns}P>0.05$ compared with the negative control group.

图7 敲低或过表达LAMB3对ESCC细胞增殖的影响
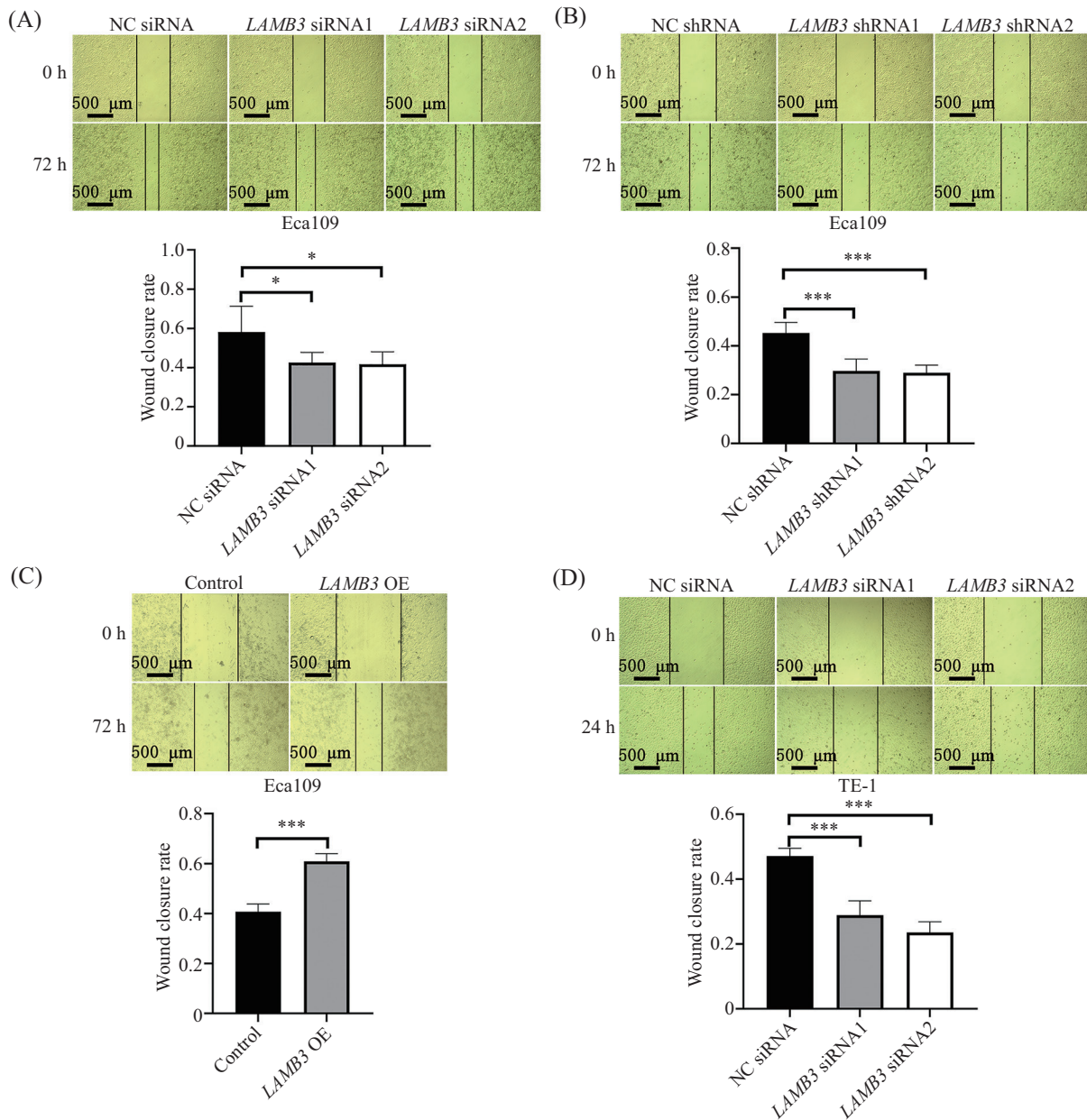**Fig.7 Effect of LAMB3 knockdown or overexpression on proliferation of ESCC cells**

细胞的裸鼠相比, 在注射LAMB3敲低组细胞的裸鼠体内更不容易形成肿瘤转移灶。

3 讨论

S1P/S1P受体(S1P receptor, S1PR)通路调控多种生理过程。S1P是一种多效性脂质介质, 在细胞内和细胞间的信号转导中发挥重要作用。存在于细胞膜上的神经酰胺(一种脂质)被神经酰胺酶水解生成鞘氨醇(sphingosine, SPH), SPH在鞘氨醇激酶(sphingo-sine kinase, SPHK)的作用下磷酸化形成S1P, 细胞外S1P与几种不同的S1PR(包括S1PR1~5)结合, 进而激活多条下游信号通路, 如Ras/ERK、PKB/Akt等, 调节淋巴细胞迁移、细胞存活、细胞凋亡, 以及促进肿瘤细胞生长、迁移、扩散[12-14]。在本研究中, 用S1P作用高表达S1PR1的TE-1细胞, 引起LAMB3表达上调, 以及在Eca109细胞中敲低或过表达S1PR1, 引起LAMB3表达下调或上调, 并且, 敲低LAMB3表

达可逆转S1PR1对Eca109细胞的促迁移作用, 可见, S1P/S1PR1能诱导LAMB3的表达, 影响ESCC细胞的生物学行为。

*LAMB3*基因编码LM-332的β3链, LM-332与整合素α3β1和α6β4相互作用, 形成黏着斑和稳定锚定接触, 促进细胞迁移、黏附[15]。LAMB3在多种肿瘤中高表达, 与肿瘤的发生发展密切相关, 但在不同肿瘤中其生物学作用尚不完全一致, 甚至完全相反。在头颈部鳞状细胞癌中LAMB3可促进肿瘤细胞的侵袭及迁移[5]; 结直肠癌中LAMB3通过激活AKT和抑制FOXO3/4的肿瘤抑制功能促进肿瘤细胞增殖和迁移[6]; 甲状腺乳头状癌中LAMB3可通过激活HGF/c-MET轴诱导的PI3K/Akt通路, 促进EMT过程及MMP9表达增高, 促进细胞的迁移、侵袭, 但对增殖无影响[8]。但HOU等[16]研究发现, 猕猴桃根提取物可诱导肝细胞癌细胞LAMB3表达增加, 进而抑制细胞增殖; LIN等[17]也发现前列腺癌中LAMB3

A、B: 敲低LAMB3后, 划痕实验检测Eca109细胞的迁移情况; C: 过表达LAMB3后, 划痕实验检测Eca109细胞的迁移; D: 敲低LAMB3后, 划痕实验检测TE-1细胞的迁移。*$P<0.05$, ***$P<0.001$。

A,B: after knockdown of LAMB3, the migration of Eca109 cells was detected by scratch assay; C: after overexpression of LAMB3, the migration of Eca109 was detected by scratch assay; D: after knockdown of LAMB3, the migration of TE-1 cells was detected by scratch assay. *$P<0.05$, ***$P<0.001$.
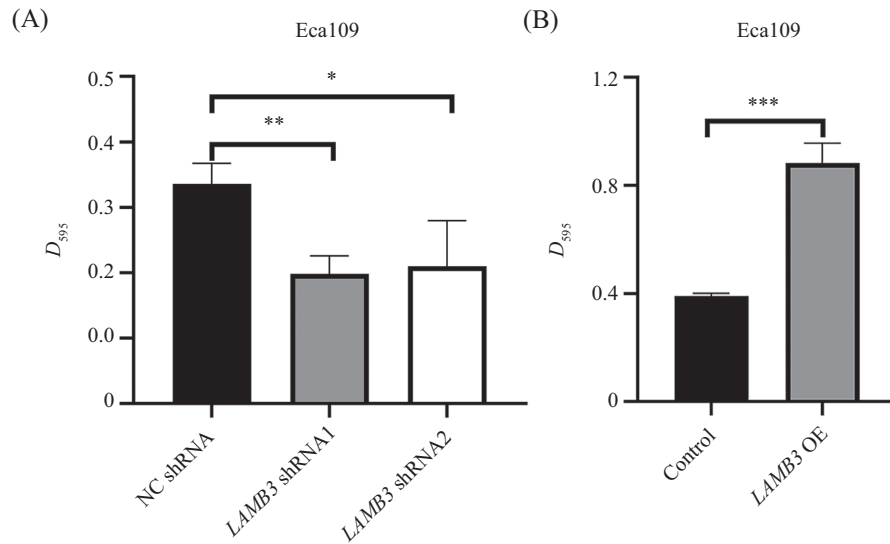
图8　敲低或过表达LAMB3对ESCC细胞迁移的影响
Fig.8　Effect of LAMB3 knockdown or overexpression on migration of ESCC cells

可以抑制肿瘤的发生与进展。本研究发现, LAMB3对ESCC细胞的增殖无明显影响, 但可促进细胞的迁移、黏附; 裸鼠体内转移模型表明, LAMB3在体内可促进ESCC细胞的多处转移, 缩短裸鼠的生存期。

在LAMB3的促肿瘤作用中, EMT过程和MMP9起关键作用。EMT是指上皮细胞分化为间质细胞的生物学和病理学过程, 与三种生物学过程有关:

发育、纤维化和伤口愈合、癌症转移。研究表明, EMT与肿瘤转移及放化疗耐药性密切相关。EMT特征是上皮分子标志物(如: E-cadherin)下调, 间充质分子标志物(如: Vimentin)上调[18-19]。MMP9是MMP家族的重要成员, 有研究发现, MMP9的表达在EMT过程中上调[20], 可促进ESCC细胞的迁移、侵袭[21]。因此, E-cadherin、Vimentin、MMP9均属于
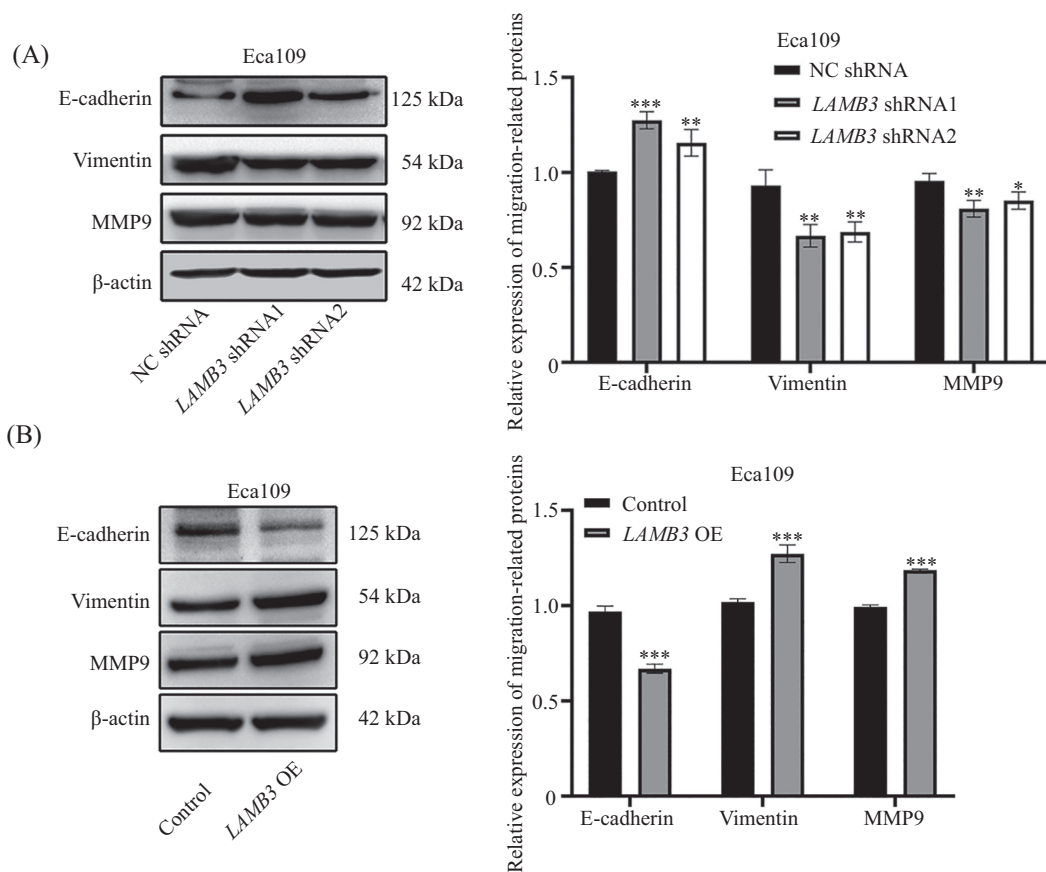
A: 敲低LAMB3对Eca109细胞黏附的影响; B: 过表达LAMB3对Eca109细胞黏附的影响。*$P<0.05$, **$P<0.01$, ***$P<0.001$。

A: effect of knockdown of LAMB3 on Eca109 cell adhesion; B: effect of overexpression of LAMB3 on Eca109 cell adhesion. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

图9　敲低或过表达LAMB3对ESCC细胞黏附的影响

Fig.9　Effect of LAMB3 knockdown or overexpression on ESCC cell adhesion

A: 敲低LAMB3对迁移相关蛋白表达的影响; B: 过表达LAMB3对迁移相关蛋白表达的影响。*$P<0.05$, **$P<0.01$, ***$P<0.001$, 与对照组比较。

A: effect of knockdown of LAMB3 on the expression of migration-related proteins; B: effect of overexpression of LAMB3 on the expression of migration-related proteins. *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared with negative control group.

图10　敲低或过表达LAMB3对EMT相关蛋白及MMP9表达的影响

Fig.10　Effect of LAMB3 knockdown or overexpression on EMT-related protein and MMP9 expression

(A)

(B)

A: Kaplan-Meier分析各组总生存率(*n*=5), **P<0.01, 与NC shRNA组比较; B: 注射Eca109细胞(NC shRNA、*LAMB3* shRNA1)的BALB/c裸鼠的肺组织及胸腹膜肿瘤组织图片和H&E染色的代表性图像。

A: Kaplan-Meier analysis of overall survival in each group (*n*=5), **P<0.01 compared with the NC shRNA group; B: pictures of lung and thoracoabdominal tumor tissues and representative images of H&E staining in BALB/c mice injected with Eca109 cells (NC shRNA, *LAMB3* shRNA1).

图11 敲低LAMB3对Eca109细胞转移的影响

Fig.11 Effect of LAMB3 knockdown on metastasis of Eca109 cells

迁移相关蛋白。采用shRNA敲低*LAMB3*, Eca109细胞稳定敲低组中E-cadherin上调, Vimentin及MMP9下调, 抑制细胞迁移与黏附, 反之, 过表达LAMB3的Eca109细胞中E-cadherin下调, Vimentin及MMP9上调, 促进细胞迁移与黏附, EMT相关蛋白及MMP9的表达在LAMB3诱导的Eca109细胞迁移中起了关键作用。

综上, 本研究发现, LAMB3在ESCC细胞及组织中表达上调, S1P/S1PR1通路能诱导LAMB3的表达, 并进一步通过促进EMT过程及MMP9的表达, 增强ESCC细胞的迁移、黏附和体内转移能力, 这可能在ESCC发生发展中发挥重要作用。本研究对S1P/S1PR1通路通过调节LAMB3的表达, 进而调控ESCC细胞增殖、迁移及黏附行为进行了初步探索, 相关信号通路有待进一步深入研究。

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