Caveolin-1 siRNA对与乳腺癌细胞共培养的成纤维细胞自噬体表达影响的研究

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摘要 为了探索乳腺癌相关的人成纤维细胞中窖蛋白-1(Caveolin-1, Cav-1)与自噬体的相关性及其对乳腺癌细胞的作用, 该研究采用siRNA技术干扰成纤维细胞株ESF表达Cav-1, qRT-PCR和Western blot确定siRNA干扰Cav-1表达的效果; Transwell insert方法共培养乳腺癌细胞株BT474和ESF细胞, 单丹磺酰戊二胺(monodansylcadaverin, MDC)染色、激光共聚焦显微镜观察Cav-1 siRNA对自噬体表达的影响; qRT-PCR和Western blot检测Cav-1 siRNA对微管相关蛋白1轻链3II(microtubule-associated protein 1 light chain 3 II, LC3II)表达的影响; CCK-8方法检测BT474细胞的增殖和活力。结果显示, 靶向Cav-1的siRNA下调了ESF细胞中Cav-1的表达; Cav-1 siRNA促进ESF细胞自噬体和LC3II的表达, 转染了Cav-1 siRNA的ESF细胞与BT474细胞共培养对自噬体和LC3II的作用更为显著; BT474细胞在ESFsiCav-1(ESF cells transfected with Cav-1 siRNA)细胞共培养条件下增殖显著加快。研究表明, Cav-1 siRNA促进了与乳腺癌细胞共培养的成纤维细胞自噬体和LC3II的表达, 同时加快了与成纤维细胞共培养的乳腺癌细胞的增殖。

关键词 Caveolin-1; 成纤维细胞; 乳腺癌; 自噬体; siRNA; LC3II

Research on the Effects of Caveolin-1 siRNA on the Expression of Autophagosome in Fibroblasts Co-cultured with Breast Cancer Cells

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Abstract In order to explore the correlation between Caveolin-1 (Cav-1) and autophagosome and its role in breast cancer cells, siRNAs were used to interfere with Cav-1 expression in fibroblast line ESF in this study. qRT-PCR and Western blot were used to determine the effect of siRNA interfering Cav-1 expression in ESF cells. Breast cancer cell lines BT474 and ESF were co-cultured by Transwell insert. The effect of Cav-1 siRNA on the expression of autophagosome in ESF cells was examined by monodansylcadaverin (MDC) staining and laser confocal microscopy. The effect of Cav-1 siRNA on the expression of microtubule-associated protein 1 light chain 3 II (LC3II) in ESF cells was examined by qRT-PCR and Western blot. The proliferation and viability of BT474 cells

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were measured by CCK-8 assay. The results showed that Cav-1 expression in ESFsiCav-1 (ESF cells transfected with Cav-1 siRNA) was downregulated by siRNA targeting Cav-1. The expressions of LC3II and autophagosome in ESF cells were increased by Cav-1 siRNA, and enhanced significantly in the co-culture of ESFsiCav-1 and BT474 cells. The proliferation of BT474 cells was significantly faster under the condition of co-culture of BT474 cells and ESFsiCav-1 cells. These findings suggested that Cav-1 siRNA promoted the expression of LC3II and autophagosome in fibroblasts co-cultured with breast cancer cells and Cav-1 siRNA accelerated the proliferation of breast cancer cell co-cultured with fibroblasts.

Keywords Caveolin-1; fibroblast; breast cancer; autophagosome; siRNA; LC3II

成纤维细胞是乳腺肿瘤微环境中最主要的基质细胞, 它通过细胞直接接触、分泌信息分子等行为与微环境中的各组分发生交互作用, 对癌变细胞的生长起着沃土或蓄电池作用, 从而影响肿瘤的发生、发展或逆转[1-3]。成纤维细胞窖蛋白-1(Caveolin-1, Cav-1)表达的下降或缺失可促使其活化成为癌相关成纤维细胞(cancer associated fibroblast, CAF), 并可以作为致死性肿瘤微环境的生物标记[4-5]。Cav-1是细胞质膜微囊(caveolae)的重要结构和功能组分, 分子量为22 kDa, 其基因位于人类染色体7q31.1位置。Cav-1通过其脚手架区域对聚集在caveolae的多种信号分子向胞内传递信息的过程发挥重要作用[6-7]。研究表明, Cav-1具有抑制肿瘤的作用, Cav-1与乳腺上皮细胞转化和乳腺癌发生发展密切相关。正常情况下, Cav-1主要在乳腺基质成纤维细胞中表达丰富, 但乳腺癌基质成纤维细胞中Cav-1表达下降甚至呈阴性[8-9]。肿瘤基质成纤维细胞中Cav-1表达下降或呈阴性的乳腺癌患者预后差, 生存率低, 而Cav-1阳性或高表达的乳腺癌患者预后好, 生存率高[10-12]。

细胞自噬是真核细胞利用溶酶体对细胞器及蛋白质进行降解的生物学过程, 自噬活性改变与多种疾病的发生发展有关, 如炎症、细胞功能失调、神经变性疾病、自身免疫病、恶性肿瘤和衰老等。成纤维细胞自噬基因表达的上调可能是成纤维细胞异常激活事件, 使成纤维细胞的自噬功能异常, 并通过自噬改变细胞的分泌功能, 从而修饰微环境, 导致微环境中细胞的遗传不稳定性及恶性选择, 促进肿瘤的发生发展。

本文采用small interfering RNA(siRNA)干扰成纤维细胞中*Cav-1*基因的表达, 在体外建立成纤维细胞和乳腺癌细胞共培养模型, 观察干扰Cav-1表达对成纤维细胞自噬体和自噬体膜标志性蛋白LC3II表达的影响及对乳腺癌细胞增殖的影响, 为深入研究乳腺癌相关的人成纤维细胞中Cav-1与自噬体相关性及对乳腺癌作用的机制提供资料。

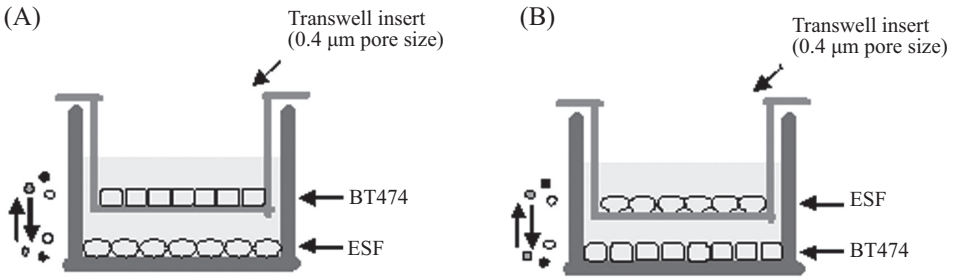
1 材料与方法

1.1 材料

人成纤维细胞株CCC-ESF-1(No.3111C0001-CCC000108, 文中简称ESF)来自中国医学科学院基础医学研究所细胞中心; 人乳腺癌细胞株BT474(No. TCHu143)来自中国科学院上海生命科学研究院细胞库; Lipofectamine™ 2000购自Life Tech-Invitrogen公司; Cav-1 siRNA设计及合成由上海吉玛公司完成; LC3II引物由宝生物工程(大连)有限公司合成; Cav-1抗体购自Santa Cruz公司; LC3II抗体购自美国ABGENT公司; DMEM-H培养液购自Life Tech-GIBCO公司; Revert aid first strand cDNA synthesis kit购自Fermentas公司; qRT-PCR试剂IQ SYBR Green Supermix和Super Real Premix Plus(with SYBR Green I)分别购自Bio-Rad和天根生化科技北京有限公司; Transwell insert(0.4 μm pore size)购自Fisher Scientific公司; 单丹磺酰戊二胺(monodansylcadaverin, MDC)购自Sigma公司; Cell Counting Kit-8试剂购自Dojindo公司。

1.2 方法

1.2.1 细胞培养和共培养 将ESF细胞和BT474细胞放入含10% FBS的DMEM-H培养液中, 添加100 U/mL青霉素和100 μg/mL链霉素, 置于37 °C、5% CO_2培养箱中培养。细胞生长至80%~90%汇合时, 进行传代培养。ESF细胞和BT474细胞共培养前, 先将培养板和Transwell insert用培养液浸湿, 根据实验要求分别接种细胞。待培养板的细胞贴壁后, 再把接种了细胞的Transwell insert放到细胞已贴壁的培养板中进行共培养, 共培养的细胞不直接接触, 但在同一培养液中, 通过培养液相互影响(图1)。



A: ESF细胞接种在培养板, BT474细胞接种在Transwell insert并插入培养板; B: BT474细胞接种在培养板, ESF细胞接种在Transwell insert并插入培养板。接种在培养板的细胞用于各实验。

A: ESF cells cultured on the bottom of culture plates with BT474 cells cultured on the Transwell inserts which was placed into the culture plates; B: BT474 cells cultured on the bottom of culture plates with ESF cells cultured on the Transwell inserts. The experiments were performed on the cells cultured on the bottom of culture plates.

图1 ESF细胞/BT474细胞共培养

Fig.1 Co-culture of ESF cells and BT474 cells

表1 Caveolin-1 siRNA序列

Table 1 The sequences of Caveolin-1 siRNA

siRNA分组 siRNA groups	序列(5′→3′) Sequences (5′→3′)
siRNA-1	GCG ACC CUA AAC ACC UCA ATT UUG AGG UGU UUA GGG UCG CTT
siRNA-2	CCU UCA CUG UGA CGA AAU ATT UAU UUC GUC ACA GUG AAG GTT
siRNA-3	GCC GUG UCU AUU CCA UCU ATT UAG AUG GAA UAG ACA CGG CTT
Negative control	UUC UCC GAA CGU GUC ACG UTT ACG UGA CAC GUU CGG AGA ATT

1.2.2 Caveolin-1 siRNA合成和转染 Cav-1 siRNA序列见表1。取0.5×10^5/孔的细胞, 悬浮于DMEM-H培养液, 接种于24孔培养板。待细胞生长至70%~80%汇合时, 用无血清培养液洗细胞3次, 按照Lipofectamine™ 2000试剂说明书进行转染。成纤维细胞Cav-1 siRNA转染分组为: siRNA-1组、siRNA-2组、siRNA-3组、NC(negative control)组、BC(blank control)组和EV(empty vector)组。转染后培养24 h, 采用qRT-PCR检测*Cav-1*的转录。转染后培养48 h, 采用Western blot检测Cav-1蛋白的表达。

1.2.3 qRT-PCR Trizol-离心柱法提取细胞总RNA, 以DEPC-H_2O为对照, 取2 μL RNA溶液, Merinton SMA4000微量分光光度计检测, 观察$D_{260/280}$、$D_{260/230}$比值及连续波长吸收峰, 计算RNA溶液浓度, 判断RNA提取质量。2.0<$D_{260/280}$<2.3, 则可以满足后续逆转录实验。逆转录合成cDNA: 总RNA溶液11 μL(约0.6~0.8 μg), 随机引物1 μL, 5×Reaction Buffer 4 μL, RiboLock™ RNase抑制剂1 μL(20 U/μL), 10 mmol/L dNTP(mix) 2 μL, Revert Aid™ M-MmLv逆转录酶1 μL, 总体积20 μL; 25 °C 5 min, 37 °C 60 min, 70 °C 5 min终止反应, –20 °C保存cDNA。PCR扩增反应: IQ SYBR Green Supermix 10 μL, 顺向引物1 μL(10 μmol/L), 逆向引物1 μL(10 μmol/L), cDNA 8 μL(10 μmol/L), 总体积20 μL。50 °C 3 min, 95 °C 3 min; 95 °C 10 s(Cav-1 siRNA)或95 °C 5 s(LC3II), 63.5 °C 30 s (Cav-1 siRNA)或60 °C 34 s(LC3II), 40个循环; 72 °C 7 min延伸; 熔解曲线65 °C~95 °C, 温度以0.5 °C/10 s的速率上升。实验结果由荧光定量PCR分析软件Bio-rad CFX Manager自动进行统计和计算。LC3II上游引物为: 5′-AAC ATG AGC GAG TTG GTC AAG-3′; 下游引物为: 5′-GCT CGT AGA TGT CCG CGA T-3′。

1.2.4 Western blot分析 细胞加入裂解液, 4 °C裂解30 min, BCA法测定蛋白, 然后根据标准曲线计算样品的蛋白浓度。8%聚丙烯酰胺凝胶电泳, 每泳道20 μg蛋白质, 转移至PVDF膜, 5%去脂牛奶封闭, 一抗分别与PBS、0.1% Tween-20和5%去脂牛奶混合, 膜放入该混合液中4 °C过夜, 洗涤后加入二抗, ECL显色, Image J软件分析各条带光密度。

1.2.5 MDC染色及激光共聚焦显微镜观察和分析 实验分组: BT474/ESF共培养组、BT474/ESFsiCav-1(ESF transfected with Cav-1 siRNA)共培养组、ESF单独培养组和ESFsiCav-1单独培养组。ESF细胞或ESFsiCav-1细胞接种于6孔培养板(下室), 爬片培养,

每孔细胞浓度为2×10^5。待细胞贴壁后, 把接种了BT474细胞的Transwell insert(上室)放到细胞已贴壁的6孔培养板进行共培养, 将单独培养的细胞接种于6孔培养板, 爬片培养。培养48 h后, 去除培养基, 4%的多聚甲醛固定细胞, PBS洗涤爬片细胞, MDC染色标记细胞自噬体[13-14], 加入50 μmol/L MDC-PBS溶液覆盖细胞, 37 ℃避光孵育30 min, PBS漂洗后在激光共聚焦显微镜下观察、拍照, Image-Pro Plus软件分析各组荧光强度。

1.2.6 Cell counting kit-8(CCK-8)实验 BT474细胞接种在24孔培养板, 将接种了ESF细胞的Transwell insert插入24孔培养板内进行共培养。实验分组为: BT474单独培养组、BT474/ESF共培养组和BT474/ESFsiCav-1共培养组。每组设5个复孔, 分别在培养第1, 2, 3, 4, 5 d, 依据CCK-8 Kit说明书, 各孔加入20 μL CCK-8溶液, 培养箱内孵育1~3 h, 从每份样品中取100 μL液体移至96孔板, 放入酶标仪, 450 nm波长处测定吸光值。

1.2.7 统计学分析 SPSS Statistics 17.0软件统计包处理实验数据, 实验结果用mean±S.D.表示, 两组间均数比较采用*t*检验, $P<0.05$为差异具有显著性。

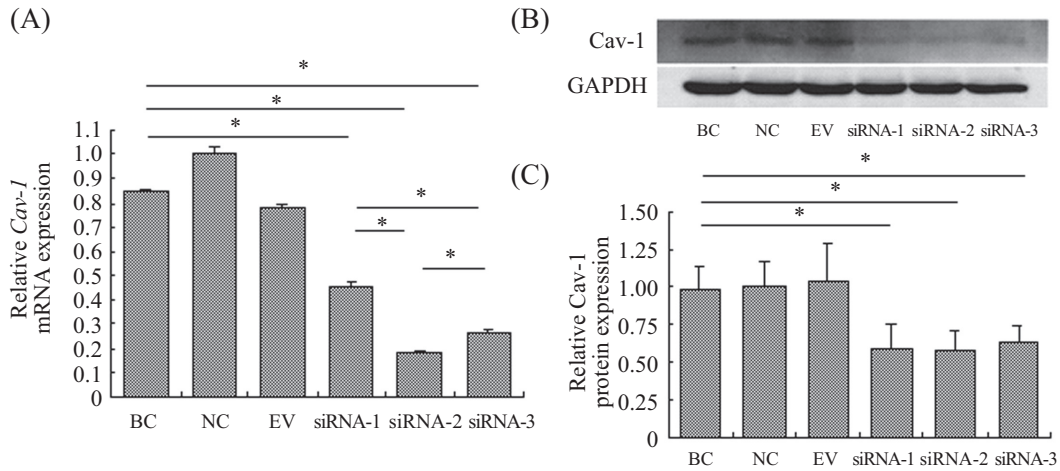
2 结果

2.1 siRNA干扰成纤维细胞ESF表达Cav-1

为了确定Cav-1 siRNA转染后对ESF细胞表达Cav-1的干扰效果, Cav-1 siRNA转染ESF细胞后培养24 h和48 h, 采用qRT-PCR和Western blot方法检测各组ESF细胞中Cav-1的表达, 确定用于后续实验的Cav-1 siRNA。图2A显示, siRNA-2实验组干扰效果尤为明显, 该组*Cav-1* mRNA表达水平显著低于其他各实验组($P<0.05$), siRNA-1组和siRNA-3组分别与siRNA-2组或BC组比较, *Cav-1* mRNA表达水平有显著差异($P<0.05$)。图2B和图2C显示, siRNA-1组、siRNA-2组和siRNA-3组的Cav-1蛋白表达水平显著低于对照组($P<0.05$), 表明siRNA有效地干扰了ESF细胞表达Cav-1。综合qRT-PCR和Western blot的结果, 选择siRNA-2实验组用于后续实验。

2.2 Cav-1 siRNA上调成纤维细胞自噬体膜标志性蛋白LC3II的表达

将ESFsiCav-1细胞与乳腺癌细胞共培养, 检测Cav-1 siRNA对成纤维细胞自噬体膜标志性蛋白LC3II表达的影响。Cav-1 siRNA转染ESF细胞后培养48 h和60 h, 采用qRT-PCR和Western blot方法检测

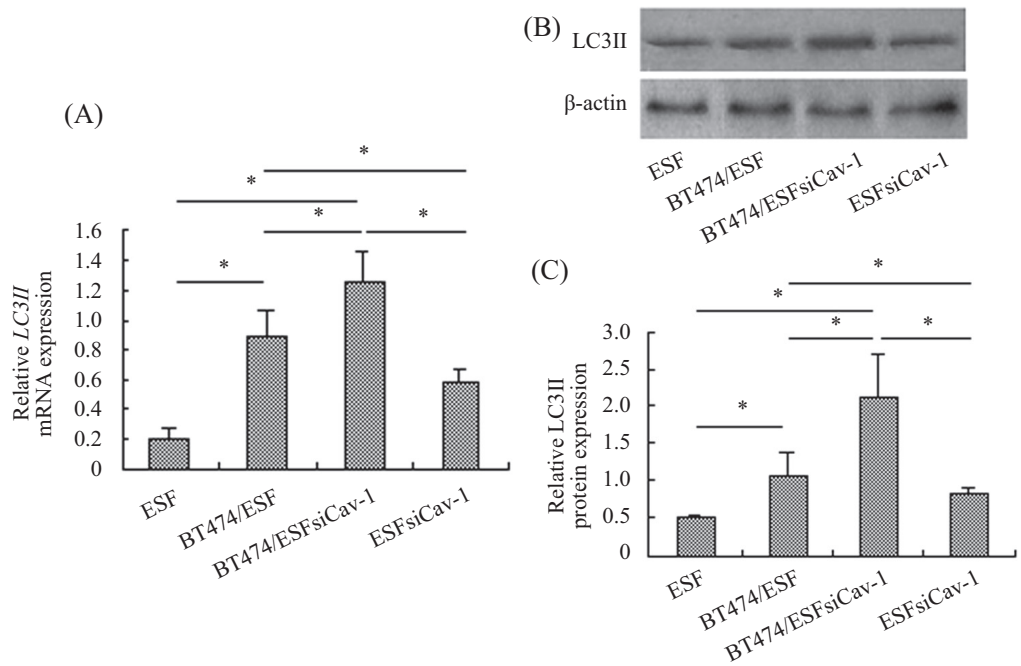


A: qRT-PCR检测转染不同的Cav-1 siRNA序列对ESF细胞表达*Cav-1* mRNA的干扰效应。与空白对照组(BC)比较, siRNA-1组、siRNA-2组和siRNA-3组*Cav-1* mRNA表达显著下调($P<0.05$), siRNA-2组*Cav-1* mRNA表达分别与siRNA-1组或siRNA-3组比较差异有显著性($P<0.05$); B、C: Western blot检测转染不同的Cav-1 siRNA序列对ESF细胞表达Cav-1蛋白的干扰效应, 与空白对照组比较, siRNA-1组、siRNA-2组和siRNA-3组Cav-1蛋白表达显著下调($P<0.05$)。BC: 空白对照组; NC: 阴性对照组; EV: 空载体组; GAPDH: 甘油醛-3-磷酸脱氢酶, 为内参对照。*$P<0.05$。

A: qRT-PCR was conducted to analyze the interfering efficiency of transfecting different sequences of Cav-1 siRNAs into ESF cells at mRNA level. The expressions of *Cav-1* mRNA were declined using siRNA in the groups of siRNA-1, siRNA-2 and siRNA-3 compared to that of blank control ($P<0.05$). Interference with Cav-1 siRNA-2 exhibited a more potent inhibitory effect than siRNA-1 or siRNA-3 ($P<0.05$). B,C: Western blot was conducted to analyze the interfering efficiency of transfecting different sequences of Cav-1 siRNAs into ESF cells at protein level. The expressions of Cav-1 protein were declined in the groups of siRNA-1, siRNA-2 and siRNA-3 compared to that of blank control ($P<0.05$). BC: blank control; NC: negative control; EV: empty vector; GAPDH: glyceraldehyde-3-phosphate dehydrogenase, which was used as an internal control. *$P<0.05$.

图2 Cav-1 siRNA下调ESF细胞Cav-1的表达

Fig.2 Cav-1 siRNA downregulated the expression of Cav-1 in ESF cells



A: qRT-PCR检测Cav-1 siRNA对ESF细胞*LC3II* mRNA表达的影响, 与未转染Cav-1 siRNA的共培养组和单独培养组比较, 转染Cav-1 siRNA的BT474/ESFsiCav-1共培养组ESF细胞*LC3II* mRNA显著上调(P<0.05); B、C: Western blot检测Cav-1 siRNA对ESF细胞LC3II蛋白表达的影响, 与未转染Cav-1 siRNA的共培养组和单独培养组比较, 转染Cav-1 siRNA的BT474/ESFsiCav-1共培养组ESF细胞LC3II蛋白显著上调(P<0.05)。β-actin为内参对照。*P<0.05。

A: qRT-PCR was conducted to analyze the effect of Cav-1 siRNA on *LC3II* expression in ESF cells at mRNA level. The expression of *LC3II* mRNA was significantly upregulated in the co-culture group of BT474/ESFsiCav-1 which was transfected with Cav-1 siRNA compared to that of the co-culture group of untransfected with Cav-1 siRNA and the mono-culture groups (P<0.05). B,C: Western blot was conducted to analyze the effect of Cav-1 siRNA on LC3II expression in ESF cells at protein level. The expression of LC3II protein was significantly upregulated in the co-culture group of BT474/ESFsiCav-1 which was transfected with Cav-1 siRNA compared to that of the co-culture group of untransfected with Cav-1 siRNA and the mono-culture groups (P<0.05). β-actin was used as an internal control. *P<0.05.

图3 Cav-1 siRNA上调ESF细胞LC3II的表达

Fig.3 Upregulation of LC3II expression in ESF cells by Cav-1 siRNA

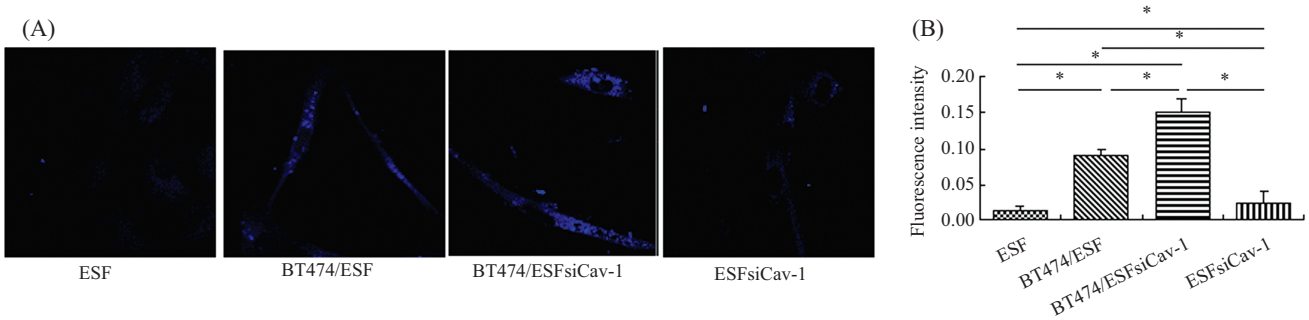
各组ESF细胞LC3II mRNA和蛋白的表达。结果显示, BT474/ESFsiCav-1共培养组LC3II的表达显著高于其他各组(P<0.05), 未转染Cav-1 siRNA的BT474/ESF共培养组LC3II的表达显著高于单独培养的ESF组和ESFsiCav-1组(P<0.05)。以上说明, Cav-1 siRNA上调成纤维细胞LC3II的表达, 乳腺癌细胞共培养能加强Cav-1 siRNA上调成纤维细胞LC3II表达的作用(图3)。

2.3 Cav-1 siRNA促进成纤维细胞ESF自噬体表达

培养板内放入细胞爬片用的玻片, 培养48 h, 进行MDC染色。MDC标记自噬体后激光共聚焦显微镜观察并拍照, Image-Pro Plus软件分析各组自噬体的荧光强度。结果显示, BT474/ESFsiCav-1共培养组自噬体表达显著高于其他各组自噬体(P<0.05), BT474/ESF组自噬体表达显著高于ESF单独培养组和ESFsiCav-1单独培养组(P<0.05), 这些结果提示, 在乳腺癌细胞共培养条件下转染Cav-1 siRNA的成纤维细胞, 自噬体表达上调幅度大(图4)。

2.4 乳腺癌细胞BT474在ESFsiCav-1细胞共培养条件下增殖加快

为了研究Cav-1表达下调对BT474细胞增殖的影响, 采用CCK-8方法检测共培养和单独培养第1 d至第5 d BT474细胞的增殖。实验结果显示, 培养后第1 d各组细胞增殖无显著差异(P>0.05), 培养后第2~5 d, BT474/ESFsiCav-1组BT474细胞增殖显著高于BT474/ESF组(P<0.05), BT474/ESF组的BT474细胞增殖显著高于BT474组(P<0.05)。根据CCK-8的实验结果, 分析了各组BT474细胞活力, BT474/ESFsiCav-1组的BT474细胞活力培养后的第2~5 d分别增加了70%、124%、96%和72%, BT474/ESF组的BT474细胞活力第2~5 d分别增加了43%、88%、64%和45%。这些结果表明, Cav-1 siRNA促进乳腺癌细胞BT474增殖(图5)。

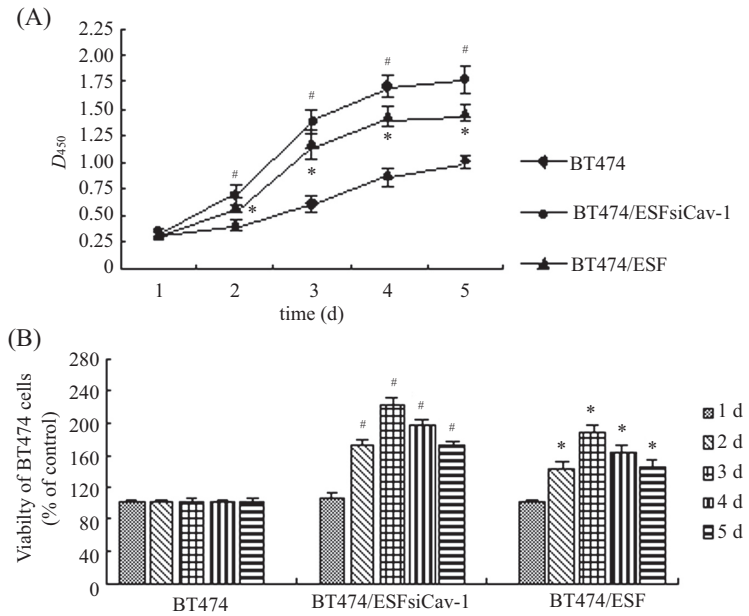


A: MDC染色激光共聚焦显微镜图, MDC染色标记成纤维细胞自噬体(蓝色颗粒); B: Image-Pro Plus软件分析各组ESF细胞自噬体荧光强度。*P<0.05。

A: the figures of MDC staining and laser confocal microscope. MDC staining (blue granules) was used to detect the expression of autophagosome in ESF cells; B: fluorescence intensity of autophagosome in ESF cells was analyzed by Image-Pro Plus. *P<0.05.

图4 Cav-1 siRNA促进ESF细胞自噬体表达

Fig.4 Promotion of the expression of autophagosome in ESF cells by Cav-1 siRNA



A: 各组BT474细胞增殖。培养第1 d, 各组BT474细胞增殖无显著差异; 培养第2~5 d, BT474/ESFsiCav-1组与BT474/ESF组的BT474细胞增殖有显著差异, BT474组与BT474/ESF组的BT474细胞增殖有显著差异; B: 各组BT474细胞活力。培养第1 d, 各组BT474细胞活力无显著差异; 培养第2~5 d, BT474/ESFsiCav-1组BT474细胞活力显著高于BT474/ESF组; 与BT474组比较, BT474/ESF组的BT474细胞活力有显著差异。*P<0.05, 与BT474组比较; #P<0.05, 与BT474/ESF组比较。

A: BT474 cell proliferation in different groups. No significant difference in cell proliferation was detected in each group on the 1st day after culture. BT474 cell proliferation was significantly greater in the BT474/ESFsiCav-1 group than in BT474/ESF group from the 2nd to the 5th day after culture. The results also showed a significant difference in BT474 cell proliferation between BT474 group and BT474/ESF group; B: BT474 cell viability in different groups. No significant difference in cell viability was detected in each group on the 1st day after culture. BT474 cell viability was significantly greater in the BT474/ESFsiCav-1 group than in BT474/ESF group from the 2nd to the 5th day after culture. A significant difference in BT474 cell viability was shown between BT474 group and BT474/ESF group. *P<0.05 compared with the BT474 group; #P<0.05 compared with the BT474/ESF group.

图5 Cav-1 siRNA促进乳腺癌细胞BT474的增殖和活力

Fig.5 Promotion of proliferation and viability of breast cancer cells BT474 by Cav-1 siRNA

3 讨论

国内外对基质成纤维细胞的自噬水平与肿瘤发生发展的关系有较多报道, 但乳腺癌相关的人成纤维细胞中Cav-1的表达与其自噬体相关性的研究较少, 本文探索了在人乳腺癌细胞共培养的条件下, 人成纤维细胞的Cav-1对自噬体膜标志蛋白LC3II和自噬体表达的影响。

Cav-1是Caveolins家族中的一员, 通过脂质转运、细胞膜运输、基因调节和信号转导等参与肿瘤的发生[15-16]。Cav-1既有抑癌基因的作用, 又可以促

进肿瘤的进展和转移, 其表达决定于肿瘤的类型和分期[17-18]。近年来, 一些研究者在乳腺癌研究中发现, 基质成纤维细胞中Cav-l表达的下降或缺失与肿瘤的预后显著相关。Witkiewicz等[19]在154例乳腺癌的研究中发现, 成纤维细胞中Cav-l的缺失可以作为一项独立的不良预后指标, 预测乳腺癌的复发、淋巴结转移及他莫昔芬抵抗, 而与雌激素受体(estrogen receptor, ER)、孕激素受体(progesterone receptor, PR)和HER2的表达无关。在ER、PR和HER2均为阴性的乳腺癌[4]和导管原位癌[10]中也发现了类似的结果, 即Cav-1表达下降或缺失的乳腺癌患者预后差, 高表达Cav-1的乳腺癌患者预后则较好。但也有报道称, Cav-l在肿瘤细胞的表达与乳腺癌预后无关[20]。成纤维细胞Cav-l的缺失也与前列腺癌的不良预后、骨和淋巴结的转移显著相关[5]。以上研究表明, 基质成纤维细胞Cav-l可能有抑癌基因的作用, 基质Cav-l的缺失有可能作为上皮性肿瘤的预后指标。Martinez-Outschoorn等[21]在人乳腺癌细胞和成纤维细胞共培养模型中发现, 成纤维细胞Cav-l表达下调, 肌成纤维细胞标志物和细胞外基质蛋白表达增多。在肿瘤形成过程中, Cav-l表达下调或缺失可能是驱动基质成纤维细胞活化的关键起始因子。

肿瘤基质成纤维细胞内的Cav-1的表达还与细胞自噬相关。自噬是一个发生在真核细胞中由细胞溶酶体处理内源性底物的重要过程, 它的特点是在细胞内形成具有双层或多层膜包裹的含有内源物的自噬体, 与自身的溶酶体结合后, 发生降解和转化。在正常情况下, 细胞保持了一种很低的、基础的自噬活性以清除损伤和衰老的细胞器从而维持自稳。自噬调控机制的失调可引起自噬功能异常, 其诱发因素有来自于细胞外的, 也有来自细胞内的。Cav-1表达的抑制或缺失诱导了成纤维细胞自噬基因表达的上调, 从而导致成纤维细胞的功能发生异常变化。由于成纤维细胞是基质微环境的主要细胞成分, 功能异常的成纤维细胞将造成微环境中细胞的遗传不稳定性或恶性选择, 促进肿瘤的发生和发展。

我们采用人工合成的3对Cav-1 siRNA序列, 通过脂质体转染成纤维细胞, 从基因转录和蛋白表达水平检测了siRNA对成纤维细胞中Cav-1表达的干扰效果, 建立Cav-1 siRNA成纤维细胞模型, 通过此细胞模型研究Cav-1对自噬体表达的影响。实验结果显示, 3对Cav-1 siRNA序列转染成纤维细胞后, 均干扰了Cav-1的表达, 尤以siRNA-2组效果最为显著, 我们选择该组用于本研究。

为了探讨与乳腺癌细胞共培养的成纤维细胞中Cav-1蛋白表达抑制对自噬体表达的影响, 本文将siRNA转入成纤维细胞后用MDC自噬体特异性染色方法染细胞爬片, 激光共聚焦显微镜观察和Image-Pro Plus软件分析荧光强度。MDC染色法是一种特异性的自体检测方法, 主要显示中晚期自噬体[13-14]。我们的实验结果显示, 在两个单独培养组中, 与ESF组比较, ESFsiCav-1组的成纤维细胞的自噬体明显增加。国外相关的实验表明, 在鼠Cav-1蛋白缺失的心肌细胞中, 电镜下可见自噬囊泡的聚集明显增多[22]。在*Cav-1*$^{-/-}$缺失的老鼠脂肪细胞中, 可见自噬体膜标志性蛋白LC3的表达上调及自噬体增多[23]。在成纤维细胞中, *Cav-1*基因的缺失可以激活核转录因子NFκB及其靶基因[24], 激活的NFκB可以转入核内并结合相应的DNA片段, 从而参与多种生物学过程, 例如细胞代谢、肿瘤的发生发展和细胞自噬等[25-26]。BT474/ESF组与BT474/ESFsiCav-1均为共培养组, 不同之处是, BT474/ESF组的ESF细胞未转染Cav-1 siRNA, BT474/ESFsiCav-1组则转染了Cav-1 siRNA, 其自噬体显著多于未转染Cav-1 siRNA的ESF细胞, 表明成纤维细胞自噬体与*Cav-1*基因表达呈负相关。与ESFsiCav-1单独培养组比较, BT474/ESFsiCav-1共培养组可见细胞自噬体显著增加; 与ESF单独培养组比较, BT474/ESF共培养组可见细胞自噬体增加。表明在共培养模式中, 乳腺癌细胞可以促进成纤维细胞自噬体增加。

微管相关蛋白LC3(MAP1-LC3)是分布于哺乳动物细胞中的自噬相关蛋白, 成熟的LC3的形成是从LC3前体开始加工的, LC3前体暴露于C-端120位的甘氨酸残基被Atg4B蛋白切除后形成胞浆型LC3(LC3I), 均匀分布于细胞质中。自噬发生时, LC3I与Atg7蛋白结合形成硫酯键, 再被传递给Atg3蛋白, 通过Atg5-Atg12-Atg16复合物的辅助连接磷脂, 形成具有与自噬体膜结合能力的LC3-磷脂共轭型(LC3II), 随之被募集于自噬体的膜上。因此, LC3II被认作是自噬体膜的一个标志分子。当哺乳动物细胞进行自噬活动时, 细胞内LC3II的含量明显上升[14,27]。我们的LC3II检测结果显示, 免疫印迹测定结果与实时荧光PCR的结果相互印证, BT474/ESFsiCav-1共培养组LC3II的表达显著高于其他各组, 表明Cav-1 siRNA可上调

与乳腺癌细胞共培养的成纤维细胞自噬体膜蛋白LC3II的表达。检测LC3II表达的qRT-PCR和Western blot的实验结果与检测自噬体表达的MDC/激光共聚焦显微镜观察的结果吻合, 转染了Cav-1 siRNA的成纤维细胞在与乳腺癌细胞共培养条件下, 自噬体和LC3II的表达显著上调。

本实验用CCK-8方法检测了与ESF细胞共培养的乳腺癌细胞BT474的增殖情况。实验结果表明, 培养后第1 d, 与BT474单独培养组比较, BT474/ESF共培养组和BT474/ESFsiCav-1共培养组的BT474细胞增殖无统计学意义。可能的原因是: BT474细胞属于贴壁生长的细胞, 细胞增殖过程包括游离期、贴壁期、潜伏期、对数期、平台期、衰退期, BT474细胞经过培养24 h, 大部分细胞仍未完全进入旺盛增殖的对数期, 细胞增殖相对缓慢, 所以各组BT474细胞的增殖未见明显统计学差异。培养后第2~5 d, 与BT474组比较, BT474/ESF组和BT474/ESF-siCav-1组均可见BT474细胞增殖上调, 但BT474/ESFsiCav-1组的BT474细胞增殖显著高于BT474/ESF组, 提示与乳腺癌细胞共培养的成纤维细胞中Cav-1的下调导致乳腺癌细胞增殖加快。

综上所述, 抑制Cav-1表达促进了与乳腺癌细胞共培养的成纤维细胞自噬体和LC3II的表达, 抑制成纤维细胞中Cav-1表达促进了乳腺癌细胞的增殖。成纤维细胞中Cav-1与自噬体的相关性及其对乳腺癌细胞作用的机制还需不断深入研究。

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