氧化低密度脂蛋白通过Wnt5a/PKCδ信号通路诱导大鼠肝星状细胞自噬

侯明粟[1] 徐军全[2]* 宋维芳[2] 崔永佳[1]

([1]山西医科大学研究生学院, 太原 030001; [2]山西医科大学汾阳学院, 汾阳 032200)

摘要 该文旨在研究氧化低密度脂蛋白(ox-LDL)对大鼠肝星状细胞(HSC-T6)自噬的影响及机制, 探讨非酒精性脂肪肝炎的发病机理。体外培养的HSC-T6细胞以不同质量浓度(0、10、20、40、60 μg/mL)的ox-LDL分别处理不同时间(0、3、6、12、24 h)后, 用Western blot检测LC3 II、Beclin1、p62的含量。不同质量浓度(0、10、20、40、60 μg/mL)的ox-LDL处理HSC-T6细胞12 h后, Western blot检测Wnt5a、p-PKCδ、p-STAT3的含量。将HSC-T6细胞分为对照组(Control)、ox-LDL组、ox-LDL+si-NC组和ox-LDL+si-Wnt5a组, 经相应处理后用Western blot、qRT-PCR分别检测LC3 II、Beclin1、p62、p-PKCδ、p-STAT3的蛋白和mRNA含量变化; 免疫荧光检测LC3 II的变化; 油红O染色观察HSC-T6脂滴含量变化; 比色法检测各组细胞培养上清中羟脯氨酸(Hyp)含量; ELISA检测细胞培养上清中透明质酸(HA)和层黏连蛋白(LN)含量。PKCδ抑制剂Rottlerin预处理细胞: 将HSC-T6细胞分为对照组(Control)、ox-LDL组、ox-LDL+DMSO组和ox-LDL+Rottlerin组, 检测方法与敲低*Wnt5a*一致。经ox-LDL处理后, HSC-T6细胞中LC3 II、Beclin1含量增加(P<0.05), p62含量减少(P<0.01), 且在ox-LDL质量浓度为20 μg/mL、作用12 h时达到峰值。ox-LDL质量浓度为20 μg/mL、作用12 h时, HSC-T6细胞中Wnt5a、p-PKCδ、p-STAT3蛋白表达显著升高(P<0.01)。敲低*Wnt5a*后, HSC-T6细胞中Wnt5a、LC3 II、Beclin1 mRNA和蛋白表达水平显著降低(P<0.001), p62蛋白表达增多(P<0.01), p-PKCδ、p-STAT3蛋白表达减少(P<0.05), 细胞内LC3 II点状聚集减少, 脂滴含量减少, 细胞培养上清中Hyp、HA、LN含量也减少(P<0.05)。抑制PKCδ后, 结果与敲低*Wnt5a*一致。ox-LDL可通过增强Wnt5a/PKCδ通路诱导HSC-T6细胞自噬。

关键词 Wnt5a/PKCδ; 肝星状细胞; 氧化低密度脂蛋白; 自噬

Oxidized Low-Density Lipoprotein Induces Rat Hepatic Stellate Cell Autophagy by Wnt5a/PKCδ Signaling Pathway

HOU Mingsu[1], XU Junquan[2]*, SONG Weifang[2], CUI Yongjia[1]

([1]*Graduate School of Shanxi Medical University, Taiyuan 030001, China*;
[2]*Fenyang College of Shanxi Medical University, Fenyang 032200, China*)

Abstract This study was aimed to investigate the effect of ox-LDL (oxidized low-density lipoprotein) on autophagy of rat hepatic stellate cell HSC-T6, and explore the pathogenesis of non-alcoholic steatohepatitis. HSC-T6 cells were cultured *in vitro* and treated with different concentrations of ox-LDL (0, 10, 20, 40, 60 μg/mL)

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*通讯作者。Tel: 13313582041, E-mail: xujq@sina.com
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*Corresponding author. Tel: +86-13313582041, E-mail: xujq@sina.com
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at different time points (0, 3, 6, 12, 24 h), and the expression levels of LC3 II, Beclin1 and p62 were detected by Western blot. HSC-T6 cells treated with different concentrations of ox-LDL (0, 10, 20, 40, 60 μg/mL) for 12 h. The expression levels of Wnt5a, p-PKCδ and p-STAT3 were detected by Western blot. HSC-T6 cells were divided into Control group, ox-LDL group, ox-LDL+si-NC group and ox-LDL+si-Wnt5a group. After treatment, the expression levles of LC3 II, Beclin1, p62, p-PKCδ and p-STAT3 in each group were detected by Western blot and qRT-PCR; the expression of LC3 II was detected by immunofluorescence assay; the lipid droplet of HSC-T6 was observed by oil red O staining; the content of Hyp (hydroxyproline) in cell culture supernatant was determined by colorimetric method and the contents of HA (hyaluronic acid) and LN (laminin) were determined by ELISA. HSC-T6 cells were pretreated with PKCδ inhibitor (Rottlerin) and were divided into Control group, ox-LDL group, ox-LDL+DMSO and ox-LDL+Rottlerin group. The detection method was consistent with *Wnt5a* knockdown. The levels of LC3 II and Beclin1 were significantly increased (P<0.05), while p62 content decreases (P<0.01) after HSC-T6 cells were treated with 20 μg/mL ox-LDL for 12 h. The levels of Wnt5a, p-PKCδ and p-STAT3 were significantly increased after HSC-T6 cells were treated with 20 μg/mL ox-LDL for 12 h (P<0.01). After *Wnt5a* knockdown, the mRNA and protein expression levels of Wnt5a in HSC-T6 cells were significantly reduced (P<0.001). Western blot showed that knockdown of *Wnt5a* reduced the expression levels of LC3 II, Beclin1, p-PKCδ, p-STAT3, and induced the expression of p62 (P<0.05). qRT-PCR showed that the expression levels of *LC3 II* and *Beclin1* mRNA were significantly decreased in ox-LDL+si-Wnt5a group compared with ox-LDL+si-NC group (P<0.05). Knockdown of *Wnt5a* reduced the LC3 II fluorescent spots, inhibited cellular lipid accumulation, while decreased the levels of Hyp, HA, and LN (P<0.05). The results of PKCδ inhibitor pretreatment were consistent with knockdown *Wnt5a*. ox-LDL induces autophagy of HSC-T6 cells through the Wnt5a/PKCδ pathway.

Keywords Wnt5a/PKCδ; hepatic stellate cells; oxidized low-density lipoprotein; autophagy

非酒精性脂肪性肝炎(non-alcoholic steatohepatitis, NASH)是非酒精性脂肪性肝病(non-alcoholic fatty liver disease, NAFLD)的一种进展形式，其特征是肝细胞脂肪变性、小叶内炎症和纤维化，其严重程度是NAFLD患者死亡率的主要决定因素[1-2]。约30%的NAFLD患者有NASH，其中20%和7%的NASH患者分别会发展为肝硬化和肝细胞癌[3]。肝纤维化是各种慢性肝病进展到肝硬化、肝癌的关键，因此，在NASH中控制肝纤维化的发生发展尤为重要。肝星状细胞(hepatic stellate cells, HSCs)在各种肝纤维化的发病机制中起核心作用，各种致病因素可以直接或间接激活HSCs，静止的HSCs被激活后细胞形态发生改变、增殖并分泌大量细胞因子和细胞外基质(extracellular matrix, ECM)，从而导致肝纤维化的发生[4]。研究表明，氧化低密度脂蛋白(oxidized low-density lipoprotein, ox-LDL)在NAFLD患者中明显升高，ox-LDL呈现剂量依赖性刺激体外培养的HSCs合成I、III型胶原及纤连素，体外培养的HSCs及肝纤维化组织中的活化HSCs均表达ox-LDL受体[5-7]，提示ox-LDL可能是NASH发生发展的重要因素，它可通过直接活化HSCs而参与NASH相关的肝纤维化，但ox-LDL活化HSCs的具体机制尚不明确。

研究表明，自噬(autophagy)在多种类型肝纤维化的发生发展中起重要作用，自噬可通过诱导HSCs活化而促进肝纤维化[8]，调节HSCs自噬被认为有望成为防治肝纤维化的新方法。Wnt5a(wingless-type MMTV integration site family member 5a)是一种分泌型糖蛋白，在NAFLD患者血清中Wnt5a异常升高[9]，其在活化的HSCs和肝脏纤维化区域高表达[10]。Wnt5a/PKCδ信号通路可介导ox-LDL诱导的巨噬细胞自噬[11]，尚未见Wnt5a是否能诱导HSCs自噬的报道。

本研究采用ox-LDL刺激体外培养的HSC-T6细胞，观察ox-LDL对HSCs自噬的影响，并使用Wnt5a小干扰RNA、PKCδ抑制剂预处理细胞，探讨ox-LDL对HSCs自噬的影响是否与Wnt5a/PKCδ信号通路有关。

1 材料与方法

1.1 实验材料

1.1.1 实验细胞 大鼠肝星状细胞系HSC-T6购自武汉普诺赛生物科技有限公司。

1.1.2 主要试剂 DMEM高糖培养基、胎牛血清、胰酶、HRP-羊抗兔IgG抗体、RIPA裂解液、蛋白酶抑制剂和BCA蛋白质定量试剂盒购自武汉博士德生物工程有限公司; Alexa Fluor 488标记山羊抗兔IgG购自上海碧云天生物技术研究所; Wnt5a干扰链和对照干扰链购自广州锐博生物科技有限公司; LC3抗体、p62抗体购自ABways公司; Beclin1抗体购自沈阳万类生物科技有限公司; Wnt5a、p-PKCδ、PKCδ、p-STAT3、STAT3抗体购自ABclonal公司; qRT-PCR相关试剂盒购自北京天根生化科技有限公司; ox-LDL购自北京协生生物科技有限责任公司; 油红O染液购自北京索莱宝科技有限公司; 羟脯氨酸测定试剂盒购自南京建成生物工程研究所; 大鼠层黏连蛋白ELISA试剂盒和大鼠透明质酸ELISA试剂盒购自江苏酶免实业有限公司。

1.2 实验方法

1.2.1 细胞培养与分组 HSC-T6细胞用含10%胎牛血清的DMEM培养基在37 °C、5% CO_2的培养箱中培养。取对数生长期的HSC-T6细胞以每孔8×10^5个细胞接种于6孔板, 分组如下: (1) 剂量效应组, 分别用不同质量浓度(0、10、20、40、60 μg/mL)的ox-LDL处理HSC-T6细胞24 h; (2) 时间效应组, 用质量浓度为20 μg/mL的ox-LDL分别处理0、3、6、12、24 h; (3) 将接种于6孔板的HSC-T6细胞随机分为对照组(不做特殊处理)、ox-LDL组(20 μg/mL ox-LDL处理12 h)、ox-LDL+si-NC组(转染si-NC 24 h后用20 μg/mL ox-LDL处理12 h)和ox-LDL+si-Wnt5a组(转染si-Wnt5a 24 h后用20 μg/mL ox-LDL处理12 h); (4) 将接种于6孔板的HSC-T6细胞随机分为对照组(不做特殊处理)、ox-LDL组(20 μg/mL ox-LDL处理12 h)、ox-LDL+DMSO(加入DMSO后给予终浓度为20 μg/mL ox-LDL处理12 h)组和ox-LDL+Rottlerin组(10 μmol/L Rottlerin溶于DMSO预处理1 h后给予终浓度为20 μg/mL ox-LDL处理12 h)。

1.2.2 细胞转染 将三段靶向Wnt5a的特异性siRNA(si-Wnt5a)以及阴性对照(si-NC)转染至HSC-T6细胞中。转染前24 h, 将细胞传代至6孔板上, 待细胞融合至40%时, 各孔加入2 mL培养基。首先在125 μL无血清培养基中加入5 μL si-Wnt5a, 轻轻混匀后再加入4 μL转染试剂Lipo8000™, 轻轻混匀后室温孵育20 min, 将转染复合物加入至培养板各孔。置于37 °C、5% CO_2培养箱继续培养48 h, 收集细胞。转染效果采用qRT-PCR、Western blot鉴定。三段干扰靶序列分别为si-Wnt5a 001: 5′-GGA CAA CAC TTC TGT CTT T-3′; si-Wnt5a 002: 5′-TCA TGA ACT TGC ACA ACA A-3′; si-Wnt5a 003: 5′-GCG TGG CTA TGA CCA GTT T-3′。

1.2.3 Western blot检测蛋白表达 用Western blot检测细胞中自噬相关蛋白LC3 II、Beclin1、p62和通路相关蛋白Wnt5a、p-PKCδ、p-STAT3的表达。按照每孔30 μg的蛋白量进行SDS-PAGE, 蛋白质分离后转移到NC膜上, 室温下用5%脱脂奶粉封闭2 h, 用相应的一抗(LC3 II抗体1:2 000稀释; p62抗体1:5 000稀释; Beclin1、Wnt5a、p-PKCδ、PKCδ、p-STAT3、STAT3抗体1:1 000稀释)于4 °C孵育过夜。TBST洗涤后, 加入HRP标记的二抗(1:6 000稀释)室温孵育2 h, TBST洗涤后, 用化学发光剂ECL显影, 并使用Image Lab软件分析蛋白质条带灰度值。

1.2.4 qRT-PCR检测*Wnt5a*、*LC3 II*、*Beclin1*、*p62*的mRNA表达 按照TRIzol试剂盒说明书提取各组细胞总RNA, 用反转录试剂盒合成cDNA, 以cDNA为模板, 采用SYBR Green进行实时荧光定量PCR反应; 以*β-actin*为内参, 采用$2^{-\Delta\Delta Ct}$法计算目的基因mRNA的相对含量。引物序列由上海生工生物科技有限公司合成: 大鼠*Wnt5a*上游引物5′-CAA CTG GCG GGA CTT TCT CAA GG-3′, 下游引物5′-CGG AAC TGG TAC TGG CAC TCT TTG-3′; 大鼠*LC3 II*上游引物5′-GAG TGG AAG ATG TCC GGC TC-3′, 下游引物5′-CCA GGA GGA AGA AGG CTT GG-3′; 大鼠*Beclin1*上游引物5′-CGT GGA GAA AGG CAA GAT TGA AGA-3′, 下游引物5′-GTG AGG ACA CCC AAG CAA GAC C-3′; 大鼠*p62*上游引物5′-CTG CTG CCT CCC TCT AAT CC-3′, 下游引物5′-TAT TCT CCG GCT CCA TCT TG-3′; 大鼠*β-actin*上游引物5′-CCC ACA CTG TGC CCA TCT ACG-3′, 下游引物5′-GCC ATC TCT TGC TCG AAG TCC-3′。

1.2.5 细胞免疫荧光检法测LC3 II的表达 以每孔1×10^5个细胞的密度将细胞接种于玻片并经相应处理后, 吸弃培养基, 用预冷的PBS洗涤后, 4%多聚甲醛固定30 min。洗涤后加入0.2% TritonX-100(PBS配制)置于4 °C通透10 min。洗涤后用5% BSA室温封闭1 h, 加入相应一抗(1:100稀释)于4 °C孵育过夜。洗涤后滴加稀释好的荧光Alexa Fluor 488标记的山羊抗兔IgG(1:400稀释), 湿盒中室温孵

育1 h, 洗涤后用抗荧光衰减封片剂封片, 荧光显微镜下观察并拍照。

1.2.6 油红O染色 将细胞接种于无菌的6孔板内, 每组经相应处理后, 用PBS洗涤, 加入预冷的4%多聚甲醛固定30 min, PBS洗涤后加入油红O染液避光染色30 min, 用60%的异丙醇溶液清洗多余染料; 苏木素染液复染细胞核后用显微镜观察。

1.2.7 细胞培养上清中Hyp、HA、LN含量的检测 将细胞接种于无菌的6孔板内, 每组经相应处理后, 收集上清, 按照说明书操作步骤用比色法检测Hyp含量, ELISA检测HA、LN含量。

1.3 统计学处理

采用SPSS 25.0软件进行统计, 结果数据以均数±标准差($\bar{x}\pm s$)表示, 多组间比较采用单因素方差分析, 两组间数据比较采用t检验。用GraphPad Prism 8.0软件进行统计图形制作。以$P<0.05$为差异有统计学意义。

2 结果

2.1 ox-LDL诱导HSC-T6细胞自噬量效和时效的关系

以浓度为0、10、20、40、60 μg/mL的ox-LDL处理HSC-T6细胞24 h后, LC3 II的表达在10、20、40 μg/mL时显著增多($P<0.05$), Beclin1的表达在20、40 μg/mL时显著增多($P<0.01$), p62的表达在20、40、60 μg/mL时显著减少($P<0.01$), 且当ox-LDL的浓度为20 μg/mL时, LC3 II和Beclin1的表达、p62的降解达到峰值(图1A); 用20 μg/mL ox-LDL处理HSC-T6细胞3、6、12、24 h后, LC3 II和Beclin1的表达、p62的降解在6 h时开始变化, 在12 h时达到峰值($P<0.001$)(图1B)。

2.2 ox-LDL诱导HSC-T6细胞Wnt5a、p-PKCδ、p-STAT3蛋白表达

以浓度为0、10、20、40、60 μg/mL的ox-LDL处理HSC-T6细胞12 h后, Western blot检测Wnt5a、p-PKCδ、p-STAT3蛋白表达上调情况(图2), 其中20 μg/mL的ox-LDL处理组变化最显著($P<0.01$)。

2.3 敲低*Wnt5a*抑制了ox-LDL诱导的HSC-T6细胞自噬

qRT-PCR(图3A)、Western blot(图3B)结果显示, 第二条干扰链002干扰效果最佳($P<0.001$)。将第二条干扰链002以及阴性对照si-NC转染至HSC-T6细胞后, Western blot结果显示, 与ox-LDL+si-NC组相比ox-LDL+si-Wnt5a组LC3 II、Beclin1含量减少($P<0.01$), p62降解减少($P<0.01$), p-PKCδ、p-STAT3含量减少($P<0.05$)(图4); qRT-PCR结果显示, 敲低*Wnt5a*后*LC3 II*、*Beclin1* mRNA含量显著降低($P<0.05$), *p62* mRNA含量没有显著差异($P>0.05$)(图5)。免疫荧光结果显示, 敲低*Wnt5a*后, LC3 II点状聚集减少(图6)。油红O染色结果显示, ox-LDL+si-Wnt5a组较ox-LDL+si-NC组相比, 细胞内脂滴显著减少(图7), 敲低*Wnt5a*后细胞培养上清中Hyp、HA、LN含量也降低($P<0.05$)(图8)。这表明敲低*Wnt5a*抑制了ox-LDL诱导的HSC-T6细胞自噬。

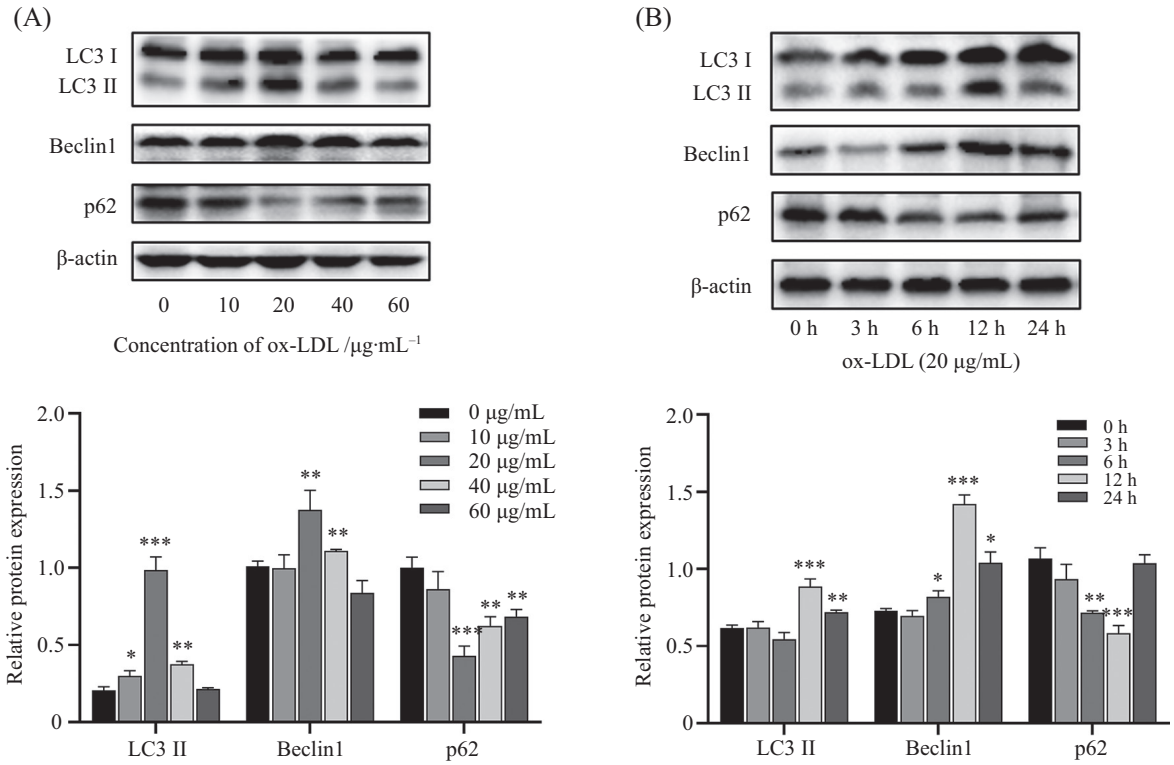
2.4 PKCδ参与ox-LDL诱导的HSC-T6细胞自噬

用PKCδ的抑制剂Rottlerin预处理细胞后, Western blot结果显示, 与ox-LDL+DMSO组相比, ox-LDL+Rottlerin组LC3 II、Beclin1蛋白表达显著降低($P<0.01$), p62降解减少($P<0.01$), p-STAT3含量减少($P<0.001$)(图9)。qRT-PCR结果显示, ox-LDL+Rottlerin组较ox-LDL+DMSO组*LC3 II*、*Beclin1* mRNA含量显著减少($P<0.01$), *p62* mRNA含量没有显著差异($P>0.05$)(图10)。免疫荧光结果显示, LC3 II点状聚集减少(图11)。油红O染色结果显示, ox-LDL+Rottlerin组较ox-LDL+DMSO组脂滴含量减少(图12)。细胞培养上清中Hyp、HA、LN含量也减少($P<0.05$)(图13)。这表明, PKCδ参与ox-LDL诱导的HSC-T6细胞自噬。

3 讨论

随着生活方式和饮食结构的变化, 高脂饮食所造成的NAFLD发生率越来越高, 估计NAFLD的全球患病率为总人口的24%~25%。NAFLD是最常见的慢性肝病原因之一, 疾病谱包括单纯性脂肪肝、NASH和其相关肝纤维化、肝硬化及肝细胞癌, 其中NASH相关肝纤维化是NAFLD进展的关键阶段, 约30%的NAFLD患者可表现为NASH, 深入研究NASH的发生发展机制有重要的理论与临床意义[12]。

脂质代谢紊乱和氧化应激是NASH发病机制“二次打击”学说的2个重要因素[13]。由于氧化应激, 低密度脂蛋白(low density lipoprotein, LDL)经脂质过氧化修饰成为ox-LDL, 被认为在肝脏炎症反应和纤维化中发挥核心作用[14]。ox-LDL可与HSCs

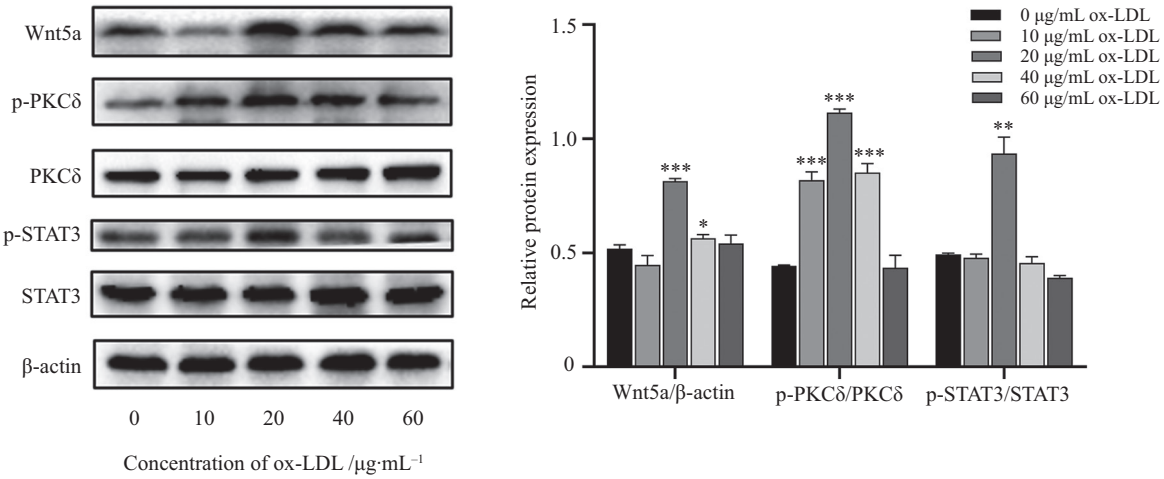


A: Western blot检测不同质量浓度的ox-LDL对HSC-T6细胞中LC3 II、Beclin1、p62含量的影响; B: Western blot检测不同作用时间的ox-LDL对HSC-T6细胞中LC3 II、Beclin1、p62含量的影响。*P<0.05, **P<0.01, ***P<0.001, 与对照组相比。

A: levels of LC3 II, Beclin1 and p62 in HSC-T6 cells treated with ox-LDL at different concentrations were detected by Western blot; B: levels of LC3 II, Beclin1 and p62 in HSC-T6 cells treated with ox-LDL (20 μg/mL) at different time points were detected by Western blot.*P<0.05, **P<0.01, ***P<0.001 compared with control group.

图1 Western blot检测ox-LDL对HSC-T6细胞自噬的影响

Fig.1 Western blot detected the effect of ox-LDL on the autophagy of HSC-T6 cells

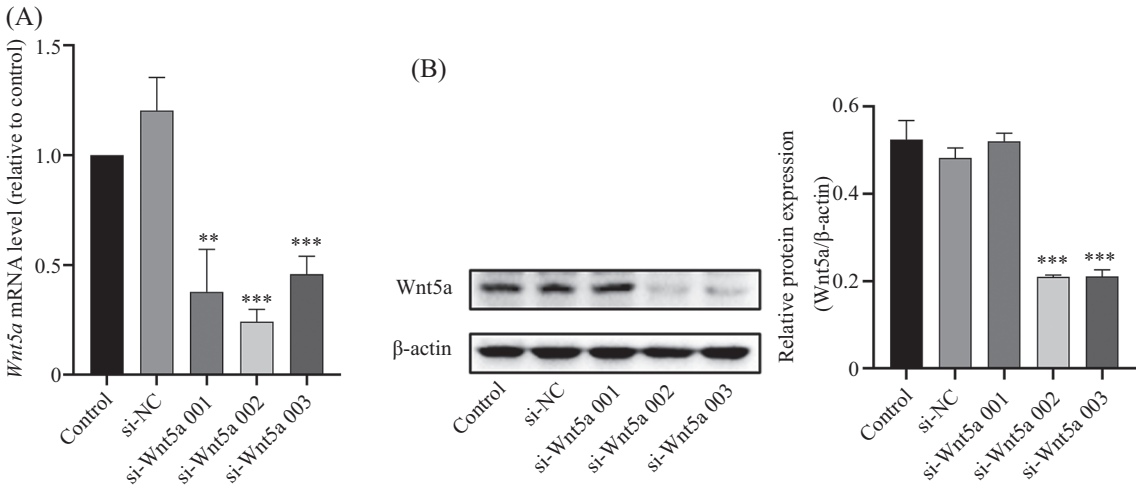


*P<0.05, **P<0.01, ***P<0.001, 与对照组相比。

*P<0.05, **P<0.01, ***P<0.001 compared with control group.

图2 Western blot检测ox-LDL对HSC-T6细胞中Wnt5a、p-PKCδ、p-STAT3含量的影响

Fig.2 Levels of Wnt5a, p-PKCδ and p-STAT3 in HSC-T6 cells treated with ox-LDL were detected by Western blot

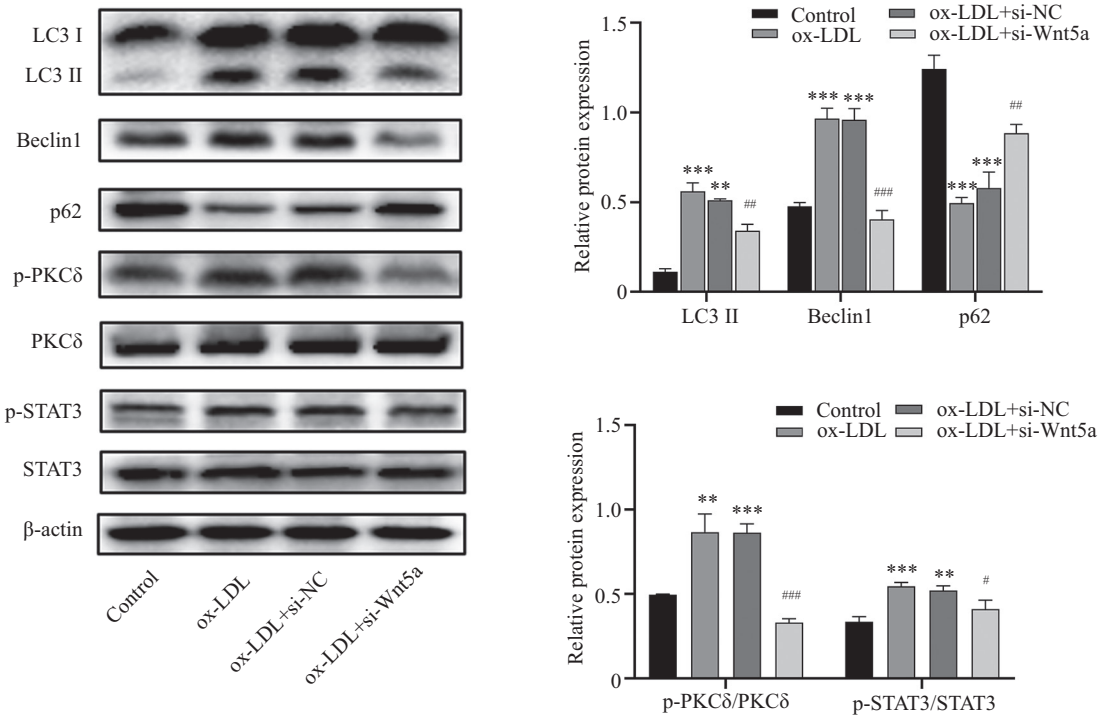


A: qRT-PCR筛选敲低*Wnt5a*效果最佳的干扰链; B: Western blot筛选敲低*Wnt5a*效果最佳的干扰链。**P<0.01, ***P<0.001, 与对照组相比。

A: qRT-PCR selected the interference chain with the best effect of knocking down *Wnt5a*; B: Western blot selected the interference chain with the best effect of knocking down *Wnt5a*. **P<0.01, ***P<0.001 compared with control group.

图3　筛选出敲低HSC-T6细胞中*Wnt5a*效果最佳的干扰链

Fig.3　Selection of the interference chain with the best effect of knocking down *Wnt5a*



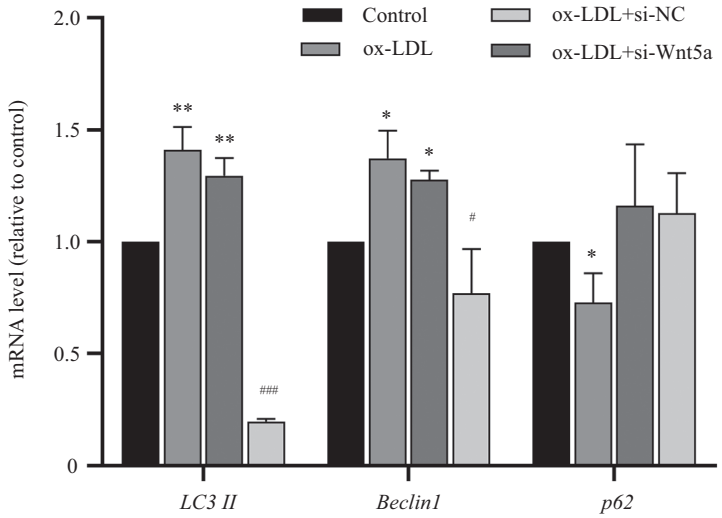
**P<0.01, ***P<0.001, 与对照组相比; #P<0.05, ##P<0.01, ###P<0.001, 与ox-LDL+si-NC组相比。

**P<0.01, ***P<0.001 compared with control group; #P<0.05, ##P<0.01, ###P<0.001 compared with ox-LDL+si-NC group.

图4　Western blot检测敲低*Wnt5a*后ox-LDL对HSC-T6细胞中LC3 II、Beclin1、p62、p-PKCδ、p-STAT3含量的影响

Fig.4　The levels of LC3 II, Beclin1, p62, p-PKCδ and p-STAT3 in HSC-T6 cells treated with ox-LDL were detected by Western blot after *Wnt5a* knockdown

的清道夫受体CD36和凝集素样氧化低密度脂蛋白受体-1(lectin like ox-LDL receptor-1, LOX-1)结合刺激其活化，并分泌胶原和其他基质蛋白，从而导致肝脏炎症和纤维化反应[15]。研究表明，HSCs自噬可促进其活化进而参与肝纤维化的发生发展[8]。Beclin1是自噬起始过程中的关键蛋白，是激活自噬体形成的标记物[16]。LC3也是一种自噬标记蛋白，有可溶性LC3 I和脂溶性LC3 II两种型式，自噬时



*P<0.05, **P<0.01, 与对照组相比; #P<0.05, ###P<0.001, 与ox-LDL+si-NC 组相比。

*P<0.05, **P<0.01 compared with control group; #P<0.05, ###P<0.001 compared with ox-LDL+si-NC group.

图5 qRT-PCR检测敲低*Wnt5a*后ox-LDL对HSC-T6细胞中*LC3 II*、*Beclin1*、*p62* mRNA含量的影响

Fig.5 *LC3 II*, *Beclin1* and *p62* mRNA expression levels in HSC-T6 cells treated with ox-LDL were detected by qRT-PCR after *Wnt5a* knockdown

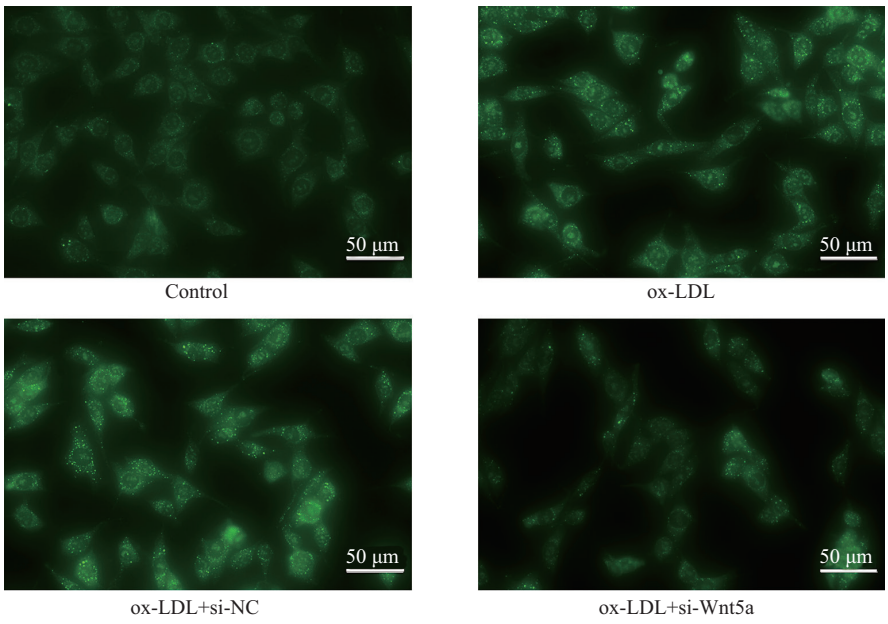


图6 免疫荧光法检测敲低*Wnt5a*后HSC-T6细胞中LC3 II点状聚集的表达

Fig.6 The expression of LC3 II in HSC-T6 cells after *Wnt5a* knockdown was detected by immunofluorescence

LC3 I与自噬泡膜表面的磷脂酰乙醇胺结合转变为LC3 II, 参与自噬体双层膜的延伸, LC3 II含量与自噬呈正相关。在形成自噬体的过程中, p62与LC3 II蛋白相结合形成复合物, 最终会在溶酶体内降解, p62的异常积累表明自噬活性降低[17]。本研究中, HSC-T6细胞经ox-LDL刺激后LC3 II、Beclin1含量明显增加, 自噬底物p62的含量减少, 且在一定范围内有量效关系, 提示ox-LDL可直接诱导HSC-T6细胞发生自噬。

近年来, Wnt信号通路在NASH发病机制中的作用受到越来越多的关注。Wnts属于一类分泌型糖蛋白家族, 与果蝇的无翅基因*wg*(Wingless)有高度同源性, 可参与胚胎发育及细胞增殖、分化、凋亡等调控过程[18]。Wnt5a为Wnt通路的主要信号分子之一, 其异常表达常与多种恶行肿瘤、炎症反应及器官纤维化相关[19]。有文献报道, NAFLD

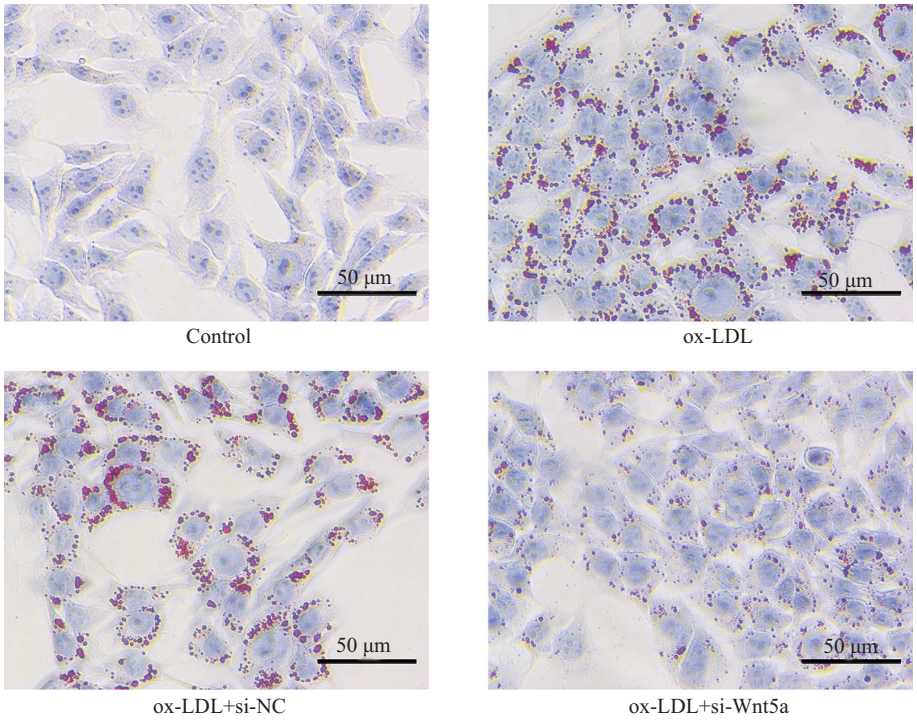
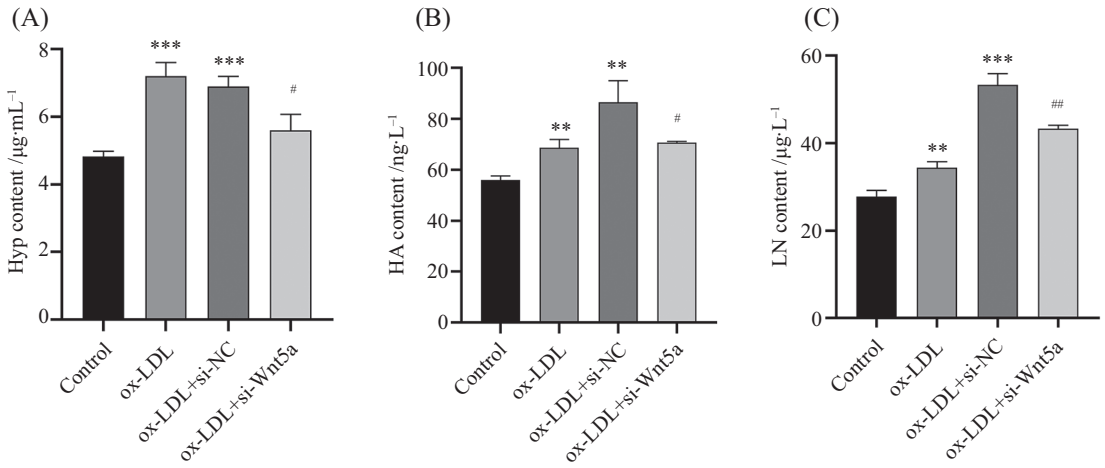


图7 油红O染色观察敲低*Wnt5a*后HSC-T6细胞脂滴含量变化

Fig.7 The change of lipid droplet content in HSC-T6 cells after *Wnt5a* knockdown was detected by oil red O staining



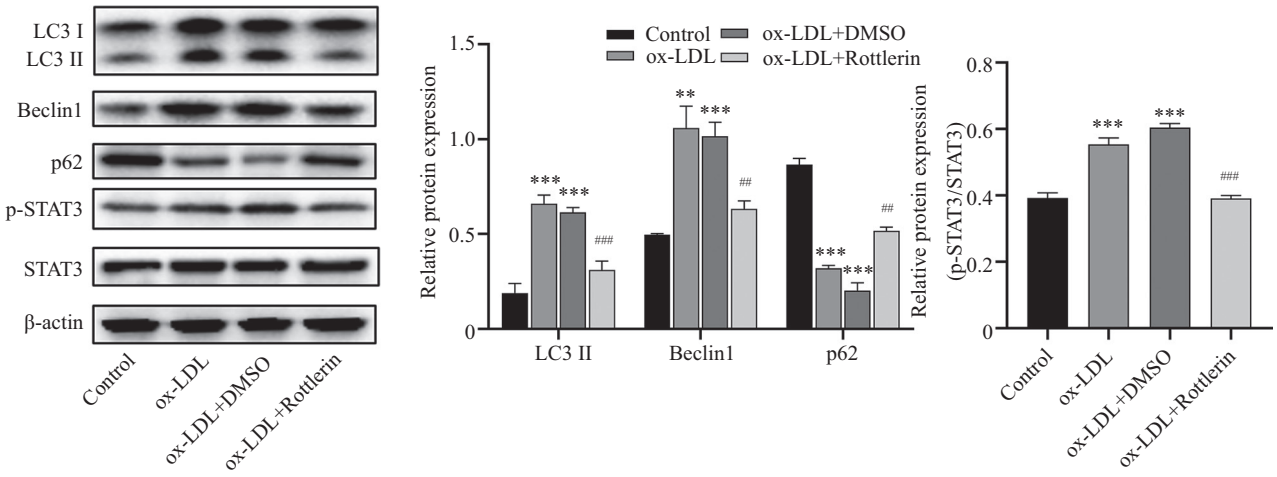
A: 各组细胞培养上清中Hyp含量; B: 各组细胞培养上清中HA含量; C: 各组细胞培养上清中LN含量。$^{**}P<0.01$, $^{***}P<0.001$, 与对照组相比; $^{\#}P<0.05$, $^{\#\#}P<0.01$, 与ox-LDL+si-NC组相比。

A: the content of Hyp in cell culture supernatant in each group; B: the content of HA in cell culture supernatant in each group; C: the content of LN in cell culture supernatant in each group. $^{**}P<0.01$, $^{***}P<0.001$ compared with control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with ox-LDL+si-NC group.

图8 细胞培养上清中Hyp、HA、LN含量

Fig.8 The contents of Hyp, HA and LN in cell culture supernatant

患者血清Wnt5a水平异常升高[9], 在高胆固醇饮食诱导的小鼠NASH中Wnt通路被激活[20]。研究人员使用微阵列分析活化和静止HSCs之间的差异表达基因, 发现活化的HSCs中*Wnt5a*表达显著上调[21], 通过建立大鼠肝纤维化模型后分析发现肝组织中Wnt5a蛋白表达增加[22], 提示Wnt5a可能参与NASH中HSCs的激活过程。另有研究发现, 在动脉粥样硬化斑块处Wnt5a可以激活巨噬细胞自噬[11], Wnt5a可专一性激活PKC亚型中的PKCδ[23], PKCδ可以直接激活STAT3从而启动自噬[24-25]。由此我们推测, 在NASH发病机制中Wnt5a也可能通过其信号通路激活HSCs自噬。本实验结果显示,

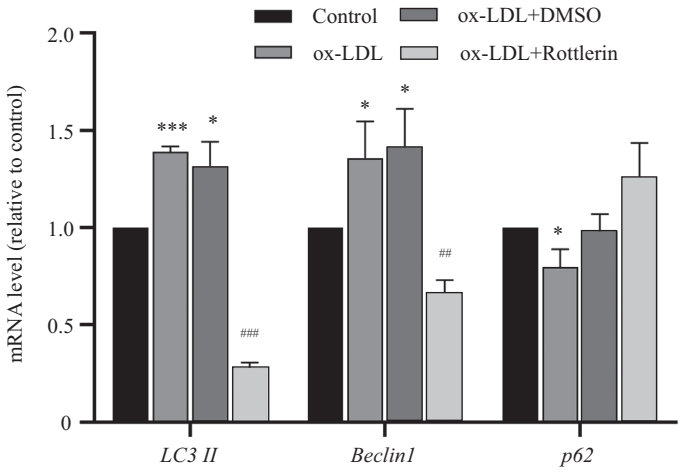


**P<0.01, ***P<0.001, 与对照组相比; ##P<0.01, ###P<0.001, 与ox-LDL+DMSO 组相比。

**P<0.01, ***P<0.001 compared with control group; ##P<0.01, ###P<0.001 compared with ox-LDL+DMSO group.

图9 Western blot检测Rottlerin预处理后HSC-T6细胞中LC3 II、Beclin1、p62、p-STAT3的表达

Fig.9 The expression levels of LC3 II, Beclin1, p62 and p-STAT3 in HSC-T6 cells after Rottlerin pretreatment were detected by Western blot



*P<0.05, ***P<0.001, 与对照组相比; ##P<0.01, ###P<0.001, 与ox-LDL+DMSO 组相比。

*P<0.05, ***P<0.001 compared with control group; ##P<0.01, ###P<0.001 compared with ox-LDL+DMSO group.

图10 qRT-PCR检测Rottlerin预处理后HSC-T6细胞中*LC3 II*、*Beclin1*、*p62* mRNA的含量

Fig.10 The mRNA levels of *LC3 II*, *Beclin1* and *p62* in HSC-T6 cells after Rottlerin pretreatment were detected by qRT-PCR

20 μg/mL ox-LDL刺激HSC-T6细胞后, 在细胞自噬增强的同时, Wnt5a、p-PKCδ和p-STAT3表达均增加, 而以siRNA敲低*Wnt5a*, 则使ox-LDL诱导的HSC-T6细胞的自噬减少, p-PKCδ、p-STAT3表达降低, 通过检测发现了ECM中Hyp、HA、LN三种成分含量也减少, 提示ox-LDL可通过增强Wnt5a/PKCδ信号通路促进HSC-T6细胞自噬(图14)。而使用Rottlerin特异性抑制PKCδ后, HSC-T6细胞的自噬减少, p-STAT3的表达降低, Hyp、HA、LN含量也降低, 表明抑制Wnt5a/PKCδ通路可下调ox-LDL诱导的HSC-T6细胞自噬水平, 这也从另一角度支持了我们的推论。

综上, 本研究发现ox-LDL可通过Wnt5a/PKCδ信号通路诱导HSC-T6细胞自噬, 证明了ox-LDL在肝星状细胞中诱导自噬的作用及具体机制, 为探讨非酒精性脂肪性肝炎的发病机理提供了参考。

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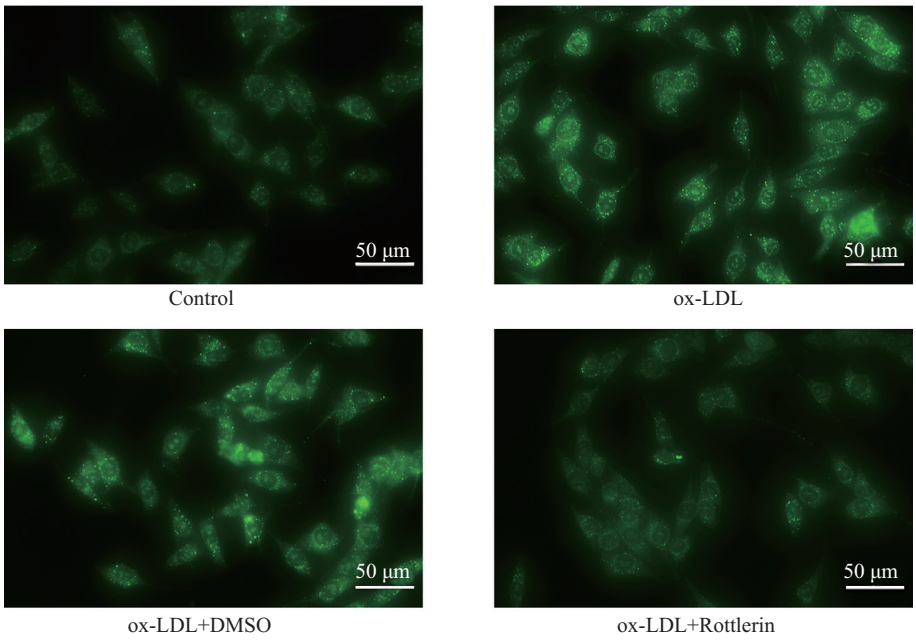
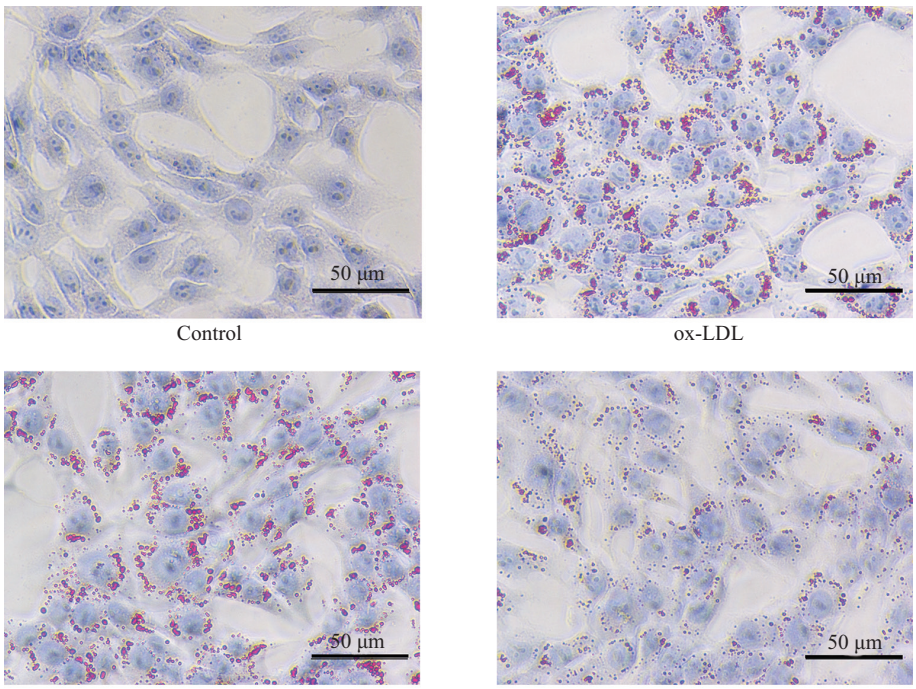


Control　ox-LDL

ox-LDL+DMSO　ox-LDL+Rottlerin

图11　免疫荧光法观察Rottlerin预处理后HSC-T6细胞中LC3 II点状聚集的表达

Fig.11　The expression of LC3 II in HSC-T6 cells after Rottlerin pretreatment was detected by immunofluorescence



Control　ox-LDL

ox-LDL+DMSO　ox-LDL+Rottlerin

图12　油红O染色观察Rottlerin预处理后HSC-T6细胞脂滴含量变化

Fig.12　The change of lipid droplet content in HSC-T6 cells after Rottlerin pretreatment was detected by oil red O staining

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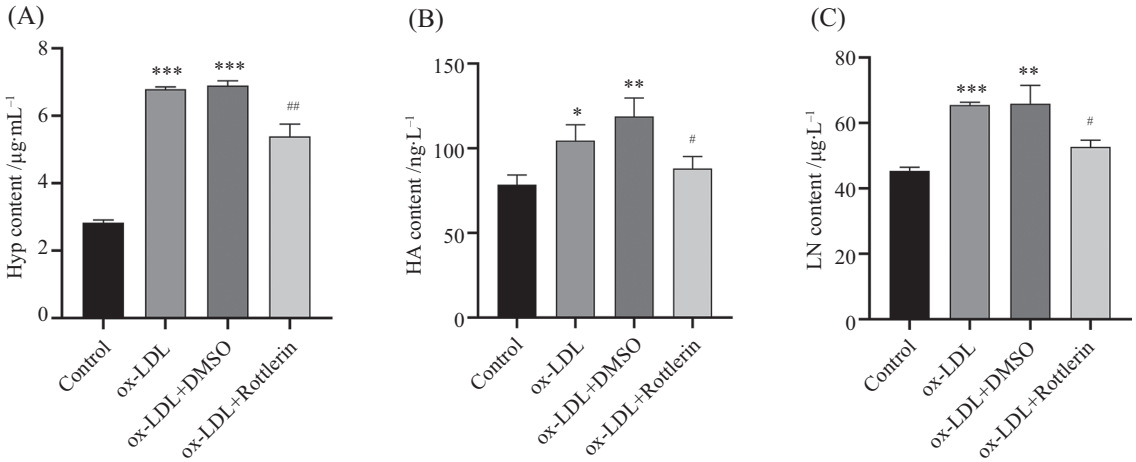
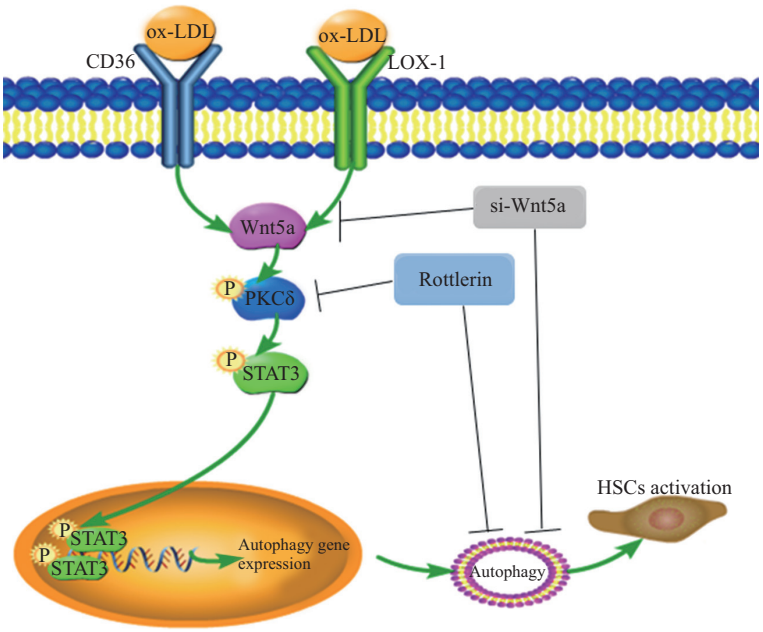


A: 各组细胞培养上清中Hyp含量; B: 各组细胞培养上清中HA含量; C: 各组细胞培养上清中LN含量。*P<0.05, **P<0.01, ***P<0.001, 与对照组相比; #P<0.05, ##P<0.01, 与ox-LDL+DMSO组相比。

A: the content of Hyp in cell culture supernatant in each group; B: the content of HA in cell culture supernatant in each group; C: the content of LN in cell culture supernatant in each group. *P<0.05, **P<0.01, ***P<0.001 compared with control group; #P<0.05, ##P<0.01 compared with ox-LDL+DMSO group.

图13 细胞培养上清中Hyp、HA、LN含量

Fig.13 The contents of Hyp, HA and LN in cell culture supernatant



P: 磷酸化; →: 分子表达或细胞增殖被促进; —|: 抑制。

P: phosphorylation; →: molecular expression or cell proliferation was promoted; —|: inhibition.

图14 ox-LDL通过Wnt5a/PKCδ信号通路诱导HSC-T6细胞自噬

Fig.14 ox-LDL induced the autophagy of HSC-T6 cells by the Wnt5a/PKCδ signaling pathway

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