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沉默 HDGF 基因抑制 HepG2 细胞的增殖和脂质代谢 *

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摘要 目的：探讨肝癌衍生生长因子（HDGF）对 HepG2 细胞增殖和脂质代谢的影响。**方法：**用脂质体包裹 siRNA 的方法沉默 HDGF 基因，用实时荧光定量 PCR 法和蛋白质免疫印迹法检测 HDGF 在 mRNA 和蛋白水平的变化，检测细胞总甘油三酯、胆固醇含量并用油红 O 染色，CCK-8 检测及琼脂糖凝胶克隆形成，实时荧光定量 PCR 法检测脂质代谢相关酶的 mRNA 表达。**结果：**将靶向 HDGF 小干扰(siRNA-HDGF)转染到 HepG2 细胞后，可明显抑制 HDGF 的 mRNA 表达(*P<0.001*)和蛋白表达。HDGF 蛋白抑制后，细胞增殖在 48 h(*P<0.01*)、72 h(*P<0.001*)和 96 h(*P<0.001*)均明显降低；细胞内总甘油三酯及胆固醇水平也明显降低(*P<0.05*，*P<0.01*)。此外，油红 O 染色显示细胞内脂滴有明显的减少。脂质代谢相关酶脂肪酸合成酶(FASN)、羟基 -3- 甲基戊二酰辅酶 A 还原酶(HMGCR)、硬脂酰辅酶 A 去饱和酶(SCD)及 ATP- 柠檬酸裂解酶(ACLY)的 mRNA 表达均明显降低(*P<0.001*，*P<0.001*，*P<0.001*，*P<0.01*)。**结论：**抑制 HDGF 的表达可明显降低 HepG2 细胞内脂质代谢水平并抑制其增殖。

关键词：肝癌衍生生长因子；脂质代谢；增殖；HepG2 细胞

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Down Regulation of HDGF Suppressed the Proliferation and Lipid Metabolism of HepG2 Cells*

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ABSTRACT Objective: To identify the effect of Hepatoma-derived growth factor (HDGF) on the proliferation and lipid metabolism of HepG2 cells. **Methods:** We silenced HDGF using siRNA. The efficiency of HDGF silencing in mRNA and protein levels were detected by real-time fluorescent quantitative PCR and Western blot, respectively. The total triglycerides and cholesterols levels were detected, and Oil red O staining, CCK-8 assay, soft agar colony formation assay were performed, real-time fluorescent quantitative PCR was used to analyze the mRNA expressions of metabolism related factors. **Results:** After the siRNA-HDGF (or siRNA-NC as control) was successfully transfected into HepG2 cells, the expression levels of HDGF mRNA (*P<0.001*) and protein were significantly downregulated. HepG2 cells transfected with siRNA-HDGF at 48 h(P<0.01), 72 h(*P<0.001*) and 96 h (*P<0.001*) were decreased; total triglycerides and cholesterols levels in intracellular were lower than those of the control group. Furthermore, Oil red O staining showed that the number of intracellular lipids droplets was obviously decreased in HepG2 cells transfected with siRNA-HDGF. The target lipogenic factors fatty acid synthase (FASN), 3-Hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR), stearoyl-CoA desaturase (SCD), and ATP citrate lyase (ACLY) were downregulated to different degrees (*P<0.001*, *P<0.001*, *P<0.001*, *P<0.01*). **Conclusions:** HDGF gene silencing dramatically inhibited the lipid metabolism level and the proliferation of HepG2 cells.

Key words: HDGF; Lipid Metabolism; Proliferation; HepG2 cells

Chinese Library Classification (CLC): R-33; R735.7 Document code: A

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前言

肝癌是最常见的恶性肿瘤之一，全球每年肝癌的发生率在所有恶性肿瘤中居第六位，而死亡率却高居第三位[1]。慢性 B 型肝炎病毒(HBV)或慢性 C 型肝炎病毒(HCV)感染、酒精性肝疾病、非酒精性脂肪肝疾病、自身免疫性肝炎、原发性胆汁性肝硬化和原发性硬化性胆管炎均可诱导肝癌发生[2]。

肝癌衍生生长因子（Hepatoma-derived growth factor, HDGF)是一个肝素结合酸性糖蛋白，基因定位于 1q21→23[3]，最初是从人肝癌细胞系 HuH-7 无血清培养的上清液中分离得到的一种生长因子，在多种细胞类型中发挥促进增殖的作用[4]。最近研究表明肝细胞肝癌[5]、非小细胞肺癌(NSCLC)[6]、胃肠道肿瘤[7]、食管癌[8]及胰腺癌[9]等多种类型的癌症都存在着 HDGF 的高表达。此外，研究表明 HDGF 的高表达与肝癌的不良预后密切相关[10]。

肿瘤代谢异常被认为是肿瘤的重要特征[10]。和其他恶性肿

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瘤一样,肝癌细胞脂质合成途径异常激活[11]。靶向脂质代谢通路及其相关调节酶已经成为非常具有前景的抗肿瘤策略。但HDGF在促进肿瘤细胞增殖的同时是否会对脂质代谢产生影响,目前尚不清楚。本研究通过RNA干扰技术,阻断肝癌细胞系HepG2中HDGF的表达,观察HDGF基因沉默后对HepG2细胞增殖和脂质代谢的影响,以期为未来开发靶向治疗药物提供实验依据。

1 材料与方法

1.1 实验材料

RNA抽提试剂盒购自美国Omega公司;反转录试剂盒购自日本Takara公司;qRT-PCR试剂盒购自日本Takara公司;qPCR引物由上海铂尚生物有限公司合成,具体见表1;siRNA-HDGF和siRNA-NC由上海吉玛制药技术有限公司合成,siRNA-NC的上游序列为5'-UUCUCCGAGCGUGUCACGUTT-3',下游序列为5'-ACGUGACACGUUCGGAGAATT-3';siRNA-HDGF的上游序列为5'- GAACGAGAAAGGAGCGUUGAA-3',下游序列为5'- UUCAACGCUCCUUUCUCGUUC-3'。脂质体转染试剂Lipofectamine 2000购自美国Invitrogen公司;细胞裂解液及蛋白浓度测定试剂盒均购自上海碧云天生物技术有限公司;硝酸纤维素膜(nitrocellulose filter membrane,NC)购自美国Millipore公司;HDGF鼠抗人抗体购自美国Abcam公司;鼠抗人β-actin抗体购自美国Cell signaling公司;荧光标记的鼠抗羊IgG(二抗)购自美国LI-COR公司。CCK8试剂盒购自日本同仁公司;油红O购自南京建成生物技术有限公司;甘油三酯测定试剂盒及胆固醇测定试剂盒购自北京普利莱基因技术有限公司。

1.2 细胞来源及其培养

人肝癌HepG2细胞购自中国科学院上海分院细胞库。HepG2细胞用含10%胎牛血清(Gibco)、青霉素(100 U/mL)和链霉素(100 μg/mL)的高糖DMEM培养基培养,待细胞密度达到80%~90%时进行传代,放在37℃,CO_2体积分数为5%的无菌恒温细胞培养箱中培养。

1.3 实时荧光定量PCR法

转染前一天,取处于对数生长期的细胞计数并铺12孔板,转染前观察细胞,细胞贴壁且生长状态良好,密度达60%~80%左右。按照脂质体转染试剂Lipofectamine 2000说明书转染细胞。48 h后收集转染了siRNA-HDGF的基因敲除组和转染了siRNA-NC的阴性对照组及空白对照组,行RNA抽提,500 ngRNA反转录成cDNA并做实时荧光定量PCR检测HDGF的mRNA表达水平,CB为内参,引物序列见表1。使用美国应用生物系统公司的QuantStudio™实时PCR系统,反应条件为95℃ 5 min;95℃ 10 s;60℃ 30 s,共40个循环,同时绘制溶解曲线。以$2^{-\Delta\Delta Ct}$值表示待测基因的mRNA水平。每个实验均有3次重复。

表1 qPCR引物序列

Table 1 Primer sequences of qPCR

Gene name	Forward primer	Reverse primer
CB	5'-AGATGTAGGCCGGGTGATCT-3'	5'-CCGCCCTGGATCATGAAGTC-3'
HDGF	5'-CTCTTCCCTTACGAGGAATCCA-3'	5'-CCTTGACAGTAGGGTTGTTCTC-3'
FASN	5'-AGTACACACCCAAGGCCAAG-3'	5'-GGATACTTTCCCGTCGCATA-3'
HMGCR	5'-GCCCTCAGTTCCAACTCACA-3'	5'-TTCAAGCTGACGTACCCCTG-3'
SCD	5'-CACTTGGGAGCCCTGTATGG-3'	5'-TGAGCTCCTGCTGTTATGCC-3'
ACLY	5'-CAGTCCCAAGTCCAAGATCCC-3'	5'-GTCTCGGGAGCAGACATAGT-3'

1.4 蛋白质印迹法

按照1.3的方法转染HepG2细胞并于48 h后收集细胞沉淀,用适量细胞裂解液裂解细胞(冰上30 min后,15000× g,4℃离心30 min)。用BCA试剂盒测量细胞蛋白浓度。取等量蛋白进行电泳,将电泳后的蛋白转移到NC膜上,并用5%脱脂奶粉室温封闭1 h,分别加入1:1000稀释的HDGF单克隆抗体(鼠抗人)和1:5000稀释的β-actin抗体(鼠抗人),4℃摇床孵育过夜;第二天加入1:10000稀释的荧光二抗(鼠抗羊),避光室温孵育1 h。在奥德赛红外激光成像系统上进行显影。

1.5 细胞生长实验

按照1.3的方法转染HepG2细胞,24 h后消化并计数,5×10^3个细胞/孔,重新铺板于96孔板中继续培养,分别于重新铺板后的48 h、72 h、96 h加入CCK-8工作液,避光反应1 h,在BIO-RAD酶联免疫检测仪上450 nm波长处检测各复孔的吸光度(D)值。每组细胞3个复孔,实验重复3次。

1.6 琼脂糖克隆形成实验

将4%的琼脂糖凝胶母液置于75℃水浴锅中水浴1 h,预热10%FBS的DMEM培养基。用预热的DMEM培养基稀释4%的母液至0.6%,24孔板中每孔加入250 μL 0.6%的下层胶,铺匀后室温静置直至凝胶凝固。消化并计数转染siRNA-HDGF,siRNA-NC 24 h后的HepG2细胞及对照HepG2细胞。将4%琼脂糖凝胶、DMEM培养基和细胞悬液混匀,使其终浓度为0.4%,作为上层胶加到24孔板中,100个细胞/孔,每组3个复孔。待上层胶凝固后,加入200 μL培养基,置于37℃,CO_2体积分数为5%的恒温细胞培养箱中培养14天。每3天更换一次培养基。14天后终止培养,4%的多聚甲醛固定细胞,PBS清洗细胞后,用结晶紫进行染色,再用去离子水洗脱,直至凝胶背景干净。用Image J软件计数克隆数量,实验均进行3次重复。

1.7 甘油三酯及胆固醇检测

按照 1.3 的方法转染 HepG2 细胞，48 h 后收集沉淀，用 PBS 清洗 3 遍之后裂解。总的蛋白浓度用 BCA 试剂盒进行测量。细胞内的甘油三酯及胆固醇水平按照说明书要求检测，并以总蛋白浓度为标准进行校正。最后在 BIO-RAD 酶联免疫检测仪上 550 nm 波长处检测各复孔的吸光度(D)值。每组细胞 3 个复孔，实验重复 3 次。

1.8 油红 O 染色

按照 1.3 的方法转染 HepG2 细胞并于 48 h 后进行染色。倒去孔中培养基，用 PBS 快速清洗细胞 3 遍。每孔加入 200 μL 4%多聚甲醛室温固定细胞 20 min，再次用 PBS 清洗细胞。用 200 μL 60%异丙醇快速轻柔地清洗每孔细胞 10 s 后，倒去异丙醇，每孔加入 200 μL油红工作液，室温下染色 30 min 后倒去染色液。用 60%异丙醇清洗细胞 2 次，PBS 清洗细胞 3 次后，在显微镜下观察细胞内红色的脂质，Zeiss Axioskop 显微镜下拍照并比较各组间油红 O 染色差异。每组实验 3 个复孔，重复 3遍。

1.9 统计学方法

实验结果用 Graphpad Prism 5 进行统计分析，组间两两比较采用 t 检验。*P<0.05* 表示差异有统计学意义。

2 结果

2.1 HDGF 基因沉默抑制 HepG2 细胞中 HDGF mRNA 及蛋白表达

在肝癌 HepG2 细胞中应用小干扰 RNA 技术进行瞬时转染，分别用实时荧光定量 PCR 法和蛋白印迹法检测 HepG2 细胞中 HDGF 的 mRNA 和蛋白表达。结果(图 1A)显示 HDGF 基因的敲减效率超过 90%(*P<0.001*)。转染 siRNA-HDGF 的实验组 HDGF 的蛋白表达水平明显低于转染 siRNA-NC 的实验组和空白对照组，结果如图 1B 所示。上述结果表明转染 siRNA-HDGF 可有效抑制 HDGF 的 mRNA 及蛋白表达。

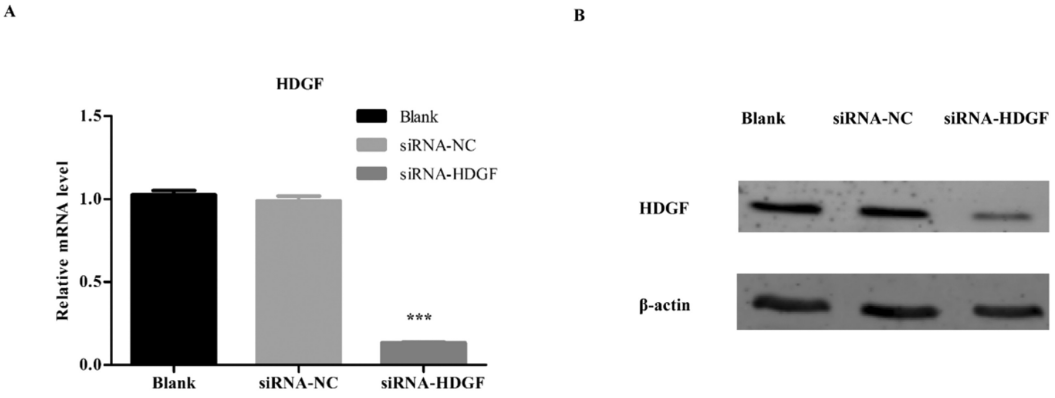

图 1 转染 siRNA-HDGF 抑制 HDGF mRNA 和蛋白的表达

Fig.1 SiRNA-HDGF decreased the mRNA and protein expression of HDGF

A. Total RNA was extracted at 48 h after transfection, and relative HDGF mRNA was analyzed by real-time fluorescent quantitative PCR. Expression of CB mRNA was used an internal control. Data represent the mean ± SD of three independent experiments, including a no-template negative control. ****P<0.001* vs. control or siRNA-NC. B. Cells were transfected with siRNA-HDGF or siRNA-NC for 48 h. Total proteins were extracted and detected by Western blotting using the indicated antibodies. β-actin as an internal control.

2.2 HDGF 基因沉默影响 HepG2 细胞的增殖

为了进一步验证 HDGF 敲除对 HepG2 细胞生长和增殖的影响，我们用细胞生长实验和琼脂糖克隆形成实验来进行验证。CCK-8 法检测结果(图 2A)显示，HDGF 对细胞生长的影响呈时间依赖性，siRNA-HDGF 转染组的 OD 值在 48、72、96 h 均明显低于 siRNA-NC 转染组及空白对照组(48 h：*P<0.01*；72 h：*P<0.001*；96 h：*P<0.001*)。此外，琼脂糖克隆形成实验结果(图 2B，C)显示 siRNA-HDGF 转染组细胞形成的克隆数目明显小于 siRNA-NC 转染组（*P<0.001*)。这些结果表明下调 HDGF 的表达之后，HepG2 细胞的增殖能力和克隆形成能力受到明显抑制。

2.3 HDGF 基因沉默影响 HepG2 细胞的脂代谢

细胞内甘油三酯检测结果(图 3A)显示 siRNA-HDGF 转染组甘油三酯水平较对照组降低了 20%~30%(*P<0.05*)，胆固醇水平也降低了 40%~50%(*P<0.01*)，结果如图 3B 所示。油红 O 染色结果(图 3C)显示细胞内脂滴数目 siRNA-NC 转染组明显高于 siRNA-HDGF 转染组。此外，如图 4 所示，siRNA-HDGF 转染组中脂肪酸合成酶（FASN)、羟基 -3- 甲基戊二酰辅酶 A 还原酶(HMGCR)、硬脂酰辅酶 A 去饱和酶(SCD)及 ATP- 柠檬酸裂解酶(ACLY)的 mRNA 水平均有显著的下降，且差异具有统计学意义(*P<0.001*，*P<0.01*)。上述结果表明，下调 HDGF 的表达可以明显抑制 HepG2 细胞的脂质代谢。

3 讨论

肝癌目前可行的治疗方法包括肝移植，手术切除，经皮穿刺消融，射频消融，经主动脉栓塞化疗和栓塞放疗[5]。由于缺乏早期诊断，肝癌患者治疗后预后仍不理想[12]。随着生物技术的发展，靶向基因治疗被认为是治疗一些恶性肿瘤的最佳方案。索拉非尼是一种口服的多重激酶抑制剂，是临床上唯一可以用于治疗进展期肝癌患者的靶向药物，可以在一定程度上延长患者的中位生存时间 3 个月 [12, 13]。因此，新的靶向分子的研究非常重要。

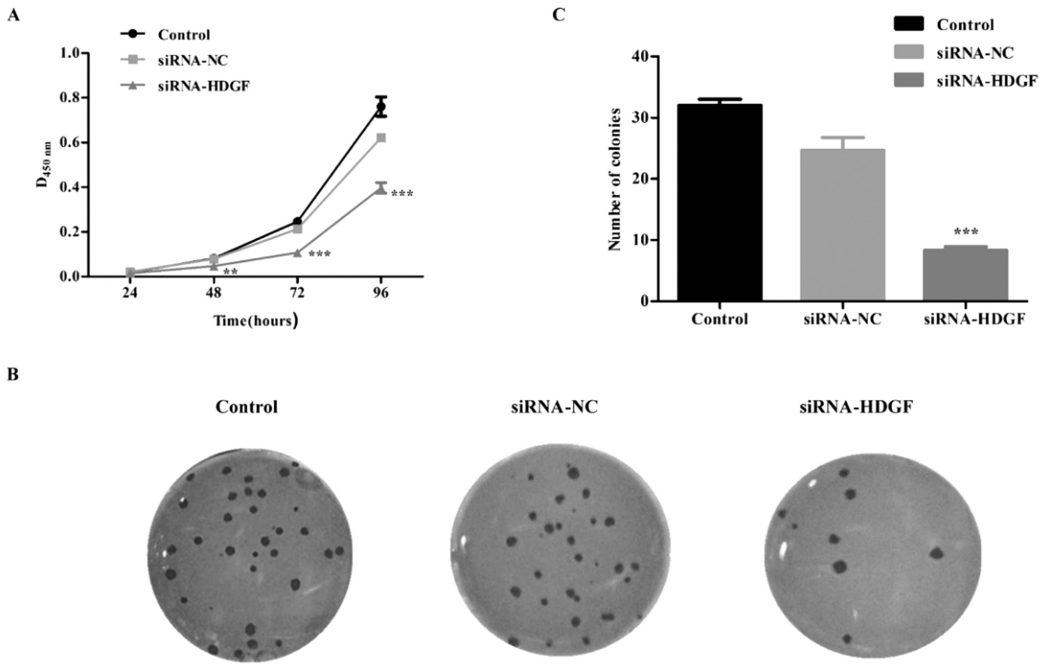

图 2 siRNA-HDGF 对 HepG2 增殖和克隆形成的影响

Fig.2 Effects of siRNA-HDGF on the proliferation and colony formation ability of HepG2 cells

A. Cells transfected with siRNA-HDGF or siRNA-NC for 24 h were countered and reseeded in 96-well plates with a density of 5 × 10^3 cells per well. Cell viability was determined using the CCK-8 for 48 h, 72 h and 96 h (***P<0.01*, ****P<0.001*, ****P<0.001*, respectively, vs. control or siRNA-NC). B.C. Cells transfected with siRNA-HDGF or siRNA-NC for 24 h were reseeded in 24-well plates (100 cells/well) and cultured for 14 days. Colonies were stained with 0.1% crystal violet and counted by Image J. ****P<0.001* vs. control or siRNA-NC.

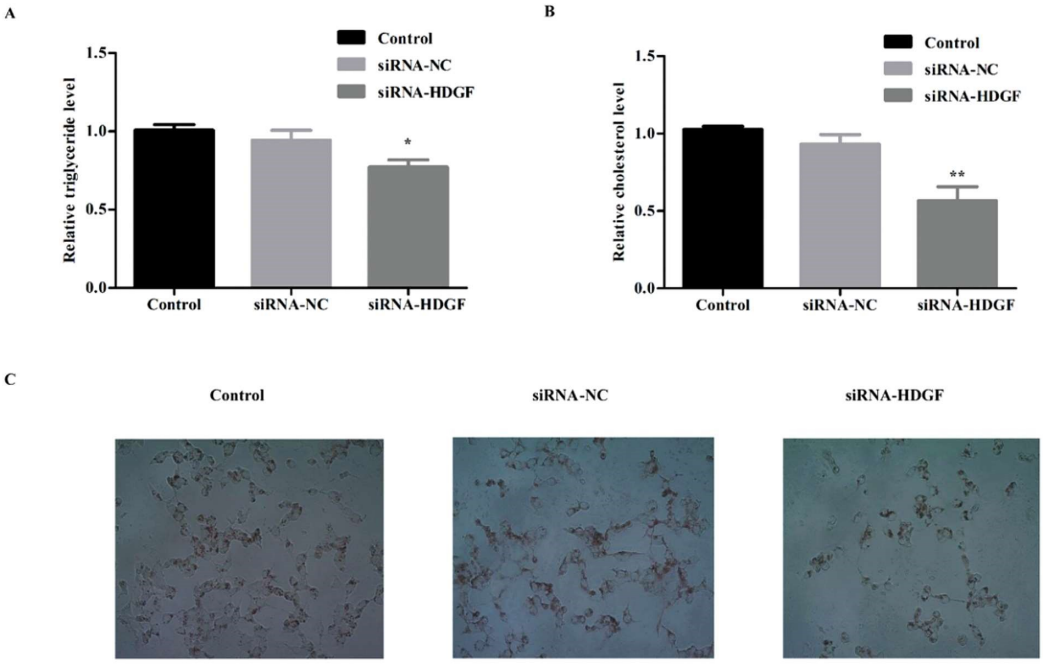

图 3 siRNA-HDGF 对 HepG2 脂质代谢的影响

Fig.3 Effects of siRNA-HDGF on the lipid metabolism of HepG2 cells

A.B. Cells were transfected with siRNA-HDGF or siRNA-NC for 48 h. Intracellular triglycerides and cholesterols were assayed and normalized by total protein levels and the levels in control cells. Data represent the mean ± SD of three independent experiments. (**P<0.05*, ***P<0.01*, respectively, vs. control or siRNA-NC). C. Cells were treated as in A.B. The pictures of representative Oil Red O staining images were recorded under a light microscope and presented (Scale bar, 10 μm).

HDGF 由一个保守的 N 端 PWWP 结构域和无序的 C 端序列构成，与高迁移率族蛋白 1(HMG-1)有同源性，可能参与转录调控过程[14]。近来研究表明 HDGF 在肿瘤转化、凋亡、血管生成、侵袭和迁移等多种癌细胞活动过程中发挥重要作用[14]。

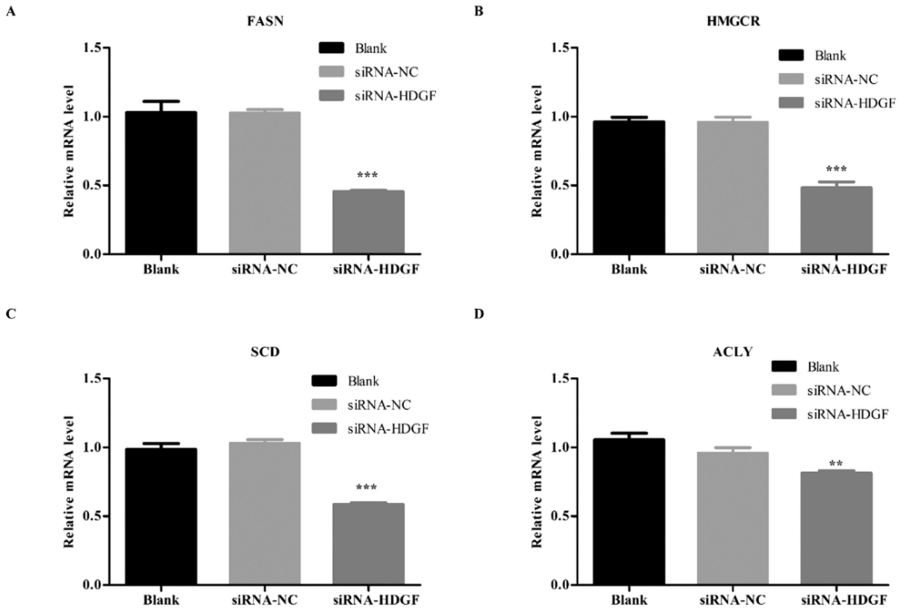

图 4 siRNA-HDGF 对 HepG2 细胞 FASN、HMGCR、SCD 和 ACLY 的 mRNA 表达的影响

Fig.4 Effects of siRNA-HDGF on the mRNA expressions of FASN, HMGCR, SCD and ACLY inHepG2 cells

A.B.C.D. Total RNA was extracted at 48 h after transfection, and expression levels of FASN, HMGCR, SCD and ACLY in mRNA were analyzed by real-time fluorescent quantitative PCR. Expression of CB mRNA was used an internal control. Data represent the mean ± SD of three independent experiments, including a no-template negative control. (****P<0.001*, ***P<0.001*, ****P<0.001*, ** *P<0.01*, respectively, vs. blank control or siRNA-NC).

HDGF 通过两种方式刺激细胞增殖：作为配体和膜受体结合及作为核蛋白和 DNA 结合[15]。研究显示下调 HDGF 的表达可以抑制胃癌、非小细胞肺癌和神经胶质瘤细胞的增殖[16-18]。本研究采用 RNA 干扰技术，最终证明下调 HDGF 后，HepG2 细胞的细胞增殖和克隆形成能力均受到抑制，与上述结果一致[12]。

增殖和代谢在细胞生长过程中是密不可分的[19]。肿瘤发生过程不仅包括失控的细胞增殖，还包括相应的能量代谢变化[20]。有氧糖酵解（瓦博格效应）及脂质从头合成高度激活是肿瘤细胞两个重要的代谢特征[19]。脂质代谢是三大物质代谢之一，具有能量储存和供应、维持细胞膜结构、构成血浆脂蛋白以及信号转导等重要生理作用。正常细胞由于生长速度缓慢，脂质从头合成受限。然而，肿瘤细胞为了满足快速增殖所需的额外脂质，脂质从头合成途径高度激活[21,22]。据报道，肿瘤细胞生长所需要的脂肪酸 90%以上来自于脂质从头合成[23]。脂质代谢包括 FASN、SCD 和 ACLY 等关键酶参与的一系列过程[24]。这些关键酶在肿瘤细胞脂质从头合成中也高度激活[25]。FASN 是肿瘤细胞中最常见的脂代谢关键酶，负责乙酰辅酶 A 和丙二酰辅酶 A 从头合成棕榈酸酯的催化步骤，在多种肿瘤中高表达[26]。SCD 催化形成短链脂肪酸，同样在多种肿瘤中高表达[25]。目前已经证实脂肪酸或胆固醇的异常增多与卵巢癌、前列腺癌、结直肠癌、神经胶质母细胞瘤等的发生和发展密切相关[27-30]。在本实验中，敲除 HDGF 基因之后，HepG2 细胞内甘油三酯和胆固醇水平及细胞内脂滴的数目明显低于对照组，表明 HDGF 在脂质合成过程中发挥重要作用。脂质代谢相关的酶，如 FASN、HMGCR、SCD、ACLY 在 mRNA 水平上有显著的降低。这些结果均表明，抑制 HDGF 表达后，可以通过直接或间接调节脂质代谢相关酶的表达，从而降低细胞内的脂质代谢，抑制细胞增殖。

综上所述，抑制 HDGF 表达可以明显降低细胞内脂质代谢水平，抑制细胞增殖。Ren 等发现在 NSCLC 异种移植模型中，抗 HDGF 抗体可以抑制肿瘤生长，提示 HDGF 可以作为肺癌治疗的靶因子[4]。深入研究 HDGF 对代谢的调控机制及其抗肿瘤效应将对肝癌患者的靶向治疗提供深远影响。

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