敲除C3G对H9C2心肌细胞增殖及凋亡的影响

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摘要 该研究构建了NT CRISPR/Cas9(阴性对照)及C3G CRISPR/Cas9质粒, 分别将其包装成重组慢病毒, 并感染H9C2心肌细胞, 以研究敲除C3G(Crk SH3域结合鸟嘌呤核苷酸交换因子)对H9C2心肌细胞增殖和凋亡的影响及其机制。将实验分为NT CRISPR/Cas9组、C3G CRISPR/Cas9组、NT CRISPR/Cas9低氧组和C3G CRISPR/Cas9低氧组。通过RT-PCR检测*C3G* mRNA的表达; Western blot检测相关蛋白表达; CCK-8法检测细胞增殖; 流式细胞术检测细胞凋亡。结果显示, C3G CRISPR/Cas9组和C3G CRISPR/Cas9低氧组的C3G mRNA和蛋白无表达; 分别与NT CRISPR/Cas9组和NT CRISPR/Cas9低氧组比较, C3G CRISPR/Cas9组和C3G CRISPR/Cas9低氧组的p-ERK1/2和Bcl-2蛋白以及细胞增殖水平均降低($P<0.05$), Bax蛋白及细胞凋亡水平均增加($P<0.05$); 与NT CRISPR/Cas9组相比, NT CRISPR/Cas9低氧组C3G mRNA和蛋白表达均降低($P<0.05$), p-ERK1/2和Bcl-2蛋白及细胞增殖水平均降低($P<0.05$), Bax蛋白及细胞凋亡水平均增加($P<0.05$); 与C3G CRISPR/Cas9组相比, C3G CRISPR/Cas9低氧组的p-ERK1/2和Bcl-2蛋白及细胞增殖水平均降低($P<0.05$), Bax蛋白及细胞凋亡水平均增加($P<0.05$)。以上结果表明, 敲除C3G能通过调控p-ERK1/2、Bcl-2及Bax抑制H9C2心肌细胞增殖并促进其凋亡。

关键词 C3G; 增殖; 凋亡; 心肌细胞

Effects of C3G Knockout on Proliferation and Apoptosis in H9C2 Cardiomyocytes

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Abstract The NT CRISPR/Cas9 (non-target), C3G CRISPR/Cas9 plasmids were constructed and packaged into lentiviruses respectively. H9C2 cardiomyocytes were infected with above lentiviruses respectively to investigate the effects of C3G [Crk SH3-domain-binding guanine nucleotide exchange factor] knockout on proliferation and apoptosis in H9C2 cardiomyocytes and their underlying mechanisms. The experiments were divided into NT CRISPR/Cas9, C3G CRISPR/Cas9, NT CRISPR/Cas9+Hypoxia and C3G CRISPR/Cas9+Hypoxia groups. *C3G* mRNA was detected by RT-PCR. C3G, p-ERK1/2, Bcl-2 and Bax proteins were tested by Western blot. Cell proliferative rate was examined by CCK-8. Apoptotic rate was determined by flow cytometry. The

接受日期: 2018-01-18 收稿日期: 2018-03-06
国家临床重点专科建设项目(批准号: 国卫办医函[2013]544号)、重庆市卫生局医学科学技术研究项目(批准号: 2009-2-290、04-2-154)和重庆市科委自然科学基金计划项目(批准号: CSTC200B527B76)资助的课题
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Accepted: January 18, 2018 Received: March 6, 2018
This work was supported by National Key Clinical Specialties Construction Program of China (Grant No.[2013]544), the Medical Science and Technology Research Fund of Health Bureau of Chongqing (Grant No.2009-2-290, 04-2-154) and the Natural Science Research Fund of Chongqing Science and Technology Commission (Grant No.CSTC200B527B76)
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网络出版时间: 2018-05-28 11:33:49 URL: http://kns.cnki.net/kcms/detail/31.2035.Q.20180528.1133.002.html

results showed that the expression of C3G mRNA and protein were absent in C3G CRISPR/Cas9 and C3G CRISPR/Cas9+Hypoxia groups. Compared with the NT CRISPR/Cas9 and NT CRISPR/Cas9+Hypoxia groups, the expression of p-ERK1/2 and Bcl-2 proteins and cell proliferative rate were decreased (P<0.05), while the expression of Bax protein and the apoptotic rate were increased in the C3G CRISPR/Cas9 and C3G CRISPR/Cas9+Hypoxia groups (P<0.05). Compared with the NT CRISPR/Cas9 group, the expression of C3G mRNA and protein (P<0.05), p-ERK1/2 and Bcl-2 proteins and the proliferative rate were decreased in the NT CRISPR/Cas9+Hypoxia group (P<0.05), while the expression of Bax protein and cell apoptotic rate were increased (P<0.05). Compared with the C3G CRISPR/Cas9 group, the expression of p-ERK1/2 and Bcl-2 proteins and the proliferative rate were decreased in the C3G CRISPR/Cas9+Hypoxia (P<0.05), while the expression of Bax protein and cell apoptotic rate were increased (P<0.05). The above results demonstrated that C3G knockout can inhibit the proliferation and promote the apoptosis of H9C2 cardiomyocytes through regulation of p-ERK1/2, Bcl-2 and Bax.

Keywords　C3G; proliferation; apoptosis; cardiomyocyte

鸟苷酸交换因子(guanine nucleotide exchange factors, GEFs)可通过激活小GTP酶来调节多种细胞功能。*C3G*(Crk SH3-domain-binding guanine nucleotide exchange factor)基因又被称为*RapGEF1*, 是鸟苷酸交换因子中的一员, 与整合素介导的信号和细胞骨架重构有关, 因此它可调控细胞的增殖、分化、转化及凋亡等过程[1]。我们前期的研究发现, 在大鼠梗死灶周围非梗死区心肌中C3G表达显著增加[2]。过表达的C3G可促进心肌细胞生存并抑制细胞凋亡[3], 而敲低C3G可抑制心肌细胞存活, 增加心肌细胞凋亡[4]。敲除C3G是否促进心肌细胞增殖、抑制心肌细胞凋亡, 目前尚不完全清楚。因此, 本实验利用CRISPR/Cas9(clustered regions of interspersed palindromic repeats/Cas9)系统构建敲除*C3G*重组慢病毒, 并用其感染H9C2心肌细胞, 以研究敲除*C3G*对H9C2心肌细胞增殖、凋亡的影响及其机制。

1　材料与方法

1.1　材料

大鼠H9C2心肌细胞株及HEK293T细胞株由本课题组保存; pLenti-Cas-Guide质粒及慢病毒包装试剂盒购自OriGene公司; 胎牛血清购自PAN公司; DMEM-F12购自HyClone公司; 无内毒素质粒小量抽提试剂盒购自Omega Bio-Tek公司; LB肉汤和琼脂购自Solarbio公司; RNA提取、逆转录和cDNA扩增试剂盒购自TaKaRa公司; 引物由Invitrogen公司合成; 蛋白提取试剂盒及CCK-8试剂盒购自江苏碧云天生物技术研究所; 兔抗大鼠GAPDH抗体购自杭州贤至生物科技有限公司; 兔抗大鼠p-ERK1/2抗体购自Cell Signaling公司; 兔抗大鼠Bcl-2、Bax抗体购自沈阳万类生物科技有限公司; 兔抗大鼠C3G抗体购自Santa Cruz公司。

1.2　方法

1.2.1　构建C3G CRISPR/Cas9质粒并包装成重组慢病毒　我们自主设计C3G CRISPR/Cas9作用的基因靶序列(表1), C3G CRISPR/Cas9和阴性对照(NT CRISPR/Cas9)质粒由重庆泽恒生物技术有限公司构建。用质粒转化感受态肠杆菌(热休克法), 将菌液及氯霉素(34 μg/mL)加入LB肉汤, 三者比例为100:1:1 000, 振摇4~6 h, 取振摇液送Invitrogen公司

表1　sgRNA作用于大鼠*C3G*靶点的核苷酸序列

Table 1　sgRNA targeted rat *C3G* nucleotide sequences

sgRNA作用于大鼠*C3G*的靶点 sgRNA targeted rat *C3G*	核苷酸序列 Nucleotide sequence
3 210-3 229	5′-GAC AAA GCC ATC TGT GCC CC-3′
	5′-GGG GCA CAG ATG GCT TTG TC-3′
3 262-3 281	5′-CTC CTT CAC CAT GAA GCT GA-3′
	5′-TCA GCT TCA TGG TGA AGG AG-3′
3 308-3 327	5′-AAG AGA ACA CCA TCC AAG AA-3′
	5′-TTC TTG GAT GGT GTT CTC TT-3′

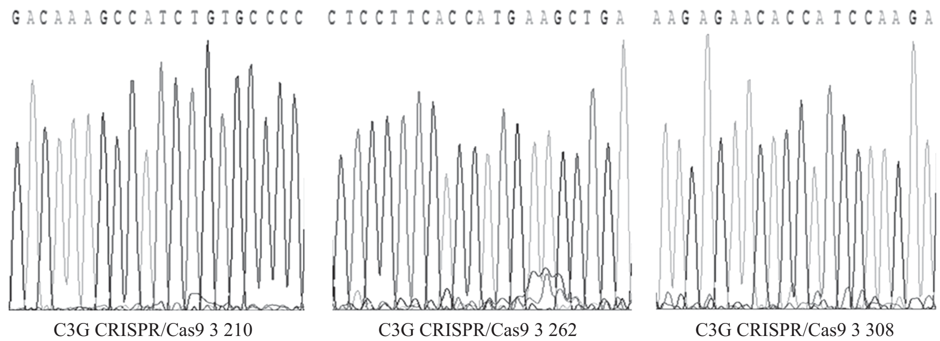


图1 重组质粒的测序

Fig.1 Sequences of recombinant plasmid

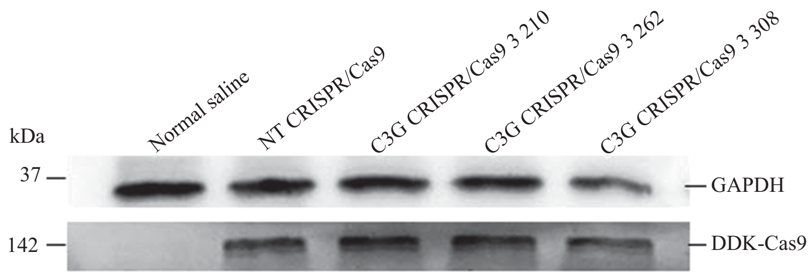


图2 包装慢病毒的验证

Fig.2 Verification of plasmid packaging into lentivirus

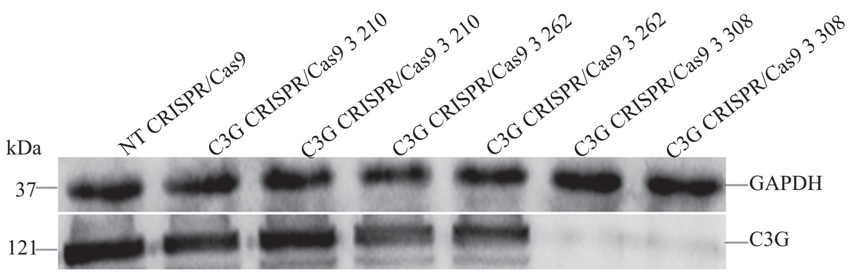


图3 C3G CRISPR/Cas9重组慢病毒筛选

Fig.3 Selecting of C3G CRISPR/Cas9 recombinant lentivirus

进行基因测序验证(图1)。抽提质粒, 并用HEK293T细胞包装重组慢病毒。

1.2.2 慢病毒包装鉴定　用含10%胎牛血清的DMEM F12培养基培养H9C2细胞。将细胞接种于6孔板中, 每孔含1×10^5个细胞, 37 ℃、5% CO_2孵箱中培养, 待细胞密度达70%时更换新鲜培养基, 分别加入生理盐水、NT CRISPR/Cas9及3种不同C3G CRISPR/Cas9重组慢病毒, 培养24 h后更换新鲜培养基, 待细胞密度达85%时提取蛋白进行Western blot检测。结果表明, 包装成功(图2)。

1.2.3 实验方案　筛选有效敲除C3G的重组慢病毒(图3)。将相同数量的H9C2细胞接种于2个孔板中, 每个孔板一半的孔感染NT CRISPR/Cas9慢病毒, 另一半的孔感染C3G CRISPR/Cas9慢病毒, 培养24 h后更换新鲜培养基, 继续培养45 h。从上述2个孔板中随机取出一份将培养基换成PBS, 置于37 ℃、1% O_2、94% N_2、5% CO_2的低氧孵箱中培养18 h(共培养87 h); 另一份继续培养18 h(共培养87 h)。此时就将细胞分成了NT CRISPR/Cas9组、C3G CRISPR/Cas9组、NT CRISPR/Cas9低氧组和C3G CRISPR/Cas9低氧组。收集细胞并进行RT-PCR、Western blot、CCK-8及流式细胞术检测。

1.2.4 RT-PCR检测*C3G* mRNA表达水平　分别提取细胞总RNA, 并行反转录、cDNA扩增及电泳。*GAPDH*上游引物为: 5′-AGA ACA TCA TCC CTG CAT CC-3′, 下游引物为: 5′-GGA TGG AAT TGT GAG

GGA GA-3′; *C3G*上游引物为: 5′-CAG GAT GGA CAG CAG ACA GA-3′, 下游引物为: 5′-CTG CGG TGT CTG GTA GAA CA-3′。反转录反应条件为37 ℃ 15 min, 85 ℃ 5 s, 4 ℃ 5 min; PCR扩增反应条件为94 ℃预变性3 min; 94 ℃变性30 s, 58 ℃退火30 s, 72 ℃延伸30 s, 共30个循环。进行琼脂糖凝胶电泳, 成像及Quantity One分析。

1.2.5　Western blot检测C3G、p-ERK1/2、Bcl-2及Bax蛋白　分别提取各组细胞蛋白, 用BCA法测定蛋白浓度。取30 μg蛋白上样于10% SDS聚丙烯酰胺凝胶中, 电泳分离后电转至PVDF膜上, 加4%牛奶37 ℃封闭2 h, 然后4 ℃孵育一抗过夜。第2天用TBST液洗膜后加二抗孵育1 h, TBST洗膜3次, 后用ECL化学发光试剂显影, 并用Fusion软件进行定量分析。

1.2.6　CCK-8检测细胞增殖　按CCK-8试剂盒说明书操作。细胞培养至24 h和87 h时分别加入CCK-8液10 μL/孔, 继续孵育2~4 h后, 用酶标仪检测各孔450 nm吸光度值。细胞增殖率(%)=(72 h吸光度值–24 h吸光度值)/24 h吸光度值×100%。

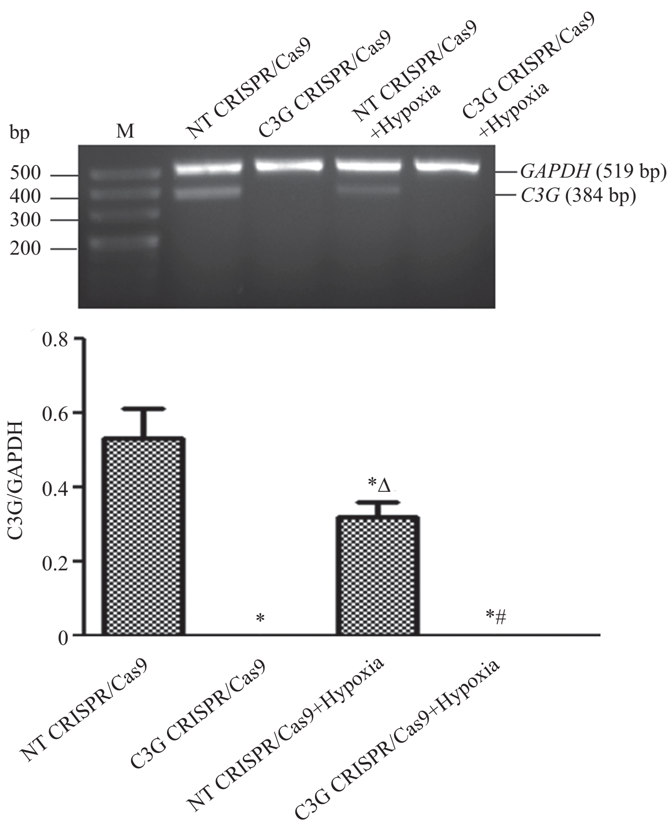
1.2.7　流式细胞术检测细胞凋亡　按细胞凋亡试剂盒说明书操作。细胞悬浮后加入Annexin V-FITC/PI双标记, 流式细胞术检测细胞凋亡。Annexin V-FITC(+)/PI(–)表示早期凋亡细胞, Annexin V-FITC(+)/PI(+)表示晚期凋亡细胞, Annexin V-FITC(–)/PI(–)表示活细胞, Annexin V-FITC(–)/PI(+)表示坏死细胞。用早期凋亡细胞计算细胞凋亡率。细胞凋亡率(%)=凋亡细胞数/总细胞数×100%。

1.3　统计学分析　用SSPS 22.0软件进行统计学分析。计量数据用“均数±标准差($\bar{x}$±*s*)”表示, 两组间比较用Tukey检验, 多组间比较用单因素方差分析(One-Way ANOVA)。*P*<0.05表示差异有统计学意义。

2　结果

2.1　H9C2细胞C3G mRNA和蛋白的表达

C3G CRISPR/Cas9组和C3G CRISPR/Cas9低氧组无C3G mRNA和蛋白表达。与NT CRISPR/Cas9组相比较, NT CRISPR/Cas9低氧组的C3G mRNA和蛋白水平均降低(*P*<0.05)(图4和图5)。



*P<0.05, 与NT CRISPR/Cas9组比较; $^{\Delta}P$<0.05, 与C3G NT CRISPR/Cas9组比较; $^{\#}P$<0.05, 与NT CRISPR/Cas9+Hypoxia组比较。

*P<0.05 *vs* NT CRISPR/Cas9 group; $^{\Delta}P$<0.05 *vs* C3G NT CRISPR/Cas9 group; $^{\#}P$<0.05 *vs* NT CRISPR/Cas9+Hypoxia group.

图4　H9C2心肌细胞*C3G* mRNA的表达

Fig.4　Expression of *C3G* mRNA in H9C2 cardiomyocytes

2.2 p-ERK1/2、Bcl-2及Bax蛋白的表达

Western blot结果显示, 与NT CRISPR/Cas9组和NT CRISPR/Cas9低氧组比较, C3G CRISPR/Cas9组及C3G CRISPR/Cas9低氧组p-ERK1/2、Bcl-2蛋白水平降低(P<0.05), Bax蛋白水平增加(P<0.05); 与NT CRISPR/Cas9组和C3G CRISPR/Cas9组比较, NT CRISPR/Cas9低氧组及C3G CRISPR/Cas9低氧组p-ERK1/2、Bcl-2蛋白水平降低(P<0.05), Bax蛋白水平增加(P<0.05)(图5)。

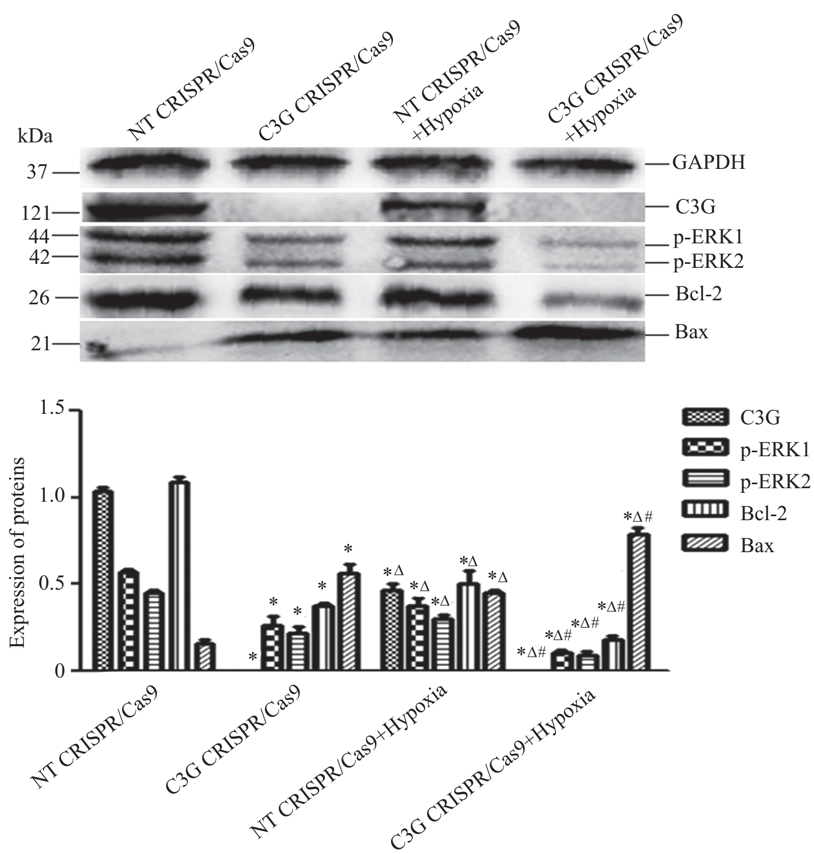
2.3 细胞增殖及凋亡

细胞CCK-8及流式结果显示, 与NT CRISPR/Cas9组和NT CRISPR/Cas9低氧组比较, C3G CRISPR/Cas9组和C3G CRISPR/Cas9低氧组增殖率降低(P<0.05), 凋亡率增加(P<0.05)。与NT CRISPR/Cas9组和C3G CRISPR/Cas9组比较, NT CRISPR/Cas9低氧组和C3G CRISPR/Cas9低氧组增殖率降低(P<0.05), 凋亡率增加(P<0.05)(图6和图7)。

3 讨论

心肌缺血、心肌梗死、扩张性心肌病及心力衰竭等心血管疾病的发生发展与心肌细胞的程序性死亡(即凋亡)的增加密切相关[5-9]。抑制凋亡可促进心肌细胞存活, 因此, 对心肌细胞凋亡及存活分子机制的研究, 有望为心血管疾病的治疗提供新的靶点。

研究发现, 整合素信号通路的β1亚基、焦点黏附激酶(focal dhesion kinase, FAK)和整合素连接激酶(integrin linked kinase, ILK)等组分可促进心肌细胞存活, 抑制其凋亡[10-12]。鸟苷酸交换因子C3G(Crk SH3-domain-binding guanine nucleotide exchange factor)是整合素通路的组分之一, 它是一个含有多结构域的蛋白, 其中央区富含脯氨酸, 能与含SH3结构域的Crk、SFKs(Src family kinases)和c-Abl等分子相互作用[13-14]。研究发现, C3G是哺乳动物胚胎发育必不可少的, 敲除C3G可导致小鼠在胚胎早期死亡[15], 且C3G亚效等位基因可使小鼠胚胎的血管发

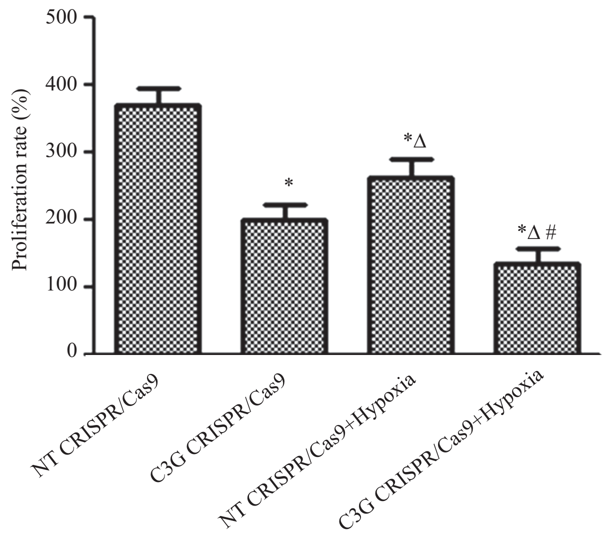


*P<0.05, 与NT CRISPR/Cas9组比较; ΔP<0.05, 与C3G NT CRISPR/Cas9组比较; #P<0.05, 与NT CRISPR/Cas9+Hypoxia组比较。

*P<0.05 *vs* NT CRISPR/Cas9 group; ΔP<0.05 *vs* C3G NT CRISPR/Cas9 group; #P<0.05 *vs* NT CRISPR/Cas9+Hypoxia group.

图5 H9C2心肌细胞C3G、p-ERK1/2、Bcl-2和Bax蛋白的表达

Fig.5 Expressions of C3G, p-ERK1/2, Bcl-2 and Bax proteins in cardiomyocytes

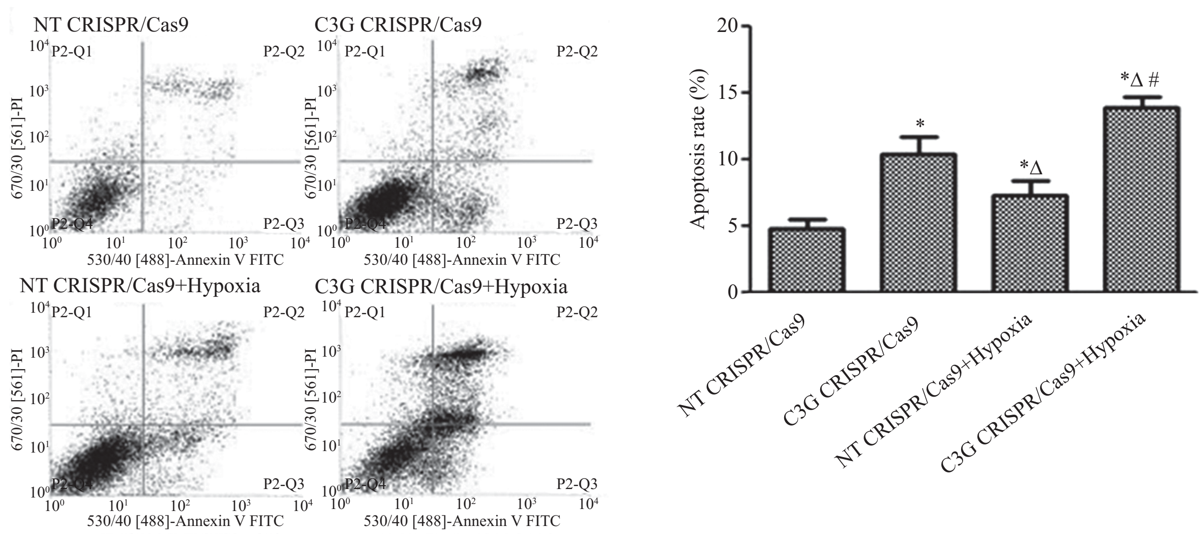


*P<0.05, 与NT CRISPR/Cas9组比较; $^{\Delta}P$<0.05, 与C3G NT CRISPR/Cas9组比较; $^{\#}P$<0.05, 与NT CRISPR/Cas9+Hypoxia组比较。

*P<0.05 *vs* NT CRISPR/Cas9 group; $^{\Delta}P$<0.05 *vs* C3G NT CRISPR/Cas9 group; $^{\#}P$<0.05 *vs* NT CRISPR/Cas9+Hypoxia group.

图6 H9C2细胞的增殖率

Fig.6 Proliferation rate of H9C2 cardiomyocytes



*P<0.05, 与NT CRISPR/Cas9组比较; $^{\Delta}P$<0.05, 与C3G NT CRISPR/Cas9组比较; $^{\#}P$<0.05, 与NT CRISPR/Cas9+Hypoxia组比较。

*P<0.05 *vs* NT CRISPR/Cas9 group; $^{\Delta}P$<0.05 *vs* C3G NT CRISPR/Cas9 group; $^{\#}P$<0.05 *vs* NT CRISPR/Cas9+Hypoxia group.

图7 H9C2心肌细胞的凋亡

Fig.7 Apoptosis rate of H9C2 cardiomyocytes

育异常[16]。C3G可参与梗死后心脏重构、纤维化、缺血性心肌病及心力衰竭等的发生发展[2], 亦可调控心肌细胞存活及凋亡, 但其潜在的机制目前仍然不完全清楚。*ERK1/2*是MAPK(mitogen activated protein kinase)家族的主要成员之一, 可促进心肌细胞存活, 抑制其凋亡, 是促存活基因[17-19]。*Bcl-2*和*Bax*是*Bcl-2*多基因家族的两个主要成员[20], 可调节线粒体死亡通路, 其中*Bcl-2*是抗凋亡基因, 能促进细胞存活; 而*Bax*是促凋亡基因, 能促进细胞凋亡[5]。我们前期的实验发现, 通过调控p-ERK1/2、Bcl-2及Bax的表达, 过表达的C3G可促进心肌细胞的存活, 抑制其凋亡[3]; 而敲低C3G可抑制心肌细胞存活, 促进其凋亡[4]。但敲除C3G对心肌细胞存活及凋亡的影响目前仍不完全清楚。

CRISPR/Cas9是一种新的基因敲除技术, 它主要通过单向导RNA(single guide RNA, sgRNA)与

靶序列碱基互补配对, 从而引导Cas9蛋白结合到靶序列处, 切断DNA双链, 然后利用细胞基因组的自主修复能力而发生错位修复, 从而完成对基因的编辑[21-22]。因此, 本研究用CRISPR/Cas9系统构建敲除C3G重组慢病毒, 感染H9C2心肌细胞, 并予以低氧处理。结果发现, 敲除C3G后p-ERK1/2及Bcl-2表达降低, Bax表达增加, 并且低氧处理后可使敲除C3G的心肌细胞的p-ERK1/2、Bcl-2表达进一步降低, 而其Bax表达进一步增加, 表明通过调节p-ERK1/2、Bcl-2及Bax的表达, 敲除C3G可抑制心肌细胞存活, 促进其凋亡。C3G调控心肌细胞存活及凋亡的作用及对心血管系统的影响有待进一步的转基因动物等实验证实。

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