

# 利用CRISPR/Cas9构建*G6PD*基因c.1388G>A突变的HEK293/K562细胞株

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**摘要** 该文旨在利用CRISPR/Cas9构建*G6PD*基因c.1388G>A突变的HEK293/K562细胞株, 为*G6PD*缺陷症及其修复研究提供细胞模型。针对*G6PD*基因c.1388G>A位点设计单链向导RNA(sgRNA)与突变同源臂, 利用CRISPR/Cas9联合同源重组修复(HDR)构建*G6PD*基因c.1388G>A突变的HEK293细胞株与红白血病K562细胞株; qRT-PCR、Western blot检测*G6PD*基因表达; CCK8检测细胞增殖; *G6PD*/*6PGD*比值法检测*G6PD*酶活性; 结晶紫染色与Annexin V-APC/7-AAD验证突变细胞株对氧化活性药物维生素K3与伯安喹的耐受情况。结果显示, 成功构建CRISPR/Cas9双质粒载体系统; 筛选单克隆细胞经测序鉴定显示, 成功构建*G6PD*基因c.1388G>A突变的HEK293与K562细胞株, 且无脱靶; 进一步发现, c.1388G>A突变不影响HEK293与K562细胞*G6PD*基因mRNA转录与蛋白翻译, 但细胞增殖减慢, *G6PD*酶活性下降; 突变HEK293细胞对维生素K3与伯安喹的耐受力减弱, 突变K562细胞对伯安喹耐受能力减弱。该研究成功构建*G6PD*基因c.1388G>A突变的HEK293与K562细胞株, 为*G6PD*缺陷症及后期基因修复研究提供细胞模型。

**关键词** *G6PD*缺陷症; c.1388G>A; CRISPR/Cas9

## Construction of HEK293/K562 Cell Strain with *G6PD* Gene c.1388G>A Mutation Using CRISPR/Cas9

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**Abstract** The aim of this study was to construct HEK293/K562 cell strain with *G6PD* gene c.1388G>A mutation using CRISPR/Cas9, and provide cell models for *G6PD* deficiency and its gene therapy research. Two sgRNAs (single-strand guide RNAs) and a mutant homology arm were designed for *G6PD* gene c.1388G>A site, the HEK293/K562 cell strain with *G6PD* gene c.1388G>A mutation were constructed through CRISPR/Cas9 combined with HDR (homologous recombination repairing) pathway. qRT-PCR and Western blot were used to detect *G6PD* gene expression. CCK8 was used to detect cell proliferation. *G6PD*/*6PGD* ratio method was used to detect *G6PD* activity. Crystal violet staining and Annexin V-APC/7-AAD were used to verify the tolerance of the mutant cell strain to the oxidative

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重庆市社会民生科技创新专项(批准号: Cstc2016shmszx130032)资助的课题

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active drug vitamin K3 and primaquine. The results showed that the CRISPR/Cas9 double plasmid vector system was successfully constructed and HEK293/K562 cell strain with *G6PD* gene c.1388G>A mutation were successfully constructed after monoclonal cell isolation and DNA sequencing, with no off-target effect. It was further found that the *G6PD* gene c.1388G>A mutation did not affect the mRNA transcription and protein translation of *G6PD* gene in HEK293 and K562 cells while the cell proliferation were inhibited and G6PD activity decreased in mutant cells. The tolerance to the oxidative active drug primaquine were weaker in both mutant HEK293 and K562 cells, and mutant HEK293 cell was less resistant to vitamin K3. This study successfully constructed HEK293 and K562 cell strain with *G6PD* gene c.1388G>A mutation, and provided cell models for G6PD deficiency and gene repair research.

**Keywords** G6PD deficiency; c.1388G>A; CRISPR/Cas9

葡萄糖-6-磷酸脱氢酶(G6PD)缺陷症是最常见的遗传性酶缺陷症,全球约4亿人患病,我国为高发区之一,主要分布于南方<sup>[1]</sup>。其发病机制是由于X染色体上*G6PD*基因突变导致G6PD酶活性降低,患者红细胞无法抵抗氧化损伤引起溶血,导致贫血、黄疸等临床症状<sup>[2]</sup>。目前全球已报道217种*G6PD*基因突变位点,其中c.1388G>A位点为最常见突变位点之一<sup>[3]</sup>。WHO根据患者G6PD酶活性残留水平将G6PD缺乏症分为I~IV类,c.1388G>A突变所致G6PD缺陷症为II类,导致严重酶活性缺乏<sup>[4]</sup>。然而目前对于G6PD缺乏相关研究主要以基因干扰与敲除为主,尚无与G6PD缺陷症发病机制一致的细胞模型<sup>[5-6]</sup>。近年来迅速发展的成簇规律间隔短回文重复序列及相关蛋白9(CRISPR/Cas9)基因编辑技术可以实现基因敲除、插入及定点突变,为细胞及动物模型的构建提供了简单易行、快捷高效的方法<sup>[7]</sup>。

本研究选取基因编辑常用模式细胞系HEK392与红白血病细胞系K562,利用CRISPR/Cas9基因编辑技术结合同源重组修复,构建*G6PD*基因c.1388G>A突变的HEK293与K562细胞株,为G6PD缺陷症及基因修复研究提供细胞模型。

## 1 材料与方法

### 1.1 实验材料

HEK293细胞、K562细胞株、pX458质粒、SEH空载质粒由本实验室保存;氨苄西林抗生素购自北京索莱宝科技有限公司; *Bbs* I、*Pme* I、*Hind* III核酸内切酶, T4 DNA连接酶, EnGen<sup>®</sup> Mutation Detection Kit 购自NEB公司; DH5 $\alpha$ 感受态细胞、血液/组织/细胞基因组DNA提取试剂盒、琼脂糖凝胶回收试剂盒均购自天根生化科技(北京)有限公司; DMEM与RPMI-

1640基础培养基购自Gibco公司; 细胞培养皿及孔板购自Corning公司; 0.25%胰酶消化液、100 $\times$ 青霉素/链霉素购自Genview公司; FBS购自Excell Bio公司; Neofect转染试剂购自零客创智(北京)生物科技有限公司; 电转仪、4 mm电转杯、定量PCR分析仪、琼脂糖凝胶电泳仪、SDS-PAGE凝胶电泳仪购自Bio-Rad公司; 定性PCR仪购自ABI公司; 流式细胞分析仪、流式细胞分选仪购自BD公司; RNA iso plus、Ex Taq购自TaKaRa公司; RIPA裂解液、糖原、BCA蛋白定量试剂盒购自上海碧云天生物技术有限公司; CCK8检测试剂、维生素K3、伯安喹购自MCE公司; Annexin V/7-AAD凋亡检测试剂盒购自江苏凯基生物技术股份有限公司; 兔抗人G6PD单克隆抗体购自CST公司; 辣根过氧化物酶标记山羊抗兔IgG抗体购自Proteintech公司; 兔抗人GAPDH抗体购自武汉三鹰生物技术有限公司; 26616蛋白marker购自Thermo公司; G6PD酶活性比值法检测试剂盒购自广州米基医疗器械有限公司; 引物合成及测序服务购自华大基因。

### 1.2 实验方法

1.2.1 sgRNA设计与pX458-sgRNA载体构建 利用在线设计工具(<https://zlab.bio/guide-design-resources>)针对*G6PD*基因12号外显子c.1388G>A位点设计两对sgRNA, 根据*Bbs* I核酸内切酶切口, 在sgRNA正向链5'端加入CACC序列, 反向链5'端加入AAAC序列, 送公司合成。将合成后Oligo稀释为1  $\mu\text{g}/\mu\text{L}$ , 取正反向各10  $\mu\text{L}$ , 95  $^{\circ}\text{C}$ 变性5 min, 自然降至室温形成双链。同时取pX458质粒用*Bbs* I内切酶37  $^{\circ}\text{C}$ 酶切30 min, 并乙醇沉淀。按载体与插入片段摩尔比1:10进行连接, T4 DNA连接酶16  $^{\circ}\text{C}$ 连接过夜。连接产物用DH5 $\alpha$ 感受态转化并接种于Amp抗性LB固体

培养基, 37 °C 孵育14 h。取菌落PCR阳性菌株摇菌扩增, 提取质粒测序验证pX458-sgRNA载体构建成功。

**1.2.2 SEH-Donor同源臂载体构建** 针对c.1388G>A位点左右各300 bp附近设计PCR扩增引物<sup>[8]</sup>, 并在正向引物5'端加入*Pme* I酶切位点, 反向引物5'端加入*Hind* III酶切位点, 引物序列如下。F: GTT GTT TAA ACC CGA GGA GTC GGA GCT GGA C; R: AAG AAG CTT CAG CTG AGG TCA ATGGTC, 产物长度706 bp。提取一例*G6PD*基因c.1388G>A突变患者外周血样本DNA, 以之为模板, 扩增同源臂目的片段, 扩增程序(1)95 °C预变性5 min; (2)95 °C变性30 s; 68 °C→58 °C梯度降温退火30 s, 每循环降温1 °C; 72 °C延伸30 s; (3)95 °C变性30 s, 58 °C退火30 s, 72 °C延伸30 s, 15个循环; (4)72 °C延伸7 min, 4 °C保存产物。乙醇沉淀PCR产物, 分别用*Pme* I和*Hind* III酶切SEH空载质粒与PCR产物目的片段, 酶切后胶回收, 并按载体与插入片段摩尔比1:10进行连接, T4 DNA连接酶16 °C连接过夜。连接产物用DH5 $\alpha$ 感受态转化接种于Amp抗性LB固体培养基, 37 °C 孵育14 h。取菌落PCR阳性菌株摇菌扩增, 提取质粒测序验证SEH-Donor载体是否构建成功。

**1.2.3 细胞培养与质粒转染** HEK293细胞培养于含10% FBS的DMEM培养基, 转染前5 h将细胞接种于10 cm培养皿中, 密度为70%左右。质粒转染使用无血清DMEM培养基, 按Neofect转染试剂说明书操作, pX458-sgRNA质粒与SEH-Donor质粒各10  $\mu$ g, 5 h后换液。K562细胞培养于含10% FBS的RPMI-1640培养基, 电转时使用 $3 \times 10^6$ 细胞重悬于300  $\mu$ L RPMI-1640培养基中, 加入pX458-sgRNA质粒与SEH-Donor质粒各8  $\mu$ g, 混匀后室温静置15 min, 电转条件: 指数衰减波、350 V、500  $\mu$ F<sup>[9]</sup>。静置20 min, 细胞接种至含20% FBS的RPMI-1640培养基, 加入80  $\mu$ mol/L ZnSO<sub>4</sub>, 24 h换液为10% RPMI-1640继续培养。

**1.2.4 流式分选GFP<sup>+</sup>细胞及单克隆细胞筛选** HEK293细胞用0.25%胰酶消化为单细胞, K562细胞直接离心取细胞沉淀, 适量PBS重悬后, BD流式细胞分选仪分选GFP<sup>+</sup>细胞, 并接种单个细胞于96孔板中, 剩余细胞接种于6孔板, 20% FBS培养基培养, 根据细胞生长状况换液。

**1.2.5 编辑效率检测及单克隆细胞基因鉴定** 设计T7E I检测引物(5'→3')F: TGA CCT ACG GCA ACA

GAT ACA A; R: GCC CTC ATA CTG GAA ACC C。分选后48 h收6孔板细胞沉淀提DNA, 扩增目的片段并胶回收, 按照EnGen<sup>®</sup> Mutation Detection Kit说明书操作, ImageJ软件分析灰度值计算编辑效率。单克隆细胞12孔板长满时收取部分细胞提取DNA, PCR扩增目的片段后将产物测序, 鉴定基因编辑是否成功。

**1.2.6 qRT-PCR与Western blot** HEK293细胞收取细胞沉淀, 加入1 mL Trizol裂解细胞; K562细胞吸取适量细胞悬液, 离心后弃培养基, 加入1 mL Trizol吹打细胞沉淀至完全裂解。苯酚/氯仿萃取法提取总RNA, 逆转录为cDNA, qRT-PCR检测*G6PD*基因相对表达量。HEK293与K562各收取 $2 \times 10^6$ 细胞, 加入200  $\mu$ L RIPA裂解液, 超声裂解5 min, 12 000 r/min离心25 min, 吸上清加入1/5体积的6 $\times$ 上样缓冲液, 100 °C变性10 min, 10% PAGE凝胶电泳检测*G6PD*蛋白表达量。

**1.2.7 G6PD酶活性检测、CCK8检测细胞增殖** 使用广州米基改良G6PD测定试剂盒检测G6PD酶活性, 各收取适量HEK293细胞与K562细胞沉淀, 200  $\mu$ L蒸馏水裂解细胞, 按照说明书配置工作液, 每管加入50  $\mu$ L工作液与50  $\mu$ L细胞裂解液, 37 °C水浴20 min, 2 mL终止液终止反应, 650 nm处检测吸光度值。CCK8检测细胞增殖: 细胞饥饿24 h后, 铺96孔板, 每孔100  $\mu$ L培养基, 接种2 000个细胞。每天加入CCK8检测试剂10  $\mu$ L/孔, 孵育2.5 h, 450 nm检测吸光度值, 连续检测5天观察细胞增殖情况。

**1.2.8 结晶紫染色与细胞凋亡检测** HEK293细胞吸去培养基, 4%多聚甲醛固定30 min, 加入结晶紫染液室温染色1 h, 自来水缓慢洗净染液。K562细胞凋亡检测使用凯基生物凋亡检测试剂盒, 细胞2 000 r/min离心5 min, 弃上清, 加入200  $\mu$ L结合缓冲液重悬细胞, 分别加入Annexin V-APC/7-AAD抗体5  $\mu$ L, 室温避光孵育20 min, 加入200  $\mu$ L结合缓冲液, BD流式细胞仪检测细胞凋亡。

**1.2.9 脱靶位点预测与检测** 使用在线软件(<http://www.rgenome.net/cas-offinder/>), 输入设计的sgRNA序列, 选取前三位概率最高的脱靶位点, 分别找到对应基因组序列并设计引物。提取目的细胞DNA, PCR扩增目的片段, 并测序验证脱靶效应。

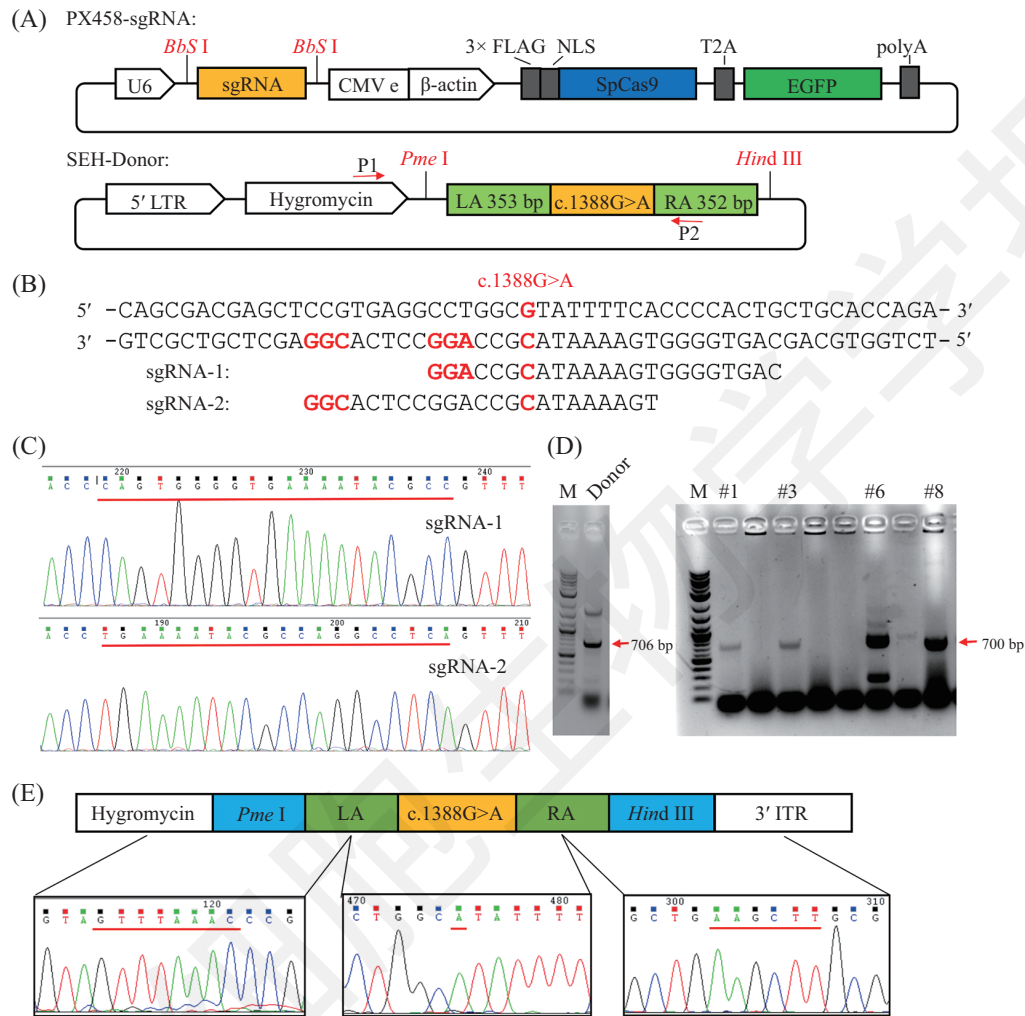
**1.2.10 统计学分析** 使用Graphpad Prism 5.01进行统计学分析, 两组间比较采用两独立样本t检验, 以P<0.05为差异有统计学意义。

## 2 实验结果

### 2.1 pX458-sgRNA与SEH-Donor载体构建

pX458-sgRNA与SEH-Donor载体结构示意图

1A所示。共设计两对sgRNA(图1B), 合成序列见表1。挑取克隆后, Sanger测序结果显示pX458-sgRNA载体构建成功(图1C)。取*G6PD*基因c.1388G>A突变



A: pX458-sgRNA与SEH-Donor载体示意图; B: 针对c.1388G>A位点设计sgRNA序列, 红色碱基代表PAM序列与目的位点; C: pX458-sgRNA载体测序结果; D: 病人基因组DNA扩增同源臂目的片段(左); SEH-Donor菌落PCR鉴定结果(右); E: SEH-Donor测序鉴定结果(LA:左臂; RA:右臂)。  
 A: schematic diagram of pX458-sgRNA and SEH-Donor plasmid; B: sgRNA sequences designed for *G6PD* gene c.1388G>A mutation site, and red bases represent the PAM sequence and the target site; C: DNA sequencing results of pX458-sgRNA plasmid; D: targeted homologous arm fragment amplified from Patient genomic DNA (left); Colony PCR identification results of SEH-Donor plasmid (right); E: DNA sequencing results of SEH-Donor plasmid (LA: left arm; RA: right arm).

图1 pX458-sgRNA与SEH-Donor载体构建

Fig.1 Construction of pX458-sgRNA and SEH-Donor vector

表1 *G6PD*基因c.1388G>A突变位点设计sgRNA序列

Table 1 sgRNA sequences designed for *G6PD* gene c.1388G>A mutation site

单链向导RNA sgRNA	序列(5'→3') Sequences (5'→3')
sgRNA-1	Forward: cac cCA GTG GGG TGA AAA TAC GCC Reverse: aaa cGG CGT ATT TTC ACC CCA CTG
sgRNA-2	Forward: cac cTG AAA ATA CGC CAG GCC TCA Reverse: aaa cTG AGG CCT GGC GTA TTT TCA

患者基因组DNA扩增同源臂706 bp(图1D左), 克隆后取PCR鉴定阳性(引物见图1A, 目的片段700 bp)菌落(图1D右), 测序结果显示, “#1”号SEH-Donor载体构建成功(图1E)。

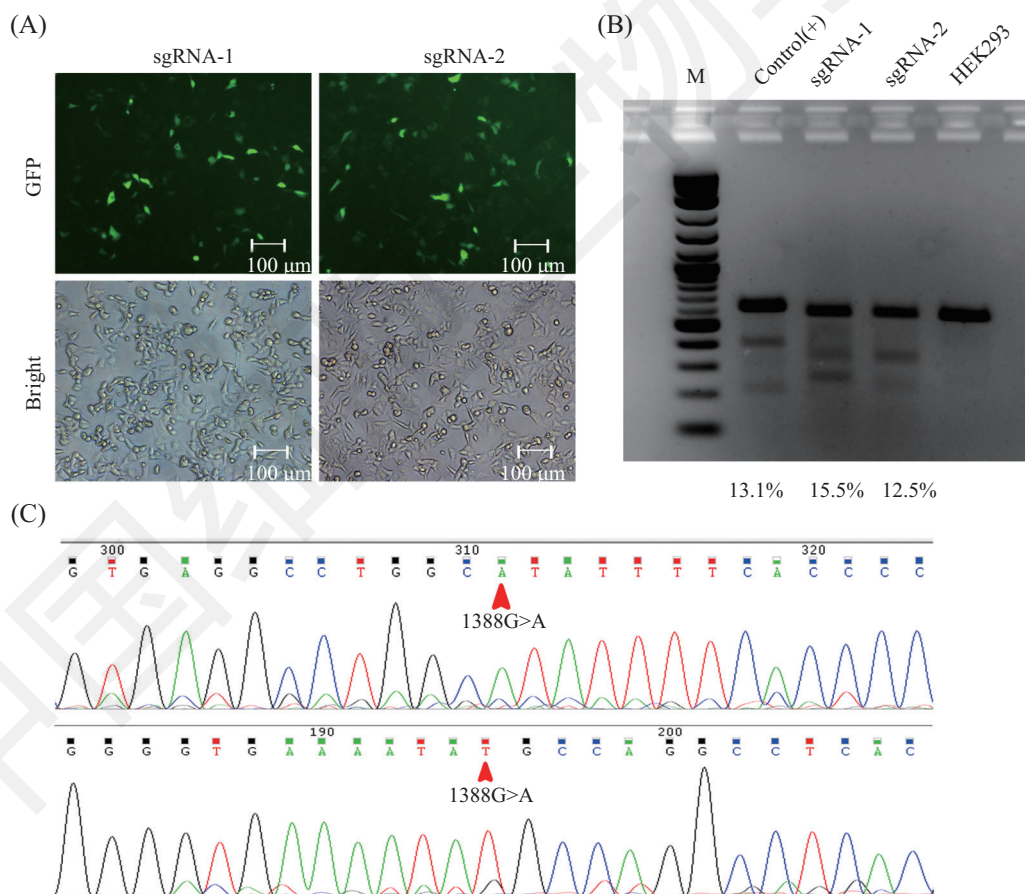
## 2.2 HEK293细胞转染、*T7E I*鉴定编辑效率及单克隆细胞筛选

将pX458-sgRNA与SEH-Donor载体共转染HEK293细胞, 48 h后显微镜下发现两对sgRNA均转染成功(图2A)。流式分选GFP<sup>+</sup>细胞, *T7E I*酶切法检测两对sgRNA编辑效率, 灰度值分析发现, 两对sgRNA均成功编辑了目的位点, 且编辑效率均较高, 分别为15.5%和12.5%(图2B)。筛选培养单克隆细胞, 提取DNA, PCR扩增目的片段。测序鉴定结果显示, 在转染sgRNA-2的细胞中成功筛选到一株*G6PD*基因c.1388G>A突变的HEK293细胞(图2C), 扩增培养后, 成

功构建*G6PD*基因c.1388G>A突变的HEK293细胞株。

## 2.3 *G6PD*基因c.1388G>A突变HEK293细胞功能检测

收取正常与*G6PD*基因c.1388G>A突变HEK293细胞沉淀, qRT-PCR检测结果显示, 突变HEK293细胞*G6PD*基因mRNA水平与正常细胞无差异(图3A); 提取突变与正常细胞总蛋白, Western blot检测发现蛋白翻译无明显差异(图3B); 比值法检测*G6PD*酶活性发现c.1388G>A突变HEK293细胞*G6PD*酶活性明显下降约50%(图3C); CCK8检测结果显示突变细胞增殖明显减慢(图3D)。维生素K3与伯安喹已被列为*G6PD*缺陷症患者禁用药物<sup>[10]</sup>, 将两种药物分别处理细胞12 h后, 结晶紫染色发现, 突变HEK293细胞在维生素K3( $\geq 15 \mu\text{mol/L}$ )和伯安喹( $\geq 200 \mu\text{mol/L}$ )处理下活细胞明显减少, 且呈剂量依赖性(图3E和图

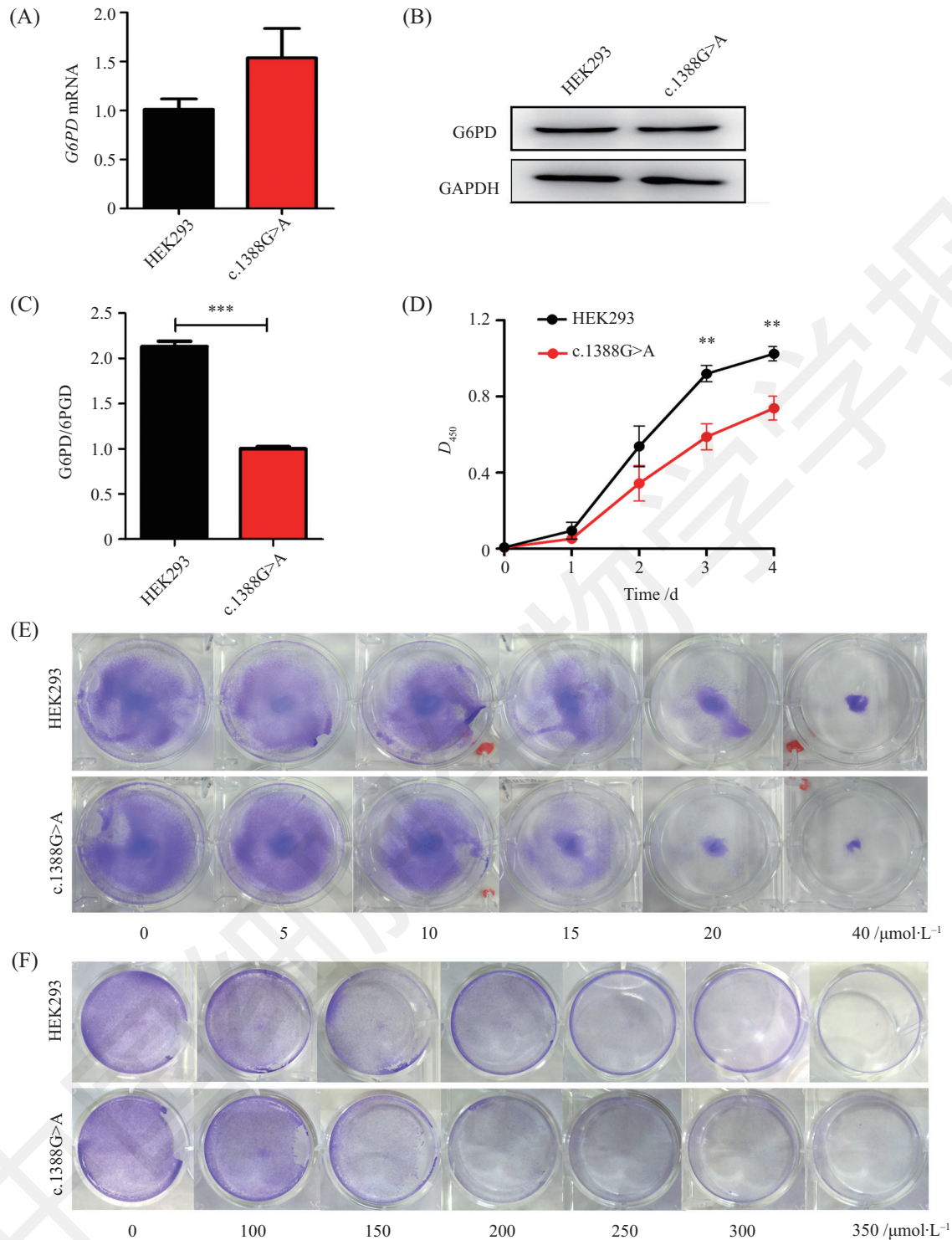


A: pX458-sgRNA与SEH-Donor载体共转染HEK293细胞48 h镜下GFP荧光; B: *T7E I*酶切法检测两对sgRNA编辑效率; C: 单克隆细胞正反向测序结果显示突变HEK293细胞株构建成功。

A: GFP fluorescence after 48h co-transfection of pX458-sgRNA and SEH-Donor plasmid into HEK293 cell; B: detection of genome editing efficiency of two different sgRNAs by *T7E I* assay; C: forward and reverse sequencing of monoclonal cells showed that the mutant HEK293 cell strain was successfully constructed.

图2 HEK293细胞转染、*T7E I*鉴定编辑效率及单克隆细胞筛选

Fig.2 HEK293 cell transfection, *T7E I* detection of editing efficiency and monoclonal cell screening

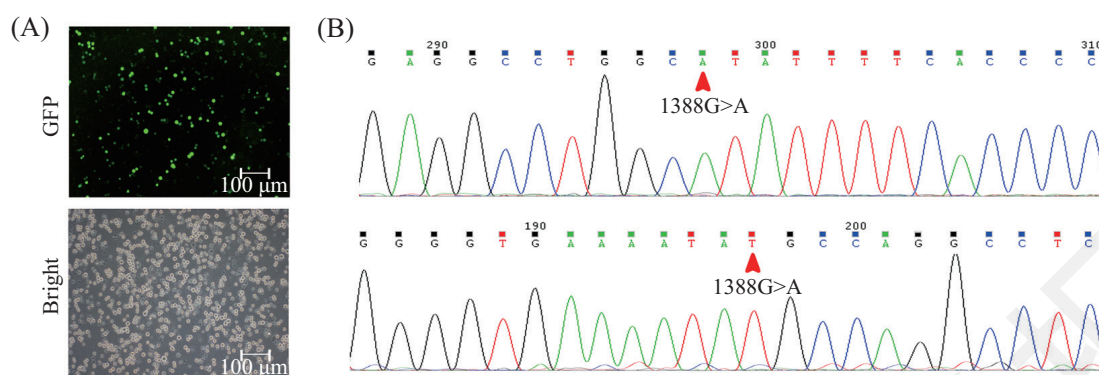


A: qRT-PCR检测突变细胞株mRNA水平; B: Western blot检测突变细胞株G6PD蛋白; C: G6PD/6PGD比值法检测突变细胞株G6PD酶活性,  $***P < 0.001$ ; D: CCK8检测细胞增殖,  $**P < 0.01$ , 与HEK293组比较; E: 不同浓度维生素K3处理细胞12 h后结晶紫染色结果; F: 不同浓度伯安喹处理细胞12 h后结晶紫染色结果。

A: qRT-PCR detection of mRNA levels in mutant cell; B: Western blot detection of G6PD protein in mutant cell; C: detection of G6PD enzyme activity in mutant cell by G6PD/6PGD ratio method,  $***P < 0.001$ ; D: cell proliferation detection by CCK8 assay,  $**P < 0.01$  vs HEK293 group; E: crystal violet staining results after treatment of cells with different concentrations of vitamin K3 for 12 hours; F: Crystal violet staining results after treatment of cells with different concentrations of primaquine for 12 hours.

图3 *G6PD*基因c.1388G>A突变HEK293细胞功能检测

Fig.3 Functional detection of *G6PD* gene c.1388G>A mutant HEK293 cells



A: sgRNA-2与SEH-Donor质粒共转染K562细胞48 h后镜下GFP荧光; B: 单克隆细胞正反向测序结果显示突变K562细胞株构建成功。

A: GFP fluorescence after 48 h co-transfection of sgRNA-2 and SEH-Donor plasmid into K562 cells; B: forward and reverse sequencing of monoclonal cells showed that the mutant K562 cell strain was successfully constructed.

图4 K562细胞电转及单克隆细胞筛选

Fig.4 K562 cell electroporation and monoclonal cell selection

3F)。上述结果提示, *G6PD*基因c.1388G>A突变不影响HEK293细胞*G6PD*基因表达, 但导致酶活性显著下降, 细胞增殖减慢, 对伯安喹与维生素K3耐受能力变差。

#### 2.4 K562细胞电转及单克隆细胞筛选

由于*G6PD*缺陷症主要影响患者红系细胞, 因此我们选取红白血病细胞系K562, 将sgRNA-2与同源臂质粒共同电转K562细胞, 48 h后显微镜下观察GFP荧光蛋白表达, 转染效率约为60%(图4A)。筛选单克隆细胞并测序验证, 结果显示成功构建*G6PD*基因c.1388G>A突变的K562细胞株(图4B)。

#### 2.5 *G6PD*基因c.1388G>A突变K562细胞功能检测

经qRT-PCR与Western blot检测, 结果显示, c.1388G>A位点突变对K562细胞*G6PD*基因mRNA转录与蛋白翻译均无明显影响(图5A和图5B)。*G6PD*基因c.1388G>A突变K562细胞*G6PD*酶活性较正常细胞下降(图5C), 细胞增殖稍受抑制, 但差异无统计学意义(图5D)。经伯安喹处理12 h后, 药物浓度为300 μmol/L时, *G6PD*基因c.1388G>A突变K562细胞晚期凋亡明显多于正常细胞(图5E), 提示突变细胞对伯安喹耐受能力稍减弱。

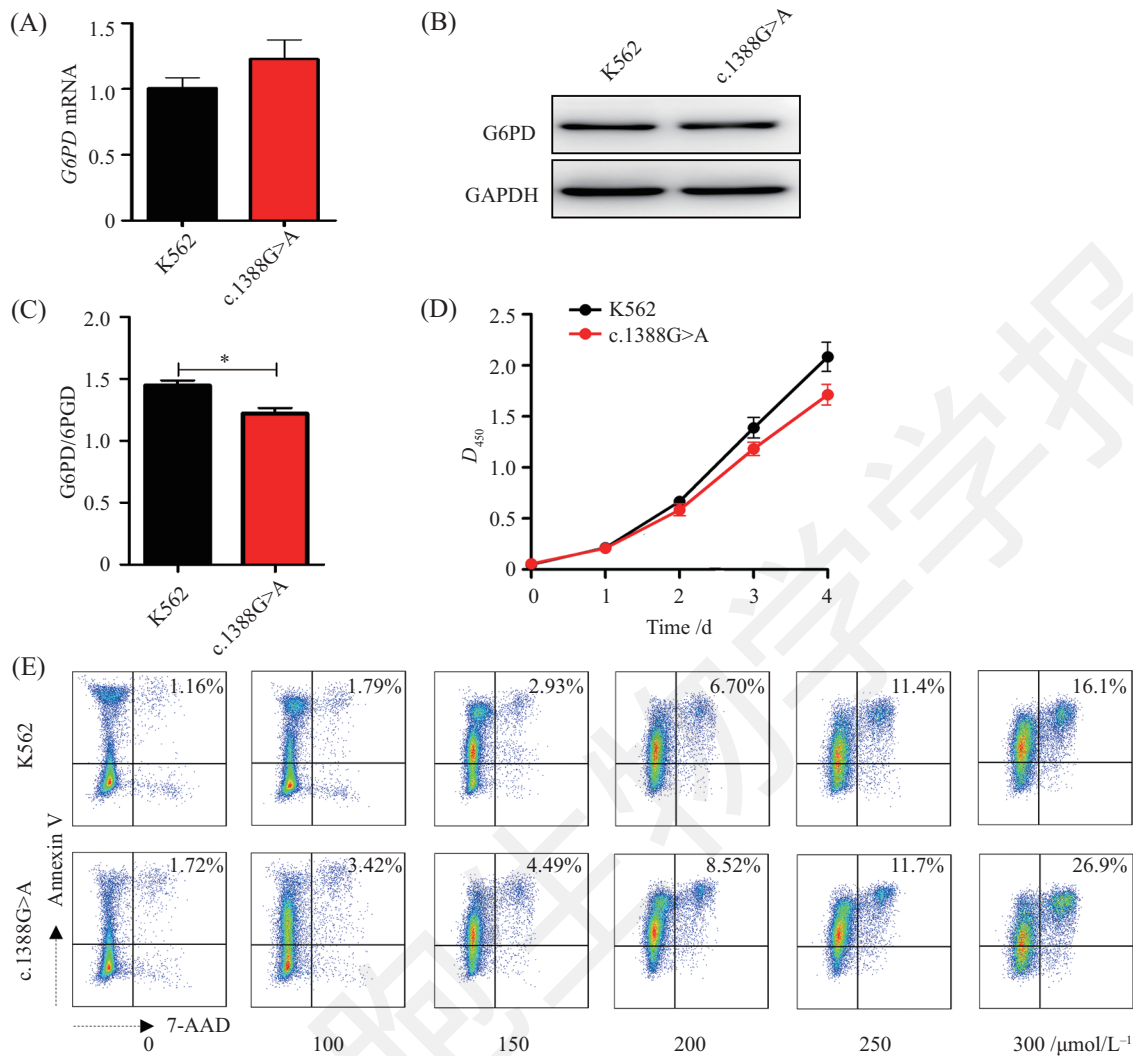
#### 2.6 脱靶效率检测

根据sgRNA-2序列找到前三位潜在脱靶序列(表2), 分别设计检测引物(表3)。提取*G6PD*基因c.1388G>A突变HEK293与K562细胞株DNA, 扩增目的片段, 测序结果显示两株细胞均无脱靶(图6A和图6B)。

### 3 讨论

糖代谢中, 磷酸戊糖途径主要生成产物为还原型烟酰胺腺嘌呤二核苷酸磷酸(NADPH)与核糖-5-磷酸, 由其生成的NADPH是成熟红细胞生成还原型谷胱甘肽的唯一来源, 核糖-5-磷酸为DNA合成的原料<sup>[11]</sup>。*G6PD*是磷酸戊糖途径的关键限速酶, *G6PD*缺陷患儿由于红细胞NADPH生成障碍, 无法维持细胞内还原型谷胱甘肽水平而使细胞无法抵抗氧化损伤引发溶血等一系列症状<sup>[11]</sup>。*G6PD*缺陷症发病机制为位于X染色体上的*G6PD*基因点突变所致, 我国目前已经报道30多种致病突变, 其中c.1388G>A、c.1376G>T、c.95A>G三种突变占70%~80%, 重庆地区*G6PD*缺陷症c.1388G>A突变发生率占突变首位, 为28.7%<sup>[12]</sup>。*G6PD*缺陷症是新生儿黄疸的主要病因, 也是*G6PD*缺陷症高发地区新生儿胆红素脑病的主要病因。此外, 食物、药物或感染也会诱发*G6PD*缺陷症患者发生急性发作性溶血, 临床表现为全身不适、乏力、发热、寒战、血红蛋白尿、黄疸、贫血, 重者可在短期内出现溶血危象、急性肾功能衰竭, 危及生命<sup>[10]</sup>。包括维生素K3与伯安喹在内的多种药物会导致*G6PD*缺陷症患者发生溶血<sup>[13-14]</sup>。*G6PD*缺陷症目前无根治手段, 只在发病时给予输血等对症支持治疗。

基因编辑技术为*G6PD*缺陷症的治疗提供了新思路, CRISPR/Cas9系统自1987年发现至今<sup>[15]</sup>, 已广泛应用于多种疾病的基因治疗研究, 如β-地中海贫血<sup>[16]</sup>、镰刀性贫血<sup>[17]</sup>、杜氏肌营养不良<sup>[18]</sup>、脆性



A: qRT-PCR检测突变细胞株mRNA水平; B: Western blot检测突变细胞株G6PD蛋白; C: G6PD/6PGD比值法检测突变细胞株G6PD酶活性, \* $P < 0.05$ ; D: CCK8检测细胞增殖; E: 不同浓度伯安喹处理细胞12 h后Annexin V/7-AAD检测细胞凋亡。

A: qRT-PCR detection of mRNA levels in mutant cell; B: Western-blot detection of G6PD protein in mutant cell; C: detection of G6PD enzyme activity in mutant cell strain by G6PD/6PGD ratio method, \* $P < 0.05$ ; D: cell proliferation detection by CCK8 assay; E: cell apoptosis detected by Annexin V/7-AAD 12 hours after treatment with different concentrations of primaquine.

图5 *G6PD*基因c.1388G>A突变K562细胞功能检测

Fig.5 Functional detection of *G6PD* gene c.1388G>A mutant K562 cells

表2 脱靶位点序列

Table 2 Off-target sequences

脱靶位点 Off-target site	基因组位置 Location	脱靶序列(5'→3') Sequences (5'→3')
Off-target site 1	Chr22:22945162	TGA AAA ATT CGC CAG GCC TCA
Off-target site 2	Chr17:29075444	TGA AAA TTC TCA CAG GCC TCA
Off-target site 3	Chr17:82813736	TGA AAA TAC GCA AGG GGC TCA

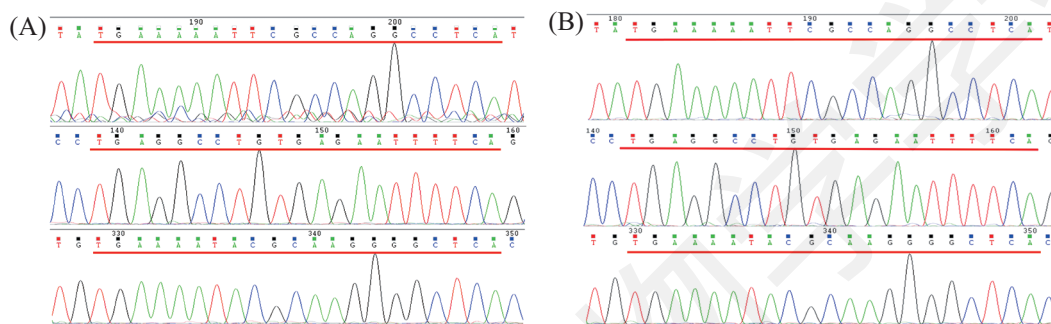
X染色体综合征<sup>[19]</sup>、重症免疫缺陷症<sup>[20]</sup>等, 相比于锌指与TALEN技术, 具有操作简便、快捷高效、特异性高的优势。然而由于CRISPR/Cas9系统所需sgRNA长度仅20 bp, 因此存在较高的脱靶效率<sup>[7]</sup>。

本研究中针对数据库预测评分前三位脱靶位点进行qRT-PCR扩增并测序验证, 结果显示未对潜在脱靶位点造成基因编辑, 确保了无脱靶效应对实验结果造成影响。



表3 脱靶位点检测qRT-PCR引物序列  
Table 3 Off-target detection qRT-PCR primer sequences

脱靶位点 Off-target site	引物序列(5'→3') sequences (5'→3')	产物长度/bp Product length /bp
Off-target site 1	Forward: AAA AGC TTC CCT GCA GAG TTC AC Reverse: TGA AAT TGC TGC TGT GTG TGA TTT A	477
Off-target site 2	Forward: CCG TGA ACC ACA TTG GCT TG Reverse: TGT GGG GCA TCA GTA GGA GA	587
Off-target site 3	Forward: TTA AGC TTG GAT CTG AGG GCG Reverse: GTA CTA ATT TCC CCG GGG TGG	692



A: *G6PD*基因c.1388G>A突变HEK293细胞脱靶位点测序结果; B: *G6PD*基因c.1388G>A突变K562细胞脱靶位点测序结果。

A: off-target site sequencing results of the HEK293 cell strain with *G6PD* gene c.1388G>A mutation; B: off-target site sequencing results of the K562 cell strain with *G6PD* gene c.1388G>A mutation.

图6 脱靶效率检测

Fig.6 Off-target efficiency detection

已有文献报道, *G6PD*基因c.1388G>A(R463H)突变会破坏其与Glu460形成的盐桥影响G6PD蛋白稳定性, 导致G6PD酶活性下降<sup>[24]</sup>。本研究首先利用CRISPR/Cas9技术成功构建*G6PD*基因最常见c.1388G>A位点突变的HEK293细胞株。检测结果显示, c.1388G>A突变使HEK293细胞G6PD酶活性下降50%左右, 且细胞增殖减慢, 提示磷酸戊糖途径核糖-5-磷酸生成障碍使DNA合成受抑制。维生素K3与伯安喹为G6PD缺陷症患者禁用药物, 常规剂量便会导致患者发生急性溶血性贫血, 在本研究中我们发现, *G6PD*基因c.1388G>A突变的HEK293细胞对两种药物的耐受均明显下降, 且呈剂量依赖性, 提示细胞NADPH生成障碍导致无法抵抗氧化损伤, 引起细胞死亡。

肿瘤细胞由于其自身的快速增殖、应对氧化应激和对生物大分子的需求会改变其自身代谢。G6PD在胞内存在活性形式(二聚体)与非活性形式(单体), G6PD的活性还与其化学修饰的情况有关, O-糖基化可以直接提高G6PD的活性而使其有利于肿瘤生长<sup>[21]</sup>, 乙酰化调控在抵抗氧化应激时也发

挥重要作用<sup>[22]</sup>。已有研究表明, 肿瘤细胞中磷酸戊糖途径异常激活, *G6PD*基因表达上调、活性升高, NADPH与核苷酸生成增加<sup>[23]</sup>。本研究构建*G6PD*基因c.1388G>A纯合突变的红白血病K562细胞株, 检测结果发现酶活性仅下降20%左右, 细胞增殖稍受抑制, 对伯安喹的耐受能力下降程度也不及HEK293细胞。这可能是由于肿瘤细胞的代偿性调控弥补了c.1388G>A突变所致G6PD蛋白的不稳定性, 具体机制尚不清楚。

目前国内外对G6PD缺陷症相关研究方法以基因敲除与基因沉默为主, 无法完全模拟G6PD缺陷症的发病机制。本研究成功应用CRISPR/Cas9联合同源重组修复技术在细胞系上进行精确基因编辑, 构建G6PD缺陷症最常见突变型细胞模型, 同时也为后续基因修复研究提供细胞模型。

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