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Ratiometric-pericam-mt 慢病毒的构建及其线粒体钙检测功能的验证 *

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摘要 目的:基于由环状异构黄色荧光蛋白(Circularly Permuted EYFP)改造的具有线粒体定位功能的 Ratiometric-pericam-mt(rp-mt) 荧光蛋白构建携带有 rp-mt 基因的慢病毒载体,验证其在线粒体钙检测中的功能,为进一步研究线粒体钙稳态在细胞生命活动中发挥的作用提供参考依据。**方法:**采用慢病毒载体 EF-1 α F-puro 和 rp-mt 质粒构建携带有 rp-mt 基因的重组慢病毒载体,经脂质体法在 293T 细胞中与包装质粒共转染,收集构建好的重组慢病毒 EF-1 α F-puro-rp-mt 并体外感染人肝癌细胞 SNU-739,验证重组慢病毒 EF-1 α F-puro-rp-mt 的活性功能。**结果:**以慢病毒载体 EF-1 α F-puro 为骨架质粒,成功构建了携带有 rp-mt 基因的重组慢病毒载体 (EF-1 α F-puro-rp-mt), 收集获得的重组慢病毒滴度为 4×10^6 TU/mL。将携带有 rp-mt 基因的慢病毒 EF-1 α F-puro-rp-mt 感染 SNU-739 细胞 48 小时后,在荧光显微镜下观察到有超过 85% 的细胞发出绿色荧光,定位于细胞线粒体内,且对 SNU-739 细胞进行 Ru360 和 CGP37157 处理后,细胞荧光强度随线粒体内钙水平改变而增强或减弱。**结论:**采用脂质体包装法成功构建了携带有 rp-mt 基因的重组慢病毒 EF-1 α F-puro-rp-mt,细胞经该慢病毒转染后可稳定表达具有线粒体定位功能的荧光蛋白 rp-mt,准确地检测细胞线粒体钙离子水平。

关键词:慢病毒载体;线粒体定位荧光蛋白;线粒体钙

中图分类号:R-33;Q244;Q78 **文献标识码:**A **文章编号:**1673-6273(2020)08-1441-05

Construction of Ratiometric-pericam-mt-lentivirus and Validation of the Function in Mitochondrial Calcium Detection*

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ABSTRACT Objective: To construct lentiviral vector carrying the rp-mt gene based on a ratiometric-pericam-mt (rp-mt) fluorescent protein modified by a circularly permuted yellow fluorescent protein (cpEYFP), verify the function of lentiviral vector in the mitochondrial calcium detection, and provide a reference for the further study on the role of mitochondrial calcium homeostasis in the cell life. **Methods:** A recombinant lentivirus vector carrying the rp-mt gene was constructed with the lentivirus vector EF-1 α F-puro and the rp-mt plasmid, and was co-transfection with package plasmid in 293T cells. The constructed recombinant lentivirus was collected and infected the HCC cells SNU-739 *in vitro*. The function of recombinant lentivirus was validated. **Results:** Are combinant lentivirus vector carrying rp-mt gene with the lentivirus vector EF-1 α F-puro was successfully constructed, the titer of recombinant lentivirus was 4×10^6 TU/mL. More than 85% of the SNU-739 cells were observed to emit green fluorescence under a fluorescence microscope after infected by the recombinant lentivirus vector for 48 hours. The fluorescence located in the mitochondria of cells and was brighter or weaker as the variation of the concentration of calcium in mitochondria with Ru360 or CGP37157 treated of the cells. **Conclusions:** Are combinant lentivirus vector carrying rp-mt gene with the liposomes packing method was successfully constructed. The cell can stably express the fluorescegenic protein which located in the mitochondrial after infected by the recombinant lentivirus, and the calcium of the mitochondrial can be accurately detected.

Key words: Lentiviral vector; Ratiometric-pericam-mt; Mitochondrial calcium

Chinese Library Classification(CLC): R-33; Q244; Q78 **Document code:** A

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前言

线粒体作为细胞中重要的细胞器,在细胞能量代谢、信号传导以及细胞周期调控中发挥重要作用^[1]。此外,多项研究表明

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线粒体具有维持细胞内钙稳态的作用^[2-4]。线粒体通过从胞质中摄取钙离子并储存在基质中,进而维持线粒体各项功能的发挥^[5]。首先,线粒体 Ca^{2+} 能够激活线粒体内三种重要能量代谢酶(α -酮戊二酸脱氢酶、丙酮酸脱氢酶、异柠檬酸脱氢酶),进而影响 ATP 的合成^[6]。

研究表明在小鼠脑神经元中,线粒体钙单向转运体 MCU 基因的敲除可导致线粒体内能量代谢向糖酵解转变,并抑制了呼吸链复合体 I 的活性^[7]。其次,线粒体作为细胞内重要的钙库,通过转运胞质内游离的钙离子,进而调节细胞内信号传导,对不同类型细胞的研究显示线粒体可以改变细胞内的 Ca^{2+} 浓度,包括局部和整个细胞质中的 Ca^{2+} 浓度^[8]。最后,线粒体内 Ca^{2+} 调节失调会影响细胞功能,破坏线粒体,导致细胞死亡^[9]。基于线粒体钙在细胞生命活动中发挥的重要作用,线粒体钙稳态重塑在疾病发生发展中的作用受到越来越多的关注。

关于线粒体钙的检测,目前使用较为广泛的是 rhod-2^[10,11],然而大量研究表明 rhod-2 存在严重缺陷,如非特异性定位,细胞毒性等^[12,13]。通过查阅文献,我们了解到 Atsushi Miyawaki 等人通过对循环倒置黄色荧光蛋白(cpEYFP)进行改构,将 cpEYFP 与钙调蛋白(CaM)和它的靶蛋白 M13 组合在一起,形成的蛋白复合物命名为 pericam 蛋白,并在此基础上改构出一种能够特异性定位在线粒体的荧光蛋白 Ratiometric-pericam-mt(rp-mt),该蛋白在 Hela 细胞中表达后,可在荧光显微镜特定波长激发下发出绿色荧光(经改构的 EYFP 黄色荧光蛋白激发波长蓝移,使得本应发黄色荧光的黄色荧光蛋白发绿光)。将编码该蛋白(rp-mt)的 cDNA 插入到质粒中,细胞经重组质粒转染后,就可表达出该蛋白,进而监测到细胞线粒体内钙离子水平^[14]。基于此,我们构建了携带有 rp-mt 基因的慢病毒,使细胞经该重组慢病毒感染后可以稳定表达该蛋白,以期为研究线粒体钙提供有利的帮助。

1 材料与方法

1.1 试验材料

Rp-mt 质粒、包装质粒(psPAX2、pMD2.G)、DH5 α 感受态细胞、293T 细胞、DMEM 培养基、胰酶和 SNU-739 均由第四军医大学生理教研室提供;慢病毒载体 EF-1 α F-puro(载体图谱如图 1)购于吉玛基因;胎牛血清购于上海生工;Lipo2000 和 mitotracker 染料购于美国 Invitrogen 公司;限制性内切酶 BamH I 和 Not I、DL10000、反转录试剂盒购于日本 Takara 公司;T4 连接酶购于美国 NEB 公司;质粒小提试剂盒、RNA 提取试剂盒购于 OMEGA BIO-TEK 公司;Ru360 和 CGP37157 购于美国 EMD Millipore 公司;激光共聚焦显微镜购于日本 Olympus 公司。

1.2 重组慢病毒载体的构建

将 EF-1 α F-puro 载体和 rp-mt 质粒用 BamH I 和 Not I 双酶切后,酶切体系进行琼脂糖凝胶电泳验证后进行胶回收 8500bp 的 EF-1 α F-puro 线性载体和 1600bp 的 rp-mt 片段,将两者用 T4 连接酶 16℃ 过夜连接。连接体系进行大肠杆菌 DH5 α 转化,挑取单克隆于 37℃ 过夜摇菌,取 5 mL 菌液提取重组载体,酶切后琼脂糖凝胶电泳鉴定重组慢病毒载体 EF-1 α F-puro-rp-mt。

1.3 慢病毒包装

将 293T 细胞种于六孔板,于 DMEM 培养基中培养 24h,待细胞汇合至 80%左右,将 EF-1 α F-puro 载体 8 μg psPAX2 质粒 3 μg pMD2.G 质粒 1 μg 溶于 300 μL 无血清培养基,为 a 溶液;将 16 μL lipo2000 溶于 300 μL 无血清培养基,为 b 溶液; a、b 溶液在室温下静置 5 min 后,混合均匀(吹打数次),室温下静置 20 min。吸弃六孔板内培养基,加入 1 mL 有血清培养基,并将 a、b 的混合液加入其中,37℃ 孵育 6h 换新的有血清培养基,转染 48h 后收集细胞上清液,6000 r/min,4℃ 离心 1h 后去除细胞沉淀,病毒上清经过滤浓缩后,于 -80℃ 保存。

1.4 病毒滴度测定

将 293T 细胞以每孔 5×10^5 个细胞种于六孔板,提前将病毒从 -80℃ 取出并放于 4℃ 过夜解冻。细胞孵育 24 h 后,取解冻的病毒用完全培养基配置终体积为 1 mL,病毒做 10^{-10} 倍稀释的病毒稀释液,分别加入到六孔板的 10 个孔中,24h 后换新的有血清培养基,48h 后在荧光显微镜下拍照,记录有荧光的最大稀释配数的 2 个孔的阳性细胞数,计算病毒滴度。

1.5 RT-PCR 鉴定 EF-1 α F-puro-rp-mt

经重组慢病毒感染 48 h 后的 293T 细胞提 RNA,取 1 μg RNA 于 20 μL 逆转录体系中,37℃,15 min 逆转录为 cDNA,取 1 μL cDNA 产物配置 25 μL PCR 体系,用 rp-mt 上下游引物(F:5'-ATGAAGAGGCGCTGGAAGAAAACTT-3';R:5'-TCA C TT CGCTGTCATCATTTGTACAAACT-3')进行 PCR 扩增,扩增产物进行琼脂糖凝胶电泳鉴定,PCR 产物送公司测序。

1.6 重组慢病毒感染人肝癌细胞

于六孔板中培养 SNU-739 细胞,每孔的密度为 4×10^5 个细胞,实验组分别加入 2 μM Ru360 (线粒体钙单向转运体 MCU 抑制剂^[15])和 1 μM CGP37157(线粒体 Na^+ - Ca^{2+} 交换转运体抑制剂^[16]),对照组加入相同体积的 DMSO。37℃ 孵育 24 h 后,根据病毒滴度为 4×10^6 TU/mL,按每孔细胞数为 1×10^6 个,以 250 μL 病毒浓缩液感染细胞,并照前一天补加相同量的 Ru360 和 CGP37157,病毒感染 48h 后,细胞在荧光显微镜下观察。

为了验证重组慢病毒的线粒体钙检测功能,用重组慢病毒感染 SNU-739 48h 后,进行 mitotracker 染色,将 mitotracker 工作液用 HBSS 以 1:5000 稀释后加入细胞 (HBSS 提前 37℃ 预热),于 37℃、5%CO₂ 下孵育 30 min,后用 $1 \times$ PBS 洗三遍,于共聚焦显微镜绿光和蓝光下拍照。

2 结果与分析

2.1 重组慢病毒载体的构建

构建好的慢病毒 EF-1 α F-puro 用 BamH I 和 Not I 进行双酶切后跑琼脂糖凝胶电泳,结果显示电泳获得了 8500bp 和 1600 bp 的片段(图 2),分别是线性化 EF-1 α F-puro 和 rp-mt,与预期结果一致,说明重组慢病毒构建成功。经慢病毒感染的 293T 细胞的 RNA 经逆转录成 cDNA 后,用 rp-mt 引物扩增出了 rp-mt cDNA 片段 (1600bp),且由 cDNA 为模板扩增出的 PCR 产物经华大基因生物公司测序确认,序列为 rp-mt 基因序列(图 3),说明 293T 细胞成功表达 rp-mt 蛋白。

2.2 病毒滴度测定

慢病毒载体与包装质粒共转染 293T 细胞 48h 后,收集细

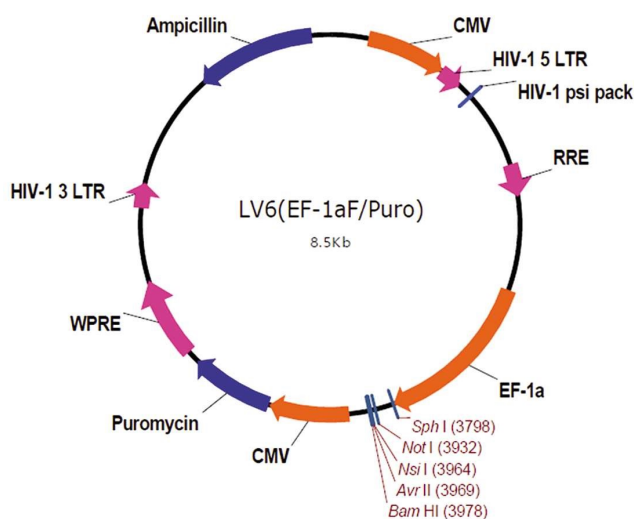


图1 慢病毒载体图谱

Fig. 1 Lentivirus vector map

胞上清液,浓缩病毒。收集好的病毒进行病毒滴度测定,在荧光显微镜下观察,能看到荧光的最大稀释倍数为 10^5 和 10^6 稀释,孔内有荧光的细胞数分别为 30 个和 5 个,因此计算 EF-1 α F-puro-rp-mt 慢病毒的滴度为 4×10^6 TU/mL。

2.3 重组慢病毒感染人肝癌细胞

人肝癌细胞 SNU-739 经慢病毒感染 48 h 后,在荧光显微镜下观察,发现实验组与对照组细胞均发出绿色荧光,均有超过 85% 的细胞发光;与对照组相比(图 4A、B),Ru360 处理组(图 4C、D)由于抑制了线粒体 Ca^{2+} 内流,荧光明显减弱;而 CGP37157 处理组(图 4E、F)由于抑制了 Na^+ - Ca^{2+} 交换,导致线粒体 Ca^{2+} 外排减少,荧光显著增强。

2.4 重组慢病毒线粒体钙特异性检测功能验证

用 EF-1 α F-puro-rp-mt 慢病毒感染人肝癌细胞 SNU-739 48 h 后,加入经 HBSS 稀释后的 mitotracker 染色 30 min;用 PBS 洗去细胞上清液中的染料,于共聚焦显微镜绿光和蓝光下观察,如图 4 所示,共定位结果显示 rp-mt 蛋白特异性聚集于

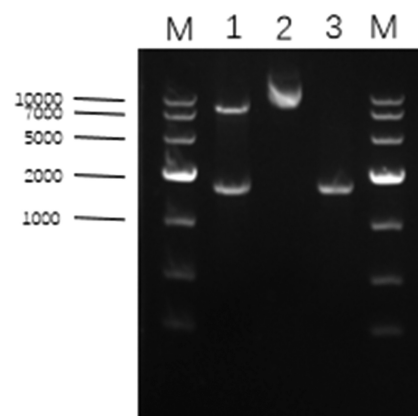


图2 重组慢病毒酶切体系及 RT-PCR 结果琼脂糖凝胶电泳

Fig. 2 The agarose gel electrophoresis of the enzyme-cutting system of the recombinant lentiviral and RT-PCR result

注:M 为 DNA marker 10000;1 为重组慢病毒载体酶切体系电泳结果;2 为重组慢病毒载体电泳结果;3 为感染重组慢病毒的 293T cDNA 为模板,rp-mt 特异性引物 PCR 产物电泳结果

Note: M: DNA marker 10000; 1: Agarose gel electrophoresis of the enzyme-cutting system of the recombinant lentiviral; 2: Agarose gel electrophoresis of the recombinant lentiviral; 3: Agarose gel electrophoresis of the PCR product by cDNA of 293T cells and specific primers of rp-mt

线粒体中(图 4C 中无共定位的为没有感染重组慢病毒的细胞),表明经 EF-1 α F-puro-rp-mt 慢病毒感染后,细胞会表达出具有绿色荧光的蛋白,且该蛋白定位于线粒体,能够发挥特异性检测线粒体钙的功能。

3 讨论

近年来,越来越多的证据表明线粒体在细胞 Ca^{2+} 稳态中发挥着重要作用。线粒体代谢的激活、细胞凋亡的诱导、胞质 Ca^{2+} 的调节,都依赖于线粒体基质内游离钙离子的动态变化^[17,18]。因此,从上个世纪五十年代起,就先后建立了各种检测细胞内钙离子的方法,如微电极法、核磁共振法、钙有机显色剂法、钙离子荧光探针法等,其中钙离子荧光探针法是目前最常用的一种

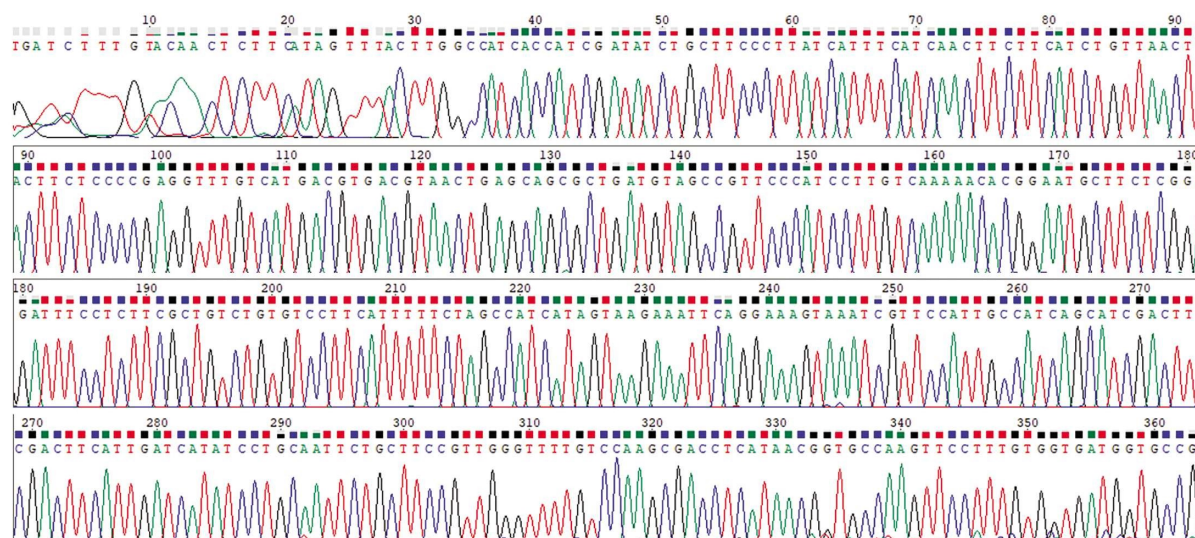


图3 感染重组慢病毒 293T 细胞 cDNA 做模板 PCR 产物测序结果

Fig. 3 Sequencing of the PCR product by cDNA of 293T cells infected with the recombinant lentiviral

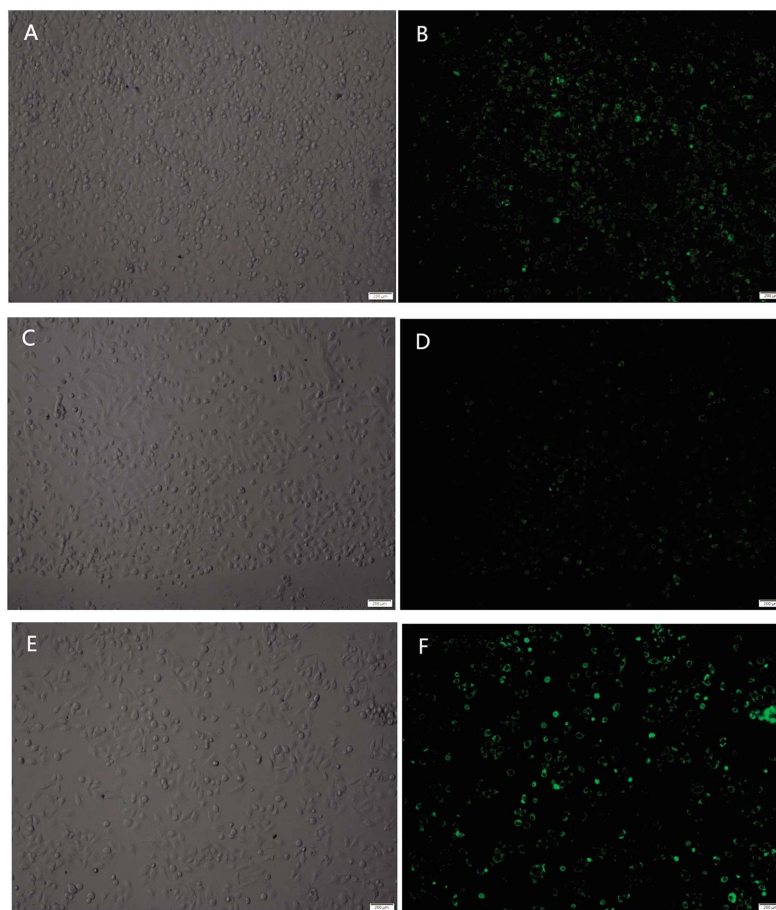


图4 重组慢病毒感染 SNU-739 细胞 48h 荧光结果

Fig. 4 Fluorescence of SNU-739 cells infected with recombinant lentiviral for 48 hours

注:A、C、E 分别为感染了重组慢病毒的 SNU-739 细胞经 DMSO、Ru360 和 CGP37157 处理后在白光下拍照结果;B、D、F 分别为感染了重组慢病毒的 SNU-739 细胞分别经 DMSO、Ru360 和 CGP37157 处理后在荧光显微镜蓝光下拍照结果

Note: A, C, E: Photograph under white light of SNU-739 infected by recombinant lentiviral for 48 hours after treated respectively with DMSO, Ru360, CGP37157; B, D, F: Photograph under blue light of SNU-739 infected by recombinant lentiviral for 48 hours after treated respectively with DMSO, Ru360, CGP37157

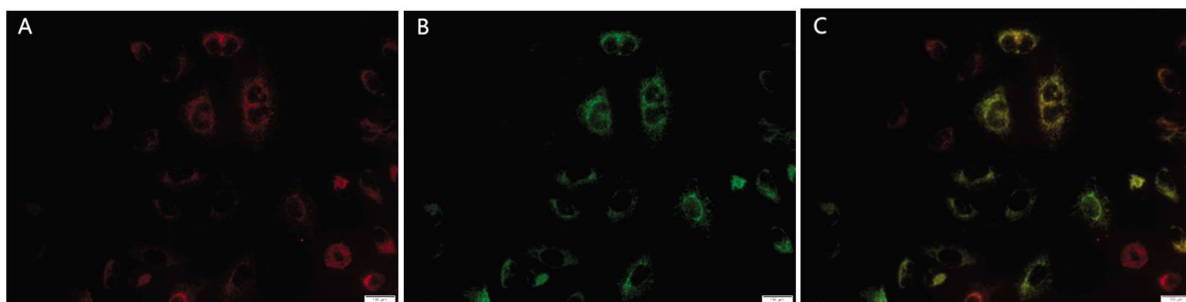


图5 Rp-mt 与 mitotracker 共定位结果

Fig. 5 Co-localization of rp-mt and mitochondria

注:A 为感染了重组慢病毒 48h 后的 SNU-739 细胞 mitotracker 染色后荧光显微镜绿光下拍照结果;

B 为感染了重组慢病毒 48h 后的 SNU-739 细胞荧光显微镜蓝光下拍照结果;C 为 A、B 结果组合结果

Note: A: Photograph of green light of SNU-739 cells infected by recombinant lentiviral for 48 hours and dyed by mitotracker;

B: Photograph of blue light of SNU-739 cells infected by recombinant lentiviral for 48 hours; C: Merging of A and B

方法。小分子荧光探针如 rhod-2^[19]、fura-2^[20]具有对 Ca^{2+} 选择性强、响应迅速等优点,被广泛用于细胞内 Ca^{2+} 浓度测定,而对于线粒体钙的检测,这类化学染料并不能满足人们的要求^[21](无法特异性检测线粒体内钙)。

应运而生的是一类 Ca^{2+} 激活的发光蛋白,如 aequorin(一

种水母蛋白,与 Ca^{2+} 结合后能够发射出蓝色荧光^[22]和以绿色荧光蛋白^[23](GFP)为基础的基因重组发光蛋白。与使用染料的方法不同,使用基因编码的钙指示物可在几个小时内对线粒体钙水平进行非侵入性监测,在分析细胞死亡过程中的线粒体钙时,这一点尤为重要^[24]。目前关于 GFP 基因重组蛋白线粒体钙

检测的经典方法为:将 GFP 相关基因(各种突变体 EGFP、EYFP 等)与钙调蛋白及 M13 多肽基因组成重组序列(如 pericam^[14]、cameleons^[25]),并在一端插入具有线粒体定位功能的蛋白(如具有线粒体靶向性的细胞色素 c 氧化酶四亚基序列^[14]),由表达该重组基因的质粒感染细胞后,表达出的 GFP 突变蛋白就可特异性检测线粒体内 Ca^{2+} 。

目前,对于利用 GFP 重组蛋白检测线粒体内钙水平,大多数的研究者选择构建重组质粒进行细胞瞬时转染细胞来表达该蛋白^[26-28],虽然这种方法操作简单、方便,但是缺点是稳定性差,一般转染后细胞传代 1-2 次后就不再表达该蛋白,因此需要筛选稳转细胞系或者通过多次转染达到实验效果。基于此,我们首次将具有线粒体靶向性的荧光蛋白 rp-mt 插入慢病毒载体,并包装成为慢病毒(病毒发挥重要活性的部分蛋白相关的序列结构去除,具有生物学安全性^[29]),细胞被重组慢病毒感染后,经过多次传代培养仍然具有目的基因表达效应(显微镜下观察显示绿色荧光),解决了普通质粒转染基因组整合能力差的问题。且对于一些较难转染的细胞,如原代细胞、干细胞和不分化的细胞等,均能使转染效率大大提高^[30]。我们通过激光共聚焦显微镜下的成像发现,细胞经我们构建的重组慢病毒 EF-1 α F-puro-rp-mt 感染后,在 24h 即可发出绿色荧光,且荧光集中于细胞质的线粒体中,胞核和胞浆内均没有绿色荧光发出;细胞经 Ru360 或 CGP37157 药物处理后,由于 rp-mt 与钙结合减少或增多,能够看到绿色荧光强度随之变化明显。该慢病毒的成功构建,为我们后续研究线粒体钙相关内容提供了巨大帮助。

本研究通过酶切连接成功构建了 EF-1 α F-puro-rp-mt 慢病毒载体质粒,经脂质体转染法包装的慢病毒感染人肝癌细胞,成功表达出 rp-mt 荧光蛋白,经验证该蛋白可以准确表征线粒体内钙离子浓度变化,为进一步研究线粒体钙稳态在细胞中发挥的作用提供了有利帮助。

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