

# 再生基因4 (REG4) 真核表达载体的构建及其蛋白在 HEK 293T 细胞中的表达、纯化

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**摘要:** **目的** 构建再生基因4 (regenerating gene 4, REG4) 的真核表达载体, 转染人胚肾细胞 293T (human embryonic kidney 293T cells, HEK 293T), 获得重组人再生胰岛衍生蛋白 IV (regenerating islet-derived protein IV, Reg IV)。**方法** 根据 NCBI 数据库 REG4 基因序列进行基因优化、合成, 将其克隆至 pCDNA3.4 载体并进行双酶切和测序鉴定, 通过转染试剂聚乙烯亚胺 (polyethylenimine, PEI) 将 pCDNA3.4-REG4 质粒瞬时转染至 HEK 293T 细胞 (实验组), 同时以 pEGFP-C1 质粒作为转染对照组, 未转染重组质粒的 HEK 293T 细胞作为空白对照组。荧光显微镜观察转染对照组转染效率, 分别收集实验组及空白对照组细胞和细胞培养液上清, 十二烷基硫酸钠聚丙烯酰胺凝胶电泳 (sodium dodecyl sulfate polyacrylamide gel electrophoresis, SDS-PAGE) 与免疫印迹试验 (Western-Blot, WB) 检测 Reg IV 蛋白表达水平。通过镍柱及丙烯葡聚糖凝胶 S-400 (Sephacryl S-400) 柱进行蛋白纯化, SDS-PAGE 及 WB 对纯化后重组蛋白进行鉴定。**结果** 经测序和双酶切鉴定, 重组质粒 pCDNA3.4-REG4 构建成功。转染对照组 (pEGFP-C1 质粒) 荧光显微镜观察结果显示转染效率约 50%, 表明转染成功。WB 结果显示仅在实验组 (pCDNA3.4-REG4 质粒) 的细胞中检测到 Reg IV 蛋白。镍柱纯化时目的蛋白无法与镍柱填料有效结合, Sephacryl S-400 凝胶柱层析纯化获得了此重组蛋白。**结论** 成功构建了 REG4 基因真核表达载体并在 HEK 293T 细胞中成功表达, 为深入研究 Reg IV 蛋白的作用机理及开发潜在的抗癌靶向药物奠定了基础。

**关键词:** 再生基因 4; 再生胰岛衍生蛋白 IV; 真核表达; 人胚肾细胞 293T; 瞬时转染; 蛋白纯化

**中图分类号:** Q786 **文献标识码:** A **文章编号:** 1671-7414 (2023) 04-035-05

**doi:**10.3969/j.issn.1671-7414.2023.04.006

## Construction of Eukaryotic Expression Vector of Regenerating Gene 4(REG4) and Expression and Purification of Its Protein in HEK 293T Cells

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**Abstract:** **Objective** To construct an eukaryotic expression vector of Regenerating gene 4 (REG4) and transfection into human embryonic kidney 293T Cells (HEK 293T) to obtain recombinant human regenerating islet-derived protein IV (Reg IV). **Methods** The REG4 gene sequence was optimized and synthesized according to the NCBI database, and it was cloned into the pCDNA3.4 vector and identified. The pCDNA3.4-REG4 plasmid was transiently transfected into HEK 293T cells by transfection reagent polyethylenimine (PEI) as the experimental group. The pEGFP-C1 plasmid was used as transfection control group, and the HEK 293T cells not transfected with the recombinant plasmid were used as the blank control group. The transfection efficiency of the transfection control group was observed by fluorescence microscope. The cells and cell culture supernatant of the experimental group and the blank control group were collected respectively. The expression level of Reg IV protein was detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western-Blot (WB). The protein was purified by nickel column and Sephacryl S-400 column, and the purified recombinant protein was identified by SDS-PAGE and WB. **Results** The recombinant plasmid pCDNA3.4-REG4 was successfully constructed by sequencing and double enzyme digestion. The transfection control group (pEGFP-C1 plasmid) showed that the transfection efficiency was about 50%, indicating that the transfection was successful. WB results showed that Reg IV protein was detected only in the cells of

**基金项目:** 兰州市科技发展指导性计划项目 (2019-ZD-131): 高性能抗 RegIV 单克隆抗体的制备及鉴定; 甘肃省自然科学基金项目 (21JR7RA866): PG, MUC1, HIK1083 联合检测在胃早癌诊断中的临床研究。

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the experimental group (pCDNA3.4-REG4 plasmid). The target protein could not effectively bind to the nickel column filler during nickel column purification, and the recombinant protein was purified by Sephacryl S-400 gel column chromatography.

**Conclusion** The eukaryotic expression vector of REG4 gene was successfully constructed and expressed in HEK 293T cells, which laid a foundation for further study of the mechanism of Reg IV protein and the development of potential anticancer targeted drugs.

**Keywords:** regenerating gene 4; regenerating islet-derived protein IV; eukaryotic expression; HEK 293T cells; transient transfection; protein purification

再生基因 (regenerating gene, REG) 家族属于钙依赖性凝集素 (C 型凝集素) 基因超家族, 有四个亚型, 该家族蛋白可能参与多种细胞和组织的再生<sup>[1]</sup>。REG4 属于该家族成员之一, 编码再生胰岛衍生蛋白 IV (regenerating islet-derived protein IV, Reg IV), 是一种小分子分泌性蛋白质, 分子量约为 18.2 KD。已有研究证明, Reg IV 能够显著促进肿瘤细胞的生长、增殖、侵袭和迁移, 抵抗凋亡, 并可提高肿瘤细胞抗药性等作用<sup>[2-5]</sup>。因此, Reg IV 在肿瘤诊断、预后及潜在治疗靶点<sup>[6]</sup>方面具有较大的应用前景。胃癌为消化系统常见恶性肿瘤<sup>[7]</sup>, 课题组前期对胃癌中 Reg IV 和性别决定区 Y 框蛋白 9 (SOX9) 的调控关系进行了研究, 通过构建真核表达载体 pEGFP/RegIV, 瞬时转染胃癌细胞系 MKN-45 和 AGS 细胞, 发现 Reg IV 可能通过调控 SOX9 的表达水平参与胃癌细胞的侵袭和迁移<sup>[8]</sup>。由于 pEGFP/RegIV 载体的标签蛋白较大及纯化问题使应用受限, 为进一步实现 Reg IV 在体内外生物功能的研究及抗体制备, 本研究拟通过构建带 His 标签的 REG4 真核表达载体, 并在人胚肾细胞 293T 细胞 (human embryonic kidney 293 T cell, HEK 293T) 细胞中表达, 以期获得可用于研究的重组人 Reg IV 蛋白。

## 1 材料与方法

**1.1 细胞及质粒来源** pCDNA3.4-REG4 质粒及克隆菌株 (南京钟鼎生物技术有限公司); HEK 293T 细胞 (本室液氮冻存, 来源于中科院上海细胞库)。

**1.2 仪器与试剂** DMEM 高糖培养基 (gibico, 美国); 新生牛血清 (兰州民海生物工程有限公司); PEI (Polysciences, Inc., 美国); PVDF 膜 (Merck Millipore, 德国); 化学显影试剂盒 (Thermo, 美国); ChemiDoc XRS+ 凝胶成像系统 (BIO-RAD, 美国); 兔源 REG4 [Cell Signaling Technology (CST), 美国]; HRP 标记的山羊抗兔 IgG [爱必信 (上海) 生物科技有限公司]; 鼠源  $\beta$ -actin, HRP 标记的山羊抗鼠 IgG (Santa, 美国); 无内毒素小量质粒提取试剂盒 II (Omega Bio-Tek, 美国); DNA Marker, 蛋白质 Marker (Takara, 日本); 本研究所用其他相关化学试剂均为国产分析纯。

## 1.3 方法

**1.3.1 pCDNA3.4-REG4 表达载体的构建与鉴定:** 根据 GenBank REG4 基因已知序列, 委托南京钟鼎生物技术有限公司进行基因合成, 按照哺乳动物蛋白表达体系进行了密码子优化, 将 REG4 原有信号肽序列转换成钟鼎信号肽序列, C 端添加 His 标签, 合成并构建在 pCDNA3.4 载体上 (酶切位点为 EcoR I / BamH I), 构建策略见图 1。将构建好的载体转化感受态大肠埃希菌 TOP10, 将转化菌涂布于 LB 平板 (含 50  $\mu$ g/ml 氨苄青霉素), 37 $^{\circ}$ C 培养过夜。用无菌牙签挑取单菌落接种于 LB 培养液 (含 50  $\mu$ g/ml 氨苄青霉素) 中, 37 $^{\circ}$ C, 200 r/min 培养过夜。提取质粒, 对重组表达质粒进行双酶切 (EcoR I / BamH I) 鉴定及序列测定, 鉴定正确的重组表达质粒命名为 pCDNA3.4-REG4。

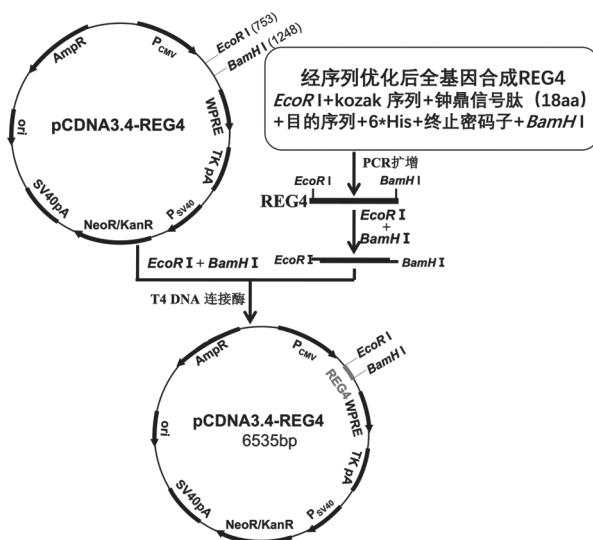


图1 重组表达质粒 pCDNA3.4-REG4 的构建策略

**1.3.2 pCDNA3.4-REG4 质粒的提取:** 取钟鼎生物交付鉴定正确的菌液, 按 1:50 比例接种 LB (含 50  $\mu$ g/ml 氨苄青霉素) 培养液, 37 $^{\circ}$ C, 200 r/min 培养过夜。根据质粒提取试剂盒操作说明提取质粒, 测定质粒浓度, 于 -75 $^{\circ}$ C 冰箱冻存。

**1.3.3 细胞培养与转染:** HEK 293T 细胞接种于含 100 ml/L 胎牛血清的 DMEM 高糖培养液中, 37 $^{\circ}$ C, 5% (v/v) CO<sub>2</sub> 培养箱中常规培养。转染前 1 天, 将生长良好的细胞消化计数铺于细胞瓶中, 细胞密

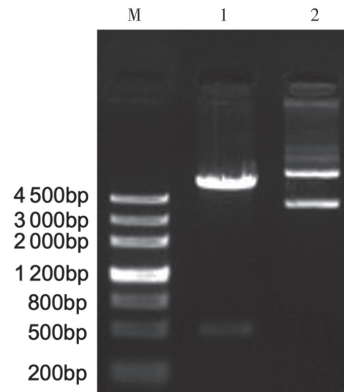
度约  $1.8 \times 10^6$  个/瓶,待细胞达到 70% ~ 80% 融合时,按照 PEI 常规转染方法将 pCDNA3.4-REG4 质粒瞬时转染至 HEK 293T 细胞(作为实验组),同时以 pEGFP-C1 质粒作为转染对照组,未转染重组质粒的 HEK 293T 细胞作为空白对照组,转染 30h 后分别收集实验组及空白对照组的细胞和细胞培养液上清,细胞悬于 0.01 mol/L PBS (pH 7.4) 缓冲液中,均于  $-75^\circ\text{C}$  冰箱冻存。荧光显微镜观察转染对照组(pEGFP-C1 质粒)转染情况。

**1.3.4 重组蛋白的 SDS-PAGE 及 WB 鉴定:** 采用三氯乙酸(trichloroacetic acid, TCA)<sup>[9]</sup> 法从细胞培养液上清沉淀蛋白,分别将收集的细胞、细胞培养液上清蛋白样品用上样缓冲液处理后进行 SDS-PAGE 电泳,然后进行蛋白染色鉴定及 WB 实验。WB 一抗为兔源 REG4、鼠源  $\beta$ -actin (均是 1:1 000 稀释),二抗为 HRP 标记的山羊抗兔 IgG (1:5 000 稀释)、山羊抗鼠 IgG (1:4 000 稀释),用 ChemiDoc XRS+ 凝胶成像系统成像, $\beta$ -actin 为内参蛋白。

**1.3.5 重组蛋白的纯化:** Reg IV 过表达 HEK 293T 细胞经  $-75^\circ\text{C}$  和常温反复冻融 3 次,超声 10 次(超声功率 100 W,超声/间隙时间 3s/3s),然后与平衡好的镍柱填料混合,  $4^\circ\text{C}$  孵育 4 h,用不同浓度咪唑缓冲液进行洗脱,留样检测。收集含目的蛋白样品,依次用 30%, 80% 饱和硫酸铵分级沉淀蛋白,沉淀用 0.01 mol/L PBS (pH 7.4) 缓冲液溶解后以 Sephacryl S-400 柱进行纯化,收集主蛋白峰,进行 SDS-PAGE 分析,再进行 WB 验证。

## 2 结果

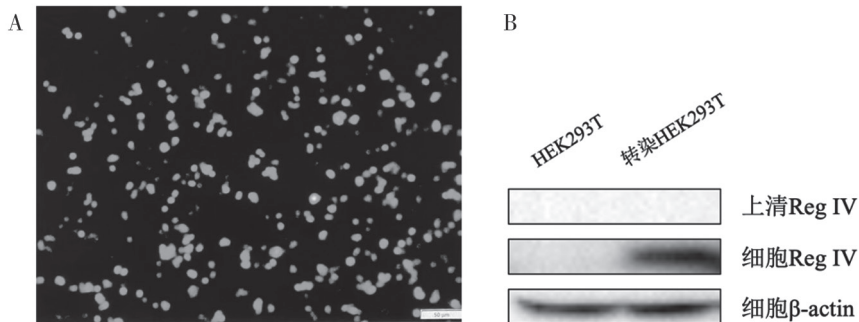
**2.1 pCDNA3.4-REG4 表达载体的鉴定** 重组表达质粒 pCDNA3.4-REG4 的双酶切产物经琼脂糖凝胶电泳分析,可见约 500 bp 的目的片段,与理论值一致(495 bp),见图 2。测序结果显示,经密码子优化的目的蛋白序列与靶蛋白序列(序列号: NP\_001152824.1)相比,除信号肽外其余氨基酸序列完全一致,共编码 Reg IV 去信号肽序列的 136 个氨基酸残基,表明重组质粒构建成功,此质粒 C 末端具有 His 标签,分子量理论值约为 16.7 KD。



M: DNA Marker; 1: pCDNA3.4-REG4 质粒; 2: pCDNA3.4 质粒

图 2 pCDNA3.4-REG4 质粒双酶切验证

**2.2 重组人 Reg IV 蛋白过表达鉴定** 转染对照组(pEGFP-C1 质粒)经荧光显微镜观察,显示转染效率约 50%,表明转染成功。WB 结果显示,与空白对照组相比,仅实验组(pCDNA3.4-REG4 质粒)的细胞在 Reg IV 蛋白预期位置( $M_r$  约 15 KD)检测到特异性条带,表明此重组蛋白过表达成功并定位于胞内,未分泌到细胞培养液中,见图 3。



A. pEGFP-C1 质粒转染 HEK 293T 细胞荧光显微镜验证; B. pCDNA3.4-REG4 质粒转染 HEK 293T 细胞 WB 验证。

图 3 质粒转染 HEK 293T 细胞验证

**2.3 重组蛋白的纯化鉴定** 镍柱收集样品经 SDS-PAGE 检测,结果显示,目的蛋白主要在与镍柱结合后的残液及流穿液中;WB 结果验证了 SDS-PAGE 的结果,WB 还显示 200 mmol/L 咪唑洗脱液仅有微弱的目的条带,说明目的蛋白无法与柱材有效结合。将含目的蛋白的残液和流穿液合并后经硫酸铵分级沉淀, Sephacryl S-400 柱纯化得到的样品

经 SDS-PAGE 检测,结果显示纯化获得了目的蛋白, WB 结果验证了 SDS-PAGE 的结果,见图 4。

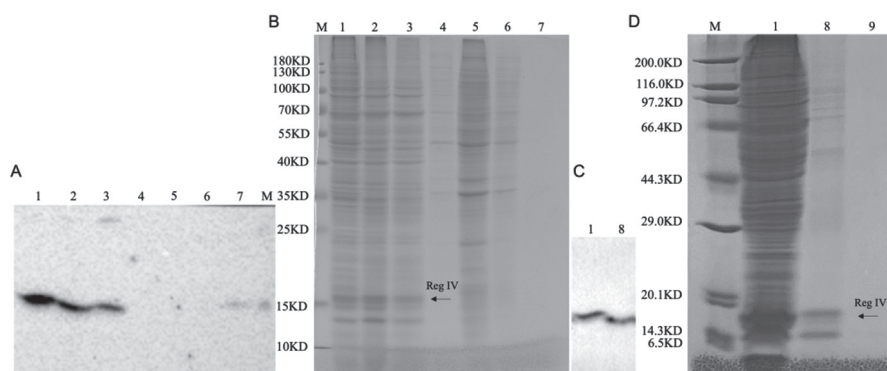
## 3 讨论

Reg IV 异常表达与感染、炎症以及多种癌症有关,其表达程度可作为预测恶性肿瘤发生、侵袭性行为和不良预后的指标<sup>[2,10]</sup>,而且它还可能是耐药患者特定亚群的治疗靶点<sup>[5]</sup>。但目前对于 Reg IV 的



生物学功能如相关受体、调节因子以及耐药机制等研究还不透彻,部分原因是生物活性蛋白的获取有限。国内外学者已进行了有关 Reg IV 在原核和酵母真核表达系统的研究<sup>[11-12]</sup>,但未见在哺乳动物细胞表达系统的相关报道,而此系统在蛋白的起始信号、加工、分泌、糖基化方面具有独特优势,能够

指导哺乳动物蛋白质的正确折叠,提供蛋白质表达所需的内源性翻译后修饰,还可提供接近天然的脂质环境,使产生的外源蛋白质在活性方面远胜于原核和酵母真核表达系统,更接近于天然蛋白质<sup>[13-14]</sup>。因此,本研究采用此表达系统进行 Reg IV 蛋白的表达。



A, B 为 Reg IV 转染细胞及镍柱纯化样品的蛋白电泳及 WB 验证; C, D 为 Reg IV 转染细胞及 Sephacryl S-400 柱纯化样品的蛋白电泳及 WB 验证。M: 蛋白 Marker; 1: 转染 HEK 293T 细胞; 2: 镍柱结合残液; 3: 流穿液; 4 ~ 6: 100 mmol/L 咪唑洗脱蛋白液; 7: 200 mmol/L 咪唑洗脱蛋白液; 8: Sephacryl S-400-1; 9: Sephacryl S-400-2

图4 蛋白纯化鉴定

本研究通过基因工程技术成功构建了 pCDNA3.4-REG4 质粒,并转染 HEK 293T 细胞,结果显示重组人 Reg IV 蛋白成功表达并定位于胞内,未分泌到细胞培养液上清中,而天然 Reg IV 蛋白为小分子分泌性蛋白质,推测此重组蛋白在上清中检测不到的原因可能是更换的钟鼎信号肽不利于蛋白分子进入分泌通路,且蛋白本身不稳定易降解所致。重组人 Reg IV 蛋白 C 端具有 6 \* His 标签,理论上可以与镍柱结合,在不同浓度咪唑缓冲液中被洗脱纯化,但实验发现此蛋白无法和柱材有效结合,一方面可能是 His 标签暴露不全、降解,另一方面可能是重组蛋白分子量小,表达量低,与镍柱结合力弱所致。考虑到影响重组蛋白表达的因素较多,且 Reg IV 蛋白自身也不稳定,蛋白表达和纯化过程仍需进一步的摸索,本研究今后拟对细胞的悬浮培养、转染后培养策略以及纯化方法等方面进行进一步系统考察,从而提高靶蛋白的表达水平及蛋白纯度,以利于下游的应用研究。

综上所述,本研究成功构建了重组人 Reg IV 蛋白的哺乳动物细胞表达体系,并对目的蛋白进行了初步纯化鉴定,为后续开发临床应用试剂盒以及研究 Reg IV 在肿瘤诊断和靶向治疗方面的应用奠定了良好的基础。

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- 收稿日期: 2022-09-21  
修回日期: 2023-04-12

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- 收稿日期: 2022-10-24  
修回日期: 2023-07-03