

pcDNA3.1⁺-HIF-1 α 载体的构建和初步表达鉴定

低氧诱导因子-1 (HIF-1) 在调节机体或细胞对缺氧的反应中起关键作用[1]。HIF-1是一种转录激活因子, 由HIF-1 α 和HIF-1 β 两种亚基组成[2]。它通过结合下游基因的低氧反应元件使下游基因的表达增加, 从而调节细胞和机体功能。HIF-1的下游基因包括促血管新生基因、促血细胞生成基因、能量代谢基因和促细胞增殖调亡基因等[1][3]。氧浓度是调节HIF-1 α 功能的重要因素。在正常氧的条件下, HIF-1 α 亚基容易被蛋白酶降解, 而 β 亚基则比较稳定; 缺氧时, HIF-1 α 的稳定性和活性大大增加[4][5][6]。本实验构建了pcDNA3.1⁺-HIF-1 α 重组载体并将其转到HEK293细胞内, 建立了HEK293/pcDNA3.1⁺-HIF-1 α 细胞株。

1 材料和方法

1.1 细胞株、菌株和质粒

HEK293细胞由本室陈哲明医生提供, 大肠癌细胞株HT29由消化科崔海宏医生提供, 大肠杆菌JM109和DH-5 α 及质粒pcDNA3.1⁺由本室周忠江博士惠赠, T载体pUC18购自美国Stratagene公司。

1.2 酶和试剂

胎牛血清为杭州四季青产品, DMEM和RPMI 1640购自美国Gibco公司, 胰酶购自上海生工生物有限公司。总RNA提取试剂TRIZOL、Lipofectin和G418购自美国Invitrogen公司。LA RT-PCR试剂盒、限制性内切酶Sma I、Kpn I 和BamH I 以及T4连接酶购自大连宝生物公司。切胶回收和质粒提取试剂盒购自德国QIAGEN公司。

1.3 引物设计与合成

从Genbank检索出HIF-1 α cDNA序列设计引物[7]。HIF-1 α cDNA片段上游引物P1: 5' GAAACCA CCTATGACCTGC 3', 下游引物P2: 5' GTCGTGCTG AATAATACCACTC 3'; 全长上游引物P3: 5' TATAG GTACCATGGAGGGCGCCGCGC3', 下游引物P4: 5' GCGCGGATCCTCAGTAACTT GATCCAA 3'。下划线处分别是Kpn I 和BamH I 酶切位点。内参照 β -actin上游引物P5: 5' AGCGGAAATCGTGCCT GACA3', 下游引物P6: 5' GTGGACTTG GGAGAGGA CTGG 3'。

1.4 总RNA的提取和RT-PCR法获取HIF-1 α 基因

采用TRIZOL试剂, 按照说明书的方法从1 \times 10⁶个大肠癌细胞HT29中提取总RNA, 琼脂糖电泳鉴定总RNA有无降解。然后以引物P3和P4用RT-PCR方法扩增HIF-1 α 基因全长, 94 $^{\circ}$ C变性30 s, 65 $^{\circ}$ C退火30 s, 72 $^{\circ}$ C延伸4 min, 循环25次, 末次循环后72 $^{\circ}$ C再延长5 min。参照LA RT-PCR试剂盒说明书。

1.5 T载体的制备及TA克隆

pUC18用Sma I 酶切, 0.8%琼脂糖凝胶电泳, 切取线性片段, 胶回收纯化试剂盒纯化。在50 μ l 反应体系中, 含dTTP 2 μ mol/L, Tap DNA 多聚酶2.5 U, 线性质粒模板1 μ g, 72 $^{\circ}$ C 3 h, 电泳, 回收纯化。20 μ l 的连接反应体系中分别加入10 \times 连接Buffer 2 μ l, 纯化的PCR产物10 μ l, T载体5 μ l, T4 DNA连接酶 2 U, 12~16 $^{\circ}$ C 16 h。连接产物转化JM109菌株。酶切、克隆、阳性重组子鉴定的具体方法见参考文献[8]。

1.6 DNA序列测定

由大连宝生物公司完成。采用双脱氧链末端中止法，以DNA全自动测序仪测定核苷酸序列，同一片段经正反两个方向重复测定。

1.7 pcDNA3.1⁺-HIF-1 α 真核表达载体的构建及鉴定

pUC18-HIF-1 α 质粒经Kpn I和BamH I双酶切，获得HIF-1 α cDNA全长，插入到pcDNA3.1⁺的Kpn I和BamH I位点，获得重组质粒pcDNA3.1⁺-HIF-1 α 。酶切鉴定得到含HIF-1 α 的表达载体pcDNA3.1⁺-HIF-1 α 。酶切、克隆、阳性重组子鉴定的具体方法见参考文献[8]。

1.8 基因转染

在6孔培养板的每一孔内接种1.5 ml含6 \times 10⁵个细胞的培养液，37 $^{\circ}$ C 5%CO₂温箱培养约24 h，至80%~90%汇合时，用无血清DMEM培养基漂洗细胞2次。每孔加入Lipofectin-pcDNA3.1⁺-HIF-1 α 复合液2 ml。每孔的转染液中含2 μ g DNA和10 μ l Lipofectin，转染时间5 h。吸除无血清转染液，换入含10%胎牛血清培养液继续培养48 h，然后按1:5的比例传代。用含800 mg/ml G418的培养液进行筛选。当未进行转染的细胞大部分死亡时(3~5 d)再更换1次培养液。14 d左右未进行转染的细胞均死亡，转染的细胞孔内有抗性的克隆团出现，待其增大后再转入另瓶增殖，传代、留种。

1.9 RT-PCR法检测重组质粒表达

用含10%胎牛血清的DMEM培养基将细胞培养至80%的密度，TRIZOL试剂提取总RNA，取1 μ g作RT-PCR。引物用HIF-1 α cDNA片段引物P1、P2和内参照 β -actin引物P5、P6。热循环条件是：94 $^{\circ}$ C变性30 s，54 $^{\circ}$ C退火30 s，72 $^{\circ}$ C延伸1 min，循环25次。反应结束后取3 μ l反应产物行琼脂糖凝胶电泳。

2 结果

2.1 HIF-1 α cDNA的扩增、克隆及鉴定

通过RT-PCR方法从HT29细胞株的总RNA扩增出一条2 500 bp左右的DNA片段(图1)，将其与pUC18 T载体连接，转化JM109。扩增，提质粒进行DNA测序，所得序列与Genbank记载的HIF-1 α cDNA序列完全一致。

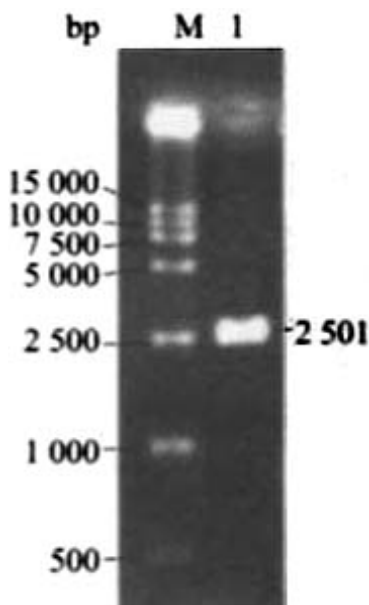


图1 RT-PCR扩增产物的鉴定

Fig.1 Analysis of RT-PCR amplification product
M: Marker (DNA Marker DL15000); Lane 1: RT-PCR product

2.2 重组真核表达质粒pcDNA3.1⁺-HIF-1 α 的鉴定

用Kpn I和BamH I分别单酶切重组质粒，得到线性质粒8 kb，与重组质粒大小一致；用这两个酶双酶切，

得到线性质粒和HIF-1 α 片段，大小为5.4 和2.5 kb，与空质粒和HIF-1 α cDNA大小一致(图2)。

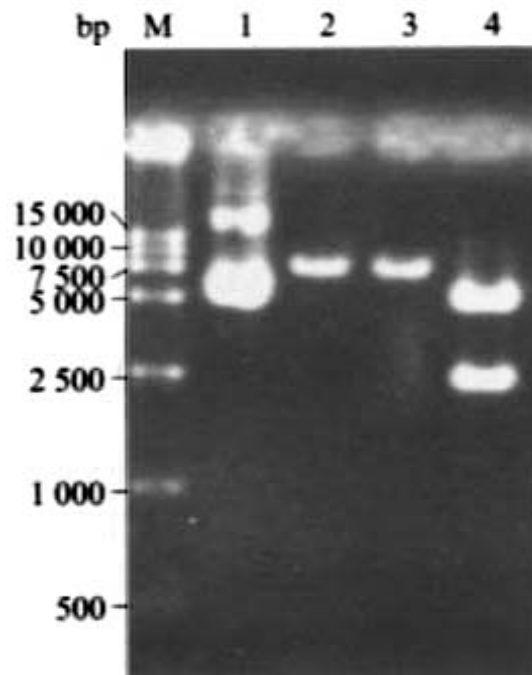


图2 重组表达载体pcDNA3.1⁺-HIF-1 α 的鉴定

Fig.2 Identification of the recombinant expression plasmid pcDNA3.1⁺-HIF-1 α
M: Marker; Lane 1: pcDNA3.1⁺-HIF-1 α ; Lane 2: pcDNA3.1⁺-HIF-1 α /Kpn I ; Lane 3:
pcDNA3.1⁺-HIF-1 α /BamH I ;
Lane 4: pcDNA3.1⁺-HIF-1 α /Kpn I +BamH I

2.3 RT-PCR检测重组质粒表达

用RT-PCR方法扩增细胞中的HIF-1 α 和 β -actin片段，电泳结果(图3)可见HEK293/pcDNA3.1⁺-HIF-1 α 细胞组HIF-1 α 条带比对照组的D(λ)值和相对D(λ)值明显大于对照组细胞，说明所构建载体能在转染细胞中转录。

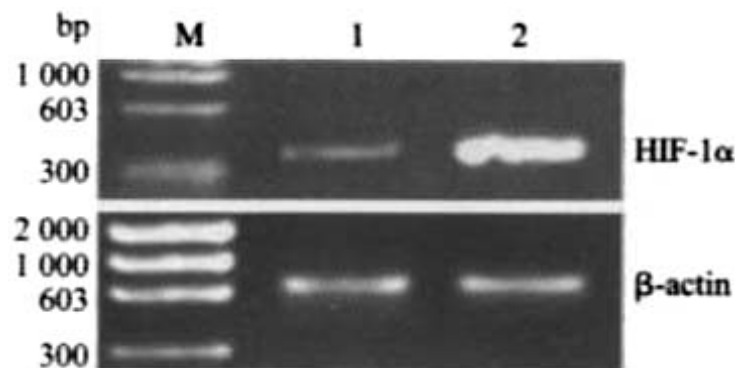


图3 RT-PCR扩增HEK293/pcDNA3.1⁺-HIF-1 α 细胞中HIF-1 α 基因片段

Fig.3 Fragment of HIF-1 α cDNA amplified by RT-PCR
M: Marker (DNA marker DL2000); Lane 1: HEK293; Lane 2: HEK293/pcDNA3.1⁺-HIF-1 α

评价HIF-1 α 的功能, 首先须将HIF-1 α 的基因编码区全长克隆到合适的载体上。我们采用RT-PCR方法, 用高保真的LA Taq酶将基因的全长扩出, 然后用TA克隆的方法, 成功将基因的cDNA克隆到T载体pUC18上, 测序证实后用双酶切法又将其克隆到真核表达载体pcDNA3.1⁺上, 并转染到HEK293细胞内, 构建了HEK293/pcDNA3.1⁺-HIF-1 α 细胞株。

心肌缺血时HIF-1 α 和血管内皮生长因子(VEGF)表达增加。VEGF是HIF-1 α 的下游基因[9]。Drake[10]报告, 在VEGF单独刺激下, 尽管血管网密度增加, 但新生的血管出现通透性增加和畸形。Elson等[11]证实了HIF-1 α 可诱导小鼠生成具有生理功能的新生血管。已经发现, 多种细胞因子或药物, 如TNF、IL-1、IFN、胰岛素、内皮素-1和血管紧张素II等能够促进HIF-1 α 在体内外的表达或转录激活功能[12][13][14][15]。研究显示, 通过用巨核细胞来源的多肽PR39可以抑制HIF-1 α 的降解, 引起心脏血管的新生[16]。

我们成功地构建了重组载体pcDNA3.1⁺-HIF-1 α , 初步结果显示, 转染到HEK293细胞后, 该载体可以在细胞内表达, 在血管紧张素II的刺激下, 蛋白表达量比对照明显增多。至于转染该基因对细胞生长的影响, 对VEGF、促红细胞生成素和葡萄糖分解酶类等HIF-1 α 下游基因表达的影响及能否在体内引起血管新生等问题, 有待进一步研究。

(责任编辑: 黄开颜)

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