适于无血清贴壁培养的抗凋亡宿主细胞系 CHO-IVB2 的构建

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摘 要 应用无血清培养基培养 CHO 细胞时，由于没有血清提供各种贴壁因子，细胞以悬浮的方式生长。在实际的大规模细胞培养中，CHO 细胞往往以贴壁方式培养，要么贴壁于悬浮的微载体中，要么贴壁于固定的聚酯盘状介质或中空纤维中，而很少直接悬浮于培养基中。在无血清培养基中，Vitronectin 单一组分可以促使 CHO 细胞的贴壁和扩增。通过双表达 *Igf*-1 和 *Bcl*-2 基因，已经构建了可以在无蛋白培养基 IMEM 中抗凋亡生长的细胞株 CHO-IB3。在此基础上，构建了可以同时表达 Igf-1、Vitronectin 和 Bcl-2 三个蛋白的三顺反子表达载体 pCI-NII-IVB。将该载体转染于 CHO-dhfr$^-$ 细胞中，构建了一个细胞株 CHO-IVB2。该细胞株可以在无蛋白培养基中抗凋亡生长，适于以贴壁的方式大规模培养，用于大量生产外源目的蛋白。

关键词 CHO 细胞，细胞工程，细胞凋亡，贴壁培养

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无血清培养基适于以悬浮方式大规模培养哺乳动物细胞。但在贴壁培养时，由于没有血清提供贴壁因子，细胞很容易从贴壁介质上脱落。Vitronectin 是众多贴壁因子中较为简单的一种，在无血清培养基中加入 Vitronectin 单一成分就可以介导 CHO 细胞的贴壁和扩展[1]。本节我们试图应用三顺反子表达载体在 CHO 细胞中同时表达 Igf-1、Vitronectin 和 Bcl-2 蛋白，而使细胞同时具有可在无血清培养基中生长、抗凋亡及贴壁培养的能力。

1 材料和方法

1.1 基因克隆

Vitronectin 基因从小鼠肝脏中克隆。分别应用 RNAgents® Total RNA Isolation System (Promega)和 SuperScript™ Preamplification System for First Strand cDNA Synthesis (Invitrogen)从 BALB/c 小鼠的肝脏中提取总 RNA，并反转录成 cDNA。RNA 的提取及反转录按试剂盒说明书操作。设计正向引物 pVtnOIF(GAGGCCTGCCATGGCATCTCTGAGG)和反向引物 pVtnIR(CTACTTCTCAGAGGTTGGGCAGC)，应用 PCR 从 cDNA中扩增出小鼠 Vitronectin 基因，条件如下：94℃ 30s，50℃ 40s(每轮退火温度增加 0.2℃)，72℃ 80s，共 40 cycles。耐热 DNA 聚合酶为 LA Taq 聚合酶(TaKaRa)。从凝胶中回收约 1.4kb 的条带，与 pGEM-T载体连接，测序。

1.2 载体构建

以引物对 pVnSmaF(GTCCCGGGCCACCATGGCATCTCTGAGG)/pVnEcoR(CCGAATTCCTACTTCTCAGAGGTTGGG)从经测序正确的 T-载体中扩增出 Vitronectin 基因，以 *Sma* Ⅰ/*Eco*R Ⅰ切割后连接进经同样酶切的 pBCIR 质粒[2]，得质粒 pBVNIR。以 *Sma* Ⅰ/*Eco*R Ⅴ从 pBVNIR 中切下 Vitronectin-IRES 片段，与经 *Sma* Ⅰ酶切并去磷的 pCI-NI-IB 连接[3]，命名为 pCI-NII-IVB(图 1)。每一步均经过酶切和序列测定加以验证。

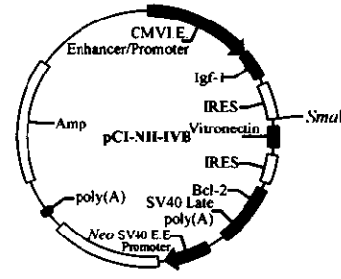

图 1 三顺反子表达载体 pCI-NII-IVB

Fig.1 Tri-cistronic expression vector pCI-NII-IVB

Driven by CMV promoter, the vector can transcribe a tri-cistronic mRNA, which contains three cDNAs spaced by two IRES sequences. The mRNA can translate three peptides: Igf-1, Vitronectin and Bcl-2

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1.3 Western blot 和细胞转染

Western blot 及细胞转染方法见参考文献[3]。

1.4 细胞在无血清培养基中贴壁性的检测

接种 CHO-dhfr⁻ 和 IVB2 细胞各 10^5 个于 6 孔细胞培养板中，于 IMDM/HT/10% FBS 培养基中培养 24h 使细胞贴壁，而后弃去培养基。加入 IMEM 无血清培养基继续培养 48h，使细胞充分分泌 Igf-1 和 Vitronectin 到培养基中。这样的培养基叫做 Conditioned 培养基。分别用胰酶消化 CHO-dhfr⁻ 和 IVB2 细胞，用 IMDM/HT/10% FBS 培养基洗涤 1 遍，以洗去并抑制残留的胰酶活性。两种细胞均以 IMEM 无蛋白培养基[3]重悬，分别计数 10^4 个细胞接种到 6 孔细胞培养板中，并分别加入等体积的 CHO-dhfr⁻ 细胞 Conditioned 培养基和 IVB2 细胞 Conditioned 培养基，继续培养。每日更换培养基，更换方法是弃去一半的 Conditioned 培养基，并加入相同体积的新鲜 IMEM 培养基。隔日计数活细胞数。

1.5 流式细胞术分析 Bcl-2 的表达

用胰酶消化下一瓶细胞，PBS 洗涤，多聚甲醛固定。穿透缓冲液(0.15% Triton X-100；0.1% BSA；PBS)室温处理 20min。离心收集细胞。细胞悬于 200μL 染色缓冲液(1% BSA；0.005% Tween-20；0.1% sodium azide；PBS)，加入一抗，4℃暗处染色过夜。以染色液洗涤 3 遍后，加入相应 FITC 标记的二抗(中山生物)，4℃暗处染色 4h。PBS 洗涤 3 次。上机分析。

1.6 细胞凋亡检测

1.6.1 通过流式细胞术检测外化磷脂酰丝氨酸而检测细胞凋亡：磷脂酰丝氨酸(Phosphatidylserine，PS)是细胞膜的正常组分，位于细胞膜的内侧。PS 与 Annexin V 有很强的结合能力。当细胞凋亡发生时，细胞膜外翻，而使 PS 暴露于细胞膜外。外露的 PS 可以与 EGFP 标记的 Annexin V(Clontech)紧密结合，从而应用流式细胞仪可以把凋亡细胞检测出来。根据 Clontech 说明书操作。

1.6.2 通过检测片段化 DNA 而检测细胞凋亡：细胞染色体片段化是细胞凋亡的一个典型特征。片段化的 DNA 可以通过凝胶电泳检测：以 Qiagen 质粒提取试剂盒从 10^6 个待检细胞中提取小片段 DNA，并用 1.5% 琼脂糖凝胶电泳分离 DNA 片段，紫外灯下观察拍照。

1.7 细胞计数

应用血球计数板对细胞进行计数，通过台盼蓝排除染色法区别活细胞和死细胞。

2 结果

2.1 基因克隆和载体构建

小鼠 Vitronectin 基因得到成功克隆，GenBank 注册号为 AF440693。三顺反子表达载体 pCI-NII-IVB 经酶切和序列测定证明为正确。

2.2 外源基因高表达克隆的筛选

应用 Lipofectamine™ 2000 Reagent (Invitrogen)转染 1μg pCI-NII-IVB 于 CHO 细胞中。以 800μg/mL G418 筛选阳性克隆。通过对 20 个 G418 抗性克隆进行 Western blot 分析，选出 3 个 Bcl-2 表达量较高的克隆，分别命名为 IVB1、IVB2 和 IVB3(图 2A)。对 IVB2 进行进一步分析，其 Igf-1 和 Vitronectin 的表达量均显著高于 CHO-dhfr⁻ 对照细胞(图 2B 和图 2C)。通过流式细胞术检测 Bcl-2 的表达量，进一步验证了图 2A 的结果(图 3)。以上结果表明，细胞株 IVB2 可以高表达 Igf-2、Vitronectin 和 Bcl-2，有可能使细胞具有可在无血清培养基中生长、可贴壁和抗凋亡的能力。

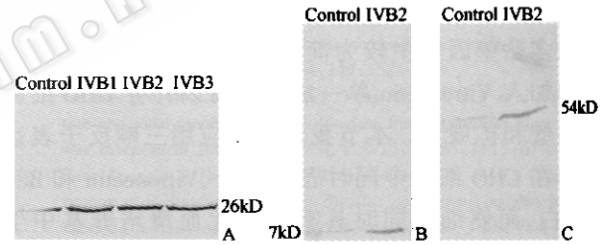

图 2 Western blot 分析基因的表达

Fig.2 Western blot analysis of expressed genes

A: total proteins were extracted with methods described in 1.3 from 20 G418-resistant clones previously transfected with pCI-NII-IVB. The proteins were analyzed for Bcl-2 expression with Western blot. Three clones that expressed highest level of Bcl-2 were shown here. They expressed higher level of Bcl-2 than the CHO-dhfr⁻ control clone.

B and C: clone IVB2 was analyzed again with Western blot for expression of Igf-1 and Vitronectin respectively. The figures showed that expression level of Igf-1 and Vitronectin in IVB2 clone were much higher than CHO-dhfr⁻ control clone

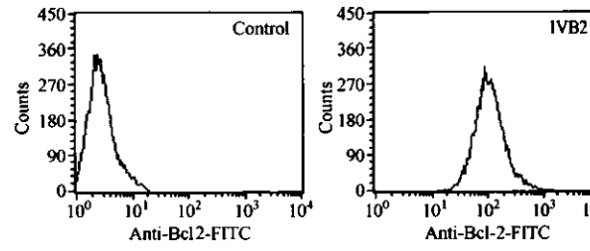

图 3 流式细胞术检测 IVB2 细胞株 Bcl-2 的表达量

Fig.3 Flow cytometry analysis of Bcl-2 expression

Flow cytometry analysis of Bcl-2 expression were conducted as described in 1.5. Clone IVB2 expressed higher level of Bcl-2 than CHO-dhfr⁻ control clone, which was consistent with Fig. 2A

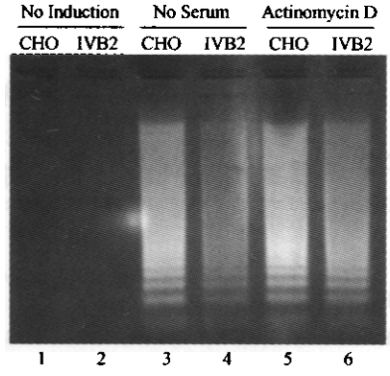

图 4　DNA Ladder 法检测撤去血清或加入放线菌素 D 诱导的细胞凋亡

Fig.4　DNA ladder detection for analysis of apoptosis induced by withdrawal of serum or addition of actinomycin D

Apoptosis was induced by withdrawal of serum (3, 4) or addition of actinomycin D (5, 6). Controls were set without apoptosis induction (1, 2). One day after apoptosis induction, small fragmented DNA was extracted from 10^6 cells with plasmid extraction kit. Electrophoresis showed that IVB2 clone (4, 6) generated less fragmented DNA than control clones (3, 5), which meant IVB2 was more resistant to apoptosis

2.3　IVB2 细胞抗凋亡能力的检测

IVB2 细胞和 CHO-dhfr$^-$ 对照细胞株均以 IMDM/HT无血清培养基培养 24h,或在 IMDM/HT/10%FBS 培养基中加入终浓度为 0.5μg/mL 的放线菌素 D (Actinomycin D)培养细胞 24h,对细胞凋亡进行诱导。应用 DNA Ladder 法或流式细胞术对细胞凋亡进行检测。结果如图 4 和图 5 所示。无论应用撤去血清还是加入放线菌素 D 诱导细胞凋亡,IVB2 细胞株显示的 DNA Ladder 均显著少于对照细胞株(图 4),表明 IVB2 具有比对照细胞株更强的抗凋亡能力。应用流式细胞术对放线菌素 D 诱导的细胞凋亡进行分析,IVB2 的凋亡率为 45.2%,而 CHO-dhfr$^-$ 对照细胞的凋亡率高达 96.5%,结果与 DNA Ladder 法相符(图 5)。

2.4　IVB2 细胞的连续培养

接种 CHO-dhfr$^-$ 和 IVB2 细胞各 1×10^4 个于 6 孔细胞培养板中,在 IMDM/HT/10% FBS 中培养 24h 使细胞贴壁,而后弃去培养基,PBS 充分洗涤细胞。把每种细胞分成 4 组,分别加入 IMDM/HT/10% FBS、IMDM/HT/1% FBS、IMDM/HT/0% FBS 以及 IMEM 无蛋白培养基。IMEM 无蛋白培养基由本室设计,为 IMDM 培养基添加补充成分而构成[3]。为使细胞易于贴壁而不致影响实验结果,IMEM 中另添加 40 μg/mL Fibronectin 和 0.5μg/mL Vitronectin。连续培养细胞 2 周,每天更换新鲜的培养基。对于 IVB2 更换方法为取走一半细胞培养上清,以保留细胞自身分泌表达的 Igf-1 和 Vitronectin,而后加入相同体积的新鲜培养基。其它细胞的换液方法相同,以具有可比性。每隔 1d 消化下在各个培养条件下的三种细胞各 3 个培养孔,进行活细胞计数。结果如图 6 所示。在 IMDM/HT/10% FBS 培养基中,两种细胞差别不明显,增殖速度都很快(A);在 IMDM/HT/1% FBS 培养基中,细胞增殖速度都显著减慢,但 IVB2 的增殖速度高于 CHO-dhfr$^-$ 对照细胞,且具有显著性(B);在 IMDM/HT/0% FBS 培养基中,两种细胞的生长都十分缓慢,在第 4 天后,细胞开始死亡,细胞数开始降低,但 IVB2 的细胞数降低趋势小于 CHO-dhfr$^-$ 对照细胞(C);在 IMEM 无蛋白培养基中,CHO-dhfr$^-$ 对照细胞生长较差,而 IVB2 细胞的生长较好,与在 IMDM/HT/1% FBS 培养基中的生长曲线相似。表明 IVB2 适于在无蛋白培养条件下大规模培养(D)。

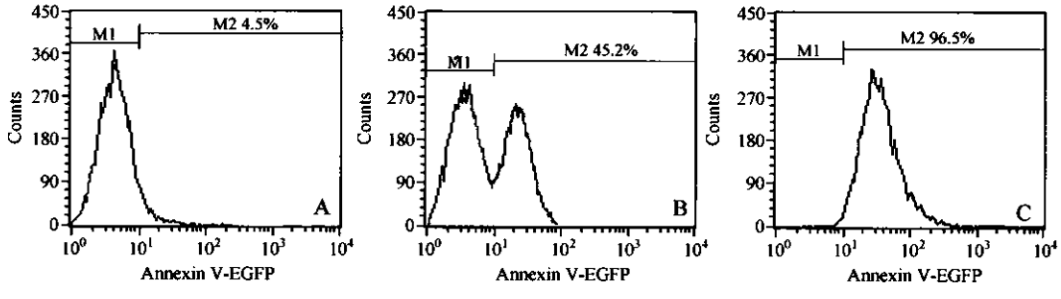

图 5　流式细胞术检测放线菌素 D 诱导的细胞凋亡

Fig.5　Flow cytometry analysis of apoptosis induced by actinomycin D

Apoptosis was induced by addition of 0.5μg/mL actinomycin D for 24h. Externalized PS was analyzed for detection of apoptosis.

A: CHO-dhfr$^-$ control without apoptosis induction, 4.5% underwent apoptosis. Apoptosis rate of un-induced IVB2 was similar (data not shown here);

B: IVB2 induced with actinomycin D, 45.2% underwent apoptosis;

C: CHO-dhfr$^-$ induced with actinomycin D, 96.5% underwent apoptosis

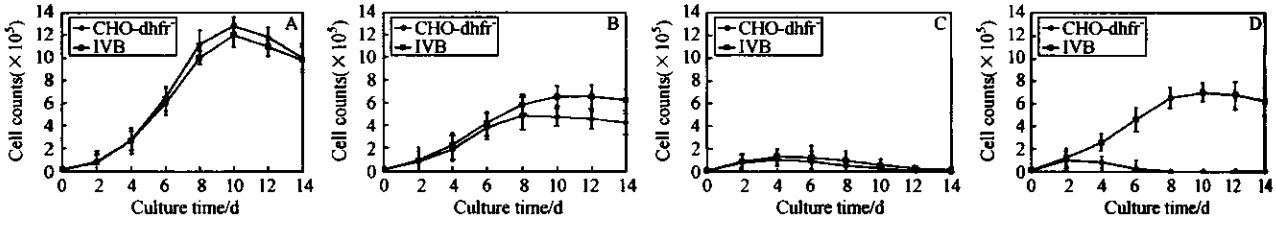

图 6 IVB2 细胞的连续培养

Fig.6 Continuous culture of IVB2

CHO-dhfr⁻ and IVB2 were cultured respectively for two weeks in 4 different media: IMDM/HT/10% FBS (A), IMDM/HT/1% FBS (B), IMDM/HT/0% FBS (C) and IMEM protein-free medium (D). Cell number was counted every other day. IVB2 exhibited much higher cell number than CHO-dhfr⁻ control cell line in IMEM medium after the 4th day ($P < 0.01$). In the figure, the bars represent standard deviation calculated from three independent tests

2.5 IVB2 细胞的无血清贴壁培养

根据方法 1.4,对三株细胞 CHO-dhfr⁻、IB3[3] 和 IVB2 进行无血清贴壁培养。结果如图 7 所示。在无蛋白培养基 IMEM 中培养时,从第 6 天之后,IVB2 的贴壁活细胞数显著高于 IB3($P < 0.01$)。尽管 IB3 已被证明可以在 IMEM 中生长,但在没有贴壁因子存在时,细胞难以贴壁生长。以上结果表明 IVB2 细胞易于无血清贴壁培养,适于大规模表达外源蛋白。

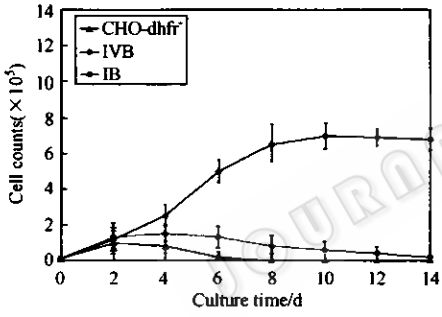

图 7 IVB2 细胞的无蛋白贴壁培养

Fig.7 Adherent culture of IVB2 in protein-free medium

IVB2 and control cell lines IB3 or CHO-dhfr⁻ were seeded into 6-well plate at 10^4 cells. They were cultured in 1:1 IMEM medium and corresponding conditioned media continuously for 2 weeks. Halves of culturing media were changed into new IMEM every day. Attached cells were counted every other day. The differences of cell numbers between IVB2 and IB2 or CHO-dhfr⁻ were significant after the 6th day ($P < 0.01$). In the figure, the bars represent standard deviation calculated from three independent tests

3 讨论

CHO 细胞是一种兼性贴壁细胞,既可以贴壁培养,又可以悬浮培养,而且两种培养方式都可以用于大规模生产外源蛋白。悬浮培养易于放大,适合超大规模的工业化培养。但悬浮培养的 CHO 细胞生长缓慢,外源蛋白表达量低[4,5];而贴壁培养的 CHO 细胞,细胞外基质可以启动一些信号通路,下调 p27 的表达,刺激细胞分裂,因而细胞生长较快,表达外源蛋白的能力也较强[6]。而且贴壁培养还有其它显而易见的优点:在连续灌流培养过程中,贴壁培养的细胞不会被洗脱;贴壁培养的细胞容易与培养基分离,表达的生物活性物质易于回收;细胞的贴壁性更易于 96 孔板大规模高通量筛选;在细胞株的克隆选择方面,贴壁细胞显然更具优势。因而在较小规模的工业化细胞培养和中试规模的细胞培养中,贴壁培养更为常用。其常用介质有微载体(Microcarrier)、聚酯盘状介质(Poly ester disc)和中空纤维(Hollow fiber)等。

在无血清培养基中,细胞只能以悬浮的方式生长。如果应用微载体等贴壁培养方式,细胞会从贴壁介质上脱落到培养基中。贴壁培养往往应用机械搅拌方式(而直接悬浮培养多用气升式搅拌),剪切力较大,脱落的细胞注定死亡。细胞内容物释放到培养基中不仅会对下游成品纯化造成困难,也会对其它细胞造成影响,促进细胞凋亡的发生。Vitronectin 是一类分子量较小、结构较简单的贴壁分子,该分子单一成分就可以介导 CHO 细胞和 293 细胞在无血清培养基中的贴壁和扩展[1,7],因而通过使 CHO 细胞表达该分子以促进自身的贴壁是现实的。Gandor 等首先做了尝试,他们把人 Vitronectin 基因置于 MMTV 地塞米松诱导型启动子的下游,转染 CHO 细胞,获得了稳定株。当培养基中加入地塞米松时,Vitronectin 获得表达,并促使 CHO 细胞贴壁[8]。

在以往工作中,我们构建了两株可以在无蛋白培养基中培养的抗凋亡细胞株:CHO-IB 和 CHO-BC[3]。这两株细胞都以悬浮的方式生长。为了易于培养,我们在培养基中添加了 40μg/mL Fibronectin 和 0.5μg/mL Vitronectin 以促进贴壁。但贴壁因子很昂贵,在大规模培养时,添加贴壁因子会极大地提高

生产成本。因此在本研究中,我们在 pCI-NI-IB 载体的基础上,插入了 IRES 序列和小鼠 Vitronecin 基因,构建了三顺反子表达载体 pCI-NII-IVB,在 CHO 细胞中同时表达 Igf-1、Vitronectin 和 Bcl-2,使细胞不仅获得了在无蛋白培养基中抗凋亡生长能力,还获得了贴壁的能力。

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Construction of CHO-IVB, A Serum-independent, Apoptosis-resistant Cell Line that can Grow in Adherence

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Abstract Without serum to provide adherent factors, CHO-dhfr$^-$ cells grow in suspension when cultured in serum-free medium. Although this offers advantages in some applications, in most production systems adherent cell growth is preferable. Gene transfection, clonal selection and amplification can be easier for adherent cells; the density of immobilized cells is often higher than those in suspension culture, which results in a higher protein productivity; washout of cells by perfused medium during continuous fermentation can be avoided; for high-throughput microplate assays, adherent cells are preferred to facilitate medium changes and cell washing. It has been proved that purified vitronectin alone was able to mediate attachment and spreading of CHO cells in serum-free medium. So we constructed a tricistronic expression vector expressing Igf-1, Vitronectin and Bcl-2 at the same time. The vector was transfected into CHO-dhfr$^-$ cells and one clone, namely CHO-IVB2, expressing high level of the three proteins was screened out by Western blot. The cell line showed similar apoptosis-resistant and serum-independent properties to CHO-IB, an engineered cell line constructed before. When cultured in IMEM protein-free medium without any components supplemented, CHO-IVB can grow adherently. The viable cell numbers and growth rate of CHO-IVB were much higher than CHO-IB, making CHO-IVB an apoptosis-resistant host for production of recombinant proteins which can grow adherently in protein-free medium.

Key words CHO cells, cytotechnology, apoptosis, adherent culture

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