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Original Article

Construction of functional bicistronic vector for gene transfer and simultaneous expression of hNkx2.2 and eGFP in human cells

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بناء ناقل وراثي مزدوج وفعال للتعبير الجيني المتزامن لعامل الاستنساخ ان اكس 2.2 والبروتين الأخضر الفوسفوري في الخلايا البشرية

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الملخص العربي

الهدف: كثير من الأبحاث التي حاولت استخدام سلسلة دخول الريبوسوم الداخلية لبناء النواقل الوراثية المزدوجة واجهت صعوبة في تحقيق تعبير جيني للبروتين الثاني في السلسلة. لذا قمنا ببناء ناقل وراثي مزدوج للتعبير الجيني المتزامن يحتوي على سلسلة دخول الريبوسوم الداخلية بين سيسترونية وسلسلة البروتين الأخضر الفوسفوري المعزز.

الطريقة: تم بناء الناقل الوراثي pIRES-2-eGFP المحتوية على سلسلة دخول الريبوسوم الداخلية بين سيسترونية المعززة للتعبير الجيني المستهدف. تم تحليل نيوكليوتيدات الناقل الوراثي الذي تم بناءه بواسطة التحليل التسلسلي للحمض النووي لتأكيد اندماج السلاسل بالشكل الصحيح. تم اختبار فعالية الناقل وقدرته على التعبير الجيني من سلسلة تحفيز السيتوميقالو فيرس بواسطة الويسترن بلوتينق و التحليل المجهري الفوسفوري.

النتائج: التحليل التسلسلي للحمض النووي للناقل الذي تم بناءه أكد اندماج سلسلة عامل الاستنساخ البشري ان اكس 2.2 في الكاسيت الثنائي وبالتالي بناء الناقل المطلوب بالصورة الصحيحة. تحاليل الويسترن بلوتينق والتحليل المجهري الفوسفوري أكدت ايضا فعالية الناقل وقدرته على التعبير الجيني وإنتاج عامل الاستنساخ البشري ان اكس 2.2 بمقاسه الصحيح والنشاط الحيوي للبروتين الأخضر الفسفوري في نموذج الخلايا المستزرعة.

الإستنتاج: سلسلة دخول الريبوسوم الداخلية تمثل اداة قيمة لإمكانية التعبير الجيني المتزامن والمتعدد السيسترون من نسخة الجين النووي المراسل الناتج من النواقل الجينية والنواقل الوراثية المتعددة الاجيال ذي تم إنتاجه سوف يكون مفيد للنقل الجيني ولدراسة تخصيص الخلايا الجذعية في المستقبل.

ABSTRACT

Objective: Many researchers using Internal Ribosome Entry Site (IRES) elements for construction of bicistronic gene transfer vector reported difficulties to achieve significant expression of the second cistron. In the present study, we have constructed an expression vector containing a bicistronic cassette composed of the human oligodendrocyte transcription factor 2 cDNA (*hNkx2.2cDNA*), an inter-cistronic sequence, the IRES and enhanced green fluorescent protein (eGFP) sequences.

Methods: The hNkx2.2 cDNA sequence together with an inter-cistronic sequence of 168bp in length was subcloned into the pIRES-2-eGFP mammalian expression vector. Expression of the transgenic proteins from the upstream cytomegalovirus (CMV) promoter was confirmed by Western and fluorescence microscopy analyses.

Results: DNA sequence analysis was performed on nascent vector DNA confirmed the integrity of Nkx2.2 cDNA in the bicitronic cassette and the construction of the desired vector. Analyses of vector containing the cassette clearly confirm the functionality of the produced vector, correct size of the generated Nkx2.2 protein and the biological activity of the eGFP reporter in the cell culture model. Transcription of the bicistronic cassette is driven by a massage from a common upstream CMV promoter and translation of the two cistrons is uncoupled.

Conclusion: IRES sequence provides a valuable tool for simultaneous expression of bicistron from a single mRNA transcript incorporated in the gene transfer and therapeutic vector. The produced bicistronic hybrid vector will be useful for gene transfer and of great value for future stem cells transdifferentiation studies.

Keywords: Diabetes, Miletus, Infection, Bacteria and fungus

INTRODUCTION

Simultaneous expression of multiple genes from a single gene transfer vector is an important requirement in molecular and cellular therapies.[1,4] There are generally three common strategies in which two genes can be co-expressed from a single vector. Firstly, two genes can be fused together in frame to produce a chimeric sequence, ensuring simultaneous expression of both genes in one protein.[5,7] However, this strategy may not work for all combinations of proteins, some of which could result in protein misfolding or mistargeting. Secondly, two transcriptional units can be constructed with separate promoters to drive expression of different genes in the same vector. The major disadvantage of constructing such a dual promoter vector is possible transcriptional interference and/or dissociated gene expression, with a fraction of the transfected cells expressing the selectable marker but not the gene of interest and vice versa.[8,10] To overcome the above shortcomings, the third strategy involve the construction of a bicistronic cassette, in which the two heterologus genes are separated by an element known as internal ribosome entry site (IRES) sequence. Transcription of both genes in the bicistronic cassette is driven by a massage from a common upstream promoter, thus eliminating promoter interferance. As a result, a single messenger unit including the bicistronic transcript of both genes spaced by IRES is produced. Translation initiation of the first cistron at the 5' of IRES is typically mediated by a cap-dependent translation initiation mechanism[11] but the second cistron at the 3' of IRES is translated via cap independent translation initiation, mediated by the IRES element which functions as ribosome-binding sites for internal initiation of translation.

Since both the first and the second genes in a bicistronic cassette are under the control of the 5' upstream promoter, detection of the protein encoded by the second cistron is theoretically the insurance that the first cistron is also being expressed. However, many researchers using IRES elements for construction of bicistronic gene transfer vector reported difficulties to achieve significant expression of the second cistron. In the present study, we have constructed an expression vector containing bicistronic cassette under control of the *CMV* promoter. This bicistronic cassette contains the human oligodendrocyte transcription factor 2 cDNA (hNkx2.2cDNA) and the IRES-eGFP cassette sequences. We then examined the efficiency of this vector with the aim to use it in future gene transfer studies for stem cells transdifferentiation and stem cells based gene therapy.

MATERIAL AND METHODS

Plasmid Construction and Cloning

The hNkx2.2cDNA insert was excised from pBatl2-Nkx2.2 plasmid by digestion with XhoI/BamHI using BamHl buffer from NEB in a total volume of 200µl reaction mix topped with 3 drops of oil and incubated at 37°C for 14 hrs. The tube was incubated at 65°C for 20mins to inactivate the endonucleases. Digested DNA was then cleaned with PCR purification column (Qiagen) and eluted in 40µl sterile DPEC treated H_2O to ensure removal of enzymes and salts. Approximately, 5µg of the pIRES-2-eGFP plasmid DNA was also digested with XhoI/BamHI enzymes, heat inactivated at 65°C for 20 mins and column purified to remove the short XhoI-BamHI flanking sequence. Purified digested vector and

insert DNA were mixed together in 1:10 ratio and ligated using T4 DNA ligase for overnight at 16°C. Ligated mix was purified again using the Qiagen column and eluted in 30µl TE buffer. Only 2ul of eluted ligation mix was used to tranform Top 10 competent cells. Transformation was performed according to invitrogen recommendations and 300µl of serially diluted SOC media containing transformed bugs was plated on *Kan* resistance plates. Colonies were allowed to grow for overnight and then subcultured on *Kan* and *Amp* resistance plates. Only *Kan* resistance colonies were screened further by insert-vector PCR method [12] using the forward primer 5'-CAC CAA AAT CAA CGG GAC TT-3' and the reverse primer 5'-TTT GTG TTG GTC AGC GAC AT-3' which anneals to the 5' and 3' restriction/ligation site. Colonies producing an amplicon 244bp were inoculated for overnight culture and the integrity of the hNKx2.2-insert in the pNKx2.2-IRES-eGFP clones was examined by restriction digestion and DNA sequence analysis.

Gene transfer into cultured cells

The human embryonic kidney epithelial (HEK 293), the Hela, and hfMSCs cells were cultured with Dulbecco's modified Eagle's medium (Invitrogen or Sigma, UK) supplemented with 10% fetal calf serum (FCS, Sigma). All cells were incubated at 37°C with 5% CO_2 in a humidified incubator. Cells were seeded into a $10cm^2$ dishes at 60-70% confluence with a density of $5x10^6$ cells and transfected the following day with 10µg of the expression vector DNA using FuGene6 (Roche, UK) according to the manufacturer's instructions. Forty-eight hours later, cellular expression of fluorescent proteins was examined with a fluorescence microscope (type). Mock transfected cells were used as controls. All transfection experiments were repeated at least three times. Approximately 48 hours post-transfection, HEK 293T cells were analysed for cellular expression of eGFP using fluorescence microscopy.

Western blot analysis of protein expression

Forty eight hours after transfection, cells were harvested by scrubbing and re-suspended in $30µl/10^6$ cells ice-cold lysis buffer (10mM Tris pH 7.5, 1mM EDTA, 1% NONIDET P-40 (Sigma, UK), protease inhibitor cocktail at indicated dilutions (Roche, UK)). Cell suspensions were then centrifuged at 13,000×*g* for 20 mins at 4°C. The protein content of cell lysate supernatants was assayed by the bicinchoninic acid method using a commercial assay kit from Pierce with bovine serum albumin as a standard. Five milligrams protein of each lysate were combined with equal volumes of Laemmli sample buffer, boiled for 5 min and were separated on a 13% or 11% SDS-PAGE gel for NKx2.2, and β-actin protein detection, respectively. Gels were then blotted onto PVDF membranes with a Hoefer apparatus (200 mA). After 1 h incubation at room temperature in blocking solution (5% dried skimmed milk in PBS-Tween-20 0.1%), membranes were incubated overnight at 4°C with specific primary antibodies, diluted as specified below with blocking solution. After three 5-min washes in PBS-Tween-20, blots were incubated at room temperature for 1h with peroxides-conjugated goat anti-rabbit antibody (Abcam) or rabbit anti-mouse polyclonal antibody (Dako, UK) diluted 1:1000 in blocking solution. Following the final wash detection on autoradiography hyper-films was performed after inducing a chemiluminescence reaction with the Amersham Supersignal detection kit. Primary antibodies used in this study were anti-NKX2.2 (polyclonal, 1:2000; Research Diagnostic, USA) and anti-GFP (polyclonal, 1:2000; abcam, UK) and anti-beta-tubulin (1:2000; Sigma, UK).

RESULTS

Structure of the bicistronic cassettes and proof of clonality

We placed the hNkx2.2cDNA sequence, at the 5' of IRES element (Figure 1-A). The hNkx2.2cDNA and the 168bp downstream spacer sequences were derived from the pBatl2-Nkx2.2 plasmid which was a kind gift of Dr Huseyin Mehmet, Imperial College London, UK. The pIRES2-eGFP expression vector was chosen because it contains the IRES of the encephalomyocarditis virus (ECMV) which has high translation efficiency compared to other IRES sequences including those from hepatitis A and C viruses, poliovirus, human rhinovirus, and foot-and-mouth disease virus.[13,16] In addition to its high efficiency, ECMV IRES possess a broad tissue tropism.[17] which makes it the most widely used in gene transfer protocols.[18,20] This vector also permits high level of plasmid DNA production and the multi-cloning site offer the possibility of convenience cloning. The eGFP incorporated in this vector is a red-shifted variant [21,22] of wild-type GFP which has been optimised for brighter fluorescence and higher expression in mammalian cells. Moreover, sequences flanking the eGFP have been converted to a Kozak consensus translation initiation site [23] to further increase the translation efficiency in eukaryotic cells. The initiator AUG codon of the eGFP is located 12 bases downstream of the IRES element. Such a short distance is nevertheless sufficient to greatly affect the eGFP expression. The eGFP was used as a marker for easy, rapid and less expensive detection and monitoring of expression.

DNA fragment containing the *hNkx2.2cDNA* was subcloned into the pIRES2-eGFP (Clontech) mammalian expression vector and its integrity was confirmed by PCR colony screening method (Figure 1-B) and DNA sequence analyses (Figure 1-C).

The correct 244bp fragment size was found in 8 colonies (Figure 1-B), indicating Nkx2.2cDNA insertion in the desired orientation. The other twelve screened colonies showed no PCR product and therefore excluded from further screening. The ligation mixture was also examined by insert-vector PCR[12] as positive control to confirm the physical presence of the expected recombinant plasmid containing the hNKx2.2cDNA in correct orientation. The PCR positive colonies were then grown individually in LB growth media under kanamycin selection. DNA sequence analysis was performed on nascent vector DNA which also confirmed the integrity of Nkx2.2 cDNA in the bicitronic cassette and the construction of the desired mammalian expression vector (Figure 1-C).

Analysis of hNKx2.2 expression by Western blotting

Analysis of gene expression was performed by Western blotting and fluorescence microscopy after cells transfected with bicistronic construct containing 5'-Nkx2.2-IRES-eGFP-3' cassette. Western analysis ensures that the expressed proteins of a correct mature sizes and the fluorescence microscopy permits accurate assessment of gene transfer and expression at a single cell level.

Immunoprobing with anti-hNKx2.2 antibody (Figure 2-A) shows the mature form of the hNKx2.2 protein from cell extracts transfected with the pNkx2.2-IRES-eGFP construct. The hNKx2.2 is migrating as one band of apparent molecular masses of 32kDa (lane 2). No signals were detected from the mock transfected negative controls (lane 1) or from extract of cell transfected with pIRES-2-eGFP mother plasmid (lane 3). Immunoprobing of the membrane with anti-β-actin antibody in Figure 2-B shows the production of β-actin protein of

the expected molecular mass of approximately 47kDa from cells. No other proteins were recognised from cell extract immunoprobed with these antibodies.

In summary, the data provided above show that the produced vector is functional and the generated proteins of correct sizes.

Analysis of eGFP expression by fluorescence microscopy

Fluorescence microscopy analysis was carried out for the detection of cellular expression of eGFP approximately 48 hours post-transfection. Transfected human kidney epithelial (HEK 293T) cells were able to express high levels of eGFP compared to mock transfected control as shown in Figure-3. Transfection efficiency of cells was estimated to be more than 90%.

Figure 1. Diagram of the constructed 5'-NKx2.2-IRES-eGFP-3' bicistronic expression cassettes and the clonality analysis. A) Schematic diagram of the constructed expression vector showing the orientation of 5'-NKx2.2-IRES-eGFP-3' transcriptional unit and the position of the upstream CMV promoter. B) Insert-vector PCR analysis for screening and selection of the desired clone. Amplicon size of 244bp generated from lane 3, 4, 5, 6, 7, 8, 9, 10, 14, indicate clonality and integrity of Nkx2.2 cDNA in the bicistronic cassette. The ligation mixture was used as positive control. Clone at lane 10 was used in the subsequent analysis. C) Sequence analysis at the ligation site from purified plasmid DNA proof construction of 5'-NKx2.2-IRES-eGFP-3' bicistronic expression cassettes.

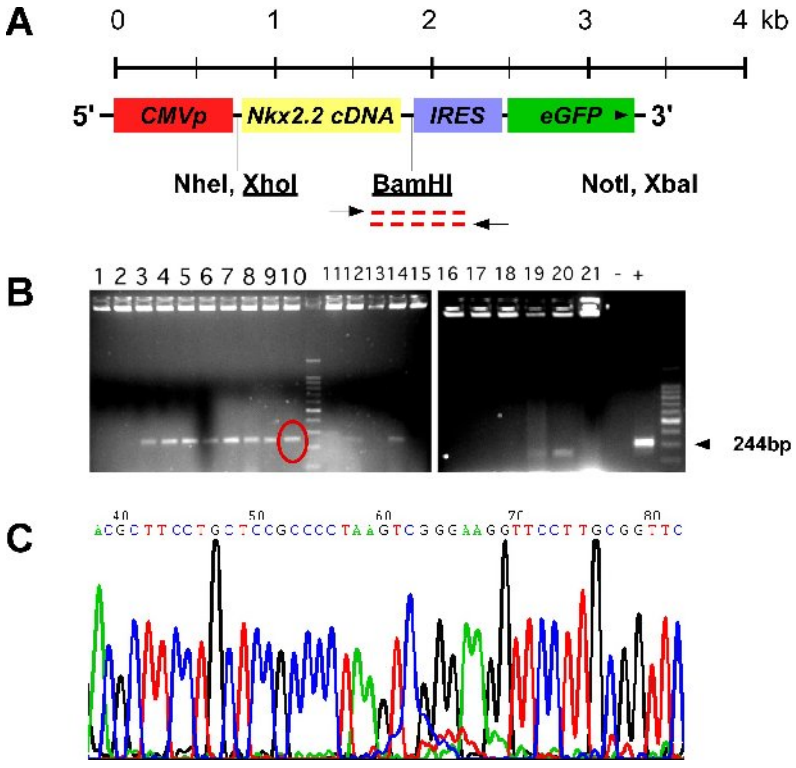

Figure 1.

Figure 2. Expression of NKx2.2 protein from HEK 293 cells transfected with pNKx2.2-IRES-eGFP constructed vector. 5µg of HEK 293 cellular extracts transfected with 10µg DNA of the pNKx2.2-IRES-eGFP construct were loaded into lane of 13% (A) and 11% (B) SDS-PAGE gel for NKx2.2, and β-actin protein detection, respectively. **A)** Immunoblotting with anti-hNKx2.2 antibody shows the mature form of the hNKx2.2 migrating as one band of apparent molecular mass of 32kDa (lane 2). No signals were detected from the mock transfected negative controls (lane 1) or from extract of cell transfected with pIRES2-eGFP mother plasmid (lane 3). Positive control is extract of primary culture of rat oligodendrocyte precursor CG4 cells (+). **B)** Immunoblotting of the membrane with anti-β-actin antibody shows the production of β-actin protein of the expected molecular mass of approximately 47kDa from cells. No other proteins were recognised from cell extract immunoprobed with these antibodies.

Do not forget a and b

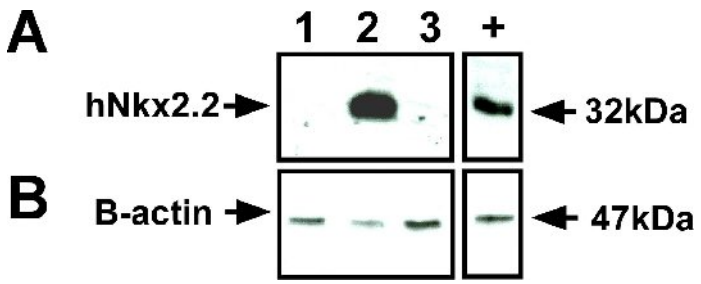

Figure 2.

Figure 3. Fluorescence microscopy analysis of IRES-mediated eGFP expression from pNKx2.2-IRES-eGFP bicistronic construct in mammalian cells.
A) Phase contrast photo of transfected HEK 293T cells. B) High levels of eGFP expression which confirms functionality of the bicistronic vector.

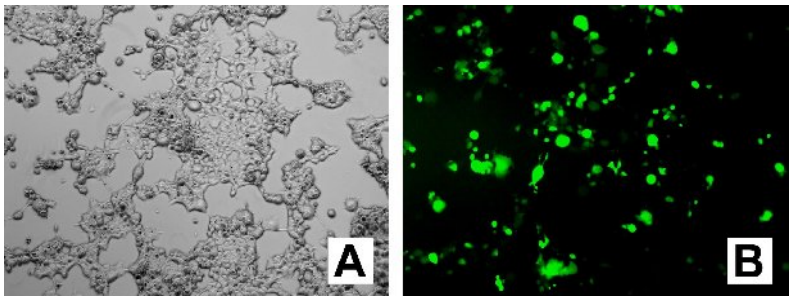

Figure 3.

DISCUSSION

When an IRES is used for the construction of a multi-cistronic cassette in gene transfer and expression vectors, it has been frequently observed that the DNA sequence subcloned at the 3' to the IRES element is poorly expressed compared to the sequence subcloned at the 5' of the IRES.[24,28] In most of the reported articles this phenomenon is attributed to the low efficiency of IRES mediated translation initiation compared to cap dependent translation. This is possibly due to the following; firstly, a higher affinity of translation factors for the cap structure than for the IRES element, therefore, transcription factors may become less available for the internal initiation of translation.[27] Secondly, the IRES may requires additional host *trans*-acting factors, which modulate its function in a cell or tissue type specific manner [(29,31)]. Thus, high levels of IRES mediated internal translation initiation can be anticipated depending on the nature of the IRES sequence and its tissue tropism.[13] Another possible explanation for this phenomenon is the length and possibly the nature of the ICS sequence flanking the IRES element at both its 5' and 3' ends. [13, 17]

In this work, an IRES derived from the ECMV genome was used to mediate eGFP marker gene translation initiation. We have successfully constructed bicistronic cassette containing the *hNkx2.2cDNA* and the *IRES/eGFP* elements. We then analysed the expression levels of the respective transgenes systematically at the DNA and protein levels. We showed functionality of the produced vector and the correct size of the transgenic proteins.

Expression efficiency of the eGFP was obtained when the ICS was 168bp in length. However, the expression levels of eGFP become completely undetectable at a spacer length of 450bp (data not shown). These results are in agreement with data published by Attal et al.[32] who found that IRES elements from ECMV as well as from poliovirus function optimally when about 100 nucleotides were added after the termination codon of the first cistron (luciferase). These authors also showed that IRES elements become totally inefficient when added after a 300-500 nucleotide spacer.[32] In a similar study using the IRES elements from poliovirus and from SV40, respectively in bicistronic cassettes containing the firefly luciferase gene as the first cistron and the *CAT* gene as the second cistron, Attal et al.[25] also showed that the expression of the second cistron was undetectable when the spacer fragment was 500 nucleotides. To exclude the effect of tissue tropism, we have also transfected our construct into Hela and hfMSCs cells and obtained similar results.

The produced bicistronic hybrid vector will be useful for simultaneous gene transfer and expression of the hNKx2.2 and the marker eGFP genes and of great value for stem cells transdifferentiation studies. The human *NKx2.2* gene encodes a homeobox protein which acts as a nuclear transcription factor involved in the morphogenesis of the central nervous system[33] and in the control of expression of myelin genes as *Nkx2.2* is able to drive gene expression of myelin proteolipid protein, an OL-specific marker. Specifically, cells hosting a proteolipid protein promoter-GFP reporter construct showed fluorescence when transfected with an Nkx2.2 encoding vector.[33] In addition to its role in neural development and differentiation, Nkx2.2 is also critical for the early pancreatic endocrine development and the following differentiation into pancreatic β cells.[34] as Nkx2.2 knockout mice are completely devoid of insulin expression.[35] In 2005, Zhang *et al.*[36] used transfection methods and expression plasmids to deliver *Nkx2.2* gene inside neural stem cells and accomplished a transient expression up to 12 days with a 60-80% efficiency of transfection. Accurate

assessment of gene transfer and expression following transfection may require a marker gene to evaluate levels and longevity of expression. In the present study, transfection of HEK 293 cells permit up to 90% transfection and eGFP expression.

We are also aware that transdifferentiation of stem cells by gene transfer may require long-lasting expression of the transgenic NKx2.2 in the relevant cell model, therefore, future work will focuses on subcloning the 5'-NKx2.2-IRES-eGFP-3' into an integrated virally based gene transfer vector system to ensure permanent expression of the integrated transgenes.

CONCLUSIONS

In conclusion**,** we have overcome the previously reported problem of insignificant second cistron expression and have produced a functional 5'-hNKx2.2-IRES-eGFP-3' expression cassette containing a 168bp spacer..

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