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Original Article

Successful construction of functional bicistronic vector for transfer and concurrent expression of human oligodendrocyte transcription factor Olig2 and DsRed2 reporter gene sequence in human cells

Dr. Faisal A. Al-Allaf , *Department of Medical Genetics, Faculty of Medicine, Umm Al-Qura University, Makkah, Saudi Arabia.*

Correspondence:
Dr. Faisal A. Al-Allaf
Department of Medical Genetics, Faculty of Medicine, Umm Al-Qura University, Al-Abdia Campus, P. O. Box 715, Makkah 21955, Saudi Arabia
Tel/Fax: +966 2 5270000 Ext: 4198
Email: fallaf@uqu.edu.sa

البناء الوظيفي الناجح لاوليق 2 والبروتين 2 الأحمر الفوسفوري في الخلايا البشرية

د فيصل العلاف – قسم المناعة كلية الطب –جامعة ام القري – مكة المكرمة

الملخص العربي:

الهدف: كثير من الأبحاث التى حاولت استخدام سلسلة دخول الريبوسوم الداخلية لبناء النواقل الوراثية المزدوجة واجهت صعوبة فى تحقيق تعبير جينى للبروتين الثانى فى السلسلة. فى هذه الدراسة قمنا ببناء ناقل وراثى مزدوج للتعبير الجينى المتزامن يحتوي على كاسيتكون من عامل الاستنساخ البشري اوليق 2 وسلسلة بين سيسترونية وسلسلة الريبوسوم الداخلية وسلسلة البروتين الأحمر الفوسفوري المعزز.

الطرائق: تم إعادة إستنساخ السلسلة المحتوية على الاستنساخ البشري اوليق 2 مع السلسلة بين سيسترونية وطولها 205 نيوكلتيد الى الناقل الوراثى للتعبير الجينى المسمى pIRES-2-DsRed2. الناقل الذي تم بناءه تم تحليله بواسطة التحليل التسلسلي للحمض النووي لتأكيد اندماج السلاسل بالشكل الصحيح. تم اختبار فعالية الناقل وقدرته على التعبير الجينى 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 اوليق 2 في الكاسيت الثنائى وبالتالى بناء الناقل المطلوب بالصورة الصحيحة. تحاليل الويسترن بلوتينق والتحليل المجهري الفوسفوري أكدت ايضا فعالية الناقل وقدرته على التعبير الجيني وإنتاج عامل الاستنساخ البشري اوليق 2 بمقاسه الصحيح والنشاط الحيوي للبروتين الأحمر الفسفوري في نموذج الخلايا المستزرعة.

الإستنتاج: سلسلة دخول الريبوسوم الداخلية تمثل اداة قيمة لإمكانية التعبير الجينى المتزامن والمتعدد السيسترون من نسخة الحمض النووي المراسل الناتج من النواقل الجينية والنواقل الوراثية العلاجية. الناقل المتعدد الذي تم إنتاجه سيكون مفيد للنقل الجيني ولدراسة تخصيص الخلايا الجذعية في المستقبل.

ABSTRACT

Objective: Internal ribosome entry site (IRES) sequences become a valuable tool for constructing gene transfer and therapeutic vectors for simultaneous expression of bicistrons from a single mRNA transcript. 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 a bicistronic cassette composed of the human oligodendrocyte transcription factor 2 cDNA, the IRES and enhanced red fluorescent protein coding sequences. Transcription of the bicistronic cassette is driven by a massage from a common upstream cytomegalovirus promoter. Translation of the two cistrons is uncoupled. Analyses of vector containing the cassette clearly confirm the functionality of the produced vector, correct size of the generated hOlig2 protein and the biological activity of the red fluorescent protein reporter in the cell culture model. The produced bicistronic hybrid vector in this configuration is intended to be used for gene transfer, gene based stem cell therapy and of great value for future stem cells transdifferentiation studies.

***Keywords*:** bicistronic vector, oligodendrocyte transcription factor, Olig2 and DsRed2

INTRODUCTION

Concurrent expression of more than one transgene from a single gene transfer vector is an important requirement in gene and cell therapy protocols {Ali, 2010 #1377;Chen, 2011 #1351;Ho, 2011 #1348;Licursi, 2011 #1368}. Concurrent expression can be achieved through the utilization of natural splicing signals of virally based vectors, in which multiple RNAs are produced from a single transcript {Kollen, 1999 #1197}. However, this is not a frequently used strategy because of the difficulties in controlling the splicing mechanism of the gene vector. Alternative approach involves in-frame fusion of chimeric sequences, ensuring concurrent expression of genes in one protein {Holt, 1999 #1188;Hoque, 2000 #1189;Kollen, 1999 #1197}. However, this strategy may not work for all combinations of proteins, some of which could result in protein misfolding or mistargeting. Instead, a vector with dual promoters expressing two separate transcriptional units can be constructed. 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 reporter but not the gene of interest and vice versa {Cullen, 1984 #1191;Emerman, 1984 #1195;Emerman, 1986 #1193}. To overcome the above limitations, heterologus chimeric gene sequences encoding the different cistrons can be separated by an element known as internal ribosome entry site (IRES) sequence. The IRES sequence is a *cis*-acting RNA element that has been found in many viral and human cellular RNAs {Fitzgerald, 2009 #1398}. Since its discovery, the IRES sequence has become a valuable tool for constructing gene therapeutic vectors for co-expressing multi-cistronic messenger RNA (mRNA) in tranfected cells and in transgenic animal models {Attal, 1999 #1203}. The function of these IRES sequences is to confer cap-independent translation initiation of internal cistrons by recruiting ribosomes directly to the mRNA without using the classical scanning mechanism suggested by Kozak {Kozak, 1995 #1201}. 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 {Kozak, 1995 #1201} 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.

Most of the described vectors utilize the IRES element from the Encephalomyocarditis virus (ECMV) for co-expression of a reporter gene together with therapeutic gene to permits titration of vector particles or to demonstrate gene transfer efficiency, and the level and longevity of gene expression {Borman, 1995 #1208;Gallardo, 1997 #1205;Licursi, 2011 #1368}. However, IRES efficiency is not absolute and it is a common occurrence for the subsequent gene in the expression cassette to fail translation or maybe translated at a lower level than the preceding gene {Attal, 2000 #1230;Mizuguchi, 2000 #1217}. Therefore, the optimal conditions for effective use of IRES element for constructing a functional expression vector are not precisely defined, but mounting evidence suggest that expression of the downstream genes can be improved by increasing or decreasing the sizes of the sequences flanking the 5' and/or 3' of IRES inserts {Attal, 1999 #1202;Romero-Lopez, 2011 #1300}.

In the present study, we have constructed an expression vector containing bicistronic cassette under control of the cytomegalovirus (*CMV)* promoter. This bicistronic cassette contains the human oligodendrocyte transcription factor 2 cDNA (hOlig2cDNA), 205bp inter cistronic sequence (ICS), the IRES element, and the DsRed2 reporter sequence. 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

1.1. Plasmid Construction and Cloning

The hOlig2cDNA insert was excised from pBluscript-Olig2 plasmid by digestion of 10µg with *SacI* (Promega) in a total volume of 200µl reaction mix topped with 3 drops of oil and incubated at 37°C for 14 hours. The tube was incubated at 65°C for 20 minutes to inactivate the *SacI* endonuclease. Digested DNA was then size fractionated on 1% agarose gel. The 1.2kb fragment containing hOlig2cDNA was cut out and DNA was purified using commercially available gel purification kit (Qiagen) and DNA was eluted in 50µl sterile Diethylpyrocarbonate (DPEC) treated H_2O. The eluted DNA was then blunt ended with Klenow enzyme (NEB), heat inactivated for 15 minutes at 65°C, column purified (Qiagen) and DNA was then eluted in 40µl sterile DPEC treated H_2O to ensure removal of enzymes and salts. Approximately, 10µg of the pIRES2-DsRed2 plasmid DNA was also digested with *BamHI/Bgl II* enzymes, heat inactivated at 65°C for 20 minutes, column purified again, blunt ended by Klenow (Roche) and alkaline phosphatase (NEB) treated for 8 hours at 37°C. Purified digested vector and insert DNA were mixed together in 1:10 ratio and ligated using T4 DNA ligase (Roche) for overnight at 16°C. Ligated mixture was purified again using the Qiagen column and eluted in 30µl molecular biology grade H_2O. Only 2µl of eluted ligation mixture was used to transform STBL2 competent bacterial cells (Invitrogen) {Sambrook, 1989 #1378}. Transformation was performed according to Invitrogen recommendations and 300µl of serially diluted SOC media containing transformed STBL2 competent cells was plated on *Kan* resistance plates. Colonies were allowed to grow for overnight and then subcultured on both *Kan* and *Amp* resistance plates. Only *Kan* resistance colonies were screened further together with ligation mixture by insert-vector PCR method {Al-Allaf, 2005 #1228} using the forward primer 5'-GCA CGG CCT ACT CAA GTC TC-3' and the reverse

primer 5'-GGA ACT GCT TCC TTC ACG AC-3' which anneals to the 5' and 3' restriction/ligation site. Colonies producing 527bp amplicon were inoculated for overnight culture and the integrity of the hOlig2-insert in the pOlig2cDNA-IRES-DsRed2 clones was examined by restriction digestion and DNA sequence analysis {Sambrook, 1989 #1378}.

1.2 Gene transfer into cultured cells

The human embryonic kidney epithelial (HEK 293), the cervix carcinoma Hela cells, and the human fetal mesenchymal stem cells (hfMSCs) were cultured with Dulbecco's modified Eagle's medium (Invitrogen or Sigma) 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) according to the manufacturer's instructions. Approximately 48 hours post-transfection, cells were analysed for cellular expression of DsRed2 using inverted fluorescence microscopy (Nikon). Mock transfected cells were used as controls. All transfection experiments were repeated at least three times.

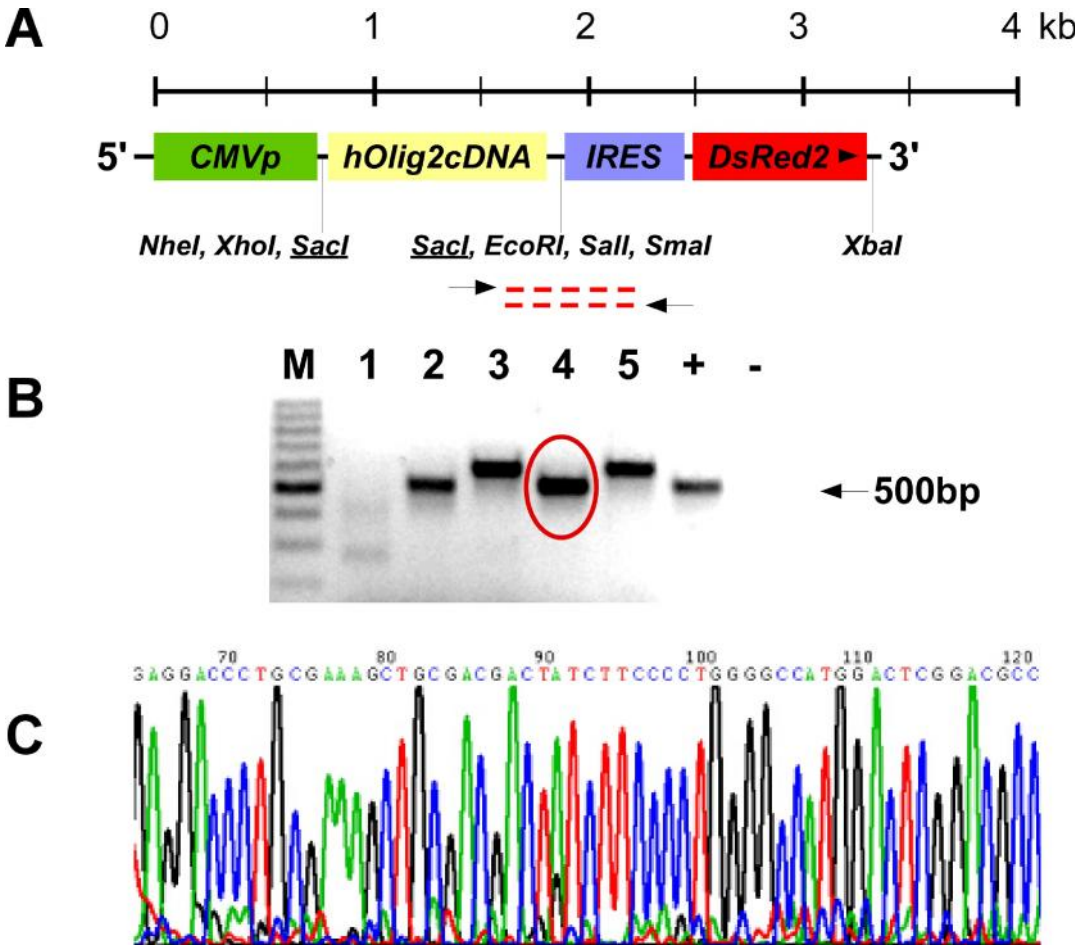
1.2. Western blot analysis for protein expression

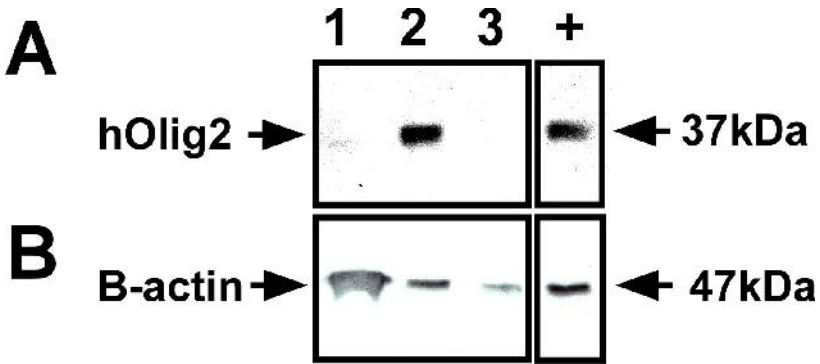
Forty eight hours after transfection, cells were harvested by scrubbing and re-suspended in 500µl/10^6 cells ice-cold lysis buffer (10mM Tris pH 7.5, 1mM EDTA, 1% NONIDET P-40 (Sigma), protease inhibitor cocktail at indicated dilutions (Roche)). Cell suspensions were then centrifuged at 13,000×*g* for 20 minutes 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 minutes and were size fractionated on a 13% or 11% SDS-PAGE gel for Olig2, and β-actin protein detection, respectively. Gels were then blotted onto PVDF membranes with a Hoefer apparatus (200 mA). After 1 hour 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 in blocking solution. After three 5-minutes washes in PBS-Tween-20, blots were incubated at room temperature for 1 hour with peroxides-conjugated anti-goat IgG horse radish peroxidase antibody (diluted 1:1000 in 5% milk solution). Following the final wash, detection on autoradiography hyper-films was performed after inducing a chemiluminescence reaction with supersignal detection kit (Amersham). Primary antibodies used in this study were polyclonal goat anti-hOlig2 antibody (diluted 1:500 in 5% milk solution in 1x TBS, Santa Cruz, USA) and anti-β-actin (1:2000; Sigma, UK) {Harlow, 1988 #1379}.

RESULTS

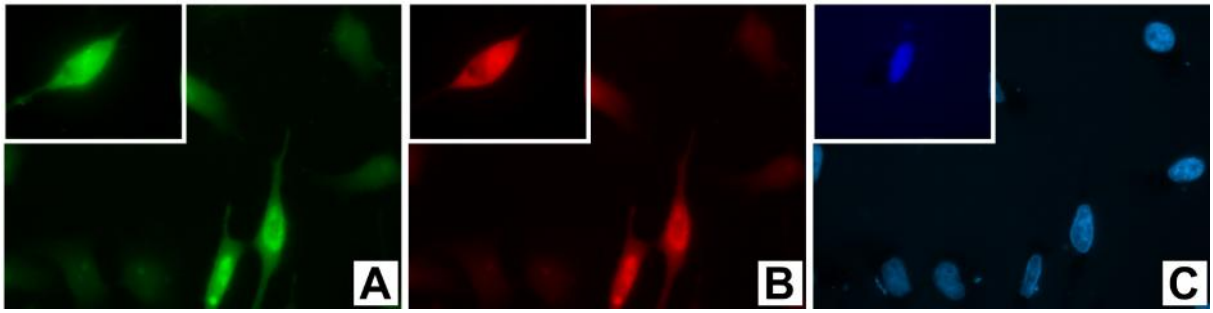
1.1 Structure of the bicistronic cassettes and proof of clonality

The pIRES2-DsRed2 expression vector was chosen as a backbone because it contains the IRES of the ECMV which has high translation efficiency compared to other IRES sequences {Borman, 1995 #1208;Borman, 1997 #1204;Gallardo, 1997 #1205;Ramesh, 1996 #1206;Saiz, 1999 #1207}. The multi-cloning site which located between the immediate early promoter of cytomegalovirus and the IRES sequence offers the possibility of convenience cloning. The hOlig2cDNA and the 205bp downstream ICS were sucloned from the pBluscript-Olig2 plasmid into pIRES2-DsRed2. The nasant pOlig2cDNA-IRES-DsRed2 vector in this configuration (Figure 1-A) permits high level of bicistronic expression. It also permits high scale of plasmid DNA production after propagation as it contains a pUC origin of replication and a bacterial promoter expresses kanamycin resistance in *E. Coli*.





During cloning protocol, the ligation mixture was examined by insert-vector PCR {Al-Allaf, 2005 #1228} as positive control to confirm the physical presence of the expected recombinant plasmid containing the hOlig2cDNA in correct orientation. The correct PCR amplicon of 527bp in size was generated from 3 randomly screened colonies (Figure 1-B), indicating Olig2cDNA insertion in the desired orientation. The other three screened colonies showed no PCR product (lane 1) or non-specific amplification (lane 3 and 5) and therefore excluded from further screening. The ligation mixture was used as positive control. 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 clonality and integrity of Olig2cDNA in the bicitronic cassette and the construction of the desired mammalian expression vector (Figure 1-C).



The vector backbone also contains an SV40 origin of replication expressing the SV40 T antigen. A neomycin-resistance cassette, consisting of the SV40 early promoter, the neomycin/kanamycin resistance gene of Tn5, and polyadenylation signals from the herpes simplex virus thymidine kinase gene, allows stably transfected eukaryotic cells to be selected using G418 {Nehlsen, 2010 #1399}.

The initiator AUG codon of the DsRed2 is located 12 bases downstream of the IRES element.

1.1. Analysis of hOlig2 expression by Western blotting

Analysis of gene expression was performed by Western blotting and fluorescence microscopy after cells transfected with bicistronic construct containing 5'-hOlig2-IRES-DsRed2-3' 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-hOlig2 antibody (Figure 2-A) shows the mature form of the hOlig2 protein migrating as one band of apparent molecular masses of 37kDa

from HEK 293 cell extracts transfected with the pOlig2cDNA-IRES-DsRed2 construct (lane 2) and from positive control lysate of MO3 cells (lane 4). No signals were detected from the mock transfected negative control (lane 1) or from extract of cell transfected with pIRES2-DsRed2 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.

1.1 In vitro analysis of Olig2 and DsRed2 expression by immunofluorescence microscopy

In order to test the functionality of the constructed vectors and its biological activity at a single cell level, the pOlig2cDNA-IRES-DsRed2 vector DNA were transfected into Hela cells and expression was analysed using Immunofluorescence microscopy (Figure 3). Cellular co-expression of Olig2 and DsRed2 was detected approximately 48 hours post gene transfer in all transfected cells. This figure proof functionality of the produced vector and absence of transcriptional interference and/or dissociated gene expression between Olig2 and DsRed2. Transfected Hela cells cells were able to co-express both Olig2 and DsRed2 cistrons in approximately 60% of cell population.

DISCUSSION

Accurate assessment of gene transfer following transfection or transduction may require co-expression of a reporter gene in addition to the transgenic sequence in question. Methods used for co-expression of more than one gene have serious drawbacks {Cullen, 1984 #1191;Emerman, 1984 #1195;Emerman, 1986 #1193}. When an IRES element 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 is poorly expressed compared to the sequence subcloned at the 5' of the IRES {Attal, 1999 #1203;Attal, 1999 #1202;Brocard, 2007 #1303;Brown, 1994 #1299;Romero-Lopez, 2011 #1300}.

In the present work, we have overcome the previously reported problem of insignificant second cistron expression and have produced a functional 5'-hOlig2-IRES-DsRed2-3' expression cassette containing the following five components: the first component of the bicistronic cassette is a cDNA encoding hOlig2 which is a member of the basic helix-loop-helix nuclear transcription factor protein family {Wegner, 2001 #1380;Zhou, 2000 #1381}. Normally, the gene encoding hOlig2 is expressed in the spinal cord, brain and the optic nerve and has been shown to be involved in both oligodendrocyte differentiation and motor neuron specification {Fu, 2002 #1382;Lu, 2002 #1383;Novitch, 2001 #1384;Rowitch, 2002 #1385;Sun, 2001 #1386;Zhou, 2002 #1387;Zhou, 2001 #1388}. This dual function of Olig2 in neurogenesis and oligodendrogenesis can be explained by the possible interaction of Olig2 with other transcription factors {Lu, 2000 #1389;Novitch, 2001 #1384;Zhou, 2001 #1388;Zhou, 2000 #1381}. The second component of the cassette is a 205bp ICS located 5' of IRES element, which derived from the pBluscript-Olig2 plasmid. The third component of the

cassette is IRES of the ECMV. The fourth component is a 12bp ICS located 3' of IRES element. The fifth component in this vector represents the human codon-optimized DsRed2 {Matz, 1999 #1397} reporter coding sequence of *Discosoma* sp. red fluorescent protein {Cormack, 1996 #1213;Yang, 1996 #1214}. This cDNA is engineered for easy, rapid and less expensive detection and monitoring of expression. It also offers brighter fluorescence, faster maturation and lower non-specific aggregation with higher expression in mammalian cells {Cormack, 1996 #1213;Jakobs, 2000 #1395;Yang, 1996 #1214}. The DsRed2 coding sequence contains nine amino acid substitutions which improve solubility of the protein and reduce the time from transfection to detection of red fluorescence. In addition, these substitutions reduce the level of residual green emission {Haas, 1996 #1393}. Although DsRed2 most likely forms the same tetrameric structure as wild-type DsRed2, with reduced tendency to aggregate {Haas, 1996 #1393}. At the 3' downstream of DsRed2, the SV40 polyadenylation signal directs proper processing of the bicistronic mRNA transcript.

We analysed the expression levels of the respective transgenes systematically at the DNA and protein levels after gene delivery which showed 90% gene transfer in HEK 293 cells following transfection. Successful co-expression of hOlig2 and dsRed2 is attributed to the following two main factors: firstly, the length of the inter-cistronic sequence flanking the IRES element at both its 5' and 3' ends {Attal, 1999 #1203;Borman, 1995 #1208;Borman, 1997 #1204}. The effect of the length of these ICS on IRES mediated internal translation initiation is well documented in the literature {Attal, 1999 #1203;Borman, 1997 #1204}. Expression efficiency of the DsRed2 was obtained when the 5' ICS is 205bp in length. However, the expression levels of DsRed2 become completely undetectable at a spacer length of 530bp (data not shown). These results are in agreement with data published by Attal et al. {Attal, 1999 #1202} 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 completely inefficient when added after a 300-500 nucleotide spacer {Attal, 1999 #1202}. 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. {Attal, 1999 #1202} also showed that the expression of the second cistron was undetectable when the spacer fragment was 500 nucleotides. This phenomenon of low efficiency of IRES mediated translation initiation compared to cap dependent translation is possibly due to higher affinity of translation factors for the cap structure than for the IRES element, therefore, translation factors may become less available for the internal initiation of translation {Brown, 1994 #1299} but can be modulated by size. To exclude the effect of tissue tropism and the requirement of additional host *trans*-acting factors, which may modulate IRES function in a cell or tissue type specific manner {Dobrikova, 2006 #1283;Lourenco, 2008 #1280;Ray, 2002 #1287}, we have also transfected our construct into Hela and hfMSCs cells and obtained similar results. The second ICS located 3' of IRES is a short sequence contains a Kozak consensus translation initiation site {Kozak, 1995 #1201} to further increase the translation efficiency of second cistron in eukaryotic cells. Thus, the initiator AUG codon of the DsRed2 is located 12 bases downstream of the IRES element. Such a short distance is nevertheless sufficient to greatly affect the DsRed2 expression. The second factor attributed to the successful co-expression of hOlig2 and dsRed2 is the use of IRES of the ECMV which has high translation efficiency compared to other IRES sequences including those from human rhinovirus, poliovirus, foot-and-mouth disease virus and hepatitis A and C viruses {Borman, 1997 #1204;Gallardo, 1997 #1205;Ramesh, 1996 #1206;Saiz, 1999 #1207}. In addition to its high efficiency, ECMV IRES possess a broad tissue tropism

{Borman, 1995 #1208} which makes it the most widely used in gene transfer protocols {Azzouz, 2002 #1209;Morgan, 1992 #1210;Pizzato, 1998 #1211}.

The Olig2 gene has been shown to drive trans-differentiation of stem cells into oligodendrocyte {Copray, 2006 #1246;Zhang, 2005 #1240;Zhou, 2001 #1388}. In 2005, Zhang *et al.* {Zhang, 2005 #1240} used transfection methods and expression plasmids to deliver *Olig2* gene inside neural stem cells and accomplished a transient expression up to 12 days with a 60-80% efficiency of transfection. In the present study, we have achieved more than 90% tranfection in HEK 293 and approximately 20% transfection in hfMSCs and accomplished a transient expression up to 14 and 9 days respectively. We are also aware that transdifferentiation of stem cells by gene transfer may require long-lasting expression of the transgenic Olig2 in the relevant cell model, therefore, future work will focuses on subcloning the successfully constructed 5'-Olig2-IRES-DsRed2-3' into an integrated virally based gene transfer vector system to ensure permanent expression of the integrated transgenes.

In conclusion, we have overcome the previously reported problem of insignificant second cistron expression and have produced a functional 5'-hOlig2-IRES-DsRed2-3' expression cassette containing a 205bp spacer.

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