SMGroup

Co-Expression Technologies in Eukaryotic Cells

Ekaterina Minskaia*, Garry A Luke and Martin D Ryan

Biomedical Sciences Research Complex, North Haugh, University of St. Andrews, Scotland, UK

***Corresponding author:** Ekaterina Minskaia, Biomedical Sciences Research Complex, North Haugh, University of St.Andrews, St.Andrews, Fife, KY16 9ST, Scotland, UK, Email: em431@st-andrews.ac.uk

Published Date: February 15, 2015

INTRODUCTION

Due to the nature of the initiation of translation in prokaryotes, co-expression of proteins with linked functions may be co-ordinated by organizing genes into operons. Here, a single polycistronic mRNA encodes multiple proteins - each encoded by a separate open reading frame (ORF). Ribosomes initiate translation of each ORF encoded by the polycistronic mRNA by simply binding to Shine-Dalgarno ribosome binding sites located in sequences linking the ORFs. The 7meG cap-dependent mode of initiation in eukaryotes precludes this strategy of co-ordination of expression at the level of translation: it is accomplished by the co-ordination of the transcription of a series of monocistronic mRNAs, each derived from a different gene.

For many (eukaryotic) biotechnological or biomedical applications, however, the desired 'gain-of-function' relies upon the effective co-expression of multiple, different, proteins: hetero-multimers, macromolecular complexes, modifications to biochemical pathways etc. In eukaryotic biotechnology, the problem of protein co-expression can be overcome by a number of strategies: (i) co-transfection of plasmids or viral co-infection expressing one protein each, (ii) assembling multiple genes into one vector molecule, or, (iii) by creating a single vector encoding a polycistronic mRNA (Figure 1).

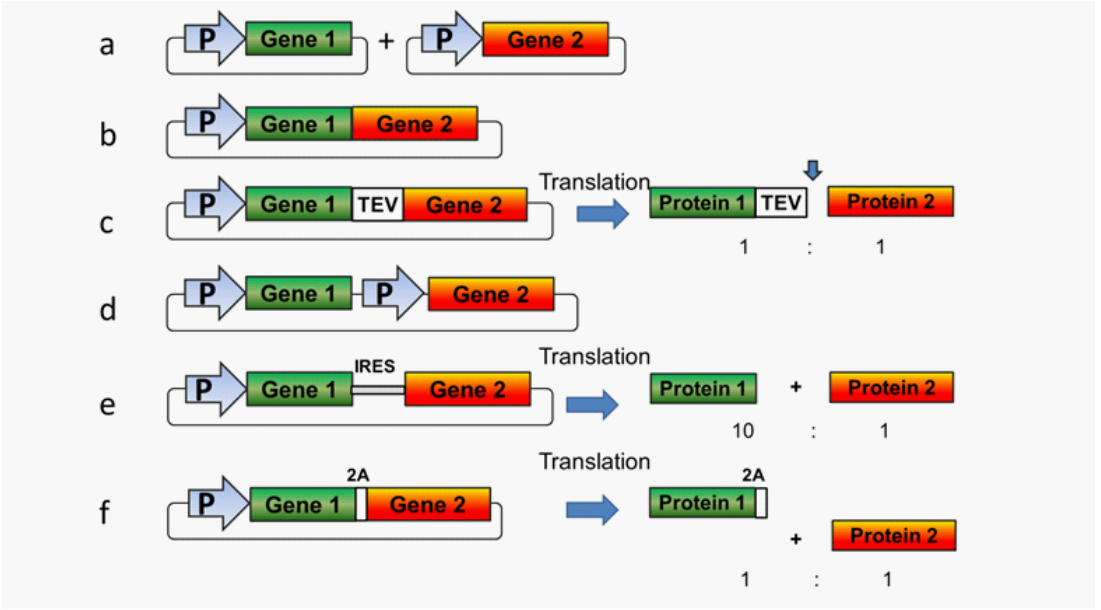

Figure 1: Co-expression strategies in eukaryotic cells.

MONOCISTRONIC VECTORS

Co-Transfection and Co-Infection

Eukaryotic cells can take up many different plasmid DNAs upon plasmid DNA transfection. The simplest way to obtain protein co-expression is by co-transfecting individual vectors, each expressing a single protein (Figure 1a). One of the advantages of this strategy is that it provides flexibility in construct generation and optimization of expression: the ratio of transient gene expression can be modulated *via* ratio between input plasmid DNAs or *via* the use of different promoters. However, considering the difference in RNA/protein stability and turnover, the appropriate ratio of input DNA usually needs to be determined empirically [1]. This approach can be successfully employed when single cells are selected for analysis but not when the whole cell population needs to be analyzed. While transfection of cells with a mixture of different plasmid DNAs may produce effective co-expression across population of cells, it leads to a heterogeneous cell population with different protein expression levels and only produces a small proportion of individual cells co-expressing all of the products. Despite the fact that this approach is considered to be efficient if only up to four genes have to be expressed, several groups successfully used this method for virus rescue. For example, replication competent influenza A virus was rescued using an eight-plasmid transfection system [2]. For stable expression, however, the sites of integration of the various transgenes into the genome have a major effect on the efficiency of co-expression within any particular cell.

Co-infection with multiple viral vectors is a similar strategy, and various viral vectors, such as lentiviral, adenoviral, retroviral and adeno-associated (AAV), are used. Lentiviral vectors stably integrate into the genome of dividing and non-dividing cells and are often used for over-expression of a gene of interest [3,4]. For example, lentiviral vectors encoding red, green or blue fluorescent proteins were used to monitor clonal expansion of cells *in vitro* and *in vivo,* and different combinations of fluorescent proteins resulted in various combinations of colours in individual cells. Strict selection of a homogenous cell population can be achieved *via* co-expression of either antibiotic resistance genes or fluorescent marker proteins followed by fluorescence-activated cell sorting (FACS: [5]) The efficiency of retroviral vectors relies upon their ability to integrate into the chromosomes of the host proliferating cells, an advantage when targeting neoplastic cells. In contrast, disabled herpes virus vectors are used for transgenesis of resting cells.

Practical constraints limiting the number of genes transferred to plants remains a significant bottleneck in multigene transfer. Iterative processes such as crosses between transgenic parental lines bearing distinct transgenes [6,7] or sequential transformation (the repetitive insertion of transgenes into a plant) [8,9] are time-consuming and/or require the use of different selectable marker genes. Co-transformation with linked genes is technically challenging while the efficiency of co-transformation with multiple plasmids (unlinked genes) decreases progressively with increasing plasmid number.

Fusion Proteins

This approach may be useful for proteins that naturally function as hetero- or homodimers, since their genes can be linked together to form a hybrid fusion gene which encodes a single polypeptide with two distinct functional domains (Figure 1b). However, suboptimal linker length and composition can lead to co-expressed proteins being unstable, insoluble, and inactive, and optimal length may need to be determined experimentally [10-12]. Furthermore, steric effects often lead to loss of function of fused proteins and the fusion protein can only be targeted to a single subcellular compartment.

Fusion Proteins Incorporating Proteinase Cleavage Sites

Proteolytic processing of polyprotein precursors with cleavable linkers is a translational strategy employed by many viruses to produce more than one protein from a single mRNA. Simple creation of a fusion protein can be modified and improved by linking the proteins *via* a proteinase cleavage site (Figure 1c). The disadvantage here is that both the polyprotein substrate and the processing enzyme must be co-expressed in the same subcellular site. The great advantage is, however, the ability to generate co-ordinated and equimolar expression through the use of one promoter. The feasibility of this approach was first demonstrated in plants by co-expressing two defence proteins separated by the tobacco etch virus (TEV) nuclear inclusion (NIa) protease recognition sequence (heptapeptide cleavage recognition sequence -ENLYFQ$^{\downarrow}$S-) together with the NIa proteinase [13,14]. The NIa protease of *Potyviridae* is the major viral protease that

processes potyviral polyproteins. The TEV-NIa-based cassette was also adopted by Liang and colleagues to express two pathogen resistance genes, a *Trichoderma harzinum* endochitinase (Ech42) and a wheat germin-like oxalate oxidase (OxO), in *Arabidopsis* [15]. A similar polyprotein vector containing the NIa protease from the tobacco vein mottling virus (TVMV) and a transit peptide was used for targeting the two enzymes, *E.coli* acetate kinase (ACK) and chloramphenicol acetyltransferase (CAT), to different cellular compartments in tobacco plants [16]. Although these two proteinases share a high degree of sequence identity (52%), they have distinct substrate specificities and do not cleave each other's recognition sites [17] - this may be useful for protein expression strategies that involve the use of more than one proteinase. While proper polyprotein processing was achieved, the utility of this system is limited due to the presence of a nuclear-localizing signal (NLS) within the protease and the amount of energy necessary to synthesize the ~48kDa protease.

As an alternative to co-expressing a viral protease as part of the polyprotein (which may prove toxic to the cell), it is possible to use endogenous plant proteases that recognize the cleavable linker. Urwin *et al*. [18] linked two different protease inhibitors effective against nematode parasites *via* a protease-sensitive "spacer" originating from a pea metallothionein-like protein and showed that the chimeric polyprotein precursor could be partially cleaved in *Arabidopsis thaliana*. François and co-workers used a linker peptide sequence (LP4) originating from a natural polyprotein occurring in seeds of *Impatiens balsamina* (-SN$^{\downarrow}$AADEVATPE$^{\downarrow}$DVEPG-) to connect two plant defensins (DmAMP1 originating from *Dahlia merckii* seeds and RsAFP2 originating from *Raphanus sativus* seeds). When expressed in *A. thaliana*the chimeric polyprotein was cleaved post-translationally and the individual, active, defensins were secreted into the extracellular space[19,20]. However, DmAMP1 was produced with two additional amino acids at its carboxy-terminus (+SN) and RsAFP2 with five additional amino acids at its amino-terminus (+DVEPG). Since the protease(s) responsible for cleavage in these studies is unknown, another approach is to use linker sequences that are putative substrates of known endogenous plant proteases. Kex2p-like proteases have been identified in eukaryotes as disparate as yeast [21], tobacco [22] and mammals [23]. These proteases reside in the *trans*-Golgi network (TGN) and function in the secretory pathway to process polypeptide prohormones. To exploit the potential of a plant native protease, Zhang *et al*. [24] expressed a red fluorescent protein (DsRed), or a cytokine (GM-CSF), linked to a GFP by three tandemly repeated Kex2 cleavage sites (-IGKR$^{\downarrow}$GIGKR$^{\downarrow}$GIGKR$^{\downarrow}$G-) in tobacco plants. In this study the kex2p-linker-based polyprotein construct achieved almost stoichiometric expression of the individual proteins encoded in the construct.

POLYCISTRONIC CO-EXPRESSION VECTORS

Multiple Promoters

Conventionally, multiple genes were expressed under control of multiple promoters (Figure 1d). Characteristics of this strategy are: (i) utilisation of identical promoters may result in

competition for the same transcription factors, (ii) homologous recombination can result in the loss of one of the genes, (iii) there is a potential for promoter attenuation interference - the expression from one promoter suppresses the activity of the other promoter[25].

Single Promoter - Transcription of Polycistronic mRNAs

The ability to co-express multiple genes within the same cell is very important in all eukaryotic cells, and polycistronic co-expression vectors solve this problem. They can be constructed by linking individual ORFs with a sequence that: (i) contains an internal ribosome entry site (IRES, Figure 1e), (ii) incorporates 2A "self-cleaving" sequences (Figure 1f), or, (iii) incorporates a proteinase cleavage site as discussed above (Figure 1c).

Internal Ribosome Entry Sites (IRESes)

IRES elements were initially identified in picornaviruses and were later found in other viral and cellular eukaryotic mRNAs [26,27-31]. IRESes are cis-acting elements that recruit the small ribosomal subunits to an internal AUG codon in the mRNA with the help of cellular *trans*-acting factors. In vectors containing IRES sequences the first gene is translated in a 5' cap-dependent manner and the second gene expression is cap-independent [32,33]. IRESes from 68 distinct viruses have been studied so far and three of these, the EMCV (encephalomyocarditis virus), FMDV (foot-and-mouth disease virus) and HCV (hepatitis C virus) IRESes, are the most commonly used [34,35]. However, other viral IRESes, such as poliovirus (PV), classical swine-fever virus (CSFV), human immunodeficiency virus (HIV), bovine viral diarrhoea virus (BVDV) and cricket paralysis virus (CrPV) are also frequently used [30,31]. While average size of efficient viral IRESes is about 450 nt, smaller viral IRESes range in size from 73 to 280 nt (Table 1: [36-38]). By 2009, 115 eukaryotic mRNAs containing cellular IRESes have been reported in the genes of yeast, the fruit fly and mammals [30], these exhibit a greater variation in length, ranging from 9nt to 1.5kb [39,40,41].

Table 1: Experimentally verified functional IRESes: viral, cellular and engineered. Average length in nucleotides (nt).

Type	Source	Length(nt)
Viral		73-904
	Bovine	866-904
	Crucifer tobacco virus	73-148
	Classical swine fever virus	359-442
	Equine rhinitis virus	674-956
	Felis silvestris virus	233-814
	Hepatitis virus (A, B, C)	338-852
	Human rhinovirus	554-614
	Human poliovirus	628-771
	Human coxsackie virus	704-750
	Encephalomyocarditis virus (EMCV)	500-588
	Foot-and-mouth disease virus (FMDV)	215-461
Cellular		22-1222
	Homo sapiens	22-1222
	Mus musculus	22-1209
	Aplysia californica	319
	Drosophila	265-846
	Engineered	58-98

Various factors influence IRES activity: RNA secondary structure, sequence complementarity to 18S rRNA, location of the AUG start codon of the downstream gene with respect to the IRES, the sequence context around the AUG codon, the distance between the stop codon of the first cistron and the IRES, the type of upstream and downstream genes and the order in which they are arranged, as well as various cellular factors [42-45].

One of the advantages of using IRESes is that they are active in situations where cap-dependent translation is inhibited, such as in the reduction in overall protein synthesis in response to stress during cell cycle or apoptosis [46,47]. However, problems arise when the expression levels of the genes upstream and downstream of the IRES are compared. A number of studies showed that the downstream gene expression was 10–50% of the upstream cap-dependent gene expression, and this discrepancy in expression levels was sequence-independent as the same gene placed before the IRES resulted in high expression [43,48,49].

Overall, picornavirus FMDV and EMCV IRESes were found to be about 5-fold better than HCV, and 10-fold better than polio or retrovirus IRES elements [50-52]. Comparison of cellular and viral IRES activities showed that vascular endothelial growth factor (VEGF) and platelet-derived growth factor 2 (PDGF2) IRESes are close to the activity of EMCV [29,53] and ~ 5-fold more active than that of BiP [54] which, in turn, was reported to be 10-fold lower than EMCV IRES [55]. Several cellular IRESes were reported to mediate better downstream gene expression compared

to the ECMV IRES. For example, c-myc IRES was found to be 3-5-fold, connexion 43 IRES - 18-fold, NRF IRES - 92-fold and eIF-4G IRES - more than 100-fold better in mediating downstream gene expression than ECMV IRES [41,45,31]. When using several IRESes, it is worth remembering that potential *in vivo* interactions may occur between different IRESs in the same vector. Using the same IRES twice in a bicistronic vector could induce recombination events and the loss of the second IRES and cistron, while using the same cistron twice could lead to a competition between the two IRESs for the binding to cell type specific translation factors [56].

While cell/tissue specific expression can be achieved by incorporation of tissue specific promoter, utilization of different IRESes can yield similar results. Various cell types have been used to determine factors influencing cell/tissue specificity of IRES activity and various viral and cellular IRESes were found to mediate downstream gene expression with different efficiency in different cell types. Most cellular IRESes are found to be active in various cell types, nevertheless, several cellular IRESes demonstrated cell type specificity. While there is evidence for dependence on cellular factors that are differentially expressed in distinct cell types [57], no definitive conclusion can be made about relative IRES activities and only an order of efficiency can be established [45]. This is due to the fact that different vectors were used for these studies: the positioning of IRES relative to AUG codon was different as were the reporter genes used, therefore, their activities were not comparable [41].

In gene therapy, depending on the cell target and the experimental goals (e.g. cellular transformation, production of transgenic animals, recombinant protein production, gene therapy, gene targeting, etc.) different types of vector are necessary. In retroviral bicistronic vectors, the use of IRES elements allows long-term simultaneous expression of proteins from a single transcription unit. The capacity of ECMV IRES to induce high levels of protein expression in different cell types was compared to the capacity of other IRESes by inserting their sequence into bicistronic or tricistronic retroviral vectors [58-64]. AAV offer the advantage of their stability upon integration and their lack of an infectious cycle [65]. However, IRES length may present a problem when small genomes are packaged - packaging limits of some of the popular viral vectors are 4.5 kb for AAV , 7.5kb for adenoviral, 8kb for retroviral, and 25kb for vaccinia. Further, truncated IRES forms should be used with caution as their activity is reduced [55].

A number of IRES motifs from animal [66-68] and plant [69,70] viruses have been tested and shown to direct the expression of multiple recombinant proteins in plant systems. As an example, a functional bicistronic plant mRNA encoding both green fluorescent protein (GFP) and firefly luciferase (LUC) was used together with *in vivo* imaging to demonstrate the conservation of IRES function of the encephalomyocarditis virus (EMCV) in plants [66]. Interestingly, an EMCV-IRES construct showed much lower efficiency than the IRES sequence from crucifer-infecting tobamovirus (crTMV), a plant virus, when tested with a bicistronic transcript expressing β-glucuronidase (GUS) linked to crTMV coat protein (CP) in tobacco protoplasts and wheat germ extracts [71]. In plants, IRES-based constructs have been used to engineer nematode pest

resistance in tobacco by the co-delivery of two distinct proteinase inhibitors [67], co-express two genes involved in the biosynthesis of carotenoids in transgenic rice endosperm [70], and to enhance salt and/or drought tolerance in potato [72] and faba bean [73] plants by linking a reporter gene to a pathogenesis-related protein. IRES sequences, however, have limitations: they are not small, adding to the size of the transgenes; internally initiated translation is generally low compared to cap-dependent translation [67,74], which means expression of a gene downstream of an IRES is typically only ~10% of the upstream gene [70].

2A Self-Cleaving Peptides

An alternative approach used in the development of polycistronic vectors employs 2A/'2A-like' linker peptides (*cis*-acting hydrolase elements - CHYSEL) to create 'self-processing' artificial polyproteins [75]. The cleavage efficiency of the 2A-based polyprotein system has been studied in various cell types using various reporter proteins such as fluorescent proteins (FPs e.g. GFP, RFP, YFP), GUS and CAT, as well as for proteins requiring discrete co- and post-translational subcellular localization [76-84].

The 2A region of the FMDV encodes a sequence (F2A) that mediates self-processing by a translational effect variously referred to as 'ribosome skipping', 'stop-go' and 'stop-carry on' translation [85,86]. Analysis of recombinant polyproteins and artificial polyprotein systems in which 2A was inserted between two reporter proteins showed that the FMDV 2A (plus the N-terminal proline of the 2B downstream protein) co-translationally 'self-cleaved' at the glycyl-prolyl pair site corresponding to the 2A/2B junction (LLNFDLLKLAGDVESNPG$^{\downarrow}$P-). 2A cleavage occurs in all eukaryotic cells tested. The use of longer versions of 2A with N-terminal extensions derived from FMDV capsid protein 1D upstream of 2A (~30aa in total) was reported to produce higher levels of cleavage [77,87-89]. The length of the F2A is important for cleavage not only *in vitro*, but also *in vivo* [77,79,89-94].

The advantages of 2A system are: (i) co-expression of proteins linked by 2A is independent of the cell type (since cleavage activity is only dependent on eukaryotic ribosomes, structurally highly conserved amongst the eukaryota), (ii) multiple proteins are co-expressed in equimolar amounts from a single transcript mRNA (single ORF) under the control of only one promoter and, (iii) 2A is smaller (54-174bp) compared to IRES elements. This makes this unique sequence an attractive substitute for previously discussed strategies for co-expression of multiple genes [78,95,96]. However, it should be noted that (i) 2A remains as a C-terminal extension of the upstream product, and (ii) proline forms the N terminus of the downstream protein. This must be taken into account if the authentic C-terminus is required for activity or subcellular targeting: such proteins should be encoded at the C-terminus of the entire polyprotein. At the same time, the presence of N-terminal proline does not seem to affect proteins which are metabolically stable [97]. The 2A extension, however, can be used to detect expression [95,78] and localization using anti-2A antibodies. In any event, cleavage sequences of the mammalian Kex2p homologue,

furin (-↓RRRR-, -↓RKRR-, -↓RRKR-), between the upstream protein and 2A can improve protein expression levels and remove the 2A "tag" [80,98].

Signal sequences for post-translational targeting can be included within 2A polyproteins, either up- or downstream of 2A. The co-translational nature of the cleavage means that polyproteins can be designed such that some proteins can be co-translationally targeted to the exocytic pathway, whereas others are post-translationally targeted to different cellular compartments. This provides a substantial advantage compared with other polyprotein-based systems, which require post-translational processing.

While the F2A sequence has been used widely, many '2A-like' sequences have been utilized successfully, including equine rhinitis A virus (E2A), porcine teschovirus-1 (P2A) and *Thosea asigna* virus (T2A) (Table 2) [78,95-104]. These '2A-like' sequences have been used in adoptive cell therapies [100-102], genetic engineering of human stem cells [103,105,106] and the co-expression of transcription factors in the induction of pluripotent stem cells[82,84,104].

Table 2: 2A and 2A-like sequences used for protein co-expression.

Abbreviation	Source	2A/2A-like sequence	Number of studies	References
F2A	Foot-and-mouth disease virus (FMDV)	-PVKQLLNFDLLKLAGDVESNPG P-	223	[107-111]
E2A	Equine rhinitis A virus	-QCTNYALLKLAGDVESNPG P-	24	[112-116]
P2A	Porcine techovirus-1	-ATNFSLLKQAGDVEENPG P-	76	[117-121]
T2A	*Thosea asigna* virus	-EGRGSLLTCGDVESNPG P-	121	[122-125]

In the case of proteins targeted to enter the exocytic pathway, successful utilization of a 24aa version of F2A with a furin cleavage site immediately upstream was reported by several groups [82,80,126,127]. For example, high levels of full-length, functional monoclonal antibodies were produced by linking the antibody heavy and light chain sequences with F2A in the context of AAV-mediated gene transfer [80], significant anti-tumour responses were observed in the clinic using monoclonal antibodies that block immune checkpoints by co-expressing CTLA-4 heavy and light chains [127] and a target Fab' fragment was expressed *via* retroviral vector [126]. Cleavage efficiency of shorter F2As in different contexts was shown to be improved by insertion of various spacer sequences, such as –SGS- or –GSG- [99,128], the V5 epitope tag (-GKPUPNPLLGLDST-) [82] or a 3xFlag epitope tag [83] immediately upstream of F2A. In γ-retroviral constructs, expression of multiple genes linked with F2A peptides was facilitated by a spacer sequence immediately upstream of $F2A_{22}$[99,128], while in lentiviral vectors expressing two-gene T-cell receptors directed against the melanoma differentiation antigens gp100 and MART-1 the addition of spacer sequences was shown to be a prerequisite for efficient synthesis and assembly of biologically active T-cell receptor complexes [82]. The space available for the insertion of foreign sequences into certain virus vectors, such as AAV, is a limiting factor, and shorter 2A-like sequences are incorporated to save vital coding capacity. For example, they were used to design a tricistronic vector that can co-express the a-L-iduronidase IDUA gene with two different reporter genes (LUC

and DsRed2) [129], to co-express four genes for assembly of the four transmembrane proteins of the CD3 complex [99] and to generate iPS cells from somatic cells by simultaneous lentiviral vector transduction of four transcription factors in a [KLF4-E2A$_{20}$-OCT3/4-T2A$_{18}$-SOX2-P2A$_{19}$-c-Myc]-IRES-hrGFP construct [130].

In plants, as well as targeting to various subcellular localizations in plant cells [131-134] the 2A co-expression system has been used to improve drought-resistance [135], disease resistance[136,137], nutritional value through metabolome engineering [70] and to produce functional recombinant proteins including vaccines and antibodies[138,139]. The most widely used 2A sequence in plants is F2A. Although part of the 2A remains on the C-terminus of upstream proteins after processing, it can be removed by incorporating the first nine amino acids of the LP4 linker ahead of the 2A peptide to generate a hybrid linker peptide (LP4/2A: -SN$^{\downarrow}$AADEVATQLLNFD LLKLAGDVESNPG$^{\downarrow}$P-) [136]. In 2012, Sun *et al*. used LP4/2A or 2A to link the *Bacillus thuringiensis* (Bt) *cry1Ah* gene, which encodes the insect-resistance protein, and the *mG$_2$-epsps* gene, which encodes the glyphosate-tolerance protein. In this comparative experiment, the expression levels of the two genes linked by LP4/2A were higher than those linked by 2A, regardless of the order of the genes within the vector [140]. Furthermore, tobacco plants transformed with the LP4/2A fusion vectors showed better pest resistance and glyphosate tolerance than the plants with the genes linked by 2A.

CONCLUSION

Various strategies have been developed for protein co-expression in eukaryotic cells and all of them have their advantages and disadvantages. Co-transfection with several plasmid DNAs leads to a heterogeneous cell population with different protein expression levels. Viral co-infection usually leads to high infection rates (up to 100%), but the expression is usually transient and the capacity of viral vectors as carriers of foreign DNA is limited. The translation of a polyprotein followed by proteolytic cleavage allows a short mRNA, but the N- and C-terminus of the mature protein are then modified which may lead to problems with proteins that need a native N- and C-terminus. The use of proteinase cleavage sites is further complicated by the tissue/species specificity of proteinase expression, and by the fact that processing of the fusion protein is post-, and not co-, translational. Co-expression using IRES results in different expression levels of upstream and downstream genes, while 'self-cleaving' 2A sequence stays at the C-terminus of the upstream gene [75,141]. Still, astonishing number of studies used IRES and 2A sequences successfully: IRES elements were used in at least 200 studies, while 2A technology was used in almost 800 studies [12,30,141,142].

References

1. Schlatter S,Stansfield SH, Dinnis DM, Racher AJ, Birch JR. On the optimal ratio of heavy to light chain genes for efficient recombinant antibody production by CHO cells. Biotechnol Prog. 2005; 21: 122-133.
2. Hoffmann E, Neumann G, Hobom G, Webster RG, Kawaoka Y. “Ambisense” approach for the generation of influenza A virus: vRNA and mRNA synthesis from one template. Virology. 2000; 267: 310-317.
3. Dull T,Zufferey R, Kelly M, Mandel RJ, Nguyen M. A third-generation lentivirus vector with a conditional packaging system. J Virol. 1998; 72: 8463-8471.
4. Naldini L,Blömer U, Gage FH, Trono D, Verma IM. Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. Proc Natl Acad Sci U S A. 1996; 93: 11382-11388.
5. Weber K, Mock U, Petrowitz B, Bartsch U, Fehse B.Lentiviral gene ontology (LeGO) vectors equipped with novel drug-selectable fluorescent proteins: new building blocks for cell marking and multi-gene analysis. Gene Ther. 2010; 17: 511-520.
6. Datta K,Baisakh N, Thet KM, Tu J, Datta SK. Pyramiding transgenes for multiple resistance in rice against bacterial blight, yellow stem borer and sheath blight. Theor Appl Genet. 2002; 106: 1-8.
7. Yang Z, Chen H, Tang W, Hua H, Lin Y. Development and characterisation of transgenic rice expressing two Bacillus thuringiensis genes. Pest Manag Sci. 2011; 67: 414-422.
8. Jobling SA, Westcott RJ, Tayal A, Jeffcoat R, Schwall GP. Production of a freeze-thaw-stable potato starch by antisense inhibition of three starch synthase genes. Nat Biotechnol. 2002; 20: 295-299.
9. Qi B, Fraser T, Mugford S, Dobson G, Sayanova O. Production of very long chain polyunsaturated omega-3 and omega-6 fatty acids in plants. Nat Biotechnol. 2004; 22: 739-745.
10. Huston JS, Mudgett-Hunter M, Tai MS, McCartney J, Warren F. Protein engineering of single-chain Fv analogs and fusion proteins. Methods Enzymol. 1991; 203: 46-88.
11. Robinson CR, Sauer RT. Optimizing the stability of single-chain proteins by linker length and composition mutagenesis. Proc Natl Acad Sci U S A. 1998; 95: 5929-5934.
12. Kerrigan JJ,Xie Q, Ames RS, Lu Q. Production of protein complexes *via* co-expression. Protein Expr Purif. 2011; 75: 1-14.
13. Marcos JF,Beachy RN. In vitro characterization of a cassette to accumulate multiple proteins through synthesis of a self-processing polypeptide. Plant Mol Biol. 1994; 24: 495-503.
14. Marcos JF,Beachy RN. Transgenic accumulation of two plant virus coat proteins on a single self-processing polypeptide. J Gen Virol. 1997; 78 : 1771-1778.
15. Liang H,Gao H, Maynard CA, Powell WA. Expression of a self-processing, pathogen resistance-enhancing gene construct in Arabidopsis. Biotechnol Lett. 2005; 27: 435-442.
16. Dasgupta S, Collins GB, Hunt AG. Co-ordinated expression of multiple enzymes in different subcellular compartments in plants. Plant J. 1998; 16: 107-116.
17. Sun P, Austin BP, Tözsér J, Waugh DS. Structural determinants of tobacco vein mottling virus protease substrate specificity. Protein Sci. 2010; 19: 2240-2251.
18. Urwin PE, McPherson MJ, Atkinson HJ. Enhanced transgenic plant resistance to nematodes by dual proteinase inhibitor constructs. Planta. 1998; 204: 472-479.
19. Isabelle EJA Françoisa, Willem F Broekaerta, Bruno PA Cammue. Different approaches for multi-transgene-stacking in plants. Plant Science. 2002; 163: 281-295.
20. François I, Dwyer G, de Bolle M, Goderis I, Van HemelrijckW, et al. Processing in transgenic *Arabidopsis thaliana* plants of polyproteins with linker peptide variants derived from the *Impatiens balsmina* antimicrobial polyprotein precursor. Plant Physiology and Biochemistry. 2002; 40: 871-879.
21. Fuller RS, Sterne RE, Thorner J. Enzymes required for yeast prohormone processing. Annu Rev Physiol. 1988; 50: 345-362.
22. Kinal H, Park CM, Berry JO, Koltin Y, Bruenn JA. Processing and secretion of a virally encoded antifungal toxin in transgenic tobacco plants: evidence for a Kex2p pathway in plants. Plant Cell. 1995; 7: 677-688.
23. Thomas G, Thorne BA, Thomas L, Allen RG, Hruby DE. Yeast KEX2 endopeptidase correctly cleaves a neuroendocrine prohormone in mammalian cells. Science. 1988; 241: 226-230.
24. Zhang B,Rapolu M, Huang L, Su WW. Coordinate expression of multiple proteins in plant cells by exploiting endogenous kex2p-like protease activity. Plant Biotechnol J. 2011; 9: 970-981.

25. Halpin C,Barakate A, Askari BM, Abbott JC, Ryan MD. Enabling technologies for manipulating multiple genes on complex pathways. Plant Mol Biol. 2001; 47: 295-310.

26. Jackson RJ, Kaminski A. Internal initiation of translation in eukaryotes: the picornavirus paradigm and beyond. RNA. 1995; 1: 985-1000.

27. Sachs AB,Sarnow P, Hentze MW. Starting at the beginning, middle, and end: translation initiation in eukaryotes. Cell. 1997; 89: 831-838.

28. Yang Q,Sarnow P. Location of the internal ribosome entry site in the 5' non-coding region of the immunoglobulin heavy-chain binding protein (BiP) mRNA: evidence for specific RNA-protein interactions. Nucleic Acids Res. 1997; 25: 2800-2807.

29. Huez I,Créancier L, Audigier S, Gensac MC, Prats AC. Two independent internal ribosome entry sites are involved in translation initiation of vascular endothelial growth factor mRNA. Mol Cell Biol. 1998; 18: 6178-6190.

30. Mokrejs M, Vopálenský V, Kolenaty O, Masek T, Feketová Z. IRESite: the database of experimentally verified IRES structures (www.iresite.org). Nucleic Acids Res. 2006; 34: D125-130.

31. Mokrejs M,Masek T, Vopálensky V, Hlubucek P, Delbos P.IRESite--a tool for the examination of viral and cellular internal ribosome entry sites. Nucleic Acids Res. 2010; 38: D131-136.

32. Jang SK,Kräusslich HG, Nicklin MJ, Duke GM, Palmenberg AC. A segment of the 5' nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during *in vitro* translation. J Virol. 1988; 62: 2636-2643.

33. Mountford PS, Smith AG. Internal ribosome entry sites and dicistronic RNAs in mammalian transgenesis. Trends Genet. 1995; 11: 179-184.

34. Jang SK,Wimmer E. Cap-independent translation of encephalomyocarditis virus RNA: structural elements of the internal ribosomal entry site and involvement of a cellular 57-kD RNA-binding protein. Genes Dev. 1990; 4: 1560-1572.

35. Belsham GJ. Dual initiation sites of protein synthesis on foot-and-mouth disease virus RNA are selected following internal entry and scanning of ribosomes *in vivo*. EMBO J. 1992; 11: 1105-1110.

36. Glass MJ, Summers DF. Identification of a trans-acting activity from liver that stimulates hepatitis A virus translation *in vitro*. Virology. 1993; 193: 1047-1050.

37. Vagner S, Waysbort A, Marenda M, Gensac M, Amalric F, et al. Alternative translation initiation of the Moloney murine leukemia virus mRNA controlled by internal ribosome entry involving the p57/PTB splicing factor. J. Biol. Chem. 1995; 270: 20376-20383.

38. Jendrach M, Thiel V, Siddell S. Characterization of an internal ribosome entry site within mRNA 5 of murine hepatitis virus. Arch Virol. 1999; 144: 921-933.

39. Chappell S, Edelman G, Mauro V. A 9-nt segment of a cellular mRNA can function as an internal ribosome entry site (IRES) and when present in linked multiple copies greatly enhances IRES activity. Proc Nat AcadSci USA. 2000; 97: 1536-1541.

40. Hudder A, Werner R. Analysis of a Charcot-Marie-Tooth disease mutation reveals an essential internal ribosome entry site element in the connexin-32 gene. J Biol Chem. 2000; 275: 34586-34591.

41. Martínez-Salas E. Internal ribosome entry site biology and its use in expression vectors. Curr Opin Biotechnol. 1999; 10: 458-464.

42. López de Quinto S,Martínez-Salas E. Parameters influencing translational efficiency in aphthovirus IRES-based bicistronic expression vectors. Gene. 1998; 217: 51-56.

43. Mizuguchi H,Xu Z, Ishii-Watabe A, Uchida E, Hayakawa T. IRES-dependent second gene expression is significantly lower than cap-dependent first gene expression in a bicistronic vector. Mol Ther. 2000; 1: 376-382.

44. Hennecke M,Kwissa M, Metzger K, Oumard A, Kröger A. Composition and arrangement of genes define the strength of IRES-driven translation in bicistronic mRNAs. Nucleic Acids Res. 2001; 29: 3327-3334.

45. Ngoi SM,Chien AC, Lee CG. Exploiting internal ribosome entry sites in gene therapy vector design. Curr Gene Ther. 2004; 4: 15-31.

46. Holcik M,Yeh C, Korneluk RG, Chow T. Translational upregulation of X-linked inhibitor of apoptosis (XIAP) increases resistance to radiation induced cell death. Oncogene. 2000; 19: 4174-4177.

47. Holcik M,Sonenberg N, Korneluk RG. Internal ribosome initiation of translation and the control of cell death. Trends Genet. 2000; 16: 469-473.

48. Zhu J,Musco ML, Grace MJ. Three-color flow cytometry analysis of tricistronic expression of eBFP, eGFP, and eYFP using EMCV-IRES linkages. Cytometry. 1999; 37: 51-59.

49. Hotta A,Kamihira M, Itoh K, Morshed M, Kawabe Y. Production of anti-CD2 chimeric antibody by recombinant animal cells. J Biosci Bioeng. 2004; 98: 298-303.

50. Ramesh N, Kim ST, Wei MQ, Khalighi M, Osborne WR. High-titer bicistronic retroviral vectors employing foot-and-mouth disease virus internal ribosome entry site. Nucleic Acids Res. 1996; 24: 2697-2700.

51. Borman AM, Le Mercier P, Girard M, Kean KM. Comparison of picornaviral IRES-driven internal initiation of translation in cultured cells of different origins. Nucleic Acids Res. 1997; 25: 925-932.

52. Sáiz J-C, López de Quinto S, Ibarrola N, López-Labrador F-X, Sánchez-Tapias J-M. et al. Internal initiation of translation efficiency in different hepatitis C genotypes isolated from interferon treated patients. Arch Virol. 1999; 144: 215-229.

53. Bernstein J,Sella O, Le SY, Elroy-Stein O. PDGF2/c-sis mRNA leader contains a differentiation-linked internal ribosomal entry site (D-IRES). J Biol Chem. 1997; 272: 9356-9362.

54. Stein I,Itin A, Einat P, Skaliter R, Grossman Z. Translation of vascular endothelial growth factor mRNA by internal ribosome entry: implications for translation under hypoxia. Mol Cell Biol. 1998; 18: 3112-3119.

55. Urabe M,Hasumi Y, Ogasawara Y, Matsushita T, Kamoshita N. A novel dicistronic AAV vector using a short IRES segment derived from hepatitis C virus genome. Gene. 1997; 200: 157-162.

56. Douin V,Bornes S, Creancier L, Rochaix P, Favre G. Use and comparison of different internal ribosomal entry sites (IRES) in tricistronic retroviral vectors. BMC Biotechnol. 2004; 4: 16.

57. Stewart S,Semler BL. RNA determinants of picornavirus cap independent translation initiation. Sem Virol. 1997; 8: 242-255.

58. Borman AM, Le Mercier P, Girard M, Kean KM. Comparison of picornaviral IRES-driven internal initiation of translation in cultured cells of different origins. Nucleic Acids Res. 1997; 25: 925-932.

59. Fussenegger M, Mazur X, Bailey JE.pTRIDENT, a novel vector family for tricistronic gene expression in mammalian cells. Biotechnol Bioeng. 1998; 57: 1-10.

60. Fan L, Owen JS, Dickson G. Construction and characterization of polycistronic retrovirus vectors for sustained and high-level co-expression of apolipoprotein A-I and lecithin-cholesterol acyltransferase. Atherosclerosis. 1999; 147: 139-145.

61. De Felipe P,Izquierdo M.Tricistronic and tetracistronic retroviral vectors for gene transfer. Hum Gene Ther. 2000; 11: 1921-1931.

62. de Felipe P,Izquierdo M. Construction and characterization of pentacistronic retrovirus vectors. J Gen Virol. 2003; 84: 1281-1285.

63. de Felipe P.Polycistronic viral vectors. Curr Gene Ther. 2002; 2: 355-378.

64. De PietriTonelli D,Mihailovich M, Schnurbus R, Pesole G, Grohovaz F. Translational control of Scamper expression *via* a cell-specific internal ribosome entry site. Nucleic Acids Res. 2003; 31: 2508-2513.

65. Fan L, Drew J, Dunckley M, Owen J, Dickson G. Efficient coexpression and secretion of anti-atherogenic human apoliprotein AI and lecithin-cholesterol acyltransferase by cultured muscle cells using adeno-associated virus plasmid vectors. Gene Ther. 1998; 5: 1434-1440.

66. Urwin P, Yi L, Martin H, Atkinson H, Gilmartin PM. Functional characterization of the EMCV IRES in plants. Plant J. 2000; 24: 583-589.

67. Urwin P, Zubko E, Atkinson H. The biotechnological application and limitation of IRES to deliver multiple defence genes to plant pathogens. Physiological and Molecular Plant Pathology.2002; 61: 103-108.

68. Groppelli E, Belsham G, Roberts L. Identification of minimal sequences of the Rhopalosiphumpadi virus 5' untranslated region required for internal initiation of protein synthesis in mammalian, plant and insect translation systems. J Gen Virol. 2007; 88: 1583-1588.

69. Toth RL, Chapman S, Carr F, Santa Cruz S. A novel strategy for the expression of foreign genes from plant virus vectors. FEBS Lett. 2001; 489: 215-219.

70. Ha SH, Liang YS, Jung H, Ahn MJ, Suh SC. Application of two bicistronic systems involving 2A and IRES sequences to the biosynthesis of carotenoids in rice endosperm. Plant Biotechnol J. 2010; 8: 928-938.

71. Dorokhov YL,Skulachev MV, Ivanov PA, Zvereva SD, Tjulkina LG.Polypurine (A)-rich sequences promote cross-kingdom conservation of internal ribosome entry. Proc Natl Acad Sci U S A. 2002; 99: 5301-5306.

72. El-Banna A,Hajirezaei MR, Wissing J, Ali Z, Vaas L. Over-expression of PR-10a leads to increased salt and osmotic tolerance in potato cell cultures. J Biotechnol. 2010; 150: 277-287.

73. Hanafy MS, El-Banna A, Schumacher HM, Jacobsen HJ, Hassan FS. Enhanced tolerance to drought and salt stresses in transgenic faba bean (*Viciafaba L.*) plants by heterologous expression of the PR10a gene from potato. Plant Cell Rep. 2013; 32: 663-674.

74. Trichas G,Begbie J, Srinivas S. Use of the viral 2A peptide for bicistronic expression in transgenic mice. BMC Biol. 2008; 6: 40.

75. Luke GA, Roulston C, Tilsner J, Ryan M. Growing Uses of 2A in Plant Biotechnology. In: Ekinci D, editor. Biotechnology. Rijeka: InTech.2015.

76. de Felipe P, Martín V, Cortés ML, Ryan M, Izquierdo M. Use of the 2A sequence from foot-and-mouth disease virus in the generation of retroviral vectors for gene therapy. Gene Ther. 1999; 6: 198-208.

77. Donnelly ML, Hughes LE, Luke G, Mendoza H, ten Dam E. The 'cleavage' activities of foot-and-mouth disease virus 2A site-directed mutants and naturally occurring '2A-like' sequences. J Gen Virol. 2001; 82: 1027-1041.

78. de Felipe P, Hughes LE, Ryan MD, Brown JD. Co-translational, intraribosomal cleavage of polypeptides by the foot-and-mouth disease virus 2A peptide. J Biol Chem. 2003; 278: 11441-11448.

79. Furler S,Paterna J, Weibel M, Bueler H. Recombinant AAV vectors containing the foot and mouth disease virus 2A sequence confer efficient bicistronic gene expression in cultured cells and rat substantianigra neurons. Gene Ther. 2001; 8: 864–873.

80. Fang J,Qian JJ, Yi S, Harding TC, Tu GH. Stable antibody expression at therapeutic levels using the 2A peptide. Nat Biotechnol. 2005; 23: 584-590.

81. Funston GM,Kallioinen SE, de Felipe P, Ryan MD, Iggo RD. Expression of heterologous genes in oncolytic adenoviruses using picornaviral 2A sequences that trigger ribosome skipping. J Gen Virol. 2008; 89: 389-396.

82. Yang S, Cohen CJ, Peng PD, Zhao Y, Cassard L. Development of optimal bicistroniclentiviral vectors facilitates high-level TCR gene expression and robust tumor cell recognition. Gene Ther. 2008; 15: 1411-1423.

83. Tan Y, Liang H, Chen A, Guo X.Coexpression of double or triple copies of the rabies virus glycoprotein gene using a 'self-cleaving' 2A peptide-based replication-defective human adenovirus serotype 5 vector. Biologicals. 2010; 38: 586-593.

84. Takata Y,Kishine H, Sone T, Andoh T, Nozaki M. Generation of iPS cells using a BacMammultigene expression system. Cell Struct Funct. 2011; 36: 209-222.

85. Martin D Ryan, Michelle Donnelly, Arwel Lewis, Amit P Mehrotra, John Wilkie, et al. A model for non-stoichiometric, co-translational protein scission in eukaryotic ribosomes. Bioorganic Chem. 1999; 27: 55-79.

86. Atkins J, Wills N, Loughran G, Wu C, Parsawar K, et al. A case for 'StopGo': Reprogramming translation to augment codon meaning of GGN by promoting unconventional termination (Stop) after addition of glycine and then allowing continued translation (Go). RNA. 2007; 13: 1-8.

87. Klump H,Schiedlmeier B, Vogt B, Ryan M, Ostertag W. Retroviral vector-mediated expression of HoxB4 in hematopoietic cells using a novel coexpression strategy. Gene Ther. 2001; 8: 811-817.

88. Luke GA, de Felipe P, Lukashev A, Kallioinen SE, Bruno EA. Occurrence, function and evolutionary origins of '2A-like' sequences in virus genomes. J Gen Virol. 2008; 89: 1036-1042.

89. Minskaia E, Nicholson J, Ryan MD.Optimisation of the foot-and-mouth disease virus 2A co-expression system for biomedical applications. BMC Biotechnol. 2013; 13: 67.

90. Groot Bramel-Verheije MH,Rottier PJ, Meulenberg JJ. Expression of a foreign epitope by porcine reproductive and respiratory syndrome virus. Virology. 2000; 278: 380-389.

91. Kinsella TM,Ohashi CT, Harder AG, Yam GC, Li W.Retrovirally delivered random cyclic Peptide libraries yield inhibitors of interleukin-4 signaling in human B cells. J Biol Chem. 2002; 277: 37512-37518.

92. Hahn H,Palmenberg AC. Deletion mapping of the encephalomyocarditis virus primary cleavage site. J Virol. 2001; 75: 7215-7218.

93. Milsom MD,Woolford LB, Margison GP, Humphries RK, Fairbairn LJ. Enhanced *in vivo* selection of bone marrow cells by retroviral-mediated coexpression of mutant O6-methylguanine-DNA-methyltransferase and HOXB4. Mol Ther. 2004; 10: 862-873.

94. Lengler J,Holzmüller H, Salmons B, Günzburg WH, Renner M. FMDV-2A sequence and protein arrangement contribute to functionality of CYP2B1-reporter fusion protein. Anal Biochem. 2005; 343: 116-124.

95. Ryan MD, Drew J. Foot-and-mouth disease virus 2A oligopeptide mediated cleavage of an artificial polyprotein. EMBO J. 1994; 13: 928-933.

96. Ryan MD, Donnelly M, Flint M, Cowton V, Luke G, et al. Foot-and-mouth disease virus proteinases. In: Sobrino F, Domingo E, editors. Foot and Mouth Disease Current Perspectives. England: Horizon Bioscience. 2004; 53-76.

97. Varshavsky A. The N-end rule. Cell. 1992; 69: 725-735.

98. Fang J, Yi S, Simmons A, Tu GH, Nguyen M. An antibody delivery system for regulated expression of therapeutic levels of monoclonal antibodies *in vivo*. MolTher. 2007; 15: 1153-1159.

99. Szymczak AL, Workman CJ, Wang Y, Vignali KM, Dilioglou S. Correction of multi-gene deficiency *in vivo* using a single 'self-cleaving' 2A peptide-based retroviral vector. Nat Biotechnol. 2004; 22: 589-594.

100. Szymczak AL,Vignali DA. Development of 2A peptide-based strategies in the design of multicistronic vectors. Expert Opin Biol Ther. 2005; 5: 627-638.

101. Huang X, Wilber AC, Bao L, Tuong D, Tolar J. Stable gene transfer and expression in human primary T cells by the Sleeping Beauty transposon system. Blood. 2006; 107: 483-491.

102. Hart DP,Xue SA, Thomas S, Cesco-Gaspere M, Tranter A. Retroviral transfer of a dominant TCR prevents surface expression of a large proportion of the endogenous TCR repertoire in human T cells. Gene Ther. 2008; 15: 625-631.

103. Hsiao EC,Yoshinaga Y, Nguyen TD, Musone SL, Kim JE. Marking embryonic stem cells with a 2A self-cleaving peptide: a NKX2-5 emerald GFP BAC reporter. PLoS One. 2008; 3: e2532.

104. Sommer CA,Stadtfeld M, Murphy GJ, Hochedlinger K, Kotton DN. Induced pluripotent stem cell generation using a single lentiviral stem cell cassette. Stem Cells. 2009; 27: 543-549.

105. Carey BW,Markoulaki S, Hanna J, Saha K, Gao Q. Reprogramming of murine and human somatic cells using a single polycistronic vector. Proc Natl Acad Sci USA. 2009; 106: 157-162.

106. Kaji K,Norrby K, Paca A, Mileikovsky M, Mohseni P. Virus-free induction of pluripotency and subsequent excision of reprogramming factors. Nature. 2009; 458: 771-775.

107. Davies SL, O'Callaghan PM, McLeod J, Pybus LP, Sung YH. Impact of gene vector design on the control of recombinant monoclonal antibody production by Chinese hamster ovary cells. Biotechnol Prog. 2011; 27: 1689-1699.

108. Maier P,Heckmann D, Spier I, Laufs S, Zucknick M. F2A sequence linking MGMT(P140K) and MDR1 in a bicistroniclentiviral vector enables efficient chemoprotection of haematopoietic stem cells. Cancer Gene Ther. 2012; 19: 802-810.

109. Hutson T, Kathe C, Menezes S, Rooney M, Bueler H, et al. The use of an adeno-associated viral vector for efficient bicistronic expression of two genes in the central nervous system. Methods in Molecular Biology. 2014; 1162: 189-207.

110. Knudsen ML,Ljungberg K, Kakoulidou M, Kostic L, Hallengärd D. Kinetic and phenotypic analysis of CD8+ T cell responses after priming with alphavirus replicons and homologous or heterologous booster immunizations. J Virol. 2014; 88: 12438-12451.

111. Ndjamen B, Farley AH, Lee T, Fraser SE,Bjorkman PJ. The herpes virus Fc receptor gE-gI mediates antibody bipolar bridging to clear viral antigens from the cell surface. PLoS Pathog. 2014; 10: e1003961.

112. Tchieu J,Kuoy E, Chin MH, Trinh H, Patterson M. Female human iPSCs retain an inactive X chromosome. Cell Stem Cell. 2010; 7: 329-342.

113. Papapetrou EP,Sadelain M. Generation of transgene-free human induced pluripotent stem cells with an excisable single polycistronic vector. Nat Protoc. 2011; 6: 1251-1273.

114. Daniels RW,Rossano AJ, Macleod GT, Ganetzky B. Expression of multiple transgenes from a single construct using viral 2A peptides in Drosophila. PLoS One. 2014; 9: e100637.

115. Mariano A, Xu L, Han R. Highly efficient genome editing *via* 2A-coupled co-expression of two TALEN monomers. BMC Res Notes. 2014; 7: 628.

116. Park K,Jeong J, Chung BH. Live imaging of cellular dynamics using a multi-imaging vector in single cells. Chem Commun (Camb). 2014; 50: 10734-10736.

117. Engelhardt J, Boldajipour B, Beemiller P, Pandurangi P, Sorensen C.Marginating dendritic cells of the tumor microenvironment cross-present tumor antigens and stably engage tumor-specific T cells. Cancer Cell. 2012; 21: 402-417.

118. Hammachi F, Morrison GM, Sharov AA, Livigni A, Narayan S. Transcriptional activation by Oct4 is sufficient for the maintenance and induction of pluripotency. Cell Rep. 2012; 1: 99-109.

119. Tittel AP,Heuser C, Ohliger C, Llanto C, Yona S. Functionally relevant neutrophilia in CD11c diphtheria toxin receptor transgenic mice. Nat Methods. 2012; 9: 385-390.

120. Matheson NJ,Peden AA, Lehner PJ. Antibody-free magnetic cell sorting of genetically modified primary human CD4+ T cells by one-step streptavidin affinity purification. PLoS One. 2014; 9: e111437.

121. Matsuzaki Y,Oue M, Hirai H. Generation of a neurodegenerative disease mouse model using lentiviral vectors carrying an enhanced synapsin I promoter. J Neurosci Methods. 2014; 223: 133-143.

122. Dempsey WP, Fraser SE, Pantazis P.PhOTOzebrafish: a transgenic resource for *in vivo* lineage tracing during development and regeneration. PLoS One. 2012; 7: e32888.

123. Alaee F, Sugiyama O, Virk MS, Tang H, Drissi H. Suicide gene approach using a dual-expression lentiviral vector to enhance the safety of *ex vivo* gene therapy for bone repair. Gene Ther. 2014; 21: 139-147.

124. Hara S, Takano T, Ogata M, Yamakami R, Sato Y. Establishment of a conditional transgenic system using the 2A peptide in the female mouse germline. J Reprod Dev. 2014; 60: 250-255.

125. Naik E, Webster JD,DeVoss J, Liu J,Suriben R. Regulation of proximal T cell receptor signaling and tolerance induction by deubiquitinase Usp9X. J Exp Med. 2014; 211: 1947-1955.

126. Camper N, Byrne R, Burden R, Lowry J, Gray B, et al. Stable expression and purification of a functional processed Fab' fragment from a single nascent polypeptide in CHO cells expressing the mCAT-1 retroviral receptor. J Immunol Methods. 2011; 372: 30-41.

127. Simmons AD,Moskalenko M, Creson J, Fang J, Yi S. Local secretion of anti-CTLA-4 enhances the therapeutic efficacy of a cancer immunotherapy with reduced evidence of systemic autoimmunity. Cancer Immunol Immunother. 2008; 57: 1263-1270.

128. Holst J,Szymczak-Workman AL, Vignali KM, Burton AR, Workman CJ. Generation of T-cell receptor retrogenic mice. Nat Protoc. 2006; 1: 406-417.

129. Osborn MJ,Panoskaltsis-Mortari A, McElmurry RT, Bell SK, Vignali DA. A picornaviral 2A-like sequence-based tricistronic vector allowing for high-level therapeutic gene expression coupled to a dual-reporter system. Mol Ther. 2005; 12: 569-574.

130. Shao L,Feng W, Sun Y, Bai H, Liu J. Generation of iPS cells using defined factors linked *via* the self-cleaving 2A sequences in a single open reading frame. Cell Res. 2009; 19: 296-306.

131. Halpin C, Cooke SE, Barakate A, El Amrani A, Ryan MD. Self-processing 2A-polyproteins--a system for co-ordinate expression of multiple proteins in transgenic plants. Plant J. 1999; 17: 453-459.

132. El Amrani A,Barakate A, Askari BM, Li X, Roberts AG. Coordinate expression and independent subcellular targeting of multiple proteins from a single transgene. Plant Physiol. 2004; 135: 16-24.

133. Samalova M,Fricker M, Moore I.Ratiometric fluorescence-imaging assays of plant membrane traffic using polyproteins. Traffic. 2006; 7: 1701-1723.

134. Samalova M, Fricker M, Moore I. Quantitative and qualitative analysis of plant membrane traffic using fluorescent proteins. Methods Cell Biol. 2008; 85: 353-380.

135. Kwon S, Hwang E, Kwon H. Genetic engineering of drought-resistant potato plants by co-introduction of genes encoding trehalose-6-phosphate synthase and trehalose-6-phosphate of *Zygosaccharomyces rouxii*. Korean J Genet. 2004; 26: 199-206.

136. François I, Van Hemelrijck W, Aerts A, Wouters P, Proost P, et al. Processing in *Arabidopsis thaliana* of a heterologous polyprotein resulting in differential targeting of the individual plant defensins. Plant Science. 2004; 166: 113-121.

137. Geu-Flores F, Olsen CE, Halkier BA. Towards engineering glucosinolates into non-cruciferous plants. Planta. 2009; 229: 261-270.

138. Smolenska L, Roberts IM, Learmonth D, Porter AJ, Harris WJ. Production of a functional single chain antibody attached to the surface of a plant virus. FEBS Lett. 1998; 441: 379-382.

139. Marconi G,Albertini E, Barone P, De Marchis F, Lico C. In planta production of two peptides of the Classical Swine Fever Virus (CSFV) E2 glycoprotein fused to the coat protein of potato virus X. BMC Biotechnol. 2006; 6: 29.

140. Sun H, Lang Z, Zhu L, Huang D. Acquiring transgenic tobacco plants with insect resistance and glyphosate tolerance by fusion gene transformation. Plant Cell Rep. 2012; 31: 1877-1887.

141. Luke G, Ryan M. The protein coexpression problem in biotechnology and biomedicine: virus 2A and 2A-like sequences provide a solution. Future Virol. 2013; 8: 983-996.

142. University of St.Andrews. 2A in Biomedicine & Biotechnology.