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Translating 2A Research Into Practice

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1. Introduction

Viruses have evolved a number of unconventional translation strategies to amplify the coding potential of their condensed genetic information. Leaky stop codons may be read-through to produce either the predicted translation product, or at a very low level an extended "read-through" protein. Overlapping (*e.g.* –UAAUG-; -UGAUG-; AUGA-), or highly proximal stop/start codons may give rise to termination accompanied by a low level of re-initiation. There are a number of cases where a single mRNA is translated into more than one protein by recoding, where the rules for decoding are altered through specific sites and signals in the mRNA such as frameshifting and readthrough. Ribosomal "skipping", first identified in the foot-and-mouth disease virus (FMDV), represents yet another translational trick to deliver multiple gene products from limited primary sequence. Briefly, when a ribosome encounters 2A within an open reading frame (ORF), the synthesis of a specific peptide bond is "skipped". The process gives rise to two alternative outcomes: either (i) translation terminates at the end of 2A, or (ii) translation of the downstream sequence occurs. In this manner discrete translation products can be synthesized from a single ORF (for in-depth reviews of recoding see Atkins & Gesteland, 2010).

2A and "2A-like" sequences have been thoroughly studied in the last 25 years. These results, as well as our current understanding of the underlying mechanism, are summarized in the first section of this review. In the next section, important considerations in the design of 2A peptide-linked vectors are discussed. The 2A peptide system has worked in all eukaryotic systems tested and has been used with some spectacular successes in a variety of biotechnology applications. In the final section we provide an overview of the literature highlighting some of these successes.

2. Basic research

2.1 The fmdv genome

The FMDV genome organization is similar to that of other picornaviruses, comprising a large single ORF flanked by highly structured 5′ and 3′ untranslated regions (UTRs) (Fig. 1). The 5′ UTR, of approximately 1,300 nucleotides (nt) contains sequence elements controlling the replication of viral RNA, packaging of RNA into capsids, and translation of the viral polyprotein. Preceding the ORF is a type II internal ribosome entry site (IRES), crucial for the cap-independent initiation of translation (for reviews see Jackson et al., 1990; Martīnez-Salas & Ryan, 2010). The 3′ UTR is about 90 nt long and is thought to contain cis-acting elements

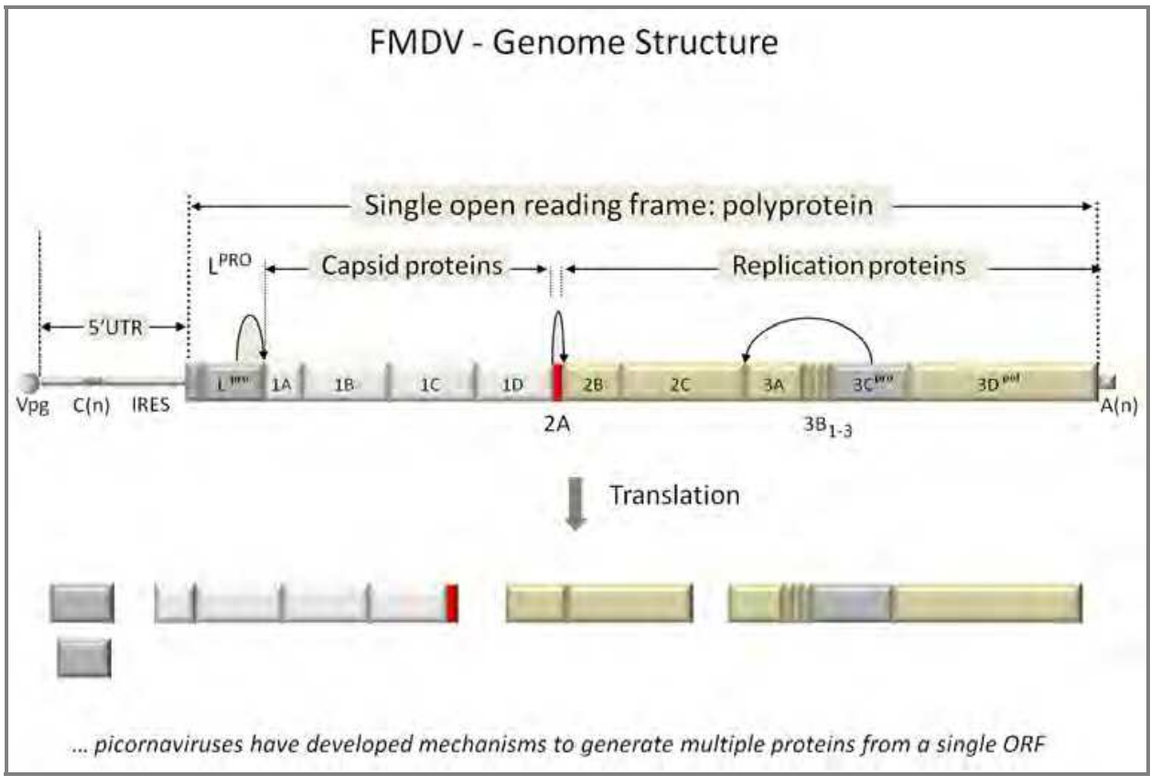

Fig. 1. The FMDV Genome. The FMDV genome is organized like a cellular mRNA: a 5′ untranslated region (5′UTR), a single open reading frame (ORF), a 3′ untranslated region (3′UTR) and a poly(A) tail. The polyprotein (~2,300aa) undergoes three "primary", co-translational cleavages; L^{pro} cleaves at its own C-terminus, 2A mediates "cleavage" at its own C-terminus and $3C^{pro}$ cleaves between [2BC] and 3A. The 2A oligopeptide is only 18aa long, mediating a "cleavage" by a translational effect "ribosome skipping".

required for efficient genome replication (Agol et al., 1999). Moreover, the 3′ end of mRNA has also turned out to be surprisingly important in regulating translation (Wells et al., 1998). The ORF encodes a large protein precursor (polyprotein) which can be divided into three regions, designated P1, P2, and P3. These correspond to the N-terminal capsid protein precursor (P1, containing four capsid proteins 1A-1D), the middle of the polyprotein containing three of the nonstructural proteins (P2, the three proteins 2A-2C), and the most C-terminal segment of the polyprotein containing four non-structural proteins (P3, proteins 3A-3D) (Palmenberg, 1987). The full-length translation product is never observed within infected cells due to co-translational, intramolecular, cleavages mediated by L^{pro}, 2A and $3C^{pro}$ domains within the polyprotein (for reviews see, Belsham, 2005; Ryan et al., 2004). Besides releasing itself from the polyprotein, L^{pro}, in common with $2A^{pro}$ of the entero- and rhinoviruses, also cleaves the translation initiation factor eIF4G (Glaser & Skern, 2000). This results in the inactivation of cap-dependent translation leading to the shutoff of cellular protein synthesis. The 2A oligopeptide is responsible for the primary cleavage which separates the region comprising the capsid proteins from domains downstream of 2A concerned with the replication of the virus (Ryan et al., 1991; Ryan & Drew, 1994). All picornaviruses encode $3C^{pro}$, which carries out a primary cleavage between 2C and 3A and secondary processing of the [P1-2A], [2BC] and P3 precursors. In FMDV, $3C^{pro}$ also cleaves between 2B and 2C (for review see

Martĩnez-Salas & Ryan, 2010). Aside from the processing sites within the viral polyprotein itself, the enzyme also modifies host cell proteins (Belsham et al., 2000; Li et al., 2001).

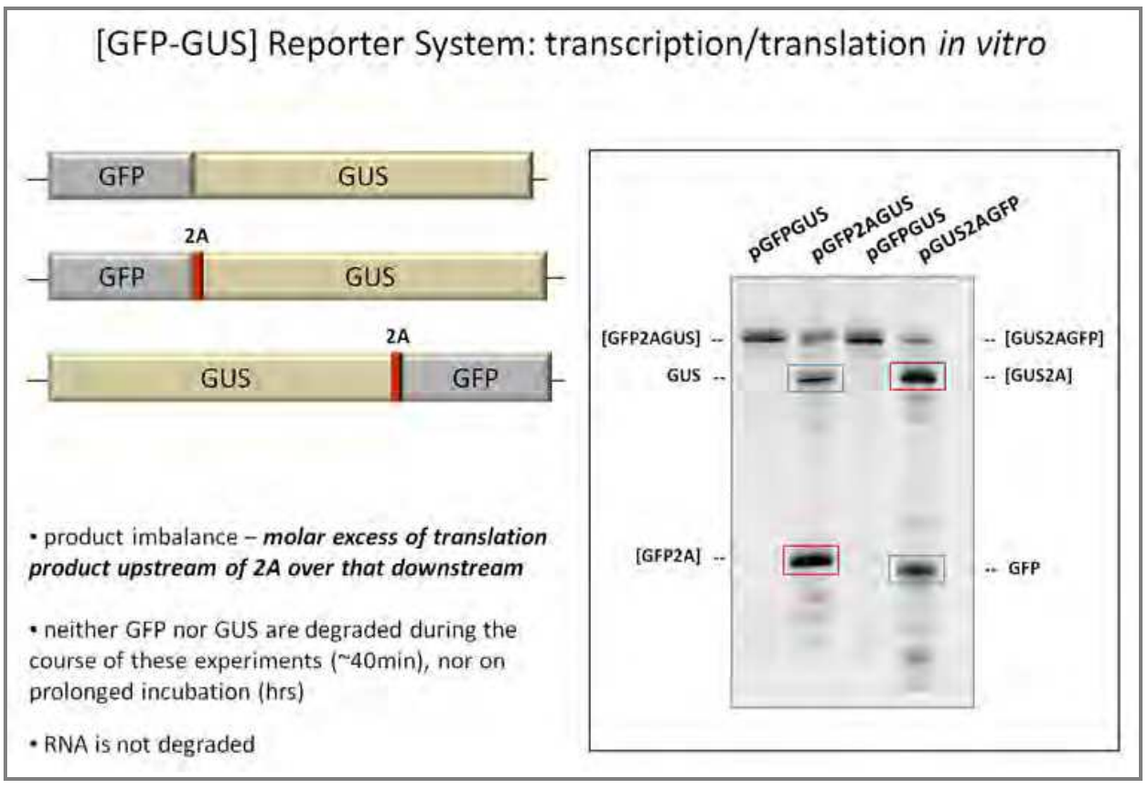

Fig. 2. Analysis of 2A-mediated "cleavage". Artificial polyprotein cDNA constructs comprising the reporter proteins green fluorescent protein (GFP) and β-glucuronidase (GUS) (left panel). SDS-PAGE of radiolabeled *in vitro* translation products (right panel). The control pGFPGUS construct produces only a single translation product – the [GFP-GUS] fusion protein. The translation profile from the pGFP2AGUS construct shows 3 major products: uncleaved [GFP2AGUS] and the cleavage products [GFP2A] and [GUS]. The profile from pGUS2AGFP also shows 3 major products: uncleaved [GUS2AGFP] and the cleavage products [GUS-2A] and [GFP]. The cleavage products upstream of 2A are highlighted in red, showing the molar excess over the downstream products shown in blue.

Secondary $3C^{pro}$ cleavage of the [1D2A] precursor protein between 1D and 2A shows the FMDV 2A segment is only 18aa long (-LLNFDLLKLAGDVESNPG-) (Belsham, 1993). Analysis of recombinant polyproteins and artificial polyprotein systems in which 2A was inserted between two reporter proteins showed that 2A alone, plus the N-terminal proline of protein 2B, was sufficient to mediate a highly efficient co-translational "cleavage" at the C-terminus of 2A (Ryan & Drew, 1994; Ryan et al., 1991; de Felipe et al., 2003). Translation *in vitro*, together with careful quantification of the products (Fig.2), provided the major finding that a molar excess of protein encoded upstream of 2A accumulated over that downstream – an observation at variance with proteolytic processing (Ryan et al., 1989; Donnelly et al., 2001a).

Extensive protein degradation studies, examining the effects of non-specific premature termination of transcription/translation, have shown that none of these effects account for this

imbalance (Ryan et al., 1999). Addition of puromycin at low concentration to translation reactions programmed with mRNA encoding a 2A containing reporter yields significant product with a size corresponding to the protein up to the 2A site, indicating a pause in translation at this position (Donnelly et al., 2001a). Employing a "toe-printing" approach, Doronina and colleagues confirmed that ribosomes pause at the end of the 2A coding sequence (-NPG↓P-), with glycine and proline in the P- and A- sites, respectively (Doronina et al., 2008b). This front end loading was due to different rates of biosynthesis of each portion of the ORF and constitutes a novel type of recoding (Baranov et al., 2002; Brown & Ryan, 2010).

2.2 The cleavage mechanism

The 2A region of the FMDV encodes a sequence that mediates self-processing by a novel translational effect variously referred to as "ribosome skipping" (Ryan et al., 1999), "stop-go" (Atkins et al., 2007) and "stop carry-on" translation (Doronina et al., 2008a). 2A-mediated cleavage occurs between the C-terminal glycine and the proline of the downstream protein 2B (-LLNFDLLKLAGDVESNPG↓P-). The upstream protein contains a short 2A peptide C-terminal fusion, whereas the downstream protein includes a single proline residue on its N-terminus (Ryan and Drew, 1994; Ryan et al., 1991). The translational model of 2A cleavage activity posited is shown in Figure 3. Briefly, the nascent 2A peptide interacts with the exit pore of the ribosome such that the C-terminal portion (-ESNPGP-) is sterically constrained within the peptidyl transferase centre of the ribosome. This inhibits nucleophilic attack of the ester linkage between 2A and $tRNA^{gly}$ by prolyl-tRNA in the A site - effectively stalling, or pausing, translation (Ryan et al., 1999; Donnelly et al., 2001a). It has been shown that this block is relieved by the action of translation release factors eRF1 and eRF3, hydrolysing the ester linkage and releasing the nascent protein (Doronina et al., 2008a & b). Thus two major outcomes are possible; either translation terminates at this point, or, translation effectively 're-initiates' to synthesize the downstream sequences. The latter case would entail; (a) egress of eRF1/3 from the A site, (b) ingress of prolyl-tRNA into the A site, (c) translocation of prolyl-tRNA to the P site and (d) entry of the next aminoacyl-tRNA (for in-depth reviews of the model see Ryan et al., 2002; Martīnez-Salas & Ryan, 2010; Brown & Ryan, 2010).

2.3 The occurrence of 2A and 2A-like sequences

Examining other picornavirus genome sequences showed the DxExNPGP motif to be present in several genera of the *Picornaviridae*: aphtho- cardio-, tescho-, erbo- and certain parechoviruses. Although cardioviruses have much longer natural 2A segments (133 to 143 amino acids) than aphthoviruses, work with *Encephalomyocarditis virus* (EMCV) and *Theiler's murine encephalitis virus* (TMEV) has shown that most of the additional 2A protein is dispensable for primary cleavage activity (Hahn & Palmenberg, 1996; Donnelly et al., 1997). Probing databases for the presence of the motif showed that "2A-like" sequences were also present in a range of non-picornavirus systems. These include a wide range of insect positive-strand RNA viruses belonging to the *Dicistroviridae* and *Tetraviridae* families and the unassigned Iflavirus genus and double-stranded RNA viruses of the *Reoviridae* (insect Cypoviruses and mammalian type C rotaviruses) (Hahn & Palmenberg, 1996; Donnelly et al., 2001b). They are also found in four nonsegmented dsRNA viruses of the *Totiviridae* (Isawa et al., 2011). Analysis of the translation products showed that in all cases these 2As had "cleavage" activity (Luke et al., 2008).

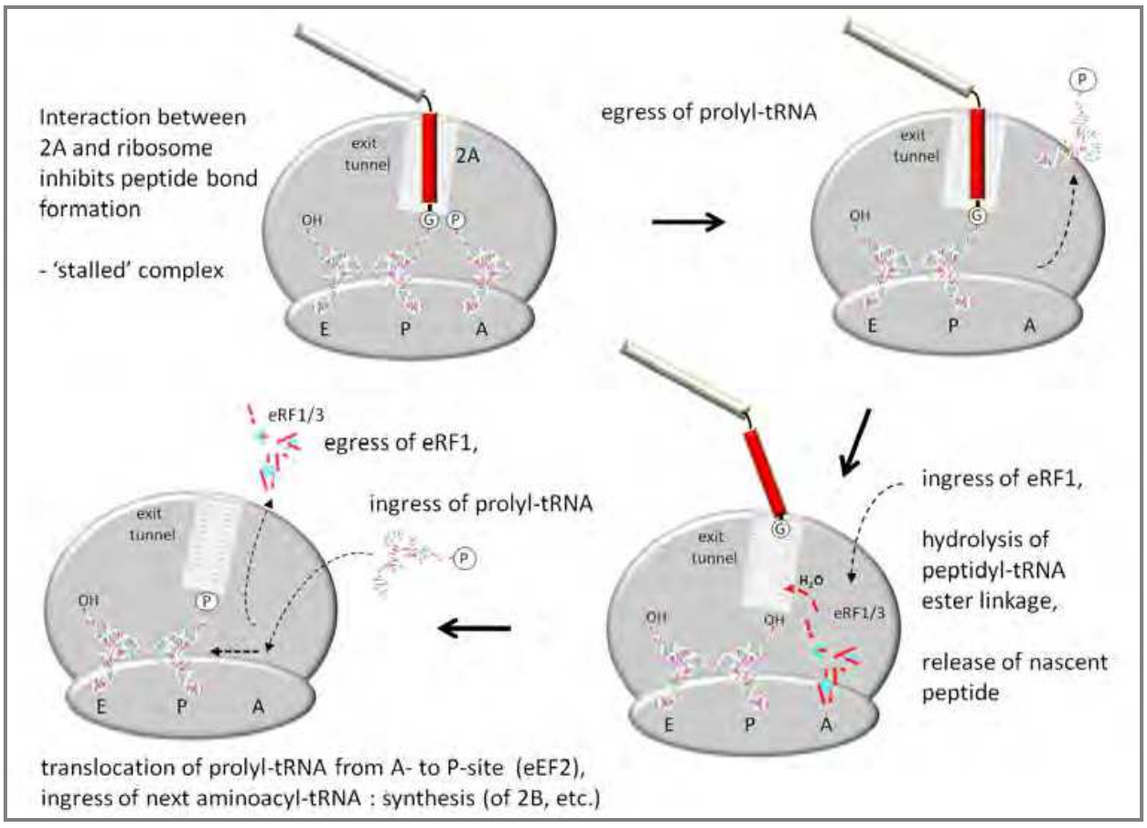

Fig. 3. Schematic representation of the translational model of 2A-mediated "cleavage".

Along with the RNA virus 2As, active 2A-like sequences were also detected in the N-terminal region of the ORFs of non-LTR retrotransposons of *Trypanosoma cruzi* and *T.brucei* – L1Tc and igni, respectively (Donnelly et al., 2001b; Heras et al., 2006). Recently we identified a range of 2As in the purple sea urchin *Strongylocentrotus purpuratus*, then demonstrated their cleavage activities (unpublished data). In this case, 2A-like sequences appear in (i) several copies of non-LTR-retroelements (like trypanosomes) and (ii) the N-terminus of nucleotide binding oligomerization domain (NOD)-like, or CATERPILLER proteins (cited in Brown & Ryan, 2010). It appears, therefore, that this method of controlling protein biogenesis is not confined to viruses or genomic sequences comprising insertion(s) of virus-related sequences (retroelements). 2A and "2A-like" sequences have been shown to function in cells from a wide variety of eukaryotes, ranging from yeast (de Felipe et al., 2003) to plants (Halpin et al., 1999) to insects (Roosien et al., 1990) to mammals (Ryan & Drew, 1994). The only requirement for 2A peptide-based cleavage appears to be translation by 80S ribosomes. The reported proteolysis activity of 1D-2A in *E.coli* cells (Dechamma et al., 2008) was not detected in equivalent constructions in our laboratory showing "cleavage" specificity for eukaryotic systems alone (Donnelly et al., 1997). Although the FMDV 2A sequence (hereafter referred to as "F2A") has been the most widely used, biotechnologists should be aware that many 2A-like sequences have been utilized successfully, including equine rhinitis A virus (ERAV, "E2A"), porcine teschovirus-1 (PTV-1, "P2A") and *Thosea asigna* virus (TaV, "T2A") (Szymczak et al., 2004; Arnold et al., 2004; Osborn et al., 2005; Szymczak & Vignali, 2005; Huang et al., 2006; Scholten et al., 2006; Hart et al., 2008; Sommer et al., 2008; Yang et al., 2008).

3. General considerations when using 2A peptide sequences

3.1 Expression of multiple genes

Conventional approaches for the production of multicistronic vectors include the use of IRES elements, multiple promoters, fusion proteins, *etc* (for a review see de Felipe, 2002). Adverse side-effects with multiple promoters on viral vectors include interference between promoters, promoter suppression and rearrangement (Cullen et al., 1984; Emerman & Temin, 1986). IRESes provided the first method of creating eukaryotic polycistronic mRNAs. The internal ribosome entry site serves as a launching pad for internal initiation of translation, allowing expression of two or more genes from a single transcript (for review see Komar & Hatzoglou, 2005). Since genes are under the control of the same promoter and integrated into the same place within the genome, transgenes expressed in this way are co-ordinately regulated. In bicistronic systems, detection of the product encoded by the second cistron is evidence that the first cistron is also being expressed. This approach has been used successfully in gene therapy research in animal systems, and IRESes from different viruses have been tested and shown to function in plant systems (Urwin et al., 2000 & 2002; Dorokhov et al., 2002; Jaag et al., 2003; Bouabe et al., 2008).

On the other hand, there are a couple of limitations using IRES elements. Firstly, the IRES is a relatively large sequence (~500bp) that can cause problems in packaging, especially for size-restricted viral and nonviral vectors. For instance, retro- and lentiviral vectors possess packaging capacities of 8kb and adeno-associated viruses can accommodate <5kb (Thomas et al., 2003). Secondly, expression of the downstream gene can be as much as 10 fold lower than the upstream gene (Mizuguchi et al. 2000; Flasshove et al., 2000; Hasegawa et al., 2007; Ha et al., 2010). In some instances, this can be useful for expressing fluorescent markers or conferring drug resistance during selection (Ngoi et al., 2004). Nevertheless, the obvious advantages of using the 2A sequence vis-á-vis the IRES are its smaller size (~60-70bp) and the stoichiometric production of both upstream and downstream protein products as measured by: i) chloramphenicol acetyltransferase (CAT) and β-glucuronidase (GUS) enzyme activity (Halpin et al., 1999); ii) cell free translation *in vitro* and Western blot (Ryan & Drew, 1994; Donnelly et al., 2001a & b; de Felipe et al., 2003; Torres et al., 2010); iii) GFP/FACS with antibiotic resistance (Lorens et al., 2004); iv) co-fluorescence reporting (de Felipe & Ryan, 2004; Samalova et al., 2006); v) fluorescence resonance energy transfer (FRET) analysis (Szymczak et al., 2004) and vi) protein segregation in genetically engineered animals (Provost et al., 2007; Trichas et al., 2008). Further, if multiple gene expression is required, different members of the 2A peptide family can be selected to disrupt sequence homology to help maintain foreign gene insert stability.

3.2 Subcellular targeting of proteins from a 2A polyprotein

A merit of this expression strategy is that individual components of the 2A-polyprotein can be targeted to a range of different sub-cellular sites using both co- and post-translational signal sequences (El Amrani et al., 2004; Lorens et al., 2004; Szymczak et al., 2004). We discovered, however, a major problem with co-expression of some proteins targeted to, or passing through, the mammalian endoplasmic reticulum (ER). When a 2A-based polyprotein comprising an upstream protein bearing an N-terminal signal sequence was followed by a protein lacking any signal sequence, both proteins were translocated into the

ER (de Felipe & Ryan, 2004). We have identified the source of this problem - the "slipstreaming" effect was due to inhibition of the 2A reaction (formation of fusion protein) by the C-terminal region (immediately upstream of 2A) of some proteins when translocated into the ER – and suggest possible solutions (de Felipe et al., 2010).

The residues that influence cleavage are predicted to reside within the translocon; this length may allow interactions between the nascent peptide and the ribosome that lead to inhibition of the 2A reaction (Ménétret et al., 2000; Beckmann et al., 2001; de Felipe et al., 2010). Solutions to the problem include the use of longer versions of 2A with extra sequences derived from the capsid protein ("1D") (Ryan et al., 1991; Groot Bramel-Verheije et al., 2000; Donnelly et al., 2001b; Klump et al., 2001). Specifically, N-terminal extension of 2A by 5aa of 1D improved "cleavage", but extension by 14aa of 1D or longer (21 and 39aa) produced complete "cleavage" and an equal stoichiometry of the up- and downstream translation products (Donnelly et al., 2001b, see fig. 4). These observations are consistent with our model in which 2A activity is a product of it's interaction with the exit tunnel of the ribosome which is thought to accommodate 30-40aa (Hardesty & Kramer, 2001). Further,

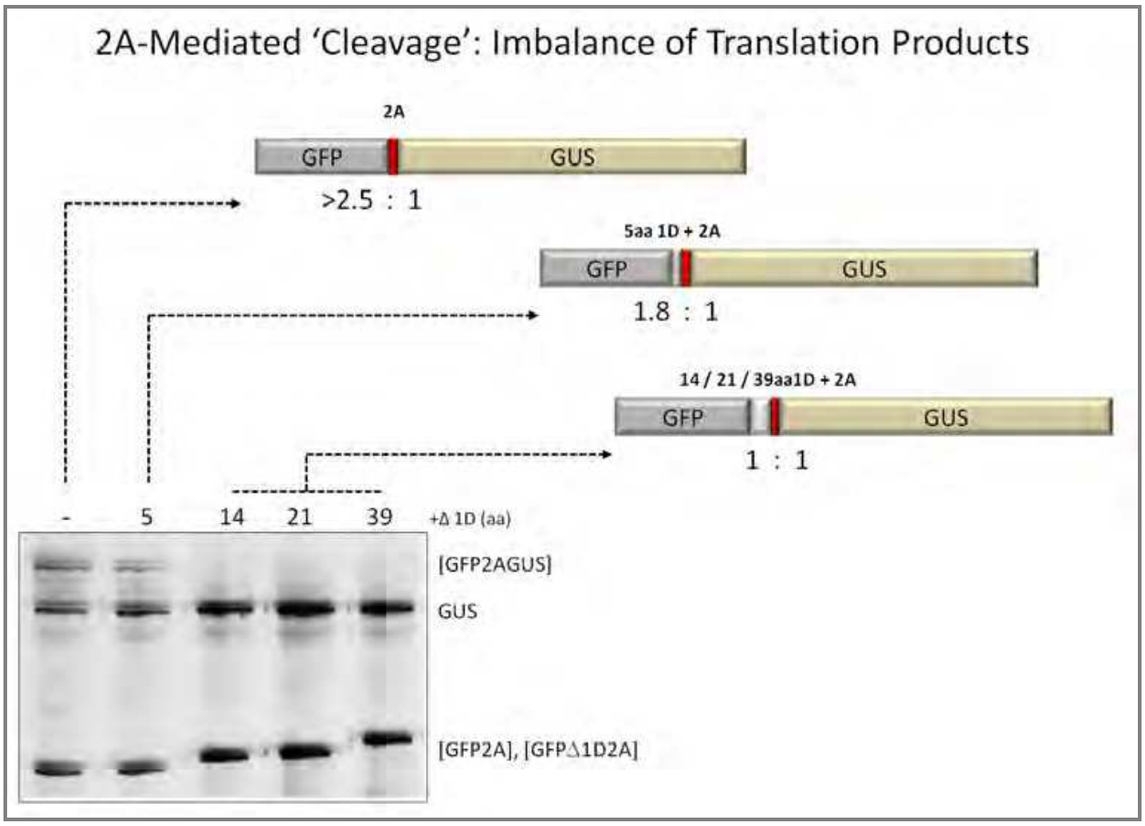

Fig. 4. Translation *in vitro*. Translation products derived from constructs encoding the wild-type 2A sequence are shown together with products derived from constructs encoding N-terminally extended forms of 2A.

the order in which the genes are expressed within the vector needs to be considered. By swapping the order of proteins in several artificial polyproteins the stoichiometry was affected by the gene upstream of 2A (Ma & Mitra, 2002; Lengler et al., 2005; Chinnasamy

et al., 2006; Rothwell et al., 2010). Cleavage activity was independent of the immediate downstream sequence (Ryan et al., 1991; Ma & Mitra., 2002). A number of studies show that cleavage efficiency is improved by incorporation of a flexible Gly-Ser-Gly or Ser-Gly-Ser-Gly linker sequence between the upstream protein and the 2A peptide (Lorens et al., 2004; Szymczak et al., 2004; Holst et al., 2006a & b; Provost et al., 2007; Wargo et al., 2009). A noteworthy caveat to attach to this review is that Yan and colleagues argue slipstreaming translocation does not occur in mammalian cells; that is, the second protein downstream of 2A still requires a signal sequence for secretory or membrane-anchored expression (Yan et al., 2010).

3.3 The unwanted tags

Cleavage occurs at the end of the 2A peptide sequence, therefore most of the 2A remains attached to the C-terminus of the upstream protein. This may affect the activity of some proteins (*e.g.* if their function is affected by the addition of other tags such as Myc, His, *etc*). In the case of proteins translocated into the ER, a strategy was adopted to include a furin proteinase cleavage site (-RA$^{\downarrow}$KR-) between the upstream protein and 2A (Fang et al., 2005). Furin is a cellular endoprotease localized on the trans-Golgi networks of virtually all cell types (Steiner, 1998). Upon entering the lumen of the ER, 2A was trimmed away from the upstream protein (in this case antibody heavy chain), leaving only a 2aa C-terminal extension (-RA). In a follow-on study, the use of alternative furin cleavage sequences consisting of only basic amino acids, which can be efficiently cut by carboxypeptidases ($^{\downarrow}$-RRRR-, $^{\downarrow}$-RKRR-, $^{\downarrow}$-RRKR-), resulted in the expression of antibodies with no residual amino acids (Fang et al., 2007). Proteins expressed in plants could have their 2A extensions removed by endogenous proteinases acting on similar hybrid linker peptides. A polyprotein precursor consisting of two different marker proteins connected by a linker peptide of Impatiens balsamina ($^{\downarrow}$-SNAADEVAT-) followed by F2A was successfully processed in *Arabidopsis thaliana* (François et al., 2002; François et al., 2004).

For biomedical applications using 2A, a concern stems from the addition of 2A-derived sequences to the upstream protein - this protein may act as a carrier to stimulate an anti-2A immune response. Any potential carrier-effects could be abolished by removal of 2A. It should be noted that this "unwanted" tag does confer two advantages. First, antibodies to the 2A-peptide have been generated, allowing detection and/or immunoprecipitation of "upstream" protein products derived from 2A-containing transgenes (de Felipe et al, 2006 & 2010). Second, a shift in protein size is observed in 2A-tagged proteins which can be useful if mutant and endogenous proteins are co-expressed and need to be identified (Szmczak et al., 2004; de Felipe et al., 2010). To our knowledge, the presence of a proline attached to the amino-terminus of the second protein, as a relic of the 2A self-cleaving process, does not interfere significantly with activity and trafficking; it does, however, confer high protein stability (Varshavsky, 1992).

4. Translational studies

4.1 Gene expression *in planta*

Currently, there are several options available for the introduction of multiple transgenes *in planta*. The different methods include sexual crossing, re-transformation, single-plasmid or

multiple-plasmid co-transformation, and IRES-based transformation. The pros and cons of each have been reviewed previously (François et al., 2002; Halpin, 2005; Luke et al., 2006 & 2010). However, these procedures all suffer from a lack of coordinated expression of the different transgenes. As an alternative, the coding sequences of the genes of interest can be linked *via* 2A in a single transcription unit (Halpin et al., 1999; Ma & Mitra, 2002). The first types of genetically modified organisms created using 2A to co-express multiple proteins were plants, initially as a research tool, but also to improve drought-resistance (Kwon et al., 2004); disease-resistance (François et al., 2004; Geu-Flores et al., 2009) and nutritional qualities (Randall et al., 2004). Plant virus vectors based on potato virus X (PVX), cowpea mosaic virus (CPMV), pepino mosaic virus (PepMV), and bean pod mottle virus (BPMV) have been engineered with F2A and used to produce functional recombinant proteins including vaccines and antibodies (Smolenska et al., 1998; Gopinath et al., 2000; Marconi et al., 2006; Zhang et al., 2010; Sempere et al., 2011).

Metabolic and combinatorial engineering of carotenoid biosynthetic pathways in plants, including those synthesizing important industrial and pharmaceutical products, provide excellent examples of the utility of this approach (Ralley et al., 2004; Ha et al., 2010). Carotenoids have attracted interest not only as a source of pigmentation but also for their beneficial effects on human health. One of the most widely known carotenoids is β-carotene, which serves as a dietary precursor of vitamin A. In developing countries, where vitamin A deficiency prevails, a promising intervention to existing strategies is to fortify the major staple food, rice, with provitamin A. Golden rice (*Oryza sativa*, GR) is the generic name given to genetically modified rice that produces β-carotene in the endosperm (Ye et al., 2000, GR1; Paine et al., 2005, GR2). Engineering the provitamin A (β-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm requires two carotenoid biosynthetic genes, phytoene synthase (*psy*) and carotene desaturase (*crtl*) (Lu & Li, 2008). As a step towards the coordinate expression of the two genes, *psy* from *Capsicum* and *crtl* from *Pantoea*, were linked *via* synthetic 2A (*psy-F2A-crtl*) or IRES (*psy-IRES-crtl*) sequences and placed under the control of the rice endosperm-specific globulin promoter (Ha et al., 2010). Collectively, the results demonstrated that the 2A construct performed better than the IRES construct in terms of carotenoid production. In addition, the use of a single promoter (GR1 and 2 require two promoters) reduces the chance of gene silencing and provides more space for transgene stacking.

4.2 Optical imaging of gene expression

In order to monitor transgene delivery and expression by optical imaging, the coding region is fused to a fluorescent/luminescent reporter. Another approach is to detect the expressed protein through its activity (conversion of a substrate in a fluorescent product as with β-galactosidase). We (Halpin et al., 1999; Funston et al., 2008; de Felipe et al., 2010) and others (Samalova et al., 2006 & 2008; Hasegawa et al., 2007; Torres et al., 2010) have successfully used the 2A sequence in a number of *in vitro* and *in vivo* heterologous systems to achieve production of various combinations of fluorescent proteins and proteins requiring discrete co- and post-translational subcellular localization. The zebrafish (*Danio rerio*) has proved to be an excellent vertebrate model system for basic and biomedical science and comparative genomics. The lauded advantage of zebrafish embryos being transparent lends itself remarkably well to the use of fluorescence. To demonstrate the utility of the 2A system in

zebrafish, reporter constructs employing eGFP and mCherry separated by the P2A sequence were designed to segregate fluorescent proteins to distinct cellular locations (Provost et al., 2007). Tissue-specific expression of both fluorophores in stably transformed embryos shows this approach could facilitate continuous expression of multiple proteins products at various stages of development in zebrafish. Likewise, Trichas et al (2008) used a bi-cistronic reporter construct containing a single coding sequence for a membrane localized red fluorescent protein (Myr-TdTomato) and a nuclear localized green fluorescent protein (H2B-GFP) separated by the T2A sequence to test 2A function in transgenic mice. Mutually exclusive localization of TdTomato and EGFP to the membrane and nucleus was observed in cultured cells and endogenous vertebrate cells, consistent with complete 2A-mediated processing. For the transgenic mice produced in this study, targeted expression was apparent in all tissues examined throughout development and into adulthood and remained constant across several generations.

In vivo bioluminescent imaging (BLI) allows a low-cost, noninvasive, and real-time analysis of biological processes at the molecular level in living systems. Cao and colleagues used BLI to visualize engraftment, survival, and rejection of transplanted tissues from a transgenic donor mouse that constitutively expresses luciferase (Cao et al., 2005). The donor mouse has a transgene comprised of a hybrid CMV-β-actin promoter, a firefly luciferase gene, a F2A and GFP gene. Isolated haematopoietic stem cells (HSC) from these mice express luciferase at the highest level among different haematopoietic cell types, and all haematopoietic lineages tested (with the exception of erythroblasts and red blood cells) express the reporter gene. As a virtually unlimited source of labelled cells this mouse line represents a valuable resource for stem cell and transplantation studies.

4.3 Immunotherapies

4.3.1 Cancer immunotherapy using heat shock protein

In an effort to extend the scope of immunotherapy for the control of advanced ovarian cancer, BLI was used to measure tumour load and distribution in mice vaccinated with irradiated tumour cells secreting heat shock protein 70 (Hsp70) (Chang et al., 2007). Hsps, including Hsp70, are highly effective in potentiating immune responses *via* interaction with several surface receptors on antigen-presenting cells (APCs). Hsp-specific receptors efficiently transport the chaperoned peptide into the major histocompatibility complex (MHC) class 1 cross-presentation pathway leading to recognition and activation of cytotoxic T cells (Udono & Srivastava, 1993; Massa et al., 2004). A retrovirus encoding sHsp70-T2A-GFP was used to introduce the gene for secreted hsp70 directly into mouse ovarian surface epithelial cells (MOSEC) that express luciferase. In summary, the tumour-secreted Hsp70 was capable of generating a potent antigen-specific "cytotoxic" $CD8^+$ T-cell response and CD40 was identified as a likely receptor for Hsp70-mediated cross-presentation.

4.3.2 Immunotherapy using monoclonal antibodies

Advances in recent years delineating the specific components of the immune system that contribute to immune responsiveness point to an important regulatory role for immunomodulators. Monoclonal antibodies (mAbs) are an important class of therapeutic agents for the treatment of cancer, autoimmune disorders, and infectious diseases. Although

satisfactory for short-term applications, antibody intravenous infusion is not appropriate in many long-term treatments. Fang et al. (2007) describe a recombinant adeno-associated virus (rAAV) gene delivery system that allows regulated long-term expression of native full-length mAbs *in vivo*. In this study a F2A sequence adjacent to a furin cleavage site (ΔK)RKRR was used to link the antibody heavy and light chain sequences. Notably, the gene expression system included a rapamycin-regulated promoter that can be used to stop mAb production if treatment needs to be terminated. This system potentially offers patients a lifelong mAb therapy that requires only a single administration of an rAAV vector.

Cytotoxic T-lymphocyte-associated antigen (CTLA-4), also known as CD152, is a co-inhibitory molecule that functions to regulate T-cell activation. Antibodies that block the interaction of CTLA-4 with its ligands CD80 (B7-1) and CD86 (B7-2) can enhance immune responses, including anti-tumour immunity (for a brief review, see Chambers et al., 2001). Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a bone marrow growth factor for APCs, which has also been shown to enhance anti-tumour immune responses. Both preclinical animial models and early clinical development indicate synergy between GM-CSF tumour cell vaccination and CTLA-4 blockade (Hurwitz et al., 2000; Hodi et al., 2003; Quezada et al., 2006). To avoid anti-CTLA-4 side effects, tumour cell lines expressing the full-length F2A anti-CTLA-4 mAb in addition to GM-CSF, were administered locally at the immunization site (Simmons et al., 2008). Preliminary results suggest that the delivery of mAbs or proteins locally from immunotherapy cells should prove useful based on promising anti-tumour responses and the reduction of toxicity or adverse immune events associated with systemic exposure.

4.3.3 Cytokines and immunotherapy

The cytokine Interleukin-12 (IL-12) is a growth and maturation factor acting on both the innate and adaptive arms of the immune system. It is produced primarily by APCs and exerts immunoregulatory effects on natural killer (NK) and T cells (Kobayashi et al., 1989; Wolf et al., 1991). The APC-derived IL-12 consists of two subunits, p40 and p35, which are covalently linked (Kobayashi et al., 1989). The expression of this cytokine has been complicated by the observation that p40 homodimers (in excess of the heterodimer) exhibit antagonistic activity (Trinchieri et al., 2003). To ensure the equal expression of both subunits, biologically active IL-12 protein was produced using F2A as a linker between the p40 and p35 subunits (Collins et al., 1998; Kokuho et al., 1999; Chaplin et al., 1999; de Rose et al., 2000; Premraj et al., 2006). Numerous studies have been done which clearly indicate that plasmid expressed F2A IL-12 can modulate and augment the immune response elicited by DNA vaccination against mycobacterial infections (Triccas et al., 2002; Palendira et al., 2002; Martin et al., 2003). Additionally, it has been reported that IL-23 (but not IL-27) increased protection after *M. tuberculosis* challenge (Wozniak et al., 2006). In this study, the genes encoding p19 and p40 chains of IL-23 and EB13 and p28 chains of IL-27 were cloned on either side of the F2A protein.

Enhanced persistence of adoptively transferred tumour-infiltrating lymphocytes has been demonstrated by the administration of growth cytokines such as IL-2 and IL-15 (for reviews see Westwood & Kershaw, 2010; Ngo et al., 2011). However, systemic toxicity and expansion of unwanted cell subsets, such as regulatory T cells limit the use of these cytokines when administered systemically. Transgenic expression of IL-2 and IL-15 has been shown to increase

antigen-specific T cell expansion *in vivo* and enhance antitumour activity without systemic toxicity in preclinical models (Quintarelli et al., 2007). The 3 genes coexpressed in the cytokine encoding vectors (*iCasp-9, ΔCD34,* and *IL-2* or *IL-15*) were linked using F2A. The truncated form of human CD34 was used as a selectable marker of transduced cells and the inclusion of an iCasp-9 "safety-switch" ensured long-term safety of adoptively transferred lymphocytes.

4.4. Gene therapy

4.4.1 *In vivo* gene therapy

Gene therapy can be defined as the introduction of nucleic acids to somatic cells for a therapeutic purpose (Ylä-Herttuala & Alitalo, 2003). Compared to traditional medicine, gene therapy offers unique possibilities to treat the genetic causes of diseases, such as fatal enzyme deficiencies. Mucopolysaccharidosis type 1 (MPS-1; Hurler syndrome) is a congenital deficiency of α-L-iduronidase (IDUA), leading to lysosomal storage of glycosaminoglycans. As accumulation and storage continue, tissue and organ damage becomes manifest as loss of function. Patients with MPS-1 present early in life with rapidly progressing disease that usually results in death due to neurological/CNS deterioration and/or cardiovascular/respiratory problems (Neufeld, 1991).

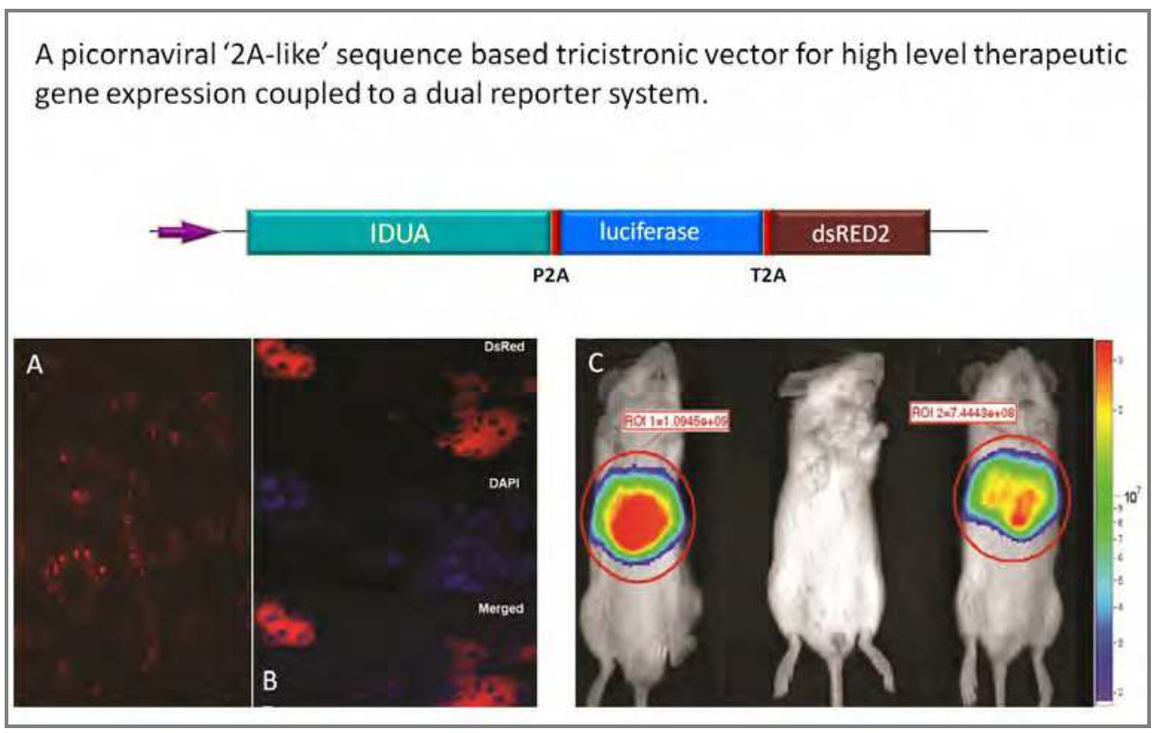

Fig. 5. Schematic of the tricistronic vector construct containing the therapeutic human iduronidase (IDUA) gene along with the firefly luciferase and DsRed2 reporter genes is shown at the top. (A) Whole-organ DsRed2 expression. (B) Cellular DsRed2 expression. (C) Whole-body *in vivo* luciferase imaging. Representative animals of tricistronic plasmid-injected (left), control (*IDUA* injected, middle), and monocistronic luciferase-injected (right) recipients are shown (adapted from Osborn et al., 2005). (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

Enzyme may be delivered by enzyme replacement therapy (ERT), haematopoietic cell transplantation (HCT) or by gene therapy vectors (Tolar & Orchard, 2008). P2A and T2A were utilized to construct a tricistronic vector bearing the human iduronidase (IDUA) gene along with the firefly luciferase and DsRed2 reporter genes (*IDUA*-P2A-luciferase-T2A-DsRed). Efficient cleavage was observed and all three proteins were functional *in vitro* and *in vivo*, leading to high-level therapeutic gene expression in NOD/*scid* mice that could be tracked by non-invasive whole-body luciferase imaging and at the cellular level using DsRed2 (Fig.5. Osborn et al., 2005).

4.4.2 *Ex vivo* gene therapy

To improve patient safety and increase the gene transfer efficiency, target cells are taken from the patient, gene-engineered and then adoptively transferred into the patient. Redirecting T cell specificity by T cell receptor (TCR) gene transfer is emerging as an attractive strategy to treat patients suffering from malignant and viral diseases. αβTCR, together with the CD3δε, γε, and ζζ signaling subunits, determines the specific $CD4^+$ and $CD8^+$ T cell responses to antigens bound to MHC molecules (Call & Wucherpfennig, 2005; Rudolph et al., 2006). Using the TCR:CD3 complex as a test system, Szymczak and co-workers reported expression of all four proteins that make up CD3 and the two proteins required to make up TCR using just two retroviral vectors (CD3δγεζ-2A and TCRαβ-F2A) (Szymczak et al., 2004; reviewed in Radcliffe & Mitrophanous, 2004). Following the seminal paper of Szymczak et al in 2004, several groups have reported efficient TCR expression using 2A peptide linkers to combine TCRα and β- chain genes (Holst et al., 2006a & b; Scholten et al., 2006; Yang et al., 2008; Leisegang et al., 2008; Wargo et al., 2009). An important consideration in redirecting T cells using TCR genes is the tendency of introduced TCR genes to mispair with endogenous TCR α- and β- chains. In this regard, "murinized" receptors improved HLA-A2/LMP2-TCR expression on the surface of human T cells and downregulated expression of endogenous TCRs (Hart et al., 2008).

The feasibility of TCR gene therapy was recently demonstrated in the first bench to bedside experiments with TCR gene-modified T cells in melanoma patients. Johnson et al., (2009) treated metastatic melanoma patients with autologous T cells genetically modified with retroviral vectors to express high-avidity TCRs recognizing tumour-associated antigens MART-1 (MART-1*TCRα*-furinT2A-MART-1*TCRβ*) and gp100 (gp100*TCRα*–IRES-gp100*TCRβ*). Objective cancer regression was observed in 30% - 19% respectively, of patients who received these high affinity TCRs. However, in the study with TCR targeting MART, some patients also experienced toxicity to normal melanocytes in the skin, eye and ear. Another interesting recent study details the first clinical trial involving the adoptive transfer of engineered lymphocytes with optimal TCR complementary determining regions (CDRs) directed against NY-ESO-1, a cancer-testis antigen frequently expressed in melanoma as well as a wide range of non-melanoma epithelial malignancies (Robbins et al., 2011). In contrast to MART-1 and gp100, which are expressed in normal tissues as well as tumours, NY-ESO-1 expression is limited to neoplastic cells and germ line tissue (Chen et al., 1997). The α- and β-chains were expressed in retroviral constructs that contained a furin cleavage site followed by a SGSG spacer and the P2A sequence between the two gene products (Robbins et al., 2008). Response rates of 45% and 67% were observed in patients with melanoma and synovial cell sarcoma, respectively, all of whom had progressive disease after extensive prior treatment.

4.5 Induced pluripotent stem cell generation

Embryonic stem (ES) cells have the ability to differentiate into any cell type of the body and to grow indefinitely while maintaining pluripotency. Remarkably, adult somatic cells can be reprogrammed and returned to the naive state of pluripotency seen in embryonic stem cells by ectopic expression of a defined set of transcription factors: Oct 3/4, Sox2, KLF4 and c-Myc (Takahashi & Yamanaka, 2006; Takahashi et al., 2007; for review see Das & Pal, 2010). The delivery of these "Yamanaka factors" to create induced pluripotent stem (iPS) cells has typically required multiple individual viral vectors, carrying the risk of both insertional mutagenesis and viral reactivation (Takahashi and Yamanaka, 2006; Aoi et al., 2008).

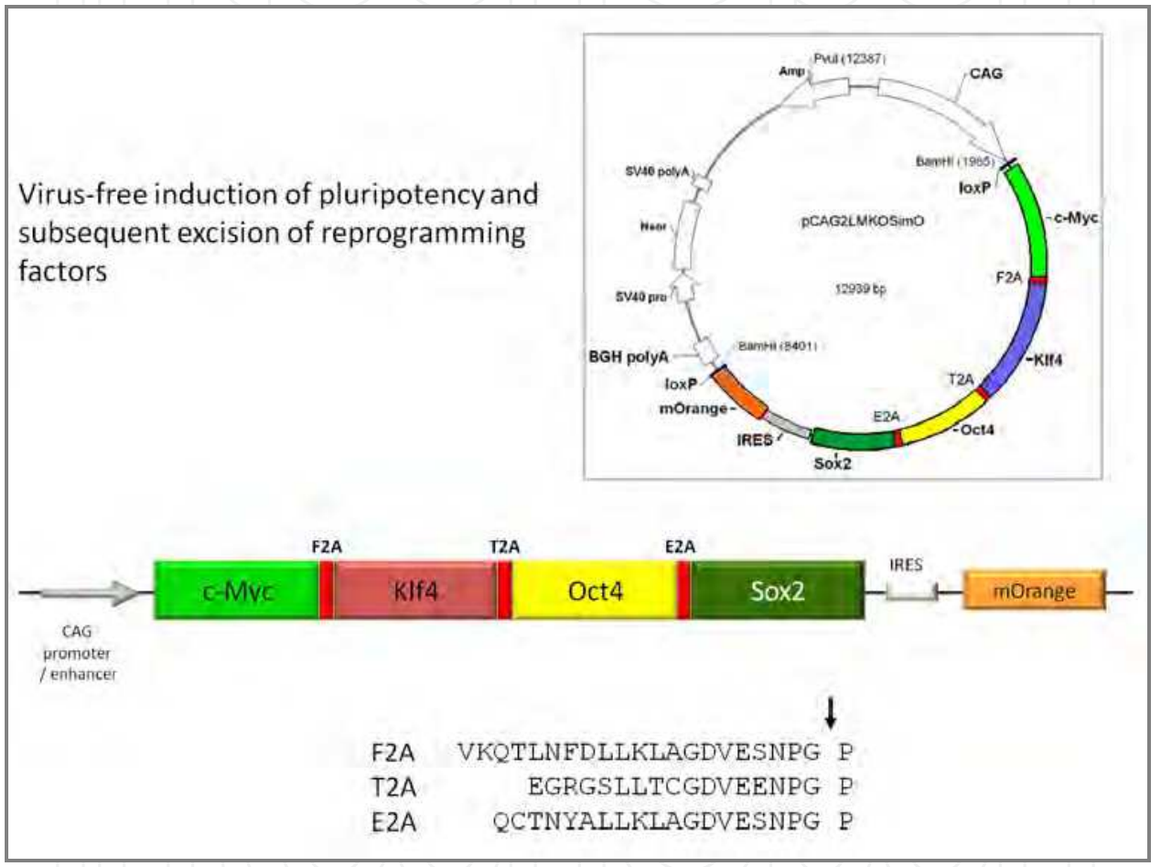

Fig. 6. Schematic diagram of reprogramming cassette. The four reprogramming factors *c-Myc*, *Klf4*, *Oct4* and *Sox2* were fused in-frame *via* 2A sequences and coexpressed as a single ORF and inset: the CAG enhancer/promoter was used to drive the 2A-linked reprogramming cassette and *mOrange* marker, flanked by *loxP* sites (Kaji et al., 2009).

Preliminary work by Okita et al. (2008 & 2010) achieved reprogramming of murine embryonic fibroblasts using repeated transient expressions of two plasmids - one encoding Oct 3/4, Sox2, KLF4 separated by F2A and the other encoding c-Myc. Although the efficiency of iPS cell generation was low, no vector DNA was stably integrated into the iPS cell genome. Sommer et al. (2008) and Carey et al. (2009) reported the derivation of iPS cells from adult skin fibroblasts using polycistronic lentiviral vectors. Sommer's team used a

single multicistronic mRNA containing an IRES element separating two fusion cistrons - Oct4 and Sox2 linked *via* F2A and KLF4 and c-Myc linked *via* E2A. In a different way, the Carey group delivered the four factors in a single vector: Oct4, Sox2, KLF4, and c-Myc separated by three different 2A peptides (P2A, T2A and E2A, respectively). Both groups demonstrated reprogramming of fibroblasts to an ES cell-like state, however, in neither case was the polycistronic vector deleted from the genome. Therefore, attempts were made to minimize genome integration by removal of the inserted genes after the reprogramming process was switched-on (Chang et al., 2009; Kaji et al., 2009; Woltjen et al., 2009; Yusa et al., 2009) and more recently by using mRNA/miRNA of the four factors rather than DNA vectors (Warren et al., 2010; Yakubov et al., 2010; Miyoshi et al., 2011). The efficient reprogramming of murine and human embryonic fibroblasts and the traceless removal of factors joined with viral 2A sequences by using the Cre/LoxP or *piggyBac* transposon/transposase systems mark important advances towards achieving clinically acceptable methods of deriving iPS cells (see Fig.6).

5. Looking ahead

Exciting work of many laboratories in the last few years has clearly established the importance of 2A for co-expression technology. Our increasing knowledge about the cleavage mechanism indicates 2A is not just a novel method of controlling protein biogenesis, but that a crucial aspect of its function is to act as a translational "sensor". Protein synthesis in eukaryotes consumes a high proportion of cellular energy, most of which is used in elongation. During times of energy and/or nutrient deprivation, 2A could act to terminate translation in a stop codon-independent manner – devoting the remainder of the cell's resources into translating only that portion of the ORF upstream of 2A. We envisage that 2A-mediated cleavage could find extra utility in the biomedical and biotechnology fields as a reporter for translational stress.

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Innovations in Biotechnology provides an authoritative crystallization of some of the evolving leading-edge biomedical research topics and developments in the field of biotechnology. It is aptly written to integrate emerging basic research topics with their biotechnology applications. It also challenges the reader to appreciate the role of biotechnology in society, addressing clear questions relating to biotech policy and ethics in the context of the research advances. In an era of interdisciplinary collaboration, the book serves an excellent indepth text for a broad range of readers ranging from social scientists to students, researchers and policy makers. Every topic weaves back to the same bottom line: how does this discovery impact society in a positive way?

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