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ORIGINAL RESEARCH PAPER

Generation of stable cell lines using readthrough expression from lentiviral integration

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Abstract Lentiviral infection is often used to integrate genetic material into cells to stably express transgenes of interest. Depending on the location of integration into the host genome, readthrough expression of the lentiviral cargo can occur via an upstream endogenous promoter, which is typically an unwanted phenomenon because it can result in dysfunctional expression. The purpose of this study was to demonstrate that readthrough expression can be a wanted phenomenon for expressing functional proteins while at the same time reducing the size of the lentiviral transfer plasmid. Readthrough expression was used to generate HEK293 cell lines stably expressing fluorescent reporter proteins, reporter protein-antibiotic resistance fusion proteins for selection, and the vascular endothelial growth factor receptor 2. The generated proteins were all functional, as demonstrated by their ability to fluoresce, confer antibiotic resistance, and participate in receptor-mediated signalling, respectively. Therefore, we suggest that the mechanism of readthrough expression may have further applications in the expression of larger genes or genetic circuits (e.g. cell-based therapeutics), where the lentiviral cargo limit is stretched to the maximum.

Keywords Promoter · Readthrough · Lentivirus · Gene expression · Cell-based therapy

Introduction

Infection with lentiviruses is a commonly used method for integration of genomic material into cells for stable transgene expression. Lentiviruses are harvested from the culture media following the transient transfection of packaging cells (e.g. HEK293T cells) with the human immunodeficiency virus 1 (HIV-1) gag, pol and rev genes to create the lentiviral packaging components, along with vesicular stomatitis virus G protein (VSVG) as the envelope protein to broaden infection to many cell types and also with the lentiviral transfer plasmid that encodes the genetic material for integration into the target cells(Mosabbir and Truong 2016; White et al. 2017). In particular, most lentiviral transfer plasmids have genetic material

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to regulate the expression of the transgene of interest under the control of the strong cytomegalovirus (CMV) constitutive promoter. Also, for selection of infected cells by the lentivirus, most transfer plasmids have an antibiotic resistance gene (e.g. a deaminase for blasticidin resistance) under the control of yet another constitutive promoter. The need to include two promoters in these transfer plasmids significantly increases the size of the genetic cargo by about 1 kb. While lentivirus have a genetic cargo of up to 8 kb, increasing genetic cargo causes decreasing viral titers and probability of genomic integration(White et al. 2017). Thus, smaller transfer plasmids are preferable for better lentiviral packaging and subsequent infection.

Although lentivirus can theoretically integrate their genetic material anywhere in the host genome, it has a preference for the transcriptional units particularly in the intron region (Yang et al. 2008). Due to this preference for integration into transcriptional units, there is a distinct possibility of readthrough expression driven by the endogenous promoter of the transcriptional unit as opposed to the CMV promoter on the transfer plasmid. Typically, this readthrough expression is unwanted in lentiviral integration as it may produce a truncated protein and potentially disrupt the transcription of the transgene of interest. Thus, to reduce readthrough expression, designs of lentiviral transfer vector may incorporate transcriptional stop sites upstream of the CMV promoter (Schambach et al. 2007; Koldej and Anson 2009). In contrast, in this paper, we show that readthrough expression from lentiviral integration can actually be used to express the transgene of interest, allowing a reduced size of the genetic cargo as we don't require any promoters in the transfer plasmid. Herein, we have generated stable cells that express reporter transgenes (i.e. fluorescent proteins) via readthrough expression. To maximize selection of cells that have readthrough expression, we created fusion proteins of our reporter transgene, 2A self-cleaving peptides and antibiotic resistance genes. The flexibility of this structure can even be used to express a functional receptor. Thus, readthrough expression can be exploited to reduce the genetic cargo of lentivirus, making readthrough not a parasitic phenomenon but potentially desired in the generation of stable cell lines.

Results and discussion

Readthrough expression can express fluorescent reporter gene

To demonstrate that readthrough expression can occur in a lentiviral infection, we created stable HEK293 cells by lentiviral infection using a transfer plasmid encoding the Venus(Nagai et al. 2002) yellow fluorescent protein without any upstream CMV promoter (Fig. 1A). Since the transfer plasmid had a self inactivating 3′LTR (i.e. LTR with a deleted U3 region) and no upstream promoter for the transgene, Venus expression requires an upstream endogenous promoter in the host cell for transcription. The transfer vector follows the 3rd generation structure using a chimeric 5′ LTR where the CMV promoter replaced the native U3 to drive greater lentiviral RNA transcription (Dull et al. 1998). Several paths of integration exists for the lentiviral cargo into the host cell (Fig. 1B): first, integration of the cargo into a transcriptional unit in a manner that results in readthrough expression of Venus (e.g. creation of a fusion protein in an exon region or creation of pseudo acceptor splice site in an intronic region); second, integration into an intron of a transcriptional unit in a manner that doesn't result in expression of Venus (e.g. intron is spliced out); third, integration into intergenic DNA should not result in any expression due to the lack of an upstream promoter. HEK293 cells were infected with lentiviruses made from this transfer plasmid and selected by puromycin to isolate cells with genomic integration. Then, these stably expressing HEK293 cells were serially diluted to select a colony grown from a single cell, thereby ensuring genotypic and phenotypic uniformity. Colonies grown from single cells had a very low likelihood of being Venus fluorescence (i.e. only 1 in 30 colonies) (Fig. 1C). Nevertheless, these cells could be isolated by serial dilution to produce a colony of cells all expressing Venus via readthrough expression, since they are derived from a single Venus-expressing cell.

Readthrough expression can be selected for using a 2A self-cleaving peptide

To remove the need for a downstream promoter to express antibiotic resistance while also improving the selection of cells expressing the transgene via

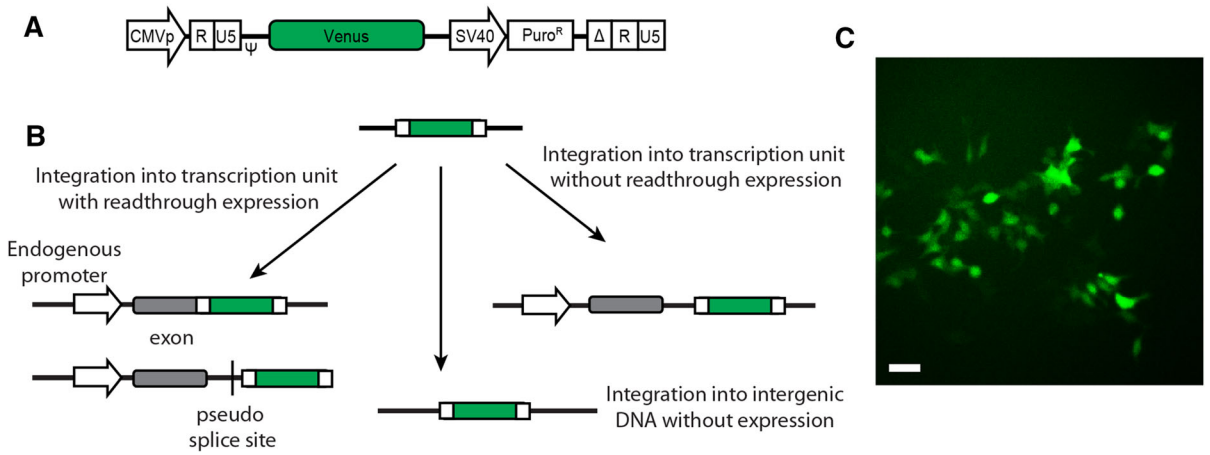

Fig. 1 **A** DNA construct with Venus expression for readthrough expression after lentiviral infection of HEK293. **B** Cartoon of genome integration possibilities of the lentivirus. **C** Fluorescence image of a stable HEK293 cell line expressing Venus by readthrough expression (×10 magnification). Scale bar represents 100 µm

readthrough expression, we created stable HEK293 cells by lentiviral infection using a transfer plasmid encoding a fusion protein (hereafter, Zeo-2a-Ceru) consisting of the zeocin resistance (Zeo) fused to the T2A self-cleaving peptide (i.e. GSGEGRGSLLTCGDVEENPGP) and the Cerulean (Rizzo et al. 2004) cyan fluorescent protein (Fig. 2A). As before, the lack of an upstream promoter in the integrated sequence ensures that expression of the Zeo-2a-Ceru only occurs as a result of readthrough expression from an endogenous promoter. In cells where genome integration occurs but there is no

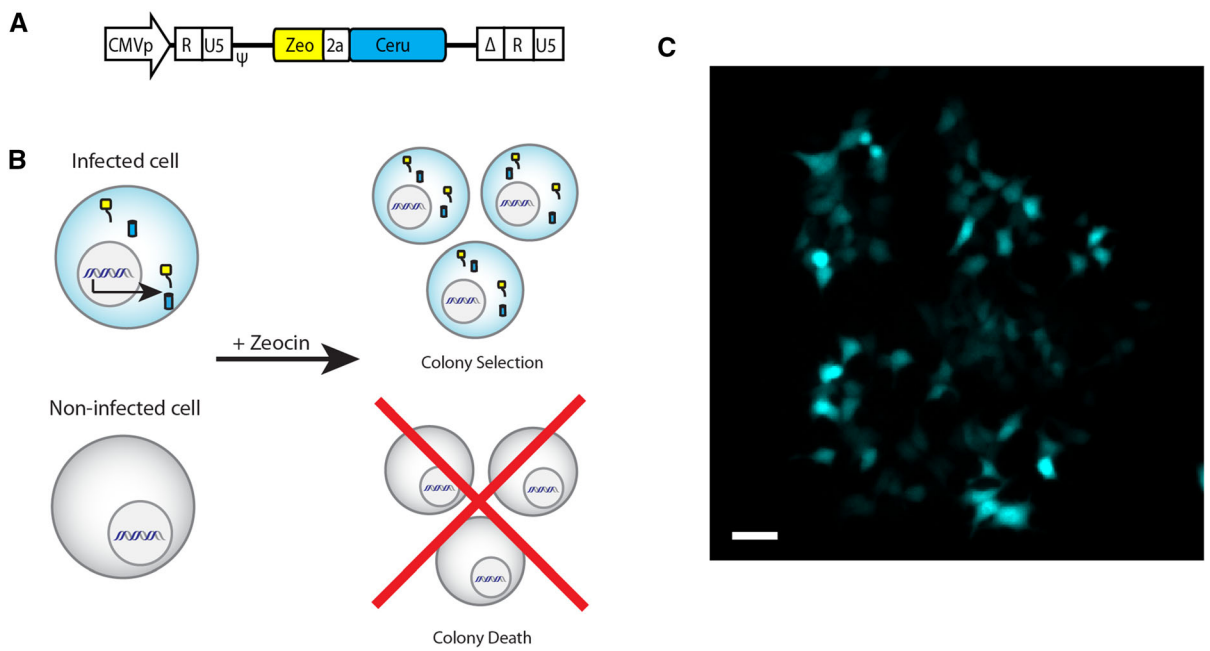

Fig. 2 **A** DNA construct with zeocin antibiotic resistance, T2A self-cleaving peptide and Ceru expression for readthrough expression after lentiviral infection of HEK293. **B** Cartoon of genome integration possibilities of the lentivirus where antibiotic resistance selects for readthrough expression. **C** Fluorescence image of a stable HEK293 cell line expressing Ceru by readthrough expression (×10 magnification). Scale bar represents 100 µm

readthrough expression, zeocin selection will kill cells. In contrast, in cells where genome integration occurs and there is readthrough expression producing the zeocin resistance in the correct reading frame, zeocin will select for those cells which should also be cyan fluorescent because Cerulean is in the same reading frame as the zeocin resistance (Fig. 2B). Again, HEK293 cells were infected with lentiviruses made from this transfer plasmid and selected by zeocin followed by serial dilution to isolate genotypically and phenotypically uniform cells. Colonies grown from single cells had a higher likelihood of being cyan fluorescent (i.e. 1 in 10 colonies) (Fig. 2C). For a colony to be counted as fluorescent, it needed to be visible on the microscope eyepiece, which precluded colonies that were too weakly fluorescent to be perceived by the human eye. Nevertheless, in the fluorescent colonies observed, there was a wide range of intensities where some colonies were brightly fluorescent and some were weakly fluorescent, likely due to the number and location of genomic integration sites. In this way, antibiotic resistance can be used to select cells for readthrough expression of a transgene, without the need of any promoters in the transfer plasmid, dramatically reducing the size of lentiviral cargo.

Readthrough expression can be used to express functional receptors

To demonstrate the versatility of lentiviral readthrough expression, we created HEK293 cells with a transfer plasmid encoding a fusion protein (Blast-2a-VEGFR2) consisting of a blasticidin resistance gene (Blast) linked to a vascular endothelial growth factor receptor 2 (VEGFR2) by a T2A self-cleaving peptide (Fig. 3A). Additionally, to detect Ca^{2+} signals from VEGFR2 activation (Mosabbir and Truong 2017), the transfer plasmid also encoded for a plasma membrane localized Ca^{2+} indicator RCaMP(Ohkura et al. 2012) under the control of the CMV promoter. Similarly, in cells where genome integration occurs but there is no readthrough expression, blasticidin selection will kill cells. In contrast, in cells where genome integration occurs and there is readthrough expression producing the blasticidin resistance in the correct reading frame, blasticidin will select for those cells which should also produce VEGFR2 in the same reading frame. Previous studies with self-cleaving peptides have shown that when these 2A peptides proceed receptors, the receptor retains the ability to trafficking through the endoplasmic reticulum (ER) (Roulston et al. 2016). When VEGF binds to the VEGFR2, it causes releases of Ca^{2+} from the ER, which we can detect using the RCaMP Ca^{2+} indicator as it increases membrane-localized red fluorescence at higher Ca^{2+} concentration (Fig. 3B). Likewise, HEK293 cells were infected with lentiviruses made from this transfer plasmid and selected by blasticidin followed by serial dilution. As expected, these cells expressing the Blast-2a-VEGFR2 fusion protein emitted red fluorescence when stimulated with 10 ng/mL VEGF, indicating that the Blast-2a-VEGFR2 fusion protein was functional when expressed via readthrough expression (Fig. 3C, D) (Supplementary Video 1). Thus, lentiviral readthrough expression is versatile enough to even express a receptor.

Conclusion

Although readthrough expression is typically an unwanted phenomenon of lentiviral infection, we have demonstrated that readthrough expression can be exploited to stably express transgenes of interest while reducing the lentiviral cargo. First, we showed that readthrough expression can be used to express the fluorescent Venus protein in HEK293 cells but the efficiency was low. To improve this efficiency, a 2a peptide was used to fuse the fluorescent Cerulean protein and the zeocin resistance gene (Zeo-2a-Ceru), which allowed antibiotic selection of cells where readthrough expression from genome integration also created the desired transgene. Lastly, to demonstrate the versatility of this structure for selecting readthrough expression, we created stable HEK293 cells expressing VEGFR2, which allowed VEGF-induced Ca^{2+} signals to be detected by the RCaMP Ca^{2+} indicator. Our results illustrate the previously unexplored utility of readthrough expression in the engineering of cells to stably express functional transgenes. Since lentiviral vectors are limited in the size of their genetic cargo, the complexity of the transgenes of interest are likewise limited. Thus, this method can be further expanded to the expression of potential therapeutic proteins, or therapeutic genetic circuits, which may previously have been too large to include in a single transfer plasmid.

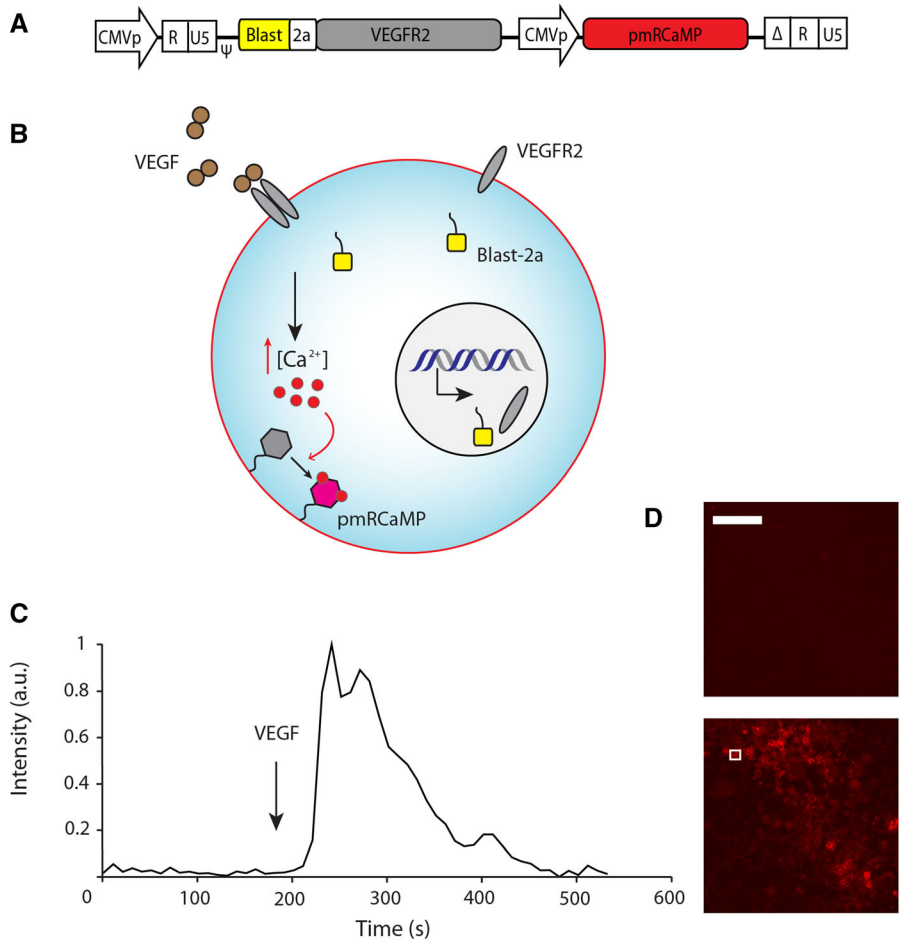

Fig. 3 **A** DNA construct with blasticidin antibiotic resistance, T2A self-cleaving peptide and vascular endothelial growth factor receptor 2 (VEGFR2) expression for readthrough expression after lentiviral infection of HEK293. On the same DNA construct, a tandem CMVp drives expression of the RCaMP Ca^{2+} indicator localized to the plasma membrane. **B** Cartoon of VEGF-induced Ca^{2+} signals measured by the RCaMP Ca^{2+} indicator after genomic integration of VEGFR2. **C** Ca^{2+} trace following 10 ng/mL of VEGF addition observed by RCaMP fluorescence. Intensity is in arbitrary units (a.u.) normalized to values between 0 and 1. **D** Fluorescent microscopy images of HEK293 cell cluster from Fig. 3C taken at time points 150 s and 240 s following bolus addition of 10 ng/mL of VEGF at 190 s. Square represents region of interest used to measure intensities for Ca^{2+} trace in Fig. 3C. Scale bar represents 300 µm

Materials and methods

Plasmid construction

All synthesis and subcloning of plasmids was done by Genscript. RCaMP was synthesized as a fusion with the plasma membrane localization peptide of Lyn kinase (^{1}MGCIKSKGKDSA^{12}). Human VEGFR2 (amino acids 1–1356), Venus and Cerulean were synthesized by Genscript. Synthesize fragments were recombined using our subcloning methodology (Truong et al. 2003) by Genscript. *E. coli* DH5-α were isolated and transformed with our plasmids using the Mini-prep kit (Invitrogen).

Stable cell culture creation

The above designed plasmids were used to create stable HEK293 cells (ATCC; CRL-1573) by lentiviral infection as previously described (Mosabbir and Truong 2016). In particular, HEK293 cells in 96-well plates at 90% confluency were transiently transfected with 100 ng of the transfer vector, 75 ng of psPAX2 (Addgene plasmid #12260) and 25 ng of pMD2.G

(Addgene plasmid #12259). Cells were then cultured for 1 week to allow sufficient time for lentivirus creation and infection. Depending on the desired cell lines, HEK293 cells were selected using 1 μg/mL of puromycin (Invivogen) or 100 μg/mL of zeocin (Invivogen) or 10 μg/mL blasticidin (Invivogen). Colonies were derived from single cells after serial dilution in 96-well plates, to ensure genotypic consistency.

Imaging

Prior to imaging, cells were plated in 96-well glass-bottom plates (Mattek). Images were taken with the Olympus IX81 microscope, using a Lambda DG4 xenon lamp as the light source, and a QuantEM 512SC CCD camera with a 10 × objective (Olympus). Excitation (EX) and emission (EM) filter bandpass specifications were detection of fluorescent proteins: Ceru (EX: 438/24, EM: 482/32), Venus (EX: 500/24, EM: 524/27), RFP/RCaMP (EX: 580/20, EM: 630/60) (Semrock). Images were analysed via ImageJ and μManager software. Time-lapse images were taken with 10 s intervals. Imaging was conducted with cells maintained in PBS. Ca^{2+} signals were stimulated by 10 ng/mL of VEGF (Cedarlane) in HEK293 cells expressing Blast-2a-VEGFR2.

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Declarations

Conflict of interest The authors declare no financial or competing interests.

Consent for publication All authors have given approval to the final version of the manuscript.

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