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MGMT enrichment and second gene co-expression in hematopoietic progenitor cells using separate or dual-gene lentiviral vectors

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ABSTRACT

The DNA repair gene O^6-methylguanine-DNA methyltransferase (*MGMT*) allows efficient in vivo enrichment of transduced hematopoietic stem cells (HSC). Thus, linking this selection strategy to therapeutic gene expression offers the potential to reconstitute diseased hematopoietic tissue with gene-corrected cells. However, different dual-gene expression vector strategies are limited by poor expression of one or both transgenes. To evaluate different co-expression strategies in the context of MGMT-mediated HSC enrichment, we compared selection and expression efficacies in cells cotransduced with separate single-gene MGMT and GFP lentivectors to those obtained with dual-gene vectors employing either encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) or foot and mouth disease virus (FMDV) 2A elements for co-expression strategies. Each strategy was evaluated in vitro and in vivo using equivalent multiplicities of infection (MOI) to transduce 5-fluorouracil (5-FU) or $Lin^-Sca\text{-}1^+c\text{-}kit^+$ (LSK)-enriched murine bone marrow cells (BMCs). The highest dual-gene expression ($MGMT^+GFP^+$) percentages were obtained with the FMDV-2A dual-gene vector, but half of the resulting gene products existed as fusion proteins. Following selection, dual-gene expression percentages in single-gene vector cotransduced and dual-gene vector transduced populations were similar. Equivalent MGMT expression levels were obtained with each strategy, but GFP expression levels derived from the IRES dual-gene vector were significantly lower. In mice, vector-insertion averages were similar among cells enriched after dual-gene vectors and those cotransduced with single-gene vectors. These data demonstrate the limitations and advantages of each strategy in the context of MGMT-mediated selection, and may provide insights into vector design with respect to a particular therapeutic gene or hematologic defect.

1. Introduction

A number of drug resistance genes have been evaluated for selective enrichment of transduced stem cells in vivo (Corey et al., 1990; Allay et al., 1997, 1998; Sorrentino et al., 1992). However, the most robust stem cell selection has been achieved using specific point mutants of MGMT (Davis et al., 2000; Bowman et al., 2003). MGMT repairs O^6-methylguanine and O^6-chloroethylguanine DNA lesions induced by chemotherapeutics such as Temozolomide and 1,3-Bis(2-Chloroethyl)-Nitrosourea (BCNU), respectively. MGMT point mutants, including P140K and G156A, are resistant to the wild-type MGMT inhibitor, O^6-benzylguanine (BG), but retain wild-type DNA repair activity (Crone and Pegg, 1993; Crone et al., 1994; Loktionova and Pegg, 1996). Thus, the endogenous MGMT in untransduced BMCs is inactivated by BG treatment, sensitizing these cells to alkylating agent damage, while mutant MGMT-transduced cells are protected from the combined BG and alkylating agent insult (Reese et al., 1996). With sequential treatments, this differential sensitivity allows transduced stem cells to survive and repopulate the hematopoietic compartment (Davis et al., 2000;

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Bowman et al., 2003; Pollok et al., 2003; Zielske et al., 2003; Beard et al., 2009, 2010). Therefore, dual-gene vectors that couple MGMT-mediated stem cell selection to therapeutic gene expression should allow diseased hematopoietic tissues to be reconstituted with functional, gene corrected cells. The feasibility of this approach has been demonstrated in animal models of β-thalassemia (Persons et al., 2003; Falahati et al., 2012), protoporphyria (Richard et al., 2004), and HIV/AIDS (Trobridge et al., 2009). However, higher MOIs are often required to compensate for the lower expression levels obtained with dual-gene vectors.

Several methods have been devised to co-express two genes from the same viral vector. Two of the most common strategies utilize either an IRES (Morgan et al., 1992; Sugimoto et al., 1994), or ribosome slippage (de Felipe et al., 1999; Klump et al., 2001) sequences for dual-gene expression. IRES elements form secondary RNA structures that act to initiate translation in a cap-independent fashion. Therefore, these elements can be used to express additional genes from the same transcript. Many endogenous and exogenous IRES sequences have been identified, but the sequence derived from the EMCV IRES is one of the most thoroughly characterized and widely used IRES elements in dual-gene expression vectors (Jang and Wimmer, 1990; Davies and Kaufman, 1992). IRES-mediated translation efficiency is typically reduced by 20–50%, compared to the cap-dependent mechanism (Mizuguchi et al., 2000; Yu et al., 2003). In addition, IRES activity can be influenced by the specific genes used, and their position with respect to the IRES sequence (Davies and Kaufman, 1992; Hennecke et al., 2001).

Whereas IRES elements function to initiate translation, ribosome-slippage sites, such as the FMDV 2A element, are active during translation. The short nucleotide sequence encoding FMDV-2A is positioned between two genes, the first of which has the stop codon removed. Thus, both genes and the 2A element are joined as one open reading frame. After the first gene and 2A sequence are translated, cis-acting hydrolase activity within the 2A residues causes the ribosome to "skip" the last peptide bond in 2A. Thus, depending on the 2A nucleotide sequence employed the first gene product is released with 17–23 residues (with up to 32 residues demonstrating increased efficiency (Donnelly et al., 2001)) from the 2A element fused to its C-terminus. The ribosome then continues translating the second gene product, which contains an N-terminal proline from the 2A sequence (de Felipe, in press). Like IRES elements, the efficiency of ribosome slippage sequences appears to be sensitive to the specific gene combinations used (Milsom et al., 2004; Chan et al., 2011). Further, the activity of the first gene product can be perturbed by the 2A residues that remain fused to its C-terminus (Lengler et al., 2005).

Herein, we evaluated the use of two separate single-gene lentiviral vectors (one encoding MGMT-P140K and the other expressing GFP) to cotransduce and selectively enrich dual-gene expressing hematopoietic populations in vivo. Cotransduction was then compared to dual-gene vectors that utilize the EMCV-IRES or FMDV-2A elements for co-expression. Comparisons were made based on enrichment of dual-gene expressing cells, individual gene expression levels, and total vector insertion averages. Although each strategy may be more or less efficient, depending on the target cells and the specific genes used, this study demonstrates the advantages and limitations of each strategy in the context of MGMT-mediated hematopoietic progenitor cell enrichment.

2. Materials and methods

2.1. Vectors

The self-inactivating lentiviral luciferase (Luc) vector, pCSO-rre-cppt-MCU3-LUC, was obtained from Donald Kohn (University of California, Los Angeles, CA). It contains the MND (myeloproliferative sarcoma virus enhancer, negative control region deleted, dl587rev primer-binding site substituted) promoter/enhancer sequence, the rev response element (RRE), and the central polypurine tract/central termination sequence (cPPT/CTS). Luc was removed by partial restriction with *Nco*I and *Eco*RI and replaced with a multiple cloning site linker. The MGMT-P140K and enhanced GFP coding sequences were separately inserted into the *Nco*I site (closest to the MND promoter) and a unique *Bam*HI site. These constructs were then restricted at unique *Bam*HI and *Eco*RI sites for insertion of the woodchuck hepatitis virus posttranscriptional regulatory element (wPRE; provided by Thomas Hope, Northwestern University, Chicago, IL) (Zufferey et al., 1999; Hlavaty et al., 2005) to generate the pMND-MGMT and pMND-GFP single-gene vectors. The EMCV IRES element was obtained from the pCITE-2a vector (Clontech, Mountain View, CA). The IRES sequence was deleted from pCITE-2a by partial *Pvu*II and *Msc*I restriction and the resulting vector fragment was religated. 5′-*Not*I and 3′-*Eco*RI sites were added onto the deleted IRES sequence by PCR and the restricted PCR product was added back to the IRES-deleted pCITE-2a vector, generating an IRES vector with both 5′ and 3′ multiple cloning sites, termed pSOB. GFP was cloned into pSOB using *Nco*I and *Bam*HI to position the GFP start codon at the site reported to maximize IRES-mediated translation (Davies and Kaufman, 1992). The IRES-GFP cassette was then removed and placed 70 nt downstream of MGMT using a unique *Not*I site in pMND-MGMT, generating pMND-MIG. The FMDV-2A sequence was generated from oligos using overlap extension PCR. An MGMT-2A PCR fragment was generated using the oligos, MGMT-FOR (5′-GTGAGCAGGGTCTGCACGAA-3′) and MGMT-2A (5′-CTGCCAACTTGAGCAGGTCAAAGCTCAAAAGCTGTTTCACCGGTGCG-TTTCGGCCAGCAG-3′). A separate 2A-GFP fragment was generated using the oligos 2A-GFP (5′-GGCAGGGGACGTCGAGTCCAA-CCCTGGGCC TATGGTGAGCAAGGGCGA-3′) and GFP-REV (5′-CTAGAGCGGCCGCTTTACTTGTACAGCTCGTCC-3′). The resulting MGMT-2A and 2A-GFP fragments were annealed, extended, and amplified with the MGMT-FOR and GFP-REV oligos. The resulting fragment was cloned into pMND-MIG using a unique *Sfi*I site within MGMT and a *Not*I site 3′ of GFP, generating pMND-MAG. All constructs were verified by sequencing.

2.2. Virus preparation and transductions

Virus was generated as previously described (Roth et al., 2012). In brief, 293T cells (ATCC, Manassas, VA) were transfected with the VSV-G pseudotyping vector (pMD.G) (Naldini et al., 1996), the packaging vector (pCMVΔR8.91) (Zufferey et al., 1997), and the pMND transducing vectors at a 3:1:3 mass ratio, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. At 24–48 h after transfection, virus was harvested in Dulbecco's modified Eagle medium (DMEM; Cellgro, Manassas, VA) containing 10% heat-inactivated (HI)-fetal bovine serum (FBS) (Cellgro) and 2 mM GlutaMAX (Invitrogen); then filtered through 0.45 μm syringe filter units (Millipore, Billerica, MA); and finally stored at −80 °C. Expression titers were determined on K562 cells (ATCC) using virus dilutions that resulted in less than 10% transduction. Titers ranged from 0.5 to 3.0×10^7 expression units (EU)/mL. Virus preps used for cotransductions were premixed at the specified MOI ratios prior to the addition of cells. K562 cells were transduced in Iscove's medium (Cellgro) containing 10% HI-FBS, 2 mM GlutaMAX, and 8 μg/mL polybrene (Sigma, St. Louis, MO). BMCs (isolated from 5-FU treated animals as previously described (Roth et al., 2012)) and sorted $Lin^-Sca\text{-}1^+c\text{-}kit^+$ (LSK) cell populations (isolated as previously described (Reese et al., 2003)) were transduced for 12 h in alpha-MEM containing 20% Hi-FBS, 2 mM GlutaMAX, 6 μg/mL polybrene, in the presence of murine IL-3 (20 ng/mL),