Transcriptomics: Open Access
ISSN: 2329-8936

Guo et al., Transcriptomics 2017, 5:1
DOI: 10.4172/2329-8936.1000140

Short Communication Open Access

Puromycin Selection Confounds the RNA-Seq Profiles of Primary Human Erythroblasts

Guo RL, Lee YT, Byrnes C, and Miller JL*

Molecular Genomics and Therapeutics Section, Genetics of Development and Disease Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland, USA

Abstract

Lentiviral transduction followed by puromycin selection is a well-recognized procedure for gene transfer and expression experiments using a variety of cell types including human hematopoietic stem and progenitor cells. Despite its widespread application, research regarding the potential effects of bacterial puromycin N-acetyltransferase (*pac*) gene expression in mammalian cell cultures is incomplete. Here the potential for puromycin selection to affect transcriptome profiles was examined using a well-studied model for human erythropoiesis. Experiments were performed using primary CD34(+) cells from six adult healthy human donors transduced with two commercially available pac-encoding lentiviral vectors and compared to non-transduced control cells. *RNA-Seq* gene expression profiles were generated at the proerythroblast stage of differentiation, then differential gene expression was analyzed with DEseq2 in R-Studio software. Inter-donor variation in the gene expression profiles and variations between puromycin selected populations after transduction of the separate lentiviral vectors was manifested by significant differences in the RNA detection levels of less than 0.1%. However, puromycin selection after *pac* gene transduction caused significant changes in over 5% of the mRNA when compared to non-transduced controls. The results suggest that consideration should be given for the potential of puromycin selection to confound the interpretation of RNA-Seq transcriptome profiles.

Keywords: Puromycin; Erythropoiesis; Drug selection; Gene transfer; RNA-seq

Short Communication

Puromycin, an amino nucleoside antibiotic, is biosynthesized by the bacterium species *Streptomyces alboniger*. The biomolecular structure of puromycin resembles that of an aminoacyl-tRNA molecule. Its main mechanism of action involves blocking protein synthesis through the interference with the peptidyl transfer procedure during translation [1,2]. Its amino group disrupts the ester bond between the nascent peptide and tRNA, allowing for the puromycin molecule to attach itself to the C-terminus. It contains an amide bond instead of ester bond, thus preventing the next aminoacyl-tRNA from appending itself. Premature chain termination thus occurs, and protein synthesis is aborted [3].

The *pac* gene encodes puromycin N-acetyltransferase, thus conferring puromycin resistance [4]. Puromycin N-acetyltransferase acetylates the nitrogen atom of the amino group in the puromycin tyrosinyl moiety, thus preventing it from breaking the ester bond in the peptidyl-tRNA. Since the puromycin molecule is no longer able to attach itself to the peptide chain, it becomes biologically inactive, and protein synthesis continues [2].

Predictable resistance to puromycin-mediated cell death after *pac* gene transduction was identified as a selection strategy for gene manipulation in eukaryotic cells [5]. More recently, lentiviral transduction followed by puromycin resistance has become an established procedure for exploring the effects of transgene expression in a variety of model systems using high-throughput RNA sequencing (RNA-Seq) with hematopoietic stem and progenitor cells [6-8]. Despite utilization of this experimental approach, the potential effect of the bacterial puromycin N-acetyltransferase gene upon the RNA-Seq profiles remains vague.

In this study, we explored the potential effects of puromycin selection upon RNA-Seq profiles using cultured primary hematopoietic cells. Human CD34(+) cells were cultured *ex vivo* from healthy adult human donors as previously described in a 3-week serum free culture system [9]. Prior to this study, written informed consent was obtained from research subjects. Consent documents regarding studies using primary erythroblasts and approval of the research protocol were obtained from the National Institute of Diabetes and Digestive and Kidney Diseases Institutional Review Board.

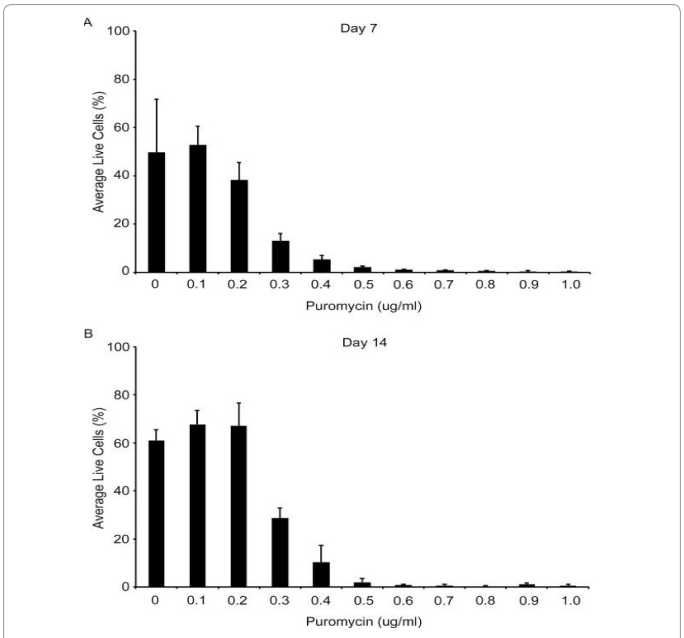

Figure 1: Puromycin titration. The percent of live cells at (A) culture day 7 and (B) culture day 14.

***Corresponding author:** Jeffery L Miller, Genetics of Development and Disease Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, 10 Center Drive, Building 10, Room 9N311, Bethesda, Maryland 20892. USA, Tel: 3014801908; E-mail: jm7f@nih.gov

Received April 01, 2017; **Accepted** April 27, 2017; **Published** May 01, 2017

Citation: Guo RL, Lee YT, Byrnes C, Miller JL (2017) Puromycin Selection Confounds the RNA-Seq Profiles of Primary Human Erythroblasts. Transcriptomics 5: 140. doi:10.4172/2329-8936.1000140

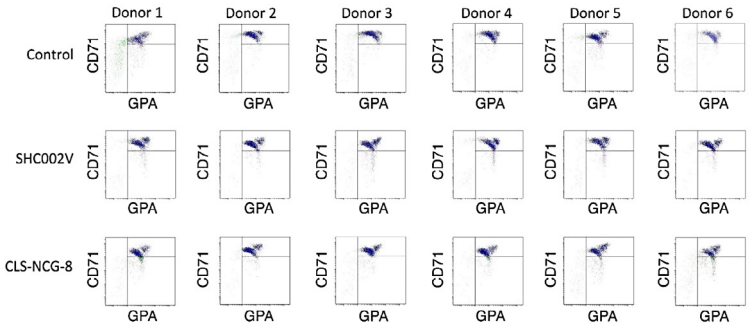

Figure 2: Flow cytometry profiles. Representative flow dot plots of control cells compared to cells transduced with either SHC002V or CLS-NCG-8 analyzed on culture day 14.

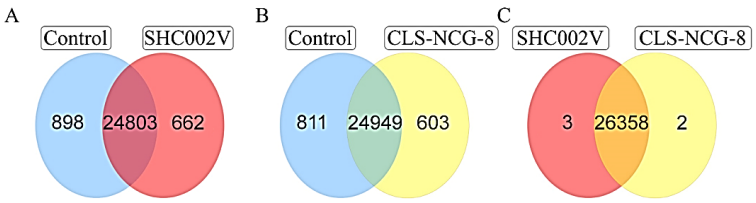

Figure 3: Venn diagram comparisons. Venn diagrams comparing the gene profiles of control (blue), SHC002V (red), and CLS-NCG-8 (yellow). (A) and (B) compare the control with each transduced sample. Genes in the blue area were significantly down-regulated by the lentivirus, genes in the red or yellow area were significantly up-regulated, and genes in the overlapped area were insignificantly differentially expressed. (C) compares the two transduced groups with each other (SHC002V vs. CLS-NCG-8).

Comparison	No. of Genes Insignificantly Differentially Expressed (FDR-adjusted p-value >0.01)	No. of Genes Significantly Differentially Expressed (FDR-adjusted p-value <0.01)	No. Down-regulated	No. Up-regulated
Between Conditions Control versus SHC002V	24803	1560	898	662
Control versus CLS-NCG-8	24949	1414	811	603
SHC002V versus CLS-NCG-8	26358	5	3	2
Inter-donor Variation Control versus Control	26359	4	2	2
SHC002V versus SHC002V	26363	0	0	0
CLS-NCG-8 versus CLS-NCG-8	26361	2	1	1

Table 1: Comparison of RNA-Seq identified genes.

An initial puromycin titration experiment was performed on cells from three donors using 0.1 μg/ml to 1.0 μg/ml dosage to determine the killing concentration after 3-day selection (puromycin selection on culture days 4-6). Puromycin was purchased from Sigma Aldrich (St. Louis, MO). At culture day 7, flow cytometry analysis was performed to determine the percent of live cells with a total of 10,000 events recorded using a BD FACSAria I flow cytometer (BD Biosciences, San Jose, CA). The cells were then transferred to phase II media without puromycin for all conditions for an additional 7 days. On culture day 14, flow cytometry was again performed to determine if any cells survived the puromycin selection. The most effective concentration of puromycin for 3-day selection was found to be 0.7 μg/ml based on less than 2% live cells on culture day 7 and no surviving cells on culture day 14 from the average of three donors (Figure 1).

For puromycin selection experiments, lentiviral transduction was performed on cells from six donors as previously described [10]. Briefly, the cells were transduced on day 3 of culture with either SHC002V (Sigma Aldrich) or CLS-NCG-8 (Qiagen, Valencia, CA). The next day, puromycin was added at 0.7 μg/ml for an additional 3 days. At culture day 7, the cells were transferred to phase II media without puromycin. On culture day 14, erythroblast differentiation was assessed using flow cytometry analysis with the BD FACSAria I flow cytometer to determine if maturation of the transduced cells was affected as compared to control. The cells were stained with antibodies directed against transferrin receptor, CD71 (Invitrogen, Carlsbad, CA) and glycophorin A (Invitrogen). There was no observed difference in cell differentiation between the control and transduced cells (Figure 2).

To explore potential effects of puromycin selection on the RNA-Seq profiles of human primary erythroblasts, live cells from six healthy adult human donors were sorted on culture day 14, and RNA was extracted using the miRNeasy mini kit (Qiagen). Globin messages were depleted from 1.0 μg of total RNA using the GLOBINclear Human Kit and rRNA was depleted using the Ribo-Zero Gold rRNA Removal Kit (Human/Mouse/Rat). After polyA-selection and depletion, the total RNA was used for cDNA library generation. An Illumina HiSeq 2000 (version 3 chemistry) was utilized to sequence the pooled libraries on multiple lanes. At least 40 million 101 base pair reads were achieved.

The Illumina sequence reads were aligned against human genome build hg19 via Illumina sequencing software, Real-Time Analysis version 1.13.48, CASAVA version 1.8.2 and Efficient Large-Scale Alignment of Nucleotide Databases (ELAND) mapping algorithm. The separate lane reads were merged into BAM files, which were loaded into the SamtoFastq tool to generate FASTQ files. Each FASTQ file was processed with FASTQ-Trimmer module and FASTQ-Masker module of FASTX-Toolkit (version 0.0.14) to trim 21 base pair from the end and mask any sequence base with a quality score less than 30 with an N to generate a final 80 base pair FASTQ file for aligning against the Human Genome hg19 build with Spliced Transcripts Alignment to a

Reference (STAR) software [11]. Quantitation of 26,363 named gene transcripts was performed using HTseq. Differential gene expression analysis was performed with DESeq2 based on applying negative binomial generalized linear models in R package and R-Studio to compare the expression levels of each transcript between SHC002V, CLS-NCG-8, and control samples. After applying the Benjamini-Hochberg procedure, genes were considered significantly up- or down-regulated if the adjusted p-value of the test statistics was less than the false discovery rate (FDR<0.01) with log2 of ≥1 for up-regulated or ≤-1 for down-regulated genes and baseMean value above 25. The same bioinformatics procedure was then repeated to quantify the effects of inter-donor variation by comparing multiple donors within SHC002V, CLS-NCG-8, and control. RNA-Seq Bam files from each experiment were deposited in Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/) and released to the public: SRP097005 (Adult CD34+), SRP097630 (Cord CD34+), SRP096196 (CLS-NCG-8), and SRP098089 (SHC002V).

The results showed that SHC002V significantly affected expression of 1560 genes (898 down-regulated, 662 up-regulated), comprising approximately 5.9% of the identified transcripts. Whereas, CLS-NCG-8 had a total of 1414 significantly differentially expressed genes (representing approximately 5.4% of the total profile), with 811 down-regulated and 603 up-regulated. Each of these vectors has a different recombinant lentivirus encoding the puromycin N-acetyltransferase gene, a 600-nucleotide fragment originating from *Lactobacillus harbinensis*. Consequently, comparisons between SHC002V and CLS-NCG-8 revealed only 5 genes to be significantly differentially expressed (less than 0.1% of the total gene IDs), suggesting that both lentiviruses had similar effects on the transcriptome profiles of the samples.

To determine the effects of inter-donor variation upon the RNA-Seq profiles, donors within the same condition were compared. The inter-donor variation tests between the control, SHC002V, and CLS-NCG-8 revealed only 4, 0, and 2 significantly differentially expressed genes, respectively, which is less than 0.1% of the total number of genes identified. Thus, neither differences in cell maturation, nor inter-donor variation in RNA-Seq profiles likely caused the significant differences between the gene profiles of puromycin selected and non-transduced samples (Table 1 and Figure 3).

These novel and unexpected results suggest that puromycin selection may cause broad and significant changes in the RNA-Seq profiles of cultured primary hematopoietic cells. Therefore, selection after expression of puromycin N-acetyltransferase can potentially be a confounding variable for experimental design, interpretation, and comparison of high-throughput sequencing data. While our experiments were limited to effects in primary human erythroblasts, similar comparisons should be considered in alternate models including other primary tissues or cancer cell lines. In this preliminary study, mechanisms responsible for the dramatic change in the transcriptome profiles were not identified. We speculate that lentiviral transduction of the *pac* gene may have off target effects that indirectly affect RNA transcription or stability. Also, puromycin itself may have effects on the cells that are not reversed by *pac* gene expression. Importantly, analogous effects from puromycin selection have been reported previously [5]. Other reports suggest that lentiviral transduction conferring puromycin resistance may lead to a misfolded protein response in human cell lines [12]. Thus, adequate controls should be incorporated in experimental designs in other model systems to more clearly interpret or compare high-throughput gene expression profiles in puromycin selected cells.

References

1. De la Luna S, Ortín J (1992) [33] *pac* gene as efficient dominant marker and reporter gene in mammalian cells. Methods Enzymol. 216: 376-385.
2. Vara J, Perez-Gonzalez JA, Jimenez A (1985) Biosynthesis of puromycin by Streptomyces alboniger: Characterization of puromycin N-acetyltransferase. Biochemistry 27: 8074-8081.
3. Palmer M, Chan A, Dieckmann T, Honek J (2012) Biochemical Pharmacology. John Wiley & Sons, Inc., Hoboken 271-273.
4. Lacalle RA, Pulido D, Vara J, Zaiacaín M, Jiménez A (1989) Molecular analysis of the pac gene encoding a puromycin N-acetyl transferase from Streptomyces alboniger. Gene 2: 375-380.
5. Lanza AM, Kim DS, Alper HS (2013) Evaluating the influence of selection markers on obtaining selected pools and stable cell lines in human cells. Biotechnol J 7: 811-821.
6. Lee YT, de Vasconcellos JF, Yuan J, Byrnes C, Noh S, et al. (2013) LIN28B-mediated expression of fetal hemoglobin and production of fetal-like erythrocytes from adult human erythroblasts ex vivo. Blood 6:1034-1041.
7. Ali N, Karlsson C, Aspling M, Hu G, Hacohen N, et al. (2009) Forward RNAi screens in primary human hematopoietic stem/progenitor cells. Blood 16: 3690-3695.
8. Sims D, Mendes-Pereira AM, Frankum J, Burgess D, Cerone MA (2011) High-throughput RNA interference screening using pooled shRNA libraries and next generation sequencing. Genome Biology 10: 104.
9. de Vasconcellos JF, Lee YT, Byrnes C, Tumburu L, Miller JL (2016) HMGA2 moderately increases fetal hemoglobin expression in human adult erythroblasts. PLoS ONE 11: 0166928.
10. Lee YT, de Vasconcellos JF, Byrnes C, Kaushal M, Miller JL (2015) Erythroid-specific expression of LIN28A is sufficient for robust gamma-globin gene and protein expression in adult erythroblasts. PLoS ONE 12:0144977.
11. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C (2013) STAR: Ultrafast universal RNA-seq aligner. Bioinformatics 29: 15-21.
12. Moran DM, Shen H, Maki CG (2009) Puromycin-based vectors promote a ROS-dependent recruitment of PML to nuclear inclusions enriched with HSP70 and Proteasomes. BMC Cell Biology 10: 32.