Nourseothricin N-Acetyl Transferase: A Positive Selection Marker for Mammalian Cells

Bose S. Kochupurakkal1, J. Dirk Iglehart1,2*

1 Department of Cancer Biology, Dana-Farber Cancer Institute; Boston, Massachusetts, United States of America, 2 Department of Surgery, Brigham and Women’s Hospital, Boston, Massachusetts, United States of America

Abstract
Development of Nourseothricin N-acetyl transferase (NAT) as a selection marker for mammalian cells is described. Mammalian cells are acutely susceptible to Nourseothricin, similar to the widely used drug Puromycin, and NAT allows for quick and robust selection of transfected/transduced cells in the presence of Nourseothricin. NAT is compatible with other selection markers puromycin, hygromycin, neomycin, blasticidin, and is a valuable addition to the repertoire of mammalian selection makers.

Materials and Methods
Reagents
Human mammary epithelial cells immortalized with human telomerase (HMEC) was a gift from Jean Zhao [10]. BT549, MDA-MB468 and U2OS were obtained from ATCC. Phoenix cells were from Orbigen. The Nourseothricin acetyl transferase (NAT) ORF was a gift from Prof. Laura Knoll, University of Wisconsin. Puromycin, Neomycin, Blasticidin, Hygromycin were sourced from Sigma Aldrich.

Drug Sensitivity Assay
HEK293T, HMEC, BT549, MDA-MB468, U2OS and A2780 cells were obtained from Sigma-Aldrich. Phoenix cells were from Orbigen. The Nourseothricin acetyl transferase (NAT) ORF was a gift from Prof. Laura Knoll, University of Wisconsin. Puromycin, Neomycin, Blasticidin, Hygromycin were sourced from Sigma Aldrich and Nourseothricin (NTC) was obtained from Jena biosciences, Germany. Tissue culture media and reagents were obtained from Sigma-Aldrich.
the plates were treated with NTC or Puromycin. Cell viability was measured using the MTS assay according to manufacturer’s protocol. Data was normalized to the MTS value obtained for untreated cells at Day 2. HMECs harboring resistance markers to Neomycin, Puromycin, Blasticidin and Hygromycin (H-NPBH) were plated in 96 well plates. After 12 hours, the cells were treated with the indicated concentration of drugs. Cell viability was measured 72 hours post treatment using MTS. The percentage of growth is plotted as a function of drug concentration.

doi:10.1371/journal.pone.0068509.g001

Figure 1. NTC efficiently kills mammalian cells and is compatible with other selection markers. (A) HEK293T, HMEC, BT549, MDA-MB-468, U2OS and A2780 cells were plated (5000 cells/well) in 96 well plates and treated with indicated drugs 12 hours post plating. Cell viability was measured using MTS on the day the drugs were added (day 0), and 24 (day 1) and 48 hours (day 2) later. The MTS values were plotted relative to cell growth devoid of drug on day 2. (B) HMEC cells harboring selection markers for Neomycin, Hygromycin, Blasticidin and Puromycin (H-NPBH cells) were plated in 96 well plates. After 12 hours, the cells were treated with the indicated concentration of drugs. Cell viability was measured 72 hours post treatment using MTS. The percentage of growth is plotted as a function of drug concentration.

doi:10.1371/journal.pone.0068509.g001

Positive Selection Marker for Mammalian Cells

Generation of Retroviral Vectors

The codon usage of the NAT ORF was analyzed using JCat [12]. Unfavorable codons situated at the ends of the ORF were replaced with codons suggested by JCat and incorporated into the exposure to drugs was measured using the MTS assay. The percentage of growth inhibition was estimated as described in the Developmental Therapeutics Program at NIH/NCI and plotted as a function of drug concentration [11].
primers to generate the NAT ORF used for cloning (Fig. S1). The ORF for Puromycin acetyl transferase in the retroviral vector pRX-Tight Puro was replaced with the NAT ORF to generate pRXTN. The DNA fragment encoding p100 and p36 with a HA-tag at the N-terminus (HA-p100, HA-p36) were cloned into the multiple cloning site of pRXTN. The sequence of the vectors was confirmed by Sanger sequencing.

**Results and Discussion**

First we compared cytotoxicity of NTC and Puromycin in a panel of six cell lines including HEK293T (Neomycin resistant; SV40 T antigen), Human Mammary Epithelial Cells (HMEC, hygromycin resistant; hTERT), BT549 (breast cancer), MDA-MB468 (breast cancer), U2OS (osteosarcoma) and A2780 (ovarian cancer). Although a higher dose of NTC compared to Puromycin was required to kill the cells, the toxicity profiles at the doses used are comparable (Fig. 1A). Susceptibility of the cells to killing with both drugs was time dependent and rapid. Therefore, similar to Puromycin, NTC kills naive cells rapidly and can be used in typical transfection or infection based experiments or screens performed 48–60 hours post transfection/infection.

Although Puromycin, Blasticidin, Neomycin, Hygromycin and NTC inhibit protein synthesis, they bind unique ribosomal sites and inhibit different steps in the polypeptide synthesis process [13]. Moreover, enzymes that inactivate these drugs are highly specific.
and therefore expected to exhibit no cross resistance. We confirmed this hypothesis using a HMEC cell line harboring resistance markers to Puromycin, Neomycin, Hygromycin and Blasticidin (H-PNHB cells) to test the cytotoxicity of NTC in the presence of resistance markers to all these drugs. Hygromycin resistance was used to introduce hTERT in H-PNHB cells. Indeed, H-PNHB cells are acutely susceptible to NTC and 50 μg/ml NTC was sufficient to kill most cells within 72 hours (Fig. 1B). Taken together, results in Fig. 1 shows that NTC is a comparable selection drug for mammalian cells.

Encouraged by this observation, we generated a Tetracycline inducible retroviral plasmid harboring the humanized sequence of NAT (pRXTN; Fig. S1). DNA fragments encoding a 36 kDa (HA-p36) or 100 kDa (HA-p100) protein with a HA-tag at the N-terminus was cloned into this plasmid and retroviruses encoding the proteins were produced using Phoenix cells. H-NH (Hygromycin and Neomycin resistant HMEC) or H-PNHB cell line previously engineered to express the rtTA under Neomycin selection was transduced with the virus. The cells were split 24 hours post infection and cultured in the presence of 50 μg/ml NTC. Cell death (Fig. 2A, dark spots) was observed 2 days after addition of NTC. At 2 weeks proliferating colonies resistant to NTC with normal morphology were easily observed (Fig. 2A). To test if NTC has any residual effect on protein expression, we induced the expression of HA-p36 in H-NH-HA-p36 cells using 0.1 and 1.0 μg/ml Doxycycline in the presence and absence of 50 μg/ml NTC. We see clear bands of HA-p36 irrespective of the presence of NTC in the H-NH cells suggesting that NAT efficiently inactivated NTC (Fig. 2B). To further confirm NTC-NAT can be used to generate stable cell lines that are already resistant to Puromycin, Hygromycin, Blasticidin and Neomycin, we introduced HA-p100 in H-PNHB cells and selected stable clones using NTC. Doxycycline-induced expression of HA-p100 in the H-PNHB cells was confirmed by Western blot (Fig. 2C). Taken together our results indicate that NTC-NAT is an excellent selection system for mammalian cells. Together with NTC-NAT, it is now possible to easily generate mammalian cells with five stable modifications.

A variety of positive selection drug-marker combinations are available for modifying mammalian cells. Among them protein synthesis inhibitors, Puromycin, Hygromycin, Neomycin and Blasticidin are used widely because they do not require specialty media, they are efficient, have minimal confounding effects and can be used together at the same time. Identifying additional drug-marker pairs will enable complex experiments targeting multiple genes at the same time to validate findings from large-scale genome analyses like TCGA. Our results show that NTC-NAT is comparable to the Puromycin-Puromycin N-acetyl transferase system and meets the requirement for multiplexing gene targeting. Moreover, since genome-wide shRNA and ORF libraries are based on Puromycin and Blasticidin resistance markers, NTC-NAT can be used in conjunction with these publically available resources to introduce an alternate gene or shRNA [14,15]. Since NTC kills cells devoid of NAT efficiently, it can be used in “transfection-analysis” experimental designs spanning 56–72 hours, typically used in genome-wide screens using shRNA or ORF libraries. High-level expression of target proteins are possible using bicistronic vectors encoding an IRES driven NAT [16]. Finally, the stability of NTC in culture medium and the broad toxicity of NTC across phyla makes it an extremely useful selection marker for combating contamination in industrial scale protein production in mammalian cells.

Supporting Information

Figure S1 Partially humanized sequence of NAT. (A) Codon usage of the NAT sequence was analyzed using the web tool JCat (http://www.jcat.de/) with “Only partly optimization in order to apply site directed mutagenesis” option to generate a humanized sequence. The Codon Adaptation Index plots for the original sequence of NAT (NAT), codon optimized sequence (NAT_COS) and the changes incorporated in the final sequence used to construct the expression vector (NAT_Final) are shown. (B) Alignment of the three sequences NAT, NAT-COS and NAT_Final are shown. (TIF)

Acknowledgments

The authors thank Prof Laura Knoll for sharing the NAT ORF and Jean-Bernard Lazaro for critical reading of the manuscript.

Author Contributions

Conceived and designed the experiments: BSK JDI. Performed the experiments: BSK. Analyzed the data: BSK JDI. Contributed reagents/materials/analysis tools: BSK. Wrote the paper: BSK JDI.

References

1. ENCODE. Available: http://encodeproject.org/ENCODE/Accessed Apr 20, 2013.
2. TCGA. Available: http://cancergenome.nih.gov/Accessed Apr 20, 2013.
3. Elohaas B, Sprio L, Koerner F, Fleming MD, Zanounj DB, et al. (2001) Human breast cancer cells generated by oncogenic transformation of primary mammary epithelial cells. Genes Dev 15: 50-63.
4. Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126: 663–676.
5. Mortensen RM, Kingston RE (2009) Selection of transfected mammalian cells. Curr Protoc Mol Biol Chapter 9: Unit9 5.
6. van Peer AF, de Bekker C, Vinck A, Wosten HA, Lagenes LG (2009) Phleomycin increases transformation efficiency and promotes single integrations in Schizosaccharomyces pombe. Appl Environ Microbiol 75: 1245–1247.
7. Van Tam T, Rooney PJ, Knoll LJ (2006) Nourseothricin acetyltransferase: a positive selectable marker for Toxoplasma gondii. J Parasitol 92: 668–670.
8. NTC_properties. Available: http://www.jenabioscience.com/cms/en/1/catalog/1136_nourseothricin_als_terned_plasmid.html Accessed Apr 20, 2013.
9. Krugel H, Fiedler G, Smith C, Baumberg S (1993) Sequence and transcriptional analysis of the nourseothricin acetyltransferase-encoding gene nat1 from Streptomyces noursei. Gene 127: 127–131.
10. Zhao J, Gjereup OW, Subramanian RR, Cheng Y, Chen W, et al. (2003) Human mammary epithelial cell transformation through the activation of phosphatidylinositol 3-kinase. Cancer Cell 3: 483–495.
11. DTP. Available: http://dtp.nci.nih.gov/docs/compare/compare_methodology.html Accessed Apr 20, 2013.
12. JCAT. Available: http://www.jcat.de/Start.jsp Accessed Apr 20, 2013.
13. Wilson DN (2009) The A-Z of bacterial translation inhibitors. Crit Rev Biochem Mol Biol 44: 393–433.
14. shRNA_Library. Available: http://www.broadinstitute.org/rnaa/trc/lib Accessed Apr 20, 2013.
15. Yang X, Boehm JS, Yang X, Salehi-Ashtiani K, Hsu T, et al. (2011) A public genome-scale lentiviral expression library of human ORFs. Nat Methods 8: 659–661.
16. Hobbs S, Jirapakdee S, Wallace JC (1998) Development of a bicistronic vector driven by the human polypeptide chain elongation factor 1alpha promoter for creation of stable mammalian cell lines that express very high levels of recombinant proteins. Biochem Biophys Res Commun 252: 368-372.