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Toward genetic transformation of mitochondria in mammalian cells using a recoded drug-resistant selection marker

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Abstract

Due to technical difficulties, the genetic transformation of mitochondria in mammalian cells is still a challenge. In this report, we described our attempts to transform mammalian mitochondria with an engineered mitochondrial genome based on selection using a drug resistance gene. Because the standard drug-resistant neomycin phosphotransferase confers resistance to high concentrations of G418 when targeted to the mitochondria, we generated a recoded neomycin resistance gene that uses the mammalian mitochondrial genetic code to direct the synthesis of this protein in the mitochondria, but not in the nucleus (mitochondrial version). We also generated a universal version of the recoded neomycin resistance gene that allows synthesis of the drug-resistant proteins both in the mitochondria and nucleus. When we transfected these recoded neomycin resistance genes that were incorporated into the mouse mitochondrial genome clones into mouse tissue culture cells by electroporation, no DNA constructs were delivered into the mitochondria. We found that the universal version of the recoded neomycin resistance gene was expressed in the nucleus and thus conferred drug resistance to G418 selection, while the synthetic mitochondrial version of the gene produced no background drug-resistant cells from nuclear transformation. These recoded synthetic drug-resistant genes could be a useful tool for selecting mitochondrial genetic transformants as a precise technology for mitochondrial transformation is developed.

Keywords: Mitochondrial codon; Recode; Mitochondrial transformation; Selection marker; G418; Neo^R

1. Introduction

Mitochondria are essential subcellular organelles in the vast majority of eukaryotic cells. Although some eukaryotes do not have apparent mitochondria, these organisms still have remnants of mitochondria in their cytoplasm (Williams et al., 2002). Mitochondria provide most of the cellular energy (ATP) required for a cell through the process of oxidative phosphorylation (Saraste, 1999). As a distinctive feature of mitochondria, they have their own genome, the mitochondrial genome (mtDNA). The mtDNA encodes critical components of the ATP-generating pathway as well as the rRNAs and tRNAs that are required for the mitochondrial translation system (Wallace, 1999).

Mutations in the mitochondrial genome and subsequent mitochondrial dysfunction in cells cause a wide range of inherited human diseases (Wallace, 1999), and the accumulation of spontaneous mutations in these genomes may play a significant role in some degenerative processes associated with normal aging (Wallace, 2001; Trifunovic et al., 2004). Although the mammalian mtDNA has been amenable to many of the standard molecular techniques used to study the nuclear genome (Yoon and Koob, 2003; Yoon et al., 2009), no practical method has yet been developed to directly modify mitochondrial sequences in mammalian cells. Although some researchers have succeeded in generating mice using naturally occurring mitochondrial genotypes from somatic cells (Irwin et al., 1999; Takeda et al., 2005), there are currently no

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available methods for direct engineering of the mtDNA genome in mammalian mitochondria.

The genetic transformation of mitochondria in mammalian cells remains a technical challenge due to the difficulty of transferring DNA into mitochondria and the limited availability of selectable genetic markers for screening and identifying mitochondrial transformants (Yoon and Koob, 2008). Although mitochondria in the yeast *Saccharomyces cerevisiae* and the green alga *Chlamydomonas reinhardtii* have been successfully transformed with both exogenous DNA and mitochondrial genes using a biolistic delivery system, the approach that is employed in these species for screening and selecting mitochondrial transformants is not feasible for transforming mitochondria in mammalian cells. The selection of mitochondrial transformants in yeast and green alga was accomplished by complementing defective mitochondrial function in mutant cells (Fox et al., 1988; Johnston et al., 1988; Boynton and Gillham, 1996). In these systems, a small DNA fragment that corrects a mitochondrial genome mutation compensates for a respiratory defect in the cells, and thus, mitochondrial transformants can be identified using selective media that requires active respiratory function for growth (Boynton and Gillham, 1996; Butow et al., 1996). However, because most mitochondrial mutants in mammalian cells carry heteroplasmic mtDNA mutations in which the mutants still contain the wild-type mitochondrial genome (Kmiec et al., 2006), it is difficult and inefficient to employ mitochondrial selection based on complementation of mutated mtDNA in mammalian cells (DiMauro and Schon, 2001; Yoon et al., 2010).

Recently, we demonstrated that the standard drug resistance genes neomycin phosphotransferase (*Neo*^{R}) and hygromycin B phosphotransferase (*Hyg*^{R}) can be used as selectable markers for mammalian mitochondrial transformation (Yoon and Koob, 2008). The drug-resistance proteins present in mitochondria are functional and confer resistance to high concentrations of G418 (aminoglycoside antibiotic) and hygromycin, respectively. The drug resistance proteins typically used in conjunction with these drugs can function in the mitochondria as well as they do in the cytoplasm because both antibiotics are part of a family of drugs known to enter the mitochondria (Rustenbeck et al., 1998). In this report, we engineered *Neo*^{R} for use as a potential mitochondrial selectable marker by recoding the gene according to mitochondrial codon usage; genes using these codons can be translated either in the nucleus and mitochondria or only in the mitochondria. We confirmed that the synthetic mitochondrial version of the *Neo*^{R} gene fused in-frame with a mtDNA-encoded gene produced no background drug resistance when the gene was transferred into mammalian cells by electroporation. This result implies that even if the mitochondrial version of the *Neo*^{R} gene is transferred into the nucleus during transfection, the gene will not produce active drug-resistant proteins to confer resistance to cells. This suggests that the recoded mitochondrial version of the gene can be a useful tool in the development of technologies for mitochondrial transformation as well as in selecting mitochondrial transformants in mammalian cells.

2. Materials and methods

2.1. Escherichia coli strains, cell lines and culture media

The *E. coli* strain DH5α was used as a host for all DNA cloning experiments. DH5α λ_{att}::*pirwt* was used to maintain plasmids containing the R6K γ-origin of replication (γ-*ori*) (Yoon and Koob, 2003; Yoon et al., 2009). The mouse cell line STO (ATCC CRL-1503) was grown in DMEM (Life Technologies, Carlsbad, CA, USA) in the presence of heat-inactivated 10% FBS at 37 °C in a humidified 10% CO_2 incubator.

2.2. Construction of the recombinant Neo^R

The synthetic *Neo*^{R} for mitochondrial expression was generated using a recombinant PCR method with the primers shown in Table 1. Three rounds of recombinant PCR were performed to produce the *Neo*^{R}universal and *Neo*^{R}mt genes: the first round (*Neo*^{R}universal: CoxINeo5′/KanTN5-2A, KanTN5-2B/KanTN5-3A, KanTN5-3B/KanTN5-4A, KanTN5-4B/KanTN5-5A, and KanTN5-5B/CoxINeo3′), and the second and third rounds (*Neo*^{R}mt: CoxINeo5′/KanTN5-2C, KanTN5-2D/KanTN5-3C, KanTN5-3D/KanTN5-4C, KanTN5-4D/KanTN5-5C, KanTN5-5D/CoxINeo3′, CoxINeo5′/KanTN5-3E, KanTN5-3F/KanTN5-4E, and KanTN5-4F/CoxINeo3′). The modified *Neo*^{R} genes (*Neo*^{R}universal and *Neo*^{R}mt) were then cloned into a mouse mtDNA clone.

2.3. Construction of a complete mouse mitochondrial genome clone containing Neo^R fused to the Cox1 gene

A transposon-inserted mouse mitochondrial genome clone, pMusMtTN-ND5 (Yoon and Koob, 2003), was modified and

Table 1
Primers used for recombinant PCR.

Primer name	Sequence
CoxINeo5	ATCTCACATGTA*TCGGGCGGAGGTGGCTCT*ATAATTGA ACAAG ATGGATTGC
KanTN5-2A	GCCGCTCCACCCAAGCCG CCGGAG
KanTN5-2B	GGCGGCTTGGGTGGAGCGGCTATTC
KanTN5-2C	ATAGCCGAATAGCCGCTCCACTCA AGCCGC
KanTN5-2D	GCGGCTATTCGGC TATGACTGAGCACAACA
KanTN5-3A	GCCAGTCCCGTCCCGCTTC
KanTN5-3B	GAAGCGGGACGGGACTGGC
KanTN5-3C	CAATAGCAGTCAGTCCCGT
KanTN5-3D	ACGGGACTGACTGCTA TTG
KanTN5-3E	CGTGGCCAGTCACGA TAGC
KanTN5-3F	GCTATCGTGACTGGCCACG
KanTN5-4A	CGGGCATCCGCGCCTTGAGCCGGGCGAAC
KanTN5-4B	GTTCGCCCGGCTCAAGGCGCGGAT GCCCG
KanTN5-4C	GCCGCCGTATTGCATCAGCTATGATGG
KanTN5-4D	CCATCATAGCTGATGCAATACGGCGGC
KanTN5-4E	GCCGTCGGGTATCCGCGCC
KanTN5-4F	GGCGCGGATACCCGACGGC
KanTN5-5A	AGCCAACGCAATGTCCTGA
KanTN5-5B	TCAGGACATTGCGTTGGCT
KanTN5-5C	GCGGTCAGCTCATTCGCCG
KanTN5-5D	CGGCGAATGAGCTGACCGC
CoxINeo3	AGTTCCGGATTA GAAGAACTCGTCAAG

Linker sequence is italicized. The modified nucleotides are underlined.