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Selection by drug resistance proteins located in the mitochondria of mammalian cells

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Abstract

Transformation of mitochondria in mammalian cells is now a technical challenge. In this report, we demonstrate that the standard drug resistant genes encoding neomycin and hygromycin phosphotransferases can potentially be used as selectable markers for mammalian mitochondrial transformation. We re-engineered the drug resistance genes to express proteins targeted to the mitochondrial matrix and confirmed the location of the proteins in the cells by fusing them with GFP and by Western blot and mitochondrial content mixing analyses. We found that the mitochondrially targeted-drug resistance proteins confer resistance to high levels of G418 and hygromycin without affecting the viability of cells.

Keywords

Mitochondria; Selectable markers; Drug-resistance proteins; mtDNA

1. Introduction

Despite the high level of interest in mitochondrial genomes, no practical means have yet been developed for introducing biologically active exogenous DNA into the mitochondrial networks of animal cells. Mitochondria in the yeast *Saccharomyces cerevisiae* and the green alga *Chlamydomonas reinhardtii* have been successfully transformed with both foreign and mitochondrial genes using a biolistic delivery system (Fox et al., 1988; Johnston et al., 1988; Boynton and Gillham, 1996). Selection of the mitochondrial transformants in these species was accomplished by their ability to grow on media that require active mitochondrial function. A short piece of DNA that corrects a DNA mutation in their mitochondrial genome leads to complementation of the respiratory defect in the cells and thus the mitochondrial transformants can be screened on selective media (Boynton and Gillham, 1996; Butow et al., 1996). Mitochondrial transformation of mammalian cells, however, is now a technical challenge due both to the difficulty of delivering exogenous DNA into mitochondria and the limited availability of selectable genetic markers for identifying mitochondrial transformants. Although human and mouse p^0 cells (i.e., cells without mitochondrial DNA) can be repopulated

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using exogenous mitochondria from the same or from closely related species, this procedure requires the use of naturally occurring mitochondrial genotypes as mitochondrial donors and the selection of these mitochondrial transformants is based on conditions that need a functional respiratory chain contributed from the entire mitochondrial genome (King and Attardi, 1989; McKenzie and Trounce, 2000).

In this report, we demonstrate that the standard drug resistant neomycin phosphotransferase (*Neo*^R) and hygromycin-B-phosphotransferase (*Hyg*^R) genes can potentially be used as selectable markers for mammalian mitochondrial transformation. Most of the standard selectable markers used in mammalian tissue culture cells are genes that chemically inactivate drugs such as G418 and hygromycin that would otherwise be toxic to these cells. The drugs G418 and hygromycin belong to a family of aminoglycosides and the bactericidal activity of aminoglycosides occurs through binding to the 16S ribosomal subunit, causing mistranslation or premature termination of protein synthesis (Davies et al., 1965; Hornig et al., 1987; Walter et al., 1999). The mechanism of toxicity of aminoglycosides in eukaryotes is not well understood but the drugs have been postulated to target intracellular processes including lipid metabolism, actin cytoskeleton and lysosome function, mitochondrial ATP production and apoptosis (Kossl et al., 1990; de Groot et al., 1990; Hashino et al., 1997; Jin et al., 2004). Moreover, the eukaryotic mitochondrial ribosome closely resembles the prokaryotic ribosome (Gutell, 1994) and may share its aminoglycoside sensitivity. Mitochondrial involvement and/or entry of aminoglycosides has been known for a long time (Duvall and Wersaell, 1964; Bagger-Sjoberg and Wersaell, 1978; Tachibana et al., 1986) and was convincingly demonstrated in the aminoglycoside susceptible patients who have altered protein synthesis in the mitochondria when exposed to aminoglycosides (Prezant et al., 1993). A mutation in the mitochondrial 12S rRNA gene was identified in these patients and this mutation was recognized to make the 12S rRNA more similar to the bacterial rRNA, the primary target of the bactericidal activity of aminoglycosides, leading to familial aminoglycoside-induced deafness (Prezant et al., 1993). Given this evidence that aminoglycosides were active within the mitochondrial matrix, we reasoned that the drug resistance proteins typically used in conjunction with G418 and hygromycin could inactivate these drugs from within the mitochondrial matrix. To test this hypothesis, we re-engineered the *Neo*^R and the *Hyg*^R genes to express proteins fused to the mitochondrial targeting leader peptide from mouse mitochondrial transcription factor A (Tfam) (Larsson et al., 1996; Yoon and Koob, 2005, 2007). We found that these proteins conferred resistance to high levels of G418 and hygromycin from within the mitochondrial matrix.

2. Materials and methods

2.1. *Escherichia coli* strains, cell lines and culture media

E. coli strain DH5 α , used as a host for all cloning experiments, was grown at 37°C in LB medium, supplemented with 50 μ g ampicillin/ml for *Amp*^R plasmid-containing strains. The human cell line HeLa229 (ATCC CCL-2.1) was cultured in MEM alpha (Life Technologies, Rockville, MD) with heat-inactivated 10% fetal bovine serum (FBS) at 37°C in a humidified 5% CO₂ incubator.

2.2. DNA manipulations

Plasmid preparation and other recombinant DNA techniques were performed essentially as previously described (Sambrook et al., 1989). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA) and used as recommended by the manufacturer. *Pfu Turbo* DNA polymerase for the recombinant PCR was purchased from Stratagene (La Jolla, CA). Deoxynucleotides were synthesized by Integrated DNA Technologies (Coralville, IA).

2.3. Construction of vectors

For the construction of mitochondria-targeting gene expression vector, the 24 N-terminal amino acids mitochondrial leader sequence of the mouse mitochondrial transcription factor A (Tfam) (Larsson et al., 1996; Yoon and Koob, 2005, 2007) was amplified by PCR using primers 5'musmtTFA (5'-AG GAATTC GCTAGC GTC GGC CCG AGC GAT GGC-3'; EcoRI and NheI restriction sites underlined, respectively) and mtTFA3'NruI (5'-GG GTACC TCGCGA GGA AAA ACA CTT CGG AAT-3'; KpnI and NruI restriction sites underlined, respectively) and cloned between NheI and KpnI sites of pcDNA6/HisA after digestion of the PCR products with NheI and KpnI. The 720-bp green fluorescent protein (*GFP*) gene was obtained by PCR amplification using primers GFP5'blunt (5'-ATG GTG AGC AAG GGC GAG GA-3') and GFP3'linker (5'-ATC AGA GCC ACC TCC GCC CGA CTT GTA CAG CTC GTC CA-3'; linker sequence underlined) and was cloned into NruI and EcoRV sites of the vector. The *Neo^R* and *Hyg^R* genes were then amplified by PCR using primers NEO5'blunt (5'-ATG ATT GAA CAA GAT GGA TTG C-3') and NEO3'SalI (5'-AGT GTCGAC TCA GAA GAA CTC GTC AAG-3', SalI restriction site underlined) or HYG5'blunt (5'-ATG AAA AAG CCT GAA CTC ACC G-3') and HYG3'SalI (5'-AGT GTCGAC CTA TTC CTT TGC CCT CG-3', SalI restriction site underlined) and were cloned between EcoRV and XhoI sites of the vectors, resulting pcDNA6-musTfamI-GFPNeo and pcDNA6-musTfamI-GFPHyg, respectively (Fig. 1). To construct expression vectors without mitochondrial targeting sequences, the *GFPNeo* and *GFPHyg* fusion genes were amplified by PCR using primers GFP5'BglII (5'-GAA AGA TCT ATG GTG AGC AAG GGC GAG GA-3', BglII restriction site underlined) and NEO3'SalI or HYG3'SalI and cloned between BamHI and XhoI sites of the pcDNA6 vector, resulting pcDNA6-GFPNeo and pcDNA6-GFPHyg, respectively.

2.4. Generation of stable cell lines

To make a stable cell line expressing GFPNeo and GFPHyg proteins targeted to mitochondria, 10 µg of the expression vectors pcDNA6-musTfamI-GFPNeo and pcDNA6-musTfamI-GFPHyg, respectively, were linearized with BglII and transfected into the HeLa229 cells. To make a cell line expressing DsRed targeted to mitochondria, pmusTfamI-DsRed (Yoon and Koob, 2007) was linearized with NotI and transfected into HeLa229 cells. Transfection of the cells was performed by a calcium phosphate method (Kingston et al., 1987) and stable cell line expressing mitochondria-targeted proteins was screened by blasticidin selection (5 µg/ml) for 2 weeks. Clonal mtGFPNeo and mtGFPHyg cells were obtained by diluting these blasticidin-resistant cells to single cell per well on 96-well plates. The cloned cells were then cultured in normal medium supplemented with 3 µg/ml of blasticidin. To make stable cells expressing GFPNeo and GFPHyg proteins in the cytoplasm of cells, 10 µg of the expression vectors pcDNA6-GFPNeo and pcDNA6-GFPHyg, respectively, were transfected into the HeLa229 cells and stably transfected clones of cells were screened by blasticidin selection (5 µg/ml).

2.5. Mitochondrial stain

Mitochondria-specific dye M429 (Mycosol Inc., Cary, NC) was used to confirm the location of mitochondrial networks in the cells (Fig. 1C and 1D). The dye was prepared according to the instructions for use. Briefly, 5 µl of the stock solution (1 mg/ml in ethanol) was added into 20 ml of the culture media (final 0.25 µg/ml). These media were then added to HeLa229 cells that were cultured on cover-glasses for microscopic analyses. After incubating for 5-10 min, the cells were washed twice with PBS and observed by microscopy.

2.6. Preparation of mitochondria

Mitochondria of tissue cultured cells were prepared as described (Gaines, 1996). Cells were harvested by centrifugation, washed twice with cell wash buffer (1 mM Tris-HCl, pH 7.0, 0.13 M NaCl, 5 mM KCl, 7.5 mM MgCl₂). The cell pellet was resuspended in half the cell volume

with $1/10 \times \text{IB}$ (4 mM Tris-HCl, pH 7.4, 2.5 mM NaCl, 0.5 mM MgCl_2) and the cells were broken using a Pellet Pestle tissue grinder (Kimble-Kontes, Vineland, NJ). The homogenate was mixed with one-ninth of the packed cell volume of $10 \times \text{IB}$ resulting in a buffer concentration of $\sim 1 \times \text{IB}$. The unbroken cells and nuclei were removed by two consecutive low-speed centrifugations (5 min at $320 \times g$). The supernatant was placed into new 1.5 ml tubes and centrifuged at full speed (10 min at $15,800 \times g$) to obtain a mitochondrial pellet. The remaining supernatant was saved for a cytosolic fraction.

2.7. Western blot analysis

The expression and localization of the GFP-fused drug resistance proteins was analyzed by Western blot using rabbit polyclonal GFP antiserum (Invitrogen, Carlsbad, CA). Cellular extracts from total cells and mitochondrial fraction were lysed in lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA and 1.0% Triton X-100 (Okumura et al., 2005). Lysates were sonicated on ice, cleared by centrifugation at $15,800 \times g$ for 10 min at 4°C and analyzed by SDS/PAGE. The primary GFP polyclonal antiserum was used at 1 : 5,000 dilution and the secondary anti-rabbit IgG conjugated with horseradish peroxidase (HRP) was used at 1 : 10,000 dilution. Immuno-reactivity was visualized by SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

2.8. Cell Fusions

In order to exam the mitochondrial fusion of cells carrying differently labeled mitochondria, cell fusion experiments were performed. The mtGFPNeo and mtDsRed cells were mixed and plated on glass coverslips 16–40 h before cell fusion. Cycloheximide (20 $\mu\text{g}/\text{ml}$) was added 30 min before fusion and kept in all solutions used subsequently to inhibit protein synthesis. The protocol for PEG-mediated fusion of adherent cells was used (Legros et al., 2002). Briefly, confluent cells in a 35-mm culture dish are washed with minimal essential medium (MEM) without serum and incubated for 1 min with 750 μl of a prewarmed (37°C) solution of PEG 1500 (50% [wt/vol] in DMEM). Cells were then washed extensively with MEM containing 10% FBS and transferred to prewarmed culture medium.

3. Results

3.1. Construction of cell lines expressing fusion proteins GFPNeo and GFPHyg targeted to the mitochondrial matrix

We constructed expression vectors to selectively deliver polypeptides into the mitochondrial matrix of cells using the mitochondrial leader sequence from the mouse *Tfam* gene (Larsson et al., 1996; Yoon and Koob, 2005, 2007). Maps of these expression vectors are shown in Figure 1. We first tested the mitochondrial targeting of the mouse *Tfam* leader sequence by fusing with GFP. We found that the GFP proteins were clearly localized in the mitochondrial networks of the cells without detectable levels of cytosolic protein (Fig. 1A). Because the mitochondrial leader sequence of mouse *Tfam* is cleaved during import into mitochondria, the GFP proteins cannot be exchanged to the cytosol through successive export and import reactions (Larsson et al., 1996). To visualize the delivery of the neomycin and the hygromycin-B-phosphotransferases to the mitochondria, the *GFP* gene was fused in-frame with the *Neo*^R or *Hyg*^R genes using the flexible linker sequences (SGGGGSD) along with the mouse *Tfam* leader sequence (Fig. 1B) (Yi et al., 2001). After transfection of HeLa229 cells with each of the constructs, stable clonal cell lines were selected with blasticidin (5 $\mu\text{g}/\text{ml}$). These cloned mtGFPNeo and mtGFPHyg cells were then cultured in medium supplemented with 3 $\mu\text{g}/\text{ml}$ of blasticidin. Since the mouse *Tfam* mitochondrial leader sequence delivers the fusion proteins to the mitochondria, we could easily observe the subcellular localization of recombinant GFPNeo and GFPHyg proteins through microscopic examination (Figs. 1C and 1D). The GFP fusion proteins expressed in these cells were clearly localized in the mitochondria, as expected,

without visibly detectable protein in the cytosol and completely matched with the mitochondrial networks stained with a mitochondrial specific dye (M429) that specifically accumulates in the mitochondria of mammalian cells (Figs. 1C and 1D).

3.2. Analysis of mitochondrial targeting of GFP-fused drug resistance proteins

To analyze the mitochondrial targeting of the GFP-fused drug resistance proteins biochemically, we isolated mitochondria from the stable cell lines expressing GFP and GFPNeo proteins and performed Western blotting using GFP antibody (Fig. 2A). We found that the GFP fusion proteins were accumulated in the mitochondrial fraction of both mtGFP and mtGFPNeo cell lines (Fig. 2A). A trace amount of the GFPNeo protein was detected in the supernatant sample of mtGFPNeo cells. This background signal, however, may have originated from broken mitochondria that were disrupted during mechanical mitochondrial preparation (See Material and Methods). We also performed an assay based on mitochondrial matrix content mixing to confirm that the mitochondrial leader sequence of the mouse Tfam selectively delivered the GFP fusion proteins into the mitochondrial matrix of cells (Fig. 2B) (Yoon and Koob, 2007). mtGFPNeo and mtDsRed cells in which mitochondria were labeled with different fluorescent markers (green and red, respectively) were fused with PEG following treatment with cycloheximide to inhibit protein synthesis. At about 40 min after PEG fusion, we observed that the mitochondria in fused cells displayed both fluorescent labels (mtGFPNeo +mtDsRed) (yellow) in a portion of network (Fig. 2B). From the rapid mixing and distribution of these green and red fluorescent labels throughout the fused mitochondria, we concluded that the GFP-fused proteins expressed in these cells were correctly targeted to the mitochondrial matrix and were not merely co-localized with the mitochondrial networks.

3.3. Mitochondrially located proteins confer drug resistance

To address the question of whether the GFPNeo and GFPHyg proteins targeted to the mitochondria in the cells can confer resistance to G418 and hygromycin, we cultured each of the stable cell lines expressing the mitochondria-targeted proteins in media containing a standard concentration of the appropriate selection drug (400 µg/ml of G418 and 200 µg/ml of hygromycin) (Fig. 3). We cultured HeLa229 cells stably transfected with pcDNA6 vector as the control. We also compared the growth of these cell lines (mtGFPNeo and mtGFPHyg) with GFPNeo and GFPHyg cells in which the fusion proteins expressed in the cytosol (nonmitochondrial targeting). We repeated the cell culture experiments twice and found that the mtGFPNeo and mGFPHyg cell lines clearly showed resistant to G418 and hygromycin, respectively, and continued to grow without difficulty for 10 days (open circles in Fig. 3A and 3B). In contrast, HeLa229 control cells stably transfected with empty vector (pcDNA6 only) could not grow in media containing these selection drugs (closed circles in Fig. 3A and 3B). The cytosolic GFPNeo and GFPHyg cells (open squares in Fig. 3A and 3B) grew faster than the mtGFPNeo and mGFPHyg clonal cell lines, respectively. However, it is difficult to compare the growth rate directly with the clonal mtGFPNeo and mGFPHyg cells because these GFPNeo and GFPHyg cells are a mix of stably transfected cells. Nevertheless, the patterns of growth in the drug-containing media were very similar for both mtGFPNeo vs GFPNeo and mtGFPHyg vs GFPHyg cells.

We also attempted to determine the highest concentration of each drug that the mtGFPNeo and mtGFPHyg cell lines can tolerate and survive. To estimate the maximal tolerated doses of drugs with regard to the mitochondria-targeted drug resistance proteins which were expressed under the control of *CMV* promoter, we cultured the cells in media containing four different levels of drugs (400 µg/ml, 600 µg/ml, 800 µg/ml and 1.6 mg/ml concentrations of G418 and 200 µg/ml, 400 µg/ml, 800 µg/ml and 1 mg/ml concentrations of hygromycin, respectively) for 10 days. The mtGFPNeo cells were grown without difficulty in concentrations up to 1.6 mg/ml of G418, which was the highest G418 concentration tested, indicating that the mtGFPNeo cells

would grow at even higher G418 concentrations. The mtGFP^{Hyg} cells grew well in up to 0.8 mg/ml of hygromycin, but we found that cell growth was delayed when we used a 1 mg/ml concentration of hygromycin.

4. Discussion

Because the drugs G418 and hygromycin are part of a family of drugs known to enter the mitochondria (Duvall and Wersaell, 1964; Bagger-Sjoberg and Wersall, 1978; Tachibana et al., 1986), we reasoned that the drug resistance proteins typically used in conjunction with these drugs could also function in the mitochondria. We re-engineered the G418 (*Neo*^R) and the hygromycin (*Hyg*^R) resistance genes to express proteins fused to the mitochondrial targeting leader peptide from mouse Tfam (Larsson et al., 1996; Yoon and Koob, 2005, 2007). To allow us to confirm the mitochondrial location of these resistance proteins, we also generated versions of these genes that express resistance proteins fused to GFP via a flexible peptide linker region (Fig. 1B). When we transfected these constructs into human or mouse tissue culture cells we found that the GFP-tagged proteins (GFP^{Neo} and GFP^{Hyg}) were detectable only in the mitochondrial networks and that they conferred resistance to high levels of these drugs (Figs. 1-3). A photograph showing the distribution of the GFP-fusion protein in human cells in which it is stably expressed and targeted into mitochondrial networks of the cells is shown in Fig. 1C and 1D. Mitochondrial networks that were stained with a mitochondrial specific probe (M429) were also well matched with the GFP-labeled mitochondria, suggesting that the GFP-tagged neomycin and hygromycin phosphotransferases were correctly targeted and accumulated in the mitochondria of the cells (Figs. 1C and 1D). In addition, we performed immuno-blot analysis using GFP antibody as well as the assay based on mitochondrial matrix content mixing and confirmed that the GFP-fusion proteins were inside the mitochondria and not merely co-localized with the mitochondrial networks. (Figs. 2A and 2B).

Survival curves of the mtGFP^{Neo} and mtGFP^{Hyg} cells grown in media containing standard levels of selective drugs (400 µg/ml of G418 and 200 µg/ml of hygromycin) clearly show that the GFP-fused drug resistance proteins in their mitochondria confers resistance to these drugs (Fig. 3). The mtGFP^{Neo} and mtGFP^{Hyg} cell lines stayed very healthy and continued to grow without any difficulty during 10 days of culture in the drug-containing media (open circles in Fig. 3A and 3B). The patterns of the growth curves of the mtGFP^{Neo} and mtGFP^{Hyg} clonal cell lines were similar with the patterns of growth shown in the GFP^{Neo} and GFP^{Hyg} cells (open circles and squares in Fig. 3A and 3B). We also found that these mtGFP^{Neo} and mtGFP^{Hyg} clonal cells were resistant to very high levels of the drugs (>1.6 mg/ml of G418 and 0.8 mg/ml of hygromycin).

Our results presented in this report demonstrate that drug resistance proteins present in mitochondria are a powerful tool for selecting mammalian mitochondrial transformants and that the *Neo*^R and *Hyg*^R genes could serve as effective markers for mtDNA transfer into the mitochondrial network if they are properly designed for expression by the mitochondrial transcription and translation systems. Successful expression of the nuclear genes using the mitochondrial genetic system has been reported in yeast (Steele et al., 1996; Golik et al., 2003). Since the genetic code used in the mitochondria is different than the universal code used in the nucleus, coding sequences of the genes were recoded to one that can generate a functional protein when translated in the yeast mitochondria. Two recoded reporter genes (*ARG8*^m and *RIP1*^m) which were integrated into the yeast mitochondrial genome have fully complemented the nuclear *arg8* and *rip1* deficiency, respectively, at the level of cell growth when these genes were expressed in the yeast mitochondria (Steele et al., 1996; Golik et al., 2003). Therefore, by recoding the prokaryotic *Neo*^R and *Hyg*^R genes based on the mammalian mitochondrial genetic system, we should be able to express these drug resistance proteins in mitochondria.

In fact, mitochondria may be a particularly good place to express the prokaryotic *Neo^R* and *Hyg^R* genes. Modern mitochondria still retain a number of features that reflect their endosymbiotic origin and the mitochondrial gene expression system still carries hallmarks of its bacterial ancestor (Wallace, 1999, 2007). For example, an *N*-formylmethionyl-tRNA (fMet-tRNA) which is involved in the initiation of protein synthesis in bacteria is employed as initiator of protein synthesis in mitochondria (Galper and Darnell, 1969; Epler et al., 1970). The bacteria-like nature of the mitochondrial transcription and translation system may therefore be more well suited to expressing prokaryotic genes such as the *Neo^R* and *Hyg^R* than the mammalian nuclear expression system. In addition, broad host range selectable markers of bacterial origin such as the *Neo^R* have evolved to function across species as part of the mobile element Tn5 and broad host range plasmids (Beck et al., 1982; Roychoudhury and Lam, 1983). They have been easily adapted to the eukaryotic nuclear transcription and cytoplasmic translation systems and are the most commonly used selectable markers for DNA transfection in tissue culture cells and the production of transgenic models (Zijlstra et al., 1989; Johnson et al., 1995). Therefore, these broad host range selectable markers expressed in the mitochondria are likely to generate functional drug resistance proteins and confer resistance to cells from within the mitochondria.

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Figure 1.

Subcellular localization of drug-resistance fusion proteins. (A) Mitochondrial targeting of GFP protein using the leader sequence of mouse mitochondrial transcription factor A (TfamL). The GFP was clearly localized only in the mitochondrial networks of HeLa229 cells. (B) Structure of mitochondrial targeting fusion vectors. The *GFP* and *Neo^R* genes or the *GFP* and *Hyg^R* genes were fused by a linker sequence along with the mitochondrial targeting sequence of mouse Tfam (TfamL). (C and D) Microscopic examination of GFP fusion proteins targeted to the mitochondria. The GFP fusion proteins expressed in the mtGFPNeo (green) (C) and mtGFPHyg (green) (D) cell lines was clearly localized in the mitochondrial networks of HeLa229 cells without visibly detectable protein in the cytosol. The mitochondrial networks

stained with a mitochondrial specific dye (M429) (red) were exactly matched with the GFP-labeled mitochondria.

Figure 2.

Examination of GFP-fused drug resistance proteins in the mitochondria. (A) Western analysis of GFPNeo fusion proteins. Mitochondria from the mtGFP and mtGFPNeo cells were prepared after mechanical cell lysis followed by differential centrifugation. Proteins from the total cells, mitochondria and supernatant were prepared and then fractionated in a 10% SDS/PAGE.

Western blot analysis was performed using the rabbit polyclonal GFP antiserum. The GFP and GFPNeo proteins were clearly accumulated in the mitochondrial matrix since the mouse Tfam mitochondrial leader sequence delivers the proteins to the mitochondria. The weak signal in the supernatant of the mtGFPNeo cells may have originated from broken mitochondria that were disrupted during mechanical mitochondrial preparation. SDS/PAGE gel was used as a

loading control for the GFP signal. (B) Fusion of mitochondrial networks between GFP- (green) and DsRed-labelled (red) mitochondria. Rapid mixing and distribution of both fluorescent proteins throughout the fused mitochondria indicated that the GFPNeo fusion proteins were correctly located in the mitochondrial matrix.

Figure 3.

Drug resistance conferred by mitochondrially located proteins. (A) Growth curve of the stable mtGFPNeo cell line in G418-containing media. The cell lines mtGFPNeo, GFPNeo and HeLa229 cells stably transfected with pcDNA6 were cultured in MEM-alpha media containing 5% FBS and standard level of G418 (400 $\mu\text{g}/\text{ml}$) for 10 days to compare the cell viability. (B) Growth curve of the stable mtGFPHyg cell line in hygromycin-containing media. The cell lines mtGFPHyg, GFPHyg and HeLa229 cells stably transfected with pcDNA6 were cultured in MEM-alpha media containing 5% FBS and standard level of hygromycin (200 $\mu\text{g}/\text{ml}$) for 10 days. Both mtGFPNeo (A) and mtGFPHyg (B) cells are highly resistant to G418 and hygromycin and continue to grow without difficulty (open circles in A and B) but HeLa229

control cells transfected with pcDNA6 in (A) and (B) quickly disappeared due to the toxicity of the drugs (closed circles in A and B). The patterns of the growth curves of the mtGFPNeo and mtGFPHyg clonal cell lines were very similar with the patterns of growth shown in the GFPNeo and GFPHyg cells (open circles and squares in A and B).