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TECHNICAL REPORT

Assessment of Risks and Benefits of Using Antibiotics Resistance Genes in Mesenchymal Stem Cell-Based *Ex-Vivo* Therapy

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Recently, *ex-vivo* gene therapy has emerged as a promising approach to enhance the therapeutic potential of mesenchymal stem cells (MSCs) by introducing functional genes *in vitro*. Here, we explored the need of using selection markers to increase the gene delivery efficiency and evaluated the potential risks associated with their use in the manufacturing process. We used MSCs/CD that carry the cytosine deaminase gene (*CD*) as a therapeutic gene and a puromycin resistance gene (*PuroR*) as a selection marker. We evaluated the correlation between the therapeutic efficacy and the purity of therapeutic MSCs/CD by examining their anti-cancer effect on co-cultured U87/GFP cells. To simulate *in vivo* horizontal transfer of the *PuroR* gene *in vivo*, we generated a puromycin-resistant *E. coli* (*E. coli/PuroR*) by introducing the *PuroR* gene and assessed its responsiveness to various antibiotics. We found that the anti-cancer effect of MSCs/CD was directly proportional to their purity, suggesting the crucial role of the *PuroR* gene in eliminating impure unmodified MSCs and enhancing the purity of MSCs/CD during the manufacturing process. Additionally, we found that clinically available antibiotics were effective in inhibiting the growth of hypothetical microorganism, *E. coli/PuroR*. In summary, our study highlights the potential benefits of using the *PuroR* gene as a selection marker to enhance the purity and efficacy of therapeutic cells in MSC-based gene therapy. Furthermore, our study suggests that the potential risk of horizontal transfer of antibiotics resistance genes *in vivo* can be effectively managed by clinically available antibiotics.

Keywords: 5-fluorocytosine, Mesenchymal stem cell, Puromycin resistance gene, Puromycin, Cytosine deaminase, Gene therapy

Introduction

MSCs are self-renewable, multipotent stem cells capable of differentiating into mesodermal lineages (1). Because of paracrine effects and trans-differentiation potential, numerous preclinical and clinical studies have used MSCs in cell-based therapies of regenerative medicine in stroke (2, 3), Parkinson's disease (4, 5), Alzheimer disease (6, 7),

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graft versus host diseases (8), myocardial infarction (9) and type 1 diabetes (10). However, despite the efforts for decades their therapeutic efficacy is yet to be fully established.

In recent years, multiple studies have demonstrated that the biological functions of MSCs can be enhanced through priming techniques, involving the use of biomaterials, culture conditions, biochemical agents, and cytokines. Given that priming methods do not involve the use of genetic materials, approval processes by regulatory agencies may be relatively straightforward. However, developing priming technology presents a challenge in ensuring the consistent quality of therapeutic cells by obtaining homogeneously primed cells on a large-scale for manufacturing in good manufacturing practice (GMP) facilities (11, 12).

Alternatively, MSCs can be genetically modified by directly transferring therapeutic genes via viral and non-viral methods. In particular, MSCs are easily transduced by retroviral and lentiviral vectors carrying functional genes without interfering with the cells' proliferative potential *in vitro*, a landmark of stem cells, and with allowing sustained expression of transduced genes over long-term expansion in a homogenous population of therapeutic cells in large quantities (13, 14).

Antibiotic resistance genes have been explored in laboratory settings to achieve the homogeneity of gene-modified cells but generally avoided in the clinical field because the transfer of antibiotic resistance genes from the vector utilized for gene delivery, such as plasmids or viruses, to the normal flora present in the gut of patients can have unintended consequences, including the development of antibiotic resistance in gut bacteria and the subsequent dissemination of antibiotics-resistant bacteria in the environment (15, 16). Therefore, regulatory agencies have implemented strict guidelines for the use of antibiotics resistance genes. Widely used selectable markers are the *NeoR, BsdR, HygR, BleoR, and PuroR* genes, which confer resistance to antibiotics G418, blasticidin, hygromycin B, zeocin, and puromycin, respectively (17-21). We chose the *PuroR* gene as our selection marker because it enables rapid selection with low doses. Additionally, using *PuroR* gene poses a low risk of horizontal transfer and environmental contamination since puromycin is not used as an antibiotic for humans. Therefore, the emergence of a puromycin-resistant bacteria is unlikely.

The *CD* gene was introduced to MSCs as a therapeutic gene along with *PuroR* as a selection marker using retroviral vectors in the form of a CD-RES-PuroR cassette (22). The resulting MSCs/CD cells catalyzes an antifungal drug, 5-fluorocytosine (5-FC) to an anti-cancer drug, 5-fluorouracil (5-FU) (23). 5-FU is highly permeable to cell membrane and can exert cytotoxic effect to neighboring proliferative cells via bystander effect. Our previous study showed that MSCs/CD cells enriched by puromycin selection effectively suppressed brain tumor growth via bystander effect in the presence of 5-FC (22) and the anticancer effect was further synergistically enhanced in combination of temozolomide (TMZ), a widely used anti-cancer drug for the treatment of glioblastoma multiforme (24).

In this study, we addressed potential risk and benefit of using *PuroR* as a selection marker in *ex vivo* gene therapy using MSCs/CD cells as an example. We showed that the *PuroR* gene is advantageous in *ex-vivo* therapy by increasing the purity of therapeutic cells while reducing the impurities of unmodified naïve cells. Additionally, we showed that the potential risk of horizontal transfer of the *PuroR* gene released from MSCs/CD into bacteria could be effectively managed by clinically available antibiotics. Finally, we showed that puromycin that was added during the selection period remains below the detectable level and can exert no harmful effect on the host cells.

Materials and Methods

Human MSCs isolation and culture

For allogenic transplantation, human MSCs were derived from the iliac crest's bone marrow of a 19-year-old healthy donor as described previously (25) with approval from the Institutional Review Board of Ajou University Medical Center (No. AJIRB-BMR-KSP-20-040) with the informed consent of the patient. Briefly, mononucleate cells were maintained as adherent cultures in Dulbecco's Modified Eagle's Medium (DMEM; Welgene, South Korea, Cat. No. LM 001-05) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA, Cat. No. 16000-044), 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco, Cat. No. 15140-122) and 10 ng/ml basic fibroblast growth factor (PEPROTECH, USA, Cat. No. 100-18B).

Viral vectors and cell lines

The *CD* gene was cloned from *Escherichia coli* K12 MG1655 (KRIBB, Daejon, Korea) as described previously (22). Briefly *CD* gene was PCR amplified using a pair of primers (forward primer: 5'-GAATTCAGGCTAGCAATGTCGAATAACGCTTTACAAAC-3'; reverse primer: 5'-GGATCCTCTAGCTGGCAGACAGCCGC-3') and subcloned into a pFIP plasmid (ViroMed, Seoul, Korea). pFIP/CD retroviral vector was produced in a FLYRD18 packaging cell line expressing the Moloney murine leukemia virus gag-pol gene and the cat endogenous virus RD114 env gene. Two days after plating of packaging cell at a density of

1.5×10^6/T-75 flask, virus soup was collected and syringe-filtered using a 0.45-μm filter. MSCs were transduced to prepare the MSCs/CD as previously described (22). The U87 human glioma cell line was purchased from ATCC (American Type Culture Collection, Mansssas, VA, USA). The U87/GFP cells were generated by transduction with lentivirus vector encoding a green fluorescence protein (GFP), Ll3.7-GFP followed by fluorescence-activated cell sorting (FACS) as described previously (24).

In vitro bystander effects

U87/GFP cells were co-cultured with MSCs or MSCs/CD at a 1 : 1 ratio (each 1×10^4 cells) in 12-well plates. Twenty-four hours after culture, 5-FC was added, and medium changed in every 2 days at the indicated concentrations of 5-FC for 7 days. The live cells were lysed in passive lysis buffer (Promega, Madison, WI, USA), and GFP fluorescence was measured using a fluorometer (Molecular Devices, Sunnyvale, CA, USA). The GFP fluorescence levels were expressed relative to the untreated control cell fluorescence as the means±SEM of at least three independent experiments.

Quantitative analysis of puromycin concentration

Standards were prepared by dissolving puromycin dihydrochloride (Cat. No. A1113803; Gibco, USA) at range of concentration such as 0, 0.05, 0.1, 0.2, 0.5, 1, 2, 4, 8 μg/ml. A linear regression equation of the standard solution was obtained using Alliance HPLC system (Waters Corporation) following manufacturer protocol. Through a total of triple experiments, the limit of detection (LOD) and limit of quantification (LLOQ) were obtained using following formula: LOD=3.3×(Standard deviation of y‒intercept)/(Average Slope) and LLOQ=10×(standard deviation of y‒intercept)/(Average Slope) respectively and the linearity of the calibration curve was confirmed. Peak area estimated from high performance liquid chromatography (HPLC) was used to estimate the concentration of puromycin in samples using following formula: Puromycin (μg/ml)=(Peak area- (y-intercept))/slope.

MSCs/CD (2×10^5 cells) were plated on a 100 mm culture dish and cultured in 10 ml growth medium (mentioned earlier for human MSCs isolation) for 24 h. Growth media containing indicated concentration of puromycin was changed and further culture for 48 h. After conditioned medium harvest, 10 ml or 2 ml Dulbecco's phosphate buffer saline (DPBS; Welgene, South Korea, Cat. No. LB 001-02) (as a washing solution) was added to each dish. Two minutes later, the DPBS was collected and subsequently 10 ml growth media was used to wash and harvested after 2 minutes. All harvested supernatants were filtered through a 0.2 μm syringe filter.

Maximum tolerated dose for puromycin

MSCs were cultured in 24 well plates at a density of 1×10^4 cells/well in media supplemented with indicated puromycin concentrations ranging from 0∼2 μg/ml for 7 days. In every 2 days, the media with the same concentration of puromycin was replaced. On 7th day, cells were washed with DPBS and final concentration of 0.5 mg/ml 3-[4,5-dimethyl-thiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT: Sigma Aldrich, USA, Cat. No. M2128) solution was added. The optical density of the reaction solution at 540 nm was measured to estimate cell viability. The viability of the treated cells was expressed relative to that of the untreated control cells as the mean±SEM of at least three independent experiments.

PuroR cloning and *E. coli/PuroR culture*

PuroR gene was PCR amplified from pFIP/CD using a pair of primer (forward primer: 5'-GGATCCAGGAGGTATGACCGAGTACAAGCCCAC-3'; reverse primer: 5'-GAATTCTCAGGCACCGGGCTTGCGGG-3' in presence of 5% DMSO. The product was cloned into the pGEM-T-easy vector. *PuroR* cDNA was inserted into multiple cloning sites in the pET-32b (+) plasmid (Merk millipore, USA, Cat. No. 69016) to obtain pET-PuroR. *E. coli* (HIT Competent Cells^{TM}-DH5α, RBC, Taiwan, Cat. No. RH617-J80) was transformed by pET-32b (+) and pET-PuroR to generate DH5α-pET and DH5α-pET-PuroR (*E. coli/PuroR*). DH5α and DH5α-pET were cultured in 3 ml terrific broth (TB) media in presence or absence of 100 μg/ml ampicillin with indicated concentration of puromycin for 16 h at 37℃, 220 rpm. The optical density of the culture solution at 600 nm (OD600) was measured for estimating the concentration of *E. coli*. The concentration of puromycin which gave same OD600 value as culture media without *E. coli* was determined as the minimum inhibitory concentration (MIC) of puromycin. The puromycin resistance *E. coli* was confirmed by culturing DH5α-pET and DH5α-pET-PuroR for 9 h and 16 h in TB media containing MIC of puromycin and subsequently by measuring OD600. The OD600 of cultured *E. coli* was expressed as the mean±SEM of at least three independent experiments.

Antimicrobial susceptibility testing

DH5α, DH5α-pET and DH5α-pET-PuroR isolates were subjected to susceptibility analysis with VITEK 2XL (BioMerieux, hazelwood, USA) using VITEK2 AST-N224 card (BioMerieux, hazelwood, USA) following the manu-

facturer's instructions and CLSI guidelines (26) in Ajou university hospital, South Korea.

Statistical analysis

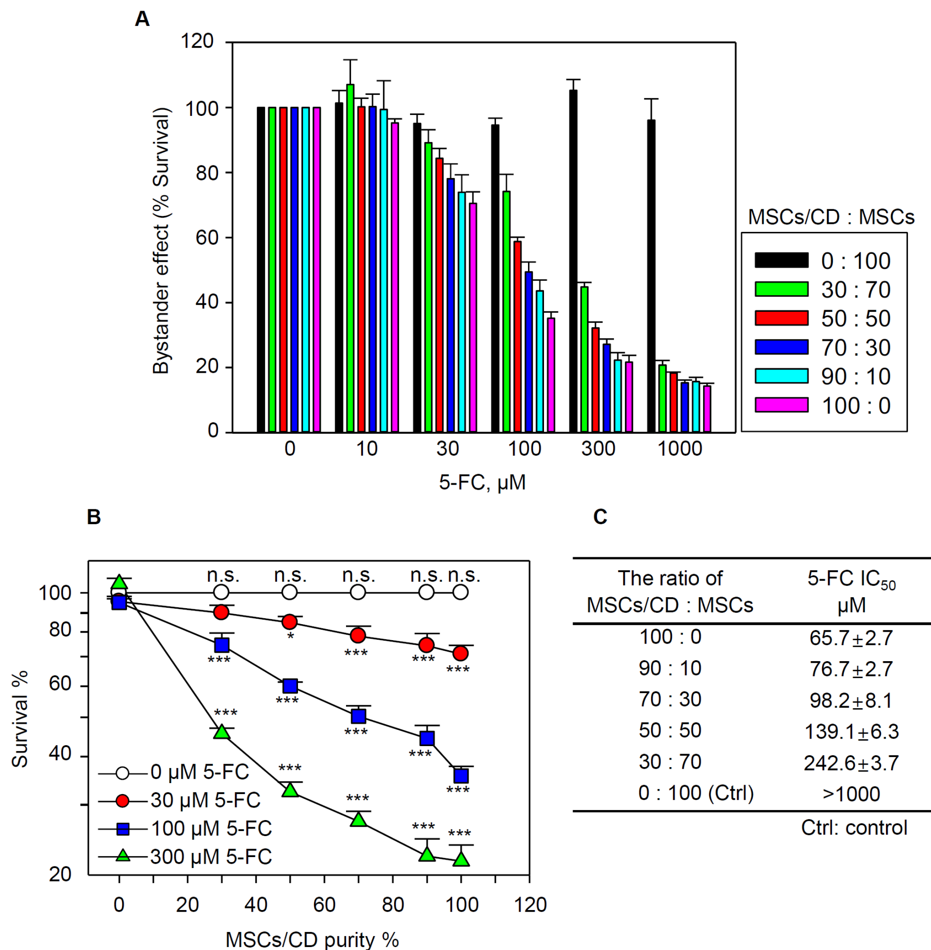
Statistical analyses were performed using SigmaPlotTM v14 software (Systat Software, USA). Data were analyzed using the Student's t-test or one-way ANOVA. Significant differences were further evaluated using Holm-Sidak method. p-values < 0.05 was considered statistically significant. All data are expressed as the mean±SEM.

Results

A *PuroR* gene is necessary as a selection marker to ensure the antitumor efficacy of MSCs/CD

In a previous study, we developed MSCs/CD as a potential treatment for brain tumors (22). In the production process of MSCs/CD, we utilized a *PuroR* gene that encodes the puromycin-N-acetyltransferase (Pac) enzyme for selecting the cells that had effectively incorporated the CD gene.

To determine how the purity of the cells impact on their therapeutic effect, we measured the anti-cancer effect of various purity levels of MSCs/CD. U87 glioma cells were engineered to express GFP (U87/GFP) for assessment tumor cell growth. Various numbers of MSCs/CD ranging



The ratio of MSCs/CD : MSCs	5-FC IC_{50} μM
100 : 0	65.7±2.7
90 : 10	76.7±2.7
70 : 30	98.2±8.1
50 : 50	139.1±6.3
30 : 70	242.6±3.7
0 : 100 (Ctrl)	>1000

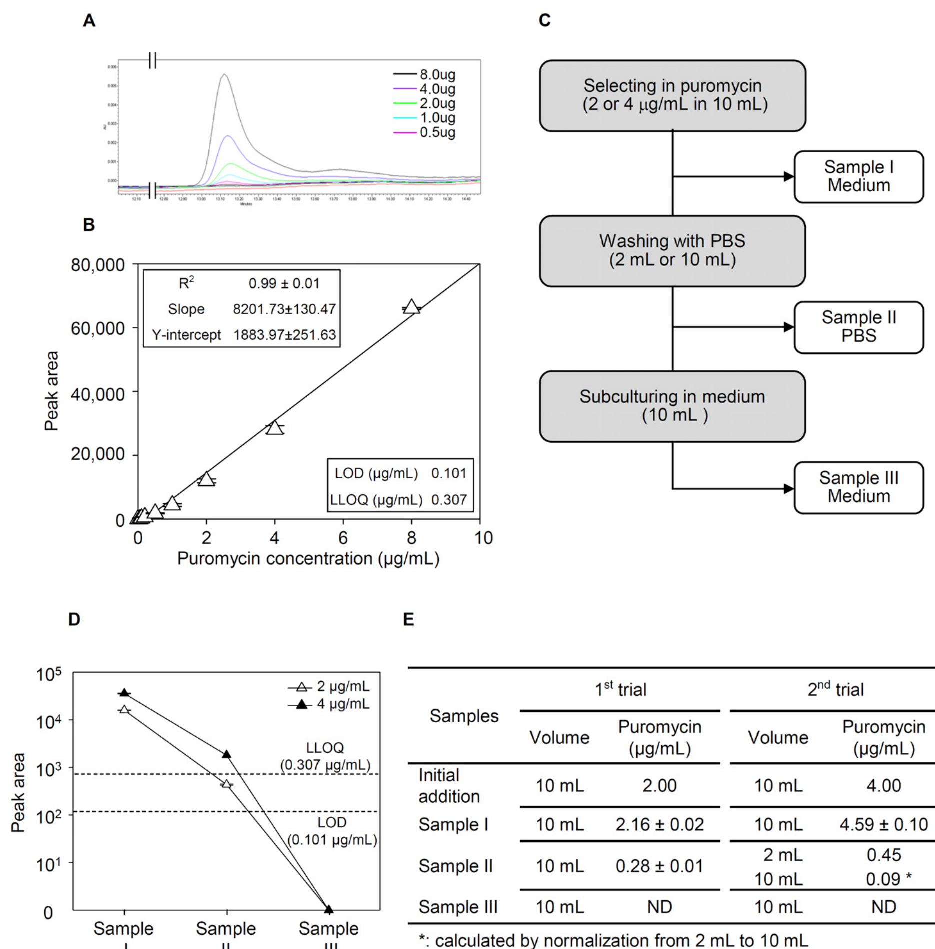
Fig. 1. MSCs/CD purity dependent antitumor effect to U87/GFP cells in presence of 5-FC. (A) Bystander effect of MSCs/CD purity level on U87/GFP cells measured by GFP fluorescence at indicated concentration of 5-FC. Note puromycin selected MSCs/CD cells were mixed with naïve MSCs to get different purity of MSCs/CD. Statistical significance (p-values) for Fig. 1A is in Supplementary Table S1. (B) Survival percentage (%) of U87/GFP cells at indicated MSCs/CD purity in presence of 30, 100, 300 μM 5-FC. (C) IC_{50} values for 5-FC in MSCs/CD at various purity level. Data are mean±SEM of at least three independent experiments (*p < 0.05, ***p < 0.001, compared to control; one-way ANOVA test). n.s.: not significant.

from 0% to 100% were co-cultured with U87/GFP cells in the presence of 5-FC (30 μM∼1 mM) for 6 days. The survival of U87/GFP glioma cells was measured as GFP intensity. During this experiment, the total number of cells was kept constant with unmodified naïve MSCs during the culture since the total number of cells affected the surviving/growth of U87 glioma cells.

The survival rate of U87/GFP glioma cells decreased in an inversely proportional manner with increasing purity in the MSCs/CD at concentrations greater than 30 μM 5-FC. At 100 and 300 μM 5-FC, the anti-cancer effect was particularly pronounced with the effect reaching saturation at a purity level of 70% at 300 μM 5-FC (Fig. 1A and Supplementary Table S1). Likewise, when the purity decreased, more 5-FC was required. The half maximal inhibitory concentration (IC_{50}) was 65.7±2.7 μM with 100% purity of MSCs/CD but increased to 98.2±8.1 and 139.1±6.3 μM with 70% and 50% purity, respectively (Fig. 1B and 1C). These results indicate that increasing the purity of MSCs/CD results in a more potent anti-cancer effect, attributed to an elevation in bystander effect. As a result, the IC_{50} value for 5-FC decreases. These outcomes validate the use of the *PuroR* gene as a selection marker to achieve higher purity cells, which enhances the efficiency of MSCs/CD and reduces the 5-FC concentration required.

Assessment of potential toxicity of residual puromycin

Next, we proceeded to investigate the potential toxicity of any remaining puromycin in the MSCs/CD culture after the selection process. We used HPLC to quantify the



Samples	1st trial Volume	1st trial Puromycin (μg/mL)	2nd trial Volume	2nd trial Puromycin (μg/mL)
Initial addition	10 mL	2.00	10 mL	4.00
Sample I	10 mL	2.16 ± 0.02	10 mL	4.59 ± 0.10
Sample II	10 mL	0.28 ± 0.01	2 mL 10 mL	0.45 0.09 *
Sample III	10 mL	ND	10 mL	ND

*: calculated by normalization from 2 mL to 10 mL

Fig. 2. Identification of residual concentration of puromycin in MSCs/CD. (A) Representative image of peaks of puromycin standard solutions containing 0.05∼8 μg/ml after injected to HPLC column. (B) Standard curve generated from standard solutions' peak areas at retention time of ∼13.5 min. (C) Schematic flow chart showing the steps of harvesting the samples to assess the residual puromycin concentration. (D) Puromycin peak area of samples I, II, III obtained from 2 and 4 μg/ml puromycin initial addition conditions. (E) Calculated puromycin concentration from 1st and 2nd trials of sample I, II, III via HPLC. Lower limit of quantification (LLOQ), Limit of detection (LOD). Data are mean±SD of at least 2∼3 independent experiments.

residual puromycin in the drug product of MSCs/CD. To establish a standard curve, we prepared reference solutions containing 0.05∼8 μg/ml using puromycin dihydrochloride powder and then injected them into HPLC column. Peak areas at retention time of approximately 13.5 min were used to calculate standard curve (Fig. 2A). The slope and Y-intercept of the curve were 8,201.73±130.47, 1,883.97±251.63, respectively. LOD and LLOQ from the standard curve were 0.101 μg/ml, and 0.307 μg/ml, respectively (Fig. 2A and 2B).

In order to determine the amount of residual puromycin, we simulated the manufacturing process, which involved adding 2 μg/ml puromycin to the culture (Sample 1), washing the cells with PBS (Sample II), and transferring the cells new culture medium for subculture (Sample III) (Fig. 2C). Samples were collected at each step and analyzed for puromycin concentration using HPLC. Initially, when 2 μg/ml puromycin was added to culture and the cells were washed with an equal volume of PBS, the puromycin amount was 0.28 μg/ml, which was below the LLOQ and therefore deemed unreliable. To obtain a more accurate measurement of the fold-reduction at each step, we modified the procedure by adding 4 μg/ml puromycin to the selection medium and then by using 2- or 10-ml PBS for washing. We were able to measure the puromycin concentration as 0.45 μg/ml in 2 ml washing buffer (Sample II), which exceeded LLOQ (Fig. 2D). After normalizing the washing volume to 10 ml, the estimated puromycin amount in 10 ml PBS was 0.09 μg/ml and no residual amount of puromycin was detected in Sample III (Fig. 2E). The results suggests that the puromycin level is diluted by approximately 50-fold in each step and the initial concentration is diluted by around 2,500-fold in subculturing medium (resulting in 0.0008 μg/ml in Sample III).

Then we tested whether this concentration would be toxic to the normal host cells. To evaluate the maximum tolerable dose of puromycin in unmodified naïve MSCs, we incubated MSCs in media containing varying concentrations of puromycin (0.002∼2 μg/ml) for one week and quantify the surviving cells with MTT assays (Fig. 3A). The maximum tolerable dose of puromycin was 0.016 μg/ml (Fig. 3B), which is 20 times higher than the estimated concentration of residual puromycin that would remain after a single subculture.

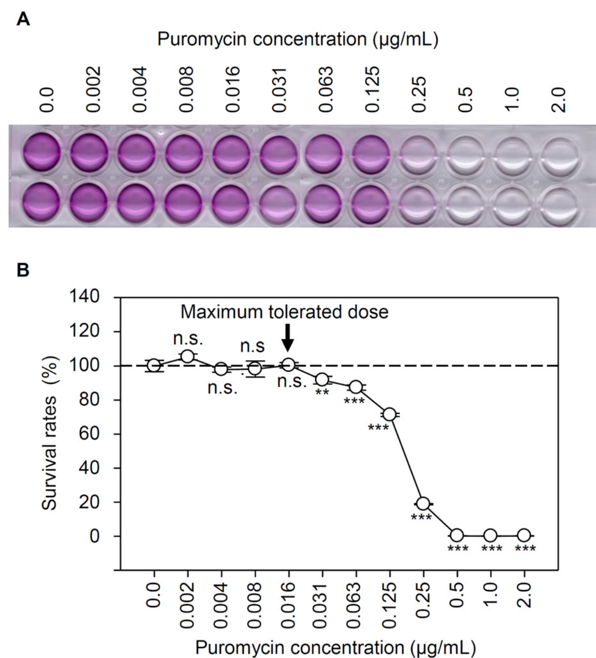


Fig. 3. Puromycin dose dependent cytotoxicity to MSCs. (A) Representative image of MTT assay at indicated concentration of puromycin showing purple colored formazan crystals after dissolving in a dimethyl sulfoxide (DMSO). (B) MTT assay indicating the cytotoxic effect of puromycin on naïve MSCs with the puromycin dose >0.016 μg/ml. Maximum tolerated dose of puromycin is indicated by arrow. Data are mean±SEM of at least 3 independent experiments (**p<0.01, ***p<0.001, compared to 0.0 μg/ml group; one-way ANOVA test). n.s.: not significant.

Hypothetical transfer of *PuroR* gene is controllable

In order to simulate the horizontal transfer of the *PuroR* gene, we first created a hypothetical puromycin resistant *E. coli* strains. We engineered a pET (AmpR) plasmid to express the *PuroR* gene (*Pac*) and obtained pET-PuroR plasmid (Fig. 4A). Then we transformed *E. coli*, DH5α with those plasmids and then obtained DH5α-pET and DH5α-pET-PuroR clones using 100 μg /ml amplicillin (Fig. 4B).

Without *PuroR* gene, both DH5α and DH5α-pET were susceptible to puromycin higher than 10 μg/ml, thus 100 μg/ml could completely block the growth of DH5α strain (Fig. 4C). The *PuroR* expression was induced by suppressing *LacI* operator with 1 mM IPTG. Only DH5α-pET-PuroR strain could grow in the presence of 100 μg/ml puromycin and 100 μg/ml. Under the same condition, the growth of DH5α-pET was inhibited (Fig. 4D). The result demonstrated that DH5α-pET-PuroR cells can effectively simulate a hypothetical scenario of horizontal transfer.

We conducted a test to determine whether hypothetical bacteria, which utilized DH5α-pET-PuroR cells, could be controlled by clinically approved antibiotics in accordance with the Performance Standards for Antimicrobial Susceptibility Testing guidelines (CLSI 31st Edition, M100 Performance Standards for Antimicrobial Susceptibility Testing, 2021). The MIC of the antibiotics was classified as Susceptible, Intermediate, or Resistant. We used un-

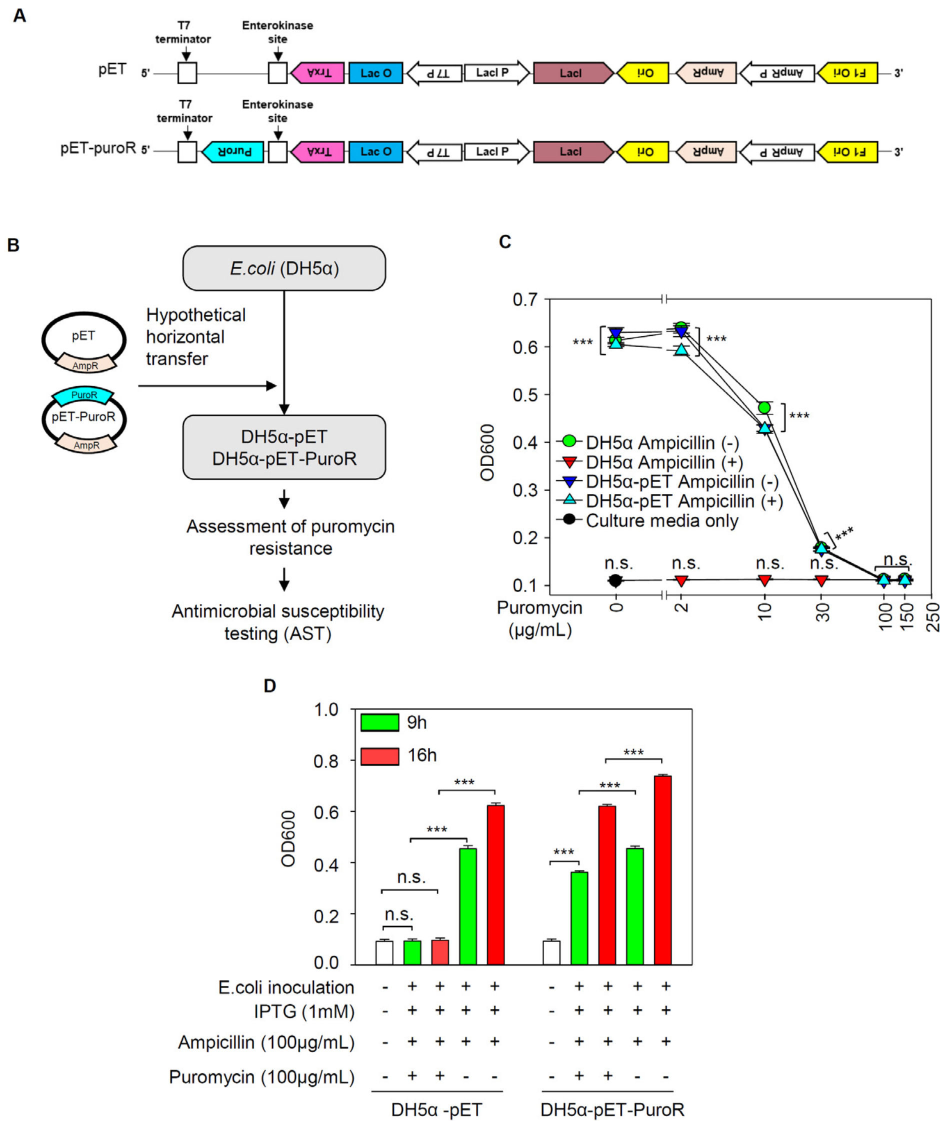


Fig. 4. Generation of puromycin resistant *E. coli*. (A) Schematic diagram of pET and pET-PuroR plasmids. (B) Flow chart representing a hypothetical horizontal transfer into *E. coli* (DH5α) to generate DH5α-pET and DH5α-pET-PuroR clones and followed by validation of puromycin resistance and antimicrobial susceptibility testing (AST). (C) Optical density measured at a wavelength of 600 nm (OD600) of DH5α, and DH5α-pET clones cultured in presence (+) or absence (−) of 100 μg/ml ampicillin at indicated concentration of puromycin to identify the minimum inhibitory concentration of puromycin. Both clones were cultured in 3 ml TB media for 16 h at 37℃, 220 rpm (rotation per minute). (D) OD600 of DH5α-pET and DH5α-pET-PuroR clones cultured for 9 h and 16 h in presence or absence of ampicillin or puromycin. Data are mean±SEM of at least 3 independent experiments (***p<0.001, compared to culture media only; one-way ANOVA test). n.s.: not significant.

transformed DH5α and DH5α-pET as controls. The responsiveness to clinically approved antibiotics was comparable across all three clones except for ampicillin and amoxicillin (Table 1). The pET plasmid used for transformation was responsible for the resistance of DH5α-pET and DH5α-pET-PuroR to these two antibiotics, thereby confirming the validity of our approach. The results indicate that commonly used antibiotics can control the hypothetical gut bacteria that acquire antibiotics resistance via horizontal gene transfer.

Discussion

Various methods are employed in gene therapy to deliver genetic materials, such as naked nucleotides, microorganisms, and viruses for *in vivo* gene therapy, or genetically

Table 1. Antimicrobial susceptibility testing of DH5α, DH5α-pET, and DH5α-pET-PuroR

Antibiotics	Samples							
	DH5α		DH5α-pET		DH5α-pET-PuroR			
					Clone 1		Clone 2	
	MIC	Interpretation	MIC	Interpretation	MIC	Interpretation	MIC	Interpretation
Ampicillin	≤2	S	≥32	R	≥32	R	≥32	R
Imipenem	≤0.25	S	≤0.25	S	≤0.25	S	≤0.25	S
Ciprofloxacin	≤0.25	S	≤0.25	S	≤0.25	S	≤0.25	S
Cotrimoxazole	≤20	S	≤20	S	≤20	S	≤20	S
Amikacin	≤2	S	≤2	S	≤2	S	≤2	S
Gentamicin	≤1	S	≤1	S	≤1	S	≤1	S
Aztreonam	≤1	S	≤1	S	≤1	S	≤1	S
Ceftazidime	≤1	S	≤1	S	≤1	S	≤1	S
Cefepime	≤1	S	≤1	S	≤1	S	≤1	S
Pip/tazobactam	≤4	S	≤4	S	≤4	S	≤4	S
Cefazolin	≤4	S	≤8	S	≤8	S	≤8	S
Cefoxitin	≤4	S	≤4	S	≤4	S	≤4	S
Amox/clavulanic acid	≤2	S	≤16	I	≤16	I	≤16	I
Cefotaxime	≤1	S	≤1	S	≤1	S	≤1	S
ESBL	NEG	-	NEG	-	NEG	-	NEG	-
Tigecycline	≤0.5	S	≤0.5	S	≤0.5	S	≤0.5	S
Ertapenem	≤0.5	S	≤0.5	S	≤0.5	S	≤0.5	S

MIC: minimum inhibitory concentration, S: susceptible, I: intermediate, R: resistance.

modified cells for *ex vivo* therapy. The efficacy of gene therapy depends on successful delivery of therapeutic genes to target cells, and manufacturing processes often use antibiotic resistance genes as selection markers. However, caution is necessary because during *in vivo* gene therapy the use of antibiotics resistance genes may result in horizontal transfer to gut microorganisms, leading to acquisition of antibiotics resistance in patients. In this study we explored the risks and benefits of using antibiotics resistance genes including the horizontal transfer in MSC-based *ex-vivo* therapy.

For this, we used the *CD* gene as a therapeutic gene and a *PuroR* gene as a selection marker. The therapeutic efficacy was highest with 100% pure MSCs/CD but proportional to the ratio of genetically modified MSCs/CD over naïve MSCs at physiologically relevant concentrations of 30∼300 μM 5-FC. Patients who orally received 75 and 150 mg/kg/day of 5-FC had plasma concentrations of 252 and 676 μmole/L, respectively (27). At a concentration of 1 mM 5-FC, GFP intensity remained at approximately 14% GFP irrespective of the MSCs/CD purity (Fig. 1). This residual GFP signal can be attributed to stable GFP proteins in dead U87/GFP cells since they barely showed signs of proliferative ability after the removal of 5-FC (data not shown). Overall, our findings highlight the benefits of using *PuroR* gene as a selection marker during the manufacturing process.

Puromycin cannot be used as an antibiotic for humans because it causes premature termination of polypeptide chains, leading to cytotoxicity to both prokaryotic and eukaryotic cells. Animal studies have shown median lethal doses (LD_{50}) of 360, 520, 678 mg/kg for intravenous, intraperitoneal, and oral administration, respectively (28). Our *in vitro* assay using human MSCs demonstrated that the maximum tolerable dose of 16 ng/ml for puromycin. Therefore, it is crucial to ensure that puromycin concentrations in the final therapeutic agents remain below 16 ng/ml if it is used during manufacturing. In our assay, puromycin was added during the selection period but excluded in the subsequent step. One cycle of removal and addition of medium leads to approximately 50-fold reduction in puromycin concentrations (Fig. 2). Therefore, the initial concentration of 2 μg/ml at the passage 4 (P4) culture is theoretically estimated to be 0.0008 μg /ml at the P5 culture. After selection, the MSCs/CD were cultured up to P10 in regular growth medium without additional puromycin selection, indicating that puromycin was entirely removed from the therapeutic product.

We assessed the risk of using puromycin as a selection

marker in the event of horizontal transfer and found that the growth of hypothetical bacteria containing the *PuroR* gene was inhibited by most clinically available antibiotics. Our simulation data, using *E. coli*, is likely applicable to other gut microorganisms as the original DH5α's responsiveness to clinically available antibiotics is not altered in DH5α-pET-PuroR and DH5α-pET. Furthermore, the use of the *PuroR* gene as a selection marker poses a low risk of horizontal transfer or environmental contamination and the emergence of puromycin-resistant bacteria is unlikely since puromycin is not used in human medicine.

Our study provides valuable insights into balancing the benefits and potential risks of associated with antibiotic resistance genes. We believe that our work can ultimately contribute to the development of effective strategies for enhancing therapeutic efficacy in MSC-based *ex-vivo* therapy while also controlling the potential risk from horizontally transferred antibiotic resistance genes.

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Potential Conflict of Interest

N.B., J.H.J., D.Y.C., and H.S.K. are employees of and stock and/or option holders in CELLeBRAIN, Ltd.

Author Contributions

N.B. and Y.J.L. wrote the manuscript. N.B., Y.J.L., J.H.J., K.W.L performed experiments. N.B., J.H.J., M.G.K., K.W.L., W.S.H., and S.S.K. analyzed the data. C.D.Y., and H.S.K. provided funding and supervised the research. All authors read and approved the manuscript.

Supplementary Materials

Supplementary data including one table can be found with this article online at https://doi.org/10.15283/ijsc23053

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