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Multiple detection of chromosomal gene correction mediated by a RNA/DNA oligonucleotide

Research Article

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Summary

Chimeric RNA/DNA oligonucleotide (RDO)-mediated gene correction of a single base mutation in a gene of an eukaryotic cell is still a controversial strategy. To better define the potential and applicability of this strategy, new systems, that allow to detect RDO-mediated gene correction in the chromosomal DNA of human cells, are needed. Here, we developed a construct containing hygromycin resistance mutant gene fused to the EGFP gene as target for correction. HeLaS3 cells were transfected with the fusion gene and clones, which had integrated one or two copies of the mutated fusion gene, were isolated and expanded. These cells were transfected with a RDO with a mismatch at the position 336 of the bacterial hygromycin resistance gene. If the gene correction occurs, the expression of both hygromycin resistance and EGFP genes is recovered. The RFLP and FACS analysis demonstrated that hygromycin resistance phenotype was due to the correction of the mutation.

I. Introduction

A chimeric RNA/DNA oligonucleotide (RDO) is a double stranded molecule consisting of RNA and DNA residues, usually 70-80 bases in length, capped at both ends by sequences which fold in a hairpin (Kmiec et al, 1994; Cervelli et al, 2002). The chimeric RDO contains a single nucleotide that differs from the target sequence and, therefore, forms a specific mismatch.

The method of targeted gene correction by specific RDO or modified DNA oligonucleotide was developed to generate or correct point mutations (Rice et al, 2001; Brachman and Kmiec, 2002; Liu et al, 2003). This strategy has been successfully used in several genetic systems both *in vitro* using mammalian cells or mammalian and plant cell free extracts, and *in vivo* using several animal models (Cole-Strauss et al, 1996; Yoon et al, 1996; Kren et al, 1997, 1999; Xiang et al, 1997; Alexeev and Yoon, 1998; Bartlett et al, 2000; Gamper et al, 2000; Rando et al, 2000; Liu et al, 2001; Kenner et al, 2002, 2004; Parekh-Olmedo and Kmiec, 2003). Recent data have contributed to understand the mechanisms and the genetic requirements of gene correction (Rice et al, 2001; Liu et al, 2001, 2002a, b; Parekh-Olmedo et al, 2002). It has been proposed that the DNA strand of RDO is responsible for gene correction activity and that the active DNA strand has to be generated inside the cell nearby the target site of correction (Andersen et al, 2002; Liu et al, 2003; Igoucheva et al, 2004). The stimulation of gene correction was also observed after DNA damage induction and following the activation of homologous recombination indicating that in mammalian cells the efficiency of gene correction may depend on the ability of the cells to undergo homologous recombination (Ferrara and Kmiec, 2004; Ferrara et al, 2004). However, the frequency of gene correction still remains highly variable and the reason for these differences is not yet clear. The lack of standardized assays for evaluating the gene correction at phenotypic level without the PCR analysis and the not yet proved mechanism that can direct the RDO-mediated correction of a chromosomal gene are the two main concerns about the applicability (Zhang et al, 1998; Rice et al, 2001; Yoon et al, 2002; Kmiec 2003). In this work, we generated two HeLa–derivative cell lines that contain in the genome a fusion construct composed by a mutated antibiotic-resistance gene (Hygromycin B) and the enhanced green fluorescence gene (EGFP). We report that, when gene

correction was measured following RDO transfection, cells both resistant to hygromycin and expressing EGFP were recovered indicating that RDO is able to induce gene correction at chromosomal level.

II. Materials and methods

A. Cell line and culture conditions

HeLaS3 cells (from Margherita Bignami, ISS, Rome, Italy) were routinely cultured in Dulbecco's medium (DMEM) supplemented with 10% fetal calf serum, 100UI/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere containing 6% CO_2.

B. Construction of the plasmid pHygNSNeo, transfection and Southern analysis

Plasmid pHygNSNeo was constructed from pHygEGFP (Clontech) (**Figure 1A**) and pMC1neo (Stratagene). pHygEGFP was restricted with *Hind*III and *Sal*I. This resulted in 2 fragments that are 2123 bp and 3669 bp long, respectively. The 2123 bp fragment was further digested with *Nco*I obtaining 2 fragments that are 714 bp and 1409 bp long. The 714 bp *Nco*I-*Hind*III fragment was PCR amplified from pHygEGFP. The forward primer, 5'-TAGAAGCTTTATTGCGGTAGTTTATCACAG-3', was designed with *Hind*III restriction site at 5'end. The reverse primer, 5'-TTTCCATGGCCTCCGCGACCGGCT**A**CA-3', was designed with *Nco*I restriction site at the 5'end such to introduce a point mutation (**A**) at the position 336 of hygB gene. This mutation produces a stop codon and the loss of the *Pst*I restriction site. Amplification was performed by denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 sec, 67°C for 45 sec, and then extension at 72°C for 2 min. The direct ligation of the new 714 bp *Nco*I-*Hind*III fragment containing the stop codon with the 1409 bp *Nco*I-*Sal*I fragment and the 3669 bp *Hind*III-*Sal*I fragment formed the plasmid pHygNS. Afterward, the neomycin resistance gene (neo) was inserted into the *Sal*I site of pHygNS by cloning the 1100 bp *Xho*I-*Sal*I fragment from pMC1neo. The new vector containing a stop codon 336 bases downstream to the ATG of the hygB-EGFP fusion and the neo marker was named pHygNSNeo. The presence of the stop codon was confirmed by sequence analysis.

Plasmid pHygNSNeo was transfected in HeLaS3 by electroporation. A sample of 3.5×10^6 exponentially growing cells and 10 µg of pHygNSNeo linearized with restriction enzyme *Cla*I were resuspended in 250 µl DMEM without serum and antibiotics. The suspension was then transferred to 50 x 4 mm cuvette (Equibio) and incubated on ice for 10 min. Afterward, the cuvette was exposed to one pulse (330 V, 1000 µF, 200) using the Electroporator II apparatus (Invitrogen) connected to a power supply (330 V, 25 mA, 25 W). The cell suspension was then cooled for 15 min on ice, resuspended in complete medium and seeded in four 100 mm diameter dishes at density of 5×10^5 cells per dish. After 24 hours, 1000 µg/ml G418 (Invitrogen) were added to every dish. After 15-21 days, one G418 resistant ($G418^R$) colony per dish was isolated, expanded to clonal population and analyzed for the presence of pHygNSNeo as follows. Genomic DNA was digested with *Hind*III and analyzed by standard Southern blot procedures. Briefly, 10µg DNA per sample was electrophoresed on 0.8% agarose gel, transferred to nylon positively charged membrane (Roche) and hybridized with digoxigenin labeled HygEGFP as probe. The labeling was carried out by Random primed DNA labeling kit (Roche).

C. Synthesis and transfection of the chimeric RNA/DNA oligonucleotide (Ch867)

The chimeric RDO, named Ch867, was obtained by using the standard phosphoramidite chemistry in an automatic synthesizer Expedite 8909 (Millipore). After ammonia deprotection, Ch867 was purified, desalted and stocked at –20°C. The structure of Ch867 is depicted in **Figure 1C**.

Cells of HeLa S3/G418R clones were seeded at density of 4×10^5 cells per 30 mm diameter dish in 3 ml of growth medium. 18 µg of Ch867 were diluted with DMEM without serum and antibiotics to a total volume of 100 µl and incubated with 22 µl of PolyFect Lipofection Reagent (Qiagen). The lipofection complex was added according to the manufacture's recommendation.

Each transfected clone was grown for 96 h in normal medium to allow the correction and the expression of hyg gene. At that time, 3×10^5 cells were seeded on 100 mm diameter dish in selective medium containing 300 µg/ml hygromycin (Roche), a selective dose derived from dose response curve carried out for HeLaS3 (data not shown). The selective medium was changed every 4 days and after 12 days, hygromycin resistant colonies were harvested, expanded as polyclonal population in complete growth medium without hygromycin, and analyzed by RFLP and FACS analysis.

D. Flow cytometry

The count of fluorescent HeLaS3 cells was performed by flow-cytometry on a fluorescence-activated cell sorting apparatus (FACScan, Lysys II software, Becton Dickinson, San Jose, CA). Briefly, 5×10^5 cells were resuspended in 100 µl PBS and the fluorescence of 10^4 cells was determined.

E. Restriction fragment length polymorphism (RFLP)

Genomic DNA extracted from polyclonal populations was amplified by PCR. The forward and reverse primers sequences were 5'-TGATGCAGCTCTCGGAGG-3' and 5'-AGTGTATTGACCGATTCCTTG-3' respectively. The PCR conditions to generate a 361-bp fragment were 94°C for 30 sec, 54°C for 30 sec, 72°C for 45 sec for 35 cycles. 10 µl PCR product was incubated overnight with *Pst*I in a final volume of 20 µl. Later on, 10 µl were loaded onto 2% agarose gel (1X TBE, EtBr 1 µg/ml), electrophoresed for 2 h at 50 V, and the migration profile analyzed.

40 ng of 361 bp PCR product were submitted to the automatic sequencing to verify the occurrence of base correction.

III. Results

To study the chimeric RDO-mediated gene correction in the chromosomal DNA of HeLaS3 cells we first constructed the plasmid pHygNSNeo containing a point mutation within the coding region of bacterial *hygB* gene at the position 336 (C T) generating a stop codon (TAG) and the loss of *Pst*I restriction site (**Figure 1A**). Therefore, the *hygB* gene is not functional and the fused EGFP gene is not translated. Thereafter, we transfected HeLaS3 cells with pHygNSNeo and then selected them in medium containing G418. Two independent G418 resistant colonies were isolated, expanded to clonal population and analyzed for the presence of *hyg*B gene. Genomic DNA was digested with *Hind*III, which cuts only once in pHygNSNeo, blotted and hybridized with DIG-labeled HygEGFP fusion gene as probe. As shown in the **Figure 1B**, the clone 20105.3A (lane 3) has at least 2 copies and the clone 20105.6A (lane 4) only one copy of the integrated vector. Furthermore, the migration profile

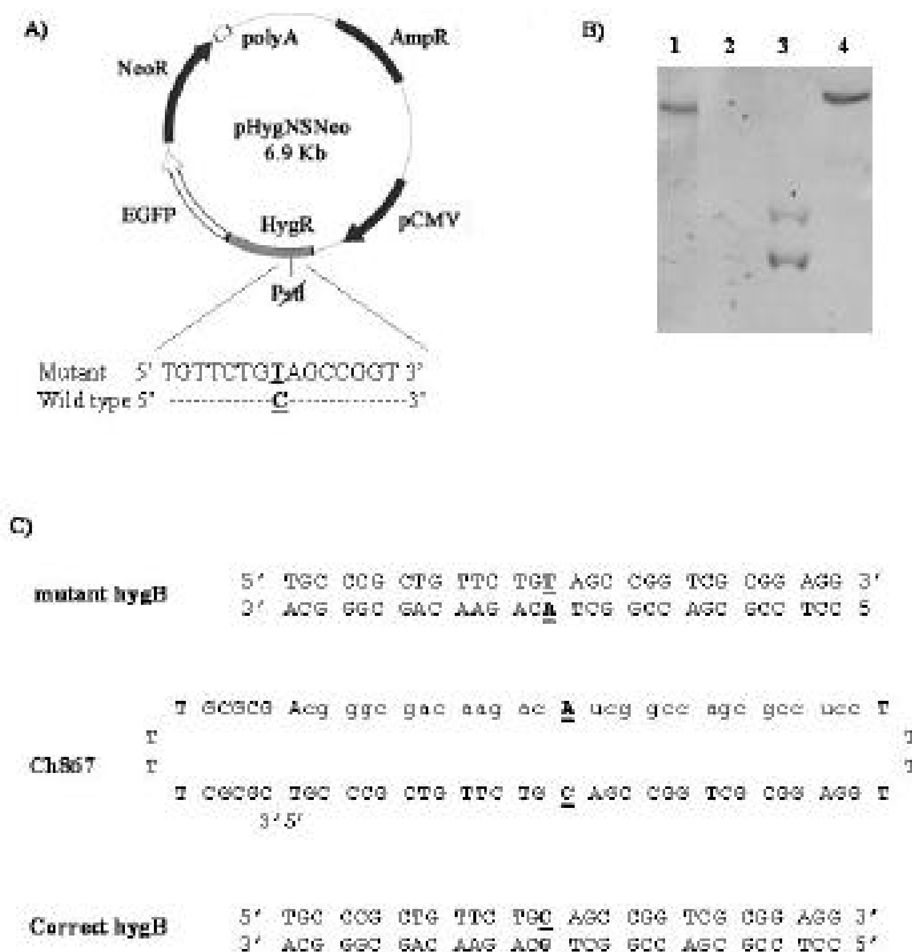

Figure 1. Plasmid pHygNSNeo and chimeric RNA/DNA oligonucleotide Ch867. (**A**) Diagram of the pHygNSNeo plasmid containing a single point mutation, thymine, at the position 336 in the coding region of hygB gene (bold letter). (**B**) Southern blot analysis of G418 resistant clones. Sample of DNA (10 µg) were digested with *Hind*III and analyzed by Southern blotting. The fused gene was used as probe. Lane 1: pHygNSNeo, lane 2: HeLaS3, lane 3: 20105.3A and lane 4: 20105.6A. (**C**) Sequences of the target site before and after correction by Ch867. Ch867 consists of a 35 bp long duplex bracket by 4 base long hairpin loops. Each RNA residue (small letters) is modified by the inclusion of a 2'-O-methyl group on ribose sugar. The DNA (capital letters) contains the designed base for correction.

analysis indicated that the integration occurred at different genomic sites. A chimeric RDO, named Ch867, was designed according to Gamper and colleagues who demonstrated that the most efficient chimeric RDO has one strand containing 2'-O-methyl RNA homologous to the target site and a DNA strand bearing the mismatched base (**Figure 1C**)(Gamper et al, 2000). A gene correction event mediated by Ch867 not only will recover the *hygB* wild type sequence, but also restore the *Pst*I site and, consequently, the right frame leading to the expression of the fusion HygEGFP. We then transfected the chimeric RDO Ch867 in the two clones 20105.3A and .6A according to the transfection protocol that gives high level of nuclear localization of the RDO (Cervelli et al, 2002). The Ch867 transfection increased significantly (p 0.01) the frequency of hyg^R clones by 6.7 fold (20105.3A) and 3.7 fold (20105.6A) above the spontaneous level (**Table 1**). Vice versa, 20105.3A and 20105.6A cells transfected with an unrelated RDO showed no increase in hygromycin resistance frequency as compared to the non-transfected control has been observed (data not shown) (Cervelli et al, 2002).

To test whether the enhancement of hygromycin resistance frequency was due to the correction of the stop mutation of *hygB* gene, hygromycin resistant colonies formed after 12 days of growth in selective medium were analyzed as a whole population (pools of 10-20 clones) for the presence of *Pst*I restriction site in the integrated *hygB* target. Therefore, genomic DNA extracted from polyclonal hyg^R populations was subjected to PCR and the amplification products were digested with *Pst*I. The digestion of the 361 bp PCR fragment with *Pst*I yielded a fragment of 98 bp and one of 263 bp as shown by the *Pst*I digestion of pHygNSNeo (**Figure 2A**, **panel 1**). As shown in the **Figure 3A**, the *Pst*I site was present in the two populations 20105.3A and 20105.6A transfected with Ch867 (**Figure 2A panel 3 and 4**). On the other hand, the *Pst*I site is not present in 361bp hygB fragment amplified

Table 1. Effect of Ch867 on hygromycin resistance frequency in HeLaS3 cells

	hygromycin resistance frequency x 10^{-5} [a]		
G418^R clones	**- Ch867**	**+ Ch867**	**Fold increase**[b]
20105.3A	0.78±0.66	5.25±0.98**	6.7
20105.6A	2.08±1.39	7.75±2.06**	3.7

Results are reported as mean±standard deviation of at least 3 independent experiments. Results are statistically analysed with the Student "*t*" test; ** p 0.01

[a] hygromycin resistance frequency has been calculated dividing the number of hyg^R colonies by the number of viable cells.

[b] Fold increase represents the ratio between the two hyg^R frequencies obtained with and without transfection of Ch867.

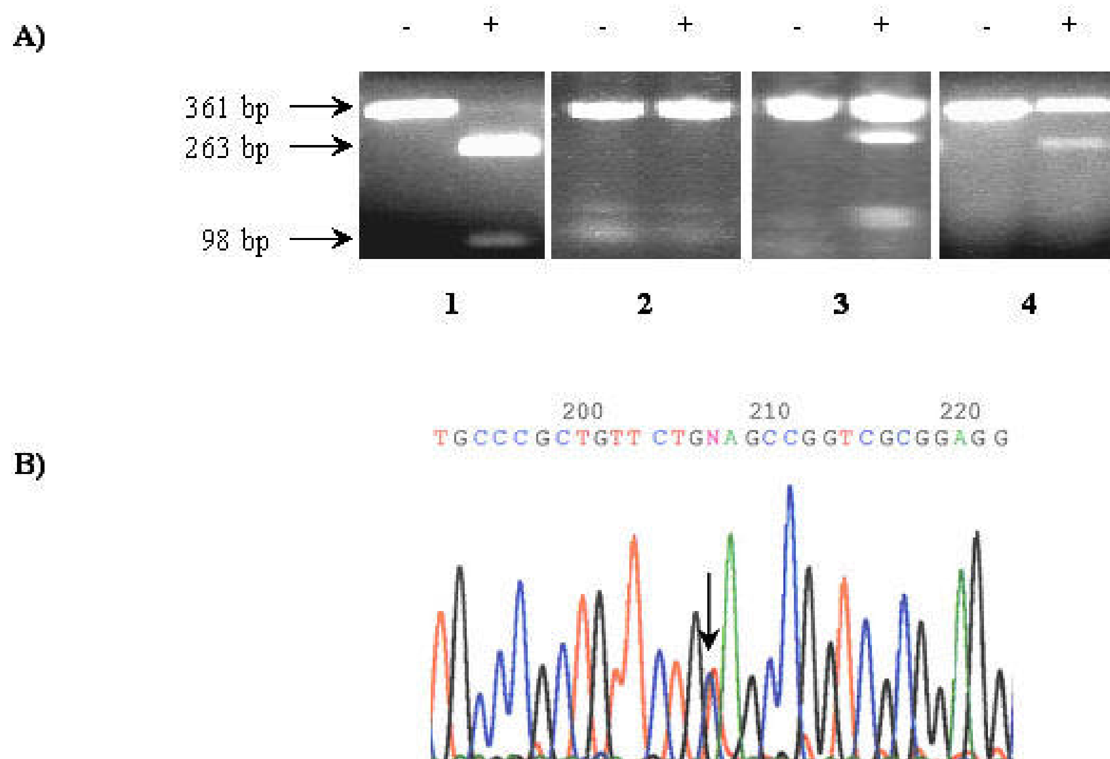

Figure 2. (**A**) RFLP analysis of the 361 bp PCR fragment from pHygNSNeo (panel 1), from hyg^R polyclonal population of 20105.3A and .6A transfected with Ch867 (panel 3 and 4), and from hyg^R polyclonal population of non transfected 20105.3A (panel 2). 500-600ng DNA were digested with *Pst*I and loaded in each lane (+). The same amount of DNA was loaded as control (-). (**B**) Sequence of the PCR fragment from a *Pst*I positive polyclonal population. Only the region flanking the nucleotide 336 is shown. Arrow indicates the targeted base for correction.

by genomic DNA extracted from the polyclonal hygromycin resistant population derived from 20105.3A non transfected (**Figure 2A, panel 2**) and 20105.6A (data not shown). Moreover, *Pst*I restriction of PCR fragment from pHygNSNeo was complete, whereas, *Pst*I restriction of PCR fragments from polyclonal transfected populations was only partial (**Figure 2A**, **panel 1, 3 and 4**). This observation was also confirmed by the sequencing of a *Pst*I-positive polyclonal population that showed a mixture of T (mutated nucleotide) and C (correct wild type nucleotide) at position 336 of hygB gene (**Figure 2B**). This indicated that Ch867 corrected the mutant sequence.

To ascertain whether the base correction, which restored the *Pst*I site, allows the expression of the fused EGFP gene, spontaneous and Ch867-induced hygromycin resistant clones were analyzed by FACS. Fluorescence profile of *Pst*I negative clone (thick line) was overlapped that of parental population (thin line), whereas that *Pst*I positive clone was only in part overlapped that of parental population (**Figure 3A and 3B**). Thus, the fluorescence of the *Pst*I positive clone was higher than *Pst*I negative clone demonstrating that the correction also restored the EGFP expression.

IV. Discussion

The reason for the differences in the gene correction rate observed in several experiments is not yet elucidated (Santana et al, 1998; Rice et al, 2001; Brachman and

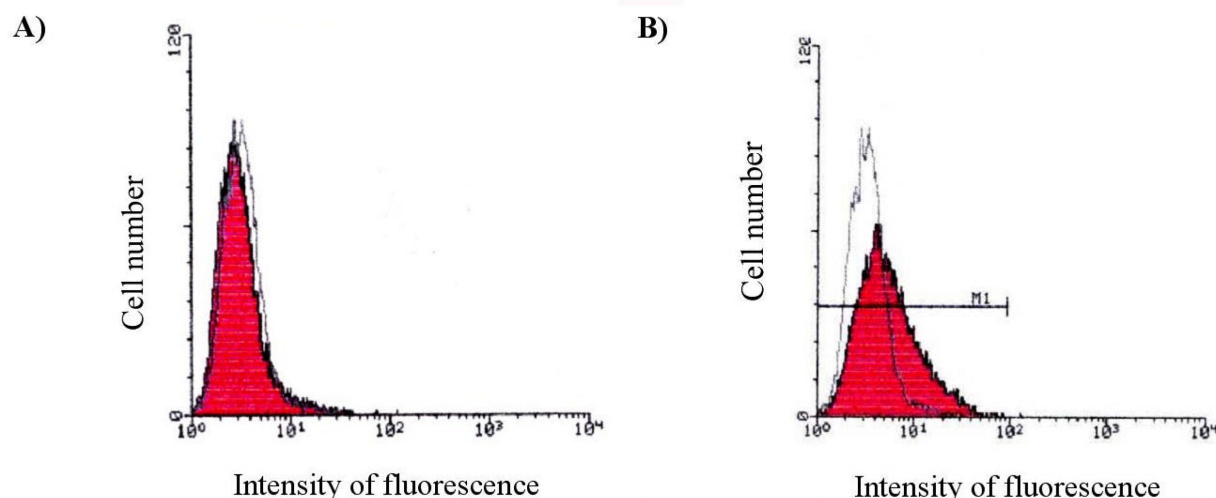

Figure 3. Flow cytometry of spontaneous *Pst*I negative hyg^R clone (**A**) and *Pst*I positive hyg^R clone of 20105.3A cells (**B**). The fluorescence profile of both clones (red area) (thick line) is compared to the profile of the parental cell population (thin line).

Kmiec 2002). The major concerns on the chimeric RDO mediated-gene correction derive from the use of PCR amplification as primary screen for the detection of the correction event (Zhang et al, 1998). The set up of new systems where the gene correction events leads to the reversion of a mutation in a gene conferring more than one phenotype is ideal to overcome the problem. Here, we described an additional eukaryotic assay to study chromosomal gene correction in human cells in which the gene correction event is screened by multiple detection. A fusion HygEGFP gene was mutated by the insertion of a stop codon in the *HygB* sequence. Therefore, cells having this construct integrated in the genome are hygromycin sensitive and do not express EGFP. We designed a chimeric RDO, named Ch867, to correct the stop mutation of the hygB gene. After transfection with Ch867, HeLaS3 containing the *hygB* mutated gene integrated as single or multiple copies showed an enhancement of hygromycin resistance frequency over the spontaneous baseline, and restored both *Pst*I site and EGFP expression. The RDO transfection increased 6.7 fold the frequency of gene correction in the cells containing at least two copies of the *hygB* mutated gene, and 3.7 fold in cells with one copy of the *hygB* mutated gene suggesting that the copy number of the integrated target may have an influence on gene correction. However, the direct comparison of the frequencies demonstrated that the difference is not statistically significant (p= 0.114).

A confounding effect in the detection of gene correction is represented by the presence of hyg^R spontaneous clones. For that, we were forced to carry out the analyses in the polyclonal populations. To rule out the possibility to get false positive results due to PCR artifact, in other words, to exclude that chimeric RDO itself could serve as primer and template in the PCR amplification (Zhang et al, 1998), we analyzed hyg^R polyclonal population of transfected and non transfected cells after growing them for 12 days in presence of hygromycin. The RFLP analysis (**Figure 2A**) and the sequencing of PCR fragments (**Figure 2B**) revealed a mixture of correct and mutant sequences in hyg^R polyclonal populations derived from Ch867 transfected cells. Fluorescence intensity of a *Pst*I positive hyg^R clone obtained from Ch867 transfection was significantly higher than that of *Pst*I negative hyg^R clone. All these results demonstrated that Ch867 precisely corrected the base mutation given that the expression of the fused HygEGFP gene was obtained. Therefore, a system in which the gene correction is tested by multiple detection, such as hygromycin resistance, RFLP and FACS analysis, may be useful to select for accurate correction event.

The results of this study confirm that the chimeric RDO strategy may be feasible to correct single base mutation and, therefore, useful to treat single gene diseases.

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