INTRODUCTION

The creation of novel phenotypes and identification of the mutations responsible has led to some of the most important advances in biology. In mammalian systems, phenotypes may be created either by modifying the germline or by modifying cultured cells, using either chemical or insertional mutagens. Germline mutagenesis can reveal phenotypes that are impossible to produce in culture (e.g., behavioral phenotypes or phenotypes related to development). However, many basic biological phenomena such as cell cycle regulation and cell death can be better explored using cultured cells. Germ-line mutagenesis is limited by the very low rate at which mutations can be produced (by present estimates, perhaps 100 times the background rate). In vitro mutagenesis in cultured cells can be expected to yield far higher rates of mutation than the germline approach. Because of the biological complexity of animal models and the time and expense involved in breeding, cells and cell lines are often preferred when cellular questions are at issue. Unfortunately, forward genetic studies in cultured mammalian cells, as presently practiced, have serious limitations (1–10).

Given that the cells used are diploid, only a single copy of each autosomal gene is generally destroyed by mutagenesis in vitro, and breeding cannot be used to achieve homozygosity for mutations as it can be in germline mutagenesis. In many instances, a dominant phenotype is not rendered and, indeed, cannot be rendered by mutation. This problem has led to develop antisense library approaches (5,9,11,12), and rescue methods have been developed to quickly identify which antisense cDNA is responsible for a given phenotype (9). But for many genes, antisense RNA expression is not effective in blocking expression. The same problem is present if a modified method, random homozygous knockout (RHKO), is used to produce a phenotype (8). In recent years, the use of small interfering RNA (siRNA) library has attracted a great deal of attention as a powerful tool for functional identification of genes (13–15). siRNA is clearly much more efficient than antisense RNA in knocking down gene expression; however, there are still some drawbacks. One study has shown that sequence identity of as few as 11 to 12 nucleotides between an interfering RNA and a messenger RNA (mRNA) may be sufficient for interference to occur (16). If this is true, then cross-reactivity, which is referred to as the off-target effect, could be a substantial problem (17). Although RNA interference (RNAi) targeting efficiency is much better than antisense, it is clear—as in the case of worms or flies—that different genes in mammalian cells are turned down with differing efficiencies. The potential interferon response to siRNA expression in mammalian cells could also interfere with some genetic screens (18–20). Recently, two groups of investigators generated embryonic stem (ES) cell libraries with genome-wide biallelic mutations (21,22). The increase in the rate of loss-of-heterozygosity (LOH) in Bloom’s syndrome gene (Blm) deficient cells was used in their strategy to generate these biallelic mutations. Blm-deficient cells carrying heterozygous mutations segregate into homozygous daughters in vitro and in vivo, presumably through mitotic recombination between nonsister chromatids. One group used Blm knockout ES cells and another group developed a tetracycline-inducible system to transiently knockout Blm.
in ES cells. The rate of LOH in Blm-deficient ES cells is $4.2 \times 10^4$ and $2.3 \times 10^4$ determined by the two groups, respectively, which is approximately a 20-fold increase from the wild-type ES cells. In order to allow the LOH to occur in most of the loci, both groups passed the mutated cells a number of generations. As a result, the libraries contained approximately $5 \times 10^8$ cells, and among them, 0.01%–0.1% cells containing biallelic mutations. Although the rates of LOH were quite low in these studies, considering the hypomorphic allele used by the first group and possible leaky inducible system used by the second group, the rate of mitotic recombination could have been higher, and therefore, using LOH to generate biallelic mutations can be a very hopeful approach. However, how the method will practically be applied to other cell types remains to be examined.

Retroviral insertion can create a single mutation in a cell, and the inserted retrovirus offers a tag with which to find the mutated gene with relative ease (23,24). In principle, insertional mutagenesis would be very effective if haploid mammalian cells could be created in culture. High rates of mutations can be most efficiently achieved by chemical mutagens because of their potency and because they can be applied to culture cells multiple times.

Here, we describe a method that utilizes the power of chemical mutagenesis in vitro to generate a pool of cells that can be regarded as quasi-haploid. We then use a specifically designed retroviral vector to randomly disrupt genes that have been rendered haploid, permitting rapid identification of each mutation that causes a phenotype of interest.

**MATERIALS AND METHODS**

**Mutagenesis with Chemical Mutagens**

N-ethyl-N-nitrosourea (ENU; Sigma, St. Louis, MO, USA) was dissolved in equal portions of 95% ethanol and PCS buffer (50 mM sodium citrate, 100 mM sodium phosphate, pH 5.0). O-6-benzylguanine (O-6-BG) was dissolved in dimethyl sulfoxide (DMSO). Approximately 50% confluence cells in a 10-mm dish were pretreated with $10 \mu M$ O-6-BG for 12 h and then treated with $0.35$ mg/mL ENU for 2 h in the presence of O-6-BG. The cells were washed and incubated with fresh medium containing O-6-BG for 24 h to recovery. The cells were plated onto a 10-mm dish at approximately 1000 cells/dish. Four dishes were used to measure mutation frequency, and the other two were used for the next round of mutagenesis. To detect hypoxanthine phosphoribosyl transferase (Hprt) null mutants, individual clones were picked from the 100-mm dish into 24-well plates. After culturing these clones for 24 h, 10 μM 6-thioguanine (6-TG) were added to the medium, and 6-TG-resistant clones were scored 1 week later. Mutagenesis with 1.5 μM acidine mutagen ICR-191 was performed the same as ENU, except the time of treatment was 24 h.

**Mutagenesis with Retrovirus**

The pDisrup 8 retroviral vector was constructed as described previously, except a ribosome sequence was incorporated into the vector (25). A blasticidin-resistant gene was used in this vector because blasticidin selection requires much less time than G418 selection. The virus preparation and infection were performed as described (25). Briefly, pDisrup 8 recombinant retroviruses were generated in Phoenix amphotropic producer cells using the calcium phosphate method of transfection. Viruses were produced at 32°C, and virus-containing medium was collected 24 h posttransfection and filtered through a 0.45-μm filter. The haploid RAW 264.7 cell pool was plated in 6-well plates at a density of $5 \times 10^5$ cells/well. Retroviral infection was performed by replacing medium with 2 mL pDisrup 8 virus (containing $4 \mu g$ Polybrene/mL; Sigma), followed by centrifugation of the 6-well plates at 859× g for 30 min at 32°C. The infection efficiency was estimated by paralleled experiments using a similar retrovirus expressing green fluorescent protein. Blasticidin (10 μg/mL) was used to select blasticidin-resistant cells.

**3′ Rapid Amplification of cDNA Ends**

The portion of the endogenous gene that was fused with the blasticidin+ gene was amplified by the 3′ rapid amplification of cDNA ends (3′-RACE) technique. Total RNA was isolated, and reverse transcription was performed with the primer 5′-CCAGTGAGCGAGTGCAG GACTCGAGCTCAAGC[T]_{7-9}-3′. A nested PCR was performed with the resulting reverse transcription product with the following primers: PI/Q1 (5′-AAAGCGATAGTGAAGGACAGT

Figure 1. Determination of chemical mutagenesis frequency and selection of lethal toxin-sensitive clones. (A) Mutation frequency in RAW 264.7 cells treated with N-ethyl-N-nitrosourea (ENU) or acidine mutagen ICR-191 or ENU plus ICR. Cells were treated with different rounds of chemical mutagens. The mutation frequency was determined by the percentage of cells that had a recessive mutation on the hypoxanthine phosphoribosyl transferase (Hprt) gene. (B) The sensitivity to lethal toxin-induced cell death of individual clones treated with or without chemical mutagens. The viabilities of each of individual clones of 50 parental and 72 chemically mutated clones treated with 500 ng/mL lethal factor (LF) and protective antigen (PA) were measured by crystal violet uptake.
GA-3′ and 5′-CCAGTGAGCAGAGT GACG-3′ and P2/Q2 (5′-TGCT GCCCTCCTGTTATGGTTGAG-3′ and 5′-GAGGACTCG AGCTCAAGC-3′). P1 and P2 are located on the \textit{blasticidin} gene, while Q1 and Q2 are on the anchor sequence of the reverse transcription, QT. The PCR products of 3′-RACE and reverse transcription PCR (RT-PCR) were directly sequenced.

Small Interfering RNA

siRNAs targeting cytohesin-4 or J κ recombination signal binding protein (Rbp-jκ), were stably expressed in RAW 264.7 cells by using pSuper vector (OligoEngine, Seattle, WA, USA) encoding a neomycin-resistant gene. All neomycin-resistant clones were pooled and analyzed for anthrax lethal toxin (LeTx) sensitivity. The sequence of the siRNAs are 5′-AAGGAGCTACAGCACATCAA -3′ for cytohesin-4 and 5′-AAGGAGGAGGTTCACAGTGT -3′ for Rbp-jκ. Predesigned siRNAs were purchased from Ambion (Austin, TX, USA) for Atp6v0c and Laptm5 and Dharmacan (Chicago, IL, USA) for hypoxia-inducible factor 1-α subunit (Hif1-α) and were transiently trans-fected in RAW 264.7 cells by using siPORT™ Amine transfection reagent (Ambion). LeTx sensitivity was analyzed 48 h after the transfection.

Cell Culture and Viability Assay

A single clone of RAW 264.7 cells was isolated and used as parental cells for the mutagenesis. The mutated cells generated at every experimental step were frozen to store at a very early passage to maintain original mutants in the event that spontaneous rever-sions occurred during cell culture. LeTx-induced cell death was measured using crystal violet uptake as described (26). Components of lethal toxin, lethal factor (LF), and protective antigen (PA) were obtained from List Biological Laboratories (Campbell, CA, USA) and used at 1 μg/mL unless otherwise indicated.

RESULTS

Development of Quasi-Haploid Cells Through High-Intensity Chemical Mutagenesis

A quasi-haploid culture can be created by generating loss-of-function mutations that affect one allele of most or all genes within a culture of limited complexity. The number of cells that are needed to create such pool depends upon the percentage of monoallelic mutations introduced within each cell. If 10% of genes are mutated on one allele in each cell, and if mutations occur at random, a pool of 40 cells should contain monoallelic mutations of 98.5% of all genes [1-(1-X)^n, X, mutation rate in each cell; n, number of cells]. Thus, it is feasible to obtain a pool of cells with a quasi-haploid background if we can create functionally null monoallelic
was fused with Jκ recombination signal binding protein (Rbp-jκ) mRNA. The insertion occurred in an intron between exon 1 and exon 10, which results in a disruption of one allele of Rbp-jκ. (B) Blasticidin + mRNA was fused with cytokinin-4 (Pscd4) mRNA. The viral insertion occurred between exon 6 and exon 7. One allele of Pscd4 is disrupted. (C) Balstatcindin + mRNA was fused with subunit c of vacuolar H+-ATPases (Atp6v0c) mRNA. The insertion is between exon 1 and exon 12. One allele of Atp6v0c was disrupted by retrovirus. (D) Balstatcindin + mRNA was fused with lysosomal-associated protein transmembrane 5 (Laptm5) mRNA. The insertion is between exon 2 and exon 3. (E) Balstatcindin + mRNA was fused with a hypothetical protein homolog to human mitochondrial carrier CGI-69 (Loc68066) cDNA. The insertion is between exon 1 and exon 2. (F) Balstatcindin + mRNA was fused with hypoxia-inducible factor 1-α subunit (Hif1-α) cDNA. The insertion is between exon 1 and exon 2.

Figure 3. The fused messenger RNA (mRNA) of blasticidin + and an endogenous gene in six anthrax lethal toxin (LeTx)-resistance RAW 264.7 clones were amplified by 3′ rapid amplification of cDNA ends (3′-RACE) and analyzed by DNA sequencing. (A) Blasticidin+ encoding by retroviral vector was fused with Jκ recombination signal binding protein (Rbp-jκ) mRNA. The incorporation occurred in an intron between exon 1 and exon 2, which results in a disruption of one allele of Rbp-jκ. (B) Blasticidin+ mRNA was fused with cytokinin-4 (Pscd4) mRNA. The viral insertion occurred between exon 6 and exon 7. One allele of Pscd4 is disrupted. (C) Balstatcindin + mRNA was fused with subunit c of vacuolar H+-ATPases (Atp6v0c) mRNA. The insertion is between exon 1 and exon 12. One allele of Atp6v0c was disrupted by retrovirus. (D) Balstatcindin + mRNA was fused with lysosomal-associated protein transmembrane 5 (Laptm5) mRNA. The insertion is between exon 2 and exon 3. (E) Balstatcindin + mRNA was fused with a hypothetical protein homolog to human mitochondrial carrier CGI-69 (Loc68066) cDNA. The insertion is between exon 1 and exon 2. (F) Balstatcindin + mRNA was fused with hypoxia-inducible factor 1-α subunit (Hif1-α) cDNA. The insertion is between exon 1 and exon 2.

mutations in 10% of genes in each cell of a relatively small population. However, it is not intuitively clear that such a high rate of mutation can be achieved.

To examine whether multiple rounds of mutagenesis can create cells with single allele mutations affecting 10% of all genes, we used a male mouse-derived monocytic cell line RAW 264.7 cells. The karyotype of RAW 264.7 cells was verified to be diploid in nature based on chromosome-spreading and counting analysis (data not shown). RAW 264.7 cells were exposed to several rounds of 0.35 mg/mL ENU or 1.5 μM ICR-191 or both ENU and ICR-191. O6-BG (10 μM) pretreatment was included to increase the mutation frequency of ENU (27). We tested for loss-of-function mutations at the hemizygous (X-linked) Hprt locus by growing cells in 10 μM 6-TG containing medium after each round of mutagenesis.

The deleterious mutation frequency at the Hprt locus reached 10⁻¹ after 6–8 rounds of exposure to ENU (Figure 1A). ICR-191-induced loss-of-function mutations of Hprt were, as previously reported (28), less frequent than loss-of-function mutations induced by ENU. The combination of the two mutagens produced no significant additive effect in RAW 264.7 cells (Figure 1A). Notably, the mutagenic efficiency was increased with repeated cycles of mutagenesis, which was peaked at the fifth cycle. This phenomenon may reflect progressive mutational degrada-
dation of repair systems (e.g., defects in DNA mismatch repair proteins) that oppose mutational pressure. The asymptotic decline in mutagenic efficiency observed after the fifth cycle presumably reflects a limitation in the amount of mutations that the cell can tolerate since we observed less cell recovery in the later cycles.

Approximately 1 month was required to complete eight rounds of mutagenesis. While some parts of the genome may be more resistant to chemical mutagenesis than others and functional heterozygosity is known to be forbidden at some loci (29) and while compound homozygosity for null mutations may have also been achieved at some loci (10,30), these should only take a very small percentage of whole genome. Therefore, based on the loss-of-
function mutations of Hprt, we consider that approximately 12% of the genome was rendered functionally haploid by repeated cycles of exposure to ENU (Figure 1A). However, the tolerance can be attributed to loss- or gain-of-function of genes other than Hprt. In addition, a month of continuous culture with repeated ENU treatments can provide ample opportunity for genetic drift, a fortuitous progressive overgrowth of a few clones marked by the resistance mutation. In this case, the saturated ENU mutation rate will be lower than we expected, and larger numbers of
cells are required to be pooled to reach a quasi-haploid background.

**Selecting the Mutated Cells for the Pool of Quasi-Haploid Cells**

The quasi-haploid background needs to be created as the starting point for insertional mutagenesis, in the expectation that most genes will be susceptible to functional knockout only by a single insertional event. In order to achieve this, the cells that constitute the pool of quasi-haploid cells should be a set of phenotypically normal clones.

In the present example, the phenotype to be identified is sensitivity to LeTx-induced cell death. Accordingly, a polyclonal population of cells with residual sensitivity to lethal toxin-induced death needs to be culled from the starting population. Seventy-two individual clones were isolated at random from the pool of cells that survived eight rounds of ENU and ICR-191 treatment. Each of these clones was tested for susceptibility to lethal toxin-induced cell death in comparison with parental wild-type RAW 264.7 cells. About 50 clones showed sensitivity to lethal toxin equal to that of the wild-type RAW 264.7 cells. The remaining clones had some alteration in their sensitivity to lethal toxin-induced cell death (Figure 1B). Forty-two lethal toxin-sensitive clones were then selected for further studies. Among them, 49 clones were exposed to lethal toxin to confirm their ability to achieve a quasi-haploid background.

**Phenotype-Based Screen of Retroviral Insertion-Induced Mutations in Quasi-Haploid Cells**

Retroviral insertion can abolish gene expression or produce a truncated gene product depending upon the site of insertion. Truncation of the gene product might yield unpredictable results, since some truncated proteins might display dominant properties, causing either a gain- or loss-of-function. In order to produce gene deletion and avoid gene truncation, we incorporated a self-cleavage ribozyme sequence into the retroviral vector pDisrup 8 (Figure 2A). The sequence of the self-cleavage ribozyme in the vector was shown in Figure 2B. Any transcript containing this ribozyme will be destroyed, because the mRNA will be self-cleaved and subsequently degraded. Previous studies have shown that this type of self-cleaving ribozyme results in a complete cleavage of RNA (31,32), and in our experience, all of the transcript incorporating the ribozyme encoding sequence, generated by a cytomegalovirus (CMV) promoter, was destroyed (data not shown). In order to select the cells that have viral insertion in gene area and to quickly find the insertion site, we adopted a poly(A) trapping strategy (33). Poly(A) trapping was selected over promoter trapping of all possible retroviral insertion sites, because the mRNA transcript initiated by the promoter of a disrupted gene. Eighteen blasticidin-resistant clones, resulting from viral infection, were analyzed by Northern blot analysis. Only one blasticidin-resistant transcript with different sizes was found in each of the different clones (data not shown), indicating that only one gene disruption was occurred in most, if not all, of the blasticidin-resistant clones. This is consistent with a report that retroviral insertion numbers can be controlled at one per cell (36).

We infected 10^7 quasi-haploid cells with the retroviral vector described above and obtained approximately 9 \times 10^3 blasticidin-resistant clones. The 9 \times 10^3 blasticidin-resistant clones were pooled and plated at about 10,000 cells in a 15-cm plate in the presence of lethal toxin. After 6–7 doubling, 98 surviving clones were isolated and re-exposed to lethal toxin to confirm their lethal toxin resistance. Among them, 49 clones which retained lethal toxin resistance were selected for further studies.

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**Figure 4. Analysis of the other allele of Rbp-jκ, Pscd4, Atp6v0c in their corresponding deficient cells.**

(A) Reverse transcription PCR (RT-PCR) was performed using a primer pairing with exon 1 sequence of Rbp-jκ and another targeting the sequence around stop codon of Rbp-jκ. The PCR product derived from messenger RNA (mRNA) of wild-type and Rbp-jκ knockout cells were analyzed on agarose gel and sequenced. Arrows and stars show the mutations sites. (B) RT-PCR was performed as in panel A using primers targeting the exon 1 and stop codon region of the Pscd4 gene. The PCR products from wild-type and Pscd4 knockout cells were analyzed on agarose gel and sequenced. Arrows and stars show the mutations sites. (C) RT-PCR was performed as in panel A using primers targeting the exon 1 and stop codon region of the Atp6v0c gene. The PCR products from wild-type and Atp6v0c knockout cells were analyzed on agarose gel and sequenced. Arrows and stars show the mutations sites.
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Laptm5, or Hif1-α of Rbp-j
viral insertion occurred in the genes of the poly(A) trap strategy. The cDNA, showing the effectiveness once, suggesting duplications occurred of them were identified more than different genes were identified. Some Search Tool (BLAST). Twenty-three database using Basic Local Alignment

Figure 3 shows six examples of the junction sequences of the fused cDNA, showing the effectiveness of the poly(A) trap strategy. The viral insertion occurred in the genes of Rbp-jk, cytohesin-4 (Pscd4), subunit c of vacuolar H+-ATPases (Atp6v0c), lysosomal-associated protein transmembrane 5 (Laptm5), hypothetical protein LOC68066, and Hif1-α. Rbp-jk is a transcription factor recognizing a consensus sequence 5′-C(T)GTGGGAA-3′ and regulating viral and cellular gene transcription (37). Pscd4 is a member of cytohesin family, which is a guanine nucleotide exchange factor regulating the ADP-ribosylation factor of small GTPases (38). Atp6v0c is a subunit of a vacuolar proton pump, which is required for intracellular vacuoles acidification (39). Laptm5 is a lysosomal-associated multispanning membrane protein with unknown function (40). LOC68066 is a hypothetical protein homolog to human mitochondrial carrier CGI-69 (41). Hif1-α is a transcription factor that regulates the adaptive response to hypoxia in mammalian cells (42). By analyzing the sequence of fused cDNA, we predicted that splice donor element downstream from the blasticidin+ sequence in pDisrupt 8 spliced to the splice acceptor of exon 2 of the Rbp-jk gene, the splice acceptor of exon 7 of the Psad4 gene, the splice acceptor of exon 2 of the Atp6v0c gene, the splice acceptor of exon 3 of the Laptm5

gene, the splice acceptor of exon 2 of LOC68066 gene, and the splice acceptor of exon 2 of Hif1-α gene, respectively (Figure 3).

Among them, the first three clones (referred as Rbp-jk, Pscd4, and Atp6v0c, respectively) were further examined whether the other alleles of Rbp-jk, Pscd4, and Atp6v0c were mutated in the corresponding cells. To specifically amplify the mRNA transcribed from the allele that was not hit by retrovirus, we amplified the cDNA region covering exon1 to the stop codon of Rbp-jk, Pscd4, and Atp6v0c by RT-PCR. The same size PCR product was obtained from wild-type and Rbp-jk cells (Figure 4A, top panel). The PCR products were recovered from agarose gel and sequenced. Three point mutations were found in the Rbp-jk cDNA amplified from Rbp-jk cell (Figure 4B). A change of E to G resulted from one of the point mutations. Pscd4 mRNA cannot be found in Pscd4 cells (Figure 4C). Reported mutation spectrum showed that ENU cannot only result in amino acid substitution, but can also affect transcription. The undetectable Pscd4 expression suggests that either the transcription of Pscd4 from the other allele was impaired by chemical mutagen or is silent naturally in RAW 264.7 cells. RT-PCR and sequence analysis revealed that Atp6v0c cells had reduced Atp6v0c mRNA (Figure 4D) but no mutation in the coding region of the mRNA, indicating that the mutation occurred only on one allele in this cell, and Atp6v0c is a haploid insufficient gene in lethal toxin-induced cell death.

To confirm the genes identified by our method were indeed responsible for the lethal toxin-resistant phenotype, we used siRNA to knockdown the five genes identified in wild-type RAW 264.7 cells to see whether lethal toxin resistance can be reproduced. As shown in Figure 5, siRNA knockdown of all the six genes rendered resistance to lethal toxin-induced cell death in RAW 264.7 cells with varying degrees. The best protection from LeTx-induced cell death was detected in Atp6v0c knockdown cells. Atp6v0c is involved in the acidification of intracellular vacuoles (43). Since endosome acidifi-
cation is known to be required for lethal toxin function (44), a requirement of Atp6v0c in lethal toxin-induced killing of RAW 264.7 cells is anticipated.

DISCUSSION

To overcome the major impediments to the use of genetic screening in cultured cells, we have successfully developed a strategy using chemical mutagenesis to generate a quasi-haploid cell population that can then be used for insertional mutagenesis to create phenotypes. However, the fact that disruption of a given gene creates a phenotype within a quasi-haploid cell line must be interpreted with some qualifications. In certain instances, the gene of interest will be essential to the process under study in any genetic background (e.g., monogenic phenotypes in this case). In other instances, the phenotype will reflect epistatic interactions between non-allelic mutations induced by the chemical mutagen and the insertional mutation that is recovered. Whether the gene identified is responsible for a monogenic or polygenic phenotype can be determined by knockdown of the gene in wild-type cells using RNAi or another method. Although monogenic phenotype has been the principal object of study to date, polygenic phenotype is undoubtedly more common in nature and better explains the differences that exist between individuals in most populations. In some respects, our method addresses the question: “What mutations are essential for a biological function given the existence of other, unspecified mutations scattered throughout the genome?” A screen for such mutations, if carried to saturation, would reveal all or most of the genes that serve as the substrate for a defined, complex phenotype and the magnitude of the effect wrought by their destruction.

We have successfully obtained approximately $9 \times 10^3$ blasticidin-resistant clones from $10^7$ quasi-haploid cells. The efficiency for generating the blasticidin-resistant clones was estimated to be about 1% of the cells infected with virus (approximately 2% insertion occurred in gene region, and only one orientation of viral insertion in gene region lead to the expression of resistant gene). Since some gene areas are transcribed in both directions and cryptic poly(A) sites could be utilized by the blasticidin transcript, the actual number of genes targeted by the retroviral vector could be an order of magnitude less than the number of apparent blasticidin-resistant clones.

Assuming that the retroviral insertion is random, approximately 2.5%–25% of genes ($9 \times 10^2$–3 out of $3.5 \times 10^4$ total genes) has been mutated once, and approximately 0.15%–1.5% (12% $\times 50\%$) hits should result in double allele mutations. The rate of biallelic mutation using this mutagenesis is superior to that generated by LOH in Bim-deficient ES cells (0.01%–0.1%) (21,22). In order to have all genes being mutated on their both alleles, $2.3 \times 10^{2}–8$ ($3.5 \times 10^4 + 0.15$ to 1.5%) blasticidin-resistant clones are required, which is clearly feasible.

There are still limitations of this method. This approach cannot be used to screen the genes that are essential for cell survival in culture. Multiple rounds of chemical mutagen treatments could also lead to unpredictable induction of polyplody and/or aneuploidy and selection of tolerant cells to chemical mutagen-induced damages, which can obscure the effects of the subsequent insertional mutagenesis. The plasticity of cultured cells is a problem associated with the use of genetic approaches in cultured cells, and this may be compounded by increased genome instability after high-intensity chemical mutagenesis. At present, the most effective way to minimize plasticity in cultured cells is to use the cells that have as few passages number as possible. In principle, the genetic approach described in this report only can be used to investigate aspects of biology that can be recapitulated in a cell culture model. However, the concept used in designing this approach may be extended to germline mutagenesis. A certain level of haploidy can be found in mice with chromosome deletions. Millions of genetic differences between different mouse strains are haploid in the hybrid offspring. Applying insertional mutagenesis in the ES cells from these mice may facilitate phenotype screens in ES cells and may be useful in studies of certain complex phenotypes.

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COMPETING INTERESTS

The authors declare no competing interests.

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