Video Article

Forward Genetic Approach to Uncover Stress Resistance Genes in Mice — A High-throughput Screen in ES Cells

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URL: http://www.jove.com/video/53062
DOI: doi:10.3791/53062

Keywords: Developmental Biology, Issue 105, Stress resistance, longevity, embryonic stem cells, piggyBac, forward genetics, functional genomics, paraquat, mouse.

Date Published: 11/11/2015

Citation: Ludwig, M., Kitzenberg, D., Chick, W.S. Forward Genetic Approach to Uncover Stress Resistance Genes in Mice — A High-throughput Screen in ES Cells. *J. Vis. Exp.* (105), e53062, doi:10.3791/53062 (2015).

Abstract

Phenotype-driven genetic screens in mice is a powerful technique to uncover gene functions, but are often hampered by extremely high costs, which severely limits its potential. We describe here the use of mouse embryonic stem (ES) cells as surrogate cells to screen for a phenotype of interest and subsequently introduce these cells into a host embryo to develop into a living mouse carrying the phenotype. This method provides (1) a cost effective, high-throughput platform for genetic screen in mammalian cells; (2) a rapid way to identify the mutated genes and verify causality; and (3) a short-cut to develop mouse mutants directly from these selected ES cells for whole animal studies. We demonstrated the use of paraquat (PQ) to select resistant mutants and identify mutations that confer oxidative stress resistance. Other stressors or cytotoxic compounds may also be used to screen for resistant mutants to uncover novel genetic determinants of a variety of cellular stress resistance.

Video Link

The video component of this article can be found at http://www.jove.com/video/53062/

Introduction

Longevity has an intimate relationship with stress resistance. In general, long-lived species often demonstrate increased resistance toward multiple stressors, such as hydrogen peroxide, paraquat (PQ), UV, heat, and heavy metals [1,2]. In contrast, increased sensitivity to stress tends to predict shortened life span and/or a more disease-prone phenotype. The anti-oxidant scavenging pathway has long been speculated to play a major role in conferring stress resistance to the animal. However, with few exceptions, studies from a variety of transgenic animals with manipulations in various anti-oxidant enzymes (*e.g.,* SOD) indicate that increasing the level of oxidant scavenging enzymes does not increase life span or health span [3]. These data suggest that the stress resistance trait consistently observed in long-lived animals is mediated by other cellular pathways yet to be uncovered.

We took an unbiased forward genetic approach to identify genes, which upon mutated, could confer a stress resistance phenotype in cultured embryonic stem (ES) cells. ES cells offer two major advantages in this study: (1) sophisticated genetic manipulations are available to modify the genome of ES cells; and (2) any stress resistant ES cells recovered from the screen can be directly used for mouse production, allowing rapid translation into whole animal studies to measure life span and health span.

In this report, we described the use of C9 ES cell line, in which the *Blm* alleles were under control by a tetracycline responsive element. Treatment of doxycycline (dox) transiently turned off the expression of Blm leading to increased incident of sister chromatid exchange. This short-term Blm knock-out allowed for the generation of homozygous mutations within the heterozygote population so that recessive mutations for stress resistance could be captured in the screening process. We also described the use of *piggyBac* (*PB*) transposon as the mutagen to randomly insert a poly-A trap cassette (PB-UPA) to mutate genes in the genome. Cells with disruption of a gene by the poly-A trap became G418 resistant and could be recovered so that a collection of gene-trap mutants (gene-trap library) could be made, and subsequently screened for mutant clones that were stress resistant.

Stress resistant clones recovered from the selection could be characterized rather rapidly by molecular techniques in regard of the number of insertions (qPCR), the site of insertion (splinkerette PCR), the identity of the disrupted gene (BLAST), and its expression level (RT-qPCR). The *PB* insertion could be remobilized by transient expression of mPB transposase in the clone to restore the wild-type DNA sequence and thus test for the loss of stress resistance. These are powerful ways to confirm causality of the mutation, which should be done prior to expensive mouse production. Previous studies showed that cells exposed to stressors lost their pluripotency [4,5]. Thus, in this protocol, the preservation of a replica set of mutant cells, which would not be treated with stressors is critical for successful mouse production.

Our lab has engineered the C9 ES cell line and the PB-UPA vector, both are available to other investigators upon request. The protocol reported here will start by the generation of de novo library of gene-trapped ES cells with PB-UPA (**Figure 1A**), followed by replica plating and stress selection to isolate stress resistant clones (**Figure 1B**). We demonstrated the selection with paraquat, a potent free radical generator inside cells. Virtually, any cytotoxic compound or toxin, for instance, ER stressors (*e.g.,* thapsigargin and tunicamycin), neuronal oxidant (*e.g.,* MPP

+, 6-hydroxy dopamine, and rotenone), heat, and heavy metals (*e.g.,* Cd, Se), could be adapted to the method to select for respective resistant mutants.

Protocol

1. Gene-trapped ES Cell Library Construction Using *piggyBac* Transposon

1. Prepare primary mouse embryonic fibroblasts (PMEF) as feeders for ES cell culture
    1. Thaw one vial of mitomycin C-inactivated PMEF (5.0 x 10^6) in a 37 °C water bath. Transfer the cells into 5 ml of ES cell medium (DMEM containing 15% FBS, 1,000 unit/ml of leukemia inhibitory factors, 100 µM nonessential amino acids, 2 mM glutamine, 55 µM 2-mercaptoethanol, and 25 unit/ml penicillin/streptomycin), and centrifuge at 100 x g for 5 min.
    2. Aspirate the supernatant and resuspend the cells in 30 ml of ES cell medium followed by distributing into two T25 flasks (5 ml each) and two 100-mm plates (10 ml each). Incubate at 37 °C, 5% CO_2 for 24 hr.

2. Culture and expand C9 ES cells
    1. Thaw one vial of C9 ES cells (2.5 x 10^6 cells in 0.5 ml) in a 37 °C water bath and transfer the cells into 5 ml of ES cell medium. Centrifuge at 100 x g for 5 min.
    2. Aspirate the supernatant and resuspend ES cells in 5 ml of ES cell medium followed by plating into the T25 feeder flask. Incubate at 37 °C, 5% CO_2. Replace ES cell medium daily.
    3. After 2 days of culture, the ES cells should become ~80% confluent, and are ready to be passaged. Wash cells with 5 ml of PBS (without Ca^{2+} and Mg^{2+}) and then add 2.5 ml pre-warmed 0.25% trypsin-EDTA. Incubate at 37 °C for 10 min. Add 2.5 ml ES cell medium to stop trypsin; pipette up and down 15 times to break up cell clumps. Centrifuge at 100 x g for 5 min.
    4. Aspirate the supernatant and resuspend cells in 4 ml of ES cell medium. Transfer 1 ml of cells per 100-mm plate containing 9 ml ES cell medium; prepare two 100-mm plates. This is a 1:8 passage. Feed the cells daily with 10 ml ES cell medium.

3. Prepare 96-well feeder plates for the library
    1. As described in 1.1.1, thaw three vials of mitomycin-C inactivated PMEF (each containing 5 x 10^6 cells) and resuspend cells from each vial with 33.3 ml ES cell medium. Combine cells to yield a 100-ml cell suspension.
    2. Plate 100 µl cells per well onto ten 96-well plates. These 96-well feeder plates should be prepared at least one day before transfecting C9 ES cells with *PB* transposon.

4. Electroporation of C9 ES cells with the PB-UPA vector
    1. Trypsinize and count the expanded C9 ES cells using an automated cell counter. Wash each 100-mm plate of C9 ES cells with 10 ml PBS. Add 8 ml pre-warmed 0.25% trypsin-EDTA and incubate at 37 °C for 10 min.
    2. Stop trypsin by adding 2 ml ES cell medium. Transfer the cell suspension to a 15-ml tube and pipette up and down 15 times to break apart cell clumps. Immediately centrifuge at 100 x g for 5 min followed by aspirating the supernatant.
    3. Resuspend cells in 10 ml ES cell medium and count the ES cells using a hemocytometer. Prepare six 15-ml tubes of cell suspension each containing 5 x 10^6 ES cells. Centrifuge at 100 x g for 5 min. Aspirate the supernatant and resuspend each cell pellet in 0.8 ml PBS.
    4. Prepare six electroporation cuvettes (4 mm gap) to which the 0.8 ml cells are transferred. To each cuvette, add 1 µg PB-UPA and 20 µg mPB transposase (mPBase); mix gently by pipetting up and down. Put the cuvettes on ice. Perform electroporation with an electroporator using the exponential setting at 250 V, 500 µF, and infinity ohm.
    NOTE: Typically, the time constant is about 10 to 12 msec for a successful electroporation, which yield about 1,500 to 2,000 G418 resistant transformants per 5 x 10^6 cells.
    5. Retrieve and pool the transfected cells to a T75 flask containing 98 ml ES cell medium. Mix the cells by gentle pipetting and then transfer cells to a reservoir (50 ml). Using a 12-channel pipette, transfer 100 µl of cells per well to the previously prepared 96-well feeder plates (10 in total). Incubate at 37 °C, 5% CO_2.
    6. After 24 hr, feed the cells daily with fresh ES cell medium containing G418 (150 µg/ml) to select for gene-trapped clones.
    NOTE: Typically each well will contain about ten G418 resistant colonies. When these colonies grow to sufficient size (typically 5-7 days), the 96-well plates (the library) will be replicated into 2 sets for storage and selection, respectively. Each plate in the library is designated as 'sub-library', so in this case, there are 10 sub-libraries.

2. Library Replication

1. Replicate the Master plates
    1. 24-48 hr prior to the G418 resistant colonies reaching 50-60% confluency, thaw another three vials of mitomycin C-inactivated PMEF onto ten 96-well plates as feeders (refer to section 1.3). Gelatinize ten 96-well plates by incubating 0.1% gelatin (100 µl/well) at 37 °C for at least 15 – 30 min; O/N incubation is acceptable.
    2. Prepare single cell suspensions in the 96-well plates by trypsinization. Aspirate medium from 96-well plates and wash once with equal volume of PBS (without Ca^{2+}, Mg^{2+}). Add 50 µl of pre-warmed 0.25% Trypsin-EDTA, incubate at 37 °C in 5% CO_2 for 10 min. Stop trypsin with 50 µl of ES cell medium. Break apart the cell clumps by pipetting up and down 15 times with the multichannel pipette.
    3. Transfer 50 µl of trypsinized cell suspension to the corresponding row of the feeder plate (Master) already containing 150 µl ES cell medium and the remaining 50 µl to the corresponding row of the gelatinized plate (replica) already containing 150 µl ES cell medium. Process each row of cells to a Master and replica plate. Incubate the plates (now containing a 200 µl culture volume) at 37 °C, 5% CO_2 O/N.
    NOTE: There are now twenty 96-well plates.

4. The next day feed the cells with 100 µl fresh ES cell medium containing 150 µg/ml G418. Replace the medium daily until the wells are 80-90% confluent.

2. Freeze the Master plates
    1. When the master plates are about 80% confluent, trypsinize the cells as described (refer to section 2.1.2).
    2. Remove the plates and stop trypsin reaction with 50 µl of 2x freezing medium (60% ES cell medium, 20% FBS, 20% DMSO). Pipette up and down 10 times with a multichannel pipette to ensure proper mixing of the freezing medium.
    3. Put the 96-well plates in a tightly sealed styrofoam box and place it in -80 °C freezer for up to four months storage. Four months will allow enough time to complete the stress selection and to identify which wells from the Master plate contain the clones of interest.
    NOTE: Storage at -80 °C longer than 4 months will result in decline of cell viability. One may utilize cryo-tubes in 96-well format that can be stored in the vapor phase of liquid nitrogen for extended period of storage.

3. Prepare DNA plates and sub-library pools from replica plates
    1. 24-48 hr prior to replicating further to DNA plates and sub-library pools, prepare ten 100-mm plates with mitomycin C-inactivated PMEF for the sub-library pool, and ten gelatinized 96-well plates for the DNA plate replicates. Refer to section 1.3 for general procedure.
    2. Trypsinize the cells in the 96-well replica plates (refer to section 2.1.2). During the 10 min trypsinization step, aspirate gelatin from the DNA plates and replace with 150 µl ES cell medium containing G418 (150 µg/ml). Also , prepare a reservoir containing 5 ml of ES cell medium for pooling of the 'sub-libraries' into the 100-mm feeder plate.
    3. Following trypsinization, stop trypsin with 50 µl of ES cell medium one row at a time using a 12-channel pipette. Pipette up and down 10 times to break apart cell clumps.
    4. Transfer 50 µl of the cell suspension to the corresponding row of the DNA plate and transfer the remaining 50 µl to the reservoir containing 5 ml ES cell medium. After completing one full 96-well plate, aspirate the medium of a 100-mm feeder plate to which the pooled cells are transferred. These pooled mutagenized cells are designated as a 'sub-library'.
    5. Repeat for the remaining nine 96-well plates. Feed the 96-well DNA plates and 100-mm pooled 'sub-library' plates daily with ES cell medium containing G418 (150 µg/ml).
    NOTE: There are now ten 96-well 'DNA' plates, and ten 100-mm 'sub-library' plates.

4. Freeze the DNA plates. When the cells in the DNA plate are 80-90% confluent, aspirate the medium, wash the cells twice with 100 µl PBS and store at -20 °C for later genomic DNA isolation [6]. These DNA plates will be screened by PCR to identify which corresponding well in the 'Master' plate contains the clone of interest.

3. Doxycycline-induced Homozygous Mutants

1. Freeze the sub-library pools and passage cells for doxycycline treatment
    1. 24 hr prior to the 100-mm 'sub-library' plates having colonies large enough to yield 70-80% confluency, prepare a set of ten gelatinized 100-mm plates for doxycycline treatment.
    2. Prepare single cell suspensions of each sub-library (refer to sections 1.4.1 and 1.4.2 for details). Transfer 5 x 10^6 cells to the gelatinized 100-mm plate with doxycycline (1 µg/ml); incubate at 37 °C, 5% CO_2. Freeze the remaining non-doxycycline treated sub-library pools at 5 x 10^6 cells per vial.

2. Passage cells in the presence of doxycycline to knock out Blm
    1. Passage the sub-libraries at 1:8 every 2-3 days in the presence of doxycycline (1 µg/ml) for up to two weeks, during which time dox-induced knock-out of Blm will promote loss of heterozygosity in the cell population, allowing for generation of homozygous mutants.

4. Stress Selection and Isolation of Resistant Clones

1. Subject doxycycline-treated cells for stress selection
    1. 24 hr prior to passaging doxycycline treated cells for stress selection, prepare ten gelatinized 150-mm plates, place in a 37 °C, 5% CO_2 humidified incubator.
    2. Prepare a single cell suspension of each sub-library by trypsinization (refer to section 1.4.1 and 1.4.2 for details). Seed 6.6 x 10^6 cells onto each 150-mm plate in 30 ml total culture volume, distribute cells evenly. Incubate cells with stressor (10 µM paraquat or omission of β-mercaptoethanol, in ES cell medium containing 7.5% heat inactivated FBS) for 7 days, after which replace with normal ES cell medium to allow surviving cells to grow into colonies for picking.
    3. Optional: Freeze the left over doxycycline-treated cells at 5.0 x 10^6 cells per vial.

2. Pick stress resistant colonies
    1. After reinstating normal media for a week, observe how many stress resistant colonies are present. Depending on the number of colonies to be picked, prepare enough wells with mitomycin C-inactivated PMEF on 96-well plates one day beforehand accordingly (refer to section 1.1 for details).
    2. On the day of picking, aspirate and replace medium in the 96-well feeder plate with 100 µl fresh ES cell medium, place back into 37 °C, 5% CO_2 humidified incubator. At this time, prepare a U-bottom 96-well plate with 50 µl 0.25% trypsin-EDTA per well. Aspirate medium from the 150-mm stress selection plate and add equal volume of PBS.
    3. Set the micropipette to 2 µl, and 'pick' surviving colonies into the U-bottom trypsin containing wells, picking one colony per well. It is acceptable to let the plate sit at RT until the desired number of colonies has been picked. Then, incubate the U bottom plate at 37 °C, 5% CO_2 for 10 min.
    NOTE: Typically, we take 1 hr to pick 96 colonies.
    4. Stop trypsin reaction one row at a time with 50 µl ES cell medium using a multichannel pipette. Pipette up and down 15 times to obtain a single cell suspension and transfer 100 µl of cells to the corresponding row of the feeder plate already containing 100 µl of ES cell

medium. Complete the transfer for the entire plate. Culture cells O/N in 200 µl. The next morning, aspirate medium and replace with 100 µl ES cell medium.

3. Replicate the 96-well plates
    1. When the wells of the picked colonies are 80-90% confluent, replicate into two matching plates, one for DNA isolation and the other for cells back-up. These plates are gelatinized and do not contain mitomycin C-inactivated PMEF.
    2. Aspirate medium, and wash with 100 µl PBS. Add 50 µl 0.25% trypsin-EDTA to each well, incubate at 37 °C for 10 min. Stop the trypsin with 150 µl ES cell medium, mix by pipetting, and transfer 100 µl of cell suspension to the corresponding row of two 96-well plates. Change medium daily.
    3. When the plates are 80-90% confluent, freeze one set of plates as a 'back-up' (refer to section 2.2 for details). The other set of plates are further expanded as described below.

4. Expanding cells for genomic DNA isolation
    1. Prepare a single cell suspension in the 96-well DNA plate (refer to 2.1.2 for details).
    2. Transfer cell suspension from each individual well of the 96-well plate to the well of a 24-well plate (4 total) using a micropipette. Ensure there is no cross contamination of any cells, as the 'picked' colonies are 'clonal' in origin. Culture cells in 0.5 ml ES cell medium, changing medium daily until wells are 80-90% confluent.
    NOTE: The 24-well plate will allow sufficient yield of genomic DNA for PCR analysis.

5. Identification of the *PB* Insertion Sites and Trapped Genes

1. Genomic DNA isolation from 24-well plate: Lyse cells in the 24-well plate and isolate RNA-free genomic DNA.
2. Perform Splinkerette PCR (SpPCR) to generate sequence fragments flanking the *PB* transposon integration site as described [7,8].
NOTE: SpPCR is a five day process, though hands-on time each day is minimal.

6. Genetic Analysis of the Mutant Clones

1. Locate master well and purify clone of interest.
    1. When the *PB* integration site has been mapped, design a forward primer upstream of the integration site to use with a *PB* reverse primer (*e.g.,* PB3'-1) [8] and screen the DNA plate corresponding to the *same* 'sub-library' from which the stress resistant clone was picked. Expect to find only one positive well across the entire 96-well DNA plate. Thaw this exact well from the 'Master' plate to a 24-well plate on feeders and grow cells until 80-90% confluency.
    NOTE: At this point, this well contains a mixture of gene-trapped clones.
    2. When the 24-well plate is 80-90% confluent, trypsinize cells and plate 1,000 ES cells in a 100-mm feeder plate (ensure single cell suspension). Passage the remaining cells to a T25 flask on feeders for expansion. Freeze the cells from the T25 flask when colonies are 80-90% confluent. Freeze 4 vials per T25 flask as back-ups.
    3. Allow the colonies to grow for 7 days and then pick 96 colonies into a 96-well feeder plate. Refer to section 4.2 for picking procedure. Replicate the plate when ready into a 'DNA' plate and freeze the other as '2nd-master' plate (refer to section 2.2 for freezing).
    4. Allow colonies to reach 80-90% confluency. Screen the DNA plate to find wells containing the purified clone of interest. Expand cells from the 96-well 2nd-master plate to 24-well and subsequently to a T25 flask (all on feeders). Freeze 4 vials per T25 flask.
    NOTE: At this point, the gene-trapped cell line is clonal, has not been exposed to the stressor, and is suitable for mouse production.

2. Determine the number of *PB* insertion: Thaw one vial of the stress resistant cell line and expand the cells in a gelatinized T25 flask. Isolate genomic DNA and check for copy number of the *PB* transposon. This can be achieved by qPCR targeting the neomycin gene.
3. Measure gene expression level: After confirming there is a single *PB* integration event in the cell line of interest, measure the level of expression of the trapped gene by RT-qPCR using specific Taqman probes for each gene.
4. Sequence the mutant mRNA: Generate a cDNA amplicon by PCR using an upstream primer annealing to an exon and a downstream primer annealing to the splice acceptor sequence of the gene trap cassette. Sequence the cDNA fragment to confirm that the expected splicing occurs in the trapped gene.
5. Remobilize the *piggyBac* transposon to test for causal relationship between the trapped gene and stress resistance.
    1. 24-48 hr prior to thawing cells for *PB* remobilization, thaw and plate DR4 feeders on one T25 flask and two 100-mm plates. At least one day after the DR4 feeders are plated onto the T25 flask, thaw one vial of the cell line containing the *PB* insertion and plate the cells in the T25 flask containing DR4 feeders. Grow the cells in ES cell medium for 48 hr, changing medium daily.
    2. Prepare a single cell suspension of the ES cells. Count cells with an automated cell counter, and transfer 5.0 x 10^6 ES cells to a 15-ml tube. Centrifuge at 100 x g, and resuspend the cells in 800 µl PBS. Transfer the suspension to a 4 mm cuvette.
    3. Add 20 µg mPBase^{Puro} to the cuvette, mix by pipetting, and electroporate cells (refer to section 1.4 for details). Distribute the transfected cells onto two 100-mm DR4 feeder plates. Culture cells O/N in ES cell medium.
    4. The next morning, feed the cells with ES medium with 1 µg/ml puromycin and culture cells for 48 hr. Withdraw puromycin selection and continue to culture the cells for 5 days. When the colonies are large enough for picking, pick into a 96-well feeder plate (refer to section 4.2).
    5. When the picked cells have reached 80-90% confluency, split 1/6th of the plate into a DNA plate and 1/6th of the plate into a G418 plate, leaving 2/3^{rds} of the cells in the original plate to be frozen (refer to section 2.2 for freezing).
    6. Culture the DNA plate in ES cell medium for later DNA isolation, and the G418 plate in 150 µg/ml G418 to test for the loss of neomycin resistance. Wells with no viable cells after 72-96 hr in culture containing G418 indicate successful remobilization of the gene-trapping *PB* transposon.
    7. The corresponding wells in the 'DNA' plate will be screened by PCR to confirm the *PB* transposon has been removed. When the DNA plate is 90% confluent, isolate the genomic DNA in 96-well plate format [4] for PCR screening for the restoration of the wild-type sequence and the absence of neo-IRES as a means to confirm reversion.

8. Thaw the matching wells from the Master plate to 24-well plate and expand to a T25 flask (all with feeders). Freeze four vials per T25 flask as back-up.

7. Generation of Mouse Mutants

1. Recover resistant ES cells from the master plates and use them for embryo injection to generate chimera. About 15–20 ES cells are injected into an individual C57BL/6 blastocyst. Transfer 15 blastocysts per mouse to the uterus of a pseudopregnant female (strain ICR). Mate the male chimera (indicated by the ES cells specific agouti coat color) recovered from the litter with several young virgin C57BL/6J females to test for germ-line transmission, and to build up a colony of mutant mice.
2. Isolate and test tail skin fibroblasts from the F1 mutant mice and wild-type littermate for reactive oxygen species (ROS) level and resistance to paraquat[9].

Representative Results

In a typical mutagenesis experiment, the transfection consists of a total of 3 x 10^7 ES cells with PB-UPA and mPB transposase. The numbers of gene-trapped ES cells generated in two independent experiments are summarized in **Table 1**. The efficiency of gene-entrapment is about 0.04%. The combined gene-trap libraries contain 22,400 independent mutants, about a full coverage of the mouse genome (23,000 coding genes). Excessive coverage could be achieved by generating more mutants through repeated mutagenesis and/or scaling up the process.

Because the gene-trap libraries contain random mutations, it is not possible to predict the number of mutants possessing a stress resistance phenotype. Two independent selections for PQ resistance were performed; the combined screening of 22,440 independent gene-trap mutations uncovered 17 mutations that confer cellular resistance to PQ (0.08%) (**Table 1**).

We have purified 3 mutant clones, the Pigl, Tiam1, and Rffl, from the master plates for further analysis and mouse production. Cells with heterozygous Rffl mutation were successfully induced to produce homozygous progeny by knocking out Blm. However, we failed to produce any homozygous mutants from the Pigl and Tiam1 mutants, presumably due to a homozygosity-linked cell lethality. The stress resistance of these purified clones was confirmed by using 2 different types of stressors: omission of 2-mercaptoethanol (2-ME) and PQ (**Figure 2**). Upon removing the *PB* from these clones, the associated stress resistance phenotype was also lost (**Figure 2**), confirming that the gene-trap mutations are the causal factor. Characterization of clones recovered from the master plates is crucial as it would rule out stochastic lesion during stress selection as the cause of the observed phenotype.

Out of 3 mutant ES cells being introduced into blastocysts for mouse production (**Table 2**), 2 lines (Pigl and Tiam1) show germline transmission. The Rffl injection produced only one female chimera which was not an optimal number of chimera to begin with. Thus, the failure of germline transmission of Rffl is likely due to one incompetent chimera. We tested the cellular phenotype of skin fibroblasts isolated from the Pigl mutant mice and showed that they maintained the reduced level of endogenous reactive oxygen species (ROS) as well as stress resistance to PQ (**Figure 3**).

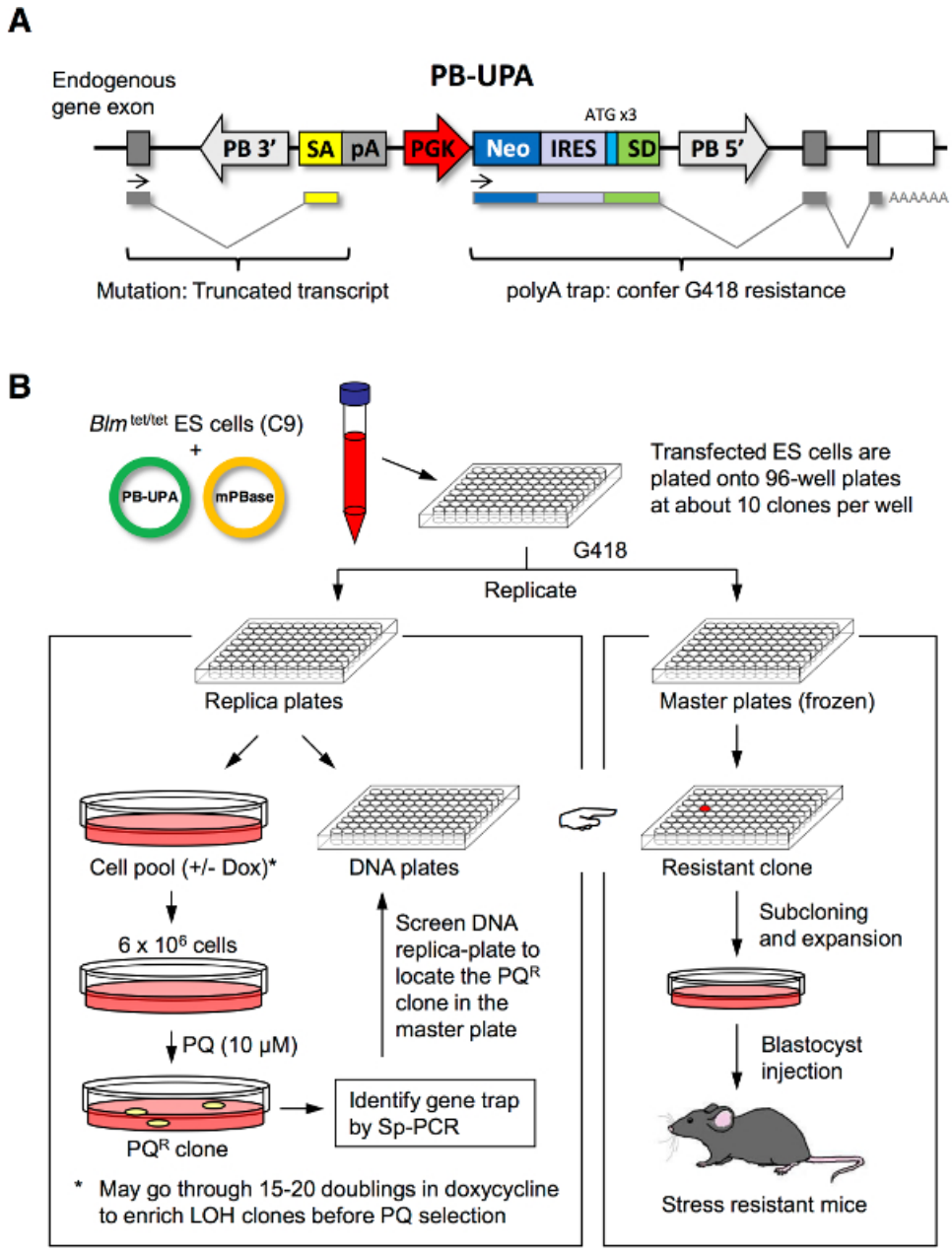

Figure 1. ES cell mutagenesis and stress selection. (**A**) PB-UPA vector. An unbiased polyA (UPA) trap vector comprising of a splice acceptor (SA), bovine growth hormone poly-adenylate signal (pA), phosphoglycerate kinase (PGK) promoter, neomycin phosphotransferase (neo), internal ribosomal entry site (IRES), and a splice donor (SD) with artificial ATG (in 3 different reading frames) was cloned into the *piggyBac* transposon, flanked by the 3' and 5' long terminal repeat (LTR). (**B**) Random mutagenesis of ES cells and selection for stress resistant clones. ES cells were co-transfected by electroporation with PB-UPA and mPBase followed by plating onto 96-well plates. Gene trapped clones were selected by G418 resistance; once confluent, they were divided into 2 replica sets. One replica set was further divided into two half for DNA isolation and stress selection by paraquat (PQ); the other replica set (master) was frozen down. The surviving ES cell colonies recovered from the stress treatment were analyzed molecularly for the *PB* insertion by Sp-PCR. Primers were then designed to screen the replica DNA plate from which the well containing the sibling PQ^R clone could be located on the master plate. These cells are for stress resistance assay on different stressors and for mouse production. Modified from Chick *et al.* [7]. Please click here to view a larger version of this figure.

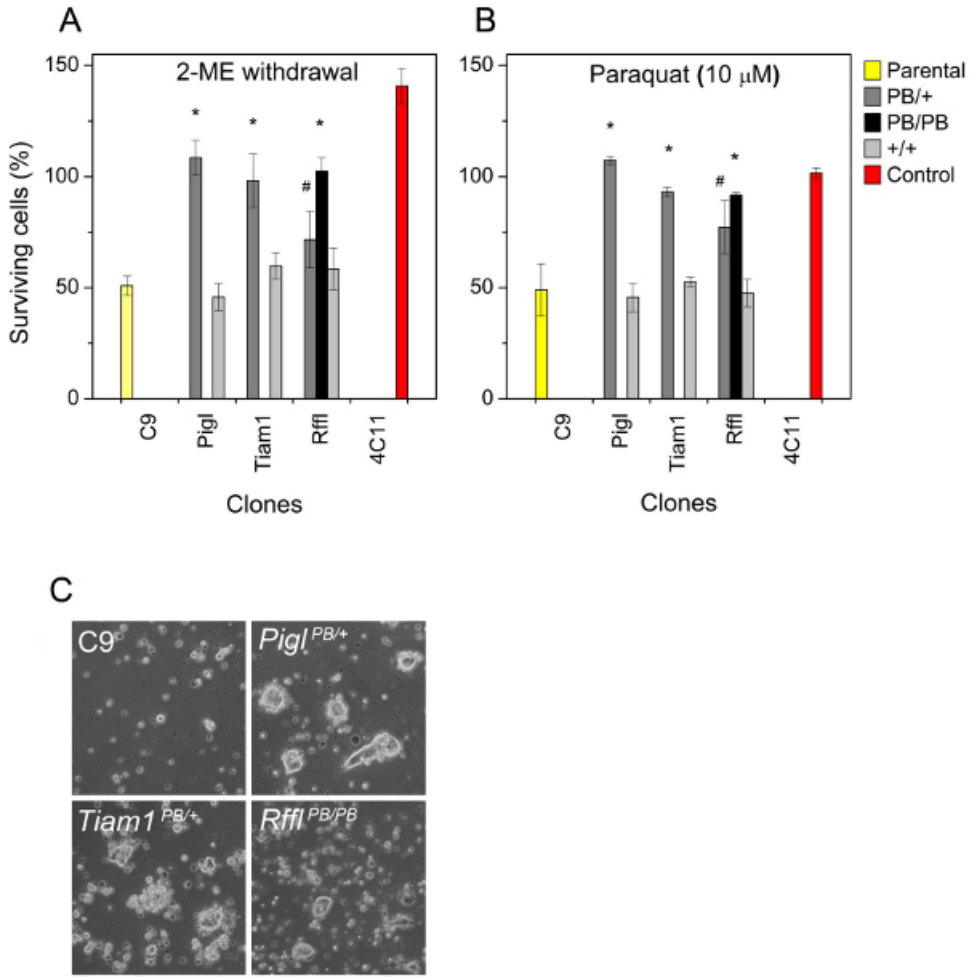

Figure 2. Stress resistance in mutant ES cells. (**A**) Resistance to 2-mercaptoethanol (2-ME) withdrawal. (**B**) Resistance to paraquat (PQ). Shown are the parental wild-type ES cells (C9: yellow), a stress-resistant control ES cell clone, (4C11: red), recovered from a previous study [5], and three gene-trap clones (grey). Cells were subjected to stress treatment for two days, after which the number of viable cells was counted. *Pigl*, *Tiam1,* and *Rffl* heterozygotes (PB/+: dark grey) exhibit resistance to both of these stressors. *Rffl* homozygotes (PB/PB: black) exhibit stronger stress resistance compared to the heterozygotes. Resistance to both stressors was lost when the *PB* insertions were removed (+/+: light grey). Error bars represent SD of mean (n=4). * $P < 0.001$, # $P = 0.01$ between the gene-trap clones and the wild-type parental C9, evaluated by Student's *t*-test. (**C**) ES cell colony formation under PQ (10 μM) treatment. Stress-resistant ES clones were able to form colonies in culture under PQ treatment while the number and size of colonies from the wild-type clone were significantly reduced. Modified from Chick *et al.* [7]. Please click here to view a larger version of this figure.

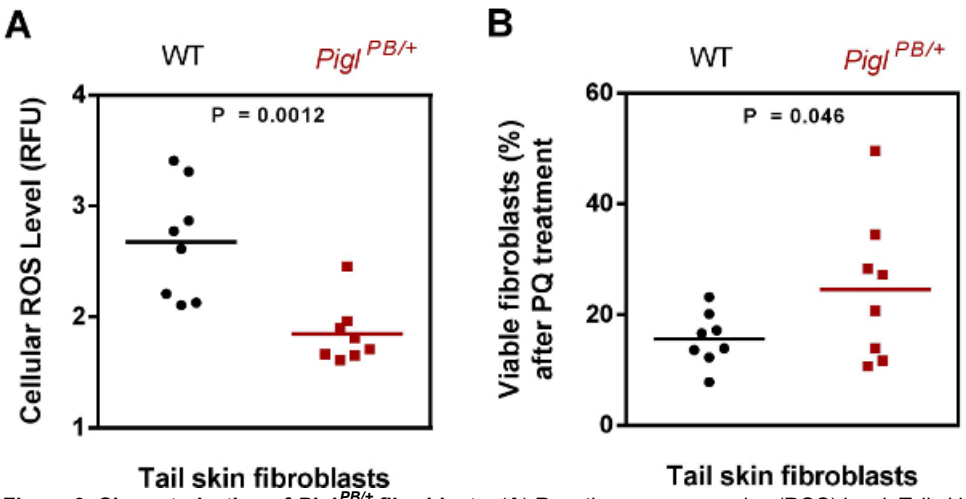

Figure 3. Characterization of *Pigl*$^{PB/+}$ fibroblasts. (**A**) Reactive oxygen species (ROS) level. Tail skin fibroblasts isolated from wild-type (WT) and *Pigl*$^{PB/+}$ mice were stained with CM-DCFCA and the fluorescence was normalized against Hoechst. The ROS content was expressed as relative fluorescence unit (RFU). The P value was evaluated by two tailed Student's *t*-test (n=8). (**B**) PQ resistance. Tail skin fibroblasts from wild-type (WT) and *Pigl*$^{PB/+}$ mice were exposed to 4 mM PQ for 6 hr, after which the viability of the cells were measured by MTT assays. The percentage of live cells after PQ treatment was calculated by the ratio of the absorbance obtained from the PQ-treated cells and that from the un-treated cells. The P value was evaluated by one tailed Student's *t*-test (n=8). Modified from Chick *et al.* [7]. Please click here to view a larger version of this figure.

Library	Sub-libraries	ES cell strain	No. of gene trapped clone	No. of PQ^{R} clones
1	C9PA01 – C9PA10	C9 (*Blm*$^{tet/tet}$)	9,000	7
2	C9PA11 – C9PA20	C9 (*Blm*$^{tet/tet}$)	13,440	10
		Total	**22,440**	**17**

Table 1. *PiggyBac* gene-trap library construction and the recovery of PQ resistant clones. Modified from Chick *et al.* [7].

ES cell clone injected	No. of chimera recovered	Germline transmission *
Pigl^{PB}/ *Pigl*$^{+}$	4	Yes
Tiam1^{PB} / *Tiam1*$^{+}$	2	Yes
Rffl^{PB} / *Rffl*$^{+}$	1	No

Table 2. Generation of mice. Modified from Chick *et al.* [7].

* Germline transmission was confirmed by inheritance of the gene-trap allele detected by PCR genotyping.

Discussion

Forward genetic analysis allows for an unbiased interrogation of the genome for genes responsible for a specific phenotype. This method is very powerful to uncover novel gene functions. It has been widely used in lower organisms but not in mammal, such as the mouse, mainly due to the extremely high cost associated with the infrastructure and logistics that would entail. Here, we moved the genetic screening process to the ES cell culture platform, greatly increasing the efficiency and throughput in generating mutants and identifying mutations.

To effectively select for resistant mutants, a lethal dose of stressor and period of treatment needs to be established such that all of the wild-type cells would be killed. The higher the stringency of the selection, the fewer occurrence of false positive will result. Fibroblast feeders have a higher tolerance to oxidative stress than ES cells; the presence of feeders would rescue the nearby ES cells which would otherwise be killed by the stressor. Thus, omission of feeders during stress selection was very critical for an effective killing. In skin fibroblasts, serum deprivation was necessary to reveal the stress resistance phenotype in long lived mice [10]. However, this application was not feasible in ES cells because they cannot survive without serum. As an alternative, we discovered that the use of heat inactivated serum at reduced level (7.5%) achieved a similar result.

In this report, we employed poly-A trap rather than a promoter trap because promoter trap will only trap actively transcribed genes, which is estimated to be 60% of the genome in ES cells, under normal conditions [11]. In contrast, a poly-A trap would trap genes regardless of their transcriptional activities, in theory giving us the potential to screen for mutations in all genes in the genome. A major drawback of the

conventional poly-A trap is the strong bias towards trapping the last exon, appeared to be mediated by an endogenous nonsense-mediated decay (NMD) mechanism [12]. By incorporating the unbiased poly-A trap (UPA) strategy in the PB-UPA transposon to suppresses NMD [13], we were able to eliminate biased trapping.

One distinguishing advantage of using ES cells is that these pluripotent cells make direct derivation of mice possible, speeding up the transition to *in vivo* studies. For this to happen, the replica plating strategy is critical, preserving a subset of clones sheltered from manipulations that can diminish pluripotency, such as knocking out of Blm and exposure to stressors. We have shown that mutant ES cells recovered from these screens were capable of participating in forming the entire mouse and transmitting the mutations to the germline. Most importantly, consistent with previous studies, the stress resistance phenotype was also transmitted and maintained in the adult mouse.

We and others showed that a variant phenotype observed in ES cells resulting from environmental induced lesion or deliberated engineered mutations was able to transmit to the differentiated progeny cells [4,5,9,14], and probably the whole mouse. This protocol describes the utilization of ES cells as surrogate cells to screen for stress resistance mutations in the mouse. In addition to screening for stress resistance genes, this method may have a broader implication to screen ES cells, identify targets, and to develop a variety of mouse models with a phenotype of interest.

Disclosures

The authors have nothing to disclose.

Acknowledgements

We would like to thank the Wellcome Trust Sanger Institute for the gifts of *piggyBac* transposon and *piggyBac* transposase. This work was supported by the Butcher grant of Colorado and the NIH R01 AG041801 (W.S.C).

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