3.1 Introduction

3.1.1 Screen for 6TG resistance mutants

The cytotoxity of 6TG and the simple methylation drug MNNG requires a functional MMR system (see Chapter1). The cytotoxic mechanism of 6TG is initiated in cells by Hprt (hypoxanthine-guanine phosphoribosyltransferase), which converts 6TG to 2'-deoxy-6-thioguanosine triphosphate. 2'-Deoxy-6-thioguanosine triphosphate can be incorporated into DNA as a guanine analogue during DNA synthesis. 6TG in DNA is methylated by cellular SAM (S-adenosylmethionine) to form S^6-methylthioguanine (S^6-mG). S^6-mG pairs with thymidine during DNA replication, forming a S^6-mG/T mismatched basepair (Swann et al., 1996). The binding of the MutSα to S^6-mG/T initiates a futile mismatch repair process and signals a G2/M cell cycle arrest and apoptosis process (Waters and Swann, 1997, Karran and Bignami, 1994)(Fig.1-4). Deficiency in four mismatch repair proteins, Msh2, Msh6, Mlh1 and Pms2, lead to a 6TG resistance phenotype in cultured cells because the mismatched S^6-mG/T is not recognized by the MMR machinery (Branch et al., 1993, de Wind et al., 1995, Abuin et al., 2000, Buermeyer et al., 1999). Based on mechanism of 6TG cytotoxity, it is conceivable that cells could mutate to 6TG resistance by alterations in 6TG transport, metabolism, mismatch repair recognition, cell cycle arrest and apoptosis. Therefore, genes involved in these pathways or processes could be phenotypically identified in a genetic screen for 6TG resistance.

3.1.2 MMR system and 6TG tolerance in ES cells

Mouse ES cells express all the known mismatch repair genes and mouse models with mutations in the mismatch repair recognition proteins have been generated through gene-targeting (Wei et al., 2002). Abuin et al (2000) generated *Msh2* deficient, *Msh3* deficient and *Msh2/Msh3* double null ES cells by

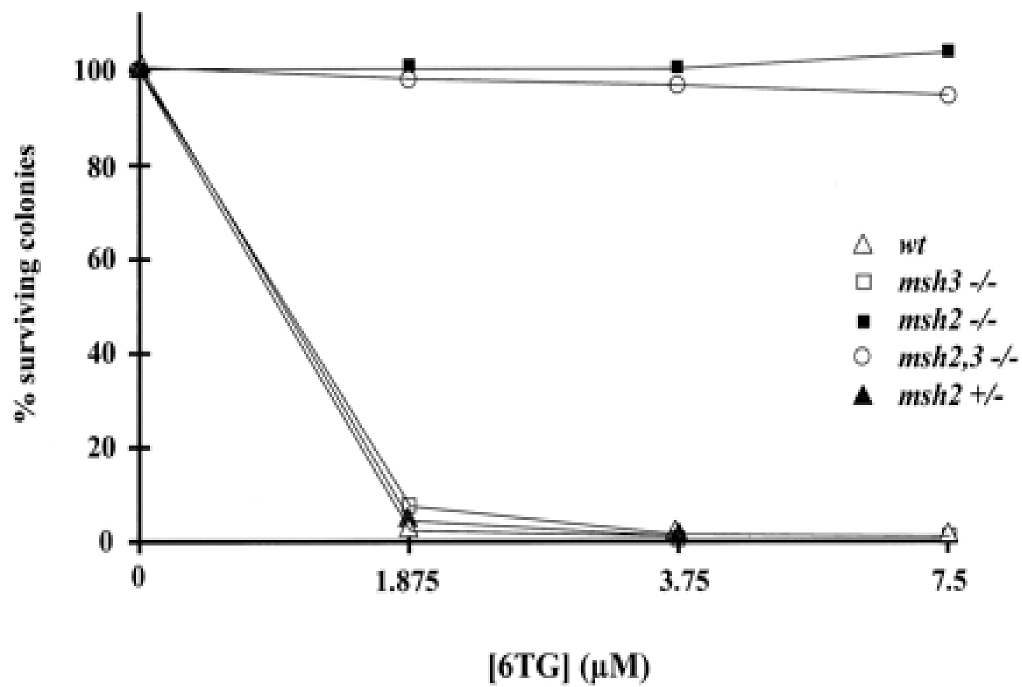


Figure 3-1. Cytotoxicity of 6TG in mouse ES cell lines.

Clonal survival of wild-type and MMR mutant ES cells in the presence of various concentrations of 6TG. Figure is taken from (Abuin et al., 2000).

sequential gene-targeting, and examined the survival of these ES cells in 6TG at various concentrations. A kill curve of 6TG on ES cells was first established (Fig. 3-1). This study revealed that *Msh2* deficient and *Msh2/Msh3* double null ES cells are able to grow in 6TG at concentrations that will kill the wild type ES cells. Another finding of this study was that ES cells with *Msh3* deficiency were not resistant to 6TG. This result is consistent with the role of Msh3 in MMR, in which Msh3 forms a protein complex with Msh2 and binds preferentially 2-4 base pair insertion/deletion lesions (Modrich, 1991). The single nucleotide mismatch (S^6-mG/T) formed by 6TG is, however, recognized by Mutα complex composed of both Msh2 and Msh6 (Waters and Swann, 1997). The effect of Msh6 and the MutL homologues, Mlh1and Pms2 on 6TG cytotoxity has not been examined directly in ES cells. Since the MMR system is highly conserved, it would be expected that mutations in these genes would confer a 6TG resistance phenotype in mouse ES cells since they do in human cells (Karran and Bignami, 1994)

3.1.3 6TG resistance caused by deficiency in *Hprt* gene

Hprt plays a central role in the mechanism of 6TG cytotoxity. It is well established that cells which have lost the function of *Hprt* gene can survive selection in 6TG. The endogenous *Hprt* gene is a single copy gene located on X-chromosome and is thus present in one copy (XY cells) or expressed from just one allele (XX cells). The mono-allelic nature of *Hprt* expression is convenient for measuring mutation rates and types since the recessive *Hprt* mutants could be selected out by 6TG (Chen et al., 2000, Munroe et al., 2000).

Despite the overall similarity of the 6TG resistant phenotype, MMR mutants and *Hprt* mutants can be distinguished in several ways. First, MMR mutants are resistant to HAT. HAT is a mixture of sodium hypoxanthine, aminopterin and thymidine. Aminopterin is a potent folic acid antagonist, which inhibits

dihydrofolate reductase blocking de novo nucleoside synthesis. Cells can only survive in HAT if the purine and pyramidine savage pathways are active. Hypoxanthine is the substrate for purine savage pathway. Thus, *Hprt* mutants are unable to utilize the purine salvage pathway and are sensitive to HAT selection, whereas MMR mutants, containing a functional *Hprt* gene, are resistant to both HAT and 6TG. Secondly, the 6TG resistance phenotype of *Hprt* mutants is affected by the genotype of the neighboring cells. It has been established that *Hprt* mutants will be killed by toxic metabolic intermediates produced by the neighboring *Hprt* positive cells, which is known as cross killing or metabolic cooperation effect. Therefore, to select *Hprt* mutants in 6TG, cells have to be plated at very low density to avoid cross killing by wild type cells (Hooper and Slack, 1977). However, MMR mutants can be selected in 6TG at high cell densities, in which *Hprt* mutants would not survive. Thirdly, It must be emphasized that the 6TG resistance phenotype exhibited by MMR deficient cells is a result of lack of recognition of the S^6-mG/T mismatch. Thus, MMR mutants will possess many mismatched S^6-mG/T nucleotides following 6TG treatment, which is likely to be mutagenic. In contrast to MMR mutants, cells without Hprt activity do not metabolize 6TG and it is not incorporated into the genome. Thus, *Hprt* deficient ES cells can grow normally in 6TG at very high concentrations. 10 μM of 6TG is routinely used to select for *Hprt* mutants. However, MMR mutants exhibit dose dependent selection (Fig. 3-1). Characteristically, *Msh2* mutants grow slower and the plating efficiency is decreased in 6TG with a concentration as low as 2 μM.

3.1.4 EMS mutagenesis in ES cells

EMS (ethyl methanosulfonate) is a monofunctional alkylation agent that ethylates DNA principally at nitrogen positions (mostly the N^7 position of guanine) as well as oxygens such as the O^6 of guanine. EMS is mutagenic in a wide variety of genetic systems from viruses to mammals (Sega, 1984). The mutagenic aspects of EMS in mouse ES cells have been investigated on the endogenous X-linked

Hprt gene. The loss of function mutations in the *Hprt* gene induced by EMS can be selected in 6TG. EMS induces predominantly point mutations, mostly G to A transitions. C to T transitions also occurs but in fewer cases. EMS is a very efficient mutagen, causing null mutations at frequencies as high as 1 mutation per locus per 1,200 treated ES cells in the 129/SvJae background with an EMS dosage of 500 μg/ml (Munroe et al., 2000).

3.1.5 *Blm* deficient ES cells in 6TG resistance screen

The rational for using *Blm* deficient ES cells to generate homozygous mutations has been discussed in Chapter 1. *Blm* deficient ES cells exhibit a high SCE and an elevated a LOH rate (Fig. 3-2 a & b). In the *Blm* deficient ES cells, *Blm* (m1/m3), the LOH rate is about $4.2x10^{-4}$(cell/ generation/ locus)(Luo et al., 2000). In practical terms, a single *Blm* deficient ES cell with an autosomal single allele mutation will have segregated several daughter cells with bi-allelic mutations by the time a single cell has expanded to an ES cell colony containing 2,000 to 5,000 cells, which requires about 14 cell population times. Therefore, It is possible to mutate *Blm* deficient ES cells, generating thousands of independent single allele mutations in different genes, and from these cells derive daughters with homozygous mutations.

The Blm deficient ES cell line used in this screen is derived from the double targeted *Blm*(m1/m3) cells that contain the Blm^{tm1Brd} and Blm^{tm3Brd} alleles (Luo et al., 2000). The Blm^{tm1Brd} allele was generated by replacement gene-targeting, in which the first coding exon, exon 2, was replace by the *loxP*-flanked PGK-*neo* cassette, resulting in a truncated message RNA lacking the initiation ATG codon (Fig. 3-3a). Blm^{tm3Brd} was derived from a complex insertional targeting event that leads to the production of an aberrant transcript with an extra copy of exon3, which causes a frameshift mutation (Fig. 3-3 a) (Luo et al., 2000). *Blm*(m1/m3) cells are *Hprt* deficient because they were established in AB2.2 ES cell, an *Hprt* deficient cell line that carries the $Hprt^{mb2}$ allele (Kuehn et al., 1987). Therefore,

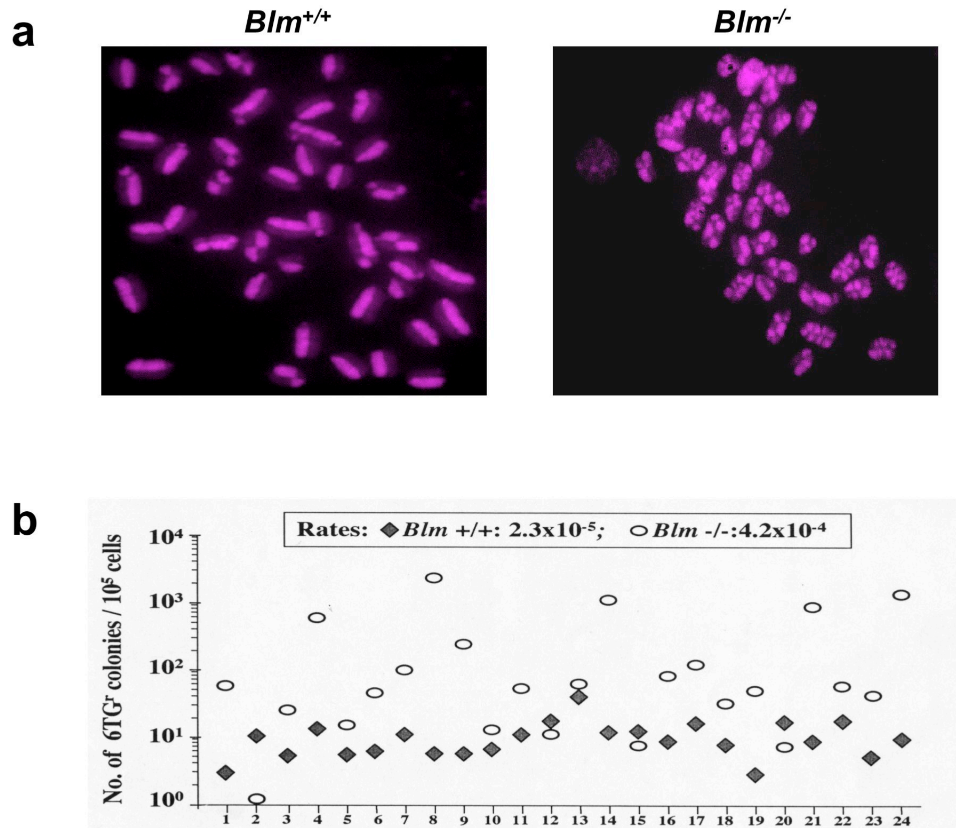


Figure 3-2. Genomic instability in *Blm* deficiency ES cells.

a. Elevated sister chromatin exchange in *Blm* deficiency ES cells illustrated by BrdU staining**. b.** Increased LOH rate in *Blm* deficiency ES cells tested by examing the loss of the single allele gene targeted *PGK-hprt* minigene. The horizontal axis shows 24 individual clones examined. The vertical axis is a log scale for the number of 6TG resistant clones per 10^5 cell. The LOH rate was calculated by Luria-Delbruck fluctuation analysis, method of means.

Figure adapted from (Luo et al., 2000)

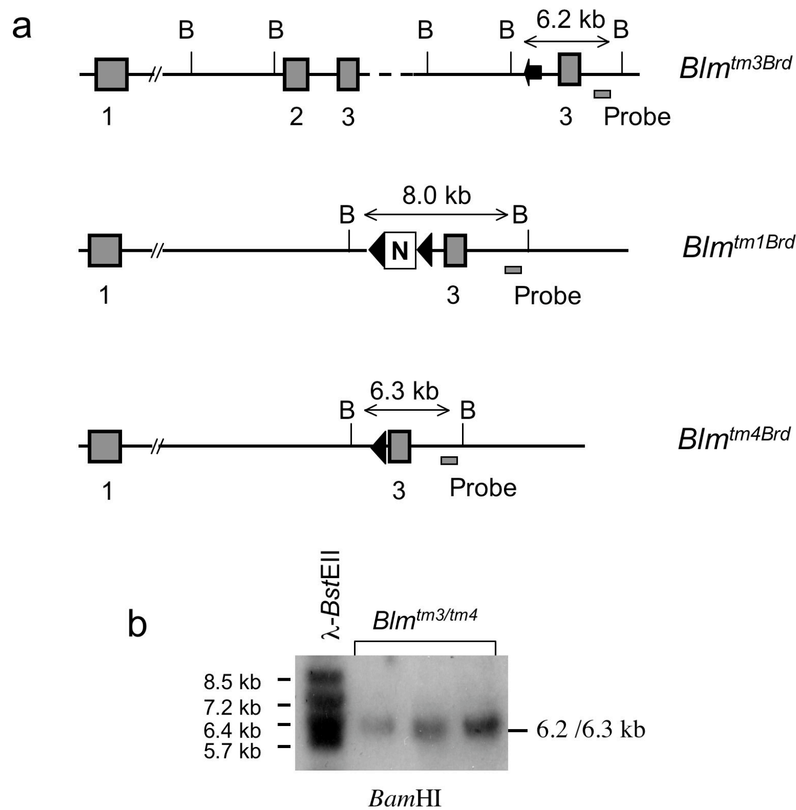


Figure 3-3. Gene-targeted *Blm* alleles

a. Schematic illustration of gene-targeted *Blm* alleles. *Blm*^{tm1Brd3}, possessing an extra exon 3, was derived from an insertional gene-targeting event. *Blm*^{tm1Brd} was generated by replacement gene-targeting, resulting in deletion of exon 2 (Luo et al., 2000). *Blm*^{tm4Brd} allele was derived from *Blm*^{tm1Brd}, in which the *loxP*-flanked *PGK-neo* cassette was removed. **b.** Southern-blot analysis of *Blm*$^{tm3/tm4}$ cells using a *Blm* probe, showing three *Blm*$^{tm3/tm4}$ cell lines that possess *Blm*^{tm3Brd} and *Blm*^{tm4Brd} alleles as indicated by 6.2 kb/6.3 kb *Bam*HI fragments. B, *Bam*HI; N, *PGK-neo* cassette; Black arrow, *loxP* site. Genomic DNA for Southern-blot analysis was digested with *Bam*HI restriction enzyme.

Blm(m1/m3) cells are 6TG resistant. To perform a screen for mutations that cause 6TG resistance in *Blm*(m1/m3) based ES cells, Hprt activity has to be provided. In this chapter, a gene-targeting strategy was adopted to introduce *Hprt* minigenes into both alleles of an autosomal gene in *Blm* deficient cells. Targeting two copies of the *Hprt* minigene also helps to reduce the background of 6TG resistant clones caused by loss of function of the targeted *Hprt* genes by spontaneous mutation or LOH events. EMS mutagenesis was performed in these *Hprt* positive and *Blm* deficient cells to verify that a 6TG-resistant screen would be successful.

3.2 Results

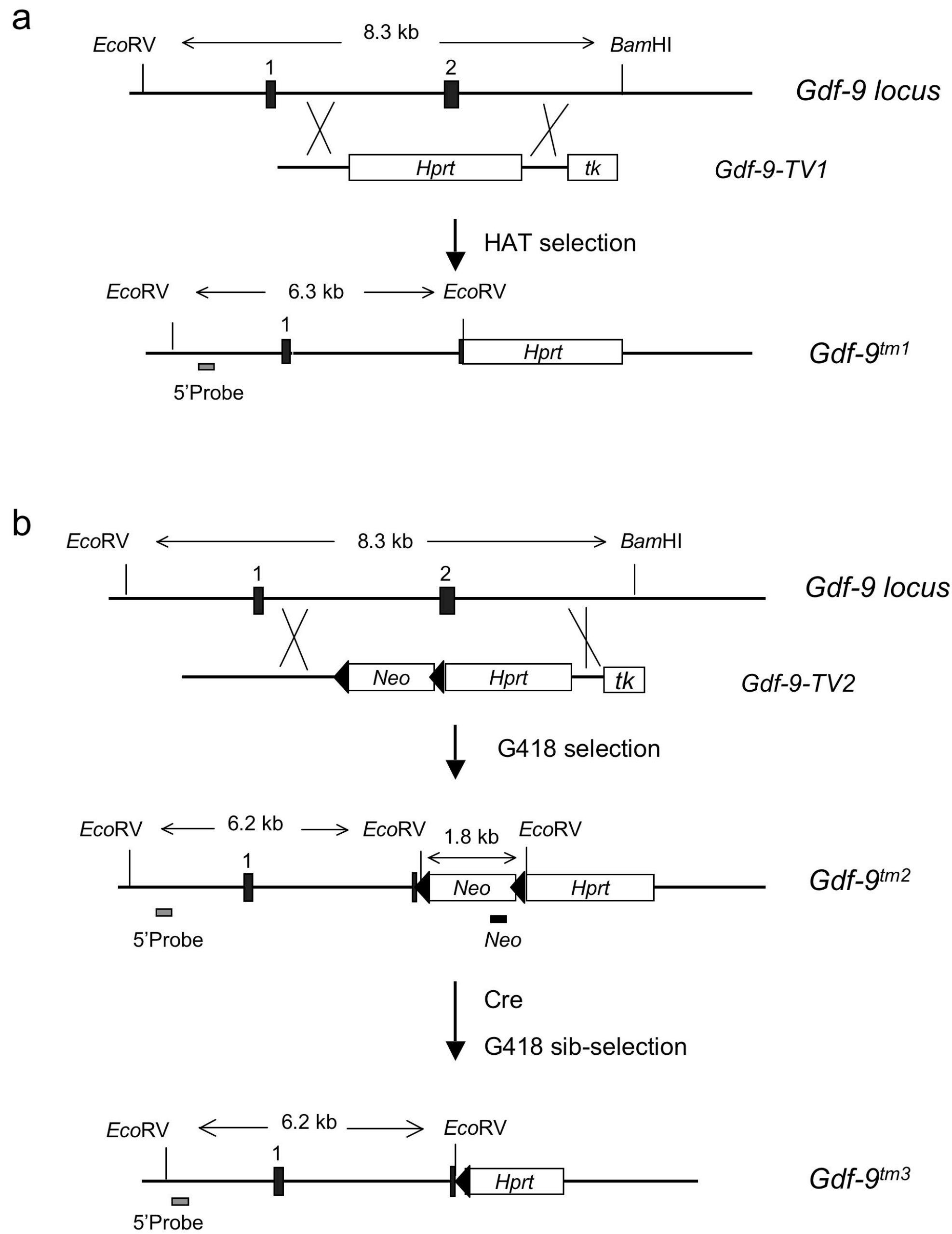
3.2.1 Construction of the *Blm* deficient ES cells carrying two copies of an *Hprt* minigene

Blm (m1/m3) ES cells contain Blm^{tm1Brd} and Blm^{tm3Brd} alleles (Fig. 3-3 a). The Blm^{tm1Brd} allele contains the *loxP*-flanked PGK-*neo* selection marker (Luo et al., 2000). To enable recycling of the *neo* selection marker (Abuin and Bradley, 1996) in subsequent aspects of our experiments, the PGK-*neo* cassette was removed by Cre-*loxP* mediated recombination from *Blm* (m1/m3) cells to generate the marker-free *Blm* deficient cells, *Blm* m3/m4) cell line. Southern-blot analysis using a 3' *Blm* external probe revealed the predicted 6.2 and 6.3 kb *Bam*HI fragments in *Blm* (m3/m4) cells (Fig. 3-3 a & b). Note that the 6.2 kb and 6.3 kb fragments can not be distinguished in the Southern-blot because of the similar size, therefore, they appear as one wild band (Fig. 3-3 a & b).

To provide the Hprt activity, PGK-*Hprt* minigenes were introduced sequentially into both autosomal *Gdf*-9 (*growth differentiation factor 9*) loci by gene-targeting technology. The *Gdf-9* locus was chosen because a *Gdf*-9 gene-targeting vector was available that contains the PGK-*Hprt* minigene and this exhibited high gene-targeting efficiency. Importantly, *Gdf*-9 is only required for sex development in

female mice and *Gdf*-9 deficiency doesn't have adverse effects on ES cell growth (Dong et al., 1996). The first *PGK-Hpr*t minigene was introduced using a previously described *Gdf*-9 targeting vector (Dong et al., 1996). This *Gdf*-9 targeting vector contains a PGK-*Hprt* cassette as the selection marker for gene-targeting and a MCI-*TK* marker for negative selection. The *Gdf*-9 targeting vector was linearized by *Pvu*I and electroporated into *Blm*(m3/m4*)* cells. HAT and FIAU double selection was applied. ES cells clones with targeted *Gdf*-9 alleles were identified by genomic Southern-blot using a 5' external *Gdf*-9 probe. For Southern-blot analysis the genomic DNA from HAT and FIAU resistant ES cells was digested with *Bam*HI and *Eco*RV. The 5' external probe recognizes a 8.0 kb *Bam*HI*/Eco*RV wild type fragment and a 6.3 kb *Eco*RV fragment for the targeted allele ($Gdf9^{tm1}$) (Fig. 3-4 a). The targeting efficiency was 50%. The targeted cell line, $Blm^{m3/m4}$/ $Gdf9^{tm1/+}$, was expanded from a 96 well tissue culture plate to 90 mm tissue culture plate for targeting of the second *Gdf*-9 allele.

The high LOH rate in *Blm* deficient cells increases the probability of losing the single targeted *Hprt* minigene via mitotic recombination. Thus, it is important to generate cells with the PGK-*Hprt* minigene targeted to both alleles. To introduce the second PGK-*Hprt* minigene into *Gdf-9* locus, the *Gdf9-TV2* gene-targeting vector was generated, in which a *loxP*-flanked PGK-*neo* cassette was inserted in front of the PGK-*Hprt* minigene as the selection marker for gene-targeting. *Gdf*9-*TV2* was linearized with *Pvu*I and electroporated into $Blm^{tm3/tm4}$ / $Gdf\text{-}9^{tm1/+}$ cells, followed by G418 selection. The second targeted allele ($Gdf\text{-}9^{tm2}$) was identified as a 6.2 kb *Eco*RV fragment by genomic Southern-blot analysis using the 5'*Gdf*-9 probe (Fig. 3-4 b & d). The PGK-*neo* selection marker was then removed by Cre-*loxP* mediated recombination from the $Gdf\text{-}9^{tm2}$ allele to generate the $Gdf\text{-}9^{tm3}$ allele (Fig. 3-4 b & d). The resultant *Blm*-deficient, *Hprt*-postive, and *neo*-negative cells were named as NGG, which have $Blm^{tm3/tm4}$ and $Gdf\text{-}9^{tm1/tm3}$ alleles (Fig. 3-4 c). NGG cells were identified by sib-selection for G418 sensitive clones. Genomic Southern-blot analysis using a *neo* probe confirmed the removal of *PGKneo* cassette. Cells with the targeted PGK-*neo* cassette displayed a 1.8 kb


a
EcoRV
8.3 kb
BamHI
1
2
Gdf-9 locus
Hprt
tk
Gdf-9-TV1
HAT selection
EcoRV
6.3 kb
EcoRV
1
Hprt
Gdf-9tm1
5'Probe
b
EcoRV
8.3 kb
BamHI
1
2
Gdf-9 locus
Neo
Hprt
tk
Gdf-9-TV2
G418 selection
EcoRV
6.2 kb
EcoRV
1.8 kb
EcoRV
1
Neo
Hprt
Gdf-9tm2
5'Probe
Neo
Cre
G418 sib-selection
EcoRV
6.2 kb
EcoRV
1
Hprt
Gdf-9tm3
5'Probe

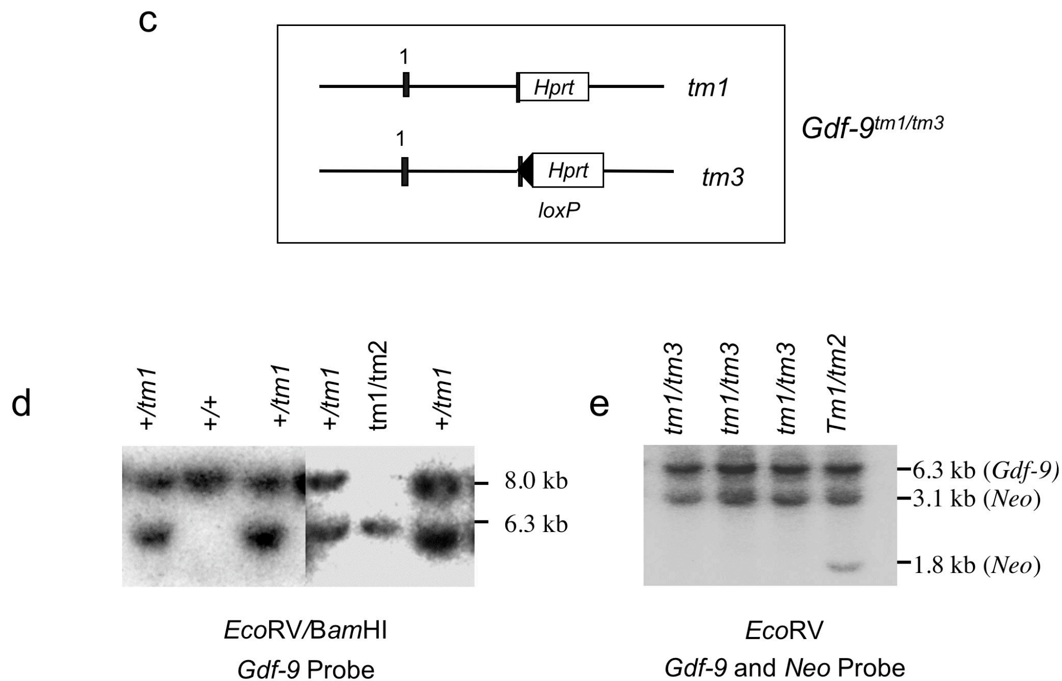


Figure 3-4. Generation of *Hprt*$^{+/+}$ *Blm* deficient ES cells.

a. Generating the *Gdf-9*^{tm1} allele by gene-targeting of wild type *Gdf-9* locus. **b.** Targeting the second *Gdf-9* allele, *Gdf-9*^{tm2} *Gdf-9*^{tm3} alleles. **c.** Schematic of the *Blm*(m3/m4) cell line that harbours the gene-targeted *Gdf-9*^{tm1} and *Gdf-9*^{tm3} alleles (the *Blm* locus is not illustrated). **d.** Southern-blot using the *Gdf-9* probe demonstrates ES cells that are single allele targeted (*Gdf-9*^{tm1}) or double targeted (with *Gdf-9*^{tm1} and *Gdf-9*^{tm2} alleles) **d.** Southern-blot showing the double gene-targeted *Gdf-9*$^{a/b}$ and *Gdf-9*$^{a/c}$ cells. The PGK-*neo* cassette(*Neo*) in the *Gdf-9*^{tm2} allele is represented as an1.8 kb *Eco*RV fragment. The Cre-*loxP* mediated recombination results in the loss of the 1.8 kb fragment (*Neo* cassette) in the *Gdf-9*$^{tm1/tm3}$ cells. The 3.1 kb fragment derived from a non-functional *Neo* gene in AB2.2 ES cells.

*Eco*RV band from the PGK-*neo* cassette and a 3.1 kb band from the X-linked non-functional *neo cassette* in the AB2.2 genetic background, while NGG cells showed only the 3.1 kb band (Fig. 3-4 e). After confirmation of the removal of PGK-*neo* cassette from NGG, one of the NGG cell lines, NGG5, was seeded at low density and allowed to form single ES cell colonies. The single cell colonies were picked and expanded. This single cell recloning process was performed to eliminate cross contamination of *Neo* positive cells during gene-targeting of the second *Gdf*-9 allele. One single cell clone, NGG5-3 was expanded and the genotype was confirmed again by Southern-blot analysis. NGG5-3 cells were then plated in G418 and confirmed to be G418 sensitive.

3.2.2 NGG cells are 6TG sensitive

Before starting a genetic screening for 6TG resistant mutants, it was important to check the PGK-*Hprt* transgenes express at a level sufficient to give 6TG toxicity. NGG cells and two other *Hprt* positive cell lines, AB1 and NG12-D were plated, separately in 5μM of 6TG. AB1cells have a normal endogenous *Hprt* gene, and are therefore sensitive to 6TG. As shown in table 3-1, AB1 cells were fully killed by 6TG. No 6TG resistant cells were recovered from plated NGG cells either, confirming that the targeted PGK-*hprt* minigenes in the *Gdf-9* loci are functional and stable. The NG-12D cell line is one of the parental *Blm*-deficient cell lines with a single targeted *Gdf*-9^{tm1} allele. Approximately 5% of these cells survived 6TG selection, which is consistent with the high LOH rate in *Blm*-deficient cells. The effect of 6TG killing was also examined in *Hprt* -deficient AB2.2 cells. AB2.2 cells were fully resistant to 5 μM 6TG with a clonal survival rate of 100%. An *Msh2* deficient cell line generated by sequential gene-targeting method (Abuin et al., 2000) was also tolerant to 5 μM 6TG, but the clonal survival rate was slightly lower (80%). The lower survival rate of *Msh2* deficient cells in 6TG suggests that 6TG may cause an adverse effect on the growth of MMR deficient cells (Table 3-1).

Cell line	Hprt♥	Survival rate in 6TG✳	Cells plated
AB1	$+^e$	0	$2.5x10^4$
NG-12D	$+^t$	5%	$2.5x10^4$
NGG	$+^t/+^t$	0	$2.5x10^4$
Msh2$^{-/-}$	$+^e$	83%	$2.5x10^2$
AB2.2	-	103%	$2.5x10^2$

Table 3-1. Survival rates of cells in 6-TG

NG12-D is a *Blm*-deficient ES cell line with single allele of *Gdf-9* targeted with a PGK-*hprt* minigene. NGG is a *Blm* deficient ES cell line with both alleles of Gdf-9 targeted with PGK-*Hprt* minigenes. *Msh2*$^{-/-}$ is a *Msh2* deficient ES cell line, generated by gene-targeting in AB1 ES cells (Abuin *et al.,*2000).

"$+^e$" represents the endogenous X-linked *Hprt* gene; "$+^t$" represents the targeted *PGK-Hprt* minigene and *Hprt* negative is illustrated as "-".

✳ Cells were plated in M15 medium with or without 6TG and allowed to grow for 10 days to form visible ES cell colonies. The Survival rate was determined by comparing the number of colonies formed with and without 6TG selection.

3.2.3 Screening for 6TG resistant mutants by EMS mutagenesis

3.2.3.1 EMS treatment of *Blm* deficent NGG cells

$2x10^6$ NGG ES cells were plated in 6-well feeder plates and treated with 600 μg/ml EMS for 15 hours. The cell plating efficiency was determined to be 31% by plating cells at low cell density. After EMS treatment, the survival cells were harvested and the number was counted. About 8,000 (0.4%) cells survived (Material and Method 2.2.8). Thus, the survival rate of EMS treatment was1.3%, which was determined as the survival rate of EMS treated ES cells versus cell plating efficiency (Table 3-2 a). Based on the established EMS mutation frequency in ES cells (about 1 mutant per locus per 1,200 cells surviving EMS treatment (Munroe et al., 2000)), it was estimated that the pool of 8,000 ES cells contains about 6 mutants for each locus. In other words, the EMS mutated cells contain 6 fold genome coverage. To allow for the segregation of homozygous mutant cells, the pool of EMS treated cells were expanded continuously for 10 days, and then a small portion of the pool was expanded for 4 more days to give time for decay of mRNA and protein in the presumptive mutants before being plated for 6TG selection (Fig. 3-5).

3.2.3.2 Screen for 6TG resistant mutants at low cell density

After 14 days passaging following EMS treatment, about $4 x10^6$ cells were plated on four 150 mm tissue culture plates at a density of $1x10^6$ ES cells per 150 mm tissue culture plate. 6TG (5 μM) selection was applied and continued for eight days. The surviving 6TG resistant clones were cultured in 6TG-free medium for four days to allow the healthy growth of the “sick” mutant cells before the colonies were picked and expanded. Eleven 6TG resistant ES cell clones were recovered in this experiment. No 6TG resistant clones were recovered in two control plates of NGG cells without EMS treatment, suggesting that the 6TG selection was efficient and the double targeted *PGK-Hprt* minigene does not

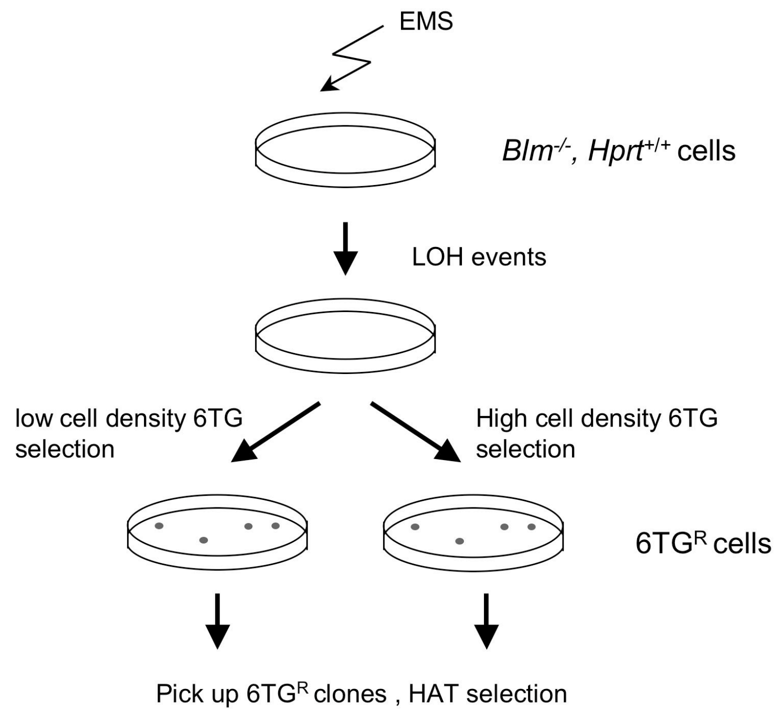


Figure 3-5 EMS mutagenesis and 6TG screens

Step1: EMS mutates NGG (*Blm*$^{tm3/tm4}$, *Gdf-9* $^{tm1/tm3}$) ES cells

Step2: Expand cells for 14 days to obtain homozygous mutants

Step3: plate cells at relevant density and apply 6TG selection

Step4: pick 6TG^R ES cell clones and apply HAT selection

Table 3-2 a. EMS treatment of NGG cells

[EMS] μg/ml	Plating efficiency	Survival rate in EMS	Mutation rate#1	No of mutants per locus#2
0	31%	-	-	-
600	0.4%	1.3%	1/1200	6

Table 3-2 b. Selection for 6TG resistant mutants

Selection	No. of cells selected	Cell density	No. of $6TG^R$ clones	No. of HAT^R clones
Low density	$4x10^6$	$1x10^6$/150 mm	11	5#3
High density	$2.3x10^7$	$2.3x10^7$/150 mm	24	24

Table 3-2. EMS mutated NGG cells and 6TG screens.

#1: This mutation rate was derived from published data (Munroe *et al*., 2001).

#2: indicates the pool of EMS mutated ES cells possesses about 6 mutants in each gene based on the assumed mutation rate and $8x10^3$ surviving cells.

#3: The number of HAT^R colonies was extrapolated from the ratio of HAT^R versus total $6TG^R$ clones obtained by examining seven of the eleven $6TG^R$ clones from the low cell density selection.

“-”: The parameters of the columns were not applied to the non-EMS treated cells.

revert. The viability of the selected clones in 6TG was confirmed by re-testing their colony forming ability in 6TG. Two groups of mutants would be expected to be recovered from the low cell density 6TG selection. One group will be mutated in the 6TG metabolism pathway, presumably the *Hprt* locus itself, while the other group should include mutants in MMR mediated DNA damage surveillance. To inspect the integrity of the *Hprt* gene, seven of the recovered 6TG resistant clones were expanded and plated on 24 well tissue culture plates in HAT. Four out of seven tested cell lines didn't grow in HAT, suggesting that they contain mutations in *Hprt*. Three out of seven 6TG resistant clones were also HAT resistant. These are potential MMR mutants with intact *Hprt* genes (Table 3-2 b).

3.2.3.3 Screen for 6TG resistance cells at high cell density

A high cell density 6TG selection was performed with 2.3×10^7 EMS treated and expanded NGG5 cells seeded on one 150 mm tissue culture plate. The 6TG selection was applied in the same way as for the low cell density 6TG selection. Twenty-four 6TG resistant clones were recovered from this screen. These 6TG resistant cells were plated in HAT supplemented cell culture medium and all were confirmed to be HAT resistant, suggesting that they are potential MMR mutants (Table 3-2 b).

3.3 Discussion

3.3.1 Construction of NGG (*Blm*$^{tm3/tm4}$, *Gdf-9* $^{tm1/tm3}$) cells

In this chapter, *Blm* deficient cell lines containing two gene-targeted PGK-*Hprt* minigenes were generated in order to establish a genetic screen for mutations in mismatch surveillance by selection in 6TG. The NGG (*Blm*$^{tm3/tm4}$, *Gdf-9*$^{tm1/tm}$) cells exhibited complete 6TG sensitivity even after long period of culture and multiple passages. This result suggests that the targeted *Hprt* minigenes are maintained stably in *Blm* deficient cells. In this regard, targeting two copies of the

Hprt genes to both alleles of one gene provides a very stable situation for a screen compared with cells with a single targeted *Hprt* minigene. For example the NG12-D cells with a single *Gdf-9* allele targeted with the PGK-*Hprt* minigene frequently segregate 6TG resistant clones (Table 3-1). Importantly, NGG and one of its single cell derivatives, NGG5-3 cell line, are devoid of the commonly used drug selection markers, such as *Puro, Neo*, *Bsd*. Thus they are amenable to further targeting based modifications.

3.3.2 Screen for 6TG resistant mutants

EMS mutagenesis was performed to mutate NGG cells in order to screen for 6TG resistant mutants. EMS is a highly efficient chemical mutagen that causes preferentially loss of function point mutations. Therefore, EMS mutagenesis is an ideal method to quickly generate a large quantity of recessive mutations. To examine the effect of cell density on 6TG killing, two screens were performed on EMS mutated NGG cells at different cell plating densities. The low cell density 6TG selection allowed the recovery of both *Hprt* mutants as well as the potential mutants in mismatch surveillance as shown recovery of the HAT resistant clones. In contrast, all clones recovered in the high cell density 6TG screen were also HAT resistant, showing that the *Hprt* mutants were killed by the metabolic cooperation under high cell density 6TG selection as expected. In the low cell density screen, 5 6TG resistant mutants were also HAT resistant. The total number of cells plated for 6TG selection in the high cell density 6TG screen was about 5 times the number of the cells plated in the low cell density screen. Thus, 25 HAT resistant mutants were expected to be recovered in the high cell density 6TG screen. In fact, 24 clones were recovered, which illustrates the effectiveness of the high cell density selection in recovering potential mismatch surveillance mutants.

These results also suggest that the *Blm* deficient ES cell is a useful tool for isolating recessive mutations. After this work was finished, Yusa et al reported a

screen for recessive mutants of glycosylphosphatidylinositol (GPI)-anchor biosynthesis using a conditional *Blm* deficient ES cell line. They mutated *Blm* deficient ES cells using ENU (*N*-ethyl-*N*- nitrosourea) mutagenesis and selected GPI anchor mutants in a drug called aerolysin, which is capable of killing GPI-anchor positive cells. By cDNA rescue, they identified mutations in 12 out of 23 candidate genes, and by sequencing they confirmed that all of these mutants are homozygously mutated (Yusa et al., 2004), demonstrating that *Blm* deficiency is indeed an efficient means to produce homozygous mutations for recessive genetic screens. Although these experiments demonstrated clearly the power of *Blm* deficiency in a recessive screen, little additional information could be determined about the novel mutated genes. The methods for identifying mutations generated by chemical mutagenesis require localization of the novel mutation by linkage analysis, cDNA rescue and sequencing of the candidate genes. These methods are not suitable to identify the molecular basis of mutants from large scale phenotype driven screens, which aim to isolate novel genes and obtain novel information. Because the major aim of my Ph.D. project is to establish a high throughput method for recessive genetic screens which include gene identification, we did not pursue chemical mutagenesis beyond the proof of principle described here. As an alternative, we used gene trap mutagenesis which does allow high throughput identification of mutated genes.