Replication and episomal maintenance of Epstein-Barr virus-based vectors in mouse embryonal fibroblasts enable synthetic lethality screens

Yulia Einav,[1] Elena Shistik,[1] Michal Shenfeld,[1] Arnold H. Simons,[1] David W. Melton,[2] and Dan Canaani[1]

[1]Department of Biochemistry, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv, Israel and [2]Sir Alastair Currie Cancer Research UK Laboratories, Molecular Medicine Centre, University of Edinburgh, Edinburgh, UK

Abstract

Recently, we demonstrated the establishment of chemical and genetic synthetic lethality screens in cultured human cells. Here, we report the establishment of this method in mouse embryonal fibroblasts (MEF). The method employs an immortalized mammalian cell line, deficient in a gene of interest, which is complemented by an episomal survival plasmid expressing the wild-type cDNA for the gene of interest and the use of a novel green fluorescent protein (GFP)-based double-label fluorescence system. The crucial part in this endeavor has been the identification of a DNA replicon that could stably replicate in MEFs while under selection for survival and gets spontaneously lost relatively fast in the absence of such a pressure. Here, we show for the first time that EBV-based replicons but not polyoma virus-based ones can replicate and be stably maintained in MEFs. In the chemical screen, selective pressure imposed by synthetic lethal drugs prevented the spontaneous loss of the GFP-marked episome, enabling drug identification. Retention or spontaneous loss over time of the episomal survival plasmid could be sensitively detected in a large-scale blind test in the presence or absence of synthetic lethal chemicals, respectively. Establishing the synthetic lethality screen should thus permit high throughput screening for chemicals, which are synthetically lethal with any mouse mutant/knockout gene of interest. Moreover, it forms the basis for a genetic synthetic lethality screen in MEFs, an important new tool for mouse functional genomics. (Mol Cancer Ther. 2003;2:1121–1128)

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Requests for Reprints: Dan Canaani, Department of Biochemistry, George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv 69978, Israel. Phone: 972-3-6408985; Fax: 972-3-6424270. E-mail: canaani@post.tau.ac.il

Introduction

The fast accumulation of mouse cDNA/expressed sequence tag sequences over the last few years ([1–3), and the completion of most of the mouse genome sequence (4),[1] has stimulated intensive initiatives to develop tools needed to close the ''gene function gap.'' Notable among which are production of mutant mouse embryonic stem (ES) cells, and their corresponding genetically modified mice, using targeted mutagenesis via constitutive homologous recombination (5), conditional DNA recombination (reviewed in 6), or combination of the two to generate chromosomal deletions (reviewed in 7). This precise gene targeting strategy was accompanied in parallel by development of random mutagenesis screens using gene-trap methodologies (8), leading eventually to whole genome screens (9–11, reviewed in 12). The availability of a large number of knockout mice is a potential rich source of immortalized mutant mouse embryonal fibroblasts (MEF) with defined gene deficiencies.

Recently, we reported the establishment of chemical and genetic synthetic lethality screens in cultured human cells (13, 14) based in part on the concept of a yeast method (15). In general, the method employs as a recipient an immortalized mammalian cell line carrying a nonlethal mutation in both alleles of a gene of interest. A mutant green fluorescent protein (GFP) gene is stably integrated into the chromosomal DNA of these cells. A chimeric episomal ''survival plasmid'' expressing both the cDNA for the gene of interest and a second GFP mutant is also introduced, allowing measurement of the double-label fluorescence ratio between the two GFPs. The ''survival plasmid'' replicates autonomously as an episome in the host cells, yet its maintenance is unstable and requires selective pressure. Monitoring double-label fluorescence ratio between the two GFPs allows detection over time of the retention or loss of the ''survival plasmid.'' Cell clones containing either a chemical or a dominant-negative mutant, synthetic lethal with the mutated gene of interest, are selected to retain the survival plasmid due to expression of the complementing cDNA.

Here, we sought to establish the principles of the synthetic lethality screening method in cultured MEFs and thus potentially gain access to a large number of mutant mouse genes of interest present in MEFs derived from their respective knockout mice. The crucial part in our endeavor has been the generation/identification of an ''imperfect episome'' that in MEFs under selection can stably replicate extrachromosomally yet in the absence of selective pressure is lost spontaneously at a reasonable rate.

[1] http://mouse.ensembl.org.

Two replicons were investigated as episomes in MEFs: initially the polyoma virus replicon and then the EBV replicon. Concerning the former, differentiated mouse cells are permissive for polyoma virus lytic replication (16). In contrast, early embryonic pluripotent cells such as embryonal carcinoma (EC) or ES cells cannot support the replication of wild-type polyoma virus unless it is mutated in the enhancer region (17–19). This enhancer region not only stimulates transcription from the early and late viral promoters but also is essential for viral replication. Whether the polyoma replicon can be stably maintained in MEFs, which are explanted from late-stage embryos (12–17 days postcoitum), has been an open question that we addressed in this work. The second line of research in MEFs employed the previously mentioned EBV-based replicon (13, 14). Early work has suggested that the EBV-based replicon cannot replicate in MEFs such as BALB/c3T3 (20, 21). Here, we report that neither wild-type polyoma replicon nor enhancer mutated polyoma replicons can replicate in MEFs. In contrast to earlier publications (20, 21), we found that the EBV-based replicon can stably propagate in MEFs while under selection and spontaneously decay in the absence of selection pressure, with a half-life of 7–14 days. These characteristics of the EBV-based replicon formed the basis for the establishment of a chemical synthetic lethality screen in MEFs. This could be useful in drug development of mouse models for human genetic diseases (22). Moreover, in analogy with the human system (14), it formed the basic setup that should enable the development of a genetic synthetic lethality screen in MEFs, an important new tool for mouse functional genomics.

Materials and Methods

Plasmids and Cell Lines

The pCEP4 and pREP4 expression vectors were purchased from Invitrogen (Carlsbad, CA). The construction of the pIS integrating sphGFP vector with a zeo^R-dominant selectable marker has been outlined (14). The episomal HPRT1-tpzGFP survival plasmid was previously described (13). The latter contains the human HPRT1 cDNA, the function of which is to complement the HPRT1 deficiency in the MEFs. Based on early cell hybrid work and the extreme sequence conservation between human and mouse HPRT1 proteins (97% identity and 99% similarity), we assumed that expression of the human cDNA is likely to complement the mouse HPRT1 deficiency. This was confirmed by complementation of HPRT1-deficient mouse cell line by human HPRT1 cDNA expression, reflected in acquisition of hypoxanthine-aminopterin-thymidine (HAT) resistance by the stable transfectants.

Three types of polyoma virus replicons were incorporated into the backbone of the human HPRT1-tpzGFP survival plasmid (13). In one, polyoma wild-type survival plasmid, the EBV nuclear antigen 1 and origin of plasmid replication elements were deleted by *Xba*I and *Bst*eII cleavage from the EBV-based survival plasmid and replaced by the polyoma early region excised from pPyLT1 DNA (23) by *Bcl*I and *Hin*cII digestion. The latter is derived from polyoma virus strain A2 and contains the wild-type polyoma origin of DNA replication joined to an early region deleted for the 5′ splice donor of middle and small T antigen, so that only large T antigen can be produced.

The second polyoma replicon survival plasmid, polyoma EC F9-5, has the same basic structure as the former, with an A2 polyoma strain mutated enhancer and duplicated origin, enabling its replication in early embryonic cells such as EC cells (18). The viral origin and mutated enhancer present in the polyoma EC F9-5 containing plasmid was isolated by *Bam*HI and *Hin*cII cleavage and ligated to the polyoma large T coding region excised from pPyLT1 by *Acy*I and *Hin*cII restriction digest. The DNA fragment bounded by *Spe*I and *Xho*I (blunt), encompassing the mutant enhancer, double origin, and large T region, was cloned in between *Xba*I and *Bst*eII (blunt) sites of the human HPRT1-tpzGFP survival plasmid.

The third polyoma survival plasmid, polyoma F101, has a polyoma strain 3 derived replicon with a mutated duplicated enhancer, allowing its replication in F9 EC cells (17) and mouse ES cells (19). The F101 polyoma origin and large T coding region were isolated from the pMGD20neo plasmid (19) by *Pvu*I cleavage, cloned to the *Eco*RV site of pBS/SK$^-$, and then ligated (bounded by *Spe*I and *Cla*I restriction sites) and cloned in between *Xba*I and *Cla*I sites of the human HPRT1-tpzGFP survival plasmid.

The HPRT1-deficient 2TGOR cells (a kind gift from H. Ozer, University of Medicine and Dentistry of New Jersey) are derivatives of the MEF BALB/c3T3 (24). The immortalized 46BR.1G1 human fibroblast cell line (25) was purchased from European Collection of Cell Cultures (Centre for Applied Microbiology and Research Center, United Kingdom). The DNA ligase I null MEF cell line PFL13 was generated by Melton's group and represents a spontaneously immortalized cell line derived from a knockout mouse (26). The NIH3T3 MEFs were purchased from the American Type Culture Collection (Manassas, VA).

Transfection and Expression of Constructs in Cells

The 2TGOR, NIH3T3, PFL13, and 46BR.1G1 cell lines were maintained in DMEM supplemented with 10% FCS and 4-mM L-glutamine. All transfections were carried out using LipofectAMINE Plus reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. For pIS, selection in zeocin (Cayla, Toulouse, France) was at 1 mg/ml. For the HPRT1-tpzGFP survival plasmid, selection in hygromycin B (Calbiochem, La Jolla, CA) was carried out at 150 μg/ml for the PFL13 cell line and at 250 μg/ml for all the other cell lines, while maintenance was at 100 μg/ml. Survival plasmid mediated HPRT1 expression was tested in HAT medium (100-μM hypoxanthine, 0.4-μM aminopterin, 16-μM thymidine; 27). Mycophenolic acid (MPA) was obtained from Eli Lilly (Indianapolis, IN), while ribavirin and mizoribine were obtained from Sigma-Aldrich (Rehovot, Israel).

Fluorescent Scanning of Microtiter Plates

For fluorescent scanning, cells were trypsinized and distributed into 96-well microtiter plates (TPP). Growth medium was changed twice a week and plates were

maintained for up to 38 days. Before scanning, the medium in the microplates was replaced with HBSS without phenol red. This procedure greatly minimized background fluorescence from the growth medium while maintaining maximal viability. Plates were scanned with a Fluoroskan Ascent CF microplate fluorescence reader using the Ascent software (Thermo Labsystems, Vantaa, Finland). Excitation for sphGFP was at 390 nm, while emission was measured at 510 nm. Excitation of tpzGFP was at 485 nm, while emission was measured at 527 nm. Integrated sphGFP was used as an internal control for the number of cells. This was achieved by dividing the relative fluorescence resulting from the episomal tpzGFP vector by the relative fluorescence from sphGFP for each well.

In the experiment testing synthetic lethality between mizoribine or ribavirin and HPRT1 deficiency, the drug was added at zero time, and fluorescent scanning was performed 4 weeks later. In contrast, in the analogue large-scale blind test experiment, the drugs were added 2 weeks after hygromycin B removal (initiating the decay of the survival plasmid), and the fluorescence reading was performed 13 days thereafter. In this way, the system is sensitized such that the HPRT1-tpzGFP plasmid retention is more pronounced.

Extraction and Characterization of Episomal DNA

Low molecular weight (LMW) DNA was extracted from 10^7 cells with Qiagen, Inc. (Hilden, Germany) mini prep kit, using the manufacturer's protocol, with minor modifications. Control pBC plasmid carrying chloramphenicol resistance gene was added to each sample. The LMW DNA was transformed into DH10B bacteria by electroporation followed by selection for ampicillin and chloramphenicol resistance. The number of bacterial colonies grown on ampicillin plates was normalized to number of colonies on chloramphenicol plates, as the latter corresponds to efficiencies of DNA extraction and bacterial transformation.

Southern blot analysis of LMW DNA for sensitivity to either the restriction endonuclease *Dpn*I, which cuts bacterially methylated DNA, or the *Mbo*I enzyme, which cleaves DNA that has lost this methylation due to replication in mammalian cells, was performed by standard techniques.

Results

Analysis of Replication and Maintenance of Polyoma Virus and EBV Replicons in MEFs

As a model system for the establishment of chemical synthetic lethality screen in MEFs, the purine biosynthesis pathway was chosen (Fig. 1). This pathway was successfully used for the chemical and genetic synthetic lethality screening in human systems (13, 14). Biosynthesis of the essential metabolite GMP is achieved in fibroblasts by either the *de novo* pathway or, when needed, the salvage enzyme HPRT1. In the HPRT1-deficient 2TGOR MEF cell line (24), chemicals such as MPA, which inhibit the IMP dehydrogenase enzyme (IMPDH, EC 1.1.1.205), should

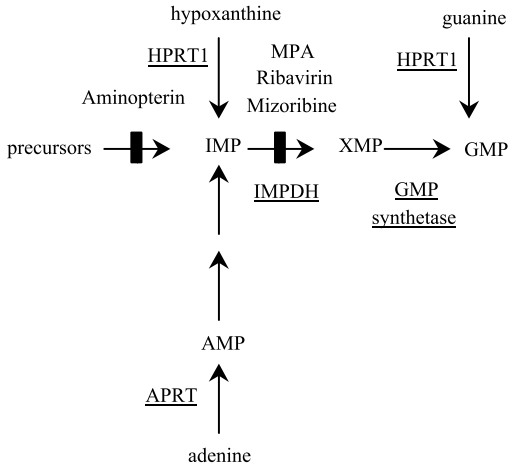

Figure 1. *De novo* and salvage pathways of purine biosynthesis. Key enzymes are *underlined*. *Arrows* indicate the action of enzymes. The sites of inhibition by MPA, ribavirin, mizoribine, and aminopterin are *marked*.

disrupt the *de novo* pathway, thus leading to synthetic lethality. We have previously shown that in human cells such selective pressure imposes the retention of an otherwise unstable EBV-based episomal plasmid expressing the HPRT1 cDNA, serving as the gene of interest (13).

To find out whether the screening for synthetic lethal chemicals could be extended to MEFs, we needed to identify a replicon that would allow the survival plasmid a stable extrachromosomal replication in MEFs while under selection. Each of the three polyoma replicon-based survival plasmids (polyoma wild-type, enhancer mutated polyoma EC F9-5, and polyoma F101) was initially transiently transfected into the 2TGOR recipient, and 4 days later, LMW DNA was isolated from the transfectants. Episomal replication was then evaluated by the sensitivity of the LMW DNA to bacterial DNA methylation. *Dpn*I cuts DNA that has been methylated in bacteria by the Dam methylase, whereas *Mbo*I cleaves DNA in which the same GATC recognition sequence is unmethylated. Accordingly, episomal DNA, which has replicated in mouse cells, should be *Dpn*I resistant and *Mbo*I sensitive. *Mbo*I and *Dpn*I digestions were carried out on the isolated plasmids and the DNA was detected by Southern blot hybridization. It appears that at 4 days post-transfection the LMW DNA is completely sensitive to *Dpn*I digestion, indicating no plasmid replication at this stage (data not shown). Then, we proceeded to the stable transfections of the three constructs into the 2TGOR cell line while selecting for hygromycin B resistance. LMW DNA was isolated 2 months later from the pool of clones for each construct and electroporated into competent DH10B bacteria. We obtained no ampicillin-resistant colonies, while control pBC plasmid carrying chloramphenicol resistance gene gave rise to several hundred colonies, demonstrating normal efficiencies of DNA extraction and bacterial transformation. In addition, we were not able to detect

polyoma origin-based plasmids by Southern hybridization of this LMW DNA. Taken together, these results indicate that the polyoma-based replicons do not replicate in the 2TGOR MEF cells.

We therefore turned to examine the replication of EBV-based vectors in MEFs and thus their potential utility for synthetic lethality screening. Two EBV-based vectors, pCEP4 and the pREP4-based HPRT1-tpzGFP plasmid (13), were each transfected into the 2TGOR MEF recipient and selection for hygromycin B resistance was instituted. Six weeks later, LMW DNA was isolated from a pool of stable clones. Episomal replication was then evaluated by the sensitivity of the LMW DNA to bacterial DNA methylation. The results are shown in Table 1. Equal amounts of the LMW DNA were nontreated, cleaved by *Dpn*I, or digested by the *Mbo*I restriction enzyme, transformed into DH10B bacteria, and scored for bacterial colony formation. We found that LMW DNA extracted from stable clones of 2TGOR is almost completely sensitive to *Mbo*I while remaining resistant to *Dpn*I. Normalizing number of bacterial transformants to the yield of DNA extraction and electroporation estimates 1–2 plasmid copies/2TGOR cell. The *Dpn*I-resistant DNA was found to be unrearranged by restriction enzyme analysis (data not shown). Very similar results were obtained with the pCEP4 plasmid DNA. Next, the LMW DNA extracted from the 2TGOR cells was tested by Southern blot analysis and found to be of the expected size and completely sensitive to *Mbo*I, while bacterially derived DNA, as a control, is completely sensitive to *Dpn*I (Fig. 2A, lanes 5 and 3, respectively). These results taken together prove that EBV-based plasmids replicate in 2TGOR MEFs while remaining unrearranged. Likewise, the pREP4-based plasmid was stably transfected into the NIH3T3 MEFs and the 46BR.1G1 human fibroblasts (25) and LMW DNA was analyzed by transformation into DH10B bacteria. This experiment determined that the NIH3T3 cell line harbors 1–2 intact plasmid copies/cell, while the human 46BR.1G1 contain 5–10 copies of this EBV-based plasmid/cell (data not shown).

The 2TGOR and NIH3T3 MEF cell lines are spontaneously immortalized MEFs that have been grown in culture for extensive periods. To assess the behavior of the EBV-based replicon in a more recently established MEF cell line, we used the immortal MEF PFL13 cell line derived from a DNA ligase I knockout mice that has been generated in Melton's laboratory (26). These cells were shown to display altered DNA replication and increased genome instability. We stably transfected the PFL13 MEF immortal cell line with an EBV-based episomal pCEP4 plasmid. LMW DNA was extracted from pool of clones following an 8-week growth period. First, the DNA was electroporated into DH10B competent bacteria as is or after digestion with *Mbo*I or *Dpn*I restriction nucleases. The

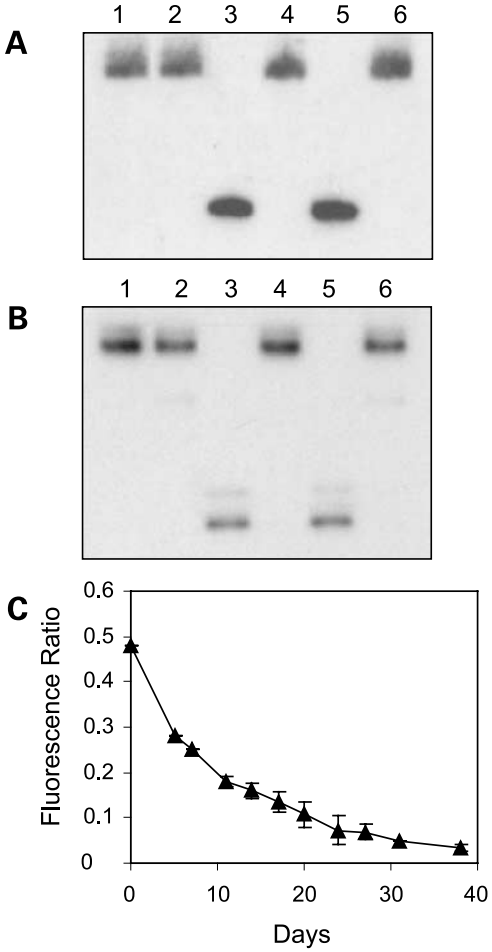

Figure 2. Episomal replication of an EBV-based plasmid in the 2TGOR and PFL13 MEF cell lines. **A,** Southern blot replication analysis of pREP4-based survival plasmid in 2TGOR cells. 2TGOR cells were transfected with HPRT1-tpzGFP survival plasmid. LMW DNA was extracted from a pool of 2TGOR stable transfectants, which had been grown under hygromycin B selection for 6 weeks (*lanes 4–6*). As a control, the survival plasmid, which was isolated from DH10B bacteria, was analyzed as well (*lanes 1–3*). DNA was digested by *Mbo*I (*lanes 2 and 5*) or *Dpn*I (*lanes 3 and 6*) restriction endonucleases. This digestion yields a 2.8-kb fragment, which hybridizes to *Nsi*I/*Dpn*I fragment of pREP4 (2.2 kb) that was used as a DNA probe. *Lanes 1 and 4,* uncut DNA. **B,** Southern blot replication analysis of pCEP4-based plasmid in PFL13 MEF cell line. Experiment design as in **A**. **C,** spontaneous loss of pREP4-based survival plasmid in 2TGOR cell clone Zeo-5. Cells were grown in 96-well plates for the entire period and read periodically using a microplate fluorescence reader. *Triangles,* average fluorescence ratio between tpzGFP and sphGFP measurements from at least three wells.

Table 1. Episomal replication of LMW DNA extracted from 2TGOR and PFL13 cell lines estimated by its sensitivity to bacterial DNA methylation

Cell line	Nontreated LMW DNA	*Mbo*I-digested LMW DNA	*Dpn*I-digested LMW DNA
2TGOR	400	7	376
PFL13	158	3	122

Note: 2TGOR and PFL13 cells were transfected with pREP4-based HPRT1-tpzGFP plasmid or pCEP4 plasmid, respectively. LMW DNA was extracted from a pool of 2TGOR stable transfectants, which had been grown under hygromycin B selection for 6 weeks. LMW DNA was electroporated into competent DH10B bacteria as is or after digestion with *Mbo*I or *Dpn*I restriction nucleases. Values were normalized to chloramphenicol-resistant control plasmid and given as number of bacterial colonies/10^6 cells.

results show almost complete sensitivity of these plasmids to *Mbo*I endonuclease and resistance to *Dpn*I (Table 1). Estimated plasmid copy number is 0.5–1/PFL13 cell. Second, Southern blot hybridization of this LMW DNA was performed, and it shows sensitivity to *Mbo*I cleavage and resistance of this episomal plasmid to digestion by *Dpn*I endonuclease (Fig. 2B, lanes 5 and 6, respectively). At the same time, bacterial control plasmid showed as expected an inverted pattern of digestion (Fig. 2B, lanes 2 and 3). Taken together, these results indicate that the immortal MEF PFL13 cell line, derived from knockout mice, can support stable episomal replication of EBV-based replicon, which also remains unrearranged after a long period of propagation in these cells.

Setup of the Experimental System and Fluorescent Measurement of Spontaneous Survival Plasmid Loss

The EBV-based episomal survival plasmid was transfected into the HPRT1-deficient 2TGOR MEFs, and stable transformants selected for hygromycin B resistance were collected as a pool of cell clones. The ectopic expression of HPRT1 protein by these transformants was demonstrated by the acquisition of resistance to HAT medium (27). This was followed by the stable integration of pIS, which encodes the sphGFP and the bacterial zeocin resistance (zeo^R) DNA into these cells while selecting for individual cell clones. We then examined whether in the absence of either HAT or hygromycin B selection pressure there is spontaneous loss of the episomal survival plasmid. For this purpose, 68 cell clones were seeded into 96-well microtiter plates and grown continuously. Following removal of hygromycin B from the medium, the tpzGFP and sphGFP fluorescence ratios were monitored periodically. The majority of the cell clones displayed spontaneous decay of the episomal survival plasmid with an average half-life of 1–2 weeks. The features of retention as an episomal plasmid while under selection and its spontaneous decay in the absence of drug selection were characteristic of each clone and were reproducible. The representing stable cell clone Zeo-5 lost about 80% of its initial survival plasmid encoded tpzGFP fluorescence within 20 days (Fig. 2C).

Detection of Synthetic Lethality Induced by Chemicals

These results demonstrate that in MEFs, under drug selection, the HPRT1-tpzGFP survival plasmid can replicate and remain as an episome while on drug removal decays spontaneously. Loss or retention of the plasmid can be determined by measuring the fluorescence intensity ratio in a microplate reader. We next tested whether these features would enable us to trace a synthetic lethal condition. The biosynthesis of GMP from IMP via xanthosine monophosphate can be efficiently blocked using MPA (Fig. 1), which inhibits IMPDH through binding to the NAD site within the enzyme (28). Under these conditions, normal HPRT1-positive cells survive by using guanine supplied in the medium to produce GMP via the salvage pathway, while HPRT1-deficient cells die. Cell clone Zeo-5, as an inherently HPRT1-deficient cell line, must retain the HPRT1-tpzGFP survival plasmid to stay alive in this synthetic lethal situation. We tested this scenario by growing Zeo-5 cells in 96-well microtiter plates containing medium supplemented with guanine, in the presence or absence of MPA, while monitoring the ratio of tpzGFP to sphGFP over time. As shown in Fig. 3, MPA caused retention of the survival plasmid over time, while cells without MPA continued to lose tpzGFP fluorescence.

IMPDH is also inhibited by nucleoside analogs, which bind to the IMP substrate site (28). Accordingly, we asked whether we could detect synthetic lethality when nucleoside analogue inhibitors were applied to cell clone Zeo-5. For this assay, we chose ribavirin and mizoribine, drugs both in use against viral infections. Zeo-5 cells grown in 96-well microtiter plates were removed from hygromycin B selection pressure, and serial dilutions of drugs (mizoribine or ribavirin) and guanine were added. Four weeks later, the microplates were read for tpzGFP and sphGFP fluorescence intensity, and the ratios were calculated for each well. The observed matrices are shown in Fig. 4. Both drugs caused retention of the EBV-based survival plasmid while displaying dependency on the concentration of each drug as well as that of guanine. Therefore, each of the three IMPDH inhibitors induced a synthetic lethal effect that could be sensitively detected by fluorescent measurement.

Next, we tested if the synthetic lethality assay in MEFs could be used in a blind test to screen for these IMPDH inhibitors. Clone Zeo-5 cells were seeded into twenty 96-well microtiter plates in medium lacking drug selection. Two weeks later, MPA, ribavirin, mizoribine, and hygromycin B were added (together with guanine) to four random wells each. AG490, an inhibitor of JAK2 kinase, and HAT + adenine (the latter promotes the adenine phosphoribosyltransferase-mediated salvage pathway of GMP synthesis) were also added as negative controls. Thirteen days later, the fluorescence levels were measured by a plate reader. As expected, all three IMPDH inhibitors and hygromycin B were clearly detected, as was one well containing false-positive cells (Fig. 5). The

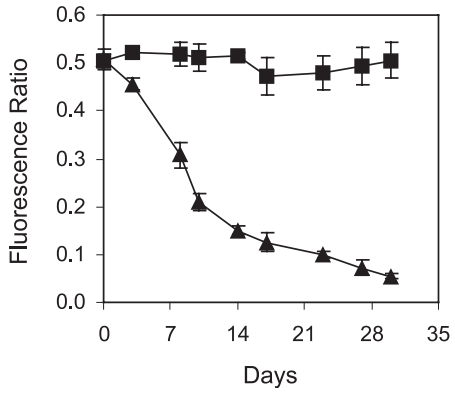

Figure 3. Synthetic lethality imposed by MPA causes retention of the survival plasmid. Zeo-5 cells in 96-well microtiter plates were maintained over the entire period in either medium with 12.5-μM guanine and 50-μg/ml MPA (*rectangles*) or guanine-supplemented medium alone (*triangles*). Plate reading and data representation are as in Fig. 2C.

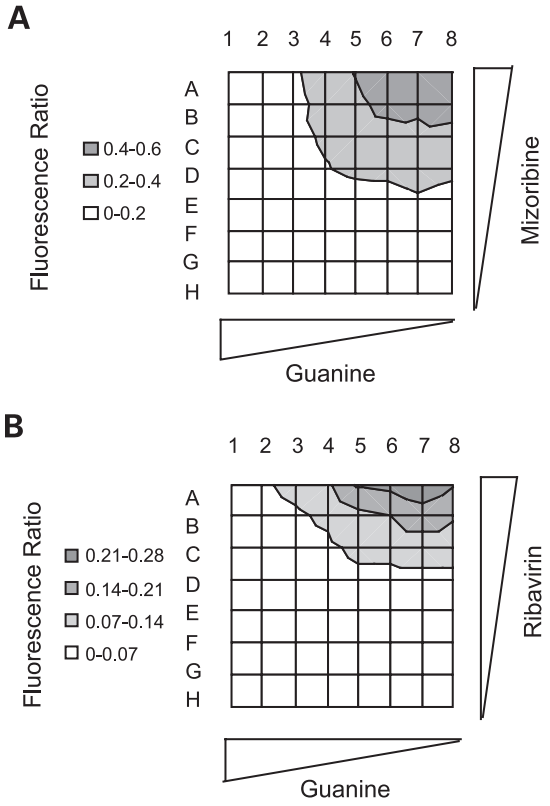

Figure 4. Chemical synthetic lethality induced by nucleoside analogue inhibitors of IMPDH. Zeo-5 cells were grown in microtiter plates in the presence of mizoribine (**A**) or ribavirin (**B**) for a period of 1 month; the fluorescence measurements were taken and the ratios are calculated. *Left,* values of the fluorescent ratios. *Rows A–H,* drug concentrations in each matrix. *Row A,* mizoribine (**A**) or ribavirin (**B**) were at 400 μg/ml, and serial 2-fold dilutions were performed in the indicated direction. Guanine was at 200 μM in *column 1*, and serial 2-fold dilutions were carried out in the indicated direction.

calculated false-positive rate for this experiment in MEFs was about 1/1920. In contrast, the survival plasmid was not retained in the presence of the nonrelevant inhibitor AG490 or HAT + adenine.

Discussion

Here, we demonstrated the feasibility of a chemical synthetic lethality screen in cultured MEFs using a double-label fluorescence system. The major challenge in the present work has been to identify a replicon that can stably replicate in MEFs while under selection and decay spontaneously at a reasonable rate on removal of selection pressure. Our initial attempt, which employed polyoma virus replicons, failed. These replicons, containing wild-type or mutated enhancer/origin of DNA replication and encoding polyoma large T antigen, were shown previously to be capable of replicating in either somatic mouse cells (16) or pluripotent embryonal mouse cells such as EC cells (17, 18) or mouse ES cells (19), respectively. However, we found out that these polyoma-based replicons could not replicate in MEFs, which are late embryonal mouse cells. In contrast, employment of the EBV-based episomal survival plasmid, previously used in human cells (13), turned out to be suitable for synthetic lethality screening in MEFs: First, either pCEP4 (Invitrogen) or pREP4-based survival plasmids recovered from stable 2TGOR transfectants were shown to be stably replicating by Southern blot analysis (Fig. 2A) and a bacterial transformation assay (Table 1). Second, analysis of stable transfectants of the EBV-based survival plasmid has shown that MEFs such as 2TGOR or NIH3T3 cells harbor 1–2 episome copies/cell, whereas, in comparison, SV40-transformed human fibroblasts such as 46BR.1G1 contain 5–10 copies/cell. Third, among 68 stable transfected clones of the 2TGOR recipient, the majority displayed spontaneous and complete loss of the survival plasmid on removal of drug selection. This is in contrast to mouse ES cells in which only about 16% of the stable clones harbor the polyoma F101 replicon vector as an episome (19). The half-life for the spontaneous decay of the survival plasmid in 2TGOR cells range between 7 and 14 days. The minority of stable clones that failed to decay completely probably had their survival plasmid DNA randomly integrated into the cellular chromosomal DNA, whereas for those 2TGOR clones displaying retention of the EBV-based survival plasmid as an episome while under selection and its spontaneous decay in the absence of drug selection, this behavior was characteristic and reproducible. Moreover, we have shown stable episomal replication of an EBV-based plasmid in the PFL13 MEF cell line, which is derived from a knockout mice. This finding forms the basis for the establishment of synthetic lethality screens using immortal MEFs derived from a large number of available knockout mice. To our knowledge, this is the first time that an EBV-based expression vector was proven capable of autonomous replication and long-term episomal maintenance in MEFs.

We then used a 2TGOR cell clone (Zeo-5) with relatively fast spontaneous decay kinetics (Fig. 2C) to test whether synthetic lethality imposed by chemical inhibitors of the rate-limiting enzyme in *de novo* GMP biosynthesis

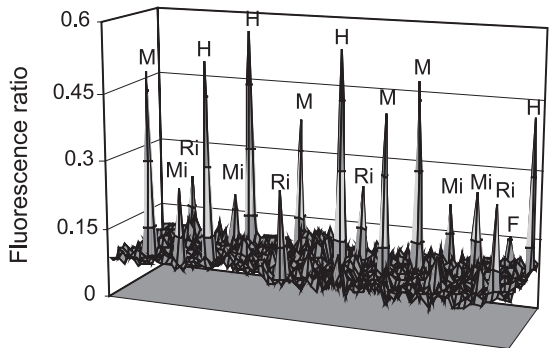

Figure 5. Detection of chemical synthetic lethality in a blind large-scale test. Zeo-5 cells were seeded into 1920 wells in 96-well microtiter plates. The cells were grown in the absence of hygromycin B for 2 weeks; then, chemicals were added at random to four wells each: hygromycin B (*H*) was at 100 μg/ml and MPA (*M*), mizoribine (*Mi*), and ribavirin (*Ri*) were at 400 μg/ml. Guanine was added at 100 μM to MPA-containing wells and at 6 μM to mizoribine- and ribavirin-containing wells. Negative controls, AG490 (an inhibitor of JAK2 kinase) at 50 μM and HAT + adenine at 100 μM, were also added. Plates were read after 13 days. The single false-positive well (*F*) is also marked.

(IMPDH) can be detected by the double-label fluorescent assay. As shown for MPA in Fig. 3 and the matrices representing the chemicals mizoribine and ribavirin (Fig. 4), synthetic lethal conditions can be sensitively detected in a manner dependent on the concentrations of the chemical inhibitor and the metabolite substrate guanine. Moreover, synthetic lethal drugs could be detected in a large-scale blind test while displaying a false-positive rate of 1/1920, which is certainly a low enough background to enable high throughput chemical screening (Fig. 5).

We propose that the application of this method should permit high throughput screening for drugs that are synthetically lethal with any mutant mouse gene of interest, the normal counterpart of which can be expressed in culture (29). In particular, one could scan a library of small organic chemical compounds (30) for reagents that kill MEFs in a defined genetic milieu. For example, this system may expedite the search for better IMPDH inhibitors, a goal that remains relevant for the findings of new immunosuppressive, antiviral, and antitumor agents (31). Furthermore, as the method selects for a lethal phenotype, it is particularly suitable for the identification of drugs that kill malignant cells in a gene-specific manner based on their predetermined cellular genotype (32).

Moreover, by establishing the principles of the method in cultured MEFs, we formed the basis for genetic synthetic lethality screens that can potentially address the genetic interactions of thousands of mouse mutant genes of interest present in MEFs derived of knockout mice. In that context, we recently reported the development of a genetic synthetic lethality screen at the candidate gene level in cultured human cells (14). For this purpose, chemicals used in the former screen were replaced by an episomal library expressing short truncated sense and antisense cDNA (33) for a gene likely to be synthetic lethal with the gene of interest. Dominant negative genetic suppressor elements (GSE) conferring synthetic lethality were selected from this library by their ability to prevent the spontaneous loss of the GFP-marked episomal survival plasmid. This allowed fluorescence-activated cell sorting enrichment for cells retaining the survival plasmid and consequently the physical isolation of the GSEs. The dominant negative nature of the GSEs was validated by several assays (14). With the discovery of the RNA interference phenomenon, several other potential tools for genetic synthetic lethality screens became available: long double-stranded RNA (34–38), short double-stranded RNA, resembling small interfering RNA (39, 40), and small inverted repeats (19–29 bp) expressed to create short hairpin RNA (41). A challenge for functional genomics would be to implement any of the mentioned tools for genetic synthetic lethality screens in MEFs so that advantage can be taken of the multitude of available knockout mice.

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