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ORIGINAL ARTICLE

Non-viral S/MAR vectors replicate episomally *in vivo* when provided with a selective advantage

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The ideal gene therapy vector should enable persistent expression without the limitations of safety and reproducibility. We previously reported that a prototype plasmid vector, containing a scaffold matrix attachment region (S/MAR) domain and the luciferase reporter gene, showed transgene expression for at least 6 months following a single administration to MF1 mice. Following partial hepatectomy of the animals, however, we found no detectable vector replication and subsequent propagation *in vivo*. To overcome this drawback, we have now developed an *in vivo* liver selection strategy by which liver cells transfected with an S/MAR plasmid are provided with a survival advantage over non-transfected cells. This allows an enrichment of vectors that are capable of replicating and establishing themselves as extra-chromosomal entities in the liver. Accordingly, a novel S/MAR plasmid encoding the *Bcl-2* gene was constructed; Bcl-2 expression confers resistance against apoptosis-mediated challenges by the Fas-activating antibody Jo2. Following hydrodynamic delivery to the livers of mice and frequent Jo2 administrations, we demonstrate that this Bcl-luciferase S/MAR plasmid is indeed capable of providing sustained luciferase reporter gene expression for over 3 months and that this plasmid replicates as an episomal entity *in vivo*. These results provide proof-of-principle that S/MAR vectors are capable of preventing transgene silencing, are resistant to integration and are able to confer mitotic stability *in vivo* when provided with a selective advantage.
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INTRODUCTION

Conventional non-integrating non-viral gene therapy vectors lack the ability to replicate their genomes after delivery to mammalian cells. This leads to the loss of vector molecules during cell division and only transient expression of the encoded transgene. Although successful in providing sustained expression, integrative non-viral vector systems based on zinc finger/nuclease, transposase or integrase systems can potentially cause insertional mutagenesis and genotoxicity. Non-viral vectors, which are maintained as nuclear episomes and therefore avoid physical integration into the genome, provide a safer alternative to these constructs.[1] However, the first developed self-replicating circular plasmid DNA (pDNA) vectors were based on viral replicons of viruses such as the Simian Virus 40 or Epstein–Barr virus.[2] Their potential as a gene therapy vehicle is limited, as Simian Virus 40 has been found to integrate into the host genome[3] and its T-antigen is a known oncogenic protein. Epstein–Barr virus, the causative agent of infectious mononucleosis, is associated with the occurrence of malignancies such as Burkitt's lymphoma and nasopharyngeal carcinoma,[4] and its EBNA 1 protein has been strongly implicated as a contributing factor to such events.[5]

By contrast, episomal vector systems based on chromosomal elements have been developed, in which the incorporation of a scaffold matrix attachment region (S/MAR) replaces the need for any virally encoded protein to ensure episomal replication and maintenance. S/MARs are DNA sequences, which bind chromatin to the nuclear matrix. They are ubiquitously present and highly sequence-conserved in eukaryotic chromosomes and therefore provide less potential immunological risk.[6] In addition, S/MAR vectors are able to prevent epigenetic silencing[7,8] by insulating the transgene sequence from heterochromatinization, thus ensuring its maintenance in a transcriptionally active chromatin environment. A pDNA vector system called pEPI, containing the mammalian S/MAR elements of the human β-interferon gene cluster, has been shown to be stably retained as episome for several hundreds of generations after initial selective pressure *in vitro* in CHO-KI,[9] HeLa[10] and K562 cell lines.[11] The mitotic stability of these plasmid vectors appears to be achieved via direct interaction of the S/MAR-DNA with components of the nuclear matrix in transcriptionally active sites of the chromosome, which bind to the scaffold attachment factor A protein, a major constituent of cellular chromatin and chromosomes.[12]

However, in contrast to the impressive ability of the S/MAR to provide mitotic stability *in vitro*, rapid cell proliferation *in vivo* as observed during partial hepatectomy results in loss of S/MAR vectors and subsequent decline of expression. For instance, after surgical removal of two-thirds of the liver and subsequent regeneration, transgene expression drops to 0.7% of the pre-hepatectomy level.[8] This loss of expression is not entirely unexpected, since *in vitro* studies also show a quick loss of S/MAR-vector pDNA within a week after transfection into dividing cells if no initial selection pressure is applied.

Remarkably, so far stable episomal S/MAR plasmid replication *in vivo* has only been achieved in pig fetuses generated by sperm-mediated plasmid gene transfer during fertilization.[13] This suggests that the unique setting as occurring physiologically during the fetal period may aid S/MARs' enrichment against non-transfected

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cells. In the present study, we created a model environment that provides S/MAR pDNA-transfected cells with a selective advantage *in vivo*[14,15] in the adult mouse. We utilized the specific induction of cell death by the Fas antigen, a transmembrane liver protein that induces apoptosis on engagement with its physiological ligand, FasL. This pathway can be activated *in vivo* by administration of an agonistic Fas antibody, Jo2. The inhibition of Jo2-mediated cell death can in turn be achieved by overexpression of Bcl-2.[16]

RESULTS

We have previously constructed a liver-specific S/MAR plasmid, pLucA1, and shown its ability to confer strong and stable levels of luciferase expression over a 6-month period in the adult liver.[8] For the experiments presented here we used this vector as a basis for the construction of a biscistronic expression cassette in which Bcl-2 is positioned as the first transcribed gene to provide high levels of Bcl-2 for protection against Fas-induced apoptosis, while expression of the second transgene luciferase permits evaluation of plasmid fate over time. The new S/MAR plasmid is called pBcLucA1 and its control without the S/MAR element is called pBcLucA1 Control. Both plasmids are driven by the liver-specific AAT promoter and are identical except for the presence or absence of the S/MAR element (Figure 1).

Selective S/MAR plasmid expression in NOD-SCID mice

The two pDNA vectors were administered by hydrodynamic tail vein injection to separate groups of NOD-SCID mice (n=8) and luciferase expression in all groups was monitored over a 3-month period using a Xenogen (Caliper Life Sciences Ltd., Runcorn, UK) *in vivo* bioluminometer. To provide S/MAR-plasmid-transfected cells with a selective advantage over resident hepatocytes, non-lethal doses of Jo2 were administered twice weekly (at a concentration of 0.12 mg/kg) to half of each cohort (n=4) to demonstrate the protective effect of Bcl-2 against Jo2. A separate negative control group of mice was injected hydrodynamically with pLucA1 (which lacks the *Bcl-2* transgene) and treated identically with Jo2 to show its apoptotic effect in the absence of Bcl-2.

As shown in Figure 2, expression 24 h after injection of all pDNA constructs was followed by a decline of levels in the initial days due to clearance of damaged transfected cells.[17] Subsequently, a sustained level of luciferase expression is observed over 60 days in the group treated with pBcLucA1 and Jo2 (pBcLucA1+Jo2), at approximately 4×10^8 photons s^{-1} cm^{-2} sr^{-1} (after a drop from the initial value of 3×10^{10} at 24 h post injection). This clearly demonstrates the protective effect of Bcl-2 expression against Jo2, comparing well with the control group treated with pBcLucA1 that did not receive Jo2 (pBcLucA1-Jo2). At day 60 the pBcLucA1+Jo2 and pBcLucA1-Jo2 groups still sustain similar levels of expression (1.4% and 2.2% of values 24 h post

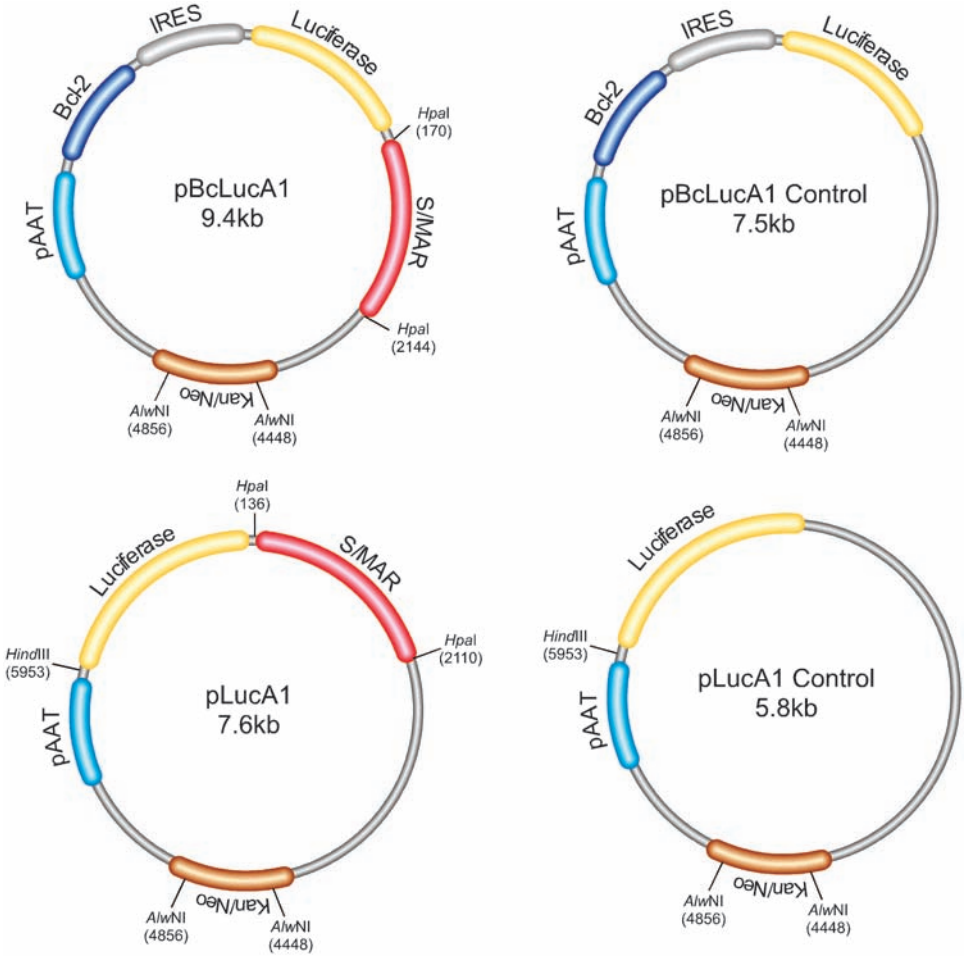

Figure 1 Plasmid vectors used in this study. The plasmids used in this study were based on pLucA1, in which luciferase expression is driven by the minimal human AAT promoter 8. The pDNA systems prepared were pBcLucA1 (*Bcl-2* gene; S/MAR element), pBcLucA1 Control (*Bcl-2* gene; no S/MAR element), pLucA1 (no *Bcl-2* gene; S/MAR element) and pLucA1 Control (no *Bcl-2* gene; no S/MAR element).

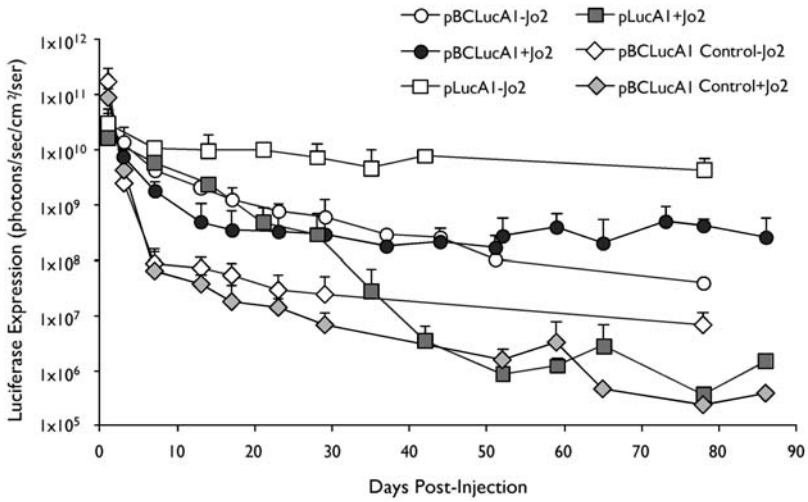

Figure 2 Longitudinal study of the expression profiles of plasmid DNA vectors, with and without treatment with Jo2. The figure illustrates the longitudinal study of the mice, treated with and without twice-weekly doses of 0.12 mg kg^{-1} Jo2 for up to 3 months, as quantified using Xenogen LivingImage software. Luciferase levels continue to fall in animals receiving Jo2, with the exception of the pBcLucA1+Jo2 group. The background level of light emission on non-treated animals is 3×10^5 photons s^{-1} cm^{-2} sr^{-1}. Mean ± s.e.m. (n=3) for each time point is shown.

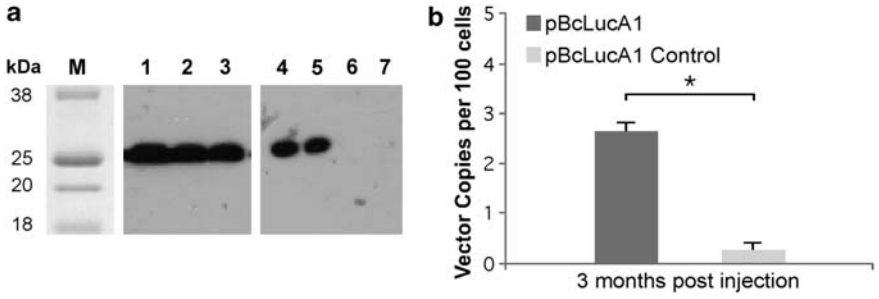

Figure 3 Sustained level of Bcl-2 expression from pBcLucA1 but not with the non-S/MAR control. (**a**) Western analysis of Bcl-2 expression from liver tissue, isolated from mice at 24 h and at day 90 following injection with pBcLucA1 and pBcLucA1 Control, and following subsequent Jo2 administrations. Western analysis of Bcl-2 expression is shown at 24 h post injection with pBcLucA1 (lanes 1 and 2) and pBcLucA1 Control (lane 3), and at 90 days following injection with pBcLucA1 (lanes 4 and 5) and pBcLucA1 Control 90 days following injection (lanes 6 and 7). The non-S/MAR plasmid is unable to maintain Bcl-2 expression over the 3-month period correlating with the loss of luciferase levels shown by bioluminescent imaging. The predicted size of the Bcl-2 protein is 26 kDa. M, Precision Plus Protein Standard. (**b**) Comparison of vector copy numbers of pDNA from animals treated with Jo2 at 3 months post-injection. DNA was extracted from livers removed at the end of the experiment and the number of vector genomes per diploid genome is shown. *$P<0.05$.

hydrodynamic delivery, respectively), and at the termination of the 3-month experiment expression in the pBcLucA1+Jo2 group is 13-fold higher than expression from pBcLucA1-Jo2, suggesting that Jo2 is able to promote proliferation of transfected hepatocytes. However, despite this, no substantial amplification of the luciferase levels was observed that would indicate repopulation of the liver with pBcLucA1 during the experimental period despite the survival advantage provided. The sustained transgene expression in the pBcLucA1+Jo2 throughout the experiment (corresponding to 24 rounds of Jo2 treatment) suggests that while there is no obvious expansion of transfected cells, the number of vector copies is not reduced.

Quantitative PCR was performed at the termination of the experiment to compare the relative copy number of plasmid molecules in the Jo2-treated groups, as shown in Figure 3b. The results reveal an approximately 10-fold higher copy number of pBcLucA1 vector over that of pBcLucA1 Control at 3 months, indicating the ability of S/MAR to maintain a higher level of vector copies over that of the non-S/MAR control plasmids, most likely by replication, as indicated by the Southern analysis (Figure 4).

The lost of expression in animals treated with pLucA1+Jo2 serves to show that Jo2 continues to exert its apoptotic effect against unprotected hepatocytes throughout the duration of the experiment in immune-compromised NOD-SCID mice. In the case of the non-S/MAR controls (pBcLucA1 Control+Jo2; pBcLucA1 Control-Jo2), luciferase levels were found to decline within the first 2 weeks after administration to roughly 0.06% of the initial values 24 h post administration regardless of Jo2 treatment as expected.

Long-term Bcl-2 expression was confirmed by a western analysis performed on liver sections taken from the animals treated with pBcLucA1+Jo2 or pBcLucA1 Control+Jo2 at the end of the 3-month experimental period (shown in Figure 3a). The loss of Bcl-2 expression from the non-S/MAR-treated animals correlates very well with the loss of luciferase expression measured by bioluminescence imaging (seen in Figure 2), further confirming the requirement of an S/MAR element to maintain transgene expression. The sustained expression of Bcl-2 from the pBcLucA1+Jo2-treated animals in contrast to those treated with pBcLucA1 Control indicates the

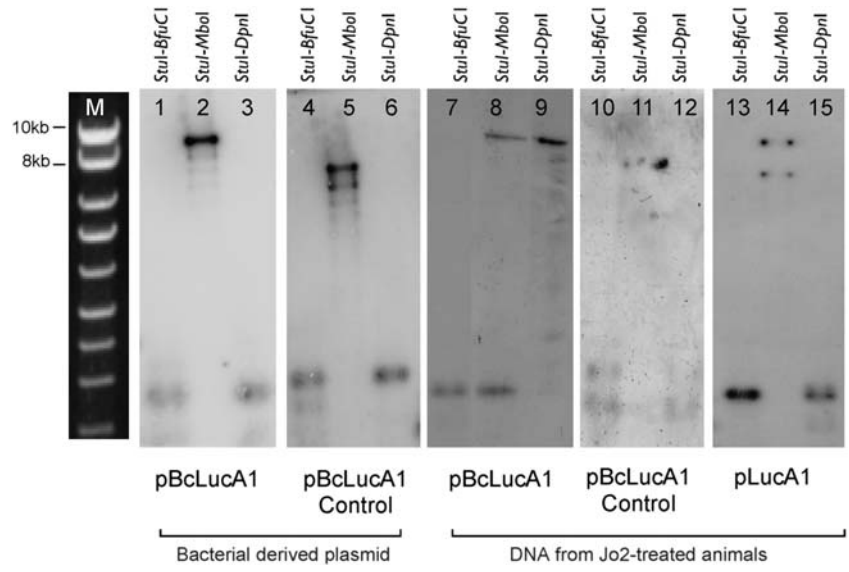

Figure 4 Plasmid pBcLucA1 replicates *in vivo*. (**a**) Replication-dependent assay of plasmids isolated from the livers treated twice weekly with Jo2. Total liver DNA (30 μg) was pooled together from each animal group (n=3) and digested with *Stu*I and further digested with *Dpn*I (lanes 3, 6, 9, 12 and 15), *Mbo*I (lanes 2, 5, 8, 11 and 14) or *Bfu*CI (lanes 1, 4, 7, 10 and 13) enzyme. The positive controls (20 ng linearized bacterial-derived pBcLucA1 and pBcLucA1 Control) are shown (lanes 1–6). M, Hyperladder I (Bioline, London, UK).

requirement of the S/MAR sequence to achieve resistance of the hepatocytes to Jo2-mediated death.

Episomal replication of pBcLucA1 in the liver

To provide physical proof that hepatocytes transfected with pBcLucA1 had replicated when challenged with Jo2, we performed a replication-dependent restriction assay. Total liver DNA was isolated from animal groups treated with pBcLucA1+Jo2 and pBcLucA1 Control+Jo2 at the end of the 3-month experiment and was digested with *Stu*I, a single cutter, to linearize these plasmids, before further digestion overnight with the methylation-sensitive enzymes *Dpn*I, *Mbo*I or *Bfu*CI. All three enzymes recognize the same sequence (GATC). *Dpn*I requires methylation of the target DNA by bacterial cells for restriction, while *Mbo*I restiction is dependent on mammalian DNA methylation and *Bfu*Cl cuts regardless and does not distinguish the source of methylation. The restriction fragments were separated on an 0.8% agarose gel, then blotted and probed with a 408-bp fragment from the kanamycin resistance gene common to all constructs (depicted in Figure 1). This replication-dependent assay was not performed on DNA from animals not treated with Jo2, as we expect passive episomal maintenance without selective pressure as previously published.[8]

The Southern analysis (Figure 4) serves to compare the restriction pattern of pBcLucA1 in liver DNA isolated from the pBcLucA1+Jo2-treated animal group (lanes 7–9) with that from the pBcLucA1 Control group (lanes 10–12) and with the restriction pattern of pLucA1 in DNA isolated from the pLucA1+Jo2 group (lanes 13–15). Lanes 1–6 show the control restrictions on pDNA prepared from bacterial cultures. A loss of bacterial methylation of the plasmids is found only in DNA isolated from the pBcLucA1+Jo2-treated group when restricted with *Stu*I/*Mbo*I or *Stu*I/*Dpn*I, respectively (lanes 8 and 9). Successful restriction by *Mbo*I is indicated by conversion of the linear *StuI* band to restriction fragments (lane 8), and lack of restriction by *Dpn*I leaves the single *Stu*I-linearized band intact (lane 9). As *Mbo*I only cuts mammalian-derived DNA, while *Dpn*I requires bacterial methylation for restriction, this indicates that pBcLucA1 plasmid has been replicated in hepatocytes of the pBcLucA1+Jo2 group. The appearance of a *Stu*I-linearized band in the *Stu*I/*Mbo*I plasmid digest of pBcLucA1+Jo2-treated animals (lane 8) represents the presence of the originally administered, not replicated plasmid, which is resistant to *Mbo*I digestion. pDNA of pBcLucA1 and pBcLucA1 Control isolated from bacteria was completely resistant to digestion with *Mbo*I (lanes 2 and 5), as indicated by the *Stu*I-linearized band of expected similar size. In all other groups, DNA digested with *Stu*I–*Dpn*I shows digestion with *Dpn*I after linearization with *Stu*I, indicating the presence of bacterial plasmids (lanes 3, 6, 12 and 15). Finally, digestion with *Stu*I-*Bfu*CI is not blocked by any kind of methylation and serves as a positive control (lanes 1, 4, 7, 10 and 13). These data indicate that the S/MAR-harbouring pBcLucA1, when transfected into hepatocytes and provided with a survival advantage, is able to replicate and subsequently express protein under selective pressure, while replication and maintenance of expression was not observed with the non-S/MAR plasmid. We conclude that, similar to studies *in vitro* in which S/MAR-endowed pDNA is able to achieve mitotic stability when untransfected cells are selectively killed by antibiotic-supplemented medium, S/MAR is also able to confer replication *in vivo* when the plasmid is maintained by a selective advantage over non-transfected cells. The correct size of the restriction bands suggests mitotic stability without gross rearrangements of the replicating plasmid.

DISCUSSION

Despite the ease by which S/MAR is able to provide mitotic stability *in vitro*,[18] rapid cell proliferation *in vivo* results in loss of pLucA1 and subsequent decline of expression.[8] Our study shows that the protection provided by Bcl-2 to S/MAR-transfected hepatocytes was sufficient to achieve mitotic stability of a small proportion of S/MAR plasmid-containing hepatocytes *in vivo*. It has been previously reported that established cell clones containing S/MAR plasmids are found exclusively in euchromatin nuclear compartments of active transcription[18] and are associated with histone markers of active

transcription (such as H3K4m3, H3K4m1 and H3K36m3) during most phases of the cell cycle.[19] During mitosis, histone modifications on the S/MAR plasmid decreases, allowing the plasmid to adopt an open permissive conformational change, similar to endogenous active genes in the S-phase.[19] The initial selection step, however, is critical to enrich the mitotically stable episomal plasmids *in vitro*, and lack of selective pressure results in less than 1% of replicating cells retaining the vector after 1 month.[7,20]

Both *in vitro* and *in vivo* the stable attachment of S/MAR plasmid to the chromatin and its subsequent replication is a very rare event. S/MAR-plasmid attachment appears to require a reorganization of chromatin during cell division. However, cell division also results in dilution or loss of non-attached plasmid. In both systems this is prevented, on penalty of cell death, by providing the plasmid with a selective advantage to the cell. This ensures plasmid maintenance over several rounds of cell division, during which a few of them acquire a self-replicating status. *In vitro* selection strategies have made it possible to achieve self-replicating S/MAR plasmid in all transfected cells. However, *in vivo,* where the level and duration of selection pressure is limited by the need to keep the animal alive, this conversion remains a rare event. This is demonstrated in our replication-dependent restriction analysis (Figure 4) by the presence of only weak bands of plasmid that has undergone mammalian methylation and the presence of plasmid that has been maintained but not replicated. Furthermore, as the usual mechanism for plasmid under selective pressure to become maintained is by genome integration, it was important to demonstrate that this is not the mechanism observed here, wherein a clear Southern blot signal was used to indicate the presence of S/MAR episomes. We cannot, however, exclude the possibility of extremely low-level integration events.

Our study demonstrates for the first time the ability of S/MAR plasmids to replicate and establish mitotic stability at a detectable level after application to an adult organism *in vivo* when provided with an initial selection. Beyond the proof of principle shown here, we would like to suggest that in some genetic diseases that cause liver cell damage and cell death, such as tyrosinaemia, Wilson's disease and alpha-1-antitrypsin deficiency (liver-type ZZ mutation), an episomal vector expressing the non-mutated protein/and or siRNA to suppress expression of the mutated protein could provide a selective advantage and may perhaps be developed into a therapeutic approach. For a broader application, a more effective and safer mechanism to provide a general selective advantage for S/MAR plasmids would need to be developed.

MATERIALS AND METHODS

Vectors used in this study

The plasmids used in this study were based on plasmid pLucA1, in which luciferase expression is driven by the minimal human AAT promoter.[8] The *Bcl-2* gene (kindly provided by Dr Georgina Lang, Cancer Research UK) was inserted into the *Sma*I site of a commercially available plasmid, pIRES-eGFP (Clontech, Mountain View, CA, USA), and the Bcl-2-IRES sequence was excised by *Eco*RI–*Nco*I digestion and inserted into the *Hin*dIII site of pLucA1 directly after the AAT promoter. The control plasmids were derived by removal of the S/MAR region (obtained from the human β-interferon gene) using *Hpa*I digestion. The plasmids were amplified in *Escherichia coli* DH10B cells (Invitrogen, Paisley, UK) and isolated using an Endotoxin free Maxiprep Kit (Qiagen, Crawley, UK). All restriction enzymes were purchased from NEB Biolabs (New England Biolabs, Hitchin, UK).

Animal work

NOD-SCID mice (n=8) (1–2 months, 18–22 g) (Harlan, Shardlow, UK) were hydrodynamically injected with 2.5 ml phosphate-buffered saline containing 50 μg of each pDNA vector using a 25-gauge needle. At 24 h and regular intervals following hydrodynamic injections, mice were injected intraperitoneally with 300 μl D-luciferin (Gold Biotechnology Inc., St Louis, MO, USA) (15 mg ml^{-1} in phosphate-buffered saline), anaesthetized by isofluorane and then imaged for bioluminescence 24 h later using the IVIS Imaging 50 Series (Xenogen). To induce apoptosis of hepatocytes, sub-lethal doses of a hamster monoclonal anti-Fas antibody called Jo2 (Pharmingen, San Diego, CA, USA) at 0.12 mg kg^{-1} were administered intraperitoneally twice weekly for 90 days beginning 48 h after hydrodynamic injection to half of each group (n=4). Bioluminiscent imaging was performed using a light-tight chamber on a temperature-controlled, adjustable stage, while isofluorane was administered by means of a gas manifold at a flow rate of 2%. The Xenogen system reports bioluminescence as photons s^{-1} cm^{-2} sr^{-1} in a 2.86-cm-diameter region of interest encompassing the liver. The autofunction was used to define the minimum for the scale at each time point. This value was 5% of the maximum in each case. Data were analysed using LivingImage 2.50 software (Xenogen). Animals were given adequate care in compliance with institutional and UK guidelines.

Western analysis

Liver tissue was lysed in protein lysis buffer (20% sodium dodecyl sulphate, 10 mM Tris, pH 7.5) and the protein concentration determined using BCA assay. Samples with 0.5 μg of total protein were resolved by sodium dodecyl sulphate-PAGE on a 4–12.5% gel and transferred to a polyvinylidene difluoride membrane. The membranes were blocked with 5% non-fat dry milk in 0.1% Tween-20 and incubated overnight at 4 °C with the mouse primary antibody against Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:200 dilution, followed by the secondary horseradish peroxidase-conjugated antibody (Dako, Ely, UK) at a 1:1000 dilution. Protein bands were visualized using an enhanced chemiluminescent kit according to the manufacturer's specifications (Piercenet, Thermo Fisher Scientific, Rockford, IL, USA).

Quantitative PCR

Amounts of isolated pDNA from hepatocyte samples were calculated by real-time PCR using an ABI PRISM 7000 sequence detector (Applied Biosystems, Warrington, UK). *PrimerExpress* software was used to design oligonucleotide primers (Invitrogen) and TaqMan probe (Eurofine MWG Operon, Ebersberg, Germany) for luciferase, to determine the amounts of S/MAR plasmid, and primers and probes specific for the mouse Titin gene to enable normalization between the samples by calculating the number of cells used as the input. The primers for the luciferase gene were as follows: forward 5′-GGCGCGTTATTTATCGGAGTT-3′; reverse 5′-CCATACTGTTGAGCAATTCACGTT-3′. The probe sequence was 5′-FAM-TGCGCCCGCGAACGACATTTATAAT-TAMRA-3′. Amplification reactions (25 μl) contained 5 μl of template DNA, 12.5 μl of Platinum Quantitative PCR Supermix-UDG with Rox (Invitrogen), 0.1 mm primers and 0.2 mm probe. Following the initial steps at 50 °C (2 min) and then at 95 °C (10 min), PCR was carried out for 40 cycles of 95 °C (15 s) and then of 60 °C (1 min). Serial dilutions of plasmids containing appropriate sequences to produce a standard amplification curve for quantification were carried out and all samples were tested in triplicate. Error bars represent mean values ± s.e.m. (*P<0.0001 between pBcLucA1 and pBcLucA1 Control vector copies).

Methylation-dependent Southern analysis

DNA was extracted from the livers at 90 days post-administration using a GenElute mammalian genomic DNA kit (Sigma-Aldrich Company Ltd., Gilingham, UK). The isolated DNA was quantified using a Nanodrop ND1-1000 spectrophotometer (Labtech International Ltd., Ringmer, UK). For the methylation-sensitive replication assay, total liver DNA (30 μg) was pooled together from each animal group (n=3) and digested with a single cutter *Stu*I and further digested with either *Dpn*I, *Mbo*I or *Bfu*CI enzyme overnight, separated on 0.8% agarose gels (20 V, 20 mA overnight) and blotted onto nylon membranes (Hybond XL, Amersham plc, Little Chalfont, UK). A 408-bp DNA fragment derived from the restriction digest of a segment of the kanamycin region, which is common to all plasmids, using enzyme *Alw*NI was labelled with ^{32}P (Rad-Prime labelling kit, Invitrogen) and applied as a probe.

The hybridization was performed in Church buffer (0.25 M sodium phosphate buffer, pH 7.2, 1 mM EDTA, 1% bovine serum albumin, 7% sodium dodecyl sulphate) at 65 °C for 16 h).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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