Adenovirus-mediated Cre deletion of floxed sequences in primary mouse cells is an efficient alternative for studies of gene deletion

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ABSTRACT

This study evaluates the utility of Cre-expressing adenovirus for deletion of floxed genes in primary cells using primary murine hepatocytes. Adenovirus infection was very efficient, even at very low MOI (>95% infection at a MOI of 6) and did not reduce viability. High level LacZ expression was cytotoxic to hepatocytes but Cre expression had no effect on viability. Cre-mediated recombination was completed within a timespan that permits experimentation during primary culture (>95% recombination after 24 h), independently of the number of floxed alleles per cell. Recombination did not induce p53 or produce cytological nuclear abnormalities (even in polyploid cells). Contrary to expectation, deletion of DNA ligase 1 did not alter cell cycle progression, although Cre expression hastens entry to S phase from G_1, independently of the presence of floxed sequences. We conclude that adenovirus-mediated deletion of floxed alleles in primary cells is a straightforward and highly efficient tool for conducting preliminary studies of conditional gene targeting. Primary cells have advantages of differentiation, relative purity and ease of experimentation within controlled conditions, while avoiding confounding problems encountered *in vivo* (i.e. target cell specificity, kinetics and level of recombination, and elicitation of inflammatory and immune responses). This system could help identify important phenotypic effects and design and interpret *in vivo* studies.

INTRODUCTION

The development of the Cre/lox system marks a milestone in mouse conditional gene targeting. In this approach, Cre recombinase excises a critical gene segment flanked by LoxP recognition sequences (floxed sequence) (1,2). However, to achieve successful tissue-specific, temporally controlled gene targeting using Cre/lox technology necessarily requires stringently regulated Cre expression in the strain with which floxed mice are mated. Indeed, while successful applications using Cre transgenic mice have been published, many failures and problems have also been reported (3). In particular, the desired tissue specificity and level and timing of induction are difficult to integrate using Cre transgenic lines: often mosaic expression, leakiness of the promoter or expression in unwanted tissues are reported and can confound the original purpose of the investigator. As an alternative to Cre transgenic mice, Cre-expressing adenoviruses have been used to overcome some of these problems (4,5). However, once again the system lacks the flexibility required for many studies: although target specificity can be achieved for some tissues by choice of the route of administration, many tissue types cannot be selectively infected. In theory, specific promoters can be inserted into the virus to achieve cell specificity, but the level of infection *in vivo* depends on the tissue. Finally, an important problem with this approach is the host immune and inflammatory response to adenoviral infection (6), which may remove the infected cells or itself cause tissue damage and disease, for example in the liver (7).

Therefore, whilst the benefits from *in vivo* study to assess the physiological function of a gene are clear, strong limitations remain to using Cre/lox technology for *in vivo* evaluations.

The use of primary cells in culture allows baseline data and principles to be established in a regulated setting without the drawbacks of *in vivo* work, but still benefiting from relatively differentiated and representative cells. From the perspective of Cre/lox conditional targeting, immune responses are avoided, target cell specificity is achieved by the isolation method, the time of recombination is dictated by the time of infection, and many experiments can be achieved with a limited number of cells, reducing to a minimum the number of animals used in subsequent *in vivo* work. However, whilst a few studies have been published that use primary cells and Cre/lox technology, to our knowledge no systematic evaluation of the applicability to primary culture of Cre-expressing adenovirus has been reported.

In the present study we show that infection of primary epithelial cells is very efficient, requiring a low multiplicity of infection (MOI) that avoids potential cytotoxicity. Furthermore, the interval from exposure to adenovirus to successful recombination is short, so the time available for biochemical or biological analysis is appropriate. Finally, we show that *in vitro* investigations revealed a significant effect of Cre recombinase on hepatocyte proliferation that would not readily have been detected *in vivo*.

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MATERIALS AND METHODS

Adenovirus propagation and titration

All adenoviruses used in this study are replication-deficient (E1 deleted), serotype 5 adenovirus. AdCA35LacZ encodes for the LacZ reporter gene (Ad-LacZ) (8) and Ad-Cre1 for Cre-recombinase (Ad-Cre) (5), under control of the CMV promoter. The control virus Ad-dl70-3 has no inserted transgene (Ad-70) (9). Viruses were propagated in 293 cells and titrated using a plaque assay as previously described (10). Stock titrations were: Ad-LacZ, 1.3×10^{10} (SEM 7.8×10^8); Ad-Cre, 3.1×10^{10} (SEM 2.1×10^9); Ad-70, 1.9×10^{11} (SEM 8.3×10^9). Virus stocks were aliquoted and stored at –70°C.

Hepatocyte isolation, culture and adenovirus infection

Primary hepatocytes from adult mice (6–12 weeks old) were isolated by a two-step retrograde perfusion procedure as previously described (11). Hepatocytes were plated onto fibronectin-coated chamber slides (Lab-Tek) or 24-well tissue culture plates (Greiner) at $0.2–0.3 \times 10^5$ per cm^2 in serum-free modified Chee's medium.

Adenovirus infections were performed 2–3 h after plating. Cells were incubated for 1 h at 37°C with a reduced volume of culture medium containing the virus at the appropriate concentration, then re-fed with fresh medium.

Mouse strains

Wild-type mice were from mixed backgrounds or littermates of the floxed mice, depending on the experiment. ROSA 26 Flox-Stop-βgeo mice (12) (Flox-Stop LacZ) have a floxed stop sequence inserted between the proviral β-geo gene (LacZ–neomycin fusion protein) and its promoter, preventing LacZ expression. Upon recombination the stop sequence is excised, and β-galactosidase expressed. LIG1$^{Flox/-}$ (13) bears one floxed ligase 1 allele and one null ligase 1 allele. Rb$^{Flox/Flox}$ are homozygous for the Rb floxed allele, in which exon 19 is flanked by LoxP sequences (14).

MTT assay

Cultures were incubated for 2 h in the presence of 1 mg/ml MTT [tetrazolium salt of 3-(4,5-dimethylthiazoyl-2-yl) 2,5-diphenyltetrazolium bromide]. The medium was removed, the well dried and crystals dissolved in DMSO. Values are average ± SEM absorbance at 490 nm of triplicate experiments.

Quantification of apoptosis

Cells were stained using Feulgen staining and light green counterstain. Briefly, cells were fixed in Boom's fixative at 4°C overnight. Slides were treated with 5 M HCl for 45 min then stained with Schiff reagent. Apoptosis was quantified according to morphology. Results are the percentage ± SEM of 500 cells counted in duplicate. Experiments were performed three times with similar results.

BrdU immunocytochemistry

Hepatocyte cultures grown on chamber slides were incubated with 40 μM BrdU (Amersham) for 6 h and fixed in 80% ethanol. Immunodetection of BrdU incorporation was performed as previously described (11) using rat anti-BrdU IgG (1/100 dilution) (Sera Labs, Sussex, UK) as primary antibody and rabbit anti-rat IgG HRP conjugate (1/100 dilution) as secondary antibody. Slides were counterstained with haematoxylin and light green. Negative controls omitted the primary antibody. BrdU incorporation was estimated by counting positive cells. Results are the percentage ± SEM of 500 cells counted in duplicate. Experiments were performed three times using cells isolated from different mice strains with similar results.

Western blotting for Cre recombinase expression

Total cell protein (2 μg) was prepared from primary hepatocytes using RIPA buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, protein inhibitor cocktail pellet) and subjected to SDS–PAGE on a 12% gel, transferred to nitrocellulose and probed with an anti-Cre rabbit polyclonal antibody (1:10 000) (no. 69050-3; Novagen) and secondary antibody donkey anti-rabbit HRP conjugate (1:2000) (sc-2313; Santa Cruz). Proteins were visualized by ECL.

Cre-mediated recombination

Cre-mediated recombination of the floxed allele was quantified by Southern blotting and PCR. Cells were lysed for DNA extraction various times after infection.

For LIG1$^{Flox/-}$, *Eco*RI digests were probed with a 1.2 kb *Eco*RI–*Hin*dIII genomic fragment. The 8.5 and 3.4 kb bands correspond to the floxed and the recombined allele, respectively. Quantification was by densometric analysis using a phosphorimager. Analysis was completed using Aida 2.0 software. Recombination was expressed as the percentage difference between the 8.5 and the 3.4 kb signals relative to the 6.7 kb band corresponding to the null allele.

For Rb$^{Flox/Flox}$, *Pst*I digests were probed with a 450 bp *Pst*I–*Pvu*II probe isolated from pHA153 (15). The 5 and 4.5 kb bands correspond to the floxed and the recombined allele, respectively (16). PCR analysis for recombination of the Rb^{Flox} allele used oligonucleotides Rb19 (5′-AACTCAAGGGA-GACCTG-3′) and Rb18 (5′-GGCGTGTGCCATCAATG-3′) as previously described (16). Thermocycling was as follows: step 1, 4 min at 94°C; step 2, 35 cycles of 1 min at 94°C, 1 min at 58°C and 1 min at 72°C; step 3, 10 min at 72°C. Amplified products from Rb^{Flox}, wild-type and recombined alleles were separated on a 1.8% agarose gel, giving bands of 748, 680 and 300 bp length, respectively.

Statistical analyses

Analyses were done with Minitab for Windows v.13.0. For counts of BrdU, p53, apoptosis and abnormal nuclei, the proportion of affected cells was arcsine transformed and differences between means were evaluated by ANOVA. Differences were taken to be significant at $P < 0.05$. Satisfactory homogeneity of variances was determined with Bartlett's test. Where a significant difference between means was identified by ANOVA, the differences between individual means were analysed further with Bonferroni simultaneous tests for making multiple comparisons. For MTT data, the differences between medians were analysed with the Kruskal–Wallis test.
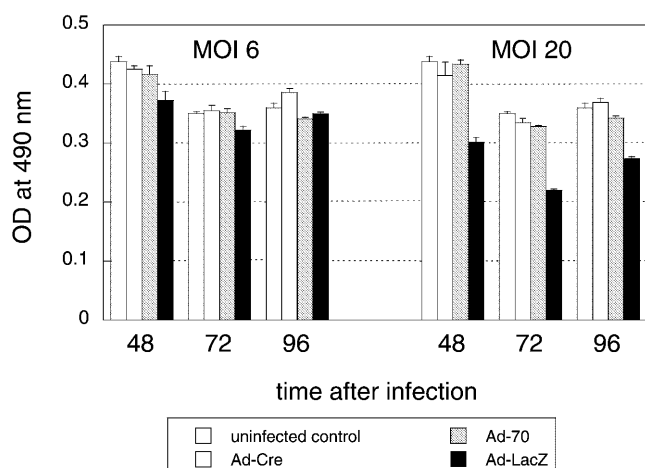
Figure 1. MTT assay performed on wild-type primary hepatocytes infected or not with adenoviral constructs at an MOI of 6 or 20. Results are the average of triplicates with standard error of the mean. The experiment was performed twice with similar results.

RESULTS

Adenoviral infection of primary hepatocytes is highly efficient and has no effect on cell viability

We used an adenovirus expressing the reporter gene LacZ to assess the efficiency of infection in primary epithelial cells. We found that a MOI as low as 4 is sufficient to infect >95% of primary hepatocytes. Similar results were obtained at our centre using Clara primary lung cells (J.M.Sallenave, personal communication) and by ourselves with primary murine renal epithelial cells (infection >95% at MOI 2, >98% at MOI 6) and epithelial colon cells (infection >95% of cells growing out of isolated crypts).

The impact of adenovirus infection and gene expression on hepatocyte health and viability was assessed with the MTT assay of mitochondrial metabolic activity over a period of 120 h. Cells infected at MOI 6 with adenovirus expressing LacZ (Ad-LacZ) or expressing Cre-recombinase (Ad-Cre) were not significantly different from the uninfected control, suggesting that neither adenovirus infection nor overexpression of an exogenous protein (Cre or β-galactosidase) had a significant effect on primary hepatocytes ($P = 0.102$, Kruskal–Wallis test) (Fig. 1). However, at higher MOI (MOI 20) infection with Ad-LacZ significantly decreased MTT staining compared with infection with the control adenovirus or Cre-expressing adenovirus ($P < 0.0001$, Kruskal–Wallis test). This suggests that high levels of expression of β-galactosidase but not Cre recombinase are toxic to hepatocytes (Fig. 1).

To investigate whether Cre-mediated recombination could cause hepatocyte apoptosis, we used wild-type and Flox-Stop LacZ primary hepatocytes infected with either Ad-70 or Ad-Cre. There were no significant differences in rates of apoptosis between the adenovirus treatments and untreated controls for cells of either genotype ($P = 0.659$ and $P = 0.554$ for Flox-Stop and wild-type, ANOVA) (a representative experiment is shown in Fig. 2). Thus there is no evidence that Cre-mediated recombination causes apoptosis.
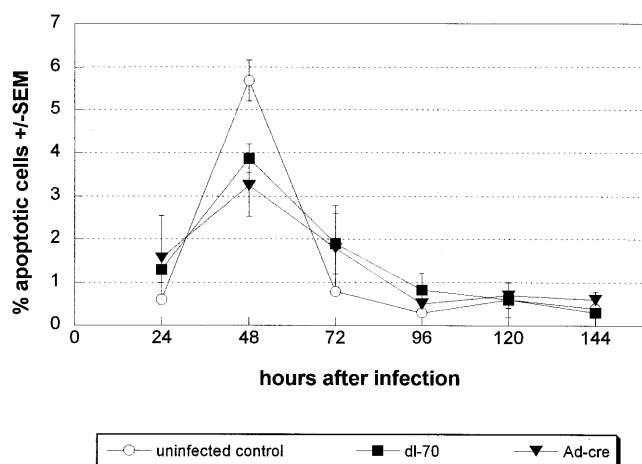

Figure 2. Percentage of apoptotic Flox-Stop hepatocytes after infection with Ad-Cre, Ad-70 or no infection. The figure shows the average percentage of apoptotic cells ± SEM in 500 cells for a typical experiment. Experiments were repeated in wild-type, Flox-Stop and other floxed hepatocytes and showed no significant effect of adenovirus infection on the level of apoptosis (the lower apoptosis level observed at 48 h in the present experiment was not observed in other experiments and is not statistically significant).
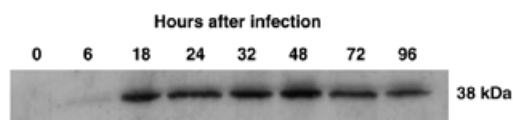

Figure 3. Western blotting for Cre recombinase expression after infection.

Adenovirus-mediated expression of Cre recombinase is rapid and accompanied by effective recombination

Expression of Cre recombinase was detected by western blotting 6 h after infection and remained high through to 96 h (Fig. 3). Cre-mediated recombination was tested in hepatocytes isolated from mice bearing three different floxed sequences. Firstly, with Flox-Stop LacZ primary hepatocytes, recombination excised the stop sequence, permitting expression of β-galactosidase. X-gal staining showed that >95% of exposed cells underwent recombination within 48 h after infection with Ad-Cre. Next, the kinetics of recombination were analysed with heterozygous and homozygous hepatocytes isolated from two different strains of mice (LIG1$^{Flox/-}$ and Rb$^{Flox/Flox}$). Recombination of the floxed LIG1 allele in primary hepatocytes heterozygous for ligase 1 (LIG1$^{Flox/-}$) was quantified by Southern blotting. Recombination occurred between 12 and 24 h after infection with Ad-Cre (MOI 6), with >95% deletion of the floxed allele (Fig. 4). A similar level of recombination was observed with MOI as low as 2. Recombination in primary hepatocytes homozygous for a floxed Rb allele (Rb$^{Flox/Flox}$) was quantified by both PCR and Southern blotting. Again, there was >95% recombination (deletion of the floxed allele), starting as early as 6 h after infection (Fig. 5). With both genotypes, recombination was complete within 24 h after infection, suggesting that homozygosity of the floxed sequence does not affect the kinetics of recombination. Moreover, most primary murine hepatocytes are polyploid (>80% of cells) (11),
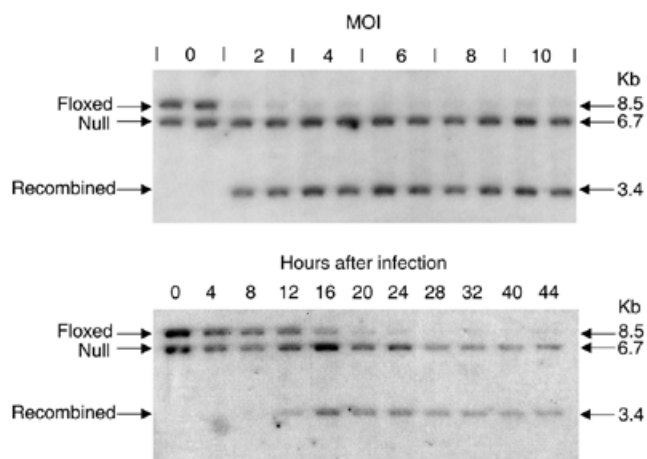
Figure 4. DNA ligase recombination. Southern blot analysis of the Lig1 locus after Ad-Cre infection. (Top) Duplicate infections at increasing MOI (0–10). (Bottom) Lanes show hours after infection with Ad-Cre at an MOI of 6.
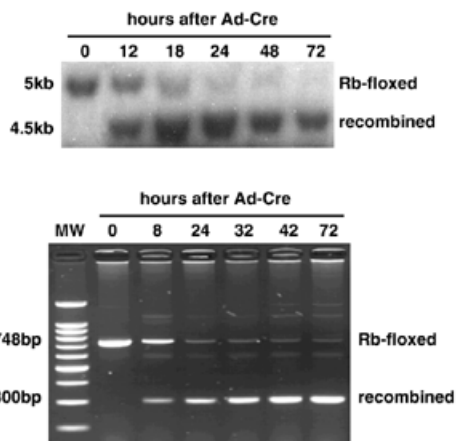

Figure 5. Kinetics of recombination of Rb$^{Flox/Flox}$. Southern blotting and PCR (top and bottom, respectively) showing the floxed and the recombined bands at indicated times after infection.

frequently with 4 and as many as 32 floxed alleles per cell. Hence, recombination between floxed sequences located on different chromosomes could be more likely than in diploid cells. However, no abnormal band was detected by Southern blotting in either genotype tested and LacZ expression was observed regardless of the cell ploidy, as assessed by the size of the nuclei, in Flox-Stop LacZ cells after infection with Ad-Cre.

It has also been reported that Cre recombinase may induce chromosomal abnormalities (17) that could lead to increased occurrence of abnormal mitosis and nuclear abnormalities. However, no abnormal mitoses were observed and the proportion of cytologically pleomorphic nuclei was similar between wild-type and Flox-Stop cells infected with Cre-expressing or control adenoviruses ($P = 0.164$, ANOVA) (Fig. 6). Taken together, these results suggest that the Cre/lox technology can be reliably used in polyploid cells (which are present in many tissues) and that the timing of recombination *in vitro* is appropriate for use in primary cultures even with primary cells with
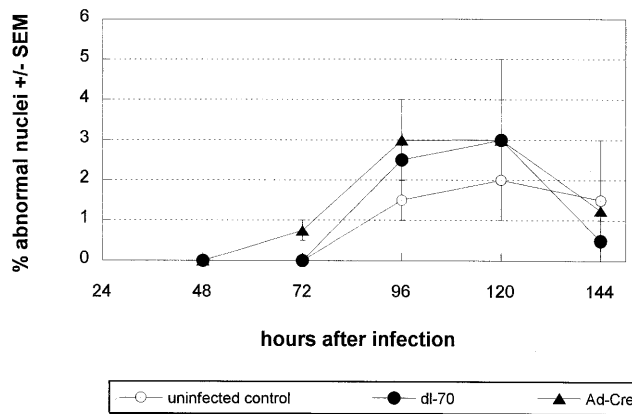

Figure 6. Abnormal nuclei in Flox-Stop hepatocytes after infection with Ad-Cre, Ad-70 or no infection. The figure shows the percentage ± SEM. Experiments were performed in wild-type and Flox-Stop hepatocytes, twice in duplicate with similar results.
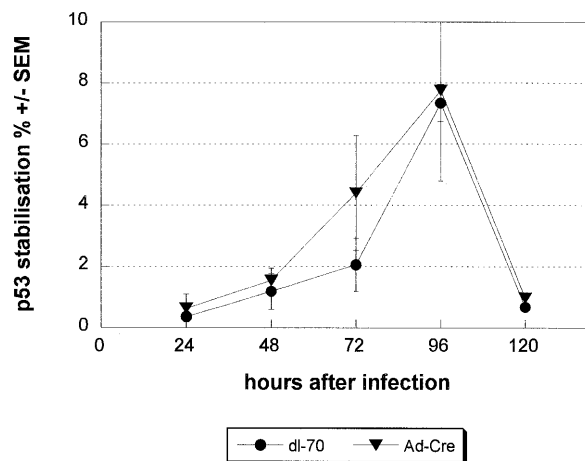

Figure 7. p53 stabilisation in Flox-Stop hepatocytes after infection with Ad-Cre or Ad-70. The figure shows the average of p53-positive cells (percentage of 500 cells ± SEM). Experiments were performed in wild-type and Flox-Stop hepatocytes, twice in duplicate with similar results.

reduced lifespans in culture, such as the primary hepatocytes used in the present study (10–14 days).

Adenovirus-mediated expression of Cre recombinase does not stabilise p53

p53 is a key protein for induction of cell cycle arrest and apoptosis in response to even very low levels of DNA damage (18). Potentially, therefore, induction of p53 by Cre-mediated recombination, which involves the creation of DNA strand breaks, could trigger unwanted cellular responses resulting from Cre-mediated targeting. We therefore investigated whether Cre-mediated recombination induced p53. Flox-Stop hepatocytes were isolated, infected with the various adenoviruses, and p53 stabilisation quantified by immunocytochemistry. There were no significant differences in p53 immunopositivity between uninfected cells, dl70 or Cre-infected hepatocytes ($P = 0.92$, ANOVA) (Fig. 7), suggesting that Cre-mediated DNA breaks during recombination are not recognised as DNA damage.
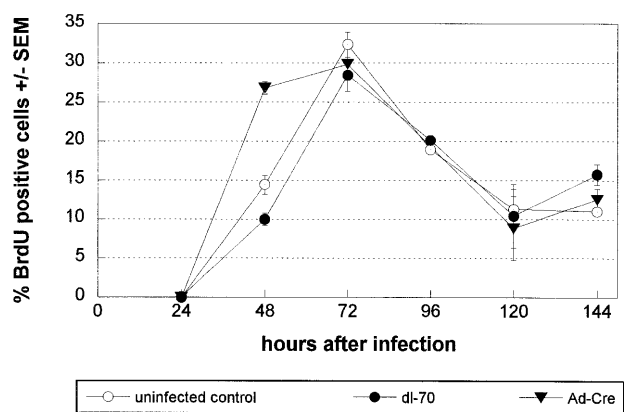
Figure 8. Proliferation of Flox-Stop hepatocytes after infection with Ad-Cre, Ad-70 or no infection. The figure shows the percentage ± SEM. Experiments were performed in wild-type, Flox-Stop and Lig$^{Flox/-}$ hepatocytes, and repeatedly, the percentage of cells incorporating BrdU at 48 h after infection was higher in Ad-Cre-infected cells ($P < 0.0001$).

Adenovirus-mediated expression of Cre recombinase accelerates entry into S phase, but DNA ligase 1 deletion has no effect on DNA synthesis

Isolation of hepatocytes from the liver stimulates them to enter the cell cycle, modelling liver regeneration. Cultured primary hepatocytes enter the cell cycle in a more or less synchronous manner, reaching an S phase peak ~72 h after isolation (11). Time courses of BrdU immunopositivity 0–144 h after adenovirus infection showed no significant differences in mean BrdU between uninfected controls, adenovirus (dl-70) infection, Cre expression or Cre-mediated recombination (ANOVA). This suggests that there were no significant differences in the total number of cells entering S phase over the time course. However, the ANOVA showed a significant interaction between adenovirus treatment and time ($P < 0.0001$), which suggests that the different adenovirus treatments behaved differently with time. Indeed, Cre-expressing adenovirus infection appeared to increase proliferation early (48 h) after infection, compared with other treatments, but not later (see for example Fig. 8). This effect was consistently apparent in different experiments regardless of genotype (wild-type, Flox-Stop or LIG1$^{Flox/-}$). Indeed, post hoc statistical analysis on 48 h time points of four experiments suggested that Cre does indeed produce an early and briefly increased BrdU positivity compared with dl-70 or uninfected controls ($P < 0.0001$ and $P < 0.0001$, respectively; ANOVA with Bonferroni simultaneous tests). We performed an additional experiment to evaluate this effect, concentrating on early time points (~48 h after plating), which gave similar results (data not shown). To test whether expression of Cre recombinase from a different vector had similar results, we attempted transfection (lipofection) of a plasmid encoding for Cre recombinase under control of the CMV promoter; however, no meaningful results were obtained due to a very low transfection efficiency (data not shown). The increased proportion of cells in S phase at 48 h in cells expressing Cre, but without significant differences in overall mean BrdU over the time course, suggests that Cre recombinase shortens G$_1$ in primary hepatocytes (i.e. hastens entry into S phase), rather than increases the total number of cells entering S phase.

There was no significant difference in overall mean BrdU immunopositivity between cultures after adenovirus-induced deletion of ligase 1, in dl-70-infected cells and in uninfected controls ($P = 0.668$, ANOVA). Hence, DNA ligase 1 does not appear, at least in hepatocytes, to be necessary for a normal pattern of DNA synthesis after stimulation to proliferate.

DISCUSSION

Cre/lox technology using adenovirus-mediated Cre expression can be used efficiently in primary cells

We have shown here that inducible gene targeting using adenovirus delivery of Cre to primary cells is efficient, rapid and without significant cytotoxicity. Rapid accumulation of reliable preliminary data on phenotypes is therefore possible, free of many confounding factors encountered *in vivo*: non-specificity and a lower rate of target cell infection, the timing of recombination and deletion of infected cells due to innate and immune responses. In this way, therefore, primary cell data can define and refine *in vivo* experiments and possibly give insights into the *in vivo* phenotype by providing a controllable and flexible study system. The high level of infection at low MOI observed in the different primary cells tested here and in other reports [pancreatic acinar cells (19) and breast epithelial cells (20)] shows that this technology is applicable to many epithelial cell types. We focused on liver cells for three main reasons. First, primary hepatocytes are commonly used in a variety of toxicological and environmental investigations, as well as cell cycle studies. Second, primary hepatocytes typically exhibit a reduced lifespan *in vitro* (10–14 days), making it a good model to investigate feasibility of Cre recombination studies. Third, although the liver is relatively easily and specifically infected *in vivo* with adenovirus to achieve conditional gene targeting (4,6,21–24), the development of hepatitis due to adenoviral infection is a major confounding factor to studies of liver cell biology (6,7,25,26). It must be acknowledged that for other study purposes hepatitis may not present a major problem, for example, a recent investigation of the consequences for blood pressure of deletion of a gene expressed in the liver (floxed human angiotensinogen trangene) (6).

β-Galactosidase is toxic to hepatocytes

The present findings that LacZ expression was toxic to hepatocytes support and extend *in vivo* observations of liver toxicity with high doses of adenovirus expressing LacZ, but where the effect of LacZ itself could not be clearly separated from toxicity due to immune or inflammatory responses to adenovirus infection (23,27). We have shown here that neither adenovirus infection nor overexpression of Cre recombinase is significantly cytotoxic, whereas β-galactosidase expression leads to lysis of primary hepatocytes *in vitro* infected with adenovirus at high MOI. We have observed similar lysis in hepatocytes when β-galactosidase is expressed at a high level from a transfected (lipofection) expression plasmid (S.Prost, unpublished observation). This is an important observation because *in vivo* direct toxic lysis of cells that express β-galactosidase could stimulate and exacerbate immune reactions to other adenovirus-infected hepatocytes (7). Indeed β-galactosidase

is known to be very immunogenic (27,28). Although Takeuchi *et al.* (29) did not observe a toxic effect of β-galactosidase in primary hepatocytes infected at an MOI of 24, the human CMV promoter used in that study is a much weaker promoter in mice than the mouse CMV promoter used here (8). Taken together, the data suggest that a high concentration of β-galactosidase is toxic to hepatocytes and could induce or at least exacerbate immune responses and hepatitis development after adenovirus infection *in vivo*. The use of an alternative reporter gene for liver studies would therefore be merited.

Polyploidy does not reduce the efficiency of Cre-mediated recombination or precipitate inappropriate recombination events

One concern about the Cre/lox technology is the possibility of unintended inter-allelic rearrangements when there are more than two intra-chromosomal LoxP sites; it has been reported that Cre recombinase under a strong promoter produced illegitimate chromosome rearrangements in haploid mouse spermatids (17), even without LoxP sites, suggested to be due to recombination between pseudo-LoxP sites (30). We did not formally investigate chromosomal rearrangement, but Cre expression in wild-type or Flox-Stop LacZ cells did not produce abnormal mitoses or nuclei.

Furthermore, no band of unexpected size was detected by Southern blotting in either LIG1$^{Flox/-}$ or Rb$^{Flox/Flox}$ cells after Cre expression, suggesting that no inappropriate inter-chromosomal recombination events took place at detectable level in these cells, which are mostly polyploid (>80%). However, a low percentage of recombination involving pseudo-LoxP and LoxP sites cannot be excluded.

One possible explanation is that the distance between LoxP sites is an important determinant of the probability of recombination. Indeed, recombination between LoxP sites on different chromosomes has been shown to occur at much lower frequency than intra-allelic recombination (31). Furthermore, whilst Cre recombinase may act at some pseudo-LoxP sites in the setting of a plasmid (30), recombination has not been shown between a pseudo-LoxP and a LoxP site and the frequency and distribution of pseudo-LoxP sites in the mammalian genome has not not reported (30). The degree and duration of Cre expression are also factors likely to influence the probability of illegitimate chromosome rearrangement (17). In the present study Cre recombinase was under the control of a strong CMV promoter. However, the study was carried out on cells in culture for ≤144 h, corresponding to a maximum of two cell cycles. This may not be enough to induce recombination from rare, less specific sites. In addition, the CMV promoter is rapidly switched off in hepatocytes (32,33), which is likely to reduce illegitimate recombination events.

It has also been suggested that recombination would be more efficient with Floxed/deleted mice than with homozygous Floxed animals, as only one allele needs to be recombined (34). However, the use of heterozygous Floxed/deleted mice may be impractical or undesirable if heterozygosity of the gene of interest has a phenotype (e.g. Rb). The present data suggest that even with polyploid cells that bear several copies of each allele there was no appreciable alteration of the kinetics of recombination.

Ligase 1 is not required for DNA synthesis but Cre affects proliferation, even in the absence of LoxP sites

We report for the first time the phenotype of deletion of DNA ligase 1 from adult hepatocytes. Homozygous germline deletion of DNA ligase 1 is embryonic lethal (35,36), so conditional gene targeting strategies are necessary to investigate the role of DNA ligase in adult somatic cells. There are suggestions that ligase 1 is critical for DNA replication (36 and references therein), however, previous work in our laboratory with a mammary gland-specific Cre transgenic failed to establish any effect of ligase 1 deletion on mammary epithelial cell proliferation (13; unpublished data). Here we have tested the hypothesis that ligase 1 is important for hepatocyte DNA synthesis. The synchronised entry into the cell cycle of primary hepatocytes in culture is an ideal system to address this question. The results indicate that, as in the mammary gland, ligase 1 is not a prerequisite for DNA synthesis.

The observation that Cre expression itself, regardless of the presence of floxed sequences, hastened the onset of DNA synthesis in hepatocytes after stimulation to proliferate is intriguing. The mechanism and its consequences for other studies using Cre recombinase are not clear. However, the observation would not have been as readily made *in vivo* without studies specifically designed to test liver regeneration and requiring considerably more animal resources.

In conclusion, we have shown that the Cre/lox technology using adenovirus infection in primary cells is an easy, reliable study system which can provide interesting and complementary information to *in vivo* studies. The cell specificity is defined by the isolation technique and the timing of Cre expression by the time of infection *in vitro*, there are no additional adverse effects due to the infection and important preliminary data can be readily accrued. Although *in vivo* studies are indispensable for physiological interactions, use of primary cells may reduce the number of transgenic animals required and may highlight or help define complex phenotypes.

ACKNOWLEDGEMENTS

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