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ORIGINAL ARTICLE

Baculovirus as delivery system for gene transfer during hypothermic organ preservation

Fernanda Murguía-Meca,[1]* Juan J. Plata-Muñoz,[2]* Richard B. Hitchman,[1,3] John O. Danquah,[1] David Hughes,[2] Peter J. Friend,[2] Susan V. Fuggle[2] and Linda A. King[1]

1 Insect Virus Research Group, School of Life Sciences, Oxford Brookes University, Oxford, UK
2 Nuffield Department of Surgical Sciences, University of Oxford, Oxford, UK
3 Oxford Expression Technologies Ltd, Gipsy Lane, Oxford, UK

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Correspondence
Professor Linda A King, Oxford Brookes University, Headington, Oxford, OX3 0BP, UK. Tel.: +44 1865 483 240; fax: +44 1865 483 242; e-mail: laking@brookes.ac.uk

Conflicts of Interest
RB Hitchman is Research Manager of Oxford Expression Technologies, Ltd.

*Both authors have contributed equally to this project

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Summary

Concerns over the safety of conventional viral vectors have limited the translation of gene transfer from an exciting experimental procedure to a successful clinical therapy in transplantation. Baculoviruses are insect viruses, but have the ability to enter mammalian cells and deliver potential therapeutic molecules with no evidence of viral replication. This study provides evidence of the ability of recombinant baculovirus to enter mammalian kidneys and livers during cold preservation. Six kidneys and six liver lobules retrieved from large pigs were perfused with University of Wisconsin (UW) solution containing a baculovirus tagged with green fluorescent protein and preserved for 8 h. In addition, six kidneys were perfused with UW containing a baculovirus expressing red fluorescent protein and preserved for 24 h. Green fluorescent virus particles were detected within transduced kidneys and livers after 8 h standard cold storage and red fluorescent protein mRNA was detected in kidneys after 24 h of cold preservation. There were no significant differences in tissue architecture, cell morphology or ATP content between experimental organs and their controls. *Ex vivo* transduction of organs with recombinant baculovirus during conventional cold preservation was demonstrated with no evidence of additional injury or reduction in cell viability.

Introduction

Gene transfer is an attractive and promising strategy for induction of graft protection and immunomodulation in experimental solid organ transplantation. Cumulative evidence underlines the potential of gene transfer to protect allografts from cold-induced injury and prolong the length of preservation [1–3] to reduce reperfusion injury after revascularization [4,5] and manipulate the innate and acquired immune responses triggered by transplantation [6,7]. However, increasing concerns over the safety of conventional viral vectors in human clinical trials [8,9], their restricted capacity to carry large or multiple genes and the length of time and resources required for their production [10] have limited the translation of gene transfer from an exciting experimental procedure to a successful clinical therapy.

Having evolved to deliver their genes into host cells, viruses are currently one of the most effective means of gene delivery into target mammalian or human cells. In organ transplantation adenoviruses, adeno-associated viruses and lentiviruses have been used almost exclusively as gene delivery vectors [11]. Unfortunately, these vectors are of limited utility in human gene therapy because of their pathogenicity, immunogenicity and the possibility of viral breakthrough [8–12]. Immune responses to adenovirus can cause severe side effects and complications, especially in immune incompetent patients [12]. Moreover,

the deaths of an 18-year-old man and a 27-year-old woman have been associated with the use of adeno-based vectors in human phase I/II gene therapy clinical trials [8]. All these problems call for a new perspective and radical thinking about future vector development for gene therapy in human organ transplantation.

Baculoviruses are insect viruses, but have the ability to enter mammalian cells and insert their genome into the nucleus with no evidence of cytotoxicity or viral replication even at high concentrations. These viruses, initially used as biological pesticides in agriculture and as effective systems for protein expression, have been proposed as an attractive delivery system for human gene therapy [13–17]. *Autographa californica* multinucleopolyhedrovirus (AcMNPV), one of the most extensively studied baculoviruses, efficiently transduces a wide range of human primary cells and cell lines with no evidence of competent virus breakthrough, changes in cell function or decrease in cell viability [3,13–17], offering a high level of biosafety for clinical transplantation. Additionally, because the natural host of baculovirus is phylogenetically distant from humans, AcMNPV-based vectors elicit a much lower innate immune response in comparison with mammalian viral vectors and do not face a pre-existing host memory immune response [3,13–17]. In addition, AcMNPV can accommodate large or multiple inserts into its genome and because AcMNPV is helper-plasmid independent, AcMNPV-based vectors are consequently easier, faster and relatively cheaper to produce than conventional viral vectors [10]. In summary, these advantages make AcMNPV-based vectors an attractive, alternative delivery system for gene therapy in human organ transplantation.

This preclinical study aimed to provide evidence of the efficiency of baculovirus as a gene delivery system during hypothermic organ preservation determining firstly, the ability of recombinant baculovirus to enter mammalian kidney and liver allografts and the effect of viral transduction on cell viability during hypothermic static cold preservation. We hypothesize that recombinant AcMNPV is able to transduce kidneys and livers from large mammals during conventional hypothermic organ preservation and does not increase the level of cell injury associated with standard hypothermic clinical preservation.

Materials and methods

Preclinical model of *ex vivo* viral transduction

Six female white-landrace pigs between 50 and 60 kg were housed and cared for in accordance with the Animal Welfare Act 2006 [18]. All pigs were premedicated and anaesthetized and their liver and kidneys were retrieved following standard clinical practice. Briefly, both organs were flushed *in situ* with cold Eurocollins solution (Soltran Kidney Perfusion Solution®; Baxter, Newbury, UK) and subsequently, the Eurocollins solution was flushed from the liver by portal and arterial perfusion and from both kidneys by arterial perfusion using cold University of Wisconsin (UW) solution (Viaspan®, Madison, WI, USA). Finally, each organ was placed in a plastic bag surrounded by fresh cold UW, immersed in ice and transported as clinical standard.

Production and analysis of recombinant baculovirus vectors, cells and viruses

Recombinant baculovirus vectors

AcVP39-eGFP is a recombinant AcMNPV in which the major structural capsid protein VP39 has been tagged with enhanced green fluorescent protein (eGFP) enabling the tracking of recombinant-virus particles in transduced cells and AcDsRed is a recombinant baculovirus in which the discosoma red (dsRed) gene has been inserted under control of the CMV gene promoter using the flashBAC system (Oxford Expression Technologies Ltd, Oxford, UK) enabling detection of gene expression in mammalian cells.

Vector design and construction

To make the recombinant baculovirus, AcEGFP-$VP39_{VP39NatP}$, a transfer vector pBacPAK8-EGFP-$EXON0_{VP39NatP}$ was made as follows. The polyhedrin gene promoter in the pBacPAK8 was replaced with AcNPV VP39 promoter. An EGFP gene was PCR amplified (JD_FP_EGFP: AAA CTG CAG AAG CTA GCG CTA CCG GTC/JD_RP_EGFP: AAC TCG AGA TCT AGT CCG GAC) from pEGFP-C1 (Molecular probe), sequenced (Geneservice Ltd, Oxford, UK) and inserted into pBacPAK8 (Molecular probe) to make pBacPAK8-$EGFP_{VP39NatP}$. A VP39 gene was PCR amplified (JD_FP_VP39: AAC TCG AGA TGG CGC TAG TGC CCG/JD_RP_VP39: AAT CTA GAG ACG GCT ATT CCT CCA CCT GC) from AcNPV C6, sequenced and inserted in-frame with EGFP to produce pBacPAK8-EGFP-$VP39_{VP39NatP}$. IPLB-Sf21 cells were transfected with pBacPAK8-EGFP-$VP39_{VP39NatP}$ and a *flash*BAC DNA according to the manufacturing instruction to produce recombinant baculovirus AcEGFP-$VP39_{VP39NatP}$ (Oxford Expression Technologies).

Cells and virus production

Spodoptera frugiperda (Sf9) cells were grown in 1L of EX-CELL 420 serum-free media (Sigma, Poole, UK) under standard conditions. Upon reaching log phase, cells were infected with recombinant baculovirus to amplify viruses to high titre. Viruses were recovered from the culture medium at 4 days postinfection incubated at 4 °C

overnight, clarified by centrifugation and re-suspended in 10 ml of cold sterile PBS. The virus were titrated by plaque assay prior to using in the experimental organ studies and evidence of green fluorescent particles and rand expression of the red fluorescent protein was confirmed in transduced insect cells (data not shown). Finally, recombinant virus with a concentration of $2–4 \times 10^9$ pfu/ml was added to the UW preservation solution and used for *ex vivo* viral transduction.

Ex vivo viral transduction

Experiment 1

After 4 h of cold preservation, three kidney pairs were infused through the renal artery with either 250 ml cold UW solution containing the recombinant baculovirus AcVP39-eGFP (Group 1) or 250 ml cold UW without baculovirus (Group 2). Similarly, after 4 h of cold preservation, three livers were surgically divided into two halves and each half was infused through the principal vein with either 500 ml cold UW containing AcVP39-eGFP (Group 3) or 500 ml UW without baculovirus (Group 4). Postinfusion, control and experimental kidneys and hepatic lobules were placed in individual plastic bags filled with fresh, cold UW solution, put in ice boxes for 8 h and serial 15-g needle biopsies were taken every 60 min post-transduction.

Experiment 2

After 4 h of cold preservation, six kidneys were infused either with 250 ml cold UW solution containing recombinant AcDsRed) (Group 5) or 250 ml cold UW without baculovirus (Group 6). Control and experimental kidneys were prepared as described above and placed on ice boxes for 24 h post-transduction during which time 15-g needle biopsies were taken every 15 min during the first 2 h post-transduction, every 30 min during the following 4 h and then hourly after that until 24 h post-transduction.

Evaluation of baculovirus entry into hepatic and kidney tissue during *ex vivo* cold preservation

Confocal microscopy

Serial 15-g needle biopsies taken from kidneys in groups 1 and 2, and hepatic lobules from groups 3 and 4, were immediately snap-frozen in liquid nitrogen and stored at −80 °C for further analysis. Subsequently, 5-μm tissue sections from each biopsy were prepared, stained with DAPI (Vectashield; Vector Lab, Peterborough, UK) and assessed for the presence of green fluorescent particles using a Zeiss LSM 510 confocal microscope (Carl Zeiss Ltd, Welwyn Garden City, UK) with a ×63 magnification oil immersion lens and numerical aperture of 1.4 Detection of VP39 by immunofluorescence. To confirm the presence of viral particles within the cells of transduced organs in groups 1–4, additional 5-μm tissue sections were stained with a monoclonal rabbit IgG anti-VP39 kindly provided by Dr Y. Ohkada from the University of California at Los Angeles. The sections were contra-stained with FITC-conjugated mouse anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) following the manufacturer's recommendations using antibody dilutions 1:100 in all cases. Presence of the structural protein VP39-eGFP was visualized using a Leica fluorescent microscope [Leica Microsystems (UK) Ltd, Milton Keynes, UK] with a ×100 magnification oil immersion lens and numerical aperture of 1.0.

Gene expression analysis

Frozen needle biopsies taken 6 h and 24 h post-transduction from kidneys perfused with UW containing either AcDsRed (Group 5) or UW alone (Group 6) were used to determine the presence of mRNA specific for DsRed. Total RNA was obtained from the kidney samples and reverse transcribed using the RNAeasy isolation kit (Qiagen Ltd, Crowley, UK) and the Super-script II kit (Invitrogen Ltd, Renfrew, UK), respectively, according to the manufacturers' specifications. The RT-PCR for DsRed was performed for 30 cycles (1 min at 95 °C, 1 min at 55 °C, and 1 min at 72 °C), using the following primers: forward 5′-ATTCCGATTCCGTCATGAGGTCTTCCAAG AATG-3′ and reverse 5′-ATTCCGATTCCGCTCGAGCT AAAGGAACAG-3′. PCR products were analysed on an ethidium bromide stained 1% agarose gel.

Evaluation of tissue morphology and cellular content of ATP

Serial 5-μm tissue sections were cut from kidney and liver samples in groups 1–4 and stained with haematoxylin and eosin (Sigma-Aldrich, Dorset, UK). Three tissue sections per sample were assessed by light microscopy using an Axio Zeiss microscope (Karl Zeiss Ltd, Hertfordshire, UK). Kidney sections were stained with haematoxylin and eosin and examined for brush border loss of the proximal tubular epithelium, proximal tubular cell oedema, proximal tubular dilatation, tubular cell vacuolation and proximal tubular cell necrosis. Each of these morphological features was evaluated separately and scored by a pathologist (DH) blinded to the groups using a semi-quantitative score validated by Diitrich and Lange in a porcine model of kidney ischaemia reperfusion injury [19]. Similarly, liver sections were stained with haematoxylin and eosin and examined for sinusoidal dilatation, steatosis, necrosis, haemorrhage, inflammation and cholestasis. In this case, the pathologist evaluated and scored the sections using a morphological score previously developed and validated in a liver transplantation porcine model by our own research group [20].

In addition, cellular ATP content was determined in groups 1–4 using a commercial colorimetric assay (CellTiter-Glo reagent; Promega, Madison, WI, USA) following the manufacturer's recommendations. Briefly, a frozen sample was homogenized and 100 μl of the resulting lysate were mixed with an equal volume of CellTiter-Glo reagent (Promega UK, Southampton, UK). After 10 min of incubation, the ATP concentration was estimated using a GloMax™ 20/20 Integrated Luminescence System (Promega).

Statistical analysis

In all the experiments, statistical analysis was performed using the SPSS.14 statistical package (SPSS Inc, Chicago, IL, USA). *t*-test and Fisher's exact tests were used as appropriate and two-tailed *P*-values <0.05 were considered to indicate statistical significance.

Results

Evaluation of baculovirus entry during *ex vivo* cold storage

Confocal microscopy

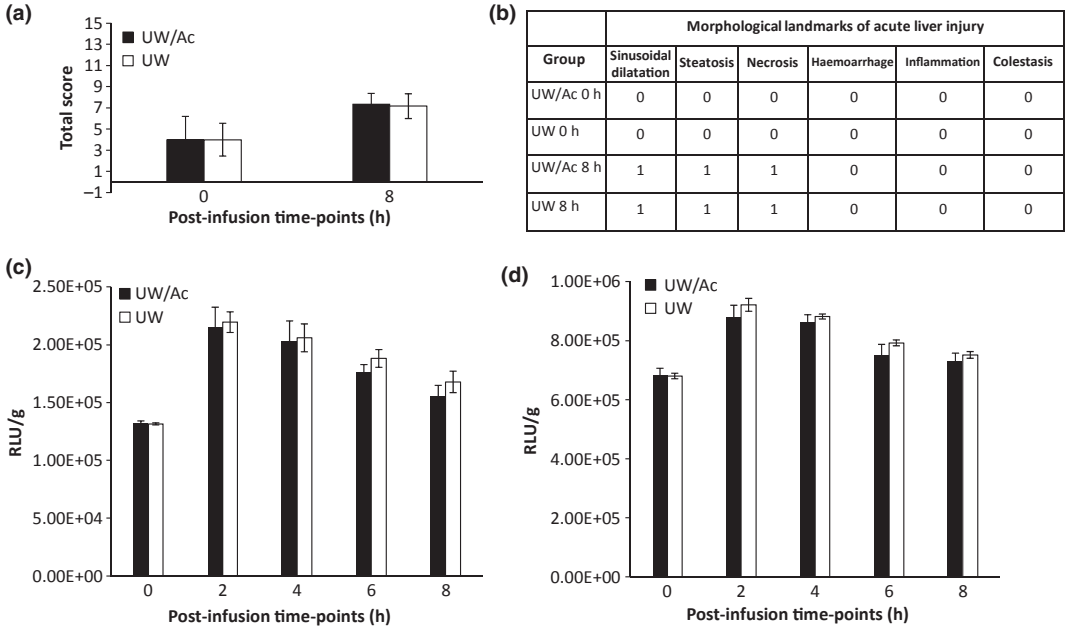
After correction for background auto-fluorescence, tissue sections taken before viral perfusion (time zero) from experimental and control kidneys and liver lobules showed no evidence of green fluorescence. Green fluorescent particles were detected in tissue sections of the livers and kidneys perfused with UW containing AcVP39-eGFP as early as 2 h after viral perfusion and their number increased progressively throughout the experiment until the last time point at 8 h post-transduction (Fig. 1).

Detection of VP39 by immunofluorescence

Immunofluorescence staining of the tissue sections with antiVP39 antiserum confirmed the presence of the major virus capsid protein VP39 within the cells 8 h post-transduction (Fig. 1), whereas no signal was detected in control samples at the same time point. In both analyses, fluorescence particles were localized mainly in the intravascular and intercellular spaces of samples from experimental organs at early time points but were observed surrounding the nuclei at 8 h post-transduction. In contrast, no fluorescence was detected in samples from control organs at similar time-points. Interestingly, there were more cells with green fluorescent particles in their cytoplasm in samples from livers perfused with UW containing Ac-VP39eGFP than in samples from kidneys perfused with the same virus (Fig. 1).

Gene expression analysis

To confirm that the virus had entered kidney cells, we also tested for the expression of mRNA specific to DsRed, which was under control of the CMV promoter, using RT-PCR for DsRed (Fig. 2). Kidney samples taken at 24 h postperfusion (experimental group 5) showed clear evidence of mRNA specific for DsRed whereas no DsRed



Group	Morphological landmarks of acute liver injury					
	Sinusoidal dilatation	Steatosis	Necrosis	Haemoarrhage	Inflammation	Colestasis
UW/Ac 0 h	0	0	0	0	0	0
UW 0 h	0	0	0	0	0	0
UW/Ac 8 h	1	1	1	0	0	0
UW 8 h	1	1	1	0	0	0

Figure 1 Evaluation of tissue morphology and cell viability after baculovirus transduction: tissue morphology in kidneys (a) and livers (b) after 8 h of viral transduction and cold preservation. Cellular content of ATP in kidneys (c) and livers (d) after 8 h post-transduction. RLU, relative luminescence units.

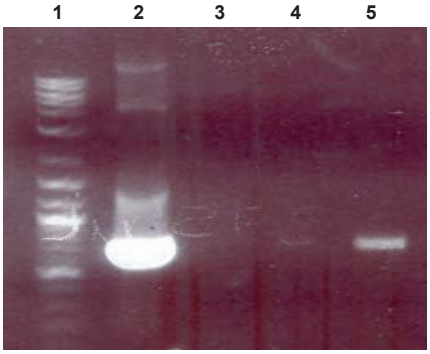


Figure 2 Analysis of gene expression after transduction with Ac-DsRed. Detection of DsRed-DNA of kidney samples after 6 and 24 h of transduction with AcMPNPV-DsRed during hypothermic preservation. RT-PCR for DsRed. Lane 1: 2 log ladder. Lane 2: positive control (DsRed DNA). Lane 3: DNA from kidney control. Lane 4: DNA from kidney 6 h post-transduction. Lane 5: DNA from kidney 24 h post-transduction.

mRNA was detected in the control group (experimental group 6) (Fig. 3). Finally, the presence of red fluorescent protein within the transduced cells was assessed by confocal microscopy. There was no evidence of red fluorescence detected in tissue sections taken from all experimental and control kidneys.

Evaluation of tissue morphology and cellular ATP concentration

Tissue morphology

There was no difference in the total score between sections from control and experimental kidneys at either 0 h [4 (1.5) vs. 4 (2.2), $P = 0.804$] or 8 h [7.1 (1.15) vs. 7.3 (1.0), $P = 0.737$] post-transduction. However, there was a significant difference in the total score between sections taken at 0 h and 8 h post-transduction in both the control [4 (1.5) vs. 7.1 (1.15), $P = 0.006$] and experimental [4 (2.2) vs. 7.3 (1.0), $P = 0.006$] samples (Fig. 1a). Similarly, there was no difference in sinusoidal dilatation, steatosis, necrosis, haemorrhage, inflammation or cholestasis between sections taken from control and experimental livers at either 0 h or 8 h post-transduction. However, there was more sinusoidal dilatation, steatosis and pericentral necrosis in liver samples taken at 8 h post-transduction from either control or experimental groups, when compared with their counterparts taken at 0 h (Fig. 1b).

Cellular ATP concentration

Overall, in controls and experimental samples, from both kidneys and livers, there was a rapid increase in the levels of ATP after perfusion of UW, followed by a progressive reduction in the ATP concentration in the subsequent

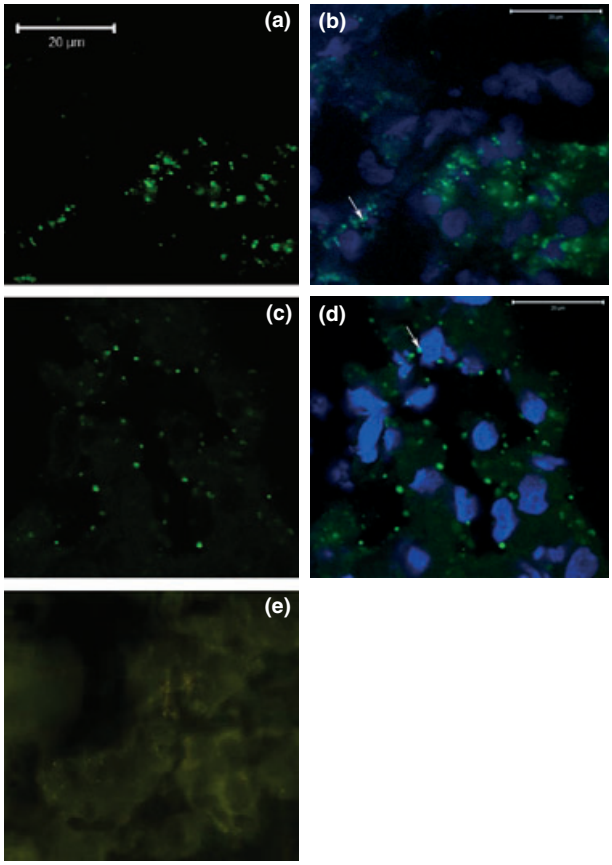


Figure 3 Kidneys and livers from large mammals successfully transduced with recombinant baculoviral vectors during hypothermic preservation. Kidney (a–c) and liver (d, e) transduced with AcMNPV vp39-eGFP (8 h postinfusion); (a, d) AcMNPV-vp39-eGFP; (b, e) Nuclear and viral DNA staining with DAPI; (×63 magnification oil immersion lens with numerical aperture of 1.4) Marker: 20 µm (c, e). Immunofluorescent staining of vp39. (×100 magnification oil immersion lens with numerical aperture of 1.0.)

time points. There was, however, no significant difference in the levels of ATP between lysates from control and experimental kidneys at 0, 2, 4 and 8 h postperfusion (Fig. 1c). Similarly, there was no difference between the levels of ATP determined in control and experimental liver samples taken at 0, 2, 4 and 8 h postperfusion (Fig. 1d).

Discussion

Gene therapy has progressed from its infancy 'proof of concept' stage, to the point at which the challenge remaining is to overcome the problems associated with its therapeutic application. Moreover, gene therapy is already proving to be an effective means of inducing graft protection [1,2,4,5] and a promising tool for the manipulation

of both innate and acquired immune responses triggered by transplantation [6,7]. So far, it is clear that the major impediment to the successful application of gene therapy in transplantation is not a scarcity of therapeutic genes, but the lack of an efficient, non-toxic gene delivery system for human clinical trials. Compelling experimental evidence shows that AcMNPV efficiently transduces a wide range of human primary cell cultures and cell lines with no evidence of competent virus breakthrough, changes in cell phenotype or decrease in cell viability, offering a high level of bio-safety [3,13–17]. Building on these *in vitro* results, this study provides evidence that recombinant baculoviruses are able to effectively transduce kidneys and livers from large mammals during conventional hypothermic static cold preservation and may lead to an effective and safe gene transfer with potential therapeutic implications in human organ transplantation.

Probably the most exciting advantage of baculoviral-based vectors as gene delivery system for gene therapy is their inability to replicate in mammalian cells. It is now well documented that although baculoviruses are able to insert their DNA into the genome of human cells, the lack of mammalian promoters in the viral DNA makes the virus unable to use the host cell machinery to synthesize viral proteins and assemble competent virus within the transduced cell [13,14]. In accordance with this evidence, a recent microarray analysis of human cells transduced with recombinant baculovirus showed that although mRNA from very early baculovirus genes was detected in the target cells, these cells did not synthesize any viral protein and the functional phenotype of the cell was maintained unchanged up to 3 months after transduction [17]. Of paramount relevance for transplantation is the fact that because the natural host of baculovirus is phylogenetically distant from humans, AcMNPV-based vectors may elicit a much lower innate immune response in comparison with conventional viral vectors and will not face a pre-existing immune memory response [3,17,21,22].

One of the limitations of cell transduction with first-generation baculovirus-based vectors compared with mammalian-based viral vectors (i.e. retrovirus and lentivirus) is the short-term gene expression achieved post-transduction [13–16]. Although engineering of third generation of recombinant baculovirus with extended gene expression is now possible, the short-life gene expression associated with conventional baculovirus-based vectors may be an additional advantage when the intended therapeutic intervention should be short-life. Transduction of allografts with putative therapeutic genes during preservation using baculovirus-based vectors may provide the organ with the therapeutic gene, and their biological effect, just the time needed for protection against a single and short-lasting insult avoiding the presence or persistence of the gene (and its effect) when this is no longer needed. This apparent disadvantage can actually be a major strength of *ex-vivo* baculovirus transduction as a therapeutic tool for allograft protection against ischaemia-reperfusion injury.

The ability of recombinant baculovirus to transduce a large variety of mammalian cell lines is well documented and fortuitously kidney and liver cells are particularly receptive to baculovirus transduction [14]. Green fluorescent virus particles were observed in tissue sections of kidneys and liver lobules perfused with UW containing AcVP39-eGFP and the percentage of transduced cells was 60–70% in kidney sections and almost 100% in liver sections. These results are in agreement with previous data published from other groups [15,16] stressing the potential of this virus as delivery vector in liver transplantation. Indeed, initial attempts for the *in vivo* application of baculovirus were performed in liver tissue because of its high transduction efficiency in liver cell lines and primary hepatocyte cells as revealed by *in vitro* studies [23]. Moreover, recent *in vivo* studies have also shown that systemic delivery of baculovirus often results in preferential uptake into certain organs, particularly the liver [24]. This evidence makes liver cell lines particularly attractive as models for baculovirus delivery and gene expression *in vitro* and highlights the opportunity of using these vectors *ex vivo* as an attractive delivery system for therapeutic intervention in liver transplantation.

Our results suggest that transduction of kidneys with recombinant baculovirus during conventional cold preservation may lead to an effective and safe gene therapy as well. Once the high transduction efficiency of baculovirus in whole livers was revealed by our *ex vivo* studies, we were particularly interested in clarifying whether recombinant baculovirus were able to efficiently deliver the sequence of a selected gene in kidneys from large mammals and whether transduction efficiency in these organs may be impaired by the hypothermic conditions associated with conventional cold storage. Our kidney model allowed the opportunity of having a challenging model of cell transduction in which an organ with a relative low avidity for baculovirus transduction can be retrieved, transduced and analysed using a natural and reliable control (the contralateral kidney). In addition, the amount of viral vector (and the logistics and costs involved in their production) required to effectively transduce kidneys, is considerably smaller than the amount needed to transduced whole livers, a convenient and cost-effective condition for a proof of concept study. Moreover, as the experiments in our study were performed in parallel using kidney and liver tissues, the differences in efficiency of gene delivery and expression in the two organs were revealed stressing that gene

therapy should be designed or tailored according to different organs to be transplanted.

Gene transfer with baculovirus-based vectors has been shown to be highly effective with transduction efficiencies of above 80% or higher reported in almost all *in vitro* and *ex vivo* transduction studies of insect and mammalian cells [14]. We did not investigate transduction efficiency quantitatively in this study. However, we were able to estimate the relative transduction efficiency of AcVP39-eGFP semi-quantitatively. An important limitation of our study is that the confocal morphological analysis was originally designed to detect the presence or absence of green fluorescence into the transduced organs and at that point the type of cell population transduced was not investigated in detail. We agree that determination of which cell populations were able to take up baculovirus, would have significant implications for the future of baculovirus as gene delivery system in organ transplantation, particularly in kidney allografts where cellular heterogeneity and compartmentalization are somewhat greater than in livers. Moreover, we recognize that a careful assessment of the differential baculovirus cell tropism and transduction efficiency should be an important part of future *in vitro* and *in vivo* baculovirus-based gene therapy studies.

A negative finding in our study was the lack of red fluorescence in kidneys transduced with a recombinant baculovirus carrying the sequence of Discosoma red (DsRed) a red fluorescent coral protein. This finding suggests that even after the cell production of DsRed-mRNA evidenced by the RT-PCR analysis of transduced kidneys, deprivation of oxygen and nutrients and the low metabolic rate associated with conventional static organ preservation may preclude the fluorescent-protein production in mammalian cells. An alternative possibility is that the activity of the mammalian promoter inserted in the baculovirus DNA was so low that the levels of protein production were difficult to detect. If this is the case, whether the low activity of the mammalian promoter was a problem of the promoter itself or an effect of the harmful conditions of cold storage over the promoter is yet unclear.

Our results also show that there was no morphological evidence of additional cell injury or reduction in the cellular content of ATP in transduced kidneys and livers compared with their controls. Interestingly, the results of this study support previous experimental and clinical evidence showing that standard cold storage induces progressive ATP depletion and cell injury irrespective of the solution used for preservation, that the level of cold ischaemic injury is related to the length of preservation and does not necessarily require the presence of reperfusion [21,25–27].

We believe that the design of our study allowed us to provide the proof of principle that baculovirus-based vectors are able to transduce organs from large mammals during conventional cold storage with no evidence of additional cell damage secondary to viral replication. However, we recognize that the lack of reperfusion, the absence of post-transplant evaluation of allograft function and assessment of the recipient immune response against the transduced allograft are important limitations in our study.

In solid organ transplantation, interruption of blood supply to the organ followed by an episode of oxygenated reperfusion are unavoidable This period of shortage of blood supply causes the tissue to become ischaemic and anoxic and can result in tissue injury and dysfunction. Paradoxically, restoration of blood flow after ischaemia can even be more damaging than the initial ischaemic insult. Reintroduction of oxygen results in inflammation and oxidative damage rather than restoration of normal function [28,29]. Non-specific immune response are generated by ischaemia and enhanced by early reperfusion and impair short- and long-term graft function through activation of allograft-selective acquire immune responses [30]. With lack of reperfusion and absence of post-transplant evaluation of the transduced allograft, the effect of baculoviral transduction on this complex cascade of events is missing and solid conclusions about the efficacy and potential of this strategy in organ transplantation are difficult to sustain. As we stated earlier, we recognize that the lack of reperfusion is an important limitation of our study. The main reason why we did not transplant the organs is because as means to elucidate the efficiency of baculovirus transduction during hypothermic conditions serial tissue biopsies and a large number of replicates were taken from all organs. In the Acvp39-eGFP experiment, needle biopsies were taken hourly while in the AcDsRed biopsies were taken every 15 min during the first 2 h post-transduction, every 30 min during the following 4 h and then hourly after that. This considerable amount of time points and replicates made highly probable that the transplanted organs develop early post-transplant complications that lead to allograft dysfunction or lost. Moreover, our main concern was that these unfavourable events could mask the actual effect of our intervention and be wrongly attributed to our preservation and transduction protocol. Moreover, these complications could increase the number of experiments needed and make the study highly expensive.

Finally, we are aware that the immunological challenge associated with allogenic implantation has a role in the amount of damage inflicted to the organ and that definitive evidence of the safety of baculovirus-based vectors in organ transplantation require the assessment of innate

and acquired immunological responses after transplantation. It has been shown that although baculoviruses do not replicate within mammalian cells [14,15] expression of baculovirus genes after transduction of human cells does occur and this may give some cause for concern [17,31,32], particularly when transduced organs are to be transplanted. DNA microarray and RT-PCR analyses identified expression of immediate-early AcMNPV genes (e.g. orf149, *ie0*, *ie1*, *ie2*, *p35*, *he65*, *pe38* and *gp64*) and change in the expression profile of host cells (HeLa, Vero6 and HEK293). Clearly, the baculovirus genome does not reside silently within mammalian cells. However, the physiology of human transduced cells was apparently not altered [17] and the effects of low-level virus gene expression on the cell phenotype are still to be determined. Moreover, there is evidence that baculoviruses (and the recombinant genes that they express) can stimulate innate and acquired immune responses in mammalian cells after *in vitro* and *in vivo* transduction [17,31–33] but which cytokines are induced and how the response is modulated is not fully understood. Furthermore, whether *ex vivo* organ therapy would elicit a significant immune activation compared to that that would cause the devastating side effects observed with other virus vectors [34] is as yet unclear. Therefore, we have designed a subsequent study in which whole organs from large mammals will be transduced with a new generation baculovirus carrying a potential therapeutic gene during organ preservation and transplanted into allogenic recipients. This study will provide evidence about the effect of *ex vivo* baculoviral transduction on the amount of ischaemia-reperfusion injury and the immunological responses associated with baculoviral transduction of functional allografts *in vivo*.

Conclusions

Ex vivo transduction of kidneys and livers with recombinant baculovirus during conventional cold preservation as current clinical practice was demonstrated with no evidence of additional disruption of tissue architecture, cell morphology or significant reduction in cell viability. Although the lack of reperfusion represents a significant limitation of this experimental study, our results suggest that baculovirus could be an attractive delivery system for gene transfer in organ transplantation and may contribute to the improvement of gene therapy in human clinical trials.

Authorship

FM-M, JJP-M, RBH, SVF and LAK: designed the research study and all the experiments. SVF and LAK: supervised the research project. FM-M, JD, RBH and LAK: designed and construct the baculoviral vectors. JJP-M and JPF: performed the surgical procedures. FM-M: performed the confocal microscopy analyses and ATP determination. JJP-M and DH: prepared the kidney and liver sections for histology and immunohistochemistry and evaluated tissue injury. FM-M and JJP-M: collected and analysed the data and wrote the manuscript. RBH, SVF and LAK: reviewed and corrected the manuscript.

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