IN VITRO COMPARISON OF FIBRE-MODIFIED AND REPLICATION- SELECTIVE ADENOVIRUSES AND ROLE FOR CD40L-ARMED ADENOVIRUSES IN THE THERAPY OF LIVER CANCER

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ABSTRACT

Liver cancer is associated with a very bad prognosis. Oncolytic virotherapy seems a promising alternative to standard treatments. Here, I compared; (1) the transduction specificity and efficacy of four fibre-modified adenoviruses and the wild type fibre virus, (2) the selectivity and oncolytic potency of three conditionally-replicating viruses, and (3) the cytotoxicity of two CD40-ligand expressing adenoviruses. The comparisons were done in vitro in liver cancer cell lines and primary hepatocytes. The long term goal is to insert the best fibre, and potentially CD40L, into the best replication-competent vector. Immunohistochemistry was also performed in liver cancer sections and surrounding non-cancer tissues to compare the levels of the viral receptors and CD40 between the tumour and the surrounding liver. The Ad5/3 fibre-chimeric virus was found to have the best overall transduction efficacy although none of the fibre-modified viruses is expected to be tumour-specific according to the results from immunohistochemistry and testing in primary hepatocytes. The conditionally replicating viruses were not found to be selective, although these results will need to be replicated. The expression of CD40L didn't have any effect on the viability of the hepatocellular carcinoma cell lines in vitro.

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Contents listings

Abbreviations and definitions table

Ad5	Adenovirus serotype 5
vp	Virus particles (calculated with the DNA assay)
IU	Infectious units (calculated with the Adeno-X Rapid Titer Kit from Clontech in 293 cells)
MOI	Multiplicity of infection. Expressed as vp/cell or IU/cell
Absorption time	The period of time for incubating the cells with the virus (from adding the virus to removing the virus-containing medium)
Absorption volume	The volume used for infection during the absorption time
HCC	Hepatocellular carcinoma
ICCA	Intrahepatic cholangiocarcinoma
Non-tumour liver	This term is preferred over “normal” or “healthy” liver as in most cases liver cancer develops from a background of chronic liver disease.
IHC	Immunohistochemistry
Mfi	Median fluorescence intensity as determined by flow cytometry
CPE	Cytopathic effect, i.e. rounding and detachment of the cells due to the adenoviral infection

See Table 1 (page 13) for the naming and a short description of the viruses used

INTRODUCTION

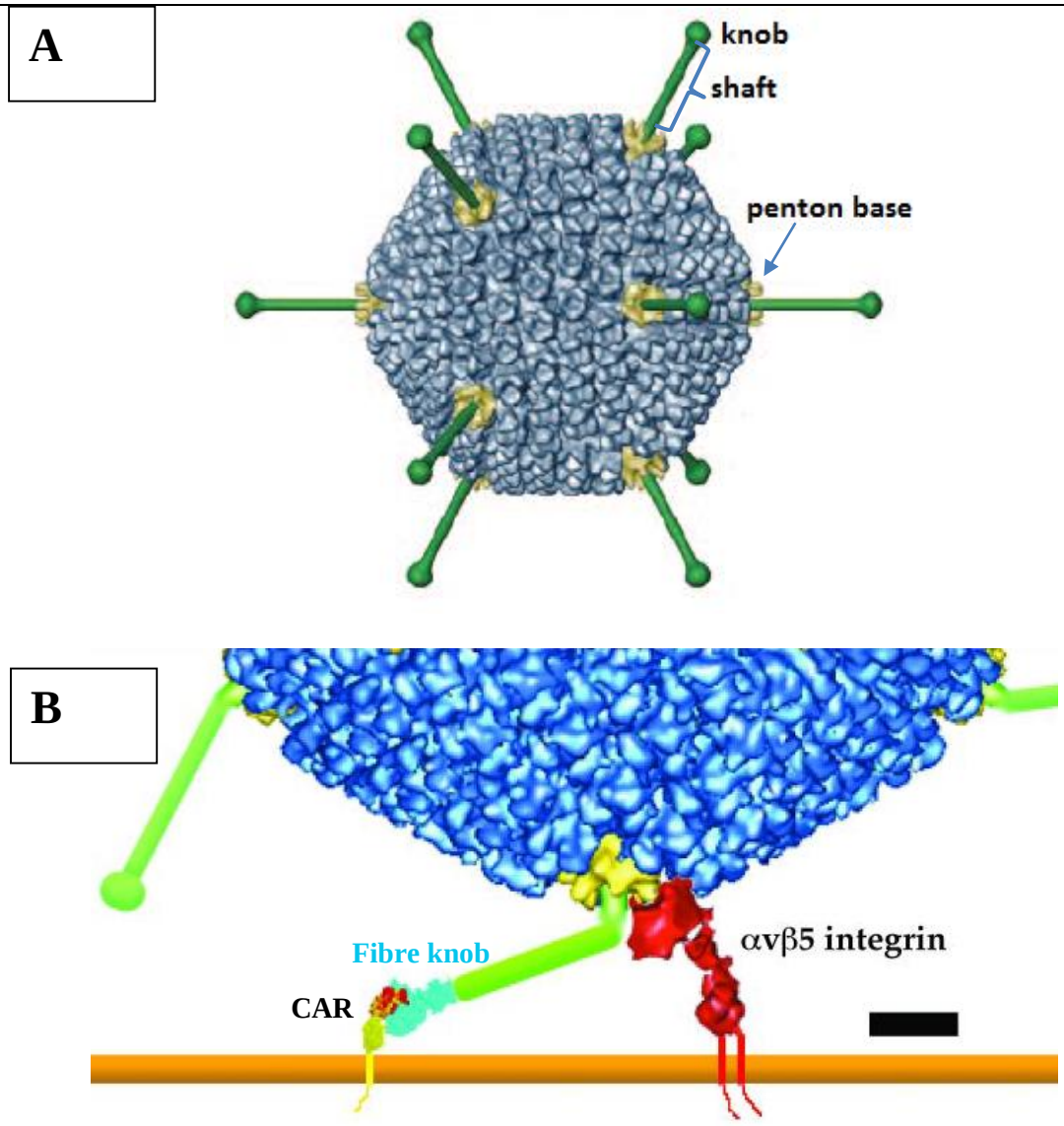
Liver cancer is a leading cause of cancer death worldwide (2nd in men, 6th in women) and its incidence is increasing in many parts of the world [1]. Most patients with hepatocellular carcinoma (HCC) are not candidates for potentially curative treatments (surgical resection or transplantation) because of the advanced stage of the disease at diagnosis [2]. Sorafenib, the standard treatment option for advanced HCC, extends survival by just a few months [3]. Therefore, novel therapies are needed. Among them, oncolytic virotherapy seems very promising. Oncolytic viruses are naturally occurring or engineered viruses that can selectively infect and kill cancer cells versus normal cells. Furthermore they can replicate intratumourally and spread. Among the oncolytic viruses, adenoviruses have been extensively studied.

The adenovirus infection involves many steps:

1) **Binding** of the viral capsid to the cell is the first. The viral capsid is an icosahedron (Figure 1A). The main proteins of the capsid are the homotrimeric hexon proteins. The penton bases and the fibre proteins are located at the 12 vertices of the icosahedron. The fibre is a homotrimer with three regions: the tail, the shaft and the knob. High-affinity binding of the fibre knob to its receptors is the first step for adenoviral infection. The attachment of adenoviruses to the cells is reviewed in more depth elsewhere (e.g. [4, 5]). Other parts of the capsid apart from the fibre knob, like the fibre shaft and the hexon proteins are also important for binding, especially in vivo after intravascular administration. Attachment of blood factors (like clotting factor X) to the hexon proteins or the fibre shaft can modify the biodistribution of the virus in vivo (e.g. [6-9]). However, the focus of this project is on modifications of the fibre protein.
2) **Internalisation** of the virus by receptor-mediated endocytosis. After binding of the fibre knob to its receptor, RGD motifs on the penton base will bind to integrins and induce

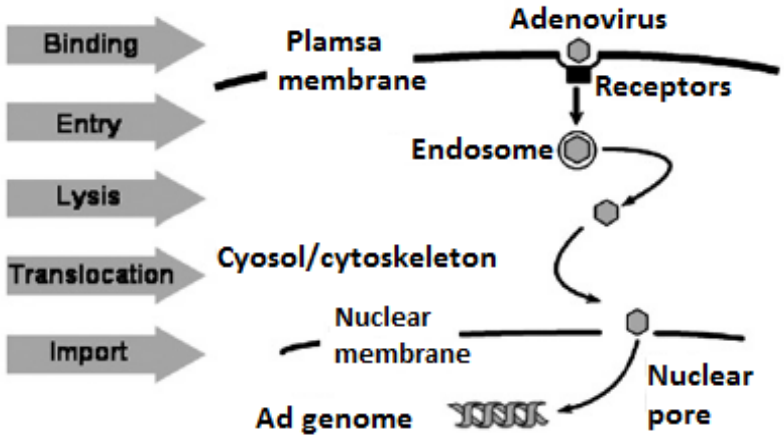
internalisation of the virus (Figure 1B), although internalisation is still possible even with RGD-deficient viruses [4, 10].

Figure 1; The viral capsid and the interactions with the viral receptors



(A) Illustration of the adenoviral capsid. The main component of the capsid is the hexon protein (blue). The penton bases (yellow) and the fibres (green) are located at the 12 vertices of the icosahedron. The fibre knob and shaft are shown. The fibre tail is not seen in this illustration. The fibre tail is the part of the fibre that interacts with the penton base. This illustration was taken from Zhang and Bergelson (2005) [4]. (B) A model of Ad5 capsid interactions with its receptors. The hexons are blue, the penton base yellow, and the fibre protein green. Flexibility of the fibre shaft is required to allow binding of the penton base RGD motif to integrins. Illustration taken from Wu et al. (2003) [11].

Figure 2; Intracellular trafficking of adenovirus serotype 5



The figure was taken from Leopold and Crystal (2007) [12].

3) The virus will then have to **escape the endosomes**, **traffic to the nucleus** and **import its genome into the nucleus (**Figure 2**)**. The intracellular trafficking of adenoviruses is reviewed by Leopold and Crystal (2007) [12]. The fibre protein plays an important role in intracellular trafficking, therefore modifying the fibre will also affect the intracellular pathway followed by the virus. For example, replacing the fibre knob of adenovirus serotype 5 with the fibre knob of adenovirus serotype 35 results in retention of the virus particles in the endosomal/lysosomal compartment for a longer period and the virus uses the lysosomes to achieve perinuclear localization [12, 13].

4) Adenovirus **transcription (early phase)** and **replication (**e.g. [14, 15]). The early phase transcription products are important to create an optimal intracellular environment for viral transcription and replication. Early region 1 (E1) is the first region to be transcribed and is essential for the adenoviral life cycle. Briefly, the E1A unit activates transcription and induces the host cell to enter the S phase, while E1B unit products block apoptosis (by binding and inactivating p53). An important interaction of E1A proteins, is binding to retinoblastoma protein (pRB) which results in release of E2F transcription factor and therefore induction of S-phase entry. Adenoviruses that have E1 region deletions or lack

expression of E1 region products (e.g. with tumour-specific promoters) are replication deficient. The early region 3 (E3) is not essential for the virus cycle, therefore transgenes are commonly inserted in place of E3 region genes. However, products of the E3 region protect the virus from the immune system in vivo, while the adenovirus death protein (ADP, E3 11.6K) is important for cytolysis and virus progeny release. Therefore, maintaining expression of E3 region genes after insertion of the transgene (e.g. [16]) is sometimes desirable.

5) Adenovirus **transcription (late phase)** and **packaging** (e.g. [14], [15], [17]). During late transcription, structural proteins are expressed and transported to the nucleus where the viral genome is packaged and new virus particles are formed.
6) Cell death and adenovirus **release**. As discussed above ADP is important for this process, therefore maintaining its expression after insertion of a transgene in the E3 region is desirable for oncolytic virotherapy. It is worth noting that the cell death induced by oncolytic adenoviruses is different from the classical apoptosis. Oncolytic adenoviruses can induce autophagy (e.g. [18]) or other immunogenic types of cell death which is essential for induction of anti-tumour immunity. The role of immunogenic cell death in oncolytic virotherapy is reviewed elsewhere (e.g. [19]).

There are many ways to achieve tumour selectivity of adenoviruses, the most popular being genetic engineering at the transductional (e.g. [20]) or transcriptional level (e.g. [21]). At the transductional level, modifications of the viral capsid have the potential to improve both the transduction efficacy and the specificity of the virus. The focus of this project is on adenoviruses with modified fibres, but other capsid modification are also possible (e.g. [20]). At the transcriptional level, tumour specificity can be achieved with tumour-specific promoter control of essential viral genes (like E1 region genes in the case of adenoviruses).

Considering the many choices available it was reasonable to start with a side-by-side comparison in vitro in liver cancer cell lines and more importantly in primary human hepatocytes. Here, four fibre-

modified and a wild-type fibre adenovirus were constructed to compare their transduction efficacy and specificity. Furthermore, three replication-selective viruses, constructed in a parallel project [22], were compared for their selectivity and cytotoxicity. See page **12** and Table 1 for a description and the naming of the viruses used.

As explained above, the adenovirus fibre is important for binding of the virus to the cell surface, the first step for adenoviral transduction. The rationale for the fibre modifications is that CAR (coxsackievirus and adenovirus receptor), the receptor for the adenovirus serotype 5 (Ad5), has been reported to be commonly downregulated in many cancers, including hepatocellular carcinoma [23, 24], therefore limiting the usefulness of the wild type virus. Retargeting the virus to other receptors by modifying the adenovirus fibre can overcome this problem, and potentially increase both the selectivity and transduction efficacy of the virus. The fibre-modifications compared here include;

- a chimeric adenovirus serotype 5/ serotype 3 fibre that uses DSG2 (desmoglein 2) as a receptor [25, 26],
- a chimeric adenovirus serotype 5/ serotype 35 fibre that binds to CD46 [27],
- an Ad5 fibre with an RGD motif insertion that binds to integrins like αvβ3 and αvβ5[28], and
- an Ad5 fibre with a peptide motif insertion that binds to integrin αvβ6 [29].

All have been reported to increase the transduction efficacy of the virus especially in cell lines with low or no expression of CAR. However improved transduction efficacy has been shown even for cells positive for CAR (e.g. for Ad5/RGD [28, 30], for Ad5/FMD [29] and for Ad5/35 [23]). Furthermore, some of these modification might result in better tumour-specificity. For example, CD46 has been reported to be overexpressed in HCC compared to normal liver [23, 31], while the integrin αvβ6 has been reported to be a very specific marker for cholangiocarcinoma [32].

Tumour selectivity of the conditionally-replicating viruses used here is achieved with tumour-specific promoter control of the viral E1A gene and/or the Δ24 deletion of the E1A gene. This deletion

ablates binding of E1A to pRB (retinoblastoma protein) therefore allowing viral replication only in cancer cells which have disrupted RB function [33]. One virus has just the Δ24 deletion. The other combines the Δ24 deletion with E2F-controlled E1A expression [34]. In the last virus, E1A expression is controlled by the hTERT (human telomerase reverse transcriptase) promoter [35]. Both promoters are highly active in cancer cells but not in normal tissues, and all three viruses have demonstrated specificity for tumour cells and cytotoxicity at least as good as that of the wild-type virus, while sparing the normal cells (e.g. [33-35]).

Oncolytic adenoviruses have been used in clinical trials with remarkably low toxicity even after intravascular or intrahepatic administration (e.g. [36-43]). However, the first generation of oncolytic adenoviruses were ineffective, at least when used as a single agent [36]. Onyx-015 is an example of the first generation oncolytic adenoviruses and features a deletion of E1B-55kd, which among other functions binds and inactivates p53, and a partial E3 region deletion. However, these deletions result in a significantly attenuated virus [36]. Newer generations of replication-selective viruses, e.g. those with tumour-specific promoters, achieve tumour specificity without loss of important viral genes, and therefore maintain cytotoxicity at least comparable to that of the wild type virus. Examples of newer oncolytic adenoviruses that have been used in clinical trials include;

1) Telomelysin: It is an Ad5 virus, with an RGD motif insertion in the fibre and is the first replication competent virus with a fully functional E3 region. To achieve tumour specificity the E1A region is controlled by the hTERT promoter and E1B is linked with an IRES. Telomelysin has been used intratumourally in a phase I clinical trial for patients with a variety of solid tumours [38].
2) ICOVIR-7: Briefly, tumour selectivity is achieved with a modified E2F promoter to control E1A and the Δ24 deletion. ICOVIR-7 is also based on Ad5 and has an Ad5/RGD fibre to enhance tumour transduction. It has been used in patients with advanced and progressive solid tumours [37].

3) Ad3-hTERT-E1A: This serotype 3, hTERT-controlled adenovirus has been used intratumourally/intravenously in patients with progressive advanced solid tumours [42].
4) Ad5-Δ24-GMCSF: A Δ24 deleted Ad5 virus expressing the immunostimulatory molecule GM-CSF. It has been used to treat patients with advanced solid tumours [43]. A similar virus with an Ad5/3 chimeric fibre has also been used intratumourally and intravenously in combination with cyclophosphamide [39].

Briefly, all the above-mentioned viruses had encouragingly low toxicity in the relevant clinical studies and demonstrated evidence of anti-tumour activity. Anti-tumour responses included mainly stabilisation of the disease, a few partial responses and rarely complete responses. Only one phase III trial has been completed so far (in China) [44]. Oncorine, an oncolytic adenovirus similar to Onyx-015, resulted in increased response rates in combination with chemotherapy and received approval by China's SFDA.

To further increase the anti-tumour efficacy, there is now more focus on combining oncolytic virotherapy with other treatments, or arming the viruses with therapeutic transgenes. More importantly the immunotherapeutic potential of oncolytic viruses has been realised (e.g.[45]) and oncolytic viruses are commonly engineered to express immunostimulatory molecules, like GM-CSF or CD40-ligand. CD40-ligand (CD40L) is a co-stimulatory molecule. Its receptor (CD40) belongs to the TNF receptor superfamily and is expressed by antigen presenting cells, like dendritic cells, macrophages and B-cells. CD40-CD40L interactions are important for the crosstalk among dendritic cells, T-cells and B-cells (reviewed in reference [46]) and expression of CD40L intratumourally by adenoviruses can overcome the immunosuppressive tumour microenvironment by shifting the T-cell response from Th2 to Th1 ([47, 48]). Furthermore, CD40L can induce apoptosis in some cancer cell lines that are expressing CD40 (e.g. [49]) and many cancers including HCC have been reported to express CD40 (e.g. [50]). In summary, oncolytic viruses expressing CD40L can have multiple therapeutic mechanisms [49]: (1) oncolysis, (2) direct anti-tumour effect by CD40L and (3) induction of anti-tumour immunity by both the CD40L and the virus-induced immunogenic cell death. A

CD40L-expressing virus, with an Ad5/3 chimeric fibre and hTERT control of E1A, has been safely used in cancer patients [48]. Furthermore, induction of antitumour immunity was demonstrated and there was some evidence of therapeutic efficacy (e.g. disease control at 3 months and improved survival compared to a historical control). Interestingly, response was seen in both injected and uninjected lesions, supporting a systemic, possibly immune-mediated, antitumour effect.

Moreover, it has been reported that membrane-presented CD40 agonists are more effective at inducing cell death compared to soluble CD40L (e.g.[51]). CD40L can be cleaved from the membrane by matrix metalloproteinases and metalloproteinase inhibitors have been shown to enhance the cytotoxicity of a CD40L-expressing adenovirus [52]. Therefore, a mutated CD40L resistant to cleavage has been generated and has been shown to have a more potent antitumour effect [52].

Here, two replication-defective (E1 and E3 deleted) adenoviruses expressing the wild type or the non-cleavable CD40L were compared either alone or in combination with one of the replication competent viruses. The viruses were tested in vitro in HCC cell lines to test whether CD40L expression can have any direct cytotoxic effect. The above-mentioned immunostimulatory functions of CD40L were not studied in this project.

AIMS OF THE PROJECT

1) Side-by-side comparison of the fibre-modified viruses for their transduction efficacy and selectivity using established liver cancer cell lines, primary tumour cells and freshly isolated primary hepatocytes. An ideal virus would show high transduction of tumour cells and low transduction of primary hepatocytes.
2) Side-by-side comparison of the replication-selective viruses for their tumour-specificity and oncolytic potency. An ideal virus would have low toxicity against primary hepatocyte but maintain oncolytic potency against tumour cells.
3) Effect of CD40 ligand-expressing viruses on cell viability of HCC (hepatocellular carcinoma) cell lines and comparison of the wild-type CD40-ligand versus the non-cleavable version of the CD40-ligand.
4) Study of the level of expression of the different viral receptors and CD40 using immunohistochemistry of formalin-fixed paraffin-embedded sections of liver tumours and surrounding non-tumour liver. The ideal scenario would be overexpression of the viral receptor and CD40 by tumours, and no or low expression by non-tumour tissues.
5) The long-term goal is to insert the best fibre, and potentially the CD40L, into the best replication-competent vector, and eventually use this virus to treat HCC patients.

MATERIALS AND METHODS

Making the constructs- propagation and quantification of the viruses

Bacterial strain and cell lines

For recombineering and preparation of the plasmid constructs the SW102 (E. coli) strain was used [53]. SW102 is readily transformable by electroporation due to its DH10B origin [54] and harbours the phage lambda-derived Red recombination system [55]. Expression of the recombination genes is controlled by a temperature-sensitive repressor and can be switched on at 42ºC and off at 32ºC. The lambda red recombination system is very efficient even with DNA homologies as short as 30-50bp.

For the transfection experiments and for propagation of the viral constructs HEK 293 cells were used [56]. 293 cells were grown in DMEM (e.g. Gibco, 41965-039) supplemented with 10% fetal calf serum (FCS), 2mM L-glutamine (1:100 from Gibco, 25030-081) and penicillin/streptomycin (1:100 from 100x stock, Gibco). For transfection/infection experiments DMEM with the HEPES modification (Sigma Aldrich, D6171) supplemented with 2% FCS and glutamine/penicillin/streptomycin (GPS) was used.

Plasmids and viruses

pPS1215A6(EGFP) (provided by Dr Peter Searle)**:** Originating from vector pUC18, this plasmid contains an EGFP (enhanced green fluorescent protein) expressing cassette flanked by the adenovirus serotype 5 (Ad5) genome (nucleotides 1 – 357 to the left of the cassette and nucleotides 3525-10593 to the right of the cassette. Nucleotide numbers are according to the Ad5 sequence from the National Center for Biotechnology Information GenBank, accession no. M73260/M29978). The EGFP coding region is from Clontech's pEGFP1. Expression is driven by the immediate early cytomegalovirus promoter (CMV). The EGFP coding region is followed by DNA containing the second intron of the human beta-globin gene (to provide splice signals), and poly-A/ transcription termination signals from human complement C2 gene.

pAdZ5-CV5-E3+ : The AdZ adenovirus cloning system was described by Stanton et al [57]. Briefly, pAdZ5-CV5-E3+ is a single copy plasmid vector of the whole Adenovirus 5 genome with the E1 region (nucleotides 478-3519) replaced by a selection cassette [amp^r(ampicillin resistance)/lacZα (β-galactosidase)/sacB (sucrose sensitivity)]. This cassette allows both positive (resistance to ampicillin and formation of blue colonies) and negative (resistance to 5% sucrose and formation of white colonies) selection. The plasmid also confers resistance to chloramphenicol. The sequence of the vector is available online: http://adz.cf.ac.uk/content/vector-maps (last accessed 15-04-2014).

pPS1399N1 rightward (provided by Dr Peter Searle): This vector contains the amp^r/lacZ/sacB cassette flanked by the Ad5 fibre N-terminus to the left (nucleotides 29511-31997) and part of the E4 region to the right (nucleotides 32826-33599).

pNKfiberRGD (provided by Ramon Allemany): This plasmid harbours the Ad5 fibre and flanking regions (from Not to KpnI sites), with the RGD-4C motif (peptide CDCRGDCFC) inserted in the HI loop of the fibre knob as described by Dmitriev et al [28].

vAd5/3-hTERT-CD40L (provided by Oncos Therapeutics): Described by Diaconou et al [49], this Ad5 virus contains a chimeric adenovirus type 5/type 3 fibre, with the Ad5 knob domain being replaced by the Ad3 knob domain. Tumour specific replication is achieved by hTERT (human telomerase reverse transcriptase) promoter-controlled E1A expression. The CD40L gene is inserted in the E3 region.

vAd5-A20FMDV2 (provided by Lynda Coughlan): In this Ad5 virus the wild type fibre is modified by insertion of a 20 amino acid peptide (derived from the foot-and-mouth disease virus) in the HI loop of the fibre knob, as described by Coughlan et al [29].

vAd5GFP-F35 (provided by Prof. Eric Blair): This Ad5 virus (described by Shayakhmetov et al [27]) contains a chimeric Ad5-35 fibre. Briefly, the adenovirus type 5 fibre tail (amino acids 1-44) was joined to the adenovirus type 35 shaft and knob domain.

Final viruses: See Table 1 for the naming of the viruses and a short description. The construction of the fibre-modified viruses is explained later. The fibres of these viruses are described in the relevant papers (Ad5/3 [58], Ad5/RGD [28], Ad5/FMD [29], Ad5/35 [27]). Briefly, the Ad5/3 fibre consists of the Ad5 fibre tail and shaft and the adenovirus serotype 3 fibre knob, and uses DSG2 (desmoglein 2) as a receptor [26]. Ad5/RGD has an RGD peptide motif inserted in the HI loop of the fibre, which allows binding to several integrins (e.g. αvβ3 and αvβ5). Ad5/FMD has a peptide derived from the foot-mouth-disease virus inserted in the HI loop, which allows binding to αvβ6 integrin [29]. Ad5/RGD and Ad5/FMD also maintain binding to CAR. Ad5/35 fibre consists of the Ad5 fibre tail and the Ad35 fibre shaft and knob domains, and uses CD46 as a receptor [27]. All are replication defective (E1-deleted) and express EGFP.

The replication-selective viruses [vAd5wt(hTERTp-E1A), vAd5wt(WTp-E1AΔ24), vAd5wt(E2Fp-E1AΔ24)] were constructed and provided by Yi Hsuan Chen and are described in more detail in her thesis [22]. All have the EGFP gene inserted in the E3 6.7K/gp19 K region similar to ONYX-304 in reference [16]. Insertion in this site isn't expected to affect the expression of neighbouring E3 genes [16]. The selectivity of the viruses is achieved by controlling the E1A transcription unit*[1] with tumour-specific promoters and/or the Δ24 deletion in the E1A region. vAd5wt(WTp-E1AΔ24) only has the Δ24 deletion which ablates binding of E1A to pRB (retinoblastoma protein) (e.g. [33]). vAd5wt(E2Fp-E1AΔ24) combines the Δ24 deletion with tumour-specific control of the E1A gene [34]. Briefly, four E2F palindromic sites and one SP-I binding site were inserted to control E1AΔ24. In normal cells, binding of E2F-pRB complexes to the E2F palindromes inhibits E1A transcription. Therefore, viral replication can only occur in cancer cells with deregulated pRB/p16 pathway. Furthermore, this modification seems to enhance the antitumour activity of the virus compared to the virus with the wild type regulation of E1A [34]. In vAd5wt(hTERTp-E1A) the E1A is controlled by

*[1] Early region 1A (E1A) is the first region to be transcribed after adenoviral infection and is necessary to create the appropriate intracellular environment for adenoviral transcription and replication.

the hTERT (human telomerase reverse transcriptase) promoter (e.g. [35]). The rest of their genome is the wild type Ad5 genome.

The CD40L viruses were propagated from a pAdZ-based (pAdZ-CV5 [59]) plasmid vector. The plasmid vectors of the CD40L viruses were constructed and provided by Dr Searle. These viruses are E1/E3 deleted due to their pAdZ-CV5 origin. They contain the CD40L expressing cassette in place of the E1 region and expression is driven by the CMV promoter. The non-cleavable version of the CD40L is resistant to cleavage by matrix metalloproteinases and has been described previously [52].

Table 1; Naming of the viruses used and short description (see text for more details and references)

Fibre-modified viruses	vAd5wt(EGFP)	Virus with the wild type Ad5 capsid	**All are replication defective** (due to lack of the E1 region). All are expressing **EGFP** under the CMV promoter.
	vAd5/3(EGFP)	Virus with an Ad5/3 chimeric fibre	
	vAd5/RGD(EGFP)	Virus with the Ad5/RGD fibre	
	vAd5/FMD(EGFP)	Virus with the Ad5/FMD fibre	
	vAd5/35(EGFP)	Virus with the Ad5/35 fibre	
Replication-selective viruses	vAd5wt(hTERTp-E1A)	E1A driven by the hTERT promoter	All have the **wild type Ad5 capsid**. All are expressing **EGFP** (inserted in the E3 6.7K/gp19 K region).
	vAd5wt(WTp-E1AΔ24)	E1A containing the Δ24 deletion, driven by the wild type promoter	
	vAd5wt(E2Fp-E1AΔ24)	E1A containing the Δ24 deletion, and controlled by E2F	
CD40L-armed viruses	vAd5wt(wtCD40L)	Virus expressing the wild type CD40-ligand	Both have the **wild type Ad5 capsid**. Both are **E1/E3 deleted**. Both are replication defective. They are not expressing EGFP. CD40-ligand is expressed under the CMV promoter
	vAd5wt(ncCD40L)	Virus expressing the non-cleavable CD40-ligand	
vAd5wt(E1AΔ24)(no EGFP)		Replicating virus not expressing EGFP. Used for the co-infection experiments.	

DNA fragments for recombineering

The DNA fragments (Table 2) were prepared by restriction digestion of the aforementioned plasmids and viruses. The digests were stained with SYBR gold (Invitrogen) followed by preparative gel electrophoresis [1% Agarose Ultrapure-Invitrogen, in Tris(40mM)-acetate(20mM)-EDTA

buffer(1mM)]. Bands were visualized on a blue light transilluminator and DNA from the relevant band was extracted with the QIAquick Gel Extraction Kit.

Table 2; Description and origin of the fragments used for recombineering	
Fragments	Description
F1(sCMV)	2189 base pairs. From SacI (Roche) digestion of pPS1215A6(EGFP). Fragment contains the EGFP gene. The left end (=the last 83 base pairs of the CMV promoter) is homologous to the CMV promoter in the amp^r/lacZ/sacB cassette. The right end is homologous to the Ad5 genome to the right of the cassette in pAdZ5-CV5-E3+ (See "construction of plasmid constructs" page 28 and Supplementary figure 3).
F(cassette-in-fibre)	Provided by Dr Peter Searle. 5727 base pairs. From AgeI+PmlI (NEB) digestion of pPS1399N1. Fragment contains the amp^r/lacZ/sacB cassette flanked by the Ad5 fibre N-terminus (nt 31042-31996*) to the left and part of the Ad5 E4 region to the right (nt 32826-33489*). See p. 28 and Supplementary figure 4.
Ad5/3 (NdeI-SspI)*	2362 base pairs. From NdeI-SspI (Roche) digestion of Ad5/3-hTERT-CD40L. Fragment containing the Ad5/3 chimeric fibre (from NdeI site in fibre tail to SspI site in the E4 region).
Ad5/RGD (AgeI-PmlI)*	2414 base pairs. From AgeI-PmlI (NEB) digestion of pNKfiberRGD. Fragment containing the Ad5/RGD fibre (from NdeI site in fibre tail to PmlI site in the E4 region).
Ad5/FMD (NdeI-AvrII)*	4442 base pairs. From NdeI-AvrII digestion of vAd5-A20FMDV2. Fragment containing the Ad5/FMD fibre (from NdeI site in fibre tail to AvrII site in the E4 region).
Ad5/35 (NdeI)*	4094 base pairs. From NdeI digestion of vAd5GFP-F35. Fragment of Ad5/35 chimeric fibre (from NdeI site in fibre tail to the end of the virus).
* according to Genbank M73260/M29978.	

Preparation of electrocompetent bacteria, electroporation and recombineering

Competent SW102 bacteria were prepared as described by Stanton et al [57] (also available at http://adz.cf.ac.uk/files/AdZ-Protocol-2.pdf). Bacteria were grown overnight at 32^{o}C in 5ml Veg LB medium (Fluka, 28713) with appropriate antibiotics (chloramphenicol 12.5 µg/ml ± ampicillin 50µg/ml). 1ml of the overnight cultures was inoculated into 50ml Veg LB with appropriate antibiotics and incubated in a shaking incubator at 32^{o}C for about 3-4 hours (ideally to an OD_{600}=0.4-0.6). Expression of the lambda Red recombination proteins was then induced by incubating in a 42^{o}C water bath for 15 minutes. Samples were then cooled on ice for 15 minutes. Cells were pelleted at

3750 rpm, 5 minutes, 0ºC (Beckman GS 6R Centrifuge, GH-3.8 rotor) and washed twice in 40ml of ice-cold ddH_2O. The pellet was resuspended in the volume left in the tube (approximately 400µl) after pouring off the supernatant. Aliquots of 25µl were mixed with 3µl of the DNA fragments (at least 10ng of DNA) and samples were electroporated (0.2 cm cuvettes, 2.5 kV- MicroPulser Electroporator, Biorad). Cells were recovered in 1ml Veg LB (no antibiotics) for 1 hour for positive selection, or in 5ml Veg LB (no antibiotics) for 4-5 hours for negative selection (to allow time for loss of sacB mRNA and proteins). Bacteria were then plated (30-100µl per plate aiming for well-spaced colonies) onto selective plates (see next paragraph) and incubated at 32ºC for 30-48 hours (bacteria grow slower at 32ºC and it takes some time for the blue colour to develop).

Selective plates

SCIX plates; For negative selection (replacement of the amp^r/lacZ/sacB cassette): 10g/L tryptone (Fluka, 95039), 5g/L yeast extract (Fisher Scientific, 10697612), 5% sucrose, 1.5% agar, 12.5 µg/ml chloramphenicol, 80µg/ml X-gal and 200µM IPTG. In the absence of salt, sucrose 5% can inhibit growth of E. Coli expressing sacB. Correct colonies should be white and sensitive to ampicillin. Sensitivity to ampicillin can be confirmed by restreaking the selected white colonies on ampicillin plates (SOB agar + ampicillin 50µg/ml). Therefore bacteria that still have the cassette cannot grow. Spontaneous loss of sacB function can also occur, without loss of the cassette, due to mutations. However, such bacteria will form blue colonies (provided that lacZ gene is still functional) (e.g. Figure 6A).

CAIX plates; For positive selection (insertion of the amp^r/lacZ/sacB cassette): SOB Agar, 12.5 µg/ml chloramphenicol, 50µg/ml ampicillin, 80µg/ml X-gal and 200µM IPTG. Correct colonies should be blue (Figure 6B).

Colonies that looked correct were then mini-prepped and confirmed with appropriate restriction enzyme digestion (see next paragraph).

Confirmation and propagation of plasmids constructs

Liquid cultures (typically 10ml) were grown from candidate colonies and the plasmid DNA was extracted with an alkaline-lysis method (see page **67** in "Supplementary methods"). The plasmid DNA was digested with appropriate restriction enzymes to confirm the expected band pattern. Confirmed bacterial stocks were expanded (typically 500ml) for higher yield preparation and the plasmid DNA was purified with caesium chloride-ethidium bromide gradient centrifugation (see page **68** in "Supplementary methods"). The constructs were confirmed once again with restriction enzyme digestion.

Propagation-quantification of the viruses

The purified plasmids were transfected to 293 cells with a calcium phosphate method (see page **69** in "Supplementary methods"). The pAdZ vector contains an expression cassette of I-SceI, a restriction enzyme whose recognition site is an 18 base-pair-long sequence, and therefore extremely rare. The I-SceI cutting sites are inserted at the ends of the adenoviral backbone of the pAdZ vector and expression of I-SceI is activated in eukaryotic cells. Therefore, after transfection in 293 cells the adenoviral backbone is released from the plasmid vector (Supplementary figure 3). Cells were harvested a few days later when enough cytopathic effect*[2] was present, and the virus was released from the cells with 3 freeze (-80ºC)- thaw (37ºC) cycles and vigorous vortexing. Then more 293 cell cultures were infected for virus propagation and the virus was purified with caesium chloride density gradient centrifugation (see page **70** in "Supplementary methods"). The DNA concentration of the virus preps was measured with a fluorometric assay based on SYBR gold staining of the DNA (see page **71** in "supplementary methods"). The virus particle concentration was calculated based on the DNA concentration and the molecular weight of the adenoviral DNA. The replication-selective and the CD40L viruses were also titrated with the Adeno-X Rapid Titer Kit (Clontech) and the infectious units (IU) concentration of the virus preps was calculated.

*[2] Cytopathic effect: rounding and detachment of the cells caused by the adenoviral infection.

Comparing the viruses

Cell lines and primary cells

To compare the viruses the following cell lines were used: two HCC-derived cell lines (HepG2 [60] and Huh7.5 [61]) and two ICCA (intrahepatic cholangiocarcinoma)-derived cell lines (CC-LP-I and CC-SW-I[62]). Cells were cultured in DMEM supplemented with 10% FCS, L-glutamine and penicillin/streptomycin. For the HCC cell lines the medium was also supplemented with non-essential amino acids (Sigma-Aldrich, M7145).

Primary tumour cells recently isolated (January 2014) by Dr E. Humphreys from an intrahepatic cholangiocarcinoma (ICCA) were also tested. The method used for their isolation and the culture medium are described in reference [63]. Fresh hepatocytes were isolated and provided by Dr E. Humphreys. One case was from a liver with cryptogenic cirrhosis and the other from a marginal liver donor. The method for isolation and the culture medium are described in reference [64].

Infection protocol

For infections of the cell lines the medium was supplemented with 2% FCS (instead of the 10% FCS that was used for culture). For the primary tumour cells and hepatocytes the same medium as for culture was used. The viruses were diluted to appropriate doses in the infection medium. For the infection, the cell culture medium was removed and the virus containing medium was added. The absorption time[3]* used for infection was 40 minutes (at 37^{o}C, 5%CO_2), with gentle rocking of the plates at 20 minutes. The absorption volume[4]* used was 600µl, 300µl or 180µl for 6-well, 12-well or 24-well plates respectively. The role of absorption time and volume, and rocking are discussed in

*[3] Absorption time: The period of time for incubating the cells with the virus (from adding the virus to removing the virus-containing medium).
*[4] Absorption volume: The volume used for infection during the absorption time

detail by Mittereder et al [65]. A short absorption time was selected based on the assumption that this might increase the sensitivity for detecting any differences on the transduction efficacy of the fibre-modified viruses. After the absorption time, the virus-containing medium was removed and the wells were washed once with PBS. Handling many wells simultaneously was avoided to prevent drying of the cells (Figure 3).

Figure 3; No fluorescent cells in a dried area of the well

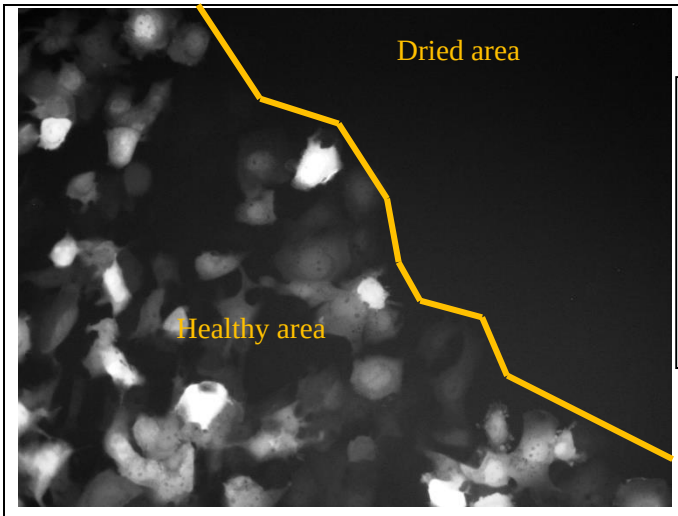


Photo from fluorescence microscope;
Three 6-well plates were handled at the same time. The medium was removed from all the wells before adding the virus. The delay resulted in drying of the last wells and the affected areas died.

Comparison of the fibre-modified viruses

The infections were performed as described above. To compare the transduction-efficacy of the fibre-modified viruses, cells were infected with the same multiplicity of infection (MOI; virus particles/cell) of each virus and the percentage of infected cells were determined with flow cytometry as described later ("Flow cytometry" section). The relative transduction efficacy was calculated as a percentage of the efficacy of the wild type virus:

$$relative\ transduction\ efficacy = \frac{\%\ \text{of positive cells infected by the fibre modified virus}}{\%\ \text{of positive cells infected by vAd5wt(EGFP)}} * 100$$

i.e. the relative transduction efficacy of vAd5wt(EGFP) is 100%

When the viruses were tested in duplicate/triplicate, the average % of positive cells was calculated for each fibre-modified virus and was divided with the average % of positive cells infected with the same MOI of vAd5wt(EGFP). This was done separately for each MOI tested and the total average for each experiment was calculated. Then results from independent experiments were averaged and summarised in a single bar chart (mean ± SD error bars).

To determine the appropriate time for harvesting, a time course experiment was done with the HCC cell lines. Cells were harvested and fixed for flow cytometry at 3 different time points after infection; at about 30 hours, 40 hours and 48 hours. vAd5wt(EGFP) and vAd5/35(EGFP) were selected for the time course experiment because of their very different infection kinetics and intracellular trafficking (e.g. see reference [13]).

Co-infection experiments

The rationale for the co-infection experiments is explained in the relevant section in "RESULTS". The co-infection experiments were done in HepG2 and Huh7.5. A replicating virus differing from the wild type Ad5 genome only at its Δ24 deletion in the E1A region [vAd5wt(WTp-E1AΔ24)(no-EGFP)] was used to complement the replication-defective fibre-modified viruses. 2*10^5 cells were seeded in 24-well plates and infected the next day with 3*10^7 virus particles of the fibre-modified viruses. After washing the viruses the cultures were incubated for 2 more hours before infecting with the replicating virus. This was done to minimize any competition between the replication-defective and the replicating viruses. Cells were infected with 3*10^7 virus particles of the replicating virus. For both infections the absorption time was 40 minutes and the absorption volume 180μl. Cells were harvested and fixed for flow cytometry at 16 hours after infection with the replicating virus. This early time point was chosen to avoid the shift of the negative population observed at high levels of EGFP (see page **73** in "Supplementary material").

Comparison of the replication-selective and CD40L-expressing viruses

The cytotoxicity of the replication-selective and CD40L-expressing viruses was compared with MTT viability assays (see below). Furthermore, for the EGFP-expressing replication-selective viruses, the median fluorescence intensity of infected cells or the mean fluorescence intensity of the total population (including the uninfected population of the sample) were measured with flow cytometry to compare the replication efficiency of the viruses. The mean fluorescence intensity of the total population was used when a shift of the negative population (see page **73** in "Supplementary material") was present therefore preventing the use of a uniform gating strategy to define the positive populations.

MTT viability assays

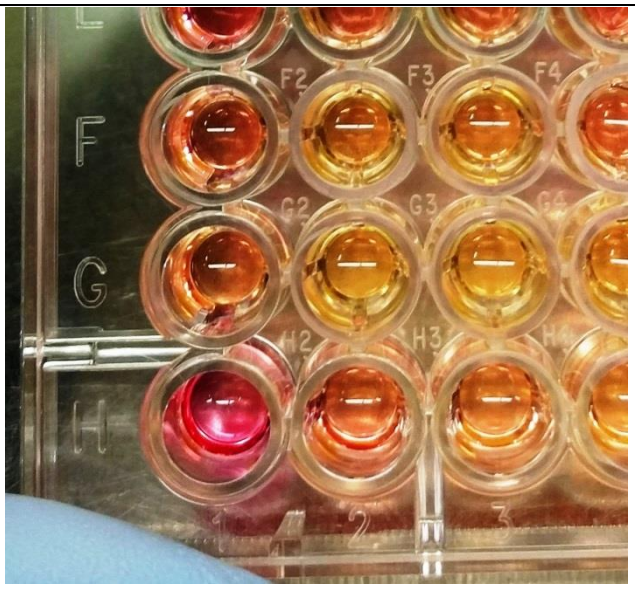
MTT assays were used to compare the cytotoxicity of the replication-selective and CD40L-expressing adenoviruses. Cells were seeded in 96-well plates one day before infection. When possible, edge and corner wells were avoided, or excluded from analysis if a significant edge effect was observed (Figure 4). On the day of the infection (day 0) the medium was removed and the cells were infected with serial dilutions of each virus in 100µl of the infection medium per well (usually each dose in triplicate or duplicate wells). 100µl of fresh infection medium were added the same or next day and plates were incubated (37ºC, 5% CO_2) until analysis at different time points (e.g. day 3, day 5, day 7). For the MTT assay, MTT stock solution (5mg/ml in PBS, from MTT powder- Sigma Aldrich M5655) was diluted 1:10 in 10% FCS medium without phenol red (Gibco, 31053-028). The medium from the wells was gently removed with a syringe and needle (to minimize any disruption of the cell monolayer) and replaced with 100µl of the MTT-medium. A well with the medium and no cells was used as a "blank" control. The plates were then incubated (37ºC, 5% CO_2) for 2 hours

(HCC cell lines) or 4 hours (primary hepatocytes). After the incubation time, 80µl*[5] of the MTT-medium were carefully removed and 100µl of DMSO (Sigma-Aldrich, D5879) were added per well to dissolve the dye. The plates were placed on a microplate shaker and analyzed within an hour with the Wallac 1420 VICTOR[2] plate reader. Absorbance was measured at a wavelength of 550nm (Huh7.5 and primary hepatocytes), or at 490nm (HepG2; absorbance was saturated at 550nm). The corrected absorbance or the percentage viability were calculated as shown below:

$$\text{Corrected absorbance} = (\text{measured absorbance}) - (\text{absorbance of the blank})$$

$$\%\ viability = \frac{\text{average corrected absorbance of infected wells}}{\text{average corrected absorbance of uninfected wells}} * 100\%$$

Figure 4; Edge effect in a seven-day-old 96-well pate for MTT assay



Note the higher pH (as indicated by the colour of the pH indicator- phenol red) of the edge wells and, to a greater extent, of the corner well. Although not obvious in this photo, the volume of the medium was much lower in the corner and edge wells due to the higher evaporation rate of these wells (see also references [66, 67]).

*[5] A variable amount of formazan crystals is lost during aspiration of the MTT medium, especially from wells infected with higher MOI. This might compromise comparisons between the viruses. It was noted that most of the crystals are lost during aspiration of the final µl of the MTT medium. Therefore, to minimize any variability due to aspiration of formazan crystals, I avoided aspirating the whole 100µl of the MTT-medium.

Flow cytometry

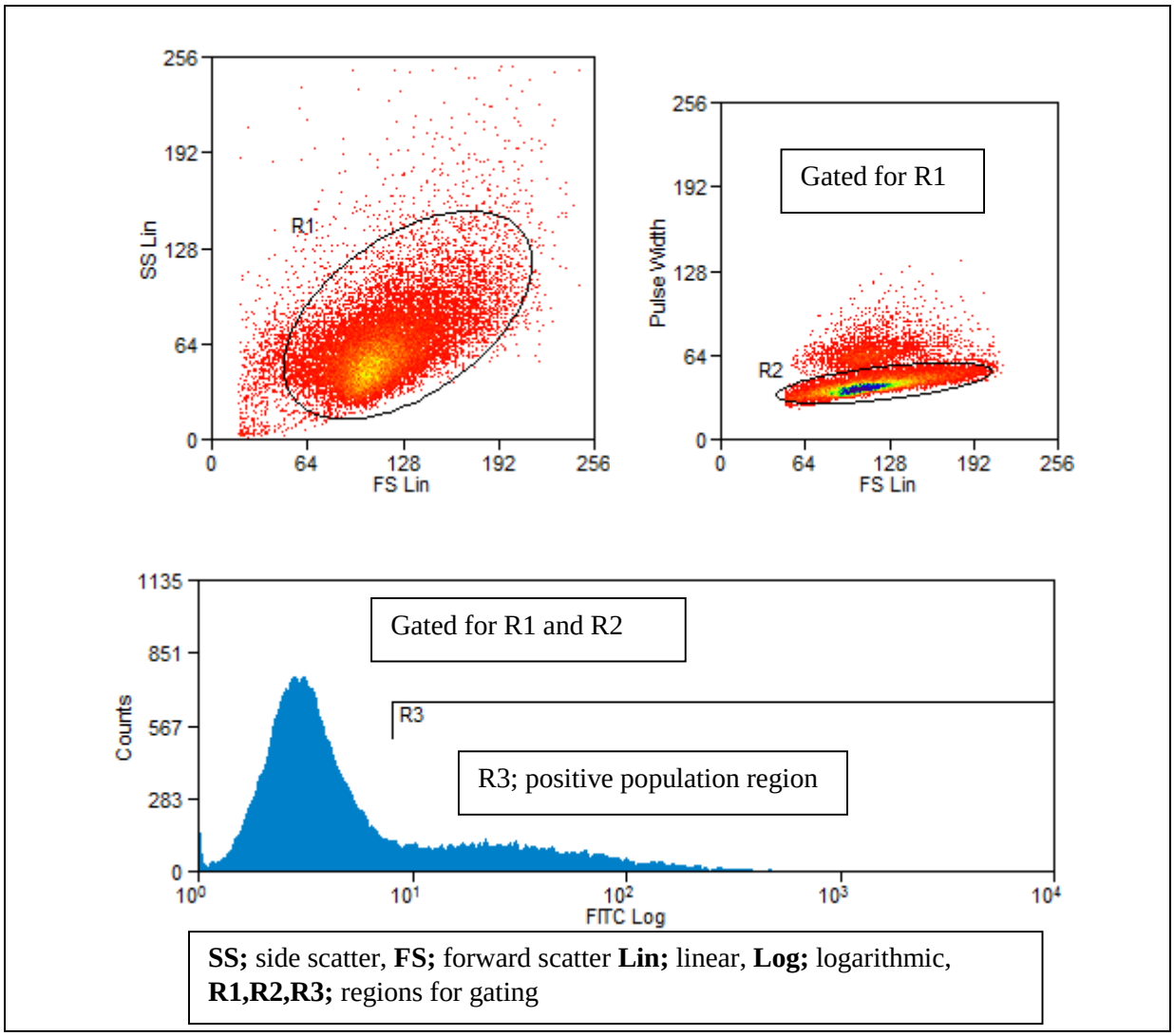
Harvesting-fixation of cells:

Cells were harvested with TrypLE-Express (Life Technologies). Trypsin was then inactivated with equal amount of 10% FCS medium and cells were washed in PBS. Cells were resuspended in 2% PFA (paraformaldehyde) and placed on ice for 20 minutes. Cells were then pelleted, the supernatant removed and then washed with 100μl PBS. Cells were resuspended in 100μl PBS +10% FCS and stored in the fridge until analysis by flow cytometry (usually the same or next day).

Flow cytometry settings, gating and analysis:

Cells were diluted in a total of 500μl PBS to about 1-5 million cells/ml. Samples were run on Cyan ADP Analyzer and analyzed with "Summit V4.3" software. An example of the settings and gating strategy is given in Figure 5. Events were gated to exclude debris and clumped cells (region R1 from "FS Lin vs SS Lin" histogram and region R2 from "FS Lin- pulse width" histogram). FITC voltage setting was adjusted so that the negative population is placed within the first decade of the logarithmic FITC histogram. Gating for the positive population (region R3, FITC-log vs event count histogram) was based on uninfected control samples. When there was very little overlap between the positive and negative distributions, the gate was set so that less than 1% of the negative control population is positive. When the overlap was significant, the gate was set so that about 2-5% of the negative control sample is positive, depending on the extent of the overlap. The same gating strategy was used for all samples for each independent experiment.

Figure 5; An example of the gating strategy used in flow cytometry



Antibodies

See **Table 3** below

Table 3; Antibodies used for immunohistochemistry, flow cytometry and blocking experiments

Antibodies	Concentration/dilution used for		
	IHC*	Flow cytometry	Blocking experiments
Mouse IgG1 isotype control (Molecular Probes, MG100-0.1mg/ml)	2 µg/ml (1:50)**	10 µg/ml (1:10)	NA
Rabbit Ig fraction isotype control (Dako, X0903)	2 µg/ml**	2 µg/ml	NA

Goat isotype control (Southern Biotech, 0109-01, 5mg/ml)	2 μg/ml**	NA	NA
Rabbit anti-ITGAV* (Sigma-Aldrich HPA004856- 0.1mg/ml)	1 μg/ml (1:100)	NA	NA
Rabbit anti-ITGB8* (Sigma-Aldrich HPA027797- 0.1mg/ml)	5 μg/ml (1:20)	5 μg/ml (1:20)	NA
Rabbit anti-ITGB5 (Thermo Scientific, PA1-37931)	1:20	1:20 or undiluted	NA
Mouse anti-CD61 (2f2 clone) (Thermo Scientific, MA5-11437)	1:25-1:50	1:25	NA
Rabbit anti αvβ6 (Biorbyt orb100289-0.5mg/ml)	6.7 μg/ml (1:75)	10 μg/ml (1:50)	10-20μg/ml
Rabbit anti-CD46 (Biorbyt orb10329-0.5mg/ml)	10 μg/ml (1:50)	10 μg/ml (1:50)	10 μg/ml (1:50)
Rabbit anti-DSG2 (Sigma-Aldrich HPA004896- 0.3mg/ml)	3 μg/ml (1:100)	6 μg/ml (1:50)***	10 to 30 μg/ml
Rabbit anti-CAR (Sigma-Aldrich, HPA030412- 0.2mg/ml)	2.7 μg/ml 1:75	NA	2 μg/ml***
Mouse anti-CD40, clone EA-5 (Calbiochem, 217590, 1mg/ml)	20μg/ml (1:50)	20μg/ml (1:50)	NA
goat anti-CD154 (AbD Serotec, AHP1029-1mg/ml)	1:50 – 1:100	NA	NA
Vector ImmPRESS HRP anti-goat, anti-mouse, anti-rabbit	Undiluted	NA	NA
Mouse anti-CAR clone RMCB (EMD Millipore, 06-644, 200μg)	NA	~ 10 μg/ml (1:100)	~ 10 μg/ml (1:100)
Mouse anti-CD46 clone MEM-258 (AbD Serotec, MCA2113, 1mg/ml)	NA	10 μg/ml (1:100)	10 μg/ml (1:100)
Mouse anti- integrin aV, clone L230 (Enzo Life Sciences, 100μg)	NA	~ 10 μg/ml	~ 10 to 20 μg/ml (1:33)
Mouse anti-DSG2, clone 6D8 (AbD Serotec, MCA2271, 0.5mg/ml)	NA	10 μg/ml (1:50)	10 to 40 μg/ml (1:50)
Rat anti- integrin αvβ6, clone 53a.2 (Abcam, ab97588, 1.52mg/ml)	NA	10 μg/ml ****	10 μg/ml
Goat anti-mouse IgG1-FITC (SouthernBiotech, 1070-02)	NA	1:250	NA
Goat anti-rabbit Ig-FITC (SouthernBiotech, 4010-02)	NA	1:250	NA

* ITG AV/B8/B5; integrins αv, β8 and β5 respectively
IHC; immunohistochemistry
** Ideally the concentration of the isotype controls should have been equal to the concentration of the primary antibodies
*** Lower concentration compared to the rest antibodies (higher concentration would require a much higher volume of antibody)
**** A secondary anti-rat IgG2a antibody was not available, so I tried the anti-mouse IgG1 as it was not crossed-absorbed against rat IgG

Immunohistochemistry (IHC)

Formalin fixed-paraffin embedded sections from hepatocellular carcinomas and surrounding non-tumour tissues were stained for the different viral receptors, CD40 and CD40L (CD154). Sections including both cancerous tissue and non-cancerous margin were preferred. This allows easier comparison of the levels of the studied antigens between the tumour and the surrounding liver. Sections were dewaxed and rehydrated in serial clearine and alcohol solutions. Endogenous peroxidase was blocked with 0.3% H_2O_2 in water for 15 minutes. For antigen retrieval a citrate low pH solution was used (Vector H-3300). The solution (10ml in 1L of dH_2O) was heated in a microwave oven for 10 minutes. The slides were then placed in the solution in a plastic rack and were heated for 20 minutes in the microwave oven. After cooling (for 10 minutes at room temperature and then under running tap water), non-specific antibody binding was blocked with 2.5% horse serum (Vector) for 20 minutes. Primary antibodies diluted in the 2.5% horse serum were then added for 1 hour at room temperature. Sections were then washed in BupH TBS (Thermo scientific) and the secondary ImPRESS peroxidase –conjugated antibodies (Vector) were added for 1 hour at room temperature. Slides were washed again in TBS for 5 minutes and then the substrate was added (DAB-Vector ; 1 drop per 1 ml diluent) for a few minutes. The slides were counter-stained in Mayers heamatoxylin for 4 minutes. Slides were then cleared and dehydrated (in serial alcohol and clearine solutions) and mounted with DPX.

Note that I personally did this work for the first 4 tumours and the rest of IHC was continued by Will Marshall. However, I examined the sections and took the photos included here myself.

Cell surface flow cytometry staining for the viral receptors and CD40

Cells (HepG2, Huh7.5, CC-LP-I, CC-SW-I and primary ICCA) were harvested with TrypLE Express (Gibco, 12605-010) and washed in PBS. Non-enzymatic harvesting with 10mM EDTA in PBS was also tried to exclude absence of staining due to trypsinization of the target antigens. 5*10^5

cells were incubated with the primary antibodies in 100 µl FACS buffer (PBS+10%FCS)*[6] for 40 minutes on ice. Cells were then pelleted by centrifugation and the supernatant was discarded. Cells were washed with 100µl PBS, and were resuspended in 100µl FACS buffer with the secondary FITC-conjugated antibodies and incubated on ice for 40 minutes. 100µl PBS were added for washing, and cells were pelleted and resuspended in 100µl PBS and then transferred to FACS tubes with 400µl PBS for flow cytometry (Cyan ADP Analyzer- Summit v4.3). All samples were analyzed using the same PMT voltage setting for FITC (480V). The isotype-corrected median fluorescent intensity was used to compare the levels of expression of the target antigens among the different cell lines:

$$corrected\ mfi\ = (total\ mfi\ of\ the\ sample) - (total\ mfi\ of\ the\ isotype\ control\ sample)$$

Where mfi refers to the median fluorescence intensity.

Note that this method is only semi-quantitative and does not represent an accurate quantification of the antigen. Truly quantitative methods using calibration beads are available (e.g. [69]). For the antibodies and concentrations used see **Table 3**. Note that for the rat anti-αvβ6 (clone 53a.2, IgG2a), an appropriate isotype control and secondary antibody were not available. As a secondary antibody I used the anti-mouse IgG1 exploiting the species cross-reactivity.

Blocking experiments

To confirm the receptor specificity of the fibre-modified viruses, blocking experiments were performed. CC-LP-I and CC-SW-I cell lines were selected for the blocking experiments for reasons discussed later. For the antibodies and concentrations used see **Table 3**. The monoclonal antibodies used were selected based on a literature review to identify antibodies that have been used successfully for similar blocking experiments in the past (RMCB [27, 70, 71], MEM-258 [72, 73], L230 [27, 70], 53a.2 [29]) . 2*10^5 cells were seeded in 24-well plates one day before the experiment. On the day of

*[6] Retrospectively, and considering that recovering cell function was not an issue, sodium azide could have been added in the FACS buffer to further minimize surface antigen loss [68. Abcam. *Indirect flow cytometry protocol*. [cited 2014 30/07]; Available from: http://www.abcam.com/protocols/indirect-flow-cytometry-protocol.].

the experiment, the medium was removed and cells were incubated with the relevant antibodies, diluted in the infection medium (2% FCS medium), for 1 hour in the cell culture incubator (37ºC, 5% CO_2). The virus was then added to the wells at an MOI of 10 vp/cell (for CC-LP-I) or 100 vp/cell (for CC-SW-I) and the plates were placed in the incubator for another hour. The medium was then removed, wells were washed twice with 0.5 ml PBS and fresh infection medium was added. Cells were harvested for flow cytometry 2 days later and the percentage transduction was calculated:

$$\% \, transduction =$$

$$= \frac{percentage \; of \; positive \; cells \; from \; wells \; preincubated \; with \; the \; mAb}{percentage \; of \; positive \; cells \; from \; control \; wells \; without \; mAb \; preincubation}$$

The percentage of positive cells was averaged from duplicate wells for each antibody or control.

mAb= monoclonal antibody

RESULTS

Construction of the plasmid vectors

Plasmid vectors of the fibre-modified viruses were made in three recombineering steps:

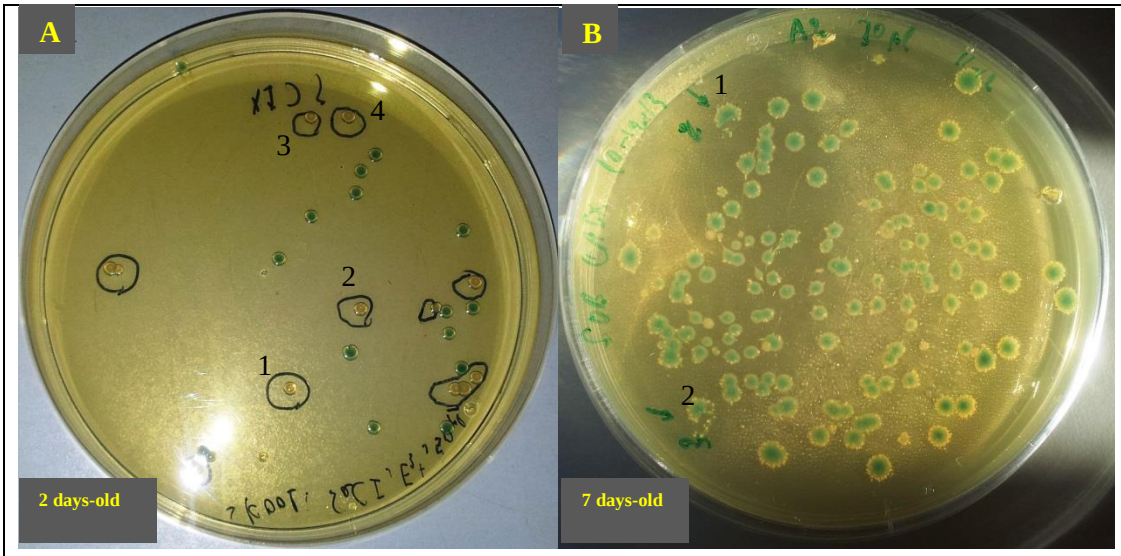
1) Insertion of the CMV-EGFP cassette in the left end of the adenoviral backbone of pAdZ5 (in place of the selection cassette) to allow monitoring of viral infection by fluorescence (Supplementary figure 3)
2) Insertion of the amp^r/lacZ/sacB cassette in the fibre region of the adenoviral backbone (allowing for selection of correct colonies after step 3) (Supplementary figure 4).
3) Insertion of the modified fibres in place of the region with the cassette (Supplementary figure 4).

For the first step, competent SW102 E. coli transformed with pAdZ-CV5-E3+ were electroporated with the DNA fragment F1(sCMV) (Table 2). Recombineering results in an AdZ plasmid vector (named as pSK1A) of the wild type virus with an EGFP expressing cassette in place of the E1 region. Bacteria were spread on SCIX selective plates (Figure 6A) and selected white colonies were mini-prepped and confirmed with HindIII digestion (Figure 7A). Sensitivity to ampicillin of the selected colonies was also confirmed by restreaking the colonies on ampicillin plates (not shown).

For the second step (Supplementary figure 4), competent SW102 harbouring pSK1A2 (= pSK1A from colony no 2) were electroporated with the DNA fragment F(cassette-in-fibre) (Table 2), resulting in a plasmid vector (named as pSK2A2) with the amp^r/lacZ/sacB selection cassette in the fibre region of the adenoviral backbone. Bacteria were inoculated on CAIX selective plates (Figure 6B) and selected blue colonies were mini-prepped and confirmed with BamHI digestion (Figure 7B).

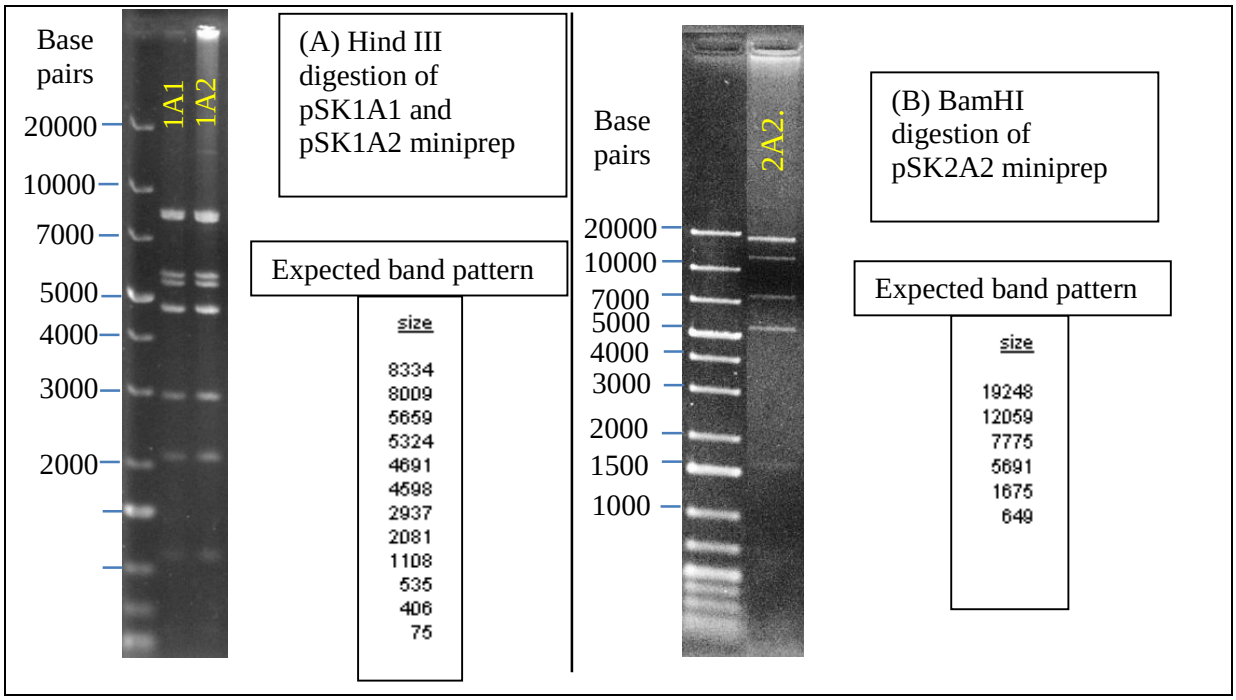
For the third step (Supplementary figure 4), competent SW102 harbouring pSK1A2 were electroporated with the modified-fibre fragments (Table 2) resulting in plasmid vectors similar to pSK1A but with the modified fibres. Bacteria were plated on SCIX plates (not shown) and selected white colonies were mini-prepped and confirmed with BamHI digestion and fibre-specific enzymes (results from digestions are not included, but the expected band pattern was confirmed).

Figure 6; Selective plates for negative and positive selection



(A) SCIX plate inoculated with SW102-pAdZ5-CV5-E3+ electroporated with the DNA fragment F1(sCMV). White colonies should harbour the recombined plasmid (pSK1A). Blue colonies represent colonies with spontaneous loss of SacB functionality. The blue colour is retained due to the presence of the lacZ gene. (B) CAIX plate inoculated with SW102-pSK1A2 electroporated with the DNA fragment F(cassette-in-fibre). Blue colonies should harbour the recombined plasmid (pSK2A2).

Figure 7; HindIII and BamHI digestion of minipreps

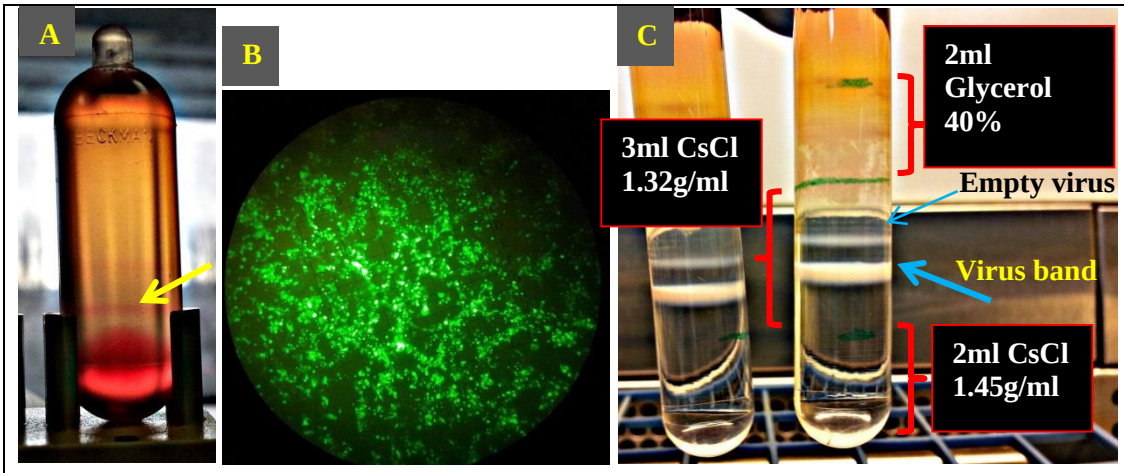


Propagation of the viruses from the plasmid vectors

Plasmid vectors of the wild type and fibre-modified viruses from confirmed colonies were bulk prepped and purified with CsCl-EtBr gradient centrifugation (e.g. Figure 8A). The purified plasmids were then confirmed again by restriction enzyme digestion with BamHI and fibre-specific enzymes. Confirmed plasmids were transfected with a calcium phosphate-based method in HEK 293 cells where the adenoviral backbone was released due to the self-excision system of the AdZ vectors. Successful transfection was confirmed under a fluorescence microscope (e.g. Figure 8B). After a few days (waiting for the cytopathic effect to develop) the viruses were harvested and further propagated in HEK 293 cultures. Virus harvested from ten T150 flasks was purified with a caesium-chloride density gradient method (e.g Figure 8C). DNA from a small aliquot was extracted and constructs were confirmed by restriction enzyme digestion. Enzymes with cutting sites in the modified parts of the fibre were preferred to further confirm the successful recombineering of the

modified fibres. DNA concentration of the virus preps was quantified with a SYBR Gold DNA assay and virus particles were calculated (Table 4). Several aliquots of each virus were then stored at -80ºC. These aliquots were used for subsequent experiments. This was done to avoid repeated freeze-thawing of the virus prep as this can affect the infectivity of the viruses.

Figure 8; Bulk prep (A), infected cells (B) and virus prep (C)



(A) Photo of a CsCl-EtBr density gradient of a plasmid bulk prep. The arrow indicates the lower band representing supercoiled plasmid DNA. (B) Photo of a 293 culture infected with vAd5/FMD(EGFP). (C) Photo of banded vAd5/RGD with the CsCl density centrifugation method.

Immunohistochemistry of HCC

According to the so far stained sections none of the viral receptors seems to be specifically expressed by the tumour compared to the surrounding liver (Figure 10). Staining for **integrins αv and β5** was consistently strongly positive in all tumour and non-tumour sections stained so far. **CD61 (integrin β3)** on the other hand was not detected in any of the sections. **Integrin αvβ6**, the receptor for the Ad5/FMD fibre, was rarely positive (2 of 8 tumours), which is similar to what previously reported [32][7]. In the 2 tumours that were positive, the non-tumour margin was also stained positive (e.g. Figure 10). **CAR** was detected in most of the tumours stained so far (6 of 7) (Supplementary photo

*[7] Although according to that study none of the HCCs (n=77) were positive.

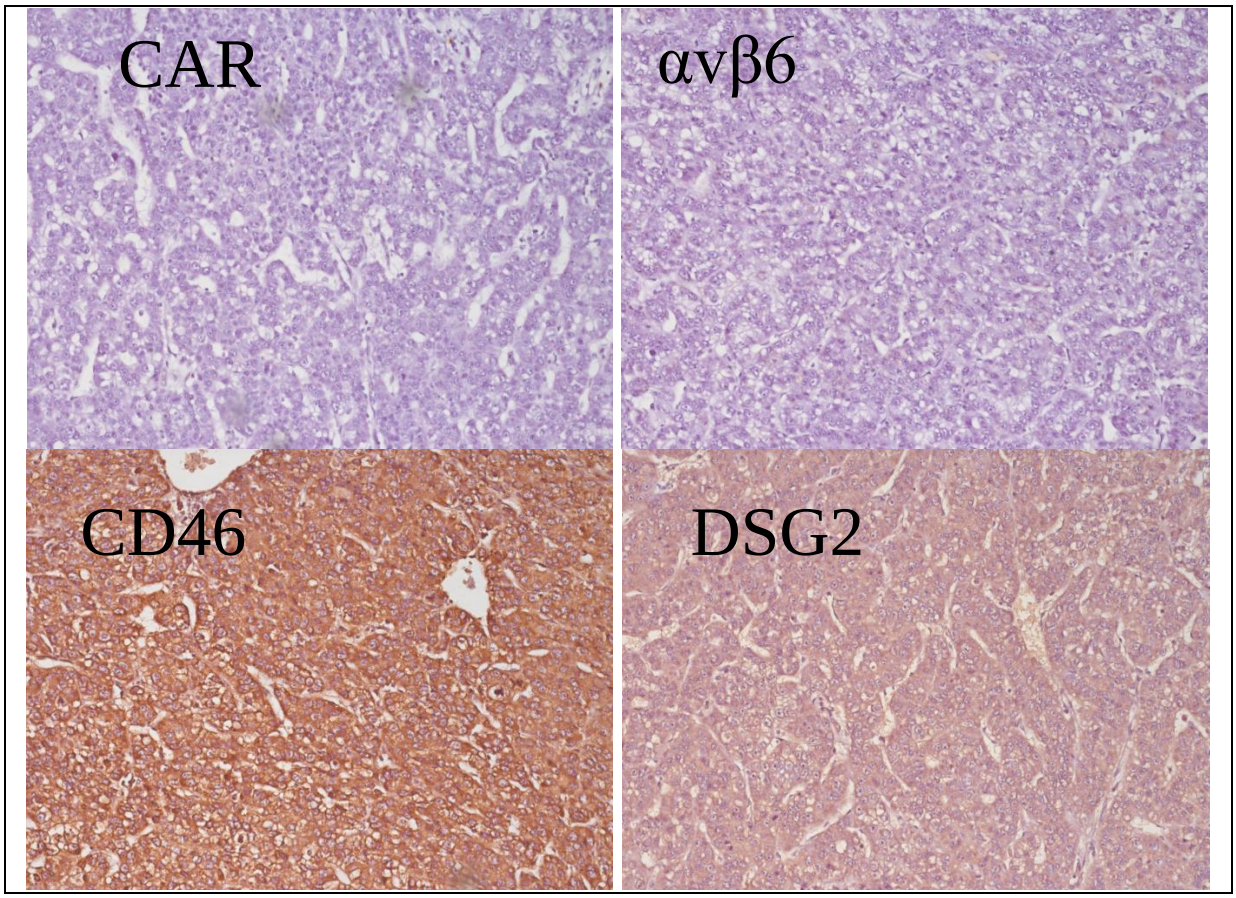
1 and 2). In one case CAR expression was lost but alternative receptors were highly expressed (Figure 9). In sections with non-tumour margin available, CAR expression by the tumour was similar to that of the margin in 3 out 5 cases, and lower but present in the other 2 cases. In one section for which the corresponding non-tumour margin was not available, CAR expression was detected mostly focally.

Table 4; Virus prep concentrations and enzymes used for confirmation of virus preps

Virus	IU/µl	Virus particles/µl	Confirmed by digestion with
vAd5wt(EGFP)	NA	$2.77*10^9$	HindIII
vAd5/3(EGFP)	NA	$1.64*10^9$	**BglII**, **ScaI**, (bulk prep also confirmed with BamHI)
vAd5/RGD(EGFP)	NA	$3.25*10^9$	**SacII** (bulk prep also confirmed with BamHI)
vAd5/FMD(EGFP)	NA	$1.88*10^9$	**BstEII**, KpnI, NdeI
vAd5/35(EGFP)	NA	$3.08*10^9$	**AflIII**, KpnI, NdeI, (bulk prep also confirmed with **Asp718** and **SpeI**)
vAd5wt(hTERTp-E1A)	2*10^7	$2.75*10^8$	The replication selective viruses where made by Yi Hsuan Chen [22]
vAd5wt(WTp-E1AΔ24)	$3.18*10^7$	$2.66*10^8$	
vAd5wt(E2Fp-E1AΔ24)	$5.24*10^7$	$8.91*10^8$	
vAd5wt(wtCD40L)	$1.06*10^8$	$1.65*10^9$	HindIII, EcoRV (bulk prep also confirmed with BglII)
vAd5wt(ncCD40L)	$9.3*10^7$	$2.57*10^9$	HindIII, EcoRV (bulk prep also confirmed with BglII)
Enzymes in bold indicate enzymes with cutting sites in the modified part of the fibre. Infectious units (IU) were calculated with the Adeno-X Rapid Titer Kit (clontech) in 293 cells			
The virus particles concentrations data for the three replication selective viruses were provided by Yi Hsuan Chen (the DNA concentration was converted to virus particles based on the conversion factor: $2.57*10^7$ vp per 1 ng of DNA).			

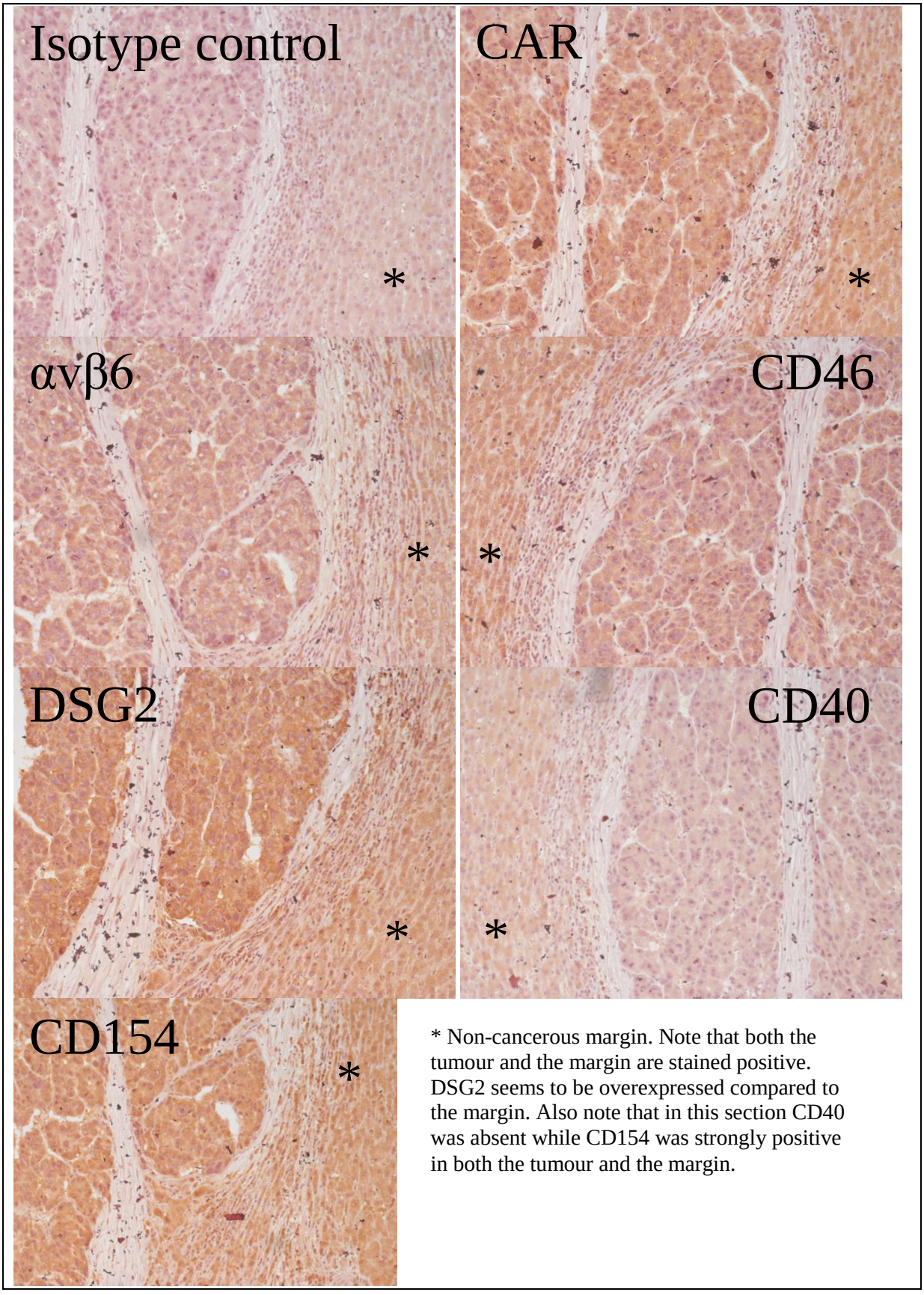
Finally, in the last section, CAR expression was concentrated mainly around the bile canaliculi, and therefore inaccessible to circulating adevovirus particles [74]. All but one tumours were positive for **CD46** (10 of 11), but non-cancerous tissues were also positive in sections with available corresponding margin (5 of 5). In 2 of the positive sections CD46 was only detected focally*[8]. **DSG2** was also consistently positive (10 of 10) but again the corresponding margin was also positive (5 of 5). **CD40** was detected in most tumours stained so far (3/4), however the surrounding diseased liver was also found positive (Supplementary photo 3 and 4). Unexpectedly, most sections (both tumour and non-tumour) stained with the CD154 (CD40L) antibody were also strongly positive (e.g. Figure 10 and Supplementary photo 3 and 4).

Figure 9; HCC that lost expression of CAR but is positive for alternative receptors like CD46 or DSG2



*[8] In one of the sections the uneven staining probably had to do with uneven deparaffinization as the same pattern was observed for all the receptors including the αv integrin.

Figure 10; Expression of the receptors is not tumour-specific



* Non-cancerous margin. Note that both the tumour and the margin are stained positive. DSG2 seems to be overexpressed compared to the margin. Also note that in this section CD40 was absent while CD154 was strongly positive in both the tumour and the margin.

Transduction efficacy of the fibre-modified viruses

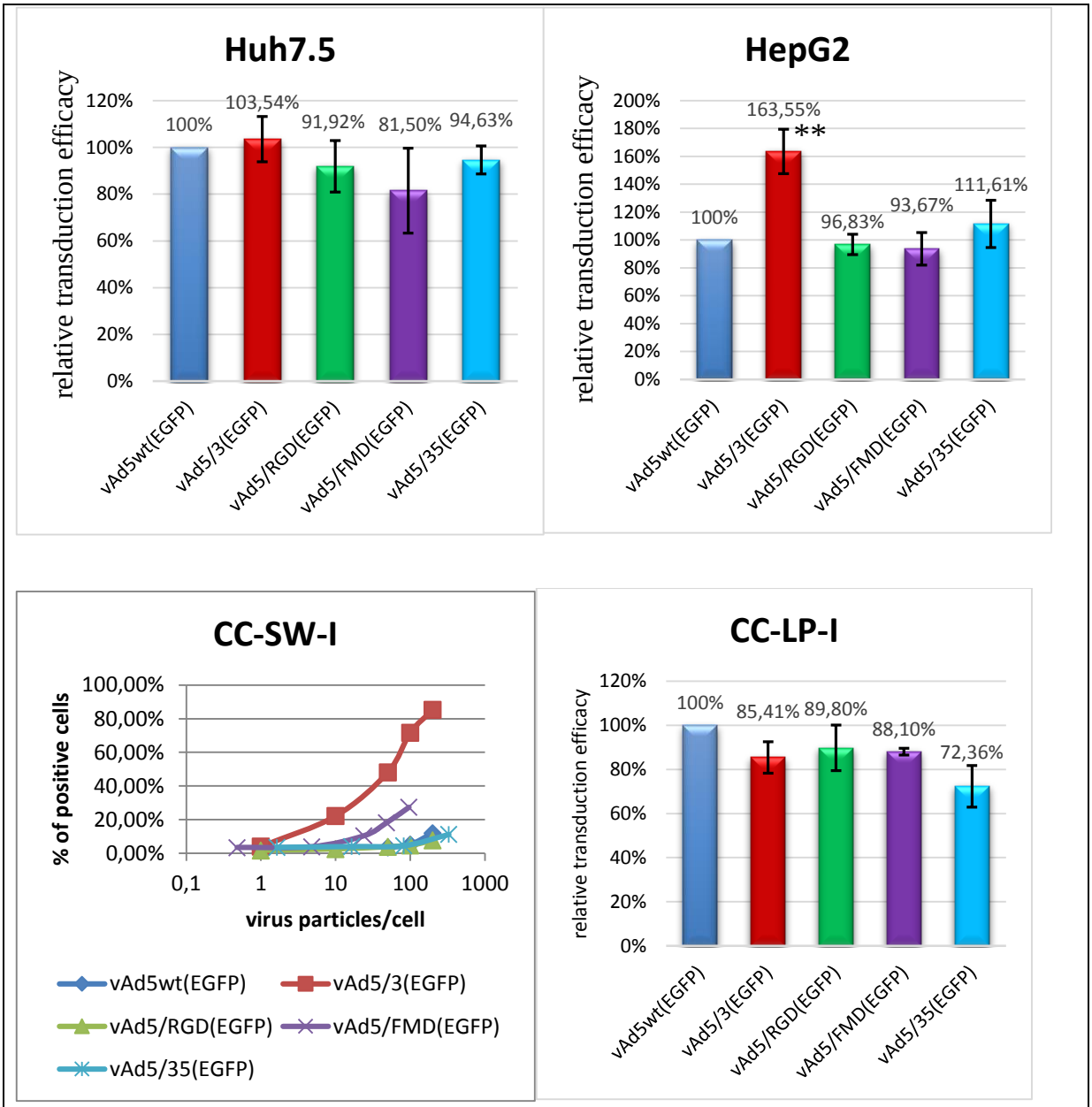
Harvesting cells at least 48 hours post infection was found as the most appropriate time point for harvesting the cells infected with the replication defective viruses*[9] (Supplementary figure 5). The transduction efficacy of the fibre-modified viruses in the different cell lines is summarised in Figure 11. Results from CC-LP-I and CC-SW-I are also included but work on these cell lines was continued in more detail by Yi Hsuan Chen [22].

No significant difference among the viruses was found in Huh7.5. In HepG2 the transduction efficacy of vAd5/3(EGFP) was on average 60% better than the rest of the viruses. In CC-SW-I the transduction efficacy of vAd5/3(EGFP) [and to a lesser degree of vAd5/FMD(EGFP)], was evidently better compared to the rest of the viruses. However, despite the significantly increased efficacy of vAd5/3(EGFP) in HepG2 and CC-SW-I, the percentage of infected cells remained much lower compared to that of Huh7.5 or CC-LP-I at the same MOI (Figure 12).

Primary hepatocytes were transduced with all the virus, although unexpectedly vAd5/RGD(EGFP) and vAd5/FMD(EGFP) were significantly less effective compared to the other viruses (Figure 13). Primary ICCA cells were found to be very resistant to adenovirus transduction and very high MOIs were required for successful transduction as measured by flow cytometry. Overall, vAd5/35(EGFP) had a markedly higher transduction efficacy, although very high MOIs were required. The very wide distribution of the autofluorescence of the uninfected control combined with the low EGFP expression by the replication-defective viruses at low MOIs or the shift of the negative population at the very high MOIs complicated accurate quantification of the relative transduction efficacies (Supplementary figure 7).

*[9] For some of the experiments that were done before the time course experiments cells were harvested at earlier time points (e.g. 38-45 hours post infection). This didn't seem to have any significant effect on the relative transduction efficacy of the viruses.

Figure 11; Comparison of the fibre-modified viruses in the different cell lines



The HepG2 and Huh7.5 bar charts represent the averaged results (± SD error bars) from at least 4 independent experiments as described in the "MATERIALS AND METHODS" section. The CC-SW-I graph illustrates a titration of the viruses. CC-LP-I and CC-SW-I cell lines are discussed in more detail in Yi Hsuan Chen's thesis [22]. **Two-tailed p value < 0.01 compared to any virus - calculated with Excel's "two sample t-test assuming unequal variance". Statistical significance was even higher ($p<0.001$) with one-way repeated measures ANOVA (pairing the means from independent experiments) followed by the Holm-Sidak method for multiple pairwise comparisons (SigmaPlot).

Figure 12; Transduction efficacy of vAd5/3(EGFP) in the different cell lines

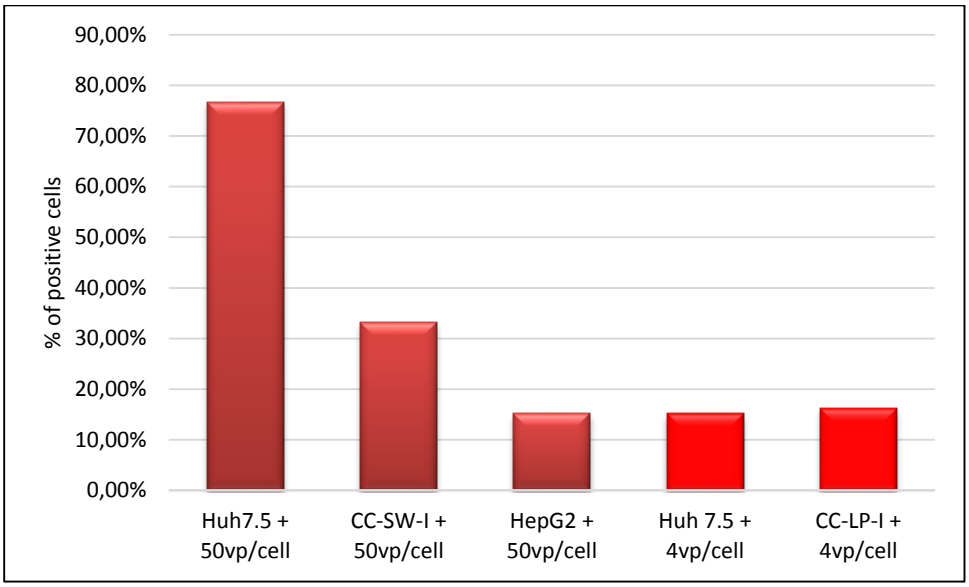
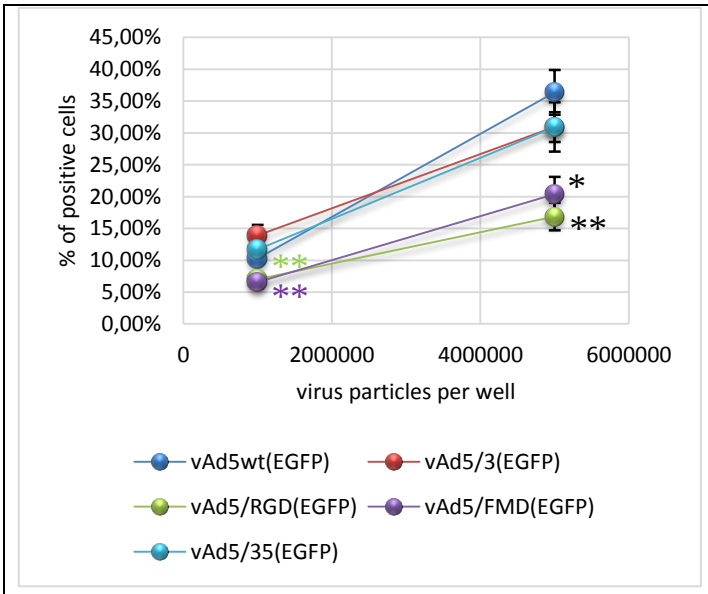
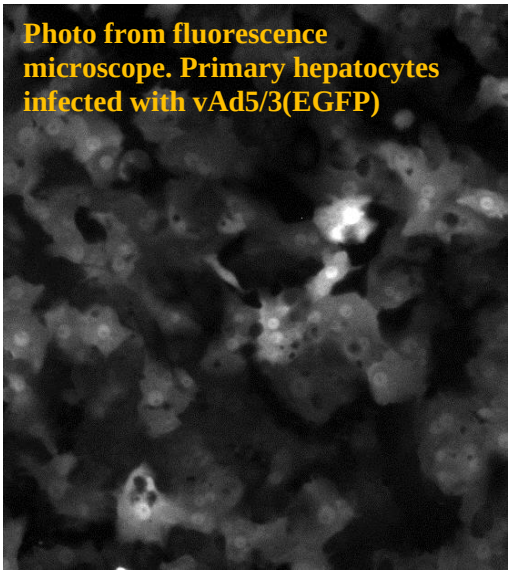


Figure 13; Transduction of primary hepatocytes





Primary hepatocytes were isolated form a marginal liver donor (see also Supplementary figure 10). 5*10^5 cells were seeded in 24 well plates, although the final cell number was probably much less. Results represent the mean (± SD error bars) from quadruplicate wells. Using SigmaPlot's Holm-Sidak method for multiple pairwise comparisons following one way ANOVA, the lower transduction by vAd5/FMD and vAd5/RGD was statistically significant comparing to the other three viruses. At the highest dose, pairwise comparisons resulted in p values of at least <0.031 (*) for vAd5/FMD, and at least <0.002 (**) for vAd5/RGD. At the lowest dose, the p values were at least < 0.003 (**), apart from the pairwise comparisons with vAd5 which weren't significant at this dose.

Co-infection experiments

As shown by the time course experiments (Supplementary figure 5), it takes at least 48 hours post-infection for the replication defective viruses to express high enough levels of EGFP. However at the same time the cells keep replicating*[10] which may result in dilution of EGFP and reduction of the calculated percentage of positive cells [especially if uninfected cells have a higher chance to replicate due to adenovirus-induced cell cycle arrest of infected cells (e.g. [75])]. The co-infection of cells with the EGFP-expressing replication defective fibre-modified adenoviruses and the replication competent, reporter-less vAd5wt(WTp-E1AΔ24)(no-EGFP) results in higher expression of EGFP and allows harvesting cells at a much earlier time point. However, the transduction of HepG2 cells remained much lower compared to the transduction of Huh7.5 (Supplementary figure 6), therefore excluding the above-mentioned problems as a cause for the low transduction observed. Similarly, lower transduction of HepG2 cells was observed with the replication-selective viruses. The relative transduction efficacy of the viruses in the co-infection experiments was similar to that shown in Figure 11.

Flow cytometry staining for the viral receptors and CD40

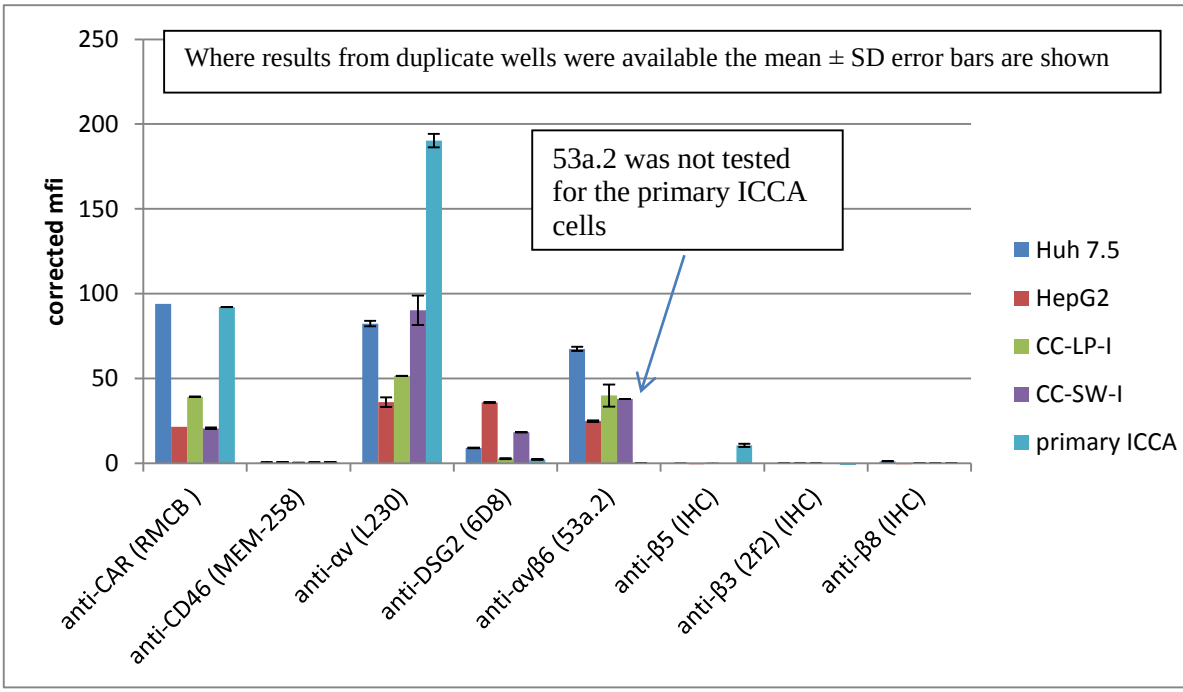
The levels of expression of the viral receptors are shown in Figure 14. Unexpectedly, CD46 was not detected with flow cytometry. Furthermore, there doesn't seem to be a good correlation between the levels of the viral receptors and the transduction efficacy. For example, CAR seems to be expressed by all the cell lines tested (Figure 15). A more detailed discussion is given in the "DISCUSSION" section. Regarding CD40 expression, CC-LP-I showed the highest level of expression followed by CC-SW-I. No or very low levels of CD40 were detected in the rest of the cell lines (Figure 16).

These results were confirmed with a second independent experiment. No significant difference was detected using non-enzymatic harvesting (with 10mM EDTA in PBS), therefore excluding sensitivity

*[10] Despite the use of 2% FCS medium, the cell count at the day of harvesting was found to be twice or more compared to the cell count at the day of the infection. Note that uninfected wells were used for the cell counts.

to trypsinization as a confounding factor (data not shown). It is worth noting that none of the polyclonal antibodies used for IHC (e.g. anti-αvβ6, anti-DSG2, anti-CD46, anti-β5, anti-β8) detected the target antigens with flow cytometry even when the corresponding monoclonal antibodies were used successfully (e.g. 6D8 versus the polyclonal anti-DSG2, or 53a.2 versus the polyclonal anti-αvβ6, Supplementary figure 12). This could suggest unsuitability of these antibodies for flow cytometry applications or a problem with the secondary anti-rabbit antibody used (e.g. lack of recognition by the secondary antibody or too low concentration).

Figure 14; Expression of the viral receptors as detected by flow cytometry



RMCB, MEM-258, L230, 6D8, 53a.2 and 2f2 represent monoclonal antibodies (name of the clone). IHC indicates antibodies that were used for immunohistochemistry, i.e. the polyclonal anti-β5 and anti-β8, and the monoclonal anti-β3.

Figure 15; CAR expression by the different cell lines as detected by flow cytometry

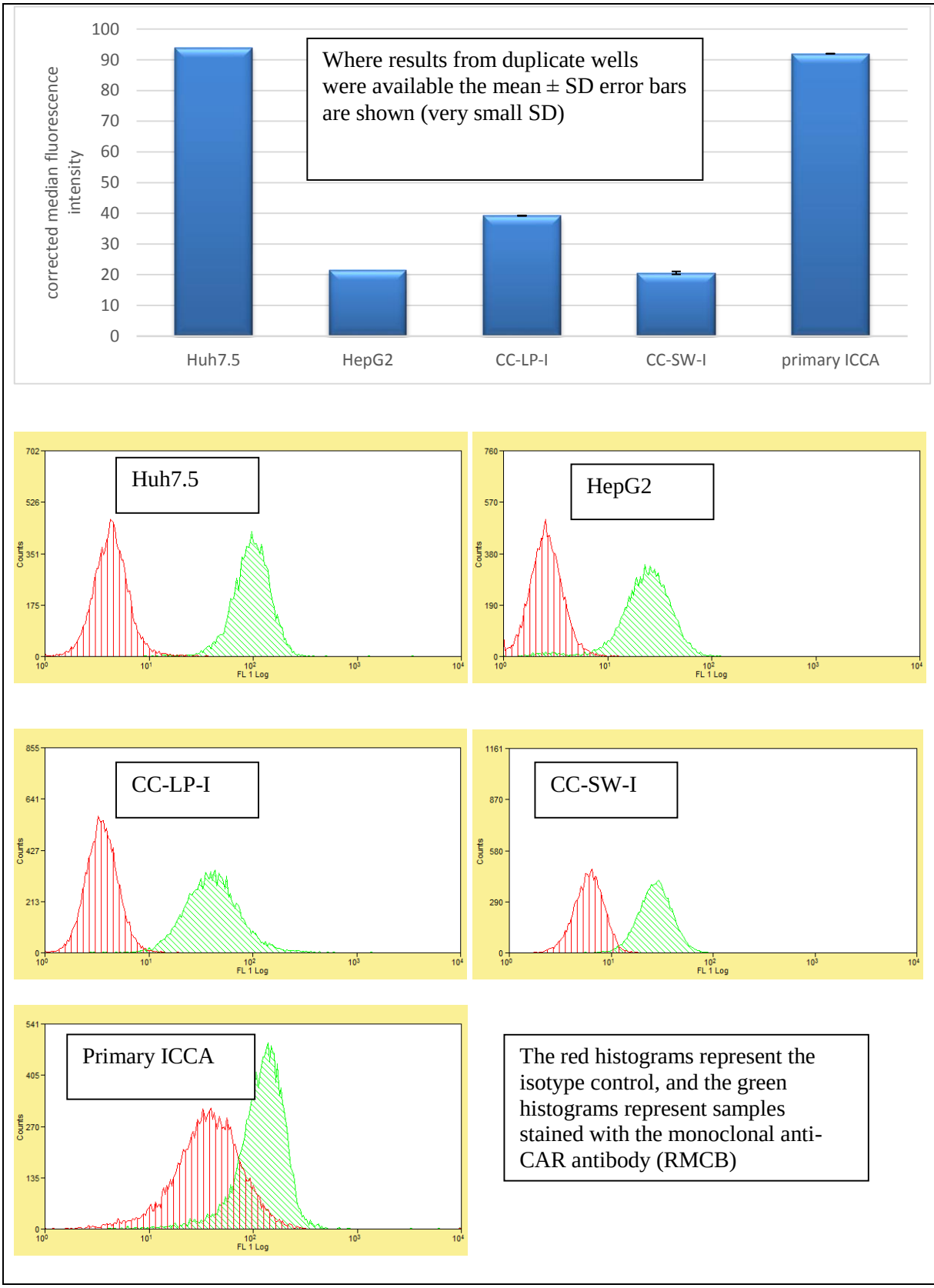
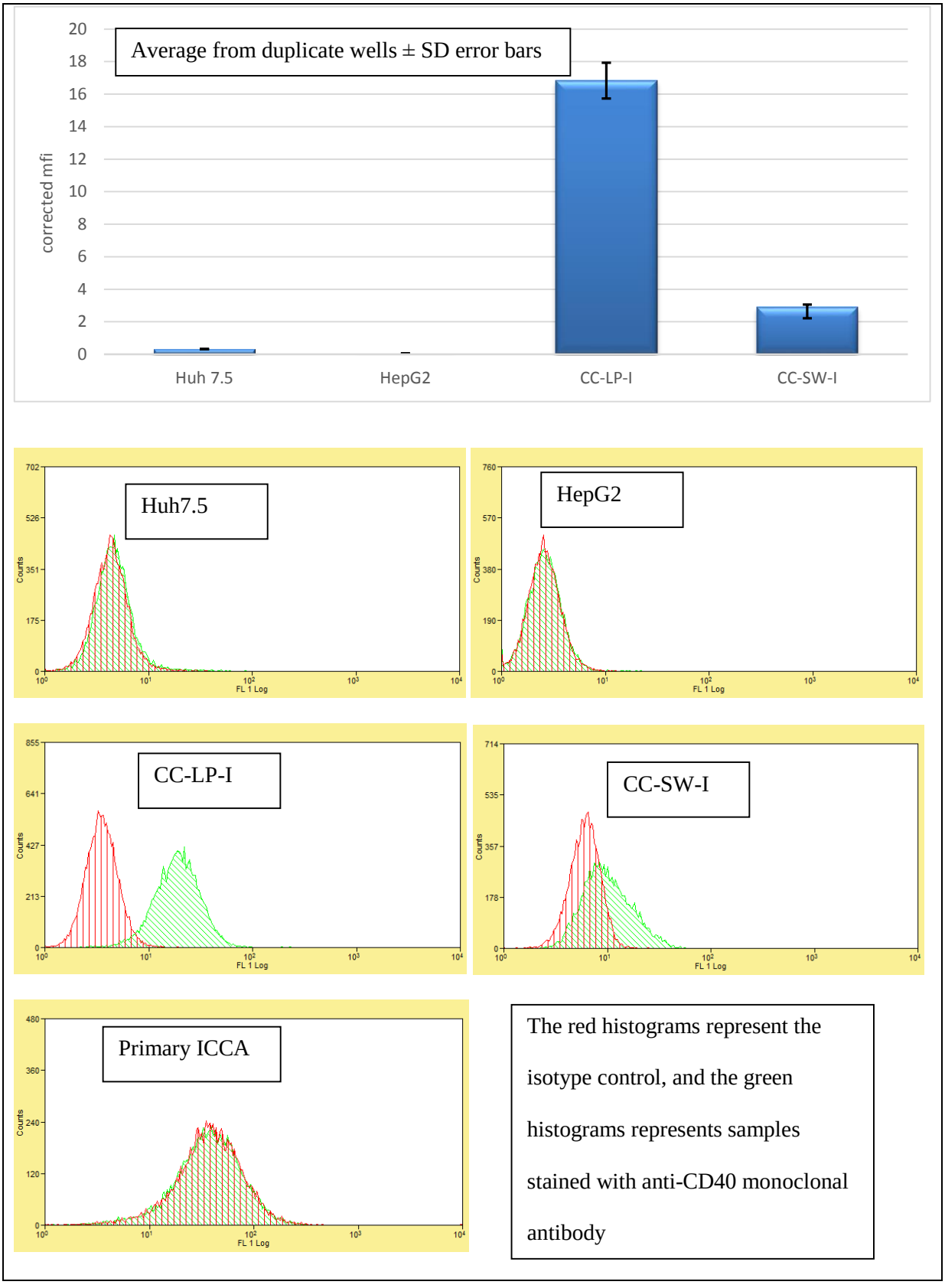


Figure 16; CD40 levels as detected by flow cytometry



Blocking experiments

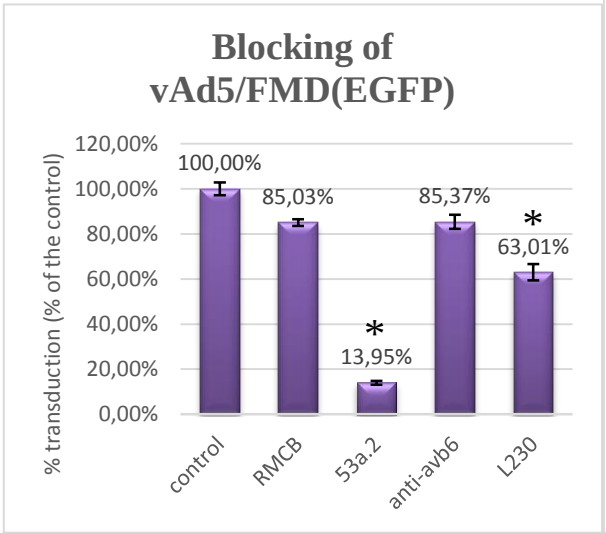
To further confirm the receptor specificity of the fibre-modified viruses blocking experiments were performed. For the blocking experiments the CC-LP-I and CC-SW-I cell lines were selected. CC-LP-I was selected because of the reproducibly low overlap between the negative and positive population in flowcytometry. CC-SW-I was selected because of the significant differences of transduction efficacy observed, as presented previously. Regarding the CC-SW-I cell line, only vAd5/3(EGFP) and vAd5/FMD(EGFP) were tested, since the rest of the viruses showed very low transduction. Ideally, similar blocking experiment should have been done with the HCC cell lines. The results are summarised in Figure 17 and Figure 18 (see also Supplementary figure 13 for some of the corresponding flow cytometry histograms). For the protocol used see page 26.

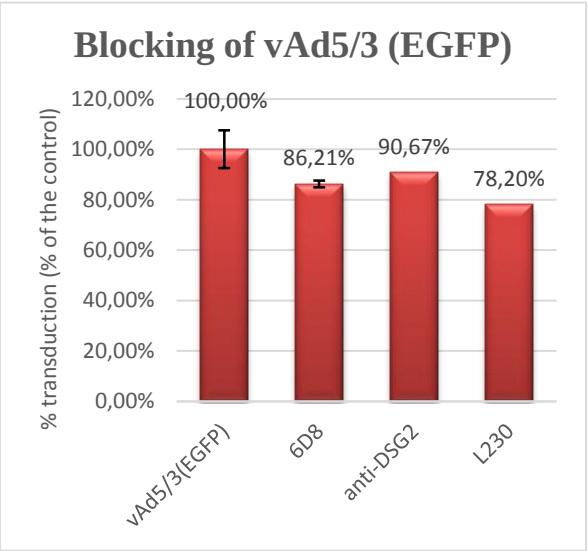
Transduction of CC-SW-I by vAd5/FMD was significantly blocked by 53a.2 (the monoclonal antibody against αvβ6) and to a lesser degree by the anti-αv monoclonal antibody (L230). RMCB (the monoclonal antibody against CAR) had a very small effect on the infection of CC-SW-I by vAd5/FMD, confirming that the primary receptor used for infection of CC-SW-I is αvβ6. Interestingly, the reverse results were found using the CC-LP-I cell line, i.e. significant blocking of infection by RMCB pre-incubation and very small effect of 53a.2 suggesting that the primary receptor for infection of CC-LP-I cells is CAR. RMCB also had a significant inhibitory effect on infection by vAd5/RGD(EGFP) while unexpectedly the effect on vAd5wt(EGFP) infection was very small. This is discussed further later ("DISCUSSION" section). The anti-CD46 monoclonal antibody (MEM-258) blocked significantly transduction of CC-LP-I by vAd5/35(EGFP) while the same antibody had very little effect on transduction by vAd5wt(EGFP) therefore confirming that vAd5/35(EGFP) uses CD46 as its primary receptor. Significant blocking of vAd5/3(EGFP) was not achieved, even with very high antibody concentration (up to 40μg/ml of 6D8, the monoclonal antibody against DSG2), but this was not unexpected as 6D8 had shown only a small inhibitory effect previously [26]. None of the polyclonal antibodies tried (anti-CAR, anti-CD46, antiDSG2, anti-αvβ6- see Table 3) had any significant blocking effect.

Figure 17; Blocking of fibre-modified viruses- CC-LP-I cell line

The data represent the mean of duplicate wells (± SD error bars). The asterisks (*) next to the antibodies indicate data averaged from two independent experiments (duplicate wells per experiment). See Table 3 for the antibodies used. Two-tailed p values compared to the control; ***<0.001,**<0.01,*<0.05. Calculated with Excel's "two sample t-test assuming unequal variances".

Figure 18; Blocking of fibre-modified viruses- CC-SW-I cell line





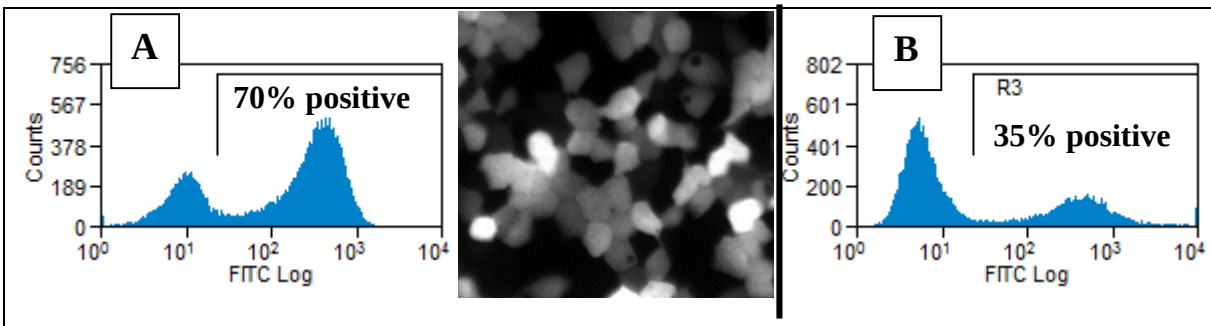
Where results from duplicate wells were available the mean (±SD error bars) are shown. *two-tailed p value < 0.05. Calculated with Excel's "two sample t-test assuming uneven variances".

Further experiments on transduction

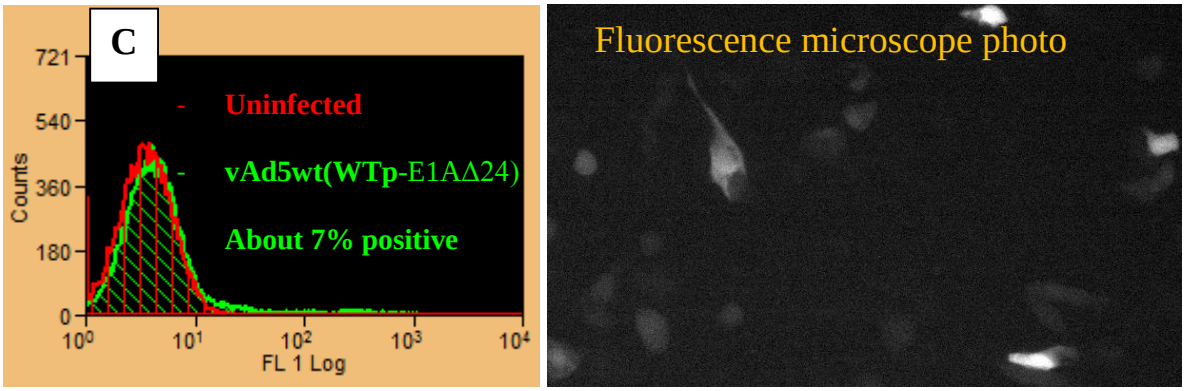
Previous experiments showed very ineffective transduction of CC-SW-I and primary ICCA cells despite expression of CAR as detected by flow cytometry. A possible explanation might be that the receptors might not be available for binding (e.g. hidden in tight junctions [76, 77]). This possibility was supported by the fact that infection of CC-SW-I cells in suspension significantly improved the transduction efficacy (Figure 19). However the same effect was not seen in primary ICCA cells, where infection with up to 5000 vp/cell resulted in a very low percentage of positive cells (Figure 19).

An MTT assay of primary ICCA cells was set up before the low transduction efficacy was yet known. Even at doses of more than 2000 vp/cell only very few fluorescent cells were detected. Therefore instead of doing the MTT assay the cells were followed to see if the virus can spread from the very few infected cells. Indeed, the virus could successfully spread from infected cells to neighbouring cells (Figure 20).

Figure 19; Infection of CC-SW-I and primary ICCA in suspension

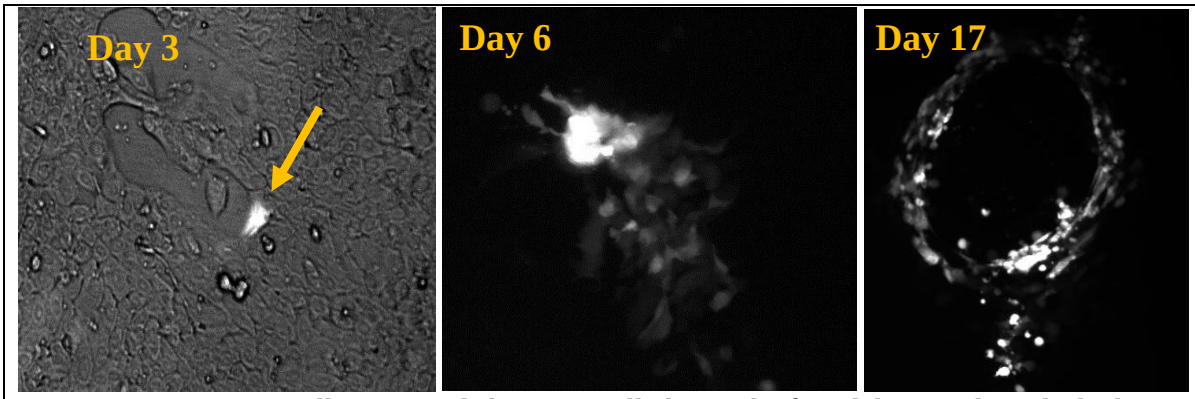


1.3*10^6 CC-SW-I cells were seeded in a 6-well plate and infected; (A) immediately with 1.3*10^8 vp of vAd5wt(WTp-E1AΔ24) or (B) the next day with 2*10^8 vp of vAd5wt(WTp-E1AΔ24). Cells were harvested and analysed with flow cytometry the next day after infection.



(C) 4*10^5 primary ICCA cells were seeded in a 24 well plate and infected immediately with the replication-selective viruses. The results shown here represent infection with 5000 vp/cell of vAd5wt(WTp-E1AΔ24).

Figure 20; Cell-to-cell spread in primary ICCA cells



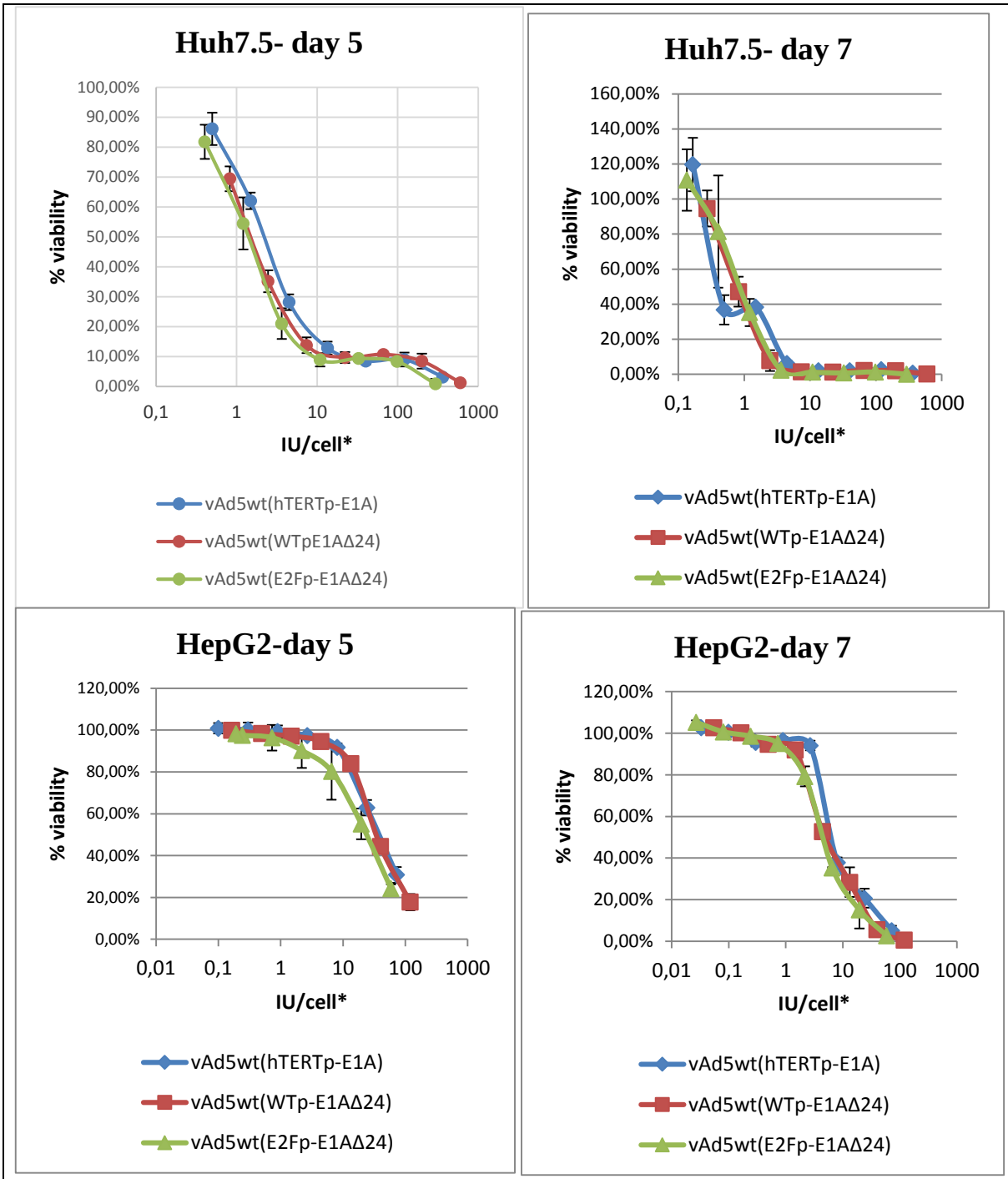
4*10^4 primary ICCA cells were seeded in a 96-well plate and infected the next day. The highest dose was 1.3*10^8 vp. The arrow in the first photo indicates one of the very rare fluorescent cells, from a well infected with the highest dose of vAd5wt(WTp-E1AΔ24). Interestingly, despite the very low initial transduction efficacy, the virus could spread efficiently to neighbouring cells.

Comparison of the replication-“selective” viruses

Analysis of the replication-selective viruses with flow cytometry was complicated by a significant and variable shift of the uninfected population suggesting uptake of EGFP by uninfected cells. This was supported by the fact that mixing cells from uninfected and infected wells during the harvesting process resulted in the same shift (see page 73). So more focus was placed on the MTT assays which are also more informative regarding the cytotoxicity of the viruses. Briefly, the flow cytometry experiments with the replicating viruses showed a similar transduction efficacy pattern to that of vAd5wt(EGFP), i.e. higher transduction of Huh7.5 compared to HepG2 cells. Furthermore, the intensity of the fluorescence of all the viruses was much higher compared to that of the replication-deficient viruses and there was a clear separation of the negative and positive population within 24 hours post-infection. This suggests that all the viruses were replicating efficiently. There were small differences in the transduction efficacy and the mean fluorescence intensity between the viruses but they did not correlate with their cytotoxicity in MTT assays.

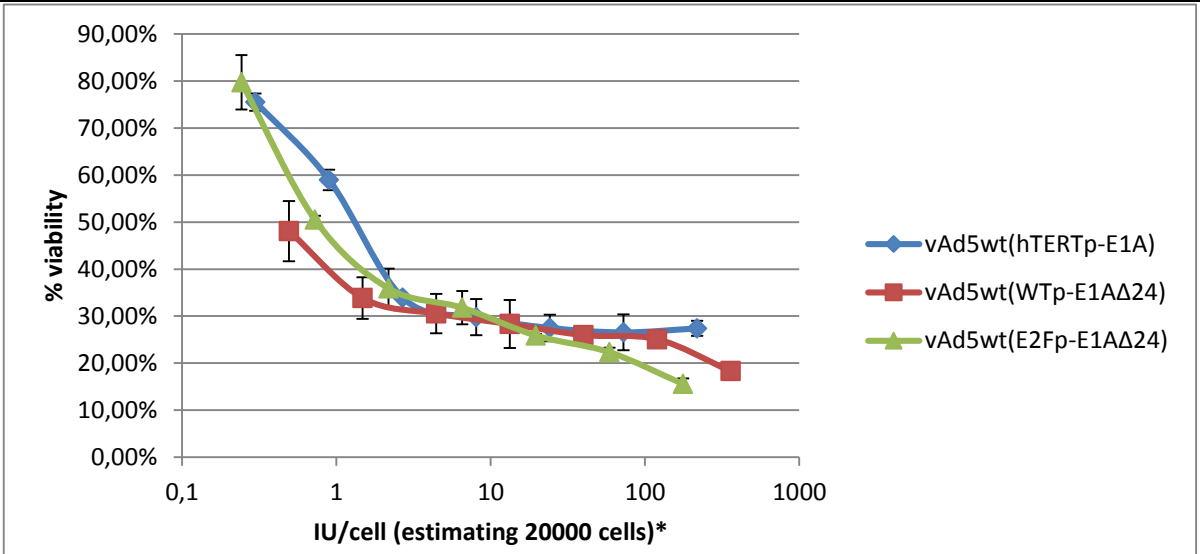
As expected from the previous results on the transduction efficacy, the MTT assays showed that the cytotoxicity of the viruses in HepG2 was lower compared to that in Huh7.5 at the same dose (Figure 21). The differences between the viruses were small. Unexpectedly, the viruses were not found to be selective in an MTT assay of primary hepatocytes isolated from a marginal liver donor (Figure 22) (see also Supplementary figure 8-9). vAd5wt(hTERTp-E1A) and vAd5wt(E2Fp-E1AΔ24) seem a bit more selective than vAd5wt(WTp-E1AΔ24) at the lowest MOIs used.

Figure 21; Comparison of the replication-selective viruses in HCC cell lines with MTT assay



10000 (Huh7.5) or 20000 (HepG2) cells were seeded in 96-well plates and infected the next day with 3-fold serial dilutions of the viruses. The MOIs (IU/cell) are calculated based on the estimation that cell number increased by about 50% from seeding to infection. Data shown represent the mean ± SD from triplicate wells.

Figure 22; MTT assay in primary hepatocytes (day 3)



* 50000 primary hepatocytes isolated from a marginal liver donor were seeded in a 96-well plate. However the wells required a lot of washing (see Supplementary figure 10) and the final number of hepatocytes was much less. Here the MOI is calculated estimating 20000 cells per well. . Data shown represent the mean ± SD from quadruplicate wells.

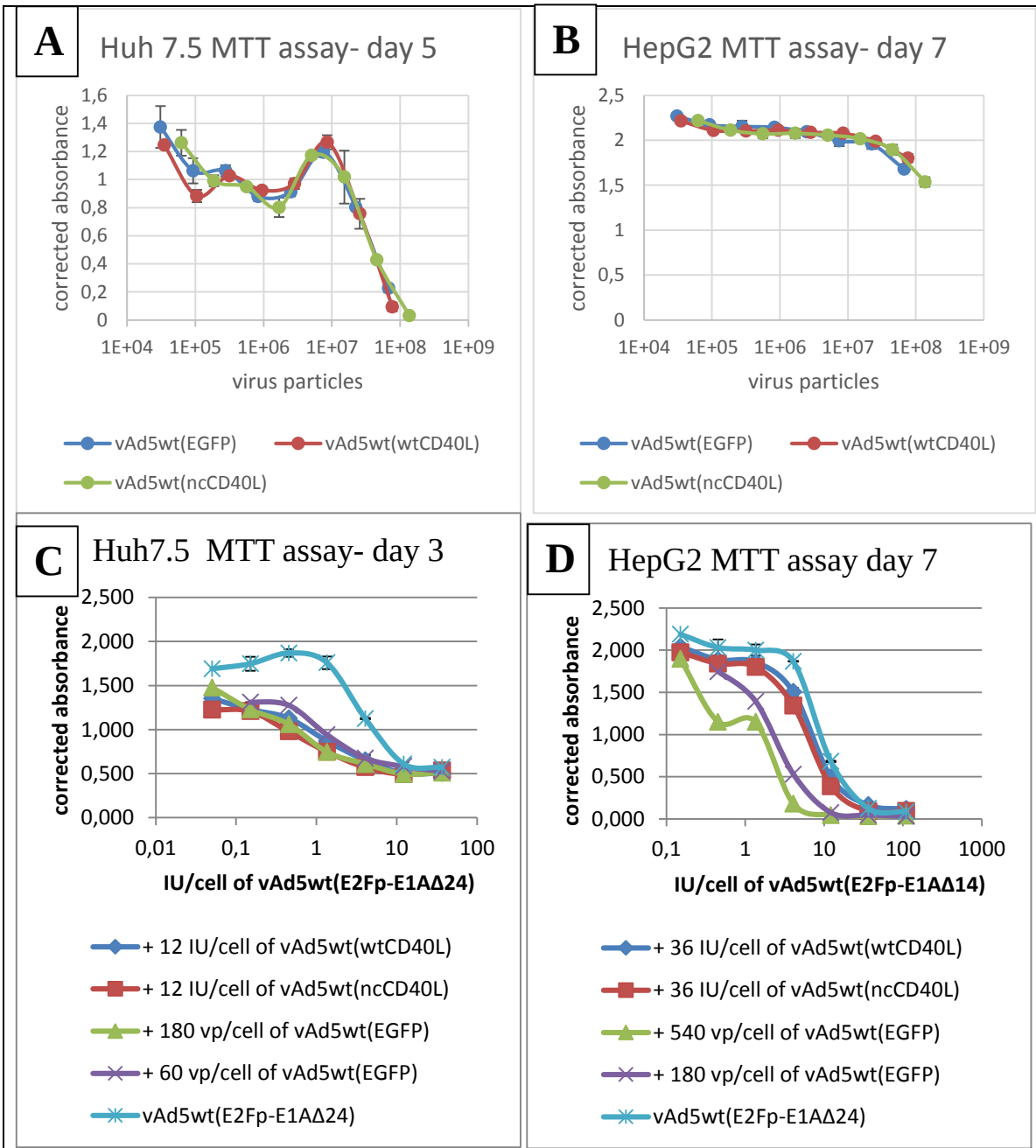
Effect of CD40L on viability and comparison of wtCD40L vs ncCD40L

The CD40L-expressing viruses*[11] were tested with MTT assays in the HCC cell lines (HepG2 and Huh7.5). A similar comparison was done by Yi-Hsuan, Chen in the CC-LP-I cell line [22]. vAd5wt(wtCD40L) and vAd5wt(ncCD40L) didn't offer any advantage in Huh7.5 and HepG2 compared to vAd5wt(EGFP) (Figure 23A and B). In HepG2 there was little cytotoxicity even at the highest dose (about 330 IU/cell), while in Huh7.5 the cytotoxicity of the CD40L-armed viruses was not greater than that of vAd5wt(EGFP). Furthermore, combining the replication-competent vAd5wt(E2Fp-E1AΔ24) with the replication-defective CD40L-expressing viruses didn't offer any advantage compared to combination with the replication-defective vAd5wt(EGFP)*[12] (Figure 23C and D). Therefore, there doesn't seem to be any synergism between oncolysis and CD40L expression

*[11] Expression of CD40L was confirmed by Dr Searle with flowcytometry staining (of A549 cells infected with increasing MOI of the CD40L viruses) with an FITC-labelled anti-human CD154 antibody (Biolegend). A dose dependent increase of CD154 was seen, confirming expression of the transgene by the viruses.
*[12] Although vAd5wt(EGFP) may not be an appropriate control as it has an intact E3 region, while the CD40-ligand viruses are E3 deleted.

in these cell lines in vitro. However, in Huh7.5 vAd5wt(ncCD40 L) seems to be slightly better than vAd5wt(wtCD40L) (Supplementary figure 11). The difference in HepG2 was much smaller.

Figure 23; MTT assay of CD40L-expressing viruses.



(A) and (B); infection with each virus alone (doses plotted as vp because the corresponding IU for vAd5wt(EGFP) is not known). (C) and (D); combination with vAd5wt(E2Fp-E1AΔ24). MTT assays were done at different time points (up to day 7). Different doses of the CD40L-armed viruses in the combination experiments were also tried (Huh7.5; 12 IU/cell, 4 IU/cell, 1.4 IU/cell, 0.5 IU/cell. HepG2; 36 IU/cell, 12 IU/cell, 4 IU/cell, 1.4 IU/cell). At all time points and doses the combination with vAd5wt(EGFP) was at least as good as the combination with the CD40L-armed viruses. One IU of vAd5wt(wtCD40L) and vAd5wt(ncCD40L) corresponds to about 16 and 28 vp respectively.

DISCUSSION-SUMMARY

Comparison of the fibre-modified adenoviruses

Four fibre-modified adenoviruses and a wild type fibre virus, all replication defective and EGFP-expressing, were constructed and compared side-by-side for their transduction efficacy and selectivity. Apart from the fibre-modification the rest of their genome, and therefore the rest of the sviral capsid, is identical for all the viruses. Therefore any differences observed can be attributed to the modified fibres.

A problem with comparing the fibre -modified viruses with each other is the lack of an appropriate control cell line. Differences in the transduction efficacy may reflect differences in the virus prep quality (particle-to-infectivity ratio) rather than the effect of the fibre modification. An ideal control would be a cell line that is transduced by all the viruses equally effectively. Despite the lack of such a control some conclusions can be made. For example, in most cases the differences between the viruses in the different cell lines are insignificant or very small, suggesting that there aren't significant differences in the virus prep quality. Therefore, the superior efficacies of vAd5/3(EGFP) in HepG2 and especially of vAd5/3(EGFP) and vAd5/FMD(EGFP) in CC-SW-I are valid, although an accurate quantification without a proper control is not possible. The results about vAd5/35(EGFP) differ from that previously published. A virus with the 5/35 chimeric fibre was reported to have a significantly improved transduction efficacy in Huh7 and HepG2 compared to a virus with the wild type fibre [23].

Despite the lack of a control to take into account differences in the virus prep quality, conclusions about the relative transduction of each virus in primary hepatocytes compared to cancer cell lines can still be made. Quantifying the transduction efficacy in primary hepatocytes was complicated by the high amount of debris, the variable number of surviving hepatocytes and the uncertainty of defining the hepatocyte population in flow cytometry. However it seems that primary hepatocytes were transduced at least as effectively if not better compared to HepG2 or CC-SW-I cells but probably less

effectively compared to Huh7.5 or CC-LP-I. Transduction of hepatocytes by vAd5/FMD(EGFP) and vAd5/RGD(EGFP) was significantly less compared to the rest of the viruses.

The tendency of vAd5/FMD(EGFP) and vAd5/RGD(EGFP) to show slightly lower transduction efficacy in most cell lines may represent a difference in the virus prep quality. Alternatively, a weaker binding to CAR receptor may explain these results and the results of the blocking experiments (transduction by these viruses was blocked with an anti-CAR antibody significantly more than the virus with the wild type fibre). Another possible explanation for the results of the blocking experiments is that the insertion of the FMD and RGD motifs in the fibre knob might allow more effective antibody-mediated steric hindrance of binding to CAR. Interestingly, reduced liver uptake in vivo of the Ad5/FMD fibre-modified has been reported in the original paper describing this modification, although this was attributed to altered electrostatic interactions with blood components and Kupffer cells, and therefore cannot explain the in vitro result reported here [29]. On the other hand, increased uptake by most organs has been reported for a virus with the RGD-modified fibre [78].

As already mentioned, a short absorption time (40 minutes) was selected for comparing the transduction efficacy assuming that this design would be more sensitive at detecting differences in the transduction efficacy. However, it would have been interesting to do similar experiments using longer absorption times as the binding affinity to the cells or internalization kinetics of the viruses might have played a significant role when using the shorter absorption time. For example, a virus with low binding affinity and slower internalization kinetics may be less likely to transduce a cell within 40 minutes and more likely to be washed away during the washing step. This might explain the unexpected lack of advantage of vAd5/RGD(EGFP) and vAd5/FMD(EGFP) (with the exception of the CC-SW-I cell line) over vAd5wt(EGFP), despite their extended receptor repertoire. As suggested by the results of the blocking experiments it is likely that these viruses bind to CAR with lower affinity. It is possible that longer absorption times would have allowed more effective utilization of the extended receptor repertoire. Nevertheless, in the original paper describing the

5/RGD modified fibre, an absorption time of just 30 minutes was used and the virus was significantly better than the corresponding wild type fibre virus [28].

Correlation with the flow cytometry staining for the receptors

Interestingly, the expression of the receptors as detected by flow cytometry showed little correlation with the transduction efficacy of the viruses. For example, CAR was highly expressed by all cell lines but transduction varied significantly, from the very ineffective transduction in the primary ICCA cells and CC-SW-I to the high transduction efficacy in Huh7.5 and CC-LP-I. Lack of correlation between expression of CAR and the transduction efficacy has been reported previously [79-81]. One possible explanation is that the receptors might not be available for binding (e.g. hidden in tight junctions [76, 77]). This might be the case with the CC-SW-I cell line for example*[13], as infection in suspension significantly improved the transduction efficacy, and resulted in very high levels of fluorescence intensity, at least comparable if not better to the more easily transducible cell lines. However the same does not apply to the primary ICCA cells which were very ineffectively transduced even at high MOIs and in suspension.

Many other factors apart from the presence and levels of the receptor can affect the transduction efficacy as defined by the percentage of cells expressing the transgene (in this case EGFP). Adenoviral transduction involves many steps; binding to the cell, internalization of the virus, endosomal escape, trafficking to the nucleus and finally expression of the transgene. Furthermore, anti-viral responses may also play a role. An obstacle in any of these steps could explain the ineffective transduction of the primary ICCA cells. For example, blocking of adenovirus trafficking to the nucleus has been reported previously as the reason for inefficient transduction in human pancreatic cancer cell lines [82]. Interestingly, despite the low efficiency of the cell-free virus to infect the primary ICCA cells, efficient cell to cell spread was demonstrated here.

*[13] Although it is questionable whether CC-SW-I cells can form an epithelial monolayer with tight junctions in the culture system used.

The flow cytometry staining results reported here have some limitation:

- Negative control cell lines should have ideally been included, e.g. for CAR since all the cell lines tested showed high positivity. However the results for Huh7.5 and HepG2 are similar to that published previously, i.e. both HCC cell lines are positive for CAR and CAR expression in Huh7 is higher than in HepG2 [23].
- Lack of an appropriate rat isotype control casts doubt on the results regarding αvβ6 receptor expression. Furthermore, b6 integrin has been previously reported to be very low in HepG2 using RT-PCR and immunocytochemistry [32]. However similar discrepancies have been reported in the literature before, as discussed later for the CD40 expression by HepG2 cells. The passage history of the cell line may play a role in such discrepancies, as the HepG2 cell line was established more than 30 years ago.
- Unexpectedly CD46 was not detected with either MEM-258*[14] or the polyclonal anti-CD46 used for immunohistochemistry (although none of the antibodies used for IHC detected the antigens with flow cytometry). The fact that MEM-258 can very effectively and specifically block transduction by Ad5/35 indicates that the receptor was present. Furthermore, Huh7 and HepG2 were found positive for CD46 with flow cytometry in a previous study [23]. Non enzymatic harvesting (with 10mM EDTA in PBS) didn't make a difference therefore excluding loss of the receptor due to trypsinization. A possible explanation is lack of recognition of MEM-258 by the secondary antibody, although the antibody worked for the rest primary antibodies of the same isotype. Alternatively, antigen internalization or capping and shedding may have caused loss of the antigen. However, doing the staining on ice should have limited these issues. Since viability of the cells was not an issue maybe sodium azide should have been added to the FACS buffer, to further minimize surface antigen loss.
- None of the polyclonal antibodies that were used for immunohistochemistry detected the target antigen in flow cytometry, even when the antigen was detected by the corresponding

*[14] The monoclonal antibody against CD46

monoclonal antibodies. Therefore, the lack of detection of integrins b3 and b5 may not be real. However, knowing the levels of these integrins could have explained the lack of correlation of transduction with the levels of CAR, as they are important for virus internalization (e.g. [80, 83]).

Blocking experiments to confirm the receptor specificity of the viruses

The results from the blocking experiments confirmed that vAd5/35(EGFP) uses CD46 as a receptor. The use of DSG2 by vAd5/3(EGFP) could not be confirmed, however this was expected as the antibody used here (6D8) had little effect on the binding of the virus in a previous study [26]. The use of integrin αvβ6 by vAd5/FMD(EGFP) was confirmed in CC-SW-I, while RMCB (the anti-CAR antibody) had a non-significant effect in this cell line. This is in agreement with what would be expected from the transduction experiments, as vAd5/FMD(EGFP) was significantly more effective than vAd5wt(EGFP) in CC-SW-I. Unexpectedly, in CC-LP-I vAd5/FMD(EGFP) infection was significantly blocked by RMCB but not by 53a.2 (the anti-αvβ6 antibody) despite that αvβ6 was detected with flow cytometry. Furthermore, vAd5/RGD(EGFP) infection was also significantly inhibited by RMCB while the effect on vAd5wt(EGFP) was much less. As discussed previously, this might have to do with lower binding affinity to CAR or more effective antibody-mediated steric hindrance in the case of the RGD and FMD modified viruses, although it is unclear why the viruses could not use the alternative receptors.

Immunohistochemistry of HCC for the viral receptors

Immunohistochemistry was performed to compare the level of expression of the viral receptors between HCC and non-tumour tissues. Expression of CAR has been reported previously to be variable in tumours, including HCC [23, 24]. Here, most tumours were found to maintain expression of CAR (6 of 7), although in 2 cases CAR was focally expressed and in another 2 the staining intensity was lower compared to the corresponding “normal” tissue. In cases where CAR expression was lost or reduced, other receptors like DSG2 or CD46 were highly expressed. However, none of the receptors was found to be specifically expressed in HCC compared to non-tumour liver. It has been

reported previously for CD46 that much higher levels are detected in HCC compared to diseased non-tumour or normal liver [23, 31]. However, this was not replicated here. According to five HCC sections that included both the tumour and the surrounding margin, CD46 was expressed by both tissues, and the intensity of the staining wasn't significantly different. The clinical relevance of IHC however is questionable considering the possible lack of correlation between receptor expression and transduction efficacy as shown for the cell lines.

Replication selective adenoviruses

Three conditionally replicating adenoviruses made in a parallel project [22], vAd5wt(hTERTp-E1AΔ24), vAd5wt(WTp-E1AΔ24) and vAd5(E2Fp-E1AΔ24), were compared side by side in HCC cell lines and primary hepatocytes. No significant differences were found among the three viruses with MTT assays in HepG2 and Huh7.5 cell lines. Furthermore, an MTT assay using freshly isolated hepatocytes from a marginal liver donor (steatotic liver), demonstrated lack of selectivity of the viruses except possibly at the very lowest dose. Moreover, the detection of good levels of fluorescence just 17 hours post-infection of primary hepatocytes (from a liver with cryptogenic cirrhosis) suggests viral replication, since expression of transgenes inserted in the E3 region (in this case EGFP) is linked with transcription of E1A and viral replication [84]). However, the quality of the hepatocytes used for the MTT assay may be questionable (Supplementary figure 10).

It is also important to note that HCC develops from a background of a chronically diseased liver. This might affect the selectivity of the viruses. The liver has a remarkable regenerative capacity and chronic liver disease might activate pathways similar to that activated in tumours and exploited to achieve tumour-specificity. Therefore, using normal hepatocytes in vitro to demonstrate the selectivity of the viruses may not be clinically relevant, especially for HCC patients with background liver disease. For example, it has been reported that hTERT promoter is activated during liver regeneration [85]. Telomerase activity or hTERT expression has also been demonstrated in humans in regenerative and precancerous liver lesions [86-88]. Furthermore, the MOI used for infection is important as higher MOIs, e.g. after intratumoural injection of the virus into the liver, may be able to

override the selectivity of the virus. It has been previously reported that an hTERT-E1A controlled adenovirus showed very little toxicity against primary hepatocytes (e.g. [35, 89]). However, only low MOIs were used and the condition of the livers from which the hepatocytes were isolated is not described. Similarly, using normal fibroblast (e.g. [35, 90]) to demonstrate selectivity might also be irrelevant to HCC patients.

Nevertheless, conditionally replicating adenoviruses have been used previously in clinical trials with little reported toxicity [36-43]. However, the lack of significant hepatotoxicity in clinical trials in cases of extrahepatic administration may have to do with the fact that the virus might not be reaching hepatocytes at a high enough MOI to override selectivity, e.g. due to elimination of the virus by tissue macrophages, like Kupffer cells in the case of the liver. Alternatively, the microenvironment of a diseased liver may not be favourable for the virus (reviewed in reference [91]). Results about safety from patients without liver disease may not be relevant to HCC patients, for the reasons explained above, but also because patients with HCC might be less tolerant to hepatotoxicity due to pre-existing liver dysfunction. Furthermore, intrahepatic intralesional administration of the viruses may pose a greater risk compared to extrahepatic administration, as a high MOI would be injected directly into the liver parenchyma. Intralesional administration of ONYX-015 in hepatobiliary tumours was reported as safe however this virus is significantly attenuated compared to the viruses tested here and patients were carefully selected (good performance status, adequate organ function, HBV excluded)[92]. Moreover, the interpretation about the safety might be questionable (3 patients died "of causes felt to be unrelated to study treatment"; serious toxicities were described as uncommon but affected 6 out of 19 patients, and cases of hepatic toxicity including one liver failure were attributed to disease progression). In an earlier study, ONYX-015 was also well-tolerated when administered intrahepatically, but no antitumour effect was seen in 4 of the 5 patients [93]. Whether the newer more potent and armed adenoviruses will be safe in HCC patients remains to be seen. An oncolytic immunotherapeutic vaccinia virus has already completed early clinical trials with promising results [94].

The CD40L-expressing viruses were not found to increase the cytotoxicity in HepG2 and Huh7.5, either alone or when co-infected with the replication-competent virus, compared to vAd5wt(EGFP). However, the comparison with vAd5wt(EGFP) is complicated by the fact that the CD40L-viruses are E3-deleted while the E3 region of Ad5wt(EGFP) is intact. This might explain the higher cytotoxicity of the co-infection with vAd5wt(EGFP) versus the co-infection with the CD40L expressing viruses, especially if the EGFP transgene in Ad5wt(E2Fp-E1AΔ24) affects the expression of other E3 region products (e.g. the adenovirus death protein [16]). Ideally an E1/E3 deleted virus not expressing the CD40L should have been used as a control. Alternatively, the CD40L transgene would be inserted into the E3 region of one of the replication-selective viruses, and the corresponding virus without this insertion would be used for comparison.

According to the flow cytometry staining, CD40 expression in the HCC cell lines was very low or undetectable, which might explain the previous results. The literature regarding the expression of CD40 by HepG2 cells is conflicting. Cruickshank et al [95] found expression of CD40 in biliary epithelial cell-derived cell lines and CC-SW-I but not in HCC-derived cell lines (including HepG2). Sugimoto et al [50] on the other hand have shown expression of CD40 by HepG2 cells with RT-PCR and flow cytometry staining. However CD40 activation did not induce apoptosis in HepG2 cells [50]. Depending on the cells and conditions, CD40 activation can both promote cell viability or induce cell death [50, 63, 96-99]. Regarding CC-LP-I and CC-SW-I, the results reported here are similar to that reported by Humphreys et al [63], i.e. both are positive and CD40 expression is higher in CC-LP-I than in CC-SW-I.

However the expression of CD40 by cell lines might not be representative of the in vivo situation as CD40 expression can be upregulated by inflammatory cytokines [50]. Also it has been reported previously that 60% of HCCs are positive for CD40 expression by immunohistochemistry [50]. While CD40 was not detected in normal or cirrhotic liver or in chronic hepatitis in that study [50], later the same authors found CD40-positive hepatocytes in HCV-associated chronic liver disease

[96]. Here CD40 was found positive in 3 of the 4 tumours stained for CD40, but was also detected in non-cancerous liver. The sections including non-tumour margin and stained for CD40 so far were from livers with chronic viral hepatitis. Unexpectedly CD154, the CD40 ligand, was also found strongly positive in most sections. Ideally, an appropriate negative control tissue or cell line should be used to exclude non-specific binding of the antibody. Nevertheless, the relevant isotype control antibody didn't show non-specific staining. Furthermore, according to the abstract of a Korean paper [100] CD154 was detected in 37.5% of 96 resected HCCs, and there were cases expressing both CD40 and CD40L.

The inefficacy of the CD40L viruses in vitro doesn't mean that the viruses will be ineffective in vivo, as has been demonstrated before [49]. Apart from any direct effect on tumour cells, CD40L expression might still be useful because of its immunostimulatory activity (reviewed by Ma and Clark [46]). Adenovirus mediated CD40L gene therapy (reviewed by Ullenhag and Loskog [101]) has been used successfully to treat HCC in vivo in animal models [102, 103] and this effect was shown to be immune-mediated rather than a direct effect on the tumour cells by CD40L. Furthermore, CD40L-armed adenoviruses have been used safely in early clinical trials and showed evidence of anti-tumour activity [104, 105]. However, it has been reported that activation of CD40 in HepG2 might facilitate immune escape by up-regulating negative co-stimulatory molecules [106] and recent studies suggested that the CD40/CD40L signalling can potentially have an immunosuppressive effect [107], although this was shown for the soluble CD40L and not the membrane bound form as it would be delivered by an oncolytic vector. For example, the membrane bound CD40L can more potently induce apoptosis, while the soluble form might promote tumour survival [52, 108]. Therefore, it is important to understand under what conditions CD40 activation can have unwanted effects.

Further discussion

- **Are the results reported here clinically relevant?** The results from the most clinically relevant experiment, the MTT assay on freshly isolated primary hepatocyte, were

unexpected. Further experiments in primary hepatocytes or primary biliary epithelial cells are needed to confirm or reject these results. However, whether freshly isolated hepatocytes are a good model for predicting the in vivo selectivity of the viruses is also questionable, as discussed previously. Another issue is that the cell lines used were established more than 20-30 years ago and have a long passage history, therefore their relevance to their tissue of origin is questionable. Furthermore, HepG2 and Huh7 are both established from well differentiated HCC, while Huh7.5 is just a subclone of the original Huh7 cell line selected for being permissive to hepatitis C virus replication. Moreover, using immunohistochemistry as a predictive factor for the transduction efficacy of a virus against a tumour does not seem appropriate considering the possible lack of correlation between the receptor expression and the transduction of the cell lines. To complicate things more, interaction of the virus with blood factors (clotting factors, complement, natural antibodies, blood cells) (e.g. [6-9]), in the case of intravascular administration of the virus, significantly reduces the relevance of the in vitro results. For example, transduction of hepatocytes in vivo, is considered to be independent of CAR or integrin binding, and more likely has to do with coating of the viruses by blood factors like the clotting factor X [8, 9]. The in vitro experiments on transduction could be made more clinically relevant e.g. by preincubation of the viruses in blood/plasma/serum before doing the infection. Selecting an appropriate anticoagulant will be important as the commonly used anticoagulants for blood collection (EDTA, citrate, heparin) can significantly affect viral transduction and the interaction with the blood factors [8, 109].

In the end, the only way to safely predict the effect of a virus in a specific tumour is to test the virus in that specific tumour. For example, ex vivo testing of oncolytic viruses in tumour biopsies is possible (e.g. [110]). Alternatively, a “window of opportunity” trial design (i.e. administering the treatment under investigation during the window period from diagnosis to planned surgery and testing the effect of the treatment on the resected tissue) could offer important and clinically relevant information, e.g. about the biodistribution of the fibre-

modified viruses or the selectivity, anti-tumour effect and immunomodulatory effects of the replication-selective and CD40L-expressing viruses. A "window of opportunity" trial of an oncolytic adenovirus in colon cancer has recently been presented in the 2014 ASCO Annual Meeting [111].

- **Oncolytic immunotherapy:** More and more emphasis is placed on the immunostimulatory effects of oncolytic virotherapy, and this has been the focus of many recent review papers (e.g. [19, 45, 112-114]). The virus mediated immunogenic cell death combined with expression of immunostimulatory molecules has the potential to overcome the immunosuppressive/ tolerogenic tumour microenvironment and induce a durable immune-mediated anti-tumour effect. Adenoviruses can be armed with many other immunostimulatory molecules apart from CD40L (e.g.[45, 112]). It would also be interesting to confirm whether (and which) immunostimulatory molecules can reverse the immunosuppressive phenotype of immune cells of the tumour microenvironment to an immunostimulatory phenotype. It has been demonstrated for example that CD40 activation can convert tolerogenic dendritic cells to immunostimulatory (e.g. [115-117]), although again the reverse effect, i.e. the induction of a tolerogenic dendritic cell phenotype by CD40L, has also been reported [118]. Combining oncolytic viruses with such immunostimulatory molecules can further enhance immunogenicity by induction of immunogenic cell death [49], and allows tumour targeted delivery and intratumoural amplification of the treatment therefore potentially minimizing systemic toxicity.
- **Sequential combination with other oncolytic viruses;** Repeated dosing of oncolytic adenoviruses is hindered by neutralising antibodies and anti-viral immunologic memory. One way of overcoming this problem is with sequential administration of antigenically different oncolytic viruses, a strategy that can also potentially have a prime and boost anti-tumour immune effect [119, 120]. An oncolytic immunotherapeutic vaccinia virus has already successfully completed early clinical trials in HCC [94]. If oncolytic adenoviruses are also proven safe for HCC then a sequential combination of the two viruses seems promising.

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SUPPLEMENTARY MATERIAL

Supplementary methods

Plasmid mini-preparation and restriction digestion

Colonies from selective plates were grown overnight in 10 ml Veg LB at 32ºC. 700µl of the overnight cultures were transferred in Sarstedt micro-tubes with 300µl of 50% glycerol in H_20 and stored at -80ºC. Plasmids were mini-prepped with an alkaline lysis method; Overnight cultures were centrifuged for 5 minutes at 3750rpm, 4ºC (Beckman GS 6R, GH-3.8 rotor). The supernatant was poured off, pellets were resuspended in the volume left and the tubes were transferred to ice buckets. Then 300µl of solution I (50mM glucose, 25mM Tris pH 8, 10mM EDTA) were added followed by 150µl of lysozyme solution (Sigma-Aldrich, L6876) (10mg/ml freshly dissolved in solution I) and tubes were left on ice for 10 minutes to digest the bacterial cell walls. Proteins and chromosomal DNA were denatured with 900µl of solution II (0.2M NaOH, 1% SDS) for 5-10 minutes on ice. Proteins and single stranded chromosomal DNA were then precipitated with 675µl of solution III (Potassium acetate; 3M K, 5M acetate; 147g KAc and 57.5 ml glacial acetic acid per 500 ml), incubating on ice for 5-10 minutes. Samples were centrifuged for 10 minutes at 3750 rpm, 4ºC (Beckman GS 6R), supernatant was transferred to clean tubes and nucleic acids were precipitated with 1 volume of isopropanol (samples were incubated at -20ºC for 20-30 minutes and then centrifuged for 10 minutes at 9000rpm- Sorvall, SM24). Pellets were re-dissolved in 300µl $T_{100}E_5N_{100}$ (Tris 100mM, EDTA 1mM, NaCl 100mM, pH 8) with 10 µg/ml heat-treated RNase A and tubes were incubated at 37ºC for 1 hour. Samples were then transferred to microcentrifuge tubes for phenol-chloroform extraction;

- 1 volume of phenol-chloroform (1:1) was added, samples vortexed and centrifuged at 13000 rpm, for 1 minute- Heraeus Biofuge Pico. The lower phase was discarded.

- 1 volume of chloroform was added and samples were vortexed and centrifuged at 13000rpm, for 1 minute. The aqueous phase was transferred to new tubes.

DNA was precipitated with 2 volumes of ethanol (incubation at -20ºC for 20-30 minutes followed by centrifugation at 13000 rpm for 10 minutes- Heraeus Biofuge Pico) and pellets were resuspended in 30µl $T_{10}E_1$ (Tris 10mM, EDTA 1mM, pH 8). Samples were then digested with appropriate enzyme followed by agarose gel electrophoresis (0.75 % Agarose MP- Roche, TAE buffer) to confirm the expected band pattern.

Plasmid bulk preparation

Confirmed stocks were cultured in 500 ml Veg LB for 30-48 hours. Cultures were centrifuged for 5 minutes at 5,000 rpm, 4ºC (Sorvall, SLA-1500) and plasmids were maxi-prepped. The method was similar to the mini-preps up to the isopropanol precipitations, but with 4ml of Solution I, 1ml of lysozyme solution, 10ml of Solution II and 7.5 ml of Solution III. Nucleic acids were precipitated with isopropanol (20 minutes at 8000rpm, SLA-1500) and pellets were redissolved in 2.5 ml of $T_{50}E_{10}$ (Tris 10mM, EDTA 10mM, pH 8). Plasmid DNA was purified with caesium chloride-ethidium bromide density centrifugation; 3.03 g of CsCl (Invitrogen, 15507-023) were added in 15ml tubes. Tubes were tared and the samples were transferred to the tubes. Liquid weight was raised to 2.75g with $T_{50}E_{10}$. 275µl EtBr (10mg/ml) were then added and tubes were placed on ice for 10 minutes. Tubes were then centrifuged for 10 minutes at 9000 rpm (Sorvall, SM24) to precipitate RNA. Supernatant was transferred to Beckman Quickseal tubes (into which 20µl of 2% Triton X-100 were previously added). Extra isopycnic CsCl was added to fill tubes marginally below the neck and balance tubes (within 0.1g, ideally less). Tubes were centrifuged in Beckman TLN100 rotor as follows; Step 1 at 100,000 rpm for 4h, Step 2 at 55,000 rpm for 30-60 minutes, Step 3 at 0 rpm brake setting 9, all at 25ºC. The lower plasmid band was drawn with a No 21 needle connected to a 2ml syringe and samples were diluted 3 times with $T_{10}E_1N_{100}$. Then a phenol-chloroform extraction was

done once (as described above but without the second step of chloroform extraction), followed by ethanol precipitation twice. Pellets were redissolved in 100µl of $T_{10}E_1$. DNA concentration was measured (SYBR gold-based DNA assay, see later, page 71) and confirmed with restriction digestion.

Transfection and infection for propagation of viruses

Hek 293 cells were seeded in T25 flasks at about 30% of confluence 2 days before the transfection. 5-15 µg of purified plasmids were ethanol precipitated one day before the transfection and re-dissolved in 175 µl of filter-sterile 10mM Tris-Cl (pH 7.5). For the transfection, 25µl of filter-sterile 2M calcium chloride were added to the DNA solution and mixed. The DNA/CaCl2 solution was then added drop-wise to 200µl of filter-sterile 2xHEBS (50mM HEPES-NaOH pH 7.05, 0.25 M NaCl, 1.5mM Na/PO4, 0.1mM EDTA) whilst gently mixing on a vortex mixer. The solution was let to stand for 20-30 minutes to allow time for the DNA/calcium phosphate co-precipitate to form. Medium of flasks was changed (4ml DMEM 10%FCS +gps) and 40µl of 10mM chloroquine were added (chloroquine is lysosomotropic, resulting in endosomal swelling/rupture and is considered to help endosomal escape of plasmids). The co-precipitate was then added and flasks were returned to incubator for 4-5 hours. Then, a glycerol shock was done to improve the transfection efficiency; Medium was removed -> 2ml of 15% glycerol in serum-free DMEM-HEPES were added-> at 90 seconds the glycerol solution was removed -> at 2 minutes DME-HEPES 2% FCS + gps was added. One extra flask was used as mock control (same transfection protocol but without any plasmid DNA) and one flask was left untransfected. Successful transfection could be confirmed the next day under a fluorescence microscope (Axiovert 25, Zeiss), for viral constructs that express EGFP. Cultures were then incubated for a few days, until enough CPE (cytopathic effect) developed to allow harvesting of cells by shaking the flasks. Harvested cells were pelleted (2000 rpm, 5 mins- Megafuge 2.0R) and redissolved in 1ml of the medium. Virus was released from cells with three freeze-thawing steps (-80^{o}C to 37^{o}C), cell debris was pelleted (4000 rpm, 10mins-Megafuge 2.0R) and supernatant

containing free virus was transferred to Sarstedt micro-tubes for storage at -80ºC. Mock controls were also harvested the same way.

The transfection pool was then titrated in 6-well plates (five 3-fold serial dilutions starting from 100µl of the transfection pool). One well was left uninfected as a negative control. Another well was "infected" with 100µl of the mock transfection pool (this was necessary to rule out contamination because CPE, although to a lesser degree, was also observed in the mock transfection control probably due to the toxicity of the transfection protocol). Viruses were harvested from the lowest dilution achieving infection of the majority of the cells to minimize carrying of defective viruses (as a result of co-infection with both a defective and a normal virus). Viruses were then propagated sequencially in one T75 flask and then in 10x T150 flasks for the virus preparation. T75 flasks were infected with 5µl of the 6-well infection pool in 2ml of medium (DMEM-HEPES, 2% FCS, gps) and T150 flasks were infected with 12 µl of the T75 pool in 4ml of medium. Flasks were incubated for 1-2 hours before adding the rest of the medium to increase the efficiency of the infection.

Virus purification and DNA extraction

Virus was harvested from 10xT150 flasks in a volume of 9-10 ml of medium. After 3x freeze-thaw cycles, debris was pelleted -(4000rpm, 10 mins, Megafuge 11, T41 rotor) and virus-containing supernatant was transferred to a new tube. 1:100 vol n-butanol was added and the tube was incubated on ice for 1 hour. The tube was then centrifuged again (4000rpm, 10 mins, Megafuge 11) and the supernatant was layered on the top of a cesium chloride step gradient. From bottom to top: 2ml of CsCl 1.45g/ml, 3ml CsCl 1.32 g/ml, 2ml glycerol 40% and about 5ml of the virus sample + extra medium if needed (tubes must be filled to about 5mm below the rim)(Table 5). Tubes were balanced and centrifuged for at least 2hours, 25k rpm, 4ºC (Beckman Coulter, SW40 Ti rotor). The virus band (e.g. Figure 8C) was withdrawn with a 2ml syringe and 21G needle and the sample was desalted with a GE PD-10 Sephadex G-25 column. For equilibration of the column and sample elution the

A195 storage buffer was used [121]. The sample was then divided in 300µl and 50µl aliquots and stored at -80ºC.

A 500µl aliquot was used for extraction of the viral DNA. The sample was heat-inactivated at 56ºC for 30 minutes. 25µl of 10% SDS + 5 µl of 0.5M EDTA + 5 µl of 0.5M NaCl + 5 µl of proteinase K were added to the sample and the tube was incubated at 37ºC for 1 hour and then at 56ºC in a water bath for 30 minutes. DNA was then purified with a phenol-chloroform extraction followed by ethanol precipitation (as described above for the minipreps). The extracted DNA was then digested with appropriate enzymes to confirm the expected band pattern.

Table 5; recipes for the gradient solutions

For 100ml CsCl 1.45 g/ml	For 100ml CsCl 1.32 g/ml	For 100ml Glycerol 40%
60.42g CsCl + 5ml 1M Tris pH 7.9	42.24g CsCl + 5ml 1M Tris pH 7.9	40g glycerol, 2ml 0.5M tris pH 7.9, 0.5ml 0.2M EDTA, H_2O to 100ml
Then dissolve with H_2O in about 90ml, adjust pH to 7.9-8.0 and then adjust final volume to 100ml.		

DNA assay

The assay is performed in a 96-well plate with flat-bottomed wells. For the standard curve 0, 10, 20, 40, 60, 80, 100, 120, 140 and 160 ng of purified calf thymus DNA were added per well in a final volume of 100µl of $T_{10}E_1$. For the samples, 2µl of appropriate sample dilutions were added in a final volume of 100µl of $T_{10}E_1$. A 200-fold dilution of a 500x SYBR gold (Invitrogen) aliquot was prepared in $T_{10}E_1$ and 100µl were added per well. Fluorescence was read on Victor plate reader, using the settings for fluorescein (ex480 nm, em535 nm).

For measuring adenovirus concentration, 50µl of the virus prep were mixed with 50µl of 0.1% SDS and were heat-inactivated at 56ºC for 30 minutes. 2µl of the sample were added per well in quadruple

using the reverse pipetting technique and the average DNA concentration was calculated. Virus particle (vp) concentration for each virus prep was calculated using the following equation:

$$\frac{vp}{\mu l} = \frac{Avogadro's\ constant}{molecular\ weight} * (DNA\ concentration)$$

- Vp = virus particles (the equation actually calculates DNA copy number),
- Avogadro's constant = 6.02214129*10^23,
- molecular weight (in ng/mol); calculated for each virus separately using an online calculator (Paul Stothard , The Sequence Manipulation Suite: DNA molecular weight, http://www.bioinformatics.org/sms2/dna_mw.html, last accessed 05/2014)
- DNA concentration (in ng/µl) calculated as described above

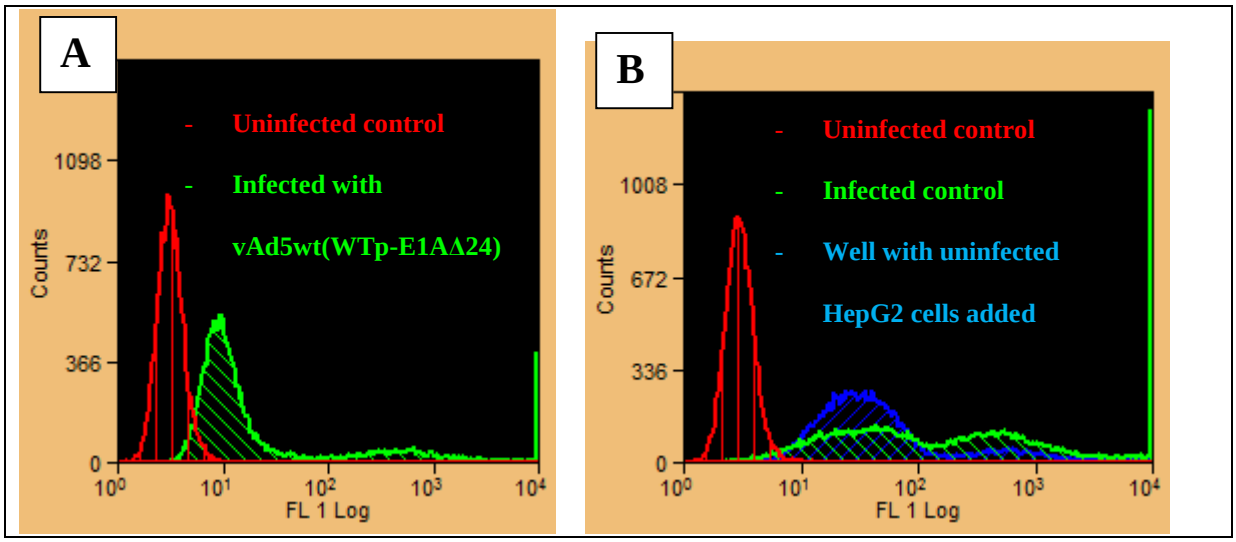
An online DNA copy number calculator (using the DNA sequence and mass as input) is also available (endmemo.com » biology » DNA/RNA copy number, http://endmemo.com/bio/dnacopynum.php, last accessed 05/2014)

Shift of the negative population in flow cytometry

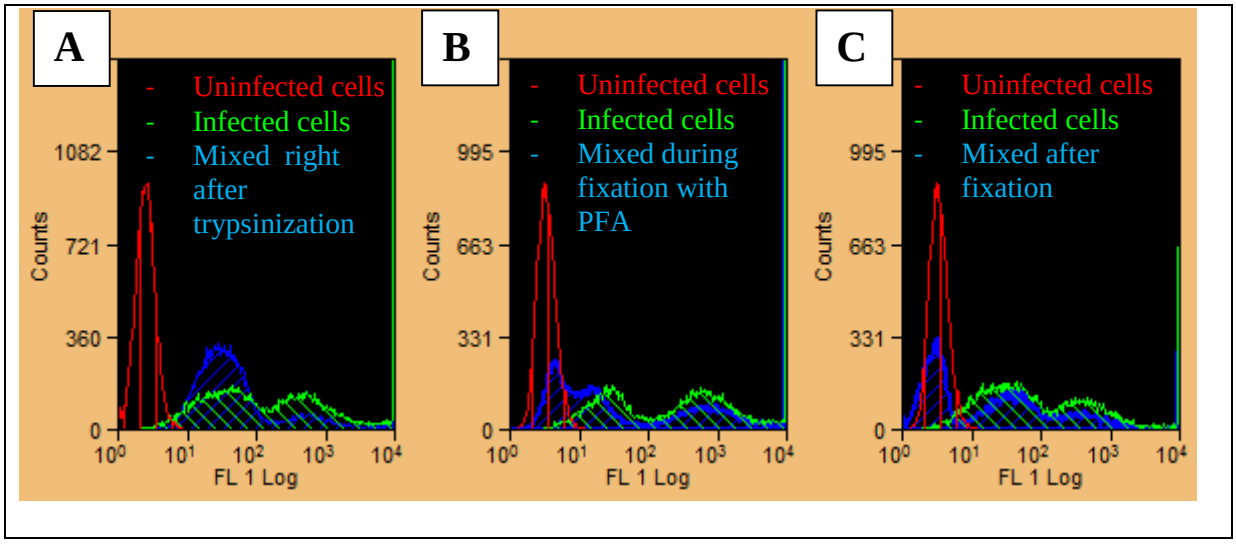
A shift of the negative population was observed in flow cytometry when using the replicating viruses (Supplementary figure 1A). To prove that this indeed represents a shift, the following experiment was done. 10^6 HepG2 cells were seeded in a 6-well plate and infected at the same time with 10^8 vp of the EGFP expressing replication-competent vAd5wt(WTp-E1AΔ24). After a few hours the infected well was washed 10 times with PBS and 10^6 more HepG2 cells were added. Cells were harvested and analysed with flow cytometry the next day. It was shown that the uninfected cells had shifted away from the control uninfected population (Supplementary figure 1B). To test if this had to do with the harvesting/fixation process, cells from uninfected wells were mixed with cells from infected wells (Supplementary figure 2): (A) right after trypsinization, (B) during fixation with 2% PFA or (C) after fixation. Interestingly, just mixing the cells after trypsinization resulted in the same shift, while mixing during or after fixation did not affect the uninfected population.

Supplementary figures and photos

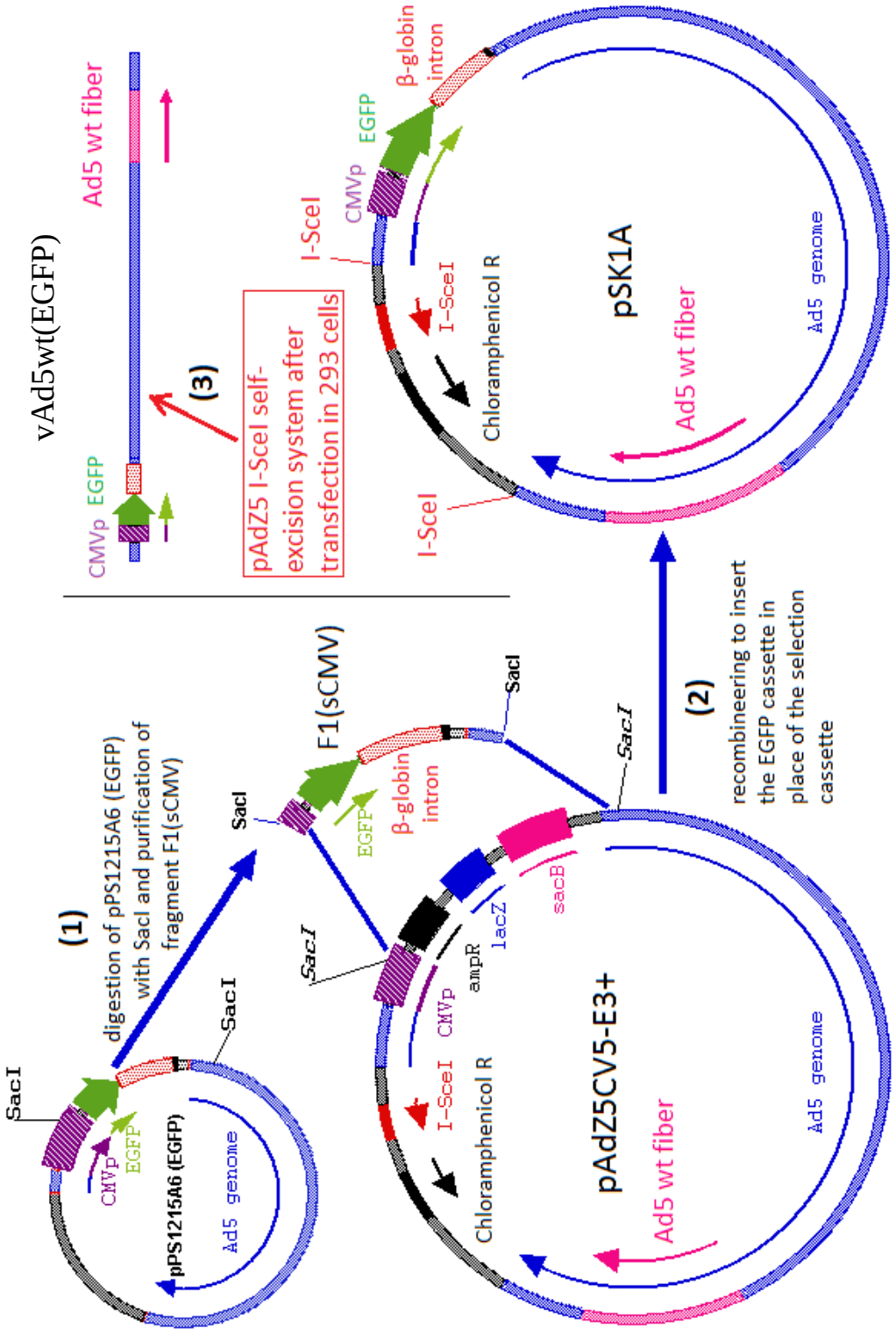
Supplementary figure 1; Shift of the uninfected population in flow cytometry



Supplementary figure 2; Shift of the uninfected population by mixing during harvesting

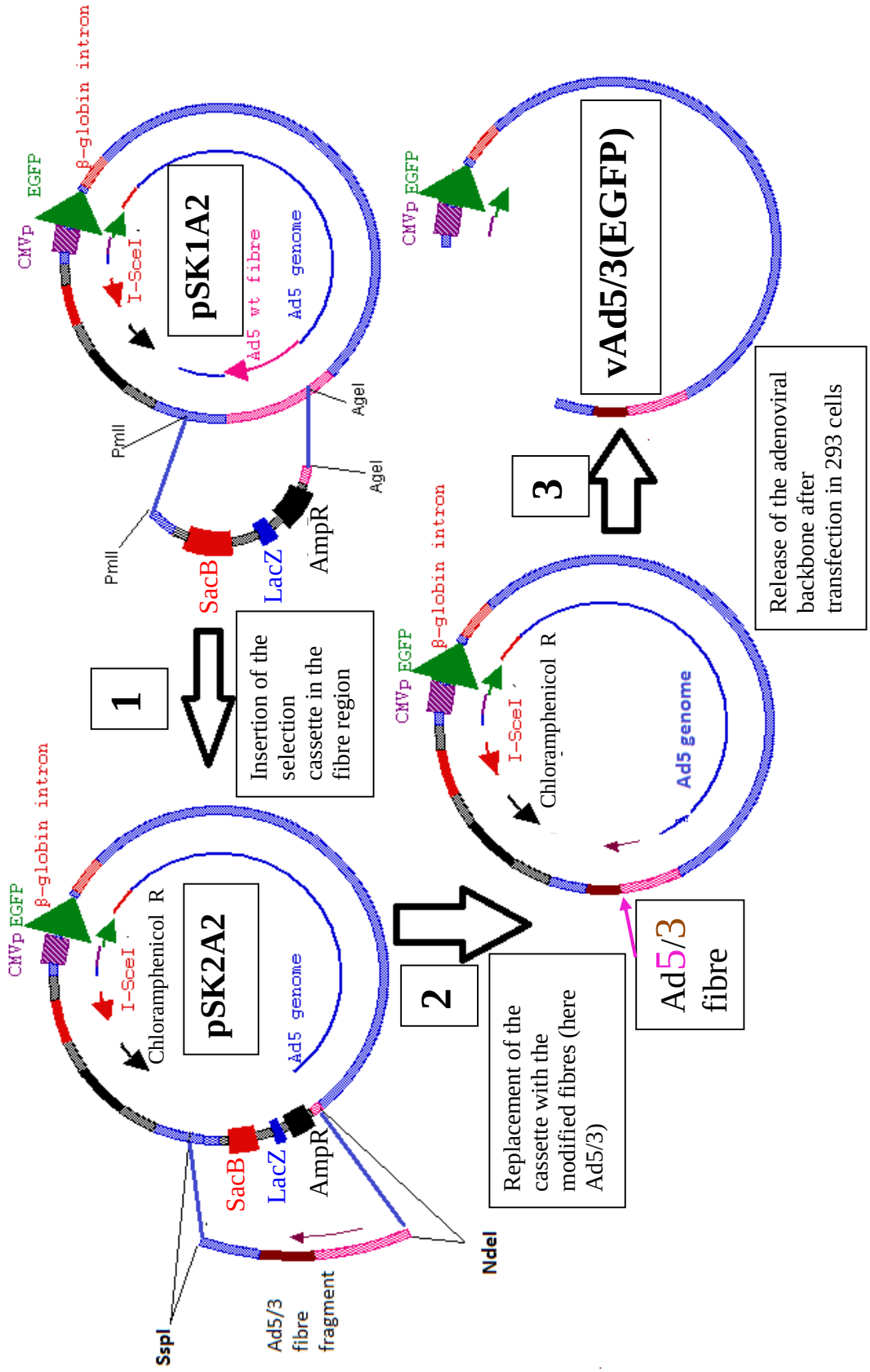


Supplementary figure 3; Insertion of CMV-EGFP cassette
(see page 28). Illustration made with Gene Construction Kit software. The scales of the plasmids and regions are modified for illustration purposes

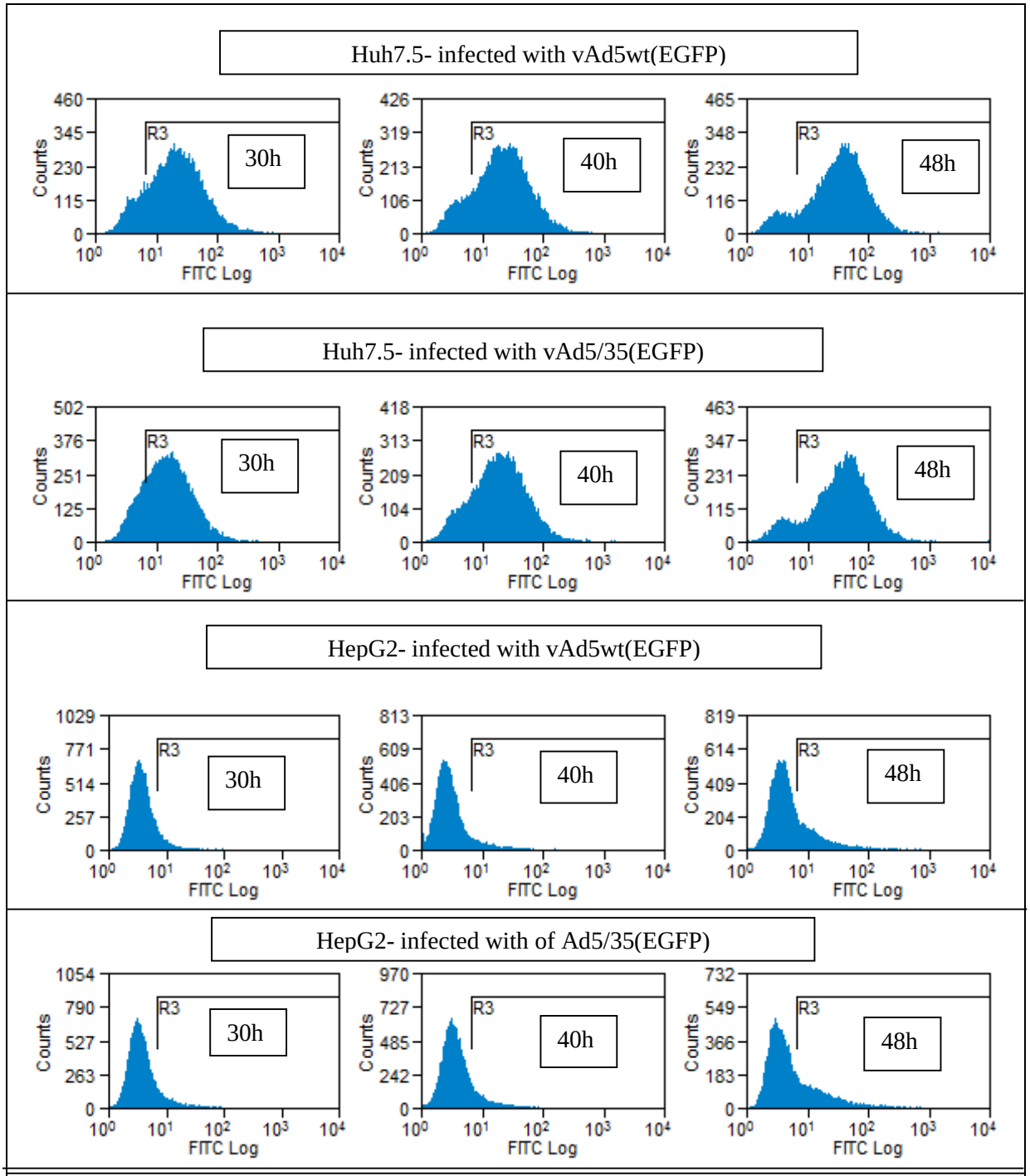


Supplementary figure 4; 2nd and 3rd recombineering step.

See page 28. The scales of the plasmids and regions are modified for illustration purposes.

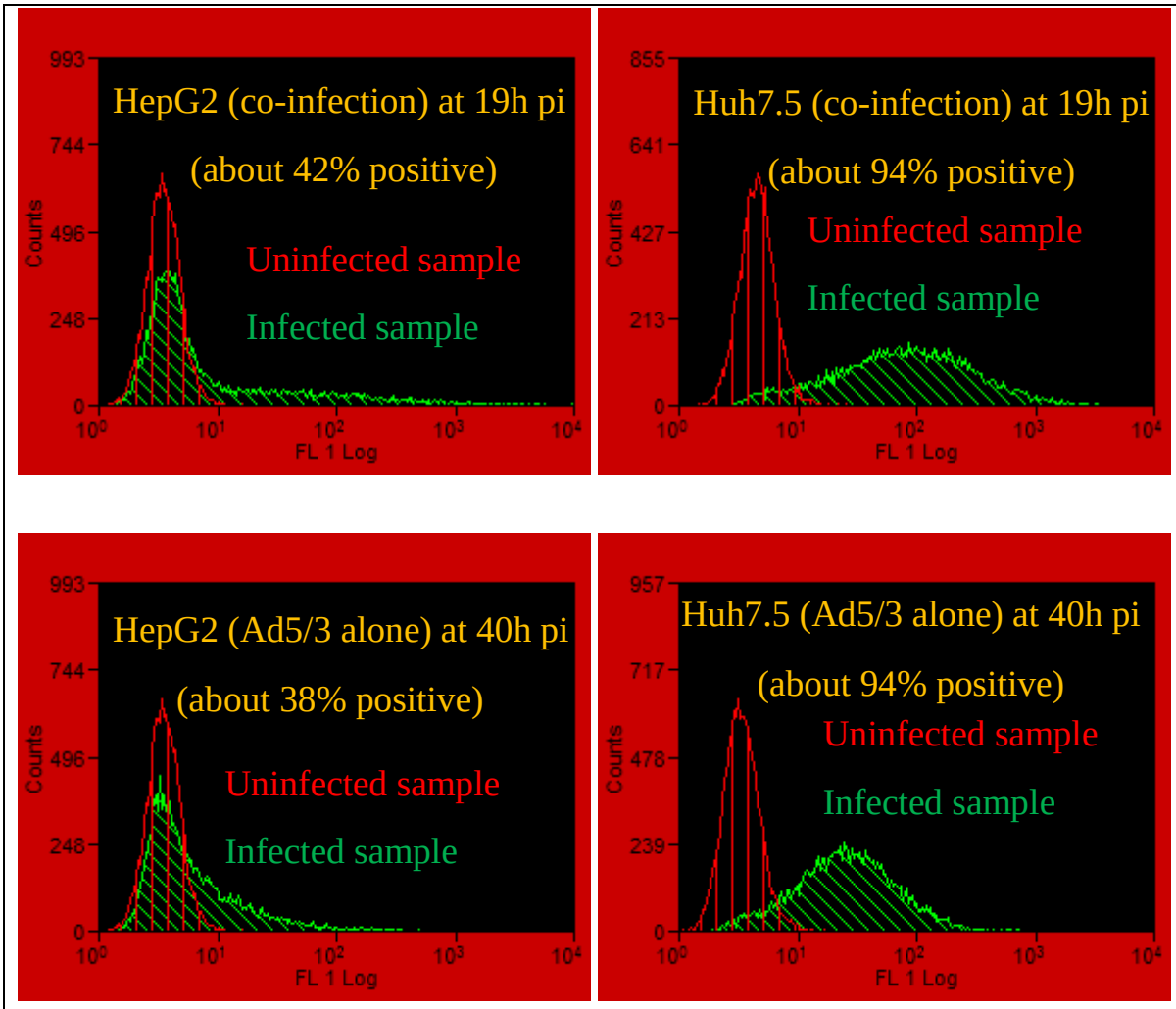


Supplementary figure 5; Time course of vAd5wt(EGFP) and vAd5/35(EGFP) in HCC cell lines



2*10^5 cells were seeded in 24-well plates and infected the next day with 3*10^7 virus particles. Cells were harvested for flow cytometry at 3 time points; 30 hours, 40 hours and 48 hours post-infection. Note the much lower transduction efficacy in HepG2 cells. Also note that even at 48 hours there is a significant overlap of the positive and negative populations in HepG2.

Supplementary figure 6; Co-infection of vAd5/3(EGFP) with vAd5wt(WTp-E1AΔ24)(no-EGFP)



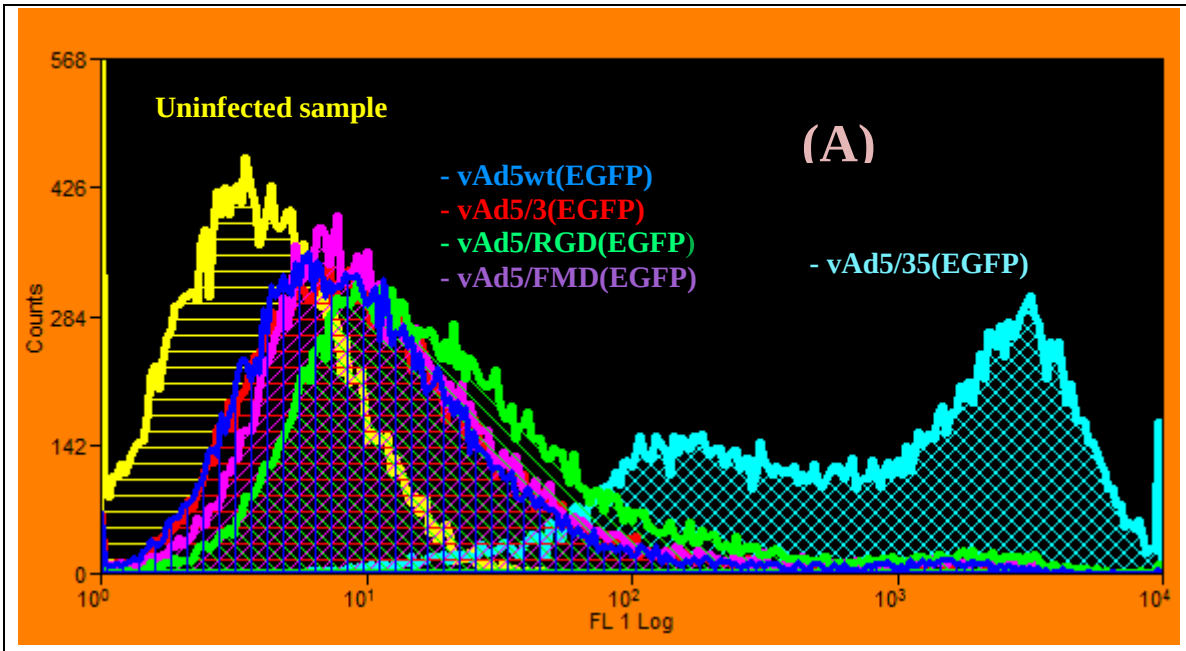
Pi; post infection

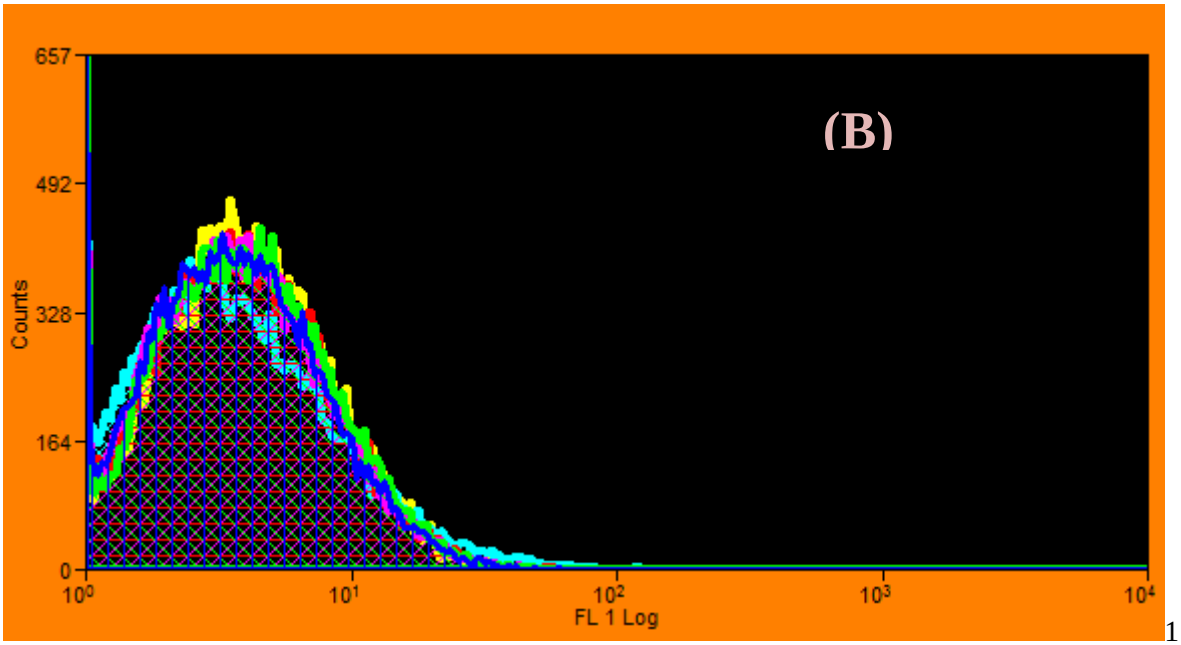
The percentage of positive cells was determined by histogram subtraction (Overton method) after normalization so that the peaks (mode count) of the negative populations match.

Unexpectedly, although the co-infection reduced the overlap between the positive and negative population the transduction efficacy remained unchanged.

The results were similar for all the fibre-modified viruses.

Supplementary figure 7; Comparison of the fibre-modified viruses in primary ICCA cells.





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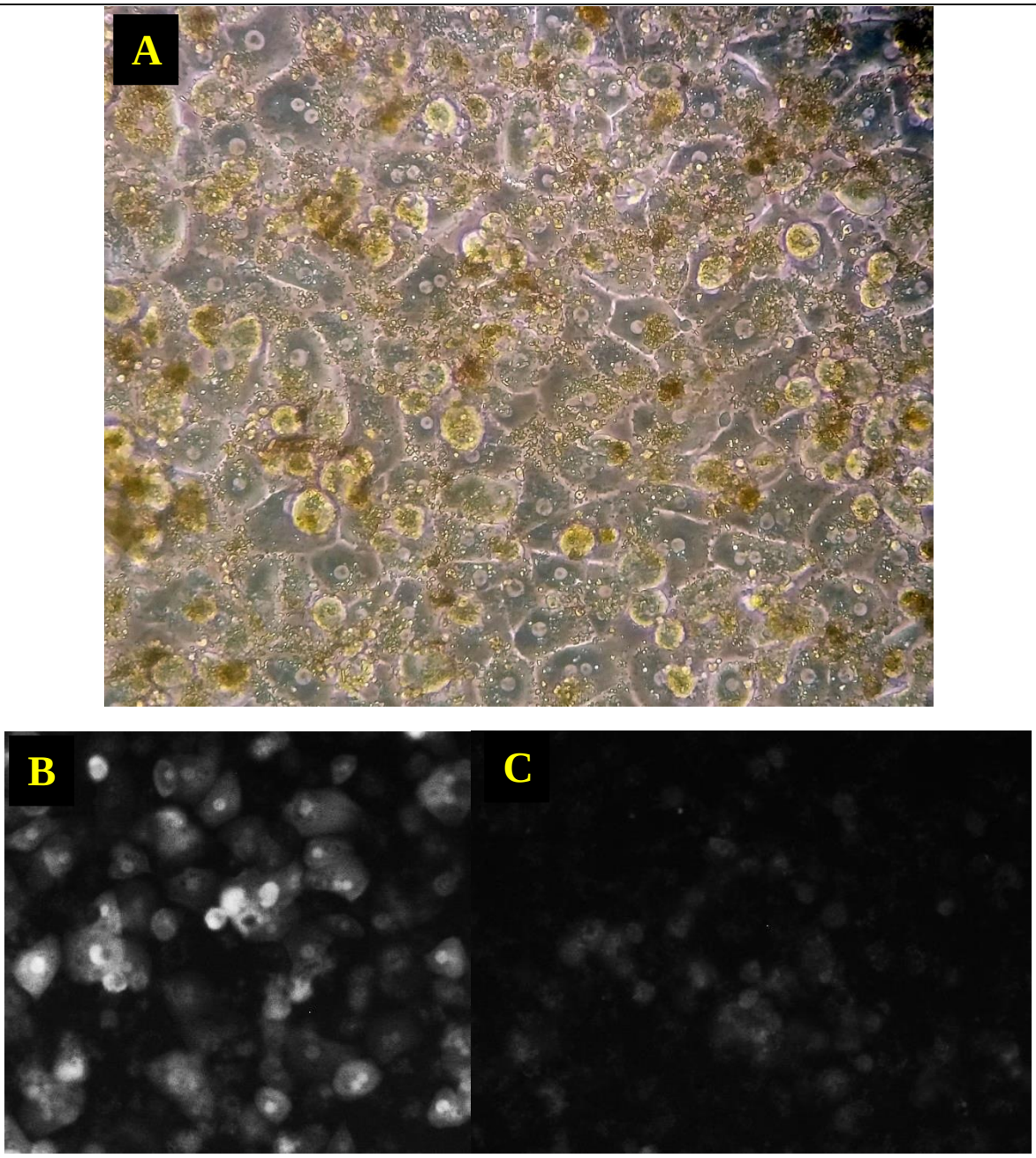
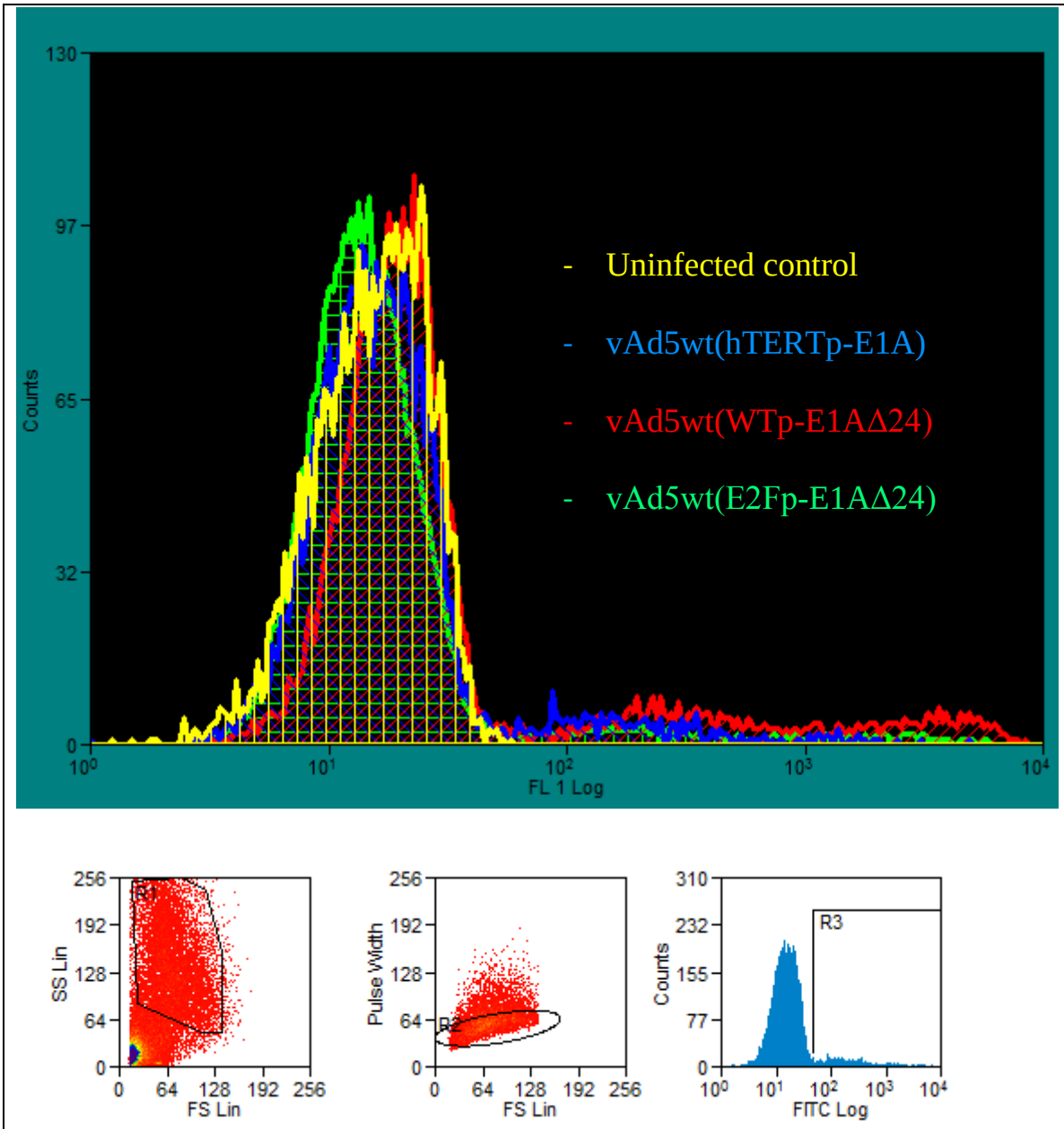
4*10^5 cells were seeded in a 24 well. 30 hours later cells were infected with four 4-fold serial dilutions of the fibre-modified viruses. The highest dose was 6.4*10^9 virus particles (figure A). Figure B represent the histograms corresponding to wells infected with 4*10^8 virus particles.

Supplementary figure 8; Primary hepatocytes (cryptogenic cirrhosis liver) infected with vAd5wt(WTp-E1AΔ24)



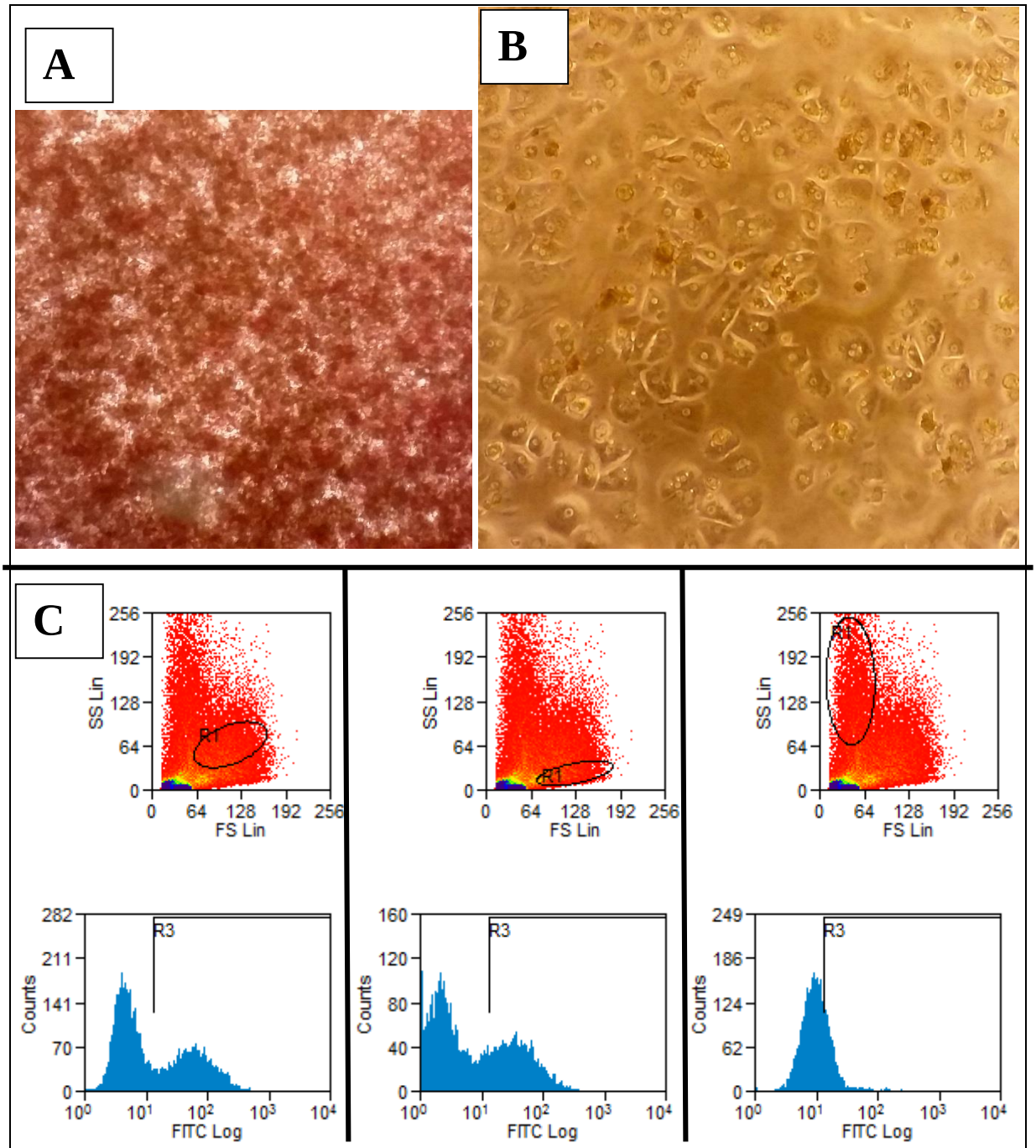
(A) Primary hepatocytes isolated by Dr E. Humphreys from a liver with cryptogenic cirrhosis. 5*10^5 cells were seeded in 24-well plates (B) 17hours post-infection with 5*10^6 virus particles of vAd5wt(WTp-E1AΔ24). (C) Uninfected control (autofluorescent debris is seen)
Note that at just 17 hours post-infection high levels of fluorescence are present suggesting virus replication. The other two viruses also resulted in visible fluorescence at 17 hours but of a lower intensity compared to that seen in (B) (Supplementary figure 9).

Supplementary figure 9; Flow cytometry histograms of hepatocytes infected with the replicating viruses



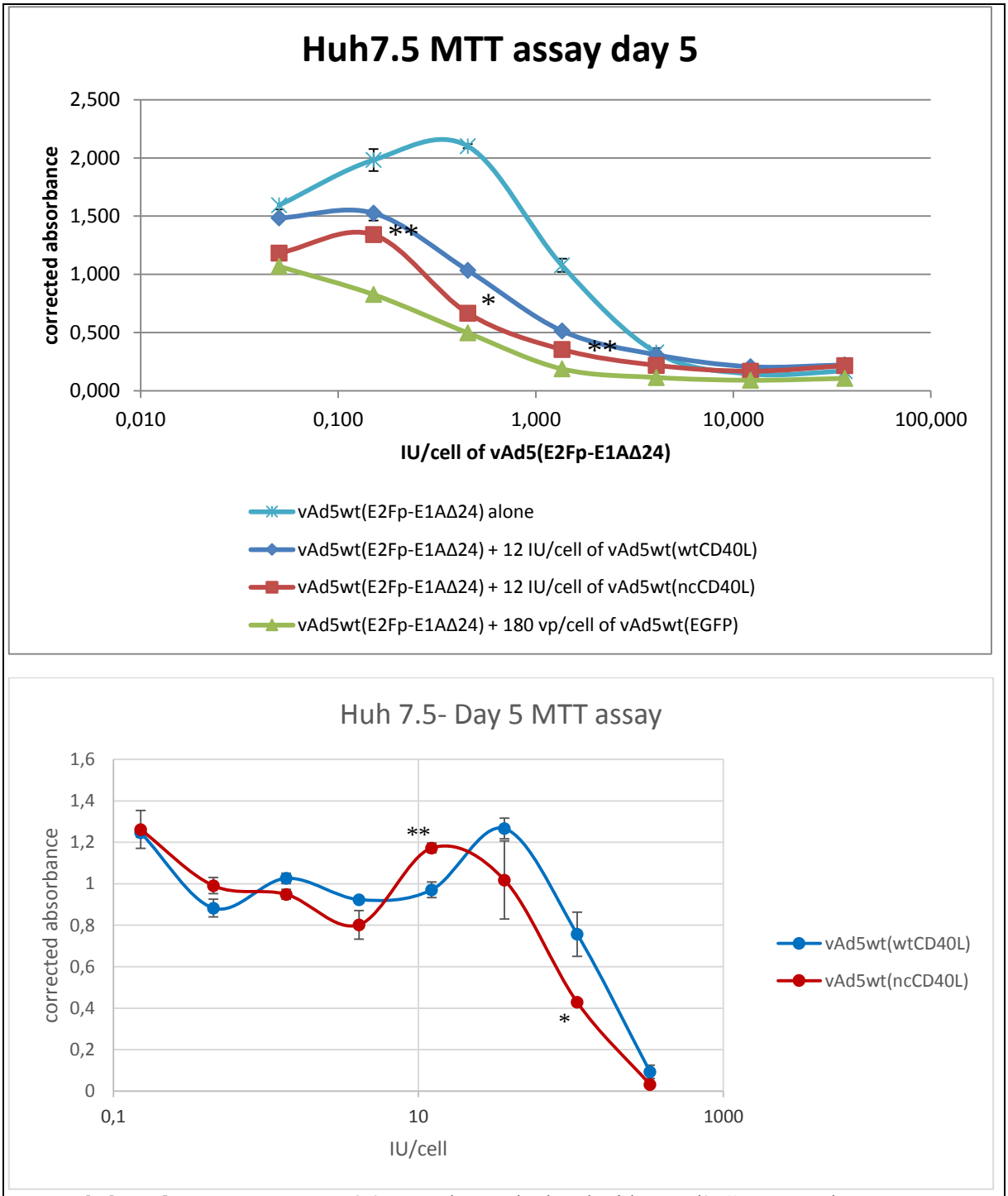
5*10^5 freshly isolated hepatocytes were seeded in a 24 well plate and infected the next day. Cells were harvested for flow cytometry 2 days later. These histograms represent infection with 5*10^6 virus particles. Note the higher fluorescence intensity of the population infected with vAd5wt(WTp-E1AΔ24). An example of the gating strategy is shown.

Supplementary figure 10; Problems with the hepatocytes isolated from the marginal liver donor.



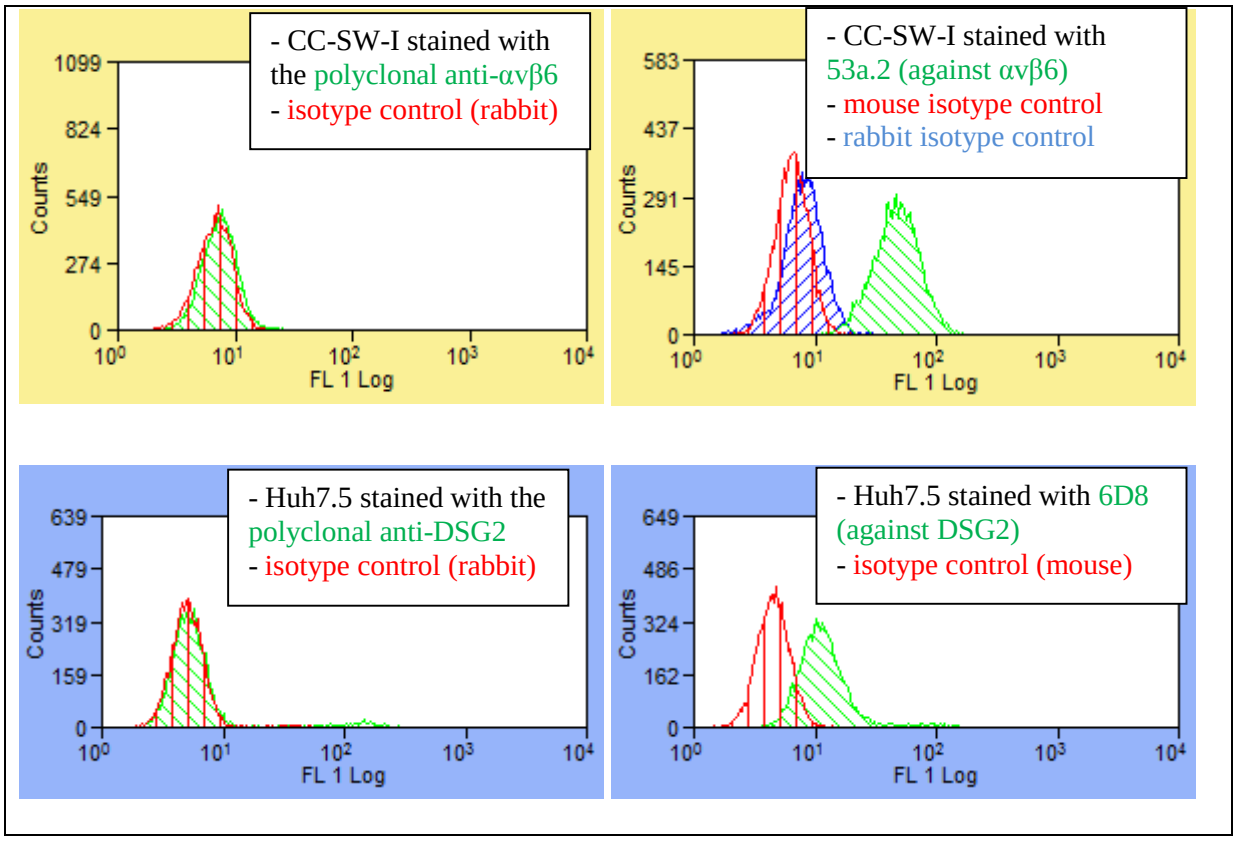
(A) A well seeded with 5*10^4 hepatocytes. The hepatocytes cannot be seen due to large amount of debris. (B) After washing the wells with PBS (a variable number of cells might have been lost during this washing) (C) Cells infected with about 10^6 vp/well of vAd5wt(EGFP) and harvested 2 days later. Different gating strategies are shown. It was unclear which region represented the hepatocyte population. The presence of other cell populations cannot be excluded.

Supplementary figure 11; ncCD40L better than wtCD40L?

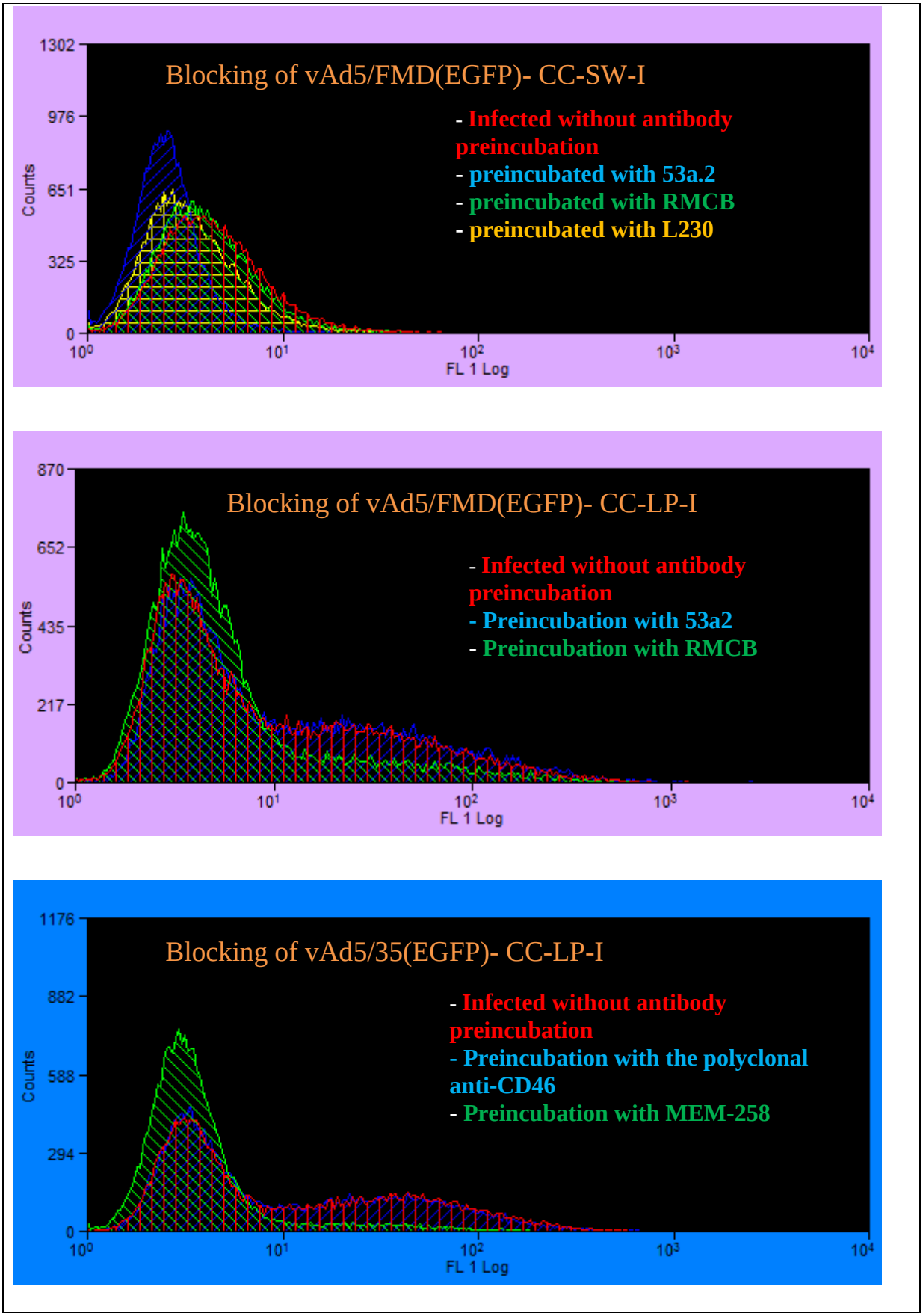


Two-tailed p values; **< 0.01, * < 0.05. P values calculated with Excel's "two sample t-test assuming unequal variances". It is possible that the small differences observed have to do with the very different particle-to-infectious units ratio of the two viruses. vAd5wt(ncCD40L) had a much higher particle-to-IU ratio according to the hexon staining titration (Table 4). Indeed, when the bottom graph was plotted based on vp/cell instead of IU/cell there was a better match between the two curves (Figure 23).

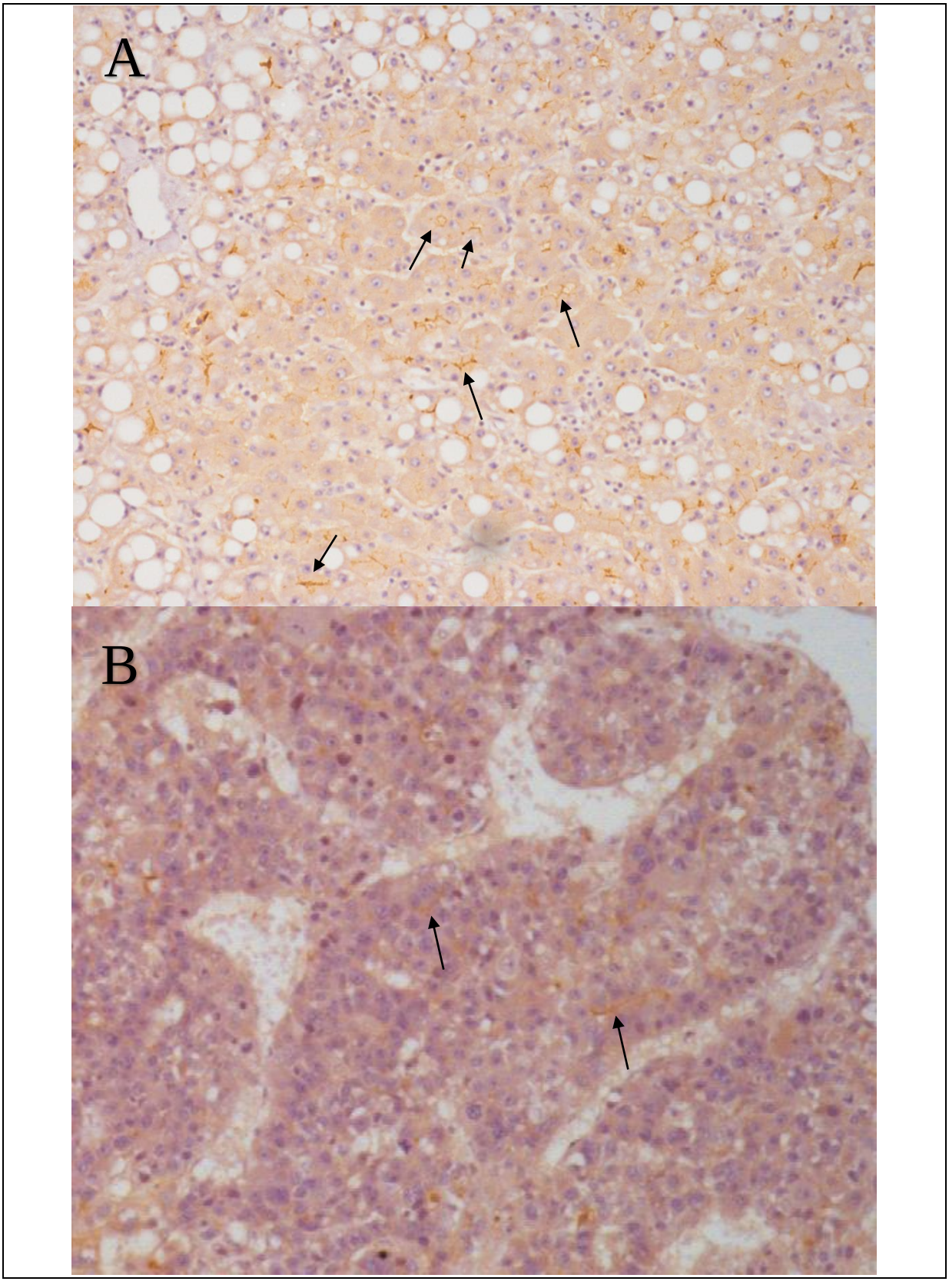
Supplementary figure 12; lack of antigen detection by the polyclonal antibodies used for IHC



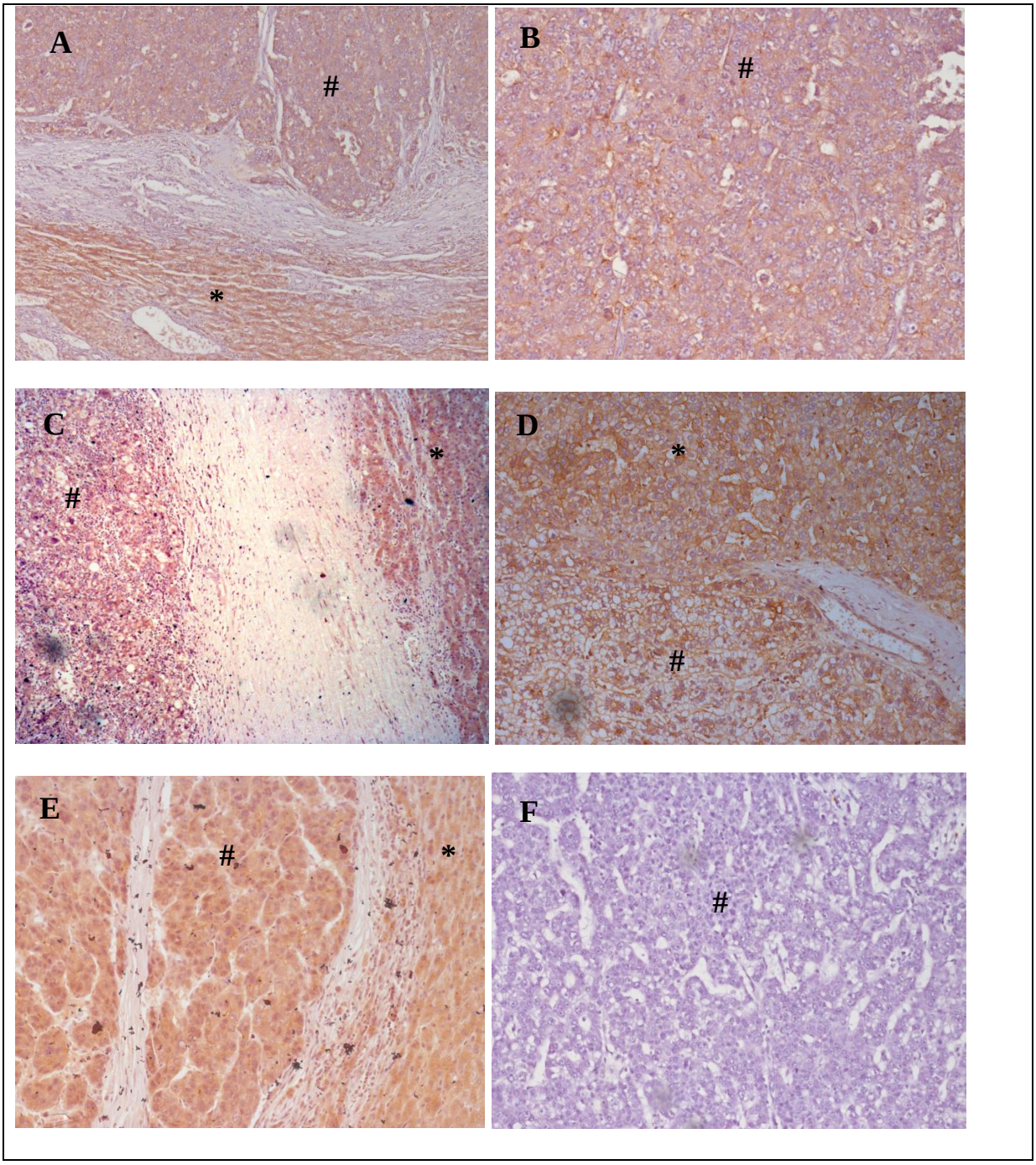
Supplementary figure 13; selected flow cytometry histograms from blocking experiments



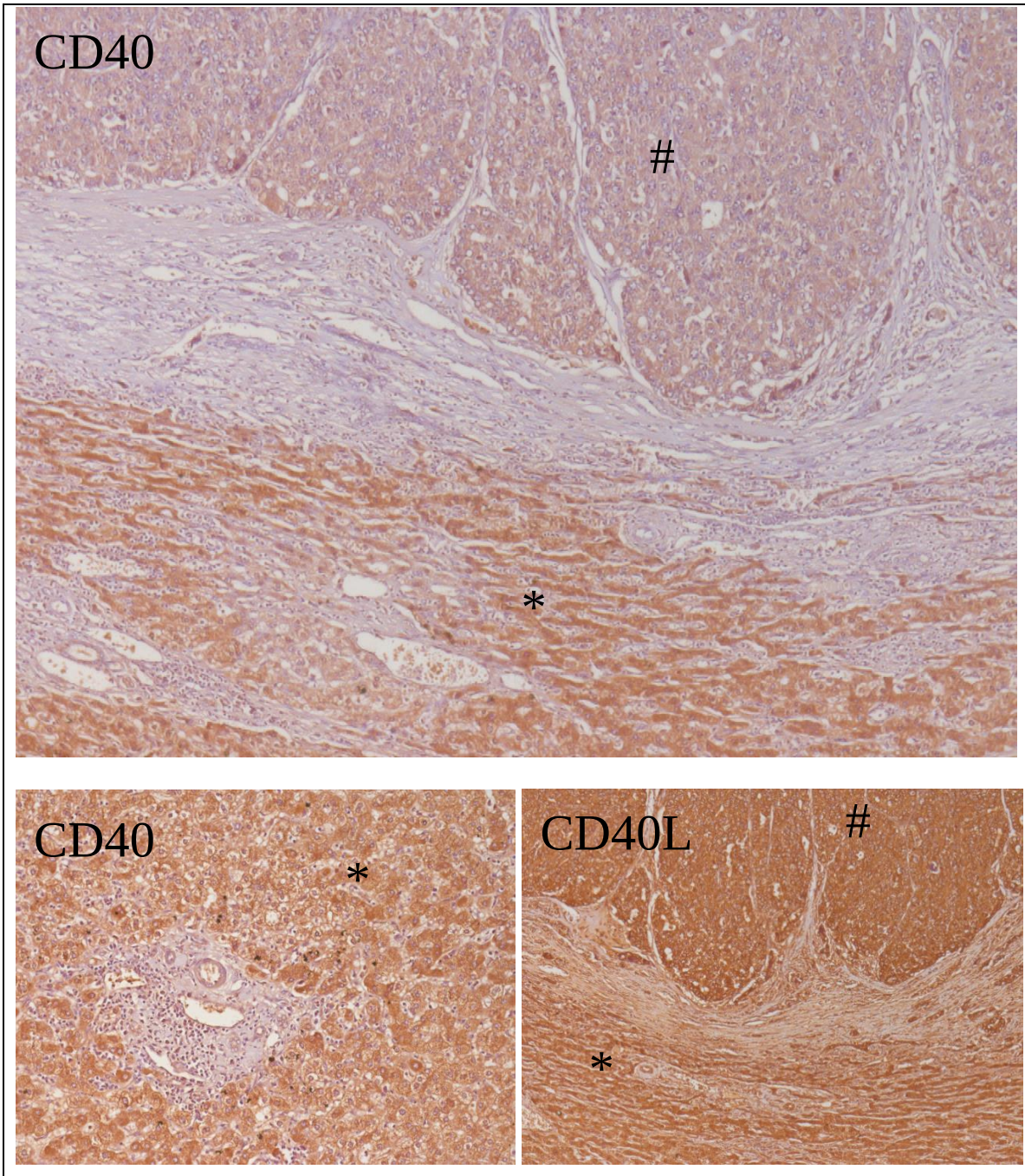
Supplementary photo 1; IHC of HCC for CAR. (A) CAR showing a bile canalicular staining pattern (arrows). (B) Focal expression of CAR (although in both cases lighter widespread cytoplasmic staining can also be seen)



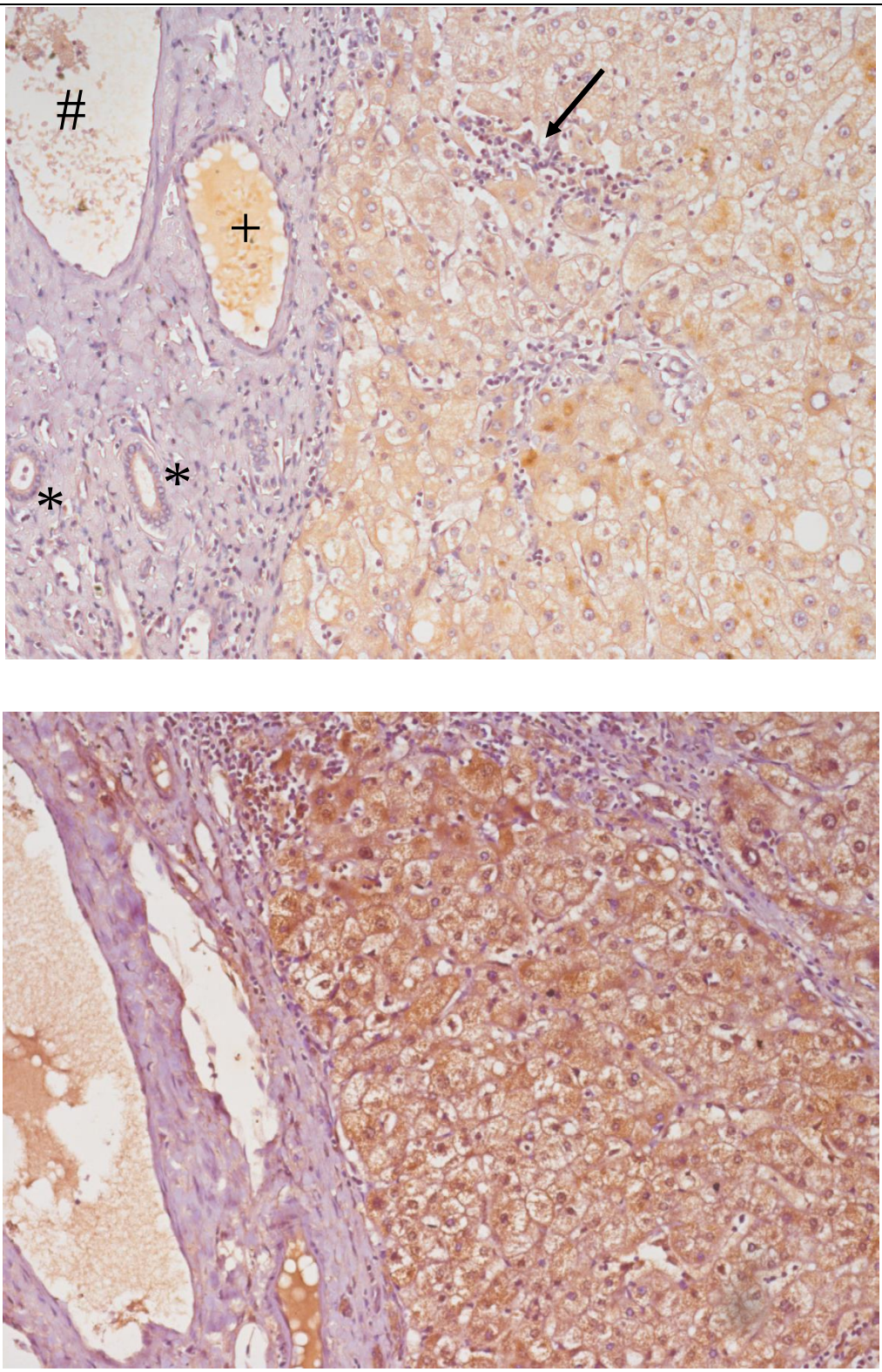
Supplementary photo 2; IHC of HCC for CAR. (A) CAR downregulated in tumour but still present. (B) Higher magnification of the tumour part of A. (C)-(E) Expression of CAR by both tumour and margin. (F) CAR was not detected in this tumour. # tumour, * margin



Supplementary photo 3; CD40 and CD40L expression by HCC (#) and margin (*) (section from a liver with HCC and hepatitis C)



Supplementary photo 4; Staining for CD40 (top) and CD40L (bottom) (section from a liver with chronic hepatitis C)



Arrow: focal intralobular necrosis with lymphocyte infiltration. Portal triad: Bile ducts (*), portal vein (#), hepatic artery (+). The membrane staining pattern seen in the section stained with for CD40L, combined with the lack of staining of the stromal tissues, argue against the possibility of non-specific binding of the antibody.