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Replicating Retroviral Vectors for Gene Therapy of Solid Tumors

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1. Introduction

Despite recent progress in the treatment of solid tumours by conventional therapeutic options including surgery, chemotherapy, and radiotherapy, development of more efficient strategies is urgently needed due to delimited efficacy and occurrence of severe side effects in current treatment regimens. Cancer gene therapy can be defined as the introduction of genetic material into the patient´s body for the purpose of reducing tumour burden, increasing life expectancy, and improving the quality of life of the treated individual. It is most commonly intended to either initiate tumour self-destruction, down-regulate angiogenesis and/or metastasis, enhance anti-tumour activity of the immune system, suppress function of an activated oncogene, or restore expression and/or function of tumour suppressor genes [1-4].

Viral vectors are the most widely used tools for the delivery of therapeutic genetic material into host cells in a clinical setting. More than 65 % of gene therapy clinical trials worldwide are making use of viral vectors (http://www.wiley.com). With almost 370 trials (~20 % of all gene therapy clinical trials), gamma-retroviruses and in particular the murine leukaemia virus (MLV)-based vectors are the second most used gene transfer system employed in recent years. These vectors are able to transduce most cell types, as long as they are actively dividing. However, most of these retroviral vectors are designed to be replication-deficient, resulting in poor transduction efficiencies in vivo. This might be one, if not the reason for the poor therapeutic success observed so far in clinical trials for cancer [5–7]. Thus, nowadays, replication-deficient retroviral vectors are mainly used in ex vivo gene transfer for the treatment of inherited monogenic disorders [8–10], rather than for in vivo tumour therapy.

However, to increase in vivo transduction efficiency and the poor therapeutic outcome observed using replication-deficient retroviral (RDR) vectors, replication-competent retroviral (RCR) vectors were created which allow vector production in the infected tumour cell and

thus, as a consequence, efficient delivery of the therapeutic gene eventually to almost all target cells (for review see [11-13]). Several research groups were involved in the design and construction of such MLV-based RCR vectors and were able to show that these vectors are well suited for efficient transduction of tumour cells and thus represent an efficacious treatment option for tumour therapy.

In the following sections we will provide an overview on MLV-derived RCR vectors and their therapeutic principle. Emphasis will be put on the different vector designs available and their influence on vector spread kinetics, vector genome stability, and transgene expression levels. Furthermore, strategies to target the vector by either selective infection of distinct cell types or selective expression and replication of the vector genome and expression of the delivered transgene in distinct cell types will be presented. Data from in vivo studies employing a set of different therapeutic genes and targeting different tumour types in various animal models will be reviewed and the therapeutic efficacy in these indications discussed. Finally issues regarding the safety of these vectors such as data from biodistribution and toxicological studies as well as potential risks associated with such a therapy are addressed in the following.

2. Biology of the murine leukaemia virus

The murine leukaemia virus belongs to the genus of gamma-retroviruses which are small, enveloped viruses carrying two copies of a single-stranded RNA genome within an icosaedric core. The unique feature of retroviruses is their replication cycle, as their RNA genome is reverse transcribed into DNA, which then integrates into the host DNA before being transcribed to give rise to new virus genomes and viral proteins. MLV is a so-called simple retrovirus carrying only 3 genes in its genome, encoding the viral Gag, Pol and Env polyproteins. The group-specific antigen Gag is processed by the viral protease (PR) to the matrix (MA), capsid (CA) and nucleocapsid (NC) proteins which all form the viral core. The surface (SU) and transmembrane (TM) proteins are processed from the Env protein and are embedded in the host-cell derived lipid-bilayer. The pol gene encodes the viral PR, the reverse transcriptase (RT) and the integrase (IN), which are delivered in the virus particle to the cell to be transduced. After release of the virus core in the cytoplasm of an infected cell, reverse transcription of the single-stranded RNA into double-stranded DNA takes place and the pre-integration complex (PIC) consisting of virus DNA and viral and cellular proteins assembles [14]. As the MLV PIC, in contrast to lentiviruses such as HIV, is not able to cross the nuclear membrane, productive infection only occurs when the nuclear membrane is disrupted, as in dividing cells. Integration of the viral DNA into the host genome occurs randomly, however an integration preference of MLV-based vectors into the 5′-proximity of transcriptionally active genes was observed [15].

During reverse transcription identical long terminal repeats (LTRs) consisting of the so-called U3, R, and U5 region and flanking the viral genes are created which carry the viral promoter in the U3 region and the poly(A) site downstream of the R region. Expression from this promoter leads to two RNA species, a genomic one also encoding the viral proteins Gag and Pol, and the subgenomic env coding message. The Gag and Pol proteins assemble together

with the genomic RNA which is recognized by Gag via a packaging signal present immediately downstream of the 5′-LTR. Newly synthesized virus particles exit the cell via budding through Env protein-rich regions of the host cell membrane without lysis of the cell.

Thus, due to its non-lytic nature retroviruses per se cannot be used as so-called oncolytic viruses, which are able to kill tumour cells by their productive infection only, but require additional gene sequences to exert a tumour destroying effect. Such therapeutic replication-competent MLV vectors can only be generated by adding therapeutic sequences in addition to the viral genes, which are all essential for virus replication, making the design of such vectors challenging and their genomic stability critical due to genomic overlength.

3. Replicating MLV vectors

### 3.1. Vector designs, spread kinetics and genome stability

Early attempts to produce replication-competent retroviral vectors have been already made in the late 80′s of the last century, when various groups inserted a transgene expression cassette into the 3′-LTR of replicating MLV to generate a research tool for analyses in whole-animal models [16-18]. During infection and reverse transcription of the proviral mRNA message, the transgene expression cassette was duplicated and, now present in the 5′- and 3′-LTR, independently expressed from the respective heterologous promoter. An RCR containing a mutant form of the dihydrofolate reductase (DHFR) gene was shown to stably transmit methotrexate resistance to infected fibroblasts upon multiple rounds of virus replication in vitro in the absence of drug selection and was produced at high titres by fibroblast cells [16].

Later, the group of Finn Skou Pedersen adopted this concept and inserted the transgene within the U3 region of the 3′-LTR of the Akv strain of MLV, mediating expression of the eGFP-transgene via an internal ribosomal entry site (IRES) of the encephalomyocarditis virus (EMCV) (Figure 1, (B)) [19]. This design again resulted in doubling of the IRES-transgene cassette in the infected cell, albeit, only the eGFP gene located in the 3′-LTR, but not the transgene present in the 5′-LTR, was expressed from the regulatory elements in the MLV 5′-LTR. Intraperitoneal injection of this vector at a concentration of 10e4 colony forming units into 3-4 days old mice led to more than 50 % eGFP-expressing spleen cells 4 days after injection. The level of eGFP-positive cells remained constant till day 7, but dramatically dropped from day 12 onwards, most likely to genetic instability of the vector and reversion to wild-type (wt) virus lacking the marker gene [20].

Due to the highly compact nature of the MLV genome, however, the positions into which heterologous sequences can be inserted without impacting on viral replication are limited. Thus, up to now only few vector designs in which the transgene is located at different positions and/or its expression is facilitated by different mechanism have been created and are currently under in-depth evaluation.

Kasahara and colleagues favoured insertion of the transgene right downstream of the envelope reading frame, as well linked via an ECMV IRES element (Figure 1, (C)) [21,22].

These RCR vectors are based on the Moloney strain of MLV and are equipped with the amphotropic MLV envelope gene, both of which are features allowing infection of human and other mammalian cells. The effect of insert size and sequence on the genetic stability and spread efficacy of the vector revealed a strong negative correlation between insert size and deletion of the introduced sequence. Insertion of 1.6 kb in length led to greatly attenuated replication kinetics relative to wild-type virus and loss of the insert within a single infection cycle, whereas inserts up to 1.3 kb were well tolerated with slightly attenuated replication kinetics. In addition, the genomic integrity was maintained over multiple serial infection cycles [21,22].
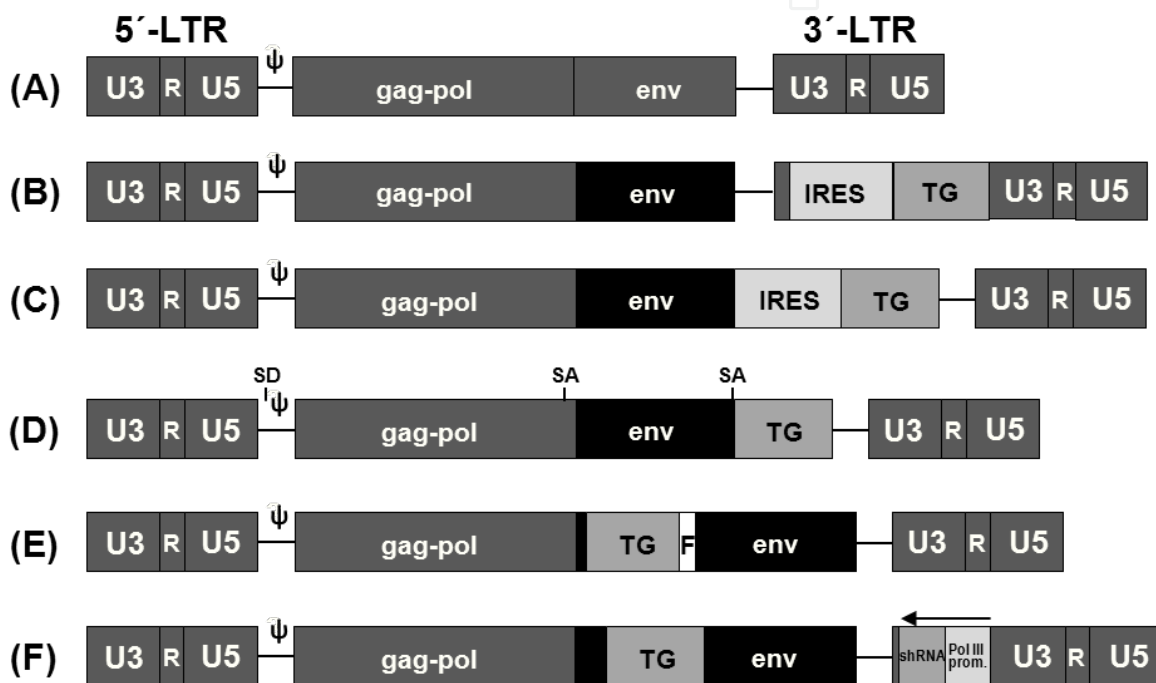
Figure 1. Schematic depiction of the different RCR vector designs ((B) – (F)). The inserted transgene is located at different positions in the viral vector and expression is facilitated by different means. (A) represents the genomic organisation of the wt-MLV provirus. LTR = long terminal repeat; gag = viral group specific antigen gene; pol = viral polymerase gene; env = viral envelope gene; IRES = internal ribosomal entry site; TG = transgene; F = protein cleavage site; SD = splice donor; SA = splice enhancer; ψ = psi packaging site; Pol III Prom = Polymerase III dependent promoter.

To further unravel the effects of viral strain and transgene position in the vector, as well as the impact the target cell type might have on spread kinetics and on the genetic stability of the virus vector in particular, we have independently compared the different parameters in serial rounds of infection in different cultured cell types as well as in vivo in tumour bearing animals [23]. To this end, various cell lines have been inoculated with RCR vectors based on the Akv and Moloney strain of MLV and carrying an IRES-EGFP transgene cassette either in the U3 region of the 3′-LTR or immediately downstream of the env gene, and passaged and monitored over time. Supernatant of the infected cells was also used to infected fresh cells for a further round of infection to allow exponential spread of virus vector until a maximum of EGFP-expressing cells was reached. Supernatant of the freshly infected cells was then used for a next

round of infection to finally end up with more than 20 rounds of infection, and virus propagation for up to 100 days. The obtained data revealed a clear advantage of the Moloney-MLV strain over the Akv-MLV strain in respect to spread kinetics, transgene expression and vector stability and demonstrated that location of the transgene immediately downstream of the env gene is preferred in respect to genomic stability of the vector. These observations have been confirmed in spread and stability analyses after virus injection in tumour xenografts of mice [23]. Unexpectedly, our results also indicated that the host cell can influence the ability of MLV-based RCR vectors to stably propagate the expression of heterologous genes, since all vectors, regardless of design, lost the ability to express eGFP in NIH-3T3 cells much more rapidly than in HEK-293 cells [23]. Differences in vector genome stability between infected cell lines seem to be dependent neither on species nor on different replication kinetics of the vector in the respective cell lines. It rather might be due to differences in other virus and/or host-cell features including fidelity of the virus reverse transcriptase linked with the p53 status of the infected cells, expression of the anti-viral mechanisms such as APOBEC and TRIM family members, availability and balance of intracellular dNTP pools, and in general, due to an overall genetic instability of certain cell types and cell lines [24].

Employing this design in which the heterologous sequences are located in the 3′-untranslated region immediately downstream of the env reading frame, expression of the transgene could also be facilitated by introducing, instead of the IRES, a splice acceptor site upstream of the transgene, which would result in a transgene specific mRNA message (Figure 1 (D)) [25]. Propagation of such vectors in cell culture however, revealed a much slower vector spread as compared to the IRES-carrying vector, which led to almost 100% infected cells after 3 days after infection [25].

RCR vectors based on Mo-MLV carrying a therapeutic gene in the 3′-untranslated region resemble currently the most advanced RCR vector design for tumour therapy and are already employed in the first clinical trial for the treatment of cancer.

A different approach in the design of RCR vectors has been pursued by the group of Christian Buchholz from the Paul-Ehrlich-Institut, as they inserted heterologous sequences including a 3′-terminally located furin cleavage site in frame into the envelope gene of the virus between the signal peptide and surface protein domain coding region (Figure 1, (E)) [26,27]. During production of the Env protein in virus vector transduced cells, the heterologous amino acid sequence will be cleaved off while the Env protein is processed through the secretory pathway and eventually will be secreted from the infected cell. Proof-of-concept for this vector design was shown with the immune stimulatory cytokine GM-CSF and the laminin-specific or T-cell specific single-chain antibody variable region fragment (scFv) [27]. The resulting viruses infected a variety of human cell lines and infectious virus particles were detected in supernatants of infected cells. Moreover, these cells were able to efficiently process the encoded Env-fusion proteins and to release reasonable amounts of protein molecules of GM-CSF, laminin-specific or T-cell specific scFvs into the cell culture media. Furthermore, the replicating viruses were genetically stable for at least 12 serial cycles of propagation. Thus, these vectors are ideally suited for production of therapeutic factors which need to be secreted, but less suitable in case the protein produced is intended to be active in the infected cell.

An additional site for integration of foreign sequences into the vector genome without impairing virus life cycle is the proline-rich region tract within the Env protein (Figure 1, (F)). Insertion of the eGFP marker gene into this site in a Mo-MLV-based RCR vector resulted in spread through almost 100% of cultured NIH-3T3 cells within one week after initial transfection with virus sequences [28,29]. PCR analysis of integrated virus vector DNA from chronically infected cells indicated no genetic recombination in the modified env gene region. An additional insertion of a Pol III promoter-shRNA expression cassette in antisense orientation into the 3′-untranslated region of the virus vector resulted in only slightly reduced spread kinetics as compared to the parental vector and in delivery and functional expression of the shRNA in most of the cells [30]. Again PCR analysis did not reveal any recombination events over 4 infection cycles.

3.2. Targeting of infection and expression

MLV-based RCR vectors can be accounted for being intrinsically tumour-selective due to the specific nature of MLV to replicate in dividing cells only. Nevertheless, it would be desirable to further improve the vector safety profile. This can be achieved by introducing transcriptional control elements that restrict RCR gene expression and subsequent virus vector replication to tumour cells - so-called transcriptional targeting; or by modulating the interaction of the RCR vector with host cells at the very early step of the infection process, known as physical targeting, via adaptation of the virus envelope glycoprotein to selectively bind to surface molecules exclusively or predominantly present on cancer cells. Alternatively, initial targeting could also be enabled by the use of delivery vehicles to facilitate transport or homing of the RCR vectors to the tumour site.

3.2.1. Transcriptional targeting

To allow transcriptional targeting of MLV-based RCR vectors, the most reasonable approach is the exchange of the ubiquitously active viral promoter located in the U3 region of the viral LTR by a tissue- or tumour-specific promoter delimiting its activity and thus virus vector replication to a specific cell type. Due to the particularities of retroviral reverse transcription, modifications of this promoter must be introduced into the U3 region of the 3′-LTR. This allows, after initial vector production and infection, duplication of the regulatory elements into the 5′-LTR (Figure 2). This strategy has been successfully employed previously in conventional replication-defective retroviral vectors to direct transgene expression to a particular cell type [31–33]. In RCR vectors however, not only expression of the transgene sequences is mediated by these regulatory elements, but also expression of viral genes which are needed to ensure efficient RCR vector replication in infected target cells and which have to be produced in an ample but well balanced manner. Moreover, as the LTR contains regulatory elements important for reverse transcription, RNA processing, and virus genome integration, modifications in this area may interfere with or may disrupt these elements and may thus negatively affect virus replication kinetics. This altogether renders the transcriptional targeting approach for RCR vectors rather complex.
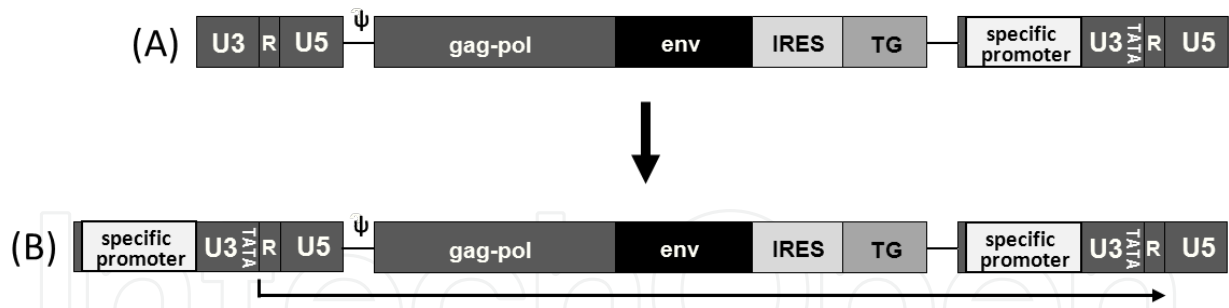
Figure 2. Principle of transcriptional targeting of RCR vectors. (A) schematically depicts the RCR proviral genome as present in a virus producer cell. The tumour- or tissue-specific regulatory sequence (specific promoter) is located in the 3´-LTR of the viral vector. After infection and reverse transcription of the virus genome, the 3´-LTR U3 region is duplicated to the 5´-LTR and is thus able to drive expression of the viral genes and of the inserted transgene (B). For abbreviations please see legend to Figure 1.

In early studies, the murine liver-specific transthyretin promoter/enhancer was inserted into the LTR U3 region, lacking the endogenous enhancer, of a replication-competent MLV [34]. When compared to wt-MLV however, the recombinant virus did not reveal an improved rate of infectivity of hepatocytes in vitro or a restricted tissue tropism in vivo [34].

Transcriptional targeting of MLV-based RCR vectors harbouring modifications in the U3 region by insertion of a heterologous promoter was demonstrated initially by Kasahara and colleagues [35]. In these vectors, hybrid LTRs were constructed by replacement of the MLV 3´-LTR U3 region from the very 5' end to either the CAAT box, the TATA box, or the transcriptional start site (TSS) by a heterologous promoter complementing the deleted boxes, respectively. Using a highly prostate-specific rat probasin (PB) proximal promoter and a synthetic variant of this promoter containing several copies of the androgen responsive region (ARR$_2$PB), respectively, virus gene expression and virus production was shown to be restricted to prostate cancer cells in vitro [35]. Replication of vectors in which the heterologous promoter was fused directly to the MLV TSS was greatly impaired relative to that of vectors in which the viral CAAT and TATA box, or the viral TATA box only, was retained. The configuration in which the MLV TATA box was preserved, but all upstream elements had been replaced by heterologous regulatory sequences was found to be ideal in respect to transgene expression, vector spread and specificity [35]. The use of the stronger ARR$_2$PB promoter resulted in a greatly improved efficacy of vector replication [35]. Moreover, results from biodistribution studies in immunocompetent and immunodeficient mice indicated that this targeting strategy prevents the productive spread of RCR vectors to spleen and bone marrow of systemically infected mice [12].

Using a different set of promoter/enhancer elements, Metzl et al. were able to demonstrate that MLV-based RCR vectors can also be targeted to liver cancer cells and to tumour cells harbouring a deregulated ß-catenin signalling pathway [36]. Vectors equipped with a chimeric promoter consisting of the hepatitis B virus enhancer II fused to the human α1-antitrypsin promoter (EIIPa1AT promoter) revealed a substantial spread in the liver cancer cell lines HepG2, AKH12, AKH13, but replicated only scarcely in the colon carcinoma cell lines SW480 and DLD-1, the cervical cancer cell line HeLa and the human embryonic kidney cell line

HEK-293 [36]. Similarly, vectors equipped with the synthetic beta-catenin/T-cell factor dependent CTP4 promoter replicated in the ß-catenin deregulated cancer cell lines HepG2, SW480, and DLD-1, but not in the cell lines AKH12, AKH13, HeLa, and HEK-293, which revealed a normal ß-catenin signalling pathway. When the heterologous promoters were used to replace almost the entire U3 region, including the MLV TATA box and TSS (TATA-replacement (TR) design), vector replication was inefficient as virus particle production from infected cells was clearly reduced by factor 100 as compared to a vector harbouring the wt-MLV 3′-LTR. On the contrary, fusion of the heterologous promoter lacking the TATA box to the MLV TATA box (TATA-fusion (TF) design) generated vectors which replicated with almost wt-MLV kinetics throughout permissive cells despite the fact that virion production from infected cells was reduced by 10-fold as compared to the prototype vector ACE-GFP. As expected, these TF-vectors exhibited low or negligible spread in non-permissive cells. The genomic stability of the TF-vectors, however, was shown to be comparable to those containing wt-MLV LTRs [36].

Both studies indicated that the precise manner in which the heterologous promoters are inserted into the U3 region of the 3′-LTR is of paramount importance. Only vectors retaining the MLV TATA box in its natural position exhibited both regulated gene expression and rapid replication kinetics [35,36].

3.2.2. Physical targeting

For decades it was generally accepted, that with MLV, and as a consequence, with MLV-based vectors, infection can only take place in dividing cells. Therefore, such vectors can be *a priori* accounted as intrinsically tumour-selective in the appropriate environment. However, very recently Liu et al. reported infection of neurons and growth arrested neuroendocrine cells with MLV-based RCR and RDR vectors harbouring an amphotropic envelope at efficiency similar to that of lentiviral vectors, which are known to infect also non-dividing cells [37]. This new and unexpected observation, if confirmed, will further raise the need for retrovirus vectors with a specific and/or targeted infection and/or replication range.

Retroviruses are unique among viral vectors in their capacity to incorporate a wide range of envelope proteins from other retroviruses and even from completely unrelated virus types. Insertion of heterologous envelope proteins into the outer shell of viral particles, so called pseudotyping, to exert an infection targeting approach has already been demonstrated in RDR vectors for a variety of envelope molecules [38–40]. The MLV-based RCR vectors used in cancer gene therapy applications to date are mostly based on the Moloney strain of MLV, which naturally expresses the ecotropic MLV envelope and thus are able to only infect rodent cells. To allow infection of human cells, the vectors are pseudotyped with the amphotropic envelope gene from the 4070A strain of MLV, which can infect most mammalian cell types via the ubiquitously-expressed Pit-2 receptor [41]. Logg and colleagues also replaced the native env sequence with that of the gibbon ape leukaemia virus (GALV) retaining a short portion of the signal peptide coding sequence of the MLV Env to avoid alteration of the MLV polymerase reading frame which is overlapping with the 5′-end of the envelope reading frame [42]. This env gene replacement greatly attenuated viral replication, most probably by a large clearance

in splicing of the viral RNA. However, employing an in vitro evolution strategy, extended passaging of cells exposed to the chimeric virus resulted in selection of virus mutants with rapid replication kinetics. Different variants arose from different sets of infection experiments. None of the revertants exhibited mutations in the GALV env gene itself, but rather in other areas of the virus to retain the ratio of spliced to unspliced viral messages which had been pertubated by the substitution of the env gene [42].

An alternative strategy for changing the tropism of MLV-based RCR vectors is via direct engineering of the specific targeting ligand sequence within the env gene. Again, several strategies have been demonstrated for RDR vectors, including the incorporation of ligands or single chain antibodies into the env gene to allow targeting to an alternative receptor [43-45]. However, these approaches were of limited success in respect to transciptional targeting, as, although retargeting of the vector was achieved, infection efficiency was greatly reduced, since the conformational change in the envelope protein necessary for proper virus-cell fusion failed to happen subsequently to the binding of the modified envelope to the alternate receptor [46].

In another approach to allow the physical targeting of RCR vectors, two tandem repeats of the immunoglobulin G-binding Z-domains of Staphylococcus protein A were inserted into the proline-rich region of amphotropic or ecotropic MLV envelope proteins present in MLV-based RCR vector particles, respectively [47]. This approach should allow virus particles to be conjugated to an antibody of choice, which can then be used for the selective binding of virus to cells (over)-expressing the chosen antibody target. Modified envelopes were efficiently expressed and incorporated into virions, while infectivity was markedly reduced by this pseudotyping [47].

For RCR vectors the most efficient physical targeting system up to now is based on protease-activatable envelope proteins [48]. Rather than attempting to redirect infection to target cells by incorporation of specific binding domains into the envelope protein, here, the virus remains non-infectious until the Env proteins become activated by cleavage by a secreted or membrane-bound cellular protease recognizing the protease target site present in the engineered Env molecule. A directed evolution-based approach was employed for the selection of retroviruses activatable by matrix metalloproteinases (MMPs) which are specifically expressed by tumour cells [26,49,50]. RCR vectors generated to express either the epidermal growth factor (EGF) or the CD40 ligand, linked via MMP cleavage sites as fusion to the N-terminal of the MLV 4070A envelope protein, were sequestered by the EGF receptor or the CD40 receptor, which are ubiquitously-expressed on potential host cells. By that the envelope protein was prevented from interacting with its natural Pit-2 receptor resulting in poor infection efficiencies and thus de-targeting of the RCR vectors from non-tumour cells [49,50]. Infection efficiency however, is restored in cells which express high levels of MMPs, such as many tumour cell types, due to Env-ligand cleavage and interaction of the Env protein with its natural receptor [50]. In a comparative study Duerner and colleagues analysed the spread of targeted and non-targeted MLV RCR variants in *s.c.* tumours derived from HT1080 and U-87MG cells, respectively, and in extratumoral organs after systemic tail vein injection of the vector into SCID mice [48]. Both virus types were able to efficiently infect tumour cells however, the non-targeted virus efficiently infected also extratumoral organs such as bone marrow, spleen, and liver. Quanti-

tative analyses revealed an up to 500-fold higher selective infection of tumour tissue with the targeted viruses than with the non-targeted counterpart [50].

3.2.3. Alternative ways for RCR vector targeting

Recently, a fully functional chimeric vector system that uses a helper-dependent adenovirus 5 (Ad) vector as a first stage carrier to express and deliver a fully functional RCR vector has been described [51]. The RCR vectors are produced *in situ* from initially adenovirus-transduced cells, thereby combining benefits of both vector systems – high titers reached with adenoviruses and stable integration of retroviruses. At equivalent initial transduction levels, more secondary RCR progeny were produced from Ad-RCR-transduced cells as compared to RCR-transduced cells, resulting in further acceleration of RCR replication kinetics [51]. In pre-established *s.c.* human breast cancer xenografts in mice, suicide gene therapy with high titre chimeric Ad-RCR vectors achieved, in a dose dependent manner, an enhanced efficacy compared to delivery of respective RCR-only vectors [52]. As the target cell binding tropism of adenoviral vectors can be altered by modifications to the fibre knob [53], the strategy of employing a chimeric Ad-RCR vector system might represent a promising step forward towards a targeted and efficacious cancer gene therapy.

As a future option, delivery of RCR vectors to tumours could also be facilitated by the use of tumour-homing cells as vector carriers. Mesenchymal stromal cells, for example, which have been shown to be able to home to malignant areas, have been loaded with replicating adenoviral vectors to deliver them to the tumour tissue to execute their oncolytic potential [54], an approach which might be also applicable to RCR vectors.

3.3. Therapeutic application of MLV-based RCR vectors

Despite the fact that the first experiments employing MLV-based RCR vectors have been commenced more than 20 years ago, the utilization of these vectors for cancer gene therapy is still at an early stage. The vectors are not oncolytic *per se* and the choice of therapeutic genes which can be used is limited, as large heterologous sequences cannot be inserted into the MLV genome without impairing vector stability and replication efficiency. Nonetheless a number of therapeutic sequences such as those encoding suicide genes, cytokines and interfering RNAs have been demonstrated to be stably propagated over several infection cycles by MLV-based RCR vectors.

3.3.1. Suicide genes

Suicide genes, also called prodrug-converting genes, encode proteins which are not toxic *per se*, but which are able to catalyse the formation of toxic metabolite(s) from a non-toxic or low-toxic prodrug. By the delivery of suicide genes to and their expression in tumour cells, conversion of a systemically administered prodrug by the respective suicide gene product results in a highly specific and effective anti-tumor therapy [55]. Although the toxic metabolites generated are quite often identical to those of classical chemotherapy, here, the local produc-

tion of metabolites in the tumour cells results in a more contained and specific effect as compared to the, in most cases, systemically given chemotherapy.

Initial data demonstrating therapeutic efficacy of an RCR vector mediated suicide therapy approach have been shown using the yeast cytosine deaminase (yCD) gene in a mouse model of human glioma [56]. In cells infected with the yCD expressing RCR vector ACE-CD, the non-toxic prodrug 5-fluorocytosine (5-FC) was converted into the toxic component 5-fluorouracil (5-FU), leading to cell death not only in infected cells but also in surrounding non-infected dividing cells due to intercellular diffusion of 5-FU. Stereotactic intratumoral injection of only 1x10e4 infectious ACE-CD particles into pre-established intracranial U-87MG human glioma xenografts in nude mice followed by daily intraperitoneal (*i.p.*) administration of 500 mg 5-FC per kg body weight eight days later for 15 days (ACE-CD + 5-FC) led to survival of all treated animals for a follow up period of 60 days, whereas mice of both control groups, vector only (ACE-CD + PBS) and prodrug only (PBS + 5-FC), died within 40 days [56]. Subsequent studies, however, revealed that this treatment regimen is insufficient to get complete eradication of U-87MG tumours in the treated animals, resulting in death of the animals after 70 and more days [57]. Retrospective immunohistochemical analyses showed that in most of the treated animals still small areas of tumour tissue were present indicating an ectopic spread of glioma cells in the brain. Despite the fact that all of the surviving tumour cells stained positive for viral envelope protein, these tumour cells had not been killed by the administration of 5-FC. This suggests that these cells either have been infected with a suicide gene deletion variant of the vector or the therapeutic gene, although present, is not expressed in these cells or that the cells are refractory to chemotherapy [57]. Tai et al. also examined whether the efficacy of this treatment could be increased by administering multiple cycles of 5-FC [57]. To this end, after injection of 1x10e4 transducing units of vector ACE-CD into preformed intracranial tumours, mice received multiple cycles of 5-FC for 8 consecutive days with 3-week intervals between the treatment periods [57]. Again, all control animals receiving virus vector only and prodrug only, respectively, died within 40 days, whereas all mice treated with vector and prodrug survived for more than 100 days, demonstrating that the multi-cycle strategy provides a significant therapeutic benefit compared to a single cycle of prodrug administration [57].

To investigate the effect of the immune system on the effectiveness of the RCR vector-mediated glioma therapy, the rat glioma cell line RG2 was used to establish syngeneic intracranial tumours in Fischer 344 rats [58]. Three days after tumour implantation, 1x10e6 infectious ACE-CD virus particles were stereotactically injected into the growing tumours. Ten days after virus injection, 5-FC at 500 mg/kg body weight was administered *i.p.* for 7 consecutive days and after a 10 days interval the treatment cycle was repeated. Animals treated with vector ACE-CD and 5-FC survived for up to 35 days, whereas control animals (ACE-CD + PBS) died within 21 days. Despite the higher initial virus vector load injected into the tumour and the shorter intervals between the treatment cycles a significantly shorter survival time of treated Fischer 344 rats was observed as compared to data of vector/prodrug treated U-87MG tumour bearing nude mice [58]. This observation might be, to a certain extent, due to reduced spread kinetics in the RG2 tumour as compared to the U-87MG tumours, as only 65 % of RG2 tumour cells got transduced after 21 days after initial virus infection. On the other hand, animals with tumours

derived from pre-infected and 100 % CD-expressing cells treated with 5-FC died due to tumour burden at a significantly later time point, however still within 50 days [58]. This indicates that other factors might contribute to the poorer treatment efficacy, such as insufficient levels of 5-FC/5-FU in the tumour, differences in resistance to 5-FU, or effects triggered by the immune system. Regarding this latter point, however, no immune response against the RCR vector in the brain has been detected [58].

Recently, we have critically analysed a panel of 15 different human and rodent glioma and glioblastoma cell lines in respect to spread of RCR vectors derived from vector ACE-CD to their sensitivity towards the 5-FC/5-FU suicide system, and to their orthotopic growth characteristics in mice to identify suitable preclinical animal models as test bed for the development and evaluation of RCR vector mediated treatment of glioblastoma [59]. Rapid virus spread was observed in eight out of nine human cell lines tested in vitro. As expected, only CD-expressing cells became sensitive to 5-FC. All LD_{50} values were within the range of concentrations obtained in human body fluids after conventional 5-FC administration. In addition, a significant bystander effect was observed in all human glioma cell lines tested, supporting the potential of this suicide gene therapy for the treatment of brain tumours [59].

This therapeutic concept has also been experimentally employed to subcutaneous and orthotopic liver metastasis of colorectal cancer in an immunocompetent rodent model [60,61]. To this end, murine CT26 cells pre-transduced with vector ACE-CD were mixed with non-transduced CT26 cells at a ratio of 1:200 prior to implantation into BALB/c mice. Twelve days later, daily 5-FC treatment at 500 mg/kg, given *i.p.* twice a day, was started and continued until the end of the experiment. 5-FC treated animals showed significant inhibition of tumour growth resulting in an average tumour size of approx. 100 mm³ at day 24, whereas in animals whose drinking water was supplemented with 0.4 mg/ml of azidothymidine (AZT) to prevent MLV vector replication, tumour growth was inhibited only moderately with an average tumour size of approx. 500 mm³ at day 24 [61]. Tumour size in untreated animals reached more than 800 mm³ at day 24 [61]. The effect on tumour size observed in the AZT-treated group most probably was due to the presence of a bystander effect, in which toxic metabolites produced by the CD-positive cells can freely diffuse into neighbouring, CD-negative cells to cause cell death and thereby eventually lead to a delay in tumour growth. These observations clearly indicate that the efficacy of the ACE-CD/5-FC treatment is dependent on a sustained vector spread. Proof-of-concept of this therapy was also shown in a multifocal hepatic metastasis model [61]. Here, CT26 cells stably expressing the firefly luciferase marker gene were infused into the portal system via intrasplenic injection, became trapped within the hepatic microcirculation, and seeded metastases. Three days later, 2x10e4 ACE-CD virus vector particles were instilled into tumour-bearing mice, again via intrasplenic injection, followed by daily 5-FC administration at 500 mg/kg given *i.p.* twice a day, initiated 14 days after tumour cells inoculation [61]. In contrast to untreated animals or animals which received vector and PBS only, and which revealed increasing bioluminescence throughout the 28 day experiment, 5 out of 7 animals treated with both vector ACE-CD

and 5-FC showed stable or decreased levels of bioluminescence, indicating that the development of metastases was inhibited [61]. The locoregional delivery of the CD suicide gene by RCR vectors infused into the portal circulation thus resulted in progressive transduction of multiple tumour foci in the liver without evidence of spread to adjacent normal parenchyma or extrahepatic tissues as shown by qPCR analyses of MLV-specific sequences in the DNA of liver tumour tissue, normal liver, and bone marrow cells [61].

For proof-of-concept in a further indication, Kikuchi and colleagues evaluated the transduction and therapeutic efficacy of intravesically administered RCR vectors in orthotopic bladder tumours in mice [62]. Tumours were established by implantation of either MBT-2 murine bladder cancer cells into immunocompetent syngeneic C3H/HeJ mice or of KU-19-19 human bladder cancer cells into nude mice. RCR vector particles carrying the eGFP transgene were delivered intravesically *via* a catheter inserted into the bladder 5 days after tumour formation. Upon injection of 3.2x10e5 infectious virus particles, vector spread in the human xenograft model reached almost all of the tumour cells within 35 days post infection [62]. In the orthotopic syngeneic model, vector spread resulted in, on average, 30 % of infected tumour cells 27 days after vector instillation, as revealed by immunohistochemical analyses of transgene expression [62]. The effects of the respective therapeutic vector ACE-CD were demonstrated in the MBT-2 bladder cancer model, whereby 3.2x10e5 infectious virus particles were instilled intravesically into the bladder of mice containing preformed tumours. Twelve days following infection, animals received daily 5-FC at 500 mg/kg given *i.p.* for 15 days [62]. A single course of 5-FC treatment led to significantly reduced tumour growth and 50 % of the animals survived for more than 70 days. In contrast, all of the animals in vector only or vehicle control groups died within 40 days. Again, the locoregional delivery of intravesically administered RCR vectors was shown to achieve significant tumour growth inhibition by efficient delivery of the therapeutic gene into the orthotopic bladder tumour cells without any evidence of spread to adjacent distant organs [62].

Recently, Kawasaki et al. investigated RCR vector mediated gene therapy for the treatment of human malignant mesothelioma [63]. Cells of this tumour type were found to be highly permissive for RCR infection [63]. After a single intratumoral injection of 1x10e4 ACE-CD virus vector particles into pre-established subcutaneous MSTO-211H human mesothelioma tumour xenografts in nude mice, followed by daily 5-FC administration at 500 mg/kg body weight given *i.p.* from day 12 to day 32 post-infection, significant inhibition of tumour growth was observed in ACE-CD/5-FC treated mice compared to control groups as indicated by a tumour volume of 200 mm^3 *vs.* 800 mm^3, respectively, at day 32 after infection. Efficacy of the treatment was also investigated in a peritoneally disseminated human mesothelioma xenograft model [63]. Here, MSTO-211H cells stably expressing the mCherry fluorescence protein and transduced with vector ACE-CD were mixed at a ratio of 1:100 with non-transduced mCherry expressing MSTO-211H cells and the mixture was injected i.p. into nude mice. After confirmation of tumour formation by in vivo imaging, daily 5-FC administration was initiated (500 mg/kg *i.p.*) for 15 consecutive days. In 4 out of 9 mice of the vector/5-FC receiving group no visible tumours were observed and mice survived until day 100 after cell injection. On the contrary, all non-treated mice had died at day 61 at the latest. In summary, the 5-FC treated

group showed a significantly prolonged median survival time as compared to the control group (81 days *vs.* 34 days) [63].

Beside the CD/5-FC suicide gene/prodrug combination, which is the most prominent and extensively studied system in context of RCR vectors, other therapeutic genes have been introduced in this vector system and have been analysed. The bacterial purine nucleoside phosphorylase (PNP) gene, for example, converts the prodrugs fludarabine phosphate (F-ara-AMP) or 6-methylpurine 2′-deoxyriboside (MeP-dR) into its toxic metabolites and represents a reasonable alternative to the CD/5-FC suicide system. Kikuchi et al. demonstrated the therapeutic efficacy of vector ACE-PNP, an MLV-based RCR vector expressing the *E.*coli PNP gene, in a subcutaneous model of KU-19-19 human bladder cancer cells in nude mice [64]. Five days following tumour cell implantation, 3.2x10e5 infectious virus particles were injected directly into the tumour and 10 days later F-araAMP at 75 mg/m² body surface was administered *i.p.* every other day for a total of seven injections. Significant tumour growth inhibition could be demonstrated with a tumour mass of 600 mm³ *vs.* ≥ 2,200 mm³ at day 26 post virus injection in treated mice *vs.* vector and vehicle control groups [64]. These results indicate that the PNP-RCR system is a reasonable alternative to the use of CD-expressing RCR vectors, in particular as enzymatic products generated by PNP seem to be more cytotoxic than 5-FU [65,66].

In in vitro experiments using human U-87MG glioma cells, Tai and colleagues were able to show that transduction of even only 1 % of cells with the ACE-PNP vector and subsequent systemic prodrug administration is sufficient to achieve significant cell killing over time [67]. Thereby, the rapidity of cell killing is highly dependent on the initial level of transduction. Treatment of pre-established *s.c.* U-87MG tumour xenografts with 1x10e5 ACE-PNP vector particles followed by F-araAMP prodrug administration 14 days after virus injection at a concentration of 80 mg/kg given *i.p.* once every other day for five times resulted in significantly inhibited tumour growth [67]. A second cycle of prodrug administration reduced tumour growth even further. The potential of an ACE-PNP RCR vector-based therapy to improve survival of nude mice bearing intracranial U-87MG tumours was evaluated by inoculating pre-established xenografts with 1x10e4 vector particles 7 days after tumour onset. Eight days later, the mice received F-araAMP (40 mg/kg given i.p. once every other day) for a total of eight treatments. The median survival time was 59 days *vs.* 30 and 28 days in treated *vs.* control groups [67]. In a second experiment, the same experimental setting was employed, but a second round of F-araAMP treatment was done after a 14 days recovery period. The median survival time of treated mice was further improved to 73 days *vs.* 33 days in the control groups [67]. This data again demonstrated the potential for additional survival benefit from multiple cycles of prodrug administration.

In summary, the published data of non-preclinical studies using suicide gene-expressing MLV-based RCR vectors have demonstrated that such vectors are therapeutically efficacious in solid tumours and/or metastases of a range of different tumour types (glioma, colorectal and bladder cancer, mesothelioma) in both immunodeficient and immunocompetent mouse and rat models, and using different therapeutic genes (yCD and PNP). On grounds of these non-clinical data the therapeutic concept of replicating retroviral vectors was moved towards clinical application. To this end, based on the design of the extensively evaluated vector ACE-

CD, a lead clinical candidate (vocimagene amiretrorepvec, Tocagen Inc., San Diego, CA) has been constructed carrying a human codon-optimised thermostable yeast CD gene [68]. Comparison with the prototype vector ACE-CD harbouring the wildtype yeast CD gene revealed a three-fold increased CD-specific conversion of 5-FC to 5-FU in infected cells and a markedly higher genetic stability of the clinical vector candidate Toca 511 [68]. To further support the production of toxic metabolites in infected cells, the modified CD gene was further linked as fusion to the gene encoding the yeast uracil phosphoribosyl transferase (UPRT) or, alternatively, to the human orotate phosphoribosyltransferase (OPRT) gene. It has been reported that expression of the UPRT gene in the CD-expressing cells leads to increased sensitivity to 5-FC, as the UPRT converts 5-FU directly to 5-FUMP, from which the active metabolites 5-FdUMP and 5-FUTP are formed [69,70]. The human OPRT protein, as part of a multifunctional UMP synthase, is a human analogue of the UPRT, converting 5-FU directly to 5-FUMP with direct impact on the cellular sensitivity towards 5-FU, since downregulation of this endogenous enzyme was found in tumour cells resistant to 5-FU [71,72]. Moreover, exogenous expression of the OPRT gene led to increased 5-FU sensitivity in cancer cell lines in vitro [73]. Despite better individual in vitro cell killing with vectors carrying these fusion genes, it remains unclear, whether this would be beneficial for therapy, as the highly efficient 5-FC salvage to phosphorylated nucleotides may diminish 5-FU diffusion and thereby the effects exerted by the bystander mechanism. Furthermore, initial killing of most of the infected cells might hinder vector spread during the 5-FC-rest period, leading to a reduced antitumor activity in vivo [68,74]. In addition, serial passaging of these infectious viruses on human U-87MG glioma cells revealed a decreased genomic stability of vectors containing fusion constructs as compared to Toca 511, probably due to the size and nature of the inserted transgene(s) sequences (~500 bp in Toca 511 vs. ~1250 bp in the fusion constructs) [68].

The therapeutic potential of Toca 511 was evaluated in two different intracranial brain tumour models in immune competent mice (CT26-BALB/c and Tu-2449-B6C3F1) [75]. Treatment of CT26 brain metastases with three different virus doses (1x10e6, 1x10e5, 1x10e4 transforming units (TU)/g brain weight) plus 5-FC administration initiated nine days after intratumoral virus injection at 500 mg/kg given i.p. twice a day for always 7 days, followed by 10 days off until termination of the study, revealed a statistically significant prolongation in median survival of mice treated with the mid (1x10e5) and high (1x10e6) vector dose as compared to their PBS-treated counterparts [75]. In a similar experiment mouse Tu-2449 gliomas were treated using different virus doses (1x10e6, 1x10e5, 1x10e4 and 1x10e3 TU/g brain weight) and 5-FC doses (500 mg/kg and 50 mg/kg body weight) [75]. 5-FC treatment or PBS treatment as control was initiated 9 days after vector injection and was given twice a day for 4 days, followed by a 10 day recovery phase. Cycles were repeated until termination of the study [75]. All vector doses in combination with 5-FC treatment at 500 mg/kg resulted in prolonged survival, as compared to PBS controls. Mice treated with vector doses of 1x10e4 and 1x10e5 and high dose 5-FC revealed a significantly prolonged survival when compared to the PBS controls [75]. However, even the 1x10e5 vector dose level with a rather low prodrug dose of 50 mg/kg 5-FC resulted in a survival advantage when compared to control. Histological analyses of Tu-2449 tumours taken before the first, second, and fourth treatment cycle with 500 mg/kg 5-FC revealed tumour

growth between the first and the second dosing. Most of the tumour tissue however, was no longer visible and gliosis was evident by the start of the fourth treatment [75].

Currently, Toca 511 is being investigated in clinical trials in the United States in subjects with recurrent high-grade glioma either as a direct intratumoral vector injection (Phase I/II Study; NCT01156584; http://www.clinicaltrials.gov) or vector injection at the time of tumour removal (Phase I Study; NCT01470794 http://www.clinicaltrials.gov).

3.3.2. Secreted therapeutic molecules

Beside direct killing of tumour cells mediated by suicide gene products, different other therapeutic approaches, e.g. based on the secretion of therapeutic molecules such as cytokines or single chain antibodies (scFv) directed against specific tumour antigens could be facilitated by RCR vectors.

To allow secretion of therapeutic molecules from infected cells, a replicating retroviral vector was constructed by inserting specific transgene sequences to the first codon of the MLV env gene via a furin cleavage site sequence [26]. The respective fusion protein will be cleaved by furin proteases in the Golgi and the scFv will be secreted upon release of the new virus particles from infected cells (for details see above). The resulting vectors were capable of efficiently transducing susceptible cells, were genetically stable for more than 12 passages and were able to efficiently mediate intracellular production and secretion of the GM-CSF cytokine and the functional laminin-specific or T-cell-specific scFv antibody, respectively [27].

Sun and colleagues demonstrated the potential of the human chemokine interferon-gamma-inducible protein 10 (IP10) gene, delivered and expressed from a MLV-based RCR vectors, to inhibit tumour growth *in vivo* [76]. IP10 is known to be a potent inhibitor of angiogenesis, tumour growth, and metastasis [77,78]. Using human fibrosarcoma HT1080 cells transduced with the IP10 RCR vector designed as described above in an *s.c.* xenograft tumour model in nude mice, significant tumour growth inhibition and a marked reduction in microvessel density was observed as compared to non-infected HT1080 control mice (tumour volume 190 mm^3 *vs.* 510 mm^3) [76]. In addition, both, growth of *s.c.* tumour xenografts established from pre-infected murine Lewis lung carcinoma (LLC) cells as well as the formation of lung metastases from pre-infected murine melanoma B16F10 cells in immunocompetent C57BL/6 mice was significantly reduced [76].

3.3.3. RNA interference

In cancer gene therapy applications, RNAi-expressing RCR vectors can be used to inhibit tumour growth, invasion and metastasis. The length of the RNA duplex required for efficient RNAi is not longer than 21-23 bp. Therefore, the insertion of RNAi expression cassettes into RCR vectors should be well tolerated in respect to genetic stability and spread kinetics of the vector. The expression of duplex RNA is usually achieved using an expression cassette consisting of the RNA Pol III promoter transcribing a sequence designed to form a short hairpin RNA structure (shRNA). MLV-based RCR vectors were constructed encompassing a tran-scription cassette consisting of an H1-RNA Pol III promoter inserted in antisense orientation

into the 3′ UTR of the vector backbone to drive expression of an shRNA sequence targeted against the epidermal growth factor receptor (EGFR) gene or the STAT3 gene [30]. To allow monitoring of infection efficiency, these vectors also contain the eGFP gene fused into the virus envelope gene. Insertion of both expression cassettes did not interfere significantly with virus fitness, and the receptor specificity of the Env protein was not impaired by the introduced eGFP sequences [28]. The modified vectors replicated rapidly and were genetically stable over several infection cycles. In addition, silencing of EGFR and STAT3 target gene expression in cells infected to levels of 80 – 95 % was shown to be highly efficient [30].

An improved, second generation MLV-based RNAi transfer vector suitable for in vivo application was recently described [79]. This RCR vector encodes miRNA modified shRNA sequences specifically targeting the eGFP and luciferase reporter genes under control of the small nuclear U6 promoter inserted in antisense orientation into the 3′ UTR of the vector. In HT1080 cells stably expressing eGFP or luciferase, marker gene expression was suppressed by more than 80 %, even when only 0.1 % of the cells were initially infected with the RCR vectors [79]. In vivo systemic tail vein administration of 2.9x10e7 of shLuc expressing vector particles in animals with pre-established subcutaneous HT1080-Luc tumours led to more than 80 % reduction in luciferase activity compared to uninfected tumours at day 25 post infection [79]. To investigate the effects of downregulation of tumour-promoting proteins, PLK1- and MMP14-specific shRNA expression cassettes were inserted into the vector. Upon infection of target cells, PLK1 and MMP14 mRNA and protein levels were reduced [79]. MLV-shPLK-infected cells were trapped in the G2-phase of the cell cycle at day 3 post infection, followed by induction of apoptosis at day 5 post infection. MLV-shMMP14 infected cells showed reduced MMP2 activity consistent with a reduced invasion capacity by ~75 % as compared to non-infected cells [79]. Tumour growth of MLV-shMMP14 infected HT1080-Rec-1 cells in immunodeficient mice was significantly and substantially reduced in comparison to controls. Similarly, direct intratumoral application of 1x10e6 of shPLK1 expressing vector particles in animals with pre-established subcutaneous HT1080 xenografts led to a significantly reduced tumour growth in comparison to the controls [79].

3.4. Safety of MLV-based RCR vectors

Up to now, a variety of different therapeutic approaches have been utilized in context of RCR vectors. High levels of vector spread, infection efficiency, and therapeutic gene expression have been detected leading to an efficacious therapeutic option for the treatment of cancer. From data on the eradication of tumour mass in animal models existing so far, a clear benefit of the RCR mediated tumour therapy is indicated. However, the use of replicating retroviral vectors also bears a number of risks which have to be identified and analysed.

In particular, 3 major concerns directly related to the use of RCR vectors have to be taken into consideration: (i) the risk of insertional mutagenesis due to integration of the vector genome into the host cell DNA, a step that can trigger the transformation of normal cells into tumour cells, (ii) the spread of viral vectors throughout the body of the patient causing viraemia, and (iii) the infection of dividing non-tumour cells and their loss due to therapeutic intervention leading to severe side effects.

The risk of insertional oncogenesis is an issue associated with the use of retroviral vectors in general, irrespective if they are replication-deficient or replication-competent. With RCR vectors this concern might be more substantial as due to the replicating nature more cells might be affected and multiple infections of the single cell might occur. On the other hand, in tumour therapy it is intended to kill the infected cells. Hence, due to this, insertional oncogenesis should not be an issue unless infected cells are resistant to treatment or got infected with an RCR vector which is reverted to wild-type due to genetic instability and thus is not able to exert its therapeutic potential.

The use of replication-competent retroviral vectors might also bear the risk of uncontrolled spread of vectors throughout the human body, resulting in infection of dividing cells other than the tumour cells itself. Non-dividing cells should not be infected as MLV-based vectors are thought to transduce dividing cells only, and, as most cells in the adult human body are non-dividing, virus spread should be limited. Moreover, some cell types, such as dividing human primary T-lymphocytes have only a low capacity to produce MLV-based RCR and, in addition, the produced virions are largely non-infectious [80]. Nevertheless, in case of unintended virus spread and high risk of viraemia, an early systemic intervention with antiretroviral drug(s) could be implemented to limit viral load [81]. Recent findings, on the other hand, suggest that the host range of MLV-based RCR vectors might include also post-mitotic and other growth-arrested cells in mammals [37]. Therefore, the issue of RCR-vector dissemination outside of the tumor mass is of particular concern in clinical studies employing RCR vectors and thus should be addressed in respective biodistribution studies.

For RCR biodistribution studies, sensitivity of the analysis is of utmost importance. Techniques based on conventional PCR, real-time PCR, flow-cytometry and immunohistochemical detection were employed so far to analyse the infection range of RCR vectors in animal models. In an early report, Logg and colleagues analysed the presence of the eGFP transgene in the DNA extracted from tumour tissue as well as from a variety of extratumoral tissues including spleen, lung, kidney, liver, and heart obtained from *nu/nu* BALB/c mice harbouring *s.c.* xenografts 7 weeks after intratumoral application of 6x10e3 RCR vector particles [22]. A PCR assay detecting 140 copies of GFP in a background of 100,000 equivalents of untransduced genomic DNA (transduction level 0.14 %) was employed [22]. Transgene sequences have been detected in tumour samples only. These data were further supported by flow cytometric analysis of the same tissues [22]. By improving this PCR assay, Wang et al. were able to detect as few as 35 copies of proviral DNA in 0.5 µg of genomic DNA. In an orthotopic glioma model in nude mice, in which animals were intratumorally inoculated with 1.2x10e4 virus particles, proviral sequences have been detected in tumour tissue, but not in contralateral brain paren-chyma, bone marrow, GI tract, liver, kidney, spleen, lung, and skin [56]. Using a PCR driven detection method, eGFP transgene sequences could not be observed in normal tissues sur-rounding the injected tumours in a *s.c.* bladder tumour xenograft model in *nu/nu* BALB/c mice [62, 64]. No signals have been obtained in distant organs such as brain, liver, spleen, lung, bladder, kidney, heart, ovary, uterus, and stomach in an orthotopic model of bladder cancer, when RCR vectors were administered intravesically [62, 64].

By detection of CD gene-specific sequences by PCR at a detection limit of 400 copies per 100,000 cell genomes (600 ng of gDNA, transduction level 0.4 %), Tai and co-workers were able to determine proviral sequences in the transduced glioma tissue, but observed no extratumoral spread to and in any of the tissue examined (lung, liver, oesophagus and stomach, intestine, spleen, kidney, skin, bone marrow, contralateral normal brain) in an orthotopic glioma model in nude mice intratumorally injected with 1.2x10e4 virus particles [57]. However, all of these studies had been based on the detection of the non-viral transgene (e.g. GFP, CD, PNP, etc.). Hence, putative spread of vectors which have lost the transgene or parts of it will not be considered in these analyses.

In an immunocompetent intracranial RG2-glioma model performed in Fischer 344 rats, tumours were injected with 1x10e6 of CD expressing RCR vector particles followed by 5-FC or PBS treatment initiated 10 days later [58]. Organs from moribund animals were collected and quantitative real-time PCR targeting the MLV env gene was performed [58]. There was no evidence of presence of the env gene from the RCR vector in systemic tissues carrying highly mitotic cells such as lung, liver, kidney, spleen, bone marrow, skin, oesophagus, intestine, and testis [58]. Using this highly sensitive technique which enables detection of 20-35 copies in 50,000 cellular genomes, Hiraoka et al. analysed RCR vector biodistribution in a mouse hepatic metastasis model using BALB/c mice and the syngeneic colon carcinoma cell line CT26 [60,61]. After locoregional delivery of 2x10e4 of RCR vector particles either expressing the eGFP or CD transgene, *via* intrasplenic injection followed by splenectomy, no detectable virus-related signals were observed in genomic DNA extracted from peritumoral normal liver tissue, bone marrow, lung, kidney, small intestine, and colon. As expected, proviral RCR signals were highly abundant in genomic DNA from RCR-transduced tumour tissues with 3,000 – 16,600 proviral copies per 50,000 cells [60,61]. The copy number was increased in DNA obtained from ACE-CD/PBS-treated control animals (11,000 – 16,600 copies/50,000 cells) compared to ACE-CD/5-FC-treated animals (8,000 – 11,000 copies/50,000 cells) [61]. Optical imaging analyses, flow cytometric analyses as well as immunohistochemistry using an anti-eGFP antibody consistently revealed strongly positive eGFP signals in the tumour masses but not in the other organs mentioned above [60].

Biodistribution of RCR vectors after intravenous injection into immune deficient as well as immune competent mice was analysed by Solly and colleagues [25]. Two weeks after RCR vector injection, a 4070Aenv-based quantitative real-time PCR revealed presence of proviral genomes in bone marrow and spleen of nude mice. On the contrary, no proviral genomes could be detected in any tissue from immunocompetent animals, which empha-sizes the potency of anti-MLV specific immune responses [25]. In vivo biodistribution of wt-MLV and MMP-activatable RCR vectors and their ability to reach tumour tissue after systemic administration was analysed in CB17-SCID mice using an optimized PCR meth-od detecting up to 50 copies of proviral DNA in 300 ng of genomic DNA [48]. After in-travenous injection of RCR vectors corresponding to 60 U of RT activity into tumour-free mice, tissues were analysed at different time points after virus injection. A strong signal in spleen and weak signals in liver and bone marrow were obtained after administration of wt-MLV vector one day after infection. After two weeks, wt-MLV sequences were

found in lung, spleen, liver, heart, bone marrow, and muscle, but not in brain. The increase in PCR signal intensity over time suggests continuous virus replication. This was further supported by the presence of infectious virus in the blood of these animals. On the contrary, no positive signals were detected in mice infected with the MMP-activatable RCR vectors [48]. A similar experiment was performed with U-87MG and HT-1080 *s.c.* tumour-bearing mice to quantify the virus load in the tumour tissue. Again, animals were injected intravenously with similar amounts of wt-MLV and MMP-activatable RCR vectors and genomic DNA was analysed 2 weeks later. For all viruses, tumour tissue revealed the highest virus load reaching 100 % of cells in case of the non-targeted wt-MLV and up to 30 % of cells for MMP-activatable RCR vectors [48]. In addition, wt-MLV infected on average 32 % of the cells in the bone marrow, 11 % of the cells in spleen, and 6 % of the cells in liver, whereas the MMP-activatable RCR vector infected only 0.02 % of cells in these organs [48]. Interestingly, the load of wt-MLV in bone marrow and the other extratumoral organs in tumour-bearing animals was generally lower compared to those obtained in tumour-free animals [48].

Recently, Ostertag et al. reported biodistribution of the clinical vector Toca 511 after intratumoral administration in an immunocompetent mouse model of brain cancer using a quantitative real-time PCR technique to detect integrated provirus sequences with high sensitivity (10-25 copies per μg of gDNA) [75]. Two animal models based on different mouse strains had been Involved; BALB/c mice permissive for virus infection, and poorly permissive C57BL/6 mice. In these animal models 10e5 and 10e6 RCR vector particles have been injected intracranially into the tumor mass. Quantitative DNA analyses were performed on samples from mice which survived for 90 days and 180 days for the CT26-BALB/c model and the Tu-2449-B6C3F1 model, respectively. In the CT26-BALB/c model, vector spread to other tissues, particularly to lymphoid organs (thymus, spleen, lymph node, blood) was detectable [75]. Up to 5x10e5 proviral copies/μg gDNA were detected in thymus, up to 1.5x10e5 proviral copies/μg gDNA in samples from salivary gland, oesophagus, lung, heart, spleen, lymph node, and blood, and less than 5x10e3 proviral copies/μg gDNA in samples from skin, ovary, intestine, liver, kidney, spinal cord, bone marrow, cerebellum and brain [75]. In the Tu-2449-B6C3F1 model vector spread was observed at low levels only. Less than 5x10e3 proviral copies/μg gDNA were detected and interestingly the oesophagus was the organ in which the proviral copy number was highest. The difference in viral distribution observed in both model systems could be explained by the presence of different APOBEC3 alleles in these mouse strains. BALB/c mice have been shown to carry an allele that does not restrict MLV, whereas B6C3F1 mice carry an active allele [82]. Both mouse strains produced antibodies against Toca 511 [75].

The issue of biodistribution of the vector to non-target sites, as well as the expression of the therapeutic gene in off-target cells could be addressed best by infection targeting of the vector or expression targeting of the therapeutic gene to cells of the tumour. This approach has already been applied using tumour- and tissue-specific regulatory sequences to drive virus replication and transgene expression, and by modifying the viral envelope protein to allow transduction in a tissue/tumour specific manner.

4. Conclusion

Very recently, RCR vectors have been employed as a novel gene transfer vehicle for the treatment of cancer. Due to their dense genome organisation and the need for presence of all virus genes to allow vector replication, an only limited capacity for the introduction of foreign sequences is available, rendering the design of such vectors rather challenging. Nevertheless, different vector designs in respect to transgene location and mode of transgene expression have been elaborated. Their analysis in in vitro and in vivo studies revealed that the vectors are genetically stable over several replication cycles and result in an efficient delivery of the therapeutic gene into solid tumours in various animal models. On the other hand, different risks are associated with the use of RCR vectors, such as the risk of insertional tumorigenesis of non-target cells or the risk of inadvertent vector spread resulting in severe side effects. Such risks need to be carefully examined in appropriate non-clinical studies. In case they can be adequately addressed and dispelled, RCR vectors will be a promising option for efficient tumour therapy in humans.

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