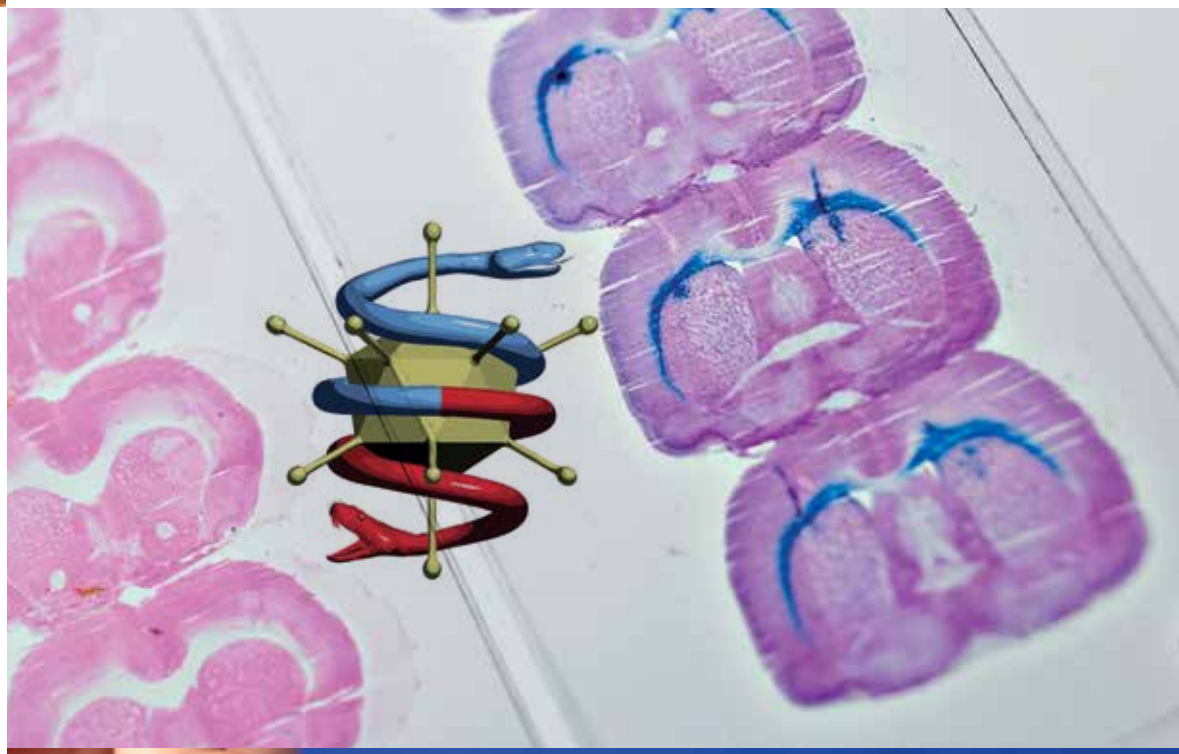
Assessment of preclinical gene therapy studies worldwide



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University Medical Center Rotterdam

Assessment of preclinical gene therapy studies worldwide

COGEM Research Report 2015-03

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Authors Dr. J. Verhagen, MDL department, Erasmus MC
Drs. P. Buijs, Surgery department, Erasmus MC
Dr. B. van den Hoogen, Viroscience, Erasmus MC
Prof. dr. C.H.J. van Eijck, Surgery department, Erasmus MC

Colofon

Cover pictures: Bart Erkamp and Ivar Pel
Cover design: Avant la Lettre, Utrecht

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Preface

Biotechnology and recombinant DNA technology have become firmly established in medicine and medical research. Genetic modification is one of the powerful tools in biomedical research and is employed all along the clinical path from diagnosis to treatment. One of the most direct clinical applications of recombinant DNA technology is experimental gene therapy. In gene therapy a limited number of the patient's cells is genetically modified in order to treat or cure the underlying disorder. In the last years research in a variety of diseases has yielded evidence of the clinical efficacy of the approach. Gene therapy works! The European Medicines Agency has approved the first gene therapy product for treatment of patients with inherited diseases. This is a fruit from Dutch research and the approval heralds a milestone for the gene therapy field.

Inevitably, the developments in the gene therapy field are attracting much attention, not only by medical specialists and researchers, but also by the general public. Therefore it is of importance that the regulatory bodies monitor the developments and the potential new treatment options, and the impact it may have. Being an independent advisory body of the Dutch government in the field of genetic modification, the COGEM must keep a close eye on the field and should signal potential implications.

The COGEM commissioned a desk study to chart the developments in the field of gene therapy. This rapport provides you the findings of the study. In it, a team of Rotterdam-based scientists describe the recent developments in the field with a focus on the basic and preclinical research stages. In these stages the new tools and techniques are developed that can shape future clinical applications. The rapport also gives insight in the preclinical research that may soon advance to the stage of clinical evaluation. It points out new technological developments and the challenges that it may pose for assessing the risks for men, society, or the environment.
The COGEM's committee that supervised the study endorses this report. It describes the diversity of developments in the field. Some examples follow.

- The technology that allows editing the DNA of human cells is advancing rapidly. The use of RNA-guided nucleases allows researchers to modify efficiently the genomes of cultured human cells with high precision. In the near future it will become possible to precisely repair the mutations in the DNA of the patient's cells, without introducing alterations at undesired places.
- New integrating viral vectors harbour a designed sequence variation in a small section of the backbone. This sequence variation provides each vector copy with a unique 'barcode' sequence. This barcode, in combination with 'deep sequencing' technology, allows precise monitoring of the patients for cell clones that expand in cell number. This can be a early signal of the transformation of a modified cell to a cancer cell. The use of barcoding therefore increases the patient safety of the gene therapy procedure.
- The field of cancer gene therapy witnesses a rapid progression. A wide diversity of new viruses is evaluated as viral oncolytic agents. Application of these oncolytic agents has yielded promising results.

These and many other developments may pose new regulatory challenges. By

signalling these early on, the COGEM facilitates the discussion on how the advancing technology can be used. On the one hand this should ensure that the research can fulfil its promises, but on the other hand we should ensure the safety of its use. In this way we hope to contribute to maintaining the public's trust in gene therapy as viable treatment modality for serious diseases.

Prof R.C. Hoeben
Chair of the advisory committee.

Advisory committee:

Prof. Dr. R.C. Hoeben (Chair)	Leiden University Medical Center
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Dr. F.H.E. Schagen	COGEM secretariat

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1 List of Abbreviations

2D	two-dimensional
3D	three-dimensional
5-FC	5-fluorocytosine
5-FU	5-fluorouracil
AAV	Adeno-associated dependoparvovirus
AcMNPV	Autographa californica multiple nucleopolyhedrovirus
aCoV	Alphacoronavirus
ADA-SCID	adenosine deaminase deficiency SCID
ADME	administration, distribution, metabolism and elimination
AdV	Adenovirus
AFP	alpha-fetoprotein
AIDS	acquired immune deficiency syndrome
ALVAC	recombinant canary poxvirus
AON	antisense oligonucleotide
ASGCT	American Society of Gene & Cell Therapy
ASLV	Avian sarcoma leukosis virus
ATMP	advanced therapy medicinal products
ATP	adenosine triphosphate
BAdV	Bovine AdV
BCL-2	B-cell lymphoma 2
BDV	Borna disease virus
BEV	Bovine enterovirus
BHV	Bovine herpes virus
BLT	bone marrow-liver-thymus
BMD	Becker muscular dystrophy
BMP	bone morphogenetic protein
BSGCT	British Society for Gene and Cell Therapy
BTV	Bluetongue virus
CAdV	Canine AdV
CAR	Coxsackie and Adenovirus receptor
Cas	CRISPR-associated
CD	cytosine deaminase
CDx	cluster of differentiation x
cDNA	circular DNA
CDV	Canine distemper virus
CEA	carcinoembryonic antigen
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
ChAdV	Chimpanzee AdV
CHMP	Committee of the Human Medicinal Products

CMV	Cytomegalovirus
CNS	central nervous system
CoDA	context-dependent assembly
COGEM	The Netherlands Commission on Genetic Modification
CPE	cytopathic effect
CPoV	Canarypox virus
cPPT-CTS	central polypurine tract/central termination sequence
crHAdV	conditionally replicating HAdV
crRNA	CRISPR RNA
CRISPR	clustered regularly interspaced palindromic repeats
CTL	cytotoxic T lymphocyte
CTLA	cytotoxic T-lymphocyte-associated protein
CVA/B	Coxsackievirus A/B
DAF	decay accelerating factor
DC	dendritic cell
DL	demyelinating leukoencephalomyelitis
DMAEMA	N,N-Dimethylaminoethyl Methacrylate
DMD	Duchenne muscular dystrophy
DNA	deoxyribonucleic acid
DSBs	double strand breaks
ds	double stranded
dsDNA	double stranded DNA
EBNA1/*oriP*	Epstein–Barr nuclear antigen 1/plasmid origin of viral replication
ECHO	Enteric Cytopathogenic Human Orphan
ECM	extracellular matrix
EEV	extracellular enveloped virus
EGFR	epidermal growth factor receptor
EHV	Equine herpes virus
EIAV	Equine infectious anemia virus
eIF4a	eukaryotic initiation factor-4A
EMA	European Medicines Agency
EMCV	Encephalomyocarditis virus
EpCAM	epithelial cell adhesion molecule
ES cell	embryonic stem cell
ETIF	EHV alpha-trans-inducing factor
EU	European Union
EV	Echovirus
F	fusion glycoprotein
FAdV	Fowl AdV
FarV	Farmington virus
FDA	Food and Drug Administration
FFoV	Feline foamy virus
FGF	fibroblast growth factor

FGFR	fibroblast growth factor receptor
FISH	fluorescent in situ hybridization
FLASH	fast ligation based automatable solid-phase high-throughput
FLP	flippase
Flt3L	FMS-like tyrosine kinase 3 ligand
FPaV	Feline panleukopenia virus
FPoV	Fowlpox virus
GADD34	growth arrest and DNA damage-inducible protein 34
GALV	Gibbon ape leukemia virus
GBM	glioblastoma multiforme
GCP	good clinical practice
GFP	green fluorescent protein
GGO	genetisch gemodificeerd organisme
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMO	genetically modified organism
GMP	good manufacturing practice
GLP	good laboratory practice
gRNA	guide RNA
gusA	β-glucuronidase gene
HA	hemagglutinin
HAC	human artificial chromosome
HAdV	Human (mast)adenovirus
HCC	hepatocellular carcinoma
HDAC	histone deacetylase
hdHAdV	helper-dependent HAdV
HDI	histone deacetylase inhibitors
HER2	human epidermal growth factor receptor 2
HGFR	hepatocyte growth factor receptor
HHV	Human herpes virus
HIF	hypoxia-inducible factor
hIFNβ	human IFN beta
HIV	Human immunodeficiency virus
HLA	human leukocyte antigen
HN	hemagglutinin neuraminidase glycoprotein
HP	high pathogenic
hPEDF	human pigment epithelium-derived factor
HPMA	N-(2-Hydroxypropyl)methacrylamide
HPRE	HBV posttranscriptional regulatory element
HR	homologous recombination
HRSV	Human respiratory syncytial virus
HSC	hematopoietic stem cell
HSP	heat shock protein
HSPG	heparan sulfate proteoglycan

HSV	Herpes simplex virus
hTERT	human telomerase reverse transcriptase
Hu-HSC	human hematopietic stem cell
HVS	Saimiriine herpesvirus
IAV	Influenza A virus
IBDV	Infectious bursal disease virus
ICAM	intercellular adhesion molecule
ICH	International Conference on Harmonisation
ICP	infected cell polypeptide
IFN	interferon
IFNAR	interferon receptor
IL	interleukin
IMPD	investigational medicinal product dossier
IMV	intracellular mature virus
iPSCs	induced pluripotent stem cells
IRES	internal ribosome entry site
IRs	inverted repeats
ISVP	infectious subviral particles
ITR	inverted terminal repeat
IV	intravenous
JAK/STAT	janus kinase and signal transducer and activator of transcription
KGD	(Lys-Gly-Asp)
kRAS	Kirsten rat sarcoma viral oncogene homolog
lacZ	β-galactosidase gene
LAT	latency associated transcript
LAM-PCR	linear amplification-mediated PCR
LM-PCR	ligation-mediated PCR
LP	low pathogenic
LPaV	LuIII parvovirus
LPL	lipoprotein lipase
LTR	long terminal repeat
MAGE	melanoma-associated antigen
MaV	Maraba virus
MC	minicircles
Mcl-1	induced myeloid leukemia cell differentiation protein 1
mCoV	Murine coronavirus
MEK	mitogen-activated protein kinase
MeV	Measles virus
MHC	major histocompatibility complex
miRNA	micro-RNA
MMP	matrix metalloproteinase
MMR	measles mumps rubella
MN	meganucleases

mORV	Mammalian orthoreovirus
mORV-T3D	mORV type 3 Dearing
mRNA	messenger RNA
MSC	mesenchymal stem cell
MTOC	microtubule organizing center
mTOR	mechanistic target of rapamycin
MuLV	Murine leukemia virus
MuV	Mumps virus
MuV-JL	Jeryl-Lynn vaccine strain of MuV
MVA	Modified vaccinia Ankara
MVM	Minute virus of mice
MyxV	Myxoma virus
NA	neuraminidase
NDV	Newcastle disease virus
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NHEJ	non-homologous end joining
NIS	sodium/iodide symporter
NK cell	natural killer cell
NPs	nanoparticles
NSCLC	non-small cell lung cancer
NS1	non-structural protein 1
NVGCT	Nederlandse Vereniging voor Gen- & Celtherapie
NYVAC	Derived Copenhagen vaccinia virus
OAdV	Ovine AdV
oHSV	oncolytic HSV
OPEN	oligomerized pool engineering
ORF	open reading frame
OrfV	Orf virus
OV	oncolytic virus
PAdV	Porcine AdV
PAMAM	poly(amidoamine)
PB	PiggyBac
PCR	polymerase chain reaction
PEG	polyethylene glycol
PEI	polyethylinimine
Ph	phage
PKR	protein kinase R
PLL	poly-L-lysine
PNP	purine nucleoside phosphorylase
PPMV	Pigeon paramyxovirus
PSC	pluripotent stem cell
PTB-1	polypyrimidine tract binding protein-1
PV	poliovirus

PVRL4	poliovirus receptor-related 4
rAAV	recombinant AAV
RCR	replication competent retrovirus
RCT	randomized clinical trial
rdHAdV	replication defective HAdV
rdHSV	replication defective HSV
RDR	replication defective retrovirus
REAL	restriction and ligation cloning
RGD	arginylglycylaspartic acid (Arg-Gly-Asp)
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
RPoV	Raccoonpox virus
rNDV	recombinant NDV
RouSV	Rous sarcoma virus
RPaV-H1	Rodent protoparvovirus 1
SAdV	Simian AdV
SAE	serious adverse event
SB	sleeping beauty
SBV	Sindbis virus
SCID	severe combined immune deficiency syndrome
sc-rAAV	self-complementary rAAV
SeV	Sendai virus
SFoV	Simian foamy virus
SFV	Semliki Forest virus
shRNA	small hairpin RNA
SIN	self-inactivating
siRNA	short interfering RNA
SIV	Simian immunodeficiency virus
SIV-agm	SIV African green monkey
SIV-mac	SIV macaque
SLAM	signaling lymphocytic activation molecule
SME	small and medium business enterprise
SNP	single-nucleotide polymorphism
ss	single stranded
ssRNA	single stranded RNA
SuHV	Suid herpesvirus
SVV	Seneca Valley virus
SV40	Simian virus 40
TAA	tumor associated antigen
TALEN	transcription activator-like effector nuclease
TAP	transporter associated with antigen processing
$TCID_{50}$	50% tissue culture infectious dose

TCR	T cell receptor
TGF	transforming growth factor
Tk	thymidine kinase
TMEV	Theiler's murine encephalomyelitis virus
TNF	tumor necrosis factor
TPoV	Tanapox virus
tracrRNA	trans-activating crRNA
TRDs	terminal repeat domains
U_L	unique long sequence
UPRT	uracil phosphoribosyltransferase
U_S	unique short sequence
USA	United States of America
USDA	USA department of agriculture
UTR	untranslated region
UV	ultraviolet
VEEV	Venezuelan equine encephalitis virus
VEGF	vascular endothelial growth factor
VGF	Vaccinia growth factor
VLA-2	very late antigen 2
VLP	virus like particle
VSV	Vesicular stomatitis virus
VV	Vaccinia virus
VZV	Varicella zoster virus
WAS	Wiskott–Aldrich syndrome
WNV-KUN	West Nile virus Kunjin
WPRE	Woodchuck hepatitis virus posttranscriptional regulatory element
X-ALD	X-linked adrenoleukodystrophy
X-CGD	X-linked chronic granulomatous disease
X-SCID	X-linked SCID
YLDV	Yaba-like disease virus
ZFN	zinc-finger nuclease

2 Management summary / management samenvatting

2.1 Management Summary

This report was commissioned by The Netherlands Commission on Genetic Modification (COGEM) and aims to reflect the current status of preclinical gene therapy and identify the trends within this area of research. The COGEM is an independent scientific advisory committee which gives statutory advice to the Dutch Ministry of Infrastructure and the Environment on the risks to human health and the environment from experiments under contained conditions (laboratories, greenhouse, production facilities) with Genetically Modified Organisms (GMOs), the release and marketing of GMOs, as well as informing the Dutch government of ethical and societal issues linked to genetic modification.

The goal of this report is to identify trends in preclinical gene therapy studies worldwide so that predictions can be made for future clinical gene therapy studies. Also, deviations from the previously identified trends are of importance since these could identify possible safety issues in certain research areas. To compare the current situation regarding preclinical gene therapy research with earlier trends, we often refer to previous COGEM reports discussing various elements of gene therapy, including CGM 2010-10 on replication competent non-human viruses in clinical gene therapy. This report features novel and promising topics concerning general research techniques (paragraph 5.1), cell-based delivery methods (paragraph 5.2), non-viral vectors (paragraph 5.3), viral vectors (paragraph 5.4), *in vitro* models (paragraph 5.5) and animal models (paragraph 5.6). For each topic the current status of research is described as well as the main areas of concern. The main points of interest per topic are summarized below.

General research techniques

There is no doubt that genome engineering is going to contribute enormously to gene therapy research in the near future. Due to the discovery of CRISPR/Cas9 it will become feasible to therapeutically exploit gene editing techniques. In connection to this, immunotherapy with genetically engineered T cells shows great promise for several disease areas. Another promising technique is RNA interference (RNAi) which will mainly be of importance for rare genetic diseases which are currently not treatable by any known drugs. In order to be able to detect insertional mutagenesis in an early stage, vector barcoding could be a promising technique. Although it has been shown that barcoding is feasible and can contribute to patient safety, it remains to be seen if and how barcoding would be seen by the regulatory authorities since barcoding could conflict with the product identity criteria.

Cell-based delivery systems

Cell-based delivery systems are becoming more popular over the years. Next to the already longer used mesenchymal stem cell (MSC) also induced Pluripotent Stem Cells (iPSC) were introduced to the gene therapy field. Although there is a lot of interest for these delivery systems there are specific safety issues concerning cellular delivery methods. Due to the still largely uncharacterized biological mechanisms involved, these type of treatments still have quite some way to go before they can actually be used in the clinic on a regular basis.

Non-viral vectors

Non-viral vectors are still of great interest to many research areas within the gene therapy field. Currently existing non-viral vectors are being improved concerning their transfection efficiency and new vectors are being introduced. These novel non-viral vectors with the greatest potential to reach the clinical testing phase include exosomes, nanoparticles like liposomes and polymers, episomal vectors and transposons.

Viral vectors

Viral vectors for gene therapy are still considered to be the most effective way to achieve high expression of therapeutic transgenes. Most research has focused on the evasion of the immune response of non-integrating vectors like adenovirus and adeno-associated virus (AAV), as well as lowering genotoxicity of integrating vectors by developing self-inactivating (SIN) strategies.

The field of oncolytic viruses has seen a tremendous progression of several platforms, leading to a possible Food and Drug Administration (FDA) approval in the near future. Current strategies focus on the use of more virulent (as compared to earlier used vectors) conditionally replicating viruses, armed with immune stimulating or tracking transgenes. Also, immune evasion is still sought after, as well as screening virus populations for possible new vectors. Accompanying this report the authors have provided an addendum with a detailed description of all known oncolytic viruses. For readability, only the most relevant and important passages from this addendum have been summarized in the main report. The addendum can be downloaded from the COGEM website (www.COGEM.net).

In vitro models

Interest for alternatives to animal models has been increasing over the past few years. Since the standard two-dimensional culture systems are not optimal with regard to cellular interactions with for example tumor stroma, three-dimensional cultures are increasingly being used in preclinical research. These 3D culture models more closely resemble physiological interactions and can therefore serve as an important link between animal models, standard cell culture models and the clinic. Although regulators are showing interest in replacing animal models with cell cultures the question remains if these 3D cell culture systems actually will be allowed to serve as a replacement system for safety risk assessments in the context of market approval.

Animal models

Animal models will still remain important for future preclinical studies. However researchers could increase the value of research results when they would use multiple animal models for their studies. Small rodent models are still the models of choice while large animal models could provide much needed information about physiological and pathophysiological responses. In addition the animal itself as a target for gene therapy is a point of interest. Although already many domestic animals have been treated using a gene therapy approach there is no legislation at the moment for tissues and cells which are not covered by the GMO regulation in the veterinary sector. Examples are cells and tissue products obtained from bone marrow and which are subsequently cultured using growth factors.

Since the last report about clinical gene therapy by Dr. L.C. M Kaptein the main factors to be considered relevant for risk assessment did not change dramatically [1]. The factors discussed in this current report include:

1. optimization of vector targeting
2. increase transduction efficiency
3. reduce immunogenicity
4. prevent insertional mutagenesis
5. develop animal models more relevant to human disease

To achieve the above mentioned aims several developments have taken place during the last five years. These developments will be summarized below.

Optimization of vector targeting

A multitude of options now exists to achieve specific targeting of vectors. However, when considering integrating gene therapy vectors, mostly local or *ex-vivo* administration is still applied since this provides the best results with limited off-target toxicity. When considering oncolytic viruses, targeting is sometimes applied and needed for specificity, although this concerns more the transcriptional or inherent targeting of cancer cells, than specific targeting through receptor binding. Since mostly no specific cancer(-type) receptors exist, this strategy seems like a logical choice as it will target as many tumor cells as possible.

Increase transduction efficiency

Integrating viruses have undergone extensive evolution since the first clinical trials, which were quite successful in terms of efficacy, but were limited by genotoxicity. Strategies to increase transgene expression from recent SIN integrating vectors include codon optimization of transgene(s), incorporation of WPRE/HPRE or heterologous polyA enhancer elements, and inclusion of the cPPT-CTS sequence.

Reduce immunogenicity

A major goal for some viral vectors and oncolytic viruses is to shield the virus from immune recognition or complement neutralization. To achieve this, a multitude of strategies have been developed, including pseudotyping of viruses, changing hexons of adenoviruses, coating of virions with polymers and cell-carrier based delivery. It is to be expected that these strategies will be used in future clinical trials.

Prevent insertional mutagenesis

After the occurrence of oncogenic transformation in patients enrolled in early clinical trials using gammaretrovirus based vectors, a general rethinking of strategy has resulted in second and third generation SIN retroviral vectors. These vectors show a similar integration pattern, but do not upregulate gene expression of neighboring genes as much as the original vectors' enhancers did. In addition, more focus has been given to agents like HIV-1 based SIN vectors instead of gammaretroviral vectors, because these have a more favorable integration pattern. Other retroviruses like alpharetroviruses and foamy virus have an even more favorable integration pattern, and it seems logical that these vectors will receive more attention in the near

future. Use of episomal vectors will circumvent the problem of insertional mutagenesis and therefore these vectors are currently under investigation, mostly for hematological conditions.

Develop animal models more relevant to human disease

Concerning small animal models, researchers are trying to develop robust models which are immune competent so that the immunogenicity of certain therapies can be tested in a correct setting. For the oncolytic field it would be of great importance to develop a mouse model in which replicating human adenoviruses can be adequately tested. Currently this is only possible in immune compromised xenografted mice which are not an ideal model. In addition many oncolytic viruses are quite species specific, and although a degree of semi-permissiveness has been proposed for certain exotic laboratory models such as adenovirus in Syrian hamsters, immunological consequences typically differ between different animals.

More and more large animal models are being developed for genetic diseases. These large animal models will be more informative concerning physiological and pathophysiological responses. In addition they are an excellent step to test whether up scaling of the gene therapy product is feasible in a Good Manufacturing Practice (GMP) compliant setting.

Based on the information given in this report we show that several developments from the past are now further developed to obtain optimal vector properties. The oncolytic virotherapy field has developed tremendously over the past five years, which in the near future could lead to a FDA approved application. Based on reports of several researchers in the field it would be desirable to adjust current regulations for cell and gene therapy in such a way that they do not delay clinical applications of promising therapies. This is also true for regulation concerning veterinary use which in its current form may limit the availability of novel therapies. The current veterinary regulation covers medicinal products consisting of or containing GMOs, however this regulation does not cover other types of gene and cell therapy yet.

2.2 Management samenvatting

Dit rapport is geschreven in opdracht van de Commissie Genetische Modificatie (COGEM) en heeft als doel om de huidige status van preklinisch gen therapie onderzoek weer te geven om zodoende de trends binnen dit onderzoeksgebied te kunnen identificeren. De COGEM adviseert de regering over mogelijke risico's van productie en handelingen met genetisch gemodificeerde organismen (ggo's) voor mens en milieu. Ook informeert de COGEM betrokken ministers of staatssecretarissen over ethisch-maatschappelijke aspecten verbonden aan genetische modificatie.

Het uiteindelijke doel van dit rapport is het identificeren van trends binnen het preklinische gentherapie onderzoek wereldwijd zodat er voorspellingen kunnen worden gemaakt betreffende de aankomende klinische gentherapie studies. In dit kader zijn ook de trendbreuken van belang aangezien deze mogelijk belangrijke veiligheidsaspecten kunnen identificeren. Om deze vergelijking te maken zetten we in dit rapport de huidige status van het gentherapie onderzoek af tegen eerder gesignaleerde trends. We hebben hier onder andere gebruik gemaakt van CGM-2010-10, een COGEM rapport uit 2010 getiteld “Niet-humane virussen in klinische gentherapie”. In het huidige rapport worden nieuwe en veelbelovende onderwerpen besproken en onderverdeeld in algemene onderzoekstechnieken (paragraaf 5.1), cel gebaseerde methoden (paragraaf 5.2), niet virale vectoren (paragraaf 5.3), virale vectoren (paragraaf 5.4), celkweek

modellen (paragraaf 5.5) en diermodellen (paragraaf 5.6). Voor ieder onderwerp wordt de huidige status beschreven van het onderzoeksveld alsmede de aandachtspunten. De algemene interesse punten worden hieronder samengevat.

Algemene onderzoekstechnieken

Zonder enige twijfel zal “genome engineering” een enorme bijdrage gaan leveren aan het toekomstige gentherapie onderzoek. Sinds de ontdekking van CRISPR/Cas9 zijn er al vele verschillende celtypen en muizen genetisch gemodificeerd zodat zij een menselijke ziekte kunnen nabootsen. Aansluitend op deze ontwikkeling zal immunotherapie met genetisch gemodificeerde T cellen voor vele ziektebeelden van toepassing kunnen worden. Een andere veelbelovende techniek is RNA interferentie (RNAi) wat vooral van belang zal zijn voor zeldzame genetische ziekten welke niet met de beschikbare huidige medicatie behandeld kunnen worden. Vector barcoding is een veelbelovende techniek welke insertie mutagenese op een vroeg tijdstip zou kunnen detecteren. Ondanks dat het is gebleken dat deze techniek toepasbaar is in de gentherapie protocollen is het nog niet toegestaan door de FDA noch de EMA omdat het mogelijk de regels betreffende de product identiteit schaadt.

Cellulaire systemen

Cellen gebruiken om transgenen in te brengen is in de afgelopen jaren steeds populairder geworden. Naast de al wat langer gebruikte mesenchymale stamcellen (MSC) worden er nu ook induceerbare pluripotente stamcellen (iPSC) gebruikt voor gentherapie. Ondanks dat er veel interesse is in dit soort systemen hebben zij hun specifieke beperkingen ten aanzien van veiligheid. Omdat de onderliggende moleculaire mechanismen nog grotendeels onduidelijk of niet gekarakteriseerd zijn zal het nog enige tijd duren voordat deze methoden daadwerkelijk op een reguliere basis in de kliniek te gebruiken zullen zijn.

Niet virale vectoren

Niet-virale vectoren zijn nog steeds in trek bij veel onderzoeksgebieden binnen de gentherapie. Reeds bestaande niet-virale vectoren worden geoptimaliseerd op het vlak van transfectie efficiëntie en nieuwe vectoren worden onderzocht. De nieuwe niet-virale vectoren met de grootste potentie om de klinische testfase te halen zijn exosomen, nanoparticles (zoals liposomen en polymeren), episomale vectoren en transposons.

Virale vectoren

Virale vectoren voor gentherapie worden nog steeds gezien als de meest effectieve manier om een hoge expressie te krijgen van transgenen. Het meeste onderzoek heeft zich gericht op de evasie van het immuun systeem door niet-integrerende vectoren zoals adenovirus en adeno-geassocieerd virus (AAV) en het verminderen van de genotoxiciteit door middel van het ontwikkelen van zelf inactiverende integrerende (SIN) vectoren.

Binnen het veld van de oncolytische virussen is er een enorme vooruitgang geboekt voor een groot aantal virussen. Deze vooruitgang zal binnenkort mogelijk leiden tot een FDA goedkeuring van een oncolytisch virus voor therapeutisch gebruik. De meeste huidige strategieën richten zich op het gebruik van meer virulente conditioneel replicerende virussen welke kunnen worden geladen met immuun stimulerende of traceerbare transgenen. Ook in dit veld is evasie van het immuun systeem een veel onderzocht aspect, net zoals het screenen van virus populaties voor mogelijk nieuwe vectoren.

Celkweek modellen

De interesse voor celkweekmodellen is in de afgelopen jaren toegenomen, mede doordat beleidsmakers de betrokken onderzoekers erop wijzen om het aantal proefdieren te verminderen. Omdat de standaard tweedimensionale modellen niet optimaal zijn met betrekking tot cel-matrix interacties is er in de afgelopen jaren veel onderzoek gedaan naar driedimensionale modellen. Deze driedimensionale modellen laten beter vergelijkbare fysiologische interacties zien en kunnen daarom dienen als een link tussen dier modellen, standaard celkweek methoden en de kliniek. De vraag blijft echter of deze modellen door beleidsmakers toegestaan zullen worden als vervanger voor diermodellen tijdens de veiligheids inschattingen die nodig zijn voor het verkrijgen van een verkoopvergunning.

Diermodellen

Diermodellen zullen ook voor toekomstige preklinische studies van belang blijven. Onderzoekers zouden de interpreteerbaarheid van hun resultaten kunnen vergroten door gebruik te maken van meerdere verschillende diermodellen. Kleine knaagdieren zijn nog steeds de meest gebruikte modellen terwijl grote diermodellen belangrijke informatie zouden kunnen opleveren betreffende fysiologische en pathofysiologische reacties. We zien ook dat het dier als patiënt momenteel in de belangstelling staat. Ondanks dat al veel huisdieren zijn behandeld door middel van cel en gentherapie is hier nog steeds een gebrek aan regelgeving voor in de veterinaire sector. Het gaat hierbij om therapieën die niet vallen binnen de klassieke GGO kaders zoals stamcellen behandeld met groeifactoren.

Sinds het laatste rapport over klinische gentherapie van Dr. L.C.M. Kaptein zijn de factoren welke relevant zijn voor een risico inschatting nauwelijks veranderd [1]. De doelen welke in het huidige rapport behandeld worden zijn:

1. optimalisatie van vector targeting
2. verbeteren van de transductie efficiëntie
3. verminderen van immunogeniciteit
4. voorkomen van insertie mutagenese
5. ontwikkelen van relevante diermodellen

Om deze doelen te behalen hebben de afgelopen jaren verschillende ontwikkelingen plaatsgevonden welke hieronder worden samengevat.

Optimalisatie van vector targeting

Er bestaan tegenwoordig vele verschillende manieren om vectoren specifiek te “targetten”. Echter wanneer het over integrerende gentherapie vectoren gaat, is het nog steeds gebruikelijk om deze lokaal dan wel via *ex-vivo* toediening te gebruiken omdat dit nog steeds de beste resultaten geeft met de laagste off-target toxiciteit. Als we kijken naar oncolytische virussen dan omvat het targetten vooral het transcriptionele of inherent targetten van kanker cellen en niet zozeer het specifiek targetten door middel van receptor binding. Dit lijkt een logische keuze aangezien er nog geen kankercel specifieke receptoren zijn ontdekt en er op deze manier zoveel mogelijk tumorcellen geraakt kunnen worden.

Verbeteren van de transductie efficiëntie

Integrerende virussen zijn enorm geëvolueerd sinds de eerste klinische onderzoeken welke qua effectiviteit voorspoedig verliepen maar minder goed betreffende de genomische toxiciteit. Strategieën om de transductie efficiëntie van de meest recente generatie zelf inactiverende integrerende vectoren te optimaliseren omvatten codon optimalisatie van het transgen, inbouwen van WPRE/HPRE en/of heterology polyA en enhancer elementen en toevoegen van cPPT-CTS sequenties.

Verminderen van immunogeniciteit

Voor verschillende virale vectoren en oncolytische virussen is het afschermen van het virus voor het immuunsysteem en complement neutralisatie erg belangrijk. Om dit te bewerkstelligen zijn er verscheidene strategieën ontwikkeld waaronder pseudotyperen van virussen, het veranderen van adenovirus hexons, coaten van virions met polymeren en cel gebaseerde afgifte van virale vectoren. Het is te verwachten dat deze strategieën gebruikt zullen gaan worden in toekomstige klinische onderzoeken.

Voorkomen van insertie mutagenese

Nadat er in patiënten, welke geïncludeerd waren in vroege klinische onderzoeken met gammaretrovirus gebaseerde vectoren, oncogene transformatie had plaatsgevonden werd de algemene strategie drastisch veranderd met als gevolg het ontwikkelen van tweede en derde generatie zelf inactiverende retrovirale vectoren. Deze vectoren hebben een zelfde integratie patroon maar beïnvloeden niet de gen expressie van naastliggende genen zoals de originele vectoren dat deden. Ook is er meer aandacht uitgegaan naar HIV-1 gebaseerde zelf inactiverende vectoren in plaats van gammaretrovirale vectoren omdat deze een beter integratie patroon vertonen. Andere retrovirussen, zoals alpharetrovirussen en foamy virussen, vertonen een nog beter integratiepatroon en het lijkt logisch dat deze vectoren meer aandacht zullen krijgen in de nabije toekomst. Het gebruik van episomale vectoren vermijdt het probleem met integratie en dit type vectoren zijn daarom momenteel ook volop in de aandacht.

Ontwikkelen van relevante diermodellen

Onderzoekers zijn momenteel bezig met het ontwikkelen en verfijnen van robuuste immuuncompetente diermodellen. Deze zijn nodig om de immunogeniciteit van bepaalde therapieën op een juiste wijze te kunnen testen. Voor het oncolytische veld is het van groot belang dat er een muismodel ontwikkeld wordt waarin replicerende humane adenovirussen zouden kunnen worden getest. Veel oncolytische virussen zijn specifiek voor een gastheer en ondanks dat er een mogelijk infectie zou kunnen plaatsvinden in niet ideale modellen zijn de immunologische consequenties vaak erg verschillend tussen diverse diersoorten.

Voor veel genetische ziekten worden tegenwoordig ook grote diermodellen ontwikkeld. Deze modellen zijn informatiever betreffende fysiologische en pathofysiologische processen. Ook kunnen deze modellen uitstekend gebruikt worden om te kijken of het opschalen van het te ontwikkelen gentherapie product haalbaar is in een GMP setting.

Gebaseerd op de informatie in dit rapport laten we zien dat er verscheidene ontwikkelingen zijn welke al vele jaren bestaan en die nog steeds verder ontwikkeld worden. Het oncolytische virotherapie veld heeft een enorme vooruitgang geboekt in de laatste vijf jaar en zal zeer waarschijnlijk in de nabije toekomst zijn eerste FDA registratie tegemoet kunnen zien. Afgaande op verschillende onderzoekers binnen het gentherapie veld zal het van groot belang zijn dat de regelgeving aangaande gentherapie-toepassingen aangepast wordt op een zodanige manier dat deze de ontwikkeling van toekomstige toepassingen niet onnodig belemmert. Dit geldt ook voor veterinair gebruik aangezien hier nog geen specifieke regelgeving bestaat ten aanzien van het gebruik van cel en gentherapie welke niet valt binnen de standaard GGO regelgeving in huisdieren.

3 General Introduction

3.1 General introduction into gene therapy

Gene therapy as defined by the European Medicines Agency (EMA) can be considered to be a biological medicinal product which consists of an active substance which in turn contains or consists of a recombinant nucleic acid used in or administered to a human being with the goal of regulating, repairing, replacing, adding or deleting a genetic sequence. In addition, its therapeutic effect must relate directly to the recombinant nucleic acid sequence it contains, or to the product of genetic expression of this sequence (adapted from [2]).

Generally, gene therapy can be categorized in two groups; germ line gene therapy, in which the genetic material is passed on to the next generation and somatic gene therapy in which only target cells are changed. Current European legislation only allows gene therapy on somatic cells. In 2012 the first gene therapy product was recommended for approval in the European Union. Glybera is an adeno-associated viral vector for the treatment of severe lipoprotein lipase deficiency.

Oncolytic viruses are a type of virus that infect and lyse cancer cells but not normal cells. Oncolytic viruses can occur naturally or can be made in the laboratory by changing viruses into oncolytic agents. They can also harbor transgenes, which can add to their oncolytic activity. As such, oncolytic viruses are regarded to be gene therapy vectors, and are discussed in detail in this report.

In 2013, cancer was by far the most common disease on which (pre)clinical gene therapy research was focused [3]. It composes over 60% of all ongoing clinical gene therapy trials worldwide and is followed by monogenetic (9%) and cardiovascular disease (8%) (Figure 1, left). The most frequently used gene transfer methods in 2013 were adenoviral (24%), retroviral (19%) and naked plasmids (18%) (Figure1, right).

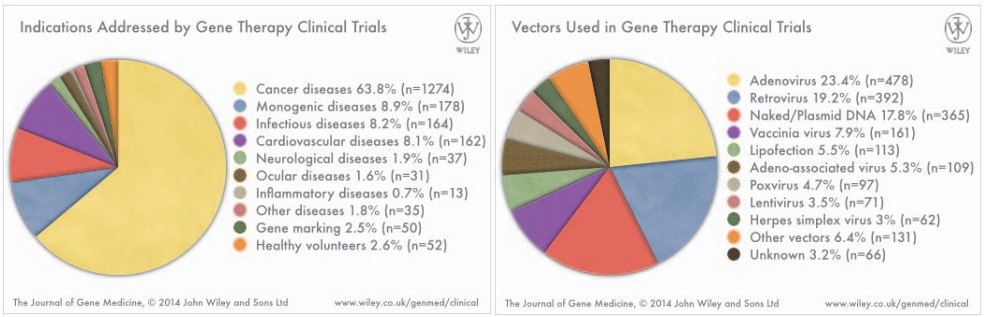

Figure 1: Distribution in gene therapy indications (left) and distribution in gene therapy vectors (right).

3.2 Cell and gene therapy regulations in the EU

Scientific progress has brought about new types of medicinal products based on gene therapy, somatic-cell therapy or tissue engineering. Every deliberate release of GMOs into the environment is subject to guideline 2001/18/EC. This is also the case for market applications concerning GMOs. In 2007, the advanced therapy medicinal products (ATMP) regulation was adopted by the European Parliament and of the Council [2]. This regulation is effective as of 30 December 2008. A transitional period was introduced for ATMPs that were already marketed before this regulation was adopted. The ATMP regulation was set up to provide a common

framework for the marketing of ATMPs as pharmaceutical products in the European Union (EU), which is supervised by the EMA. The ATMP regulation builds on the procedures, concepts, and requirements designed for chemical-based medicinal products. However, this may not be the most ideal starting point for gene therapy products. In contrast to chemical-based products, research using ATMPs is mostly conducted by academia, non-profit organizations, and small and medium business enterprises (SMEs), which only have limited financial resources and often lack exposure to the regulatory system that governs medicines. In addition, ATMPs represent a wide variety of products, all with different characteristics.

The goal of the ATMP regulation is to protect patients by providing a high standard of quality, efficacy and safety before a product is made available to them. However, the requirements could have unfavorable consequences for public health. The vast ATMP regulations could prevent the appearance of valid treatments for unmet medical needs. The ATMP regulations should contribute to market conditions which facilitate the appearance of new medical products, while ensuring a high level of safety. In addition, it is of great importance that the existing ATMP regulation can be rapidly adapted to stay in line with scientific progress.

EU member states are allowed to authorize the use of custom-made ATMPs as long as they are prepared for an individual patient, in a hospital, and under the strict responsibility of a medical practitioner. This so-called hospital exemption requires the application of national requirements on quality, traceability, and pharmacovigilance equivalents to those required for EMA authorized medicinal products. The hospital exemption enables patients to receive an ATMP under controlled conditions in cases where no EMA authorized medicinal product is available. Additionally, it facilitates research and development in advanced therapies by non-profit organizations (such as academia and hospitals) and it can be a valuable tool to obtain information prior to seeking EMA marketing authorization.

In December 2012 stakeholders in the gene therapy regulations were invited to provide their view on the ATMP regulation [4]. Although the common framework was generally seen as a positive step, the stakeholders face quite a number of obstacles to comply with the ATMP regulations. These include: the variability of the source material, small batch sizes, short half-lives, difficulty to set up randomized clinical trials (RCT) and lack of financial aids. The lack of a harmonized approach on aspects such as the classification of products or the application of the hospital exemption was generally perceived as a problem. The European Commission will now have to debate on how to act upon the defined problem areas.

3.3 Outline of the report

This report discusses the preclinical gene therapy studies roughly in four different categories:

- General research techniques
- Cell-based delivery systems
- Non-viral gene therapy vectors
- Viral gene therapy vectors (including oncolytic viruses)

Each category will describe several techniques or vectors which are currently of importance. General information will be given as well as a description of the preclinical status, clinical status and safety. The safety category will address patient safety, germ line transmission and the environmental issues (specifically transmission/shedding and mutagenesis/reversion).

In addition the report summarizes the *in* vitro as well as the animal models currently available within the gene therapy field and discusses the potential of domestic animals as target for gene therapy.

We would like to point out that this report is not a systematic review in such a way that it covers all techniques and vectors used in preclinical research. It focuses primarily on the products which are currently of importance and which have the potential to influence future clinical gene therapy studies, with a special attention for environmental and patient safety.

In Chapter 8 a summary of all conducted expert interviews can be found. In the separate addendum to this report general information, preclinical data, clinical data and safety details can be found concerning oncolytic viruses. This addendum can be downloaded from the COGEM website (www.COGEM.net).

4 Methods

4.1 Literature search

In cooperation with the Medical Library of the Erasmus Medical Center a literature search was performed. Four different databases were used to assemble the starting database: Embase, Medline on OvidSP, Web of Science and Google Scholar. To search within these four databases the following queries were used:

Embase.com
('animal experiment'/exp OR 'animal model'/exp OR ((animal* OR mouse OR mice OR rat OR rats) NEAR/3 (model* OR experiment*)):ab,ti) AND ('gene therapy'/exp OR 'gene therapy agent'/exp OR 'gene transfer'/exp OR virotherapy/exp OR 'virotherapy agent'/exp OR (((gene* OR dna) NEAR/3 (therap* OR transfer OR target*)) OR virotherap*):ab,ti) AND ('treatment response'/de OR 'treatment outcome'/de OR (((treatment* OR therap*) NEAR/3 (response* OR outcome*)) OR patient* OR trial* OR (clinical NOT 'pre clinical')):ab,ti) AND diseases/exp AND [2009-2014]/py NOT ([Conference Abstract]/lim OR [Conference Paper]/lim OR [Conference Review]/lim) NOT ('clinical trial'/exp)

Medline (OvidSP)
("Animal Experimentation"/ OR exp "Models, Animal"/ OR ((animal* OR mouse OR mice OR rat OR rats) ADJ3 (model* OR experiment*)).ab,ti.) AND (exp "Genetic Therapy"/ OR exp "Gene Transfer Techniques"/ OR "Oncolytic Virotherapy"/ OR (((gene* OR dna) ADJ3 (therap* OR transfer OR target*)) OR virotherap*).ab,ti.) AND (exp "treatment outcome"/ OR (((treatment* OR therap*) ADJ3 (response* OR outcome*)) OR patient* OR trial* OR (clinical NOT "pre clinical")).ab,ti.) NOT (Congresses).pt. NOT ("clinical trial"/)

Web of science
TS=((((animal* OR mouse OR mice OR rat OR rats) NEAR/3 (model* OR experiment*))) AND ((((gene* OR dna) NEAR/3 (therap* OR transfer OR target*)) OR virotherap*)) AND ((((treatment* OR therap*) NEAR/3 (response* OR outcome*)) OR patient* OR trial* OR (clinical NOT "pre clinical"))) NOT (Conference* OR congres*))

Google Scholar
"animal|mouse|mice|rat|rats model|models|experiment" "gene|dna|genetic therapy|transfer|target"|virotherapy "treatment|therapy response|outcome"|patient|trial|clinical

In total 7492 unique hits were found after removal of 3516 duplicates. The starting database was categorized into several topics which were then again divided into subgroups corresponding to the paragraphs described in this report. To search for more specific information additional searches were performed in Pubmed using keywords relevant to the topic at hand. Key publications were also identified by looking at reference lists of publications. Also, editorial publications in journals publishing on gene therapy were evaluated to identify trends in the field. General web searches were also conducted, especially for the legislation issues around gene therapy.

4.2 Scientific meetings

To obtain information about current trends several scientific meetings were attended. During these meetings, state-of-the–art and most recent (pre)clinical research was presented, in several cases not yet published. The following scientific meetings were attended:

- NVGCT Spring Symposium: Lunteren, the Netherlands, 13-14th March 2014
- Annual conference of the BSGCT: London, UK, 28th March 2014
- 8th oncolytic virus conference: Oxford, UK, 10-13th April 2014
- Targeted gene editing using CRISPR/CAS9 and ZFN technologies seminar, Erasmus MC Rotterdam, the Netherlands, 14th May 2014
- 17th annual meeting of the ASGCT: Washington DC, USA, 19-24th May 2014

4.3 Interviewing experts

Several experts in the field of gene therapy were invited for an interview to share their vision on the current trends and the future of gene therapy research. We aimed to interview researchers with interest in different fields within the gene therapy research community. The people who were able to participate are listed below:

- Dr. J. Hiscott, Vaccine & Gene Therapy Institute of Florida, *oncolytic viruses*. Dr. Hiscott is an internationally recognized molecular biologist and virologist, program director, principal investigator and full member of the Vaccine and Gene Therapy Institute of Florida (VGTI Florida®). Dr. Hiscott's research has provided major contributions to the understanding of the immune response to infectious diseases, cancer and human retrovirus pathogenesis. He is also investigating the use of oncolytic vaccine vectors as novel experimental cancer therapeutics.
- Dr. M. Brugman, LUMC, *vector barcoding*. Dr. Brugman is currently working as a postdoc at the Immunohematology and blood transfusion department of the LUMC in Leiden. His research focusses on the molecular signatures of HSC and MSC and he has expertise in the field of retroviral gene therapy.
- Prof. Dr. A. Vulto, Erasmus MC, *legislation of gene and cell therapy*. Prof. Vulto is professor of hospital pharmacy in the Erasmus MC. He is the qualified person for biotechnological medicines and in this respect he has expertise in the field of ATMP regulations.
- Dr. A. Aartsma-Rus, LUMC, *exon skipping*. Dr. Aartsma-Rus is an associate professor at the Department of Human Genetics of the LUMC in Leiden. She currently works as project leader of the Duchenne Muscular Dystrophy Genetic Therapy group and aims to optimize antisense-mediated exon skipping towards clinical application.

A summary of the interviews can be found in Chapter 8.

4.4 Intermediate meetings with COGEM committee

During the writing process the authors have had two intermediate meetings concerning the progress of the report. At these meetings several topics were discussed to obtain information about which topics would be described in the final report and which were not of interest to the committee. Based upon these meetings the outline of the report was compiled and the contents was adjusted to the wishes of the committee.

5 Results

5.1 General research techniques

5.1.1 Genome Engineering Technologies

Advanced genetic engineering techniques allow very specific genetic manipulations such as gene insertion, gene removal or gene targeting. Before 2009 the only genome engineering method available for most animal and plant species was random mutagenesis with screening. This method involved radiation, chemicals or transposons to generate low levels of random mutations, followed by screening at individual genotype or phenotype level for the desired mutation. However these methods are highly inefficient because most of the alterations occur off target, requiring treatment of very large populations to obtain enough targeted mutations in a complex genome. Following these random mutagenesis methods, homologous recombination was discovered in the 1980s. This method uses many copies of an exogenous donor DNA molecule with an insertion cassette flanked by long regions of homology to the desired target site. The cells homologous recombination (HR) DNA repair mechanisms could then introduce the insertion cassette at the target site in a precise and predictable manner [5]. Due to this discovery it was now routinely possible to make alterations from a single base-pair to large conditional deletions. Even today the homologous recombination technique is still used in laboratories all around the world for developing targeted knock-out or knock-in mice.

In the past decade, true targeting has been made possible by so called genome editing technology. This technology is based on the use of engineered nucleases composed of sequence-specific DNA-binding domains fused to a nonspecific DNA cleavage module. These chimeric nucleases enable efficient as well as precise genetic modifications by inducing DNA double strand breaks (DSBs) which activate both homologous recombination (HR) as well as error-prone non-homologous end joining (NHEJ) DNA repair mechanisms [6]. In 2011, Nature Methods declared targetable nucleases as the “Method of the Year” which shows how powerful this technology actually is [7]. Currently several novel methods of genome editing technologies are being used which will be discussed in more detail below.

5.1.1.1 Zinc-Finger Nucleases (ZFN)

A zinc-finger nuclease (ZFN) subunit consists of a non-specific endonuclease domain fused to a specific DNA-binding domain composed of engineered zinc-finger motifs which target the nuclease domain to a specific preselected chromosomal site [8, 9]. Each module recognizes three nucleotides [10]. When two ZFN subunits dimerize at the target site, the ZFN pair specifically cleaves the DNA thereby generating a DSB which can be repaired by endogenous DNA repair mechanisms. In most circumstances NHEJ is the predominant repair pathway for ZFN induced DSBs since HR requires a homologous DNA template, unless an exogenous donor sequence is provided which may lead to site-specific insertion of this sequence (see Figure 2).

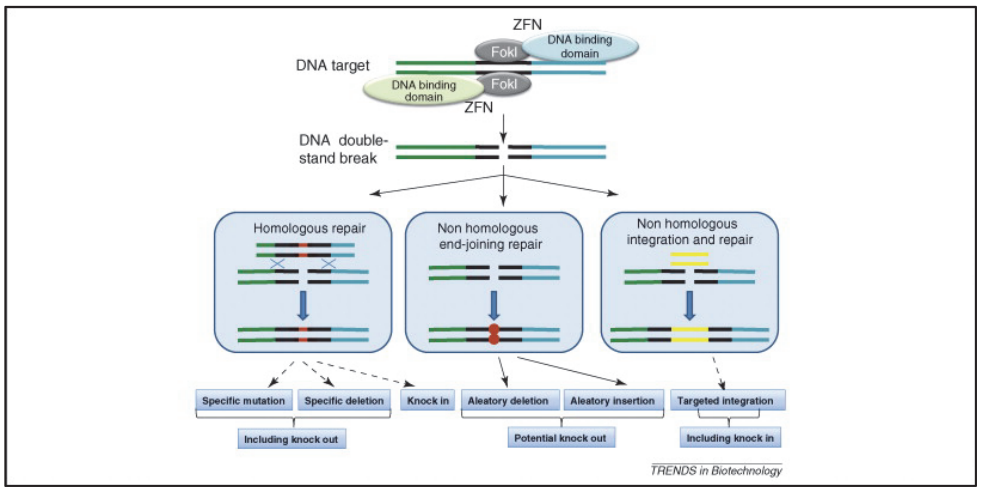

Figure 2. Potential genome manipulations using ZFNs. ZFN-mediated targeted genome modification relies on gap repair mechanisms. In ZFNs, the nonspecific cleavage domain of the *Fok*I endonuclease is combined with specific DNA-binding domains of zinc finger proteins, which leads to the generation of a double-strand break. This strategy offers a number of potential genome modifications as presented. Homologous repair (bottom left) is based on a double crossing-over event that occurs between the two regions of the genome that flank the double DNA strand break and the injected homologous regions. The inserted sequence (shown in red) can induce specific mutations and/or deletions in the targeted sequence that can result in gene invalidation or allelic mutations, or insert a gene open reading frame (knock-in). Non-homologous end-joining repair is based on the cellular gap repair mechanism that can induce aleatory deletions or insertions (red circles), some of which will induce gene inactivation (knockout). Finally, this mechanism can also be used to insert injected non-homologous sequences (yellow lines) during the repair process, which allows targeted integration, including knock-in events (bottom right). Dotted arrows refer to possible applications yet to be performed in transgenic mammals. Figure adapted from Le Provost *et al* [11].

Up till now most of the successes in genome engineering have been achieved by use of ZFNs. However, it is still very difficult to engineer active ZFNs. Publicly available methods for engineering zinc finger domains include: Context-dependent Assembly (CoDA), Oligomerized Pool Engineering (OPEN), and Modular Assembly. Approximately half of the OPEN/CoDa engineered ZFNs fail to cleave at the endogenous target site, optimized techniques such as extended modular assembly and an optimized two-finger archive show a success rate of about 80% [12, 13].

Although the ZFN technique can virtually target any sequence there are also a few restrictions to be mentioned. The most important restriction is that the position of cleavage site is determined by the DNA itself and not by the investigator which results in the fact that all currently available ZFN technologies lack sufficient resolution to target single-nucleotide polymorphisms (SNPs), enzyme active sites or precise boundaries of genetic elements.

5.1.1.2 Transcription activator-like effector nucleases (TALEN)

Transcription activator-like effector nucleases (TALENs) are naturally occurring proteins from the plant pathogenic bacteria genus *Xanthomonas*. They contain DNA-binding domains composed of a series of 30-35 amino-acid repeat domains that can each recognize a single base pair (see Figure 3). The value of these proteins for genome engineering was discovered in late 2009, when the TALE-DNA-binding code was discovered [14, 15]. TALENs are currently the only class of DNA-binding proteins which possess a useful DNA recognition code. The first TALEN was reported in 2010 [16].

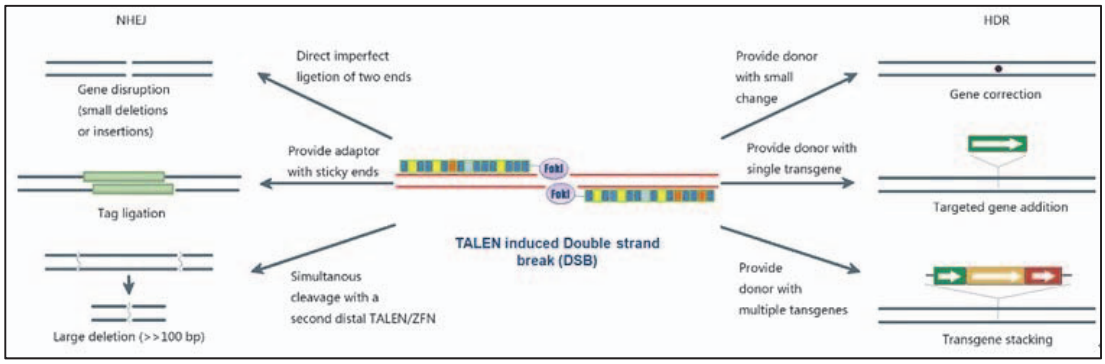

Figure 3. Potential applications of TALEN engineering technology. Like the zinc finger nucleases, TALENs work in pairs to efficiently create double strand DNA breaks (DSB) in the target genome site. The NHEJ repair process includes error-free repair and error-prone repair, the later will result in mutations, such as deletion and insertion, and will thus cause shifts in the reading frame. Since error-free repair will restore the TALEN cutting site and make it subject to cutting again, the selection process will favor cells containing mutations or knockout clones. Researchers can simultaneously provide donor DNA templates and by utilizing the HDR pathway they can create gene corrections, gene additions and transgene insertion. Figure adapted from Cell Inspire Bio (www.cib.cc).

Due to the simple TALE-DNA code TALENs can be easily designed and constructed in such a way that they can bind any unique sequence in the genome. However due to the high sequence similarity between each TALEN repeat it is a challenge to assemble several TALEN repeats in the same construct. Several methods have been developed to overcome this difficulty; restriction enzyme and ligation cloning (REAL) [17], type II restriction enzyme cloning (Golden Gate cloning) [18], fast-ligation based automatable solid-phase high-throughput method (FLASH) [19] and a ligation-independent high-throughput cloning method [20].

When comparing TALEN technology to the above mentioned ZFN technology it is apparent that TALEN is the superior technology. Where not all ZFNs show activity, almost all TALENs show at least some activity on their chromosomal target sites, and in general this activity is also greater. When directly comparing ZFNs with TALENs in a zebra fish model of mutagenesis it is clear that the mutation rate using TALENS is much higher (20-77%) compared to ZFNs (1-3%) [21]. When looking at cytotoxicity caused by off-target mutagenesis TALENs would be expected to show binding of fewer off-target sequences due to their broader recognition site. Although no extensive off-target analysis is done yet it is shown that the cytotoxicity level of TALENs is similar or reduced as compared to ZFNs [22]. As already mentioned, TALENs can be designed for almost any sequence which makes its application spectrum much broader than ZFNs. With TALEN technology it will be possible to target SNPs, enzyme active sites and precise

boundaries of genetic elements, something that is still not achieved with ZFN technology, even after 20 years of engineering.

5.1.1.3 Clustered regularly interspaced short palindromic repeats/CRISPR associated endonuclease (CRISPR/Cas9)

A recently discovered alternative to ZFNs and TALENs is the CRISPR/Cas9 system. This system is based on bacterial genomes encoding loci known as clustered regularly interspaced palindromic repeats (CRISPR), which consist of an array of short direct repeats interspaced with short intervening spacers which code for the CRISPR RNA (crRNA). While the repeats are identical, the spacers vary in sequence. The CRISPR locus is surrounded by a cohort of CRISPR-associated (Cas) genes. In the vicinity of these Cas genes so called trans-activatingcrRNA (tracrRNA) loci are located. tracrRNA are complementary to the CRISPR repeats and thus a tracrRNA:crRNA hybrid is formed. Cas9 binds these hybrids and the complex is guided to the target DNA which is complementary to the spacer sequence. Cas9 subsequently cleaves the DNA to create a DSB which can be repaired by NHEJ or HR as described for ZFN and TALEN (see Figure 4). For genome editing purposes, a fused version of crRNA and tracrRNA is often used as a single guide RNA (sgRNA) [23].

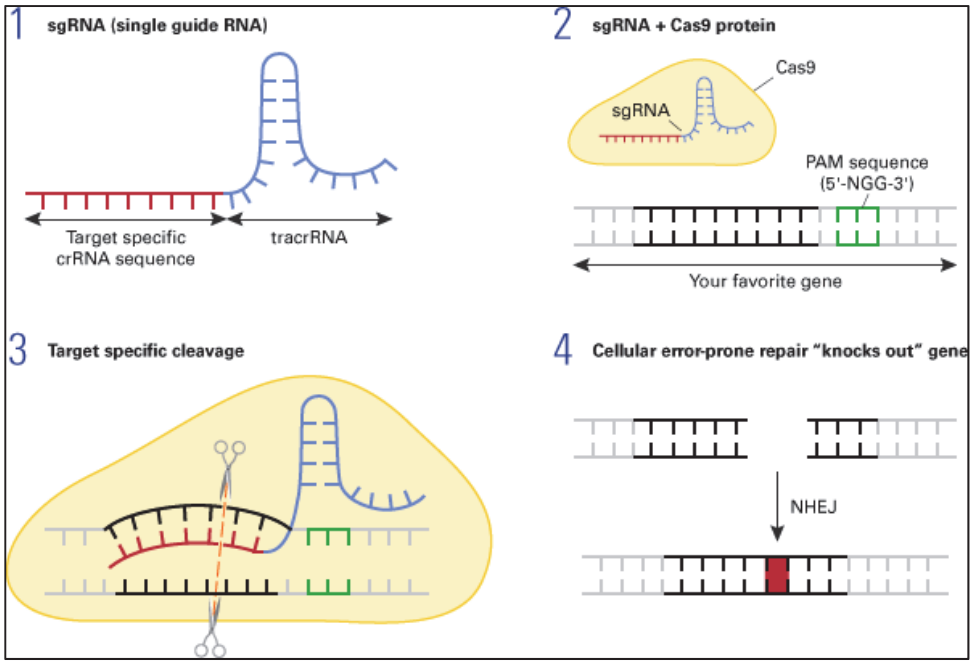

Figure 4. The principle of CRISPR/Cas9-mediated gene disruption. (1) A single guide RNA (sgRNA), consisting of a crRNA sequence that is specific to the DNA target, and a tracrRNA sequence that interacts with the Cas9 protein, binds to a recombinant form of Cas9 protein that has DNA endonuclease activity (2). The resulting complex will cause target-specific double-stranded DNA cleavage (3). The cleavage site will be repaired by the non-homologous end joining (NHEJ) DNA repair pathway, an error-prone process that may result in insertions/deletions (INDELs) that may disrupt gene function (4). Figure adapted from Clontech (www.clontech.com).

The CRISPR/Cas system can be retargeted to cleave virtually any DNA sequence by redesigning the crRNA. Instead of engineering new proteins for each cleavage site (e.g. for TALEN approx. 1800 bp), it is only necessary to synthesize a new DNA-complementing region of the sgRNA (approx. 20 new nt) to program the Cas9 nuclease. It already has been shown that the system is usable in a human setting by co-delivering plasmids expressing the Cas9 endonuclease and the necessary crRNA components [24-26]. It is also a potential easy multitasking technology since it has already been shown that multiple loci can be changed in a single step procedure [27, 28].

Cas-9 mediated genome editing has enabled accelerated generation of transgenic models and expands biological research beyond the traditional, genetically tractable animal model organisms [29]. By recapitulating genetic mutations found in patient populations, CRISPR-based editing could be used to rapidly model the causal roles of specific genetic variation instead of relying on disease models that only phenocopy a particular disorder. This approach has been used in generation of a novel cynomolgus monkey model [30], showing the potential for more accurate modeling of complex human diseases.

In the last year several novel applications of CRISPR-Cas9 have emerged. Cas9-mediated pooled sgRNA screens have shown to be more sensitive as well as more consistent than conventional RNAi screens. These screens can be designed in such a way that they can target nearly any DNA sequence [31]. In addition, epigenetic control, live imaging techniques and inducible regulation of Cas9 activity are being studied [32]. In Figure 5 a timeline with key studies within the CRISPR-Cas9 field are shown.

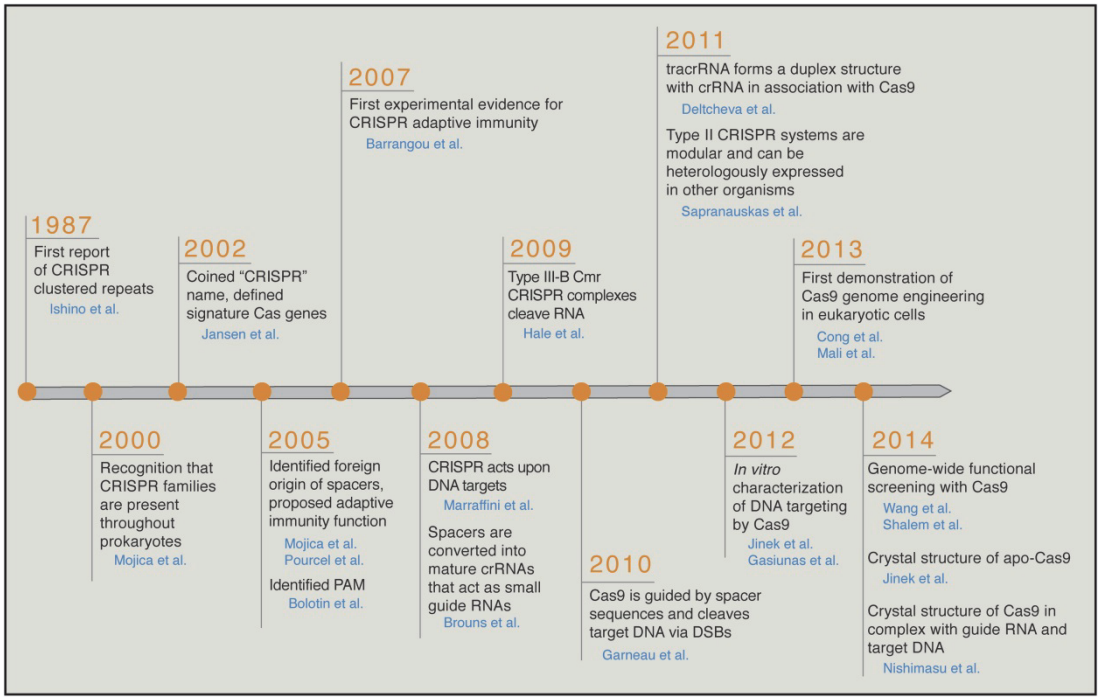

Figure 5. Timeline of the key events and publications within the CRISPR-Cas9 field. Figure adapted from Hsu *et al* [32].

5.1.1.4 Use of designer nucleases in gene therapy

Recent advances in the techniques described above have greatly broadened the ability to easily and effectively manipulate human genome sequences. In 2005 the first study was published which showed that ZFNs can be used in human cells to restore a mutation of X-linked SCID in the *IL2Rγ* gene [33, 34]. The strategies used to correct genes can be categorized in four main groups: disruption of endogenous genes, frame shift induction, insertion of a foreign sequence and substitution of a mutated sequence. Most of the strategies used up to now are still in the proof-of-concept stage. Currently there is only one phase1/2 clinical trial which uses a ZFN based gene knockout strategy to prevent human immunodeficiency virus (HIV) infection [35].

Although this might seem discouraging there are many applications possible for use of designer nucleases in gene therapy approaches. Currently, most experimental gene therapy for Duchenne muscular dystrophy (DMD) is based on exon skipping. This strategy involves restoring the dystrophin reading frame by skipping particular exons, which contain nonsense mutations and frame shift deletions. This is accomplished by administrating antisense RNA analogous or morpholino oligomers. Clinical trials have been initiated using this technique [36, 37], however the effect is transient because the antisense reagents are only stable for a few months *in vivo* [38, 39]. Engineered nucleases could be used to create a more permanent genetic change which would last far longer than manipulation of the post-transcriptional step [40]. Currently several researchers are showing that this technique is promising in proof-of-principle studies in mouse models of DMD by using meganucleases (MN), ZFNs or TALENs [41-44]. However applying this technique in humans will be challenging due to the low proliferative capacity of primary myeloblasts.

Another possibility for using engineered nucleases in gene therapy is the upcoming approach of using induced pluripotent stem cells (iPSCs) for *ex vivo* gene therapy. iPSCs can be isolated directly from the patient where after they can be manipulated to acquire an unlimited self-renewal capacity and the ability for multi-lineage differentiation, yet retaining their normal karyotype [45]. A variety of gene editing methods can be used to manipulate iPSCs among which are ZFNs [46], TALENs [47] and CRISPR [48]. iPSCs can be genetically manipulated in several ways, for example single amino acid substitution, removal of premature stop codons, footprint free gene correction, selection free gene correction, chromosomal modification or the reduction of immunity. For more details on iPSCs the reader is referred to paragraph 5.2.1.

5.1.2 Vector barcoding

Retroviral vectors have been widely used to deliver therapeutic genes in the context of gene therapy clinical applications for monogenic disorders, cancer, and infectious diseases providing stable and efficient expression of the transgene to patients [49]. Although clinical trials for primary immunodeficiencies have clearly demonstrated the therapeutic benefit of retroviral-based approaches [50, 51] the field of gene therapy was significantly impacted by the sudden occurrence of severe adverse events linked to insertional mutagenesis due to aberrant vector-on-host interactions [52, 53]. Thus, insertional profiling, aimed at identifying vector integration sites and studying their potential impact in preclinical and clinical samples, has become an important tool to evaluate the global safety profile of clinical trials [54, 55].

To date, ligation-mediated PCR (LM-PCR) or linear amplification-mediated PCR (LAM-PCR) are the most exploited methods to retrieve integration sites from transduced cells. Both the

technologies are based on the digestion of genomic DNA with restriction enzymes, the ligation of a linker cassette and the exponential amplification of vector-genomic junctions through primers annealing on the final long terminal repeat (LTR) portion and the linker cassette itself [56]. The final PCR products are then sequenced in order to collect and map the regions flanking the vector LTR, retrospectively identifying the integration sites on the genome of reference. The early protocols based on shotgun cloning into competent bacteria and Sanger sequencing have been replaced with more efficient and cost-effective methods such as the barcode tagging of LAM-PCR products from different cell sources followed by pyrosequencing of the pooled samples [57].

However, LAM-PCR has been associated with significant biases resulting in the selective amplification of some insertion sites and loss of others [58, 59]. To overcome these limitations, researchers constructed a retroviral plasmid library that consisted of vectors containing a variable random sequence tag or “barcode” [60]. On stable chromosomal integration, this barcode introduces a unique, identifiable, and heritable mark into the genome, allowing the clonal progeny of the host cell to be tracked over time. Given that several preconditions such as sufficient complexity of the barcode library are met, barcode marking should allow unbiased and precise analyses of quantitative contributions of marked cells to any population of interest (see Figure 6). Owing to the compact size of typical barcodes, the approach is amenable to readout using deep sequencing platforms.

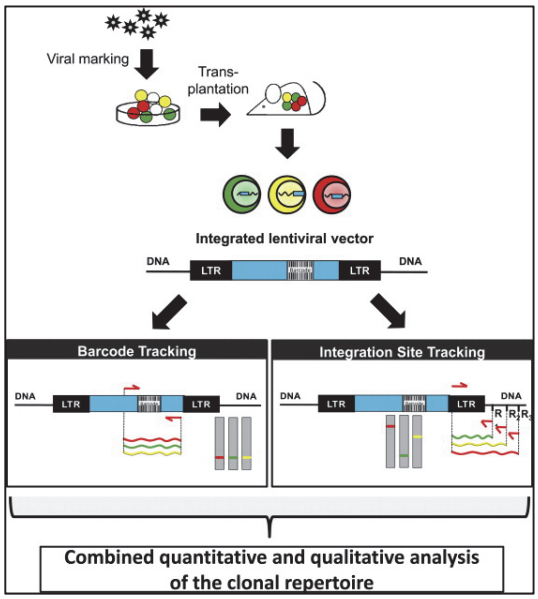

Figure 6. Clonal Tracking of Virally Marked Stem Cell Clones.
The progeny of individual stem cell clones that were marked by integrating viral vectors can be tracked after transplantation using two strategies. Barcodes introduced into the marking vector can be efficiently amplified genome-wide. This strategy allows an unbiased quantification of clonal contribution (left). On the other hand, the exact genomic location of vector integration enables deciphering the influence of vector integration on the stem cell clones' biology (right). Figure adapted from Glimm *et al* [61].

5.1.2.1 Use of barcoding in gene therapy

In some of the initial human clinical trials, gene-marking vectors were used in an attempt to investigate the source of relapse in patients undergoing high-dose chemotherapy and autologous stem cell rescue for a number of malignancies. These pioneering studies provided absolutely definitive evidence, perhaps not surprisingly, that at least one source of relapse following autologous transplantation can be the graft itself.

DNA barcoding can be used to improve the safety of viral gene therapy. In retroviral gene therapy, leukemia caused by inserting vectors is the largest problem. Barcoding can be useful for *ex vivo* gene therapy applications. By putting a short barcode sequence into the viral vector, clonality of expanding cells within a treated patient can be monitored with high specificity and sensitivity, allowing earlier discovery of leukemic clones.

5.1.3 Therapeutic fields of interest

5.1.3.1 (Antisense) Oligonucleotide-based therapy

Oligonucleotides are macromolecules which target DNA or RNA (including pre-mRNA and mRNA). These (pre)-mRNA molecules are the carriers of genetic information before it is translated into proteins. Because mRNAs encode all cellular proteins, oligonucleotides targeting mRNA could be effective for targets and diseases which are not treatable by current drugs. A good example are genetic diseases in which the defect in a gene can be best repaired by manipulating DNA or RNA rather than the protein they encode. Translation of RNA may be suppressed by a broad range of agents which include short interfering RNAs (siRNAs), small hairpin RNAs (shRNAs), ribozymes, DNAzymes, antisense oligonucleotides (AON) and decoys. Antisense oligonucleotides can be used for exon skipping to restore open reading frames, blocking gene expression, alternative splicing, cryptic splicing and exon inclusion.

The agents mentioned above all mediate suppression of RNA translation however they do not all have the same impact on the development of therapeutic solutions. siRNAs and shRNAs are generally bundled in the term RNA interference (RNAi). siRNA are double-stranded RNA fragments which interact with a multiprotein RNA-induced silencing complex (RISC). Within the RISC the siRNA is unwound, the sense strand is discarded and the antisense strand binds to the mRNA. When the siRNA is fully complementary to its target the mRNA is cleaved by an endonuclease (see Figure 7). shRNA is developed as a long-lasting alternative to the siRNA molecule. shRNAs are short double-stranded RNA fragment with overhanging nucleotides at the phosphorylated 5'- and hydroxylated 3' end. They incorporate directly into RISC, where its guide-strand binds to and cleaves the complementary mRNA with a perfect match. When the cleaved mRNA is released the guide-strand bound RISC binds to another mRNA and start a new round of cleavage.

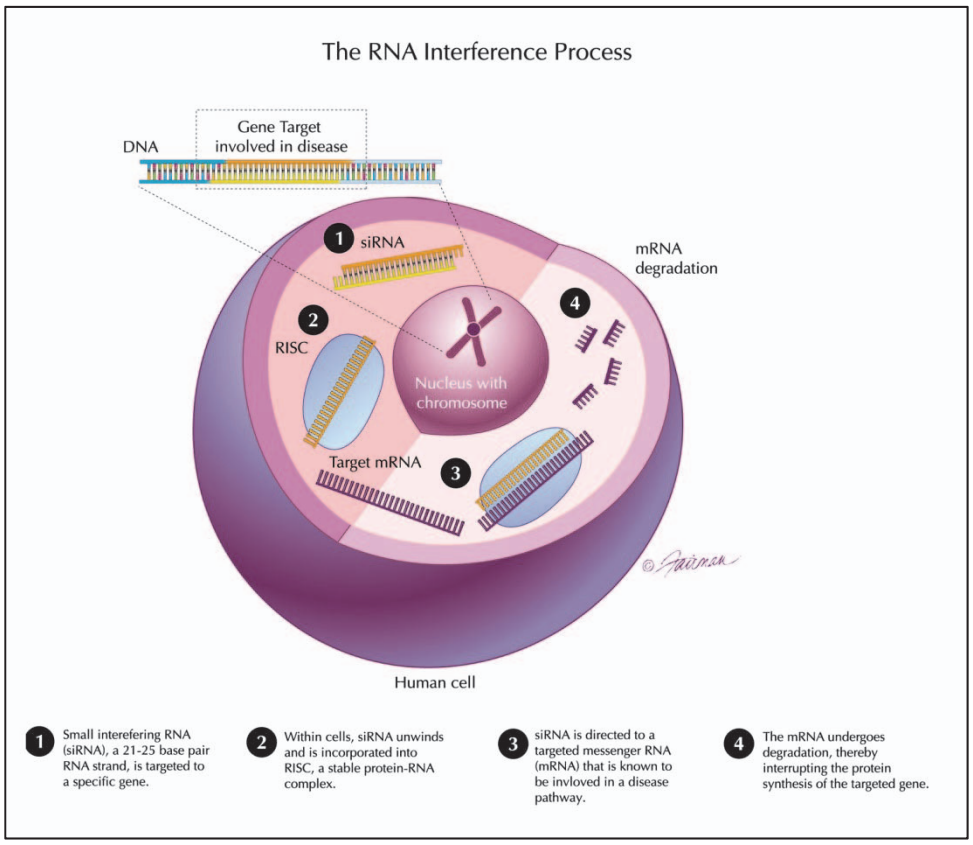

Figure 7: The working mechanism of RNAi.
Short interfering RNA (siRNA) is designed to correspond to the target gene (1). Once inside the cell the siRNA is incorporated into the RISC complex and is unwound (2). The antisense strand of the siRNA is directed to the target messenger RNA (mRNA (3)). If the mRNA is completely complementary, the mRNA will become degraded by an endonuclease thereby interrupting protein systhesis of the targeted gene (4). Figure adapted from www.alnylam.com.

DNAzymes and ribozymes are oligonucleotides which possess catalytic activity for either DNA or RNA, respectively [62]. Both types can down-regulate gene expression by binding and cleaving DNA or RNA, in addition some ribozymes can repair RNA by trans-splicing. Several types of ribozymes are observed in nature, however this is not the case for DNAzymes [63]. AONs are single-stranded fragments of DNA or RNA generally 15-25 bp long and designed to bind target mRNA via base pair complementarity, thereby inhibiting its translation into protein. Decoys are short double-stranded DNA molecules that contain binding elements which competitively inhibit promoter binding and gene expression.

Antisense oligonucleotide-mediated exon skipping has great potential for rare diseases such as Duchenne muscular dystrophy (DMD). A phase III clinical trial has recently been completed for DMD utilizing AON mediated exon skipping. Recently the Committee of the Human Medicinal Products (CHMP) of the EMA gave a positive advice to the European Commission to conditionally approve Ataluren which treats DMD patients with nonsense mutations who are >5 years old and still ambulant. Ataluren is an orphan designated medicine and is an achiral, orally bioavailable compound which has no structural similarity to aminoglycoside antibiotics or other clinically developed drugs. Ataluren enables the protein-making apparatus in cells to move past the defect, allowing the cells to produce a functional dystrophin protein. This conditional approval

is of great importance for the field since it would then be known where the bar is set for clinical benefit for ambulant DMD patients (30 meters improvement in one year compared to a placebo group).

Generally, therapeutic intervention using synthetic siRNA aims to achieve selective blockage of a protein expressing gene thereby ultimately silencing a gene involved in cell abnormalities. A large number of preclinical studies have presented favorable outcomes after *in vivo* siRNA delivery and further gene silencing of components critical for disease staging, modulating cell functions implicated in survival, invasion, angiogenesis, apoptosis, senescence and chemoresistence.

Between 2004 and 2014 three antisense drugs have been approved by the FDA for age-related macular degeneration (pegaptanib, an RNA aptamer), cytomegalovirus retinitis (fomivirsen, an antisense oligonucleotide) and familial hypercholesterolemia (mipomersen, an RNA aptamer).

5.1.3.2 Immunotherapy using genetically modified T cells

Recognition of and responses to diseased cells are mediated by the T cell receptor (TCR) upon engagement with antigens presented by major histocompatibility complex (MHC) molecules. Among the requirements for full activation of T cells are the need for TCR specificity against tumor antigens as well as the activation and expansion of large numbers of tumor antigen-specific T cells. Using genetic engineering, T cells can be produced which are reactive against specific tumors. This involves genetic engineering of the TCR itself or using chimeric antigen receptors (CARs) which are synthesized using molecular biology techniques (see Figure 8). Because CARs are derived from antibodies, the recognition of tumor associated antigens (TAA) is not MHC restricted. Irrespective of the approach used a functional TAA must be identified.

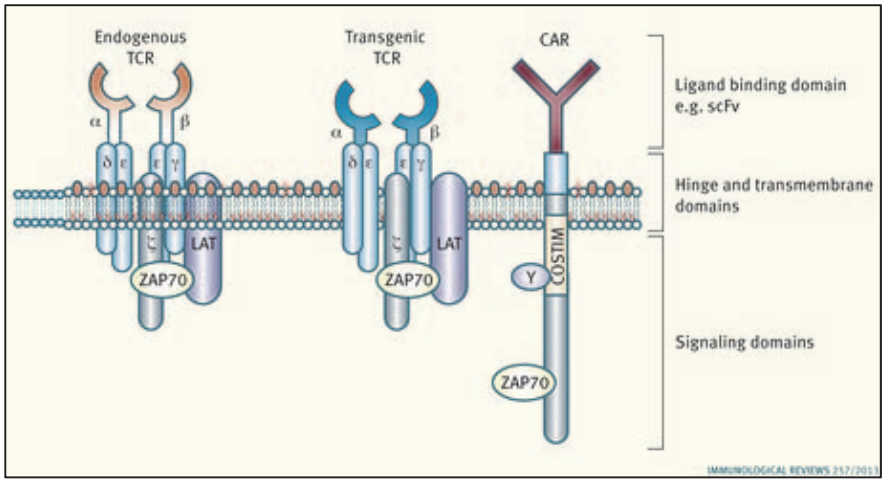

Figure 8. T cells can be engineered to have retargeted specificity for tumors.
Bispecific T cells are created by introduction of genes that encode T-cell receptors (TCRs) and chimeric antigen receptors (CARs) of desired specificity and affinities for tumors. CARs target surface antigens in an MHC-independent fashion. The T cells retain expression of the endogenous TCR, unless this is knocked down by various approaches. Figure adapted from Vonderheide *et al* [64].

An advantage of using TCR genes to enable specificity is that the TAA can be derived from the entire protein composition of tumor cells, including intracellular proteins. In addition there is potential for truly tumor-specific responses with the identification of specific mutant proteins which are restricted to tumor cells [65]. However each TCR gene can only be used in a subset of patients, due to the MHC-restricted nature of TCR function. To broaden the application of genetically engineered T cells, genes encoding CARs have been generated. It is quite simple to generate single-chain variable fragments from cells that produce monoclonal antibodies, and therefore a large number of CARs have been produced which target many different TAAs [66].

T cells can be isolated from the blood of patients and can then be subsequently cultured *in vitro* for a period of time. In culture they can be activated and expanded using several different cytokines such as interleukin-2 (IL-2), IL-7, IL-15 and IL-21. During this period the T cells can be genetically modified using vectors that contain nucleic acids encoding for a range of molecules which are important for T cell recognition of, and response to, cancer cells.

The most frequently used vectors are γ-retroviral and lentiviral vectors which have been rendered replication incompetent by removal of genes encoding for crucial viral proteins. Alternative vectors are DNA-plasmid based vectors which are composed of a transposon containing the gene of interest, and a transposase which mediates integration within the genome. Using either approach the genetic modification is stable and passed down through generations of cells [67].

Genetic engineering strategies up to now include:

- optimization of specificity
- enhancing activity
- enhance T cell survival
- enhance proliferation
- enhance trafficking
- mediate immune suppression in the tumor microenvironment
- improve safety

After the first reports with moderate anti-tumor responses in clinical trials with genetically modified T cells, the first encouraging results are now accumulating. A summary of published reports of genetically modified T cells in clinical trials can be found elsewhere [68]. The most recent success was reported in HIV/AIDS patients where the CCR5 gene of T cells was mutated using genetic engineering techniques [69]. Patients receiving an autologous T cell transplant with modified T cells showed a rapid and significant drop in viral load. This study showed that a HIV patient's own T cells can safely and effectively be engineered to mimic a naturally occurring resistance to the virus, infuse those engineered cells, have them persist in the body, and potentially keep viral loads at bay without the use of drugs.

5.2 Cell-based delivery systems

5.2.1 Induced Pluripotent Stem Cells (iPSCs)

Stem cells are able to differentiate into lineage-specific cell types while they also self-renew extensively to generate more stem cells. Due to their differentiation capacity they are regarded as a novel cell replacement therapy to regenerate damaged tissues and/or organs which are damaged by injury or disease. Transplantation of stem cells is considered one of the most promising remedies for many incurable diseases. Bone marrow transplantation for treatment of leukemia is the best-known application of this strategy [70]. Unfortunately, immune compatible cells are hardly obtainable for patients because of the specificity and complexity of the human immune system. In this regard, human iPSCs are believed to offer an unprecedented solution for obtaining sufficient healthy autologous cells.

iPSC reprogramming is a technology used to convert differentiated somatic cells back to embryonic-stem-like cells via the ectopic expression of four transcription factors (see Figure 9). This expression can be achieved by integrating viral vectors (lentiviral or gamma retroviral), non-integrating adenoviral vectors, transposons, minicircles, episomal plasmids, RNA vectors, conventional plasmids, protein transduction, mRNA transfection or use of IRES sequences.

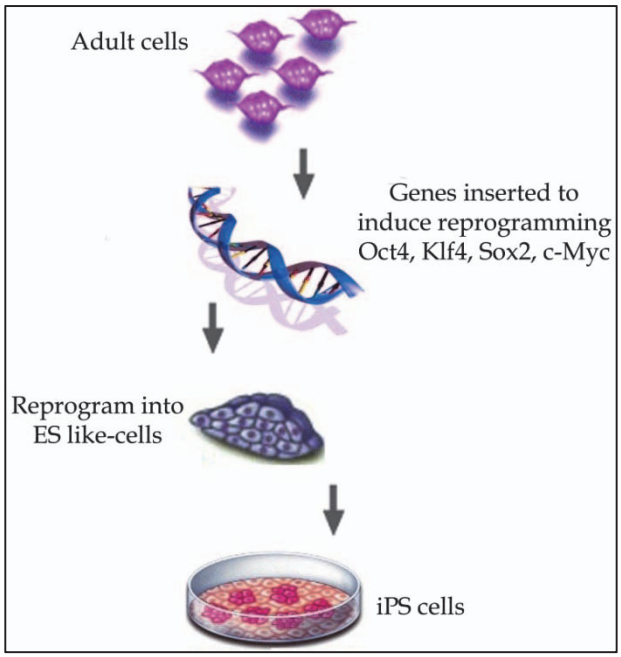

Figure 9: mechanism of iPSC generation and reprogramming.
Generation of iPS cells. Reprogramming of adult stem cells in iPS cells mediated by Oct-4, Klf4, Sox2 and c-Myc give rise to ES like cells with embryonic potential. Figure adapted from Meregalli *et al [71]*

The possibility of reprogramming somatic cells was first demonstrated in murine [45] and human [72] cells. After that a wide variety of other cell types was used to show the conserved nature of the mechanisms which induce pluripotency among mammalian species. Among these cell types are non-human primate [73], rat [74], rabbit [75], dog [76] and several domestic hoofed animals (reviewed in [77]).

The field is still in a relatively early stage regarding clear understanding of underlying developmental processes, cellular behavior and biological effects after cell engrafting experiments. Therefore the use of iPSCs in a clinical setting poses many new challenges from both the experimental and regulatory view. A better understanding of the nature of similarities and differences between human and animal stem cells and simulation of the behavioral, cellular and molecular characteristics seen in human diseases using animal models should lead to interpretable testing of efficacy and prediction of major complications and off-target effects of iPSC-based therapies. Currently iPSCs are used in several animal disease models recapitulating liver diseases [78], eye diseases [79], diabetes [80], heart disease [81] and neurological diseases [82].

5.2.2 Mesenchymal stem cells (MSCs)

Mesenchymal stem cells (MSCs) are self-renewing, multipotent progenitor cells which have multilineage differentiation potential. They can differentiate into cell types of mesodermal origin (e.g. adipocytes, osteocytes and chondrocytes [83]), ectodermal origin (e.g. neuronal cells [84]) and endodermal origin (e.g. hepatic, pancreatic and respiratory epithelium cells [85, 86]). MSCs can be isolated form several tissues like bone marrow [87], adipose tissue [88], placenta [89], amniotic fluid [90] and umbilical cord blood [91]. They are seen as a promising candidate for cellular therapy due to their ability to differentiate across various lineages beyond the conventional mesodermal lineages, their ability to secrete soluble factors with an immunomodulatory effect and their capacity to migrate towards sites of injury and tumor microenvironments (see Figure 10). Furthermore they can be genetically engineered to express certain receptors on their surface which can increase migration capacity.
Currently MSCs are being tested for clinical application in four main areas:

- tissue regeneration for cartilage, bone, muscle, tendon and neuronal cells
- cell vehicles for gene therapy
- enhancement of hematopoietic stem cell engraftment
- treatment of immune diseases such as graft-versus-host disease, rheumatoid arthritis and acute pancreatitis

Before broad scale clinical application becomes a fact more research needs to be done concerning their biological characteristics in order to obtain therapeutic effects. Concerning MSCs four properties are considered the most important to guarantee clinical efficacy: the ability to home to the site of injury after intravenous injection, the ability to differentiate into various cell types, the ability to secrete molecules capable of inhibiting inflammation and stimulating recovery and lastly the lack of immunogenicity and the ability to perform immune modulatory functions [92].

The first clinical trial which used MSCs was performed in 1995. 15 patients treated with autologous stem cells were included in this study. Since 2009 a total of 342 studies have been registered on the public clinical trials database ClinicalTrials.gov which use MSCs for a wide variety of therapeutic applications. Only a very small number of studies (< 10%) are phase 3 studies. Most trials reported only minor adverse events like transient peri-injection effects which seems to show that MSCs are well tolerated in patients [92]. Many completed clinical trials have demonstrated the efficacy of MSC infusion for diseases like liver cirrhosis (MSCs reduced fibrotic index thereby improving liver function [93]) and amyotrophic lateral sclerosis (decreased

inflammation in the spinal cord after MSC infusion [94]) . However long-term effects still need to be assessed.

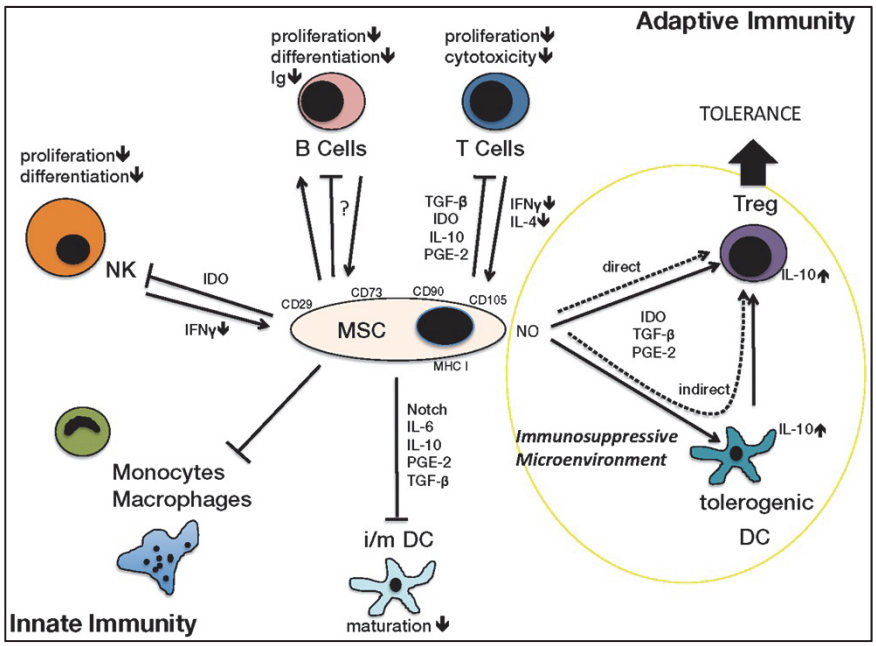

Figure 10. Immunological Function of MSCs on different cell types of the innate and adaptive immunosystem. Arrows indicate activation or induction, T-bars indicate blockade of function or activation, in particular inhibition of proliferation, differentiation, cytotoxicity, maturation. Figure adapted from Plock *et al* [95].

5.3 Non-viral vectors

Non-viral gene delivery systems were introduced as an alternative method to the viral-based systems. In general, they are potentially safer, cheaper, more reproducible and they can contain larger DNA fragments [96]. The main limitation of non-viral vectors is their low transfection efficiency although over the last years significant improvements have been made [97]. These improvements have led to an increased number of gene therapy products entering clinical trials [98]. The most widely used non-viral methods for gene delivery are DNA condensing agents, liposomes, microinjection, electroporation, nanoparticles and gene guns. Recently new methods are being investigated for non-viral gene delivery in the form of exosomes, transposons, nanoparticles, bacterial vectors and episomal vectors. These novel methods will be discussed below.

5.3.1 Exosomes

Exosomes belong to the family of nanoparticles and are secreted by most cell types in the human body [99]. Up to 1996 they were regarded as waste-disposal vessels or byproducts of cell homeostasis. This view changed when it was found that B cells could release functional antigen-transferring exosomes [100]. Of all the membrane type vesicles which cells can secrete, the exosome is the only one small enough to evade clearance by the mononuclear phagocyte system, thereby maximizing their circulation time [101]. Exosomes can contain defined sets of lipids and proteins and can function as carriers of nucleic acids, including mRNA and miRNA [102] (see Figure 11).

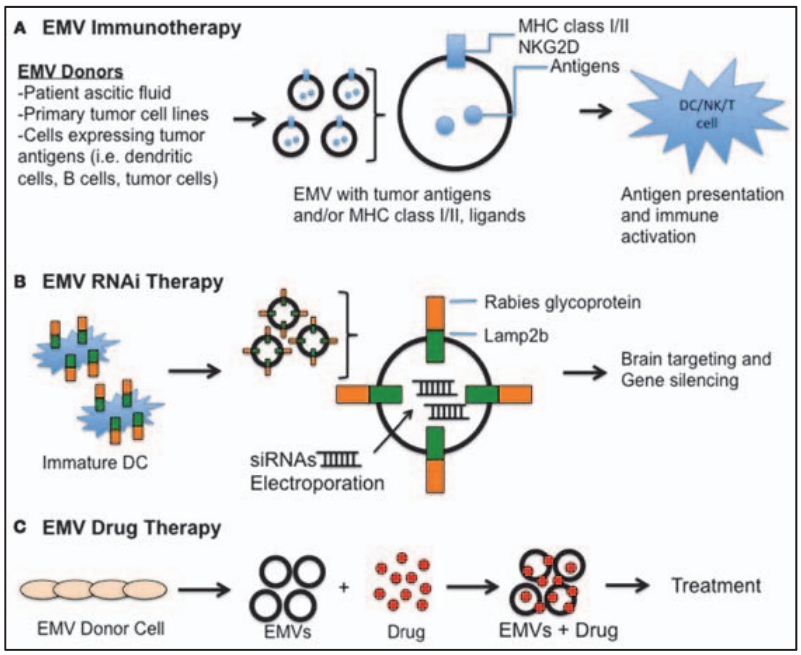

Figure 11. Extracellular membrane vesicles-based (EMV) therapies. **(A)** EMV immunotherapy. EMVs containing tumor-antigen within and/or on the membrane surface are isolated from different sources and introduced *in vivo* to elicit targeted immune responses. **(B)** EMV RNAi therapy. EMVs derived from immature dendritic cells (DCs) expressing Rabies glycoprotein-Lamp2b fusion protein were electroporated with siRNAs for targeting against neurons, microglia, and oligodendrocytes for subsequent gene silencing. **(C)** EMV drug therapy. Therapeutic compounds can be packaged into/onto EMVs isolated from donor cells to minimize degradation and increase delivery to intended sites. Figure adapted from Lai *et al* [103].

For some applications unmodified exosomes could be used since they natively exhibit therapeutic activity. Exosomes derived from dendritic cells can be used for vaccination [104] and exosomes derived from MSCs can reduce ischemia/reperfusion injury in mice [105].

A key benefit of exosomes is their potential to mediate gene delivery without inducing adverse reactions. Since exosomes can be derived from the patient's own cells there is no additional risk of immunogenicity. This may be a potential benefit since repeated administration of the exosomes could be a possibility. Exosomes can either have a stimulatory or inhibitory effect on the immune system [106]. They can function as shuttles for antigen presentation [107] and can mediate immune tolerance via dendritic cells, T and B cells [108]. These features have led to research looking into the potential of exosome-based cancer vaccines [109]. Three Phase 1 clinical trials have been conducted in which it was shown that these vaccines were well-tolerated in patients without major side effects [110-112]. These trials also showed that repeated administration was well-tolerated. Although therapeutic benefits are not yet proven, several patients exhibited a halt in disease progression after exosome vaccination [110, 112]. In addition exosomes are non-replicating therefore the probability of virulence is on theoretical grounds extremely low.

5.3.2 Transposons

Transposons are discrete segments of DNA that have the ability to move and replicate within genomes. They were discovered in the 1940s and have been found to be present ubiquitously in almost all living organisms [113]. Transposons can be best seen as molecular parasites which propagate themselves by using resources of the host cell. Unlike viruses they are not infectious and their activity is therefore restricted to the intracellular space. Due to this restriction transposons have to coexist with the host cell to survive. Transposable DNA elements have become a promising non-viral delivery system for persistent gene delivery [114]. The simple gene integration machinery of cut-and-paste transposons provides possibilities to insert transgenes into the target cell genome (see Figure 12). The *Sleeping beauty (SB)* transposon has been shown to show significantly high activity in vertebrates [115]. In addition to SB, *piggyBac (PB)* transposons are being evaluated for use in gene therapy [116].

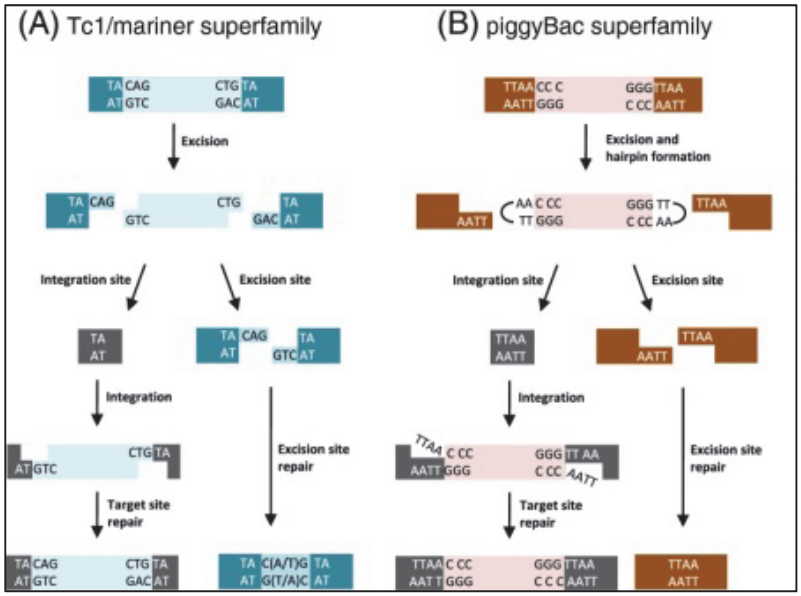

Figure 12. Schematic representation of cut-and-paste transposition.
(A) Transposition of Tc1/*mariner* elements (like *Sleeping Beauty*) leads to double-stranded breaks and formation of a 2 or 3 bp 3′-overhang at the excision site (a 3 bp overhang is shown). DNA repair by host-encoded enzymes creates a characteristic footprint at the excision site. Integration occurs at TA dinucleotides which are duplicated upon transposition. The single-stranded gaps are repaired by host-encoded enzymes. (B)*PiggyBac*-mediated excision is followed by hairpin-formation at the transposon ends. After integration into TTAA target sites that are consequently duplicated, the single-stranded breaks are repaired by ligation. The 5′ TTAA overhangs created at the excision site anneal, thus repairing the double-stranded break without leaving any footprint. Figure adapted from Skipper *et al* [117].

5.3.2.1 Sleeping Beauty (SB) transposase

The SB transposon consists of a single gene encoding the transposase polypeptide, the enzymatic factor for transposition, flanked by terminal inverted repeats (IRs) containing binding sites for the transposase [118]. The transposase gene can be physically separated from the IRs and replaced by other DNA sequences since they can mobilize transposons *in trans* as long as they retain the IRs. The transposase can be located on the same DNA molecule [114], supplied on another DNA molecule [119] or supplied in the form of mRNA or protein [120].

The natural size of SB is 1.6 kb and the sequences which are minimally required are included in the 230 bp long IRs. To be able to use SB as a vector for gene transfer it should be able to move DNA which is several kb's in length. Similar to other vectors, efficiency of SB transposition decreases with increased transposon length [119]. The maximum capacity lies around 10 kb. However, by using special PCR-based DNA shuffling techniques hyperactive SB was produced which is 100-fold more active than the original SB transposase [121].

5.3.2.2 PiggyBac (PB) transposase

The PB transposon consists of two terminal repeat domains (TRDs), two internal domain sequences and an open reading frame (ORF) encoding the PB transposase. The TRDs and internal domains are crucial for effective integration of the transposon in the host genome. The 5' terminal domain serves as a native promoter for transposase expression. During transposition the terminal domains are integrated exclusively at a TTAA integration site into the host cell genome. The natural size of PB is 2.5 kb.

5.3.2.3 Use of transposon systems for gene therapy

Naked DNA and plasmids are popular vectors for gene therapy because of their low immunogenicity and low risk of insertional mutagenesis. However due to the transient gene expression they are not suitable for gene therapy when long-term therapeutic gene expression is required for treatment. DNA transposons have all the desired features of naked DNA as well as the ability to insert transgenes into host genomes for long-term expression. To use DNA transposons as a gene delivery tool, a two plasmid system is used. This system consists of a helper-plasmid expressing the transposase and a donor plasmid which carriers the gene of interest flanked by 2 TRDs or IRs.

Stable insertion has been achieved in human and murine primary cells as well as *in vivo* in mice, rats and pigs for a variety of diseases including hemophilia [122], glioblastoma [123], ovarian cancer [124], Fanconi Anemia [125] and skin inflammation [126]. Using an *ex vivo* gene delivery approach efficient transfection and stable transgene expression was achieved in several human stem cell types, including cord blood-derived $CD34^+$ hematopoietic progenitors [127, 128], primary T cells [129] and human embryonic stem cells [130].

The first phase 1 clinical trial using the SB transposon system was directed against B-lineage malignancies [131]. The vector carried a CAR to direct T cell toxicity specifically towards $CD19^+$ B-lineage tumors. In the second generation of clinical trials the CAR was combined with a CD28-CD3-z fusion to provide T cells with an endodomain to achieve CD19-specific activation events [132].

5.3.3 Nanoparticles

Pharmaceutical nanoparticles (NPs) were first described in the 1970s. Their size offers one of the most attractive advantages for gene therapy and can allow more efficient delivery of therapeutic agents into target sites. In addition NPs can provide targeted delivery of genes into tissues and cells, protect the therapeutic agents from enzymatic degradation by nucleases and provide a sustained effect in the target tissue. The most frequently studied strategy for non-viral gene delivery is the incorporation of DNA into condensed particles based on cationic lipids or cationic polymers. In addition, other carriers such as peptides, dendrimers and magnetic NPs have also been investigated. In general NPs can be split into 2 categories: synthetic (inorganic) polymers and natural (organic) polymers.

Synthetic polymers encompass NPs made of metal, metal oxide, semiconductors, earth minerals and silica. These NPs often possess unique electric, magnetic, optical and plasmonic properties due to quantum mechanical effects at nanometer scales. Most of these synthetic NPs can be generated with great control over size, shape, composition and physical properties. Small molecules including dyes, therapeutic agents and targeting ligands can be conjugated to NPs. In addition they can be coated to improve physical/chemical adsorption of small molecules to have sufficient payload amounts. As such, numerous multifunctional NPs can be generated by combining existing NPs with small molecules (reviewed in [133]).

Natural NPs can be prepared by using several biodegradable materials such as polylactide-polyglycolide and polycaprolactones as well as proteinaceous materials such as albumin and collagen. Optimization of several nanoparticle characteristics has been used to enhance the functionality of these vectors (see Figure 13). These strategies include:

- functionalization with polyethylene glycol (PEG) for stealth capacity [134]
- addition of cell penetrating peptides for increased uptake [135]
- addition of polycationic peptides and ligands for endosomal escape [136]
- conjugation of nuclear localization signals [137]
- addition of antibodies and peptides for targeting [138]

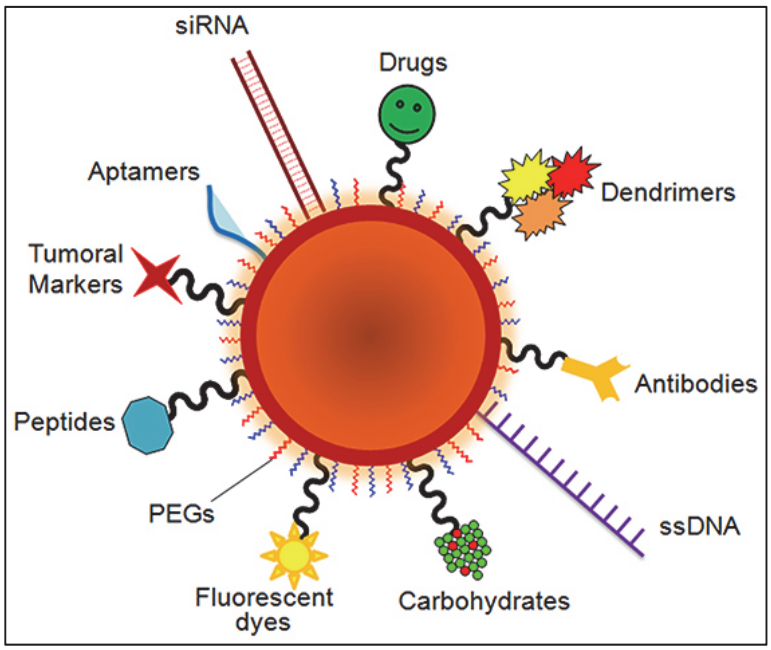

Figure 13. Schematic representation of a multifunctional nanocarrier. These innovative NPs comprise nucleic acids such as RNA and DNA used for gene silencing approaches and in colorimetric assays, respectively. Aptamers and anticancer drug molecules are also used for delivery to the target tissue. Carbohydrates may be useful as sensitive colorimetric probes. PEG is used to improve solubility and decrease immunogenicity. Responsive nanocarriers can also trigger reaction upon external stimuli through the functionality of valuable tumor markers, peptides, carbohydrates, polymers and antibodies that can be used to improve nanocarrier circulation, effectiveness, and selectivity. Multifunctional systems can also carry fluorescent dyes that are used as reporter molecules tethered to the particle surface and employed as tracking and/or contrast agents. Figure adapted from Conde *et al* [139].

Nanoparticles can provide efficient delivery of therapeutic agents to target sites due to their size dependent ability to cross fine capillaries and enter deep tissues [140]. In addition NPs can be controlled to modulate the kinetics of release from the nanoparticles [141]. The first FDA approval of inorganic nanoparticles dates back to the mid-1990s. Since then a limited number of inorganic NPs have successfully entered the market and are used in (mainly cancer related) clinical trials.

In the last few years, many groups have reported the use of nanoparticles to complex and deliver viral vectors (e.g. adenoviruses, retroviruses) [142] as well as nucleic acids [143], leading to the emergence of new approaches known as magnetofection and theranostics. Magnetofection is a viral and non-viral approach that uses superparamagnetic nanoparticles to improve gene delivery under a magnetic field [144]. Theranostics combines therapeutics with diagnostics and covers several fields, including personalized medicine [145], pharmacogenomics [146], and molecular imaging [147] to develop efficient new targeted therapies with an adequate risk/benefit ratio. Furthermore, theranostics aims to monitor the response to treatment and to increase efficacy and safety. In the following two paragraphs liposomes and polymeric nanoparticles will be discussed in more detail.

5.3.3.1 Liposomes

Liposomes are defined as unilamellar or multilamellar microvehicles consisting of a phospholipid bilayer. Liposomal formulations historically have been the most popular nanoparticle delivery system and they have been extensively used for enhancing efficiency of drug delivery by systemic administration. Liposomes have the ability to deliver a broad range of payloads such as chemotherapeutic drugs, oligonucleotides and protein and this makes them the most successful method for delivery of therapeutic agents [148].

They recently gained interest as carriers for siRNA because they prevent degradation of the siRNA, accumulate preferentially in tumor tissues, deliver high payload concentrations and in addition they have proven to be safe in both animals and humans. Liposomes can be coated with polymers like polyethylene glycol (PEG) to avoid detection and elimination by the reticuloendothelial system. These type of liposomes are called stealth liposomes and have a longer circulating half-life [149]. The PEGylation also provides a linker for the addition of targeting ligands to specifically target liposomes and enhance cell surface receptor interaction [150]. However, a major drawback of liposomes is their lack of structural integrity and instability during storage [151].

Several general techniques emerged from the rapidly increasing number of literature which are summarized below.

- Positive charge, e.g., cationic lipid, is needed for efficient association of nucleic acids with lipids.
- A positive charge on liposomes results in their rapid elimination by the mononuclear phagocyte system and non-specific cell binding.
- To increase the circulation half-life of liposomal nucleic acids, they should have a near-neutral surface charge: two approaches have been used to achieve this, the formation of coated cationic liposomes and the use of ionizable lipids.
- Ligands are needed for specific binding and internalization.
- Efficient endosomal release following internalization is needed for therapeutic activity, and this can be provided by ionizable cationic lipids with optimized bilayer destabilizing capacities and pKa.

A number of products are on the market, with many more in clinical development [152]. AmBisome and Doxil, in particular, have both achieved considerable clinical success. Although many routes of administration have been used for liposomal and lipid-based products, parenteral administration is the predominant one for clinically approved products, in particular intravenous administration. Currently liposomes are under investigation for several gene therapies including cystic fibrosis [153] and melanoma [154].

5.3.3.2 Polymeric nanoparticles

One of the earliest used polymers in gene therapy was polyethylinimine (PEI) [155]. Although PEI has great potential to transfect dividing cells it is not feasible to achieve transfection in non-dividing cells. In addition a major drawback is cytotoxicity *in vivo* which precludes use in clinical trials [156]. In the past decades many alternatives emerged for PEI, which are better for transfecting as well as possessing a lower cytotoxicity level. The most commonly used polymers include: PLL, Poly(β-aminoesters), DMAEMA, pluronic, PEG, dendrimers (PAMAM) and Chitosan. The reader is referred to Aied *et al* for details [157]. Unlike conventional polycation-based systems, decationized polyplexes are based on hydrophilic and neutral polymers. They are obtained by a three-step process: charge-driven condensation followed by disulfide crosslinking stabilization and finally polyplex decationization. Decationized polyplexes could be a platform for safer polymeric vectors with improved biodistribution properties when used in systemically administered gene therapy [158].

5.3.4 Bacteria

Invasive bacteria can internalize and replicate within tumor cells, while non-invasive bacteria grow externally to tumor cells, within the tumor microenvironment [159]. The tumor selective growth of bacteria has made them an attractive vehicle for delivery of reporter genes, therapeutic genes or inhibitory RNA. An increasing amount of protocols for modification of the bacterial genome leads to creation of bacteria which directly express therapeutic genes and/or which can internalize into tumor cells with subsequent therapeutic nucleic acid release for tumor cell expression, so called bactofection. Bactofection of mammalian cells applies to both active invasion of non-phagocytic mammalian cells (e.g. tumor cells) and passive uptake by phagocytic immune cells (DNA vaccination). Co-expression of reporter genes in these bacteria can be used for *in vivo* imaging of localization and spread. To date the following bacterial species have been investigated for use in clinical treatment: *Salmonella* [159, 160], *Clostridium* [161], *Listeria* [162], *Bifidobacterium* [163] and *Lactococus* [164].

The first modern attempts at using bacteria for therapeutic purposes were made already 50 years ago [165]. However these findings remained largely unexplored until the turn of the 20th century when oncolytic bacteria capable of lysing host cells were first identified [166-168].
Bacteria may be favorable over other microorganisms such as gene therapy vectors derived from viruses. Several bacterial species are motile and have the ability to swim against pressure or diffusion gradients created within the abnormal tumor environment. Since viruses rely on convection to spread within a tumor this limits their penetration significantly [169]. In addition, bacteria can adhere to or even invade tumor cells and are capable of proliferating within the tumor thereby establishing extracellular colonies. Furthermore their large genome allows for insertion of a variety of exogenous therapeutic genes. From safety perspectives they can be eliminated using antibiotics in case of complications.

Therapeutic strategies include direct cell killing (via pro-apoptotic genes or oncolysis), anti-angiogenic therapy and immune therapy (via upregulation of the immune system or DNA vaccination). Use of clostridial species for targeted tumor killing and attenuated *S. typhimurium* vectors for oral vaccination or tumor gene delivery represent the most widely applied bacterial vectors at clinical trial level.

5.3.5 Episomal vectors

Episomal vectors offer several advantages over integrating vectors by persisting in the nucleus in an extra chromosomal state. Therefore the inserted gene of interest will not be disrupted or subjected to regulatory constraints. Due to the non-integrating nature there is also no risk of cell transformation. Since episomal vectors can persist in multiple copies per cell this results in high expression of the gene of interest. In addition they display a high insert capacity which allows for the delivery of entire genomic DNA loci and subsequent physiological levels of transgene expression.

5.3.5.1 Human artificial chromosomes

Human artificial chromosomes (HAC) could represent an alternative to virus mediated gene transfer systems (see Figure 14). All HACs contain a functional centromere that provides them with several advantages over currently used other episomal viral vectors [170, 171]. Firstly, the presence of a functional centromere enables long-term maintenance of HACs as a single copy episome without integration into the host genome thereby minimizing the chance of transgene silencing. Secondly, there is no upper size limit to the DNA which is cloned into the HAC. Entire genomic loci with all regulatory elements can be used which can truly mimic the normal pattern of natural gene expression. Thirdly, HACs can be transferred from one cell to another. And lastly, because of the lack of viral sequences, HAC vectors minimize adverse host immune responses and the risk of cellular transformation.

Several studies have shown the use of top-down and bottom-up generated HACs for delivery and expression of genes in human gene-deficient cell lines as well as for animal transgenesis (reviewed in [172]). However in almost all cases, the copy number of the gene inserted in the HAC was not precisely controlled. This was either because of the presence of multiple gene acceptor sites in the HAC or because the gene was inserted into the HAC during its de novo formation. No clinical trials have been done up to now which utilized artificial chromosomes for gene delivery.

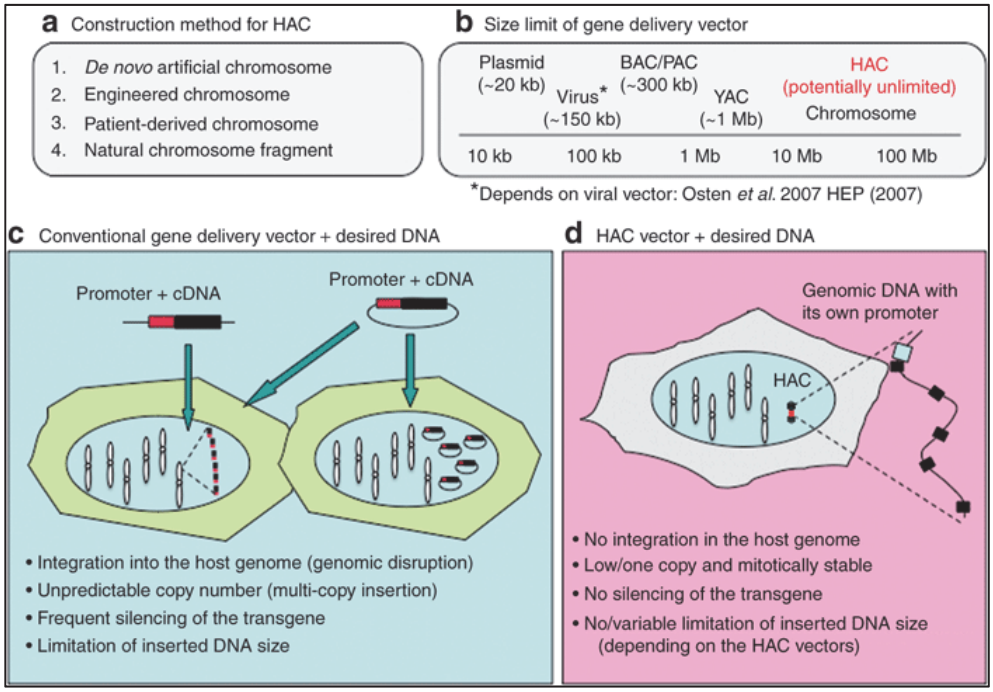

Figure 14. Potential characteristics of human artificial chromosomes (HACs). (a) Method for constructing HACs. (b) Size limits for gene delivery vectors. Maximum deliverable DNA size in each vector is described. HAC vectors as well as chromosomes, can carry DNA larger than 1 Mb. The size limits depend on each vector. (c,d) Limitations and consequences of gene delivery with conventional vectors such as a virus or plasmid, and with HACs, respectively. Figure adapted from Kazuki *et al* [170].

The greatest example of correction of gene deficiency in a mouse model utilizing patient-derived cells by a top down generated HAC is the use of 21HAC containing the dystrophin gene. Mutations in this gene lead to Duchenne muscular dystrophy [173]. Bottom-up HACs are used for regulated correction of genetic deficiencies in patient-derived cells from Nijmegen breakage syndrome patients [174].

In addition HACs could be used to generate iPSCs (for more information on iPSCs see paragraph 5.2.1). HACs represent a potential system for the delivery of reprogramming factors and their robust expression for as long as required without integration into the target cell genome [175]. Derivatives of the alphoid^{tetO}-HAC, without the necessary transfection step, are a promising system for use in a new iPSC protocol which meets all safety and efficacy requirements.

In respect to iPSCs, HACs could also be used as a tool for removal of residual embryonic stem cell (ES)/iPSC cells following their differentiation. Residual stem cells have been shown to become tumorigenic. Genetic sensitization via the thymidine kinase (TK) gene may be an approach to selectively kill undifferentiated ES/iPS cells. The TK gene can be driven by either an ubiquitous promoter [176] or by ES/iPS-specific regulatory elements such as the Nanog [177] or Oct4 enhancer [178]. Besides teratoma control this technique may also be useful for tissue replacement therapies with cell types that are not derived in sufficient amounts *in vitro* from ES/iPS cells.

5.3.5.2 S/MAR containing vectors

S/MARs are short, usually AT-rich regions with extended base unpairing regions. They are involved in various biological activities compatible with their interaction with the nuclear matrix. These functions include augmentation of transcription [179], insulator function [180] and long-term maintenance of transcription and they are always found in close proximity to mapped mammalian origins of replication [181]. Efficient maintenance of episomes require authentic replication. The process is initiated during an “establishment” phase defined as the interval between vector transduction and its functional association with nuclear substructures providing replication potential.

Any modifications of the prototype vector pEPI attempted to improve its functioning with respect to delivery, expression and establishment efficiency. Since the expression cassette-S/MAR module is essential for episomal replication and maintenance, any modifications only apply to the vector backbone and the insertion of various promoters [182]. Modification of the vector backbone led to the development of minicircles.

Minicircles (MC) are plasmid-based supercoiled DNA vectors from which the bacterial sequences are excised and which thus only contain the expression cassette consisting of a promoter linked to an S/MAR sequence, transgene and polyadenylation signal [183]. In principle, a parental plasmid is constructed consisting of the eukaryotic expression cassette flanked by recombination sites. Outside these sites lie all sequences needed for plasmid propagation in bacteria (e.g. origin of replication and antibiotics resistance genes). Induction of recombination produces a MC devoid of bacterial sequences containing only the gene of interest with suitable regulatory sequences (see Figure 15). MCs show higher gene transfer efficiency by chemical methods compared to conventional plasmid DNA [184]. MCs are in theory very suitable for gene therapy since regulatory agencies recommend avoiding antibiotic resistance genes in DNA vectors for clinical trials. In addition the FDA recommends an 80% supercoiled plasmid fraction. Currently no clinical trials are ongoing in which minicircles are being utilized to achieve gene transfer.

Although S/MAR vectors have been used successfully in many *in vitro* studies, their use *in vivo* proved to be much more challenging. The main limitation is rapid silencing of transgene expression when the vectors are delivered to differentiated tissues. To overcome this a vector was constructed which encodes bcl-2 which provides a selective survival advantage of transfected cells which have the vector stably established in their nucleus. Despite their limitations S/MAR systems are used in the monitoring of tumor growth and tracking [185, 186] as well as in the transdifferentiation of liver cells towards pancreatic β cells [187].

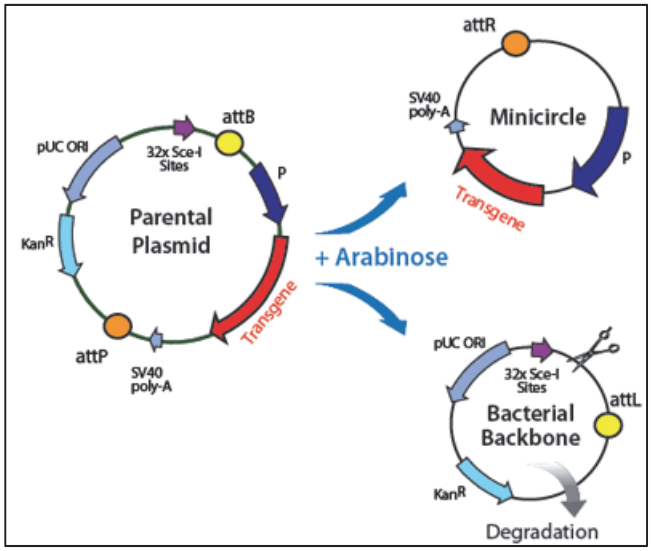

Figure 15. Production of a minicircle (MC). The MC DNA elements are generated by intramolecular (cis-) recombination from a parental plasmid (PP) mediated by PhiC31 integrase. The full-size MC-DNA construct is grown in a special host E. coli bacterial strain ZYCY10P3S2T harboring an Arabinose-inducible system to express PhiC31 integrase and I-SceI endonuclease simultaneously. Adding arabinose to the media turns on the integrase and endonuclease genes. The PhiC31 integrase produces the MCDNA molecules as well as PP-DNA backbone from the full-size MC-DNA construct. The Sce-I endonuclease then degrades the PP-DNA backbone preventing immune responses. Figure adapted from BioCat (www.biocat.com).

The most promising approach to improve efficiency of S/MAR vectors is the construction of “hybrid vectors”. These vectors aim to combine the advantages of viral vectors with those of episomal replicating vectors. These constructs were used successfully for non-integrating lentiviral vectors and gene deleted adenoviral vectors ([188, 189]). Currently no clinical applications involving S/MAR based vectors are being tested, however due to the progress which is currently made it is very likely that these expression systems will lead to safe and efficient vectors which can be used in gene therapy.

5.4 Viral vectors and oncolytic viruses

In this section we give an overview of developments in the field of viral vectors and oncolytic viruses for (cancer) gene therapy.

Viruses are ordered based on the most recent virus taxonomy as published by the International Committee on Taxonomy of Viruses. For a more detailed description, including general information and (pre)clinical studies, the authors refer to the addendum belonging to this report (available at the COGEM website: www.COGEM.net). Also, several vectors will not be described here, since no significant development has been made recently. However, those vectors are described in detail in the addendum. Furthermore, only key references are used in the following sections, more detailed references can also be found in the addendum.

5.4.1 Bacteriophages

Most phages possess a large packaging capacity, e.g. lambda vectors up to 53 kb and M13 vectors even undefined larger [190-192]. Coat proteins of phages can be engineered or selected using phage display to incorporate targeting and cell internalization enhancing ligands, which is needed for efficient gene transfer to eukaryotic cells [193-196]. Phagemid vectors are plasmids with a bacteriophage origin of replication that are packaged into phage particles upon addition of helper phages, making the genetic modification of bacteriophages easier, and allowing for the development of phage-like particles [197, 198]. Phage virus like particles (VLPs) have been used to package exogenous RNA, nanoparticles, chemotherapeutic drugs and protein cocktails [196, 199-201]. By inserting a eukaryotic gene cassette from AAV in an intergenomic region of a phage clone displaying RGD-4C, a chimeric AAV/Ph virus has also been created, improving transduction rates significantly [202-205].

Attempts have also been made to evaluate bacteriophages for oncolytic activity [206]. More recently, genetically engineered bacteriophages have also been evaluated for efficacy in mouse models for melanoma and were shown to induce tumor regression by activating local antitumor immune response [207, 208]. Also, phage based lambda nanobioparticles were successful in transfecting tumor cells with the apoptin gene from chicken anemia virus, which induced cytotoxicity *in vitro* and tumor regression in a mouse xenograft model for breast cancer [209].

The application of bacteriophages as therapeutic agents seems safe for patients. However, no information is readily available regarding shedding and transmission. Moreover, bacteriophages can easily adapt to passaging [210, 211]. This has strangely not been perceived as a potential safety issue.

5.4.2 Herpesvirus (HSV)

Human herpesviruses have been developed as viral and oncolytic gene therapy vectors. Deletion of viral genes essential for lytic replication, reactivation and/or immune evasion (i.e. ICP0, ICP4, ICP27, and/or ICP47), while leaving latency genes intact, has resulted in recombinant replication defective HSV (rdHSV) particles with transgene expressing capacity of equal size as the viral genome deletions. Transduction with rdHSV vectors causes a latent-like infection in (non-)neural tissue, which leads to long lasting transgene expression. rdHSV vectors expressing (pre)opioid or anti-inflammatory peptides have been developed for treatment of pain

syndromes [212-214].

Because HSV is neurotropic and causes a latent infection, most genetic modifications of oncolytic HSV (oHSV) have first focused on this potential safety issue. To generate tumor-specific oHSVs, three main strategies have been used: attenuation by conditional replication in tumor cells [215-218]; arming with transgenes (most notably immune stimulators like GM-CSF, but also therapeutic and imaging transgenes) [219-224]; and targeting by tropism or transcriptional specificity [225-227]. Most recent strategies use less attenuated oHSVs and retarget them to tumor cells for safety. This leads to an intrinsic raise in environmental safety issues, since shedding of less attenuated strains is more likely. Spontaneous reversion of oHSV deletion mutants is not possible, however mutants can acquire compensatory mutations, while still being highly attenuated. Especially these compensatory mutations can be of concern when evaluating the safety of second and third generation oHSVs.

rdHSV vectors have been evaluated for safety of intradermal injection in patients who have intractable pain due to malignant disease located below the angle of the jaw, which showed some objective results without SAEs [228]. Several oHSVs (talimogene laherparepvec, HSV1716, NV1020, G207, M032, rRp450 and other) have already been used in clinical trials [229]. Most of these trials demonstrated a good safety profile of oHSV and treatment benefits were observed. A phase III clinical trial using talimogene laherparepvec has just been concluded in patients with advanced melanoma, and indications are that this will be the first oncolytic virus to obtain FDA approval [230]. The use of newer oHSVs, especially vectors not fully attenuated but rather retargeted to tumors, will have to be evaluated in human-like models to exclude accidental neurotoxicity and occurrence of latent infection. Given their promise of better oncolytic activity, it seems likely that these oHSVs will also proceed to clinical trials in the near future.

Preclinical evaluation employing intra-organ (brain or prostate) injection in non-human primates demonstrated no shedding of virus, which points to limitation of oHSV to injection sites [231-233]. This was confirmed by early clinical trials in patients injected intratumorally with oHSVs: sometimes shedding of HSV in saliva was noted, but this was shown to be wildtype virus as opposed to the injected oHSV [234, 235]. Other studies have observed limited leakage of oHSV from injection sites up to 2 weeks post treatment, without other excreta containing viable oHSV [236-240]. New generations of oHSVs should be evaluated for increased shedding. Other (non-)human herpesviruses that have been investigated earlier, without significant progress in recent years include BHV-1, EHV-1, VZV, SuHV-1, HHV-6B, BHV-4 and HVS-2.

5.4.3 Newcastle disease virus (NDV)

In more recent years, the interest in oncolytic NDV has revived with the advent of recombinant viruses (rNDV). Several approaches have been used to improve oncolytic efficacy of rNDV, including increasing virulence [241-248], expression of immunomodulating or apoptotic transgenes [241-243, 248-251], targeting of tumor cells with modified attachment proteins [252, 253] and combinations with other treatment modalities, most recently immune checkpoint blockade [254, 255]. NDV has been shown to be very safe in tumor models using mice or rats, even when used in high dose and injected intravenously.

Early clinical trials in humans also have demonstrated wildtype NDV to be a safe oncolytic agent with minimal side effects upon administration. No recent clinical trials have been reported.

Virulent strains pose an environmental risk. Relevant shedding of live virus was observed

in non-human primates and early clinical trials [256]. Future research should focus on either limiting virulence for susceptible hosts [257], targeting NDV to human (tumor) cells, or optimizing non-virulent rNDV for oncolytic activity.

5.4.4 Measles virus (MeV)

Most (pre-)clinical research with oncolytic MeVs has been done using recombinant MeV of the attenuated vaccine Edmonston strain [258, 259]. Infection of cells with MeV leads to viral replication and expression of glycoproteins on the cell surface, which results in a typical cytopathic effect called syncytia formation. Cancer selectivity stems from CD46 overexpression on malignant cells, which also explains the choice for a lab adapted strain to be used as oncolytic vector [260]. Tumor selectivity may also be facilitated by defects in innate immune response in cancer cells. Oncolytic MeV has been shown to be a potent antitumor agent in a wide array of malignancies [259].

Recombinant MeV can accommodate and maintain large sizes of foreign genetic material with good genetic stability *in vitro* and *in vivo* [261-264]. Like with other oncolytic vectors that have been under extensive study, efforts have been made to express transgenes from MeV (most notably CEA and NIS) [265-269]. Also, alternative strategies to improve oncolytic effect and selectivity have been evaluated, including receptor retargeting, selective F protein cleavage, miRNA based selectivity, carrier cell delivery, exchanging envelope glycoproteins and various combination therapies. Recently, a new strategy has been described by making a chimeric oncolytic MeV/VSV vector, which was shown to outperform both parental viruses [270].

Completed and ongoing clinical trials have first used non-recombinant MeV and later recombinant MeV-CEA and MeV-NIS [258, 271-273]. Intratumoral, intraperitoneal and intravenous administration have been done using doses up to 10^9 infectious viral particles without dose limiting toxicity or immunosuppression, despite low or absent neutralizing antibodies in some patients.

Spread of oncolytic MeV in the general population is highly unlikely since most individuals living in industrialized countries are immune to measles, although herd immunity is waning with declining vaccination percentages. There was no evidence of shedding in mouth gargle and urine samples in patients injected intraperitoneally with MeV-CEA [272].

Generally, the use of MeV as an oncolytic agent is safe. However, strategies that revert an attenuated MeV vaccine strain to more virulent forms by e.g. blocking innate immunity - combined with retargeting to cancer cells to increase safety - should be evaluated properly to exclude higher pathogenicity of these oncolytic MeVs [274-276]. This also applies to the newly described MeV/VSV chimeric viruses [270].

5.4.5 Sendai virus (SeV)

SeV-based virosomes can incorporate exogenous plasmid DNA or other (therapeutic) substances, and can fuse with target cell membranes by means of their HN and F glycoproteins to release their contents into target cells [277]. SeV virosomes loaded with siRNA, tumor antigens, immune stimulatory cytokines or cytotoxic drugs have demonstrated antitumor efficacy *in vitro* and *in vivo* [278-288].

By deleting M and/or F genes from the viral genome, SeV also becomes non-transmissible [289-292]. These non-transmissible SeV vectors have also been armed with

immunogenic or therapeutic transgenes and retargeted to tumors [293-300]. More recently, a new strategy has been applied where deleting accessory interferon-antagonizing viral proteins, in combination with including broad tumor cell retargeting, created attenuated recombinant SeV vectors [301].

Previous clinical studies have demonstrated safety of a wildtype SeV vaccine administered intranasally and a non-transmissible SeV vector expressing FGF2 injected intramuscular in patients with critical limb ischemia [302, 303]. A phase I/IIa clinical trial using UV-inactivated SeV vector is currently in progress in patients with advanced melanoma [304].

5.4.6 Farmington virus (FarV)

Using high-throughput screens of dozens of novel rhabdoviruses to identify new oncolytic vectors, FarV was selected for its superior oncolytic efficacy in a panel of tumor cell lines [305]. FarV is non-neurotoxic in adult mice and demonstrated oncolytic efficacy in immune deficient and competent mouse models of glioblastoma multiforme (GBM). Besides being restricted to IFN-defective cells, replication of FarV also appears to be restricted to dividing cells, adding to safety. Armed recombinant FarVs are being developed and systematically evaluated in syngeneic mouse models of GBM [306].

Given the obscure origin and uncertain host range of FarV, a thorough evaluation is needed in different (human-like) animal models to gain more information on pathogenesis and environmental risks.

5.4.7 Maraba virus (MaV)

After the initial report of oncolytic MaV [307], two more reports have been published using genetically altered MaV strain MG1. First, MG1 was reported to be a potential oncolytic vaccine boost vector for melanoma in a syngeneic mouse model [308], which was later demonstrated to depend on NK and dendritic cell activation [309]. In this latter study, single-cycle or non-replicating mutants were also evaluated, which were shown to be attenuated, also for oncolytic activity.

Quite surprisingly, the results reported in aforementioned studies have prompted the planning of a phase I/IIa clinical trial employing heterologous prime-boost vaccination with AdMAGE3 and MaV-MAGE3 [310]. Before this planning, toxicology studies in non-human primates have demonstrated safety of MaV strain MG1.

5.4.8 Vesicular stomatitis virus (VSV)

In the last decade, a great number of recombinant VSVs have been generated, aiming to optimize oncolytic potency and abolish neurotropism. To summarize, VSVs have been generated that express reporter genes, have attenuating mutations, express retargeting or fusogenic foreign glycoproteins, use miRNA retargeting and/or express other therapeutic transgenes (most notably immunomodulators) [311]. Furthermore, combination therapy has been described with a plethora of options. Finally, optimizing delivery and distribution of oncolytic VSVs has been evaluated using cell-based carriers, aptamer coating or PEGylation of virions.

A relatively new strategy employed to increase the oncolytic activity of VSV consists of so-called semi-replication-competent vectors. This is based on two *trans*-complementing

propagation deficient VSV vectors [312]. Using this method, the genes that are essential for viral replication are divided between two separate viral genomes, so that only double-infected host cells will produce infectious progeny. This results in VSV vectors that are attenuated for neuropathology, but still maintain good oncolytic efficacy.

A recent study in purpose-bred beagle dogs showed that a dose up to 10^{10} $TCID_{50}$ of VSV-hIFNβ was well tolerated, with mild adverse events with the exception of one dog that received 10^{11} $TCID_{50}$ which developed severe hepatotoxicity and shock leading to euthanasia [313]. A following study testing VSV-hIFNβ on rhesus macaques via intrahepatic injection did not result in neurological signs and is considered to be safe enough to proceed into phase I clinical trials, which are currently ongoing in humans and pet dogs [314, 315]. No VSV RNA was detected in buccal swabs taken from non-human primates after intrahepatic injection with VSV-hIFNβ.

VSV is an RNA virus with an inherent higher viral polymerase error rate due to the lack of proofreading, resulting in quasispecies populations [316]. Theoretically, VSV mutants harboring mutations in their M or G gene (making them oncoselective and abolishing neurotropism) could revert to wildtype VSV upon passaging, and recombinant VSVs expressing attenuating transgenes like hIFNβ can acquire mutations in this transgene, and these more wildtype-like mutants can also be selected for [317, 318]. Indeed, there is evidence present for reversion to wildtype VSV upon passaging of non-oncolytic mutants [319, 320], and loss of foreign gene expression has also been observed [321], which seems to depend on site of insertion [322]. Furthermore, recombinant oncolytic VSVs have been shown to optimize targeted glycoproteins upon passaging in tumor cells [323], and to mutate expressed transgenes to optimize replication [324]. These examples have strangely not been perceived as a safety problem.

As with other heavily researched oncolytic viruses, more recent strategies for oncolytic VSV include reversion towards more wildtype-like retargeted viruses, and combination therapies that potentially alter the outcome of VSV infection dramatically. We would therefore strongly advise to evaluate these new strategies thoroughly before approval for future clinical trials, as holds true for other oncolytic viruses.

5.4.9 Coxsackievirus A/B (CVA/CVB)

Similarly to rhinoviruses, CVA-21 binds to ICAM-1 and additionally needs DAF-attachment for productive viral infection. Given that ICAM-1 and DAF are overexpressed in melanoma cells, efforts to evaluate the oncolytic potential of CVA-21 (and other coxsackieviruses) have mainly focused on this disease [325]. CVA-13, CVA-15 and CVA-18 were also found to be effective in immune deficient xenograft mouse models for melanoma [326], which can be an attractive alternative option given the fact that a high percentage of people have neutralizing antibodies against CVA-21.

By screening 28 enteroviral strains, wildtype CVB-3 was recently added to the arsenal of oncolytic coxsackieviruses and evaluated as an option to treat NSCLC [327]. However, given the fact that CVB-3 is associated with acute and chronic cardiomyositis and pancreatitis [328], wildtype CVB-3 seems unsafe for administration to patients. Other groups have evaluated attenuated CVB-3 vectors for gene transfer to cardiomyocytes [329].

Currently ongoing phase I/II clinical trials employing intratumoral injection of CVA-21 (CAVATAK) in patients with advanced melanoma in Australia are showing promising results [330].

No information is available regarding shedding. When considering non- or low-pathogenic coxsackieviruses for oncolytic virotherapy, environmental risks can also considered to be low. However, when using viruses that do cause (severe) disease in humans, care should be taken to evaluate and/or attenuate these new vectors.

CVA/B, like other picornaviruses, possesses genomic instability and variability. Serial passage of CVB vectors expressing HIV-epitopes resulted in the truncated expression of larger inserted transgenes, although smaller (<20 kDa) transgenes where stably expressed after passaging [331]. Similarly, expression of GFP from an attenuated CVB-3 vector was maintained up to 10 passages, although 23 viral nucleotide changes resulting in 10 amino acid mutations where present after 5 passages, without reversion to wildtype virulence [332]. As with shedding, these findings are important to include into the evaluation of pathogenic CVA/B (oncolytic) vectors.

5.4.10 Poliovirus (PV)

Most preclinical research with poliovirus has been performed with PVS-RIPO, a recombinant PV type 1 (Sabin vaccine) strain with the IRES element of human rhinovirus type 2. PVS-RIPO has shown oncolytic efficacy in immune-deficient xenograft rat and mouse models of malignant glioma [333, 334].

Currently, a phase I clinical trial is ongoing with intratumoral infusion of PVS-RIPO in patients with recurrent GBM is showing durable responses [335]. Extensive evaluation in non-human primates has shown PVS-RIPO to be safe for either intraspinal or intrathalamic injection [336, 337]. No serious adverse events have been observed so far in an ongoing phase I clinical trial [335]. No observations of extraneural replication or shedding have been made in preclinical evaluation employing intraspinal or intrathalamic injection in non-human primates [336, 337].

One of the biggest concerns with PV is the inherent genomic instability of picornaviruses and thus the possible reversion to wildtype pathogenicity. PVS-RIPO has been evaluated extensively by e.g. serial passaging *in vitro* and *in vivo* and it was shown that escape mutants reverting to neuropathogenic virulence in the CD155-transgenic mouse model do arise [338]. Similar mutants have not been observed in human-like systems, which makes it unclear what the importance of this preclinical finding is in relation to clinical trials in humans.

5.4.11 Seneca Valley virus (SVV)

Since its introduction as oncolytic virus in 2007, SVV has shown preclinical efficacy in immune deficient mouse xenograft models for various cancer types [339-344]. Interestingly, in these studies SVV has been shown to cross the blood-brain-barrier and is effective in cerebral tumor eradication when injected intravenously. More recently, a recombinant SVV expressing GFP has been generated [345]. Another recent approach has been the development of a prodrug that can be activated by specific cleavage of the SVV 3C protease [346].

Published results from a phase I clinical trial employing an intravenous dose escalation in patients with neuroendocrine tumors show that SVV is safe to administer even in high dose (10^{11} viral particles/kg) [347]. Also, intratumoral replication was observed as well as (marginal) treatment benefits. A phase II RCT in patients with extensive stage NSCLC and a phase I dose escalation trial in pediatric patients with neuroblastoma, rhabdomyosarcoma or rare tumors with neuroendocrine features are currently underway.

More evidence has now been gathered that SVV, although its natural host is still uncertain, is a safe virus to use for oncolytic virotherapy in (pediatric) patients. Analysis of researchers in close contact with phase I trial patients revealed no detectable neutralizing antibody titers, which points to lack of effective viral transmission [347]. However, detailed evaluation of shedding was not performed. Like other picornaviruses, SVV has an inherent genomic instability. Variable loss of GFP expression after numerous passages of recombinant SVV-GFP in culture without plaque purification was noted due to partial deletion of inserted transgenes at common RNA break points [345].

5.4.12 Human adenovirus (HAdV)

Because of its association with mild disease and relatively easy to manipulate genome, most work on HAdV as vector for (cancer) gene therapy has been done with serotype 5 (HAdV-5) of species C.

In the first generation replication defective HAdVs (rdHAdVs), the viral genome was modified by deleting E1A and/or parts of E1B and E3 genes, and these viral genome deletions could be filled with transgenes (up to 8 kb) [348]. However, low-level viral replication still induced cellular immune responses against transduced cells, resulting in limited duration of gene expression *in vivo*. Second generation rdHAdVs therefore have been created which lack E2A and harbor mutations/deletions in E4, although controversy exist whether these vectors are more effective *in vivo* [349-351]. In another approach, third generation high-capacity 'gutless' or so-called helper-dependent HAdV vectors (hdHAdVs) have been created by removing the complete viral coding regions and leaving only the ITRs, permitting insertion of up to 37 kb of foreign sequences [351, 352]. This approach can also be used to create hybrid rdHAdV-retrovirus vectors, combining high infectivity with integration capacity [353].

In 1999, a dramatic fatal case of a systemic inflammatory response following intra-hepatic arterial administration of a second generation rdHAdV-5 in an 18-year-old patient enrolled in a phase I clinical trial put an abrupt stop to HAdV-5 procedures and led to a general rethinking of the approach [354]. More recent rdHAdV gene therapy vectors are being based on serotypes without pre-existing immunity in patients (most notably type 3), or modified capsids to prevent liver sequestration [355].

In the case of oncolytic HAdV vectors, replication is thought to be advantageous because of cancer cell killing by viral replication, thereby reducing the number of administrations needed for effective treatment. As such, efforts have been made to develop conditionally replicating HAdVs (crHAdVs), which specifically replicate (better) in cancer cells [356-362]. A different approach for creating crHAdVs is to use cancer or tissue specific promoters to limit expression of essential early HAdV genes to specific cells and/or tissues [363, 364]. Like other oncolytic viruses that have undergone extensive development, crHAdVs have also been armed with transgenes, often under the control of a tissue/cancer specific promoter as mentioned above.

Despite the capacity to achieve tumor infection in animal models, the therapeutic efficacy of crHAdVs has been disappointing. Off-target toxicity by transduction of mainly the liver is a serious concern, even when crHAdVs are blinded for CAR CAR [365-368]. This has been shown to be due to blood factors opsonization of crHAdV virions for Kupffer cell uptake, which can be counteracted by ablating the fiber region that interacts with these blood factors [369], although other studies have implicated the viral hexon to be a more potent binder of blood coagulation factor X [370, 371]. Hexon mutations or even complete exchange of hexons have been shown to

reduce liver sequestration and transduction dramatically [372-374]. Other strategies used are PEGylation or polymer/dendrimer coating of crHAdV virions, and cell-based or magnetic/liposomal nanoparticle delivery techniques.

To circumvent the limitation of low CAR expression in (tumor) cells, retargeting has also been applied to crHAdVs, permitting CAR-independent infection [375]. The strategy of retargeting can also circumvent existing humoral immunity for HAdV-5 in the general population, and aid in prevention of liver sequestration as described above. Examples include conjugation with anti-knob or anti-penton/hexon antibodies or adapters with retargeting ligands, pseudotyping or xenotyping with (chimeric) fiber knobs or capsids, peptide presentation (RGD or other), Affibody targeting, knob-less HAdVs and genetically modified capsids and/or fiber knobs.

More recently, efforts have also been made to develop crHAdVs based fully on other serotypes, most notably HAdV-3 [376-389], or even non-human AdVs (see paragraph 5.4.13). Using 'directed evolution' or 'accelerated evolution' strategies, other groups have reported the development of ColoAd1 and other crHAdVs which appear to be more potent than parental HAdV-5 based vectors [390-393]. Another interesting strategy is to develop genetically modified capsids incorporating marker proteins to visualize crHAdV infection and biodistribution [394, 395].

Combinations of strategies for rdHAdV and crHAdV retargeting, expression of (multiple) viral and/or transgenes under control of specific promoters, delivery options and combination with other treatment modalities make for a virtually infinite number of treatment options to be evaluated for a large number of different types of cancer and models. However, studies describing direct comparisons between different prototype HAdV vectors are scarce, which makes it difficult to predict which HAdV will make it into clinical trials [396, 397].

458 clinical trials employing HAdV-mediated gene therapy have been reported to date. Clinical trials employing third generation (hybrid) rdHAdVs or rdHAdVs based on other serotypes can be expected in the near future. In China, Gendicine (rdHAdV-5 expressing p53) was approved for clinical use in patients with head and neck cancer in 2003 [398]. However, its American and European alternative, Advexin, has never been approved for clinical application, due to insufficient safety and efficacy data [399]. Similarly, the assessment of Cerepro (rdHAdV-5 expressing HSV-tk in combination with gancyclovir) for marketing authorization in patients with high-grade glioma resulted in a negative advice from EMA, after having failed to show improvement in overall survival [400, 401].

ONYX-015, H101 (Oncorine) and other first-generation crHAdVs have gone through several phase I/II trials without relevant signs of toxicity [402]. However, the therapeutic effect was also disappointing, resulting in the discontinuation of further trials with these first generation oncolytic crHAdVs. Although Oncorine is registered for use in head and neck cancer patients in China, ONYX-015 was never approved by the FDA.

More recent clinical trials employing new generations of crHAdVs like RGD retargeted oncolytic crHAdVs, crHAdV-5/3 chimeric vectors, ColoAd1, hTERT-promoter driven crHAdV-5 vector Telomelysin, E2F-1-promoter driven CG0070 and crHAdV vectors expressing immunomodulating genes have shown safety with some promising preliminary results [403-419]. Furthermore, combination therapy with low-dose cyclophosphamide, temozolomide or verapamil has been evaluated, and cell-based carriers have also been used [420-423].

In general, the use of first and second generation crHAdVs appears to be reasonably safe when administered locally and at lower doses systemically. However, the development of new crHAdVs expressing transgenes, with altered capsids, or different promoters can dramatically alter this perceived safety. Therefore, it is important to thoroughly evaluate newer

crHAdVs for their patient safety in human-like animal models, since it is likely that these new agents will proceed into (more) phase I/II/III clinical trials.

Shedding of rdHAdVs and crHAdVs from injection sites and patient excretions, although certainly not always reported, has been observed in several (pre)clinical trials, and increases with dosage and systemic administration [399, 403, 424-432].

Homologous recombination between AdVs of the same subgroup occurs with high efficiency during growth in co-infected cultured cells, and there is evidence of recombination events in humans too [433-438]. Theoretically, homologous recombination between wildtype AdVs and recombinant crHAdVs could lead to new wildtype AdVs that e.g. possess transgenes, or worse, have expanded tissue tropism due to retargeting strategies. Such recombination has never been detected in any clinical trial to date.

5.4.13 Non-human adenovirus

During the quest for less immunogenic adenoviral vectors, several non-human adenoviruses have been evaluated to date: BAdV-3, CAdV-2, PAdV-3, SAdV, OAdV-7 and FAdV-1. As compared to human adenoviruses, development has a long way to go, but can be expected to continue in the future.

5.4.14 Baculovirus

Virtually all cell types (including stem cells), both nondividing and dividing, from several species can be transduced by baculoviruses [439-443]. Adding transgenes into the AcMNPV genome is theoretically without limit, given that the viral capsid extends to accommodate its (larger) genome. AcMNPV infects human cells and expresses transgenes under control of mammalian (BacMam) or constitutionally active viral promoters, but does not replicate or cause cytotoxicity in these cells [444-446]. Modification of the virus surface through capsid modification, xenotyping (e.g. with VSV-G) or coating can further augment the infectivity of AcMNPV, while simultaneously overcoming the limitation of complement inactivation [447-454]. AcMNPV cancer gene therapy has been shown effective in mouse models for several tumor types [455-458].

5.4.15 Influenza A virus (IAV)

IAV infection induces apoptosis or necrosis in cultured (tumor) cells [459, 460]. This observation has lead several groups to evaluate IAV as on oncolytic virus. Deletion or truncation of NS1 from IAV (ΔNS1-IAV) leads to a virus that is non-replicating in cells that express normal PKR. However, tumor cells can either have defective PKR or overexpression of Ras that leads to inhibition of PKR. These tumor cells are susceptible to ΔNS1-IAV oncolysis and expression of IL-15 from this ΔNS1-IAV results in more oncolysis *in vitro* [461-465].

Seasonal IAV viruses spread very efficient from human to human. No information is available on the spread of ΔNS1-IAV viruses. Although IAV is an RNA virus with an inherent higher viral polymerase error rate due to the lack of proofreading, spontaneous reversion of ΔNS1-IAV to wildtype IAV seems unlikely. However, recombination with other wildtype IAVs could theoretically lead to reassortant chimeric IAVs with wildtype-like properties expressing transgenes.

5.4.16 Adeno-associated dependoparvovirus (AAV)

Since the first description of AAV-mediated transduction of human and mouse cells [466], the field of AAV gene therapy has undergone extensive evolution towards successful clinical trials [467, 468]. Recombinant AAV vectors (rAAVs) can be produced in helper cell lines by supplying *in trans* rep, cap and helper genes in addition to a plasmid with a transgene cassette incorporated between the ITRs, without the need for replication competent helper or AAV virus in 2- or 3-plasmid systems [469]. Self-complementary rAAV vectors (sc-rAAV) were developed to circumvent rate-limiting second-strand DNA synthesis [470-472]. sc-rAAVs display enhanced transduction in comparison with conventional rAAV vectors, although it limits the transgene size by half [473-475].

The host immune response to AAV-2 hinders the efficient systemic delivery and persistence of rAAV-vectored transgenes, mainly due to cellular and humoral immunity [476-478]. Efforts have been made to overcome this immunity by restricting the transgene expression to the target tissue, codon-optimization, manipulating the rAAV capsid by (directed) mutagenesis, peptide display and chemical conjugation [479-483]. The discovery of novel AAV serotypes has led to the use of tissue specific AAVs, that also can evade pre-existing AAV-2 immunity [484-489].

To date, 109 registered clinical trials in humans have been conducted or are underway [490-493]. rAAVs have shown efficacy of transgene expression in tissues such as liver, retina and brain. However, host and vector-related immunity not observed in animal models have limited duration and strength of transgene expression when administered systemically [494-498]. Therefore, more recent clinical trials are evaluating several other serotypes besides AAV-2 as gene transfer vectors [490]. Glybera (alipogene tiparvovec; an AAV-1 vector expressing LPL) has been approved for treatment of patients with LPL deficiency by the EMA and FDA. It was the first gene therapy product to be approved in Europe and the only one in the USA. Of note, intramuscular injection of Glybera has not led to systemic or local immune responses limiting transgene expression [499].

Generally, rAAV administration appears to be safe. Sometimes, dose limiting toxicity is encountered, but this mainly relates to rAAV-2 vectors or other vectors with pre-existing immunity. Also, random integration events have thus far not resulted in oncogenic transformations in patients. New and generated rAAV serotypes should be evaluated in a human-like model for off-target toxicity, although no good animal model exists for predicting safety. rAAV is replication defective, and as such not directly transmissible. Shedding of rAAV however, can occur around the time of high dose administrations [432].

5.4.17 Rodent protoparvovirus 1 (RPaV-H1)

Recent research has focused on the importance of the immune system in oncolytic activity [500-502], combination with other therapies [503-507], retargeting by modifications of the viral capsid [508] and also arming with anti-angiogenic chemokines or pro-drug converting enzymes [509, 510].

Currently, one clinical trial is active in patients with progressive or recurrent GBM [511]. Also, a case has been reported of compassionate use in an eight year old patient with metastatic neuroblastoma [512]. As with other oncolytic viruses, strategies that include retargeting and expression of immunomodulating or therapeutic transgenes can alter the safety of RPaV-H1, and

should be evaluated accordingly.

5.4.18 Fowlpox virus (FPoV)

Like CPoV, FPoV has been extensively evaluated as a (cancer) vaccine vector. FPoV boosts immune responses against foreign transgenes encoded by the virus and induces a strong T-cell immune response. FPoV vectors can accommodate large amounts of foreign DNA [513]. Also, antisera against orthopoxviruses (like vaccinia) do not neutralize FPoV, and FPoV itself does not elicit high levels of neutralizing antibodies, making it possible to administer boost vaccines without losing potency.

Most recent clinical trials have employed prime boost tumor-antigen vaccination schemes using vaccinia virus prime and multiple FPoV boosts [514-526]. A phase III trial with Prostvac +/- GM-CSF for metastatic castration-resistant prostate cancer is currently recruiting patients [527]. Trials employing intratumoral injection of FPoV vectors harboring immune-stimulatory molecules have also been conducted, with limited T-cell responses [528].

Theoretically, no replication of FPoV in humans is expected and thus no shedding can occur beyond the administration site. Spontaneous recombination between FPoV vectors and wild-type viruses or mutation events could theoretically lead to restoration of replication competence. During the design of FPoV vectors, the aim was to introduce at least two gene deletions critical for viral replication, limiting this risk. Reversion to wild-type virus has not been observed in clinical trials of FPoV vectors.

5.4.19 Myxoma virus (MyxV)

Recent research has added more evidence for oncolytic activity of wild-type MyxV in mouse models for leukemia, multiple myeloma, pancreatic cancer, brain tumors and ovarian cancer [529]. Genetic modification of MyxV has mainly been used to study host range restriction, although oncolytic efficacy of deletion mutants has also been evaluated [530-532]. Recombinant MyxV producing vaccinia virus F11L gene, which is related to virus exit and spread, was shown to produce bigger and faster growing plaques, resulting in higher viral titers and better oncolytic activity [533, 534].

5.4.20 Vaccinia virus (VV)

VV infection is highly immunogenic and produces a strong cytotoxic T cell (CTL) response and neutralizing antibodies [535, 536]. As such, VV vaccine vectors have been evaluated for infectious diseases (beyond the scope of this report) and cancer [537-543].

Several strategies have been described to target oncolytic VV to tumor cells [544-553], as well as arming of VV with transgenes. Although VV can travel systemically through the blood, efforts have also been made to enhance the ability of VV to evade premature removal by the hosts innate (complement) and adaptive immune responses after first delivery. Interestingly, different strains can be used as backbone for oncolytic VV, which can differ in their efficacy, although direct comparison between strains has not been described very often [554].

GLV-1h68 (GL-ONC1) was created by inserting three reporter proteins, RUC-GFP, lacZ and gusA into the F14.5L, J2R and A56R loci of the VV genome, respectively, while harboring several other attenuating mutations [555, 556]. GLV-1h68 has undergone extensive preclinical

testing in models for several cancer types with promising results[555, 557-570].

GLV-1h68 (GL-ONC1) is currently active in several phase I clinical trials [571, 572]. JX-594 (TK gene deleted, GM-CSF expressing VV Wyeth; Pexa-Vec) [573-575] has been evaluated in phase I-II clinical trials for patients with metastatic melanoma, (primary) liver tumors, lung, colorectal and various other solid cancer types [576-582]. A phase II clinical trial for patients with peritoneal carcinomatosis of ovarian origin is not yet recruiting patients. Clinical trials with oncolytic VV thus far have reported good safety with minor side effects like transient low-grade fever and local pain, although reactive tumor swelling did result in dose-limiting hyperbilirubinaemia in patients with injected liver tumors [579].

Commonly, live vaccinia virus is shed from skin injection after vaccination [583, 584]. Also, in clinical trials, live JX-594 was detected in throat swabs and skin pustules of patients up to one week after administration [582]. Theoretically, recombination between oncolytic recombinant VV and wildtype VV is possible, however, since VV vaccination is not practiced on a large scale anymore, this event is highly unlikely. Spontaneous mutation rates for VV have been shown to be very low [585].

5.4.21 Reovirus (mORV)

mORV-T3D replicates in cells with dysfunctional cell signaling cascades, most importantly (but not exclusively) kRAS-overexpression and subsequent PKR inhibition, making it an inherent oncolytic virus [586-603]. A multitude of cancer types have been shown to respond to mORV-T3D treatment in (animal) models. Combination therapies have been evaluated, as well as carrier cell delivery. Cellular immunity has been found to be important for anti-tumor efficacy [604-610]. Other mORV subtypes and attenuated strains have also been evaluated for oncolytic efficacy [611, 612].

The absence or inaccessibility of the JAM-A/1 receptor is perceived as a possible limitation for mORV-T3D infection of tumor cells [613-615]. As such, bio-selection through passaging has been attempted to retarget mORV-T3D to other receptors, although this strategy is probably limited by the quasispecies presence in mORV-T3D isolates [616-620]. The segmented genome of mORV-T3D makes reverse genetics challenging, but several techniques have been described to overcome this challenge [614, 621-626]. Only one study using recombinant oncolytic mORV-T3D has been described thus far, and more can be expected in the near future, probably focusing on receptor retargeting and expression of therapeutic or imaging transgenes [627].

At this time, 16 clinical trials employing intratumoral or intravenous injection of mORV-T3D (REOLYSIN®: pelareorep) have been conducted and more (n=15) are currently underway or planned to start in the near future. The already conducted trials have shown that administration of mORV-T3D in patients is safe for various solid tumors without dose limiting toxicities, while at the same time the virus is having some appreciable anti-tumor effects in phase II/III trials [628, 629]. Limited mORV shedding has been observed in clinical trials in patient samples of urine, saliva and feces, mostly with high i.v. administrations [629].

As an RNA virus with a viral RNA polymerase, mORV genome replication is prone to errors. Furthermore, since wild-type isolates are in use, these probably represent several quasispecies [630]. Even so, since mORV-T3D does not seem to cause disease in human subjects, the relevance of this mutation rate is low.

5.4.22 Rous sarcoma virus (RouSV)

Similar to HIV-1 development (see paragraph 5.4.25), SIN-RDR-RouSV vectors have been recently developed by eliminating enhancer and promoter elements from the LTRs, by using split packaging systems and removing additional viral-coding sequences and retroviral splice sites from the vector [631, 632]. This SIN-RDR-RouSV vector has been evaluated in preclinical (animal) models for X-linked chronic granulomatous disease (X-CGD) [633]. Integration profiles of alpharetroviruses including RouSV are much more neutral than gamma- or HIV-1 based retroviruses [631, 634-636], and it is possible that these retroviruses will receive more attention and development in the near future.

5.4.23 Murine leukemia virus (MuLV)

MuLV development has been focused on non-replicating as well as more recently replicating vectors. The capacity of MuLV and other retroviruses to integrate into the host genome of dividing cells carries the risk of insertional mutagenesis/oncogenesis. Reducing this risk was an important goal in designing the first (and more recent) retroviral vectors [637-639].

RDR-MuLV vector genomes generally have a *cis* retroviral genome sequence driving transgene expression and packaging, and virions are produced by providing structural viral genes (i.e. Gag, Pol and Env) in *trans* in vector-producing cells [640]. RDR-MuLV virions are capable of infecting and integrating their genomic transgene into host cells, while viral replication is not possible due to the lack of structural viral genes in the host cell. This results in a fairly limited gene transduction rate, which can be enough for correction of (mono)genetic diseases, but mostly will not result in proper anti-cancer activity [641]. Clinical trials using these first generation RDR-MuLV vectors to correct monogenic diseases like X-linked SCID (X-SCID) in children have been complicated by cases of acute leukemia due to proviral integration in (the proximity of) proto-oncogenic loci [52, 54, 642, 643]. Similar observations have been made in clinical trials for patients with X-chromosome linked chronic granulomatous disease (X-CGD) and Wiskott-Aldrich syndrome (WAS) [53]. This has led to a general reconsideration of RDR-MuLV vector design.

So-called self-inactivating (SIN-)RDR-MuLV vectors lack enhancer regions and use an internal promoter and enhancer to drive transgene expression [644-646]. However, also these SIN-RDR-MuLV vectors have been shown to possess oncogenic activity (although considerably lower), and their integration profile does not differ from RDR-MuLV vectors [645, 647-652]. Besides strong ubiquitously active viral promoters, other weaker (cellular) promoters can be used in SIN-RDR-MuLVs to achieve transcriptional targeting [648, 650, 653-655]. Incorporation of WPRE or codon optimization leads to increased transgene mRNA stability, export and translatability, needed to optimize transgene expression from these relatively weak (targeted) cellular promoters [656-658].

Integration of RDR-MuLV vectors favors promoter and enhancer regions [659-661]. Targeting of RDR-MuLV integration to pre-selected locations of the host genome by swapping integrases between other retroviruses or fusing the integrase with sequence specific DNA-binding domains has been evaluated with limited success [662, 663]. Another safety issue of (SIN-)RDR-MuLV vectors consists of an increased polyA signal read-through in the 3'-LTR, which may result in the activation of normally silent oncogenes upon integration [664]. The deletions in SIN-RDR-MuLV LTRs actually increase this risk [665, 666].

To circumvent genotoxic events associated with integration, non-integrating (episomal)

RDR-MuLV vectors have also been developed to achieve transient gene expression by mutating the viral integrase, leading to a marked reduction of viral (but not spontaneous) integration, which can be combined with strategies like ZFN or SB [667-671]. Chromatin insulators have also been described as means to dampen genotoxicity [672].

Regarding the application of MuLV vectors in cancer patients, the replication capacity of RCR-MuLVs is considered to be beneficial to optimize gene expression in tumors. This stems from the fact that for oncolytic activity, gene expression does not have to be long lasting, but preferably strong. More recent RCR-MuLV vector genomes consist of an intact viral genome including an IRES-transgene immediately after the stop codon of the *env* gene, which results in more genetic stability, while retaining good replication capacity [673-675]. The fact that RCR-MuLVs can only infect and integrate in dividing cells results in an inherent onco-selectivity. In contrast to most other oncolytic viruses, the oncolytic activity of RCR-MuLVs depends solely on the transgene that is carried by the virus, since infection itself is not cytolytic. The transgene of choice to date has mostly been CD, which converts the antifungal drug 5-FC into active chemotherapeutic agent 5-FU. Oncolytic activity of RCR-MuLV-CD (Toca 511) has been evaluated in preclinical (animal) models for several cancer types [676-682].

RDR-MuLV vectors have been used in relatively successful clinical trials for monogenic diseases like X-SCID, ADA-SCID, X-CGD, WAS, epidermolysis bullosa, and also multigenic disease like melanoma. However, as noted, oncogenic integration has led to the redesign of RDR-MuLV vectors, and in the meantime lentiviral vectors have taken the lead due to their more favorable integration profiles and ability to transduce non-dividing cells. Even so, the clear clinical benefit of MuLV-vector treated patients with ADA-SCID and SCID-X1 outperforms the results obtained after allogenic stem cells transplantation with HLA-mismatched donors, which make risk-evaluation and patient education important in the possible continued application of this strategy.

RCR-MuLV-CD Toca 511 (vocimagene amiretrorepvec) is being investigated in clinical trials in the United States in subjects with recurrent high-grade glioma. Up to now, over 70 patients have been treated without dose limiting toxicity and with evidence of clinical oncolytic efficacy [683].

5.4.24 Equine infectious anemia virus (EIAV)

Oxford Biomedica uses SIN-RDR-EIAV vectors with transgenes under the control of a CMV promoter and pseudotyped with VSV-G for different indications (LentiVector). Several clinical trials are currently ongoing. The results of the first phase I/II trial of ProSavin (LentiVector expressing normal tyrosine hydroxylase, aromatic L-amino acid decarboxylase, and guanosine 5'-triphosphate cyclohydrolase 1) injected into the putamen of Parkinson's disease patients have recently been reported and showed a very good safety profile with some improvement in motor behavior [684, 685].

In the toxicity study of RetinoStat (LentiVector expressing angiostatic proteins endostatin and angiostatin), very low amounts of vector particles were detected in a minority of non-human primates on day 2 in saliva and eye swabs [686]. In a similar study evaluating StarGen (LentiVector expressing normal ABCA4 gene), no shedding was observed [687]. The clinical study of ProSavin noted only limited detection of viral sequences in urine samples [685].

5.4.25 Human immunodeficiency virus (HIV)

HIV-1 based vectors have been developed most extensively, and were the first retroviral vectors to transduce non-dividing neurons when injected into rat brains [688]. Several generations have now been developed. Transfer vectors can be optimized by incorporation of HPRE, heterologous polyA enhancer elements like SV40 or β-globulin, or the use of different internal promoters [689-692]. Three other major modifications include: firstly substitution of the 5' U3 viral promoter for a heterologous promoter, enabling Tat-independent transcription; secondly deletion of the enhancer/promoter sequence in the 3' U3, resulting in SIN vectors; and thirdly inclusion of the cPPT-CTS sequence exerting a positive effect on transduction efficiency [691, 693-700].

Pseudotyping of HIV-1 vectors has benefited to their tissue tropism, infectivity and selectivity [701-708]. SIV/HIV-2 Vpx protein enhances transduction of myeloid cells by HIV-1 vectors markedly [709-712]. HIV-1 vectors show a preferential integration into actively transcribed genes, and integrate through a largely stochastic process, driven by an active process targeting open chromatin regions in the host cell genome [634, 713-717]. This lowers the risk of genotoxicity [650, 718-720]. Modifications of HIV-1 vectors to more selectively target proviral integration are relatively new and results obtained so far disappointing [721-724]. As an alternative strategy, non-integrating HIV-1 vectors have also been developed to produce transient episomal vectors [725, 726].

Clinical trials using SIN HIV-1 based vectors have been performed for monogenic diseases like β-thalassaemia, WAS, X-ALD and metachromatic leukodystrophy [727-730]. To date, no oncogenic transformation has been reported in these clinical trials with good clinical benefits. An additional trial for ADA-SCID is currently underway [731]. Also, multiple clinical trials have evaluated HIV-1 vectored chimeric antigen receptors expressed by T-cells targeted to CD19 as treatment for advanced leukemia [732-734].

5.4.26 Simian immunodeficiency virus (SIV)

Using similar strategies as HIV-1 vectors, several (SIN) SIV vectors have been created based on SIV-agm and SIV-mac [735-739]. Pseudotyping has been applied to change cell/tissue tropism [740-742]. SIN SIV-agm based vectors have been evaluated for retinal gene transfer in (animal) models for retinitis pigmentosa, as well as for X-CGD, hemophilia and cystic fibrosis [742-748]. Toxicity testing in non-human primates of intraocular administration of a SIV-agm based vector expressing hPEDF showed no dose limiting toxicity, and no shedding [749].

5.4.27 Foamy virus (SFoV)

The most recent generation of SFoV vectors is self-inactivating (SIN), with transgene expression driven by chimeric 5' LTRs with CMV immediate early promoters, and harboring safety deletions in the 3' LTR [750, 751]. SFoV vectors have a much more random integration pattern than gammaretroviruses and lentiviruses, adding to their safety for insertional mutagenesis [752, 753]. In addition, polyA read-through is less as compared to these other retroviral vectors [754]. Therefore, genotoxicity of SFoV vector integration seems to be limited [755].

SFoV vectors have been shown to transduce a multitude of cell types, including

neuronal, hematopoietic and embryonic stem cells [756-760]. In addition, efficacy for treatment of monogenic diseases like SCID, pyruvate kinase deficiency, WAS or Fanconi anemia has been shown in animal models [761-766]. *In vivo* gene therapy for canine SCID-X1 has recently also been described [767]. Non-integrating SFoV vectors with mutations in their integrase have also been developed [768].

In dogs, SFoV vectors have been used to treat different monogenic diseases. Currently, no clinical trials in humans have been performed or are in progress, although a protocol is active for transduction of blood stem cells of healthy volunteers. Zoonotic events do not cause apparent disease in humans. Insertional oncogenesis has not been observed in a clinical trial in dogs, even though the vector was sometimes integrated in the vicinity of growth-promoting sites [769-771].

5.4.28 Semliki Forest virus (SFV)

Oncolytic SFV has been evaluated in (animal) models of hepatocellular carcinoma (HCC), ovarian cancer, melanoma and (metastatic) colon cancer [772-777]. Arming with immune stimulators and TAAs has been described [772-777]. Also, combination therapy with oncolytic VV and miRNA targeting has been attempted [774, 778, 779]. In addition, non-replicating SFV gene therapy vectors (SFV RNA replicons) [780] have been evaluated for stroke, glioma, breast cancer, and melanoma. VV/SFV and hAdV/SFV chimerics have been developed to produce non-replicating SFV replicon delivery systems. No clinical trials has been reported or announced, although clinical trials employing SFV based vaccines are underway (beyond the scope of this report).

5.4.29 Sindbis virus (SBV)

SBV has been evaluated as replicating oncolytic virus in (mouse) models for colorectal cancer, ovarian cancer, and hematopoietic malignancy [781-784]. NK cells have been found to be important for antitumor effects, with improved efficacy of SBV-IL-12 vectors [782, 783], and there are clues that defective IFN pathways relate to SBV oncolytic susceptibility [785]. Arming of an oncolytic SBV with TAAs and miRNA targeting have also been evaluated [786, 787]. Also, more studies have been published on SBV as a non-replicating (cancer) gene therapy vector [788, 789]. Of note, production of replication-incompetent SBV vectors also results in low-level production of replication-competent wild-type SBV through recombination [790].

5.4.30 Venezuelan equine encephalitis virus (VEEV)

VEEV based replicons have been evaluated mostly for vaccination for infectious diseases (beyond the scope of this report), as well as for use as tumor vaccines [791-802]. The apparent superiority of VEEV replicons over other alphavirus vectors in vaccination could be related to the inherent tropism of VEEV for dendritic cells (DCs) [803]. Addition of VEEV vectored IL-12 augmented the anti-tumor efficacy of a VEEV-CEA replicon vaccine [804].

VEEV replicons have been evaluated in clinical trials mainly for infectious diseases, as well as a tumor vaccine, although the cancer vaccine trials described in literature have reported relatively disappointing results [805, 806]. Subsequent clinical trials are currently ongoing.

5.5 *In vitro* models

5.5.1 Three-dimensional cell culture models

The past decades have provided great advances in the understanding of complex human diseases, many of which are currently leading to development of novel treatments with high clinical relevance. Three dimensional (3D) *in vitro* models are increasingly being used to study (cancer) cell biology and the interaction between cells within a microenvironment which is more similar to an *in vivo* situation than the standard bidimensional (2D) cultures. This technology may provide an important link between *in vivo* experimental models, standard *in vitro* cultures and the clinic, thereby possibly accelerating and facilitating the translation of basic advances into innovative treatments.

The types of 3D culture systems to date include: cell spheroids [807], scaffolds like gels, films and fibers [808], organs on a chip [809], organotypic cultures [810] and explant cultures [811]. For a more detailed discussion the reader is referred to the review of Leong *et al* [812].

3D tumor spheroids were initially developed to address radio- and chemo-sensitivity of cancer cells *in vitro* under conditions mimicking *in vivo* features [813]. Most data available on tumor interactions in humans have been obtained by culturing cells isolated from peripheral blood or tumor tissue in conventional 2D *in vitro* cultures. However, they fail to account for critical aspects of the tumor microenvironment which most likely plays a decisive role *in vivo* as suggested by the frequently detected discrepancies between *in vitro* data and the outcome of clinical trials [814]. Murine models have emphasized the poor immunogenicity of tumor tissue fragments, as compared to single cell suspensions derived from the same tumor, thus suggesting a pro-tumoral role for the tissue architectures [815]. It has recently been shown that recombinant adenoviruses are able to effectively deliver transgenes into cultured 3D mini-gut organoids [816]. It is conceivable that effective gene manipulations in such organoids, including overexpression, knockdown and gene editing with TALEN and CRISPR/Cas9 systems, may allow for reconstruction of disease processes and/or development of novel therapeutics.

5.6 Animal models

No currently available *in vitro* system can adequately model whole body physiology or immune system functionality; therefore preclinical studies will still need to be performed in an appropriate animal model. No species is likely to provide the best model for all human diseases, however it would be useful to use a combination of several different animal species in preclinical studies. Comparative analysis of the obtained data can identify evolutionary conserved networks of expression and gene regulation, thereby unraveling the complex interactions between genetic, environmental and lifestyle factors which influence disease pathology.

5.6.1 Small animal models

5.6.1.1 Rodents

Mice, and to a lesser extend rats, are the most common and often the only animal species used for preclinical studies before trials proceed in human subjects. However their usefulness can be debated since they considerably differ in size, general physiology, anatomy and lifespan. In the recent decades genetically modified mice dominated the research into cancer and many other human diseases. This is mostly not because of their predictive value but rather due to their low costs and technical ease with which you can genetically engineer them. A wide variety of research areas is revealing limitations and shortcomings of current mouse models, for example in inflammatory diseases [817]. Preclinical studies can be greatly improved by reducing the overreliance on mouse models. Rats could be an alternative, however they have only recently been genetically modified [818]. Nevertheless, rats still share size, diet and physiology challenges similar to mice.

For the past several decades, investigators have created “humanized” mice to bridge the gap between small animal models and human studies and to examine the human immune system in an experimental setting. Humanized mice were first generated by transplanting human cells and/or tissues into mice with severe deficiencies of their own immune systems [819-821], and this basic approach has continued to the present. Advances in these models have come from gradual improvements in the ability of the strains of immunodeficient mice to engraft donor human cells and tissues, and from refinements in the procedures used to engraft those cells and tissues [822]. One of the major recent advances in this field came from the development of immunodeficient mice with a null mutation in the interleukin-2 (IL-2) receptor common γ-chain locus (IL2Rγc), the use of which has resulted in far higher levels of human cell engraftment than previously possible [823]. The newest addition to these models is the generation of humanized mice that utilize IL2Rγc-null immunodeficient mice, particularly BLT (bone marrow-liver-thymus) and Hu-HSC (human hematopoietic stem cell) mice. BLT mice are generated by co-transplantation of human fetal liver and thymus fragments under the mouse renal capsule followed by intravenous injection of CD34+ HSCs isolated from the same fetal liver [824]. Hu-HSC mice are generated by injecting newborn mice with human HSCs isolated from umbilical cord vein blood [825], granulocyte colony-stimulating factor-mobilized adult blood [826], or fetal liver [827]. However, incomplete B-cell development in both BLT and Hu-HSC mice has been reported [828], which most likely will need to be overcome to further improve the capacity of these mice to model human humoral immunity although human B-cell maturity in humanized mice improves with increasing time following their human cell reconstitution. Several investigators have

already reported strategies that can improve B-cell maturation in humanized mice, including increasing the length of time prior to their use [829], improving T-cell help by adoptive transfer of autologous mature T cells [830], and providing additional human cytokine support [831]. Improving humoral responses in humanized mice will contribute greatly to the ultimate goal of creating a small animal system that can model the human immunity well enough to meaningfully assess candidate human vaccines and other immunotherapeutic strategies.

5.6.1.2 Rabbits

In the context of biomedical research, rabbits are most often thought of as bioreactors for the production of monoclonal and polyclonal antibodies and more recently recombinant proteins. However, rabbits are increasingly becoming a valuable experimental model in their own right and are in some cases, the translational model of choice. Some of the fields for which the rabbit often serves as a primary experimental model include atherosclerosis [832], Alzheimer's disease [833], eye research [834] and osteoarthritis [835]. Rabbits are phylogenetically closer to primates than rodents and further offer a more diverse genetic background than inbred and outbred rodent strains, which makes the model a better overall approximate to humans.

5.6.1.3 Cats

Cats have been considered to be less frequently affected by hereditary diseases. However, at the time of writing this report a total of 318 disorders have been listed of which 182 might be a potential model for human disease [836]. Compared to traditional murine models, the cat demonstrates features in common with humans, including many anatomic and physiologic similarities, longer life span, increased size, and genetically more heterogeneous background. The development of genomic resources in the cat has facilitated mapping and further characterization of feline models. Feline models are mostly used for mimicking retinal diseases such as congenital glaucoma [837]. Only in recent years have specific mutations been elucidated for hereditary retinal diseases in cats [838, 839]. In addition cat models exist for motor neuron transduction [840] and lysosomal storage disease [841].

5.6.2 Large animal models

5.6.2.1 Dogs

Over 50% of genetic diseases present in the dog are true orthologues of human diseases caused by mutations in the same genes. The enormous genetic diversity of canine breeds (many of which have extensive pedigree information) and the broad range of spontaneously occurring canine diseases give researchers opportunities to examine genetic etiologies and explore the possibility of gene therapies. In addition to the more comparable longevity and size of humans they also in part have a similar immune system [842]. Canine models have already served to advance human medicine in a number of areas and have been instrumental in some, such as narcolepsy, hemophilia, retinal degeneration, and muscular dystrophy [843].

Canine oncology is providing a useful complementary perspective in the human cancer field [844]. Dogs spontaneously, and with high frequency, develop the same types of cancers that humans do and are often even treated with the same therapeutic strategies. Additionally, the

centuries of selective breeding of dogs confers opportunities to examine polymorphisms specific to particular breeds that have exaggerated incidences of cancer subtypes. Finally, because dogs cohabitate with their owners, they are both exposed to the same environmental factors that may potentiate the development of cancer. This offers an exceptional opportunity to look at the interactions between genetics and environment in the etiologies of various forms of cancer. A wide variety of cancers are being studied in dogs including soft tissue sarcomas, mammary carcinomas, primary and secondary lung carcinomas, malignant melanomas, and cancers of the prostate, bladder, intestine, brain, mouth, and many others [845]. Naturally occurring tumors in dogs have many clinical and biologic similarities to human cancers that are difficult to reproduce in other model systems [846].

Unfortunately, the genetic diversity is also the main disadvantage of canine models as there are breed-specific differences in physiology and metabolism, especially unusual features in pharmacodynamics and pharmacokinetics, which can introduce complications when interpreting or translating results. In addition, dogs have a slow breeding rate, are scarce and expensive to house. The high cost-high risk aspect can be partially alleviated by first establishing proof-of-principle studies in smaller models and later complement these studies by extending them into a canine model. From the researcher point of view, dogs are particularly difficult to work with since they bond quickly with humans which makes it difficult to maintain objectiveness and can pose psychological dilemmas.

5.6.2.2 Pigs

Pigs are becoming increasingly important in preclinical research. They share many similarities with humans, such as body size, anatomy and physiological and pathophysiological responses. They are also relatively long lived, thereby enabling longitudinal studies in individual animals under conditions which mimic a human patient.

Of the large animal species used for biomedical research, pigs are easily the most popular. There are hundreds of breeds available worldwide, some of which are classified as miniature swine and commonly known as minipigs. Pigs reach sexual maturity early, breed year-round, and deliver as many as 10 to 12 piglets in a single litter. Pigs are large enough and robust enough to tolerate complex experimental protocols over an extended period of time that require multiple interventions, repeated tissue or fluid sampling, and imaging using technologies standard to hospitals. There is broad availability of a range of established cell lines derived from a variety of swine tissues and the offering of pig-specific reagents is expanding. Further, swine genomics and proteomics are more advanced than nearly every other large animal model. Pigs are very similar to humans in various aspects of their anatomy and physiology, diet, metabolism, and histopathology and pharmacokinetics. A wide variety of diseases are studied in pigs including cardiovascular disease, wound healing, melanoma, diabetes, cystic fibrosis and neurodegenerative disease [847, 848]. For cancer studies their longevity means that important clinical parameters can be followed such as tumor progression and remission, response, toxicity or treatment failure as well as developing drug-resistance [849]. For cell and gene therapy pigs are mostly used to model cardiovascular disease and ischemia [850].

5.6.2.3 Sheep

In the case of sheep, the potential for their use in biomedical research lies, above all, within respiratory diseases. The anatomy and physiology of the sheep respiratory system is more similar to that of humans than rodents, and has been proposed as a good model for vaccines, asthma pathogenesis and inhalation treatments. Recently sheep have also been used in gene therapy strategies for cystic fibrosis [851]. Furthermore, it is a large animal (30-90 kg, depending on sex and race) with well-studied anatomy and physiology, is easy to cannulate, and provides ease in taking frequent and/or large samples. Also, it provides a very useful specimen for surgical trials, measuring certain respiratory parameters and many other processes which cannot be carried out in rodent models.

5.6.2.4 Horses

Horses are mainly used as models for osteoarthritis. This is a naturally occurring disease in this species. Adenoviral gene therapy approaches have been used to investigate therapeutic effects *in vivo* [852]. Another disease for which horses are used as an animal model is melanoma. Recently a placebo-controlled study using equine IL-12 and IL-18 reported activation of the immune system and significant tumor regression [853]. Other investigators have developed an experimental protocol involving cytokine-enhanced tumor vaccination plus suicide gene therapy. This led to a significant reduction in tumor size (50- 100%) and perceived improvement in quality of life [854].

5.6.2.5 Non-human primates

The most common non-human primate models are the macaque, rhesus macaque, owl monkey and the common marmoset. Because of their physiological similarities to humans, non-human primates can serve as a valuable translational research model in moving toward early phase clinical trials in humans. The rhesus macaque has been a very valuable model organism for development of novel cell and gene therapies, and analysis of the immune system. Physiological similarity including size and life span, phylogenetic similarity to humans, cross-reactivity of human cytokines and antibodies, completed genome sequencing, and knowledge gained from three decades of macaque immunologic studies stimulated by the HIV epidemic contribute to the utility of this model [855]. Moreover, rhesus iPSCs resemble human iPSCs in terms of morphology, marker expression, and growth factor dependency to maintain their pluripotency [856]. Modeling of all steps in iPSCs derivation, characterization, differentiation, and autologous delivery can be accessed via a new macaque model [857]. The macaque immune system has been well characterized due to the utility of macaques as models for HIV/AIDS and is highly predictive of the human immune response [858]. The size and prolonged life expectancy of macaques allow relevant long-term observation with repeated sampling of administered iPSC-derived cells, which is very difficult in murine models or in a clinical setting.

Although nonhuman primates seem a perfect model there are some drawbacks. For example, endemic viruses can pose a problem in viral gene therapy trials. The presence of wildtype virus in an animal model poses a risk of recombination between wildtype virus and the administered viral vector. It may also mean that the infected primates have circulating serum antibodies to the administered vector. These antibodies may cross-react and it is currently

unclear which effect this may have on the efficient engraftment of gene-modified cells.

5.6.2.6 The general weaknesses of using large animal models

In general, there is a lack of biological reagents for many large animal models compared to rodents. However, more are becoming available, particularly for sheep and swine. Recently, many of the large animal genomes have been sequenced, or are in the process of being sequenced, which will further advance research. Larger animal models are not inbred, so there is more genetic variation and “noise” in an experiment. Also from a genetic standpoint, the ability to manipulate gene function in rodent models is easier than in most domestic species. However, comparing conserved sequences and identifying conserved gene function across a variety of mammalian species may be beneficial in extrapolation of this information to function in humans.

Housing facilities for large animals may also be an issue at some institutions, and costs per animal are usually greater than in rodents. These are variable, but prices are in the hundreds of euros per animal, about ten times that of mice. It is good to keep in mind that in some cases multiple samples can be taken from the same animal without sacrificing them, and a greater amount of sample material may be obtained, which could allow research to be conducted with fewer numbers of animals compared to a situation in which rodents are used. Particularly in the case of horses, animal costs can be significant and having an adequate number of animals can be a problem for some projects. Animal care and use protocols and regulations are similar to those for rodent models. Lastly, large animals have significantly longer generation intervals, which can be problematic for some projects.

5.7 Animals as target for therapy

5.7.1 Small domestic animals

Cancer is among the top fatal diseases in both domestic as well as wild dogs and cats [859]. Incidence of canine or feline cancer ranges from 1% to 2% and cancer currently accounts for about half of the deaths of domestic animals older than 10 years [860]. The most common forms of cancer in dogs and cats are skin, lymphoma, mammary, bone, connective tissue, and oral cancers [860]. The traditional and established methods for pet cancer treatment include surgery, radiation therapy, chemotherapy, hyperthermia and photodynamic therapy. However, the available treatment options for pet patients with advanced-stage disease are limited and the prognosis for such patients is very poor. Therefore, developing novel therapies, which may also work synergistically in combination with the conventional treatment options, is crucial. One promising new therapeutic approach is oncolytic virotherapy. In contrast to the progress of human oncolytic virotherapy, there are very few clinical trials using OVs for canine or feline cancer patients [861]. Since many forms of canine or feline neoplasms resemble their human counterparts in histological appearance, tumor genetics, biologic behavior, pathologic expression, recognized risk factors and response to therapy [862], it is reasonable to expect that the human clinical protocols will be transferred directly to the treatment of pet cancer patients.

Many wildtype or recombinant viruses have been tested as oncolytic agents for treatment of canine or feline cancer. They include human and canine adenoviruses, canine distemper virus (CDV), reovirus and members of the poxvirus family such as vaccinia virus, recombinant canary poxvirus (ALVAC), NYVAC (derived from the Copenhagen vaccinia strain) and myxoma virus. The major obstacles that restrict the optimal use of oncolytic viruses as therapeutics for canine or feline cancer patients are viral toxicity, ineffective delivery of virus to tumor and inefficient spread of OVs throughout the tumor mass. Oncolytic viruses also raise new biosafety and risk management issues [863]. The risk assessment for trials with these agents must take into account and mitigate the potential risk of transmission of the infectious agent to other pets and persons in contact with the treated patient. The zoonotic aspects or risk to pet owners and general public has to be monitored. The spectrum of diseases caused by parental viral strains in dogs or cats is an important safety factor for consideration. If necessary, the risk of disease or adverse effects from a viral therapeutic could be countered with antiviral agents effective against the viral strains considered for cancer treatment.

5.7.2 Large domestic animals

Cattle and other livestock will mostly benefit from DNA vaccination strategies, which lies out of the scope of this report. However horses are animals which could therapeutically benefit from gene therapy. Degenerative joint disease is a common cause of lameness in horses. Stem cell therapy for tissue regeneration is rapidly gaining momentum as the treatment of choice for many equine orthopedic lesions. Lameness due to osteoarthritis has long been regarded as a leading cause of reduced or lost performance in horses and a significant reason for economic hardship to the equine industry. MSCs have been in clinical use for equine orthopedic injuries as early as 2003, with only a handful of research publications in print at that point. Since that time, the clinical use of MSCs has exploded, with thousands of horses treated all over the world [864]. Basic research has also expanded, but it lags substantially behind the rapid product development

and clinical experimentation. MSCs are used to treat acute and chronic, primarily orthopedic, lesions, including tendinopathies, ligament injuries, fractures, laminitis, and joint diseases, such as subchondral bone cysts, meniscal tears, and cartilage defects. Similar to the human setting, MSCs are being genetically modified in the hope of augmenting their healing capacity or as a specific way to deliver gene therapy [865]. For example, the osteogenic potential of BM-MSCs may be augmented by genes encoding bone morphogenetic proteins (BMPs).

Gene therapy for equine corneal disease is a relatively new application [866]. Common equine corneal diseases include traumatic, inflammatory, neoplastic, and infectious keratidities. For many of these conditions, particularly when severe, therapeutic goals are directed at preserving the globe and relieving ocular discomfort and to a lesser extent toward re-establishing clear corneal transparency. Many of these keratidities require labor-intensive treatments which can be difficult for both horse and owner. Gene therapy could provide an alternative to traditional small molecule therapy or be used adjunctively to improve corneal transparency and long-term visual success.

6 Discussion and Conclusion

To complete the picture concerning the topics described in this reports some points of concern will be discussed. These concerns will further clarify the risks which are involved when these techniques or vectors are used within a gene therapy setting.

6.1 General research techniques

6.1.1 Engineered nucleases (general information in paragraph 5.1.1)

Immunogenicity

Since engineered nucleases are derived from foreign species it is possible that they can trigger an immune reaction in the host (humans in case of gene therapy). This could happen by direct recognition of the foreign protein components itself, for instance the RNA component of the CRISPR/Cas9 system has potential to induce native immunity by Toll-like receptors. In addition, the corrected gene product itself could be immunogenic, this could be especially true for correction of genetic defects present since birth. Another possible immunogenic trigger is repeated exposure to engineered nucleases which can lead to build up of immunogenicity.

Delivery to the target site

Gene delivery is one of the most challenging processes in gene therapy. To achieve maximum therapeutic efficacy the engineered nucleases must be delivered in a non-toxic, and preferably, non-invasive way. For *in vivo* gene therapy, viral vectors are currently most effective. MNs [43, 44], ZFNs [33] and CRISPR/Cas9 [31] can be delivered using retroviral or lentiviral vectors, however, TALENs consist of too repetitive DNA sequences due to which there is an increased risk of rearrangement during viral packaging [867, 868]. Although AAV vectors have a very high infection rate in most tissues and are the preferred vectors for gene therapy trials they are probably of limited use for packaging TALENS and CRISPR/Cas9 due to their small packaging capability of 4.7 kb (including promoter and expression components). Adenoviruses have a bigger packaging capacity and reasonable infectivity but they possess relatively high immunogenicity and cell toxicity, especially when higher doses needs to be administered. For *ex vivo* gene therapy cells could be transduced using electroporation- or lipofection based DNA transfection methods. The important factor to keep in mind is that the transfection method needs to be determined depending on which nuclease is used and which cell type needs to be targeted.

Off-target mutagenesis

The biggest concern regarding the use of engineered nucleases in a clinical setting is the possible toxicity which can arise due to off-target mutagenesis. The indels generated by target-site dependent and independent cleavage may damage tumor suppressor genes and induce a growth advantage which could lead to tumorigenicity. Off-target disruption of genes important for normal cellular activity is less likely to be a problem since these cells will probably be eliminated by cellular homeostasis. Random integration of donor DNA is also a concern since HR mediated targeting is not 100% efficient.

6.1.2 Barcoding (general information in paragraph 5.1.2)

General

The main issue with barcoding at the moment is that during the registration process it is not allowed to jeopardize the therapeutic identity of the gene therapy product in any way. Since adding a short barcode tag elicits changes in the genetic makeup of the vector this is currently not allowed for clinical application. To overcome this problem the FDA as well as the EMA should engage in a discussion with researchers and physicians in order to see if it is possible to integrate barcoding in such a way that every party will be satisfied.

6.1.3 Antisense oligonucleotides (general information in paragraph 5.1.3.1)

General

The attractiveness of RNAi relies on its efficient and specific gene silencing. However, a number of barriers still need to be overcome before broad application will become a reality. Instability and low bioavailability, off-target effects, immune responses and delivery problems are the main barriers. Although siRNA nanocarriers could render powerful cell modulators, loss of activity and low transfection efficiency have not yet permitted their way to routine clinical practice. Viral vectors, despite the recognized efficacy, have been related to immunogenicity triggering toxicity and thus require a reassessment of their utility. Non-viral based carriers can also present toxicity problems such as hematotoxicity, complement activation, carcinogenicity, teratogenicity and immunogenicity, however cell-based delivery systems might be the next generation strategy for systemic delivery of RNAi therapeutics.

Delivery

Systemic delivery of nucleic acids exposes them to nuclease degradation, which makes systemic delivery of RNAi complicated and challenging. In addition it has been shown that systemically delivered siRNA preferentially accumulates in the kidney and is excreted in the urine within one hour. As a result, target site accumulation to the level of a therapeutic dosage will be a major problem in the clinic [869]. Delivering the RNAi within a nanoparticle shields the RNA from degradation, however these NPs increase non-specific uptake by innate immune cells such as monocytes and macrophages [870]. The major bottleneck however is the delivery of the molecules to the target cells. siRNAs do not readily cross the cellular membrane due to their negative charge and large size. Endocytosis is thus the major route of cellular entry for non-viral siRNA delivery. siRNAs which fail to be released from the acidic endosomes or lysosomes will be degraded. Therefor delivery strategies must provide possibilities for endosomal escape.

Off-target effects

Like other gene silencing methods RNAi also has off-target effects. miRNA-like binding in the 3'UTRs is a major cause for this [871]. Another problem is saturation of the miRNA machinery which leads to dysfunction of the therapeutic miRNAs [872]. These unwanted effects can be influenced by chemically modifying residues within the siRNA itself or by lowering the concentration of the given siRNA.

Immunogenicity

Systemic nucleic acid delivery activates innate immune responses leading to unexpected toxicities and significant undesirable side effects. Systemic administration of miRNA duplexes can trigger secretion of inflammatory cytokines and type I interferons through Toll-like receptors [873]. The issues described above can be overcome by dose reduction, the use of biocompatible carriers or chemical modification of the siRNAs. Despite the improvements infusion-related reactions or complement activation are still the most commonly observed adverse effects in clinical trials [874].

6.1.4 Genetically engineered T cells (general information in paragraph 5.1.3.2)

Off-target effects

The side effects observed in the setting of adoptive T cell therapy with genetically engineered T cells can be classified in 3 major categories: (1) on-target toxicity due to the expression of the CAR or TCR recognized target antigen in normal tissues, (2) off-target toxicity resulting in damage to tissues and organs that do not express the CAR or TCR recognized antigen and (3) conditioning toxicity due to side effects of the lympho-depleting treatment used to facilitate the engraftment of the adoptively transferred T cells.

Recently there were several reports of lethal cases in TCR trials including cardiotoxicity and neurotoxicity. These may be caused by *in vitro* mutagenesis and subsequent cross-reactivity as well as unrelated protein expression [875-877]. Two recent CAR-engineered T cell trials reported serious adverse effects associated with a fatal outcome. Of these fatal outcomes one was most likely due to on target toxicity [878] and the other cause of death is still unknown [879].

Several strategies have been pursued to enhance safety of genetically engineered T cells, among which are suicide gene strategies which can eliminate T cells upon certain drug administration, reduction of TCR mispairing by using disulphide bonds or ZFN manipulation, decrease risk of oncogene activation by using alternative vectors, inducible promoters to be able to turn off transgene expression, dual target antigens so that an effective response can only be achieved by two antigens and combinatorial signaling strategies which separate two signals in two CAR .

In addition to safety issues there are also several approaches to enhance effectiveness of the engineered T cells. These include:

- modifying other T cell features such as proliferation capacity
- co-stimulation of T cells with cytokines in such a way that T cells are directed against a more primitive phenotype which can result in greater expansion and persistence *in vivo*
- pre-selection of T cell subsets prior to gene transfer to obtain predefined cell pools with predictable behavior
- modifying the host environment by pre-conditioning before T cell transfer in order to enhance efficacy
- modifying the host environment by supportive treatment after T cell transfer to increase survival of the administered T cells

6.2 Cell-based delivery systems

6.2.1 iPSCs (general information in paragraph 5.2.1)

General

There are many challenges which should be addressed before iPSC-based therapy will become available in a clinical setting. Preclinical evaluation should include:

- selection of the animal species which are used for preclinical experiments. Both choice of disease model as well as immune competence or immunosuppressed status should be considered
- safety studies which assess toxicity, genetic/epigenetic stability, proliferation behavior (including excessive proliferation as well as de-differentiation), tumorigenicity, immunogenicity, disease transmission, off-site effects and contamination
- biodistribution studies including engraftment and survival of introduced cells
- proof-of concept studies which will show delivery options, optimal dosing, biomarkers, endpoints, mechanism of action, clinical indications and efficacy

Current concerns for the clinical application of iPSCs are their low reprogramming efficiency, the use of reprogramming factors which are associated with cell proliferation and tumorigenesis, potential leaky expression and the use of integrating viral vectors for reprogramming. These issues are currently under investigation and further optimization of iPSC generation techniques should address these problems in the future. Optimization will include:

- changes in reprogramming factors [880]
- increasing efficiency of reprogramming by use of specific pathway inhibitors (e.g. miRNA [881] or lincRNA [882])
- use of different reprogramming vectors (e.g. non-integrating vectors [883], Cre/Lox systems, piggyback transposon systems, recombinant proteins or synthetic RNA technology [884])

Mutagenesis

Genomic mutations represent a serious risk for clinical applications, however complete prevention of mutations is hardly possible. The task is to come up with strategies to monitor and evaluate tolerable levels of genetic changes and to evaluate their consequences. Numerous studies have compared mutation rates between the original somatic cells and the derived iPSCs to be able to see at which stage reprogramming affects genomic stability [885]. The major source of mutations seem to be carryover aberrations from the original cell source, mutations acquired during cell reprogramming, insertional mutagenesis due to the transgenes which are used for reprogramming and passages in cell culture [886, 887]. It has been shown that mouse derived iPSCs have a significantly lower mutation rate compared to human cells [888] therefore it is of importance to do comparative analysis of cell derived from different species to design useful preclinical studies which can predict the outcome in human trials. Epigenomic instability has also been reported for iPSCs [889, 890]. Recently it was shown that there were residual specific epigenetic marks from the somatic cells of origin (probably due to incomplete reprogramming [891]) as well as new methylation patterns detectable in iPSCs [892].

iPSCs share significant similarities with cancer cells such as self-renewal capacity, rapid unlimited proliferation, high telomerase activity, expression profiles and epigenetic signatures [893]. Since iPSCs themselves are not intended to be used for therapy the major concern relates mainly to the possible contamination of differentiated progenitors with mutated pluripotent cells. Development of highly sensitive methods for detecting and separating undifferentiated cells will be needed to overcome this [894]. Another approach could be increasing the copy number of tumor suppressors [895] or the use of specific drugs as metformin [896] and pluripotent cell-specific inhibitors [897]. An additional way to safeguard iPSC derived cells from overproliferation or teratoma formation after transplantation is the insertion of inducible suicide genes that can be specifically regulated by using prodrugs [177, 898, 899].

6.2.2 MSCs (general information in paragraph 5.2.2)

Future application in the clinic seems promising however there are still some outstanding issues which need to be resolved before actual application is possible. The main challenges include: poor MSC retention *in vivo*, poor engraftment, viability and function *in vivo*, unclear mechanism of action, safety assessment, and lack of standardized clinical trials.

Mutagenesis

There is some evidence that MSCs can enter early stages of carcinogenesis through spontaneous transformation. Both *in vitro* and *in vivo* studies in rodents have shown that during long-term culture of MSCs chromosomal aberrations are acquired which subsequently lead to malignant transformation [900]. Also human derived MSCs are able to undergo spontaneous transformation after long-term expansion *in vivo* [901]. This of course raises concerns that transplanted human MSCs could undergo transformation in patients. Although results from clinical trials performed up till now do not show any tumor formation in treated patients the numbers of patients who are treated is still too low to definitively state that there is no risk of transformation. Larger numbers of clinical trials, longer follow-up times, improved registries and predefined follow-up protocols will need to address this issue in the future [902].

Since MSCs can be genetically engineered this poses a biological safety concern. The random integration of vectors could increase the risk of insertional mutagenesis. However, the rapid development of non-viral vectors and targeted delivery strategies may overcome this problem in the near future.

In vitro expansion of MSCs is conventionally achieved in medium containing FBS and additional growth factors. However, for broad clinical application serum contact must be minimized due to the risk of viral contamination and genetic instability. Currently expansion of MSCs in serum-free medium is not a solution since attachment to the culture dish needs the addition of fibronectin which contains components of human origin. The production of safe cell products will require quality approved process which makes sure that the cells maintain overall phenotype, functional potential, are not contaminated with microbes and remain untransformed.

6.3 Non-viral vectors

6.3.1 Exosomes (general information in paragraph 5.3.1)

Delivery

Successful delivery of substantial amounts of therapeutic cargo highly depends on an efficient loading method. The approaches for loading external cargo into exosomes include both classical cell transfection methods (e.g. electroporation) as well as transfection or activation of the exosome producing cells [903]. The most common method for encorporating of cargo is via genetic fusion of the cargo-encoding gene to a gene known to encode a protein which localizes to exosomes. Several exosomal targeting genes are used for this, e.g. lactadherin [904] or Lamp2 [905], however these fusion proteins are displayed on the outside of the exosomes, fusion of peptides to proteins that localize to the exosome lumen has not yet been investigated.

The mechanisms of exosome uptake include endocytosis, macropinocytosis and phagocytosis [906]. Cargo of exosomes is mainly delivered to the cytoplasm of the recipient cell. Enhancing uptake starts with correct targeting as described above, and can be further optimized by functionalizing exosomes with cell-penetrating peptides [907]. These peptides may induce direct fusion between exosomes and the recipient cell's outer plasma membrane leading to escape from the endosomal system thereby increasing delivery of cargo to the cytoplasm. When the mechanisms mediating intracellular delivery of exosomes become more clear it may be possible to develop synthetic exosomes [908]. Liposomes, or other lipid vesicles, could be altered to match lipid composition of exosomes or they could be fused with proteins that confer immunosuppressive properties. These synthetic vectors could be a valuable tool for future gene therapy strategies.

Targeting

The next challenge after loading the exosomes with the cargo of choice is to target them to specific cells types or cellular sites. Several strategies could be used to achieve this. Exosomes can be tagged with virus-derived proteins and peptides which are developed to precisely target to specific cell types [905, 909]. Although no immune responses have been observed yet, it must be kept in mind that viral components could be able to promote an immune response against therapeutic exosomes. A non-viral alternative could be using engineered peptide ligands such as antibody fragments for specific epitopes displayed on target cells. This strategy is currently being applied to target nanoparticle drugs in clinical trials [910] and could be a good candidate for exosome targeting as well. Another alternative could be phage display in which the displayed peptide targets specific cells [911].

Production

It is known that large-scale production of exosomes can be achieved by oncogenic immortalization of human stem cells [912]. However this technique relies on the use of an oncogenic lentivirus which abolishes the idea of using exosomes as a non-viral carrier in the first place.

Exosome content is strongly influenced by the producer cell from which the exosome is derived. Even exosomes derived from a single source can exhibit a multitude of effects on recipient cells. Therefore, developing safe and effective exosome-based techniques will require both a correct choice of producer cells as well as analysis of exosome content and their biological

effects on receipt cells. Currently most clinical strategies for biological gene transfer make use of autologous cell-based therapies. However it is not yet clear if autologous cells must be used when considering exosomes since there is some evidence that exosomes from other species may be tolerated to some extent [105]. It is highly probable that hybrid exosomes will be engineered where the source of exosomes would be the patient's own cells, but these would be optimized *ex vivo* by incorporating specific receptors or modifying the payload [913, 914].

6.3.2 Transposon systems (general information in paragraph 5.3.2)

As with all gene transfer methods several points will have to be considered before transposon systems can be extensively used for gene therapy. Important points are transposition efficiency, site specific targeting, genotoxicity, gene expression and potential silencing.

Delivery

In addition to the above mentioned concerns, the need of the helper plasmid which is needed for carrying of the transposase gene can be another drawback. During transposition the terminal domains are integrated into the host cell genome alongside the delivered transgene. Therefore, similar to integrating viruses, they deliver a significant amount of DNA to the target cell genome. These sequences may potentially increase the risk of cell transformation due to their retained promoter and enhancer activity. Previous attempts to minimize the size of the terminal domains resulted in a substantial decrease in transposition efficiency [915]. Recently a minimal PB vector has been engineered with very small terminal domains and an extra PB sequence which no longer incorporates into the host genome and thus reduces the risk of target cell transformation [916]. This modified plasmid can also be used as a single-plasmid system which makes the vector potentially useful for *in vivo* application. To further optimize transposition efficiency different promoters can be used which drive transposase expression.

Off-target effects

To minimize adverse events due to random integration of transgenes, transposon systems are engineered for site-directed integration. The most used method for engineering is fusion of DNA binding domains to the transposase. SB has been engineered to bias integration into plasmids containing target sites [917] and near selected (repeat) elements in the genome [918]. The PB system seems to be more suitable for modifications to the transposase since the addition of domains to the transposase does not alter the efficiency [919]. PB has been engineered by Gal4 fusion to bias integration near Gal4 sites in the genome [920], zinc finger protein has been fused to create a chimeric transposase [919] and transcription factor DNA binding domains have been fused to label nearby transcription factor binding sites in the genome [921]. In the coming few years further engineering of both the transposase and the transposon may overcome the need for targeting machinery to integrate the transposase.

Once they are integrated into the genome, transgenes may undergo silencing due to epigenetic effects [922]. It is possible to circumvent this by integrating chromatin control elements, such as insulators, into the transposon cassette. Insulators prevent silencing of the transgene, thereby mediating higher or more persistent levels of expression. Several insulator sequences have been assessed in various systems, however a comparison of their potency is not yet available [672].

6.3.3 Nanoparticles (general information in paragraph 5.3.3)

General

While NPs show enormous potential to enhance gene therapy they also pose some toxicity associated risks. Because of their size, NPs are able to move through the circulatory and lymphatic systems, ultimately ending up in body tissues and organs. Depending on their composition and size they could possibly induce irreversible cell damage by oxidative stress and/or organelle injury [923].

Toxicology

In vitro there seem to be little barriers to the application of NPs due to the simple and well controlled environment. However translating this to a highly complex and variable *in vivo* environment requires a thorough understanding of kinetics and toxicology of the NPs. Although there is an increasing number of toxicity studies performed *in vitro*, the wide range of NP concentrations, the various cell lines, incubation times and assays used makes it difficult to interpret the physiological relevance of the cytotoxic results [924]. There is however some evidence that exposure to NPs could be harmful to the brain [925], lungs [926], cardiovascular system [927], gastro-intestinal system [928] and the skin [929]. It is important to keep in mind that not all particles lead to adverse effects and that *in vitro* results can differ significantly from what is observed *in vivo*.

Mediators of NP toxicity include size, chemical composition and shape. Due to the size of NPs they can have the same dimensions as some biological molecules such as proteins and nucleic acids. Key factors in this interaction includes NP dosage, solubility and spreading potential. Some NPs dissolve easily and their effects will be comparable to the chemical they are manufactured from. However, non-degradable or non-dissolvable NPs may accumulate in the body and persist there for a long time, which makes using these kind of NPs risky.

Targeting

One of the greatest challenges which limits the success of NPs is their ability to reach the therapeutic site at the intended and necessary dose while minimizing accumulation at undesired sites. The biodistribution of NPs is mainly determined by the body's biological barriers which consist of the reticuloendothelial system, the endothelial barrier, the cellular barrier and the skin/mucosal barrier. Optimization of NPs via several methods may overcome these biodistribution limitations. It is of importance to make sure that the NPs are not engulfed in the liver or spleen, due to reduction of therapeutic efficacy but also because this leads to effects on organ function (e.g. inflammatory responses or transient cell alterations).

Animal models

The use of healthy animal models for toxicology studies of NPs may hamper the interpretation of the results as some of the effects may only be a risk for susceptible and predisposed individuals, but not for healthy individuals. For instance, age, respiratory tract problems and pollutants can modify the inflammation and oxidative stress responses induced by NPs [930].

6.3.3.1 Liposomes (general information in paragraph 5.3.3.1)

General

The formulation and use of multi-functional, multi-component liposomal nanoparticles, sometimes referred to as theranostics, is increasingly being explored. Formulations that are carried within an individual lipid nanoparticle can be used for site-specific targeting, biomarkers and imaging capabilities, delivery of combinations of therapeutics, and response to external or internal triggers to control drug release. As the complexity of lipid nanoparticles increases, so do the expenses and difficulties associated with their manufacture, quality control, and control over the intellectual property. To compensate for the additional expense, the gains in therapeutic benefits must be substantial. Multi-functional formulations that show only marginal clinical benefits are unlikely to be successful.

Delivery

Furthermore a number of technical problems have to be overcome before liposome mediated gene therapy can be fully exploited. Liposomes are still significantly less efficient than viral vectors in their transfection ability. In addition, the DNA-lipid complexes are not stable in size for longer periods of time. Where targeting is concerned, optimization of targeting techniques is still ongoing, this is also of importance for reducing cytotoxicity when liposomes are systemically administered in patients. In this respect plasmid-liposomes may be more suited for delivery of genetic material via local administration.

Safety

When looking at patient safety the identification of a suitable sterilization method is a major challenge since phospholipids are sensitive to sterilization procedures which involve heat, radiation and/or chemical sterilizing agents. The current method of sterilization after production is filtration through sterile membranes, however this is not suitable for larger vesicles and it is also not possible to remove viruses using this technique.

6.3.3.2 Polymers (general information in paragraph 5.3.3.2)

General

Although polymeric vectors are easy to generate and modify they still have some limitations when applied for gene therapy. Biocompatibility of polymers is influenced by molecular weight, charge density and type of cationic functionalities, structure and sequence as well as conformational flexibility.

Cytotoxicity

Cytotoxicity of polymers is directly related to increased molecular weight. Polycationic polymers constituting nanoparticles undergo strong electrostatic interaction with membrane proteins which can lead to destabilization and ultimately rupture of the cell membrane [931]. Molecular weight of PEG or HPMA co-polymers can be tailored, however these co-polymers are non-biodegradable and thus have the potential to accumulate intracellularly, thereby presenting the risk of a “lysosomal storage disease” syndrome [932]. This is especially a concern when non-degradable polymers are used at high doses and/or repeatedly to treat indications where chronic parenteral administration is required. Preclinical evidence of the induction of intracellular

vacuolation by certain PEG-protein conjugates has increased awareness of the potential problem, and the potential risk of any non-biodegradable polymer (used as a drug or component of a conjugate or supramolecular system) should be carefully considered in respect of dose administered, frequency of dosing and clinical setting.

Challenges

The specific challenges for industrial development and regulatory consideration relating to polymer therapeutics are discussed in the review by Gaspar et al [933].The main points currently under discussion are (adapted from [934]):

Quality

- There are always safety concerns regarding use of novel polymers or block co-polymers (not previously approved for use in man). There is a need to consider critical attributes in respect to safety on a case by case basis.
- Definition of, and minimization of, heterogeneity within complex multicomponent products; even the simplest tripartite constructs comprise a polymer, linker, and therapeutic. Others can additionally contain targeting residues, imaging agents and/or use of complex block copolymers or dendritic architectures. Control of synthetic methods is essential.
- Control of manufacturing on an industrial scale to ensure an acceptable/reproducible specification must be achieved. Scale-up manufacture and purification bring different challenges compared to low molecular weight chemical entities.
- Development of an appropriate formulation (needs to relate to route of administration), to ensure stability on storage and appropriate form at point of administration (e.g. absence of particulates during intravenous administration).
- Development of validated analytical techniques to characterize the polymer therapeutic (drug), the formulation of the polymer therapeutic (drug product), and its stability.
- Understanding the distinction between the “polymer therapeutic” (drug) and the excipients (also potentially polymeric) used as additions during formulation development.

Safety and efficacy

- To ensure meaningful results, only well-characterized polymer therapeutics should be used for all biological studies.
- Definition of and optimization of the critical product attributes controlling safety and efficacy.
- Understanding that the administration, distribution, metabolism and elimination (ADME) of polymer therapeutics will be substantially different from a low molecular weight chemical entity.
- Establishing and validating new *in vitro* and *in vivo* methods to adequately characterize the critical product attributes in preclinical development.
- Design of preclinical safety studies on a product by product, route of administration and clinical setting basis to address any polymer therapeutic-specific safety issues.
- Development of appropriate *in vitro* and *in vivo* models to define efficacy. Models should be validated for the relevant biomarkers of polymer therapeutic performance (not just reliance on existing models typically used to assess low molecular weight chemical entities exhibiting totally different cellular and whole body pharmacokinetics).

6.3.4 Bacterial vectors (general information in paragraph 5.3.4)

Immunogenicity

For some bacterial species significant safety concerns exist concerning their severe pathogenicity and immunogenicity and due to these characteristics they are known to cause life-threatening infections in a clinical setting. Efforts to refine these characteristics have involved pre-treatment measures to make the tumor environment more hypoxic, combination therapies and genetic engineering (e.g. addition of transgenes for use in pro-drug strategies). However some bacterial strains are particularly difficult to genetically modify which hampers their development in terms of expression or delivery of heterologous genes. Nonetheless, there is still cause for optimism with this treatment strategy since genetic technologies are improving rapidly.

Some bacteria are toxic at therapeutic dosages due to their accumulation in liver and spleen after administration. This is true for separate administration but also in combination with radiation or chemotherapeutics. Reducing the dose does reduce toxicity but also lowers efficacy. Other bacteria can effectively colonize tumors but do not produce any therapeutic effect due to their harmless nature. This can be overcome by arming the bacteria with genes encoding for proteins which can induce cytotoxicity.

Gene transfer

However, for any use of recombinant bacteria in humans, care must be taken to prevent lateral gene transfer to other bacteria and to limit environmental spread of the vector. Biological containment strategies may aid in overcoming these issues, where the vector is engineered to survive in the host but not in the external environment where specific nutrients are limiting [935].

6.3.5 Human artificial chromosomes (general information in paragraph 5.3.5.1)

Delivery

Like many gene delivery techniques HACs have several drawbacks which primarily arise due to their large size (> 1 Mb). First they can't be amplified in significant amounts outside eukaryotic cells. Secondly, due to rapid degradation they can't be readily delivered to target tissues or organs via injection into the pericellular space or bloodstream. To overcome this, carrier cells must be used in the form of adult stem cells, embryonic stem (ES) cells or iPSCs. However, there are many issues, particularly concerning the genetic and epigenetic status of these cells following the withdrawal of immortalizing activities which remain to be addressed. Third, the efficiency of the HAC transfer into the desired host cells remains problematic due to their large size.

Future research should focus on the efficiency and safety of delivering HAC vectors *in vitro* as well as in animal models. In addition, transfer protocols should be optimized to make transfer of HACs between cells more easy. Other types of studies will include analysis of mitotic HAC stability and gene expression from the HAC in different types of non-transformed human cells. Furthermore the effect of the extra chromosome on replication and segregation of the endogenous chromosomes needs to be established.

6.3.6 S/MAR based minicircles (general information in paragraph 5.3.5.2)

Delivery

The principle limitation of current S/MAR vectors in general is the low establishment within cells. The establishment of S/MAR minicircles in general is estimated to be less than 5% [936]. This appears to be regulated at the epigenetic level, since treatment of cells with histone deacetylase (HDAC) inhibitors prior to transfection can improve the establishment rate [936]. Further work needs to be done to explore the effects of HDAC inhibitors or other epigenetic factors in this system.

Confirmation of episomal persistence is of great importance for future clinical applications. Currently this is done by either full-length PCR, Southern blot, extrachromosomal DNA extraction or metaphase-FISH. The most comprehensive approach is the FISH method but this method is also the most labor intensive. Future work will need to address a more rapid method to detect persistence.

Off-target effects

Like other gene delivery techniques episomal S/MAR vectors also show integration. This integration can be detected in up to 40% of clones. This poses a risk for tumorigenicity, however the development of minicircles may overcome this problem, since integration is rarely seen in these type of vectors.

Animal models

Very little has been reported on attempts to produce animals that ubiquitously express episomes. Manzini et al. [100] generated transgenic pig fetuses by sperm mediated gene transfer and showed the episome to confer expression of the transgene marker GFP in most cells and tissues. To our knowledge, though, no live animals with episomes have been generated. Recent experiments try to fill this gap, and will allow addressing the question as to whether episomes are stably and ubiquitously expressed and passed on to the next generation through the germ line.

6.4 Viral vectors

For readability, only a selection of viral vectors and especially oncolytic viruses has been included in the main text of this report. For a more detailed description of all relevant vectors we refer to the addendum (available at the COGEM website: www.COGEM.net), which also includes all references for this section about viral and oncolytic vectors.

6.4.1 Non-integrating vectors (general information in paragraph 5.4)

More detailed general information can be found in the addendum. The most widely used non-integrating viral vectors are mostly adenoviruses. Helper dependent pseudotyped HAdV vectors could be of interest for applications of transient gene expression, although the indications for such a strategy are limited.

6.4.2 Integrating vectors (general information in paragraph 5.4)

After successful marketing application of Glybera, more AAV-based vector products can be anticipated to follow in the future. Capsid modifications and coating of virions have been evaluated to overcome pre-existing immunity.

Rous sarcoma virus has a much more neutral integration pattern than other vectors, and SIN RouSV vectors can be expected to be developed towards new clinical trials.

SIN Murine leukemia vectors are currently overshadowed by other vectors with more favorable integration patterns.

SIN EIAV vectors are being developed as basis for different gene therapy trials by Oxford Biomedica, who seem to be fairly successful to date while using local administration. No real vector developments have been reported recently.

SIN HIV-1 vectors are currently the most used integrating vectors in clinical trials. Having a more favorable integration profile than MuLV vectors, while harboring similar expression levels of transgenes, this seems a logical choice. However, other vectors could be expected to overtake SIN HIV-1 in the future, due to more favorable integration patterns and less immunogenicity. Similarly, SIN SIV are currently evaluated in preclinical trials, and clinical trials could arise if found to be safer than SIN HIV-1 vectors.

Foamy viruses have also undergone evolution towards SIN vectors and preclinical evaluations are promising with better integration patterns than SIN HIV-1 vectors. Clinical trials in dogs are currently ongoing, and a clinical trial in humans can be anticipated in the near future.

6.4.3 Oncolytic viruses (general information in paragraph 5.4)

A magnitude of (pre)clinical studies have shown the value of oncolytic viruses as new treatment modality for cancer patients. Of these viruses, several have taken the lead, including an oHSV that is expected to receive FDA marketing approval in the near future.

Human oHSVs are at the front of the line, and have recently undergone a gradual evolution towards more virulent, but targeted agents. Expression of different transgenes, especially immune stimulating has been shown to be very beneficial for immunotherapeutic potential. With a successful phase III trial completed for early generation oHSV, more candidates of newer generations can be expected to follow this lead. Several non-human herpesviruses have been evaluated to circumvent (pre-existent) immune responses, but none seem to fulfill this promise. More virulent oHSVs should be evaluated for patient and environmental safety.

Virulence of NDV remains an important biosafety issue. Several attempts have been made to optimize oncolytic activity of non-virulent strains with limited success. A recent study has shown immunotherapeutic efficacy of non-virulent NDV, and future strategies of conditionally replicating NDVs could revive the interest for the strong oncolytic virus.

Attenuated measles virus is also already in use in clinical trials, and the developers have wisely chosen to incorporate tracking transgenes like CEA and NIS into the virus. Chimeric MeV/VSV viruses are relatively new and could receive more attention and development in the near future, but should also undergo patient and environmental safety testing.

Sendai virus vectors are used as UV-inactivated virions in clinical trials. However, efforts have also been made to render the virus non-transmissible, while arming and retargeting have also been described. Possibly, non-transmissible SeV vectors will be used as oncolytic virus.

High throughput screen of dozens of novel rhabdoviruses has identified Farmington virus

as a new oncolytic agent. Although the origin and nature of FarV is very obscure, a serious effort is now made to develop the virus as treatment for GBM. Obviously, serious patient and environmental safety issues have to be evaluated first.

Similarly, Maraba virus has surprisingly undergone rapid development into clinical trials, without any problem concerning safety testing in non-human primates. However, the information on environmental safety is (very) limited.

VSV has undergone extensive preclinical evaluation and is currently used in phase I clinical trials in humans and dogs. VSV-hIFNβ has been chosen as prime candidate, having shown added safety and immune activation in preclinical studies.

Literally somewhat under the radar, Australian groups have mastered to get apathogenic Coxsackievirus A-21 into clinical trials. Other apathogenic serotypes with less pre-existing immunity in humans can be expected to proceed into new clinical trials in the future.

Also somewhat surprising, poliovirus PVS-RIPO is currently used in a phase I clinical trial for patients with GBM. Exchange of the IRES element with that of rhinovirus type 2 has been shown to attenuate the virus. However, preclinical testing has also shown escape mutants upon passaging, and it is unclear how this relates to ongoing and future clinical trials.

SVV is also under evaluation in several clinical phase I/II trials. Patient and environmental safety have now been shown to be very low, even though also for this virus the origin and nature is very obscure.

Human adenoviruses have undergone an evolution after disappointing results of clinical trials employing early generation conditionally replicating vectors. New generation promoter targeted HAdV can be expected to proceed into (more) clinical trials, and the expression of immune stimulatory transgenes also provides an addition to oncolytic efficacy. Changing viral hexons or pseudotyping of HAdV virions seems important to circumvent pre-existing immunity and liver sequestration, while optimizing (tumor) cell targeting. Non-human AdVs have also been evaluated for this strategy, but so far these type of vectors need a lot more work to come to par with the progress for HAdVs.

Rodent protoparvovirus 1 is undergoing evaluation in a phase I trial for treatment of GBM. Retargeting and immune modulation are new strategies for this virus that could evolve towards the future, also depending on the currently running clinical trial.

Myxoma virus seems to have progressed steadily through preclinical trials, and this might result in clinical trials in the near future. Recombinant viruses with increased virulence and oncolytic efficacy warrant further patient and environmental safety evaluations.

Vaccinia virus is also being evaluated in several clinical trials, with one product expressing GM-CSF. Shedding of VV is commonly seen, and should be taken into account when considering (further) clinical trials with more virulent strains.

Reovirus development has proceeded to include a multitude of clinical trials, all employing wild-type strains. Recently, a reverse genetic system has become available, and in the future it is foreseeable to have more (pre)clinical trials with modified reoviruses.

Replication competent retrovirus Toca 511 is another enigma in the list of oncolytic viruses. Although theoretically dangerous, adequate safety measures have been taken to prevent reversion to wild-type retrovirus and the product is currently undergoing evaluation in clinical trials for GBM.

6.5 *In vitro* models (general information in paragraph 5.5)

3D culture holds great promise for basic preclinical research as well as for drug development. However there are still many hurdles and unmet needs. Many novel 3D culture systems focus on a very specific application [33,34]. By contrast, pharmaceutical industry is searching for a universal standardized 3D culture system for drug development. While in academia the main goal is to create 3D systems with excellent biological relevance, industrial application relies on efficient read-out, automation and acceptable costs. The current drawbacks are summarized below.

- Most of the existing systems fail to mimic the biomechanical characteristics of tissue *in vivo* and thus only represent a static condition [35].
- Animal-derived or human-derived scaffold materials risk the potential transmission of diseases.
- For scaffold-based culture systems, reproducibility between different batches is unsatisfactory, especially if animal-derived components are used. In order to circumvent the batch to batch variability of naturally derived materials, many fully synthetic or chemically defined scaffolds have been developed.
- Commonly used fully synthetic scaffolds are often PEG-based. PEG is cell-compatible but inert. Embedded cells are not able to attach to the matrix without modifications like RGD-sites covalently attached to PEG hydrogels.
- Methods to gently and rapidly recover encapsulated cells (e.g. for isolation of RNA or protein) are missing or still need to be optimized, particularly in scaffold-based systems. However, many different enzymatic and non-enzymatic reagents have meanwhile become available to specifically digest the scaffold without harming the cells, indicating that this may not be an issue in 3D culture anymore in the near future. In addition for assays like luminescent ATP content measurements recovery of cells is obsolete because the reagent penetrates the scaffold to produce a luminescent signal.
- Methods directly applying screening and bioprocessing in 3D culture systems like imaging tools are scarce and face scaffold-typical problems such as autofluorescence of collagenous scaffold.
- Limitations of the scaffold-based 3D culture systems are potential interactions of screening compounds with the scaffold. Scaffold absorption of compounds strongly relies on the compounds properties (hydrophilic, hydrophobic). Therefore, it is important to compare different scaffold- based systems for their absorption properties of compounds or to switch to scaffold-free 3D culture systems without additives

6.6 Animal models and animals as target for therapy (general information in paragraph 5.6)

In the field of small animal models there is special interest for generating robust immune competent models which adequately mimic human disease. Also the development of models which can be used for testing oncolytic virotherapy is currently under investigation. In addition, many new rodent models are expected to be developed in the near future due to the ease with which the genome now can be manipulated using CRISPR/Cas9.

Large animal models have been increasingly used in the last few years. While non-human primates were already a well-known model for several hematological studies there is an

expected increase in the use of other large models for a wide variety of disease areas. Due to the newly emerging CRISPR/Cas9 technology it is now possible to easily manipulate cells from larger animals. The large models which are currently most widely used for gene therapy testing, next to non-human primates, are dogs, sheep and pigs. In addition, many researchers see the need to test their products in large animal models to ensure that upscaling of the production process is possible.

An emerging field of interest is the animal as a target for therapy. Domestic pets are rapidly becoming patients in the field of cell therapy. In the veterinary field the cells used for cell therapy are also often genetically modified to achieve optimal effects. The EMA has regulated veterinary use of medicinal products consisting of or containing GMOs in guideline 2001/18/EC as well as in Regulation EC No 726/2004, if they fall into the following category [207]: “Medicinal products for veterinary use intended primarily for use as performance enhancers in order to promote the growth of treated animals or to increase yields from treated animals”.

The 726/2004 regulation states that all products which apply for marketing authorization must be accompanied by a consent of the competent authority concerning the deliberate release of GMOs in the environment, a complete technical dossier, environmental risk assessment and an overview of all investigations performed. In addition, similar to human medicines, all regular safety measures need to be undertaken such as registration of pharmocovigilance.

However, there is a growing interest to develop innovative products which do not fall into the classical categories of pharmaceuticals and immunologicals covered by the current veterinary medicines legislation. Examples are cell and tissue products such as stem cells obtained from bone marrow which are cultured using growth factors, treatment of different kinds of diseases in horses and dogs. Very recently the U.S. department of Agriculture has granted a license for the therapeutic DNA based vaccine ONCEPT to treat melanoma in dogs. This vaccine contains a gene encoding for human tyrosinase. A further field of innovation is the development of nanotechnology medicinal products also for veterinary applications.

Since 2005 four DNA products have been licensed by the FDA for prophylactic and therapeutic purposes in veterinary medicine [937]. In 2005, the first vaccine for West Nile virus was licensed for use in horses. Almost immediately after this a DNA vaccine for farmed Atlantic salmon was licensed against infectious hematopoietic necrosis vector. In 2007, a gene therapy for swine was approved to increase the number of piglets in a single litter. And finally, as mentioned above, in 2010 ONCEPT was licensed for melanoma treatment in dogs.

Other innovative products are certain immunologicals developed for food-producing animals, for example the development of a vaccine to immunize cattle against certain *E.coli* strains, which do not cause illness in the animals, but can affect humans seriously. Preventing growth of these microbes in animals helps to limit the contamination of meat, and reduces the shedding of the microbes into the manure and the environment.

For the time being there is no legislation at all for tissues and cells in the veterinary sector which do not fall within the GMO regulation. Advanced therapy veterinary medicinal products have not been addressed when the Annex I to Directive 2001/82/EC was revised in 2009. There is a regulatory gap for these kinds of products in the veterinary medicines legislation, which leads to uncertainties on both the industry and the regulators side concerning marketing authorization, classification, GMP rules, manufacturing/import authorization.

It is expected that the scientific progress in the veterinary field in combination with the experience already gained in the human sector will trigger in first instance the development of tissue/cell products and later on of more complex advanced therapy medicinal products to treat

different diseases in animals. Currently tissue/cell products but also complex tissue engineered products (in the understanding of the definition given in article 2 of regulation (EC) No 1394/2007) are under research/development and have the potential to enter the market within the next years. Therefore it should be explored to what extend the current veterinary medicines legislation can cover these 'borderline' products and to what extend new legislation is needed. However, it is clear that the human legal framework cannot be implemented equally in the veterinary field, and in addition specific veterinary needs and the smaller markets should be taken into account [208].

6.7 Conclusion

In the future non-viral vectors will be of interest for many applications. The just discovered naturally existing exosomes are likely to progress into clinical development over the coming time period if they are proven safe for use after further characterization. The knowledge acquired from this characterization can then in turn be used to develop new synthetic nanoparticles with improved properties. The scope of application for iPSCs is rapidly increasing. Although characterization of iPSCs is far from complete they have the potential to become the cell type of choice in autologous cell transplantations.

Looking at the strategies used to modify viral vectors these have not dramatically changed over the last 5 years. Concerning viral and oncolytic vectors, SIN HIV-1 based integrating vectors are currently most used in clinical trials, while other retroviral vectors with more favorable integration patterns can be expected to gain more attention in the near future. Oncolytic virotherapy has a new prime candidate in oHSV, with some other viruses not lagging far behind. Oncolytic adenoviruses seem to be losing some popularity. General strategies include the use of more virulent strains, expression of immune stimulating transgenes, (transcriptional) tumor targeting, virion shielding, and the search for (obscure) new oncolytic viruses. When focusing on safety aspects barcoding may become an important technique to detect, *in vivo*, clonal expansion early on.

Furthermore it is expected that due to the discovery of the CRISPR/Cas9 technology the number of available animal models for complex diseases will increase quickly, thereby enabling further expansion of gene therapy applications in new disease areas.

7 Trend analysis

The following trend analysis has been constructed by comparative reading of relevant literature, scientific meeting abstracts, editorials, research updates and research highlights. In addition the views of several experts in the field of gene therapy have been taken into account.

7.1 General techniques

In the field of general research techniques, the following trends have been identified:

- In the last few years more frequent use of advanced genetic engineering techniques such as TALEN and the more recently discovered CRISPR/Cas9 to easily and effectively manipulate the genome is observed. Especially the CRISPR/Cas9 technique is developing at such a fast rate that in the coming year this technique will be drastically optimized. It is to be expected that the use of this technique will drastically influence the research done on gene therapy, more complex diseases can be modelled which will lead to more preclinical gene therapy research.
- The safety profile of viral vectors could be increased by embedding barcodes in the viral backbone. This barcoding will make it possible to track clonality of cell populations *in* vivo thereby making real time monitoring of possible clone formation possible. When this strategy will be approved for use by FDA and EMA it is expected to be introduced in gene therapy vectors for several indications. Especially hematological indications will benefit from direct *in vivo* monitoring of cell clonality to monitor the development of leukemic clones.
- Oligonucleotide-based therapy and RNA interference are becoming more popular in several research fields. Especially in the field of antisense mediated exon skipping, the recent conditional approval of Ataluren by the EMA has boosted confidence of the field. This conditional approval set the bar for what is seen as clinical benefit in ambulant DMD patients.
- Immunotherapy using genetically modified T cells will become of specific interest since the latest successes in HIV/AIDS research. Currently, clinical trials are being conducted for several tumor types and preliminary results show that T cell-based immunotherapy seems to be more effective in hematological tumors rather than in solid tumors. It is to be expected that viro-immunotherapy will become more popular within the oncology field over the coming years, with several preclinical successes in the past year.

7.2 Cell-based delivery systems

In the field of cell-based delivery systems, the following trends have been identified:

- iPSC reprogramming to obtain sufficient numbers of bone marrow cells for autologous transplantation is currently under investigation to overcome the shortage of suitable bone marrow donors. It is not expected that iPSCs will move to clinical trials in the near future because safety profiling still needs to be carried out.
- MSCs are still a popular delivery vehicle and candidate for cellular therapy. Although some preliminary clinical successes have been achieved, the routine clinical application is still not around the corner. Safety profiles need to be assessed in more detail as well as the actual mechanism of action.

7.3 Non-viral vectors

In the field of non-viral vectors, the following trends have been identified:

- Naturally secreted exosomes as a delivery vehicle for lipid, protein or gene delivery will become increasingly investigated in the near future. The possibility of exploiting a naturally existing nanoparticle for clinical use which can also be genetically modified to obtain a more desirable effect would be of great benefit to the clinical community. A few clinical trials have been started and it is expected that this number will steadily increase in the coming year.
- Transposons to stably insert transgenes into the genome thereby obtaining long-term expression have been around for a few years already. However they have regained interest in the last year as a non-viral delivery method for gene therapy. Several studies have been done in animal models and slowly the number of clinical trials is increasing as well.
- Synthetic nanoparticles to complex and deliver nucleic acids are still popular. This is mainly due to the great possibility of controlling their characteristics such as size, shape and composition. Currently the possibility of complexing and delivering viral vectors with NPs is of special interest. This approach is especially used in the field of theranostics where diagnostics are combined with a therapeutical agent.
- Optimization of several liposomal gene delivery systems with or without the addition of polymers are gaining interest especially in the field of siRNA delivery. Currently liposomes are under investigation for several gene therapy indications and therefore it is expected that this will lead to several new clinical trials in the future.
- Bacteria for delivery of therapeutic genes or RNAi are mainly of interest in the context of oral gene therapy for cancer. It is an elegant way of introducing genes which have anticancer properties when expressed. Several preclinical trials are being conducted at the moment and it could be well possible that some of these strategies will be continued into clinical trials.
- Episomal vectors like human artificial chromosomes and minicircles to correct genes without integrating into the genome are under investigation as an alternative way of delivering genes. Until now only preclinical work has been done and still some issues are to be resolved, such as controlling the delivered copy number. However, results from mouse studies are encouraging and it could thus be expected that the amount of preclinical work will increase eventually leading to a small number of phase 1 clinical trials.

7.4 Viral vectors and oncolytic viruses

In the field of viral gene therapy vectors, the following trends have been identified:

- Human adenovirus vectors with less prevalent serotypes than type 5 (most notably type 3) and/or with capsid modifications to circumvent pre-existing immunity and liver sequestration have been evaluated successfully in preclinical trials, with some early clinical trials underway. The relatively disappointing results of the first clinical trials with adenovirus vectors have led to the development of new adenovirus vectors which either possess a modified capsid or knob or are based on a completely different type or species. Both strategies have resulted in higher transduction efficiency in preclinical trials due to more immune evasion and less liver sequestration, leading to less toxicity.
- Other serotypes of AAV (besides type 2) have been developed to specifically target certain

organs or tissues. As with adenoviruses, this approach has been used to evade immune responses. This strategy has resulted in longer lasting gene expression of transduced organs in preclinical studies, with some early clinical trials underway.

- SIN Rous sarcoma virus vectors with very neutral and favorable integration patterns have received more attention and development in recent years.
- The development of SIN or non-integrating murine leukemia virus (MuLV) vectors have led to lower genotoxicity associated with integration. (SIN-)RDR-MuLV vectors lack enhancer regions and use an internal promoter and enhancer to drive transgene expression. However, also these SIN-RDR-MuLV vectors have been shown to possess oncogenic activity (although considerably lower), and their integration profile does not differ from RDR-MuLV vectors. Non-integrating (episomal) RDR-MuLV vectors have also been developed to achieve transient gene expression by mutating the viral integrase, leading to a marked reduction of viral (but not spontaneous) integration, which can be combined with strategies like ZFN or SB.
- Based on a proprietary platform, Oxford Biomedica has progressed several SIN equine infectious anemia virus (EIAV) vectors for correction of monogenic diseases into early clinical trials.
- A trend of using SIN or non-integrating HIV-1 vectors as opposed to first/second generation vectors has been observed, with several clinical trials already underway employing SIN vectors.
- SIN simian immunodeficiency virus vectors, similar to HIV-1, have taken the lead in the field of SIV vectors, but no clinical trials are underway yet.
- SIN or non-integrating simian foamy virus vectors with neutral integration patterns have undergone more development. As with alpharetroviruses, spumaviruses have a favorable integration profile leading to less genotoxicity. As such, it is possible that these viruses will receive more attention in the near future, with multiple clinical trials in dogs already underway.

<u>In the field of oncolytic viruses, the following trends have been identified:</u>

- There is a shift from attenuated to more virulent conditionally replicating viruses. In response to the relatively disappointing results of early clinical trials, most oncolytic viruses have been developed towards more wild-type like viruses, harboring safety measures like conditional replication, either from exploitation of tumor-specific signaling pathways or promoters, or retargeting (see also below).
- The expression of transgenes from recombinant oncolytic viruses, with most notable examples:
    - Immune stimulators: mainly GM-CSF, IFN, IL-12
    - Therapeutic genes
    - Pro-drug converting enzymes: cytosine deaminase
    - Tumor associated antigens
    - Tracking/imaging related genes: fluorophores/luciferase, CEA, NIS
    - Radiovirotherapy: NIS
- Enveloped viruses are being targeted towards specific tumor cells or tissues, or detargeted from cells in which they cause potential side effects, by means of envelope modifications.
- As described above, the trend towards more wild-type like viruses has stimulated the use of retargeting by means of tumor specific transcription and/or miRNA regulation.
- Combination with standard therapies, like chemo-, radio- and latest immunotherapy has been

shown effective and safe in numerous preclinical, and some early clinical trials. After defining an effective oncolytic virus, evaluation in combination with other (standard) therapies is often described. It is likely that these evaluations will continue, since oncolytic virotherapy has to take its place within these therapies.

- There is now a general notion that the efficacy of oncolytic viruses is more related to immunological effects rather than direct oncolysiss. As a result, there is a shift of mechanistic studies from direct oncolytic efficacy to immune stimulation and the use of the immune system for tumor eradication and immunologic memory. This goes in sync with the recent discoveries on immune checkpoint blockade. Oncolytic viruses might present an interesting agent to overcome this blockade, especially when used as a delivery vehicle for immune-modulating transgenes.
- Carrier-cell delivery to shield viruses from innate and/or pre-existing adaptive immunity has been shown effective in multiple preclinical studies, and also in a few early clinical trials, although researchers prefer to use viruses that do not require this delivery method.
- Hybrid/chimeric viruses to combine oncolytic effects and/or integration have been evaluated in early preclinical studies. Combining favorable treats of different oncolytic viruses to optimize oncolytic efficacy has been shown, and surely is an interesting option for the future. However, it also holds an inherent risk of creating non-natural viruses with a difficult to predict behavior.
- Human adenovirus vectors with less prevalent serotypes than type 5 and/or with capsid modifications to circumvent pre-existing immunity and liver sequestration have been developed and successfully evaluated in preclinical studies, with a number of early clinical trials underway. Being the group of oncolytic viruses that has seen most development, this new branch holds promise for the years to come. Adenovirus type 5 seems not to fulfill its promise in most clinical trials.
- The screening of virus family groups for new, less known oncolytic viruses, has led to the discovery and development of new (obscure) agents. Employing ‘library screens’ on viruses also holds the inherent risk of these more obscure virus types and their ill-defined natural habitat. Examples of such viruses are:
    - Farmington virus (obscure nature, but fast development)
    - Maraba virus (obscure nature, but already proceeded into clinical trial)
    - Other serotypes of coxsackievirus to circumvent pre-existing immunity
    - Seneca Valley virus proceeding into phase II clinical trials
    - Semliki Forest virus, preclinical work expanding
- Recombinant myxoma virus with increased virulence has been developed and has shown better oncolytic potential as compared to the reference strain. Having shown oncolytic efficacy as wild-type virus, recombinant myxoma virus will be used in the future to further improve upon.
- Progression of wild-type reovirus in clinical trials and recent development of a recombinant virus has stimulated the development of this agent. As with myxoma virus, reovirus has proven its efficacy (also in clinical trials). Genetic modifications can now be made that will add to the development of more oncolytic reoviruses.
- Clinical trials in pet animals, most notably dogs, are being carried out more frequently. Trailing slightly behind clinical trials in humans, clinical trials in pet animals have provided viable information on potential safety issues for humans or the environment, while efficacy in humans can also be predicted with better accuracy.
- Clinical trials with some oncolytic viruses have proceeded into phase III, with one or more

possible FDA approvals to be expected in the near future. Possibly the biggest achievement in the field of oncolytic viruses, the approval of the first product (talimogene laherparepvec) will pave the way for more oncolytic viruses to come.

7.5 *In vitro* models

In the field of *in vitro* models, the following trends have been identified:

- Use of 3D culture systems and organoids in preclinical research is becoming more popular over the years. Especially organoids are being investigated as models for human disease like colon cancer and pancreatic cancer. However it is unclear if these models will be able to completely replace current animal models and if they will be accepted by regulatory authorities for safety profiling of novel drugs applying for market approval.

7.6 Animal models

In the field of animal models, the following trends have been identified:

- Generation of animal models which more closely resemble the human disease situation will soon become a reality due to the upcoming CRISPR/Cas9 genome editing technique. This technique makes it possible to easily target multiple genes thereby enabling modelling of complex human genetic diseases.
- Optimization of immune competent mouse models is becoming increasingly important since testing of several vaccines and other immunotherapy strategies require a functional immune system.
- Large animal models such as dogs, sheep, pigs and non-human primates are being used more often in preclinical studies. It is expected that this trend will further increase in the future. Especially for upscaling of therapeutic production these models are of great value. In addition it is becoming more clear that rodent models are not always the best choice in several diseases.
- In the last few years domestic animals like dogs, cats and horses are increasingly being treated with gene therapy. Although most of these therapies fall within several regulations (2001/18/EC and EC/726/2004) not every type of gene therapy is clearly regulated. It is expected that in the coming years the development of tissue and cell products will increase dramatically, followed by development of complex advanced therapies. These kind of therapies have the possibility to enter the veterinary market within the coming four years.

8 Summary of interviews with experts

8.1.1 Summary of Interview with Dr. John Hiscott, 6th of May 2014

- **Which oncolytic viruses are new and upcoming?**
  The following viruses have the most potential of reaching clinical application:
  1. *Herpes virus derived oncolytic virus*, modified such that it is non-replicating and can stimulate immune system via GM-CSF expression (completed phase III trials).
  2. *Measles virus*: derived from viruses that are already in use for human vaccination (phase II trials).
  3. *Vaccinia virus:* derived from viruses that are already in use for human vaccination (phase II trials ongoing).
  4. *Reovirus:* one of the first generation oncolytic viruses (phase III trials ongoing)

In addition to these 4 viruses also NDV (Newcastle Disease Virus) and VSV (Vesicular Stomatitis Virus) are being used as prototypic oncolytic viruses. The issue with these viruses is that they can cause disease in poultry and cattle respectively, and thus pose a threat to important agricultural industries. The exact mechanism of oncolysis should be defined so that the risk can be determined.

- **What are novel modifications to already used viruses?**
  Several modifications are being tested in preclinical models:
  1. Insertion of a tracking gene such as GFP (green fluorescent protein) or NIS (sodium-iodine symporter). This approach is clinically interesting because these transgenes permit a precise tracking of the virus, and thus allow analysis of the localization and multiplication of the virus within the tumor. This strategy will also determine if the virus mislocalizes or replicates in off-target sites.
  2. Insertion of immune modulators (e.g. GM-CSF or IFN-β). By expressing GM-CSF an immune enhancement occurs because GM-CSF stimulates dendritic cell maturation and thus increases the bridging of the early innate and adaptive immune responses (antibody production, cell mediated immunity). This enhanced immune response will increase the recognition of tumor antigens and stimulate immune mediated killing of the tumor. Insertion of IFN-β gene in VSV increases the probability that the virus will infect and replicate in tumor cells (which are often insensitive to IFN) and not in healthy cells (which are sensitive to IFN and block virus multiplication via the release of IFN)
  3. Introduction of enzymatic activities. Insertion of enzymatic activities into OV permit the conversion of a pro-drug chemotherapy into an active drug at the site of the tumor. For example, some studies have use OV delivery to introduce the enzymes CD and UPRT into the tumor. These enzymes convert 5FC (5'fluor-cytosine - pro-drug) into the active drug 5FU (5'fluoro-uracyl – the active drug) locally within the tumor cells. By using this mechanism, healthy cells are not affected because they will not produce the toxic 5-FU.
  4. Incorporation of genes that induce cell death (suicide genes, pro-apoptotic genes).

Insertion of tissue specific promoters is possible, so that expression of the therapeutic gene occurs only in the tumor cell environment, but the strategy has not been used extensively as yet with oncolytic viruses.

- **What is the "bottleneck" for oncolytic vaccines?**
  1. *Delivery.* Currently, most clinical trials deliver oncolytic therapeutics by direct intratumoral inoculation, mainly to solid tumors or easily detectable tumors (head and neck cancers, melanoma, for example). To facilitate wide use of oncolytic virus strategies, it will be necessary to improve the delivery route, and ideally intravenous delivery instead of intratumoral. By using IV as delivery route, metastasis will also be targeted.
  2. *Immune response.* Immune responses are generated against both virus and tumor antigens, often with viral antigens predominating. The strong OV-generated immune response against tumor antigens is one of the important goals for OV research and development of such a capability would boost the therapeutic potential of OV and would also broaden the range of tumors that could be targeted.
  3. *Regulatory issues.* Patients who are currently enrolled in clinical trials usually have already failed other treatment options and as a consequence are highly compromised in terms of their immune system. To adequately test oncolytic vaccines (or any immunotherapy strategy), it will be important to include patients at early stages of their disease and/or treatment regimens, so the immune response can be adequately evaluated i.e. clinical trials in patients who are more healthy and can still induce near normal immune responses.

- **Is it conceivable that oncolytic vaccines will be used for veterinary purposes?**

There will certainly be a market in the veterinary field. The regulations for animal testing are less stringent, compared to human clinical trial regulations. Therefore it is possible that the veterinary field will move forward faster to clinical application. Diseases which could be good candidates are feline leukemia (cats) and bone cancer (dogs).

- **What are specific risks for the environment?**

When used in animals there is a certain risk of transmissibility; however it should be remembered that many candidate oncolytic viruses are not human pathogens and pose no known risk to the population. It will be important to choose your virus candidate wisely.
In human clinical trials, strict regulations and safety measures are applied to eliminate the risk of transmissibility.

- **Is it possible to administer multiple dosages over time?**

One would expect that after initial inoculation of a viral vaccine that the immune response would recognize a second administration and neutralize the virus. However, in dose escalation studies in patients (phase 1), this does not seem to be the pattern. Multiple inoculations with increasing concentrations of OV have shown clinical benefit, and side effects are minimal compared to standard chemotherapy. During dose escalation studies it was seen that repeated high dosage administration still has clinical benefit. It might be possible that these high dosages flood the immune system, and the immune response is not sufficient to inhibit the high dose of virus.

High dose delivery of OV therapy is very well tolerated; compared to traditional chemotherapeutics, patients do very well and side effects are confined to mainly flu-like responses.

This effect may be closely related to the therapeutic index of OV therapy – the number of tumor cells killed relative to healthy cells. With standard chemotherapy the therapeutic index is

often ~5:1 (5 tumor cells killed per normal cell). This high rate of cell death in normal cells accounts in part for the severe side-effects often seen with chemotherapy, while with OV treatment, the therapeutic index is often >1000:1, indicating a much greater proportion of tumor cell killing compared to normal cell death. This fact is one of the most encouraging aspects of OV immunotherapy – the potential for specific selective killing of tumor cells, with minimal death of normal cells and tissue.

- **Is it possible to modify viruses in such a way that they spread systemically and in that way target metastasis?**

  This point was already touched upon during a previous question. Modification of virus may be possible in which cell specific markers will be expressed that cause the OV therapeutic to 'home into the site of the tumor. Modifications include surface markers such as immunoglobulins.

- **How do you see clinical application of oncolytic viruses?**

  There are several potential applications of OV therapy:

  1. *As a complementary therapy to surgery***.** For example, remove the bulk of the tumor via surgery, and then deliver OV to the site of the tumor, as a therapy that would identify, target and kill remaining metastatic cells.
  2. *Combination OV strategies***.** A number of studies in pre-clinical models have shown that OV therapy can be accompanied by other therapeutic strategies with synergistic benefit. Histone deacetylase inhibitors (HDI) for example, have been shown to increase OV replication and killing at the site of the tumor. An advantage here is that the HDI Zolinza (Vorinostat) is already FDA approved for cutaneous T cell lymphoma and thus can potentially be used in combination with OV. Other combinations include small molecule chemotherapies that stimulate apoptosis (cell death), or other immunotherapies that boost the immune response (anti-CTLA4, anti-CD40, anti-PD-1).
  3. *Pre-transplantation use:* OV therapy could be adapted to purge the bone marrow of remaining cancer cells *in vitro* prior to reinfusion of marrow cells during autologous bone marrow transplantation.
  4. *Use of OV therapy to target cancer stem cells*; some preliminary work demonstrates that OV will infect and destroy cancer stem cells – cells that give rise to the heterogeneous population of cancer cells.

8.1.2 Summary of Interview with Dr. M.H. Brugman, 12th of May 2014

- **Which viruses are currently being used for hematological gene therapy purposes?**

Retroviral vectors are mostly being used since they can stably mark hematopoietic stem cells and progenitor cells of the T and B cell lineages. The first gene therapy trials for XSCID, XCGD and WASP showed genotoxicity related to integration sites in patients. Since 2001 researchers are looking into viruses which integrate in a more desirable fashion. Although lentiviral vectors have been proven to be less oncogenic than the gamma-retroviral vectors used in the first gene therapy trials, they were also shown to have oncogenic potential. At the same time, the field moved to vectors which are self-inactivating (SIN), which means that they inactivate their viral promoter upon insertion in the host genome. Current estimates are that SIN vectors or lentiviral vectors with inserted endogenous promoters are 10-100 times safer than retroviral vectors (Zychlinski Mol Ther 2008, Montini JCI 2009). However, (SIN) lentiviral vectors present some procedural difficulties, due to their slow insertion kinetics. In addition, they are harder to produce at a clinical scale. Therefore, people are also looking into other viruses such as alpha retroviruses and foamy viruses. Foamy viruses received a lot of attention for a period of time but this seems to have passed without these vectors going into the clinic.

- **Which new virus has potential to go towards clinical application?**

Alpha retroviruses could be a good candidate for clinical application. ASLV is a chicken virus which has desirable insertion properties for gene therapy purposes and is less oncogenic than gamma retroviral vectors while maintaining the production advantages. By utilizing codon optimization and removing all irrelevant viral genes from the vector there is almost no overlap with the wild type virus and thus the risk of reactivation by recombination with wild type virus or the risk of infecting poultry is reduced to a minimum, similar to the risk which is seen with gamma retroviral and lentiviral vectors.

- **For what applications is DNA barcoding used?**

DNA barcoding can be used to improve the safety of viral gene therapy. In retroviral gene therapy, it can be used for *ex vivo* gene therapy applications where either bone marrow, cord blood or peripheral cells are isolated from the patient and subsequently infected with either gamma retroviral or lentiviral vectors to obtain transduced cells which can then be given back to the patient. By putting a short barcode sequence into the viral vector, clonality of expanding cells within a treated patient can be monitored, allowing earlier discovery of leukemic clones. Leukemia caused by inserting vectors has been the largest problem in retroviral gene therapy and the use of DNA barcodes in gene therapy vectors allows us to directly monitor clonal outgrowth resulting from an oncogenic insertion during the study, rather than retrospectively.

- **What is the principle of barcoding?**

The barcode tag is ~37 bases long of which 16-21 bases are variable. Therefore a large number of possible tags can be generated (4.39×10^{12} of total combinations) to track clonality. The tag is placed in a defined region of the vector outside of the coding sequence (either in the LTR or after the stop codon). This barcode can be read via deep sequencing methods.

- **Why is barcoding necessary?**

Vector toxicity is determined by the vector backbone and the desired transgene which is used. Risks of retroviral gene transfer have been studied for many years. Retroviruses integrate into the host genome with a preference to integrate in sites near internal promoters/enhancers. This can lead to (proto) oncogene expression and subsequent leukemia development. In a XSCID (Hacein Bey Abina Science 2001) and more recent WASP trial (Braun, Science Translational Medicine 2014) subjects developed leukemia. Retrospectively it was found that the insertion site of the vector is important for disease development, because only a small set of genes is involved when leukemia develops from retroviral gene therapy. It is of great importance to keep track of what happens real time in the patient so that we are able to clinically intervene when necessary.

- **Are there any known risks of using barcodes?**

Until now there are no known risks for using a barcode. Of course insertion of unknown material into a vector is undesirable, however the amount of unknown (~37 bp) material is very small. For pharmaceutical products there is a problem since the addition of a barcode jeopardizes the product identity criteria. The question is if this variation weighs up to the possibility to be able to continuously keep track of what is happening with the patient after gene therapy. By using barcoding, clonality of the re-infused cells can be determined in the peripheral blood of the patient. In this way the condition of the patient can be monitored during the treatment and more frequent samples can be taken when a specific clone seems to outgrow the other marked cells.

- **What is the future of DNA barcoding?**

In the last few years the costs of detecting DNA barcodes has decreased about 10 fold due to the development and wide acceptance of deep sequencing technology. Before deep sequencing was available, costly and labor intensive methods were used to track clones.
The concept of the DNA barcode (variable bases with known bases as an anchor) will probably not change much because the current design allows the generation of sufficiently complex variations.

- **What techniques in hematopoietic gene therapy are promising in the coming few years?**
    1. *Generation of hematopoietic stem cells from iPS cells*. This technique is promising, because the generation of HSC from iPS cells would allow gene repair (homologous recombination, zinc finger nucleases, CISPR/CAS9 mediated repair) rather than gene addition strategies, but the technique is in its infancy and still needs to be validated.
    2. *Episomal vectors.* Plasmids which dock to the genome and which can possibly replicate without integrating into the genome. This method might also be applicable for *in vivo* gene therapy (direct injection into the patient). These vectors have not yet been used therapeutically.
    3. *Directed expression of integrating vectors*. While the use of tissue specific promoters to direct tissue specific expression is usually difficult in retroviral gene therapy, tissue specific miRNA sequences have shown to be able to restrict expression to a specific cell type. This could prevent toxic effects of the transgene in cells which do not belong to the desired target population.

8.1.3 Summary of interview with Prof. dr. A.Vulto, 27th of May 2014

- **What is your field of expertise/interest within the gene therapy field?**

Prof. Vulto is directly engaged in gene therapy research related to Pompe Disease conducted in the Erasmus MC. Within the Erasmus MC he is involved in quality control of the vectors which are used in gene therapy research (in close collaboration with the biological safety officer) and he is a member of the Erasmus MC Ethical Committee. Furthermore he is a member of an expert group which has combined their expertise in safety regulations concerning gene therapy. This group is involved in generation of the upcoming revision of the Advanced Therapy Medicinal Product (ATMP) regulation under which gene therapy is situated.

- **Which regulations are currently in place for gene therapy research/products?**

All gene therapy trials in patients must adhere to, among others, the ATMP regulations. These regulations came into force on the first of January 2008 and are primarily focused on industrial development of medication for a large set of patients. For large scale medicinal applications an Investigational Medicinal Product Dossier (IMPD) is mandatory. This IMPD describes the exact quality and safety guarantees of the medicinal product and usually spans around a 1000 pages.

Legislation takes into account the following topics:

  - Environment
  - Safe working conditions/containment (ATMP, GMP and GLP)
  - Patient safety (ICH-GCP)

- **Why is translational gene therapy research difficult under the existing regulations?**

Legislation is holding back the translational research in gene therapy. Gene therapy is primarily focused on individual patient treatment and is not intended for use in large numbers of patients and thus official registration of the gene therapy treatment is not the primary goal. Due to the restrictions in production- and distribution methods of large scale medications a vast amount of safety measures is brought into place which are disproportionate for gene therapy. Examples are the IMPD requirement and the need for specially validated laboratory which may produce the viral vectors used in gene therapy. For the Erasmus MC this means that only vectors used for rodents are produced in house, for clinical application this is outsourced to external companies which are also obliged to prepare the IMPD for the produced vectors.

- **Should ATMP legislation be adapted to improve research on gene therapy?**

Currently governments are not investing enough in gene therapy research. It seems like governments are afraid of the possible risks which gene therapy may imply. However if they would be better informed about the actual risks and benefits it may be possible to clarify the current issues. It would be of great importance to uncouple academic research from drug registration so that patients can benefit from all the technical improvements which have been made in the past years. In October 2014 a meeting will be organized to try and improve the knowledge around gene therapy research by ways of several speakers who will talk about important topics in biosafety.

- **What is the future of gene therapy?**

Since the discovery of gene therapy a lot of technological progression is made. In addition a lot is now know about vector technology and engineering. The ultimate goal is to generate a vector which integrates predictably and which has a predictable effect on the patient. Currently we do not have these kinds of vectors yet.

In the future, vector expression will become better and safer as expression will become more and more directed. In general researchers will try to keep their multiplicity of infection (MOI) as low as possible, however it is not always possible to have optimal functionality with a low MOI. Using a higher MOI will increase the risk of integrational mutagenesis in the genome but by steering the expression to certain cells this may be overcome. The biological effect of treatment depends on the oncolytic effect, the changes which are made to the cell repertoire (receptors or enzymes) and the promoters used.

There will be a shift from direct gene therapy towards directly targeted oncolytic therapy. *Ex vivo* stem cell therapy, in which gene therapy and traditional stem cell therapy are combined, will probably reach the clinic within 5-10 years from now.

iPSCs will not be used in the near future due to the fact that they have an instable genome and therefore have increased safety issues. These issues will first have to be further studied and resolved before clinical application may proceed.

8.1.4 Summary of interview with Dr. A.M. Aartsma-Rus, 3rd of June 2014

- **How does exon skipping work?**

For Duchenne muscular dystrophy (DMD) exon skipping aims to correct the reading frame of the dystrophin protein at the pre-mRNA level. This can be achieved by using antisense oligonucleotides (AONs). AONs hide certain exons from the splicing machinery during the splicing process, thereby these exons are skipped and the open reading frame is restored and a truncated, but partially functional protein can be formed (instead of the non-functional protein that is normally formed). This approach is in general mutation specific, depending on their location different mutations may require the skipping of different exons. The overall aim is to convert the DMD phenotype into the less severe Becker muscular dystrophy (BMD) phenotype. Therefore it will be necessary to obtain more information about the natural history of BMD patients so that prediction of the potential beneficial effects of exon skipping would become better understood. AONs are delivered via intravenous injection or via injection under the skin. Currently delivery to muscle tissue is limited, but sufficient to induce exon skipping and protein restoration. Delivery may be improved in the future.

- **Are there other approaches under investigation for treatment of DMD?**

Gene therapy is also a possible way of correcting the gene defect seen in DMD. However due to the large amount of muscle tissue in the human body this is a challenge. In addition the muscles are surrounded by layers of connective tissue, this connective tissue strongly reduces the efficiency of gene delivery. Muscle is hard to transduce using viral vectors, AAV vectors are an exception however they do not possess enough loading capacity to carry the dystrophin gene.

To overcome this problem minidystrophins were developed which consist of only the minimal required functionality domains. In animal models this was shown to improve the phenotype, however in a clinical trial no effect was seen. This was most likely due to the presence of antidystrophin T cells. In addition up scaling of viral production is an issue for viral therapy and delivery thus far is only possible for isolated muscles or muscle groups.

Another approach is to deliver an antisense gene using small nuclear ribonucleoproteins (snRNPs). snRNPs are expressed under their own promoter which decreases the chance of an immune response. Results in mouse models are promising, showing expression and phenotype rescue. However, snRNPs need to be delivered by viral vectors and thus suffer from the same challenges as regular gene therapy. Currently snRNPs are being developed for use in clinical trials.

- **What is the current status within your field of work?**

Proof of concept for the exon skipping approach has been obtained in patient-derived cell cultures and animal models. This preclinical work showed targeted exon skipping, dystrophin restoration and functional improvement. The largest subset of patients would benefit from exon 51 skipping, therefore clinical application is first tested for this exon. Multiple clinical trials are currently ongoing with 2 AON formulations (Drisapersen and Eteplirsen) for skipping of exon 51.

Current preclinical work focuses mainly on improvement of AON delivery to muscle (especially heart muscle). Although heart muscle targeting is possible, the formulations used are arginine-rich and not well tolerated in primates and humans. Future work may reveal formulations which improve heart muscle targeting and display a better safety profile.

- **Are AONs still good candidates for clinical application now new technologies like genome editing are upcoming?**

Exon skipping utilizes RNA modification and not DNA modification therefore it does not fall under the classical gene therapy description. Genome editing currently only works *in vitro*. Another problem is that about 40% of the human body consists of muscle which makes it difficult to repair sufficient amounts of cells. *Ex vivo* stem cell therapy would be a solution to overcome this problem however due to the large amounts of cells needed it is almost not feasible. In addition, the cells which are isolated from the patient are not of high quality because they usually come from fibrotic muscles and as such are more geared towards fibrotic tissue formation than muscle tissue formation after they are transplanted back into the patient. In the US research is done to improve gene therapy for DMD patients. They locally inject the virus in the quadriceps. Current problems are that you can only treat 1 body part at a time and that due to immune reactions limbs can only be treated once.

- **Which characteristics of the current AONs can still be improved?**

Currently physiologic salt solutions are used as a carrier for AONs. Improvement can be made on the formulation of the AONs. Additional sugar groups or proteins can be added to improve delivery efficiency. However this is all done in preclinical settings because changes to the current clinical formulas will need new trials and this will severely delay availability to the patients. In addition, clinical safety is of utmost importance so any changes made to the existing formulas must be tested vigorously.

Is there any interest in other animal models besides mice?

Currently dog models are available which carry spontaneous mutations and a pig model has been developed. However there are some problems concerning these models. There is a lot of variation between different dogs, and because you can only perform an experiment in a small number of animals the variation will lead to inconsistent results. Also ethically dogs are more problematic due to the fact that they are domestic pets and people get emotionally attached to them during the experimental period. Pig models are relatively new and will mostly be used for PK and PD modeling.

It is important that in the future other models will be used in an earlier stage of research. Mice are relatively unpredictable in their response to antisense oligonucleotides and it would be useful to use rats at an earlier stage, since they have more predictive value concerning toxicity.

- **What are the major hurdles for clinical application within the field of exon skipping?**

1. *Lack of approved medicines.* Approved medicines would set the benchmark for which outcomes are good enough for approval (i.e. which outcomes are considered as a 'clinical benefit' by the regulators). Since 2 weeks the Committee of the Human Medicinal Products (CHMP) of the European Medicine Agency (EMA) gave a positive advice to the European Commission to conditionally approve Ataluren which treats DMD patients with nonsense mutations who are >5 years old and still ambulant. This is of great importance for the field since it would then be known where the bar is set for clinical benefit for ambulant DMD patients (30 meters improvement in one year compared to a placebo group).
2. *Mutation specificity.* Due to the fact that several different mutations are involved in DMD multiple AONs will need to be developed. Would it be possible to extrapolate in the future so that multiple AONs can be approved within a short period of time (like vaccines)? This

would be the best option, however long term effects are not known so extrapolation is less straightforward. Approval of the first AON will be needed before any other assumptions can be made.

3. *Outcome measures.* Currently the 6 minute walk test is the only validated and approved outcome measure. EMA is open also to other outcome measures as long as they reflect clinical benefit. This means that a test which involves testing the ability to bring a hand to your mouth (independent feeding) could also be used. However these tests are currently in development and the field is collaborating currently to study the new tests in patients and controls over time for validation.

- **What are the regulatory issues within the field of exon skipping and how can these be overcome?**

As mentioned above the lack of additional outcome measures besides the 6 minute walk test is a big issue, but work is ongoing to solve this. In addition, the way clinical studies need to be set up is also a limitation. Most clinical trials are initiated by companies, little investigator initiated trials are done. This is mostly due to the fact that there are not a lot of patients available per hospital which necessitates the development of multicenter clinical trials. These type of trials are difficult to perform without help of the pharmaceutical industry and are also hampered by the fact that approval to conduct these type of clinical trials has to be done on a national level rather than a European level.

The collaboration within the DMD field is quite intense and much of the needed infrastructure has been or is being generated (see www.treat-nmd.eu). This is of importance for international standardization of clinical trials and research methods. The involvement between industry and academic research is strongly present. Researchers need the industry to help them correctly perform clinical trials as well as providing necessary information on registration and reimbursement conditions. On the other hand the industry needs researchers for their expertise and ongoing preclinical work which will benefit clinical research as well.

- **What techniques will become key players in the near future?**

1. Viral vectors for eye and central nervous system applications. Currently a lot of new developments are seen in this field.
2. Genome editing for ex vivo hematopoietic stem cell therapy. For other diseases this technique will need to be developed further.

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