Giridhara R. Jayandharan *Editor*

# Gene and Cell Therapy: Biology and Applications

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*Editor*
Giridhara R. Jayandharan
Department of BSBE
IIT-Kanpur
Kanpur, Uttar Pradesh, India

ISBN 978-981-13-0480-4 ISBN 978-981-13-0481-1 (eBook)
https://doi.org/10.1007/978-981-13-0481-1

Library of Congress Control Number: 2018950550



This Springer imprint is published by the registered company Springer Nature Singapore Pte Ltd.
The registered company address is: 152 Beach Road, #21-01/04 Gateway East, Singapore 189721, Singapore

# Preface

Recent advances in stem cell biology, nanotechnology, and gene therapy have created new avenues for therapeutics. The availability of molecular therapeutics that rely on delivery of DNA, RNA, or proteins, harnessing enhanced delivery with nanoparticles, and the regenerative potential of stem cells (adult, embryonic, or induced pluripotent stem cells) have made a tremendous impact on translational medicine. The attached book chapters cover the various strategies for molecular and cellular therapies for human disease, including their advantages and challenges to their widespread applications. Potential solutions to these issues have been discussed elaborately. This book will provide an overview of advances on novel therapeutics and provide specific examples of disease conditions where these strategies have been translated to the clinic. The chapters also highlight state of the art into current research aspects of molecular and cell therapies.

A summary of the chapters covered in this book is given below.

## Part I: Gene Therapy

### Chapter 1. Retroviral Vectors in Gene Therapy

Chitra Gopinath · Trupti Job Nathar · Everette Jacob Remington Nelson
Gene Therapy Laboratory, SMV124A, Department of Integrative Biology, School of Biosciences and Technology, Vellore Institute of Technology, Vellore, TN, India

Viral vectors are the most effective means to deliver genes into cells. Evolution of viruses over the years has enabled them to adopt several strategies not only to enter but also infect a wide range of cells. Making use of this property, scientists have manipulated viruses to express therapeutic genes. These viruses serve as vehicles for gene delivery and are referred to as viral vectors. The ones that are currently used in gene therapy include retroviral vectors, lentiviral vectors, adenoviral vectors, adeno-associated viral vectors, and foamy viral vectors. Each of them has their own salient features that are both beneficial and harmful. Retroviral vectors belonging to the family *Retroviridae* were among the first viral vectors used in gene therapy clinical trials. Their genetic material is in the form of RNA. The ability of

retroviruses to integrate into the host genome makes them a permanent resident of the cell. The *Retroviridae* family is further divided into two subfamilies and seven genera, out of which gammaretrovirus, lentivirus, and foamy virus are the most widely used. Gammaretroviral vectors which have been part of nearly 21% of gene therapy clinical trials were developed from the prototype Moloney murine leukemia virus and hence referred to as MoMLV-based retroviral vectors. The gammaretroviral vector genome ranges in size from 9 to 11 kb and is composed of two long terminal repeats (LTRs) one each at the 3′ and 5′ ends and three essential genes, namely, *gag*, *pol*, and *env*, which code for proteins required for the viral packaging. Reports of insertional mutagenesis and clonal proliferation due to the integration of gammaretrovirus into the LMO2 proto-oncogene raised concerns about the safety of its application for human gene therapy. Another drawback of this virus is that it can transduce only rapidly proliferating cells. So, in order to successfully target terminally differentiated or largely quiescent cells such as stem cells, lentiviruses are used as they could infect both dividing and nondividing cells the fairly efficiently. Unlike gammaretroviruses, lentiviruses do not require cells to be in the active mitosis while entering. Over the past three decades, three different generations of lentiviral vectors have been developed, each generation significantly improved over the preceding one. Safety has always been a concern with the use of all viral vectors due to the adverse events reported such as immune response in the case of adenoviral vectors and insertional mutagenesis in the case of retroviral vectors. Recently, third-generation self-inactivating (SIN) lentiviral vectors have been proven to be very efficient and also safer when compared to gammaretroviral vectors which were used in earlier clinical trials. Despite integrating into active transcription units, lentiviral vectors were reported to have a safer integration profile compared to gammaretroviral vectors. Foamy viruses belong to the genera of *Spumavirus*. Analysis of integration sites of foamy viruses in HSCs has shown a unique and safe integration profile compared to both gamma and lentiviruses. Reports from ongoing clinical trials might answer emerging questions related to their safety and efficacy in human gene therapy applications.

## Chapter 2. Adeno-associated Virus Vectors in Gene Therapy

Bertin Mary · Nusrat Khan · Sathyathithan Arumugam · Himanshi Saxena
Mohit Kumar · Paramasivam Manimaran · Sourav Chattopadhyay
G. R. Jayandharan
Department of Biological Sciences and Bioengineering,
IIT-Kanpur, Kanpur, Uttar Pradesh, India
e-mail: jayrao@iitk.ac.in

Adeno-associated virus (AAV) belongs to family Parvoviridae and the genus Dependovirus. To date 13 different human serotypes (AAV1 to AAV13) and more than 100 serotypes from nonhuman primates have been discovered. The

nonpathogenic nature of this virus, the persistence of viral infection in dividing and nondividing cells, and the availability of multiple serotypes have enhanced the utility of AAV as a delivery vehicle for gene therapy applications. However, the recent success in phase I/II clinical trials has also highlighted the issues related to the host- and vector-related immune responses that preclude the universal application of this promising vector system. A fundamental insight into the mechanism by which AAV infects the host cell and a thorough understanding of the immediate and long-lived cellular responses to AAV infection will offer clues and help design better intervention strategies to improve the therapeutic efficiency of AAV vectors. This chapter will review the biology of AAV-host cellular interactions and outline their application in the development of improved AAV vector systems for human gene therapy.

## Chapter 3. Nanointerventions for Gene Therapy

K. Uma Maheswari
Centre for Nanotechnology & Advanced Biomaterials (CeNTAB),
School of Chemical & Biotechnology,
SASTRA University,
Thanjavur, Tamil Nadu, India
e-mail: umakrishnan@sastra.edu

Vadim Annenkov
Limnological Institute, Siberian Branch of the Russian Academy of Sciences,
Irkutsk, Russia

Translation of gene therapy from bench to bed has become a reality due to nanoparticle-mediated delivery of the therapeutic genes. The therapeutic potential of naked oligonucleotides has been limited by their poor stability as well as off-targeting effects. However, complexation of the anionic oligonucleotides in cationic nanodimensional carriers has enhanced their stability. Moreover, modification of the nanocarrier surface with targeting ligands has reduced off-targeting effects and enhanced site-specific delivery. The carriers can also be programmed to release their cargo over an extended period of time. In addition, the choice of the carrier material or selective incorporation of molecules that can facilitate endosomal escape ensures higher transfection efficiency for the carriers. Though non-viral carriers have been in general considered less efficient than viral carriers, an emerging paradigm in the field of nanotechnology has introduced hybrid carriers. Here, a viral vector is encapsulated in a non-viral nano-dimensional carrier thereby retaining the high transfection efficiency of viral-based delivery systems and also poses a lower risk to the biological system. This chapter elaborates on the various types of nanocarriers and surface modifications that have been attempted for efficient delivery of the therapeutic gene to the target. Shortcomings of the existing systems and future perspectives for nanoparticle-mediated gene delivery have been discussed. A separate section on clinical trials involving nanoparticles for gene delivery has also been included.

## Chapter 4. Viral- and Non-viral-Based Hybrid Vectors for Gene Therapy

Manohar Mahato · Praveen Kumar Vemula
Institute for Stem Cell Biology and Regenerative Medicine (inStem),
Bangalore, India
e-mail: praveenv@instem.res.in

Giridhara R. Jayandharan
Department of Biological Sciences and Bioengineering,
IIT-Kanpur,
Kanpur, UP, India

Gene therapy offers a great potential for the treatment of genetic diseases as well as acquired diseases by means of delivering therapeutic nucleic acids inside the cell. To deliver nucleic acids, broadly two strategies have been employed by using viral vectors and non-viral vectors. The viral vectors exhibited high transduction efficacy both in vitro and in vivo. The non-viral vectors composed of mainly cationic polymers and lipids which provide efficient condensing capability against negatively charged nucleic acids and low cytotoxicity. Till date, >2300 clinical trials for gene therapy are going on worldwide, approximately 70% using viral vectors and remaining with non-viral vectors. The immunogenicity and non-targeting abilities are the biggest hurdles in terms of safety and efficiency for successful therapy with these vectors. These two classes of vectors have their own advantages as well as disadvantages which hinder their therapeutic endpoint in clinical trials. Now, researchers have made attempts to form virus encapsulated in chemical vectors which are called as hybrid vectors. These hybrid vectors have immense potential to evade host immune system by masking the immunogenic epitopes present on viral vectors. The molecules or scaffold which is used for encapsulating virus enhance their targeting ability and sustained release to the targeted tissue. The hybrid vectors, a combination of viral and chemical vectors, form a new class of gene delivery vectors which overcome the limitations of each vector and simultaneously augment desirable features such as targeting ability, low immunogenicity, cytotoxicity, higher payload, and ability to deliver more than one transgene. The hybrid vectors should retain characteristics of each vector in order to achieve optimal tissue targeting and gene delivery with minimal toxicity. To achieve therapeutic endpoint with the hybrid vectors, development of such hybrid vectors requires extensive understanding of physicochemical properties after coating virus with chemical analogues and their optimal ratio as well. These aspects will be discussed in this chapter.

## Chapter 5. Pharmaco-Gene Therapy

Martin H. M. Sailer
Department of Biomedicine, Pharmacenter,
University of Basel, Basel, Switzerland

Ganesh Ram Sahu
GROW Research Laboratory, Narayana Nethralaya Foundation,
Bangalore, India

Vellore Institute of Technology, Vellore, India

Arkasubhra Ghosh
GROW Research Laboratory, Narayana Nethralaya Foundation,
Bangalore, India
e-mail: arkasubhra@narayananethralaya.com

There are numerous disease conditions emerging from mutations in genes or alterations in gene expression for which gene-based therapies provide the most promising treatment modality. However, expression of therapeutic genes and proper localization of the expressed proteins require a complex interactive environment within the target cells or tissues for ultimately being clinically applicable. Our knowledge of molecular pathways, cell biology, and the immune system has helped in the development of a variety of drugs that can modulate these processes. Historically, gene therapies and pharmacologic therapies have remained isolated methods of tackling a disease. In this chapter, we will provide an alternate perspective that converges both schools of thought. In specific cases, if conducive to the disease mechanism and the underlying pathology, a pharmacologic drug can help enhance and regulate a gene therapy or vice versa. Such combined approaches can hence increase the specificity and efficiency of the final therapy, which may be much better than the individual ones. There are many hurdles to such dual-modality processes and hence must be employed after careful evaluation of the proposed positive and negative outcomes at a molecular level. We will provide specific examples of such collaborative pharmacologic and gene-based therapeutic strategies and discuss how similar strategies may be beneficial in different diseases.

## Chapter 6. Aptamer as Therapeutics for Cancer with Focus on Retinoblastoma

Nithya Subramanian
PNAC Department,
MRC – Laboratory of Molecular Biology, Cambridge, UK

Akilandeswari Balachandran
Department of Nanobiotechnology,
Vision Research Foundation, Chennai, India

Krishnakumar Subramanian
L&T Department of Ocular Pathology, Department of Nanobiotechnology,
Vision Research Foundation, Chennai, India
e-mail: drkk@snmail.org

Aptamers are composed of oligonucleotide moiety either deoxyribonucleotides (DNA aptamer) or ribonucleotides (RNA aptamer). This single-stranded oligonucleotide molecule folds in specific three-dimensional conformations that enables them to bind specifically to target molecules. These aptamers are highly specific which enables them to differentiate between different conformations of the same protein. Aptamers have several advantages such as smaller size, less immunogenicity, less toxicity, increased stability upon modifications, less production cost compared to antibodies, long shelf life, and highly specific towards the target. The selections of these aptamers are done mainly against cell surface receptors. These aptamers are selected in vitro against targets based on SELEX (systematic evolution of ligands by exponential enrichment) approach. They can also be chemically modified to reduce their size, increase specificity, and also conjugate other molecules. These aptamers can also be modified with fluorescent molecules at 3′ and 5′ positions enabling their use in imaging purposes. The first aptamer approved by FDA is pegaptanib (Macugen) which is used for treating age-related macular degeneration. For cancer therapy, many aptamers are in clinical trials which include anti-PSMA aptamer (A10), anti-nucleolin aptamer (AS1411), anti-mucin aptamer (MUC-1), and anti-stromal cell-derived factor-1 aptamer (NOX-A12). Other than cancer therapeutics, aptamers find major applications in the field of biosensors and bioimaging as well.

## Part II: Cell Therapy – "With Emphasis on Gene-Modified Cell Therapies"

### Chapter 7. SETting up Methylation in Mammalian Cells: Role of Histone Methyltransferases in Disease and Development

Abhishek Mohanty · Shravanti Rampalli
Centre for Inflammation and Tissue Homeostasis,
Institute for Stem Cell Biology and Regenerative Medicine (inStem),
Bangalore, India
e-mail: shravantird@instem.res.in

It is now increasingly evident that the alterations in epigenetic factors have a major impact on human development. Therefore, a substantial amount of efforts are invested at academia and industry toward understanding the mechanism and treatment of such diseases. Embryonic stem cells and, their counterparts, induced pluripotent cells have revolutionized the field of regenerative medicine. Specifically, iPSC technology provides us with the unique tools that are needed for personalized medicine. This chapter will highlight the role of epigenetic regulators in the induction of pluripotency and how their perturbations impact development and disease.

## Chapter 8. Hope or Hype: Stem Cells as Therapeutics in Retinal Degenerative Diseases

Parameswaran Sowmya
Radheshyam Kanoi Stem Cell Laboratory, KNBIRVO,
Vision Research Foundation, Sankara Nethralaya,
Chennai, India
e-mail: drpsowmya@snmail.org

"Netram Pradhanam Servendriyanam"; "Eyes are the precious gift given to humans by the Almighty" – verse from Bhagavad Gita emphasizes the importance of vision for the mankind. The most recent statistics from the World Health Organization (WHO) reveals that 39 million are blind worldwide, signifying the problem and a need to initiate formidable approaches to address the issue. Almost all the ocular diseases involving the retina, the innermost layer of the eye composed of light-sensitive tissue, is characterized by degeneration of retinal cells. The treatment for retinal degenerative diseases is impeded for the want of suitable cells to replace those that are getting degenerated in response to the disease. Stem cell therapy offers a unique opportunity to replace the damaged cells with new ones. Stem cell-based therapeutic approaches can be broadly classified as exogenous and endogenous. The former utilizes exogenous stem cells, such as mesenchymal stem cells, neural progenitors, embryonic stem cells, and induced pluripotent stem cell-derived retinal progenitors that are transplanted into the degenerating retina. The latter approach utilizes activation of endogenous stem cells present in the retina for replacing the degenerating cells. In this book chapter, the key concepts involving both the exogenous and endogenous stem cells for retinal degenerative diseases and their potential advantages and the limitations will be discussed. The outcome of the recent clinical trials along with the future directions and the challenges of stem cell-based therapies will be briefly covered.

## Chapter 9. Haploidentical Stem Cell Transplantation

Narendra Agrawal · Dinesh Bhurani
Department of Hematology, Rajiv Gandhi Cancer Institute &
Research Centre,
Delhi, India

The use of hematopoietic stem cells for transplantation has become standard treatment modality for a wide variety of acquired and congenital disorders of the hematopoietic system. HSCT not only replaces defective/diseased hematopoietic tissue but also provide antitumor immunity in malignant conditions.

Over the span of five decades, there have been many developments in the field of HSCT like high-resolution HLA typing, unrelated donor pools, cord blood banks, preferable use of peripheral blood stem cells and marrow stem cells for different sets of diseases, tailored conditioning regimes to suit different diseases and different ages, better preventive measures for graft versus host disease (GvHD) and opportunistic infections, and managing transplant across HLA disparate pairs. We will discuss the promise and implications of this form of therapy for various hematological disorders.

# Contents

# About the Editor

**Dr. Giridhara R. Jayandharan** is currently working as an Associate Professor at Department of Biological Sciences and Bioengineering, Indian Institute of Technology, Kanpur, India. Dr. Jayandharan completed his Ph.D. from Christian Medical College, Vellore, India, in the year 2006 and subsequently worked as post-doctoral fellow at University of Florida, Gainesville, USA, between the year 2007 and 2009 where he worked extensively on gene therapy. Before joining the coveted Indian Institute of Technology, he worked as a faculty member in the Department of Hematology and also at Center for Stem Cell Research, Christian Medical College, Vellore, India. His main area of research includes gene transfer and therapy, human molecular genetics, and virology.

Dr. Jayandharan is a prolific publisher as he has over 60 papers in peer-reviewed high quality journals. He is recipient of several awards and honors. Most notable awards received by him are Wellcome Trust DBT senior fellowship, Swarnajayanthi fellowship from Department of Science and Technology, Government of India, Young scientist award, YIM Boston, USA; Senior Innovative Young Biotechnologist award, Department of Biotechnology, Government of India; Bayer Hemophilia Early Career Investigator Award, Bayer Inc, USA etc.

Dr. Jayandharan is a member of several notable academies/societies such as International Society for Thrombosis and Hemostasis, European Society for Gene and Cell Therapy, American Society for Gene and Cellular Therapy.

# Part I

# Gene Therapy

# Retroviral Vectors in Gene Therapy

1

Chitra Gopinath, Trupti Job Nathar,
and Everette Jacob Remington Nelson

Retroviruses evolve rapidly to be able to infect a host, replicate, and propagate successfully. Exploiting these viral properties, scientists have genetically engineered the viral genome in order to carry exogenous genetic sequences. The life cycle of a virus is essentially divided into six major stages such as attachment, penetration, uncoating, replication, assembly, and release. Different types of viruses are being tested for their potential in delivering genes into cells or tissues for transient or stable transgene expression [1]. Thus, viral vectors serve as vehicles for gene delivery and currently represent the most efficient means to specifically manipulate cells or tissues for therapeutic applications. Construction of viral vectors for gene delivery applications should typically involve the following steps: (1) removal of genes encoding viral proteins that could be potentially pathogenic; (2) preservation of cis-acting elements of the viral genome such as the packaging signal, ψ essential for viral assembly; (3) expression of essential viral genes involved in viral replication either through helper plasmids, i.e., packaging plasmids, or virus-producing cells; and (4) prevention of immune responses to viral vectors [2, 3].

Six major classes of viruses have entered advanced stages of clinical experimentation for various human diseases by gene therapy, which include gammaretroviruses, lentiviruses, adenoviruses, adeno-associated viruses [4], herpes simplex viruses, and foamy viruses.

C. Gopinath · T. J. Nathar · E. J. R. Nelson (✉)
Gene Therapy Laboratory, SMV124A, Department of Integrative Biology,
School of Biosciences and Technology, Vellore Institute of Technology,
Vellore, TN, India
e-mail: everette.nelson@vit.ac.in



G. R. Jayandharan (ed.), *Gene and Cell Therapy: Biology and Applications*,
https://doi.org/10.1007/978-981-13-0481-1_1

## 1.1 Classification of Retroviruses

The *Retroviridae* family is broadly categorized into three subfamilies, based on their morphological features by electron microscopy and their genome architecture, as oncoretroviruses, lentiviruses, and spumaviruses. Oncoretroviruses are further grouped into five genera such as alpha, beta, gamma, delta, and epsilon retroviruses. Not all retroviruses classified as oncoretroviruses are oncogenic in nature. While some are harmless, others such as the Mason-Pfizer monkey virus (betaretrovirus, type D) are as pathogenic as human and simian immunodeficiency viruses (HIV and SIV, respectively) [5]. Foamy viruses from the genus of spumavirus have so far not shown any reports of pathogenicity and are safer to use as viral vectors for gene therapy [6–8].

### 1.1.1 Retrovirus Structure

Retroviruses contain a viral protein core and a surrounding viral envelope made up of cell membrane-derived lipid bilayer. The diameter of an enveloped virion is 80–120 nm. The core structure consists of two identical single-stranded RNA molecules along with the nucleocapsid protein (NC) which is further surrounded by the capsid protein (CA). Two viral envelope proteins such as surface (SU) and transmembrane (TM) are located on the plasma membrane. The viral envelope is linked to the capsid by the matrix protein (MA) (Fig. 1.1).

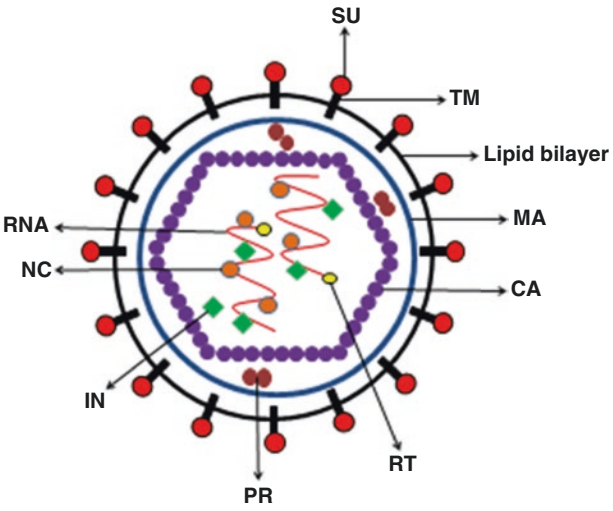


**Fig. 1.1** Structure of a retrovirus**.** The viral genome consists of two identical single-stranded RNA molecules protected by nucleocapsid proteins (NC). Each RNA molecule is associated with the functions of a reverse transcriptase (RT), which is an RNA-dependent DNA polymerase, a protease (PR) and an integrase (IN). The RNA-protein nucleocapsid is enclosed within a capsid which consists of viral capsid proteins (CA). The outermost plasma membrane consists of two proteins, transmembrane (TM) and surface (SU). The virus envelope is connected to the capsid by matrix protein (MA)

## 1.2 Vectors Based on Gammaretroviruses

Vectors derived from one of the prototype gammaretroviruses, namely, Moloney murine leukemia virus (Mo-MLV), were the first viral vectors to be constructed more than three decades ago. Since then, they have been widely used as efficient tools for gene delivery into a wide range of host cells. They are evolutionarily optimized gene delivery vehicles, which naturally adapt to their host environment in order to stably integrate their genetic material into the host genome overcoming natural cellular barriers [9, 10]. The hallmark feature is their ability to reverse transcribe their single-stranded RNA genome into a double-stranded DNA material that can be integrated. They act along with several host cell factors to efficiently deliver their genetic material and further utilize the host cell machinery for their replication and hence sustained, long-term transgene expression.

Since the biology of *Retroviridae* family is well understood, the initial use of these vectors in the treatment of human diseases by gene therapy over the past decade has paved way to the more recent and safer viral vectors such as lentiviral and foamy virus vectors.

### 1.2.1 Genome Organization

The genome of a prototype gammaretrovirus, Mo-MLV, is 9–11 kb long. The reverse transcribed and integrated proviral DNA is flanked by two identical sequences of each 400–700 bp at both 5′ and 3′ ends known as the long terminal repeats (LTR). The U3, R, and U5 regions together constitute the LTR. The U3 region of the 5′ LTR has a promoter that drives the expression of the entire viral transcript. Transcription begins at the first nucleotide of the 5′ LTR's R region and ends at the polyadenylation signal in the 3′ LTR's R region.

The three essential genes, namely, *gag*, *pol*, and *env*, are located between the two LTRs. Proteins such as matrix (MA), capsid (CA), and nucleocapsid (NC) that are essential for viral packaging are encoded by the *gag* gene. The *pol* gene encodes three viral enzymes, namely, reverse transcriptase (RT), integrase (IN), and protease (PR), which play a critical role in the characterization of all members of the *Retroviridae* family. Reverse transcriptase converts single-stranded RNA genome into its double-stranded DNA form and is responsible for the following three enzymatic activities: RNase H, RNA-dependent DNA polymerase, and DNA-dependent DNA polymerase. Polyproteins generated during the process of translation of viral genes are cleaved into individual proteins by protease (PR). Integration of proviral DNA into the host genome is mediated by integrase (IN). The *env* gene encodes two key proteins that appear initially on the surface of infected cells and again on the envelope of budding virions. Surface protein (SU) helps to recognize cellular receptors on the surface and is bound to transmembrane protein (TM) present across the cell membrane.

The proviral DNA has a primer binding site (PBS) for reverse transcription initiation, a splice donor (SD) site, and a packaging signal (ψ). All these regions are

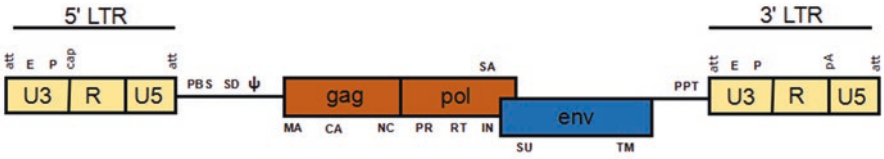


**Fig. 1.2** Genomic organization of the gammaretrovirus. The structure of the murine leukemia virus (MLV) genome consists of 5′ and 3′ LTRs each of which contains the U3, R, and U5 regions (beige). Open reading frames for gag, pol, and env (brown and blue) encoding the corresponding viral proteins (MA, CA, NC, PR, RT, IN, SU, TM as mentioned in Fig. 1.1) are shown. *LTR* long terminal repeat, *att* attachment site, *E* enhancer, *P* promoter, *cap* 5' RNA capping site, *PBS* primer binding site, *SD* splice donor site, *ψ* packaging signal, *SA* splice acceptor site, *PPT* polypurine tract, *pA* polyadenylation site

located upstream of the transcriptional start codon of *gag/pol*. Downstream of *gag/pol* is the *env* region, whose expression is facilitated by a splice acceptor site (SA) situated in the *pol* region [11]. There is a polypurine tract (PPT) just upstream of the 3′LTR which also contains a polyadenylation signal followed by a polyA (pA) tail. The presence of a 5′ cap and a 3′ pA tail in the viral genome makes it similar to the cellular mRNA. Reverse transcription ensures restoration of complete LTRs before integration of the provirus into the host genome.

The location of the packaging signal, ψ, immediately downstream of the major SD site ensures that the unspliced viral genomic RNA preferentially binds the viral structural proteins for proper packaging, whereas the subgenomic RNA lacking the ψ signal can only be translated into envelope proteins encoded by the *env* gene [12] (Fig. 1.2).

### 1.2.2 Replication Cycle

*Adsorption/Binding* The first step in the retroviral replication cycle is the active adsorption of virions onto the surface of the host cell. This interaction is mediated by cellular plasma membrane proteins acting as receptors for the viral surface proteins. The molecules involved in attachment are distinct from the receptors responsible for viral entry [13, 14]. The early attachment of virions to the cell surface is brought about by a wide range of cell surface-associated molecules such as lymphocyte function-associated antigen 1, LFA1 [15], heparin sulfate proteoglycan (16) and nucleolin. Different members of the *Retroviridae* family have shown specificity for different receptors, for example, cationic amino acid transporter CAT1 for ecotropic MLV, T cell surface marker CD4 for HIV1, and glucose transporter GLUT1 for HTLV. Although a specific receptor for the foamy virus is still unknown, heparin sulfate is considered to be one of the cellular attachment factors required for its entry [16].

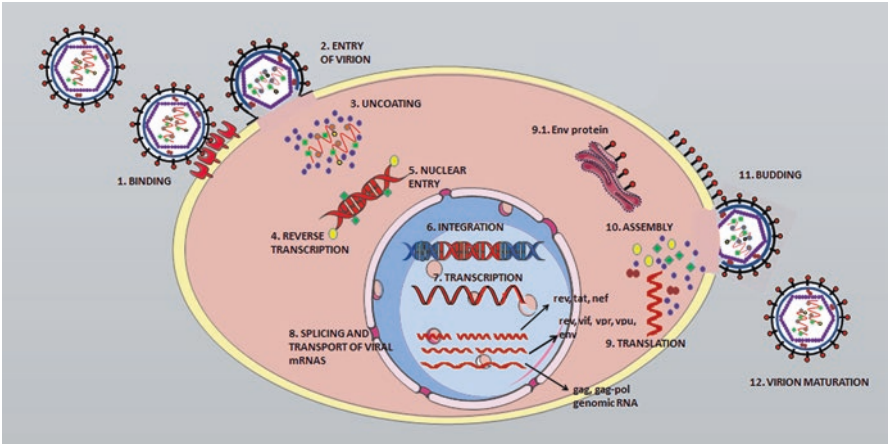


**Fig. 1.3** Retroviral replication cycle. The sequential steps involved in the replication of retrovirus are indicated. Binding of the virus to the membrane is facilitated by interaction of SU with a cell surface receptor followed by entry mediated by TM and subsequent uncoating (steps 1–3). Reverse transcription in the cytosol is catalyzed by RT followed by nuclear entry with the help of pre-integration complex (steps 4 and 5). Integration into the host genome is mediated by IN (step 6). Following transcription and mRNA processing, the viral mRNA is transported back to the cytosol for translation into viral proteins (steps 7–9). Assembly of virions is primarily driven by the Gag polypeptide followed by budding and virion maturation (steps 10–12)

*Entry of the Virion* Retroviral entry is a complex multistep process. The interaction of the surface protein with the cellular receptor leads to a conformational change in the transmembrane. This causes fusion of the viral envelope with the plasma membrane of the host cell and subsequent release of the viral core into the host cell cytoplasm [17–19] (Fig. 1.3).

*Uncoating and Reverse Transcription* Fusion of virus with the host cell membrane allows the viral core to enter the cytoplasm where reverse transcription of viral RNA mediated by the virion-packaged RT takes place. Immediately after the release of the viral core into the cytoplasm, it undergoes a partial and progressive disassembly or uncoating, which results in the formation of reverse transcription complexes (RTCs) and pre-integration complexes (PICs). Initiation of reverse transcription is often seen to be coupled with the onset of uncoating of the viral core [20–22]. This process is further subdivided into nine steps:

1. Primer binding site (PBS), a specific sequence that is located downstream of the 5′LTR hybridizes to the cellular tRNA. Different viruses of the *Retroviridae* family use different tRNAs such as $tRNA^{Pro}$ by both HTLV-1 and Mo-MLV, $tRNA^{Lys1,2}$ by Visna, Spuma, Mason-Pfizer monkey viruses and foamy viruses and $tRNA^{Lys3}$ by HIV-1. The 3′ OH group of the tRNA initiates the synthesis of the complementary DNA strand, as it corresponds to the negative (−) strand of

the RNA genome. R region at the 5′ end is thus added to the strand through polymerization. This leads to the formation of an intermediate DNA fragment, known as the minus-strand strong-stop DNA (−sss) [23].
2. The RT enzyme by virtue of its RNase H activity spans across the DNA-RNA hybrid and digests the RNA moiety, whereas the polymerase synthesizes a new DNA strand known as the complimentary DNA (cDNA). Thus, the RNA is removed, and the newly synthesized cDNA is retained, thereby allowing the single-stranded R region within the newly synthesized –sss DNA to be exposed.
3. As the R region is complementary to the 5′ end, −sss DNA translocates and thus hybridizes to the 5′ end region.
4. Negative (−) strand is thus synthesized, originating from the 3'OH end to the PBS region.
5. Simultaneously, the RT enzyme's RNase H activity digests the viral RNA at the 3′OH end of the genome and stops when it comes in contact with PPT, which is a 10-nucleotide-long purine-rich sequence.
6. Positive strand (+) is synthesized toward the 3′ end, where PPT acts as a primer. It includes the complete LTR and the PBS region that is present downstream of the LTR, represented as +sss DNA.
7. Removal of tRNA primer from the 5′ end of (−) strand by RNase H activity.
8. Excision of tRNA makes the +sss DNA available for hybridization with the (−) strand. This hybridization results in the formation of a circular intermediate.
9. Both strands are elongated up to the two LTRs by RT, thereby substituting the circular intermediate with a linear proviral cDNA.

*Nuclear Entry* Formation of provirus by integration of the viral cDNA into the genome of the host is a crucial step in the life cycle of a retrovirus. To achieve this, the cDNA along with viral Vpr, matrix, and integrase proteins forms a pre-integration complex (PIC) that must enter the nucleus. PICs of most retroviruses such as Mo-MLV are too large to pass through an intact nuclear membrane and hence require host cell division for viral integration to occur [24]. These PICs get imported to the nucleus with the help of nuclear pore complexes. This is the reason why retroviruses such as Mo-MLV are capable of infecting only actively dividing cells.

*Integration* Integration of PICs into the host cell genome is facilitated by integrase (IN). Several other host cellular factors also contribute to the process of integration [25, 26]. The mechanism by which IN mediates integration into the host genome is as follows: IN removes two terminal nucleotides at the 3′ ends of both the strands of the newly synthesized viral cDNA thus generating nucleophilic 3′OH ends. These nucleophilic 3′OH ends are inserted into the host genome through a transesterification reaction. Though integrations are generally observed to be random, they are mostly seen occurring in the active transcription units. The exact reason behind this specificity still remains unknown. The relaxed nature of the chromatin during mitosis is assumed to be one of the reasons for its easy accessibility [24].

*Transcription* After integration of the proviral cDNA into the host genome, it becomes a part of it as an additional protein-coding gene of the infected host cell. Transcription of the provirus is carried out by RNA polymerase II along with a host of cellular transcription factors. The promoter activity is located within the U3 region of the 5′ LTR. This sequence varies among different members of the *Retroviridae* family due to the presence of various transcription factor-binding sites which is responsible for expression of the provirus in specific cell types.

*Splicing and Transport of Viral mRNAs* The primary transcript generated by RNA polymerase II after transcription extends from the first nucleotide of the R sequence in the 5′LTR to the pA site in the U5 region of the 3′LTR. This transcript undergoes further processing before the synthesis of viral proteins by translation. Since mRNAs of all eukaryotic cells are monocistronic, they undergo posttranscriptional splicing to produce different shorter mRNAs. These fragments are directed for translation into a specific protein. In Mo-MLV, one of the two mRNAs is the full-length primary transcript, and the other is a shorter fragment with a deleted large intron at the 5′ end of the primary transcript. The primary transcript is involved in the translation of *gag* and *pol* genes that occurs in the cytosol, whereas the shorter mRNA fragment goes into translating the *env* gene with the help of ribosomes associated with the ER. The spliced products are also transported to the cytosol for translation. Factors such as Rex and Rev bind to the cellular protein Crm1 at the nuclear pores and promote translocation of the incompletely spliced mRNA products into the cytosol by retaining the RRE and RXRE sequences [27, 28].

*Translation* Gag, Pol, and Env which emerge as products of alternative splicing of viral mRNAs are first translated into precursor polyproteins and further cleaved to produce the individual polypeptides. The Gag polyprotein produces three proteins such as MA, CA, and NC; the Gag-Pol polyprotein produces the enzymes RT, PR, and IN; and the Env polyprotein produces SU and TM proteins. The viral PR enzyme cleaves the Gag and Pol polyproteins and a furin protease cleaves the Env polyprotein inside the Golgi apparatus. The Env polyprotein is translated from a single or several specific mRNAs, whereas the Gag and Gag-Pol are translated from the same mRNA sequence. They are present on the same reading frame and are merely separated by a stop codon between the Gag and Pol sequences. Translation is initiated at the first AUG codon to produce the Gag protein, which further continues if the translation initiation complex does not recognize the Gag stop codon due to the presence of an additional amino acid instead of the stop codon thereby leading to the generation of the Gag-Pol polyprotein.

*Assembly, Budding, and Virion Maturation* Assembly of virions occurs at different places for different classes of the *Retroviridae* family. For instance, in type C viruses, the assembly occurs on the plasma membrane, and in type B and D viruses, it takes places in the cytosol. Gag polypeptide is mainly involved in the assembly process. The packaging signal (ψ) that is present in the 5′ region of the *gag* gene packages the genome-length viral mRNA into the virions. Each virion comprises of

1200–1800 Gag and 100–200 Gag-Pol polyproteins. Translation of the Env polyprotein occurs individually in the ER, which undergoes further glycosylation and maturation into SU and TM proteins in the Golgi apparatus. It is then translocated to the plasma membrane of the infected host cell. During viral budding, protein binds to the virions through the N-terminus of the Gag polypeptide. The virions functionally mature outside the cell, and both Gag and Gag-Pol polyproteins undergo cleavage to produce their respective peptides.

### 1.2.3 Structure of Gammaretroviral Vectors

To generate a gammaretroviral vector which is replication deficient, the following modifications are carried out. All *trans*-acting elements of the viral genome (*gag*, *pol*, and *env* genes) are excised, retaining only the LTRs, the packaging signal (ψ), and the elements crucial for viral gene expression. Further, a gene of interest or a transgene cassette could easily replace *gag*, *pol*, and *env* genes. This improves the safety as virus replication would only occur when trans-acting elements such as *gag*, *pol*, and *env* are expressed either by co-transfection using helper plasmids [29] or by a packaging cell line (Fig. 1.4).

Viruses are usually produced or packaged in cultured mammalian cells. The recombinant vector or transfer plasmid is obtained by inserting a transgene by standard molecular cloning techniques followed by amplification of the plasmid DNA via bacterial transformation. The transfer plasmid is introduced into a packaging cell line which is mostly of murine or human origin. A packaging cell line is usually generated by stable co-transfections of the plasmids encoding sequences for Gag-Pol and Env proteins, which could constitutively express the respective proteins. The *gag* and *pol* genes are cloned into a single plasmid, whereas the *env* gene is cloned separately into another plasmid. This strategy is employed to avoid integration of the three essential genes within close proximity at a specific genomic locus. This would

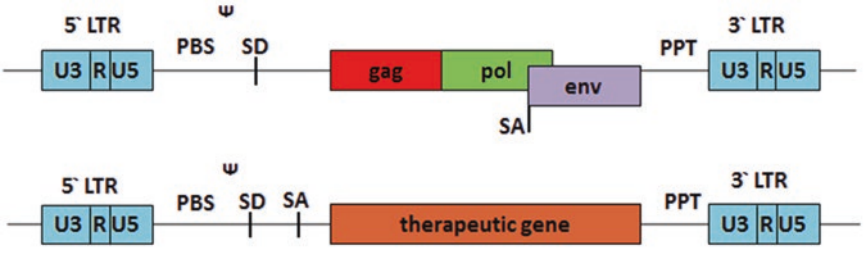


**Fig. 1.4** Schematic structure of wild-type and recombinant retroviral vectors. Top – genetic elements of a wild-type gammaretroviral vector, namely *LTR* long terminal repeat consisting of U3, *R* U5 regions, *PBS* primer binding site, *PPT* polypurine tract, *Ψ* packaging signal, *SD* 5′ splice donor site, *SA* 3′ splice acceptor site; gag-pol and env regions. Bottom – recombinant retroviral vector containing a therapeutic gene inserted between the 5′ and 3′ LTRs after the replacement of the gag, pol, and env genes

negate chances of any recombination leading to the generation of infectious replication-competent retroviruses (RCRs), thereby improving the safety [30].

Once the transfer plasmid is introduced into the packaging cell line, transcription is initiated from the 5′ LTR region generating a proviral mRNA. This mRNA consists of a packaging signal (ψ) that helps *gag* gene to recognize the vector followed by its inclusion into a virion. This virion which imitates the wild-type virus is now fully capable of infecting a host cell. The enzyme RT along with *cis*-acting PBS and PPT sequences reverse transcribes the mRNA producing a cDNA which will eventually integrate into the host genome mediated by the enzyme IN. After integration, the proviral DNA is rendered noninfectious as all virulent retroviral proteins are made unavailable. This design would enable numerous generations of the recombinant vectors to be assembled without the risk of RCRs being formed [31, 32].

### 1.2.4 Gammaretroviral Vector Variants

Over the last few years, several modifications have been incorporated into the gammaretroviral vector backbone both to increase its efficacy as well as safety. These modifications were necessary either to allow the insertion of additional genes or a new promoter instead of relying on the LTR for expression of the transgene [33]. Detailed descriptions of these modifications are stated below:

*Vectors with an Internal Promoter* Viral vectors are already known to possess promoter activity within its 5′ LTR region. Addition of another promoter to the vector backbone was thought to be beneficial for transgene expression. Hence, an additional internal promoter which escapes silencing and is strong, inducible, or even tissue-specific could be considered [34, 35]. This promoter is placed in a region downstream of the 5′ LTR and immediately upstream of the transgene. As a result, two unique mRNAs are transcribed, one initiated by the 5′ LTR, which also includes the internal promoter sequence and another that is initiated by the internal promoter itself. This could increase the possibility of transcriptional interference between the two promoters, thus affecting the production of the desired mRNA species. As virus production in the packaging cells almost exclusively depends on viral LTR-mediated transcription, the presence of a strong internal promoter could lead to a reduction in the viral titers. Hence, an internal promoter such as a tissue-specific promoter that is weak and inactive in packaging cells but becomes strong and active in the final target cells could be effective in resolving this issue.

*Vectors Expressing Two Genes* Co-expression of two or more genes is valuable in many experimental settings. To accomplish this, scientists have used a number of techniques such as using multiple or bidirectional promoters, co-transfecting two or more plasmids, or creating bicistronic or multicistronic vectors. Unlike promoters that create unique mRNA transcripts for each protein, multicistronic vectors

simultaneously express two or more separate proteins from the same mRNA transcript [9]. The following strategies can be adopted for the simultaneous expression of two or more genes:

1. Cloning one gene between SA and SD sites and a second one downstream of the SD site. This arrangement is very similar to that of the retroviruses where the second gene expression depends on splicing of the primary mRNA transcript.
2. By cloning one gene downstream of the 5′ LTR and a second gene downstream of the internal promoter. Thus could also result in transcriptional interference between both the promoters.
3. By cloning two different genes under the influence of the viral 5′ LTR and separating them by introducing an internal ribosomal entry site (IRES). This is considered to be one of the most efficient strategies as it not only increases the viral titer but also leads to high-level transgene expression. Viral IRESs are derived either from *Picornaviridae* or hepatitis C viruses (HCV). This strategy would allow production of a single transcript that could translate into two different proteins from two independent AUG start codons.
4. The last strategy involves cloning the coding sequences of both the genes together thereby producing a fusion gene which would lead to the production of a fusion mRNA transcript and protein. However, it is important to retain the function of both the proteins.

*Double Copy Vectors* This strategy involves cloning of the transcriptional cassette expressing the gene of interest within the U3 region of the 5′ LTR without disrupting the transcriptional activity. The mRNA is produced from the 5′ R region to 3′ U5 region thus containing a modified U3 region. During reverse transcription, the modified U3 sequence is transferred to the 5′ LTR region, duplicating the gene of interest. Genes such as a selectable marker gene can also be placed inside the vector which will be transcribed normally by the 5′ LTR of the vector [36].

*Self-Inactivating Vectors* A self-inactivating (SIN) vector contains an inactive or modified 3′ LTR sequence. During integration, the modified 3′ LTR sequence is replicated on the 5′ end of the vector genome thereby inactivating the promoter activity associated with the LTRs. The modifications incorporated into a SIN vector are as follows: (i) U3 region of the 5′ LTR is replaced with a CMV or RSV promoter/enhancer, and (ii) binding sites for Sp1, NF-kB, and the TATA box transcription factors present in the U3 region of the 3′ LTR are deleted. After reverse transcription, this deletion gets transferred to the 5′ LTR thereby eliminating the transcriptional unit from the LTRs of the provirus. Thus, a SIN modified vector is capable of driving efficient transgene expression and at the same time is safe as it avoids transcriptional interference of the strong viral LTR with the desired internal promoter [37, 38].

## 1.3 Vectors Based on Lentiviruses

Lentiviruses belong to a different genus under the *Retroviridae* family, and are classified as complex retroviruses based on the complexity of its viral genome [39]. Lentiviruses include a variety of primate viruses like the human and simian immunodeficiency viruses (HIV and SIV, respectively), as well as non-primate viruses like feline immunodeficiency virus (FIV), equine infectious anemia virus (EIAV), maedivisna virus (MVV), caprine arthritis encephalitis virus (CAEV), and bovine immunodeficiency virus (BIV). The human immunodeficiency virus type 1 (HIV1) is considered to be the most commonly known prototype lentivirus. One of the characteristic features that make lentiviruses more attractive for gene therapy applications is their ability to infect both actively dividing and nondividing cells and integrate into the host cell genome [40]. Nondividing cells such as hepatocytes, myocytes, macrophages, neurons, and stem cells are potential targets for gene therapy using lentiviruses. These viruses are often used to treat diseases involving organs like the brain and retina which are mostly composed of quiescent cells [41]. Their unique feature is largely due to the fact that their PIC components can interact directly with the nuclear pore proteins and enter the nucleus without the need for dissolution of the nuclear membrane and eventually integrate into the host genome. This is of paramount importance especially with respect to hematopoietic stem cells where ex vivo transduction could be done without the need for cell division [42]. Lentiviruses that are currently in development as gene therapy vectors are derived from HIV1 and 2, SIV, FIV, EIAV, and CAEV (Fig. 1.5).

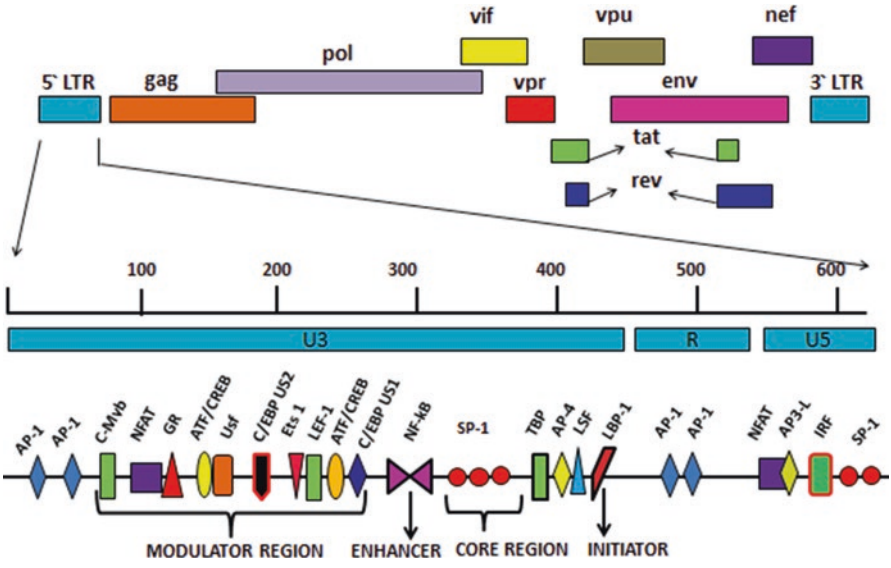


**Fig. 1.5** Structure of HIV1 LTR. An array of putative regulatory elements that make up the LTR is shown. Protein-binding sites indicated as modulator region function as negative regulatory elements. The HIV1 LTR also contains a TATA box-binding protein (TBP), two direct repeats of NF-kB-binding site, three Sp1-binding sites, and a LBP-1 initiator-binding site

### 1.3.1 Genome Organization

As stated earlier, the genome organization of lentiviruses is complex. In addition to structural genes such as *gag*, *pol*, and *env* that are universal to all retroviruses, they also contain other regulatory elements (*rev* and *tat*) as well as accessory genes such as *vif*, *vpr*, *vpu*, and *nef*. These genes are not essential for viral replication and hence termed as accessory genes. The roles of each of these accessory genes are as follows: *vif*, necessary for HIV-1 replication; *vpu*, enhances release of HIV-1 from cell membrane to cytoplasm; *vpr*, mediates HIV infection of nondividing cells; and *nef*, disrupts T cell activation and stimulates HIV infectivity (Fig. 1.6).

These genes are mainly involved in the regulation of viral gene expression, assembly of viral particles and also responsible for structural and functional changes in the infected host cell. The *cis*-acting genetic sequences, which do not code for any protein, are indispensable for replication, integration, and packaging of the lentiviruses. These *cis*-acting elements play a crucial role in the lentiviral vector design and hence retained in the transfer vector, whereas the *trans*-acting elements that encode structural, regulatory, and accessory proteins are dispensed with. The steps involved in the lentiviral life cycle such as attachment, entry, reverse transcription, and integration are exclusively retained as they are replication deficient and hence do not multiply in the host organism. Most of the *trans*-acting elements are eliminated as the earlier steps do not require viral protein synthesis. This allows transfer vector to exclusively express the gene(s) of interest. *Trans*-acting elements are provided in trans through the helper plasmids, which are co-transfected with the transfer vector during packaging.

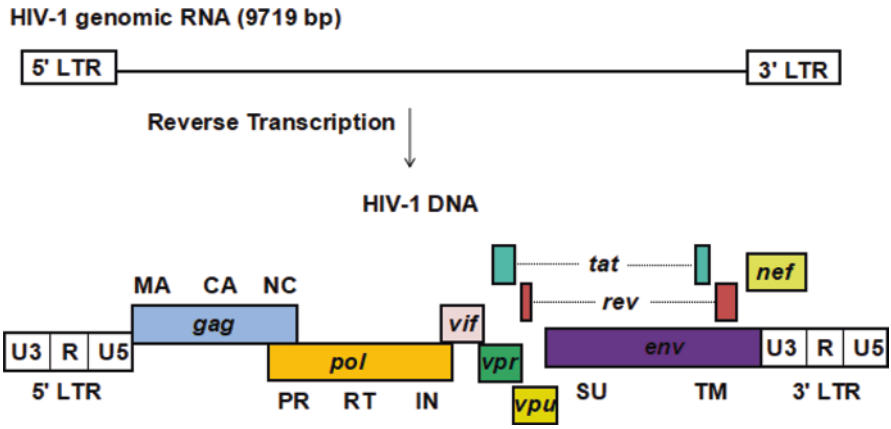


**Fig. 1.6** Genome organization of HIV-1**.** Essential genes such as *gag*, *pol*, and *env* flanked between the 5′ and 3′ LTRs are shown. The six additional open reading frames that correspond to the regulatory and accessory genes such as *vif*, *vpr*, *vpu*, *tat*, *rev*, and *nef* are also indicated

The regulatory genes include *tat* and *rev* that encode for transactivator proteins that are important for viral replication. The *rev and tat* genes are universal to all lentiviruses except FIV where only the *rev* gene is present. Both of these genes augment the synthesis of viral mRNAs. The *rev* sequence consists of a nuclear export signal (NES) which facilitates nuclear export of partly and unspliced mRNAs encoding viral structural proteins. In the absence of *rev*, the only mRNAs found in infected cells are the doubly spliced transcripts. Since it is a key protein involved in viral replication, it serves as a target for protein- and RNA-based anti-HIV therapeutic strategies. In HIV1-based vector systems, the presence of *rev* is crucial unless it is replaced with a functionally equivalent element. The other viral protein Tat is shown to upregulate viral transcription by associating with the Tat activation region (TAR) on the 5′ end of viral mRNAs. Additionally, binding of Tat to the cyclin-dependent kinase (Cdk)-Cyclin T complex is necessary for the activity of Tat and TAR decoys. To generate a potential gene therapy vector, the requirement for Tat is overcome by the addition of a promoter/enhancer of the human cytomegalovirus (CMV) or Rous sarcoma virus (RSV) which functions as strong constitutive promoters in the transfer vector.

### 1.3.2 Replication Cycle

Since both gamma and lentiviral viruses are closely related, their life cycle and various other components are essentially the same. The glycoproteins of the viral envelope have an affinity toward specific cellular receptors, which then fuse with the host cell membrane, thus facilitating the core to be released into the cytoplasm. Immediately after internalization, the ssRNA is converted to a double-stranded genome (cDNA) by the viral proteins and gets incorporated into the host genome. Despite these similarities, some major variations do exist in the life cycle of the lentivirus. First, gene expression occurs during both the early and late phases, which are distinguished based on the binding of the Rev protein. Secondly, lentivirus is capable of infecting nondividing cells mediated by PICs consisting of the viral *Vpr*. The *tat* gene that is necessary for HIV-1 replication is found only in complex retroviruses such as the lentivirus.

### 1.3.3 Structure and Production of Lentiviral Vectors

Early HIV1-based vectors were constructed for the purpose of investigating the infective nature of HIV-1 rather than its applications in gene therapy. Three different generations of HIV1-based lentiviral vectors have emerged over the past decade or so, each comparatively safer to the previous generation(s) as described below:

*First-generation lentiviral vectors* involved generation of viral particles by co-transfection of three plasmids. First is the transfer plasmid that carries the gene of interest or therapeutic gene where the cDNA of the desired gene is cloned in the 5′ to 3′ orientation. This plasmid consists of the following genetic elements: wild-type 5′ LTR; leader region with PBS sequence and a 5′ splice donor (SD) site; about

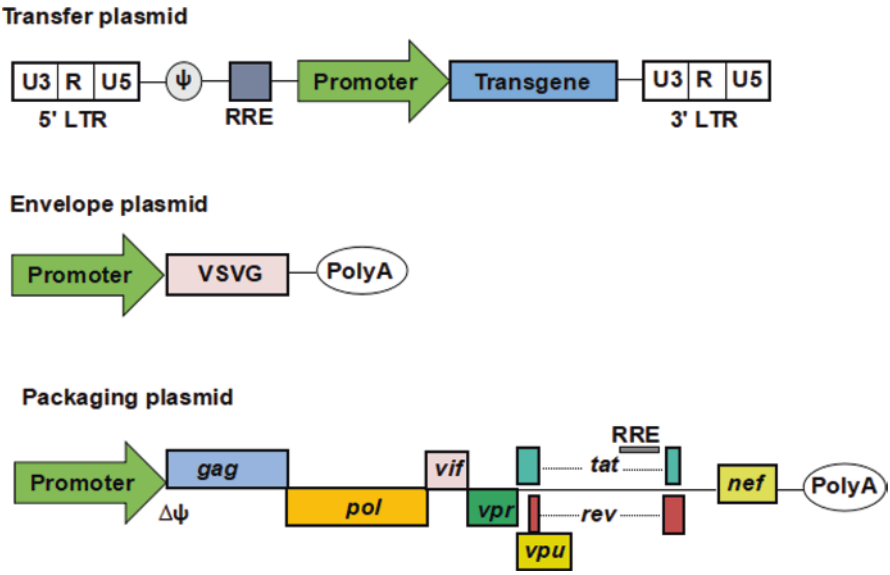


**Fig. 1.7** First-generation lentiviral vectors**.** Transfer plasmid carrying the therapeutic gene driven by an internal promoter, RRE, packaging signal ψ, and modified LTRs; envelope plasmid coding for the VSV-G protein for broader tropism; packaging plasmid derived from HIV-1 driven by a constitutive promoter such as CMV immediate early genes, mutated ψ (Δψ) devoid of the *env* gene

350 bp of the *gag* gene which includes the packaging signal (ψ), stop codon at the 3′ end of the gene's open reading frame; about 700 bp of the *env* gene containing the RRE sequence and 3′ splice acceptor (SA) site; internal promoter driving the gene of interest as the HIV1 promoter is silenced due to the absence of the viral Tat protein; and viral 3′ LTR immediately upstream of the PPT sequence (Fig. 1.7).

The second plasmid which is also derived from the HIV1 genome is a packaging plasmid. All other viral genes are retained with the exception of the *env* gene. It also carries a mutation in the packaging signal (ψ) to avoid packaging of the encoded RNA into the virion assembly. It is devoid of the 3′ LTR and is replaced with a heterologous polyA sequence. The plasmid consists of a strong constitutive promoter of the cytomegalovirus immediate early genes (CMV-IE) to drive its expression. The third plasmid codes for the vesicular stomatitis virus glycoprotein (VSV-G) to improve the tropism of the vector [43].

First-generation lentiviruses are produced by transient co-transfection of all three plasmids into human embryonic kidney 293 T (HEK293T) cells. Similar to gammaretroviruses, virions containing the mRNAs are released into the culture supernatant. First-generation lentiviral vectors raised some important safety concerns in terms of viral packaging and its clinical utilization. During production, replication-competent lentiviruses (RCL) could be generated due to recombination events occurring between the packaging plasmids and the lentiviral vector whose infectivity could be further enhanced by the presence of VSV-G protein. Also during

reverse transcription, a similar recombination event could happen in the target cells where the virion would carry two RNA copies, one from the transfer vector and another from the packaging plasmid. The chances of wild-type lentiviruses recombining with the first-generation lentiviral vectors leading to the production of novel viruses which can be pathogenic are given serious consideration keeping in mind their possible use in a clinical trial. To overcome these safety issues, further deletions were carried out wherein all the genes responsible for the production of viral particles were removed altogether [44].

The *second-generation lentiviral vectors* also belong to the three-plasmid system; however in the packaging plasmid, all accessory genes were removed except *rev* and *tat*. This was done to reduce the chances of recombination; however, the presence of intact LTRs and (ψ) packaging signal could still lead to recombination with the wild-type HIV1 if used to treat a HIV1-infected patient (Fig. 1.8).

*Third-generation or SIN lentiviral vectors* were designed to overcome the safety issues posed by the previous two generations. This was largely achieved by reducing the chances of recombination with the wild-type HIV1 and vector mobilization inside the organism. This generation involves a four plasmid system. The first plasmid is a transfer plasmid with SIN modification. The U3 region of 3′ LTR is deleted to inactivate transcription of the proviral DNA following reverse

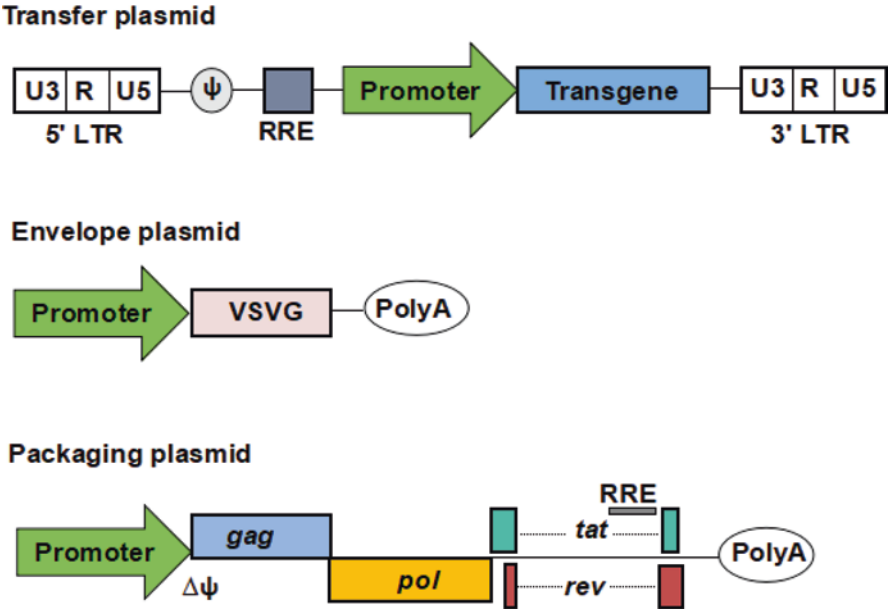


**Fig. 1.8** Second-generation lentiviral vectors**.** This involves the use of three plasmids similar in design to the first-generation vectors; however, in the packaging plasmids, all accessory genes are eliminated except *tat* and *rev*. Second-generation vectors carry intact LTRs and ψ region. Transfer plasmid of the second-generation lentiviral vectors must be used along with second-generation helper plasmids as transgene expression from the LTR is *tat* dependent

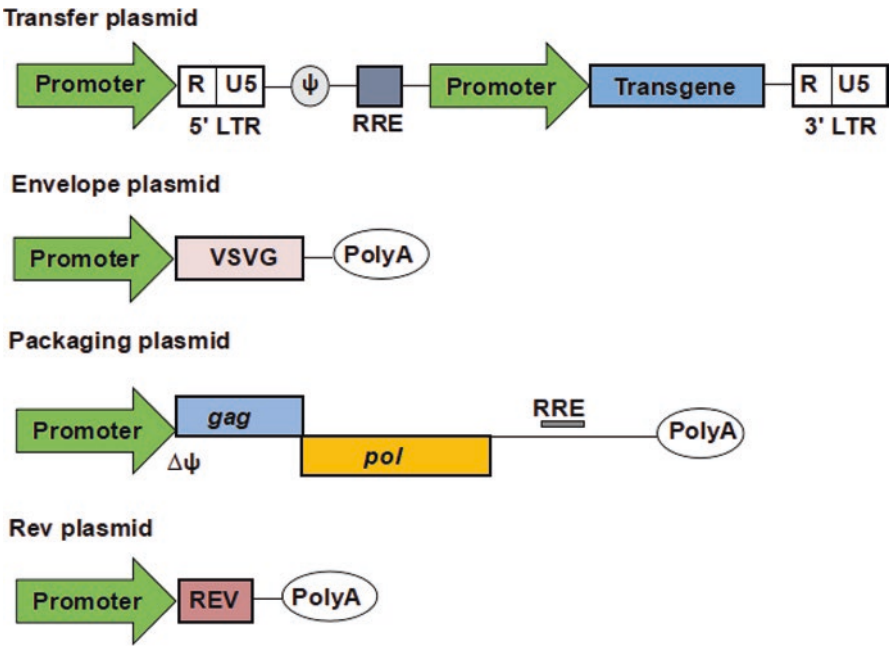


**Fig. 1.9** Third-generation lentiviral vectors**.** Transfer plasmid consists of CMV/HIV or RSV/HIV LTR hybrids with deletions of *tat* and SIN 3′ modifications to the LTR regions for enhanced safety. Expression of transgene from this promoter system is no longer dependent on *tat* transactivation. The *rev* element is separated on a different regulatory plasmid. This four plasmid system is safer as they are now replication incompetent. They can be packaged either with second- or third-generation packaging systems

transcription. As it inactivates its own transcriptional activity it is referred to as a self-inactivating (SIN) lentiviral vector. Therefore in the packaging cells, transcription is carried out by an active heterologous promoter placed upstream of the 3′ LTR's R region. Also, in the *pol* gene, a new HIV1 sequence called the central polypurine tract (cPPT) was added to increase the viral titers. This sequence was shown to act as both secondary to PPT to drive the synthesis of (+) strand strong-stop DNA and aid in the nuclear transport of PICs. As the transcription of the transfer plasmid relies solely on the heterologous promoter, the *tat* gene is therefore unnecessary. Hence, the packaging plasmid now has only *gag* and *pol* genes, with the rev gene being included on an additional plasmid as it is required for the transport of viral mRNAs back into the cytosol of the host cell. Finally, similar to the previous two generations, the VSV-G is retained in the envelope plasmid. Thus, third-generation lentiviral vectors are considered to be comparatively safer to the previous generations and gammaretroviral vectors based on the preference of the site of integration [45–49] (Fig. 1.9).

## 1.4 Vectors Based on Foamy Viruses

Human foamy virus (HFV) is a complex retrovirus belonging to the genus of spumavirus. It was first described in 1954 and later isolated in 1971 [50]. Its genomic organization is found to be more similar to lentiviruses like HIV1 than any other retroviral vectors [51]. The name foamy comes from its characteristic "foamy" appearance due to the cytopathic effect induced in the infected cells. They are found to be nonpathogenic in accidentally infected humans [52]. The human prototype foamy virus is the human foamy virus (HFV), which was isolated from a culture of cells obtained from a patient of African origin with nasopharyngeal cancer. It was called as human foamy virus because of its origin and named SFVcpz (hu) as the prototypic laboratory strain, also due to its resemblance to the simian foamy virus. Later, it was identified to be a distinct type of foamy virus as it was very similar to SFV types 6 and 7 isolated from chimpanzees. Another claim was that SFVcpz (hu) was not a distinct type of the foamy virus but merely a variant strain of chimpanzee foamy virus [53]. In 1994, the foamy virus genome was finally cloned and sequenced, and it was shown that both SFV and HFV genomes share 86%–95% homology in their amino acid sequences [54].

### 1.4.1 Genome Organization

The genome organization of the human foamy virus is complex very similar to other complex retroviruses like HIV1. In addition to the canonical *gag*, *pol*, and *env* genes, they also encode various open reading frames (ORFs) at the 3′ end of their genome, among which only two ORFs are known to encode proteins. As already known, the Gag protein plays an important role in various stages of the retroviral replication cycle. They not only help in the viral assembly but also interact with various other host cell proteins. They are also involved in the regulation of viral gene expression, more importantly in viral intracellular trafficking and budding. Unlike other complex retroviruses, the foamy viral Gag protein lacks the characteristic orthoretroviral domains like the membrane-binding domains (M domains), which are major homology regions (MHR), and the signature Cys-His motifs, and contain unique domains such as the Gag-Env interaction domain and the glycine and arginine-rich boxes (GR boxes). Additionally, a foamy viral Gag protein undergoes finite maturation and follows an atypical nuclear translocation pathway [55].

Foamy viruses possess large LTR regions, the full-length of the HFV LTR being 1769 bp [50]. Certain sequences of the viral 3′ LTR's U3 region are deleted, which lead to a selective advantage for virus replication only in some cell types, those of the lymphoid tissue being an exception. These deleted regions seem to contain few negative regulatory elements, but their exact nature is still unknown. Similarly, the U5 region also contains a negative element. Foamy viruses also

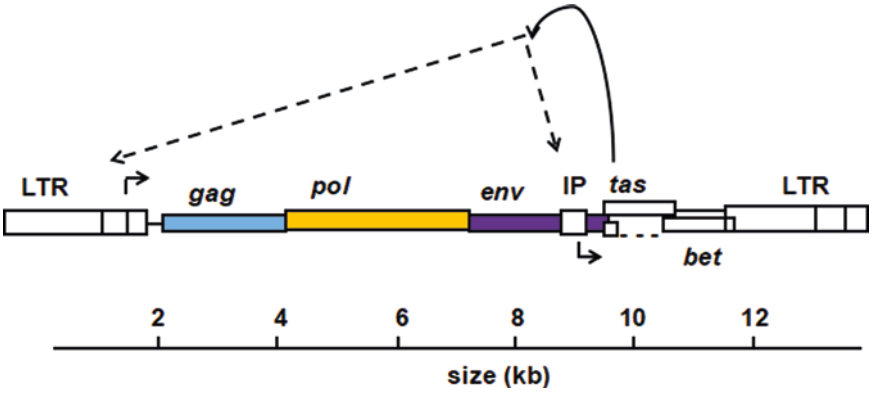


**Fig. 1.10** Genomic organization of the foamy virus**.** Genetic elements of a prototypic foamy virus are shown. Two additional accessory genes, *tas* and *bet*, are expressed by an internal promoter (IP) located within the env gene. Transcription from both the 5′ LTR and the IP is transactivated by Tas

contain several *cis*-acting sequences like the PPT for positive-strand priming and packaging sequence at the 5′ end of the genome. Some additional *cis*-acting sequences have been mapped to the *pol* gene that is required for the transfer of HFV vectors. These sequences could either be required for packaging or for other posttranscriptional modifications like RNA stability or efficient nuclear export. If, indeed, these *pol* sequences are involved in the packaging, this could attribute to a unique feature of the foamy virus genome. Tas protein, a DNA-binding transactivator, is indispensable for transcription from the LTR promoter/enhancer. In the viral particles that are devoid of Tas, the initiation of infection remains a question, which could be resolved by using multiple promoters. This mechanism is observed only in complex DNA-based viruses and not in retroviruses. The 3′ end of the *env* gene contains an internal promoter that is necessary for viral infectivity of cells grown in culture. The level of expression driven by such a promoter is higher than that of the LTR promoter; thus its use could lead to the generation of transcripts encoding the proteins, Tas and Bet. Once enough Tas levels are produced, the transcription would be taken over by the LTR promoter. The LTR promoter has less affinity for Tas when compared to the internal promoter resulting in the accumulation of *gag*, *pol*, and *env* transcripts. Among members of the *Retroviridae* family, only foamy viruses show such temporal transcriptional regulation (Fig. 1.10).

**Tas (Bel1) Protein**

The transcription driven by both the LTR promoter and the internal promoter requires the Tas protein for transcription [56]. The sequence of the Tas protein,

which was formerly called Bel1, is quite different from that of other known transcriptional transactivators, and it mediates transcription by a unique mechanism. It is a 36 kDa phosphoprotein that has a centrally located DNA-binding domain and an acidic transcription activation domain at its C-terminus [57]. The Tas protein has affinity toward both the LTR promoter and the internal promoter although these regions contain different DNA sequences, and hence the Tas-specific DNA-binding sequence is said to be degenerate. Sequence alignment results showed a 25 bp binding site in both the LTR and the internal promoter with conserved purine residues, as identified by methylation patterns with less sequence identity [58]. The higher affinity of Tas for the internal promoter leading to the greater level of initial transcription displays the temporal transcription regulation initiated from the internal promoter and the eventual transition to the LTR at higher Tas concentrations. The Tas protein is different from the Tax protein, a transcriptional activator of human T cell lymphotropic virus (HTLV) [59], and the Tat, a transcriptional activator of HIV, in that only Tas is a sequence-specific DNA-binding protein. Elaborate studies are required to understand the details of the protein-DNA interactions. It is notable that only foamy viruses are atypical among complex retroviruses that do not encode posttranscriptional regulatory genes [60, 61].

**Bet Protein**

The Bet protein is generated from a dual-spliced mRNA which is translated from the Tas and Bel2 open reading frames [50, 62]. It is made up of 88 amino acids of the Tas protein which is fused to the complete Bel2 ORF. Infected cells generate the Bet protein both in vitro and in vivo, whose function remains unclear [63]. Sequence analysis does not yield any clue about its function. Bet is highly conserved among other members of foamy viruses, but surprisingly does not show significant sequence similarity with any known proteins or protein motifs. The presence of conserved histidine and cysteine residues in Bet points to its role analogous to that of HIV1 Vif protein [64]. Deletion of a portion of the Bel2 ORF from Bet has led to the production of infectious virus particles even at titers of around 10% that of the wild-type. This decrease in viral titers was only noticed in cell-free virus systems, whereas cell lysates from both Bet1 and Bet2 viruses yielded very similar titers [65]. However, other explanations are possible as it is difficult to conclude from these small effects. There is a possibility that Bet may play a significant role in in vivo infections as suggested by a recent report that states that overexpression of Bet in cultured cells may prevent infection by the wild-type foamy virus. Though the exact mechanism is unknown, blocks are seen downstream of the viral entry and upstream of viral gene expression, most likely at the viral integration site [66]. Due to these reasons, the role for Bet is not very clear. Further studies would be required to demonstrate if Bet could be a key player in maintaining persistent low-level infections in vivo.

### 1.4.2 Replication Cycle

A closer look into the foamy virus replication cycle revealed mechanistic differences in nearly all the steps when compared to other retroviruses. For example, the reverse transcription occurs late in foamy viruses or even starts late directly before the release of the virus from the cell [67]. Therefore, in all likelihood the genome of the foamy virus is DNA instead of RNA or DNA-RNA hybrid [68]. The full-length RNA mostly serves as a pre-genome as the cells transfected with DNA isolated from foamy viruses produce infectious virus particles. The nature of the genome in vivo is still questionable. The overall genome organization of the foamy viruses is very similar to those of other complex retroviruses and encodes two regulatory genes for *bet* and *tas*, in addition to *gag*, *pol*, and *env* (Fig. 1.11).

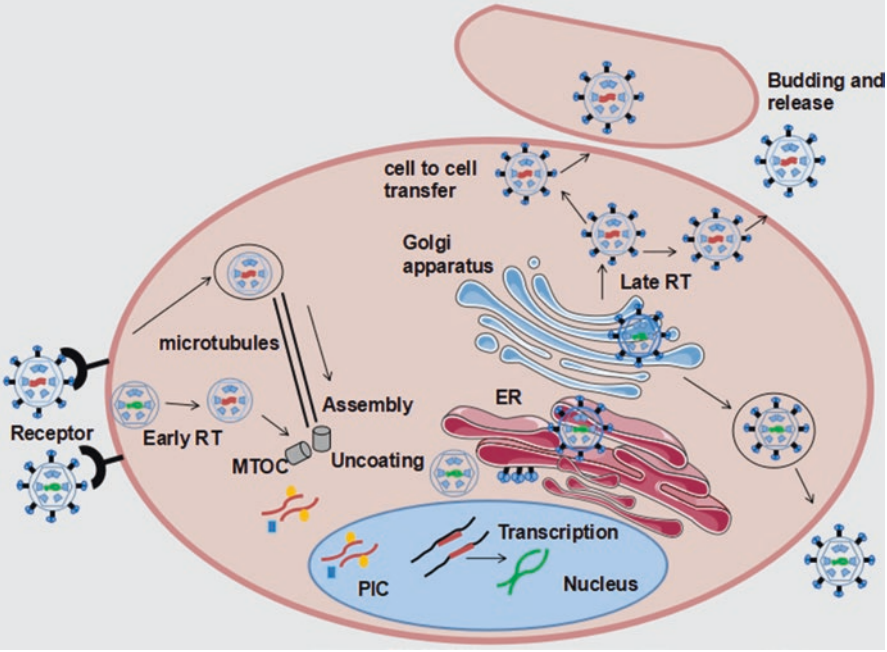


**Fig. 1.11** Replication cycle of foamy virus**.** Foamy virus binds to an unknown cellular receptor following which the viral core passes along microtubules toward the microtubule-organizing center (MTOC) and early reverse transcription is initiated. The Gag protein is cleaved with the foamy virus protease which leads to disassembly of the core at the MTOC. After integration, viral mRNAs and proteins are synthesized and the foamy virions assemble in the cytoplasm. ER retention signal targets *env* to the ER which is crucial for foamy virus budding to occur. Late reverse transcription occurs before viral budding, giving rise to about 20% of virions consisting of infectious genetic material [69]

The replication cycle of foamy viruses is different from that of other retroviruses in the following ways [69]:

1. Reverse transcription occurs late during viral morphogenesis, and the infectious particles produced may contain DNA genomes.
2. Unlike other retroviruses, mature virions lack MA, CA, or NC proteins and rather consist of two large Gag proteins which are unidentical at the C-terminus due to the removal of 3 kDa.
3. Gag and Env proteins are both essential for viral budding.
4. Majority of viral budding occurs from the ER rather than from the plasma membrane.
5. Most of the virus produced is found to be intracellular and perhaps accounts for most of the unintegrated DNA observed in infected cells.
6. Frequently, infected cells carry an excess of integrated DNA.

Intracellular recycling pathway could be one the reasons for the above, although no evidence of that occurring during foamy viral infection in vitro or in vivo has been reported. Certain characteristics of foamy viruses are found to be like those of hepadnaviruses, but in these viruses, the process of reverse transcription is completed before budding. The viral core, S proteins, and a recycling pathway all seem to be essential for viral budding as the core does not get cleaved into smaller polypeptides. This pathway allows the accumulation of several copies of covalently closed circular viral DNA inside the nucleus. Undoubtedly, foamy viruses like other retroviruses differ from hepadnaviruses as they synthesize both proteases and integrases, integration being a necessary event in the viral life cycle.

### 1.4.3 Development of Foamy Virus Vectors

Foamy virus vectors have been widely studied and could have great potential for gene transfer into hematopoietic repopulating cells. Development of vectors derived from several foamy viruses is discussed in the following sections [70].

**First-Generation or Early Replication-Competent Foamy Virus Vectors**

The first-generation foamy virus vectors were replication-competent [71] and were derived from an infectious plasmid clone of a prototype foamy virus where the reporter genes were incorporated into the *bet* region. They demonstrated great potential for relatively high titers ($10^5$ transducing units/ml) and were shown to deliver a chloramphenicol acetyltransferase (CAT) transgene to human fibroblasts. Later, development of non-replicating vectors was shown to cause infiltration of the wild-type foamy virus in the vector preparation [72]. Despite these limitations, efforts to efficiently transduce hematopoietic cells for long-term and stable transgene expression required very high titers [73].

*Second-Generation Tas-Independent Foamy Virus Vectors* During the development of second-generation foamy virus vectors, high titer helper-independent vector preparations were achieved. These foamy virus vectors consist of two independent helper plasmids incorporating *gag*, *pol*, and *env* under the control of strong CMV promoters and were transfected into HEK293T cells [74]. Like in the case of other retroviral vectors, the vector transcripts in the U3 region of the LTR were substituted with a constitutive CMV promoter (CMV-R-U5) in order to initiate transcription at the start of the R region in the 5′ LTR. This modification resulted in a system where vector transcription is independent of Tas (Bel1), which could now be totally excluded from the system. Foamy virus vectors are thus made self-inactivating (SIN) due to the elimination of Tas. Second-generation vectors are capable of yielding titers in the order of $10^5$ transducing units/ml, with the removal of the *tas* gene leading to the formation of replication-deficient viruses in the preparation. These high titer helper-independent foamy virus vector preparations were useful in evaluating the transduction efficiency of hematopoietic stem cells (HSCs) in mouse models [70].

**Third-Generation Foamy Virus Vectors**

The cis-acting sequences (CAS) of length 2.2–2.5 kb retained in the third-generation foamy virus vectors were shown to facilitate gene transfer. These sequences encompass the region between the 5′ LTR and env, a proximal sequence in the 5′ region of gag (CAS I) and a distal sequence, CAS II spanning the 3′ region of pol and the 5′ region of env. The third-generation foamy viruses include a deletion of the *tas* gene, formerly known as *bel1*, that functions at the viral LTR in the wild-type foamy virus. These vectors are SIN considering the deletion of *tas* and an additional deletion surrounding the TATA box and enhancers in the U3 region of the 3′ LTR of the plasmid. During reverse transcription this deletion is forged to the 5′ end of the viral genome, thereby silencing both LTRs in the integrated provirus. Unlike in the lentiviral vector systems, the foamy viral *gag* and *pol* are translated from two independent mRNAs. Moreover, *gag, pol*, and *env* genes are provided in *trans* using three independent helper plasmids, whereas the *cis*-acting regions (CAR) are retained on the vector backbone. CAS I was mutated to stop the expression of *gag* gene from the plasmid with further modifications made to foamy viral *cis*-acting regions. To reduce or avoid recombination events involving helper and transfer plasmids that could result in the generation of replication-competent viruses, the *gag*, *pol*, and *env* genes are placed onto individual plasmids, each expressed by a CMV promoter with an intron to obtain high level expression. Thus, the deletion of *tas* and *bet* from both the helper and transfer plasmids totally eliminates the possibility of producing a wild-type foamy virus by recombination. The third-generation foamy virus vectors do not have detectable RCRs and, along with being non-pathogenic in humans, make it relatively safer for gene therapy applications [70, 75, 76] (Fig. 1.12).

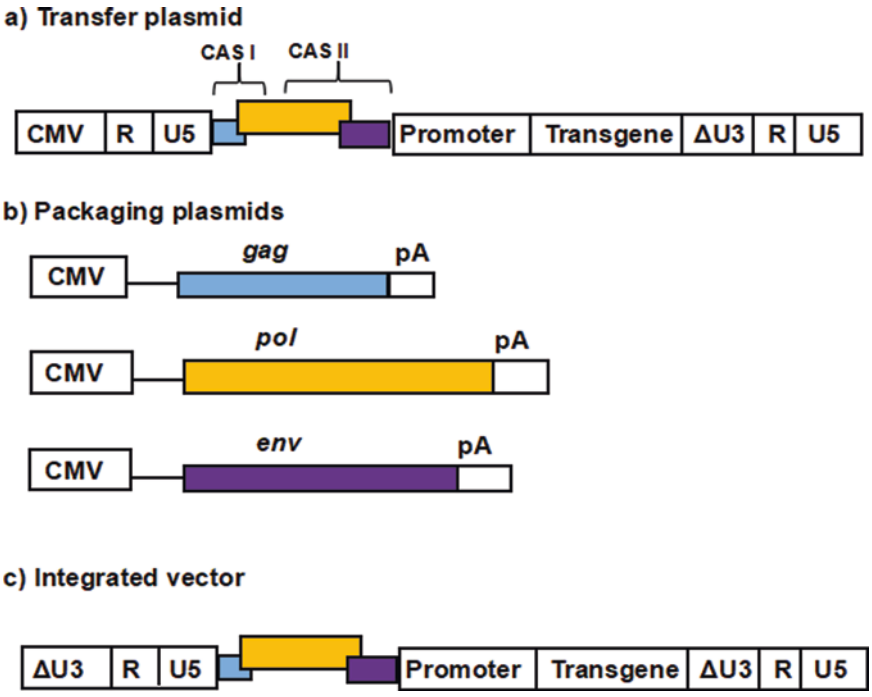


**Fig 1.12** A third-generation foamy virus vector system is shown**.** (**a**) The U3 region of 3'LTR has targeted deletions as mentioned. The *gag*, *pol*, and *env* regions are removed, while *cis*-acting such as LTRs and CAS I and II that are essential for efficient transduction have been retained. CMV-LTR fusion promoter drives transcription. (**b**) The *gag*, *pol*, and *env* genes are expressed by a CMV promoter on three independent plasmids. These also include a heterologous intron and a polyadenylation (pA) site. Integrated vector provirus. (**c**) Deletion in the plasmid's 3′ LTR U3 region which occurs during reverse transcription is copied to the vector provirus 5′ LTR. Both LTRs are transcriptionally nonfunctional; therefore an internal promoter is required to drive transcription of the desired transgene

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# Adeno-associated Virus Vectors in Gene Therapy

2

Bertin Mary, Nusrat Khan, Sathyathithan Arumugam, Himanshi Saxena, Mohit Kumar, Paramasivam Manimaran, Sourav Chattopadhyay, and G. R. Jayandharan

## 2.1 Introduction

Adeno-associated viruses (AAVs) are small, non-enveloped viruses containing a single-stranded genome, which is approximately 4.7 kb in length. It belongs to the *Parvoviridae* family due to its small (240 Å) size and non-enveloped nature. Due to the wide host preference of AAV, they are classified into the *Parvoviridae* subfamily *Parvovirinae*, whereas the other subfamily *Densovirinae* has a preference for insects. In addition, due to the satellite nature of the virus, marked by its dependence on other helper viruses for its infectivity led to its classification in genus *Dependoparvovirus*.

AAV was discovered in 1965, when Atchinson et al. observed this virus as contaminating particles during electron microscopy screening for simian adenovirus type 15 (SV15) in rhesus monkey kidney cell cultures [1]. The virus had a hexagonal profile and was not able to induce cytopathic effect either in in vitro or in vivo. The coinfection experiments done along with SV15 showed that AAV could not replicate in the absence of SV15. Conversely, their ability to replicate in the presence of the SV15 suggested AAV as defective virus which needs a helper virus function for its productive life cycle [2]. The defective nature of the virus was later confirmed by Hoggan group in 1966 by exploiting the physical, biological, and immunological aspects of AAV, after separating it from adenovirus particles by cesium chloride gradient centrifugation at a density range of 1.395 gm/cm$^3$ [3].

Initially AAV genome was believed to be in double-stranded form by biochemical analysis using acridine orange staining [4]. Later, based on physicochemical and renaturation kinetics studies, Heather D. Mayor (1969) showed that AAV genome is

B. Mary · N. Khan · S. Arumugam · H. Saxena · M. Kumar · P. Manimaran
S. Chattopadhyay · G. R. Jayandharan (✉)
Department of Biological Sciences and Bioengineering, IIT-Kanpur, Kanpur, Uttar Pradesh, India
e-mail: jayrao@iitk.ac.in



G. R. Jayandharan (ed.), *Gene and Cell Therapy: Biology and Applications*,
https://doi.org/10.1007/978-981-13-0481-1_2

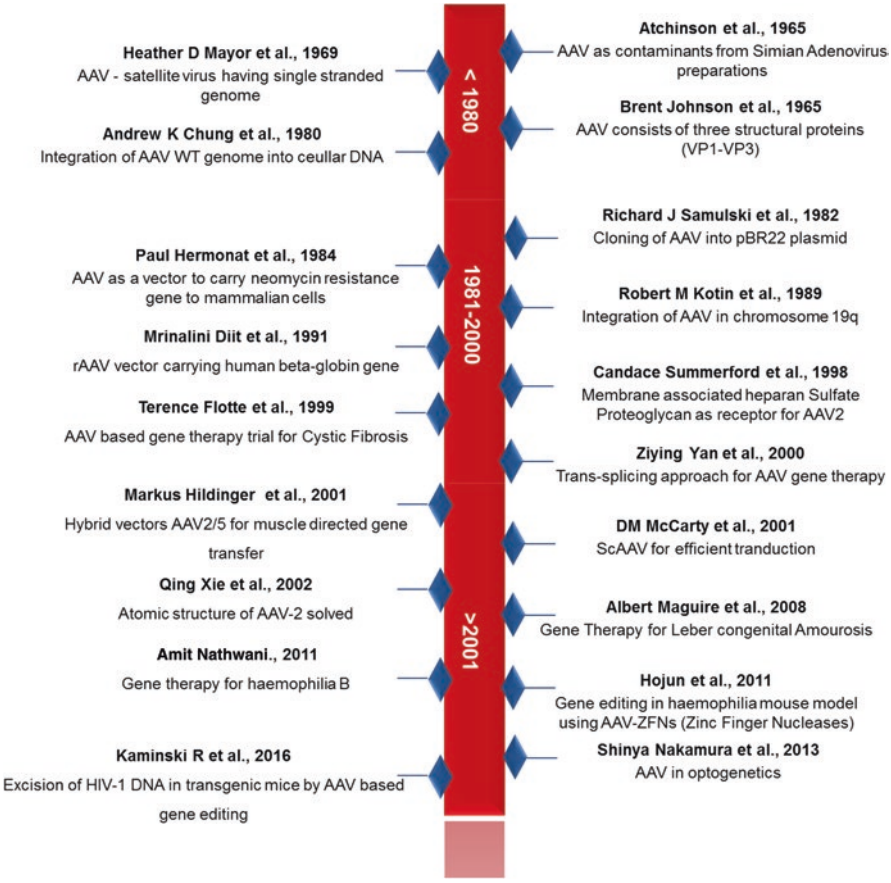


**Fig. 2.1** Milestones in development of AAV vectors

a single-stranded molecule which exists either in a (−) or a (+) strand form [5, 6]. Further studies during 1984 on the genome and structure of AAV paved way for the development of AAV as gene transfer vector in mammalian cells [7]. Since then, the unique properties of AAV such as their nonpathogenic nature have made them as a vector of choice in many gene therapy clinical trials [8]. The first clinical trial using AAV was carried out to correct cystic fibrosis in 1999 [9]. Since then the field has rapidly progressed, and in 2012, Glybera (alipogene tiparvovec) was approved to be the first gene therapy drug by the European Medicines Agency. Glybera is a human lipoprotein lipase gene variant (LPLs447x) produced using insect cells [10]. Since then, the AAV-based vector system has been utilized for a variety of disease states and across frontier areas of research including in gene-editing strategies (Fig. 2.1). Recently, a study conducted by Kaminski et al. demonstrated that AAV engineered to carry Cas9 gene from *Staphylococcus aureus* was able to suppress HIV-1 viral replication by excision of integrated HIV-1 virus genome in transgenic mice and rat

genome [11]. Though AAV-based gene therapy is promising, certain challenges, pertaining to host- and vector-related immune complications, exist, which demands further characterization of the life cycle of the virus for its utility as a better vector system.

## 2.2 AAV Genome

The single-stranded genome of AAV contains two open reading frames (ORF) *rep* and *cap* and inverted terminal repeat (ITR) elements that are 145 bp in length, flanking the two ends of the genome. The ITR includes a palindromic sequence of 125 bases that forms a hairpin structure, while the remaining 20 bases, which are not paired, are designated the D-sequence [12–14]. Two features in the ITR [Rep-binding elements (RBEs) and a terminal resolution site (TRS)] are critical for the viral replication process. Thus the ITR contains various *cis*-acting elements minimally essential for multiple aspects including the integration and rescue of AAV, its replication, and packaging both in its native and recombinant forms [14].

The left ORF contains the Rep gene that generates the following Rep proteins, Rep78, Rep68, Rep52, and Rep40. The Rep proteins are synthesized from two promoters, p5 and p19. These promoters are activated and regulated by a concomitant infection with another helper virus. The P5 promoter generates the larger Rep proteins (Rep78 and Rep68), whereas the P19 promoter generates the smaller Rep proteins (Rep52 and Rep40). Of these, Rep78 and Rep68 are important in regulating multiple aspects of the AAV life cycle. The p19 promoter is ~5 times stronger than the p5 promoter which reflects in the relative abundance of Rep52/40 to Rep78/68 proteins (5:1).

The right ORF includes the Cap gene, which utilizes the P40 promoter to generate the VP1, VP2, and VP3 viral capsid proteins that constitute the icosahedral capsid. The two shorter capsid proteins, VP2 and VP3, 72KDa and 61 KDa proteins, respectively, are encoded by the same mRNA and generated by alternative splicing. VP2 is synthesized from an upstream ACG codon, while VP3 is synthesized from the first ATG codon (*). The major VP1 component is an 87KDa protein encoded by an alternatively spliced mRNA that uses an upstream splice acceptor site [12, 15, 16]. Recently an overlapping ORF within the *cap* gene was described which encoded for assembly-activating protein (AAP), involved in capsid formation [17]. The genomic organization of AAV2 is shown in Fig. 2.2.

## 2.3 Capsid Structure

AAV has a T = 1 icosahedral capsid, made of 60 units each containing the VP1, VP2, and VP3 proteins in a stoichiometric ratio of 1:1:10. These three proteins share approximately ~530 amino acids of VP3 C-terminal region but differ in their amino termini [18]. In the presence of AAP, the VP3 protein assembles the capsid by two-, three-, and fivefold symmetry-related interactions [17]. The three-dimensional

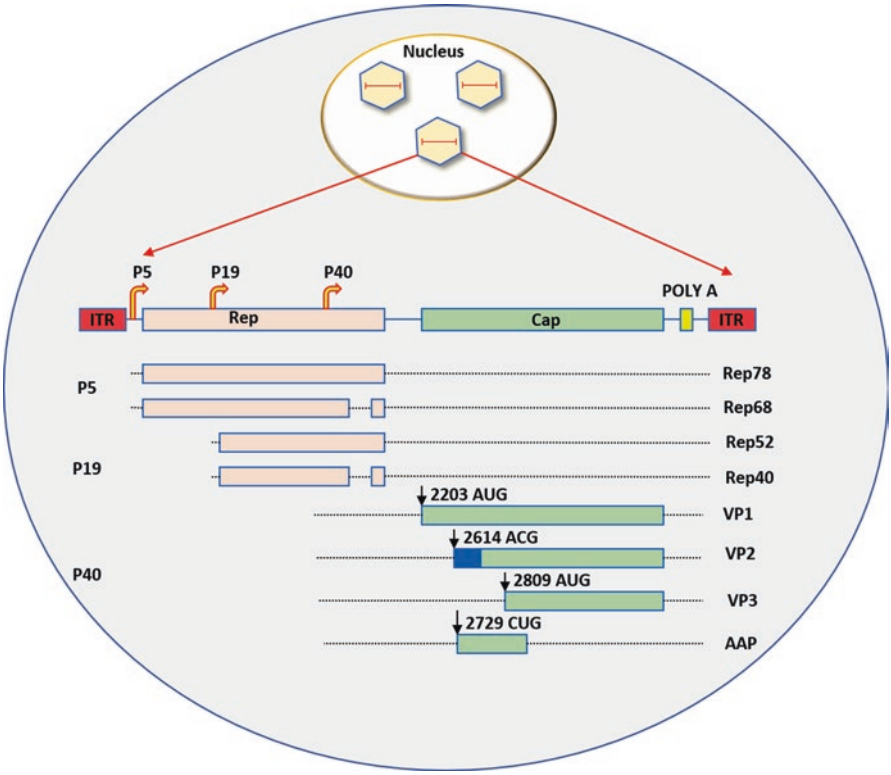


**Fig. 2.2** Organization of AAV2 genome. The ITRs flanking the two ORFs, Rep and Cap, and the genes encoding the respective proteins responsible for replication of AAV (Rep78, Rep68, Rep52, Rep 40) and capsid structure (VP1, VP2, VP3) are shown. The position of the three promoters P5, P19, and P40 and start codons of cap genes are indicated. *AAP* assembly-activating protein

structures of AAV1–9 serotypes and other AAV variants have been determined by either X-ray crystallography or a combination of cryo-electron microscopy and pseudoatomic model building. The VP protein basically consists of a conserved α-helix and a conserved eight-stranded antiparallel β-barrel motif at the core, with large loops inserted between the strands of the β-barrel. These loops comprise the variable regions, which determine the topological differences in capsid surface and functional differences in receptor binding, transduction potential, and immunological profile (by altering antigenic epitopes) between the various AAV serotypes/variants [19–22]. The AAV2 capsid structure, which is very similar to other serotypes, is well characterized and consists of depressions (dimple) at the twofold axes, surrounding a cylindrical channel (canyon) at the fivefold axes and protruding spikes surrounding the threefold axes. Between the depression at the twofold axis and surrounding the fivefold channel, a wall or plateau is located which is termed as the "2/5-fold wall" [23]. The threefold axis of the AAV2 capsid dictates the capsid/receptor binding. Two arginines at positions 585 and 588, located at this symmetry, bind to heparan sulfate proteoglycan (HSPG), the primary receptor of AAV2 [24].

## 2.4 Life Cycle

### 2.4.1 AAV Infection

AAV infection of the target cell is a complex process which involves multiple steps such as receptor attachment and internalization, endosome trafficking and release, nuclear translocation, capsid uncoating, and gene expression (Fig. 2.3) [13]. In the first step, during infection, AAV binds to specific receptors on the cell surface, which are unique to different serotypes. Heparan sulfate proteoglycan (HSPG) is the primary receptor utilized by AAV2. The broad tropism of AAV2 in various cells of human or other organisms is explained by the ubiquitous presence of HSPG in several species [25]. The process of viral internalization is further facilitated by the presence of co-receptors [e.g., $\alpha_v\beta_5$ integrin and laminin] [26]. AAV5 and AAV4 use N- and O-linked sialic acids, respectively, as their receptors. Further platelet-derived growth factor receptor also serves as a receptor for AAV5 transduction [27]. AAV1 and AAV6, which are closely related serotypes, require α2,3- and α2,6-N-linked sialic acids for cell surface attachment and efficient transduction [28]. Among the

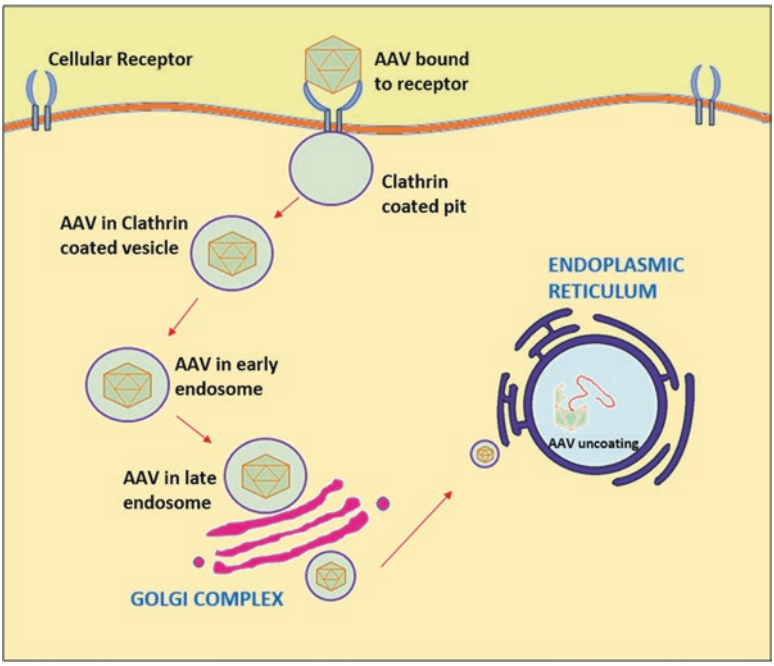


**Fig. 2.3** AAV infection in host cell. This multistep process starts with the binding of AAV to specific cell surface receptor, localized adjacent to clathrin-coated pits. Endocytosis and intracellular trafficking to the Golgi complex are shown. After endosomal escape, the virus particle traverses from Golgi to ER by a retrograde transport mechanism. Finally AAV enters the nucleus and undergoes uncoating and genome release, followed by gene expression or chromosomal integration

six amino acid residues which differ between AAV1 and AAV6, the presence of lysine at position 531 was found to impart additional HSPG-binding properties to AAV6 [29]. Laminin receptor acts a cellular receptor for AAV serotypes 2, 3, and 8, while AAV9 utilizes terminal N-linked galactose as receptor for cellular entry [30].

Receptor binding is followed by viral internalization through the plasma membrane by receptor-mediated endocytosis into clathrin-coated vesicles [31]. Dynamin, a GTPase protein, is required for the formation and pinching of the clathrin-coated pits from the cell membrane [32]. Subsequent intracellular trafficking of AAV2 particles through the cytosol to the nucleus is dependent upon the activation of PI3K pathway as well as microtubules and microfilaments [31]. A recent study utilizing chlorpromazine, a drug which inhibits the formation of clathrin-coated vesicles, has demonstrated that AAV2 endocytosis can be independent of clathrin-mediated process [33]. The release of virions from early endosomes takes place upon acidification of the vesicles, following which they accumulate perinuclearly and slowly penetrate into the nucleus. Whether the viral capsid uncoating happens in the nucleus or the cytoplasm is still unclear. Most of the evidence accumulated from studies based on immunofluorescence microscopy and subcellular trafficking strongly suggest that AAV uncoating happens within the nucleus [34]. Using fluorophore-labeled AAV2 particles, it was shown that within 2 h of infection, virus translocates into the nucleus possibly through the nuclear pore complex [35]. Cathepsins B and L, which are endosomal cysteine proteases, have been shown to bind and cleave the capsids of AAV2 and AAV8 differently, thus acting as uncoating factors for these serotypes [36]. The higher rate of transduction for AAV8 compared to AAV2 is postulated to be due to the faster rate of capsid processing by cathepsins for the former. The PLA2 motif within the N-termini of VP1 protein protrudes through the fivefold pore of AAV2 capsid as a result of conformational changes during infection, whereby the phospholipase activity is induced and plays a key role in the nuclear trafficking of AAV genome from the endosomes and subsequent viral gene expression [37].

### 2.4.2 AAV Life Cycle: Lytic and Lysogenic Phases

Once inside the nuclear compartment, AAV can follow either the lytic or the lysogenic cycle (Fig. 2.4). In the presence of a helper virus such as adenovirus or herpesvirus, AAV adopts the lytic stage, which involves the rescue of the viral genome and its replication and further generation of viral proteins to generate infectious virions. The adenoviral genes which provide the helper functions for AAV-productive infection include E1a, E1b, E2a, E4, and VA RNA, while herpesvirus can regulate AAV gene expression by supplementing DNA polymerase/helicase, thus creating a permissive intracellular milieu for AAV transduction [38, 39]. The lytic phase also ensues in response to cellular genotoxic stress like UV irradiation and metabolic inhibitors, albeit with low efficiency [40, 41]. AAV replication is believed to take place by a rolling hairpin model. The 3′- hydroxyl end of ITR

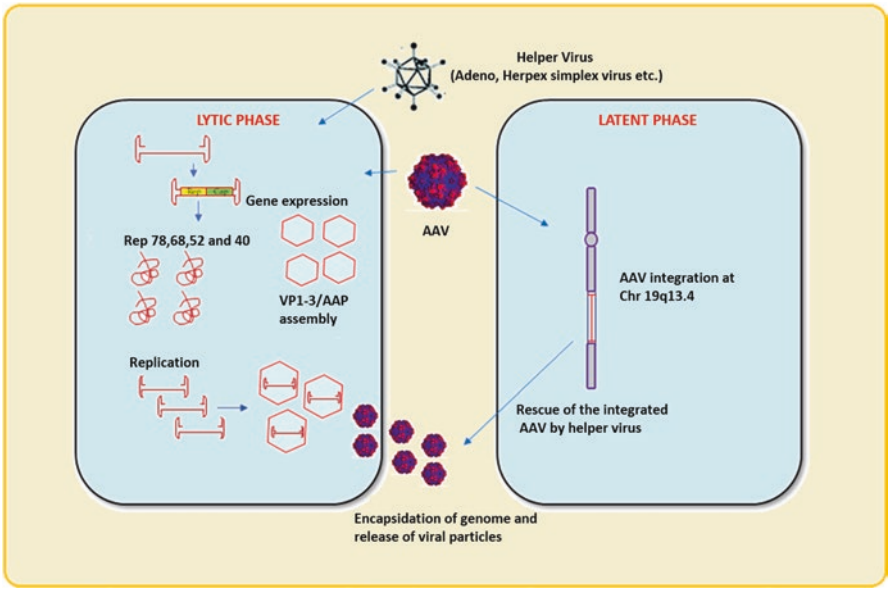


**Fig. 2.4** AAV Life cycle

serves as a primer for DNA polymerization leading to the formation of duplex monomeric and dimeric replicative forms. These duplex concatenated replicative intermediates are resolved by strand displacement mechanism to generate single-stranded progeny DNA with the aid of Rep proteins. Both "plus" and "minus" single-stranded AAV genomes produced are encapsidated into virions with equal efficiency [42]. During virus assembly, VP3 capsid protein translocates from the cytosol into the nucleus by associating with VP1 and VP2 which possess nuclear localization signals [43]. AAV capsids get assembled within the nucleoli, enter the nucleoplasm, and then package the genome in a Rep-dependent manner to produce infectious AAV particles [44].

AAV undergoes latent infection when helper virus functions are not available, by repressing viral gene expression and integrating its genome largely into human chromosome 19 (q13.4) AAVS1 site, by a nonhomologous end-joining (NHEJ) pathway [45]. In 1975, Berns KI et al. first demonstrated this phenomenon by latently infecting *Detroit 6* fibroblast-like cells with AAV2 and showing the persistence of the viral DNA for nearly 47 passages [46]. The site-directed integration of AAV is directed by a 33 bp sequence at AAVS1, which has an RBE-like and trs-like sequence within a gap of eight nucleotides [47]. Rep78/Rep68 proteins have been shown to bind GCTC repeat elements in the AAVS1 site and mediate the complex formation between AAVS1 site and the ITR [48]. Targeted integration at AAVS1 site is shown to enhance upon co-transfection with a plasmid encoding Rep78; however its application is limited by the cytotoxicity of host cells induced by this Rep

protein [49]. The P5 promoter integration efficiency element (IEE) is also identified as a viral factor important for AAV integration [50]. The frequency of site-specific integration at AAVS1 site is estimated to be 0.05% in tissues of humans and rhesus monkeys [51]. The switch to lytic phase in the presence of helper virus infection is brought about by the rescue of the integrated provirus by the rep proteins.

## 2.5 Serotypes

Based on the phylogenetic classification, 13 AAV serotypes are characterized each with variable tissue tropism. However they share some common features like the genome size and capsid organization with little variation in the end palindromic region. Gao et al. compared the phylogenetic relationship between serotypes that were initially isolated from human and nonhuman samples. They observed that AAV serotypes 4 and 5 were the most divergent sharing only 45% capsid sequence similarity with the AAV2 serotype. Thereafter human serotypes (AAV1, AAV2, AAV6, and AAV9) or rhesus-specific serotypes (AAV7) or a combination of both (AAV8) was identified. Interestingly, the serotype variability is not reflected to capsid protein sequence, but localized to the looped-out domains on the capsid surface [52]. Although AAV serotype 2 has been characterized extensively, other alternate capsids are very attractive for gene delivery. The availability of a repertoire of serotypes makes AAV-mediated gene therapy versatile as they offer improved infectivity rates and tissue targeting abilities.

## 2.6 Tropism

The biodistribution of multiple AAV serotypes in murine models have been characterized [53], of which AAV9 has the broadest tissue tropism, except the kidney. Target organs like the liver showed the presence of all the serotypes except AAV3. AAV7 showed strong tropism for the hepatic tissue and, to a lesser extent, the muscle. Meanwhile, AAV6 was more cardiotropic, in comparison to other tissues. AAV4 was found to be abundant in the lungs and the heart tissue. Such variation in the tissue tropism can be attributed to use of different surface receptors. AAVs exploit several different types of glycans as primary attachment receptors and use a broad range of cell surface receptors to mediate cellular uptake. The first receptor identified for AAV2 was heparan sulfate proteoglycan (HSPG). Later several other receptors like different linkage variants of sialic acid in case of AAV1 and AAV5, galactose for AAV9, 37/67 KDa laminin receptor, etc. were identified for different capsid variants [29, 36]. Recently a cellular protein receptor AAVR which is a predicted type I transmembrane protein has been described as a key universal receptor [54]. All the receptors identified for various serotypes of AAV thus far are listed in Table 2.1.

**Table 2.1** Cellular Receptors for AAV

| Serotype | Primary receptor | Secondary receptor |
|---|---|---|
| AAV1 | N-linked sialic acid, AAVR | |
| AAV2 | HSPG, AAVR | FGFR1; HGFR; CD9; integrins; LamR |
| AAV3 | HSPG, AAVR | HGFR; LamR |
| AAV4 | O-linked sialic acid, AAVR | |
| AAV5 | N-linked sialic acid, AAVR | PDGFR |
| AAV6 | N-linked sialic acid; HSPG | |
| AAV7 | Unknown | |
| AAV8 | LamR, AAVR | |
| AAV9 | N-linked galactose AAVR | |
| AAV10 | Unknown | |
| AAV11 | Unknown | |
| AAV12 | Unknown | |
| AAV13 | HSPG | |

*FGFR1* fibroblast growth factor receptor, *HGFR* hepatocyte growth factor receptor, *HSPG* heparan sulfate proteoglycan, *PDGFR* platelet-derived growth factor receptor, *LamR* 37/67 KDa laminin receptor, *AAVR* adeno-associated receptor

## 2.7 Recombinant AAV

### 2.7.1 Design and Production

The intact genome of AAV was first cloned into a bacterial plasmid in 1982 and for vector packaging, the viral DNA rescue was achieved by infecting HEK 293 cells with adenovirus 5, thus recapitulating the lytic phase of AAV infection [55]. The knowledge that ITRs contain all the *cis* functions required for AAV genome replication and virion assembly paved way for the production of recombinant AAV (rAAV) vectors. The design of rAAV follows the concept of "gutless" vectors which retain only the ITRs in *cis*, while the viral encoding genes meant for replication and capsid structure formation are replaced by the foreign DNA of choice [13]. The rep-cap functions are provided in *trans* as a separate plasmid during vector packaging. This negates the possibility of generation of wild-type AAV and any undesirable effects associated with it during gene transfer. Unlike the wild-type virus, the lack of rep protein prevents the site-specific integration of rAAV vectors which usually exist in episomal form within the target tissues although there are reports which claim that a rep-independent integration event can also take place [56]. The classical rAAV vector based on native virus contains the single-stranded genome, which needs to undergo the second-strand synthesis during replication and hence produces delayed and low-level gene expression [57]. To overcome this limitation, self-complementary (sc) AAV vectors were designed with a mutation in terminal resolution site *(trs)* of right ITR, which facilitated their spontaneous re-annealing, bypassing the second-strand synthesis to form transcriptionally active genomes with enhanced transgene

expression [58]. Nonetheless the packaging capacity of scAAV was reduced to 2.2 Kb in comparison to 4.4 Kb with ssAAV vectors.

A pseudotyping approach (wherein the ITRs of one serotype, usually AAV2, flank the Cap genes of another serotype) can enhance the already broad tissue tropism of AAV. Most serotypes can transduce a wide range of tissues. Based on this, a hierarchy of transduction efficiencies for the major tissue types with AAV has been suggested [29]. The liver is best infected by AAV8 [59]. AAV7 and AAV6 can also efficiently transduce the skeletal muscles [60, 61]. AAV7 has also been reported to be an effective vector for the long-term transduction of retinal and anterior chamber structures [62]. AAV4 can transduce the mammalian central nervous system with the ability to transduce the ependymal, although it could not transduce the cells in the parenchyma [63].

Earlier methods of rAAV production involved the transfection of HEK 293 packaging cell line with the two plasmids, namely, the transgene plasmid with the ITRs and the rep-cap plasmid, and coinfection with adenovirus (Ad) [55]. The helper function, which is necessary for rescue and synthesis of AAV particles, is provided by the adenoviral genes [E1a, E1b, E2a, E4, and VA RNA]. However there are issues associated with Ad coinfection. The most serious of them is the elimination of contaminating Ad particles from the final vector preparation which is done using physical methods like cesium chloride, column chromatography, and heat denaturation, none of which can guarantee absolute purity of the AAV vector stock [64]. Additionally, there could be potential competition between both the viruses for the essential genes for their life cycle, adversely affecting the AAV vector yield. A triple plasmid transfection method for rAAV production where Ad coinfection is replaced by a plasmid carrying the Ad helper genes was introduced in 1998 [65]. Several modifications on this protocol were then reported to further improve the process of vector production using either calcium phosphate or PEI as the transfection reagent [66]. In addition, baculovirus expression systems based on insect cell lines such as SF9 cells have been utilized to scale up the production of rAAV vectors [16] (Fig. 2.5).

## 2.8 Gene Therapy Using Recombinant AAV

AAV has been a promising candidate for gene therapy of a variety of disease states. It must be noted that years of experience with preclinical studies in animals provided substantial information on biodistribution, toxicity, and efficacy of AAV vectors that ultimately facilitated its translational use in humans. A brief summary of the recent success with this vector system is presented below.

The first recombinant AAV2 vector, carrying the CFTR gene, was tested in cystic fibrosis patient with mild lung disease [70]; since then several hundred trials with this vector have been initiated. The most recent feat with this vector is the development of Luxturna, an AAV type 2-based vector for the treatment of RPE65-mediated inherited retinal dystrophy, which is now approved by US FDA [71]. Other diseases include hemophilia B, a hereditary bleeding disorder caused due to the lack or

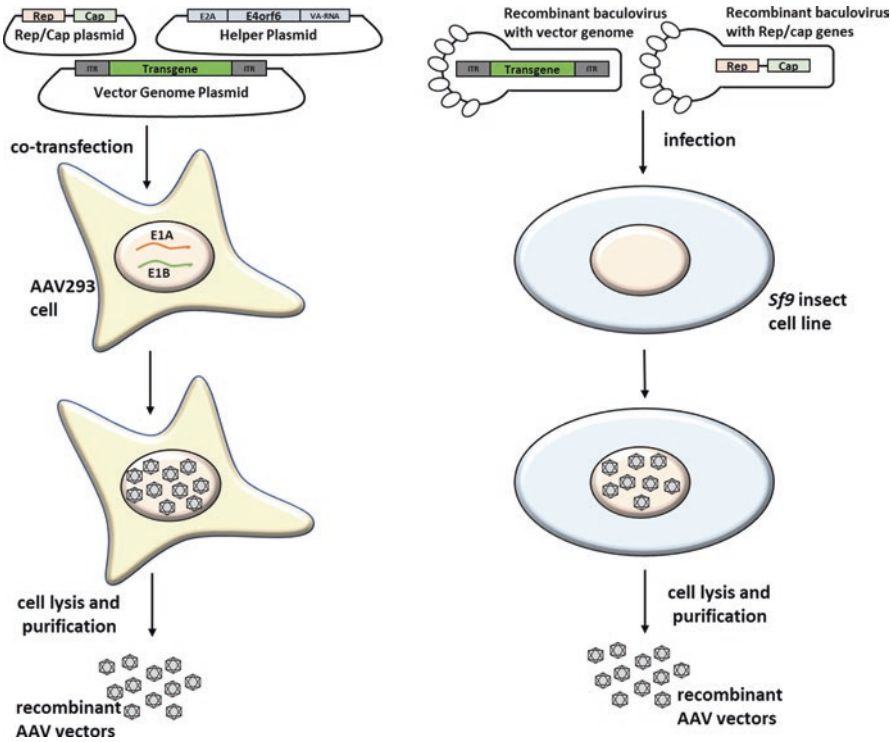


**Fig. 2.5** Triple transfection method for packaging AAV vector: AAV production involves the triple transfection of plasmids into AAV permissive cell lines such as HeLa and HEK 293 cells, followed by cell harvesting and purification of virus after 48–72 h. The three plasmids used for the transfection protocol involve (1) AAV-ITR-transgene plasmid (the transgene along with a heterologous promoter and poly A tail is cloned into AAV backbone by removing the AAV genome leaving the core *cis*-acting 145 bp left and right ITR); (2) plasmid carrying AAV rep and cap gene; and (3) pHelper – plasmid carrying early *Ad5* gene elements such as VA RNA, E2A, and E4. The permissive cell lines were engineered to stably express Ad5 gene elements such as E1a and E1b [48, 67–69]. The packaged virus carrying the desired transgene is harvested by cell lysis and further purified by density gradient centrifugation as well as by affinity chromatographic procedures [56, 57, 67]. The schematic on the left represents the production of rAAV vectors by transient transfection of AAV293 cells. On the right is a representation of the production of rAAV vectors by infection of SF9 insect cell line with recombinant baculoviruses containing vector genome and rep/cap genes

absence of coagulation factor IX. The current clinical trials with a single dose of recombinant AAV8 vector containing a gene expressing human factor IX in patients with severe hemophilia B have resulted in long-term therapeutic factor IX expression and no toxic effects [72]. AAV is also being used to target Duchenne and Becker muscular dystrophy (DMD/BMD), which is a common inherited disease with defective expression of dystrophin protein. Phase 1 clinical trial for DMD was performed with chimeric AAV vector (AAV2.5) incorporated with

dystrophin-producing gene; the results for the same established the vector to be safe [73]. Another success story of AAV relates to the clinical trials of lipoprotein lipid deficiency gene therapy via administration of alipogene tiparvovec or popularly known as Glybera (AAV1-LPL$^{S447X}$) [10]. Similar attempts were performed with Leber congenital amaurosis type 2 (LCA2) patients where an AAV2 vector expressing RPE65 gene delivered subretinally improved visual function [74–76].

## 2.9 Immune Response to AAV Vectors

Despite the accelerating pace of use of AAV for clinical gene therapy, one major rate-limiting aspect is the immune responses triggered against it in the humans. For example, in case of hemophilia B trials, a reduction in F.IX expression and transient transaminitis was observed due to destruction of hepatocytes that were transduced with AAV via cell-mediated immunity targeting the capsid [77, 78]. Preexisting antibodies to AAV or the memory cell response generated due to the wild-type virus infection can augment host immune response and affect the therapeutic delivery.

The occurrence of such immune response against AAV in clinical trials has renewed efforts to understand its immunological manifestations so as to facilitate its widespread application [79].

### 2.9.1 Innate Immunity

Innate immune response is the first line of defense that acts against any foreign particle. In the context of AAV-mediated gene therapy, it majorly targets the capsid or the transgene [80, 81]. The host immune components triggered during an AAV infection have been studied by various groups and listed in Table 2.2. There is a

**Table 2.2** Generation of innate immunity in the form of activation, infiltration, or augmentation of various components of the innate immune system like Toll-like receptors, interferon cascades, TNF-alpha, etc. in in vitro and in vivo systems

| Host system | Innate immune response | Reference |
|---|---|---|
| Murine and human plasmacytoid dendritic cells (in vitro) | TLR 9/MyD88<br>Type I interferon cascade (INF α/β) | [85, 86] |
| Mouse liver | NF-kB-dependent cytokines and chemokines<br>TLR-9-dependent inflammatory cytokines<br>TNF-alpha, RANTES, IP-10, MIP-1beta, MCP-1, and MIP-2<br>Infiltration of neutrophils and CD11b(+) | [82] [87] |
| Human Kupffer and liver sinusoidal epithelial cells | TLR2 | [88] |
| THP-1 cells | RANTES, IP-10, MIP-1, MIP-1, MCP-1, and IL-8 | [84] |

clear demarcation between the immune response generated for ssAAV vector and scAAV vectors, with the reduced capsid stability of scAAV leading to more exposure of the genome to immune sensors such as TLR9 and activation of MyD88 pathway [82]. However ssAAV leads to minimal increase in infiltration of neutrophils and macrophages, whereas the scAAV genome led to a substantial rise in the infiltration of the same neutrophils, macrophages, and NK cells [82]. The complement cascade is also known to be stimulated by the AAV capsid which binds to the C3 complement proteins and complementary regulator H, thus enhancing phagocytic activity [83, 84].

The unfolded protein response (UPR) pathway genes, specifically IRE1α, are known to activate NF-kB and its target pro-inflammatory genes. The retrograde movement of AAV into ER (endoplasmic reticulum) is known to activate the UPR and its cognate inflammatory cytokines to maintain homeostasis and manage ER stress [89].

In summary, the interactions of AAV vector with the host innate immune system may flare and augment the inflammatory response leading to its clearance or the activation of a specific adaptive immune response. Due to their pronounced impact on the success of gene therapy, these immunological processes need to be overcome without conceivable disadvantage to the host.

### 2.9.2 Adaptive Immune Response

#### 2.9.2.1 Humoral Response

Humans are natural hosts, and infection of AAV at an early age leads to the generation of neutralizing antibodies to AAV. Studies have shown that neutralizing antibody (NAb) titers as low as 1:5 can completely block AAV transduction in murine liver [90]. The route of delivery is another major determinant of the neutralization activity. In an intravenous administration targeted to the liver, AAV vectors are prone to prolonged NAb exposure and its neutralization by these circulating antibodies. However, vectors injected into the eye have shown relatively longer transgene expression due to immune privileged nature of the ocular compartment [91]. Due to environmental exposure, humoral immunity develops in healthy humans against almost all the serotypes within the first 2 years of life [92]. AAV2 is the most common serotype infecting humans, and thus >70% of human population are known to be seropositive to AAV2, while antibodies to AAV5 and AAV8 have least prevalence (<30%) in humans [92].

Furthermore, B-cell activation against viral capsid might be T-cell dependent or independent [93]. In general, B-cell activation follows T-cell-dependent pathway wherein, after viral peptides are presented on MHC-II molecules, a co-stimulatory response occurs which tends to express CD4+ T cells along with CD40L (ligand) and cytokine release. This ligand interacts with CD40 on B cells which then differentiate into plasma cells or initiate the formation of germinal center, crucial for antibody expression and memory cell generation [94]. The antibodies generated during AAV infection, particularly against those targeting the viral capsid, are

predominantly of IgG class, specifically IgG1 with lower levels of IgG2, IgG3, and IgG4 [81, 95, 96]. AAV-specific antibodies are known to contribute to failure of gene transfer protocols, such as the decline noted in circulating FIX levels in a patient with Nab titer of 1:17 and infused with high vector dose of AAV2-FIX vectors in a clinical trial for hemophilia B [97].

#### 2.9.2.2 Cell-Mediated Immunity

Numerous studies have shown that T-cell-mediated immunity can eliminate AAV-transduced cells. The lack of viral proteins can also produce weak T-cell response and infiltration of CD4+ and CD8+ T cells in the target organ. The mechanism follows the classical route of presentation of viral peptides via the MHC-II pathway following an activation of CD4+ T helper cells, which in turn activate CD8+ cytotoxic T cells. Furthermore such responses are also cross-reactive against multiple AAV serotypes [98].

During AAV-mediated targeting of a target tissue/cell, degradation of AAV particles within the host cell initiated by the inflammatory mediators leads to the generation of immunogenic capsid peptides which is then sensed and cross-presented on MHC-I molecule to induce a cytotoxic response [99]. On the other hand, if the viral particles are encountered by antigen-presenting cells (APCs) during their systemic administration, they can present the AAV peptide antigens by MHC-II to activate CD8+ T cells or CD4+ T helper cells, respectively.

## 2.10 Strategies to Improve Efficiency of AAV Vectors

The major challenges of using AAV in humans for gene therapy include low transduction of the viral vector, restricted tropism, limited packaging capacity and host immune response. Some of the strategies to overcome these barriers are discussed in detail in the next section.

### 2.10.1 Bioengineering of AAV

AAV bioengineering involves a directed evolution approach and rational design approach of the vector. Directed evolution approach depends on the knowledge of the diversity found in the capsid surface among all the serotypes and further evolution of those variants. The latter approach involves the sequence and structural information of the capsid region of different AAV serotypes and their further optimization by downstream methods. Both the approach use PCR-based methods such as insertion mutagenesis, site-directed mutagenesis, library construction, and screening methods for the development of novel-engineered vectors [100–104].

### 2.10.2 Directed Evolution Approach

Directed evolution approach is a forward genetic approach to create a designer AAV vector (Fig. 2.6). This involves creation of viral particle library by inducing mutations in the capsid region by error-prone PCR/insertion mutagenesis, followed by their cloning into a packaging plasmid and transfection into viral packaging cells. Thus the viral particle library created will be further screened for any novel vector with new property or function followed with sequence analysis [105–107]. One of the interesting features of this approach is screening for immune evasion mutants. It involves the screening of whole capsid mutant viral library against neutralizing sera and selection of infectious particle. Multiple rounds of selection can be made to get highly efficient AAV variants having immune evasion properties [108].

Directed evolution approach can also be used to identify vectors that have the ability to target specific cell types by designing random peptide libraries. The random peptides were inserted at the capsid surface particularly at the host cell-capsid surface natural interaction site, to eliminate their inherent tropism profile. The modified viral capsids were then screened against target tissue to identify and characterize viral capsid showing higher transduction efficiency [109].

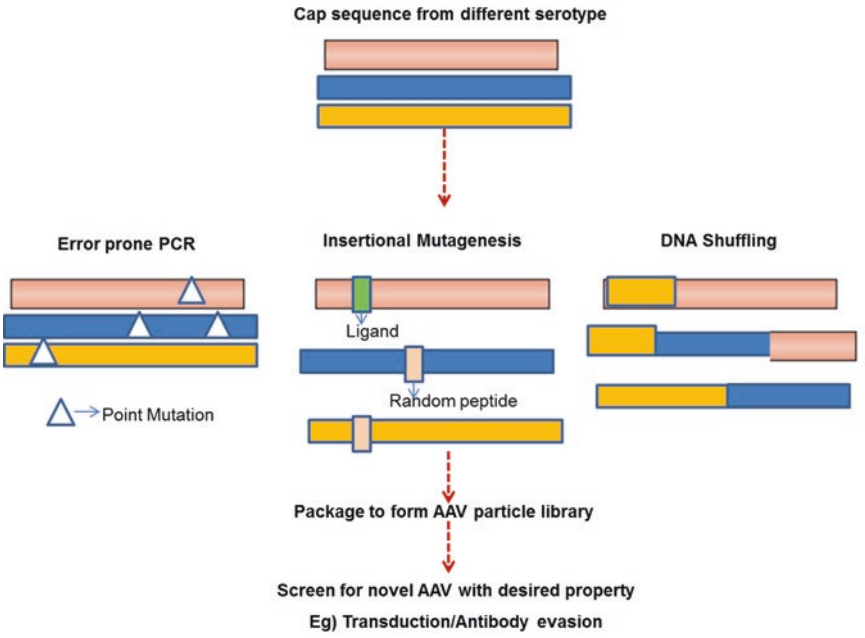


**Fig. 2.6** Schema for directed evolution approach for generating a novel AAV vector

## 2.10.3 Rational Design Approach for Engineering AAV

### 2.10.3.1 Improving the Packaging Capacity

To overcome the constraints of limited packaging ability (4.4 kb) of AAV vectors, a trans-splicing approach has been proposed to increase the packaging limit of the transgene up to 9 kb. In trans-splicing approach, the transgene cassette containing splice intron donor and acceptor sites will be split into two plasmids each containing ITRs. Upon co-transfection of these two plasmids, a functional transgene product will be formed by the intermolecular recombination between the 5′ITR of one plasmid and the 3′ITR of the other [110–112]. The pictorial representation of trans-splicing strategy is given below (Fig. 2.7).

### 2.10.3.2 Self-Complementary AAV

One of the major drawbacks of using rAAV as a gene delivery vector was the synthesis of a double-stranded intermediate to be used as a template for transcription activation after infection of the target cells. This is due to the dependence on the host DNA polymerases for synthesis of the leading strand [113]. For efficient transduction (~100%) of hepatocytes, vector doses of $10^{13}$ were required for a successful gene therapy of the liver [114]. Such high viral doses can place a burden on virus production strategies, not to mention the increased toxicity it can cause in vivo and the increased immune response.

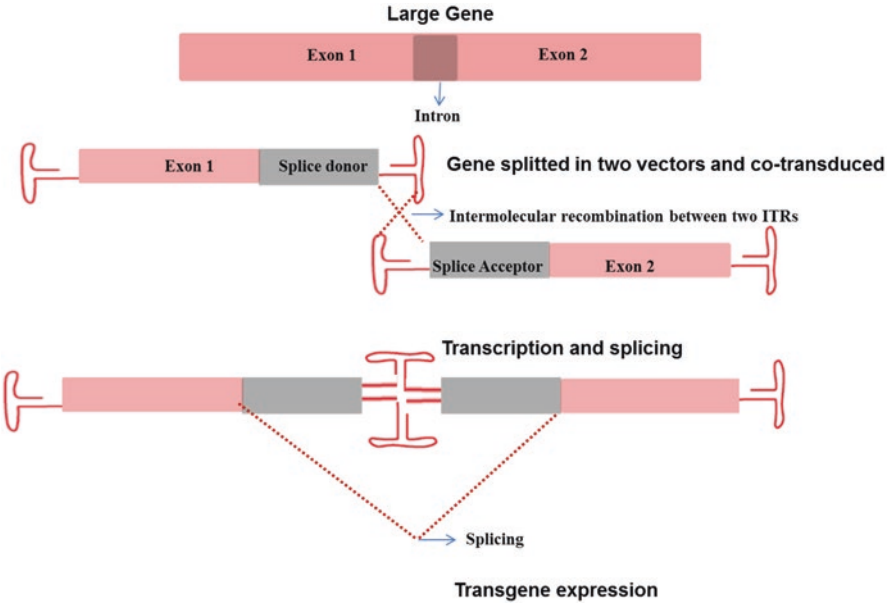


**Fig. 2.7** Trans-splicing strategy to generate vectors with improved packaging ability

Self-complimentary AAV (scAAV) was first described in 2001 [59], and the rationale is when the AAV genome is shortened to half, the scAAV can fold upon itself to form transcriptionally active double-stranded DNA, thereby removing the need for a rate-limiting second-strand synthesis. The scAAV, however, limits the size of the transgene to be incorporated by 50% (~2.4 kb can be encapsidated), but they showed 5–140 folds higher transduction efficiency as compared to ssAAV [59, 115, 116].

## 2.11 Approaches to Overcome Immunotoxicity to AAV Vectors

The major targets of host immune response against AAV are the capsid and/or the protein expressed from the encapsidated transgene. AAV gene transfer to immune-privileged sites such as the eye and brain has been associated with minimal or no detectable immune response, even though vector doses tested were lower compared to those administered in the liver or muscles. AAV vectors have been directly administered into the brain to treat Parkinson disease, Canavan disease, and late infantile neuronal ceroid lipofuscinosis and into the eye for the correction of LCA2 and choroideremia, establishing the immunological unresponsiveness of these target tissues [117]. Other possible strategies that have been tested to prevent the immune clearance of AAV vectors during gene transfer to the other tissues are discussed below.

During systemic gene delivery, the immune response to AAV is largely dependent on the vector dose, as noted from several clinical trials [118] (Fig. 2.8). Thus efforts are underway to enhance the transduction of vectors and thus utilize significantly lower doses for gene transfer into target organs.

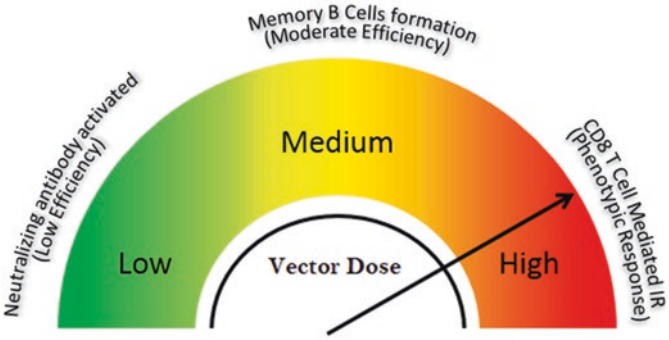


**Fig. 2.8** Correlation between vector capsid dose and outcome of gene transfer**.** Low capsid doses are efficiently neutralized by anti-AAV antibodies. This results in lack of therapeutic efficacy in hosts. However, higher capsid dose can achieve therapeutic efficacy but also cross activates the capsid-specific T-cell immune response (IR). This does not affect therapeutic outcome until a critical threshold, beyond which immune-mediated clearance of vector-infected target cells results in loss of phenotypic outcomes [98]

To improve vector transduction, several variations of AAV capsid engineering have emerged. Specific mutations of viral capsid involving proteasomal or phosphorylation sites, including tyrosine (Y) to phenylalanine (F) mutations on surface-exposed regions of the AAV2 [117, 119] and mutant AAV1, 2, and 8 vectors with alterations of serine/threonine/lysine (S/T/K) residues [120–123], significantly increased the transduction potential of these vectors.

Alternatively, modification of the ITR containing transgene construct by mutagenesis of NF-κB-repressing factor (NRF)-binding site from one of the ITRs of ssAAV vectors improved the transgene expression in vitro and in vivo [124].

To overcome immune manifestations in the host, several strategies have been described. The use of empty/decoy capsids to react with anti-AAV antibodies reduces the risk of neutralization of transgene containing AAV vectors [95]. Modification of the capsid B-cell or T-cell epitopic regions is another effective approach. Screening of a randomized AAV capsid library allowed the characterization of two vectors with significant reduction in neutralization with human sera or pooled IVIG (intravenous immunoglobulin) [108]. Alternatively, nanoparticle-mediated shielding of immunogenic epitopes has also yielded AAV vectors, which evade the humoral response [125].

Apart from this, many immunosuppressive drugs targeting T-cell reactivity and specific immune regulatory proteins (e.g., PARP-1) which are approved for human use have been tested to mitigate the cellular immune response [126]. Similarly, the induction of immune tolerance to the transgene via regulatory T cells (Tregs) following AAV gene transfer into the liver is another approach that leads to suppression of CD4+/CD8+ T cell responses against the antigen [127]. A recent study has exploited this strategy in modulating immune response to AAV vectors by expression of Treg-specific epitopes in AAV1 vector [128]. MR1, which is a monoclonal antibody to CD40L in mice, and CTL4AIg, a soluble CTLA4 immunoglobulin that binds to CD80 ligand on APCs, have been shown to be efficient [129]. The use of glucocorticoids prior to systemic delivery is another approach that reduces innate immune response to AAV without affecting the efficiency of the viral transduction [130].

## 2.12 Gene Editing using AAV

In the early 1990s, it was demonstrated that AAV-based approach yields significantly higher frequencies of gene repair [105, 131–133]. AAVs are particularly suitable for in vivo genome-editing strategies owing to its unique abilities. These include their relatively low immunogenic profile in immune-competent animal models, amenable for delivery with specific tissue tropism based on engineered capsids, and also their ability to transduce nondividing cells as well. In the process of rAAV-mediated gene editing, the transgene serves as a repair sequence that exhibits homologous arms to the target site with the desired modification preferably placed in the middle of the homology sequence [134]. Despite the feasibility of this approach, off-targeted insertions of the gene-editing cassettes combined with the

requirement of large multiplicities of infection (>$10^4$ MOI) have required designer nuclease-assisted DNA targeting. This approach has been successfully tested in a murine model of hemophilia B [135]. Similarly, Nelson et al. used AAV9 to deliver CRISPR/*Cas*9 gene-editing system to restore dystrophin protein expression in vivo [136]. These results underscore the potential of AAV-based gene editing to correct disease-causing mutations at the level of the host chromosome, especially when a selection scheme is employed.

### 2.12.1 CRISPR-*Cas*9

The clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein (*Cas*9) is a strategy that makes use of the cellular mechanisms that bacteria (*Streptococcus pyogenes*) use to recognize and edit the DNA of invading phage particles [137, 138]. This is basically a nuclease system which cuts the DNA molecule or RNA molecule. *Cas*9 is an endonuclease enzyme that can cut double-stranded DNA, while the guide RNA (gRNA) helps guide the *Cas*9 enzyme to bind with a specific sequence on the genomic DNA where the CRISPR-*Cas*9 nuclease complex will make a specific cut.

### 2.12.2 Nomenclature and Types

CRISPR systems are categorized into three types (I–III) depending on the *cas* protein. The *cas*1 and *cas*2 are found in all three types of CRISPR-*cas* system, but some specific *cas* protein such as *cas*3 is present in type I, *cas*4 present in types I and II, *cas*5 present in type I, and *cas*6 present in types I and III [139, 141].

Among them, the type II CRISPR system is well characterized because of its high utilization in gene-editing tools. Type II CRISPR requires the following three components: cas9 protein with crispr RNA (crRNA) and trans-activating crRNA (tracrRNA). The crRNA part of the type II CRISPR system contains two parts – a guide RNA and tracrRNA that processes the crRNA. Each crRNA unit then contains a 20 nt guide RNA sequence and a partial direct repeat, where the former directs Cas9 to a 20 bp DNA target. In 2012, Prof. Doudna and Prof. Charpentier collaboratively developed a single synthetic small guide RNA (sgRNA) by combining tracrRNA and crRNA. This sgRNA increases the widespread utility of type II CRISPR-Cas9 system [141]. Further, the type II CRISPR-Cas9 system has been subgrouped into three different variants depending on the cas9 [142–144].

### 2.12.3 PAM Sequence

The sgRNA sequence which is complementary to the target DNA molecule is essential for CRISPR/Cas9 system. This sgRNA sequence serves a very crucial job as it

helps to recognize complementary sequences of target DNA. sgRNA sequence that is complementary to the target sequence is known as a protospacer adjacent motif or PAM sequences. PAM sequence is a 2–6 base pair DNA sequence present in protospacers part of target DNA. The protospacers are short sequences (~20 bp) of known foreign DNA separated by a short palindromic repeat. The PAM sequence is very much essential for Cas9 function. A specific PAM sequence is essential for a specific type of Cas9 enzyme activity. The most common Cas9 nuclease, derived from *S. pyogenes*, recognizes a PAM sequence of "NGG." This PAM sequence is not present in the sgRNA sequence but just next to the downstream of the target site in the genomic DNA.

### 2.12.4 Mechanism

Recognition of the PAM by the CRISPR-Cas9 nuclease allows cross-examination of the sequence by the sgRNA and its subsequent complexing. The Cas9-sgRNA complex acts as a scissor that can cut the target DNA molecule at a specific location in the genome. The gRNA will only specifically bind to the target sequence. The cas9 follows the gRNA to the same location in the DNA and cut both strands of the target DNA molecule.

The resulting double-strand break (DSB) is then repaired by Non-homologous end joining (NHEJ) pathway or the Homology directed repair (HDR) pathway. The NHEJ pathway rapidly renovates the DSB in the DNA molecule. But during renovation, NHEJ pathway makes small nucleotide insertions or deletions at the DSB site and induces frame amino acid deletions, insertions, or frameshift mutations. This is an efficient but error-prone repair process. NHEJ-mediated DSB repair is defective and often results in interference of the open reading frame of the gene [142]. HDR can be used to generate specific nucleotide changes. In the HDR pathway, a homologous sequence is inserted in between the DSB. During renovation, HDR pathway does not make frequent insertions or deletions at the DSB site, which makes HDR the less efficient but a high-fidelity repair process. The repair template can be of multiple forms of DNA depending on the specific application. Though HDR has lower efficiency in DNA repair, it has several important practical implications in creating mutations with the gene-editing system [145].

AAV-based vectors are excellent vehicles for CRISPR-Cas9 gene delivery because of their low pathogenicity and an ability to infect numerous human cell types in vivo [146]. Collectively, CRISPR-Cas9 and AAV technologies have the potential to speed up both basic research and clinical applications of genome engineering.

The advancements in the last few decades in AAV vector biology and their steadfast application in the field of gene therapy has increased hope for the otherwise incurable patients. Nonetheless, a variety of problems particularly related to vector-host biology needs to be characterized more diligently to overcome the currently existing barriers for the universal application of this promising vector system.

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# Nanointerventions for Gene Therapy

3

K. Uma Maheswari and Vadim Annenkov

## 3.1 Introduction

Modifications in genes present in the cells result in the onset of diseases. The genetic alterations can be hereditary or can be acquired due to exposure to mutagenic factors. These disorders are seldom corrected using conventional chemotherapy. Hence, an emerging paradigm in treatment of diseases is to address the genetic defect through silencing of the rogue genes (gene silencing) or constitutively expressing an under-expressed gene [1]. Both cases involve introduction of exogenous oligonucleotides into the cell. The recent advances made in gene editing also require exogenous introduction of the editing tools, namely, enzymes and oligonucleotides, into the target cell. The introduction of free oligonucleotides has been found inadequate in many cases due to certain physiological barriers. A large body of literature is available on different attempts to deliver therapeutic genes into the cell using a carrier system known as vectors [2]. Several of these attempts have been successful both in vitro and in vivo and have moved on to clinical trial phase. About 2145 clinical trials are underway in the world for various types of gene therapy, out of which nearly 82% involve the use of vectors. Among them, nearly 70% employ viral vectors. The major non-viral vector used in these clinical trials is the liposomal carriers that comprise 5% of the total trials that are underway [3]. These numbers, however, represent only a small fraction of the total number of therapeutic clinical trials that are underway across the globe. Gene therapy though promising is limited by several roadblocks. The increasing knowledge on viral vectors and their mode of gene transfection as well as the advent of nanotechnology have helped in designing

K. Uma Maheswari (✉)
Centre for Nanotechnology & Advanced Biomaterials (CeNTAB), School of Chemical & Biotechnology, SASTRA University, Thanjavur, Tamil Nadu, India
e-mail: umakrishnan@sastra.edu

V. Annenkov
Limnological Institute, Siberian Branch of the Russian Academy of Sciences, Irkutsk, Russia



G. R. Jayandharan (ed.), *Gene and Cell Therapy: Biology and Applications*,
https://doi.org/10.1007/978-981-13-0481-1_3

strategies to overcome several of these limitations in recent times. The past decade has seen the emergence of numerous non-viral vectors, and it may be possible that these systems may aid the clinical translation of gene therapy.

## 3.2 Challenges in Gene Therapy

The delivery of free oligonucleotides is limited by several physiological barriers. Systemic delivery of naked oligonucleotides is ineffective due to its poor stability against nucleases. Further, the charged nature of oligonucleotides attracts opsonization, thereby triggering immune recognition and subsequent degradation [4]. Another challenge in the delivery of oligonucleotides is 'off-targeting' where the oligonucleotide may localize in a nontarget cell. This can lead to disastrous changes in normal cells leading to undesirable adverse effects. Further, the highly anionic oligonucleotide also experiences electrostatic repulsion from the anionic cell membrane, thereby limiting its internalization into cells. Even if some oligonucleotides manage to enter the cells, in most cases, the oligonucleotides are directed to the endosomes that gradually acidify and fuse with lysosomes where the oligonucleotides are degraded. These factors independently or in combination limit the transfection efficiency of the oligonucleotides [4]. It is evident that if the oligonucleotides are delivered to the target cell using a suitable delivery vehicle, it may serve to offset several of these limitations.

## 3.3 Requisites of a Delivery Vehicle for Gene Therapy Applications

Nano-dimensional carriers are preferred as gene delivery vehicles as their small size favours internalization into the cells [5, 6]. As oligonucleotides are negatively charged, it is important that the nanocarrier is polar in nature, preferably with cationic charges to favour complexation through electrostatic attractive forces. An important parameter that requires optimization is the binding affinity of the oligonucleotide and the carrier. If the binding is very strong, it ensures that the nucleic acid cargo is stable from enzymatic degradation. However, its release in the cell will be retarded that reduces its therapeutic efficiency. In contrast, if the binding affinity is low, then the complex will be loosely bound which may compromise the stability of the oligonucleotide in serum. Hence, the carrier must exhibit switchable characteristics wherein it should tightly bind the oligonucleotide when in circulation and must loose affinity to the oligonucleotide once inside the target cell [7, 8] . The N/P (nitrogen from the protonated amines in the carrier and phosphorus from phosphate of oligonucleotides) ratio therefore is a critical parameter when designing gene delivery systems [9].

As cationic carriers tend to be immunogenic, their circulation time is reduced, thereby limiting their transfection ability. Hence, modifications to enhance their circulation time may be required. However, most of these modifications retard cell

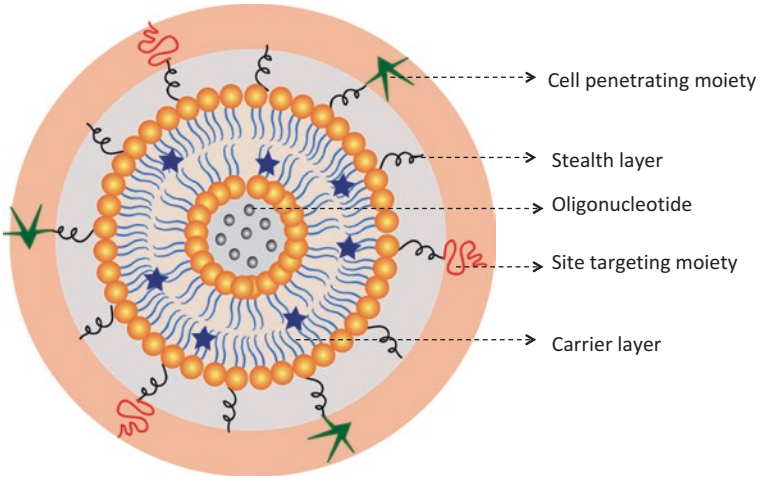



**Fig. 3.1** Schematic representation of the key components required in a gene delivery system

internalization. Hence additional modifications to ensure cellular uptake are necessary. Further, it is essential that the nanocarrier accumulates in the target cell. Hence, it is essential that the delivery system incorporates a target recognition moiety for site specificity. The delivery system must also incorporate in its design a component to either bypass the endosomal pathway or escape from the endosome once it is internalized. This will ensure that the therapeutic gene is not lost due to lysosomal degradation. If the therapeutic cargo is a *si*-RNA, then the site of action will be the cytosol. However, in the case of suicide gene therapy or plasmid DNA delivery, the cargo has to be delivered to the nucleus. Delivery systems designed for such types of oligonucleotides require a nucleus-targeting entity to ensure entry and also transcription of the transgene [10, 11]. Most importantly, the delivery system should be biocompatible and should not cause any toxic effects to the biological system. Figure 3.1 depicts the schematic representation of a delivery agent with the necessary modifications for gene delivery.

A wide range of delivery systems that comply with these requirements to various degrees have been explored. Each system has its own pros and cons. The following sections provide a brief overview of different strategies involving nano-dimensional systems that have been developed for gene delivery applications.

## 3.4 Viral Carriers

Viruses represent the most potent gene delivery systems in nature. Viruses are nano-dimensional structures that lie at the interface between living and inanimate systems as they cannot replicate by themselves. But they possess a well-organized system comprising proteins and genes that are programmed to enter into specific host cells where they integrate their genetic material into the host genome and successfully

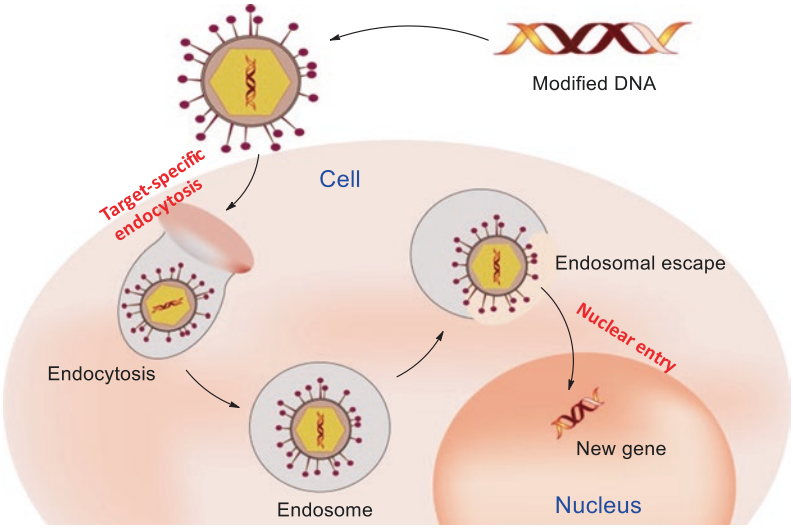



**Fig. 3.2** Schematic representation of the transfection by a typical viral vector

produce more copies of themselves utilizing the host cell's replication machinery [12]. Thus, viruses display target specificity, effective internalization into the cell and entry into the nucleus bypassing the intracellular barriers (Fig. 3.2). In fact, the first clinical trial for gene delivery employed retrovirus as the carrier [13]. The use of viruses for gene delivery has been prompted by the unsuccessful attempts to deliver naked oligonucleotides into the body. As viruses are pathogenic, recombinant viral vectors have been selectively engineered to carry the gene of interest and with their infective domains removed leaving behind the essential machinery for recognition, entry and integration of the gene.

Though a large number of viruses exist in nature, most of the gene delivery applications have centred around lentiviruses, retroviruses, adenoviruses, adeno-associated viruses and herpes simplex viruses [14]. Lentiviruses and retroviruses are integrating vectors that can infect proliferating cells; also, other types are nonintegrating and have been more effective in nonproliferating cells. Adeno-associated viruses are least toxic and have been widely engineered for customized delivery of the genetic cargo but are limited by the packaging size of the gene that can be delivered [1]. Table 3.1 summarizes the salient features of various viral vectors commonly employed for gene therapy.

A large number of studies have employed viral vectors for gene therapy, and most of the currently ongoing clinical trials also have used viruses for gene delivery. Recently, gene therapy has elicited attention due to the development of several gene editing strategies that enable molecular level therapy for treatment of genetic disorders at a pre-diseased state as well as in the diseased state. These include the CRISPR-Cas9 technique as well as the use of zinc finger nucleases and transcription activator-like effector nucleases (TALENs) for cutting the DNA at specific

**Table 3.1** Salient characteristics of viral vectors commonly employed for gene delivery [3]

| Viral vector | Type | Packaging capacity | Tropism | Salient features |
|---|---|---|---|---|
| Retrovirus | Enveloped, integrating | 8 kb | Proliferating cells | Contains RNA as genetic material |
| | | | | Has low inflammatory potential |
| | | | | Persistent gene transfer |
| | | | | Risk of oncogenesis |
| Lentivirus | Enveloped, integrating | 8 kb | Broad | Contains RNA as genetic material |
| | | | | Has low inflammatory potential |
| | | | | Persistent gene expression |
| | | | | Risk of oncogenesis |
| Herpes simplex virus-1 | Enveloped, nonintegrating | 40 kb (replication defective) | Neurons | Contains double-stranded DNA as genetic material |
| | | 150 kb (amplicon) | | Has high inflammatory potential |
| | | | | Transient gene expression |
| Adeno-associated virus | Non-enveloped, mostly nonintegrating (>90%) | <5 kb | Broad (except hematopoietic cells) | Contains single-stranded DNA as genetic material |
| | | | | Has low inflammatory potential |
| | | | | Non-pathogenic |
| | | | | Low packaging capacity |
| Adenovirus | Non-enveloped, nonintegrating | 8 kb (replication defective) | Broad | Contains double-stranded DNA as genetic material |
| | | 30 kb (helper dependent) | | Has high inflammatory potential |
| | | | | Efficient transduction in most tissues |

location followed by insertion of the gene of interest [15]. These techniques have employed AAVs for delivering the gene of interest to the editing site. The target specificity, long-term safety and efficacy of these strategies are currently being investigated, and if the results turn out to be encouraging, they might usher in a new era of gene therapy. The world's first gene editing inside a body has been performed on November 2017 on a 44-year-old patient suffering from Hunter syndrome using zinc finger nucleases [16]. The progress made by the patient in the coming months will provide valuable insights in the field of gene therapy and delivery.

Each virus class employs its own unique mode of cell entry and hence may not always be effective against all cell types. In a study carried out to investigate the ability of two serotypes of adeno-associated virus, namely, AAV2 and AAV9, to cross the blood-brain barrier, it was found that the AAV9 employed an active mode of transport to cross the blood-brain barrier without disrupting its integrity, while the AAV2 exhibited better transfection, and its entry was mediated through its interactions with the heparan sulphate proteoglycans expressed on the basolateral side of the endothelial cells [17]. This resulted in differences in localization of the two serotypes within the cell. Several studies have revealed that the recombinant vector may not always produce persistent transgene expression, which is a drawback in virus-mediated gene delivery. Immunogenicity of the viral vectors is another major stumbling block in gene therapy [18]. Surface modification of the viruses to reduce their immunogenicity and to alter their tropism offers possible solutions to these problems. Further, the high frequency of mutations encountered in viruses may lead to deleterious effects that may risk the life of the individual. Recently, gene delivery employing a combination of viral vectors such as integrating adenovirus genome in adeno-associated virus has been explored to reduce the risk of insertional mutagenesis [3]. Alternately, non-viral vectors that do not display such effects have been explored for gene delivery applications.

## 3.5 Non-viral Carriers

The serious safety concerns associated with viral transduction approaches have paved the way for the development of non-viral gene delivery methods. These include the use of physical methods such as the use of gene gun, electroporation, ultrasonication and hydrodynamic delivery into highly perfused organs. These strategies introduce transient defects on the membrane to deliver the oligonucleotide into the cell [19]. They are, however, limited to ex vivo and in vitro studies and are not very effective in vivo due to poor targeting, low transfection efficiency and possible irreversible damage to the cell and tissue architecture. The use of cationic molecules that can complex the anionic oligonucleotides and deliver the oligonucleotide into specific site through membrane fusion or receptor-mediated endocytosis has been a widely explored non-viral approach. These systems have been engineered to mimic the viruses for better efficacy [5, 20, 21]. Despite numerous efforts to develop and improve upon existing non-viral vectors, their transfection efficiencies are yet to match those of viral vectors probably due to limited ability to escape

the intracellular barriers as well as reaching the intracellular target. Nevertheless, with better understanding of the mechanism of viral transduction coupled with advances in synthetic chemistry and nanotechnology, novel non-viral vectors have emerged that exhibit the potential to circumvent the drawbacks in the present generation of non-viral vectors. The following sections provide an overview of some of the most widely explored non-viral vectors for gene delivery applications.

## 3.6 Lipoplexes

Cationic lipids have been extensively explored for complexation of oligonucleotides. The most commonly employed gene delivery system based on cationic lipids is the liposomes. Liposomes are bilayer vesicles comprising an aqueous core and polar head groups on the periphery sandwiching a hydrophobic layer comprising of the acyl chains of the phospholipids [6]. Liposomes are biocompatible, easily prepared by self-assembly and are amenable to surface modification. These advantages make liposomal carriers among the most extensively investigated delivery systems. However, their poor stability limits their translational potential. A large body of literature exists on different liposomal systems for gene delivery applications [22]. A common aspect in all these reports is that most of the cationic lipids exhibit toxic side effects that need to be overcome for successful translation of the therapeutic strategy from bench to bed. The structure of the lipids employed for the preparation of the nanocarrier exerts a major influence on the magnitude of toxicity. Cationic lipids with single acyl chain such as cetyltrimethylammonium bromide (CTAB) have been found to exert more toxicity and lower transfection efficiency when compared with their counterparts with two acyl chains such as dioctadecyldimethylammonium bromide (DDAB). However, there are several exceptions such as 6-lauroxyhexyl ornithinate that has demonstrated better transfection and lower toxicity than cationic lipids with two acyl chains. Several cholesterol derivatives of cationic lipids have been demonstrated to inhibit protein kinase C (PKC) which in turn contributes to the toxic effects of the carrier. The polar group bearing the positive charge also influences the magnitude of toxicity exhibited by the cationic lipids containing cholesterol. Quaternary ammonium groups have been found to exhibit greater toxicity than the tertiary ammonium groups. Distribution of the positive charges through conjugation has been suggested as a potential solution to overcome this challenge. Indeed, lipids containing imidazole and pyridine head groups have been found to display lower cytotoxicity and better endosomal escape and transfection efficiency [23]. A cationic liposomal system was developed based on a novel pyrrolidinium lipid, distearoyl phosphatidylcholine (DSPC), CTAB and cholesterol. The carrier was employed to co-deliver erlotinib and IL36α *si*-RNA to HEK293 cells and psoriatic mice models. Significant decrease in the levels of STAT3 and other inflammatory markers indicates the promise of this carrier for gene therapy applications [24].

The spacer that bridges the polar and hydrophobic moieties also has a major influence on the performance of the lipidic carrier. While ester links are easily

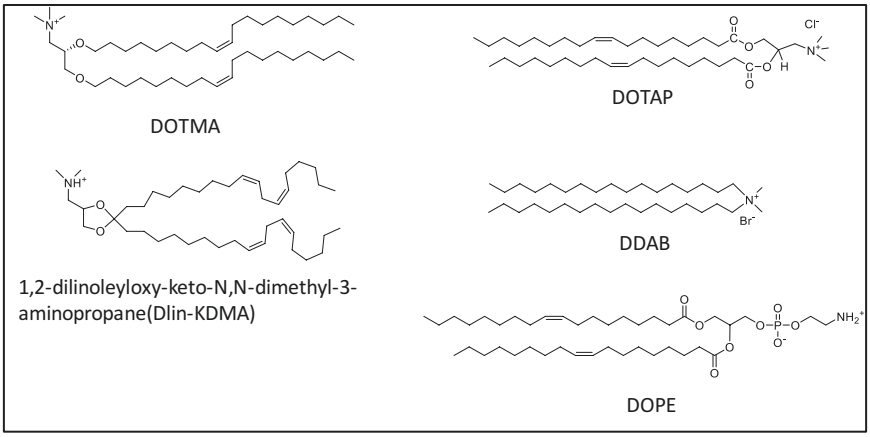



**Fig. 3.3** Structures of commonly employed cationic lipids in gene delivery

hydrolysed, amide bonds are degraded by proteases and peptidases in vivo. But ether bonds are stable and do not degrade in the biological *milieu*, thereby increasing the risk of toxic effects. Recently, carbamate links have been shown to possess low cytotoxicity, and lipids with carbamate spacers exhibit stability in circulation while they are degraded in the intracellular *milieu*. Longer spacers have been suggested to exhibit lower toxicity [23]. Commonly employed cationic lipids for fabricating gene delivery systems are depicted in Fig. 3.3.

The net charge on the liposomal carrier determines its safety, complexation, internalization and transfection efficiencies. Therefore, optimization of the N/P ratio arising due to the number of cationic amine groups and phosphate groups in the system is important. Systems with high N/P ratios display higher toxicity to cells manifested through cell shrinkage, disrupted mitosis, inhibition of key metabolic enzymes and increased vacuolation in the cytoplasm [23]. The use of neutral or anionic lipids may circumvent these toxicity issues but at the cost of complexation and cell internalization. Therefore, hybrid liposomal systems incorporating cationic polymers for complexing the oligonucleotide cargo have been employed. Helper lipids such as dioleoyl phosphatidylethanolamine (DOPE) have been incorporated in liposomes to impart better fusogenic characteristics to the carrier. The presence of small amounts of DOPE has been found to enhance cellular uptake and endosomal escape, thereby improving the transfection efficiency. Hybrid liposomes comprising neutral phospholipid egg phosphatidylcholine, the helper lipid DOPE, and distearoyl phosphatidylethanolamine-poly(ethylene glycol) (DSPE-PEG) were used to encapsulate poly(L-lysine)-*si*-RNA complex to successfully silence the expression of epithelial cell adhesion molecule in MCF-7 cells both in vitro and in tumour-bearing mice [25]. No local and systemic toxicity was reported, and the carrier exhibited good silencing efficacy. Similarly, PEI-complexed miR-29b was delivered successfully in transferrin-conjugated liposomes made from DOPE, linoleic acid and dimyristoylglycerol-methoxypoly(ethylene glycol) into acute myeloid leukemic cells. This approach

improved the treatment prognosis for acute myeloid leukaemia as it sensitized the cells to the chemotherapeutic agent decitabine [26]. Numerous reports are available on the synthesis of novel cationic lipids towards development of cationic liposomes for effective transfection. Conjugation of oleoyl chains to both the carboxylic acid groups of the amino acid glutamic acid resulted in a cationic lipid N,N′-dioleoylglutamide denoted as DG that exhibited superior transfection characteristics than Lipofectamine and conventional cationic liposomes [27].

Site-specific targeting to tumour cells has been achieved by introduction of hyaluronic acid, a negatively charged polysaccharide commonly found in the extracellular matrix, to the carrier. Hyaluronic acid enables site-specific internalization through CD44 receptor-mediated endocytosis. Most cancer cells have been found to over-express CD44, and hence this strategy has been successfully exploited to target triple-negative breast cancer cells. Hyaluronic acid linked poly(lactide-co-glycolide)-poly(ethylene glycol) was used to encapsulate lipoplex formed between DOTAP (dioleoyl trimethylammoniumpropane) and plasmid DNA. The hyaluronic acid conferred target specificity by recognizing CD44-expressing breast cancer cells and was demonstrated to be poorly internalized in nontarget cells [28]. In a similar strategy, liposomes formed using anionic phospholipids, and PEGylated lipids were used to encapsulate a poly(ethyleneimine)-*si*-RNA polyplex. This strategy ensured that the inherent toxicity of the cationic poly(ethyleneimine) was masked by the lipid layer while retaining the high complexation efficiency as well as pH buffering capabilities of the polymer. The hybrid system displayed good silencing efficiency against survivin in prostate cancer cells both in vitro and in vivo [29].

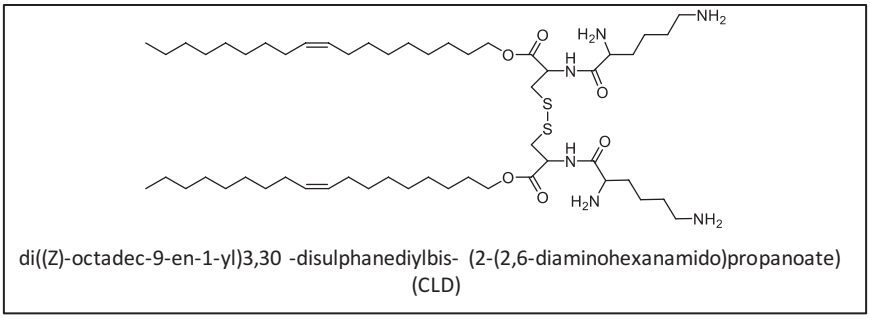
Solid lipid nanoparticles (SLNs) have been favoured in recent years for gene delivery owing to their ease of preparation by emulsion method, solvent evaporation or homogenization techniques. They exhibit better stability when compared to liposomal carriers. They also retain the biocompatibility and amenability to surface modification as liposomes [30]. The composition of solid lipid nanoparticles has been found to exert a pronounced effect on the complexation, internalization and transfection. Surprisingly, there are very few instances of gene delivery employing solid lipid nanoparticles. SLNs have been explored for ocular gene delivery applications by many groups due to their ease of administration, production in large scale, stability in biological fluids, good shelf life, surface modifiability and amenability for post-processing methods like autoclaving, sterilization and freeze drying [31]. SLNs prepared by solvent emulsification were modified through incorporation of protamine and dextran or hyaluronic acid for delivery of plasmid DNA encoding for retinoschisin to retinal pigment epithelial cells. The hyaluronic acid-modified system displayed better transfection efficiency in vitro as well as in vivo. Both subretinal and intra-vitreal injections were found to result in gene expression in several layers. While the dextran-modified system exhibited lower diffusivity and the gene expression by this system was confined near the site of administration, the hyaluronic acid-modified SLN displayed good diffusivity throughout the layers. This difference was ascribed to the surface charge on the particles with the more cationic systems exhibiting lower mobility [32, 33]. The efficacy of the hyaluronic acid-SLN system to transfect the plasmid DNA opens up new avenues for gene delivery

applications in the treatment of ocular disorders. In another study, cationic 3β-[N-(N′,N′-dimethylaminoethane)carbamoyl] cholesterol or DC-Chol was used to form SLN along with tricaprin for delivery of plasmid DNA encoding for p53 to lung cancer cells. Administration of the SLN resulted in tumour inhibition and death of cancer cells by initiation of the apoptotic pathway [34]. Other cationic components such as DOTAP, DDAB, cetylpyridinium chloride and CTAB have been introduced in SLN for complexation of oligonucleotides [31]. However, the toxicity of these cationic components limits the widespread use of SLNs for gene therapy.

Variants of liposomal vesicles have also been explored for gene and drug delivery applications. A hollow vesicle made of neutral lipids dipalmitoyl phosphatidylcholine (DPPC), DOPE and cholesterol similar to liposomes was fabricated. The vesicle referred to as the protocell was used to encapsulate the 'sleeping beauty' transposon system which comprises plasmids for transposon and transposase genes. This system can introduce the gene of interest into the target cell facilitating long-term transgene expression. A unique aspect of the protocell-based delivery system is that it was also loaded with dNTPs, primers and *Taq* polymerase for amplification of the genes encapsulated inside the protocell using polymerase chain reaction. This amplification step resulted in high encapsulation levels of the transposon system. PEGylated lipids were introduced to improve the circulation time as well as to conjugate folic acid for target-specific internalization into cancer cells that over-express folic acid receptors. The anti-inflammatory drug dexamethasone was loaded into the carrier to promote nuclear targeting. The in vitro studies performed using HeLa cells and normal cells revealed greater internalization in the cancer cells predominantly by clathrin-mediated endocytosis when the folic acid-conjugated system was employed. In vivo studies also confirmed the specific accumulation of the folic acid-conjugated protocells in the tumour tissue [35].

## 3.7 Gemini Surfactants

Amphiphilic cationic surfactants and lipids have been traditionally employed as nanocarriers due to their ability to self-assemble in aqueous medium. However, their stability on encountering the membrane bilayer is poor, and hence dissociation of the oligonucleotide complex occurs before their entry into the cell. Consequently, their therapeutic efficacy is reduced. Gemini surfactants were introduced to overcome this problem. Gemini surfactants typically comprise of two identical units containing cationic groups for complexation and acyl chains to confer amphiphilicity. These units are bridged by a linker/spacer [36]. One of the earliest Gemini lipids that was reported is $C_{10}CysG^{+}$ where $C_{10}$ denotes the carbon acyl chain length, Cys refers to the cysteine residue, and $G^{+}$ refers to the guanidino group that confers positive charge for complexing oligonucleotides. When this system was introduced into an aqueous medium, micellization occurred which brought the cysteine residues in close proximity. The thiol functional groups in cysteine underwent spontaneous oxidation to form disulphide links that stabilized the self-assembled structure. Once inside the cell, the disulphide bonds will be cleaved by the reduced glutathione




di((Z)-octadec-9-en-1-yl)3,30 -disulphanediylbis- (2-(2,6-diaminohexanamido)propanoate)
(CLD)

**Fig. 3.4** Structure of a typical Gemini surfactant for gene delivery. The acyl chains confer amphiphilicity for self-assembly, while the amine functionalities enable effective complexation of the oligonucleotide. The disulphide links provide stability in circulation and are cleaved by the intracellular reduced glutathione for release of the oligonucleotide

(GSH) present in the cells, thereby enabling the dissociation of the complex. It was found that the length of the acyl chain influenced the internalization of the complex. A minimum length of 14 carbons was desired for internalization into the cells. In order to increase the complexation efficiency, the guanidino group has been replaced with ornithine or spermine giving rise to a series of Gemini lipids such as $C_{16}$CysO and $C_{18}$CysSper. Figure 3.4 depicts the structure of a typical Gemini lipid for gene complexation and delivery.

Recently, partially fluorinated Gemini surfactants based on pyridinium framework were developed along with dioleoyl phosphatidylethanolamine (DOPE) for delivery of plasmid DNA into cells. The length of the spacer that bridged the two identical units was found to influence the internalization of the surfactant. Short spacers were better than longer spacers. In order to improve the cellular uptake of the Gemini surfactant, the DOPE component was introduced. The partial fluorination imparted stability and better complexation ability to the system. This system was proposed to be a potential candidate for gene delivery to treat lung disorders like cystic fibrosis [37].

## 3.8 Polyplexes

Poly(ethyleneimine) (PEI) is a cationic polymer whose complexation efficiency is influenced by its molecular weight and branching. It has been employed for gene delivery applications due to its ability to escape from the endosome by employing the proton sponge mechanism wherein the polymer offsets the acidification of the endosome leading to the activation of the proton pump. This causes swelling of the endosome leading to its disruption, thereby enabling endosomal escape of the complex. Comparison of PEI with other cationic carriers has demonstrated that PEI-induced transfection maintains long-term expression of plasmid DNA unlike cationic lipid carriers [38]. But PEI has been mainly limited by its cytotoxicity.

Hence, attempts to utilize its complexation efficiency and endosomal escape properties while effectively masking its cytotoxicity have been attempted. Poly(ethylene glycol) (PEG)-modified PEI grafted with poly(L-succinimide) exhibited low cytotoxicity but high transfection efficiency when employed to deliver plasmid DNA to HeLa cervical cancer cells. The improvement in the performance of the PEI-based nanocarrier has been attributed to the presence of the PEG chains on the surface that sterically stabilizes the nanoparticles from aggregation [39].

The N/P ratio has been found to be a major factor in influencing the complexation efficiency as well as the cytotoxicity of the complex. High N/P ratios improve complexation but also increase cytotoxicity, while low N/P ratios reduce complexation efficiency but also display low cytotoxicity [40]. Hence, identification of an appropriate N/P ratio that balances adequate complexation efficiency with low cytotoxicity is the need of the hour. Apart from N/P ratios, surface modification of PEI has been found to exert a profound influence on its internalization and transfection efficiency. In a recent study, branched PEI was complexed with fluorescent *si*-RNA and gamma-polyglutamate moiety encapsulating plasmid DNA encoding for green fluorescent protein. The system exhibited good stability and high transfection efficiency. The incorporation of polyglutamate promotes internalization into hepatic cancer cells by the gamma-glutamyl transpeptidase pathway [41]. Though in vitro results are promising, in vivo studies need to be performed to establish the true potential of this modified PEI carrier. Tyrosine-modified low molecular weight branched PEI employed for delivery of *si*-RNA against survivin to cancer cells was found to exhibit good silencing efficiency in vitro. In vivo studies revealed that the modified PEI was found to effectively silence survivin and reduce the tumour volume. Complete regression of the tumour was not observed which was attributed partly to the rapid growth kinetics of the tumour as well as to the absence of a cytotoxic molecule to support the gene silencing strategy. However, the results from the study demonstrate that tyrosine conjugation to low molecular weight PEI exhibits improved silencing efficiency when compared with low molecular weight PEI that displays poor transfection efficiency [42]. Hyaluronic acid conjugation to PEI polyplexes containing plasmid DNA has shown specific internalization into CD44-expressing cancer cells [43]. The polyplex also incorporated epigallocatechin gallate, a potent antioxidant flavonoid from green tea for effective treatment of colon cancer both in vitro and in vivo.

Poly(β-amino esters) (PBAEs) represent another widely investigated polymeric system for gene delivery applications. These polymers incorporate cationic amino groups for DNA complexation and hydrolysable ester bonds. A series of PBAEs based on poly(1,4-butanediol diacrylate-co-4-amino-1-butanol), having its end modified with the moiety 1-(3-aminopropyl)-4-methylpiperazine, were synthesized for delivery of the suicide gene HSV-Tk (herpes simplex virus thymidine kinase) to glioma cells through convection-enhanced diffusion. The survival time of the animals were found to nearly double after administration of the PBAE-suicide gene complex followed by systemic administration of the drug ganciclovir. This polyplex represents the first instance of a non-viral vector employed for suicide gene delivery

to treat glioblastoma, and it showed better prospects than the earlier reports on its viral counterparts for the same condition [44].

The triblock polymer Pluronics has been explored for gene delivery applications due to its ability to internalize into cells. In order to impart oligonucleotide complexation ability, it was incorporated with PEGylated (polyethylene glycol) cationic segment poly(N-[N-(2-aminoethyl)-2-aminoethyl] aspartamide). This nanoparticle was employed to deliver plasmid DNA encoding for the reported gene luciferase to human breast cancer and lung cancer cells. The in vitro results reveal the potential of this system for gene delivery applications, but the complex was found to display a tendency to undergo salt-induced aggregation that may limit its in vivo efficiency [45]. However, when a redox-sensitive disulphide link was introduced between Pluronics and aspartamide moiety, the colloidal stability of the complex significantly improved along with the transfection properties of the carrier [46]. This improvement may be attributed to the lysis of the disulphide bond in the intracellular *milieu*, thereby facilitating faster release of the gene in the cytosol. The free polymer was found to display higher toxicity when compared with the polyplex. Hence, chemical modifications of the polymer were attempted to reduce toxicity to nontarget cells. Incorporation of cholesterol and cyclic RGD peptide on either end of the polymer chain before micellization was found to significantly decrease nonspecific toxicity of the carrier, while its ability to transfect plasmid DNA into target endothelial cells and cancer cells remained unaffected in vitro. Studies in mice models of pancreatic cancer revealed no toxic effects and good tumour regression properties suggesting that the Pluronics-based cationic carriers can be employed for superior gene transfection [9]. The cationic component used in the above studies by itself was found to complex plasmid DNA encoding from brain-derived neurotropic factor effectively. The cationic polymer has been demonstrated to undergo autocatalysed degradation in vivo, thereby limiting long-term toxic effects. The polyplex was employed successfully to treat spinal cord injury through an intrathecal route of administration in mice models. A single injection of the polyplex showed significant attenuation of neuro-inflammation at the damaged site and improved motor neuronal function restoration [47]. A similar micellar system of PEG-poly[N-[N-(2-aminoethyl)-2-aminoethyl]aspartamide] was successfully employed in another study to deliver mRNA for brain-derived neurotropic factor through the intranasal route for treatment of olfactory nerve dysfunction [48]. Incorporation of a thermoresponsive poly(N-isopropylacrylamide) (PNIPAM) segment in the polymer was found to impart 'on-demand' release of the anti-angiogenic gene sFlt1 to hepatoma cells H22 [49]. Thus, it is evident that poly[N-[N-(2-aminoethyl)-2-aminoethyl] aspartamide]-based systems have the potential to be employed for gene therapy applications and can be suitably tailored for specific tissues and requirements.

Chitosan is a partially deacetylated form of the polysaccharide chitin that is present in the exoskeletons of crustaceans. Chemically, chitosan is a random mix of N-glucosamine and N-acetyl glucosamine units that are linked through β-1,4 glycosidic links. The amine groups in chitosan impart pH responsiveness to the polymer and also facilitate conjugation with other functional molecules. The protonated amine functionalities also favour complexation with negatively charged

oligonucleotides. Chitosan-DNA complexes are easily formed, and the extent of complexation depends on the N/P ratios used [50]. Chitosan exhibits mucoadhesive properties that have promoted studies on its incorporation with solid lipid nanoparticles for possible ophthalmic applications [51]. In order to improve target-specific localization, chitosan carriers have been modified with folic acid to target cancer cells of epithelial origin [52], while lactosylated and galactosylated chitosan have been developed for selective uptake into liver cancer cells [53]. Transferrin-modified chitosan carriers have been explored for delivering the therapeutic gene across the blood-brain barrier [54]. Complexation with chitosan improves the stability of the oligonucleotide and also enhances the cellular uptake due to the fusogenic nature of chitosan nanoparticles. Comparison of the performance of chitosan and PEI for gene delivery applications reveals that the transfection efficiency of PEI is very high but it is also cytotoxic, whereas in comparison, chitosan has lower transfection efficiency but is relatively less cytotoxic. The lower transfection efficiency of chitosan when compared with PEI is due to PEI's superior ability to escape from the endosome by the proton sponge mechanism [38]. Hence attempts to incorporate endosomal escape strategies to chitosan-based carriers have been reported. Urocanic acid-modified chitosan nanoparticles have been developed for delivery of plasmid DNA to HeLa cells and 3T3 cells. The urocanic acid moiety was introduced to impart endosomal escape characteristics to the carrier through the proton sponge mechanism [55].

Poly(lactide-co-glycolide) (PLGA) is a biodegradable polymer that has been widely employed as a drug delivery system. The content of lactide in this copolymer influences the degradation profile of the polymer. Higher lactide contents tend to retard the degradation. The encapsulation in PLGA offers a protective environment for the oligonucleotides. However, the hydrophobic interior and slightly anionic surface of PLGA impede interactions with oligonucleotides, thereby necessitating additional modifications. PLGA nanoparticles coated with cationic polymers such as chitosan or PEI have been employed for adsorbing *si*-RNA that were successfully delivered to lung cancer cells [56]. A note of caution is required during the fabrication of PLGA nanoparticles for gene delivery. The surfactant used in the preparation of PLGA nanoparticles may be cytotoxic, and hence the choice of surfactant is a major determinant in the efficacy of PLGA gene delivery systems. A peptide-linked PLGA system containing a cocktail of *si*-RNA against myc, VEGF and bcl-2 was successfully delivered to neuroblastoma cells both in vitro and in vivo. The peptide sequence was derived from the rabies viral glycoprotein that shows high specificity to neuroblastoma cells [57]. Other biodegradable polymers like poly(caprolactone) conjugated with poly(ethylene glycol) and PEGylated poly(lactide) have also been explored for gene delivery applications after appropriate modifications with a cationic component and a targeting moiety for site-specific delivery [58].

## 3.9 Dendriplexes

Dendrimers are hyperbranched polymers that possess numerous functional groups at the terminus of the branches. Each dendrimer comprises of a central core from which branches originate. Each branched layer defines the generation of the dendrimer. As the generation increases, steric repulsion between the functional groups in the branches also increases, thereby reducing the mobility of the molecule and increasing the possibility of immune recognition. Hence, generations beyond five are rarely employed with most of the work restricted to two–three generations. The core group, type and number of terminal functional groups as well as the type of branching can be tailored by regulating the molecular architecture of dendrimers [59]. The unique advantage possessed by dendrimers is that the numerous functional groups at the branch tips can be conjugated to different molecules that can confer special properties to the system. The use of cationic end groups such as amine functionalities has been used for complexing oligonucleotides, and the resulting complex is referred to as a dendriplex. Dendrimeric systems are promising gene delivery carriers as they are capable of endosomal escape by the 'proton sponge' effect, thereby displaying better transfection efficiency [60].

A wide range of dendrimeric systems have been reported for gene delivery applications. Some of the dendrimeric systems that have been explored for gene delivery are shown in Fig. 3.5. The most extensively investigated dendrimer is the poly(amidoamine) (PAMAM) system first reported in the mid-1980s. Both unmodified and surface-modified PAMAM dendriplexes have been investigated for gene silencing applications. It has been observed that surface conjugation of a targeting moiety such as folic acid or luteinizing hormone-releasing hormone (LHRH) improved the internalization and silencing efficiency of the dendriplex [61]. PAMAM dendrimer in combination with arginine-conjugated poly(cystamine-bisacrylamide-diaminohexane) was used to successfully deliver anti-VEGF *si*-RNA to different cancer cell lines [62]. The cystamine moiety favoured the formation of disulphide bonds that promoted complexation at much lower concentrations and also ensured the release of the oligonucleotide cargo in the cells when they are cleaved by intracellular glutathione. The silencing efficiency displayed by this hybrid system was found to be better than PEI-*si*-RNA polyplexes.

PAMAM-pullulan conjugates were used to deliver plasmid DNA to liver cells successfully. The liver targeting ability of pullulan was exploited to achieve target specificity in this study [63]. Angiopep peptide-conjugated PEGylated PAMAM dendrimer has been demonstrated to exhibit superior transfection efficiency in the brain due to the ability of angiopep to cross the formidable blood-brain barrier by a combination of receptor-mediated endocytosis and macropinocytosis [64]. The major drawback of PAMAM systems is its cytotoxicity which arises due to the amine functionalities. Hence, attempts to mask the amine groups by acetylation have been

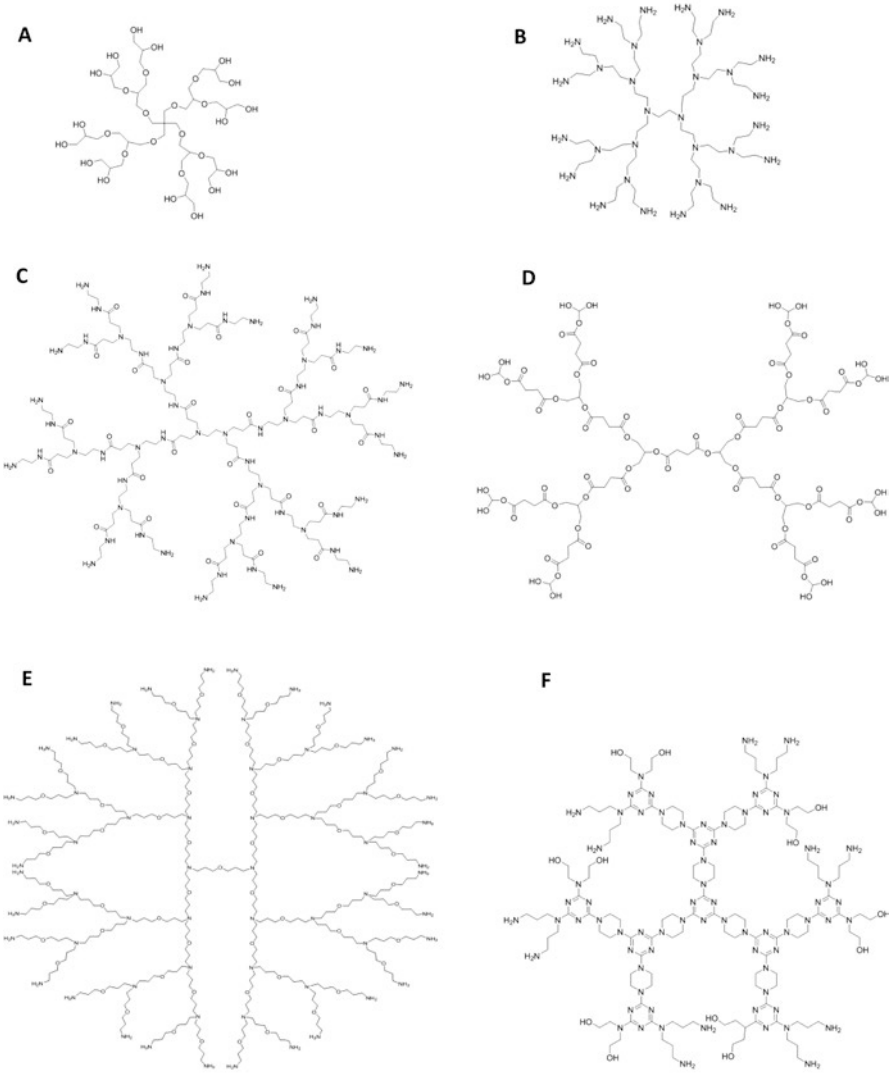



**Fig. 3.5** Structure of different dendrimers explored for gene delivery applications. (A) Polyglycerol dendrimer, (**b**) polypropylene imine dendrimer, (**c**) PAMAM dendrimer, (**d**) polyglycerol-succinic acid dendrimer, (**e**) polyetherimine (PETIM) dendrimer, (**f**) triazine dendrimer

reported. However, as the degree of acetylation increased, the gene complexation efficiency was compromised [65]. Modifications of the PAMAM dendrimer core group from the conventional ethylene diamine to triethanolamine or beta-cyclodextrin or pentaerythritol have been reported in literature for altering the stability and branching characteristics to achieve better transfection efficiencies [59].

Polypropyleneimine (PPI) dendrimers are other widely explored systems for gene delivery applications. These carriers are synthesized using ethylene diamine core and diaminobutane as the branching moieties. The PPI dendrimers are more compact and more hydrophobic than their PAMAM counterparts and display better biocompatibility [66]. PPI dendrimers modified with disulphide linkers, poly(ethylene glycol) and LHRH targeting moiety have been developed for cytoplasmic delivery of bcl2 *si*-RNA to ovarian cancer cells [66]. The dendriplex ensured better stability of the *si*-RNA against nuclease degradation, while the LHRH moiety favoured specific internalization of the carrier in the cancer cells. The *si*-RNA release was mediated by the lysis of the disulphide bond by intracellular glutathione.

Silicon-containing dendrimers have received attention in recent decades due to the exceptional stability of Si-O and Si-C bonds against hydrolytic degradation [67]. Water-soluble carbosilane dendrimers containing Si-C bonds have been developed for nucleic acid delivery to hepatocellular cancer cells [68]. Triazine dendrimers with tunable flexibility have been explored for gene delivery applications in HeLa cancer cells [69]. Similarly, plasmid DNA was successfully delivered using triazine dendrimers with flexible architecture to melanoma cells more effectively than PAMAM dendrimers indicating the potential of these molecules for gene delivery [70]. Another novel class of dendrimers that have been explored for gene delivery applications in HeLa cells are the polyglycerol dendrimers [69]. A series of polyglycerol dendrimers with different oligoamine functionalities were explored for gene delivery efficacy in HeLa cells. It was found that the pentaethylenehexamine carbamate modification resulted in superior transfection efficiency and low toxicity.

Peptide dendrimers have also been explored for gene delivery. Polymeric dendrimers with nonstandard amino acid functional groups or dendrimers with peptide branches or dendrimers with an amino acid core and various polypeptide chains as the branching units have been described in the literature [71]. The most widely reported peptide dendrimers are poly(L-lysine) dendrimers. These molecules have higher surface charge and reproducible sizes. A poly(L-lysine) dendriplex of apolipoprotein *si*-RNA was successfully used to silence apolipoprotein B in mice models without any discernible toxic manifestations [71]. However, their poor in vivo stability and persistent nonspecific toxicity limit their extensive applications. A poly(L-lysine) dendrimer was modified with lipoamino acid chains and employed for delivery of vascular endothelial growth factor (VEGF) *si*-RNA to human retinal epithelial cells [59]. The system exhibited good penetration into the different retinal layers and silencing of VEGF. Recently, an interesting system comprising of polyglycerol dendrimer with dendritic poly(L-lysine) was employed for the co-delivery of the chemotherapeutic agent docetaxel and the *si*-RNA against MMP9 for treatment of breast cancer [72]. The system displayed lower toxicity and better serum stability but slightly lower transfection efficiency when compared with poly(ethyleneimine) dendrimers. Thus, it is evident that dendrimeric systems can be interesting candidates for gene delivery due to their excellent complexation and ability to escape the endosome and internalize into the target cells. However, a

major concern with these systems is their toxicity and poor hemocompatibility. Hence, the field remains wide open for the development of novel hyperbranched molecular architectures to overcome these lacunae.

## 3.10 Exosomes

Exosomes are nano-dimensional spherical multivesicular bodies that are secreted by cells into the extracellular space. The membrane of exosomes is rich in cholesterol, sphingolipids, ceramides and phospholipids with long saturated fatty acyl chains and phosphatidylethanolamine. In addition, several proteins such as flotillins, integrins, Alix, annexins, GTPases, phospholipases, tetraspanins and several heat shock proteins are present in exosomes [73]. It is now understood that exosomes have an endosomal origin. Exosomes are formed during the maturation of endosomes from the early to the late endosomal phase, when, instead of fusing with the lysosomes, they fuse with the plasma membrane resulting in their release in the extracellular fluid as small vesicular structures now referred to as the exosomes [74]. Exosomes are found in biological fluids such as saliva, blood, urine, breast milk, amniotic fluid, pleural effusions and synovial fluid. Exosomes have been implicated in transporting cargo such as RNA, proteins and prostaglandins in the biological systems. They are also reported to have a major role in cell-cell communication pathways.

Ever since their discovery in 1981, exosomes have been explored for various biomedical applications. They have been investigated as biomarkers for several inflammatory disorders and pathogenic conditions [75]. They have also been used as a therapeutic entity for immunotherapy against various forms of cancer [75]. Their non-toxic nature and nano-dimensions have been key factors that have promoted their use as drug carriers for delivery of curcumin and anti-Stat3 inhibitors [76]. Exosomes have dimensions that are comparable to viruses and fall in the range of 30–100 nm. Their small size permits them to accumulate in cancer tissue through enhanced permeation and retention (EPR) effect. They also have been demonstrated to cross the blood-brain barrier effectively. Hence exosomes have also been explored as nanodelivery systems to treat brain disorders. Exosomes also have a remarkable tissue-homing property that is determined by the cells from which they are produced. The cell surface markers exhibited by the exosomes are highly origin specific and hence confer their tissue specificity. It was shown that HEK293 cells that were engineered to over-express the Epstein-Barr virus glycoprotein gp350 produced exosomes that specifically targeted CD21-expressing cells [73]. This homing ability was conferred to the exosomes due to the gp350 that specifically interacts with CD21. Recent research has suggested that the tetraspanin family of transmembrane proteins found on the exosomal membrane may play a major role in regulating the tissue-specific homing properties of exosomes. The composition of the exosomal membrane and its nucleic acid cargo is also dependent on their cell of origin.

The discovery of the fact that exosomes shuttle coding and non-coding RNA between cells has led to new research focusing on the use of exosomal carrier for gene therapy. While genetic transfer mediated by native exosomes has positive effects such as modulating the stem cell plasticity, mediating inflammatory responses and facilitating the transfer of neurotropic and neuroprotective factors important for the development of the neuronal network, recent research has also revealed the negative aspects of exosomal RNA transfer in biological systems especially in pathological conditions [75]. These include promotion of cancer metastasis, immunosuppression and acceleration of neurodegeneration in aberrant systems. It was demonstrated that non-infected B-cells when cocultured with B-cells infected with Epstein-Barr virus expressed EBV-*mi*-RNAs that was transferred by exosomes produced by the infected cells [77]. Such exosomal transmission of viral *mi*-RNA may also be the reason for the spread of retroviral infections such as AIDS.

In the context of the therapeutic use of exosomes, several studies have shown that mesenchymal stem cell-derived exosomes have been able to promote myocardial repair and regeneration due to the delivery of *let7 mi*-RNA isoforms *let7b* and *let7g* [74]. Exploring the native exosomes derived from stem cells thus has a huge potential in regenerative applications and continues to be investigated by several groups across the globe. The first report of engineering exosomes to deliver exogenous gene cargo appeared in 2011 when modified exosomes expressing the brain-targeting peptide RVG derived from the rabies virus glycoprotein were generated from immature dendritic cells. The RVG modification on the exosomal surface enabled them to permeate the formidable blood-brain barrier through acetylcholine receptor-mediated endocytosis. The engineered exosome carried *si*-RNA to silence GAPDH and BACE-1. The successful delivery of the cargo was confirmed from the GAPDH and BACE-1 (β-secretase enzyme) knockdown observed in the brain cells of mice [78].

Adeno-associated viral (AAV) vectors have several limitations for use as gene delivery systems due to their poor transfection efficiency as well as poor target specificity. Recently, it was found that some AAV are associated with exosomes. These vector-associated exosomes known as vexosomes were found to exhibit superior transfection efficiencies when compared to the AAV systems that were not associated with exosomes [79]. In another study, hepatic cells were transfected with lentiviral constructs expressing *si*-RNA against CD81. These cells were introduced into immune-compromised mice where specific knockdown of CD81 was observed [80]. This silencing of CD81 in mice hepatic cells was attributed to the *si*-RNA-rich exosomes produced by the transplanted cells that effectively transferred the anti-CD81 *si*-RNA to the hepatic cells in the vicinity. In a similar strategy, mesenchymal stem cells were transfected with lentiviral vectors designed to express sh-RNA against a mutant Huntingtin protein. When these mesenchymal stem cells were cocultured with glioma or neuroblastoma cells, specific silencing of the Huntingtin protein mRNA was observed [80]. This indicates that the exosomes produced by the engineered mesenchymal stem cells had effectively served as a gene transfer agent. An interesting observation in many studies on exosomes is that they have very low accumulation in the liver unlike other vesicular carriers such as liposomes. The mechanism by which

exosomes bypass the hepatic elimination remains unknown, but this characteristic could probably be the reason for the low toxicity of the exosomal carrier in vivo. Though exosomes represent an interesting option for gene delivery applications, further in-depth studies are required on its immunomodulatory properties and other yet unexplored physiological effects before translational studies.

## 3.11 Virosomes

Though cationic liposomal carriers are extensively investigated for gene delivery applications, their widespread use is limited by their immunogenicity and also their inability to escape from the endosomes resulting in their degradation in the lysosomal compartment. To circumvent these issues, a novel strategy incorporating viral fusion proteins from the viral envelope to the liposomal membrane was attempted. The viral fusion proteins incorporated liposomes are referred to as 'virosomes'. Essentially, a virosome contains the membrane lipids, the spike glycoproteins and the fusion proteins of a virus but is devoid of the viral genetic material. This strategy helps in achieving efficient delivery without the danger of infections associated with viral delivery systems.

The two most important fusion proteins that have been extensively explored for gene delivery applications are hemagglutinin and neuraminidase found in many viruses including the influenza virus. The hemagglutinin stabilizes the virosomes and prevents aggregation. It further enables the fusion of the virosome with the plasma membrane. The virosome internalizes into the cell through endocytosis [81]. In the acidic pH of the endosome, the hemagglutinin undergoes a conformational change to expose peptide sequences that fuses with the endosomal membrane and facilitates the release of the cargo into the cytosol. Neuraminidase catalyses the hydrolysis of N-acetylneuraminic acid from the sugar residues on the membrane surface, thereby promoting the fusion process with the cell membrane. For more efficient complexation of the negatively charged oligonucleotides, cationic lipid components such as DOTAP, DODAC (dioleoyldimethylammonium chloride), DC-Chol, DMRIE (dimyristyloxypropyl-3-di-methyl-hydroxyethyl ammonium), GAP-DLRIE ((±)-N-(3-aminopropyl)-N,N-dimethyl-2,3-bis (dodecyloxy)-1-propanaminium bromide) and TMAG (N-(alpha-trimethylacetyl)-didodecyl-D-glutamate chloride) have been used to form the vesicles [81].

Apart from the influenza virus, membrane fusion components from the envelopes of the *Semliki Forest virus*, *Sendai virus* (F-protein), *Vesicular stomatitis virus* (G-protein), *Rubella virus* (E1 and E2 glycoproteins) and *Sindbis virus* have been employed for developing virosomes. Early studies have shown that vesicular stomatitis viral protein containing virosomes exhibited high transfection efficiency of a green fluorescent protein bacmid in sf9 cells [82]. The experiments revealed that the endosomal escape of the virosomes was facilitated by the viral proteins through a conformational change in the acidic pH of the endosome. When the endosomal acidification was inhibited using ammonium chloride, the transfection was found to be reduced confirming the need for acidic pH to trigger endosomal escape of the

virosome. Along with similar lines, virosomes comprising cationic DODAC lipids, hemagglutinin and neuraminidase from influenza virus were found to display good transfection efficiency [83]. When these virosomes were exposed to acidic pH prior to the transfection, the hemagglutinin was inactivated, and it resulted in a dramatic reduction in the transfection efficiency. This result clearly indicates that hemagglutinin is essential for internalization into the cell as well as endosomal escape without which the transfection is severely compromised.

Virosomes with influenza viral proteins hemagglutinin and neuraminidase loaded with *si*-RNA were found to effectively silence the expression of constitutively expressed green fluorescent protein both in vitro and in vivo [84, 85]. Virosomes with similar constituents have also been reported for effective delivery of a gene encoding parathyroid hormone-related protein in Balb/c mice [86]. Virosomes containing carcinoembryonic antigen plasmid along with a plasmid encoding the antigen CD40 have also been used as a vaccine to confer anticancer properties in Balb/c mice models [87]. In another study, antisense therapy was successfully demonstrated in non-small cell lung cancer through suppression of *L-myc* expression using virosomal carriers [86]. Similarly, virosomal delivery of the gene encoding for β-galactosidase was successfully achieved in BHK-21 cells (baby hamster kidney fibroblasts) [86] employing virosomes containing the cationic lipid DODAC. The outer envelope of the inactivated hemagglutinating virus of Japan (HVJ) was successfully used to introduce fluorescent oligonucleotides into the target cells both in vitro and in vivo. The viral envelope ensured high internalization efficiency into the cells but did not display toxic effects [88, 89]. However, the presence of viral proteins causes immune response, and hence virosomes have been employed in immunotherapy and as adjuvants to vaccines as well as carriers for peptide and nucleic acid-based vaccines. Several studies employing the fusion peptide, hemagglutinin and neuraminidase components of the *Sendai virus* or HVJ have shown that virosomes constituted using anionic lipids such as phosphatidylserine showed lower immunogenicity when compared to their cationic counterparts [89]. Interestingly, in another independent study, it was shown that cationic vesicles with Sendai (HVJ) viral protein components exhibited superior transfection efficiency of LacZ gene in chick embryo and rat models [89], while the anionic virosomes containing phosphatidylserine were found to exhibit less specificity.

Tissue specificity for these virosomes will require additional surface modifications with cell-specific ligands. In some instances, it has been shown that the viral protein components may confer cell-specific homing properties. Sendai viral components have been demonstrated to induce higher internalization of the virosomes into hepatocytes when compared to other tissues [90]. Similarly, the G-protein from the vesicular stomatitis virus has been found to selectively fuse with membranes rich in phosphatidylserine [89]. Virosomes containing protein components from Epstein-Barr virus internalize preferentially in B-cells, while introduction of HIV glycoprotein components favours their uptake into lymphocytes and $CD4^+$ cells. Hepatitis B viral proteins confer liver-homing properties to the virosomes. Altered tissue tropism was observed when different protein components from adenoviral vectors were incorporated in virosomes [89].

## 3.12 Inorganic Carriers

Though a plethora of polymeric and liposomal carriers have been investigated for drug and gene delivery applications due to their degradability in the biological environment that facilitates the release of the therapeutic cargo, this characteristic also presents several problems. The degradability of these 'soft' carriers results in premature release of the cargo in nontarget compartments. These carriers are also largely ineffective in the highly acidic environment presented in the stomach. Hence, more structurally stable carriers have been explored to circumvent these issues. It is in this context that inorganic carriers have emerged as promising gene delivery systems. The major categories of inorganic carriers that have been explored for gene delivery are the calcium phosphates, gold nanoparticles and silica-based systems.

## 3.13 Calcium Phosphate Carriers

Calcium phosphate is a non-toxic, biocompatible and resorbable material that has recently gained importance for gene delivery applications. The cationic calcium ions favour complexation of the oligonucleotides. The major advantage of calcium phosphate nanoparticles is that they are capable of endosomal escape which is a major limitation of many organic carrier systems. When calcium phosphate nanoparticles are endocytosed, the acidic pH of the endosomes promotes their dissolution into calcium and phosphate ions. This results in an increase in the osmotic pressure leading to the lysis of the endosome, thereby releasing the contents into the cytosol. The rate of dissolution of the calcium phosphate nanoparticles in acidic pH is therefore the critical factor in the endosomal escape mechanism [91]. There are different forms of calcium phosphate such as tetracalcium phosphate, tricalcium phosphate, monetite, brushite, hydroxyapatite, octacalcium phosphate, etc., which differ in their dissolution rates in acidic pH, and hence the type of calcium phosphate employed will influence the transfection efficiency. In a study employing calcium phosphate nanoparticles for delivery of plasmid DNA encoding for luciferase in HeLa cells, two types of magnesium-doped calcium phosphate were employed – one containing 14% brushite and the other 50% brushite. It was found that the nanoparticles containing 50% brushite exhibited superior transfection. This was attributed to the difference in brushite content and location of magnesium ions in the crystal lattice resulting in variation in the dissolution rate at acidic pH [92]. Along similar lines, silicon-incorporated hydroxyapatite nanoparticles have been reported for gene delivery applications. Incorporation of silicon distorts the hydroxyapatite crystal lattice, thereby facilitating faster dissolution in the intracellular *milieu*. However, high levels of silicon beyond 60% dramatically reduced plasmid DNA

complexation. Silicon incorporation of 8.3% was found to be most effective and exhibited higher transfection efficiencies in breast cancer cells when compared with their unsubstituted counterparts [93].

Another advantage of the calcium phosphate nanoparticles in the context of gene delivery is the fact that they play a major role in the transfer of the oligonucleotide cargo into the nucleus. The nuclear pore complex present in the nuclear membrane regulates the entry of molecules into the nucleus. It remains open when calcium ions are present in the cisterna located between the nuclear membrane. During the regulatory process, phospholipase is activated which then forms inositol triphosphate that binds to the inositol triphosphate receptor in the nuclear membrane. This alters the conformation of the nuclear pore complex blocking the pore. High concentrations of calcium ions in the cytosol that are commonly encountered in calcium phosphate nanoparticle-mediated delivery systems inhibit the formation of inositol triphosphate, thereby keeping the nuclear pore complex open facilitating the entry of the exogenous oligonucleotide into the nucleus [91].

The mechanism of cell internalization of the calcium phosphate nanoparticles was demonstrated through the use of specific inhibitors of endocytosis pathways. It was found that the calcium phosphate nanoparticles internalized into the cell primarily through the clathrin- and caveolae-mediated endocytic pathways [94]. Though calcium phosphate delivery systems overcome several limitations associated with other gene carrier systems, they also have several drawbacks due to their tendency to form polydisperse aggregates that limit their internalization into the cells. Therefore, preparation of monodisperse calcium phosphate nanoparticles below 100 nm is essential to realize their beneficial properties towards gene delivery. Micelle-based synthesis of calcium phosphate nanoparticles has been reported to achieve monodisperse particles below 100 nm [92].

Another important consideration during the synthesis of calcium phosphate nanoparticles is the calcium-to-phosphate ratio. It has been demonstrated that the nanoparticles with high Ca/P ratio between 100 and 300 exhibit high transfection efficiency, but Ca/P ratios greater than 500 exhibited poor complexation efficiency due to deficiency in the amount of phosphate ions that limits the calcium phosphate nanoparticles available for complexation [95]. Ca/P ratios between 30 and 70 were reported to exhibit poor transfection efficiency in spite of demonstrating high complexation efficiency. This anomalous behaviour was attributed to the large sizes of these complexes that retarded the internalization of these particles [95]. Thus, the preparation method employed plays a key role in determining the gene transfection efficiency of calcium phosphate nanoparticles. Stabilization of the calcium nanoparticles with PEGylated bisphosphonate was found to improve cell internalization and transfection efficiency as the bisphosphonate coating associated well with calcium phosphate and ensured the formation of nano-dimensional particles [96]. Chitosan- and lipid-coated calcium phosphates have also been reported in the literature to improve dimensional stability [91].

## 3.14 Mesoporous Silica Nanoparticles

Mesoporous silica represents a family of carriers with highly ordered porous network whose dimensions can be controlled by modifying the preparation conditions. They have a rigid silica framework and high surface area due to its porous network. This confers high entrapment efficiencies to mesoporous silica. The surface and pore walls of the mesoporous carrier are amenable to functionalization and hence can be tailored to accommodate a wide range of guest molecules. Recently, mesoporous silica carriers have been functionalized with molecular gatekeepers that serve as 'caps' over the pores preventing premature release of the cargo. The lysis of these 'caps' in the target site enables site-specific release of the therapeutic agent [97]. Such smart release systems represent the next generation of delivery systems that can overcome the limitations presented by the present generation of carriers. The pore size of the mesoporous silica can be regulated by the preparation conditions and typically range from 2 to 50 nm. Though their mechanism of elimination and long-term toxicity in the biological system are yet to be completed and elucidated, these systems have elicited sufficient interest for gene and drug delivery applications due to their unique characteristics.

A dendrimer-capped mesoporous silica loaded with Texas red dye was reported to exhibit good transfection efficiency without any cytotoxicity commonly associated with PAMAM dendrimers. The study employed second-generation PAMAM dendrimer to complex plasmid DNA encoding for green fluorescent protein when compared to the more toxic fifth-generation dendrimers conventionally used for gene delivery. The mesoporous silica offered superior protection of the DNA from nuclease degradation and also ensured better cell internalization with reduced cytotoxicity [98]. Yet another report describes glutathione-responsive hollow manganese mesoporous silica particles with large pores containing *si*-RNA for modulating p-glycoprotein along with doxorubicin. The glutathione responsiveness was conferred through a disulphide linked poly(beta amino ester) cap over the pores. This system demonstrated the ability to reverse multidrug resistance in cancer cells during in vitro trials [99]. In an interesting study, mesoporous silica modified with an amine-terminated organic moiety known as ormosil was surface modified with fibroblast growth factor receptor-1 (FGFR1). This system displayed excellent transfection efficiency in neuronal cells and also promoted differentiation of the neuronal progenitor cells due to the FGFR1. Brain delivery of the gene was also recorded in mouse models which was comparable to the efficiencies achieved using viral vectors. However, ormosil-mediated gene delivery did not cause toxic effects unlike its viral counterparts [100]. A recent report has highlighted the promise of a poly(ethyleneimine)-conjugated mesoporous silica system for gene delivery applications. This hybrid carrier did not exhibit cell toxicity which is commonly encountered with poly(ethyleneimine). In addition, the high complexation efficiency and endosomal escape characteristics of poly(ethyleneimine) are retained in the hybrid system [101]. Amine-modified mesoporous silica with pore dimensions >15 nm was used to entrap plasmid DNA encoding for green fluorescent protein or luciferase enzyme and was successfully used for transfecting HeLa cells [102]. The pores

protected the plasmid DNA from degradation and also displayed better transfection efficiency when compared with mesoporous structures with small pores, thereby highlighting the importance of pore dimensions.

## 3.15 Gold Nanoparticles

Gold nanoparticles have generated considerable interest for gene delivery applications due to their ease of formation, tunable shape and size, amenability for surface modification through covalent linkages and biocompatible nature [103]. In addition, they also have been demonstrated to exhibit size-dependent luminescence due to surface plasmon resonance and produce heat when exposed to specific wavelengths of electromagnetic radiation [104]. Studies have also shown that gold nanoparticles display excellent cell internalization properties [104].These diverse range of properties have made gold nanoparticles a sought after system for drug and gene delivery applications both independently and in combination with other carriers to augment their effectiveness as a delivery vehicle. The near-infrared (NIR)-responsive nature of gold nanoparticles has been exploited for gene delivery where gold nanorods complexed with DNA were triggered to release their cargo upon stimulation by NIR [105]. This opens up new avenues for stimuli-responsive release of the complexed DNA from gold nanorods through laser light of specific wavelengths. The shape and size of the gold nanoparticles alter its absorption wavelength. This property has been employed for smart release of specific DNA from gold nanorods of different sizes. Two different DNA sequences were conjugated to gold nanorods of different lengths [106]. The shorter nanorods responded to wavelength of 800 nm, and the longer nanorods were responsive towards 1100 nm. When a mixture of these nanorods were exposed to electromagnetic radiation of 800 nm, only those DNA sequences bound to the shorter rods were released, and for the release of the DNA complexed with the longer rods, irradiation with 1100 nm source was required. Such tunable release gives rise to the possibility of delivery of multiple sequences to the target cell/tissue using different irradiation wavelengths. The photothermal properties of the gold nanoparticles have also been explored for multimodal cancer therapy by several groups [104].

One of the major limitations of gene delivery systems is their inability to escape the endosome. To overcome this limitation, citrate-capped gold nanoparticles conjugated with PEG and *si*-RNA through thiol links were covalently modified with hydroxychloroquine by esterification [107]. This system displayed superior endosomal escape properties due to proton sponge effect when compared to the particles without hydroxychloroquine. A layer-by-layer self-assembly of the cationic poly(ethyleneimine) (PEI) and the charge reversal polymer poly(allylamine) functionalized with cis-aconitic anhydride were employed over gold nanoparticles for delivery of oligonucleotides [108]. The PEI facilitated endosomal escape through proton sponge mechanism, while the allylamine enabled the release of the oligonucleotide through charge reversal in the cytosol. This system was evaluated in vitro using HeLa cells for transfection of green fluorescent protein (GFP) and luciferase

in a proof-of-concept study. Amino acid-functionalized gold nanoparticles were also used as a substrate for complexing *si*-RNA [109]. It was found that cationic amino acids like lysine promoted transfection while glycine modification was ineffective. Similar results were also obtained when the *si*-RNA was complexed to gold nanoparticles surface modified with lysine and generation 1 (G1) dendrimer containing amine groups. Gold nanorods linked with G1 PAMAM dendrimer modified with thiol groups were used to deliver sh-RNA against survivin in MCF-7 breast cancer cells. The nanoparticles accumulated in the target cells within half an hour and released the sh-RNA on irradiation with near-infrared radiation. The gold nanoparticles conferred the photo-responsiveness, while the dendrimer enabled complexation, internalization and endosomal escape [105]. In a seminal work reported recently, five different *si*-RNA sequences were linked to gold nanoparticles along with an aptamer sequence for site-specific delivery [110]. The target sequences were BAG1, MDM2, Bcl-2, survivin and XIAP, while the aptamer was designed with specific affinity towards CD33 and CD34. The penta-*si*-RNA complexed gold nanoparticles were co-administered with doxorubicin and evaluated for their therapeutic efficacy against acute myeloid leukaemia cells (AML-M2). The effect of the combination was compared with naked gold nanoparticles and free doxorubicin. The combination of gene silencing and chemotherapy offered superior therapeutic efficacy when compared to the other combinations. A generation 5 (G5) dendrimer with amine terminals conjugated with gold nanoparticles was used to complex plasmid DNA for GFP [111]. It displayed nearly 100 times greater transfection than their counterparts without gold nanoparticles. The authors attribute this improvement to the gold nanoparticles which facilitated better interactions between the dendrimers and oligonucleotides.

## 3.16 Magnetic Nanocarriers

Magnetic nanoparticles respond to an external magnetic field that can be used to guide the nanoparticles to the desired location. In addition, in the presence of an alternating magnetic field, the magnetic nanoparticles get heated up which can be used to increase the temperature of the environment where the magnetic nanoparticles are localized. When the magnetic field is switched off, the remnant magnetism present in the particle may lead to variations in its mobility and interactions within the physiological *milieu*. The size of the magnetic nanoparticle therefore becomes critical. Magnetic nanoparticles below 20 nm exhibit a property known as 'superparamagnetism' where the particle tends to magnetize instantaneously in the presence of a magnetic field and demagnetizes immediately on the removal of the field. There is no discernible hysteresis recorded for these particles. This property is beneficial for biomedical applications as instantaneous response to the applied magnetic field, and immediate capture or release of magnetic nanoparticles can be achieved by controlling the applied magnetic field. Introduction of exogenous genes into the cell employing an external magnetic field and magnetic nanoparticles has been referred to as 'magnetofection'. Repeated application of magnetic field to

enhance gene expression has also been reported, and this method is denoted as 'multifection' [112]. Magnetic iron oxide ($Fe_3O_4$) nanoparticles coated with silica were grafted with ethanolamine containing poly(glycidyl methacrylate) and used to complex plasmid DNA encoding for the reporter gene luciferase. The carrier exhibited better internalization and transfection in the presence of an applied magnetic field. On application of an alternating external magnetic field, the magnetic nanoparticles undergo hysteresis leading to the release of heat which in turn enhances permeability and release of the genetic cargo from the polymer shell. In addition, the ability of the iron oxide nanoparticles to influence the relaxation of neighbouring water molecules has been beneficially used to enhance the negative contrast of the magnetic resonance image of the tumour tissue, thereby enabling simultaneous visualization of the nanoparticles in hepatoma cells both in vitro and in vivo [113].

Magnetic nanoparticles have also been explored for transfection of neurosphere suspensions through multifection approaches for long-term expression of the transgene [112]. Ethylenediamine- and spermine-modified pullulan incorporating iron oxide magnetic nanoparticles was used for gene complexation. This magnetic-gene complex was found to be effective in transfection of the gene of interest into mesenchymal stem cells with higher efficiency in the presence of applied magnetic field. The complex also exhibited better serum stability and did not adversely affect the proliferation and differentiation ability of mesenchymal stem cells [114]. Magnetic nanoparticles have also been employed in combination with polymers for improving the transfection efficiency and target specific and triggered release of the oligonucleotide. Plasmid DNA encoding for luciferase was complexed in the cationic layer of PEI-poly(caprolactone) containing iron oxide nanoparticles. The magnetic polyplex system responded to external magnetic field and exhibited better transfection when compared with the polyplex alone. This indicates that the magnetic stimulus promotes nuclear internalization of the plasmid DNA that may be invaluable for gene therapy [115]. Indeed, several reports have also demonstrated that magnetic stimulus circumvents the hypoxic barrier that commonly limits the efficiency of conventional delivery systems, and therefore the transfection effect is not limited to certain locations but is realized throughout the target tissue [116]. The ease of preparation and surface modification of magnetic nanoparticles have made them attractive candidates for gene delivery applications. However, it should be ensured that the surface modifications do not lead to excessive masking of the magnetic properties. Very low magnetization values necessitate application of very high magnetic fields which are not practically feasible in biological systems due to alteration of the rheological parameters of the blood [117].

## 3.17 Stimuli-Responsive Carriers

Gene delivery systems have to complex oligonucleotides with high affinity to ensure their stability in circulation. At the same time, the complex has to dissociate in the intracellular environment. These two diverse properties are difficult to be integrated in the same system [118]. This requirement has resulted in the emergence of

stimuli-responsive carriers for gene delivery. Triggered release of therapeutic agents has attracted much attention in recent years as the amount of therapeutic cargo released can be tuned according to the need. In this context, several attempts to tailor the release of oligonucleotides from nanocarriers have been reported in the recent literature. Both internal stimuli, such as pH, molecules, ions, etc., and external stimuli like temperature, magnetic field and electric field have been explored for controlled release of the oligonucleotide.

pH-responsive systems have been widely explored for gene delivery. As the carriers employed for complexing the oligonucleotides are generally charged, it is evident that their charge status can be altered with varying pH. Some of the extensively investigated pH-responsive nanocarriers include cationic liposomes, chitosan, poly(ethyleneimine) (PEI), polylysine and polyketals [119]. These carriers have amine functionalities that become protonated in acidic pH leading to swelling or specific interactions with the membranes. These interactions in turn favour endosomal escape through membrane perturbation or proton sponge mechanism which augurs well for gene therapy. A salicylamide-grafted PEI polyplex was employed for gene delivery. The modified PEI self-assembled at physiological pH to form micelles that enhanced the nucleic acid stability and dissociated in the intracellular milieu when exposed to acidic pH in the endosomes, thereby releasing the nucleic acid [120]. Though the therapeutic gene transfected in this study targeted Duchenne muscular dystrophy, the same platform can be useful for cancer therapy too. In another study, lipoplexes containing *si*-RNA against polo-like kinase (PLK) were surface modified with activatable cell-penetrating peptide containing an octaarginine sequence linked with glutamate and histidine sequence through hydrazone linker [121]. This modified lipoplex when incubated with MCF-7 breast cancer cells and A549 lung cancer cells was found to exhibit pH-responsive lysis of the hydrazone bond, protonating the histidine residues that favoured fusion with the endosomal membrane components mediating the release of the *si*-RNA. Acid-responsive designer peptides introduced in PEI-DNA complex have been found to enhance the transfection efficiency of plasmid DNA encoding for luciferase gene [122]. The acid-responsive peptide was derived by replacing the cationic residues in the antimicrobial peptide derived from melittin by glutamate residues that became protonated in acidic pH, thereby altering its charge status. The glutamate residues augmented the proton sponge mechanism of PEI and facilitated the endosomal escape of the plasmid DNA.

Many studies have explored chitosan as pH-responsive delivery system due to its amine groups that switch between a charged protonated state and an uncharged deprotonated state. Chitosan nanoparticles tagged with antibodies against transferrin and bradykinin B2 have been successfully employed for delivering *si*-RNA across the blood-brain barrier and specifically endocytose into astrocytes. The pH-responsive nature of chitosan favours endosomal escape and resulted in the inhibition of HIV replication [123]. An interesting strategy attempting to mimic the efficiency of viral vectors to escape the endosome was reported recently. The ‘virus-inspired polymer for endosomal release (VIPER)’ system was designed using poly(oligo(ethylene glycol) monomethyl ether methacrylate)-co-poly(2-(dimethylamino)ethyl methacrylate)

[p(OEGMA-DMAEMA)], a cationic segment for complexing the oligonucleotide and a pH-responsive segment comprising poly(2-diisopropylaminoethyl methacrylate)-co-poly(pyridyl disulphide ethyl methacrylate) [p(DIPAMA-PDSEMA)] that transformed from a hydrophobic to hydrophilic state at pH 6.3 [124]. The pore-forming peptide from bee venom, melittin, was grafted to the pH-responsive segment via disulphide linker. The oligonucleotide cargo used was plasmid DNA encoding for luciferase gene in the in vivo studies and for green fluorescent protein in the in vitro studies. The system self-assembled to a micellar form with the hydrophobic melittin present in the core. In the acidic environment of the endosome, the DSEMA segment underwent a transition from hydrophobic to hydrophilic revealing the melittin to the external environment. Melittin inserts into the endosomal membrane resulting in the disruption of the membrane. The system exhibited superior transfection efficiency when compared with the peptide-free system and PEI-based system in different cancer cell lines as well as in mouse models injected with KB tumour cells. The advantage of this system over other melittin-based gene delivery systems is reduced toxicity to normal cells. As melittin is masked in the core at physiological conditions and is exposed only in the acidic endosomal pH akin to protein VI in adenoviral systems, this system has lower adverse effects when compared to other melittin-containing systems.

Polyketals belong to a class of pH-responsive polymers that degrade into neutral by-products at acidic pH. These by-products are easily metabolized and do not cause any inflammatory or adverse response when compared with polyesters that result in acidic by-products [125]. The hydrolysis rate of the polyketals can be tailored based on the type of diol employed for the synthesis of the polyketal [126]. The pH responsiveness and benign nature of the polymer have been explored for gene delivery applications. In a seminal work, polyketal nanoparticles were successfully employed to deliver the *mi*-RNA PK3-*miR* for reprogramming bone marrow-derived mononuclear cells into induced pluripotent stem cells [127]. This work opens up numerous vistas for generating stem cells from blood cells for therapeutic applications. Polyketal-mediated delivery of *si*-RNA to silence the expression of nitric oxide synthase (Nox2) gene in macrophages as well as in ischemic heart tissue of mice post infarction has been found to promote better recovery due to abrogation of the inflammatory environment [128].

Temperature-responsive polymeric and liposomal systems have been explored for stimuli-responsive release of therapeutic agents including oligonucleotides. Poly(N-isopropyl acrylamide) (PNIPAM) is the most widely employed thermo-responsive element in nanocarriers. This polymer exists in the swollen state at temperatures below its critical solution temperature (CST) which lies around 32 °C, while it transforms into a gel phase above this temperature. Copolymerization of PNIPAM with other polymers alters its critical solution temperature. In a remarkable demonstration of the effectiveness of temperature-responsive gene delivery, PNIPAM was copolymerized with N,N′-dimethyl ethylamine methacrylate and n-butyl methacrylate [118]. The amine moiety conferred cationic character for complexing the plasmid DNA encoding for β-galactosidase, while the n-butyl moiety imparts hydrophobic nature that causes a negative shift of the CST of PNIPAM to

21 °C. When COS1 cells were incubated with this nanocarrier at different temperatures, it was found that incubation at 37 °C for 20 h followed by incubation at 20 °C for 3 h and finally at 37 °C for 24 h displayed the highest transfection efficiency when compared with those cells that were incubated at 37 °C for 47 h and those that were initially incubated at 20 °C for 3 h and then at 37 °C for 44 h. The incubation at 37 °C ensures internalization of the carrier, but as the incubation temperature is far higher than the CST of PNIPAM, it remains in the gel phase preventing the release of the plasmid DNA. However, when the cells were incubated initially at 20 °C, it retards the internalization of the carrier and promotes the release of the plasmid in the extracellular medium which is reflected in the low transfection efficiency. Initial incubation at 37 °C ensures that PNIPAM will be in the gel form and hence the complex stability is intact, and internalization into the cell is not compromised. Once inside the cell, when the temperature is reduced to 20 °C, that is, well below the CST of PNIPAM, hence it transforms to the sol state leading to the dissociation of the complex and release of the plasmid DNA. When the temperature is then increased to 37 °C, transport of the plasmid into the nucleus and subsequent transfection occur. In order to confirm that the increased transfection was due to the enhanced release of the plasmid DNA from the sol phase, the temperature in the second phase was changed from 20 °C to 25 °C which is above the CST of PNIPAM; the transfection efficiency was reduced. This proof-of-concept study clearly demonstrates the potential of temperature-responsive release in gene therapy. However, it is imperative that the temperature range to which the carrier responds is in the practically feasible range when applied in vivo.

Apart from pH and temperature, novel intrinsic stimuli have also been explored for gene delivery applications. Alkylated poly(ethyleneimine) was conjugated to 2-nitroimidazole and was employed for delivering *si*-RNA against survivin and luciferase enzyme in the mouse breast cancer cell line 4 T1 [129]. The system self-assembled to form a micelle in aqueous medium. Under hypoxic conditions, the hydrophobic 2-nitroimidazole was reduced to form the water-soluble 2-aminoimidazole which results in the dissociation of the micelle. As hypoxia is a hallmark of all solid tumours, this strategy may serve to be effective in treatment of most solid cancers. The *si*-RNA was complexed with the poly(ethyleneimine) that facilitated endosomal escape through the proton sponge mechanism. Both in vitro experiments performed in 3D spheroids and in vivo experiments in Balb/c mice model revealed that this carrier exhibited *si*-RNA release in hypoxic conditions.

Enzyme-responsive nanocarriers offer a versatile platform for triggered release of the therapeutic cargo in the target cells. In the context of gene delivery, a cationic liposomal system encapsulating a plasmid DNA encoding for luciferase enzyme was developed [130]. Apart from the cationic lipid 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), the lipid carrier also contained the fusogenic lipid dioleoylphosphatidyl ethanolamine (DOPE) and polyethylene glycol (PEG)-modified lipid that contained the peptide sequence GFLG which is recognized by the enzyme cathepsin B. The lipid nanoparticles on internalization into the cell entered the endosome where the GFLG was cleaved by cathepsin B which destabilized the lipoplex. The fusogenic DOPE perturbed the endosomal membrane leading to the release of the

plasmid DNA into the cytoplasm. The enzyme-responsive system displayed 100-fold increase in the transfection efficiency when compared with the unmodified carrier indicating its potential in cancer gene therapy. Another study reports a polyacrylate copolymer of ethoxyethylamine and secondary butyl acrylate modified with a PEG-phenylalanine-citrulline-p-aminobenzyloxycarboxyl moiety [131]. The *si*-RNA against factor VIII – a blood clotting agent released by hepatocytes in the cancerous state – was conjugated through a disulphide linked to the polymer. N-acetylgalactosamine (NAG) linked to maleic anhydride was employed as the targeting moiety for specific localization in hepatocytes. The NAG ensured internalization into liver cancer cells through endocytosis. Once inside the endosome, the acidic pH resulted in the cleavage of the maleamate to form maleic acid and the amine groups in the polymer become free. The cathepsin B in the endosome cleaves the phenylalanine-citrulline link leading to the release of PEG that prevented the endosomal membrane interactions. Both events lead to the exposure of the amine groups of the polymer to interact with and disrupt the endosomal membrane. The reduced glutathione in the cells cleaves the disulphide bond resulting in the release of the *si*-RNA into the cytosol that resulted in effective silencing both in vitro and in vivo. The system was found to be very stable in circulation, and this coupled with the superior silencing efficiency makes this multi-stimulus-responsive system a promising candidate for gene therapy applications.

Ultrasound-mediated gene delivery (UMGD) also known as sonoporation has garnered much attention in the past couple of decades due to its unique advantages. These include spatially and temporally controlled release of the gene and increased blood vessel permeability. In addition, the application of ultrasound results in generation of free radicals that may further exert cytotoxic effects on the cancer cells [132]. Experiments had revealed that the administration of cationic microbubbles decreased the cavitation threshold significantly both in vitro and in vivo and reduced the dose of DNA required for the same effect when compared to free DNA and those co-delivered along with neutral microbubbles [133]. The microbubbles are gas-filled structures that implode rapidly even on application of low-intensity ultrasound. To improve the target-specific accumulation of the microbubbles in C6 glioblastoma multiforme cells, folic acid-modified cationic microbubbles were employed [134]. The targeted system performed better than the free plasmid DNA, lipoplex and unmodified microbubble groups. Bubble liposomes modified with AG73 peptide derived from laminin were found to be effective in transfecting plasmid DNA selectively into the human umbilical vein endothelial cells (HUVECs). The advantage of these systems was that application of ultrasound induces cavitation in both the unassociated liposomes and the membrane-bound AG73 liposomes resulting in better transfection efficiencies when compared with conventional bubble liposomes and naked plasmid DNA [135]. Nanobubbles have better internalization properties than microbubbles owing to their size advantage. Chitosan nanobubbles complexed with plasmid DNA encoding for green fluorescent protein exhibited superior transfection efficiency in COS9 cells only when insonated and remained ineffective in the absence of ultrasound [136]. In another study, plasmid DNA for β-galactosidase was complexed with cationic poly(ethyleneimine), and

these nanoparticles were co-administered with anionic PLGA bubbles in prostate cancer-bearing nude mice. The ultrasonication triggered the release of the plasmid DNA with an eightfold higher efficiency when compared with the unsonicated samples [137]. Adenoviral delivery systems have also been entrapped in lipid-encapsulated perfluorocarbon microbubbles for transfection of athymic mice with transgenes encoding for p53, Rb, Rb2 (retinoblastoma genes) and Mda7/IL-24 (an anticancer gene of IL-10 family) [138]. The application of ultrasound enabled good transfection efficiency, while the complexation of the viral particle with the microbubbles reduced undesirable immune responses. Transfection was found to be maximum in the cells at the site of sonication. Ultrasound-mediated gene delivery is influenced by the intensity, frequency and pulse duration. In addition, the lag time between injection and insonation also determines the transfection efficiency. Though the transducer design can be modified to alter the focus area of ultrasound, it is now known that different tissues absorb ultrasound to various magnitudes. This leads to variations in the transfection efficiency. Despite these challenges, ultrasound-mediated gene delivery offers promise for site-specific and triggered release of the oligonucleotide cargo.

Photo-responsive systems have been explored for drug delivery applications, but not many instances have been reported for gene delivery. In a recent report, a peptide-based system has been developed for photo-switchable conformational changes that alter the binding affinity of the peptide to the oligonucleotide. The cationic peptide PVBLG-8 containing benzyl ester groups was conjugated to the uncharged and hydrophobic peptide segment 4,5-dimethoxy-2-nitrobenzyl glutamate (DMNBLG). The peptide sequence complexed DNA due to the cationic residues in PVBLG-8. When irradiated with light, cleavage of the ester moiety in DMNBLG occurs resulting in the formation of anionic carboxylate groups. The electrostatic attraction between the oppositely charged segments in the peptide distorts the helical conformation as well as destabilizes the peptide-oligonucleotide complex resulting in the release of the oligonucleotide. This photo-responsive system was successfully demonstrated to release its gene cargo in the target cells when exposed to specific wavelength of light. The light-induced switching of the oligonucleotide-binding affinity could bring about selective release of the cargo in the target site alone [139].However, the reported system responds to ultraviolet wavelengths and hence may not be clinically relevant owing to the poor penetration of ultraviolet radiation. Attempts to shift the wavelength that trigger to the more acceptable near infrared are underway, which if successful can represent a new paradigm in stimuli-responsive gene delivery.

The fact that both DNA and the carriers are charged indicate the possibility of employing electric field for eliciting a response from these entities. Electrical stimulus has been explored in the field of gene delivery for many decades and is commonly referred to as electropermeabilization or electroporation. In an earlier report, it was demonstrated that when the skeletal muscle was electrically stimulated, the transfection efficiency of plasmid DNA administered via intramuscular route in rats was increased [140]. This increase was due to the permeabilization of the muscle membranes by the applied field. Combining the benefits of nanocarriers and

electroporation offers a promising strategy to bring about site-specific transfection. A cationic PEGylated gold nanoparticle complexed with plasmid DNA was found to exhibit increased expression of the gene in the liver lobe that was subjected to electric pulses [141]. The application of the electric field causes redistribution of the charges across the membrane, and hence the membrane polarity and permeability characteristics are altered. It has also been found that above a particularly applied potential that just exceeds the dielectric strength at the junctions of cells, cell fusion accompanied with membrane deformation can occur, which can promote exchange of materials between the cells [142]. Though electric pulses have been widely employed during in vitro studies, the feasibility of employing such stimuli for in vivo requires extensive optimization of various parameters that include the strength, frequency and duration of electric pulse apart from the cell type and size. Moreover, accessibility of the target tissue to the electric stimuli is another challenge in clinical translation of this strategy.

The field of stimuli-responsive delivery has now become more complex with the development of nanocarrier systems that respond to multiple stimuli. A dendrimeric system that responded to two different types of molecular stimuli has been recently reported. Vascular endothelial growth factor (VEGF) was silenced using *si*-RNA that was delivered to cancer cells using a system comprising PAMAM dendrimer linked with arginine-conjugated poly(cystamine-bisacrylamide-diaminohexane) [62]. The *si*-RNA release was triggered by the lysis of the disulphide bond by thiol functionalities such as dithiothreitol. In addition, the same work demonstrated the release of the VEGF *si*-RNA by competitive displacement from the cationic dendrimer by the more anionic heparin. The dendriplex displayed low cytotoxicity and more effective silencing of VEGF when compared with conventionally employed poly(ethyleneimine) against three different types of cancer cells. In a novel attempt to decrease the expression levels of survivin in pancreatic cancer, a polyethylene glycol-based hyperbranched dendrimer was synthesized for co-entrapment of paclitaxel and the *si*-RNA against the nuclear receptor target TR3 [143]. The redox stimuli system released its therapeutic cargo only in PanC-1 pancreatic cancer cells where a large content of reduced glutathione was present. In another attempt also involving a dendrimeric carrier, a PAMAM dendriplex cross-linked by the peptide sequence GFLG was developed. This sequence served as the substrate for cathepsin B enzyme. The dendrimer was also modified with the basic histidine-arginine dipeptide to promote endosomal escape by the proton sponge mechanism. The system demonstrated enzyme-specific release of plasmid DNA only in the target HeLa cells, thereby avoiding adverse effects due to off-targeting [144].

Ammonium salt modified cationic mesoporous silica with conical pores and two stimuli-responsive linkages that were lysed in the presence of high concentrations of reduced glutathione and in acidic pH was developed for gene silencing applications [145]. The system was found to successfully silence in HeLa cells, the expression of indoleamine-2,3-dioxygenease 1, an over-expressed target in many cancers. The study also showcased the ability of the carrier to deliver plasmid DNA by demonstrating the simultaneous delivery of pDNA encoding GFP as well as the *si*-RNA against the GFP into 293T cells. In an interesting strategy, a novel polymeric system

comprising of phenylboronic acid-modified poly(ethyleneimine) conjugated through an ester bond with the catechol-conjugated poly(ethylene glycol) [146]. The phenylboronic acid was also found to interact with the *si*-RNA through the hydroxyl groups in the ribose sugar. These linkages conferred stability to the polyplex in serum and reduced off-targeting as the poly(ethylene glycol) chain retarded cell internalization. However, in the tumour microenvironment which is more acidic, the borate ester link between phenylboronic acid and catechol cleaves releasing the poly(ethylene glycol) chain. This enables internalization of the polyplex into the cancer cells. Once inside the endosomal compartment, the highly acidic pH cleaves the phenylboronic acid-*si*-RNA ester bond resulting in its release into the cytosol. The proton sponge mechanism of poly(ethyleneimine) facilitates the endosomal escape of the *si*-RNA. The phenylboronic acid enabled specific recognition of the sialic acid residues over-expressed on the surface of lung and liver cancers. The carrier was found to successfully deliver *si*-RNA against survivin specifically to 4T1 cells both in vitro and in Balb/c mice models.

Magnetic stimuli have been extensively employed for triggered release of oligonucleotide in different cells both independently and in combination with several other stimuli. Oleic acid-capped iron oxide nanoparticles were coated with thermoresponsive and pH-responsive N-isopropyl acrylamide-acrylic acid derivative that was used to entrap short hairpin RNA against alpha-synuclein and neural growth factor. The system responded to temperature, pH and magnetic stimuli and exhibited significant reduction in the alpha-synuclein levels in Parkinson's diseased animal model. Consequently, dopaminergic neuron numbers significantly reduced, and an improvement in the gait of the treated animals was reported. Prussian blue staining revealed that the nanoparticles exhibited greater localization in the brain tissue when compared with other organs [147].

A dual-stimuli-responsive system that responds to both photo- and redox stimuli has been reported recently [148]. The system employed poly(cyclodextrin) conjugated to cationic azobenzene through a disulphide bond. While the disulphide bond responds to the reduced glutathione levels in the cells, the presence of azobenzene confers photo-responsiveness due to its ability to undergo cis-trans isomerization when irradiated with ultraviolet radiation. The nanocarrier was found to successfully deliver plasmid DNA encoding for luciferase enzyme to HeLa cells, HepG2 hepatocellular carcinoma cells and HEK293T cells. Though the system displayed lower toxicity than poly(ethyleneimine) without compromising the gene delivery characteristics, the use of UV as the stimuli may limit the applicability of this system for deep-seated tumours due to penetration issues. Recently, a chitosan-based composite system has been reported for multimodal cancer therapy. Magnetic mesoporous silica nanoparticles were loaded with the photosensitizer chlorine and chemotherapeutic agent doxorubicin. The surface of the nanoparticles was modified with adsorbed alternating layers of chitosan and alginate that were used to complex short hairpin RNA (shRNA) targeting the multidrug-resistant gene p-gp. The system displayed pH-responsive release of the shRNA that served to reverse the multidrug resistance and also resulted in the release of the photosensitizer and chemotherapeutic agent leading to high levels of cytotoxicity both in vitro and in vivo [149].

## 3.18 Peptide Carriers

Peptide sequences have been employed as surface-modifying agents to impart specific characteristics such as cell penetration or site specificity to nanocarriers. For instance, sorbitan monooleate nanoparticles modified with polyarginine were employed for delivering plasmid DNA encoding for the pre-mRNA processing factor gene PRPF31 for treatment of complications arising due to retinitis pigmentosa. The system exhibited excellent internalization into the retinal cells due to the polyarginine sequence on the surface and improved retinal function [150].

A new class of nanocarriers known as multifunctional envelope-type nanodevice (MEND) have emerged for gene delivery applications. These systems primarily comprise of a nucleotide complex with cationic polymers or lipids that are surface modified with fusogenic lipid components, targeting ligands, poly(ethylene glycol) for enhanced residence time and cell penetration [151]. Usually, octaarginine residue is employed as the peptide component in MEND as it uses a non-endosomal pathway to enter the target cell [152]. Lipidated sorbitol framework was used to develop a series of molecules that can be employed as surface modifiers in MENDs or independently as gene complexing molecules based on their acyl chain lengths [153]. Peptides have also been employed independently as gene delivery systems as they are biodegradable and can be tailored to impart unique properties favourable for efficient transfection.

Peptides from natural origin as well as synthetic peptides have been employed for gene delivery applications. Synthetic peptides offer the versatility of introducing domains for specific functions to augment the complexation and target specificity of the sequence. The cationic peptide sequence RALA was employed to complex the plasmid DNA encoding for iNOS (inducible nitric oxide synthase) to treat aggressive form of breast cancer. The choice of RALA was based on its cationic nature that enabled complexation with the plasmid DNA as well as its fusogenic character that facilitated cell internalization and endosomal escape. The peptide-DNA complex exhibited good transfection efficiency as was evident from the cytotoxicity to cancer cells due to excess levels of nitric oxide generated in the transfected cells. In addition, the gene delivery sensitized the cancer cells to the cytotoxic effects of the chemotherapeutic agents taxol and cisplatin [154]. Similarly, another endosome-disrupting peptide sequence GALA was incorporated in a sequence containing dendronic lysine residues for complexing *si*-RNA and the virus-derived cell-penetrating *tat* peptide sequence. This designer peptide was further modified by conjugation of stearic acid moiety that promoted greater membrane interactions of the complex resulting in better cellular uptake and silencing efficiency when compared with free *si*-RNA and those complexed with non-stearylated peptides [155]. In a similar approach, stearylated peptide comprising *tat* for internalization and an endosomal escape sequence $(LLKK)_3$ or $(LLHH)_3$ apart from the stearyl group was designed for gene delivery. The endosomal escape sequence was flanked by cysteine residues that improved stability and maintained alpha-helical content required for internalization. The L-amino acids were substituted by their D counterparts to protect the peptide from enzymatic degradation. The peptide with histidine residues complexed

with plasmid DNA exhibited the best transfection efficiency. This is because the imidazole groups of histidine contribute to endosomal escape through the proton sponge mechanism. The peptides did not show any adverse effects on normal cells indicating the potential of such designer systems for gene delivery [156]. It is evident from these studies that peptide amphiphiles with components for improved complexation, cell internalization and endosomal escape can be effective gene delivery systems.

The self-assembled structure obtained from two branched peptide amphiphiles bis(FLIVIGSII)-K-K4 and bis(FLIVI)-K-K4 was used to complex DNA encoding for an oncoprotein of human papilloma virus (HPV). The peptide-DNA complex was employed as a DNA vaccine to treat tumour induced by HPV through immunomodulation. The complex activated dendritic cells in mice models revealing the efficacy of the gene delivery by the peptide system [157]. Cationic peptides have been traditionally employed as cell-penetrating agents due to their ability to endocytose readily, perturb the membrane architecture and form pores [158]. However, due to their short sequences, such peptides have insufficient binding associations with the oligonucleotides, thereby compromising the stability of the complex. To overcome this limitation, a branched polyarginine sequence CRRRRRRRRRCRRRRRRRRRC having disulphide links at the branching points was used to complex plasmid DNA encoding for green fluorescent protein as well as *si*-RNA against vascular endothelial growth factor (VEGF). The complexes exhibited good stability both in physiological pH and in the presence of serum proteins. In vitro studies using different cancer cell lines revealed that the complexes exhibited good internalization and transfection efficiency. The disulphide links are cleaved in the intracellular environment leading to better transfection. In vivo biodistribution studies showed that the complexes were found to accumulate in the tumour tissue due to enhanced permeation and retention (EPR) [159].

A novel 30-mer peptide was obtained through fusion of a sequence derived from the cytochrome c oxidase of yeast with a series of nine lysine-histidine pairs. This fusion peptide referred to as Cytcox-KH was amphipathic in nature and displayed excellent transfection of plasmid DNA encoding for green fluorescent protein in the mitochondria of HEK293 cells. The transgene expression was stable and was discernible at 24 h and up to108h after transfection, but the magnitude of expression gradually declined with time as the cells proliferated. Structural elucidation of the peptide-plasmid DNA complex using spectroscopic tools revealed that the KH domains loosely complexed with the DNA enabling its release in the intracellular *milieu*. The hydrophobic Cytcox sequence in the peptide retained its α-helical conformation even after complexation with DNA, and this property along with its hydrophobic nature contributed to mitochondrial localization of the peptide-DNA complex [160]. Recombinant peptides containing sequences derived from the DNA condensing domains of histone H1 protein and a fusion domain derived from the HIV glycoprotein 41 and SV40 large T-antigen NLS were engineered for delivery of plasmid DNA to target cells. The histone-derived sequences contained abundant lysine residues that enabled complexation of the plasmid DNA. The glycoprotein 41-derived motif enabled endosomal escape, while the NLS sequence from SV40

facilitated the transfer of the plasmid DNA from cytosol into the nucleus. The results revealed that the structure and spatial orientation of the various domains exert a major influence on the transfection efficiency [161]. All the recombinant proteins were non-toxic. Further studies on the immunogenicity of these proteins and in vivo stability are yet to be performed to assess the true potential of these classes of gene delivery systems. Two cell-penetrating peptides pepM and pepR derived from the capsid protein of the dengue virus that were employed to complex *si*-RNA as well as interfere with the signaling pathways in myeloid leukaemia cells have been reported [162]. The peptide pepM inhibited the expression of Bcr-Abl1 gene for 120 h and also inhibited associated pathways. The peptide was shown to inhibit the expression of cytoskeletal elements, thereby augmenting the anticancer effects brought about by gene silencing. Such multifunctional delivery systems could well represent the next generation of smart delivery systems.

Other than peptides, proteins also have been employed as gene carriers. Gelatin is a protein that has been extensively employed for drug and gene delivery applications due to its biocompatibility, biodegradability and amenability for functionalization [163]. Gelatin modified with cationic components such as ethylenediamine, spermidine and spermine was used to complex plasmid DNA encoding for luciferase. The system exhibited superior internalization into the cells and consequently exhibited better transfection efficiency when compared with free plasmid DNA [164]. On similar lines, gelatin was transformed to a more cationic moiety through conjugation of cholamine on the surface. This modified gelatin was used to successfully deliver *si*-RNA against AEG-1 gene that has been identified to trigger metastasis in MCF-7 breast cancer cells [165]. To improve the residence time of the carrier, PEGylated gelatin nanoparticles have been employed for delivering plasmid DNA encoding for β-galactosidase to Lewis lung carcinoma cells. The encouraging results obtained in vitro and in vivo suggest the potential of such systems for treatment of solid tumours [166].

Nanoparticulate delivery has also been employed to augment the effects of gene therapy in cell lines. In a proof-of-concept study carried out in lung epithelial cells, the prodrug 5-fluorocytosine was delivered over an extended period of time using glutaraldehyde-cross-linked albumin nanoparticles into transformed cells expressing the suicide genes cytosine deaminase (CD) and uracil phosphoribosyl transferase (UPRT). While CD transformed 5-fluorocytosine to the cytotoxic 5-fluorouracil, the UPRT enzyme converted 5-fluorouracil into 5-fluorodeoxyuridine monophosphate, which in turn interfered with DNA synthesis by inhibiting thymidylate synthase enzyme. In cells where this enzyme is absent, 5-fluorouracil is converted to the non-toxic β-alanine. Albumin is an amphipathic protein that is easily degraded in the physiological environment and is well tolerated by the biological systems and hence was used as the vehicle for delivering the prodrug in the study [167]. This approach employing nanodelivery systems results in better therapeutic outcome in suicide gene therapy against cancer and in the future may evolve into an integrated delivery system for sustained release of the prodrug and the suicide gene components for transfecting target cells.

Peptides and nucleic acids are capable of self-assembly, and these self-assembled structures exhibit different surface charge and stability in serum that influence their

gene delivery efficiency. In a novel approach, four arginine residues were conjugated with a palmitic acid moiety and a tetraphenylethene. The modified peptide denoted as TR4 complexed plasmid DNA through the short arginine sequence and formed short fibre-like structures due to the hydrogen bonding and π-π stacking interactions. The tetraphenylethene moiety displayed highly intense fluorescence in the aggregated state, thereby enabling visualization of the gene delivery system using confocal microscopy. The system exhibited better transfection efficiency in different cell lines including stem cells when compared with PEI [168]. Further studies on the safety and biocompatibility of this modified peptide are warranted for exploring its potential in a clinical setting.

## 3.19 Hybrid Nanocarriers: An Emerging Paradigm

Several attempts have been made to create new carriers through integration of the positive aspects of two different carrier molecules towards gene delivery applications. Hybrids of dendrimers with liposomes have been reported to enhance the complexation efficiency while simultaneously reducing toxic effects [60]. This is because the hybrid tends to accumulate more in the tumour due to enhanced permeation and retention. Dendrimer hybrids with quantum dots, gold nanoparticles and magnetic nanoparticles have been reported in literature [169]. Carbon nanotubes linked with PAMAM dendrimers have been found to overcome the aqueous dispersivity of carbon nanotubes. The hybrid system was employed to deliver the *si*-RNA cargo efficiently [170]. Similarly, a composite of magnetic nanoparticles and fifth-generation PAMAM dendrimer was successfully employed to deliver anti-survivin *si*-RNA to breast cancer and liver cancer cell lines [171].The composites exhibited a rapid internalization within 15 min, and a dose-dependent toxicity was recorded in the cancer cells.

Gold nanoparticles were coated on γ-$Fe_2O_3$ magnetic nanoparticles in the presence of polyvinyl alcohol. These composite nanoparticles were found to bind adenovirus efficiently through the formation of thiol links between gold nanoparticles and the cysteine residues found on the surface proteins of the virus [172]. These hybrid nanoparticles displayed excellent cell internalization in response to an external magnetic field. Consequently, the transfection efficiency of these particles assessed through the expression of GFP was superior to that achieved using the adenovirus alone.

Silicon oxide-coated magnetic microbubbles containing lentiviral systems were employed to induce the expression of vascular endothelial growth factor (VEGF) in aortic endothelium through application of ultrasound. The presence of magnetic iron oxide nanoparticles enabled enhanced targeting of the vascular endothelial cells in flow conditions as these particles closely resemble the heme containing erythrocytes. While lentiviruses are known to internalize into the target cells using

the clathrin-mediated endocytosis, the silicon oxide-coated magnetic microbubble system with lentivirus used the caveolae-mediated pathway for internalization [173]. This study clearly demonstrates that the composition of the nanocarrier system influences its transfection, internalization route as well as performance under dynamic conditions.

## 3.20 Cerasomes

Liposomes and silica-based systems have both been explored independently for gene delivery applications. However, both systems suffer from several drawbacks. Liposomal carriers are biocompatible but have a very poor colloidal and chemical stability, while silica nanocarriers are chiefly limited by their rigidity and nondegradable nature. To overcome the drawbacks of these two families of carrier systems, a novel hybrid system known as cerasome has been fabricated. Cerasomes essentially comprise of a lipid bilayer over which a polyorganosiloxane network is coated. The siloxane network confers stability to the underlying lipid bilayer and also facilitates surface modification through the formation of covalent linkages with the molecule being introduced. The liposomal vesicle permits encapsulation of hydrophilic, hydrophobic and amphipathic molecules and also reduces the rigidity of the entire system when compared to pristine silica nanoparticles [174].These cerasomes did not exhibit any reduction in the oligonucleotide complexation ability of the cationic liposomes when compared with the cationic liposomes sans the silica coating.

Cerasomes were employed to transfect HeLa and HepG2 cells with a plasmid encoding a gene for luciferase, and it was found to exhibit higher transfection efficiency than their liposomal counterparts [175]. Moreover, the transfection efficiency of the cerasomes remained intact in serum, whereas the liposomal carrier exhibited a drastic reduction in serum due to poor stability. Similar results have been demonstrated by the same group using *si*-RNA [174]. Recently, cerasomes were immobilized in a solid glass substrate over which HEK293T cells were cultured. The immobilized cerasomes were found to be more efficient transfection agents when compared to the same system in solution. The improved transfection efficiency displayed in the solid phase was attributed to the constant contact of the cells with the cerasome immobilized solid surface [176]. However, the size control of cerasomes has been found to be a critical factor in determining their fusion with cell membranes. Cerasomes with sizes greater than 100 nm have been found to undergo cross-linking, especially at higher concentrations. This leads to a reduction in their ability to internalize into cells. Similar problems have also been encountered when the large plasmids are complexed with cerasomes. The plasmids are found on the outer layer of the lipids and therefore induce cross-linking between the cerasomes, thereby rendering them ineffective [174].

## 3.21 Lipopolyplexes

Lipopolyplexes are hybrid systems that contain lipids and a cationic component that can be a polymer or a peptide for complexing oligonucleotides [177]. Lipopolyplexes integrate the low cytotoxicity of lipoplexes and the high transfection efficiency of polyplexes in a single system. Several types of lipopolyplexes have been explored for gene delivery. These include PEI, poly(L-lysine), poly(2-(dimethylethylamino) ethyl methacrylate) and cationic peptides like K16, K8 and pK17 [177]. For instance, a pH-responsive transferrin-conjugated lipopolyplex system comprising distearoyl phosphatidylethanolamine and protamine was successfully employed to silence ribonucleotide reductase gene in cancer cells [178]. The length of the acyl chain and the degree of unsaturation in the phospholipids have been found to influence the internalization of the lipopolyplex. Unsaturation and carbon chain length of 14 seem to enhance the internalization though the exact mechanism remains to be deciphered. Erythrocyte membrane was employed to encapsulate the cationic polymers PEI and, poly(caprolactone) containing complexed DNA encoding for green fluorescent protein was successfully employed for transfecting different cancer cell lines with minimal cytotoxicity and increased transfection efficiency [179]. All these systems exhibited superior transfection efficiency when compared with the corresponding lipoplexes and polyplexes in vitro. Further trials in vivo are required to confirm the potential of these systems.

## 3.22 Nano-dimensional Polymer-Virus Hybrids

Viral vectors possess their own advantages of high transfection efficiency and excellent endosomal escape that remain unmatched. However, their immunogenicity and risk of infections owing to high frequency of mutations necessitate alternate strategies for delivery of the therapeutic gene. Non-viral vectors have comparatively lower risk but display poor transfection efficiency. Therefore, hybrid systems that integrate the advantages of both classes of vectors have emerged. The viral vectors are encapsulated in polymer nanoparticles for delivery of the therapeutic gene. While the polymer masks the viral vector thereby reducing undesirable interactions in the biological system, the virus ensures endosomal escape and excellent transfection efficiency once inside the cell. As the viruses are masked by the polymer coat, cell-specific internalization will be mediated by the surface modification of the polymer. This may serve to be a benefit or demerit depending on the cell and hybrid nanosystem being investigated. Adenoviruses tend to internalize into target cells through the coxsackie-adenovirus receptors. However, these are not expressed on mesenchymal stem cells, and hence they are ineffective in transfecting the genetic cargo. However, poly(ethyleneimine)-coated adenoviruses have been successfully employed to transfect mesenchymal stem cells owing to the better cell permeability of PEI-based systems [180]. In another work, adeno-associated viruses were successfully delivered through porous silicon microparticles that not only shielded the viral particles from eliciting an immune response but also ensured delivery of large

number of viral particles into the target endothelial cells both in vitro and ex vivo. The promising results from this study serves as a proof-of-concept for future applications involving the delivery of immunomodulatory genes [181]. Other coatings that have been explored include zinc oleate nanoparticles loaded with adenoviral vectors for localized gene delivery in stented arteries [182]. Poly(ethylene glycol) [183] and poly[(N-2-hydroxypropyl)methyacrylamide] nanoparticles encapsulating adenoviruses bypassed the CAR-mediated entry into A549 cancer cells [184] and displayed decreased cytotoxicity in mouse models [185]. The polymer, poly[(N-2-hydroxypropyl)methyacrylamide], was surface modified with epidermal growth factor to mediate site-specific internalization of the adenovirus cargo into ovarian cancer cells [186]. A similar strategy was used to entrap poly(L-lysine)-DNA complexes by poly(N-2-hydroxypropyl)methacrylamide conjugated with transferrin for selective transfection of human leukaemia cells K562 [187]. Though the therapeutic cargo did not involve any viral particles, this study provides proof for transfection of specific cell targets. Similarly, polyketal nanoparticles surface modified with sialic acid were employed to cloak adeno-associated viruses for specific delivery of the gene of interest to $CD22^{+}$ cells [188]. Alternately, external stimulus has also been used for site-specific gene delivery using hybrid vectors. Iron oxide core-shell particles have been used for magnetic guidance of lentiviral vectors into endothelial cells [189]. Though this approach represents a promising option for gene delivery, further studies are warranted to establish the short-term and long-term safety and efficacy of these hybrid systems both in preclinical and in clinical models.

## 3.23 Other Strategies Explored for Gene Delivery

Bacteria have been employed as a non-viral vector for delivering genes to cells – a process referred to as 'bactofection'. *Listeria monocytogenes* has been the most widely employed bacteria for gene transfection. Attenuated strains of pathogenic bacteria such as *Shigella*, *Salmonella typhimurium*, *Escherichia coli*, *Clostridium*, *Bifidobacterium* and *Vibrio cholerae* have been also employed for gene delivery. *Listeria* is the most preferred vector due to its ability to permeate through membrane barriers as well as innate ability to escape from the endosomal and lysosomal compartments using the pore-forming molecule listeriolysin. Bactofection requires the use of antibiotics to disrupt the bacterial vector for the release of the oligonucleotide cargo or to regulate the expression of the transgene. However, a recent approach for delivering genetic cargo into cells involved the use of bacteria conjugated with nanoparticles that eliminated the need for antibiotics. These hybrid bacterial systems were termed 'microbots' and were conjugated to polystyrene nanoparticles complexed with luminescent gene using the well-known biotin-avidin chemistry. The nanoparticle-decorated bacteria internalized into the target KB (nasopharyngeal carcinoma) cells where the gene was released and successful transfection was achieved. The system also displayed excellent transfection efficiency in vivo [190]. Incorporation of a targeting moiety in this system may further improve

its site-specific homing efficiency, and this system represents an attractive alternate to viral vectors.

Self-assembled DNA structures and DNA cages have also been explored for gene delivery. The therapeutic oligonucleotide is introduced through complementary base pairing with the DNA sequences. The resultant self-assembled structure has been found to exhibit better serum stability when compared to the single-stranded and duplex forms. A recent study employed a self-assembled triangular DNA formed by hybridizing with three copies of *si*-RNA against mTOR for transfecting H292 cells. The triangular DNA structure internalized into the cells predominantly through macropinocytosis and clathrin-mediated endocytosis. The authors report that the self-assembled structure showed better stability and silencing efficiency when compared to the free *si*-RNA suggesting the potential of such systems as novel gene delivery systems [191]. However, further evaluation of these systems in vivo will reveal their true potential in circumventing the physiological barriers.

An organic-metal complex of calcium-zoledronate has been successfully employed to complex plasmid DNA encoding for green fluorescent protein. The resultant complex exhibited good colloidal stability. The system was used to transfect HeLa cells in vitro. In vivo studies revealed that the organic-metal complex protected the plasmid DNA from enzymatic degradation in the tumour tissue [176]. Other complexes such as calcium-PEG-bisphosphonate have also been explored for gene delivery applications. Further studies in this direction are required for the development of this novel class of materials for gene delivery applications.

Other novel nanomaterials such as carbon nanotubes (CNTs) have also been explored for gene delivery. But these materials are highly hydrophobic and therefore have to be functionalized with amine, hydroxyl or carboxylate groups for better aqueous dispersivity and association with the oligonucleotides. In order to conjugate the oligonucleotide, many studies have wrapped the carbon nanotube structures with cationic polymers such as allylamine [192]. But several concerns still persist on the biocompatibility of CNTs, thereby limiting its applications for gene therapy. Another interesting material that has gained attention in recent years for gene delivery applications is cationic cyclodextrin. Cyclodextrin (CD) is a cyclic oligomer of α-D-glucose units. The interior of CD is relatively hydrophobic as the hydroxyl groups are all present in the exterior surface. The abundant hydroxyl functionalities present on the outer surface of CD make it amenable for chemical modification. Cationic derivatives of CD have been explored for complexing oligonucleotides. The surface of CD has been further modified with lipid coating to protect the oligonucleotide and promote cell internalization. Cationic CD was used to deliver plasmid DNA encoding luciferase to COS-9 cells that have been derived from monkey epithelial cells and are widely employed to investigate transgene expression. The transfection of the CD derivatives was found to be dependent on the hydrophile-lipophile balance presented by an alkyl chain modification on the surface groups. Butanoyl and hexanoyl chains were the most efficient in transduction as they imparted stability to the oligonucleotide and also contributed to the endosomal escape by augmenting the proton sponge mechanism [193]. Similarly, a surface-modified CD system with anti-CD123 to home into acute myeloid leukemic cells

KGI was successfully employed to deliver *si*-RNA to silence bromodomain-containing protein 4 (BRD4) both in vitro and ex vivo samples obtained from patients with acute myeloid leukaemia [194]. The cyclodextrin carrier was also used to co-deliver the chemotherapeutic agent cytarabine for improved therapeutic efficacy due to synergistic action of gene silencing and chemotherapy. In order to target prostate cancer, anisamide-modified cationic CD was employed for silencing PLK1 gene using *si*-RNA in a 3D model of bone metastasis. The cell penetration was improved by introduction of octaarginine, a well-known cell-penetrating peptide [195].

## 3.24 Concluding Remarks

A plethora of nanocarriers have been explored as non-viral strategies to improve the stability of oligonucleotides and ensure site-specific delivery of the therapeutic gene to target cells. Modifications of the carriers to respond to specific stimuli and/or enhance their circulation time have also been reported. Majority of the carriers have been designed to facilitate endosomal escape that has been a major limitation in non-viral vectors. One of the key aspects that still accounts for the lower transfection efficiency of non-viral vectors when compared to their viral counterparts is the extremely high transcription efficiency of viral vectors that is not exhibited by many nanocarriers. The recent emergence of hybrid vectors comprising a polymer-coated virus may offer a solution to circumvent these issues. As newer gene editing techniques are being introduced, there exists a need for appropriate nano-dimensional carriers that facilitate highly efficient transfection in the target cell without inducing cytotoxic effects to nontarget tissues. The notable progress made in engineering nano-dimensional gene delivery systems to mimic viral transduction promises to transform gene therapy for personalized therapy in the near future.

**Acknowledgement** The authors wish to acknowledge funding towards development of novel gene delivery systems from DBT (BT/PR-11210/NNT/28/421/2008) (UMK), DST's Indo-Russia collaborative programme (INT/RUS/RSF/P-10) (UMK & VA), Nanomission (SR/NM/PG-16/2007) (UMK) and SASTRA Deemed University (UMK).

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# Viral- and Non-viral-Based Hybrid Vectors for Gene Therapy

4

Manohar Mahato, Giridhara R. Jayandharan, and Praveen Kumar Vemula

## 4.1 Introduction

Paradigm changing recent developments in the field of nanotechnology offer the platform where Physicochemical and biological properties of metallic and nonmetallic molecules can be modulated for the wide range of applications in several areas of communications, basic sciences, engineering, medicine, robotics, etc. [1–4]. Since the introduction of the term nanotechnology, various modifications have been implemented to develop novel variants of nanoparticles with diverse properties [5, 6]. Specifically, nanoparticles are in the size range of 5–100 nm and possess high surface area to volume ratio which renders them to bind molecules with bi-specific conjugate or specific targeting peptides [7]. Based on the composition of nanoparticles, they can be categorized as polymeric (synthetic or natural polymers), Q-dots, nanoemulsions, ceramic (silica) particles, metallic (gold, silver, iron oxide), liposomes, and graphene [8]. Accordingly, nanoparticles exhibit specific optical, magnetic, chemical, and physical properties that lead to their application in various biomedical applications such as in vivo imaging, tissue-specific drug delivery, etc. [9–11]. The major advantage of nanoparticles containing tissue-specific moiety as a delivery vehicle is the ability to bypass side effects associated with therapeutics such as antibiotics or chemotherapeutic agents [12]. Thus, such properties make them useful for both vaccination and therapeutic strategies to circumvent an immune response or for gene delivery, respectively [13]. Delivery of a normal copy of genes inside the cells is a promising approach for the treatment of various genetic or acquired disorders and is also called as replacement gene therapy. Viruses are the

M. Mahato · P. K. Vemula (✉)
Institute for Stem Cell Biology and Regenerative Medicine (inStem), Bangalore, India
e-mail: praveenv@instem.res.in

G. R. Jayandharan
Department of Biological Sciences and Bioengineering, IIT-Kanpur, Kanpur, UP, India



G. R. Jayandharan (ed.), *Gene and Cell Therapy: Biology and Applications*,
https://doi.org/10.1007/978-981-13-0481-1_4

potential candidates for delivering therapeutic genes inside the cells with higher efficiency; to achieve the therapeutic gene expression, various recombinant vectors have been used including adenovirus, retro-/lentivirus, and adeno-associated virus (AAV) [14]. Till date, >2300 clinical trials for gene therapy are going on worldwide out of which 70% are using viral vectors, but none of them have reached desired therapeutic endpoints [14–16]. The primary objective of gene therapy is to maintain stable transgene expression, but the major hurdle for gene therapy is host immunity which hinders persistent high levels of transgene expression [17]. Since the viruses are a coating of proteins, thus they are recognized as foreign bodies by host immune system and lead to their activation. The host immunity serves as a major side effect for gene therapy through viral vectors. Recently, some reports showed using the combination of nanoparticle and viral vectors to optimize therapeutic delivery, for example, viral vectors encapsulated in nanomaterials rescued gene therapy vectors from adverse immunity events [18, 19]. Taking a cue from these kinds of studies, this chapter summarizes the data available on hybrid vector-based delivery systems which consist of nanoparticle and viral vectors, the strategies to enhance the potential of the hybrid system, and their advantages.

## 4.2 Concept of Gene Therapy

The technique of delivering a therapeutic gene into host cells to ameliorate the genetic disorder or acquired disease is called gene therapy. The gene therapy can be classified into two categories as (1) somatic cell gene therapy, limited to individual, and (2) germline gene therapy – modified gene is inheritable. Due to ethical issues, insufficient knowledge, and risk to future generations, germline gene therapies are prohibited in many countries. Thus, most of the gene therapy programs have been focused on somatic gene therapy. Since its discovery in 1980, the first clinical trial was established in 1990 using retrovirus as a vector for functional adenosine deaminase [20]. Till date, >2300 clinical trials have been conducted using various viral vectors in different parts of the world [14]. As advancement in the field of viral vectors, alternative approaches have been implemented and showed promising potential by knocking down the mutated gene (suicide gene therapy) or editing faulty gene (nuclease-mediated gene editing) to reach therapeutic endpoint [21]. These delivery systems have been used in two distinct modes: (1) ex vivo, where viral vector with therapeutic gene is transduced in recipient cells (e.g., hematopoietic cells) and then transduced cells are introduced into the host body, and (2) in vivo, where the viral vectors are administered directly into host body [22]. The choice of approach is mainly based on disease and target tissue to be treated from gene delivery. An ideal vector should exhibit sustained transgene expression and tissue specificity with low immunogenicity for higher therapeutic efficacy through critical designing of viral vectors; they have been used with fair success for various genetic disorders like cystic fibrosis [23], hemophilia [24], Leber's congenital amaurosis [25], and various severe combined immunodeficiency (SCID) [26, 27]. Currently, three kinds of viruses, i.e., adenovirus, retrovirus/lentivirus, and adeno-associated

virus (AAV), have been employed as vectors for delivering therapeutic genes to the desired cells [14]. These vectors have shown promising results and success in gene therapy up to some extent. Thus, there is a scope for further advancement for improving the therapeutic efficacy.

## 4.3 Viral and Non-viral Hybrid Vectors

Since the 1980s, both viral and non-viral vectors (synthetic) have been developed to overcome the limitations associated with both delivery systems for making gene therapy a viable technology in clinics (Fig. 4.1). Although viral vectors have a high transduction efficacy for DNA, they are immunogenic. On the contrary, non-viral vectors have low transfection efficacy than viral vectors, but generally, they have low immunogenicity (as they have been designed from biocompatible material). Thus, to leverage the advantages from both types of vectors, hybrid vectors were developed by the combination of both vectors to achieve higher gene delivery efficacy than individual vector alone with minimal side effects (i.e., immunogenicity). In an attempt to develop hybrid vectors, viral vectors (AAV, adenovirus, retro-/lentivirus) have been encapsulated within synthetic materials such as liposomes, dendrimers, and hydrogels (Figs. 4.3 and 4.4). Some of hybrid vectors that have shown significant efficacy in delivering genes are listed in Table 4.1. Among all the viral vectors, adenovirus showed promising potential for development of hybrid vector systems as they were able to target tumor tissues efficiently [28–32]. Moreover, it can function effectively with different non-viral vectors (Table 4.2) such as alginate

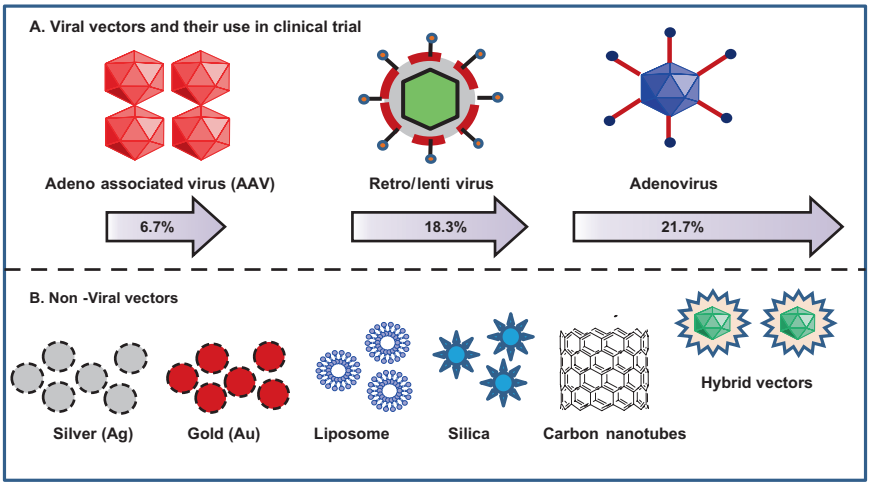



**Fig. 4.1** Various nanoparticle and gene therapy vectors. (**a**) Viral vectors, e.g., adenovirus, adeno-associated virus, and lentivirus, which have been dominantly used in clinical trials. (**b**) Non-viral vectors and metallic and nonmetallic nanomaterials have been developed with targeting capability

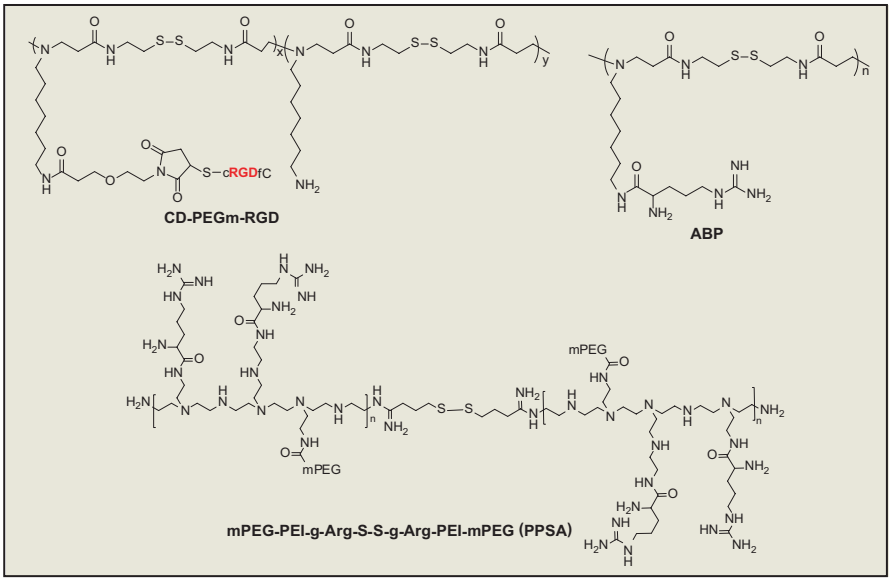
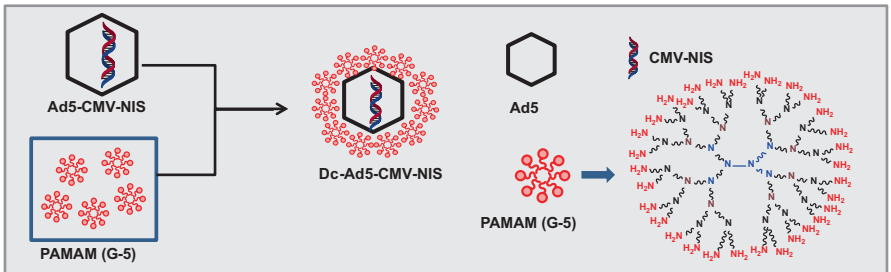



**Fig. 4.2** Chemical structure of bioreducible polymer used for coating over adenoviruses to develop various hybrid vectors




| Experiments | Naked adenovirus (Ad5-CMV-NIS) | Dendrimer coated adenovirus (Dc-Ad5-CMV-NIS) |
|---|---|---|
| *In vitro* | | |
| ➢ HuH7 (CAR-positive cells) | 70-fold transduction | 70-fold transduction |
| ➢ U87 MG (low CAR-positive cells) | Low transduction | 5.5-fold transduction |
| ➢ SKOV-3 (CAR-negative cells) | No transduction | 22-fold transduction |
| ➢ Adenovirus neutralizing antibodies (IgG) | Rapid neutralization | Effective protection |
| *In vivo* | | |
| ➢ Virus biodistribution (hepatic pooling) | High | ~70% lower |
| ➢ hepatic NIS mRNA expression | High | ~16-fold lower |
| ➢ tumoral iodide accumulation | 3.5% ID/g | 13.5% ID/g |
| ➢ NIS mRNA expression in tumor cell | 2.5 fold increase | 10-fold increase |

**Fig. 4.3** Schematic representation of formation of PAMAM-G5-coated adenovirus and their efficacy in in vitro and in vivo

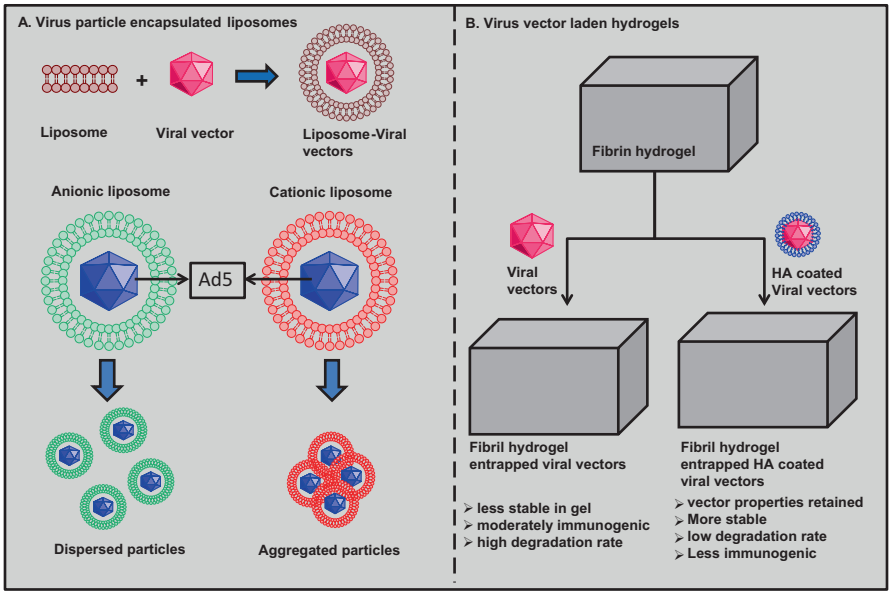



**Fig. 4.4** Hybrid viral nanoparticles. The combination of viral vectors and nanomaterials offers many advantages such as delivery of multiple payloads, targeting ability to specific tissue, and escape from host immune system. The schematic representation of the advantages of hybrid vectors. (**a**) Variations between the cationic liposomally bound viral particles and anionic liposomally bound nanoparticles. (**b**) Viral particles encapsulated in fibrin hydrogels. Difference between naked viral particles and hydroxyapatite (HA)-coated viral particle-loaded in fibrin hydrogels is highlighted

beads [33], chitosan [34, 35], chitosan-PEG-folate conjugate [36], polyethyleneimine [37, 38], etc. To develop tissue targeting hybrid vectors, conjugation of moieties like arginine graft [39], RGD [40], and Herceptin [41] and even surface charge modification [38, 42] have shown precise targeting by vectors (Fig. 4.2 and Table 4.3). The arginine-grafted bioreducible polymers (ABP) were synthesized, and hybrid vectors were developed with adenovirus to overcome the immune response from the host with minimal cytotoxicity. In vitro results showed after electrostatic coating of ABP over adenovirus resulted in enhancement of six-fold transduction efficiency in coxsackievirus and adenovirus receptor (CAR)-negative cells as compared to naked Ad vectors [39]. These results suggested after cationic polymer coating, hybrid vectors internalize within the cell through CAR-independent pathway. Moreover, ABP-Ad vector showed 83.1% of transduction efficiency in the presence of 30% serum in A549 cells, while naked Ad vectors showed 47.49% efficiency. Further, an innate immune response was evaluated after treating RAW264.7 macrophage cells with naked Ad and ABP-Ad vectors. Pro-inflammatory cytokine IL-6 release was significantly reduced after treatment with ABP-Ad (38.57 ± 0.5 pg/mL) as compared to naked Ad (70.35 ± 0.5 pg/mL). These results strongly suggested that shielding of the viral proteins with cationic polymers can enhance

**Table 4.1** List of nanomaterial-coated hybrid viral vectors that have been tested either in vitro or in vivo

| S. No. | Vector | Nanomaterial | Transgene | Assay endpoint | Reference |
|---|---|---|---|---|---|
| 1. | Adenovirus | PAMAM-G5 | Sodium-iodide symporter | $^{123}$I Scintigraphy$^{+}$ (radiovirotherapy) | [45] |
| 2. | Lentivirus | Fibrin hydrogel (+/− hydroxyl apatite) | Luciferase | Bioluminescence | [53] |
| 3. | Lentivirus | Collagen hydrogel (+/− hydroxyl apatite) | Luciferase | Bioluminescence | [52] |
| 4. | AAV | Elastin-like polypeptide (ELP) | GFP | In vitro transduction | [54] |
| 5. | AAV | Heparin-coated superparamagnetic iron oxide | GFP | In vitro transduction | [55] |
| 6. | AAV | Glyceraldehyde tag | GFP | In vitro transduction | [86] |
| 7. | AAV | Elastin-like polypeptide + poly(ε-caprolactone) | GFP | In vitro transduction | [87] |
| 8. | AAV | Polyethylene glycol (PEG) | GFP | In vitro transduction | [88] |

**Table 4.2** Hybrid vectors composed of polymer/Ad

| Polymer | Adenovirus | Efficacy |
|---|---|---|
| Reducible PEI | RdB/shMet | Enhanced transduction efficiency – increased viral entry and production in vitro [38] |
| Bile acid-conjugated PEI | KOX | Enhanced antitumor therapeutic efficacy and antiangiogenic effect than cognate control virus in vitro and in vivo [37] |
| PNLG | Δ B7-U6ShIL8 | Preserved Ad's biological activity at 37 °C – significantly enhanced antitumor efficacy than either Δ B7-U6ShIL8 or Δ B7-U6ShIL8/ABP in HT1080 and A549 tumor models in vitro and in vivo [29] |
| PPSA | DWP418 | Enhanced transduction efficiency and antitumor efficacy than Ad/ABP in vitro and in vivo [30] |
| PAMAM | E1/AFP-E3/NIS | Synergistic therapeutic effect by combining oncolytic Ad and therapeutic dose of $^{131}$I in vitro and in vivo [39] |
| PEG | Ad-GL | More potent antitumor effect and less hepatotoxicity by 20-kDa PEGylated oncolytic Ad than 5-kDa PEGylated oncolytic Ad in vitro and in vivo [38] |
| ABP | YKL-1001 | Increased blood circulation time and safety profiles – enhanced antitumor therapeutic efficacy than cognate control in hepatoma xenograft model in vitro and in vivo [35] |

**Table 4.3** Modified polymers used for development of targeting hybrid vectors

| Polymer | Targeting ligand | Oncolytic adenovirus | Targeted receptor/cell | Efficacy |
|---|---|---|---|---|
| Chitosan-PEG-FA | Folic acid | HmT | Folate receptor overexpressed cancer | Folic acid-mediated antitumor efficacy of Ad/polymer is higher than EPR-mediated delivery [32] |
| CD-PEG-cRGD | cRGD | Δ B7-U6ShIL8 | αβ integrin positive cancer | Greater antitumor efficacy than naked Ad in A549 lung orthotopic model [36] |
| PEG-HER | Herceptin | DWP418 | Her2/neu overexpressed cancer | Her2 targeted specific transduction and antitumor efficacy [37] |
| PAMAM-GE11 | GE11 | E1/AFP-RSV/NIS | EGFR-positive cancer | EGFR targeted specific antitumor efficacy by combination of polymer-coated Ad and $^{131}$I [46] |

circulation period for hybrid vectors and reducing innate immune response. In a similar study, a cationic biodegradable polymer, methoxy poly(ethylene glycol)-b-poly{N-[N-(2-aminoethyl)-2-aminoethyl]-L-glutamate} [PNLG], was synthesized, and hybrid vectors were developed with adenoviruses [29]. The transduction efficiency of developed PNLG-Ad vector was compared to ABP-Ad vectors in vitro and in vivo. The PNLG-Ad vectors exhibited high stability at 37 °C and pharmacokinetics due to the formation of smaller particle size (~130–140 nm), while ABP-Ad vector formed 400–1300 nm size particles. The tumor growth was reduced in various xenograft models such as 57.5% (HT1080)/47.0% (A549), whereas ABP-Ad showed reduction up to 24.8% (HT1080)/16.4% (A549). The innate immune response was also evaluated by quantifying the IL-8 and vascular endothelial growth factor (VEGF) released after treatment with ABP-Ad, PNLG-Ad, and naked Ad vectors. The results showed significant inhibition of IL-8 or VEGF secretion, 76.6% or 79.7%, respectively, on treatment with PNLG-Ad while 47.7% or 60.7% with ABP-Ad. Moreover, systemic administration of PNLG-Ad vector exhibited a 1229-fold increase in tumor to the liver ratio as compared to naked Ad. These studies revealed that biophysical property of hybrid vectors such as particle size and surface charge plays a crucial role in their therapeutic efficacy. Similarly, several reports have been published using cationic bioreducible polymers (exclusively polyethylenimine) [37, 38] and mPEG-PEI-g-Arg-S-S-Arg-gPEI-mPEG [30] for the development of hybrid vectors.

After successful development of hybrid vectors which can internalize inside the cell through CAR-independent pathway without triggering the immune response, researchers focused on developing hybrid vector with targeting ability. In an attempt to develop targeting hybrid vector against folate receptor (FR)-positive cancer, adenovirus was electrostatically complexed with chitosan [36]. Then, polyethylene

glycol (PEG)/folic acid (FA) or PEG-FA was chemically conjugated to the surface of chitosan-Ad to develop various nanocomplex such as chitosan-Ad, chitosan-PEG-Ad, chitosan-FA, and chitosan-PEG-FA-Ad. The vectors consisting of FA on the surface (chitosan-FA-Ad, chitosan-PEG-FA-Ad) exhibited significant selectivity against folate receptor-positive cells (HeLa and KB cells) and showed cell viability up to ~45% in KB and HeLa cells while ~70–80% in FR-negative cells (U343 and A549 cells). Systemic administration of chitosan-PEG-Ad and chitosan-PEG-FA-Ad significantly increased the blood circulation time after 24 h of injection, resulting in 9.0-fold and 48.9-fold increase, respectively, as compared to naked Ad. Moreover, these hybrid vectors showed ~75% decrease in generation of adenovirus-specific neutralizing antibodies in mice when treated with chitosan-PEG-FA-Ad as compared to naked Ad. The administration of chitosan-PEG-FA-Ad exhibited 378-fold reduction in liver tissues and 285-fold increase in tumor tissue as compared to naked Ad; hence the hybrid vector was able to enhance the tumor-to-liver ratio. The targeting hybrid vector exhibited 52.8% inhibition of tumor growth as compared to naked Ad. Thus, conclusively chitosan-PEG-FA-Ad showed promising potential for further development of targeting hybrid vectors in terms of efficacy and safety. To develop targeting vectors against endothelial cells of tumor capillaries and neointimal tissues, a bioreducible cationic polymer CD was conjugated to cyclic RGD peptide (Fig. 4.2). These tissues inherently overexpresses αvβ3 and αvβ5 integrin proteins which selectively bind to RGD peptides. Two hybrid variants were synthesized with different molecular weights of PEG chains, viz., $PEG_{500}$ and $PEG_{2000}$ to generate CD-$PEG_{500}$-RGD-Ad and CD-$PEG_{2000}$-RGD-Ad [40]. The results showed RGD-tethered polymer-coated hybrid vectors were specifically killing the cancer cells having integrin protein on cell membrane, irrespective of CAR. The CD-$PEG_{500}$-RGD-Ad hybrid vector was efficiently able to express shRNA against IL-8 mRNA. There was significant reduction of IL-8 expression in cancer cells was observed as compared to naked Ad, such as 79.6% decrease in HT1080 and 77.2% decrease in MCF7 cells. Further, exploiting the cell surface biomarker as a target which is overexpressed on cancer cells, various potential ligands have been investigated. Her2/neu is widely known as human epidermal growth factor 2 receptor and overexpressed in 20–30% of breast cancer patients. This receptor plays an important role as an oncogene in cancer cells. Drugs which target these receptors like trastuzumab and lapatinib are in clinical use; trastuzumab (Herceptin), a monoclonal antibody specific for Her2/neu, is also being used widely for treatment of both early and metastatic breast cancer [43, 44]. To develop Her2/neu targeting hybrid vector, adenovirus (Ad) was chemically conjugated with bioreducible PEG chain, and Herceptin was tethered terminally, HER-PEG-Ad [41]. Specificity and CAR-independent cellular uptake of these Herceptin-conjugated hybrid vectors were evaluated in vitro using Her2-positive (MDA-MB435, SK-OV3, and MDA-MB231) and Her2-negative (SK-Her1 and HeLa) cells. Further, innate response and stability of the HER-PEG-Ad were evaluated after systemic administration in BALB/c mice. The results showed after administration, IL-6 secretion level was found to be 77 pg/mL, 14 pg/mL, 411 pg/mL, and 46 pg/mL for HER-PEG-Ad, PEG-Ad, naked Ad, and PBS, respectively. After 1 h of administration, HER-PEG-Ad and PEG-Ad

were six-fold higher than naked Ad in blood circulation. The ligand-modified hybrid vector showed significant higher targeting ability for tumor in xenograft model, and HER-PEG-Ad showed $10^{10}$-fold increase in tumor-to-liver ratio with minimal hepatic toxicity. These reports suggest that the development of hybrid systems from nanomaterial-coated viral vector using nonpathogenic viruses like AAV serves as excellent candidates for higher efficacy with minimal side effects.

## 4.4 Dendrimer-Coated Virus Particles

In a recent study, the hybrid vector (as shown in Fig. 4.3) was developed for gene transfer in liver cancer xenograft model from adenovirus coated with poly(amidoamine) dendrimer generation 5 (PAMAM-G5) [45]. The transduction efficacy and tissue tropism of coated adenovirus particles (Ad5-CMV/NIS) which consist of hNIS transgene (sodium-iodide symporter) were tested by radioactive iodine isotope ($^{123}$I) scintigraphy. The in vitro results have shown a significant decrease in antibody-mediated neutralization and increase in the CAR-negative cell (extent in adenovirus infection). Further, when this hybrid vector was administered in mice, it showed sustained transgene expression and reduction in tumor size as well. The study showed such delivery systems using adenovirus hybrid vectors indicate high therapeutic potential. Moreover, to incorporate targeting ability to the dendrimer-based hybrid vectors, dendrimer was conjugated to the peptide as a ligand specific for epidermal growth factor receptor (EGFR), PAMAM-GE11 [46]. In this study, PAMAM-G2 and PAMAM-G5 were used, but PAMAM-G2-GE11 showed better efficiency due to improved covering of adenoviral surface epitopes by smaller diameter of dendrimers. This hybrid vector also showed CAR-independent cellular uptake with low hepatic accumulation as well as an increase in transduction efficiency over tumor cells in the xenograft model.

## 4.5 Virus Particles Encapsulated Liposomes

Viral gene therapy holds great potential in treating cancer using oncolytic replication-selective viruses (OVs) as they selectively replicate within cancer cells and causes apoptosis [47]. The use of OV-based gene therapy showed significant alleviation of cancer in human clinical trials even with advanced stages of cancer [48]. However, their efficacy has been limited by rapid clearance through reticuloendothelial (RE) system in liver and neutralization by antibodies which affect their distribution into the tumor cells [49]. To overcome the issue of neutralization of OV by antibodies, Yotnda P. et al. have encapsulated adenovirus vectors in bilamellar cationic liposomes consists of DOTAP (1,2-dioleoyloxypropyl)-N,N,N-trimethylammonium chloride) and cholesterol [50] (Fig. 4.4a). This hybrid vector was able to efficiently transfect the cells which either lacks adenoviral receptors or in the presence of receptor, as compared to naked adenovirus. However, their clinical application was hindered due to systemic toxicity, low targeting efficacy, and poor serum stability. To address these issues, adenoviral vectors (adenovirus 5, Ad5) were encapsulated in

anionic bilamellar liposomes composed of phosphatidylcholine, phosphatidylethanolamine, inositol phosphatides, cholesterol, PEG-2000, and nontoxic lecithin (Fig. 4.4a) [51]. These anionic liposome-encapsulated adenoviral-based hybrid vectors have shown superior transfection efficacy in cancer cells than naked Ad5 and were able to administer repeatedly without any immunogenic response in vivo. Moreover, the anionic liposomal virus particles have shown stability for 32 h as a monodisperse solution, while cationic liposomal virus particle got aggregated within a couple of hours (Fig. 4.4A). The anionic liposomal-based encapsulated viral particles have shown promising results for further use in clinical application [51].

## 4.6 Virus Vector-Laden Hydrogels

In a study to develop a better transduction profile with lentiviruses, lentiviruses were encapsulated in hydrogels composed of collagen and hydroxyapatite [52]. The effect of material used for hydrogel formation and their degradation kinetics for transgene expression was evaluated both in vitro and in vivo. The encapsulated lentivirus showed ~80% of transfection efficiency in invasive C6 glioma cells. Further, the virion release and cell migration from the surrounding tissue was depending on the composition of collagen hydrogel (0.05%, 0.15%, 0.3%). While the efficacy of lentivirus loaded in hydroxyapatite containing collagen-gels was marginal (~33% increase in luciferase gene expression) as compare to only collagen-containing gels in an animal model (CD-1 male mice). Similarly, another study was carried out using fibrin and hydroxyapatite hydrogel encapsulated lentiviruses for localized vector transduction in CD-1 mice (Fig. 4.4b), but this strategy did not affect the virus infectivity or their cellular infiltration [53]. To develop high-performance delivery systems, researchers have used AAV vectors combined with elastin-like polypeptides (ELP) and evaluated for their infectivity on human neural stem cells (NSCs) and murine fibroblasts (NIH3T3) [54]. This study was carried out using AAV variant r3.45 which showed a significant increase in transduction efficacy when conjugated to ELP as compared to control groups. The results showed potential use of these hybrid vectors in NSCs for the treatment of various neurodegenerative disorders. To maximize the AAV contact with tissue for efficient and sustained gene transfer, AAVs were encapsulated in a nanofiber scaffold composed of ELP and poly(ε-caprolactone) (PCL) through electrospinning [18]. The super paramagnetic iron oxide nanoparticles (SPION) were coated with heparin and combined with AAV variant r3.45 which showed enhanced gene delivery in different types of cell lines, e.g., HEK293T and PC12 cell lines [55]. Even a short incubation period of <180 min was sufficient in transducing the target cells with the same efficiency achieved with conventional 24-hour incubation of naked virus. Moreover, the magnetically driven AAV transduction improved some of the critical phenotypes such as the neurite extension and expression for nerve growth factor in PC12 cells. These reports suggest that the hybrid vectors have several advantages over the convention delivery vectors, but its development requires exhaustive understanding related to host immune response, tissue specificity, and kinetics of nanoparticle-vector hybrid delivery under in vivo settings.

## 4.7 Challenges

The cellular uptake of nanoparticles in a large quantity inside the host cells generates concentration gradient across vascular endothelium which leads to inhibition of further uptake [56]. Moreover, this phenomenon stimulates the residential monocytic-phagocytic system and results in an aberrant distribution of nanoparticles (Fig. 4.5). However, there are various properties of hybrid nanoparticles such as size, surface charge, stability, and route of delivery which decide the efficiency of these vectors inside the cell [57, 58]. Under in vivo condition, the interaction of natural multivalent biomolecules in blood circulation and viral vectors generates the immune response and has been described below [59–61]. Many reports are suggesting that the generation of immune response associated with administering vaccines and drug delivery vehicles [10]. The extent of innate response mainly depends on targeted tissue (e.g., skin, lungs, gut), as each of tissue having a different number of residential immune cells [61, 62]. After cellular uptake, hybrid vectors got fragmented and stimulate either innate or adaptive immune response through a cascade of events initiating from antigen generation by antigen-presenting cells (APCs) to

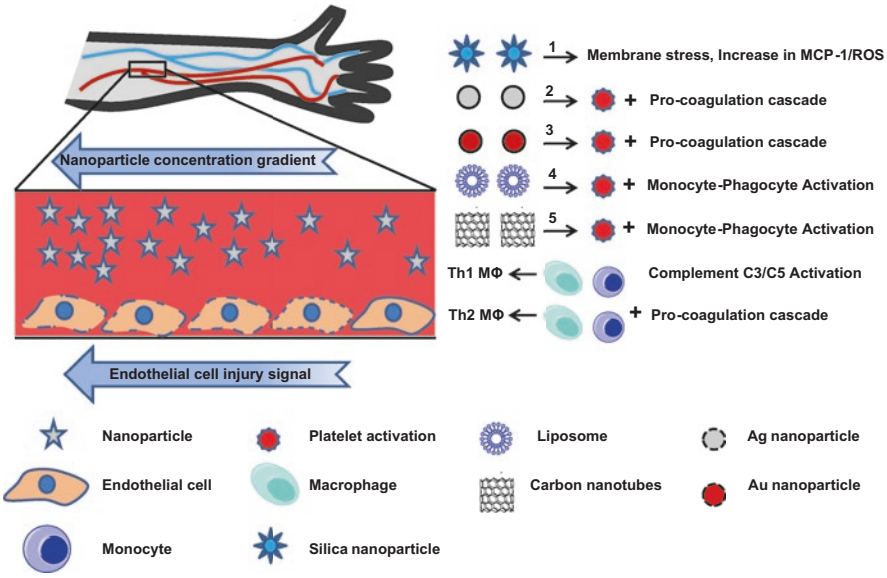



**Fig. 4.5** Innate immune response against nanoparticles. The entry of nanoparticles through endothelial cells is a primary event that triggers a cascade of signals toward nanoparticles or their fragments [75]. Innate immune response toward them differs substantially as it depends on the physical characteristics of nanoparticle such as size, shape, charge, and associated ligand/peptide molecules as well as route of entry [76–78]. The invasion of nanoparticle through endothelial cells leads to disruption of endothelial membrane and eventually activates vascular system along the nanoparticle concentration gradient. Events 1–5 show five different nanoparticles, i.e., silica, silver, gold, liposome, and carbon nanotubes, and the innate response observed against them. Macrophage-mediated phagocytosis of nanoparticle/ fragment involves multiple events including macrophage migration and differentiation in response to cytokines/chemokines that activates Th1Mϕ /Th2Mϕ cells [79–81]

**Table 4.4** Immune response reported with various different nanoparticle formulations

| S. No. | Nanomaterial | Primary events lead to immune response |
|---|---|---|
| 1. | Gold nanoparticles | Platelet activation, plasma membrane disruption [60, 89] |
| 2. | Silver nanoparticles | Induce cytotoxicity to endothelial cells, pro-inflammatory cytokine, chemokine production, NF-KB pathway activation, free radical production [64] |
| 3. | Metal oxide nanoparticles | Chemokine receptor molecule (type 4, CXCR4), adhesion molecule expression levels [90] |
| 4. | Silica nanoparticles | Nitric oxide and peroxynitrite production; upregulation of ICAM1, VCAM1, IL-8, and IL-6; NF-KB activation [91]; reactive oxygen species generation; apoptotic signal molecule generation and transcription factor upregulation; release of tissue factor, IL-6, IL-8, MCP-1, and ROS [92] |
| 5. | Carbon nanotubes | Complement-mediated opsonization, C3/C5 and membrane attack complex formation [93]<br>Endothelial membrane leakage [94]<br>Platelet activation and aggregation, degranulation, ATP release [95]<br>Oxidative stress induction, cytokine production (TNF-α), IL-1β, and IL-8 [63, 96–99]<br>Inflammation [100–104] |
| 6. | Dendrimers | Endothelial cytotoxicity, endotoxin-induced procoagulant activity [98, 105, 106] |
| 7. | Liposome | Expression of macrophage maturation marker and polarization of monocyte<br>Inhibition of macrophage migration [107]<br>Endothelial cell cytotoxicity [79] |
| 8. | Cationic lipids (RPR206252) | TNF-α, IL-1β, IL-6, IFN-γ production<br>NF-KB activation, TLR-2 and NLRP3 activation [108] |
| 9. | Polystyrene latex particle | Platelet activation and aggregation, upregulation of adhesion receptor [109] |
| 10. | 1,3-β-Glucan chitosan shell with poly(lactide-co-glycolide) | Reactive oxygen species, reactive nitrogen species, pro-inflammatory cytokine secretion, increased expression of TNF-α and IFN-γ [110] |
| 11. | Perfluorocarbon emulsion | Complement system activation [111, 112] |

exocytosis or leading to cellular apoptosis [63]. The invasive property of hybrid vectors which causes endothelial cell injury and malfunction acts as the first sign for toxic effects on vascular system (Fig. 4.5) [64]. The immune response associated from various nanoparticle has been summarized in Table 4.4, but the overlap between the response generated is also frequently observed. In Fig. 4.6, detailed schematic representation of a possible number of events which lead to adaptive immunity in the presence of nanoparticles has been described. The nanoparticle antigens are captured by immature dendritic cells from closest lymph nodes which lead to the activation of T-cell differentiation and stimulating B cell as well [65]. Several inflammatory cytokines (TNF-α and IL-1β) and co-stimulatory receptor ligands CD80 (B7-1) and CD86 (B7-2) are responsible for activation and

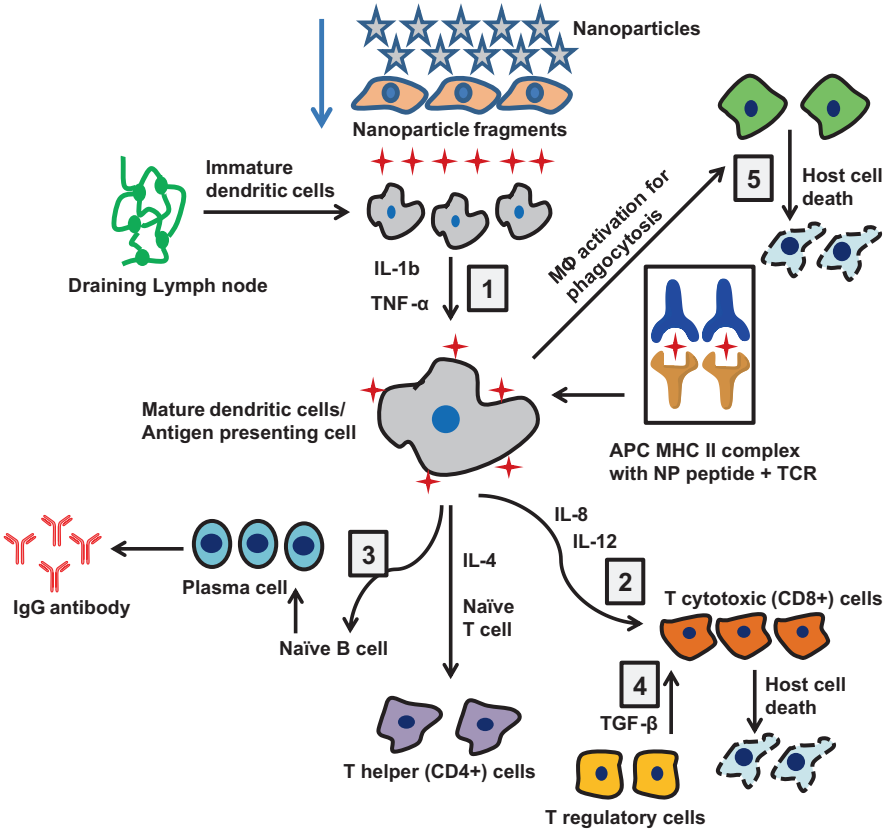



**Fig. 4.6** This schematic representation depicts the adaptive immunity observed against the nanoparticles. (1) Dendritic cells act as a link between innate and adaptive immune system and regulate their cross activation through several signals (MHCI/II-peptide complex, CD80-CD80L, etc.) [66–69]. Movement of DCs bearing the peptide/MHC complex toward lymph nodes [82] is a critical factor that determines the magnitude of this activation. (2) In response to MHCI/II complex, cytokines (IL-4, IL-6, IL-12, TGF-β) and chemokines are released by naïve T cells that further activate downstream effectors such as the residential macrophages/monocytes which capture and destroy nanoparticle containing host cells. (3) Nanoparticle interaction with adaptive immune cells/molecules that leads to activation of host dendritic and cytotoxic T-cell population [83]. Some reports suggested that the nanoparticle coated with peptide ligand can also activate B cell and generate antibodies. (4) Nanoparticle-mediated response through T cell or B cell has been bypassed through activation of T-regulatory cells and suppression of pro-inflammatory molecules (IL-2, IL-6, TNF-α, etc.). (5) Macrophage activation and differentiation by dendritic cells and nanoparticle phagocytosis leads to the expulsion of nanoparticle from the host cells [84, 85]

functionalization of DCs with antigen [66]. These activated DCs perform a cascade of signals along with MHC class I and II molecules to naive T cells having T-cell antigen receptor (TCR) [67]. Co-stimulatory signals CD80/86 which are generated from APCs interact with CD28 (T-cell receptor), and simultaneously secretion of cytokines (IL-12, IL-14, IL-16, TGF-β) also takes place which stimulates naive T

cells to differentiate into Th1, Th2, or Th17 cells. The antigen functionalization involves MHC class II loading pathway [68] which leads to the generation of a limited number of CD8+ T cells, and thus antigens can only be presented to only specific groups of DCs in the spleen or lymph nodes [69]. These pathways are suggesting the possibility to modify strategies against induction of immunological tolerance associated with hybrid vectors. In a study using modified PEI/DNA complex, nanoparticles have suppressed the antigen-specific T-cell responses and lead to regulatory T-cell activation via IFN-αβ-mediated DC activation [70]. However, experimental variations by using different animal strains (C57BL/6 and BALB/c) were also affecting clearance of nanoparticles in mice strains [71]. These reports suggest that further extensive studies are needed to determine the fate of nanoparticles during in vivo administration of vectors. Moreover, the targeting ability of hybrid vectors needs further improvement for efficient gene delivery. The major drawback of viral vectors is their ability to induce oncogenicity and lack of gene transfer specificity [72, 73]. Among other viral vectors, lentiviral vectors can integrate the foreign gene into the host genome and activates proto-oncogenes [74]. Thus, there is a need for further systematic studies for hybrid vectors (viral vector and nanomaterial) to overcome the barriers of individual vectors which hinder their use in the clinical applications.

## 4.8 Conclusions

Till now, viral and non-viral vectors have been extensively used to deliver a gene of interest to multiple target tissues. Combining both the vectors, hybrid vectors offers immense potential to deliver more than one transgene with tissue specificity. These vectors impart shielding of viral epitope surface to evade host immune response and provide a platform for conjugation of receptor-specific ligands on the surface to enhance targeting ability. However, the development of hybrid vector systems needs exhaustive knowledge of virus structure and the effect of nanomaterial coating on physicochemical properties of vectors. Further, most of the synthetic nanomaterials are also immunogenic in nature which cannot be overlooked. Thus, to generate hybrid system and for other biomedical applications, it is necessary to alleviate the immunogenicity of the synthetic nanomaterial. It is essential to select an appropriate non-immunogenic nanomaterial to develop hybrid vector systems and achieve higher efficacy with minimal side effects.

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# Pharmaco-Gene Therapy

5

Martin H. M. Sailer, Ganesh Ram Sahu,
and Arkasubhra Ghosh

## 5.1 Introduction

In the past few decades, the field of medicine has been evolving rapidly primarily driven by a deeper understanding of disease pathology, mechanisms of progression, and genetic and molecular factors leading to the emergence of advanced treatment modalities beyond the traditional surgery, chemotherapy, and radiation therapy: gene and cell therapy. The efficiency of the individual traditional therapies to combat diseases such as malignancies still remains poor and often fails in advanced or life-threatening disease stages [1–3]. Such observations have been made in many diseases such as chemo-resistant cancer, as well as infectious diseases where multidrug-resistant organisms evolve due to antibiotic treatments [4–6]. Hence, the development of more targeted and effective treatment strategies for those diseases represent a critical unmet clinical need. Combinatorial approaches have been gaining attention in recent years to overcome the lacunae of individual therapies [7]. Pharmaco-gene therapy is one such strategy where pharmacological drugs or formulations are combined with gene therapy for targeted delivery or expression of the transgene in specific diseases. This chapter summarizes different types of pharmaco-gene therapy approaches and disease applications (Fig. 5.1).

Monotherapy is a traditional approach toward the treatment of disease where a single modality of the treatment procedure is followed, which includes either drug

M. H. M. Sailer
Department of Biomedicine, Pharmacenter, University of Basel, Basel, Switzerland

G. R. Sahu
GROW Research Laboratory, Narayana Nethralaya Foundation, Bangalore, India

Vellore Institute of Technology, Vellore, India

A. Ghosh (✉)
GROW Research Laboratory, Narayana Nethralaya Foundation, Bangalore, India
e-mail: arkasubhra@narayananethralaya.com



G. R. Jayandharan (ed.), *Gene and Cell Therapy: Biology and Applications*,
https://doi.org/10.1007/978-981-13-0481-1_5

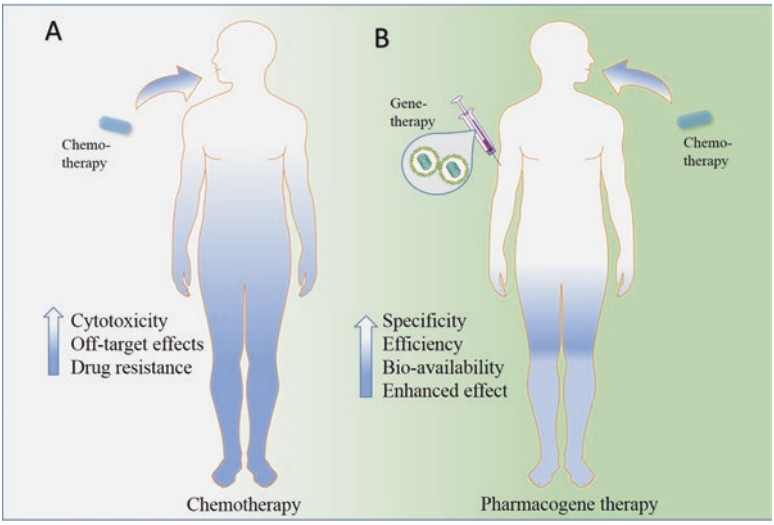


**Fig. 5.1** A pharmacogene therapy approach as advanced treatment modality: (**a**) Chemotherapy alone could increase the chances of treatment failure due to an off-target effect, cytotoxicity to normal cells and the development of drug resistance. (**b**) Combination of Chemo and gene therapy complements each other by enhancing the specificity, efficiency, bio-availability and final efficacious dosage of the treatment modality

therapy, surgery, radiation therapy, or gene therapy alone. Apart from the failure to achieve clinically significant benefits, many monotherapies (such as in cancers) also have significant side effects that are dose dependent [7, 8]. Antibiotic-resistant bacteria are an example illustrating the failure of monotherapy where pathogenic microorganisms develop resistance against the available antibiotics by accumulating mutations [9]. A multidrug therapy was sought as a better option to overcome such situation where it was adopted for tuberculosis, leprosy, and many infectious diseases [10, 11]. Similarly, although various treatment procedures continue to be developed for treatment of cancer, many chemotherapies show poor disease resolution or have been shown to possess higher level of cytotoxicity against even the normal cells and systemic side effects [12]. Surgical resection despite maintaining morphologically normal cut margins also does not guarantee nonrecurrence of the tumor in the future [13]. Similarly, radiotherapy also possesses off-target effects which are harmful for the normal tissues [14]. Gene therapy has emerged as a treatment modality for a number of genetic disorders as well as complex diseases such as cancers and metabolic disorders [15]. However, diseases such as cancers employ multiple pathways for their survival and proliferation, making single gene therapy prone to failure [16, 17]. Targeting multiple genes using gene therapy is highly challenging due to challenges of delivery as well as temporal and location-specific expression [18].

The inherent limitations associated with the individual therapeutic modalities could be overcome by combination therapies [19–21]. Researchers have been using

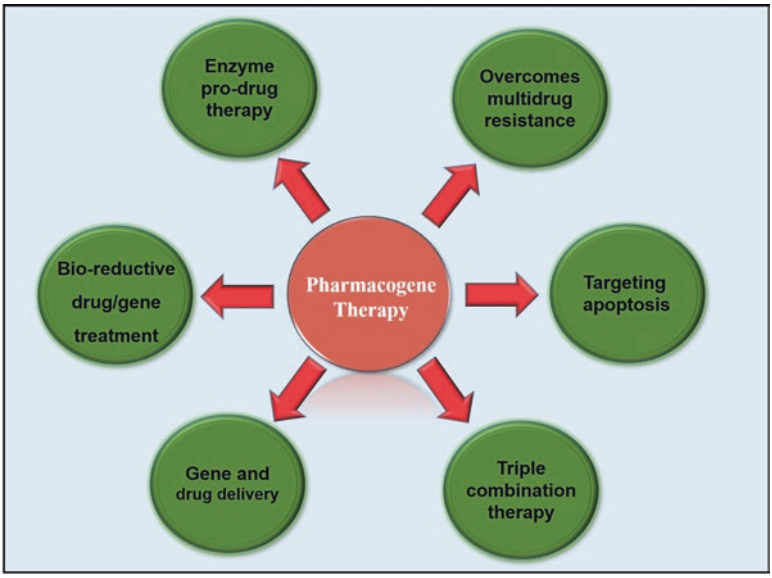


**Fig. 5.2** Different pharmacogene therapy approaches to achieve therapeutic benefit in different disease conditions

combinations of different therapies such as chemoradiation therapy, hyperthermia, surgical resection with chemoradiation therapy, gene therapy, and the use of different pharmacological agents for the delivery of drugs and genes, gaining remarkable success in the initial studies [22–24]. We discuss these combination therapies in the following sections. Since the topic "gene therapy" is being covered in other chapters, we will not expand on the gene therapy and vector delivery discussion in this chapter (Fig. 5.2).

## 5.2 Pharmaco-gene Therapy

Pharmaco-gene therapy entails the use of pharmacological agents or drugs to either supplement the gene therapy or its delivery and function in targeted disease treatment. The knowledge of gene functions from extensive research in various disease models has led us to design rational treatment strategies tailored to disease progression processes and organismal responses. Here, we broadly categorize all the gene function-based therapeutic strategies under the gene therapy moniker. There are many different forms of pharmaco-gene therapy where individual entities were used for different purposes based on the treatment requirements, delivery modalities, and molecular mechanisms of drug/therapeutic agent to achieve the desired outcome. The following sections discuss some of the key approaches and applications of pharmaco-gene therapy.

## 5.3 Overcoming Multidrug Resistance

Drug resistance is one of the major causes for the failure of chemotherapy against local and metastatic cancer [25]. The combination of multiple drugs and neoadjuvant therapy [26] is being used to overcome this problem; however, there remains a high probability for the patient to develop resistance even to other drugs or develop a relapse of the disease. A well-known mechanism attributing to multidrug resistance is the overexpression of multidrug resistance genes (MDRs) encoding for the efflux transporters which reduces the accumulation of drug molecule by pumping them out of the cell [27]. Inhibition of the expression of these genes by RNA interference followed by cytotoxic antitumor drug is an important strategy of pharmacogene therapy for the better efficacy of the therapy against cancer [28]. P-glycoprotein (Pgp) is the first identified MRD protein also known as MRDP1 encoded by the MDR1 gene used as a target against a number of drug-resistant cancers [29]. Inhibition of Pgp expression using siRNA along with the pharmaceutical drugs has been tried in vitro as well as in vivo by many researchers against the number of diseases. MDR1 siRNA with doxorubicin (DOX) was used in vitro and in vivo against human breast carcinoma, human cervical carcinoma, and human breast cancer [30–34]. Further, the combination of MRD1 siRNA with paclitaxel (PTX) was studied in human ovarian adenocarcinoma and mouse mammary gland adenocarcinoma with success [35, 36]. Additional MDR family members including MRP1, MRP2, and BCRP are also promising targets against drug-resistant cancer [37]. Certain cell signaling pathways such as Notch and c-Met among others also impart resistance to the cancer cell and targeted for the treatment against drug-resistant cancers [38] (Fig. 5.3).

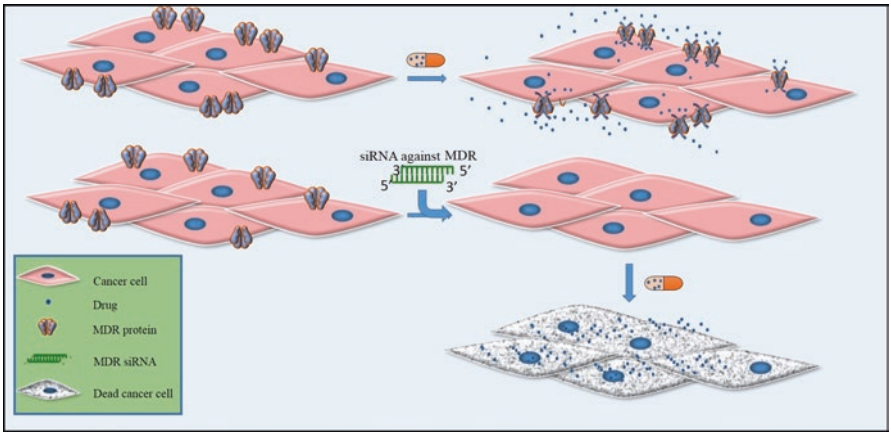


**Fig. 5.3** Pharmacogene therapy to overcome multidrug resistance: cancer cells expressing multidrug resistance genes (MDRs) encoding efflux transporters to pump out the drug used for its treatment and develops resistance against it. siRNA against MDRs inhibits the expression of these efflux transporters and enhances the therapeutic effect of chemotherapy

## 5.4 Targeting Apoptosis

Apoptosis is an important phenomenon through which cells undergo programmed cell death to maintain cell number and the healthy cellular population [39]. If cells are no longer required or if they encounter any kind of stress, or mutations which are beyond the control of the cellular machinery, the process of apoptosis is initiated leading to the death of the cell [40]. There are a number of pro-apoptotic and anti-apoptotic genes which regulate this process; however, they fail to do so in cancer resulting in an uncontrolled proliferation of cancer cells and growth of tumor mass which is characteristic of cancer [41]. One of the approaches of pharmaco-gene therapy against cancer is to target pro- and anti-apoptotic genes in combination with apoptotic drugs [42–44]. Most of the research involves either overexpression of tumor suppression genes such as p53, Rb (retinoblastoma protein), proteins inducing apoptosis like TRAIL (tumor necrosis factor-related apoptosis-inducing ligand), TNF-α (tumor necrosis factor-alpha), and BAX or suppression of anti-apoptotic genes such as survivin, Bcl-2, and cIAP (cellular inhibitor of apoptotic protein) [45]. P53 is a very common pathway found to be deregulated in many types of the cancer [46]. Herrero et at. 2016 used adenoviral-mediated p53 gene transfer with cisplatin and DOX chemotherapy which showed significant restoration of p53 function resulting in reduced cell viability. Increased sensitivity was observed in the p53 overexpressing cells when treated with cisplatin and DOX in the Saos-2, HOS, KHOS/NP, and MNNG cell lines [47] (Fig. 5.4).

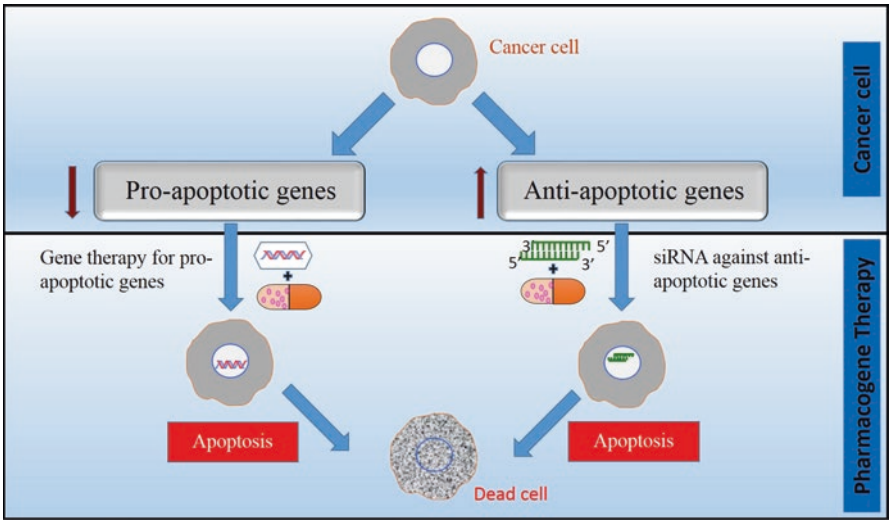


**Fig. 5.4** Pharmacogene therapy to induce apoptosis: Cancer cells overcome apoptosis either by over-expressing antiapoptotic genes or downregulating proapoptotic genes. A genes therapy approach towards pro-apoptotic genes or silencing anti-apoptotic genes using RNA interference in combination of chemotherapy induces apoptosis in the cancerous cells

Some researchers have also tried to over-express pro-apoptotic genes along with chemotherapy for cancer treatment. Zheng et al. 2011 delivered TRAIL to head and neck squamous cell carcinoma (HNSCC) cells using an AAV-2 vector and treatment with cisplatin. In vitro data showed enhanced apoptosis in the HNSCC cell lines when pretreated with cisplatin. Similarly, in a xenograft tumor model of nude mice, a significant reduction of tumor growth as well as higher sensitivity toward cisplatin chemotherapy was observed after AAV-2/TRAIL gene therapy [48]. Overexpression of the TRAIL gene in combination with DOX was also used to treat human ovarian cancer, liver cancer, and glioblastoma [49–51].

Another approach using TNF-α gene therapy in combination with DOX chemotherapy was done by Baowei et al. 2013 in vivo in the murine neuroblastoma, human hepatoma, and human colon carcinoma [52]. TNF-α is an antitumor cytokine which kills tumor cells directly and also by promoting blood vessel formation in the tumor making them more susceptible for the antitumor drugs by its accumulation in the tumor tissue. The TNF-α gene was delivered using polyamine-based polyplex targeting epidermal growth factor receptor (EGFR); DOX was delivered using lipoplexes: This combination showed an efficient treatment of tumor metastases [52].

Overexpression of anti-apoptotic genes was often associated with the failure of tumor chemotherapy. Inhibition of anti-apoptotic gene expression resulted in remarkable treatment outcomes when combined with chemotherapy. Nakamura et al. 2011 evaluated the therapeutic benefit of pharmaco-gene therapy by silencing Bcl-2 RNA in combination with chemotherapy of 5-fluoreuracil (5-FU) for the treatment of colorectal cancer in the DLD-1 cell lines as well as in an in vivo model of DLD-1-xenografting. The inhibition of DLD-1 cells was observed after transfection with Bcl-2 siRNA followed by 5-FU treatment. An oral formulation of tegafur, which is a prodrug of 5-FU, was used along with siRNA against Bcl-2 (coated on polyethylene glycol (PEG)-coated lipoplex) in a xenograft model. This showed a remarkable suppression of tumor growth compared to individual agents. It was observed that the daily treatment with tegafur enhanced the accumulation of PEG-coated siBcl-2 lipoplex in the tumor tissue, which clearly indicates that chemotherapy enhances intratumoral siRNA delivery and the delivered siRNA enhances the chemosensitivity of tumors [53]. Silencing of Bcl-2 in combination with either cisplatin, PTX, or DOX chemotherapy was studied in different studies in vitro and in vivo against human hepatic cancer, rat glioma, Ehrlich ascites carcinoma, human ovarian cancer, and human breast cancer [44, 54–57].

Similarly, silencing of survivin using lentiviral or non-viral vectors, combined with cisplatin or PTX chemotherapy, was also studied in HNSCC and mouse ovarian cancer and exhibited increased apoptosis and reduced tumor mass compared to their individual effect [58, 59].

## 5.5 Triple Combination Therapy

Conde et al. have applied the combination of gene, chemo, and phototherapy for the treatment of colon cancer to overcome the off-target effects of the conventional cancer treatment agents. They used prophylactic hydrogel patches to deliver the combined therapeutic agents to a murine cancer model which led to tumor remission when directly treated as well as led to prevention of tumor recurrence when applied following tumor resection [60]. Prophylactic hydrogel patch leads served as a better scaffold for the delivery of the therapeutic agents due to its adhesiveness and ability to provide a stable matrix for targeted delivery of embedded nanoparticles/drug moieties. The K-Ras oncogene was targeted using siRNA delivered by spherical gold nanoparticles, whereas rod-shaped gold nanoparticles facilitated the conversion of near-infrared radiation to heat that induced cytotoxicity and the induction of release of chemotherapeutic agent [60].

A combinatorial approach of pharmaco-gene therapy was made by Zhang et al., 2004, in his in vitro study on human primary cystic fibrosis (CF) bronchial epithelial cells, where pharmacological drugs were used to enhance the delivery of functional CFTR gene using rAAV and also suppressed ENaC activity through the mechanism independent of viral transduction. They used proteasome-modulating agents such as N-acetyl-L-leucyl-L-leucyl-L-norleucinal (LLnL) and doxorubicin which improved rAAV2 or rAAV2/5-mediated CFTR gene transduction and its function significantly. They also observed a dramatic decrease in the ENaC activity which was mediated by doxorubicin through the downregulation of γ-ENaC subunit mRNA expression and methylation of the promotor of γ-ENaC. This was the first report of utilization of pharmaceutical agent for their dual therapeutic action where the efficacy of gene therapy was enhanced as well as the pathophysiology of CF was also improved [61].

## 5.6 Gene Therapy to Enhance Bioreductive Drug Treatment

Many tumor cells are found to exist in very low levels of oxygen, termed “hypoxia,” which is thought to be one of the reasons for the failure of chemo- and particularly radiation therapy [62]. This is due to the fast dividing tumor cells and growing tumor mass which lacks the appropriate vasculature [63]. It was observed that hypoxia imparts tumor cells with a three times higher resistance to ionized radiation compared to normal well-oxygenated cells. When ionizing radiation hits oxygen, peroxides are generated causing tumor cell death [64]. Due to hypoxia there is a lack of oxygen molecules in the tumor parenchyma leading to inadequate outcome of radiation therapy [65]. Chemotherapy also has a poor outcome due to the lack of vasculature in those tumor sites. Consequently, the availability of drugs at the tumor site is reduced, impairing the desired therapeutic effect. Further, most of the

currently available chemotherapeutics target rapidly dividing cells which are considered to be lower in hypoxic tumor tissue. Another important adaptation of tumor cells in hypoxic conditions imparts the upregulation of hypoxia-mediated survival genes which enable tumor cell proliferation and survival. It also provides resistance against many cytotoxic drugs used for the treatment of solid tumors [66, 67].

Mitomycin C (MMC) is an indolequinone alkylating drug used for the treatment of a number of cancers such as lung, bladder, prostate, and breast cancer. Interestingly, MMC may be transformed (called bioreduction) into another substance which was not known for a long time. The bioreductive property of MMC is low but significant and selective toward hypoxia. Under hypoxic conditions the bioreduction of MMS is catalyzed by the one-electron reductase P450R leading to the generation of semiquinone, a free radical which covalently binds and cross links with DNA and induces cell death [68].

## 5.7 Enzyme Prodrug Therapy

Enzyme prodrug therapy is another aspect of pharmaco-gene therapy where gene-encoding enzymes are delivered with the tumor-specific promotor. This allows the conversion of a nontoxic or minimal toxic prodrug to the cytotoxic drug inside the tumor cell for the targeted killing of the tumor. This reduces the toxic effect of drug to normal cells and enhances the efficacy of the drugs against tumor [69]. Caution is warranted for choosing the optimal enzymes and prodrugs. Enzymes must express exclusively or relatively high levels in the tumor cells compared to normal cells to avoid off-target expression of enzyme. Similarly, prodrugs should have high affinity for the enzyme transduced than the endogenous enzyme and to be taken up effectively by the tumor cells. It should also produce potent cytotoxic metabolite with long half-life for their prolonged effect and should be having good distribution to maximize bystander effect for effective outcome [70]. Four well-studied and famous enzyme/prodrug systems are herpes simplex virus thymidine kinase (HSV-TK) with ganciclovir (GCV), cytosine deaminase (CD) of *Escherichia coli* with 5-fluorocytosine (5-FC), cytochrome P450 with cyclophosphamide/ifosfamide (CPA/IFA), and nitroreductase with CB1954 [69] (Fig. 5.5).

## 5.8 Herpes Simplex Virus Thymidine Kinase/Ganciclovir System

HSV-TK/GCV system is most widely studied enzyme prodrug system which involves the transfer of HSV thymidine kinase gene and GCV prodrug for the treatment of a number of cancers such as glioma, leukemia, bladder cancer, liver cancer, colon adenocarcinoma, and oral cancers [71–76]. Expression of viral thymidine kinase catalyzes the conversion of the prodrug GCV to monophosphoGCV which further gets converted to triphosphoGCV (an analogue of deoxyguanosine triphosphate (dGTP)), a cytotoxic drug which inhibits DNA polymerase or gets incorporated into the DNA

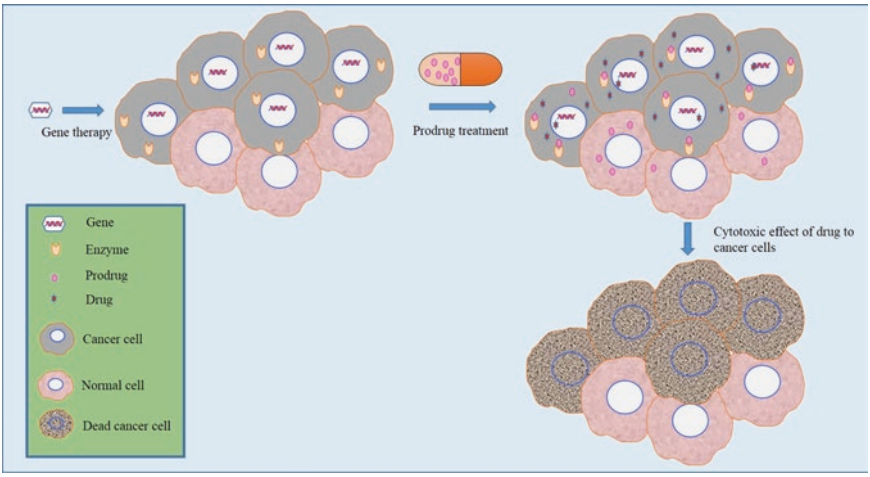


**Fig. 5.5** Gene-directed enzyme prodrug therapy: gene for the enzyme converting prodrug to the active drug is introduced to the cancer cells using gene therapy techniques with cancer-specific promotor which expresses enzymes only to the cancer cells. The prodrug is administered and gets converted to an active cytotoxic drug inside the cancer cells by the enzyme which was introduced by gene therapy. Active cytotoxic drugs specifically kill the cancer cells without harming the normal cells

during the replication leading to premature strand termination, replication failure and finally programmed cell death [77]. Another alternative of GCV is acyclovir which was also used as a prodrug for the treatment of ovarian cancer cell line and has better bystander effects compared to GCV [78].

## 5.9 Cytosine Deaminase/5-Fluorocytosine System

Cytosine deaminase is an enzyme found in bacterial and fungi which promotes the enzymatic deamination of cytosine to uracil. This property of the enzyme was utilized for the therapeutic purpose to convert 5-flurocytosine (5-FC) to the highly cytotoxic cancer drug 5-fluorouracil (5-FU). 5-FC is the only prodrug which is used in this system. Introduction of CD gene to the tumor cells expresses enzyme CD which deaminates the prodrug 5-FC to 5-FU which is then converted to the 5-fluorodeoxyuridine 5-monophosphate (5-FdUMP), 5-fluorodeoxyuridine 5-triphosphate (5-FdUTP), and 5-fluorouridine 5-triphosphate (5-FUTP) by cellular enzymes. Cytotoxic effects are attributed either by inhibiting thymidylate synthase or by the formation of 5-FU RNA and DNA complex [79, 80]. This system was studied in a wide variety of in vitro and in vivo animal models of cancers such as MDA-MB-231 breast carcinoma cells, breast carcinoma xenografts in nude mice, and intracranial human glioma xenografts in severe combined immunodeficiency (SCID) mice, hepatic metastases of colon carcinoma, and prostate cancer [81–83].

## 5.10 Cytochrome p450/Oxazaphosphorine System

CYP enzymes play a vital role in drug metabolism in the liver and are utilized to activate a number of prodrugs used for the treatment of cancer. Oxazaphosphorines cyclophosphamide (CPA) and ifosfamide (IFA) are the common prodrugs which get activated by CYPs. CYP2B6 and CYP3A4 catalyzes the hydroxylation of CPA and IFA, respectively, to form their 4-hydroxy derivative which further gives rise to a cytotoxic phosphoramide mustard and acrolein. Though phosphoramide mustard does not diffuse across the cell membrane and shows a reduced bystander effect, the 4-hydroxy product of CPA and IFA can cross the cell membrane and is used for therapeutic purpose. Overexpression of CYPs genes using a gene therapy approach can efficiently catalyze the hydroxylation of CPA and IFA with enhanced bystander effect yielding a significant therapeutic benefit. CPA is mostly used for the treatment of lymphoma, leukemia, multiple myeloma, neuroblastoma, retinoblastoma, ovarian cancer, breast cancer, and endometrial cancer; IFA is commonly used for the treatment of soft tissues sarcomas, testicular, ovarian, and breast cancer [69].

## 5.11 Delivery Vehicle for Drug and Gene

Cancer formation is considered to be caused by mutations in multiple genes and involving a multitude of signaling pathways. As a consequence, monogenic therapies have a high risk of failure. Monogenic therapies are almost always combined with chemotherapy, radiotherapy, and other therapeutic modalities. Delivery of the gene or siRNAs and drug moieties together is a big challenge as the physicochemical properties of both of them are very different. Apart from their physicochemical properties, other parameters which should be considered are (a) kinetics of the individual moieties, (b) order of releases either simultaneous or in sequence, and (c) their mutual compatibilities. The conventional delivery vectors such as lentiviruses, adenoviruses, and adeno-associated viruses on one hand have a high transduction efficiency and expression of the transgene. On the other hand, it is difficult to conjugate pharmaceutical agents to them directly for simultaneous exposure. Therefore, non-viral vectors have been studied to allow easy chemical manipulation and the delivery of both, gene and drug. They also serve the purpose of controlled release of individual agents with the desired sequence and are safer than the conventional viral vectors. Jing Li et al. have reviewed nanocarriers such as polyplexes, liposomes, micelles, and nanoparticles for the delivery of drug and nucleic acids combination for the treatment of cancer [24].

## 5.12 Co-encapsulation of Nucleic Acids and Drugs in Biodegradable Polyester Particles

Polyesters have achieved remarkable significance in the field of drug delivery due to the possibility to manipulate their chemical and physical properties as desired, allowing controlled release of therapeutics, multi-route of administration and

biodegradability which makes it safer to use as delivery vehicle for a number of therapeutic applications [84]. Its ability to deliver drugs as well as genes was tested in vitro and in vivo for the treatment of tumor resistant to chemotherapy by Patil et al., 2010. They used functionalized poly (D,L-lactide-co-glycolide) (PLGA) nanoparticles with biotin on the surface for the targeted delivery of siRNA against MDR1 gene to overcome the drug resistance and paclitaxel an antitumor drug. The in vivo data showed a significant reduction in tumor growth [36]. Another approach to co-deliver doxorubicin (DOX), an anticancer drug, and p53 gene (tumor suppressor gene) was made using double-walled microspheres composed of a PGLA core encircled by a poly-lactic acid shell. Tumor suppressor gene p53 was encapsulated in the shell by using chitosan-DNA nanoparticles, whereas, DOX was encapsulated in the core to get sequential release of p53 gene followed by DOX at close to zero order rate [42]. Zhang et al., 2016 used epidermal growth factor (EGF)-modified monomethoxy (polyethylene glycol)-poly (D, L-lactide-co-glycolide)-poly(L-lysine) (mPEG-PLGA-PLL, PEAL) nanoparticles (EGF-PEAL) for the co-delivery of siRNA for Bcl-2 gene, a well-known inducer of apoptosis in the cancer cells, and DOX for the treatment of lung cancer. They observed a gradual and sustainable release of both DOX and Bcl-2 siRNA and apoptosis in H1299 cells and reduced tumor growth in the H1299 xenograft mice using EGF-PEAL nanoparticles for the co-delivery of DOX and Bcl-2 siRNA [43].

## 5.13 Delivery Systems Based on Complexes of Nucleic Acids with Cationic Lipids and Polymers

Cationic lipids and polymers form complexes with nucleic acids which are known as lipoplexes and polyplexes, respectively, which do not encapsulate small drug molecules. However, increasing the hydrophobicity of these complexes allowed the drug to be incorporated in the form of inclusion complexes, also directly conjugating them to the polymeric vectors through a biodegradable linker [30, 51]. Han et al. 2012 have tried functionalized polyamidoamine (PAMAM) with hyaluronic acid (HA) for the delivery of siRNA against MVP (major vault protein) gene and DOX for the treatment of drug-resistant breast cancer. Enhanced cytotoxic effect of DOX has been observed in breast tumor cells because of the inhibition of MVP expression which involved the drug resistance in the in vivo model [85].

## 5.14 Chimeric Peptide for Delivery of Gene and Drug

Kai Han and team have developed and characterized an amphiphilic chimeric peptide $(Fmoc)_2KH_7$-TAT with pH-responsibility (dependency on specific pH levels) for use as a vehicle for combined gene and drug delivery. They found that $(Fmoc)_2KH_7$-TAT exhibits faster release of doxorubicin (DOX) at pH5 which is the pH of tumor cells (compared to normal tissue-pH7). $(Fmoc)_2KH_7$-TAT also served as a non-viral vector for gene delivery with satisfactorily transfecting 293T and

HeLa cells with the pGL-3 reporter plasmid (with or without serum). It also displayed endosome escape capability of the peptide/DNA complex. The efficiency of $(Fmoc)_2KH_7$-TAT for co-delivery of p53 plasmid and DOX for their synergistic effect was assessed for the treatment in tumor cells using in vitro and in vivo studies. In vitro studies showed a significant inhibition in the growth of HeLa cells, which was also reproduced in an in vivo experiment where tumor growth was reduced in the H22 tumor cells implanted in mouse model [86].

## 5.15 Summary

Pharmaco-gene therapy has the potential to harness the best of both worlds, the efficacy and reduced side effects of targeted gene delivery and the easy delivery and broad effectivity of small molecule drugs. Until today only few studies are available in this area. The obvious ingenuity of such combinatorial approaches is gaining relevance in treatment of a number of conditions, particularly cancers. These approaches also have potential applications in advanced monogenic inherited disorders where a small molecule drug may increase the effectiveness of a gene or cell therapy by modulating the local tissue degenerative and inflammatory processes. In summary, these combination strategies require additional exploration and are the way of effective future treatment modalities.

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# Aptamer as Therapeutics for Cancer with Focus on Retinoblastoma

6

Nithya Subramanian, Akilandeswari Balachandran, and Krishnakumar Subramanian

## 6.1 Introduction

In spite of the remarkable advances in the understanding of the disease, a tangible solution for cancer is yet to be attained. The current chapter tries to briefly summarize various methods available for cancer therapy followed by comprehensive analysis of aptamers and its implication in cancer therapy. Later this chapter deals with recent advancements in the aptamer-based applications in ocular diseases majorly focusing on the eye cancer, retinoblastoma.

### 6.1.1 Conventional Chemotherapy

Combinational chemotherapy is the main strategy in the management of both solid and liquid cancers. This is complemented with other forms of therapy where the bulk of the tumor mass is removed in some forms of solid cancers in addition to other forms of therapies such as cryotherapy [14], brachytherapy [179, 182], and hormonal therapy [9, 89, 260]. However, there are limitations in the current chemotherapy drugs because of the side effects which add to the morbidity of chemotherapy such as cytopenias and damage to the gastrointestinal tract and hair. Some of the chemotherapy drugs such as etoposide induce secondary malignancies such as acute

N. Subramanian
PNAC Department, MRC – Laboratory of Molecular Biology, Cambridge, UK

A. Balachandran
Department of Nanobiotechnology, Vision Research Foundation, Chennai, India

K. Subramanian (✉)
L&T Department of Ocular Pathology, Department of Nanobiotechnology, Vision Research Foundation, Chennai, India
e-mail: drkk@snmail.org



G. R. Jayandharan (ed.), *Gene and Cell Therapy: Biology and Applications*,
https://doi.org/10.1007/978-981-13-0481-1_6

myeloid leukemia [206]. The nonspecific delivery-mediated side effects of chemotherapy urged researchers to focus on alternate targeted strategies.

### 6.1.2 Targeted Therapy

Targeted therapy is now accepted as standard of care in oncology. A few examples of currently given targeted therapy drugs include Herceptin for a subset of breast cancer expressing HER2 [29] and rituximab, a monoclonal antibody for CD20-positive lymphomas [213]. However, with the confidence gained by these targeted therapy drugs over the years and the expanding knowledge gained by studying the genomics [111, 114, 194, 217, 334] and proteomics of the primary tumor samples and the relevant cell lines, newer targets are being identified [167, 175, 268, 269, 323, 338, 344]. Newer techniques like the OncoScan® array that has been introduced as a tool for identification of copy number variation and loss of heterozygosity in formalin-fixed paraffin-embedded oncology samples [8, 135, 209, 252, 253, 327] will enable identification of more novel targets for therapy in cancer.

### 6.1.3 Novel Ligands for Cancer Therapy

Ligands are developed for specific targets to deliver the payloads or functionally block the target's activity or alternatively used for diagnosis. The cellular targets identified could be either mRNA or miRNA or proteins toward which novel ligands are developed. Until now antibodies regard as best ligand for cancer therapy and they include humanized recombinant antibodies [6, 38, 133] or single-chain fragment variable (scFv) fragment [5]. The antibody fragments can also be useful in the conjugation and delivery of immunotoxins, therapeutic genes, and anticancer intrabodies for therapeutic purposes [352].

Further research and investigation resulted in the next generation of antibodies such as the affibodies and lambodies. Affibodies are small protein domain that has the ability to bind specifically to different target proteins and are selected using different combinatorial approaches [65, 196, 198]. They are used in biochemical research also in the imaging and therapy of cancers and are being developed as potential new biopharmaceutical drugs [7, 97, 256].

Lambodies are unique variable lymphocyte receptors (VLR) monoclonal antibodies developed using lampreys that has high affinity and target specificity for glycan determinants. Glycan-binding proteins play vital role in the various disciplines of basic research and clinical applications [99]. Lambody VLRB.aGPA.23 preferentially detects cancer-associated carbohydrate antigen in cancers. Lambodies with excellent affinity and selectivity for glycans may find innumerable uses in biomedical research [289].

The next category of ligands is the designed ankyrin repeat proteins (DARPins). DARPins are non-immunoglobulin proteins that possess several advantages over

antibodies for target binding in drug discovery and development [90, 100, 261, 266]. DARPins are engineered against the target proteins such as HER2 [255] and EpCAM [161]. DARPins offers advantage over antibodies by exhibiting elevated accumulation in the tumor with low tissue contrasts, whereas intermediate-sized proteins like scFvs show quick clearance but only moderate tumor accumulation [265, 335].

## 6.2 Aptamer

Aptamer (apto, meaning "to fit," derived from Latin) is a single-stranded nucleic acid molecule and acts similar to antibodies. These molecules are 20–80 nucleotides in length that binds its targets with high affinity and selectivity.

### 6.2.1 Discovery of Aptamer

Professor Larry Gold and Craig Tuerk and Andrew Ellington and Jack Szostak artificially selected nucleic acid ligands from a diverse pool of RNA sequence that binds T4 DNA polymerase. Professor Larry Gold and Craig Tuerk called this approach as Systematic Evolution of Ligands by EXponential enrichment (SELEX) [299]. Independently Andrew Ellington and Jack Szostak worked on in vitro selection procedure for the generation of nucleic acid moieties against small molecules and they coined the term "aptamer" [66]. Bruce Sullenger also elucidated individually that decoy RNA can compete with HIV-1 TAR sequence to bind tat protein [279]. Ellington and Szostak later did the selection of single-stranded DNA (ssDNA) ligand and observed that ssDNA was able to bind to targets similar to RNA [67].

### 6.2.2 Advantage of Aptamers

Aptamers have advantage over other ligands, including ease of synthesis, low molecular weight, high stability, lack of immunogenicity, and possibilities of modification and manipulation compared to their protein equivalents. Aptamer development has been significantly facilitated by recent developments such as automated solid-phase synthesis that allows commercial scale of synthesis at relatively low cost [301]. Finally, the activity of aptamers can be easily modified by an antidote (e.g., a complementary oligonucleotide), a feature that holds beneficial for drug design and development [230]. Aptamers fold into a special three-dimensional (3D) confirmation due to intramolecular interaction, and this allows their binding to target proteins with increased affinity and specificity [229]. These features enable aptamers as ideal candidates for molecular probes, to identify extracellular matrix signatures of cancer cells and target cell-specific ligands for therapeutic purposes [154].

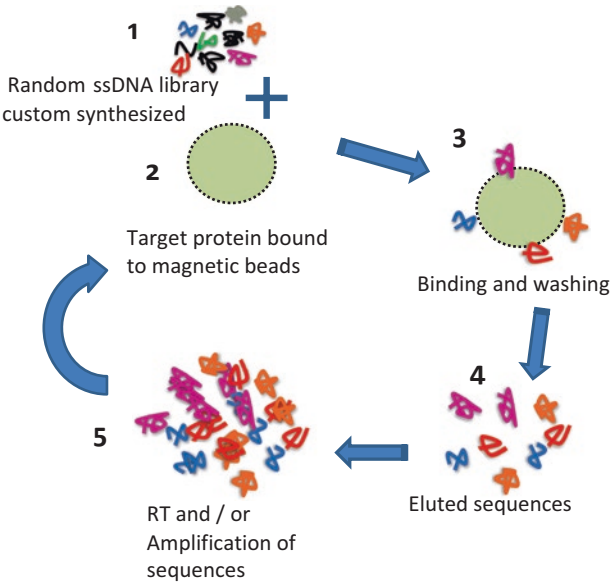


**Fig. 6.1** In vitro SELEX procedure. The steps involved in the in vitro method use random library (1) and magnetic beads or beads coated with target protein (2). The protein and library are left to interact (3) followed by eluting the bound sequence (4) for reverse transcribing and PCR amplifying (5). The steps 2 to 5 are repeated until the enrichment is attained

### 6.2.3 Methods of Aptamer Selection

The SELEX procedure is well standardized for selecting DNA or RNA ligands against recombinant proteins. Automation of this process had led to aptamer selection very rapid [72]. It involves rounds of standard steps, while the RNA ligand development includes reverse transcription step in each round. The SELEX procedure basically involves iterative cycles of selection, resulting in the generation of high-affinity ligands to a specific target. The development of magnetic beads has enormously shortened the SELEX procedure. The sequence of steps in SELEX begins with (1) synthesis or preparation of nucleic acid library for selection; (2) preparing the target, either coating the magnetic beads covalently with small molecule or protein or nucleic acid molecule of interest or preparing bacterial or mammalian cells; (3) binding of the library with the target; (4) eluting the bound molecules; and (5) reverse transcribing and/or amplification and generation of next round library for the selection. The second round repeats the step 2 to step 5 followed by consecutive rounds (Fig. 6.1), while the libraries of different rounds are assessed for the enrichment before proceeding for sequencing.

Once the selection is assessed to have enrichment, sequencing of the various rounds will yield sequences to further work on post-SELEX modification. The post-SELEX modifications include truncation of the flanking primer sequences, nuclease resistance modification, and modifications to increase affinity. Modifying the

sequences for nuclease resistance may lead to changes in the structure, thus abrogating its activity or decreasing its affinity. The nuclease resistance modifications include 2'fluoro, 2'O-methyl, and 2'C-4'C locked nucleic acid [122]. Hence, the use of nuclease-resistant modified libraries for selection is to yield better resistant aptamers. This also includes a challenge of replicating these nuclease-resistant modifications, which is addressed by some groups by developing mutant T7 RNA polymerase and DNA polymerases [49, 168].

### 6.2.4 Improvement in Aptamer Technology Is Constant

SELEX is an in vitro procedure that employs repeated rounds of selection to bind several targets such as proteins, nucleic acids, small molecules, mammalian cells, bacterial cells, and other biomolecules. Initially in vitro SELEX procedure was used for the selection of DNA/RNA ligands using nucleic acids, recombinant proteins, or small molecule targets. Later due to the growing needs, aptamer selection protocols were constantly modified to generate therapeutic aptamers specific for a disease. Aptamer selected against a recombinant protein versus an aptamer selected in vivo and with primary tumor cells are better due to their in vivo characteristics. This process of aptamer selection using cells is referred as cell-SELEX technology. Numerous aptamers were identified using the cell-SELEX technology. Many of these aptamers are useful for molecular imaging and in biomarker targeting [17, 41, 139, 140, 149, 207, 225, 237–239, 244, 245, 286–288, 312, 315, 324, 347]. A noteworthy application includes the use of in vivo SELEX for the development of aptamers targeting metastatic tumor model and brain tissue [44, 169].

#### 6.2.4.1 Microfluid-Based SELEX

SELEX can be integrated into microfluidic chip using magnetic capture-based systems. This enables identification of aptamers with high affinity and a lower KD [46, 199]. Next-level advances in aptamer screening have completely engineered chip-based in vitro selection platform, based on magnetic separation of target coupled with magnetic beads using a continuous-flow magnetic separation device to select aptamers automatically against the protein targets. This setup efficiently combines the magnetic separation, microfluidics, mixers, and temperature control systems for enzymatic replication/amplification reactions. This unified novel platform helps to discover aptamers against complex target proteins [103].

#### 6.2.4.2 Cell-SELEX

Cell-SELEX is a modification of SELEX technology which allows the selection of oligonucleotide (DNA/RNA) aptamers based on the binding affinity with the target proteins present on cell surface (Fig. 6.2). This technology has several advantages over the native SELEX where the aptamers are developed to specific recombinant protein. (i) The technology does not require prior knowledge of target proteins expressed. (ii) The aptamers are raised against cell surface protein in its native conformation as against the recombinant proteins hence a better binding affinity can be

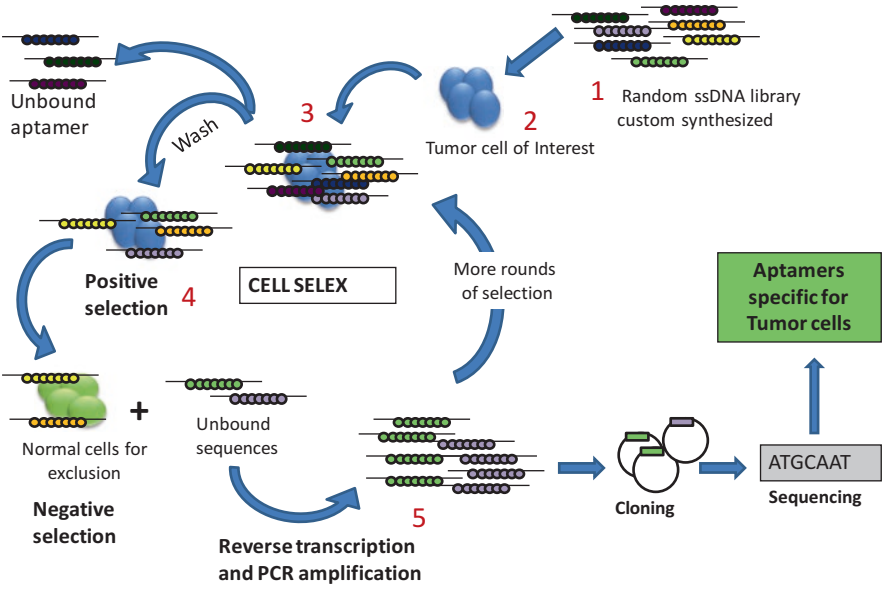


**Fig. 6.2** A schematic illustration of cell-SELEX method for isolating aptamers against tumor cells. Preparation of library (1) cells (2) and incubating together (3) followed by washing and collection of bound sequences (4). The sequences are reverse transcribed and/or PCR amplified (5) for further rounds of selection. Finally the selected library is subjected for cloning or sequencing

expected. (iii) The aptamers designed may be more specific and efficient in tumors that are heterogenous, as they are not designed against a single protein and selected based on their affinity to the tumors as against the normal cells. One of the complexities with cell-SELEX is that high-end molecular biological methods have to be employed to decipher the targets to which it can bind. However, this technology is preferred when a large amount of heterogeneity is expected within the tumor and the number of positive and negative screens can be increased to select the best possible aptamers to a particular cell population.

The sequence flanking the random region has possibility to interact with it and hence the post-SELEX modifications were carried out. The post-SELEX modifications include truncating or reducing the flanking region sequence [204], modifying the nucleotides for nuclease stability and hence they are laborious. Numerous strategies are now used to minimize this and newer techniques like capture-SELEX are evolving, which reduces the need for complex post-SELEX modification [262]. Aptamer conformation and optimization are done with the help of next-generation sequencing data analysis tools [46, 95, 104, 293–295].

Aptamer discovery have been improved with the high-throughput sequencing and computational analysis of SELEX data. Software tools, such as COMPAS, FASTAptamer, APTANI, AptaTools, etc., enable processing of high-throughput sequencing data generated from SELEX in the labs lacking bioinformatic expertise.

Combining diverse applications and their integration in one platform in spite being a bigger challenge will enable the discovery of more aptamers in the future [19].

#### 6.2.4.3 Aptamer Microarray

Aptamers can be used for developing aptamer microarray. This will have many advantages over antibody-based protein microarrays. Aptamer has many advantages due to cost-effective synthesis and so on. Aptamer microarray can be used for studying protein-protein interactions [228]. Huh et al. used aptamer microarray in colon cancer and were able to stratify the disease to high risk and low risk [108]. SomaLogic Incorporation has developed aptamer microarray for discovery of biomarkers and also for clinical applications [203, 318]. Thus aptamer-based microarray technology holds application in proteomics and metabolomics research.

## 6.3 Aptamer in Cancer

Aptamers have found varied applications from use in infectious disease diagnosis to toxin detection, and use in defense for explosive detections [125, 136, 188]. This part focuses on the applications of aptamers in cancer diagnosis and therapy.

### 6.3.1 Aptamer Isolated to Potential Cancer Targets

Both RNA and DNA aptamers have found to have functional properties. This means these aptamers bind to a surface protein expressed on the cancer cells and intercept the signaling pathways [73]. Thus these aptamers are desired for further studies. The other important desired properties of these aptamers are that they should be biocompatible and nontoxic and there must be no off-target effects or elicit immune responses. Another class of aptamers is the binding aptamer, which does not intercept with cellular processes or elicit cytotoxicity. The illustration shown in Fig. 6.3 summarizes the receptor ligand interaction and use of aptamers to inhibit the cellular pathways. Also the binding aptamers are widely utilized for imaging purposes in cancer and other diseases [277]. Some of the important functional and binding aptamers in cancer therapy are being discussed in the following sections.

#### 6.3.1.1 G-Quadruplex Aptamer

The significance of G-quadruplex structure in aptamer was elucidated against thrombin [313]. G-quadruplex aptamers reported so far with clinical importance include thrombin aptamer, nucleolin, HIV-1 integrase, VEGF, and so on. G-quadruplex structure is crucial for the function and stability of these aptamer. G-quadruplex is classified into two topologies – parallel and antiparallel. Parallel G-quadruplexes have four strands in the similar direction, and antiparallel quadruplexes comprise four strands in opposite direction [298].

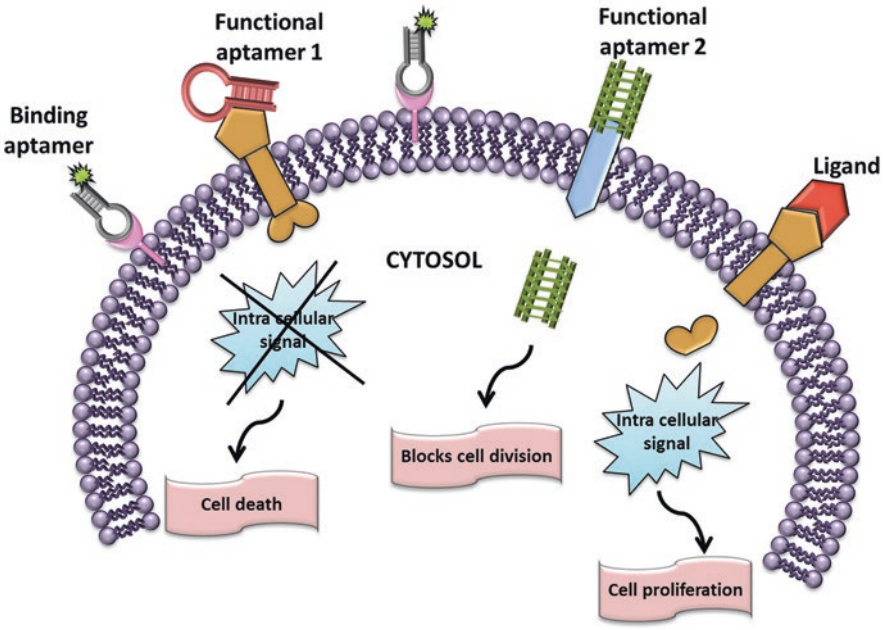


**Fig. 6.3** Functional and binding aptamers. Binding aptamers selected against membrane proteins can be used for cancer cell imaging purposes. Functional aptamer 1 binds to the receptors and prevents intracellular signaling pathways leading to apoptosis or necrosis of cancer cells. Functional aptamer 2 binds and gets internalized in cancer cells through receptors and blocks the signaling pathways related to cell division/proliferation

### AS1411-Nucleolin Aptamer

AS1411 is a **G-quadruplex** 26-mer DNA aptamer. AS1411 targets the protein nucleolin [15] which plays essential part in cell growth and death through its association in rRNA transport and DNA replication, transcription, and recombination [283]. Nucleolin, a protein that resides in the nucleus and cytoplasm in normal cells, is found to be overexpressed in the plasma membrane in several types of cancer, including breast cancer, lung cancer, prostate cancer, hepatocellular carcinoma, and lymphocytic leukemia [348]. AGRO100, though being developed at Aptamera, was renamed as AS1411 after procurement by Antisoma. In May 2011, AS1411 was further acquired by Advanced Cancer Therapeutics (ACT) and later regarded as ACT-GRO-777. AS1411 inhibits the proliferation of extensive range of cancer cell lines by potentially disrupting the interaction of nucleolin with its binding partners. Cancer cell death induced by AS1411 has been anticipated to include aptamer internalization through membrane or cell surface nucleolin followed by interference with DNA replication leading to S-phase arrest and by the stabilization of the mRNA of B-cell lymphoma protein 2 (BCL-2) [15, 348]. AS1411 exhibits minimal toxicity in patients with progressive solid tumors [134] and is currently in phase II clinical trials for both acute myeloid leukemia (AML) and metastatic renal cell cancer [348].

#### Stat3 Aptamer: T40214

Signal transducer and activation of transcription 3 is an oncogene which is triggered in many malignant epithelial cancers [23]. Activated stat3 upregulates the expression levels of antiapoptotic proteins such as Bcl-xl and MCL-1 thereby reducing spontaneous apoptosis in cancer [26]. A G-quadruplex aptamer was observed to inhibit DNA-binding activity of Stat3 with an IC50 of 7 μM and reduced Stat3 signaling [117]. The Stat3 G-quadruplexes were modified later and were observed to inhibit tumor progression in prostate, breast, and non-small cell lung cancer [116, 118].

### 6.3.1.2 Non-G-Quadruplex Aptamers

#### Platelet-Derived Growth Factor Receptor β-Gint4.T Aptamer

PDGFR β belongs to platelet-derived growth factor signals (PDGRs). PDGFRβ overexpression, deletions, point mutations, and translocations play a crucial role in tumorigenesis [81, 202]. Targeted inhibition of PDGFRβ signaling cascades showed significant anticancer effects [243]. PDGFR β signaling is observed in glioblastoma multiforme (GBM) [31]. This tyrosine kinase receptor present on the cell surface plays significant role in cancer cell proliferation, migration, and tumor angiogenesis. PDGFR β depletion reduces the glioma stem cell population. Currently monoclonal antibodies such as imatinib are available for targeting PDGFR β. However, there are limitations due to the toxicity and the development of drug resistance [50, 53, 222].

Camorani et al. fabricated a 2′-fluoropyrimidine (2′F-Py) RNA aptamer which is resistant to nuclease using highly tumorigenic U87MG GBM cells by a differential cell-SELEX approach. Gint4.T is a truncated RNA aptamer (Kd: 9.6 nmol/l) and is truncated to 33 mer from the original aptamer sequence. The RNA aptamer is bound to PDGFR β extracellular domain and strongly inhibits receptor activation and downstream signaling in GBM cells using both in vitro and in vivo model. Thus Gint4.T aptamer is a potential therapeutic aptamer [31].

#### Axl Aptamer: GL21.T Aptamer

The TAM receptors comprising of unique family of receptor tyrosine kinases like Axl, Tyro3, and Mer are potential targets [141]. Axl is an oncogenic kinase and signaling pathways such as FOS and YAP are involved and helps in cancer cell survival, proliferation, invasion, and migration in several solid epithelial cancers [234, 328]. Currently small molecules and recombinant antibody are available for targeting Axl receptor [96, 332] that can interfere with Axl signaling and regards as novel tumor therapeutic candidate [307].

Cerchia L et al. isolated an RNA aptamer to Axl using cell-SELEX approach on human glioma cell lines (U87MG cells). The first aptamer isolated was GL21 a 2′-F Py-modified RNA aptamer that was 92 mer that bound to glioma cells with a *Kd* of 221 nmol/l [35]. It was later truncated to generate a 34 mer aptamer called GL21.T retaining the GL21 active site and the high binding affinity for extracellular domain of-Axl ($K_d$ of 13 nmol/l) to the glioma cells. The aptamer inhibited cell invasion and migration and interfered with spheroid formation by abrogating the Axl-mediated signaling in cancer cells and inhibited tumor proliferation in a human non-small cell

lung xenograft mouse model [34]**.** GL21.T aptamer is a propitious RNA-based molecule alternative to existing Axl inhibitors.

### HER2 (ErbB2) Aptamer

HER2 refers to human epidermal growth factor receptor 2 which is expressed in a subset of the breast cancer and associated with poor prognosis [254, 255]. Recombinant humanized antibody trastuzumab (Herceptin) is currently used for HER2-positive patients. However there are limitations due to the development of drug resistance and side effects such as cardiotoxicity [242, 310]. Three RNA and two DNA aptamers targeting HER2 protein are reported so far.

#### S6 RNA Aptamer

Kang et al. developed RNA aptamers using cell-SELEX strategy, which are specific for HER-2-overexpressing breast cancer cell line, SK-BR-3. The screened RNA aptamers exhibited strong affinity to SK-BR-3, but not to HER-2-underexpressing breast cancer cell line MDAMB-231. The Kd value of S6 aptamer was determined as 94.6 nmol/l and had 40 nucleotides. This aptamer was not found to have any functional property [121]**.**

#### SE15-8 and Mini-aptamer

Kim et al. isolated a RNA aptamer longer than 50 bases for extracellular domain of ErbB2/HER2 protein; RNA aptamer SE15-8 exhibited a binding affinity of 10 nmol/l. The truncated aptamer is called as mini-RNA aptamer (mini-aptamer) with 34 nucleotides. The binding affinity of the mini-aptamer is $3.49 \pm 1.3 \times 10$ nmol/l. This aptamer does not show any functional property in the in vitro experiments [126]. Another HER2 RNA aptamer was isolated using mouse mammary carcinoma cell line which expresses rat-HER2 using cell-internalization SELEX. This aptamer did not have any functional property [293].

#### HeA2_1 and HeA2_3

Gijs et al. generated a 40-mer DNA aptamer specific to HER2 from whole cell-SELEX using SKBR3 human breast cancer cells. The binding affinity coefficient (Kd) was determined as 28.9 nM for aptamer HeA2_1 and 6.2 nM for aptamer HeA2_3. Both the aptamers showed affinity to HER2 protein at nanomolar range. They were shown to bind to cells overexpressing HER2 and primary tumor samples. The aptamers showed specificity for the tumor tissue in the animal model and they reduced tumor growth suggesting they had functional property over HER2 signaling [80].

### EGFR Aptamer: CL4 Aptamer

EGFR (epidermal growth factor) is a family of receptor tyrosine kinases and has four members: EGFR/ErbB1/HER1, ErbB2/HER2, ErbB3/HER3, and ErbB4/HER4 [107]. It contains three regions, an extracellular region, a single membrane region, and an intracellular tyrosine kinase domain. Epidermal growth factor binding to EGFR [331] leads to EGFR dimerization, thereby activating multiple intracellular pathways that promote survival and proliferation by inhibiting apoptosis [152]**.**

Overexpression of EGFR or constitutive activation stimulates tumor progression including angiogenesis and metastasis. EGFR receptor is expressed in colorectal cancers, head and neck squamous cell carcinomas, and non-small cell lung cancer (NSCLC) [107]. Currently FDA-approved monoclonal and small molecule inhibitors are not specific and they influence multiple pathways, and the patients on these medications develop acquired drug resistance [58, 138, 260]**.**

Esposito et al. developed a new RNA aptamer referred as CL4 using cell-SELEX which has the potential to bind and inhibit EGFR. CL4 aptamer binds to EGFR expressed on the tumor cell surface as well as the soluble EGFR receptor extracellular domain with an affinity of 10 nmol/l. The aptamer is very specific to inhibit the activation of EGFR and EGFR-mediated signaling pathways in cancer and inhibited the growth of tumor in xenograft mouse model of human non-small cell lung cancer (NSCLC). Aptamer treatment in addition to cetuximab clearly showed synergistic effect in inducing apoptosis both in vitro and in vivo [71]. This aptamer is a promising biomolecule which can be developed as an alternative to the existing EGFR inhibitors.

**Osteopontin Aptamer (OPN-R3)**

Osteopontin (OPN), an acidic hydrophilic glycophosphoprotein, is overexpressed in tumors and in advanced metastatic cancers and also found to be secreted by malignant cells [4, 25, 47, 54, 59, 74, 86, 87]. OPN is an important molecule playing a vital role in migration of tumor cells, and it signals using two cell adhesion molecules $\alpha_v\beta_3$ integrin and CD44 [60, 61, 319]. OPN is considered as a potential therapeutic target for the regulating metastasis in cancer. Zhiyong Mi et al. developed a RNA aptamer OPN-R3 which was identified against human OPN using SELEX technology. It was reported with a kd of 18 nmol/l, and it prevents the binding of the OPN to its cell surface receptors CD44 and $\alpha_v\beta_3$ integrin receptors. As a result of exposure to OPN-R3, many components of the OPN signaling pathways like PI3K, JNK1/2, Src, and Akt, mediators of extracellular matrix degradation, matrix metalloproteinase 2 (MMP2), and uroplasminogen activator (UPA) are very much reduced. OPN-R3 reduces breast cancer progression in animal models [172]. Subsequently, Talbot et al. showed the efficacy of OPN-R3 against the breast cancer cells by reversing its growth with favorable PK stability for clinical application [285]. Thus, OPN-R3 aptamer looks promising.

**C-Met Inhibiting Aptamer: ssDNA Aptamer CLN3 and CLN6 RNA Aptamer**

The c-Met receptor, also referred as hepatocyte growth factor receptor, belongs to the receptor tyrosine kinase family [20] and consists of ligand-binding extracellular domain and intracellular kinase domain, respectively. C-Met receptor is triggered by its ligand hepatocyte growth factor (HGF) followed by dimerization and phosphorylation within the intracellular kinase domains [105]. The C-Met receptor is expressed or deregulated in human cancer and it plays an important role in cellular signaling pathways.

Boltz et al. earlier had fabricated CLN3, ssDNA aptamer specific to c-Met that might efficiently mediate cell lysis in a bi-specific manner with CD16α aptamer

[21]. Later Piater et al. generated a RNA aptamer CLN 64 by using SELEX technology. CLN 64 $K_D$ value is 7 nM. They observed that CLN64 and CLN3 showed higher specificity and affinity to both recombinant and cellular expressed c-Met. These aptamers inhibited HGF-dependent c-Met activation, signaling, and cell migration effectively [211].

#### PSMA Aptamer: A9 Aptamer

Prostate-specific membrane antigen (PSMA) is expressed on the surface of prostate cancer cells. PSMA levels correlate with clinicopathological features including the histological grade and pathological stage of the cancer, and it serves as potential therapeutic target for prostate cancer [267]. Anti-PSMA antibody which binds to the intracellular region of PSMA had limited success as an imaging agent for prostate cancer [147]. Lupold et al. fabricated two unique RNA aptamers to extracellular portion of the PSMA termed as xPSM-A9 and xPSM-A10. Truncation of the A10 aptamer to 56 nucleotides from 71 nucleotides (A10–3) retained the ability to obstruct PSMA enzymatic activity wherein A9 aptamer lost its functionality [151]. Later Rockey et al. used RNA structure prediction and docking algorithms to rationally truncate A9. This truncated PSMA aptamer (A9g (43 mer) ($K_D$ = 5 nM) retained binding activity and functionality in inhibiting PSMA enzyme activity [223]. Dassie et al. performed the preclinical studies of an RNA aptamer (A9g) by exploring the functional activity on the inhibition of the PSMA enzyme, thereby showing that A9g is safe in vivo and it is not immunogenic to human cells [55].

#### DNA Aptamer to Advanced Glycation End Products (AGE)

In diabetes and cancer, higher levels of formation of advanced glycation end products (AGE) are reported [101, 219, 342]. The interaction of AGE with its receptor (RAGE) contributes to the development and progression of several diabetes- or age-related disorders, which includes cardiovascular disease, cancer growth, and metastasis [2, 284, 329]. Kaida et al. generated a DNA aptamer against AGE. AGE-aptamer was modified with phosphorothioate for stability, and affinity of AGE-aptamer is 1.38 × 10 micromol/l [119]. Ojima A et al. investigated the efficiency of this DNA aptamer on melanoma cells. AGE-aptamer significantly reduced the number of tumor-associated vessels. AGE-aptamer-treated G361 melanoma exhibited reduced RAGE and vascular endothelial growth factor levels. AGE-aptamer also repressed the proliferation and endothelial cell tube formation and cell proliferation of the G361 in vitro. Their findings suggested that this aptamer inhibits AGE-RAGE axis in G361 melanoma which resulted in tumor growth suppression in nude mice by blocking the angiogenesis [200]**.** Therefore targeting AGE-RAGE axis is ideal for slowing the growth of cancer in diabetic patients and hence further studies are need for this promising aptamer.

#### Glutathione-Binding RNA Aptamer

Glutathione (GSH) is a ubiquitously expressed natural tripeptide with antioxidant properties. Under different circumstances, it plays protective and pathogenic roles as well [91]. Elevated glutathione levels provide chemotherapeutic drug resistance

in cancer cells. Inhibiting glutathione synthesis sensitizes the cells for apoptosis and enhances the effect of chemotherapeutic drugs [282]. Yadav's research group fabricated three aptamers GSHapt8.17 (class III), GSHapt 5.39 (class IV), and GSH apt (class I) that had Kd values of 8.17, 5.39, and 1.2 nmol/l. In vitro experiments showed that the GSH aptamers had functional properties [12]. Due to their functional activity, further in vivo studies would be of future interest.

**BAFF Receptor Aptamers**

The B-cell-activating factor (BAFF), a member of the tumor necrosis factor (TNF) family of cytokines, was shown to improve survival and maturation of peripheral B cells [88, 181, 296]. BAFF trimerizes and binds to the BAFF-R on surface of the cell and gets internalized by receptor-mediated endocytosis [153, 193]**.** Therefore, the interaction of BAFF and BAFF-R is vital in B-cell survival, proliferation, and maintenance [16, 251, 296]**.** Increased BAFF and its receptor expression has been identified in numerous B-cell non-Hodgkin's lymphoma (NHL) [92, 124, 197, 292]. Among the NHL, mantle cell lymphoma (MCL) and diffuse large B-cell lymphoma (DLBCL) are difficult to treat with chemotherapy and rituximab. BAFF-R is highly expressed in MCLs than in the DLBCLs [77, 185].

Various 2'-F-modified RNA aptamers that target B-cell-specific BAFF-R with nanomolar affinity were isolated using in vitro SELEX approach. R-1 aptamer (54 nM of $K_d$) bound efficiently to BAFF-R on B-cell surface, blocked BAFF-mediated B-cell proliferation, and also gets internalized into B cells. The R-1 aptamer-mediated delivery of signal transducer and activator of transcription 3 (STAT3) siRNA was found to be processed by dicer and proficiently reduced target mRNA and protein levels in the cancer cells. These aptamer-siRNA conjugates are capable of delivering siRNAs and also block ligand-mediated processes, proving to be an ideal combinatorial therapeutic candidate for B-cell malignancies [347].

## 6.3.2 Aptamers to Cellular Organelles

### 6.3.2.1 Aptamers to Nucleus

Cell internalization and further localization to intracellular compartments are proven to be lesser efficient by aptamers; also they are restricted in their capability to target intracellular proteins. Mi et al. fabricated two aptamers that localize to the nucleus. One was an RNA aptamer DHX9 that was identified using in vivo selection [170], and the second was a functional RNA aptamer expressed under the H1 promoter using an adenoviral vector, intracellularly expressed to target NF-kappaB p50 protein [171].

Kaur and Tiko identified a RNA aptamer using in vitro SELEX to oncogenic transcription factor that localizes to the nucleus, expressed in many metastatic cancer cells. A diverse range of solid tumors including epithelial tumors, sarcomas, and astrocytomas overexpress Ets1. Ets1 plays pivotal role in the invasive behavior by regulating the genes such as metalloproteases MMP-1, 3, and 9 and urokinase-type

plasminogen activator. The Ets1 aptamer was successful in targeted delivery of drug-encapsulated nanoformulation to cancer cells in vitro and in vivo [123].

#### 6.3.2.2 Nucleophosmin

Jian et al. generated a RNA aptamer toward nucleophosmin (NPM) using in vitro SELEX. NPM is a multifunctional protein that negatively regulates p53 and aids in proliferation and apoptosis. Its overexpression is frequently reported in various types of cancers. NPM-specific aptamers caused displacement of NPM in the nucleoplasm and upregulation of p53 in the cells expressing NPM aptamers. Also NPM aptamers expressing cancer cells undergo apoptosis induced by DNA-damaging agents. Thus inhibiting NPM function by obstructing NPM oligomerization using aptamers serve as prospective mode of anticancer drug development [113].

#### 6.3.2.3 RNA Aptamers Against the Nup358 Protein

Shrivastava et al. fabricated a RNA aptamer to nucleoporin 358 (Nup358), a member nucleoporin family using protein SELEX. They modified the aptamer with liposome and called it Apt-PEG-DSPE [aptamer-lipid conjugate]. Two aptamers were identified NupApt01 and NupApt02 with a dissociation constant of 36 and 70 nmol/l and were better than the aptamer-modified liposomes [250].

#### 6.3.2.4 NAS-24

Zamay et al. fabricated a DNA aptamer to intracellular organelles and nucleus of malignant cells and reported that an aptamer NAS-24 mixed with natural polysaccharide arabinogalactan as a carrier reagent binds to vimentin which leads to apoptosis of mouse ascitic adenocarcinoma cells both in vitro and in vivo [337].

### 6.3.3 Aptamers in Tumor Immunology

Aptamers do not elicit immune response when given lesser than 60 to 100 nucleotides; however when they are functional aptamers, they can induce immune response through activation or inhibition of specific target receptors.

#### 6.3.3.1 CD28 Aptamer

Naïve lymphocytes need two signals for its proper activation: first, by activation of T-cell receptor and second by means of costimulatory signal activation from CD28 receptor [142]. CD28 receptor is expressed on naïve lymphocytes, whereas the surface of the activated antigen-presenting cells (APC) expresses its ligands B7.1 and B7.2 [137]. Lack of costimulation turns the lymphocyte into a stage of anergy, thus being unable to respond to its antigen [22]. Under cancerous conditions, there are multiple factors that lead the cancer cells to escape immune surveillance.

Cancer cells have low antigenicity and APCs within the tumor are continuously maintained in a state of tolerance as it express immunosuppressive receptors and release immunosuppressive cytokines [351]. For the immune rejection of the tumor

cells, one could introduce an artificial costimulatory ligand to the tumor antigen-specific lymphocytes. Pastor et al. isolated two aptamers which specifically bind to CD28 receptor. The CD28 agonistic aptamer showed enhanced cellular immune response against a lymphoma idiotype in vivo and has the ability to increase the survival of mice which received the aptamer along with an idiotype vaccine. Thus the immune responses in cancer can be modified using the CD28 aptamers by either blocking the interaction with B7 or improving vaccine-induced immune response [208].

#### 6.3.3.2 Aptamer Targeting 4-1BB and OX40

There are two agonistic aptamers which show costimulatory effects. 4-1BB is a major costimulatory receptor that promotes survival and expansion of activated T cells. McNamara et al. identified 4-1BB targeting RNA aptamer [163]. This aptamer binds to 4-1BB expressed on activated mouse T cells surface and costimulated T-cell activation in vitro using multivalent configurations and mediated tumor regression in mice.

OX40 is a member of the TNF family of receptors and is found to be expressed on the surface of activated T cells. OX40 interaction with its ligand leads to increased immune function by eliciting T-cell proliferation and cytokine production [317, 320]. Dollins et al. generated an RNA aptamer that recognizes OX40. This aptamer activated OX40 receptor and was capable of inducing nuclear localization of NFκB, cytokine production, and cell proliferation. When systemically delivered to mice, it improved the potency of dendritic cell-based tumor vaccines [63].

#### 6.3.3.3 CTLA-4 Aptamer

Cytotoxic T lymphocyte-associated antigen (CTLA)-4 engagement reduces T-cell responses by increasing the threshold of signals required for T-cell activation [36, 232]. Santulli-Marotto et al. developed RNA aptamers that bind specifically to CTLA-4 with high affinity. These CTLA-4 aptamers repressed its function in vitro and improved tumor immunity in mice, and future methods can increase their bioactivity in vivo. They concluded that aptamers could be used effectively to manipulate immune system for therapeutic applications and also the multivalent versions of aptamers may be predominantly considered as potential agents in vivo [233].

#### 6.3.3.4 Aptamer-siRNA Conjugates for Immunotherapy

Immunotherapy is getting importance, as this avoids the toxicities of chemotherapy and drug resistance. The tumor antigens are not expressed in cancer cells such a way that they can be identified by the immune system as "foreign" and hence the tumors are not regulated by the immune system of the cancer patient. Aptamers have application in this emerging field. In this novel strategy, tumor rejection antigens could be expressed on the tumor cells by obstructing nonsense-mediated messenger RNA decay (NMD). PSMA aptamer-siRNA chimera with SMG, UPF1, UPF, and UPF3 siRNA resulted in upregulation of numerous products determined by premature termination code [165] containing mRNAs. This resulted in immune response in the tumor cells and that will reduce tumor growth [83].

#### 6.3.3.5 Aptamer Targeting Viral Glycoprotein

Aptamers are developed against viruses such as HIV-1, HBV, HCV, and HSV-1 [85]. In the case of HSV-1 viral infection, glycoprotein (gD) is essential for cellular target binding and entry. Two groups Gopinath et al. and Moore et al. individually developed RNA aptamers against gD of HSV-1 and HSV-2 using cell culture and recombinant protein, respectively (ref 12 and 13 from the paper). Tejabhiram et al. developed a DNA aptamer (DApt) harboring the mini-1 aptamer motif required for functional activity with a binding affinity of 53.4 nmol/L. This DApt was able to significantly lower the HSV-1 infection therapeutically and loss of infection under prophylactic ocular model under in vivo condition in mice.

### 6.3.4 Aptamer Chimerizations

#### 6.3.4.1 Aptamer-siRNA/Aptamer-miRNA Conjugates

**Antisense Oligos**

The classes of antisense oligos currently studied extensively include short/small interfering RNAs (siRNAs), microRNA (miRNA), short hairpin RNAs (shRNAs), DNAzyme (Dz), and ribozyme (Rz). Unlike the aptamers, these antisense oligos play catalytic role due to their catalytic activity. Both siRNAs and miRNAs are considered novel class of therapeutics in cancer. They are endogenously synthesized small noncoding RNAs with significant role in gene regulation, and both have advantage over the chemotherapy [24, 45]. Due to their rapid degradation in vivo, the efficacy of gene silencing nucleic acids is relatively short [28]. This is currently addressed by incorporating stability confirming modifications. Chimerization of these catalytic small RNAs with aptamer harboring nuclease-resistant modification will aid in cell-targeted gene silencing (Fig. 6.4).

siRNA therapy has proven promising and has entered clinical trial. The first siRNA to enter clinical trial, for treating metastatic skin melanoma, was delivered using cyclodextrin nanocarriers with transferrin ligand [57]. shRNAs are similar to siRNAs but they are constitutive expressed, thus complementing the half-life issues of siRNA. Extended silencing is achieved due to the cellular synthesis guided by RNA Pol III promoters. At times, they saturate the small RNA biogenesis in the cell and result in severe toxicity [32]. Thus alternately they are expressed under RNA polymerase II promoter in multicistronic format or with other non-RNA interference therapies like ribozymes and small RNA decoys [340].

miRNA-based approaches have benefits as it includes both miRNA inhibition and miRNA replacement [300]. miRNA inhibition is done using antagomirs or anti-microRNAs by sequestering the target miRNA. miRNA replacement or miRNA mimics strategy is used to expedite the function of low-expressing target miRNA. siRNA and miRNAs offer the advantage of acting on target-like proteins which have conformational changes. They are similar to aptamers, ssRNA, or rarely dsRNA shorter sequences, easy to synthesis. Identifying the best cell internalizer or a cell surface-binding aptamer would be vital for the delivery of siRNA and miRNAs.

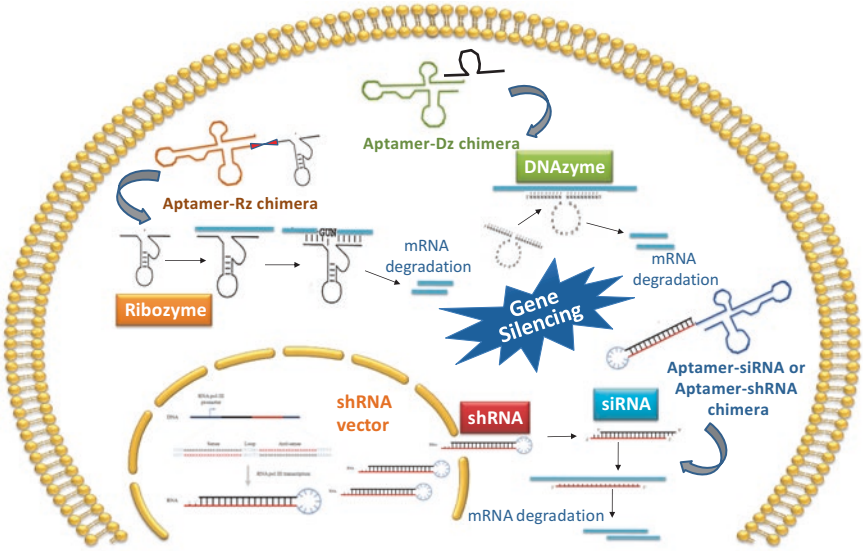


**Fig. 6.4** Aptamer-mediated catalytic RNA delivery. Aptamers are chimerized with ribozyme or DNAzyme or siRNA or shRNA for their cell-specific delivery. The delivered catalytic oligo undergoes processing further to liberate from the aptamer to exert gene silencing

### Aptamer-siRNA and Aptamer-miRNA Conjugates

Aptamers offer a potential opportunity for cell-specific delivery of siRNA and miRNA [33, 69, 70]. siRNA/miRNA molecules are processed by the ribonuclease DICER leading to generation of short double-stranded RNAs (dsRNAs) of 20–24 base pairs which in turn are recognized by the RNA-induced silencing complex (RISC) in the cytoplasm. The RISC guides the strand that directs silencing (guide strand) to the target mRNA, whereas the other strand (passenger strand) is degraded. Based on the degree of complementarity, the hybridization of guide strand to mRNA inhibits translation or induces degradation [220]. siRNAs that are perfectly complementary to their target mRNA lead to gene silencing through a sequence-specific target RNA cleavage, whereas microRNAs, which show a limited complementarity, mediate translational repression or transcript degradation.

Aptamers which are developed against the cell membrane proteins are ideal for siRNA delivery. McNamara et al. developed an aptamer-siRNA chimera for intracellular siRNA delivery. The aptamer (A10) of the chimera binds to PSMA-expressing cells. This results in intracellular delivery of the siRNA and it inhibited the cell survival genes resulting in reduction in tumor cell proliferation in animal model. This aptamer-siRNA chimera showed specific inhibition of the tumor growth without off-target effects and without stimulating the inflammatory cytokines. This strategy allowed the use of numerous aptamers generated against surface protein to deliver siRNA [162].

Numerous aptamers binding the cell surface proteins have been used for the delivery of siRNA and miRNA. Some of these aptamers include prostate-specific

membrane antigen (PSMA) to deliver polo-like kinase 1 (*PLK1*) and *BCL2 siRNA* in prostate cancer model [56, 162] and to deliver radiation sensitization DNAPK siRNA in vitro and in vivo prostate cancer models [190, 191]. Epithelial cell adhesion molecule (EpCAM) aptamer was used for delivering PLK1 siRNA to study lipid changes in cancer cells using desorption electrospray ionization mass spectrometry [112]; EpCAM aptamer for delivering siRNA to breast cancer cells using in vivo model [276], for delivering survivin siRNA using breast cancer model [314], and for delivering *PLK1* siRNA in breast cancer model [82]; and HER2 aptamer for delivering bcl2 siRNA in breast cancer model and for delivering chemosensitizing *BCL2* siRNA in breast cancer model [293]. CTLA-4 aptamer was also used for the delivery of siRNA specific to STAT3 into tumor-associated and malignant T cells [93].

Numerous research groups have fabricated aptamer miRNA conjugates that inhibit the growth of cancer. Axl aptamer-let-7g miRNA was fabricated successfully to target let-7g target genes and showed efficacy in both in vitro and in vivo lung adenocarcinoma models [69]**.** Multifunctional aptamer Axl and PDGFRβ aptamer conjugates were used for delivery of tumor suppressor antimiR-222 [33]. A RNA aptamer Axl (GL21.T) was chosen to deliver miR-212 to human lung cancer cells expressing Axl [108]. MUC1 aptamer-miR-29b chimera was used to deliver to ovarian cancer and to lung carcinoma cells [51, 52, 145, 210]. Transferrin receptor aptamer (TRA) has been used for the delivery of miR-126 (pre-miR-126) to breast cancer cells [224].

#### 6.3.4.2 Aptamer Drug Conjugates

Aptamers being the carrier molecules with increased specificity toward target cells are used in delivery of cytotoxic payloads in cancer therapy. This restricts the drugs from damaging healthy cells.

Doxorubicin (dox) referred initially as Adriamycin is an FDA-approved anthracycline class drug which is widely administered for treating leukemia and solid tumors. Due to its cardio- and nephrotoxicity, alternative strategies for delivering this molecule specifically to cancer cells are sought [330]. One among the means for cell-specific delivery of dox is using oligonucleotides as doxorubicin has the potential to intercalate in the double bonds of G-C or C-G. The fluorescent property of dox is quenched upon intercalation which can be used for characterizing its binding to the aptamer and release into the cells [11]. Both DNA and RNA aptamers have been checked so far in evaluating their efficacy, as carriers of dox specifically to cancer cells.

The first aptamer dox conjugate was prepared using prostate-specific membrane antigen (PSMA) and prostate-specific RNA aptamer A10 which was subjected to binding with LnCaP and PC3 prostate cancer cells, positive and negative for PSMA expression, respectively. The A10-dox conjugate was found to have enhanced cytotoxicity in LnCaP cells than in PC3 reflecting the target-specific delivery of dox by this conjugate [11]. EpDT3, an EpCAM RNA aptamer, was conjugated with dox for targeting retinoblastoma. The EpDT3-dox conjugate was subjected to treatments in Y79 and WERI-RB1 retinoblastoma cell lines and also in **M**üller glial cells. The

efficacy of this aptamer conjugated dox in killing retinoblastoma cell lines and not the Müller glial cells showed that this drug can be made to target cancer cells specifically [271].

Similarly several aptamers specific for different cancers have been evaluated for their dox delivering efficacy to cancer cells. Dox conjugated to MA3 (MA3-dox), an 86 nucleotide DNA aptamer against MUC1, was found to be effective in delivering dox to MUC1-positive MCF-7 breast cancer and A549 lung cancer cell lines sparing MUC1-negative HepG2 liver cancer cell line and L02 normal liver cells [102]. HER2-specific aptamer HB5 conjugated with dox (HB5-dox) selectively delivered dox to HER2-positive breast cancer cells with minimal cytotoxic effect in HER2-negative cells [146].

The FDA-approved G-rich DNA aptamer AS1411, specific to nucleolin, was utilized for the synthesis of AS1411-dox conjugate. This conjugate tested in vitro in Huh7 hepatocellular cancer cells and in vivo in mice bearing Huh7 tumors showed cytotoxicity and inhibition of tumor progression, respectively. This AS1411-dox conjugate was reported for its protective activity against nontarget heart and kidney tissues [297]. Similarly, c2C-dox, HCA#3-dox, and #1S-dox conjugates were tested against pancreatic, hepatocellular, and multiple myeloma cell lines, respectively, for its target-specific delivery [216, 321, 333].

Other than doxorubicin, methotrexate (MTX), a chemotherapy agent, was conjugated with CD117-specific aptamer and tested against acute myeloid leukemia (AML). This conjugate was specific to AML cells leaving healthy bone marrow cells unharmed [345].

#### 6.3.4.3 Aptamer and Nanocarriers

Drug delivery using nanocarriers has ushered a new era in cancer therapy. The nanocarriers being so small and similar in size range to the cellular components such as mitochondria and Golgi body. Thus nanocarriers utilize two important features of tumor such as leaky expression and high metabolic activity than the normal tissues. The leaky expression in the aberrant blood vessels of the tumors [231] mediated nanocarrier accumulation even without ligand. The tumor lobules have rapidly dividing cells, requiring high metabolic activity. Thus there is always shortage of nutrient and the tumor cells undergo necrosis. These events make the tumor environment acidic and lower the pH. However, the tumor cells have adapted to this and have evolved mechanisms to survive. Thus the smart nanocarriers can be programmed to deliver in the acidic pH [166].

Another important aspect in targeted therapy is the surface property of the cancer cells. Surface proteins/receptors must be selected for delivery of nanocarriers which have been conjugated to specific ligand [231]. The most desired properties of nanocarrier are biocompatibility and stability and nontoxic post-degradation. Various materials have been used for fabricating nanocarriers such as PLGA polymer, liposomes, dendrimer, cyclodextrin, albumin, chitosan, gold, silver, and carbon nanoparticles. The most common chemistry used for conjugating aptamers to nanocarriers includes amine modification of the ligand for further linkage to COOH in the PEG/counter material through EDC/NHS chemistry. Different aptamers which bind cell surface proteins/receptors are used as ligands. Examples include EpCAM, PSMA, NCL, etc. [143].

### 6.3.5 Aptamer and Diagnostics

Aptamers carry enormous potential as diagnostic agent due to their specific binding. The aptamer microarray would be an excellent platform for screening disease, biomarker, etc. Aptamer-based imaging, tumor cell capture, and device fabrication for the later are of current interests.

#### 6.3.5.1 Aptamer Imaging

Aptamers because of their small size are better ligands than the antibodies for cancer imaging. Aptamers coupled with diagnostic agents such as fluorescent molecules, magnetic resonance imaging (MRI) contrast-enhancing agents, and radioisotopes used for positron emission tomography are made use for detecting both malignant and metastatic cancer. Excellent reviews are available for the readers to look at [79, 115, 280, 311, 326].

#### 6.3.5.2 Circulating Tumor Cells Detection and Its Application in Oncology

Circulating tumor cells (CTCs) in cancer provide important information on the tumor load and response to therapy [227]. Thus monitoring the CTCs is considered important. CTC detection also provides a possible way to monitor patient response to certain anticancer therapies [128, 343]. Detecting CTCs and isolation is important for single-cell sequencing for detecting mutation-like KRAS and for molecular diagnostics [48] and also enables the monitoring of metastatic burden for clinical management in breast cancer [246] and for following patients during treatment with chemotherapy [325] or antibody targeting in lung cancer [192]. Switching treatments based on CTC status instead of relying on traditional clinical signs of progression may provide an opportunity for patients to receive more beneficial treatment [174].

**Challenges in Circulating Tumor Cells Analysis**

There are many challenges in analyzing CTCs. These include the heterogeneity of the CTC and low CTC number (<100 cells/ml) in the blood. Majority of the CTC detection techniques depends on antibody-based capture and staining of cytokeratins and tumor-specific antigens like EphB4, EpCAM, EGFR (Her/ErbB1), HER2, MUC-1, and CEA/CEACAM5 [164]. It is not possible to capture and detect all rare CTCs using a single antibody. For example, EpCAM is not a perfect CTC selection marker due to increased variation in its gene expression within tumor subtypes and leukocytes [129]. Most CTCs express epithelial, mesenchymal, and stem cell markers. DNA aptamers have been generated to identify known and unknown CTC markers. Several studies have revealed prospective applications of DNA and RNA aptamers for tumor cell detection [144, 173, 247]. Mesenchymal cancer cells also circulate in the blood. However the current strategies to identify are based on few markers such as EpCAM and likely they could miss the circulating tumor cells which have EMT transformation.

Zhang et al. fabricated a BC-15 aptamer to identify rare CTCs. BC-15 aptamer was screened against human breast cancer tissue from a random oligonucleotide library. This aptamer-based detection was compared with an established anti-cytokeratin method utilizing 15 pancreatic cancer patient blood samples, and enumeration showed no remarkable difference between these two methods. This new approach to identify CTCs using an aptamer probe is similar to the anti-cytokeratin-based CTC identification method [339].

Zamay et al. have done cell-SELEX on the postoperative lung tumor tissues and generated a battery of aptamers without aforementioned knowledge of biomarkers and then used same aptamers for detecting the tumor cells in the blood stream and developed aptamer-associated protein biomarkers for lung cancer such as vimentin, histone 2B, annexin A2, annexin A5, neutrophil defensin, and clusterin. This shows that aptamers can be generated for individual patients targeting specifically tumor tissue, thus opening up the chances of personalized diagnostics [336].

#### 6.3.5.3 Aptamer-Based Devices

##### Advances in Devices for Circulating Tumor Cells

**CellSearch™ Assay**

The present FDA-cleared CellSearch™ assay is based on immunomagnetic separation of CTCs. Because of its unsatisfactory efficacy and high cost, researchers have been developing new technologies, for example, flow cytometry, size-based filtration systems, and microfluidic devices, that might offer improved sensitivity and low cost for CTC detection [84].

Prof Kanwar's team had fabricated simple, flat channel polydimethylsiloxane (PDMS-based) microfluidic devices that are functionalized with locked nucleic acid [LNA]-modified EpCAM and nucleolin aptamer for rapid and efficient capture of CTCs and cancer cells. With improved flow rates (10 μl/min), the aptamer-modified devices offered reusability of the device up to six times while holding optimal capture efficiency (>90%) and specificity. This device was validated in head and neck cancers [159].

Shen et al. developed a new-generation NanoVelcro Chip, which is capable of capturing NSCLC CTCs from blood with high efficiency, and also recover the nanosubstrate-immobilized NSCLC CTCs upon nuclease treatment. Aptamer-grafted NanoVelcro Chips aided selection of two single-stranded DNA aptamers (**Ap-1** and **Ap-2**) by cell-SELEX process as a substitute for conventional antibody-based capture agents, allowing precise capture and release of NSCLC cells from blood. The capturing and releasing features enable separation of circulating tumor cells (CTCs) with minimal of white blood cell (WBC) contamination and minor disruption to CTCs' viability and functions, thus leading to molecular and functional analyses of CTCs [247].

## 6.4 Aptamer in Ophthalmology

The immune privilege of the eye and the possibility to deliver drug locally made it an ideal organ for aptamer-based therapeutics [64]. Aptamers will find more application in ophthalmology, oncology, and inflammation and disorders with vascularization. Currently there are three aptamers in use for clinical application of ophthalmic care. The anti-vascular endothelial growth factor (VEGF) aptamer, pegaptanib (Macugen), remains the only US Food and Drug Administration-approved aptamer to date. It was originally selected by NeXstar Pharmaceuticals (later taken over by Ophthotech) for the treatment of neovascular (wet) age-related macular degeneration [187]. Aptamers such as pegpleranib/Fovista and Zimura/ARC1905 are in phase 3 and in phase 2 development, respectively [64].

Aptamers are finding application in glaucoma which is one of the main causes of blindness in the world [218]. Zhu et al. generated two aptamers, aptamer S58 and aptamer S68, selected to bind the extracellular region of TGF-β receptor II (TβRII) which were evaluated for their efficiency to transdifferentiate the fibroblast induced by the TGF-β. The S58 aptamer was found to be functional which inhibited TGF-β2-induced myofibroblast transdifferentiation in human tenon fibroblasts. They later showed the efficacy of the aptamer and nanocomplex in a rat glaucoma filtration surgery (GFS) [43, 349, 350]. Aptamers also finds major application in treatment of retinoblastoma (RB). RB is a childhood eye cancer that comprises approximately 3% of childhood malignancies globally. RB if untreated leads to complete loss of vision and might even lead to mortality if not enucleated at advanced stages. Therefore, improved treatment modalities are desired for eye salvage and good life.

**Limitations of Current Chemotherapy**

One of the most extensively used protocols for the chemotherapeutic treatment of RB comprises a triple-drug therapy of carboplatin, vincristine, and etoposide [37]. Focal therapies combined with the triple-drug combination are not so effective in as many as 40% of bilateral retinoblastoma patients [76]. Furthermore, etoposide is found to increase the secondary malignancies probably due to DNA damage triggered by this topoisomerase II inhibitor [195]. The drugs etoposide and vincristine are effluxed out by the P-glycoprotein in the tumor cells [37, 130]. To circumvent the MDR, cyclosporine A has been advocated. However, the toxicity of cyclosporine A adds to the morbidity of the chemotherapy. Carboplatin is ineffective in advanced retinoblastoma or as a monotherapy due to the downregulation of serine-/arginine-rich protein-specific kinase 1 (SRPK1, a cisplatin-/carboplatin-sensitive protein) leading to drug resistance [131]. Intra-arterial chemotherapy is now adopted for retinoblastoma; however there are concerns with toxicity [110, 308]. Thus targeted therapy is gaining importance in retinoblastoma toward differentially regulated proteins and signaling pathways.

### 6.4.1 Aptamer Applications in Retinoblastoma

The identification of cancer stem cells in retinoblastoma suggests that chemotherapy drugs could be complemented with specific targeting of the cancer stem cells or drug-resistant proteins using aptamer-siRNA/miRNA therapy or functional aptamers that could be delivered to the eye specifically thus reducing the toxicity of chemotherapy and also selectively targeting proteins which contribute to resistance to therapy. Further research would be done to show reduced tumor viability in vitro and decreased target mRNA levels in human RB cells in vivo in an orthotopic mouse xenograft model using these oligonucleotides. Alternatively the functional aptamers could be delivered as an eye drop using nanoformulations for posterior segment delivery [40].

### 6.4.2 Functional Aptamers

#### 6.4.2.1 HMGA Aptamer

HMGA, a DNA-binding *architectural transcription factor*, is associated with the expression of several genes that could play a vital role in carcinogenesis, including those responsible for cell proliferation, apoptosis, immune response, and DNA repair. It is well known that both HMGA1 and HMGA2 bind to the minor groove of AT-rich DNA [68, 221, 258, 263].

HMGA1 and HMGA2 proteins are expressed in retinoblastoma and contribute to tumor invasion and proliferation [39, 252, 306] and could be targeted for therapy using shRNA [39] or siRNA [304] or miRNA mimics [305]. HMGA1 protein has been targeted earlier with Spiegelmer NOX-A50 in pancreatic cancer using in vitro and in vivo model [155].

Watanabe et al. generated phosphorothioate-substituted ssDNA aptamers that specifically bind HMGA proteins. These aptamers contained multiple HMGA AT-hook binding sites (AT-sDNA) which would compete with HMGA protein binding to genomic DNA and thereby directly inhibit the activity of HMGA protein in pancreatic cancer cells. Since both HMGA1 and HMGA2 proteins bind AT-rich DNA, this aptamer inhibited the activity of both the proteins [316]. Later Nalini et al. used the HMGA aptamer to block HMGA2 protein function in RB cells. HMGA2-aptamer internalization in RB cells (Y79, Weri Rb1) and nonneoplastic human retinal cells (MIO-M1) were studied. Aptamer-induced dose-dependent cytotoxicity in RB cancer cells with increased expression of TGFβ, SMAD4, CDH1, BAX, CASP3, and PARP mRNA and decreased SNAI1 and Bcl2 mRNA levels in aptamer-treated RB cells suggests the activation of TGFβ-*SMAD4*-mediated apoptotic pathway. Synergistic effect of the aptamer with etoposide was reported in treated RB cells (p value $\leq 0.05$) with insignificant toxicity in nonneoplastic retinal cells [186].

#### 6.4.2.2 Nucleolin Aptamer

Nithya et al. showed that NCL protein is differentially expressed in retinoblastoma primary tumor and cell lines. AS1411, a nucleolin aptamer, was used to inhibit the RB cell proliferation. Later this aptamer was modified to harbor LNA nucleotides. Nucleolin aptamer reduced proliferation of the tumor cells in vitro and in vivo models of retinoblastoma. The molecular changes underlining aptamer treatment were found to affect the tumor miRNA and serum onco-miRNA levels. The levels of phosphatidylcholine lipid were reduced in vitro in aptamer-treated cells and in vivo in tumor tissues which was shown using desorption electrospray ionization mass spectrometry (DESI MS)-based imaging [278].

#### 6.4.2.3 Nucleolin Aptamer and DNAzyme Chimerization

Nucleolin aptamer can be used for both delivery and as a functional aptamers. DNAzymes, a new group of catalytic nucleic acids composed of deoxyribonucleotides, were selected using an in vitro selection system. The therapeutic potential of these RNA-cleaving DNAzymes has been reported both in vitro and in vivo. Although they compete the activity and stability of synthetic ribozymes, the effective use of these molecules is limited due to their inefficient delivery to the intracellular targets. Recent achievements in clinical testing of the DNAzymes in cancer patients have rejuvenated the potential clinical utility of DNAzymes [78]. Thus developing delivery strategies for DNAzyme is necessary. Zhang et al. used a survivin DNAzyme to inhibit survivin in breast cancer cells [341]. Subramanian et al. delivered the survivin DNAzyme to retinoblastoma cells using AS411 aptamer. This was for the first time a DNAzyme was delivered using an aptamer. NCL-APT was conjugated with Sur_Dz using the aptamer with a poly T linker at the 5′ end with complementary sequence to Sur_Dz. This NCL-APT linker was used for the conjugation with Dz using the denaturing and annealing principle. The aptamer DNAzyme was reported to be stable and functionally effective thereby inhibiting the proliferation in RB cells [272]. Further use of the chimeric construct in xenograft model will be of great interest.

#### 6.4.2.4 Pri-miRNA Aptamer

MicroRNAs (miRNAs) are small (18–25 nt) noncoding RNAs which are involved in regulating several biologic processes by silencing specific target genes [30]. Many mature miRNAs reported to be involved in cancer may be generated from the same pri-miRNA transcript. An example for this is the *miR-17 ~ 92* oncogenic cluster (also denoted as *OncomiR-1*), where six mature miRNAs such as *miR-17*, *miR-18a*, *miR-19a*, *miR-19b*, *miR-20a*, and *miR-92* are derived from the same pri-miRNA transcript [98, 201]. This *miR-17 ~ 92* cluster is reported to be overexpressed in several cancers such as colon, breast, prostate, pancreas, lung, stomach, and medulloblastoma [42, 302, 309].

Earlier our group reported *miR-17 ~ 92* overexpression in RB. The expression of *miR-17 ~ 92* cluster was observed in both the primary RB tumor samples and RB cell lines [120] and also in serum of RB patients [18]. Further, this *OncomiR-1* cluster was targeted individually in RB using its antagomirs [120]. An RNA aptamer

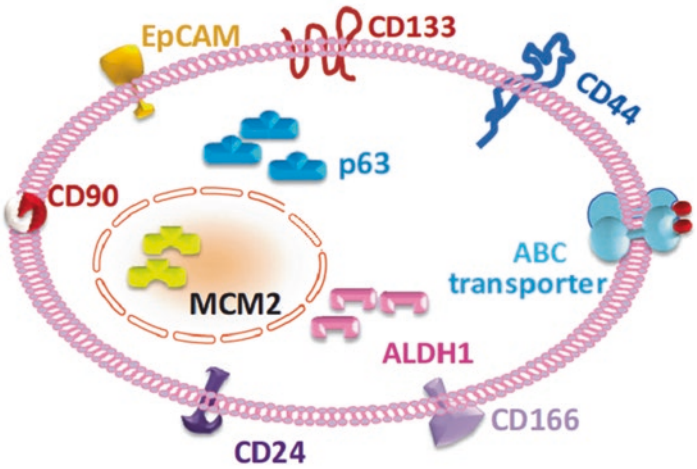


**Fig. 6.5** Cancer stem cell markers present in RB. RB tumor contains heterogeneous population with subset of cells expressing stem cell markers. The CSC markers reported so far are EpCAM, CD133, CD44, CD90, CD24, ALDH1, p63, MCM2, and ABC transporters

which specifically targets the primary transcript of *miR-17 ~ 92* was selected by SELEX to collectively inhibit the biogenesis of miRNAs in this cluster [150].

The functional effect of this pri-aptamer was evaluated on the RB cell lines by Subramanian et al. and was found to be capable of inhibiting the mature miRNAs of the miRNA-17 ~ 92 cluster with upregulation of mRNA targets of this cluster which in turn induced apoptosis and inhibited cell proliferation. The in vitro study shows that the pri-aptamer has higher prospective for application in other cancers and further in vivo work and strategies for delivery of this aptamer are needed [274].

### 6.4.3 Aptamers Targeting Cancer Stem Cell (CSC) Markers

Retinoblastoma tumor expresses stem cell markers such as EpCAM, CD133, ABGC2, CD44, CD24, ALDH1, CD166, MCM2, CD90, SCA-1, and p63 [3, 13, 176, 180, 240]. Some of these markers are potential targets for the identification of cancer cells, imaging, and targeted delivery of anticancer agents. The CSC markers reported in RB are illustrated in Fig. 6.5.

#### 6.4.3.1 CD44

CD44 is a transmembrane glycoprotein actively involved in regulating growth, survival, differentiation, and cell motility [215]. CD44 being a receptor of hyaluronan (HA) binds to HA and promotes matrix-dependent migration [160].Variants of CD44 represented as CD44v are transiently expressed in embryonic development, lymphocyte maturation, and activation. The splice variants CD44v4-7 were reported for its contribution in metastatic phenotype of tumor cells.

RNA aptamers developed against full length recombinant CD44 and the binding efficacy was checked in CD44 expressing breast cancer cells [1]. Later DNA aptamers specific for variant 10 of CD44 protein, reported for cancer metastasis and invasion, were also studied in breast cancer cell lines [109]. The HA binding domain, being conserved in all splice variants, was selected for the generation of specific thioaptamers which bound selectively to $CD44^{+ve}$ ovarian cancer cell lines rather than $CD44^{-ve}$ cell lines [260].

The thioaptamers reported earlier were truncated and synthesized without thiol modifications (TA1 and TA6). The binding affinity of TA6 aptamer was retained wherein affinity of TA1 was reduced post-truncation. This was due to the different three-dimensional conformation of the TA1 aptamer after truncation. The TA1 and TA6 aptamers binding in retinoblastoma and breast cancer cell lines and $ABCG2^{+ve}$ MCF spheroid cells showed better uptake of both TA6 and TA1 in $ABCG2^{+ve}$ cells. Similarly proliferation and colony formation assays showed significant effect in both the aptamer treatments [270].

#### 6.4.3.2 CD133

CD133 also referred as AC133 and prominin1 is a pentaspan transmembrane protein identified initially as CD34+ hematopoietic stem cell marker. CD133 is a prominent cancer stem cell marker located in the glycolipid-enriched membrane domains which is composed of a short extracellular amino terminal, two large extracellular domains, five transmembrane domains, two small intracellular domains, and an intracellular carboxy-terminal. The AC133 epitope reported to be masked upon differentiation is located on the extracellular domain 2 of the protein.

The RNA aptamer against CD133 was selected using HEK293T cells overexpressing recombinant CD133 protein. Two aptamers specific for the protein were isolated and truncated further to yield smaller aptamers. Both the aptamers A15 and B19 bound to CD133-positive cells, and also the A15 aptamer was reported to interact specifically with the AC133 epitope of the CD133 protein. The aptamers subjected to binding with different cancer cell lines including colorectal, breast, ovarian, lung, liver, and prostate cancers showed similar binding efficacy as of AC133-specific CD133 antibody [250].

With the A15 aptamer subjected to binding in retinoblastoma and breast cancer cell lines, $CD133^{+ve}$primary retinoblastoma tumors showed better uptake in $CD133^{+ve}$ primary tumors deciphering the aptamer specificity toward CD133. The effect of A15 aptamer on cancer cell proliferation by MTT assay and colony formation assay showed significant cell proliferation inhibition with reduction in number of colonies. The structure prediction of the CD133 extracellular domain and the molecular docking of A15 aptamer with the protein showed interacting residues which can be further explored as potential targets [270].

#### 6.4.3.3 ABCG2

ATP-binding cassette (ABC) group G member 2 (ABCG2) also referred as breast cancer-resistant protein (BCRP), a subfamily of ABC transporter superfamily, is reported to contribute for multidrug resistance (MDR) in several solid and liquid

tumors. ABCG2 is a potent CSC marker and its expression is related to tumorigenicity, chemoresistance, cell proliferation, and metastasis [322].

ABCG2 expression in cancer cells leads to increased efflux and decreased drug accumulation leading to MDR. This protein plays vital role in prognosis of cancer and in drug resistance to cancer stem cells [178]. ABCG2 offers resistance to a wide variety of drugs including anthracyclines, anthracenes, camptothecin derivatives, nucleoside analogs, and topoisomerase inhibitors [158].

To target MDR breast cancer stem cells (BCS), DNA aptamers were screened against ABCG2 overexpressing baby hamster kidney (BHK) cells. The resulting aptamers A12 specific to ABCG2 protein and A35 specific to BCS bound specifically and internalized into cells obtained from mammospheres and not with differentiated MCF7 breast cancer cells. Cells sorted using these aptamers resulted in mammosphere formation depicting the stemness nature of the MDR BCS cells [205].

ABCG2 expression in RB primary tumor cells correlated with the tumor recurrence and metastasis with higher levels of ABCG2 in invasive RB tumors [180]. The A12 and A35 aptamers subjected to binding in retinoblastoma cell lines, breast cancer cell line, and retinal cell line showed specific binding only in cancer cell lines. Also these aptamers bound with higher affinity toward ABCG2$^{+ve}$ cells isolated from MCF7 mammospheres. The functional effect of these aptamers evaluated using cytotoxicity assay and colony formation assays showed promising results in reducing the cell proliferation of cancer cells [270].

#### 6.4.3.4 EpCAM

Epithelial cell adhesion molecule (EpCAM) also referred as ESA or CD326 is expressed in membranes of epithelial cells, in stem and progenitor cells, and also in cancer cells. Since this molecule is expressed in the intercellular spaces between the normal cells and expressed homogenously in cancer cell surface, targeting cancer cells through EpCAM is superior. This protein has three domains, an extracellular, a transmembrane, and a small intracellular domain [160]. Retinoblastoma primary tumor samples subjected to immunohistochemistry showed more than 50% percent positivity for EpCAM with elevated expression levels in invasive tumors. This suggests that EpCAM can be effectively used for targeting retinoblastoma cells [133].

The RNA aptamers against EpCAM generated using recombinant EpCAM protein were found to bind specifically to breast, gastric, and colorectal cancers expressing EpCAM. Serial truncation of the aptamers resulted in 19 nucleotide RNA aptamer EpDT3, with uncompromised binding as of the initially selected aptamers. Being internalized, post-binding makes this aptamer a superior molecule for cancer cell-targeted delivery and imaging approaches [248].

##### Targeting EpCAM in Retinoblastoma

Epithelial cell adhesion molecule (EpCAM) is an eminent CSC marker expressed on cell surface and considered as a tumor-associated antigen [182]. Epithelial cell adhesion molecule (EpCAM) is highly expressed in epithelial cancers [10] and in RB [132]. Both noninvasive and invasive retinoblastoma primary tumor cells

express EpCAM, and it also contributes to proliferation in retinoblastoma cells [177]. Thus EpCAM is an ideal molecule to target.

**EpCAM Aptamer Drug Conjugate**

EpDT3, a RNA aptamer that binds with EpCAM, was conjugated with dox for targeting retinoblastoma. The EpDT3-dox conjugate was subjected to treatments in Y79 and WERI-RB1 RB cell lines and also in Müller glial cells. The efficacy of aptamer conjugated dox in killing retinoblastoma cell lines and not the normal retinal Müller glial cells showed that this drug can be made to target cancer cells specifically [271].

**EpCAM Aptamer-siRNA Conjugate**

EpCAM has an extracellular domain (EpEx), a transmembrane domain (EpTM), and an intracellular domain (EpICD). It plays a major role in oncogenic signaling by proteolysis of EpCAM and translocation of EpICD into the nucleus [156, 235]. Proteolysis of EpCAM results in EpICD complex formation with FHL2, β-catenin, and Lef1. This whole complex binds to the Lef1 binding site of the target genes thereby modulating transcription [156]. Subramanian et al. using an EpCAM targeting aptamer-siRNA chimera showed the efficacy of the chimera in reducing the proliferation of the RB cells and also showed its EpICD-mediated signaling in epithelial cancers. Knockdown of EpICD in RB primary tumors resulted in the suppression of pluripotency markers such as SOX2, NANOG, OCT4, and CD133. In vivo studies revealed complete regression of tumor growth without any toxicity in animals ($P < 0.001$). Their results showed that EpApt-siEp is effective in eradicating EpCAM-positive cancer cells while sparing normal cells [276]. EpCAM aptamer-siRNA was further modified to harbor LNA bases that give superior serum stability. LNA-modified chimeric constructs showed increased silencing of target genes (stathmin and survivin). Future studies in tumor model will be interesting to access if the LNA-modified chimera would have better in vivo activity [275].

**EpCAM Aptamer Conjugated to Nanocarrier with Encapsulated siRNA.**

Subramanian et al. developed an aptamer polyethyleneimine (PEI) siRNA nanocomplex for targeting EpCAM. PEI nanocomplex synthesized with EpCAM aptamer (EpApt) and EpCAM siRNA (SiEp) unveiled decreased cell proliferation in the retinoblastoma and breast cancer cells using in vitro model [273].

## 6.5 Next-Generation Aptamers

### 6.5.1 Spiegelmer

Spiegelmers are aptamers that are based on L-ribose units and remain unaffected by nuclease as such. L-ribose is an artificial "mirror-image" molecule of the naturally occurring D-ribose, hence the name Spiegelmer. The word "Spiegel" is derived from German which means "mirror" [303]. Spiegelmers are generated against

synthetic selection targets which are the mirror image of the respective biological target [128]. The resulting L-aptamers bind specifically to the natural target with same affinity as the D-aptamers bind to the mirror-image selection target.

### 6.5.2 L-RNA Aptamers/AptamiR

L-aptamers, termed "aptamiRs," bind their corresponding pre-miR target via highly specific tertiary interactions instead of Watson-Crick pairing. Formation of a pre-miR-aptamiR complex inhibits Dicer-mediated processing of the pre-miRNA, which is required to form the mature functional microRNA. Pofahl et al. synthesized and reported on an LNA antimiR targeting oncomir miRNA-21 [214]. Sczepanski et al. generated an aptamiR which bound oncogenic pre-miR-155 and inhibited Dicer processing under simulated physiological conditions, with an IC50 of 87 nM [236]. L-RNAs are intrinsically resistant to nuclease degradation and aptamiRs are a new class of miR inhibitors for obesity.

### 6.5.3 NOX-A12

NOX-A12 is a L-configuration 45-mer (Spiegelmer) RNA aptamer which is developed by Noxxon Pharma and is conjugated to polyethylene glycol (PEG). This aptamer targets CXCL12/SDF-1 (CXC chemokine ligand 12/stromal cell-derived factor-1) [189], a chemokine which acts via binding to CXCR4 and CXCR7 chemokine receptors and has several roles in cancer biology, comprising regulation of migration of leukemia stem cell to the bone marrow [184] and tumor growth and metastasis [94].

## 6.6 Future Prospects

The current protein-based in vitro selection is getting replaced with other advanced methods and in vivo cell-SELEX. Slowly other advances in microfluidic technology would be applied for aptamer selection. This would be complemented with next-generation sequencing which would be used to analyze the different rounds of selection. Novel bioinformatics tools which will help in post-SELEX truncation would be adapted. The current limitations in the RNA aptamers such as the reduced in vivo stability would be addressed with further modifications using LNA technology or totally novel aptamers may be generated.

Aptamer chimerization with siRNA or miRNA has opened doors for siRNA and miRNA therapeutics. Functional aptamers against oncogenic proteins and pri-miRNA will contribute to new opportunities in therapy both in oncology and other areas. Similar to any drug development, the in vitro functionality vs the in vivo characteristics of aptamer varies, thus leading to challenging compromised activity in vivo. An example is nucleolin aptamer wherein the data generated were not much

exciting at the phase 2 study with renal cell carcinoma. Among the 35 renal cell carcinoma patients, only one patient demonstrated a significant response to treatment [226]. In another phase 2 trial in AML patients, AS1411 in combination with cytarabine demonstrated only limited improvement over cytarabine alone [264]. Some aptamers such as BAX499, formerly ARC19499, that binds to tissue factor pathway inhibitor and helps in hemophilia management were not taken for further development after clinical trials because the increased levels of tissue factor pathway inhibitor could not be controlled by aptamer administration [62].

Macugen is used less because of its limitations in VEGF isoform recognition, when compared to its protein competitors Avastin, Lucentis, and Eylea [75, 148, 241]. Fovista, commonly referred as pegpleranib, is an aptamer that targets platelet-derived growth factor and is currently under clinical trials. Fovista is currently under development by Ophthotech and is undergoing simultaneous phase 2/3 clinical trials. The aptamers like Macugen are designed to be delivered through intravitreal injection and are intended to work synergistically with VEGF inhibitors, such as Macugen, Lucentis, Avastin, and Eylea in the treatment of age-related macular degeneration. However the concern is that these aptamers have taken more than two decades to reach clinical trials.

Newer technology and refinement in selection methodology and nucleotide modification has generated newer version of aptamers like SOMAmers and mirror-image aptamers, or so-called Spiegelmers. The SOMAmers show increased binding affinity and binding kinetics compared to current aptamers. Similarly Spiegelmers which has overcome degradation and immunogenicity will soon come to clinical application [157]. Newer chemistry methods like creation of novel polymerases that allows incorporation of wide variety of sugar backbone and base structures will improve aptamer stability and clinical translation [27]. Holliger and colleagues have developed a variety of mutant DNA polymerase and reverse transcriptase to generate a novel next generation of aptamers called xeno nucleic acid (XNA) aptamers [212]. The future of aptamer technology strongly depends on such development as it can generate synthetic material with striking functionalities [290, 291]. Thus we have come a long way from discovery of aptamers to their clinical applications with further promising developments for future.

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# Part II

# Cell Therapy – "With Emphasis on Gene Modified Cell Therapies"

# SETting up Methylation in Mammalian Cells: Role of Histone Methyltransferases in Disease and Development

7

Abhishek Mohanty and Shravanti Rampalli

## 7.1 Introduction

The sequencing of human genome and its completion in the year 2003 has led to enormous progress in understanding the genetic basis of diseases and other genetic processes in humans. Notwithstanding the rapidity in the functional analysis of the human genetic code that has been relatively straight forward, an improved understanding of the human disease and development turns out to be far more complex than as expected originally. One of the complexities which are implicated in the expression of human genetic traits is epigenetics. The epigenetic changes essentially involve processes which alter gene activity without modifying the actual DNA sequence that codes for the various inherited traits and predispositions in humans and other organisms. These epigenetic regulatory changes include the DNA methylation, histone modifications, and expression of noncoding RNAs. Thus, studying such epigenetic and epigenomics, the genome-wide distribution of epigenetic change effects has become imperative considering their vital roles in cancer, genome imprinting, influencing the effects of environment on maternal behavior such as in vitro fertilization risks, and, thus, reawakening their previously unrecognized function nature and nurture interrelationship to determine human traits.

Many types of epigenetic processes such as posttranslational modifications (PTMs) of histones have been identified including methylation, acetylation, phosphorylation, ubiquitylation, and sumoylation. Epigenetic programming in controlling cell differentiation is achieved by regulating gene expression in a tissue-specific manner thereby regulating developmental processes. Accordingly, the dynamic regulation of histone posttranslational modifications governing the local chromatin state is evolving as a binding epigenetic mechanism that tunes all the DNA-template

A. Mohanty · S. Rampalli (✉)
Centre for Inflammation and Tissue Homeostasis, Institute for Stem Cell Biology and Regenerative Medicine (inStem), Bangalore, India
e-mail: shravantird@instem.res.in



G. R. Jayandharan (ed.), *Gene and Cell Therapy: Biology and Applications*,
https://doi.org/10.1007/978-981-13-0481-1_7

processes, including transcription, DNA compaction, replication, and repair. Therefore, the expression of genes in mammalian systems that is primarily dependent upon the chromatin structure and function is being regulated by posttranslational modifications on the core nucleosomal histones.

The nucleosome is created by an octamer of histone proteins H2A, H2B, H3, and H4. Interactions among the histone proteins are structurally stabilized by hydrophobic bridges between internal globular domains and by long, flexible, basic N-terminal tails that protrude out from the nucleosomal core. Further to the prevalence of the PTMs reported for the histone N-terminal tails, including the methylation of lysine and arginine residues, acetylation and ubiquitinylation of lysine residues, and phosphorylation of serine residues [1], such modifications are also seen in the core of histones and in the C-terminal regions [2, 3]. The histone modifications which form the crux of the epigenetic landscape in mammalian genomes are referred to as "marks," and the catalysts mediating the deposition of these marks onto either the histone tails or the DNA itself are commonly termed the "epigenetic writers." The proteins that recognize these specific histone posttranslational marks or series of epigenetic marks are called "readers," and due to the reversibility of these epigenetic modifications, the "erasers" can remove them as well.

Owing to our increased understanding of PTMs in histones over the years, methylations of lysine and arginine residues in histones in particular have emerged to be the better characterized epigenetic marks involved in maintaining the cell and tissue-specific gene expression programs critical for mammalian development, for establishment of cell lineage memory, and even for the epigenetic maintenance of altered transcription after cell division [4]. The enzymatic methylation of histones is brought about by writers of histone methylation marks, "the histone methyltransferases" displaying exclusive specific activity with respect to the degree of methylation (mono-, di-, or trimethylated lysine, respectively) depicted in Fig. 7.1 [5]. Accordingly, they are classified as lysine methyltransferases (KMTs) and arginine methyltransferases (PRMTs), utilizing S-adenosyl-L-methionine (SAM) as the methyl donor. Hence, the precise effect of these histone marks is relatively dependent on the context of where methylation happens which makes identification of these epigenetic signatures and their correlations with disease and development a real daunting task.

However over the last decade, rapid analytical and technological advances have led to a stir in the interest of understanding the roles of histone methyltransferase in early developmental gene expression programs. Owing to the distinctive nature of epigenetic alterations as compared to genetic mutations specifically in view of the reversibility of histone methylations or acetylations maintaining epigenetic marks, the histone methyltransferases are now considered a new class of targets for treatments. The development of therapeutics against epigenetic molecules has been heralded by small-molecule inhibitors targeting these multifunctional methylating enzymes that have been shown to contribute to tumorigenesis and cancer cell renewal and many other diseases.

Here, we review the status of histone methylations in mammalian genomes with a special focus on histone methyltransferase complexes in the regulation of

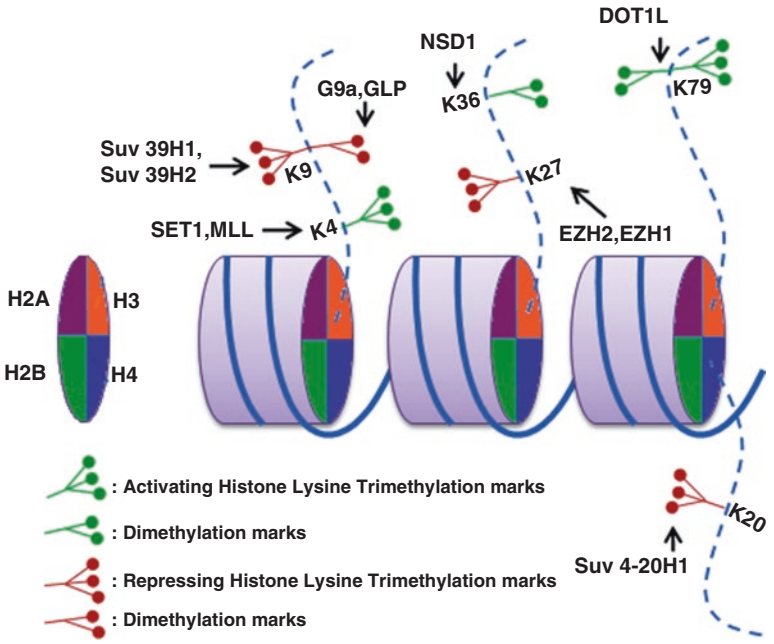


**Fig. 7.1** The histone lysine methyltransferases (HKMTs) deposit the activating histone lysine methyl marks (shown in green) and repressing histone lysine methyl marks (shown in red) on the amino-termini of histones H3 (orange) and H4 (blue) which are depicted here. The numbers represent methylated lysine residue on each histone

chromatin structure, function, and proper execution of gene expression programs. This would further highlight central paradigms regarding the family of histone methyltransferases in transcription, in disease-like cancer, and in development and differentiation providing examples of the non-histone or aberrant histone methylations and their relationship to diseases and mammalian development.

## 7.2 The Histone Methyltransferases as Methyl Mark Readers and Writers

The discovery of histone methyltransferases that made the deposition of methylations in histones a reality descends down to the early genetic screens in *Drosophila*. Such kind of powerful screens were designed to understand the effect of transcriptional silencing of genes inserted near heterochromatin, an event which was popularly coined as position-effect variegation (PEV). Not only did these studies end up in discovering the suppressor genes like Su (var) and the activator genes E(var) but also the repressing group of polycomb proteins (PcG) and activating group of trithorax (TrxG) proteins [6]. Among the proteins identified in such screens, three separate *Drosophila* proteins, *s*uppressor of variegation (Su(var)3-9) [7], the polycomb-group protein *e*nhancer of zeste [E(z)] [8], and *t*rithorax [9], comprised

of a highly conserved, 130 amino acid stretch, defined as the *SET* domain, named after the initials of these three genes, firstly discovered to express such domains. Later in the year 2000, these observations got a boost with the identification of a specific methyltransferase activity associated with the SET domain of SuV39h in *Drosophila* as well as its homologues in higher organisms [10].

It has been seen in multiple studies that especially in mammals, the functional domains catalyzing the histone methylation are conserved thereby reflecting the size and complexity of the genomes involved. This is also evident for the SET domain family members which exhibit a near fivefold expansion in mammals relative to yeast. Extensive structural and functional analyses have attributed specific SET domain-dependent enzymatic functions to a number of histone lysine and arginine methyltransferases. In other words, the histone lysine methylation is almost exclusively catalyzed by enzymes in the SET domain-containing family of methyltransferases. Thus, based on these studies, the histone methyltransferases are usually subdivided into three classes: (i) SET domain lysine methyltransferases (PKMTs), (ii) non-SET domain lysine methyltransferases (e.g., DOT1L [11], which has a catalytic domain with similarities to those found in arginine and unrelated small-molecule methyltransferases), and (iii) arginine methyltransferases (PRMTs).

The methylation status of histone tail residues is subject to dynamic modifications orchestrated by the specific activities of the writers, histone methyltransferases with the assistance of a diverse recognition motifs in highly evolved families of methyl-lysine, and methylarginine-binding domains which are known as the "readers" of histone methylation. These methyl-lysine-binding domains are mostly found in large effector multiprotein complexes which recognize modified residues with high specificity. Apparently, these protein modules acting as methyl mark readers are accountable for attracting the effector proteins harboring them to the chromatin sites bearing the histone modifications leading to a variety of outcomes [12]. Royal family of proteins including MBT, Agenet, Tudor, Chromo, and PWWP domains [13–16] harbors methyl-lysine-binding modules which are also seen in the PHD family and WD40 repeat protein WRD5 [17] and the ankyrin repeats [18].

### 7.2.1 Lysine Methylations of Mammalian Histone Proteins

Lately a lot of genome-wide profiling of histone PTMs have put forth the relationship between chromatin structure and genome function. Prominently, lysine methylation is emerging as a dynamic PTM serving as a mark of both transcriptionally active and inactive chromatin. The position of the methylated nucleosome within the gene and the genome defines the methylation traits. The most widely studied histone lysine methylation sites are those of H3K4, H3K9, H3K27, H3K36, H3K79, and H4K20 (Table 7.1). In addition several methylated lysine residues have been found on H1, H2A, and H2B and in further positions within H3 and H4. Some of the lysine methylation marks are preferentially associated with actively transcribed regions (like H3K4, H3K36, and H3K79) or with poorly transcribed heterochromatin (H3K9, H3K27, and H4K20) [19]. Frequently it has been observed that the

**Table 7.1** Activating/repressing histone methylations of lysine residues with biological function

| Target sites/ methyl marks | Writers/HMTs (KMT family in brackets) | Methylated residues (me3/ me2/me1) | Readers/ binding domains | Biological functions | References |
|---|---|---|---|---|---|
| H3K4 | SET1 (KMT2) | H3K4me3 | Chd1 | Activation of transcription | [36] |
| | MLL (KMT2) | H3K4me3 | WDR5 | Elongation of transcription | |
| | SET7/9(KMT7) | H3K4me1 | JMJD2A | Silencing transcription | |
| | SMYD2(KMT3) | H3K4me1 | MBT | | |
| | SMYD3(KMT3) | H3K4me3 | PHD finger | | |
| | ASH1L(KMT2) | H3K4me3 | | | |
| H3K9 | Suv 39H1(KMT1) | H3K9me3 | (Chromo domain), HP1 | Activation of transcription | [36] |
| | Suv 39H2(KMT1) | H3K9me3 | CDY1 | Repression of transcription | |
| | G9a (KMT1) | H3K9me2 | JMJD2A | DNA methylation | |
| | GLP(KMT1) | H3K9me2 | Ankyrin | Euchromatic silencing | |
| | EZH2(KMT6) | H3K9me3 | | Heterochromatic silencing | |
| | SETDB1(KMT1) | H3K9me3 | | | |
| | RIZ (KMT8) | H3K9me3 | | | |
| H3K27 | EZH1(KMT6) | H3K27me3 | Pc | Silencing transcription | [36] |
| | EZH2(KMT6) | H3K27me3 | | Euchromatic silencing | |
| | G9a (KMT1) | H3K27me1 | | Transcriptional silencing | |
| | NSD2(KMT3) | H3K27me3 | | X-chromosome inactivation | |
| H3K36 | NSD1(KMT3) | H3K36me1 | Eaf3 | Silencing transcription | [36] |
| | NSD2(KMT3) | H3K36me2/1 | | Elongation of transcription | |
| | NSD3(KMT3) | H3K36me2/1 | | Regulation of transcription | |
| | ASH1L(KMT2) | H3K36me2 | | | |
| | SETD2(KMT3) | H3K36me2/3 | | | |
| H3K79 | DOT1L(KMT4) | H3K79me2/3 | 53BP1 | DNA repair | [36] |

(continued)

**Table 7.1** (continued)

| Target sites/ methyl marks | Writers/HMTs (KMT family in brackets) | Methylated residues (me3/ me2/me1) | Readers/ binding domains | Biological functions | References |
|---|---|---|---|---|---|
| H4K20 | Suv4-20H1(KMT5) | H4K20me3 | Crb2 | Silencing transcription | [36] |
| | Suv4-20H2(KMT5) | H4K20me3 | JMJD2A | Activation of transcription | |
| | NSD1(KMT3) | H4K20me2/1 | | Regulation of transcription | |
| | SET9(KMT7) | H4K20me1 | | Cell cycle-dep. silencing | |
| | SET8(KMT5) | H4K20me1 | | Mitosis and cytokinesis | |

ultimate consequence on chromatin is given an effect to by the interplay of several histone modifications together or "histone cross talk" [20]. Majority of the SET domain KMTs contain an additional motif which confers them with ability to identify various PTMs including the epigenetic marks and thereby facilitating the coupling of writer properties with PTM reader properties. Thus, the lysine methyltransferases recognize certain histone PTMs in tandem through the catalysis of methylation. This is how these enzymes justify protein cross talk mechanism for the establishment and/or propagation of different histone marks.

### 7.2.2 Lysine Methyltransferases

The human genome encodes for approximately 66 methyltransferases with demonstrated or predicted abilities to methylate histone, lysine, and arginine residues based on which they serve as emerging candidate clinically important drug targets, thus making the studies on histone methylations a research hot spot and focus of attention in epigenetics, molecular biology, and oncology. Predominantly the occurrence of these enzymes is in the form of lysine methyltransferases harboring a canonical SET domain or a homologous domain [11]. The catalytic motif in the SET domain is accountable for the catalytic activity/histone methylation activities of the HMTs and is flanked on either side by the cysteine-rich pre-SET domains which preserve the structural stability of the protein and the post-SET domain offering a hydrophobic channel to participate in composition of parts of active sites of the enzyme [21]. The presence of SET domain renders the HMTs their own unique folding structure making them different from other methyltransferases. For instance, the histone methyltransferases make use of a common cofactor, S-adenosyl-L-methionine (SAM), as the methyl donor. It has been seen that SAM displays a common extended conformation in DOT1L and the RMTs [22] and depicts a structural homology between the catalytic domains of DOT1L with the arginine

methyltransferases (RMTs), in contrast to the U-shaped conformation that SAM adopts in the catalytic domain of the SET domain KMTs [23].

On the basis of sequence homology in and around the catalytic SET domain and on similar protein domain architecture and evolutionary history, seven subfamilies of lysine methyltransferases (KMTs) exist and one KMT family without SET domain:

(1) KMT1 family: Suppressor of variegation 3–9 (SUV39) family [24]
(2) KMT2 family: SET1/mixed lineage leukemia (MLL) family [25]
(3) KMT3 family: SET2/nuclear receptor SET domain-containing (NSD) family [26] and SET and MYND domain-containing (SMYD) family [27, 28]
(4) KMT4 family: Dot1L the human, non-SET domain KMT [29, 30]
(5) KMT5 family: Suppressor of variegation 4–20 (SET8 and SUV4-20) family [31–33]
(6) KMT 6 family: The enhancer of zeste (EZ) family [34]
(7) KMT7 family: (SET7/SET9/SETD7 family)
(8) Retinoblastoma-interacting zinc-finger (RIZ)/PR domain-containing (PRDM) family [35]

A brief summary of the activating/repressing histone methylations of lysine residues with biological functions is represented in Table 7.1 {Reprinted from reference: [36] with copyright permission}.

#### 7.2.2.1 KMT1 Family: Suppressor of Variegation 3–9 (SUV39) Family

The KMT1 family comprises of the SUV39 family that specifically methylates H3K9 (KMT1A/SUV39H1, KMT1B/SUV39H2, KMT1C/EHMT2/G9a, KMT1D/EHMT1/GLP, KMT1E/SETDB1 or SET domain bifurcated 1, and KMT1F/SETDB2). The members of the SUV39 subfamily are named after the first characterized mammalian KMTs, SUV39H1 and SUV39H2, and homologues of *D. melanogaster* Su(var)3-9 are defined by the presence of SET domain flanked by two cysteine-rich modules: the pre-SET and post-SET domains on either side [10] that are indispensable for their methylation activity. However, SETDB1 and SETDB2 contain a fully functional large insertion, 347 amino acids long within their SET domains to create a "bifurcated domain" that is conserved across worms, flies, and mammals, unseen in any other histone methyltransferase [21, 37]. SUV39H1 and SUV39H2 are the dominant enzymes mediating the bulk of H3K9 trimethylation (H3K9me3) at a highly compact, transcriptionally silent pericentric heterochromatin domain [38]. Contrarily G9a and GLP mono- and di-methylate H3K9 at euchromatin, and therefore known as euchromatic histone methyltransferases, EHMT1 and EHMT2 respectively [39].

Additionally, SUV39 subfamily members harbor certain unique motifs in their proteins that aid in the specific recognition of epigenetic marks like methylated H3K9 which allows the H3K9 methyltransferases to be both efficient writers and readers of histone marks and for the nucleation and spreading of H3K9 methylation along chromatin.

While SUV39H1/SUV39H2 have a SET domain adjacent, chromatin-organization modifier domain (chromodomain) that recognizes methylated histones lysines [40, 41], the G9a and GLP contain an ankyrin repeat domain (ANK) possessing a methyl-lysine-binding module that allows binding to preexisting H3K9me1 and H3K9me2 marks, independently of SET domains [18], and even spreading these methyl marks to the neighboring nucleosome. Moreover, some adaptor proteins help in spreading methyl marks by recruitment of writers as in the case of heterochromatin protein 1 (HP1), which is an H3K9me2 and H3K9me3 reader that recruits the H3K9me3 writer SUV39H1 [13, 42].

Consequently, the loss of SUV39H1 and SUV39H2 reduces H3K9me3 levels genome-wide [43] with a particular loss at constitutive heterochromatin [44] and facultative heterochromatin (such as the inactive X-chromosome) [45] leading to activation of transcription. Contrastingly, in mammals, the levels of H3K9me1 and H3K9me2 in euchromatic regions were drastically reduced by loss/knockout of the genes encoding G9a and/or GLP leading to embryonic lethality without affecting H3K9me3 levels due to the likely activity of SUV39H1 and/or SETDB1, justifying the vitality of G9a/GLP in mammalian development [38, 46]. Adding up, G9a knockdown leads to a global reduction of H3K9me2 at 67 % of promoters [47].

Two of the most well-studied H3K9 methyltransferases, G9a and GLP, are very strongly expressed in multiple tissue types, including skeletal muscles, peripheral blood leukocytes, and bone marrow and do not rely on any cross talk between neighboring marks for their catalytic activity but only on the presence of an un- or monomethylated lysine [48]. Thus, G9a and GLP can independently exert their catalytic activity either as homodimeric and heterodimeric complexes through their SET domains [39, 46]. It has been always debatable as to how KMTs are (co)-recruited to the target sites in mammals. In this context, a set of transcription factors such as E2F6 within the GLP complex was found to target H3K9 methyltransferases particularly GLP to silence proliferation of genes in quiescent cells [49]. Very lately G9a and GLP were reported to show prioritized occupancy to promoters by widely interspaced zinc-finger-containing protein (WIZ) and ZNF664 [50].

Another class of H3K9 methyltransferases, SETDB1 and SETDB2, facilitate transcriptional repression by trimethylation of histone H3K9 [51, 52] and harbor a methyl-CpG-binding domain which has affinity toward methylated DNA and an extra Tudor domain in SETDB2, functioning as a reader for methyl-lysines [15, 53]. Interestingly, SETDB1 null mutants are embryonic lethal between 3.5 and 5.5 days post coitum, signifying its role in early development [54]. H3K9me3-mediated repression through SETDB1 silences some spontaneous mutations scattered throughout euchromatic regions of the genome due to endogenous retroviruses [55, 56].

#### 7.2.2.2 KMT2 Family: SET1/Mixed Lineage Leukemia (MLL) Family

The KMT2 family includes *Drosophila* trithorax homologues, MLL family proteins (MLL1–MLL5, named KMT2A–KMT2E, respectively), and two proteins KMT2F/SET1A and KMT2G/SET1B and KMT2H/ASH1 and exhibit H3K4 methyltransferase activity. The MLL family of KMTs (MLL1-MLL5) is a large group of proteins harboring multiple kinds of domains which include (i) cysteine-rich domain or

the CxxC that acts as a homologue of DNMTs, (ii) DNA-binding AT hooks, (iii) plant homeodomain finger (PHD) and Win motifs, (iv) a bromodomain (BD), (v) a transactivation domain (TAD), (vi) nuclear receptor interaction motif (NR box), and ( vii) SET domain at the C-terminal that bestows MLL its histone methyltransferase (HMT) activity [57]. Even though MLL1 group of proteins mainly participate in the hematopoiesis and embryonic development by regulating homeobox (HOX) gene expression, other MLL proteins can also mark non-HOX genes and are implicated in cellular memory and heritable changes through chromatin modifications [58]. Also, the regulation of transcriptional activity by MLL 2 and MLL3 is mediated through their association with H3K27 demethylase KDM6A/UTX which occupies the promoters of HOX gene [59]. Recurrent rearrangement of the MLL is prevalent in human acute leukemias [60]. The enzymatic/H3K4me3 activity of the MLL complexes comprises of three subunits mainly WDR5 (WD repeat domain 5), RBPB5 (retinoblastoma-binding protein 5), and ASH2L (*Drosophila* ASH2-like) [61, 62].

#### 7.2.2.3 KMT3 Family: SET2/Nuclear Receptor SET Domain-Containing (NSD) Family and SET and MYND Domain-Containing (SMYD) Family

The KMT3 family comprises of the yeast Set2 homologue SETD2/KMT3A, nuclear receptor-binding SET domain protein 1, NSD1/KMT3B, and the SET and MYND domain-containing proteins SMYD2/KMT3C, SMYD1/KMT3D, and SMYD3/KMT3E. The principal methyltransferase activity attributed to this group is histone H3K36, but their KMT activities are not just limited to H3K36. For instance, SETD2, a Huntington interacting protein, has H3K36 trimethylation activity which is a specific epigenetic mark for activation of gene expression [63, 64]. However, unlike SETD2, NSD1 via its dimethylation activity at H3K36 is involved in modulating gene activation [65]. In addition to its unexpected methylation activity at H4K20, it is also shown to be implicated in early postimplantation development [66]. NSD1 has been associated with cancers with oncogenic properties as obviously visible in intermittent translocation of NSD1 fused to nucleoporin-98 in childhood acute myeloid leukemia [65, 67] and association of its two homologues, NSD2/MMSET/WHSC1 and NSD3/WHSC1L1 with multiple cancers. Another representative of KMT 3 family member, SMYD2, has methylation activity toward both H3K4 and H3K36 [28, 68] compared to only H3K4 KMT activity of SMYD1 and SMYD3 [27, 69].

#### 7.2.2.4 KMT4 Family: DOT1L, the Human, Non-SET Domain KMT, Only Example of SET Independent Lysine Methylation in Mammals

DOT1L or disruptor of telomeric silencing-1 (Dot1), a gene found in *Saccharomyces cerevisiae* [70], is the only lysine methyltransferase that lacks a SET domain. This gene is the mammalian homologue but is a domain similar to that of Gly N-methylase [11]. DOT1L is the only enzyme known till date to methylate mono-, di-, and trimethylation of the ε-amino group on H3K79, an activating mark with respect to gene transcription as reported from recent studies in yeast, flies, and mice [29, 30].

So far, due to the sluggish turnover of this methyl mark, no lysine demethylase (KDM) has been instrumental in removing this mark [71].

Evidence from the studies on knockout of DOT1L gene in mice ends up in complete loss of H3K79 methylation exposed the essential role of DOT1L in various pathophysiological events. This kind of methylation by DOT1L mainly regulates the transcriptional regulation, cell-cycle regulation, DNA damage response (DDR) and repair, embryonic development, hematopoiesis, cardiac function, and leukemia development [11, 72, 73]. Cells deficient in DOT1L exhibit severe loss of H3K79 methylation with loss of dimethylated H3K9 (H3K9me2) and H4K20me3 marks at centromeres and telomeres [72] leading to aneuploidy, elongation of telomeres, and cell proliferation abnormalities. Such precedence has surfaced the relevance of H3K79 methylation by DOT1L being vital for heterochromatin formation and chromosome integrity. The in vitro experimental results to potentially identify substrate specificity of DOT1L revealed nonbinding activity of DOT1L on free histones or methylated peptides but specific binding activity only on nucleosomal substrates, attributed to a stretch of positively charged residues at the carboxy-terminal part of human DOT1L [11].

#### 7.2.2.5 KMT 5 Family: Suppressor of Variegation 4–20 (SET8 and SUV4-20) Family

The KMT5 family primarily consisting of enzymes that methylate H4K20 is represented by KMT5A/SET8, specifically exhibits H4K20me1 activity, and is related to gene repression and activation, depending on its chromatin context. For instance, SET8-dependent H4K20me1 activity represses gene expression and is critical for mitosis [74]. It is also essentially associated with WNT target gene activation [75] and other chromatin-associated processes, including DNA replication and damage response [76–81]. The other members of KMT5 family, SUV420H1 and its homologue SUV420H2 though their di- and trimethylation of H4K20, along with H3K9me2/3, establish transcriptionally silent pericentric and telomeric regions [32, 82]. Dimethylation of H4K20, recognized by the Tudor domain of 53BP1 in mammals, mediates the targeting of these proteins to DNA damage sites [83].

#### 7.2.2.6 KMT 6 Family: The Enhancer of Zeste (EZ) Family

*The two H3K27 methyltransferases, KMT6A/EZH2 and KMT6B/EZH1, are the only mammalian histone methyltransferases identified so far which exhibit H3K27 di- and trimethyltransferase activity. These two methyltransferases are also known to be catalytic components of polycomb repressive complex 2 (PRC2) in mammals* [84]. It has been well documented that the obligatory association of EZH1 and EZH2 with the other three PRC2 members, suppressor of zeste 12 homologue (SUZ12), embryonic ectoderm development (EED), and the retinoblastoma-binding proteins RBBP7 or RBBP4, is mandatory for executing the methyltransferase activity in spite of the existence of SET domain in these enzymes [85, 86]. *The co-occupancy of the active*

*methyl marks H3K4me3, H3K36me2, and H3K36me3 with PRC2 is speculated to inhibit the activity of PRC2 as well* [87]. Intriguingly, H3K27 methylation activity both in vitro and in vivo was also reported to be associated with the KMT1 family members G9a and GLP (Tachibana et al., 2001), which when depleted or knocked out in embryonic stem (ES) cells resulted in a significant decrease in H3K27me1 levels [88]. Apart from these studies, recent literature also highlights a functional and physical interaction of G9a and GLP with PRC2 to maintain the silencing of developmental genes raising the plausibility of them being inserted together onto a shared common genomic loci by a common recruiting factor [89]. EZH2, being a component of PRC2, maintains the repressive state of chromatin owing to its high expression and high H3K27me3 levels in human cancers and rapidly dividing cells such as embryonic stem cells and cancer stem cells [90, 91]. Excitingly, EZH1 was found to be present in a noncanonical (PRC2-EZH1) complex as an H3K27 methyltransferase and functionally redundant with EZH2 [92].

#### 7.2.2.7 KMT 7 Family: (SET7/SET9/SETD7 Family)

The KMT7 family is composed of one protein, SET7/9, which monomethylates histone at H3K4 [93]. Conversely, SET7/9 was seen to methylate non-histone proteins such as p53, DNA methyltransferase 1 (DNMT1), estrogen receptor alpha (ERa), nuclear factor kappaB (NFkB), and components of the TATA-binding protein (TBP) complex, TBP-associated factors TAF10 and TAF7 [94]. On the contrary, H3K4 demethylase KDM1A/LSD1, via a recognized consensus sequence in substrates for SET7-mediated lysine methylation, K/R–S/T–*K*, is able to remove the methyl mark on most of these substrates [95, 96]. This activity thus establishes the KMT7/KDM1A as a classic emerging model for dynamic lysine methylation of both histone and non-histone proteins.

#### 7.2.2.8 KMT 8 Family: Retinoblastoma-Interacting Zinc-Finger (RIZ)/ PR Domain-Containing (PRDM) Family

*Currently, the KMT 8 family or PRDM family of methyltransferases comprises of 16 members, of which 4 are H3K9 methyltransferases. These methyltransferases are defined by a conserved amino-terminal catalytic positive regulatory domain called PR (PRDI–BF1–RIZ1 homologous)* [97] which is quite conserved and shows 20–30% identity to the SET domain. *Along with the zinc-finger repeats, the presence of PR domain makes the PRDM proteins to function as bona fide transcription factors and simultaneously as histone-modifying enzymes* [98]. *Some members of KMT8 family, including PRDM2, PRDM3, PRDM6, PRDM8, PRDM9, and PRDM16, show a well-defined histone methyltransferase activity, whereas almost all the members show chromatin regulatory functions by recruiting the chromatin modifiers* [35]. *Two members of the family, PRDM6 and PRDM9, showed methylation of H4K20 and H3K4, respectively, in contrast to H3K9 methylation as reported for PRDM2, PRDM8* [98], *PRDM3, and PRDM16* [99].

### 7.2.3 Consequences of Histone Lysine Methylations on Silencing of Genes

"Silent" heterochromatin is characteristically typified by high levels of DNA and histone H3K9 and H3K27 methylation, low levels of H3K4 methylation, and generally low levels of histone acetylation. Heterochromatin in general is categorized into facultative and constitutive regions comprised of developmentally regulated genes and repetitive sequences located at centromeric and telomeric regions, respectively. *Lysine methyltransferases ensure the repression of genes by transcriptional silencing associated with the heterochromatin. Generally, the levels of lysine methylation mainly H3K9, affecting the promoters or the non-genic regions, have a positive correlation with silencing of genes. For example, there seems to be preferential occurrence of H3K9 methyltransferases such as SUV39H1 and SUV39H2 which show extended binding at constitutive heterochromatin* [43, 45] *w.r.t. other methyltransferases involved in transcriptional repression like SETDB1* [37]*, G9a, and GLP* [39]. Adding on, in case of histone H1 methyl mark deposition that induces condensation of chromatin via decreased nucleosome spacing, the major role is played by G9a-mediated methylation at Lys26 of histone H1 isotype 4. This also creates a tethering site for chromatin-binding proteins HP1 and L3MBTL1(lethal 3 malignant brain tumorlike protein 1) [100]. Furthermore, the automethylation of G9a and GLP expedites their own recruitment to chromatin and binding of HP1 to H3K9me2 marks [42]. From previous studies it was evident that SUV39H1 is recruited by HP1 [101], and therefore the di- or trimethyl marks (H3K9me2/3) deposited by G9 and GLP enforce a stronger silencing platform at both euchromatic and facultative heterochromatic regions. The generation and maintenance of facultative heterochromatin also require H3K27me3 methyl mark deposition by the conserved EZH2 complex known as polycomb repressive complex 2 (PRC2) [45]. Constitutive heterochromatin requires catalytic activity of SUV39H1 and SUV39H2, as well as H3K9 monomethylation on the nucleosomes via PRDM3 and PRDM16 [99]. This phenomenon is further powerfully corroborated by the knockout of PRDM3 and PRDM16 in mice that causes the heterochromatin to be dispersed and disrupts the nuclear lamina thereby indicating the essence of PRDM in heterochromatin integrity through its H3K9 monomethylation activity.

Finally, a genome-wide study has unveiled the existence of large and diffuse regions of H3K9me2 modification covering up to 4.9 Mb, termed as large organized chromatin "K" modifications (LOCKs). These regions are confined in larger regions of euchromatin and conserved between mice and humans and show an increment in size subsequent to cellular differentiation [102]. There has been an overlap of around 80% of LOCKs in differentiated tissues with nuclear lamin B1-associated domains (LADs) [103]. Recent investigations in mammals and *C. elegans* have linked H3K9 methylation and the tethering of chromatin to nuclear lamina [104, 105] and also identifying G9a in the regulation of chromatin contacts with nuclear lamina in human cells. H3K9 methylation has emerged to be an evolutionarily conserved mechanism that promotes peripheral localization and silencing of chromatin. Thus, it is quite tempting to envision that H3K9me2 could play a major role in

docking LOCKS at nuclear periphery with most of the G9a-repressed genes, marked with H3K9me2 in ES cells, found to be localized to the nuclear periphery [106]. Put together, lysine methylations specifically H3K9 methylation might be emerging as a determining factor in establishing well-structured and well-ordered chromosome with an orchestrated and organized nuclear architecture, through a heightened association of transcriptionally inactive genomic regions with the nuclear periphery.

### 7.2.4 Lysine Methylation of Non-histone Targets in Mammalian Cells

The first evidence of the methylation of a lysine residue came into sight from the work of Ambler and Rees in the year 1959 [107], in the flagellin protein of *Salmonella typhimurium*. Although the origin and physiological relevance of the methyl-lysine residue were mysterious at that time, with the discovery of methylations of histone proteins in the 1960s and 1970s [108], prevalence of this modification was established and, followed by the predominance of this PTM in both prokaryotes and eukaryotes, was confirmed by subsequent findings on the methylation of a wide range of proteins other than histones [109–115]. Historically, the research on protein lysine methylation was initiated to decipher its epigenetic functions stressing on histone methylation, which is of course undoubtedly well documented and well known too. Nevertheless, with the progress of functional assays and proteomic analysis, using mass spectrometry has enabled the identification of methylation sites to become feasible both in vitro and in vivo, allowing the recent reports to expand the language of covalent histone modifications to non-histone proteins.

In the twenty-first century, the discovery of several lysine methyltransferases (PKMTs) and the demethylases (PKDMs) has also expedited the understanding of the role of protein lysine methylation on non-histone proteins as an emerging PTM with important biological consequences. The processes influenced by such PTMs encompass protein-protein interactions, localization, stability, and enzymatic activity which affect indirectly the chromatin structure and remodeling, apoptosis [116], gene transcription [117], signal transduction, and DDR [15, 118, 119]. An important rising concept is that a constant cross talk between methylation and other PTMs (such as phosphorylation, ubiquitylation, and acetylation) and the integration of methylation signals from histones with those from non-histone proteins are essential for regulating such cellular functions for ensuring desired biological outcomes.

Owing to such a growing interest in this burgeoning field of non-histone protein methylation and due to the brisk advances in experimental techniques like peptide arrays coupled with peptide-/domain-based pull-down assays, integrated with the tremendous surge in analytical proteomics, particularly mass spectrometry [120–123], the list of methylated proteins has been escalating at a rapid pace. In this regard, the current PhosphoSitePlus database enlists 1005 lysine methylation sites in 974 human proteins with most being non-histone substrates [124]. Thus, a comprehensive list of a large number of non-histone substrates/proteins which have

been identified as substrates of lysine methyltransferases with severe impact on many biological functions with insurmountable number of biological consequences has been provided in Table 7.2 with the associated references {Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology [125] Dec 10, 2014}.

As evident from the enlisted precedence in Table 7.2, lysine methylations encompass the common PTMs of non-histone proteins with a wide range of cellular functions, and as the methylproteome in mammalian cells is just beginning to be delineated, a clear understanding of the full spectrum of cellular processes that it can regulate will also be unveiled soon. From whatever is known currently, expectedly the size of the methylproteome can be comparable to that of the phosphotyrosine proteome. Nonetheless, approximately 21,000 phosphotyrosine sites in mammalian cells and tissues have been listed with respect to around 4,000 lysine and arginine methylation sites combined together [125]. Functionally annotating the residues getting methylated whether lysine or arginine could be an answer to exploit this mushrooming field of protein methylation for therapeutic purposes, keeping in mind that anomalous methylations effect many human diseases, markedly cancer [116, 126, 127]. Subsequently this would lead to targeting the lysine methyltransferases or demethylases deregulated in diseases like cancer as attractive therapeutic strategies or drug targets [128, 129].

A methodical identification coupled with functional and mechanistic characterization of lysine methylation of non-histone proteins would facilitate the development of innovative strategies for disease diagnosis and therapy and show the way to a complete, or nearly complete, understanding of the mammalian methylproteome to better appreciate the functions of noncanonical protein methylations.

### 7.2.5 Small-Molecule Inhibitors of Histone Lysine Methyltransferases

The epigenetic landscape of human genome is packed up with gradual alterations which, apart from being engrossed in varying patterns of gene expression, also open up opportunities to invent strategies that can be designed toward the preliminary stages of diseases. In view of the fact that such epigenetic alterations owing to their reversible nature are found to be quite distinctive from genetic mutations, the development of epigenetic therapeutics is therefore based on exploring the enzymes responsible for establishing and maintaining these epigenetic marks, as a new class of potential drug targets for treatment and curing diseases. Many of these epigenetic enzymes encompassed by the DNA methyltransferases (DNMTs), protein methyltransferases (PMTs), demethylases, histone acetyltransferases, histone deacetylases, ubiquitin ligases, and the kinases are encoded in the human genome and are classified by the nature of the covalent modifications they catalyze and by the substrates upon which they act.

Even though HDACs and DNMTs have been successful targets of both natural or synthetic and small-molecule inhibitors for treatment of human cancers and many

**Table 7.2** Non-histone substrates of lysine methylation

| Biological function/ cellular processes | Substrate/ protein | Lysine(s)/residue | KMT | Other SET domain-containing proteins | Biological consequence | SET domain present (+) or absent (−) | References |
|---|---|---|---|---|---|---|---|
| Transcription | p53 | 370 | | SMYD2 | p53-dependent transcription-inhibited | (+) | [130, 131] |
| | | 372 | SETD7 | | p53 activation (challenged by two new reports) | (+) | [132–134] |
| | | 373 | GLP | | Inhibition of p53 | (+) | [135, 136] |
| | | 382 | SETD8 | | Promotes interaction with L3MBTL1, represses p53 activity | (+) | [137] |
| | RB | 810/873 | SETD7 | | Cell cycle arrest, interaction with HP1, transcriptional repression | (+) | [138, 139] |
| | | 810/860 | | SMYD2 | Elicits E2F1-mediated transcription, enhances L3MBTL1 binding to RB1 | (+) | [140, 141] |
| | E2F1 | 185 | SETD7 | | Enhanced proteasomal degradation, prevention of apoptosis | (+) | [142] |
| | RELA | 37 | SETD7 | | Enhanced promoter binding, activation of NF-κB target gene | (+) | [143, 144] |
| | | 314/315 | SETD7 | | Enhanced proteasomal degradation, repression of NF-κB target genes | (+) | [138, 139] |

(continued)

**Table 7.2** (continued)

| Biological function/ cellular processes | Substrate/ protein | Lysine(s)/residue | KMT | Other SET domain-containing proteins | Biological consequence | SET domain present (+) or absent (−) | References |
|---|---|---|---|---|---|---|---|
| | | 218/221 | NSD1 | | Activation of NF-κB | (+) | [145] |
| | | 310 | SETD6 | | Repression of NF-κB target genes via GLP | (+) | [117] |
| | TAF10 | 5 | SETD7 | | Triggers TAF 10-dependent transcription | (+) | [146] |
| | STAT 3 | 140 | SETD7 | | Downregulation of STAT3-dependent transcription | (+) | [147] |
| | | 180 | EZH2 | | Increased STAT 3 activity due to its high phosphorylation | (+) | [148] |
| | FOXO3 | 271 | SETD7 | | Decrease protein stability | (+) | [149] |
| | YAP | 494 | SETD7 | | Cytoplasmic retention | (+) | [150] |
| | GATA4 | 299 | EZH2 | | Decreased GATA4-dependent transcription | (+) | [151] |
| | MEF2D | 267 | G9a | | Inhibit transcription | (+) | [152] |
| | MYOD | 104 | G9a | | Inhibit transcription | (+) | [153] |
| | C/EBP | 39 | G9a | | Inhibit transcription | (+) | [154] |
| | WIZ | 305 | G9a | | Unknown | (+) | [48] |
| | RUVBL2 | 67 | G9a | | Inhibit hypoxia-inducible genes | (+) | [155] |
| | MTA1 | 532 | G9a | | Unknown | (+) | [156] |
| | KLF12 | 313 | G9a | | Unknown | (+) | [48] |
| | RORα | 38 | EZH2 | | Enhanced proteasomal degradation | (+) | [157] |
| | MYPT1 | 442 | SETD7 | | Unknown | (+) | [158] |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | IRF1/2 | 126/134 | SETD7 | | Unknown | (+) | [159] |
| | PIAS2 | 2076 | SETD7 | | Unknown | (+) | [159] |
| | AR | 630/632 | SETD7 | | AR-dependent transcription | (+) | [160] |
| | FXR | 206 | SETD7 | | Enhanced binding to response elements and transactivation | (+) | [161] |
| | ER | 302 | SETD7 | | Stabilization of and increased transactivation by ERα | (+) | [162] |
| | UBF | 232/254 | SETDB1 | | Nucleolar chromatin condensation, decreased rDNA transcription | (+) | [163] |
| Methyltransferases/ Demethylase | DNMT1 | 70 | G9a | | Unknown | (+) | [48] |
| | | 142/1094 | SETD7 | | Enhanced proteasomal degradation | (+) | [164] |
| | DNMT3a | 47(human)/44 (mouse) | G9a/GLP | | Binding of MPHOSPH8 to DNMT3A | (+) | [165] |
| | SUV39H1 | 105/123 | SETD7 | | Heterochromatin relaxation and genome instability | (+) | [166] |
| | G9a | 165 | G9a | | Binding of HP1 and CDYL | (+) | [42] |
| | | 239 | G9a | | Binding of HP1 and CDYL | (+) | [42] |
| | GLP | 174 | G9a | | Binding of HP1 and CDYL | (+) | [167] |
| | | 205 | GLP | | Binding of MPP8 to GLP | (+) | [167] |
| | MAM | 16 | G9a | | Binding of HP1 | (+) | [167] |
| Acetyl transferases/ deacetylases | PCAF | 78/89 | SETD7 | | Unknown | (+) | [168] |
| | CDYL1 | 135 | G9a | | Decreased interaction with H3K9me3 | (+) | [48] |
| | SIRT1 | 233, 235, 236, 238 | SETD7 | | Unknown | (+) | [169] |
| | HDAC1 | 432 | G9a | | Unknown | (+) | [48] |
| | ACINUS | 654 | G9a | | Unknown | (+) | [48] |

(continued)

**Table 7.2** (continued)

| Biological function/ cellular processes | Substrate/ protein | Lysine(s)/residue | KMT | Other SET domain-containing proteins | Biological consequence | SET domain present (+) or absent (−) | References |
|---|---|---|---|---|---|---|---|
| Chromatin/ chromosome regulation | EED | 66/197/268/284 | G9a | | Regulates the activity and specificity of PRC2 | (+) | [170] |
| | CENPC | 414 | SETD7 | | Unknown | (+) | [159] |
| | MECP2 | 347 | SETD7 | | Unknown | (+) | [159] |
| | CBX4/PC2 | 191 | SUV39H1 | | TUG1 ncRNA-dependent recruitment to polycomb bodies | (+) | [171] |
| DNA replication, synthesis, and repair | PCNA | 248 | SETD8 | | Stabilization of PCNA, proper DNA replication | (+) | [172] |
| | PARP1 | 508 | SETD7 | | Stimulates poly-ADP-ribose formation after oxidative stress | (+) | [173] |
| | KIN17 | 135 | METTL22 | | Unknown | (+) | [174] |
| | PPARBP | 1006 | SETD7 | | Unknown | (+) | [159] |
| Cellular signaling | NUMB | 158/163 | SETD8 | | Diminished association with p53, ubiquitination-dependent p53 degradation | (+) | [116] |
| | MAP3K2 | 260 | | SMYD3 | Increase in MEK1 and MEK2 and ERK1 and ERK2 activities | (+) | [126] |
| | VEGFR | 831 | | SMYD3 | Enhance VEGFR1 kinase activity | (+) | [175] |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Viral proteins | TAT(HIV protein) | 51 | SETD7 | | Stimulation of HIV transcription | (+) | [176] |
| | | 50/51 | SETDB1 | | Inhibition of HIV transcription | (+) | [177] |
| Chaperones | HSP90 | 209/615 | | SMYD2 | Myofilament organization | (+) | [178] |
| | HSP70 | 561 | SETD1A | | cancer cell proliferation enhanced by activating Aurora kinase B | (+) | [179] |
| | | | | | Methylation-dependent nuclear localization | | |
| | DNAJC8 | Unknown | SETD6 | | Unknown | (+) | [180] |
| Metabolism | CAMK | 115 | CaKMT | | Regulating CaM signaling-dependent pathways | (+) | [181] |
| Kinases/phosphatase | TTK | 708/710 | SETD7 | | Unknown | (+) | [159] |

other diseases, additional families of histone-modifying enzymes like the lysine methyltransferases due to their reversible modifications can also be amenable to small-molecule modulation. The desire to control epigenetic transcription through chemical probe development or by pharmacological means has generated a lot interest in the medicinal chemists and many drug discovery scientists to target the protein lysine methyltransferases (PKMTs). This stir in the interest has been principally due to selective mechanisms of catalysis involving an organic cofactor S-adenosylmethionine (SAM) and emerging rich literature base of crystallographic and enzyme kinetic studies of these enzymes. SAM, instrumental in being the architect of the catalytic activity of these methyltransferases, consists of a SAM-binding pocket accessible from one face of protein and a narrow hydrophobic acceptor (i.e., lysine) channel forming the opposite face of the protein permitting the two substrates to enter the active site from opposite sides of the surface of the enzyme.

Hence, from the time the first histone lysine methyltransferases were characterized in the year 2000 [10], considerable progress by the pharmaceutical industry and many academic groups has resulted in discovery of many improved inhibitory compounds with regard to potency and selectivity toward different families of lysine methyltransferases. A broad list of the reported small-molecule inhibitors discovered till date, with their respective $IC_{50}$ values, the target lysine methyltransferase, and the modification or substrate with associated and significant references has been enlisted in the Table 7.3 {Reprinted by permission from Springer Publishers Heidelberg from Springer reference} [182].

Hence, owing to the successful discovery of the small-molecule inhibitors targeted against the histone lysine methyltransferases (HKMTs) with some of these drugs already in clinical trials to be launched as anticancer drug targets and potential therapeutic tools for diseases, it has been feasible to block the process of epigenetic modification of deposition of methyl marks by these HKMTs as being shown in Fig. 7.2.

### 7.2.6 Targeting Histone Methyltransferases: Profound Implications in Disease and Development

A complete survey of human genome has brought into limelight the impact of epigenetic modifications especially histone modifications such as lysine methylation affecting the chromatin structure and function on mammalian gene expression. The enzymes that catalyze methylation of lysine residues (protein lysine methyltransferases, PKMTs) have gained prominence and have become molecules of substantial interest due to expanding levels of biochemical and biological data available indicative of PKMT involvement in having pathogenic functions in many neurodegenerative diseases. The PKMTs have thus been reported to participate in human growth and development such as nervous system, cognition, behavior, lymphocyte development and immune function, cellular pluripotency, and cellular differentiation. It is interesting to note that such conversion of enzymatic activities of PKMTs

**Table 7.3** List of lysine methyltransferases inhibitors discovered till date

| Inhibitor | Target enzyme | Modification | $IC_{50}$ values | Comments | References |
|---|---|---|---|---|---|
| BIX-01294 | G9a/ GLP | H3K9me1/2 | G9a(0.21.7μM) GLP(0.03–0.38μM) | First selective inhibitor of G9a | [183] |
| Bunazosin | G9a | H3K9me1/2 | Unknown | Alpha adrenoceptor antagonist | [184] |
| UNC-0224 | G9a/ GLP | H3K9me1/2 | $K_i$ 2.5 nM | Essentially equipotent against G9a and GLP | [185] |
| UNC0638 | G9a/ GLP | p53K373 | $K_i$ 2.5 nM | Inhibitor reduces H3K9Me2 in MDA-MB231 cells with an IC 50 of 81 nM | [186] |
| UNC0321 | G9a/ GLP | H3K9me1/2 | $K_i$ 63 pM | Improved potency over both BIX-01924 and UNC0224 | [185] |
| E72 | G9a/ GLP | H3K9me1/2 | G9a inhibitor ($IC_{50}$100 nM) | Higher affinity for GLP | [187] |
| UNC0642 | G9a/ GLP | H3K9me1/2 | $IC_{50}$ : 2.5 nM | Increased in vitro and cellular potency, low cell toxicity, and excellent selectivity with improved in vivo PK properties, suitable for animal studies | [188] |
| A-366 | G9a/ GLP | H3K9me1/2 | $IC_{50}$ : 3.3 M | Selectively inhibits G9a/GLP | [189] |
| BRD9539 (active form ofBRD4770) | G9a | H3K9me1/2 | $IC_{50}$ :6.3 μM | Reduced H3K9me2 and H3K9me3 and induced senescence in pancreatic cancer cell lines | [190] |
| DZNep | EZH2 | H3K27me1/2/3<br>H4K20me3 | $K_i$ 50 pM | Induces apoptosis, reactivates PRC2 silenced genes in cancer cells with low cytotoxicity in normal cells | [191] |

(continued)

**Table 7.3** (continued)

| Inhibitor | Target enzyme | Modification | $IC_{50}$ values | Comments | References |
|---|---|---|---|---|---|
| EPZ005687 | EZH2 | H3K27me1/2/3 | $K_i$ 24 nM | Reduces H3K27 methylation in lymphoma cells | [192] |
| GSK126 | EZH2 | H3K27me1/2/3 | $IC_{50}$ = 9.9 nM | Reduction of H3K27me3 is due to direct selective inhibition of EZH2 as reported for in vivo studies | [193] |
| EPZ004777 | Dot1L | H3K79 | $IC_{50}$ :0.4 nM | First selective DOT1L inhibitor with selective activity in treating MLL-rearranged leukemias | [194] |
| AZ505 | SMYD2 | p53K370, RbK860 | $IC_{50}$ :0.12 μM | A potent and highly selective inhibitor of SMYD2 | [195] |
| PDB4E47 | Set7/9 | H3K4me1, Tat ER | $IC_{50}$ :100nM | Not determined | [196] |

with deposition of methyl marks can have desired biological outcomes and there are examples that demonstrate the significance of maintenance of such methylation going awry resulting in the onset of many diseases and subsequently enforcing developmental defects.

#### 7.2.6.1 Histone Lysine Methyltransferases in Cancer

Mammalian epigenome is subjected to a dynamic orchestration of developmental gene expression programs precisely controlled by various local posttranslational modifications (PTMs) on histone tails but affecting chromatin globally. Among a range of PTMs identified to be occurring on chromatin, methylation of histones is a key epigenetic mark with fundamental roles in virtually all DNA-templated processes, including gene transcription. This particular PTM on histones catalyzed by a group of enzymes called lysine methyltransferases (PKMTs) is structurally stabilized by formation of macromolecular complexes which also aid in their functional activities. Such macromolecular complexes help in understanding the molecular basis of regulation of cell fate during development with even dire consequences also observed with aberrant histone methylations at the onset and progression of the disease state, which either directly inactivate or activate causal mutations thus either compelling the pathogenesis or acting indirectly as facilitators bringing about cancer-related pathways.

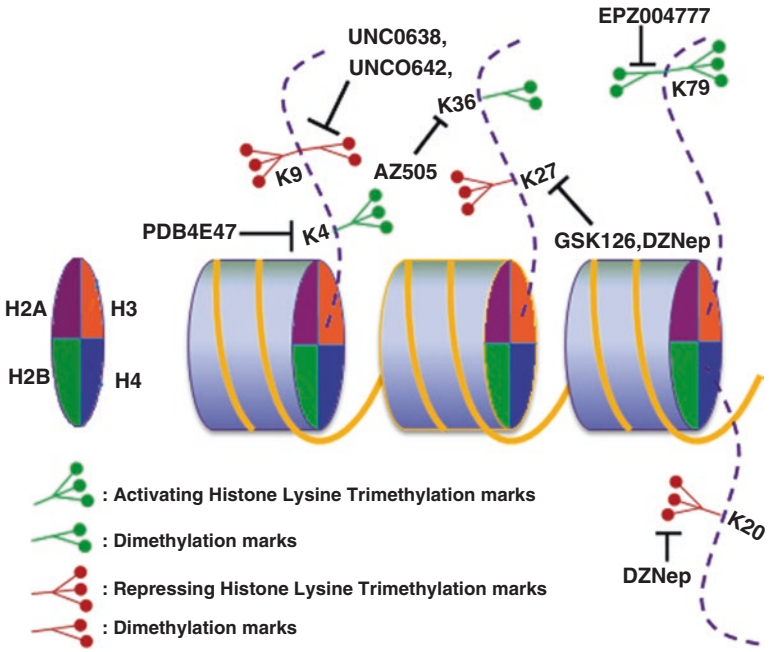


**Fig. 7.2** The small-molecule inhibitors targeted against the histone lysine methyltransferases (HKMTs) which inhibit the deposition of histone lysine methyl marks are depicted here

One of the members of the KMT6 family is EZH2, a SET domain-containing protein that forms the core of PRC2 complex and catalyzes the H3K27me3 marks. Overexpression of EZH2 or SUZ12 (another PRC2 subunit) has been associated with numerous human cancer types including prostrate, breast, bladder, colon, skin, liver, endometrial, lung, and gastric cancers as well as lymphomas and myelomas [197]. Due to high levels of EZH2 seen responsible for the invasiveness of breast carcinomas, EZH2 could be a clear indicator of patient outcome and prognosis for breast cancer [198]. Notably higher H3K27 methylation determines the phenotypic effects of EZH2 overexpression which is in turn dependent on an intact SET domain, implying a role for EZH2 enzymatic activity in pathogenesis [197, 198]. Similarly SUV39H1, which is associated with gene repression, was found in high levels in colorectal cancers [199], and another family member, Suv39H2, shows heightened correlative levels in various cancers such as bladder cancer, cervical cancer, NSCLC, esophageal cancer, osteosarcoma, prostate cancer, and STT [200]. MLL, a methyltransferase depositing H3K4 marks from KMT2 family, has severe correlation with chromosomal aberrations and is a cause of acute leukemias where the SET domain is lost in translocation [201–203]. In support of this, Dot1L, a non-SET domain KMT displaying H3K79 activity, is associated with MLL-rearranged leukemias and recruited by MLL fusion partners MLLT1, MLLT2, MLLT3, and MLLT10 to

homeobox genes and associated with transcriptional activation and elongation [60, 204, 205]. The KMT3 family members comprised of NSD family including NSD1, NSD2, and NSD3 have been implicated in many leukemias like AML (translocation fuses NSD1 to nucleoporin 98 in human acute myeloid leukemia) [65], myeloma (NSD2 translocated and increased expression in myeloma) [65, 206, 207], and lung and breast cancer and childhood acute myeloid acute leukemias (NSD3 amplified in lung and breast cancers and translocated in myeloid leukemia) [208, 209]. Another member of KMT3 family, SMYD3 with its H3K4 methyl transferase is found to be over-expressed in multiple cancers like breast, liver, colon and gastric cancers [27, 210].

The role of G9a/EHMT2 cannot be neglected in cancers owing to its role in cellular proliferation and its elevated levels in many cancers like hepatocellular, colon, prostrate, bladder, and lung and invasive cell carcinomas and B-cell chronic lymphocytic leukemias with respect to normal tissue [135]. A plethora of information on the role of G9a in activating tumorigenesis based on upcoming literature has revealed its functions in suppression of growth and increased apoptosis with increase in sub-G1 population upon its downregulation in bladder and lung cancer cell lines [211] and there are also reports about the prevalence of centrosome disruption, chromosomal instability, inhibition of cell growth, and increased cellular senescence in G9a downregulated prostate cancer cells [212]. Also, surprisingly deleting SET domain from G9a impedes the repression of Runx3 under both normoxic and hypoxic conditions in gastric cancer cells [213].

In view of the increasing reports citing the overexpression of many KMTs apart from G9a in many cancers, in addition to their responsible roles in various facets of tumorigenesis like cellular proliferation and differentiation, the KMTs have emanated as potential but powerful targets for cancer therapy. Hence, deregulating the human epigenome through these inhibitors could probably be a prime contributing mechanism for oncogenesis, and this undoubtedly supports the ongoing global efforts to develop inhibitors for KMTs as therapeutic avenues with a pursuit to design commercially available and efficient small-molecule inhibitors as anticancer drugs in the future.

#### 7.2.6.2 Histone Lysine Methyltransferases in Nervous System, Cognition, and Brain Behavior

The mammalian nervous system principally comprises of neurons and glial cells with the myelinating glia ensheathing the neurons being made up of the oligodendrocytes in the central nervous system (CNS) and Schwann cells in the peripheral nervous system (PNS), providing insulation with faster conduction of electric signals. Neural crest cells serve as progenitors of the Schwann cells and sensory neurons, and hematopoietic stem cells give rise to the microglia, while the other types of neurons, oligodendrocytes, and astrocytes are derived from neural stem cells. The neural development program encompassing the specification of precursor cells into distinct neural subtypes is regulated by a complex network of transcription factors orchestrated by a group of chromatin remodeling enzymes like the histone methyltransferases (HMTs) and the demethylases. Interestingly, a lot of efforts have

emphasized the role of histone methylation in the maturation and differentiation of neural cells.

Prominent examples of HMTs involved in such developmental phenomena include the enhancer of zeste homologue 2 (EZH2) HMT, which through the methylation of H3K27 is instrumental in maintaining the proper balance between self-renewal and differentiation of neural stem cells [214, 215]; the H3K4 HMT MLL1 that promotes neurogenesis in the sub-ventricular zone of the mouse post-natal brain by accelerating the expression of Dlx2, a homeodomain-containing transcription factor [216]; and the histone demethylases (HDMs), the H3K27me3 demethylase JMJD3, eliciting the expression of neuronal genes such as Dlx5, Gad1/2, and Dcx [217] and playing a definitive role toward the commitment of embryonic stems cells to neuronal lineage [218] and the H3K4 HDM KDM5C that via its association with the REST complex represses neuronal genes in nonneuronal tissues [219].

Histone methylation is yet another PTM tightly associated with learning and stabilization of long-term memory as evident from the mice deficient for the H3K4 HMT, MLL, or fore brain-specific knockout of MLL displaying deficits learning function [220] and in fear memory formation indicating regulation of active histone methylation in hippocampus facilitating long-term memory [221]. The same phenotype has also been prevalent upon the inhibition of the G9a/GLP complex which catalyzes H3K9me2 in the hippocampus that impacted long-term memory in mice while that of G9a in entorhinal cortex interfered with memory consolidation [222].

Fascinatingly, H3K9me2 has not been extensively studied in the brain until of late, till the regulation and roles of G9a or GLP came into the forefront using the GLP/G9a post-natal, neuron-specific conditional knockdown mice that displayed a significant decrease in global H3K9me2 levels and improper expression of genes resulting in behavioral phenotypes such as a deficit in learning, reduction in exploratory behaviors, motivation and environmental adaptations such as contextual fear conditioning as an outcome of an increase in both nonneuronal and neuronal progenitor genes [39, 223–225]. Nevertheless, in humans, deletions or loss-of-function mutations of G9a or the haploinsufficiency of the H3K9me2 HMT GLP/EHMT1 results in Kleefstra syndrome (9q subtelomeric deletion syndrome) [226–228], a genetic disorder having severe outcomes in learning disability, developmental delay, severe mental retardation, hypotonia (reduced muscle strength), brachycephaly (flat head syndrome), epileptic seizures, flat face, hypertelorism, and many other developmental and heart defects. Moreover, the heterozygous GLP knockout mice develop autistic-like behavior that resembles the human pathology [229], and reduced levels of G9a-mediated H3K9 dimethylation were implicated in neuronal plasticity induced by cocaine assumption [230], thereby providing precedence suggestive of histone methylation regulating behavioral plasticity and molecular mechanisms of drug addiction. In fact, one of the most up-to-date reports by Dr. Kleefstra and his colleagues in 2016 has highlighted the autonomous significance of Kleefstra syndrome-associated protein GLP in synaptic scaling (a kind of synaptic plasticity wherein single neurons can control their overall action potential firing rate) [231]. It was observed in this study that lowering G9a or GLP by genetic knockdown and pharmacological blockade prevented the increase of H3K9me2 and synaptic scaling

up that is suggestive of an H3K9me2-mediated repressive program that controls synaptic scaling.

Various other lysine methyltransferases have also been implicated in neurological disorders such as heightened expression of SETDB1 with elevated H3K9me3 levels is visible both in the neurons of Huntington disease patients and transgenic Huntington mice [232] and EZH2 was found to be directly inhibited by the kinase ataxia telangiectasia mutated (ATM) with elevated EZH2-mediated H3K27me3 bringing about neurodegeneration in ataxia telangiectasia patients [233]. In continuation, postmortem brain of a patient suffering from Alzheimer's disease (AD) exhibited enhanced levels of H3K9me3 compared to the same in the brain of his healthy twin brother [234]. The observations were replicated in a transgenic mouse model of AD demonstrating an increase of histone H3 methylation in the presence of beta-amyloid (Aβ) precursor protein in the prefrontal cortex.

Owing to the scarcity of expanded knowledge on the functions of histone methylations in the biology of the nervous system, it is still elusive as whether the aberrant or normal histone methylations regulating chromatin remodeling are actually a cause or consequence of the neurological diseases or pathologies, and the future certainly looks promising to unfold the hidden mechanisms of these aberrant histone modifications. But, from the current state of knowledge, it is apparent that if not a single modification, a combination of several histone modifications as drug targets could be the clue to the success of future epigenetic-based therapeutic strategies for neurological disorders.

#### 7.2.6.3 Histone Lysine Methyltransferases in Lymphocyte Development and Immune Function

The fine-tuning of immune systems in mammalian cells through directed expression of transcription programs determines stable equilibrium between beneficial and harmful immunity. Such transcriptional regulation of immune system is invaluable for the lineage specification of effector T cells, the release of effector cytokines and molecules, and the development and constant turnover of memory T cells. Posttranslational modification of histones particularly by methylation plays a principal function in coordinating the expression of these transcriptional programs in T cells. Histone methylation signatures for effector molecules and transcription factors in T cells and the functional importance of histone methyltransferases in regulating T-cell immune responses are essential to tightly controlled expression of transcriptional programs to regulate the balance of beneficial versus harmful immunity.

Histone methylations especially occurring at lysine residues, which may be monomethylated, dimethylated (me2), or trimethylated (me3), are deeply involved in establishing cell type-specific gene expression [235–237]. Multiple literature evidences have documented the presence of methyl mark depositions on histones influencing lineage-specific effector genes in differentiation [238–241]. For instance, the IFNG gene (coding for IFN-γ) in Th1 cells and the IL4 gene in Th2 cells are marked with permissive H3K4me3 marks, whereas silenced lineage promiscuous genes, such as the IL4 gene in Th1 cells and the IFNG gene in Th2 cells, are marked with

repressive H3K27me3 [242, 243] clearly indicating the role of histone methylation in the dynamic changes during development of Th1 and Th2 cells.

Genome-wide mapping analysis has also exposed the presence of histone methylation signatures such as increased H3K27me3 levels for transcription factors like PRDM1 gene encoding for Blimp-1 in naive CD8+T cells, in contrast to higher levels of H3K4me3 and lower levels of H3K27Me3 in memory CD8+Tcells at the PRDM1 locus [242, 243] thereby, justifying the up regulation of PRDM1 during transition from naive cells to effector and memory cells. Further insights into how histone methyltransferases functionally regulate these transcriptional factors have illustrated the crucial roles of MLL in regulating the development and maintenance of memory Th2 cells [240], the association of Suv39H1 and EZH2 with the stability and differentiation of distinct subsets of effector T cells such as Th2 cells [244], and, intriguingly, the vitality of histone methylation pathways for maintaining effector functions in memory T cells and their implications in promoting the survival of memory T cells [238, 240, 241, 245]. Hence, the histone methyltransferases and the resulting regulation of histone methylation pathways may orchestrate a group of transcriptional programs important for memory cell development and maintenance, for prevention of inappropriate expression of lineage-specific genes, or for converting the stimulation of TCR and cytokine signals to the T-cell-intrinsic transcriptional programs. Furthermore, some of the histone methyltransferases (HMTs) like MLL and SUV39H1 regulate T-cell-mediated airway inflammation [240, 244, 246]; the HMTs-G9a can regulate production of Th2 cytokines independent of its histone methyltransferase enzyme activity effecting the Th2 cell-mediated immunity against *Trichuris muris* [247]. Similarly, EZH2 can mediate cell signaling through methylation of other signaling proteins like interacting with NF-κB [248] or recruiting DNMT3a and DNMT3b (which mediate DNA methylation) [249] to regulate gene expression, thus giving instances of histone methyltransferases functioning at levels beyond catalyzing histone methylation.

Histone lysine methylation has also been known to participate in macrophage homeostasis primarily through Ash1L, a mammalian homologue of the *Drosophila* Ash1 gene, functioning as an H3K4 methyltransferase and enhancing the A20 expression in macrophages for the production of IL-6 [250]. Put together, loss-of-function studies on epigenetic transformers have proven DNA methylation and histone modifications to be key for cell fate decisions, lineage stability, and multiple functions of immune cell subsets [251]. Therefore a fine-tuning of epigenetic basis of immune cells and factors defining it is pivotal to sustain immune homeostasis. Imperatively, many of the external factors and molecular players like cytokines, hormones, diets, stress, etc. now probably defining the epigenetic code of immune cells owing to the inheritable nature of epigenetic alterations to daughter cells can be identified which might be provoking a lifelong inflammatory response.

#### 7.2.6.4 Histone Lysine Methyltransferases in Pluripotency and Differentiation

Profiling of mammalian epigenomes in the context of disease and development reveals key informative insights into the epigenetic underpinnings driving

multicellular development including cell fate determination, lineage commitment of cells, and even organogenesis. The fine-tuning of such complex mammalian developmental processes is brought about by means of a precise temporal order of gene expression involving both activation and repression of genes during embryonic development and is properly ensured by epigenetic regulation of chromatin structure and function. Modern concepts of epigenetic regulation driving current areas of research implicated in maintenance of normal cellular states are based on certain key biological processes such as DNA methylation, posttranslational histone tail modifications (PTMs), noncoding RNA control of chromatin structure, and nucleosome remodeling. Predominantly, posttranslational modifications of histone tails (PTMs) such as methylations, acetylations, and phosphorylations have emerged to be combinatorial, essential mechanisms used by eukaryotic cells to regulate local chromatin structure, diversify and extend their protein functions, and dynamically coordinate complex signaling networks involved in the regulation of cellular pluripotency and cellular differentiation. Not underestimating the great advancement in recent years about the understanding of cellular pluripotency and its utility in biomedical research and clinical therapy, it still remains to be explored as to how the human pluripotent stem cells (hPSCs), including embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs), are modulating their self-renewal and differentiation into functional somatic cells during cellular reprogramming and differentiation. In this regard, the consequences of posttranslational protein alterations in the hPSCs via protein lysine methyltransferases (PKMTs) in regulation of pluripotency or in the alteration of cellular states are a rapidly growing interest in the field of stem cell biology. So far so good, it is now also evident that KMTs play a crucial role in shaping the mammalian epigenome in diverse cellular and developmental contexts.

As well documented, the methylation of histone H3 lysine 4 (H3K4), H3K36, and H3K79 is related with active gene expression [252], and the methylation of histone H3K9, H3K27, and H4K20 is concerned with gene silencing [86]. The presence of bivalent chromatin bearing both the active mark H3K4me3 and the repressive mark H3K27me3 in hESCs and hiPSCs is controlled via the activity of polycomb proteins (PRC1 and PRC2) [253] that is instrumental in establishing the fate of ESC development. Demethylation of H3K27me3 mark which is deposited by PRC2 [92] drives the hESC/hiPSC differentiation. Significant correlation between gene expression and the methylation states of H3K4 and H3K27 has been illustrated in the commitment of hpSCs (hESCs and hiPSCs) [254–256]. In fact, both human and mouse ESCs exhibit high levels of association of activating H3K4me3, and the repressive H3K27me3 methylation patterns termed as bivalent domains are spotted at the untranscribed lineage-specific gene loci [257]. In this regard, it has been exclusively reported that such state of bivalency of histone modifications is a direct consequence of assaying a subset population of hESCs showing hallmarks of pluripotency and harboring unmatched developmental potentials and necessarily not a state of individual hPSCs [254]. Thus, histone methylation and chromatin remodeling have been proved to be critical for the cardiac and pancreatic differentiation and are highly similar between hESCs and hiPSCs. Interestingly, one

third of human genome differs in chromatin structure which could be due to differences in transcription factor binding and chromatin state remodeling during differentiation. This is indicated by this observation that differentiated fibroblasts and T lymphocytes have larger regions marked by H3K9me3 and H3K27me3 when compared with ESCs with similar levels of H3K36me3 between them [258, 259] and also undergo subsequent remodeling of these large expanded H3K27me3 domains to an ESC-like state during reprogramming. The histone lysine N-methyltransferase (MLL) mediating the transcriptionally active H3K4me3 marks has been found to be potential regulators in reprogramming by virtue of their subunits WRD5 getting activated by exogenous OCT4 in mouse embryonic fibroblasts upon transfection with the four Yamanaka factors and reestablishing H3K4 methyl marks [260].

The peculiar role of individual transcription factors in reorganizing the chromatin landscape during iPSC generation has increasingly been documented. In this regard, a lot of precedence is existing highlighting the essentiality of chromatin regulator EZH2 in mouse and human fibroblast reprogramming [261–263] with mechanistic details of EZH2's role in altering epigenome missing thereby leading to a great focus of interest. In a significant contribution toward defining the role of EZH2, it has been shown to be driving mesenchymal to epithelial transition (MET) during human iPSC generation. Interestingly, by using the gain-/loss-of-function approaches and specific small-molecule inhibitor of H3K27 activity, it has been reported that the H3K27me3 activity of EZH2 facilitates the induction of pluripotency by transcriptionally targeting the TGF-b signaling pathway [264]. These findings ascertain a transcriptional and miRNA modulation-based restriction of the somatic program during the initial phases of fibroblast reprogramming and thus promoting iPSC generation, covering up the mechanistic loopholes which were vigilant before.

Lysine methylation inhibitors have also been used to facilitate reprogramming of fibroblasts by treating them with inhibitors of G9a and GLP that methylate H3K9 thereby efficiently creating pluripotent cells and bypassing the need for Myc and KLF4 [265] indicating that reversal of silencing induces pluripotency. To suffice, it has also been reported that the small-molecule inhibitor of the G9a histone methyltransferase BIX-01294 (BIX) [183] can improve the reprogramming of iPSCs from neural progenitor cells (NPCs) transduced with OCT3/4-KLF4 to a level comparable to transduction with all four [266] and compensate for the exogenous expression of Sox2 or Oct4 and suggesting that loss of function of certain genes may be an effective mechanism for generating iPS cells. Furthermore, it has been illustrated that G9a can assist in fusion-based reprogramming of adult neuronal cells [267]. Interestingly, G9a and GLP have been involved in differentiation of various tissue and cell types in mice including blood, adipose, cardiac, retina, and germ cells as revealed by the cell-specific knockout experiments [247, 268–272]. Depleting G9a in differentiated ES cells allows these cells to revert back easily to a pluripotent state hinting toward the vitality of G9a in maintenance of differentiated state in these cells [273, 274]. Adding on, Oct3/4 and Nanog have an extended expression in G9a mutant embryos [275]. Lineage specification of hematopoietic stem and progenitor cells (HSPCs) is determined by progressive H3K9me2 patterning via a G9a/

GLP-dependent manner [276]. An elegant knock-in approach has been recently used to highlight the heterochromatinization of Oct3/4 in response to RA that is dependent upon H3K9me1 activity of GLP and can be reversed by loss of this binding ability delaying pluripotent gene silencing in RA-induced ES cells [277] and leading to death of such knock-in mice shortly.

Hence, dynamic epigenetic changes accompanying cellular pluripotency and cellular diversity have become a hallmark of hPSCs with emerging evidences suggesting the vital roles of PTMs like lysine methylations regulate such local chromatin changes ending up effecting gene expression levels. Owing to complex but distinctive properties and functionalities associated with each of the PTMs, a general description on the effect of different PTMs on various types of proteins affecting the pluripotency reprogramming and differentiation of human ESCs and PSCs are not possible. However, complexity of PTMs offers many accessible targets and more possibilities for characterizing hPSCs and directing their differentiation opening up many novel and important protein functions that rely on appropriate PTMs like lysine methylations but may not be feasibly identified at the transcriptional or translational levels in stem cells. This imposes widespread investigations of PTMs in hPSCs to unearth the unknown but critical mechanisms, fine-tuning pluripotency-associated signaling and cellular plasticity in hESCs and hPSCs which still continue to hold great promise for the success of regenerative medicine. With the advent of more and more sensitive and cheaper genome-wide, single-cell profiling methods and technologies hitting the researchers, a multifold information on human development and stem cell differentiation is awaiting to come.

One important insight which is worth considering is that in addition to the different posttranslational modifications of histones or non-histone proteins in mammalian cells with their consequences in disease and development being discussed more frequently, there is always a cross talk with other types of PTMs (e.g., ubiquitination and SUMOylation) occurring in human cells which are well documented to have integrated roles in the maintenance of normal biological function of various proteins and the integrity of cell signaling pathways. This is also well supported by the close proximity of the lysine residues being methylated or acetylated in core histones to several serine phosphorylation sites and constant cross talk between that of lysine and arginine termed as arginine/lysine-methyl/methyl switch [278] indicating such cross talks between PTMs are actual mechanisms underlying protein methylations and its phenotypic consequences in context of effecting biological functions in mammalian cells. Therefore, even if the information of lysine methylations of mammalian proteins is humongous compared to limited information on arginine methylation, the impact of arginine methylations cannot be underrated nor neglected seeing their relevance in cross talk with lysine methylations along with plethora of their independent functional roles and consequences on transcriptional regulation, cell signaling pathways, DNA damage, and repair with many other physiological processes being increasingly added with time.

## 7.3 Arginine Methylations of Mammalian Histones

Arginine methylation on histones and other proteins is present as one of the ubiquitous cellular posttranslational modifications (PTMs), found in both the nucleus and cytoplasm of eukaryotic cells [279] and mainly mediated by the family of protein arginine methyltransferases (PRMTs). The impact of PRMTs is determined on numerous essential biological pathways including epigenetic regulation, RNA processing, DNA repair, hormone-receptor signaling, etc. with such biological outcomes being accomplished by methylating different nuclear, cytoplasmic, and membrane protein substrates. By virtue of depositing methyl marks onto the nucleosomal histone tails, PRMTs function in conjunction with other PMTs in regulating the dynamic transitions between transcriptionally active or silent chromatin states affecting activation and suppression of gene expression [280, 281]. Thus, PRMTs may act as either transcriptional coactivators or repressors, depending on the modification site and status. More than 60 PMTs including 11 known PRMTs and more than 50 protein lysine methyltransferases (PKMTs) influencing histone methylations are encoded by the human genome prominently serving as hallmarks of the complex epigenetic makeup in human beings.

PRMTs, for their catalytic activity, methylate the guanidine nitrogen of arginine via the universal methyl donor S-adenosylmethionine (SAM) as a cofactor leaving S-adenosyl homocysteine (SAH or AdoHcy) as the side product and methylated arginine as the final product. Apparently, modifying these guanidine groups will impinge on the interaction of the arginine on the substrate with corresponding hydrogen bond acceptors like DNA, RNA, and proteins that could eventually result in altered physiological functions. The methylated arginine exists in three forms in mammals: monomethyl arginine (MMA), asymmetric dimethylarginine (aDMA), and symmetric dimethylarginine (sDMA).

Currently nine members of PRMTs (PRMTs 1–9) have been identified with established arginine methylation activity in human cells [127] and have been classified into three major types. Type I enzymes convert arginine to MMA and further to ADMA and include the PRMT1, 2, 3, 4, 6, and 8 [127, 278]. PRMT4 is also named as coactivator-associated arginine methyltransferase 1, or CARM1. Type II enzymes generate the MMA and SDMA and are comprised of PRMT5 [282] and PRMT9 [283], and Type III enzymes are represented solely by PRMT7 that creates MMA [284]. It has been reported that around 0.5% of arginine moieties are susceptible to these kind of posttranslational methylations which principally occur at glycine-, alanine-, and arginine-rich portions (GAR motifs) [285] and preferentially get methylated by PRMT1, 3, 5, 6, and 8 [286, 287]. Predominantly, the histone arginine methylation sites which have been identified include H2AR3 (PRMT1, 5, 6), H2AR29/R31 (PRMT7), H3R2 (PRMT6), H3R8 (PRMT5), H3R17/R26 (CARM1), H4R3 (PRMT1, 5, 6), and H4R17/R19 (PRMT7) [278, 288, 289], while more still remain uncovered.

A comprehensive overview of selected target histone substrates for the nine mammalian arginine methyltransferases (PRMTs) and the primary biological functions of these PRMTs with their implications are summarized below:

### 7.3.1 PRMT1

The first mammalian protein arginine methyltransferase [290] to be identified was PRMT1 [291] with the responsibility of carrying out bulk (85%) of total protein arginine methylation activity [292]. The diversity in the protein function of PRMT1 is exemplified by the lethality of the disruption of this enzyme in mice which die shortly after implantation [293]. The target specificity of PRMT1 has been determined to be on histone H4 at arginine 3 generating a H4R3me2a mark [294] making PRMT1 a transcriptional coactivator [295] and participating in signal transduction, RNA splicing, and DNA repair [296]. The PRMT1 has been reported to be overexpressed or aberrant in breast, prostate, lung, colon, and bladder cancer and leukemia [127] and pulmonary diseases like pulmonary fibrosis, pulmonary hypertension, COPD, and asthma [297] with evidences of regulatory roles in cardiovascular and renal disease [298] and diabetes [290].

### 7.3.2 PRMT2

The PRMT2 arginine methyltransferase comprises of a methyltransferase and a SH3 domain [299] that binds the N-terminal domain of PRMT8 which might also target it to the substrates [300] which happens to be H3R8 [301]. PRMT2 has also been found to act as a coactivator of both the androgen receptor and the estrogen receptor alpha [302, 303]. In addition, it can also promote apoptosis and inhibit NF-kappaB function by blocking the nuclear export of IkappaB-alpha [304]. Overexpression or aberrant expression of PRMT2 is seen in case of breast cancer [305] and pulmonary inflammation [306].

### 7.3.3 PRMT3

The presence of a zinc-finger domain at its N-terminus marks the substrate recognition module of the third arginine methyltransferase PRMT3 [307]. The target substrate is defined by the 40S ribosomal protein S2 (rpS2) that is a zinc-finger-dependent substrate [308]. The targeted null mice of PRMT3 exhibit smaller mouse embryos which survive after birth and attain normal size in adulthood and also hypomethylation of rpS2 demonstrating it as an in vivo substrate [308]. PRMT3 is overexpressed in coronary heart disease [309] and chronic kidney disease [310].

### 7.3.4 CARM1 (PRMT4)

CARM1 also referred to as PRMT4 was initially discovered as a steroid coactivator, and this finding also supported the first observation of arginine methylation regulating transcription [311]. Its recruitment to the promoters also ends in the methylation of histone H3 (H3R17me2a and H3R26me2a) and also in reinforcing many other transcription factor pathways, mediating splicing factors thereby coupling transcription and splicing [312]. The CARM1 null mice succumb to death just after birth and are smaller than their wild-type counterparts [313], and detailed analysis of these null mice has also exposed the vital in vivo roles of CARM1 in early T-cell development [314], in adipocyte differentiation [315], in chondrocyte proliferation [316], and in the proliferation and differentiation of pulmonary epithelial cells [317].

### 7.3.5 PRMT5

Generally known as a strong transcriptional repressor [318], PRMT5 is the principal Type II arginine methyltransferase in mammals. It is initially identified as a Jak2-binding protein and has been shown to methylate histones H2A, H3, and H4 [282, 319]. Binding of the PRMT5 to COPR5 (cooperator of PRMT5) in the nucleus aids in its transcriptional corepressor functions by altering specificity of PRMT5 leading to methylation of H4R3 over H3R8 [320]. Recruitment of PRMT5 is facilitated by numerous transcription factors and repressors like Snail [321], ZNF224 [322], and Ski [323] and also at the globin locus [324]. Whereas the role of PRMT5 in the cytoplasm is in snRNP biogenesis via its methylation activity on a number of Sm proteins [325], the Piwi proteins [326] help in regulating a class of small noncoding RNAs [326]. PRMT5 is found to be overexpressed in gastric, colorectal, and lung cancer, lymphoma, and leukemia [127] and mis-localized in prostate cancer cells [327] with supportive results revealing regulatory roles in renal cardiovascular disease [328], Huntington's disease [329], and Alzheimer's disease [330].

### 7.3.6 PRMT6

PRMT6 is primarily localized to the nucleus and methylates GAR motifs [331]. The principal mammalian enzyme for H3R2 methylation is PRMT6 {[332], [333]}, and this methylation counteracts the H3K4me3 activation mark, making it a transcriptional repressor. One of the first transcriptionally repressed targets of PRMT5 is Thrombospondin-1 (TSP-1) [334]. PRMT6 is known to suppress HIV-1 activity [335] and aberrantly expressed in COPD and asthma [336].

### 7.3.7 PRMT7

The arginine methyltransferase PRMT7 that characterizes this family of PRMTs harbors two putative AdoMet-binding motifs [337]. PRMT7 methyltransferase activity has been seen to have been linked indirectly with variable levels of sensitivity toward DNA-damaging agents [338, 339, 340] and that of the response of the kidney toward certain antibiotics and the damage caused by them [341]. PRMT7 via its interaction with CTCFL has been shown to be involved in genomic imprinting in male germ line which is owing to the associated gene methylation and ensuing symmetrical methylation of H4R3 [342]. Interestingly there exists a speculation in literature about the role of PRMT7 in embryonic stem cell (ESC) pluripotency based on the loss of expression of PRMT7 upon differentiation of ESCs [343]. Lastly there have been implications of PRMT7 and its activity in breast cancer metastasis through EMT transition [344].

### 7.3.8 PRMT8

PRMT8 exhibits a great similarity with respect to its sequence identity with PRMT1, a high degree of sequence identity with PRMT1 [345]. Its unique N-terminal end harboring a functional myristoylation motif enhances its association with plasma membrane [346]. The localization of endogenous protein might get affected due to the alternate usage of initiator methionine residues. Additionally, the expression of PRMT8 is limited to the brain specifically in neurons [345] with somatic mutations observed in ovarian, skin, and large intestinal cancer [127].

### 7.3.9 PRMT9

PRMT9 (4q31) was also identified with PRMT8 and contains two putative AdoMet-binding motifs like PRMT7. Along with these two motifs, N-terminalend harbors two tetratricopeptide repeats (TPR), mediating protein-protein interactions [347]. PRMT9 though is yet to be fully characterized and is reported to be associated with lymphoma, melanoma, testicular, and pancreatic cancers [348].

As precedented in the literature, protein arginine methyltransferases (PRMTs) through their methylating action on arginines in mammalian histones actively contribute in influencing cellular development and physiology leading to disease and tumorigenesis. Arginine methylation being omnipresent on both nuclear and cytoplasmic proteins has recently gained the attention of epigenetic researchers. Owing to escalating evidences concerning the post-translational modifications of non-histone targets by PRMTs, there has been a surge in appreciating the importance of PRMTs in various cellular functions.

### 7.3.10 Arginine Methylation of Non-histone Targets in Mammalian Cells

Nine different family members of protein arginine methyltransferase (PRMT) with several substrates have been documented in mammalian cells. While major focus has been on the histone methylation, chromatin remodeling and transcriptional regulation, along with novel non-histone substrates methylated by these PRMTs, have been identified and also being added to the existing list with time. Recent literature highlights the existence of many such non-histone arginine methylations affecting variety of biological processes such as protein-protein interaction, protein-DNA or protein-RNA interaction, protein stability, subcellular localization, or enzymatic activity with target non-histone substrates identified including many coactivators, transcription factors, and signal transducers, DNA damage repair proteins, chaperones, viral protein-DNA methyltransferases, chromatin, and RNA-binding proteins among other proteins, leading to a plethora of biological consequences as reflected in the Table 7.4 {Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology [125] Dec 10, 2014}.

Thus, protein arginine methylations along with the non-histone modifications have received widespread interest in the epigenetic community owing to its detrimental and beneficial implications in diseases like various cancers impacted by the crucial biological functions they execute and ensure in mammalian cells. Notwithstanding, the discovery of independent roles for these PTMs, unfortunately the information on arginine methylation owing to the freshness of these PTMs, is relatively scarce as compared to the other PTMs in eukaryotic systems. However, this does not deter the medicinal chemists and drug discovery scientists to develop potent and selective PRMT inhibitors to further understand the biological impact of inhibiting arginine methylation. Of late, reasonable success has been achieved in this area with lots still to come in the pursuit for discovering small-molecule inhibitors of histone arginine methyltransferases.

### 7.3.11 Small-Molecule Inhibitors of Histone Arginine Methyltransferases

In the pursuit of carrying out biological studies to enlighten the physiological functions of PRMT, a well-timed research endeavor in both academic laboratories and pharmaceutical industry has been to design selective PRMT inhibitors. Due to the occurrence of multiple PRMT isoforms in the cell with each being distinctive individually in its functions, a focused attention in the campaign of PRMT drug discovery is to find out effective and efficient lead compounds exhibiting prominent isoform specificity, though at varying degrees. Along this line, in the last decade or so, quite a number of PRMT inhibitors have been identified through the efforts of researchers worldwide from different research laboratories and pharmaceutical companies. Thus, since the discovery of the first non-nucleoside-specific inhibitors of PRMTs following a screening of 9000 compounds, leading to the identification

**Table 7.4** Non-histone substrates of arginine methylation

| Biological function/cellular processes | Substrate/ protein | Arginine(s)/residue | PRMT | Biological consequence | References |
|---|---|---|---|---|---|
| Transcription | RUNX1 | No specific site defined (NS) | PRMT4 | Blocks myeloid differentiation | [350] |
| | | 206/210 | PRMT1 | Increases transcription activity of RUNX1 | [351] |
| | E2F1 | 109 | PRMT1 | Controls growth regulation by E2F1 | [352] |
| | | 111/113 | PRMT5 | Governs growth control by E2F1 | [353] |
| | TRF2 | 17 | PRMT1 | Regulates telomere length and stability | [354] |
| | TLS2 | NS | PRMT1 | Unknown | [291] |
| | ILF3 | COOH-terminal region | PRMT1 | Regulation of PRMT1 is done by ILF3 | [355] |
| | ER | 260 | PRMT1 | Regulation of estrogen signaling | [356] |
| | | AF-1(DNA-binding and hormone-binding domain) | PRMT2 | PRMT2 identified as a novel ER-alpha coactivator | [303] |
| | TAF15 | RGG repeats | PRMT1 | Positively regulates function and localization of TAF15 | [357] |
| | P300 | 2142 | PRMT4 | Unknown | [358] |
| | SOX9 | High-mobility group (HMG) domain | PRMT4 | Regulates endochondral ossification | [316] |
| | TAF2S | Proline-, glycine-, methionine-rich (PGM) | PRMT4 | Regulates the coupling of transcription and mRNA processing | [312] |
| | p53 | 333/335/337 | PRMT5 | Growth control and development | [359] |
| | HOXA | 140 | PRMT5 | Required for endothelial ell expression of leukocyte adhesion molecules | [360] |
| | RELA | 30 | PRMT5 | Activation of NFκB | [361] |
| Chromatin/chromosome regulation | SMARCC1 | 1064 | PRMT4 | Enhances tumor progression and metastasis | [362] |
| | HMGA1 | 57/59 | PRMT6 | | [363] |
| DNA replication, synthesis, and repair | 53BP1 | GAR | PRMT1 | Modulates DNA-binding activity of 53BP1 | [119] |
| | MRE11 | GAR | PRMT1 | Essential for DNA damage checkpoint control | [296] |
| | POLL | 83/152 | PRMT6 | Methylation regulates DNA polymerase beta | [364] |

| | | | | | |
|---|---|---|---|---|---|
| Protein synthesis | PABP1 | 455/460 | PRMT4 | Regulates transcription and translation | [365] |
| Chaperones | SERPINH1 | 234 | PRMT5 | Negatively regulates DNA damage-induced apoptosis | [366] |
| Metabolism | PFKFB3 | 131/134 | PRMT1 | Shunts glucose toward the pentose phosphate pathway | [367] |
| | GST | GAR | PRMT2 | Negatively regulates basal p65 NF-kappaB activity | [368] |
| Viral proteins | TAT | 49–63 | PRMT6 | Regulates human immunodeficiency virus Type I gene expression | [369] |
| Cellular signaling | BTG1 | No specific site defined | PRMT1 | Unknown | [291] |
| | IFN α/β | Intracytoplasmic domain (IC) | PRMT1 | Mediates signal transduction. Signaling mechanism complementing protein phosphorylation | [370] |
| | BCR | 198 | PRMT1 | Promotes differentiation | [371] |
| Apoptosis | PDCD4 | 110 | PRMT5 | Alters tumor suppressor function and accelerates tumor growth in breast cancer | [372] |
| Cytoskeletal-related substrates | MBP | 107 | PRMT5 | Helps in maintaining the integrity of myelin protein | [373] |
| Methyltransferases/ demethylases | ASH2L | 296 | PRMT1 | Being a shared component of mammalian histone H3K4 methyltransferase complexes, aids in methyltransferase activity | [374] |
| | | 296 | PRMT5 | Being a shared component of mammalian histone H3K4 methyltransferase complexes, aids in methyltransferase activity | [374] |
| RNA binding, processing | CPSF6 | GAR | PRMT1 | Accelerates tumor growth by arginine methylation of the tumor suppressor programmed cell death | [375] |
| | | GAR | PRMT5 | Accelerates tumor growth | [375] |
| | CPSF7 | GAR | PRMT1 | Accelerates tumor growth by arginine methylation of the tumor suppressor programmed cell death | [375] |
| | HNRNP A1 | 194 | PRMT1 | Unknown | [376] |

(continued)

**Table 7.4** (continued)

| Biological function/cellular processes | Substrate/ protein | Arginine(s)/residue | PRMT | Biological consequence | References |
|---|---|---|---|---|---|
| | SPT5 | 681/696/698 | PRMT1 | Regulates interaction with RNA polymerase II and transcriptional elongation | [377] |
| | HNRNPU | 778–793 | PRMT1 | Transcriptional regulation & RNA and protein trafficking | [378] |
| | FMRP | 544/546 | PRMT1/3/4 | Regulates target mRNA translation of FMRP | [379] |
| | KHDRBS1 | 280–339 | PRMT1 | Negatively regulates their poly(U) RNA-binding activity | [380] |
| | SLM | GAR | PRMT1 | Negatively regulates their poly(U) RNA-binding activity | [380] |
| | RPS2 | GAR | PRMT3 | Impacts ribosomal assembly and protein biosynthesis | [381] |
| | SNRPB | PGM | PRMT4 | Regulates the coupling of transcription and mRNA processing | [312] |
| | SNRPC | PGM | PRMT4 | Regulates the coupling of transcription and mRNA processing | [312] |
| | SF3B4 | PGM | PRMT4 | Regulates the coupling of transcription and mRNA processing | [312] |
| | LSM4 | No specific site defined | PRMT5 | Form a major B-cell epitope for anti-sm autoantibodies | [382] |
| | SNRPD1 | GAR | PRMT5 | Form a major B-cell epitope for anti-sm autoantibodies | [382] |
| | SNRPD3 | GAR | PRMT5 | Form a major B-cell epitope for anti-sm autoantibodies | [382] |
| | EBNA1 | 325–376 | PRMT5 | Regulation of the EBNA1 Epstein-Barr virus protein | [383] |
| | EBNA2 | 325–376 | PRMT5 | Promotes binding to the survival motor neuron protein | [384] |
| | SPT5 | 698 | PRMT5 | Regulates interaction with RNA polymerase II and transcriptional elongation | [377] |
| | EWSR1 | Arginine-glycine-glycine repeats (RGG) | PRMT8 | Unknown | [385] |

of compounds with a low micromolar inhibition of PRMT1 (0.19–16.3 μM) named arginine methyltransferase inhibitors (AMIs) [349], a lot of improvisations with further redesign and optimization of these lead molecules AMIs resulted in the generation of many other more potent and selective analogs such as allantodapsone, stilbamidine, RM65, MHI-21, DB75, MethylGene compound 7a, and Bristol-Myers Squibb compound 7f as described and summarized in the Table 7.5 with the corresponding $IC_{50}$ values and relevant biological effects.

Thus, the histone lysine methyltransferases and arginine methyltransferases specifically represent an inherently tractable class of epigenetic molecules as targets for small-molecule drug intervention as many genetic modifications can be traced back to epigenetic writers, erasers, and readers. Small molecules can take advantage of this relationship by blocking enzymatic activity or chromatin recognition, and this forms the basis of research for many pharmaceutical companies such as Epizyme Inc. and GlaxoSmithKline and academic setups over the globe like Boehringer Ingelheim and Research Institute of Molecular Pathology (IMP) Vienna, which have independently designed small-molecule inhibitors and chemical probes for PKMTs like PRC2 and EZH2, DOT1L, PRC2, and G9a/GLP. Hence, this clearly demonstrates in a concentration-dependent fashion the feasibility to reduce intracellular levels of the relevant histone methyl marks resulting in some of these drug discoveries already in clinical trials to be used as successful drug interventions against various types of cancers. However, certain issues still remain a center of controversy. Like, for instance, if these methyl marks are really contributing or just merely symptomatic of chromatin accessibility and hence transcriptional activation [393] or it is due to redundant nature of HMTs, it is unclear if broad or selective inhibitors would be needed for therapy, given that key lysine arginine residues in histone tails can be methylated by several methyltransferase isotypes. Nevertheless, it should be only a matter of time until histone methyltransferase inhibitors are successfully investigated in a clinical study and launched in the drug market as potent drug targets for human cancers and many debilitating diseases.

## 7.4 The Roads Less Traveled in Journey of Eukaryotic Methyltransferases

The emanating proteomic tools have exposed the conservation of posttranslational methylations of lysine and arginine residues across the species ranging from yeasts to humans. Deciphering the precise biological functions of protein methylation will necessitate devising better proteomic and high-throughput tools to analyze global methylation and methylation dynamics coupled to in-depth investigation engaging traditional approaches in molecular biology. Important issues of immediate concern encompass looking into mechanistic insights of specific methylation events that govern protein function or protein interactions and interactions with nucleic acids or other biomolecules in addition to characterization of novel methyltransferases and demethylases being implicated in such methylation events.

**Table 7.5** List of arginine methyltransferases inhibitors discovered till date

| Inhibitor | Target enzyme | Substrate | $IC_{50}$ values | Comments | References |
|---|---|---|---|---|---|
| | Arginine methyltransferases | | | | |
| AMI-1 | PRMT1 | H4R3, hnRNP A1BTG1, TIS2, IFNα/β, | $IC_{50}$:0.3–8 μM | Inhibition of arginine methylation of both Npl3 protein and Sam68 protein, by in vivo radiometric methylation assay in HeLa cells | [386] |
| AMI-6 | | ILF3, SPT5, SAF-A, p53, MRE11 FMRP, Sam68, SLMERα, RUNX1, TAF15 | | | |
| AMI-8 | | BCR, CF, Ash2L, nuclear (PABP1) | | | |
| AMI-9 | | | | | |
| Allantodapsone | PRMT1 | Same as above | $IC_{50}$PRMT1:1.7 μM | In HepG2 cells, inhibition on H4R3 methylation | [387] |
| Stilbamidine | PRMT1 | Same as above | $IC_{50}$:57 μM | Reduces the transcriptional activation of an estrogen-dependent gene in MCF-7-2a cells. | [388] |
| RM65 | PRMT1 | Same as above | $IC_{50}$:55 μM | High dose is needed to achieve 50% inhibition of arginine methylation in HepG2 cells | [389] |
| MHI-21 | PRMT1 | Same as above | $IC_{50}$:4.1 μM | Concentrated in the nucleus, arrests the cell growth in the S phase | [390] |
| DB75 | PRMT1 | Same as above | $IC_{50}$:9.4 μM | Cell permeable and inhibit methylation of ALY protein in 293 T cells | [391] |
| MethylGene compound 7a | CARM1 | H3R2, H3R17, H3R26, PABP1, SAP49 (CBP)/p300, FMRP, Sox9, CA150, SmB, U1C, and SF3b4 | $IC_{50}$:60 nM | No cellular activity reported | [392] |
| Bristol-Myers Squibb compound 7f | CARM1 | Same as above | $IC_{50}$:40 nM | No cellular activity reported | [392] |

Now, with increasing evidences suggestive of methylation occurring across a variety of non-chromatin histone proteins, histone methylations cannot be considered as the only sphere of chromatin regulation. A systematic approach to identify and functionally characterize the lysine and arginine methylation of non-histone proteins coupled with tools to map methylation signaling networks using proteomics, protein microarrays, and chemical biology would facilitate the development of innovative strategies for diagnosis of disease and therapy. For instance, employing a peptide array to determine substrate specificity of a KMT and then based on specificity profile of KMTs, potential substrates [48] can be identified as in the case of studies done to discover additional non-histone substrates of KMT7 and KMT1C [394]. Even though advanced mass spectrometry has made the mapping of entire human proteome possible [395], systemic identification of protein methylations remains a challenge.

To determine the epigenetic landscape of the protein methylation or the methyl proteome, an integrated approach involving specific high affinity antibodies to mono-, di-, and trimethylated-lysine or arginine residues for enrichment of methylated proteins needs to be designed. In this regard a number of methyl-lysine and methyl-arginine-specific antibodies were developed [121] but with limitations of being used in large-scale experiments. Alternatively methyl-lysine-binding modular domains enriching methylated proteins have been used before tandem mass spectrometry analysis or multiple reaction monitoring (MRM) analysis [120, 396]. In view of this, metabolic labeling methods such as heavy methyl silac [397] are now being designed and made to use in de novo, high-throughput discovery of chromatin-specific methylation sites [398]. The need of the hour is to use a combinatorial approach that integrates conventional molecular, cellular approaches in combination with proteomic and bioinformatic analyses to address fundamental issues such as specificity of methyltransferases and demethylases, the dynamics of protein methylation, and the cross talk between two or more methylation events or between methylation and other PTMs in order to dissect the role of lysine and arginine methylation in normal cellular function, physiologic homeostasis, animal development, and disease. Owing to aberrant methylations implicated in several complex human diseases, notably in cancer [127], functional annotation followed by targeting lysine or arginine methyltransferases deregulated in cancer [116] would pave the way to take advantage of protein methylation for therapeutic purposes. A reasonable success has been achieved in inhibiting DOT1L and EZH2 in cancerous cell lines and xenograft models. Such initiatives by pharmaceutical companies worldwide have gained momentum paving the way for the discovery of several more potent, selective inhibitors of methyltransferases although the impact and consequences of inhibiting histone methylation are unclear and need more investigation. Hence, in view of the fact that methyltransferases and demethylases ensure reversible epigenetic modifications in eukaryotic cells, there has been a spur in the interest in developing pharmacological inhibitions to these enzymes with an aim to overturn the pathological epigenetic states.

Conclusively, the role of histone methyltransferases in cellular pluripotency, a fascinating feature of hPSCs (pluripotent stem cells) and hiPSCs (induced

pluripotent stem cells) which holds great promise for the success of regenerative medicine, cannot be sidelined. In the years to come, the profound influence of protein methylations as primary PTMs in offering targets and possibilities to characterize hPSCs and driving their differentiation will be unveiled in addition to the ongoing efforts, involving genomics, epigenetics, and transcriptomics to demystify the critical mechanisms responsible for tuning pluripotency-associated signaling and cellular plasticity in human pluripotent stem cells (hPSCs).

## 7.5 Summary and Conclusions

It can be indisputably claimed from the ever-increasing information available that the histone methyltransferases play seminal roles in shaping the mammalian epigenome in various cellular and physiological states. The ongoing efforts and investigations being carried out in profiling the mammalian epigenome and its perturbations have opened up mechanistic and exhaustive insights into the epigenetic secrets of mammalian development, cell fate, and diseases. The central concepts and emerging paradigms from such efforts have cemented the composition and functionalities of already established histone methyltransferases in regulating gene expression through determining chromatin structure and compaction of the nucleosomes. A lot more research is also being done to map the protein-protein, protein-RNA, and protein-DNA interactions and networks to obtain an entire landscape of mechanisms that surround the methyltransferase activity of these enzymes. With the coupling of novel discoveries of non-histone methylation substrates along with emerging importance of histone methyltransferases as druggable target of therapeutic significance, the changing era of epigenetic and epigenomics would be reigned by these enzymes laying the foundation of future clinical strategies in the days to come.

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# Hope or Hype: Stem Cells as Therapeutics in Retinal Degenerative Diseases

8

Parameswaran Sowmya

"Netram Pradhanam Servendriyanam"; "Eyes are the precious gift given to humans by the Almighty" – verse from Bhagavad Gita emphasizes the importance of vision for the mankind. The most recent statistics from the World Health Organization (WHO) reveals that 39 million are blind worldwide, signifying the problem and a need to initiate formidable approaches to address the issue. Almost all the ocular diseases involving the retina, the innermost layer of the eye composed of light sensitive tissue, is characterized by degeneration of retinal cells. The treatment for retinal degenerative diseases is impeded for the want of suitable cells to replace those that are getting degenerated in response to the disease. Stem cell therapy offers a unique opportunity to replace the damaged cells with new ones. Stem cell-based therapeutic approaches can be broadly classified as endogenous and exogenous. The former approach utilizes activation of endogenous stem cells present in the retina for replacing the degenerating cells. The latter utilizes exogenous stem cells, such as mesenchymal stem cells, neural progenitors, embryonic stem cells, and induced pluripotent stem cell-derived retinal progenitors that are transplanted into the degenerating retina. In this book chapter, the key concepts involving both the endogenous and exogenous stem cells for retinal degenerative diseases and their potential advantages and the limitations will be discussed. The outcome of the recent clinical trials along with the future directions and the challenges of stem cell-based therapies will be briefly covered.

Vision is considered the most important sensory modalities that we possess. Blindness or loss of vision is a debilitating condition that affects the quality of life. The most recent statistics from the World Health Organization (WHO) reveals that nearly 285 million people are visually impaired in the world, with 39 million totally blind [117]. Retinal degenerative diseases are the leading causes of untreatable

P. Sowmya (✉)
Radheshyam Kanoi Stem Cell Laboratory, KNBIRVO, Vision Research Foundation, Sankara Nethralaya, Chennai, India
e-mail: drpsowmya@snmail.org



G. R. Jayandharan (ed.), *Gene and Cell Therapy: Biology and Applications*,
https://doi.org/10.1007/978-981-13-0481-1_8

blindness in the world [35]. Unlike cataract or corneal disorder, where replacement of either a synthetic implant or live tissue is possible, the restoration of visual function due to retinal degeneration is precarious, owing to the complex structure of the retinal tissue and its neural nature.

## 8.1 Retina: The Delicate Screen in the Eye

Until studies by Cajal, the retina was considered as an inextricable membrane consisting of reticular and granular layers of uncertain significance. His studies revealed that the retina is a complex structure composed of different classes of neural cells that convey visual message toward the encephalic centers along well-defined paths [121].

Today, retina is defined as a light-sensitive membrane at the back of the eyeball which triggers nerve impulses that pass via the optic nerve to the brain, where a visual image is formed. The retina is organized into three layers of cell bodies and two layers of neuropil.

The three layers of cell bodies are the outer nuclear layer (ONL) which comprise the primary sensory neurons/photoreceptors (rods and cones), the inner nuclear layer (INL) which homes the interneurons (horizontal, amacrine, and bipolar cells), and the ganglion cell layer which contains the output neurons (retinal ganglion cells (RGCs)). In addition to the interneurons, the INL contain sole glia of the retina, the Muller glia [77]. Figure 8.1 shows a schematic representation of different layers of the retina and the diseases associated with each layer.

There are several subtypes of each type of neuron with at least 30 known types of RGCs and 33 types of amacrine cells. These subtypes show variations in their morphological characters, functions, and also in their spatial distributions and frequencies. The fundamental schema of the retina is conserved across vertebrates, and in all species, the light traverse the whole thickness of the retina before reaching the photoreceptors. Retinal pigment epithelium (RPE), which interfaces the retina and choroid, by definition, is not a part of the retinal tissue. However, it is primarily involved in the health of the retinal tissue by providing trophic support and hence for the purpose of the book chapter will be considered as part of the retina. Unlike cornea which possesses resident stem cells in the limbal region, the adult retina does not harbor authentic resident stem cells hence do not have the potential to replenish the retinal cells that are lost during degeneration.

## 8.2 Retinal Degenerative Diseases: Causes and Mechanisms

Retinal degeneration is defined as the deterioration of the retina caused by the progressive loss of the retinal cells. Retinal degenerative diseases are broadly classified as inherited monogenic or multifactorial depending on the cause of the disease. Classical examples of monogenic diseases include Leber's congenital amaurosis and retinitis pigmentosa, while the most common multifactorial retinal degenerative diseases are age-related macular degeneration, diabetic retinopathy, and glaucoma.

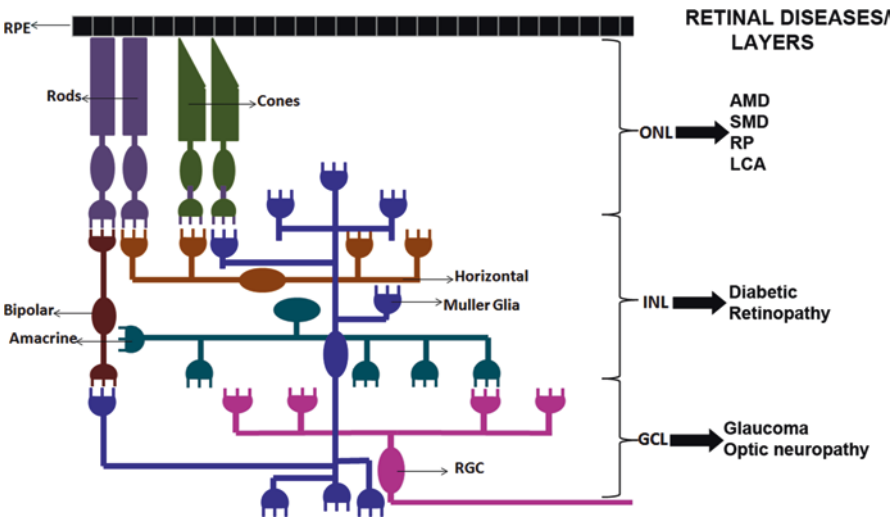


**Fig. 8.1** Schematic representation of different layers of the retina with the most common retinal diseases associated with each layer. Abbreviations: *RPE* Retinal pigment epithelium, *RGC* Retinal ganglion cells, *ONL* Outer nuclear layer, *INL* Inner nuclear layer, *GCL* Ganglion cell layer, *AMD* Age-related macular degeneration, *SMD* Stargardt macular degeneration, *RP* Retinitis pigmentosa, *LCA* Leber's congenital amaurosis

Table 8.1 provides a list of common retinal degenerative diseases, the cells they affect and known causative factors. It can be realized from the table that the most vulnerable cell populations in the retina are the photoreceptors, retinal ganglion cells, and the retinal pigment epithelial cells.

The vulnerability of the photoreceptors to cell death is attributed to its unique physiology and biochemical properties. There seems to be a delicate balance maintained between the survival and death of the photoreceptors, and any minute defect seems to tip them toward cell death [171]. The well-known factors that make the photoreceptors vulnerable to cell death are the presence of photosensitizing molecules [1] and its lipid-rich outer segments [153]. These molecules on exposure to light, in the presence of oxygen, lead to the generation of reactive oxygen and nitrogen species that ultimately lead to cell damage. This is also one of the primary reasons for macular damage since the light has to focus on the central macula to ensure high visual acuity. The mechanism of the photooxidative damage suggests that blue light in the range of 450–495 nm in wavelength leads to activation of rhodopsin with the release of all-trans-retinal. Any defect in the visual cycle which inhibits the conversion of all-trans-retinal to 11-cis retinal lead to a buildup of toxic bisretinoids which upon photooxidation lead to the generation of reactive oxygen and nitrogen species [146, 173]. The presence of polyunsaturated fatty acid-rich outer segment of the photoreceptors leads to accumulation of lipid peroxidation products such as 4-hydroxy-2-nonenal and malondialdehyde. These form protein adducts which compromise the lysosomal proteolysis thereby inhibiting the exocytosis of

**Table 8.1** List of the common retinal degenerative diseases with the cells affected and causative factors

| Diseases | Cell types affected | Causative factors |
|---|---|---|
| Age-related macular degeneration | Rods, cones, retinal pigment epithelium | Complex, multiple genetic/ environmental factors |
| Stargardt disease | Rods, cones, retinal pigment epithelium | Genetic mutations in ABCA4; ELOVL4 |
| Retinitis pigmentosa | Rods more than cones retinal pigment epithelium (progressive) | Genetic mutations in more than 150 genes |
| Leber's congenital amaurosis | Rods, cones retinal pigment epithelium (progressive) | Genetic mutations in RPE65; CRX; AIPL1; TULP1; CABP4; CEP290; RDH12 |
| Diabetic retinopathy | Retinal blood vessel | Diabetes greater than 20 years |
| Glaucoma | Retinal ganglion cells, optic nerve | Complex, multiple genetic/ environmental factors |
| Optic neuropathy | Retinal ganglion cells, optic nerve | Ischemia, trauma, mutations in mitochondrial genes |

undegraded protein adducts. The accumulation of these adducts ultimately leads to degeneration of RPE [79]. In addition, the protein adducts are also shown to activate the complement pathway thereby initiating the pathophysiology in disease such as age-related macular degeneration [61].

Unlike the photoreceptors and the RPE, photooxidative stress does not seem to be the primary cause of RGC cell death. RGCs have been shown to be susceptible to various modes of injury, particularly intraocular pressure elevation and ischemia. [174] suggested that the mechanism and extent of injury in the RGCs differ based on their subcellular components. In this study, the RGCs were divided into subcellular components based on their structure, composition of their extracellular environment, and energy consumption, all of which play an important role in their response to the insults [174]. The four subcellular components of RGCs are as follows: (1) RGC dendrites and synapse in the inner plexiform layer, (2) RGC cell bodies in the ganglion cell layer, (3) nonmyelinated axons of the RGCs in the retina and the optic nerve head, and (4) myelinated axons in the orbit and the cranial region [174]. Based on several studies, it is now accepted that the optic nerve head is the primary site of RGC damage in glaucomatous optic neuropathy [19, 105, 164]. It is suggested that increase in IOP and ischemia interfere with axonal transport and likely damage the mitochondria in the axons of the optic nerve head [24, 69, 78]. This leads to deprivation of neurotrophic factors from the cell body and synapse. Chronic deprivation of these factors initiates apoptosis in RGC ultimately leading to cell death [3].

Although the causative factors and mechanisms of retinal degeneration are profoundly different, they converge toward a single aspect, i.e., loss of retinal cells. Hence, therapeutic approaches which can replace the lost retinal cells can aid in

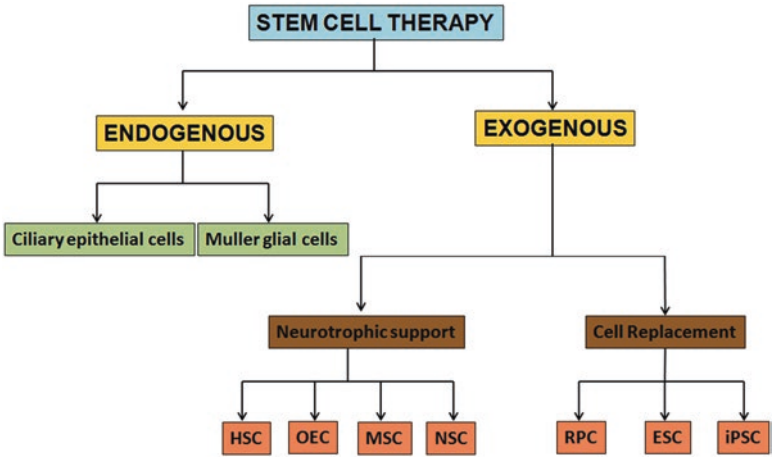


**Fig. 8.2** Schematic representation of stem cell-based therapeutic strategies for retinal degenerative diseases. (1) Endogenous strategy involves activation of retinal stem cells/progenitors present in ciliary epithelium and Muller glial cells by growth factors or small molecules, and (2) exogenous strategy involves replacement of degenerated cells by transplantation of stem cells. The cells that are transplanted either provide neurotrophic support and prevent further degeneration (HSC; OEC; MSC; NSC) or transdifferentiate and form functional retinal cells (RPC; ESC; iPSC). Abbreviations: *HSC* hematopoietic stem cells (adult), *OEC* olfactory ensheathing cells (adult), *MSC* mesenchymal stem cells (adult), *NSC* neural stem cells (fetal/adult), *RPC* retinal progenitor cells (fetal), *ESC* embryonic stem cells (Embryonic), *iPSC* induced pluripotent stem cells (reprogrammed cells)

treating retinal degenerations. Figure 8.2 shows a broad representation of the two therapeutic strategies: (1) endogenous, activation of retinal stem cells/progenitors, and (2) exogenous, cell replacement therapies.

## 8.3 Regeneration of the Retina: Repairing the Retina from Within

Regeneration of either entire or a part of the retina has been observed in several organisms. The extent of regeneration seems to vary between different organisms and also on the developmental stage. Upon injury, retinal regeneration can follow either of the two strategies: (1) transdifferentiation, wherein differentiated cells dedifferentiate to a primitive progenitor type and redifferentiate into a new retinal cell, and (2) direct differentiation of quiescent population of stem cells.

While retinal regeneration is highly hampered during the adulthood in the mammals, it is widely demonstrated in adult fish, amphibians, and birds. In fact, fishes and amphibians regenerate the retina throughout their lives. This property is an absolute requirement in these species since their eyes grow throughout their life, and in order to meet the ever-increasing size of the eye, the retina also grows. This is accomplished by the presence of retinal stem and progenitor cells in the periphery

of the neural retina called the ciliary marginal zone (CMZ) [120]. It is also suggested that these stem/progenitor cells also contribute toward regeneration during retinal damage in the adulthood of lower vertebrates. However, the studies have indicated that different mechanisms are involved in the retinal development from CMZ during embryonic stages and that during the retinal regeneration during adulthood [40, 104, 151]. It is also noticed that in the teleost fish, the truly regenerated cells post-injury in the retina shows a "clumped" pattern, while the normal cells that are made from the CMZ as part of the normal retinal development (in uninjured sites) were more "random" suggesting difference in spatiotemporal patterns of regeneration [149–151]. In addition, several studies suggested that a complete destruction of the entire retina or at least greater than 30% of the retina was a requirement to initiate the regenerative response in the teleost fishes [13, 133]. Although the stem cells in the CMZ have the capacity to contribute to normal retinal development throughout adulthood and also post-injury, the regeneration was observed to be restricted to more peripheral regions of the retina. Several studies indicated that the regeneration of more central portions of the retina was not contributed by CMZ but by another set of putative stem cells [39, 41, 70, 163].

Initial studies in the direction of finding the other putative stem cells' contribution to the retinal regeneration in injured retina suggested that these stem cells were predominantly harbored in the inner nuclear layer (INL) of the retina. These cells were initially identified as cells of rod lineage and named as proliferative inner nuclear cells (PINC) [38, 70]. However, further studies failed to demonstrate the association of these cells to retinal regeneration. In 2000, the study by Vihtelic and Hyde using light-induced photoreceptor damage in zebrafish suggested that the proliferating cells within the INL post-injury could be the Muller glial cells [163].

Fischer and Reh [41], using chick eye, convincingly proved that these proliferating cells in the INL are Muller glial cells, which upon injury dedifferentiate and reenter the cell cycle to give rise to retinal progenitor-like cells capable of differentiating into new retinal neurons [41]. The mechanism of Muller glia-directed retinal regeneration is very well established in the zebrafish model. It is now widely accepted that in response to the injury, growth factors and cytokines such as heparin-binding epidermal growth factor and tumor necrosis factor alpha are released by Muller glia in the site of injury which leads to an intrinsic genetic reprogramming of these cells [10, 74, 108, 131, 148, 165]. The reprogramming of these cells leads to interkinetic nuclear migration wherein the nucleus of these reprogrammed cells which usually lies in the INL migrates to the ONL. The interkinetic nuclear migration is then followed by an asymmetric cell division which leads to the generation of retinal progenitors (sometimes referred to as neurogenic clusters) [107]. These retinal progenitors are multipotential and migrate to different retinal layers giving rise to almost all the retinal neuron types. The signaling cascades involved in different steps of the retinal regeneration via activated Muller glial cells are varied and difficult to comprehend. The variation seen in different studies arises from the fact that the nature and the intensity of the injury vary immensely between these studies. The signaling cascades that are involved in light-induced damage are different from that involved in the chemical and mechanical damage. Knowledge from various

studies point toward activation of four main signaling pathways: the MAPK-ERK, Wnt – β catenin, Notch, and JAK-STAT pathways [109, 131, 165]. In addition to the activation of these pathways, inhibition of the TGFβ and GSK3β using pharmacological inhibitors also increased the efficiency of regeneration in the injured retina [83, 103, 131]. Although these signaling pathways contribute to the overall reprogramming of the Muller glial cells and its differentiation to different retinal neurons, it is not very clear as to the stages at which each signaling pathways specifically play a role. There is paucity in the studies demonstrating the role of different signaling pathways for directed differentiation of Muller glial cells to specific retinal neurons. Nevertheless, the factors involved in specific retinal neuron generation are emerging. For instance, Insm1a, a transcriptional repressor, is indicated in overall differentiation of Muller glial-derived retinal progenitors; signal transducers such as FGFR1 and Notch are implicated in rod photoreceptor differentiation [60, 126, 132, 165]; and Mps1, a regulator of mitotic checkpoint, and N-cadherin, cell adhesion molecules, have been attributed to cone photoreceptor and inner retinal neurons [107, 125], respectively.

These studies clearly demonstrate that both ciliary marginal zone cells and the Muller glia regenerate retina in lower vertebrates and chicks, and the intensity of this regeneration can be modulated by regulating specific signaling pathways. However, although this capacity is clearly evident in these nonmammalian animals, most studies have suggested that this capacity is very much reduced and hidden in the mammals. In the case of mammals, the regenerative ability of ciliary epithelial cells is controversial [2, 25, 160]. In addition, the mammalian Muller glial cells post-injury tends to undergo a different phenomenon known as reactive gliosis.

## 8.4 Muller Glia: A Ray of Hope for Endogenous Retinal Repair in Mammals

In the mammals, the Muller glial cells post-injury undergoes either a proliferative or a nonproliferative gliosis. The gliosis aids in neuroprotection by releasing neurotrophic factors which enable the retinal neurons to survive the injury [16]. However, chronic gliosis leads to neurodegeneration and interferes with the proper functioning of the retina [16, 50]. As stated earlier, the Muller glia in mammals is in the threshold of retinal regeneration and reactive gliosis. Any stimuli which tip off the balance from the gliotic response to retinal regeneration would authenticate Muller glial cells as an endogenous source for retinal repair.

In the studies involving rodents, it is shown that the Muller glial cells which are cultured in vitro under the influence of growth factors such as EGF, bFGF, and/or Shh dedifferentiate into retinal progenitor-like cells [26, 48, 72, 161, 166]. These cells have the potential to proliferate and differentiate into photoreceptors and retinal ganglion cells when specific cues are provided. In addition, these retinal progenitor-like cells, when transplanted into damaged rodent retina, were able to orchestrate retinal repair, albeit at a lower efficiency. These observations persuaded studies toward establishing external stimuli that are required to coax the Muller glial cells to reenter cell cycle and differentiate into other retinal cells that are lost during the injury.

In vivo stimulation of Muller glial cells in the rodents has been attempted by several groups with limited success. Different methods such as pharmacological damage and genetic engineering have been attempted to achieve this. These studies reveal that Notch-, Shh-, EGF-, Wnt-, and Ascl-1-dependent signaling could indeed stimulate proliferation of the Muller glial cells in vivo and also lead to neural regeneration in the injured rodent retina; however, these events were very rare [26, 29, 30–32, 72, 161, 166].

The well-studied model of retinal regeneration in mammals involves cytotoxicity induced by N-methyl-D-aspartate (NMDA), an amino acid derivative and an agonist of NMDA receptor with functions similar to glutamate [72]. In this model of retinal degeneration, the introduction of EGF growth factor led to the proliferation of Muller glia by activation of MAPK, PI3K, and BMP signaling. The activation of EGFR was observed upstream of these signaling pathways [161]. Low doses of glutamate also led to proliferation and neural regeneration in the retina, suggesting that retinal injury in the mammals might lead to increased glutamate secretion at the site of injury thereby stimulating the Muller glia to break quiescence and reenter cell cycle [157]. Compared to the signaling pathways involved in zebrafish or chick retinal regeneration post-injury, in mammals, the expression of Ascl-1, one of the immediate features in proliferating Muller glial cells, was not observed. Gain-of-function experiments with Ascl-1 in mouse retina post-injury increased the proliferation and differentiation toward bipolar neurons [123].

Taken together, these studies postulate Muller cells as a cellular source for healing the retina from within mammals. However, the findings in the mammalian studies have also left some pressing questions that are yet to be answered. (1) Why does the retina of lower vertebrates regenerate more readily than mammals'? (2) Although the Muller glial cells in the injured retina in mammals readily proliferate and dedifferentiate, what are the mechanisms that prevent their differentiation into other retinal neurons? Future studies involving high-throughput sequencing will enable identification of genetic and epigenetic differences between the lower vertebrate and mammalian retina in terms of their regenerative capacity. Those that are identified can then be tested in the mammalian models of retinal regeneration to improve the efficiency of endogenous repair.

The advantages of the endogenous repair are the following: (1) the approach does not entail transplant of genetically or chemically modified cells; (2) no risk of the immune response, chromosomal aberrations, or tumorigenicity; and (3) does not require cell infiltration or grafting to specific layer for functionality. The major drawbacks of the approach are (1) inefficient delivery of growth factor or small molecules to activate endogenous stem cells, (2) lower stability and bioavailability of the growth factor or small molecules that necessitate repeated injections, (3) increased possibility of unexpected physiological responses depending on the nature of the injury which cannot be predicted by any in vitro or in vivo models, and (4) the most regenerative potential of the Muller glial cells that is limited to outer retinal layers, i.e., photoreceptors. In view of the abovementioned limitations, cell replacement strategies involving exogenous stem cells are currently being explored.

## 8.5 Exogenous Stem Cell Therapy: Tackling Retinal Degeneration Using Non-native Cells

The rationale for stem cell therapy stems from the following facts of the inner retina: (i) the organ is supposedly immune-privileged (with some reservation); (2) earlier observations suggesting long-term survival of neural grafts; (3) possibility of intraocular route of administration of the exogenous cells, and (4) demonstration of migration and integration of transplanted cells into specific layers. Hence, the only constraint is to find the most reliable source of cells to replace the once that are lost during the retinal degeneration.

The ideal characteristics of the source of the stem cells for cell-based therapies are (i) renewable, available in sufficient amounts; (ii) versatile, a single source that can generate different kinds of retinal cells; (iii) ethical and legal, both in accordance with accepted principles and beliefs; (iv) autologous, devoid of immune rejection; (v) cost- effective; and (vi) technically less challenging to isolate, culture, or transplant.

Several types of stem cells have been studied for their ability to replace or rejuvenate the degenerating retinal cells which have one or more of the ideal characteristics mentioned above. These cells can be broadly classified as adult stem cells, embryonic or fetal stem cells, and reprogrammed stem cells.

## 8.6 Adult Stem Cells

### 8.6.1 Hematopoietic Stem Cells

Hematopoietic stem cells (HSCs) are the first stem cells that were discovered in 1963 by Ernest McCulloch and James Till [159]. These cells were predominantly found in the adult bone marrow and had the ability to give rise to the complete spectrum of blood and immune cells. HSCs are usually obtained from bone marrow aspirates collected from the hip bones (bone marrow-derived stem cells; BMSC) or by mobilization of these in peripheral blood using cytokines such as granulocyte colony-stimulating factors (G-CSF) [23, 80, 98]. Recently, HSCs have also been isolated from the umbilical cord blood and placenta [20].

With respect to retinitis pigmentosa mouse models rd1 and rd10, intravitreal injection of HSCs was shown to improve the visual parameters [112]. The improvement in the visual parameter was also attributed to the neurotrophic factors that are secreted by the lineage negative HSCs. In addition, this study also showed that the HSCs which are intravitreally injected in these models rescued mostly the cone photoreceptors which would have otherwise died due to the bystander effect of degenerating rod photoreceptors. Hence, this study provided the proof-of-principle that autologous BMSCs can slow down the progression of retinitis pigmentosa by secreting neurotrophic factors which orchestrate its function by upregulating anti-apoptotic genes and factors [112]. These preclinical studies eventually led to clinical trials involving the intravitreal injection of BMSCs in three individuals with

retinitis pigmentosa (discussed under the subheading stem cell-based clinical trials in retinal degenerative diseases).

### 8.6.2 Olfactory Ensheathing Cells

Olfactory ensheathing cells (OECs) are specialized glial cells that ensheath the axons of the olfactory nerve which connects the nose to the brain. They are predominantly present in the olfactory bulb and nasal mucosa. Studies have revealed that transplantation of OECs has potential in RGC regeneration in rat optic nerve transaction models. Analysis of the transplanted animals 6 months posttransplantation revealed that the transplanted cells had the ability to survive, migrate, and integrate into the optic nerve [84, 86]. They also increased the survival of the RGCs post-axotomy and promoted their axonal growth. It was assumed that the effect of OECs on RGC survival and regeneration was due to the neurotrophic factors that they secreted.

The factors responsible for the effect of OECs on RGC survival was analyzed using an in vitro model of RGCs. In this study, scratch-insulted RGCs were exposed to adult OEC conditioned medium with and without neutralizing antibodies to brain-derived neurotrophic factor (BDNF). The study revealed that neutralization of BDNF in the conditioned medium attenuated the survival and neuroprotective effect of the OEC conditioned medium [168, 172]. Currently, the clinical trials of OECs are limited to spinal cord injury and ischemic strokes and have not been initiated with respect to retinal and/or optic nerve injury.

### 8.6.3 Adult Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are non-hematopoietic multipotent progenitors originally isolated and identified in the bone marrow by [42]. Although MSCs were initially isolated from the bone marrow, currently, several tissue sources of MSCs have been identified [62, 64, 95, 152]. Sources of mesenchymal stem cells other than the bone marrow are adipose tissue and peripheral blood [106]. MSCs from the lung and heart have also been isolated [63, 135]. In addition to the authentic adult MSCs, several birth-associated tissues such as umbilical cord, cord blood, placenta and amnion, and Wharton's jelly have been used as sources for isolating and enriching MSCs [12, 58, 75, 90, 93, 124, 147, 158]. Currently, MSCs have been identified from almost all the tissues in the body. MSCs have also been reported in the limbal and corneal tissues [14, 15, 122]. Since there is no concurrence with either morphology or functionality of MSCs identified in various tissues, a minimal set of criteria have been laid by the International Society for Cellular Therapy to define a set of cells as MSCs: (1) selective adherence of the cells to plastic surface of culture dishes; (2) minimum 95% positive for cell surface markers CD105, CD90, and CD73 and negative for CD34, CD45, CD14 or CD11b, CD79 or CD19, and

HLA-DR; and (3) capacity to differentiate into osteocytes, adipocytes, and chondrocytes [34].

MSCs are favored in the field of regenerative medicine because of the ease of logistics involved in isolation and expansion from the bone marrow and adipose tissues of adult patients to facilitate autologous transplantation. Thus, these cells do not entail ethical and immunological issues associated with many other stem cell sources. With respect to retinal degeneration, although few studies have indicated transdifferentiation potential of MSCs into photoreceptors or RPE cells, the results were not reproducible, and hence the potential of MSCs in generating retinal cells is controversial [7, 8, 66, 177]. However, the neuroprotective aspect of MSCs in various retinal degeneration model is well accepted. Unlike the OECs, the neuroprotective effect of MSCs are not restricted to inner retinal layer such as GCL but also reported in outer retinal layers such as photoreceptors and RPE. Both subretinal and intravitreal transplantations of bone marrow-derived MSCs have been attempted in RCS rats and mouse models of retinitis pigmentosa [7, 66]. The studies showed significant improvement in the retinal functionality attributed to the increased survival of photoreceptor and RPE cells compared to the untreated or sham-treated controls. In addition to the subretinal and intravitreal routes, intravenous administration of these MSCs has also been shown to improve the visual function, which makes the procedure less complex. It is assumed that the MSCs that are injected intravenously can home into the sites of injury, as observed in other central nervous system-related injuries [167]. More recently, intravenous injection of MSCs genetically engineered to secrete pigment epithelium-derived factor (PEDF) has been shown to be effective in treating laser-induced choroidal neovascularization in mice. The authors of the study suggest that the injected MSCs can be a source of long-term anti-angiogenic factor that otherwise is provided to patients as part of intermittent intravitreal injections [8].

With respect to inner retinal degenerations, MSC-based therapies have been evaluated in animal models of glaucoma, ischemic retinopathy, and diabetic retinopathy [37, 68, 130, 136, 179]. A preclinical rat model of ocular hypertension has been extensively used as a model to assess the effect of MSC-based therapy in glaucoma. Intravitreal transplantation of MSCs into the ocular hypertension models irrespective of the method employed for inducing hypertension (episcleral vein cauterization or laser photocoagulation of trabecular meshwork) showed significant RGC protection. [59] showed that the vitreous humor is impermeable to differentiation of BMSCs to neurons [59]. However, the study by [100] substantiated that the effect of intravitreal MSCs was due to the neurotrophic factors such as BDNF and NT-3 that diffused through the vitreous thereby conferring neuroprotection to RGCs in the optic nerve injury model [100]. It was confirmed that the dental pulp MSCs were more effective than the bone marrow in the RGC protection, and this was attributed to the increased level of neurotrophic factors secreted by dental pulp MSCs. In addition to BDNF, CNTF and bFGF have been found to be secreted by intravenously injected MSCs in ischemic retina model. The cells were shown to secrete these neurotrophic factors for at least 4 weeks [100].

Both local and systemic routes of administration have been shown to be effective in different studies. The most tested routes are intravitreal, subretinal, and intravenous. Although systemic administration proved to be effective in choroidal neovascularization model, it did not confer neuroprotection in glaucoma model [67, 68]. Several pros and cons of the routes of administration have been postulated. Intravitreal injection is localized, easy to perform, and less complex; however, it does not ensure close contact of the transplanted cells to the retina. In addition, inner limiting membrane seems to be a potential barrier for the migration and integration of the cells. Subretinal injection provides more close contact of transplanted cells to the retinal tissue; however, it is difficult to perform and is associated with higher risk. Intravenous route of administration is safest, however; it requires a higher number of cells to be injected. In addition, the cues that would allow the MSCs to migrate to the retinal site of injury may not be profound in systemic conditions such as diabetes and age-related macular degeneration, where the MSCs may be recruited to other sites of injury and may not be available in sufficient numbers to fix the retinal injury.

Several studies with respect to "homing" of MSCs to specific organs have revealed that the process is orchestrated by a complex association of chemokines, chemokine receptors, cell adhesion molecules, and proteolytic enzymes [73]. More studies on the abovementioned factors in the normal and degenerating retina are essential for gaining insights and devise potential methods for systemic delivery of MSCs. Some studies have also recommended delivering MSCs with specific cocktails of growth factors or genetic modification for increased neurotrophic factors [8]. However, it must be noted that some conditions such as glaucoma may not be conducive for such modifications, since higher than optimal level of neurotrophic factors can lead to apoptosis in these conditions, owing to the increased expression of low-affinity proapoptotic neurotrophic factor receptors.

In an open-label phase 2a proof-of-concept clinical trial in patients with secondary progressive multiple sclerosis with visual pathway involvement, [27] found that the intravenous administration of autologous bone marrow-derived MSCs improved several neuro-ophthalmic parameters including visual acuity and visual-evoked responses. The clinical trial suggested that the procedure was safe and led to visual improvement in subjects owing to some neuroprotection [27].

## 8.7 Embryonic/Fetal Stem Cells

### 8.7.1 Neural Stem Cells

Neural stem cells are self-renewing multipotent cells that give rise to neurons, glia, and oligodendrocytes. Several studies have reported isolation and culture of neural stem cells, and they vary to a great extent with respect to the source of the neural stem cells and the culture protocols [46, 53, 134, 162]. Although listed under embryonic or fetal stem cells for the purpose of this chapter, neural stem cells have been identified in both fetal and adult forebrain structures. Initial research established the

possibility of isolation of neural stem cells from the adult hippocampus and maintaining them in long-term cultures without compromising their self-renewal and differentiation properties [4, 5, 44, 45, 113]. These cells have been shown to differentiate into retinal neurons in the presence of specific growth factors and culture matrices. In addition, NSCs, when transplanted into the neonatal fetal retina, have been shown to integrate into the retinal tissue and expressed pan-neuronal and glial markers [71, 99, 156]. However, these cells did not show a retinal morphology nor they expressed retinal markers. Another study showed that subretinal transplantation of the NSCs led to an efficient migration of cells to nerve fiber layer as well as the optic nerve. The study also showed that these NSCs were capable of penetrating the lamina cribrosa layer of the optic nerve [54].

However, in one study, the intravitreal transplantation of NSC after optic neuropathy did not improve the retinal function even though the transplanted cells differentiated and integrated into the inner retinal layers [52]. This observation was confirmed by another study which utilized a mice model lacking RGC which showed that the transplanted NSCs integrated well and also expressed βIII-tubulin+ markers, however, did not form mature and functional RGCs [101].

A study by [99] on subretinal transplantation of human fetal neural stem cells isolated from the brain of medically terminated fetuses of 16–20 weeks gestation into RCS rats showed that the cells migrated and survived in the retina [99]. In addition, the procedure revealed substantial improvement in the visual parameters. Analysis of retinal histology showed significant improvement in the treated eyes. It is confirmed that the transplanted cells do not transdifferentiate into retinal phenotypes; however, it is hypothesized that the transplanted cells could provide trophic support thereby preventing further retinal degeneration. It was also suggested by further studies that the protection of the photoreceptors could be orchestrated by NSC-directed phagocytosis of photoreceptor outer segments, which is usually compromised due to RPE degeneration in the RCS rats [28] and also by the induction of CNTF expression by Muller glia [87].

The commercial version of the neural stem cells has been developed by StemCells Inc. (Newark, CA, USA) and called as HuCNS-SC. Recently, a clinical trial has been initiated by StemCells Inc. in partnership with the Retina Foundation of the Southwest (Dallas, TX, USA) for dry AMD. In this clinical trial, the HuCNS-SC is provided as a subretinal injection in patients with geographic atrophy secondary to AMD. The study has been completed and the results are yet to be published.

### 8.7.2 Fetal Retinal Progenitor Cells

Retinal progenitor cells are multipotent progenitors that are isolated from dissociated embryonic or neonatal retina. These cells have the potential to give rise to almost all the retinal cells. Several studies have been carried out with transplantation of fetal retinal tissues either as whole retinal sheets or as dissociated cells in various animal models [33, 85, 88, 141]. Almost all the studies have found that the fetal retinal tissue can successfully engraft and can differentiate into photoreceptors.

Some studies have shown spontaneous ganglion cell activity posttransplantation of fetal retinal tissue in retinal degeneration model which is attributed to the partial functional integration of graft with host retina [127–129]. However, unlike the animal studies, the studies on humans with retinitis pigmentosa did not show evidence of visual improvement post-fetal retinal tissue transplants. This was mainly attributed to the failure of the graft to survive in the host retina for an appropriate period of time and also the inefficient transplantation methods involved in the whole fetal retinal transplant.

### 8.7.3 Embryonic Stem Cells

Embryonic stem cells are pluripotent stem cells that are derived from the inner cell mass of the blastocysts. These cells have the potential to self-renew indefinitely for a longer period and also can theoretically differentiate into almost all the cells that exist in the body except for the extraembryonic tissues. One of the most important advantages of the embryonic stem cells over other multipotent stem cells is that these cells can be coaxed to differentiate into specific lineages by recapitulating normal developmental mechanisms. Most of the differentiation protocols involving the ESCs are designed based on the knowledge and information available in developmental biology. With respect to the retinal tissue, the protocols involve activation and inhibition of signaling pathways known to be modulated during early optic cup development. In most protocols, the ESCs are differentiated in the presence of inhibitors of Wnt and BMP/Nodal pathways to orient the cells toward cells that are akin to the progenitors found in the optic cup. Culturing the cells in the presence of DKK1 (Wnt inhibitor) and Lefty (Nodal inhibitor) conditions seems to propel the ESCs toward early retinal progenitor phenotypes which express most eye field genes such as Pax6, Rx, Six3, Six6, Lhx2, and Chx10 [110, 178]. In some studies, the inclusion of insulin-like growth factors (IGF) seems to increase the efficiency of this differentiation [82]. In addition, studies have also emerged, where the recombinant endogenous inhibitors and growth factors are replaced with small molecules modulating the signaling pathways thus making the process less expensive and clinically more feasible [111]. Interestingly, Meyer et al. devised a strategy which did not employ any signaling pathway modulators included in the culture. This protocol involved culturing human ESCs (hESCs) in the absence of bFGF for promoting its differentiation and involved manual selection of optic vesicle-like embryoid bodies and their preferential differentiation toward retinal cells [102].

This study proved the fact that cells within the embryoid bodies during differentiation modulate the original developmental signaling pathways to instruct retinal differentiation which can be enriched by manual selection without the need for any extraneous factors. Although different protocols showed that the retinal progenitors can be efficiently generated from the ESCs and also proved that these retinal progenitors in the presence of specific factors can give rise to mature retinal cells such as photoreceptors, RGCs, and RPE, it was in 2011 that a study showed that a three-dimensional retinal tissue can be generated in a culture dish. Using an ultralow

adhesion round-bottomed, 96-well plate and Matrigel as cell matrix, [36] showed that ESCs could form clusters or aggregates that autonomously generate retinal primordial structures. These aggregates in long-term cultures could form stratified neural retinal tissue akin to that seen in vivo during retinal development. This study provided the first evidence for the possibility of in vitro retinogenesis from ESCs [36].

Several studies have fine-tuned the protocols for differentiating hESC-derived retinal progenitors to RPE and other retinal neural cells such as rod and cone photoreceptors and RGCs [6, 18, 21, 49, 56, 57, 65, 76, 81, 89, 97, 175]. Among these, ESC-derived RPE cells are the best characterized. In most studies, the hESC-derived RPE had global genetic profiles similar to fetal RPE cells with all the functional attributes established by in vitro assays such as rod outer segment phagocytosis, PEDF and VEGF secretion, and analysis of activities of the enzymes involved in visual cycles. In addition, several preclinical studies established that subretinal transplantation of hESC-derived RPE significantly improves the visual acuity in a variety of retinal degeneration models such as RSC rats (model of AMD) and Evolv4 and ABCA4 knockout mice (models of Stargardt disease) [138]. The most recent clinical trial establishes subretinal transplant of hESC-derived RPE to be safe and effective for AMD and Stargardt disease (expanded under clinical trials section).

With respect to the rod and cone photoreceptors, although, several studies have reproducibly observed that in vitro differentiation of these cells from hESCs is possible; however, very few studies have shown that hESC-derived photoreceptor precursors survive, migrate, and integrate into retinal degenerative models. Most studies have found limitations with respect to the outer segment formation of the photoreceptors and with their synaptic connections. It is also suggested that the success of transplantation may depend on the specific stage of the differentiated photoreceptors [92] and also on the recipient host retinal environment [51, 118, 119, 169, 170].

Although these studies demonstrate the feasibility of hESC-based retinal cell transplantation for retinal degenerative diseases, the use of hESCs poses significant limitations that need to be addressed. As already stated, hESCs is confounded with ethical issues and immunogenic barrier, which has been recently addressed by a different set of stem cells which are reprogrammed from somatic stem cells – the induced pluripotent stem cells (iPSCs).

## 8.8 Reprogrammed Cells

### 8.8.1 Induced Pluripotent Stem Cells

In 1962, the study by Sir John Gurdon provided the first evidence that fully mature somatic cells could be reprogrammed to their earlier developmental stage. In this experiment, the nucleus of the egg of the frog was replaced with the nucleus of a mature intestinal cell, which allowed normal tadpole to develop [55]. This provided the proof that the major factors responsible for the somatic reprogramming are present in the cytoplasm of the oocyte. In addition, isolation and maintenance of ESCs

by [96] and subsequent work by others on the molecular biological aspects of ESCs allowed the possibility of establishing the factors that are involved in the maintenance of ESCs in the pluripotent state [96]. Based on the abovementioned studies, in 2006, nearly 42 years later, Takahashi and Yamanaka hypothesized that introduction of the factors that are involved in maintaining the ESCs in a pluripotent state might also be capable of reprogramming somatic cells into a pluripotent state. They initiated the study with a set of 24 factors and, by careful titration of different combinations, identified that a combination of 4 factors Oct4, Sox2, Klf4, and cMyc (now addressed as Yamanaka factors) is sufficient to induce pluripotency in adult fibroblasts. These somatic cells which are reprogrammed are named induced pluripotent stem cells (iPSCs) [155]. These reprogrammed cells are similar to ESCs in the fact that they can theoretically be differentiated into any type of cells following the same protocols as established for the ESCs. However, they do not have the limitations that are associated with the hESCs, such as the ethical issues and immune rejection. The iPSCs showed morphology similar to ESCs, express pluripotent and other cell surface markers, and exhibit similar genetic and epigenetic profiles.

Several studies have emerged in the field of iPSC pertaining to different sources of somatic cells that are amenable to reprogramming and also in terms of the methods employed for delivering the pluripotency factors. These studies confirmed that the iPSCs can be reproducibly generated from almost all kinds of somatic cells including complex cells such as lymphocytes and primordial germ cells [11, 17]. In addition, several methods of reprogramming such as viral/nonviral, integrative/non-integrative, and mRNA/protein-based protocols have been developed for delivering the pluripotent factors into the somatic cells. Of these, skin tissues and peripheral blood have been reported in numbers much higher than other sources. Recently, non-integrative approaches such as episomal plasmid-based delivery and Sendai viral-based methods have been widely employed as reprogramming method of choice [9, 43, 91, 94, 140] .

With respect to the retinal differentiation, several studies have employed the protocols already established in ESCs to aid retinal differentiation with iPS cells. Most of the studies provide evidence that iPSCs have potential similar to ESCs in terms of retinal cell differentiation. The reports with regard to differentiation of iPSC-derived retinal progenitors toward RPE, photoreceptors, and retinal ganglion cells are encouraging. [22, 114] have established the possibility of differentiating iPSCs to RGCs [22, 114]. While the former studies utilize an approach wherein the environmental cues that promote normal RGC histogenesis are adapted in RGC differentiation, the latter study utilized overexpression of exogenous transcription factor MATH5 to achieve the differentiation. Nonetheless both the studies provide substantial proof that iPSCs can be used as an autologous source for generating RGCs. The study by [115] also revealed that in addition to expressing the differentiation markers, the iPSC-derived RGCs when transplanted intravitreally migrate and integrate into the GCL layers of a rat model of glaucomatous neuropathy [115]. In addition, the study also provided substantial evidence that these iPSC-derived cells do not cause teratoma, establishing the safety of the procedure. Transplantation of iPSC-derived RPE and photoreceptors is also well documented and shows safety

and efficacy profiles similar to those observed with the ESCs. Recently, a clinical trial utilizing iPSC-derived RPE cells has been initiated in Japan for treating patients with AMD.

## 8.9 Stem Cell-Based Clinical Trials in Retinal Degenerative Diseases

A data search for stem cell-based clinical trials in retinal degenerative diseases in the clinical trials registry databases of the National Institute of Health (https://clinicaltrials.gov/), European Medicines Agency (https://www.clinicaltrialsregister.eu), Indian Council of Medical Research (http://www.ctri.nic.in/), and Japanese Ministry of Health (http://www.umin.ac.jp/) reveal that there are at least 37 clinical trials registered (Table 8.2). Thirty-two of the 37 clinical trials are either ongoing or completed. Figure 8.3 shows the distribution of these clinical trials in relation to the source of stem cells that are transplanted and the disease of interest. hESC-RPE and BMSCs are the most predominant source of stem cells, and macular degeneration and retinitis pigmentosa are the most common diseases where the stem cell-based clinical trials are currently being conducted.

The US Food and Drug Administration granted orphan drug designation to hESC-derived RPE cells for Advanced Cell Technology, Inc. (Santa Monica, CA, USA) in 2010, for initiating the phase 1/2 clinical trials to treat patients with SMD. In addition, in 2011, an approval in same lines was received from the committee for orphan medicinal products of the European Medicines Agency. The results of this clinical trial have been recently published [139]. This study provided the first evidence of long-term safety and graft survival in dry AMD and SMD patients who underwent subretinal transplantation of hESC-RPE. The study reported no serious adverse events that could directly be associated with the hESC-RPE. The common drawbacks attributed to transplantation of cells derived from hESC such as teratoma formation, immune rejection, and risk of differentiation into unwanted cellular phenotype were not noted in the study. However, the authors report complications with systemic immunosuppression in older patients and recommend modified immunosuppressant protocols. Although the predominant aim of the study was to establish the safety of the procedure, the authors also reported that they could appreciate a visual improvement in 8 out of 18 patients. Overall, the study suggested that the subretinal transplantation of hESC-RPE was relatively safe and was effective in the cohort tested.

This study was received more positively, as reflected by the news and commentaries published. However, the following concerns were raised on the outcome:

1. The true visual improvement posttransplantation versus the spontaneous improvement during the course of the disease must be distinguished.
2. Insufficient size of the cohort (9 AMD and 9 SMD) to draw either safety or efficacy of the treatment.
3. Actual duration and dose of the immunosuppressive regimen.

**Table 8.2** Stem cell-based clinical trials for retinal diseases registered under FDA/CTRI/Japanese Ministry of Health

| Source cell | Funding source | Location | Ocular condition | Route of administration | Status | Published | Identifier |
|---|---|---|---|---|---|---|---|
| ADSC | CCHOHCBAPRF | Russia | Glaucoma | Sub-Tenon | Ongoing | No | NCT02144103 |
| ADSC | Bioheart Inc. | USA | AMD (dry) | Intravitreal | Withdrawn | No | NCT02024269 |
| BMMNCs | Chaitanya Hospital | India | RP | Not provided | Ongoing | No | NCT01914913 |
| BMSC (autologous) | AIIMS | India | AMD (dry) and RP | Intravitreal | Ongoing | No | CTRI/2010/091/000639 |
| BMSC (autologous) | Al-Azhar University | Egypt | AMD | Intravitreal | Ongoing | No | NCT02016508 |
| BMSC (autologous) | Retina Associates of South Florida, Al Zahra Hospital, Dubai | USA, UAE | Retinal disease; AMD; hereditary retinal dystrophy; optic nerve disease; Glaucoma | Retrobulbar; Sub-Tenon intravenous intravitreal intraocular | Ongoing | No | NCT01920867 |
| BMSC (autologous) | University of Sao Paulo | Brazil | Glaucoma | Intravitreal | Ongoing | No | NCT02330978 |
| BMSC (autologous) | University of Sao Paulo | Brazil | RP | Intravitreal | Completed (phase 1) | No | NCT01068561 |
| BMSC (autologous) | University of Sao Paulo | Brazil | RP | Intravitreal | Completed (phase 2) | [142] | NCT01560715 |
| BMSC (autologous) | University of Sao Paulo | Brazil | AMD and SMD | Intravitreal | Ongoing | No | NCT01518127 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| BMSC (autologous) | University of Sao Paulo | Brazil | Ischemic retinopathy | Intravitreal | Ongoing | No | NCT01518842 |
| BMSC (autologous) | Clinical University Hospital | Spain | RP | Intravitreal | Ongoing | No | NCT02280135 |
| BMSC (autologous) | Stem Cells Arabia | Jordan | RP | Intravitreal | Ongoing | No | NCT02709876 |
| BMSC (autologous) | University of California | USA | Retinopathy | Intravitreal | Ongoing | No | NCT01736059 |
| hESC-RPE | AIRM[a] | UK | SMD | Subretinal | Completed | [137, 139] | NCT01469832 |
| hESC-RPE | AIRM[a] | USA | SMD | Subretinal | Completed | [137, 139] | NCT01345006 |
| hESC-RPE | AIRM[a] | USA | SMD | Subretinal | Ongoing | [137, 139] | NCT02445612 |
| hESC-RPE | AIRM[a] | USA | AMD (dry) | Subretinal | Ongoing | [137, 139] | NCT01344993 |
| hESC-RPE | AIRM[a] | USA | AMD (dry) | Subretinal | Ongoing | [137, 139] | NCT02463344 |
| HESC- RPE | CHA Biotech | Korea | SMD | Subretinal | Ongoing | [145] | NCT01625559 |
| hESC-RPE | CHA Biotech | Korea | AMD (dry) | Subretinal | Ongoing | [145] | NCT01674829 |
| hESC-RPE | Southwest Hospital | China | AMD and SMD | Subretinal | Ongoing | No | NCT02749734 |
| hESC-RPE | Chinese Academy of Sciences | China | AMD (dry) | Subretinal | Ongoing | No | NCT02755428 |
| hESC-RPE | Cell Cure Neurosciences Ltd. | Israel | AMD (dry) | Subretinal | Ongoing | No | NCT02286089 |
| hESC-RPE | Pfizer, UCL | UK | AMD (wet) | Subretinal | Ongoing | No | NCT01691261 |
| hESC-RPE | University of California, Los Angeles | USA | Myopic macular degeneration | Subretinal | Withdrawn | No | NCT02122159 |
| hRPC | ReNeuron Ltd. | USA | RP | Subretinal | Ongoing | No | NCT02464436 |
| hRPC | jCyte Inc. | USA | RP | Intravitreal | Ongoing | No | NCT02320812 |

(continued)

**Table 8.2** (continued)

| Source cell | Funding source | Location | Ocular condition | Route of administration | Status | Published | Identifier |
|---|---|---|---|---|---|---|---|
| HSC (autologous) | University of Calgary | Canada | Neuromyelitis optica | Intravenous | Ongoing | No | NCT01339455 |
| HUCNS SC | Stem Cells Inc. | USA | AMD (GA) | Subretinal | Completed | No | NCT01632527 |
| HuCNS SC | Stem Cells Inc. | USA | AMD (GA) | Subretinal | Terminated | No | NCT02467634 |
| HuCNS SC | Stem cells Inc. | USA | AMD (GA) | Subretinal | Terminated | No | NCT02137915 |
| iPSC-RPE | Riken | Japan | AMD (wet) | Subretinal | Ongoing | [47] | UMIN000011929 |
| MSCs (autologous) | Tianjin Medical University | China | Neuromyelitis Optica | Intravenous | Ongoing | No | NCT02249676 |
| MSCs (autologous) | Mahidol University | Thailand | RP | Intravitreal | Ongoing | No | NCT01531348 |
| UTSC | Janssen Research & Development | USA | AMD (GA) | Subretinal | Ongoing | No | NCT01226628 |
| UTSC | Centocor Inc. | USA | RP | Subretinal | Terminated | No | NCT00458575 |

**Abbreviations:** *FDA* Food and Drug Administration, *CTRI* Clinical Trials Registry India, *CCHOHCBAPRF* Central Clinical Hospital with Outpatient Health Center of Business Administration for the President of Russian Federati, *AIIMS* All India Institute of Medical Sciences, *AIRM* Astellas Institute for Regenerative Medicine, *UCL* University College London, *USA* United States of America, *UAE* United Arab Emirates, *UK* United Kingdom, *AMD* age-related macular degeneration, *RP* retinitis pigmentosa, *SMD* Stargardt macular degeneration, *GA* geographic atrophy, *NCT* National Clinical Trial, *UMIN* University Medical Information Network

[a]Previously known as Advanced Cell Technology Inc. and Ocata Therapeutics

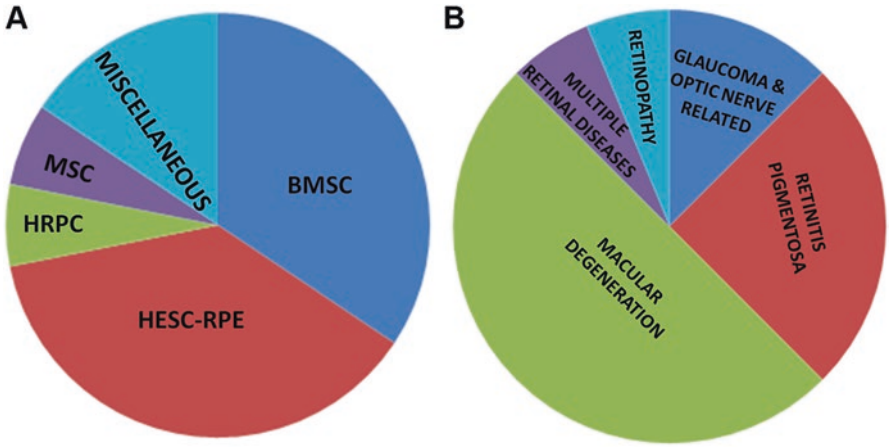


**Fig. 8.3** Graphical representation of the distribution of the clinical trials. (**a**) Distribution in relation to the source of stem cells. (**b**) Distribution in terms of retinal diseases

4. The need and possibility of transplanting RPE along with photoreceptors for actual visual improvement have been suggested, since most photoreceptors degenerate during the course of disease which might dampen the visual improvement parameters.

Sunness [154] suggested that microperimetry and low-vision training should be performed before the trial to evaluate the actual visual improvement due to the subretinal transplantation of hESC-RPE [154]. The authors of the clinical trial, although agreed with the potential of microperimetric analysis concluded that the microperimetric analysis would not have changed the results [137]. In addition, they agreed on other points provided in the commentary by [176] and its incorporation in future clinical trials will provide an unbiased and improved assessment of the treatment [137, 176]. A similar clinical trial was also initiated in South Korea by CHA Biotech (Seoul, South Korea) in collaboration with Ocata Therapeutics, and the results obtained in this study confirmed the earlier findings that hESC-RPE transplantation was safe without any evident adverse events [145].

Other prominent clinical trials with reported outcome are available on the safety and efficacy of intravitreal injection of autologous BMSCs in a range of retinal diseases, including advanced RP [116, 144] dry AMD, SMD, and retinal vascular occlusions. The first clinical study was reported from the University of Sao Paulo, Brazil, by [144] wherein the safety and beneficial effect on RP-associated macular edema were assessed. The trial suggested that the procedure was safe; however, the visual parameter did not improve significantly. This study provided an impetus for the phase 2 study incorporating questionnaire-based evaluation of a cohort of 20 patients. The phase 2 study revealed an improvement in the quality of life of the patient; however, the effect was transient and was not evident after 1 year of injection [142–144]. The second clinical study utilizing BMSC for retinopathy was

carried out at the University of California, Davis. The preliminary clinical findings from the phase 1 patients was published recently. This study which had the results of six patients with 6 months follow-up [116] reported that the autologous BMSCs were well-tolerated post-intravitreal injection. In addition, the treated eye showed evidence of improvement over the baseline. Similar, to the study by the University of Sao Paulo, the effect was transient in this trial as well.

## 8.10 Other Imminent Clinical Trials

In Japan, the first clinical trial utilizing subretinal transplantation of iPSC-RPE in wet AMD was initiated in 2014. This was the first use of iPS technology in a clinical setting after the regulatory approval by the Ministry of Health, Japan. The iPSC-RPE cells were transplanted as sheets of 1.3–3.0 mm into the subretinal space, without any surgical complications. Recently, a report published [47] revealed that the trial was suspended owing to the regulatory change in the clinical trial and suggested that the trial protocol is revised to use allogeneic iPSC-RPE cells for evaluation. It added that this change in the regulation might have been brought about in wake of the identification of mutations in the iPSC lines generated and its confirmation by whole genome sequencing that these mutations were acquired during the reprogramming process. It is stated that the Center for iPS Cell Research and Application (CiRA) at Kyoto University, Japan, is creating a bank of iPSCs from HLA-typed peripheral blood and cord blood samples from normal individuals after obtaining their consent. These iPSC lines will serve as a renewable and well-characterized source of cells which will be differentiated into the cells of interest and transplanted after matching the patient's major HLAs which determines the transplant rejection. It is assumed that the RPE differentiated from the HLA-matched allogeneic iPSCs could be used with immunosuppressive therapy which would provide an advantage over the hESC-RPE. In addition, it will also eliminate the issues of genetic stability and the expense involved in generating and characterizing iPSCs from all the patients. In addition to the abovementioned trial, regulatory approvals have been provided for a number of related trials utilizing hESC-RPE for AMD, namely, the London Project (London, UK) and California Project to Cure Blindness (USA).

## 8.11 Hype About Stem Cell Treatments: Blooming Stem Cell Clinics

Although several clinical trials are ongoing, none of these are approved stem cell-based therapies for retinal degenerative diseases. The realm of the stem cell trials has been besieged by the hype created from the predicted gains to patients desperately looking for a cure for otherwise incurable diseases. In addition, the profit motive companies and clinicians associated with these companies have provided additional thrust even though the reproducibility in terms of safety and efficacy has not been

established. The community expectations have added to the woes by allowing these companies and clinicians to propel the hype further. A quick search on the internet reveals information on clinics throughout the world offering unscientific stem cell treatments. Several news articles refer to the stem cells as "magic bullets" or "only hope," and this has created unquestionable faith in the potential of stem cells in the public which is exploited by these clinics. Almost there is no clinical evidence available on the effect of the treatments offered, and the results are published as patient testimonials. The analysis of individual patient testimonials is difficult since improvement cannot be directly attributed to the treatment since almost any disease has a variable course. So, it is an absolute need that a clinical trial is conducted for all these new treatments with a large number of patients to make sure that they are really effective even before considering them as a treatment option. In addition, any untoward incidence associated with the trials is reported without any bias.

## 8.12 Conclusion

Currently, stem cell-based therapies for retinal degenerative diseases are on the cusp between preclinical research and phase 1/2 clinical trials. Although the approaches involving endogenous activation of innate stem cells/progenitors in the degenerating retina are ongoing, its progress in the level of human studies is slow compared to the cell replacement therapies involving transplantation of exogenous stem cells. As already discussed, several clinical trials are ongoing, and the results of these are eagerly awaited in terms of their safety and efficacy. In addition, the requirement of an immunosuppression regimen versus an autologous approach, specific cell replacement versus paracrine effect, and the best route of administration needs to be compared to arrive at the best treatment options**.** Immense hope has been created by the success of the ongoing clinical trials on the therapeutic application of stem cells for retinal disease. An unbiased assessment of the results needs to be communicated, and an awareness must be created among the professionals in the ophthalmology field on institutions that might commercialize unproven stem cell therapy, who can then counsel their patients on the real state of the science.

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# Haloidentical Stem Cell Transplantation

9

Narendra Agrawal and Dinesh Bhurani

## 9.1 Introduction

Allogeneic haematopoietic stem cell transplantation (alloHCT) has emerged as a curative treatment modality for a variety of disorders including haematological malignancies, inherited and acquired bone marrow failure syndromes, congenital immunodeficiencies and errors of metabolism.

HLA identical sibling has been the donor of choice if available and is considered to be associated with best outcome, but the probability of finding an HLA-matched donor in the family is only 25% considering the small family size in most parts of the world. In case of non-availability of HLA identical family member, an alternative stem cell source has to be looked upon which can be unrelated to patient or a HLA partially matched family donor.

Currently available, though limited, studies have shown feasibility of haploidentical donor as graft source upfront in case of non-availability of HLA identical donor in certain conditions. A recent randomized study of haploidentical versus matched unrelated adult donor (MUD) in patients with AML showed comparable OS and DFS while better GvHD profile with haploidentical transplants over MUD [1]. The decision of which donor source is to be selected upon largely depends upon the clinical situation (mainly urgency of transplant) and the transplant centre's experience.

Several advantages of haploidentical donor over other alternative donor sources are universal and rapid availability of a motivated (often multiple) donor, low cost of graft acquisition, ready availability of donor for subsequent cell collections if needed and lastly a possible stronger graft versus leukaemia effect.

DNA is inherited from both of the parents. Usually a full haplotype with some crossovers is acquired by progeny from both parents. Most of family members share a haplotype of chromosomes/genes with each other with a 1 in 4 (25%) probability

N. Agrawal · D. Bhurani (✉)
Department of Hematology, Rajiv Gandhi Cancer Institute & Research Centre, Delhi, India



G. R. Jayandharan (ed.), *Gene and Cell Therapy: Biology and Applications*,
https://doi.org/10.1007/978-981-13-0481-1_9

amongst siblings of sharing haplotypes from both parents while 2 in 4 (50%) probability of sharing a haplotype from same 1 parent. Family members having minimum of one antigen each on HLA A, B, C, DR and DQ locus are considered to be haplotype matched or haploidentical with each other. With currently available HLA testing technique, it's not possible to ascertain the relatedness of all the matching alleles to single chromosome. In case of family members sharing haplotype, it is assumed that all the matching antigens are acquired from one of the parents (either mother or father). An unrelated person, even if sharing a full haplotype with patient, can't be considered a haploidentical donor as it's not possible to ascertain the presence of all matched antigens on single chromosome, though with future next-generation sequencing it will probably be possible to exactly determine relation of all the antigens with each other too [2].

So haploidentical donor is defined as a person who has, by a common inheritance, one haplotype similar to the recipient and can have mismatches at 1–5 (if tested at HLA A, B, C, DR and DQ loci) HLA loci on unshared haplotype. Nearly all (>90%) of the patients who need an alloHCT have haploidentical donor available in his/her family (parents, offspring and some siblings).

In the absence of HLA-matched family donor, a haploidentical transplantation is a readily available option and has been explored in the last three decades with substantial increase in numbers and improvement in outcome.

Transplant across HLA barrier has usually been associated with higher rates of complications like increase incidences of graft rejections and GvHD due to bidirectional alloreactivity, infections due to intense immunosuppression/delayed immune recovery and even relapses. In a report from CIBMTR data of more than 2000 transplants performed between 1985 and 1991, HLA haploidentical transplants (haplo) as compared to matched sibling donor transplants(MSD) were associated with higher graft failure (16 vs 1%), severe acute GvHD (36 vs 13%) and chronic GvHD (60 vs 42%) and a higher transplant-related mortality (55 vs 21%) [3].

Achieving successful engraftment without significant GvHD has been a challenge in HLA haploidentical transplants. Several approaches to successfully achieve engraftment while avoiding severe GvHD in haploidentical transplants explored graft engineering and pharmacologic prophylaxis of rejections and GvHD and made haploidentical transplant a viable option in the last decade.

## 9.2 Donor Selection

In most of the cases, we can have more than one person having haplotype match with the patient. Selection of a suitable donor is very important for optimal outcome. It's always better to have more than one potential donor available to select out from.

Like HLA identical transplants, preference is given to younger, male donor or if female then preferably nulliparous. Other factors which can be considered are ABO compatibility with patient or at least to avoid ABO major mismatch and the CMV

serostatus that matches with patient (CMV seropositive donor for CMV seropositive patient).

There are certain unique considerations for haploidentical donor:

1. Donor HLA specific antibody (DSA)
2. Donor relationship
3. Degree of HLA mismatch
4. NIMA (non-inherited maternal antigens)
5. NK cell alloreactivity

**Donor HLA-Specific Antibody (DSA)** Patients can have anti-HLA antibodies (HLA allo-immunization), and some of these antibodies may be directed to potential donors' HLA. These are called donor-specific antibodies (DSA). Presence of DSA has been found to be significantly associated with graft rejection (60% and 75% in two small case series) [4, 5]. With that, the presence of DSA is an absolute contraindication for selecting that particular haplo donor. And if a patient is having DSA for all potential haplo donors, then an alternative donor (matched or mismatched unrelated or cord blood) should be looked for. If there is no other alternative donor available, then desensitization to lower concentrations of DSA can be tried using plasma exchange, IVIg, tacrolimus and MMF regime [6].

Common causes of HLA DSA are multiple blood transfusions, directed blood transfusions from family donors, females more than males and multiparity. DSA can be detected by complement-dependent cytotoxicity assay. Nowadays a more sensitive and very rapid (results in 2 h) technique of DSA detections is single-antigen flow beads (SAFB) allowing detection of antigen-/allele-specific DSAs using different colour fluorescent microspheres coated with specific HLA allele/antigen. The reactivity is determined by Luminex-based technology and is reported in MFI units [7].

Evidence-based guidelines on exact upper limit above which the DSA may lead to graft failure are lacking. For a successful haplo transplant, a DSA of 1000 MFI is usually taken as cut-off above that a donor is not be considered for transplant.

In various studies, the incidences of anti-HLA antibodies are 20–23% and that of DSA are 15%. Incidences of DSA in males, nulliparous females and parous female recipients were 5%, 13% and 43% [4, 5, 8].

**Donor-Recipient HLA Mismatch** Though increasing HLA disparity has traditionally been considered detrimental for transplant outcome, with currently available protocols of graft engineering or pharmacologic prophylaxis of GvHD/Rejection, the deleterious effect of HLA mismatch is almost eliminated amongst haplo transplants [9] with GvHD and rejections almost comparable to HLA identical transplants [10–12]. Currently there is no need to select a donor with a higher match, and a donor with 3/6 or 5/10 is considered equally good to >3/6 or >5/10. There can be certain specific antigen mismatch associated with detrimental effect.

Huo MR et al. have shown in their study of 481 patients an increased rate of severe acute GvHD and NRM in pairs with HLA-B mismatches as compared to others. There was no impact of degree of HLA disparity on NRM or disease progression [13]. In another study by Kasamon Y L et al., the increasing HLA disparity was not found to have significant correlation with dismal outcome; rather DRB1 mismatch in graft versus host direction had lower incidence of relapse [9]. Other studies using post-transplant cyclophosphamide (PTCy) [10, 11] have also confirmed no detrimental effect of increasing HLA disparity on transplant outcome.

**Non-inherited Maternal Antigens (NIMA)** HLA haploidentical siblings share inheritance of either the paternal or the maternal haplotype. If both the siblings in a pair of recipient-donor share paternal haplotype, then we can call them having a mismatch at non-inherited maternal antigens. As we all are exposed to maternal antigens (inherited as well as non-inherited) in utero, we all have some immune tolerance to non-inherited maternal antigens. So in this pair of siblings, who share paternal haplotype with each other, the recipient will behave as more than haplo match (sharing paternal haplotype while reduced intolerance to the mismatched maternal haplotype) and can better tolerate the graft. In an early study from Japan involving 35 patients having refractory haematological malignancies, patients received T cell replete graft after myeloablative or reduced intensity conditioning and tacrolimus for GvHD prophylaxis. Acute and chronic GvHD were present in 56 and 57% of patients, respectively. NIMA mismatch in graft versus host direction was found to have lower incidence of severe acute GvHD in comparison to paternal haplotype mismatch grafting ($p = 0.03$) [14]. Selection based on NIMA requires HLA testing of parents to determine the inheritance of haplotypes.

Significance of maternal antigen exposure in utero in immune tolerance also comes from an observational study of 118 patients receiving T cell-depleted haplo graft from either of the parents. The 5-year EFS was significantly better for patients receiving graft from mother as compared to those who received grafts from father (51% versus 11%) [15].

A retrospective CIBMTR study involving 269 patients showed a higher incidence of acute GvHD in patients transplanted with graft from maternal haplotype match versus maternal haplotype mismatch (RR 1.86, 95% CI 1.15–3.05), though the TRM was not different. In the same study, it was concluded that father-to-child transplant had significantly higher incidences of chronic GvHD versus mother-to-child transplant (RR 2.44, 95%CI 1.12–5.34) and the transplant from either parent was associated with higher TRM as compared with transplant from a sibling donor [16].

Similarly, in haplo transplants with a pharmacologic prophylaxis for GvHD (ATG, cyclosporin A, mycophenolate mofetil and methotrexate) after a myeloablative conditioning (usually in Chinese studies, the GIAC strategy – mentioned below), involving more than 1200 patients, the NIMA-mismatched sibling donor graft was associated with lowest incidences of acute GvHD as compared to parents or paternal haplotype-mismatched sibling graft [17].

**Donor Relationship** Above-mentioned studies can guide us in selecting a donor from all of the family members. If all other factors are favourable (specially, the DSA should be negative), then a NIMA-mismatched sibling is preferred over maternal haplotype-matched sibling and that over mother and lastly the father.

**NK Cell Alloreactivity** NK cell surface has immunoglobulin-like receptors which identify specific ligands, and antigens attached to those ligands are then subjected to NK cell-induced lysis. So NK cells are part of our innate immunity. There are at least 15 different gene coding for these immunoglobulin-like receptors on NK cells which are called killer immunoglobulin-like receptors (KIRs). KIRs can be activating or inhibitory. The ligands for KIRs are HLA A, B and C (class 1 HLAs). KIR haplotypes are broadly classified into haplotype A (includes mainly inhibitory receptors) and B (includes mainly activating receptors).

NK cell alloreactivity has been shown to have great significance in viral infections and anti-tumour immunity. Patients with AML receiving HLA-matched graft from KIR-BB donor (homozygous for B receptors) resulted in lower rates of relapse [18].

In cases of haploidentical transplants, theoretically there should be heightened NK cell alloreactivity (against non-self HLA class-1 haplotype) leading to NK cell-mediated graft versus leukaemia (GvL) effect. The NK cell-mediated GvL effect was found in T cell-depleted transplants initially and was thought to be overcome by remaining alloreactive T cells after T cell replete transplants. With currently prevalent haplo transplant strategy (PTCy approach), the presence of KIR mismatch with KIR-A haplotype recipient and KIR-B haplotype donor confers survival advantage and reduced risk of relapse too [19]. The protective effect of KIR-B haplotype and KIR mismatch is seen in AML cases and not in ALL in most of studies.

The issue of KIR-based donor selection needs to be addressed in prospective studies. KIR typing can readily be done along with HLA typing.

## 9.3 Transplant Strategies

As mentioned above, transplant across HLA barrier has been a challenge due to intense bidirectional alloreactivity leading to a very high TRM in initial reports. Preventing GvHD and at the same time reducing risk of rejection and infections have been the goals in designing strategies for haplo transplants. There has been remarkable improvement in haploidentical transplant strategies and outcome in the last decade. Three most commonly used and developed strategies are T cell depletion (TCD), post-transplant high-dose cyclophosphamide (PTCy) and GIAC strategy (described below).

**T Cell Depletion Strategies** Various studies utilizing T cell depletion have shown higher rejections and infections. To overcome rejections, a mega dose of stem cells was employed to successfully achieve engraftment. Also stem cells act as veto cells

and therefore can significantly reduce the alloreactivity of T cells which are cytotoxic in nature [20, 21]. Hence, theoretically immune tolerance can be induced by high doses of stem cells in haplo transplants. This strategy is known as *TCD with mega dose CD34+ cell* graft.

T cell depletion can be achieved by mechanical methods and pharmacologic methods. Initially used mechanical methods (graft engineering) of TCD were negative selections of CD3 cells from the harvest by soya bean agglutination or sheep's erythrocyte resetting. Later studies used positive selection of CD34+ stem cells from the harvest [22–24] or a combination of both the negative selection of T cells and positive selection of CD34+ cells. Conditioning used was usually myeloablative combining with strong immunosuppressants like either of cyclophosphamide or fludarabine and either ATG or alemtuzumab. As the graft was TCD, there was no other GvHD prophylaxis used with significant acute GvHD in less than 10% and chronic GvHD in 14% but a very high 2-year NRM of up to 66%. The 2-year DFS was 48% for AML in first CR, while it was also very low at 1% and 7% for advanced AML and ALL, respectively [25]. Overall there was a very poor outcome after TCD allograft for advanced haematological malignancies due to high NRM as well as relapses [26–29].

Pharmacologic technique of ex vivo TCD is adding alemtuzumab (anti-CD52 monoclonal antibody) in the harvest. Alemtuzumab specifically depletes expressing CD52 antigen-expressing cells and has higher activity on CD4 cells in comparison to CD3 and CD8 cells, which are depleted in excess of two logs when added to the harvest bag. The minimum optimal concentration of alemtuzumab is 0.001 mg/ml and up to 0.01 mg/ml in bag containing $10 \times 10^9$ cell per 100 ml. Alemtuzumab is more effective when used in incremental concentrations, although it did not show much effect on the numbers of granulocyte-macrophage colony-forming units [30]. Addition of complement had also shown significant reduction in helper and cytotoxic T cells.

Efforts to improve the outcome after TCD transplant largely aimed at reducing infections and NRM. Advances in graft engineering or manipulation have made it possible to select specific population of cells out of harvest. Few of these strategies are CD3-/CD19-negative selection and negative selection of alpha/beta T cells but not affecting gamma/delta T cells in the harvest [31–34].

Numerous strategies have been investigated to deplete alloreactive T cells in selective manner which can prevent prolonged T cell deficiency. For this, alloactivation is achieved by incubating donor cells with host cells as a large mixed lymphocyte reaction. On activation, alloreactive donor T cells upregulate interleukin-2 receptor (CD25) on their surface, and then these donor T cells are selectively depleted by anti-CD25 antibody immunotoxin [35, 36], immunomagnetic bead separation or photodynamic purging [25, 37]. The clear significance of this strategy of selective removal of alloreactive T cells has been shown in clinical studies in haplo-HSCT [38]. Recently, a new approach has been developed which utilizes a photosensitizing compound, which accumulates in alloreactive T cells which would

be eliminated by light exposure in vitro [39, 40]. Although it seems to be an effective method, immune recovery is delayed in these recipients [40].

The graft engineering/manipulation is not widely applicable because it's an expensive technique and requires special equipments with expertise.

The immune reconstitution post-transplant depends upon degree of T cell depletion in the graft. TCD transplants usually have slower immune reconstitution and very high rates of infection leading to mortality of 40% [23, 24]. Efforts to improve infection outcome include infusion of viral-specific T cells (adoptive immunotherapy) post-transplant [41, 42]; sparing gamma/delta T cells in the harvest also helps in controlling CMV reactivation and also in graft versus leukaemia effect.

**The High-Dose Post-transplant Cyclophosphamide (PTCy) Strategy** This strategy does not require any graft manipulation, equipment or extra cost. Here a high dose of cyclophosphamide is given post-transplant on two consecutive days, on the third and fourth day post-transplant and thereafter tacrolimus and mycophenolate mofetil from the fifth day post-transplant onwards. Immediately post-transplant, there is proliferation of selective T cells which are alloreactive to donors' antigens. This proliferation of alloreactive T cells is checked by cyclophosphamide which largely spares non-proliferating T cell subsets and also stem cells. So, with this strategy, the cyclophosphamide given on day +3 and +4 preferentially targets alloreactive T cells with minimal effect on other T cell subsets and stem cells. This strategy does not harm T cell-mediated immunity much as evidenced by low rates of infections and no case of post-transplant lympho-proliferative diseases (PTLD) reported in initial series of cases [43].

Initially, PTCy strategy included a non-myeloablative conditioning with low-dose cyclophosphamide 14.5 mg/kg on day -6 and -5, fludarabine 30 mg/m$^2$ from day -6 till day -2 and low-dose single-fraction total body irradiation with 200 cGy on day -1 to achieve maximum immunosuppression followed by a bone marrow infusion on day 0 [44, 45]. With this approach, a significantly high number of graft rejections and relapses too were reported.

In an effort to decrease relapses, intensification of the conditioning regime was employed and also GCSF-mobilized PBSC used in some centres instead of bone marrow. In a report of myeloablative conditioning followed by PBSC with PTCy, the incidences of GvHD and NRM remain unchanged, while relapse rate decreased (22%) [10, 11]. Commonly used myeloablative agents are busulfan, thiotepa, melphalan or TBI [46]. There was no increase in incidences of chronic GvHD with use of PBSC instead of bone marrow graft [47, 48].

Immune reconstitution post-transplant with PTCy approach is slightly slower than that of post-matched sibling donor (MSD) transplant. Raiola AM et al. compared immune reconstitution after PTCy haplo transplants versus all other types of transplants [11]. The CMV reactivation rates were 58% after MSD versus 74% after haplo, CD4+ cell counts at day +100 were 229/μL vs 190/μL, and infection-related mortality was 4% vs 11% after MSD and haplo, respectively, though the NRM was

similar in both groups. Considering the lower infection-related mortality, the PTCy approach was superior to TCD approach in terms of immune recovery.

**The 'GIAC' Strategy** Acronym GIAC comes from four main components of this strategy: *G*, GCSF to donor; *I*, intensified immunosuppression for GVHD prophylaxis (involving cyclosporin A, MMF and small doses of methotrexate); *A*, ATG with conditioning; and *C*, combination of BM and PBSC grafts. Conditioning regime used is myeloablative using busulfan, cyclophosphamide, cytarabine, semustine and ATG with T cell replete graft from both GCSF-stimulated bone marrow and PBSC. Most of the reports on this approach are from China. This strategy is found to be associated with high engraftment rates with low NRM (20%), relapse (12%) rates and DFS of 68% at 2 years for standard risk patients. But the incidences of GvHD have been very high with 100-day acute GvHD of 55% (severe acute GvHD 23%) and 2-year chronic GvHD of 74% (extensive in 47%) [12, 49–51].

Considering the very high rates of severe GvHD, the protocol was modified by Italian investigators by utilizing only bone marrow as stem cell source and incorporating basiliximab with resultant lower rates of chronic GvHD (17%) and higher rates of NRM (36%) [52].

Immune recovery after GIAC strategy is slower than MSD transplants but better than TCD transplants considering lower infection-related deaths. In a report of comparison between MSD ($n$ = 25) and haplo using GIAC strategy ($n$ = 50), CMV reactivation was seen in 13 versus 50% of patients and a lower T cell counts at day 90 in haplo group [53].

## 9.4 Relapses After Haploidentical Transplants

Relapses are still common after haplo transplants, specially in high-risk patients/advanced leukaemia patients. Relapses can occur early or late after transplant with different mechanisms of leukaemia escape from donor immunity.

In cases of early relapses (occurring in less than 9 months or till immunosuppression is going on), theoretically, the residual leukaemia blasts get chance to multiply due to non-establishment of GvL. The reason can be either ongoing immunosuppression or not having a suitable target epitope on blasts for graft's immunity to establish an immune reaction. Such patients can be treated with chemotherapy and DLI.

Relapses occurring more than 9 months after haplo transplant may not only be because of non-establishment of GvL effect but also due to loss of mismatched HLA antigens from patients' cells and subsequent loss of GvL effect in up to 25% of relapses. The mechanism of mismatched HLA loss is loss of heterozygosity with subsequent acquisition of uniparental disomy on chromosome 6p, in which the HLA locus from shared chromosome replaces the mismatched HLA on unshared chromosome. Such relapses usually do not respond to DLI as the blasts now lack mismatched HLA. Before planning a DLI in late relapses, it is important to establish

the presence of mismatched HLA antigens. Patients who have lost those mismatched HLAs can be considered for a second haplo transplant from a different donor who should be having mismatch with original donor.

Relapses in acute leukaemias or aggressive relapses in other malignancies require chemotherapy first and then DLI immediately with a starting CD3+ cell dose of 1x10^6/kg and up to a maximum of 1x10^7/kg. Indolent relapses or only molecular relapses may be salvaged with DLI alone. With this approach, the complete remission rates of 30% with response duration of nearly 12 months were achieved in patients with relapsed leukaemia post-haplo with PTCy approach. Acute and chronic GvHD were 30% and 8%, respectively [54].

In another report of DLI after haplo transplant with PTCy approach, 42 patients received 108 DLIs, showing best response in patient with Hodgkin lymphoma (2-year survival 80%) followed by molecular relapse leukaemia (2-year survival 43%) and haematological relapse leukaemia (2-year survival 19%), suggesting feasibility of DLI in haplo transplants [55].

## 9.5 Recent Data About Haploidentical Transplant in Acute Leukaemias

In a study of 31 older patients (>55 years age) with haematological malignancies, who underwent haplo transplant with PTCy approach, patients were compared with MRD and MUD transplant of the same age group. Incidence of grade II–IV aGvHD was 23%, 21% and 44% respectively, while a significant difference in severe cGvHD with 0, 16% ($p = 0.02$) and 14% ($p = 0.03$), respectively. Relapse incidences were similar between three groups, while NRM was threefold higher in MUD group. There was no statistically significant difference in 2-year OS (70%), PFS (67%) and progression and severe chronic GVHD-free survival (67%) in comparison to MRD transplantation (78%, 64% and 51%, respectively), but significantly better than UD transplantation (51% [P = .08], 38% [$P = .02$] and 31% [$P = .007$]) [56].

In another large study of haplo (PTCy approach) ($n = 192$) versus MUD transplant ($n = 1982$) for AML, the 3-month incidence of grade II–IV aGvHD was lesser in haplo transplants ($p = <0.0001$ and 0.05 after myeloablative and RIC, respectively). Also 3-year incidence of cGvHD were significantly low after haplo transplants ($p = <0.0001$ and 0.002 after myeloablative and RIC, respectively). Three-year probability of OS was 45–50% in all groups of patients and was not different significantly [1].

In another study of 372 consecutive patients with haematological malignancies undergoing haploidentical transplant using non-myeloablative conditioning, T cell replete marrow or PBSC grafting and PTCy approach were retrospectively evaluated for risk-stratified outcome according to refined disease risk index (DRI) [57]. The group showed 6-month probability of NRM and severe aGvHD of 8% and 4% only. Three-year probability of relapse, PFS and OS were 46%, 40% and 50% with median follow-up of 4.1 years. According to DRI group risk stratification, different risk groups had shown significant difference in PFS and OS. Three-year PFS

estimates in low ($n = 71$)-, intermediate ($n = 241$)- and high/very high ($n = 60$)-risk groups were 65%, 37% and 22% ($P < .0001$), respectively, which corresponded to 3-year OS estimates of 71%, 48% and 35% ($P = .0001$), respectively. On multivariable analyses, the DRI was statistically significantly associated with relapse, PFS and OS (each $P < .001$) [58].

Another prospective study of haplo ($n = 231$) (GIAC approach) versus matched sibling transplant ($n = 219$) in AML showed 3-year DFS and OS of 74% vs 78% ($p = 0.34$) and 79% vs 82% ($p = 0.36$) with similar relapses and NRM in both arms. This further proves the non-inferiority of haplo transplant for AML patients [59].

A study of ALL patients ($n = 183$) with haplo transplant with GIAC approach showed 3-year OS 74.9% and 72.7% and DFS 67.6% and 68.2% for high-risk and low-risk patients, respectively [60].

## 9.6 Haploidentical Transplant for Non-malignant Conditions

Most commonly encountered conditions in clinical practice are haemoglobinopathies and severe aplastic anaemia and other less common conditions like congenital metabolic or immunodeficiency disorders. Non-malignant conditions are mostly non-urgent conditions too for a transplant. For example, a patient with thalassemia major may not be in a hurry to get a transplant. Patients with aplastic anaemia may be benefitted with earliest possible transplant but only recommended if they have a matched sibling donor. Using haploidentical approach in a patient with thalassemia or aplastic anaemia upfront can be a difficult decision considering paucity of data and availability of effective alternative treatment options in both diseases. Apart from that, occasionally, a matched sibling donor can be born to patients' parents in due course of time who can provide a better graft source.

For haemoglobinopathies, initial data on haploidentical transplant showed EFS of 40–60% with more than 30% graft failures. A study of haploidentical stem cell transplant utilizing reduced intensity conditioning followed by PTCy approach in patients with sickle cell disease showed high incidences of graft failures and unstable mixed chimerisms requiring prolonged immunosuppressive therapy [61]. Another study of TCD haplo grafting after myeloablative conditioning in patients with thalassemia reported similar results [62].

More recently, a report from Thailand, on 31 patients (age 10 years, range 2–20 years) with thalassemia (majority having class III high-risk features), applied 2 courses of pre-transplant immunosuppression with fludarabine and dexamethasone and thereafter a myeloablative and intensely immunosuppressive conditioning including busulfan, fludarabine and ATG. This was followed by a higher dose of stem cells (target of more than $10 \times 10^{-6}$/kg) and GvHD prophylaxis based on PTCy with MMF and tacrolimus. The study showed engraftment in 29 of 31 patients (93.5%) and projected 2-year OS and DFS of 95% and 94%, respectively, and can show a way for haplo transplant in high-risk thalassemia patients [63].

For severe aplastic anaemia, a haploidentical transplant can be considered in the absence of an HLA-matched donor for patients who fail immunosuppressive therapy [64].

A prospective, multicentre study of haploidentical transplant for 101 severe aplastic anaemia (SAA) patients after failing IST between June 2012 and October 2015 and compared with 48 contemporaneous recipients of matched family donor grafts reported engraftment in all cases surviving for more than 28 days. The recipients of haploidentical transplant had higher cumulative incidence of grade II–IV acute GvHD (33·7% vs. 4·2%, $P < 0·001$) and chronic GVHD (22·4% vs. 6·6%, $P = 0·014$) at 1 year at median follow-up of 18·3 (3·0–43·6) months, although there was no significant difference in grade III–IV aGvHD (7·9% vs. 2·1%, $P = 0·157$), 3-year estimated OS (89·0% vs. 91·0%, $P = 0·555$) and failure-free survival (FFS, 86·8% vs. 80·3%, $P = 0·659$) [65].

## 9.7 Conclusion

Clinical outcomes after haploidentical stem cell transplantation have improved significantly with introduction of newer graft engineering and better pharmacologic immunosuppression with improvement in infections and NRM. With currently prevalent PTCy-based approach or the GIAC approach, the outcome after haploidentical transplantation matches that of matched unrelated donor and also to matched related donor transplant. Careful selection of donor and the recipient is paramount for the successful haplo transplant.

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