to their "targeting activity." To reduce trapping by organs with a highly developed reticuloendothelial system (RES), such as the liver, lung, and spleen (phagocyte-rich organs), the surface of nanocarriers have been modified with polyethylene glycol (PEG). The formation of a hydrated phase minimizes recognition by the RES and maximizes the circulating levels, thus increasing the so-called stealth activity of nanocarriers (Matthews and McCoy, 2004). However, the repetitive administration of PEGylated nanomedicines can elicit the production of anti-PEG IgM antibodies that can accelerate blood clearance (Ishida et al., 2006). Thus, new strategies need to be developed to incorporate stealth activity into nanocarriers while minimizing immunogenicity. Furthermore, since the subcellular targets of nanocarriers (e.g., interstitium, cell membrane, endosomes, cytoplasm, and nucleus) should be optimized for incorporated therapeutic materials to maximize their efficacy, the nanocarriers should possess "cell membrane–penetrating activity," "endosome-escaping activity," and "nuclear-localizing activity," concurrently within extremely small space without any interference.

15.3 Lessons from Viruses as Natural Nanocarriers

Viruses that infect humans harbor a nanostructure, circulate in the body, and adsorb onto specific target cells and tissues, which they then invade to establish infection. Through the mutation and adaptation that occurred during the evolution of viruses in the human body, viruses have successfully acquired the ability to target specific cells and tissues. Examples of viruses and their target cells include Japanese encephalitis virus and poliovirus targeting the brain, hepatitis B and C viruses targeting the liver, rotavirus and noroviruses targeting the intestine, human papilloma virus targeting the endocervix, and human immunodeficiency virus and Epstein–Barr virus targeting lymphocytes. Unlike nonviral nanocarriers, viruses are able to establish infection even at low titers, suggesting that they are innate and efficient nanocarriers. By analyzing the surface structures of viruses, we have been able to elucidate the molecular mechanisms underlying the following functions (see Figure 15.1) that are invaluable for their development as nanocarriers:

1. Optimal entry (administration) route
2. Stealth activity
3. Nonimmunogenicity, nonantigenicity, and nontoxicity
4. Active targeting to specific cells and tissues
5. Cell membrane–penetrating activity and endosome-escaping activity
6. Nuclear-localizing activity

In principle, nanocarriers displaying surface proteins of a particular virus could simulate the above viral activities *in vivo*. Liposomes displaying viral surface proteins—termed virosomes—were first used as immunogens for vaccines (Almeida et al., 1975). Since then, Kaneda (2000) demonstrated that virosomes composed of liposomes containing Sendai virus envelope proteins could deliver drugs and genes into cells and tissues. However, Sendai virus–derived virosomes have shown high immunogenicity and less targeting activity, thus hampering the development of these virosomes as specific DDS nanocarriers.

15.4 Bio-nanocapsules: Hybrids of Human Liver–Specific Hepatitis B Virus and Liposomes

Hepatitis B virus (HBV) is a DNA virus of approximately 42 nm that specifically infects the liver of humans and chimpanzees. The highly infectious virions can be transmitted by body fluids, and even at low titers they can evade the RES to establish infection in hepatocytes. The HBV surface antigen (HBsAg) L protein, comprising pre-S1 (108 amino acids in HBV serotype y), pre-S2 (55 amino acids), and S (226 amino acid) regions, plays a central role in the infection machinery of HBV. When expressed in eukaryotic cells, L proteins self-aggregate to form hollow nanoparticles of 30–40 nm in diameter stabilized by the S region, which contains three transmembrane segments (Figure 15.2) (Kuroda et al., 1992; Yamada et al., 2001; Jung et al., 2011). The N-terminal half of the pre-S1 region (amino acid residues 10–36) contains a human liver–specific binding site (Neurath et al., 1986), whereas the central domain of the pre-S2 region contains a receptor for polymerized human albumin (pHSA) (Itoh et al., 1992); both of these binding sites are exhibited on the surface of HBV and L protein particles. In 2003, we successfully produced large amounts of L protein particles in *Saccharomyces cerevisiae*, and we incorporated drugs and genes by electroporation into these particles. When administered systemically into a mouse xenograft model, the L protein nanoparticles delivered their payloads specifically and efficiently to human hepatic tumors (Yamada et al., 2003), demonstrating liver-specific targeting. We have since showed that the uptake of L protein particles by human hepatic cells is comparable to that of HBV in magnitude, and it is pre-S1 dependent (Yamada et al., 2012). We have designated these nonviral human liver–specific nanoparticles as bio-nanocapsules (BNCs).

The structure of the BNC is very similar to that of the immunogen for conventional hepatitis B vaccines, which have also been produced in *S. cerevisiae* during the last three

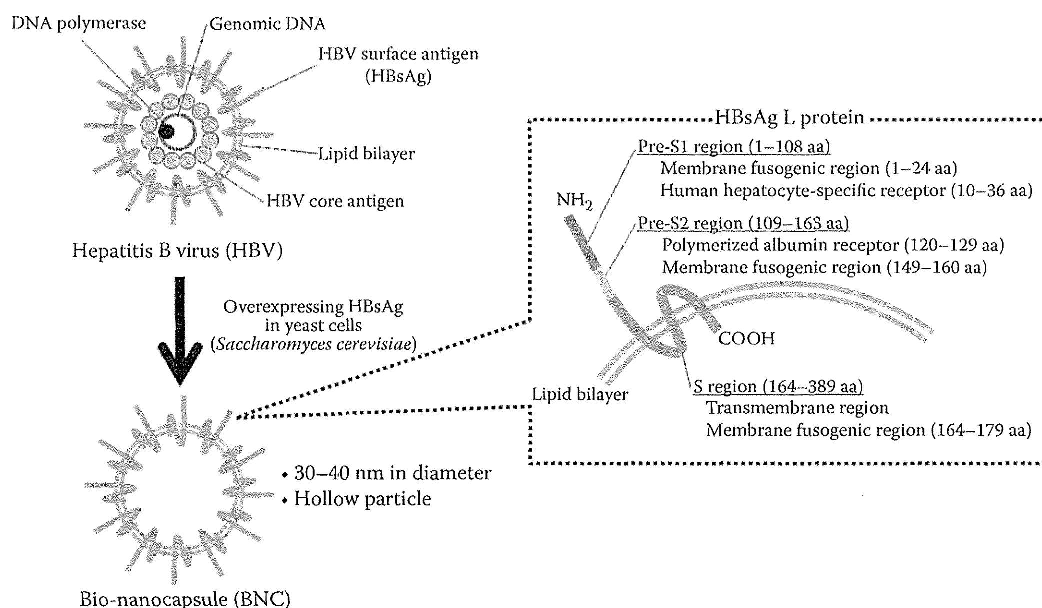

FIGURE 15.2
Structures of hepatitis B virus and bio-nanocapsule.

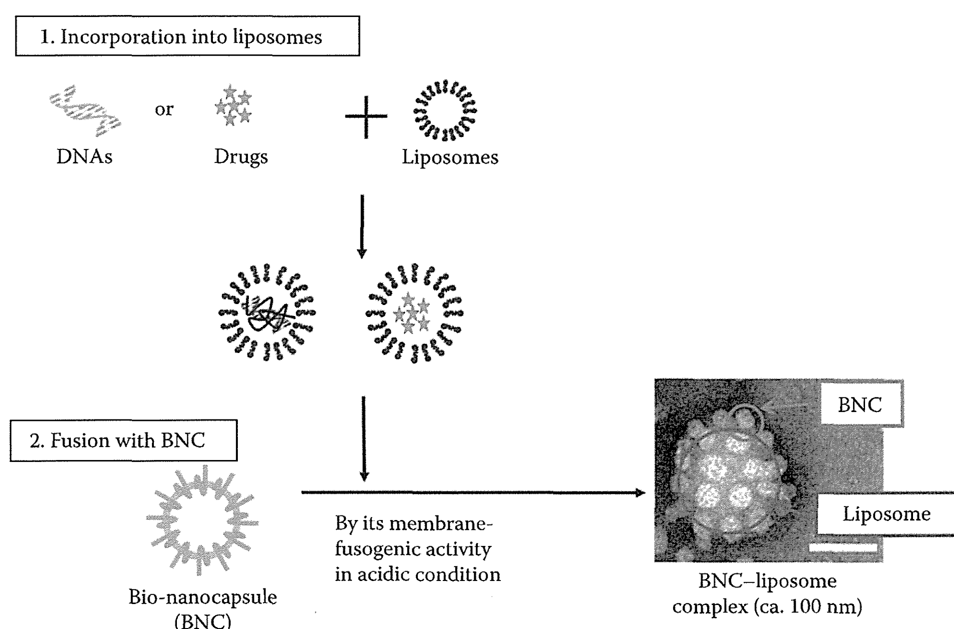

FIGURE 15.3
Process flowchart of the preparation of BNC–liposome complexes.

decades. The immunogenicity of BNC without adjuvant is not as high as that of the hepatitis B vaccine; nevertheless, studies are under way to reduce it further by engineering L protein mutations based on those identified in HBV escape mutants (see Section 15.5). Using good manufacturing practice (GMP) similar to that for recombinant hepatitis B vaccines, large amounts of BNC can be produced for clinical use.

[see below] was changed to [see Section 15.5]. Correct section cited?

Difficulties of using electroporation to produce BNC-based nanomedicines on a large scale led us to develop an alternative method for incorporating therapeutic agents into BNCs. In 2008, we discovered that the membrane fusogenic activity of BNCs facilitated the spontaneous formation of stable complexes of BNCs with liposomes (Jung et al., 2008). Transmission electron microscopy revealed that the liposomes are surrounded by 10–20 BNCs, suggesting that the complex has a similar structure to virosomes. We have shown that BNC–liposome complexes containing small molecules or genes, approximately 100 nm in diameter, are able to deliver their payloads to human hepatic cells and tumors *in vitro* and *in vivo* (Figure 15.3). The specificity and incorporation rate of BNC–liposome complexes into human hepatic cells were comparable to those of BNC alone and HBV.

15.5 Bio-nanocapsules as Candidate Nanocarriers

Minimizing or eliminating the immunogenicity and antigenicity of nanocarriers is necessary for their long-term administration. This is particularly important in humans since viral proteins are highly immunogenic and antigenic. While liposomes can attenuate the immunogenicity and antigenicity of some conjugated proteins, this is still a major

drawback of virosomes, including BNC–liposome complexes. Recently, we compared the amino acid sequences of various HBV escape mutants, which could propagate in recipients of the hepatitis B vaccine without being cleared by immune system in humans, and found two common mutations in the L protein. After repetitive injections of BNCs harboring the two mutations into mice (at 4-week intervals during 28 weeks), more than half of the mice did not develop anti-BNC antibodies (Jung et al., submitted). Thus, further study of HBV escape mutants as potential nanocarriers, as well as other mutants of viruses, is warranted.

Studies have shown that BNCs can achieve high systemic delivery. When BNC–liposome complexes containing the anticancer drug doxorubicin were injected into a mouse xenograft model, the circulating levels were comparable to those of PEGylated liposomes containing the same drug (Kasuya et al., 2009). These data suggest that coating liposomes with BNCs (i) increases the stability of liposomes in blood to levels comparable to the ones seen with PEGylated liposomes, and (ii) facilitates evasion from the RES. Recent evidence points to a role for the pHSA receptor in the pre-S2 region in conferring this "stealth" activity on BNC–liposome complexes, BNCs, and HBV. Specifically, HSA-coated polystyrene nanospheres injected intravenously into rats remained in the circulation longer than non-coated nanospheres and exhibited significantly reduced hepatic clearance (Ogawara et al., 2004). Ogawara et al. postulated that HSA prevented the binding of other serum proteins that would render the nanospheres more susceptible to opsonization. Moreover, in severe combined immunodeficiency mice harboring normal human liver tissues under the kidney capsule, BNC was demonstrated to accumulate only in the human liver tissue without being trapped by other tissues (Matsuura et al., 2011). Recently, we have shown that 100-nm polystyrene beads displaying a synthetic peptide functioning as a receptor for pHSA efficiently escape from the RES in a mouse xenograft model (Takagi et al., submitted).

Once endocytosed by cells, HBV requires a low pH within the endosomes to fuse with the endosome membrane and to release its natural payload (i.e., core proteins, genomic DNA, or polymerase) into the cytoplasm. Others have identified membrane fusogenic domains at the N-terminal half of the S region and the C-terminal half of the pre-S2 region (Rodríguez-Crespo et al., 1995; Oess and Hildt, 2000); we have recently found a new membrane fusogenic domain at the N-terminal 20 amino acids of the pre-S1 region (Oeda et al., submitted). Deleting the pre-S1 region or blocking this region with anti-pre-S1 antibodies completely inhibited the formation of the BNC–liposome complex, suggesting that the membrane fusogenic domain in the pre-S1 region is indispensable for the movement of payloads of the BNC–liposome complex, BNC, and HBV from endosomes to the cytoplasm (endosomal escape).

We have shown in several studies using a mouse xenograft model that BNCs or BNC–liposome complexes are capable of delivering nucleic acids encoding and expressing a variety of genes. Specifically, intravenous injection of a BNC containing an expression vector for blood coagulation factor IX elicited the production of factor IX for at least 1 month at a level sufficient to treat moderate levels of hemophilia B (Yamada et al., 2003). In a separate study, the size of human hepatocyte–derived tumors, but not of human colon cancer–derived tumors, was significantly reduced by the intravenous injection of a BNC containing an expression vector for the herpes simplex virus–derived thymidine kinase (HSV-tk), along with the subcutaneous injection of ganciclovir, an inhibitor of HSV-tk (Iwasaki et al., 2007). Moreover, intravenously injected BNC–liposome complex containing doxorubicin (4 mg/kg) caused a significant reduction in the size of human hepatocyte-derived tumors; doxorubicin alone and PEGylated liposomes containing doxorubicin produced a lower effect at the same dose (Kasuya et al., 2009). These findings show that BNCs

and BNC–liposomes possess increased functionality over other nanocarriers by their ability to introduce drugs and genes to be expressed.

15.6 Retargeting of Bio-nanocapsules

Because the pre-S1 region represents a specific ligand for a receptor on hepatocytes, BNCs were originally developed to target liver cells. The liver is often a target of viral infections and subject to various metabolic disorders; therefore, the human liver-specific targeting ability of BNCs may have therapeutic value. Nevertheless, it is necessary to target BNCs (and BNC–liposome complexes) to other tissues and organs as well to expand their clinical utility. To this end, we and others have carried out studies to change the target tissue specificity of the BNC by replacing the pre-S1 region with other molecules through genetic recombination. Specifically, we modified the BNC to display a tandem form of the Z domain (IgG Fc-binding motif) from *Staphylococcus aureus* protein A, to which we could tether IgGs of interest; these modified BNCs were designated as ZZ-BNC (Iijima et al., 2011). Tsutsui et al. (2007) conjugated an anti-epidermal growth factor receptor (EGFR) IgG to the ZZ-BNC; this complex was injected intracerebroventricularly into mice harboring an EGFR-expressing glioma, where it was found to accumulate specifically in the tumor (Tsutsui et al., 2007). When ZZ-BNCs conjugated with anti-selectin IgG were mixed with liposomes containing either a green fluorescent protein expression vector or 100-nm fluorescent polystyrene beads and were applied intravenously in mouse models of either uveitis or arthritis, the BNCs were found localized to the retina or knee joint, respectively, suggesting successful targeting into the inflamed tissues. These complexes also delivered fluorescent dye to infarcted myocardium in a rat model of myocardial ischemia/reperfusion injury (Jung et al., submitted). Recently, we made ZZ-BNCs conjugated with anti-CD11c IgG and mixed them with liposomes containing a Japanese encephalitis virus (JEV)-derived envelope protein. Mice receiving two intravenous injections 4 weeks apart exhibited a higher titer of anti-JEV antibodies 2 weeks after the second injection compared with mice receiving control vaccines. This suggests that the anti-CD11c antibody can retarget the ZZ-BNC–liposome complex to $CD11c^+$ mouse splenic dendritic cells to elicit the efficient production of anti-JEV antibodies (Matsuo et al., 2012).

In addition to antibodies, other conjugates have also been tested for their ability to target BNCs to specific tissues. *Phaseolus vulgaris* agglutinin-L4 (PHA-L4) isolectin recognizes β1–6 branching *N*-acetylglucosamine (β1–6GlcNAc), which is abundantly expressed as a part of high-mannose glycans in various highly metastatic cancers. ZZ-BNCs were conjugated with PHA-L4 isolectins and mixed with a liposome complex containing fluorescent dyes or luciferase expression vector. Following injection of the isolectin–ZZ-BNC–liposome complex into mice harboring tumors with or without β1–6GlcNAc (i.e., malignant or benign tumors respectively), payloads were found only in malignant, but not benign, tumors (Kasuya et al., 2008). These data demonstrate that antibodies and lectins are useful for the *in vivo* pinpoint targeting of nanocarriers. Finally, Laakkonen et al. (2008) have reported on the use of homing peptides to specifically recognize various stages of lymphatic tumors. However, our laboratory has not been successful in using homing peptide-displaying BNCs for the *in vivo* targeted delivery of payloads. For accomplishing *in vivo* pinpoint delivery of BNC-based nanomedicines, it would be important to

display targeting molecules that can specifically recognize the complicated structures on the surface of the targeted sites.

15.7 Future of Bio-nanocapsules

To date, >30 patent applications have been filed in Japan related to BNCs; >20 have been filed internationally. Basic patents for BNCs have been registered in Japan, Korea, United States, and Europe. BNCs fulfill all of the requirements necessary to effectively deliver therapeutic materials, both small molecules and genes, to target cells, and thereby hold much promise as nanocarriers. However, since BNCs can mediate the movement of payloads only from extracellular space via endosomes to the cytoplasm, the machinery for the transport of genes from the cytoplasm to the nucleus must be installed for maximizing the transgene expression, which is a common problem for every nonviral nanocarrier (Hama et al., 2006). Furthermore, the BNC–liposome complex is composed of biologics and chemical products that complicate CMC (chemistry, manufacturing, and controls), retard the production of BNC-based nanomedicines under current GMP conditions, and increase the cost of the drug. Finally, the idea of BNCs and the BNC–liposome complex should not be limited to self-aggregating proteins from HBV but should be expanded to proteins from other viruses to yield a wider array of BNCs selective for a variety of tissues. Moreover, the use of nanocarriers other than liposomes needs to be explored. As demonstrated by the targeting of polyplex, a complex of polyethyleneimine and DNA (Somiya et al., 2012), BNCs could be used in combination with various nanocarriers.

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Chapter 7
MicroRNAs and Oncogenic Human Viruses

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Abstract MicroRNAs (miRNAs) are small, non-coding RNAs that regulate mRNA expression by post-transcriptional mechanism in eukaryotic cells. Some viruses also encode primary transcripts containing miRNA-like structures, and such transcripts are subjected to host miRNA processing pathway to generate viral miRNAs. Viral miRNAs derived from oncogenic viruses are often associated with tumor progression. Moreover, infections with oncogenic viruses alter the expression of host miRNAs, increasing the risk of tumor progression and viral escape from the host immune mechanism. In this chapter, we discuss the roles of virally-regulated cellular miRNAs in the respective viral life-cycles and in virus-related tumors.

Keywords microRNA • Oncogenic viruses • Tumorigenesis • Immune system

1 Introduction

1.1 Discovery of Viral miRNAs

MicroRNAs (miRNAs) are 18–25 nucleotides (nt) non-coding small RNAs derived from double-stranded RNAs, and play an important role in eukaryotic cells by post-transcriptional repression of mRNAs. It has been shown that some viruses encode primary transcripts containing miRNA-like structures. In 2004, Pfeffer et al. (2004) reported that Epstein-Barr virus (EBV) strain B95-8 encodes 5 viral pre-miRNA-like structures, and that viral miRNAs were detected in infected cells. Moreover, Cai et al. (2006) reported that wild-type EBV encodes 13 more pre-miRNAs than EBV B95-8 strain as the 12 kb region that is deleted in EBV B95-8 strain is rich in pre-miRNA genes. The expression of viral miRNAs are very common in cells that are infected

with other Herpesviruses (Pfeffer et al. 2005) (http://www.mirbase.org) including Kaposi's sarcoma-associated herpesvirus (KSHV), human cytomegalovirus, herpes simplex viruses (HSVs), and also observed in simian virus 40-infected cells. It is speculated that viral miRNAs may suppress viral transcripts or host-specific genes. However, the pathophysiological role of viral miRNAs is not clearly understood.

1.2 Viral Infection and miRNAs

Oncogenic viral infections induce the expression of several miRNAs that are associated with cancer progression. Virally induced miRNAs play the role of oncogenes when they target tumor suppressor genes. Moreover, when viral infections involve regulation of oncogenes, they repress some host miRNAs with tumor suppressive functions. Some herpesviruses such as EBV and KSHV encode pri-miRNA-like structures that are tolerated as self-entities by the host machinery. Virally derived-factors repress host miRNA cascade and are called "RNA-silencing suppressors" (RSSs) (de Vries and Berkhout 2008). RSSs were originally identified in plant viruses and oncogenic viruses origin interact with miRNA pathway (de Vries and Berkhout 2008).

2 MicroRNAs in Epstein-Barr Virus; Expression, Regulation and Function Epstein-Barr Virus

2.1 EBV Encoded miRNAs

EBV is a ubiquitous human herpesvirus that establishes life-long latent infection in human B lymphocytes and pharyngeal epithelial cells (kieff 2007). EBV has quite a large genome (~170 kb) and encodes >70 open reading frames. While many of the virally encoded proteins are immunogenic in the human body, miRNAs can affect gene expression in the host without stimulating an immune response. Therefore, encoding miRNAs work to the advantage of the virus. EBV miRNAs were the first virally encoded miRNAs to be identified (Pfeffer et al. 2004). A Burkitt's lymphoma cell line harboring EBV B95-8 strain, a laboratory strain with 12 kb deletion in its genome, (Baer et al. 1984) was used as a source of RNA. Five miRNAs were identified in the study. Later studies revealed that there are far more miRNAs in the wild-type EBV, and the region deleted in the EBV B95-8 strain is rich in pre-miRNA genes (Lo et al. 2012) (Fig. 7.1). Currently, 44 mature miRNAs that are encoded at two different loci in the EBV genome have been identified: 4 mature miRNAs encoded at the BHRF1 locus and 40 mature miRNAs encoded at the BART locus (Pfeffer et al. 2004; Cai et al. 2006; Grundhoff et al. 2006; Zhu

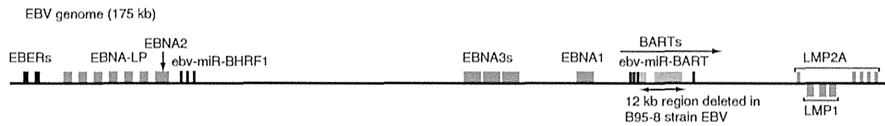

Fig. 7.1 Schematic illustration of EBV genome. The positions of EBV miRNA genes are indicated together with those of EBV latent genes, including BARTs. The 12-kb region missing in the EBV B95-8 strain is also indicated

et al. 2009) (Fig. 7.1). The presence of such a high number of miRNAs in EBV indicates the evolutionary selection of these miRNAs. A complete listing of EBV miRNAs (ebv-miR-BHRF1 and ebv-miR-BART) with both mature and precursor sequences can be found at www.mirbase.org. EBV miRNAs have no notable sequence similarity with known host (human) cell miRNAs, and no orthologous miRNAs are identified in other human Herpesviruses (Pfeffer et al. 2005). In comparison with Rhesus lymphocryptovirus, it is apparent that many of EBV miRNAs are evolutionarily conserved (Cai et al. 2006).

The expression levels of EBV miRNAs in various EBV-infected cells have been examined using various strategies, including the stem-loop PCR method (Amoroso et al. 2011; Chen et al. 2005; Cosmopoulos et al. 2009; Pratt et al. 2009) and direct sequencing of small RNA libraries, either through traditional or high-throughput sequencing method (Lung et al. 2009; Zhu et al. 2009; Chen et al. 2010). The results revealed that EBV miRNAs are expressed at markedly different levels among cell lines (Pratt et al. 2009). Four miRNAs encoded within the BHRF1 locus (hereafter referred to as miR-BHRFs) are highly expressed in cells with latency type III (Xia et al. 2008; Cai et al. 2006) [expressing all EBNAs (EBNA1, 2, 3A-C), LMP1, LMP2A, EBERs, and BARTs (BamHI A Rightward Transcripts)]. The miR-BHRFs are also highly expressed in primary EBV-associated AIDS-related diffuse large B-cell lymphomas (DLBCL) (Xia et al. 2008), but they are undetectable in B cells or epithelial cells with latency type I (expressing EBNA1, EBER, and BARTs) or latency type II (expressing EBNA1, LMP1 and LMP2A, EBER, and BARTs). On the other hand, miR-BART miRNAs (miR-BARTs) are expressed not only in B cells with type III latency, but also in epithelial cells with latency type I or type II (Cai et al. 2006). The miR-BARTs are of particular interest as they are highly expressed in nasopharyngeal carcinomas (Zhu et al. 2009; Cosmopoulos et al. 2009), gastric carcinoma cells (Kim do et al. 2007), and NK/T lymphomas-derived cell lines (Ramakrishnan et al. 2011). Therefore, it is likely that miR-BARTs somehow contribute to the tumorigenesis (Lo et al. 2012; Marquitz and Raab-Traub 2012; Raab-Traub 2012). Transcripts now referred to as BARTs originally identified from nasopharyngeal carcinoma cells (Hitt et al. 1989), have remained enigmatic for many years. However, it is now clear that BARTs most likely serve as primary transcripts that are processed to generate miR-BARTs. Interestingly, the currently identified all miR-BARTs are encoded in the introns of the transcripts of BART, and are subject to highly complicated splicing (Edwards et al. 2008).

Table 7.1 Targeting genes of EBV encoded miRNAs

Target genes	EBV miRNAs	Reference
Viral target genes		
BALF5 (DNA polymerase)	miR-BART2-5p	Barth et al. (2008)
LMP1	miR-BART1-5p, -16, -17-5p	Lo et al. (2007)
	miR-BART9	Ramakrishnan et al. (2011)
	miR-BART19-5p, -5-5p	Riley et al. (2012)
LMP2A	miR-BART22	Lung et al. (2009)
BHRF1	miR-BART10-3p	Riley et al. (2012)
Cellular target genes		
Bim	miR-BART cluster 1 and cluster 2	Marquitz et al. (2011)
PUMA	miR-BART5	Choy et al. (2008)
DICER1	miR-BART6-5p	Iizasa et al. (2010)
CXCL-11	miR-BHRF1-3	Xia et al. (2008)
IPO7	miR-BART3	Vereide et al. (2013)
CASP3	miR-BART16	Vereide et al. (2013)
GUF1, SCRN1	miR-BHRF1-1	Skalsky et al. (2012)
CAPRIN2	miR-BART13-3p	Riley et al. (2012)

2.2 *Pathophysiological Roles of EBV Encoded miRNAs*

Viral miRNAs can either target other EBV transcripts or cellular transcripts. The viral and cellular targets of EBV miRNAs so far identified are listed in Table 7.1. MiR-BART2-5p, which is located directly antisense to the 3′-UTR of BALF5 (a viral polymerase) can down-regulate the expression of BLAF5, inhibiting the transition from latent to lytic viral replication (Barth et al. 2008). Several miR-BARTs suppress the expression of viral oncoproteins LMP1 (Riley et al. 2012; Lo et al. 2007; Ramakrishnan et al. 2011) and LMP2A (Lung et al. 2009). Cellular targets of EBV miRNAs so far identified include proapoptotic proteins Bim (Marquitz et al. 2011) and BBC3/PUMA (Choy et al. 2008), a Dicer (Iizasa et al. 2010), an interferon-inducible T-cell-attracting chemokine CXCL-11/I-TAC (Xia et al. 2008), IPO7, and CASP3 (Vereide et al. 2013). Genome-wide searches for the targets of EBV miRNAs (miRNA targetome) have been conducted using either human Burkitt's lymphoma cell lines (Dolken et al. 2010), primary effusion lymphoma cell lines (co-infected with EBV and KSHV) (Gottwein et al. 2011), or EBV-transformed lymphoblastoid cell lines (Skalsky et al. 2012; Riley et al. 2012).

It is now technically feasible to utilize recombinant viruses, having miRNA genes either deleted or restored in the EBV genome, to clarify the biological significance of viral miRNAs. It was shown, by two independent studies, that disruption of genes encoding miR-BHRF1 results in slightly attenuated outgrowth of infected primary B cells (Feederle et al. 2011; Seto et al. 2010). The EBV B95-8 strain lacks 17 pre-miRNAs of miR-BARTs. Research groups attempted to reconstitute the

expression of all EBV-encoded miR-BARTs by ectopically inserting the missing pre-miRNA genes that were driven by heterologous promoters (Vereide et al. 2013; Seto et al. 2010). However, the displaced miR-BARTs were not expressed as efficiently as the endogenous miRNAs (Seto et al. 2010). The efficient expression of miR-BARTs may require primary transcripts under the control of native BART promoter, followed by proper processing of the primary transcripts.

It was also shown that EBV miRNAs were secreted from infected B cells and that they were functional upon transfer via exosomes in primary monocyte-derived dendritic cells (Pegtel et al. 2010). Another study recently demonstrated that certain plasma EBV miRNAs did not copurify with exosomes, implicating non-exosomal transport of miRNAs into plasma (Gourzones et al. 2013). Further studies are required to clarify the functional significance of viral miRNAs secreted into plasma via exosomal or non-exosomal mechanisms.

2.3 *Alteration of Human miRNA Pathway by EBV Infection*

Regulating host gene expression is crucial for viruses to survive in host cells, and it is now becoming apparent that viral miRNAs significantly contribute to such regulations, especially in latently infected cells where a few viral proteins are expressed. Viral miRNAs can affect the expression of cellular miRNAs. Specific cellular miRNAs, namely, miR-21, miR-155, and miR-146a, were found to be up-regulated in B lymphocytes transformed by EBV B95-8 strain (Godshalk et al. 2008; Mrazek et al. 2007), while other cellular miRNAs were dramatically down-regulated following EBV infection of primary B cells (Godshalk et al. 2008). It is tempting to speculate that the up-regulation of miR-21 plays critical roles in EBV-mediated transformation, as miR-21 is a well-characterized oncomir (Gabriely et al. 2008). Therefore, it appears that viral and cellular miRNA regulatory networks affect each other, and virus-host interactions are apparently far more complicated than previously thought.

3 MicroRNAs in Kaposi's Sarcoma-Associated Herpesvirus; Expression, Regulation and Function

3.1 *KSHV Encoded miRNAs*

KSHV belongs to the human herpesvirus family and is implicated in human diseases such as Kaposi's sarcoma (KS), AIDS-related primary effusion lymphoma (PEL), and multicentric castle-man's disease (Boshoff and Weiss 2002). KSHV exists as a latent or lytic infection in host cells. Pfeffer et al. and other groups discovered

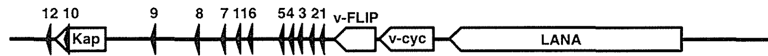

Fig. 7.2 Location of KSHV-encoded miRNAs in KSHV genome *Black triangle*: miRNA, *Kap*: kaposin

KHSV-derived miRNAs in latently infected cells (Pfeffer et al. 2004, 2005; Cai et al. 2005; Samols et al. 2005). KSHV encodes 12 miR-K12 pre-miRNAs (24 miRNAs) and A-to-I RNA edited mir-K12-10a is registered as a mir-K12-10b on miRBase (http://www.mirbase.org) (Pfeffer et al. 2005; Umbach and Cullen 2010; Lin et al. 2010). Most miR-K12s are localized in the intron of K12 (Kaposin) and two pre-miRNAs are localized in the protein- coding region and 3′-UTR of K12, respectively (Fig. 7.2).

3.2 Pathophysiological Roles of KSHV Encoded miRNAs

MiR-K12s are expressed in latently infected cells; however their role in the viral life cycle is largely unknown. MiR-K12-9 suppresses the expression of RTA, which is an essential transcription factor for KSHV lytic infection (Bellare and Ganem 2009; Lin et al. 2011). Transfection of miR-K12-7 or miR-K12-5 also represses RTA-expression (Lin et al. 2011; Lu et al. 2010). Moreover, mutated KSHV that lacks miR-K12s, except miR-K12-10 and miR-K12-12, increased lytic protein expression by enhancing NF-κB activation (Lei et al. 2010). These reports indicate that miR-K12s suppress lytic reactivation and maintain latent infection in host cells.

Seed sequences of miR-K12s are similar to human miRNAs (KSHV-K12-11 and human miR-155, miR-K12-6-5p and human miR-15a and miR-16) (Skalsky et al. 2007; Gottwein et al. 2007). These reports suggest that miR-K12s may target human genes to maintain latent infections. MiR-K12s repressed thrombospondin1 (THBS1), a tumor suppressor, via inhibition of angiogenesis and down-regulation of THBS1 expression, was also previously observed in KS lesion (Samols et al. 2007; Taraboletti et al. 1999). MiR-K12-5, -9, -10a, and -10b repress Bcl-2-associated transcription factor 1 (BCLAF1), which is a repressor of Bcl2 family and induces apoptosis (Ziegelbauer et al. 2009). MiR-K12-11 targets the xCT-negative regulator BACH-1 (Qin et al. 2010a). xCT is an amino acid transporter that protects cells from environmental oxidative stress. KS legions show high expression of xCT (Qin et al. 2010a), and interestingly, xCT is reported to be a regulator of cancer stem cells (Ishimoto et al. 2011). MiR-K12-1 represses cyclin-dependent kinase inhibitor p21. Inhibition of miR-K12-1 results in cell cycle arrest by p53 activation (Gottwein and Cullen 2010). These miR-K12-targeting genes are related to the pathogenesis of KSHV-associated diseases.

3.3 Immune Defense and KSHV Encoded miRNAs

Latent infection of KSHV were observed in 40–50 % of the population in south part of Africa, 10 % of the north American population and 4 % of the Japanese population; however, most of these people were kept healthy (Fujii et al. 1999). MiR-K12s are expressed in latently infected cells. To escape from host immune mechanism, miR-K12s may regulate this mechanism. MiR-K12-7 inhibits the expression of MHC class I polypeptide-related sequence B, which is recognized by NK cells (Nachmani et al. 2009). miR-K12-10a represses the tumor necrosis factor receptor superfamily member 12A, which regulates apoptosis and inflammatory response (Abend et al. 2010). MiR-K12s also alter human cytokine expression via targeting of the cytokine repressor C/EBPβ p20 (Qin et al. 2010b). C/EBPβ p20 is a repressor of IL-6 and IL-10. MiR-K12s induce the expression of these cytokines in murine macrophages (Qin et al. 2010b). A bioinformatics sequence analysis revealed that this could be attributed to viral miRNA-mediated expression of a known repressor of these cytokines.

To identify target genes of miR-K12s, a bioinformatics approach was used. Identifying target genes of viral miRNAs is much more difficult than identifying those of mammalian miRNAs, because species conservation of 3′-UTR miRNA targeting site is not useful for viral miRNAs. Recently, high-throughput sequencing of RNA isolated by cross-linking immunoprecipitation (HITS-CLIP) was developed to identify miRNA target gene by immunoprecipitation of Ago2-miRNAs and associated mRNAs. This method recovered approximately 1,000 cellular targeting genes of miR-K12s, including THBS1, BACH1, and C/EBPβin PEL cell lines (Haecker et al. 2012). Interestingly, HITS-CLIP revealed that the miR-K12s predominate Ago2-associated miRNAs and miR-K12smay contribute to global alteration of the human miRNAs pathway in KSHV-infected cells (Haecker et al. 2012). KSHV-positive KS is derived from endothelial cells and the mRNA expression profile of endothelial cells is not to the same as that of PEL. In the near future, the pathophysiological role of miR-K12s in endothelial cells will be identified using the HITS-CLIP method.

4 MicroRNAs in High-Risk Human Papillomavirus; Expression, Regulation and Function

4.1 Basic Knowledge of HPV Infection

Human papillomaviruses (HPVs) have small double-stranded circular genomic DNA that encode early genes (E1, E2, E4, E5, E6, and E7) and late genes (L1 and L2) (Zheng and Baker 2006). HPVs infect squamous epithelium, and then integrate into the epithelial stem cells on the basal membrane. HPV early genes

are expressed in the epithelial stem cells; however, expression of viral late genes and viral DNA replication are observed in differentiated epithelial layers. The E6 and E7 genes of high-risk HPVs (HPV16 and HPV18) have oncogenic activity and inactivate p53 and pRb, respectively (Scheffner et al. 1990; Dyson et al. 1989; Gonzalez et al. 2001).

4.2 MiRNAs Expression Profile of HPV Infected Cells and Pathophysiological Role of miRNAs in HPV Infection

DNA viruses encode viral miRNAs and therefore are able to regulate viral life cycle or human immune defense. However, HPVs do not have viral miRNAs because they have small genome (size, 8 kb). Infection with high-risk HPVs leads to tumorigenesis in the epithelial stem cells by the inactivation of tumor suppressive factors. The component p53 interacts with the Drosha-DGCR8 complex component p68 and regulates part of miRNA processing (Suzuki et al. 2009). The effect of high-risk HPVs infection in miRNA expression is largely unknown.

Dreher et al. (2011) reported that the expression level of miR-145 was increased in high-risk- HPV infected cells compared to low-risk HPV-infected cells. High-risk HPV component E6 is one of the key factors for tumorigenesis, it suppresses the expression of miR-145 (Shi et al. 2012; Gunasekharan and Laimins 2013), miR-218 (Martinez et al. 2008), miR-34a (Wang et al. 2009b; Xie et al. 2013) and miR-23b (Au Yeung et al. 2011). Previously, miR-145 was identified as a tumor suppressive miRNA (called "anti-oncomir") (Cho et al. 2009) as it suppresses the expression of c-Myc (Sachdeva et al. 2009), MUC1 (Sachdeva and Mo 2010) and stem cell-related transcription factors (Xu et al. 2009). Tumor-related target genes of miR-218 are Robo1 (Tie et al. 2010), survivin (Alajez et al. 2011), Runx2 (Zhang et al. 2011b), and the mTOR component Rictor (Uesugi et al. 2011). Moreover, miR-34a and miR-23b repress c-Myc (Christoffersen et al. 2010; Gao et al. 2009) and these miRNAs are induced by p53. These reports suggest that the high-risk HPV E6 gene represses tumor suppressive miRNAs via p53 inactivation.

High-risk HPV E7 can inactivate pRb and induce the activation of the transcription factor E2F (Scheffner et al. 1990; Dyson et al. 1989; Gonzalez et al. 2001). Interestingly, E7 suppresses miR-203 expression and induces p63 expression; p63 is an enhancer of cancer stem cells (Melar-New and Laimins 2010; Keyes et al. 2011). MiR-203 is a repressor of dermal stem cells, but the molecular mechanism underlying the transcriptional regulation of miR-203 is unknown (Yi et al. 2008). Moreover, high-risk HPV E5 regulates miR-146a, miR-203, and miR-324-5p (Greco et al. 2011). High-risk HPV infection itself may regulate cell differentiation by repressing the expression of human miRNAs.

5 MicroRNAs in Hepatitis C Virus; Expression, Regulation and Function

5.1 *Basic Knowledge of HCV*

Hepatitis C virus (HCV) has a 9.6 kb genome that encodes a single positive-strand polyprotein, which is organized in structural and the non-structural (NS-) replication proteins. The open reading frame is flanked by the 5′- and 3′ -UTRs that contain the cis-signals for the translation and replication of the viral RNA. The structural proteins, which form the viral particle, include the core protein and the envelope glycoproteins E1 and E2. The non-structural proteins include p7 ion channel, NS2-3 protease, NS3 serine protease and RNA helicase, NS4A polypeptide, NS4B and NS5A proteins, and NS5B RNA-dependent RNA polymerase (RdRp) (Appel et al. 2006; Moradpour et al. 2007) (Fig. 7.3). HCV infection is a cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) (Wasley and Alter 2000).

5.2 *The Expression and Role of miRNAs in Liver*

The miRNA expression pattern differs dramatically among internal organs. miR-122 constitutes ~70 % of the hepatic miRNAs (Landgraf et al. 2007), and its function in the liver is varied. MiR-122 maintains the hepatic function by down-regulating genes involved in cholesterol synthesis like HMG-CoA reductase, amongst others (Esau et al. 2006). The serum lipid profiles of both liver-specific and germline knockouts of miR-122 induced a 30 % reduction in the total cholesterol, LDL, HDL, and serum triglyceride level. Against expectations, the knockout miR-122 mice had progressive steatohepatitis (Hsu et al. 2012; Tsai et al. 2012). MiR-122 can also regulate lipid synthesis in the liver by controlling expression of *Agpat1* and *Cidec* (Hsu et al. 2012; Tsai et al. 2012). These genes are a part of the triglyceride biosynthesis pathway (Kim et al. 2008). Gatfield et al. (2009) showed that miR-122 is associated with circadian rhythm as the circadian metabolic regulators of the PPAR family are regulated by the miR-122-mediated metabolic control.

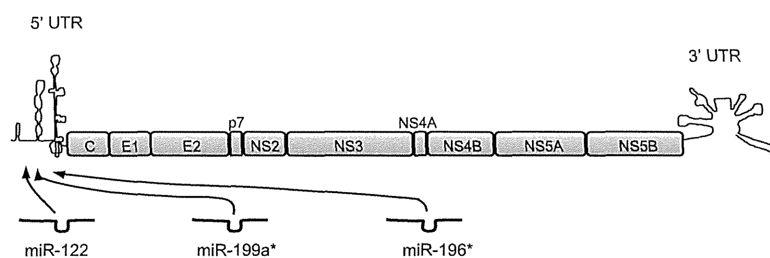

Fig. 7.3 Structure of the HCV genome. The recognition sites of miR-122, miR-199a*, and miR-196* on the HCV genome and in miRNAs are shown. This confirms the *in vitro* replication of HCV

In miR-122-knock out animals, steatohepatitis and liver fibrosis were observed (Hsu et al. 2012; Tsai et al. 2012; Gatfield et al. 2009). MiR-122 is also related to liver inflammation (Lanford et al. 2010). Several researchers showed that the expression level of miR-122 is reduced in experimental models and clinical samples of HCC, and loss of miR-122 is associated with tumor invasiveness and cancer progression (Hsu et al. 2012; Tsai et al. 2012; Wu et al. 2009; Coulouarn et al. 2009; Bai et al. 2009; Cheung et al. 2008; Wang et al. 2012).

5.3 MiRNA and HCV Associated Liver Disease

It was demonstrated that HCV replication is controlled by miR-122 (Jopling et al. 2005), and since then, the function of miR-122 in the hepatic tissue is mostly analyzed in relation to HCV replication. The reasons why HCV replication is controlled by suppressing the function of miR-122 are (1) The binding site of miR-122 is downstream to the internal ribosomal entry site, which controls duplication in the early stages of HCV infection (Henke et al. 2008), (2) It is possible that the isoprenoid biosynthetic pathway, controlled by miR-122, regulates HCV replication.miR-122 can directly regulate HCV replication when used as a target gene (Henke et al. 2008), (3) The recognition site of miR-122 in HCV is located in both the 5′-UTR and 3′-UTR domains. miR-122 forms an oligomeric complex in which one miR-122 molecule binds to the 5′-terminus of the HCV RNA, masking the 5′-terminal sequences of the HCV genome while the 3′ nucleotides are overhanging (Machlin et al. 2011). We highlight the most recent findings regarding the role of miRNAs in viral hepatitis, liver fibrosis, and HCC by analyzing the possible mechanisms by which they contribute to the progression of chronic liver disease. MiR-122, which is liver-tropic, can control HCV by stimulating and accelerating translation during replication of HCV (Jopling et al. 2005) and inhibition of miR-122 can block HCV replication.

Lohmann et al. (1999) have developed the HCV subgenomic replicon system, in which an HCV subgenomic replicon autonomously replicates in Huh-7 cells (HCV replicon cells). This technology has contributed greatly to the development of anti viral agents, and helped us to monitor the effect of miRNA on the replication of HCV. The algorithms that search miRNAs responsible for HCV-targets were demonstrated. MiRNAs, except miR-122, can also control the replication of HCV (Hsu et al. 2007) (Fig. 7.4). MiR-199a* can recognize the 5′-UTR region so overexpressing or inhibiting miR-199a* can respectively suppress or enhance HCV replication (Murakami et al. 2009). MiR-196, a HCV protein repressor, can recognize HCV genome as target gene (Hou et al. 2010) (Fig. 7.3).

MiR-130a expression was significantly higher in HCV-infected hepatocytes and liver biopsy specimens than in controls. MiR-130a can regulate interferon-induced trans-membrane 1 (IFITM1). Up-regulation of miR-130a in HCV infections reduces the expression level of IFITM1. This can inhibit HCV replication (Bhanja Chowdhury et al. 2012). The hepatic miRNA expression pattern that exists in

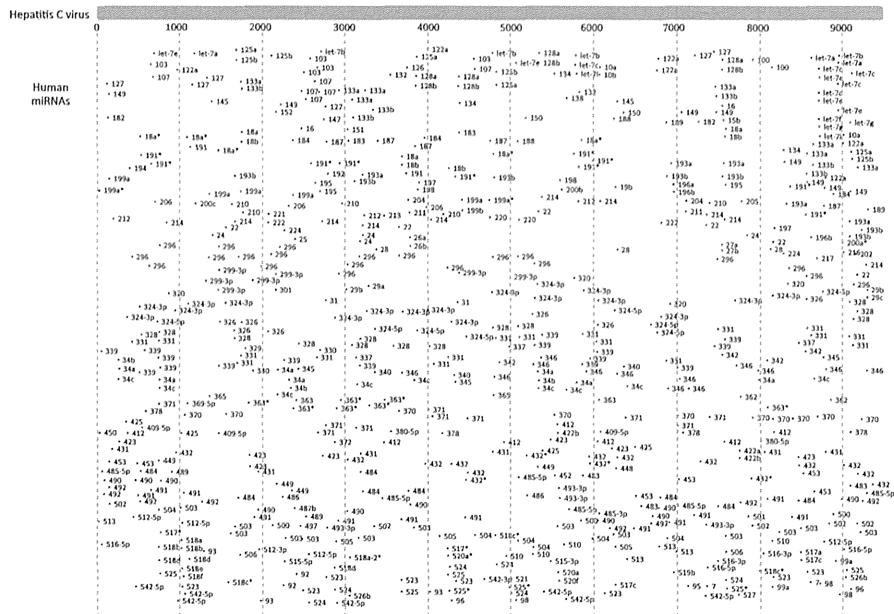

Fig. 7.4 Hypothetical miRNAs target sites on HCV genome

chronic hepatitis c (CHC) patients before pegylated interferon and ribavirin combination therapy is associated with their therapeutic outcome. The expression level of nine miRNAs was significantly different in the sustained virological response (SVR) and non-responder (NR) groups. The accuracy of this diagnosis is 70.5 % (Murakami et al. 2010). Viral species may have different expression patterns for miRNA; for example, expression patterns of miRNAs are unique in HBV and HCV infections and are closely related to liver disease progression. When seventeen miRNAs are down-regulated in HCC, cancer –associated pathways such as cell cycle, adhesion, proteolysis, transcription, and translation are enhanced. However, when miRNAs are up-regulated in HCC, the anti-tumor immune response is suppressed (Murakami et al. 2010).

The miRNAs can recognize HCV genome by using the *in silico* target search algorithm (ViTa: http://vita.mbc.nctu.edu.tw). The number above the bar indicates the nucleotide number.

The paragraphs written above summarize the close relationship between miRNA and HCV infection and chronic liver disease. The accumulated information between expression pattern of miRNAs and HCV infection can pave the way for clinical application. This knowledge has opened the path to clinical applications of miRNA analysis. Many researchers have attempted to diagnose cancer using the miRNA expression in serum or plasma (Kosaka et al. 2010). Expression pattern in circulating miRNAs were used to diagnose chronic liver disease (Bihrer et al. 2011; Cermelli et al. 2011; van der Meer et al. 2013; Shrivastava et al. 2013; Murakami et al. 2012). The second phase of the clinical trial for chronic hepatitis C involves a complementary