3.2 Routine Culturing of hADSCs

When cells reach 70–90 % confluence, passage them as follows.

1. Wash the cells twice in PBS(−).
2. Add 1 mL accutase to each 100 mm dish. Incubate each dish for 5 min at 37 °C.
3. Tap the dish, use 5 mL/dish of MesenPRO RS™ complete medium to collect cells into a tube, and centrifuge cells at 220 × *g* for 5 min at room temperature.
4. Resuspend the cells in MesenPRO RS™ complete medium, count the cell number, and seed the cells into new CellBIND™ dishes at a concentration of 5×10^3 cells/cm^2.

3.3 Intravenous Administration of hADSCs in the CCl_4-Induced Mouse Model of Acute Liver Disease

1. To ensure the acquisition of sufficient numbers of cells, plate hADSCs 2–5 days prior to the intravenous injection of these cells into mice.
2. Prepare diluted CCl_4 solution by mixing one volume CCl_4 with nine volumes olive oil.
3. Intraperitoneally inject mice with 100 μL diluted CCl_4 solution/20 g body weight (10 μL CCl_4/20 g body weight).
4. To establish a sham operation, intraperitoneally inject mice with 100 μL olive oil/20 g body weight.
5. Twenty-four hours after CCl_4 injection, perform the intravenous injection of hADSCs as follows.
6. Wash the cells twice in PBS(−).
7. Add 1 mL accutase per 100 mm dish, and incubate dishes at 37 °C for 5 min.
8. After tapping the dishes, use 5 mL/dish of MesenPRO RS™ complete medium to collect cells into a tube.
9. Remove cell aggregates by filtering the cell suspension through a 40 μm cell strainer into a new tube.
10. Centrifuge the cells at 220 × *g* for 5 min at room temperature.
11. Resuspend these cells in 0.5–1 mL PBS(−), and count the number of cells obtained.
12. Use PBS(−) to dilute the cell suspension to 7.5×10^6 cells/mL, and store the suspension on ice until it is injected into mice (*see* **Note 5**).
13. Load the hADSC suspension into a 1 mL syringe and equip this syringe with a 27-G needle (*see* **Note 6**).
14. Slowly inject 200 μL ADSC suspension/mouse (1.5×10^6 cells/mouse) into the tail veins of the mice (*see* **Notes 7** and **8**).

3.4 Blood and Tissue Sampling

1. Use isoflurane to anesthetize mice 24 h after the injection of hADSCs.
2. Open the chest of each mouse with surgical scissors to expose the heart.

3. Insert a syringe with a 24-G needle into the left ventricle. Slowly collect blood into the syringe (*see* **Note 9**).
4. After collecting this blood sample, extract the liver of the mouse. Wash the liver once in PBS(−), and fix the extracted liver by soaking it in 10 % formalin.
5. Collect blood into a 1.5 mL tube and incubate this tube at room temperature for 30 min.
6. Incubate the tube at 4 °C for 1 h.
7. Centrifuge the tube at 2,200 × *g* for 20 min at 4 °C.
8. Transfer the supernatant into a new tube.
9. Centrifuge this tube at 2,200 × *g* for 5 min at 4 °C.
10. Carefully collect the supernatant (serum) and transfer it into a new tube (*see* **Note 10**).
11. Use serum samples for blood tests, or store these samples at −20 °C until use (*see* **Note 11**).

3.5 Histological Analyses of Liver Tissue

1. Fix the collected liver tissue in PBS(−) containing 10 % formalin, and prepare a paraffin block. Use a general sectioning procedure to obtain 3–5 μm sections.
2. Utilize a generally accepted procedure to perform hematoxylin and eosin (HE) staining (*see* **Note 12**).
3. To detect human hADSCs in the livers of immunodeficient mice, perform immunofluorescent staining of mouse liver tissue using an anti-HLA-1 antibody (*see* **Note 13**).

4 Notes

1. Animals are maintained in an isolator unit at a constant temperature of 20 °C and subjected to a 12 h light–dark cycle. Mice receive a standard sterilized diet and water ad libitum. All experiments were performed in accordance with national laws and institutional regulations.
2. Dissolve 0.015 g collagenase in 10 mL PBS(−) by layering the powder on the surface of the liquid to avoid clumping. After the powder has completely dissolved, sterilize the solution by filtration through a 0.22 μm filter.
3. Prepare MesenPRO RS™ complete medium by supplementing 500 mL of basal medium with 10 mL growth supplement, 5 mL Antibiotic-Antimycotic, and 5 mL GlutaMAX.
4. The cells exhibit a spindle-shaped morphology that is characteristic of MSCs (Fig. 1a) and express CD105 (endoglin) (Fig. 1b). CD105, which is a component of the receptor complex of transforming growth factor (TGF)-β, is involved in various cellular

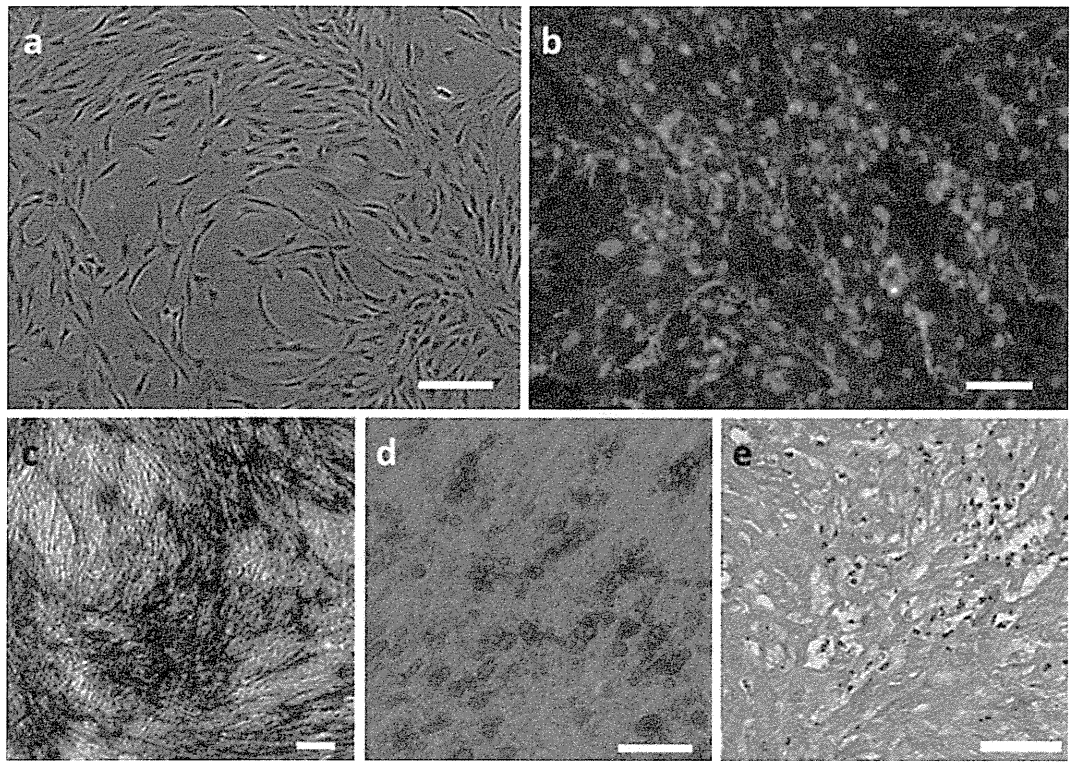

Fig. 1 Characterization of isolated hADSCs. (**a**) Phase contrast images of isolated hADSCs indicate the spindle-shaped morphology of these cells, which is a characteristic feature of MSCs. Scale bar: 200 μm. (**b**) hADSCs are positive for CD105 (*green*), an important molecule for maintaining MSC characteristics [9]. Nuclei are counterstained with Hoechst 33342 (*blue*). Scale bar: 100 μm. (**c**) Alkaline phosphatase staining reveals the osteogenic differentiation of hADSCs. Scale bar: 100 μm. (**d**) Oil red O staining reveals the adipogenic differentiation of hADSCs. Scale bar: 100 μm. (**e**) Alcian blue staining reveals the chondrogenic differentiation of hADSCs. Scale bar: 50 μm

events, including proliferation, differentiation and migration. The cultured cells are also multipotent. In particular, these cells can differentiate into adipocytes, osteoblasts, and chondrocytes (Fig. 1c–e). In accordance with the manufacturer's instructions the following commercial kits are used for the differentiation of hADSCs into three mesodermal lineages: hMSC Mesenchymal Stem Cell Adipogenic Differentiation Medium (Lonza), hMSC Mesenchymal Stem Cell Chondrocyte Differentiation Medium (Lonza), and hMSC Mesenchymal Stem Cell Osteogenic Differentiation Medium (Lonza). Surface marker characterization by flow cytometry indicates that these cells are positive for CD105, CD73, CD90, and CD44 but negative for CD45, CD31, and CD34 [23].

5. hADSCs are likely to aggregate at room temperature. This aggregation may cause pulmonary embolisms in mice injected with these cells.

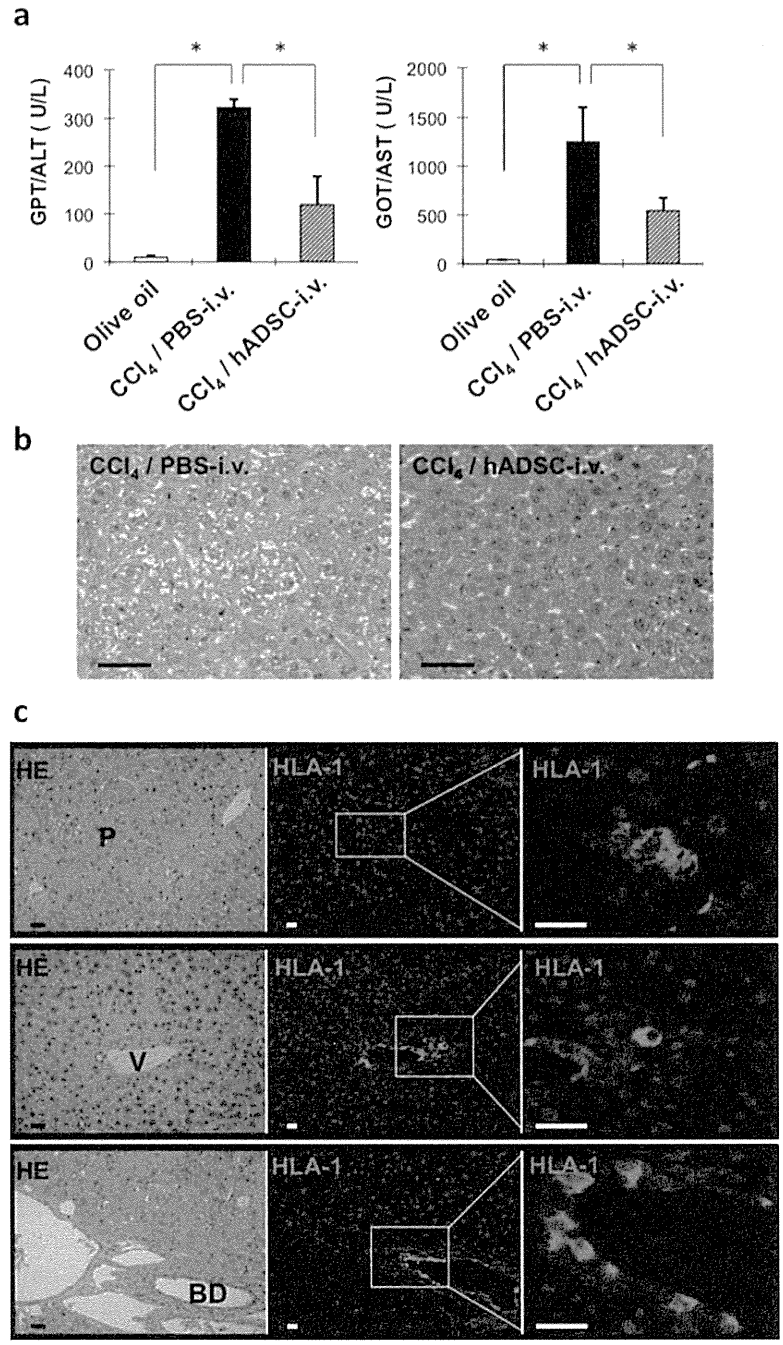

Fig. 2 Therapeutic efficacy of systemically transplanted hADSCs in the CCl4-induced acute hepatitis mouse model. (**a**) Biochemical analysis of mouse blood serum samples for the liver injury markers GPT/ALT (*left*) and GOT/AST (*right*). Immunodeficient mice received an intraperitoneal injection of either olive oil (a control for the CCl_4 injection) or 10 μL CCl_4/20 g body weight. At 24 h after this injection, the CCl_4-injected mice received an intravenous injection of either PBS(–) (a control for hADSC administration) or 1.5×10^6 hADSCs/mouse. Data are expressed as means ± S.D. and subjected to analysis using the Bonferroni

6. When loading cells into a 1 mL syringe, thoroughly mix the cell suspension by pipetting because the cells will tend to fall to the bottom wall of the tube. Do not attach a needle to the syringe prior to loading the cells because the use of a syringe with an attached needle may damage the cells.
7. In our experience, a tail vein injection of more than 2×10^6 cells/mouse is associated with an increased risk of pulmonary embolism.
8. If more than 5 min are required to complete an injection, reloading of the cell suspension is recommended to avoid precipitation of cells.
9. Rapid drawing of the syringe may cause hemolysis.
10. Leave a small portion of the supernatant in the tube to ensure that the serum samples are not contaminated by the pellet.
11. In our laboratory, we use the DRI-CHEM system (Fuji) to measure blood markers of liver injury, such as serum levels of GPT/ALT, GOT/AST, ammonia, uric acid, and blood urea nitrogen. We have observed significant improvement in liver injury markers, particularly with respect to GPT/ALT and GOT/AST levels (Fig. 2a).
12. This staining reveals that hADSC administration produces significant morphological changes in hepatocytes in non-necrotic regions (Fig. 2b). Relative to injured livers from control mice, injured livers from mice that received hADSCs exhibit lower levels of vacuolar degeneration caused by the dilatation of mitochondria and the rough endoplasmic reticulum.
13. We detected hADSCs within the injured mouse liver 24 h after these cells were injected into the mice. HLA-1 positive cells were found in various areas of the examined mouse livers, including the parenchyma, vessels, and bile ducts (Fig. 2c).

Fig. 2 (continued) correction; $n=3$. (*$p<0.05$). (**b**) Histological analysis of CCl_4-injured liver sections. This figure presents HE-stained images of mouse livers 24 h after an intravenous injection of either PBS(–) (*left*) or 1.5×10^6 hADSCs/mouse (*right*). These mice received an intraperitoneal injection of CCl_4 24 h prior to the administration of hADSCs. Scale bars: 100 μm. (**c**) Immunohistochemical analyses for human leukocyte antigen 1 (HLA-1)-positive cells in mouse liver sections after the administration of hADSCs. HLA-1-positive cells are present in different areas of the liver, including the parenchyma (P), vessels (V), and bile duct (BD). The left side of this figure presents the results of HE staining for these areas of the examined liver sections. Scale bars: 500 μm. This figure is reproduced from ref. 10

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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

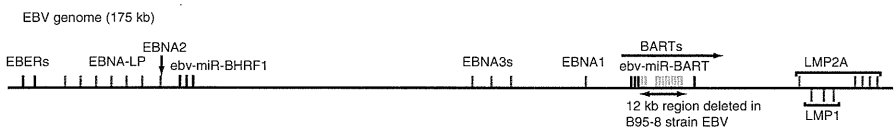

Fig. 7.1 Schematic illustration of EBV genome. The positions of EBV miRNA genes are indicated together with those of EBV latent genes, including BARTs. The 12-kb region missing in the EBV B95-8 strain is also indicated

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

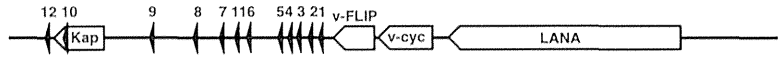

Fig. 7.2 Location of KSHV-encoded miRNAs in KSHV genome *Black triangle*: miRNA, *Kap*: kaposin

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.

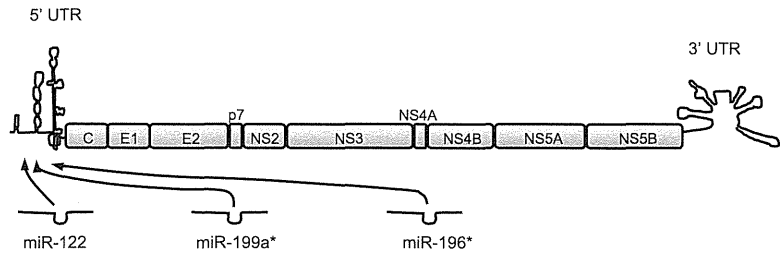

Fig. 7.3 Structure of the HCV genome. The recognition sites of miR-122, miR-199a*, and miR-196* on the HCV genome and in miRNAs are shown. This confirms the *in vitro* replication of HCV

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 over-expressing 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

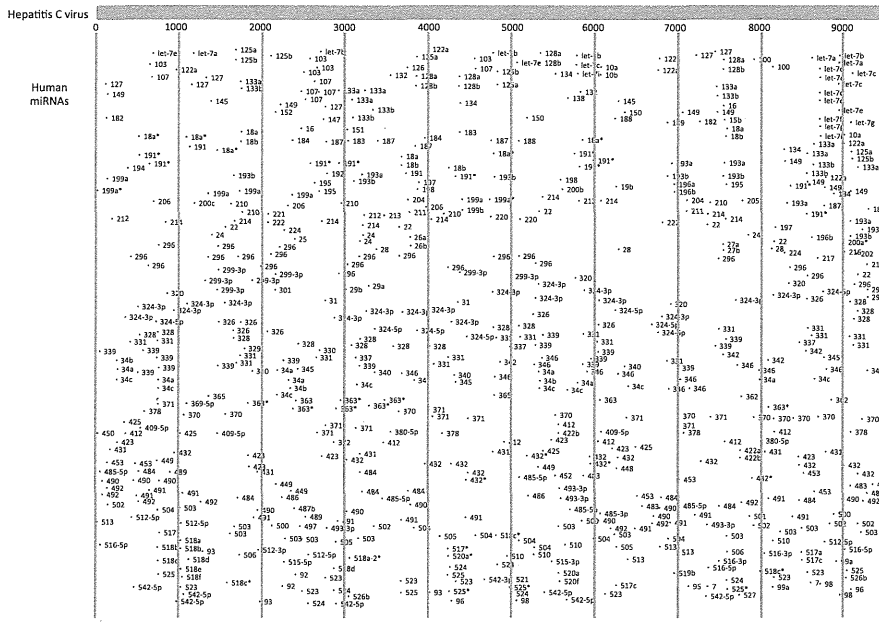

Fig. 7.4 Hypothetical miRNAs target sites on HCV genome

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

based sequence of miR-122 and the uses of locked nucleic acid-miR-122 to control the function of liver miR-122 (Janssen et al. 2013). Biomarkers and nucleic acid drugs will be applied clinically in the near future.

6 MicroRNAs in Hepatitis B Virus; Expression, Regulation and Function

6.1 Basic Knowledge of HBV Infection

The hepatitis B virus (HBV) is a small enveloped partially double-stranded DNA virus that belongs to the *Hepadnaviridae* family. This virus primarily infects hepatocytes and causes acute and chronic liver disease. Among the 2,000 million people worldwide infected with HBV, more than 350 million remain chronically infected and become carriers of the virus (Ganem and Prince 2004). Epidemiological studies have revealed that chronic HBV infection is the major etiological factor in the development of HCC. Indeed, more than a half of the HCC patients are HBV carriers (Parkin 2006). Despite the availability of an efficacious vaccine, persistent HBV infection remains a challenging global health issue that requires a better understanding of the virus biology and pathogenesis for improved control and treatment.

The life cycle of HBV is complex (Fig. 7.5a). The initial stages of the acute HBV infection, including virion attachment, uncoating and nucleocapsid transport to the cell nucleus, are still poorly understood (Seeger and Mason 2000; Yan et al. 2012). Once delivered into the nucleus, the 3.2 kb relaxed circular DNA genome is converted into a covalently closed circular DNA (cccDNA) from which all the viral RNAs are transcribed. These transcripts include the pregenomic RNA (pgRNA) that will serve as template for reverse transcription and the subgenomic mRNAs that derive from the four overlapping gene sequences composing the viral genome. These sequences comprise the pre-S and surface genes, the precore and core genes, the polymerase gene, and the X gene. The newly formed nucleocapsids can either assemble with envelope proteins in the endoplasmic reticulum and form mature virion that will be secreted, or return to the nucleus to maintain the cccDNA amplification. When the immune system fails to clear the virus, the HBV infection becomes chronic (Fig. 7.5b). Eventually, the viral genetic material or sequences can integrate into the host cellular DNA. The integration has been frequently observed and is associated with HCC (Brechot et al. 1980; Paterlini-Brechot et al. 2003).

MiRNAs play key roles in the regulation of almost every cellular process in all multicellular eukaryotes (Bartel 2009). As intracellular pathogens, viruses are affected by these post-transcriptional modulators and have found a way to subvert their effects. Several viruses, especially the herpesviruses, encode for miRNAs that increase their replication potential and/or allow the evasion from the innate immune system (Skalsky and Cullen 2010). This chapter will outline the implication of miRNAs in the HBV biology and the associated pathogenesis, including HCC development. We will also outline the present and future miRNA-based strategies for the diagnosis, prognosis and treatment of the HBV-related HCC.