specific CTL levels was not indicated. These results suggest that Vif-specific CTL induction may contribute in part to acquisition of the potential to suppress SIV-G64723mt replication efficiently.

Discussion

We have previously shown that *90-120-Ia*-positive macaques eliciting Gag-specific CTL responses by vaccination can control SIVmac239 replication but are unable to contain a challenge with a mutant SIV, SIV-G64723mt, carrying multiple *gag* mutations that result in escape from recognition by $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTLs [24]. The present study revealed, by in-vitro viral suppression assay, that those *90-120-Ia*-positive vaccinees can acquire, after wild-type SIVmac239 challenge, $CD8^+$ cells able to suppress the mutant SIV replication. Induction of these $CD8^+$ cell responses may have some supportive effect on the maintenance of viral control after the initial viral containment [4,26,27]. Such dynamics of anti-SIV responses have not been shown clearly even in live attenuated SIV infection [41–44]. Recently, HIVs have been suggested to accumulate mutations escaping from dominant CTL responses [45–51], but our results imply a possibility of induction of cellular immune responses effective against even those HIV variants escaping from dominant CTL responses.

The group I animals induced multiple Gag epitope-specific CTL responses after boost (before challenge) and after challenge, whereas the group II animals elicited only $Gag_{206-216}$-specific or $Gag_{241-249}$-specific CTL responses before challenge and showed induction of additional CTL responses directed against Gag epitopes other than $Gag_{206-216}$ and $Gag_{241-249}$ after challenge. Furthermore, both groups elicited SIV non-Gag-specific CTL responses after challenge. These results indicate post-challenge accumulation of broader CTL responses. The in-vitro anti-SIVmac239 efficacy levels correlated with Vif-specific and Nef-specific CTL as well as $Gag_{206-216}$-specific and $Gag_{241-249}$-specific CTL levels but not with total Gag-specific or total SIV-specific CTL levels, suggesting that not all but some particular epitope-specific CTL responses were involved in suppression of SIVmac239 replication. Nef-specific CTL responses were detected more frequently than Vif-specific ones, whereas the latter showed stronger correlation with antiviral efficacy levels (Fig. 5). We did not find common CTL epitopes in Vif or Nef. These may imply higher frequencies of effective CTLs in Vif-specific ones; conversely, Nef-specific CTLs may include effective ones but with higher frequencies of ineffective ones.

Postboost $CD8^+$ cells able to suppress SIVmac239 replication failed to show suppressive effect on SIV-G64723mt replication. We confirmed it also in two *90-120-Ia*-positive vaccinated animals that had failed to control the mutant SIV challenge in our previous studies [24] (data not shown). However, $CD8^+$ cells in the chronic phase suppressed SIV-G64723mt replication efficiently. This indicates postchallenge induction of $CD8^+$ cells with the potential to suppress SIV-G64723mt replication in vaccine-based SIVmac239 controllers, although it remains unclear whether these $CD8^+$ cells with antimutant SIV efficacy are responsible for the control of mutant SIV superchallenge *in vivo*. The in-vitro anti-SIV-G64723mt efficacy levels correlated with Vif-specific CTL levels and $CD8^+$ cells with detectable Vif-specific CTL responses showed suppressive effect on SIV-G64723mt replication. These results implicate Vif-specific CTL responses in the suppression of SIV-G64723mt replication *in vitro* by $CD8^+$ cells in the chronic phase, although other factors may also be involved in this suppression. Preservation of memory $CD4^+$ T cells by vaccine-based SIV control [26] may contribute to induction of these effective CTL responses.

We found dynamics of cellular immune responses during viral control in vaccine-based SIV controllers, but the exact mechanism for broadening or changes in dominance patterns of CTL responses remains unclear. All the group I animals and macaque R04-015 showed rapid selection of a CTL escape *gag* mutation, L216S, at week 5 after challenge, whereas no *gag* mutations were selected at week 5 in macaques R04-016 or R06-007 (data not shown). We failed to recover viral genome cDNAs for sequencing from plasma after week 5 due to undetectable viral loads, but selection of viral CTL escape mutations and reversions [23,28,52–57] under undetectable levels of viral replication may contribute to induction of broader CTL responses in SIV controllers.

It is difficult to directly compare anti-SIVmac239 and anti-SIV-G64723mt efficacy of $CD8^+$ cells because of difference in their replicative ability, but the ratios of the latter level to the former 1 year after challenge were higher than those after boost in all animals. Indeed, $CD8^+$ cells 1 year after challenge in macaques R03-012 and R02-003 showed suppressive effect on SIV-G64723mt but not on wild-type SIVmac239 replication, although R03-012 $CD8^+$ cells at 5 months and 1 year after challenge efficiently suppressed SIVmac239 replication at higher E/T ratio of 1 : 1 (R02-003 $CD8^+$ cells in the chronic phase for this analysis were unavailable). Because no SIV controllers elicited CTL responses specific for peptides with mutated amino acid sequences (data not shown), all CTLs specific for SIV-G64723mt antigens in SIV controllers are expected to recognize SIVmac239 antigens also. Thus, our observation that some post-challenge $CD8^+$ cells showed efficient suppressive effect on SIV-G64723mt but not on SIVmac239 replication *in vitro* may be explained by higher replicative ability of SIVmac239 compared with SIV-G64723mt; it could

be more difficult for $CD8^+$ cells to suppress replication of the wild-type SIVmac239 than the mutant SIV-G64723mt, implying a possible requirement of more potent CTL responses for SIVmac239 control than for SIV-G64723mt control.

In summary, this study showed dynamics of postchallenge cellular immune responses in vaccine-based SIV controllers. Our results suggest that, during persistent viral control, vaccine-based SIV controllers can acquire $CD8^+$ cells with the potential to suppress replication of SIV variants carrying CTL escape mutations. Elucidation of the mechanism for induction of broader responses in these controllers may contribute to development of a vaccine effective against highly diversified HIV infection.

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A structural constraint for functional interaction between N-terminal and C-terminal domains in simian immunodeficiency virus capsid proteins

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Abstract

Background: The Gag capsid (CA) is one of the most conserved proteins in highly-diversified human and simian immunodeficiency viruses (HIV and SIV). Understanding the limitations imposed on amino acid sequences in CA could provide valuable information for vaccine immunogen design or anti-HIV drug development. Here, by comparing two pathogenic SIV strains, SIVmac239 and SIVsmE543-3, we found critical amino acid residues for functional interaction between the N-terminal and the C-terminal domains in CA.

Results: We first examined the impact of Gag residue 205, aspartate (Gag205D) in SIVmac239 and glutamate (Gag205E) in SIVsmE543-3, on viral replication; due to this difference, $Gag_{206-216}$ (IINEEAADWDL) epitope-specific cytotoxic T lymphocytes (CTLs) were previously shown to respond to SIVmac239 but not SIVsmE543-3 infection. A mutant SIVmac239, SIVmac239Gag205E, whose Gag205D is replaced with Gag205E showed lower replicative ability. Interestingly, however, SIVmac239Gag205E passaged in macaque T cell culture often resulted in selection of an additional mutation at Gag residue 340, a change from SIVmac239 valine (Gag340V) to SIVsmE543-3 methionine (Gag340M), with recovery of viral fitness. Structural modeling analysis suggested possible intermolecular interaction between the Gag205 residue in the N-terminal domain and Gag340 in the C-terminal in CA hexamers. The Gag205D-to-Gag205E substitution in SIVmac239 resulted in loss of in vitro core stability, which was recovered by additional Gag340V-to-Gag340M substitution. Finally, selection of Gag205E plus Gag340M mutations, but not Gag205E alone was observed in a chronically SIVmac239-infected rhesus macaque eliciting $Gag_{206-216}$-specific CTL responses.

Conclusions: These results present in vitro and in vivo evidence implicating the interaction between Gag residues 205 in CA NTD and 340 in CA CTD in SIV replication. Thus, this study indicates a structural constraint for functional interaction between SIV CA NTD and CTD, providing insight into immunogen design to limit viral escape options.

Background

One of the characteristics of human immunodeficiency virus (HIV) is to induce persistent viral replication resulting in AIDS progression. HIV has enormous capacity to mutate and escape from host immune recognition, driving genetic diversification of the circulating viruses [1-3]. The Gag capsid (CA), comprising the N-terminal (NTD) and the C-terminal domains (CTD) [4-6], is one of the most conserved proteins in highly-diversified HIVs [7]. Understanding structural constraints in such viral proteins could provide valuable information for immunogen design in AIDS vaccine development.

Virus-specific cytotoxic T-lymphocyte (CTL) responses play a central role in the control of immunodeficiency virus infection [7-12]. CTLs exerting strong suppressive pressure on HIV replication select for viral mutations resulting in escape from CTL recognition [13-16]. Escape mutations in viral proteins with structural constraints are often selected with viral fitness costs, possibly facilitating subsequent immune control

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[3,17-23]. Thus, conserved viral proteins such as CA can be a promising antigen for vaccine-based CTL induction toward HIV control.

We previously showed vaccine-based control of a simian immunodeficiency virus mac239 (SIVmac239 [24]) challenge in a group of Burmese rhesus macaques possessing the major histocompatibility complex class I (MHC-I) haplotype *90-120-Ia* [19,25]. $Gag_{206-216}$ (IINEEAADWDL) epitope-specific CTL responses play an important role in this control and select for a CTL escape mutation, GagL216S, leading to a leucine (L)-to-serine (S) substitution at the 216th amino acid (aa) in Gag (CA) with the cost of viral fitness [26]. However, *90-120-Ia*-positive vaccinees failed to control a challenge with another pathogenic SIV strain, SIVsmE543-3 [27], that has the same $Gag_{206-216}$ epitope sequence with SIVmac239; $Gag_{206-216}$-specific CTLs did not show responses against SIVsmE543-3 infection due to an aspartate (D)-to-glutamate (E) change, GagD205E, at Gag residue 205 [28].

Thus, the GagD205E substitution in SIVmac239 could result in viral escape from $Gag_{206-216}$-specific CTL recognition. However, in our previous analyses of *90-120-Ia*-positive animals eliciting $Gag_{206-216}$-specific CTL responses for one or two years postchallenge, we observed selection of GagL216S, but not GagD205E mutation in SIVmac239 infection, suggesting a possibility that the GagD205E substitution results in larger reduction of viral replicative ability than GagL216S. In the present study, we first constructed a mutant SIVmac239, SIVmac239Gag205E, with the GagD205E substitution and examined its replication ability in vitro. We found that this amino acid change in the CA NTD results in loss of viral fitness, which can be recovered by an additional amino acid change in the CA CTD. Further analyses presented in vitro and in vivo evidence for a structural constraint in the functional interaction between SIV CA NTD and CTD.

Results

Compensation for loss of viral fitness in SIVmac239Gag205E by additional GagV340M substitution

We first constructed a mutant SIVmac239 molecular clone DNA with a mutation of a D-to-E substitution at the 205th aa in Gag (CA NTD) to obtain the mutant virus, SIVmac239Gag205E (Figure 1). Analysis of viral replication kinetics on HSC-F, a macaque T cell line, revealed delayed peak of the mutant SIVmac239-Gag205E replication, indicating its lower replicative ability compared to the wild-type SIVmac239 (Figure 2).

We further followed up SIVmac239Gag205E replication on HSC-F cells and explored a possibility of viral reversion or additional mutations (Figure 3). No additional gag mutation became dominant on day 10 after SIVmac239Gag205E infection. Interestingly, however, in the second culture after passage of the first culture supernatants on day 10 into uninfected HSC-F cells, an additional mutation, GagV340M, resulting in a valine (V)-to-methionine (M) substitution at the 340th aa in Gag (CA CTD), became dominant in two of four sets of experiments; SIVmac239 has V while SIVsmE543-3 has M at the Gag residue 340. The GagD205E mutation remained dominant, and no other mutations were detected in the CA-coding region even in the second culture.

We then constructed a mutant SIVmac239 molecular clone DNA by introducing the GagV340M mutation into the SIVmac239Gag205E CA-coding region to obtain SIVmac239Gag205E340M (Figure 1). This mutant SIV showed similar replication kinetics on HSC-F cells with the wild-type SIVmac239, indicating compensation for loss of viral fitness in SIVmac239Gag205E by addition of the GagV340M substitution (Figure 2). These results imply that SIV CA with Gag205D-340V or Gag205E-340M combination is functional whereas the CA with Gag205E-340V is less functional.

Possible interaction between Gag residues 205 and 340 in SIV CA hexamers

Recovery of viral fitness of SIVmac239Gag205E by the GagV340M substitution suggests a possibility of interaction between Gag residues 205 in the NTD and 340 in the CTD. Modeling of CA monomer structure, however, showed that the Gag 205th residue is located in the helix 4 of CA NTD, while the 340th is in the loop between helices 10 and 11 of CTD, which does not support a possibility of intramolecular contact between Gag residues 205 and 340 (data not shown).

CA molecules are known to form hexamer lattice in mature virions [29-33]. Modeling of CA hexamer structure revealed that the Gag 205th residue in the NTD is located in close proximity to the 340th in the CTD of the adjacent CA molecule (Figure 4). These observations support a possibility of intermolecular interaction between Gag residues 205 and 340 in CA hexamers.

In addition, the 312th residue in the loop between helices 8 and 9 of CTD is located in close proximity to the 205th in the NTD of the adjacent CA molecule. Because SIVmac239 and SIVsmE543-3 have different amino acids at this residue 312, alanine (A) in the former and proline (P) in the latter, we also constructed a mutant SIVmac239 molecular clone DNA by introducing the GagA312P mutation resulting in A-to-P substitution at the 312th aa in Gag into the SIVmac239Gag205E CA-coding region to obtain SIVmac239Gag205E312P (Figure 1). Analysis of replication kinetics on HSC-F cells indicated recovery of viral fitness by the additional GagA312P substitution in SIVmac239Gag205E (Figure 2).

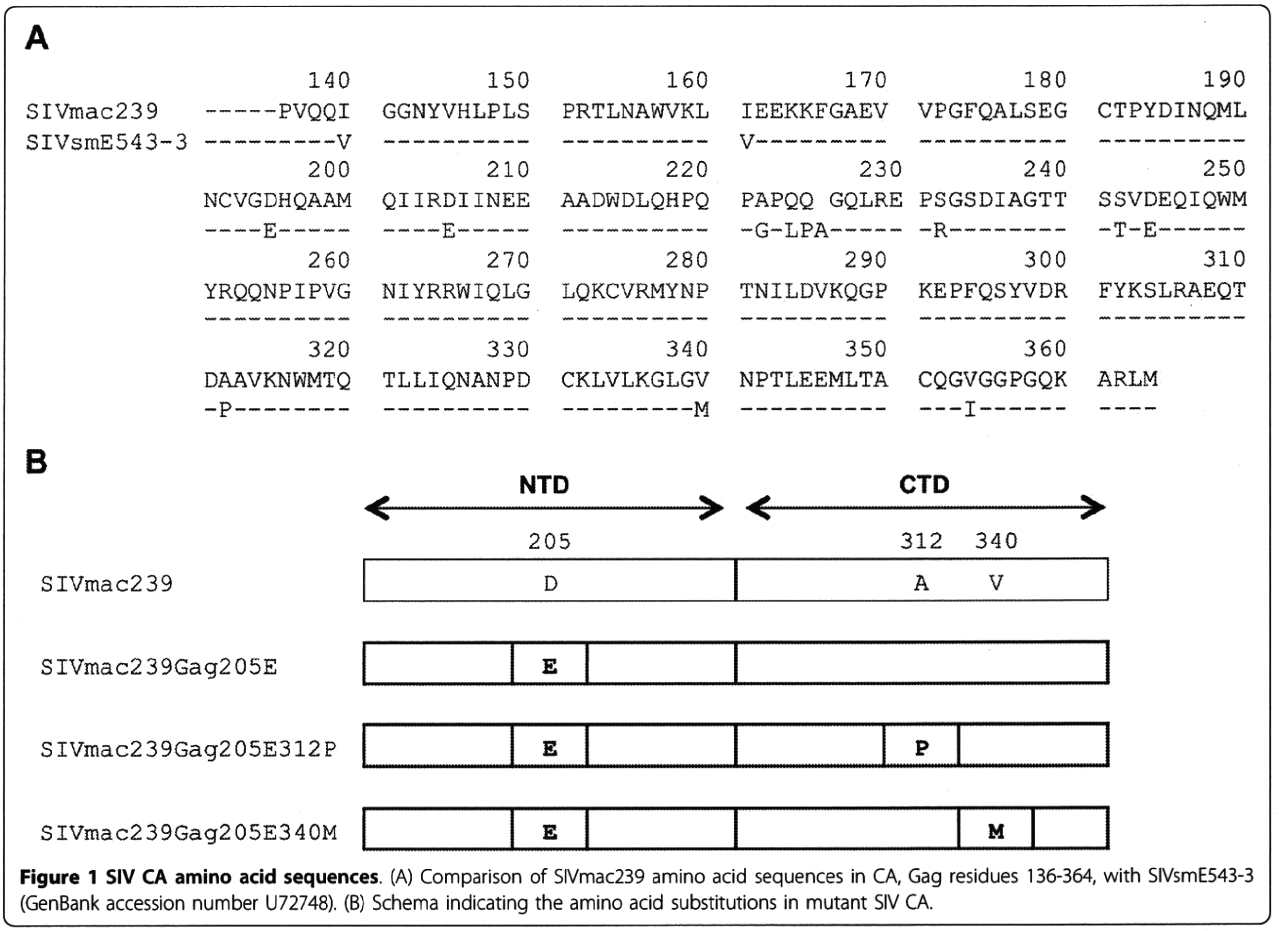

Figure 1 SIV CA amino acid sequences. (A) Comparison of SIVmac239 amino acid sequences in CA, Gag residues 136-364, with SIVsmE543-3 (GenBank accession number U72748). (B) Schema indicating the amino acid substitutions in mutant SIV CA.

Full recovery of viral fitness in SIVmac239Gag205E340M

We then focused on analyzing the possibility of functional interaction between Gag residues 205 in CA NTD and 312/340 in CA CTD. To confirm differences in viral fitness among SIVmac239, SIVmac239Gag205E, SIVmac239Gag205E312P, and SIVmac239Gag205E340M, we compared their replicative ability by viral competition assay (Table 1). The competitions confirmed lower viral fitness of SIVmac239Gag205E compared to wild-type SIVmac239, SIVmac239Gag205E312P, and SIVmac239Gag340M. SIVmac239Gag205E312P showed lower viral fitness than SIVmac239, whereas replication ability of SIVmac239Gag205E340M was no less than the wild-type. These results indicate that the GagD205E substitution in SIVmac239 reduced viral fitness, which was recovered partially by an additional GagA312P and fully by an additional GagV340M substitution. The competition between SIVmac239 and SIVmac239Gag205E340M at the ratio of 1:1 resulted in selection of the latter, suggesting that SIV CA with Gag205E-340M combination observed in SIVsmE543-3 may be slightly more functional than that with Gag205D-340V in SIVmac239.

Inhibition of the early phase of SIVmac239Gag205E replication

We examined whether the GagD205E substitution affects the early or late phase of SIVmac239 replication. On LuSIV cells, SIVmac239Gag205E infection showed significantly lower luciferase activity compared to wild-type SIVmac239, SIVmac239Gag205E312P, or SIVmac239Gag205E340M, indicating suppression of the early phase of SIVmac239GagD205E replication (Figure 5). In contrast, we did not find a significant difference in viral production among SIVmac239, SIVmac239Gag205E, SIVmac239Gag205E312P, and SIVmac239Gag205E340M (Figure 6). These results indicate that the loss of viral fitness by the GagD205E substitution is mainly due to inhibition of the early phase of viral replication.

Loss of in vitro core stability in SIVmac239Gag205E

If the GagD205E substitution disturbs intermolecular CA interaction for hexamer formation, it may affect SIV core stability. To assess the core stability in vitro [34], concentrated viruses were separated into three fractions by ultracentrifugation under gradient sucrose

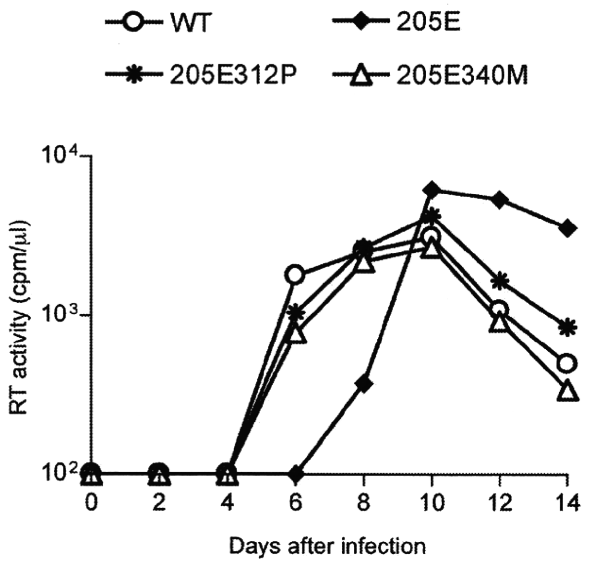

Figure 2 Wild-type and mutant SIV replication kinetics in HSC-F cells. HSC-F cells were infected with SIVmac239 (WT, open circles), SIVmac239Gag205E (205E, closed diamonds), SIVmac239Gag205E312P (205E312P, asterisk), or SIVmac239Gag205E340M (205E340M, open triangles). Virus production was monitored by measuring RT activity in the culture supernatants. A representative result from five sets of experiments is shown.

concentrations in the presence of Triton X-100 and each fraction was subjected to Western blot analysis to detect CA p27 proteins (Figure 7). In the absence of Triton X-100, CA proteins were detected in the bottom fraction, whereas those in the presence of 1% Triton X-100 were sensitive to the detergent and detected not in the bottom but only in the top fraction (data not shown). We compared the in vitro viral core stability between SIVmac239 and SIVmac239Gag205E in the presence of 0.6%, 0.9%, and 1.35% Triton X-100, respectively, and found a difference in the presence of 0.6% Triton X-100. Additional experiments revealed that SIVmac239Gag205E core was more sensitive to 0.6% Triton X-100 treatment than SIVmac239, SIVmac239Gag205E312P, and SIVmac239Gag205E340M (Figure 7). These results suggest that viral core stability may be reduced by GagD205E substitution but can be recovered by additional GagA312P or GagV340M substitution.

Selection of GagD205E plus GagV340M mutations in a SIVmac239-infected macaque

The GagD205E substitution results in viral escape from $Gag_{206\text{-}216}$-specific CTL recognition. Finally, we examined whether this substitution can be selected in the chronic phase of SIVmac239 infection in *90-120-Ia*-positive macaques eliciting $Gag_{206\text{-}216}$-specific CTL responses using plasma samples obtained in our previous experiments [35,36]. SIVmac239-infected *90-120-Ia*-positive macaques select the GagL216S mutation resulting in viral escape from $Gag_{206\text{-}216}$-specific CTL recognition, but we found selection of both GagD205E and GagV340M mutations in viral genomes in one animal, R01-007 (Table 2). In this animal, GagD205E and GagV340M mutations were undetectable at week 123 after SIVmac239 challenge, but both became detectable at week 137 and were dominant at week 150. In contrast, the GagL216S mutation dominant at week 123 was not detected at week 150. These results present in vivo evidence indicating functional interaction between the Gag 205th residue in NTD and the 340th in CTD of SIV CA.

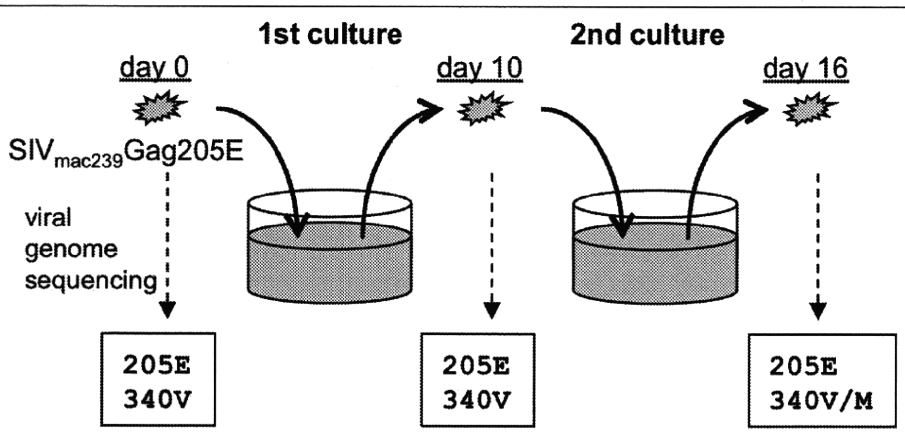

Figure 3 Passage of SIVmac239Gag205E culture supernatants. HSC-F cells were infected with SIVmac239Gag205E. The culture supernatant on day 10 was added to fresh HSC-F cells to start the second culture. Viral RNAs were extracted from the first culture supernatant on day 10 and the second culture supernatant on day 16 after the initial infection and subjected to sequence analyses. Dominant amino acid at the 340th residue remained V on day 10 in all cases but was M on day 16 in two of four sets of experiments (Gag340M was detectable on day 10 in these two sets of experiments). No other amino acid change was observed in the CA-coding region.

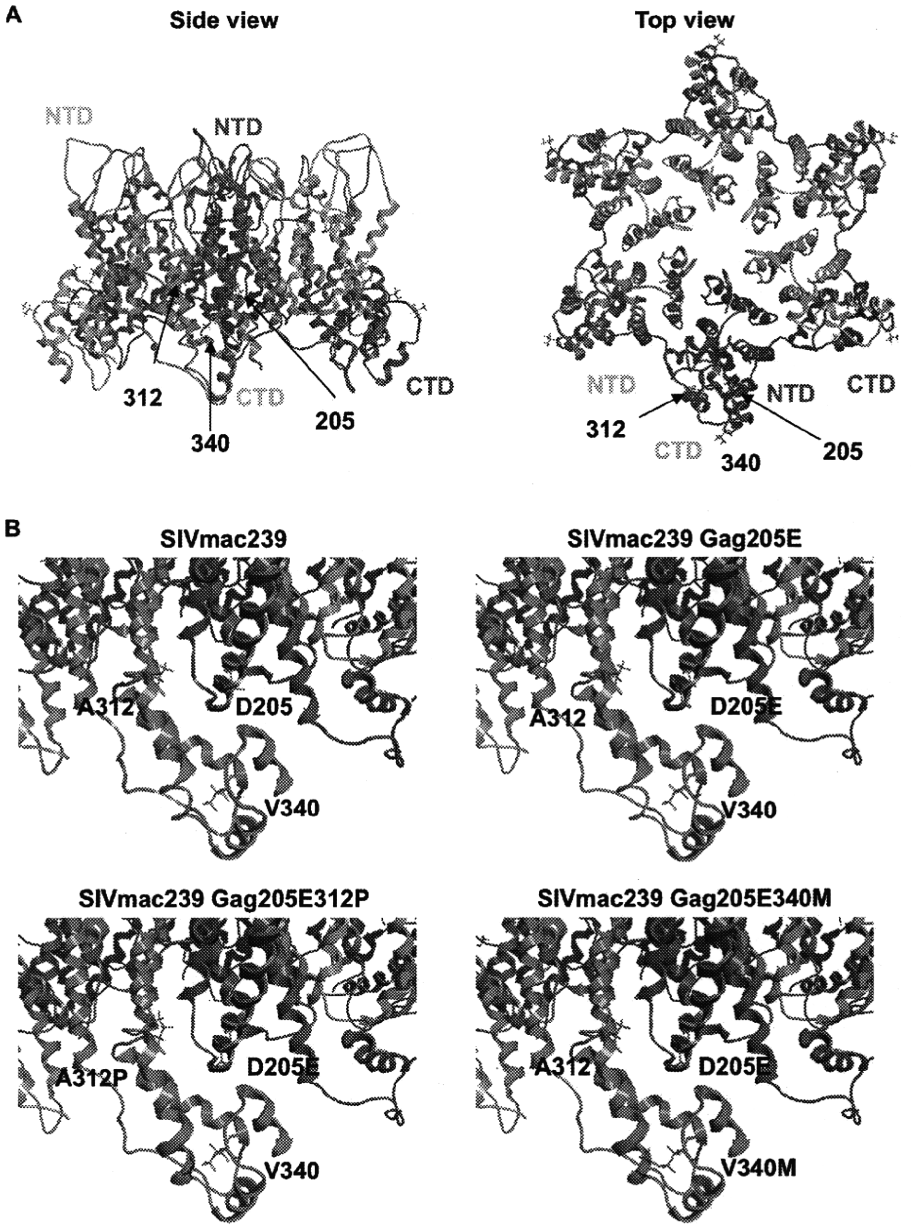

Figure 4 Structural models of SIVmac239 CA hexamer. The hexameric SIVmac239 CA models were constructed by homology-modeling using a crystal structure of the hexameric HIV-1 CA at a resolution of 1.90 Å (PDB code: 3H47[33]) as a modeling template. "MOE-Align" and "MOE-Homology" in MOE version 2008.1002 were used for the modeling. The side chains of the 205th, 312th, and 340th aa in Gag are shown as orange sticks. (A) Overall structure of SIVmac239 CA hexamer. (B) The hexameric structures near positions 205, 312, and 340 of wild-type and mutant SIVmac239 CAs.

Discussion

The Gag CA which is one of the most conserved proteins in HIV and SIV may be a promising immunogen for CTL-based AIDS vaccines. However, the limitations imposed on amino acid sequences in CA are not fully understood. In the present study, we found that the GagD205E substitution in SIVmac239 CA NTD reduces viral fitness, which is recovered by additional GagA312P or GagV340M substitution in the CTD. SIVmac239-Gag205E passaged in cell culture often resulted in selection of an additional GagV340M mutation. Furthermore, selection of Gag205E plus Gag340M mutations, but not Gag205E alone, was observed in a chronically SIVmac239-infected rhesus macaques. These results provide evidence indicating a functional interaction between Gag residues 205 in CA NTD and 340 in CA CTD,

Table 1 Competition between SIV mutants[a]

SIVs in competition	Ratio of inoc. titers[b]	Exp. no.	Dominant aa sequences[c]			
			day 6		day 18	
SIVmac239 & SIVmac239Gag205E	4:1	#1	205D		205D	
		#2	205D		205D	
	1:1	#1	205D		205D	
		#2	205D		205D	
	1:4	#1	205D		205D	
		#2	205D		205D	
SIVmac239 & SIVmac239Gag205E312P	4:1	#1	205D	312A	205D	312A
		#2	205D	312A	205D	312A
	1:1	#1	205D	312A	205D	312A
		#2	205D	312A	205D	312A
	1:4	#1	205D	312A	205D	312A
		#2	205D	312A	205D	312A
SIVmac239 & SIVmac239Gag205E340M	4:1	#1	205D	340V	205D	340V
		#2	205D	340V	205D	340V
	1:1	#1	205D/E	340V/M	205E	340M
		#2	205D/E	340V/M	205E	340M
	1:4	#1	205E	340M	205E	340M
		#2	205E	340M	205E	340M
SIVmac239Gag205E & SIVmac239Gag205E312P	4:1	#1	205E	312P	205E	312P
		#2	205E	312P	205E	312P
	1:1	#1	205E	312P	205E	312P
		#2	205E	312P	205E	312P
	1:4	#1	205E	312P	205E	312P
		#2	205E	312P	205E	312P
SIVmac239Gag205E & SIVmac239Gag205E340M	4:1	#1	205E	340M	205E	340M
		#2	205E	340M	205E	340M
	1:1	#1	205E	340M	205E	340M
		#2	205E	340M	205E	340M
	1:4	#1	205E	340M	205E	340M
		#2	205E	340M	205E	340M

[a]HSC-F cells were coinfected with two kinds of SIVs indicated. Viral *gag* fragments were amplified by RT-PCR from viral RNAs from the culture supernatants on days 6 and 18 postinfection and then sequenced. Results from two sets of experiments (Exp. #1 and #2) are shown.

[b]The ratio of the dose (RT activity) of the virus indicated at the top to that at the bottom at coinfection.

[c]Dominant amino acid sequences at the positions where mutations were included in the inoculums are shown. 205D/E, D and E were detected equally at the 205th aa in Gag; 340 V/M, V and M were detected equally at the 340th aa in Gag.

presenting a structural constraint for functional interaction between SIV CA NTD and CTD.

HIV and SIV Gag proteins are expressed as unprocessed polyproteins, which are assembled and incorporated into the virions. Concomitant with viral budding, incorporated Gag polyproteins are proteolytically cleaved by viral protease into processed proteins including MA (matrix), CA, and NC (nucleocapsid), participating in mature infectious virion formation [37,38]. Recent structural analyses [31-33,39-41] indicated that CA proteins form hexamer lattice in matured virions; in the mature CA core, the intermolecular NTD-NTD and NTD-CTD interfaces are involved in the formation of CA hexamers, while the intermolecular CTD-CTD interface connects neighboring hexamers. Our modeling analyses did not support a possibility of intramolecular interaction but indicated possible intermolecular interaction between Gag205 in CA NTD and Gag312/340 in CA CTD, which may affect CA hexamer formation during viral maturation. This is consistent with our results in Figure 5 indicating that the GagD205E substitution results in inhibition of the early phase of SIVmac239 replication, which can be overcome by additional GagA312P or GagV340M substitution. This possibility is supported also by our results on viral core stability in vitro, although it remains unclear how much extent the

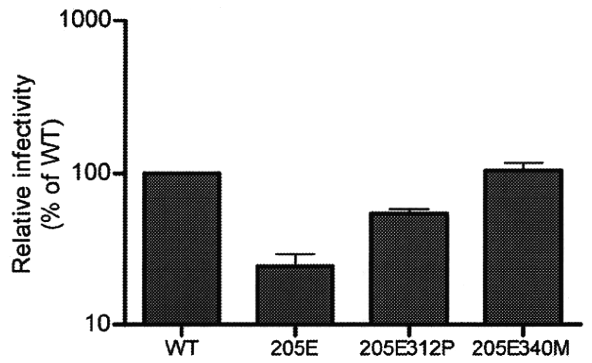

Figure 5 Mutant SIV infection. LuSIV cells were infected with SIVmac239 (WT), SIVmac239Gag205E (205E), SIVmac239Gag205E312P (205E312P), or SIVmac239Gag205E340M (205E340M). Luciferase activity was measured 24 hr after infection. Relative infectivity is shown as the ratio (%) of the luciferase activity to that of SIVmac239 (WT). Mean values in three sets of experiments are shown.

core stability in vitro can reflect the one in vivo [42]. There has been no report suggesting the influence of the Gag 205 residue on SIV sensitivity to tripartite interaction motif 5α (TRIM5α). A previous report on HIV CA lattice [31,43] indicated a potential interaction between the helix 4 of NTD and the loop connecting helices 10 and 11 of CTD in the adjacent molecule. Our results suggest the possible involvement of Gag205 and Gag340 residues in this intermolecular NTD-CTD interaction in CA hexamers.

The molecular model of CA hexamers incorporating the GagD205E substitution suggested shortening of the distance between Gag205 and Gag340 residues, which looked to be compensated by GagV340M substitution (Figure 4). The modeling can draw a hydrophobic pocket between Gag205 and Gag340 residues in

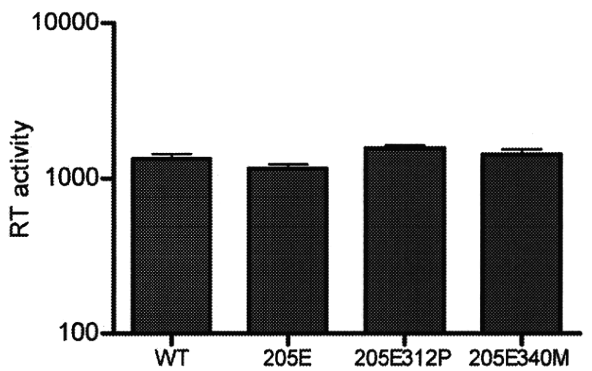

Figure 6 Mutant SIV production. COS-1 cells were transfected with molecular clone DNAs of SIVmac239 (WT), SIVmac239Gag205E (205E), SIVmac239Gag205E312P (205E312P), or SIVmac239Gag205E340 M (205E340 M). RT activity of the culture supernatants two days after transfection was measured. Mean values in five sets of experiments are shown.

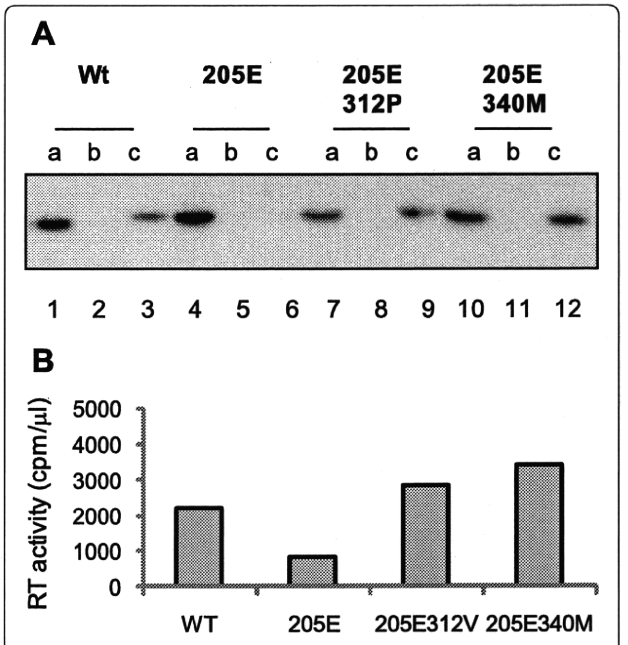

Figure 7 SIV core stability in vitro. Concentrated SIVmac239 (Wt; lanes 1-3), SIVmac239Gag205E (205E; lanes 4-6), SIVmac239Gag205E312P (205E312P; lanes 7-9), or SIVmac239Gag205E340M (205E340 M; lanes 10-12) was separated into three fractions (top [a], middle [b], and bottom [c]) by ultracentrifugation under gradient sucrose concentrations in the presence of 0.6% Triton X-100. Each fraction was subjected to Western blot analysis to detect SIV CA p27 proteins (A). A representative result from three sets of experiments is shown. The bottom (c) fractions were also subjected to RT assay (B).

SIVmac239Gag205E340M as well as SIVmac239, but not in SIVmac239Gag205E CA hexamers. Thus, this pocket may be a target candidate for anti-viral drugs.

Both GagL216S and GagD205E mutations can result in escape from $Gag_{206-216}$-specific CTL recognition [19,28], but the former is usually selected in SIVmac239-infected *90-120-Ia*-positive macaques probably

Table 2 Viral gag sequences in macaque R01-007 infected with SIVmac239[a]

Wks after challenge	Amino acid sequences[b]		
	at 205th	at 216th	at 340th
123	D	S	V
137	D (E)	S (L)	V (M)
150	E	L	M

[a]Viral RNAs were extracted from plasma obtained from a 90-120-Ia-positive macaque R01-007 at weeks 123, 137, and 150 after SIVmac239 challenge. Viral *gag* fragments were amplified by RT-PCR from viral RNAs and then sequenced. This animal showed efficient $Gag_{206-216}$-specific CTL responses and vaccine-based control of a SIVmac239 challenge with rapid selection of the GagL216S escape mutation (at week 5), but accumulated viral mutations in the chronic phase, leading to reappearance of plasma viremia around week 60 after challenge as described previously [19,35].

[b]Dominant amino acid sequences at the 205th, 216th, and 340th aa in Gag are shown. Parentheses indicate the sequences that are not dominant but detectable.

because the latter reduces viral fitness more severely than the former. In this study, we found selection of GagD205E plus GagV340M mutations in the chronic phase of SIVmac239 infection in a *90-120-Ia*-positive macaque. In this animal, the CTL escape GagL216S mutation first selected after SIVmac239 challenge became undetectable and was replaced with the CTL escape GagD205E mutation in combination with GagV340M in the chronic phase. This may imply that the GagD205E plus GagV340M mutations might be more advantageous than the GagL216S mutation for SIVmac239 replication in the presence of $Gag_{206-216}$-specific CTL pressure.

We observed the addition of GagV340M mutation but not a Gag205E-to-Gag205D reversion in SIVmac239-Gag205E passage. This may be due to difference in frequencies between purine-to-purine (guanine-to-adenine) change in the former and purine-to-pyrimidine (adenine-to-thymine) change in the latter. The appearance of additional GagV340M mutation in SIVmac239-Gag205E passaged in cell culture as well as the selection of GagD205E plus GagV340M mutations in an animal provides key evidence indicating functional interaction between Gag residues 205 in CA NTD and 340 in CA CTD. The Gag is a promising candidate as a vaccine immunogen for CTL induction, because cumulative studies have indicated the efficacy of Gag-specific CTL responses against HIV and SIV infection [7,25,44,45]. However, viral mutational escape from CTL recognition is a major challenge for AIDS vaccine design. Thus, the information on the structural constraint presented in this study might be helpful for immunogen design in AIDS vaccine development.

Conclusions

Our results present in vitro and in vivo evidence implicating the interaction between Gag residues 205 in CA NTD and 340 in CA CTD in SIV replication. SIV CA with Gag205D-340V (observed in SIVmac239) or Gag205E-340M combination (observed in SIVsmE543-3) is functional whereas the CA with Gag205E-340V is less functional. Thus, the present study indicates a structural constraint for functional interaction between SIV CA NTD and CTD, providing valuable information for immunogen design to limit viral escape options.

Methods

Analysis of mutant SIV replication

SIV molecular clone DNAs with gag mutations were constructed by site-directed mutagenesis from the wild-type SIVmac239 molecular clone DNA [24]. Virus stocks were obtained by transfection of COS-1 cells with wild-type or mutant SIV molecular clone DNAs using Lipofectamine LTX PLUS (Invitrogen, Tokyo, Japan). Viral titers were measured by reverse transcription (RT) assay as described previously [46]. For analysis of viral replication kinetics, HSC-F cells (herpesvirus saimiri-immortalized macaque T-cell line) [47] were infected with wild-type or mutant SIVs (normalized by RT activity), and virus production was monitored by measuring RT activity in the culture supernatants. To examine viral infectivity, LuSIV cells, which are derived from CEMx174 cells and contain a luciferase indicator gene under the control of the SIVmac239 long terminal repeat, were cultured for 24 hr after viral infection and then lysed in a reporter lysis buffer (Promega Corp., Tokyo, Japan) for measurement of the luciferase activity in a luminometer (GloMax™ 96 Microplate Luminometer, Promega Corp.).

Viral competition assay

HSC-F cells were coinfected with two SIVs at a ratio of 1:1 or 1:4, and the culture supernatants harvested every other day were used for RT assays. On day 6, the supernatant was added to fresh HSC-F cells to start the second culture. Similarly, on day 12 after the initial coinfection, the second culture supernatant was added to fresh HSC-F cells to start the third culture. RNAs were extracted using the High Pure viral RNA kit (Roche Diagnostics, Tokyo, Japan) from the initial culture supernatant on day 6 and from the third culture supernatant on day 18 post-coinfection. The fragment (nucleotides 1231 to 2958 in SIVmac239 [GenBank accession number M33262]) containing the entire gag region was amplified from the RNA by RT-PCR and sequenced to determine dominant sequences as described previously [19].

Molecular modeling of hexameric SIVmac239 CA

The crystal structures of HIV-1 CA NTD at a resolution of 2.00 Å (PDB code: 1M9C[48]), HIV-1 CA CTD at a resolution of 1.70 Å (PDB code: 1A8O[5]), and hexameric HIV-1 CA at a resolution of 1.90 Å (PDB code: 3H47 [33]) were taken from the RCSB Protein Data Bank [49]. Three-dimensional (3-D) models of monomeric SIVmac239 CA were constructed by the homology modeling technique using 'MOE-Align' and 'MOE-Homology' in the Molecular Operating Environment (MOE) version 2008.1002 (Chemical Computing Group Inc., Quebec, Canada) as described [50,51]. We obtained 25 intermediate models per one homology modeling in MOE, and selected the 3-D models which were the intermediate models with best scores according to the generalized Born/volume integral methodology [52]. The final 3-D models were thermodynamically optimized by energy minimization using an AMBER99 force field [53] combined with the generalized Born model of aqueous solvation implemented in MOE [54]. Physically unacceptable

local structures of the optimized 3-D models were further refined on the basis of evaluation by the Ramachandran plot using MOE. The structures of hexameric SIVmac239 CA were generated from the monomeric structures by MOE on the basis of the assembly information of hexameric HIV-1 CA crystal structure [33].

Analysis of viral CA core stability in vitro

Detergent treatment of wild-type and mutant SIV particles was performed essentially as described previously [34]. Briefly, viruses from COS-1 cells transfected with viral molecular clone DNAs (normalized by RT activity) were concentrated by ultracentrifugation at 35,000 × rpm for 75 min at 4°C in a SW41 rotor (Beckman Instruments, Tokyo, Japan) through a cushion of 20% sucrose in phosphate buffered saline (PBS). The concentrated viral pellets were suspended in PBS. Sucrose step gradients were prepared in SW55 centrifuge tubes with the 2.0 ml layer of 60% sucrose on the bottom and 2.1 ml layer of 20% sucrose overlaid. Then, 0.1 ml of Triton X-100 in PBS and 0.5 ml of concentrated viruses were overlaid and ultracentrifuged at 35,000 × rpm for 60 min at 4°C in a SW55Ti rotor (Beckman Instruments). Three fractions (top [a], middle [b], and bottom [c]) of 1.1 ml each were collected from the top and subjected to Western blot analysis using plasma from a simian-human immunodeficiency virus 89.6PD-infected rhesus macaque [55] and RT assay.

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Authors' contributions

NI and TM designed the study. NI, HT, and AR performed virological analyses in vitro. MY and HS performed structure modeling analyses. HY and MK examined viral genome sequences. NI and TM analyzed the data and wrote the paper. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Mammalian microRNAs: post-transcriptional gene regulation in RNA virus infection and therapeutic applications

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RNA silencing mediated by microRNAs (miRNAs) is a recently discovered gene regulatory mechanism involved in various aspects of biology, such as development, cell differentiation and proliferation, and innate immunity against viral infections. miRNAs, which are a class of small (21–25 nucleotides) RNAs, target messenger RNA (mRNA) through incomplete base-pairing with their target sequences resulting in mRNA degradation or translational repression. Although studies of miRNAs have led to numerous sensational discoveries in biology, many fundamental questions about their expression and function still remain. In this review, we discuss the dynamics of the mammalian miRNA machinery and the biological function of miRNAs, focusing on RNA viruses and the various therapeutic applications of miRNAs against viral infections.

Keywords: mammalian microRNAs, RNA virus infection, host–virus interaction, gene therapy

INTRODUCTION

The human genome is about 3,300 Mb in size and comprises 1% exons and 24% introns (the remainder being transposons (45%) and repetitive sequences). It is assumed that less than 40,000 protein-coding genes are present (Lewin, 2004), so why do we have such a large genome containing so many non-coding regions? Historically, the idea that RNA regulates the gene expression profile of each cell was proposed by Britten and Davidson (1969). This hypothesis was realized in the 1990s, especially after the first key finding (in *Caenorhabditis elegans*) that double-stranded RNA is cleaved by the ribonuclease (RNase), Dicer, into small, ~22 nucleotide (nt) RNAs, and induces gene silencing (RNA silencing) (Fire et al., 1998). Subsequent extensive studies and computer-based bioinformatics approaches shed light on "the world of small RNAs", which consist of small RNAs (~20–30 nt) with a variable origin, including exogenous and endogenous short interfering RNAs (siRNA), Piwi (P-element-induced whimpy testes)-interacting (pi)RNAs and microRNAs (miRNAs) (see review in Zamore and Haley, 2005; Choudhuri, 2009).

The post-transcriptional gene silencing (PTGS) mechanism mediated by these small RNAs plays a fundamental role in development, differentiation, proliferation, and transposon mobility (Bartel, 2004; Zamore and Haley, 2005; Berkhout and Jeang, 2007). Currently, nearly 1,000 mature human miRNAs are registered in the miRNA data base (http://www.mirbase.org/). Although our knowledge of small RNAs is expanding rapidly, the expression profiles and function of many of these miRNAs remains unclear. In this expanding field of science, it seems impossible to cover all the aspects of miRNA research and there are already numerous reviews of miRNA focusing, for example, on the immune system (Baltimore et al., 2008; Lodish et al., 2008; Xiao and Rajewsky, 2009; O'Connell et al., 2010), cancer (Croce, 2009), and virus-encoded miRNAs (Gottwein and Cullen, 2008; Boss et al., 2009; Cullen, 2009). Here, we discuss recent topics regarding miRNAs (mostly human) with a focus on RNA virus infection and the development of novel technologies that take advantage of the particular characteristics of miRNA for therapeutic purposes.

BASIC KNOWLEDGE OF MAMMALIAN miRNA

STRUCTURE OF miRNA PROCESSING MACHINERY

At present, siRNAs, which are exogenously induced double-stranded or short hairpin RNAs, are utilized as an essential tool for gene-specific silencing. In contrast, miRNAs play an important role in both post-transcriptional and translational regulation (Bartel, 2004). To obtain insights into the molecular mechanisms underlying RNA silencing pathways, the three-dimensional structures of proteins participating in miRNA biosynthesis have been deduced (see review in Jinek and Doudna, 2009). The protein structure of the human miRNA processing machinery is illustrated in **Figure 1**.

Drosha, a nuclear RNaseIII enzyme, has two RNaseIII catalytic sites with a double strand RNA binding domain (dsRBD) at the C terminus (Lee, 2003) and a proline-rich domain and arginine/serine-rich domains at the N terminus. DGCR8 has two dsRBDs and a WW domain containing two conserved tryptophan (W) residues. DGCR8 binds to the base of the long primary transcript (pri-)miRNA hairpin, positioning Drosha to cleave the pri-miRNA stem at a distance of 11 base pairs from the junction between the dsRNA stem and the flanking ssRNA regions (Han et al., 2006). The core region of human DGCR8 has been crystallized (Sohn et al., 2007).

Dicer, cytoplasmic RNaseIII, has ATPase and helicase domains at the N-terminus, followed by DUF283 (unknown function) and PAZ (PIWI/Argonaute/Zwille) domains, in addition to two RNaseIII domains and one dsRBD (Bernstein et al., 2001; Jinek and Doudna, 2009). The PAZ domain binds specifically to the 3′ end of single-stranded RNA. The crystal structure of *Giardia* Dicer suggests that it functions as a molecular ruler by anchoring the 3′ dinucleotide of the dsRNA bound to the PAZ domain, cleaving it at

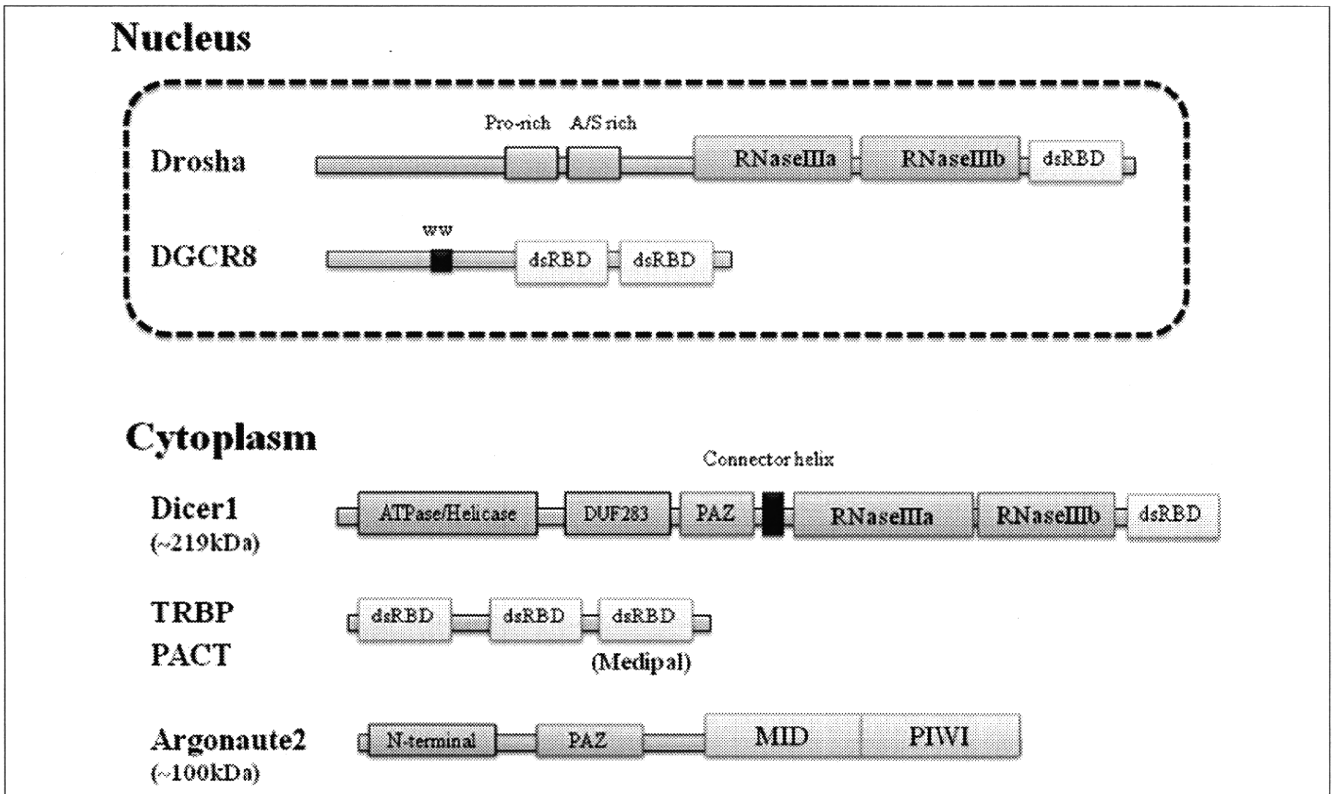

FIGURE 1 | Structure of the human miRNA processing machinery. The predicted structures of Drosha and DGCR8 in the nucleus are shown in the upper panel. Dicer1, TRBP, and PACT interact in the cytoplasm and recruit Argonaute2. TRBP and PACT have a similar structure with the three dsRBDs (green). Two RNase III domains (red) are common in Drosha and Dicer1. PAZ domain (blue) is common in Dicer1 and Argonaute2. In Argonaute2, MID domain provides a RNA binding pocket and PIWI domain has endonuclease activity (light blue) (see the text in detail).

a fixed distance from that end, and generating products of defined length (Macrae et al., 2006). A connector helix may be the main determinant of product size.

TAR RNA-binding protein (TRBP) and PACT have similar structures, with three dsRBDs that directly interact with each other and with Dicer (Kok et al., 2007). Interestingly, PACT is an activator of protein kinase R (PKR), whereas TRBP is an inhibitor. One dsRBD known as Medipal in TRBP is a protein–protein interaction domain that binds Merlin, Dicer, and PACT. The TRBP C-terminus of Madipal interacts with Dicer via its ATPase/Helicase domain (Daniels et al., 2009).

Like Dicer, the Argonaute protein also has a PAZ domain, which binds to the 3′ end of guide RNA (Wang et al., 2008). The MID domain provides a binding pocket for the 5′-phosphate of guide RNA and the PIWI domain adopts an RNaseH fold and has endonuclease activity (Boland et al., 2010). In humans, there are four genes encoding Argonaute and only one of these, Ago2, has endonuclease activity. The mechanism by which the Ago protein mediates translational repression remains controversial and may be different in different cell types (for details, see review in Peters and Meister, 2007).

BIOGENESIS OF miRNA

Although there are considerable variations in miRNA biogenesis and regulatory pathways (Winter et al., 2009), a representative pathway is shown in **Figure 2**.

Human miRNAs are present within the introns of coding genes and the introns and exons of non-coding transcripts (Berkhout and Jeang, 2007). pri-miRNA, which is transcribed by RNA polymerase II, forms a distinctive hairpin structure with a 5′-capped poly(A) tail. This characteristic hairpin structure is recognized by Drosha (Bartel, 2004). Drosha forms a complex with DGCR8 and cleaves pri-miRNA at the unpaired flanking regions, leaving a monophosphate group at the 5′ ends and a two-nt overhang at the 3′ ends (Han et al., 2006). The products of these processed pri-miRNAs are ~70-nt stem-loop RNA intermediates (pre-miRNA). The pre-miRNA is exported from the nucleus to the cytoplasm by exportin 5 in a Ran guanosine triphosphate-dependent manner (Lund et al., 2004), where it is processed by Dicer.

Human Dicer works together with its dsRNA-binding partner proteins, TRBP and PACT, to cleave pre-miRNA into ~22-nt long dsRNAs, though TRBP and PACT are not essential for cleavage (Haase et al., 2005; Lee et al., 2006). The complex formed by Dicer, dsRNA, TRBP, and PACT then recruits Argonaute to form the RNA-induced silencing complex (RISC) loading complex (Chendrimada et al., 2005). Finally, Argonaute delivers mature miRNA to the processing bodies (PBs) or stress granules (SGs), where the target messenger RNA (mRNA) is located (Peters and Meister, 2007).

Mature miRNA, carried by Argonaute, can destroy target mRNA with complementary sequences or repress protein synthesis by partially binding to the 3′ untranslated region (UTR) of target

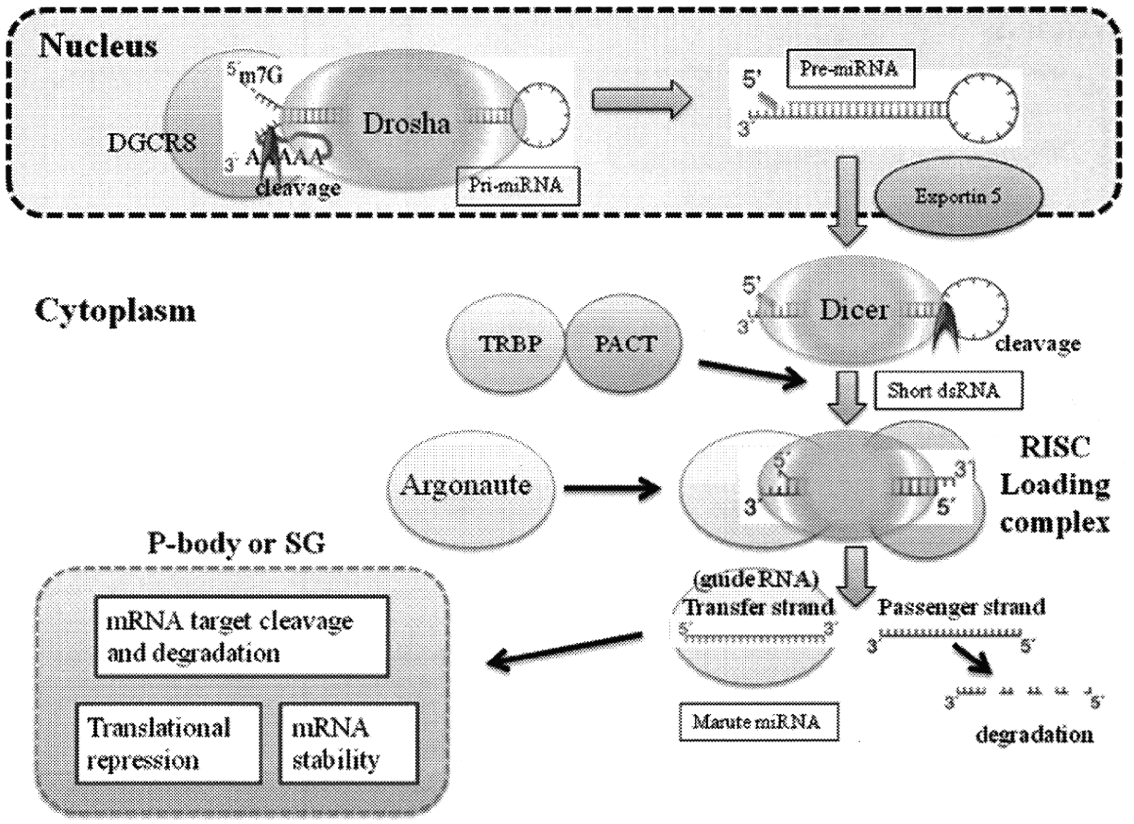

FIGURE 2 | Biogenesis of miRNA. Drosha recognizes the specific hairpin structure of pri-miRNA and, in the presence of the cofactor DGCR8, cleaves the un-stemmed ssRNA region of pri-miRNA to generate pre-mRNA. Pre-miRNA is exported by exportin 5 to the cytoplasm, where Dicer recognizes the hairpin structure of dsRNA and cleaves, and short deRNA is generated. TRBP and PACT bind to Dicer, and Argonaute is recruited to form the RISC loading complex. Mature miRNA, complexed with Argonaute, is known as the transfer strand and is carried to the P-body or stress granules (SG), depending on the condition of the cell. The mRNA is rich in P-bodies, and either degradation or translational repression of target mRNA occurs. The red arrowheads in Pri- and Pre-miRNA indicate cleavage sites.

mRNA. Because seed sequences of only 7–8 nt are required to loosely base-pair with the target sequence, a single miRNA is estimated, on average, to broadly target ~200 species of RNA (Lewis et al., 2005; Bartel, 2009).

HIGH THROUGHPUT PROFILING ANALYSIS

Expression of miRNAs may change temporally, in a spatial and tissue- or cell-type-specific manner. Large scale cloning and expression analyses of mammalian miRNAs were carried out by Landgraf et al. (2007) and the expression profiles of distinct human and mouse mature miRNAs were characterized. Comparison of miRNA expression between the hematopoietic system and all other organ systems indicated that only five miRNAs are highly specific to cells of the hematopoietic lineage (miR-142, miR-144, miR-150, miR-155, and miR-223). It is worth noting that species-specificity is also demonstrated by the data; miR-150 and miR-155 are highly hematopoietic-specific in mice, but not in humans. They also observed that the miRNA expression profiles of different sorted mature T cell types were similar and clustered together, and that no striking differences in the miRNA expression profiles of the different myeloid sub-lineages existed, except in granulocyte/monocyte-specific miR-223 expression. Thus, exclusive expression of miRNA by particular tissues or cell types is rare.

To identify the functionally important targets of miRNA, Selbach et al. (2008) employed a novel isotope pulse-labeling method of proteins (pulsed stable isotype labeling with amino acids in cell culture, pSILAC) in combination with mass-spectrometry-based proteomics. They evaluated changes in production of ~5000 proteins associated with the overexpression of several miRNAs in HeLa cells, which included tissue-specific miRNAs not expressed in HeLa cells (miR-1 and miR-155) and those expressed ubiquitously (miR-16, miR-30a, and let-7b). This pSILAC technology was quite useful in identifying functionally important targets of miRNAs at the protein level. They demonstrated that a single miRNA can directly downregulate the production of hundreds of proteins, and identified the seed sequence in the 3′ UTR as the primary motif for miRNA-mediated regulation of protein synthesis, probably acting via both mRNA degradation and translational repression. Notably, the repressive effect on individual proteins was relatively small, which may reflect the paucity (less than 3) of seed sites for each miRNA in individual target 3′ UTRs. Importantly, the results obtained from miRNA overexpression systems are equivalent to those obtained from knock down systems, confirming that their conclusions were not experimentally biased.

Baek et al. (2008) demonstrated the proteomic impact of overexpressed miRNAs (miR-1, miR-181, and miR-124) using the same SILAC technology. They also analyzed the relationship between

seed sequence matches and protein repression in HeLa cells and found that mRNAs with single 7-mer or 8-mer site matches, but not 6-mers, were significantly downregulated by miRNA. The seed sites within the 3′ UTRs were generally more effective than those in coding regions. Again, they demonstrated that the targeting principles elucidated from overexpressed exogenous miRNAs also apply to endogenous miRNA targeting at the level of protein downregulation, as argued by Selbach et al. (2008). We assume that coordinated post-transcriptional gene regulation by miRNAs contributes to the fine tuning of protein expression by cells in a tissue-, lineage-, differentiation-, proliferation-, or cytokine signal-specific manner. Accumulating knowledge about miRNA expression profiles and their biological functions will be used to build sophisticated bioinformatics programs to clarify various aspects of miRNA biology. Such computer-based tools for predicting the target genes of miRNAs are available at http://www.targetscan.org/, http://www.microrna.org/ and other sites (Bartel, 2009), and will prove helpful for the identification of targets with physiological relevance.

VIRUS–HOST INTERACTIONS AND miRNA

Recently, miRNAs were broadly implicated in viral infection of mammalian cells, having either positive, or negative effects on virus replication and host immune responses. Herpes-family viruses, which have a large DNA genome, are known to encode viral miRNA (v-miRNA), probably for the regulation of their own replication and latency and to control cellular defense mechanisms. However, the existence of v-miRNA in smaller DNA and RNA viruses remains controversial. It is argued that small viruses have size constraints that mean a greater proportion of their genome is required for coding purposes and is, therefore, less likely to retain non-coding RNAs (Berkhout and Jeang, 2007). In contrast, long-term, latently infected viruses such as herpes viruses derive a greater survival benefit from utilizing the cellular miRNA machinery (Cullen, 2010).

Because DNA virus-encoded v-miRNAs have been reviewed in detail elsewhere (see review in Gottwein and Cullen, 2008; Boss et al., 2009; Cullen, 2009), we will focus on the interaction of cellular miRNAs with RNA viruses as a host defense mechanism against viral infection.

IMPLICATIONS OF miRNA CONTRIBUTIONS TO ANTIVIRAL ACTIVITY

In plants, worms and flies, RNA silencing suppresses the mobilization of endogenous retroviruses. Therefore, it is expected that the miRNA silencing machinery may be involved in defense against viral infections in vertebrates. Triboulet et al. (2007) demonstrated that, by silencing RNase III, Dicer, or Drosha, HIV-1 replication is inhibited in PBMCs from HIV-infected donors and in latently infected U1 cells in which HIV-1 is not efficiently transcribed through its promoter (the long terminal repeat, LTR) due to a Tat mutation. They analyzed the level of miRNA in Jurkat cells using microarray analysis and found that a polycistronic miRNA cluster, miR-17/92, was markedly decreased upon HIV-1 infection. Although miR-17/92 does not directly target the viral genome, the histone acetylase, PCAF, which is a cofactor for Tat transactivation, has four potential target sequences for miR-17-5p and 20a in its 3′ UTR. Triboulet et al. clearly demonstrated the relationship between HIV-1 replication and the expression of miRNA and PCAF/Tat. Thus, the miRNA biogenesis machinery contributes to host defense against HIV-1. However, additional cellular miRNAs, or other inhibitory mechanisms, may also be involved in virus–host interactions.

Mammals have a single Dicer gene, Dicer1. Inactivation of Dicer1 in mice causes embryonic lethality or severe damage to tissue morphogenesis, cell differentiation and development (Bernstein et al., 2003). Otsuka et al. (2007) managed to produce such Dicer1-deficient mice expressing low levels of Dicer in some tissues and almost none in peritoneal macrophages. Using these mice, they demonstrated for the first time *in vivo* that miRNAs target the RNA genome of vesicular stomatitis virus (VSV). Interestingly, these Dicer1-deficient mice were susceptible to VSV, but not to other RNA viruses, including encephalomyocarditis virus (EMCV), lymphocytic choriomeningitis virus (LCMV), and Influenza A virus. Computer prediction models allowed them to select 24 candidate miRNAs to potentially target the positive-strand sequence of VSV. They demonstrated that miR-93 and miR-24 suppress VSV replication by targeting the P (polymerase cofactor)- and L (RNA-dependent RNA polymerase)-encoding regions of VSV, respectively.

CELLULAR miRNAs DIRECTLY TARGET VIRAL RNA

For the inhibition of virus replication, the seed sequences of miRNA must be complementary to the viral targets for silencing (Gottwein and Cullen, 2008). Although a number of computer-based programs that search for homology between human miRNAs and viral genomes can predict complimentarity to various regions of viral sequences, the effects of RNAi need to be demonstrated in infected cells. The following are examples of successful approaches used to identify cellular miRNAs that target viral RNA.

Lecellier et al. (2005) found no evidence of virus-derived siRNAs within primate foamy virus type 1 (PFV-1), which is a complex retrovirus closely related to HIV and encodes two accessory factors, Bet and Tas. Instead, by fusing viral fragments to the UTR of a GFP-tagged reporter gene, they found that the 3′ end fragment of the PVF-1 genome, which encodes Bet and Env/Bet proteins (and is also within the 3′ UTR), reduced GFP levels and was a target for miR-32. Thus, miR-32 exerts a direct, sequence-specific effect against PFV-1. Moreover, they demonstrated that the viral Tas protein interacts with cellular miRNAs and acts as a broadly effective silencing suppressor. Similar, virally coded, RNA silencing suppressor (RSS) activity was shown for HIV-1 Tat (Bennasser et al., 2005), vaccinia virus E3L, influenza A virus NS1 and Ebola virus VP35 proteins (Haasnoot et al., 2007).

Huang et al. (2007) inserted DNA fragments from the 3′ UTR of HIV-1 RNA into the 3′ UTR of the pEGFP vector and identified the region targeted by a potential miRNA-mediated mechanism in resting primary $CD4^+$ T cells. They found that miRNAs, such as miR-28, miR-125b, miR-150, miR-223, and miR-382, are abundant in resting T cells, but not in activated T cells, and the replication of latent viruses in patients on HAART was prevented by inhibiting these miRNAs. However, there are more than 100 cellular mRNA targets for each miRNA (Lewis et al., 2005) and the consequences of modulating the expression of these miRNAs may differ between T-cell subpopulations at distinct differentiation stages (see below).

Furthermore, because transfection of RNA or DNA into resting T cells is a very inefficient procedure, applying miRNA expression in a therapeutic context may be very difficult.

Using microarray technology, Pedersen et al. (2007) analyzed RNA derived from interferon (IFN)-stimulated cells and found that eight miRNAs induced by IFN had seed sequences that were 100% complementary with hepatitis C virus (HCV). Of these putative miRNAs, five (miR-196, miR-296, miR-351, mir-431, and miR-448) were able to attenuate HCV replication in Huh7 cells. miR-196 and miR-448 did not prevent the replication of HCV containing mutant target sequences, indicating that the antiviral effect is not induced by non-specific alterations in cellular gene expression. Furthermore, miR-122, which is essential for HCV replication (Jopling et al., 2005), was downregulated in response to IFN-β. Thus, these results suggest that the modulation of the expression levels of these miRNAs has an important role in the antiviral effects of IFN-β against HCV. However, a recent analysis of liver biopsy samples from chronic hepatitis C patients revealed no correlation between miR-122 expression and viral load (Sarasin-Filipowicz et al., 2009). Moreover, most of the miRNAs that mediate the effects of IFN-β on HCV replication were present only at very low levels, even after IFN treatment, arguing against a protective role for these miRNAs. Nevertheless, the finding that IFN-treatment significantly reduces miR-122 expression in non-responders, as opposed to complete responders, indicates the usefulness of miRNA profiling as a prognostic marker for IFN therapy.

ALTERED EXPRESSION OF CELLULAR miRNA UPON VIRAL INFECTION: PATHOGENICITY

The involvement of RNAi in influenza virus infection was identified by knocking down Dicer in Vero cells, which lack type I IFN genes (Matskevich and Moelling, 2007). Any acute virus infection may alter the expression pattern of both mRNA and miRNA in infected cells. Li et al. (2010) analyzed miRNA expression profiles in the lungs of mice infected with a highly pathogenic 1918 influenza A virus and compared them with those of mice infected with a non-lethal seasonal influenza A virus. By analyzing the inverse correlation between altered miRNA expression and the expression of predicted mRNA targets, they demonstrated that the target mRNAs of one altered miRNA, miR-200a, were associated with the type I IFN signaling pathway, which plays an important role in the pathogenesis in 1918 influenza A virus infection in mice (Kash et al., 2006). They also found that miR-223 indirectly downregulated CREB activity, which is required for the maintenance of cell survival and growth.

Another example can be found in HIV-1 infection. Cells of the monocyte/macrophage lineage are susceptible to HIV-1 infection; however, only macrophages can support massive virus production. This differentiation-dependent restriction mechanism has been attributed to the distinct expression of the APOBEC3 subfamily by monocytes (Peng et al., 2007). Recently, Wang et al. (2009) reported that high expression of miR-28, miR-150, miR-223, and miR-382 was correlated with low susceptibility of monocytes to HIV-1 infection. Although the target mRNAs of these miRNAs were not identified, the results suggest that monocyte differentiation and HIV-1 susceptibility are linked by a common set of miRNAs. In relation to monocyte/macrophage differentiation, the expression of Cyclin T1, required for transactivation by HIV-1 Tat, increases during macrophage differentiation and enhances HIV-1 replication within macrophages (Liou et al., 2002). A recent report indicates that miR-198-mediated repression of Cyclin T1 may contribute to HIV-1 replication during monocyte differentiation into macrophages (Sung and Rice, 2009).

These results indicate that RNA viruses utilize cellular miRNAs to manipulate the expression of cellular genes to enhance their own survival and expansion. The known interactions between cellular miRNA and RNA viruses are summarized in **Figure 3**.

EFFECTS OF miRNAs ON IMMUNE CELL DIFFERENTIATION AND VIRAL INFECTION

The conditional knockdown of Dicer in murine T or B lymphocytes causes abnormal lymphocyte differentiation (Cobb et al., 2005; Muljo et al., 2005). Antigen-induced immune cell differentiation is a key feature of host defense and elicits coordinated immune responses to protect the host from pathogens (Baltimore et al., 2008). For example, profound changes in gene expression occur during antigen-induced $CD8^+$ T cell differentiation in mice (Wu et al., 2007). Wu et al. identified seven miRNAs (miR-16, miR-21, miR-142-3, miR-142-5, miR-150, miR-15b, and let-7f) frequently expressed in all T cells and observed alterations in the miR-21 expression signature as cells differentiated from naïve to effector cells. We have also shown the existence of similar, but not identical, differentiation-associated miRNA expression profiles in human $CD8^+$ T lymphocytes. The function of these miRNAs requires further investigation.

Recent microarray analysis studies show that monocyte differentiation into dendritic cells is regulated and coordinated by miR-34a and miR-21 (Hashimi et al., 2009). JAG1 and WNT1 were identified as targets for these miRNAs using a bioinformatic target ranking system. Considering that monocyte-derived dendritic cells (MDDCs) are poor producers of HIV-1 compared with monocyte-derived macrophages (Tsunetsugu-Yokota, 2008), it is highly likely that miRNA expression associated with cell differentiation from monocytes to either macrophages or MDDCs may also regulate HIV replication in monocytes (Wang et al., 2009). Although such differentiation-associated miRNAs have any impact on virus replication within infected cells remains to be addressed, the results of extensive miRNA profiling analyses of viral infections both *in vitro* and *in vivo* will be highly informative for the diagnosis and treatment of viral infections in humans.

THERAPEUTIC APPLICATION OF miRNAs TO VIRAL INFECTIONS

Knowledge regarding the basic biology and function of mammalian miRNAs has increased greatly in recent times. The idea of utilizing RNA interference by miRNA for genetic manipulation is already being realized. Taking advantage of the tissue-specificity of miRNAs, Kelly et al. inserted the target sequences of muscle-specific miRNAs (miR-133a and miR-206) into the 3′ UTR of Coxsackievirus A21 (CVA21), a pathogenic picornavirus that causes lethal myositis and oncolysis in tumor-baring mice (Kelly et al., 2008). The result was tissue-specific attenuation that allowed the virus to replicate well in muscle cells without being pathogenic. Likewise, by incorporating target sites for the neuron-specific miR-124a into the 3′ end of

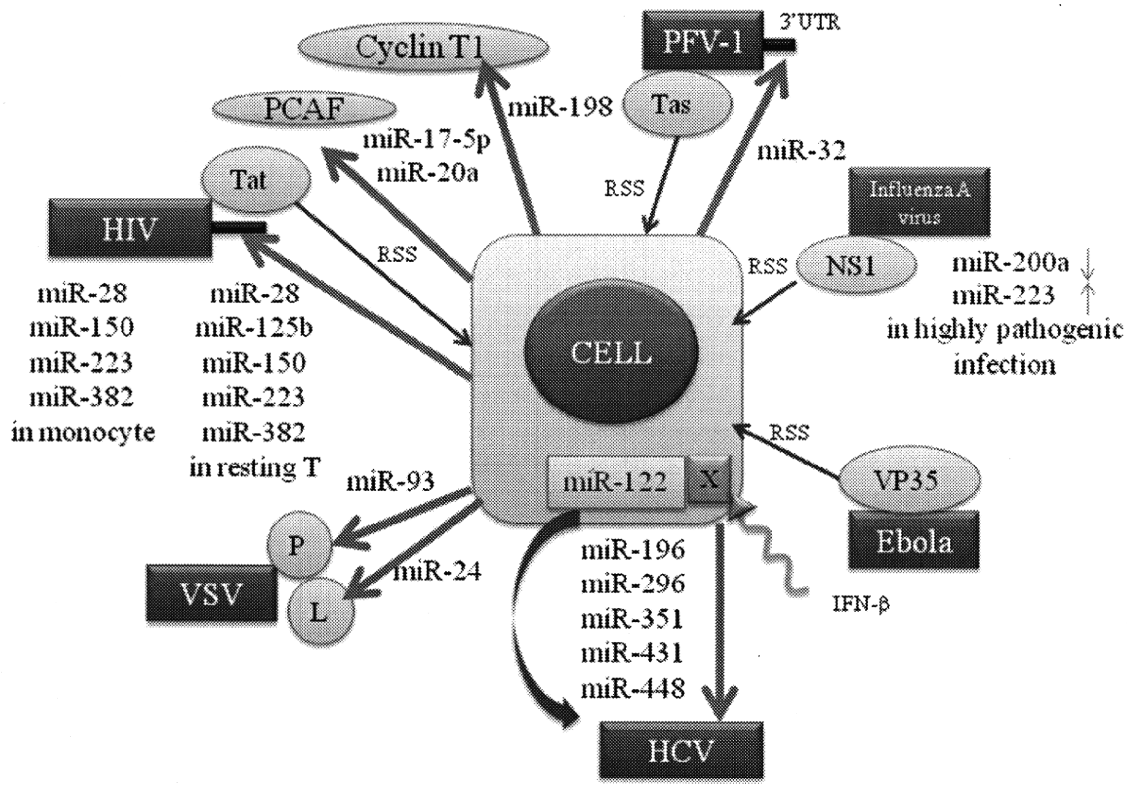

FIGURE 3 | Cellular miRNAs known to interact with RNA viruses. The direct and indirect interactions between cellular miRNA molecules or cellular proteins (blue) with the viral genome (black bar) or viral proteins (red) are depicted. Blue arrows from the cell indicate negative effect of miRNAs on the virus, and a dotted blue line indicates the indirect interaction (see the text in detail). The IFN-β treatment (an orange winding arrow) induces several miRNAs, whereas it downregulates (X) miR-122 essential for HCV replication (a red ribbon arrow). Red thin arrows indicate the RNA silencing suppressor (RSS) activity of viral proteins.

the 5′ UTR and between the structural and non-structural genes within the coding region of the poliovirus, an attenuated poliovirus vaccine lacking any neurovirulence was developed (Barnes et al., 2008). Similar engineering strategies were also applied to the VSV by inserting neurotropic miR-125 target sequences into the 3′ UTR of the viral polymerase gene (Kelly et al., 2010) and to adenoviruses by inserting a hepatocyte-selective miR-122 target site into the 3′ UTR of the E1A transcription cassette (Cawood et al., 2009). The structures of these attenuated viruses are illustrated in **Figure 4**. In this context, Perez et al. successfully attenuated the influenza A virus by incorporating species-specific, non-avian microRNA (miR-93) targets into nucleoproteins. The result was attenuated viral activity in mice, but not in eggs as expected (Perez et al., 2009). Thus, miRNA-mediated control of viral replication is a promising technology that can be used to develop safe attenuated viruses for cancer therapy and vaccines.

The RNAi-based gene delivery systems using lentivirus vectors have been developed for the control of HIV-1 infection, and such lentivirus can be utilized as potential AIDS vaccine candidates (Morris and Rossi, 2006; Rossi et al., 2007; Yamamoto and Tsunetsugu-Yokota, 2008). Likewise, considering the therapeutic applications of miRNA, efficient *in vivo* miRNA delivery systems by lentivirus vectors are a promising gene-transfer medium. However, because the VSV envelope glycoprotein used to encapsulate the transfer gene is pantropic, transgene expression in all cells could be problematic. To overcome the problem of non-specific gene transfer, Brown et al. (2007) systemically treated a mouse model of hemophilia B with a lentivirus vector expressing clotting factor IX (F.IX) under the control of a hepatocyte-specific promoter. Although F.IX is only expressed in the liver, an anti-F.IX immune response was elicited, probably due to off-target expression by the hepatocyte-specific promoter in hematopoietic cells. By inserting four tandem-repeat target sequences of the hematopoietic-specific miR-142-3p into the 3′ UTR of the transgene expression cassette, they were able to achieve sustained F.IX production in hemophilia B mice. In these mice, miR-142-3p levels were 50-fold higher in the spleen than in the liver. Importantly, the introduction of lentivirus containing miR-142-3p target sequences did not affect the normal *in vivo* level of miRNA, including that of miR-142-3p. Thus, by combining a highly cell lineage-specific promoter with PTGS using cell state- or lineage-specific miRNAs, the engineered lentivirus delivery system should make it possible to achieve specific expression of transgenes within the desired cells or tissues (Brown et al., 2007).

As described in the previous section (4.2), the abundantly expressed, liver-specific miRNA, miR-122, binds to two closely spaced target sites within the 5′ UTR of the HCV genome and is required to maintain high HCV RNA abundance in liver cells (Jopling et al., 2005, 2008). Interestingly, the same miR-122 binding site placed in the 3′ UTR of a reporter mRNA downregulated

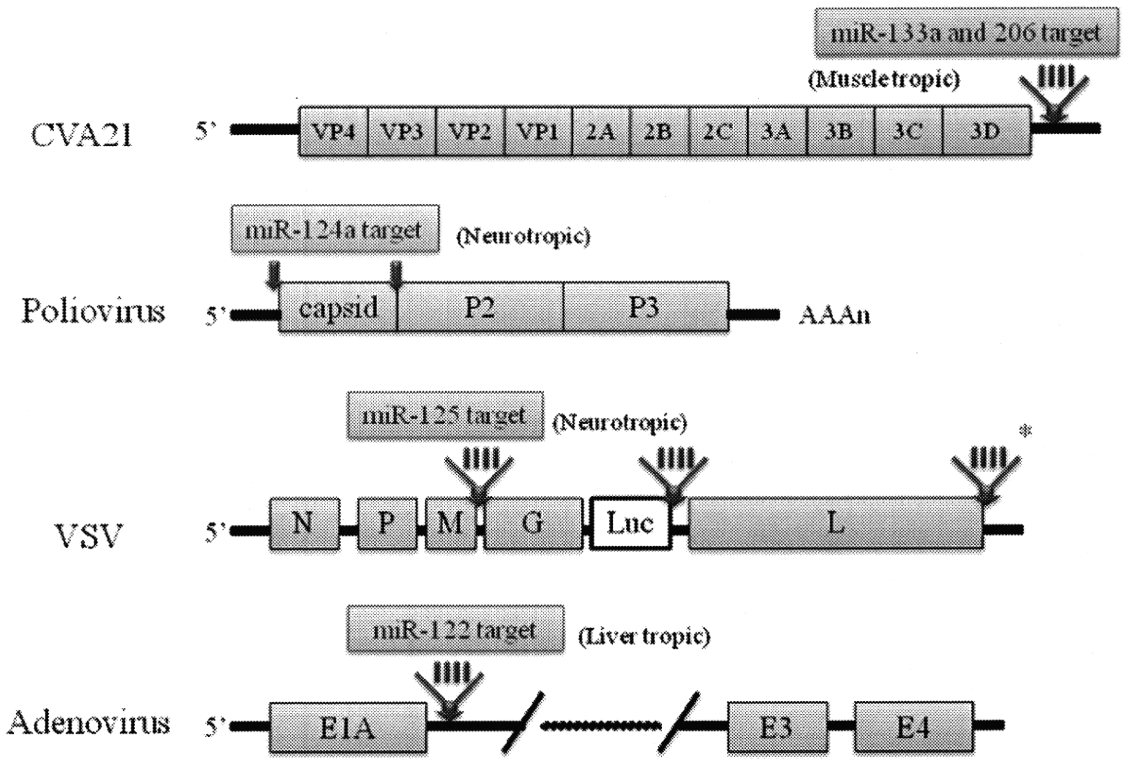

FIGURE 4 | Schematic genome structures of attenuated viruses. The localization of miRNA target insertion site in each virus is depicted (red arrow). In the case of CVA21, VSV and adenovirus, 4-tandem repeat target sequences (red bars) were inserted. Asterisk indicates the most effective site for attenuation of VSV.

mRNA expression, which clearly demonstrates location-dependent gene regulation by miRNA (Jopling et al., 2008). Based on these findings, Lanford et al. (2010) utilized a locked nucleic acid (LNA)-modified phosphorothioate oligonucleotide (SPC3649) complementary to miR-122 to treat chimpanzees chronically infected with HCV. Four animals (two each) received a high or low dose (5 or 1 mg/kg) of SPC3649 intravenously once a week for 12 weeks and were observed over a 17-week treatment-free period. They showed long-standing suppression of HCV viremia without obvious disease, side effects, virus mutation, or virus rebound, which are all consistent with potent and sustained suppression of miR-122 in the liver. Their results clearly show the feasibility and future potential of LNA drug therapy to antagonize specific miRNA functions.

All these studies indicate a promising future for antiviral therapy via the regulation of cellular miRNA expression pathways. By further understanding the biological functions of miRNAs and applying them for diagnostic and therapeutic purposes, we may yet achieve unexpected and exciting discoveries.

CONCLUSION

In the post-human genome era, our research efforts have shifted more toward the regulation of gene expression and to understanding the function of non-coding regions. Now, accumulating evidence indicates that the coordinated expression of distinct sets of miRNAs regulate many aspects of biological events occurring in cells, such as differentiation, cell-cycle progression, and proliferation, in a cell- or tissue-specific manner. We are just beginning to understand the physiological and pathological roles played by miRNAs in viral infections. We expect that, as we learn more about the targets of miRNAs and their regulatory function in cell physiology, we may be able to develop more sophisticated technologies to treat infectious diseases in humans.

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