Chapter 16

Utility of Potent Anti-viral MicroRNAs in Emerging Infectious Diseases

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Additional information is available at the end of the chapter

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Abstract

MicroRNAs (miRNAs) are small, noncoding RNA molecules that have emerged as important posttranscriptional regulators of gene expression. miRNA provides intracellular immune defense when the body is faced with challenges from transgenes, viruses, transposons, and aberrant mRNAs. miRNA molecules trigger gene silencing in eukaryotic cells. To date, more than 3,000 different human miRNAs (*hsa-miRs*) have been identified, and it is generally agreed that cellular gene regulation is significantly impacted by the presence of miRNAs. A single miRNA has the complex capacity to target multiple genes simultaneously. In a viral infection context, miRNAs have been connected with the interplay between host and pathogen, and occupy a major role in the host–parasite interaction and pathogenesis. While numerous viral miRNAs from DNA viruses have been identified, characterization of functional RNA virus-encoded miRNAs and their potential targets is still ongoing. Here, we describe an in silico approach to analyze the most recent Ebola virus (EBOV) genome sequences causing West African epidemics. We identified numerous "candidate" miRNAs that can be utilized to quell the Ebola virus. Future approaches will focus on experimental validation of these miRNAs during quelling the Ebola target transcripts for further elucidating their biological functions in primates and other animal models.

Keywords: Ebola virus, gene alignment, miRNA, prevention, vaccine

1. Introduction

1.1. Inhibition of Ebola virus by anti-Ebola miRNAs In silico

Since the HIV-1 pandemic of the 1980s and more recent outbreaks of bird flu, severe acute respiratory syndrome (SARS), and Middle East respiratory syndrome-Corona Virus (MERCoV), it has been widely believed that there would be new pandemics of highly pathogenic

viruses [1]. Fortunately, until recently, many of the new emerging pathogenic agents, such as Ebola virus (EBOV) and Marburg virus (MARV), have failed to demonstrate the transmissibility or animal reservoirs required to become true pandemic threats [2, 3]. Few, if any, antivirals can claim to be specific enough to halt the epidemic. Recently, a whole EBOV replication-defective vaccine—EBOVdVP30—has been found to be very effective in nonhuman primates, while two others are in Phase II trials [4].

The testing of the recently developed replication-defective recombinant chimpanzee adenovirus type 3–vectored ebolavirus (cAd3-EBO) vaccine is based on a demonstration of efficacy in a nonhuman primate model [5]. However, a curious finding has puzzled the investigators —preexisting neutralizing antibodies against cAd6 and cAd68 in human serum samples were found in ~40% of Ugandans and in 15% of the US and European volunteers. The increased prevalence of neutralizing antibodies against chimpanzee adenoviruses in sub-Saharan Africa may indicate cross-species transmission of these viruses from chimpanzees to humans. The possibility of fairly high levels of neutralizing antibodies against cAd3 may complicate the evaluation of the effectiveness of Ebola vaccines currently underway.

1.2. Virology of Ebola virus

Filoviruses are taxonomically classified within the order *Mononegavirales*, a large group of enveloped viruses whose genomes are composed of a nonsegmented, single-stranded minus [2, 3] RNA molecule. Following their discovery, filoviruses were originally grouped with rhabdoviruses, as the appearance of these virus particles appeared similar [3]. However, subsequent filamentous morphology and extensive genetic, physiochemical, and virologic studies of Marburg virus (MARV) and Ebola virus (EBOV) revealed distinctive characteristics, and these viruses were placed into a separate family, the *Filoviridae* [3]. Further characterization of these agents demonstrated that EBOV and MARV represent divergent lineages of filoviruses, and that their variances were significant enough to warrant the formation of the two genera, MARV and EBOV [4]. Subsequent to the International Committee on Taxonomy of Viruses recommendation, the MARV genus contains a single species, the Lake Victoria Marburg virus, as this strain exhibits only limited genetic variation.

EBOV is the causative agent of Ebola virus disease (EVD) [6]. The mortality rate can vary from 40% to 93% depending on the strains [6]. The viral life cycle begins with host cell entry through a mechanism that is still poorly understood. The incubation period for EVD is 2–21 days, and typical early symptoms include fever, chills, malaise, and myalgia (all of which could be misdiagnosed as malaria, which is highly prevalent in West African nations), followed by the onset of symptoms indicative of multi-organ stress and subsequent failure, sometimes followed by hemorrhagic episodes that can easily be misdiagnosed as Lassa fever.

EBOV is a negative, single-stranded RNA virus with an unusual, variable-length, filamentous, branched morphology whose helical capsid is enclosed inside a membrane. The mechanism of attachment and entry into the cell is still not completely defined (see discussion below). Once inside, the viral RNA polymerase (L protein) begins to copy the negative strand (–ve) RNA to make the positive strand (+ve) transcripts that mimic the structure of mRNA and are translated by host ribosomes. Replication is thought to occur in the cytoplasm. An unusual

http://dx.doi.org/10.5772/61687

feature of the transcription and translation of the Ebola genes is the fact that the glycoproteins (GP) are only expressed through transcriptional editing. The genome of the Zaire Ebola virus (EBOV), the most pathogenic among all species of EBOV, is 18,959 nucleotides (nts) in length and contains seven transcriptional units that guide synthesis of at least nine distinct primary translation products: the nucleoprotein (NP), virion protein (VP) 35, VP40, glycoprotein (GP), soluble glycoprotein (sGP), small soluble glycoprotein (ssGP), VP30, VP24, and the large (L) protein. L is the catalytic subunit of the viral polymerase complex (Figure 1). Similar to other nonsegmented negative-sense (NNS) RNA viruses, EBOVs encode a multiprotein complex to carry out replication and transcription. In the case of EBOV, viral RNA synthesis requires the viral NP, VP35, VP30, and L proteins. Transcription of filovirus mRNAs is presumed to occur as in other NNS viruses, where there is a gradient of viral mRNAs with the abundance of each mRNA transcript decreasing as the polymerase transcribes toward the 5′ end of the template [7]. Each EBOV mRNA is presumed to be efficiently modified with a 5′–7′-methylguanosine (m^7G) cap and a 3′p (A) tail [8].

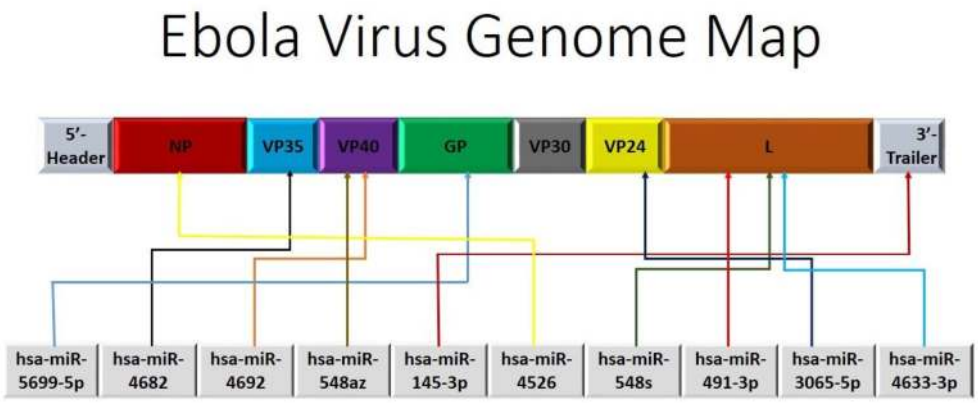

Figure 1. The genome of EBOV, 18 9 kb in length, has the following gene order: 3′ leader nucleoprotein (NP), virion proteins (VP) VP35-VP40, membrane glycoprotein (GP), viral polymerase (VP) VP30-VP24, viral polymerase L protein, and 5′ trailer.

The Ebola virus genus possesses greater diversity, and four viral species have been recognized: Zaire Ebola, Sudan Ebola, Reston Ebola, and Ivory Coast Ebola (EBOV-Z, EBOV-S, EBOV-R, and EBOV-IC, respectively). Each of the EBOV species has a different degree of pathogenicity and mortality rate [9]. Therefore, EBOV-S and EBOV-Z, which are the predominant EBOVs associated with known outbreaks, are more pathogenic than EBOV-R and EBOV-IC [10]. EBOV-IC has only caused a single nonfatal human infection, but EBOV-R has caused fatal infection in nonhuman primates [8]. However, EBOV-S, EBOV-Z, and EBOV-B often cause severe hemorrhagic diseases with markedly high case fatality rates (40–90%) [10]. The EBOV genome is 18.9 kb in length with the following gene order: 3′ leader nucleoprotein (NP), virion protein (VP) 35-VP40, glycoprotein (GP), VP30, VP24, polymerase (L), and 5′ trailer. The GP differences between any two species range from 37% to 41% at the nucleotide level and from

34% to 43% at the amino acid level [40]. However, variations within EBOV-Z species are very low (~2–3%) [11]. Thus, GP nucleotides are usually used in the phylogenetic analysis of EBOV (Figure 2).

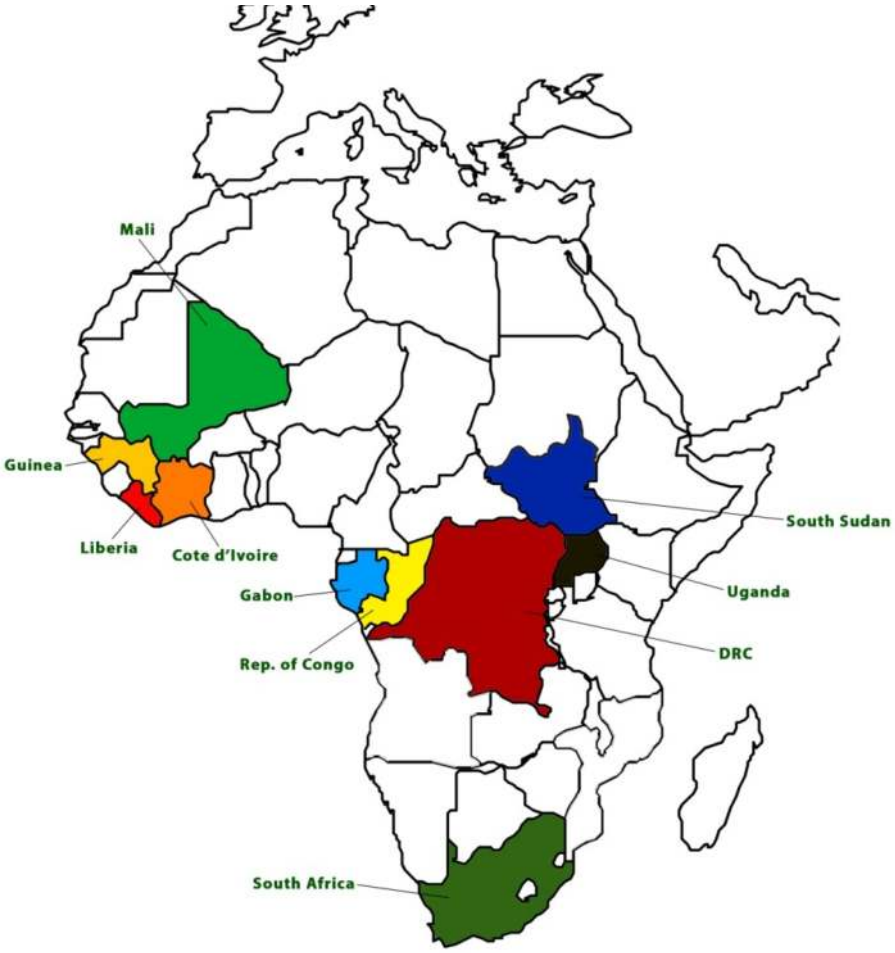

Figure 2. The Ebola Pandemic Map depicts the history of Ebola in Africa. The sporadic cases of Ebola were common in the central African countries such as the DRC (988 cases with 767 fatalities), Uganda (606 cases with 283 fatalities), South Sudan (335 cases with 180 fatalities), Gabon (214 cases with 150 fatalities), the Republic of the Congo (248 cases with 210 fatalities), and South Africa (2 cases with 1 fatality).

Because of their high mortality rate, which can vary from 40% to 93%, and their potential for person-to-person transmission and lack of an approved vaccine or antiviral therapy, MARV and EBOV are classified as biosafety level 4 (BSL-4) viruses by World Health Organization [12, 13]

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1.3. History of MARV and EBOV

Viruses are obligate intracellular parasites and essentially rely on host cells for raw materials, replication, transcription, and translations of their genetic codes. Until a few years ago, we assumed that the major intracellular defenses against viral pathogens were interferons [13]. Since the discovery of RNA interference (RNAi) and miRNAs, we know that one of the fundamental functions of miRNAs is to prevent replication of foreign viruses by pre- and posttranscriptions and suppressions of viral expression [12]. Therefore, besides endogenous gene regulation, miRNAs are the primary intracellular immune defense system [13]. Viruses have also evolved to counter the antiviral effects of miRNAs by viral miRNAs (vmiRNAs).

Recently, Li and Chen [14] have conducted molecular epidemiologic analyses of presently extant Ebola viral genomes to ascertain their evolutionary viral history. Of considerable potential importance are interpretations derived from a dataset that is between 1,000 and 2,100 years old and includes four Ebola species (EBOV-Z, EBOV-S, EBOV-TF, EBOV-R) [15]. Logically, one could assume that over the past 2,000 years, humans have evolved countermeasures to the Ebola virus via innate, adaptive, and miRNA-based immunity. The identification in a human database of 71 miRNAs capable of potentially quelling EBOV strongly suggests that *Homo sapiens* already have developed primary intracellular defenses to quell EBOV infection [16]. This raises a question: Why have EBOVs been circulating for about 2,000 years, and yet they seem to have emerged only recently? The earliest known cases of Ebola date to the 1970s. One theory proposes that EBOV-Z experienced a recent genetic bottleneck [17]. Before Ebola viral strains were introduced to primates, they had already been circulating among small mammals, including bats, rodents, marsupials, shrews, and so on [16]. Although these bats and other animals were infected [15, 16], no evidence demonstrated that such infections were fatal to them [18]. This indicates that a natural balance had been achieved between the viruses' pathogenicity and the host's immune system, especially at the intracellular levels where miRNAs provide immunological protection [19]. This homeostasis, this balance, apparently was broken in 1900, when EBOV genetic diversity experienced a dramatic drop [16]. Accordingly, most lineages of the various EBOV species became extinct because of such influences as threatening human activities, climate change, and a steep decline in the number of animals to serve as a reservoir for viral replication. Probably due to altered patterns of positive selection in the glycoprotein (GP), which diversified substantially and was found to be part of fusion and receptor binding within cellular membranes, infection patterns through direct exposure were changing. Therefore, by about 1970, few lineages that possessed broader tropism and enhanced fitness had the capacity to infect primates via direct exposure [16]. Similar examples can be seen in the emergence of HIV-1, which appeared to have surfaced in the 1950s through a zoonotic event that involved common infections among chimpanzees (i.e., SIV) and then accidentally jumped to humans [16–18]. Due to the paucity of significant differences in EBOV genetic diversity since 1970, the decreased number of surviving viruses may have become the only circulating lineages in primates and viral reservoirs. EBOV-Z has the ability to traverse a long distance through bats, which serve as a migratory reservoir. Outbreaks with their epicenter in Congo have been caused by the EBOV-Z species [20–23].

Through analysis of miRNA numbers that demonstrate high homologies in seed sequences and that show high identity to EBOV species, we have deduced that the genetic variations at the GP may serve as a type of Achilles' heel. After all, only one miRNA showed identity to GP, while eight proved capable of blocking polymerase steps. This indicates that minor variations within the GP amino acid sequence could allow for viral entrance into host target cells in humans. The subsequent transcription of negative-stranded RNA viruses into positive RNA strands occurs amid a struggle to overcome the miRNAs with quelling potential that can halt this process. It is possible that at the time of exposure to EBOV, all of the protective miRNAs may not be present in the target cells, or may be present, but not in sufficient quantities to block early EBOV replication [24].

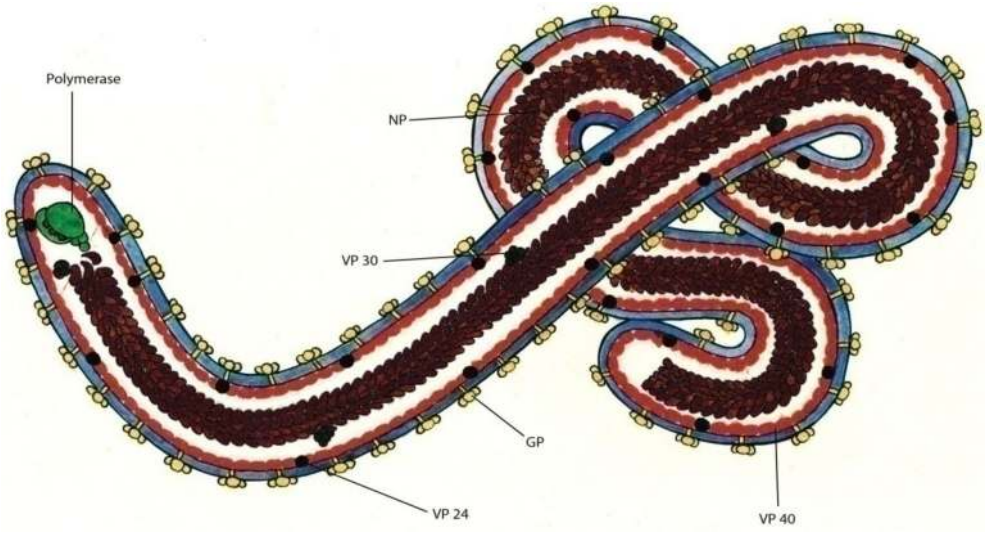

Figure 3. The illustration depicts a simplified structure of Ebola virus. The functions of various viral proteins are described in the text. GP, glycoprotein; NP, nucleoprotein; VP40, matrix protein; VP30, transcription factor; and polymerase enzyme.

Figure 3 shows the VP24, VP30, VP35, VP40, and L nucleoproteins that constitute the nucleocapsid, which is crucial in both the transcription and viral replication processes [25, 26]. The glycoprotein is located in the lipid membrane of the Ebola virus; this is also the place in the host target cells where receptors that facilitate viral entry are embedded [27]. Viral matrix proteins VP40 and VP24 are essential to viral budding, stability, and structure. VP40 is the primary matrix protein, and is the viral protein that is expressed most abundantly. It plays a central role in the process of Ebola budding from the plasma membrane. For example, in mammalian cells, the mere expression of VP40 is sufficient to create virus-like particles (VLPs) with morphological characteristics that are similar to those of the actual Ebola virus [28, 29]. Given VP40's absence, studies have found that the nucleocapsid was not transported effectively into the plasma membrane, and as this membrane is the site of assembly, budding, and

http://dx.doi.org/10.5772/61687

incorporation into the virions, considerable attention should be given to the role of this matrix protein [30]. The utilization of miRNAs that specifically target VP40 mRNA degradation is important to our understanding of just how VP40 functions and what potential roles it might play in the regulation of VLP assembly in both in vitro and live cell settings. *hsa-miR-4692* and *hsa-miR-548-az* effectively target VP40; therefore, the overexpression of these particular miRNAs within host cells could totally disrupt the viral life cycle and may have a decisive impact in the categorization of therapeutic targets (data unpublished). The tendency of Ebola VP40 to assemble virus-like particles (VLPs) presents an appealing model for analysis of the Ebola viral assembly at biosafety level 2 made possible by the noninfectious nature of genetically engineered VLPs [31].

VP40's association with the plasma membrane is of fundamental importance [30]; it is here that assembly is initiated as well as oligomerization [31], and nucleoprotein recruitment. Besides membrane association, VP40 also associates or otherwise interacts with host cell factors, including the endosomal sorting complex that supports transport (ESCRT) machinery [27, 29], the vesicle coat II proteins (COPII) [24], as well as the protein actin [25, 30]; these host cell factors, respectively, have been shown to enable VP40 budding, transport, and movement. Moreover, host cell protein kinases could contribute to Ebola infectivity as c-Abl1 can phosphorylate Tyr^{13} in VP40 [32, 33]. Still we have an inadequate understanding of how VP40 actually assembles on the plasma membrane before virion release occurs. Localization of VP40 in the plasma membrane is believed to be important as studies give evidence that hydrophobic residues located within the C-terminal domain, including Leu^{213}, are essential in the localization and budding processes [32]. Detection of VP40 oligomers in VLPs and UV-inactivated virions has occurred [34, 35]; they have been detected mainly in filamentous structures stemming from the plasma membrane [36]. Therefore, VP40 oligomerization apparently occurs on the same plasma membrane in which oligomers selectively have found to reside [37]. In terms of structure, VP40 has predominantly been found to oligomerize into either hexamers or octamers [38, 39]. These share a comparable monomer–monomer (or intradimeric) antiparallel interface. However, the detection of oligomeric structures in live cells suggests that these structures, too, could exert a critical influence on both viral assembly and egress [40]. We discovered that *hsa-miR-4692* and *hsa-miR-548-az* both target VP40 (data unpublished).

The formation of virus-like particles (VLPs) requires VP40 oligomers; these are associated with membranes that are resistant to detergent [41], which underscores the active part that the plasma membrane may play in VP40 oligomerization. Moreover, on the plasma membrane, matrix protein oligomerization may function as a scaffold in host protein recruitment, and also supply the force needed to effect the formation of virus particles and the deformation of membranes. A comprehension of VP40 plasma membrane association thus becomes crucial to our understanding of how the formation of protein buds occurs on the plasma membrane. Gupta K [42] recently investigated the role that the VP40 C-terminal domain plays in membrane association as well as in membrane penetration. These investigators utilized the monolayer penetration methodology to conduct in vitro research into the molecular basis of the penetration of the VP40 membrane. To study VP40 assembly and its associated egress in cells, they employed a multipronged methodology that blended cellular imaging, number and

brightness (N&B) analysis, analysis of the egress of virus-like particles, site-directed mutagenesis, and total internal reflection (TIRF) microscopy. N&B analysis permitted them to ascertain the average number of molecules and also the brightness within each pixel within a fluorescence microscopy image. This permitted them to detect the oligomeric status of proteins that are labeled fluorescently. They concluded that within the VP40 C-terminal domain, a hydrophobic interface actually penetrates the plasma membrane, which plays a key role in the oligomerization of VP40. The knocking out of plasma membrane penetration by hydrophobic mutants also substantially reduces the egress of VLPs [38, 39]. Therefore, degradation of VP40 mRNA by a two-pronged attack from *hsa-miR-4692* and *hsa-miR-548-az* can stop Ebola.

A distinguishing characteristic of filovirus genomes is their 3′- and 5′-UTRs that are long related to other RNA viruses of the nonsegmented negative-strand (NNS) variety [40]. Of particular note, Kochetov AV [41] concentrated on the 5′-UTRs in the mRNA of seven EBOV viruses, due to the critical importance of the 5′-UTRs in translation initiation. In four of these seven mRNAs, small alternate upstream open reading frames (uORFs) were identified, but their significance is yet to be fully characterized. In cellular mRNAs, uORFs are known to be a common feature; they are critical in modulating translation of primary ORFs (pORFs), which was accomplish by reducing the efficiency and quantity of the scanning ribosomes associated with the reinitiation that occurs at the start codon of pORFs [42]. At a uAUG, rather than a pAUG, translation initiation frequency is affected by a variety of factors, including the strength of the Kozak consensus sequence that surrounds the uAUG. Moreover, between the pAUG and the upstream open reading frame (uORF) is an intercistronic space that, combined with the phosphorylation status of and the eIF-2α [43, 44], controls whether translation takes place at the principal protein initiation site (pAUG) or at the termination codon (uAUG).

When eIF-2$\alpha\sim$P is absent, cap-dependent translation has been found to be efficient, which permits higher ribosome initiation rates at the uORF [45]. When eIF2$\alpha\sim$P is enhanced, impairment of translation initiation occurs, which causes a ribosome to continue scanning beyond the uAUG; in this case, initiation occurs at the pAUG. In short, when cell stress occurs, eIF2$\alpha\sim$P facilitates translation initiation of select mRNAs that possess uORFs at the primary open ready frame (pORF) [46].

They characterized how the EBOV 5′-UTRs modulate translation. Mutating any of the four uAUGs present in the EBOV genome enhances translation at the corresponding pORF. The most dramatic effect was with the L gene where the L uAUG can potently suppress pORF translation; however, in response to eIF2$\alpha\sim$P, the L uAUG maintains L translation. Modulating viral polymerase levels is biologically significant as ablating the L uORF in a recombinant EBOV reduces viral titers 10- to 100-fold in cell culture, severely impairs viral RNA synthesis, and functions to maintain virus titers in cells treated with stress-inducing agents. These data suggest that a uORF in the EBOV L mRNA regulates polymerase expression in response to the status of the cellular innate immune response and is required for optimal virus replication.

It would be relatively easy to incorporate a combination of relevant miRNAs in a miRNA-expression vector to test the utility of these miRNAs in genetically engineered VLP cell models in vitro that can be performed in a BSL-2 facility, and then to extend these studies in animal models utilizing safe vectors in a BSL-4 environment.

http://dx.doi.org/10.5772/61687

Currently, there are several genetically engineered vaccines containing genes for surface proteins (GP) that are in clinical trial. The first among these is a vaccine that Ebola GP genes stitched into a weakened chimpanzee adenovirus that serves as a vector. The second vaccine contains the Ebola surface protein gene inside a weakened version of vesicular stomatitis virus (VSV), which commonly infects farm animals. The potential dangers of employing of VSV are obvious: it can save men but potentially harm livestock in West Africa. The chimpanzee adenovirus will be a zoonotic event itself, and its potential danger cannot be underestimated [47, 48].

The third vaccine uses a vector known as MVA, a modified version of the smallpox vaccine virus, and involves protection from an Ebola virus "challenge" 10 months after the last vaccination.

We noted that none of these three approaches mentioned a simple and well-tested method of human and animal vaccination. What happened to the simple, whole formalin-killed or UV-killed, less pathogenic EBOV vaccines that have been tried in so many viral vaccinations [49, 50]?

With viruses like the major Ebola strands, where the mortality rate is over 50%, it will be difficult to find a reasonable and ethical way to carry out an unbiased clinical trial. However, if one can prepare a "dead Ebola virus" with antigenicity intact, it would be easy to immunize "high risk groups" without utilizing unusual vectors as exemplified by "harmless" chimpanzee adenovirus, VSV or MVA (modified smallpox virus), each with unknown long-term risk factors and accompanied by immediate concerns of viral vector-induced antigenic competition that may potentially quell proper immune responses to the Ebola antigens [51]. We believe that a dead vaccine may induce the protective miRNAs and quell the pandemic. Increasingly, miRNA-induced intracellular immunity is being better understood, and several clinical trials are under way to treat viral diseases and cancers [52–55]. The cost of each of these vaccines would run into millions of dollars and would be prohibitively expensive to any of the individuals who are predicted to be infected with the virus in West African nations. In contrast to the proposed recombinant vaccines, each of the more traditional "killed vaccines" has been very inexpensive to produce and has benefited billions of humans [56].

2. Conclusions

The current ongoing Ebola outbreaks in West Africa that began almost three years ago in March 2013 have already claimed 11,000 lives and over 27,000 cases. The rapid spread of the infection demands the need for rapid prevention methods. Currently, there are several vaccines that are in different phases of clinical trials. In this report, we highlight an alternative to the standard vaccine for Ebola prevention. We show that a preventive method based on miRNAs could be utilized and tested in nonhuman primates. Some of the lessons that we have learned from the recent West Africa Ebola outbreaks is to test the vaccine and other preventive methods that are currently available against Ebola before the major outbreaks occur. Therefore, we recommend that vaccines and preventive methods must be developed to the point that the measures

correlate for human protection (Phase I level), so when the outbreaks occur, the vaccine and other measures can be rolled out quickly to prevent the spread of the disease.

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