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Original Article

Inhibition of Ebola Virus by Anti-Ebola miRNAs *in silico*

Zhabiz Golkar[1], Roshan Battaria[2], Donald Gene Pace[3], Omar Bagasra[2]

[1] *Department of Biology, School of Health and Natural Science, Voorhees College, Denmark, SC, United States*
[2] *South Carolina Center for Biotechnology at Claflin University, Orangeburg, SC, United States*
[3] *School of Sociology and Humanities, Claflin University, Orangeburg, SC, United States*

Abstract

Introduction: MicroRNAs (miRNAs) are small, noncoding RNA molecules that regulate transcriptional and posttranscriptional gene regulation of the organisms. miRNA provides immune defense when the body is faced with challenges intracellular agents. miRNA molecules trigger gene silencing in eukaryotic cells. More than 3,000 different human miRNAs (hsa-miRs) have been identified thus far. During ontogenesis, viral or intracellular parasitic infections, miRNAs are differentially expressed to protect the host from intracellular invaders. In a viral infection context, miRNAs have been connected with the interplay between host and pathogen, and occupy a major role in pathogenesis.

Methodology: An *in silico* approach was used to analyze the four major Ebola Virus genome sequences including the recently characterized Ebola virus responsible for West African epidemic that has killed over 10,000 people. All totaled, 2,543 mature human miRNA sequences were retrieved through an miR-database, and the identification of mature miRNAs were aligned with full length sequences of the four major Ebola viruses via computational tools.

Results: We identified 32 miRNAs that exhibited significant inhibitory capacity to block more than one EBV strains. miR-607 showed capacity to quell all four major EBVs. Ten putative miRNAs were found to have near perfect identity at seed sequences with numerous targets of Ebola virus that may completely degrade the viral transcripts.

Conclusion: We hypothesize that a miRNA-based vaccine can quell Ebola virus infection. Future approaches will focus on validation of these miRNAs in quelling the Ebola virus to further elucidate their biological functions in primate and other animal models.

Key words: filoviruses; DNA-directed RNA Polymerases; metabolism; Ebola virus; *in silico*; screening; microRNA precursor.

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Introduction

Filoviruses are taxonomically classified within the order *Mononegavirales*, a large group of enveloped viruses whose genomes are composed of a non-segmented, single-stranded minus [1] RNA molecule. Following their discovery, Filoviruses were originally grouped with rhabdoviruses, since the appearance of virus particles appeared similar [2]. 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, Lake Victoria Marburg virus, since this strain exhibits only limited genetic variation. 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 rates [4]. Therefore, EBOV-S and EBOV-Z, which are the predominant EBOVs associated with known outbreaks, are more pathogenic than EBOV-R and EBOV-ICV [5]. EBOV-IC has only caused a single non-fatal human infection, but EBOV-R has caused fatal infection in non-human primates [2]. However, EBOV-S, EBOV-Z, and EBOV-B often cause severe hemorrhagic diseases with markedly high case fatality rates (40–90%) [5,6]. 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 [7]. However,

variations within EBOV-Z species are very low (~2–3%) [6,7]. Thus, GP nucleotides are usually used in the phylogenetic analysis of EBOV (Figure 1).

The recent Ebola pandemic (2014) is the largest yet reported in the history of Ebola and, according to World Health Organization (WHO), over 14,000 individuals have been infected with this new strain, which may be an underestimation since many of the previous victims might have been misdiagnosed as malaria, cholera, or even Lassa fever [8]. Recently, a whole EBOV replication defective vaccine –EBOVdVP30 has been found to be very effective in non-human primates and two other are in Phase II trials [9]. In this report, we present the pathobiology, epidemiology, therapeutics and vaccinology of Ebola and explain how these miRNAs may be utilized to contain the pandemic

Materials and Methods

At the time of our studies (November 2014), mature *hsa-miRs* were listed in the Sanger database. By utilizing the human miRBase sequences database http://microrna.sanger.ac.uk/sequences_/version 20.0), the *hsa-miR* sequences were first downloaded from the database and then aligned with major Ebola genomes (accession number: NC_002549.1). In addition, since alignment tools are generally programmed in the FASTA format, all the genomic sequences were annotated in FASTA format before the alignment process. The reference genome sequences of all four viruses were obtained from http://www.ncbi.nlm.nih.gov/. Following this, we utilized multiple alignment tools to search for miRNAs that shared identities with all the viruses, as described previously by us [9] (Table 1).

Determination of miRNA alignment to viral sequences

To determine the suitability of each of the *hsa-miRNAs* as a potential post-transcription silencing agent, we developed and refined an algorithm that incorporates the three critical elements that increase the suitability of a miRNA as a successful silencing agent [10]. These include the length of the complementary pairing between hsa-miRNAs and their target sites in viral genomes. Generally, in plants miRNA targets exhibit complete homology at the ORFs, whereas the binding between animal miRNAs and their targets show incomplete homology in base-pairing, binding sites at 3'UTRs, 5'UTRs and the coding regions of target genes. Therefore, it is important that the length of the targeting miRNAs must be 19 bp or above for silencing to take place. In this case, we downloaded the available miRNAs from miRbase and aligned the most recently sequenced member of the Ebola virus published to date [11]. These sequences were analyzed for homologies in each of the genes of the Ebola genome for 1) seed sequence complementarity: a near-perfect alignment at miRNA seed sequences located at the 3'-untranslated region (UTR) base pair 2 to 8 that signals a successful silencing match [12] and 2) a high degree of complementarity: an 80%-90% degree level of homology of the sequences of miRNAs with each of the Ebola genes was considered as highly significant ($p < 0.001$) and reported to significantly reduce the "off-target" silencing of other genes [13].

Table 1. EBOV-Accession numbers used in this study

S.N.	EBOV	Accession Number
1.	Zaire Ebolavirus isolate	KJ660346.2
2.	Reston Ebolavirus isolate	NC_004161.1
3.	Sudan Ebolavirus isolate	NC_006432.1
4.	Tai Forest (Ivory Coast) Ebolavirus isolate	NC_014372.1

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

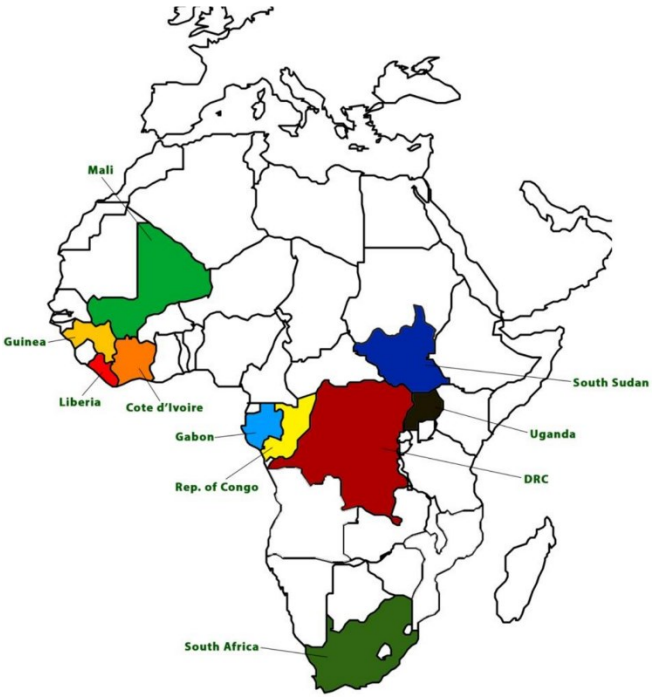
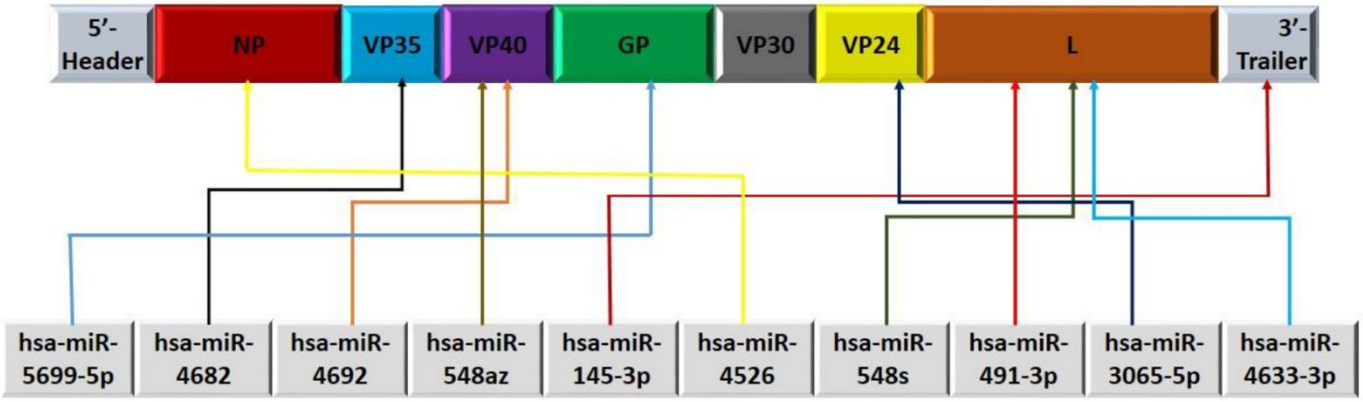


Figure 2. The genome of EBOV, 18·9 kb in length, has the following gene order: 3' leader nucleoprotein (NP), virion proteins (VP) VP35-VP40, membrane glycoprotein (GP), viral polymerase (VP) VP30-VP24, viral polymerase L protein, and 5' trailer.



Results

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 2). Similar to other nonsegmented negative-sense (NNS) RNA viruses, EBOVs encode a multi-protein 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 towards the 5′ end of the template [14,15]. Each EBOV mRNA is presumed to be efficiently modified with a 5′-7-methylguanosine (m^7G) cap and a 3′ p(A) tail [16].

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 [17]. 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 post-transcriptions and suppressions of viral expression [18]. Therefore, besides endogenous gene regulation, miRNAs are the primary intracellular immune defense system [19]. Viruses have also evolved to counter the anti-viral effects of miRNAs by viral miRNAs (vmiRNAs).

We searched for miRNAs that exhibited over 80% identity to the EBOV genome and found 71 miRNAs in a human miR database. Many of them showed significant homologies at the seed sequences that are considered to be an important specific gene silencing motifs (Table 1**).**

Here, we show that several miRNAs can specifically bind target certain key Ebola genes. Therefore, hsa-miR-5699-5p specifically targets Ebola-GP, hsa-miR-4682 targets *VP35, hsa-miR-4692* and *hsa-miR-548-az* both target *VP40, hsa-miR-4526* targets *NP, hsa-miR-3065-5p* targets *VP24, hsa-miR-145-3p* targets the trailer portion of the virus genome, whereas, *hsa-miR-491-3p, hsa-miR-4633-3p, hsa-miR-491-3p, and hsa-miR-548-3p* all target *L (polymerase gene)*. We believe that the last miRNAs that target the viral polymerase gene are significant.

All of the above miRNA showed near 100% homology at the seed sequences of Ebola Virus (Table 2).

Discussion

Recently, Li and Chen [20] 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) [21]. Logically, one could assume that over the past 2000 years, humans have evolved counter measures 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 [22]. 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 [23]. Before Ebola viral strains were introduced to primates, they had already been circulating among small mammals, including bats, rodents, marsupials, shrews, etc. [22]. Although these bats and other animals were infected [22,23], no evidence demonstrated that such infections were fatal to them [24]. 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 [25]. This homeostasis, this balance, apparently was broken about the year 1900, when EBOV genetic diversity experienced a dramatic drop [23]. 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 is 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 [24]. 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 [21,24]. Since 1970, 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 [25,26].

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 minus-strand RNA viruses into +RNA strands occurs amidst 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 [27].

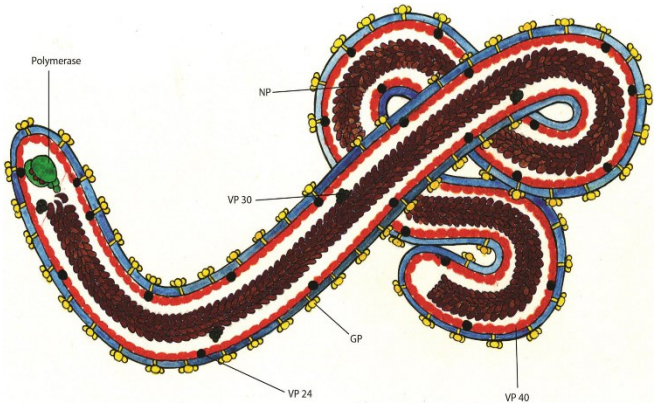
Table 2. Sequence Alignment of human miRNAs that exhibited over 80% identity to the EBOV genome in human miRNA database. Many of them showed significant homologies at the seed sequences that are considered to be an important specific gene silencing motifs.

No.	miRNAs	Sequence	Homology
1	hsa-miR-5699-5p	6595- TGCCCCAAGCTAAGAAGGAC- 6614 1- TGCCCCAAGCAAGGAAGGAC- 19	%85
2	hsa-miR-4682	3910- CTGAGTTCCAGGCCAGCCTGG- 3390 2- CTGAGTTCCTGGCCAGCCTGG- 20	%85
3	hsa-miR-4692	4675- CAGGCAGTGT—GTCATCAG- 4692 2- CAGGCAGTGTGGGTCATCAG- 20	%85
4	hsa-miR-548az	2801- AAAAGTGATTCTTATTTTTG- 2820 2- AAAAGTGATTGTGGTTTTTG- 21	%85
5	hsa-miR-145-3p	18891- GGATTCCTGGAAA-AATGGTC- 18910 1- GGATTCCTGGAAATACTGTTC- 21	%85
6	hsa-miR-4526	543- TGACAGCAGGGCTGGCCGTT- 562 3- TGACAGCAGGGCTGGCCGCT- 22	%85
7	hsa-miR-548s	12488- TGGCCAAAA-TTCAATTAT- 12505 2- TGGCCAAAACTGCAGTTAT- 20	%84
8	hsa-miR-491-3p	11902- ATGCAAGATGCTCTCTTCT- 11920 4- ATGCAAGATGCTCCCTTCT- 20	%84
9	hsa-miR-3065-5p	10296- CAACAAAAT-ATTGATACT- 10313 2- CAACAAAATCACTGATGCT- 20	%84
10	hsa-miR-4633-3p	15846- CGAGCTAGCCAAGACCATCATGCA- 15869 2- GGAGCTAGCCAGGACCATCATGCA- 22	%83

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 [28-32]. 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 [33,34]. Viral matrix proteins VP40 and VP24 are essential to viral budding, stability, and structure [6,7]. VP40 is the primary matrix protein, and is 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 [35,36]. Given VP40's absence of VP40, studies have found that the nucleocapsid was not transported effectively into the plasma membrane, and since this membrane is the site of assembly, budding, and incorporation into the virions, considerable attention should be given to the role of this matrix protein [37]. 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. We show that *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. 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 [38].

VP40's association with the plasma membrane is of fundamental importance [35]; it is here that assembly is initiated as well as oligomerization [38], 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 [39,40], the vesicle coat II proteins (COPII) [41], as well as the protein actin [42, 43]; 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 since c-Abl1 can phosphorylate Tyr^{13} in VP40 [42]. Still we have inadequate understanding of how VP40 actually assembles on the plasma membrane before virion

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.



release occurs. Localization of VP40 in the plasma membrane is believed to be important since studies give evidence that hydrophobic residues located within the C-terminal domain, including $Leu,^{213}$ are essential in the localization and budding processes [43]. Detection of VP40 oligomers in VLPs and UV-inactivated virions has occurred [44-46]; they have been detected mainly in filamentous structures stemming from the plasma membrane [42]. Therefore, VP40 oligomerization apparently occurs on the same plasma membrane in which oligomers selectively have found to reside [40]. In terms of structure, VP40 has predominantly been found to oligomerize into either hexamers or octamers [38,46-47]. 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 [48]. We discovered that *hsa-miR-4692* and *hsa-miR-548-az* both target *VP40.*

The formation of virus-like particles (VLPs) requires VP40 oligomers; these are associated with membranes that are resistant to detergent [40], 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 become crucial to our understanding of how the formation of protein buds occurs at the plasma membrane. Adu-Gyamfi *et al*. [49] recently investigated the role that the VP40 C-terminal domain

plays in membrane association as well as membrane penetration. These investigators utilized 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 [39,40]. Therefore, degradation of VP40 mRNA by a two prong 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 relative to other RNA viruses of the nonsegmented negative-strand (NNS) variety [48-51]. Of particular note, Shabman *et al*. [12] 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 yet their significance still has 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 they accomplish by reducing the efficiency and quantity of the scanning ribosomes associated with the reinitiating that occurs at the start codon of pORFs [50-52]. 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 [53-56], controls whether translation takes place at the principal protein initiation site (pAUG) or at the termination codon (uAUG).

When eIF-2α~P is absent, cap-dependent translation has been found to be efficient, which permits higher ribosome initiation rates at the uORF [57]. When eIF2α~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, eIF2α~P facilitates translation initiation of select mRNAs that possess uORFs at the primary open ready frame (pORF) [55,58,68].

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 eIF2α~P, the L uAUG maintains L translation. Modulating viral polymerase levels is biologically significant since 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. Here, we show that *hsa-miR-145-3p* targets the 5' portion of the virus genome, potentially blocking the crucial step of the virus.

In conclusion, we present computation analyses based data that identify 10 human miRNAs that can be potentially used to block Ebola virus pandemic. 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 a genetically engineered VLP cell models *in vitro* that can be performed in a BSL2 facility, and then to extend these studies in animal models utilizing safe vectors in a BSL4 environment. 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 [59]. 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 in none of these three approaches was a simple and well-tested method of

human and animal vaccination mentioned. What happened to the simple, whole formalin killed or UV killed less pathogenic EBOV vaccines that have been tried in so many viral vaccinations? [60]

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 [61-63]. We believe that a dead vaccine may induce the protective miRNAs and quell the pandemic. Increasingly, miRNA-induced intracellular immunity is becoming better understanding, and several clinical trials are underway to treat viral diseases and cancers [64]. The cost of each of these vaccines would run into the 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 [65].

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Authors Contribution

Drs. Golkar and Bagasra were responsible for the creative concepts, direction of the computational analysis and writing the manuscript. Mr. Battaria completed the computational analysis and Drs. Pace and Bagasra were responsible for editing of the manuscript.

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Corresponding author
Zhabiz Golkar, PhD
481 Porter Drive,
Denmark, SC 29042, USA
Phone: (803)-780-1056
Email: zgolkar@voorhees.edu

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