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Brief Communication

Development of a reverse genetics system to generate a recombinant Ebola virus Makona expressing a green fluorescent protein ☆

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ABSTRACT

Previous studies have demonstrated the potential application of reverse genetics technology in studying a broad range of aspects of viral biology, including gene regulation, protein function, cell entry, and pathogenesis. Here, we describe a highly efficient reverse genetics system used to generate recombinant Ebola virus (EBOV) based on a recent isolate from a human patient infected during the 2014–2015 outbreak in Western Africa. We also rescued a recombinant EBOV expressing a fluorescent reporter protein from a cleaved VP40 protein fusion. Using this virus and an inexpensive method to quantitate the expression of the foreign gene, we demonstrate its potential usefulness as a tool for screening antiviral compounds and measuring neutralizing antibodies.

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Introduction

The first outbreak of Ebola virus disease (EVD) caused by Ebola virus (EBOV) was detected in 1976 in Zaire (now the Democratic Republic of the Congo), and received significant international attention due to its high case fatality rate (Johnson, 1978; Johnson et al., 1977). During the early months of 2014, a large EVD outbreak was detected in the Republic of Guinea (Baize et al., 2014; Gatherer, 2014) and spread quickly to the neighboring countries of Liberia and Sierra Leone (Dixon et al., 2014). To date, this continuing outbreak has been the largest EVD outbreak ever recorded, with over 23,000 cases and over 10,000 deaths in Western Africa as of March 2015. A limited number of cases were also recorded in Mali, Nigeria, Senegal, USA, Spain, Germany, and the United Kingdom (Baize, 2015; CDC, 2015).

Several viruses of the *Filoviridae* family, including EBOV, Sudan virus, Bundibugyo virus, Tai Forest virus, Marburg virus (MARV), and Ravn virus, cause sporadic outbreaks of viral hemorrhagic fevers (VHFs) with high case fatality rates in sub-Saharan Africa (Albariño et al., 2013a; Feldmann et al., 2013; Hartman et al., 2010; Leroy et al., 2011). Filoviruses are enveloped viruses that carry a single-strand RNA genome with negative-sense polarity (Feldmann et al., 2013). The filoviral genome is approximately 19 kb, and encodes 7 genes, NP, VP35, VP40, VP30, VP24, and L, which are transcribed in sequential order from the 3′ end of the viral genome and are separated by intergenic untranslated regions (Fig. 1A).

For more than a decade, reverse genetics technology has been a useful tool for screening antiviral compounds and for studying different aspects of filovirus biology, including virulence factors in the viral genome, cell entry, mechanisms of transcription and replication, and pathogenesis (Ebihara et al., 2005; Falzarano and Feldmann, 2014; Hoenen and Feldmann, 2014; Hoenen et al., 2011; Neumann et al., 2002; Theriault et al., 2005). Several reports also describe modifying the genomes of EBOV and MARV by inserting reporter genes, such as those coding for fluorescent proteins and luciferase (Albariño et al., 2013b; Ebihara et al., 2007; Hoenen et al., 2013; Schmidt et al., 2011; Schudt et al., 2013; Towner et al., 2005; Uebelhoer et al., 2014).

In order to start studies on the biological characteristics of the EBOV variant responsible for the current Western Africa outbreak, we developed a reverse genetics system for an isolate (Makona) that was obtained from a patient in 2014. Here, we report the generation of recombinant viruses that would constitute an appropriate tool for future studies on this particular EBOV variant.

Results and discussion

Comparisons of full-length genomes from EBOV samples isolated from 1976 to 2014 show a high degree of sequence identity (Fig. 1A). As expected, representative isolates of the Makona variant from the 2014 outbreak in Western Africa, obtained from

☆The findings and conclusions in this report are those of the author(s) and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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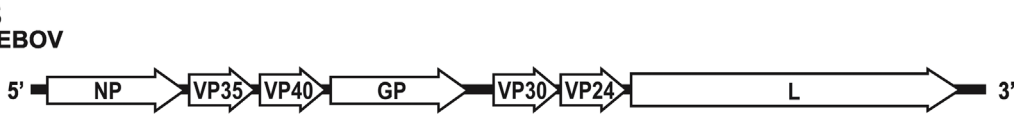
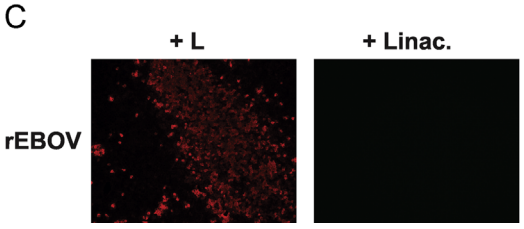
A

	1	2	3	4	5	6	7	8	9	10	11
LBR-2014 KP178538	1		3	9	610	544	602	653	595	641	561	561
SLE-2014 KM233072	2	99.98		8	609	545	603	654	596	642	562	562
GIN-2014 KJ660346	3	99.95	99.96		605	537	595	646	588	634	554	554
COD-2014 KM519951	4	96.78	96.79	96.81		417	474	189	148	174	236	236
COD-2007 KC242784	5	97.13	97.13	97.17	97.80		410	468	408	453	361	361
GAB-2002 KC242800	6	96.82	96.82	96.86	97.50	97.84		516	460	502	414	414
GAB-1996 KC242794	7	96.56	96.55	96.59	99.00	97.53	97.28		193	69	278	278
COD-1995 KC242796	8	96.86	96.86	96.90	99.22	97.85	97.57	98.98		180	222	222
GAB-1994 KC242792	9	96.62	96.61	96.66	99.08	97.61	97.35	99.64	99.05		263	264
COD-1977 KC242791	10	97.04	97.04	97.08	98.76	98.10	97.82	98.53	98.83	98.61		2
COD-1976 NC_002549	11	97.04	97.04	97.08	98.76	98.10	97.82	98.53	98.83	98.61	99.99	

B



C



D

	Rescue Efficiency (pos/total)	Average Titer (tcid50/ml)
rEBOV	11/11	$1x10^7$

Fig. 1. (A) Sequence comparison. Full-length genomes of representative Ebola virus (EBOV) isolates compared at the nucleotide level. Sequence identity (%) is indicated in the lower diagonal half, while the number of differing residues is indicated in the upper diagonal half. (B) Schematic representation of recombinant EBOV (rEBOV) genome. Genome of recombinant EBOV in the viral complementary sense, with the 7 ORFs depicted in the 5′ to 3′ orientation. (C) Rescue of rEBOV. Wild-type rEBOV was rescued in Huh7 cells using the full-length clone shown in B, T7 polymerase expression plasmid, and codon-optimized support plasmids that express NP, VP35, VP30, and L or inactive L (L-inac). Supernatants from transfected cells were used to infect fresh monolayers of Huh7 cells. The infected cells were fixed 4 days post infection, and stained for immunofluorescence assays (IFA) using a polyclonal rabbit anti-EBOV antibody followed by anti-rabbit Alexa-Fluor 594. (D) Efficiency of rescue. Rescue efficiency is shown as the number of positive wells (pos)/total wells; average titer was assessed by standard $TCID_{50}$ assay.

patients in Liberia, Guinea, and Sierra Leone, showed over 99% sequence identity with each other across the full length of the genome (Baize et al., 2014; Gire et al., 2014). In contrast, these isolates showed ~97% sequence identity with the Mayinga isolate of the Yambuku variant collected during the 1976 outbreak, and with representative isolates of the Kikwit variant from 1995. Until now, all published studies using reverse genetics technology with EBOV have used only the historic Mayinga isolate (Groseth et al., 2012; Hartman et al., 2008, 2006; Martinez et al., 2011; Neumann et al., 2002; Volchkov et al., 2001). Despite the limited divergence between the Makona and Yambuku variant isolates (Fig.1A: 97% identities, 561 different nucleotides), concerns have been raised about differences in pathogenicity, virulence, or transmission associated with the 2014 variant (Dowall et al., 2014; Gire et al., 2014; Stadler et al., 2014). For this reason, we developed a new reverse genetics system based on an isolate from the current EVD outbreak so that detailed studies on this 2014 variant or comparison studies with previous variants could be performed.

After obtaining confirmation of the sequence identity of the viral RNA termini by standard 5′ and 3′ RACE methods (data not shown), we constructed a full-length recombinant clone (Fig. 1B) based on the Makona variant isolate (Ebola virus/H.sapiens-wt/LBR/2014/Makona-201403007; an isolate of the Makona variant (GenBank accession # KP178538). Similarly to previously reported constructs for EBOV and MARV (Albariño et al., 2013b; Enterlein et al., 2006; Neumann et al., 2002; Volchkov et al., 2001), the viral complementary (anti-genomic) sense genome was cloned into a standard T7 transcription vector, flanked at the 3′ end by the HDV ribozyme and the T7 terminator.

Different cells lines, including 293T, BSRT7 and BHK, have been traditionally used to rescue EBOV and MARV (Albariño et al., 2013b; Neumann et al., 2002; Towner et al., 2005; Volchkov et al., 2001). Since human liver cells are an important target of EBOV infection, we decided to use Huh7 cells, a fully differentiated human liver cell line, for viral rescue due to their high transfection efficiency and the high titers obtained by propagating wild-type filoviruses in these cells. In addition, Huh7 cells minimize selection of variant viruses generated by reverse genetics (Tsuda et al., 2015). To rescue the wild-type recombinant EBOV (rEBOV), we co-transfected Huh7 cells with the full-length genomic plasmid, with codon-optimized support plasmids expressing EBOV NP, L (or inactive L; see below), VP35, and VP30, and with another plasmid expressing a codon-optimized version of the T7 RNA polymerase (Uebelhoer et al., 2014). For the negative control, we built a new plasmid expressing an inactive form of the EBOV L polymerase (L-inac), in which the GDN motif was removed from the L ORF. Four days post transfection, we applied the clarified supernatants from this transfection onto fresh monolayers of Huh7. We assessed viral rescue 5 days later by performing an immunofluorescence assay to detect EBOV antigens (Fig. 1C). As shown in Fig. 1D, the rescue of rEBOV was successful in 11 of 11 replica wells, which demonstrated the high efficiency of our new approach to rescue rEBOV.

After confirming the successful rescue of rEBOV, we developed a new recombinant virus expressing a reporter gene that could be used to detect viral infection in live cells or to facilitate screening antiviral agents against the 2014 EBOV variant. Historically, the most common approach for introducing a reporter gene into the filoviral genome has been to insert an additional transcription unit into any of the 6 intergenic regions (Albariño et al., 2013b; Ebihara et al., 2007; Hoenen et al., 2013; Schmidt et al., 2011; Schudt et al., 2013; Towner et al., 2005; Uebelhoer et al., 2014). Unfortunately, recombinant viruses generated using this approach exhibited attenuated phenotypes in animal models (Ebihara et al., 2007) or

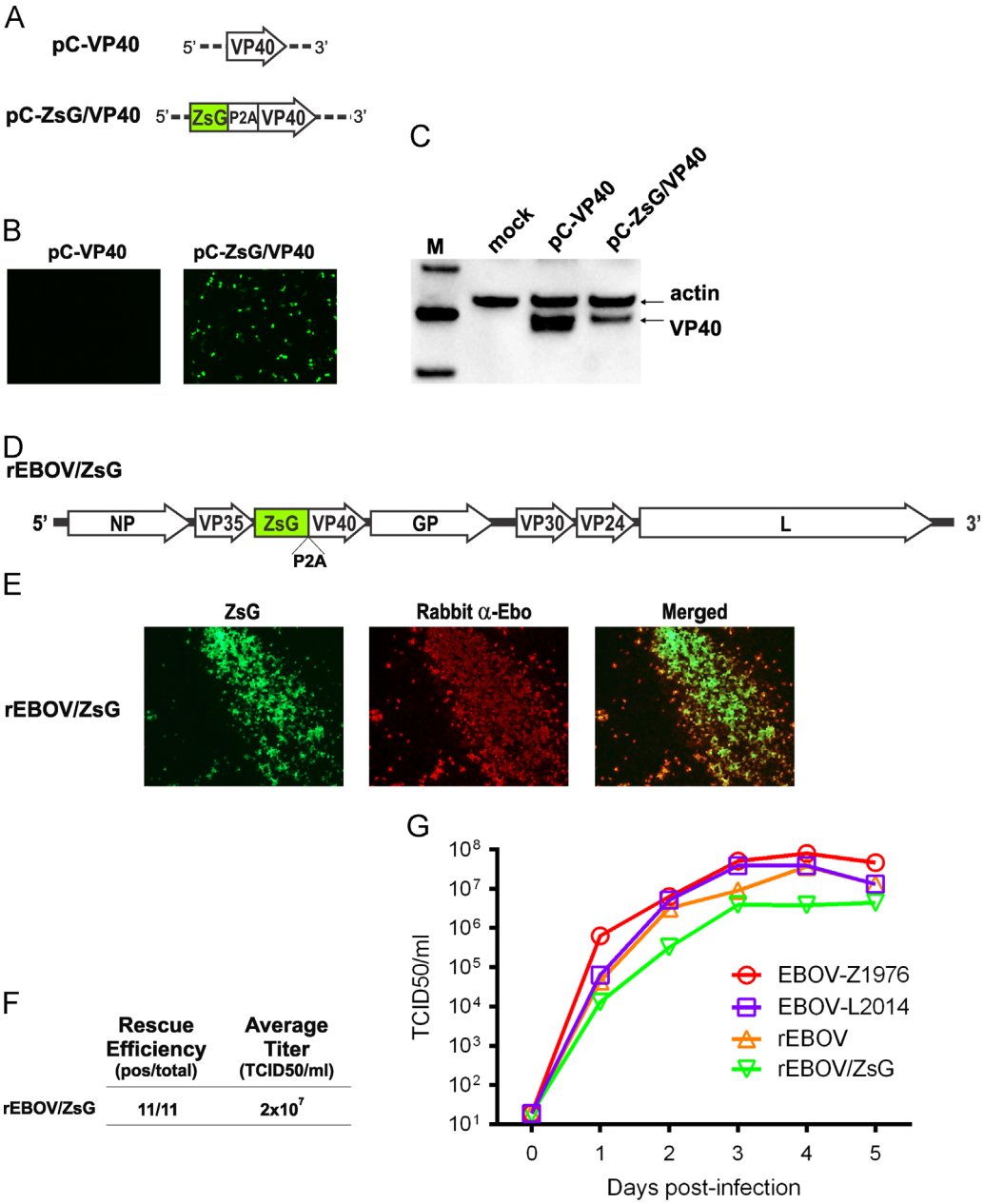


Fig. 2. (A) VP40 fusion protein. Schematics of plasmids expressing VP40 or ZsG/P2A/VP40 fusion protein are shown. (B) ZsGreen (ZsG) expression. Huh7 cells were transfected with pC-VP40 or pC-ZsG/VP40. Images shown were taken 3 days post transfection on an inverted fluorescent microscope using the GFP channel. (C) VP40 expression. Huh7 cells were transfected as above and harvested 3 days post transfection. Protein lysates were analyzed by western blotting using a Mab against EBOV-VP40 (lower panel). (D) Schematic representation of rEBOV genome expressing ZsG. Genome of rEBOV expressing ZsG fusion protein (rEBOV/ZsG) is shown in the viral complementary sense. (E) Rescue of rEBOV/ZsG. rEBOV/ZsG was rescued using the same conditions as wild-type virus (see Fig. 1B). Fresh monolayers of Huh7 cells were infected with transfection supernatants. ZsG expression was visualized on an inverted fluorescent microscope using the GFP channel (left panel), or via IFA as in Fig. 1D (middle panel, and merged images panel at right). (F) Rescue efficiency. Efficiency of rescue and average titer of rEBOV/ZsG were assessed as in Fig. 1. (G) Growth kinetics of wild-type and recombinant viruses. Huh7 cells were infected with wild-type EBOV Mayinga (EBOV-Z1976), or wild-type human isolate of the Makona variant (EBOV-L2014), or with the 2 recombinant viruses, rEBOV or rEBOV/ZsG, with an MOI=0.1. Growth kinetics were assessed by determining viral titers in cell supernatants using a standard $TCID_{50}$ assay.

reduced growth in interferon-competent cells (Albariño et al., 2013b). An alternative strategy, used for non-segmented negative strand viruses, has been to fuse the foreign gene to one of the viral genes (Chambers and Takimoto, 2010; Duprex et al., 2002; Hoenen et al., 2012; Lo et al., 2014; Schudt et al., 2013; Silin et al., 2007). A particularly interesting report by Lo and colleagues describes the successful generation of a fully functional recombinant Nipah virus in which the reporter gene, green fluorescent protein (GFP), had been fused to the matrix protein, M (Lo et al., 2014). In this particular construct, M and GFP are released through the action of self-cleaving peptides, such as F2A derived from foot and mouth disease virus, or P2A derived from porcine teschovirus 2A (Kim et al., 2011). We therefore decided to test this latest strategy by fusing the modified green fluorescent protein, ZsGreen1 (ZsG), to the self-cleaving peptide P2A and EBOV VP40.

To verify that VP40 is released from the protein fusion, ZsG/P2A/VP40 was initially cloned into a standard expression vector (Fig. 2A) and used to transfect Huh7 cells. As shown in Fig. 2B, the fusion protein yields bright green fluorescence in transfected cells. Moreover, protein analysis by western blotting showed that EBOV VP40 is released from the fusion protein, although at lower expression levels than wild-type VP40 (Fig. 2C).

After confirming that ZsG and EBOV VP40 can be expressed from the fusion cassette, we modified our new full-length clone to carry this cassette in the same locus as wild-type VP40 (Fig. 2D). Using the conditions described above to rescue wild-type rEBOV, we successfully generated rEBOV/ZsG in all 11 replica wells (Fig. 2E and F). Both rEBOV and rEBOV/ZsG were successfully rescued with 100% efficiency (11 of 11 wells) in 2 successive rescue attempts.

As shown in Fig. 2E, rEBOV/ZsG recombinant virus expressed ZsG and spread through the cell monolayer in a pattern similar to that of wild-type rEBOV.

We compared the growth kinetics of the most relevant wild-type viruses, EBOV Mayinga isolate and the human isolate of the Makona variant, and of the 2 recombinant viruses, rEBOV and rEBOV/ZsG. Interestingly, the wild-type Mayinga virus (EBOV-Z1976) exhibited a slight growth advantage over the Makona isolate (EBOV-L2014), while EBOV-L2014 and rEBOV exhibited very similar growth characteristics (Fig. 2G). Moreover, rEBOV grew slightly faster than rEBOV/ZsG (Fig.2G).

Quantifying the fluorescence signal from GFP- or RFP-expressing viruses has an intrinsic limitation because it requires expensive, high-content, sophisticated imaging techniques (Panchal et al., 2010, 2012). To avoid this need, we sought to validate an alternative method described by Lo et al. (2014), which quantifies viral growth by measuring the fluorescence signal from live cells infected with rEBOV/ZsG. In this experiment, we infected Huh7 cells with rEBOV/ZsG at a low multiplicity of infection (MOI), and captured daily micrographs to record the progress of infection (Fig. 3A). Concurrently, we also measured the ZsG-specific signal on a multi-mode microplate reader and removed sample aliquots to determine viral titers over 5 days of infection. As shown in Fig. 3B, the fluorescence signal of ZsG could be easily quantified in a non-destructive way, and these data correlated with the spread of virus shown in the captured images (Fig. 3A). Moreover, the gradual increase of the ZsG signal also matched with increases in viral titers (Fig. 3B).

Using this straightforward approach for quantifying the fluorescence signal, we conducted a proof of principle experiment using rEBOV/ZsG as a rapid tool for screening antiviral compounds and measuring neutralizing antibodies in sera collected from convalescent rhesus monkeys. As shown in Fig. 3C, we used rEBOV/ZsG virus in Huh7 cells to measure the antiviral effect of 6azaU, a known inhibitor of viral replication (Crance et al., 2003; Morrey et al., 2002; Pyrc et al., 2006; Smee et al., 1987; Uebelhoer et al., 2014). Consistent with our previous report (Uebelhoer et al., 2014), 6azaU used at the maximum concentration tolerated without toxicity significantly reduced ZsG signal: 94% and 97% signal reduction at 62.5 μM and 125 μM concentrations of 6azaU, respectively

As a second proof of principle experiment, we measured the neutralizing effect of sera collected from convalescent rhesus monkeys infected with EBOV Mayinga on rEBOV/ZsG (Fig. 3D). In this experiment, we pre-incubated rEBOV/ZsG with different dilutions of an IgG+ or an IgG− serum, and then used the treated virus to infect Huh7 cells. As shown in Fig. 3D, the ZsG signal was reduced by 98% after using a 1:400 dilution of the IgG+ serum.

Conclusions

Here, we describe a highly efficient reverse genetics system to generate a recombinant EBOV (rEBOV) based on a virus isolated from a patient during the 2014–2015 Western Africa EVD outbreak. We also report the successful rescue of a recombinant EBOV expressing a ZsG reporter protein that is initially fused to the EBOV protein VP40, but is released by a cis-acting proteolytic cleavage event. The fusion of this reporter gene did not adversely affect

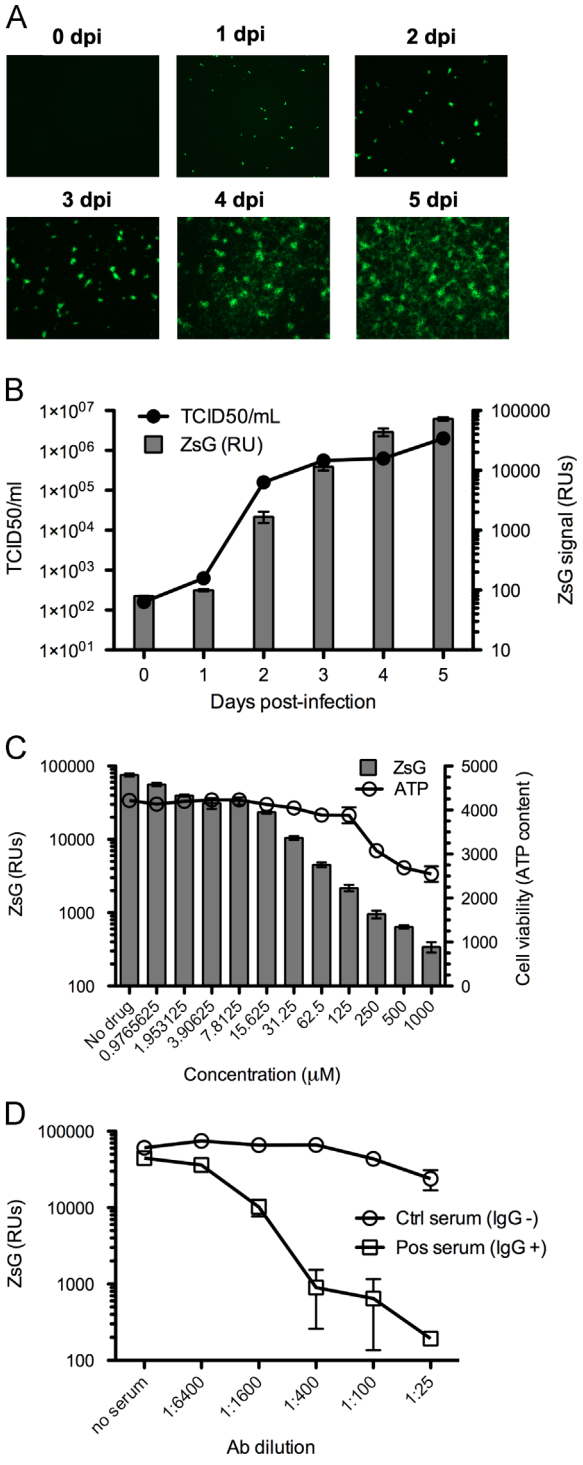


Fig. 3. (A) Characterization of rEBOV/ZsG. Vero-E6 cells were infected with rEBOV/ZsG viruses at MOI of 0.01, and pictures were taken as in Fig. 2C on indicated days post infection (dpi). (B) Quantitation of ZsG expression and viral titers. Huh7 cells were infected as indicated above, and fluorescence resulting from ZsG expression was measured daily for 5 days using a multi-mode microplate reader (BioTek Synergy). Fluorescence is reported as relative fluorescence units (RU). Viral titers were determined as in Fig. 2G. (C) Effects of antiviral agent 6azaU. Huh7 cells growing in 96-well plates were pre-treated with 6azaU at indicated concentrations for 1 h, and then infected with rEBOV/ZsG at MOI of 0.1. Three days post infection, ZsG expression (bars) was measured as indicated above. Mean and SEM of ZsG expression from 4 wells are displayed (left *Y*-axis). Cytotoxicity was assayed by measuring cellular ATP content (circles, right *Y*-axis) in uninfected cells treated with 6azaU at indicated concentrations. (D) Serum neutralization assay. rEBOV/ZsG was pre-incubated for 1 h with the indicated dilutions of an IgG-positive or control rhesus monkey serum, and then used to infect Huh7 cells growing in 96-well plates. Expression of ZsG was measured as indicated above; mean and SEM from 4 wells are depicted.

viral replication in vitro. Moreover, we show a straightforward approach to quantify virus growth of rEBOV/ZsG in live infected cells, and demonstrate that this virus can be used to screen antiviral drugs and to measure the neutralizing effect of antibodies in sera from convalescent animals. Finally, we consider that the tools described here could have other potential applications, such as testing the effects of novel mutations that could arise in nature, specifically directed at the variant responsible for the largest outbreak of EVD ever reported.

Materials and methods

Cell culture and biosafety

All work with recombinant viruses was performed in a biosafety level 4 (BSL-4) facility. Huh7 and Vero-E6 cells were propagated in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Grand Island, NY, USA) supplemented with 5% fetal bovine serum (FBS) and penicillin–streptomycin (Pen–Strep).

Plasmid construction

(A) *Support plasmids*. The construction of EBOV support plasmids (pC-L, pC-NP, pC-V35, and pC-v30) has been described before (Uebelhoer et al., 2014). Briefly, these clones were designed to express rodent codon-optimized synthetic genes corresponding to those of the EBOV Mayinga isolate. The original pC-L plasmid was also modified to express an inactive form of the EBOV L polymerase (L-inac) by removing the GDN motif from the L ORF. An expression cassette containing ZsG/P2A/VP40 was made using a previously described strategy (Lo et al., 2014). Briefly, ZsGreen1 (ZsG) ORF (Clontech, Mountain View, CA, USA) was fused to the self-cleaving P2A peptide and to EBOV VP40, and cloned into the standard Pol II expression vector pCAGGS (Niwa et al., 1991). (B) *Full-length clone*. Viral RNA from Ebola virus/H.sapiens-wt/LBR/2014/Makona-201403007 (GenBank accession KP178538) was used as template to amplify by RT-PCR 2 overlapping fragments of similar size spanning the full-length genome. These fragments were gel-purified and used to assemble a full-length clone into the T7 transcription vector. The final plasmid contained the full-length anti-genome (viral complementary sense) preceded by the T7 RNA polymerase promoter and followed by the hepatitis delta virus ribozyme and T7 polymerase terminator. A spurious nucleotide change in the GP-VP30 intergenic region was kept to differentiate the recombinant viruses rEBOV and rEBOV/ZsG from the wild-type virus.

The full-length clone was later modified by replacing the VP40 ORF with the ZsG/P2A/VP40 cassette described above. Both full-length clones were sequenced to completion. Details regarding construction strategies of support expression plasmids and plasmids encoding the full-length clone are available upon request.

Rescue of infectious viruses

Rescue of recombinant viruses was performed in Huh7 cells as described previously (Albariño et al., 2013b). Briefly, a ~70% confluent monolayer of Huh7 cells grown in 12-well plates was transfected with 1 μg pEBOV, 0.5 μg pC-L (wells # 1–11) or pC-L-inac (well # 12), 0.5 μg pC-NP, 0.05 μg pC-VP35, 0.05 μg pC-VP30, and 1 μg of codon-optimized pC-T7

Supernatants from transfected cells were harvested 4 days post transfection, clarified by low-speed centrifugation, and passaged twice in fresh monolayers of Huh7 cells. The rescue events were confirmed by immunostaining Huh7 cell monolayers (also grown in 12-well plates) infected with the first passage of the virus. A 100% rescue efficiency (positive detection in 11 of 11 replica wells) was obtained in 2 successive rescue experiments.

Both recombinant viruses were sequenced to completion, and complete genomic sequences were deposited in GenBank (accession KR781608 and KR781609 for rEBOV and rEBOV/ZsG, respectively). The viral genomic sequences were identical to those in the full-length plasmids.

Virus titration and growth curves

To characterize the growth kinetics of wild-type and recombinant viruses, $\sim 2 \times 10^6$ Huh7 cells were infected at MOI = 0.1. After 1 h of adsorption, cell monolayers were washed with PBS. Aliquots of the supernatant were taken daily, and viral titers were determined by tissue culture infective dose 50 ($TCID_{50}$) assay, as described previously (Uebelhoer et al., 2014).

Protein expression

Expression of ZsG in transfected or infected live cells was determined by direct UV microscopy using the GFP channel. Expression of cleaved VP40 was detected by western blotting using a monoclonal anti-EBOV-VP40 antibody followed by anti-mouse HRP secondary antibody. Staining of $TCID_{50}$ plates was done using a polyclonal rabbit anti-EBOV antibody followed by anti-rabbit Alexa-Fluor 594 antibody.

Quantitation of ZsG

The protocol described by Lo et al. (2014) was used to quantitate ZsG fluorescence in infected cells. Briefly, $\sim 4 \times 10^4$ Huh7 cells were seeded in 96-well flat-bottom black plates (Corning) in 100 μL Fluorobrite medium (Life Technologies) per well. The medium was removed on the following day, and cells were pre-treated with 100 μL media containing various concentrations of 6azaU for 1 h. Virus was added in an additional 100 μL media (final MOI = 0.1). Three days post infection, ZsG fluorescence was measured in 4 replicates for each antiviral concentration using a multi-mode microplate reader (BioTek Synergy) with a gain/sensitivity preset of 90. Cell viability was determined by measuring ATP content using CellTiter-Glo Luminescent Cell Viability reagent (Promega) as previously described (Uebelhoer et al., 2014).

Serum neutralization

About 300 $TCID_{50}$ of rEBOV/ZsG were incubated for 1 h at 37 °C with indicated dilutions of an IgG+ or a control (IgG−) serum from rhesus monkeys, and then used to infect Huh7 cells growing in 96-well flat-bottom black plates with Fluorobrite medium. Three days post infection, ZsG fluorescence was measured in 4 replicates for each serum concentration, as described above.

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References

Albariño, C.G., Shoemaker, T., Khristova, M.L., Wamala, J.F., Muyembe, J.J., Balinandi, S., Tumusiime, A., Campbell, S., Cannon, D., Gibbons, A., Bergeron, E., Bird, B., Dodd, K., Spiropoulou, C., Erickson, B.R., Guerrero, L., Knust, B., Nichol, S.T., Rollin, P.E., Ströher, U., 2013a. Genomic analysis of filoviruses associated with four viral

hemorrhagic fever outbreaks in Uganda and the Democratic Republic of the Congo in 2012. Virology 442, 97–100.
Albariño, C.G., Uebelhoer, L.S., Vincent, J.P., Khristova, M.L., Chakrabarti, A.K., McElroy, A., Nichol, S.T., Towner, J.S., 2013b. Development of a reverse genetics system to generate recombinant Marburg virus derived from a bat isolate. Virology 446, 230–237.
Baize, S., 2015. Ebola virus in West Africa: new conquered territories and new risks-or how I learned to stop worrying and (not) love Ebola virus. Curr. Opin. Virol. 10C, 70–76.
Baize, S., Pannetier, D., Oestereich, L., Rieger, T., Koivogui, L., Magassouba, N., Soropogui, B., Sow, M.S., Keita, S., De Clerck, H., Tiffany, A., Dominguez, G., Loua, M., Traore, A., Kolie, M., Malano, E.R., Heleze, E., Bocquin, A., Mely, S., Raoul, H., Caro, V., Cadar, D., Gabriel, M., Pahlmann, M., Tappe, D., Schmidt-Chanasit, J., Impouma, B., Diallo, A.K., Formenty, P., Van Herp, M., Günther, S., 2014. Emergence of Zaire Ebola virus disease in Guinea. N. Engl. J. Med. 371, 1418–1425.
CDC, 2015. Update: Ebola virus disease epidemic – west Africa. MMWR Morb. Mortal. Wkly. Rep. 64, 109–110.
Chambers, R., Takimoto, T., 2010. Trafficking of Sendai virus nucleocapsids is mediated by intracellular vesicles. PLoS One 5, e10994.
Crance, J.M., Scaramozzino, N., Jouan, A., Garin, D., 2003. Interferon, ribavirin, 6-azauridine and glycyrrhizin: antiviral compounds active against pathogenic flaviviruses. Antiviral Res. 58, 73–79.
Dixon, M.G., Schafer, I.J., Centers for Disease, C., Prevention, 2014. Ebola viral disease outbreak – West Africa. MMWR Morb. Mortal. Wkly. Rep. 63, 548–551.
Dowall, S.D., Matthews, D.A., Garcia-Dorival, I., Taylor, I., Kenny, J., Hertz-Fowler, C., Hall, N., Corbin-Lickfett, K., Empig, C., Schlunegger, K., Barr, J.N., Carroll, M.W., Hewson, R., Hiscox, J.A., 2014. Elucidating variations in the nucleotide sequence of Ebola virus associated with increasing pathogenicity. Genome Biol. 15, 540.
Duprex, W.P., Collins, F.M., Rima, B.K., 2002. Modulating the function of the measles virus RNA-dependent RNA polymerase by insertion of green fluorescent protein into the open reading frame. J. Virol. 76, 7322–7328.
Ebihara, H., Groseth, A., Neumann, G., Kawaoka, Y., Feldmann, H., 2005. The role of reverse genetics systems in studying viral hemorrhagic fevers. Thromb. Haemost. 94, 240–253.
Ebihara, H., Theriault, S., Neumann, G., Alimonti, J.B., Geisbert, J.B., Hensley, L.E., Groseth, A., Jones, S.M., Geisbert, T.W., Kawaoka, Y., Feldmann, H., 2007. In vitro and in vivo characterization of recombinant Ebola viruses expressing enhanced green fluorescent protein. J. Infect. Dis. 196 (Suppl. 2), S313–S322.
Enterlein, S., Volchkov, V., Weik, M., Kolesnikova, L., Volchkova, V., Klenk, H.D., Muhlberger, E., 2006. Rescue of recombinant Marburg virus from cDNA is dependent on nucleocapsid protein VP30. J. Virol. 80, 1038–1043.
Falzarano, D., Feldmann, H., 2014. Possible leap ahead in filovirus therapeutics. Cell Res. 24, 647–648.
Feldmann, H., Sanchez, A., Geisbert, T.W., 2013. Filoviridae: marburg and Ebola viruses. In: Knipe, D.M.a.H., P.M (Ed.), Fields Virology, 6th ed. Lippincott, Williams and Wilkins, Philadelphia, pp. 923–956.
Gatherer, D., 2014. The 2014 Ebola virus disease outbreak in West Africa. J. Gen. Virol. 95, 1619–1624.
Gire, S.K., Goba, A., Andersen, K.G., Sealfon, R.S., Park, D.J., Kanneh, L., Jalloh, S., Momoh, M., Fullah, M., Dudas, G., Wohl, S., Moses, L.M., Yozwiak, N.L., Winnicki, S., Matranga, C.B., Malboeuf, C.M., Qu, J., Gladden, A.D., Schaffner, S.F., Yang, X., Jiang, P.P., Nekoui, M., Colubri, A., Coomber, M.R., Fonnie, M., Moigboi, A., Gbakie, M., Kamara, F.K., Tucker, V., Konuwa, E., Saffa, S., Sellu, J., Jalloh, A.A., Kovoma, A., Koninga, J., Mustapha, I., Kargbo, K., Foday, M., Yillah, M., Kanneh, F., Robert, W., Massally, J.L., Chapman, S.B., Bochicchio, J., Murphy, C., Nusbaum, C., Young, S., Birren, B.W., Grant, D.S., Scheiffelin, J.S., Lander, E.S., Happi, C., Gevao, S. M., Gnirke, A., Rambaut, A., Garry, R.F., Khan, S.H., Sabeti, P.C., 2014. Genomic surveillance elucidates Ebola virus origin and transmission during the 2014 outbreak. Science 345, 1369–1372.
Groseth, A., Marzi, A., Hoenen, T., Herwig, A., Gardner, D., Becker, S., Ebihara, H., Feldmann, H., 2012. The Ebola virus glycoprotein contributes to but is not sufficient for virulence in vivo. PLoS Pathog. 8, e1002847.
Hartman, A.L., Bird, B.H., Towner, J.S., Antoniadou, Z.A., Zaki, S.R., Nichol, S.T., 2008. Inhibition of IRF-3 activation by VP35 is critical for the high level of virulence of Ebola virus. J. Virol. 82, 2699–2704.
Hartman, A.L., Dover, J.E., Towner, J.S., Nichol, S.T., 2006. Reverse genetic generation of recombinant Zaire Ebola viruses containing disrupted IRF-3 inhibitory domains results in attenuated virus growth in vitro and higher levels of IRF-3 activation without inhibiting viral transcription or replication. J. Virol. 80, 6430–6440.
Hartman, A.L., Towner, J.S., Nichol, S.T., 2010. Ebola and marburg hemorrhagic fever. Clin. Lab. Med. 30, 161–177.
Hoenen, T., Feldmann, H., 2014. Reverse genetics systems as tools for the development of novel therapies against filoviruses. Expert Rev. Anti-Infect. Ther. 12, 1253–1263.
Hoenen, T., Groseth, A., Callison, J., Takada, A., Feldmann, H., 2013. A novel Ebola virus expressing luciferase allows for rapid and quantitative testing of antivirals. Antiviral Res. 99, 207–213.
Hoenen, T., Groseth, A., de Kok-Mercado, F., Kuhn, J.H., Wahl-Jensen, V., 2011. Minigenomes, transcription and replication competent virus-like particles and beyond: reverse genetics systems for filoviruses and other negative stranded hemorrhagic fever viruses. Antiviral Res. 91, 195–208.
Hoenen, T., Shabman, R.S., Groseth, A., Herwig, A., Weber, M., Schudt, G., Dolnik, O., Basler, C.F., Becker, S., Feldmann, H., 2012. Inclusion bodies are a site of ebolavirus replication. J. Virol. 86, 11779–11788.
Johnson, K.M., 1978. Ebola haemorrhagic fever in Zaire, 1976. Bull. World Health Organ. 56, 271–293.
Johnson, K.M., Lange, J.V., Webb, P.A., Murphy, F.A., 1977. Isolation and partial characterisation of a new virus causing acute haemorrhagic fever in Zaire. Lancet 1, 569–571.
Kim, J.H., Lee, S.R., Li, L.H., Park, H.J., Park, J.H., Lee, K.Y., Kim, M.K., Shin, B.A., Choi, S.Y., 2011. High cleavage efficiency of a 2A peptide derived from porcine teschovirus-1 in human cell lines, zebrafish and mice. PLoS One 6, e18556.
Leroy, E.M., Gonzalez, J.P., Baize, S., 2011. Ebola and Marburg haemorrhagic fever viruses: major scientific advances, but a relatively minor public health threat for Africa. Clin. Microbiol. Infect. 17, 964–976.
Lo, M.K., Nichol, S.T., Spiropoulou, C.F., 2014. Evaluation of luciferase and GFP-expressing Nipah viruses for rapid quantitative antiviral screening. Antiviral Res. 106, 53–60.
Martinez, M.J., Volchkova, V.A., Raoul, H., Alazard-Dany, N., Reynard, O., Volchkov, V.E, 2011. Role of VP30 phosphorylation in the Ebola virus replication cycle. J. Infect. Dis. 204 (Suppl. 3), S934–S940.
Morrey, J.D., Smee, D.F., Sidwell, R.W., Tseng, C., 2002. Identification of active antiviral compounds against a New York isolate of West Nile virus. Antiviral Res. 55, 107–116.
Neumann, G., Feldmann, H., Watanabe, S., Lukashevich, I., Kawaoka, Y., 2002. Reverse genetics demonstrates that proteolytic processing of the Ebola virus glycoprotein is not essential for replication in cell culture. J. Virol. 76, 406–410.
Niwa, H., Yamamura, K., Miyazaki, J., 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene 108, 193–199.
Panchal, R.G., Kota, K.P., Spurgers, K.B., Ruthel, G., Tran, J.P., Boltz, R.C., Bavari, S., 2010. Development of high-content imaging assays for lethal viral pathogens. J. Biomol. Screen. 15, 755–765.
Panchal, R.G., Reid, S.P., Tran, J.P., Bergeron, A.A., Wells, J., Kota, K.P., Aman, J., Bavari, S., 2012. Identification of an antioxidant small-molecule with broad-spectrum antiviral activity. Antiviral Res. 93, 23–29.
Pyrc, K., Bosch, B.J., Berkhout, B., Jebbink, M.F., Dijkman, R., Rottier, P., van der Hoek, L., 2006. Inhibition of human coronavirus NL63 infection at early stages of the replication cycle. Antimicrob. Agents Chemother. 50, 2000–2008.
Schmidt, K.M., Schumann, M., Olejnik, J., Krahling, V., Muhlberger, E., 2011. Recombinant Marburg virus expressing EGFP allows rapid screening of virus growth and real-time visualization of virus spread. J. Infect. Dis. 204 (Suppl. 3), S861–S870.
Schudt, G., Kolesnikova, L., Dolnik, O., Sodeik, B., Becker, S., 2013. Live-cell imaging of Marburg virus-infected cells uncovers actin-dependent transport of nucleocapsids over long distances. Proc. Natl. Acad. Sci. USA 110, 14402–14407.
Silin, D., Lyubomska, O., Ludlow, M., Duprex, W.P., Rima, B.K., 2007. Development of a challenge-protective vaccine concept by modification of the viral RNA-dependent RNA polymerase of canine distemper virus. J. Virol. 81, 13649–13658.
Smee, D.F., McKernan, P.A., Nord, L.D., Willis, R.C., Petrie, C.R., Riley, T.M., Revankar, G.R., Robins, R.K., Smith, R.A., 1987. Novel pyrazolo[3,4-d]pyrimidine nucleoside analog with broad-spectrum antiviral activity. Antimicrob. Agents Chemother. 31, 1535–1541.
Stadler, T., Kuhnert, D., Rasmussen, D.A., du Plessis, L., 2014. Insights into the early epidemic spread of ebola in sierra leone provided by viral sequence data. PLoS Curr. 6, Oct 6 . Edition 1. 10.1371/currents.outbreaks.02bc6d927ecee7bbd33532ec8ba6a25f.
Theriault, S., Groseth, A., Artsob, H., Feldmann, H., 2005. The role of reverse genetics systems in determining filovirus pathogenicity. Arch. Virol. Suppl. 19, 157–177.
Towner, J.S., Paragas, J., Dover, J.E., Gupta, M., Goldsmith, C.S., Huggins, J.W., Nichol, S.T., 2005. Generation of eGFP expressing recombinant Zaire Ebola virus for analysis of early pathogenesis events and high-throughput antiviral drug screening. Virology 332, 20–27.
Tsuda, Y., Hoenen, T., Banadyga, L., Weisend, C., Ricklefs, S.M., Porcella, S.F., Ebihara, H., 2015. An Improved reverse genetics system to overcome cell-type-dependent Ebola virus genome plasticity. J. Infect. Dis., Mar 24. pii: jiu681. [Epub ahead of print].
Uebelhoer, L.S., Albariño, C.G., McMullan, L.K., Chakrabarti, A.K., Vincent, J.P., Nichol, S.T., Towner, J.S., 2014. High-throughput, luciferase-based reverse genetics systems for identifying inhibitors of Marburg and Ebola viruses. Antiviral Res. 106, 86–94.
Volchkov, V.E., Volchkova, V.A., Muhlberger, E., Kolesnikova, L.V., Weik, M., Dolnik, O., Klenk, H.D., 2001. Recovery of infectious Ebola virus from complementary DNA: RNA editing of the GP gene and viral cytotoxicity. Science 291, 1965–1969.