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Retroviral self-inactivation in the mouse vagina induced by short DNA

Lina Wittmer-Elzaouk[a], Jiunshan Jung-Shiu[a], Jochen Heinrich[a], Karin Moelling[a,b,*]

[a] Institute of Medical Virology, University of Zurich, Gloriastrasse 30/32, CH-8006 Zurich, Switzerland
[b] Institute for Advanced Study, Wallotstraße 19, D-14193 Berlin, Germany

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ABSTRACT

Human immunodeficiency virus (HIV) has been shown to undergo self-destruction upon treatment of cell-free virions with partially double-stranded oligodeoxynucleotides targeting the polypurine tract (PPT) of the viral RNA in the virus particle. The ODN forms a local hybrid with the PPT activating the viral RNase H to prematurely cleave the genomic RNA. Here we are describing the self-destruction of a recombinant lentivirus harboring the PPT of HIV in a mouse vagina model. We showed a decrease in viral RNA levels in cell-free virus particles and a reduction reverse transcribed complementary DNA (cDNA) in virus-infected human and primary murine cells by incubation with ODNs. In the vagina simultaneous, prophylactic or therapeutic ODN treatments led to a significant reduction in viral RNA levels. Our finding may have some relevance for the design of other viral self-destruction approaches. It may lead to a microbicide for reduction of sexual and mother-to-child transmission.

1. Introduction

We have previously demonstrated that human immunodeficiency virus (HIV-1) can be in activated in vitro and rendered non-infectious by an oligodeoxynucleotide (ODN). The ODN A is a partially double-stranded 54-mer hairpin-loop-structured DNA designed to bind specifically to the polypurine tract, PPT, of HIV-1, a highly conserved region of the HIV genome. The DNA forms a local RNA–DNA hybrid with the viral RNA genome and mimics a natural replication intermediate (Jendis et al., 1998, 1996; Moelling et al., 2006). The hybrid is a substrate for the RT/RNase H (Moelling et al., 1971) and is specifically cleaved to generate the primer for the (+) strand DNA synthesis (Wöhrl and Moelling, 1990). The RT/RNase H forms a heterodimer, which is located inside the virus particles and then carried into the cell during infection. Therefore the inhibitory action of ODN A can be exerted already in the viral particles before they infect the cell (Matskevich et al., 2006).

Recently, we demonstrated in a retrovirus mouse model that an ODN that targets the PPT of the retrovirus can reduce disease progression, prevent infection or reduce the viral load in the blood (Matzen et al., 2007). However, this murine oncogenic retrovirus model does not reflect the situation of sexual HIV transmission. Therefore we tested an application of ODN against a lentiviral vector applied to the mouse vagina as a model for sexual transmission. The lentiviral vector FUGW (Flap, ubiquitin promoter, GFP and WRE vector) contains the HIV–PPT sequence, which is essential for its single replication round. Here we demonstrate that FUGW present in the mouse vagina or in human vaginal or cervical cell lines can be treated with ODN A. We applied chemical modifications of the ODN such as phosphorothioates or 2′-*O*-methyl groups to protect against nucleases and increase stability. In all cases, statistically significant reduction of FUGW virus RNA copies was observed with ODNs compared to their respective randomized sequence serving as negative controls. The approach we are using differs from previous ones, since it is based on the activation of a retroviral enzyme for destruction of the virus, instead of inhibition. This may be worth developing further.

2. Materials and methods

2.1. Oligodeoxynucleotides

All oligodeoxynucleotides (ODNs) were phosphorothioated at each end and in the T4 linker and have the same length (54-mer). ODN A consists of an antisense strand of PPT and a passenger strand. ODN AM is the methylated version of the ODN A (Fig. 1A). ODN S2 consists of a randomized sequence of both strands serving as a control for non-specific activity of ODN A. ODN S2 M is therefore the methylated sequence of the ODN S2 (Fig. 1A). The ODNs were purchased from Operon or Integrated DNA Technology.

* Corresponding author. Tel.: +41 44 634 1929; fax: +41 44 634 49 17.
E-mail address: moelling@imm.uzh.ch (K. Moelling).

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2.2. Lentiviral vector production

FUGW is a lentiviral self-inactivating vector with a VSV-G coat, which allows transduction of many cell-types but does not replicate. This lentiviral vector contains the PPT sequence that is identical to that of HIV. For generating high titer lentiviral vectors, 7×10^6 human embryonal kidney (HEK) 293T cells in 10 cm plates were co-transfected with cFUGW, pCMVR8.9, and pHCMV-G (5 μg each) using lipofectamine 2000 (Invitrogen). Six hours post-transfection 8 ml fresh medium replaced the original medium. Two days post-transfection the culture medium was collected and filtered through a 0.45 μm filter. The filtrated medium was then ultracentrifuged twice for 90 min using Polyallomer tubes. Titers of viral preparations were assessed by infecting 5×10^5 C81–66 cells with serial dilution of the virus and determining the fraction of GFP-positive cells 1 day after infection by FACS. Titers of $5–9 \times 10^7$ transducing units (TU) per ml were routinely obtained.

2.3. RT/RNase H cleavage assay

HIV–PPT RNA was in vitro transcribed, dephosphorylated and 5′-phosphorylated as described (Matskevich et al., 2006). Different ODNs (10 nM) and HIV–PPT RNA (10 nM) in RT buffer were annealed (2 min 90 °C, 10 min 37 °C) and cleaved with 0.1 U/μl RT/RNase H (GE Healthcare, Piscataway, NJ) for 30 min at 37 °C. The samples were subjected to electrophoresis in 10% polyacrylamide containing 8 M urea, together with HIV–PPT RNA partially digested with RNase T_1 (Ambion) as described by the manufacturer.

2.4. Virions studies

FUGW virions (10^4 TU) were either treated with different concentrations of ODN A (0–25 μM) for titration or different ODNs for 4 h at 37 °C. These virions were also titrated using either 10^4 IU or 10^5 IU with 5 μM ODN A for also 4 h at 37 °C. Viral RNA was then extracted using the viral RNA mini kit (Qiagen) and eluted with 50 μl elution buffer. Then 5 μl RNA was used for cDNA synthesis in a reaction volume of 12.5 μl and 5 μl of the cDNA was used for qPCR.

2.5. In vitro studies

Both cell lines VK2/E6E7 and Ect1/E6E7 were purchased from the American Type Culture Collection (ATCC). The VK2/E6E7 (ATCC CRL-2616) cell line was established from normal vaginal mucosal tissue taken from a premenopausal woman undergoing anterior–posterior vaginal repair surgery. The ectocervical Ect1/E6E7 (ATCC CRL-2614) cell line was established from normal epithelial tissue taken from a premenopausal woman undergoing hysterectomy for endometriosis (Fichorova et al., 1997). These two cell lines were seeded in a 24-well-plate at a density of 1×10^5 cells/well in Keratinocyte-Serum Free medium (Invitrogen) supplemented with 0.1 ng/ml human recombinant epidermal growth factor (EGF, Sigma–Aldrich), 0.05 mg/ml bovine pituitary extract (Invitrogen), and additional 44.1 mg/ml calcium chloride (final concentration 0.4 mM). Cells were infected with 10^4 IU of FUGW resulting in a multiplicity of infection (MOI) of 0.1, for 2 days at 37 °C. Cells were washed twice with PBS and then genomic DNA (gDNA) was extracted using the blood DNA mini kit (Qiagen) and eluted in 30 μl elution buffer. For qPCR analysis, 100 ng of total gDNA was used in a reaction volume of 25 μl. Primary vaginal cells extracted from mouse vaginal lavage fluid were cultured in epithelial cell medium (ECM) as described (Macartney et al., 2000). ECM consists of equal volumes of phenol-red-free DME (Sigma–Aldrich) and Ham's F-12 medium (Invitrogen) supplemented with 10% FCS, 100 mg/ml streptomycin, 100 IU/ml penicillin, 1 mmol/ml L-glutamine, and 10 ng/ml EGF. The vaginal cells at a density of 5×10^4 cells in 500 μl volume were infected with 10^4 FUGW TU corresponding to a MOI of 0.2 for 1 day at 37 °C. Cells were washed twice with PBS and then gDNA was extracted and eluted in 30 μl elution buffer. For PCR, 40 ng of total gDNA was used in a reaction volume of 25 μl.

2.6. Mouse model and FUGW virus challenge

Six- to eight-week old female C57BL/6 mice purchased from Harlan (Zeist) were used throughout these studies. Animal studies were performed according to Swiss Animal Rights in the animal facilities of the Institute of Medical Virology, University of Zurich, with permission by the Zurich-Veterinary-Office (213/00). Mice were kept in conventional conditions with full access to food and water. All mice were treated s.c. with 2.5 mg of progestin (Depo-Provera, Pfizer) to synchronize the estrus cycle (Khanna et al., 2002). One week later, the progestin-treated mice were anesthetized with isoflurane (ABBOTT AG) and challenged with an intravaginal inoculum of 20 μl 3% carboxymethyl cellulose sodium (CMC) medium (Roberts et al., 2007) (Sigma–Aldrich) containing 10^4 IU FUGW with or without 25 μM ODNs. Four hours later the mice were euthanized and vaginal lavage with 100 μl of sterile PBS was performed. Viral RNA was then extracted from these vaginal lavage fluids using the viral RNA mini kit (Qiagen).

2.7. Detection of FUGW by Real-Time PCR

For qRT-PCR RNA was reverse transcribed into cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems) according to the Manufacturer's instructions.

Primers and Probes flanking the PPT sequence of FUGW were: 5′-GAGGAGGTGGGTTTTCCAGT-3′ (forward), 5′-GGGAGTGAATTAGCCCTTCC-3′ (reverse), and FAM-5′-ACCTTTAAGACCAATGACTTACAAGGCAGC-3′-TAMRA (probe); for mouse glyceraldehyde-3 phosphate dehydrogenase (mGAPDH), 5′-CTTCACCACCATGGAGAAGGC-3′ (forward), 5′-GGCATGGACTGTGGTCATGAG-3′ (reverse). FAM-5′-CCTGGCCAAGGTCATCCATGACAACTTT-3′-TAMRA; for human glyceraldehyde-3 phosphate dehydrogenase (hGAPDH), 5′-GTTCCAATATGATTCCACCC-3′ (forward), 5′-GAAGATGGTGATGGGATTTC-3′ (reverse), FAM-5′-CAAGCTTCCCGTTCTCAGCCTAMRA-3′ (probe). All primers and probes were purchased from Microsynth. The cycling conditions were 50 °C for 2 min (1 cycle), 95 °C for 10 min (1 cycle), 95 °C for 15 s and 60 °C for 1 min (50 cycles). The results are presented in the figures as FUGW RNA or DNA corresponding to absolute copy numbers per assay.

2.8. Statistical analysis

The statistical significance of the antiviral activity of ODN A and ODN AM in virions and in vitro was determined by Student's *T*-test and the results were expressed as mean ± SEM (error bars in graph). These *P*-values were for two-tailed significance test. For the in vivo studies, viral RNA levels after treatment were compared by analysis of variance with Bonferroni post-hoc test applied to logarithmically transformed and PBS normalized data using SPSS 13.0 (SPSS Inc., IL). Differences were considered to be significant at $P < 0.01$.

3. Results

3.1. Analysis of ODNs and lentiviral system

In order to establish an animal model for the antiviral activity of ODN we used the lentiviral vector FUGW (Fig. 1A). FUGW is a lentiviral self-inactivating vector, which carries a green fluorescent protein, GFP, reporter driven by an internal ubiquitin promoter.

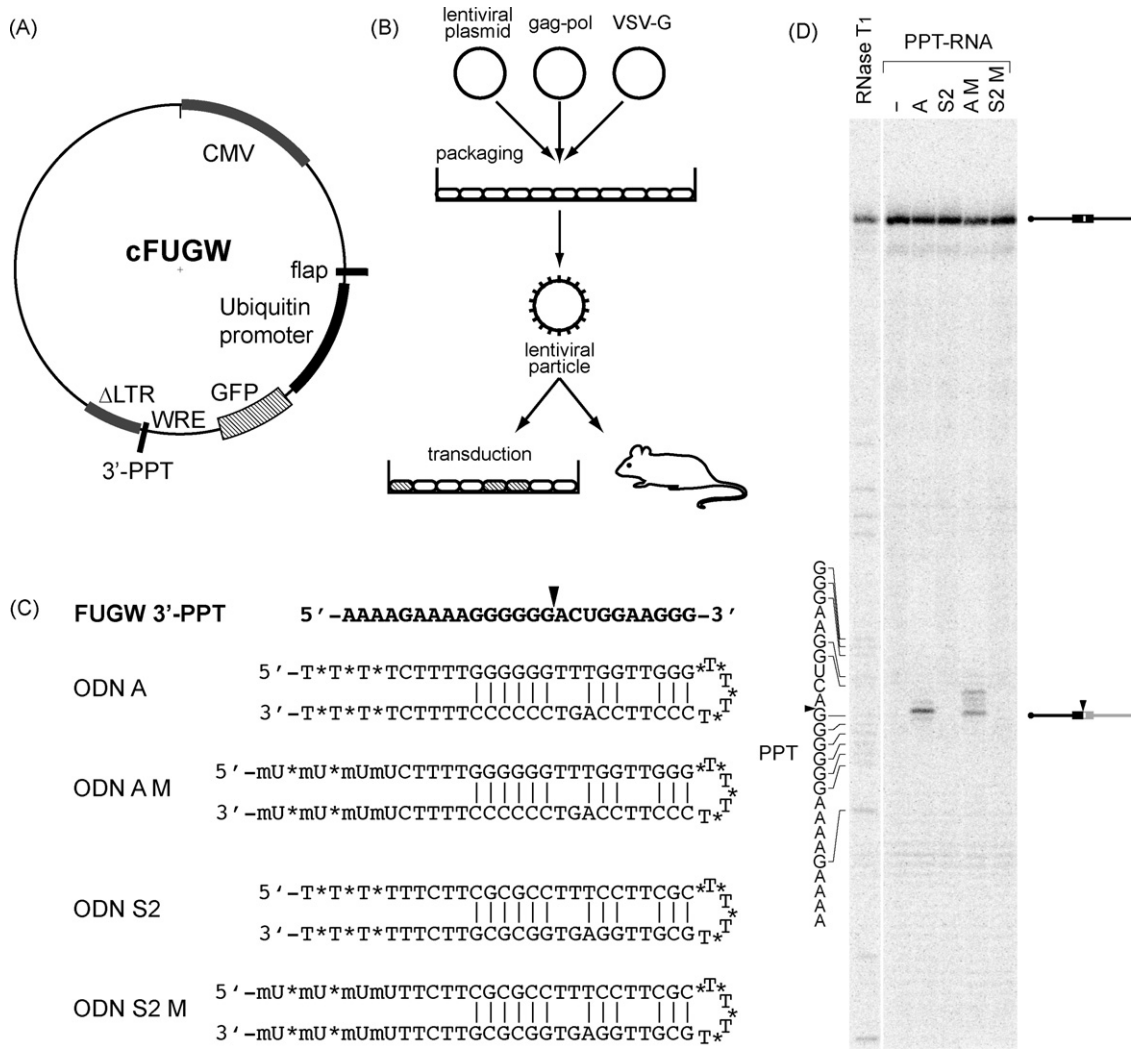

Fig. 1. Oligodeoxynucleotides and FUGW system and RT/RNase H. (A) Recombinant lentiviral vector cFUGW harboring the HIV-3′-PPT sequence. The vector contains the cytomegalovirus enhancer (CMV), the HIV-1 flap region, the human ubiquitin C promoter for transcription of GFP, the woodchuck hepatitis virus post-transcriptional regulatory element (WRE) and an inactive 3′-ΔLTR. (B) Schematic representation of the three-plasmid expression system used for generating lentiviral particles by transient transfection consisting of cFUGW (lentiviral plasmid), pHCMV-G (VSV-G), and pCMVΔR8.9 (gag–pol). Viral particles produced were used either for transduction of cells or for in vivo application. (C) Sequences of the FUGW-3′-PPT and of ODNs. The cleavage site by RT/RNase H is indicated by an arrowhead (phosphorothioates, *; 2′-*O*-methyl, m). (D) Cleavage of PPT–RNA by RT/RNase H. PPT–RNA annealed to ODNs was incubated with recombinant RT/RNase H and subjected to denaturing PAGE. In parallel PPT–RNA partially digested with RNase T_1 was used as a marker.

Lentiviral particles are produced by co-transfection of the lentiviral vector plasmid cFUGW, the packaging plasmids pCMVΔR8.9 encoding gag and pol, and pHCMV-G encoding the vesicular stomatitis virus envelope glycoprotein VSV-G (Fig. 1B). FUGW particles with the VSV-G envelope protein are infectious, but allow only one round of infection with no new virus production. These particles were used to infect cells or to conduct experiments in the mouse vagina. We are targeting the PPT of FUGW with a hairpin-loop-structured ODN, which hybridizes with one strand to the PPT and thereby presents a substrate for the RT/RNase H, which leads to cleavage of the RNA moiety in the local hybrid. The PPT of FUGW is identical to that of HIV as shown in Fig. 1C. The ODN contains phosphorothioates at the ends and in the central linker regions to protect against nucleases and to increase its stability and longevity. Additional modifications were 2′-*O*-methylated nucleotides, ODN AM. We designed control ODNs, which differed in the sequence of C and G (Fig. 1C).

To demonstrate ODN A-mediated hydrolysis by the RT/RNase H, we cleaved labelled PPT–RNA, which contains 167 nucleotides of the PPT region of FUGW. The PPT–RNA was hybridized to ODNs and incubated with RT/RNase H for 0.5 h at 37 °C. In parallel, the PPT–RNA was sequenced by RNase T_1 to determine the specific cleavage site. Only ODN A and ODN AM, but not ODN S2 or ODN S2 M led to specific cleavage 5′ to ACU, which is the natural cleavage site (Fig. 1D).

3.2. ODN A and ODN AM have an inhibitory effect on virions

To test for cleavage of viral RNA in lentiviral particles, purified FUGW virions were treated with different concentrations of ODN A for 4 h at 37 °C. Then the viral RNA was purified and determined by qRT-PCR using primers flanking the PPT. These primers do not yield an amplification product, when the genomic RNA has been cleaved by RT/RNase H upon binding of ODN A to the PPT. The viral RNA levels in the virions were significantly reduced in a dose-dependent manner. With 5 μM ODN A the reduction was 71% ($P < 0.01$) and was used for further experiments. Similarly, when different virus titers were used, the reduction was less pronounced for higher virus titers (60%, $P = 0.023$, Fig. 2B) in comparison to lower titers (80%, $P = 0.008$). To assess the effect of all different ODNs on virions, cell-

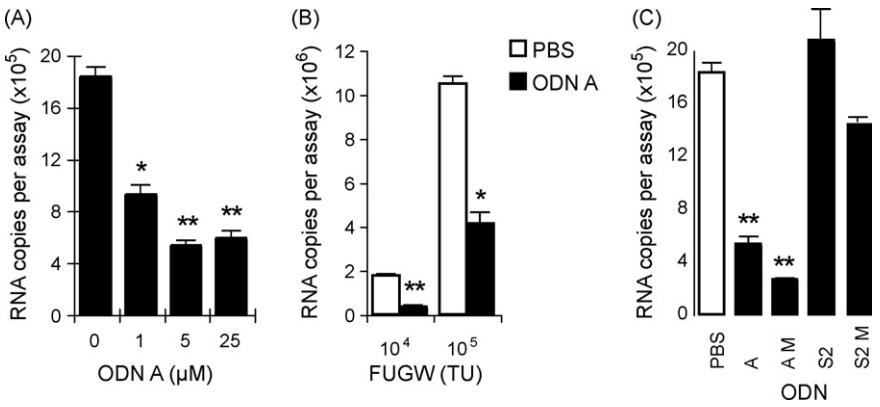

Fig. 2. Effect of ODNs on FUGW virions. (A) Cell-free FUGW virions were incubated with ODN A at the indicated concentrations for 4 h at 37 °C. The genomic RNA of lentiviral particles was purified and quantified by PPT-specific qRT-PCR. Bars represent mean values ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$. $n = 3$. (B) Titration of FUGW-virions (10^4 or 10^5 TU) with 5 μM ODNs for 4 h at 37 °C. RNA was quantified by qRT-PCR. Bars represent mean values ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$. $n = 2$. (C) Effect of 5 μM ODNs on 10^4 FUGW TU for 4 h at 37 °C. RNA was quantified by qRT-PCR. Bars represent mean values ± SEM. $^{**}P < 0.01$. $n = 3$.

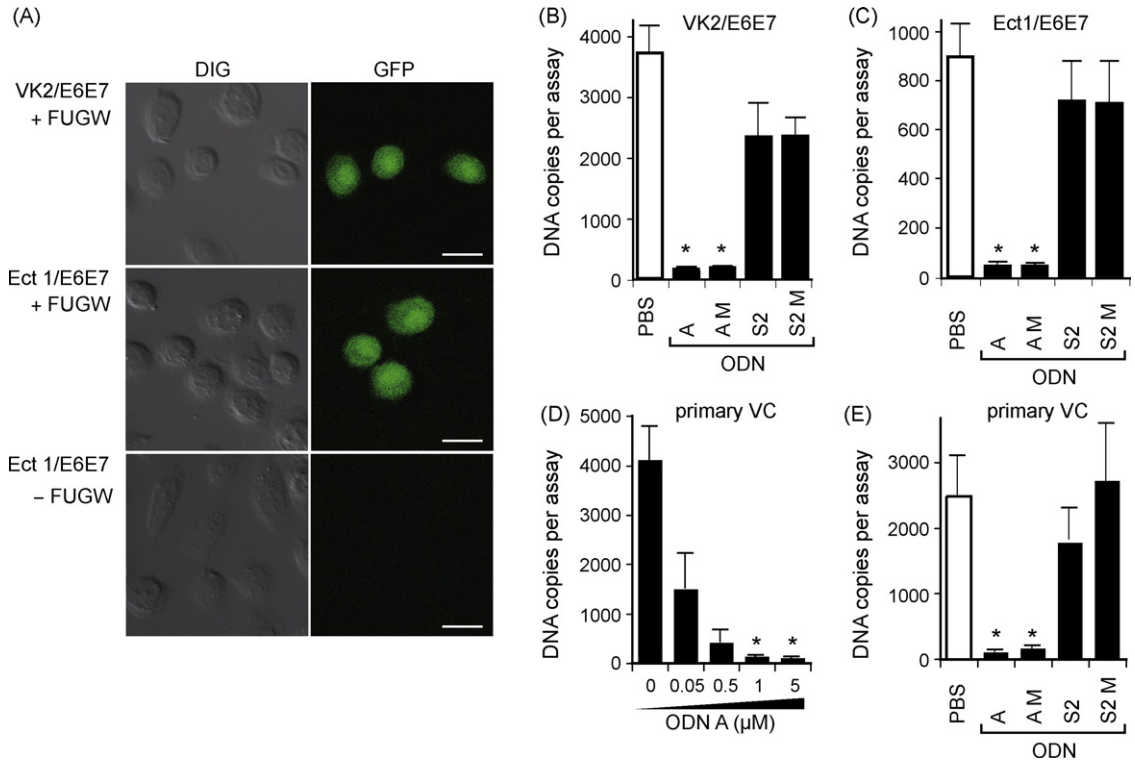

Fig. 3. Effect of ODNs on FUGW-infected cells. (A) Expression of GFP in infected human vaginal VK2/E6E7 and cervical Ect1/E6E7 cell lines. The cells were transduced with FUGW at a multiplicity of infection MOI = 0.2. After 2 days the expression of GFP was detected by confocal microscopy. The differential interference contrast (DIC, left panel) and the GFP signal (right panel) are shown. Bars, 20 μm. (B–E) Cells were infected with 10^4 FUGW TU in the presence of 1 μM ODN as indicated and incubated for 1 or 2 days. DNA was isolated and quantified by qPCR using human or mouse GAPDH for standardization. Data are shown as mean ± SEM. $^{*}P < 0.05$. VK2/E6E7 (B, $n = 6$) and Ect1/E6E7 (C, $n = 6$). Primary vaginal cells extracted from vaginal lavage fluid were infected in the presence of the indicated concentrations of ODN A (E, $n = 3$) or different ODNs (D, $n = 6$) for 1 day.

free FUGW particles were treated with ODN A, –AM, –S2, and –S2 M for 4 h at 37 °C (Fig. 2C). A reduction of FUGW RNA was observed for ODN A (71%, $P = 0.0063$ versus PBS, $P = 0.0337$ versus ODN S2) and ODN AM (86%, $P = 0.0037$ versus PBS, $P = 0.0015$ versus ODN S2 M) but not for their respective controls, ODN S2 and ODN S2 M. ODN AM was slightly more active than ODN A.

3.3. Effect of ODNs on FUGW-infected cells

FUGW transduces cells through one round of replication leading to provirus-containing cells, which then express GFP. However, no new virus progeny is produced from these cells, since FUGW is replication-defective.

In order to analyze the effect of ODNs in human vaginal cells, we transduced the human vaginal (VK2/E6E7) and cervical (Ect1/E6E7) cell lines with FUGW (Fig. 3A). The cells were tested in the absence and presence of different ODNs. Since we and others noticed that siRNA can reduce GFP expression from integrated provirus without reducing transduction frequency (Westerhout et al., 2006), we decided to measure relative DNA copy numbers of the lentivirus, which directly correlate with the number of transduced cells. Genomic DNA (gDNA) was purified from the infected cells after

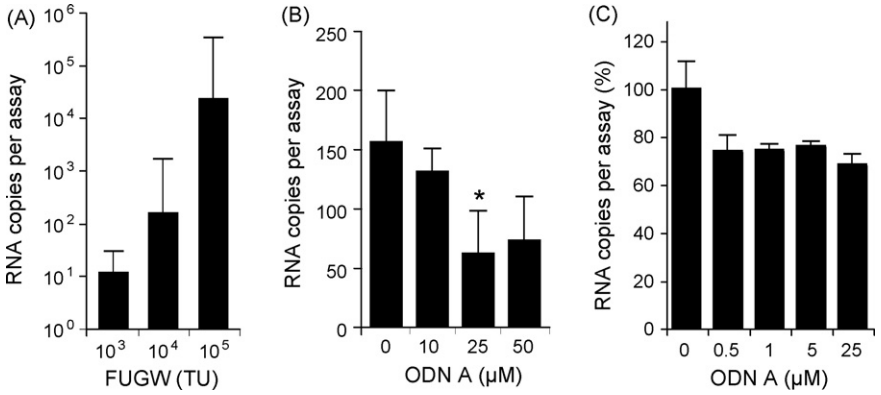

Fig. 4. Establishing the lentivirus mouse vagina model. (A) Different amounts of FUGW were applied in the mouse vagina. After 4 h a vaginal lavage was performed and the RNA isolated from the lavage fluid and analyzed by PPT-specific qRT-PCR. Bars represent mean values ± SEM of RNA. $n = 7$ mice per group. (B) Titration of ODN A intravaginally with 10^4 FUGW TU. FUGW was applied together with increasing concentrations of ODN A for 4 h and FUGW RNA was measured as described in (A). Bars represent mean values ± SEM. $^*P < 0.05$. $n = 4$ mice per group. (C) ODN does not interfere with RT-PCR assays. Purified FUGW RNA was incubated for 1 h with different concentrations of ODN A, repurified and RNA levels determined by qRT-PCR performed. Bars represent mean values ± SEM. $n = 3$.

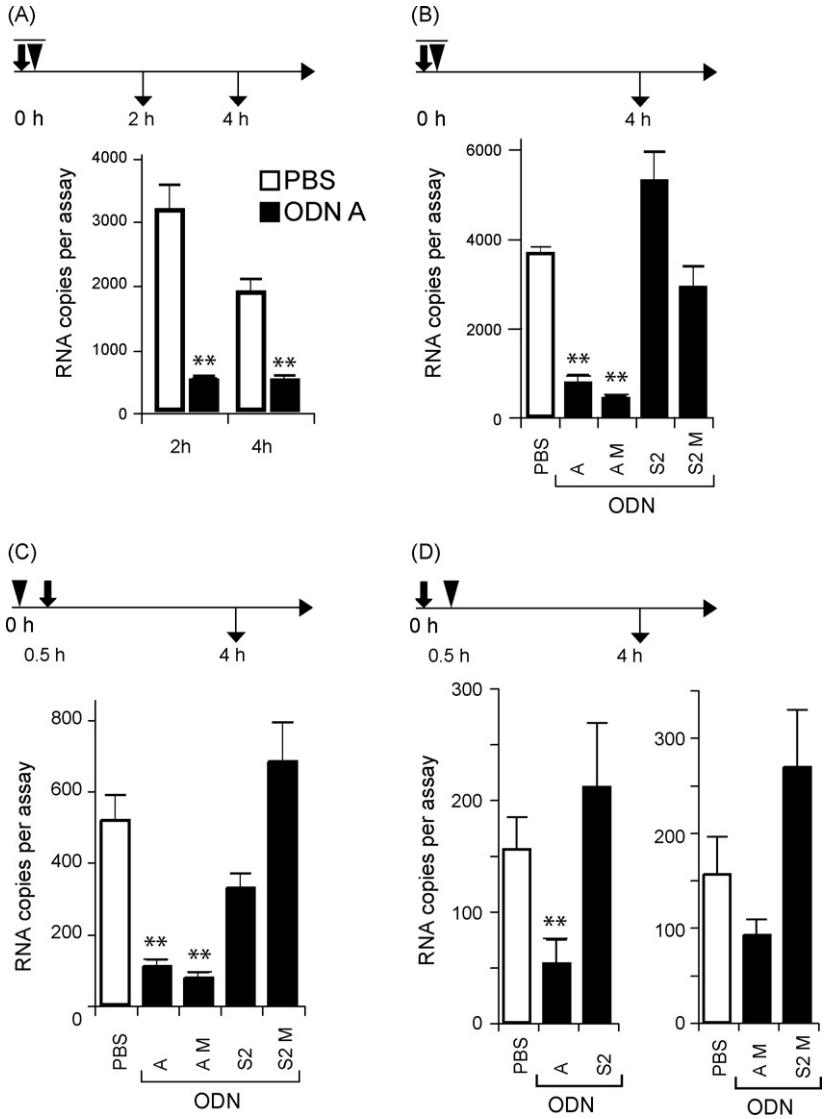

Fig. 5. Effect of ODN treatment on FUGW in the mouse vagina. C57BL/6 mice were treated with 10^4 FUGW TU and ODN in the vagina. Two or four hours later RNA was extracted from vaginal lavage fluids and FUGW RNA levels were determined by qRT-PCR. Thick arrow, FUGW infection; arrowhead, treatment with ODN; thin arrow, vaginal lavage. Bars indicate mean values ± SEM of RNA. $^{**}P < 0.01$. (A, B) Co-application of FUGW and 50 μM ODN A (A, $n = 50$ mice per group) and with different ODNs at 25 μM (B, $n = 16$ mice per group). (C) For the prophylactic regimen 25 μM ODNs were applied 0.5 h before FUGW. $n = 13$ mice per group. (D) In a therapeutic setting FUGW was applied 0.5 h before treatment with 25 μM ODNs. $n = 13$ mice per group.

Table 1
Reduction of FUGW RNA levels upon treatment with ODNs in the mouse vagina.

ODN	*n*	Mean RNA level (%)	Std. error (%)	95% Confidence interval		*p*-values in comparison to				Percentage of more than 10-fold reduced RNA levels
				Lower bound (%)	Upper bound (%)	ODN AM	ODN S2	ODN S2 M	PBS	
Co-application										
ODN A	87	30.36	0.09	21.64	42.59	1	5.12×10^{-14}	6.96×10^{-5}	7.49×10^{-13}	33
ODN AM	30	18.95	0.10	11.87	30.32	n.a.	1.30×10^{-12}	1.37×10^{-5}	1.78×10^{-10}	38
ODN S2	37	210.70	0.10	136.04	325.90		n.a.	0.736	0.276	1
ODN S2 M	20	110.58	0.10	64.34	190.03			n.a.	1	4
PBS	87	100.00	0.09	71.28	140.30				n.a.	3
Prophylactic										
ODN A	40	20.88	0.12	15.11	28.86	1	0.006	1.36×10^{-5}	1.22×10^{-8}	30
ODN AM	15	14.65	0.13	8.72	24.63	n.a.	0.001	3.71×10^{-6}	5.94×10^{-8}	35
ODN S2	30	53.69	0.12	37.17	77.43		n.a.	0.336	0.128	0
ODN S2 M	15	106.88	0.13	63.66	179.73			n.a.	1	2
PBS	40	100.00	0.12	72.35	138.21				n.a.	1
Therapeutic										
ODN A	15	22.25	1.04	14.46	34.22	0.013	4.49×10^{-6}	2.56×10^{-8}	1.98×10^{-6}	17
ODN AM	15	61.43	1.04	39.94	94.48	n.a.	0.340	0.012	0.696	2
ODN S2	15	118.96	1.04	77.28	182.82		n.a.	1	1	0
ODN S2 M	15	171.55	1.04	111.48	263.74			n.a.	0.452	0
PBS	30	100.00	0.98	73.75	135.59				n.a.	0

The data of all in vivo experiments including experiments not shown are summarized and statistically analyzed. The number of mice, the mean RNA level and standard error, the lower and upper bound of the 95% confidence interval as well as *P*-values for each combination of ODNs are shown. $P < 0.01$ is considered as significant. From the distribution of differences the percentage of responders with a more than 10-fold reduced FUGW RNA levels was calculated. n.a., not applicable.

2 days incubation at 37 °C and analyzed by FUGW-specific qPCR (Fig. 3B and C) to measure the reverse transcribed cDNA or proviral DNA. A significant decrease of 95% of FUGW DNA copies was determined for ODN A and ODN AM in comparison to the controls for both cell lines.

Next we used murine primary vaginal cells (VC), which were obtained by lavage of mouse vagina and infected them with FUGW using different concentrations of ODN A from 0.05 to 5 μM (Fig. 3D). The isolated DNA was analyzed by qPCR. The results indicate that ODN A exhibited a dose-dependent reduction of FUGW DNA copies with the strongest antiviral effect ($P = 0.0297$) at 1 μM. Subsequently, all ODNs were tested under the same conditions (Fig. 3E). As can be seen, a significant decrease in FUGW DNA copies was observed for ODN A (96%, $P = 0.02$ versus PBS and $P = 0.0249$ versus ODN S2) and ODN AM (94%, $P = 0.0233$ versus PBS and $P = 0.0406$ versus ODN S2 M) compared to their respective controls (Fig. 3E).

3.4. Assessment of ODNs in vivo

In order to establish the conditions for ODN treatment in the in vivo model, we applied three increasing doses of FUGW (10^3, 10^4 and 10^5 TU) into the vagina in 20 μl containing 3% carboxymethyl cellulose, in order to prevent leakage of the virus. Four hours after infection, a vaginal lavage was performed, viral RNA purified and genomic FUGW RNA detected by qRT-PCR using PPT-flanking primers. The lavage fluid contained FUGW RNA levels, which correlated with the dose of the applied lentivirus (Fig. 4A). Based on these results, we used 10^4 TU FUGW for further experiments. First we assessed the effect of ODN A on FUGW in the mouse vagina by co-application of three concentrations of ODN A for 4 h. The reduction of FUGW cDNA was dose-dependent. At a concentration of 25 μM the reduction was 61% compared to absence of ODN ($P = 0.0269$ shown in Fig. 4B).

In order to exclude the possibility that ODN A interfered with the RT-PCR system, we performed a control. We used purified FUGW RNA from 10^4 TU and added increasing ODN A concentrations and subsequently purified the amount of RNA. It was then tested by RT-PCR and amplification was only weakly affected and this effect was independent on the amount of ODN A (Fig. 4C). A similar control has been described previously (Matzen et al., 2007).

3.5. Use and effect of ODNs in lentiviral mouse vaginal model

For the evaluation of ODN-mediated inhibition of FUGW in vivo we decided to apply three different treatment regimens, namely co-application, prophylactic and therapeutic regimens. First FUGW and 50 μM ODN A were co-applied and FUGW RNA levels were determined after 2 and 4 h. A significant four- to seven-fold decrease ($P < 10^{-6}$) of viral RNA levels in the presence of ODN A was observed (Fig. 5A). For the following treatments, 10^4 FUGW TU and 25 μM ODNs were used. The co-application into the vagina of FUGW and different ODNs for 4 h showed that ODN A and ODN AM exerted a strong reduction of viral RNA in comparison to the controls ODN S2 and ODN S2 M (Fig. 5B). In the prophylactic treatment regimen ODNs were applied 30 min before lentivirus infection (Fig. 5C). Also under these conditions ODN A and ODN AM, but not ODN S2 or ODN S2 M led to significantly decreased viral titers. Finally, in the therapeutic treatment (Fig. 5D) the lentivirus was applied 30 min before ODNs. In the case of ODN A the intravaginal titer of FUGW RNA was reduced by 78%. A reduction by ODN AM was also detected (Fig. 5D).

4. Discussion

Here we are demonstrating the role of the RNase H in the antiviral effect of ODNs in the mouse vagina. We first demonstrated the cleavage of the PPT–RNA in presence of ODN A and ODN AM in vitro and mapped the cleavage site (Fig. 1D), which is adjacent to the ACU site and identical to the natural RNase H cleavage site for generation of the RNA primer for the second strand DNA synthesis. Using lentiviral particles (Lois et al., 2002) harboring the HIV-1IIIB-type 3′-PPT (Fig. 1A, B and C), we demonstrate the antiviral effect by incubating these particles with ODN A or ODN AM in vitro (Fig. 2). Accordingly, also the infectivity of the treated virions was significantly reduced with ODN A and ODN AM in human vaginal or cervical cell lines as well as in mouse primary vaginal cells (Fig. 3). Next we established an in vivo model to test the reduction of lentiviral genomic RNA in the mouse vagina and excluded the possibility that ODN A would interfere with the PCR reaction (Fig. 4). ODN A, ODN AM and control ODNs are not toxic up to 50 μM according to a cell proliferation assay (data not shown). The inhibitory effect of ODN A and ODN AM was dose-dependent and control ODNs exhib-

ited only a very weak or no inhibition at low doses as described before (Heinrich et al., 2009; Matskevich et al., 2006; Matzen et al., 2007).

In the lentiviral mouse vaginal model, we evaluated different therapeutic regimens. We treated mice first by applying FUGW and ODNs simultaneously (Fig. 5A and B). Then we infected the mice in the vagina with FUGW and applied ODNs earlier (Fig. 5C) or later (Fig. 5D). In these different treatment regimens, simultaneous, prophylactic or therapeutic, ODN A and ODN AM led to reduction of lentiviral load as summarized in Table 1. ODN A significantly induced reduction of FUGW in all settings. Also ODN AM led to a reduction. It was slightly more active in simultaneous and prophylactic ODN applications. In these simultaneous and prophylactic settings, based on the statistical analysis of the distribution of our data, we expected between 30% and 38% responders with more than 10-fold reduction of the RNA levels when treated with ODN A and ODN AM, whereas in the therapeutic treatment with ODN A, we expected 17% responders with 10-fold reduction of RNA levels (Table 1). Thus, in this study we were able to show a proof of concept that ODNs are able to induce self-destruction of the viruses by degrading the viral RNA in the mouse vagina. Double-stranded ODNs are more stable than single-stranded DNA and this stability is increased by phosphorothioate-modifications in ODNs and by additional 2′-*O*-methyl groups in ODN AM. Other chemical modifications are feasible and may improve the stability of the ODN significantly. Recently locked nucleic acid (LNA) DNA has been shown to be stable for weeks (Crinelli et al., 2004).

We described a novel approach, which is based on the activation rather than the inhibition of a retroviral enzyme. This may be worth testing for other substances and in other cases as well. The activation of the viral RNase H and thereby induced self-destruction of the RNA by treatment of virus particles suggests that our approach may lead to the inactivation of viral infectivity outside of the cell. One of the most prominent examples where this is relevant would be prevention of sexual and mother-to-child transmission.

Previously we showed in vivo efficacy against a murine retrovirus, the spleen focus forming virus, SFFV, in mice (Matzen et al., 2007). In this case, the ODN used was designed to the PPT sequence of SFFV and resembles ODN A. In the study of Matzen et al., chronically infected mice exhibited either transient or long-term reductions of virus titer depending on the therapeutic regimen. This treatment destroys the viral RNA template in virus particles in plasma as well as early retrovirus replication intermediates in infected cells (Jendis et al., 1998, 1996; Matzen et al., 2007). However, SFFV is an oncogenic retrovirus, which reflects only some of the properties of HIV. Therefore we analyzed here the lentiviral vector FUGW.

In all cases tested the decrease of viral RNA and infectivity was not complete, raising the question, whether a population of viruses exists, which is resistant against ODN A. Putative mutations may reduce the antiviral effect but could theoretically be overcome by homologous ODN mixtures. So far very few studies have achieved reproducible protection against intravaginal viral challenge (Abel et al., 2003; Miller and Abel, 2005). The vaginal milieu has lead recently to unexpected results in Phase III-trial with a polyanion as microbicide, which enhanced the HIV-infection in the vagina (www.aidsinfo.nih.gov). Instead of unspecific microbicides used (McCormack et al., 2001; Stone, 2002), we believe that self-destruction of HIV particles before or early during infection, as described here, could stimulate the design of novel approaches for the control of HIV.

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