[The following lines are extracts for the purposes of this exercise]

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Gene- and Protein-Delivered Zinc Finger–Staphylococcal Nuclease Hybrid for Inhibition of DNA Replication of Human Papillomavirus
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Previously, we reported that artificial zinc-finger proteins (AZPs) inhibited virus DNA replication in planta and in mammalian cells by blocking binding of a viral replication protein to its replication origin. However, the replication mechanisms of viruses of interest need to be disentangled for the application. To develop more widely applicable methods for antiviral therapy, we explored the feasibility of inhibition of HPV-18 replication as a model system by cleaving its viral genome. To this end, we fused the staphylococcal nuclease cleaving DNA as a monomer to an AZP that binds to the viral genome. The resulting hybrid nuclease (designated AZP–SNase) cleaved its target DNA plasmid efficiently and sequence-specifically in vitro. Then, we confirmed that transfection with a plasmid expressing AZP–SNase inhibited HPV-18 DNA replication in transient replication assays using mammalian cells. Linker-mediated PCR analysis revealed that the AZP–SNase cleaved an HPV-18 *ori* plasmid around its binding site. Finally, we demonstrated that the protein-delivered AZP–SNase inhibited HPV-18 DNA replication as well and did not show any significant cytotoxicity. Thus, both gene- and protein-delivered hybrid nucleases efficiently inhibited HPV-18 DNA replication, leading to development of a more universal antiviral therapy for human DNA viruses.

Transient replication assays were performed essentially as previously described [3]. A total of 8×10^5 cells of the human cell line 293H (Invitrogen) were plated onto a BioCoat poly-D-lysine 12-well plate (Becton Dickinson) and maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 0.1 mM nonessential amino acids and 10% fetal bovine serum (Invitrogen). Three plasmids necessary for transient replication, pRL-E1 (1.5 μg), pRL-E2 (0.17 μg), and pOri derivative (0.17 μg), were cotransfected with pCMV-AZP–SNase, pCMV-AZP, or a vacant plasmid, pcDNA3.1 (0.17 μg) by using Lipofectamine 2000 (Invitrogen) according to the protocol accompanying the reagent.

Each Hirt-extracted DNA sample from the transient replication assays described above was treated with T4 polynucleotide kinase to remove 3′-phosphoryl groups generated by AZP–SNase cleavage [29] and added 5′-phosphates at 37°C for 30 min, and then treated with T4 DNA polymerase to blunt staggered DNA lesions at 12°C for 15 min. After phenol extraction, the blunted DNA was ligated with DNA Ligation Kit Ver.2.1 (Takara, Shiga, Japan) to 100 pmol of a double-stranded blunt-ended linker, which was generated by annealing *5′-GCGGTGACCCGGGAGATCTGAATTC-3′* and *5′-GAATTCAGATC-3′* [40], in a total volume of 10 μl overnight at 16°C. The ligated DNA sample (0.5 μl) was amplified by PCR with the primer set of the linker-specific primer *5′-GCGGTGACCCGGGAGATCTGAATTC-3′* and biotin-labeled pOri-specific primer *5′-CAGCTGGCACGACAGGTTTCCCGACTGGAA-3′*. The PCR conditions were as follows: initial denaturation at 94°C for 3 min and 10 cycles of heating (94°C, 30 s), annealing (66°C, 2 min), and elongation (72°C, 1 min). The PCR product was purified by using a QIAGEN PCR purification kit (Qiagen, Valencia, CA, USA) and further purified with Dynabeads M-270 Streptavidin (Invitrogen, Carlsbad, CA, USA) according to the protocol accompanying the reagent. The purified sample was used as the template for a second round of PCR with the primer set of the linker-specific primer and pOri-specific primer. The PCR conditions were initial denaturation at 94°C for 3 min and 30 cycles of heating (94°C, 30 s), annealing (66°C, 2 min), and elongation (72°C, 1 min).

The cytotoxicity of AZP–SNase–R9 was evaluated by an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. A total of 6×10^4 of 293H cells were plated onto a BioCoat™ poly-D-lysine 96-well plate (Becton Dickinson, Franklin Lakes, NJ, USA) and maintained in 0.1 ml of Dulbecco's modified Eagle's medium supplemented with 0.1 mM nonessential amino acids and 10% fetal bovine serum. An AZP–SNase–R9 solution (10 μl in Opti-MEM) was added to the culture medium. After incubation for 3 days, 10 μl of the solution from a Cell Counting Kit-8 (Dojin, Tokyo, Japan) was added to each well, and the plate was incubated at 37°C for 2 h. The cytotoxicity of AZP–SNase–R9 was evaluated by measuring the absorbance at 450 nm with an ARVO SX 1420 multilabel counter (Wallac, Freiburg, Germany).