

RESEARCH ARTICLE

Development of immortalized *Callithrix jacchus* kidney cell lines supporting infection with a panel of viruses

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OPEN ACCESS

Citation: Gärtner S, Stomberg P, Pöhlmann S, Winkler M (2026) Development of immortalized *Callithrix jacchus* kidney cell lines supporting infection with a panel of viruses. PLoS One 21(3): e0337680. <https://doi.org/10.1371/journal.pone.0337680>

Editor: Juan Carlos de la Torre, The Scripps Research Institute, UNITED STATES OF AMERICA

Received: December 4, 2025

Accepted: February 12, 2026

Published: March 2, 2026

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Data availability statement: All relevant data are within the manuscript and its [Supporting Information](#) files.

Funding: We thank the Bundesministerium für Bildung und Forschung (BMBF) – now Bundesministerium für Forschung, Technologie

Abstract

Common marmosets (*Callithrix jacchus*) are valuable non-human primate (NHP) animal models in biomedical research, including infectious diseases modelling. However, for in vitro studies only a few immortalized cell lines have been generated, and additional lines are needed to comply with the 3R principles of replacement, reduction and refinement. Here, we present the generation and characterization of three cell lines derived from kidney tissue, which were immortalized by transduction of SV40 large T antigen. The cell lines display an epithelioid morphology, show differential podoplanin expression and are likely of pericyte origin, as deduced from expression profiles of marker genes obtained by RNA sequencing analysis (RNA-seq). All cell lines had a functional interferon (IFN) system, as shown by responsiveness to human IFN β and marmoset IFN α 14 and the induction of interferon-stimulated genes (ISG). Infection with retroviral pseudotypes demonstrated susceptibility to entry driven by glycoproteins from a wide range of human pathogenic viruses. Finally, these cell lines are highly permissive for Zika virus, for which marmosets are a model organism, and Herpes simplex virus 1, which causes a deadly disease in marmosets. We believe that these cell lines are a valuable resource for in vitro studies on marmosets.

Introduction

Common marmosets (*Callithrix jacchus*) are small, neotropical NHP that have emerged as a valuable model organism in biomedical research, particularly in the fields of immunology, neuroscience, and infectious disease modeling [1–3]. Their small size, short lifespan, and close phylogenetic relationship to humans make marmosets an attractive alternative to larger primates, such as rhesus macaques, as well as to traditional rodent models like mice and rats. Unlike rhesus macaques, marmosets do not carry pathogens harmful to humans, such as Macacine alphaherpesvirus 1 (Herpes B virus) [3]. Conversely, marmosets are naturally susceptible to numerous human pathogenic viruses [1,3,4]. This includes highly pathogenic viruses,

und Raumfahrt (BMFTR; https://www.bmfr.bund.de/DE/Home/home_node.html) – for funding (031L0155 to SP). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

such as Lassa virus (LASV) [5,6], Ebola virus (EBOV) and Marburg virus (MARV) [7], emerging viruses, such as Zika virus (ZIKV) [8,9], and the respiratory pathogens SARS-CoV-1 [10], SARS-CoV-2 [11] and MERS-CoV [12], but also herpesviruses such as Epstein-Barr virus [13] and Herpes simplex virus 1 (HSV-1), which can cause life threatening infections in common marmosets [14–16].

The use of animals in research is strictly regulated by laws, and animal experiments need to be conducted under an ethical framework, which includes adherence to the 3R principles of replacement, reduction and refinement [17]. Immortalized, well-characterized cell lines are powerful tools that can be integrated into experimental workflows, for example to evaluate virus entry or innate immune responses in vitro, and thus help to comply with the 3R principles. However, for marmosets, in contrast to humans, only a small number of cell lines have been described, including immortalized fibroblasts [18,19], ovarian granulosa and theca cells [20] and hepatic progenitor cells [21], with a single marmoset cell line being available from ATCC. Therefore, more immortalized cell lines are needed, particularly for in vitro studies in virology, oncology and drug metabolism [22]. So far no immortalized kidney cell lines from *Callithrix jacchus* have been described, despite the fact that kidney cell lines from other species (most notably Vero from African green monkey and HEK293 from humans) are easily cultured, susceptible to a wide range of viruses and have been widely used for antiviral drug development and vaccine production [23]. Biologically, many viruses infect kidneys, causing kidney disease, and are shed in urine [24–27].

In this study, we describe the establishment and characterization of three immortalized cell lines from marmoset kidney tissue, which will serve as a valuable resource for the marmoset research community. The cell lines are highly related to pericytes as evidenced by RNA-seq analysis and retained a functional IFN system. The cell lines were susceptible to the entry of pseudoparticles bearing the glycoproteins of multiple highly pathogenic viruses and supported growth of the RNA virus ZIKV as well as the DNA virus HSV-1.

Materials and methods

Plasmids and oligonucleotides

The previously described plasmids HIV gag-pol(SCA), pLenti-IP-large T, MLV-gag/pol and MLV-luc were used for lentiviral or retroviral transduction [28–30]. Additionally, expression plasmids for various viral glycoproteins were used, including those from vesicular stomatitis virus (*Vesiculovirus indiana*, VSV-G), Ebola virus (*Orthebolavirus zairense*, EBOV-GP), Marburg virus (*Orthomarburgvirus marburgense*, MARV-GP), influenza A virus strain WSN (*Alphainfluenzavirus influenza*, HA and NA), Lassa virus (*Mammarenavirus lassaense*, LASV-GP), Machupo virus (*Mammarenavirus machupoense*, MACV-GP), Lymphocytic choriomeningitis virus strain Armstrong (*Mammarenavirus choriomeningitidis*, LCMV-GP), La Crosse virus (*Orthobunyavirus lacrosseense*, LACV-GPC) or severe fever with thrombocytopenia syndrome virus (*Bandavirus dabieense*, SFTSV-Gn/Gc) have been previously described [31–38].

Expression plasmids for IFNs were newly generated. For this, human IFNB1 was amplified from genomic DNA of A549 cells using primer hIFNb1-5Acc

(5-CCGGTACCATGACCAACAAGTGTCTCC-3)/ hIFNb1-3Bgl (5-GGAGATCTTCAGTTTCGGAGG-3) and cloned into pCAGGS [39] via Acc65I and BglII to obtain pCAGGS-hIFNB1. *Callithrix* IFNa14 was amplified from genomic DNA of CajaK-D4 cells using primer cjIFNa2-5Acc (5-CCGGTACCATGRCCTTGACCTTTCCTTTA-3)/cjIFNa2-3Sal (5-CCCG TCGACTCATTCTTACTTCTTAAMCC-3) and cloned into pCAGGS via Acc65I and SalI to obtain pCAGGS-cjIFNA14. This clone has an amino acid exchange A161T with respect to the cjIFNa14 sequence in GenBank (LOC100406466; XM_002763506.4).

Cell culture

Cell lines 293T (human kidney, female; DSMZ catalogue no. ACC-635, RRID: CVCL 0063), Vero76 (African green monkey kidney, female; ATCC catalogue no. CRL-1586; RRID: CVCL 0574; kindly provided by Andrea Maisner) and A549 (human lung, male; CRM-CCL-185, ATCC, RRID: CVCL_0023; kindly provided by Benjamin tenOever) were cultured in DMEM supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin. Human cell line identity was verified through short tandem repeat (STR) analysis [40], and regular mycoplasma contamination screenings were performed on all cell lines.

Establishment of primary cultures

Primary cultures were established from kidney tissue obtained from a 12-year and 10-month-old male animal. The animal has not been challenged with any pathogen and has not received any substance treatment. The tissue was finely chopped into small pieces (<1mm) and 2–3 pieces were transferred to Eppendorf tubes containing a 50 µl digestion mixture (100 mg/mL collagenase IV dissolved in PBS). After incubation on a shaker (37°C, 800 rpm) for 1–1.5 h, cell suspensions were transferred into 6-well plates containing DMEM supplemented with 10% FCS and antibiotics (penicillin, streptomycin, gentamycin, nystatin and amphotericin B).

Retroviral transduction

For immortalization, cells were transduced with a lentiviral vector expressing SV40 large T antigen. For preparation of transducing particles, 293T cells were seeded in T25 flasks and transfected with a mixture of 6 µg lentiviral vector pLenti-IP-large T, 3 µg HIV gag-pol(SCA) and 3 µg pHIT/G expressing VSV-G, using the calcium phosphate method. Transduced cells were subsequently selected with 1 µg/mL puromycin. Established cell lines were named as *Callithrix jacchus* kidney (CajaK) cells with a suffix for distinction (CajaK-C3, CajaK-D4, CajaK-E5).

Microscopy

Brightfield images were taken of cells grown in 24-well plates using a ZOE cell imaging system (Bio-Rad, Feldkirchen, Germany) at 20x magnification. Images were processed (cropping, scale bar) using Fiji/ImageJ 1.54f [41].

RNA-seq analysis

Cells were seeded in 6-well plates at a density of 250,000 cells/well. After overnight growth, total cellular RNA was isolated using Trizol reagent (Ambion, Carlsbad, CA, USA) following the manufacturer's instructions. The isolated RNA was subjected to 50bp single-end bulk RNA sequencing (Illumina HiSeq 4000) at the Core Unit NGS-Integrative Genomics (NIG), University Medical Center Göttingen (UMG). The NIG provided raw count profiles and normalized TPM values. To visualize the expression of marker genes, heatmap graphs were generated using the R platform (version 4.5.1; <https://www.R-project.org/>) and the pheatmap package [42,43]. Marker genes were chosen based on a published single-cell RNA sequencing (scRNA-seq) analysis of human adult kidney samples [44]. For our analyses, we used canonical marker genes (Suppl. 3B in S1 Fig. in [44]) and a panel of genes specifically expressed in pericyte-2 cells or fibroblasts (Suppl. S3 Table in [44]). In the latter case, only genes with highest significance and expression in our cell line samples were selected.

Flow cytometry

The CajaK cell lines generated here were seeded in 6-well plates at 200,000 cells/well and incubated for two days. The cells were then detached using PBS containing 5 mM EDTA. After centrifugation (1,200 rpm, 5 min) cell pellets were washed in FCS buffer (PBS, 5% FCS, 2 mM EDTA) and reacted with either rat anti-podoplanin antibody (1:100; Origene AM01133PU-N, Rockville, MD, USA) or rat IgG2a isotype control antibody (Antikoerper-online ABIN5675832, Aachen, Germany) in a volume of 50 μ L for 30 min on ice. After three washes in FCS buffer, cells were incubated (30 min, room temperature) with Alexafluor488-conjugated donkey anti-rat antibody (1:100; Invitrogen A21208, Carlsbad, CA, USA), followed by three final washes in FCS buffer and fixation in 2% PFA. Samples were analyzed on an ID7000 Spectral Cell Analyzer (Sony, San Jose, CA, USA). Diagrams for the figures were prepared using EasyFlowQ version 1.7 [45].

Quantitative real-time PCR

CajaK cell lines and A549 control cells were seeded in 12-well plates at 150,000 cells/well. The next day, the cells were either left untreated, treated with pan-IFN (PBL Assay Science 11200–2, Piscataway, NJ, USA) at 100 U/mL, or infected with VSV ncp* at MOI 0.1 [46]. Human pan-IFN is a recombinant chimera of human IFN α 1 (IFN D) and IFN α 2 (IFN A), with activity on many species ranging from humans and monkeys to mice [47,48]. After 24 h, cells were harvested in Trizol (Ambion, Carlsbad, CA, USA) and RNA purified according to instructions of the manufacturer. For each sample, 1 μ g RNA was treated with 0.5 U RNase-free DNase I for 10 min at 37°C and stopped with EDTA. After denaturation at 75°C (10 min), cDNA synthesis was performed with random hexamers using the SuperScript™ III First-Strand Synthesis System (Thermo, Waltham, MA, USA). For quantitative PCR analysis, 1 μ L of cDNA and primers against IFNB1 (forward 5-CAGCAATTTTCAGTGTGTCAGAAGC-3, reverse 5-TCATCCTGTCCTTGAGGCAGT-3), MX1 (forward 5-TTCAGCACCTGATGGCCTATC-3, reverse 5-TGGATGATCAAAGGGATGTGG-3), and the housekeeping gene 18S rRNA (forward 5-GATCCATTGGAGGGCAAGTCT-3, reverse 5-CCAAGATCCAACCTACGAGCTT-3) were used [49–51]. The reactions were performed on a Rotorgene Q platform (Qiagen, Hilden, Germany) using the QuantiTect SYBR Green PCR kit (Qiagen, Hilden, Germany). Calculation of IFNB1 and MX1 induction was done using the $2^{-\Delta\Delta CT}$ method using 18S rRNA as reference [52].

Conditioned IFN supernatants

For generation of conditioned supernatants, 293T cells were seeded in T25 flasks at 1,500,000 cells/flask. The next day, cells were transfected with 5 μ g of pCAGGS-hIFNB1 or pCAGGS-cjIFN α 14 using polyethyleneimine (PEI) [53]. For transfection, 12 μ g DNA and 36 μ L of PEI (Polyscience, Inc., 23966; 0.1% w/v) were separately diluted in 250 μ L OptiMEM, mixed and incubated at room temperature before adding the mixtures to the cells. After overnight incubation, medium was replaced with fresh medium (DMEM containing 10% FCS) and cells were incubated for additional 48 h. Cell culture supernatant was collected, filtered through 0.2 μ m filters, aliquoted and stored at –80°C.

IFN activity was determined using a VSV strain (VSV* Δ G(Fluc)) essentially as described in Berger Rentsch and Zimmer [54]. Briefly, both A549 and CajaK-E5 cells were seeded in 96-well plates at 10,000 cells/well and incubated overnight. Two-fold dilution series were prepared for IFN-containing supernatants as well as for pan-IFN (starting concentration 400 U/mL) and cells treated with 100 μ L/well for 24 h. On the next day, medium was removed and cells were infected with VSV* Δ G(Fluc) at MOI 5 (100 μ L volume). After 6 h incubation, cells were lysed in 50 μ L Luciferase Cell Culture Lysis Reagent (Promega, Madison, WI, USA) for 20 min at room temperature. The lysates were transferred to white 96-well plates, mixed with 50 μ L Beetle Juice reagent (PJK, Kleinblittersdorf, Germany) and luciferase activity measured in a Hidex Sense luminescence reader (Hidex, Turku, Finland). After subtraction of background (untreated, uninfected cells) the infection rate was calculated. Assuming 1 unit IFN resulting in 50% inhibition, the activities in the sample solutions (titrated on CajaK-E5 cells) were calculated. Titration of pan-IFN on A549 cells served as control.

Immunoblot

For analysis of large T expression after IFN treatment, CajaK cell lines, A549 and 293T control cells were seeded in 6-well plates at 250,000 cells per well. After 48 h, the cells were lysed in SDS sample buffer (30 mM Tris pH 6.8, 1 mM EDTA, 10% glycerol, 2% SDS, 0.1% bromophenol blue, 5% beta-mercaptoethanol), heated to 95°C for 15 min and separated using SDS-PAGE. The proteins were transferred onto nitrocellulose membranes (Protran; Amersham, Freiburg, Germany) using a Mini-Protean electrophoresis system (Bio-Rad, Hercules, CA, USA). Filters were blocked in PBS-T/MP (PBS with 0.1% Tween-20 and 5% skim milk powder) for 1 h at room temperature followed by incubation with primary antibodies overnight at 4°C. Primary antibodies mouse anti-large T monoclonal PAb108 (BD Biosciences 554150, San Diego, CA, USA) and mouse anti-beta-actin (Sigma A5441, Taufkirchen, Germany) were diluted 1:1,000 in PBS-T/MP. After washing three times in PBS-T (PBS with 0.1% Tween-20), filters were incubated for 1 h at room temperature with horseradish conjugated anti-mouse antibodies (Dianova, Hamburg, Germany) diluted 1:10,000 in PBS-T/MP. After three final washes in PBS-T, filters were incubated with Westar Antares chemiluminescence substrate (Cyanagen XLS0142,0250; Bologna, Italy) and imaged using an Azure 600 Imager (Azure Biosystems, Singapore).

For analysis of IFITM3 expression after IFN treatment, CajaK cell lines and A549 control cells were seeded in 24-well plates at 50,000 cells per well. The next day, the cells were treated with conditioned medium of either hIFNB1 or cJIFNa14 at a concentration of 1,000 U/mL for 24 h. After treatment, the cells were lysed in SDS sample buffer heated to 95°C for 5 min and separated using SDS-PAGE (15%). After transfer and blocking, filters were processed as described above, using primary antibodies rabbit anti-IFITM (Abgent AP1153a, San Diego, CA, USA) and mouse anti-beta-actin (Sigma A5441, Taufkirchen, Germany) diluted 1:1,000 and 1:500, respectively, and horseradish conjugated secondary antibodies anti-mouse or anti-rabbit (Dianova, Hamburg, Germany) diluted 1:10,000. For detection, filters were incubated with HRP Juice (PJK Biotech, Kleinblittersdorf, Germany) and imaged using a ChemoCam Imager (Intas, Göttingen, Germany). Immunoblots were quantified using the Gel Analyzer function of Fiji/ImageJ 1.54p [41].

Transduction with retroviral pseudoparticles

Production of retroviral pseudoparticles bearing glycoproteins of multiple viruses followed established protocols [29,30,55,56]. Briefly, 293T cells were seeded into T25 flasks and transfected with 6 µg vector MLV-luc, 3 µg MLV-gag-pol and 3 µg expression plasmid for the respective viral glycoprotein. After two days, cell culture supernatants were harvested, filtered through 0.45 µm filters, aliquoted and stored at -80°C. Transduction efficiency was first quantified on 293T cells by measuring luciferase activity.

For comparative experiments, CajaK cell lines and 293T control cells were seeded in 96-well plates at 10,000 cells/well in 50 µL medium. Before infection, pseudoparticle preparations were adjusted for comparable luciferase yield as measured in 293T cells. For infection, 50 µL adjusted pseudoparticle supernatant was added to the cells, incubated for 4–6 h until an additional 100 µL medium was added. After 3 days, cells were lysed using 50 µL Luciferase Cell Culture Lysis Reagent (Promega, Madison, WI, USA) for 20 min at room temperature. After transfer to white 96 well plates, samples were mixed with 50 µL Beetle Juice reagent (PJK, Kleinblittersdorf, Germany) and luciferase activity measured in a Hidex Sense luminescence reader (Hidex, Turku, Finland).

Virus

ZIKV (*Orthoflavivirus zikaense*) strain SPH2015 has been previously cloned from RNA and rescued from plasmid in Vero76 cells [57]. HSV-1 (*Simplexvirus humanalpha1*) strain F was rescued from bacterial artificial chromosome (BAC) pHSVF-CREin (kindly provided by Greg Smith) [58]. To obtain a HSV-1 reporter virus expressing Gaussia luciferase (HSV-1-cmvGluc), an expression cassette consisting of human cytomegalovirus enhancer-promoter, Gaussia luciferase and bovine growth hormone poly A signal present in pcDNA3-Gluc-en [59] was amplified using primers ep-HSV1-UL3 BpA-5 (5-CACTGCCCGTCGCGCGTG

TTTGATGTTAATAAATAACACATAAATTTGGCTCCCCAGCATGCCTGCTATT-3) and ep-HSV1-UL4cmv-3 (5-AAATGCCCCCCCCCTTGCGGGCGGTCCATTAAAGACAACAAACAACCAGTTGACATTGATTATTGACT-3). The purified PCR product was electroporated into *E. coli* GS1783 containing the BAC pHSVF-CREin and integrated using recombineering as previously described [58–60], followed by rescue on Vero76 cells. The Papiine alphaherpesvirus 2 (*Simplexvirus papiinealpha2*, PaHV-2) containing Gaussia luciferase reporter gene driven by the human cytomegalovirus enhancer-promoter (PaHV-2-cmvGluc) was previously described [59]. Likewise, the Cercopithecine alphaherpesvirus 2 (*Simplexvirus cercopithecinealpha2*, CeHV-2) containing Gaussia luciferase linked via a 2A sequence to ICP4 (CeHV-2-ICP4-Gluc) has been described [60].

Virus replication kinetics and titration

For single step growth curves, CajaK cell lines or Vero76 control cells were seeded in 24-well plates at 50,000 cells/well and infected on the next day with either ZIKV or HSV-1 at MOI 1. For infection, medium was removed and inoculum added in triplicates, incubated for 1 h at 37°C and replaced with fresh medium. After 1, 24, 48 and 72 h, supernatants were harvested, centrifuged at 4,000 rpm for 5 min and the cleared supernatants frozen at –80°C. Titration of HSV-1 by plaque assay and ZIKV by focus forming assay, were performed on Vero76 as previously described [29,60].

For multicycle growth experiments, marmoset cell lines were seeded in 6-well plates at 250,000 cells. On the next day, cells were infected with HSV-1-cmvGluc, PaHV-2-cmvGluc or CeHV-2-ICP4-Gluc in triplicates with 100 pfu/well (MOI 0.0004). At time intervals of 6–18 h aliquots of 25 µl were collected until 96 h postinfection and stored at –20°C. After collection of all samples, luciferase activity was measured in a Hidex Sense plate reader (Hidex, Turku, Finland) using coelenterazine (PJK, Kleinblittersdorf, Germany) as substrate, diluted to a final concentration of 1.5 mM in D-PBS (with Ca and Mg) [61].

Statistical analysis

For statistical analyses and preparation of graphs, GraphPad Prism software (version 10.6.1; GraphPad Software, Boston, MA, USA) was used. Significance was tested by one-way ANOVA with Tukey's correction for multiple comparisons assuming a lognormal distribution [62].

Ethical statement

For this study only leftover material (post-mortem samples) from a 12-year and 10-month-old male animal were used.

The German Primate Center is a non-profit independent research and service institute since 1977. It is registered and authorised by the local and regional veterinary governmental authorities (Ref. no.122910.3311900, PK 36674). All works regarding animals, animal research and sampling followed valid national and international guidelines. Good veterinary practice was applied to all procedures whenever animals were handled. According to the Animal Welfare Committee, the post-mortem use of samples from non-human primates derived from animals with a permit in accordance with §4, §7 and §11 of the Animal Welfare Act is in line with the German legal and ethical requirements for appropriate animal experiments with non-human primates. The consultation of the Animal Welfare Committee and the Animal Welfare Officer is documented under no. E3-25.

For the care of marmoset monkeys, legal guidelines for the use of animals and the institutional guidelines of the DPZ were strictly followed. Experienced veterinarians and animal care attendants daily controlled health and well-being of the animals. As social tree-living New World monkeys the animals were pair-housed in a temperature- ($25 \pm 1^\circ\text{C}$) and humidity-controlled ($65 \pm 5\%$) facility, and the parameters controlled daily. Illumination by daylight and additional artificial lighting followed 12:12 hour light:dark cycles. Cages consisted of stainless steel, had a vertical orientation with size of 180x65x80 cm (h, w, d) and were furnished with wooden branches, hammock and shelves for environmental enrichment. Cages were cleaned with water and disinfectant at weekly intervals. The animals had ad libitum access to water and a

pelleted marmoset diet. The diet was supplemented daily with 20g mash per animal (served in the morning) and 30g cleanly cut fruits or vegetables mixed with noodles or rice (served in the afternoon). In addition, the daily diet was varied by adding, for example, mealworms, nuts, seeds, and eggs. For sacrifice, the animal was narcotized with Midazolam (0.05 mL) and Alfaxalon (0.4 mL) intramuscularly by a veterinarian and euthanized with an intracardial injection of 0.5 mL Pentobarbital.

Results

Establishment of immortalized marmoset kidney cell line

To establish marmoset kidney cell lines, we obtained leftover material from a male animal aged 12 years and 10 months that had been sacrificed as part of an unrelated study ([Fig 1a](#)). The kidney tissue sample was dissected into small fragments and cultivated in vitro until cultures of growing adherent cells were established. These cells were then transduced with a lentiviral vector expressing SV40 large T antigen and subjected to puromycin selection [30]. Individual puromycin-resistant colonies were picked and grown as individual cultures and named as *Callithrix jacchus* kidney (CajaK) cells with a suffix for distinction (CajaK-C3, CajaK-D4, CajaK-E5). Morphological examination revealed that the CajaK cells exhibited an epithelioid morphology, with CajaK-D4 cells displaying a more spread-out appearance and CajaK-C3 and CajaK-E5 cells showing a more rounded morphology ([Fig 1b](#)). All cell lines expressed the immortalization marker large T antigen, as demonstrated by immunoblot ([Fig 1c](#)). Quantification of large T expression levels showed that CajaK cell lines consistently expressed lower levels as compared to 293T cells, with highest levels measured for CajaK-D4 cells ([Fig 1d](#)) but these differences were not statistically significant. Further analysis of these cell lines revealed differential expression of the kidney marker podoplanin ([Fig 1e](#)), with CajaK-E5 cells exhibiting clear surface expression, CajaK-C3 cells showing no expression, and CajaK-D4 cells displaying an intermediate phenotype. This pattern was corroborated by subsequent RNA sequencing (RNA-seq) analysis ([Fig 1f](#)).

Marmoset kidney cell lines display pericyte identity

For analysis of cell identity, total RNA was isolated from duplicate samples and subjected to RNA-seq (Suppl. [S2 Table](#)). Principal component analysis demonstrated distinct identities for each cell line (Suppl. [S1 Fig](#)). To infer the cell type identity, we first analyzed expression of canonical marker genes based on scRNA-seq analysis of human adult kidney samples [44]. As shown in [Fig 2a](#), all the cell lines demonstrated strong expression of the pericyte marker COX4I2 (Suppl. [S3 Table](#)). Using panels of genes specifically expressed in individual cell types, we confirmed medium to high expression of genes specific to pericytes ([Fig 2b](#)), while genes specific for fibroblasts typically showed low expression in our cell lines (Suppl. [S4-S5 Tables](#)). Thus, RNA-seq analysis indicates that our immortalized cell lines were likely derived from cells of pericyte origin.

Immortalized marmoset kidney cells lines have a functional IFN system

Next, we characterized whether the marmoset cell lines expressed interferon β (IFNB1) or the ISG MX1 in response to treatment with IFN α or infection with VSV. The human lung adenocarcinoma cell line A549 has a functional IFN system and was used as positive control [29,63]. Cells were incubated for 24 h with either pan-IFN, a chimeric human IFN α , or VSV-ncp*, a VSV variant which strongly induces IFN [46]. Human pan-IFN was chosen due to its activity in mammalian species ranging from humans to mice [47,48]. Induction of IFNB1 ([Fig 3a](#)) and MX1 ([Fig 3b](#)) was analyzed by quantitative RT-PCR. Infection with VSV-ncp* clearly induced both IFNB1 and MX1 ([Fig 3a](#)). Induction of IFNB1 expression by VSV treatment was significantly stronger in CajaK-C3 cells than in A549, CajaK-D4 or CajaK-E5 cell lines. Induction of MX1 expression by VSV treatment was significantly reduced in CajaK cell lines compared to A549 cells, while induction in CajaK-C3 cells was significantly stronger than in CajaK-D4 or CajaK-E5 cell lines. Treatment with pan-IFN did not induce IFNB1, as expected [29], but unexpectedly also did not induce MX1 in marmoset cell lines, while a strong induction was

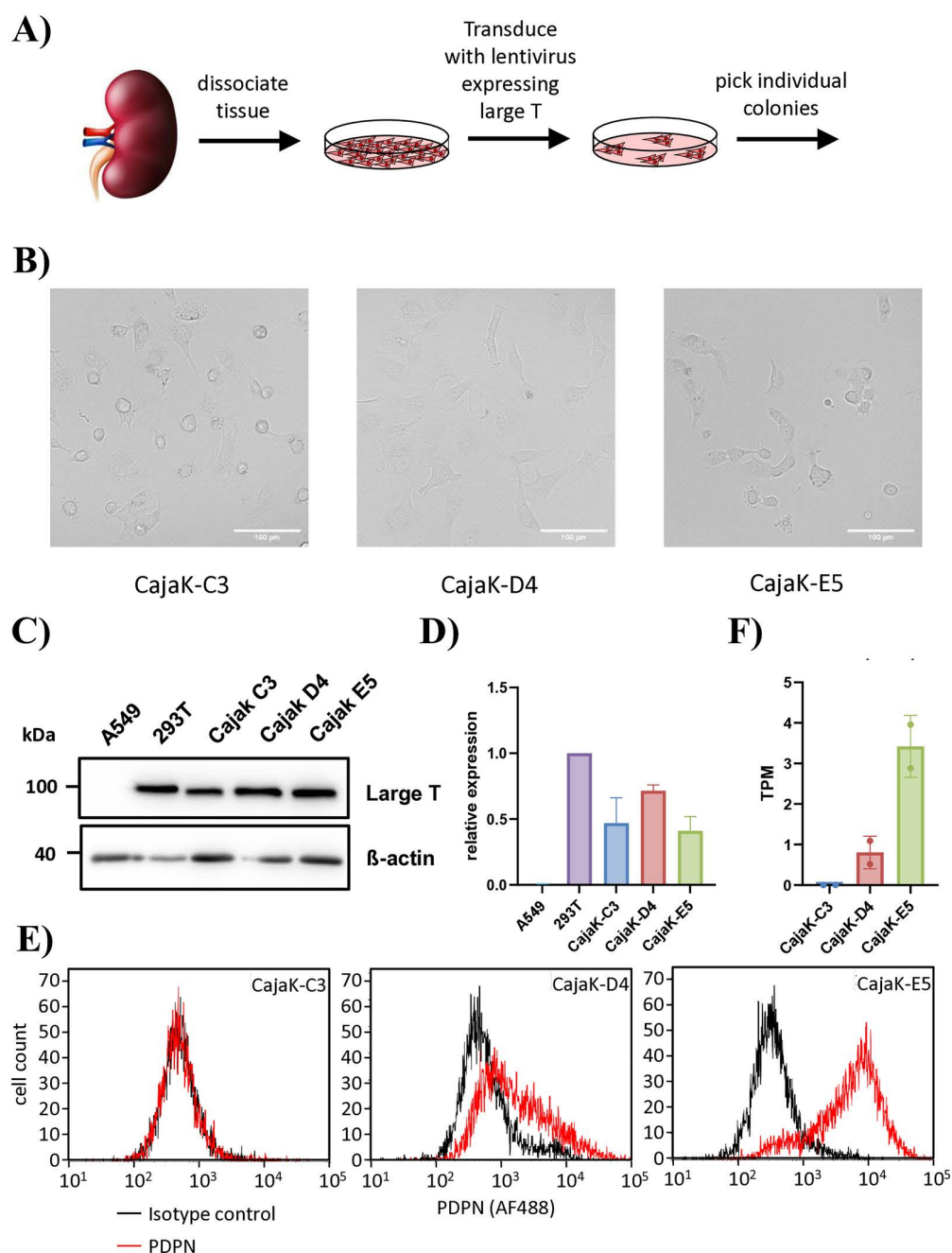


Fig 1. Generation and initial characterization of marmoset kidney cell lines. (A) Overview of the strategy used to generate marmoset kidney cell lines (image of kidney designed by brgfx/Freepik). (B) Morphology of marmoset kidney cell lines. Cell lines were seeded in 24-well plates and bright field images were taken at 20x magnification using a ZOE cell imaging system. Scale bars indicate 100 μ m. (C) Immunoblot analysis of expression of immortalization marker large T in marmoset kidney cell lines. A549 and 293T cells served as negative or positive control, respectively. Beta-actin served as loading control. Results were confirmed in a separate experiment. (D) Quantification of large T expression in immunoblot experiments. Protein bands were normalized to beta-actin control bands and to the large T signal of 293T cells. Error bars indicate standard deviation (SD). (E) Analysis of podoplanin (PDPN) surface expression by flow cytometry. Cell lines were detached and stained with anti-podoplanin antibody (red) or isotype control antibody (black). Data were filtered to include only intact singlet cells. The results were verified through a separate, independent experiment. (F) Comparison of PDPN expression at transcriptional level. Cell lines were subjected to RNA-seq, transcripts per million (TPM) are shown. Error bars indicate SD.

<https://doi.org/10.1371/journal.pone.0337680.g001>

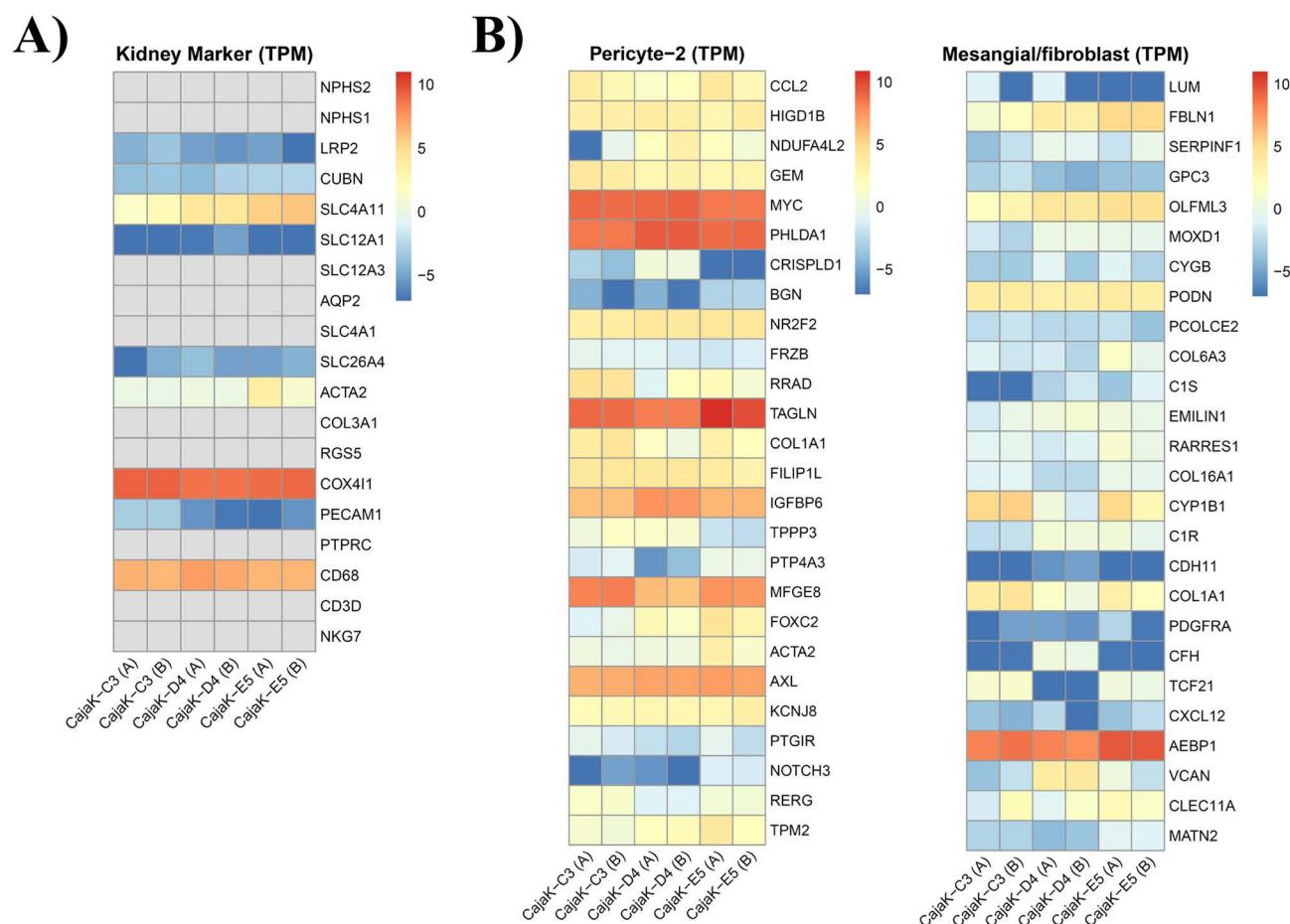


Fig 2. Cell type identification by transcriptome sequencing. Marmoset kidney cell lines were subjected to RNA-seq analysis. For each cell line total RNA was isolated from two independent samples. **(A)** Expression in marmoset kidney cell lines of canonical markers for human kidney cell types as defined by Subramanian et al, 2019 Suppl. Fig 3B [44]. Shown is a heatmap of log2-transformed TPM data derived from marmoset kidney cell lines. Grey boxes indicate that no signals were obtained. **(B)** Expression in marmoset kidney cell lines of genes selectively expressed in pericytes (left panel) and fibroblasts (right panel), as defined by Subramanian et al, 2019 Suppl. S3 Table [44]. Shown are heatmaps of log2-transformed TPM data derived from marmoset kidney cell lines.

<https://doi.org/10.1371/journal.pone.0337680.g002>

measured in A549 cells (Fig 3b). This unresponsiveness of marmoset cells to human IFN α is in agreement with a previous report on marmoset hepatocytes [64].

To analyze if the marmoset cell lines were responsive to IFN molecules other than pan-IFN, we cloned human IFN β (hIFNB1) and marmoset IFN α 14 (cjIFNa14). Conditioned media containing these IFNs were generated by transfecting 293T cells and tested for antiviral activity. Subsequently, marmoset cell lines were treated with conditioned media containing either hIFNB1 or cjIFNa14 and analysed for expression of the ISG IFITM3 as shown in Fig 3c. The two IFITM3-specific bands detected in the Cjalk cell lines likely constitute different IFITM3-like genes, since multiple IFITM3-like genes have been annotated in the *Callithrix* genome but have so far not been characterized on the protein level [65,66]. Compared to A549 cells all Cjalk cell lines showed higher constitutive expression of IFITM, but IFITM expression was clearly induced by both hIFNB1 and cjIFNa14. This indicates that the cells lines are responsive to IFN, although certain alpha IFNs (e.g., pan-IFN) might not work.

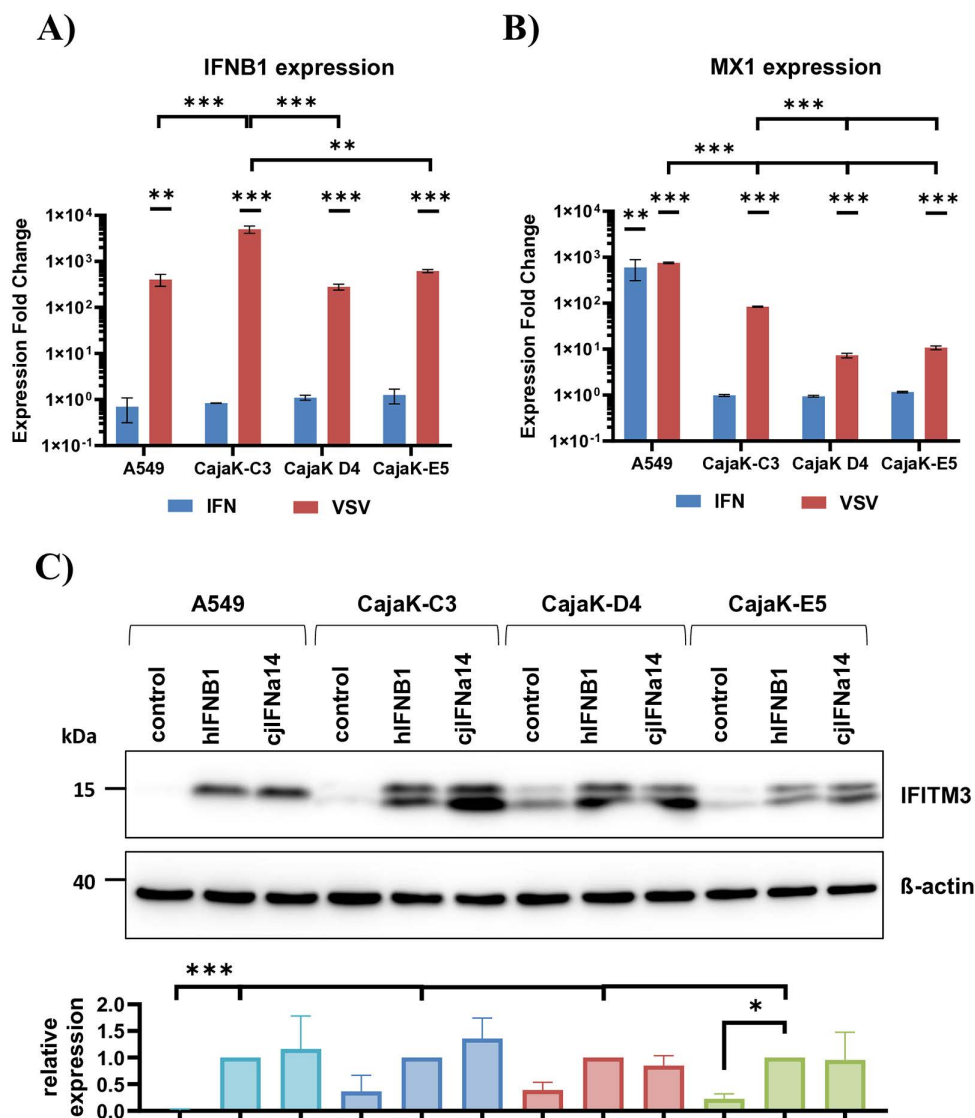


Fig 3. Immortalized marmoset kidney cells respond to virus infection and IFN. (A, B) Marmoset kidney cell lines or control cells (A549) were treated with pan-IFN (100 U/mL) or infected with VSV ncp * at MOI 0.1 for 24 h. Untreated cells served as control. Total RNA was isolated and expression of IFNB1 (A) or interferon-stimulated gene MX1 (B) quantified by qRT-PCR. Transcript levels were normalized against 18S rRNA and expression fold change calculated with respect to control cells. The data presented represent the means of two experiments conducted in triplicate with error bars in the graph indicating the standard error of the mean (SEM). Statistical significance was tested by one-way ANOVA with Tukey's correction: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$. (C) Marmoset kidney cell lines or A549 control cells were treated with conditioned medium from 293T cells expressing human IFNB1 or *Callithrix jacchus* IFNA14. Untreated cells served as control. After 24 h, cell lysates were harvested and analyzed by immunoblot using antibodies against the ISG IFITM3 or beta-actin (as loading control). Results were confirmed in two separate experiments. Quantification of IFITM3 expression from three immunoblot experiments is shown in the lower panel. Protein bands were normalized to beta-actin control bands and to the IFITM3 bands of IFNB1 treated samples for each respective cell line. Error bars indicate standard deviation. Error bars indicate SD. Statistical significance was tested by one-way ANOVA with Tukey's correction: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

<https://doi.org/10.1371/journal.pone.0337680.g003>

Immortalized marmoset kidney cells lines are susceptible and permissive for virus infection

To analyze the susceptibility of the cell lines to virus infection, we used MLV-based particles pseudotyped with a large panel of viral glycoproteins of rhabdoviral (VSV-G), filoviral (EBOV-GP, MARV-GP), arenaviral (LCMV-GP, LASV-GP,

MACV-GP), peribunyaviral (LACV-GPC), phenuiviral (SFTSV-Gn/Gc) and orthomyxoviral (WSN-HA/NA) origin. Most of the pseudoparticles infected all three cell lines with high efficiency (Fig 4). Notable exceptions were particles bearing influenza A virus WSN-HA/NA, or Lassa Virus GP which showed significantly reduced entry on all CajoK cell lines, when compared to 293T cells. This was most pronounced for influenza A virus entry into CajoK-D4 and CajoK-E5 cells. In summary, all immortalized cell lines were susceptible to infection with pseudotypes bearing glycoproteins from a wide range of viruses, including several highly pathogenic ones.

Finally, we tested whether the cell lines were permissive to an RNA virus (ZIKV) or a DNA virus (HSV-1). Cells were infected with MOI 1 and virus titer determined in supernatants harvested at regular intervals using focus-formation assay (ZIKV) or plaque assay (HSV-1). In these experiments, Vero76 cells were used as control cells, as these are typically preferred for propagation and titration of these viruses [67–69].

Both viruses grew on all CajoK cell lines to high levels comparable to Vero76 cells (Fig 5A, 5B), indicating efficient and permissive infection. In addition, we used a HSV-1 reporter virus expressing Gaussia luciferase and two closely related reporter viruses of PaHV-2 and CeHV-2 in multicycle infection experiments. Infection was performed at low MOI 0.0004 and replication was quantified using Gaussia luciferase. Here, HSV-1 (Fig 5C) and PaHV-2 (Fig 5D) replicated efficiently, but significantly reduced compared to Vero76 cells. Among CajoK cell lines, replication was most efficient on CajoK-C3 cells, while CajoK-D4 cells showed lowest levels of replication. Despite these differences, both HSV-1 and PaHV-2 reached plateau levels of replication with only slight delay, while CeHV-2 (Fig 5E) demonstrated a profound delay of virus growth on all three CajoK cell lines. In summary, the immortalized CajoK cell lines supported efficient growth of the RNA and DNA viruses tested.

Discussion

In the present work, we describe the generation of three marmoset cell lines of likely pericyte origin, which have a functional IFN system and are susceptible and permissive to infection by multiple viruses. They expand the limited set of immortalized cell lines derived from the common marmoset, a highly valuable NHP model for biomedical research, thus supporting the 3R principles in helping to replace, reduce or refine animal experiments.

So far only few immortalized marmoset cell lines have been described and none were derived from kidney tissue [22]. Our cell lines, derived from marmoset kidney tissue and immortalized by transduction of SV40 large T, therefore markedly expand the repertoire of available marmoset cell lines. Based on RNA-seq analysis (Fig 2) and comparison with marker genes (COX4I1) and expression profiles derived from human scRNA-seq analyses [44,70], we identified these lines as pericyte-derived, mural cells of the microvasculature involved in maintenance of vessel integrity and regulation of vascular integrity [71]. All three cell lines share similar expression profiles, but clearly have distinct identities as revealed by PCA (Suppl. S1 Fig). Since cell lines were derived from individual colonies, they should constitute homogenous cultures, but we did not assess this by single cell analyses. Our FACS analyses of a single marker, podoplanin, point toward some heterogeneity in the expression of this protein, but it is unclear, whether this expands to other genes. The differences between cell lines may be derived from the phenotypic and transcriptomic diversity of cell types in tissues, but may also be caused in part by expression of the immortalization gene SV40 large T.

One limitation of our cell lines is that they were all generated from a single male animal. Sex based differences have been noted to impact disease progression and drug development, and may yield measurable differences in cell culture, which may limit the conclusions derived from a male-only cell line [72,73]. In addition, it should be stated that most experiments were done with cells not exceeding 10–30 passages and formally we cannot exclude that highly passaged cells might yield slightly different results.

The IFN pathway serves as a crucial innate barrier that impacts viral replication. However, many cell lines, for example Vero cells, derived from cancerous tissue lack a functional IFN system [74]. We could show that central steps such as sensing of virus and induction of ISGs are functional in our cell lines (Fig 3). Interestingly, initial treatment with pan-IFN, a chimeric molecule based on human IFN α 1 and α 2, did not induce the ISG MX1 (Fig 3b) despite its activity on

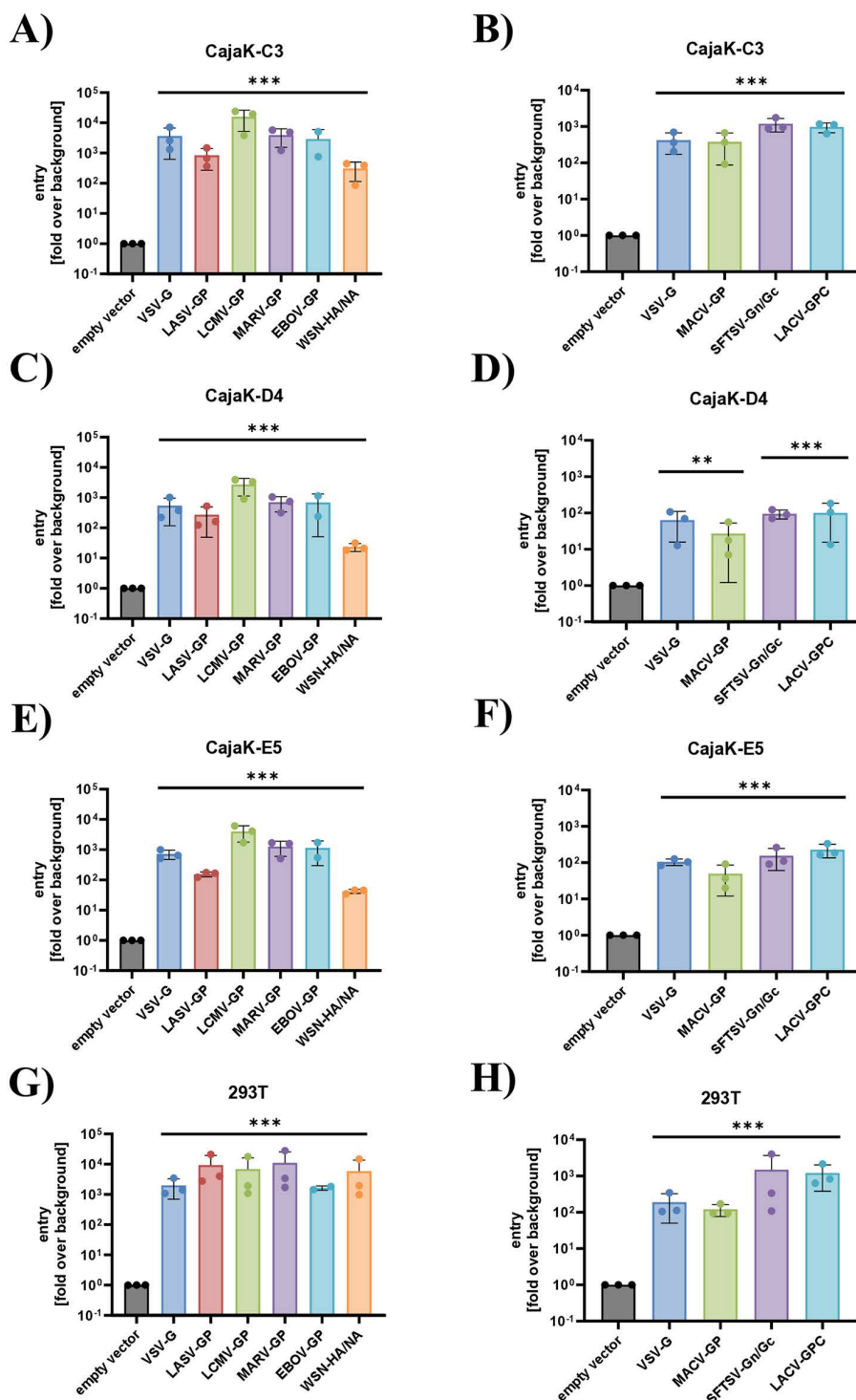


Fig 4. Immortalized marmoset kidney cells are susceptible to entry driven by diverse viral glycoprotein. Marmoset kidney cell lines CajaK-C3 (A, B), CajaK-D4 (C, D) or CajaK-E5 (E, F) as well as 293T control cells (G, H) were infected with MLV-based particles pseudotyped with glycoproteins of rhabdoviral (VSV-G), filoviral (EBOV-GP, MARV-GP), arenaviral (LCMV-GP, LASV-GP, MACV-GP), peribunyaviral (LACV-GPC), phenyuviral (SFTSV-Gn/Gc) and orthomyxoviral (WSN-HA/NA) origin and diluted for comparable luciferase activity upon infection of 293T cells. Two different doses, high (10⁶ cps; A, C, E, G) and low (10⁵ cps; B, D, F, H), were used for infection. After 72 h, cell lysates were harvested and luciferase activities measured. The average of the means of two (EBOV-GP) to three independent experiments (all other) each performed in triplicates is shown. Error bars indicate SEM. Statistical significance was tested by one-way ANOVA with Tukey's correction: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

<https://doi.org/10.1371/journal.pone.0337680.g004>

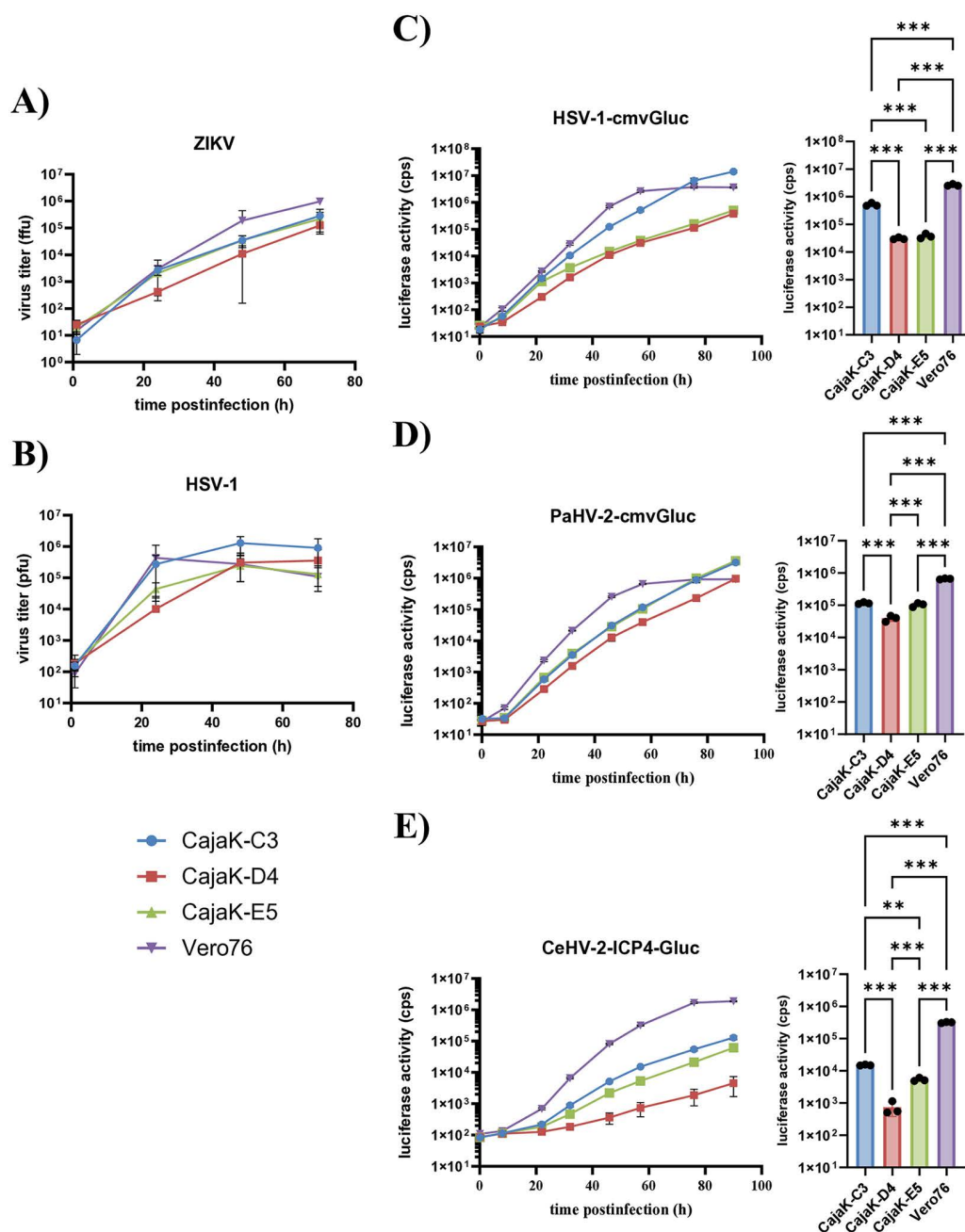


Fig 5. Immortalized marmoset kidney cells support growth of Zika virus and human and primate simplexviruses. (A, B) Infection of cell lines with ZIKV (A) or HSV-1. Marmoset kidney cell lines and Vero76 control cells were infected with MOI 1 and supernatants harvested at the indicated time points post infection. Virus titers were determined on Vero76 cells using focus-forming assay (ZIKV) or plaque assay (HSV-1) and are shown as focus forming units (ffu) or plaque forming units (pfu), respectively. The means of two (ZIKV) or three (HSV-1) independent experiment are shown. Error bars indicate SEM. (C-E) Multicycle growth curves using reporter viruses HSV-1-cmvGluc, PaHV-2-cmvGluc [59] or CeHV-2-ICP4-Gluc [60]. Marmoset kidney cell lines or Vero76 control cells were infected at MOI 0.0004 and supernatant was collected at regular intervals. Infection was quantified by measuring Gaussia luciferase activity in the collected supernatants. The results of single representative experiments are shown (left panels) and were confirmed in two independent experiments. Differences in infection levels were analyzed at 57 h, a time point late in the exponential growth phase (right panels). Error bars indicate SD. Statistical significance was tested by one-way ANOVA with Tukey's correction: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

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both human and mouse cells as stated by the manufacturer. This may be due to low responsiveness to IFN α 2, as has been reported for marmoset hepatocytes treated with human or marmoset IFN α 2 [64]. A reason for this may be a general high ISG expression levels in marmoset hepatocytes. Indeed, when analyzed in immunoblot, we observed high levels of constitutive IFITM3 expression in our kidney cell lines (Fig 3c), as compared to the highly IFN responsive human A549 cell line. However, upon treatment with IFNB1 or cjlIFN α 14 we clearly noted induction of IFITM3 expression in our marmoset cell lines. In summary, crucial steps of the IFN system are functional in the newly described cell lines.

All cell lines were susceptible to entry driven by glycoproteins of multiple human pathogenic viruses (Fig 4). This includes glycoproteins from viruses causing hemorrhagic fevers, such as LASV, EBOV and MARV, for which marmoset models have already been established [5–7], where cell lines may help to reduce and refine animal experiments. Further, infection by LCMV was found to cause hepatitis in marmosets [75,76]. The cell lines were also susceptible to entry driven by the glycoproteins of MACV or SFTSV, for which marmoset models have not been described so far. However, we note that the ability of pseudoparticles to enter cells does not indicate successful viral replication. Therefore, it will be interesting to determine within future studies the permissiveness of these cell lines to authentic viruses in a laboratory with appropriate biosafety levels. Finally, we could demonstrate that the cell lines were permissive to infection by ZIKV and HSV-1 and related primate simplexviruses (Fig 5), albeit replication of CeHV-2 was strongly delayed on all three CajaK-cell lines as compared to Vero cells. Herpesviruses have adapted to their respective host species due to coevolution resulting in multiple interactions with the host proteome to reprogram cellular pathways or to counteract innate immune pathways. The delayed growth of CeHV-2 on CajaK cell lines may therefore reflect suboptimal interaction with proviral factors or inefficient evasion of innate antiviral pathways. Notably, HSV-1 causes lethal systemic infections in common marmosets after epizootic transmission likely from humans [14,16,77] and in a recent outbreak in black-tufted marmosets (*Callithrix penicillata*) virus could be detected in kidney among other organs [27], suggesting that our cell lines model natural target cells.

In summary, we established immortalized kidney cell lines with pericyte identity, which retain a functional IFN system and are permissive to infection with ZIKV, HSV-1 and primate simplexviruses and likely a wide range of other viruses. This broadens the available base of marmoset cell lines and contributes valuable tools for translational research or comparative infection research.

Supporting information

S1 Fig. Principal component analysis. Principal component analysis based on RNA-seq profiles of cell lines CajaK-C3 (red), CajaK-D4 (green) and CajaK-E5 (blue). Duplicate samples are labelled A and B.
(TIF)

S2 Table. Full gene list normalized counts TPM.
(XLSX)

S3 Table. Expression of kidney marker in normalized counts TPM.
(XLSX)

S4 Table. Expression of pericyte marker in normalized counts TPM.
(XLSX)

S5 Table. Expression of fibroblast marker in normalized counts TPM.
(XLSX)

S6 Raw. Images for blots in Fig. 1c and Fig. 3c.
(PDF)

Acknowledgments

We thank Martina Bleyer (German Primate Center, Göttingen, Germany) for providing the kidney tissue sample, Berit Roshani and Katharina Decker (German Primate Center, Göttingen, Germany) for help with flow cytometry and Gabriela Salinas-Riester and Maren Sitte (Core Unit NGS-Integrative Genomics NIG, Göttingen, Germany) for RNA-seq analysis. We thank Greg Smith (Northwestern University Feinberg School of Medicine, Chicago, USA), Thomas von Hahn (Leibniz University Hannover, Germany), Andrea Maisner (Philipps University of Marburg, Germany), Graham Simmons (UCSF, San Francisco, USA), Birke Bartosch (Lyon, France), Michael Farzan (The Scripps Research Institute, USA), David Brown and Matthew Jones (Public Health England, UK) for their kind gift of material.

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Supervision: Stefan Pöhlmann, Michael Winkler.

Writing – original draft: Michael Winkler.

Writing – review & editing: Stefan Pöhlmann, Michael Winkler.

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