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Research Paper

The Human Cytomegalovirus Strain DB Activates Oncogenic Pathways in Mammary Epithelial Cells

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ABSTRACT

Background: Human cytomegalovirus (HCMV) establishes a persistent life-long infection and increasing evidence indicates HCMV infection can modulate signaling pathways associated with oncogenesis. Breast milk is an important route of HCMV transmission in humans and we hypothesized that mammary epithelial cells could be one of the main cellular targets of HCMV infection.
Methods: The infectivity of primary human mammary epithelial cells (HMECs) was assessed following infection with the HCMV-DB strain, a clinical isolate with a marked macrophage-tropism. The impact of HCMV-DB infection on expression of p53 and retinoblastoma proteins, telomerase activity and oncogenic pathways (c-Myc, Akt, Ras, STAT3) was studied. Finally the transformation of HCMV-DB infected HMECs was evaluated using soft agar assay. CTH cells (CMV Transformed HMECs) were detected in prolonged cultures of infected HMECs. Tumor formation was observed in NOD/SCID Gamma (NSG) mice injected with CTH cells. Detection of long non coding RNA4.9 (lncRNA4.9) gene was assessed in CTH cells, tumors isolated from xenografted NSG mice and biopsies of patients with breast cancer using qualitative and quantitative PCR.
Results: We found that HCMV, especially a clinical strain named HCMV-DB, infects HMECs *in vitro*. The clinical strain HCMV-DB replicates productively in HMECs as evidenced by detection of early and late viral transcripts and proteins. Following infection of HMECs with HCMV-DB, we observed the inactivation of retinoblastoma and p53 proteins, the activation of telomerase activity, the activation of the proto-oncogenes c-Myc and Ras, the activation of Akt and STAT3, and the upregulation of cyclin D1 and Ki67 antigen. Colony formation was observed in soft agar seeded with HCMV-DB-infected HMECs. Prolonged culture of infected HMECs resulted in the development of clusters of spheroid cells that we called CTH cells (CMV Transformed HMECs). CTH cells when injected in NOD/SCID Gamma (NSG) mice resulted in the development of tumors. We detected in CTH cells the presence of a HCMV signature corresponding to a sequence of the long noncoding RNA4.9 (lncRNA4.9) gene. We also found the presence of the HCMV lncRNA4.9 sequence in tumors isolated from xenografted NSG mice injected with CTH cells and in biopsies of patients with breast cancer using qualitative and quantitative PCR.
Conclusions: Our data indicate that key molecular pathways involved in oncogenesis are activated in HCMV-DB-infected HMECs that ultimately results in the transformation of HMECs *in vitro* with the appearance of CMV-transformed HMECs (CTH cells) in culture. CTH cells display a HCMV signature corresponding to a lncRNA4.9

Abbreviations: ChIP, chromatin immunoprecipitation; HMECs, human mammary epithelial cells; HCMV, human cytomegalovirus; MOI, multiplicity of infection; UV, ultraviolet rays; HI, heat inactivated; Rb, retinoblastoma; hTERT, human telomerase reverse transcriptase; IE, immediate early; LA, late antigen.

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genomic sequence and give rise to fast growing triple-negative tumors in NSG mice. A similar lncRNA4.9 genomic sequence was detected in tumor biopsies of patients with breast cancer.

1. Introduction

Worldwide breast cancer is the most common cancer diagnosed among women (Warner, 2011). Notably, majority of the breast cancers classified as carcinomas have been found to be originated from the mammary epithelial cells lining the duct responsible for converting most precursors into milk constituents and transporting them to the mammary lumen (Dimri et al., 2005). Breast cancer exhibits heterogeneous molecular characteristics and utilizing gene expression patterns several types of breast cancer have been identified including a normal breast epithelial-like group, a luminal epithelial cell type A, a luminal epithelial cell type B, an ErB2-overexpressing group, a basal-like group and a claudin low group (Lehmann et al., 2011). Etiological factors involved in breast cancer include genetic and environmental risk factors (Hüsing et al., 2012), and among these latter viruses could be involved with close to one-fifth of all cancers in the world caused by infectious agents (Zur Hausen, 2009).

The human cytomegalovirus (HCMV), a highly host specific pathogen, is a member of *Betaherpesviridae* family. HCMV generally causes asymptomatic to mild infection in immunocompetent host. However, its infection in immunocompromised host may result in serious complications (Coaquette et al., 2004). HCMV infects a broad range of cells including monocytes, macrophages, fibroblasts, endothelial cells, epithelial cells, stromal cells, hepatocytes, smooth muscle cells, and neural stem/progenitor cells (Belzile et al., 2014; Khan et al., 2009; Lepiller et al., 2013; Wang and Shenk, 2005). Although HCMV clinical isolates display a broad cellular tropism infecting among others fibroblasts and epithelial cells, the growth of laboratory HCMV strains is restricted to fibroblasts (Wang and Shenk, 2005). In infected patients, the blood monocytes and tissue macrophages are regarded as an important HCMV cellular reservoir responsible for the dissemination of virus and may also act as a site for the establishment of latency (Hargett and Shenk, 2010; Khan et al., 2009; Smith et al., 2004). Noteworthy, HCMV has the ability to induce a distinct inflammatory (M1) and immunosuppressive (M2) macrophages polarization (Chan et al., 2009). In addition, macrophage polarization into M1/M2 phenotype is associated with the secretion of cytokines that could play a pivotal role in viral replication and fitness, and favor breast cancer promotion (Grivennikov et al., 2010; McKinney et al., 2014; Teng et al., 2012).

Role of HCMV in inflammatory diseases and cancer has been well speculated (Cobbs et al., 2002; Lepiller et al., 2011; Söderberg-Nauclér, 2006). Earlier studies demonstrated that HCMV was able to induce the *in vitro* transformation of human embryo lung fibroblasts (Clanton et al., 1983; Geder et al., 1976). More recently, HCMV DNA or antigen has been found in tumor tissues from brain (glioblastoma, medulloblastoma), colon, prostate, liver and breast cancer (Banerjee et al., 2015; Baryawno et al., 2011; Bhattacharjee et al., 2012; Harkins et al., 2010; Samanta et al., 2003; Taher et al., 2013). Besides a direct role of HCMV in cellular transformation, HCMV could infect the tumor tissue and acts as a cofactor in amplifying mechanisms of oncogenesis, a paradigm called as oncomodulation (Michaelis et al., 2009).

Previously, we isolated a clinical HCMV strain from a 30-year-old pregnant woman named as HCMV-DB (KT959235), which is highly macrophage-tropic, triggers a M2 phenotype and upregulates the proto-oncogene Bcl-3 (Khan et al., 2009). HCMV-DB strain is close from other primary clinical isolates which also infect macrophages such as PH and TR strains (Suppl. Fig. S1) (Michaelis et al., 2009). There is scarcity of direct evidence suggesting the involvement of HCMV in transformation of human mammary epithelial cells (HMECs) (Herbein and Kumar, 2014). Here, we assessed the potential direct oncogenic role of HCMV in primary human mammary epithelial cells (HMECs) *in vitro* and *in vivo*.

2. Materials and Methods

2.1. Reagents

Anti-p53, anti-Rb, anti-Ras, anti-pAktThr308, anti-pAktSer473, anti-Akt and anti-cyclinD1 antibodies were purchased from Cell signaling (Danvers, MA, USA). Anti-pSTAT3, anti-STAT3 and anti-Myc antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-actin antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-pp65, anti-pp85 (pUL25), anti-pp71 (pUL82), anti-IE1 and anti-IE2 antibodies were purchased from Santa Cruz Biotechnology. Recombinant Raf1-GST was purchased from Millipore (Molsheim, France).

2.2. Cell Cultures

Human primary mammary epithelial cells (HMECs) were obtained from Life Technologies (Carlsbad, CA, USA). MDA-MB-231 and MCF-7 cells were provided by Institut Hiscia (Arlesheim, Switzerland). HMECs were cultivated in HMEC medium (Life Technologies, Carlsbad, CA, USA) supplemented with HMEC supplement and bovine pituitary extract (Life Technologies, USA). Cell viability assay was performed as previously described (Khan et al., 2009). Cultures were free of mycoplasma.

2.3. HCMV Infection of HMECs

AD169 is a highly passaged laboratory strain of HCMV originally isolated from the adenoids of a child (Murphy et al., 2003). The clinical isolate HCMV-DB was isolated in our laboratory from a cervical swab specimen from a 30-year-old pregnant woman (Khan et al., 2009). Cell-free virus stocks were prepared by propagating AD169 in MRC5 human fibroblasts, meanwhile HCMV-DB were grown in macrophages, as described previously (Khan et al., 2009). The TB40/E strain was isolated from a throat wash of a bone marrow transplant recipient (Tomasec et al., 2005). MRC5 human fibroblasts were cultured as described previously (Coaquette et al., 2004; Khan et al., 2009). HMECs (1×10^6) and MRC5 cells (1×10^6) were infected at a multiplicity of infection (MOI) of 1 or 10 for 2 h at 37 °C, washed thoroughly (three times with 1× PBS), and covered with fresh medium. Where specified, HCMV heat-inactivated at 95 °C for 10 min or treated with UV (1200 μJ/cm^2 for 15 min) were used as controls. Supernatants were clarified by centrifugation and stored at −80 °C until use. Virus titers were determined by plaque-forming assay in MRC5 human fibroblasts as described previously (Khan et al., 2009). The purity of our HCMV stocks was confirmed by the absence of detection of other viruses (HSV-1, HSV-2, varicella-zoster virus, Epstein-Barr virus, adenovirus, BK virus) using PCR screening (data not shown). Following HCMV infection of HMECs, viral replication was assessed by the appearance of a cytopathic effect (CPE) in the cultures and by detection of IE1, IE2, pp65 and pp85 by western blotting and IE1 antigen (clone E13, Argene-Biosoft, Varihes, France) using immunofluorescence microscopy (Nikon Eclipse E400, Kanagawa, Japan). For the detection of HCMV mRNA using RT-PCR assay, total RNA was extracted from uninfected, UV-treated and HCMV-DB infected HMECs with RNeasy mini kit (Qiagen). Total 2 μg of RNA was reverse transcribed into cDNA with Superscript III RT (Life Technologies) using oligo (dT) primers. The 5 μl of reverse transcription reaction product was amplified using primers against IE1, US28 and UL82 primers as

described previously (Lepiller et al., 2013). The beta-globin gene was amplified as an internal control (sense, 5′-TCCCCTCCTACCCCTACTTTC TA-3′; antisense, 5′-TGCCTGGACTAATCTGCAAGAG-3′). The PCR product was electrophoresed on a 2% agarose gel containing ethidium bromide. Quantification of viral titer in cell culture supernatants was performed by qPCR as previously described (Khan et al., 2009). The UL128, UL131, UL133 and UL138 genes were amplified using the following primers: UL128 (sense, 5′-GATTCGCGGGATCGTCACCA-3′; antisense, 5′-TCACTGGAGCATATAGCCCA-3′); UL131 (sense,5′-ATGTGT ATGATGTCTCATAATAAAGC-3′; antisense, 5′-TCAACGTGACGTCCAC GAGC-3′); UL133 (sense, 5′-AGACTCCGTAATCGACCTCC-3′; antisense, 5′-GATGACCGTATTGGACCATGTC-3′) and UL138 (sense, 5′-ACGATC TGCCGCTGAATGTC-3′; antisense, 5′-ACAGCTCGCAACAGCGGATC-3′). Quantification of c-Myc (MYC) and cyclin-D1 (CCND1) transcripts in HMECs infected with HCMV-DB (moi = 1, day 1 postinfection) and uninfected HMECs was performed using the human breast cancer RT^2 profiler PCR assay (PAHS-131ZA) (Qiagen). HCMV entry assays were performed as previously described (Khan et al., 2009).

2.4. Western Blotting

Cellular extracts of HMECs either uninfected or infected with HCMV or UV-treated HCMV strains were used to examine the expression of p53, Rb, pRb, c-Myc, Ras, pAkt(Thr308), pAkt(Ser473), Akt, pSTAT3, STAT3, cyclin D1, IE1, IE2, pp65, pp85 and β-actin protein by western blotting as described previously (Khan et al., 2009). Where specified, protein levels were quantified by densitometry using ImageJ 1.40 software (National Institutes of Health, Bethesda, MA, USA).

2.5. Pull-Down Assay

For GST-pull down assay, 20 μg of GST-Raf (Millipore) proteins were immobilized on glutathione agarose beads (Thermo Fisher Scientific, Rochester, NY), washed five times with 1× PBS, and were incubated overnight at 4 °C with 500 μg of lysates. The suspension was then washed three times with 1× PBS, denaturized and the expression of active-Ras was analyzed by SDS-PAGE and autoradiography.

2.6. Immunoprecipitation

HMECs were mock-treated or were infected with HCMV strains (MOI = 1). At day 3 post infection, the cell lysates were precleared by adding 50 μl of protein A magnetic beads (Millipore) for 1 h at 4 °C. The clear supernatants were removed, combined with anti-p53 antibody (Cell Signaling) or isotype control antibody (Millipore) and incubated overnight at 4 °C. The lysates were further incubated with 50 μl protein A magnetic beads (Millipore) at 4 °C for 2 h. Immune complex were washed in the presence of protease inhibitors (Roche, Meylan, France) and bound protein was eluted with sample buffer and run on 10% SDS-PAGE gels. Expression of IE2 was determined by western blotting.

2.7. Chromatin Immunoprecipitations Assay

p53 and H3K9me3 chromatin immunoprecipitations (ChIPs) were performed with EZ-Magna ChIP™ A - Chromatin Immunoprecipitation Kit (Millipore). Briefly, 10^7 HMECs were infected with HCMV-DB and at day 1 post infection cells were fixed and sonicated as per manufacturer's instructions. Sonication conditions were as follows: 9 pulses, 40% amplitude, pulse duration 9 s and gap between each cycle 30 s. p53 and H3K9me3 ChIP assays were performed as described previously (Soria et al., 2010). DNA was purified and analyzed using PCR followed by agarose gel electrophoresis.

2.8. Flow Cytometry Analysis

For proliferation assays, HMECs were left uninfected or were infected with HCMV. Proliferation was measured using the quantification of Ki67 Ag expression by intracellular flow cytometry as described previously (Lepiller et al., 2013). To discriminate between infected and uninfected HMECs, the pp71 HCMV expression was detected by flow cytometry using an anti-pp71 antibody.

2.9. Assessment of Telomerase Activity

Telomerase activity was assayed by using TRAPEZE Telomerase detection kit as recommended by the manufacturer (Chemicon, Temecula, CA). Control and HCMV-infected HMECs were suspended in 3-[(3-cholamidopropyl) dimethyl ammonio] propanesulfonic acid (CHAPS) lysis buffer (10 mMTris – HCl [pH 7.5], 1 mM $MgCl_2$,1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM P3-mercaptoethanol, 0.5% CHAPS, 10% glycerol) for 20 min on ice and centrifuged at 20,000*g* for 20 min at 4 °C. The amount of protein in the supernatants was determined using a DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). For each assay of telomerase activity, 1 μg of protein was used, and 30 PCR cycles were performed after the elongation reaction using telomerase primers. The PCR products were resolved by polyacrylamide gel electrophoresis and visualized by staining with SYBR Green I (Roche). For the detection of hTERT transcripts, total cellular RNA was isolated from uninfected or HCMV-infected HMECs with an RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized with OligodT20 primers using a SuperScript III First-Strand Synthesis System (RT-PCR; Invitrogen Life Technologies, Carlsbad, CA). hTERT mRNA expression was analyzed by RT-PCR with primers specific for hTERT mRNA (accession no. AF015950): 5′-CGGAAGAGTGTCTGGA GCAA-3′ and 5′-GGATGAAGCGGAGTCTGGA-3′.

2.10. Soft Agar Colony Formation Assay

Colony formation in soft agar seeded with uninfected HMECs, HMECs infected with wild-type or heat-inactivated HCMV, MCF-7 cells and MDA-MB-231 cells was assayed using Cell Biolabs Cytosolic Cell Transformation Assay kit (Colorimetric assay, CB135; Cell Biolabs Inc., San Diego, CA) as per the manufacturer's protocol. Starting 1 day post infection, cells were incubated for 14 days (15 days post-infection) in the semisolid agar medium before solubilization and detection using the provided MTT solution for quantification of the formation of colonies in soft agar (Cayman Chemical, Ann Arbor, MI) and a microplate reader (A_{570nm}). Colonies were observed under an Olympus microscope (Center Valley, PA). Following DNA extraction from soft agar colonies, the HCMV major immediate early promoter (MIEP) and beta-globin sequences were amplified using the following primers: MIEP (sense, 5′-TGGGACTTTCCTACTTGG-3′; antisense, 5′-CCAGGCGATCTGACGGTT-3′) and beta-globin (sense, 5′-TCCCCTCCTACCCCTACTTTCTA-3′; antisense, 5′-TGCCTGGACTAATCTGCAAGAG-3′).

2.11. Isolation and Growth of CTH Cells

HMECs cultures were infected with HCMV-DB at a MOI of 1. Several clusters of spheroid-cells were observed in HMECs infected with HCMV-DB around day 20 post infection in some of the cultures. These spheroid-cell clusters detected in HMEC cultures infected with HCMV-DB were gently detached and the floating detached cells named CTH cells were cultured in HMEC Ready medium (Cat#12752010, Gibco, Grand Island, NY) for numerous passages, currently >150 passages.

2.12. Animals

Six-week-old female NOD/SCID Gamma (NSG) mice purchased from Charles River Laboratories (L'Arbresle, France) were kept under strict

pathogen-free conditions at the Central Animal facilities of University of Franche-Comté (notification d'autorisation n° 05085.02). Cell viability was determined by trypan blue staining and cells were counted using haemocytometer. After harvesting and during injection, cells were maintained at 4 °C. Two million of CTH cells, five million cells of uninfected HMECs, two million of MCF-7 cells and MDA-MB-231 cells suspended in 100 μl of serum-free Dulbecco's modified Eagle's medium together with 100 μl of Matrigel at the day 0, were injected into the mammary fat pad of NSG mice. Mice were checked twice in a week for tumor growth. Once tumor development was detectable by eye, tumor dimensions were measured using vernier caliper. Tumor volume was calculated using the formula: volume (cm^3) = (d × d × D)/2 where d is the shortest diameter and D is the longest diameter. On day 37 post injection, mice were killed according to the guidelines of the animal ethical committee. Tumors were retrieved from the mice and analyzed for the presence of MIEP and lncRNA4.9 sequences from HCMV-DB using PCR.

2.13. Immunohistochemistry of Mice Tumors

Formalin-fixed tumors retrieved from the mice were embedded in wax and sections (5 μm) were prepared using standard methods and stained with eosin and hematoxylin. Sections were processed and stained individually for hematoxylin and eosin staining, ER (1:1, Roche), PR (1:1, Roche), HER2 (1:1, Roche), vimentin (1:400, Leica), E-cadherin (1:50, Dako), CK5/6 (1:50, Dako), GATA3 (1:100, Cell Marque), CK20 (1:5000, Biocare), and GCDFP (1:1000, Cell Marque) before observation by microscopy.

2.14. Detection of HCMV lncRNA 4.9 Genomic Sequence in CTH Cells, Xenografted Mice Tumor and Human Breast Cancer Tissue

Total DNA from uninfected HMEC, HCMV-DB infected HMEC, CTH cells and MRC5 cultures was isolated. The retrieved mice tumors were grinded in liquid nitrogen and DNA was isolated using QIAamp DNA mini kit (Qiagen, Valencia, CA) as per manufacture's guidelines. Genomic DNA isolated from patient breast tumor biopsies and from healthy human breast tissue was provided by the regional tumor bank (BB-0033-00024 Tumorothèque Régionale de Franche-Comté).

Presence of HCMV was determined by qualitative and quantitative PCR where specified using a set of HCMV-DB lncRNA 4.9 gene primers (sense, 5′-GTGAACCGATACGGGTGCAG-3′; antisense, 5′-CATTTGAACAGAGAAAGGTGG-3′). An amplicon of 126 bp corresponding to HCMV-DB lncRNA4.9 gene was amplified as confirmed by Sanger's sequencing (Genoscreen, Lille, France and GATC, Köln, Germany). We also screened CTH cells, mice tumors and human breast biopsies (tumor and healthy tissue) for the presence of MIEP sequence using PCR assay (sense, 5′-TGGGACTTTCCTACTTGG-3′; antisense, 5′-CCAGGCGATCTGACGGTT-3′). As a positive control, DNA isolated from HCMV-DB infected HMECs and from HCMV-DB viral stock was included in the study. Equal amount of DNA was analyzed by PCR using HCMV-DB MIEP and lncRNA 4.9 primers. As equal loading control β-globin gene was amplified (sense, 5′-TCCCCTCCTACCCCTACTTTCTA-3′; antisense, 5′-TGCCTGGACTAATCTGCAAGAG-3′). Amplified product was electrophoresed in 2% agarose gel stained with Sybr green I nucleic acid stain.

Table 1
Phylogenetic analysis based on *UL144* gene was determined among several HCMV strains.

	Strain name	Abbreviation	Accession number
1	HHV5 strain DB, complete genome	DB	KT959235
2	HHV5 transgenic strain Towne, complete genome	Towne	GQ121041
3	HHV5 strain AD169, complete genome	AD169	FJ527563
4	HHV5 strain TB40/E clone Lisa, complete genome	TB40/E	KF297339
5	HHV5 transgenic strain Merlin, complete genome	Merlin	GU179001
6	HHV5 strain Toldeo, complete genome	Toledo	GU937742
7	HHV5 strain VR1814, complete genome	VR1814	GU179289
8	HHV5 strain Davis, complete genome	Davis	JX512198
9	HHV5 strain JP, complete genome	JP	GQ221975
10	HHV5 strain TR, complete genome	TR	KF021605
11	HHV5 PH-BAC isolate, complete genome	PH	AC146904

2.15. Phylogenetic Analysis

Phylogenetic analysis was determined among several HCMV strains (described in Table 1) with respect to the *UL144* gene as previously reported (Waters et al., 2010). Multi-sequence alignments (MSA) were performed using CLUSTAL W with following parameters: a gap opening penalty of 15 and gap extension penalty of 6.66. Phylogenetic tree was constructed using the neighbor-joining method. Each clustering was confirmed by the bootstrap method with 1000 replicates. The analysis was conducted using MEGA7 (http://www.megasoftware.net/).

2.16. Statistical Analysis

The reported values are the means and SD of independent experiments. Statistical analysis was performed using Mann Whitney *U* test, and differences were considered significant at a value of $P < 0.05$. Microsoft Excel was used to construct the plots.

3. Results

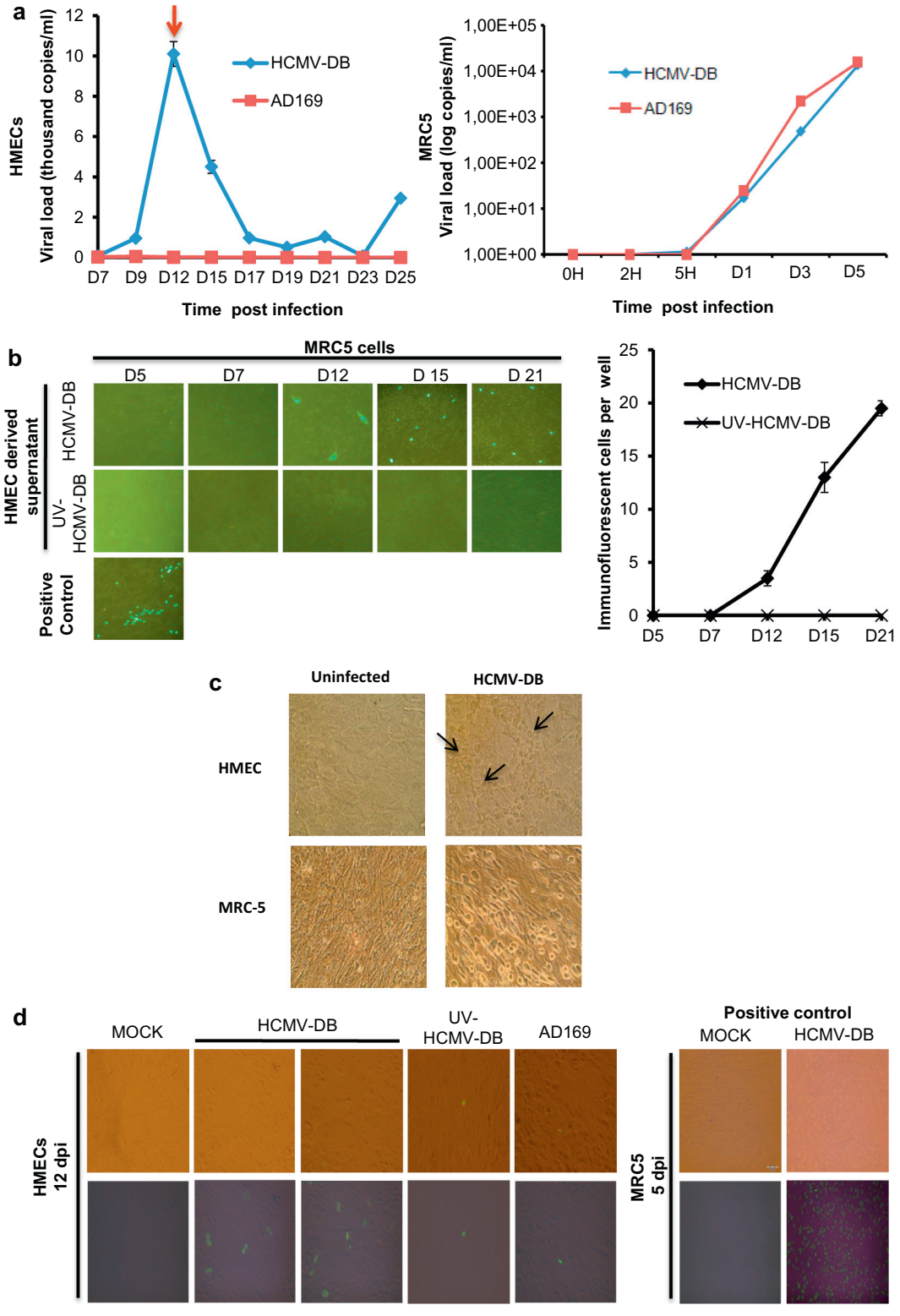
3.1. HCMV Permissively Infects and Completes its Life Cycle in HMECs

Although HCMV proteins and DNA have been reported in breast cancer tissue (Harkins et al., 2010; Taher et al., 2013), so far only few attempts have been made to investigate HCMV replication in HMECs *in vitro* (Twite et al., 2014). We infected HMECs with two strains of HCMV, HCMV-DB a clinical isolate which is macrophage-tropic, close to PR and TH strains and belongs to UL144 genotype C (Suppl Fig. S1) (Khan et al., 2009), and the extensively passaged laboratory strain AD169 that has lost the ability to efficiently replicate in endothelial and epithelial cells (Sinzger et al., 2008; Wang and Shenk, 2005). We observed productive replication of HCMV-DB in infected HMECs with peak viral titer at day 12 post infection (Fig. 1a, left panel). AD169 did not replicate in HMECs (Fig. 1a, left panel). The level of productive infection of the HCMV-DB strain in HMECs was much lower than what was

Fig. 1. HCMV-DB infects and replicates in HMECs. a. Growth kinetics of HCMV in HMECs and MRC5 cells. Left panel. HMECs were infected individually with two HCMV strains (HCMV-DB and AD169) (MOI = 1). At 2 h post infection inoculum was removed and cells were washed three times with 1× PBS followed by three washes with HMEC media. At several time points (up to day 25) supernatants were collected and viral growth kinetics were determined by realiversity of Bourgogne France-Comté (UBFC), F-25030 time qPCR. Right panel. MRC5 cells were infected with HCMV-DB and AD169 (MOI = 1) and supernatant was collected at several time points (up to day 5). Viral growth kinetics was determined using real time qPCR. Results represent means (±SD) of three independent experiments. b. Replicative virus is detected in supernatants harvested from HMECs infected with HCMV-DB. Supernatants collected from HMECs infected with HCMV-DB at several time points (up to day 21) were used to infect MRC5 cells and at day 5 post infection cells were fixed. Expression of IE1 antigen was determined by immunofluorescence as described in Materials and Methods. Direct infection of MRC5 cells by HCMV-DB (MOI = 1) was used as a positive control. Supernatants harvested from HMECs infected with UV-inactivated HCMV-DB (MOI = 1) were used as a negative control. Right panel. The curves represent the number of IE1 positive foci detected in MRC5 cultures treated with supernatants harvested from HMECs infected with HCMV-DB and UV-inactivated HCMV-DB respectively. Results represent means (±SD) of three independent experiments. c. Detection of a typical cytopathic effect at day 15 post-infection in HMECs infected with HCMV-DB (MOI = 1) (Upper right panel). MRC5-infected with HCMV-DB were used as a positive control (Lower right panel). Mock-infected HMECs and MRC5 cells were included as negative controls. Magnification 100×. d. Detection of IE1 antigen in HMECs infected with HCMV-DB at day 12 post infection. Presence of IE1 antigen was assessed in HMECs cells infected with HCMV-DB (two positive cultures are shown) and AD169 strains at day 12 post infection by immunofluorescence assay as described in Materials and Methods. As controls, uninfected HMECs and MRC5 cells, HMECs infected with UV-treated HCMV-DB and MRC5 cells infected with HCMV-DB are shown. Magnification 20×.

observed in MRC5 fibroblasts, using a PCR assay (Fig. 1a, right panel). To determine whether HCMV DNA detection by PCR assay represents genuine replicative virus, we harvested supernatants of HMECs infected with HCMV-DB strain up to day 21 post infection. Harvested supernatants were used to infect MRC5 cells and the number of infected cells was determined by using IE1 immunofluorescence staining (Fig. 1b). Using harvested supernatants to infected MRC5 cells, we observed low levels of HCMV-DB replication in HMECs (Fig. 1b). In contrast supernatants of HMECs infected with UV-inactivated HCMV-DB failed to replicate in MRC5 cells (Fig. 1b). MRC5 cells directly infected with HCMV-DB were used as a positive control (Fig. 1b).

Although limited, a typical cytopathic effect was observed in HMEC cultures directly infected with HCMV-DB (Fig. 1c). In contrast to AD169, HCMV-DB was able to start its life cycle in HMECs as evidenced by expression of IE1 genes determined by immunofluorescence assay (Fig. 1d). We detected both viral transcripts and proteins of various



phases of HCMV replication cycle (immediate early to late phases) in HMECs infected with HCMV-DB but not in AD169 infected cultures. We observed the transcripts of IE1, US28 and UL82 at two time points, day 1 and day 3 of post infection (Fig. 2a). The immediate early proteins (IE1 and IE2), the tegument protein pp65 and the late antigen pp85 were detected in HMECs infected with HCMV-DB up to 5 days post infection using western blotting (Fig. 2b). The detection of the pp65 tegument protein at early time-points post infection is most likely due to the translocation of pp65 after virus entry as previously reported (Hensel et al., 1995). Of note, in contrast to AD169 we found that HCMV-DB has an intact UL*b′* region that has been shown to govern tropism of HCMV strains, especially for efficient infection of epithelial cells (Fig. 2c). Taken together our data indicate a tropism of the clinical isolate HCMV-DB for HMECs.

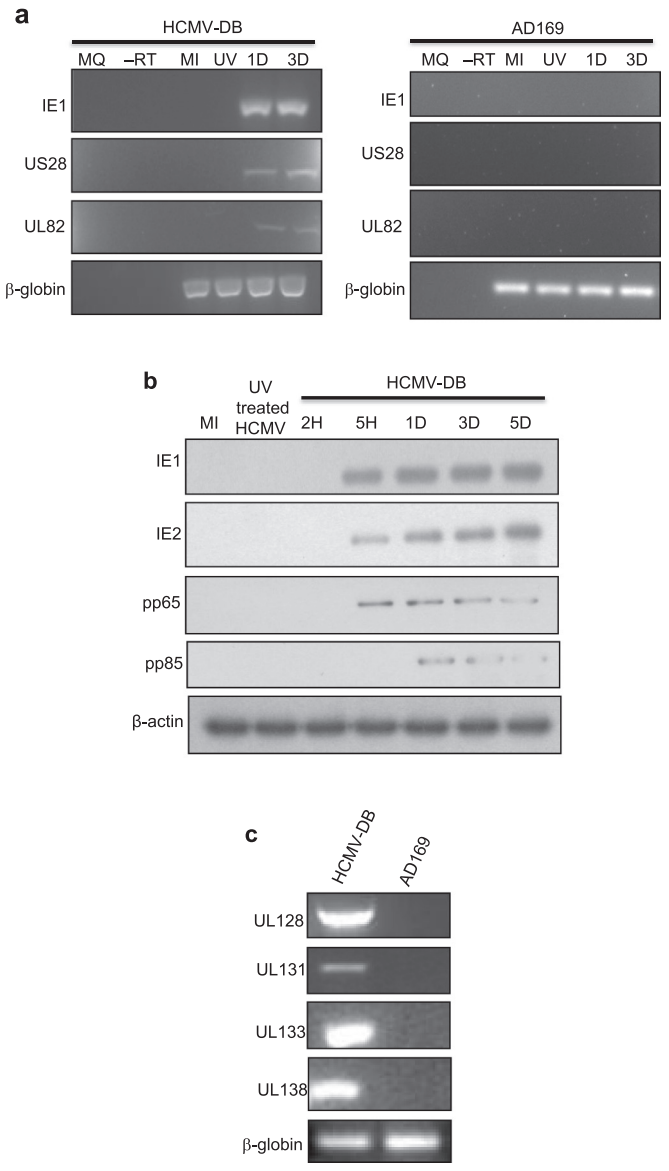


Fig. 2. Detection of HCMV transcripts and proteins in infected HMECs. a. Detection of IE1, US28 and UL82 transcripts in HMECs infected with HCMV-DB, but not in HMECs infected with AD169, at day 1 and day 3 post infection using RT-PCR. Beta-globin was used as an internal control. Results are representative of three independent experiments. b. Detection of immediate early (IE1 and IE2) and late (pp65 and pp85) viral proteins in HMECs infected with HCMV-DB using western blotting. Results are representative of three independent experiments. c. Determination of an intact ULb′ locus in HCMV-DB, but not in AD169, using conventional DNA PCR. Results are representative of three independent experiments.

3.2. HCMV Infection Promotes Oncogenic Environment in HMECs

A limited set of growth-deregulating changes is required for oncogenic conversion of HMECs. These changes minimally involve the inactivation of the p53 and retinoblastoma protein (Rb) pathways, telomere maintenance conferred by the *hTERT* gene, and acquisition of a constitutive mitogenic signal provided by oncogenic H-Ras (Elenbaas et al., 2001).

HCMV is known to upregulate the cellular levels of p53 in infected cells (Hannemann et al., 2009). We also observed the upregulation of p53 protein in HMECs infected with the HCMV-DB strain as measured by western blot (Fig. 3a). In addition, we observed the binding of p53 to HCMV-IE2 viral protein in infected HMECs by co-immunoprecipitation assay (Fig. 3b), indicating a potential inhibitory effect of IE2 on p53 transcriptional activity as previously reported (Hsu et al., 2004). In HMECs infected with HCMV-DB, we observed decreased binding of p53 to endogenous p53-responsive promoters of *p21* and *MDM2* genes as shown by chromatin immunoprecipitation (Fig. 3c).

Sustained human telomerase reverse transcriptase (hTERT) activity is one of the prerequisite for the immortalization and potentially transformation of HMECs *in vitro* (Elenbaas et al., 2001). HCMV has been reported to activate telomerase in normal human fibroblasts and human malignant glioma cell lines (Stråat et al., 2009). Therefore, we determined the expression of hTERT mRNA and telomerase activity in HMECs infected with HCMV-DB. We observed overexpression of hTERT mRNA and enhanced telomerase activity in HMECs infected with HCMV-DB as compared to uninfected HMEC cells (Fig. 4a and b). Overexpression of hTERT mRNA and enhanced telomerase activity observed in HMECs infected with HCMV-DB could contribute actively to the pro-oncogenic environment in HCMV-DB infected cells.

DNA tumor viruses encode gene products which can functionally inactivate Rb, promoting cellular proliferation and viral DNA synthesis (Helt and Galloway, 2003). The inactivation of the Rb protein results from direct degradation of the Rb protein in the 26S proteasome by HCMV pp71 and/or from its hyperphosphorylation by UL97 protein which inhibits the tumor suppressor activity of Rb (Hume et al., 2008; Kalejta and Shenk, 2003). We observed that Rb is hyperphosphorylated at Ser 780 residue after infection of HMECs with HCMV-DB (Fig. 5) and that Rb expression is downregulated at day 3 post infection (Fig. 5) which is consistent with previous findings (Hume et al., 2008; Kalejta and Shenk, 2003). Thus, both p53 and Rb could be inactivated in HMECs infected with HCMV-DB therefore potentially abolishing cellular senescence and hence promoting unchecked cell division (Dimri et al., 2005).

The acquisition of a mitogenic signal provided by the proto-oncogene Myc and/or activated Ras has been reported to be critical to observe complete transformation of HMECs *in vitro* (Elenbaas et al., 2001; Wang et al., 2011). We observed overexpression of c-Myc protein and transcript in HMECs infected with HCMV-DB by western blotting and RT-PCR microarray, respectively (Fig. 5, Suppl. Fig. S2). In HMECs infected with UV-treated HCMV-DB, we also observed higher levels of c-Myc protein as compared to uninfected controls (Fig. 5). In addition, we observed higher expression of activated Ras (GTP-Ras) on day 3 postinfection in HMECs infected with HCMV-DB (Fig. 5).

Upregulation of c-Myc and activated Ras coupled with constitutively activated PI3K/Akt pathway can lead to anchorage-independent growth and transformation of HMECs (Zhao et al., 2003). Therefore, we further assessed the PI3K/Akt signaling in HMECs infected with HCMV-DB. We observed that Akt undergoes serine-473 and threonine-308 phosphorylation in HMECs infected with HCMV-DB (Fig. 5). We did not observe any significant increase in the levels of Akt protein in HMECs infected with HCMV-DB as compared to controls using western blotting (Fig. 5).

In our previous study, we have observed the activation of JAK/STAT3 axis in primary human hepatocytes and HepG2 cells infected with HCMV (Lepiller et al., 2013). In addition STAT3 activation has been

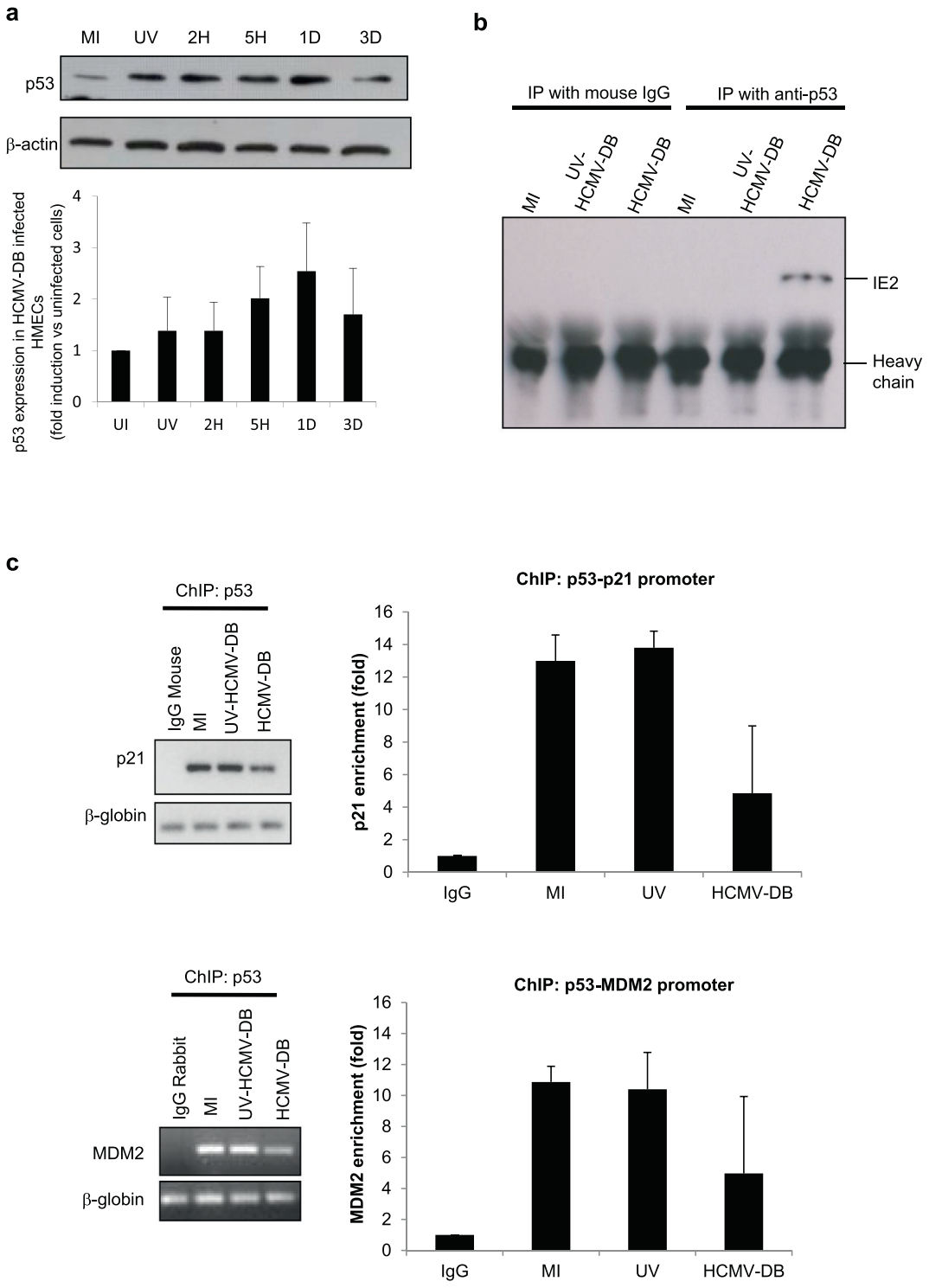


Fig. 3. Functional inactivation of p53 in HMECs infected with HCMV-DB. a. HCMV-DB upregulates the expression of the p53 protein in infected HMECs. HMECs cells were left uninfected or infected with either HCMV-DB or UV-inactivated HCMV-DB (MOI = 1). Cell lysates were prepared at several time points and expression of the p53 protein was determined using western blotting. Beta-actin was included as an internal control. Results are representative of three independent experiments. The histogram represents means (±SD) of three independent experiments. b. The interaction between HCMV-DB IE2 protein and p53 was detected by co-immunoprecipitation as mentioned in Materials and Methods. Results are representative of two independent experiments. c. Decreased binding of p53 to its target genes promoters *p21* and *MDM2* in HMECs infected with HCMV-DB. p53 ChIPs were assessed by semi-quantitative as well as by quantitative PCR for p21 and MDM2 promoter sequences as described in Materials and Methods. Results represent mean (±SD) of three independent experiments.

described in breast cancer biopsies (Diaz et al., 2006). We assessed the expression of STAT3, phospho-STAT3 and cyclin D1 in HMECs infected with HCMV-DB. We observed the enhanced phosphorylation of STAT3 protein (P-STAT3) and the upregulation of cyclin D1 protein and its transcript in HMECs infected with HCMV-DB as compared to uninfected and UV-treated controls (Fig. 5, Suppl. Fig. S2). Our data indicate that HCMV-DB activates STAT3 in HMECs that leads to upregulation of cyclin-D1, a key regulator of cell proliferation. Therefore, we assessed the proliferation of HMECs infected with HCMV-DB. We measured the expression of the nuclear antigen Ki67, a hallmark of cell proliferation

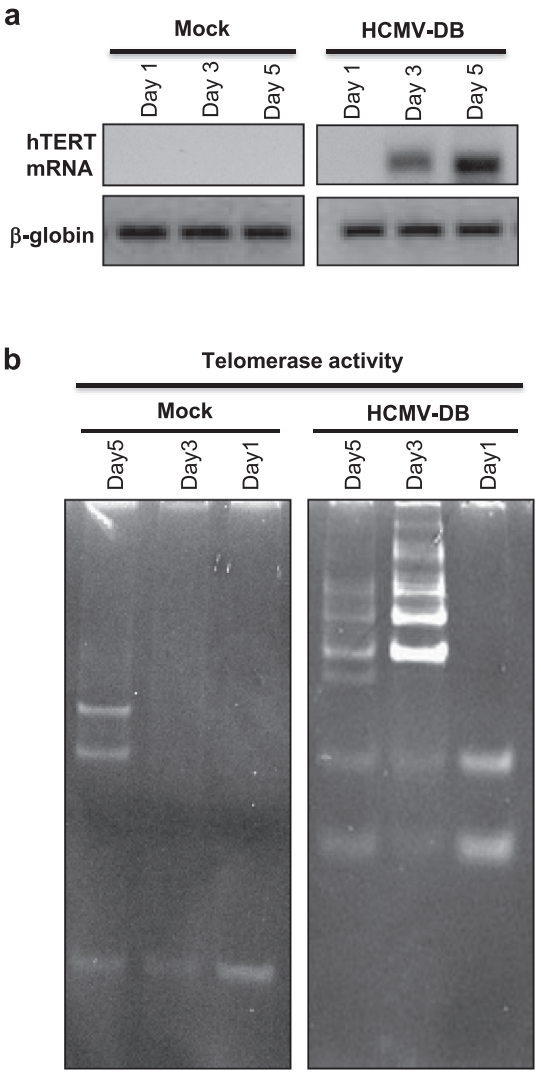


Fig. 4. Upregulation of telomerase transcript and telomerase activity in HMECs infected with HCMV-DB. Upregulation of telomerase mRNA (a) and telomerase activity (b) in HMECs infected with HCMV-DB compared to mock infected HMECs. HMECs were infected with HCMV-DB (MOI = 1) and cells were harvested at different time points. Mock infected cells were included as a negative control. a. RNA was isolated from the cells and expression of hTERT mRNA was determined by RT-PCR as described in Materials and Methods. b. Increased telomerase activity in HMECs following infection with HCMV-DB. Telomerase activity was determined using TRAPEZE Telomerase detection kit as described in Materials and Methods. Results are representative of three independent experiments.

by flow cytometry. We observed significant increase in Ki67 Ag expression upon HCMV-DB infection of HMEC cells (Fig. 6). As an additional control, viral supernatants were filtered through 0.2 μm filter and were added to HMECs (Fig. 6a). The increase in Ki67 antigen expression upon HCMV-DB infection of HMEC cells was abolished by UV-inactivation of the virus (Fig. 6b). The cellular proliferation was observed primarily in infected HMECs labelled with an anti-pp71 HCMV antibody compared to uninfected (pp71-negative) cells (Fig. 6c).

Altogether, our results indicate that, in HMECs infected with the clinical isolate HCMV-DB, the molecular requirements for oncogenic immortalization and transformation of primary HMECs *in vitro* are fulfilled, namely inactivation of p53 and Rb, telomere maintenance, acquisition of constitutive mitogenic signals provided by Ras/cMyc, Akt activation, STAT3 activation and cyclin D1 overexpression with enhanced cellular proliferation.

3.3. Cellular Transformation of HMECs by HCMV-DB

Above-mentioned observations indicate that HCMV can challenge HMECs to proceed towards oncogenic transformation *in vitro*, preferentially with the clinical isolate HCMV-DB. Thus to further test whether these observations can be translated into malignant transformation of HMECs, we proceed to perform soft agar assay which is the most stringent *in vitro* assay for the detection of transformation. HMECs were infected individually with HCMV-DB and AD169 and at day 1 post infection HMECs were seeded in soft agar medium for 14 days. In parallel, uninfected cells and cells infected with heat-inactivated HCMV and UV-treated HCMV were seeded as negative controls, and tumoral MCF-7 cells and MDA-MB-231 cells were seeded as positive controls. As additional controls, viral supernatants were filtered through 0.2 μm filter and were used to infect HMECs. In addition, HMECs were treated with ganciclovir followed by HCMV infection. After 14 days of culture (*i.e.* on day 15 post-infection), we observed the formation of colonies in soft agar that had been seeded with HMECs infected with HCMV-DB (Fig. 7). Colonies were also observed for established representative breast cancer cell lines such as MCF-7 cells and MDA-MB-231 cells (Fig. 7). We did not observe the formation of colonies in soft agar that had been seeded with HMECs infected with AD169 (Fig. 7) or with MRC5 cells infected with HCMV-DB or AD169 (data not shown). Additionally, we isolated DNA from soft agar colonies at day 15 post infection (after 14 days of culture in soft agar) and assessed the presence of HCMV DNA, namely the major immediate early promoter (MIEP) sequence, using a PCR assay. Surprisingly, we did not detect HCMV MIEP DNA in colonies grown in soft agar seeded 14 days earlier with HMECs infected with HCMV-DB (Suppl. Fig. S3).

3.4. Emergence of Clusters of Spheroid Cells Termed "CMV-Transformed HMECs" or CTH Cells in HCMV-DB-Infected HMEC Cultures

HMEC cultures were individually infected with HCMV-DB, Epstein-Barr virus (EBV), herpes simplex virus type 1 (HSV-1) and varicella zoster virus (VZV). As negative controls uninfected HMECs, UV-inactivated HCMV-DB and HCMV-DB supernatant passaged through 0.2 μm were used to infect HMECs. Several clusters of spheroid cells called CMV-transformed HMECs or CTH cells were observed in HMECs infected with HCMV-DB around day 20 post infection in some of the cultures (Fig. 8a). No such cell clusters were observed in cultures of HMECs infected with other members of *Herpesviridae* family tested such as EBV, HSV-1 and VZV, and in negative controls such as HMEC cultures infected with HCMV-DB filtrate and UV-treated HCMV-DB (Fig. 8a and data not shown). These clusters of spheroid CTH cells were gently detached and cultured in HMEC Ready medium for several passages (currently up to >150 passages) (Fig. 8a) and suggested to have undergone immortalization. We detected the presence of a HCMV-DB lncRNA4.9 genomic sequence of 126 bp in CTH cells using both qualitative and quantitative PCR (Fig. 8b and c). The sequence of the amplified 126 bp fragment of HCMV-DB lncRNA4.9 DNA detected in CTH cells was confirmed to match the lncRNA4.9 gene sequence from HCMV-DB (Suppl. Fig. S4). We did not detect the 126 bp amplicon of lncRNA4.9 gene in uninfected HMEC and MRC5 cells (Fig. 8b and c). Using both qualitative and quantitative PCR assays, we did not detect the presence of HCMV-DB MIEP sequences in CTH cells (Fig. 8b and c), indicating that only part of the HCMV-DB genome is present in CTH cells.

3.5. Injection of CTH Cells in NSG Mice Results in Tumor Formation With a HCMV lncRNA4.9 Signature

With previously mentioned encouraging results, we sought to determine the oncogenic potential of CTH cells *in vivo*. We injected two million CTH cells in the mammary fat pad of NOD SCID Gamma (NSG) mice. As controls uninfected HMECs, MCF-7 and MDA-MB-231 cells were used. No tumor formation was observed in mice injected with

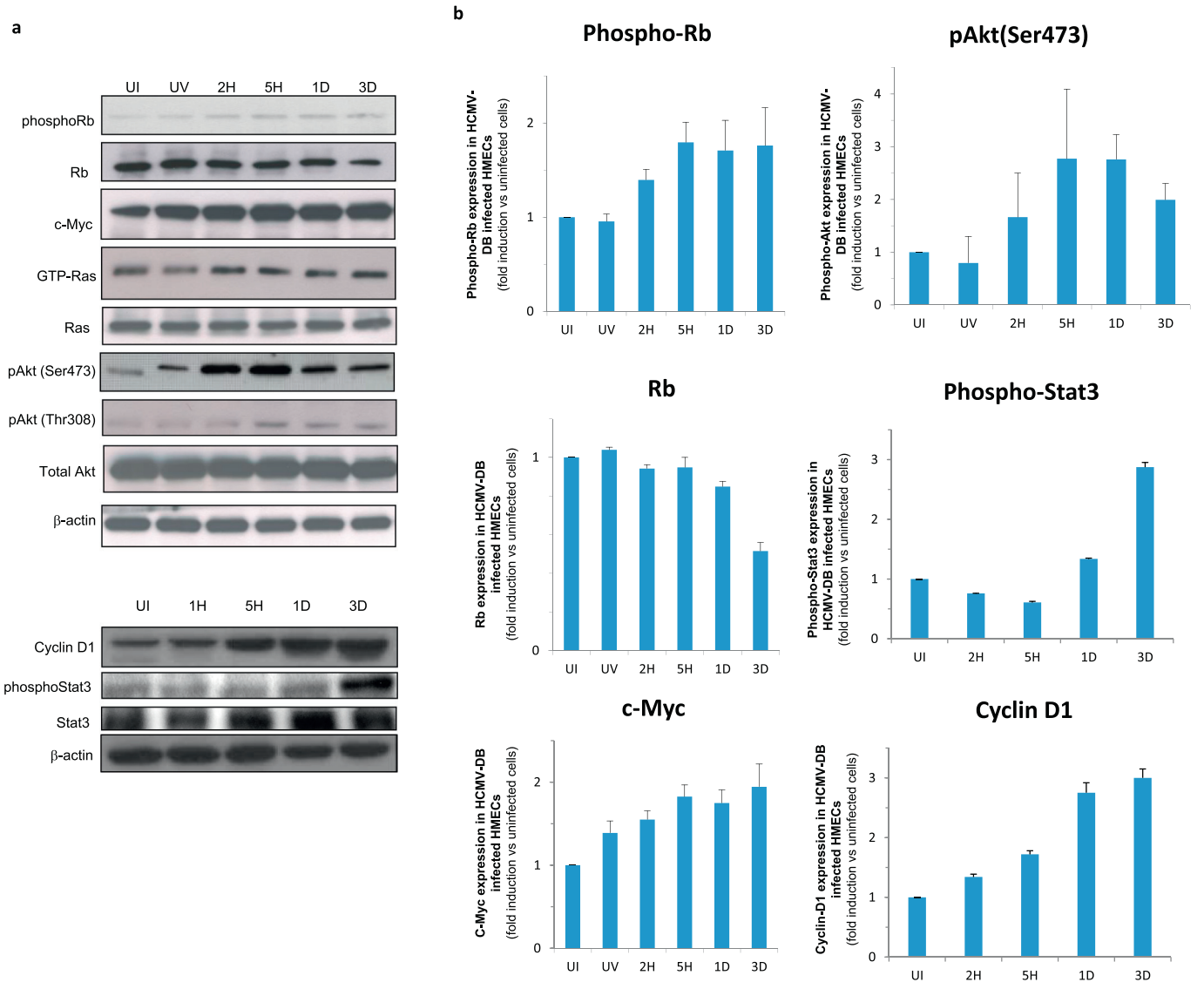


Fig. 5. Increased expression of phospho-Rb, c-Myc, phospho-Akt, phospho-STAT3 and cyclin-D1 in HMECs infected with HCMV-DB. a. Expression of phospho-Rb, c-Myc, phospho-Akt (Ser473 and Thr308), phospho-STAT3 and cyclin D1 is upregulated in HMECs infected with HCMV-DB as determined by western blotting. HMECs were infected with HCMV-DB and cells were harvested at described time points. As controls, HMECs were either left uninfected or infected with UV-inactivated HCMV-DB. β-actin was used as an internal control. To determine the active Ras (GTP-Ras) expression in HMECs, GST-pull down assay was performed as described in Materials and Methods. Results are representative of three independent experiments. b. Histograms represent the expression of phospho-Rb, Rb protein, c-Myc, phospho-Akt, phospho-Stat3, and cyclin-D1 quantified by densitometry using ImageJ 1.40 software. Results are the means (±SD) of three independent experiments.

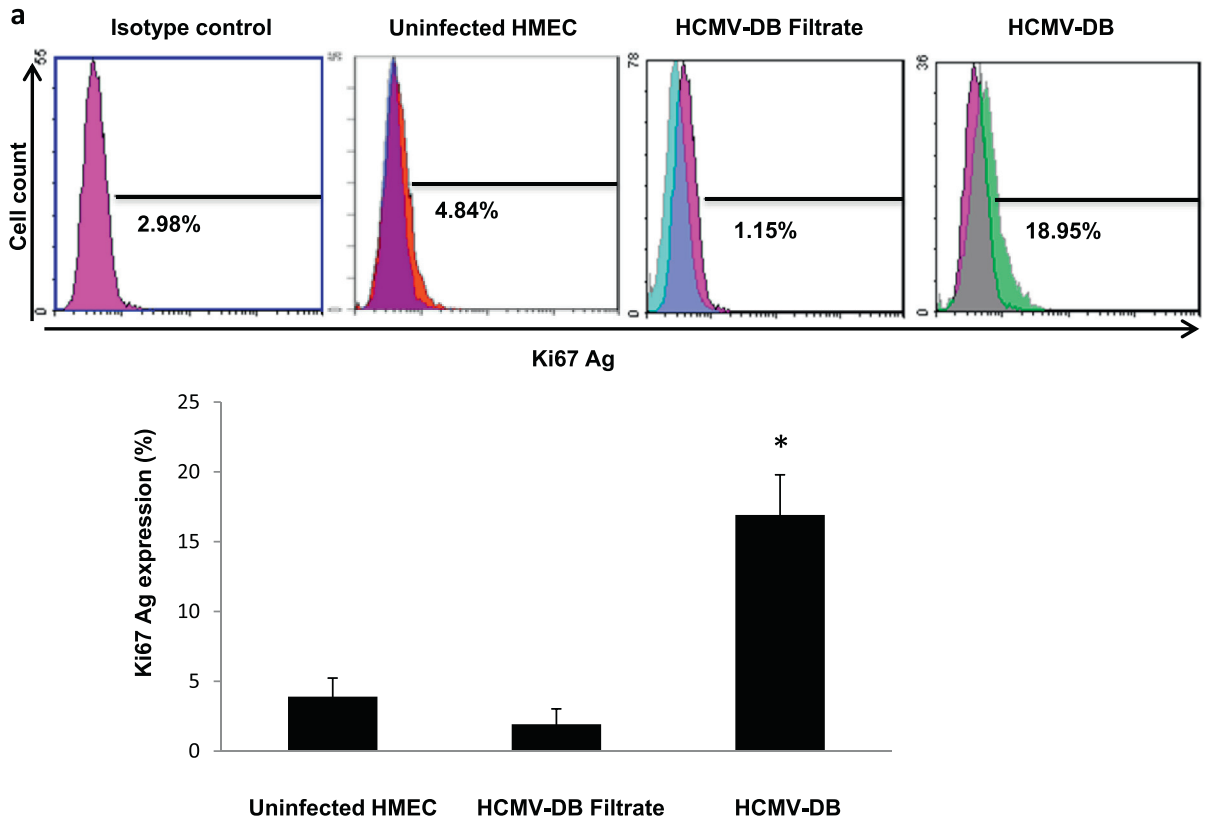
uninfected HMECs (Fig 9a and b). On the other hand, tumor begins to appear two weeks post injection in mice injected with CTH cells (Fig 9a and b). Surprisingly, tumor growth arising from CTH cells was rapid as compared to tumor growth resulting from injection of MDA-MB-231 cells (Fig. 9a). As a negative control no tumor formation was observed in mice injected with MCF-7 cells in the absence of estrogen supplementation (Fig. 9a). Using immunohistochemistry staining, the tumor biopsies of mice injected with CTH cells displayed a triple negative phenotype with the absence of ER, PR and HER2 protein, were negative for E-cadherin, CK5/6, GATA3, CK20 and GCDFP (Fig. 9c). Interestingly vimentin expression was upregulated in the tumor biopsies, indicating EMT traits (Fig. 9c). Interestingly, we detected the presence of lncRNA4.9 DNA, but not of MIEP DNA, in all tumor biopsies using PCR assay (Fig. 9d).

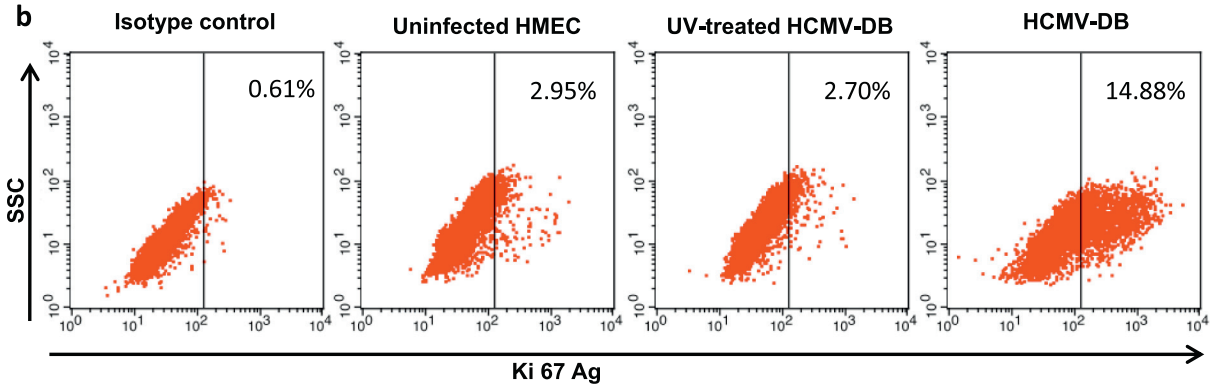
3.6. Detection of HCMV lncRNA4.9 DNA in Biopsies From Patients With Breast Cancer

Genomic DNA isolated from patient breast tumor and healthy breast tissue biopsies was provided by the regional tumor bank (BB-0033-00024 Tumorothèque Régionale de Franche-Comté). Using qualitative PCR, we were able to detect HCMV sequences of lncRNA4.9, but not of MIEP, in the genomic DNA preparation from biopsies of patients with breast cancer (Fig. 9e). Neither lncRNA4.9 nor MIEP sequences were detected in biopsies of healthy breast tissue (Fig. 9f).

4. Discussion

Our results indicate that the clinical isolate HCMV-DB replicates productively in HMECs. Infection of HMECs with HCMV-DB results in a pro-oncogenic cellular environment with decreased p53 functional activity and Rb hyperphosphorylation, overexpression of *hTERT* mRNA and enhanced telomerase activity, upregulation of c-Myc and activation of Akt and STAT3, and upregulation of cyclin D1 leading to enhanced cellular proliferation. We also observed the potential of the clinical isolate HCMV-DB in transforming primary HMECs as assessed by colony formation in soft agar. Interestingly, we observed spheroid-cell clusters in HMEC cultures infected with HCMV-DB resulting in the growth of floating rapidly proliferating transformed cells which we named CTH cells.





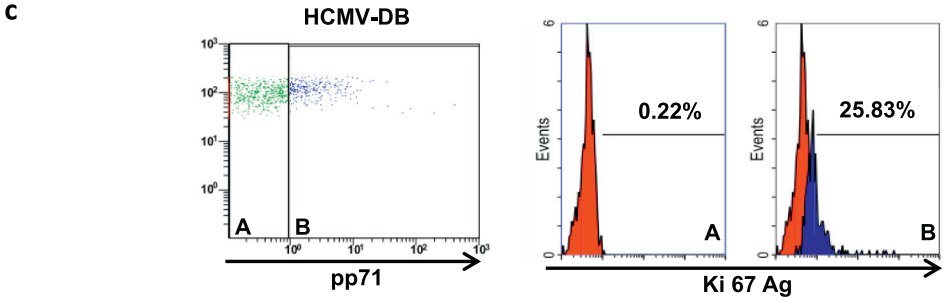


Fig. 6. Enhanced cellular proliferation following HCMV-DB infection of HMECs. a. HMECs were either left uninfected or infected with filtrate or wild type HCMV-DB. At day 3 post infection cells were harvested and expression of the Ki-67 antigen was determined using flow cytometry as described in Materials and Methods. The histogram represents means (±SD) of three independent experiments. *$P < 0.05$. b. HMECs were either left uninfected or infected with UV-treated or wild type HCMV-DB. At day 3 post infection cells were harvested and expression of the Ki-67 antigen was determined using flow cytometry as described in Materials and Methods. The results are representative of three independent experiments. c. Enhanced expression of the Ki-67 antigen in infected (pp71-positive) cells compared to uninfected (pp71-negative) cells. Results are representative of three independent experiments.

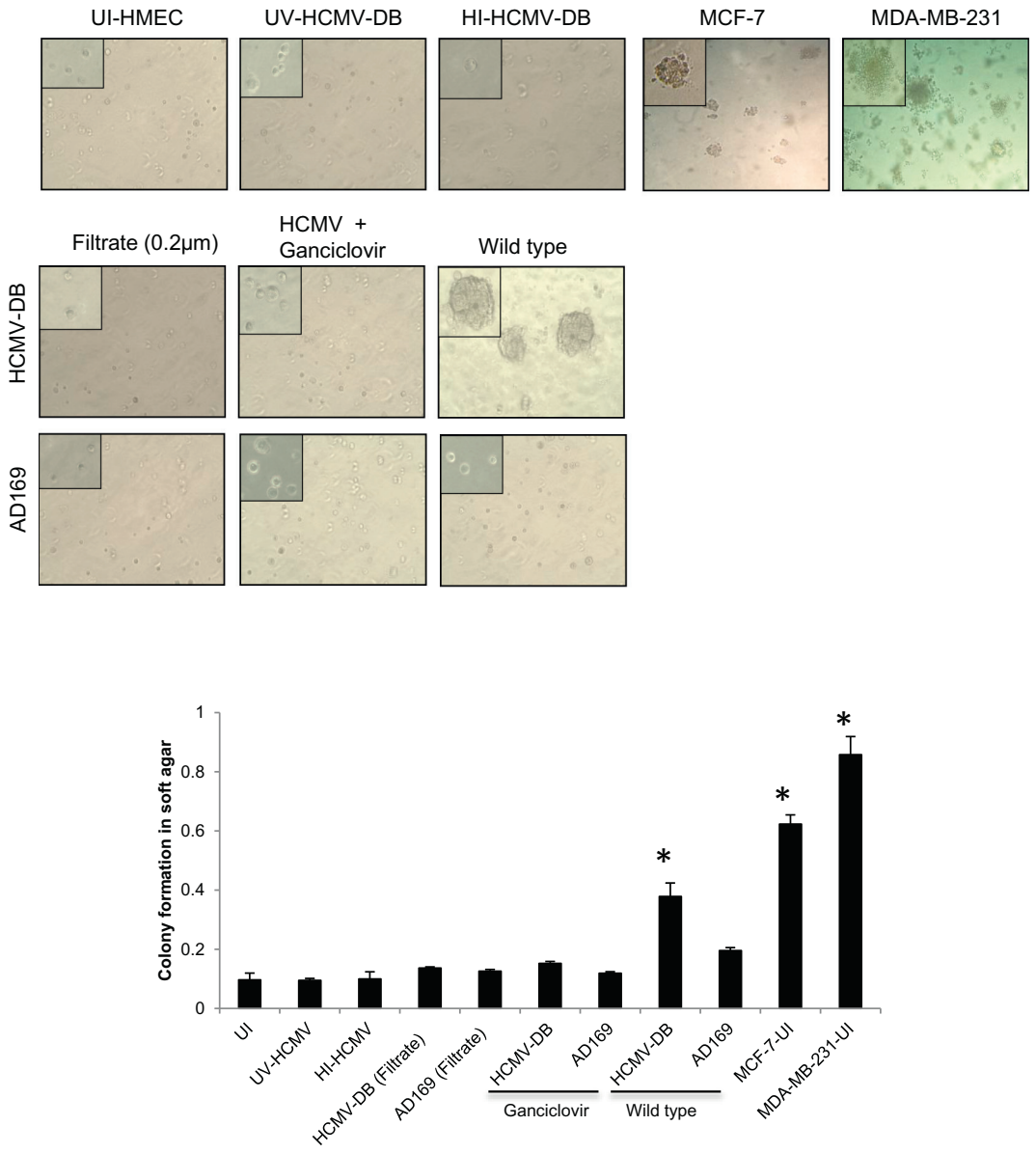


Fig. 7. Colony formation in soft agar seeded with HMECs infected with HCMV-DB. HMECs were either left uninfected or infected with UV-inactivated HCMV, heat-inactivated HCMV or wild-type HCMV strains (DB and AD169) at MOI = 1. As controls viral supernatants were passed through 0.2 μm filter and filtrates were used to infect HMECs. In addition HMECs were infected with ganciclovir-treated HCMV. At day 1 post infection, cells were seeded in soft agar as per manufacturer's instructions. After 14 days in soft agar (day 15 post infection) soft agar colonies were observed under an Olympus microscope (magnification 100× and 200×). As positive controls, breast cancer cell lines MCF-7 and MDA-MB-231 were used. Results are representative of three independent experiments. Histogram shows quantification of colony formation in soft agar seeded with HMECs at day 15 post infection. Histogram represents means (±SD) of three independent experiments. *$P < 0.05$.

We detected the presence of HCMV-DB lncRNA4.9 genomic sequence in CTH cells using both qualitative and quantitative PCR. The CTH cells when injected into mammary fat pad of NSG mice form fast growing triple-negative breast tumors *in vivo* which express the genomic sequence of HCMV-DB lncRNA4.9. In breast cancer biopsies from patients we also observed the presence of lncRNA 4.9 genomic sequence, indicating the potential *in vivo* relevance of our HCMV-DB model. Our results indicate that some HCMV strains such as HCMV-DB display oncogenic properties, transform HMECs, result in fast growing triple-negative tumors in NSG mice and could favor the appearance of human breast tumors.

We observed the most efficient and sustained replication of HCMV-DB in HMECs as compared to laboratory passaged strain AD169 (Fig. 1). We observed the complete life cycle of the clinical isolate HCMV-DB in HMECs as determined by the detection of early and late viral transcripts and proteins by RT-PCR and western blotting, respectively (Fig. 2). In agreement with our results, laboratory adapted HCMV strains and low passage clinical strains show different cell tropism *in vitro*. Low passage clinical HCMV isolates have been shown to preferentially replicate in epithelial cells, endothelial cells and myeloid cells (O'Connor and Shenk, 2012; Wang and Shenk, 2005). In addition to infecting HMECs, the clinical isolate HCMV-DB (Accession number KT959235) infects macrophages and we demonstrated previously its ability to induce M2 phenotype and upregulation of proto-oncogene Bcl-3 (Khan et al., 2009). In contrast to laboratory adapted strains, some HCMV clinical strains including HCMV-DB but also PH and TR strains (Fig. S1), display a tropism for monocytes/macrophages (O'Connor and Shenk, 2012; Wang and Shenk, 2005). Thus, the macrophage-tropic HCMV strains might play a role in the development of tumor-associated macrophages (TAM), a marker of poor prognosis in human breast carcinomas and glioblastomas (Dziurzynski et al., 2011; Medrek et al., 2012). Recently, using high throughput sequencing and population genetics approaches,

a detailed map of HCMV *in vivo* evolution was built and provided evidence that viral populations can be stable or rapidly differentiate, depending on host environment, and ultimately could result in tissue compartment colonization (Renzette et al., 2013). Positive selection could be a strong driver of evolution associated with compartmentalization (Renzette et al., 2013). Taken together these data indicate that the tissue (tumor) environment can drive the viral fitness and that clinical isolates such as the macrophage-tropic HCMV-DB strain isolated from a pregnant women could be well adapted to this environment and in turn might ultimately favor the development of breast cancer.

Our data show that HCMV-DB not only productively infects HMECs but also shapes the cellular environment in favor of the transformation. We observed upregulation of p53 in HMECs infected with HCMV as previously reported in other cell types (Hannemann et al., 2009) (Fig. 3). In addition to IE2 binding to p53 in HMECs infected with HCMV as reported previously in human fibroblasts (Hsu et al., 2004), we observed decreased binding of p53 to its target gene promoters (p21 and MDM2) in the HMECs infected with the clinical isolate HCMV-DB (Fig. 3b and c). This could explain why p53 response mounted in HMECs upon HCMV-DB infection, failed to efficiently protect HCMV-infected cells against cell cycle promotion and cellular proliferation. Furthermore, recently it has been shown the role of viral proteins in inducing heterochromatin state in the region of p53 targets genes (Soria et al., 2010). In order to investigate the potential of HCMV-DB infection in inducing heterochromatin at the region of p21 and MDM2 promoter, we performed a ChIP assay using anti-H3K9me3 antibody. However, we did not observe any significant change in lysates from HMECs infected with HCMV-DB compared to uninfected control lysates (data not shown).

Immortalization is perquisite for the cancer development. Continuous cell growth without cellular senescence, one of the hallmarks of cancerous cells, is governed by telomerase activity which usually declines in the normal cells (Hahn and Meyerson, 2001). We observed that infection with HCMV-DB results in upregulation of telomerase activity in infected HMEC cells (Fig. 4) that usually leads to cell immortalization. Recently the role of STAT3 in activating catalytic subunit of telomerase has been shown (Chung et al., 2013) which could explain increased telomerase activity parallel to elevated levels of activated STAT3 in HMECs infected with HCMV-DB.

Several molecular mechanisms have been described contributing to oncogenesis including modifications in cell cycle progression and apoptosis (Alibek et al., 2013). It is a generalized notion that more than one proto-oncogene and/or deregulation of tumor suppressor proteins are involved in the tumor progression. In accordance with previous findings, we observed elevated levels of phosphoRb (at Ser 780 residue) upon infection of HMECs with HCMV-DB (Fig. 5) (Hume et al., 2008). The role of HCMV UL97 in phosphorylating and inactivating Rb family proteins and promoting cell cycle progression has been shown (Iwahori et al., 2017). Furthermore, the downregulation of retinoblastoma family of proteins by pp71 (pUL82) has been also reported

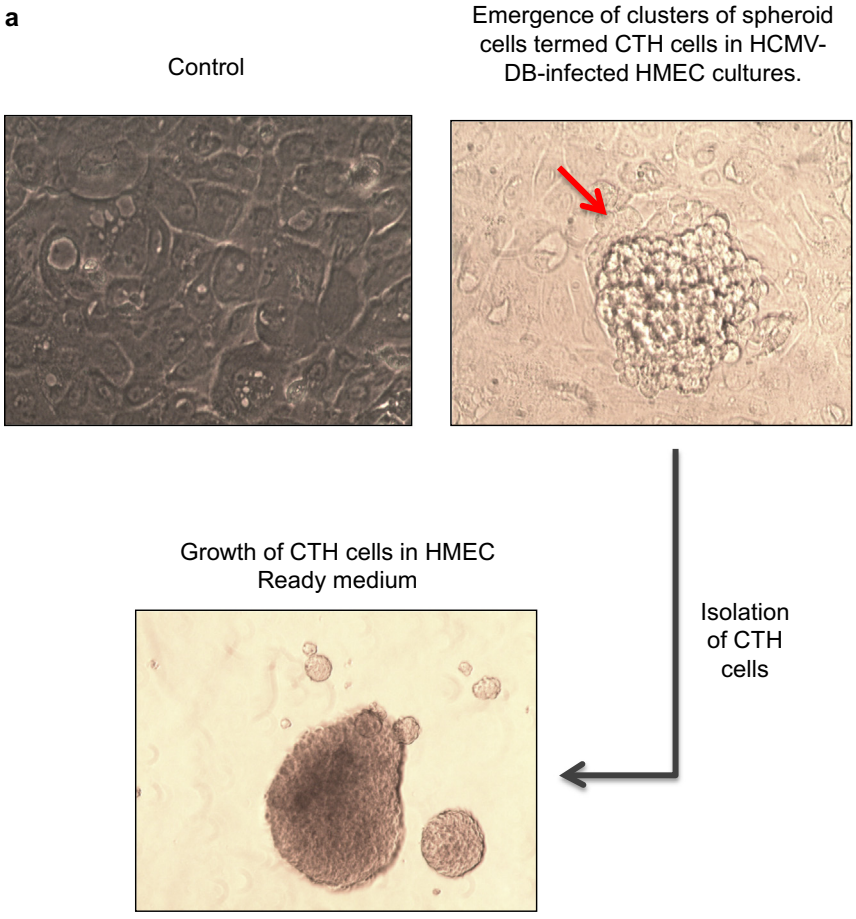


Fig. 8. Emergence of clusters of spheroid cells named CTH cells in HMECs infected with HCMV-DB at day 20 post infection which display a HCMV-DB lncRNA4.9 signature. a. Right panel. Clusters of spheroid cells appeared in cultures of HMECs infected with HCMV-DB (MOI = 1) at day 20 post-infection. Left panel. HMECs treated with HCMV-DB filtrate were used as a negative control. Magnification 200×. Lower panel. The clusters of spheroid CTH cells were gently detached and cultured in HMEC Ready medium (currently up to >150 passages). b., c. We detected the presence of a 126 bp HCMV-DB RNA4.9 DNA sequence, but not of MIEP sequence, in CTH cells using (b) qualitative PCR and (c) quantitative PCR. Uninfected HMECs and MRC5 cells were used as negative controls. HCMV-DB viral stock (HCMV-DB) and HMECs infected with HCMV-DB (HMEC + HCMV-DB) were used as positive controls. Beta-globin was used as an internal control. Results are representative of three independent experiments.

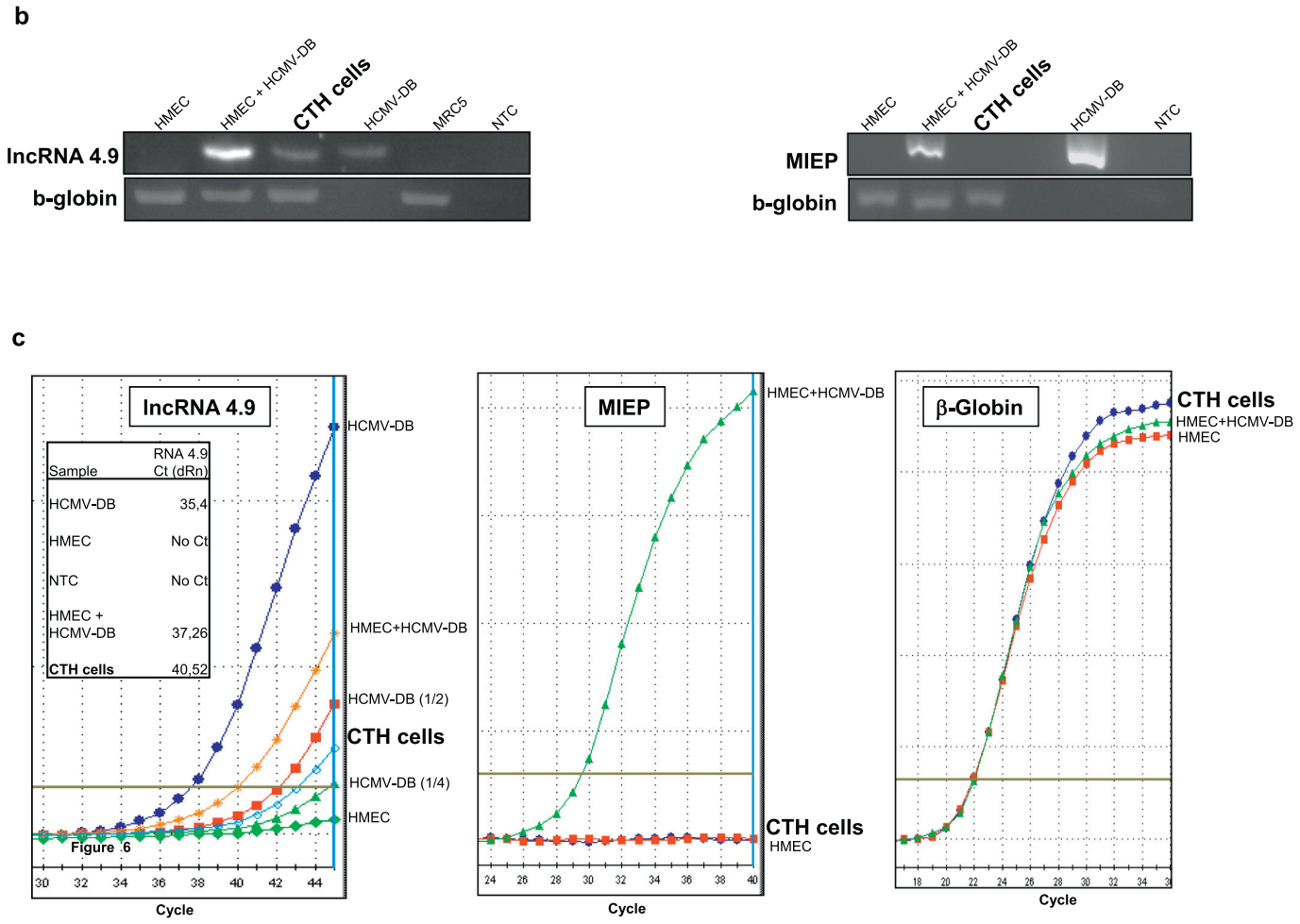


Fig. 8 (*continued*).

(Kalejta and Shenk, 2003). In HMECs infected with HCMV-DB we observed the presence of the UL82 transcript at day 1 and day 3 (Fig. 2a) parallel to the decrease in the expression of Rb protein at day 3 (Fig. 5). We also observed the up-regulation of c-Myc, elevated levels of phospho-Akt, phospho-STAT3, and cyclin-D1 expression following infection of HMECs with HCMV-DB (Fig. 5) (Boldogh et al., 1991, 1990; Chan et al., 2009; Hagemeier et al., 1992; Slinger et al., 2010; Soroceanu et al., 2011). Data are consistent with previous reports where HCMV has been shown to induce the expression of proto oncogenes including c-Myc, c-Fos and c-Jun in human embryo lung cells (Boldogh et al., 1991, 1990). In addition, we observed increased expression of Ki67 antigen in infected HMECs upon HCMV-DB infection (Fig. 6). To rule out the possibility that the involvement of serum factors present in HCMV-DB supernatant resulted in enhanced proliferation, we passed HCMV-DB viral supernatant through 0.2 μm filter and also exposed HMECs to the filtrate. We observed only marginal increase in Ki67 antigen expression with the filtrate as compared to HCMV-DB viral supernatant, linking the presence of HCMV-DB virus itself to increased expression of Ki67 antigen (Fig. 6).

Soft agar assay is widely recognized as the most stringent and reliable assay to determine the malignant transformation *in vitro* (Gilmore et al., 2001). We observed that HCMV-DB, but not AD169, was able to induce colony formation in soft agar seeded with infected HMECs (Fig. 7). In contrast, no colonies were observed neither in uninfected, heat-inactivated, UV-treated, ganciclovir-treated and filtered HCMV controls (Fig. 7) nor in HMECs infected with EBV, HSV-1 and VZV (data not shown). The proposition that HCMV has the potential to contribute to oncogenesis by inducing mutations in cellular genes has been evoked previously indicating a close collaboration between HCMV IE1 and IE2 and the adenovirus E1A proteins to generate transformed foci of primary baby rat kidney cells (Shen et al., 1997). In addition, primary HCMV infection can cause chromosomal breaks or mutations (Fortunato et al., 2000). Accumulation of such mutations in infected cells may trigger tumor initiation.

We were not able to amplify the MIEP sequence in the colonies harvested from soft agar seeded with HMECs infected with HCMV-DB. At least two possibilities could account for the observed result. First, the HCMV genome is not present in colonies after 14 days in soft agar cultures and the sustained cellular transformation does not require anymore the presence of viral genes/proteins at this time. Second, only part of the HCMV-DB genome excluding the MIEP sequence is still present in the soft agar transformed HMECs after 14 days in culture, and is sufficient for the sustained transformation of the infected HMECs. This latter hypothesis seems to be the most plausible since we detected HCMV lncRNA4.9 sequence, but not MIEP sequence, in CTH cells. Our results are in agreement with the transformation of keratinocytes by the human papilloma virus (HPV) where only a few viral genes (*e.g.* E6 and E7 genes) are detected and required for the sustained transformation of infected cells (Zur Hausen, 2009).

To confirm the results of soft agar assay, HMECs infected with HCMV-DB were cultured for a prolonged period of time. On the 20th day of infection we observed several spheroid-cell clusters (Fig. 8a). These spheroid-cell clusters were absent in uninfected HMECs cultures and cultures infected with HCMV-DB filtrate and UV-inactivated HCMV-DB. These spheroid-cell clusters composed of CTH cells exhibit rapid growth as compared to the surrounding cells, were easily detached

and were cultured in HuMEC Ready Medium containing epidermal growth factor, hydrocortisone, isoproterenol, transferrin, insulin and bovine pituitary extract (Fig. 8a). These CTH cell clusters grow in suspension and fails to attach the surface (Fig. 8a), a sign of anchor independent growth and epithelial mesenchymal transition (Larue and Bellacosa, 2005).

To demonstrate a close link between HCMV-DB infection and HMEC transformation it was critical to demonstrate that part of the HCMV-DB genome was present in CTH cells. In CTH cells we detected the lncRNA4.9 DNA sequence of HCMV-DB by both qualitative and quantitative PCR that amplified a 126 bp region of the lncRNA 4.9 gene from HCMV-DB, as confirmed by Sanger's sequencing (Fig 8b and c, Suppl. Fig. S4). Since we did not detect the MIEP sequence in CTH cells, the presence of a limited region of the HCMV-DB genome in CTH cells including the 126 bp lncRNA4.9 sequence might be a critical factor for the sustained transformation of these cells during time. Interestingly, a small number of loci of HCMV, centered on long non coding RNA including lncRNA4.9 show evidence of selective sweeps and these loci are targets of positive selection indicating a strong conservation within HCMV (Renzette et al., 2016). The detection of HCMV-DB lncRNA sequence in CTH cells rules out the possibilities of spontaneous generation of transformed cells (Soule et al., 1990).

Our data indicate the potential of a clinical HCMV strain, namely the HCMV-DB isolate, to fulfill all requirements to transform HMECs *in vitro* possibly through limited viral gene expression including the lncRNA4.9 gene. Lnc RNAs have recently gained increased interest in cancer and in viral infections (Huarte, 2015; Fortes and Morris, 2016). Interestingly, HCMV lncRNA4.9 has been reported to be involved in HCMV latency

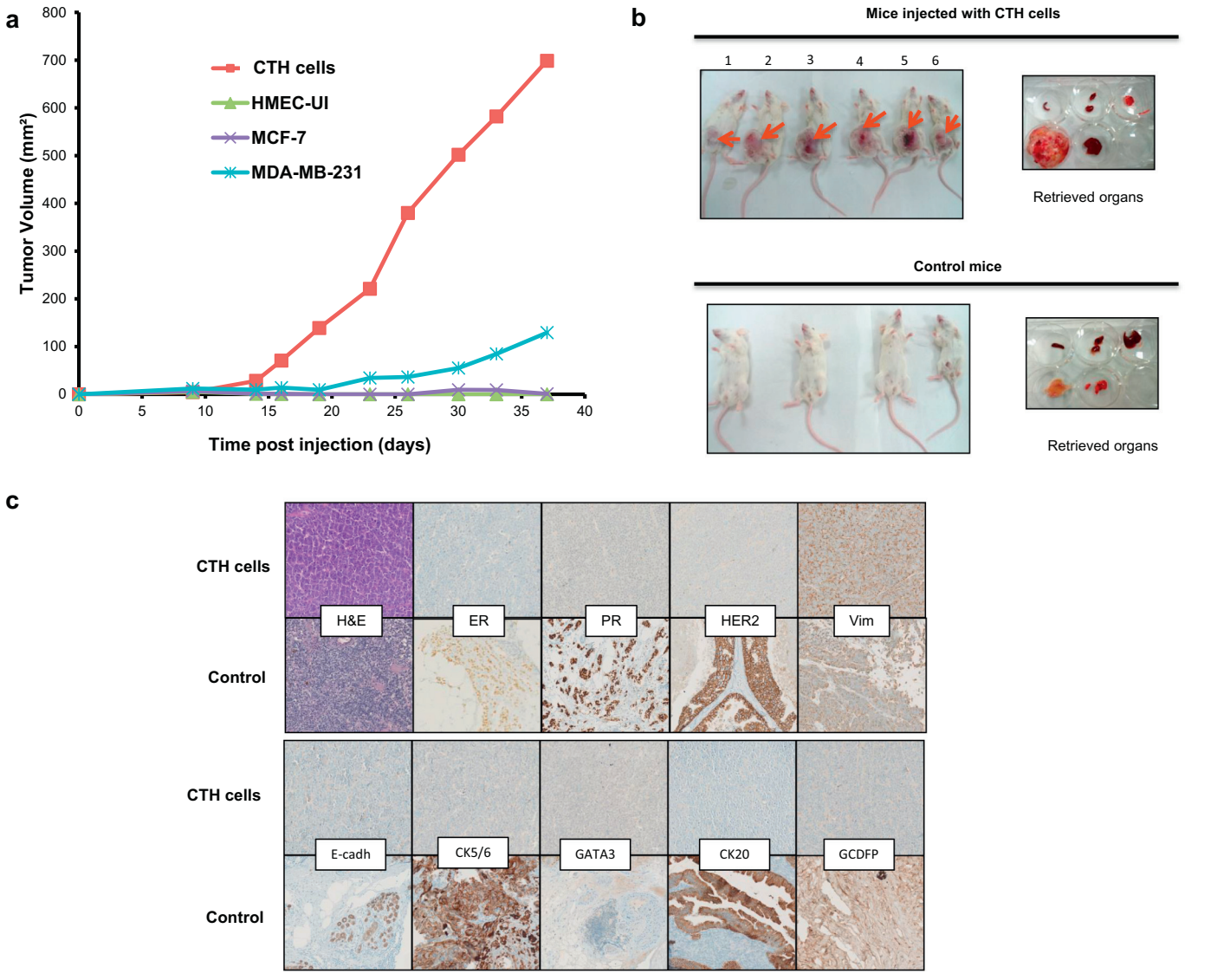


Fig. 9. Appearance of tumors with HCMV lncRNA4.9-signature in NSG mice injected with CTH cells and detection of the HCMV lncRNA4.9 gene in tumor biopsies of human breast cancer. a. Injection of CTH cells in NSG mice results in tumor formation. Two million of CTH cells, five million of uninfected HMECs and two million of MCF-7 and MDA-MB-231 cells were injected in the mammary fat pad of NSG mice in the absence of hormone supplementation. Uninfected HMECs, MCF-7 cells and MDA-MB-231 cells were used as controls. Tumor dimensions were recorded as shown in the figure. Results are means of experiments with six mice in each group. b. After mice sacrifice, tumors and organs (liver, lung, kidney, spleen) were retrieved. c. Using immunohistochemistry staining, tumor biopsies display a triple negative phenotype with the absence of ER, PR and HER2 protein, are negative for E-cadherin, CK5/6, GATA3, CK20 and GCDFP, but positive for vimentin. d. Detection of the presence of lncRNA4.9 DNA, but not of MIEP DNA, in all tumor biopsies from mice injected with CTH cells using PCR assay ($n = 6$). DNA extract of HMECs infected with HCMV-DB (Control HMEC+HCMV-DB) was used as a positive control. Beta-globin was used as an internal control. e. Tumor biopsies of patients with breast cancer display a lncRNA4.9 signature. Using qualitative PCR, we were able to detect HCMV sequences of lncRNA4.9 gene, but not of MIEP, in the genomic DNA preparation from tumor biopsies of patients with breast cancer. DNA extract of HCMV-DB viral stock (Control HCMV-DB) was used as a positive control. f. lncRNA4.9 and MIEP DNA sequences were not amplified from human healthy breast tissue. Beta-globin was used as an internal control.

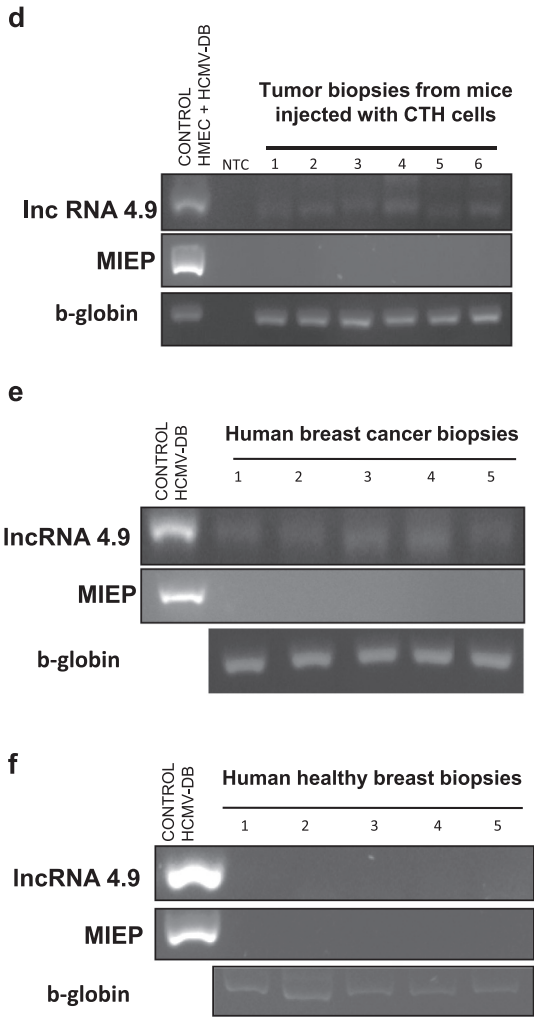


Fig. 9 (*continued*).

and also to interact with the polycomb repressive complex (PRC), especially the PRC2 complex which is abundant in highly proliferative cells including cancer cells (Rossetto et al., 2013; Van Damme and Van Loock, 2014).

We also assessed the expression of HCMV lncRNA4.9 gene in the genomic DNA obtained from human breast cancer biopsies. In contrast to the absence of the MIEP sequence, we detected the presence of the lncRNA4.9 gene (126 bp amplicon) in the tumors isolated from women with breast cancer (Fig. 9e). We did not detect lncRNA4.9 and MIEP sequences in biopsies of healthy human breast tissue (Fig. 9f). Altogether our results point to a critical role of HCMV in the appearance and development of breast cancer. Nevertheless additional studies are required to decipher the exact role of lncRNA4.9 and/or other yet to identified viral genes and proteins in the pathophysiology of the disease.

Finally, other HCMV strains especially low passaged clinical isolates may also activate oncogenic pathways in HMECs. Interestingly, the TB40/E strain isolated from a throat wash of a bone marrow transplant recipient, a low passage clinical isolate, enters into HMECs (data not shown), overexpresses hTERT mRNA and enhances the telomerase activity in infected HMECs as compared to uninfected HMECs (Suppl. Fig. S5a and b), and ultimately favors the apparition of colonies in soft agar (Suppl. Fig. S5c). The enhancement of telomerase activity and the appearance of colonies in soft agar is less pronounced for HMECs infected with TB40/E strain compared to cells infected with HCMV-DB. We are currently screening other primary HCMV isolates for their ability to activate oncogenic pathways in HMECs *in vitro* and potentially to lead to the appearance of tumors *in vivo*.

5. Conclusion

In conclusion, our data indicate that the infection of HMECs with HCMV-DB results in a pro-oncogenic cellular environment with decreased p53 functional activity and Rb hyperphosphorylation, overexpression of *hTERT* mRNA and enhanced telomerase activity, upregulation of c-Myc and activation of Akt and STAT3, and upregulation of cyclin D1 leading to enhanced cellular proliferation. We also observed the potential of the clinical isolate HCMV-DB in transforming primary HMECs as assessed by colony formation in soft agar. We observed the appearance of transformed cells in HMEC cultures infected with HCMV-DB, namely the CTH cells which display a lncRNA4.9 viral signature. The injection of CTH cells in NSG mice resulted in the appearance of triple-negative breast tumors. Finally the HCMV lncRNA4.9 signature is present in the tumor biopsies of human breast cancer *in vivo*. Future studies will decipher the molecular mechanisms involved in the transformation of HMECs infected with HCMV-DB and to determine the exact relevance of such a model in the pathogenesis of breast cancer.

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Conflicts of Interest

The authors declare no conflict of interest.

Availability of Data and Materials

The data sets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Author Contributions

AK, MKT, WA, KAK, FAM, SP, LC, LR, MPA, SVD, OA performed research; GH and SMB designed research; AK and GH wrote the paper.

Consent for Publication

Not applicable.

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