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

Amit Kumar a,1, Manoj Kumar Tripathy a,1, Sébastien Pasquereau a,1, Fatima Al Moussawi a,b, Wasim Abbas c, Laurie Coquard d, Kashif Aziz Khan e, Laetitia Russo d, Marie-Paule Algros d, Séverine Valmary-Degano d, Olivier Adotevi f,g, Stéphanie Morot-Bizot c, Georges Herbein a,g,*

a Department Pathogens & Inflammation-EPILAB, UPRES EA4266, University of Franche-Comté (UFC), University of Bourgogne France-Comté (UBFC), F-25030 Besançon, France
b Lebanese University, Beirut, Lebanon
c Apex Biosolutions, F-25000 Besançon, France
d Department of Pathology, CHRU Besançon, F-25030 Besançon, France
e Department of Medical Oncology, CHRU Besançon, F-25030 Besançon, France
f Department of Virology, CHRU Besançon, F-25030 Besançon, France

corresponding author at: Department Pathogens & Inflammation-EPILAB, EA4266, University of Bourgogne Franche-Comté, 16 route de Gray, F-25030 Besançon, Cedex, France.
E-mail addresses: sebastien.pasquereau@univ-fcomte.fr, MP Almogas@chu-besancon.fr, MP. Almogas@chu-besancon.fr, M-P. Almogas@chu-besancon.fr, S. Valmary-Degano, olivier.adotevi@univ-fcomte.fr, (O. Adotevi), smorot@apexlabo.com, (S. Morot-Bizot). Georges.herbein@univ-fcomte.fr, (G. Herbein).

1 AK, MKT, SP contributed equally to the work.

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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 the 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., 2006). 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 (Cobb et al., 2002; Lepiller et al., 2011; Süderberg-Naulé, 2006). Earlier studies demonstrated that HCMV was able to induce the in vitro transformation of human embryo lung fibroblasts (Clayton 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; 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, pp56 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-D8 infected HMECs with RNeasy mini kit (Qiagen). Total 2 μg of RNA was reverse transcribed into cDNA with Superscript III RT (Life Technologies, USA) using oligo (dT) primers. The 5 μl of reverse transcription reaction product was amplified using primers against IE1, US28 and UL82 primers as 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. © 2018 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
described previously (Lepiller et al., 2013). The beta-globin gene was amplified as an internal control (sense, 5′-TCCCCCTTACCCCTACTTTCTTA-3′; antisense, 5′-TGCTCTGACTAATCTGCAAGAG-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′-GATTCGCGGGATCGTCAACCA-3′; antisense, 5′-TGATCCGAGACTAGATAG-3′); UL131 (sense, 5′-ATGGTGTCTCATAATAAAGC-3′; antisense, 5′-TCAACCTGAGCTCCACGACG-3′); UL133 (sense, 5′-AGACTCCGTAATGACTGCTC-3′; antisense, 5′-GATGACCTGATGTCGTAGC-3′); and UL138 (sense, 5′-ACGATC TGGCGCTGAATGTC-3′; antisense, 5′-ACAGTCCGGACACGGCGATCT-3′). Quantification of c-Myc (MYC) and cyclin-D1 (CCND1) transcripts in HMECs infected with HCMV-DB (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. For the detection of hTERT transcripts, total cellular RNA was isolated from infected 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′-CGAAGATGTTCTTGGA CCAA-3′ and 5′-GGATCAGCAGTCTGGCA-3′.

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) propane sulfonic acid (CHAPS) lysis buffer (10 mMTris – HCl [pH 7.5], 1 mM MgCl2, 1 mM EGTA, 0.1 mM phenylmethysulfonyl fluoride, 5 mM P3-mercaptoethanol, 0.5% CHAPS, 10% glycerol) for 20 min on ice and centrifuged at 20,000g 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 DNA was amplified using primers with 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′-CGAAGATGTTCTTGGA CCAA-3′ and 5′-GGATCAGCAGTCTGGCA-3′.

2.10. Soft Agar Colony Formation Assay

Colony formation in soft agar seeded with 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 (Asyst, Chicago, IL). Colonies were observed under an Olympus microscope (Center Valley, PA). Following DNA extraction from soft agar colonies, the HMEC major immediate early promoter (MIEP) and beta-globin sequences were amplified using the following primers: MIEP (sense, 5′-TGGGACTTCTTACTTTG-3′; antisense, 5′-CCACCGATGATCAGGGTT-3′) and beta-globin (sense, 5′-TCCCTCTTACCCCTACTTCTA-3′; antisense, 5′-TGCGCTGTTCTCAAAGGAG-3′).

2.11. Isolation and Growth of CTH Cells

HMECs cultures were infected with HCMV-D8 at a MOI of 1. Several clusters of spheroid-cells were observed in HMECs infected with HCMV-D8 around day 20 post infection in some of the cultures. These spheroid-cell clusters detected in HMEC cultures infected with HCMV-D8 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 of 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 (cm³) = (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 IncRNA4.9 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 (1:50,000, Biocare), and GCDFP (1:1000, Cell Marque) before observation by microscopy.

2.14. Detection of HCMV IncRNA 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 mouse tumors were grinded in liquid nitrogen and DNA was isolated using QIAamp DNA mini kit (Qiagen, Valencia, CA) as per manufacturer's guidelines. Genomic DNA isolated from patient breast tumor biopsies and from healthy human breast tissue was provided by the regional tumor bank (BB-0033-00002).

Presence of HCMV was determined by qualitative and quantitative PCR where specified using a set of HCMV-DB IncRNA 4.9 gene primers (sense, 5′-GTGAACCAGATCCGCTGCAGC-3′; antisense, 5′-CATTGGAAAGAGAAGGCTTC-3′). An amplicon of 126 bp corresponding to HCMV-DB IncRNA4.9 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′-TGGGACTTCTTCATCTTG-3′; antisense, 5′-CCAGCCGACTCTGAGCCTG-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 IncRNA 4.9 primers. As equal loading control β-globin gene was amplified (sense, 5′-TCTCCCTCTCCTTACCTTCTT-3′; antisense, 5′-TGCTTGGACATACGTCC-3′). Amplified product was electrophoresed in 2% agarose gel stained with Sybr green I nucleic acid stain.

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 (Foot 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 (Tuite 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 passed laboratory strain AD169 that has lost the ability to efficiently replicate in endothelial and epithelial cells (Singer 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 expected.
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 region that has been shown to govern tropism of HCMV (Lepiller et al., 2013). In addition STAT3 activation has been shown to be a crucial component of the JAK/STAT3 axis in primary human hepatocytes and HepG2 cells infected with HCMV (Helt and Galloway, 2003). The inactivation of the Rb protein results in the upregulation of p21, 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 uncheckd cell division (Dimri et al., 2005).

The acquisition of a mitogenic signal provided by the protooncogene Myc and/or activated Ras has been reported to be critical to observe complete transformation of HMECs in vitro (Elenbaas et al., 2001). We observed the 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 HMEC cells (Fig. 4a and b). 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.

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 (Strååt 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 protooncogene 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).

Uprogulation 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
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. 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. 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 semiquantitative 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.

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. 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 semiquantitative 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.

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...
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 Termined “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 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 IncRNA4.9 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 IncRNA4.9 DNA detected in CTH cells was confirmed to match the IncRNA4.9 gene sequence from HCMV-DB (Suppl. Fig. S4). We did not detect the 126 bp amplicon of IncRNA4.9 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-DB IncRNA4.9 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
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 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 GCDP (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.
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 phosphorH (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.

![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.](image)

**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.
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\(\mu\)m 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 IncRNA4.9 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 comprised of CTH cells exhibit rapid growth as compared to the surrounding cells, were easily detached.

Fig. 8 (continued).
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 fail 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 IncRNA4.9 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 IncRNA 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.
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 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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