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Human cytomegalovirus envelope glycoprotein B as pathogenicity factor for congenital infection

Dissertation

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List of abbreviations

HCMV	Human cytomegalovirus
gB	glycoprotein B
VZV	Varicellazoster virus
HSV	Herpes simplex virus
EBV	Epstein–Barr virus
MPR	Membrane-proximal region
TM	Transmembrane domain
Cyto	Cytoplasmic tail
BACs	Bacterial artificial chromosomes
DSP	Dual split protein
IE	Immediate-early
ATCC	American Type Culture Collection
EIPA	5-(N-Ethyl-N-isopropyl)-Amiloride
PBS	Phosphate-buffered saline
HA	Homology arm
ORF	Open reading frame
IU	Infectious unit
hpi	Hour post-infection
Intden	Integrated density
gO	glycoprotein O
FFWO	Fusion from without
FFWI	Fusion from within
FP	Fusion peptide
N-Term	N-terminal region
CTD	Cytoplasmic domain

Presentation of the publication

Infections in pregnancy can vertically transmit to the fetus resulting in congenital infection or even severe disease, such as growth restriction, hearing loss, and stillbirth [1] (Figure 1). Human cytomegalovirus (HCMV), along with several other pathogens, is known collectively as “TORCH” (Toxoplasma, Other pathogens, Rubella, Cytomegalovirus, Herpes simplex virus). The term “TORCH “refers to pathogens known to pass through the placenta barrier and cause congenital disease in the fetus or newborns with similar symptoms [2]. In the USA, more than 7000 children per year are born with diseases caused by congenital HCMV infection, a far greater number than other disorders for which interventions are available [3]. However, no vaccine is currently available, and the genotype-associated pathogenicity is still unknown.

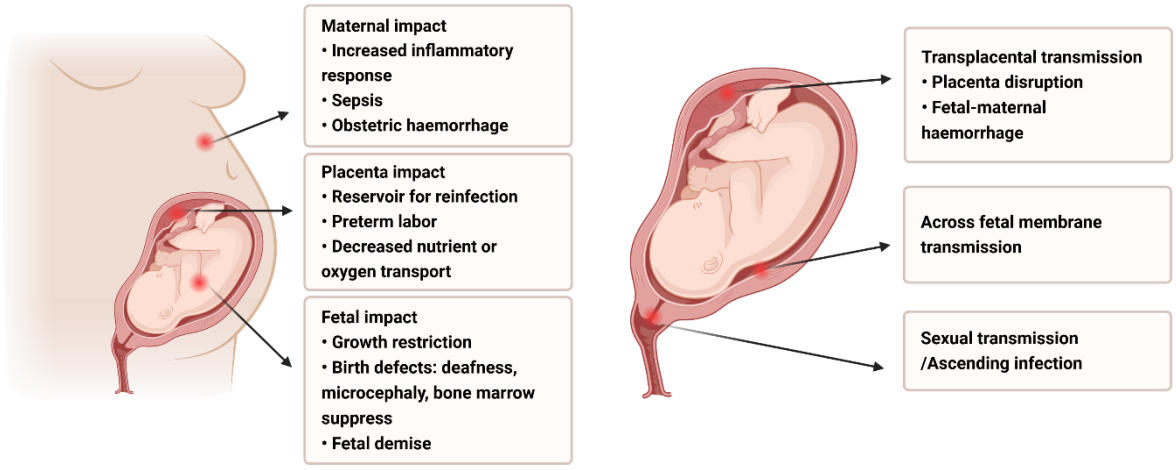

Figure 1: Transmission through the placenta and impact of the infection. Modified from Megli, C.J., Nat Rev Microbiol, 2022.

HCMV has a large double-stranded DNA genome with a length of about 235 kb encoding over 200 open reading frames (ORF). The genome is surrounded by the nucleocapsid that contains five core proteins: the major capsid protein, the minor capsid, the smallest capsid protein that decorates MCP tips, and the portal protein. The nucleocapsid itself is embedded in the tegument containing abundant virus-encoded proteins. Then there is an envelope enclosing the tegument that includes many kinds of glycoproteins expressed (gB, gH, gL, gO, UL128-131 locus, etc.) [4, 5] (Figure 2).

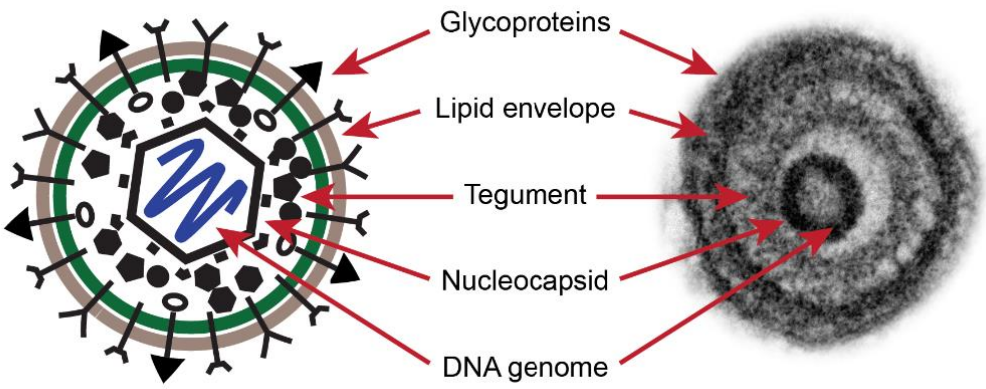

Figure 2: Structure of an HCMV virion. From Close WL, Adv Exp Med Biol. 2018.

Viral envelope glycoproteins that can mediate membrane fusion and interact with cell receptors for entry have been identified as factors in viral genotypes and their associated pathogenicity [6, 7]. Cell-cell fusion, as a complex process, exists in various pathological and physiological situations, including viral infections [8-10]. The trigger of this event requires the interaction between fusion proteins of viral particles expressed on infected cell membranes and the cell receptors present on the uninfected cells, leading to the fusion of the two adjacent plasma membranes, resulting in the formation of multinucleated cells called syncytia [11]. The membrane fusion process driven by the viral fusion machinery requires sequential steps (Figure 3) [12-16]: A. The fusion mechanism is activated, which exposes the fusion peptide (FP). B. The FP is set in the neighboring membrane, which approaches membranes on both sides more closely. C. The fusion protein refolding induces the deformation of the membrane. D. The outer leaflet of the membrane merges, forming a stalk. E. The stalk expands, creating a transient hemifusion diaphragm. F. A fusion pore opens and then completes the merger of both membranes. This mechanism allows the virus to spread widely and rapidly through cells and escape from host defenses, facilitating viral dissemination and pathogenicity [11]. The fusogenic proteins of the virus can reach the cellular membrane in two ways: “fusion from without (FFWO)” and “fusion from within (FFWI)” [17, 18]. In FFWO, the glycoproteins expressed on the envelope of viral particles are left on the plasma membrane upon virus entry (Figure 3 A, B). Fusion proteins on the membrane can then bind with cell receptors present on adjacent cell membranes, driving the fusion called FFWO. This fusion type requires a large number of viral particles. However, it does not involve newly produced viral proteins. It is difficult to

observe because the fusogenic proteins expressed on the viral envelope are changed by the high-speed centrifugation which is required to have viral stock. FFWI requires the newly produced viral proteins (Figure 3 C). The newly produced proteins are translocated to the plasma membrane and can then participate in the mediation of cell fusion. Therefore, it occurs hours or even days after infection. It has been known that cell-cell fusion necessitates a set of conserved glycoproteins, such as gB, gH, and gL, which form the core fusion machinery of the virus [19-23].

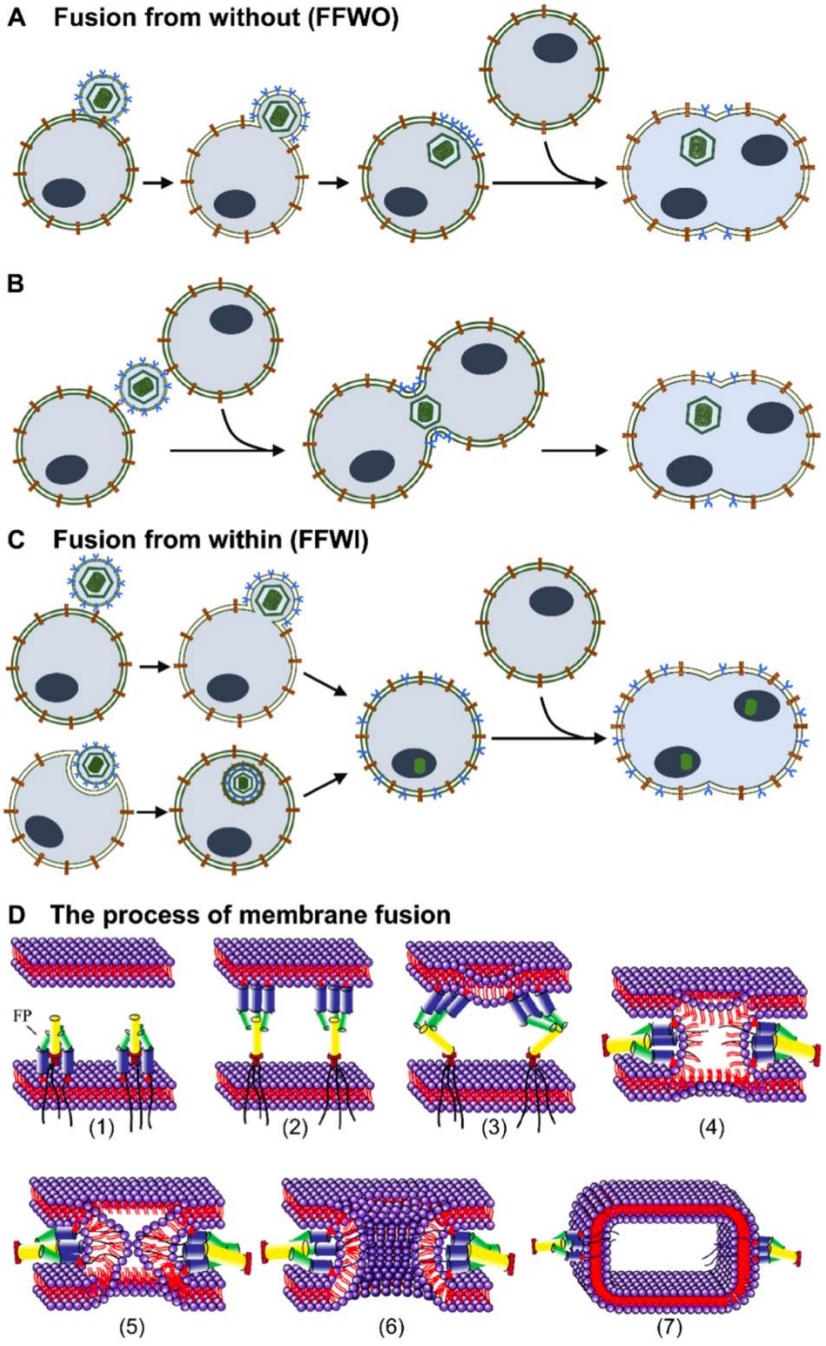

Figure 3. A.B.C: the different ways of membrane fusion induces by the virus. (A) FFWO: The envelope of viral particles is retained on the membrane of the infected cell. The fusion is mediated by viral glycoproteins between the envelope of the virus and the neighboring cellular membrane. (B) When

the viral particle fuses with two cells at the same time, FFWO also can occur. (C) FFWI: The viral genes are expressed after the virus enters the cells, leading to the synthesis of glycoproteins. These proteins are transferred to the cell surface, where they bind with receptors on neighboring cells, triggering syncytium formation. D: The process of membrane fusion [18]. I. Activation of the fusion mechanism and then the FP exposed. II. The FP inserts into the adjacent membrane. III. The fusion protein refolding induces membrane deformation. IV. The stalk expands and then forms a hemifusion diaphragm. V. The fusion pore is open, completing the merging of both cell membranes. VI. The expansion of fusion pore. VII. postfusion. From Tang J, Frascaroli G, Zhou X, Knickmann J, Brune W, Viruses, 2021 [18].

HCMV gB is encoded by the UL55 ORF. It is the most abundant glycoprotein expressed on the envelope of the viral particle [24]. gB plays a crucial role in both virus entry into cells and cell–cell fusion [25-27]. The gB protein consists of approximately 900 amino acids (aa) and is comprised of distinct regions. It includes a signal peptide N-terminal region (N-Term; aa 1-87), an ectodomain with five distinct antigenic domains (domain I to V; aa 1-705), a membrane-proximal region (MPR; aa 706-751), a transmembrane domain (TM; aa 752-796), and a cytoplasmic domain (CTD; aa 797-906) [28-30] (Figure. 4). Among HCMV strains, the amino acid sequence is highly conserved. However, there are high polymorphisms in the N-terminal region and scattered sequence variations throughout the entire protein. gB exists in two different conformations: post-fusion conformation (low energy) [28, 30, 31] and pre-fusion conformation (high energy) [29] (Figure 4B). The pre-fusion gB is a compact trimeric structure with a size of about 100 Å in diameter and 110-130 Å in length. But the post-fusion gB elongates and shapes with a diameter of about 70 Å and a height of approximately 170 Å [28, 31, 32]. During the fusion process, the fusion loop is positioned at the base of the trimer which is close to the infected cell membrane before fusion. However, after fusion, gB appears more elongated and the central helix pushes the FP in the direction of the target membrane, thus synergistically mediating the cell fusion [18, 29].

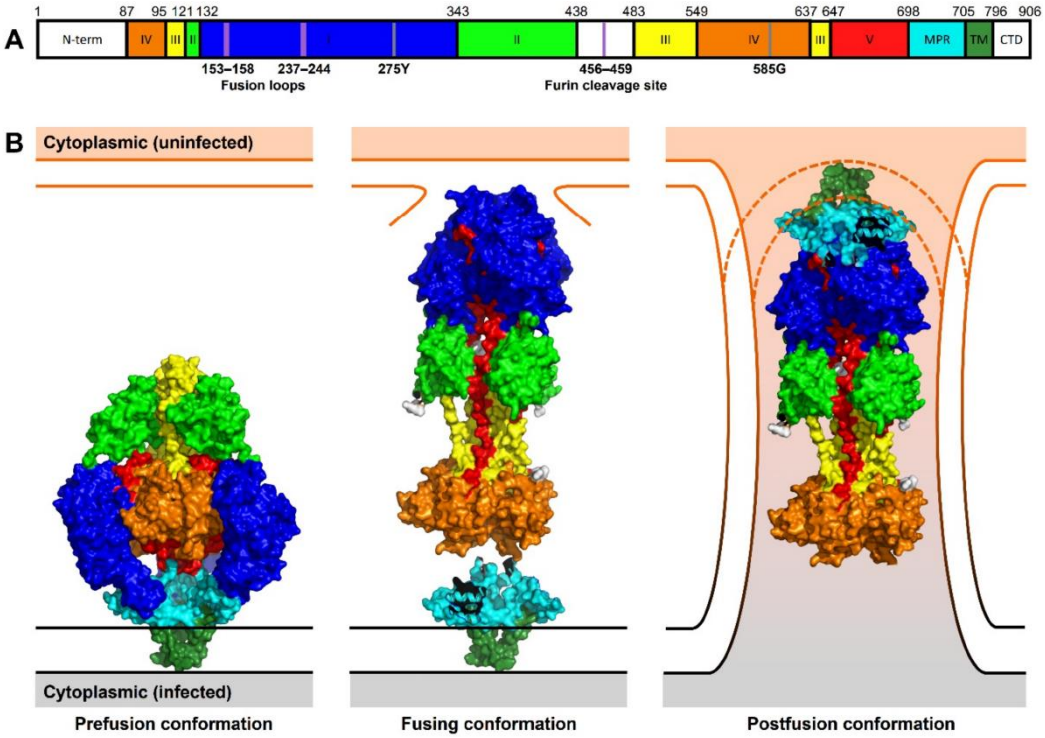

Figure. 4 The structure of HCMV gB. (A) The diagram illustrates the distribution of gB's structural domains. Amino acid indices are shown above the sequence representation. The violet lines are fusion loops and furin cleavage sites. The grey lines marked two known residues that affect gB fusion activity. (B) The conformations of gB protein among the steps of cell–cell fusion. The pre-fusion conformation, displayed on the infected cellular membrane, is described as a compressed structure (PDB 7KDP). The color scheme represents the structural domains: N-term and CTD are in white, domains I to V are in blue, green, yellow, orange, and red, respectively; MPR is shown in cyan, and TM is in dark green. Generated using PyMOL. Adapted from Tang J, Frascaroli G, Zhou X, Knickmann J, Brune W, Viruses, 2021 [18].

Viral glycoproteins have different functions in viral entry and fusion. However, the relation between strain-specific variations and pathogenicity remains poorly known. Our lab has described the strain-specific polymorphisms in gB. Fusogenic gB cause faster cell entry, syncytium formation, and cellular genome instability [25]. These results suggest that functional diversity in viral glycoproteins could potentially underlie the strain-specific discrepancy observed in pathogenicity. Clinical isolates are highly variable and show different growth capacities and properties according to the infected cell type. Interestingly, some HCMV strains isolated from congenitally infected newborns can induce syncytium formation and cell-cell fusion in a fashion that is both virus- and cell-dependent [33]. This raises the question of whether HCMV strains that can induce cell–cell fusion might be associated with increased transmission or pathogenicity.

The aim of my thesis project was to identify the viral genetic determinants responsible for syncytium formation in various cell types. To this end, firstly, I wanted to know if fusogenic gB variants occur in congenitally HCMV-infected isolates frequently. After analyzing the alignment of the gB sequences among the Chinese strains and the consensus sequences (AD169, VR1814, TB40/E, and TR), I found that it is a challenge to determine whether these differences had any functional impact on gB. The problem was that none of these variations corresponded to a previously characterized variant. Therefore, I developed a system that allows me to compare the function of glycoprotein variants in the context of viral infection. To address this, two well-characterized HCMV BAC clones (TB40-BAC4 and TR) were decided to be used as vector backbones for detecting and analyzing the functional differences of gB. With this system, I constructed eight TB40 recombinants carrying the whole length UL55 of the six congenitally HCMV-infected strains from Nanjing. Recombinational TR strains were also obtained using the same method (Figure 5). For investigating the fusogenicity of the obtained gB variants as well as the reference strains (VR1814, TB40, and TR), I used a reporter system (the Renilla luciferase–GFP dual split protein system) that allows me to easily visualize and quantify cell–cell fusion. Most of the gB variants from congenital strains were founded can promote cell-cell fusion of infected fibroblasts. Since gB is essential for viral infectivity and entry, I also tested the kinetics of viral immediate-early (IE) gene expression for addressing whether TB40 and TR recombinants show differences. There are large differences in the amounts of tegument protein pp150 that were brought into the cells by the viral input. Furthermore, to investigate the impact of EIPA (a virus entry inhibitor) treatment on virus entering into cells, I infected MRC-5 cells with TB40 recombinants as well as reference strains (VR1814 and TB40) treated with or without EIPA. All strains tested in the experiment were inhibited to some extent by EIPA. Nonetheless, the most significant inhibition was detected in TB40_gB(NAN13) and TB40_gB(TR), which are less likely to induce cell-cell fusion.

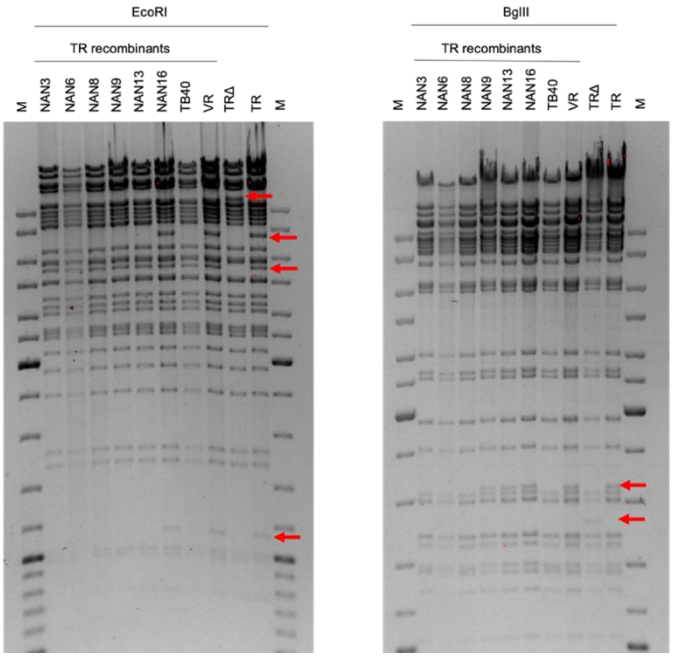

Figure 5: Restriction fragments of the TR BAC, TR_ΔgB, and TR recombinants. Arrows show the difference in the restriction patterns.

The ability to induce cell–cell fusion has shown to be a pathogenicity factor in α-herpesvirus infections, like HSV [34, 35]. However, research on HCMV promoting cell–cell fusion is still in its early stages. Several recent studies have demonstrated the association between phenotypes which can induce cell–cell fusion and genotypes in HCMV strains isolated from congenitally/postpartum-infected newborns [33, 36]. This raises the question of if strains that form syncytia be linked to increased transmission and pathogenicity. While the knowledge of the viral determinants associated with syncytium formation remains uncompleted, what is clear, is that envelope glycoproteins, particularly gB, play an important role among the viral factors. gB, as a viral fusion protein, not only facilitates fusion between the envelope of the virus and host cell membranes but also promotes it between infected cells and neighboring uninfected cells. Nevertheless, more research needs to be done to gain further knowledge of the molecular determinants of syncytium formation and the significance of fusogenic variants in the context of infection/pathogenicity.

Abstract

Cell-cell fusion, as a complex process, plays a role in various physiological and pathological situations containing infectious diseases. It can be induced by the expression of glycoproteins on the infected cells where they bind with cell receptors on the adjacent cells. The fusion of adjacent plasma membranes results in the formation of multinucleated cells called syncytia. This allows viruses to spread efficiently to neighboring cells and escape from the host defenses, thereby enhancing viral dissemination and pathogenicity. Human cytomegalovirus (HCMV), as a herpesvirus, is the main cause of congenital infection in the world and is characterized by a broad cell tropism. HCMV clinical isolates are highly variable and show different growth properties dependent on the infected cell type. A recent study has shown that some HCMV isolates from congenitally infected newborns are capable of inducing cell–cell fusion and syncytium formation, suggesting that this property might be a pathogenicity factor. In the present study, I wanted to identify the viral genetic determinants responsible for cell–cell fusion in various cell types. To this end, I established a method for cloning candidate genes from clinical HCMV isolates and introducing them into the genomes of HCMV reference strains. The GFP-luciferase dual-split protein reporter system was used to quantify the syncytia induced by the TB40 and TR recombinants as well as the reference strains. Using these methods, I analyzed variants of the viral envelope glycoprotein B (gB, UL55) from several congenital and reference strains and found that most of the gB variants from congenital strains promoted cell-cell fusion of infected fibroblasts. The gB variants also affected the efficiency of virus entry into fibroblasts. These findings suggest that highly fusogenic gB variants can facilitate virus entry and trigger cell-cell fusion.

Zusammenfassung

Die Zell-Zell-Fusion ist ein komplexer Prozess, der in verschiedenen physiologischen und pathologischen Situationen, die auch Infektionskrankheiten beinhalten, eine Rolle spielt. Sie kann durch die Expression von Glykoproteinen auf den infizierten Zellen ausgelöst werden, welche sich mit Zellrezeptoren benachbarter Zellen verbinden. Die Verschmelzung benachbarter Plasmamembranen führt zur Bildung mehrkerniger Zellen, die Synzytien genannt werden. Auf diese Weise können sich die Viren effizient auf benachbarte Zellen ausbreiten und der Wirtsabwehr entkommen, wodurch die virale Verbreitung und Pathogenität gefördert wird. Das humane Cytomegalovirus (HCMV) ist als Herpesvirus die Hauptursache für kongenitale Infektionen in der Welt und zeichnet sich durch einen breiten Zelltropismus aus. Klinische HCMV-Isolate sind sehr variabel und zeigen je nach infiziertem Zelltyp unterschiedliche Wachstumseigenschaften. Eine kürzlich durchgeführte Studie hat gezeigt, dass einige HCMV-Isolate von kongenital infizierten Neugeborenen in der Lage sind, eine Zell-Zell-Fusion und Synzytiumbildung zu induzieren, was darauf hindeutet, dass diese Eigenschaft ein Pathogenitätsfaktor sein könnte. In der vorliegenden Studie wollte ich die viralen genetischen Determinanten identifizieren, die für die Zell-Zell-Fusion in verschiedenen Zelltypen verantwortlich sind. Zu diesem Zweck habe ich eine Methode zur Klonierung von Kandidatengenen aus klinischen HCMV-Isolaten entwickelt und sie in die Genome von HCMV-Referenzstämmen eingeführt. Das GFP-Luciferase-Dual-Split-Protein-Reportersystem wurde zur Quantifizierung der von den TB40- und TR-Rekombinanten sowie von den Referenzstämmen induzierten Synzytien verwendet. Mit diesen Methoden analysierte ich Varianten des viralen Hüllglykoproteins B (gB, UL55) von mehreren Kongenital- und Referenzstämmen und stellte fest, dass die meisten gB-Varianten von kongenitalen Stämmen die Zell-Zell-Fusion von infizierten Fibroblasten förderten. Die gB-Varianten beeinflussten auch die Effizienz des Viruseintritts in Fibroblasten. Diese Ergebnisse legen nahe, dass hoch fusogene gB-Varianten den Viruseintritt erleichtern und die Zell-Zell-Fusion auslösen können.

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Authors' contribution

Own contribution:

After literature searching, I conceptualized the information into a project outline. I established some methods for investigating the functional difference of gB, performed the experiments shown in the thesis, and collected the data. In the end, I wrote the manuscript.

Contribution of co-authors:

Yihua Zhou provided DNA of congenitally CMV-infected fetuses. Giorgia Cimato provided initial help with some repetitions of experiments. Giada Frascaroli and Wolfram Brune supervised me and helped with the preparation of the manuscript.

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2. Tang J, Frascaroli G, **Zhou X**, Knickmann J, Brune W. Cell Fusion and Syncytium Formation in Betaherpesvirus Infection. Viruses. 2021,13(10):1973.

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