Chapter I

General Introduction
Glioblastoma Multiforme

Glioblastoma multiforme (GBM) is the most common and malignant type of primary brain tumor. Gliomas represent 60-75% of all astrocytomas, a type of brain tumor originating from astrocytes in the cerebrum, and 15.4% of all primary brain tumors [1]. Gliomas usually comprise of a mixed population of cells, complicating successful treatment and eradication. Therapy usually consists of a combination of several approaches, including surgery, radio-, and chemotherapy. Complete surgical removal of GBM is not possible as a result of the highly infiltrative characteristics into adjacent healthy tissue. Residual tumor cells are often resistant to radiotherapy and possess the ability of unlimited self-renewal and proliferation [2]. While the rest of the tumor consists of phenotypically diverse cells with limited proliferation and tumorigenic potential, it is believed that a specific subpopulation of cells within solid tumors, referred to as glioma cancer stem cells (CSCs), are responsible for tumor recurrence.

Human Cytomegalovirus

The human cytomegalovirus (HCMV), also known as human herpesvirus 5 (HHV-5) is a member of the β-herpesvirus family [3]. In case of an adequate immune system, such as in immunocompetent individuals, HCMV infection usually remains asymptomatic. During this phase, HCMV resides in latency reservoirs in blood and bone marrow, where it is able to avoid recognition by the immune system [4]. When symptoms do arise, it mostly is a general infection alongside other viruses and bacteria causing disease symptoms, such as hepatitis or retinitis, and therefore not characteristic for HCMV-associated pathologies, which include accelerated atherosclerosis or graft rejection [4], [5]. However, in immunocompromised individuals, neonates, or patients on immune suppressive therapies, HCMV can cause significant morbidity and mortality [3]. HCMV has two life cycle phases: a productive phase, where new virions are produced and a latent phase, where there is a restricted gene transcription profile and no new virion production [3]. Upon primary infection, HCMV induces a chronic pro-inflammatory state in both healthy and immunocompromised individuals [6]. The virus is not cleared after primary infection but persists for the lifetime of the host. Latency transcripts protect the virus from endogenous cellular anti-viral protective mechanisms, including cellular apoptosis and antiviral alpha-interferon production [7]. Following the establishment of latency, the viral major immediate early promoter enhancer (MIEP), which drives
initial expression of the major immediate early genes (major IEs) required for lytic infection, is associated with repressive chromatin markers e.g. methylated histone marks [3]. During latent infection the viral MIEP is heavily suppressed by histone posttranslational modifications essentially preventing lytic infection, thereby preserving latency. Aside from HCMV’s role in morbidity and mortality in immunocompromised individuals, HCMV infection has also been associated with several types of cancer, including glioblastoma (GBM) [8]–[12]. Like the well described Kaposi’s sarcoma (KS)-associated herpesvirus (KSHV), which is associated with primary effusion lymphoma and multicentric Castleman’s disease by expressing viral proteins within the host [13], HCMV encodes for viral proteins, which are able to hijack cellular communication. These viral proteins stimulate inflammation and modulate signaling in transformed cells (i.e. oncomodulation), thereby deregulating key cellular pathways involved in mutagenesis, cell cycle, apoptosis, angiogenesis, cell invasion, and host immune response [14]–[18].

**Virally encoded G Protein-Coupled Receptor US28**

One class of proteins expressed in the host cells by HCMV are viral G protein-coupled receptors (GPCRs). GPCRs are seven transmembrane receptors, consisting of a complex of α-, β-, and γ-subunits. They are essential mediators of cellular communication due to their easy access and activation by extracellular ligands. GPCR activation is involved in a wide range of biological processes, from gene transcription to cellular migration and proliferation. HCMV encodes for four known viral GPCRs, among which US28, UL33, US27, and UL78. These share great homology to human chemokine receptors. Human chemokine receptors are cytokine binding receptors and are best known for their role in regulating the migration of various cells in the body. Unlike mammalian chemokine receptors, viral GPCRs are able to signal in a ligand-independent manner. This constitutively signaling by viral GPCRs contributes to virus survival and host invasion [19].

US28 shows high homology to mammalian chemokine receptors CCR1, CCR2, and CX3CR1 [19]. It can be activated by several CC chemokines [20] and is known to couple to a variety of G protein α subunits, which differs depending on the bound ligand and cellular context (Figure 1) [21]. This promiscuous coupling promotes ligand-specific and ligand-nonspecific signaling in a highly cell-specific manner. It is believed that the capacity of HCMV infected cells to sequester CC chemokines
by binding chemokines, such as CCL5 (formerly known as RANTES), CCL3 (formerly known as MIP-1α), and CCL2 (formerly known as MCP-1), is involved in the perturbation of local immune responses. As chemokines are involved in inflammation and infiltration by immune cells, altering their concentration in the proximal extracellular milieu might affect the immune response [22], [23]. Chemokine receptors are mostly G\(\alpha_i\)-coupled i.e. they inhibit adenylyl cyclase, limit the level of intracellular cAMP, and activate protein kinase A (PKA). However, US28 is able to bind to several G proteins. Coupling of US28 to G\(\alpha_{q,11}\) induces inositol triphosphate (IP\(3\)) accumulation, activates the release of calcium from the endoplasmic reticulum (ER) and activates protein kinases e.g. protein kinase C (PKC). Coupling to G\(\alpha_{12/13}\), signals to focal adhesion kinases (FAK), extracellular signal-regulated kinases (ERK), and RhoA pathway. Finally, activation of G\(\alpha_i\) signals towards adenylyl cyclases. As a result of US28’s ability to promiscuously couple to various G proteins, it is not surprising that US28 activity has been associated with oncomodulatory properties.

**US28 in Glioblastoma Multiforme**

HCMV proteins have been found in several types of cancer. Cobbs C. et al. Cancer Res 2002 were the first to show an association between HCMV and malignant gliomas, thereby suggesting that HCMV plays a role in glioma pathogenesis. Although a correlation of HCMV in the aggravation of several types of cancer remains a debate to this day due to challenging and elaborate detection methods, in 2012, researchers came to a consensus that HCMV indeed plays a role in GBM progression [13]. This is supported by previous observations, where upon treatment of GBM patients with the anti-viral drug, valganciclovir, GBM patient survival increased [24]. Although, this study used a relatively small cohort, several studies followed, sharing similar observations of a role for CMV in GBM [25].

The contributing role of US28 has been further emphasized by its ability to signal via several pathways in transformed cells [19], [21]. US28 has been detected in GBM tissue specimen [26] and its constitutive activity has been linked to increased angiogenesis, migration, and invasion by e.g. stimulating vascular endothelial growth factor (VEGF) secretion [20] and promoting the activity of COX2 and STAT3 [22]. As a result, US28 is an interesting target for novel therapies to inhibit cancer growth and proliferation.
**Hypothesis: HCMV, via US28, is involved in maintaining the Cancer Stem Cell niche in Glioblastoma Multiforme**

The cancer stem cell hypothesis describes the existence of a specific subset of stem cells in cancer, which are believed to be responsible for tumor regrowth and have also been associated with resistance to therapy [27]. It supports the contribution of CSCs to the malignancy of GBM. Prenatal development requires tight regulation of pluripotency genes and transcription factors (TFs). These control the development of the brain from the ectodermal lineage, including neural stem cells [28]. Glioma stem cells (GSCs) show all characteristics to qualify as neural stem cells; the establishment and expansion of glioblastoma multiform-like tumors and expression of stem cell markers, like cell surface marker CD133, and...
transcriptional factors SRY-box 2 (Sox2) and POU5F1 (OCT4) [29], [30]. The latter cellular factors are involved in the regulation of self-renewal, proliferation, survival, and differentiation into astrocytes, oligodendrocytes, and neurons, and found to be negatively correlated to the malignancy of GBM [31].

One of the key factors in the infiltrative nature of GBM, other than regulating pluripotency, is the ability of CSCs to promote the development of their own perivascular niche through the secretion of proangiogenic factors [32]. In fact, previous research have reported that breast cancer cells and malignant glioma may give rise to endothelial cells and vascular smooth muscle-like cells to enable neo-vascularization [33]–[35]. This plays a crucial role in the promotion of tumor growth by providing sufficient oxygen and nutrients.

**HCMV and Glioma Stem Cells**

Several indications point towards the involvement of HCMV and US28 in particular in controlling signaling in GSCs. Not only is HCMV more efficient in infecting cancer stem cells, viral genes were found to be expressed for longer periods of time compared to infected differentiated cells [36]. Additionally, HCMV infection of GBM cells upregulated GSC marker CD133 and transcription regulators, including Sox2 and OCT4 [37]. HCMV IE proteins were even found to co-localize with these GBM stemness regulators able to actively regulate their expression [38]. It remains unclear whether tumor neuronal stem cells in GBM originate from the transformation of normal human neuronal cells or whether they arose from mature brain cells after transformation or dedifferentiation. In the last few years, more evidence arises where a unidirectional mechanism is suggested i.e. non-stem cancer cells give rise to cancer stem cells [39], [40]. We therefore speculate that HCMV, with US28 in particular, might play a role in glioma cell (de)differentiation.

**Epithelial-Mesenchymal-Transition and Mesenchymal-Epithelial Transition**

Epithelial cells form layers of cells lining all organs. They are closely joined by membrane structures and are motile within their own epithelial layer. Mesenchymal cells on the other hand are not associated with a basal lamina and migrate freely as chains of cells or single cells [41]. Epithelial cells can convert into mesenchymal cells, a process also known as epithelial-mesenchymal transition (EMT), and vice versa, mesenchymal cells can convert into epithelial cells, a process known as
mesenchymal-epithelial transition (MET) (Figure 2). These processes play an important role during the course of embryonic development and wound healing. During EMT, cell-cell junctions are disassembled and cells lose their polarity to become more migratory and invasive, resistant to apoptosis, increase secretion of degradative enzymes, and digest underlying extracellular matrix in order to migrate to specific sites [42]. Upon reaching these sites, the mesenchymal-like cells re-differentiate via MET, and form specific epithelial tissue structures.

EMT has been widely accepted as an essential part of establishing extravasation by tumor cells during metastasis. Tumor cells are required to increase their invasive potential and become anchorage independent by undergoing EMT. Because of the heterogeneity of tumors, this process is less organized as the EMT cells undergo during embryogenesis [42], [43]. Although limited evidence exists for the role of MET in extravasation and establishing at tumor on a distant site, it is required for efficient co-localization of pivotal EMT regulators e.g. miR-200 in secondary organs [44]. Furthermore, depending on the type of cancer and the route of dissemination – via the lymphatic system or vascular system – cancer cells require either more or less invasive potential, as lymphatic capillaries lack a basement membrane [45], [46].

**EMT and MET in GSCs**

For years, the role of cancer stem cells and EMT/MET in cancer have been studied in parallel to each other. Upon switching to EMT and gaining the ability to disseminate, a vast majority of the cancer cells reduce the ability of self-renewal. It has been suggested that stem cell properties are required to successfully establish a secondary tumor [40]. For this reason, studies focusing on the effect of EMT on the formation of these so-called migrating cancer stem cells have been increasing for the last few years. In fact, direct evidence for the existence of a link between EMT and CSC i.e. overlapping signaling pathways and similar regulatory mechanisms, has been found. One of these studies, conducted in breast cancer showed that the induction of EMT increased the CD44\textsuperscript{high}/CD24\textsuperscript{low} cell population [40], [42]. This specific cell population is a characteristic of breast CSCs and has previously been associated with a shorter metastasis-free over-all survival in patients [47]. Despite the increased interest, little is known about EMT and MET in GSCs and the progression of GBM. In view of the described effects of HCMV on GSCs, we
aimed at gaining more insight into the role of HCMV and more particular US28 in the mechanism underlying EMT and MET in GBM.

**Aim of the Thesis**
The constitutive activity of the HCMV encoded chemokine receptor US28 has been associated with an invasive and pro-angiogenic phenotype in GBM and activates various signaling pathways involved in the aggravation of cancer [48]–[51]. As a result of the complexity of GBM, it remains incurable to this day. Therefore, new treatment options are of dire need. Research into small molecules to target and mitigate US28-mediated effects in cancer is ongoing and an inverse agonist VUF2274 has been developed previously [52]. However, due to limited affinity and specificity, the developed small molecules targeting US28 are not suitable for in vivo use. This thesis primarily aims to understand the role of HCMV-encoded chemokine receptor US28 signaling in glioblastoma on several regulatory levels i.e. RNA level, transcription factor and protein level, and morphological/cellular level. We start with an overview of all viral GPCRs and their involvement in various cancer hallmarks in Chapter II. There we provide a general overview of what is known about these viral receptors and how they modulate various signaling pathways involved in cancer. In Chapter III, we focus on setting up more clinical relevant in vitro and in vivo models; an inducible US28 GBM cell line and orthotopic animal models. Furthermore, in Chapter IV we describe the development of novel therapeutics, US28 nanobodies, with enhanced affinity and specificity. In chapter V, we aim at elucidating the contribution of US28 in aggravating GBM tumor growth via cancer cell dedifferentiation and stem cell regulation. In chapter VI, we look into the Hippo pathway and the death receptor ligand (PD-L1) modulated by US28. Finally in chapter VII, we discuss how our findings can be linked together, what value we can add to the current knowledge, and where we currently stand. With this, we hope our studies provide additional insight into a role for the HCMV encoded receptor US28 in GBM and will contribute to improving the understanding of glioblastoma progression and finding of novel therapeutic targets.
References


