Cytomegalovirus pp71 Protein Is Expressed in Human Glioblastoma and Promotes Pro-Angiogenic Signaling by Activation of Stem Cell Factor

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Abstract

Glioblastoma multiforme (GBM) is a highly malignant primary central nervous system neoplasm characterized by tumor cell invasion, robust angiogenesis, and a mean survival of 15 months. Human cytomegalovirus (HCMV) infection is present in >90% of GBMs, although the role the virus plays in GBM pathogenesis is unclear. We report here that HCMV pp71, a viral protein previously shown to promote cell cycle progression, is present in a majority of human GBMs and is preferentially expressed in the CD133+ cancer stem-cell population. Overexpression of pp71 in adult neural precursor cells resulted in potent induction of stem cell factor (SCF), an important pro-angiogenic factor in GBM. Using double immunofluorescence, we demonstrate in situ co-localization of pp71 and SCF in clinical GBM specimens. pp71 overexpression in both normal and transformed glial cells increased SCF secretion and this effect was specific, since siRNA mediated knockdown of pp71 or treatment with the antiviral drug cidofovir resulted in decreased expression and secretion of SCF by HCMV-infected cells. pp71-induced upregulation of SCF resulted in downstream activation of its putative endothelial cell receptor, c-kit, and angiogenesis as measured by increased capillary tube formation in vitro. We demonstrate that pp71 induces a pro-inflammatory response via activation of NFκB signaling which drives SCF expression. Furthermore, we show that pp71 levels and NFκB activation are selectively augmented in the mesenchymal subtype of human GBMs, characterized by worst patient outcome, suggesting that HCMV pp71-induced paracrine signaling may contribute to the aggressive phenotype of this human malignancy.

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Introduction

Stem cell factor (also known as SCF, kit-ligand, or steel factor) is a cytokine that binds to the c-kit receptor tyrosine kinase (CD117) and is involved in hematopoiesis, spermatogenesis, and melanogenesis. The c-kit receptor, a proto-oncogene activated in several human tumors, is expressed on hematopoietic stem cells, germ cells, and progenitor cells derived from the neural crest [1]. SCF, which exists in both a membrane-bound and a secreted form, is produced by fibroblasts and endothelial cells and promotes cell survival, proliferation, and differentiation by activating multiple signaling cascades downstream of c-kit, including the RAS/ERK, PI3-kinase, Src kinase, and Jak/STAT pathways [1]. Importantly, SCF/c-kit activation has been shown to promote recruitment of endothelial progenitor cells to stimulate angiogenesis in ischemic environments, a process which is essential to the growth and maintenance of tumors [2].

Malignant gliomas are an extremely aggressive type of brain tumor, which are highly vascular and invasive. Because tumor-induced angiogenesis is a pathological hallmark of this malignancy, it is believed that anti-angiogenic strategies have a great potential in improving the treatment and survival of glioma patients [3,4]. SCF is abundantly expressed in high-grade gliomas and in neurons following traumatic brain injury, and plays an important role in tumor- and host-induced angiogenesis [5].

Human cytomegalovirus (HCMV) is a member of the betaherpesvirus subfamily that has been found to be associated with several human malignancies, including glioblastoma [6,7,8,9,10,11,12,13,14,15,16,17]. HCMV, like all other herpesviruses, has the ability to remain latent within the body for the lifetime of the host and can contribute to chronic inflammation, immunosuppression, and metabolic disruption of associated tissues. Several lines of evidence support the idea that HCMV may act as a promoter of the neoplastic process in glioma by modulating the survival, invasive potential, angiogenic properties, chromosome stability, DNA damage response, immune evasion, and cell cycle control of associated cells [18]. To date, several HCMV proteins have been identified in glioma that display oncogenic features. For example, the viral chemokine receptor US28 can promote a pro-angiogenic and proliferative phenotype...
through IL6-Stat3 signaling activation and the viral glycoprotein gB can bind and activate the cellular PDGFRα receptor, which drives proliferation and tumorigenesis of glial precursors through persistent PI3K-Akt activation [19,20,21].

The HCMV tegument protein pp71 performs many functions to enhance the efficiency of viral gene expression and replication. At the start of infection, pp71 stimulates viral immediate early gene expression by degrading the cellular receptor protein Daxx [22,23]. Degradation of Daxx in conjunction with sumoylation of PML by the viral protein IE1 results in the dispersal and disorganization of associated cellular regulatory proteins during infection [24]. pp71 has also been demonstrated to degrade the hypophosphorylated form of the retinoblastoma (Rb) tumor suppressor protein in fibroblasts, thus promoting cell cycle progression into S phase [25,26]. In addition, pp71 downregulates MHC class I cell surface expression in glioblastoma cells, thus facilitating immune evasion [27]. Based on these proposed activities of pp71, we hypothesized that the pp71 protein may mediate additional oncomodulatory effects of HCMV in human GBMs. Here, we investigate the role of HCMV pp71 in modifying SCF and NFKB signaling, as they relate to glioblastoma pathogenesis.

Materials and Methods

Ethics Statement

All human brain tissues (including glioblastoma samples processed as described below) used in these studies were obtained from the CPMC Neurosurgery Department, under an IRB approved protocol (Protocol # 25.125-1). All patients provided written consent stating that they allowed for their tumor samples to be used for basic research. The California Pacific Medical Center Institutional Review Board Panel#1 approved the tissue collection protocol, including the patient consent forms (Current IRB Assurance NO: FWA00009921). Samples have been de-identified before being processed, to protect patient privacy. NPC cells were a gift from Dr. Dennis Steindler (please see reference 28 for details about how the line was produced).

Cell Culture

U87 human glioblastoma cells were obtained from ATCC and grown in DMEM/F12+10% FBS. The NPC cell line was characterized by immunofluorescence and found positive for Nestin, GFAP, TuJ1, and Olig 2. NPCs were grown in DMEM-F12+5% FBS+Bovine Pituitary Extract (Invitrogen)+N2 supplement (Invitrogen) +20 ng/ml EGF +20 ng/ml bFGF. HUVEC cells were obtained from Invitrogen and were cultured in Medium 200PRF with or without low serum growth supplement (LSGS) (Invitrogen) according to the manufacturer’s protocol, and were used at passages 1–3. Human normal astrocytes (Lot #0000199740) were obtained from Lonza and grown in astrocyte basal medium supplemented with SingleQuots growth factors, cytokines, and supplements. Primary glioblastoma derived cultures were processed as follows: tissues were dissociated using papain and single cell suspensions were cultured using neural basal medium+N2 supplement, 20 ng/ml EGF, 20 ng/ml bFGF, and 1 ug/ml laminin (Sigma). GBM cell lines 3832 and 387 were obtained from Dr. Jeremy Rich (Cleveland Clinic) and grown in complete neurobasal medium [28]. Where indicated, CD133+ cells were isolated using the MACS CD133 Microbead kit and an autoMACS magnetic separator (Miltenyi). For ELISA, HUVEC activation, transwell migration, and tube formation assay supernatant collection, cells were cultured in the absence of serum and growth factors for at least 48 hours prior to media collection.

RNA and Protein Isolation from Whole Tissue

Brain tissue was homogenized and lysed in 1 mL QIAzol reagent (Qiagen) using a TissueRuptor homogenizer (Qiagen). RNA was then chloroform extracted and purified using the RNeasy lipid tissue mini kit (Qiagen). The quality of the RNA was verified by spectrometry and visualization of ribosomal RNA bands on an agarose gel. DNA was precipitated from the remaining interphase and organic phase with 75% ethanol, and the protein in the supernatant was then isopropanol precipitated, denatured with 0.3 M guanidine hydrochloride, and resuspended in 1% SDS. Protein fractionation was performed on cells homogenized in cold PBS from frozen tissue using a subcellular protein fractionation kit (Pierce) according to the manufacturer’s instructions.

PCR Analysis

For standard end-point PCR experiments, 1 ug of each RNA was reverse transcribed into cDNA using the iScript cDNA synthesis kit (BioRad) or the SuperScript II kit (Invitrogen) and PCR amplified using the Taq PCR core kit (Qiagen) with an input of 50 ng of cDNA for each experimental sample and water only for the negative control. Control RNA from fetal brain and adult normal cortex were commercially obtained (BioChain). The primers used for PCR analysis are as follows: pp71F 5′-GAGAACACGCTGTGCGCGG-3′, pp71R-5′, CCGGCGGCGGAAGAAAAATCG-3′, Rab14-F-5′-GCAA-
GATTGGGATACAGCAGG-3′, R-5′-CAGTGTTG-
GATTTGAGATATC-3′. All PCR reactions were performed in 50 uL volumes with 50 amplification cycles, and an annealing temperature of 60 degrees for the pp71 primers and 58 degrees for the Rab14 primers. 20 uL of each amplification reaction was resolved on a 1% agarose gel and the size of each amplicon (pp71 = 390 base pairs, Rab14 = 167 base pairs) was verified relative to a 1 Kb DNA ladder (Fermentas). The DNA from the remainder of each PCR reaction was then isolated using the MiniElute PCR Purification kit (Qiagen) and sequenced. TaqMan analysis was performed using custom pp71 and US28 primers and probes (pp71R-5′-TCAGGGGTTCATTTGGAAGAACCCACGGCCGGGAAAAAG-3′, US28R-5′-ACATTGTCGACGACCCGGATGAG-3′) and standard curves were generated with serial dilutions of Ad169 viral DNA (Advanced Biotechnologies) or human genomic DNA (Applied Biosystems). Cytokines RANKL (40 ng/mL) and TNFα (20 ng/mL) were obtained from R&D systems, and the drug Bay11-7085 (5 μM) was obtained from Santa Cruz Biotechnology. Drug and cytokine treatments were performed in serum-free conditions for 24 hours. For quantitative analysis of gene expression using the oncogenes and tumor suppressor genes RT2 Profiler PCR array (SA Biosciences), RNA was extracted from adenovirus transduced NPCs using the RNeasy kit (Qiagen) and 1ug RNA was reverse transcribed into cDNA using the RT2 first strand kit (SA Biosciences) according to the manufacturer’s instructions. Resulting cDNA was then mixed with the RT2 Sybr Green/ROX master mix and water, and 25 uL of the experimental cocktail was added to each PCR well. Real-time PCR was then performed using an Applied Biosystems 7500 Fast thermocycler according to the manufacturer’s instructions, and data was
analyzed using the SA Biosystems online RT² Profiler PCR Array Data Analysis software.

Serology
Cytomegalovirus IgG and IgM ELISA kits were used for determination of serostatus from patient serum according to the manufacturer’s instructions (IBL International).

Viruses
Recombinant adenovirus expressing HA-tagged pp71 (rAD-pp71) was kindly provided by Dr. Robert Kalejta (University of Wisconsin, Madison) and has been previously described [25]. The control adenovirus expressing GFP was obtained from Vector Biolabs. All infections were done at 10,000 particles per cell for 48 hours and in the absence of serum where indicated. Retroviruses pLXSN and pLSNX-pp71 were transfected into PT67 packaging cell lines and supernatants were harvested at 32 degrees and used to transduce U87 cells with 6 ug/mL polybrene. U87 cells were selected with 500 ug/mL G418 for 1 week and then G418 was removed. All HCMV infections were performed at an MOI of 3 with either the laboratory strain Towne (ATCC) or the clinical strain TR (gift from Dr. Lec Fortunato, University of Idaho). Presence of the clinical cassette region UL b’ in TR was confirmed by PCR analysis after propagation.

Antibodies
Mouse antibodies to pp71 have been previously described [29] and were kindly provided by Dr. Tom Shenk (Princeton University). Antibody 2H10-9 was used for western blot at a 1:10 dilution and 10G11 was used for immunofluorescence at a 1:2 dilution. The following antibodies were obtained from commercial sources: anti-actin, anti-HA (Sigma), anti-SCF (Epitomics), anti-eKit, anti-phospho-eKit, anti-p30, anti-phospho-p30, anti-Cox2 (Cell Signaling), anti-CD31, anti-p100/p52 (Abcam), anti-ReB, anti-p65/RelA, anti-NIK (Santa Cruz Biotechnology), anti-IE1 (mAB810), anti-Olig2, anti-CD44 (Millipore) and anti-RB (BD Biosciences).

ELISA
Supernatants from cultured cells were subjected to ELISA using the DuoSet human SCF kit Dyc55 (R&D Systems) according to the manufacturer’s instructions.

RNA Interference
Knockdown of pp71 was achieved with 2 custom siRNA oligonucleotide duplex designed and synthesized by Dharmacon. The sense sequences for the siRNAs are: A = GCATACATCCCGAGTGACATT, B = AGGAAGAGGGAGCAGGAGAU. A non-targeting control pool was used as a negative control (Dharmacon D-001810-10-05). Transfections were performed for 72 hours according to Dharmacon’s suggested protocol with Lipofectamine 2000 (Invitrogen) transfection agent. For U87 cells the optimal amount of Lipofectamine 2000 was used (10 uL per well in a 6-well dish). Half this amount was used for primary cultures to alleviate toxicity. For ReB knockdown, a pool of 3 siRNAs specific to ReB was used (sc-36402) according to the manufacturer’s instructions (Santa Cruz Biotechnology).

Immunostaining
Cells were grown on glass slides in 24-well tissue culture dishes and treated as indicated. Prior to staining, cells were rinsed with PBS, fixed for 10 minutes in cold methanol, and then blocked for 30 minutes with protein free blocking buffer (Thermo Fisher).
Microarray Analysis

Affymetrix Human Gene 1.0 ST arrays were processed and analyzed as previously published [21]. The raw and RMA normalized microarray data were deposited in the Gene Expression Omnibus database (accession number http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=hxutbkkiwaschc&acc=GSE42618).

Luciferase Reporter Assay

5000 U87 cells were seeded in white 24-well plates and transfected the next day with a luciferase reporter construct driven either by a scrambled promoter (R01 negative control), a RPL13 promoter (positive control) or the SCF promoter (KITLG-PROM light switch promoter) at a 1:3 DNA to FuGENE HD transfection reagent (Switch Gear Genomics). Cells were treated as indicated (72 h HCMV infection, 48 hour adenovirus infection or knock-down, and 24 hour cytokine or drug treatment) and then the LightSwitch assay reagent was added for 30 minutes and plates were read with a luminescent plate reader. Each condition was performed in triplicate for each experiment.

Results

To determine if HCMV pp71 gene products are expressed in human glioblastoma cells, we performed a RT-PCR based screening of RNA obtained from fresh frozen primary brain tissue specimens. We determined that 10/15 GBM specimens had detectable pp71 gene expression, while none of the normal brain tissues (CPMC-083, 084, and 074) and 1/2 samples from low grade astrocytomas (LGA, CPMC-080 and 087) were positive (figure 1A). RT-PCR for the Rab14 gene product, which is a cellular gene that has very little expression variability in GBMs served as a control (figure 1A). The identity of the PCR products in GBM samples was confirmed by sequence analysis, and comparison to HCMV-infected U251 cells provided confirmation that amplified sequences were not laboratory contaminants (figure S1A). For a more sensitive approach to detect low copy numbers of pp71, we performed TaqMan analysis on a subset of samples and were able to detect various levels of pp71 expression in 14 specimens with greater specificity (figure 1B). Three tumors were negative for pp71 by TaqMan (CPMC-41, -080, and -113) and were not plotted. Due to the low level of expression of viral genes in these samples, we tested the effects that the cDNA synthesis reaction (iScript versus SuperScript II) as well as cDNA dilution (72 h HCMV infection, 48 hour adenovirus infection or knock-down, and 24 hour cytokine or drug treatment) and then the LightSwitch assay reagent was added for 30 minutes and plates were read with a luminescent plate reader. Each condition was performed in triplicate for each experiment.

We then performed western blot analysis on protein lysates extracted from fresh primary brain specimens to confirm the presence of the pp71 protein in GBMs. A representative blot is shown in Figure 1C, demonstrating pp71 expression in several primary brain samples (7/10) and the positive control HCMV-infected U87 glioma cells, but not in the negative control cell lysates from normal human astrocytes and uninfected U87 cells. Equivalent volumes of each lysate were loaded since quantification of primary protein lysates is often inaccurate due to necrotic cellular debris; therefore the blot was reprobed for the cellular gene actin to account for loading differences. Table 1 summarizes results showing pp71 expression for several primary samples analyzed and corresponding patient HCMV serology status. Due to the variable sensitivity of these different assays few cases of discordance in pp71 detection were noted; however 8 cases were positive by both TaqMan and western blot and were also HCMV seropositive (table 1). Furthermore TaqMan analysis (more sensitive than RT-PCR) displayed a good correlation with protein expression in the same samples.

Our laboratory has found evidence that HCMV gene products are preferentially expressed in the GBM cancer stem cell subpopulation that are often characterized by expression of the CD133 cell surface marker [30]. Therefore we performed RT-PCR on the CD133 positive and negative subpopulations sorted from three fresh, primary GBM samples. As shown in figure 1D, we observed that pp71 RNA was more highly expressed in the CD133 positive subpopulation compared the CD133 negative fraction. To verify this finding, we performed pp71-specific TaqMan on cDNA from an HCMV negative primary GBM cell line (3832) infected with a clinical HCMV strain (TR) in culture, followed by CD133 sorting (figure 1E). We observed a 13-fold higher expression of pp71 in the CD133+ fraction compared to the CD133- negative fraction when normalized to Rab14. These data indicate that expression of pp71 may occur preferentially in cells with cancer stem-like characteristics.

Given the propensity for pp71 expression in glioma stem like cells, we hypothesized that HCMV pp71 expression may represent an early event in gliomagenesis promoting a microenvironment conducive to tumor progression. To investigate this concept, we transiently expressed pp71 (rAD-pp71) or GFP (rAD-GFP) in adult neural precursor cells (NPCs – a glioma cell of origin model system) and measured changes in human oncogenes and tumor suppressor genes using a real-time PCR array. Multiple cellular gene products were specifically induced by pp71, especially the gene encoding stem cell factor (SCF/KITLG), with >50- fold induction (figure 2A). There was no induction of the gene encoding the SCF receptor, c-KIT; however several genes involved in growth and survival, cell cycle regulation, and DNA damage and apoptosis were upregulated by pp71 (figure 2A). We also observed a significant induction of the retinoblastoma gene (RB1), which is consistent with pp71’s ability to activate the E2F transcription factor [25].

To determine if the increased transcription of SCF correlated with production of the secreted SCF protein, we performed quantitative ELISA analysis using supernatant from NPCs, U87 glioma cells, and normal human astrocytes (NHA) infected with either rAD-pp71 or rAD-GFP. As shown in figure 2B, expression of pp71 resulted in a greater than two-fold induction of secreted SCF in both NPCs (p = 0.042) and glioma cells (p = 0.03), and a greater than four-fold induction in normal human astrocytes (p = 0.013), which have very low endogenous levels of SCF. This finding was confirmed by immunofluorescence in NPCs, where robust expression of SCF was observed in association with pp71 staining in HCMV-infected (TR) and pp71- transduced cells but not in the mock infected or GFP-transduced control cells (figure 2C).

To determine if pp71 expression is correlated to SCF expression in primary, endogenously infected GBM tumor samples, TaqMan analysis for SCF was performed on primary tissue samples previously analyzed for pp71 expression by TaqMan in figure 1B. Though SCF expression was variable, the average expression of SCF in pp71-positive GBMs was 2.4-fold higher than SCF expression in pp71-negative tumors (figure 2D). We also performed double immunofluorescence on passage zero cells cultured from primary GBMs to determine the localization of these proteins in situ (n = 6 primary cultures analyzed). Figure 2E shows a representative example, where pp71 and SCF protein expression are co-localized in a subset of primary GBM cells. As negative controls cells were stained with secondary antibody only or with anti-mouse and anti-rabbit isotype controls (figure S1C).
Cells with HCMV and then treated the cells with the antiviral drug expression using a clinically relevant paradigm, we infected U87 secretion in the same cells by ELISA (p = 0.003, figure 3C, bottom panels), we were able to detect a significant decrease in SCF (12.5%) due to the required mild transfection conditions (figure 3C, in an endogenously infected primary GBM culture was modest the expression of another viral gene, IE1. While pp71 knockdown of pp71 protein expression in infected NPCs measured by ELISA (figure 3B). These siRNAs had no effect on knockdown of pp71 in both HCMV infected NPCs and a primary GBM culture. Using two siRNA sequences targeting pp71, we achieved a potent knockdown of pp71 protein expression in infected NPCs (figure 3A), which resulted in a decrease in SCF secretion as described in [21], demonstrating that SCF was more highly specificity of detection. The extent of pp71 and SCF co-localization was quantified in a small number of cells (n = 7) as displayed for 3 independent experiments, *p < 0.05 by student t-test. doi:10.1371/journal.pone.0068176.g001

Double immunofluorescence of primary GBM tissue sections for pp71 and SCF further demonstrates co-localization of the two proteins in situ (figure 2F). Negative controls (i.e., immunostaining of frozen tissue sections using secondary antibody alone) confirmed specificity of detection. The extent of pp71 and SCF co-localization was quantified in a small number of cells (n = 7) as described in [21], demonstrating that SCF was more highly expressed in pp71 positive GBM cells (Pearson co-efficient = 0.69, figure 2G). These data suggest a biologically relevant link between the presence of HCMV pp71 and SCF expression in human glioblastoma.

To confirm that pp71 is specifically involved in the upregulation of SCF expression, we utilized RNA interference to knock down pp71 in normal human astrocytes and mock-infected or HCMV-infected U87 glioma cells were used as controls. The N.C. negative control (NC) is PCR performed with water instead of cDNA. E: HCMV-infected primary GBM cell line (3832) was sorted using CD133 labeled antibody at 72 hours post-infection and analyzed by TaqMan for pp71 compared to a mock-treated control. The relative expression of pp71 normalized to Rab14 is displayed for 3 independent experiments, *p < 0.05 by student t-test.

Table 1. pp71 status, HCMV serology and IF analysis of primary GBM samples.

| Subclass | RT-PCR | TaqMan | Western | IgG | IgM | pp71 IF | SCF IF | CD31 IF |
|----------|--------|--------|---------|-----|-----|---------|--------|--------|
| CPMC-074 | BC     | –      | +       | N.D. | N/A | N/A     | N.D.   | N.D.   |
| CPMC-080 | LGA    | –      | –       | –   | N/A | N/A     | N.D.   | N.D.   |
| CPMC-087 | LGA    | +      | –       | –   | N/A | N/A     | –      | –      |
| CPMC-076 | PN     | –      | +       | +   | –   | –       | –      | –      |
| CPMC-092 | MES    | +      | +       | +   | –   | +       | +      | +      |
| CPMC-047 | MES    | +      | +       | +   | –   | +       | +      | +      |
| CPMC-050 | CLAS   | +      | +       | +   | –   | +       | +      | +      |
| CPMC-085 | MES    | +      | +       | +   | –   | +       | +      | +      |
| CPMC-086 | MES    | –      | +       | N.D. | N/A | N/A     | N.D.   | N.D.   |
| CPMC-093 | MES    | –      | +       | +   | –   | –       | +      | –      |
| CPMC-098 | CLAS   | +      | +       | N/A | N/A | N.D.    | N.D.   | N.D.   |
| CPMC-102 | MES    | +      | +       | +   | –   | N.D.    | N.D.   | N.D.   |
| CPMC-101 | PN     | +      | +       | +   | –   | N.D.    | N.D.   | N.D.   |

Serology for IgG and IgM was performed and charted relative to pp71 expression status as determined by RT-PCR, TaqMan and western blot in figure 1. Flash frozen tissue sections were analyzed for the presence of pp71, SCF and CD31 by immunofluorescence (IF) in selected tumor cases, as indicated. GBM patient samples without available plasma were labeled N/A. Tumors were classified as low grade astrocytoma (LGA) or GBM by pathology, and the GBM samples were further subclassified as proneural (PN), mesenchymal (MES), or classical (CLAS) based on their gene expression signatures (data not shown and figure 6C). BC refers to a non-tumor surgical specimen from a blood clot. ND signifies not determined.

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Figure 1. Detection of pp71 RNA and protein in primary glioma specimens. A: RNA was extracted from 20 different primary brain tissues, synthesized into cDNA, and amplified using pp71 and Rab14-specific PCR primers. RNA from U251 glioma cells mock infected or infected with HCMV Towne strain and commercially available RNA samples from normal fetal and adult human brain were used as controls. The N.C. negative control PCR contained water in place of cDNA. B: Several cDNA samples described in A were analyzed by TaqMan using primers and probes specific for the pp71 gene and normalized to Rab14. Copy number was determined using Ad169 viral DNA standard curve. C: Western blot analysis for pp71 from 10 different primary brain tissues. Lysates from normal human astrocytes and mock-infected or HCMV-infected U87 glioma cells were used as controls. D: Cells from 3 freshly resected GBM were sorted using CD133 labeled antibody and analyzed by RT-PCR for pp71 and Rab14. Negative control (NC) is PCR performed with water instead of cDNA.
can induce proliferation of NPC cultures, we performed a BrdU incorporation assay. NPCs that were untreated, infected with rAD-GFP, or incubated with rh-SCF did not show a significant increase in DNA synthesis (figure S2A). In contrast, pp71-transduced NPCs demonstrated an ∼8-fold increase in BrdU incorporation, which is likely due to pp71-mediated degradation of the hypophosphorylated form of Rb [25] (figure S2B).

Since SCF-mediated c-Kit signaling can promote activation of the mitogen-activated protein kinase (MAPKs) and phosphoinositide 3-kinase (PI3K)/Akt pathways in target cells [31], we sought to determine if pp71-induced SCF could activate these downstream pathways in cultured human umbilical vein endothelial (HUVEC) cells. As shown in figure 4A, when HUVECs were stimulated with conditioned medium from NPCs transduced with pp71 (15 min), SCF-mediated phosphorylation of the c-Kit receptor was observed to a similar extent as that induced by authentic recombinant SCF ligand. This effect was specific, since c-Kit phosphorylation was not observed in HUVECs stimulated with conditioned medium from pp71-transduced NPCs treated with a SCF neutralizing antibody. Consistent with the downstream signaling pathway for c-Kit, we also observed increased phosphorylation of p38-MAPK in cells treated with conditioned medium from pp71-expressing NPCs or recombinant SCF (Figure 4A).

Secreted SCF can directly activate brain microvascular endothelial cells (ECs) in vitro and induce a potent angiogenic response in glioblastoma in vivo [5]; therefore we hypothesized that pp71 expression could promote angiogenesis and used an in vitro endothelial tube formation assay to test this hypothesis. As shown in figure 4B, conditioned medium from NPCs that expressed pp71 induced a dramatic increase in capillary tube formation in HUVEC cells. Indeed, the extent of tube formation elicited by pp71 expression was comparable to that of the positive control media and to recombinant SCF. Overexpression of pp71 in U87 GBM cells also induced SCF secretion (Figure 2C), which promoted HUVEC tube formation (figure S2C). Taken together these data demonstrate that pp71-induced SCF derived from either NPCs or glioma cells is biologically active.

Both endothelial cell recruitment and capillary tube formation are essential features of glioma-induced angiogenesis, and since studies of the developing human central nervous system suggest that SCF may play a role in angioblast recruitment [32], we hypothesized that GBM cells that express pp71 may also increase endothelial migration via SCF secretion. As shown in figure 4C, untreated U87 cells or conditioned medium from these cells increased HUVEC transwell migration compared to the medium only negative control (which was expected, since U87 cells naturally secrete SCF). However, both pp71-expressing U87 cells and conditioned medium from these cells caused a significant increase in the transwell migration of HUVECs compared to control treated U87 cells (p = 0.015 and p = 0.032, respectively). HUVEC transwell migration induced by pp71 was comparable to that elicited by recombinant SCF, and was inhibited by pretreatment with a SCF neutralizing antibody, demonstrating specificity. Overall, these data indicate that the SCF secreted by pp71-expressing GBM cells can directly promote endothelial cell migration.

Our data support a mechanism whereby pp71 expressed in GBM tumor cells, particularly the cancer stem-like cells, stimulates secretion of SCF in the tumor microenvironment, resulting in the activation and migration of nearby endothelial cells. To determine if pp71-expressing cells are proximal to endothelial cells in primary tumor specimens, we performed double immunofluorescence on frozen GBM tissue samples and found that pp71 positive GBM cells were indeed located adjacent to and surrounding the CD31 positive endothelial cells, further supporting our hypothesis for the pp71-induced paracrine signaling promoting GBM angiogenesis (figure 4D).

To investigate the molecular mechanism underlying HCMV pp71 induction of SCF expression in glioblastoma, we generated a pp71-expressing stable U87 glioma cell line (figure S3A) and pp71 induction of SCF expression in glioblastoma, we generated a pp71-expressing stable U87 glioma cell line (figure S3A) and interrogated changes in gene expression between pp71-expressing and control cells using Affymetrix arrays, followed by Ingenuity pathway analysis (IPA) (raw Affymetrix data is available at the following link http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=luxubkkiswaa&acc=GSE42618). We found that NFKB-stimulated genes were significantly upregulated in the pp71-expressing glioma cells (figure 5A). Among the 170 cancer-related molecules with altered expression, the top five activated regulators are all components of the NFKB pathway (table 2). Since it has been reported that transcription of the SCF gene can be activated by NFKB in a pro-inflammatory environment [33], we sought to investigate whether pp71 can activate NFKB signaling.

To this end, we used NPCs either infected with whole virus (HCMV, TR clinical isolate) or transduced with rAD-pp71 and evaluated the status of NFKB pathway activation using immunostaining. We observed a mild activation of the canonical NFKB component p100/p52 (NFKB2) in both pp71-transduced and HCMV-infected cells when compared to rAD-GFP or mock controls (figure 5B). The p52 heterodimer RelB was also induced in HCMV-infected cells and re-localized to the nucleus upon pp71 expression (figure S3B). It has been reported that RelB is upregulated by canonical NFKB signaling, which is known to be strongly activated by other viral genes [34,35]. Our data demonstrate that pp71 was sufficient to
Figure 3. SCF induction by HCMV can be blocked by pp71 knockdown and Cidofovir. A: NPCs infected with HCMV (TR) were transfected with either negative control siRNA or a pp71-specific siRNA (designated A and B). pp71 protein levels were measured 72 hours later using western blot analysis (left panel). IE1 expression was determined to ensure specificity of the knockdown. B: Conditioned medium samples from the cells described in A were analyzed by ELISA for SCF. The percent decrease in SCF is displayed. (* p = 0.07 for pp71 B siRNA compared to negative control siRNA). C: HCMV positive, primary GBM cells transfected with control non-targeting siRNA or a siRNA specific to pp71 were analyzed by western blot for pp71 and actin 72h later. Percentage of pp71 knockdown (12.5%) from the western blot in C was calculated by normalization to actin (top panel). Conditioned medium from the same samples was analyzed by ELISA for secreted human SCF (bottom panel). *p = 0.003 by student t-test when pp71 siRNA was compared to negative control siRNA treatment. D: U87 cells mock or HCMV-infected were treated with 10uM cidofovir (CDV) or vehicle for 72 hours. RNA was collected and analyzed by TaqMan for pp71 and SCF and expression was normalized to Rab14 (top panel). Conditioned medium from the cells in E were analyzed by ELISA in triplicate. The percent decrease in SCF after CDV treatment compared to vehicle control is displayed (bottom panel). *p<0.05 as determined by a student t-test. E: Endogenously infected primary GBM cells (CPMC-145) were sorted for CD133 and the positive fraction was sub-cultured and treated with vehicle only or 10 uM CDV for 72 hours. RNA was collected and analyzed by TaqMan for pp71, US28 (another viral gene), SCF and Rab14.

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Figure 4. SCF induced by pp71 in NPCs and glioma cells is biologically active. A: HUVECs stimulated with either recombinant human SCF or conditioned medium from NPCs transduced with rAD-GFP, rAD-pp71, or rAD-pp71+ SCF neutralizing antibody were analyzed by western blot for phosphor-c-Kit, total c-Kit, phosphor-p38, total p38, and actin. B: HUVECs were grown overnight in gel matrix supplemented as indicated. Capillary tubes that formed in each condition were visualized by microscopy (top panel), counted and plotted (bottom panel). *p = 0.05 as determined by student t-test when rAD-pp71 was compared to rAD-GFP and when rAD-pp71+Nab was compared to rAD-pp71. Each condition was run in duplicate and the experiment was repeated three times. C: HUVEC cells were subjected to a 4 h transwell migration assay using either 20,000 U87 cells treated as indicated (dark grey bars) or supernatant obtained from the same cells (light grey bars) on the bottom wells. For negative and positive control we used serum-free medium or rhSCF (1 ug/mL) respectively. HUVECs that migrated to the bottom of the wel were stained and counted. *p = 0.015 for cells and 0.032 for supernatant when compared to untreated control, or 0.058 and 0.075 when compared to rAD-GFP, respectively by student t-test. Each condition was run in triplicate and the experiment was repeated twice. D: Frozen GBM tissue sections were immunostained for pp71 alone (figure 2F) or co-immunostained for pp71 (green) and the endothelial cell marker CD31 (red). Tissues were counterstained with DAPI. Two regions from a representative example from a GBM sample are shown. Magnification of the region of interest is shown in the last panel. Bar = 200μm. Negative controls can be found in figure 2F. doi:10.1371/journal.pone.0068176.g004
Figure 5. pp71 stimulation of SCF secretion requires activation of the NFKB signaling pathway. A: U87 cells stably transduced with a retrovirus encoding pp71 (pLXSN-pp71) or control (pLXSN) were used for transcriptome profiling using an Affymetrix Gene 1.0 ST array. Ingenuity pathway analysis (IPA) for differentially expressed genes identified several targets of the NFKB signaling pathway as being upregulated (fold-increase in expression is displayed next to the molecule icon). B: NPCs transduced with rAD-pp71, vector control, or HCMV-infected were co-immunostained for pp71 and markers of NFκB activation (p100/p52, Cox2, and RelB). Cells were counterstained with DAPI. C: Primary GSC neurospheres were treated as indicated and total RNA was analyzed by TaqMan for SCF, CXCL12, IL8, and Myb expression levels. Rab14 levels were used for normalization. The percent change in expression relative to untreated control is displayed. The experiment was repeated three times with similar results. D: U87 cells were transfected with an SCF-promoter driven luciferase construct and then treated as indicated. Luminescence readouts from a representative experiment are shown. Each condition was run in triplicate and the experiment was repeated three times. Positive (RPL13) and negative (scrambled) control promoter driven luciferase constructs were used. Differences were not statistically significant by student t-test.

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activate processing of the inactive p100 protein to the active p52 form, as shown by its nuclear localization (for pathway diagram see figure S3C).

To corroborate nuclear re-localization with functional activation of the non-canonical NFκB pathway, we performed TaqMan for two NFκB-stimulated genes using primary GBM cells transduced with pp71. CXCL12 (SDF-1) gene expression is activated by the non-canonical NFκB signaling, while IL8 is a target of the canonical NFκB pathway. Treatment with the cytokines RANKL or TNFα were used as positive controls since both are known to activate NFκB signaling to varying degrees. Both CXCL12 and IL8 were moderately activated by RANKL and TNFα strongly induced IL8, indicating the NFκB signaling pathway is intact in this GBM cell line (figure 5C). Transduction with the control adenovirus (rAD-GFP) had a negligible effect on gene expression, while transduction with pp71 led to a robust increase in expression of CXCL12 and SCF and a modest increase in expression of IL8. To verify specificity of the pp71 effect on the NFκB signaling, we used Taqman analysis of HCMV-infected GBM cells treated with a combination of two siRNA oligonucleotides targeting pp71, or a scrambled, non-targeting siRNA. Upon treatment with pp71 siRNA, we measured a significant decrease in expression of SCF and CXCL12, indicating that pp71 is directly responsible for their activation. The effect on IL8 expression was modest, likely due to other viral genes which can stimulate canonical NFκB signaling and thus compensate for the loss of pp71.

Since pp71 only partially activates canonical NFκB signaling (known to activate the SCF promoter) we hypothesized that additional mechanisms underlying SCF stimulation exist. It has been reported the c-myb proto-oncogene binds the SCF promoter. Interestingly, our initial PCR-based screening shows 40-fold upregulation of c-myb after pp71 expression (figure 2A) [36]. Subsequent Taqman validation showed that c-myb expression was induced both by pp71 and HCMV, and that pp71 knockdown completely negated the whole virus-induced activation (figure 5C). RelB is essential for expression of the full-length c-myb transcript, therefore it is possible that NFκB and c-myb pathways are complementary mechanisms leading to SCF activation [37]. To confirm the role of NFκB in regulating SCF activation, we performed a promoter- based Luciferase reporter assay using U87 cells to measure activity of the SCF promoter (figure 5D). Luminescence measurements demonstrated a trend of SCF promoter activation upon stimulation with RANKL, TNFα, rAD-pp71 expression, or HCMV infection. pp71 knockdown or direct inhibition of NFκB using Bay11-7085 or RelB knockdown (figure S3D) reversed HCMV-induced activation of the SCF promoter. These results indicate that pp71 specifically upregulates SCF via activation of the non-canonical NFκB pathway.

| Table 2. U87-pp71 IPA pathway analysis summary. |
|---|---|---|---|
| **Top Molecules** | **fold change** | **Top Biological Functions** | **p-value** | **# of molecules** |
| IL8 | 21.39 | Diseases and Disorders | | |
| MMP3 | 17.09 | Cancer | 2.96E-15–4.34E-04 | 170 |
| MMP12 | 15.06 | Dermatological Diseases and Conditions | 8.57E-12–2.43E-04 | 33 |
| LIPH | 14.36 | Gastrointestinal Disease | 2.62E-09–3.22E-04 | 89 |
| FDCSP | 11.69 | Inflammatory Response | 1.03E-08–4.10E-04 | 72 |
| SERPINB2 | 8.20 | Cardiovascular Disease | 1.85E-08–2.44E-04 | 69 |
| PI3 | 9.43 | Molecular and Cellular Functions | 9.23E-09–3.88E-04 | 87 |
| SERPINB3 | 8.20 | Cellular Movement | 1.40E-09–3.44E-04 | 33 |
| IL1B | 8.03 | Cell Morphology | 1.40E-09–4.34E-04 | 118 |
| ZNF626 | 6.68 | Cellular Development | 2.14E-08–4.34E-04 | 64 |
| FABP3 | –4.83 | Cell-to-Cell Signaling and Interaction | 2.24E-08–4.34E-04 | 127 |
| RNU5A-1 | –4.83 | Cell Death and Survival | 2.24E-08–3.88E-04 | 64 |
| SCD | –4.52 | Top Upstream Regulators | | |
| INSIG1 | –3.91 | p-value | | |
| HMGC51 | –3.40 | Outcome | | |
| LDB2 | –3.36 | TNFα | 8.07E-20 | Activated |
| TME229B | –3.30 | IL8 | 5.81E-16 | Activated |
| NR2E1 | –3.10 | Lipopolysaccharide | 3.77E-17 | Activated |
| PTP4A3 | –2.92 | IL1A | 1.73E-14 | Activated |
| GABRG3 | –2.90 | RELA | | |
viral gene expression characteristic of a lytic infection. In this GBM sample, the canonical NFKB component p65/RelA appears largely inactive based on its localization in the cytoplasmic and membrane fractions; the p100 (inactive) subunit of the noncanonical pathway was detected only in the cytoplasm, indicative of clean fractionation (Figure 6A). However the activated p52 subunit was abundant in the nucleus as well as the chromatin, suggesting the noncanonical NFKB pathway is highly activated in this tumor. To corroborate this result in additional GBM specimens, we performed western blot analysis of whole cell lysates prepared from primary frozen GBM samples (figure 6B); pp71 was expressed in all tumor samples examined, but was much more abundant in tumors of the mesenchymal subclass, which are known to be more aggressive, invasive, and angiogenic (Figure 6B–C) [38,39]. GBM sample classification was verified using Taqman for specific proneural and mesencymal markers and confirmed by western blot analysis for CD44 (mesenchymal marker) and Olig2 (proneural marker, Figure 6 B–C). The tumors with the highest expression of pp71 also displayed highest levels of expression of the activated noncanonical NFKB components p52 and NFKB inducing kinase (NIK), suggesting that pp71 expression may contribute to sustained activation of NFKB signaling in GBMs belonging to the mesenchymal molecular subtype.

Discussion

Glioblastomas are characterized by a very high degree of vascular proliferation and angiogenesis, which promotes their aggressive growth and invasiveness [40]. Our group first reported that HCMV infection was present in a high percentage of GBMs [41]. Since HCMV encodes for multiple gene products that might enhance tumor aggressiveness, we have begun to examine these tumors for expression of viral gene products which may contribute to the complex GBM phenotypes.

Recent work by our group and others indicates that HCMV infection of human gliomas may promote mitogenic signaling, angiogenesis, invasion and other pathways that are mediated by HCMV gene products including IE1, gB, and US28 [reviewed in [42]]. Given the potential oncomodulatory properties of the HCMV pp71 gene product [43], we sought to determine whether this viral gene was present in primary GBMs in situ and whether ectopic expression of pp71 in adult NPCs (the putative cells of origin of glioma) or glial cells could mimic the pathogenesis of this disease. Here, we demonstrate that HCMV pp71 RNA and protein are expressed in a majority of GBM specimens examined.

Our functional studies support a novel role for pp71 with important implications for glioma progression. We demonstrate that pp71 expression in adult NPCs and glial cells induces SCF expression in a NFKB dependent manner. Importantly, SCF was recently shown to be a potent angiogenic factor in human GBM; furthermore, SCF expression levels are positively correlated with glioma grade and inversely correlated with patient survival [44]. Induction of SCF by pp71 in glia precursor stem cells or transformed cells is likely to promote a pro-angiogenic microenvironment, which is critical for tumor progression.

We demonstrate here that pp71 expression in NPCs, U87 GBM cells, and normal human astrocytes increased secretion of biologically active SCF, capable of c-Kit receptor activation on endothelial cells. Knockdown of pp71 expression using siRNA or the anti-viral drug cidofovir in both in vitro infected cells and endogenously infected primary glioblastoma cells resulted in decreased SCF secretion, indicating a specific role for pp71 in driving SCF mediated pro-angiogenic signaling. Furthermore, conditioned media from pp71-expressing NPCs or U87 GBM cells induced endothelial tube formation and cell migration to levels similar to those induced by recombinant SCF, and these activities were specifically blocked with a neutralizing antibody to SCF. Overall, these data strongly suggest that pp71 expression in human GBM cells in vivo may contribute to endothelial cell migration and angiogenesis, typically associated with an aggressive tumor phenotype.

Recent evidence has shown that a subpopulation of tumor cells, designated cancer stem cells, plays a critical role in GBM initiation and contributes to resistance to radiation and chemotherapy [45,46]. This population of cells has also been demonstrated to express high levels of SCF in other solid tumors [47]. Importantly, we found pp71 preferentially expressed in the stem-like (CD133+) primary GBM cells in three cases we interrogated. Furthermore, immunostaining of frozen GBM sections revealed that pp71 is expressed in tumor cells surrounding the CD31 positive endothelial cells, an area of the tumor known to be enriched in glioma stem-like cells [48]. Of additional relevance to GBM stem cell survival is our finding that pp71 induces c-myb expression, c-myb is required for the maintenance and expansion of progenitor cells in the bone marrow and neurogenic regions of the adult brain [49]. Therefore, induction of c-myb by pp71 may contribute to the survival and expansion of glioma stem cells while also stimulating SCF secretion [49].

Using complementary approaches, we show that pp71 activated both the canonical and non-canonical NFKB pathways, leading to up-regulation of SCF as well as sustained overexpression of several pro-inflammatory cytokines (IL8, IL1B, IL6, LIF, PTGS2, IL1A), tissue remodeling matrix metalloproteinases (MMP3, 12, 1, 7), and angiopoietins, which are important for vasculogenesis (ANGPT1, ANGPTL4). These results (shown in table 2) suggest that persistent pp71 expression could contribute to glioma progression by activation of tumor cell survival pathways and increased invasion, in addition to pro-angiogenic signaling.

The findings we present here regarding the pro-angiogenic effects of pp71 in GBM may represent one component of a broader HCMV strategy in promoting angiogenesis in GBM. Recently, we and others have determined that HCMV US28 is expressed in human GBMs, and promotes tumor angiogenesis by upregulating vascular endothelial growth factor (VEGF) [19,50], suggesting that US28 and pp71 may have complementary roles in driving pro-angiogenic signaling.

Taken together our studies describe a novel role for HCMV pp71 as a tumor promoter and possible therapeutic target in glioblastoma. In addition, these data suggest that CMV-specific antiviral therapy could be a potentially efficacious approach to mitigate the oncomodulatory effects of HCMV in glioma patients.
Supporting Information

Figure S1 Detection of pp71 in primary GBM samples. A: Sequence alignment of pp71 PCR products obtained from 8 different primary GBM specimens compared to 2 HCMV lab-adapted strains Ad169 and Towne. Sites where nucleotide changes were observed are underlined. The sites within pp71 that were mapped for Rb interaction (LXCXD motif) and DAXX interaction (hDAXX interaction domain) are displayed. B: pp71 and GAPDH TaqMan analysis of cDNA synthesized from fetal brain or 2 primary GBMs (CPMC101 and 102) using either the iScript or SSII cDNA synthesis kits. cDNA from the SSII kit was also diluted 1:10 with water to alleviate the reported inhibitory effects of the cDNA synthesis components on downstream qPCR [51]. Bars represent the copy number of each transcript as determined by standard curve per ug of input DNA, therefore the effect of dilution is accounted for. C: Primary passage 0 GBM cells were fixed and immunostained with anti-mouse and anti-rabbit isotype control primary antibodies and counterstained with DAPI.

Figure S2 SCF does not induce autocrine proliferation but does stimulate HUVEC tube formation. A: NPCs were untreated, transduced with rAD-GFP or rAD-pp71 adenoviruses for 48 hours, or incubated with recombinant human SCF (1 ug/mL) for 24 hours in 0.1% serum and then labeled with BrdU for 60 minutes. Cells were then fixed, stained for BrdU, and counterstained with propidium iodide. The percentage of BrdU positive cells in each treatment group was calculated and plotted. (* p < 0.007 for rAD-pp71 compared to control adenovirus transduced cells). B: NPCs were mock treated or transduced with rAD-pp71 and were immunostained for total RB protein (green), pp71 (blue), and counterstained with propidium iodide (left panel). Cells lysates were also subjected to western blot analysis, where the faster migrating band represents the hypophosphorylated form of Rb (middle panel). Quantification of the two Rb bands was performed and normalized to actin (right panel). C: HUVECs were grown overnight in gel matrix and either negative control medium (serum and growth factor free), positive control complete medium, negative control medium plus recombinant SCF (rHSCF, 1 ug/mL), or conditioned medium from U87 cells transduced with rAD-GFP, rAD-pp71, or rAD-pp71 followed by 1 hour preincubation with neutralizing antibody to SCF. Capillary tubes that were formed in each condition were visualized by microscopy (left panel), counted and plotted (right panel).

Figure S3 Modulation of NFKB signaling by pp71. A: U87 cells were stably transduced with a pp71 expressing retrovirus (pLXSN-pp71) versus an empty vector control (pLXSN) and pp71 expression was confirmed by immunostaining and western blot. B: NPCs were mock treated or transduced with rAD-pp71 and immunostained for RelB and pp71 and counterstained with propidium iodide. C: Ingenuity systems pathway analysis software was used to diagram components of both the canonical and non-canonical NFKB pathways predicted to be activated by pp71. D: U87 cells were tested for RelB expression by western blot with or without TNFα treatment to induce expression or after RelB siRNA treatment to knockdown expression. Actin was used as a loading control.

Acknowledgments

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Author Contributions

Conceived and designed the experiments: LM LS. Performed the experiments: LM LH VB LS. Analyzed the data: LM VB LS. Wrote the paper: LM LS CSC.

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Author Contributions

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