Investigation into the Possible Mitogenic Role of an Epidermal Growth Factor Ortholog Expressed by Ectromelia Virus

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Candidate for the degree

Bachelor of Sciences

Submitted in partial fulfilment of the requirements for

College Honors

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Abstract

It has been demonstrated that poxviruses express proteins with high sequence homology to epidermal growth factor (EGF). Upon analysis of the ectromelia virus genome, a gene with sequence homology to EGF has been identified. The product of this gene is predicted to be shorter than EGF and other poxvirus growth factors, but may still retain functionality due to the presence of six conserved cysteine residues in its primary structure. This gene and its product have not been previously studied, so in this study, we seek to investigate the possible mitogenic role of the growth factor homolog encoded by ectromelia virus during infection. Using 5-ethynyl-2'-deoxyuridine assays, we observe an upregulation of DNA synthesis in cells infected with ectromelia and vaccinia viruses, indicating the viruses likely have mitogenic properties.

Introduction

Viruses are disease-causing agents that can infect every form of life on Earth. They are only able to replicate inside of living cells and are therefore termed obligate intracellular parasites[1]. Throughout their evolution, viruses have acquired different machineries to enter and thrive within cells, and they are categorized into virus families accordingly[1]. One such family is *Poxviridae* (commonly referred to as the poxvirus family), a group of viruses characterized by their brick or oval-shaped virus particles (virions) and double-stranded DNA genomes[1,2].

The poxvirus family is divided into two subfamilies: *Entomopoxvirinae*, which infect insects, and *Chordopoxvirinae*, which infect vertebrate animals[2]. *Chordopoxvirinae* is further divided into multiple genera, one of which is the orthopoxvirus genus[2]. Orthopoxviruses are of particular interest because they include variola virus, the causative agent of smallpox[1,2]. Other members of this genus include vaccinia virus, the virus used as a vaccine for the eradication of smallpox, and ectromelia virus (ECTV), the causative agent of mousepox disease[2].

In the 1940's ECTV was proposed as a model system for the study of smallpox and other skin-related diseases[3]. ECTV has high genetic similarity to variola virus, and the resulting diseases of these viruses share common features[4]. Additionally, because the natural host for ECTV is the mouse, a commonly used laboratory animal, it is easy to observe the virus directly in its native host[4]. This is significant because a large area of interest in poxvirus research is viral-host interaction[2]. By observing ECTV in mice, mechanisms of viral pathogenesis and host immune defenses have been elucidated. ECTV infections in laboratory mice have also been used to test antiviral drugs and candidate vaccines for multiple orthopoxviruses[4].

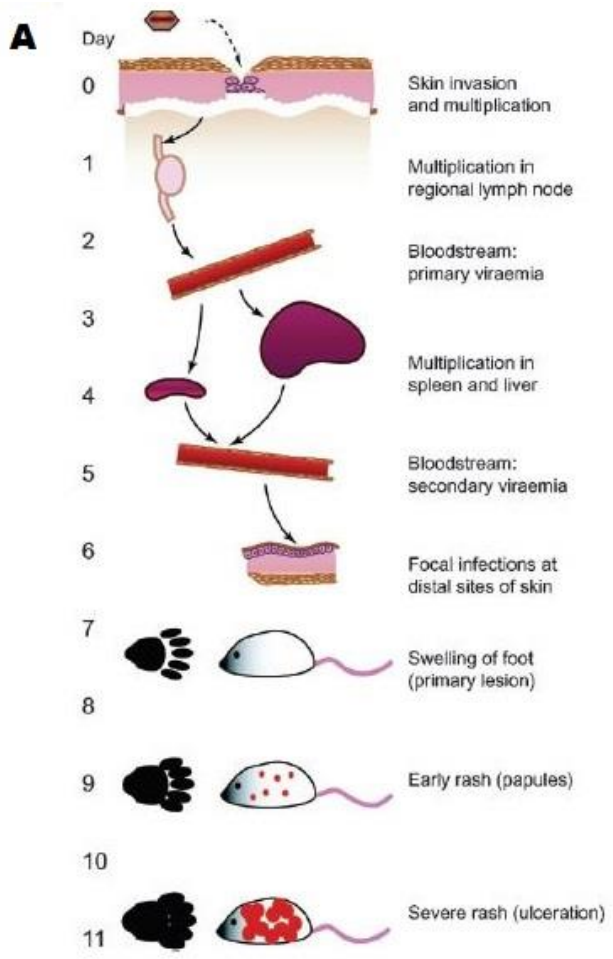

Figure 1. Infection with ectromelia virus. **A)** Spread of ectromelia after entry into the body using the footpad model; **B)** A mouse infected with ectromelia virus showing characteristic lesions of the disease.

Figure adapted from Esteban and Buller (2005) (A) and McFadden (2005) (B).

Clinically, ECTV shows the same general pattern as smallpox in humans[4]. It is generally believed that the virus enters the body through the skin[2,4]. Microscopic abrasions allow the virus access to the dermal and epidermal layers, and the virus spreads from there[2,4] (Figure 1). Therefore, the footpad model, a technique in which virus is injected into the epidermal and dermal layers of a mouse's foot, is the most commonly used technique to infect mice with ECTV[2,4]. From the foot, the virus reaches the draining lymph node located in the knee, and from there, the virus enters the bloodstream causing viremia[2]. The virus then accumulates in the spleen as it filters the mouse's blood[2]. Once inside this organ, the virus continues to replicate and causes tissue death[2,4]. This necrosis liberates the virus from the spleen, and it is released into the bloodstream a second time (secondary viremia) [2,4]. This time, the virus spreads to virtually all

organs in the body, but it replicates most rapidly in the skin which leads to the characteristic lesions associated with poxviruses[4]. From these lesions, it leaves the mouse in order to infect a new host[2,4].

The transmission of ECTV depends on the replication of its virions within host cells[1,2]. In order for any virus to replicate, it must first enter host cells. The mechanism to do so varies between different viruses depending on their structure[1]. ECTV has the same general structure as other poxviruses: brick-shaped virions with unique biconcave cores flanked by lateral protein bodies[2] (Figure 2a). The core and lateral bodies are enclosed in a lipid bilayer membrane which is then surrounded by the viral envelope[2] (Figure 2b). The core of the virus contains the viral genome as well as various enzymes needed for replication[2]. Within the core, the genome is coated with the nucleosome, a protective layer of proteins[1]. Lateral bodies have been shown to contain protein, but their function is unknown[1,2]. The envelope of the virion consists of a lipid bilayer studded with multiple viral proteins[2] (Figure 2b). While the outer membrane of the virus is sufficient for infectivity of the virus, the viral envelope increases infectivity through multiple viral proteins important in host cell attachment and entry[1,2].

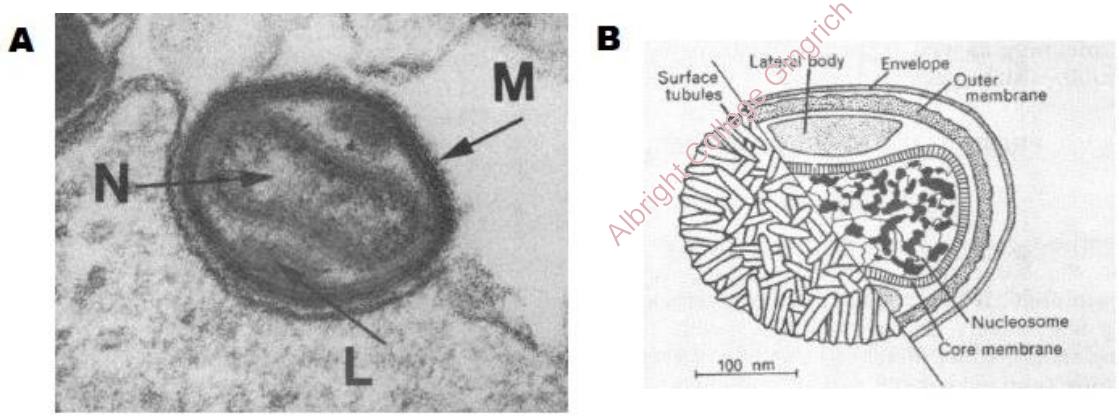

Figure 2. Structural characteristics of poxviruses. **A)** Electron micrograph of a cowpox virion, M = membrane, L= lateral body, N = nucleosome; **B)** Detailed schematic of virion structure. Figure adapted from Buller and Palumbo (1991).

Entry of poxviruses into host cells is facilitated by their viral envelopes[1]. This occurs through membrane fusion with the host cell membrane[1,2]. The envelope of poxvirus virions consists of a lipid bilayer studded with various non-glycosylated viral proteins[6]. The host cell membrane also consists of a lipid bilayer and multiple proteins, so the viral envelope is able to mix with the host cell membrane, creating a pore for the virus to enter through[1,6]. This mixing event is facilitated by receptors and other proteins on the cell surface that facilitate virus attachment[1]. There have been a great number of these proteins found to be important in poxvirus entry, contributing to the large host range of these viruses[2]. Virions attach to these proteins on the cell, bringing them within close proximity to the membrane[1]. This leads to membrane fusion and virus entry into the cytoplasm for replication to occur.

Poxvirus replication is a multi-step process with each step dependent on the preceding step[6]. Most DNA viruses traffic to the cell nucleus to make use of host cell replication enzymes, but poxviruses are able to encode and package their own enzymes[1]. Upon entry, these enzymes are activated within the core, and proteins needed for the replication of the genome are synthesized[1,6]. Once this occurs, the viral genome is replicated. Then, proteins needed for virus assembly are produced from these replicates[6]. The pieces of the poxvirus virions can then assemble and leave the host cell to infect new cells.

The multiple steps of poxvirus replication are an easy target for the immune system. Because each step is dependent on the former, if the virus is inhibited at one step, it will not replicate at all[6]. Therefore, poxviruses have gained different mechanisms to evade immunity[2]. ECTV is a prime example, as it encodes multiple host-response modifiers (HRMs) which disrupt immune system function[4]. Most HRMs are involved in inhibition of cytokine signaling and disruption of receptor-ligand interactions and subsequent signaling pathways[4]. The overall effect

of these disturbances is the attenuation of innate immune function, the part of the immune system that monitors the body for pathogens[4]. This weakened immune surveillance allows the virus to replicate and spread within the host without being slowed down by the immune system[4].

Apart from blocking host defenses against viral infection, poxviruses have also been shown to manipulate their environments in other ways to enhance transmission[2]. One example of this is poxvirus growth factors[2]. Multiple poxviruses have been shown to express proteins with a high degree of similarity to mammalian epidermal growth factor (EGF)[2]. EGF is a hormone that has important roles in cellular pathways leading to proliferation, migration, cell cycle progression, and cell survival[7]. Multiple EGF-like proteins have been discovered, all of which share three main characteristics: high affinity binding to the EGF receptor (EGFR), the presence of six conserved cysteine residues within the primary structure of the protein, and the activation of EGFR signaling pathways upon binding[8]. Most of the effects of EGF and related proteins are related to cell growth and proliferation, making the EGF family a family of mitogenic proteins[9].

Like most protein hormones, EGF induces changes in cells through binding to a receptor protein on the surface of cells[9]. The EGFR is a receptor tyrosine kinase, meaning upon activation, the protein phosphorylates other proteins in order to cause a cellular response[8,9]. The EGFR signaling pathway has been known to involve numerous proteins, leading to many different effects for the cell[7] (Figure 3). Its involvement in the cell cycle and cell proliferation control has made the EGFR an important target for cancer therapy, but the discovery of viral growth factors has made EGFR signaling an area of interest for virus biology as well[10].

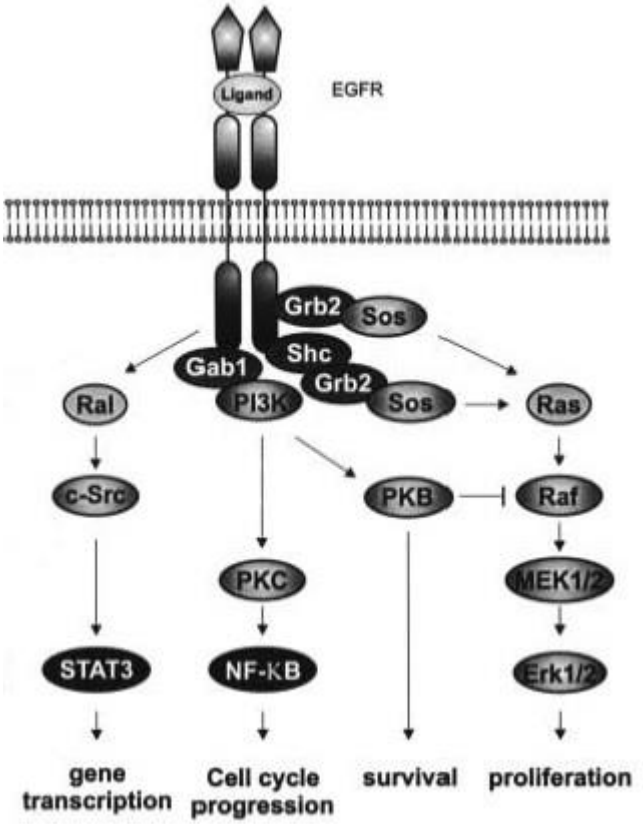

Figure 3. Signaling pathways of the epidermal growth factor receptor. Figure adapted from Prenzel et al. (2001)

EGF homologs have been found in the genomes of all sequenced orthopoxviruses and leporipoxviruses[3]. The functional role of these proteins in viral infections has not been fully elucidated, but there are three main theories that have been suggested[2,11,12]. The first and probably most intriguing theory for the function of viral growth factors is that the EGF homologs cause upregulation of growth and metabolic activity in uninfected cells surrounding the primary site of infection, therefore facilitating the spread of infection and improving the efficiency of replication[2]. One pathway activated by EGFR is the MAP kinase pathway[9]. This pathway is important for the transition of the cell into S-phase, the phase of the cell cycle in which DNA replication occurs[10]. In this phase, there are higher amounts of critical macromolecules inside the cell including nucleotides, the building blocks of DNA[10]. Nucleotides are one of the major

macromolecules needed for viral replication, so a cell with higher amounts of nucleotides would in theory increase the efficiency of viral replication and therefore facilitate viral transmission[2].

This model also explains the proliferative nature of some poxvirus lesions[2,11]. To date, this possibility has only been studied in leporipoxviruses, such as Shope Fibroma virus (SFV) and myxoma virus[2,11]. Many studies have been conducted using malignant rabbit virus (MRV), which is a hybrid virus of SFV and myxoma virus[11]. The formation of fibromas (areas of increased cell proliferation, similar to a tumor) were observed upon infection with MRV[11]. This prompted researchers to analyze the genome of MRV to discover which genes were causing this effect[11]. It was found that MRV includes a growth factor gene originating from SFV, and it was concluded that this gene most likely led to the formation of fibromas upon infection[11]. More work is being done with other poxviruses known to have proliferative lesions in order to confirm this theory[11].

A second theory that has been proposed involves the precursor molecule of EGF[2]. EGF is a secreted protein, so it leaves the cell in which it was synthesized to act on neighboring cells[8]. However, EGF must mature from its precursor molecule, prepro-EGF, before it can be exported from the cell[8]. Prepro-EGF contains a hydrophobic sequence near its C terminus which is a common characteristic of transmembrane proteins[8]. Therefore, it is hypothesized that EGF can take both secreted and transmembrane forms[8]. This hypothesis is also made for the EGF homologs found in poxviruses, and it has been suggested that the membrane-bound forms of the growth factors could aid in viral attachment and entry into host cells[8,12]. It is thought that a transmembrane form of the growth factor would reside in the envelope of the virus, and it would attach to cells bearing the EGFR[12]. Thus, the virus would be effectively attached to the host cell,

facilitating viral entry[12]. However, this theory has been disputed, and no clear evidence has been presented thus far[2,12].

The final theory for the function of growth factors during poxvirus infections deals with the host immune response to infection[13]. A defense mechanism used by cells to combat viral infections is the induction of apoptosis, or programmed cell death[1,13]. Through this mechanism, infected cells are destroyed, effectively halting viral replication and spread. In response to this, many viruses have evolved to inhibit apoptosis in their host cells[13]. Poxviruses have been shown to express homologs of anti-apoptotic Bcl-2 proteins, such as the F1L protein in vaccinia virus that works to inhibit mitochondrial-dependent apoptosis[13]. The overall effect of these proteins is cell survival, so viruses can continue to use their cellular machineries to replicate and spread[13].

Interestingly, one downstream effect of EGFR signaling is also cell survival[7]. This led Postigo et al. (2009) to investigate if the two mechanisms could be related in vaccinia virus. They synthesized a mutant virus that lacks both F1L and growth factor proteins. When they infected cells with this virus, they saw an increased amount of cell death in comparison to the wild-type virus. When each protein was added back in, there was a lower amount of apoptosis, indicating that both F1L and the vaccinia growth factor are important for cell survival during vaccinia virus infection. Additionally, they demonstrated that signaling through the EGFR was needed to achieve maximum cell survival, thus indicating the role of the growth factor homolog binding in the inhibition of apoptosis. This data, along with that of other studies, shows that viral growth factors enhance cell survival during infection, leading to a more favorable environment for viruses to replicate[13].

The most extensively studied poxvirus growth factor has been vaccinia virus growth factor (VGF). This protein was discovered through a computer-aided analysis of the vaccinia

virus genome[14]. It was shown to have similar structural characteristics to EGF, including the six conserved cysteine residues found in EGF-related proteins[14]. In competitive binding assays, it was shown that VGF competes with EGF for binding to the EGFR[14]. It was also observed that the presence of this protein leads to the phosphorylation of tyrosine residues on the EGFR, indicating VGF is capable of activating EGFR signal pathways[15]. Later studies with vaccinia virus showed that upon deletion of the VGF gene, the virus formed smaller plaques *in vitro* and had a decreased virulence *in vivo*[16]. It was also shown that VGF$^-$ vaccinia virus lead to a smaller amount of cell proliferation than the wild-type virus[12]. Altogether, these studies show the role of VGF in EGFR activation and cell proliferation in vaccinia virus infections, and these findings set precedents for future studies with other viral growth factors.

In the early 2000's, the genome of ECTV was sequenced, revealing a gene with high sequence homology to VGF[3]. Termed open reading frame 10.5 (ORF 10.5), this gene shares 78% DNAsequence identity with the gene for VGF[3] (Figure 4). The predicted gene product of ORF 10.5 is about 65 amino acids long[3]. Although the protein is shorter than the 77 amino acid VGF, the ECTV protein may still retain function because the protein maintains the six highly conserved cysteine residues important for the structure of EGF homologs[3] (Figure 4). Therefore, this protein is predicted show the same experimental characteristics as VGF, including binding to the EGFR, triggering tyrosine phosphorylation, and inducing cell proliferation. In this study, we aim to show the mitogenic effect of infection with ECTV by a secreted viral factor.

Figure 4. Sequence alignment of VACV and ECTV growth factors. Black shading indicates identical amino acids between the two polypeptides and asterisks denote conserved cysteine residues.

Materials and Methods

Cell Culture

In this study, two immortal cell lines were utilized: M2-10B4 and BS-C-1. M2-10B4 is an immortal cell line derived from the bone marrow of mice, where BS-C-1 is another immortal cell line derived from monkey kidney cells. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin. Cultures were maintained at 37°C in a 6% CO_2 incubator and split weekly.

Viruses

Four viruses were used throughout the course of this study: wild-type ECTV, wild-type vaccinia (VACV), and vaccinia with a deletion in the VGF gene (VACVΔVGF, gift of Dr. Bernard Moss). Additionally, we constructed a mutant ectromelia virus with a green fluorescent protein (GFP) gene in place of the growth factor gene, termed mousepox growth factor (ECTVΔMGF), using previously described methods[17]. A plasmid containing the sequence for GFP with a poxviral p7.5 promoter flanked by the ECTV sequence from either side of the growth factor gene was synthesized by Invitrogen (Figure 5a). The plasmid was transfected into BS-C-1 cells, which were subsequently infected with ECTV in order for homologous recombination to occur. After this initial infection/transfection, one viral plaque expressing GFP was isolated and underwent four rounds of passage. At this point, 100% of plaques from the virus expressed GFP, indicating full recombination of the virus. The region in the viral genome was amplified using PCR and analyzed using agarose gel electrophoresis (Figure 5b). The region was also sequenced to confirm the presence of GFP (data not shown) and deletion of the desired viral sequence. All viruses used in this study were quantified using BS-C-1 cells.

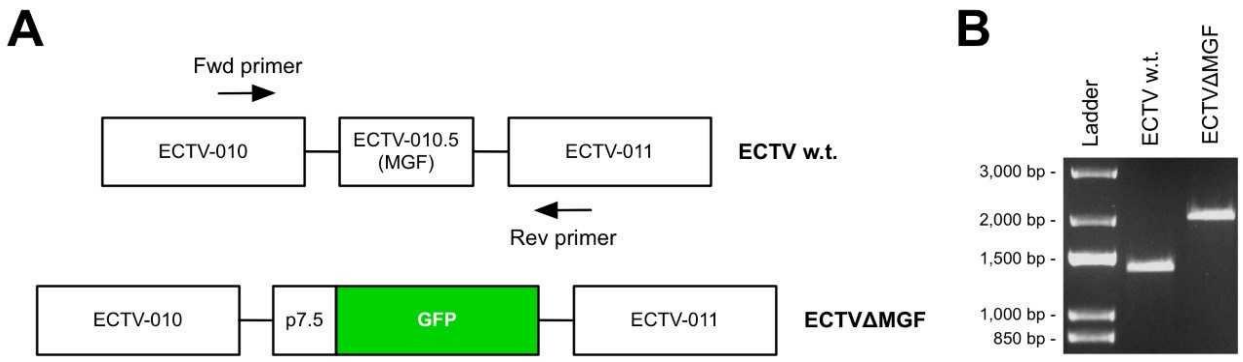

Figure 5. Construction of a mutant ectromelia virus lacking the gene for mousepox growth factor. **A)** Schematic comparing the genetic sequence of wild-type ECTV to ECTVΔMGF. The gene was replaced with the gene for GFP preceded by a p7.5 promoter through homologous recombination. **B)** Comparison of ORF 10.5 region of wild-type ECTV and ECTVΔMGF through agarose gel electrophoresis. DNA from both viruses were extracted and the ORF region was amplified using PCR. The resulting PCR products were run on a 1% agarose gel and visualized using ethidium bromide.

Fluorescence Microscopy

5-ethynyl-2'-deoxyuridine (EdU) assays were utilized in this study to assess the mitogenic effects of ECTV. The assays were performed using the Click-iT™ EdU Imaging Kit from Invitrogen. Cells were grown in four-well chamber slides and infected with ECTV, VACV, ECTVΔMGF, or VACVΔVGF for the indicated time. EdU was added to the cells and the mixture was allowed to incubate for 1 hour. The cells were then fixed with 5% formalin for 15 minutes at room temperature. After a 1x PBS wash, the cells were incubated in a Triton™ X-100 solution (0.5% in 1x PBS) for 20 minutes at room temperature to permeabilize. After a wash with 1x PBS and 3% bovine serum albumin (BSA) in PBS, 1 mL of the EdU reaction mixture (EdU reaction buffer, copper sulfate, Alexa Fluor azide 594, and EdU buffer additive; prepared according to Invitrogen protocol) were added to the slides and they were allowed to incubate at room temperature in the dark for 30 minutes. The slides were then washed in 1x PBS and water, dried, and mounted using ProLong Gold antifade reagent with DAPI.

In some of the experiments, the cells were stained for the viral protein B5 to track the location of the virus in addition to the EdU assay. In these cases, the slides were placed in blocking buffer (2% BSA and 2% FBS in 1x PBS) for 30 minutes following incubation with the EdU reaction mixture. Then anti-B5 monoclonal antibody diluted in blocking buffer was added to the slides and the cells were allowed to incubate for 30 minutes. Following a 1x PBS wash, Alexa Fluor 488 secondary antibody diluted in blocking buffer was added to the cells, and they were allowed to incubate for an additional 30 minutes. The slides were then washed with 1x PBS and water, dried, and mounted with DAPI. All slides were allowed to set overnight before visualization. Fluorescence microscopy was done using a Zeiss Axiostar microscope equipped with an Optronics camera system. Captured images were processed using ImageJ software.

Flow Cytometry

Cells were prepared for flow cytometric analysis using the Click-iT™ Plus EdU Flow Cytometry Assay Kit from Invitrogen. Cultured cells were washed with 1% BSA in PBS, then incubated with Click-iT™ fixative for 15 minutes in the dark. After a BSA wash, the cells were permeabilized in 1X saponin-based Click-iT™ permeabilization buffer for 15 minutes. The cells were then incubated with EdU reaction mixture (1X PBS, copper protectant, Fluorescent dye picolyl azide, and reaction buffer additive) for 30 minutes protected from light. The cells were then washed with 1X saponin-based Click-iT™ permeabilization buffer and suspended in 1% BSA in PBS for flow cytometric analysis. All flow cytometry readings were obtained using a FACSCalibur flow cytometer.

Results

In a recent study by Schmidt et al. (2015), EdU assays were used to analyze the DNA synthesis of cells infected with herpes simplex virus (HSV)[18]. EdU is an analog of thymidine, one of the four bases of DNA[19]. When cells enter S-phase, they begin to replicate their DNA, and when EdU is present, it will be incorporated into newly synthesized DNA[19]. From there, EdU can be labelled through 'click chemistry' reactions with fluorescent azide molecules, effectively labelling all cells in S-phase[19]. In a similar fashion to Schmidt et al. (2015), we analyzed cells infected with ECTV, VACV, and VACVΔVGF using EdU assays.

We began by analyzing VACV-infected cells using fluorescence microscopy. BS-C-1 and M2-10B4 cells were grown to confluency and infected with VACV for two days. Upon analysis with fluorescence microscopy, we found that VACV infection induced an upregulation of DNA synthesis in surrounding cells (Figure 6). There were increased levels of EdU staining in cells infected with VACV in comparison to the mock-infected cells. Similar assays were conducted using 3T3, L929, HFF, and A431 cells (data not shown), but the results were inconclusive.

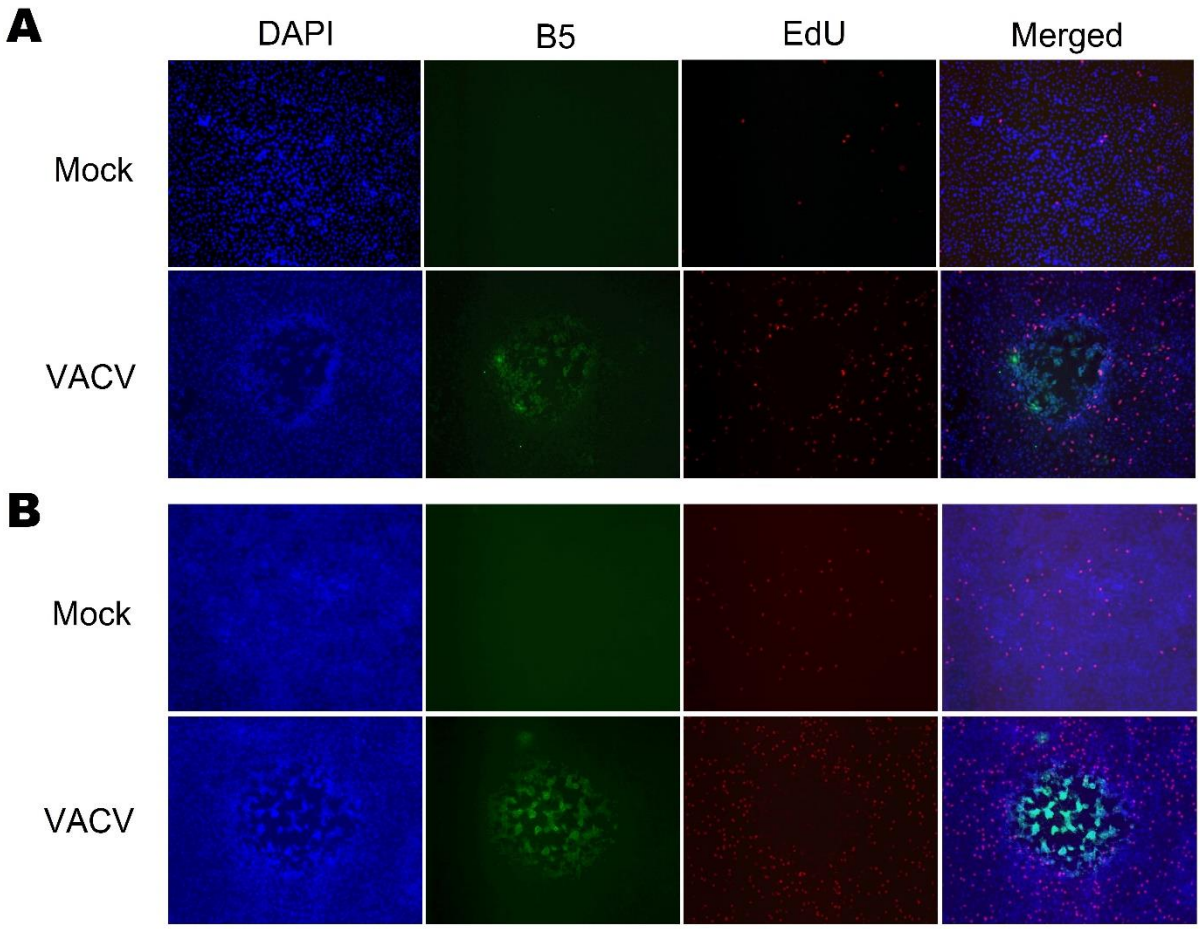

Figure 6. Vaccinia infection induces an upregulation of DNA synthesis. BS-C-1 cells **(A)** and M2-10B4 cells **(B)** were infected with wild-type VACV and were stained two days post-infection using an EdU assay, immunofluorescence against the viral protein B5, and DAPI. All images were taken under 50x magnification.

We next examined the effect of ECTV infection on DNA synthesis. M2-10B4 cells and BS-C-1 cells were infected with wild-type ECTV, and two days post-infection, fluorescence microscopy was used to analyze the levels of EdU staining (Figures 7a and 8a). Both cell lines experienced an upregulation of DNA synthesis after ECTV infection, as shown by the EdU stains. The number of EdU-positive cells in each cell type were counted over 3 independent trials and the average percentage of EdU-positive cells was calculated (Figures 7b and 8b). After an ANOVA was performed, it was found that there was a significant increase in the amount of

EdU-positive cells after infection with ECTV in both cell types (BS-C-1, P= 0.0074; M2-10B4, P= 0.00035).

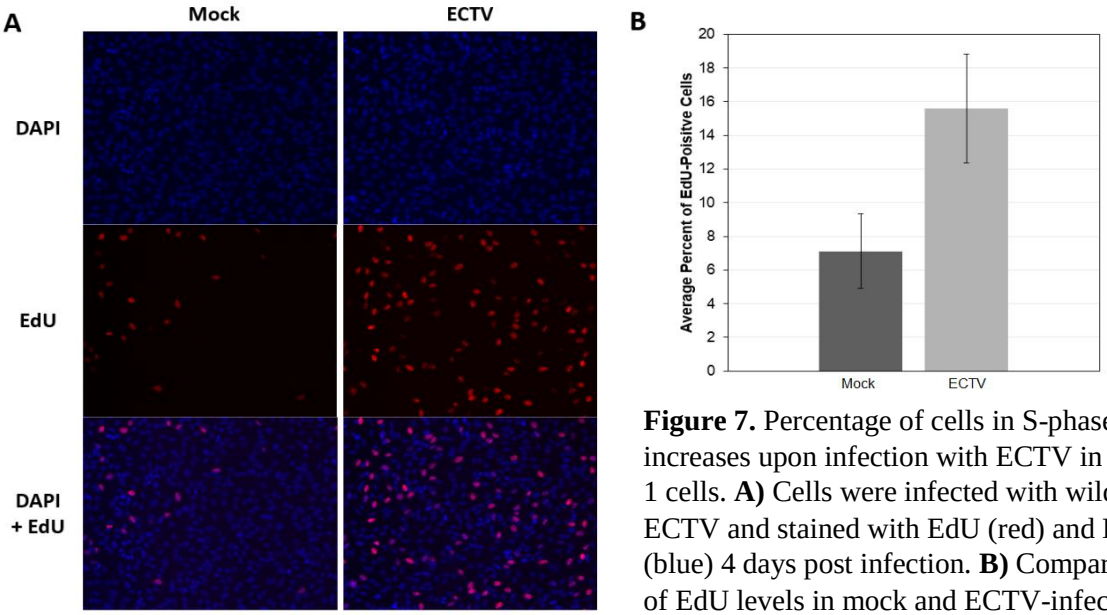

Figure 7. Percentage of cells in S-phase increases upon infection with ECTV in BS-C-1 cells. **A)** Cells were infected with wild-type ECTV and stained with EdU (red) and DAPI (blue) 4 days post infection. **B)** Comparison of EdU levels in mock and ECTV-infected cells in BS-C-1 cells. All images were taken under 100x magnification. Error bars represent standard deviation.

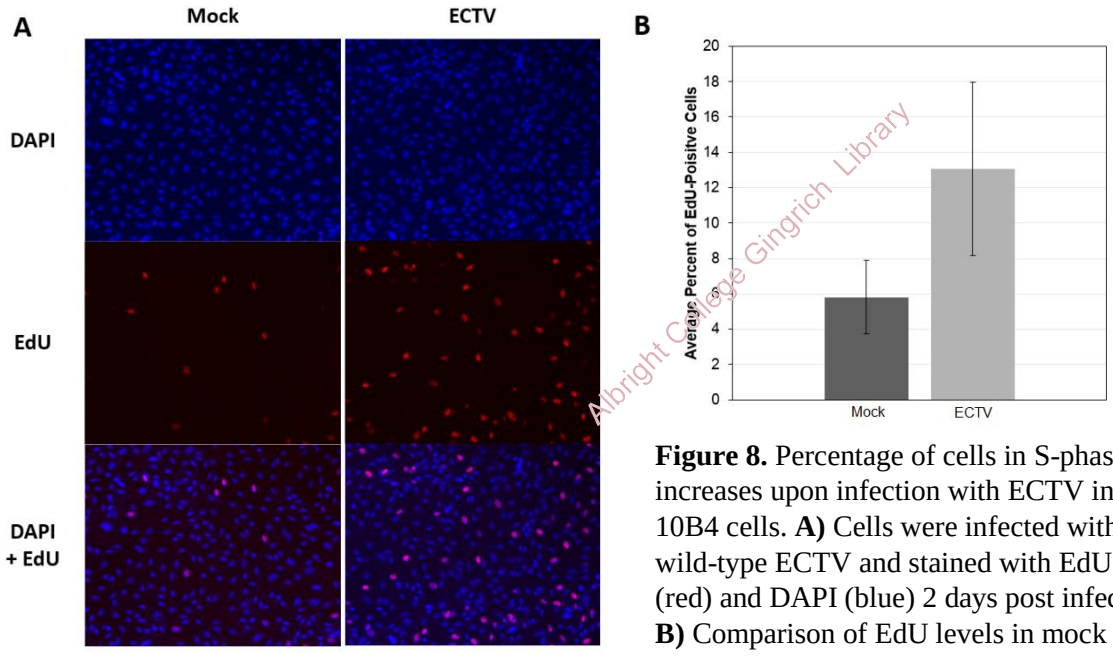

Figure 8. Percentage of cells in S-phase increases upon infection with ECTV in M2-10B4 cells. **A)** Cells were infected with wild-type ECTV and stained with EdU (red) and DAPI (blue) 2 days post infection. **B)** Comparison of EdU levels in mock and ECTV-infected cells. All images were taken under 100x magnification. Error bars represent standard deviation.

To obtain a more quantitative measurement of DNA synthesis in infected cells, we performed flow cytometry using ECTV- and VACV-infected cells. M2-10B4 cells and BS-C-1 cells were infected with each virus, and two days post-infection, they were stained and analyzed via flow cytometry. Only cells that were not directly infected were analyzed (Figures 9a and 10a). It was observed that both ECTV-infected and VACV-infected cells had a higher percentage of EdU-positive cells than the mock-infected cells (Figures 9b and 10b). However, the VACV-infected cells still showed higher levels than their ECTV-infected counterparts. Furthermore, the M2-10B4 cells showed a larger difference in percentages between infected and mock-infected cells than the BS-C-1 cells (Figures 9b and 10b).

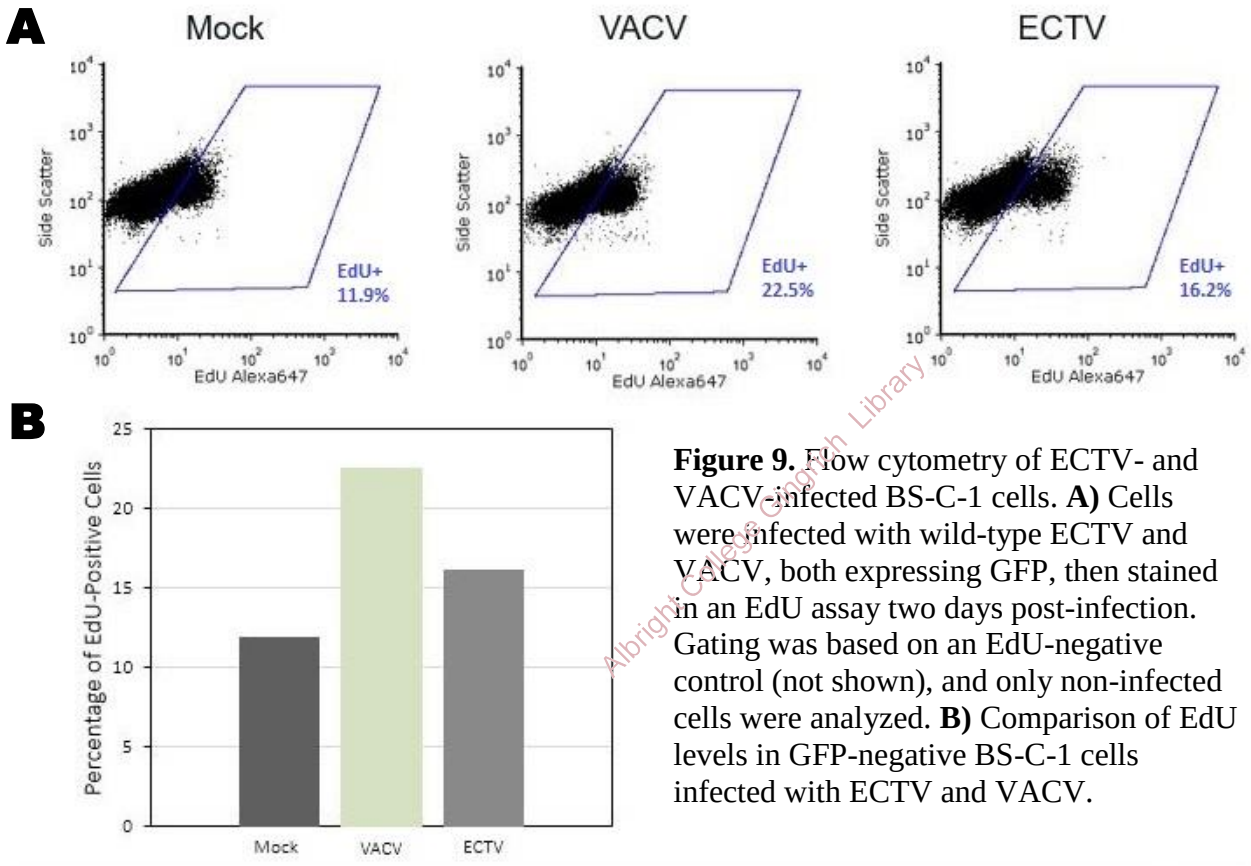

Figure 9. Flow cytometry of ECTV- and VACV-infected BS-C-1 cells. **A)** Cells were infected with wild-type ECTV and VACV, both expressing GFP, then stained in an EdU assay two days post-infection. Gating was based on an EdU-negative control (not shown), and only non-infected cells were analyzed. **B)** Comparison of EdU levels in GFP-negative BS-C-1 cells infected with ECTV and VACV.

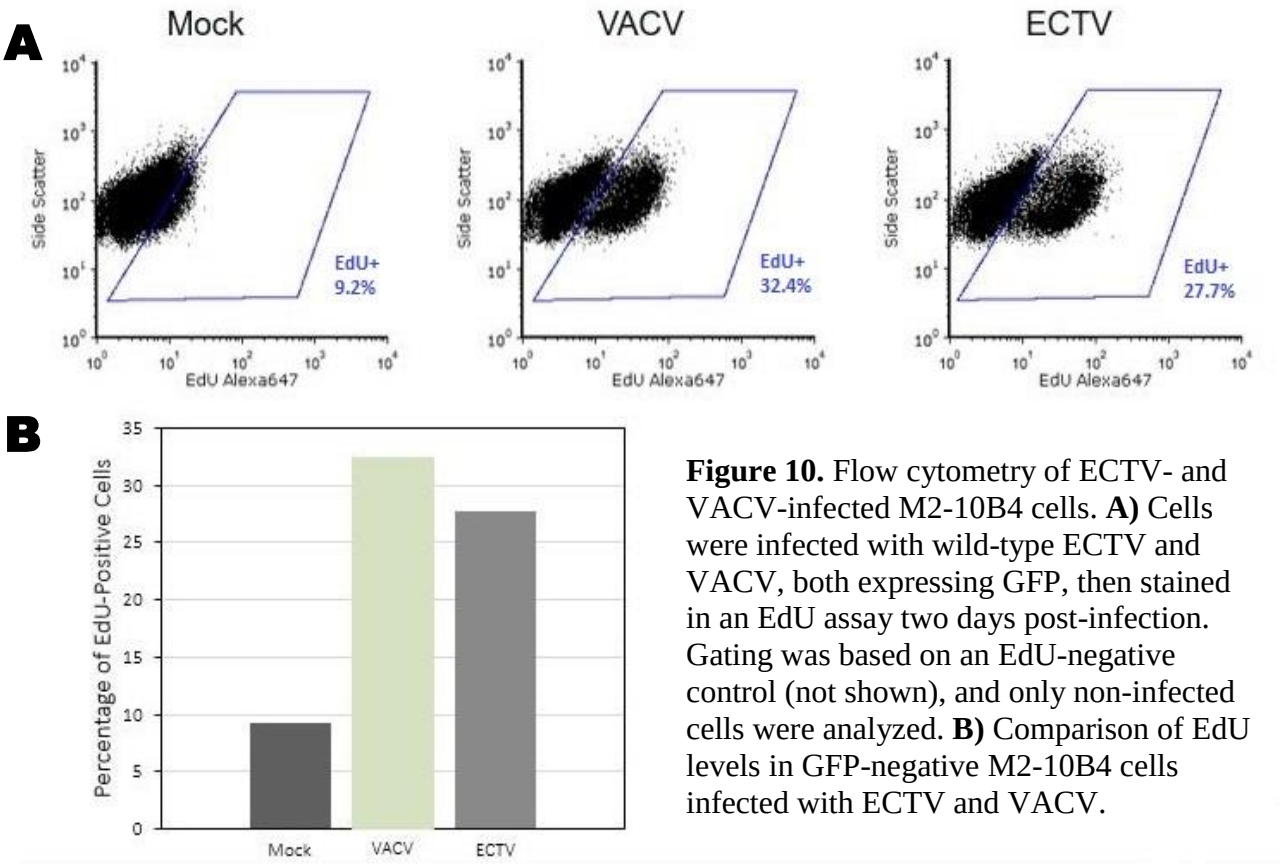

Figure 10. Flow cytometry of ECTV- and VACV-infected M2-10B4 cells. **A)** Cells were infected with wild-type ECTV and VACV, both expressing GFP, then stained in an EdU assay two days post-infection. Gating was based on an EdU-negative control (not shown), and only non-infected cells were analyzed. **B)** Comparison of EdU levels in GFP-negative M2-10B4 cells infected with ECTV and VACV.

To better investigate the function of the growth factors expressed by poxviruses, cells were infected with VACVΔVGF. For VACVΔVGF infection, BS-C-1 cells were infected with VACVΔVGF and wild-type VACV and assayed two days post-infection. Fluorescence microscopy revealed that, once again, VACV infection led to increased levels of EdU compared to the control (Figure 11). However, VACVΔVGF infection had little to no effect on DNA synthesis in the cells (Figure 11). Furthermore, there appears to be more EdU labelling in the mock-infected cells than in the VACVΔVGF-infected.

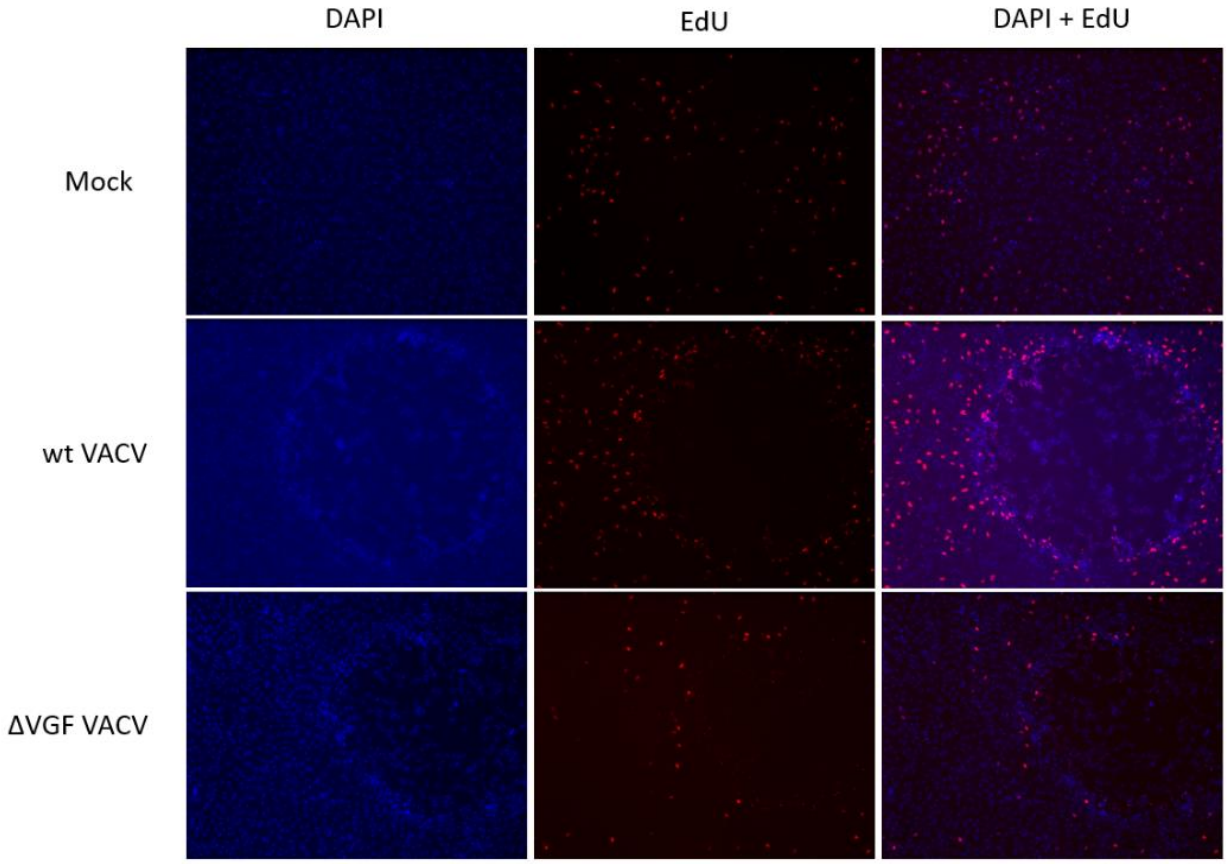

Figure 11. Infection with wild-type VACV induces increased levels of DNA replication in BS-C-1 cells, but infection with VACVΔVGF does not. Cells were infected with VACV or VACVΔVGF and stained with EdU (red) and DAPI (blue) 4 days post infection. All images taken under 50x magnification.

Discussion

Multiple poxviruses have been shown to encode growth factor homologs in order to enhance replication and spread[2]. ECTV has been shown to include a gene with high sequence homology to other viral growth factors, but it is unknown if this gene is functional[3]. In this study, we have demonstrated the possible mitogenic effect of a growth factor encoded by ECTV (mousepox growth factor, MGF) during infection.

Because vaccinia virus has been shown to encode a functioning viral growth factor and MGF is closely related to VGF, we used it as a positive control in our experiments[3,12]. We

observed an overall increase in DNA synthesis with VACV infection, indicating a functional viral growth factor increases levels of EdU. These findings are consistent with those of Schmidt et al. (2015) in which they saw an increase in EdC (an EdU-related compound) levels upon infection with herpes simplex virus, indicating the virus had a functioning viral growth factor[18]. Therefore, it can be concluded analysis of possible mitogenic effects of viruses through EdU assay is a valid method.

Our results with VACV also provide an additional means to characterize VGF during VACV infection. Not only is this virus an important vaccine strain for smallpox but it has also been recently studied for its possible applications for cancer therapy[2,20]. VGF stimulates the MAP kinase pathway, which is also utilized by cancer cells for their replication and spread throughout the body[7]. Therefore, vaccinia virus has been engineered to target cancer cells through the use of this pathway[20]. The process for such alteration involves manipulation of the VGF gene, so it is important to have an easy and accurate characterization of gene function[20]. Our findings suggest EdU analysis may be a candidate.

When ECTV-infected cells were subjected to EdU analysis, we observed an increase in DNA synthesis in the cells. This indicates ECTV infection has a mitogenic effect on cells. However, the increase in S-phase cells was only seen in cells between viral plaques, where infection with VACV increased the number of cells in S-phase in the immediate vicinity of the plaques. This could be due to the truncation of MGF in comparison to VGF, making MGF a less potent growth factor. More work will need to be done on the structural and biochemical properties of MGF in order to more fully understand the biology of this protein. Nonetheless, our findings show a mitogenic effect from ECTV infection which has not been previously demonstrated.

In this study, we also describe the construction of a virus lacking the MGF gene, ECTVΔMGF. Advances in molecular techniques have made viral gene knockout an almost standard method for investigating the impact of certain genes on viral replication and pathogenesis[17]. With poxviruses, this method has been used to identify genes whose functions enable viruses to manipulate their host environment[17]. Therefore, we constructed the ECTVΔMGF virus in order to more fully understand the role of MGF in ECTV infection. Future work with the virus include comparison of wild-type ECTV and the mutant in similar EdU assays as shown here, as well as the construction of a revertant virus.

All of our work has been *in vitro*, but an observation of MGF *in vivo* is still needed. In studies with vaccinia virus, infection was observed in rabbits and chick embryos in order to fully elucidate the effects of VGF[12]. Because mice are the natural host for ECTV, it is more convenient to observe the effects of MGF and its interactions with the host. Additionally, a comparison of wild-type ECTV with ECTVΔMGF in mice would further our understanding of MGF and its role in ECTV infection in its natural host.

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