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Identification of host range mutants of myxoma virus with altered oncolytic potential in human glioma cells

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The authors have recently demonstrated that wild-type myxoma virus (MV) tagged with gfp (vMyxgfp) can generate a tumor-specific infection that productively infects and clears human tumor-derived xenografts when injected intratumorly into human gliomas transplanted into immunodeficient mice (Lun *et al*, 2005). To expand the understanding of MV tropism in cancer cells from a specific tissue lineage, the authors have screened a series of human glioma cells (U87, U118, U251, U343, U373) for myxoma virus replication and oncolysis. To assess the viral tropism determinants for these infections, the authors have screened myxoma virus knockout constructs that have been deleted for specific host range genes (M-T2, M-T4, M-T5, M11L, and M063), as well as a control MV gene knockout construct with no known host range function (vMyx135KO) but is highly attenuated in rabbits. The authors report wide variation in the ability of various vMyx-hrKOs to replicate and spread in the human glioma cells as measured by early and late viral gene expression. This differential ability to support vMyx-hrKO productive viral replication is consistent with levels of endogenous activated Akt in the various gliomas. The authors have identified one vMyx-hrKO virus (vMyx63KO) and one nonhost range knockout construct (vMyx135KO) that appear to replicate in the gliomas even more efficiently than the wild-type virus and that reduce the viability of the infected gliomas. These knockout viruses also inhibit the proliferation of gliomas in a manner similar to the wild-type virus. Together these data, as well as the fact that these knockout viruses are attenuated in their natural hosts, may represent environmentally safer candidate oncolytic viruses for usage in human trials. *Journal of NeuroVirology* (2007) **13,** 549–560.

Keywords: glioblastoma; host range; poxvirus; tropism; virotherapy

Introduction

Oncolytic viruses are defined as those viruses that can infect, selectively replicate, and kill cancer cells while at the same time sparing normal cells (Liu *et al*, 2007; Merrill *et al*, 2006; Roberts *et al*, 2006; Woo *et al*, 2006). Naturally oncolytic viruses have been identified in both RNA and DNA viral families, including vesicular stomatitis virus and adenovirus, and are in varying stages of development for oncolytic virotherapy in man (Roberts *et al*, 2006; Woo *et al*, 2006).

Myxoma virus (MV) is a poxvirus that productively infects only rabbits in the wild. Our laboratory has

demonstrated that MV can also infect and spread in a variety of nonleporid cell lines derived from other mammalian species in vitro. More surprising still was the observation that MV could productively infect and kill the majority (15 of 21) of human cancer cell lines from the reference NCI-60 panel (Sypula *et al*, 2004). The same study demonstrated that the permissive human cancer cells could be separated into two groups: those that required the expression of the MV host range protein, M-T5 (type II) and those cancer cells for which the expression of M-T5 by MV was not required (type I). We later demonstrated that the requirement of the M-T5 ankyrin-repeat host range protein was based on the ability of M-T5 protein to bind, and activate, host cell Akt (Wang *et al*, 2006). This interaction between cellular Akt and the MV host range protein M-T5 was critical for infection of type II cancer cells and was similar to the ability of cellular PIKE-A to bind and activate the kinase activity of Akt (Werden *et al*, 2007). Recently, we have also shown that manipulation of the cellular Akt signaling pathway, for example with the drug rapamycin, also improves the oncolytic potential of MV in type II human cancer cells (Stanford *et al*, 2007).

Malignant glioblastomas are human brain cancers that have proven particularly refractory to treatment and generally result in death within a year of diagnosis (King *et al*, 2005; Merrill *et al*, 2006; Wollmann *et al*, 2005). Recently, an enhanced green fluorescent protein (EGFP)–tagged wild-type version of MV (vMyxgfp) was shown to exhibit significant antitumor activity against experimental gliomas grafted into mice (Lun *et al*, 2005). The majority of human gliomas tested (seven of eight) were fully permissive for vMyxgfp replication and were efficiently killed as a consequence of the viral infection. As well, intracerebral injections of vMyxgfp into control mice were well tolerated and produced only minimal inflammatory changes. Finally, orthotopic xenografts of human glioma cells into nude mice were used to test MV efficacy. Most animals (92%) injected with vMyxgfp were "cured" and survived to the end of the study (>130 days) (Lun *et al*, 2005).

Recent analysis of viral mutants has demonstrated improved oncolytic potential (Gaddy and Lyles, 2007; Stojdl *et al*, 2003; Wollmann *et al*, 2007). We have generated a growing collection of tagged wild-type MV and targeted gene knockout variants based on the parental Lausanne strain of MV, a normally rabbit-specific poxvirus. Some of these gene knockout viruses were also shown to exhibit host range defects in specific classes of rabbit cells and most are dramatically attenuated for myxomatosis when tested in the susceptible host, the European rabbit (Johnston and McFadden, 2004). In an attempt to evaluate these attenuated MV candidates as possible viral oncolytics, we have selected a series of these constructs (Table 1) to screen their ability to infect and kill human glioma cells in the hope of identifying safer MV oncolytic candidates. This collec-

tion of test viruses represents two controls (vMyxlac and vMyxgfp) that express markers under either the vaccinia synthetic early/late promoter (vMyxgfp) or the vaccinia late P11 promoter (vMyxlac). The gene knockout viruses all express either β-galactosidase or enhanced green fluorescence protein (EGFP; Table 1). The most extreme gene knockout in this panel of viral constructs is vMyx63KO, which is unable to replicate in any rabbit cell type tested to date and is completely nonpathogenic in rabbits (Barrett *et al*, 2007a). The other vMyx-hrKOs include vMyxT2KO, which is defective in the viral tumor necrosis factor (TNF) receptor homolog (Upton *et al*, 1991); vMyxT4KO, which is defective in an endoplasmic recticulum (ER)–localized protein important for apoptosis regulation (Barry *et al*, 1997); vMyxT5KO, which lacks an ankyrin-repeat host range protein (Mossman *et al*, 1996); and vMyxM11LKO, which lacks a key apoptosis regulator that localizes to the mitochondria (Opgenorth *et al*, 1992). These latter MV gene knockout constructs are all tissue-specific host range mutants that have lost the ability to productively replicate in rabbit T lymphocytes (RL5 cells). In infected European rabbits, the progression of myxomatosis is highly attenuated and the levels of virulence reduction differ according to the mutant (Johnston and McFadden, 2004). vMyx135KO (Barrett *et al*, 2007b) is used as a control attenuated MV that is defective in a cell-surface viroceptor, and functions as a virulence factor in virus-infected rabbits but is not yet known to exhibit any host-range defect in cultured cells.

An ideal live oncolytic virus candidate is one that is not pathogenic to any vertebrate species, including man. In this study, we screen five human glioblastoma cell lines (U87, U118, U251, U343, U373) with a variety of myxoma viruses in which a single viral gene has been specifically deleted (Johnston and McFadden, 2004). These gene knockout viruses were screened in vitro for their ability to infect, replicate, and kill the human glioma cell lines. We report that several of the host range gene knockout viruses (vMyx-hrKOs), which were originally shown to be defective for replication in subsets of rabbit cells, were also unable to establish a productive infection in some, or all, of the tested human glioblastomas. This result is in contrast to what was previously observed in human cancer cells from other nonglioma tissue origins (Sypula *et al*, 2004) and may shed light on unique intracellular signaling properties of human glioblastomas. Surprisingly, several vMyx-hrKOs appeared to be as effective as vMyxgfp at infecting and killing human glioblastoma cells. Because vMyx-hrKO variants also exhibited an attenuated *in vivo* phenotype in rabbits, they represent a safer alternative to wild-type MV as an oncolytic therapeutic in settings where the virus might come into contact with susceptible rabbits. This study also points the direction for future work on the oncolytic potential of vMyxhr-KO viruses for other human cancers.

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Table 1 Description of the myxoma virus host range mutants and controls

Marker	Virus name	Deleted gene	Lost gene function	Host range restriction	Reference
Viruses express beta-galactosidase	vMyxlac	None	Marked wildtype virus, β-gal under VV late promoter	None, control	Opgenorth *et al*, 1992
	vMyxT2KO	M-T2	Binding of rabbit TNF; inhibition of apoptosis	RL-5	Upton *et al*, 1991
	vMyxT4KO	M-T4	ER retention	RL-5	Barry *et al*, 1997
	vMyxT5KO*	M-T5	Binding partner for cullin-1 and activates cellular Akt	RL-5	Mossman *et al*, 1996; Johnston *et al*, 2005; Wang *et al*, 2006
	vMyxM11LKO	M11L	Inhibitor of mitochondrial dependent apoptosis	RL-5	Opgenorth *et al*, 1992; Everett *et al*, 2000; Wang *et al*, 2004; Su *et al*, 2006
Viruses express fluorescent marker	vMyx63KO	M063	Critical for MV replication in rabbit cells	All rabbit cells	Barrett *et al*, 2007a
	vMyx135KO	M135	Critical virulence factor	ND	Barrett *et al*, 2007b
	vMyxgfp	None	Marked wildtype virus, EGFP under VV synE/L promoter	None, control	Mansouri *et al*, 2003

ND, not determined.

*vMyxT5KO is also available in an EGFP-expressing version.

Results

Myxoma viruses with targeted gene knockouts exhibit variable levels of replication efficiency in human glioma cells

To test the ability of various myxoma virus gene knockout constructs to infect and kill human glioma cells, we first began by screening the ability of the various viruses (Table 1) to productively replicate in the panel of gliomas. The infectivity of each virus was quantified by single-step growth yields on the various glioma cells and compared to control baby green monkey kidney (BGMK) cells, which are fully permissive for all the knockout constructs used in this study. Viral titers derived from the gliomas were compared to those obtained on the control BGMK cell line and the viral replication efficiency was calculated. Some of the tested viruses replicated to levels several logs lower than that observed for the control cell line (BGMK) (data not shown). In an effort to evaluate the "relative" replication efficiency of each virus in a specific glioma, we calculated the progeny viral titer relative to the observed titer of vMyxgfp for that specific glioma. This meant that the titer of vMyxgfp was defined to be 100% in any particular glioma cell line and the various viruses were graphed as a percent value relative to their replication compared to vMyxgfp (Figure 1). In all of the gliomas, except U118, the knockout virus vMyx63KO replicated to higher titers and was more replication competent than other knockouts or the wild-type controls. Also, some glioma lines (U87, U343, U373) supported more robust replication levels than the other glioma cells tested (data not shown). The glioma cell lines U343 and U373 (Figure 1, lanes 4 and 5) allowed for detectable levels of replication of all viruses except for vMyxT5KO (both EGFP and β-gal versions) and vMyxT4KO relative to vMyxgfp (Figure 1). The least permissive glioma cell for all viruses tested was the U118 cell line (Figure 1, lane 2). In U118 cells, the most efficient virus was the control wildtype virus, with vMyx63KO, vMyx135KO, and vMyxM11LKO replicating to detectable levels (Figure 1). Virus replication efficiency was also very low for U251 (Figure 1, lane 3) cells, with all viruses replicating to levels $100\times$ below what was measured on the control BGMK cells. However, when we correct for the relative efficiency against vMyxgfp replication, we find that only vMyxT5KO (both EGFP and β-gal versions) and vMyxT4KO were below detectable levels and that vMyx63KO replicated to titers 2x as high as vMyxgfp (Figure 1).

Some vMyx-hrKOs are unable to transit from early to late gene expression

A rapid measure of poxvirus initiation of the viral replication cycle is early viral gene expression, which begins following virus entry and uncoating. For these studies we used an antibody against M-T7, the viral interferon-γ receptor (IFN-γR) homolog that is expressed robustly and secreted at early times post infection (Upton *et al*, 1992). For late MV gene expression, we employed monoclonal antibody against Serp1, a late, secreted serpin (Macen *et al*, 1993; Nash *et al*, 2000). Human glioblastomas were infected with various MV controls or knockout viruses at a multiplicity of infection (m.o.i.) = 3, and at 24 hour post infection the supernatants were collected. The results suggested that several vMyx-hrKOs were restricted in their transition from early (M-T7) to late (Serp1) gene expression (Figure 2A). These restricted viruses included vMyxT2KO (U118,
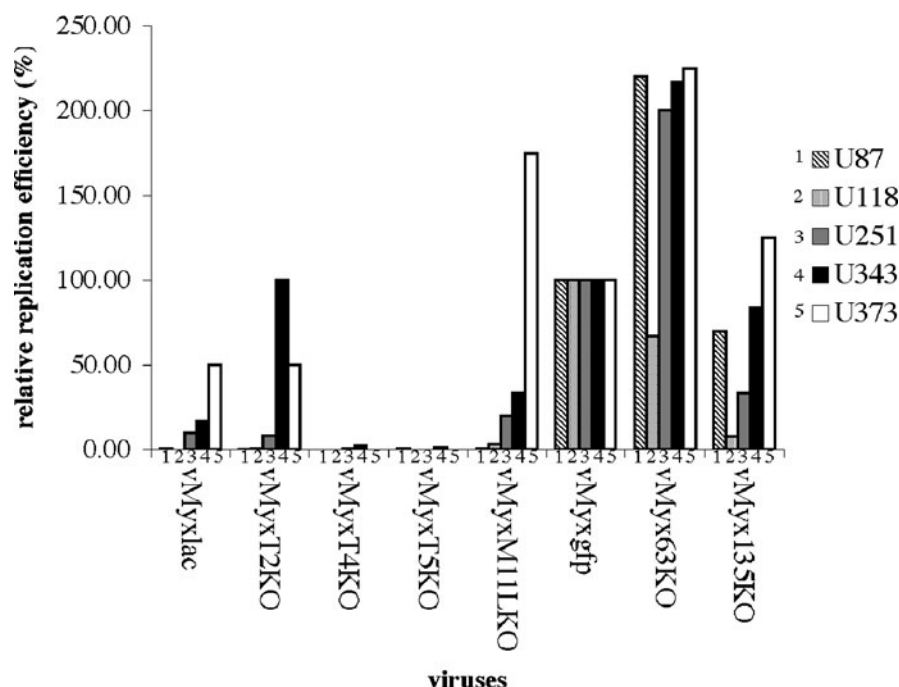
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Figure 1 Titers of various myxoma viruses in selected glioma cell lines. Absolute titers were converted to relative measures of replication efficiency for each virus against the tagged wild-type myxoma virus (vMyxgfp) for each glioma cell line. The *y*-axis represents percent replication compared to vMyxgfp.

U251; Figure 2A, lane 3), vMyxT4KO (all gliomas; Figure 2A, lane 4), and vMyxT5KO (U251; Figure 2A, lane 5). Surprisingly, vMyxT4KO did not even undergo successful early gene expression in two of the glioma cell lines (U87, U373) (Figure 2A) and exhibited significantly decreased early expression in the other lines (U118, U251, U343). M-T7 protein expression following vMyxT4KO infection was only consistently detected in the control cell line (BGMK), indicating a more generalized defect for this virus KO construct in all human glioma cells tested.

To determine whether the reduced levels of secreted MV protein detected in the supernatants were because of decreased viral expression, as opposed to reduced secretion of the expressed viral protein, we examined whole-cell lysates of the specific protein marker (Figure 2B). We observed two bands for each of M-T7 and Serp1, detected in whole-cell lysates from infected gliomas. These two bands represent the native, unglycoslated (lower band) and the processed, glycosylated (larger band) forms of the protein. Generally we observed both the unglycosylated and glycosylated forms of the Serp1 and M-T7 from the lysates collected from the gliomas infected with the majority of the viruses. However, those viruses that exhibited poor secretion of M-T7 or Serp1 (Figure 2A) also exhibited low intracellular expression of the unglycosylated protein. The most dramatic effect was observed for vMyxT4KO infection of U251 cells, in which we were not able to detect the early

gene product, M-T7, expressed from the lysates (Figure 2B, lane 4). In fact, we noted reduced M-T7 expression following vMyxT4KO virus infection in all the gliomas tested, except for the U118 cells. vMyxT2KO and vMyxT5KO also exhibited restricted expression of Serp1 protein in several of the infected gliomas. For example, in U251 cells, both viruses expressed reduced levels of Serp1 in the glycosylated form specifically, suggesting that some aspect of the ER-to-Golgi transit may be impaired in these cells. This would also explain the low levels of Serp1 in the supernatants collected from the gliomas infected with these viruses. In contrast, two of the knockout viruses, vMyx63KO and vMyx135KO, exhibited levels of early and late protein expression and secretion comparable to the wild-type virus infection (Figure 2).

Single-step growth curves
Comparison of the replication efficiency of the various viruses suggested that several virus constructs, including vMyx63KO and vMyx135KO, had the most optimal replication kinetics for several of the cell lines. To examine this further, we performed single-step growth curves of vMyxgfp, vMyx63KO, and vMyx135KO. Although viral replication of the tested viruses was observed in all the gliomas tested, the highest progeny amplification appeared to occur in the U343 cells and the poorest virus replication occurred in the U251 and U118 cells (Figure 3). U87 and U373 cells appeared to be similar and all three
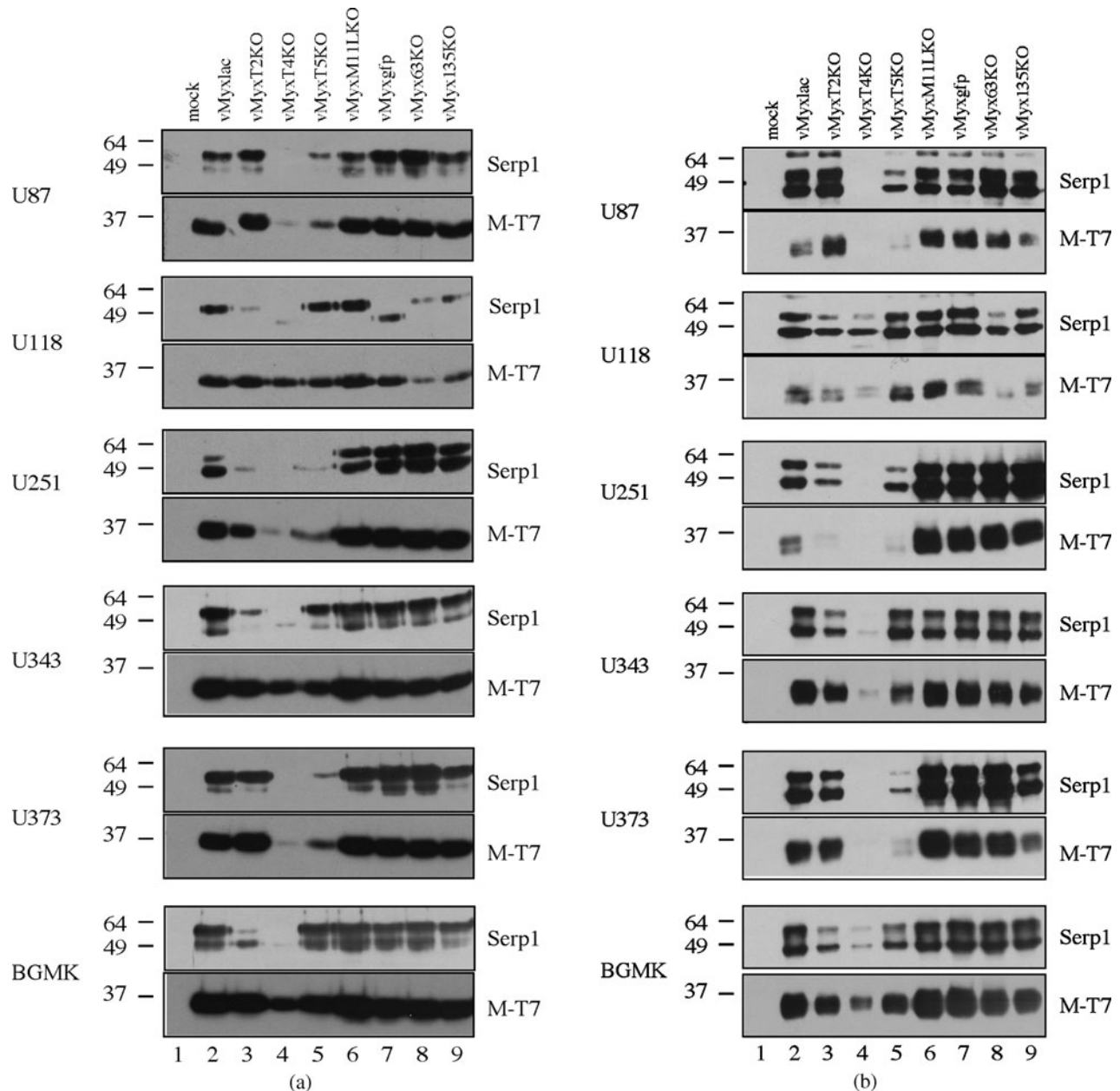
Figure 2 Early and late gene expression from infected glioma cells. Gliomas were infected with indicated viruses or left uninfected. Supernatants (**a**) or infected cells (**b**) were collected 20 hpi. (**a**) Supernatants were concentrated 10× in microconcentrators and then separated on 10% SDS-PAGE gels. Membranes were probed with anti-Serp1, a late, secreted MV protein. Following detection the membranes were striped and probed with anti-M-T7, a strongly expressed and secreted, early gene product. (**b**) Whole-cell lysates were prepared from the samples collected above and 50 μg of total protein were separated on 10% SDS-PAGE gels and then probed with the antibodies above. The various viruses are described in Table 1.

viruses responded in a similar fashion; exhibiting 2 logs of amplification over 48 h. The vMyxgfp titer increased by 3 logs in U343 cells and this was the most permissive host cell for replication of this control virus. However, the titer of vMyxgfp only increased by less than 0.5 logs in U251, thus we classify this cell (U251) as semipermissive. A similar trend was observed for vMyx63KO, which increased in titer by 100-fold in U343 cells but by less than 0.5 logs in U251 cells. In contrast, vMyx135KO increased by 4

logs in U343 and amplified 100-fold in U251 cells (Figure 3).

MV knockout viruses exhibit differential killing of gliomas
To evaluate and compare the effects of various myxoma virus constructs on glioma cell viability, we used a WST-1 assay to assess the metabolic status of infected glioma cells. The results suggest that the various viral constructs exhibit one of three phenotypes
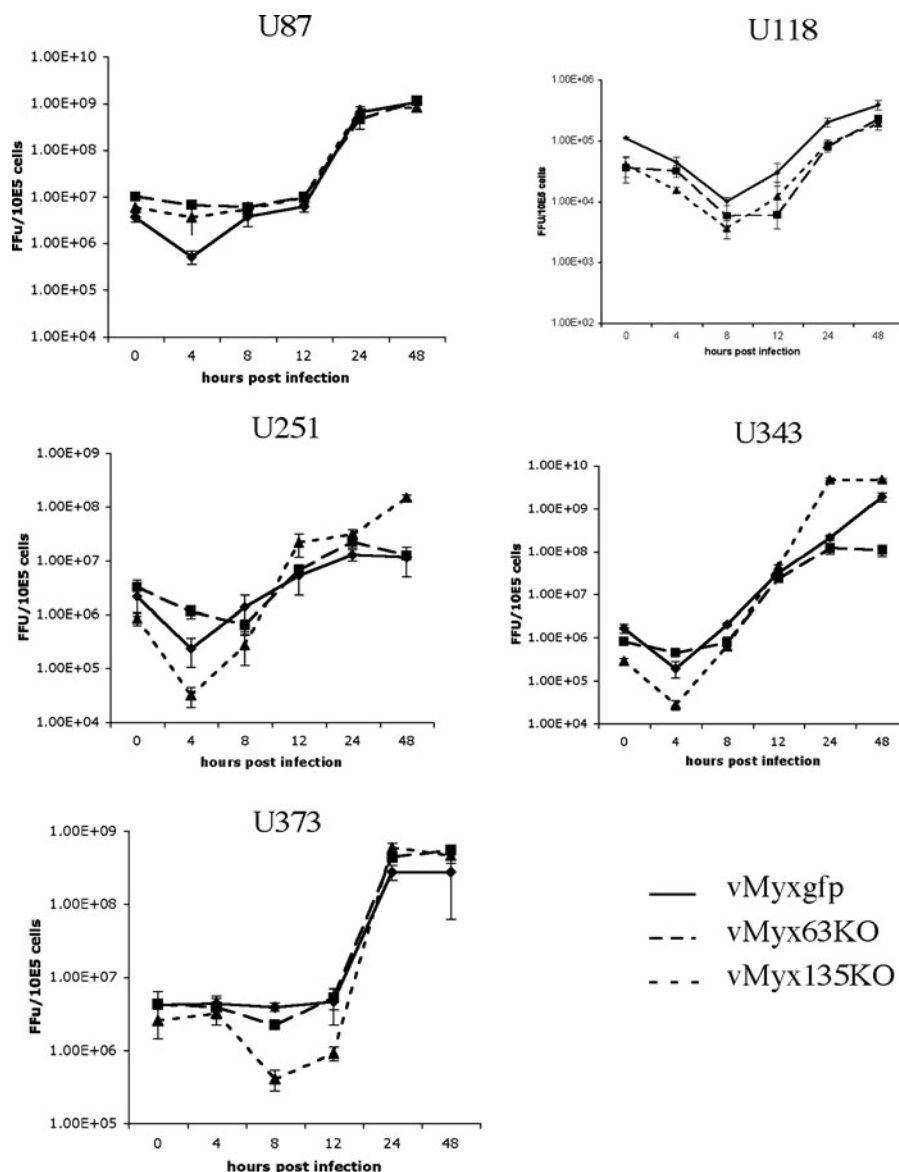
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Figure 3 Single-step growth curves to evaluate viral replication were performed with selected viruses (vMyxgfp [*solid line*], vMyx63KO [*long dash*], and vMyx135KO [*short dash*]) on select glioma cell lines. Infected cells were collected at the times indicated post infection. The virus was released from the infected cells and then recovered virus was titrated on BGMK cells.

(Figure 4A). The viruses could be grouped into those viruses that had little effect on cell viability, those viruses that caused a minor decrease in cell viability and those viruses that efficiently killed the target glioma cells. Our results suggest that this first cluster of viruses included vMyxT4KO and vMyxT5KO and to a lesser extent vMyxT2KO and vMyxM11LKO (data not shown). In contrast, infection of human glioma cells with vMyx135KO or vMyx63KO resulted in greater loss of viability and death than even the killing effects following infection with the wild-type virus (vMyxgfp) (Figure 4A). This difference between the cell viability and virus used for infection was most dramatic at a high m.o.i. (10). At a low m.o.i. (0.1 and 1), the differences between the

various viruses were less dramatic as the infections progressed (data not shown). These results suggest that there may be some aspect about vMyx135KO or vMyx63KO cytotoxicity that leads to a decrease of glioma cell viability and suggests that either of these two viruses could represent a more potent and safer oncolytic agent than the parental wild-type virus, at least for treating gliomas.

Clonogenic assay of gliomas in vitro
Infection of glioblastoma monolayers by the virus constructs reduced the number and size of the clonal populations when compared to uninfected controls (Figure 4B, C, and data not shown). When clonal populations that had been infected with viruses
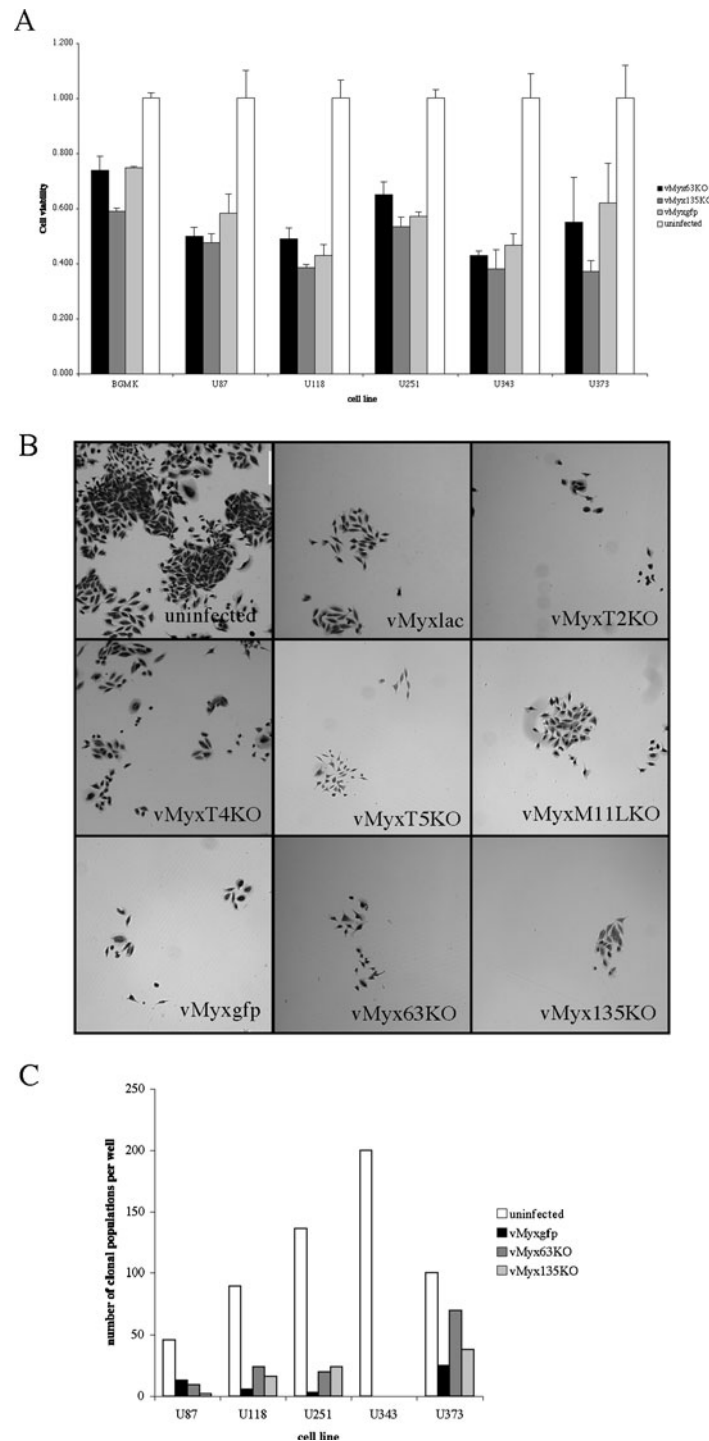
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Figure 4 Cell-based cytotoxicity and clonogenic assays. (**A**) Select control (BGMK) or glioma cells were seeded into 96-well dishes and left uninfected or infected at an m.o.i. of 0.1, 1 (these m.o.i.s not shown), or 10 with the viruses indicated. Seventy-two hours post infection, the metabolic dye WST-1 (Roche) was added to the cultures and then change in color was monitored every 60 min for 4 h. The plates were measured at 450 nm using a Multiskan plate reader (Thermo Labsystems). Each m.o.i. and virus infection was done in triplicate. Colorimetric values were corrected against the uninfected samples ± SD. (**B**) U343 monolayers were infected at an m.o.i. = 10 with various viruses and 6 hpi the monolayer was trypsinized and either 100 or 1000 cells were plated back onto fresh plates. Ten days post infection the cells were fixed and stained with crystal violet. Clones of more than 50 cells were enumerated and graphed. (**C**) Monolayers of the glioma cells were infected at an m.o.i. = 10, with the indicated virus and 6 hpi the monolayer was trypsinized and either 100 or 1000 cells were plated back onto fresh plates. Ten days post infection the cells were fixed and stained with crystal violet, and representative fields were photographed.

expressing EGFP were examined for the presence of fluorescently expressing cells, indicating presence of virus, no EGFP-expressing cells were observed. This suggests that individual cells that were able to establish clonal populations were able to do so because the initial progenitor cell had not been infected. This may explain why the best results were obtained at the highest multiplicity of infection because for such aggressive cancers, myxoma virus and its various knockouts must be able to establish a productive infection within each cell to prevent tumor enlargement and spread. If infection is less than 100%, the virus appears to inhibit cell proliferation and spread, as observed in the clonogenic assay, and reduce the size of the clonal expansion but not to block the tumor completely.

Generally, all the viruses tested were least effective at controlling U373 cells. The clonal populations were smaller than the uninfected controls (most less than 50 cells) but were still numerous. The exception was vMyxgfp, which was able to control both the size and number of clones most effectively. Most of the viruses tested were able to control clonal establishment of U87 and U118; and at the highest m.o.i. also control U343. In contrast, vMyxT4KO exhibited the most limited ability to inhibit glioma reestablishment following infection. This was true for all glioblastomas, but was particularly evident for U87, U343, and U373 (data not shown) because monolayers were quickly formed. This result is in agreement with the poor levels of early and late viral gene expression (Figure 2A and B) for this virus.

For three of the gliomas, the wild-type virus (vMyxgfp) blocked proliferation most effectively (Figure 4C; U118, U251, and U373). However, the ability of vMyx63KO and vMyx135KO to block clonal development was not much different (Figure 4C). There was little difference between any of the three viruses (vMyxgfp, vMyx63KO, or vMyx135KO) in the U87 and U343 cells. However, in contrast to most glioma cell lines, U343 cells were able to quickly establish numerous clonal populations if initially infected at an m.o.i. less than 10 (data not shown).

Human glioma cells exhibit variable levels of endogenous Akt levels
We have proposed previously that the ability of myxoma virus (MV) to productively infect human tumor cells is correlated to the levels of phosphorylated Akt expression, either constitutively expressed or induced as a consequence of the virus infection (Wang *et al*, 2006). To confirm whether this correlation is maintained in human glioma cells, we screened five glioma lines for their levels of endogenous phospho-Akt at both threonine 308 (T308) and serine 473 (S473). Three of the five gliomas exhibited high levels of Akt protein, which is constitutively phosphorylated at T308 but variably phosphorylated at S473 (Figure 5A). Thus, the same three cells lines that exhibited detectable P-Akt S473 endogenously

also expressed P-Akt T308 (Figure 5A). Based on these observed profiles of endogenous phospho-Akt, and a previous report of permissive MV infection (vMyxgfp) of these lines (Lun *et al*, 2005), we suggest that the human gliomas may still be subgrouped into type I (U87, U118, U343) or type II (U251, U373) cells. There was no evidence of any type III phenotype in the tested gliomas, in which activated Akt was not detectable. This observation caused us to predict that, if the correlation reported by Wang *et al* (2006) was correct for all cancer cells, then the gliomas should all be infected and killed by wild-type MV; however, there may be a difference in the ability of the knockout viruses, particularly vMyxT5KO, to productively infect some of the type II gliomas.

Human glioma cell permissiveness to infection by myxoma virus is correlated with endogenous levels of activated Akt
Consistent with earlier observations, we found that infected type I gliomas (U87, U118, U343), maintained high levels of activated P-Akt S473 and T308 following infection with various MV constructs (Figure 5B and data not shown). In contrast, MV infection of Type II gliomas (U251, U373) induced an increased level of activated Akt (S473 and T308) when compared to the level of phosphorylated Akt (S473 or T308) observed in the mock infected samples (Figure 5A and B). P-Akt S473 and T308 levels comparable to that observed following wt MV infections were also noted following infection with either Myx135KO or Myx63KO viruses.

Discussion

Rapid progress in the area of virotherapy for human cancer has initiated the search for new candidate viruses that are potently oncolytic but also safe for humans and other vertebrate animal hosts (Liu *et al*, 2007; Liu and Kirn, 2007; Merrill *et al*, 2006; Parato *et al*, 2005; Roberts *et al*, 2006; Tong, 2006; Woo *et al*, 2006). A wide range of candidate viruses from both human and animal reservoirs (Roberts *et al*, 2006), which encompass a wide variety of antitumor mechanisms, are now in various stages of clinical and preclinical development (Liu *et al*, 2007; Liu and Kirn, 2007; Merrill *et al*, 2006; Parato *et al*, 2005). An important attribute desired for oncolytic virus candidates is the ability to infect and kill human cancer cells without being pathogenic to man or causing unacceptable levels of infection of normal tissues (Parato *et al*, 2005). Several studies have demonstrated that the rabbit specific poxvirus, myxoma virus (MV), is capable of infecting and killing many human tumor cells (Sypula *et al*, 2004) in which the activated levels of Akt are sufficiently high (Wang *et al*, 2006). *In vivo* studies using a GFP-tagged version of wild-type MV, vMyxgfp, have demonstrated that vMyxgfp was effective at
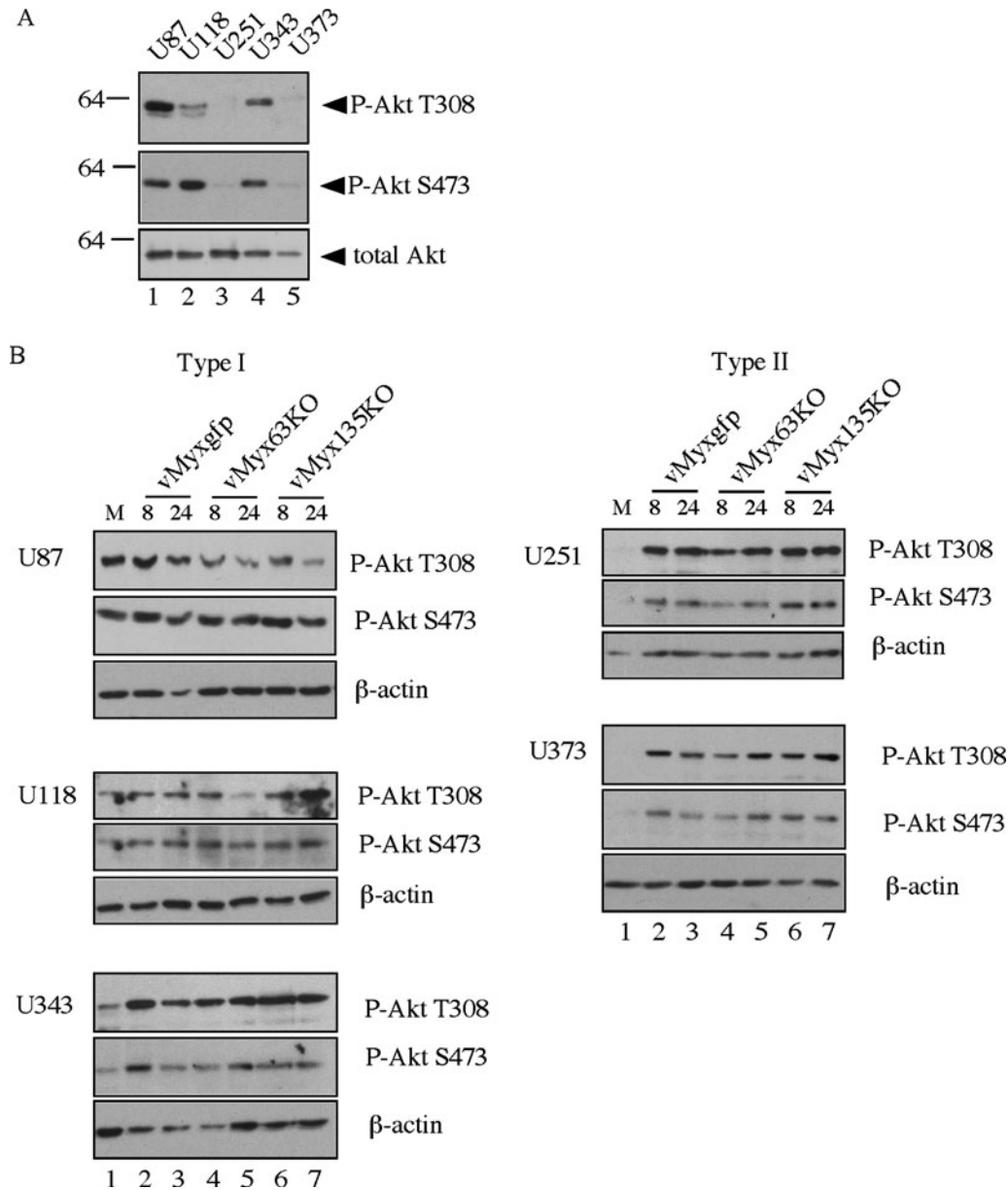
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Figure 5 Levels of endogenous and virally activated phospho-Akt in human glioblastoma cells. (**A**) Whole-cell lysate (50 μg/lane) prepared from uninfected cells was separated on a 10% SDS-PAGE gel and probed overnight at 4ºC with the appropriate antibody. Total Akt was used as a loading control. (**B**) Each glioma was left uninfected (M) or infected with one of the MV constructs. The various viruses are described in Table 1. Samples were collected at 8 and 24 hpi and proteins were separated on a 12% SDS-PAGE gel.

eliminating human glioblastoma xenografts in nude mice (Lun *et al*, 2005).

Our objective in this study was to identify targeted gene knockout variants of oncolytic MV that had the potential to be as oncolytic as the parental virus but would also be nonpathogenic to rabbits that might come into contact with patients receiving virotherapy. Previous studies have examined the ability of wild-type MV and a few select host range mutants of MV to infect and kill a wide range of cancers and tissues *in vitro* (Stanford *et al*, 2007; Sypula *et al*, 2004; Wang *et al*, 2006). Here we have focused on a

selection of cell lines derived from a single type of cancer, namely human gliomas, where MV has been shown to be effective in an animal cancer model (Lun *et al*, 2005). A previous study had reported that MV (vMyxgfp) infection was most robust in U87 cells and that U118 and U251 were semipermissive (Lun *et al*, 2005). The results of this study support and confirm that MV and various knockouts were most productive in U87, U343, and U373 and restricted in U118 and U251 cells (Figure 1). Also, the data presented here demonstrate for the first time that productive infection of the three most permissive

human gliomas was dramatically enhanced when vMyx63KO or vMyx135KO was used, compared to even the wild-type control MV (Figures 3 and 4).

We have also observed considerable variability amongst the MV constructs in their ability to infect and kill human glioma cells. Most of the viruses, including the tagged wild-type versions (vMyxlac, vMyxgfp), were unable to replicate in any of the glioma cell lines to levels identical to the reference BGMK cells, which support maximal progeny MV replication levels. The progeny MV levels in MV-infected glioma cells were consistent with the level of MV replication we have previously reported in other human tumor cell lines (Sypula *et al*, 2004). The most productive of all the viruses tested were vMyx63KO and vMyx135KO. However, even these infections reached only about 20% to 30% of their amplification titers observed on BGMK cells (data not shown), but still were highly efficient at initiating virus infection and killing the target cells (Figure 4).

Generally, the viruses that expressed fluorescent markers (vMyxgfp, vMyx63KO, vMyx135KO) were easier to quantify than the lacZ-tagged constructs, and observed titers ranged from 5% or less in U118 and U251 to 20% to 30% in U87, U343, and U373 to those titers obtained in BGMK cells. This difference in the ability of the various viruses to replicate on the gliomas does not seem to be related to a benefit conferred by the fluorescent marker over β-gal, as the same knockout virus expressing β-gal (vMyxT5KO) or the green fluorescent protein (vMyxT5KO:gfp) appeared to replicate and respond in similar manner (data not shown). Rather, the observed differences may simply reflect the ease of visualizing fluorescent foci versus counting X-gal–stained foci in the glioma cell monolayers.

The endogenous levels of activated Akt, observed in the gliomas (Figure 5A), suggest that human glioma cells also correlate with the pattern of phospho-Akt levels reported for other human tumor cells (Wang *et al*, 2006). Based on those observations, we predicted that tagged wild-type MV such as vMyxlac and vMyxgfp should productively infect all of the gliomas. In contrast, we would predict that vMyxT5KO would produce a productive infection only in type I (U87, U118, U343) cells but not in type II (U251, U373) glioma cells. vMyxgfp appears to infect and replicate equally well in all glioma cells lines we tested. Several of the knockout viruses exhibit restrictions on early and/or late gene expression (Figure 2), supporting their poorer replication efficiencies (Figure 1). vMyxT5KO exhibited reduced late gene expression in two gliomas. The block between expression of early and late gene products was observed in U251 and U373 (Figure 2) and is consistent with the prediction of infection based on endogenous levels of activated Akt (Figure 5). The most sensitive virus knockout construct to infection of gliomas was vMyxT4KO, which only produced detectable infection in U343 cells but was to varying degrees com-

pletely nonpermissive in the other glioma cell lines. The MV M-T4 protein is localized in the ER by virtue of a C-terminal RDEL retention sequence, but otherwise the basis for its host-range defect is unknown (Barry *et al*, 1997; Hnatiuk *et al*, 1999). vMyxT2KO, which fails to express a TNF inhibitor (Macen *et al*, 1996; Schreiber *et al*, 1997; Upton *et al*, 1991), also exhibited some replication restriction in U251 gliomas (Figure 2A and B). Further, study on how these gene products interact with glioma-specific host proteins might provide insights into how MV is able to show cross-species tropism and the ability to productively infect human cancer cells. Glioblastoma cells exhibit a uniquely restrictive tropism and MV likely manipulates multiple signaling pathways that require viral micromanagement to allow for optimal viral replication (McFadden, 2005). Although we have focused on the role of Akt in MV permissiveness in gliomas, this is only one pathway of many that may be involved or altered. For example, previous studies have demonstrated that glioblastomas exhibit variable abilities to produce or respond to interferon (Stojdl *et al*, 2003) and so numerous other factors may be involved.

We would expect that glioma cell lines should be an ideal tumor type to test the oncolytic potential of vMxy-hrKOs because many gliomas express aberrant levels of phosphatase and tensin homolog (PTEN) as a result of mutations or deletions in this control molecule (Hill *et al*, 2003; Rao and James, 2004). A search of the catalogue of somatic mutations in cancer cells at the Sanger Institute (http://www.sanger.ac.uk/perl/genetics/CGP/cosmic) indicate that primary gliomas of the five types of human gliomas tested in this study have been tested and found to contain either mutations to the coding region that are silent or result in alteration to the coding sequence for three genes. U118, U251, and U373 all contain mutations in the CDKN2A gene and U251 also has a mutated P53 gene, but all five gliomas contain mutations of the PTEN gene (Ishii *et al*, 1999; Li *et al*, 1997; Steck *et al*, 1997). The consequence of these PTEN mutations would result in activated Akt in human glioblastoma cells. Activated Akt is a critical pathway that MV utilizes for productive infection of cancer cells (Stanford *et al*, 2007; Wang *et al*, 2006; Werden *et al*, 2007). The correlation between a set of known mutations within a cancer and levels of activated Akt might lead to the development of "personalized medicine" in which patients with gliomas that exhibit activated Akt could receive therapeutic MV whereas glioma patients with wild-type PTEN and could be treated by over expression of the EGFR variant EGFRvIII and EGFR kinase inhibitors (Mellinghoff *et al*, 2005).

Twelve percent of all human glioblastomas have been shown to up-regulate a cellular protein called PIKE-A (Knobbe *et al*, 2005). We have previously demonstrated that human PIKE-A can functionally

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replace the MV gene product M-T5 by activating Akt and improving MV replication in nonpermissive human cancer cell lines (Werden *et al*, 2007). However, we were unable to detect any endogenous PIKE-A expression from the gliomas tested in this study (data not shown).

Based on the results of this study, and because vMyx63KO and vMyx135KO infect and kill glioma cells more effectively than the control wild-type viruses and both viruses are nonpathogenic in their susceptible natural host (European or domestic rabbits), we propose that these two virus constructs represent safer candidates for development of MV as an oncolytic virus candidate in man. These knockout viruses will still need to be tested in animal cancer models, but because of their dramatically reduced pathogenicity in rabbits, they are a safer alternative to wild-type MV for human oncolytic virotherapy trials in the future.

Materials and methods

Viruses and cells
Glioma cell lines were obtained from ATCC. Baby green monkey kidney (BGMK) cells were a gift of Sam Dales (University of Western Ontario). All cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U penicillin/ml, and 100 μg streptomycin/ml. Cells were routinely treated with prophylatic levels (2.5 mg/ml) of plasmocin (Invivogen) to prevent mycoplasm contamination. The viruses

employed in this study have all been described previously (see Table 1).

Single-step growth curves
Cells were seeded into 6-well dishes (6×10^{-5}) and infected at an m.o.i. of 5. Infected cells were collected at the times indicated. Virus was released from the collected cell pellets and titrated back onto BGMK cells. Fluorescent foci were scored 48 h post infection (hpi).

Cell-based cytotoxicity
Cells were seeded into 96-well dishes and 24 h later were infected with the various viruses at a multiplicity of infection (m.o.i.) of 10, 1, 0.1, or were uninfected. Seventy-two hours post infection the infected cells were treated with the WST-1 reagent (Roche) as a measure of cell viability. Color changes were measured at 450 nm every 60 min for 4 h using a Multiskan Ascent plate reader (Thermo Labsystems).

Clonogenic assay
To measure the ability of the infected gliomas to reestablish clonal populations and therefore demonstrate the oncolytic potential of the various knockouts, we developed a clonogenic assay. Glioblastoma monolayers were infected at various multiplicity of infection and then at 6 hpi the infected monolayers were trypsinized and reseeded at densities of 100 or 1000 cells in fresh wells. Five to 10 days post infection, the cell populations were fixed and stained with crystal violet. Clonal populations with at least 50 cells (Franken *et al*, 2006) were enumerated.

References

Barrett JW, Shun Chang C, Wang G, Werden SJ, Shao Z, Barrett C, Gao X, Belsito TA, Villenevue D, McFadden G (2007a). Myxoma virus M063R is a host range gene essential for virus replication in rabbit cells. *Virology* **361:** 123–132.

Barrett JW, Sypula J, Wang F, Alston LR, Shao Z, Gao X, Irvine TS, McFadden G (2007b). M135R is a novel cell surface virulence factor of myxoma virus. *J Virol* **81:** 106–114.

Barry M, Hnatiuk S, Mossman K, Lee SF, Boshkov L, McFadden G (1997). The myxoma virus M-T4 gene encodes a novel RDEL-containing protein that is retained within the endoplasmic reticulum and is important for the productive infection of lymphocytes. *Virology* **239:** 360–377.

Franken NA, Rodermond HM, Stap J, Haveman J, van Bree C (2006). Clonogenic assay of cells in vitro. *Nat Protoc* **1:** 2315–2319.

Gaddy DF, Lyles DS (2007). Oncolytic vesicular stomatitis virus induces apoptosis via signaling through PKR, Fas, and Daxx. *J Virol* **81:** 2792–2804.

Hill C, Hunter SB, Brat DJ (2003). Genetic markers in glioblastoma: prognostic significance and future therapeutic implications. *Adv Anat Pathol* **10:** 212–217.

Hnatiuk S, Barry M, Zeng W, Liu L, Lucas A, Percy D, McFadden G (1999). Role of the C-terminal RDEL motif of the myxoma virus M-T4 protein in terms of apoptosis regulation and viral pathogenesis. *Virology* **263:** 290–306.

Ishii N, Maier D, Merlo A, Tada M, Sawamura Y, Diserens AC, Van Meir EG (1999). Frequent co-alterations of TP53, p16/CDKN2A, p14ARF, PTEN tumor suppressor genes in human glioma cell lines. *Brain Pathol* **9:** 469–479.

Johnston JB, McFadden G (2004). Technical knockout: understanding poxvirus pathogenesis by selectively deleting viral immunomodulatory genes. *Cell Microbiol* **9:** 695–705.

King GD, Curtin JF, Candolfi M, Kroeger K, Lowenstein PR, Castro MG (2005). Gene therapy and targeted toxins for glioma. *Curr Gene Ther* **5:** 535–557.

Knobbe CB, Trampe-Kieslich A, Reifenberger G (2005). Genetic alteration and expression of the phosphoinositol-3-kinase/Akt pathway genes PIK3CA and PIKE in human glioblastomas. *Neuropathol Appl Neurobiol* **31:** 486–490.

Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI, Puc J, Miliaresis C, Rodgers L, McCombie R, Bigner

SH, Giovanella BC, Ittmann M, Tycko B, Hibshoosh H, Wigler MH, Parsons R (1997). PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science* **275:** 1943–1947.

Liu TC, Galanis E, Kirn D (2007). Clinical trial results with oncolytic virotherapy: a century of promise, a decade of progress. *Nat Clin Pract Oncol* **4:** 101–117.

Liu TC, Kirn D (2007). Systemic efficacy with oncolytic virus therapeutics: clinical proof-of-concept and future directions. *Cancer Res* **67:** 429–432.

Lun X, Yang W, Alain T, Shi Z-Q, Muzik H, Barrett JW, Mc-Fadden G, Bell J, Hamilton MG, Senger DL, Forsyth PA (2005). Myxoma virus is a novel oncolytic virus with significant antitumor activity against experimental human gliomas. *Cancer Res* **65:** 9982–9990.

Macen JL, Graham KA, Lee SF, Schreiber M, Boshkov LK, McFadden G (1996). Expression of the myxoma virus tumor necrosis factor receptor homologue (T2) and M11L genes is required to prevent virus-induced apoptosis in infected rabbit T lymphocytes. *Virology* **218:** 232–237.

Macen JL, Upton C, Nation N, McFadden G (1993). SERP1, a serine proteinase inhibitor encoded by myxoma virus, is a secreted glycoprotein that interferes with inflammation. *Virology* **195:** 348–363.

McFadden G (2005). Poxvirus Tropism. *Nature Reviews Microbiology* **3:** 201–213.

Mellinghoff IK, Wang MY, Vivanco I, Haas-Kogan DA, Zhu S, Dia EQ, Lu KV, Yoshimoto K, Huang JH, Chute DJ, Riggs BL, Horvath S, Liau LM, Cavenee WK, Rao PN, Beroukhim R, Peck TC, Lee JC, Sellers WR, Stokoe D, Prados M, Cloughesy TF, Sawyers CL, Mischel PS (2005). Molecular determinants of the response of glioblastomas to EGFR kinase inhibitors. *N Engl J Med* **353:** 2012–2024.

Merrill MK, Selznick LA, Gromeier M (2006). Oncolytic viruses fro the treatment of malignant glioma. *Expert Opin Ther Patents* **16:** 363–371.

Mossman K, Lee SF, Barry M, Boshkov L, McFadden G (1996). Disruption of M-T5, a novel myxoma virus gene member of poxvirus host range superfamily, results in dramatic attenuation of myxomatosis in infected European rabbits. *J Virol* **70:** 4394–4410.

Nash P, Barry M, Seet BT, Veugelers K, Hota S, Heger J, Hodgkinson C, Graham K, Jackson RJ, McFadden G (2000). Post-translational modification of the myxomavirus anti-inflammatory serpin SERP-1 by a virally encoded sialyltransferase. *Biochem J* **347:** 375–382.

Opgenorth A, Graham K, Nation N, Strayer D, McFadden G (1992). Deletion analysis of two tandemly arranged virulence genes in myxoma virus, M11L and myxoma growth factor. *J Virol* **66:** 4720–4731.

Parato KA, Senger D, Forsyth PA, Bell JC (2005). Recent progress in the battle between oncolytic viruses and tumours. *Nat Rev Cancer* **5:** 965–976.

Rao RD, James CD (2004). Altered molecular pathways in gliomas: an overview of clinically relevant issues. *Semin Oncol* **31:** 595–604.

Roberts MS, Lorence RM, Groene WS, Bamat MK (2006). Naturally oncolytic viruses. *Curr Opin Mol Ther* **8:** 314–321.

Schreiber M, Sedger L, McFadden G (1997). Distinct domains of M-T2, the myxoma virus TNF receptor homolog, mediate extracellular TNF binding and intracellular apoptosis inhibition. *J Virol* **71:** 2171–2181.

Stanford MM, Barrett JW, Nazarian SH, Werden S, McFadden G (2007). Oncolytic virotherapy synergism with signaling inhibitors: Rapamycin increases myxoma virus tropism for human tumor cells. *J Virol* **81:** 1251–1260.

Steck PA, Pershouse MA, Jasser SA, Yung WK, Lin H, Ligon AH, Langford LA, Baumgard ML, Hattier T, Davis T, Frye C, Hu R, Swedlund B, Teng DH, Tavtigian SV (1997). Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat Genet* **15:** 356–362.

Stojdl DF, Lichty BD, tenOever BR, Paterson JM, Power AT, Knowles S, Marius R, Reynard J, Poliquin L, Atkins H, Brown EG, Durbin RK, Durbin JE, Hiscott J, Bell JC (2003). VSV strains with defects in their ability to shutdown innate immunity are potent systemic anti-cancer agents. *Cancer Cell* **4:** 263–275.

Sypula J, Wang F, Ma Y, Bell J, McFadden G (2004). Myxoma virus tropism in human tumor cells. *Gene Ther Mol Biol* **8:** 103–114.

Tong A (2006). Oncolytic viral therapy for human cancer: challenges revisited. *Drug Dev Res* **66:** 260–277.

Upton C, Macen JL, Schreiber M, McFadden G (1991). Myxoma virus expresses a secreted protein with homology to the tumor necrosis factor receptor gene family that contributes to viral virulence. *Virology* **184:** 370–382.

Upton C, Mossman K, McFadden G (1992). Encoding of a homolog of the IFN-gamma receptor by myxoma virus. *Science* **258:** 1369–1372.

Wang G, Barrett JW, Stanford M, Werden SJ, Johnston JB, Gao X, Sun M, Cheng JQ, McFadden G (2006). Infection of human cancer cells with myxoma virus requires Akt activation via interaction with a viral ankyrin-repeat host range factor. *Proc Natl Acad Sci U S A* **103:** 4640–4645.

Werden SJ, Barrett JW, Wang G, Stanford MM, McFadden G (2007). M-T5, the ankyrin repeat, host range protein of myxoma virus, activates Akt and can be functionally replaced by cellular PIKE-A. *J Virol* **81:** 2340–2348.

Wollmann G, Robek MD, van den Pol AN (2007). Variable deficiencies in the interferon response enhance susceptibility to vesicular stomatitis virus oncolytic actions in glioblastoma cells but not in normal human glial cells. *J Virol* **81:** 1479–1491.

Wollmann G, Tattersall P, van den Pol AN (2005). Targeting human glioblastoma cells: comparison of nine viruses with oncolytic potential. *J Virol* **79:** 6005–6022.

Woo Y, Adusumilli PS, Fong Y (2006). Advances in oncolytic viral therapy. *Curr Opin Investig Drugs* **7:** 549–559.