6-2017

Oncolytic Tanapoxvirus for Melanoma Therapy

Tiantian Zhang

Western Michigan University, guojiansf8@gmail.com

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Oncolytic viruses (OVs), which preferentially infect cancer cells and induce host anti-tumor immune responses, have emerged as an effective melanoma therapy. Tanapoxvirus (TPV), which possesses a large genome and causes mild self-limiting disease in humans, is potentially an ideal OV candidate. The purposes of our studies are to engineer TPV into effective OVs via arming immuno-stimulatory proteins and/or manipulating the virokines, and to explore the immuno-modulatory activities of TPV.

Interleukin-2 (IL-2) plays a critical role in activating T cells, natural killer cells and macrophages in both the innate and adaptive immune systems. In our study, a recombinant TPV expressing mouse IL-2 (TPVΔ66R/mIL-2) was generated, where the viral thymidine kinase (TK) gene (66R) was replaced with the mIL-2 transgene. We demonstrate that IL-2 inhibits virus replication through intracellular components and without activating the interferon-signaling pathway. The anti-tumor potential of TPVΔ66R/mIL-2 was studied in athymic (T cell deficient) nude mice carrying human melanoma xenografts. Introduction of mIL-2 into TPV remarkably increased its anti-tumor activity, resulting in a more extensive cell degeneration with a significantly increased peri-tumor accumulation of mononuclear cells present in the tumors, compared with that in those treated with wtTPV or TPVΔ66R.
Neuregulin (NRG), an epidermal growth factor is known to promote the growth of various cell types, including human melanoma cells through ErbB family of tyrosine kinases receptors. TPV encoded protein TPV-15L, a functional mimic of NRG, also acts through ErbB receptors. We show that the TPV-15L protein promotes melanoma proliferation. TPV recombinant generated by deleting the 15L gene (TPVΔ15L) showed replication ability similar to that of wtTPV. Whereas, a TPV recombinant with both 15L and 66R genes ablated (TPVΔ15LΔ66R) replicated less efficiently than TPVΔ15L and the parental virus. TPVΔ15L exhibited more robust tumor-regression in the melanoma-bearing nude mice than other TPV recombinants.

Matrix metalloproteinases (MMPs), which are involved in degradation of extracellular matrix, are critical regulators in tumor metastasis. We report that infection with TPV promotes the expression of MMP-9 in the melanoma cells. In addition, we show that MMP-9 exerts an antiviral effect on TPV replication and plays a protective role in TPV-infected melanoma cells in vitro. Moreover, the neutralization of MMP-9 in melanoma cells remarkably enhances the TPV infection and leads to a significant reduction in cell survival.

In summary, our results suggest that 1) TPVΔ66R/mIL-2 is potentially therapeutic for human melanomas in the absence of T cells, and IL-2 expression results in an overall increase of therapeutic efficacy despite its viral inhibitory effects; 2) deletion of TPV-15L gene product which facilitates the growth of human melanoma cells can be an effective strategy to enhance the oncolytic potential of TPV for the treatment of melanoma; 3) in regard of the role played by MMP-9 in virus replication, identifying mechanisms that suppress MMP-9 expression upon TPV infection can potentially improve its used as a melanoma virotherapy.
ACKNOWLEDGEMENTS

I would like to express my gratitude to Dr. Karim Essani and my mom for being my academic mentor and life mentor in guiding me through my Ph.D career. I sincerely want to thank Dr. Karim Essani for all his advice, help and support for completion of my Ph.D degree. I would also like to thank the members of my committee, Dr. Chris Fisher, Dr. Bruce Bejcek and Dr. Rob Eversole for their advice and input in my projects. Additionally, I am thankful to Dr. Charles Mackenzie for his assistance in the immuno-histopathological studies.

It is worthwhile to acknowledge Dr. Steven Kohler and his assistance and guidance. I am greatly in debt to past and present members in Essani lab, including Dr. David Jeng, Yih Wen Goh, Esaw Kurban, Dennis Kordish, Yogesh Suryawanshi and Helene Woyczesczyk. I also wish to thank all the friends that I did not mention here that helped me complete my Ph.D projects. Moreover, I am grateful to the NIH grant 1R15CA156262-01 and FRACAC grant from Western Michigan University to KE.

Last, I would like to mention my family, Xianxia Shang, Chaoyu Zhang, Xiaoren Zhang and all other family members, who have stood by me and supported me on this path. I appreciate from the bottom of my heart.

Tiantian Zhang
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS ........................................................................................................... ii

LIST OF TABLES ................................................................................................................... v

LIST OF FIGURES ................................................................................................................ vi

CHAPTER

I. INTRODUCTION .................................................................................................................. 1

   References .......................................................................................................................... 3

II. ONCOLYTIC TANAPOXVIRUS EXPRESSING INTERLEUKIN-2 IS CAPABLE OF INDUCING THE REGRESSION OF HUMAN MELANOMA TUMORS IN THE ABSENCE OF T CELLS ........................................................................................................... 5

   Background ....................................................................................................................... 5

   Materials and methods ..................................................................................................... 8

   Results ............................................................................................................................... 13

   Discussion ........................................................................................................................ 29

   References ....................................................................................................................... 33

III. TANAPOXVIRUS LACKING A NEUREGULIN-LIKE GENE REGRESSES HUMAN MELANOMA TUMORS IN NUDE MICE ......................................................................................... 38

   Background ....................................................................................................................... 38

   Materials and methods ..................................................................................................... 41

   Results ............................................................................................................................... 46

   Discussion ........................................................................................................................ 53

   References ....................................................................................................................... 57
CHAPTER

IV. NEUTRALIZATION OF MATRIX METALLOPROTEINASE-9 ENHANCES THE PRODUCTIVITY OF TANAPOXVIRUS IN MELANOMA CELLS IN VITRO ...............................................................62

Background ..................................................................................................................62

Materials and methods ...............................................................................................65

Results ..........................................................................................................................69

Discussion ....................................................................................................................76

References ....................................................................................................................79

V. CONCLUSION ........................................................................................................83

Oncolytic virus armed with immuno-stimulatory protein ...........................................83

Oncolytic potency of TPV with deletions of immunomodulators ...............................84

Immuno-modulatory activities related to TPV ...........................................................85

APPENDICES

A. Institutional Biosafety Committee (IBC) .................................................................86

B. Institutional Animal Care and Use committee (IACUC) ........................................87
LIST OF TABLES

1. Semi-quantitative score of the general histopathological characteristics of the tumors in the different test groups .......................................................... 27

2. Immunocytochemical studies........................................................................ 28
LIST OF FIGURES

1. Verification of gene expression and deletions in TPV recombinants................................. 14
2. TPVΔ66R/mIL-2 replication compared to wtTPV and TPVΔ66R in tissue culture ............ 16
3. Co-infection of TPVGFP with TPVΔ66R/mIL-2 or TPVΔ66R........................................ 17
4. Analysis of the mechanism of IL-2’s antiviral activity. ................................................. 19
5. Replication curves of TPVΔ66R/mIL-2 in melanoma cells ........................................... 20
6. Efficacy of TPV recombinants in tumor regression in melanoma tumor models in vivo...... 23
7. General histopathological and immunocytochemical staining presentation of melanoma tumor grafts........................................................................................................... 26
8. Effect of TPV-15L protein and NRG on the proliferation of human melanoma SK-MEL-3 cells......................................................................................................................... 46
9. Verification of gene ablations in TPV recombinants......................................................... 48
10. TPVΔ15L and TPVΔ15LΔ66R replication compared to wtTPV and TPVΔ66R............... 49
11. Replication of TPVΔ15L and TPVΔ15LΔ66R in melanoma cells ..................................... 50
12. Effectiveness of mutant viruses in tumor suppression in melanoma tumor models in vivo... 52
13. Effect of MMP-9 on the replication of TPVGFP and proliferation of human melanoma SK-MEL-3 cells......................................................................................................................... 70
14. Verification of MMP-9 expression in SK-MEL-3 cells ...................................................... 72
15. Effect of anti-MMP-9 on the replication of TPVΔ15L and the cell survival of infected SK-MEL-3 cells......................................................................................................................... 75
CHAPTER I

INTRODUCTION

Although melanoma accounts for only 3-5% of all skin cancers, it is responsible for nearly 80% of all skin-cancer related deaths [1-3]. Like most other types of cancer, melanoma arises from a combination of genetic and epigenetic abnormalities. The genetic mutations such as over-activation of BRAF and Ras, which are found in respectively around 50% and 20% of metastatic melanomas, favor melanoma cell over-proliferation and tumor progression [4]. In addition, the innate and adaptive immune suppression further contributes to melanoma survival and metastasis, such as the loss of the expression of tumor specific antigens and tumor associated antigens (TAAs), the lack of co-stimulatory signals for T cell activation and the inefficient presentation of antigen to antigen presenting cells (APCs). Conventional cytotoxic therapies are not always effective for melanoma [5]. For example, the application of dacarbazine or vermurafenib resulted in less than 20% response rate in patients with malignant melanoma [6]. Although interleukin (IL)-2 seems beneficial to a selected group of patients, it is associated with significant toxicity [7].

Oncolytic viruses (OVs) emerge as a promising approach for melanoma therapy by possessing two closely-related properties. These include OVs’ ability to preferentially target and lyse the tumor cells and their capability to enhance antitumor immune responses [8]. While the antiviral mechanism exists in normal cells, the malignantly activated or abnormally regulated pathways in tumor cells often favor OVs’ infection and replication, therefore generating natural tumor selectivity for some viruses. For example, melanoma cells over-expressing Ras and harboring defective interferon (IFN)-signaling pathways are effectively infected by reovirus and vesicular stomatitis virus (VSV) [9]. In addition, the abnormal characteristics of tumors can be manipulated for generating or increasing virus oncoselectivity. For example, the promoters of tyrosinase or survivin genes that are over-expressed in melanoma, have been incorporated into virus genomes and used for the oncospecific targeting of engineered OVs [10, 11]. Further, OVs have been engineered to express immunostimulatory or immunomodulatory genes to increase their immunoreactivity and antiproliferative efficacy. Genes of cytokines such as granulocyte monocyte colony-stimulating factor (GM-CSF), IL-2, IL-12, IL-18 and IFN-γ have been
incorporated into OVs [12, 13]. Among these cytokines, GM-CSF has been the most widely engineered into OVs to recruit immune cells, enhancing the OVs’ clinical benefit. Talimogene laherparepvec (T-vec) and JX-594, the two most advanced OVs approved for treating melanoma and head and neck cancer respectively, are modified herpes simplex virus (HSV) and vaccinia virus (VV) both expressing human GM-CSF [14]. By causing tumor lysis and immune cells recruitment, OVs armed with immunostimulatory genes exhibit higher efficacy in regressing tumors than immunostimulatory gene products alone. For example, the objective response rate was 26% including the complete response in 11% of patients with treatment of T-vec; while an objective response rate of 6% with 1% complete response rate was observed with treatment of GM-CSF [15, 16].
References

CHAPTER II

ONCOLYTIC TANAPOXVIRUS EXPRESSING INTERLEUKIN-2 IS CAPABLE OF INDUCING THE REGRESSION OF HUMAN MELANOMA TUMORS IN THE ABSENCE OF T CELLS

Background

Melanoma is one of the most common skin cancers. It is known for its propensity for causing metastatic spread throughout the body leading to poor prognoses in affected patients [1]. Although melanoma accounts for approximately 3-5% of all skin cancers, it is responsible for nearly 80% of all skin-cancer related deaths [1-3]. The current therapies for melanoma such as chemotherapy and radiotherapy have limited efficacy and significant side effects. The unresponsiveness of melanoma to the traditional therapies is mainly attributed to immune suppression, which includes loss of expression of tumor-specific antigens (TSAs) on tumor cells, the lack of costimulatory signals for T cell activation and the inefficient presentation of antigens to antigen-presenting cells (APCs) [4]. A number of immunological therapies such as interleukin-2 (IL-2) and adoptive cell therapy (ACT) have been devised to enhance the immune responses and overcome immune suppression for melanoma treatment [5, 6].

Oncolytic viruses (OVs), which preferentially infect and destroy cancer cells and employ host immune responses, have emerged as effective therapies for melanoma [7]. A number of viruses, such as Newcastle disease virus (NDV), herpes simplex virus (HSV) and vesicular stomatitis virus (VSV) have been engineered for oncolytic virotherapy of melanoma [8-10]. These viruses have shown tremendous potential in tumor regression by causing tumor lysis and expressing immuno-stimulatory gene products, which lead to enhanced tumor-specific immune response [11-13]. An encouraging example is talimogene laherparepvec (T-vec), a modified HSV strain expressing human granulocyte monocyte colony-stimulating factor (GM-CSF) that has been approved by U.S. Food and Drug Administration (FDA) for melanoma therapy in 2015 [14]. One source of OV selectivity for cancer cells is by virtue of mutations in viral enzymes involved in nucleotide metabolism, such as thymidine kinase (TK) and ribonucleotide reductase (RR) [15, 16]. As functional similarity exists between viral and cellular enzymes that are upregulated in cancerous cells, viruses with mutations in these enzymes
replicate in a relatively more targeted manner in the cancer cells. Tanapoxvirus (TPV), which belongs to the family Poxviridae (genus Yatapoxvirus), is a relatively benign and geographically limited virus that only causes self-limiting and mild illness, resulting in fever, skin lesions and lymph node enlargement in humans and monkeys [17, 18]. TPV potentially serves as an ideal candidate for OV, due to benefits that include a large genome (approximately 144 kbp), antigenic distinction, no reported human-to-human transmission and thermo-stability.

IL-2 is a pleiotropic cytokine that plays a key role in both the innate and adaptive immune systems. IL-2 is secreted by T cells and induces differentiation and development of thymic lymphocytes to become effector T cells [19]. IL-2 has been shown to promote the survival of memory CD8+ T cells and enhance MHC-II expression on tumor cells [20]. In adoptive T cell transfer therapy, which has demonstrated consistent efficacy in treating melanoma, IL-2 has been used for expanding T cells in vitro before reinfusing the T cells back into cancer patients [6, 20]. In addition, IL-2 possesses substantial efficacy in activating the innate immune system. It has been demonstrated that IL-2 activates natural killer (NK) cells to acquire enhanced cytotoxic functions (known as lymphokine-activated killing [LAK]) [21]. NK cells activated by IL-2 have been shown to target tumor cells in a broader spectrum with an increased affinity of perforin for the tumor cells, which resulted in more significant cell lysis [22]. Additionally, it has been demonstrated that macrophages, after being activated by IL-2, become larger, more granular and conglomerated on the cancer cells with enhanced cytotoxicity [23]. IL-2 has also been shown to activate macrophages to induce tumor necrosis factor (TNF) and other cytotoxic molecules such as free radicals [24]. Moreover, rapid activation of neutrophils has been observed after exposure to IL-2 [25].

IL-2 and interferon-α (IFN-α) are the two cytokines approved by FDA for melanoma immunotherapy [26, 27]. IL-2 transgene has also been engineered into viruses such as NDV and reovirus for combining virotherapy and immunotherapy [28, 29]. Studies have clearly demonstrated that OVs expressing IL-2 successfully increase the adaptive anti-tumor immune response including the proliferation of regulatory CD4+ and cytotoxic CD8+ T cells and development of memory T cells, resulting in effective tumor reduction [28, 30]. However, few studies have explored the involvement of the innate immune response elicited by OVs armed with IL-2, which might also be responsible for tumor regression. It is desirable to examine if the viral lysis together with the innate anti-tumor immune response mediated by the IL-2-expressing
OVs, is therapeutically sufficient for melanoma control and elimination. If so, it would be an appealing treatment option for melanoma patients with T cell suppression/deficiencies, such as those with human immunodeficiency virus (HIV) positivity.

Studies regarding the role of IL-2 in virus replication have generated conflicting results. Some studies suggest that IL-2 possesses a protective role against viral infection through a mechanism potentially involving both CD4\(^+\) and CD8\(^+\) T cells [31]. It has also been observed that the replication of mammalian viruses such as NDV expressing IL-2 was attenuated [28, 31, 32]. However, other research suggests that IL-2 likely plays a pathogenic role supporting viral infection [33]. Thus the exact effect of IL-2 on virus replication and infection is of great interest as it still remains unclear.

Based on these findings, we introduced a sequence that encodes mouse IL-2 into the TPV genome to replace 66R gene that encodes TK, therefore generating TPVΔ66R/mIL-2. The in vitro study shows that TPVΔ66R/mIL-2 replicates less effectively than wtTPV and TPVΔ66R in owl monkey kidney (OMK) cells, human lung fibroblast cells (WI-38) and human melanoma cells (SK-MEL-3), especially at a low multiplicity of infection (MOI). In addition, we demonstrate that IL-2 expression attenuates virus replication potentially through an intracellular mechanism without activating IFN signaling pathway. Our in vivo experiments show that TPVΔ66R/mIL-2 exhibits remarkably higher efficacy in reducing xenografted melanoma tumors in athymic nude (T cell deficient) mice than wtTPV and TPVΔ66R. Histopathological observations show that treatment with TPVΔ66R/mIL-2 results in more extensive tumor cell degeneration and increased mononuclear cells accumulation, in comparison to treatment with the other TPVs. These data provide compelling evidence that IL-2 induces a host anti-tumor immune response to regress tumor in the absence of mature T cells. Taken together, our results suggest that TPVΔ66R/mIL-2, which causes tumor cytolysis and also elicits T cell-independent anticancer immune response, is a promising virotherapy for melanoma.
Materials and methods

Cell lines, virus, and reagents

OMK cells, human lung fibroblasts WI-38 and human melanoma cell line SK-MEL-3 were purchased from American Type Culture Collection (ATCC product numbers CRL-1556, CCL-75, HTB-69 respectively). OMK and WI-38 were cultured in complete growth medium containing Earle’s Minimum Essential Medium (EMEM) supplemented with 10% (vol/vol) fetal bovine serum (FBS) (Atlanta Biologicals), 2 mM L-glutamine (Sigma-Aldrich) and 50 µg/ml gentamicin sulfate (AMRESCO). SK-MEL-3 were cultured in growth medium consisting McCoy’s 5A medium (Sigma-Aldrich) with 10% FBS. All cell monolayers infected with virus were maintained in maintenance medium with all components being the same as the growth medium except for FBS concentration being 2%. All cell lines were grown at 37°C in a 5% CO₂ atmosphere. The wtTPV (Kenya strain) was originally a gift from Dr. Joseph Espósito (Centers for Disease Control, Atlanta, GA, USA). It was genetically modified in the laboratory of G. McFadden to express the fluorescent reporter enhanced green fluorescent protein (EGFP), and the recombinant virus was named as TPVGFP. EGFP gene was expressed under a synthetic early/late (E/L) promoter and inserted between 5L and 6L in TPV-kenya genome [34]. IL-2 protein (mouse) was purchased from Sino Biological, China. Antibodies including anti-IFNα, anti-IFNβ and anti-IL-2 were purchased from R&D Systems, MN.

Construction of the recombinant TPV containing the transcription cassette of mouse IL-2

The recombinant TPVs were derived from wtTPV. A plasmid p2KO vector was constructed based on the commercially available cloning vector pBluescript II KS(+), which is a high-copy number plasmid featuring ampicillin selection and containing multiple cloning sites with unique restriction sites. Genomic sequences flanking the left and right sides of TPV-66R gene (left flank (LR) and right flank (RF)) were inserted between unique Sac I and Not I restriction sites (LR) and unique EcoRI and Hind III restriction sites (RF) into the p2KO vector. Two identical synthetic E/L promoters [35] were inserted between Xba I and BamH I sites and the Xma I and EcoRI sites, in each case long primers were designed to include ribosome binding sequence that would be between the 3’- end of the promoter and the start codon on the transgene. By using viral genomic flanking sequences (66R LF and RF), the p2KO expression vector could
be guided to a specific point where a targeted ablation occurs (ablation of 66R gene) with the simultaneous replacement of a fluorescent reporter (mCherry) and a desirable expressed transgene. The mIL2 open reading frame (ORF) complementary DNA (cDNA) clone was purchased as an ORF-bearing plasmid pGEM-T vector (Sino Biological, Incorporated). The mIL2 ORFs were amplified from their vectors via polymerase chain reaction (PCR) method, using a pair of designed primers with the forward primer being as BamH I forward (CTAGGCCTGGGATCCGATCCACCGTGCCACCATGTACAGCATGCAGCTCGC) and XmaI reverse (CTAGGCCTGCCCCGGATATGGTCGACCTGCAGCTCGC). The PCR products were ligated between BamH I and XmaI sites in p2KO vector having 66R left and right flanks, and the resulting plasmid was named as p2KO-66RLFmIL-2-66RRF. The resulting plasmid was confirmed by DNA sequencing.

This plasmid was transfected (Superfect transfection reagent; Qiagen) into 35-mm tissue culture dishes plated with OMK cell monolayer infected with wtTPV at 1 MOI. The cells with transfection and infection were incubated at 37 °C incubator with 5% CO2 until visible fluorescence caused by mCherry expression appeared. The virus was harvested, and recombinant virus TPVΔ66R/mIL-2 was purified from wtTPV by using a serially diluted plaque assay, with the selection enabled by the expression of mCherry. Recombinant virus was plaque purified at least 3 times until no visible non-fluorescence plaques was observed, and no relevant gene of wtTPV was detected by confirmation PCR. Plaque assays were carried out as described earlier [36].

Replication of recombinant TPVs

Virus replication was determined in OMK, WI-38 and SK-MEL-3 cells. Cell monolayers in 24-well plates were infected with wtTPV or one of the TPV recombinants (TPVΔ66R and TPVΔ66R/mIL-2) at 0.1 MOI and 5 MOI. Infected cells were harvested at 48, 96 and 240 hours post-infection (hpi) and total virus titer was determined by viral plaque assay using OMK monolayers in 6-well plates. Each experiment was repeated 3 times.

Virus co-infection assay

OMK cell monolayers were infected with 5 MOI of both TPVΔ66R/mIL-2 and TPVGFP, controlled with co-infection of 5 MOI of both TPVΔ66R and TPVGFP
simultaneously. Cytopathic effect (CPE) caused by TPVΔ66R or TPVΔ66R/mIL-2 expressing mCherry protein was distinguished from that of TPVGFP expressing EGFP, via using fluorescence microscope with different filter settings. The infected cells were harvested at 48 and 96 hpi, and the titer of each virus was determined by viral plaque assays. Each experiment was repeated 3 times.

**Western blot analysis**

Cell supernatant and cell lysate from virus-infected cells were collected. The samples were mixed with 5× SDS gel loading buffer (25% glycerol, 5% SDS, 0.002% bromophenol blue, 15% β-mercaptoethanol) and boiled for 3 min. Protein samples were separated on SDS-PAGE gels and transferred to Immobilon-P polyvinylidene difluoride (PVDF) membrane using a semi-dry transfer apparatus (Bio-Rad Trans-Blot SD) at 14V for 1.25 hs. Membranes were blocked in TBST (20 mM Tris, 137 mM NaCl [pH 7.6] plus 0.1% Tween 20) containing 5% nonfat dry milk for 2 hs at room temperature and then incubated with 1:1000 dilution of primary antibody goat anti-mIL2 (R&D systems) overnight at 4°C. The membranes were washed five times for 10 min each with TBST before incubation with a 1:7500 dilution of anti-goat IgG-horseradish peroxidase (HRP) (Sigma) in TBST plus 5% nonfat dry milk at room temperature for 1 h with gentle agitation. The membrane was washed five times for 10 min each with TBST, and the signal was detected by using chemiluminescence reagents (Thermo Scientific) [37].

**Animals**

Female athymic nude mice (Crl:NU(NCr)-Foxn1nu) were purchased from Charles River company (Wilmington, Massachusetts, US). Animals were received at 6-8 weeks of age and were housed individually with food and water available in pathogen-free animal facility at Western Michigan University. Animals were allowed to acclimate for one week before experimentation. All protocols for treatment and manipulations were approved by the Institutional Animal Care and Use Committee of Western Michigan University (IACUC protocol number 13-07-01).
Animal studies

To induce tumors, \(5 \times 10^6\) SK-MEL-3 cells were injected subcutaneously on the dorsal surface. Each injection was followed by cell viability assay which was conducted using trypan blue to test the cell viability after the time of injection. Once visible, tumor volume was calculated by using the digital calipers to measure three dimensionally according to the formula \(\frac{4}{3} \times 3.1415 \times (\text{length}/2) \times (\text{width}/2) \times (\text{height}/2)\) in \(\text{mm}^3\). When the tumor size reached \(45 \pm 4.5\) \(\text{mm}^3\), mice were randomized into control and experimental groups, with each group containing 5 mice (\(n=5\)). A single injection of \(5 \times 10^6\) pfu virus particles in 100 ul medium was given intratumorally to each mouse in experiment group, while in control group a mock treatment of only 100 ul medium was used for each mouse. The day of treatment administration was considered as day 0. Mouse weights and tumor volumes were measured and recorded daily. All the mice were sacrificed at 35 days post treatment, and the tumors were harvested. The blood of each mouse was collected for the serum. Tumors were removed, weighed and stored for histological analyses.

Histopathology

Harvested tumors were fixed in 10% neutral-buffered formalin for 3 days at room temperature and then placed in 50% ethanol at 4 °C until processed by routine histopathology techniques as previously described [38]. The removed tumors were sectioned at the point of greatest diameter and the resulting cut surface was used for morphometrics, using an Image. The H&E sections were stained using standard practice. The Ki67 staining was carried out using a polyclonal rabbit anti-Ki67 (Cell Marque. Cat #275R-15) with Gill 2 haematoxylin (Richard Allen-Kalamazoo, MI, USA) counter staining for 5 seconds using a 3% \(\text{H}_2\text{O}_2\)/meth and an avidin/biotin block with heat-treatment for 30/10 minutes at 100/25 degrees centigrade. A system negative control without the anti-K67 antibody was used as well as a positive internal control using normal mouse tissues. F4/80 and CD3 were also tested with similar methodology using rabbit polyclonal anti-CD3 (AbD Serotec, Raleigh NC), rat anti-mouse F4/80 (Abcam Cambridge, MA). The mitotic rate was counted at x40 magnification. The number of cells positive to Ki67 staining was assessed using a Chalkey Point Array graticule and measured under a magnification of x40. Counts of Ki667 positive cells and F4/80 cells were established by
counting all cells in x40 objective field in different defined zones of the tumor and expressed as %.

Following a general appraisal of the tissues for major change and characteristics the main features of the tissues were identified and these semi-quantitatively assessed, using the scoring system: - = feature/form not present, + = limited amounts of feature/form present, ++ moderate amounts of the feature/form present, +++ = feature/form commonly present in the tumor.

Statistical analyses

All in vitro experiments were done in triplicate and the measurement were presented as mean ± SD. The two-sample unequal variance Mann-Whitney U test analysis was applied for testing the differences. The significance level used was P < 0.05.

Mann-Whitney U test was used for assessing the treatment effect of each experimental virotherapy group compared to the mock group in vivo. The tumor volumes and counts of Ki67 positive cells and F4/80 cells of each experimental group were compared with those in the mock group. The significance was considered if P < 0.05. For each mouse, the change in tumor volume over time was fit to an exponential growth model: $Vol_t = Vol_o e^{kt}$, where $Vol_t$ is tumor volume on day $t$, $Vol_o$ is the initial tumor volume, $k$ is the growth rate coefficient, and $t$ is time in days. $K$ for each mouse was estimated from a linear regression of log$_e$ (tumor volume) on time. We then used one-way ANOVA to test whether tumor growth rate ($k$) differed among treatments. We made post hoc comparisons of treatment means using Tukey’s HSD. All analyses were performed using R 3.3.2 core [39].
**Results**

**Generation of mutant TPV expressing mIL-2**

The mIL-2 ORF was inserted into p2KO plasmid under the control of an E/L synthetic poxvirus promoter, and flanked by 66R left and right flanking sequences for the deletion of the core regions of TPV-66R gene. This plasmid also included a mCherry fluorescent reporter gene under another synthetic E/L poxvirus promoter. The p2KO expression cassette including 66R right and left flankings, mCherry gene and mIL-2 gene, was transferred to the viral genome through homologous recombination double-crossover event during the simultaneous infection with wtTPV and transfection with the engineered p2KO in OMK cells (Fig 1A). The region in between the 66R flanking sequences on the p2KO vector was inserted into TPV genome, replacing the 66R gene and resulting in the recombinant virus TPVΔ66R/mIL-2. TPVΔ66R/mIL-2 was separated from wtTPV by plaque purification at least 3 times, until no visible wtTPV plaques were present in cell culture and no relevant fragments of wtTPV genomic DNA were detected by confirmation PCR. Fig 1B demonstrates that compared with wtTPV, TPVΔ66R/mIL-2 contained mIL-2 transgene in the genome. Additionally, wtTPV contained 66R gene which was absent in TPVΔ66R/mIL-2. ORF 136R was used as a positive control and was present in both wtTPV and TPVΔ66R/mIL-2. As indicated in Methods and Materials, verification of IL-2 expression was conducted by western blot with the supernatant and cell lysate samples of the virus-infected cells. As shown in Fig 1C, mIL-2 was expressed in both supernatant and cytoplasm of the cells infected with TPVΔ66R/mIL-2 rather than TPVΔ66R. Results showed that a recombinant virus expressing mIL-2 was constructed with 66R deleted from the virus genome (Fig 1B and C).
Figure 1. Verification of gene expression and deletions in TPV recombinants.

(A) Homologous recombination of TPV and engineered vector p2KO. Two identical poxvirus E/L synthetic promoters allow the simultaneous expression of the fluorescent reporter mCherry protein and an additional transgene product (mIL-2). The ORF 66R left and right flanks were amplified from wtTPV genome using PCR, with specific restriction sites allowing for directional ligation of PCR amplicons of both flanks into p2KO vector. OMK cells were simultaneously infected with wtTPV and transfected with engineered p2KO. Through homologous recombination, 66R gene was replaced with transgene (mIL-2) and mCherry gene. (B) Confirmation PCR of TPVΔ66R/mIL-2. Genomic DNA from TPVΔ66R/mIL-2 and wtTPV was isolated and probed for sequences internal in mIL-2, 66R and 136R genes. Genes ablated showed no bands. As indicated, the mIL-2 gene expected to be 598 bp is shown in TPVΔ66R/mIL-2 viral DNA (Lane b) instead of in wtTPV DNA (Lane a). The 66R gene predicted to be 379 bp is contained in wtTPV (Lane c) but ablated from TPVΔ66R/mIL-2 viral genome (Lane d). A region of TPV-136R gene used as a control has a predicted length of 531 bp (Lane e: wtTPV; Lane f: TPVΔ66R/mIL-2). (C) Western blot analysis of mIL-2 expression. OMK cells were infected
with TPVΔ66R/mIL-2, TPVΔ66R and medium (mock) separately. The cells were harvested after 48 hours and centrifuged to collect the supernatant samples. The cell pellets were subsequently frozen and thawed three cycles to release the mIL-2 from the cytoplasm collected as the cell lysate samples. Both supernatant and cell lysate samples were subjected to 12% SDS-PAGE gel and western blot analysis using a goat antibody against mIL-2 and the secondary anti-goat HRP conjugated antibody. ECL was applied for visualization of the bands. Lane a represents the positive control, recombinant mIL-2 protein with a predicted apparent molecular mass of 19 kDa. Supernatant samples are represented by lanes (b) TPVΔ66R/mIL-2, (c) TPVΔ66R and (d) Mock. Cell lysate samples include (f) TPVΔ66R/mIL-2, (g) TPVΔ66R and (h) Mock. Lane e represents an empty lane with no protein sample loaded.

Replication of recombinant TPVs in cell culture

To evaluate the replication kinetics of TPV and TPV recombinants, virus titers were determined by infecting OMK and WI-38 cells with wtTPV, TPVΔ66R and TPVΔ66R/mIL-2 in triplicate at 0.1 MOI and 5 MOI. The virus was harvested at 48, 96 and 240 hpi, and the yields of infectious virus particles were quantitated by plaque assays. Consistent with our previous study [37], the infection of wtTPV and recombinant TPVs reached to the maximum titer at approximately 96 hpi (Fig 2). At an MOI of 0.1 in OMK cells, TPVΔ66R/mIL-2 exhibited approximately 5-fold lower virus yields than wtTPV and TPVΔ66R (P < 0.05) at 96 and 240 hpi. At 5 MOI, the yield of TPVΔ66R/mIL-2 from infected OMK cells was significantly less than that of wtTPV and TPVΔ66R at 48, 96 and 240 hpi (P < 0.05) (Fig 2A). The yield of virus from infected WI-38 cells was time dependent at 0.1 MOI and 5MOI (Fig 2B). At 0.1 MOI, the maximum yield of TPVΔ66R/mIL-2 was nearly 3-fold lower than that of TPVΔ66R and 10-fold lower than wtTPV yield at 96 hpi (P < 0.05). At 5 MOI, the yield of TPVΔ66R/mIL-2 was around 2-fold lower than that of TPVΔ66R and 4-fold lower than wtTPV yield at 96 hpi (P < 0.05). The above data showed that replication of TPVΔ66R/mIL-2 was attenuated compared to the parental virus and wtTPV in the cell culture.
Expression of IL-2 impedes virus replication

Similar to what our study shows, previous studies have revealed that the replication and virulence of the viruses, such as HSV and NDV expressing IL-2, was attenuated [31, 32]. However, the exact mechanism was unclear and deserved further exploration. Thus, we sought to test whether the insertion of IL-2 transgene into viral genome or the IL-2 gene product reduced virus replication. Simultaneous infection of OMK cell monolayer with TPVA66R/mIL-2 and TPVGFP both at 5 MOIs was administered. As a control, OMK cell monolayer was coinfected with 5 MOI of both TPVA66R and TPVΔ66R/mIL-2 simultaneously. While TPVA66R and TPVΔ66R/mIL-2 were constructed to express mCherry protein, TPVGFP was engineered to express EGFP. Therefore, CPE caused by TPVΔ66R and TPVΔ66R/mIL-2 was visibly distinguished from that of TPVGFP, via using fluorescence microscope with different filter settings. The progress of the viral infection was monitored by harvesting the virus-infected cells at two time points (48 and 96 hpi). The yield of infectious virus particles was quantitated by using a plaque assay as described earlier [36]. As shown in Fig 3, TPVGFP co-infecting with TPVΔ66R/mIL-2 displayed approximately 4-fold lower virus replication than that co-infecting with TPVΔ66R at 48 and 96 hpi (P < 0.01) (Fig 3A, C, D, E and F). Consistent with the viral
replication kinetics shown in Fig 2, TPVΔ66R/mIL-2 co-infecting with TPVGFP maintained less effective replication than TPVΔ66R co-infecting with TPVGFP at 5 MOI (Fig 3B). The results suggest that compared to TPVΔ66R, TPVΔ66R/mIL-2 impedes the replication of other viruses when infecting simultaneously. This further provides evidence that IL-2 expression is related to decreased viral replication.

Figure 3. Co-infection of TPVGFP with TPVΔ66R/mIL-2 or TPVΔ66R.

OMK cells were infected with a mixture of 5 MOI TPVGFP and 5 MOI TPVΔ66R/mIL-2, or a mixture of 5 MOI TPVGFP and 5MOI TPVΔ66R/mIL-2 as indicated. CPE of TPVGFP expressing GFP was visualized by fluorescence microscope at 48 hours [(C) and (E)] and 96 hours [(D) and (F)]. TPVΔ66R/mIL-2 and TPVΔ66R expressed mCherry protein, which were distinguished from TPVGFP. The virus was harvested at 48 and 96 hpi and titrated on OMK
monolayers. The number of plaques formed by each virus was counted. (A) The replication of TPVGFP co-infecting with TPVΔ66R/mIL-2 in comparison to that of TPVGFP co-infecting with TPVΔ66R. (B) The replication of TPVΔ66R/mIL-2 co-infecting with TPVGFP compared to that of TPVΔ66R co-infecting with TPVGFP.

Neutralization of IL-2 expressed in the cell culture exhibits no significant efficacy in increasing virus replication

To further investigate the mechanism by which IL-2 inhibited virus replication, SK-MEL-3 cells in 24-well plates were infected with TPVΔ66R/mIL-2 at 0.1 MOI and incubated with anti-mIL-2 in serial dilutions (0 ug, 0.01 ug, 0.1 ug, 1 ug) separately. The cells were harvested at two time points (48 and 96 hpi) to determine the virus yield. Different from the results achieved by Ghiasi H et al. [31], our results showed that addition of anti-mIL-2 in the cell culture exerted no significant effect in boosting virus replication (Fig 4A). The result suggests that IL-2 expression reduces virus replication through a mechanism which likely occurs intracellularly instead of extracellularly. Additional experiments clearly demonstrate that addition of IL-2 in the cell culture medium neither boosts nor impedes the virus replication (results not shown).

Previous research has demonstrated that IL-2 stimulates extracellular signal regulated protein kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) in various immune cell populations [21]. In addition, IL-2 has been shown to trigger the phosphorylation and activation of members of Janus kinases (JAKs) and the signal transducer and activator of transcription (STAT) families, which overlaps the signaling pathway of IFN [40]. Thus, we sought to examine if IL-2 impeded virus replication by eliciting the activation of IFN signaling pathway and the secretion of IFN. As IFN release mediated by the activation of JAK-STAT pathway has been observed in human lung fibroblasts [41-43], WI-38 cells were infected with TPVΔ66R/mIL-2 at 0.1 MOI with addition of anti-IFNα (2.5 ug/ml) or anti-IFNβ (2.5 ug/ml) in the cell culture. The virus replication was determined by harvesting the cells at 48 and 96 hpi, and the virus titer was quantitated using plaque assays. Results showed that addition of anti-IFN had no significant effect in elevating the virus replication (Fig 4B). Moreover, we have not detected IFNα or IFNβ mRNAs in WI-38 cells infected with TPVΔ66R/mIL-2 using reverse transcriptase PCR (data not shown). These results strongly suggest no involvement of IFN signaling pathway in decreased
virus replication caused by IL-2. The exact mechanism by which IL-2 attenuates viral replication still deserves further investigation.

Figure 4. Analysis of the mechanism of IL-2’s antiviral activity.

(A) TPVΔ66R/mIL-2 replication with addition of anti-mIL-2 in cell culture. SK-MEL-3 cells in 24-well dishes were infected with TPVΔ66R/mIL-2 at 0.1 MOI, followed by addition of respectively 0, 0.01, 0.1 and 1 ug anti-mIL-2 into the cell culture. The virus was harvested at 48 and 96 hpi and titrated in OMK monolayers. Shown here represents the average of 3 independent
experiments. (B) TPVΔ66R/mIL-2 replication with addition of anti-IFN in cell culture. WI-38 cells in 24-well dishes were infected with TPVΔ66R/mIL-2 at 0.1 MOI, followed by addition of anti-IFNα or anti-IFNβ (2.5 ug/ml) into the cell culture. The virus was harvested at 48 and 96 hpi and titrated in OMK monolayers. Shown here represents the average of 3 independent experiments.

Production of progeny virus in malignant melanoma SK-MEL-3 cells

As we sought to analyze the therapeutic effect of TPVΔ66R/mIL-2 for melanoma, target human melanoma SK-MEL-3 cells were infected respectively with wtTPV, TPVΔ66R and TPVΔ66R/mIL-2 at 0.1 MOI and 5 MOI in vitro. Infected cells were harvested and virus titer was determined at 48, 96 and 240 hpi. As shown in Fig 5, at 5 MOI, TPVΔ66R/mIL-2 exhibited similar replication kinetics as the parental virus and wtTPV. At 0.1 MOI, TPVΔ66R/mIL-2 achieved the maximum titer at 96 hpi that was approximately 3.5-fold lower than that of TPVΔ66R, and 10-fold lower than that of wtTPV. Consistent with the results shown before, IL-2 expression likely inhibited the virus replication in SK-MEL-3 cell culture.

![Replication curves of TPVΔ66R/mIL-2 in melanoma cells.](image)

Figure 5. Replication curves of TPVΔ66R/mIL-2 in melanoma cells.
Melanoma cell line SK-MEL-3 cells in 24-well plates were infected with wtTPV, TPVΔ66R and TPVΔ66R/mIL-2 at 0.1 MOI and 5 MOI. The virus was harvested at 48, 96 and 240 hpi and titrated on OMK cells. Shown here represents the average of 3 independent experiments.

**Oncolytic effectiveness of TPVs in xenografted tumor models in athymic mice**

Previous studies have shown that IL-2 possesses the capability of enhancing the innate immune responses for tumor regression, such as activating the NK cells and macrophages and augmenting their lysing ability [23, 44]. Herein, we aimed to evaluate the efficacy of TPVΔ66R/mIL-2 in melanoma regression, compared to that of wtTPV and the parental virus. Also, we sought to determine if IL-2 expressed from TPVΔ66R/mIL-2 could assist eliciting innate immune cells such as NK cells, macrophages and neutrophils. Melanoma tumors were induced in athymic nude mice via injecting 5X10^6 SK-MEL-3 cells subcutaneously on the dorsal surface in the inter-scapular region. When the tumor sizes developed to 45 ± 4.5 mm^3, the mice were randomized into 4 groups and treated respectively with intratumoral injection of mock (only medium), wtTPV, TPVΔ66R and TPVΔ66R/mIL-2. As shown in Fig 6A-C, the average tumor sizes in the animals treated with TPVΔ66R/mIL-2 were significantly smaller than those in the mice treated with mock injection. Furthermore, the volumes of tumors in TPVΔ66R/mIL-2-treated mice were smaller than those in wtTPV- or TPVΔ66R-treated mice, and the difference was statistically significant (P < 0.05). The mean tumor volume of the tumors treated with TPVΔ66R/mIL-2 increased from around 45 mm^3 in day 0 (the day of treatment) to 64 mm^3 in day 35, while the mean tumor volume of tumors treated with mock medium injection increased from 46 mm^3 in day 0 to 310 mm^3 in day 35. The mean tumor volume of the tumors treated with wtTPV increased from 45 mm^3 in day 0 to 153 mm^3 in day 35, while the mean tumor volume of tumors treated with TPVΔ66R increased from 45 mm^3 in day 0 to 155 mm^3 in day 35. The mean tumor growth rate (k) differed significantly among treatments (Fig 6A-C; F_3, 16 = 4.35, P = 0.020). On average tumor volume increased by 4.49%/day in the mice treated with mock injection but only by 0.06%/day in the TPVΔ66R/mIL-2-treated mice (Fig 6D). The mean tumor growth rate in the animals treated with TPVΔ66R/mIL-2 was significantly lower than in the mice treated with mock injection (Tukey’s HSD, P = 0.016). Furthermore, mean tumor growth rate in TPVΔ66R/mIL-2-treated mice was not significantly different from 0 (Fig 6A-C; t-test, t_4 =0.061, P = 0.954). Mean tumor growth rate in the wtTPV- or TPVΔ66R- treated mice was not
significantly lower than in the mice treated with mock injection (Tukey’s HSD; wtTPV: $P = 0.887$; TPVΔ66R: $P = 0.606$). In light of the attenuated replication kinetics of TPVΔ66R/mIL-2 compared to wtTPV and TPVΔ66R indicated by \textit{in vitro} studies, the \textit{in vivo} results strongly suggest the immuno-stimulatory effect of IL-2 in eliciting innate immune response for tumor reduction, in addition to the direct viral cytolysis.
Figure 6. Efficacy of TPV recombinants in tumor regression in melanoma tumor models in vivo.
SK-MEL-3 cells (5x10⁶) were injected into the dorsal surface of 6-8 weeks nude mice to induce the tumors. Mice were randomly segregated into the control or experimental groups when the tumor size reached 45 ± 4.5 mm³ and each group contained 5 mice. Tumor volumes were calculated by using the digital calipers everyday. Average tumor volumes are shown, with all groups [(A) wtTPV, (B) TPVΔ66R, (C) TPVΔ66R/mIL-2] compared to the mock group in which the mice were treated with only medium. Bars show the standard error of the mean (±1 SEM). Points indicated with an asterisk (*) refer to tumor volumes that were significantly reduced compared with the control (P ≤ 0.05). Asterisks were marked every three days if there was significance. (D) Tumor growth rate k. The changes in tumor volume over time were fit to an exponential growth model: \( Vol_t = Vol_o e^{kt} \), where \( Vol_t \) is tumor volume on day \( t \), \( Vol_o \) is the initial tumor volume, \( k \) is the growth rate coefficient, and \( t \) is time in days. \( K \) for each mouse was estimated from a linear regression of \( \log_e \) (tumor volume) on time.

**General histopathological presentation**

Overall preservation and presentation of the experimental tissues was judged as satisfactory. The tumors in the four treatment groups examined (i.e. mock, wtTPV, TPVΔ66R or TPVΔ66R/mIL-2) all carried a range of distinct histopathological features although these changes were found in different degrees across all the different groups for almost all assessed criteria (Table 1). The primary melanocyte tumor cell line was the predominate cell type present in all groups, although considerable pleomorphism existed in the form of this cell (Fig 7); this primary cell form differed in its content of melanin with the majority of cells in all groups being melanin-free cells (Fig 7A-D), in size, and also in shape (the majority of this cell type being essentially a rounded cell, however a more stellate form was seen in some tumors). In many tumors the primary cell line was clearly undergoing degeneration (the cells were swollen and lytic, and in some cases early acidification was occurring); in some treatment groups distinct areas of tissue necrosis (Fig 7E) and of early calcification were present. Few obvious mitotic figures were seen and when present were almost exclusively in the outer areas of the tumor body (Fig 7F), a location where commonly there were more cells per unit than in the central regions. Blood vessels were relatively uncommon and those present were nearly always small in size. A fibrous capsule was present in certain groups and these capsules, when most obvious, were several cell-fiber layers thick. Strikingly, there was a distinct lack of inflammatory cells infiltrating the main body of the tumor tissue; however, inflammatory cells, mostly of mononuclear morphology were present in the capsule of all groups. These capsular area
mononuclear cells contained degenerating melanin pigment in many instances (Fig 7D); most of these cells were F4/80 positive. Notably, in the tumors in one group, the TPVΔ66R/mIL-2 treated animals, the number of cells accumulating in the capsular area of this group was significantly greater than in all other groups (Fig 7L), and there was a difference in the immunocytochemical and morphological profile in the peri-tumor cellular population in this group compared to the others. The cells in the TPVΔ66R/mIL-2 treated tumors, aside from being greater in overall number than in other groups, also contained more F4/80 negative cells; these cells were mononuclear and relatively small in size possessing only sparse cytoplasm.

A general summary of the major histopathological characteristics in each group is presented in Table 1. The most extensive degeneration was seen in the TPVΔ66R/mIL-2 group, and the capsule area in this group appeared more cellulary active than the other groups; in contrast, however, there was less development of a fibrous capsule in this group compared to other groups. The amount of degenerating melanin in the capsular f4/80 positive cells was greatest in this particular group, which corresponds to the lack of melanin pigment positive tumor cells in the main body of these tumors. Other characteristics e.g. vascularity, and extent of necrosis, were similar between the different treatment groups (Fig 7A-F).

Quantification of the immunocytochemical staining showed differences between cell types and between groups (Table 2). There was no positivity to anti-CD3 staining in any of the groups. Both Ki67 positive cells and F4/80 positive cells were present in all tumor groups although in different proportions in the different groups (Fig 7G-L). Strikingly, virtually no melanin pigment containing tumor cell line was positive for Ki67, whereas a significant proportion of the non-melanin pigment containing tumor cell were Ki67 positive, although less so in the TPVΔ66R/mIL-2 group. There was a higher number of cells and a much higher proportion of Ki67 positivity in the outer areas of virtually all groups (Fig 7G). A few of the host-derived cells in the capsule were Ki67 positive but unlike the positive cells in the tumor body some of these contained degenerating melanin. F4/80 cell positivity was present only in the tissues surrounding tumors and not in the body of any tumor group; positive cells were virtually all mononuclear in form but were of various cellular shapes, e.g. stellate, round, etc.; the vast majority of these cells contained degenerating melanin. Mononuclear cells that were F4/80 negative were also present in focal areas of the capsules of the tumors in the TPVΔ66R/mIL-2 group, but this was not seen to any extent in any of the other groups. The cellularity of the
capsule in this group was considerably more that in others, and the phenotype of these cells remained predominantly F4/80 positive; the amount of degenerating pigment present in the capsule was also higher in this group, but in contrast to other test groups there was a lower amount of fibrous layers in the capsule (Fig 7G-L).

Figure 7. General histopathological and immunocytochemical staining presentation of melanoma tumor grafts.

A. Healthy, pleomorphic, tumor cells, B. Degenerating tumor cells, C. Melanin pigment containing cells, D. Capsule seeded with cells containing degenerating melanin (arrow). E. Area of gross necrosis (N) in the tumor body, F. Outer area of tumor with higher density of tumor cell present. G. Ki67 positivity higher toward the outer edge of tumors, H. Melanin containing cells in body of tumor are Ki67 negative, I. Mitotic figures stained with anti-Ki67 more common in outer areas of the tumors, J & K. Anti-F4/80 positivity seen in cells outside the tumor, cells that often contain degenerating melanin (arrow), L. H&E stain of capsule showing an accumulation of mononuclear cells and the occasional granulocyte seen in TPVΔ66R/mIL-2 treated tumors (arrow).
<table>
<thead>
<tr>
<th>Tumor Groups</th>
<th>Extent of degenerating individual melanocytes ²</th>
<th>Extent of necrotic areas</th>
<th>Extent of extratumor melanin ³</th>
<th>Quantity of melanin containing tumor cell lines</th>
<th>Extent of vascularity</th>
<th>Extent of invading inflammatory cells ⁴</th>
<th>Cellularity of extratumor capsule ⁴</th>
<th>Extent of fibrous capsule</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOCK</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>wtTPV</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>TPVA66R</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+/+</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<td>-/+</td>
<td>+</td>
<td>-</td>
<td>+++</td>
<td>+</td>
</tr>
</tbody>
</table>

¹ Assessment score: The 5 standardized areas in each tumor were scored using the following criteria: - = form not present, + = limited amounts of form present, ++ moderate amounts of the form present, +++ = form commonly present in the tumor.

² Presence of individual tumor cells lines undergoing degeneration.

³ Extent of extra-tumor cells (macrophages) containing melanin products.

⁴ Presence of inflammatory cells (*e.g.* neutrophils, lymphocytes, macrophages, *etc.*)
Table 2. Immunocytochemical studies. Assessment of cells undergoing proliferation (Ki67 positive) and the presence of macrophage cell lines (F4/80 positivity).

<table>
<thead>
<tr>
<th>Tumor Groups</th>
<th>Ki67 positivity in melanin pigment containing melanocytes ¹</th>
<th>Ki67 positivity in melanin pigment negative melanocytes ¹</th>
<th>Overall Ki67 positivity in main tumor tissue ²</th>
<th>% of F4/80 positive cells in the capsule ³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>0</td>
<td>19.6</td>
<td>16.1</td>
<td>82.8</td>
</tr>
<tr>
<td>wtTPV</td>
<td>0.1</td>
<td>16.9</td>
<td>13.8*</td>
<td>85.8</td>
</tr>
<tr>
<td>TPVΔ66R</td>
<td>0.1</td>
<td>15.4</td>
<td>14.9</td>
<td>84.1</td>
</tr>
<tr>
<td>TPVΔ66R/mIL-2</td>
<td>0</td>
<td>8.0***</td>
<td>8.9***</td>
<td>69.2</td>
</tr>
</tbody>
</table>

Mann-Whitney U test

* P ≤ 0.05, vs. Mock group
*** P ≤ 0.01, vs. Mock group

¹ Average % positive cells in 10 sites within the main body of each tumor within the respective group that include areas in the center, the mid zone and the outer zone maintaining the same formulae for selection between tumors.
² Using the same approach as in ¹, although increasing the number of sites assessed to 20, and including all cell types present in the main tumor body.
³ Average % of positive cells present in 10 areas of the capsule forming around the tumors; the presence and extent of the capsule varies depending on the tumor group.
Discussion

OVs have emerged as a promising melanoma therapy. Viruses can specifically infect tumor cells and cause tumor lysis with the release of TSAs, which will be taken up by APCs for cross-presenting to T cells and priming of anti-tumor immune response [13]. In addition, OVs can overcome the limitations of traditional systemic therapies by functioning as vectors carrying immuno-stimulatory and/or immuno-modulatory transgenes, which is likely to increase the local concentration of gene product and minimize the significant side effects caused by systemic exposure [45, 46]. For example, T-vec, which has been approved by FDA for the melanoma treatment, is a modified HSV expressing human GM-CSF which promotes immune cells recruitment and anti-tumor responses [47, 48].

TPV appears as a great candidate for melanoma virotherapy with advantages including (a) only mild disease caused by TPV infection, (b) no reported human-to-human transmission, (c) large viral genome for genetic engineering, and (d) thermo-stability for transportation and storage of the vaccines. Our previous studies have shown that TPV recombinants expressing immuno-stimulatory proteins such as flagellin. C (fli.C), GM-CSF and chemokine ligand-2 (ccL-2) exerted durable therapeutic effect in regressing colon tumor xenografts in nude mice, which strongly supported TPV as a potentially effective virotherapy [38]. Viruses including reovirus, VSV and NDV, considered as natural OVs, possess innately oncoselective replication and preferentially target and kill the melanoma cells with dysregulated genetic properties [49-51]. In comparison, viruses such as poxviruses are not considered as having high native oncoselectivity [52]. A variety of genetic modification strategies, such as transcriptional and transductional targeting, have been devised to enhance viral tumor selectivity and immuno-stimulatory capability. For example, the promoters of tyrosinase, survivin and Cox-2 genes, which are overexpressed in melanoma, have been engineered into the viral genome to drive the expression of virus genes, resulting in increased oncoselectivity for melanoma [53-56]. Deletion of genes encoding TK and RR has also been widely used, and it has been shown that ablation of TK increases cancer cell selectivity for vaccinia virus (VV) [57]. Interestingly, in our studies, TK ablation from TPV (TPVΔ66R) failed to show the effect of enhancing tumor specificity in vivo [38]. It is likely due to the decreased replication of TPVΔ66R compared to wtTPV and the intratumoral injection of virus inoculums.
Although many studies have investigated viruses expressing IL-2, the effect of IL-2 in virus replication and infection has not been fully uncovered. In this study, we demonstrated that IL-2 expressed from TPVΔ66R/mIL-2-infected cells inhibited virus replication in vitro. At both 0.1 and 5 MOIs, the replication of TPVΔ66R/mIL-2 was significantly decreased than that of parental virus and wtTPV. This is in accordance with the results drawn from previous studies that NDV and HSV expressing IL-2 replicate less efficiently than the parental viruses [26, 31]. We further showed that it was the expressed IL-2 in the tissue culture rather than the insertion of IL-2 transgene that caused the decrease of virus replication, as TPVGFP replicated less effectively when replicating in the presence of TPVΔ66R/mIL-2 in the same cell culture. In contrast to the study of Ghiasi H et al. showing that anti-IL-2 increased the replication of HSV-IL-2 in tissue culture [31], our results demonstrated that addition of anti-IL-2 in cell culture had no significant effect in enhancing the replication of TPVΔ66R/mIL-2. In addition, our studies showed that addition of the recombinant IL-2 protein in the cell culture supernatant exhibits no efficacy in attenuating the virus replication (data not shown). These results strongly suggest that the expressed IL-2 inhibited virus replication, presumably via activating certain intracellular components of the virus-infected cells. As IL-2 has been shown to activate JAK and STAT proteins [40], it is possible that the decreased replication of TPVΔ66R/mIL-2 is due to increased activation of IFN signaling pathway or IFN secretion by IL-2. However, our in vitro study showed that addition of antibody anti-IFNα or anti-IFNβ in cell culture exerted no significant effect in increasing the virus replication, indicating that the virus inhibition effect of IL-2 likely involves no immune components of IFN signaling pathway.

Similar to our study, the incorporation of other immuno-stimulatory cytokines such as IFN and GM-CSF into OV's has also been shown to cause a reduced capacity for viral replication and earlier clearance of the therapy, despite the fact that expression of these chemokines and cytokines results in overall increase of therapeutic effect [58-60]. In light of these observations, approaches to maintain the viral replicating oncolysis while increasing the immuno-therapeutic efficacy are highly desirable. Chen H et al. have devised an externally controlled system that the transgene of interest is fused with a destabilizing or degradation domain which leads to rapid proteasomal degradation of the protein. The addition of a molecule (such as Shield-1) which binds to the degradation domain and shields it from proteasomal degradation and thus restores
the protein function [61]. Regulation of the time for destabilizing the cytokine potentially permits enhanced virus delivery before cytokine functional activity exerts.

It is well established that IL-2 has pleiotropic effects and is required for the activation and proliferation of many cell types, including T cells, NK cells and macrophages [62, 63]. Different from T cells, NK cells and macrophages recognize and target cancer cells in a non-MHC restricted manner [23]. Once activated by IL-2, NK cells acquire increased lysing capability with enhanced expression of activation markers such as CD25 and CD69 on the cell surface [64], while macrophages become larger and more granular with increased cytotoxicity [23, 24]. Although IL-2 has been shown to function as the immuno-regulatory link between adaptive and innate immune cells, previous studies of cancer virotherapies expressing IL-2 have mainly focused on the involvement of adaptive immune cells. It has been shown that IL-2 expression from OVs assists in recruiting CD4$^+$ and CD8$^+$ T cells and upregulating IFN-$\gamma$ release [26, 33, 62]. In contrast to these studies, we focused on the substantial efficacy of IL-2 in potentiating innate immune cells and therefore performed the study in athymic nude mice in which only innate immune system is intact. Our results demonstrated that TPV$\Delta$66R/mIL-2 produced a robust therapeutic effect in regressing melanoma xenografts in the nude mice. TPV$\Delta$66R/mIL-2 showed statistically significant reduction, with the final tumor volumes after 35 days of treatment being only 48% larger than those in day 0 (initiation of treatment) and nearly 5-fold smaller than those in the mock group. Although both wtTPV and TPV$\Delta$66R also exhibited remarkable tumor regression, the efficacy was less significant than that of TPV$\Delta$66R/mIL-2 (Final tumor volumes after 35 days of treatment with wtTPV were nearly 255% larger than day 0 tumor volumes; final tumor volumes after 35 days of treatment with TPV$\Delta$66R were around 242% larger than day 0 tumor volumes). These results are in accordance with previous studies reporting that viruses expressing IL-2 decreased the tumor volumes and increased the survival time of the tumor-bearing animals [33]. Moreover, our histopathological analyses clearly showed that mononuclear cells, potentially both lymphocytes and macrophages, were more abundantly present in the tumors treated with TPV$\Delta$66R/mIL-2. While anti-CD3 staining showed no positivity in tumor samples of all groups, indicating the absence of T cell response, anti-Ki67 staining showed more cell degeneration and greater amount of degenerating melanin present in TPV$\Delta$66R/mIL-2-treated tumors. Our results provide compelling evidence
that IL-2 plays a robust protective role in eliciting host innate immune responses for tumor regression in addition to the direct viral cytolysis.

While OVs have been used as cancer monotherapy, more and more groups are actively exploring strategies to combine OVs with other anti-tumor treatments to fully realize OVs’ therapeutic potential. An emerging promising strategy consists of combining viral oncolysis and adoptive cell transfer. It has been shown that adoptively transferred CD8$^+$ T cells exhibits melanoma regression efficacy, and further intra-tumoral injection of oncolytic VSVs causing localized inflammation and activation of T cells remarkably increases the cell therapy’s effectiveness [13]. Also, clinical studies involving the combinational administration of oncolytic adenovirus and adoptive T cell transfer have shown the overall objective response of 38.5% in melanoma patients [65]. An alternative approach of this combinational strategy has been achieved by loading the oncolytic viruses onto the T cells serving as carriers, intending to minimize the virus neutralization and increase the virus tumor localization [66-68]. Based on the previous studies and our work [69-71], it is appealing to consider the combination of virotherapy armed with IL-2 and adoptive T cell transfer. Besides the innate anti-tumor response and viral lysis caused by IL-2 expressing virotherapy, this strategy would also potentially elicit T-cell-mediated antitumor efficacy as well as IL-2-mediated T cell proliferation.
References


34

35

CHAPTER III

TANAPOXVIRUS LACKING A NEUREGULIN-LIKE GENE REGRESSES HUMAN MELANOMA TUMORS IN NUDE MICE

Background

Melanoma is a common skin cancer with significant morbidity. It accounts for approximately 4% of all skin cancers, but for nearly 80% of all skin-cancer related deaths [1-3]. If not detected at the early stage, metastatic melanoma is highly aggressive with the five-year survival rate of approximately 15% [4]. Significant progress has been made in the development of treatment options for melanoma where current therapeutic options include surgery, chemotherapy, radiotherapy, immunotherapy, cryotherapy, and targeted drugs (such as BRAF inhibitors). These advances have not significantly changed the survival of melanoma patients with advanced disease [5]. In the continuous quest for development of new therapeutic agents for melanoma, apoptosis inducing agents and oncolytic viruses (OVs) are relatively new additions.

The OVs, which either selectively or preferentially infect and destroy tumor cells by lysis and possibly by inducing an anti-tumor immune response, appear to be one of the most promising approaches to treat melanoma [6-8]. Some of the common broad strategies used during the development of OVs include increasing tumor selectivity and induction of a strong anti-cancer immune response. Infecting and replicating in tumor cells in a selective manner is an inherent feature of certain viruses. For viruses without significant native tumor specificity, oncoselectivity can usually be achieved by targeted genetic engineering to increase the viral tropism and/or the efficiency of replication in the cancerous cells [9-10]. For example, thymidine kinase (TK) and ribonucleotide reductase (RR) are important for DNA synthesis and their levels of expression are elevated in cancerous cells as compared to normal cells [11]. Therefore, deletion of genes encoding TK and RR in OVs is a commonly used strategy to increase tumor selectivity of DNA viruses [9]. Further, OVs can be genetically modified and equipped to express immune stimulatory proteins, such as granulocyte monocyte-colony stimulating factor (GM-CSF), macrophage chemotactic protein-1, flagellin and interleukin 2 (IL-2), to activate the host innate and adaptive immune systems to trigger tumor regression [9, 11-12]. Several DNA and RNA viruses, including vesicular stomatitis virus (VSV), adenovirus and Newcastle disease
virus (NDV), have been genetically modified and/or “armed” with immune modulatory genes for use in advanced clinical trials [6, 13-16]. A genetically engineered adenovirus (H101) was approved by China’s State Food and Drug Administration (CFDA) in 2005 for treating head and neck cancer [17-18]. Talimogene laherparepvec (T-VEC), a herpes simplex virus-1 (HSV) strain modified to express human GM-CSF, was approved by the U.S. FDA for melanoma treatment in October 2015 [19].

Tanapoxvirus (TPV), which belongs to the family *Poxviridae* (genus *Yatapoxvirus*), is a large virus containing double-stranded (ds) DNA genome (approximately 144 kbp) [20]. TPV, which is endemic in equatorial Africa, causes a mild self-limiting disease in humans, and most of the global population (with the exception of Africa) is immunologically naïve. Additionally, no man-to-man transmission has been reported [21-23]. With a large genome for genetic modification and highly attenuated virulence, TPV can potentially serve as an ideal candidate for oncolytic virotherapy. Previous studies have revealed that poxviruses employ various strategies to modulate the host anti-viral immune responses, including the secretion of cytokine and cytokine- and/or chemokine-binding receptor homologs [24-27]. It has been shown that a 45-kDa protein is encoded by the *TPV-2L* gene, which functions as a high-affinity inhibitor of human tumor necrosis factor (TNF) [28-29]. Our previous studies have also shown that TPV-15L encoded protein biologically mimics neuregulin (NRG). As one of epidermal growth factors (EGF), NRG is the ligand for ErbB3 and ErbB4 that are associated with therapeutic resistance in many cancers, such as melanoma, breast cancer and prostate cancer [30-33]. We have also demonstrated that TPV-15L protein is capable of binding and phosphorylating the NRG receptor heterodimer ErbB2/3 in an established NRG bioassay [34].

The ErbB family of tyrosine kinases receptors includes ErbB1 (epidermal growth factor receptor [EGFR]), ErbB2, ErbB3 and ErbB4 [35-36]. The binding of ligands to the ErbB receptors leads to the formation of homo- or hetero-dimers and the activation of the intrinsic kinase domain. These activities, in turn, initiate a signal transduction cascade that ultimately leads to DNA synthesis and cell proliferation [37-39]. NRG specifically binds to ErbB3 and ErbB4 receptors. Binding of NRG to ErbB3/ErbB4 changes the conformation of receptors and induces hetero-dimerization with ErbB2, which results in the activation of downstream signaling [40-41]. ErbB3 expression has been found to be enhanced in malignant melanoma cells and is associated with poor prognosis and reduced patient survival rate [42-44]. NRG-treated
melanocytes show increased proliferation and invasion, altered morphology, and increased expression of progression and metastasis genes [40]. Further, it has been shown that the knockdown of NRG results in significant inhibition of melanoma growth and that melanoma tumors treated with anti-ErbB3 antibody showed slower proliferation [45]. Recent research advances have shown success with melanoma therapies targeting activated BRAF. It has been demonstrated that NRG is highly expressed in dermal fibroblast and cancer associated fibroblasts (CAF), and enhances the growth of RAF-inhibited BRAF V600E/D melanoma cells. Further, the safeguarding effects of fibroblast-derived NRG on melanoma cells treated with RAF inhibitors can be impeded effectively by ErbB2 and ErbB3 neutralizing antibodies. Upregulation of ErbB3 and enhanced responsiveness to NRG was observed as a form of adaptive resistance to RAF/MEK inhibitors in mutant BRAF melanoma [46-50]. These studies provide a solid theoretical basis for targeting ErbB signaling in combination with RAF inhibitors in mutant BRAF melanoma [51].

In this study, we show that the NRG mimicking protein encoded by the TPV-15L gene exhibits a similar growth promoting effect on human mutant BRAF melanoma cells SK-MEL-3 in vitro. In light of this, we genetically engineered TPV with a 15L gene deletion and without/with the deletion of 66R gene that encodes TK, thus generating TPVΔ15L and TPVΔ15LΔ66R, respectively. TPVΔ66R, with 66R/TK gene deletion was generated earlier in our laboratory [11]. Deletion of the 15L gene had no significant effect on TPV replication in vitro. However, TPVΔ15LΔ66R replicated less efficiently than wtTPV, TPVΔ66R and TPVΔ15L. Our in vivo study showed that TPVΔ15L regressed human melanoma tumors in nude mice more significantly than that of wtTPV, TPVΔ66R or TPVΔ15LΔ66R. Taken together, our results suggest that TPVΔ15L can be a promising candidate for oncolytic virotherapy of melanoma.
Materials and methods

Cell lines, virus, and reagents

Owl monkey kidney (OMK) cells, human lung fibroblasts (WI-38) and human melanoma cell line SK-MEL-3 were purchased from American Type Culture Collection (ATCC product numbers CRL-1556, CCL-75, HTB-69 respectively). OMK and WI-38 were cultured and propagated in complete growth medium containing Earle’s Minimum Essential Medium (EMEM) supplemented with 10% (vol/vol) fetal bovine serum (FBS) (Atlanta Biologicals), 2 mM L-glutamine (Sigma-Aldrich) and 50 µg/ml gentamicin sulfate (AMRESCO). SK-MEL-3 were cultured in growth medium consisting of McCoy’s 5A medium (Sigma-Aldrich) with 15% FBS. McCoy’s 5A medium with no serum was used as starving medium for melanoma growth. All cell monolayers infected with virus were maintained in maintenance medium with all components being the same as the growth medium except for FBS concentration being 2% vol/vol. All cell lines were incubated at 37°C in a 5% CO₂ atmosphere. All cell counting and viability assays were conducted using an Improved Neubauer hemacytometer and 0.2 % (wt/vol) trypan blue in a normal saline solution. The wtTPV (Kenya strain) was originally a gift from Dr. Joseph Esposito (Centers for Disease Control, Atlanta, GA, USA). NRG1 was purchased from R&D Systems, MN.

Construction of the recombinant TPVs

A plasmid derived from a commercially available cloning vector pBluescript II KS (+) was used to include the genomic sequences flanking the right and left sides of TPV-15L open reading frame (ORF). The left flanking was generated by using polymerase chain reaction (PCR) with forward primer 5’-TAGGTACTCGAGAAAAACACCAATA-3’ (XhoI) and reverse primer 5’-GTTTAAATCGATGGACCTG-3’ (ClaI). Two primers were designed for amplifying the right flanking as forward primer 5’-CATATTTTGCGGCGCGCGTAAACATT-3’ (NotI) and reverse primer 5’- GTTAAAAATGGAAAAAGAGCTCTAATTTTAACAACAG-3’. A synthetic poxvirus early/late (E/L) promoter and a green fluorescence protein (GFP) gene were in between left and right flanking sequences. The plasmid was named as p15LGFP plasmid for generating a TPV-15L knockout virus (TPVΔ15L). This plasmid was transfected (Superfect transfection reagent; Qiagen) into 35-mm tissue culture dishes plated with OMK cell monolayer infected with
wtTPV at 1 multiplicity of infection (MOI). The cells with transfection and infection were incubated at 37 °C incubator with 5% CO₂ until visible fluorescence caused by GFP expression appeared. The virus was harvested, and recombinant virus TPVΔ15L was plaque purified at least 3 times until no visible non-fluorescence plaques, and no 15L gene was detected by confirmation PCR. Plaque assays were carried out as described earlier [52].

A second plasmid based on pBluescript II KS (+) was generated to produce a TPV-66R gene knock-out virus. The left flanking and right flanking of 66R ORF were inserted in the plasmid, with an E/L synthetic promoter driving the expression of mCherry in between two flanking sequences. The left flanking was amplified using PCR with Sacl forward 5’-AATGGATCACATAAAGGAGCTCTTAACG-3’ and NotI reverse 5’-CAGAAAAACATGCGGCCGCATATAATCT-3’. The right flanking was generated using EcoRI forward 5’-GGAGATGAACAAGAAATAGAATTGAG-3’ and HindIII reverse 5’-CTGTTCTTTATCACAAAGCTTCTATCGGGTG-3’. The generated plasmid was named as p66RmCherry plasmid. By using TPV genomic flanking sequences for either 15L or 66R, the engineered plasmid could be guided to a specific point where a targeted ablation occurs (ablation of 15L or 66R gene) with the simultaneous replacement of a fluorescent reporter (GFP/mCherry). All plasmids were confirmed by DNA sequencing.

This p66RmCherry plasmid was transfection into OMK cell monolayer infected with wtTPV at 1 MOI. Via the process of transfection and infection, the recombinant virus TPVΔ66R was generated with visible fluorescence caused by mCherry expression. The virus was harvested, and TPVΔ66R was plaque purified at least 3 times by using a plaque purification assay, with the selection enabled by the expression of mCherry as described earlier [53]. Also, the p66RmCherry plasmid was transfection into TPVΔ15L-infected OMK cells, and recombinant TPV with both TPV-15L and TPV-66R ablated (TPVΔ15LΔ66R) was generated. TPVΔ15LΔ66R was plaque purified with the selection by both mCherry and GFP expression. The purity of the generated recombinant viruses was determined by confirmation PCR [11]. The TPV-15L deletion was confirmed by amplifying using internal forward primer 5’-CACACCTTTTTCCGTAAATTGCC-3’ and internal reverse primer 5’-GTTTTTTACTTTATCATGTGTCATTATTAGC-3’. TPV-66R deletion was confirmed by amplifying internal forward primer 5’-CGGTATCAAATTGCTAGGTATACCTTG-3’ and internal reverse primer 5’-CTCCAATTCGTTTTAGAAAACGATGC-3’. Internal primers for
**TPV-136R** gene are forward 5’-GTATTATGTACTGTTTCAACTAAACAAAGC-3’ and reverse 5’-CCTTTAGGTGTTAGGATATATCAATTATACAG-3’. The primers for amplifying **ampicillin** gene in the plasmid are forward primer 5’-CTCACGTAAAGGGATTGTCATGAG-3’ and reverse primer 5’-CCGCTCATGAGACAATAACCCTG-3’.

**Expression and purification of TPV-15L protein**

**TPV-15L** gene was amplified using forward primer 5’-GGGGATCCATGAAAAACAAATTTATG-3’ (BamHI) and reverse primer 5’-CGCTCGAGATTTACTATTTTTCAC-3’ (XhoI). The 15L amplicon was cloned into pcDNA3.1/myc/His ver C (Invitrogen), producing a fusion-tagged construct which was subsequently cloned into the pFastBac-Dual-eGFP cassette. Recombinant baculovirus was generated using Bac-to-Bac system (Invitrogen). The recombinant baculovirus was used to infect sf21 insect cells and the cell supernatant was collected and TPV-15L protein was purified using a hexa-His Co²⁺ chelate resin affinity column (BD sciences) routinely employed in our laboratory [34].

**Growth of human melanoma cells in the presence of TPV-15L protein**

Human melanoma cells (SK-MEL-3) were plated in a 96 well plate in McCoy’s 5A medium with 15% FBS and incubated overnight at 37°C with 5% CO₂ in humidified chambers. The next day, cells were switched to the serum-free McCoy’s 5A medium and incubated for 24 hours before the addition of NRG or TPV-15L protein. Cells were treated with either TPV-15L purified protein (500ng/ml) or NRG (500ng/ml). Untreated cells were used as mock. Total number of viable cells were determined in each group at day 1, 3 and 5 post-treatment, by counting cells on a hemocytometer chamber using trypan blue stain to exclude non-viable cells. Each experiment was repeated three times independently and standard deviations were calculated.

**Replication of TPV recombinants**

Virus replication was determined in OMK, WI-38 and SK-MEL-3 cells. Cell monolayers planted in 24-well plates were infected with wtTPV or one of the recombinants of
TPV (TPVΔ66R, TPVΔ15L and TPVΔ15LΔ66R) at 0.1 and 5 MOI. Infected cells were harvested at 48, 96 and 240 hours post infection (hpi) and total virus titer was determined by viral plaque assay on OMK monolayers in 6-well plates. Each experiment was repeated 3 times.

**Animals**

Male athymic nude mice (Crl:NU(NCr)-Foxn1\textsuperscript{nu}) were purchased from Charles River company (Wilmington, Massachusetts, US) at 6-8 weeks of age. Mice were housed individually with food and water available in pathogen-free animal facility at Western Michigan University. Animals were allowed to acclimatize for one week before experimentation. All protocols for treatment and manipulations were approved by the Institutional Animal Care and Use Committee of Western Michigan University (IACUC protocol number 13-07-01).

**Virotherapy of human melanoma tumor xenografts**

Melanoma tumors were induced in nude mice by injecting \(5 \times 10^6\) viable SK-MEL-3 cells suspended in 100 µl of phosphate saline buffer (PBS), subcutaneously on the dorsal surface in the inter-scapular region. Cell viability assays were conducted using trypan blue following each injection to test the cell viability. Tumor volume was calculated by using the digital calipers to measure three dimensionally according to the formula \(\frac{4}{3} \times 3.1415 \times \frac{(\text{length}/2) \times (\text{width}/2) \times (\text{height}/2)}{}\) in mm\(^3\). Mice were randomly segregated into control and experimental groups, when the tumor size reached \(45 \pm 4.5\) mm\(^3\), with each group containing 5 mice (n=5). The number of animals per group was determined by power analysis and n of 5 is standard procedure for in vivo experiments including quantitative pathology. Each mouse in experimental group received a single intratumoral injection of \(5 \times 10^6\) pfu virus particles in 100 ul medium. Animals in mock group received 100 µl medium only. The day of treatment administration was considered as day 0, and mouse weights and tumor volumes were measured and recorded every day. All the mice were sacrificed at 35 days post treatment, and the tumors were harvested. The blood sample of each mouse was collected and centrifuged for the collection of the serum. Tumors were weighed and stored for histological analyses.
Statistical analysis

All *in vitro* experiments were done in triplicates and the measurements were presented as mean ± SD. The two-sample unequal variance Student’s t test analysis was applied for testing the differences. The significance level used was P < 0.05. Student’s t test was used for assessing the treatment effect of each experimental virotherapy group compared to the mock group *in vivo*. The tumor volumes of each experimental group were compared with those in the mock group, and significance was considered if P < 0.05.
Results

TPV-15L protein and NRG increase melanoma cell proliferation

We have shown that TPV-15L is a secreted early protein that phosphorylates NRG receptors and binds to heparin [34]. Since NRG has also been shown to promote melanoma progression and metastasis [40], we sought to investigate if TPV-15L protein could have a similar effect on melanoma cells. SK-MEL-3 cells in the serum-free medium were treated with NRG or TPV-15L protein (500ng/ml) and incubated for 1, 3 and 5 days. Cells in the control wells received no treatment. Compared to control, cell numbers of those treated with NRG and TPV-15L were significantly higher as early as on day 1 (P < 0.05). At day 3, SK-MEL-3 cells treated with NRG and TPV-15L increased by 110% and 86%, respectively, demonstrating continued proliferation, while those with no treatment achieved only 2.5% growth. At day 5, NRG- and TPV-15L-treated cells achieved 125% and 120% growth, respectively, while those with no treatment gained only 69% growth (Fig 1). These results provide compelling evidence that TPV-15L protein, like NRG, promoted melanoma growth. The proliferative efficacy of TPV-15L was indistinguishable from NRG (P < 0.05).

Figure 8. Effect of TPV-15L protein and NRG on the proliferation of human melanoma SK-MEL-3 cells.
Each well in 96-well plate was planted with $2 \times 10^4$ SK-MEL-3 cells. Growth medium was replaced with serum-free (starving) medium containing purified TPV-15L protein (500 ng/ml) or NRG (500 ng/ml). Viable cells were counted on day 1, 3 and 5 following treatment. Untreated cells served as mock. Each experiment was repeated three times independently and standard deviations are shown.

**Generation of TPVΔ15L and TPVΔ15LΔ66R**

In the light of functional similarities between TPV-15L protein and NRG in enhancing melanoma proliferation, we sought to delete 15L gene from TPV (TPVΔ15L) and to test as an OV for melanoma virotherapy. The recombinant virus TPVΔ15L was plaque purified at least three times until no visible wtTPV plaques appeared. Deletion of 15L ORF was then confirmed by PCR. Similarly, ORF 66R which encodes TK was deleted from TPVΔ15L. TK is present in abundant quantities in cancerous cells and its deletion from virus has widely been used to enhance tumor selectivity of DNA viruses [9]. For this purpose, TPVΔ15L-infected cells were transfected with p66R-mCherry plasmid containing mCherry reporter gene under E/L synthetic promoter. The resulting TPVΔ15LΔ66R was plaque purified at least 3 times as previously described. The exhibition of both mCherry and GFP was confirmed using fluorescence microscopy. The deletions of both 15L and 66R genes were further confirmed by PCR, using primers described in Materials and Methods. Fig 2 demonstrates that wtTPV contained both 15L and 66R ORFs, while ORF 15L was deleted from TPVΔ15L. Similarly, ORF 15L and ORF 66R were absent in TPVΔ15LΔ66R. ORF 136R was used as a positive control in these experiments and was present in all three viruses, namely wtTPV, TPVΔ15L and TPVΔ15LΔ66R. Ampicillin gene contained in plasmid was amplified in all three viruses to exclude single crossover recombinants. These experiments provide compelling evidence that 15L and/or 66R gene were deleted and that these viruses were generated as a result of double crossover recombination events during transfection and infection.
PCRs were used for verifying the ablations of 15L and 66R genes. Genomic DNA from each virus was isolated and probed for sequences internal in 15L, 66R, 136R and ampicillin genes. Genes ablated showed no bands in the gel. TPV-15L internal fragment is 197 bp; 66R region is 379 bp and 136R internal fragment used as a control has a predicted amplicon length of 531 bp. Ampicillin gene was probed for determining the insertion of ampicillin or other genes from plasmid into viral DNA during the transfection-infection process when homologous recombination occurs. Lanes: wtTPV (control); TPVΔ15L (15L is ablated); TPVΔ15LΔ66R (both 15L and 66R are ablated). Moreover, no insertion of ampicillin gene is shown in the viral DNA of TPVΔ15L and TPVΔ15LΔ66R.

Replication of TPV recombinants in human and monkey cells

Replication curves of wtTPV, TPVΔ66R, TPVΔ15L and TPVΔ15LΔ66R were generated using 0.1 and 5 MOI in OMK cells and human fetal lung fibroblasts (WI-38) and shown in Fig 3. The infected cells were harvested and virus was titrated on OMK monolayers at 48, 96 and 240 hpi. The first time point for harvesting (48 hpi) was decided based on the eclipse phase period of TPV which is around 48 hpi, as TPV unlike vaccinia virus (VV) is a slow replicating virus [52]. Results in Fig 3 demonstrate that wtTPV and recombinant TPVs achieved the maximum titer at around 96 hpi as expected [34, 52-53]. At 0.1 and 5 MOI in OMK cells, TPVΔ15L and wtTPV exhibited similar replication kinetics. At 0.1 MOI, the yield of TPVΔ15LΔ66R was time dependent, with the titer being lower than wtTPV and TPVΔ66R at 48
hpi. By 96 hpi, however, the titer had attained levels similar to those of wtTPV and TPVΔ66R. At 5 MOI on OMK cells, the replication of TPVΔ15LΔ66R was not significantly lower than wtTPV and TPVΔ66R. At 0.1 MOI in WI-38 cells, TPVΔ15L showed similar replication kinetics as wtTPV and more effective replication than TPVΔ66R. TPVΔ15LΔ66R exhibited less efficient replication than TPVΔ15L, with the maximum yield at 96 hpi being 7-8 fold lower than that of TPVΔ15L. When infecting WI-38 cells at 5 MOI, TPVΔ15L, TPVΔ66R and TPVΔ15LΔ66R showed similar titers which were lower than those of wtTPV. The above data supports the earlier results that 1) TPV-15L is non-essential for the replication of TPV in cell culture and that 2) deletion of TPV-66R adversely affects the viral replication.

Figure 10. TPVΔ15L and TPVΔ15LΔ66R replication compared to wtTPV and TPVΔ66R.

OMK and WI-38 cell monolayers in 24-well plates were infected with wtTPV, TPVΔ66R, TPVΔ15L and TPVΔ15LΔ66R at 0.1 and 5 MOI. The virus was harvested at 48, 96 and 240 hpi.
and titrated on OMK monolayers. Each experiment was repeated three times independently. (a) viral infection at 0.1 MOI in OMK cells, (b) viral infection at 0.1 MOI in WI-38 cells, (c) viral infection at 5 MOI in OMK cells, (d) viral infection at 5 MOI in WI-38 cells.

Replication of TPVΔ15L and TPVΔ15LΔ66R in SK-MEL-3 cells

The replication characteristics of TPVΔ15L and TPVΔ15LΔ66R were also examined in BRAF mutant melanoma SK-MEL-3, and compared with wtTPV and TPVΔ66R. SK-MEL-3 cells were infected at 0.1 and 5 MOI and the total virus titer was determined at 48, 96 and 240 hpi. As shown in Fig 4, at 0.1 MOI, the replication of TPVΔ15L displayed a slight delay in the onset of the replication. However, the virus titer of TPVΔ15L reached a similar level as wtTPV at 96 hpi. In contrast, TPVΔ66R and TPVΔ15LΔ66R replicated less efficiently than wtTPV and TPVΔ15L. At 96 hpi, the titer of TPVΔ15LΔ66R was 5-6 fold lower compared to TPVΔ15L and the titer of TPVΔ66R was 3-4 fold lower than that of TPVΔ15L. At 5 MOI, TPVΔ15L exhibited similar replication kinetics with wtTPV. At 96 hpi, virus titers reached the maximum, with the titer of TPVΔ15L being 2-3 fold higher than that of TPVΔ66R and 3-4 fold higher than that of TPVΔ15LΔ66R. The results showed that deletion of 66R gene but not 15L gene resulted in a significant delay of TPV replication in SK-MEL-3 melanoma cells *in vitro.*

![Figure 11. Replication of TPVΔ15L and TPVΔ15LΔ66R in melanoma cells.](image)

SK-MEL-3 cell monolayers in 24-well plates were infected with wtTPV, TPVΔ66R, TPVΔ15L and TPVΔ15LΔ66R at (a) 0.1 MOI and (b) 5 MOI. The virus was harvested at 48, 96 and 240 hpi and titrated on OMK monolayers. Each experiment was repeated three times independently.
Oncolytic virotherapy of human melanoma tumors xenografted in nude mice

To evaluate the oncolytic effectiveness of wtTPV and TPV recombinants, human melanoma tumors were induced by injecting BRAF mutant SK-MEL-3 cells subcutaneously on the dorsal surface in the inter-scapular region of athymic nude mice. Treatments consisted of intratumoral mock or virus injections, when the tumor volumes reached $45 \pm 4.5 \text{ mm}^3$. While mock-treated tumor xenografts continued to grow exponentially, xenografts treated with viruses exhibited continued but slowed tumor growth. As shown in Fig 5, there were significant differences in the tumor growth between virus-treated groups and mock group ($P < 0.05$). Compared to mock-treated tumors achieving nearly 552% growth, tumors treated with wtTPV and TPVΔ66R displayed 250% and 244% growth, respectively, after 35 days of treatment. TPVΔ15L produced significantly greater tumor growth delay than wtTPV and TPVΔ66R ($P < 0.05$). At 35 days post treatment, TPVΔ15L-treated tumors exhibited only 68% growth compared to day 0. Interestingly, TPVΔ15LΔ66R showed markedly less tumor growth inhibition efficacy (286% tumor growth) compared to TPVΔ15L, perhaps due to the poor replication of the virus when ORF 66R was deleted (Fig 3 and Fig 4). These results showed that TPVΔ15L was able to significantly reduce the tumor progression at more time points in comparison to the mock than other TPVs. We believe that the enhanced oncolytic potential of TPVΔ15L was due to deletion of TPV-15L gene which encodes a viral protein that contributes to melanoma proliferation, as demonstrated by our in vitro studies. As TPV replicates only in humans and monkeys, nude mice with xenografted melanoma tumors were not suitable for studies related to the bio-distribution and safety of the virus.
Figure 12. Effectiveness of mutant viruses in tumor suppression in melanoma tumor models in vivo.

Melanoma tumors were induced in 6-8 week old athymic nude mice by subcutaneously injecting $5 \times 10^6$ SK-MEL-3 cells onto the dorsal surface. Mice were randomly segregated into the control or experimental groups with each group containing 5 mice ($n=5$) when the tumor size reached $45\pm4.5$ mm$^3$. Mice in the experimental groups were treated with intratumoral injections of $5 \times 10^6$ pfu of virus, while the mice in the mock group were intratumorally injected with medium only. Tumor volumes were measured using the digital calipers every day. In each graph shown here, the y-axis represents the average percentage of tumor growth (%) and the x-axis is time (days post virotherapeutic treatment). All experimental groups are compared to the mock group in which the mice were treated with only medium injection. Bars show the standard error of the mean ($\pm1$ SEM). Points indicated with an asterisk (*) refer to tumor growth that was significantly reduced from the control ($p\leq0.05$). Asterisks were marked every three days if there was significance. (a) wtTPV, (b) TPVΔ66R, (c) TPVΔ15L, (d) TPVΔ15LΔ66R.
Discussion

In the search for novel anti-cancer strategies, OV s have emerged as one of the most promising therapeutic options to eliminate cancer cells. They also overcome tumor-induced immune evasion in host and elicit an effective anti-tumor immune response. Viruses which are under evaluation for therapeutic purposes can be divided into two major groups: (a) viruses that possess a degree of innate oncoselectivity, such as reovirus, VSV and NDV [10, 54-56], and (b) viruses which require genetic modifications to enhance their tumor selectivity and immune stimulatory capability, such as VV with ablation of TK gene [57-58]. Although OV s have shown encouraging results in experimental and clinical trials, the efficacy of an OV is still potentially limited by several factors. First, the anti-viral immune response developed by the host immune system, such as the induction of neutralizing anti-viral cellular and humoral immune responses, inactivates the virus and impedes the effectiveness of virus administered systemically. The “barriers” such as neutralization and sequestration of the virus make it uncertain for OV s to be “one shot” therapeutic agents, and also make that virus less effective during successive treatment cycles. Strategies have been devised to prime the immune response with one viral vector and boost with another vector, in order to focus the immune response on the tumor antigens and avoid anti-viral immune responses [59-60]. Although, an effective virus-specific immune tolerance strategy will ultimately solve this issue, presently it may be necessary to have a set of antigenically distinct OV s for serial injections to avoid this problem. Second, the safety profile of a virus determines its feasibility as the virotherapy, and transmission from human to human in combination of continued virus evolution pose a serious concern for virus application. Only a small number of viruses such as HSV and adenovirus have been demonstrated as safe for application in clinical trials [54]. In light of these concerns, TPV appears to be an ideal OV candidate due to its characteristics. These include: (1) most of the global population is immunologically naïve to TPV, (2) TPV causes a mild, self-limiting febrile disease in humans making it relatively safer to use, (3) TPV is not transmitted from human to human, and (4) the large genome size of TPV (144 kbp) allows incorporation of several immune stimulatory transgenes.

At present, advanced malignant melanoma is one of the diseases with very few effective treatments. Although significant advances have been made in monitoring and treatment of
melanoma, the mortality associated with the disease remains largely un-altered [5]. Due to the limited efficiency and significant side effects of chemotherapies, a variety of immunological approaches have been devised, among which interferon-α (IFN-α) and IL-2 have shown significant efficacy and have been approved by U.S. FDA for melanoma treatment [61-63]. The OVs appear to be an appealing addition to melanoma therapies, due to the viral oncoselectivity and lysis of tumor cells [13]. Combination of virotherapy and other therapies, such as chemotherapy, have also shown synergistic effects [64].

Our previous studies of TPV genomic analyses revealed that TPV-66R gene encodes TK and that TPV-15L gene encodes EGF-like growth factor often referred to as NRG [34, 65]. Further, we have demonstrated that TPV-15L protein functions as a mimic of NRG, capable of binding and phosphorylating the most potent NRG heterodimer receptor ErbB2/3 [34]. Elevated NRG levels have been shown to contribute to melanoma proliferation by binding to ErbB3, while melanoma treated with anti-ErbB3 has shown reduced cell proliferation [30, 45]. Thus, it would be interesting and worthwhile to further explore the application of neutralizing agents against ErbB receptors or NRG for melanoma therapy.

A variety of virokines carrying EGF-like sequence, such as vaccinia virus growth factor (VGF) and myxoma virus growth factor (MGF), have been demonstrated to act on ErbB receptors and promote cell proliferation [66-67]. In this study, we showed that TPV-15L protein and NRG both significantly increased the proliferation of the human melanoma cells (SK-MEL-3) *in vitro*. This further supports our earlier claim about the functional similarity between TPV-15L and NRG. In the light of these findings, we deleted TPV-15L gene both with and without the deletion of TPV-66R (TK) gene, generating two TPV recombinants TPVΔ15L and TPVΔ15LΔ66R. The replication kinetics of TPVΔ15L and TPVΔ15LΔ66R was studied in OMK, WI-38 and SK-MEL-3 cells. We demonstrated that TPVΔ15L had similar replication efficacy as wtTPV and that TPVΔ15LΔ66R replicated less efficiently than TPVΔ15L and wtTPV. The data supports our earlier results showing that TPV-15L is a nonessential gene for TPV replication and that TPV-66R exerts more significant effect on viral replication [34].

We further studied whether deletion of TPV-15L gene (encoding NRG mimicking protein) will abolish the growth promoting effect of TPV-15L protein on human melanoma cells (SK-MEL-3), and enhance oncolytic efficacy of TPV in melanoma-bearing nude mice. Tumor xenografts were established using SK-MEL-3 cells, which were treated with intratumoral
injection of wtTPV, or one of the TPV recombinants (TPVΔ66R or TPVΔ15L or TPVΔ15LΔ66R). Our results demonstrated that TPVΔ15L exhibited a more robust tumor reduction compared to wtTPV, TPVΔ66R and TPVΔ15LΔ66R. TPVΔ15LΔ66R was less effective in regressing tumor, possibly due to its slower replication, which has been demonstrated in vitro. Moreover, the deletion of TPV-66R gene failed to make the virus tumor-selective. The greater tumor reduction efficacy of TPVΔ15L compared with wtTPV and other TPV recombinants suggests that (1) the reduced melanoma proliferation possibly relates to the absence of tumor-enhancing properties caused by TPV-15L protein and that (2) deletion of viral genes encoding NRG-like proteins is potentially an effective strategy to be used in genetic engineering of OVs for melanoma. Considering the pathological power of abnormal ErbB signaling and its contribution to oncogenesis in cancer biology, deletion of EGF-like growth factor genes from certain OVs and/or blocking the ErbB receptors would likely offer additional tumor suppression effects for oncolytic virotherapies. Similarly, Saydam et al [68] have shown that HSV expressing small interference RNA (siRNA) directed against EGFR inhibits human glioblastoma cell growth more significantly than the control virus both in vitro and in vivo.

The results of this study warrant further investigation of TPVΔ15L as an effective vector for oncolytic virotherapy, while more strategies could be incorporated to further enhance its oncolytic efficacy in the therapy of melanoma and other cancers. TPV-2L gene encodes a 38kDa secretory protein which binds and neutralizes TNFα [29] with high affinity [28]. It has been shown that TNF downregulates the expression of ErbB2 in pancreatic tumor cells, which demonstrates the inverse relation between TNF and ErbB2 [69]. In addition, ErbB2 overexpression induces resistance of breast cancer cells to the cytotoxic effect of TNFα [70]. Therefore, it would be appropriate to consider the construction of an oncolytic TPV recombinant with both 15L and 2L genes ablated, which will synergistically decrease the ErbB-related tumor proliferation and increase the viral oncolysis. Also, it is conceivable to delete TPV-136R gene encoding type I IFN binding receptor from TPVΔ15L backbone, as host IFN will potentially be “saved” from being neutralized and exert antiproliferative efficacy.

Coupling viral oncolytic activity with immune cells recruitment proves to be another attractive strategy for attaining the optimal clinical efficacy. Oncolytic VV JX-594 and more recently Amgen’s oncolytic HSV, T-vec, are both armed with GM-CSF, suggesting the clinical benefit of enhancing host antitumor immunity [8]. In addition to virotherapies expressing GM-
CSF, many other viruses expressing cytokines such as IL-2, IL-12 and IL-24 have shown encouraging tumor regression efficacy [9, 71-72]. Other strategies such as promoting virus spread and increasing cytotoxicity have also been applied in OV modification. Engineered virus expressing relaxin and decorin known as extracellular matrix modulating proteins, have shown enhanced penetration, persistence and spread compared to the control virus in melanoma, glioma and lung cancer [73-74]. Introduction of suicide genes such as FCU1 encoding bifunctional fusion protein which converts nontoxic 5-FC to toxic 5-fluorouracil and 5-fluorouridine monophosphate, has also been proven to be a feasible strategy for enhancing OV’s effectiveness in different cancers [75]. Based on these findings and the oncolytic efficacy of TPVΔ15L shown in this study, we speculate that the modifications described above to TPV or TPVΔ15L may further improve the outcome of TPV as an oncolytic virotherapy.
References

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CHAPTER IV

NEUTRALIZATION OF MATRIX METALLOPROTEINASE-9 ENHANCES THE PRODUCTIVITY OF TANAPOXVIRUS IN MELANOMA CELLS IN VITRO

Background

Melanoma is one of the most aggressive skin cancers, with respect to tumor cell invasion and metastasis [1, 2]. If not detected early, metastatic melanoma has approximately a 15% five-year survival rate [3]. As the primary cause of the mortality of patients, metastasis is a complex process including the invasion of the tumor cells to the surrounding tissues and basement membranes, the penetration into the blood and lymphatic vessels, and the re-penetration in other organs to form detectable tumors [4]. Different proteolytic enzymes, which degrade the extracellular matrix (ECM) proteins and basement membranes, have been shown to promote the liberation and seeding of the tumor cells, therefore increasing melanoma cell migration and metastasis [5-7]. For instance, the positivity of matrix metalloproteinase-1/3 (MMP-1/3) in melanoma has been associated with increased malignancy and decreased overall survival [5].

MMPs are a family of calcium-dependent zinc-containing endopeptidases that include collagenases, gelatinases, stromelysins and others [8]. Several MMPs, such as MMP-1, MMP-2, MMP-9 and MMP-13, are directly involved in the melanoma progression and are indicative of a poor prognosis [9-13]. In addition, tissue inhibitors of metalloproteinase (TIMPs), which inhibit the activation of latent enzymes or the proteolytic ability of active MMPs, decrease experimental and spontaneous tumor growth and metastasis, and possess an anti-metastatic effect on a number of cancer model systems [4]. Approximately 22 MMPs have been characterized and cloned to date, and each targets specific protein substrates [4]. For instance, MMP-9, also known as gelatinase B, degrades type IV and V collagens and gelatin, which are essential components of the ECM [14].

Increased MMP-9 expression is correlated with many diseases and pathological abnormalities, including arthritis [15], atrial fibrillation [16] and cancers including melanoma [4]. Expression of MMP-9 has been shown to enhance melanoma growth and lung colonization [17]. In addition, in transgenic mice MMP-9 contributed to both keratinocyte proliferation and
skin carcinogenesis [18]. Studies have also demonstrated that MMP-9 is expressed in tumor and stromal cells as well as in tumor-infiltrating immune cells, such as neutrophils and macrophages. The expression of MMP-9 by the bone marrow-derived inflammatory cells has been shown to induce the angiogenesis and enhance the tumor growth in animal models [18]. Therefore, host MMP-9 may contribute to cancer cell metastasis [19].

Several virus infections and their accompanying pathogenesis are associated with the induction of MMP-9. For instance, respiratory syncytial virus (RSV) infection is a common cause of bronchiolitis and pneumonia in premature infants, resulting in substantial morbidity and mortality. It has been shown that RSV infection increases MMP-9 expression in human epithelial cells and reduced MMP-9 results in decreased virus replication in vitro [20]. Increased MMP-9 expression is also observed in the influenza virus-infected kidney cells in vitro [21], as well as in flu-infected human patients [22]. Moreover, infection of macrophages with human immunodeficiency virus (HIV) also results in increased MMP-9 production [23]. Although induction of MMP-9 is frequently observed during virus infections and elevated MMP-9 levels are often correlated with disease severity, the functional role of MMP-9 in viral replication and infection still remains controversial. For example, neuroblastoma cells transduced with MMP-9 showed significantly enhanced distribution and replication of herpes simplex virus (HSV) as a viral vector [24], but an antiviral effect of MMP-9 has been demonstrated in RSV infection in the airway epithelial cells in vivo [25].

Tanapoxvirus (TPV) is a benign human virus, which causes self-limiting febrile illness [26-28]. Belonging to the family Poxviridae (genus Yatapoxvirus), TPV is a double-stranded (ds) DNA virus containing a genome of approximately 144 kbp [29]. With attenuated virulence and a large genome for manipulation, TPV has proved itself as an appealing oncolytic virus (OV) candidate. In our previous studies, recombinant TPV armed with flagellin. C (Fli.C) was shown to effectively reduce the human colorectal tumors xenografted in athymic nude mice [30]. In addition, we demonstrated that TPV-15L gene product, a mimetic of neuregulin (NRG) [31], stimulates melanoma cell proliferation. Recombinant TPV lacking TPV-15L gene significantly regressed melanoma tumors in animal models [32].

In this study, we show that TPV replication in melanoma cells is significantly decreased with the addition of MMP-9. In addition, the infection of human melanoma SK-MEL-3 cells with wtTPV and TPV recombinants remarkably induced the expression of the MMP-9,
while blocking of MMP-9 significantly increased the TPV replication in human melanoma SK-MEL-3 cells and decreased the cell survival. Discovery of a mechanism that will suppress MMP-9 expression upon TPV infection will potentially improve the use of TPV as an oncolytic virotherapy.
Materials and methods

Cell lines, virus, and reagents

Owl monkey kidney (OMK) cells and human melanoma cell line SK-MEL-3 were purchased from the American Type Culture Collection (ATCC product numbers CRL-1556 and HTB-69 respectively). OMK cells were cultured in complete growth medium containing Earle’s Minimum Essential Medium (EMEM) supplemented with 10% (vol/vol) fetal bovine serum (FBS) (Atlanta Biologicals), 2 mM L-glutamine (Sigma-Aldrich) and 50 µg/ml gentamicin sulfate (AMRESCO). SK-MEL-3 were cultured in a growth medium consisting of McCoy’s 5A medium (Sigma-Aldrich) and 15% (vol/vol) FBS. Macoy’s 5A medium without FBS was used as starving medium for melanoma growth. All cell monolayers infected with virus were maintained in a medium that is identical to the growth medium except that it contained 2% (vol/vol) FBS. All cell lines were incubated at 37°C in a 5% CO₂ atmosphere. All cell counting and viability assays were conducted in a normal saline solution containing 0.4 % (wt/vol) trypan blue using an Improved Neubauer hemacytometer. The wtTPV (Kenya strain) was originally a gift from Dr. Joseph Esposito (Centers for Disease Control, Atlanta, GA, USA). TPVGFP is a recombinant TPV that was modified to express green fluorescent protein (GFP) and provided by Dr. Grant McFadden. MMP-9 purified protein was purchased from Sino-Biological, China and anti-MMP-9 antibody was purchased from R&D Systems, MN.

Generation of the recombinant TPVs

The p66R-mCherry plasmid was derived from a commercially available cloning vector pBluescript II KS (+) and was engineered to produce a virus in which the TPV-66R gene was knocked out through homologous recombination in the process of transfection and infection. The genomic sequences flanking the left and right sides of 66R open reading frame (ORF) were inserted in the plasmid. The left flanking sequence was amplified using PCR and primers containing SαI or NotI restriction endonuclease (RE) cut sites: 5’-AATGGATCACAATAAGGAGCTCTTAACG-3’ (forward) and 5’-CAGAAACATGCGCCGATATAATCT-3’ (reverse). The right flanking sequence was isolated using primers containing EcoRI or HindIII RE cut sites: 5’-GGAGATGAACAAGAATAGAATTTCATAGG-3’ (forward) and 5’-

65
CTGTTCTTTATCACAAGCTTCTATCGGGTG-3’ (reverse). An early/late (E/L) synthetic poxvirus promoter [33] and a mCherry gene for expression were inserted in between the two flanking sequences. OMK cell monolayers plated in 35-mm tissue culture dishes were transfected (Superfect transfection reagent; Qiagen) with p66R-mCherry plasmid and infected with wtTPV at a multiplicity of infection (MOI) of 5. Transfected and infected OMK cells were incubated at 37 °C with 5% CO2 until mCherry fluorescence was observed. The virus was harvested, and recombinant virus TPVΔ66R was plaque purified until all plaques displayed mCherry fluorescence and no 66R gene was detected by confirmation PCR. Plaque assays were performed as described earlier [30].

The p15L-GFP plasmid was also derived from pBluescript II KS (+) and engineered to produce a virus in which the TPV-15L gene was knocked out (TPVΔ15L) by homologous recombination in transfection and infection. The procedures described for p66R-mCherry plasmid construction were used. The left flanking sequence of TPV-15L ORF was generated using primers that contained XhoI or ClaI RE cut sites: 5’-TAGGTACTCGAGAAAAACACCAATA-3’ (forward) and 5’-GTTTTAATCGATGGGACCTG-3’ (reverse). The right flanking sequence was isolated using primers containing NotI or SacI RE cut sites: 5’-CATATTTCGCGCGCGGTAACCAATT-3’ (forward) and 5’-GTTTAAATGAGAAAAGAGCTCTTATTTAACAACAG-3’ (reverse). A synthetic poxvirus early/late (E/L) promoter and a GFP gene were in between left and right flanking sequences. All plasmids were confirmed by DNA sequencing.

**MMP-9 antiviral assay**

Human melanoma cells (SK-MEL-3) were plated in a 48-well plate in McCoy’s 5A medium with 15% FBS and incubated overnight at 37°C with 5% CO2 in humidified chambers. The next day, cells were infected with TPV-GFP at 0.1 or 5 MOIs. After virus adsorption for 1 hour, the virus inocula were replaced with 250 µl serum-free McCoy’s 5A medium containing 0.1 µg/ml, 1 µg/ml, or 10 µg/ml purified human MMP-9 proteins. Infected cells with no MMP-9 treatment were used as mock. Virus replication and cell viability were determined in each group at day 4 post-treatment. The total number of viable cells was evaluated by counting cells on a hemocytometer chamber, and trypan blue stain was used to exclude non-viable cells. Viral
replication was assessed using plaque assays. Each experiment was repeated three times independently and standard deviations were calculated.

**Reverse transcriptase-polymerase chain reaction (RT-PCR)**

SK-MEL-3 cells plated in T75 flasks were infected with 1 MOI of wtTPV, TPVΔ66R or TPVΔ15L respectively. A mock-infected culture served as a control. After 72 hours, cells were harvested and total RNA was extracted using the Purelink RNA mini kit (Thermo Fisher Scientific). Each RNA sample was then dissolved in DEPC-treated water and adjusted to the same volume. MMP-9 cDNA was amplified using RT-PCR with the 5’-GAGACCGGTGAGCTGGATAG-3’ forward primer and the 5’-CAAACTGGATGACGATGTCTGC-3’ reverse primer. The primers used to amplify GADPH cDNA were 5’-CTCTGATTGCTGATTGGG-3’ (forward) and 5’-TGATTTTGGAGGGATCTCGC-3’ (reverse). After 20, 25, 30, 35, 40 cycles, PCR products amplified from each RNA sample were separated on a 2% agarose gel containing ethidium bromide.

**Western blot analysis**

SK-MEL-3 cell monolayers were cultured on 35 mm petri-dishes and infected with wtTPV, TPVΔ66R or TPVΔ15L at 5 MOI respectively. Mock-infected cells served as a control. Infected SK-MEL-3 cells were incubated in serum-free medium at 37°C until the monolayer was destroyed. Cells were harvested and lysed with sterile ice-cold 1% NP-40 and proteins were collected. Samples were mixed with 5× SDS gel loading buffer (25% glycerol, 5% SDS, 0.002% bromophenol blue, 15% β-mercaptoethanol) and boiled for 3 min. Protein samples were separated on SDS-PAGE gels and transferred to Immobilon-P polyvinylidene difluoride (PVDF) membrane using a semi-dry transfer apparatus (Bio-Rad Trans-Blot SD) at 14V for 1.25 hours. Membranes were blocked in TBST (20 mM Tris, 137 mM NaCl [pH 7.6], 0.1% Tween 20) containing 5% nonfat dry milk for 2 hours at room temperature, and subsequently incubated with 1:1000 dilution of the goat anti-MMP-9 primary antibody (R&D systems) at 4°C overnight. The membranes were washed five times for 10 min each with TBST before incubation with a 1:7500 dilution of anti-goat IgG-horseradish peroxidase (HRP) (Sigma) in TBST with 5% nonfat dry milk at room temperature for 1 hour with gentle agitation. The membrane was washed five times
for 10 min each with TBST, and the signal was detected by using enhanced chemiluminescence reagents (Thermo Scientific).

**Anti-MMP-9 assay**

Human melanoma cells (SK-MEL-3) were plated in a 48-well plate in McCoy’s 5A medium with 15% FBS and incubated overnight at 37°C. The following day, cells were switched to a serum-free medium containing 0.1 µg/ml, 1 µg/ml, or 10 µg/ml anti-MMP-9 and incubated for 2 hours at 37°C. Cells incubated with medium containing no anti-MMP-9 served as mock. Following incubation, the cells were infected with TPVΔ15L at 0.1 or 5 MOI. Virus replication and cell viability were assessed at 2 and 4 days post-treatment. Total numbers of viable cells were evaluated by counting cells on a hemocytometer chamber using trypan blue stain to exclude non-viable cells. Viral replication was determined via using plaque assays. Each experiment was repeated three times independently and standard deviations were calculated.

**Statistical analysis**

All the *in vitro* experiments were done in triplicates and the measurements were presented as mean ± SD. The two-sample unequal variance Student’s t test analysis was applied for testing the differences. The significance level used was P < 0.05.
Results

MMP-9 reduces the TPV replication in melanoma cells

The role of MMP-9 in virus replication varies between viruses. MMP-9 can have antiviral activity and inhibit the replication of some viruses [25], while MMP-9 expression will enhance the infectivity of other viruses and increase the severity of the associated symptoms [20]. The effect of MMP-9 on TPV replication is unknown and requires further investigation as enhanced MMP-9 expression is observed in many cancers and TPV is a potential oncolytic therapy. To investigate the effect of MMP-9 on TPV replication, SK-MEL-3 cells were infected with TPVGFP at 0.1 and 5 MOI and incubated in the serum-free medium containing MMP-9 protein (0.1, 1, 10 µg/ml respectively) for 96 hours. The infected cells in the mock wells received no treatment of MMP-9. The yield of TPV-GFP was significantly reduced upon treatment with MMP-9. Infection with a MOI of 0.1 was not significantly affected by 0.1 µg/mL MMP-9 treatment, but 1 and 10 µg/ml MMP-9 treatments did result in significant decrease in the number of plaques (p < 0.05) (Fig 1). Compared to the replication of TPVGFP with no MMP-9 treatment, the 10 µg/ml MMP-9 treatment resulted in a ~10-fold decrease in virus titer. Incubation with MMP-9 also protects infected melanoma cells, since there is a significant increase in cell survival (P < 0.05). The percentage of infected cell survival upon treatment with 10 µg/ml MMP-9 was approximately 2-fold greater than that of those in mock wells. At 5 MOI in SK-MEL-3 cell, TPVGFP replication was significantly decreased only with treatment of 10 µg/mL MMP-9, and was around 3 fold lower than that with no MMP-9 treatment (P < 0.01). Cell survival, however, was significantly increased with both 1 and 10 µg/mL treatments (P < 0.05). These results provide compelling evidence that MMP-9 exerts an antiviral activity on TPV replication and protects TPV-infected cells.
Figure 13. Effect of MMP-9 on the replication of TPVGFP and proliferation of human melanoma SK-MEL-3 cells.

Each well in 48-well plate was planted with $5 \times 10^4$ SK-MEL-3 cells. Cells were infected with
TPV infection induces the expression of MMP-9

Elevated expression of MMP-9 has been observed in the cells and organisms infected with different viruses including RSV [20, 34], influenza virus [21, 35] and coxsackievirus [14]. In addition, a variety of cytokines, such as interleukin (IL)-8, IL-6, transforming growth factor (TGF) and MMPs, are associated with melanoma progression and metastasis. Therefore, we sought to test the effect of TPV infection on the expression of MMPs in melanoma cells. The expression of MMP-9 was monitored using RT-PCR and western blot analyses. Both analyses reveal that MMP-9 expression was significantly enhanced in SK-MEL-3 cells infected with wtTPV, TPVΔ66R and TPVΔ15L, relative to control samples that were mock infected. Moreover, the MMP-9 expression appeared even more remarkable in TPVΔ15L-infected cells, compared with that in wtTPV- and TPVΔ66R-infected cells (Fig 2). While MMP-2, the other gelatinase, has been shown to degrade type IV collagen and promote cancer invasion and metastasis [36-38], we have been unable to detect MMP-2 in SK-MEL-3 cells, either infected or uninfected (data not shown). Moreover, expression of mRNAs of TGF-β, protein kinase R (PKR), interferon-stimulating gene (ISG)-15 and IL-8 has also been detected in the SK-MEL-3 cells, and elevated TGF-β was also observed in the SK-MEL-3 cells infected with TPVs compared with that in uninfected cells (data not shown). MMP-9 expression is clearly enhanced by TPV infection, even when 15L and 66R genes are knocked-out of the genome.
Figure 14. Verification of MMP-9 expression in SK-MEL-3 cells

(a) RT-PCR amplification of MMP-9. PCR products were amplified using the mRNAs extracted from the cells with mock infection, infection with wtTPV, TPVΔ66R and TPVΔ15L. The PCR products amplified after 20, 25, 30, 35 and 40 cycles were separated on a 2% agarose gel with ethidium bromide. Marker is the 500 bp DNA ladder. No PCR products were detected in the negative controls, in which either no RNA or reverse transcriptase was present (data not shown).

(b) Western blot analysis of MMP-9 expression. SK-MEL-3 cells were infected with wtTPV, TPVΔ15L, TPVΔ66R and medium (mock) separately. The cells were harvested after 72 hours and centrifuged. The cell pellets were subsequently frozen and thawed three cycles to release the MMP-9 from the cytoplasm, which were collected as the cell lysate samples. The protein samples were subjected to 12% SDS-PAGE gel and western blot analysis using a goat antibody against MMP-9 and the secondary anti-goat HRP conjugated antibody. ECL was applied for visualization of the bands.

Neutralization of MMP-9 enhances TPV replication in melanoma cells

In light of the antiviral effect exerted by MMP-9 on TPV replication and the induced expression of MMP-9 by TPV infection, the effect of blocking MMP-9 on the replication of TPV in melanoma cells was assessed in vitro. A neutralizing anti-MMP-9 antibody was used at different concentrations (0.1, 1, 10 µg/mL respectively) to block MMP-9 activity in SK-MEL-3 cells infected with TPVΔ15L at MOIs of 0.1 and 5 (Fig 3). The infected cells with no
antibody treatment served as mock. The total virus titer and percentage of cell survival was assessed at 48 and 96 hours post-infection (hpi). At 0.1 MOI of infection, the incubation of infected cells (0.1 MOI) with 10 µg/mL anti-MMP-9 significantly increased replication of TPVΔ15L at both 48 and 96 hpi (P < 0.05). Cell survival was significantly decreased at 48 hpi upon treatment with 1 ug/ml anti-MMP-9, while the 10 ug/ml anti-MMP-9 treatment significantly reduced cell survival at both 48 and 96 hpi, relative to the controls (P < 0.05). At a MOI of 5, TPVΔ15L virus replication at 48 and 96 hpi was enhanced by approximately 2 fold when treated with 10 ug/ml of anti-MMP-9 (P < 0.05). Infected cell survival at 48 hpi was significantly decreased upon treatment with 1 and 10 ug/ml anti-MMP-9 (P < 0.05), while a significant decrease in cell survival at the 96 hpi was only observed with the 10 ug/ml anti-MMP-9 treatment (P < 0.05). The results reveal that blocking MMP-9 expression in TPV infected melanoma cells significantly increased virus replication and cellular apoptosis.
Figure 15. Effect of anti-MMP-9 on the replication of TPVΔ15L and the cell survival of infected SK-MEL-3 cells.

Each well in 48-well plate was planted with 5 x 10^4 SK-MEL-3 cells. Following adhesion, cells were incubated in the serum-free medium containing anti-MMP-9 at 0.1, 1 and 10 ug/ml for 2 hours in 37°C incubators. Following incubation, the cells were infected with TPVΔ15L at 0.1 or 5 MOI. The infected cell incubated with medium containing no anti-MMP-9 served as mock. Fluorescence and light images were taken at 48 hpi (a and b) and 96 hpi (g and h). The titer of TPVΔ15L at 0.1 MOI and 5 MOI was evaluated at 48 and 96 hpi using plaque assay and shown as (c) 0.1 MOI at 48 hpi; (d) 5 MOI at 48 hpi; (i) 0.1 MOI at 96 hpi and (j) 5 MOI at 96 hpi. The number of viable infected cells in each treatment group was counted on hemocytometer chamber using trypan blue stain at 48 and 96 hpi. The percentage of cell survival was determined at 48 hpi and 96 hpi and calculated as 100 × (number of viable cells in each treatment group/viable cell number in the wells with no infection and no MMP-9 treatment). The percentage of cell survival of cells infected with 0.1 MOI TPVΔ15L is shown in (e) (48 hpi) and (k) (96 hpi), while that of cells infected at 5 MOI is shown in (f) (48 hpi) and (l) (96 hpi). Each experiment was repeated three times independently and standard deviations are shown.
Discussion

In this study, the interplay between MMP-9 expression and TPV replication is investigated in melanoma cells. TPV infection stimulates elevated expression of MMP-9 in melanoma SK-MEL-3 cells (Fig 2), which is similar to the effect observed in the cells or organisms infected with other viruses, such as RSV [20], dengue virus [39] and coxsackievirus [14]. Nevertheless, some other viruses, such as human cytomegalovirus (HCMV), decrease the MMP-9 expression and instead increase the production of TIMPs [40]. The study also revealed that TPV replication is inhibited by the presence of MMP-9. The addition of MMP-9 to TPV infected melanoma cells decreased the virus titer and increased melanoma cell survival (Fig 1). Also, specifically inhibiting MMP-9 in TPV-infected melanoma cells resulted in a significant increase in virus replication and decreased cell survival (Fig 3). The effect of MMP-9 on TPV is opposite to what is observed for other viruses. For example, MMP-9 has been shown to increase the syncytia formation of RSV which contributes to the virus replication and spread [34].

MMPs have been demonstrated to protect the tumor cells and promote cancer cell survival in many ways. For example, MMP-7 has been shown to protect the sarcoma and colon cancer cells by cleaving Fas-ligand and reducing the Fas-mediated cell apoptosis [41]. It has also been shown that MMP-9 promotes the tumor cell survival and proliferation via coordination with TGF-β and CD44 [42]. Similarly, we show here that MMP-9 treatment protects SK-MEL-3 cells infected by TPV and increases the cell survival. Inhibition of MMP-9 further decreased cell survival of the infected cells (Fig 1 and 3). In light of the contribution of MMPs to cancer aggressiveness and the corresponding poor prognosis, a variety of MMP inhibitors have been designed to block the activity of MMPs [43]. The use of MMP inhibitors as an oncotherapy has led to unsatisfactory outcomes in clinical trials, likely due to several reasons. First, although MMPs are essential in promoting tumor progression at early stages, targeting MMPs for treatment is probably less effective for cancers that have metastasized. Second, since expression levels of different MMPs vary among cell types, the inhibitors used may not target the MMPs responsible for cancer progression. For example, SK-N-AS neuroblastoma and U373 glioma cells have been identified as lower expressers of MMP-9, compared to SNB-19 glioblastoma and U251 neuroblastoma cells [24]. Third, more and more studies have revealed that certain MMPs possess highly complicated functional activities. While MMPs have been correlated with cancer
metastasis and elevated angiogenesis, some recent studies have shown that MMPs in some cancers plays a protective role against tumor progression, therefore serving as tumor suppressors. Although over-expression of MMP-9 is often associated with cancer progression, it induced tumor regression and generation of anti-angiogenesis components in breast cancers [44, 45]. In addition, increased expression of MMP-8 has also been shown to prevent lung metastasis of melanoma cells through modulating tumor cell adhesion and invasion [46]. Therefore, based on the functionally diverse roles of MMPs, it is necessary to determine the effect of a MMP on particular cancer types before administering MMP inhibitors.

OVs have emerged as an appealing option for cancer therapies, as they can selectively replicate in the tumor cells, cause the cell lysis, and stimulate the host anti-tumor immune responses. A variety of viruses, such as HSV [47], coxsackievirus [48], reovirus [49] and vaccinia virus (VV) [50], have been demonstrated for their enormous oncolytic potential and tested in the clinical trials for cancer treatment. Talimogene laherparepvec (T-vec) and JX-594, respectively the recombinant HSV and VV both expressing granulocyte monocyte colony-stimulating factor (GM-CSF), have been approved for treatment of melanoma and head and neck cancers [51]. While tumor microenvironment plays a critical role in tumor progression and metastasis and affects OVs’ delivery and spread in the tumors, strategies have been designed to target tumor microenvironment in order to enhance the efficacy of OVs. Transgenes of collagenase, hyaluronidase and MMPs, which are enzymes that degrade ECM components, have been incorporated into OVs to assist the virus replication and spread [52-54]. Since MMPs have variable functions and effects on cancer cells, careful consideration of the incorporated MMP is required. For example, while both MMP-8 and MMP-2 show the ECM-degrading activities, only MMP-8 has been shown to suppress melanoma growth [54]. Consequently, an OV that incorporates MMP-8 may be better as a melanoma therapeutic than one that incorporates MMP-2. Nevertheless, a comparison between MMP-8 and MMP-2 expressing viruses would be critical to determine their relative oncolytic efficacies. To identify MMP-expressing OVs that have maximal efficacy, the MMPs’ efficacy in enhancing virus spread, and the effects of the MMP on tumor progression and oncolytic virus replication must be considered.

Our previous studies have clearly demonstrated the oncolytic efficacy of TPVs [30, 32, 55]. While a recombinant TPV that expressed Fli.C remarkably regressed colorectal tumors, a TPVΔ15L variant significantly reduced melanoma tumors xenografted into athymic
nude mice using SK-MEL-3 cells [30, 32]. Since the presence of MMP-9 can reduce the efficacy of oncolytic viruses used as chemotherapies, studies into the interplay between MMP-9 and TPV are necessary, and prompted the current investigation. The results reveal that TPVΔ15L infection significantly elevates MMP-9 expression, and that MMP-9 protects SK-MEL-3 melanoma cells infected with TPV by reducing virus replication and promoting cell survival. Confirmation that MMP-9 was responsible for protecting the cells was obtained by neutralizing MMP-9 in SK-MEL-3 cells. The suppression of MMP-9 activity resulted in significantly increased replication of TPVΔ15L and increased cell lysis. In light of the antiviral effect of MMP-9 on TPV replication, it is reasonable to speculate that the oncolytic efficacy of TPVΔ15L was partially attributed to MMP-9’s effect of enhancing virus spread. The evaluation of a combined TPVΔ15L and anti-MMP-9 therapy of melanoma *in vivo* would be interesting, since neutralization of MMP-9 would potentially increase virus yield but also reduce virus spread, relative to the to TPVΔ15L monotherapy.

While expression of cytokines and enzymes such as MMP-9, TGF-β, PKR and IL-8 has been confirmed in the melanoma SK-MEL-3 cells, elevated TGF-β expression following TPV infection was also observed in this work (data not shown). TGF-β regulates the expression of MMPs, through the TGF-β-inhibitory element (TIE) binding site found in the promoters of MMP-1, 7, 9 and 13 [56, 57]. MMP-9 proteolytically activates the latent TGF-β via cleaving the latency-associated peptide (LAP) from TGF-β precursor [42]. Therefore, TGF-β and MMP-9 function in a bidirectional manner to regulate each other in cancer progression. It would be interesting to postulate that the MMP-9 induced by the TPV infection subsequently stimulated the elevated expression of TGF-β. Similarly to MMP-9, TGF-β has been demonstrated as either tumor suppressor or tumor promoter. While TGF-β plays a critical role in inducing apoptosis and antitumor responses in the pretumoral stage, it is highly expressed and secreted in the advanced stages of a variety of cancers, such as colorectal cancer, breast cancer and prostate cancer [57]. Further studies are required to assess if the elevated level of TGF-β in infected SK-MEL-3 cells is associated with the over-expression of MMP-9, and results in enhanced or reduced melanoma cell proliferation.


In this study previously described in Chapter II, our data indicates that IL-2 plays a protective role in inhibiting virus replication, via a mechanism occurring intracellularly without the involvement of IFN signaling pathway. In addition, we demonstrated that TPV expressing IL-2 was therapeutically effective in regressing melanoma without the involvement of the adaptive immune responses. Taken together, our results strongly support that TPVΔ66R/mIL-2 is potentially a promising melanoma therapy, and warrant the further investigation of TPVΔ66R/mIL-2 as oncolytic virotherapy and immunotherapy in future clinical applications.
Oncolytic potency of TPV with deletions of immunomodulators

In this study previously described in Chapter III, we show that the NRG mimicking protein encoded by the *TPV-15L* gene exhibits a similar growth promoting effect on human mutant BRAF melanoma cells SK-MEL-3 *in vitro*. In light of this, we genetically engineered TPV with a 15L gene deletion and without/with the deletion of 66R gene that encodes TK, thus generating TPVΔ15L and TPVΔ15LΔ66R, respectively. Deletion of the 15L gene had no significant effect on TPV replication *in vitro*. However, TPVΔ15LΔ66R replicated less efficiently than wtTPV, TPVΔ66R and TPVΔ15L. Our *in vivo* study showed that TPVΔ15L regressed human melanoma tumors in nude mice more significantly than that of wtTPV, TPVΔ66R or TPVΔ15LΔ66R. Taken together, our results suggest that TPVΔ15L can be a promising candidate for oncolytic virotherapy of melanoma.
Immuno-modulatory activities related to TPV

As was previously described in Chapter IV, we show that TPV replication in melanoma cells is significantly decreased with the addition of MMP-9. In addition, the infection of human melanoma SK-MEL-3 cells with wtTPV and TPV recombinants remarkably induced the expression of the MMP-9, while blocking of MMP-9 significantly increased the TPV replication in human melanoma SK-MEL-3 cells and decreased the cell survival. Discovery of a mechanism that will suppress MMP-9 expression upon TPV infection will potentially improve the use of TPV as an oncolytic virotherapy.
Appendix A

Institutional Biosafety Committee

2016
Project Approval Certification

For Institutional Biosafety Committee Use Only

Project Title: Oncolytic Tanapoxvirus

Principal Investigator: Karim Essani

IBC Project Number: 16KEb

Date Received by the Institutional Biosafety Committee: November 25, 2015

☑ Reviewed by the Institutional Biosafety Committee

☑ Approved
☐ Approval not required

I [Signature]
Chair of Institutional Biosafety Committee Signature

12/11/2015
Date
Appendix B

Date: June 29, 2015

To: Karim Essani, Principal Investigator
Bruce Bejcek, Co-Principal Investigator

From: Lisa Baker, Chair

Re: IACUC Protocol Number 13-07-01

This letter will serve as confirmation that the change to your research project “Oncolytic Tanapoxvirus” requested in your memo received June 28, 2015 (to add student investigator Dennis Kordish) has been approved by the Institutional Animal Care and Use Committee.

Approval Termination: June 28, 2016