
Electronic Thesis and Dissertation Repository

4-21-2016 12:00 AM

Oncolytic Virus Therapy for the Treatment of Metastatic Ovarian Cancer

Jessica Tong
The University of Western Ontario

Supervisor
Dr. Trevor Shepherd
The University of Western Ontario

Graduate Program in Anatomy and Cell Biology
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy
© Jessica Tong 2016

Follow this and additional works at: <https://ir.lib.uwo.ca/etd>



Part of the [Cancer Biology Commons](#), [Cell Biology Commons](#), [Medical Cell Biology Commons](#), [Medical Microbiology Commons](#), [Obstetrics and Gynecology Commons](#), [Oncology Commons](#), and the [Virology Commons](#)

Recommended Citation

Tong, Jessica, "Oncolytic Virus Therapy for the Treatment of Metastatic Ovarian Cancer" (2016).
Electronic Thesis and Dissertation Repository. 3707.
<https://ir.lib.uwo.ca/etd/3707>

This Dissertation/Thesis is brought to you for free and open access by Scholarship@Western. It has been accepted for inclusion in Electronic Thesis and Dissertation Repository by an authorized administrator of Scholarship@Western. For more information, please contact wlsadmin@uwo.ca.

Abstract

The management of patients with epithelial ovarian cancer (EOC) faces two major challenges which standard treatments fail to effectively address: 1) Diffuse metastasis as a consequence of late stage diagnosis and 2) intra-tumoral heterogeneity, which fuels tumor evolution and drives the acquisition of chemotherapeutic resistance. In this thesis, we tested new therapeutic strategies using a 3-dimensional *in vitro* spheroid culture model that mimics key steps of epithelial ovarian cancer metastasis; and another model that mimics both temporal and cellular heterogeneity by establishing multiple cell lines from a single patient over the course of disease progression. Using these models, we investigated the therapeutic efficacy of three oncolytic viruses for treatment of ovarian cancer: Myxoma virus (MYXV), a modified Vaccinia virus (vvDD), and Maraba virus (MRBV). We determined that all three viruses were capable of inducing some level of oncolysis, but that spheroid formation limited the replication efficiency of poxviruses (MYXV and vvDD), which heavily rely on cell proliferation. However, upon spheroid reattachment, poxvirus oncolysis was restored and prevented cell dispersion. MRBV was least affected by spheroid formation, although there was a capacity for some cell lines to develop resistance to MRBV upon spheroid formation. We discovered MRBV uses the low-density lipoprotein receptor (LDLR) to gain entry to host cells and that entry into spheroids was affected by dynamic changes in LDLR expression. However, we observed that this was only a partial mediator of MRBV tropism. In our *in vitro* assays of tumor heterogeneity, we observed temporal changes that directly impact MRBV oncolysis and we identified two major groups of subclones in our patient derived cell lines: one that was highly susceptible to MRBV, and another set which exhibited 1000-fold reduced susceptibility to MRBV-

mediated oncolysis. Differential susceptibility to MRBV virus oncolysis did not strictly depend on LDLR expression. Furthermore, co-culture of virus-sensitive and virus-resistant cells conferred sensitization of virus-resistant cells to MRBV oncolysis. We therefore sought other mechanisms which could impact MRBV tropism and found that oncolysis could be significantly increased through the induction of TGF β signaling and epithelial-to-mesenchymal transition, commonly activated pathways found during ovarian cancer metastasis. Taken together, these findings not only define the differential therapeutic efficacies between oncolytic viruses for metastatic EOC, but also identify key trophic factors which impact MRBV oncolysis that can be exploited to enhance MRBV-mediated oncolysis for EOC in future therapeutic strategies.

Co-Authorship

Portions of this thesis were completed with the experimental guidance of Yudith Ramos-Valdes and Samah Rafehi. Quantification of some data generated was performed by Rachel Dales, Nicole Lesmeister, Milani Sivapragasam, and Yingke Yang.

Acknowledgements

I would like to thank my supervisor Dr. Trevor Shepherd for his support over the course of my PhD and for giving me the opportunity to work on this project, which I have come to develop a deep passion for. I would also like to acknowledge the advice and guidance provided by my graduate advisory committee, especially Dr. Gabriel DiMattia, who have made significant contributions to this project's success. In addition to my superiors, I owe a lot of my personal development to my lab mates and friends, Yudith, Samah, Adrian, and Parima. They made every day a fun and pleasurable experience – I learned a lot from them. All of the aforementioned people have directly influenced my development, both in critical scientific thought and personal growth.

I would also like to express my gratitude to my parents and family who supported my personal ambition to pursue higher education and encouraged me to excel in both my professional and personal life. I am incredibly fortunate to have such a loving, nurturing, and selfless family. This includes my husband, Matt Rytelewski, whose emotional support and pragmatism has helped me get through difficult times when I felt like quitting. The culmination of my work as reflected in the completion of this thesis is in part a reflection of all the encouraging people in my life, both professional and domestic, who have created a security net that made it impossible for me to fail. Thank you.

Table of Contents

Abstract.....	i
Acknowledgements.....	iii
List of Tables	vii
List of Figures.....	viii
List of Supplementary Figures.....	ix
List of Abbreviations	x
CHAPTER 1: General Introduction	1
1.1 Clinical presentation, frequency, and treatment of epithelial ovarian cancer ..	1
1.2 Origin and pathogenesis of epithelial ovarian cancer.....	4
1.3 Genome instability, heterogeneity, and disease recurrence in EOC.....	7
1.3.1 Genomic instability of EOC- the contribution of TP53 and BRCA1/2	8
1.3.2 Tumor heterogeneity in EOC	11
1.3.3 The development of chemotherapeutic resistant EOC.....	12
1.4 Biology of ovarian cancer metastasis	14
1.4.1 Exfoliation of EOC cells from the primary tumor and epithelial to mesenchymal transition	15
1.4.2 Transit of ovarian cancer cells throughout the peritoneum.....	18
1.4.3 Spheroid reattachment and secondary metastasis	20
1.5 Oncolytic virotherapy for the treatment of cancer.....	22
1.5.1 Basics of virology.....	23
1.5.2 Inherent oncolytic virus selectivity for cancer cells.....	25
1.5.3 Engineered viruses that improve tumor targeting and oncolytic efficacy	28
1.5.4 Clinical trials with oncolytic viruses.....	31
1.5.5 Clinical trials of oncolytic viruses in ovarian cancer	34
Adenovirus	34
Measles virus.....	35
Reovirus	37
Vaccinia Virus.....	38
1.5.6 Pre-clinical development of novel oncolytic viruses for the treatment of metastatic heterogeneous EOC	39
Myxoma virus	39
Double deleted vaccinia virus (vvDD).....	43

General poxvirus replication lifecycle	46
Maraba Virus.....	47
VSV replication lifecycle.....	50
MRBV cancer cell tropism.....	51
1.6 Experimental objectives and hypothesis.....	53
1.7 References	54
CHAPTER 2: Evidence for differential viral oncolytic efficacy in an <i>in vitro</i> model of epithelial ovarian cancer metastasis	78
2.1 Introduction	78
2.2 Results	80
2.3 Discussion.....	92
2.4 Materials and methods.....	99
2.5 Supplementary information	104
2.6 References	109
CHAPTER 3: Spatial and temporal epithelial ovarian cancer cell heterogeneity.....	112
can impact Maraba virus oncolytic potential	112
3.1 Introduction	112
3.2 Results	114
3.3 Discussion.....	122
3.4 Materials and methods.....	128
3.5 Supplementary Information.....	134
3.6 References	138
CHAPTER 4: Transforming growth factor-beta signaling mediated induction of epithelial-to-mesenchymal transition in ovarian cancer ascites-derived spheroid cells augments Maraba virus-mediated oncolysis	140
4.1 Introduction	140
4.2 Results	141
4.3 Discussion.....	151
4.4 Materials and methods.....	155
4.5 References	160
CHAPTER 5: General Discussion	163
5.1 Summary of overall findings	163
5.2 Oncolytic virus replication and tumor dormancy	163

5.3	Primary cells vs. established cell lines for <i>in vitro</i> assays.....	167
5.4	Tumor heterogeneity and precision therapy in oncolytic virotherapy.....	171
5.5	Antitumor immunity and oncolytic viruses	175
5.6	Conclusions	177
5.7	References	178
	Curriculum Vitae	184
	Appendix.....	192

List of Tables

Table 1.1 Oncolytic viruses used in clinical trials with ovarian cancer patients	41
Table 2.1. Quantification of oncolytic virus production in infected adherent ovarian cancer cells and spheroids.....	87
Table 2.2 Summary of overall results for MYXV, vvDD and MRBV oncolytic efficacy in ovarian cancer adherent cells and spheroids.....	95

List of Figures

Figure 1.1 EOC tumor heterogeneity.....	9
Figure 1.2. Mechanism of EOC metastasis.....	16
Figure 1.3. Preferential viral replication in cancer cells.....	30
Figure 1.4. Rhabdovirus structure and genome organization.....	49
Figure 2.1 In vitro three-dimensional spheroid model system of ovarian cancer metastasis.....	81
Figure 2.2 Analysis of MYXV, vvDD, and MRBV oncolytic-mediated killing of EOC cell lines in adherent and spheroid culture.....	83
Figure 2.3 Rapid kinetics of MRBV-mediated killing of EOC cells and spheroids compared with MYXV and vvDD.....	85
Figure 2.4 MYXV and vvDD oncolysis is reactivated after spheroid reattachment.....	89
Figure 2.5 Reattached EOC spheroids are susceptible to MRBV infection.....	91
Figure 2.6 Efficient MRBV entry into ovarian cancer cells requires LDLR expression..	93
Figure 3.1. Temporal changes in tumor biology differentially impacts MRBV oncolysis.....	116
Figure 3.2. Intratumoral cellular heterogeneity differentially impacts MRBV oncolysis.....	117
Figure 3.3. Differences in sensitivity between subclones are partially due to differences LDLR expression and MRBV entry.....	121
Figure 3.4. Extracellular factors do not confer sensitivity to MRBV.....	123
Figure 3.5. MRBV sensitivity can be conferred to resistant cells through co-culture and cell-cell contact.....	125
Figure 4.1. Spheroid formation sensitizes ascites-derived cells to MRBV oncolysis....	143
Figure 4.2. TGF β treatment sensitizes adherent ascites-derived cells to MRBV oncolysis.....	146
Figure 4.3. Inhibition of signaling through TGF- β type I receptor in spheroids prevents sensitization to MRBV oncolysis.....	148
Figure 4.4. Sensitization to MRBV by TGF β treatment and spheroid formation is not due to increased virus entry, changes in LDLR expression, or IFN β signaling.....	150
Figure 4.5 Sensitivity to MRBV correlates with markers of EMT and knockdown of Snail prevents ascites derived spheroid sensitization.....	152

List of Supplementary Figures

Supplementary Table A2.1. Summary of gene mutations identified in the three cell lines used in this study.....	104
Supplementary Figure A2.1. Differential oncolytic efficacy in ovarian cancer patient ascites-derived primary cells.....	105
Supplementary Figure A2.2. Time course of infected spheroid reattachment	108
Supplementary Figure A3.1 The LDLR gene is altered in a significant fraction of ovarian serous cancers	134
Supplementary Figure A3.2 siLDLR smart pool knockdown and impact on clone viability	135
Supplementary Figure A3.3-LDLR expression does not consistently correlate with MRBV sensitivity across heterogeneous ascites cells or its subclonal populations.	136
Supplementary Figure A3.4- LDLR expression in adherent and spheroid iOvCa147 parental line and F8 and G4 clones.	137

List of Abbreviations

Abbreviation	Term
Ad	Adenovirus
AML	Acute myeloid leukemia
APC	Antigen presenting cell
CAF	Cancer associated fibroblast
CEA	Carcinoembryonic antigen
CPE	Cytopathic effect
DC	Dendritic cell
ds	double stranded
EMT	Epithelial-to-mesenchymal transition
EOC	Epithelial ovarian cancer
FGF2	Fibroblast growth factor 2
G	Glycoprotein
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HGSOC	High-grade serous ovarian cancer
HLA	Human leukocyte antigen
HRR	Homologous recombination repair
HSV	Herpes simplex virus
IEV	Intracellular enveloped virus
IFN	Interferon
IMV	Intracellular mature virion
L	Large polymerase protein
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
M	Matrix protein
MET	Mesenchymal-to-epithelial transition
MOI	Multiplicity of infection
MRBV	Maraba virus MG1 construct
MV	Measles virus
MYXV	Myxoma virus
N	Nucleoprotein
NIS	Sodium-iodide importer
NK	Natural killer cell
OSE	Ovarian surface epithelium
OV	Oncolytic virotherapy
P	Phosphoprotein
PAK1	p21-activated Kinase
RdRp	RNA dependent RNA polymerase
RIG-I	Retinoic acid-inducible gene
ss	Single stranded

STIC	Serous tubal intra-epithelial carcinoma
TGF β	Transforming growth factor β
TK	Thymidine Kinase
T β R	Transforming growth factor β receptor
ULA	Ultra-low attachment plate
UPR	Unfolded Protein Response
VACV	Vaccinia virus
VGF	Vaccinia growth factor
VSV	Vesicular stomatitis virus
vvDD	Double deleted vaccinia virus construct (deletion in virus thymidine kinase and vaccinia growth factor)

CHAPTER 1: General Introduction

1.1 Clinical presentation, frequency, and treatment of epithelial ovarian cancer

Epithelial ovarian cancer (EOC) represents the 8th most commonly diagnosed cancer among women in Canada and ranks 7th globally [1, 2]. Approximately 2,800 Canadian women (1 in 71 women) and 239,000 women worldwide will be diagnosed every year with EOC, which represents ~3.6% of all cancers diagnosed in females [1, 2]. Despite this relatively low rate of diagnosis, EOC represents the most lethal gynaecologic malignancy in industrialized countries, with approximately 1,750 deaths in Canada yearly or 1 in every 91 women [1]. This is largely due to its advanced stage presentation and limited therapeutic advances, despite extensive efforts over decades to identify more effective alternatives to conventional treatment regimens [3, 4].

EOC survival rates progressively decline with advanced staging of the disease. When the disease remains confined to the ovary, survival rates are particularly favorable with approximately 91% of women surviving beyond 5 years post diagnosis. However, the majority of women are not diagnosed until widespread intraperitoneal metastasis has occurred and survival rates are dramatically reduced to 27% [3, 5]. Hence, there has been a tremendous effort to develop early detection methods for the diagnosis of EOC. This is exceptionally difficult as the physical symptoms indicative of ovarian cancer are subtle and often overlap with more common disorders including dyspepsia, irritable bowel syndrome, menstruation, and menopause [6]. These symptoms include abdominal or pelvic pain, increased abdominal size or bloating, and feeling full or difficulty eating. Although not one of these symptoms alone is sufficient, a combination of symptoms present are more effective for diagnosing EOC [6]. Symptom score systems have been developed to aid in the identification of specific individuals at risk of ovarian cancer based on the presentation

of single or combinations of symptoms, however the validation of such systems has not been effectively performed and the integration of other risk factors including menstrual or family history have not been incorporated into their design [6]. In general, although combinations of symptoms have higher sensitivity than individual symptoms, their specificity is still inadequate as an effective screening tool for diagnosis. Based on the current systems criteria, in a population of 10,000 women, 3 out of 5 cancers may be detected, but 500 tests would result in false positives. This can result in patient anxiety, as well as unnecessary procedures such as ultrasound of the ovaries, and possibly a surgical biopsy. Thus, the positive predictive value would be only 3 of 503 or 0.6% [6]. Therefore, there is currently inadequate evidence to recommend the implementation of symptom scoring systems for general use.

A subpopulation of EOC results from inherited mutations, most commonly in *BRCA1* or *2*. Approximately 5-15% of ovarian cancers are associated with the inheritance of *BRCA1* or *2* germline mutations. Therefore, genetic screening can be useful to identify those at risk [7]. However, *BRCA1* or *2* mutations have roughly only a 10-44% penetrance rate for ovarian cancer and the age at which the cancer occurs can be widely variable [8, 9]. In short, the utility of predictive genetic testing can be limited and positive identification of *BRCA1* or *2* mutations does not lead to straightforward measures that reduce risk. This in combination with poorly defined symptomology make detection of early stage ovarian cancer very difficult.

Since early detection is relatively uncommon, the treatment of ovarian cancer typically involves management of late stage advanced metastatic disease. Standard treatment heavily relies on surgical intervention and optimal de-bulking of metastatic

tumors. Optimal cyto-reduction is typically defined by the removal of tumors greater than 1 cm in diameter with no or few amounts of residual disease apparent after surgery [10-12]. This often requires radical resection procedures to remove metastatic lesions including intestinal resection, diaphragm stripping or resection, splenectomy and liver resection. However, complete cyto-reduction of disseminated tumors is possible in only 25% of cases [12, 13]. Therefore, a combination of surgery and platinum based chemotherapy (carboplatin and paclitaxel) is almost universally applied, although controversy remains over the timing of surgical and chemotherapeutic intervention. Neoadjuvant chemotherapy, in which chemotherapy is applied prior to cyto-reductive surgery, is considered more favorable when patients present with comorbidities or with *a priori* non-debulkable tumors. Studies which have compared neoadjuvant chemotherapy vs. primary cyto-reductive surgery (surgery prior to chemotherapy) demonstrate decreased surgical morbidity and mortality with decreased estimated blood loss, shorter surgical times, less intensive-care-unit admissions, fewer bowel resections, and reduced overall lengths of stay with neoadjuvant treatment regimens. Likewise, neoadjuvant therapy has been associated with a greater ability to achieve complete resection or optimal debulking and improved toxicity [14-19]. Likewise, it has been suggested that patients who initially present with large volumes of disease may fare better with neoadjuvant therapy [19]. However, many challenge this view by suggesting that although surgical morbidities may be decreased, both progression free survival and overall survival in neoadjuvant treatment groups are much poorer compared to primary cyto-reductive surgery [20-22]. Moreover, Chi et al. argue that patients undergoing primary de-bulking surgery fare much better than the neoadjuvant group and that the outcomes documented for neoadjuvant therapy in many of

the studies more closely resemble those of patients with large amounts of residual disease after debulking [23]. Thus, there is an ongoing debate between the application of neoadjuvant and primary cytoreductive surgery which remains unresolved [4].

1.2 Origin and pathogenesis of epithelial ovarian cancer

There has been difficulty in identifying the origins of ovarian cancer from traditional epidemiological and pathologic observations due to the fact that these approaches assumed that EOC was one disease. With greater advancement in the molecular techniques used to characterize cancer cells, it is now understood that EOC is a general term for a larger subset of tumors which may or may not manifest in the ovary and are varied in their clinical manifestation and behavior [24, 25]. Traditionally EOC is divided into two types based on cell phenotype and underlying genetic traits: type I (low-grade) and type II (high-grade)[26]. These two larger groups can be further subdivided into a number of histologic subtypes: serous, mucinous, endometrioid, clear cell, transitional cell, or any combination of these (mixed) [27]. Type I tumors include low-grade serous, low-grade endometrioid, clear cell and mucinous carcinomas. These tumors are typically slow growing or indolent and usually present at a lower stage [28]. Type II tumors however can present as high-grade serous, high-grade endometrioid and undifferentiated histologies and differ from that of Type I tumors in both their morphology, (type II typically exhibit papillary, glandular, and solid patterns) as well as their behavior [29]. Type II tumors are much more aggressive and typically diagnosed at an advanced stage and are unfortunately far more common, as they account for over 75% of ovarian carcinoma [24]. Perhaps the most distinguishing difference between the two types of ovarian cancer exist at the genetic level [30, 31]. The prototypical type II histotype, high-

grade serous, which alone accounts for 70% of all ovarian cancers, almost always exhibits *TP53* mutations, and in fact has the highest rate of *TP53* mutations compared to all other solid tumors [32]. Type I tumors rarely have mutations in *TP53*, but do exhibit mutations in *KRAS*, *BRAF*, or *ERBB2* in over two-thirds of low-grade cancers [33-38]. Due to their mutation profiles, high grade tumors display much higher degrees of genomic instability and cellular heterogeneity than low grade tumors which are typically more genomically stable [35, 39].

There is much debate regarding the cell of origin from which EOC is derived. To best understand the basis of these arguments, comprehension of the embryonic development of the reproductive system is required. The ovary is derived from multiple embryonic structures, including the coelomic epithelium, the subcoelomic mesoderm, and the primordial germ cells from the yolk sac endoderm. The rest of the female genital tract, including the fallopian tubes, uterus, cervix, and upper vagina, are derived from the Müllerian ducts [40]. However, the ovary itself does not contain epithelial cells. Instead, a thin layer of ovarian surface epithelium (OSE) derived from the coelomic epithelium covers both the ovarian surface and the serosa of the fallopian tubes, uterus, and the peritoneal cavity [41]. Thus, it is difficult to know the origins of the epithelial nature of EOC when no truly differentiated epithelium actually exists in the ovary. In fact, on pathological assessment of these tumors, many appear to be derived from the Müllerian ducts [42]. For example, serous tumors resemble the cells found in the fallopian tube epithelium and endometrioid tumors resemble the cells of the endometrium both of which are Müllerian in nature [42]. Thus, the debate over the origins of EOC exists as two major theories: 1) the original cancer cell arises from the ovarian surface epithelium (OSE) and

that metaplastic changes lead to the development of the cell types observed in the different EOC histotypes; and 2) tumors with a Müllerian phenotype (serous, endometrioid and clear cell) are derived from Müllerian-type tissue not the OSE (or mesothelium).

Perhaps the strongest evidence that supports the cell of origin belonging to the OSE is the distinct association between number of ovulations and risk of EOC. This relationship was first demonstrated in hens that were raised to produce excessive numbers of eggs without breaks in ovulation. High rates of ovarian adenocarcinomas were documented in these hens due to incessant ovulation [43]. It was hypothesized that the OSE cells became damaged during ovulation and internalized into the ovary to form cortical inclusion cysts, which undergo metaplasia to become differentiated Müllerian-like epithelium and ultimately lead to ovarian carcinoma [44]. A similar correlation between number of ovulations and ovarian cancer has been observed in women [45]. Particularly, women who have decreased ovulatory cycles due to pregnancy, breast-feeding, or oral contraceptive use have a significantly reduced lifetime risk of developing ovarian cancer by almost 50% [46-48]. However, it must also be noted that this epidemiological evidence to support ovulations and risk of EOC is not entirely correlative. Women with polycystic ovarian syndrome, characterized by infrequent ovulatory cycles, are at increased risk for EOC [49]. Moreover, formulations of oral contraceptives which do not reduce the number of ovulations also have observed protective effects [50]. Although there are many theories about how the OSE can undergo metaplasia and dysplasia, perhaps the most difficult aspect of this theory to accept is that the identification of a true precursor lesion for EOC has not been found in the ovary [51, 52].

Due to the complex and heterogeneous nature of EOC, it is likely that not one single location or etiology can be used to explain all types of EOC. The majority of mucinous tumors display intestinal differentiation or could be of appendiceal or other gastrointestinal origin [53]. Transitional cell tumors appear to be derived from urothelium of the urinary tract [53]. Endometriosis has also been more definitively linked to both endometrioid and clear cell EOCs [54]. For high-grade serous ovarian cancer, it is becoming more widely believed to originate from the fallopian tube. Studies conducted of tubal segments derived from women with *BRCA* mutations or family history of EOC undergoing prophylactic bilateral salpingo-oophorectomy (removal of both fallopian tubes and ovaries) showed evidence of dysplasia or hyperplastic lesions histologically resembling high-grade serous ovarian cancer (HGSOC) at a frequency of 1–5% at the time of the surgery [55-58]. The majority of these malignancies were located in the distal fimbriated end of the fallopian tube. It was hypothesized that these tubal epithelial cells then spill onto the surface of the ovary and therefore create the appearance of ovarian origin [59]. These regions of dysplasia within the fallopian tube have since been named “serous tubal intra-epithelial carcinoma” (STIC) and in most cases, these areas demonstrated high levels of p53 accumulation, even in benign lesions which are now believed to be an even earlier precursor lesion than STICs [60]. The examination of fallopian tubes from advanced-stage HGSOC showed that 75% had evidence of STIC lesions. Moreover, in a subset of these cases, the exact same *TP53* mutation can be observed in both the fallopian tube and the cancer tissue, further supporting the site of origin and clonality of the STIC and the tumors [61].

1.3 Genome instability, heterogeneity, and disease recurrence in EOC

The pathophysiology of EOC is diverse and can manifest as multiple histotypes with varied disease origins and differential behaviors and clinical outcomes. Thus, it is clear that EOC is not a single disease, but a category of malignancies with a very heterogeneous nature. Not only are the clinical manifestations of the disease diverse, EOC is highly heterogeneous, both spatially and temporally. In high-grade EOCs, the overwhelming rate of *TP53* mutations (>95%) bears a significant contribution to intratumoral heterogeneity observed (Fig. 1.1).

1.3.1 *Genomic instability of EOC- the contribution of TP53 and BRCA1/2*

In HGSOC, mutations in prototypical oncogenes are relatively uncommon [62]. However, mutations in *TP53* and *BRCA1* and 2 are the primary contributors of a significant level of genome structure variation and render HGSOC highly genomically unstable [63]. The tumor suppressor encoded by *TP53*, p53, has a central role in maintaining genome stability within normal cells. Under stress-free conditions, p53 is monoubiquitinated predominantly through the RING-finger ubiquitin ligase, MDM2 and rapidly degraded [64, 65]. In the event of genotoxic stress, p53 becomes stabilized through phosphorylation by many different kinases, the most commonly described being ATM [66]. Additionally, acetylation further promotes conformational changes in p53 structure to augment DNA binding capacity and induces the transcriptional upregulation of a number of genes involved in DNA damage repair, cell cycle arrest, and apoptosis [67-70].

In normal cells, functioning p53 prevents the accumulation of DNA damage and mutation [71]. A loss of functioning p53, either through mutation or deletion, may result in the loss of G2/M checkpoint arrest, translocations, amplifications, and aneuploidy leading to genomic instability [69, 71-73]. Genomic instability is defined as a high

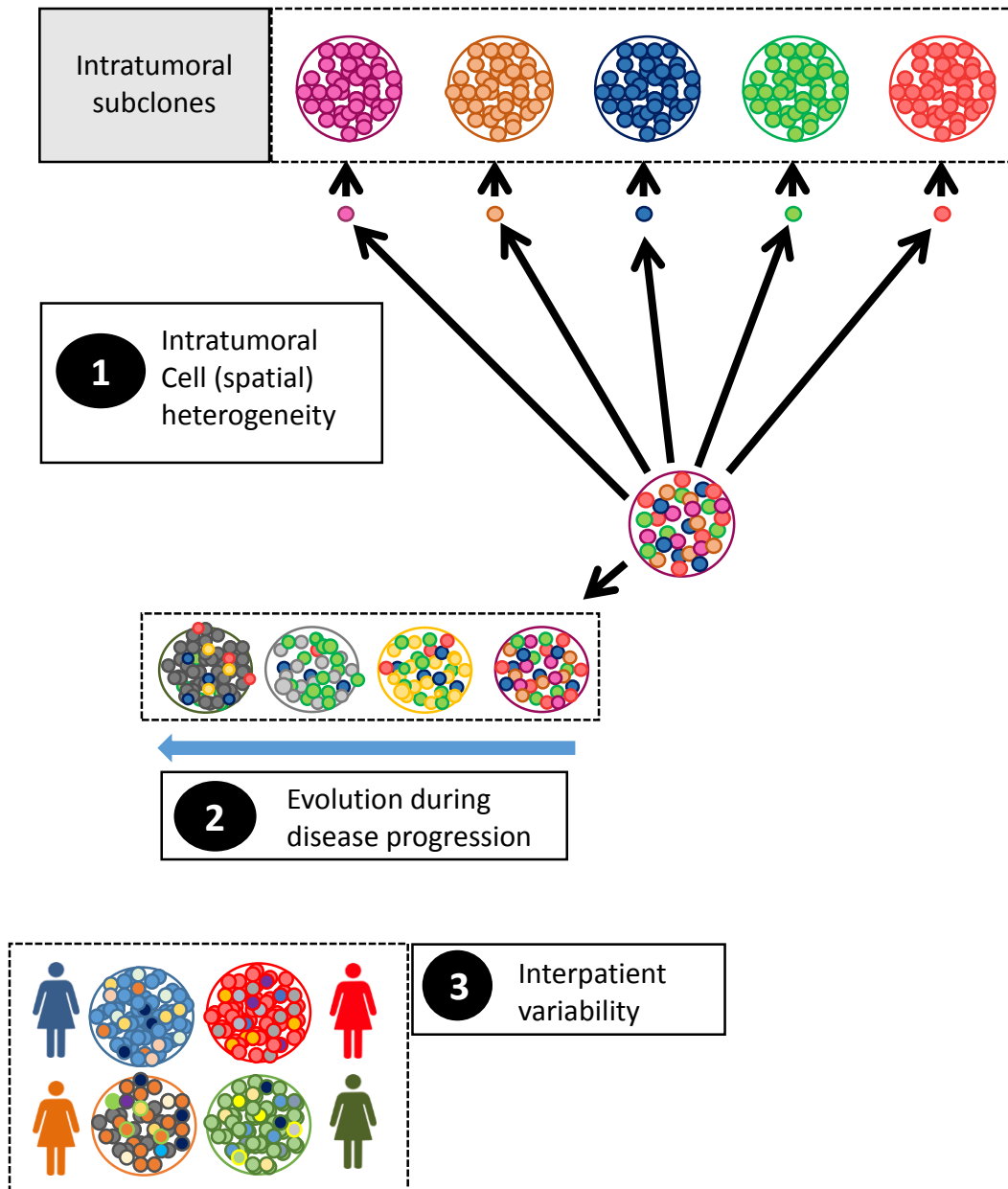


Figure 1.1 EOC tumor heterogeneity- 1) Genome instability drives intracellular heterogeneity within a tumor. Each subpopulation of cells are characterized by their own set of mutations and aberrant signaling pathways. Each subclonal population may respond differently to treatment. 2) Changes in the tumor over time due to changes in both tumor microenvironment, perhaps as a consequence of therapy, puts selective pressure on EOC tumors. The fittest subclones are selected in a Darwinian fashion to repopulate tumors causing changes in behavior of tumors over the course of disease. 3) Differences in histotype and genetic mutations between patients create different degrees of genomic instability, affect degrees of spatial heterogeneity, and the rate of temporal changes, resulting in differences between patient responses to therapy.

frequency of DNA alterations within the genome resulting in mutations, chromosomal rearrangements or aneuploidy [74]. The majority of *TP53* mutations in HGSOC are single nucleotide substitutions in the DNA binding domain of the protein resulting in hyper-stabilization of the protein but inactivation of WT functions [75-79]. Approximately 92% of *TP53* mutations in HGSOC render the protein non-functional [79].

The consequence of such mutations and the loss of genomic stability is the development of a large number of small regions of copy number gain and loss, which often encompass genes that are involved in ovarian tumor progression, such as the small deletions found within the retinoblastoma 1, *RBI*, locus [73]. However, certain p53 mutations result not only in the loss of WT tumor suppressor functions, but in some instances can promote a gain of function change which results in p53 acting as an oncogene (via a mechanism completely independent of its WT functions) [80]. Genome instability caused by mutations in p53 can promote further alterations in genes that contribute to invasion and metastasis by activating metastasis associated signaling pathways such as TGF β and EGFR[81, 82]. It is possible that up to 20% of *TP53* mutations in EOC tumors may be oncogenic [80]. Other mutations associated with EOC, such as *BRCA1* and 2, which occur in approximately 10% of EOC, further contribute to the high degree of genomic instability observed as both *BRCA1* and 2 share vital roles in repairing DNA damage [7]. The prevalence of *BRCA1* and 2 mutations is 1/400 to 1/800 in the general population, which varies depending on ethnic background, and puts individuals at increased risk for both breast and ovarian cancer (and may be associated with prostate cancer) [83-85]. The penetrance rate for these heritable mutations in either of these two genes is between 6-65% for ovarian cancer, depending on the specific mutation [86]. Over 90% of ovarian cancer patients with a

BRCA1 mutation develop HGSOC, as opposed to 70% in EOC patients that do not have the mutation [87, 88]. Loss of functional *BRCA1* and 2 result in defects in homologous recombination repair (HRR), a process involved in repairing double stranded breaks in the genome that occur during cellular replication [89]. It has been reported that in up to half of ovarian tumors HRR is defective through mutations or even methylation of *BRCA1* or 2 and other putative DNA repair genes, including *ATM*, *RAD50*, *RAD51C*, *RAD51D* and *PALB2* [62, 90-93]. Thus, mutations in either *BRCA1* or 2 result in further accumulation of DNA damage and genome instability within cells. Furthermore, there is a significant amount of evidence to suggest that both p53 and *BRCA1* physically interact in response to DNA damage and repair [94-99]. In fact, germline mutations in *BRCA1* are often associated with the causal mutations in *TP53* that lead to the accumulation of non-functional P53 and may mark an important step in the malignant transformation of EOC cells [100].

1.3.2 *Tumor heterogeneity in EOC*

Genetic diversity or heterogeneity within a tumor is the result of an accumulation of DNA damage and genomic instability accompanied by an escape from apoptosis in individual tumor cells. These genetic differences result in a high degree of phenotypic variability between cells, some of which may be functionally significant and promote tumor development, while others represent random genetic mutations with little or no biological consequence. These cell populations may be differentially affected and selected for by the tumor microenvironment. Through this selection process, tumors evolve temporally in response to changes in the tumor microenvironment via the Darwinian process of natural selection [101]. Therefore, greater genomic instability promotes

increased intratumoral cellular heterogeneity, or spatial heterogeneity, resulting in greater fitness and survivability of a tumor. Genetic studies using both microsatellite and single-nucleotide polymorphism analysis in EOC have identified extensive intratumoral heterogeneity [102]. Phylogenetic investigation of the heterogeneous cell populations within different regions of EOC tumors shows that the origin of each clonal population is derived from a common ancestor, with subsequent evolutionary divergence to create genetically distinct cell populations within a tumor [102]. Similar phylogenetic screens of EOC metastases also show lineage tracing back to the primary tumor. These data support a model in which EOC cells have a common clonal origin, but become polyclonal with both metastatic and primary tumours developing new clones, distinct from one another [103]. The extent of this clonal expansion between patients can vary significantly with a greater degree of clonal expansion correlating with poorer survival [104]. This is particularly true in the context of chemotherapeutic resistance. Given the substantial degree of intratumoral heterogeneity, both intercellularly and temporally, that exists in EOC and that the rate of clonal expansion varies throughout the course of disease, not only is it likely that no two tumours within a patient are identical, but no two cancers between individuals are alike either. This is a significant problem in designing effective treatment regimens in which a single therapy can be applied unilaterally across the patient spectrum.

1.3.3 *The development of chemotherapeutic resistant EOC*

The majority of patients who undergo chemotherapy will almost inevitably develop chemotherapeutic resistance and succumb to recurrent disease. The time between initial diagnosis and the development of resistance to platinum based chemotherapy is typically 3 years [105, 106]. Recurrence with platinum resistant disease is almost always incurable

and death is eventual within 5 years after initial diagnosis [107, 108]. It is during this time between disease diagnosis and relapse that clonal expansion is most significant [104]. Cancers with the greatest degree of genomic instability possess the greatest genetic diversity and this heightens the possibility of the emergence of drug resistant clones. Phylogenetic studies of patients with recurrent disease document the outgrowth and enrichment of cells with a mutation in *NFI* after treatment, which has been associated with the development of chemotherapeutic resistance [109]. Histologic samples of matched fallopian tube and ovary tissue acquired prior to the start of treatment showed the same mutation in 1.2% of the primary invasive carcinoma of the fallopian tube and in 7.9% of the tumor biopsy, meaning that the resistant disease arose from the clonal expansion of a minor subclone that was present prior to treatment [104]. A larger cohort study of similarly matched primary tumour and recurrent disease samples demonstrated that only 6% of inactivating mutations in *NFI* could be seen in primary samples, but the same inactivating *NFI* mutations were observed in 20% of recurrent disease [109]. This is common example of temporal heterogeneity in EOC that is driven by high levels of genome instability and selective pressures from the tumour microenvironment. Interestingly, this selective pressure can be so strikingly robust that *BRCA2* reversion mutations can be observed post chemotherapy in *BRCA2* mutated patients to yield functioning *BRCA2* capable of repairing double stranded DNA breaks induced by treatment [110, 111]. Re-acquisition of *BRCA2* function decreases the effectiveness of chemotherapy and is a strong predictor of platinum resistant EOC [110, 111]. A deep sequencing study of primary tumour and metastases samples isolated during an autopsy of a patient with a *BRCA2* germline mutation showcases this reversion phenomenon. At the time of death, the patient no longer responded to

olaparib or carboplatin treatment after the recurrence of her disease. Upon sequencing of the different tumor metastases, 12 different reversion events in *BRCA2* could be documented contributing to her resistance to both treatments [109]. In the limited studies conducted on this phenomenon, reversion in mutated *BRCA 1 or 2* occurs in 6% - 46% of patients with germline mutations [109, 110].

1.4 Biology of ovarian cancer metastasis

The majority of ovarian cancer patients do not die from primary disease, and instead ultimately succumb to metastatic spread of tumors throughout the body. However, unlike many other cancers which disseminate to distal sites in the body via the circulatory system (which requires intravasation, migration, extravasation and invasion), EOC metastasis is not typically blood borne. Rather, EOC cells detach from the primary tumour as either single cells or clusters and are passively carried by peritoneal fluid to seed secondary sites throughout the peritoneal cavity [112, 113]. Typical sites for metastases include the omentum (a large fold of fatty peritoneal tissue descending from the stomach connecting it to other organs), the bladder, large bowel, and colon [113]. Staging of ovarian cancer is directly related to the degree of dissemination of metastases through the peritoneum. Stage I refers to cancers being restricted to one or both ovaries. Stage II refers to cancer that has spread from the ovaries to the pelvis, including the uterus and fallopian tubes. In stage III EOC, the cancer has spread from the ovaries microscopically (less than 2cm) to the abdominal area outside the pelvis or to pelvic lymph nodes. Whereas in stage IV, tumors have spread to distal organs [114]. Approximately 85% of EOC patients are not diagnosed until stage III or IV [115]. It is therefore very important from a treatment standpoint to

understand the biological changes in both EOC cells and the tumor microenvironment that facilitate the migration of EOC tumors to distal sites (Fig. 1.2).

1.4.1 *Exfoliation of EOC cells from the primary tumor and epithelial to mesenchymal transition*

One of the initial events triggering the exfoliation of EOC cells from the primary tumor into the peritoneum is an epithelial-to-mesenchymal transition (EMT) [116-118]. During this process, cells lose their epithelial characteristics, including the loss of cell-cell junctions and apical-basal polarity, and acquire mesenchymal features, characterized by stress fiber formation and actin reorganization causing a spindle-shaped morphology [119]. This process decreases cellular connection to the basement membrane and interaction with neighboring cells, which eases detachment from the primary tumor [120]. A loss of E-cadherin (a glycoprotein present at adherens junctions between cells) is considered fundamental to the EMT process [121]. E-cadherin is a transmembrane protein with both an extracellular and cytoplasmic region. Its cytoplasmic domain forms a complex with β -catenin and α E-catenin, which together binds to actin cytoskeleton [122]. Its extracellular domain binds with other proteins related to cell-cell adhesion, including other cadherins and nectins, which thereby connect the cytoskeletons of neighbouring cells and facilitate the formation of different cell-cell junctions (including tight junctions, adherens junctions, and desmosomes) [123, 124]. Thus, the loss of E-cadherin has a significant role in the acquisition of a motile phenotype in cancer cells and helps facilitate metastasis. In ovarian cancer, the absence of E-cadherin is associated with greater invasiveness and is a predictor of poor patient survival [125, 126].

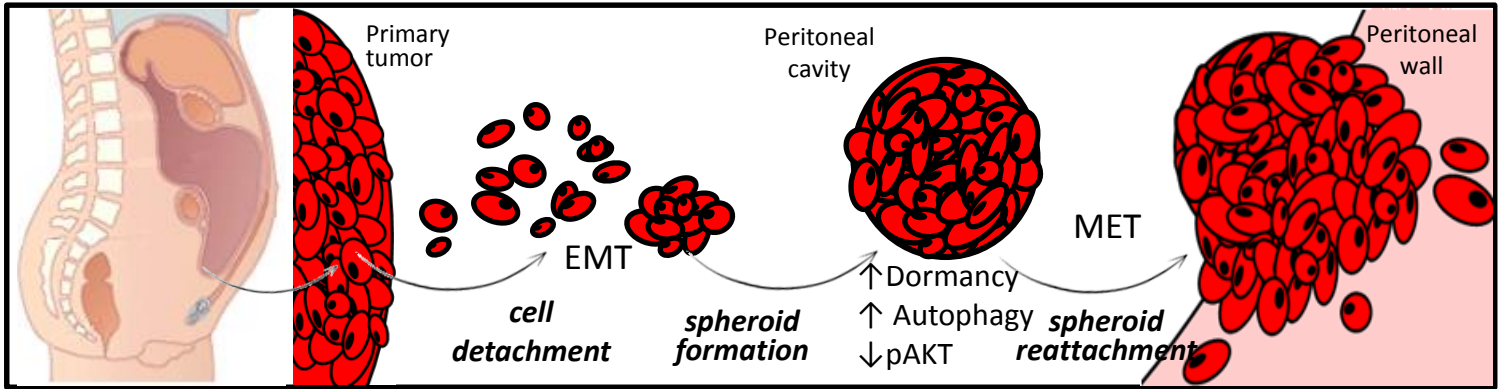


Figure 1.2. Mechanism of EOC metastasis – Malignant cells slough from the primary tumor due to decreased cell- cell adhesion. While in suspension in the abdominal fluid/ ascites, EOC cells adhere to each other and undergo epithelial-to-mesenchymal transition to become spheroids. Spheroids decrease cellular pathways associated with proliferation to conserve energy consumption and undergo dormancy to survive low-nutrient conditions. Autophagy is induced to promote survival by consuming unnecessary proteins and recycling amino acids for the synthesis of proteins necessary for survival during cell stress and starvation. Spheroids are able to reattach and disperse to form secondary tumor metastases within the abdominal cavity via mesenchymal-to-epithelial transition. Omental metastases are common due to high levels of energy rich lipids which promote EOC cell survival.

The loss of E-cadherin can be triggered by the activation of a number of transcriptional repressors which suppress the transcription of E-cadherin's encoding gene, *CDHI* [127]. This may be initiated by different signaling events occurring in the tumor microenvironment or tumor-associated stroma. Transforming growth factor β (TGF β) has been shown to have dual functions in human cancers, acting as both a tumor suppressor and a promoter of metastasis [128, 129]. In normal cells and in early carcinogenesis, TGF β acts as a growth inhibitor, inducer of apoptosis, and inhibitor of cell immortalization [130]. In ovarian and other cancers, TGF β is an important modulator of EMT. TGF β signaling is initiated when the ligand binds to a receptor complex that possess serine/threonine kinase activity. TGF β binds to the TGF β type II receptor (T β RII), followed by the recruitment of the TGF β type I receptor which is trans-phosphorylated by T β RII [131]. Activation of the TGF β type I receptor is the main component of the TGF β receptor complex and controls downstream signaling of various pathways, including the SMAD dependent pathway from which many EMT-mediated effects are driven (including the loss of E-cadherin) [128]. Two receptor regulated SMADs (SMAD 2 and 3) are phosphorylated by the TGF β type I receptor, causing their disassociation from the receptor complex and their interaction with SMAD4 in the cytoplasm [128, 132]. This SMAD complex then translocates into the nucleus where it associates with a number of DNA binding partners and acts as a transcriptional regulator of TGF β target genes, including SNAIL, SLUG, and ZEB2 which act as a transcriptional repressors of *CDHI* [127]. Furthermore, SNAIL repression of E-cadherin expression allows for the dissociation of desmosomes and the dissolution of cell junction complexes [125, 133].

TGF β induction of EMT is further exemplified by the loss of other epithelial markers including ZO-1, occludins, claudins, cytokeratins and the induction of more mesenchymal markers, including N-cadherin, vimentin, and fibronectin [134]. The transformed cells, which look more like fibroblasts upon undergoing EMT, acquire a more invasive phenotype [120]. The reorganization of the actin cytoskeleton leads to the formation of actin stress fibers, which are anchored to focal adhesion complexes and aid in the formation of filipodia which promotes cell migration [120]. After the cells have detached from the primary tumor as single cells or clusters, they spread to the peritoneum and are carried by the physiological movement of the peritoneal fluid [113].

1.4.2 *Transit of ovarian cancer cells throughout the peritoneum*

A consequence of advanced metastasis is the formation of an exudative fluid within the peritoneum called ascites. This fluid accumulates and aids in the passive transit of ovarian cancer cells to distal sites within the abdomen [135]. More than one-third of patients present with ascites at the time of diagnosis and almost all have ascites with recurrent disease [136]. Clinically, the presentation and progression of ascites is correlated with poor survival [136, 137]. The presence of ascites strongly impacts the quality of life of patients as it causes debilitating symptoms such as abdominal swelling and pain, early satiety, and compromised gastrointestinal, urinary, and respiratory systems [138]. Under normal physiological conditions, capillary membranes of the peritoneum continuously produce lubricating fluid to cover serosal surfaces which allows the easy passage of solutes between the peritoneum and adjacent organs. Two-thirds of this fluid is reabsorbed by the lymphatic channels of the diaphragm and is directed to the subclavian vein to allow drainage [139]. In advanced metastasis, these lymphatic channels often become obstructed

with tumor cells and heightened fluid production is induced by tumor cells due to the increased leakiness of tumor vasculature from angiogenesis [139-141]. Thus, there is an accumulation of abdominal fluid due to defects in drainage and increased fluid production.

As single cells floating in suspension in the peritoneal fluid, EOC cells become susceptible to a process of cell death called anoikis. Anoikis acts as a protective mechanism induced when epithelial cells detach from the extracellular matrix and helps prevent ectopic cell growth [142]. As a mechanism to avoid death due to detachment or as a consequence of clustered detachment from the primary tumor, EOC cells in suspension will form multicellular aggregates called spheroids [143]. It is believed that spheroids may survive anoikis through the upregulation of the anti-apoptotic protein Bcl-xL [144]. EMT plays an important role in the efficiency of spheroid formation by EOC cells. Blocking SMAD dependent TGF β signaling and EMT particularly through the Snail transcription factor, profoundly inhibits the ability of EOC cells to form spheroids and reduces their metastatic potential [145].

Spheroids undergo a number of biological changes in their behavior to help promote EOC cell survival while in suspension. As another mechanism of avoiding death due to starvation within the low-nutrient conditions of the ascites, spheroids have been shown to undergo a period of dormancy to support nutrient conservation. This occurs due to the downregulation of signaling pathways associated with metabolism and cell proliferation, such as the AKT pathway [146, 147]. Furthermore, spheroids can undergo autophagy, a cellular process initiated under periods of stress in which vesicles engulf cytoplasmic organelles and fuse with lysosomes to promote hydrolysis, thus utilizing intracellular proteins and lipids to temporarily sustain energy production [148, 149]. If

prolonged, autophagy of cells is lethal. However acute induction of autophagy can not only promote survival in low nutrient conditions, but also permit cancer cell survival in response to cytotoxic drugs [150, 151]. For example, the mechanisms of action for platinum based chemotherapies is to target key components of the cell cycle and induce apoptosis in proliferating cell populations [152]. Thus, targeting dormant EOC cells in spheroids is considerably more challenging [148]. Furthermore, the expression of Bcl-xL in spheroids, which allows EOC cells to escape anoikis, contributes to their resistance to chemotherapy induced apoptosis [144]. Some studies suggest EOC spheroids are enriched for cells with stem-like properties marked by their gene expression profiles of Notch1, Nanog, Cdc1, CD34, and Myc, which are upregulated by 10-2000 fold and have increased ALDH activity [153]. This may also contribute to chemotherapeutic resistance, increased invasion abilities, and resistance to hypoxic conditions.

1.4.3 *Spheroid reattachment and secondary metastasis*

Although EOC spheroids are capable of reattaching throughout the peritoneum onto any mesothelial lined site, the location for reattachment is not entirely random. The most common sites for distant metastases are the omentum, diaphragm, and small bowel [113]. The first steps of invasion require an interaction between the EOC cells and the mesothelium. Integrins exhibit a key functional role in this process. β 1-integrins on EOC cells can heterodimerize with a number of α -integrin subunits found on the mesothelium to promote adhesion [143, 154]. Similarly, blocking vascular cell adhesion molecule 1 (VCAM-1) on mesothelial cells or α 4 β 1-integrins on EOC cells can restrict adhesion and prevent metastasis in xenograft models [155]. The interaction of integrins between the mesothelium and EOC cells is aided by an upregulation of matrix metalloproteinase 2

(MMP-2), which cleaves the extracellular matrix proteins vitronectin and fibronectin into smaller pieces on the mesothelial cells. Thus, binding to these smaller integrins on the mesothelium is enhanced [156, 157]. The CD44 cancer stem cell marker has also been shown to aid in adhesion to the peritoneal mesothelium, which could support the model that spheroids enriched for stem cells have enhanced metastatic abilities. This may be a possible mechanism for the observed selection of more highly aggressive cancer cells in secondary tumors [158].

Interestingly, since EMT has been shown to be important in spheroid formation and migration, mesenchymal-to-epithelial transition (MET) has been demonstrated as a key morphological modification that promotes EOC spheroid invasion and adhesion [116, 145]. Transcriptional changes in reattaching spheroids parallel those of cells undergoing MET [116, 145]. Likewise, metastasis and the dispersion of cells from reattaching spheroids was decreased when MET was restricted [145].

After EOC spheroids attach to a secondary site and invade, effective growth of these newly seeded tumors must be supported by the transformation or modification of the secondary site. For example, after reattachment and dispersion of EOC spheroids, EOC cells promote the production of vascular endothelial growth factors (VEGF) in both an autocrine and paracrine fashion to develop new vasculature to support their growth [159]. High levels of VEGF within the ascites and in serum are associated with EOC tumor progression and poor prognosis [160]. Therefore, the microenvironment of disseminated tumors plays a significant role in the success of secondary tumor growth. Implantation of EOC spheroid metastases to the omentum is enhanced through TGF β production by EOC cells, which transforms normal fibroblasts in the omentum into cancer associated

fibroblasts (CAFs) that contribute to EOC adhesion and invasion [161]. The preferential cultivation of the omentum is supported by a microenvironment rich in adipocytes, which aid in homing, invasion, and growth of EOC cells [162]. Inhibition of the IL-8 receptor using a neutralizing antibody markedly reduced the amount of omental metastases in an *in vivo* mouse model of EOC metastasis, emphasizing the importance of IL-8 secretion by adipocytes to increase omental homing of EOC cells [162]. Furthermore, because adipocytes store triglycerides, the omentum provides energy-dense lipids to EOC cells to promote their growth. Indeed, omental metastases display an increase in the expression of FABP4, which binds long chain fatty acids on adipocytes. Inhibition of FABP4 in an *in vitro* co-culture of EOC cells with adipocytes strongly reduced lipid accumulation in cancer cells and invasion were dramatically reduced [162]. Likewise, *in vivo* metastasis models in FABP4^{-/-} mice showed significant reductions in tumor burden paralleled by a reduction in microvessel density and tumor cell proliferation [162]. These results further support the notion that adipocytes act as major mediators of ovarian cancer metastasis to the omentum as they fuel rapid tumor growth through fatty acid metabolism.

1.5 Oncolytic virotherapy for the treatment of cancer

The use of viruses as therapeutic agents against cancer has distinct advantages over current chemotherapeutic strategies and other conventional small molecule drugs. The biological dysregulation often observed in cancer cells can provide a favorable environment for virus replication. Viruses are often dependent on cell cycling of host cells to produce progeny. Thus, uncontrolled cellular replication and resistance to apoptosis in cancer cells provides a suitable niche for virus amplification. Moreover, the tumor microenvironment of many cancers is highly immunosuppressive, and is associated with

decreased immune surveillance from lymphocytes due to downregulation of antigen presentation and decreased immunostimulatory cytokine production. These same features provide a concomitant loss in host cell defenses to pathogens, thereby making them more susceptible to viral infection. Furthermore, virus efficacy can be augmented, either through genetic modifications that allow better targeting of cancer cells, or through combinations with specific adjuvants [163, 164].

Before the concept of using viruses for cancer therapy, there had been a slow trickle of reports of cancer regression coinciding with virus infection [165-167]. The first clinical use of a virus for cancer treatment was in the early 20th century when rabies virus was used to vaccinate a woman with cervical cancer [168]. However, the dangers in using native wild-type (WT) viruses limited their use in a clinical setting. With modern advances in genetic modification techniques, engineered viruses have been designed with cancer selective tropism and restricted replication, thus spawning a resurgence in the popularity of viral oncolytics over the past 20 years. The following sections review both advances in basic research and the clinical application of viruses for the treatment of cancer and specifically EOC.

1.5.1 *Basics of virology*

A virus is an obligate intracellular parasite with a genome consisting of either DNA or RNA that may be single stranded (ss) with either positive or negative sense (+/-) or it may be double stranded (ds) [169]. Virus genomes contain genes required for the infection and replication of a virus in a host cell. The production of viral proteins promote the synthesis of viral components, assembly, maturation and packaging of virus particles or virions. Viruses are acellular genetic parasites that hijack cellular machinery (eg.

polymerases, ribosomes) to provide the resources required for their own replication. The appropriation of host cell systems in the replication of viruses varies depending on the viral genome content, size, and proteins packaged with the virus [169]. A minimal virus consists of a virus genome and a proteinaceous coat called the capsid [170]. Virus infection begins with entry into a host cell. This involves virus attachment to the cell, penetration into the cytoplasm, and uncoating of the virus to release its genome into the cell [171]. Enveloped viruses typically mediate attachment through the expression of virus glycoproteins that bind to cell surface receptors, whereas non-enveloped viruses may rely on a single protein or multiprotein structure to attach [170, 172]. Typically, non-enveloped viruses enter the cytoplasm through the endocytic pathway or disruption of cell membrane integrity [171, 173, 174]. Enveloped viruses enter either through membrane fusion or through endocytic vesicles followed by the fusion of the virus envelope with the vesicle membrane, which can mediate the release of the capsid and genome into the cell [171, 172]. For other viruses, penetration sometimes triggers a change in the microenvironment, such as acidification of the endocytic compartment, to allow the uncoating of the virus which releases its genome and capsid into cytoplasm [171, 175]. The specific details in location of virus uncoating and the process varies depending on the virus. Most DNA viruses are transported to the nucleus through a nuclear pore to allow transcription of virus genomes using cell replication machinery, such as RNA polymerase II [176]. For this reason, viruses are often dependent on the host cell cycle. The mechanism for production of viral mRNA, translocation, and translation vary but for dsDNA viruses, it occurs in a similar fashion as in the host cell using host cell machinery [177]. ssDNA viruses may use cellular enzymes to generate dsDNA before proceeding. For some RNA viruses (+ssRNA), their genome

can act as mRNA and interact directly with ribosomes to be translated with the subsequent polyprotein being cleaved to produce individual viral proteins. -ssRNA or dsRNA require packaging of a viral polymerase to transcribe viral mRNA [177]. Due to their small genome sizes, viruses often maximize their coding potential by including overlapping genes and can be alternatively spliced to permit more than one protein to be produced from a single genome region [171].

After the production of viral proteins, virus assembly usually occurs in the cellular compartment where genome replication takes place. Virus components include the virus genome, any accessory proteins, capsid/structural proteins, and envelope proteins (if it is an enveloped virus). Typically, assembly centers on capsid and structural proteins forming a scaffold for the virus genome to enter [178]. Viral glycoproteins may incorporate into cell membranes for viruses to acquire upon egress [179]. The specific mechanisms for viral protein packaging and assembly differ for each virus but generally rely on intricate localization signals and chance [180]. The release of non-enveloped viruses typically occurs during lysis of infected cells [181]. For enveloped viruses, budding occurs through a cell membrane after virus assembly leading to the acquisition of the envelope [182]. This may be the plasma membrane in which case this budding releases the mature infectious virus and may or may not induce lysis, or it may be a membrane acquired from an intracellular organelle membrane [183]. If it is the latter, the nascent virion is then transported in a vesicle to the plasma membrane for release by fusion of the vesicle to the plasma membrane [183].

1.5.2 *Inherent oncolytic virus selectivity for cancer cells*

Cancer cells acquire a number of generally observed adaptations during the process of becoming malignant. As reviewed above, this includes uncontrolled entry into S-phase, loss of functional apoptotic pathways, mutations in tumor suppressors including p53, suppression of tumor targeting cell-mediated immune surveillance, and decreased production of immunostimulatory effectors molecules [184]. These same features of cancer cells which may make them resistant to many conventional chemotherapeutics or small molecule inhibitors are in fact favorable characteristics for viral infection and production. Viruses often require cycling host cells to replicate and benefit from immune evasion strategies to avoid systemic clearance [164]. Most if not all of these characteristics are capable of being modulated by viral proteins in order to replicate effectively in a host cell. However, many viruses do not encode additional genes that modulate these systems and instead will preferentially infect host cells which possess such features; this endows them with inherent cancer cell selectivity.

A list of naturally “cancer-specific” viruses includes reovirus, vesicular stomatitis virus (VSV), Newcastle disease virus, and parvovirus [164]. As these viruses do not encode genes which alter the host cell’s response to virus, they rely on the inherent defects in anti-viral immunity through tumor-specific inactivation of protein kinase R (PKR) and interferon (IFN) response pathways [185]. PKR is a highly conserved cytoplasmic RNA virus sensor which signals through the eIF2- α protein to terminate the translation of viral transcripts. Furthermore its activation stimulates the production of a family of antiviral cytokines, IFNs, which trigger a cascade of antiviral responses within the cell and in neighboring cells to shut down replication or induce apoptosis to prevent virus spreading. In cancers with inactivating RAS mutations, PKR signaling is typically impaired, allowing

productive replication of reovirus, VSV, and Newcastle disease virus [185-189]. Impaired IFN signaling, either through inactivating mutations or epigenetic silencing, is found in a diverse range of cancers at both early and late stages of disease, meaning it is likely an early mechanism of immune dysfunction that persists through tumor progression and metastasis [190]. This defect renders cancer cells significantly more vulnerable to virus infection and can help oncolytic viruses selectively replicate in cancer cells, while safeguarding normal cells with intact IFN-signalling from virus infection. This selective feature permits cancer specific oncolysis by a number of viruses including, Myxoma virus (MYXV) [191].

Aside from direct infection and oncolysis of tumor cells, viruses have been strategically applied to target other aspects of tumor growth. A secondary feature of some oncolytic viruses not only involves direct oncolysis of tumor cells but also targeting and inducing lysis of the tumor vasculature, thereby restricting nutrient supply to growing tumors. This could be due to direct infection of tumor endothelial cells and the expression of viral proteins with antiangiogenic properties, although the mechanism is still unclear [192].

Many malignancies also demonstrate an overexpression of virus receptors compared to normal cells, which increases permissiveness to virus infection. For example, many melanoma tumors upregulate both I-CAM and DAF which are used by coxsackievirus to mediate virus infection of host cells [193, 194]. Furthermore, echovirus demonstrates specificity for ovarian cancer cells with upregulated expression of $\alpha_2\beta_1$ integrin, while poliovirus requires the overexpression of CD55 [195, 196]. Despite abundant expression of these receptors on normal cells, coxsackievirus, echovirus, and

poliovirus are likely limited in their ability to infect non-cancerous cells via additional factors, such as a functional IFN response.

Interestingly, some viruses also display anti-tumoral effects independent from direct infection and cell killing. For instance, although the parvovirus NS1 protein can induce apoptosis of transformed cells, it also exhibits oncosuppressive behavior by preventing further transformation and tumorigenesis [197, 198]. Infection of BALB/c mice with parvovirus prior to engraftment with syngeneic sarcoma cells led to graft rejection despite the fact these sarcoma cells are resistant to parvovirus infection [199]. Similarly, replication defective cytomegalovirus was able to prevent tumor growth and metastasis in mice despite the absence of a productive virus infection [200]. The mechanisms by which these viruses are able to induce an anti-tumoral effect is most likely modulated by increased immune surveillance caused by virus infection acting as an adjuvant to stimulate anti-tumor immunity (Fig. 1.3).

1.5.3 *Engineered viruses that improve tumor targeting and oncolytic efficacy*

Many virus genes necessary for replication in normal tissues are redundant (not necessary) for replication in cancer cells (due to the inherent changes in cancer cells, described in previous sections). Therefore much effort has been placed into creating virus deletion mutants defective in these replication genes (essential for replication in normal cells) to improve their tumor cell selectivity. Thymidine kinases (TKs; TK-1 and TK-2) are highly conserved enzymes which catalyze the phosphorylation of deoxythymidine into deoxythymidine monophosphate, a precursor to deoxythymidine triphosphate, which is incorporated into replicating DNA. [201]. TKs are therefore important for cell division, and in non-malignant cells, are expressed only during the cell cycle [202]. Thus, many

viruses encode their own TKs to provide nucleotides to promote the replication of their own genomes, even when the cells are not dividing and cellular TKs are not expressed. The creation of TK deletion mutants in various oncolytic viruses, including herpes simplex virus (HSV) and vaccinia virus (VACV), aids in restricting virus replication to cycling cells that have expression of cellular TK and have high phosphorylated deoxynucleotide pools for the virus to usurp [203, 204]. Furthermore, the overexpression of TK can be observed in many malignancies including bladder, breast, and colorectal cancers leading to significantly higher expression of TK compared to normal cycling cells. This provides more favorable conditions for virus replication and subsequent oncolysis [205-207].

The introduction of non-viral genes in an oncolytic virus is another strategy to help improve virus efficacy. Typically, the addition of non-viral DNA (when not for improving tumor cell specificity) is done either to increase bystander cytotoxicity of the virus to neighboring tumor cells, enhance anti-tumor immunity, introduce pro-drug converting enzymes, or include safeguarding suicide genes that restrict uncontrolled virus infection and killing. Perhaps the most common and rapidly expanding field in oncolytics research is the activation of the immune system to stimulate anti-tumor immunity and improve bystander cytotoxicity after virus infection. A commonly introduced gene designed to stimulate enhanced anti-tumor immunity is CD40L, the ligand which binds to the CD40 receptor found on many monocytes, dendritic cells, and B lymphocytes. Viruses which have been engineered to express this ligand include adenovirus and VACV [208, 209]. Oncolysis of tumor cells by these viruses induces a massive release in tumor associated antigens and provides co-stimulatory danger signals. Moreover, arming oncolytics with CD40L further aids in inducing direct apoptosis of tumor cells and triggers a cascade of

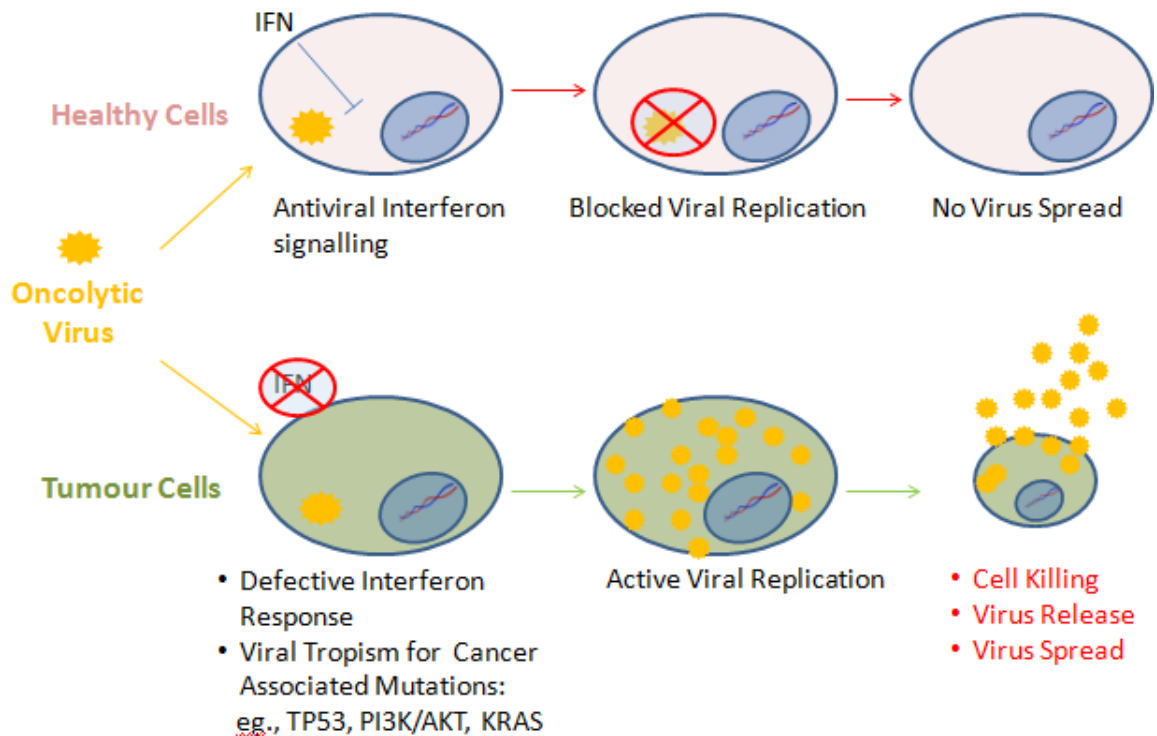


Figure 1.3. Preferential viral replication in cancer cells- viral infection and replication in healthy tissues is normally restricted by highly conserved antiviral signaling through IFN. Many tumor cells decrease IFN and other antiviral signaling molecules to help in immune evasion rendering them more susceptible to virus infection. Selection of viruses with natural tropism for cancer associated signaling pathways and mutations, or engineering viruses to have improved cancer selective properties promotes virus replication that is more restricted to cancer cells. Productive virus infection results in cell killing, virus release, and spread to neighboring cancer cells.

immunostimulatory signaling events to reduce the immunosuppressive environment of a tumor. *In vivo* xenograft models of endometrial cancer using CD40L armed adenovirus showed increased potency over WT adenovirus infection via three mechanisms: **1)** Increased recruitment and activation of antigen presenting cells (APCs) leading to the production of T-cell associated cytokines which stimulated robust cytotoxic T cell responses against tumors, **2)** direct apoptosis of CD40L tumor cells by CD40 expressing cells caused by infection with CD40L expressing adenovirus; and **3)** Direct oncolysis from adenovirus infection [208]. Thus, viruses can also be applied as a way to counteract tumor mediated immune evasion and immune suppression.

1.5.4 *Clinical trials with oncolytic viruses*

The first engineered virus to enter clinical trials in humans was a strain of adenovirus with two deletion mutations called, ONYX-015 or Adenovirus dl1520. This strain of adenovirus was engineered as a hybrid of adenovirus serotypes 2/5 and expressed mutations in the E1B-55K and E3B genome regions, thus rendering it only able to replicate in cancer cells with p53 mutations [210]. Over 10 clinical trials have been performed using the virus, leading all the way up to phase III which demonstrated that the virus was generally well-tolerated with patients experiencing flu like symptoms [211, 212]. Importantly, the virus was shown to be tumor selective, but replication was fairly transient and typically lasted less than 10 days. Moreover, replication was highly variable and was affected by tumor histology with a 0-14% tumor regression rate [211]. However, the clinical findings were important indicators of the strengths of oncolytics and of the hurdles that needed to be overcome. A broad range of adenoviral serotype 5 vectors have been assessed clinically with no serious toxicity. However, strong antibody mediated

neutralization of the virus has repeatedly been observed with intravenous injections [213, 214]. A majority of patients have pre-existing antibodies to adenovirus serotype 5 thus limiting its clinical utility [213, 215].

Newer strategies with engineered viruses almost always contain an immunostimulatory component to augment the antitumor response. The most clinically advanced construct of herpes simplex virus, OncoVex^{GM-CSF} contains deletions in both the γ -34.5 gene, which renders the virus susceptible to PKR and eIF2 α antiviral responses and also results in neuroattenuation, and ICP47 which re-enables MHC I presentation in host cells [216-221]. Furthermore, OncoVex^{GM-CSF} also contains Granulocyte-macrophage colony-stimulating factor (GM-CSF), a glycoprotein secreted by macrophages, T cells, mast cells, and NK cells, and functions as a cytokine to stimulate stem cell differentiation into granulocytes and monocytes for greater immunogenicity [217]. Phase II studies with OncoVex^{GM-CSF} in stage IIIc and IV melanoma showed that it was well tolerated with only transient flu like symptoms. Importantly, 26% of the 50 patients enrolled showed either complete response (n=8) or partial response (n=5) [222]. OncoVex^{GM-CSF} was rapidly inactivated in the blood as many people have pre-existing immunity to many strains of herpes viruses and this likely contributed to the relatively low success rate. However, of patients who responded, regression of both intratumorally injected and distal lesions was observed and maintained for 7- 31 months with an additional 10 patients who exhibited stable disease for 3 months [222]. Overall, survival after 1 year was 58% and 51% after two years thus stimulating the progression of OncoVex^{GM-CSF} to ongoing phase III trials with melanoma and squamous cell carcinoma of the head and neck [223].

Improvements in vector delivery and design have propelled further advancement of oncolytics through clinical trials. An oncolytic pox virus, JX-594, designed using a TK deletion mutant of vaccinia virus and armed with GM-CSF has demonstrated significant therapeutic utility in phase I-II dose escalation trials of hepatocellular carcinoma patients. Infection with this virus resulted in virus replication and successful expression of GM-CSF in tumors, which induced tumor necrosis, devascularisation, systemic dissemination of the virus and targeting of distant tumors [224]. Importantly, these studies also demonstrated an ability to treat patients with JX-594 despite having high neutralizing antibody titers. Phase I-II dose escalating trial in patients with advanced liver cancer showed significant improvement in overall survival of patients correlating with high doses of JX-594 [225]. In this dose group, 35% of patients had a long-term survival benefit of greater than 2 years after 4 weeks of dosing suggesting a durable therapeutic benefit. The mechanisms of action for these antitumor effects were direct oncolysis of tumor, acute vascular disruption, and immune stimulation through antibody generation and increased tumor-infiltrating lymphocytes [225]. Thus phase III clinical trials with JX-594 began in late 2015 and are ongoing with hepatocellular carcinoma patients actively being enrolled [226].

The advent of oncolytic virotherapy as a new and viable therapeutic option for a breadth of malignancies has sparked tremendous enthusiasm among not only researchers but patients as well. In 2005, China approved an oncolytic adenovirus called H101 for the treatment of head and neck cancers [227]. This brought with it a wave of medical tourism to the country after claims it could shrink tumors but never went so far as to say it could improve overall survival. Late in 2015, the Food and Drug Administration approved its first oncolytic virus for use in the United States called, TVEC, an oncolytic herpes simplex

virus highly similar to OncoVex^{GM-CSF} with an additional insertion of the US11 gene, encoding a tegument protein. This alteration increases the efficiency of virus replication [228]. Phase III trials demonstrated clinical effectiveness through a significant reduction in tumor size and an increase in overall survival by 4.4 months although the result fell just short of being statistically significant [229]. However, therapeutic efficacy and survival may be improved with combinations with other cancer immunotherapies. More than likely, these represent just two of many oncolytic viruses that are making their way down the pipeline for clinical approval.

1.5.5 *Clinical trials of oncolytic viruses in ovarian cancer*

Adenovirus

Due to the almost universal presence of *TP53* mutations in HGSOC, it is logical to investigate the therapeutic efficacy of oncolytic viruses which selectively replicate in tumor cells harboring this mutation. Among the first clinical trials in EOC patients using an oncolytic virus was a phase I clinical trial using the adenovirus construct ONYX-015, which was designed to preferentially replicate in p53-deficient tumor cells. Repeated intraperitoneal injections of virus were delivered in 16 patients ranging from 1×10^9 pfu- 1×10^{11} pfu. Although the treatment appeared safe (symptoms were generally mild, and a maximum tolerated dose was not reached), no clear therapeutic benefit was observed despite successful virus replication. This may be due to the presence of neutralizing antibodies in 46% patients prior to the start of treatment, which increased significantly over the treatment, potentially limiting viral replication. Moreover, 6/7 of the remaining patients without prior existence of neutralizing antibodies developed them over the course of treatment [230]. It is also possible that the minimal therapeutic benefit observed could be

due to variable expression of the coxsackie adenovirus receptor required by adenovirus to bind and enter cells. To improve delivery and uptake of adenovirus to target EOC cells, other strains of adenovirus have been engineered with modified viral capsid proteins, including an integrin binding motif to target other overexpressed integrin receptors on EOC [231]. In a phase I clinical trial with the capsid protein modified adenovirus, Ad5- Δ 24-RGD, stable disease was observed in 15/21 patients. However, no partial or complete responses were noted [232].

Although little therapeutic efficacy was demonstrated in reducing tumor size or improving overall survival, other adenoviruses have demonstrated an ability to improve quality of life. The clinically approved adenovirus construct, H101, which too is restricted to replicating in *TP53*-mutated cells, was shown in a phase I clinical trial to reduce ascites burden in EOC patients. Outcomes were grouped as either complete response (no recurrence of ascites), partial response (recurrence of ascites <50% of volume), or no response (recurrence of ascites > 50%, requiring paracentesis). Of 9 patients treated over a 30 day trial, 3 patients had complete responses, 2 had partial responses, and 4 had no response with no adverse effects. The interval between paracenteses before and after H101 treatment went from a mean of 12.4 \pm 3.4 days to 39.9 \pm 11.6 days thus demonstrating H101's potential in improving patient quality of life [233].

Measles virus

Rather than engineering viruses to have increased tumor cell selectivity, other clinical trials have focussed on viruses with innate tropism for tumor associated proteins. Measles virus requires expression of CD46 cell surface receptor to bind and enter target cells and this is highly overexpressed in many carcinomas, including EOC [234, 235]. To

examine virus replication in real time, a measles virus strain with an engineered carcino-embryonic antigen (MV-CEA) was generated such that CEA levels could be used as a surrogate for virus replication. Intraperitoneal injection of MV-CEA was performed in 6 cycles over 4 weeks at 7 different doses in 21 patients with platinum resistant ovarian cancer. Although 13 of 15 tumors which were immunohistologically positive for CD46 showed overexpression of the receptor, the best objective response was disease stabilization in 14/21 patients with effects lasting from 54-277 days. Although no statistically significant assessments could be made on the effects on overall survival, the median survival rate was 12 months (ranging between 1.3- 38.4 months) and is relatively favorable compared to historic averages of 6 months in this patient population. With no dose limiting toxicities or treatment-induced immunosuppression, no increase in anti-CEA antibodies, no increase in anti-MV antibody titers, or virus shedding in urine or saliva, these findings are promising for the continued development of measles virus as an oncolytic for EOC [236].

Additional phase I studies have been conducted with other strains of measles that were modified to facilitate *in vivo* imaging of virus infection and replication. In particular, a specific measles virus strain has been altered to express a sodium-iodide importer (MV-NIS). Unlike MV-CEA, which was engineered to allow for assessment of viral replication using CEA protein levels as a surrogate marker, the MV-NIS strain permits the visualization of virus replication in tissues *in vivo*. In a similar treatment regimen as described for MV-CEA, 16 patients with platinum and taxol resistant EOC were treated for 4 weeks with 6 cycles of high dose virus treatment (1×10^8 - 1×10^9 vpu). Median survival was 26.5 months, and 2/3 patients treated with 1×10^8 vpu and 11/13 patients treated with

1x10⁹ vpu exhibited stable disease. This was in agreement with patients from the previous MV-CEA trial treated with the same doses, and improvement in overall survival correlated with higher dosing of both MV-CEA and MV-NIS. NIS uptake was observed for 3/13 patients treated with the higher dose of MV-NIS and the intratumoral expression of NIS correlated with longer progression free survival.

Interestingly, although no measles virus specific antibodies were developed over the course of treatment, tumor specific T cell activation and IFN γ production was induced to two EOC antigens, FR α and IGBP2, and continued even after treatment stopped. Thus, not only is measles potentially a highly effective oncolytic for EOC based on its direct targeting and oncolysis of tumor cells, it may also possess immunotherapeutic advantages to augment virus oncolysis. Thus phase II trials comparing MV-NIS intraperitoneal administration to chemotherapy in patients with recurrent EOC and low disease burden have been designed and other phase I/II trials which test MV-NIS infected mesenchymal cell carrier delivery systems are being assessed [237, 238].

Reovirus

One of the earliest oncolytics to be used to treat ovarian cancer in clinical trials was reovirus serotype 3 also known as, Reolysin. This relatively small and quickly replicating virus preferentially infects cells with activated RAS. There are two ongoing clinical trials with Reolysin in EOC. A randomized phase II clinical study with Reolysin in combination with paclitaxel vs paclitaxel alone in patients with recurrent EOC, fallopian tube, or primary peritoneal cancers. Although the study is ongoing, it is no longer recruiting patients and results have not been published [239]. The second clinical trial with Reolysin is a phase I/II trial in patients with metastatic ovarian, peritoneal, or fallopian tube cancers

using intravenous and intraperitoneal injection [240]. As of 2010, viral replication in peritoneal and ovarian cancer cells could be observed after IV injection and is the first observation of reovirus to penetrate and replicate within the peritoneum and ascites [241]. However, official conclusions from the study have not been published. Recently the FDA gave orphan drug designation to Reolysin for the treatment of ovarian cancer [242]

Vaccinia Virus

The phase I clinical trial with JX-594 previously described in [Section 1.5.3](#) included 2 patients with recurrent ovarian cancer. One of the two patients received a low dose of JX-594 and did not have observable virus present in tumors but did exhibit stable disease for more than 4 weeks after treatment. The other patient received the second-highest dose and had replicating virus present in her tumors and exhibited stable disease for more than 16 weeks [243]. A phase II clinical study was designed to specifically investigate the potential therapeutic efficacy of JX-594 for ovarian cancer patients, but was withdrawn prior to the enrollment of patients [244].

Other vaccinia virus vectors have been engineered to induce the expression of tumor associated antigen NY-ESO1 (vaccinia-NY-ESO1) in infected tumor cells and have been used in phase II clinical trials as a vaccination strategy against both melanoma and ovarian cancer followed by booster vaccinations with fowl pox-NY-ESO1. In melanoma and ovarian cancer patients, both cytotoxic T cells and T helper cells were induced in a high proportion of patients (20/22). Patients who were seronegative for viral antibodies and remained seronegative, but developed T cell responses, and/or patients who seroconverted and developed T cell responses showed improved overall survival of 52.4 months and 48.4 months respectively compared to those that elicited no response (median

survival of 14.5 months). Furthermore, cytotoxic T cells extracted from EOC patients were able to induce lysis of NY-ESO1 positive tumor cells, thus providing clinical evidence and promising efficacy for the application of poxvirus oncolytics as immunotherapeutic vaccines against EOC [245]. Similar vaccination strategies with other modified vaccinia viruses are currently undergoing clinical trials. One example includes the p53 expressing P53MVA strain, which induces the expression of p53 in vaccinia virus infected tumors. Used in combination with gemcitabine, P53MVA was designed to help build more effective immune responses to tumor cells expressing mutated P53. This phase I clinical trial is ongoing and actively recruiting patients [246]. (Table 1.1).

1.5.6 *Pre-clinical development of novel oncolytic viruses for the treatment of metastatic heterogeneous EOC*

Although progress is being made in the development of viruses for the treatment of ovarian cancer, few agents have been evaluated in metastatic disease models, and none to our knowledge have been investigated in the context of both spatial and temporal heterogeneity. It is clear that both tumor heterogeneity and metastasis act as major hurdles in the development of clinically efficacious therapies to treat EOC. Therefore we sought to investigate the therapeutic potential of three oncolytic viruses, Myxoma virus, a strain of vaccinia virus (vvDD), and Maraba virus (MRBV), to overcome the most commonly neglected and lethal aspects of EOC.

Myxoma virus

Myxoma virus (MYXV) is a dsDNA enveloped virus and a member of the *Poxviridae* family. The Lausanne strain of MYXV contains a large 161.8 kbp genome and

encodes 171 genes, a large proportion of which produce immunomodulatory and host-interactive factors, which aid in its subversion of the host immune system and other antiviral responses [247-249]. Unlike other poxviruses such as VACV, which is capable of infecting a broad range of hosts, MYXV replication is restricted to lagomorphs and only causes pathogenesis in European rabbits [250-252]. MYXV virus co-evolved with the *Sylvilagus* genus (cottontail rabbit) which can be found endemically in Brazil. Although it replicates robustly and may be transferred efficiently from host to host, it causes no serious disease in its normal host [253]. The virus is passively transmitted through mosquitoes and other biting arthropods [254-257]. In its natural host, the cotton tail rabbit, MYXV causes a cutaneous fibroma at the site of inoculation. However in European rabbits, MYXV infection is lethal, causing disseminated disease called, myxomatosis [258]. In the 1950s, MYXV was introduced into Australia as a pesticide against encroaching European rabbits. With lethality rates as high as 99.8% huge declines in the rabbit population were observed making MYXV one of the most successful examples of a biological agent as a pesticide [253]. Lethality from myxomatosis is typically observed between 8-12 days after infection with virus shedding from ocular and nasal discharge and multiple cutaneous swellings and lesions which can be transmitted through contact or biting insects [259, 260]. MYXV's success in rabbits derives from its ability to effectively circumvent a wide range of host innate and adaptive immune responses. MYXV encodes many mimetics of host immune receptors (also known as viroceptors) which sequester immune responses to infection. This includes the viroceptor M-T7, which antagonizes the effects of rabbit IFN- γ [261]. Moreover, M-T7 can promiscuously bind to the heparin-binding domain of a broad range of chemokines to significantly reduce macrophage and other immune cell influx to the site

Table 1.1 Oncolytic viruses used in clinical trials with ovarian cancer patients

Virus	Name	Design	Phase	# Patients	Response	Toxicity
Measles Virus	MV-CEA	IP injection	I	21	14/21 SD	mild
	MV-NIS	IP injection	I	13	11/13 SD	mild
	MV-NIS	MV-NIS vs chemo	II	Recruiting		
	MV-NIS	MV-NIS infected Mesenchymal stem cell carrier	I/II	Recruiting		
Adenovirus	ONYX-015	IP injection	I	16	NR	mild
	Ad5-Δ24-RGD	IP injection	I	21	15/21 SD	mild
	H101	IP H101 to reduce ascites burden	I	9	3 CR, 2 PR 4 NR	mild
	Ad5-Δ24-GMCSF	Compassionate use		4	1 CR, 1 PR, 1 SD, 1 NR	mild
	Ad5/3-Δ24-GMCSF	Compassionate use		4	1 SD, 3 NR	mild
	Vaccinia Virus	JX-594	IV JX-594	I	2	2 SD
VV-NY-ESO1		Vv-NY-ESO1 →FP-NY-ESO1	II	22	20/22 increased antitumor T Cells; improved OS	mild
p53MVA		IV p53MVA + gemcitabine	I	recruiting	N/A	N/A
GL-ONC1		IP injection	I/II	Completed; unpublished	N/A	N/A
Reovirus		Reolysin	Paclitaxel +/- Reolysin	II	Active, not recruiting	N/A
	Reolysin	IP vs IV reolysin	I	Active, not recruiting	N/A	N/A

SD- stable disease, CR- Complete response, PR- partial, response, NR- no response, OS overall survival

of infection [261, 262]. However, the MYXV host range is extremely narrow. It is virtually non-pathogenic for any host outside the lagomorph family and fails to replicate efficiently in any non-rabbit host, even severely immunodeficient mice [263, 264].

Despite its narrow host range, MYXV is able to broadly infect human cancer cells. This is primarily due to two factors: **1)** many cancer cells are unable to produce a potent anti-viral response through the synergistic interaction between IFN and tumor necrosis factor α (TNF α), which normally blocks MYXV virus replication and transmission in non-cancerous human cells [265, 266]; and **2)** malignant transformation of many cancers induces constitutive activation of a number of intracellular pathways including the activation of AKT [267]. Pharmacological manipulation of AKT phosphorylation directly impacts MYXV tropism for human cancer cells [268]. It is thought that because MYXV has such broad cancer cell specificity that these two factors are the main determinants of MYXV tropism for cancer cells, rather than the expression of any particular cell surface receptor [269, 270]. For these reasons, and its otherwise highly restricted species specificity, MYXV can act as a highly specific oncolytic virus for cancer cells with a promising safety profile. MYXV demonstrates cancer cell-specific viral replication in a number of cancer types, including glioma, medulloblastoma, melanoma, pancreatic cancer, acute myeloid leukemia (AML), and ovarian cancer [271-276]. In AML, MYXV has been shown to effectively kill primary leukemic hematopoietic stem and progenitor cells derived from patients without inducing lysis or altering the behavior of normal hematopoietic stem cells [271]. Furthermore, MYXV has been shown to work synergistically with chemotherapeutic agents in models of pancreatic cancer that have acquired resistance to gemcitabine [277]. Resistance to gemcitabine is associated with increased levels of pAKT,

thereby selecting for cells with greater MYXV permissivity. Combination treatment regimens of MYXV and gemcitabine demonstrate enhanced survival in disseminated pancreatic cancer models than either treatment alone and may suggest novel therapeutic alternatives for this disease [277].

The activation of PI3K/AKT pathway can be found in up to 70% of ovarian cancers and is associated with poor overall survival and progression free survival [278-282]. Thus, we postulate that MYXV may act as a potent oncolytic in the most aggressive EOC cells which harbor activating mutations or dysregulated signaling of AKT. Our lab has demonstrated that MYXV can effectively replicate and kill EOC cell lines and has varying oncolytic efficacy for cells isolated directly from EOC-patient ascites [275]. Furthermore, infection of spheroids formed from EOC cell lines demonstrate decreased reattachment and dispersion in our *in vitro* models of EOC metastasis [275]. These oncolytic effects were correlated with changes in activation of AKT between adherent and spheroid EOC cells [275]. MYXV replication is also directly modulated by the mutational status of *TP53* and preferentially replicates in cancers with dysfunctional or deleted *TP53* (which is found in >95% of HGSOC cases) [283]. These findings suggest that MYXV may work as a targeted therapeutic for the treatment of metastatic EOC and possibly for type II EOCs in which pAKT is most highly induced and *TP53* mutations are ubiquitous.

Double deleted vaccinia virus (vvDD)

Similar to MYXV, VACV belongs to the *poxviridae* family, is enveloped, and is also has a genome composed of dsDNA. However, unlike MYXV, VACV is capable of replicating in a diverse range of mammalian hosts including humans, allowing for abundant preclinical research to be conducted in many different animal systems [269]. Much of its

genome encodes for enzymes required for its replication, such that it can replicate with little dependence on host cell machinery. This includes a DNA dependent RNA polymerase, numerous transcription factors, a number of capping and methylating enzymes, a poly(A) polymerase, and many immunomodulatory proteins [269, 284]. To encode all these proteins, VACV has a very large genome with over 190kbp needed for approximately 270 genes [285]. Moreover, by encoding most of the genes required for its replication, VACV does not need to enter the nucleus and its replication is entirely cytoplasmic, thereby eliminating any risk of viral gene integration in the host genome [286]. It is this large genome that makes poxviruses effective gene therapy vehicles for the delivery and expression of up to 25kb foreign DNA without significantly compromising virus particle integrity [287]. However, despite its large genome, VACV completes its replication cycle within 24hrs of infection and produces relatively high titers of virus [288]. Furthermore, its historical use as the vaccination agent in the eradication of smallpox has allowed for extensive characterisation of clinical effects in human (compared to any other virus) [289]. Thus, VACV pathogenesis in both preclinical and clinical settings is well defined, with clear symptomology and safety profiles outlined.

VACV is capable of inducing robust immune responses mediated through both T cell activation and antibody responses, which can induce potent bystander effects to augment anti-tumor immunity [290]. For this reason, VACV is considered a promising oncolytic for the treatment of cancer. VACV has typically been applied in cancer therapy as either a gene therapy vehicle to deliver tumor specific therapeutic genes, a direct oncolytic that can replicate and kill within cancer cells specifically, or as a cancer vaccine which expresses tumor antigens or immunostimulatory agents.

VACV entry into target cells occurs via the expression of a multi-protein fusion complex carried by the virion and may be dependent on interaction with cell surface glycosaminoglycans or other undefined cell moieties that promote direct virus membrane fusion with the plasma membrane [291]. Alternatively, some strains of VACV can also use the endocytic pathway under low pH conditions, however inhibition of endosomal acidification prevents virus entry [292]. Upon entry and initial rounds of viral mRNA translation and protein production, vaccinia growth factor (VGF) is produced, which binds the EGF receptor and signals through the ERK1/2 pathway to stimulate the production of thymidine kinase (TK) in the host cell and increase nucleotide pools to provide sufficient availability of nucleotides for the production of new virions [293-295]. In addition, VACV encodes its own TK (vTK) to further promote phosphorylation of deoxythymidine, contributing to higher nucleotide pools for the incorporation into replicating VACV genomes [296]. In replicating cancer cells, available nucleotides for genome incorporation are abundant, thus the need for vTK and VGF are considered dispensable for VACV replication [297]. Therefore, to promote cancer specific viral replication, a construct of VACV with a double deletions of vTK and VGF was created, called vvDD. This created enhanced cell tropism for cells with constitutive EGFR or ERK1/2 activation [298]. By this same logic, cancers with activating mutations in KRAS downstream of EGFR also promote vvDD replication [299]. Infections of both resting and dividing NIH-3T3 cells demonstrated restricted vvDD replication compared to WT in resting cells but equivalent virus production in actively dividing cells [298]. Currently, strains with additional modifications to the vvDD backbone have gone on to clinical trials in patients with superficial injectable tumors [300].

General poxvirus replication lifecycle

Poxviruses encode the necessary enzymes and proteins to allow replication within the host cytoplasm. The binding of poxvirus virions is mediated by the expression of surface glycosaminoglycans on target cells or by components of the extracellular matrix [301-305]. The full replicative cycle depends on three waves of viral mRNA and protein synthesis known as early, intermediate, and late [284, 306]. After cell surface binding, poxvirus membranes fuse with cell membranes to release the virus core into the cytoplasm [307]. Along with the virus, packaged viral RNA polymerase and transcription factors initiate the first wave of viral gene expression (including the production of vTK in VACV). Approximately half of the 200+ genes encoded by VACV are transcribed during early infection [284]. These genes include intracellular modulators that inhibit apoptosis and block IFN signaling and prevent anti-viral responses [308]. Through a poorly understood mechanism, core uncoating is initiated causing dissolution of the core structure, releasing viral DNA into the cytoplasm which can be used as a template for DNA replication and the subsequent intermediate and late stages of virus lifecycle. Unlike the early stage, the subsequent intermediate and late stages require host transcription factors to contribute to the efficiency of viral gene expression [299, 309-312]. Late genes are required for the assembly of virus particles. The assembly of virions occurs in compartmentalized regions of the cytoplasm known as viral factories, which largely exclude cell organelles [286]. The process of forming infectious progeny is known as morphogenesis. The first visible structures are crescent shaped and made from lipid and protein, which subsequently elongates to form an oval or spherical structure that encloses the virus core components, creating an immature virion [313]. The viral DNA genome becomes packaged within the

immature virion, and as the core proteins are cleaved the virus acquires a characteristic brick-shaped structure which is fully infectious. These are known as intracellular mature virions (IMV). For the majority of progeny virions, morphogenesis ends here and IMV are released after cell lysis [313]. However, a subset of IMV will be transported away from the viral factories via microtubule trafficking to acquire an envelope, typically from golgi-derived membranes forming intracellular enveloped viruses (IEV) [314-319]. During egress of the virus from the cell, the IEV loses one of its outer membranes and fuses to the cell membrane to form cell-associated enveloped virus [320-323]. This articulated form of the virus can either help aid spread of the virus through direct cell-cell contact, or it may be released freely as extracellular enveloped virus [324]. For both MYXV and VACV based oncolytics, the IMV and IEV are used, since the majority of virions remain in these forms.

Maraba Virus

Maraba virus (MRBV) is an insect virus originally derived from female Brazilian sand flies in 1983. Following the initial characterization of MRBV in 1984, MRBV was believed to be a strain of vesicular stomatitis virus (VSV), belonging to the *Rhabdoviridae* family, based on a number of complement fixation assays. It is antigenically related to the New Jersey, Indiana, Cocal, Alagoas, and Carajas strains of VSV [325]. Rhabdoviruses are a family of bullet-shaped, enveloped viruses whose genomes consist of negative sense single stranded RNA and encode only 5 genes. The prototypical Rhabdovirus, VSV, is made up of 11,161 nucleotides. The outer most layer consists of a lipid envelope with incorporated glycoproteins (G), which allows virus binding and entry into the host cell [326, 327]. Currently, it is unknown what cell surface receptor the MRBV G protein binds

to mediate virus entry. However, due to the diverse species tropism, it is believed that the receptor is ubiquitously expressed. The middle layer consists of a matrix (M) protein which forms a bridge between the viral envelope and its genome [328]. The inner core is made up of the virus RNA genome tightly coiled around nucleocapsid protein (N) [329]. This core associates with both the large polymerase protein (L) and the phosphoprotein (P) [330]. (Fig. 1.4)

Interestingly, MRBV, Indiana, Alagoas, and Cocal are so antigenically similar that they were indistinguishable from one another based on the laboratory methods used at the time [331]. Isolations from human sera revealed high neutralizing antibody titers indicating that human infection with these viruses was common, but this only manifested in 3-4 day illness with fever, headache, myalgia, and malaise [332]. With improvements in genome sequencing over many years, stratification of VSV strains became more feasible with VSV becoming divided into two serotypes, New Jersey and Indiana. Based on the sequences of its glycoprotein and phosphoprotein genes, MRBV was later classified as a vesiculovirus with a distinct lineage within the Indiana serotype [333]. In the burgeoning field of oncolytic virotherapy, VSV demonstrated potent oncolytic activity and specificity for cancer cells in both *in vitro* and *in vivo* models [334-336]. However, the concern of pre-existing neutralizing antibodies against VSV raises the possibility of diminished systemic therapy for human cancer. Serological studies with VSV in both human and non-human animal models demonstrates differential serum neutralization of VSV between cancer patients and those of healthy subjects. Interestingly, both MRBV and pseudotyped VSV with MRBV glycoprotein were able to evade both non-immune and immune mediated serum neutralization and retained parental virus growth kinetics and tropism.

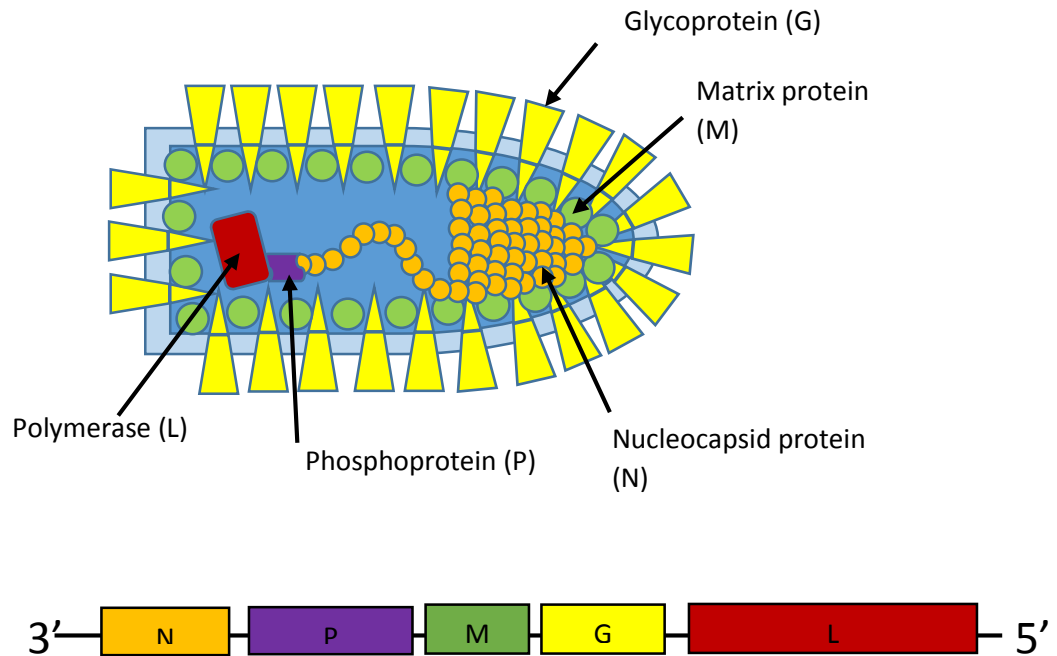


Figure 1.4. Rhabdovirus structure and genome organization- Rhabdoviruses are transcribed in a sequential manner with genes at the 3' end being transcribed in higher numbers than those at the 5' end. Nucleocapsid proteins are transcribed at the highest level to maintain RNA genome stability and viral packaging. Phosphoproteins play an important role in initiating and stabilizing L and N during transcription, translation, and genome replication during virus replication. Matrix protein maintains structural integrity of the virus and acts as an anchor for G protein incorporation in the virus membrane. Glycoprotein expression at the virus surface is required for virus binding and entry into the host cell. Packaged polymerases are important for transcription and replication of virus genomes.

Therefore, although there is high genetic sequence similarity between these two viruses, it is clear that MRBV is a serologically distinct member of the *Rhabdoviridae* family [337].

VSV replication lifecycle

Rhabdovirus binding to the host membrane is pH dependent as G protein folding is influenced by pH [327]. Entry is facilitated by clathrin-dependent endocytosis of virions and trafficked through the cytoplasm via microtubule transport [338, 339]. Through an undefined mechanism, the endosome becomes multi-vesicular, causing fusion of the virus envelope with the internal vesicles in a pH dependent fashion [340, 341]. These internal vesicles then fuse back with the membrane of the endosome releasing the virus core into the cytoplasm of the cell [342]. The genome serves as both a template for genome replication and for transcription of virus genes. Upon entry into the cytoplasm, the virus is uncoated and the M protein dissociates from the virus core leaving the N-RNA complex associated with both L and P proteins [343, 344]. The P protein is post translationally modified to promote homodimer formation, which is important for the interaction with the N and L proteins [330, 345]. Interaction of the L protein with the N-RNA is mediated through the P protein to initiate transcription [330, 346]. The L protein acts as the catalytic subunit of the viral polymerase and with the P protein forms an RNA dependent RNA polymerase (RdRp). Together with host cell elongation factor 1 α , guanylyl transferase, and heat shock protein 60, this complex facilitates transcription of viral genes [347, 348]. As the RNA genome is non-segmented, transcription is sequential with genes at the 3' end being transcribed in greater abundance than those at the 5' end [349, 350].

The fundamental difference for genome replication is the requirement of newly synthesized L, N and P protein complexes, whereas transcription requires predominantly

just L and P complexes [351]. During replication, the full genome is duplicated to produce an anti-genome, which subsequently acts as a template for the production of the full-length genome [352]. Because the promoter region of the anti-genome is more efficient than that of the genome promoter, more genomic copies of the RNA are made than anti-genome [352]. It is thought that the P protein maintains the N protein in a soluble encapsidated form [353, 354]. Both the genome and anti-genome contain localization signals to promote their replication and incorporation into new virions [355]. Virus assembly occurs at the cell membrane, where viral proteins are transported through different mechanisms. G protein is transported to the plasma membrane through the secretory route, however the mechanism for M protein transport remains undefined [356, 357]. The core comprised of N-RNA, P, and L proteins is transported in a microtubule dependent manner to the plasma membrane, where it associates with M proteins [339, 358]. Finally, the M protein recruits a number of cellular proteins which help facilitate M protein ubiquitination and virus budding from the membrane [359, 360].

MRBV cancer cell tropism

Maraba virus was first discovered to possess cancer cell specific tropism in a screen of a number of Rhabdoviruses in a panel of cancer cell lines. Maraba was discovered to be the most potent of these viruses, even more so than VSV. Further modifications of the virus were introduced through point mutations in both the M (L123W) and G (Q242R) proteins, which rendered the virus more virulent but also more vulnerable to IFN responses. With a 100x greater maximum tolerated dose than WT MRBV, the MG1 construct of MRBV showed durable curative responses in syngeneic murine models of colon carcinoma and ovarian xenograft models [361]. Additional modifications to MRBV MG1 have been

engineered to produce an IFN decoy receptor (MG1^{IDE}) upon successful replication of the virus, thereby reducing the capacity for IFN mediated resistance to MRBV in IFN-responsive cancer cells. Furthermore, normal cells were protected against MG1^{IDE} oncolysis as only a small subpopulation were capable of being infected. Thus, without productive virus replication, insufficient levels of the IFN decoy receptor could be produced to render the uninfected cells sensitive to virus infection [362]. Based on its broad acting cancer cell specificity and the relatively cell independent nature of replication, it is unclear whether additional trophic factors exist which may affect MRBV replication other than IFN response.

It is abundantly clear that effective MRBV based oncolysis is not strictly due to its direct infection and oncolysis of cancer cells, but also because of its immunostimulatory activity. In syngeneic murine models of metastatic melanoma, MG1-induced dendritic cell (DC) and natural killer (NK) cell activation was vital to effective oncolysis of cancer cells and reducing lung metastases. Infection and replication of MG1 in DCs induced DC maturation, which allowed for priming of NK cells to mediate NK cell cytotoxicity of tumor metastases [363]. However, MRBV alone is a relatively weak inducer of anti-tumoral adaptive T cell immune responses. Therefore MRBV application has since evolved to be utilized as a vaccine vector. Other syngeneic melanoma models have applied adenovirus vector based delivery of melanoma tumor associated antigens (Ad-hDCT) to prime CD8⁺T cell responses prior to MRBV administration. Although no hDCT-specific T cells could be observed 10 days after Ad-hDCT was administered, administration of MG1-hDCT 12 days after Ad-hDCT elicited a strong recall response from CD8⁺ T cells. Optimal administration of secondary MG1-hDCT 9 days after Ad-hDCT showed reduction

of melanoma metastases and tumor burden, which significantly improved survival and 20% of mice were cured [364]. Thus, current phase I/II clinical trials being carried out have adopted a similar prime boost vaccine strategy with adenovirus vectors engineered to express the MAGE A3 tumor associated antigen (AdMA3) followed by MG1MA3 administration [365].

1.6 Experimental objectives and hypothesis

Oncolytic virotherapy represents a burgeoning field in cancer therapy. The development of engineered viruses with improved cancer cell tropism, specificity, and efficacy represent the most recent advances in virotherapy. Furthermore, many different pre-clinical and clinical trials have made it evident that viruses have central roles in immunostimulatory functions aside from strictly inducing oncolysis of tumors. However, important questions remain unresolved in establishing the clinical utility of viruses for cancer therapy, specifically ovarian cancer therapy. Few studies have investigated the therapeutic effect of viruses in intraperitoneally disseminated EOC metastases. EOC spheroids undergo substantial morphological and signaling changes in pathways which are known to directly impact virus replication. Thus it remains to be determined what impact spheroid mediated metastasis has on viral oncolysis. Currently, only one study conducted by our research program has attempted to investigate how these factors impact MYXV oncolysis, and it remains unclear whether or not MYXV represents the best virotherapy for the treatment of metastatic EOC or whether more effective virotherapies exist. Previous trials with MYXV, vvDD, and MRBV have demonstrated that all three viruses have significant oncolytic activity and potential clinical benefit. However, the trophic factors which dictate their specificity are differentially represented within EOC tumors during

metastasis and across the patient population. Therefore we sought to determine how dynamic changes in EOC biology during metastasis and between EOC cell lines impact their viral kinetics in an *in vitro* metastasis model.

Additionally, we sought to investigate cell mediators of MRBV infection and replication as they have yet to be elucidated. Published research on VSV has shown that few host cell dependent factors are required for virus replication. This suggests that VSV and other highly similar viruses (such as MRBV) may be less susceptible to the various biological changes that occur in EOC cells. Studies with MRBV have shown diverse cell tropism for a number of homogenous cancer cell lines, but it is unclear whether or not MRBV oncolysis will be impacted by intercellular differences within a heterogeneous tumor. Thus, we sought to discover potential cell mediators that may impact MRBV and whether modulation of those factors could improve MRBV oncolysis. *Based on the complex and transforming nature of EOC cells during spheroid formation and metastasis as well as the high degree of intratumoral heterogeneity between cells within a tumor and over time, we hypothesize that these biological differences will directly impact oncolytic virus killing by MYXV, vvDD, and MRBV MG1 in EOC cells.*

1.7 References

1. Advisory, C. C. S. s., and Statistics, C. o. C. (2015). Canadian Cancer Statistics 2015. Canadian Cancer Society.
2. Ferlay J, S. I., Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray, F. (2012). GLOBOCAN 2012: Estimated Cancer Incidence, Mortality, and Prevalence Worldwide in 2012. International Agency for Research on Cancer.
3. Jelovac, D., and Armstrong, D. K.(2011) Recent progress in the diagnosis and treatment of ovarian cancer. *CA: a cancer journal for clinicians* 61: 183-203.
4. Coleman, R. L., Monk, B. J., Sood, A. K., and Herzog, T. J. (2013) Latest research and treatment of advanced-stage epithelial ovarian cancer. *Nature reviews* 10: 211-224.

5. Benedet, J. L., Bender, H., Jones, H., 3rd, Ngan, H. Y., and Pecorelli, S. (2000). FIGO staging classifications and clinical practice guidelines in the management of gynecologic cancers. FIGO Committee on Gynecologic Oncology. *International journal of gynaecology and obstetrics: the official organ of the International Federation of Gynaecology and Obstetrics* 70: 209-262.
6. Ebell, M. H., Culp, M. B., and Radke, T. J. (2015) A Systematic Review of Symptoms for the Diagnosis of Ovarian Cancer. *American journal of preventive medicine*.
7. Claus, E. B., Schildkraut, J. M., Thompson, W. D., and Risch, N. J. (1996). The genetic attributable risk of breast and ovarian cancer. *Cancer* 77: 2318-2324.
8. Ford, D., Easton, D. F., Bishop, D. T., Narod, S. A., and Goldgar, D. E. (1994). Risks of cancer in BRCA1-mutation carriers. Breast Cancer Linkage Consortium. *Lancet (London, England)* 343: 692-695.
9. Struewing, J. P., et al. (1997). The risk of cancer associated with specific mutations of BRCA1 and BRCA2 among Ashkenazi Jews. *The New England journal of medicine* 336: 1401-1408.
10. Hoskins, W. J., et al. (1994). The effect of diameter of largest residual disease on survival after primary cytoreductive surgery in patients with suboptimal residual epithelial ovarian carcinoma. *American journal of obstetrics and gynecology* 170: 974-979; discussion 979-980.
11. Bristow, R. E., Tomacruz, R. S., Armstrong, D. K., Trimble, E. L., and Montz, F. J. (2002). Survival effect of maximal cytoreductive surgery for advanced ovarian carcinoma during the platinum era: a meta-analysis. *J Clin Oncol* 20: 1248-1259.
12. du Bois, A., Reuss, A., Pujade-Lauraine, E., Harter, P., Ray-Coquard, I., and Pfisterer, J. (2009). Role of surgical outcome as prognostic factor in advanced epithelial ovarian cancer: a combined exploratory analysis of 3 prospectively randomized phase 3 multicenter trials: by the Arbeitsgemeinschaft Gynaekologische Onkologie Studiengruppe Ovarialkarzinom (AGO-OVAR) and the Groupe d'Investigateurs Nationaux Pour les Etudes des Cancers de l'Ovaire (GINECO). *Cancer* 115: 1234-1244.
13. Eisenhauer, E. L., Abu-Rustum, N. R., Sonoda, Y., Aghajanian, C., Barakat, R. R., and Chi, D. S. (2008). The effect of maximal surgical cytoreduction on sensitivity to platinum-taxane chemotherapy and subsequent survival in patients with advanced ovarian cancer. *Gynecologic oncology* 108: 276-281.
14. Inciura, A., et al. (2006). Comparison of adjuvant and neoadjuvant chemotherapy in the management of advanced ovarian cancer: a retrospective study of 574 patients. *BMC cancer* 6: 153.
15. Le, T., Faught, W., Hopkins, L., and Fung Kee Fung, M. (2005). Primary chemotherapy and adjuvant tumor debulking in the management of advanced-stage epithelial ovarian cancer. *Int J Gynecol Cancer* 15: 770-775.
16. Ivanov, S., Ivanov, S., and Khadzhiolov, N. (2004). [Prognostic factors and better survival rate after the treatment of advanced ovarian cancer with neoadjuvant chemotherapy]. *Akusherstvo i ginekologiya* 43: 17-19.
17. Lee, S. J., Kim, B. G., Lee, J. W., Park, C. S., Lee, J. H., and Bae, D. S. (2006). Preliminary results of neoadjuvant chemotherapy with paclitaxel and cisplatin in

- patients with advanced epithelial ovarian cancer who are inadequate for optimum primary surgery. *The journal of obstetrics and gynaecology research* 32: 99-106.
18. Schwartz, P. E. (2002). Neoadjuvant chemotherapy for the management of ovarian cancer. *Best practice & research* 16: 585-596.
 19. Vergote, I., Trope, C. G., Amant, F., Ehlen, T., Reed, N. S., and Casado, A. (2011) Neoadjuvant chemotherapy is the better treatment option in some patients with stage IIIc to IV ovarian cancer. *J Clin Oncol* 29: 4076-4078.
 20. Bristow, R. E., and Chi, D. S. (2006). Platinum-based neoadjuvant chemotherapy and interval surgical cytoreduction for advanced ovarian cancer: a meta-analysis. *Gynecologic oncology* 103: 1070-1076.
 21. du Bois, A., et al. (2012) Neoadjuvant chemotherapy cannot be regarded as adequate routine therapy strategy of advanced ovarian cancer. *Int J Gynecol Cancer* 22: 182-185.
 22. Steed, H., et al. (2006). A retrospective analysis of neoadjuvant platinum-based chemotherapy versus up-front surgery in advanced ovarian cancer. *Int J Gynecol Cancer* 16 Suppl 1: 47-53.
 23. Chi, D. S., et al. (2012) An analysis of patients with bulky advanced stage ovarian, tubal, and peritoneal carcinoma treated with primary debulking surgery (PDS) during an identical time period as the randomized EORTC-NCIC trial of PDS vs neoadjuvant chemotherapy (NACT). *Gynecologic oncology* 124: 10-14.
 24. Kurman, R. J., and Shih Ie, M. (2010) The origin and pathogenesis of epithelial ovarian cancer: a proposed unifying theory. *The American journal of surgical pathology* 34: 433-443.
 25. Seidman, J. D., and Kurman, R. J. (1996). Subclassification of serous borderline tumors of the ovary into benign and malignant types. A clinicopathologic study of 65 advanced stage cases. *The American journal of surgical pathology* 20: 1331-1345.
 26. Shih Ie, M., and Kurman, R. J. (2004). Ovarian tumorigenesis: a proposed model based on morphological and molecular genetic analysis. *The American journal of pathology* 164: 1511-1518.
 27. Gilks, C. B. (2004). Subclassification of ovarian surface epithelial tumors based on correlation of histologic and molecular pathologic data. *Int J Gynecol Pathol* 23: 200-205.
 28. Smith Sehdev, A. E., Sehdev, P. S., and Kurman, R. J. (2003). Noninvasive and invasive micropapillary (low-grade) serous carcinoma of the ovary: a clinicopathologic analysis of 135 cases. *The American journal of surgical pathology* 27: 725-736.
 29. Burks, R. T., Sherman, M. E., and Kurman, R. J. (1996). Micropapillary serous carcinoma of the ovary. A distinctive low-grade carcinoma related to serous borderline tumors. *The American journal of surgical pathology* 20: 1319-1330.
 30. Cho, K. R., and Shih Ie, M. (2009). Ovarian cancer. *Annual review of pathology* 4: 287-313.
 31. Kuo, K. T., et al. (2009). Analysis of DNA copy number alterations in ovarian serous tumors identifies new molecular genetic changes in low-grade and high-grade carcinomas. *Cancer research* 69: 4036-4042.

32. Kobel, M., et al. (2010) The biological and clinical value of p53 expression in pelvic high-grade serous carcinomas. *The Journal of pathology* 222: 191-198.
33. O'Neill, C. J., Deavers, M. T., Malpica, A., Foster, H., and McCluggage, W. G. (2005). An immunohistochemical comparison between low-grade and high-grade ovarian serous carcinomas: significantly higher expression of p53, MIB1, BCL2, HER-2/neu, and C-KIT in high-grade neoplasms. *The American journal of surgical pathology* 29: 1034-1041.
34. Russell, S. E., and McCluggage, W. G. (2004). A multistep model for ovarian tumorigenesis: the value of mutation analysis in the KRAS and BRAF genes. *The Journal of pathology* 203: 617-619.
35. Singer, G., Kurman, R. J., Chang, H. W., Cho, S. K., and Shih Ie, M. (2002). Diverse tumorigenic pathways in ovarian serous carcinoma. *The American journal of pathology* 160: 1223-1228.
36. Singer, G., Shih Ie, M., Truskinovsky, A., Umudum, H., and Kurman, R. J. (2003). Mutational analysis of K-ras segregates ovarian serous carcinomas into two types: invasive MPSC (low-grade tumor) and conventional serous carcinoma (high-grade tumor). *Int J Gynecol Pathol* 22: 37-41.
37. Singer, G., et al. (2005). Patterns of p53 mutations separate ovarian serous borderline tumors and low- and high-grade carcinomas and provide support for a new model of ovarian carcinogenesis: a mutational analysis with immunohistochemical correlation. *The American journal of surgical pathology* 29: 218-224.
38. Ho, C. L., Kurman, R. J., Dehari, R., Wang, T. L., and Shih Ie, M. (2004). Mutations of BRAF and KRAS precede the development of ovarian serous borderline tumors. *Cancer research* 64: 6915-6918.
39. Staebler, A., et al. (2002). Micropapillary serous carcinoma of the ovary has distinct patterns of chromosomal imbalances by comparative genomic hybridization compared with atypical proliferative serous tumors and serous carcinomas. *Human pathology* 33: 47-59.
40. Dubeau, L. (2008). The cell of origin of ovarian epithelial tumours. *The Lancet* 9: 1191-1197.
41. Blaustein, A. (1984). Peritoneal mesothelium and ovarian surface cells--shared characteristics. *Int J Gynecol Pathol* 3: 361-375.
42. Cheng, W., Liu, J., Yoshida, H., Rosen, D., and Naora, H. (2005). Lineage infidelity of epithelial ovarian cancers is controlled by HOX genes that specify regional identity in the reproductive tract. *Nature medicine* 11: 531-537.
43. Fathalla, M. F. (1971). Incessant ovulation--a factor in ovarian neoplasia? *Lancet (London, England)* 2: 163.
44. Auersperg, N., Wong, A. S., Choi, K. C., Kang, S. K., and Leung, P. C. (2001). Ovarian surface epithelium: biology, endocrinology, and pathology. *Endocrine reviews* 22: 255-288.
45. Casagrande, J. T., Louie, E. W., Pike, M. C., Roy, S., Ross, R. K., and Henderson, B. E. (1979). "Incessant ovulation" and ovarian cancer. *Lancet (London, England)* 2: 170-173.

46. Soegaard, M., et al. (2007). Different risk factor profiles for mucinous and nonmucinous ovarian cancer: results from the Danish MALOVA study. *Cancer Epidemiol Biomarkers Prev* 16: 1160-1166.
47. Risch, H. A., Weiss, N. S., Lyon, J. L., Daling, J. R., and Liff, J. M. (1983). Events of reproductive life and the incidence of epithelial ovarian cancer. *American journal of epidemiology* 117: 128-139.
48. Rosenblatt, K. A., and Thomas, D. B. (1993). Lactation and the risk of epithelial ovarian cancer. The WHO Collaborative Study of Neoplasia and Steroid Contraceptives. *International journal of epidemiology* 22: 192-197.
49. Schildkraut, J. M., Schwingl, P. J., Bastos, E., Evanoff, A., and Hughes, C. (1996). Epithelial ovarian cancer risk among women with polycystic ovary syndrome. *Obstetrics and gynecology* 88: 554-559.
50. Rosenberg, L., et al. (1994). A case-control study of oral contraceptive use and invasive epithelial ovarian cancer. *American journal of epidemiology* 139: 654-661.
51. Risch, H. A. (1998). Hormonal etiology of epithelial ovarian cancer, with a hypothesis concerning the role of androgens and progesterone. *Journal of the National Cancer Institute* 90: 1774-1786.
52. Maccio, A., and Madeddu, C. (2012) Inflammation and ovarian cancer. *Cytokine* 58: 133-147.
53. Seidman, J. D., and Khedmati, F. (2008). Exploring the histogenesis of ovarian mucinous and transitional cell (Brenner) neoplasms and their relationship with Walthard cell nests: a study of 120 tumors. *Archives of pathology & laboratory medicine* 132: 1753-1760.
54. Veras, E., et al. (2009). Cystic and adenofibromatous clear cell carcinomas of the ovary: distinctive tumors that differ in their pathogenesis and behavior: a clinicopathologic analysis of 122 cases. *The American journal of surgical pathology* 33: 844-853.
55. Piek, J. M., et al. (2001). Dysplastic changes in prophylactically removed Fallopian tubes of women predisposed to developing ovarian cancer. *The Journal of pathology* 195: 451-456.
56. Callahan, M. J., et al. (2007). Primary fallopian tube malignancies in BRCA-positive women undergoing surgery for ovarian cancer risk reduction. *J Clin Oncol* 25: 3985-3990.
57. Cass, I., Holschneider, C., Datta, N., Barbuto, D., Walts, A. E., and Karlan, B. Y. (2005). BRCA-mutation-associated fallopian tube carcinoma: a distinct clinical phenotype? *Obstetrics and gynecology* 106: 1327-1334.
58. Leeper, K., Garcia, R., Swisher, E., Goff, B., Greer, B., and Paley, P. (2002). Pathologic findings in prophylactic oophorectomy specimens in high-risk women. *Gynecologic oncology* 87: 52-56.
59. Piek, J. M., Verheijen, R. H., Kenemans, P., Massuger, L. F., Bulten, H., and van Diest, P. J. (2003). BRCA1/2-related ovarian cancers are of tubal origin: a hypothesis. *Gynecologic oncology* 90: 491.
60. Lee, Y., et al. (2007). A candidate precursor to serous carcinoma that originates in the distal fallopian tube. *The Journal of pathology* 211: 26-35.
61. Lee, Y., Medeiros, F., Kindelberger, D., Callahan, M. J., Muto, M. G., and Crum, C. P. (2006). Advances in the recognition of tubal intraepithelial carcinoma:

- applications to cancer screening and the pathogenesis of ovarian cancer. *Advances in anatomic pathology* 13: 1-7.
62. (2011) Integrated genomic analyses of ovarian carcinoma. *Nature* 474: 609-615.
 63. Ciriello, G., Miller, M. L., Aksoy, B. A., Senbabaoglu, Y., Schultz, N., and Sander, C. (2013) Emerging landscape of oncogenic signatures across human cancers. *Nature genetics* 45: 1127-1133.
 64. Wu, L., and Levine, A. J. (1997). Differential regulation of the p21/WAF-1 and mdm2 genes after high-dose UV irradiation: p53-dependent and p53-independent regulation of the mdm2 gene. *Molecular medicine (Cambridge, Mass* 3: 441-451.
 65. Haupt, Y., Maya, R., Kazaz, A., and Oren, M. (1997). Mdm2 promotes the rapid degradation of p53. *Nature* 387: 296-299.
 66. Canman, C. E., et al. (1998). Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. *Science (New York, N.Y)* 281: 1677-1679.
 67. Gu, W., and Roeder, R. G. (1997). Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. *Cell* 90: 595-606.
 68. Kastan, M. B., Onyekwere, O., Sidransky, D., Vogelstein, B., and Craig, R. W. (1991). Participation of p53 protein in the cellular response to DNA damage. *Cancer research* 51: 6304-6311.
 69. Hirao, A., et al. (2000). DNA damage-induced activation of p53 by the checkpoint kinase Chk2. *Science (New York, N.Y)* 287: 1824-1827.
 70. Sakaguchi, K., et al. (1998). DNA damage activates p53 through a phosphorylation-acetylation cascade. *Genes & development* 12: 2831-2841.
 71. Hanel, W., and Moll, U. M. (2012) Links between mutant p53 and genomic instability. *Journal of cellular biochemistry* 113: 433-439.
 72. Overholtzer, M., et al. (2003). The presence of p53 mutations in human osteosarcomas correlates with high levels of genomic instability. *Proceedings of the National Academy of Sciences of the United States of America* 100: 11547-11552.
 73. Goringe, K. L., et al. (2007). High-resolution single nucleotide polymorphism array analysis of epithelial ovarian cancer reveals numerous microdeletions and amplifications. *Clin Cancer Res* 13: 4731-4739.
 74. Storchova, Z., and Pellman, D. (2004). From polyploidy to aneuploidy, genome instability and cancer. *Nat Rev Mol Cell Biol* 5: 45-54.
 75. Hainaut, P., and Hollstein, M. (2000). p53 and human cancer: the first ten thousand mutations. *Advances in cancer research* 77: 81-137.
 76. Tommasino, M., et al. (2003). The role of TP53 in Cervical carcinogenesis. *Human mutation* 21: 307-312.
 77. Kohler, M. F., Kerns, B. J., Humphrey, P. A., Marks, J. R., Bast, R. C., Jr., and Berchuck, A. (1993). Mutation and overexpression of p53 in early-stage epithelial ovarian cancer. *Obstetrics and gynecology* 81: 643-650.
 78. Marks, J. R., et al. (1991). Overexpression and mutation of p53 in epithelial ovarian cancer. *Cancer research* 51: 2979-2984.
 79. Petitjean, A., et al. (2007). Impact of mutant p53 functional properties on TP53 mutation patterns and tumor phenotype: lessons from recent developments in the IARC TP53 database. *Human mutation* 28: 622-629.

80. Brachova, P., Thiel, K. W., and Leslie, K. K. (2013) The consequence of oncomorphic TP53 mutations in ovarian cancer. *International journal of molecular sciences* 14: 19257-19275.
81. Adorno, M., et al. (2009). A Mutant-p53/Smad complex opposes p63 to empower TGFbeta-induced metastasis. *Cell* 137: 87-98.
82. Muller, P. A., et al. (2009). Mutant p53 drives invasion by promoting integrin recycling. *Cell* 139: 1327-1341.
83. Petrucelli, N., Daly, M. B., and Feldman, G. L. (2010) Hereditary breast and ovarian cancer due to mutations in BRCA1 and BRCA2. *Genet Med* 12: 245-259.
84. Agalliu, I., Gern, R., Leanza, S., and Burk, R. D. (2009). Associations of high-grade prostate cancer with BRCA1 and BRCA2 founder mutations. *Clin Cancer Res* 15: 1112-1120.
85. Narod, S. A., et al. (2008). Rapid progression of prostate cancer in men with a BRCA2 mutation. *British journal of cancer* 99: 371-374.
86. (2000). Prevalence and penetrance of BRCA1 and BRCA2 mutations in a population-based series of breast cancer cases. Anglian Breast Cancer Study Group. *British journal of cancer* 83: 1301-1308.
87. Rubin, S. C., et al. (1996). Clinical and pathological features of ovarian cancer in women with germ-line mutations of BRCA1. *The New England journal of medicine* 335: 1413-1416.
88. Aida, H., et al. (1998). Clinical features of ovarian cancer in Japanese women with germ-line mutations of BRCA1. *Clin Cancer Res* 4: 235-240.
89. Liu, Y., and West, S. C. (2002). Distinct functions of BRCA1 and BRCA2 in double-strand break repair. *Breast Cancer Res* 4: 9-13.
90. Meindl, A., et al. (2010). Germline mutations in breast and ovarian cancer pedigrees establish RAD51C as a human cancer susceptibility gene. *Nature genetics* 42: 410-414.
91. Loveday, C., et al. (2011). Germline mutations in RAD51D confer susceptibility to ovarian cancer. *Nature genetics* 43: 879-882.
92. Thorstenson, Y. R., et al. (2003). Contributions of ATM mutations to familial breast and ovarian cancer. *Cancer research* 63: 3325-3333.
93. Ramus, S. J., et al. (2015). Germline Mutations in the BRIP1, BARD1, PALB2, and NBN Genes in Women With Ovarian Cancer. *Journal of the National Cancer Institute* 107.
94. Zhang, H., et al. (1998). BRCA1 physically associates with p53 and stimulates its transcriptional activity. *Oncogene* 16: 1713-1721.
95. Chai, Y. L., Cui, J., Shao, N., Shyam, E., Reddy, P., and Rao, V. N. (1999). The second BRCT domain of BRCA1 proteins interacts with p53 and stimulates transcription from the p21WAF1/CIP1 promoter. *Oncogene* 18: 263-268.
96. Ouchi, T., Monteiro, A. N., August, A., Aaronson, S. A., and Hanafusa, H. (1998). BRCA1 regulates p53-dependent gene expression. *Proceedings of the National Academy of Sciences of the United States of America* 95: 2302-2306.
97. Jiang, J., et al. (2011). p53-dependent BRCA1 nuclear export controls cellular susceptibility to DNA damage. *Cancer research* 71: 5546-5557.
98. Brugarolas, J., and Jacks, T. (1997). Double indemnity: p53, BRCA and cancer. p53 mutation partially rescues developmental arrest in Brca1 and Brca2 null mice,

- suggesting a role for familial breast cancer genes in DNA damage repair. *Nature medicine* 3: 721-722.
99. Sourvinos, G., and Spandidos, D. A. (1998). Decreased BRCA1 expression levels may arrest the cell cycle through activation of p53 checkpoint in human sporadic breast tumors. *Biochemical and biophysical research communications* 245: 75-80.
 100. Buller, R. E., et al. (2001). The p53 mutational spectrum associated with BRCA1 mutant ovarian cancer. *Clin Cancer Res* 7: 831-838.
 101. Nowell, P. C. (1976). The clonal evolution of tumor cell populations. *Science (New York, N.Y)* 194: 23-28.
 102. Khalique, L., Ayhan, A., Weale, M. E., Jacobs, I. J., Ramus, S. J., and Gayther, S. A. (2007). Genetic intra-tumour heterogeneity in epithelial ovarian cancer and its implications for molecular diagnosis of tumours. *The Journal of pathology* 211: 286-295.
 103. Khalique, L., et al. (2009). The clonal evolution of metastases from primary serous epithelial ovarian cancers. *International journal of cancer* 124: 1579-1586.
 104. Schwarz, R. F., et al. (2015). Spatial and temporal heterogeneity in high-grade serous ovarian cancer: a phylogenetic analysis. *PLoS medicine* 12: e1001789.
 105. Pfisterer, J., et al. (2006). Gemcitabine plus carboplatin compared with carboplatin in patients with platinum-sensitive recurrent ovarian cancer: an intergroup trial of the AGO-OVAR, the NCIC CTG, and the EORTC GCG. *J Clin Oncol* 24: 4699-4707.
 106. Parmar, M. K., et al. (2003). Paclitaxel plus platinum-based chemotherapy versus conventional platinum-based chemotherapy in women with relapsed ovarian cancer: the ICON4/AGO-OVAR-2.2 trial. *Lancet (London, England)* 361: 2099-2106.
 107. du Bois, A., et al. (2003). A randomized clinical trial of cisplatin/paclitaxel versus carboplatin/paclitaxel as first-line treatment of ovarian cancer. *Journal of the National Cancer Institute* 95: 1320-1329.
 108. Ozols, R. F., et al. (2003). Phase III trial of carboplatin and paclitaxel compared with cisplatin and paclitaxel in patients with optimally resected stage III ovarian cancer: a Gynecologic Oncology Group study. *J Clin Oncol* 21: 3194-3200.
 109. Patch, A. M., et al. (2015) Whole-genome characterization of chemoresistant ovarian cancer. *Nature* 521: 489-494.
 110. Norquist, B., et al. (2011). Secondary somatic mutations restoring BRCA1/2 predict chemotherapy resistance in hereditary ovarian carcinomas. *J Clin Oncol* 29: 3008-3015.
 111. Swisher, E. M., Sakai, W., Karlan, B. Y., Wurz, K., Urban, N., and Taniguchi, T. (2008). Secondary BRCA1 mutations in BRCA1-mutated ovarian carcinomas with platinum resistance. *Cancer research* 68: 2581-2586.
 112. Tarin, D., Price, J. E., Kettlewell, M. G., Souter, R. G., Vass, A. C., and Crossley, B. (1984). Mechanisms of human tumor metastasis studied in patients with peritoneovenous shunts. *Cancer research* 44: 3584-3592.
 113. Lengyel, E. (2010). Ovarian cancer development and metastasis. *The American journal of pathology* 177: 1053-1064.
 114. Saida, T., Tanaka, Y. O., Matsumoto, K., Satoh, T., Yoshikawa, H., and Minami, M. (2015). Revised FIGO staging system for cancer of the ovary, fallopian tube,

- and peritoneum: important implications for radiologists. *Japanese journal of radiology*.
115. Siegel, R., Naishadham, D., and Jemal, A. Cancer statistics, 2012. *CA: a cancer journal for clinicians* 62: 10-29.
 116. Elloul, S., Vaksman, O., Stavnes, H. T., Trope, C. G., Davidson, B., and Reich, R. (2010). Mesenchymal-to-epithelial transition determinants as characteristics of ovarian carcinoma effusions. *Clinical & experimental metastasis* 27: 161-172.
 117. Turley, E. A., Veiseh, M., Radisky, D. C., and Bissell, M. J. (2008). Mechanisms of disease: epithelial-mesenchymal transition--does cellular plasticity fuel neoplastic progression? *Nature clinical practice* 5: 280-290.
 118. Vergara, D., et al. (2010) Epithelial-mesenchymal transition in ovarian cancer. *Cancer letters* 291: 59-66.
 119. Kalluri, R., and Weinberg, R. A. (2009). The basics of epithelial-mesenchymal transition. *The Journal of clinical investigation* 119: 1420-1428.
 120. Ahmed, N., Thompson, E. W., and Quinn, M. A. (2007). Epithelial-mesenchymal exception to the norm. *Journal of cellular physiology* 213: 581-588.
 121. Huber, M. A., Kraut, N., and Beug, H. (2005). Molecular requirements for epithelial-mesenchymal transition during tumor progression. *Current opinion in cell biology* 17: 548-558.
 122. Desai, R., Sarpal, R., Ishiyama, N., Pellikka, M., Ikura, M., and Tepass, U. (2013) Monomeric alpha-catenin links cadherin to the actin cytoskeleton. *Nature cell biology* 15: 261-273.
 123. Tanaka, Y., et al. (2003). Role of nectin in formation of E-cadherin-based adherens junctions in keratinocytes: analysis with the N-cadherin dominant negative mutant. *Molecular biology of the cell* 14: 1597-1609.
 124. Hartsock, A., and Nelson, W. J. (2008). Adherens and tight junctions: structure, function and connections to the actin cytoskeleton. *Biochimica et biophysica acta* 1778: 660-669.
 125. Kurrey, N. K., K, A., and Bapat, S. A. (2005). Snail and Slug are major determinants of ovarian cancer invasiveness at the transcription level. *Gynecologic oncology* 97: 155-165.
 126. Blehshmidt, K., Sassen, S., Schmalfeldt, B., Schuster, T., Hofler, H., and Becker, K. F. (2008). The E-cadherin repressor Snail is associated with lower overall survival of ovarian cancer patients. *British journal of cancer* 98: 489-495.
 127. Elloul, S., et al. (2005). Snail, Slug, and Smad-interacting protein 1 as novel parameters of disease aggressiveness in metastatic ovarian and breast carcinoma. *Cancer* 103: 1631-1643.
 128. Do, T. V., Kubba, L. A., Du, H., Sturgis, C. D., and Woodruff, T. K. (2008). Transforming growth factor-beta1, transforming growth factor-beta2, and transforming growth factor-beta3 enhance ovarian cancer metastatic potential by inducing a Smad3-dependent epithelial-to-mesenchymal transition. *Mol Cancer Res* 6: 695-705.
 129. Sun, L., et al. (1994). Expression of transforming growth factor beta type II receptor leads to reduced malignancy in human breast cancer MCF-7 cells. *The Journal of biological chemistry* 269: 26449-26455.

130. Lafon, C., Mathieu, C., Guerrin, M., Pierre, O., Vidal, S., and Valette, A. (1996). Transforming growth factor beta 1-induced apoptosis in human ovarian carcinoma cells: protection by the antioxidant N-acetylcysteine and bcl-2. *Cell Growth Differ* 7: 1095-1104.
131. Lebrun, J.-J. (2012). The Dual Role of TGF in Human Cancer: From Tumor Suppression to Cancer Metastasis. *ISRN Molecular Biology*, 2012: 28.
132. Nakao, A., et al. (1997). TGF-beta receptor-mediated signalling through Smad2, Smad3 and Smad4. *The EMBO journal* 16: 5353-5362.
133. Leroy, P., and Mostov, K. E. (2007). Slug is required for cell survival during partial epithelial-mesenchymal transition of HGF-induced tubulogenesis. *Molecular biology of the cell* 18: 1943-1952.
134. Xu, J., Lamouille, S., and Derynck, R. (2009). TGF-beta-induced epithelial to mesenchymal transition. *Cell research* 19: 156-172.
135. Ahmed, N., and Stenvers, K. L. (2013). Getting to know ovarian cancer ascites: opportunities for targeted therapy-based translational research. *Frontiers in oncology* 3: 256.
136. Ayantunde, A. A., and Parsons, S. L. (2007). Pattern and prognostic factors in patients with malignant ascites: a retrospective study. *Ann Oncol* 18: 945-949.
137. Parsons, S. L., Lang, M. W., and Steele, R. J. (1996). Malignant ascites: a 2-year review from a teaching hospital. *Eur J Surg Oncol* 22: 237-239.
138. Kipps, E., Tan, D. S., and Kaye, S. B. (2013). Meeting the challenge of ascites in ovarian cancer: new avenues for therapy and research. *Nat Rev Cancer* 13: 273-282.
139. Feldman, G. B., and Knapp, R. C. (1974). Lymphatic drainage of the peritoneal cavity and its significance in ovarian cancer. *American journal of obstetrics and gynecology* 119: 991-994.
140. Adam, R. A., and Adam, Y. G. (2004). Malignant ascites: past, present, and future. *Journal of the American College of Surgeons* 198: 999-1011.
141. Feldman, G. B., Knapp, R. C., Order, S. E., and Hellman, S. (1972). The role of lymphatic obstruction in the formation of ascites in a murine ovarian carcinoma. *Cancer research* 32: 1663-1666.
142. Gilmore, A. P. (2005). Anoikis. *Cell death and differentiation* 12 Suppl 2: 1473-1477.
143. Shield, K., Riley, C., Quinn, M. A., Rice, G. E., Ackland, M. L., and Ahmed, N. (2007). Alpha2beta1 integrin affects metastatic potential of ovarian carcinoma spheroids by supporting disaggregation and proteolysis. *Journal of carcinogenesis* 6: 11.
144. Frankel, A., Buckman, R., and Kerbel, R. S. (1997). Abrogation of taxol-induced G2-M arrest and apoptosis in human ovarian cancer cells grown as multicellular tumor spheroids. *Cancer research* 57: 2388-2393.
145. Rafehi, S., et al. (2016) TGFbeta signalling regulates epithelial-mesenchymal plasticity in ovarian cancer ascites-derived spheroids. *Endocrine-related cancer*.
146. Correa, R. J., Peart, T., Valdes, Y. R., DiMattia, G. E., and Shepherd, T. G. (2012) Modulation of AKT activity is associated with reversible dormancy in ascites-derived epithelial ovarian cancer spheroids. *Carcinogenesis* 33: 49-58.

147. Peart, T., et al. Intact LKB1 activity is required for survival of dormant ovarian cancer spheroids. *Oncotarget* 6: 22424-22438.
148. Lu, Z., et al. (2008). The tumor suppressor gene ARHI regulates autophagy and tumor dormancy in human ovarian cancer cells. *The Journal of clinical investigation* 118: 3917-3929.
149. Correa, R. J., et al. (2015). Combination of AKT inhibition with autophagy blockade effectively reduces ascites-derived ovarian cancer cell viability. *Carcinogenesis* 35: 1951-1961.
150. Sui, X., et al. (2013). Autophagy and chemotherapy resistance: a promising therapeutic target for cancer treatment. *Cell death & disease* 4: e838.
151. Bao, L., et al. (2014). Induction of autophagy contributes to cisplatin resistance in human ovarian cancer cells. *Molecular medicine reports* 11: 91-98.
152. Chen, X., Wu, Y., Dong, H., Zhang, C. Y., and Zhang, Y. (2013). Platinum-based agents for individualized cancer treatment. *Current molecular medicine* 13: 1603-1612.
153. Liao, J., et al. (2014). Ovarian cancer spheroid cells with stem cell-like properties contribute to tumor generation, metastasis and chemotherapy resistance through hypoxia-resistant metabolism. *PloS one* 9: e84941.
154. Casey, R. C., et al. (2001). Beta 1-integrins regulate the formation and adhesion of ovarian carcinoma multicellular spheroids. *The American journal of pathology* 159: 2071-2080.
155. Slack-Davis, J. K., Atkins, K. A., Harrer, C., Hershey, E. D., and Conaway, M. (2009). Vascular cell adhesion molecule-1 is a regulator of ovarian cancer peritoneal metastasis. *Cancer research* 69: 1469-1476.
156. Kenny, H. A., Kaur, S., Coussens, L. M., and Lengyel, E. (2008). The initial steps of ovarian cancer cell metastasis are mediated by MMP-2 cleavage of vitronectin and fibronectin. *The Journal of clinical investigation* 118: 1367-1379.
157. Kenny, H. A., and Lengyel, E. (2009). MMP-2 functions as an early response protein in ovarian cancer metastasis. *Cell cycle (Georgetown, Tex)* 8: 683-688.
158. Strobel, T., and Cannistra, S. A. (1999). Beta1-integrins partly mediate binding of ovarian cancer cells to peritoneal mesothelium in vitro. *Gynecologic oncology* 73: 362-367.
159. Spannuth, W. A., et al. (2009). Functional significance of VEGFR-2 on ovarian cancer cells. *International journal of cancer* 124: 1045-1053.
160. Nishida, N., Yano, H., Komai, K., Nishida, T., Kamura, T., and Kojiro, M. (2004). Vascular endothelial growth factor C and vascular endothelial growth factor receptor 2 are related closely to the prognosis of patients with ovarian carcinoma. *Cancer* 101: 1364-1374.
161. Cai, J., et al. (2012). Fibroblasts in omentum activated by tumor cells promote ovarian cancer growth, adhesion and invasiveness. *Carcinogenesis* 33: 20-29.
162. Nieman, K. M., et al. (2011). Adipocytes promote ovarian cancer metastasis and provide energy for rapid tumor growth. *Nature medicine* 17: 1498-1503.
163. Russell, S. J., Peng, K. W., and Bell, J. C. (2012). Oncolytic virotherapy. *Nature biotechnology* 30: 658-670.

164. Thorne, S. H., Hermiston, T., and Kirn, D. (2005). Oncolytic virotherapy: approaches to tumor targeting and enhancing antitumor effects. *Seminars in oncology* 32: 537-548.
165. Dock, G. (1904). The influence of complicating disease upon leukaemia *The American Journal of the Medical Sciences* 127: 563-592.
166. Pelner, L., Fowler, G. A., and Nauts, H. C. (1958). Effects of concurrent infections and their toxins on the course of leukemia. *Acta medica Scandinavica* 338: 1-47.
167. Kelly, E., and Russell, S. J. (2007). History of oncolytic viruses: genesis to genetic engineering. *Mol Ther* 15: 651-659.
168. De Pace, N. (1912). SULLA SCOMPARSA DI UN ENORME CANCRO VEGETANTE DEL COLLO DELL'UTERO SENZA CURA CHIRURGICA. *GINECOLOGIA* 9: 82-89.
169. Cann, A. J., Ed. (2005). *Principles of Molecular Virology*, Elsevier Inc.
170. Lodish H, B. A., Zipursky S, et al. (2000). Section 6.3, Viruses: Structure, Function, and Uses. In *Molecular Cell Biology, 4th Edition* (W. H. Freeman, Ed.). W. H. Freeman and Company, New York.
171. Pellet, P. E., Mitra, S., Holland, T.C (2014). Basics of virology. In *Handbook of Clinical Neurology* (A. C. T. a. J.Booss, Ed.).
172. Haywood, A. M. (2010). Membrane uncoating of intact enveloped viruses. *Journal of virology* 84: 10946-10955.
173. Tsai, B. (2007). Penetration of nonenveloped viruses into the cytoplasm. *Annual review of cell and developmental biology* 23: 23-43.
174. Moyer, C. L., and Nemerow, G. R. (2011). Viral weapons of membrane destruction: variable modes of membrane penetration by non-enveloped viruses. *Current opinion in virology* 1: 44-49.
175. Mire, C. E., Dube, D., Delos, S. E., White, J. M., and Whitt, M. A. (2009). Glycoprotein-dependent acidification of vesicular stomatitis virus enhances release of matrix protein. *Journal of virology* 83: 12139-12150.
176. Le Sage, V., and Mouland, A. J. (2013). Viral subversion of the nuclear pore complex. *Viruses* 5: 2019-2042.
177. Schmid, M., Speiseder, T., Dobner, T., and Gonzalez, R. A. (2013). DNA virus replication compartments. *Journal of virology* 88: 1404-1420.
178. Dokland, T. (1999). Scaffolding proteins and their role in viral assembly. *Cell Mol Life Sci* 56: 580-603.
179. El Najjar, F., Schmitt, A. P., and Dutch, R. E. (2014). Paramyxovirus glycoprotein incorporation, assembly and budding: a three way dance for infectious particle production. *Viruses* 6: 3019-3054.
180. Stockley, P. G., et al. (2013). Packaging signals in single-stranded RNA viruses: nature's alternative to a purely electrostatic assembly mechanism. *Journal of biological physics* 39: 277-287.
181. Bird, S. W., and Kirkegaard, K. (2015). Escape of non-enveloped virus from intact cells. *Virology* 479-480: 444-449.
182. Cadd, T. L., Skoging, U., and Liljestrom, P. (1997). Budding of enveloped viruses from the plasma membrane. *Bioessays* 19: 993-1000.
183. Welsch, S., Muller, B., and Krausslich, H. G. (2007). More than one door - Budding of enveloped viruses through cellular membranes. *FEBS letters* 581: 2089-2097.

184. Hanahan, D., and Weinberg, R. A. (2000). The hallmarks of cancer. *Cell* 100: 57-70.
185. Stojdl, D. F., et al. (2000). Exploiting tumor-specific defects in the interferon pathway with a previously unknown oncolytic virus. *Nature medicine* 6: 821-825.
186. Reichard, K. W., et al. (1992). Newcastle disease virus selectively kills human tumor cells. *The Journal of surgical research* 52: 448-453.
187. Bergmann, M., et al. (2001). A genetically engineered influenza A virus with ras-dependent oncolytic properties. *Cancer research* 61: 8188-8193.
188. Stojdl, D. F., et al. (2003). VSV strains with defects in their ability to shutdown innate immunity are potent systemic anti-cancer agents. *Cancer cell* 4: 263-275.
189. Gong, J., and Mita, M. M. (2014). Activated ras signaling pathways and reovirus oncolysis: an update on the mechanism of preferential reovirus replication in cancer cells. *Frontiers in oncology* 4: 167.
190. Critchley-Thorne, R. J., et al. (2009). Impaired interferon signaling is a common immune defect in human cancer. *Proceedings of the National Academy of Sciences of the United States of America* 106: 9010-9015.
191. Wang, F., et al. (2004). Disruption of Erk-dependent type I interferon induction breaks the myxoma virus species barrier. *Nature immunology* 5: 1266-1274.
192. Angarita, F. A., Acuna, S. A., Ottolino-Perry, K., Zerhouni, S., and McCart, J. A. (2013). Mounting a strategic offense: fighting tumor vasculature with oncolytic viruses. *Trends in molecular medicine* 19: 378-392.
193. Au, G. G., Lindberg, A. M., Barry, R. D., and Shafren, D. R. (2005). Oncolysis of vascular malignant human melanoma tumors by Coxsackievirus A21. *International journal of oncology* 26: 1471-1476.
194. Shafren, D. R., et al. (2004). Systemic therapy of malignant human melanoma tumors by a common cold-producing enterovirus, coxsackievirus a21. *Clin Cancer Res* 10: 53-60.
195. Ochiai, H., et al. (2006). Targeted therapy for glioblastoma multiforme neoplastic meningitis with intrathecal delivery of an oncolytic recombinant poliovirus. *Clin Cancer Res* 12: 1349-1354.
196. Shafren, D. R., Sylvester, D., Johansson, E. S., Campbell, I. G., and Barry, R. D. (2005). Oncolysis of human ovarian cancers by echovirus type 1. *International journal of cancer* 115: 320-328.
197. Cornelis, J. J., Lang, S. I., Stroh-Dege, A. Y., Balboni, G., Dinsart, C., and Rommelaere, J. (2004). Cancer gene therapy through autonomous parvovirus--mediated gene transfer. *Current gene therapy* 4: 249-261.
198. Iseki, H., et al. (2005). Parvovirus nonstructural proteins induce an epigenetic modification through histone acetylation in host genes and revert tumor malignancy to benignancy. *Journal of virology* 79: 8886-8893.
199. McKisic, M. D., Paturzo, F. X., and Smith, A. L. (1996). Mouse parvovirus infection potentiates rejection of tumor allografts and modulates T cell effector functions. *Transplantation* 61: 292-299.
200. Erlach, K. C., Bohm, V., Seckert, C. K., Reddehase, M. J., and Podlech, J. (2006). Lymphoma cell apoptosis in the liver induced by distant murine cytomegalovirus infection. *Journal of virology* 80: 4801-4819.

201. Dou, Q. P., and Pardee, A. B. (1996). Transcriptional activation of thymidine kinase, a marker for cell cycle control. *Progress in nucleic acid research and molecular biology* 53: 197-217.
202. Sherley, J. L., and Kelly, T. J. (1988). Regulation of human thymidine kinase during the cell cycle. *The Journal of biological chemistry* 263: 8350-8358.
203. Martuza, R. L., Malick, A., Markert, J. M., Ruffner, K. L., and Coen, D. M. (1991). Experimental therapy of human glioma by means of a genetically engineered virus mutant. *Science (New York, N.Y)* 252: 854-856.
204. Puhlmann, M., et al. (2000). Vaccinia as a vector for tumor-directed gene therapy: biodistribution of a thymidine kinase-deleted mutant. *Cancer gene therapy* 7: 66-73.
205. Bolayirli, M., et al. (2013). Serum thymidine kinase 1 activity in solid tumor (breast and colorectal cancer) patients treated with adjuvant chemotherapy. *Journal of clinical laboratory analysis* 27: 220-226.
206. Chen, G., et al. (2013). Nuclear TK1 expression is an independent prognostic factor for survival in pre-malignant and malignant lesions of the cervix. *BMC cancer* 13: 249.
207. Rausch, S., et al. (2015). Muscle-invasive bladder cancer is characterized by overexpression of thymidine kinase 1. *Urologic oncology* 33: 426 e421-429.
208. Diaconu, I., et al. (2012). Immune response is an important aspect of the antitumor effect produced by a CD40L-encoding oncolytic adenovirus. *Cancer research* 72: 2327-2338.
209. Autio, K., et al. (2014). Safety and biodistribution of a double-deleted oncolytic vaccinia virus encoding CD40 ligand in laboratory Beagles. *Molecular Therapy* 16: 14002.
210. Bischoff, J. R., et al. (1996). An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. *Science (New York, N.Y)* 274: 373-376.
211. Kirn, D. (2001). Clinical research results with dl1520 (Onyx-015), a replication-selective adenovirus for the treatment of cancer: what have we learned? *Gene therapy* 8: 89-98.
212. Kirn, D., Hermiston, T., and McCormick, F. (1998). ONYX-015: clinical data are encouraging. *Nature medicine* 4: 1341-1342.
213. Ganly, I., et al. (2000). A phase I study of Onyx-015, an E1B attenuated adenovirus, administered intratumorally to patients with recurrent head and neck cancer. *Clin Cancer Res* 6: 798-806.
214. DeWeese, T. L., et al. (2001). A phase I trial of CV706, a replication-competent, PSA selective oncolytic adenovirus, for the treatment of locally recurrent prostate cancer following radiation therapy. *Cancer research* 61: 7464-7472.
215. Khuri, F. R., et al. (2000). A controlled trial of intratumoral ONYX-015, a selectively-replicating adenovirus, in combination with cisplatin and 5-fluorouracil in patients with recurrent head and neck cancer. *Nature medicine* 6: 879-885.
216. Fruh, K., et al. (1995). A viral inhibitor of peptide transporters for antigen presentation. *Nature* 375: 415-418.
217. Hu, J. C., et al. (2006). A phase I study of OncoVEXGM-CSF, a second-generation oncolytic herpes simplex virus expressing granulocyte macrophage colony-stimulating factor. *Clin Cancer Res* 12: 6737-6747.

218. York, I. A., Roop, C., Andrews, D. W., Riddell, S. R., Graham, F. L., and Johnson, D. C. (1994). A cytosolic herpes simplex virus protein inhibits antigen presentation to CD8+ T lymphocytes. *Cell* 77: 525-535.
219. Todo, T., Martuza, R. L., Rabkin, S. D., and Johnson, P. A. (2001). Oncolytic herpes simplex virus vector with enhanced MHC class I presentation and tumor cell killing. *Proceedings of the National Academy of Sciences of the United States of America* 98: 6396-6401.
220. Martuza, R. L. (2000). Conditionally replicating herpes vectors for cancer therapy. *The Journal of clinical investigation* 105: 841-846.
221. Berger, C., et al. (2000). Expression of herpes simplex virus ICP47 and human cytomegalovirus US11 prevents recognition of transgene products by CD8(+) cytotoxic T lymphocytes. *Journal of virology* 74: 4465-4473.
222. Senzer, N. N., et al. (2009). Phase II clinical trial of a granulocyte-macrophage colony-stimulating factor-encoding, second-generation oncolytic herpesvirus in patients with unresectable metastatic melanoma. *J Clin Oncol* 27: 5763-5771.
223. Eager, R. M., and Nemunaitis, J. (2011). Clinical development directions in oncolytic viral therapy. *Cancer gene therapy* 18: 305-317.
224. Liu, T. C., Hwang, T., Park, B. H., Bell, J., and Kirn, D. H. (2008). The targeted oncolytic poxvirus JX-594 demonstrates antitumoral, antivascular, and anti-HBV activities in patients with hepatocellular carcinoma. *Mol Ther* 16: 1637-1642.
225. Heo, J., et al. (2013). Randomized dose-finding clinical trial of oncolytic immunotherapeutic vaccinia JX-594 in liver cancer. *Nature medicine* 19: 329-336.
226. SillaJen, I. Hepatocellular Carcinoma Study Comparing Vaccinia Virus Based Immunotherapy Plus Sorafenib vs Sorafenib Alone (PHOCUS). pp. 2015-[cited Jan 2016]. In: ClinicalTrials.gov [Internet] Bethesda (MD): National Library of Medicine (US).
227. Garber, K. (2006). China approves world's first oncolytic virus therapy for cancer treatment. *Journal of the National Cancer Institute* 98: 298-300.
228. Ledford, H. (2015). Cancer-fighting viruses win approval. *Nature* 526: 622-623.
229. Andtbacka, R. H., et al. (2015). Talimogene Laherparepvec Improves Durable Response Rate in Patients With Advanced Melanoma. *J Clin Oncol* 33: 2780-2788.
230. Vasey, P. A., et al. (2002). Phase I trial of intraperitoneal injection of the E1B-55-kd-gene-deleted adenovirus ONYX-015 (dl1520) given on days 1 through 5 every 3 weeks in patients with recurrent/refractory epithelial ovarian cancer. *J Clin Oncol* 20: 1562-1569.
231. Bauerschmitz, G. J., et al. (2002). Treatment of ovarian cancer with a tropism modified oncolytic adenovirus. *Cancer research* 62: 1266-1270.
232. Kimball, K. J., et al. (2010). A phase I study of a tropism-modified conditionally replicative adenovirus for recurrent malignant gynecologic diseases. *Clin Cancer Res* 16: 5277-5287.
233. Liu, H. L., and Chen, J. (2009). Oncolytic virus as an agent for the treatment of malignant ascites. *Cancer biotherapy & radiopharmaceuticals* 24: 99-102.
234. Dorig, R. E., Marcil, A., Chopra, A., and Richardson, C. D. (1993). The human CD46 molecule is a receptor for measles virus (Edmonston strain). *Cell* 75: 295-305.

235. Anderson, B. D., Nakamura, T., Russell, S. J., and Peng, K. W. (2004). High CD46 receptor density determines preferential killing of tumor cells by oncolytic measles virus. *Cancer research* 64: 4919-4926.
236. Galanis, E., et al. (2010). Phase I trial of intraperitoneal administration of an oncolytic measles virus strain engineered to express carcinoembryonic antigen for recurrent ovarian cancer. *Cancer research* 70: 875-882.
237. Galanis, E., et al. (2014). Oncolytic measles virus expressing the sodium iodide symporter to treat drug-resistant ovarian cancer. *Cancer research* 75: 22-30.
238. Galanis, E. MV-NIS Infected Mesenchymal Stem Cells in Treating Patients With Recurrent Ovarian Cancer. In: *ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2000- [cited 2016 Jan 22]. Available from: <https://clinicaltrials.gov/ct2/show/NCT02068794>. NLM identifier: NCT02068794.*
239. Cohn, D. Paclitaxel With or Without Viral Therapy in Treating Patients With Recurrent or Persistent Ovarian Epithelial, Fallopian Tube, or Primary Peritoneal Cancer. In *In: ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine(US).2010-[cited2016Jan22].Available From:<https://clinicaltrials.gov/ct2/show/NCT01199263>.NLMIdentifier: NCT01199263.*
240. Cohn, D. Viral Therapy in Treating Patients With Ovarian Epithelial Cancer, Primary Peritoneal Cancer, or Fallopian Tube Cancer That Did Not Respond to Platinum Chemotherapy. In *In: ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2008-[cited 2016 Jan 22] Available from:<https://clinicaltrials.gov/ct2/show/study/NCT00602277>. NLM identifier:NCT00602277, NLM Identifier:NCT00602277.*
241. Phelps, M. A., Cohn, D.E., O'Malley, D.M., Wei, L., Wilkins,D., Campbell, A., Schaaf, L. J., Coffey, M.C., Villalona-Calero, M.A., Grever, M.R., Nuovo, G.J, Zwiebel, J.A. (2010). Abstract 2594: Reovirus replication in ovarian and peritoneal tumors after intravenous administration. *Cancer research* 70.
242. (2015). Oncolytics Biotech® Inc. Announces Receipt of Orphan Drug Designation from the U.S. FDA for Ovarian Cancer. Oncolytics Biotech Inc.
243. Breitbach, C. J., et al. (2011). Intravenous delivery of a multi-mechanistic cancer-targeted oncolytic poxvirus in humans. *Nature* 477: 99-102.
244. McCart, A. Phase 2 Study of JX-594 in Patients With Peritoneal Carcinomatosis of Ovarian Cancer Origin. In: *ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2000- [cited 2016 Jan 22]. Available from: <https://clinicaltrials.gov/ct2/show/NCT02017678>. NLM identifier: NCT02017678.*
245. Odunsi, K., et al. (2012). Efficacy of vaccination with recombinant vaccinia and fowlpox vectors expressing NY-ESO-1 antigen in ovarian cancer and melanoma patients. *Proceedings of the National Academy of Sciences of the United States of America* 109: 5797-5802.
246. Cristea, M. C. p53MVA Vaccine and Gemcitabine Hydrochloride in Treating Patients With Recurrent Ovarian Epithelial Cancer. In: *ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2000- [cited 2016 Jan 22]. Available from:<https://clinicaltrials.gov/ct2/show/NCT02275039>. NLM identifier: NCT02275039.*

247. Cameron, C., et al. (1999). The complete DNA sequence of myxoma virus. *Virology* 264: 298-318.
248. Barrett, J. W., Cao, J. X., Hota-Mitchell, S., and McFadden, G. (2001). Immunomodulatory proteins of myxoma virus. *Seminars in immunology* 13: 73-84.
249. Zuniga, M. C. (2002). A pox on thee! Manipulation of the host immune system by myxoma virus and implications for viral-host co-adaptation. *Virus research* 88: 17-33.
250. Fenner, F. (2000). Adventures with poxviruses of vertebrates. *FEMS microbiology reviews* 24: 123-133.
251. Andrewes, C. H., and Harisijades, S. (1955). Propagation of myxoma virus in one-day old mice. *British journal of experimental pathology* 36: 18-21.
252. McCabe, V. J., Tarpey, I., and Spibey, N. (2002). Vaccination of cats with an attenuated recombinant myxoma virus expressing feline calicivirus capsid protein. *Vaccine* 20: 2454-2462.
253. Fenner, F. (1959). Myxomatosis. *British medical bulletin* 15: 240-245.
254. Grodhaus, G., Regnery, D. C., and Marshall, I. D. (1963). Studies in the epidemiology of myxomatosis in California. II. The experimental transmission of myxomatosis in brush rabbits (*Sylvilagus bachmani*) by several species of mosquitoes. *American journal of hygiene* 77: 205-212.
255. Marshall, I. D., and Regnery, D. C. (1960). Myxomatosis in a California brush rabbit (*Sylylagus bachmani*). *Nature* 188: 73-74.
256. Kilham, L., and Woke, P. A. (1953). Laboratory transmission of fibromas (Shope) in cottontail rabbits by means of fleas and mosquitoes. *Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y)* 83: 296-301.
257. Dalmat, H. T. (1959). Arthropod transmission of rabbit fibromatosis (Shope). *The Journal of hygiene* 57: 1-30.
258. Kerr, P. J., Liu, J., Cattadori, I., Ghedin, E., Read, A. F., and Holmes, E. C. (2015) Myxoma virus and the Leporipoxviruses: an evolutionary paradigm. *Viruses* 7: 1020-1061.
259. Best, S. M., and Kerr, P. J. (2000). Coevolution of host and virus: the pathogenesis of virulent and attenuated strains of myxoma virus in resistant and susceptible European rabbits. *Virology* 267: 36-48.
260. Silvers, L., et al. (2010). Host-specificity of myxoma virus: Pathogenesis of South American and North American strains of myxoma virus in two North American lagomorph species. *Veterinary microbiology* 141: 289-300.
261. Mossman, K., Nation, P., Macen, J., Garbutt, M., Lucas, A., and McFadden, G. (1996). Myxoma virus M-T7, a secreted homolog of the interferon-gamma receptor, is a critical virulence factor for the development of myxomatosis in European rabbits. *Virology* 215: 17-30.
262. Lalani, A. S., et al. (1997). The purified myxoma virus gamma interferon receptor homolog M-T7 interacts with the heparin-binding domains of chemokines. *Journal of virology* 71: 4356-4363.
263. Kerr, P. J. (2012). Myxomatosis in Australia and Europe: a model for emerging infectious diseases. *Antiviral research* 93: 387-415.

264. Lun, X., et al. (2010). Myxoma virus virotherapy for glioma in immunocompetent animal models: optimizing administration routes and synergy with rapamycin. *Cancer research* 70: 598-608.
265. Bartee, E., and McFadden, G. (2009). Human cancer cells have specifically lost the ability to induce the synergistic state caused by tumor necrosis factor plus interferon-beta. *Cytokine* 47: 199-205.
266. Bartee, E., Mohamed, M. R., Lopez, M. C., Baker, H. V., and McFadden, G. (2009). The addition of tumor necrosis factor plus beta interferon induces a novel synergistic antiviral state against poxviruses in primary human fibroblasts. *Journal of virology* 83: 498-511.
267. Wang, G., et al. (2006). Infection of human cancer cells with myxoma virus requires Akt activation via interaction with a viral ankyrin-repeat host range factor. *Proceedings of the National Academy of Sciences of the United States of America* 103: 4640-4645.
268. Werden, S. J., and McFadden, G. (2010). Pharmacological manipulation of the akt signaling pathway regulates myxoma virus replication and tropism in human cancer cells. *Journal of virology* 84: 3287-3302.
269. McFadden, G. (2005). Poxvirus tropism. *Nat Rev Microbiol* 3: 201-213.
270. Villa, N. Y., Bartee, E., Mohamed, M. R., Rahman, M. M., Barrett, J. W., and McFadden, G. (2010). Myxoma and vaccinia viruses exploit different mechanisms to enter and infect human cancer cells. *Virology* 401: 266-279.
271. Kim, M., et al. (2009). Myxoma virus targets primary human leukemic stem and progenitor cells while sparing normal hematopoietic stem and progenitor cells. *Leukemia* 23: 2313-2317.
272. Lun, X., et al. (2005). Myxoma virus is a novel oncolytic virus with significant antitumor activity against experimental human gliomas. *Cancer research* 65: 9982-9990.
273. Lun, X. Q., et al. (2007). Targeting human medulloblastoma: oncolytic virotherapy with myxoma virus is enhanced by rapamycin. *Cancer research* 67: 8818-8827.
274. Stanford, M. M., et al. (2008). Myxoma virus oncolysis of primary and metastatic B16F10 mouse tumors in vivo. *Mol Ther* 16: 52-59.
275. Correa, R. J., et al. (2012). Myxoma virus-mediated oncolysis of ascites-derived human ovarian cancer cells and spheroids is impacted by differential AKT activity. *Gynecologic oncology* 125: 441-450.
276. Tong, J. G., et al. (2015). Evidence for differential viral oncolytic efficacy in an in vitro model of epithelial ovarian cancer metastasis. *Molecular Therapy* 23: 1501-1513.
277. Wennier, S. T., Liu, J., Li, S., Rahman, M. M., Mona, M., and McFadden, G. (2012). Myxoma virus sensitizes cancer cells to gemcitabine and is an effective oncolytic virotherapeutic in models of disseminated pancreatic cancer. *Mol Ther* 20: 759-768.
278. Huang, J., et al. (2011). Frequent genetic abnormalities of the PI3K/AKT pathway in primary ovarian cancer predict patient outcome. *Genes, chromosomes & cancer* 50: 606-618.

279. Altomare, D. A., et al. (2004). AKT and mTOR phosphorylation is frequently detected in ovarian cancer and can be targeted to disrupt ovarian tumor cell growth. *Oncogene* 23: 5853-5857.
280. Kato, M., Yamamoto, S., Takano, M., Matsubara, O., and Furuya, K. (2012). Aberrant expression of the mammalian target of rapamycin, hypoxia-inducible factor-1alpha, and glucose transporter 1 in the development of ovarian clear-cell adenocarcinoma. *Int J Gynecol Pathol* 31: 254-263.
281. Mabuchi, S., et al. (2009). mTOR is a promising therapeutic target both in cisplatin-sensitive and cisplatin-resistant clear cell carcinoma of the ovary. *Clin Cancer Res* 15: 5404-5413.
282. Cai, J., et al. (2014). The role of the PTEN/PI3K/Akt pathway on prognosis in epithelial ovarian cancer: a meta-analysis. *The oncologist* 19: 528-535.
283. Kim, M., et al. (2010). The viral tropism of two distinct oncolytic viruses, reovirus and myxoma virus, is modulated by cellular tumor suppressor gene status. *Oncogene* 29: 3990-3996.
284. Broyles, S. S. (2003). Vaccinia virus transcription. *The Journal of general virology* 84: 2293-2303.
285. Goebel, S. J., Johnson, G. P., Perkus, M. E., Davis, S. W., Winslow, J. P., and Paoletti, E. (1990). The complete DNA sequence of vaccinia virus. *Virology* 179: 247-266, 517-263.
286. Tolonen, N., Doglio, L., Schleich, S., and Krijnse Locker, J. (2001). Vaccinia virus DNA replication occurs in endoplasmic reticulum-enclosed cytoplasmic mini-nuclei. *Molecular biology of the cell* 12: 2031-2046.
287. Smith, G. L., and Moss, B. (1983). Infectious poxvirus vectors have capacity for at least 25 000 base pairs of foreign DNA. *Gene* 25: 21-28.
288. Hraby, D. E., Guarino, L. A., and Kates, J. R. (1979). Vaccinia virus replication. I. Requirement for the host-cell nucleus. *Journal of virology* 29: 705-715.
289. Shen, Y., and Nemunaitis, J. (2005). Fighting cancer with vaccinia virus: teaching new tricks to an old dog. *Mol Ther* 11: 180-195.
290. Thorne, S. H. (2014). Immunotherapeutic potential of oncolytic vaccinia virus. *Frontiers in oncology* 4: 155.
291. Carter, G. C., Law, M., Hollinshead, M., and Smith, G. L. (2005). Entry of the vaccinia virus intracellular mature virion and its interactions with glycosaminoglycans. *The Journal of general virology* 86: 1279-1290.
292. Townsley, A. C., Weisberg, A. S., Wagenaar, T. R., and Moss, B. (2006). Vaccinia virus entry into cells via a low-pH-dependent endosomal pathway. *Journal of virology* 80: 8899-8908.
293. Martin, S., Harris, D. T., and Shisler, J. (2012). The C11R gene, which encodes the vaccinia virus growth factor, is partially responsible for MVA-induced NF-kappaB and ERK2 activation. *Journal of virology* 86: 9629-9639.
294. Postigo, A., Martin, M. C., Dodding, M. P., and Way, M. (2009). Vaccinia-induced epidermal growth factor receptor-MEK signalling and the anti-apoptotic protein F1L synergize to suppress cell death during infection. *Cellular microbiology* 11: 1208-1218.

295. Andrade, A. A., et al. (2004). The vaccinia virus-stimulated mitogen-activated protein kinase (MAPK) pathway is required for virus multiplication. *The Biochemical journal* 381: 437-446.
296. Weir, J. P., Bajszar, G., and Moss, B. (1982). Mapping of the vaccinia virus thymidine kinase gene by marker rescue and by cell-free translation of selected mRNA. *Proceedings of the National Academy of Sciences of the United States of America* 79: 1210-1214.
297. Chen, W., et al. (2014). Reciprocal regulation of autophagy and dNTP pools in human cancer cells. *Autophagy* 10: 1272-1284.
298. McCart, J. A., et al. (2001). Systemic cancer therapy with a tumor-selective vaccinia virus mutant lacking thymidine kinase and vaccinia growth factor genes. *Cancer research* 61: 8751-8757.
299. Katsafanas, G. C., and Moss, B. (2004). Vaccinia virus intermediate stage transcription is complemented by Ras-GTPase-activating protein SH3 domain-binding protein (G3BP) and cytoplasmic activation/proliferation-associated protein (p137) individually or as a heterodimer. *The Journal of biological chemistry* 279: 52210-52217.
300. Zeh, H. J. Safety Study of Modified Vaccinia Virus to Cancer. In: ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2000- [cited 27 Jan 2016]. Available from: <https://clinicaltrials.gov/ct2/show/NCT00574977>. NLM identifier: NCT00574977.
301. Blasco, R., Sisler, J. R., and Moss, B. (1993). Dissociation of progeny vaccinia virus from the cell membrane is regulated by a viral envelope glycoprotein: effect of a point mutation in the lectin homology domain of the A34R gene. *Journal of virology* 67: 3319-3325.
302. Lin, C. L., Chung, C. S., Heine, H. G., and Chang, W. (2000). Vaccinia virus envelope H3L protein binds to cell surface heparan sulfate and is important for intracellular mature virion morphogenesis and virus infection in vitro and in vivo. *Journal of virology* 74: 3353-3365.
303. Hsiao, J. C., Chung, C. S., and Chang, W. (1999). Vaccinia virus envelope D8L protein binds to cell surface chondroitin sulfate and mediates the adsorption of intracellular mature virions to cells. *Journal of virology* 73: 8750-8761.
304. Chung, C. S., Hsiao, J. C., Chang, Y. S., and Chang, W. (1998). A27L protein mediates vaccinia virus interaction with cell surface heparan sulfate. *Journal of virology* 72: 1577-1585.
305. Hsiao, J. C., Chung, C. S., and Chang, W. (1998). Cell surface proteoglycans are necessary for A27L protein-mediated cell fusion: identification of the N-terminal region of A27L protein as the glycosaminoglycan-binding domain. *Journal of virology* 72: 8374-8379.
306. Assarsson, E., et al. (2008). Kinetic analysis of a complete poxvirus transcriptome reveals an immediate-early class of genes. *Proceedings of the National Academy of Sciences of the United States of America* 105: 2140-2145.
307. Senkevich, T. G., Ward, B. M., and Moss, B. (2004). Vaccinia virus entry into cells is dependent on a virion surface protein encoded by the A28L gene. *Journal of virology* 78: 2357-2366.

308. Deng, L., Dai, P., Ding, W., Granstein, R. D., and Shuman, S. (2006). Vaccinia virus infection attenuates innate immune responses and antigen presentation by epidermal dendritic cells. *Journal of virology* 80: 9977-9987.
309. Sanz, P., and Moss, B. (1999). Identification of a transcription factor, encoded by two vaccinia virus early genes, that regulates the intermediate stage of viral gene expression. *Proceedings of the National Academy of Sciences of the United States of America* 96: 2692-2697.
310. Rosales, R., Sutter, G., and Moss, B. (1994). A cellular factor is required for transcription of vaccinia viral intermediate-stage genes. *Proceedings of the National Academy of Sciences of the United States of America* 91: 3794-3798.
311. Gunasinghe, S. K., Hubbs, A. E., and Wright, C. F. (1998). A vaccinia virus late transcription factor with biochemical and molecular identity to a human cellular protein. *The Journal of biological chemistry* 273: 27524-27530.
312. Broyles, S. S., Liu, X., Zhu, M., and Kremer, M. (1999). Transcription factor YY1 is a vaccinia virus late promoter activator. *The Journal of biological chemistry* 274: 35662-35667.
313. Roberts, K. L., and Smith, G. L. (2008). Vaccinia virus morphogenesis and dissemination. *Trends in microbiology* 16: 472-479.
314. Ward, B. M. (2005). Visualization and characterization of the intracellular movement of vaccinia virus intracellular mature virions. *Journal of virology* 79: 4755-4763.
315. Sodeik, B. (2000). Mechanisms of viral transport in the cytoplasm. *Trends in microbiology* 8: 465-472.
316. Sanderson, C. M., Hollinshead, M., and Smith, G. L. (2000). The vaccinia virus A27L protein is needed for the microtubule-dependent transport of intracellular mature virus particles. *The Journal of general virology* 81: 47-58.
317. Tooze, J., Hollinshead, M., Reis, B., Radsak, K., and Kern, H. (1993). Progeny vaccinia and human cytomegalovirus particles utilize early endosomal cisternae for their envelopes. *European journal of cell biology* 60: 163-178.
318. Hiller, G., and Weber, K. (1985). Golgi-derived membranes that contain an acylated viral polypeptide are used for vaccinia virus envelopment. *Journal of virology* 55: 651-659.
319. Schmelz, M., et al. (1994). Assembly of vaccinia virus: the second wrapping cisterna is derived from the trans Golgi network. *Journal of virology* 68: 130-147.
320. Zhang, W. H., Wilcock, D., and Smith, G. L. (2000). Vaccinia virus F12L protein is required for actin tail formation, normal plaque size, and virulence. *Journal of virology* 74: 11654-11662.
321. Hollinshead, M., et al. (2001). Vaccinia virus utilizes microtubules for movement to the cell surface. *The Journal of cell biology* 154: 389-402.
322. van Eijl, H., Hollinshead, M., Rodger, G., Zhang, W. H., and Smith, G. L. (2002). The vaccinia virus F12L protein is associated with intracellular enveloped virus particles and is required for their egress to the cell surface. *The Journal of general virology* 83: 195-207.
323. van Eijl, H., Hollinshead, M., and Smith, G. L. (2000). The vaccinia virus A36R protein is a type Ib membrane protein present on intracellular but not extracellular enveloped virus particles. *Virology* 271: 26-36.

324. Payne, L. G. (1979). Identification of the vaccinia hemagglutinin polypeptide from a cell system yielding large amounts of extracellular enveloped virus. *Journal of virology* 31: 147-155.
325. Travassos da Rosa, A. P., Tesh, R. B., Travassos da Rosa, J. F., Herve, J. P., and Main, A. J., Jr. (1984). Carajas and Maraba viruses, two new vesiculoviruses isolated from phlebotomine sand flies in Brazil. *The American journal of tropical medicine and hygiene* 33: 999-1006.
326. Altstiel, L. D., and Landsberger, F. R. (1981). Lipid-protein interactions between the surface glycoprotein of vesicular stomatitis virus and the lipid bilayer. *Virology* 115: 1-9.
327. Gaudin, Y., Ruigrok, R. W., Knossow, M., and Flamand, A. (1993). Low-pH conformational changes of rabies virus glycoprotein and their role in membrane fusion. *Journal of virology* 67: 1365-1372.
328. Mebatsion, T., Weiland, F., and Conzelmann, K. K. (1999). Matrix protein of rabies virus is responsible for the assembly and budding of bullet-shaped particles and interacts with the transmembrane spike glycoprotein G. *Journal of virology* 73: 242-250.
329. Blumberg, B. M., Leppert, M., and Kolakofsky, D. (1981). Interaction of VSV leader RNA and nucleocapsid protein may control VSV genome replication. *Cell* 23: 837-845.
330. Mondal, A., Victor, K. G., Pudupakam, R. S., Lyons, C. E., and Wertz, G. W. (2014). Newly identified phosphorylation site in the vesicular stomatitis virus P protein is required for viral RNA synthesis. *Journal of virology* 88: 1461-1472.
331. Tesh, R. B., et al. (1987). Natural infection of humans, animals, and phlebotomine sand flies with the Alagoas serotype of vesicular stomatitis virus in Colombia. *The American journal of tropical medicine and hygiene* 36: 653-661.
332. Cline, B. L. (1976). Ecological associations of vesicular stomatitis virus in rural Central America and Panama. *The American journal of tropical medicine and hygiene* 25: 875-883.
333. Pauszek, S. J., Barrera Jdel, C., Goldberg, T., Allende, R., and Rodriguez, L. L. (2011). Genetic and antigenic relationships of vesicular stomatitis viruses from South America. *Archives of virology* 156: 1961-1968.
334. Paglino, J. C., and van den Pol, A. N. (2011). Vesicular stomatitis virus has extensive oncolytic activity against human sarcomas: rare resistance is overcome by blocking interferon pathways. *Journal of virology* 85: 9346-9358.
335. Lemay, C. G., et al. (2012). Harnessing oncolytic virus-mediated antitumor immunity in an infected cell vaccine. *Mol Ther* 20: 1791-1799.
336. Barber, G. N. (2004). Vesicular stomatitis virus as an oncolytic vector. *Viral immunology* 17: 516-527.
337. Tesfay, M. Z., et al. (2014). Vesiculovirus neutralization by natural IgM and complement. *Journal of virology* 88: 6148-6157.
338. Sun, X., Yau, V. K., Briggs, B. J., and Whittaker, G. R. (2005). Role of clathrin-mediated endocytosis during vesicular stomatitis virus entry into host cells. *Virology* 338: 53-60.

339. Das, S. C., Nayak, D., Zhou, Y., and Pattnaik, A. K. (2006). Visualization of intracellular transport of vesicular stomatitis virus nucleocapsids in living cells. *Journal of virology* 80: 6368-6377.
340. Superti, F., Seganti, L., Ruggeri, F. M., Tinari, A., Donelli, G., and Orsi, N. (1987). Entry pathway of vesicular stomatitis virus into different host cells. *The Journal of general virology* 68 (Pt 2): 387-399.
341. Matlin, K. S., Reggio, H., Helenius, A., and Simons, K. (1982). Pathway of vesicular stomatitis virus entry leading to infection. *Journal of molecular biology* 156: 609-631.
342. Cernescu, C., Constantinescu, S. N., and Popescu, L. M. (1990). Electron microscopic observations of vesicular stomatitis virus particles penetration in human fibroblasts. *Rev Roum Virol* 41: 93-96.
343. Li, Y., Luo, L. Z., and Wagner, R. R. (1989). Transcription inhibition site on the M protein of vesicular stomatitis virus located by marker rescue of mutant tsO23(III) with M-gene expression vectors. *Journal of virology* 63: 2841-2843.
344. Rigaut, K. D., Birk, D. E., and Lenard, J. (1991). Intracellular distribution of input vesicular stomatitis virus proteins after uncoating. *Journal of virology* 65: 2622-2628.
345. Ding, H., Green, T. J., Lu, S., and Luo, M. (2006). Crystal structure of the oligomerization domain of the phosphoprotein of vesicular stomatitis virus. *Journal of virology* 80: 2808-2814.
346. Grdzlishvili, V. Z., Smallwood, S., Tower, D., Hall, R. L., Hunt, D. M., and Moyer, S. A. (2005). A single amino acid change in the L-polymerase protein of vesicular stomatitis virus completely abolishes viral mRNA cap methylation. *Journal of virology* 79: 7327-7337.
347. Das, T., Mathur, M., Gupta, A. K., Janssen, G. M., and Banerjee, A. K. (1998). RNA polymerase of vesicular stomatitis virus specifically associates with translation elongation factor-1 alphabeta for its activity. *Proceedings of the National Academy of Sciences of the United States of America* 95: 1449-1454.
348. Qanungo, K. R., Shaji, D., Mathur, M., and Banerjee, A. K. (2004). Two RNA polymerase complexes from vesicular stomatitis virus-infected cells that carry out transcription and replication of genome RNA. *Proceedings of the National Academy of Sciences of the United States of America* 101: 5952-5957.
349. Abraham, G., and Banerjee, A. K. (1976). Sequential transcription of the genes of vesicular stomatitis virus. *Proceedings of the National Academy of Sciences of the United States of America* 73: 1504-1508.
350. Ball, L. A., and White, C. N. (1976). Order of transcription of genes of vesicular stomatitis virus. *Proceedings of the National Academy of Sciences of the United States of America* 73: 442-446.
351. Whelan, S. P., and Wertz, G. W. (2002). Transcription and replication initiate at separate sites on the vesicular stomatitis virus genome. *Proceedings of the National Academy of Sciences of the United States of America* 99: 9178-9183.
352. Li, T., and Pattnaik, A. K. (1997). Replication signals in the genome of vesicular stomatitis virus and its defective interfering particles: identification of a sequence element that enhances DI RNA replication. *Virology* 232: 248-259.

353. Masters, P. S., and Banerjee, A. K. (1988). Complex formation with vesicular stomatitis virus phosphoprotein NS prevents binding of nucleocapsid protein N to nonspecific RNA. *Journal of virology* 62: 2658-2664.
354. Masters, P. S., and Banerjee, A. K. (1988). Resolution of multiple complexes of phosphoprotein NS with nucleocapsid protein N of vesicular stomatitis virus. *Journal of virology* 62: 2651-2657.
355. Li, T., and Pattnaik, A. K. (1999). Overlapping signals for transcription and replication at the 3' terminus of the vesicular stomatitis virus genome. *Journal of virology* 73: 444-452.
356. Brown, E. L., and Lyles, D. S. (2003). Organization of the vesicular stomatitis virus glycoprotein into membrane microdomains occurs independently of intracellular viral components. *Journal of virology* 77: 3985-3992.
357. Swintek, B. D., and Lyles, D. S. (2008). Plasma membrane microdomains containing vesicular stomatitis virus M protein are separate from microdomains containing G protein and nucleocapsids. *Journal of virology* 82: 5536-5547.
358. Flood, E. A., and Lyles, D. S. (1999). Assembly of nucleocapsids with cytosolic and membrane-derived matrix proteins of vesicular stomatitis virus. *Virology* 261: 295-308.
359. Harty, R. N., Paragas, J., Sudol, M., and Palese, P. (1999). A proline-rich motif within the matrix protein of vesicular stomatitis virus and rabies virus interacts with WW domains of cellular proteins: implications for viral budding. *Journal of virology* 73: 2921-2929.
360. Jayakar, H. R., Murti, K. G., and Whitt, M. A. (2000). Mutations in the PPPY motif of vesicular stomatitis virus matrix protein reduce virus budding by inhibiting a late step in virion release. *Journal of virology* 74: 9818-9827.
361. Brun, J., et al. (2010). Identification of genetically modified Maraba virus as an oncolytic rhabdovirus. *Mol Ther* 18: 1440-1449.
362. Le Boeuf, F., et al. (2013). Model-based rational design of an oncolytic virus with improved therapeutic potential. *Nature communications* 4: 1974.
363. Zhang, J., et al. (2014). Maraba MG1 virus enhances natural killer cell function via conventional dendritic cells to reduce postoperative metastatic disease. *Mol Ther* 22: 1320-1332.
364. Pol, J. G., et al. (2014). Maraba virus as a potent oncolytic vaccine vector. *Mol Ther* 22: 420-429.
365. NCIC Clinical Trials Group; Ottawa Hospital Research Institute. MG1 Maraba/MAGE-A3, With and Without Adenovirus Vaccine, With Transgenic MAGE-A3 Insertion in Patients With Incurable MAGE-A3-Expressing Solid Tumours. In *ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2000- [cited 2016 Jan 28]*. Available from: <https://clinicaltrials.gov/ct2/show/NCT02285816>. NLM identifier: NCT02285816.

CHAPTER 2: Evidence for differential viral oncolytic efficacy in an *in vitro* model of epithelial ovarian cancer metastasis

Tong, J. G., Vales, Y.R., Barrett, J.W., Bell, J.C., Stojdl, D., McFadden, G., McCart, J.A., DiMattia, G.E., Shepherd, T.G

A version of this chapter has been published in Molecular Therapy – Oncolytics

2.1 Introduction

Epithelial ovarian cancer (EOC) is the most lethal gynaecologic malignancy and represents the sixth most commonly diagnosed cancer amongst women in the developed world [1]. A lack of effective therapeutic options, coupled with the highly heterogeneous nature of EOC, and being typically diagnosed at an advanced metastatic stage, contribute to the lethality of EOC [2, 3]. Current therapeutic strategies involve exhaustive cytoreductive surgery and post-operative platinum- and taxane-based chemotherapy [4-6]. However, effective treatment is complicated by the manifestation of EOC as multiple histotypes, which are differentially responsive to platinum- and taxane-based combination chemotherapy treatments [7]. Furthermore, patients that initially respond well to platinum therapy almost inevitably relapse with chemo-resistant disease resulting in reduced overall survival. Thus, there is a critical need for targeted and durable therapeutic alternatives beyond the standard first-line chemotherapeutic agents [8-10].

Oncolytic virotherapy promotes viral infection and lysing of cancer cells. The specific nature of oncolytic virus therapy stems from the selection of non- or lowpathogenic non-human viruses that display tropism for cancer-associated genetic mutations or aberrant signaling [11]. Myxoma virus (MYXV) is a European rabbit-specific poxvirus that has not been shown to cause disease in humans and is used as a pesticide to control Australian rabbit populations [12]. MYXV displays tropism for cancer cells with up-regulation in active AKT signaling and dysfunctional p53, which is found in essentially all high-grade

EOC [13]. Conversely, vvDD is an engineered poxvirus with deleted vaccinia growth factor and viral thymidine kinase genes, which limit its infection to cells harbouring upregulated EGFR/RAS signaling commonly observed in low-grade EOCs [14]. Point mutations in the strain of MRBV used in this study modify the matrix protein (M) and glycoprotein (G) effectively boosting its replicative capacity in cancer cells while rendering it unable to counteract an antiviral type I interferon response in healthy cells. Though its specific tropism for cancer cells is relatively undefined, MRBV has been shown to have potent oncolytic effects in a broad range of cancer cells, including EOC [15].

The mode of EOC metastasis is unique among most solid malignancies, and therefore it likely possesses distinct and novel mechanisms. EOC metastasis occurs via the shedding of malignant cells from the primary tumor into the peritoneal cavity; this can occur in the context of ascites, an exudative fluid commonly associated with advanced-stage disease. Single cells in suspension within the ascites are susceptible to death through anoikis; thus aggregation of single cells into multicellular spheroids facilitates escape from cell death [16, 17]. Furthermore, EOC spheroid survival is maintained in the low-nutrient environment of the ascites by undergoing cellular quiescence and autophagy [18, 19]. This tumour cell dormancy phenotype within spheroids is thought to allow persistence of microscopic EOC secondary deposits after treatment with first-line chemotherapeutics and support growth under more favorable conditions [20]. In addition, spheroids have an enhanced capacity to attach and invade mesothelial-lined surfaces in the peritoneal space promoting the formation of secondary tumor nodules [16].

In our current study, we hypothesize that in the context of metastatic ovarian cancer, the ability to kill dormant tumour cells is essential to eradicate the potential for disease recurrence. In this study, we compare three oncolytic viruses, MYXV, vvDD and MRBV,

in an *in vitro* spheroid culture model of ovarian cancer metastasis to determine whether they have the potential to kill dormant tumour cells residing in spheroids.

2.2 Results

Oncolytic effects of MYXV, vvDD and MRBV in ovarian cancer cell lines.

To begin to define the optimal oncolytic viral approach to the eradication of dormant EOC cells in spheroids, we applied three different viruses in an *in vitro* three-dimensional spheroid culture system, which we have established to model metastatic EOC (Fig. 2.1). Distinct molecular characteristics typify the lifecycle of metastatic ovarian cancer cells as they move from a proliferative state in the solid tumour to the resting state in ascites-suspended spheroids and finally when these structures attach to a secondary site and cells proliferate to form a metastatic lesion. Herein, we performed oncolytic infections using proliferating adherent EOC cell lines, spheroids cultured in suspension, and spheroid reattachment to tissue culture substratum to determine whether molecular and cellular changes at these specific steps would affect oncolytic virus cell killing efficacy. We selected the established HEYA8, SKOV3 and OVCAR8 cell lines since they have been well-characterized genomically (Suppl. Fig. A1) and have been predicted to represent different EOC subtypes based on this data [21].

First, we performed parallel viral infections of adherent EOC cell lines with established spheroids in suspension (Fig. 2.2a). Even in proliferating adherent cultures, we found that MYXV, vvDD, and MRBV were capable of inducing oncolysis of EOC lines with differential killing capacities among the three viruses and across cell lines (Fig 2.2). MYXV displayed the least potent killing and was unable to induce significant oncolysis at less than an MOI 1 in all cell lines (Fig 2.2b). vvDD exhibited oncolysis at similar

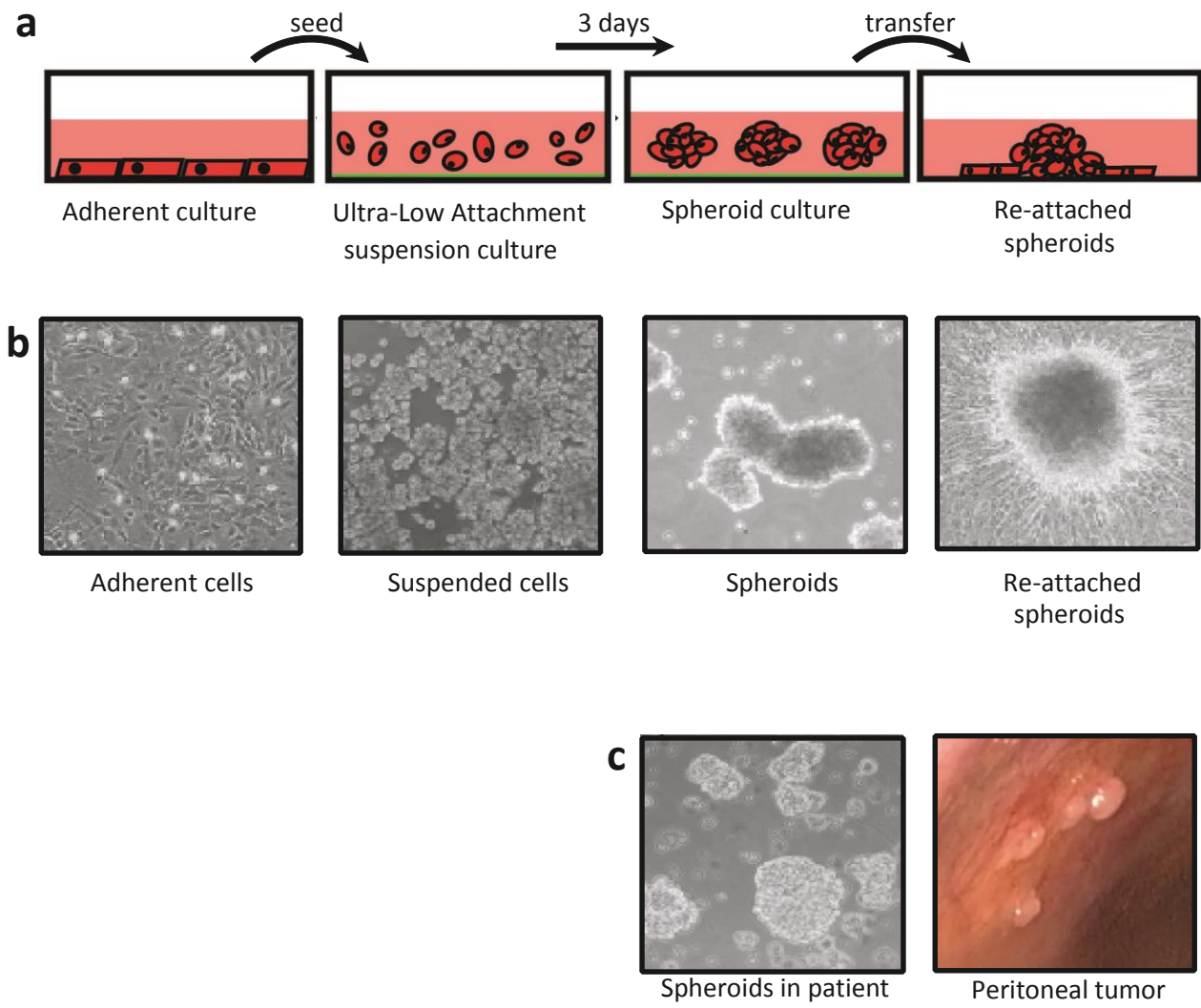


Figure 2.1 In vitro three-dimensional spheroid model system of ovarian cancer metastasis. (a) Ovarian cancer cells are grown as adherent proliferating monolayer cultures, and are transferred to Ultra-Low Attachment tissue culture plastic-ware where they naturally form multicellular aggregates, or spheroids, when in suspension culture. Spheroids are subsequently transferred back to standard tissue culture plastic to facilitate adhesion and growth of cells out of viable spheroids. (b) Phase contrast images of ovarian cancer cells in each of the culture conditions outlined in panel a. (c) Left: Phase contrast microscopic image of freshly-collected ovarian cancer patient ascites indicating the presence of spheroids in suspension. Right: Image representing ovarian tumour nodules implanted on the peritoneal wall of an ovarian cancer patient at the time of laparoscopic surgery.

concentrations as MYXV, but was able to induce greater loss of viability in comparison. Among the three EOC cell lines, OVCAR8 cells displayed greatest sensitivity to vvDD and MYXV infection whereas SKOV3 cells were most resistant in adherent culture infections. In a similar fashion, when tested using ovarian cancer patient ascites-derived primary cultures, vvDD yielded better oncolytic activity than MYXV in the majority of clinical samples (Suppl. Fig. A2.1). Although both MYXV and vvDD were able to induce modest oncolysis of EOC cell lines in adherent culture, infection-mediated cell killing was dramatically reduced in EOC spheroids for all cell lines tested (Fig. 2.2c). vvDD was completely ineffective at inducing oncolysis of SKOV3 spheroids, but it did maintain some oncolytic activity in HEYA8 and OVCAR8 spheroids.

These findings using MYXV and vvDD were in stark contrast to results of MRBV infection, which induced robust oncolysis-mediated cell killing across all EOC cell lines in adherent culture even at an MOI of 0.001 (Fig. 2.2b). In contrast, MRBV infection of heterogeneous patient-derived cells yielded highly variable oncolytic effects with one sample exhibiting robust MRBV-mediated loss in cell viability similar to established EOC cell lines (Suppl. Fig. A1). However, a potent oncolytic effect was observed in MRBV-infected HEYA8 and SKOV3 spheroids where dramatic loss of cellular viability was evident with as little as MOI 0.01 at three days post-infection. Interestingly, we observed a significant reduction in MRBV oncolytic effects in OVCAR8 spheroids compared with adherent cells suggesting that EOC cells may have the capacity to acquire resistance against MRBV infection in three-dimensional spheroid form (Fig. 2.2c).

MRBV is faster at inducing oncolysis of EOC cells than MYXV and vvDD.

We hypothesized that the potent oncolysis of EOC cells by MRBV may be due to a rapid ability to replicate its small RNA-based genome, allowing it to complete multiple

rounds of infection within the experimental time frame of 72 hours. MRBV contrasts the large poxviruses, MYXV and vvDD, which have been shown to take up to several days to complete their life cycle, thus they may only complete a single round of infection within 72 hours.

To assess this directly in our system, we sought to compare viral infection kinetics among MYXV, vvDD, and MRBV in both adherent and spheroid cultures. Cells were infected with an MOI 10 to maximize infection of all cells at the initial time point. We then assayed cell viability as an initial surrogate to observe virus infection over five days. In adherent culture, we found that MRBV was able to induce oncolysis in both HEYA8 and OVCAR8 cells within 24 hours of infection (Fig. 2.3a). In support of our previous findings (Fig. 2.2b), MRBV exhibited a significant delay of infection in adherent SKOV3 cells taking over 48 hours to die from MRBV infection. In contrast, complete oncolysis of adherent EOC cells by MYXV and vvDD required up to five days.

We have previously shown that MYXV replication is attenuated in EOC spheroids compared to infection of adherent monolayer cells [22]. Therefore, we tested the kinetics of MYXV, vvDD, and MRBV infection in spheroids to compare directly with our results using proliferating adherent cell lines. We found that MYXV was largely ineffective at inducing detectable oncolysis in EOC spheroids by three days, but cell viability was reduced in HEYA8 and OVCAR8 spheroids by five days post-infection (Fig. 2.3b). Although a 72-hour infection of EOC spheroids with vvDD yielded little oncolysis, extending the time course to five days was sufficient for marked loss of HEYA8 and OVCAR8 spheroid cell viability. Time course infections with MRBV resulted in cell death between 48 and 72 hours for both HEYA8 and SKOV3 spheroids. Interestingly,

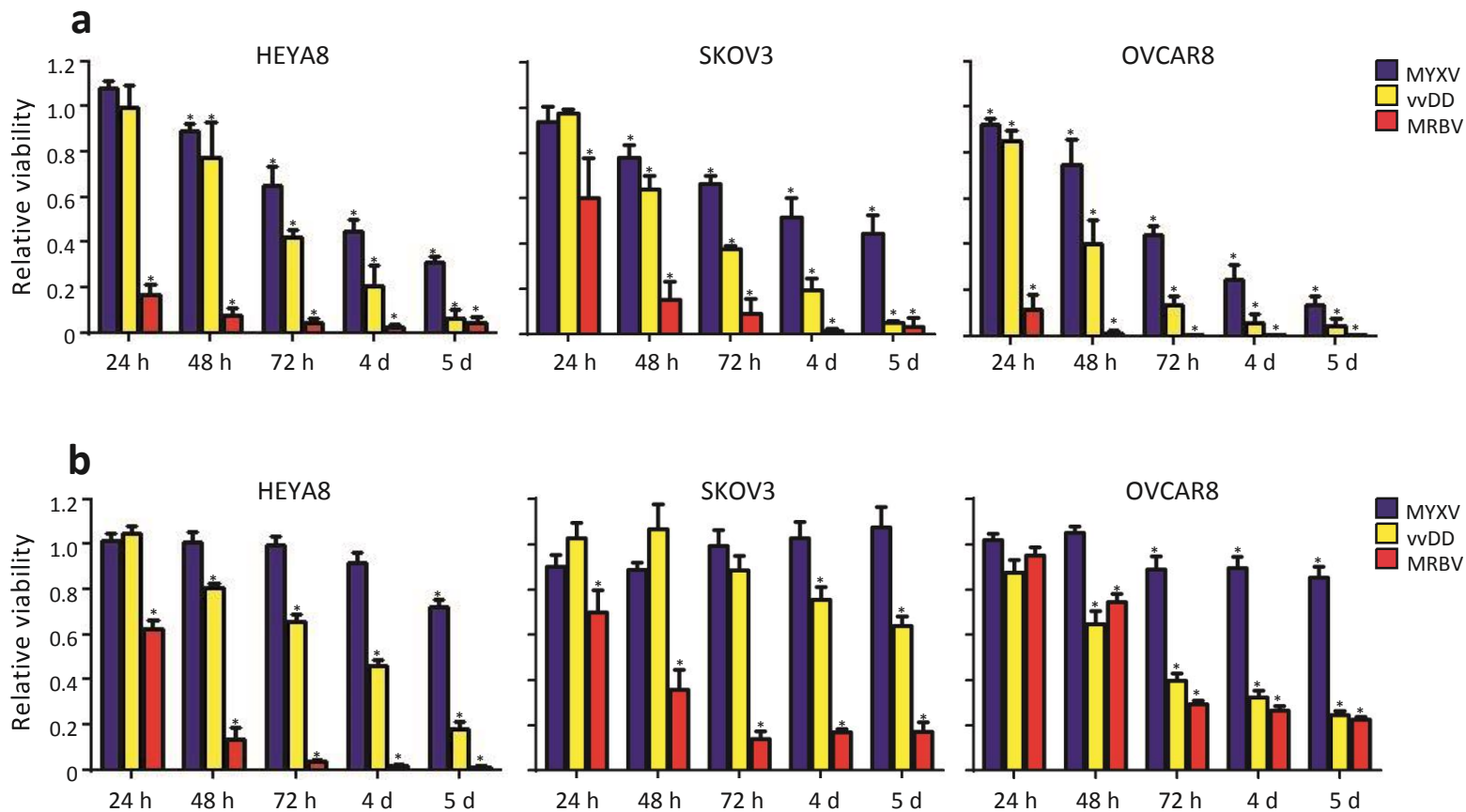


Figure 2.3 Rapid kinetics of MRBV-mediated killing of EOC cells and spheroids compared with MYXV and vvDD. (a) MRBV-mediated cell killing is observed within 24 h in adherent EOC cells, but requires longer incubation in SKOV3 cells. Oncolysis of adherent cells by MYXV and vvDD is considerably slower. (b) MRBV produces rapid cell killing in spheroids, but there is an incomplete oncolytic effect in SKOV3 and OVCAR8 spheroids. EOC spheroids have reduced viability due to vvDD infection by 5 d, yet remain relatively resistant to MYXV infection. (* $p < 0.05$)

OVCAR8 spheroids were relatively resistant to MRBV mediated cell killing with a limited extent of cell death similar to what we observed for vvDD. This significantly contrasts MRBV infections of adherent EOC cell lines, including OVCAR8 cells, which displayed significant cell death within 24 hours. These unexpected results reinforce the idea that the potential underlying mechanisms governing oncolytic efficacy in EOC cells may be quite dynamic, and stress the importance of preclinical testing in complementary *in vitro* model systems.

MRBV produces significantly more virus progeny than MYXV and vvDD.

We postulated that the observed differences in oncolytic effect among the three viruses in our spheroid culture system were also impacted by the efficiency of total virus production. To assess this directly, we infected adherent cells and spheroids and titrated total infectious virus particles. MRBV infection of adherent EOC cell lines yielded significantly more infectious viral progeny compared to both vvDD (7- to 17-fold increase) and MYXV (90- to 400-fold increase) (Table 2.1). Moreover, the number of viral progeny produced from MRBV infection in adherent culture was relatively similar among the three EOC cell lines. In spheroids, however, infectious progeny produced by MRBV infection was more variable among cell lines. MRBV infection of SKOV3 spheroids yielded 16-fold less virus and OVCAR8 spheroids yielded 69-fold less than HEYA8 spheroids (Table 2.1). These MRBV titers were congruent with our results of cell viability demonstrating reduced MRBV oncolysis of OVCAR8 spheroids (Fig. 2.2c). The number of viral progeny produced from vvDD infections was similarly reduced in spheroids among cell lines tested. In fact, both MYXV and vvDD were able to produce only a 0.2- to 3.2-fold increase in viral progeny than what was used to initiate infection. Again, this result suggests that EOC spheroids possess physical or molecular changes in cells that significantly impact the

Table 2.1. Quantification of oncolytic virus production in infected adherent ovarian cancer cells and spheroids.

	Adherent culture ^a		
	HEYA8	SKOV3	OVCAR8
MYXV	1.38±0.08 x10 ⁵	5.37±0.21 x10 ⁵	1.30±0.13 x10 ⁵
vvDD	7.78±1.22 x10 ⁶	2.78±0.35 x10 ⁶	2.83±0.59 x10 ⁶
MRBV	5.52±1.55 x10 ⁷	4.82±1.11 x10 ⁷	2.38±0.43 x10 ⁷
	Spheroid culture ^b		
	HEYA8	SKOV3	OVCAR8
MYXV	8.54±2.07 x10 ⁵	1.75±0.33 x10 ⁵	1.12±0.34 x10 ⁵
vvDD	1.60±0.21 x10 ⁶	3.12±1.58 x10 ⁵	6.04±1.39 x10 ⁵
MRBV	1.09±0.15 x10 ⁸	6.80±0.78 x10 ⁶	1.58±0.41 x10 ⁶

a- In adherent culture, 2.5 x 10⁴ cells were infected in 24-well dishes at MOI 10 (2.5 x 10⁵ pfu). b- In spheroid culture, 5 x 10⁴ cells were infected in 24-well ULA cluster plates at MOI 10 (5 x 10⁵ pfu).

replicative life cycle or amplification for both MYXV and vvDD in these dynamic structures.

Activation of MYXV and vvDD oncolysis upon spheroid reattachment.

We use spheroid reattachment as a general method to assess cell viability within these structures, as well as to model metastasis formation due to adhesion of spheroids to secondary sites. To this end, EOC spheroids were infected for 72 hours prior to transfer to adherent culture for spheroid reattachment (Fig. 2.4a). Spheroid cells are allowed to reattach and disperse for another 72 hours, after which the dispersion area of cells from infected spheroids was quantified (Fig. 2.4b). Despite lacking a significant oncolytic cell-killing effect in spheroids while in suspension, oncolysis mediated by both MYXV and vvDD was activated upon spheroid reattachment and significantly reduced the ability of cells to disperse from spheroids and form a viable monolayer (Fig. 2.4c and 2.4d). Reduced dispersion was apparent within 24 hours after reattachment and sustained for up to 4 days (Supplementary Fig A2.2). Due to its dramatic impact on spheroid cell viability, MRBV infection completely prevented reattachment of HEYA8 and SKOV3 spheroids (Fig. 2.4c and Supplementary Fig. A2.2). Although our previous results of cell viability indicated a marginal effect of vvDD infection on OVCAR8 spheroids (Fig. 2.2c), vvDD completely prevented OVCAR8 spheroid reattachment suggesting a significant reduction of cell viability in these structures. Interestingly, and in marked contrast to vvDD, MRBV-infected OVCAR8 spheroid cells were still capable of reattaching and dispersing, but to a lesser extent than mock-infected controls (Fig. 2.4c and 2.4d, and Supplementary Fig. A1).

We next sought to examine the direct oncolytic effect of all three viruses on established reattached spheroids (Fig. 2.5a). This facilitates our ability to evaluate their potential to target metastases, as well as determine whether insensitivity to oncolytic virus infection observed in suspension spheroids also existed in reattached spheroid nodules.

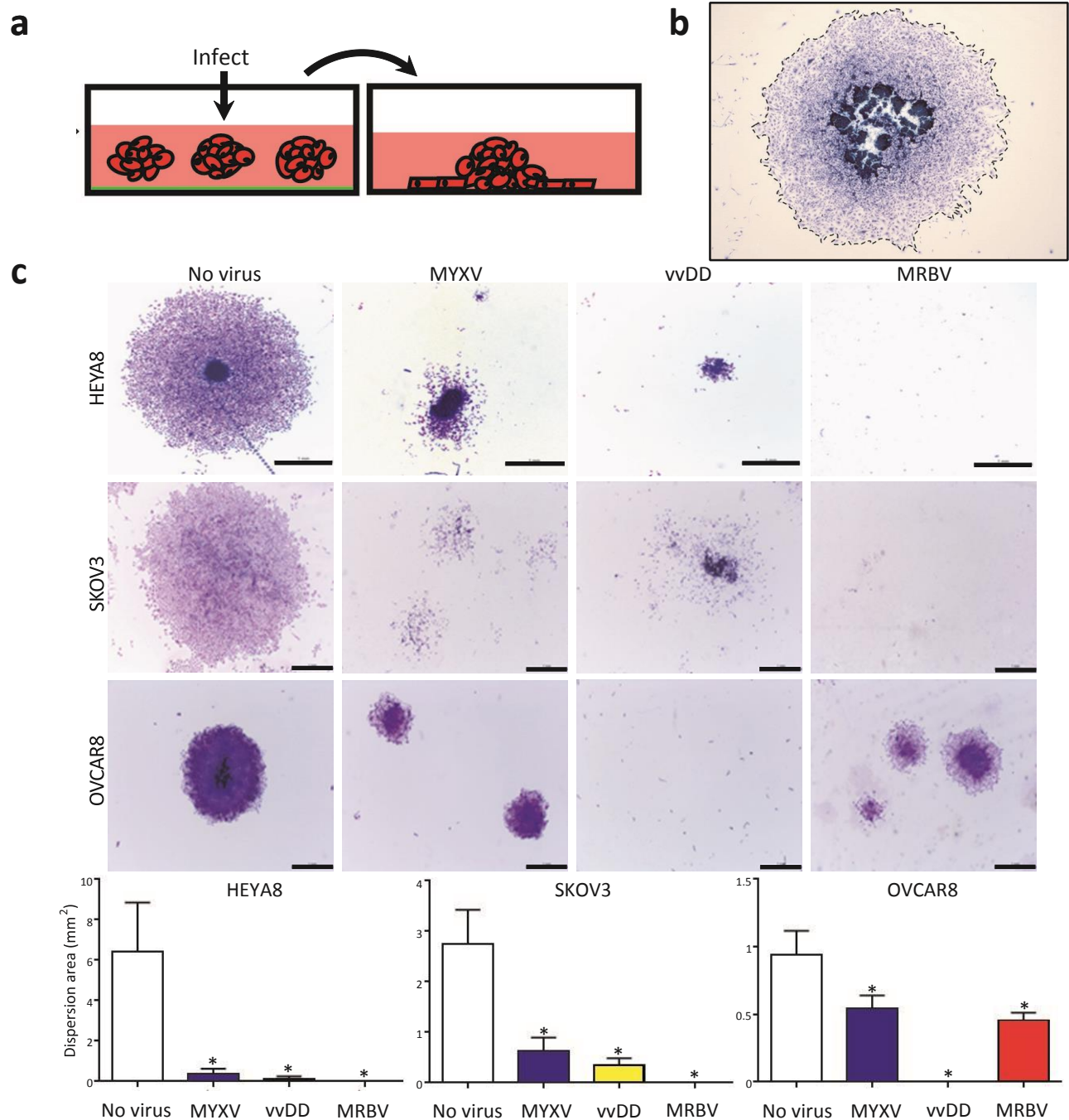


Figure 2.4 MYXV and vvDD oncolysis is reactivated after spheroid reattachment. (a) Schematic representation of spheroid infection followed by reattachment to standard tissue culture-treated plastic. (b) Representative image of dispersion area quantification as denoted by dashed outline. (c) HEYA8, SKOV3 and OVCAR8 cells were seeded to form spheroids then infected with MYXV, vvDD, or MRBV for 72 h. Infected spheroids were transferred to adherent culture to allow reattachment for 72 h, then fixed and stained. Spheroid reattachment was either completely absent (indicating lack of viable cells), or dispersion of cells from the attached spheroid was significantly reduced (indicating re-activation of oncolytic activity upon reattachment to adherent culture). Scale bar: 1mm. (d) Quantification of mean dispersion area was performed using *ImageJ* software. (* $p < 0.05$).

We observed virus infection of attached spheroids with treatment of MYXV, vvDD, and MRBV within 24 hours using green fluorescent protein (GFP) expression as a marker (Fig. 2.5b); this resulted in cytopathic effect in the dispersing adherent cells emanating from attached spheroids while leaving the spheroid cores relatively intact (Fig. 2.5c). These findings further emphasize the requirement of cells to be adherent to promote MYXV and vvDD induced oncolysis. Similar to reduced cytopathic efficacy observed in MRBV infection of OVCAR8 spheroids, we observed reduced GFP expression in MRBV-infected OVCAR8 attached spheroids when compared with MRBV-infected HEYA8 and SKOV3 attached spheroids, suggesting decreased viral entry or replicative potential in OVCAR8 spheroids.

MRBV entry into EOC spheroid cells is significantly reduced.

Since we had observed an appreciable difference for MYXV and vvDD to induce oncolysis of spheroids, and slower infection kinetics of spheroids by MRBV particularly in OVCAR8 spheroids, we sought to determine the efficiency of virus entry into adherent cells and spheroids. To achieve this end, we titrated both the amount of virus remaining in the supernatant and that which had entered the cell. Although we had observed a significant reduction in oncolytic efficacy in EOC spheroids for MYXV and vvDD (Fig. 2.2b and 2.2c), there was no significant difference for either MYXV or vvDD to enter adherent or spheroids cells (Fig. 2.6a). In contrast, we observed a significant reduction in the ability of MRBV to enter spheroids for all three EOC cell lines when compared with adherent cells.

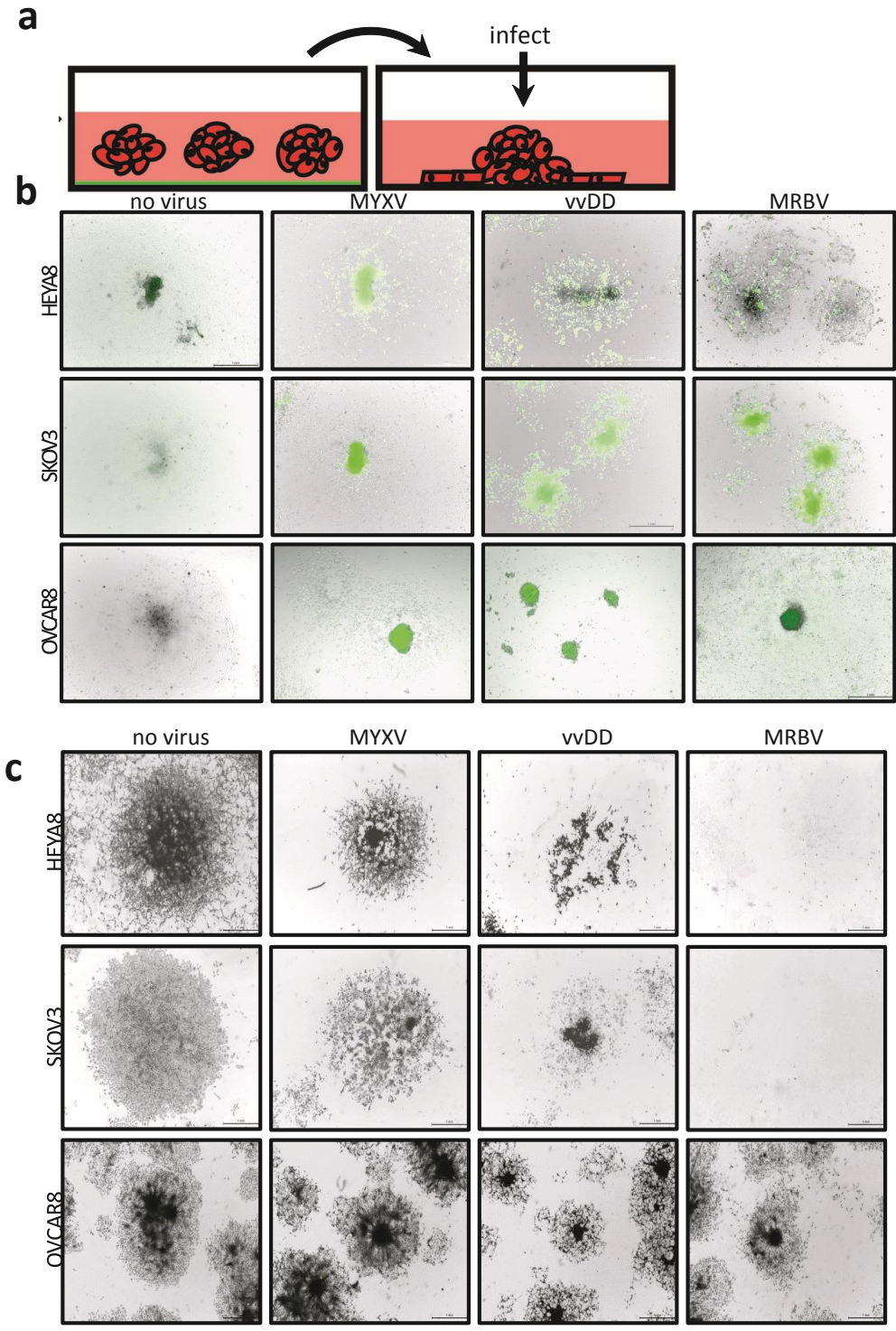


Figure 2.5 Reattached EOC spheroids are susceptible to MRBV infection. (a) Schematic representation of the infection of reattached spheroids. (b) HEYA8, SKOV3 and OVCAR8 cells were seeded to form spheroids, transferred to adherent culture to attach and disperse for 72 h, and then infected with MYXV, vvDD, or MRBV. Bright field and fluorescence images were captured at 24 h post-infection and images were merged using Adobe Photoshop software. (c) After 72 h of infection, SKOV3 and HEYA8 spheroids and dispersing cells are completely eradicated by MRBV infection, yet OVCAR8 spheroids and dispersing cells exhibit less oncolytic-mediated cell death. Infection by vvDD is more effective to reduce viable cells than MYXV for all reattached spheroids. Scale bar: 1 mm.

MRBV binding and infection of EOC spheroids requires low density lipoprotein receptor (LDLR) expression.

To investigate the acquired mechanism determining the enhanced ability of MRBV to enter adherent EOC cells compared with spheroids, we postulated this was due to changes in the expression of a cell surface receptor required for MRBV entry. Previous studies have identified the LDLR as a cell surface receptor that is used by the closely-related vesicular stomatitis virus (VSV). Since the glycoprotein responsible for binding and entry of host cells by VSV shares 80% amino acid sequence homology with MRBV glycoprotein, we sought to determine if the mechanism of MRBV entry in EOC cells was LDLR mediated. Indeed, we observed a consistent decrease in the expression of LDLR protein expression in day-3 spheroids when compared with adherent cells among multiple EOC cell lines (Fig. 2.6b). To test this mechanism further, we performed siRNA knockdown of *LDLR* to validate whether LDLR is required by MRBV to gain entry to host cells. *LDLR* knockdown in SKOV3 cells (Fig. 2.6c) resulted in a significant decrease in MRBV entry (Fig. 2.6d), and a resultant increase in cell viability in MRBV infected cells as compared with knockdown control SKOV3 cells (Fig. 2.6e). In contrast, knockdown of *LDLR* had no effect on cell viability due to infection with MYXV or vvDD.

2.3 Discussion

Most ovarian cancer patients are diagnosed with late-stage metastatic disease, are subjected to multiple successive recurrences, and will eventually succumb to chemotherapy-resistant disease. Thus, the first objective of this study was to test the potential of MYXV, vvDD, and MRBV as therapeutic alternatives to conventional chemotherapeutics for the treatment of metastatic EOC. A second important objective was

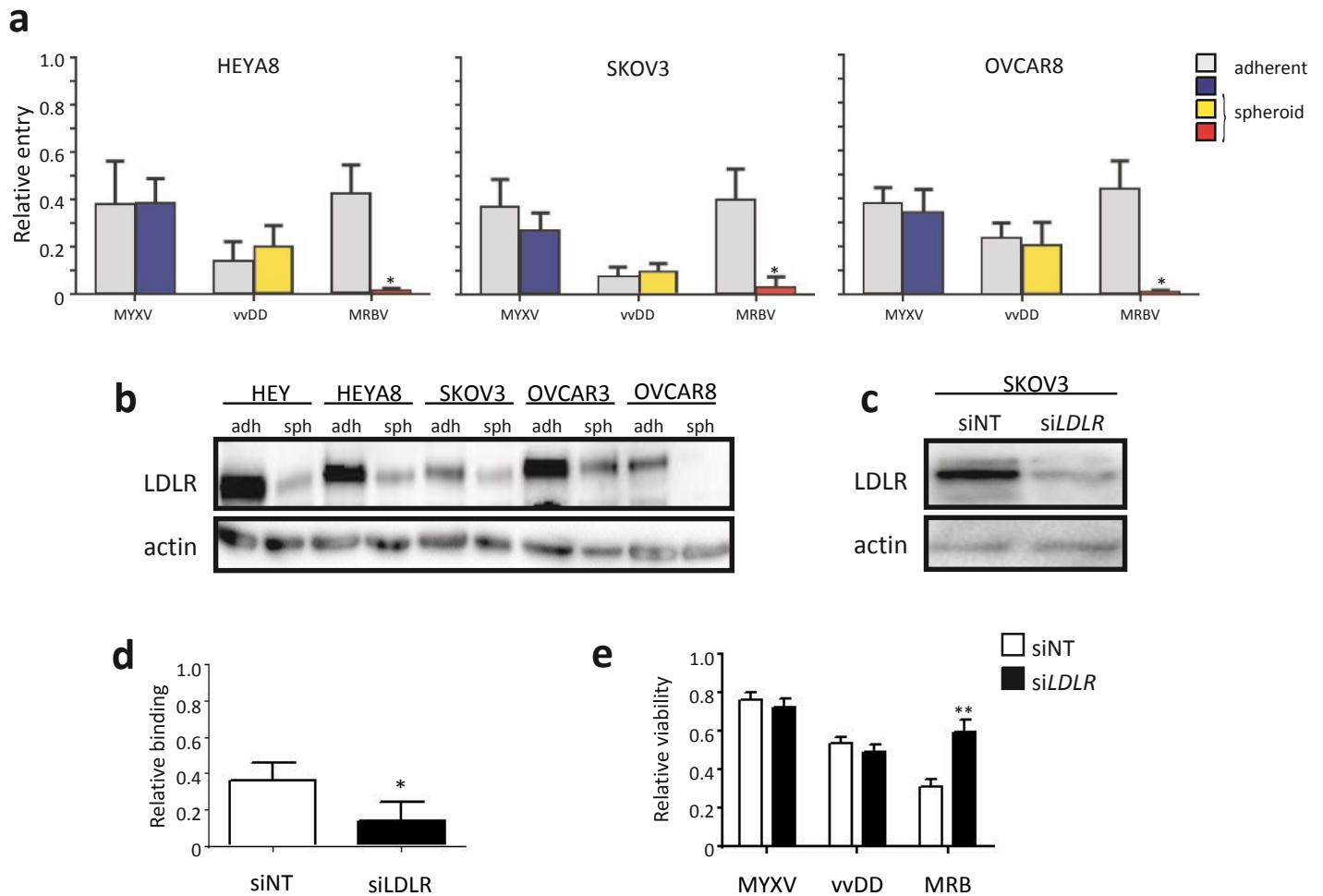


Figure 2.6 Efficient MRBV entry into ovarian cancer cells requires LDLR expression. (a) HEYA8, SKOV3 and OVCAR8 cells were seeded to adherent and spheroid culture, and infected with MRBV, vvDD and MYXV for 1 h at 4°C to allow adsorption. Virus titers from cell lysates are shown relative to total virus collected (*i.e.*, supernatant + cells). MRBV titers were determined using supernatant only. Virus treatment in the absence of cells were used as controls. MRBV binding was significantly reduced in EOC spheroids for all three cell lines tested when compared with MYXV and vvDD. (b) LDLR protein expression is reduced in ovarian cancer day-3 spheroids as compared with proliferating adherent cells; this results was also observed in the HEY and OVCAR3 cell lines. (c) Western blot demonstrating siRNA mediated LDLR knockdown performed by transient transfection of SKOV3 cells. (d) MRBV binding is reduced in SKOV3 cells with *LDLR* knockdown as compared with siNT control transfected cells. (e) *LDLR* knockdown significantly decreases MRBV-mediated oncolytic SKOV3 cell death as determined by CellTiter-Glo after 24 h, whereas there is no effect on viability after MYXV and vvDD infection. (* $p < 0.05$; ** $p < 0.01$)

to demonstrate that testing viral oncolytics in a uniquely different culture-based model system, *i.e.* three-dimensional spheroids, can elicit unforeseen results and uncover important mechanisms controlling virus infection and efficacy. MRBV is clearly the most potent oncolytic virus among the EOC cell lines that we tested in both proliferating adherent cells and quiescent 3D spheroids. In addition, we are the first to discover that endogenous down-regulation of LDLR protein expression in spheroids has the potential to reduce MRBV oncolytic efficacy. Although the larger and slower poxviruses MYXV and vvDD were less infectious and produced less virus progeny in ovarian cancer cell lines and spheroids, virus-infected spheroids displayed reduced capacity to reattach and grow due to reactivation of virus infection. Given our results, we propose that early *in vitro* testing of viral oncolytic agents should consider using an experimentally-tractable cell culture system such as ours that mimics unique mechanisms of disease metastasis.

We observed significant differential effects of the three viruses among the three cell lines and when assessing the different steps of metastasis as modeled in our culture system, particularly when comparing adherent to spheroid cells (summarized in Table 2.2). In adherent culture, MRBV clearly exhibited the highest oncolytic activity. Adherent monolayer cultures represent proliferating ovarian cancer cells with intact growth factor signaling. In contrast, overall efficacy was reduced among the three viruses in spheroids. In particular, the almost universal MRBV oncolytic efficacy was dramatically reduced in OVCAR8 spheroids. We demonstrate that one of the key receptors MRBV utilizes to bind and enter ovarian cancer cells is LDLR; surprisingly, we also show that the LDLR receptor is down-regulated in spheroids compared with adherent proliferating ovarian cancer cells. This could be related to the dormant phenotype and the fact that the overall anabolic metabolism is reduced in spheroids [18, 19, 23]. It has been reported previously that

Table 2.2 Summary of overall results for MYXV, vvDD and MRBV oncolytic efficacy in ovarian cancer adherent cells and spheroids.

	HEYA8		SKOV3		OVCAR8	
	ADH	SPH	ADH	SPH	ADH	SPH
MYXV	+	-	+	-	+	-
vvDD	++	+	+	-	++	++
MRBV	+++++	+++++	+++++	+++++	+++++	+

- No killing
 + EC₅₀ MOI 10
 ++ EC₅₀ MOI 1
 +++ EC₅₀ MOI 0.1
 ++++ EC₅₀ MOI 0.01
 +++++ EC₅₀ MOI 0.001

densely-packed cells down-regulate LDLR resulting in decreased cholesterol metabolism in gynaecologic cancer cell lines [24, 25]. It is possible that this same effect of LDLR down-regulation is occurring in densely-packed cells comprising EOC spheroids thereby reducing initial virus entry. It is important to note, however, that after this initial delay in virus entry, MRBV is capable to infecting spheroid cells and producing infectious viral progeny albeit with slower kinetics compared with adherent proliferating cells. We postulate that the dormant phenotype observed in cultured spheroids is analogous to microregions of tumours that are avascular or lack essential growth factor and nutrient availability. To that end, it would be important to address LDLR expression level in ovarian tumours directly, and assess whether modifications can be made to increase MRBV binding and entry to the potentially resistant subpopulations due to altered metabolism.

MRBV is the most potent of the three oncolytic viruses tested. It exhibited the greatest killing in the three cell lines. It had the fastest kinetics and generated the most infectious progeny. This is most likely supported by the fact that MRBV is a rhabdovirus with a short life cycle and small genome. This has also been observed for MRBV in other cancer cell systems, and its related family members, such as the most widely-studied rhabdovirus, VSV [15, 26]. MRBV was originally identified from a large biorepository of rhabdoviruses as having potent activity in several different cancer cell lines [15]. This group also developed the double point mutant MRBV MG1, which exhibited enhanced growth in cancer cells and reduced effects in normal cells. MRBV is rapidly entering clinical trials with engineered vectors expressing tumour-associated antigens (TAAs), such as MAGE A3 [27]. We postulate that identifying similar TAAs specific for ovarian cancer, perhaps other MAGE proteins [28], could be rapidly applied to develop clinically-useful MRBV oncolytic viral vectors. It has become increasingly evident that oncolytic virus

efficacy *in vivo* relies on eliciting an active immune response, which may lead to more durable anti-tumour effects in the long-term [29]. We recognize that the cell culture-based system used in this report is unable to assess the contribution of the immune system; however, we argue that our *in vitro* ovarian cancer spheroid metastasis model is a useful, rapid and widely-amenable experimental tool for initial testing of novel oncolytic vectors across histologic and molecularly-defined cancer subtypes, particularly using patient-derived malignant tumour cells.

Generally speaking, ovarian cancer spheroids are more restrictive to viral oncolysis. One argument could be made regarding the general physical structure of spheroid that may make them less readily infected by viruses. Interestingly, we found that for MYXV and vvDD viruses there was no difference in binding of these viruses to adherent monolayer cultured cells compared with 3D spheroids in suspension. However, the ability of these two different viruses to complete their lifecycle was dramatically restricted in spheroids. In this case, this is most likely due to the inherent phenotype of ovarian cancer cells in spheroids, namely down-regulation of AKT signaling, induction of autophagy, and a cellular quiescent phenotype [18, 19, 23]. Oncolytic viruses typically rely on overactive or mutant growth factor signaling to promote their life cycle and this constitutes a critical cancer-specific tropism [30]. We have shown in other reports that the AKT signaling pathway is markedly down-regulated in ovarian cancer cell spheroids, and this directly affected MYXV oncolytic efficacy [22]. Soares and colleagues have shown that increased levels of phosphorylated AKT are required for late-stage vvDD morphogenesis and production of virus progeny [31]. Likewise, phosphorylated AKT is also required for permissive infection of MYXV; however, the specific stage of the requirement is currently undefined [32]. In our previous study we demonstrated that activated AKT levels are

significantly reduced within spheroids as compared with adherent EOC cells, but this activity is re-instated upon spheroid reattachment [19]. Our present results are in agreement with this phenomenon, since reattachment of spheroids triggered the re-activation of MYXV and vvDD-mediated oncolytic killing of dispersing cells.

MYXV and vvDD reach late-stage virus production in spheroids yet are restricted to cause cell death. This restriction is quickly relieved upon spheroid reattachment when the dormant-to-proliferative switch occurs [19] and cells are again susceptible to viral-mediated oncolytic cell death. This result is consistent with our previous study using MYXV in patient-derived spheroids [22]. We use spheroid reattachment as a model of intraperitoneal metastatic seeding of malignant cells akin to what is observed in patients [16, 17, 33, 34]. In fact, OVCAR8-generated spheroids were dramatically susceptible to vvDD-induced cell death when using spheroid re-attachment as an assay. It would be interesting to determine what mechanisms are utilized by vvDD to affect OVCAR8 spheroid cells compared with MRBV, an agent that was largely ineffective only in OVCAR8 spheroids. This knowledge may uncover novel strategies to engineer MRBV to make it more widely applicable to ovarian tumours of different histologies and pathobiologies across the ovarian cancer patient spectrum. Given our results, we propose that tumour-homing oncolytic viruses could be potent therapeutic agents with particularly high tropism and efficacy to seek and destroy these persistent microscopic structures in a patient after surgical debulking of macroscopic disease.

2.4 Materials and methods

Cell culture

HEYA8, SKOV3, HEY, Vero, HeLa, and BGMK cell lines were cultured in DMEM (Wisent) supplemented with 5% fetal bovine serum (FBS; Wisent). OVCAR8 and OVCAR3 cells were cultured DMEM/F12 (Wisent) containing 10% FBS. All cell lines were cultured in a humidified environment at 37°C with 5% CO₂.

Ascites fluid obtained from ovarian cancer patients at the time of debulking surgery or paracentesis was used to generate primary cell cultures as described previously [22]. Cells were cultured in DMEM/F12 media supplemented with 10% FBS and grown in a 37°C humidified atmosphere of 95% air and 5% CO₂. Since these represent primary cell cultures, all experiments were performed between passages 3 and 5. All patient-derived cells were used in accordance with institutional human research ethics board approval (UWO HSREB 12668E).

Virus production

MYXV, vvDD, MRBV were amplified in BGMK, HeLa, and Vero cell lines, respectively. BGMK cells were infected with MYXV at MOI 10 for 1 h. After 48 h of infection, cells were harvested and lysed, cell debris was pelleted by centrifugation and supernatant with MYXV were purified [35]. HeLa cells were infected with vvDD at MOI 0.1 and 60 h after infection cells and virus were harvested and purified similar to MYXV. Vero cells were infected with MRBV at MOI 0.01. After 20 h of infection, supernatant was collected and virus was purified using a 0.2 µM filter. All virus constructs have been engineered previously to express green fluorescent protein (GFP) from endogenous viral promoters: MYXV [36, 37], vvDD[14], MRBV [26]. The MRBV MG1 mutant strain used in these experiments has been described previously [26].

Viral titer quantification

Quantification of MYXV, vvDD, and MRBV titers were performed using BGMK, HeLa, and Vero cells, respectively. Virus titers were determined through limiting dilutions of virus on BGMK, HeLa, or Vero. Agarose overlays and plaque assays were performed to determine virus concentration.

Adherent culture HEYA8, SKOV3, and OVCAR8 cells were seeded at 25 000 cells/well of a 24-well plate and infected with MYXV, vvDD, or MRBV at MOI 10. After 48 h MRBV infection, and 4 d after MYXV and vvDD infection, cells and supernatant were harvested together for virus content. MYXV, vvDD, and MRBV were titrated on BGMK, HeLa, and Vero cell lines, respectively.

Spheroid culture HEYA8, SKOV3, and OVCAR8 cells were seeded at 50 000 cells/well of a 24-well ultra-low attachment (ULA) cluster plate and allowed to form spheroids for 3 d. Spheroids were then infected while in suspension with MYXV, vvDD, or MRBV at MOI 10. Seventy-two hours after MRBV infection, and 5 d after MYXV and vvDD infection, spheroids and supernatant were harvested together for virus content. Spheroids were triturated using a 26-gauge needle and titered as described above.

Virus infection of EOC cells

HEYA8, SKOV3, and OVCAR8 were seeded at 5 000 cells/well of a 96-well plate and were infected the following day with MYXV, vvDD, or MRBV at a multiplicity of infection (MOI) 0.001, 0.01, 0.1, 1, and 10. The appropriate UV-inactivated virus at MOI 10 or no virus (mock infected) was used as controls. Seventy-two hours post infection, viability was assayed using CellTiter-Glo® Luminescent Cell Viability Assay (Promega).

For infection of EOC spheroids, cells were seeded at 50 000 cells/well of a 24-well ULA cluster plate (Corning) and spheroids were allowed to form over 72 h. Spheroids were then infected at MOI 0.01, 0.1, 1, and 10, using the same controls as described for adherent cell infections.

For infection of reattached spheroids, spheroids were formed as previously described, in the absence of virus, and transferred to 6-well tissue culture-treated plates for reattachment. Forty-eight hours after reattachment, spheroids were then infected at MOI 10 based on the initial seeding of 50 000 cells/well of a 24-well ULA plate. Spheroids were imaged 24 h after infection then fixed and stained at 72 h post-infection using HEMA3 (Fisher HealthCare™). Phase contrast and fluorescence images of infected cells and spheroids were captured during each experiment using a Leica DMI 4000B inverted microscope. Fluorescence and phase contrast overlays were generated using Adobe Photoshop.

Kinetics of infection and cell viability

HEYA8, SKOV3, and OVCAR8 cells were seeded as described above for both adherent and spheroid cultures. Cells were then infected with an MOI 10 to allow for maximum virus infection and achieve synchronous virus lifecycle among all cells within the culture. Viability was then assayed using CellTiter-Glo® at 12, 24, 36, 48, 72 h, and 4 and 5 d after infection for adherent cultures and at 24 h intervals for up to 6 d with spheroids. To assay cell viability in spheroids, spheroids were collected and pelleted, followed by resuspension in CellTiter-Glo® and trituration with a 26-gauge needle. Luminescence was measured using a Wallac Plate Reader (PerkinElmer).

Spheroid reattachment quantification

Cells were seeded at 50 000 cells/well of a 24-well ULA cluster plate to form spheroids over 72 h. Spheroids were infected with MYXV, vvDD, or MRBV at MOI 10. Spheroids were reattached by transferring to 6-well tissue culture plates. Spheroids were permitted to attach and disperse for an additional 72 h prior to fixing and staining using HEMA3™. Dispersion areas were calculated using *ImageJ* 1.48 software (NIH) by subtracting the area of the core spheroid from the total area of the dispersion zone.

Virus entry quantification

Adherent cells and spheroids were infected with MOI 10 MYXV, vvDD, and MRBV for 1 h at 4°C to allow virus infection of cells, but to prevent virus uncoating that would affect subsequent quantification of infectious virus titers. After 1 h of virus adsorption, supernatant and cells were separated and cell pellets were washed twice with PBS. Spheroids and adherent cells were triturated as described above to ensure that all non-adsorbed virus was released. Virus content from supernatants and cell pellets were titrated separately to quantify the proportion of virus that had entered adherent cells and spheroids.

Immunoblotting

Cell lysates were generated using a modified radioimmunoprecipitation assay (RIPA) buffer [50 mM HEPES pH7.4, 150 mM NaCl, 10% glycerol, 1.5 mM MgCl₂, 1 nM EGTA, 1 nM sodium orthovanadate, 10 mM sodium pyrophosphate, 10 mM NaF, 1% Triton X 100, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 1× protease inhibitor cocktail (Roche, Laval, QC)] as described previously [38]. Lysates were incubated on ice for 20 minutes and vortexed to ensure complete lysis. Lysates from day-3 spheroids were triturated using a 26-gauge needle to help facilitate lysis. Protein concentrations were then determined by Bradford assay using Protein Assay Dye Reagent (BioRad, Mississauga, ON). Thirty micrograms of each lysate was run on an 8% SDS PAGE gel and transferred

to a polyvinylidene difluoride membrane (PVDF; Roche). Blots were blocked with 5% skim milk in Tris-buffered saline with Tween 20 (TBST; 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20). After 1 h of blocking, blots were incubated on a rocking platform shaker at 4°C overnight with specific antibodies at 1:1000 dilution in bovine serum albumin (BSA)/TBST [anti-LDLR (Abcam, ab14056; Cambridge, MA); anti-actin (Sigma)]. Blots were washed using TBST and incubated with peroxidase-conjugated anti-rabbit IgG (GE Healthcare) at 1:10 000 dilution, 5% skim milk/TBST (LDLR) or 5% BSA/TBST (actin), for 1 h at room temperature. Blots were washed again using TBST followed by incubation with Luminata™ Forte Western HRP Substrate (Millipore) and visualization of bands with the ChemiDoc™ MP System (BioRad).

LDL Receptor Knockdown

SKOV3 cells were seeded in 48-well dishes and transfected the next day with si*LDLR* SMARTPool RNA or the siNT non-targeting control RNA using DharmaFECT 1 transfection reagent (Dharmacon). At 48 h post-transfection, cells were used for infection experiments [virus entry (MRBV at MOI 0.1) and cell viability (MYXV and vvDD at MOI 1; MRBV at MOI 0.1)] as described above.

Statistical Analysis

Statistical significance was determined by unpaired two-tailed Student's *t*-test or one-way analysis of variance (ANOVA) using GraphPad Prism 6. Statistical significance was set at $p < 0.05$.

CONFLICT OF INTEREST

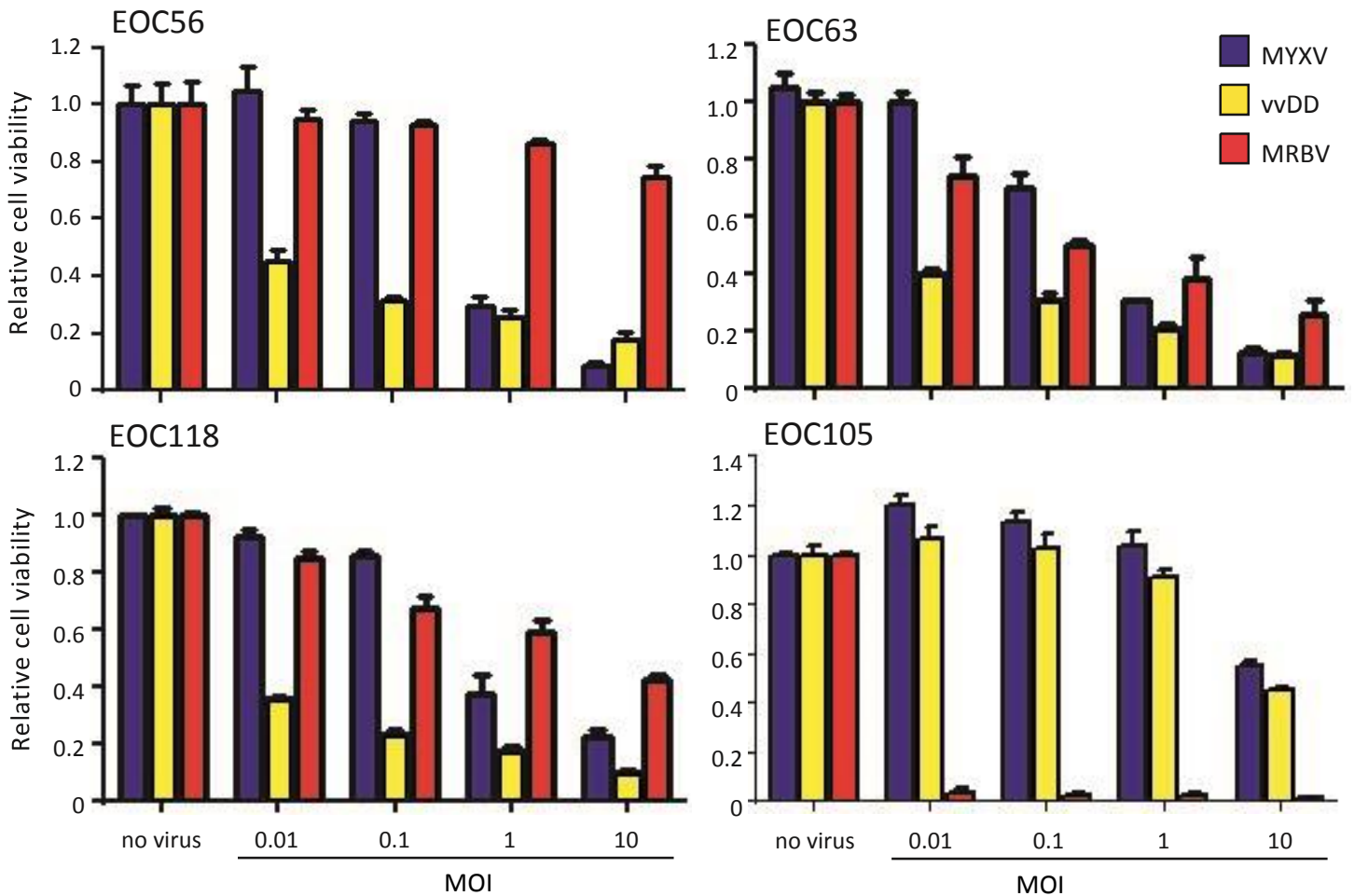
The authors of this article have no conflicts of interest to declare.

2.5 Supplementary information

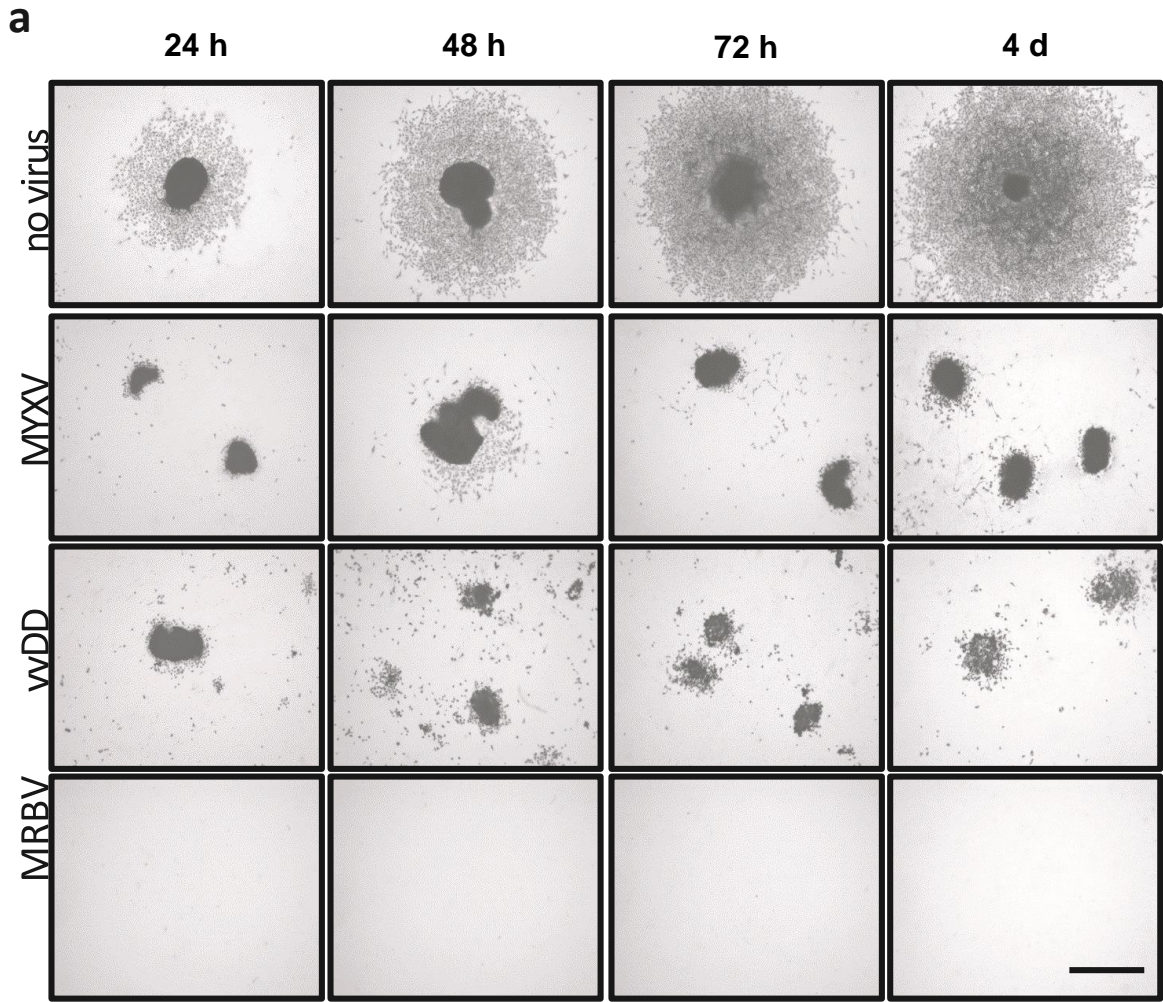
Supplementary Table A2.1. Summary of gene mutations identified in the three cell lines used in this study.

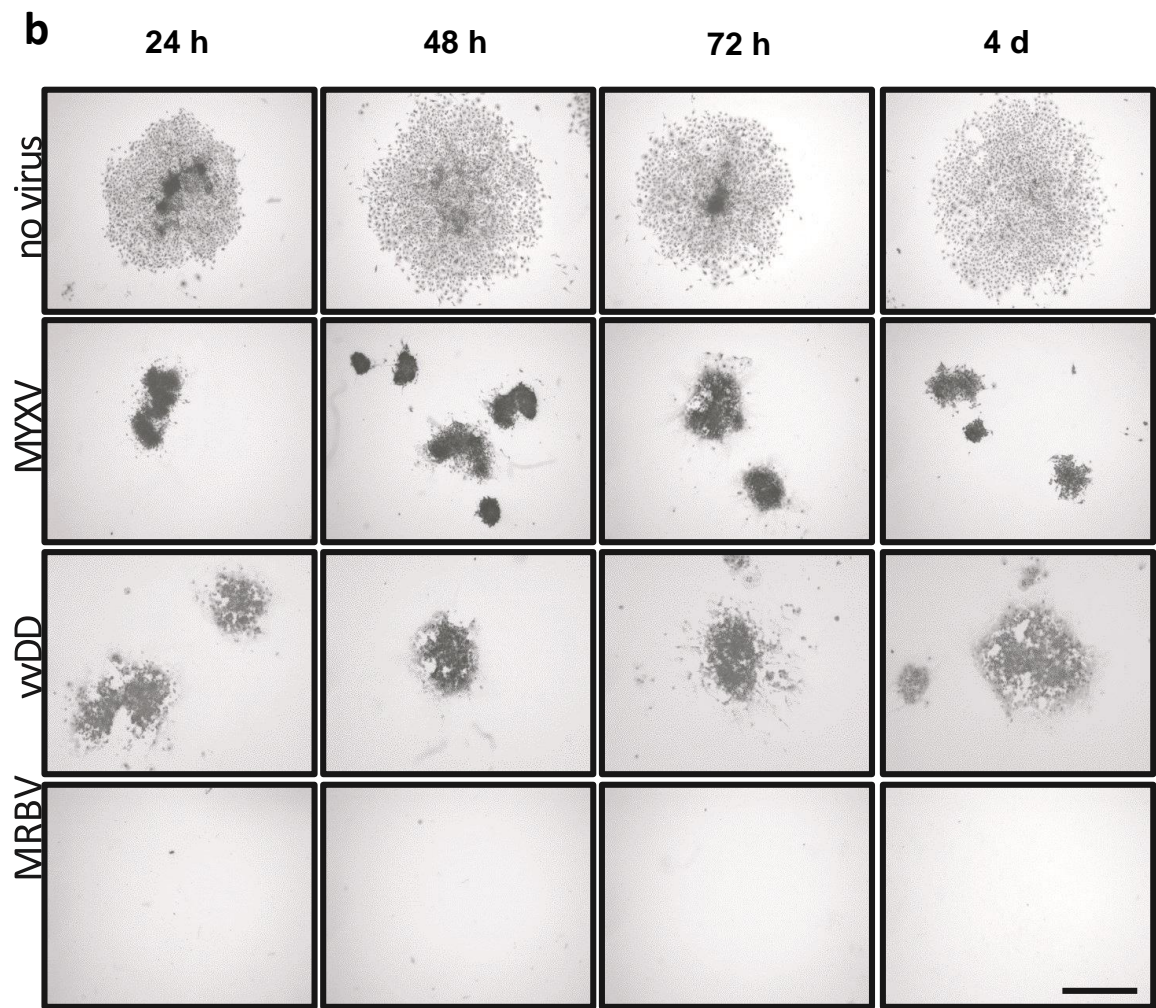
	<i>TP53</i>	<i>KRAS</i>	<i>BRAF</i>	<i>PIK3CA</i>	<i>ERBB2</i>	<i>ARID1A</i>	<i>CTNNB1</i>
HeyA8		G12D; AMP	G464E	AMP			
SkOV3		AMP		H1047R	AMP	Q586*	
OVCAR8	Y126 splice	P121H			G776V		Q26R

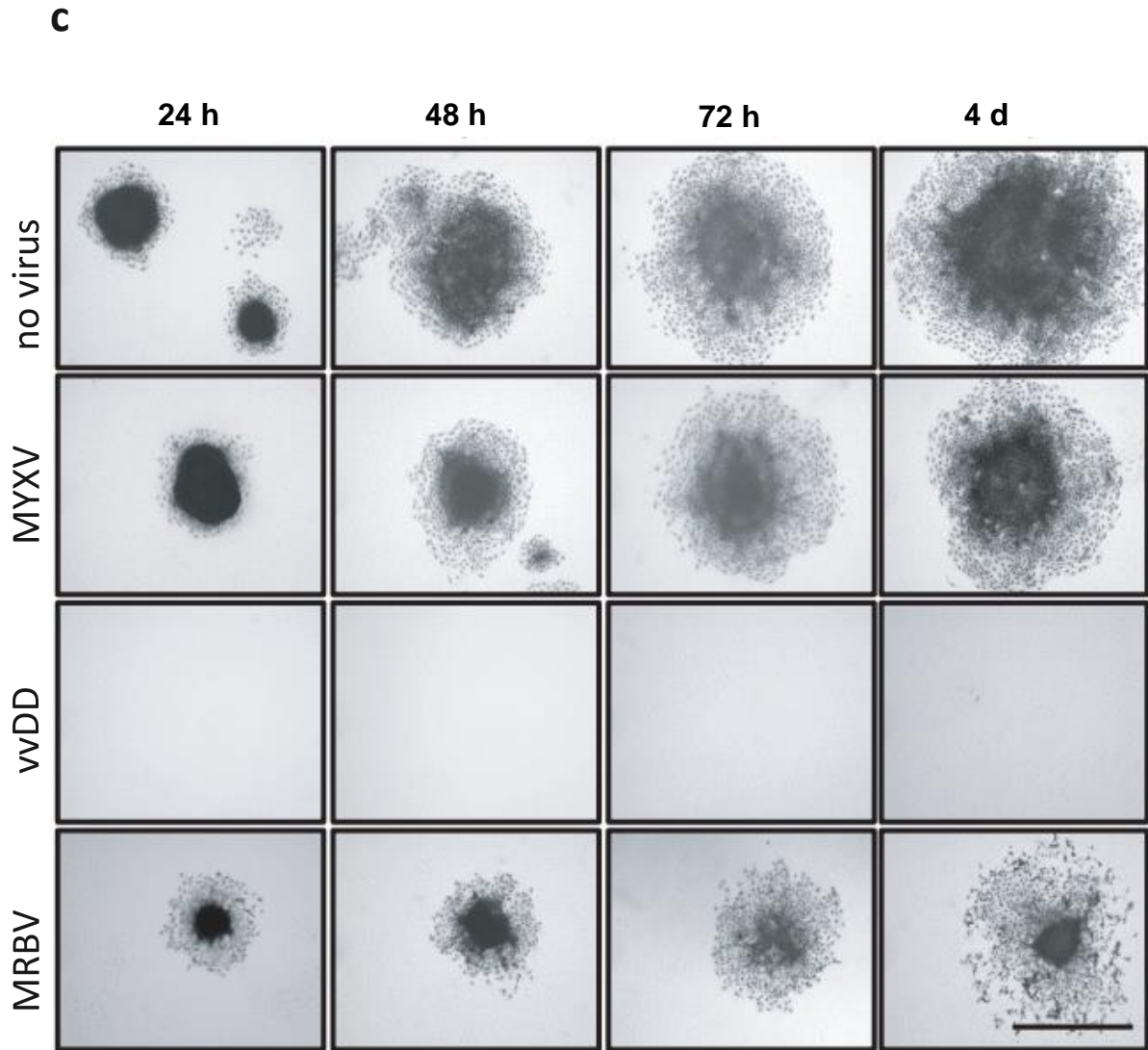
Data from Domcke *et al.* (2013) Ranking ovarian cancer cell lines by suitability as HGSOC models. *Nat Commun* 4: 212



Supplementary Figure A2.1. Differential oncolytic efficacy in ovarian cancer patient ascites-derived primary cells. Primary cultures of four independent ascites samples (EOC56, EOC63, EOC118 and EOC105) were infected with MYXV, vDD and MRBV in adherent culture at increasing concentrations to a maximum of MOI 10, as indicated. UV-inactivated virus was used at an MOI of 10. Cell viability was measured after 72 h of infection using CellTiter-Glo®.







Supplementary Figure A2.2. Time course of infected spheroid reattachment. Spheroids were formed using HEYA8 (a), SKOV3 (b) and OVCAR8 (c) cells for three days, infected, then followed by reattachment to adherent culture as described in Figure 4. Spheroids were fixed and stained every 24 h for up to 4 d. Spheroid attachment and cell dispersion area was significantly reduced by all virus infections, however OVCAR8 spheroids exhibited reduced oncolytic efficacy by MYXV and MRBV infection. Scale bar: 1 mm.

2.6 References

1. Canadian Cancer Statistics 2014. (Public Health Agency of Canada, Ed.), pp. 1-132. Government of Canada.
2. The Cancer Genome Atlas Research Network. (2011) Integrated genomic analysis of ovarian carcinoma. *Nature* 474: 609-615.
3. Howlader, N., Noone, A. M., Krapcho, M., Garshell, J., Miller, D., Altekruse, S. F., Kosary, C. L., Yu, M., Ruhl, J., Tatalovich, Z., Mariotto, A., Lewis, D. R., Chen, H. S., Feuer, E.J., Cronin, K.A. (2014). SEER Cancer Statistics Review, 1975-2011. (US Dept. of Health and Human Services, Ed.). National Cancer Institute, National Institutes of Health.
4. Griffiths, C. T. (1975). Surgical resection of tumor bulk in the primary treatment of ovarian carcinoma. *National Cancer Institute monograph* 42: 101-104.
5. Hacker, N. F., Berek, J. S., Lagasse, L. D., Nieberg, R. K., and Elashoff, R. M. (1983). Primary cytoreductive surgery for epithelial ovarian cancer. *Obstetrics and gynecology* 61: 413-420.
6. Coleman, R. L., Monk, B. J., Sood, A. K., and Herzog, T. J. (2013) Latest research and treatment of advanced-stage epithelial ovarian cancer. *Nature reviews* 10: 211-224.
7. Ferrandina, G., et al. (1999). p53 overexpression is associated with cytoreduction and response to chemotherapy in ovarian cancer. *British journal of cancer* 81: 733-740.
8. Kurman, R. J., and Shih Ie, M. (2010) The origin and pathogenesis of epithelial ovarian cancer: a proposed unifying theory. *The American journal of surgical pathology* 34: 433-443.
9. Tothill, R. W., et al. (2008). Novel molecular subtypes of serous and endometrioid ovarian cancer linked to clinical outcome. *Clin Cancer Res* 14: 5198-5208.
10. Kurman, R. J., and Shih Ie, M. (2011) Molecular pathogenesis and extraovarian origin of epithelial ovarian cancer--shifting the paradigm. *Human pathology* 42: 918-931.
11. Russell, S. J., Peng, K. W., and Bell, J. C. (2012) Oncolytic virotherapy. *Nature biotechnology* 30: 658-670.
12. Kerr, P. J. (2012) Myxomatosis in Australia and Europe: a model for emerging infectious diseases. *Antiviral research* 93: 387-415.
13. Kim, M., et al. (2010) The viral tropism of two distinct oncolytic viruses, reovirus and myxoma virus, is modulated by cellular tumor suppressor gene status. *Oncogene* 29: 3990-3996.
14. McCart, J. A., et al. (2001). Systemic cancer therapy with a tumor-selective vaccinia virus mutant lacking thymidine kinase and vaccinia growth factor genes. *Cancer research* 61: 8751-8757.
15. Brun, J., et al. (2010) Identification of genetically modified Maraba virus as an oncolytic rhabdovirus. *Mol Ther* 18: 1440-1449.
16. Burleson, K. M., Casey, R. C., Skubitz, K. M., Pambuccian, S. E., Oegema, T. R., Jr., and Skubitz, A. P. (2004). Ovarian carcinoma ascites spheroids adhere to extracellular matrix components and mesothelial cell monolayers. *Gynecologic oncology* 93: 170-181.

17. Burleson, K. M., Hansen, L. K., and Skubitz, A. P. (2004). Ovarian carcinoma spheroids disaggregate on type I collagen and invade live human mesothelial cell monolayers. *Clinical & experimental metastasis* 21: 685-697.
18. Lu, Z., et al. (2008). The tumor suppressor gene ARHI regulates autophagy and tumor dormancy in human ovarian cancer cells. *The Journal of clinical investigation* 118: 3917-3929.
19. Correa, R. J., Peart, T., Valdes, Y. R., DiMattia, G. E., and Shepherd, T. G. (2011) Modulation of AKT activity is associated with reversible dormancy in ascites-derived epithelial ovarian cancer spheroids. *Carcinogenesis* 33: 49-58.
20. Makhija, S., Taylor, D. D., Gibb, R. K., and Gercel-Taylor, C. (1999). Taxol-induced bcl2 phosphorylation in ovarian cancer cell monolayer and spheroids. *International journal of oncology* 14: 515-521.
21. Domcke, S., Sinha, R., Levine, D. A., Sander, C., and Schultz, N. (2013) Evaluating cell lines as tumour models by comparison of genomic profiles. *Nature communications* 4: 2126.
22. Correa, R. J., et al. (2012) Myxoma virus-mediated oncolysis of ascites-derived human ovarian cancer cells and spheroids is impacted by differential AKT activity. *Gynecologic oncology* 125: 441-450.
23. Liao, J., et al. (2014) Ovarian cancer spheroid cells with stem cell-like properties contribute to tumor generation, metastasis and chemotherapy resistance through hypoxiaresistant metabolism. *PloS one* 9: e84941.
24. Kruth, H. S., Avigan, J., Gamble, W., and Vaughan, M. (1979). Effect of cell density on binding and uptake of low density lipoprotein by human fibroblasts. *The Journal of cell biology* 83: 588-594.
25. Gal, D., MacDonald, P. C., Porter, J. C., Smith, J. W., and Simpson, E. R. (1981). Effect of cell density and confluency on cholesterol metabolism in cancer cells in monolayer culture. *Cancer research* 41: 473-477.
26. Stojdl, D. F., et al. (2000). Exploiting tumor-specific defects in the interferon pathway with a previously unknown oncolytic virus. *Nature medicine* 6: 821-825.
27. Institute, N. C. T. G. O. H. R. (2014). MG1 Maraba/MAGE-A3, With and Without Adenovirus Vaccine, With Transgenic MAGE-A3 Insertion in Patients With Incurable MAGE-A3-Expressing Solid Tumours (I214). (C. g. [Internet]. Ed.). Bethesda (MD): National Library of Medicine (US).
28. Daudi, S., et al. (2014) Expression and immune responses to MAGE antigens predict survival in epithelial ovarian cancer. *PloS one* 9: e104099.
29. Lichty, B. D., Breitbach, C. J., Stojdl, D. F., and Bell, J. C. (2014) Going viral with cancer immunotherapy. *Nat Rev Cancer* 14: 559-567.
30. Chiocca, E. A. (2002). Oncolytic viruses. *Nat Rev Cancer* 2: 938-950.
31. Soares, J. A., et al. (2009). Activation of the PI3K/Akt pathway early during vaccinia and cowpox virus infections is required for both host survival and viral replication. *Journal of virology* 83: 6883-6899.

32. Werden, S. J., and McFadden, G. (2010) Pharmacological manipulation of the akt signaling pathway regulates myxoma virus replication and tropism in human cancer cells. *Journal of virology* 84: 3287-3302.
33. Shield, K., Ackland, M. L., Ahmed, N., and Rice, G. E. (2009). Multicellular spheroids in ovarian cancer metastases: Biology and pathology. *Gynecologic oncology* 113: 143-148.
34. Burleson, K. M., Boente, M. P., Pambuccian, S. E., and Skubitz, A. P. (2006). Disaggregation and invasion of ovarian carcinoma ascites spheroids. *Journal of translational medicine* 4: 6.
35. Smallwood, S. E., Rahman, M. M., Smith, D. W., and McFadden, G. (2010) Myxoma virus: propagation, purification, quantification, and storage. *Current protocols in microbiology* Chapter 14: Unit 14A 11.
36. Opgenorth, A., Graham, K., Nation, N., Strayer, D., and McFadden, G. (1992). Deletion analysis of two tandemly arranged virulence genes in myxoma virus, M11L and myxoma growth factor. *Journal of virology* 66: 4720-4731.
37. Johnston, J. B., et al. (2003). Role of the serine-threonine kinase PAK-1 in myxoma virus replication. *Journal of virology* 77: 5877-5888.
38. Shepherd, T. G., Mujoomdar, M. L., and Nachtigal, M. W. (2010) Constitutive activation of BMP signalling abrogates experimental metastasis of OVCA429 cells via reduced cell adhesion. *Journal of ovarian research* 3: 5.

CHAPTER 3: Spatial and temporal epithelial ovarian cancer cell heterogeneity can impact Maraba virus oncolytic potential

Tong, J. G., Valdes, Y.R., Barrett, J.W., Bell, J.C., Stojdl, D., DiMattia, G.E., Shepherd, T.G.

A version of this chapter is in preparation for submission to Gynecologic Oncology

3.1 Introduction

Diagnosis of high grade serous epithelial ovarian cancer, the most common ovarian cancer, late in metastasis and the inevitable acquisition of chemotherapeutic resistance are the major contributors to the lethality of this disease [1, 2]. This chemoresistance is fueled by profound genomic instability caused by DNA repair pathway deficiencies and universal loss of TP53 which also results in a high degree of intratumoral cellular heterogeneity [3, 4]. As seen in many cancers, intratumoural heterogeneity results in a high degree of phenotypic variability which can manifest as differential responses to different therapies. Debulking surgery either before or after adjuvant chemotherapy is the standard treatment for ovarian cancer patients with metastatic disease. Under this treatment regimen, epithelial ovarian cancer (EOC) is still the most lethal gynaecologic malignancy in the developed world with a 5-year survival rate of less than a 30% [5-7]. Following chemotherapy, the selection and expansion of platinum-resistant EOC cells results in the recurrence of aggressive disease that is largely incurable with second-line treatment options [8-10]. Thus, there is significant clinical need for more effective therapeutics that target disease heterogeneity more effectively, thereby increasing progression-free survival for these patients.

Cancer cells not only gain survival- and growth-enhancing properties through the selection and expansion of specific clones within a tumour, they also lose many intracellular pathogen defense mechanisms and induce immunosuppressive mechanisms.

Oncolytic virotherapy (OV) exploits these defects in intracellular defense to selectively replicate in malignant cells. Additional changes in the tumour microenvironment, such as decreased immune surveillance, also enhance virus targeting of cancers. For example, mutations in interferon (IFN) and in other proteins in this signaling pathway are frequently seen in cancer cells as they are major drivers of antitumor immunity [11-13]. However, type I IFNs are also key antiviral signaling molecules found in all somatic cells, thereby allowing cancer cells to be selectively infected and killed by OVs [14]. Many rhabdoviruses, including Maraba virus (MRBV), represent promising OV vectors because of their susceptibility to IFN signaling, as well as other innate and adaptive immune responses, making these viruses relatively non-pathogenic in humans. Thus, the immunological deficits in cancer cells deficient confer increased susceptibility to these viruses. Currently, a construct of MRBV armed with the MAGE A3 tumor associated antigen, is being evaluated in a phase I/II clinical trial in conjunction with a similarly armed adenovirus to investigate their immunostimulatory activity and oncolytic potential.

In a previous cross-comparison of several OVs, we observed potent oncolytic effects of MRBV in several EOC cell lines [15]. Infections of EOC cell lines cultured as either adherent cells or three-dimensional spheroids in suspension revealed that MRBV was the most potent of the viruses tested at inducing oncolysis. Furthermore, we identified the low-density lipoprotein receptor (LDLR) and its family members as partial mediators of MRBV entry, which may be used to predetermine MRBV oncolysis of cancer cells. However, the potential for resistance to MRBV treatment has yet to be determined in a heterogeneous tumor model.

Our previous analyses described the variability of virus oncolysis of EOC cells derived from independent patients. Herein, our objective was to examine the efficacy of MRBV infection and oncolytic killing in the context of the temporal and spatial heterogeneity of malignant EOC cells present in a patient with metastatic disease. Direct analysis of multiple isolates from an individual patient with metastatic high-grade serous ovarian cancer (HGSOC) of the ovary may provide evidence for intratumoral heterogeneity impacting MRBV oncolytic efficacy. Moreover, it is unclear whether temporal changes in a tumour cell population, particularly after chemotherapy, may cause molecular and cellular changes that affect MRBV infection and oncolysis. Thus, we hypothesized that the high degree of tumour cell heterogeneity in HGSOC of the ovary would yield differential MRBV oncolytic efficacy.

3.2 Results

MRBV oncolysis can be impacted by HGSOC tumour cell heterogeneity

We commonly isolate cancer cells from the ascites of patients with metastatic EOC to perform *in vitro* cell culture experimentation [16]. In one case, we received four independent isolates from a single patient over 14 months of disease progression and treatment (Fig. 3.1a). To investigate the effects of temporal heterogeneity on EOC cell susceptibility to MRBV, we used these newly-derived continuous HGSOC cell lines to assess oncolytic infection and cell killing. Using a range of MOI from 0.001 to 10, we observed that iOvCa105 and iOvCa147 cell lines, which were the first and last isolates received, were highly sensitive to MRBV with complete oncolysis achieved by an MOI 0.01 after 3 d post-infection. This result was in stark contrast to iOvCa131 and iOvCa142 cell lines isolated from the same patient, where complete oncolysis was not achieved at any

virus concentration and partial oncolysis was observed only at MRBV concentrations as high as MOI of 1 after 3 d (Fig. 3.1b).

These results indicated that the HGSOc metastatic cell population in this patient was dynamic and heterogeneous with respect to MRBV sensitivity. We had generated several subclones from the iOvCa147 cell line by limiting-dilution subculturing. Using a set of seven different subcloned cell lines, we performed MRBV infections as described above and viability was measured 3 d post-infection. We observed two distinct responses to MRBV infection from the clones: one group of four subclones exhibited complete oncolysis at less than or equal to an MOI of 0.1, and another group of three subclones (iOvCa 147-B3, G4, G7) exhibiting 1000-fold reduced sensitivity to MRBV, where complete oncolysis was not achieved even at an MOI of 10 (Fig 3.2a). Indeed, we readily observed a widespread cytopathic effect (CPE) and readily detectable green fluorescent protein (GFP) expression from MRBV infected cells, signifying productive MRBV replication in iOvCa147-F8 sensitive cells. However, we observed only modest CPE and GFP-positivity in the resistant iOvCa147-G4 cell line (Fig. 3.2b). Taken together, these results represent an important example of both temporal and spatial heterogeneity in metastatic HGSOc of the ovary and its impact on MRBV oncolytic efficacy.

Low-density lipoprotein receptor (LDLR) is required for efficient MRBV entry of sensitive HGSOc cells

As a first step to determine factors affecting differential MRBV oncolysis in HGSOc cell subpopulations, we sought to determine whether MRBV entry was altered between sensitive and resistant subclone cell lines. Since we have previously established

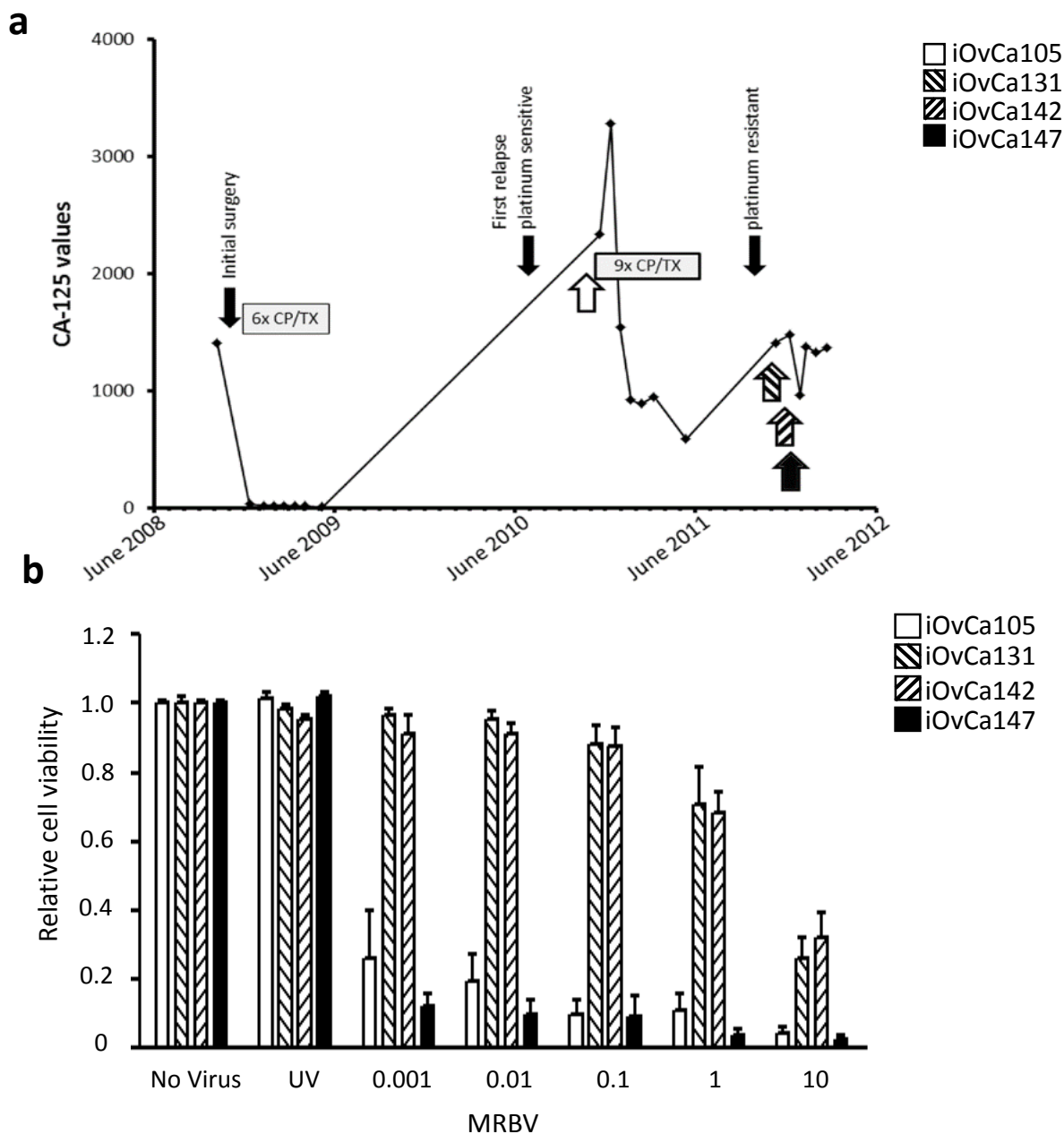


Figure 3.1. Temporal changes in tumor biology differentially impacts MRBV oncolysis. a) iOvCa105, iOvCa131, iOvCa142, and iOvCa147 patient samples were derived from multiple ascites isolates over the course of one year of a single patients' disease. iOvCa105 were isolated October 2010 after first relapse with platinum sensitive disease. iOvCa 131, 142, and 147 were collected one year later between October 2011 to December 2011 after the acquisition of platinum resistance. b) Cells were seeded at 10,000 cells/well of a 96-well plate and infected with replication competent or UV inactivated MRBV. Cells were assayed for viability using CellTiter-Glo® reagent at 72 h after infection.

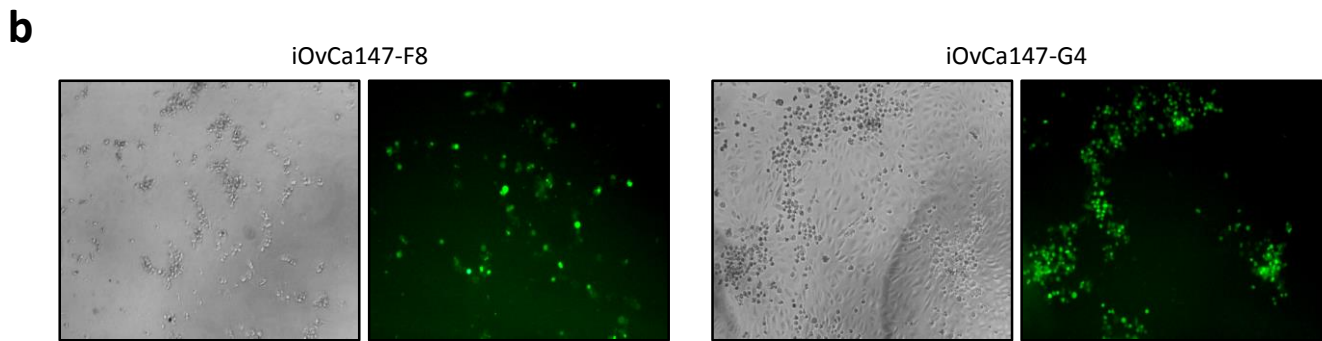
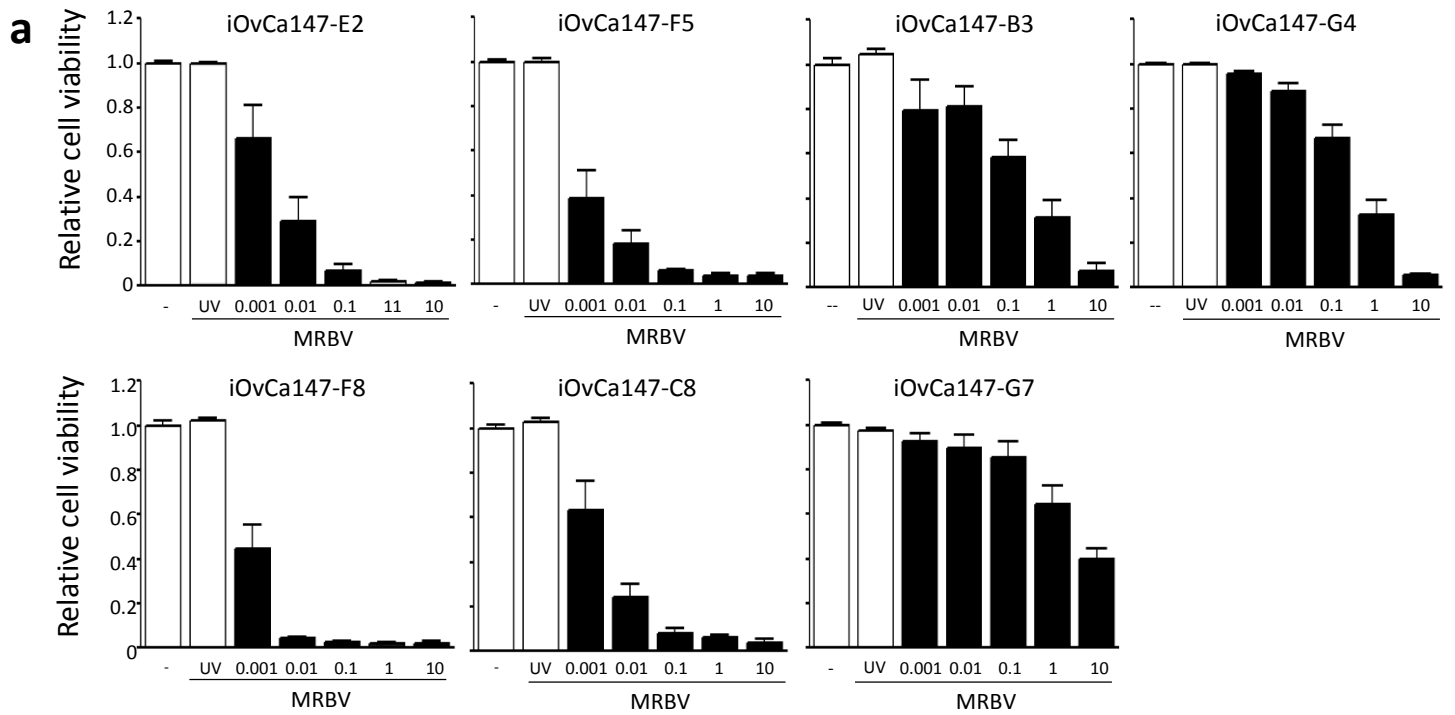


Figure 3.2. Intratumoral cellular heterogeneity differentially impacts MRBV oncolysis. a) Subclones from iOvCa147 were generated through limiting dilution assay and single cell expansion to create homogeneous cell lines from a heterogeneous cell population. Cells were seeded at 10,000 cells/well of a 96-well plate and infected 24 h after seeding. Cells were assayed for viability using CellTiter-Glo at 72 h after infection. b) Images of infected cells were taken at 72 h post infection using bright field and fluorescence microscopy at 5x magnification.

a link between the expression of LDLR and MRBV entry [15], we also sought to determine if differences in the levels of LDLR expression between the iOvCa147-F8 and iOvCa147-G4 subclones could affect MRBV entry and cell susceptibility to MRBV. We infected iOvCa147-F8 and -G4 cell lines with MRBV for one hour after which we quantified the remaining virus in the media. We observed that nearly 25% of MRBV entered iOvCa147-F8 cells, yet only 5% of MRBV entered iOvCa147-G4 cells (Fig. 3.3a).

We previously reported reduced LDLR expression in EOC cell lines during spheroid formation which correlated with decreased MRBV entry into spheroid cells, and this was confirmed by siRNA-mediated knockdown of LDLR expression in adherent cells [15]. In fact, analysis of gene copy-number alterations in serous ovarian adenocarcinoma using The Cancer Genome Atlas data revealed that approximately 14% of HGSOC tumours show LDLR gene amplification with elevated mRNA expression levels (Suppl. Fig. A3.1). Therefore, we determined whether LDLR was differentially expressed between sensitive and resistant clonal lines. We examined LDLR expression in iOvCa147-F8 and iOvCa147-G4 cells with and without MRBV infection. Indeed, iOvCa147-F8 cells expressed two-fold higher LDLR as compared with iOvCa147-G4 cells. In response to both UV-inactivated MRBV (binds and enters cells, but does not replicate) and replication-competent MRBV, LDLR expression was decreased in iOvCa147-F8 cells. This may indicate virus-binding to the LDLR, followed by endocytosis and lysosomal degradation of the internalized receptor [17]. This result was distinct from iOvCa147-G4 cells as the lower LDLR expression in this line did not change in response to virus (Fig 3.3b).

After observing similar correlative data linking LDLR expression with virus infectivity in iOvCa147-F8 and -G4 cells, we sought to determine if decreased LDLR

would impact the susceptibility of sensitive HGSOC cells to virus infection. As expected, siRNA-mediated LDLR knockdown in two MRBV-sensitive subclone cell lines, iOvCa147-F8 and -E2 (Fig. 3.3c), was sufficient to significantly decrease MRBV entry (Fig. 3.3d, Suppl. Fig. 3.2). An inhibitor of LDL-related receptors, RAP, was sufficient to impact MRBV entry in iOvCa147-E2 clones but not F8 (Fig. 3.3d). Thus, we suspect that LDLR is the primary mediator controlling virus entry over its related family members.

Given that EOC cells require sufficient LDLR expression to mediate efficient MRBV entry, we sought to determine whether endogenous LDLR expression was sufficient to predict HGSOC cell susceptibility to MRBV oncolysis. We assessed LDLR expression in the four independent isolates, iOvCa105, iOvCa131, iOvCa142, and iOvCa147, and all seven subclone cell lines generated from iOvCa147, yet failed to observe a correlation between LDLR expression and sensitivity or resistance (Suppl. Fig. A3.3). This suggests that although LDLR regulates MRBV entry into HGSOC cells, its expression alone is not sufficient to predict sensitivity to MRBV oncolytic infection.

Secreted factors do not impact MRBV infection of sensitive or resistant HGSOC cells.

We next sought to determine whether MRBV-resistant HGSOC produce secreted factors that would reduce infection of sensitive cells. Since type I IFN response to MRBV infection can elicit a robust anti-viral response in normal cells, we investigated whether MRBV-resistant HGSOC cells possessed an intact IFN response. We performed quantitative RT-PCR analysis of *IFNB1* mRNA expression in sensitive iOvCa147-F8 cells, and two resistant subclones, iOvCa147-G4 and -B3, in response to MRBV infection. Neither MRBV-sensitive nor -resistant HGSOC subclone cell lines elicited a potent IFN

antiviral signaling response after MRBV infection, whereas the positive control A549 human lung adenocarcinoma cell line responded with a robust increase in IFNB1 expression (Fig 3.4a). Thus we sought to determine if any other secreted factors elicited an effect on cell sensitivity to MRBV. We performed treatments of iOvCa147-F8 and -G4 cell lines with conditioned media (reciprocal media swap; media change or no media change served as controls) immediately prior to MRBV infection. Forty-eight hours after infection, we observed that none of the conditions altered sensitivity or resistance to MRBV oncolytic infection (Fig 3.4b). Since extracellular factors other than IFNB1 may affect HGSOC cell sensitivity to MRBV infection, we performed conditioned media experiments as described above, but after MRBV infection. Again, we detected no differences in cell viability that would indicate transfer of resistance factors between iOvCa147-F8 and -G4 subclone cell lines (Fig 3.4c).

Direct contact in MRBV-sensitive and -resistant cell co-cultures can restore efficient oncolysis.

Lastly, we sought to determine whether or not direct interaction of MRBV-sensitive and -resistant cells within a heterogeneous tumour cell population might impact oncolytic efficacy. We predicted that efficient MRBV infection and oncolysis would be restored since the original iOvCa147 cell line was quite sensitive to MRBV oncolytic infection (Fig. 3.1b). To recapitulate various iterations of a heterogeneous tumour population, we co-cultured iOvCa147-F8 and iOvCa147-G4 cells at different ratios. Indeed, we observed increased sensitization to MRBV-mediated cell death at each co-culture ratio compared with the expected cell viability for each individual subclone cell line (Fig. 3.5a). In fact, where co-cultures consisted of an equal ratio of sensitive and resistant cells the viability

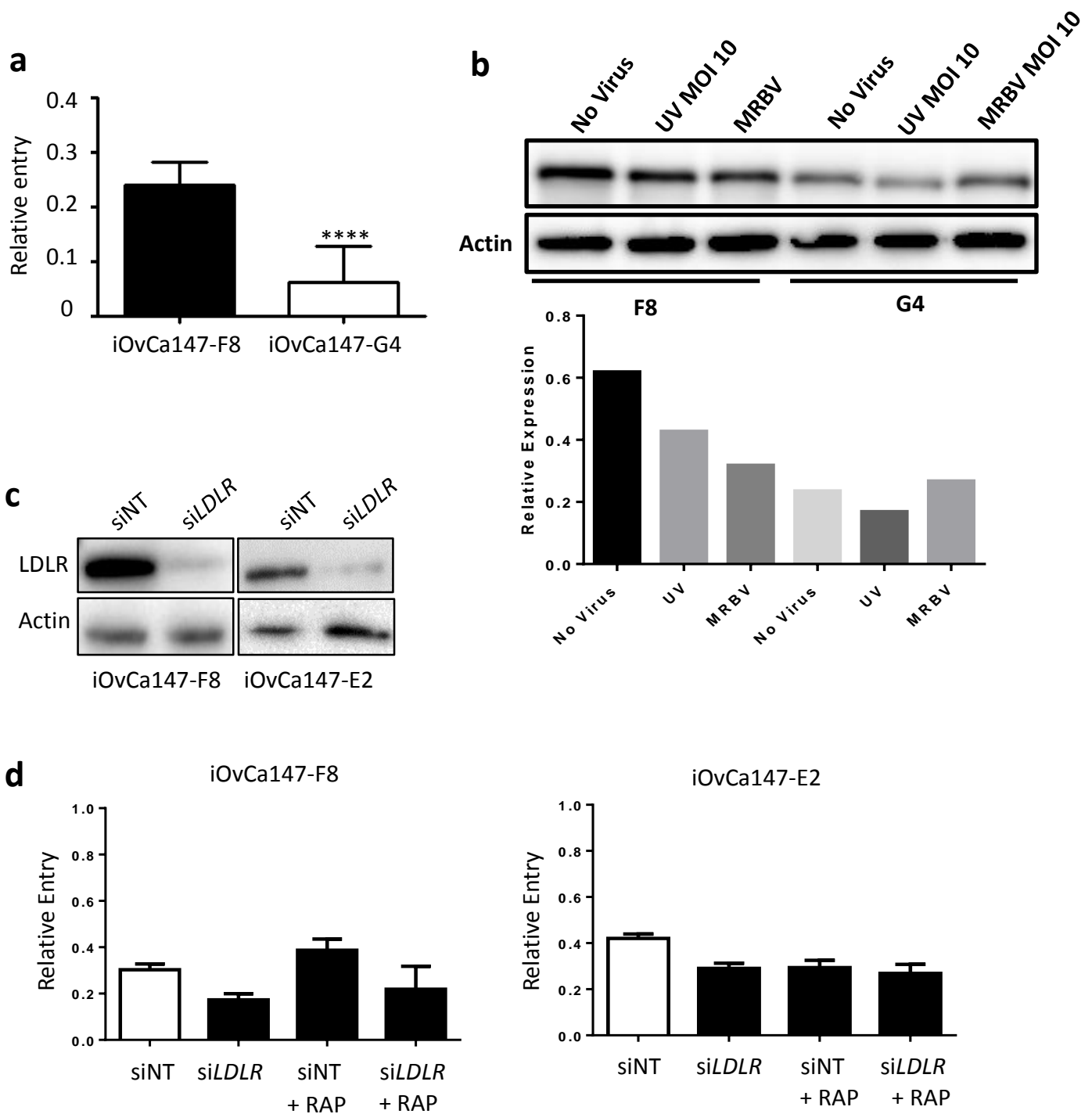


Figure 3.3. Differences in sensitivity between subclones are partially due to differences in LDLR expression and MRBV entry. a) F8 and G4 cells were seeded at 75,000 cells/ well of a 24 well plate. 24 h after seeding, cells were infected with MOI 1 MRBV. 1 h after infection, supernatants were collected and uninfected virus was titered on vero cells. Supernatants from a no-cell infection were titered as a control for total inoculated virus. b) Cells were seeded at 300,000 cells/well of a 6-well plate. Cells were infected with MRBV at MOI 1 and cells were harvested for protein at 20 h after infection. c) Cells were seeded at 20,000 cells/well of a 48-well plate. Cells were transfected for with siNT or siLDLR. 48hrs after transfection, wells were harvested for lysates; and unharvested cells were d) treated with 100nM of RAP for 1hr then infected with MRBV for 1hr. Media was collected for titring of uninfected virus. No-cell infections were performed as controls. Virus was titered on vero cells via plaque assay.

was reduced to the level routinely achieved with the MRBV-sensitive iOvCa147-F8 cells alone. Individual fluorescence labeling of iOvCa147-F8 cells (DiI) and iOvCa147-G4 cells (CMAC) prior to co-culture and infection (MRBV expressing GFP) confirmed the increased presence of MRBV-infected iOvCa147-G4 cells (both CMAC and GFP positive) when cultured with infected iOvCa147-F8 cells (DiI and GFP positive) (Fig. 3.5b). Indeed, quantification of co-stained GFP and CMAC cells revealed a 9 fold increase in the number of infected iOvCa147-G4 cells when co-cultured at an equal ratio with iOvCa147-F8 cells when compared to MRBV infection of iOvCa147-G4 cells alone (Fig. 3.5c). These observations were further validated when iOvCa147-F8 cells were co-cultured with iOvCa147-G4 cells, but physically separated by a porous Transwell membrane to allow transfer of secreted factors and virus during active infection (Fig. 3.4b and c). We observed no enhanced sensitivity of iOvCa147-G4 cells when cells were cultured separately from the iOvCa14-F8 cells confirming the need for physical contact between sensitive and resistant cells to mediate sensitization (Fig. 3.5d). These findings support MRBV as an effective therapeutic agent to infect and kill HGSOC cells throughout a heterogeneous tumour, as long as sufficient subpopulations of sensitive cells are present to support oncolytic replicative infection.

3.3 Discussion

There is currently a dire and unmet need for therapeutic alternatives for the treatment of metastatic EOC due to the high rate of therapeutic resistance. This is largely a result of genomic instability driving disease heterogeneity. We previously showed MRBV to be a potent oncolytic in EOC cells in both adherent and spheroid culture, thus we sought to evaluate the impact of disease heterogeneity on MRBV oncolysis. Using 4

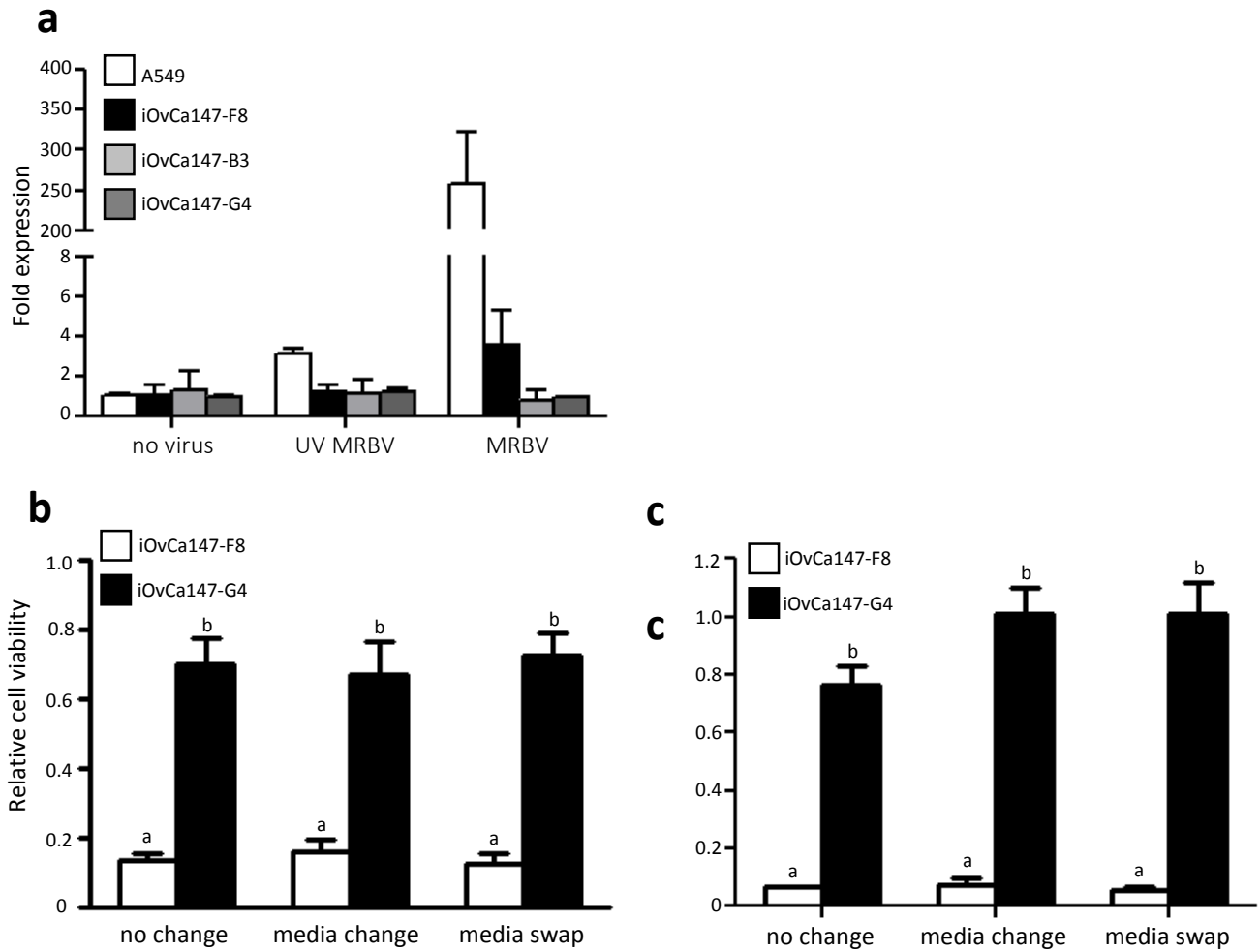


Figure 3.4. Extracellular factors do not confer sensitivity to MRBV a) Cells were seeded at 500,000 cells/well of a 6-well plate. The next day, cells were infected for 6 h with MRBV MOI 1 or UV MOI 1. RNA was isolated and qRT-PCR was performed using human-specific *IFNB1* primers and SYBR Green detection. b) Cells were seeded at 10,000 cells/well of a 96-well plate. 16 h later, fresh media was either replaced, swapped, or unchanged between F8 and G4 subclone cells. Cells were then infected with MRBV at MOI 0.1 for 1 h followed by media change. CellTiter-Glo® assays were performed for cell viability 48 h after infection. c) Cells were seeded at 10,000 cells/well of a 96-well plate. 16 h later, cells were infected with MRBV at MOI 0.1 for 1 h followed by media change. At 12 h, fresh media was either replaced or media was swapped between F8 and G4 subclone cells. CellTiter-Glo® assays were performed for cell viability 48 h after infection

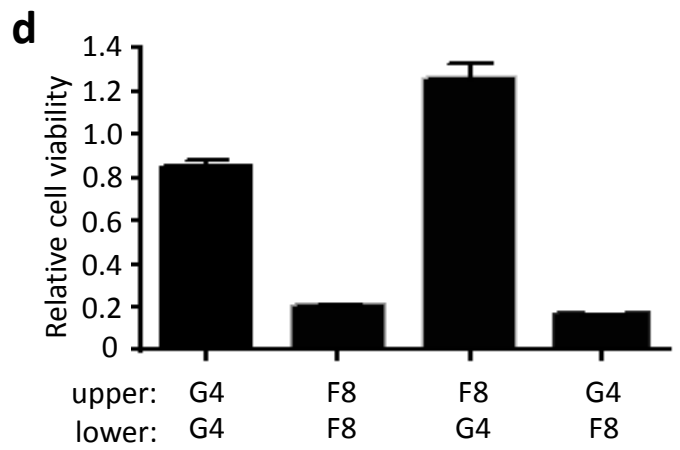
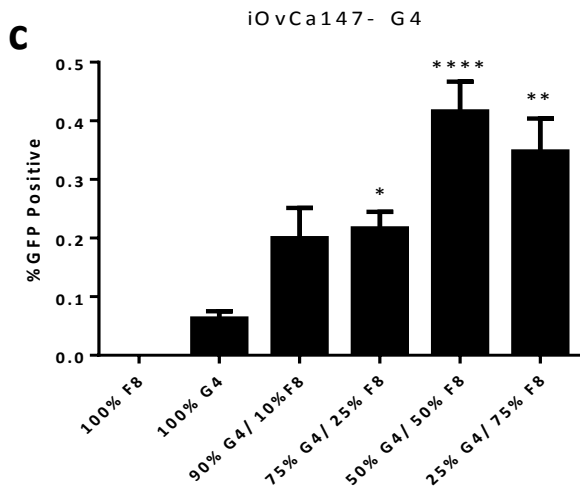
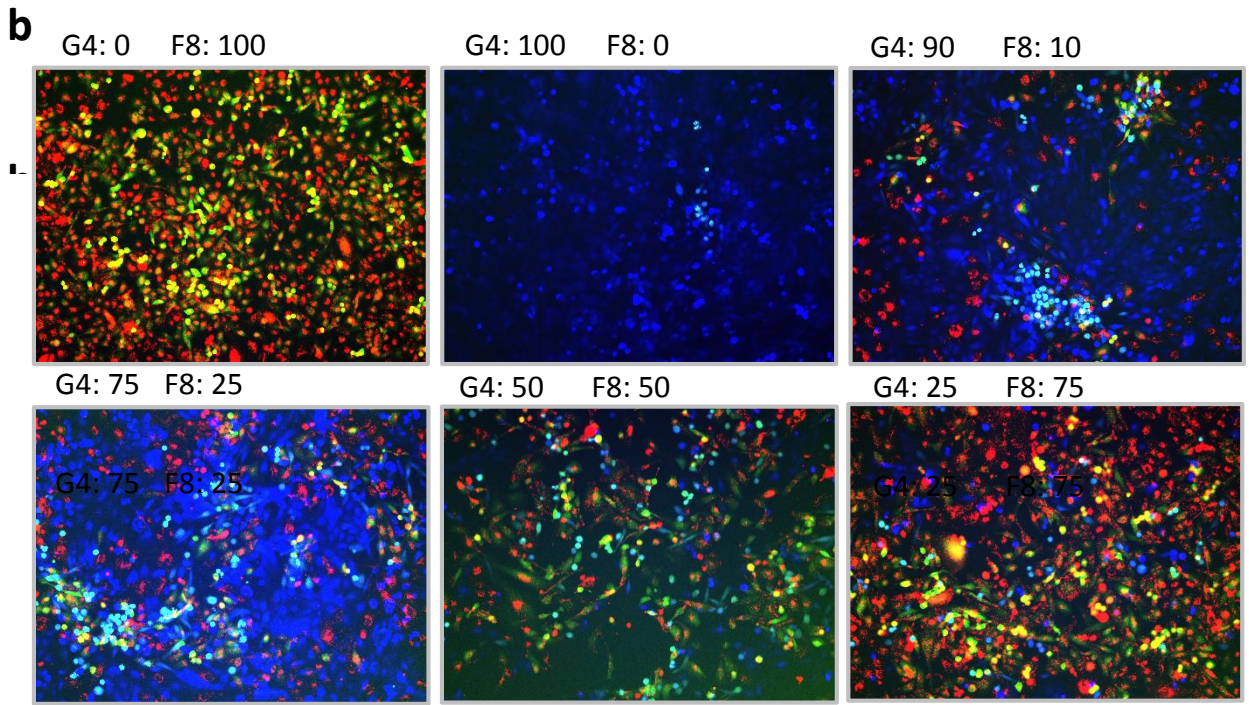
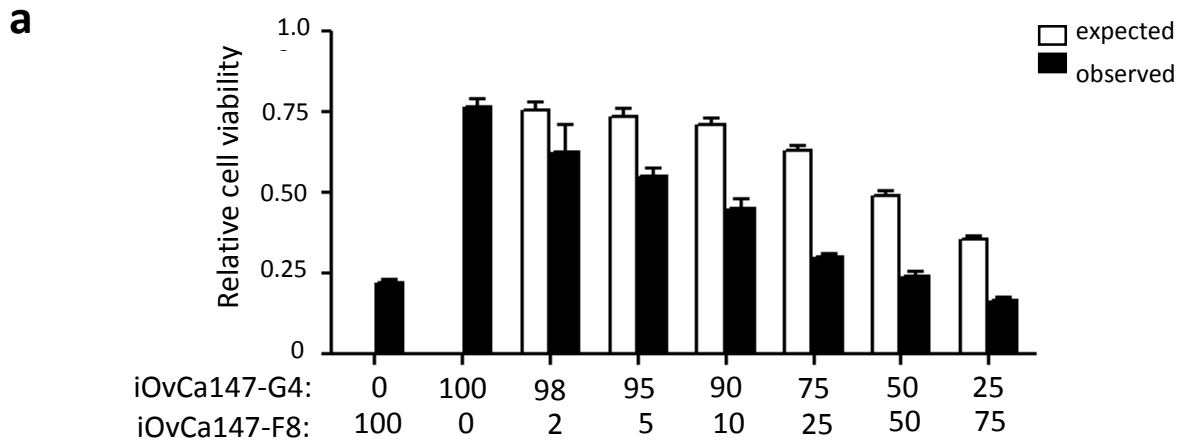


Figure 3.5. MRBV sensitivity can be conferred to resistant cells through co-culture and cell-cell contact. a) F8 and G4 cells were seeded to a total of 100,000 cells/well of a 24well plate. No virus controls of each cell mixture were used as controls to determine relative viability. Viability was assessed 48hrs after infection using CellTiter-Glo®. b) Fluorescence images of co-cultured cells F8 (red) and G4 (blue) were taken 16 h after infection with MRBV MOI 0.1. Infected F8 cells appear yellow. Infected G4 cells appear turquoise. c) Infected G4 cells were counted for each co-culture concentration and normalized to the total number of G4 cells to determine percent infectivity. d) Physical separation of cells was achieved through culturing cells in 0.4uM pore transwell plates. Media was shared between both top and bottom chambers. 100,000 cells total were seeded in a 24-well transwell plate. 25,000 cells were seeded in the top chamber while 75,000 cells were seeded in the bottom chambers. 24 h after seeding, media was changed and cells were cultured and infected with 2.5ml media containing 50,000 vpu (MOI 0.5) of MRBV. 48 h after infection, viability of cells in the bottom chamber was measured using CellTiter-Glo®.

ascites-derived primary EOC cell lines, which were isolated directly from the same patient, we observed differential oncolytic efficacies for MRBV in EOC from a temporal perspective. Likewise, clones generated from one of these heterogeneous samples were differentially sensitive to MRBV oncolysis despite having been cloned from a sample that is largely sensitive to MRBV. These findings highlight the potential impact that disease heterogeneity can have on therapeutic efficacy particularly in disease models in which genomic instability is an outstanding feature. Our data represent the first evidence to highlight the impact of both temporal and spatial heterogeneity in EOC on oncolytic virus therapy. Given our results, we propose that *in vitro* testing of therapeutics should consider using cell culture systems which recapitulate the disease heterogeneity found in EOC from a temporal and spatial perspective. These findings not only imply that disease heterogeneity can impact MRBV, but also show that in a largely sensitive heterogeneous tumour, there can exist resistant cells which could potentially be selected for to give rise to MRBV resistant tumours after treatment.

Previously, we have reported that expression of LDLR could mediate the entry of MRBV in target cells [15]. Although we further validated that this receptor is in fact important for mediating MRBV entry, we found that LDLR expression across a heterogeneous tumour did not solely dictate cell sensitivity to MRBV. We found that we could sensitize G4 cells to MRBV mediated oncolysis when co-cultured with as little as 10% F8 cells and achieve oncolytic effects of 100% infected F8 cells when as little as 25% of the co-culture is made up of sensitive F8 cells. As physical contact between these cell populations is needed to facilitate this sensitization, we speculate that perhaps the formation of a virological synapse to allow direct cell-cell transmission could permit

MRBV infection of resistant cell populations. Extensive studies with HIV have shown more efficient virus transmission through direct cell-cell contact than cell-free virus spread [18]. This occurs when virus assembly components polarize at the site of the synaptic junction allowing for direct transfer of mature virus to an adjacent cell rather than having large excesses of cell-free virus [19, 20]. This is true for other viruses including vesicular stomatitis virus (VSV) which can spread directly between cells [21]. In fact, intravenously injected VSV is rarely observed as cell-free particles *in vivo* and are normally rapidly sequestered by cells (either by infection or adhesion) [22]. Surface attachment of VSV has been shown to modulate infectivity by augmenting viral passage between cells. VSV-G (glycoprotein) pseudotyped HIV-derived particles exhibit prolonged retention on the cell surface which mediate direct cell-cell transfer of virus and increase infectivity by 20-130 fold [23]. Therefore we speculate that MRBV, which has greater than 80% sequence homology with the VSV glycoprotein and was initially thought to be a vesiculovirus may be transmitted in a similar fashion between sensitive and resistant clones [24, 25]. These findings imply that perhaps only a subpopulation of cells need to be initially infected to obtain oncolysis of an entire heterogeneous tumour.

Moreover, our findings support the notion that MRBV may be more effective and broad acting than other precision therapies since its infectivity is not entirely dependent on the expression of a single tropic factor, such as LDLR expression. Likewise, the capacity for resistance to MRBV treatment in heterogeneous cell populations can arise independently of LDLR expression as observed in the iOvCa 131 and 142 patient samples. This suggests that these heterogeneous populations initially have too few sensitive cells permissive to infection to facilitate sensitization of resistance cells and that the resistance

of the overall tumour may be insurmountable. Alternatively, it could be that other undefined intracellular factors affect productive virus replication. Ilkow et al. recently reported that fibroblasts could be sensitized to VSV killing through pretreatment with TGF β . This led to production of fibroblast growth factor 2 (FGF2) and subsequent reduction in retinoic acid-inducible gene I (RIG-I) which is responsible for virus detection, IFN production, and induction of innate antiviral responses against RNA viruses [26]. It is possible that upregulated signaling within the TGF β pathway, which is commonly observed in ovarian cancer, may promote or sensitize certain EOC clones to MRBV oncolysis. It would be interesting to determine what, if any, impact endogenous TGF β signaling during EMT in ovarian cancer metastasis would have on MRBV oncolysis. This approach could uncover novel strategies to better exploit MRBV for broader therapeutic use in treating EOC over the course of disease progression and in preventing disease resistance to MRBV therapy. Given our results, we propose that perhaps the use of cell carrier systems for MRBV may be more efficient in transmitting MRBV to resistant cells in heterogeneous populations via direct cell-cell contact rather than cell-free spread [22].

3.4 Materials and methods

Cell Culture

Ascites fluid obtained from ovarian cancer patients at the time of debulking surgery or paracentesis was used to generate primary cell cultures as described previously [27]. iOvCa-105, 131, 142, and iOvCa147 lines were isolated after removal of non-cancer cells by differential trypsinization from the mixed ascites-derived cultures from the same patient. Histopathological assessment concluded that this patient's ovaries displayed a mixed morphology tumour consisting of 70% serous and 30% clear cell adenocarcinoma.

She presented with metastasized stage IIIC disease. The iOvCa147 line was used in a limiting dilution series with each well of two 96-well cluster dishes seeded with 0.3 cells/well. Clonal lines isolated in this manner were subjected to STR analysis, which verified that they originated from the iOvCa147 line. STR analysis confirmed that all four lines were derived from the same patient. The resulting lines were cultured in Dulbecco's Modified Eagle medium/ F12 (Wisent) supplemented with 10% FBS (Wisent). Cells were grown in a 37°C humidified atmosphere of 95% air and 5% CO₂. Adherent cells were maintained on tissue culture treated polystyrene (Sarstedt, Newton, NC). Non adherent cells and spheroids were maintained on Ultra-Low Attachment (ULA®) cultureware (Corning, Corning, NY), which is coated with a hydrophilic, neutrally charged hydrogel to prevent cell attachment. All patient-derived cells were used in accordance with institutional human research ethics board of approval (UWO HSREB 12668E).

Generation of clones

Clones were generated through limiting dilution of iOvCa147 cells in a 96-well plate. Cells were seeded at 1 cell/well and cultured in DMEM/F12 (Wisent) supplemented with 10% FBS. Cells were expanded to generate all 7 clones used, which included - B3, C8, E2, F5, F8, G4, and G7.

Virus production

Vero cells were infected with MRBV at MOI 0.01. Twenty hours after infection, supernatant was collected and virus was purified using a 0.2 micron filter. The MRBV MG1 mutant strain used in these experiments has been described previously.

Virus infection of EOC cells

Primary EOC cells were seeded at 10,000 cells/well of a 96-well plate and were infected the following day at MOIs of 0.001, 0.01, 0.1, 1, or 10. The appropriate UV-inactivated virus at an MOI of 10 or no virus (mock infected) was used as controls. Seventy-two hours after infection, viability was assayed using the CellTiter-Glo® Luminescent Cell Viability Assay, which is based on quantitation of ATP levels (Promega, Madison, WI). For infection of EOC spheroids, cells were seeded at 50,000 cells/well of a 24-well ULA cluster plate (Corning, Corning, NY) and spheroids were allowed to form over 72 h. Spheroids were then infected at MOIs of 0.01, 0.1, 1, or 10 using the same controls as described for adherent cell infections. Phase contrast and fluorescent images of infected cells and spheroids were captured during each experiment using a Leica DMI 4000B inverted microscope.

Virus entry quantitation

iOvCa147-F8 and iOvCa147- G4 cells were infected with MRBV at an MOI 1 at 4°C to allow virus infection of cells. 1hr after infection, supernatants containing uninfected virus was removed and titrated on vero cells. Virus titers were determined through limiting dilutions of virus. Agarose overlay and plaque assay was performed to determine virus concentration. A no cell infection was performed as a negative control to determine virus concentration at 0% infection.

LDL receptor knockdown

iOvCa147-F8 and E2 cells were seeded in 48-well dishes and transfected 16 h after seeding with siLDLR sMARTPool RNA or with siNT nontargeting control RNA using

DharmaFECT1 transfection reagent (Dharmacon). At 48 h post-transfection, cells were used for infection experiments (virus entry and cell viability) as described above.

Media swapping experiments

Media swap after infection

Cells were seeded at 10,000 cells/well of a 96-well plate. Sixteen hours post seeding, cells were infected with MRBV at an MOI of 0.1 for 1 h followed by media change. At 12 h, fresh media was either replaced, swapped between iOvCa147- F8 and iOvCa147- G4 subclone cells, or were unchanged. CellTiter-Glo® assays were performed for cell viability 48 h after infection.

Media Swap before infection

Cells were seeded at 10,000 cells/well of a 96-well plate. Sixteen hours after seeding, conditioned media from both iOvCa147- F8 and iOvCa147- G4 was either replaced with fresh medium, swapped between the two clones, or remained unchanged. Cells were then infected at MOI 0.1 and viability was assessed 48 h after infection.

Quantitative RT-PCR

iOvCa147- F8, G4, and B3 clones were seeded at 500,000 cells / well of a 6-well plate. Sixteen hours post seeding, cells were infected with MRBV at an MOI of 1 or UV inactivated MRBV at an MOI of 1 for 6 h. The A549 lung cancer epithelial cell line was used as a positive control for an IFN response. Total RNA was isolated from both non-infected and infected clones and A549 cells using Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). Purified RNA was quantified using an ND-1000 spectrophotometer

(NanoDrop technologies, Wilmington, DE). Reverse transcription was performed using total RNA isolated and Superscript II reverse transcriptase (Invitrogen) as per manufacturer's instructions. PCR reactions were carried out using Brilliant SYBR Green QPCR Master Mix (Agilent Technologies/Stratagene) and a Stratagene Mx3000P machine with data exported to Microsoft Excel for analysis. *IFN β 1* and *GAPDH* primers were used and were previously described [28]. *GAPDH* served as an internal control for RNA input and quantification was performed using the $\Delta\Delta C_t$ method (Livak and Schmittgen 2001).

Co-culture experiments

Co-cultures of iOvCa147-F8 and G4 cells were seeded in 24-well plates at a total of 100,000 cells/ well. Wells of 100% iOvCa147- F8 and G4 cells were used as positive controls for MRBV effects on viability. An increasing proportion of iOvCa147- F8 cells were titrated into the G4 co-culture (G4:F8 ratio: 98:2, 90:10, 75:25, 50:50, and 25:75). Sixteen hours post seeding, cells were infected with MRBV at an MOI of 0.05 and viability was measured 48 h after infection using CellTiter-glo.

Stained co-culture images

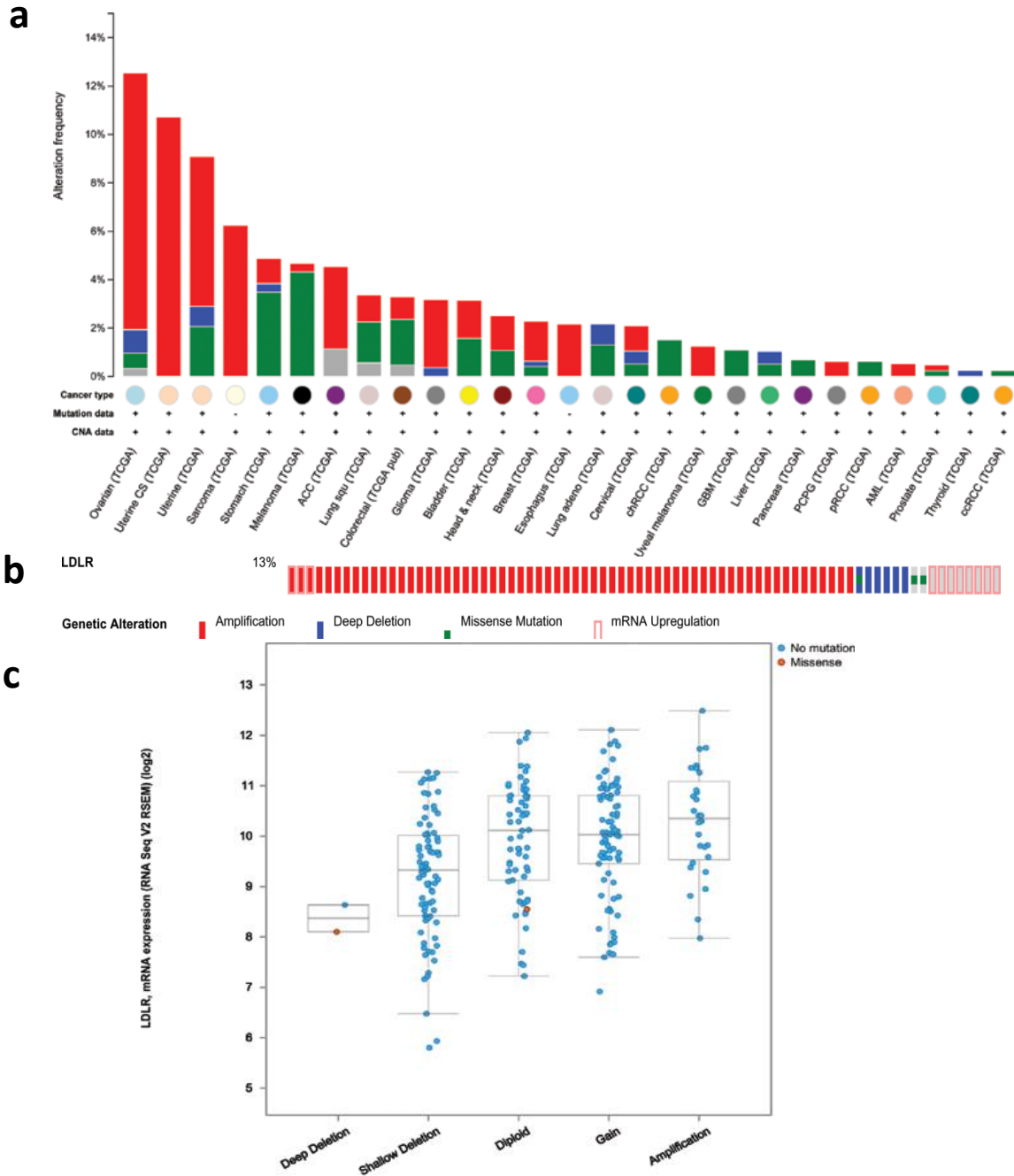
Confluent 10cm plates of iOvCa147-F8 cells were stained with Molecular Probes™ Lipophilic Tracer DiI at 1:500 (ThermoFisher Scientific, Waltham, MA). Confluent 10cm plates of iOvCa147-G4 cells were stained with CellTracker™ Blue CMAC Dye 1:500 (ThermoFisher Scientific, Waltham, MA). Cells were stained for 1 h. Subsequent seeding and infection of cells was performed as described above. Images were captured at 24hrs post infection using a Leica DMI 4000B inverted microscope.

Immunoblotting

Cell lysates were generated using a modified radioimmunoprecipitation assay (RIPA) buffer ((50mM HEPES pH 7.4, 150mM NaCl, 10% glycerol, 1.5mM MgCl₂, 1nM ethylene glycol tetraacetic acid, 1nM sodium orthovanadate, 10mM sodium pyrophosphate, 10mM sodium fluoride, 1% triton X 100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1mM phenylmethylsulfonyl fluoride, 1x protease inhibitor cocktail (Roche, Laval, QC)) as described previously [15]. Lysates were incubated on ice for 20 minutes and vortexed to ensure complete lysis. Protein concentrations were determined by Bradford assay using Protein Assay Dye Reagent (BioRad, Mississauga, ON). Thirty micrograms of lysates were run on an 8% sodium dodecyl sulphate-polyacrylamide electrophoresis gel and transferred to a polyvinylidene difluoride membrane (Roche, Mississauga, ON). Blots were blocked with 5% skim milk in Tris-bffered saline with Tween 20 (TBST; 10mM Tris-HCl, pH 8.0, 150mM NaCl, 0.1% Tween 20). After 1 hr of blocking, blots were incubated overnight on a rocking platform shaker at 4°C with specific antibodies at 1:1000 dilution in BSA/ TBST (anti-LDLR (Abcam, ab14056; Cambridge, MA); anti-actin (Sigma)). Blots were washed using TBST and incubated with peroxidase- conjugated anti-rabbit IgG (GE Healthcare) for 1hr at 1:10,000 dilution, in 5% skim milk/TBST for the LDLR antibody, or 5% BSA/TBST for actin at room temperature. Blots were washed again using TBST followed by incubation with Luminata Forte Western horseradish peroxidase substrate (Millipore, Etobicoke, ON) and visualized with the ChemiDoc MP System (BioRad, Mississauga, ON).

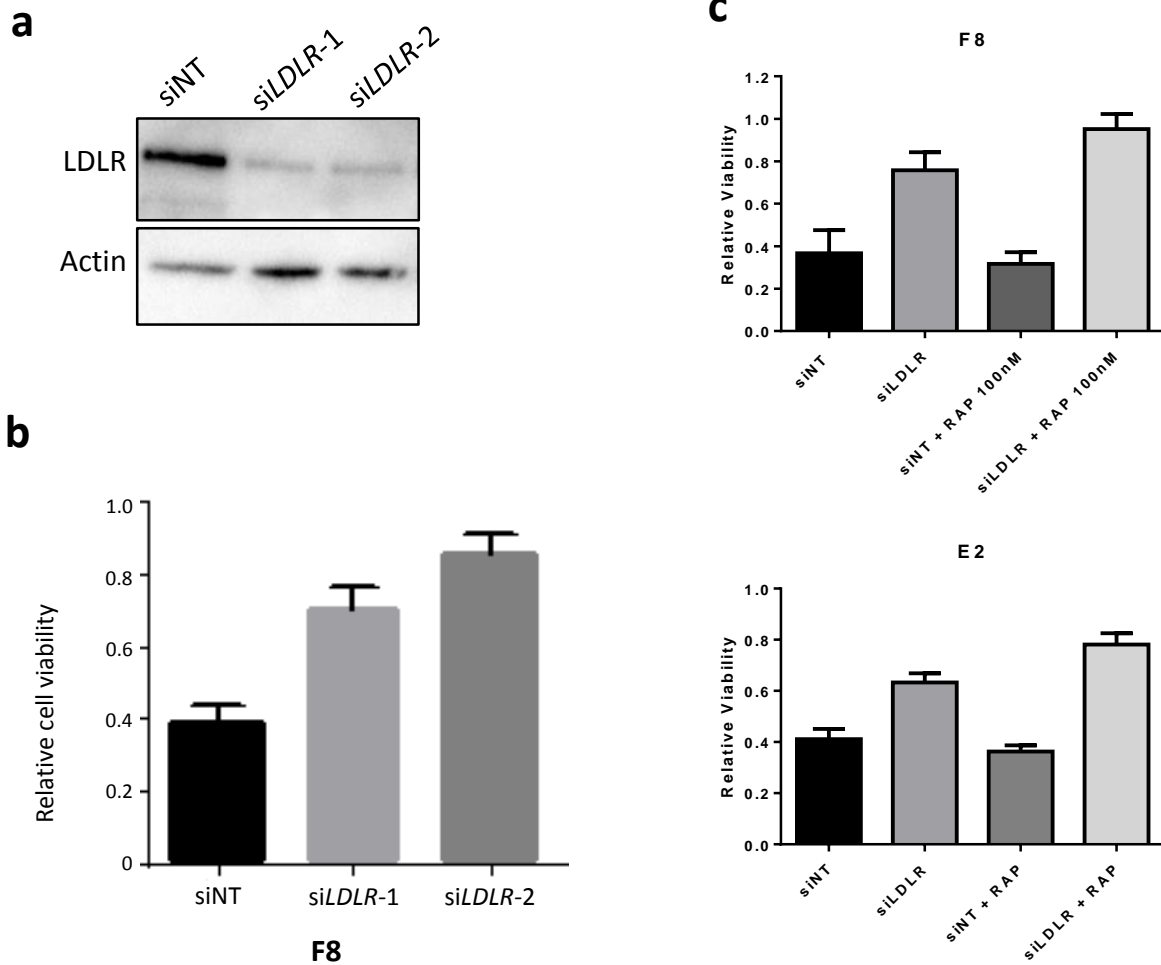
Statistical Analysis Statistical significance was determined by either unpaired two-tailed Student's t-test or one-way analysis of variance using GraphPad Prism 6 (GraphPad Software, San Diego, CA). Statistical Significance was set at $p < 0.05$.

3.5 Supplementary Information

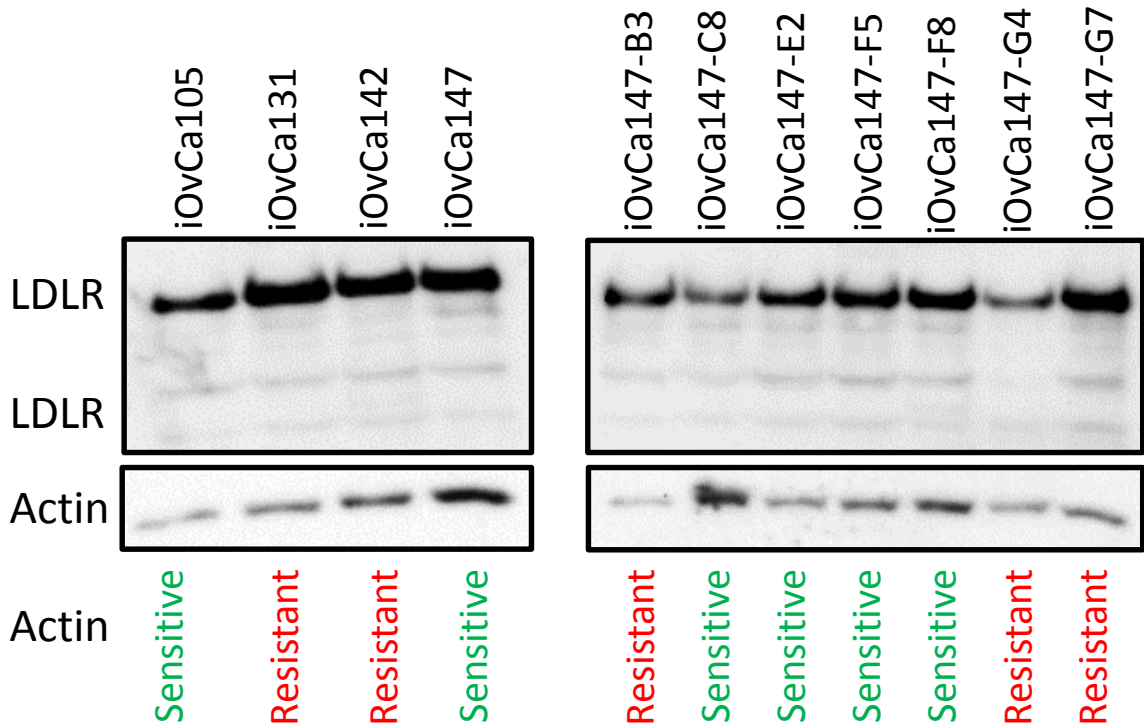


LDLR, Putative copy-number alterations from GISTIC

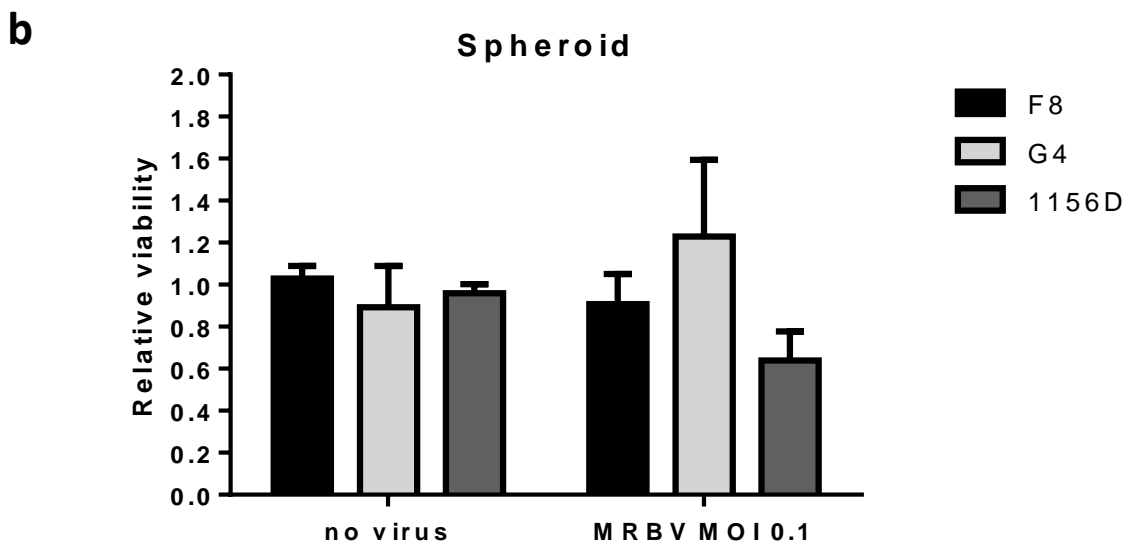
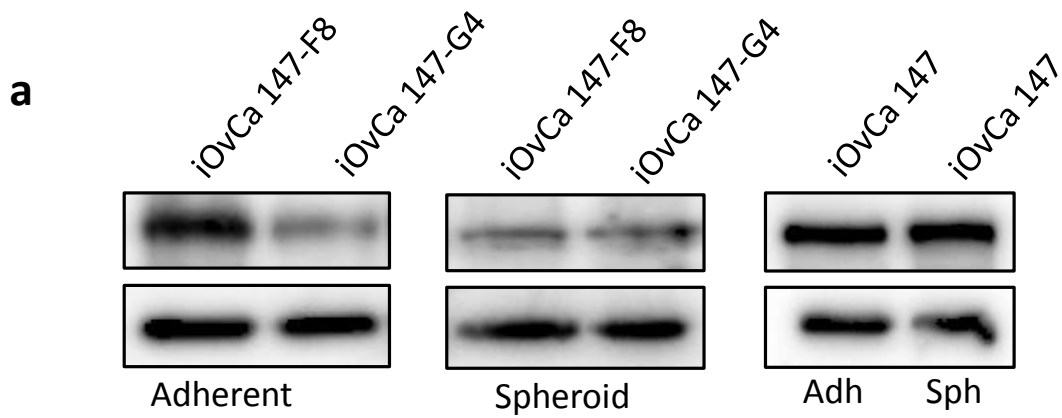
Supplementary Figure A3.1 The LDLR gene is altered in a significant fraction of ovarian serous cancers. (A) Mutation and gene copy-number status for *LDLR* across human cancers as determined used The Cancer Genome Atlas (TCGA) Provisional datasets accessed from cBioPortal as of November 12, 2015. Ovarian cancer (serous adenocarcinoma) has the highest rate of *LDLR* gene mutation and copy-number changes as compared with all other malignancies characterized to date. (B) Oncoprint of serous ovarian tumours harbouring *LDLR* mutations, copy-number changes, and gene expression changes (z-score > 2) from the TCGA Provisional dataset. Only tumour samples with alterations (13%; 78/599 samples) are displayed for clarity. (C) *LDLR* gene expression as compared with copy-number status among all serous ovarian tumours from the TCGA Provisional dataset.



Supplementary Figure A3.2 siLDLR smart pool knockdown and impact on clone viability- a) Individual siLDLR siRNA from the siLDLR smartPool. Cells were seeded at 20,000 cells/well of a 48 well dish. Cells were transfected with each siLDLR siRNA for 48 h. 4 wells were harvested for each protein lysate b) and remaining wells were infected with F8 MRBV MOI 0.05 for 48 h then viability was measured using CellTiter-Glo®. c) Cells were seeded and transfected as described in a). 48 h after transfection, 100nM of RAP was added for 30 minutes followed by infection with MRBV for 1h. Media was then removed. 48 h after infection, viability was measured by CellTiter-Glo®.



Supplementary Figure A3.3-LDLR expression does not consistently correlate with MRBV sensitivity across heterogeneous ascites cells or its subclonal populations. Cells were seeded at 500,000 cells/ well of a 6 well plate. Lysates were generated 24hrs after seeding. Parallel wells were used for infection to verify MRBV sensitivity.



Supplementary Figure A3.4- LDLR expression in adherent and spheroid iOvCa147 parental line and F8 and G4 clones. A) Cells were seeded at 500,000 cells/ well of 6-well plate and harvested for lysate 24 h after seeding. B) cells were seeded at 50,000 cells/ well of a 24-well ULA plate and spheroids were formed over 72 h. Spheroids were then infected with MRBV MOI 0.1 for 48 h and viability was assessed using CellTiter-Glo®

3.6 References

1. Howlander, N., Noone, A. M., Krapcho, M., Garshell, J., Miller, D., Altekruse, S. F., Kosary, C. L., Yu, M., Ruhl, J., Tatalovich, Z., Mariotto, A., Lewis, D. R., Chen, H. S., Feuer, E. J., Cronin, K. A. (2014). SEER Cancer Statistics Review, 1975-2011. (U. S. D. o. H. a. H. Services, Ed.). National Cancer Institute, National Institutes of Health.
2. Tan, D. S., et al. (2011) Genomic analysis reveals the molecular heterogeneity of ovarian clear cell carcinomas. *Clin Cancer Res* 17: 1521-1534.
3. Wang, Z. C., et al. (2012) Profiles of genomic instability in high-grade serous ovarian cancer predict treatment outcome. *Clin Cancer Res* 18: 5806-5815.
4. Lord, C. J., and Ashworth, A. (2012) The DNA damage response and cancer therapy. *Nature* 481: 287-294.
5. Coleman, R. L., Monk, B. J., Sood, A. K., and Herzog, T. J. (2013) Latest research and treatment of advanced-stage epithelial ovarian cancer. *Nature reviews* 10: 211-224.
6. Griffiths, C. T. (1975). Surgical resection of tumor bulk in the primary treatment of ovarian carcinoma. *National Cancer Institute monograph* 42: 101-104.
7. Hacker, N. F., Berek, J. S., Lagasse, L. D., Nieberg, R. K., and Elashoff, R. M. (1983). Primary cytoreductive surgery for epithelial ovarian cancer. *Obstetrics and gynecology* 61: 413-420.
8. Cooke, S. L., et al. (2010) Genomic analysis of genetic heterogeneity and evolution in high-grade serous ovarian carcinoma. *Oncogene* 29: 4905-4913.
9. Schwarz, R. F., et al. (2015) Spatial and temporal heterogeneity in high-grade serous ovarian cancer: a phylogenetic analysis. *PLoS medicine* 12: e1001789.
10. Martin, L. P., Hamilton, T. C., and Schilder, R. J. (2008). Platinum resistance: the role of DNA repair pathways. *Clin Cancer Res* 14: 1291-1295.
11. Critchley-Thorne, R. J., et al. (2009). Impaired interferon signaling is a common immune defect in human cancer. *Proceedings of the National Academy of Sciences of the United States of America* 106: 9010-9015.
12. Colamonici, O. R., Domanski, P., Plataniias, L. C., and Diaz, M. O. (1992). Correlation between interferon (IFN) alpha resistance and deletion of the IFN alpha/beta genes in acute leukemia cell lines suggests selection against the IFN system. *Blood* 80: 744-749.
13. Wong, L. H., et al. (1997). Interferon-resistant human melanoma cells are deficient in ISGF3 components, STAT1, STAT2, and p48-ISGF3gamma. *The Journal of biological chemistry* 272: 28779-28785.
14. Stojdl, D. F., et al. (2000). Exploiting tumor-specific defects in the interferon pathway with a previously unknown oncolytic virus. *Nature medicine* 6: 821-825.
15. Tong, J. G., et al. (2015) Evidence for differential viral oncolytic efficacy in an in vitro model of epithelial ovarian cancer metastasis. *Molecular Therapy* 23: 1501-1513.
16. Shepherd, T. G., Theriault, B. L., Campbell, E. J., and Nachtigal, M. W. (2006). Primary culture of ovarian surface epithelial cells and ascites-derived ovarian cancer cells from patients. *Nature protocols* 1: 2643-2649.
17. Grove, J., and Marsh, M. (2011) The cell biology of receptor-mediated virus entry. *The Journal of cell biology* 195: 1071-1082.

18. Jolly, C., Kashefi, K., Hollinshead, M., and Sattentau, Q. J. (2004). HIV-1 cell to cell transfer across an Env-induced, actin-dependent synapse. *The Journal of experimental medicine* 199: 283-293.
19. Chen, P., Hubner, W., Spinelli, M. A., and Chen, B. K. (2007). Predominant mode of human immunodeficiency virus transfer between T cells is mediated by sustained Env-dependent neutralization-resistant virological synapses. *Journal of virology* 81: 12582-12595.
20. Hubner, W., et al. (2009). Quantitative 3D video microscopy of HIV transfer across T cell virological synapses. *Science (New York, N.Y)* 323: 1743-1747.
21. Vassalli, J. D., Lombardi, T., Wohlwend, A., Montesano, R., and Orci, L. (1986). Direct cell-to-cell transmission of vesicular stomatitis virus. *Journal of cell science* 85: 125-131.
22. Willmon, C., Harrington, K., Kottke, T., Prestwich, R., Melcher, A., and Vile, R. (2009). Cell carriers for oncolytic viruses: Fed Ex for cancer therapy. *Mol Ther* 17: 1667-1676.
23. Skinner, A. M., Chakkaramakkil Verghese, S., and Kurre, P. (2013) Cell-cell transmission of VSV-G pseudotyped lentivector particles. *PloS one* 8: e74925.
24. Travassos da Rosa, A. P., Tesh, R. B., Travassos da Rosa, J. F., Herve, J. P., and Main, A. J., Jr. (1984). Carajas and Maraba viruses, two new vesiculoviruses isolated from phlebotomine sand flies in Brazil. *The American journal of tropical medicine and hygiene* 33: 999-1006.
25. Brun, J., et al. (2010) Identification of genetically modified Maraba virus as an oncolytic rhabdovirus. *Mol Ther* 18: 1440-1449.
26. Ilkow, C. S., et al. (2015) Reciprocal cellular cross-talk within the tumor microenvironment promotes oncolytic virus activity. *Nature medicine* 21: 530-536.
27. Correa, R. J., et al. (2012) Myxoma virus-mediated oncolysis of ascites-derived human ovarian cancer cells and spheroids is impacted by differential AKT activity. *Gynecologic oncology* 125: 441-450.
28. Fonseca, G. J., et al. (2012) Adenovirus evasion of interferon-mediated innate immunity by direct antagonism of a cellular histone posttranslational modification. *Cell host & microbe* 11: 597-606.

CHAPTER 4: Transforming growth factor-beta signaling mediated induction of epithelial-to-mesenchymal transition in ovarian cancer ascites-derived spheroid cells augments Maraba virus-mediated oncolysis

In preparation for submission to Cancer Biology & Therapy

Tong, J.G., Rafehi, S., Valdes, Y.R., Bertrand, M., McGee, J., Prefontaine, M., Sugimoto, A., Bell, J.C, Stojdl, D., DiMattia, G.E., Shepherd, T.G.

4.1 Introduction

Epithelial ovarian cancer (EOC) metastasis is unique amongst carcinomas in that its primary route is not via the bloodstream or lymphatics. Rather it occurs through the direct shedding of malignant cells from a primary tumour on the ovary into the peritoneal fluid (ascites) and subsequent dissemination of these cells throughout the peritoneum [1, 2]. During this process, shed cells are capable of forming multicellular aggregates, or spheroids, with the concurrent induction of epithelial-to-mesenchymal transition (EMT) [3]. EMT in ovarian cancer enhances metastasis by a general decrease in cell adhesion within the primary tumour yet promotes cell migration and invasion during secondary tumour formation. EMT is characterized by reduced epithelial features, including the loss of cell-cell junctions and apical-basal polarity, and acquired mesenchymal features, such as stress fiber formation and actin reorganization causing a spindle-shaped morphology [4, 5]. Loss of E-cadherin is considered fundamental to this process and can occur through the activation of a number of transcriptional repressor proteins, such as Snail, that actively repress expression from the *CDH1* gene encoding E-cadherin [1, 6-8]. Snail expression is robustly induced through endogenous transforming growth factor-beta (TGF β) signalling activation in ascites-derived cells to promote EMT during EOC spheroid formation [3, 9]. The activation of an EMT-like phenotype has been associated with greater chemotherapy resistance, cell motility, and cancer stem-cell characteristics; thus, EMT is considered to be reflective of more aggressive tumour behaviour[10]. Therefore, the development of

therapies which can better target EOC tumour cells which undergo EMT during tumour progression and metastasis is essential to improving clinical outcomes.

We have previously demonstrated that a derivative of Maraba virus (MG1, herein referred to as MRBV), is a potent inducer of oncolysis in EOC lines and that the low-density lipoprotein receptor (LDLR) can mediate efficient MRBV infection [11]. Thus, MRBV oncolysis is delayed and in some cases completely abrogated due to the down-regulation of LDLR expression in EOC spheroids [11]. However, LDLR expression was not the sole determining factor for susceptibility to MRBV infection since we failed to observe any correlation between LDLR expression and MRBV oncolysis (Tong et al, in preparation). Furthermore, we were able to increase sensitivity to MRBV independently from LDLR expression through co-culturing of sensitive and resistant cells. Therefore we speculate that intracellular signaling events downstream of virus entry could also influence MRBV replication. Ilkow et al., recently demonstrated that cancer associated fibroblasts could have enhanced sensitivity to a number of oncolytic viruses, including VSV, when pre-treated with TGF β [12]. This is due to an observable decrease in antiviral transcripts, including RIG-I, rendering cancer associated fibroblasts more susceptible to virus oncolysis. Since induction of EMT is a canonical response of epithelium-derived cells to TGF β , we sought to determine whether MRBV oncolytic activity is modulated by endogenous TGF β signalling in ovarian cancer ascites-derived spheroids during this robust EMT response.

4.2 Results

Spheroid formation of EOC ascites-derived cells promotes robust MRBV-mediated oncolysis

We have demonstrated previously that by treating several ovarian cancer cell lines with three different oncolytic viruses—Myxoma virus, double-deleted vaccinia and Maraba virus (MRBV)—the greatest oncolytic effect was observed using MRBV, yet it yielded less cancer cell killing in cells cultured as three-dimensional spheroids [11]. This finding was similar to our results using MYXV infection of primary cells isolated directly from the malignant ascites of ovarian cancer patients, where MYXV had impaired oncolytic efficacy in cultured spheroids [13]. To extend these studies, we sought to determine whether MRBV oncolytic potential would also be hindered in ovarian cancer ascites-derived spheroids. The MG1 strain of MRBV [14] was used to infect (MOI of 0.1) ascites cells isolated from nine independent ovarian cancer patients that were cultured as both adherent cells and spheroids. Surprisingly, we observed a significant reduction in cell viability in all nine ascites-derived cells due to MRBV-mediated oncolysis, but only when cultured as spheroids (Fig. 4.1a). This effect was in stark contrast to MRBV infection of adherent cells where minimal or no oncolysis was observed. Collectively, spheroids generated using EOC ascites-derived cells exhibited a nearly 4-fold greater degree of MRBV-mediated oncolysis in comparison with matched cells in monolayer culture (79.2% vs. 21.5% cell killing, respectively) (Fig. 4.1b). This MRBV oncolytic effect was readily evident with a decrease in overall spheroid integrity (Fig. 4.1c), which corresponded directly with our observed decrease in cell viability in these structures. This indicates that the majority of cells present in the malignant ascites of ovarian cancer patients can be infected effectively by MRBV, but may require one or more acquired properties of spheroids to render them susceptible to MRBV-mediated oncolysis.

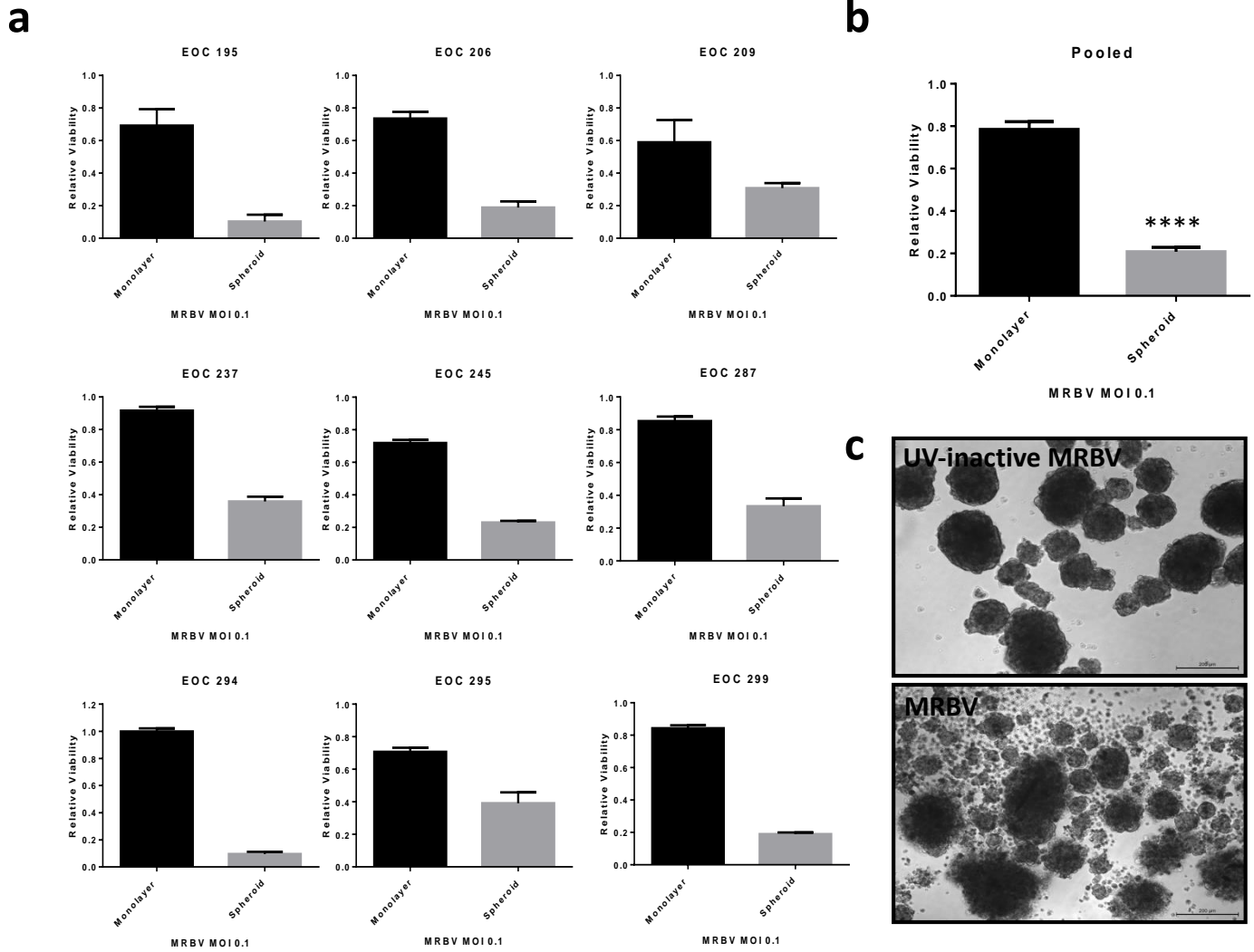


Figure 4.1. Spheroid formation sensitizes ascites-derived cells to MRBV oncolysis. a) Ascites-derived cells were used at passage 3. Cells were seeded at 50,000 cells/ well of a 24 well adherent plate or in ULA plate to form spheroids. Adherent cells were infected with either UV inactivated or MRBV at an MOI of 0.1 24 h after seeding. Spheroids were infected at the same concentrations 72 h after seeding. 48 h after infection, adherent and spheroid cells were assayed for viability using CellTiter-Glo® reagent. Relative viability was determined by normalizing MRBV infected cells to their own UV infected adherent or spheroid control. b) Individual patient samples assayed were pooled as biological replicates and t-test was performed to determine statistical significance between adherent and spheroid MRBV infected cells. c) Images of spheroids were taken 48 h after infection when viability was being assayed.

TGF β treatment sensitizes adherent ascites-derived cells to MRBV-induced oncolysis.

There are numerous alterations that can occur in ovarian cancer ascites-derived spheroids as compared with cells in monolayer culture, including the induction of epithelial-to-mesenchymal transition (EMT) due to endogenous activation of transforming growth factor-beta (TGF β) signaling in these structures [3]. The presence of TGF β signalling in ovarian cancer primary cell spheroids may directly impact MRBV infectivity since the presence of this pathway in tumour stroma has been recently implicated in modulating MRBV oncolysis in animal models [12]. To directly test whether TGF β signaling could sensitize ascites-derived monolayer cells to MRBV oncolysis, we pre-treated the cells with 5 ng/mL of TGF β for 48 h prior to infecting with MRBV at an MOI of 0.1. This is a time point at which we have observed robust transcriptional and translational changes in EMT-associated proteins and the resultant morphological changes characteristic of EMT [3]. In almost every patient-derived sample (9 out of 10), we observed enhanced MRBV-mediated oncolysis in TGF β -treated cells (Fig. 4.2a), with a significant reduction in overall cell viability (Fig. 4.2b). We verified TGF β induction of EMT cell morphological changes, and readily observed a robust cytopathic effect due to MRBV infection in these co-treated cell samples (Fig. 4.2c).

TGF β signaling inhibition prevents MRBV oncolysis of EOC ascites-derived spheroids.

Inhibition of TGF β receptor signalling using small molecule inhibitors is an effective approach to block its downstream effects, including EMT [15]. Indeed, we have demonstrated that treatment of ascites-derived spheroid cells with the type I receptor inhibitor SB-431542 effectively blocks endogenous TGF β signalling and the induced EMT

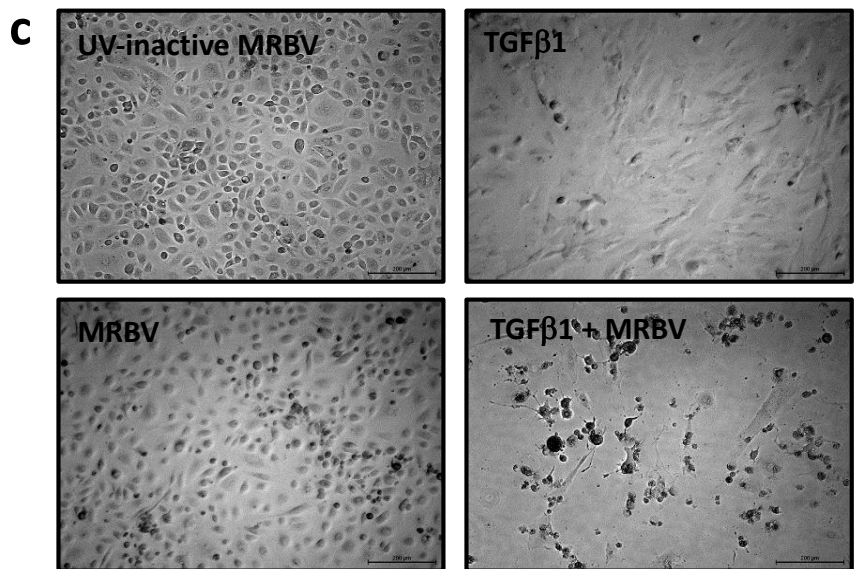
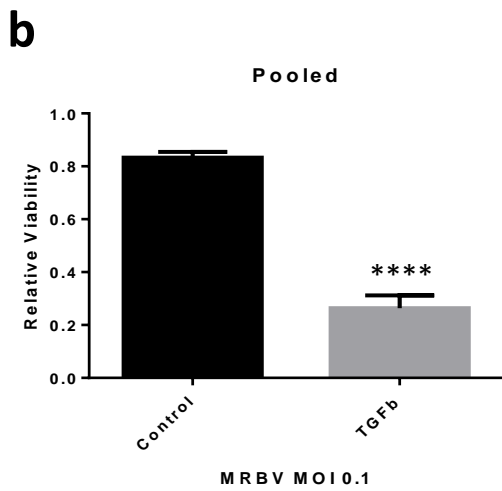
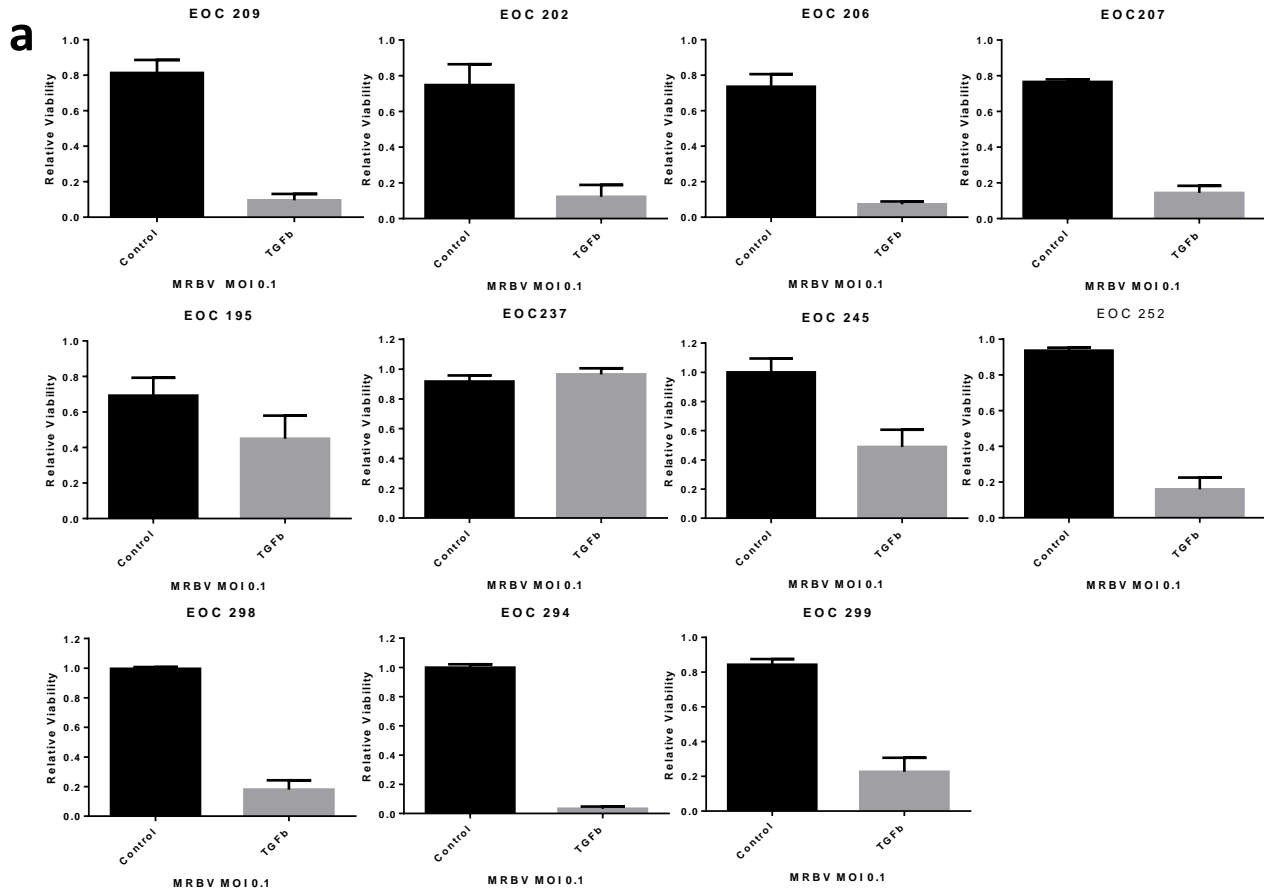


Figure 4.2. TGF β treatment sensitizes adherent ascites-derived cells to MRBV oncolysis. a) Ascites-derived cells were seeded at 50,000 cells/ well of a 24 well plate. 24 h after seeding, cell were Treated with 10nM of TGF β or DMSO as a control. Forty-eight hours after treatment, cells were then infected with MRBV or UV inactivated MRBV at an MOI of 0.1 for 48 h after which cell viability was assayed using CellTiter-Glo®. Relative viability was determined by normalizing TGF β treated cells to the DMSO control. b) Individual assays from different patient samples were pooled as biological replicates and statistical significance was determined by student's t-test. C) Images were captured at 48h after infection when cell viability was being performed.

phenotype in these structures [3]. To determine whether the sensitization of EOC ascites-derived spheroids to MRBV oncolysis is due to endogenous TGF β signalling, we treated with 5 μ M SB-431542 at the time of seeding cells to form spheroids followed by MRBV infection (MOI of 0.1) at 72 h. In all four patient samples tested, we observed a complete blockade in the ability of MRBV to achieve effective killing of EOC ascites-derived spheroids, as compared with infections of untreated cells (Fig. 4.3a). In line with previous findings, we observed significant effects on spheroid formation due to SB-431542 treatment alone with smaller and less dense clusters (Fig. 4.3b), but this has little effect on overall spheroid cell viability on its own (data not shown, and [3]). Thus, the enhanced oncolytic effect due to MRBV infection that we observed in EOC ascites-derived spheroids is largely controlled by endogenous TGF β receptor signalling.

Enhanced MRBV oncolysis is not a result of enhanced virus entry, changes in LDL receptor expression, or changes in IFN- β 1 antiviral responses.

Since MRBV is highly related to vesicular stomatitis virus [16-19], it utilizes the low density lipoprotein receptor as its primary means for binding and entering host cells [11]. In fact, we have evidence that this is at least one mechanism by which MRBV may have differential ability to gain access to ovarian cancer cells for efficient oncolysis [11] (and Tong et al, in preparation). To address this further in the current study, we first determined whether activated TGF β signalling influences MRBV entry of ascites-derived cells. Direct MRBV entry assays were performed in which we infected adherent cells pre-treated with 5 ng/mL TGF β 1 ligand and compared them to untreated controls. After only 1 h of MRBV infection, we removed culture supernatant and titrated virus that remained unbound and available for infection using Vero cells. We observed no significant

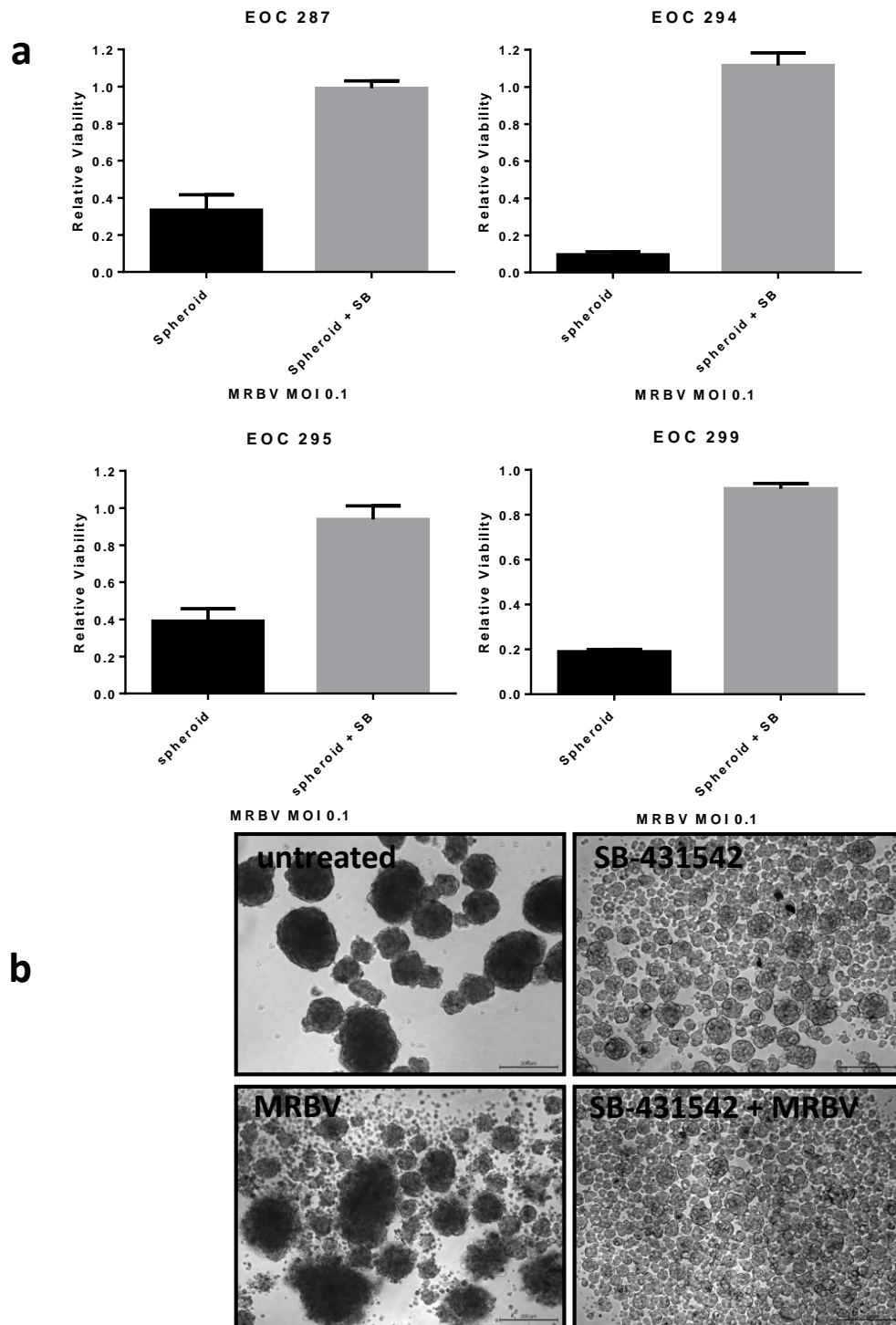


Figure 4.3. Inhibition of signaling through TGF- β type I receptor in spheroids prevents sensitization to MRBV oncolysis. a) EOC ascites-derived cells were seeded at 50,000 cells/well ULA plate. At the time of seeding, 5nM of the TGF β inhibitor, SB-431542, was used to treat cells or DMSO as control. Seventy-two hours after seeding, spheroids were infected with MRBV or UV inactivated MRBV at MOI 0.1 for 48 h after which viability was assayed using CellTiter-Glo®. b) Images of treated spheroids were taken 48 h after infection at the time viability was being assessed.

differences between TGF β -treated and untreated samples (Fig. 4.4a), thus strongly suggesting that activated TGF β signaling did not increase MRBV oncolytic efficacy via enhanced virus entry. In addition, we did not observe either increased LDLR expression in TGF β -treated monolayer cells or decreased LDLR expression in SB-431542-treated spheroids (Fig. 4.4b).

A common mechanism by which cells can potently block MRBV infection is by the induction of a robust type I interferon response, a hallmark of which is the rapid expression of IFN β 1 from infected cells [14]. However, we observed no significant differences in *IFNB1* mRNA expression among TGF β -treated monolayer cells and SB-431542-treated spheroids and their respective controls after 8 h of MRBV infection (Fig. 4.4c); we confirmed a robust induction of *IFNB1* expression in A549 cells, which are known to elicit a type I interferon response to virus infection [20]. Although anti-viral responses may be regulated by TGF β signalling in some tumour contexts to affect MRBV infectivity [12], our results imply that this is not likely to be the mechanism utilized by ovarian cancer ascites-derived spheroids.

TGF β -induced EMT via Snail is required for efficient MRBV oncolysis of ovarian cancer ascites-derived spheroids.

Both the endogenous TGF β signalling during spheroid formation and TGF β -treatment of monolayer ascites-derived cells induced an EMT response as defined by decreased E-cadherin and increased Snail expression (Fig. 4.5a), in line with our previous report [3]. Since Snail is the most consistently upregulated EMT product in both spheroids and TGF β -treated monolayer cells and is a key regulator of EMT induction in these cells

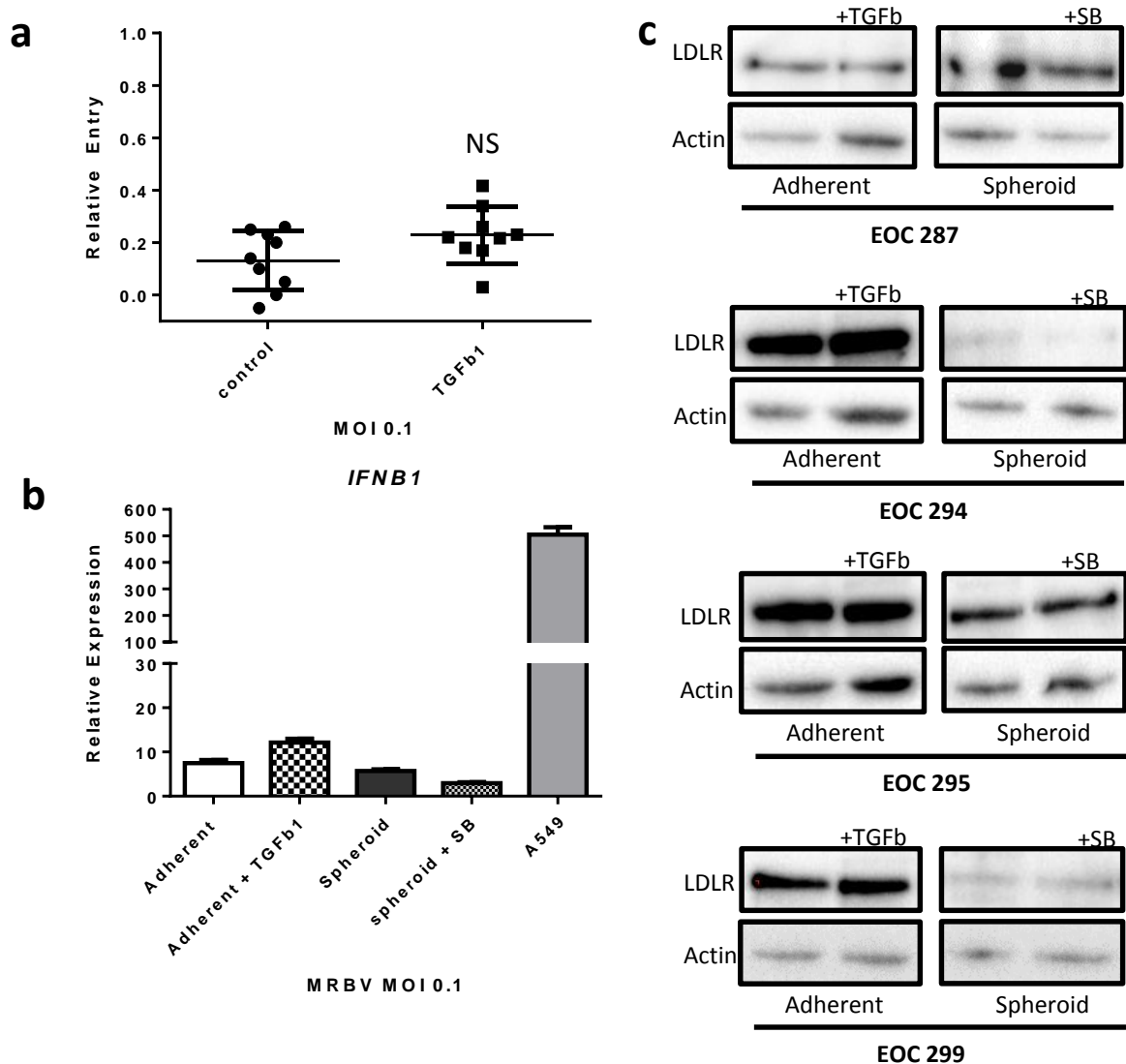


Figure 4.4. Sensitization to MRBV by TGF β treatment and spheroid formation is not due to increased virus entry, changes in LDLR expression, or IFN β signaling. a) Ascites-derived cells were seeded at 50,000 cells/well of a 24 well plate. Twenty-four hours later, cells were then treated with 10nM of TGF β or DMSO. Forty-eight hours after treatment, cells were then infected with MRBV at an MOI of 0.1 for 1 h. After 1 h, supernatant was collected with uninfected virus. No cell control infection was also performed as a negative control to mimic 0% infection. Unadsorbed virus was titrated via plaque assay on Vero cells. b) Cells were seeded at 50,000 cells/well of a 24 well ULA plate to form spheroids or in adherent culture to harvest for protein and RNA. SB treated spheroids were treated at the time of seeding. TGF β treated cells were treated 24 h after seeding. Forty-eight hours after treatment wells were harvested for protein. c) Unharvested wells were then infected with MRBV at an MOI of 0.1 or a no virus control. Eight hours after infection, cells were harvested for RNA and qPCR was performed for human IFN β 1. Infection of A549 cells in monolayer were used as a positive control for IFN β 1 response

(Rafehi & Shepherd, unpublished), we performed siRNA-mediated knockdown experiments of *SNAIL* in EOC ascites-derived spheroids. In three out of the four patient samples tested, we observed significant reduction of MRBV-mediated oncolysis as a result of *SNAIL* knockdown in spheroids (Fig. 4.5b). In fact, the sample that failed to elicit a Snail-dependent response via knockdown (EOC287) did not respond to TGF β 1 to induce Snail in monolayer cells, nor did it decrease Snail expression upon SB-431542 treatment of spheroids (Fig. 4.5a), perhaps indicating that this sample has uncoupled TGF β signalling regulation of Snail and EMT. Taken together, it is likely that the EMT phenotype as regulated by TGF β signalling in ovarian cancer ascites-derived spheroid cells is a chief regulator of MRBV oncolysis of cells found in these structures.

4.3 Discussion

The process of spheroid formation in EOC has been postulated to create important reservoirs for malignant EOC cells associated with increased metastasis in advanced stages of the disease [21-23]. We have previously shown that the induction of EMT through endogenous activation of TGF β signaling can increase spheroid migration and invasiveness and confer greater resistance to standard chemotherapies such as carboplatin [3]. We have identified a novel tropic factor of MRBV that promotes the oncolysis of cells which are upregulated in the specific factors that make EOC spheroids more aggressive, invasive, and chemoresistant.

Similarly to what has previously been observed, we found that endogenous TGF β signaling in EOC ascites-derived spheroids could dramatically sensitize EOC cells to

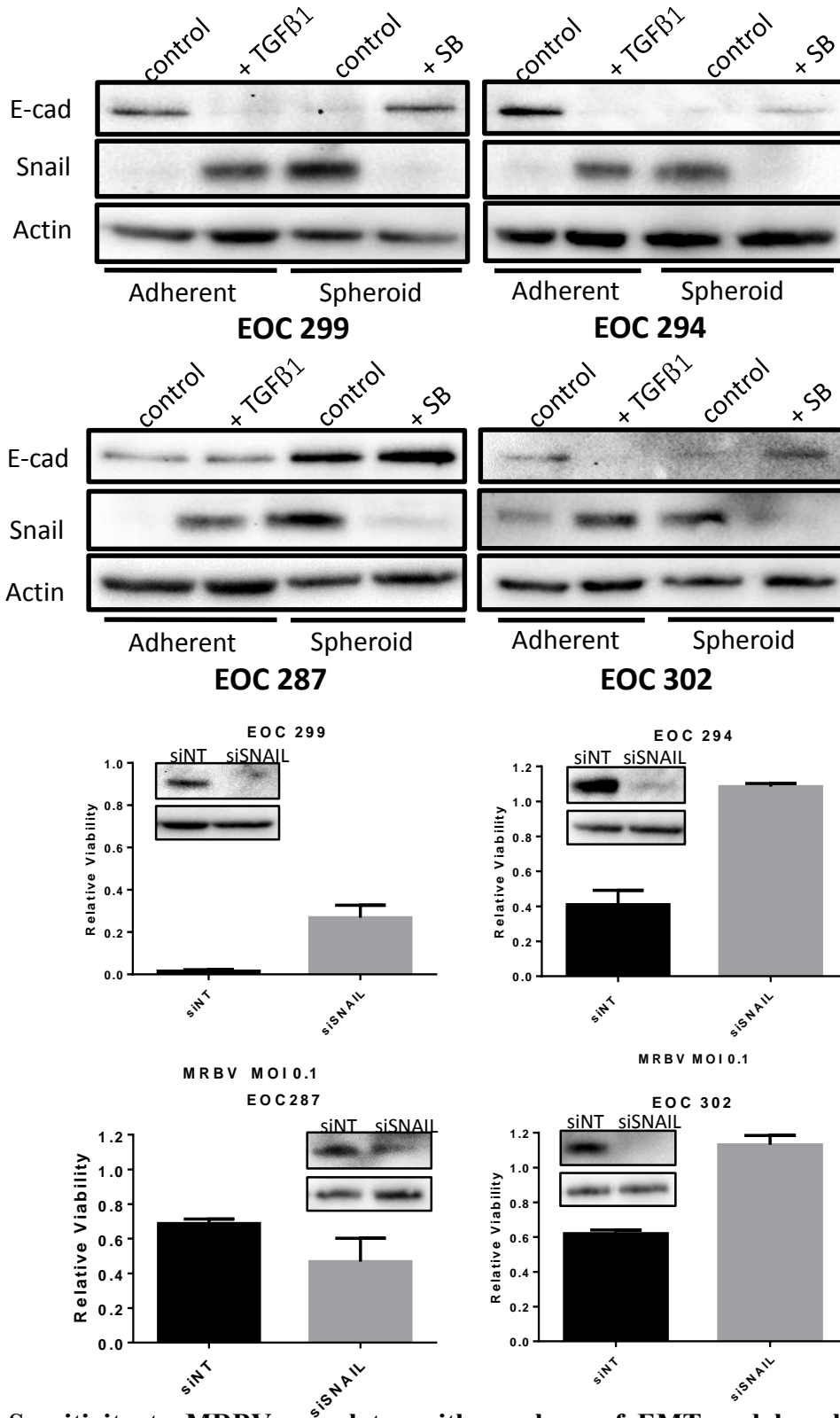


Figure 4.5 Sensitivity to MRBV correlates with markers of EMT and knockdown of Snail prevents ascites derived spheroid sensitization. a) Adherent and spheroid cells were seeded as previously described. Lysates were generated and probed with human E-cadherin, Snail, and Actin antibodies. b) Cells were seeded at 300,000 cells/ well of a 6 well plate and transfected with siSnail or non-targeting control. Seventy-two hours after transfection, cells were trypsinized and re-seeded at 50,000 cells/well of a 24 well ULA plate to form spheroids. Seventy-two hours later, cells were infected with MRBV at an MOI of 0.1 and viability was assessed 48 h after infection using CellTiter-Glo®

MRBV induced oncolysis [12]. However, unlike what was described by Ilkow et al., we did not observe significant differences in antiviral responses of ascites-derived cells to MRBV infection upon TGF β treatment or spheroid formation. Instead, we specifically identified that through TGF β type I receptor activation, the subsequent EMT response induced through SNAIL upregulation is capable of sensitizing EOC ascites cells to MRBV.

It is important to note that enhanced viral oncolysis of EOC spheroids is context dependent as previously we have documented that MRBV oncolysis is delayed or even abrogated in spheroids form from EOC cell lines [11]. Likewise, TGF β treatment of EOC cell lines does not result in sensitization to MRBV. It remains to be determined the precise biological differences between ascites-derived EOC cells and that of established cell lines which may be important in determining their sensitivity to MRBV. It is likely that the restricted ability for EOC cell lines to induce a complete EMT response with corresponding morphological changes and transcriptional activation of EMT associated genes prevents EOC cell lines from being sensitized to MRBV when cultured as spheroids or when treated with TGF β [24-26]. Decreased expression of SMAD4, which is an essential factor in EMT, has been described in several ovarian cancer cell lines which results in a loss of sensitivity to TGF β treatment and corresponding growth inhibition [27]. Furthermore, it is unlikely that the heterogeneous population of cells from the ascites are entirely cancerous, but instead likely represents a combination of cancerous cells, cancer associated fibroblasts, and normal cells and therefore may have differential responses to TGF β [28-30]. However, QPCR of IFN β 1 transcripts from MRBV infected EOC ascites derived cells shows little upregulation in IFN β 1 in response to infection suggesting a defect in initiating an antiviral response which is characteristic of abnormal cells. Aside from the direct oncolysis of

cancer cells, targeting cancer associated fibroblasts in the ascites would also be vital in limiting the aggressive metastatic behavior of EOC tumours [31]. Specifically, TGF β 's contributions to ovarian tumour invasiveness and motility are largely controlled by affecting the transcriptional profile of cancer associated fibroblasts in the tumour microenvironment [32].

Although previous studies have shown an association of EMT with greater sensitization of cancer cells, including ovarian, to oncolytic virus induced cell death by adenovirus and herpes virus, the mechanisms by which the viruses sensitize cancer cells are distinctly different. Both adenovirus and herpes virus show decreases in virus entry in epithelial cells due to virus receptors required for entry being trapped in tight-junctions. During EMT however, the mesenchymal phenotype promoted a loss of tight and adherens junctions, allowing more freely accessible receptors to increase virus entry [34, 35]. We observed no difference in entry of MRBV in patient samples which had been treated with TGF β in monolayer or SB-431542 in spheroids, nor increases in LDLR expression from adherent to spheroid culture conditions. Therefore we believe the sensitization to MRBV occurs intracellularly and is imparted by active TGF β signaling and EMT rather than increased entry to mesenchymal-like cells. TGF β ligands are present and active in malignant ascites from ovarian cancer patients [26]. We have demonstrated that active maintenance of endogenous TGF β signaling promotes EMT in spheroids and is required for efficient re-attachment and dispersion [8]. Furthermore, spheroids that have undergone EMT have been found to be enriched for cells with stem-like characteristics with increased resistance to apoptosis and contribute to more aggressive secondary tumour growth [10, 33]. Therefore, a heightened mesenchymal and stem-like phenotype of EOC tumors and

metastases could serve as important predictive biomarkers for MRBV to act as a potent therapeutic against the invasive and chemo-resistant cells.

The use of MRBV in combination with current therapeutic approaches may be advisable to best tackle advanced metastatic disease. Current therapeutic strategies centered on surgery and platinum based chemotherapy could be applied to enrich for cell populations which may have chemoresistant disease due to elevated TGF β signaling. This process could select for cell populations which may be best suited and better targeted by MRBV to promote oncolysis of the most aggressive tumour cell types which contribute to therapy resistant and recurrent disease.

4.4 Materials and methods

Cell Culture

Vero and A549 cells were cultured in Dulbecco's Modified Eagle Medium (Wisent) and supplemented with 5% fetal bovine serum (Wisent). Cell lines were cultured in a humidified environment at 37°C with 5% CO₂. Both cell lines were generously donated by Dr. Joseph Myrmyk.

Ascites fluid obtained from ovarian cancer patients at the time of debulking surgery or paracentesis was used to generate primary cell cultures as described previously [13]. All patient samples were cultured in Dulbecco's Modified Eagle medium/ F12 (Wisent) supplemented with 10% FBS (Wisent). Cells were grown in a 37°C humidified atmosphere of 95% air and 5% CO₂. Adherent cells were maintained on tissue culture treated polystyrene (Sarstedt, Newton, NC). Non-adherent cells and spheroids were maintained on Ultra-Low Attachment (ULA®) cultureware (Corning, Corning, NY), which is coated with

a hydrophilic, neutrally charged hydrogel to prevent cell attachment. All patient-derived cells were used in accordance with institutional human research ethics board of approval (UWO HSREB 12668E).

Virus production

MRBV MG1 was generated as previously described [14]. Vero cells were infected at MOI 0.01. Twenty hours after infection, supernatant was collected and virus was purified using a 0.2micron filter. MRBV MG1 mutant strain used in these experiments has been described previously [14].

Virus infection of EOC cells

Primary EOC cells were seeded at 50,000 cells/well of a 24-well plate and were infected the following day at MOI 0.1. Spheroids were formed using 50,000 cells/ well in a 24-well ULA plate (Corning, Corning, NY) over 72 h prior to infection. The appropriate UV-inactivated virus at MOI 0.1 or no virus (mock infected) was used as controls. Forty-eight hours after infection, viability was assayed using CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI). Phase contrast images of cells and spheroids were captured during each experiment using a Leica DMI 4000B inverted microscope.

TGF β signaling modulation

Recombinant TGF β 1 was purchased from Millipore (Temecula, CA), prepared in distilled water, and used at a concentration of 5 ng/mL [38]. Adherent cells were treated with recombinant human TGF β 1 24 h after seeding for 48 h to induce and EMT response. Images were captured using a Leica DMI 4000B inverted microscope prior to cells being harvested for analysis. The TGF β type I receptor small molecular inhibitor, SB-431542

(Sigma) was prepared in DMSO and used at a concentration of 5 μ M [39]. Spheroids were treated with SB-431542 or DMSO vehicle control at the time of seeding to ULA plates. Seventy-two hours after seeding and treatment, spheroids were imaged and harvested for analysis.

Cell Viability

Adherent culture

Cells were kept in their original 24-well plates and 75ul of CellTiter-Glo® reagent (Promega, Madison, WI) was added to each well and diluted at a 1:1 ratio with media as per manufacturer's instructions. Ten minutes after the addition of the diluted reagent, cell lysates were harvested and placed in white walled 96-well microplates and luminescence signal was detected using a Wallac 1420 Victor 2 Spectrophotometer (Perkin-Elmer, Waltham, MA). Results were normalized to vehicle treated adherent controls.

Spheroid culture

Spheroids were collected, pelleted, and left in 100ul of media. CellTiter-Glo® reagent was added at a 1:1 ratio with media as previously described and as per manufacturer's instructions. Samples were triturated using a 26 ½ guage needle until spheroids were no longer visible. Samples were then added to a white-walled 96 well microplate and luminescence was detected as described above and normalized to vehicle-treated control spheroids.

Quantitative RT-PCR

EOC ascites-derived cells cultured as both adherent and spheroids were harvested from 24-well plates seeded at 50,000 cells/well. The A549 lung cancer epithelial cell line was used as a positive control for an IFN- β 1 response. Total RNA was isolated from both non-

infected and infected clones and A549 cells using Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). Purified RNA was quantified using an ND-1000 spectrophotometer (NanoDrop technologies, Wilmington, DE). Reverse transcription was performed using total RNA isolated and Superscript II reverse transcriptase (Invitrogen) as per manufacturer's instructions. PCR reactions were carried out using Brilliant SYBR Green QPCR Master Mix (Agilent Technologies/Stratagene) and a Stratagene Mx3000P machine with data exported to Microsoft Excel for analysis. *IFN β 1* and *GAPDH* primers were used and were previously described [40]. *GAPDH* served as an internal control for RNA input and quantification was performed using the $\Delta\Delta$ Ct method [41].

Virus entry quantitation

iOvCa147-F8 and iOvCa147- G4 cells were infected with MRBV at an MOI of 1 at 4°C to allow virus infection of cells. One hour after infection, supernatants containing uninfected virus was removed and titrated on vero cells. Virus titers were determined through limiting dilutions of virus. Agarose overlay and plaque assay was performed to determine virus concentration. A no cell infection was performed as a negative control to determine virus concentration at 0% infection.

Immunoblotting

Adherent and spheroid cells were washed once in cold phosphate-buffered saline (PBS), dissolved in lysis buffer (50 mM HEPES pH7.4, 150 mM NaCl, 10% glycerol, 1.5 mM MgCl₂, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 10 mM NaF, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 1x protease inhibitor cocktail (Roche, Laval, Quebec, Canada)), clarified by centrifugation

(20 min at 15,000g), and quantified by Bradford analysis (Bio-Rad Laboratories, Mississauga, Ontario, Canada). Western blotting of protein lysates was performed as described previously [3]. Antibodies against E-cadherin (#3195) and SNAIL (#3879) were purchased from Cell Signaling technologies (Danvers, MA). LDLR antibody was purchased from Abcam (ab14056; Cambridge, MA). Anti-actin antibody (A2006) was purchased from Sigma (Mississauga, ON). Blots were washed using TBST and incubated with peroxidase-conjugated anti-chicken IgY (Ab97135) for 1 h at 1:10,000 dilution, 5% skim milk/TBST for the LDLR antibody, or peroxidase-conjugated anti-rabbit IgG 1:10,000 dilution 5% BSA/TBST for actin, E-cadherin, and SNAIL at room temperature. Blots were washed again using TBST followed by incubation with Luminata Forte Western horseradish peroxidase substrate (Millipore, Etobicoke, ON) and visualized with the ChemiDoc MP System (BioRad, Mississauga, ON).

***SNAIL* knockdown**

Ascites-derived EOC cells were seeded at 500,000 cells/well of a 6-well dish. After 16 h, cells were transfected with siSNAIL SMARTPool RNA or siNT non-targeting control siRNA using DharmaFECT1 transfection reagent (Dharmacon). At 72 h post-transfection, cells were trypsinized and used to form spheroids at 50,000 cells/ well in 24-well ULA plates. Virus infection and viability assays were performed as described above. Protein was harvested from replicate wells at the time point when cell viability was assessed to verify Snail protein knockdown.

Statistical Analysis

Statistical significance was determined by unpaired two-tailed Student's t-test or one-way analysis of variance using GraphPad Prism 6 (GraphPad Software, San Diego, CA).

Statistical Significance was set at $p < 0.05$.

4.5 References

1. Lengyel, E. (2010) Ovarian cancer development and metastasis. *The American journal of pathology* 177: 1053-1064.
2. Shield, K., Ackland, M. L., Ahmed, N., and Rice, G. E. (2009). Multicellular spheroids in ovarian cancer metastases: Biology and pathology. *Gynecologic oncology* 113: 143-148.
3. Rafahi, S., et al. (2016) TGFbeta signalling regulates epithelial-mesenchymal plasticity in ovarian cancer ascites-derived spheroids. *Endocrine-related cancer*.
4. Ahmed, N., Thompson, E. W., and Quinn, M. A. (2007). Epithelial-mesenchymal interconversions in normal ovarian surface epithelium and ovarian carcinomas: an exception to the norm. *Journal of cellular physiology* 213: 581-588.
5. Do, T. V., Kubba, L. A., Du, H., Sturgis, C. D., and Woodruff, T. K. (2008). Transforming growth factor-beta1, transforming growth factor-beta2, and transforming growth factor-beta3 enhance ovarian cancer metastatic potential by inducing a Smad3-dependent epithelial-to-mesenchymal transition. *Mol Cancer Res* 6: 695-705.
6. Peinado, H., Ballestar, E., Esteller, M., and Cano, A. (2004). Snail mediates E-cadherin repression by the recruitment of the Sin3A/histone deacetylase 1 (HDAC1)/HDAC2 complex. *Molecular and cellular biology* 24: 306-319.
7. Batlle, E., et al. (2000). The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. *Nature cell biology* 2: 84-89.
8. Cano, A., et al. (2000). The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nature cell biology* 2: 76-83.
9. Cao, L., Shao, M., Schilder, J., Guise, T., Mohammad, K. S., and Matei, D. (2012) Tissue transglutaminase links TGF-beta, epithelial to mesenchymal transition and a stem cell phenotype in ovarian cancer. *Oncogene* 31: 2521-2534.
10. Liao, J., et al. (2014) Ovarian cancer spheroid cells with stem cell-like properties contribute to tumor generation, metastasis and chemotherapy resistance through hypoxia-resistant metabolism. *PloS one* 9: e84941.
11. Tong, J. G., et al. (2015) Evidence for differential viral oncolytic efficacy in an in vitro model of epithelial ovarian cancer metastasis. *Molecular Therapy* 23: 15013.
12. Ilkow, C. S., et al. (2015) Reciprocal cellular cross-talk within the tumor microenvironment promotes oncolytic virus activity. *Nature medicine* 21: 530-536.
13. Correa, R. J., et al. (2012) Myxoma virus-mediated oncolysis of ascites-derived human ovarian cancer cells and spheroids is impacted by differential AKT activity. *Gynecologic oncology* 125: 441-450.

14. Brun, J., et al. (2010) Identification of genetically modified Maraba virus as an oncolytic rhabdovirus. *Mol Ther* 18: 1440-1449.
15. Akhurst, R. J. (2006). Large- and small-molecule inhibitors of transforming growth factor-beta signaling. *Curr Opin Investig Drugs* 7: 513-521.
16. Travassos da Rosa, A. P., Tesh, R. B., Travassos da Rosa, J. F., Herve, J. P., and Main, A. J., Jr. (1984). Carajas and Maraba viruses, two new vesiculoviruses isolated from phlebotomine sand flies in Brazil. *The American journal of tropical medicine and hygiene* 33: 999-1006.
17. Tesh, R. B., et al. (1987). Natural infection of humans, animals, and phlebotomine sand flies with the Alagoas serotype of vesicular stomatitis virus in Colombia. *The American journal of tropical medicine and hygiene* 36: 653-661.
18. Pauszek, S. J., Barrera Jdel, C., Goldberg, T., Allende, R., and Rodriguez, L. L. (2011) Genetic and antigenic relationships of vesicular stomatitis viruses from South America. *Archives of virology* 156: 1961-1968.
19. Tesfay, M. Z., et al. (2015) Vesiculovirus neutralization by natural IgM and complement. *Journal of virology* 88: 6148-6157.
20. Voigt, E., Inankur, B., Baltés, A., and Yin, J. (2013) A quantitative infection assay for human type I, II, and III interferon antiviral activities. *Virology journal* 10: 224.
21. Burleson, K. M., Hansen, L. K., and Skubitz, A. P. (2004). Ovarian carcinoma spheroids disaggregate on type I collagen and invade live human mesothelial cell monolayers. *Clinical & experimental metastasis* 21: 685-697.
22. Burleson, K. M., Casey, R. C., Skubitz, K. M., Pambuccian, S. E., Oegema, T. R., Jr., and Skubitz, A. P. (2004). Ovarian carcinoma ascites spheroids adhere to extracellular matrix components and mesothelial cell monolayers. *Gynecologic oncology* 93: 170-181.
23. Kenny, H. A., Nieman, K. M., Mitra, A. K., and Lengyel, E. (2011) The first line of intra-abdominal metastatic attack: breaching the mesothelial cell layer. *Cancer discovery* 1: 100-102.
24. Yamada, S. D., Baldwin, R. L., and Karlan, B. Y. (1999). Ovarian carcinoma cell cultures are resistant to TGF-beta1-mediated growth inhibition despite expression of functional receptors. *Gynecologic oncology* 75: 72-77.
25. E, O. h., Quartuccio, S. M., Cheng, W., Ahmed, R. A., King, S. M., and Burdette, J. E. (2014) Mutation or loss of p53 differentially modifies TGFbeta action in ovarian cancer. *PloS one* 9: e89553.
26. Dunfield, L. D., Dwyer, E. J., and Nachtigal, M. W. (2002). TGF beta-induced Smad signaling remains intact in primary human ovarian cancer cells. *Endocrinology* 143: 1174-1181.
27. Hu, W., Wu, W., Nash, M. A., Freedman, R. S., Kavanagh, J. J., and Verschraegen, C. F. (2000). Anomalies of the TGF-beta postreceptor signaling pathway in ovarian cancer cell lines. *Anticancer research* 20: 729-733.
28. Ahmed, N., and Stenvers, K. L. (2013) Getting to know ovarian cancer ascites: opportunities for targeted therapy-based translational research. *Frontiers in oncology* 3: 256.
29. Wintzell, M., Hjerpe, E., Avall Lundqvist, E., and Shoshan, M. (2012) Protein markers of cancer-associated fibroblasts and tumor-initiating cells reveal subpopulations in freshly isolated ovarian cancer ascites. *BMC cancer* 12: 359.

30. Ho, C. M., Chang, S. F., Hsiao, C. C., Chien, T. Y., and Shih, D. T. (2012) Isolation and characterization of stromal progenitor cells from ascites of patients with epithelial ovarian adenocarcinoma. *Journal of biomedical science* 19: 23.
31. Zhang, Y., et al. (2011) Ovarian cancer-associated fibroblasts contribute to epithelial ovarian carcinoma metastasis by promoting angiogenesis, lymphangiogenesis and tumor cell invasion. *Cancer letters* 303: 47-55.
32. Yeung, T. L., et al. (2013) TGF-beta modulates ovarian cancer invasion by upregulating CAF-derived versican in the tumor microenvironment. *Cancer research* 73: 5016-5028.
33. House, C. D., Hernandez, L., and Annunziata, C. M. (2015) In vitro enrichment of ovarian cancer tumor-initiating cells. *J Vis Exp*.
34. Strauss, R., et al. (2009). Epithelial phenotype confers resistance of ovarian cancer cells to oncolytic adenoviruses. *Cancer research* 69: 5115-5125.
35. Chen, C. H., Chen, W. Y., Lin, S. F., and Wong, R. J. (2014) Epithelial-mesenchymal transition enhances response to oncolytic herpesviral therapy through nectin-1. *Human gene therapy* 25: 539-551.
36. Rangel, L. B., et al. (2003). Tight junction proteins claudin-3 and claudin-4 are frequently overexpressed in ovarian cancer but not in ovarian cystadenomas. *Clin Cancer Res* 9: 2567-2575.
37. Agarwal, R., D'Souza, T., and Morin, P. J. (2005). Claudin-3 and claudin-4 expression in ovarian epithelial cells enhances invasion and is associated with increased matrix metalloproteinase-2 activity. *Cancer research* 65: 7378-7385.
38. Vergara, D., et al. Epithelial-mesenchymal transition in ovarian cancer. *Cancer letters* 291: 59-66.
39. Inman, G. J., et al. (2002). SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. *Molecular pharmacology* 62: 65-74.
40. Fonseca, G. J., et al. (2012) Adenovirus evasion of interferon-mediated innate immunity by direct antagonism of a cellular histone posttranslational modification. *Cell host & microbe* 11: 597-606.
41. Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods (San Diego, Calif)* 25: 402-408.

CHAPTER 5: General Discussion

5.1 Summary of overall findings

Viral oncolytic therapy cannot be regarded as a universally efficacious treatment for cancer, but rather a broad strategic category of therapies that have vastly different impacts on the cells they infect, the tumor microenvironment, and the immune system which results in a spectrum of outcomes for patients. The efficacy of oncolytic virotherapy is highly dependent on a number of dynamic variables which are differentially regulated during EOC metastasis and disease progression. Thus, the efficacy of a virus is highly contextual and may not be universally effective across all disease stages or a broad patient population. Within a single tumor, we observed variance in susceptibility to oncolysis which suggests that tumor cells could be selected for to give rise to cells resistant to viral therapy. This implies that virus therapy faces the same challenges that most cancer therapies have to conquer: tumor heterogeneity and preventing treatment resistance. However, by defining the specific factors that regulate effective virus replication, oncolytic viruses are unlike conventional chemotherapies in that they can be genetically engineered and designed to improve their oncolysis in contexts which would normally limit their replication. In characterizing both virus and host factors that influence MXYV, vvDD, and MRBV replication in an *in vitro* model of EOC, we found virus size, dynamic signaling processes during spheroid formation, dormancy, and both intercellular and temporal heterogeneity could impact virus infectivity, replication, and oncolysis. These findings lay a foundation for the strategic design of future oncolytic viruses which can best circumvent conventional treatment barriers to effectively treat EOC.

5.2 Oncolytic virus replication and tumor dormancy

Tumor dormancy in EOC is thought to allow the persistence of microscopic tumor lesions after front-line treatment with chemotherapeutic drugs. In the context of metastatic ovarian cancer, the ability to target and kill dormant cancer cells is essential to eradicate disease recurrence, and decrease the high proportion of patients which experience relapse. Multi-cellular spheroids are not only a delivery mechanism to promote metastasis, but a reservoir for supporting tumor cell dormancy in EOC [1, 2]. In our assessment of oncolytic virus efficacy in dormant EOC spheroids, we observed significant reduction in the replication efficiency and induction of lysis from large poxviruses, including MYXV and vvDD. However smaller viruses, like MRBV were able to efficiently replicate, albeit to a slightly reduced capacity in EOC spheroids made from cell lines [3]. Given that the oncolysis by MYXV and vvDD was induced immediately upon spheroid reattachment rather than requiring multiple days to complete replication as was observed in adherent cell infections, it is likely that viral replication is progressing within spheroids but is restricted at a late stage such that completion of oncolysis can only be observed upon reattachment. Similarly, expression of GFP by MYXV and vvDD in both adherent and spheroid EOC cells implies at least early gene expression of the viruses in both culture conditions as viral GFP expression is under the control of a synthetic early/late promoter. Given that the first wave of poxvirus transcription is driven by RNA polymerases and transcription factors packaged with the virus, it is logical that virus replication in spheroids may be restricted in later stages that are more heavily dependent on host cell factors [4-8]. This would imply restriction possibly during intermediate or late stages of the viral life cycle perhaps during, morphogenesis, packaging, or egress in both MYXV and vvDD.

During morphogenesis, assembling virions require abundant lipid and protein within cytoplasmic viral factories to enclose the virus core components [9]. We have previously documented the role of autophagy in the degradation of many cellular components during quiescence in spheroids [10]. It is possible that the digestion of cellular organelles and proteins that are non-essential to cell survival through autophagy is sequestering vital components required for membrane wrapping of maturing virions. Similarly, intracellular acquisition of poxvirus envelopes requires trafficking through golgi-derived membranes. However, golgi-derived membranes are commonly redirected for the formation of autophagosomes during autophagy and this may curtail viral envelope incorporation in spheroids [11]. Electron microscopy based studies of the intracellular components of spheroids is one future experiment which could be performed to determine the stage of poxvirus replication within spheroids.

Independent of the potential affect that autophagy may have on late stage viral replication in spheroids, it is possible that poxviruses do not represent an effective oncolytic therapy for the treatment of dormant cancer cells regardless of their morphology (adherent or spheroid). It is likely that poxvirus replication is highly dependent on cell cycle progression [12, 13]. For instance, effective replication of MYXV requires the activation of mammalian p21-activated kinase 1 (PAK1) which is phosphorylated during mitosis and is important for cell cycle regulation [14]. Decreased phosphorylation of PAK1 does not impact virus entry or early virus gene expression but impedes MYXV before late gene expression and viral DNA replication, thus restricting the production of virus progeny [15]. Furthermore, a number of poxviruses have been shown to encode genes which act as viral mimetics of cellular anaphase-promoting protein complexes [13].

This regulates the progression of the host cell through mitosis and promotes poxvirus growth, thus emphasizing the significance of cellular replication for poxvirus replication. Dependence of MYXV on AKT signaling and vvDD on EGFR/ERK activation and abundant deoxynucleotide pools are unfavorable requirements for virus replication in dormant cells, particularly for viruses with sizeable genomes [16, 17]. Unfortunately, not only are these unfavorable conditions found in EOC spheroids but both AKT and ERK are among the most common signaling pathways to be suppressed in many dormant and resilient forms of pancreatic, colorectal, head and neck, and breast cancer metastases [18-21]. Likewise, the expression of both host cell TK (on which vvDD is dependent) and ribonucleotide reductase, which is integral for the synthesis of dNTP, are nearly undetectable in resting G₀ cells, and this represents as an important rate limiting step in the synthesis of the large DNA genomes of progeny virions [22, 23]. dNTP pools required for DNA synthesis are 20x lower in resting cells than those that are actively replicating [22]. Thus, although there may be potential clinical therapeutic benefits of these larger poxviruses for highly proliferative cancer cells, they may be subject to the same limitations as chemotherapeutic drugs in indolent cancers.

Interestingly, MRBV oncolysis of spheroids was relatively resistant to tumor dormancy. Thus far, no host cell factors have been identified for MRBV replication and its replication may be relatively independent of the host cell cycle. Genome-wide siRNA screens to identify potential host factors necessary for VSV replication did not link any genes associated with cell cycle [24]. Furthermore, efficient replication of MRBV in dormant EOC ascites-derived spheroids suggests complete independence of MRBV from host cell replication. The comparatively small genome size of MRBV implies a reduced

dependence on abundant ribonucleic acid pools for mRNA synthesis and genome replication for each progeny virion, potentially allowing for more virus progeny to be produced per round of infection, as we observed. Thus, it is likely MRBV represents a more viable therapeutic option for the treatment of slow growing, indolent cancers which are associated with greater chemotherapeutic resistance.

5.3 Primary cells vs. established cell lines for *in vitro* assays

Comparative analysis of MRBV infected primary cells versus established cell lines reveals remarkable incongruencies in their overall response to infection. Not only did we observe relative resistance of primary EOC ascites-derived cells to MRBV in adherent culture when compared to EOC cell lines, but there was also dramatic sensitization to MRBV-induced oncolysis of spheroids formed from ascites-derived cells, compared to EOC cell line spheroids. These seemingly contradictory results raise many questions regarding the nature of cells that are used during *in vitro* assays which aim to recapitulate clinically relevant responses.

Cell lines are enriched populations of immortalized cancer cells and therefore are a favorable model for understanding the behavior of tumor cells. However most have been long removed from patients and have transformed significantly over decades of *in vitro* culture. In contrast, the nature of ascites-derived cells and other primary cell isolates is heterogeneous. The cellular component of malignant ascites consists of variable proportions of tumor cells, mesothelial cells, fibroblasts, macrophages, white blood cells, and red blood cells which perhaps more accurately mimics the microenvironment of tumors [25]. It is evident that fibroblasts have significant contributions to the microenvironment of tumors and can alter behavior of cancer cells to promote increased metastasis and

aggressiveness. Therefore, the absence of these cells from cell line models potentially limits our ability to understand the complex interaction between tumor stroma and malignant cells and therefore the context in which a treatment can function. However, in heterogeneous ascites-derived cell populations it is unclear what proportion of cells are truly malignant thereby obscuring the interpretation of the results from molecular analyses. To accurately interpret our conflicting data between EOC cell lines and primary ascites infected with MRBV, further characterization of the ascites-derived cells is necessary.

Given that ascites contains both malignant and non-malignant cellular components it is logical to expect greater death in transformed cell lines compared to ascites-derived cells from MRBV infection. However, it is unclear why spheroid formation of ascites cells significantly increases susceptibility to MRBV-induced death over both adherent culture of ascites cells and even spheroids formed from cell lines. In comparing TGF β responses between adherent and spheroid EOC cell lines, we observed inconsistent changes in EMT markers across the lines and often only partial effects (unpublished data), whereas the induction of EMT in EOC ascites-derived cells was almost universal upon spheroid formation or TGF β treatment [26]. This may be due to cellular defects in the TGF β /SMAD signaling pathway in many EOC cell lines [27-30]. It is well established that cell line models fail to respond to TGF β signalling or fully emulate the morphological and behavioral changes of EMT [31-36]. We speculated that this key difference between ascites-derived cells and EOC cell lines may be a mechanism governing differential MRBV responses.

TGF β has dynamic functions in cancer and its behavior is highly contextual [37]. In pre-malignancy, TGF β functions as a tumor suppressor and induces apoptosis or

cytostasis in malignant cells. However in malignant progression, cells can circumvent the tumor suppressive effects of TGF β either through inactivation of core pathway components, such as TGF β receptors, or through alterations downstream of TGF β that disable only the tumor-suppressive arm of this pathway. This latter mode of circumvention allows cancer cells to freely usurp the remaining TGF β functions to acquire invasive properties (EMT), produce autocrine mitogens, and release pro-metastatic cytokines. The induction of EMT through TGF β signaling has essential functions in promoting metastasis, migration, chemo-resistance, and stem-like behavior in diverse cancers and many of its markers can act as predictive features for patient prognosis [26, 38-44]. For this reason, we used primary ascites-derived cells which consistently recapitulates this biologically relevant phenomenon to investigate the impact of TGF β and EMT on MRBV replication.

It is likely that the sensitization to MRBV by TGF β treatment or spheroid formation observed in ascites-derived cells may due to the oncolysis of non-cancerous that have undergone EMT rather than strictly tumor cells given that the non-cancerous cell population comprises up to 99% of the cellular component of ascites (although it is uncertain how generalizable this is across samples) [25]. However, it is unclear why non-cancerous cells become sensitive to oncolytic death after adopting a mesenchymal phenotype. Other groups that have described an association between virus infection and EMT have proposed increased receptor availability for virus binding after acquiring a mesenchymal phenotype. However, we observed that neither LDLR expression nor virus entry are changed in TGF β treated cells, so it remains unclear exactly what promotes sensitization of mesenchymal-like cells to MRBV. Treatment of fibroblasts with TGF β or FGF2 has been shown to induce desmoplasia (proliferation of reactive fibroblasts), a

hallmark of many epithelial tumors which promotes the transformation of normal fibroblasts into cancer-associated fibroblasts [45]. Cancer-associated fibroblasts demonstrate decreased IFN β production and antiviral signaling compared to their normal counterparts. Infection of normal fibroblasts with VSV demonstrates a robust induction of IFN β and associated antiviral genes, while pre-treatment of the same fibroblasts with TGF β show reduced antiviral responses [46]. However, while TGF β pre-treatment can negatively impact IFN β production, we demonstrated that the MRBV resistant ascites-derived cells grown in adherent culture lack an effective antiviral IFN β response to MRBV infection without TGF β pre-treatment. Thus this explanation is still insufficient to explain the sensitization of ascites-derived cells to activated TGF β signaling. Moreover, it suggests a potentially unconventional mesenchymal-like phenotype of the cells we culture directly from the ascites.

One study has illustrated an important function of TGF β in inducing ER stress and the upregulation of a number of proteins associated in the unfolded protein response (UPR), XBP1, ATF6, and GRP-78 in lung fibroblasts [47]. ER stress induced by the accumulation of unfolded proteins from TGF β treatment also causes EMT and pulmonary fibrosis [47, 48]. Interestingly, inhibition of these same UPR pathways causes mild ER stress due to an accumulation of unfolded proteins similar to that observed during TGF β treatment in lung fibroblasts [49]. Mild ER stress caused by unfolded protein accumulation prior to MRBV infection has been shown to rewire cancer cells to become sensitized to caspase-2 dependent apoptosis upon MRBV infection [49]. This sensitization was independent of changes in antiviral cytokines such as IFN β [49]. Further investigation into the mechanisms of TGF β induced sensitization of ascites-derived cells to MRBV oncolysis

should include the characterization of ER stress and UPR responses after TGF β treatment and spheroid formation to determine their functional relevance in mediating sensitivity to MRBV.

5.4 Tumor heterogeneity and precision therapy in oncolytic virotherapy

The development of personalized treatment regimens for individuals with cancer, including EOC, has been a major therapeutic directive in order to combat the heterogeneous nature of cancer across broad patient populations. We selected SKOV3, OVCAR8, and HEY A8 based on their characterized genetic mutations and therefore predicted susceptibility to killing based on the known viral trophic factors for each virus. We predicted both OVCAR8 and SKOV3 cells with *TP53* and *PIK3CA* mutations to be most susceptible to MYXV killing, while cells with mutations in *KRAS*, OVCAR8 and HEY A8, to be more susceptible to vvDD [50]. Interestingly, we found no correlation between known viral trophic factors in the EOC cell lines and susceptibility to virus killing. This was not surprising as intratumoral heterogeneity in EOC may be quite substantial and viral tropism is dependent on multiple factors and not tumor suppressor mutation/oncogene activation status alone. This is clearly exhibited in our temporal and spatial heterogeneity models of EOC and the differential oncolytic efficacy of MRBV.

Many *in vitro* models demonstrate diverse tumor cell heterogeneity which can give rise to differential treatment responses even in cell lines classified as being homogenous. For example, single nucleus genome sequencing of just 50 cells from the breast cancer cell line, SK-BR-3, which has previously been determined to be monoclonal and genomically stable[51, 52], reveals significant heterogeneity between cells with single nucleotide variants, copy number alterations, and structural variants. Further characterization of these

variants revealed 409 non-synonymous variants and 1,452 structural variants, many of which occurred in cancer genes [53]. Additionally, unique barcoding of individual cells from the non-small cell lung cancer cell line HCC827 revealed a common mechanism for acquired resistance to erlotinib treatment. HCC827 harbor an activating mutation in EGFR conferring sensitivity to the EGFR inhibitor erlotinib. Of a total of approximately 1 million cells each with a distinct barcode, 462 barcodes were expanded and enriched after erlotinib treatment to give rise to erlotinib resistance. If this resistance was driven mostly by *de novo* mutations, distinct barcodes would emerge as independent replicates. Instead, this enrichment and expansion of a limited number of barcodes suggests the existence of resistant cell populations prior to treatment and extensive heterogeneity within the cell line [54]. Furthermore, the study shows that acquired tumor resistance to erlotinib treatment can arise from a starting subclonal population of cells which make up only 0.05% of the population. These data suggest that the application of oncolytic viruses strictly as targeted therapies for the direct oncolysis of tumors with specific mutations or aberrant signaling pathways may be subject to similar Darwinian selective pressures and result in acquired resistance. The possibility of acquired resistance through this mechanism would be more likely in genomically unstable cancers with high intratumoral heterogeneity such as EOC, where the probability of spontaneously developing a mutation to confer virus resistance would be greater [55].

Alternatively, we demonstrated that resistance to MRBV could be overcome by coculturing resistant EOC cells with sensitive cells. Therefore, it is reasonable to believe that genomic instability may in fact benefit MRBV oncolysis as it increases the probability of having MRBV sensitive clones that can confer sensitivity to neighbouring resistant cells.

Under our co-culture conditions we demonstrated that as few as 25% of co-cultured cells need to be sensitive to confer MRBV sensitivity to resistant cells. Likely, genomic instability will differentially impact patients depending on the random nature of tumor cell mutagenesis. However, the acquisition of specific mutations which support tumor survival and metastasis, such as activating mutations in TGF β [56], may be more common across many cancers and consequentially aid in mediating MRBV oncolysis. Many breast, melanomas, and prostate cancers acquire mutations in the SMAD transcriptional complexes which alter cytostatic gene responses but maintain the core signaling components of the TGF β pathway to enhance migration, invasion, and immune evasion. CD44⁺/CD24^{lo} cells, which have been associated with cancer stemness and poorer metastasis-free survival, have been shown in human breast cancers to be driven by activations in the TGF β pathway [57]. Therefore there is also reason to believe that heterogeneity could increase tumor cell susceptibility to MRBV infection. Future studies investigating the dynamics of TGF β signaling across heterogeneous EOC cells and mediating MRBV sensitization should be considered and perhaps assessed via co-culture viability assays of MRBV-sensitive TGF β -responsive cells and MRBV-resistant TGF β -unresponsive cells.

Although we showed that MYXV had variable success in EOC cells, it may be possible to apply the virus as an adjuvant to promote greater MRBV infection and replication in a combination therapeutic approach similar to AdMA3 and MRBV [58]. Subverting the challenges of tumor heterogeneity in MRBV replication through modulating cancer cell gene expression with an initial infection with MXYV may theoretically be feasible. Though MYXV demonstrated the least potent oncolytic activity,

it may alternatively function as a potent gene delivery vehicle [59-61]. In the three EOC cell lines cultured as both adherent and spheroids, MYXV demonstrated the highest degree of infection in of all three viruses tested. We broadly observed MYXV infection of approximately 40% of EOC cells and GFP expression indicative of early gene expression. This suggests the differential oncolytic efficacy of MYXV for EOC may be a result of restriction later in the virus lifecycle and that MYXV entry and early gene expression may not be as strongly impacted by tumor heterogeneity or cell morphology. By comparison, EOC spheroids formed from cell lines and clones which were resistant to MRBV oncolysis had as little as 5% of cells permissive to infection. Identification of both LDLR expression and TGF β activation as trophic factors which impact MRBV replication could support the concept of engineering either an LDLR or TGF β type I activated receptor expressing MYXV under the control of an early/late promoter [62]. Given the large cloning capacity of poxviruses, both constructs would be feasible to engineer. This strategy may broaden tumor cell tropism for a subsequent MRBV infection. The expression of LDLR or TGF β type I activated receptor after MYXV infection and early gene expression could support greater replication and oncolysis by a subsequent MRBV infection in the absence of MYXV oncolysis. Given that we have demonstrated complete oncolysis of heterogeneous tumor populations with as little as 25% of cells being permissive to MRBV, a pre-treatment of tumor cells with MYXV-LDLR or MYXV-TGF β type I activate receptor could proportionally increase the number of cells permissive to MRBV infection and induce complete oncolysis in heterogeneous tumors or even across patients largely resistant to MRBV. In theory, this strategy could induce oncolysis of tumor cells regardless of the cell cycle stage so long as MYXV production of LDLR or TGF β type I receptor is under the

control of the early/late promoter [62]. Furthermore, a dual virus administration strategy may enhance immune responses to both viruses and tumor cells. Investigation on the feasibility of this conceptual treatment regimen would have to be explored in future studies.

5.5 Antitumor immunity and oncolytic viruses

The growing field of oncolytic therapy is increasingly recognizing the importance of immune stimulation in promoting cancer cell death [63]. The majority of newly developed applications of viral therapies incorporate some form of immune stimulation by the virus. Thus, the host-pathogen interaction is an important limiting factor in our ability to fully assess the potential therapeutic benefit of the viruses we have evaluated in EOC cells *in vitro*. Although we have identified important viral trophic factors impacting MRBV oncolysis and the impact intratumoral heterogeneity may have on virus killing, the biological significance of tumor heterogeneity in MRBV killing would need to be assessed in the context of an active immune response. Perhaps MRBV-induced oncolysis of a subpopulation of tumor cells may promote robust antitumor immunity which may be sufficient to overcome the cellular complexity in tumor heterogeneity. Assessing this may be particularly difficult in pre-clinical models of cancer as mouse xenograft models of EOC using heterogeneous primary human cancer cells use immunocompromised animals. Syngeneic models using murine cancer cell lines are little better as the tumors are much less heterogeneous. In immune competent syngeneic murine melanoma models using B16-F10 cells, Pol et al. show relatively poor induction of both cytotoxic and helper T cell activation after infection with armed MRBV-hDCT (melanoma associated antigen, dopachrome tautomerase). This is likely due to an intact type I IFN response in B16 cells, which limits productive viral replication *in vivo* and restricts robust activation of the

adaptive immune system against the tumor [64]. Antitumor immune stimulation in heterogeneous tumor models in which a mixed population of virus-sensitive and virus-resistant cells would be more informative in comprehending MRBV induction of antitumor immunity. Transgenic mouse models of EOC may be one of the few pre-clinical models to fully evaluate the potential impact of tumor heterogeneity on MRBV in the context of an active immune system. However, few if any, biologically relevant HGSOC transgenic mouse models have been characterized in terms of their ability to recapitulate tumor heterogeneity or stimulate immune function [65, 66]. Alternatively, humanized mouse models with xenografted human immune systems and tumor tissues may be another model to assess MRBV oncolysis in a heterogeneous tumor model in the context of a competent immune system [67, 68]. However, mismatching human leukocyte antigen (HLA) from tumors and hematopoietic cells and stem cells (used to recapitulate human immune system) is known to cause severe graft vs. host disease or lack of response [69]. Therefore, either matching HLA is required or co-transplantation of human tumors and human peripheral blood mononucleocytes into immunodeficient mice are required for the analysis of the immune response to tumor [70]. Thus, assessing the impact of tumor heterogeneity on MRBV oncolysis in the context of a fully competent immune system is exceedingly challenging given the systems that currently exist to model EOC.

Though the oncolytic activity for both MYXV and vvDD may not be particularly potent in tumor dormancy, their ability to induce early gene transcription and large cloning capacity permits the application of poxviruses in diverse contexts aside from direct cancer cell infection and oncolysis. Poxviruses could be used as vectors armed with tumor-associated antigens to aid in tumor cell recognition by the immune system even in the

absence of virus amplification and oncolysis [71-73]. For EOC patients, increased anti-tumor immunity as measured by tumor infiltrating lymphocytes is a robust prognostic indicator of outcome [74]. Furthermore, their restricted replication within the cytoplasm eliminates the risk of viral gene integration into the host genome and potential oncogenesis of host cells. Recombinant MYXV armed with an IL15R α -IL15 fusion protein, which functions as an immunostimulatory cytokine, demonstrates potent stimulation of both innate and adaptive immune responses. Through cytotoxic lymphocyte proliferation and significantly greater NK cell tumor infiltration in a murine melanoma model, a cessation of tumor growth and prolonged survival was achieved [61].

Prime-boost administration of sequential infections with armed poxviruses has been evaluated in clinical trials with promising results. Infection of EOC and melanoma patients with vaccinia virus armed with the tumor-associated antigen NY-ESO1, followed by a booster vaccination with fowl pox virus armed with the same antigen, elicited enhanced cytotoxic and helper T cell responses against tumors in 20/22 patients in a phase II clinical trial. Patients who developed T cell responses in the presence or absence of neutralizing antibodies demonstrated improved overall survival compared to those who did not develop T cell responses (52.4 months vs. 14.5 months). T cells extracted from responsive patients demonstrated immune recognition and cytotoxicity of NY-ESO1 expressing tumors in *ex vivo* models providing evidence for the efficacy of poxvirus based immunotherapeutic vaccines against EOC [75].

5.6 Conclusions

This work highlights the aspects of cancer which continue to plague the development of effective therapies and perhaps limited the therapeutic benefit of early

clinical trials with oncolytic viruses. The use of oncolytic viruses in *in vitro* models of EOC which aim to recapitulate the most common hurdles for effective therapy emphasize ongoing challenges but also new opportunities for improved treatment. Although both metastasis and tumor heterogeneity remain major obstacles, characterization of viral trophic factors and their kinetics within EOC cells reveal new contexts in which viruses can be applied to combat these treatment barriers and hopefully improve outcomes. New insights into the syngeneic relationship between viruses and the immune system provide novel avenues to develop virus therapy to help overcome these longstanding impediments. The expanding repertoire of oncolytic viruses and their application in combination with each other provides a growing arsenal of agents to diversify the current options for cancer therapy and stimulate anti-tumor immunity in ways that standard chemotherapy and surgery fail to confront. The challenges and opportunities presented here for oncolytic virus use in EOC reveal a new framework for designing viruses to surmount the complexity of EOC tumors and heterogeneity.

5.7 References

1. Correa, R. J., Peart, T., Valdes, Y. R., DiMattia, G. E., and Shepherd, T. G. (2012) Modulation of AKT activity is associated with reversible dormancy in ascites-derived epithelial ovarian cancer spheroids. *Carcinogenesis* 33: 49-58.
2. Peart, T., et al. (2015) Intact LKB1 activity is required for survival of dormant ovarian cancer spheroids. *Oncotarget* 6: 22424-22438.
3. Tong, J. G., et al. (2015) Evidence for differential viral oncolytic efficacy in an in vitro model of epithelial ovarian cancer metastasis. *Molecular Therapy* "Oncolytics" 2: 15013.
4. Broyles, S. S. (2003). Vaccinia virus transcription. *The Journal of general virology* 84: 2293-2303.
5. Deng, L., Dai, P., Ding, W., Granstein, R. D., and Shuman, S. (2006). Vaccinia virus infection attenuates innate immune responses and antigen presentation by epidermal dendritic cells. *Journal of virology* 80: 9977-9987.
6. Sanz, P., and Moss, B. (1999). Identification of a transcription factor, encoded by two vaccinia virus early genes, that regulates the intermediate stage of viral gene

- expression. *Proceedings of the National Academy of Sciences of the United States of America* 96: 2692-2697.
7. Rosales, R., Sutter, G., and Moss, B. (1994). A cellular factor is required for transcription of vaccinia viral intermediate-stage genes. *Proceedings of the National Academy of Sciences of the United States of America* 91: 3794-3798.
 8. Gunasinghe, S. K., Hubbs, A. E., and Wright, C. F. (1998). A vaccinia virus late transcription factor with biochemical and molecular identity to a human cellular protein. *The Journal of biological chemistry* 273: 27524-27530.
 9. Roberts, K. L., and Smith, G. L. (2008). Vaccinia virus morphogenesis and dissemination. *Trends in microbiology* 16: 472-479.
 10. Correa, R. J., et al. (2014) Combination of AKT inhibition with autophagy blockade effectively reduces ascites-derived ovarian cancer cell viability. *Carcinogenesis* 35: 1951-1961.
 11. Geng, J., and Klionsky, D. J. (2010) The Golgi as a potential membrane source for autophagy. *Autophagy* 6: 950-951.
 12. Yoo, N. K., Pyo, C. W., Kim, Y., Ahn, B. Y., and Choi, S. Y. (2008). Vaccinia virus-mediated cell cycle alteration involves inactivation of tumour suppressors associated with Brf1 and TBP. *Cellular microbiology* 10: 583-592.
 13. Mo, M., Fleming, S. B., and Mercer, A. A. (2009). Cell cycle deregulation by a poxvirus partial mimic of anaphase-promoting complex subunit 11. *Proceedings of the National Academy of Sciences of the United States of America* 106: 19527-19532.
 14. Thiel, D. A., Reeder, M. K., Pfaff, A., Coleman, T. R., Sells, M. A., and Chernoff, J. (2002). Cell cycle-regulated phosphorylation of p21-activated kinase 1. *Curr Biol* 12: 1227-1232.
 15. Johnston, J. B., et al. (2003). Role of the serine-threonine kinase PAK-1 in myxoma virus replication. *Journal of virology* 77: 5877-5888.
 16. Wang, G., et al. (2006). Infection of human cancer cells with myxoma virus requires Akt activation via interaction with a viral ankyrin-repeat host range factor. *Proceedings of the National Academy of Sciences of the United States of America* 103: 4640-4645.
 17. Andrade, A. A., et al. (2004). The vaccinia virus-stimulated mitogen-activated protein kinase (MAPK) pathway is required for virus multiplication. *The Biochemical journal* 381: 437-446.
 18. Endo, H., Okuyama, H., Ohue, M., and Inoue, M. (2014) Dormancy of cancer cells with suppression of AKT activity contributes to survival in chronic hypoxia. *PLoS one* 9: e98858.
 19. Aguirre Ghiso, J. A., Kovalski, K., and Ossowski, L. (1999). Tumor dormancy induced by downregulation of urokinase receptor in human carcinoma involves integrin and MAPK signaling. *The Journal of cell biology* 147: 89-104.
 20. Aguirre-Ghiso, J. A., Estrada, Y., Liu, D., and Ossowski, L. (2003). ERK(MAPK) activity as a determinant of tumor growth and dormancy; regulation by p38(SAPK). *Cancer research* 63: 1684-1695.
 21. Zhang, X. H., et al. (2009). Latent bone metastasis in breast cancer tied to Src-dependent survival signals. *Cancer cell* 16: 67-78.

22. Hakansson, P., Hofer, A., and Thelander, L. (2006). Regulation of mammalian ribonucleotide reduction and dNTP pools after DNA damage and in resting cells. *The Journal of biological chemistry* 281: 7834-7841.
23. Sutterluety, H., Bartl, S., Karlseder, J., Wintersberger, E., and Seiser, C. (1996). Carboxy-terminal residues of mouse thymidine kinase are essential for rapid degradation in quiescent cells. *Journal of molecular biology* 259: 383-392.
24. Lee, A. S., Burdeinick-Kerr, R., and Whelan, S. P. (2014) A genome-wide small interfering RNA screen identifies host factors required for vesicular stomatitis virus infection. *Journal of virology* 88: 8355-8360.
25. Sheid, B. (1992). Angiogenic effects of macrophages isolated from ascitic fluid aspirated from women with advanced ovarian cancer. *Cancer letters* 62: 153-158.
26. Rafehi, S., et al. TGFbeta signaling regulates epithelial-mesenchymal plasticity in ovarian cancer ascites-derived spheroids. *Endocrine-related cancer* 23: 147-159.
27. Dunfield, L. D., Dwyer, E. J., and Nachtigal, M. W. (2002). TGF beta-induced Smad signaling remains intact in primary human ovarian cancer cells. *Endocrinology* 143: 1174-1181.
28. Lynch, M. A., et al. (1998). Mutational analysis of the transforming growth factor beta receptor type II gene in human ovarian carcinoma. *Cancer research* 58: 4227-4232.
29. Wang, D., et al. (2000). Analysis of specific gene mutations in the transforming growth factor-beta signal transduction pathway in human ovarian cancer. *Cancer research* 60: 4507-4512.
30. Chen, T., et al. (2001). Transforming growth factor-beta receptor type I gene is frequently mutated in ovarian carcinomas. *Cancer research* 61: 4679-4682.
31. Osada, H., et al. (2001). Heterogeneous transforming growth factor (TGF)-beta unresponsiveness and loss of TGF-beta receptor type II expression caused by histone deacetylation in lung cancer cell lines. *Cancer research* 61: 8331-8339.
32. Kalkhoven, E., et al. (1995). Resistance to transforming growth factor beta and activin due to reduced receptor expression in human breast tumor cell lines. *Cell Growth Differ* 6: 1151-1161.
33. Nicolas, F. J., and Hill, C. S. (2003). Attenuation of the TGF-beta-Smad signaling pathway in pancreatic tumor cells confers resistance to TGF-beta-induced growth arrest. *Oncogene* 22: 3698-3711.
34. Ijichi, H., et al. (2001). Systematic analysis of the TGF-beta-Smad signaling pathway in gastrointestinal cancer cells. *Biochemical and biophysical research communications* 289: 350-357.
35. Simeone, D. M., Pham, T., and Logsdon, C. D. (2000). Disruption of TGFbeta signaling pathways in human pancreatic cancer cells. *Annals of surgery* 232: 73-80.
36. Kretschmar, M. (2000). Transforming growth factor-beta and breast cancer: Transforming growth factor-beta/SMAD signaling defects and cancer. *Breast Cancer Res* 2: 107-115.
37. Massague, J. (2008). TGFbeta in Cancer. *Cell* 134: 215-230.
38. Pang, M. F., et al. TGF-beta1-induced EMT promotes targeted migration of breast cancer cells through the lymphatic system by the activation of CCR7/CCL21-mediated chemotaxis. *Oncogene*.

39. Vo, B. T., Morton, D., Jr., Komaragiri, S., Millena, A. C., Leath, C., and Khan, S. A. (2013) TGF-beta effects on prostate cancer cell migration and invasion are mediated by PGE2 through activation of PI3K/AKT/mTOR pathway. *Endocrinology* 154: 1768-1779.
40. Fuchs, I. B., et al. (2002). The prognostic significance of epithelial-mesenchymal transition in breast cancer. *Anticancer research* 22: 3415-3419.
41. Nitta, T., et al. (2014) Prognostic significance of epithelial-mesenchymal transition-related markers in extrahepatic cholangiocarcinoma: comprehensive immunohistochemical study using a tissue microarray. *British journal of cancer* 111: 1363-1372.
42. Dave, B., Mittal, V., Tan, N. M., and Chang, J. C. (2012) Epithelial-mesenchymal transition, cancer stem cells and treatment resistance. *Breast Cancer Res* 14: 202.
43. Comijn, J., et al. (2001). The two-handed E box binding zinc finger protein SIP1 downregulates E-cadherin and induces invasion. *Molecular cell* 7: 1267-1278.
44. Postigo, A. A., Depp, J. L., Taylor, J. J., and Kroll, K. L. (2003). Regulation of Smad signaling through a differential recruitment of coactivators and corepressors by ZEB proteins. *The EMBO journal* 22: 2453-2462.
45. Lohr, M., et al. (2001). Transforming growth factor-beta1 induces desmoplasia in an experimental model of human pancreatic carcinoma. *Cancer research* 61: 550-555.
46. Ilkow, C. S., et al. (2015) Reciprocal cellular cross-talk within the tumor microenvironment promotes oncolytic virus activity. *Nature medicine* 21: 530-536.
47. Baek, H. A., et al. (2012) Involvement of endoplasmic reticulum stress in myofibroblastic differentiation of lung fibroblasts. *American journal of respiratory cell and molecular biology* 46: 731-739.
48. Tanjore, H., et al. (2011) Alveolar epithelial cells undergo epithelial-to-mesenchymal transition in response to endoplasmic reticulum stress. *The Journal of biological chemistry* 286: 30972-30980.
49. Mahoney, D. J., et al. (2011) Virus-tumor interactome screen reveals ER stress response can reprogram resistant cancers for oncolytic virus-triggered caspase-2 cell death. *Cancer cell* 20: 443-456.
50. Domcke, S., Sinha, R., Levine, D. A., Sander, C., and Schultz, N. (2013) Evaluating cell lines as tumour models by comparison of genomic profiles. *Nature communications* 4: 2126.
51. Kytola, S., et al. (2000). Chromosomal alterations in 15 breast cancer cell lines by comparative genomic hybridization and spectral karyotyping. *Genes, chromosomes & cancer* 28: 308-317.
52. Navin, N., et al. (2011) Tumour evolution inferred by single-cell sequencing. *Nature* 472: 90-94.
53. Wang, Y., et al. (2014) Clonal evolution in breast cancer revealed by single nucleus genome sequencing. *Nature* 512: 155-160.
54. Bhang, H. E., et al. (2015) Studying clonal dynamics in response to cancer therapy using high-complexity barcoding. *Nature medicine* 21: 440-448.
55. (2011) Integrated genomic analyses of ovarian carcinoma. *Nature* 474: 609-615.

56. Lei, X., Wang, L., Yang, J., and Sun, L. Z. (2009). TGFbeta signaling supports survival and metastasis of endometrial cancer cells. *Cancer management and research* 2009: 15-24.
57. Shipitsin, M., et al. (2007). Molecular definition of breast tumor heterogeneity. *Cancer cell* 11: 259-273.
58. NCIC Clinical Trials Group; Ottawa Hospital Research Institute. MG1 Maraba/MAGE-A3, With and Without Adenovirus Vaccine, With Transgenic MAGE-A3 Insertion in Patients With Incurable MAGE-A3-Expressing Solid Tumours. In *ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2000- [cited 2016 Jan 28]*. Available from: <https://clinicaltrials.gov/ct2/show/NCT02285816>. NLM identifier: NCT02285816.
59. Stanford, M. M., Barrett, J. W., Gilbert, P. A., Bankert, R., and McFadden, G. (2007). Myxoma virus expressing human interleukin-12 does not induce myxomatosis in European rabbits. *Journal of virology* 81: 12704-12708.
60. Kirn, D. H., and Thorne, S. H. (2009). Targeted and armed oncolytic poxviruses: a novel multi-mechanistic therapeutic class for cancer. *Nature reviews* 9: 64-71.
61. Tomic, V., et al. (2014) Myxoma virus expressing a fusion protein of interleukin-15 (IL15) and IL15 receptor alpha has enhanced antitumor activity. *PloS one* 9: e109801.
62. Chakrabarti, S., Sisler, J. R., and Moss, B. (1997). Compact, synthetic, vaccinia virus early/late promoter for protein expression. *BioTechniques* 23: 1094-1097.
63. Kaufman, H. L., Kohlhapp, F. J., and Zloza, A. (2015) Oncolytic viruses: a new class of immunotherapy drugs. *Nat Rev Drug Discov* 14: 642-662.
64. Pol, J. G., et al. (2014) Maraba virus as a potent oncolytic vaccine vector. *Mol Ther* 22: 420-429.
65. Sherman-Baust, C. A., et al. (2014) A genetically engineered ovarian cancer mouse model based on fallopian tube transformation mimics human high-grade serous carcinoma development. *The Journal of pathology* 233: 228-237.
66. Kim, J., Coffey, D. M., Creighton, C. J., Yu, Z., Hawkins, S. M., and Matzuk, M. M. (2012) High-grade serous ovarian cancer arises from fallopian tube in a mouse model. *Proceedings of the National Academy of Sciences of the United States of America* 109: 3921-3926.
67. Wege, A. K., et al. (2011) Humanized tumor mice--a new model to study and manipulate the immune response in advanced cancer therapy. *International journal of cancer* 129: 2194-2206.
68. Aryee, K.-E., Shultz, L., Greiner, D., Brehm, M., and Jurczyk, A. (2015) Development of humanized mouse models to study human immune system-tumor interactions (TUM10P.1055). p. 211.236.
69. Greenblatt, M. B., Vrbanac, V., Tivey, T., Tsang, K., Tager, A. M., and Aliprantis, A. O. (2012) Graft versus host disease in the bone marrow, liver and thymus humanized mouse model. *PloS one* 7: e44664.
70. Ito, R., Takahashi, T., Katano, I., and Ito, M. (2012) Current advances in humanized mouse models. *Cellular & molecular immunology* 9: 208-214.
71. Liu, T. C., Hwang, T., Park, B. H., Bell, J., and Kirn, D. H. (2008). The targeted oncolytic poxvirus JX-594 demonstrates antitumoral, antivascular, and anti-HBV activities in patients with hepatocellular carcinoma. *Mol Ther* 16: 1637-1642.

72. Kaufman, H., Schlom, J., and Kantor, J. (1991). A recombinant vaccinia virus expressing human carcinoembryonic antigen (CEA). *International journal of cancer* 48: 900-907.
73. Tsang, K. Y., Zaremba, S., Nieroda, C. A., Zhu, M. Z., Hamilton, J. M., and Schlom, J. (1995). Generation of human cytotoxic T cells specific for human carcinoembryonic antigen epitopes from patients immunized with recombinant vaccinia-CEA vaccine. *Journal of the National Cancer Institute* 87: 982-990.
74. Hwang, W. T., Adams, S. F., Tahirovic, E., Hagemann, I. S., and Coukos, G. (2012) Prognostic significance of tumor-infiltrating T cells in ovarian cancer: a meta-analysis. *Gynecologic oncology* 124: 192-198.
75. Odunsi, K., et al. (2012) Efficacy of vaccination with recombinant vaccinia and fowlpox vectors expressing NY-ESO-1 antigen in ovarian cancer and melanoma patients. *Proceedings of the National Academy of Sciences of the United States of America* 109: 5797-5802.

Curriculum Vitae

Jessica Tong, BSc, MSc., PhD

EDUCATION

University of Western Ontario (PhD. Anatomy and Cell Biology) 2011- Present
London, ON

University of Western Ontario (MSc. Microbiology and Immunology) 2009 - 2011
London, ON

McMaster University (H.BSc. Life Sciences) 2004 - 2009
Hamilton, ON

De La Salle College 1998- 2004
Toronto, ON

ACHIEVEMENTS / AWARDS

Poster Award June 2015
Oncology Research & education Day- London, ON

Women's Health Research Foundation Award June 2015
Canadian Student Health Research Forum & CIHR – Winnipeg, MB

Selected for CIHR National Student Research Competition June 2015
For top 5% of graduate students as chosen by their institution
Canadian Student Health Research Forum & CIHR - Winnipeg, MB

1st Place Poster Award in Cellular & Cancer Biology April 2015
London Health Research Day- London, ON

CIHR-STP Cancer Research and Technology Transfer Scholarship September 2014- August 2015
Canadian Institutes of Health Research

Poster Award June 2014
Oncology Research & Education Day- London, ON

1st Place Poster Session III May 2014
Canadian Conference on Ovarian Cancer Research- Victoria, BC

1st Place Poster Award May 2014
Obstetrics & Gynaecology Research Day- London, ON

CIHR Institute Community Support Travel Award April 2014
Canadian Institutes of Health Research

CIHR-STP Cancer Research and Technology Transfer Scholarship Canadian Institutes of Health Research	September 2013 - August 2014
CIHR-STP in Cancer Research and Technology Transfer Scholarship Canadian Institutes of Health Research	September 2012 - August 2013
Ovarian Cancer Canada Teal Heart Scholarship Ovarian Cancer Canada	August 2012
Poster Award Oncology Research & Education Day – London, ON	June 2012
1st Place Poster Session III- <i>New therapies in animal models and humans, clinical trial reports, surgical outcomes, survival psychosocial issues</i> Canadian Conference on Ovarian Cancer Research – Quebec City, QUE	May 2012
Western Graduate Research Scholarship University of Western Ontario	September 2009 - Present
Dean’s Honour Roll McMaster University	September 2008 - April 2009
Entrance Scholarship McMaster University	September 2004 - April 2005
Ontario Scholars Award Ministry of Education	June 2004

RESEARCH

Refereed Research:

Tong, J.G., Rafahi, S., Valdes, Y.R., Bertrand, M., McGee, J., Prefontaine, M., Sugimoto, A., Bell, J.C, Stojdl, D., DiMattia, G.E., Shepherd, T.G., “Transforming growth factor-beta signaling induction of epithelial-to-mesenchymal transition in ovarian cancer ascites-derived spheroid cells augments Maraba virus-mediated oncolysis” In preparation for submission to *Cancer Biology & Therapy*, 2016.

Tong, J. G., Valdes, Y.R., Barrett, J.W., Bell, J.C., Stojdl, D., DiMattia, G.E., Shepherd, T.G. “Spatial and temporal epithelial ovarian cancer cell heterogeneity impact Maraba virus oncolytic potential” In preparation for submission to *Gynecologic Oncology*, 2016.

Tong, J. G., Vales, Y.R., Barrett, J.W., Bell, J.C., Stojdl, D., McFadden, G., McCart, J.A., DiMattia, G.E., Shepherd, T.G, “Evidence for differential viral oncolytic efficacy in an *in vitro* model of epithelial ovarian cancer metastasis” *Molecular Therapy Oncolytics*, 2015; 2: 15013.

Rytelewski, M., **Tong, J.**, Buensucesco, A., Leon, H., Maleki-Vareki, S., Figueredo, R., Di Cresce, C., Wu, S., Herbrich, S., Baggerly, K., Romanow, L., Shepherd, T., Deroo, B., Sood, A., Chambers, A., Vincent, M., Ferguson, P., "BRCA2 inhibition enhances cisplatin-mediated alterations in tumor cell proliferation, metabolism, and metastasis" *Molecular Oncology*, 2014; 8(8):1429-40.

Woods, M.W., **Tong, J.G.**, Tom, S.K., Szabo, P.A., Cavanagh, P.C., Dikeakos, J. D., Haeryfar, S. M., Barr SD, " Interferon-induced HERC5 is evolving under positive selection and inhibits HIV-1 particle production by a novel mechanism targeting Rev/RRE-dependent RNA nuclear export." *Retrovirology*, 2014; 11(27): 1-16.

Li, S., **Tong, J.**, Rahman, M.M., Shepherd, T.G, McFadden, G., "Oncolytic Virotherapy for Ovarian Cancer", *Oncolytic Virotherapy*, 2012; 1: 1 - 21

Correa, R. J., Komar, M., **Tong J. G. K.**, Sivapragasam, M., Rahman, M. M., McFadden, G., Dimattia, G. E., Shepherd, T. G., "Myxoma virus-mediated oncolysis of ascites-derived human ovarian cancer cells and spheroids is impacted by differential AKT activity", *Gynecol Oncol*, 2012; 125(2): 441-50

Woods, M.W., Kelly, J.N., Hattlmann, C. J., **Tong, J. G. K.**, Xu, L. S., Coleman, M., Quest, G. R., Smiley, J. R., Barr, S. D., "Human HERC5 restricts an early stage of HIV-1 assembly by a mechanism correlating with the ISGylation of Gag", *Retrovirology*, 2011; 8(1): 95

Book Chapters:

Jenna Kelly, **Jessica G. K. Tong**, Clayton Hattlmann, Matthew Woods, and Stephen Barr, "Cellular Restriction Factors: Can We Exploit the Body's Natural Antiviral Proteins to Combat HIV/AIDS?," *HIV and AIDS - Updates on Biology, Immunology, Epidemiology and Treatment Strategies*, INTECH open access publisher, October 2011, p143-182. ISBN 978-953-307-665-2.

Abstracts:

Jessica Tong, Yudith Ramos-Valdes, John Barrett, Milani Sivapragasam, Monique Bertrand, Jacob McGee, Michel Prefontaine, Akira Sugimoto, Grant McFadden, John C Bell, David Stojdl, Gabriel E DiMattia, Trevor G. Shepherd (2015). *Ovarian tumour cell heterogeneity impact Maraba virus infection and oncolytic efficacy*. Oral Presentation: 34th Annual Meeting of the American Society for Virology, London, ON.

Jessica Tong, Yudith Ramos-Valdes, John Barrett, Milani Sivapragasam, Monique Bertrand, Jacob McGee, Michel Prefontaine, Akira Sugimoto, Grant McFadden, John C Bell, David Stojdl, Gabriel E DiMattia, Trevor G. Shepherd (2015). *Ovarian tumour cell heterogeneity impact Maraba virus infection and oncolytic efficacy*. Poster Presentation: Oncology Research & Education Day, London, ON.

Jessica Tong, Yudith Ramos-Valdes, John Barrett, Milani Sivapragasam, Grant McFadden, John C. Bell, David Stojdl, Gabriel E. DiMattia, Trevor G. Shepherd (2015) *Ovarian tumour cell heterogeneity impact Maraba virus infection and oncolytic efficacy*. Poster Presentation: Canadian Student Health Research Forum, Winnipeg, MB.

Jessica Tong, Yudith Ramos-Valdes, John Barrett, Milani Sivapragasam, Monique Bertrand, Jacob McGee, Michel Prefontaine, Akira Sugimoto, Grant McFadden, John C Bell, David Stojdl, Gabriel E DiMattia, Trevor G. Shepherd (2015). *Ovarian tumour cell heterogeneity impact Maraba virus infection and oncolytic efficacy*. Oral Presentation: 9th International Conference on Oncolytic Virus Therapeutics, Boston, MA, USA.

Jessica Tong, Yudith Ramos-Valdes, John Barrett, Milani Sivapragasam, Grant McFadden, John C. Bell, David Stojdl, Gabriel E. DiMattia, Trevor G. Shepherd (2015) *Ovarian tumour cell heterogeneity impact Maraba virus infection and oncolytic efficacy*. Oral Presentation: Obstetrics & Gynaecology Research Day, London, ON.

Jessica Tong, Yudith Ramos-Valdes, John Barrett, Milani Sivapragasam, Grant McFadden, John C. Bell, David Stojdl, Gabriel E. DiMattia, Trevor G. Shepherd (2015) *Ovarian tumour cell heterogeneity impact Maraba virus infection and oncolytic efficacy*. Poster Presentation: London Health Research Day, London, ON

Jessica G.K. Tong, Yudith Ramos-Valdes, Milani Sivapragasam, John Barrett, John C. Bell, Grant McFadden, David Stojdl, Gabriel DiMattia, Trevor Shepherd (2014). *Differential viral oncolytic efficacy is impacted by tumour heterogeneity using a three-dimensional model of ovarian cancer metastasis*. Poster Presentation: New Zealand Society of Oncology, Tauranga, New Zealand.

Jessica G.K., Tong, Milani Sivapragasam, John C. Bell, Grant McFadden, David Stojdl, Gabriel DiMattia, Trevor G. Shepherd (2014). *Differential viral oncolytic efficacy is impacted by tumour heterogeneity using a three-dimensional model of ovarian cancer metastasis*. Poster Presentation: 7th Canadian Conference on Ovarian Cancer Research, Victoria, BC.

Jessica G.K., Tong, Milani Sivapragasam, John C. Bell, Grant McFadden, David Stojdl, Gabriel DiMattia, Trevor G. Shepherd (2014). *Differential viral oncolytic efficacy is impacted by tumour heterogeneity using a three-dimensional model of ovarian cancer metastasis*. Poster Presentation: Oncology Research & Education Day, London, ON.

Jessica G.K., Tong, Milani Sivapragasam, John C. Bell, Grant McFadden, David Stojdl, Gabriel DiMattia, Trevor G. Shepherd (2014). *Differential viral oncolytic efficacy is impacted by tumour heterogeneity using a three-dimensional model of ovarian cancer metastasis*. Poster Presentation: Obstetrics & Gynaecology Research Day, London, ON.

Jessica G.K., Tong, Milani Sivapragasam, John C. Bell, Grant McFadden, David Stojdl, Gabriel DiMattia, Trevor G. Shepherd (2014). *Differential viral oncolytic efficacy is*

impacted by tumour heterogeneity using a three-dimensional model of ovarian cancer metastasis. Oral Talk: London Health Research Day, London, ON.

Rytelewski, M., **Tong, J.**, Buensucesco, A., Maleki-Vareki, S., Ferguson, P.J., Figueredo, R., Vincent, M., Shepherd, T., Deroo, B.J., Koropatnick, D.J (2013) "A novel BRCA2 targeting antisense oligonucleotide sensitizes human tumor cells to chemotherapy and radiotherapy - the induction of 'complementary lethality' by targeting DNA repair." Poster Presentation: AACR Molecular Targets and Cancer Therapeutics, Boston, MA.

Jessica G.K., Tong, Milani Sivapragasam, John C. Bell, Grant McFadden, David Stojdl, Gabriel DiMattia, Trevor G. Shepherd (2013). *Cross-comparison of oncolytic viruses using a three-dimensional in vitro model of ovarian cancer metastasis.* Poster Presentation: Anatomy & Cell Biology Research Day, London, ON.

Jessica G.K. Tong, Milani Sivapragasam, John C. Bell, Grant McFadden, David Stojdl, Gabriel DiMattia, Trevor G. Shepherd (2013) *Cross-comparison of oncolytic viruses using a three-dimensional in vitro model of ovarian cancer metastasis.* Oral Talk: Oncology Research & Education Day, London, ON.

Jessica G.K. Tong, Milani Sivapragasam, John C. Bell, Grant McFadden, David Stojdl, Gabriel DiMattia, Trevor G. Shepherd (2013) *Cross-comparison of oncolytic viruses using a three-dimensional in vitro model of ovarian cancer metastasis.* Oral Talk: Paul Harding Research Day, London, ON.

Jessica G.K. Tong, Milani Sivapragasam, John C. Bell, Grant McFadden, David Stojdl, Gabriel DiMattia, Trevor G. Shepherd (2013) *Cross-comparison of oncolytic viruses using a three-dimensional in vitro model of ovarian cancer metastasis.* Poster Presentation: London Health Research Day, London, ON.

Jessica G. K. Tong, Monica Komar, Grant McFadden, Gabriel DiMattia, Trevor Shepherd (2012) *Investigating the mechanisms for restrictive myxoma virus-mediated oncolysis in epithelial ovarian cancer spheroids.* Poster Presentation: Anatomy & Cell Biology Research Day, London, ON.

Jessica G. K. Tong, Monica Komar, Grant McFadden, Gabriel DiMattia, Trevor Shepherd (2012) *Investigating the mechanisms for restrictive myxoma virus-mediated oncolysis in epithelial ovarian cancer spheroids.* Poster Presentation: Oncology Research & Education Day, London, ON.

Jessica G. K. Tong, Monica Komar, Grant McFadden, Gabriel DiMattia, Trevor Shepherd (2012) *Investigating the mechanisms for restrictive myxoma virus-mediated oncolysis in epithelial ovarian cancer spheroids.* Poster Presentation: 6th Canadian Conference on Ovarian Cancer Research, Quebec City, QUE.

Jessica G. K. Tong, Monica Komar, Grant McFadden, Gabriel DiMattia, Trevor Shepherd (2012) *Investigating the mechanisms for restrictive myxoma virus-mediated oncolysis in*

epithelial ovarian cancer spheroids. Poster Presentation: Paul Harding Research Day, London, ON.

Jessica G. K. Tong, Monica Komar, Grant McFadden, Gabriel DiMattia, Trevor Shepherd (2012) *Evaluation of viral oncolytics for treatment of epithelial ovarian cancer and metastatic control*. Poster presentation: London Health Research Day, London, ON.

Matthew W. Woods, **Jessica G. K. Tong**, Jenna N. Kelly, Clayton J. Hattlmann, Stephen D. Barr (2010) *Interferon-induced Herc5 restricts HIV-1 particle release*. Oral presentation: Infection and Immunity Research Forum (IIRF), London, ON.

Varun Anipindi, **Jessica G. K. Tong**, Mohammad Khan, Allison Holloway, and Geoff Werstuck (2008) *Vascular Effects of High Fat Diet Along With Fetal/Neonatal Exposure of Nicotine in Wistar Rats*. Poster Presentation: Canadian Cardiovascular Congress (CCC), Toronto, ON.

RELEVANT ACADEMIC CONTRIBUTIONS

Chair of CaRTT Speaker Selection Committee **April 2013- April 2014**
Organization of meetings and Selection of speakers for the Cancer Research and Technology Transfer (CaRTT) seminar series.
Department of Oncology
University of Western Ontario

The National Scholarship Panel Member **March 2013- 2016**
Evaluation of incoming undergraduate student applications for the Faculty of Science Entrance Awards and President's Entrance Awards
Faculty of Science
University of Western Ontario

PhD Candidate **September 2011-Present**
Viral oncolytic therapies for ovarian cancer and metastasis
Dr. Trevor Shepherd
Department of Anatomy and Cell Biology
University of Western Ontario

Research Project Mentor **September 2010 – April 2014**
Supervision of an undergraduate research project
Adrian Chan (Dr. Barr Lab) & Milani Sivapragasam (Dr. Shepherd Lab)
Department of Microbiology & Immunology / Anatomy and Cell Biology
University of Western Ontario

Teaching Assistant **September 2010 – December 2010**
Instructing undergraduate microbiology student labs
Micro&Imm 2100A: Biology of Prokaryotes
Dr. Susan Koval
Department of Microbiology & Immunology

University of Western Ontario

Infection and Immunity Research Forum Committee **September 2009 – August 2011**

Plan annual conference and arrange keynote speaker

Select poster presenters and oral speakers

Department of Microbiology & Immunology

University of Western Ontario

MSc.

September 2009 - August 2011

Innate antiviral immune responses to HIV

Dr. Stephen Barr

Department of Microbiology & Immunology

University of Western Ontario

Undergraduate Research Student

September 2008 – April 2009

Nicotine exposure in utero and development of diabetes

Dr. Geoffrey Werstuck

Department of Biochemistry

McMaster University

Clinical Research Assistant

May 2008 – August 2008

Developing alternatives for protection against cold weather

Intelligent Design for Adaptation, Participation,
and Technology (iDAPT)

Toronto Rehabilitation Institute

Laboratory Maintenance - Volunteer

September 2007 – April 2008

Dr. Jianping Xu Lab

Department of Biology

McMaster University

EXTRACURRICULAR

Canadian Cancer Society

Research Information Outreach Team

March 2013- January 2015

- Community education on progress and promise of cancer research
- Contributing to: monthly columns in the Londoner newspaper, public speaking engagements, panel presentations, and videos.

AIDS Committee of London

January 2010 – August 2012

- Safe sex education
- Fundraising

Society of Graduate Students Council Member

September 2009 – October 2011

- Representative for Microbiology and Immunology
- Raise funds for Department of Microbiology and Immunology

- Policy making for graduate students

Infection and Immunity Research Forum Committee **September 2009 – August 2011**

- Plan annual conference
- Arrange keynote speaker
- Select poster presenters and oral speakers

Microbiology and Immunology Social Committee **September 2009 – August 2011**

- Organize social events for the department
- Fundraising

McMaster Science for Peace **September 2007 - April 2009**

- Organizing Annual Conference
- Fundraising
- Arranging keynote speaker

Biodiversity Gallery Facilitator (*Royal Ontario Museum*) **May 2007 - August 2009**

- Communicating biological concepts to gallery visitors

Appendix

Chapter 2 was used under the Creative Commons License as attribution/non-commercial.