Novel Treatment of Colonic Dysplasia with an Oncolytic Vaccinia Virus

by

Fernando Andres Angarita, MD

A thesis submitted in conformity with the requirements for the degree of Masters of Science
Institute of Medical Science
University of Toronto

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2013

Abstract

Colonic dysplasia is a non-invasive intraepithelial neoplastic process that can eventually become cancer. Novel therapies are needed for dysplastic lesions not amenable to standard treatment. Oncolytic viruses selectively kill tumours, but the effect on dysplasia is unknown. This study determined if oncolytic vaccinia virus (vvDD) infects colonic dysplasia. After chemically inducing colonic dysplasia, mice received intraperitoneal (IP) or intracolonic (IC) vvDD expressing red fluorescent protein (vvDD-RFP) or control. RFP signal was apparent at 24h post-virus infection (pvi), peaking at 72h (IC) and 120h (IP) pvi. vvDD-RFP infected high-grade dysplasia more so than low-grade dysplasia; normal tissue was unaffected. vvDD-RFP infected larger surface areas of dysplasia when administered IC than IP. Viral titres peaked earlier and higher with IC than IP delivery. vvDD-RFP-treated mice had less polyps and dysplasia and had higher survival rates than mock-treated animals. This study suggests that oncolytic virotherapy may have a role in treating colonic dysplasia.
Contributions

Dr. Fernando A. Angarita designed and carried out the experiments, analysed the data, and wrote the thesis. Dr. Hala El-Zimaity (Department of Pathology, Toronto General Hospital, University Health Network, Toronto, ON, Canada) graded the histopathology. The Pathology Research Program (PRP) Laboratory at the Department of Pathology, Toronto General Hospital, University Health Network, ON, Canada performed the sectioning and staining for histopathology. Dr. Siham Zerhouni assisted in designing experiments, randomized animals for treatment, and maintained the database for survival analysis. Kathryn Ottolino-Perry and Dr. Sergio A. Acuna assisted in designing experiments and carrying out experiments. Nan Tang made the red fluorescent protein expressing double deleted vaccinia virus (vvDD-RFP). Dr. J. Andrea McCart conceived the idea, designed the experiments, analysed the data, and supervised the study.
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This project would not have been possible without the participation of Dr. Hala El-Zimaity who generously dedicated her time to take me on as a student. Her passion for pathology was contagious from the start as she taught me how to review slides, shared her knowledge and experience, and provided significant intellectual input. During my moments of hopelessness her amazing advice, which was always accompanied by a warm smile and a great sense of humour, helped me put things into perspective and continue working hard.

I am also very grateful for the friends and colleagues with whom I have shared a great deal of experiences. Dr. Siham Zerhouni made my work environment and personal life more enjoyable as she kept me at ease during the difficult times by reassuring me and helping me deal with problems. Her sense of commitment, work ethic, and pursuit of excellence were a constant source motivation. Moreover I am thankful because she generously dedicated her time to exchange ideas, read and comment on my work, and help me practice for presentations. Dr. Sergio A. Acuña committed himself to helping me out whenever needed, offering his time to plan and carry out experiments. I am thankful for the countless valuable discussions we had on this and other projects over recent years. As an early source of motivation when we started
working on projects together I will be forever thankful for his friendship. Kathryn Ottolino-Perry was always a very bright source of knowledge who I considered the go-to expert. Her willingness to read my work was always appreciated because her input taught a lot about presenting and discussing basic science information. I would also like to thank Nan Tang for teaching me the technical skills necessary to carry out many of the experiments. I would also like to express profound gratitude to Dr. Carla Rosario for her very strong and valuable words of encouragement. Her willingness to hear me out and provide me with academic and professional advice were always very appreciated.

These past years could not have been possible without the generous support and unconditional love of both my parents and grandmother. I am forever indebted for their many sacrifices to give me this and many other opportunities in life. From them I have learned about hard work, dedication, and perseverance, all of which have been particularly instrumental in these past years. Finally I would like to express profound gratitude to Artur Iatsko for being so patient and understanding during this time. His company, support, and, more importantly friendship, were important sources of motivation, which kept me working hard and focused on my goals. His immense willingness to hear me out and provide words of encouragement will be forever appreciated.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AD</td>
<td>Autosomal dominant</td>
</tr>
<tr>
<td>AFP</td>
<td>Alpha fetoprotein</td>
</tr>
<tr>
<td>AJCC</td>
<td>American Joint Committee on Cancer</td>
</tr>
<tr>
<td>AOM</td>
<td>Azoxymethane</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>AR</td>
<td>Autosomal recessive</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>CAR</td>
<td>Coxsackievirus and adenovirus receptor</td>
</tr>
<tr>
<td>CEV</td>
<td>Cell-associated enveloped virus</td>
</tr>
<tr>
<td>CHRPE</td>
<td>Congenital hypertrophy of the retinal pigment epithelium</td>
</tr>
<tr>
<td>CIN</td>
<td>Chromosomal instability</td>
</tr>
<tr>
<td>CRC</td>
<td>Colorectal cancer</td>
</tr>
<tr>
<td>DAMP</td>
<td>Danger-associated molecular pattern molecule</td>
</tr>
<tr>
<td>DCC</td>
<td>Deleted-in-colon-cancer</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
</tr>
<tr>
<td>DMH</td>
<td>Dimethylhydrazine</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxyribonucleoside triphosphate</td>
</tr>
<tr>
<td>dpi</td>
<td>Days post-infection</td>
</tr>
<tr>
<td>DSS</td>
<td>Dextran sodium sulfate</td>
</tr>
<tr>
<td>dTTP</td>
<td>Deoxynucleoside triphosphate</td>
</tr>
<tr>
<td>EEV</td>
<td>Extracellular enveloped virus</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EMA</td>
<td>Epithelial membrane antigen</td>
</tr>
<tr>
<td>EMR</td>
<td>Endoscopic mucosal resection</td>
</tr>
<tr>
<td>ESD</td>
<td>Endoscopic submucosal dissection</td>
</tr>
<tr>
<td>FAP</td>
<td>Familial adenomatous polyposis</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FOBT</td>
<td>Fecal occult blood testing</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycans</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphatase</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and eosin</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks’ balanced salt solution</td>
</tr>
<tr>
<td>HGD</td>
<td>High-grade dysplasia</td>
</tr>
<tr>
<td>HNPCC</td>
<td>Hereditary nonpolyposis colorectal cancer</td>
</tr>
<tr>
<td>hSSTR2</td>
<td>Human somatostatin receptor type 2</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes simplex virus</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>IC</td>
<td>Intracolonic</td>
</tr>
<tr>
<td>IC mock</td>
<td>Intracolonic mock</td>
</tr>
<tr>
<td>IC vvDD</td>
<td>Intracolonic virus</td>
</tr>
<tr>
<td>IEV</td>
<td>Intracellular enveloped virus</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
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</tbody>
</table>
IL  Interleukin
IMV  Intracellular mature virus
IP  Intraperitoneal
IP mock  Intraperitoneal mock
IP vvDD  Intraperitoneal virus
IV  Immature virion
JPS  Juvenile polyposis syndrome
LD50  Lethal dose for 50% of the population
LEF  Lymphocyte enhancer factor
LGD  Low-grade dysplasia
MAM  Methylazoxymethanol
MAP  MYH-associated polyposis
MAPK–ERK  Mitogen-activated protein kinase-extracellular signal-regulated kinase
MHC  Major histocompatibility complex
MMP  Matrix metalloproteinase
MMR  Mismatch repair
MVA  Modified vaccinia Ankara
NCDV  Newcastle disease virus
NFκB  Nuclear factor κB
NYCBH  New York City Board of Health
OV  Oncolytic virus
PAMP  Pathogen-associated molecular pattern molecule
PBS  Phosphate buffered saline
pfu  Plaque forming units
PJS  Peutz-Jeghers syndrome
PKr  Protein kinase R
pRb  Retinoblastoma protein
PRNT  Plaque reduction neutralization test
pvi  Post-virus infection
RFP  Red fluorescent protein
RNR  Ribonucleotide reductase
RPMI  Roswell Park Memorial Institute
R-SMAD  Receptor-regulated SMAD
SA  Surface area
SCID  Severe combined immunodeficiency
SEM  Standard error of the mean
SSA/P  Sessile serrated adenoma/polyp
TCF  T-cell factor
TGF-β  Transforming growth factor-β
TK  Thymidine kinase
TNK  Tumour necrosis factor
TSA  Traditional serrated adenoma
UDP  Uridine diphosphate
VEGF  Vascular endothelial growth factor
VEGFR  Vascular endothelial growth factor receptor
VSV  Vesicular stomatitis virus
VV  Vaccinia virus
vvDD  Double deleted vaccinia virus
<table>
<thead>
<tr>
<th>WHO</th>
<th>World Health Organization</th>
</tr>
</thead>
<tbody>
<tr>
<td>WR</td>
<td>Western Reserve</td>
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</tbody>
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Chapter 1
Introduction

1.1 Precancerous colorectal lesions

1.1.1 Overview of colorectal cancer

Cancer is defined as the abnormal proliferation of cells that typically show loss of structural differentiation and function (anaplasia) and invade nearby tissues and spread to other parts of the body. Cancer is alternatively known as a malignant tumour. The biological hallmarks of cancer comprise six characteristics, which are acquired during carcinogenesis\(^1\); these include: sustaining proliferative signaling as many oncogenes are upregulated and mimic the growth signals that stimulate proliferation in normal cells; evading external and internal tumour growth suppressors that regulate cell proliferation; resisting cell death by circumventing apoptosis; maintaining a replicative immortality; inducing angiogenesis with the aim of obtaining nutrients and oxygen; and becoming invasive in order to continue growing and spreading to other tissue and organs. A less aggressive form of abnormal proliferation is known as benign tumours, which typically show all the features of cancers, except that of invasion.

In the colon and rectum, 96% of neoplasms arise from the epithelial cells lining the glands of the mucosa (carcinoma) and are therefore histologically classified as adenocarcinomas\(^2,3\). Because nearly all colorectal malignant tumours are primarily adenocarcinomas, the term colorectal cancer (CRC) generally refers to this histologic subtype. Moreover, it should be noted that in order to render a diagnosis of CRC the tumour must have at least disrupted the muscularis mucosa and invaded into the submucosa; this differs from the definition of cancer in other parts of the gastrointestinal tract such as the oesophagus, stomach, and small intestine where invasion into the lamina propria is sufficient to diagnose cancer\(^4\). Given the relative paucity of lymphatic vessels, colorectal adenocarcinomas confined to the lamina propria and muscularis mucosa have no risk of nodal or distant metastasis and should therefore be considered benign\(^4\). Invasive
adenocarcinoma and CRC will be used interchangeably throughout this text and will refer to a neoplasia that has invaded through the muscularis mucosa.

CRC develops sporadically, through hereditary cancer syndromes, or in association with inflammatory bowel disease (IBD). The most common type is sporadic CRC, accounting for 88-94%5. Environmental and, to some extent, genetic risk factors play a role in the development of sporadic CRC5. The second most common type of CRC (5–10%) are hereditary cancer syndromes such as hereditary nonpolyposis colorectal cancer (HNPCC), familial adenomatous polyposis (FAP), Peutz-Jeghers syndrome (PJS), juvenile polyposis syndrome (JPS), and MYH-associated polyposis (MAP)5 (Table 1.1). The remaining 1-2% of CRC is associated with IBD such as ulcerative colitis and Crohn’s disease5.

As a leading cause of morbidity and mortality, CRC has considerable impact on public health. Worldwide, it is the third (663,000 cases and 10% of the total number of cases of cancers) and second (571,000 cases and 9.4% of the total number of cases of cancers) most commonly diagnosed cancer in males and females, respectively6. Incidence rates vary 10-fold in both sexes worldwide; with the highest rates in Australia/New Zealand and Western Europe, intermediate in Latin America and the lowest rates in Africa and South-Central Asia6. CRC causes an estimated 600,000 deaths worldwide, which accounted for 8% of all cancer deaths and made it the fourth most common cause of cancer-related death in 20086.

Reduction in incidence and mortality rates are reported in longstanding, economically developed countries such as Canada, Australia, and the United States7,8. In Canada the incidence rate has significantly declined by 0.8% annually since 20009. In Canada cancer-related mortality rates are continuously declining by 2.6% per year in males since 2003 and 1.8% per year in females since 19989. Although the improvements in cancer awareness and treatment in recent decades have facilitated better patient outcomes, screening with fecal occult blood testing (FOBT) and endoscopy (including flexible sigmoidoscopy and colonoscopy) are the largest,
Table 1.1 Summary of hereditary colorectal cancer syndromes.

Reprinted from Surgical Oncology Clinics of North America, 18 (1), Learn PA and Kahlenberg, Hereditary colorectal cancer syndromes and the role of the surgical oncologist, 121-144, 2009, with permission from Elsevier10.

<table>
<thead>
<tr>
<th>Syndrome</th>
<th>Heritability</th>
<th>Underlying gene</th>
<th>Gene function</th>
<th>Lifetime risk of CRC</th>
<th>Extracolonic neoplasms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hereditary nonpolyposis colorectal cancer (HNPCC) (Lynch syndrome)</td>
<td>AD with high penetrance</td>
<td>MLH1 and MSH2 (85%); MSH6 (10%); PMS2 (5%)</td>
<td>DNA base MMR</td>
<td>80%</td>
<td>Cancers of the uterus, small bowel, and urothelial tract</td>
</tr>
<tr>
<td>Familial adenomatous polyposis (FAP)</td>
<td>AD with high penetrance</td>
<td>APC</td>
<td>Control of apoptosis through Wnt signaling pathway</td>
<td>100%</td>
<td>CHRPE; desmoid tumours; osteomas; hepatoblastoma; thyroid tumour; brain neoplasms</td>
</tr>
<tr>
<td>Peutz-Jeghers syndrome (PJS)</td>
<td>AD with variable penetrance</td>
<td>LBK1/STK11 (50% to 60% of cases)</td>
<td>Serine-threonine kinase</td>
<td>39%</td>
<td>Cancers of the breast, pancreas, stomach, testicles, ovaries, and cervix</td>
</tr>
<tr>
<td>Juvenile polyposis syndrome (JPS)</td>
<td>AD with variable penetrance</td>
<td>MADH4/SMAD4 (20%); BMPR1A (20%)</td>
<td>Serine-threonine Kinases involved in TGF-β signaling</td>
<td>60%</td>
<td>Cancers of the stomach, small bowel, pancreas, breast, and thyroid</td>
</tr>
<tr>
<td>MYH-associated polyposis (MAP)</td>
<td>Both AD and AR patterns observed</td>
<td>MYH</td>
<td>Base excision repair for G:C to T:A transversions</td>
<td>80% (biallelic carriers) and 8% for (monoallelic carriers)</td>
<td>Gastroduodenal neoplasms</td>
</tr>
</tbody>
</table>

Abreviations: AD, autosomal dominant; APC, adenomatous polyposis coli; AR, autosomal recessive, CHRPE, congenital hypertrophy of the retinal pigment epithelium; CRC, colorectal cancer; MMR, mismatch repair; TGF, transforming growth factor
contributor. In the USA, from 1975 to 2000 the overall observed decline in CRC mortality was 26% of which 14%, 9%, and 3% was due to screening with FOBT and endoscopy, risk factor modification, and treatment alone, respectively. Similarly, in Canada the use of screening colonoscopy is associated with a 48% and 81% reduction in the incidence and mortality of CRC, respectively.

1.1.1.1 Colorectal cancer screening

Screening is the presumptive identification of unrecognized disease by applying tests to patients at risk that lack signs or symptoms. Screening improves patient outcomes by detecting it at a curable stage (early detection) or preventing the disease from occurring. In the case of CRC, screening is beneficial in two ways. First, if the tumour is diagnosed in early stages before it invades beyond the submucosa (Tis-T1) (Appendix 1), then survival rates are more favourable as treatment prevents locoregional spread and metastasis. Second, and more importantly, by detecting and treating precancerous lesions the risk of CRC is eliminated. CRC prevention is feasible because the transformation process from precancerous to cancerous lesion occurs over 10–15 years. The target of modern CRC screening is adenomatous polyps because they give rise to the majority of tumours (~95%). The remaining ~5% of CRCs arise from serrated polyps.

CRC screening is carried out either with stool-based tests, namely FOBTs, or anatomical tests, which include either radiological imaging or endoscopy. Non-anatomical screening tools, such as FOBT, require further workup to localize the lesion. Both flexible sigmoidoscopy and colonoscopy are preferred over other screening tools because not only do they localize the lesion, they prevent CRC. Endoscopic screening tools allow for macroscopic in vivo assessment of the mucosa, carrying out targeted biopsy, and providing treatment through local excision of macroscopically visible abnormalities.

Given that the entire colon and rectum can be thoroughly examined by using colonoscopy, its role in CRC screening is greater than that of sigmoidoscopy, which only detects left-sided lesions. The concern over flexible sigmoidoscopy screening missing lesions proximal to the sigmoid colon was highlighted by a recent meta-analysis that reported 58% of patients with
proximal advanced adenomatous polyps do not have a distal lesion which would warrant workup with colonoscopy\textsuperscript{25}. Colonoscopy provides a 67\% reduction in CRC incidence and a 65\% reduction in mortality\textsuperscript{26} whereas flexible sigmoidoscopy reduces the incidence rate by 18-23\% and the mortality rate by 22-31\%\textsuperscript{27}. The advantages of flexible sigmoidoscopy over colonoscopy include less bowel preparation, no need to sedate the patient, lower cost, and lower morbidity rate\textsuperscript{28}. Colonoscopy is also described as having the highest sensitivity rate (96.7\% for cancers, 85\% for large polyps, and 78.5\% for small polyps) and specificity rate (98\% for all lesions) relative to other means of screening\textsuperscript{29}. Remarkably, the presumed superiority of colonoscopy as a screening tool is brought into question by the fact that the reduction in mortality associated with its use is more notable in left- rather than right-sided tumours\textsuperscript{30, 31}. However the discrepancy between left- and right-sided CRC has been explained by the fact that “complete” colonoscopies do not always evaluate the entire right colon, bowel preparation is not ideal in the right colon, and left- and right-sided tumours are biologically different\textsuperscript{30, 32}.

Screening colonoscopy is recommend by multiple professional organizations, such as the Canadian Task Force on Preventive Health Care, the Canadian Association of Gastroenterology, the Canadian Cancer Society, the United States Preventive Services Task Force and the combined organizations of the American Cancer Society, US Multi-Society Task Force on Colorectal Cancer representing multiple gastroenterology societies, and the American College of Radiology\textsuperscript{17, 33-36}. Some organizations such as the American College of Gastroenterology\textsuperscript{37}, the American Society for Gastrointestinal Endoscopy\textsuperscript{38}, and the National Comprehensive Cancer Network\textsuperscript{39} grant it a “preferred” status over other screening tools. The general consensus amongst professional organizations is that colonoscopy should be offered every ten years starting at age 50 years to individuals who are at average risk of developing CRC\textsuperscript{34, 36-41}. Average risk patients are defined as those individuals 50 years or older with a negative family history and no history of adenomatous polyps, CRC, or IBD\textsuperscript{39}. The rationale for setting the screening age at 50 years is based on the fact that it is more probable to detect adenomatous polyps than CRC at this age\textsuperscript{42}.
1.1.2 Colorectal polyps

1.1.2.1 Overview

Colorectal polyps are defined as benign neoplastic proliferations that protrude into the bowel lumen\(^\text{43}\). Polyps are classified by the World Health Organization (WHO) into four general histologic categories: (1) adenomatous; (2) serrated, which include hyperplastic, sessile serrated adenomas/polyps (SSA/Ps) and traditional serrated adenomas (TSAs); (3) hamartomatous, and (4) inflammatory\(^3\). Adenomatous polyps are the most common type, accounting for 50–67% of all polyps followed by hyperplastic polyps (25%), SSA/Ps (0.2–9%), and hamartomatous polyps (<1%)\(^{20}\).

The characteristic feature of adenomatous polyps is that they harbour dysplasia, which is defined as a benign intraepithelial neoplasm that cannot grow into the basement membrane\(^{44,45}\). Because colonoscopic detection and treatment of adenomatous polyps prevents 64–90% of CRC cases\(^{26,46-48}\) and reduces CRC-associated mortality rate by 53%\(^{49}\), they are the main target of CRC screening\(^{17,34,36-38,40,41,50}\). Owing to their major role in colorectal carcinogenesis adenomatous polyps are the focus of this study.

Serrated polyps are a group of lesions that all have saw-toothed infolding (serration) of the crypt epithelium, but differ in terms of their molecular profile and role in colorectal carcinogenesis\(^\text{22}\). Approximately 5 to 20% of sporadic CRCs derive from serrated polyps\(^{19-22}\). Serrations result from decreased apoptosis and delayed migration of the epithelial cells from the crypt to the surface\(^\text{51}\). Hyperplastic polyps, which account for 70-95% of serrated polyps, are epithelial elevations with a minimal risk of becoming cancerous and therefore generally do not warrant treatment or further workup after diagnosis except under specific conditions (e.g., >1 cm, proximal lesions, multiple lesions, and/or history of serrated polyposis syndrome)\(^{17,38,40,41,50,52}\). SSA/Ps, which account for <25% of serrated polyps, are precancerous flat or slightly elevated lesions that tend to develop in the proximal colon\(^\text{22,53,54}\). TSAs, which account for 2–3.5% of serrated polyps, are precancerous pedunculated lesions that usually develop in the distal colon and rectum, particularly in elderly patients\(^\text{51}\). Approximately 11% of SSA/Ps and TSAs develop
dysplasia with histological features similar to that of adenomatous polyps\textsuperscript{55}. In this case, these serrated polyps are considered equivalent to advanced polyps because of the higher risk of developing CRC\textsuperscript{22, 40, 56, 57}. The CRC preceded by SSA/Ps and TSAs is considered to be biologically different from that induced by adenomatous polyps because it derives through a molecular pathway characterized by hypermethylation and microsatellite instability\textsuperscript{22, 51} and rarely has mutations in genes such as adenomatous polyposis coli (APC) and p53\textsuperscript{58}.

Hamartomatous polyps are lesions composed of tissues from any of the layers of the colonic lining that are abnormally arranged and are non-dysplastic\textsuperscript{43}. This type of polyp is considered to have minimal cancerous potential except when associated with hereditary polyposis syndromes such as JPS, PJS, multiple endocrine neoplasia (MEN) syndrome 2B, hereditary mixed polyposis syndrome, Cronkhite–Canada syndrome, basal cell nevus syndrome, and neurofibromatosis 1\textsuperscript{20}. Inflammatory polyps are elevations of normal or apparently normal colonic mucosa and submucosa bordered by denuded or abnormal colonic lining that result from chronic inflammatory disease such as IBD or regeneration of the colonic wall after inflammatory, infectious, or ischemic diseases\textsuperscript{20}. These lesions are not considered to be associated with CRC, but rather make screening in patients with IBD difficult because of their potential to mask underlying malignant changes in the mucosa.

1.1.2.2 Adenomatous polyps

The majority of CRC (80-95\%) develops from adenomatous polyps\textsuperscript{20, 52}. Several epidemiologic, clinical, and pathologic findings support the development of CRC from adenomatous polyps including: (1) adenomatous polyps and CRC have a similar anatomical distribution; (2) adenomatous polyps and CRC express similar antigens and activated oncogenes; (3) presence of adenomatous polyps increases a patient’s lifetime risk of developing CRC; (4) the risk of CRC is higher with increasing number of adenomatous polyps; (5) adenomatous polyps are generally diagnosed earlier than CRC, which is enough time to acquire several of the genetic changes that promote growth and malignant transformation; (6) “de novo” CRC is extremely rare; (7) surgical specimens containing CRC also harbour one or more adenomatous polyp; (8) adenomatous polyps may contain foci of CRC; (9) endoscopic removal of adenomatous polyps reduces the
incidence of CRC; and (10) patients who refuse treatment for adenomatous polyps develop CRC at an increasing rate over time⁴³, ⁵⁹, ⁶⁰.

1.1.2.2.1 The adenoma-carcinoma sequence

Adenomatous polyps arise from the upper portion of a single colonic crypt through a “top-down” mechanism⁶¹. Normally, more epithelial cells (29-47%) at the base of the crypt are in S-phase than at the top of the crypt (0.1-2.0%), but with the “top-down” mechanism there is a switch⁶². In the early stages of colorectal carcinogenesis, dysplastic cells appear near the top of the crypt and acquire genetic mutations that lead to a deregulated proliferation⁶¹, ⁶³, ⁶⁴. As dysplastic cells replicate, they spread laterally and downward to form new crypts. Initially they connect to preexisting normal crypts, then replace them over time and eventually increase the number of proliferating cells throughout the crypt (Figure 1.1)⁶¹. Continuous growth of dysplastic cells leads to “tenting up” of the mucosa and formation of a polyp.

**Figure 1.1. Models of morphogenesis of sporadic adenomatous polyps.**

(A) Transformation of a single epithelial cell occurs at the base of the crypt (arrow) by virtue of adenomatous polyposis coli (APC) inactivation. The transformed cell proliferates and passively migrates upward as a result of routine epithelial turnover. Once the transformed cells reach the superficial portion of the mucosae, they continue to proliferate and migrate and begin to populate the superficial mucosae of the adjacent crypts. The adenomatous epithelium thereby confronts the normal epithelium of the adjacent crypts, pushing the latter downward and gradually replacing it from top-to-bottom. (B) Similar to A except that the initial transformation event occurs in an epithelial cell in the intercryptal zone lying between crypt orifices (arrow). (C) En face view of surface, indicating spread of intercryptal dysplastic epithelial cell to adjacent crypts.

Adenomatous polyps develop through the adenoma-carcinoma sequence, a multistep model describing the progressive accumulation of distinct mutations in growth regulatory genes that manifests histologically with proliferative, non-invasive epithelial lesions that eventually become invasive adenocarcinoma65 (Figure 1.2). Although the adenoma-carcinoma model continues to be refined, several key principles have been established66: (1) multiple genetic mutations are necessary, (2) there are discrete intermediate lesions in the progression to cancer, and (3) the temporal acquisition of the genetic changes is important because not all mutations can initiate or promote carcinogenesis independently67.

Figure 1.2. The adenoma-carcinoma sequence. (Angarita FA, 2013)
genetic alterations may vary; therefore it seems most likely that the accumulation of multiple genetic alterations both in oncogenes and tumour suppressor genes is more important than the order of the alterations.

The molecular basis of CRC determines factors that initiate tumour development, drive its progression, and ultimately determine how it will respond to antitumour agents. The loss of genomic stability is a key feature in colorectal carcinogenesis that drives the acquisition of multiple tumour-associated mutations. There are at least three distinct pathways in colorectal carcinogenesis: chromosomal instability (CIN), microsatellite instability, and CpG island methylator phenotype pathways. CIN is the most common type of genomic instability in CRC and is responsible for the molecular and histological features observed in the adenoma-carcinoma sequence. CIN acts as an efficient mechanism that causes a characteristic set of mutations in specific tumour suppressor gene and oncogenes. It is unknown whether CIN creates the appropriate environment for the accumulation of these mutations or vice versa.

In the initiation phase of sporadic CRC, colonic epithelial cells sporadically acquire somatic mutations and deletions that inactivate both copies of the APC gene (chromosome 5q21-q22). The prevalence of APC mutations in adenomatous polyps and CRC is 30–70% and 34–72%, respectively. The APC protein destabilizes free β-catenin thereby acting as a tumour suppressor in the cell proliferation Wnt signaling pathway. Mutations in the APC gene lead to a defective APC protein that cannot bind and degrade β-catenin protein. As a result β-catenin translocates to the nucleus and binds to the T-cell factor (TCF)/lymphocyte enhancer factor (LEF) family of transcription factors, which then targets c-myc, cyclin D1 and c-jun genes to promote cell proliferation. In a small subgroup of CRCs with intact APC, gain-of-function mutations of the β-catenin gene CTNNB1 (chromosome 3p21) are responsible for activating the Wnt signaling pathway. The percentage of β-catenin activating mutations in adenomatous polyps and CRC is about 2.4%–12.5% and 1.4%, respectively. Deletion of a complete exon or individual amino acids encoded by the exon allows β-catenin to escape degradation from the APC protein. β-catenin activating mutations are not found in conjunction with APC mutations suggesting that changes in the two genes act as alternative pathways. Histologically, this phase is characterized by an upward shift of the proliferative zone of the colonic crypt, which leads to the development of a single-crypt adenoma.
During promotion, the initiated epithelial cells undergo further clonal expansion as a result of somatic mutations that activate \textit{KRAS} (chromosome 12p12) and, to a lesser extent, \textit{BRAF} (chromosome 7q34). Both \textit{KRAS} and \textit{BRAF} mediate transduction of signals from extracellular ligands, such as epidermal growth factor (EGF), by activating the mitogen-activated protein kinase-extracellular signal-regulated kinase (MAPK–ERK) pathway. Activated ERK translocates to the nucleus and activates transcription factors to alter the expression of genes critical in the regulation of cell growth. \textit{KRAS} mutations are common in adenomatous polyps (15-68\%) and CRC (40-65\%)\(^\text{70, 77}\). \textit{KRAS} encodes a 21-kilodalton guanosine triphosphatase (GTP)-binding protein called p21\textsuperscript{ras}, which is responsible for regulating cell growth signals\(^\text{78}\). Single nucleotide point mutations of \textit{KRAS} are responsible for activating p21\textsuperscript{ras}, leading to continuous growth stimulus\(^\text{77}\). \textit{BRAF}, which is downstream of \textit{KRAS}, is deregulated in 2.8\% of adenomatous polyps through a V599E (valine 599 to glutamic acid) mutation in the activation segment\(^\text{79}\). This mutation results in the insertion of a negatively-charged residue next to a regulatory site of phosphorylation, which mimics phosphorylation and constitutively activates \textit{BRAF} independently of \textit{KRAS}. Activating \textit{BRAF} induces the same biological effect observed with \textit{KRAS}, as both increase mitosis. Histologically, \textit{KRAS} or \textit{BRAF} mutations lead to exophytic growth, in which the epithelial cell proliferation predominates along the luminal surface without extending to the base of the crypt. Further expansion of the single-crypt adenoma occurs by crypt fission, a process by which it undergoes basal bifurcation followed by longitudinal division to ultimately form two daughter crypts\(^\text{80}\). Typically, the rate of crypt fission is dramatically increased and poorly regulated, leading to asymmetrical and disorganized tissue architecture. Clusters of dysplastic crypts are known as microadenomas\(^\text{80}\).

In the progression stage, cells acquire mutations that make them resistant to growth inhibitors and apoptosis, thereby allowing microadenomas to grow and become larger lesions known as adenomatous polyps. The \textit{deleted-in-colon-cancer (DCC)} gene (chromosome 18q21) was initially proposed to encode a tumour suppressor, but after its initial characterization the product was identified as the transmembrane neuronal protein netrin-1, which plays a key role in axon guidance in the developing nervous system\(^\text{81}\). Approximately 50\% of all CRCs carry an allelic loss mutation in the \textit{DCC} gene in comparison to only 12.5\% of adenomatous polyps\(^\text{77}\). Studies later showed that when engaged by netrin ligands, \textit{DCC} may activate downstream signaling pathways and that in settings where netrin is downregulated or absent, \textit{DCC} promotes
apoptosis\textsuperscript{81}. Other genes with tumour suppressor function have been reported on 18q, including \textit{SMAD2} and \textit{SMAD4}, which are intracellular mediators of the transforming growth factor (TGF)-\(\beta\) pathway\textsuperscript{66}. Given the potent inhibitory effects of the TGF-\(\beta\) pathway on colonic epithelium, which include regulation of cell growth, differentiation, and apoptosis, inactivation of both \textit{SMAD2} and \textit{SMAD4} may have an important role in colorectal carcinogenesis. Homozygous deletion or mutation of these genes are reported in <20\% and 10\% of CRCs, respectively\textsuperscript{66}.

\textit{p53} mediates cell cycle arrest in response to DNA injury in order to allow for DNA repair or apoptosis. \textit{p53} is known as the “guardian of the genome” because it regulates the transcription of hundreds of genes required for DNA metabolism, apoptosis, cell cycle regulation, angiogenesis, immune response, cell differentiation, motility, and migration\textsuperscript{66}. The prevalence of \textit{p53} mutations in adenomatous polyps and CRC is 25\%\textsuperscript{70} and 34-80\%\textsuperscript{70, 82, 83}, respectively. During the adenoma-carcinoma sequence colonic epithelial cells acquire inactivation mutations in both alleles of the \textit{p53} gene (chromosome 17p13) usually through a combination of a missense mutation that inactivates its transcriptional activity and a 17p chromosomal deletion that eliminates the second allele\textsuperscript{68}. These mutations lead to the synthesis of an inactive protein with an abnormally long half-life\textsuperscript{66}. Mutations in \textit{p53} facilitate genomic instability because cells will continue to replicate despite genetic errors as DNA repair and apoptosis are not carried out. The inactivation of \textit{p53} often coincides with the transition of large adenomatous polyps into invasive adenocarcinomas\textsuperscript{84}.

In order to invade nearby tissues, cancer cells secrete extracellular matrix-degrading proteases, such as matrix metalloproteinases (MMPs) and metalloproteinases, which degrade collagens that constitute the basement membrane\textsuperscript{85}. At the same time, cancer cells also stimulate normal cells to aid in the process. The nuclear accumulation of \(\beta\)-catenin, which is retained throughout the adenoma-carcinoma sequence, plays a role in stromal invasion because it increases MMP promoter activity\textsuperscript{86}. CRC cells invade either as single cells or by moving collectively as epithelial sheets, forming focal adhesions on which they can generate traction and move\textsuperscript{87}. Traction force is provided by integrins \(\beta1, \beta3, \alpha2\beta1, \text{and} \alpha6\beta3\) expressed at the invading tip, which connect to extracellular matrix components, such as fibronectin, collagen, and fibrin-rich surfaces\textsuperscript{87}. Although cancer cells disintegrate the basement membrane, as they disarrange the extracellular matrix they can also stimulate \textit{de novo} synthesis of basement membrane
components because nearby stromal cells sometimes react to the disintegration of the basement membrane by repairing it\(^8\).

Historically, \textit{de novo} CRC was defined as colonic mucosa that directly became invasive adenocarcinoma without the intervening steps of an adenomatous polyp because at that time CRC usually presented as large symptomatic masses that rarely contained adenomatous tissue\(^9\). This concept has changed in recent years because there is no clear evidence to support a separate truly \textit{de novo} pathway. Instead, studies have shown an acceleration of the molecular events that lead to invasive adenocarcinoma, including overexpression of \textit{APC}\(^9\), \textit{DCC}\(^9\), \textit{KRAS}\(^9\), and \textit{MMP}\(^9\) as well as down-regulation of the apoptosis inhibitor bcl-2 protein\(^9\) and the cell-to-cell adhesion molecule E-cadherin\(^9\). Altogether, these characteristics imply that as cancer cells rapidly grow, pre-existing adenomatous features are lost and the tumour acquires what appears to be a \textit{“de novo”} appearance\(^1\). Nonetheless, truly \textit{de novo} carcinomas are reported, accounting for 0.7\% of CRC\(^9\). Currently, \textit{de novo} CRC are defined as small (<1.0 cm in diameter) rapidly invasive tumours without any obvious adenomatous or \textit{“in situ”} component\(^1,9\). This definition is based on the fact that the chance of invasive adenocarcinoma having arisen in an adenomatous polyp and then the adenomatous features being destroyed is extremely low at this small size\(^1\).

\subsection*{1.1.2.3 Epidemiology of adenomatous polyps}

In general, the prevalence and age distribution of adenomatous polyps tend to parallel that of CRC\(^9\). Prevalence is low in Africa and in some Asian countries, such as the Philippines, but extremely high in Western countries where 22.2\%-58.2\% of the adult population at average risk for sporadic CRC will have one or more adenomatous polyp\(^7\). The prevalence of adenomatous polyps increases with age and peaks at 70–74 years of age\(^8\). The estimated risk of adenomatous polyps doubles from 50-54 to 70-74 years of age [odds ratio (OR), 2.00; 95\% confidence interval (CI), 1.76 –2.26]\(^8\). Adenomatous polyps are more common in men (24.9-30.6\%) than in women (14.8-29.2\%)\(^8,9\), a characteristic that is seen across all ages and races/ethnic groups\(^8\). Total prevalence of adenomatous polyps is similar across all races/ethnic groups\(^8\). Adenomatous polyps are more commonly detected distal to the juncture of the splenic flexure as 65\% are found in the distal colon\(^4\). In the last two decades, the distribution of polyps has shown
a “proximal shift” as the incidence of proximal adenomatous polyps increased from 19.2–48.5% to 26.0–66.3%\textsuperscript{100-102}. The mechanism of this right-side shift is uncertain, but the fact that rightsided adenomatous polyps increase with age may have a significant role\textsuperscript{103}. Additional explanations include the increase in screening colonoscopy and the “see and sampling” strategy implemented since the early 1990s\textsuperscript{100}.

The mean number of sporadic adenomatous polyps detected in patients is as follows: 1 (55.1%), 2-4 (38.4%) and >4 (6.6%)\textsuperscript{99}. The likelihood of a synchronous adenomatous colonic polyps ranges from 31 to 40%\textsuperscript{104, 105}. Individuals with a distal lesion are more likely to have proximal adenomatous polyps\textsuperscript{106}. Size distribution of adenomatous polyps is as follows: <5mm (60.3%), 5-10mm (29.2%), 11-20mm (7.5%), and >20mm (3.1%)\textsuperscript{99}.

1.1.2.4 Clinical presentation of adenomatous polyps

Adenomatous polyps are asymptomatic in two-thirds of patients while the remaining third has symptoms that are either coincidental (e.g.: haemorrhoids or anal fissures) or polyp-induced\textsuperscript{107}. Rectal bleeding is the most common symptom reported, especially when the lesions are in the distal colon\textsuperscript{43}. The presence of bleeding is most likely related to size and location\textsuperscript{108}. The incidence of bleeding is more common in large polyps and once CRC develops\textsuperscript{43}. Less than half of these polyps, particularly large ones, cause occult fecal blood\textsuperscript{108, 109}. Other potential symptoms include abdominal pain and change in bowel habits\textsuperscript{110}. Adenomatous polyps are rarely large enough to cause intussusception or prolapse as reflected by the occasional case reports in the literature.

1.1.2.5 Histopathological features of adenomatous polyps

Histologically, the defining feature of all adenomatous polyps is the presence of dysplasia\textsuperscript{44, 45}. Adenomatous polyps are macroscopically classified into three categories: (1) pedunculated, which grow on a stalk, (2) sessile, which lack a stalk and have a broad base, and (3) flat or depressed. In general, 61.8% of adenomatous polyps are sessile followed by flat (24.7%) and pedunculated (13.5%)\textsuperscript{99}. Histologically, adenomatous polyps can also be classified into villous,
tubular, and tubulovillous. Villous polyps are composed of \( \geq 80\% \) of finger-like processes of the lamina propria (papillary configuration) and the remaining portion is composed of dysplastic tubular tissue. Tubular polyps contain dysplastic epithelium that occupies \( \geq 80\% \) of the polyp while the remaining portion is composed of \(< 20\%\) of villous components. Tubulovillous polyps are composed of dysplastic tubular crypts and villous processes, each of which contribute to \( > 20\% \) of the polyp’s configuration. The most commonly reported growth pattern is tubular (65-87%) followed by tubulovillous (8-25%) and villous (5-15%)\(^{42,111-113}\).

1.1.2.5.1 Dysplasia

Colonic dysplasia is an intraepithelial neoplasia that lacks the ability to grow into the basement membrane. Histologically, dysplasia is different from carcinoma \textit{in situ} because in the latter, the tissue architecture changes are more severe. Carcinoma in situ can either involve the entire thickness of the epithelium (intraepithelial carcinoma \textit{in situ}) or it can invade the lamina propria without extending beyond the muscularis mucosa (intramucosal carcinoma \textit{in situ}) (\textbf{Appendix 1})\(^{114}\). In clinical practice distinguishing dysplasia and carcinoma \textit{in situ} has no significance. Given the relative scarceness of lymphatic vessels in the colonic lamina propria, lesions confined to this area have theoretically no risk of nodal or distant metastasis; therefore carcinoma \textit{in situ} is clinically equivalent to the severe form of dysplasia, i.e. high-grade dysplasia (HGD)\(^{4}\).

Dysplasia and carcinoma in situ differ from T1 CRC because in the latter the cells have disrupted the muscularis mucosa and invaded the submucosa (\textbf{Appendix 1}). Because the basement membrane is not visible on standard histological techniques, indirect evidence of invasion into the submucosa is used to verify if the basement membrane has been breached. Generally submucosal invasion is accompanied by desmoplasia, a densely fibrous proliferation of connective tissue that surrounds cancer cells\(^{4,115}\). Another difficulty in establishing a diagnosis of invasive adenocarcinoma is pseudoinvasion, in which adenomatous features are misplaced or herniated into the muscularis mucosae or beyond giving an illusion of invasion into the submucosa\(^{4,115}\). Pseudoinvasion is observed in 3-10% of adenomatous polyps, particularly those arising in the sigmoid colon\(^{116,117}\). Pseudoinvasion results from either a reparative down-growth of the mucosa or hypertrophy of the muscularis mucosa in response to traumatization of the polyp’s stalk by twisting and torsion. Histologic features that distinguish pseudoinvasion from
invasive adenocarcinoma include herniated elements with lobular configuration, lack of high grade atypia, presence of a rim of lamina propria inflammatory cells around entrapped elements, lack of desmoplasia, lack of direct contact with submucosal muscular blood vessels, and presence of hemosiderin or haemorrhage⁴.

Histopathologically, colonic dysplasia is characterized by several cytological abnormalities including: (1) variations in the size and shape of cells and their nuclei (pleomorphism); (2) abnormal nuclear morphology such as abundant chromatin, dark staining (hyperchromatism), coarsely clumped chromatin distributed along the nuclear membrane, variation in nuclear position throughout the crypt (stratification), 1:1 nuclear-to-cytoplasm ratio rather than the normal 1:4 or 1:6, variable and irregular nuclear shape, and large nucleoli; (3) loss of polarity, defined as disturbed cell orientation that leads to a disorganized colonic crypt pattern; (4) increased mitotic count, which may or not be accompanied by bizarre mitotic figures (e.g., tripolar, quadripolar, or multipolar spindles); (5) reduction in the number of goblet cells; and (6) mucin inside the goblet cells is displaced to the basal side of the nucleus (dystrophic goblet cells)¹¹⁴,¹¹⁵. Architecturally, the main abnormality is an increase in the thickness of the mucosa, either by crypt elongation (villous pattern) or budding (tubular pattern)¹¹⁴,¹¹⁵.

Dysplasia is graded based on the severity of the cytological and architectural abnormalities previously described. Three categories of dysplasia (mild, moderate, and severe) as well as carcinoma in situ were described by O’Brien et al as part of the National Polyp Study¹¹⁸. Currently these have been condensed into a two-tiered grading system that classifies lesions into either low-grade dysplasia (LGD) (mild/moderate dysplasia) or HGD (severe dysplasia/carcinoma in situ) (Table 1.2).
Table 1.2. Histological features and classification of colonic dysplasia. Adapted from O’Brien MJ et al.118.

<table>
<thead>
<tr>
<th>Histological features</th>
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<tr>
<td><strong>Low-grade dysplasia</strong> (LGD)</td>
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<tr>
<td>- Crypts show branching or elongation and some reduction of interglandular stroma.</td>
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<tr>
<td>- Cell nuclei are oval, regularly overlapping, and basally located.</td>
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<tr>
<td>- Nuclear membranes are regular, the chromatin pattern is fine, and nucleoli are inconspicuous.</td>
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<tr>
<td><strong>Moderate dysplasia</strong></td>
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<tr>
<td>- Glands show a loss of basal polarization of nuclei.</td>
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<tr>
<td>- Nuclear membranes are more irregular and chromatin patterns are uneven with more prominence of nucleoli.</td>
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<tr>
<td><strong>Severe dysplasia</strong></td>
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<tr>
<td>- Marked reduction of interglandular stroma with complex irregularity of glands and papillary infolding.</td>
</tr>
<tr>
<td>- Marked irregularity of nuclear membranes; nuclear chromatin cleared, vesicular, irregularly clumped, or densely hyperchromatic; and large and irregular nucleoli.</td>
</tr>
<tr>
<td><strong>Carcinoma in situ</strong></td>
</tr>
<tr>
<td>- Severe architectural disturbance of glands, such as cribriform or back-to-back patterns or growth in solid sheets in association with the cytological features of dysplasia.</td>
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*Note that invasive adenocarcinoma was defined as carcinoma invasion beyond the muscularis mucosa.

1.1.2.6 The role of adenomatous polyps in colorectal cancer

Despite the fact that adenomatous polyps are common, the lifetime cumulative incidence of CRC is about 5.5%; therefore many of these polyps do not progress towards CRC and instead stabilize or regress. In fact, comparison of the prevalence of adenomatous polyps on autopsy and the reported incidence of CRC in a defined population yields a conversion rate of 0.25% per year119. By definition all adenomatous polyps contain as a minimum LGD, but HGD, the most
severe histologic form that precedes cancer, is present to a lesser extent. Approximately 5-7% of adenomatous polyps harbour HGD. The importance of HGD derives from a multivariate analysis in which polyps with HGD were more likely to harbour previously undetected CRC (adjusted OR 4.60 and 95% CI(2.91–7.27). The incidence of HGD increases with increasing size, villous pattern, and multiple lesions. For example, the OR for HGD is 20 for adenomatous polyps >1 cm and for those with a >75% villous component. HGD incidence rates in single tubular, multiple tubular, single villous, and multiple villous adenomatous polyps are 2.8%, 4.6%, 16%, and 22%, respectively.

Detection and removal of adenomatous polyps prevents CRC and reduces mortality. Screening and surveillance intervals take into consideration several characteristics to determine what patient is at higher risk of developing CRC after detecting an adenomatous polyp. Evidence-based risk stratification relies on using several histopathologic characteristics, specifically the predominant type of histology, size (diameter), and/or the number of polyps, to predict the risk of interval CRC. Patients at low-risk for CRC have 1-2 tubular adenomatous polyps that measure <10mm. In these patients, colonoscopy is repeated every 5-10 years. High-risk patients are diagnosed with either one of the following lesions: tubular adenomatous ≥10 mm, ≥3 adenomatous polyps, or adenomatous polyps with either villous histology or HGD. Similarly, high-risk adenomatous polyps are known as advanced polyps as they represent the most serious type of lesion. In this case, patients are recommended to undergo colonoscopy within 3 years of the baseline screening. The prevalence of low- and high-risk adenomatous polyps in patients at average risk of CRC is 17.7% and 5.7%, respectively.

1.1.2.7 Treatment of colorectal polyps

Colorectal polyps detected through screening of patients at average risk for CRC should be removed and assessed histopathologically in order to confirm the diagnosis, exclude concurrent malignancy, exclude the potential need for further intervention, and prevent progression to invasive disease. Colorectal polyps are either removed endoscopically (e.g.: forceps polypectomy removal with or without electrocautery, snare polypectomy, or mucosectomy) and/or surgically. The polyp’s morphology and size determine the treatment modality.
example, small polyps <5mm are amenable to polypectomy with cold forceps, pedunculated polyps benefit from polypectomy with a snare, and sessile polyps may be treated by piecemeal excision with a snare.

Endoscopic polypectomy is the standard treatment modality because it has significantly lower morbidity, mortality, and costs than surgical excision\textsuperscript{123, 124}. Despite the existence of safe and effective techniques for removing almost all colorectal polyps, deciding whether a particular polyp should undergo endoscopic polypectomy is dependent on the endoscopist’s level of expertise as well as their estimation of the potential for malignancy that would require formal segmental resection with lymphadenectomy\textsuperscript{123, 125}. The recommendation is that if the endoscopist considers they cannot safely and completely remove the polyp then the procedure should be aborted and the patient should be referred to a tertiary care center where experts in endoscopic management of “complex” polyps can most likely prevent surgical excision, if appropriate\textsuperscript{126}.

1.1.2.8 “Complex” polyps

“Complex” colorectal polyps (alternatively known in the literature as “difficult” or “defiant” polyps) are polyps that are considered more technically challenging and/or associated with an increased risk of complications (e.g., perforation and bleeding) and, therefore, are deemed unsuitable for routine endoscopic polypectomy\textsuperscript{125, 127}. The following criteria are described as characteristics that typically define “complex” polyps\textsuperscript{125, 126, 128}: (1) large size >2.0 cm for either sessile or pedunculated polyps; (2) location in a wall that is difficult to access with a colonoscope, such as areas of severe diverticulosis or at the base of a diverticulum or the appendix; and (3) unfavourable distribution (e.g.: polyps wrapping around a fold in a clam-shell fashion, occupying greater than one third of the colonic circumference, or crossing over two haustral folds).

An estimated 2-15% of colorectal polyps are “complex”\textsuperscript{125, 129, 130}. Although any type of polyp can be “complex”, the majority are adenomatous (78.0-90.3%)\textsuperscript{121, 127, 131, 132}. Other common histopathological types include SSA/P (1.9-19.68%) and hyperplastic polyps (0.8-3.17%)\textsuperscript{121, 127}. Invasive adenocarcinoma is reported in 4.4-22.0% of “complex” polyps with no particular stage
Approximately 74% of patients with “complex” polyps have some grade of dysplasia. On average, “complex” polyps are >2 cm (average: 23-32mm and range 5-100mm). Generally they are reported in the right colon. The anatomical distribution of “complex” polyps is as follows: ileo-cecal valve (3.49%), cecum (21.9-46.0%), ascending colon (14.0-38.1%), hepatic flexure (5.0-8.25%), transverse colon (1.90-7.0%), splenic flexure (1.0-1.59%), descending colon (3.49-5.0%), sigmoid (13.02-12.0%), and rectum (8.25-9.0%).

“Complex” polyps are technically challenging to remove because they are associated with haemorrhage (10-13%) and perforation (0.4-0.5%). Although some experts advocate that these lesions be treated with standard endoscopic techniques, this practice is generally not advised. Alternative measures include advanced endoscopic techniques and/or surgery. Essentially, the patient’s age and comorbidities should be considered in the risk versus benefit analysis dictating the treatment approach. There are three treatment options: (1) advanced endoscopic procedures, namely endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD), (2) laparoscopic or open colorectal resection, or (3) basic endoscopic procedures with surgery known as laparoscopic assisted endoscopic polypectomy.

“Complex” polyps amenable to advanced endoscopic treatment should be treated at the time of discovery if the endoscopist is trained in these techniques. “Complex” sessile polyps are treated with EMR in 90% of cases while the remaining 10% undergo surgery. EMR consists of injecting 0.9% saline solution in the submucosa to create a fluid cushion that facilitates excision of the polyp in piecemeal fashion. Alternatively, ESD uses the same principals as EMR but the polyp is removed through en-bloc resection by using a cutting device. Surgical treatment of “complex” polyps is only recommended when the lesion is not technically removable through advanced endoscopic techniques or because the patient chooses this option. The distribution of types of surgical procedure reveals that the majority of patients undergo right hemicolectomy (72.4%) followed by sigmoidectomy (7.5%), ileocecectomy (6.9%), left hemicolectomy (4.1%), transverse colectomy (3.9%), extended right colectomy (2.0%), anterior resection (1.2%), subtotal colectomy (1.3%), or other non-anatomic resection (0.7%). Laparoscopic-colonoscopic “rendezvous” techniques, such as laparoscopically-
assisted endoscopic transluminal resection, which allow for intra- and extraluminal manipulation of the bowel wall and facilitate endoscopic polypectomy, are being studied, but have not gained widespread use\textsuperscript{137, 138}.

1.1.2.8.1 Treatment gap

Despite the fact that current treatment options for “complex” adenomatous polyps prevent progression towards CRC, two limitations exist. First, advanced endoscopic techniques are associated with significantly high failure and recurrence rates, which oblige patients to undergo multiple treatment sessions and/or ultimately surgery. EMR fails to completely remove these lesions in 13.3–23.3\% of patients\textsuperscript{139-141}. Moreover when EMR is successful, residual tissue is detected on subsequent follow ups in 27\% of patients\textsuperscript{127} and lesions recur in 3.1–46\% of patients\textsuperscript{136, 142-144}. With this in mind, treatment must improve because recent evidence has shown that the occurrence of advanced adenomatous polyp and CRC is particularly common in adenomatous polyps ≥ 2.0 cm\textsuperscript{145}. The risk of a metachronous advanced adenomatous polyps is high when adenomatous polyps measure ≥ 2.0 cm in size. Additionally, relative to patients with a baseline adenomatous polyp of <5mm, the adjusted odds for CRC for those with at least one adenomatous polyp ≥ 20 mm is 2.99 (95\% CI, 2.24–4.00).

Secondly, resorting to surgery for an adenomatous polyp, which is by definition precancerous, may seem aggressive. Historically, endoscopically challenging polyps are treated as high risk lesions with the assumption that they are at significant risk of cancer even when the biopsy reports benign disease\textsuperscript{133}. This assumption is based on the fact that certain characteristics, namely size >2 cm, increases the risk of a metachronous advanced adenoma and/or CRC\textsuperscript{145}. Accordingly referral to a surgeon for “complex” polyps generally results in surgical procedures with oncological parameters\textsuperscript{133}. The benefits of surgery to prevent progression to CRC are not without associated morbidity and mortality, which are present even with elective laparoscopic colon surgery. The perioperative morbidity and mortality rate are reported to be 20.8\% and 0.9\%, respectively\textsuperscript{131}. 
The fact that a select population of patients with adenomatous polyps is undergoing ineffective procedures and/or is being treated with what some patients may consider to be aggressive procedures emphasizes the need for a novel non-invasive therapeutic approach.

1.2 Animal models of colorectal carcinogenesis

1.2.1 Overview

*In vitro* and *in vivo* models of colorectal carcinogenesis that mimic sporadic human CRC provide researchers with the means to study novel therapeutics. Despite the fact that *in vitro* models allow for fast and simplified analyses of specific aspects of cancer cell biology, they are of limited use because they lack tissue organization, stroma, and vasculature and the environment in which they grow is non-representative of an organism due to lack of environmental factors.

Three categories of murine models of colorectal carcinogenesis are available\(^{146}\): (1) genetically engineered mouse strains, (2) orthotopic models, and (3) chemically-induced models. Genetically engineered models carry germline mutation in specific genes, such as the Min/ΔAPC-mouse strain which has a mutated *APC* gene. These mice develop benign polyps that do not progress to invasive adenocarcinoma\(^{147}\). Orthotopic models consist of implanting CRC cells, either of human or murine origin, in the colon and rectum. This model provides metastasis to the lymph nodes and liver\(^{148}\), but lacks the development of precancerous lesions and may require the use of immunodeficient mouse strains if human cells are used\(^{146}\). Chemically-induced models use carcinogens to induce precancerous lesions. This type of model recapitulates several of the key histopathological findings of early phases of colorectal carcinogenesis, but rarely produces invasive adenocarcinoma\(^{146}\). Owing to their high reproducibility and relatively simple and affordable means of production, chemically-induced animal models have become a useful platform to study sporadic colorectal carcinogenesis and to test potential therapeutics and chemoprevention agents.
1.2.2 Chemically-induced models of colorectal carcinogenesis

Colorectal carcinogenesis is induced by four classes of carcinogens\textsuperscript{146}: (1) heterocyclic amines such as 2-amino-33-methylimidazo [4,5-f]quinoline and 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine; (2) aromatic amines such as 3,2’-dimethyl-4-aminobiphenyl\textsuperscript{149}; (3) alkylnitrosamide compounds, including methylnitrosourea and N-methyl-N’-nitro-N-nitrosoguanidine; and (4) dimethylhydrazine (DMH) and its metabolite azoxymethane (AOM). Carcinogens are classified into direct- or indirect-acting agents based on the need for enzymatic activation in order to form the reactive species that will alter nucleic acids and lead to genetic mutations.

DMH and its metabolite AOM are preferred because they do not have the drawbacks of other carcinogens. Heterocyclic and aromatic amines have multi-target-organ specificity, which means they induce lesions in the colon and elsewhere, such as mammary gland and prostate\textsuperscript{150, 151}. Alkylnitrosamide compounds require elaborate modes of administration such as intrarectal injections\textsuperscript{151, 152}. Heterocyclic amines have long administration schedules (52–104 weeks) and low tumour incidence (5–55\%\textsuperscript{150, 153}). AOM has been the carcinogen of choice because of its high reproducibility and potency, simple mode of application, excellent stability in solution, and low cost\textsuperscript{154}.

1.2.2.1 The azoxymethane colorectal carcinogenesis model

AOM is an indirect-acting carcinogen that requires multiple steps of metabolic activation before it forms the DNA-reactive metabolite methylazoxymethanol (MAM)\textsuperscript{155-158} (Figure 1.3). MAM is relatively stable in blood\textsuperscript{159} and has a half-life of approximately 12 hours\textsuperscript{160}. 
Dimethylhydrazine (DMH) and its metabolite, azoxymethane (AOM), are indirect carcinogens that require metabolic activation, mainly through the cytochrome P-450 isoform CYP2E1, to form the DNA-reactive metabolite methylazoxymethanol (MAM). Multiple xenobiotic-metabolizing enzymes are required to activate these compounds through several N-oxidation and hydroxylation steps. MAM yields a methyl diazonium ion, which adds methyl groups at the O6 or N7 position of guanine (O6-methyl-deoxyguanosine and N7-methyl-deoxyguanosine) and causes a guanine to adenosine transition.

Several AOM-based protocols exist in the literature all of which describe how intraperitoneal (IP) administration in rodents causes a high incidence of colorectal lesions\textsuperscript{146}. Oral administration of AOM has no effect in inducing colon carcinogenesis\textsuperscript{162}. After IP injection, AOM is absorbed into the portal system where it reaches the liver. The metabolic activation of AOM begins when it is hydroxylated in the liver to form MAM, a process mediated by the cytochrome P-450 isoform enzyme CYP2E1\textsuperscript{163}. MAM is also activated via non-P450-dependent mechanisms, such as direct oxidation within the colon\textsuperscript{163, 164}. After MAM is created in the liver, it is excreted via two routes: (1) into the bile after which it enters the small intestine where it can
occasionally induce tumours distal to the entrance of the bile duct and (2) into systemic blood circulation through which it eventually reaches the colon. Once MAM reaches the colonic crypt it yields methylidiazonium ions. The ions then methylate DNA bases by adding methyl groups at the O6 or N7 position of guanine (O6-methyl-deoxyguanosine and N7-methyl-deoxyguanosine), causing a guanine to adenosine transition that has promutagenic effects.

AOM-induced mutations result in colorectal carcinogenesis by affecting several genes typical of the adenoma-carcinoma sequence. AOM induces β-catenin mutations at codons 33 and 41, which leads to an accumulation and activation of Wnt signalling pathway. AOM also causes a KRAS gene transversion mutation from G:C to A:T at codon 12 that changes glycine to aspartic acid and activates the KRAS protein p21. This protein regulates the PI3K/Akt and MAPK-ERK pathway, two intracellular signal pathways that control cell proliferation. Activated PI3K/Akt increases cell survival pathways by phosphorylating the downstream targets nuclear factor κB (NFκB), and Bcl-xl, decreases apoptosis by blocking p53 and the forkhead/Fas-ligand, and increases cell proliferation by promoting cyclin D1 and myc. MAPK and ERK promote cell proliferation through proteins such as c-myc, CREB, RSK, Mcl1, p16, Rb, and cyclins. AOM also disrupts the anti-tumour and apoptosis effects of the TGF-β pathway. AOM decreases the active form of TGF-β thereby decreasing its regulatory effects on receptor-regulated SMAD (R-SMAD), death associated protein 6, and the p85 subunit of PI3K/Akt.

Dose-dependent effects and variations between mouse strains, such as A/J, SWR/J, FVBN, C57Bl/6, BALB/cJ, and AKR/J, sex, and age have been described in the literature. AOM is also described to cause dose-dependent fulminant hepatic failure in C57BL/6J mice at doses of 50-200 μg/g as early as 28.5±1.4 hours after administration. Despite the high level of reproducibility and similarities with human disease, using AOM alone has a major limitation: it takes 20 to 50 weeks to induce tumours (latency period).
1.2.2.1.1 The azoxymethane and dextran sodium sulfate animal model

AOM was combined with dextran sodium sulfate (DSS) in order to accelerate carcinogenesis. DSS is a polymer of sulfated glucose that directly induces an intestinal inflammatory response. After mice received AOM they were exposed to a short course of DSS (<1 week), dissolved in the drinking water and provided *ad libitum*, in order to accelerate the tumour-inducing properties of AOM, thus reducing the latency time to 10 weeks. The sequential administration of AOM followed by DSS did not alter the tumour multiplicity and tumour histopathological and molecular features that are induced by AOM alone. The AOM/DSS model is reproducible in both inbred and outbred strains of mice.

DSS induces colitis through two sequential mechanisms of action; first by inducing erosion of the colonic mucosa and then by stimulating an acute inflammatory response against it. The earliest histologic effects of DSS are focal destruction of mucin content with subsequent cytotoxicity of the epithelium. The process begins with focal damage to the basal one-third of the crypt on day 3 followed by complete erosion. Regeneration of the colonic mucosa is a relatively slow process that begins at about two weeks after suspending DSS. Parallel to these changes is a progressive increase in intestinal permeability, which further facilitates distribution of DSS.

An acute inflammatory response begins once the mucosa has been disrupted. DSS leads to increased levels of macrophage-derived inflammatory cytokines, including interleukin (IL)-1β, IL-6, and tumour necrosis factor (TNF), granulocyte-macrophage colony-stimulating factor (GM-CSF). Secondary recruitment of auxiliary effector cells, mainly neutrophils, amplifies the initial immune response and cause excessive non-specific injury. The intestinal microflora plays no role in this process because DSS induces colitis in germ-free mice. The acquired immune system is also not involved in acute DSS-induced colitis because the same effects are observed in both immunocompetent and severe combined immunodeficiency (SCID) mice. Acute DSS colitis is characterized by weight loss, bloody diarrhoea, colon shortening, and histopathological changes, including distorted and irregularly distributed colonic crypts, ulcerations, and dense infiltration by polymorphonuclear and mononuclear cells.
DSS-induced inflammation promotes the carcinogenic effects induced by the AOM through several mechanisms of action. These include induction of genomic instability, alterations in epigenetic events and subsequent inappropriate gene expression, enhanced proliferation of initiated cells, resistance to apoptosis, and production of inflammation-induced reactive oxygen and nitrogen species\(^1\). DSS also stimulates the production of several inflammatory factors with cancer-promoting properties, such as NFκB and TNF-α\(^1\).\(^2\).\(^3\).

Colons of mice exposed to AOM and DSS develop precancerous lesions that mimic both micro- and macroscopically human colorectal carcinogenesis\(^1\). Single-crypt adenomas cluster together and display dysplastic features to form microadenomas. As microadenomas grow, they become grossly visible nodular, polypoid, or caterpillar-like adenomatous polyps, which are limited to the middle and distal third\(^1\). The middle third of the colon acts as an area of transition because fewer polyps develop than in the distal colon. Microscopically, the polyps display both LGD and HGD with histopathological characteristics resembling human disease. Notably LGD tends to develop in the periphery of polyps adjacent to normal colonic mucosa whereas HGD predominates in the central part\(^1\). Despite the fact that adenomatous polyps give rise to invasive adenocarcinoma in humans, AOM and DSS-induced adenomatous polyps rarely show signs of invasiveness into the submucosa\(^1\). The lack of submucosal invasion is not an effect of DSS as models that only use AOM also lack invasive adenocarcinoma\(^1\).\(^2\).

1.3 Oncolytic virotherapy

1.3.1 Overview

Oncolytic virotherapy is an innovative therapeutic that uses replication-competent viruses to eradicate tumours. Oncolytic viruses (OVs) preferentially infect cancer cells due to innate and/or genetically engineered properties. OV infection is further facilitated by the fact that tumours have impaired anti-viral defense mechanisms to impede viral protein translation or induce apoptosis after infection\(^1\).\(^2\).\(^3\). OVs kill cancer cells through various mechanisms of action, such as direct virus-mediated cytotoxicity, tumour-specific vascular shutdown\(^1\), or induction of
innate and adaptive immune responses\textsuperscript{204}. Normal cells are unharmed because they can suppress viral gene expression and induce apoptosis to prevent viral replication.

Viruses have been pursued as anti-cancer agents for more than a hundred years. Ever since the early observation in the 1900s that patients with leukemia, Burkitt’s lymphoma, and Hodgkin’s lymphoma exhibited complete regression after spontaneous viral infection or vaccination\textsuperscript{205-208} exploiting viruses as an anti-cancer therapy has been appealing. Initial studies were rudimentary and consisted of exposing cancer patients with infectious body fluids or tissue harvested from other patients with active viral disease\textsuperscript{209-211}. Investigations were eventually abandoned because of unfavourable results. Treatment failed to impact tumour growth, but when tumours did regress, more notably in immunosuppressed patients, virus-related morbidity was generally observed. Several hallmark advances in genetic engineering were necessary before researchers could further develop the notion of using viruses to eradicate tumours in a safe manner. In 1991, the first application of genetic engineering to develop an oncolytic agent from herpes simplex virus (HSV) for the treatment of glioma\textsuperscript{212} gave rise to modern day oncolytic virotherapy. Since then oncolytic virotherapy has grown, as reflected in the number of publications in the indexed literature, which has grown from only a few in the 1990s to over 200 annually in recent years. This growing body of evidence has consistently demonstrated that OVs can eradicate tumours \textit{in vitro} and \textit{in vivo} animal models with minimal toxicity. Currently, a growing number of phase I and II clinical trials with OVs from at least ten different virus families are being carried out\textsuperscript{213}. To date patients with advanced cancers that have received OVs as part of this clinical trials have generally shown minimal and transient signs of toxicity such as flu-like symptoms although with transient transaminitis and occasionally thrombocytopenia\textsuperscript{214}.

1.3.1.1 Mechanisms of cancer cell specificity

Replication-competent viruses infect cells in order to replicate as they lack organelles. OVs are tropic to cancer cells (oncotropism) in several ways. Some viruses, such as the H1 parvovirus, reovirus, Newcastle disease virus (NCDV), and mumps virus naturally infect cancer cells, whereas other viruses such as measles, adenovirus, vesicular stomatitis virus (VSV), vaccinia virus (VV), and HSV are engineered to make them tumour-specific\textsuperscript{213}. 
The first generation of OVs arose from naturally occurring viruses that innately infect cancer cells. Natural oncotropism derives from two biological features within tumours. First, entry is facilitated because some cancer cells overexpress certain receptors required by viruses. Examples of receptor-dependant OVs and their target include adenoviruses and the coxsackie adenovirus receptor (CAR)\(^{215}\), sinbis virus and laminin\(^{216}\), poliovirus species and CD155\(^{217}\), and measles virus and the membrane cofactor protein CD46 and the signaling lymphocytic activation molecule CD150\(^{218}\). Secondly, tumours have impaired anti-viral defense mechanisms, a consequence of their need to avoid immune detection and destruction\(^{202}\). For example, NCDV, VSV, myxoma virus, parvovirus H1, and raccoonpox are naturally tumour selective because of defects in the INF pathway\(^{219-221}\).

Genetic engineering addressed the need to obtain a higher level of oncotropism and brought forth the second generation of OVs. In general three strategies have been used to date\(^{222}\). The first entails deleting viral genes critical for virus replication in healthy cells, but expendable in cancer cells. The second strategy involves adding tumour-specific promoters upstream of critical virus replication genes. The third approach is transductional targeting in which viral surface proteins are modified in order to make them dependent on receptors overexpressed in cancer cells.

1.3.1.2 Mechanisms of action of oncolytic viruses

OVs kill cancer cells through various mechanisms of action each of which produces direct or indirect cancer cell death (Figure 1.4). These mechanisms of action are either innate or derive from genetic engineering. Some mechanisms of action are common to all OVs irrespective of the species or strain, while others are specific to the genetically engineered construct.
Vaccinia delivered to the tumour through the vascular system can produce an antitumour effect through multiple mechanisms, which include viral infection and tissue destruction. This leads to release of cytokines (blue symbols), danger signals (yellow symbols) and antigens (red symbols) that can stimulate the innate and adaptive immune responses. Viral infection of tumour cells leads to replication of the virus and viral spread through and between tumours. Viral infection in and around tumour endothelial cells leads to vascular collapse. Endothelial cells are destroyed either as a result of direct infection with virus, or subsequent to infection of surrounding tumour cells, which leads to infiltration of neutrophils into the tumour and thrombosis.
OVs were developed to specifically grow in cancer cells with the primary objective of killing them via oncolysis. In this process, a cancer cell hosting OV replication dies because its cell membrane is disrupted as an excessive amount of viral particles bud from it and cytotoxic viral proteins and virions accumulate in the cytoplasm\textsuperscript{224}. These cytotoxic proteins also increase the tumour’s sensitivity to TNF-mediated death\textsuperscript{225}. Oncolysis is necessary for viral progeny to spread throughout the tumour microenvironment and reach uninfected cells.

Indirect cancer cell death occurs through virus-mediated effects on the tumour vasculature and immune system. Recently, innate anti-tumour vascular effects have been identified for several OVs, including adenovirus, VV, HSV, and VSV\textsuperscript{203}. These properties include the ability to directly infect tumour endothelial cells, express viral proteins with anti-angiogenic properties, or induce virus-mediated immune responses that cause neutrophil accumulation inside blood vessels. All of these mechanisms lead to decreased perfusion and necrosis in the tumour.

Despite the existing local immunosuppression inside the tumour, OVs are capable of stimulating an immune response against the tumour\textsuperscript{226}. After the cancer cell is lysed cellular danger signals [danger-associated molecular pattern molecules (DAMPs)], viral danger signals [pathogen-associated molecular pattern molecules (PAMPs)], as well as virus- and tumour-associated antigens are released\textsuperscript{223}. The presence of tumour antigens induces an adaptive immune response against the tumour. Additionally, the anti-viral cytokines and IFNs released by tumour-infiltrating immune cells kill neighbouring uninfected cancer cells, a process known as the bystander effect\textsuperscript{204}. Together these factors are expected to clear minimal residual disease and provide long-term surveillance against relapse\textsuperscript{223, 227}.

1.3.1.3 The tumour microenvironment and oncolytic virotherapy

The tumour microenvironment impacts the delivery of OVs as they travel through a complex network of cells and a dense extracellular matrix. Delivery is limited by the interlocked meshwork of secreted proteins and glycoproteins. Tumour vasculature, which typically results in poor perfusion\textsuperscript{228}, can also limit systemic delivery of certain OVs. Hypoxia and acidosis, which develop throughout the tumour as a result of vascular abnormalities, can also decrease replication of some OVs\textsuperscript{229}. 
Despite the fact that tumours have impaired innate anti-viral responses, they contain immune cells that are competent at controlling an infectious process\textsuperscript{204}. Upon infection, tumour-infiltrating immune cells respond by secreting potent anti-viral cytokines such as IFN-\textgreek{a} and \textgreek{b}\textsuperscript{204, 230, 231}. This innate activation creates a pro-inflammatory state that facilitates the influx of natural killer cells and dendritic cells\textsuperscript{232}. OV-activated macrophages and dendritic cells secrete additional anti-viral and pro-inflammatory cytokines, including IL-12, IL-6, and TNF-\textgreek{a}\textsuperscript{233}, which in turn recruit and activate more immune cells\textsuperscript{234}. Altogether this directly limits viral replication, lysing ability, and spread throughout the tumour.

1.3.2 Oncolytic vaccinia virus

1.3.2.1 Overview

VV is one of several viruses currently being developed for oncolytic virotherapy. The distinctive characteristics that make VV more attractive and safer over other candidate agents are listed as follows:

- There is extensive knowledge about this virus as it was the first animal virus species observed microscopically, grown in tissue culture, accurately titered, physically purified, and chemically analyzed\textsuperscript{235}.

- VV has an extensive host species range, enabling it to infect and replicate in almost all types of human and many other species’ cells\textsuperscript{236}; therefore it can be studied in various syngeneic animal models.

- Because VV completely replicates in the cytoplasm, its genome never enters the host cell nucleus\textsuperscript{235}; consequently the possibility of chromosomal integration is eliminated.

- VV can be engineered with standard DNA manipulation techniques to create recombinants that carry and efficiently express up to 25kb of transgenes\textsuperscript{237, 238}.
• VV has biological characteristics that favour its spread in permissive cells\textsuperscript{223}. The virus is highly infectious, replicates specifically and quickly in permissive cells, and spreads efficiently from cell to cell after lysis\textsuperscript{239}. Spread to distant permissive tissues is possible because the virus has intravenous stability. As VV replicates, it has different infectious forms some of which hide the viral antigens, thereby providing resistance to antibody\textsuperscript{240} and complement-mediated neutralization\textsuperscript{241}.

• Mass production is feasible because it produces relatively high titers and is stable over time\textsuperscript{222}. VV can be stored in frozen solution or dry powder for prolonged periods of time without significantly losing infectivity\textsuperscript{222}.

• Finally, VV has the longest and most extensive history of use in humans of any virus due to its role in eradicating smallpox\textsuperscript{222}; therefore there is extensive information on adverse events. Additionally, in the unlikely case of uncontrolled VV replication several clinically approved and experimental anti-viral agents are available, including vaccinia immunoglobulin, cidofovir, ST-246, and tyrosine kinase inhibitors\textsuperscript{238}.

1.3.2.1.1 Classification, history, and origin of vaccinia virus

VV is a member of the \textit{Poxviridae} family. Poxviruses are large (\(\sim 360 \times 270 \times 250\) nm) brick-shaped viral particles that replicate in the cytoplasm\textsuperscript{235, 242}. Members of this family have a single linear double-stranded DNA genome of 130 – 300 kilobase pairs. It encodes approximately 176 to 266 proteins, including enzymes and factors critical for self-replication and maturation\textsuperscript{243}. The central region of the genome contains highly conserved genes that are essential for viral replication while the terminal regions carry less conserved genes that are important for virus-host interactions\textsuperscript{243}. VV is a member of the subfamily \textit{Chordopoxvirinae}, which specifically infect vertebrates, and the genus \textit{Orthopoxvirus}, which infect mammals. Notable examples of other orthopoxviruses include variola virus, cowpox virus, monkeypox virus, and raccoonpox virus\textsuperscript{235}.

Orthopoxviruses are antigenically related\textsuperscript{243}; therefore prior infection with one orthopoxvirus provides protection against other members of the genus\textsuperscript{244-246}. This feature provided the basis for preventing smallpox, an acute, highly contagious, and often fatal disease caused by variola virus.
It was only during a worldwide campaign led by the WHO that smallpox was eradicated by 1980 through VV-based vaccines\textsuperscript{247}.

Infection with VV results in overt diseases in humans, which can be mild and self-limited, such as generalized vaccinia, or severe and life-threatening as eczema vaccinatum, progressive vaccinia, and postvaccinial encephalitis\textsuperscript{248}. Infection with VV only results from direct inoculation\textsuperscript{248}; no consistent data indicates that the virus is transmitted through aerosolization\textsuperscript{249}. Because VV is not present naturally, cases of infection occur in smallpox vaccinees\textsuperscript{250}. Other populations at risk include close contacts of vaccinees inadvertently inoculated through an unhealed vaccination site\textsuperscript{248}, research or laboratory staff\textsuperscript{251}, and dairy workers in areas where zoonotic outbreaks are occasionally reported\textsuperscript{252}.

1.3.2.1.2 Replication cycle of vaccinia virus

VV’s replication cycle occurs in the cytoplasm of infected cells (\textbf{Figure 1.5}). A full replication cycle takes approximately 24 hours, and as many as 10,000 new virions are released from an infected cell\textsuperscript{235}. The cycle is characterized by three waves of viral mRNA and protein synthesis, which are named early, intermediate, and late transcription. In each one of these phases the virus acquires different morphogenesis\textsuperscript{236}. The cycle beings when VV binds to the cell surface. Several viral proteins are crucial for binding of the virion to the cell surface. The cell determinants of binding are ubiquitously expressed carbohydrate moiety of glycoproteins such as heparin sulfate and chondroitin sulfate\textsuperscript{253-257}. After binding, VV enters the cell through ubiquitous macropinocytosis with subsequent fusion of the plasma membrane via a low pH-dependent endosomal pathway\textsuperscript{258}. The virus core is released into the cytoplasm, a process mediated by the virion protein VV-A28\textsuperscript{259}. After virus uptake, infection-specific cytoplasmic domains, known as “viral factories”, are created. These discrete granular foci lacking cellular organelles act as a site where viral DNA replicates and early progeny are assembled\textsuperscript{260}.
All poxviruses replicate in the cytoplasm of infected cells by a complex, but largely conserved, morphogenic pathway. Two distinct infectious virus particles — the intracellular mature virus (IMV) and the extracellular enveloped virus (EEV) — can initiate infection. The IMV and EEV virions differ in their surface glycoproteins and in the number of wrapping membranes. The binding of the virion is determined by several virion proteins and by glycosaminoglycans (GAGs) on the surface of the target cell or by components of the extracellular matrix. Fully permissive viral replication is characterized by three waves of viral mRNA and protein synthesis (known as early, intermediate and late), which are followed by morphogenesis of infectious particles. The initial IMV is transported via microtubules (not shown in the figure) and is wrapped with Golgi-derived membrane, after which it is referred to as an intracellular enveloped virus (IEV). The IEV fuses to the cell surface membrane to form cell-associated enveloped virus (CEV; not shown), which is either extruded away from the cell by actin-tail polymerization (not shown) or is released to form free EEV. EEV might also form by direct budding of IMV, therefore
bypassing the IEV form. Poxviruses also express a range of extracellular and intracellular modulators, some of which are defined as host-range factors that are required to complete the viral replication cycle. Poxviruses can be markedly diverse in their portfolio of specific modulators and host-range factors, which determine tropism and host range. Non-permissive poxvirus infections generally abort at a point downstream of the binding/fusion step.

In the next step, early viral gene expression begins as the endogenous RNA polymerase and encapsidated transcription factors synthesize viral mRNA under the control of early promoters. Translation products of early mRNA in conjunction with host factors induce the dissolution of the core structure, a process known as core uncoating. This releases the viral DNA into the cytoplasm where it subsequently acts as the template for DNA replication as well as intermediate and late transcription.

Unlike early transcription, which is controlled by viral transcriptosome factors encapsulated in the virus core, both intermediate and late transcription require host-derived transcription factors. Intermediate mRNAs are translated to produce late mRNAs, which are then translated into structural and nonstructural viral proteins. These proteins, in conjunction with DNA concatemers formed early on during the replication cycle, are assembled into genomic DNA. At this point the virus exists in two states, a non-infectious precursor known as crescents and the immature virion (IV).

During replication, VV evolves through four mature forms, each differing in the number of membranes, types of surface antigens, and role in the infection process. Inside the cytoplasmic factories, crescents and IVs become intracellular mature virus (IMV) virions. IMVs have a single lipid bilayer envelope and are the earliest and most abundant infectious form. This form is only infectious after the host cell is lysed and contributes to close range infection. Although the majority of IMVs remain inside the cell until lysis, some travel throughout the cytoplasm via microtubules where they are wrapped with a double layer of Golgi-derived membranes. At this point the virus acquires the form of intracellular enveloped virus (IEV). The IEV then moves to the cell surface where it loses one of its outer membranes as it fuses with the cell membrane. In this stage, VV takes the form of cell-associated enveloped virus (CEV), which remains attached to the cell or induces polymerization of actin to form filaments that directly
transfer it to adjacent cells. Once the CEV becomes dissociated from the cell membrane it becomes known as extracellular enveloped virus (EEV). Notably, EEV can also form by direct budding of IMV, bypassing the IEV form. Both the CEV and EEV have an extra lipid bilayer that derives from the host cell. The importance of this is that it hides the majority of viral antigens and provides the virus with host complement control proteins. Because of this the immune system cannot recognize CEVs and EEVs and the virus gains the ability of long-range infection.

1.3.2.1.3 Cellular tropism of vaccinia virus

VV’s tropism is considerably different from that of other viruses. VV does not require specific host-cell receptors for adsorption and fusion events, internalization of the core, or initiation of early transcription. Instead tropism is mediated by several intracellular characteristics that pertain to the host’s cell cycle status, complementing host factors, and signal transduction pathways. Because of this feature, VV can infect a wide range of animal hosts that include species of many mammalian orders, including rodents, rabbits, and primates. Despite being able to infect many cells, replication varies between cells. Once infected with VV, cells either allow full replication, and are known as permissive cells, or impede the process, making them restrictive cells. Permissive cells are in S phase of the cell cycle and provide VV with the necessary components to assemble and shed progeny, including core un-coating factors, nucleotides, factors for intermediate and late transcription, and machinery for microtubule and actin-based motility. Non-permissive poxvirus infections are stopped downstream of the binding/fusion step.

VV has the potential to counteract restrictive cells. For example, VV expresses growth factor homologues of EGF that act in a paracrine manner on neighbouring uninfected cells in order to change them from quiescence into S-phase. Another property is the ability of the virus to overcome the host cell and immune system’s anti-viral responses by encoding factors with immunomodulatory or anti-apoptotic affects. Anti-IFN strategies include inhibitors of IFN induction, receptors that mimic scavenger IFN ligands, phosphatases that block the STAT mediated signal-transduction pathway, and inhibitors of the IFN-induced protein mediators of the anti-viral state, such as PKR. VV inhibits the activation of pro-inflammatory signalling
cascades, such as those transduced by NFκB\textsuperscript{280, 281} and encodes proteins that inhibit apoptosis, including M-T7/B8R, B18R, E3L, K4L\textsuperscript{282}.

1.3.2.1.4 Developing vaccinia virus as an oncolytic agent

1.3.2.1.4.1 Overview

Wild type VV naturally infects and destroys tumours as it replicates. In the past, it was tested as a treatment for human cancers in several case reports and clinical trials. Intratumoural injections in patients with melanoma led to regression of most of the injected lesions and, in some cases, non-injected lesions\textsuperscript{283-287}. Patients with bladder cancer\textsuperscript{288}, metastatic lung and kidney cancer\textsuperscript{289}, as well as multiple myeloma\textsuperscript{290} were reported to have similar results. Given the noteworthy observations that VV could naturally kill cancer cells, attaining a higher level of specificity was the next step. By genetically modifying VV, not only would oncolysis become more efficient but it would also enhance its safety profile.

VV’s oncolytic potential depends on the strain from which it derives. Each strain differs in terms of pathogenicity and host range\textsuperscript{223} (Table 1.2). Different strains of VV, including Lister, New York City Board of Health (NYCBH), Wyeth, Western Reserve (WR), and modified vaccinia Ankara (MVA), are available because each vaccine manufacturing centre developed its own production scheme in response to the growing demand during the smallpox vaccination campaign\textsuperscript{260}. Strains with no oncolytic potential include MVA and NYVAC because they do not replicate in mammalian cells. Lister, Wyeth, and WR strains have oncolytic potential. The Lister strain was used in conjunction with the NYCBH strain during the WHO global eradication campaign\textsuperscript{250}. The Wyeth strain is a NYCBH derivative produced by Wyeth laboratories for experimental smallpox vaccines\textsuperscript{291}. Both the Lister and Wyeth strain have low virulence, but pose difficulties for mass production. Many OV research groups have opted for the WR strain, a laboratory derivate of the NYCBH strain after passage in mouse brains because it produces more efficient tissue destruction than other VV strains\textsuperscript{292}. In vitro, the WR strain forms large plaques, reflecting its ability to spread efficiently and cause large areas of destruction \textsuperscript{222}. Additionally there is potentially a lower risk of anti-viral immune response with this strain because it produces
a larger amount of CEV relative to the other strains. The only disadvantage of the WR strain is that because it was not used as a vaccine strain there is limited information on its human safety profile.

Table 1.3. Wild type vaccinia virus strains used as backbones for oncolytic viral agents.


<table>
<thead>
<tr>
<th>Strain</th>
<th>Background</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wyeth or NYCBH</td>
<td>North American vaccine strain</td>
<td>Most commonly used clinical strains</td>
<td>Minimal inherent tumour selectivity <em>in vitro</em></td>
</tr>
<tr>
<td>Lister</td>
<td>European (UK) vaccine strain</td>
<td>Extensive use during smallpox eradication</td>
<td>No inherent tumour selectivity <em>in vitro</em></td>
</tr>
<tr>
<td>Copenhagen</td>
<td>Northern European vaccine strain</td>
<td><em>In vitro</em> tumour selectivity; history as a vaccine</td>
<td>Withdrawn during smallpox eradication programme owing to toxicity</td>
</tr>
<tr>
<td>Western Reserve</td>
<td>Primarily laboratory strain derived from Wyeth after passage in mice</td>
<td>Increased lytic ability and inherent selectivity <em>in vitro</em></td>
<td>Minimal clinical use in humans</td>
</tr>
<tr>
<td>Tian Tan</td>
<td>Chinese vaccine strain</td>
<td>Extensive use during smallpox eradication</td>
<td>Unknown potential as oncolytic agent (some use as cancer vaccine)</td>
</tr>
<tr>
<td>MVA</td>
<td>Vaccine strain derived from Ankara strain after passage in avian cells</td>
<td>Increased safety but retains vaccine potential</td>
<td>Will not replicate in mammalian cells, so has no oncolytic potential</td>
</tr>
<tr>
<td>NYVAC</td>
<td>Vaccine strain derived from Copenhagen with 18 gene deletions</td>
<td>Increased safety but retains vaccine potential</td>
<td>Will not replicate in mammalian cells, so has no oncolytic potential</td>
</tr>
</tbody>
</table>

Abbreviations: NYCBH, New York City Board of Health; MVA, modified vaccinia Ankara

Of the three general approaches used in oncolytic virotherapy to attain oncotropism, only one has been feasible for VV. Attenuated oncolytic VVs were developed by deleting viral genes
critical for its replication in normal cells, but expendable in cancer cells. Deletions in one or more of the following nonessential genes have been used in to create oncolytic VVs: *J2R* encoding thymidine kinase (TK)\(^{293, 294}\), *C11R* encoding vaccinia growth factor (VGF)\(^{294}\), *A56R* encoding for hemagglutinin\(^{295}\), and *B22R/B13* encoding serpin-1 and serpin-2\(^{239}\). Of these deletions, TK or VGF, or a combination of both, have been the most widely used thus far.

### 1.3.2.1.4.2 Deletion of thymidine kinase

TK is a family of enzymes that regulate the synthesis of deoxyribonucleotides\(^{296}\). TK exists in two isoforms: (1) TK1 is present in the cytoplasm in anticipation of mitosis (cell cycle-dependent) and (2) TK2 is located in mitochondria for mitochondrial DNA synthesis (cell cycle-independent). Given that nucleotides are essential components of DNA synthesis and that the amount of nucleotides ingested by cells is not enough to meet their needs, cells, whether they are normal or not, recycle nucleotides to guarantee a source\(^{297}\). Ribonucleotide reductase (RNR) is an enzyme that catalyzes the formation of deoxyribonucleoside triphosphate (dNTP) from ribonucleotides. dNTPs are used for DNA synthesis whereas deoxythymidine triphosphate (dTTP) regulates RNR’s specificity\(^{298}\). In order for DNA replication to adequately occur, dTTP is continuously required. Aside from the complex *de novo* synthesis of dTTP through uridine diphosphate (UDP) reduction, dTTP is provided through salvage of deoxyribonucleoside thymidine, a process catalyzed by TK.

Deleting the viral TK gene attenuates VV by making it specifically replicate in cells that are actively dividing and can provide it with a continuous supply of TK. Cancer cells become the optimal host as they constitutively overexpress TK irrespective of their proliferation status\(^{297}\). In contrast, normal cells have a strict control over their TK levels and only express it during S phase of the cell cycle\(^{296}\). Additionally; viral replication is inhibited in normal cells as their antiviral defense mechanisms are intact. Biodistribution analyses in several tumour animal models confirm that this deletion significantly improves tumour selectivity\(^{222, 293, 294, 299, 300}\). When compared to wild type VV, TK negative VV significantly prolongs the survival of mice bearing subcutaneous tumours\(^{294}\).
1.3.2.1.4.3  Deletion of the vaccinia growth factor

VGF, a secretory, glycosylated protein encoded by one of VV’s early genes C11R, shares amino acid sequence homology and functional properties with cellular growth factors EGF and TGF-α. VGF acts as a mitogen on uninfected cells in order to facilitate the spread of the infection. VGF is produced and secreted early on after the host cell is infected. After secretion it binds to EGF receptors (EGFRs) on surrounding non-infected cells where it induces phosphorylation of the receptor and enhancement of its tyrosine-specific kinase activity thereby stimulating cell growth. It is unknown whether VGF is capable of autocrine signaling.

Deletion of VGF attenuates VV by limiting the spread of the virus. Although VGF negative VV yields the same amount of virus in vitro as wild type VV in replicating cells, it significantly reduces the virus yield in non-proliferating cells. Deletion of VGF has a greater impact in vivo. Measurement of the level of virus infectivity in the brain and the lethal dose for 50% of the population (LD50) reveals that VGF negative VV has lower levels of infectivity and a greater LD50 than wild type virus. Moreover, higher doses of VGF negative VV than wild type VV are necessary to produce skin lesions in rabbits. In vivo experiments with chicken embryos indicate that when compared to wild type virus, VGF negative VV-induced lesions contain fewer proliferating cells, antigen, and amount of virus infection.

1.3.2.1.4.4  Double deleted vaccinia virus (vvDD)

Given the success with individual deletions of TK and VGF genes it was hypothesized that combining both deletions would enhance oncotropism through synergistic mechanisms as well as improve its safety profile. Replication of VV would only occur in actively dividing cells, notably cancer cells, because in the absence of viral TK VV will only obtain TTP from dividing cell and the lack of VGF will impede the virus from priming resting cells to undergo mitosis. WR strain VV deleted of both its TK and VGF genes is known as the double deleted VV or vvDD.
Wild type vaccinia virus (VV), encodes viral thymidine kinase (vTK), a nucleotide synthesizing enzyme, which works in conjunction with cellular TK (cTK) to increase the nucleotide pool necessary for DNA replication. The virus also encodes vaccinia growth factor (VGF), an epidermal growth factor (EGF)-homologue that acts as a mitogen to prime neighboring uninfected cells with the aim of preparing them to host newly developed viral progeny. Double deleted vaccinia virus (vvDD) has been genetically modified to increase its cancer specificity by deleting TK and VGF. These deletions make this OV specifically replicate in cells with upregulated proliferation pathways. By deleting the vTK gene VV can only replicate in cells that are actively dividing and constitutively overexpress cTK during all phases of the cell cycle. Normal cells are not permissive for VV replication because cTK is generally down-regulated during the majority of the phases of the cell cycle. Even when cTK is expressed during the S phase of normal cells, they are capable of shutting down the viral replication as their viral defense mechanisms are intact. By deleting the VGF, the virus has no effect on uninfected cells.

vvDD has significant oncotropism relative to VVs with a single deletion in either TK or VGF\(^ {294}\). Biodistribution analyses confirm tumour selectivity over normal tissue. Remarkably significantly high viral titres were seen in ovarian follicles. This phenomenon is suggested to result from increased virus delivery because ovaries, like tumours, have hyperpermeable vasculature\(^ {306,307} \). Infection of the ovaries induced minimal to severe necrosis and/or fibrinous inflammation and haemorrhage\(^ {294} \). Although vvDD can potentially replicate in normal cells undergoing mitosis as their TK activity increases during G1/S phase\(^ {296} \), other tissue with high proliferation rates, such as bone marrow, are not prone to infection. vvDD’s short- and long-term safety profile was described in rhesus macaques\(^ {308} \). At doses up to \(10^9\) plaque forming units
(pfu), vvDD did not result in visible pox lesions, clinical signs or symptoms of viremia, or shedding in tissues, serum, saliva, urine, or feces, suggesting it may be a good candidate for systemic treatment of human tumours.

Deleting both TK and VGF does not impair VV’s anti-tumour effect. In fact vvDD has significantly higher anti-tumour activity than viruses with single deletions\(^{294}\). The killing capacity is directly linked to virus replication because vvDD does not carry therapeutic genes. The effects are not a result of a bystander anti-tumour immune response as vvDD was tested in athymic/nude mice and significantly prolonged survival rates. The direct cytopathic effects of this virus have been confirmed in both human and animal cancer cell lines\(^{309-314}\). In vitro and in vivo animal models of peritoneal carcinomatosis\(^{311}\), glioma\(^{313}\), myeloma\(^{315}\), and malignant mesothelioma\(^{310}\) demonstrate that vvDD specifically infects cancer cells and leads to improved survival. Recently the anti-tumour vascular effects of this vvDD have been described\(^{203}\). Immunohistochemical analyses confirm that vvDD infects tumour vascular endothelial cells.

### 1.3.3 Oncolytic virotherapy for the treatment of precancerous lesions

Several of the histopathological and molecular features that facilitate replication of vvDD in cancer cells are also present in pre-cancerous lesions, such as colonic dysplasia. Colonic dysplasia, like invasive adenocarcinoma, is a highly proliferative tissue with upregulated growth factor signaling pathways, such as EGFRs\(^{316-318}\). For example, EGFR tyrosine kinase is increased in colonic mucosa by 35.2% in patients with adenomatous polyps\(^{317}\). This is paralleled by upregulation of TK1\(^{319, 320}\) and high concentrations of nucleotides in dysplastic cells\(^{321}\). Immunohistochemistry (IHC) staining with Ki-67, a marker of proliferation, confirms that the number of actively dividing cells significantly increases from LGD to HGD\(^{322-324}\). Altogether these features suggest that vvDD may have the capacity for tissue-specific killing of colonic dysplasia.

To date, oncolytic adenovirus is the only virus that has been tested as a treatment against pre-cancerous lesions, specifically oral dysplasia. Oncolytic adenoviruses are mainly derived from the serotype 5 of species C. Modifications to make it tumour specific are generally made to
capsid proteins, particularly the fibre knob. Two studies have used oncolytic adenovirus in the treatment of oral dysplasia at a pre- and clinical level.

Gaballah et al developed an in vitro model from patient-derived normal tissue and dysplastic oral epithelium of different histological grades. This study used dl922-947, a pRb-binding deficient conditionally replicating oncolytic adenovirus with deletion of its E1A proteins. As a result dl922-947 only replicates in cells where E2F is deregulated such as dysplastic and cancer cells. dl922-947 resulted in lysis only of oral dysplasia; the normal tissue remained resistant to treatment. Rudin et al reported on a phase I clinical trial in which a cohort of patients with oral dysplasia were treated with multiple 30-minute treatment sessions of mouthwash containing ONYX-015. ONYX-015 is an oncolytic adenovirus designed to preferentially replicate in and destroy p53-mutant cells. Efficacy was measured through histologic response. Toxicity did not exceed grade 2 (febrile episode in one patient) and the maximum tolerated dose was $10^{11}$ pfu/day. Disease resolution, as confirmed by histology, was observed in 7 (37%) of 19 patients. In addition, one patient had improvement in the grade of dysplasia.
1.4 Hypotheses and specific aims

vvDD is a modified WR strain VV deleted of its TK and VGF genes that specifically lyses cancer cells. Histopathological and molecular features of cancer that facilitate vvDD replication are present in colonic dysplasia. Notable characteristics include a high proliferation rate, increased cellular TK levels, and a large pool of nucleotides secondary to upregulated growth factor signaling pathways such as EGFR. Hence the hypothesis of this project is that vvDD will specifically infect, replicate within, and lyse dysplastic colonic cells, similarly to how it acts in cancer cells. In order to test this hypothesis, this study was carried out in three specific aims:

1. Specific aim 1: Characterize the murine model of colonic dysplasia induced by azoxymethane and 2% dextran sodium sulfate

2. Specific aim 2: Determine the specificity of red fluorescent protein expressing double deleted VV (vvDD) towards murine colonic dysplasia

3. Specific aim 3: Determine the efficacy of red fluorescent protein expressing double deleted VV (vvDD) as a treatment for murine colonic dysplasia
Chapter 2
Material and Methods

2.1 Animal model

2.1.1 Animal housing

All animal experimental protocols were approved by the Animal Care Committee of the University Health Network, Toronto, ON, Canada and followed the Canadian Council on Animal Care guidelines. Three-week old male CD-1 mice were purchased from Charles River (Charles River Canada, Saint-Constant, QC, Canada). All animals were housed under standard conditions in cages (≤ 5 mice per cage) with a 12 hour light-day cycle and ad libitum access to 5% irradiated food and water. Mice acclimated for at least 5 days before the start of the experiment.

2.1.2 Experimental protocol

The AOM and 2% DSS model was previously described in detail by Suzuki et al\textsuperscript{199}. In brief, mice received a single IP injection of AOM (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) at a dose of 10 mg/kg body weight, which was diluted in distilled autoclaved water. One week later, mice were exposed to a seven day cycle of 2% (w/v) DSS with a molecular weight of 36,000-50,000 (MP Biomedicals, LLC, Solon, OH, USA) in the drinking water to which they had ad libitum access. DSS drinking water was changed every other day. The AOM and DSS used for each cohort of animals were obtained from a single production lot. Normal food was available throughout the experiment. Mice returned to regular drinking water after the DSS cycle.
2.1.3 Animal health

Animals were evaluated every other day except when they were on DSS, during the two weeks post-DSS, and when signs of morbidity developed, in which case they were evaluated daily. Attention was focused primarily on (1) body weight; (2) clinical signs of dehydration such as skin lacking elasticity; (3) characteristics of stool to monitor for presence of gross blood; and (4) presence and type of prolapse (reducible versus irreducible) as well as the characteristics of the rectal mucosa when the rectal prolapse developed. Fluid volume loss was corrected with a subcutaneous injection of 0.5-1.0 mL of 0.9% sodium chloride solution. Animals were euthanized with carbon dioxide (CO₂) overdose in the following cases: hunched or abnormal posture; failure to groom; lethargy; weight loss of >20% relative to baseline; anorexia and dehydration that could not be alleviated; disease burden that compromised normal behaviour, ambulation, food and water intake; ulcerated rectal mucosa; permanent rectal prolapse; and excessive, persistent rectal bleeding, as per the animal usage protocol.

2.1.4 Histopathological characterization of the animal model

To characterize the histopathological features of the animal model, twenty-one animals were exposed to AOM and 2% DSS as previously described. Three mice were randomly selected and euthanized by CO₂ overdose at weeks 4, 6, 8, 10, 12, 14, and 16 (Figure 2.1). For the purpose of referring to timing in this thesis, the number of the week is relative to AOM injection.

Figure 2.1. Timeline used to characterized the AOM and 2% DSS animal model

On autopsy, the colon was excised, dissecting it from the surrounding mesentery, terminal ileum, and anal verge. The colon was measured from the cecum to the anal verge; the length was recorded in centimeters. The colon was flushed from proximal to distal with ice-cold phosphate buffered saline (PBS) by inserting the tip of an 18G needle attached to a 5 mL syringe into the
The colon was slowly flushed until completely clear of stool. The colon was fixed as “sausages” in 10% formalin phosphate for 48 hours at room temperature, as described by Whittem et al.\textsuperscript{329} In brief, the colon was sealed by tying off the distal end with monofilament polypropylene 5-0 suture (Ethicon Inc, Johnson and Johnson, Somerville, NJ, USA). Next, 5 mL of 10% buffered formalin phosphate were infused using only the plastic catheter of a 27G IV catheter needle (Becton Dickson Infusion Therapy systems, Sandy, UT, USA); the needle was discarded before use. As the colon expanded into a “sausage” the catheter was slowly pulled out and the proximal end was sealed with suture. Colons were individually stored in 15 mL tubes with 10% buffered formalin phosphate (Sigma Aldrich, St. Louis, MO, USA). Twenty-four hours later, the formalin was replaced with 70% ethanol (Commercial Alcohols, Brampton, ON, Canada) and stored at room temperature until further processing.

To macroscopically characterize lesions, the colon was opened through a longitudinal cut along the anti-mesentery axis and pinned to a dissecting board. Using a ruler, the colon was divided into three equally long segments named proximal, middle, and distal by making marks on the dissecting board. Visible polyps were counted, noting their localization in each segment. Polyps that were in between two segments were recorded as half for each segment.

To prepare samples for histopathological assessment, colons were cut into the previously marked divisions (proximal, middle, and distal segments). Each segment was then cut into halves for a total of six samples per colon (Figure 2.2).

**Figure 2.2. Distribution of segments and histological sections**
Samples were transferred to individual cassettes and embedded in paraffin. From each sample, three 4-μm longitudinal sections approximately 100 μm apart from one another were obtained (Figure 2.2) and stained with haematoxylin and eosin (H&E). A total number of 18 sections were obtained per mouse. All the slides for this study were prepared by the Department of Pathology, Toronto General Hospital, University Health Network.

Slides were initially graded by FAA and then confirmed by an experienced gastrointestinal pathologist (Dr. Hala El-Zimaity, Department of Pathology, Toronto General Hospital, University Health Network, Toronto, ON, Canada). Each observer evaluated the slides once. Sections were classified as negative for dysplasia or positive for LGD, HGD, and/or invasive adenocarcinoma. The cytological and architectural criteria used for grading are summarized in Table 2.1. The diagnosis of HGD was based primarily on architectural features, supplemented by appropriate cytology. The data for each slide per mouse per time point were recorded.
Table 2.1. Histopathological criteria for the diagnosis of colonic dysplasia

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Architectural features</th>
<th>Cytological features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Non-branching gland, basal nuclei</td>
<td>Low nuclear cytoplasmic ration; basal nuclei with no loss of cell polarity.</td>
</tr>
<tr>
<td>Low-grade dysplasia (LGD)</td>
<td>Glands have parallel sides with little branching and minimal gland budding</td>
<td>High nuclear cytoplasmic ration; basal nuclei with no loss of cell polarity.</td>
</tr>
<tr>
<td>High-grade dysplasia (HGD)</td>
<td>Branching cribriform glands &quot;back to back&quot; glands, prominent glandular budding and intraluminal papillary tufting with no maturation towards surface</td>
<td>Loss of cell polarity nuclear stratification through the entire thickness of the epithelium, markedly enlarged nuclei, often with open, dispersed chromatin and prominent nucleoli, atypical mitotic figures, dystrophic goblet cells and prominent apoptosis imparting the &quot;dirty&quot; appearance.</td>
</tr>
<tr>
<td>Invasive adenocarcinoma</td>
<td>Branching cribriform, irregular, solid glands with desmoplastic stromal reaction</td>
<td>Markedly enlarged nuclei, often with open, dispersed chromatin and prominent nucleoli, atypical mitotic figures, dystrophic goblet cells and extensive tumour budding.</td>
</tr>
</tbody>
</table>

In order to characterize potential changes in surface area (SA) of dysplasia, slides from an early (week 8) and late (week 12) time point were compared. Stained slides were scanned at 20x magnification using a ScanScope XT (Aperio Technologies, Vista, CA, USA). Eighteen histological sections per mouse were assessed. Slides were analyzed using ImageScope 11.0. Areas containing dysplasia were graded as previously described. For each section, the SA (mm²) of regions of interest displaying normal mucosa, LGD, and HGD was measured using the imaging software.
2.2 Cell lines

The *Cercopithecus aethiops* monkey kidney fibroblast cell line CV-1 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). The C57BL/6 mouse sarcoma cell line 24JK was a kind donation from Dr. David L. Bartlett, University of Pittsburgh, Pittsburgh, PA, USA. Cells were grown in Dulbecco’s modified Eagle medium (DMEM) (Sigma Aldrich, St. Louis, MO, USA) supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS) (PAA Laboratories, Etobicoke, ON, Canada) and 1% antibiotics – antimycotic (Invitrogen, GIBCO, Grand Island, NY, USA). Cell lines were maintained in an incubator at 37°C with 5% CO₂ and serially passaged every 3–4 days. Mycoplasma-negative status was confirmed before starting experiments by using MycoProbe (R&D Systems, Minneapolis, MN, USA). Cells were passaged up to 10 times.

2.3 Double deleted vaccinia virus expressing red fluorescent protein

vvDD-RFP is a double deleted vaccinia virus expressing the human somatostatin receptor type 2 (hSSTR2) and a red fluorescent protein (RFP) (DsRed) (Figure 2.3). The hSSRT2 gene is a reporter gene that was not utilized in this experiment. The RFP is also a reporter gene that allows for monitoring of viral infection through fluorescent microscopy. vvDD-RFP was previously made and adapted from McCart et al as described by Ottolino-Perry et al.

**Figure 2.3. Linear diagram of vvDD-RFP structure (Angarita FA, 2013)**

![Linear diagram of vvDD-RFP structure](image-url)
2.4  *Ex vivo* infection of animal-derived samples with vvDD-RFP

2.4.1  Experimental setup

In order to select appropriate parameters of viral pre-infection time for intracolonic (IC) *in vivo* delivery of vvDD-RFP, a small pilot study was carried by infecting samples obtained from mice bearing adenomatous polyps. *Ex vivo* infections were adapted from Diallo *et al*\textsuperscript{334}. Male CD1 mice (n = 11) were exposed to AOM and 2% DSS as previously described. Animals were euthanized in groups of three or four as of week 8 with CO\textsubscript{2} overdose. Colons were harvested as previously described. To prevent contamination, sterile instruments were used and resterilized in between animals with a hot bead sterilizer (Fine Science Tools Inc., North Vancouver, BC, Canada). After excising the colon, the length was measured and each colon was cut into three equally long segments and named proximal, middle, and distal.

From each segment, one sample (~3 cm) was obtained. Samples from each segment were placed in sterile 15 mL tubes previously filled with Roswell Park Memorial Institute (RPMI) (Sigma Aldrich, St. Louis, MO, USA) culture media supplemented with 20% (v/v) FBS (Sigma Aldrich, St. Louis, MO, USA) and placed on ice. Using a sterile scalpel blade, samples from each segment were individually sliced into 10 smaller samples (~2-3 mm) and then washed with Hank’s balanced salt solution (HBSS) (Invitrogen, GIBCO, Grand Island, NY, USA). Each sample was placed in one well of a sterile 96-well round bottom plate. A total dose of $10^9$ pfu of vvDD-RFP was suspended in 1.5 mL of RPMI supplemented with 2.5% FBS and equally divided amongst 15 samples (5 proximal, 5 middle, and 5 distal) (100 μL per well). The 15 remaining samples (5 proximal, 5 middle, and 5 distal) were treated with blank 2.5% FBS RPMI media (100 μL per well), which acted as a negative control. Samples from the first and second set of mice were incubated for 60 and 30 minutes, respectively, at 37°C. After the incubation period, 1.5 mL of RPMI supplemented with 10% FBS and 1% antibiotics – antifungal were added to each well. Infection was monitored by fluorescent microscopy at 24 and 48 hours post-virus infection (pvi). Forty-eight hours pvi, samples were fixed in buffered formalin phosphate for 24 hours and then transferred to 70% ethanol until processed for histopathology. RFP signal and histology was compared between samples that were incubated for 30 and 60 minutes.
2.4.2 Fluorescence microscopy

Viral infection were imaged under fluorescent light using a Zeiss AxioObserver microscope (Carl Zeiss, Oberkochen, Germany) equipped with a Series 120Q Fluorescence Illumination unit (EXFO, Quebec City, QC, Canada) and a 100W halogen lamp. Images were acquired with a CoolSnap HQ camera (Roper Scientific, Tucson, AZ, USA) under a 10x objective lens.

2.4.3 Histology

Samples were individually transferred to cassettes and embedded in paraffin. Three sets of longitudinal sections (4-μm thick cuts) were obtained from each paraffin block. Each set consisted of two sequential sections. Sets of slides were 100μm apart from the next set of slides. From each set, one section of tissue was used to make a slide for staining with H&E. This slide was initially evaluated by FAA and then confirmed by a gastrointestinal pathologist (Dr. El-Zimaity) who confirmed the presence of dysplasia according to established criteria. If dysplasia was present then the second section of tissue was used for IHC staining. IHC slides were stained using a primary anti-body polyclonal rabbit anti-VV (ab35219, dilution 1/1000; Abcam, Cambridge, MA, USA). Stained slides were scanned at 20x magnification using a ScanScope XT and viewed with ImageScope 11.0.

2.5 Specificity experiments

2.5.1 Experimental set up

Twenty-seven CD-1 male mice were exposed to AOM and 2% DSS and monitored as previously described. Treatment was delivered either intraperitoneally or intracolonically. IP delivery consisted of a single dose of 10⁹ pfu of freshly sonicated vvDD-RFP diluted in 1.0 mL of vehicle solution (HBSS) supplemented with 0.1% of bovine albumin (BioShop, Burlington, ON, Canada) or mock (vehicle solution only) administered through a 27G needle connected to a 1.0 mL syringe. IC delivery was in the form of a retention enema. In order to prepare mice for IC treatment, evacuant enemas were first performed. The evacuant enema consisted of instilling 2.0
mL of warm 0.9% sodium chloride solution (Baxter Corporation, Toronto, ON, Canada) into the rectum through the plastic catheter of a 20G Angiocath needle, that was previously lubricated with Muko lubricating jelly (Source Medical Corporation, Mississauga, ON, Canada). Note that the needle of the Angiocath was discarded and only the plastic catheter was used to deliver the enema. Animals were allowed to spontaneously evacuate the normal saline solution and feces; the evacuant enema was repeated until the effluent was clear. After confirming absence of stool, mice were anesthetised. General anaesthesia was provided with a single IP injection of a mixture of ketamine (Bimeda- MTC Animal Health Inc., Cambridge, ON, Canada) and xylazine (Bayer Inc., Toronto, ON, Canada) at a dose of 90mg/kg and 10mg/kg, o respectively. An additional one third of the dose was administered if the animal woke up during the procedure. Under general anaesthesia, $10^9$ pfu of freshly sonicated vvDD-RFP diluted in 2.0 mL of vehicle solution or mock (vehicle solution only) was instilled through a 30 minute retention enema. The plastic catheter of a 20G Angiocath lubricated with Muko lubricating jelly was inserted through the anus and slowly mobilized so that the length (~4.7 cm) of the catheter was in the colon. The solution was slowly instilled as the catheter was withdrawn. In order to prevent the solution from being evacuated, the tip of a sterile cotton swab covered in Muko lubricating jelly was used as a seal. During the retention enema, animals rested at 45° with the head down. After 30 minutes the cotton swab was removed and animals were monitored until they fully recovered from the general anaesthesia.

At 11 weeks a total of 26 animals were available; one animal was euthanized early on because it developed irreducible rectal prolapse. Treatment was rescheduled from week 12 to 11 in order to prevent having to sacrifice more animals from rectal prolapse, which would have decreased the sample size. Animal distribution was as follows: intraperitoneal virus (IP vvDD) n=12, intraperitoneal mock (IP mock) n=1, intracolonic virus (IC vvDD) n=12, and intracolonic mock (IC mock) n=1. It should be noted that the ratio of 12 virus- to 1 mock-treated mice may have led to an unbalanced assessment; however the aim of this experiment was to determine specificity and not efficacy; we only wanted to know if the virus would only infect colonic dysplasia. Because we confirmed that animals in this model did not develop dysplasia in the proximal colon, each mouse acted as its own control, whether they were treated with virus or mock solution. In order to characterize viral infection, animals were euthanized at specific time points (Figure 2.4). Animals were euthanized at 24, 72, 120, and 168h pvi. Three mice from the IP and
IC groups were euthanized at every time point and mock-treated animals were euthanized at 72 hours pvi.

Figure 2.4. Timeline used to characterize virus infection after delivery of vvDD-RFP to CD-1 mice bearing colonic polyps induced by AOM and 2% DSS

Colons were harvested as previously described (Page 47). To prevent contamination, sterile instruments were used and resterilized in between animals with a hot bead sterilizer. After excising the colon, the length was measured and each colon was cut into three equally long segments as previously described. These segments were then used to assess viral infection through fluorescent microscopy, histopathology, and viral titres. For assessment through fluorescent microscopy, two samples (size: 1.5 – 2.0 cm width) were obtained from each of the proximal, middle, and distal segments. These samples were transported in DMEM supplemented with 1% antibiotics – antimycotic on ice. Imaging with fluorescent microscopy was carried as previously described. After imaging sessions, the samples were stored at -80°C in order to be used for viral titration. The remaining tissue from each segment was individually fixed and stored in 10% buffered formalin phosphate for twenty-four hours and then transferred to 70% ethanol until processed for histopathology.

2.5.2 Viral titration

Viral titration was carried out through standard plaque assay. CV-1 cells were harvested from flasks using 0.25% trypsin – EDTA solution (Sigma-Aldrich, St. Louis, MO, USA) and seeded at 5 x 10⁵ cells/per well in 6-well plates. Cells were incubated overnight to allow them to adhere. Prior to infection, samples were thawed on ice and three 4mm round biopsies were obtained from each sample using a sterile biopsy punch (Integra Miltex, Plainsboro, NJ, USA). Individual biopsy samples were suspended in 0.5 mL HBSS. Biopsy samples were homogenized using sterile 200mm beads in an oscillatory tissue homogenizer (Tissue Lyser II, Qiagen, Hilden,
Germany) at 25Hz for 3 minutes. Samples were then exposed to three freeze-thaw cycles followed by sonication on ice until fully dissolved. Serial dilutions of the homogenized sample were prepared in triplicate in low-serum (2.5% FBS) DMEM supplemented with 1% antibiotics – antimycotic. Before infecting plates, media was removed and 0.5 mL of each dilution was added to a well. Culture plates were incubated at 37°C and 5% CO₂ for 2 hours with shaking every 10 minute. Cells were supplemented with 2.0 mL of DMEM containing 10% FBS and 1% antibiotics – antimycotic and incubated for 48 hours. Plates were stained with crystal violet and plaques were quantified. Titers were calculated as pfu/mm² of tissue.

2.5.3 Measuring colocalization

Slides were scanned at 20x magnification using a ScanScope XT and analyzed with ImageScope 11.0. In the H&E slide, regions of interest were drawn around each type of tissue (normal mucosa, LGD, or HGD). The imaging software calculated the SA (mm²) for each type of tissue. The same areas of interest were drawn in the corresponding IHC slide and analyzed using the software’s positive pixel algorithm to confirm positive staining. If the region of interest was positive, then the SA was measured. Colocalization was quantified as a percentage using the following formula:

\[
\text{Colocalization} = \frac{\text{SA of region of interest in IHC slide with positive staining}}{\text{SA of region of interest in H&E slide}} \times 100
\]

2.6 Efficacy experiments

2.6.1 Single dose experiment set up

One cohort of fifty-five male CD-1 mice were exposed to AOM and 2% DSS and monitored as previously described. Mice were treated with IP or IC vvDD-RFP or control (HBSS) as previously described. Animal distribution was as follows: IP vvDD n=14, IP mock n=14, IC vvDD n=13, and IC mock n=14. Treatment was administered at week 8 because one of the objectives of this experiment was to determine the impact of treatment on development of polyps.
and survival (Figure 2.5). Efficacy was evaluated by comparing the number of polyps, the SA of dysplasia, and survival between virus and mock-treated animals.

**Figure 2.5. Time line to assess the efficacy of single dose treatment of vvDD-RFP in mice bearing adenomatous polyps induced by AOM and 2% DSS**

2.6.1.1 Histopathologic assessment of efficacy

At 10 days post-infection (dpi), mice from each treatment group were euthanized by CO$_2$ overdose. Animal distribution was follows: IP vvDD n=4, IP mock n=4, IC vvDD n=5, and IC mock n=4. Colons were harvested and prepared for H&E staining as previously described. In order to assess changes in the burden of dysplasia, the total SA (mm$^2$) of LGD, HGD, and total dysplasia were measured and compared between virus- and mock-treated groups. Stained H&E slides were scanned and analyzed as previously described.

2.6.1.2 Survival analysis

Survival analysis was carried out on the remaining animals from the cohort of 55 animals. Animal distribution for this experiment was as follows: IP vvDD n=10, IP mock n=10, IC vvDD n=8, and IC mock n=10. In order to prevent any bias in euthanizing animals, another member of the laboratory coded the cages after administering the treatment and kept a list of the treatment groups to which each cage belonged. Mice were euthanized by CO$_2$ overdose when they developed signs of morbidity as previously described. The person euthanizing the mice did not know to which group each animal belonged.
2.6.1.3 Plaque reduction neutralization test for anti-vaccinia virus neutralizing antibodies

In order to determine if variability in infection rates between IP and IC delivery was a result of different kinetics of immune response against the virus, anti-VV neutralizing antibodies levels were measured through a plaque reduction neutralization test (PRNT) over time as described by Newman et al. Tail vein blood samples (maximum 200µL per blood draw) were obtained from the animals in the survival analysis group. Animal distribution for the purpose of this experiment was IP vvDD n=3, IP mock n=2, IC vvDD n=3, and IC mock n=2. Blood samples were collected over the course of seven time points every four days, starting at 6dpi and ending at 30 dpi (Figure 2.6). Blood was consecutively drawn from the same mouse at every time point.

Figure 2.6. Time line used to obtain blood samples to measure anti-vaccinia virus neutralizing antibodies.

Blood was centrifuged at 1,500 revolutions per minute (rpm) for 5 minutes. The supernatant was discarded and serum was stored at -80°C prior to testing. All serum samples were heat inactivated at 56°C for 30 min before being assayed. The reference vvDD-RFP used in the assay was prepared as previously described. The negative-control serum sample was serum from mice that did not receive vvDD treatment (IP mock and IC mock groups). The positive-control serum sample was obtained from a female macaque 137 days post-IP treatment with vvDD-RFP from another study. A blank control was prepared by mixing 60 pfu of the reference virus with serum-free DMEM supplemented with 1% antibiotics – antimycotic.

One day before the PRNT, CV-1 cells were seeded at 2.5 x 10^5 cells/per well into 24-well tissue culture plates. The next day, serial dilutions (1:10, 1:50, 1:100, 1:500, and 1:1000) of heat-inactivated study sera and control sera were prepared in duplicate in serum-free DMEM
supplemented with 1% antibiotics – antimycotic. Each dilution was mixed with 60pfu of freshly sonicated vvDD-RFP. Serum-virus mixtures were incubated for 1 hour at 37°C with 5% CO₂. The previously prepared culture plates of CV-1 cells were washed with DMEM supplemented with 1% antibiotics – antimycotic. After the hour of incubation, serum-virus mixtures were added to the washed CV-1 cells and allowed to adsorb for 1 hour in a 37°C incubator with 5% CO₂. Cells were then supplemented with DMEM containing 2.5% FBS and 1% antibiotics – antimycotic and incubated at 37°C for 48 hours. Plates were stained with crystal violet and plaques were quantified. The number of plaques for each well was compared to the number obtained from the blank control. The endpoint serum neutralizing antibody titer for each sample was defined as the highest dilution with at least a 60% reduction in the number of plaques with respect to the blank control. The titre reported for each sample was the highest dilution obtained by at least half the samples. The percentage of samples obtaining the highest dilution is also reported.

2.6.2 Multiple dose experiment set up

One cohort of fifty-five male CD1 mice were exposed to AOM and 2% DSS and monitored as previously described. Mice were treated with IP or IC vvDD or control (HBSS) as previously described. Animal distribution was as follows: IP vvDD 14, IP mock 14, IC vvDD 14, and IC mock 13. Treatment was administered through three doses, starting on week 8 and thereafter every six days until day 12 (Figure 2.7). Efficacy was evaluated as was done in the single dose therapy experiment. In this experiment, the effect on the number of polyps and SA of dysplasia were assessed on mice that were euthanized 5 days after the last dose of treatment. The distribution of animals that were euthanized in each treatment group was as follows: IP vvDD n=4, IP mock n=4, IC vvDD n=4, and IC mock n= 4. The number of animals followed for survival analysis was as follows: IP vvDD n=10, IP mock n=10, IC vvDD n=10, and IC mock n=9. This experiment was performed using the same blinded experimental design previously described. Mice were euthanized by CO₂ overdose when they developed signs of morbidity as previously described.
2.7 Statistics

Data were analyzed using SPSS Version 20 (IBM, Armonk, NY, USA). All data are presented as mean ± one standard error of the mean (SEM) except for prevalence rates, which are reported as a percentage. Means were compared using a two-tailed unpaired or paired student’s t tests. Survival analysis was assessed on Kaplan-Meier plots and compared with log-rank analysis. Statistical significance was set at p<0.05.
Chapter 3
Characterize the murine model of colonic dysplasia induced by azoxymethane and 2% dextran sodium sulfate

3.1 Objectives

The objective of this first aim was to characterize and acquire experience with a murine colonic dysplasia model using AOM and 2% DSS. To carry this out we used a dosing schedule proposed by Suzuki et al\textsuperscript{99}. Two specific aims were established for this study: (1) to confirm the macroscopic and histological characteristics of adenomatous polyps in the colons of mice exposed to AOM and 2% DSS and (2) to establish a specific time point in which both LGD and HGD were present concomitantly.

3.2 Results

3.2.1 Azoxymethane and 2% dextran sodium sulfate are not associated with severe morbidity or mortality

To assess the morbidity associated with AOM and 2% DSS, animals were monitored for body weight and clinical signs associated with the development of large polyps, specifically bloody stool and rectal prolapse. Mean body weights at different time-points are shown in Figure 3.1. In week 2 mice weighed significantly less than the previous week (32.9±0.2g versus 31.1±0.4g, p=0.003) and after week 2 they started to gain weight again. At week 2 mice had a mean weight loss of 7.6±1.1% relative to the week 1. No remarkable changes in body weight were observed after week 8. Bloody stool was observed in 76%, 57%, and 33% of 21 mice during weeks 2, 3, and 4, respectively. The amount of bloody stool was minimal and did not induce lethargy or any limitations in their activity. Bloody stool was not observed outside of this period of time. By week 12 mice developed spontaneously reducible rectal prolapse secondary to the development of large polyps in the rectum. On examination, the rectal mucosa of mice that developed rectal
prolapse did not have signs of ulceration or necrosis. Mice with rectal prolapse did not have bloody stool. Overall, no mice developed significant signs of morbidity that justified euthanasia outside of the established time points.

Figure 3.1. Changes in body weight over time in the AOM and 2% DSS animal model.

![Graph showing body weight changes over time](image)

Four week-old CD-1 male mice \((n=21)\) were exposed to a single injection of AOM (Week 0) and seven days later to a one week cycle of 2% (w/v) of DSS (Week 1). Weights were obtained on the first day of each week as well as prior to administering chemicals and sacrifice. Error bars represent ± two SEM.

3.2.2 Azoxy-methane and 2% dextran sodium sulfate induce polyps in the distal and middle colon

Initially to characterize the localization and extent of disease, colons were harvested and inspected macroscopically every two weeks after AOM and 2% DSS. Mean lengths of colons at different time points are shown in Figure 3.2A. No remarkable changes in the lengths of colons were noted. On gross examination colons had an increase in the thickness of the wall secondary to the development of macroscopic lesions. The increase in wall thickness was notable as of week 8. Initially this feature was limited to the distal third, but, over time, it progressively extended to the middle segment (Figure 3.2B). After colons were fixed in 10% formalin phosphate, macroscopic inspection revealed that as of week 6 polyps were observable in the middle and distal colon (Figure 3.3C). The proximal third of the colon did not have macroscopically visible polyps. The number of polyps was quantified per segment. The mean
number of polyps per segment at different time points is shown in **Figure 3.3D**. A paired t test was performed between the number of polyps in the middle and distal segment at each time point in order to determine if there was any difference in the number of lesions between these two segments. Overall, more polyps developed in the distal than middle segment, a distribution that was statistically significant as of week 8 (middle 2.0±1.2 versus distal 9.0±0.6, p=0.01) and continued until week 16 (middle 6.7±2.2 versus distal 17.2±2.0, p=0.03).

**Figure 3.2.** Macroscopic features of the colon of mice exposed to AOM and 2% DSS.

A. 

<table>
<thead>
<tr>
<th>Week</th>
<th>Mean length of colon (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>10.0±1.0</td>
</tr>
<tr>
<td>6</td>
<td>10.5±1.0</td>
</tr>
<tr>
<td>8</td>
<td>11.0±1.0</td>
</tr>
<tr>
<td>10</td>
<td>11.5±1.0</td>
</tr>
<tr>
<td>12</td>
<td>12.0±1.0</td>
</tr>
<tr>
<td>14</td>
<td>12.5±1.0</td>
</tr>
<tr>
<td>16</td>
<td>13.0±1.0</td>
</tr>
</tbody>
</table>

**B**

**C**
3.2.3 Azoxymethane and dextran sodium sulfate induce low- and high-grade dysplasia, but not invasive adenocarcinoma

To evaluate the type of disease in the colorectal mucosa that developed as a result of AOM and 2% DSS, representative histological sections of each segment of the colon were graded in collaboration with an experienced gastrointestinal pathologist (Dr. Hala El-Zimaity) using defined diagnostic criteria. LGD was characterized by glands with little branching and budding, high nuclear cytoplasmic ration, and basal nuclei with no loss of cell polarity. (Figure 3.3B). Relevant features of HGD included branching cribriform glands "back to back" glands, prominent glandular budding and intraluminal papillary tufting with no maturation towards the
luminal surface, loss of cell polarity nuclear stratification throughout the entire thickness of the epithelium, markedly enlarged nuclei, often with open, dispersed chromatin and prominent nucleoli, atypical mitotic figures, dystrophic goblet cells, and prominent apoptosis (Figure 3.3C). Invasive adenocarcinoma was not detected at any time point in any of the segments. To characterize the prevalence of LGD and HGD, samples from each segment of the colon were assessed over time. Prevalence of dysplasia per time point is depicted in Figures 3.3D-F. Dysplasia was not detected in the proximal segment at any time point (Figures 3.3D). Colons at week 4 were normal. The prevalence rate of each type of dysplasia varied over time in the middle and distal segments. LGD preceded HGD as it was first detected on week 6 in 100% of mice in both the middle and distal segment. HGD was first detected on week 8 in 67% and 100% of animals in the middle and distal segments, respectively. In the middle segment, simultaneous presence of LGD and HGD was first detected at week 8 (67%) and fluctuated thereafter (week 10: 0%, week 12: 0%, week 14: 67%, and week 16: 33%). One third of mice at weeks 8, 10, 12, and 14 had normal mucosa in the middle segment. In the distal segment, LGD and HGD were simultaneously diagnosed in 66.7% of mice from week 8 to 14 and reached 100% of animals at week 16.
Figure 3.3. Histopathological features of the colonic mucosa of mice exposed to AOM and 2% DSS.

A

B

C
Representative pictures of normal colonic mucosa (A), LGD (B), and HGD (C) at week 14 (Left: 5x, Right: 20x, bar represents 100µm). Prevalence of normal mucosa, only LGD, only HGD, or both HGD and LGD is depicted for the proximal (D), middle (E), and distal (F) segment over time. Three mice were euthanized and analyzed at each time point.

3.2.4 Surface area of low- and high-grade dysplasia increases over time in the azoxymethane and 2% dextran sodium sulfate model

To evaluate the burden of dysplasia over time, the mean SA of normal and dysplastic (LGD and HGD) mucosa were measured and compared in slides obtained between an early (week 8) and late (week 12) time point. Figure 3.4 depicts the distribution of SAs of each type of lesion at each time point. An independent t test was performed between the SA of samples obtained at week 8 and 12 in order to determine if there was any difference in the amount of dysplasia between these two time points. The mean SAs of week 12 were significantly higher than those of week 8 for LGD (0.8±0.1 mm² versus 0.06±0.02 mm², p<0.0001) and HGD (2.6±0.8 mm² versus 0.3±0.1 mm², p=0.006).

Figure 3.4. Comparison of the surface area of normal and dysplastic mucosa between an early (week 8) and late time (week 12) point in the AOM and 2% DSS animal model.
The surface area of regions of interest were measured and averaged per slide. Three mice were euthanized and analyzed at each time point. Six sections were obtained from each segment of the colon for a total of 18 sections per mouse. Bars represent data from the middle and distal segment combined. Error bars represent ± two SEM. Brackets represent the independent t test performed between the SA of samples obtained at week 8 and 12. **, p<0.01; ***, p≤0.001.

3.3 Summary

The AOM and 2% DSS model is reproducible and consistently induces adenomatous polyps and dysplasia. Adenomatous polyps, which were detected as of week 6, were exclusively detected in the middle and distal segment, significantly more so in the latter. Polyps increased in number and size over time. Dysplasia, which was detected as early as week 6, was also limited to the middle and distal segments of the colon. Microscopically, there were no histopathological changes in the proximal segment. The fact that the proximal third of the colon was consistently normal in all animals is of particular benefit to this study because it can be used as an internal negative control when measuring the specificity of OV infection. LGD preceded HDG at week 6, but eventually both types of dysplasia were simultaneously present at later time points. Invasive adenocarcinoma was not detected at any time point. As the number of polyps increased so did the SA of each type of dysplasia. A significantly larger SA of dysplasia was detected at a later time point (week 12) than early on (week 8). At week 8, there was no significant difference between the mean percentages of LGD and HGD whereas at week 12, there was a significantly larger percentage of HGD than LGD.

3.4 Discussion

The results of this study demonstrate that a IP-delivered AOM at a dose of 10mg/kg body weight followed 7 days later by a one-week course of oral exposure to 2% DSS induces adenomatous polyps and dysplastic mucosa with human-like characteristics in CD-1 mice.

Animals tolerated both AOM and DSS, showing only modest signs of toxicity immediately after exposure and at later time points. Exposing 4 week-old mice to AOM did not affect their growth
as mean body weight before (28.7±0.2g) and after (32.8±0.2g) AOM were in line with the expected growth of this mouse strain. Only after oral exposure to a one week cycle of 2% DSS did mice begin developing signs of morbidity (Figure 3.1). The change in body weight is attributed to the sequential administration of AOM and DSS as mice exposed to either agent alone generally do not have significant changes in weight\cite{336, 337}. On average, mice had a mean weight loss of 1.8g at week 2 relative to the prior week (32.9±0.2g versus 31.1±0.4g, p=0.003). This corresponded to a mean loss of 7.6±1.1%, which is in line with the expected weight loss of 5-10% that is typically described in this animal model\cite{337}. Nonetheless, mice regained the weight during the subsequent week and only started to present a weight loss once again as of week 12. In this case, the weight loss was not statistically significant and it only represented a decrease of 1.7±0.5% relative to week 11. Weight loss varies significantly between mouse strains as shown by Suzuki et al\cite{336}. Mice strains such as Balb/c and C57BL/6N can lose up to 7.3 and 3.7g, respectively, when exposed to DSS. In comparison, the weight loss observed in CD-1 mice is more subtle as shown in this study in which mice only lost 1.8g in the week following DSS. A similar level of weight loss of 1.3g was reported in another study using CD-1 mice\cite{199}.

Another early sign of morbidity was the presence of bloody stool. Typically bloody stool is anticipated during the first couple of weeks after exposure to DSS\cite{337}. In our study bloody stool was observed over the course of the first three weeks after exposure to DSS (week 2 – 4). Initially the proportion of mice with bloody stool was high (76%), but it decreased on weeks 3 (57%) and 4 (33%) and eventually disappeared. Bloody stool is directly related to oral exposure to DSS because it induces ulceration of the colonic mucosa\cite{187} through focal destruction of the mucin content, which subsequently leads to cytotoxicity of the epithelium\cite{187-190}. The presence of bloody stool is therefore an indicator that mice are consuming sufficient levels of DSS.

Rectal prolapse is a sign that presented late in this animal model. Mice developed rectal prolapse as of week 11.5 (56%) and affected 100% of the animals by week 16. There is a lack of information in the literature regarding incidence rates of rectal prolapse in the AOM and DSS model. Generally studies only report that a few mice develop such sign\cite{199, 338, 339}. In these studies rectal prolapse developed earlier at week 7 and 8. It is difficult to understand why in those studies, rectal prolapse developed so early. In our study we were able to note that the development of rectal prolapse paralleled the increase in mean number of polyps. By weeks 12 –
16, the distal segment contained a higher number of polyps than at earlier time points (Figure 3.2C). On another note, the rectal prolapse observed in this study was always spontaneously reducible, the mucosa did not have ulcers or necrosis, and mice appeared to be overall healthy, therefore they were not euthanized outside of the scheduled time points.

Adenomatous polyps specifically developed in the middle and distal segment of the colon. This distribution is a known characteristic of the AOM and DSS model\textsuperscript{146, 199, 337}. This characteristic underlines how well the model mimics human disease\textsuperscript{42}. Certain histological characteristics favour the carcinogenic effects of AOM and DSS in the middle and distal segment of the colon. Notably, these characteristics are not species-specific as murine and human colorectal mucosa share similar histological characteristics\textsuperscript{340}; therefore any characteristic can be extrapolated from one to the other.

Several features of the colon’s histology and physiology may have favoured the development of lesions in the distal colon. First, the cell cycle duration of rectal epithelium is different from that of other segments of the colon. Murine mucosa has slower renewal rates in the sigmoid and rectum relative to other regions of the gastrointestinal tract\textsuperscript{321, 341}. Because the normal rectosigmoid mucosa renews slower it may have been in more contact with the carcinogens AOM and DSS. Second, the distal part of the colon tends to have a longer transit time as a whole relative to the proximal colon\textsuperscript{342}; therefore the cells in that distal colon may have had a longer exposure time to the DSS and the MAM secreted through the bile duct. Third, metabolic activation of the DNA reactive molecule MAM requires the presence of alcohol dehydrogenase\textsuperscript{343, 344}, which is significantly more concentrated in the distal colon than the proximal colon\textsuperscript{345}.

Previous publications described a sequential progression of dysplasia to invasive adenocarcinoma in the AOM and DSS model\textsuperscript{186, 199, 336, 346}. These studies indicated that the incidence rate of invasive adenocarcinoma increased over time. For example, Suzuki et al reported that adenomatous polyps and invasive adenocarcinoma were first observed in 40% of mice at week 3 and 4, respectively and by week 9 both types of lesions were present in 100% of mice\textsuperscript{199}. Nonetheless in our study invasive adenocarcinoma was not detected at any time point. The discrepancy between our results and other groups’ work lies not only in the fact that AOM
and DSS rarely lead to invasive disease\textsuperscript{146}, but more importantly in the definition of CRC being used. The previously mentioned studies\textsuperscript{186, 199, 336, 346} were carried out in Japan. The discrepancies in the concept of “cancer” between Japanese and Western pathologists is well-known and it is explained by differences in interpretation of cytological and architectural criteria and nomenclature\textsuperscript{347}. Using Western diagnostic criteria, which is in accordance with the WHO definition\textsuperscript{3}, CRC is diagnosed when neoplasia has at least invaded the submucosa. In contrast Japanese pathologists do not use submucosal invasion as an obligatory criterion for the diagnosis of CRC and instead they use nuclear and structural criteria\textsuperscript{347}. Consequently lesions with severe cytological or architectural distortion that are confined to the mucosa, which would be classified as HGD by Western pathologists, are diagnosed as CRC by Japanese pathologists. Accordingly, the Japanese publications using the AOM and DSS model reported high incidence rates of CRC when in fact what might have been observed was a lesion that met the Western definition of HGD. Certainly, the lack of invasive adenocarcinoma in the AOM and DSS model is an advantage to our study because the objective is to determine if oncolytic VV will infect and replicate in dysplastic mucosa as its selectivity for invasive disease has already been well documented.

AOM and DSS induced both LGD and HGD in a sequential manner. This is in accordance with what was previously described in the literature\textsuperscript{199}. In general, dysplasia was detected in all mice as of week 6 (Figure 3.3D–F). Our macroscopic and histological findings validate the selective effect of AOM and DSS on the colorectal mucosa, as described by others\textsuperscript{146, 186, 199, 336, 337, 346}. LGD preceded HGD as it was detected on week 6 while HGD was detected at week 8 in both the middle and distal segment. Concomitant presence of LGD and HGD was detected at week 8, but varied depending on the segment. Although the distal segment consistently presented both LGD and HGD, the middle segment did not, particularly at week 10 and 12. Although this inconsistency may have been due to sample bias, variations in the incidence of each type of dysplasia are commonly described in this animal model\textsuperscript{346}. Variability in the consumption of the drinking water with DSS may be an explanation. As previously described, 23.8% of mice (n=5) at week 2 did not present bloody stool and when each mouse was assessed post mortem at different sacrifice time points (week 8 – 16), four of these mice did not develop polyps (Figure 3.2D) or dysplasia (Figure 3.3E) in the middle segment at week 8 – 14.
As the objective of this study is to determine if oncolytic VV has a potential role in treating dysplasia, determining a time point to treat mice was necessary. Ideally, the time point should have both LGD and HGD in significant amounts in order to test the hypothesis that oncolytic VV will infect in a grade-dependent manner. With the aim of selecting a suitable time point to treat, the SA of dysplasia was compared between an early (week 8) and late (week 12) time point. As expected, a significant difference in SA between week 8 and 12 was noted for both LGD (0.06±0.02 mm$^2$ versus 0.8±0.1 mm$^2$, $p<0.0001$) and HGD (0.3±0.1 mm$^2$ versus 2.6±0.8 mm$^2$, $p=0.006$). Given these results, treatment was scheduled at a late time point; however timing of treatment was adjusted to week 11 as animals started to develop rectal prolapse as of week 12.

Based on the results from these experiments we conclude that AOM and 2% DSS induce a predictable pattern of adenomatous polyps in CD-1 mice as of week 6. Adenomatous polyps are limited to the middle and distal segment, and eventually lead to the development of rectal prolapse. Nonetheless, this complication was not associated with substantial morbidity or mortality. Furthermore, dysplasia progresses from LGD to HGD over time without developing invasive adenocarcinoma. The consistent lack of disease in the proximal segment is beneficial to our study. The proximal segment can be used as an internal control in the second aim of this project in which having normal colonic mucosa will be useful in determining if oncolytic VV specifically infects dysplasia. Additionally, the SA of each type of dysplasia increases over time.
Chapter 4
Determining the specificity of red fluorescent protein expressing double deleted vaccinia virus (vvDD) towards murine colonic dysplasia

4.1 Objectives

The objective of the second aim was to determine if vvDD-RFP could specifically infect murine colonic dysplasia while leaving normal mucosa unaffected. A small pilot study was carried out to determine the optimal viral pre-infection duration, which would be necessary for IC delivery. To evaluate the specificity of the virus, mice bearing adenomatous polyps induced by AOM and 2% DSS were treated with a single dose of vvDD-RFP. Three specific aims were established for this objective: (1) to determine if vvDD-RFP specifically infects colonic dysplasia; (2) to determine if vvDD-RFP replicates in dysplastic colonic cells; and (3) to determine if vvDD-RFP infects and replicates in colonic dysplasia in a grade-dependent manner.

4.2 Results

4.2.1 vvDD-RFP specifically infects the middle and distal segment of the colon while leaving the proximal segment uninfected after ex vivo infection

Viral pre-infection parameters for local in vivo delivery of vvDD-RFP were assessed through a small pilot study in which representative samples obtained from colons of mice exposed to AOM and 2% DSS were infected ex vivo with vvDD-RFP. To mimic local delivery, the total dose of $10^9$ pfu/mL of vvDD-RFP was divided equally amongst the samples. Equal numbers of samples from the same segments were treated with mock solution (RPMI) in order to act as a negative treatment control. Samples were incubated with virus for 30 or 60 minutes and then monitored by fluorescent microscopy at 24 and 48h pvi (Figure 4.1). A diffuse, but well-defined RFP signal was detected in samples from the middle and distal segment, increasing thereafter at 48h pvi (Figure 4.1A and B). The RFP signal was equally as intense with 30 (Figure 4.1A) and 60
(Figure 4.1B) minutes of incubation. Proximal samples that were treated with vvDD-RFP (Figure 4.1A and B) as well as those that received mock treatment lacked an RFP signal at both 24 and 48h pvi (Figure 4.1C). Inspection of the culture media revealed a considerable amount of well-defined clusters of free-floating cells with positive RFP signal (Figure 4.1D). H&E slides from all virus-infected samples revealed that the colonic mucosa had undergone autolysis or had been shed; therefore the histology of the tissue examined under fluorescent microscopy could not be determined (Figure 4.1E). H&E staining of mock-infected samples revealed no damage to the tissue histology or architecture. Despite not having histological confirmation of viral infection, RFP signal on fluorescent microscopy suggested that the virus specifically infected tissue with polyps. Based on the results of this pilot study we selected a 30-minute dwell-time following IC delivery in the subsequent experiments.

Figure 4.1. Ex vivo infection of samples obtained from colons of mice exposed to AOM and 2% DSS.

A
B

24 hours  48 hours

Distal

Middle

Proximal

C

Distal  Proximal

24h pvi  48h pvi  24h pvi  48h pvi
Representative images of samples from the proximal, middle, and distal segment of the colon were infected with vvDD-RFP, incubated for 30 (A) and 60 (B) minutes, and then examined on fluorescent microscopy at 24h and 48h pvi. Images depict an increase in RFP signal over time except for proximal samples. (C) Representative images of mock (RPMI)-treated samples from the distal and proximal segment of the colon that were examined after fluorescent microscopy at 24h and 48h pvi. Images depict a lack of RFP signal at both time points. (D) Representative images taken at 48h pvi depicting free floating vvDD-infected cells that had detached from the distal (first and second) and middle (third) colon samples and were found floating in the culture media. (Obj.: 10x for all fluorescent microscopy images) (E) Representative H&E slides obtained from the distal third of the colon illustrating a lack of mucosa in vvDD-infected samples (left) while a non-infected sample from the same segment did not undergo any change in tissue architecture at 48h pvi (right) (Bar represents: 500µm).
4.2.2 vvDD-RFP specifically infects colonic dysplasia and not normal mucosa

_in vivo_

Eleven weeks after injection of AOM, CD-1 male mice were treated with a single IP or IC dose of vvDD (10^9 pfu) or mock solution. To evaluate the specificity of vvDD-RFP, segments of colons bearing polyps induced by AOM and 2% DSS were compared with the proximal segment, which served as a normal control. Representative samples from each segment were inspected under fluorescent microscopy (Figure 4.2). The infection was characterized over time by sacrificing animals at different time points (24h, 72h, 120h, and 168h pvi) for each treatment group. In both the middle and distal segments in which polyps were notable, viral RFP signal appeared as early as 24h in mice treated either intracolonically or intraperitoneally (Figure 4.2A). At 24 hours, 42% IP-treated and 83% IC-treated samples had a well-defined RFP signal in samples from the middle and distal colon. At 72 hours, 67% IP-treated and 100% IC-treated samples virus had a positive RFP signal. At 120 hours, 100% of both IP-treated and IC-treated samples had a well-defined RFP signal. By 168 hours, the RFP signal was decreasing in both IP- and IC-treated samples. At all four time points no RFP signal was observed in any of the 6 sections of the proximal colon for either route of delivery (Figure 4.2B). The samples from mock-treated mice did not have an RFP signal in any segment of the colon (Figure 4.2C).

With regards to distribution and intensity of RFP signal, IC delivered vvDD induced a more intense signal over a larger area at all four time points relative to IP delivery (Figure 4.2A). Moreover, the kinetics of peak fluorescence varied between the two routes of delivery. With IC delivery, the signal peaked at 72h pvi and maintained the intensity at 120h pvi. In contrast the RFP signal peaked at 120h pvi following IP delivery; this intense signal was short-lived, as it was not observed at 168h pvi.
Figure 4.2 vvDD-RFP infects colonic mucosa from the middle and distal segment of colons of mice exposed to AOM and 2% DSS

A.

B.
**vvDD-RFP infection of colonic mucosa was characterized over time by obtaining representative samples from each segment of the colon from mice treated with $10^9$ pfu of virus delivered either IP or IC. Imaging was compared to samples obtained from animals treated with mock solution. Images of post-mortem colon samples examined under fluorescent microscopy from the (A) distal and (B) proximal segment of vvDD-RFP-treated animals. (C) Images of post-mortem colon samples examined under fluorescent microscopy from the distal and proximal segment of mock-treated animals. Images obtained with 10x objective.**

Histological analysis was performed on samples to confirm the specificity of viral infection. IHC was used to detect VV infection and standard H&E staining was used to confirm what type of tissue (normal, LGD, and HGD) was infected by the virus. Samples harvested from both IP- and IC-treated mice at 24, 72, 120, 168h pvi showed diffuse colocalization of VV staining with areas of LGD and HGD (Figure 4.3). Notably, normal mucosa and submucosa did not show immunoreactivity with the VV antibody. Similar patterns of specificity were observed with both IP (Figure 4.3A) and IC (Figure 4.3B) delivery overtime.
Figure 4.3 Histological assessment of specificity of vvDD-RFP infection of colonic tissue in mice exposed to AOM and 2% DSS

A

H&E

Normal
LGD
HGD

Anti-VV
4.2.3 vvDD-RFP infects colonic dysplasia in a grade-dependent manner

After confirming that vvDD specifically infected dysplasia the next objective was to determine if the virus infected in a grade dependent manner. To answer this question, the extent of infection was assessed for each type of tissue by quantifying colocalization; this was calculated as the percentage of SA of each type of tissue (normal mucosa, LGD, HGD, and total dysplasia) with positive anti-VV staining relative to the SA of the same tissue on the corresponding H&E slide. Figure 4.4A shows the mean colocalization for each type of tissue at each time point. To determine if there was a grade-dependent infection, the mean colocalization of LGD and HGD were compared at each time point for each route of delivery. The bars with asterisks represent the paired t test performed between the colocalization rates of LGD and HGD for each route of delivery. vvDD-RFP significantly infected HGD more than LGD at 24h, 72h, 120h, and 168h pvi when administered either intraperitoneally or intracolonically. For example, at 120h pvi HGD was infected more than LGD with IC (14.4±4.5% versus 5.5±4.2%, p<0.01) and IP (12.9±7.7% versus 2.0±2.9%, p<0.01) delivery. Overall, normal mucosa was insignificantly infected in all segments of the colon at all four time points. No statistically significant difference was noted between the infection rates of normal mucosa in the disease-free (proximal) and disease-bearing (middle and distal) segments (p>0.05 for all time points.).

The second aim was to determine what route of delivery induced greater infection rates. To determine this we compared the mean colocalization rates between IP and IC delivery for each type of tissue at each time point (Figure 4.4A). The asterisks without bars represent the independent t test performed between the colocalization rates of each route of delivery. Comparison of the mean values of colocalization of each type of dysplasia between the two routes of delivery indicated that overall IC delivery infected a higher SA of LGD and HGD.
In order to confirm that the variability in infection rates of dysplasia were due to an increase in replication and not the result of the availability in the SA of susceptible tissue, the total mean SA of each type of tissue was measured over time (Figure 4.4B). Overall, there was no statistically significant difference in the SA of normal and dysplastic tissue in the middle and distal segment over the course of this experiment; however there was a trend for the SA of dysplasia to decrease overtime. The SA of normal mucosa in proximal segment did not change over time.
Figure 4.4. Quantification of vvDD-RFP infection of colonic dysplasia over time.

A

B
Mice bearing AOM and 2% DSS-induced adenomatous polyps were treated with vvDD-RFP (10⁹ pfu), which was administered either intraperitoneally or intracolonically. **(A)** Quantification of VV infection in areas of normal tissue, LGD, HGD, and total dysplasia expressed as the percentage of tissue SA staining positive VV by IHC (colocalization) over time. **(B)** Distribution of mean total SA (mm²) of tissue per histological slide over time. Bars labeled ‘proximal’ represent the normal tissue from the proximal segment. Bars labeled ‘normal, LGD, HGD, and dysplasia’ represent the results of samples from the middle and distal segment combined. ‘Dysplasia’ represents the sum of LGD and HGD. Three mice from each treatment group were euthanized at each time point. Six sections were obtained from each segment of the colon for a total of 18 sections per mouse. Error bars represent ± two SEM. The brackets with asterisks represent the paired t test performed between the colocalization rates of LGD and HGD. The asterisks without brackets represent the independent t test performed between the colocalization rates of each route of delivery. *, p<0.05.

### 4.2.4 vvDD-RFP specifically replicates in the middle and distal colon

To quantify the rate of replication over time, representative samples from each segment of the colon were used to quantify viral titres. **Figure 4.5** shows the changes in viral titres over time in the middle and distal colon. Data from the proximal segment are not shown because virus was not detected at any time point on standard plaque assay. Changes in viral titres over time were similar in the middle and distal segment for both IP and IC delivery. Viral titres increased from 24 to 120h pvi followed by a marked decrease at 168h pvi. Peak viral titres were higher and occurred earlier with IC relative to IP delivery in both the middle (IC 3.0x10⁶ pfu/mm² at 72h pvi versus IP 8.7 x10⁵ pfu/mm² at 120h pvi, p=0.1) and distal (IC 2.7x10⁷ pfu/mm² at 72h pvi versus IP 3.9x10⁶ pfu/mm² at 120h pvi, p=0.04) colon. Overall, higher peak viral titres were observed in the distal compared to middle segment with IC delivery.
Figure 4.5. vvDD-RFP viral titres over time in the middle and distal segment of the colon.

Two representative samples (size: 1.5 – 2.0 cm width) were obtained from the middle and distal segments of colons of mice exposed to AOM and 2% DSS at different time points after treatment with vvDD-RFP (10⁹ pfu). Results represent the titration of two representative samples from the middle (left) and distal (right) segment of the colon at each time point. Samples were obtained from three mice per treatment group, which were euthanized at each time point. Titration was carried out in triplicate. Error bars represent ± two SEM. The asterisks represent the independent t test comparing the mean pfu/mm² for each route of delivery at each time point. *, p<0.05.

4.3 Summary

VV specifically infected colonic dysplasia as shown by fluorescent microscopy and immunohistochemistry. In the pilot study, ex vivo infection of samples showed a dispersed and well-defined RFP signal starting at 24h pvi, increasing thereafter. Ex vivo samples were not assessed by IHC because H&E staining revealed that the mucosa of virus-treated samples had undergone autolysis while that of mock-treated samples was unaffected. Post-mortem examination under fluorescent microscopy of samples obtained from the middle and distal colon of mice treated in vivo with vvDD-RFP revealed an RFP signal as of 24h pvi. RFP signals were more intense and diffuse with IC than IP delivery. The RFP signal peaked earlier when virus was administered intracolonically (72h pvi) than intraperitoneally (120h pvi). Samples from the proximal colons lacked an RFP signal, as did the samples from mock-treated mice. IHC of in vivo-treated samples confirmed that vvDD-RFP specifically infected dysplasia, HGD more so
than LGD. Normal mucosa was unaffected by the virus; this confirmed that the lack of RFP signal of samples from the proximal segment was due to the virus not replicating in this tissue. Viral titres confirmed the fluctuations observed on fluorescent microscopy in that infection rates increased from 24 to 120h pvi, significantly decreased at 168h pvi, and were higher with IC delivery.

4.4 Discussion

vvDD-RFP demonstrated a high degree of disease-specific infection of AOM and 2% DSS-induced adenomatous polyps and dysplastic mucosa as shown on fluorescent microscopy and histopathological analysis. These results are the first to illustrate that vvDD-RFP has the ability to differentiate a precancerous lesion from its corresponding normal tissue and that the former is permissive to viral replication.

Precancerous lesions are a recently described target of OVs and have been the topic of only three studies, including our own. The degree of dysplasia-specific infection obtained with vvDD-RFP is comparable to that described by the other research groups using oncolytic adenoviruses. Gaballah et al reported that dl922-947, an oncolytic adenovirus defective in pRb-binding, specifically infected patient-derived oral dysplasia. At doses ranging from 10 to 1,000 viral particles per cell, dl922-947 had minimal effect on the viability of normal oral keratinocytes, whereas oral dysplastic cells were significantly lysed. Rudin et al described topical administration of ONYX-015, an attenuated oncolytic adenovirus that selectively infects and destroys cells in which p53-dependent signaling pathways are non-functional, leading to complete histologic response in patients with oral dysplasia.

Ex vivo infection studies offer the advantage of using representative samples and placing them in direct contact with the OV so that one can monitor the infection process continuously over time via fluorescence microscopy. The fact that the RFP signal was limited to virus-treated samples from the middle and distal segment of the colon and that no signal was detected in the mock samples suggested that vvDD-RFP had some degree of specificity. Because colon segments that harbored adenomatous polyps also had foci of normal tissue, as noted during the animal model
characterization experiments, IHC staining was necessary to confirm which were actually being infected.

Although the *ex vivo* infection protocol we followed has been extensively used in our laboratory and *ex vivo* infected tumour samples can remain viable and support OV replication for up to 6 days\textsuperscript{334}, there were challenges in maintaining tissue architecture. The mucosa in virus-treated samples was not present after 48 hours of infection because it had been shed into the culture media before the samples were fixed in formalin (Figure 4.1D); therefore immunohistochemical confirmation of VV infection was impossible. In contrast, mucosa of mock-treated or proximal colon samples was present after 48 hours of treatment. The difference in tissue architecture was likely virus infection-related and not because of experimental conditions. Ischemia per se induces cell loss from the upper half of the colonic crypt while cells in the basal portion of the crypt increase cell proliferation by shortening the cell cycle \textsuperscript{348}. These acute changes may have increased the lytic activity of the virus in the basal portion of the crypt thereby disintegrating the connection between the mucosa and submucosa.

From the *ex vivo* experiments we were able determine the amount of time the treatment solution needed to be in contact with the colonic mucosa in order to produce infection, also known as dwell time. This result was used to determine the duration of dwell time for *in vivo* IC delivery of vvDD-RFP. We tested a dwell time of 60 minutes based on the dwell time used in the standard plaque assay, an *in vitro* assay that measure viral replication, and 30 minutes because it was used in an *in vivo* study that reported histological resolution of oral dysplasia with ONYX-015 mouth wash\textsuperscript{328}. Fluorescence microscopy results revealed that vvDD-RFP produced similar patterns of distribution and intensity with 30 and 60 minutes of dwell time (Figure 4.1); hence we chose to use 30 minute for IC delivery because it appeared to be as effective as 60 minutes. Additionally it decreased the amount of time animals had to be anesthetised. Notably, other studies that administered intracavitary, namely intravesical, oncolytic virotherapy have reported that using dwell times as low as 15 minutes can lead to optimal viral uptake by cancer cells\textsuperscript{349, 350}.

*In vivo* experiments confirmed that vvDD-RFP specifically replicated in dysplasia. There are several possible explanations for this selectivity, including, most importantly the disruption of
the VV TK gene\textsuperscript{222, 293, 294, 299, 300, 351}. Wild type VV synthesizes its own nucleotides and replicates irrespective of the phase which the host cell is in. However, when the TK gene is deleted, viral replication becomes dependent on the host cell’s nucleotide pool. Normal mucosa cells lack a continuous supply of TK because they only express it during the S phase of the cell cycle; in contrast, dysplastic cells are replicating at an abnormal rate and have an increase nucleotide reservoir\textsuperscript{319, 323, 324}. Consequently dysplastic cells offer a permissive environment for viral replication. Successful vvDD-RFP replication requires the host cell to be actively in S-phase of the cell cycle in order to provide the virus with necessary factors to create and assemble progeny\textsuperscript{236}. The molecular features of dysplasia that likely facilitate this include upregulation of growth factor signaling pathways that allow the virus to take advantage of cells, which will provide it with an abundant amount of replication and transcription factors\textsuperscript{316-320}. Other cellular factors that are typically upregulated in adenomatous polyps and facilitate VV replication may have played a role in the specificity. For example, TK deleted VV preferentially replicates in cells with upregulated EGFR/KRAS signaling\textsuperscript{352, 353}. EGFR is upregulated in adenomatous polyps by 35.2% relative to normal mucosa\textsuperscript{317}. The prevalence of KRAS mutations in adenomatous polyps ranges from 15-27% and increases to 33-50% if the lesion is \textgeq2 cm and contains HGD\textsuperscript{354-356}. Although the molecular features that facilitate OV infection of cancer cells are expressed at a lower concentration in dysplastic cells relative to cancerous cells, they are apparently sufficient to allow complete replication of vvDD.

A third possible reason why normal mucosa was unaffected by vvDD-RFP derives from how cells normally grow in the colonic mucosa. The epithelial cells composing the normal colorectal mucosa proliferate in the lower third of the colonic crypt through a well-regulated and fairly slow process every 5 to 6 days\textsuperscript{357}. Consequently, if the virus reached these cells the replication rate is extremely slow and it would not amount to vast viral replication. Additionally, as normal epithelial cells reach the luminal side of the crypt they start to undergo apoptosis in order to counterbalance the growing number of cells at the base of the crypt. In this case, if the virus were to infect these cells, it would not be able to replicate to a significant amount. These characteristics may explain why normal mucosa was unaffected by vvDD-RFP as shown by the lack of RFP signal (\textbf{Figure 4.2}) and viral titres (\textbf{Figure 4.5}) as well as low infection rate (\textbf{Figure 4.4}).
Different infection rates of LGD and HGD were noted on histological analysis. A possible explanation for this derives from differences in cell self-renewal between LGD and HGD, which may have affected the efficiency with which the virus replicated. Dysplastic cells are under a continuous growth stimulus and have defective apoptotic molecules (e.g.: p53)\textsuperscript{59, 358}. These cells continue proliferating even when they reach the luminal side of the crypt and progressively accumulate until forming a mass\textsuperscript{63, 359}. The balance between proliferation and apoptosis varies between the different histological grades of dysplasia. LGD has slightly more cells undergoing apoptosis than HGD\textsuperscript{323, 360, 361}. Based on this, HGD has a more permissive cellular environment that would lead to higher levels of vvDD replication than LGD. For both routes of delivery, vvDD had significantly higher percentages of co-localization in HGD relative to LGD (IC: 14.4±4.5% versus 5.5±4.2% and IP: 12.9±7.7% versus 2.0±2.9%, for each route of delivery $p<0.01$).

Other factors that may have facilitated the specificity towards colonic dysplasia include the size of the virus and specific receptors within adenomatous polyps. The relatively large size of VV prevents its extravasation through normal blood vessels but not through abnormal tumour vasculature, which is generally considered more permeable than normal blood vessels\textsuperscript{242}. Like tumours, dysplasia is perfused by structurally abnormal and hyperpermeable blood vessels\textsuperscript{362, 363}. Additionally, VV binding to cell surfaces significantly increases in the presence of heparin sulfate proteoglycans\textsuperscript{256}. Adenomatous polyps typically have a higher concentration of heparin sulfate proteoglycans relative to normal mucosa and are even comparable to that of CRC\textsuperscript{364}.

Anti-viral defense mechanisms \textit{per se} have not been completely evaluated in colonic dysplasia; however there are some known characteristics that may enhance an already permissive cellular environment. vvDD-RFP may have thrived in dysplasia given that the number of intraepithelial CD8+ T cells, which are responsible for killing virus-infected cells, is normally lower in the descending colon relative to the ascending colon\textsuperscript{365}.

Although we hypothesize that there may be a local pro-viral state that facilitated vvDD-RFP replication in areas of dysplasia, these animals were immunocompetent and therefore it is undeniable that the systemic immune system cleared the virus over time. It has previously been reported that VV replicates for up to 8 days inside immunocompetent hosts\textsuperscript{366}. Similar results
were seen in this study with indicators of viral replication, such as RFP signal intensity, infection rate, and viral titres, present but significantly decreasing by 168h (7d) pvi. A lack of susceptible tissue was not the cause of the decrease in viral replication, as the SA of dysplasia was measured and dysplastic areas were still present over the course of this experiment (Figure 4.4B). Given the level of specificity observed in these experiments, vvDD holds great potential as a treatment for colonic dysplasia; however the innate and adaptive immune system will invariably affect its delivery and efficacy. Overcoming immune system barriers such as antibodies, cytotoxic cells, and the complement system will be necessary in order to enhance its effects on dysplasia.

The route of delivery affected the extent of vvDD-RFP infection. The difference in infection rates between IP and IC delivery were attributed to differences in viral delivery to the target tissue as the viral dose and disease burden were the same between the two treatment groups (Figure 4.4B). Lesions confined to a cavity or hollow structures are ideal for intracavitary delivery. Directly administrating an OV agent has the advantage of increasing the probability of delivering a higher dose concentration because more viral particles come in direct contact with the target rather than if it is administered systemically. Furthermore, despite the fact that systemic delivery is more clinically practical than a retention enema, only a small fraction of the initial viral dose extravasates and reaches target lesions because OVAs are rapidly cleared from the bloodstream by the immune system via the complement system. Virus and virus-infected cells are directly lysed by the complement system or by an antibody-directed response mediated by the complement system. While the relative replication rates were comparable between IP- and IC-delivered virus (Figure 4.5) the amount of virus reaching the tumours at the earliest time point (24h pvi) was significantly decreased following IP administration. Overall IC-delivered vvDD-RFP resulted in a superior infection of colonic dysplasia than IP-delivered virus as shown by the fact that the intensity of the RFP signal peaked earlier (72h versus 120h pvi) and more diffusely, SAs of infected dysplasia (LGD and HGD) were larger, and viral titres were 1-2 logs higher at several time points. Our results are comparable to another study that compared intracavitary versus systemic delivery of oncolytic virotherapy for tumours confined to the pleural cavity. The therapeutic effects observed in nude rats bearing a pleura-based human non-small cell lung cancer were attributed to the direct injection of NV1020, an attenuated, replication-competent HSV-1 into the pleural space as this theoretically increased the concentration of virus at the site of disease.
Chapter 5
Determining the efficacy of red fluorescent protein expressing double deleted vaccinia virus (vvDD) as a treatment for murine colonic dysplasia

5.1 Objective

The objective of this third aim was to determine if vvDD-RFP could effectively treat murine colonic dysplasia. Efficacy was evaluated by treating mice bearing AOM and 2% DSS-induced adenomatous polyps with either virus or mock solution and then comparing the disease burden, residual SAs of dysplasia, and the survival between the treatment groups. Additionally, the effect of single and multiple doses on therapeutic efficacy was studied. Four specific aims were established: (1) to determine changes in the number of polyps and SA of dysplasia; (2) to determine if treatment with vvDD-RFP changed the survival of mice bearing adenomatous polyps; (3) to determine if the rate of the anti-VV humoral immune response was different between the routes of delivery; and (4) to determine if multiple dose therapy was more effective than single dose therapy in treating murine colonic dysplasia.

5.2 Results

5.2.1 Single dose treatment with vvDD-RFP reduces the number of polyps and surface area of dysplasia in mice bearing azoxymethane and 2% dextran sodium sulfate-induced adenomatous polyps

Mice bearing AOM and 2% DSS-induced adenomatous polyps were treated at week 8 with a single IP or IC dose of either vvDD-RFP (10⁹ pfu) or mock solution and then comparing disease burden post-treatment. Treatment was administered at week 8 as this was the earliest time point they would reliably have LGD and HGD. The effects of vvDD-RFP were assessed macroscopically by comparing the number of polyps. SA of dysplasia was also compared
between the treatment groups. From each mouse six histological sections from each segment of the colon were obtained. Slides were scanned and analyzed using imaging software that calculated the SA of areas of interest.

**Figure 5.1** summarizes disease burden of each treatment group. No significant differences were noted in the mean length of colons at 10 days post-treatment (**Figure 5.1A**). To determine if treatment with vvDD-RFP reduced the number of polyps, an independent t test was performed between the mean number of polyps between virus- and mock-treated animals. Mice that received IP treatment with vvDD had a tendency to have fewer polyps than those that received mock treatment in both the middle (IP 5.8±0.5 versus 8.7±1.5, p=0.2) and distal (IP 9.4±0.5 versus 12.8±1.1, p=0.05) segment (**Figure 5.1B**). In contrast, mice that received IC treatment with vvDD had significantly fewer polyps than those that received mock treatment in both the middle (4.3±0.8 versus 10.3±0.5, p=0.001) and distal (5.3±1.1 versus 14.5±1.8, p=0.008) segment (**Figure 5.1B**).

Another aim of this experiment was to determine the impact of vvDD-RFP on the surface area of dysplasia. To do so an independent t test was performed between the mean surface areas of dysplasia between virus- and mock-treated animals. Treatment with vvDD significantly reduced the SA of dysplasia relative to mock treatment when administered intraperitoneally (vvDD 2.1±0.06 mm$^2$ versus mock 2.6±0.1 mm$^2$, p=0.003) or intracolonically (vvDD 1.9±0.05 mm$^2$ versus mock 2.8±0.09 mm$^2$, p<0.0001) (**Figure 5.1C**). Mice that received IP vvDD had significantly lower SA of LGD than their mock counterparts (0.5±0.03 mm$^2$ versus 0.8±0.02 mm$^2$, p<0.0001). In contrast, mice that received IC vvDD had significantly lower SA of HGD relative to mock treatment (1.2±0.01 mm$^2$ versus 1.9±0.08 mm$^2$, p<0.0001).
Figure 5.1. Efficacy of single dose treatment as measured by disease burden in mice bearing AOM and 2% DSS-induced adenomatous polyps.
Eight weeks post-AOM, mice were treated with a single IP or IC dose of vvDD (10^9 pfu) or mock and euthanized 10 days post-infection (dpi) in order to analyze disease burden. Number of animals per treatment group used for this analysis was as follows: IP vvDD: n=4, IP mock: n=4, IC vvDD: n=5, IC mock: n=4. (A) Comparison of the mean colon length between the different treatment groups. (B) Distribution of mean number of polyps per segment of colon between treatment groups. (C) Comparison of the mean surface area (mm^2) of normal tissue, LGD, HGD, and total dysplasia between different treatment groups. Bars represent results obtained from the middle and distal segment combined. Six sections were obtained from each segment of the colon. Error bars represent ± two SEM. Brackets with asterisks represent an independent t test performed between the data from virus- and mock-treated animals. *, p≤0.05, **, p≤0.01, ***, p≤0.001.

5.2.2 Single dose treatment with vvDD-RFP prolongs the survival of mice bearing azoxymethane and 2% dextran sodium sulfate-induced adenomatous polyps

CD-1 mice bearing AOM and 2% DSS-induced adenomatous polyps received either an IP or IC dose of vvDD-RFP (10^9 pfu) or vehicle eight weeks after injection of AOM. The number of animals that were randomized per treatment group was as follows: IP vvDD n=10, IP mock n=10, IC vvDD n=8, and IC mock n=10. This experiment was performed using a blinded experimental design in which the individual who euthanized the animals did not know the
treatment group to which the anima belonged. Animals were clinically examined every other day except when signs of morbidity developed, in which case animals were evaluated daily. Animal health was assessed by monitoring body weight and the development and characteristics of rectal prolapse. If rectal prolapse developed and it was spontaneously reducible the animal was monitored daily, but if it was irreducible the animal was euthanized. The characteristics of the rectal mucosa were also examined and if persistent bleeding, necrosis, or ulcers developed then the animal was euthanized. **Figure 5.2** shows the Kaplan-Meier survival curves comparing the different treatment groups. The main indication to euthanize animals was the development of irreducible rectal prolapse. Median survival dates for IP vvDD, IP mock, IC vvDD, IC mock was 63, 61, 102, and 56 days post-treatment, respectively. IC virus-treated mice survived significantly longer than IC mock-treated controls (median survival 102 versus 56 days, p=0.005), IP mock-treated controls (median survival 102 versus 61 days, p=0.005), and IP virus-treated animals (median survival 102 versus 63 days, p=0.02). IP virus treatment did not improve survival over IP mock-treated controls (median survival 63 versus 61 days, p=0.2).

**Figure 5.2** Impact of single dose vvDD-RFP treatment on survival of mice bearing AOM and 2% DSS-induced adenomatous polyps.
Kaplan-Meier survival curves of mice bearing AOM and 2% DSS-induced adenomatous polyps treated with vvDD-RFP (109 pfu) or mock solution (HBSS) at week 8. Treatment was administered either intraperitoneally or intracolonically. The number of animals per treatment group was as follows: intraperitoneal vvDD n=10, intraperitoneal mock n=10, intracolonic vvDD n=8, and intracolonic mock n=10. Median survival was compared using the log-rank test.

5.2.3 Kinetics of the immune response against vvDD-RFP with intraperitoneal and intracolonic delivery

With the aim of carrying out a multiple dose therapy experiment we wanted to determine if there was a difference in the humoral immune response between the IP and IC delivery. Anti-VV neutralizing antibodies levels were measured through a PRNT from blood samples obtained every four days over the course of 30 days. Samples were consecutively obtained from the same mouse (n=3 for each virus treatment group and n=2 for each treatment mock group). Serial dilutions (1:10, 1:50, 1:100, 1:500, and 1:1000) of heat-inactivated study sera and control sera were prepared in duplicate and mixed with 60 pfu of freshly sonicated vvDD-RFP. The endpoint serum neutralizing antibody titer for each sample was defined as the highest dilution with at least a 60% reduction in the number of plaques with respect to the blank control. Table 5.3 summarizes the highest dilution for each sample; the percentage of samples with that result is also indicated. Despite the fact that the absolute number of plaques for each sample varied (data not shown) the percentage of samples with the indicated end point titre was consistent and relatively high. Detectable levels of anti-VV antibodies were observed at 10 dpi for both routes of delivery; at this time point both IP and IC delivery induced similar levels of antibodies (1:10). IP delivery induced a quicker anti-VV response as antibodies peaked at 18 dpi (1:500) and with IC delivery the same peak occurred at 22 dpi. Serum from a nonhuman primate 137 days post-IP treatment with vvDD-RFP from another study was used as a positive control; the highest positive antibody titre was 1:500. No plaque reduction was observed with the internal negative control (HBSS). The antibody titres for mock-treated animals, both IP and IC, were negative at each time point.
Table 5.1. Anti-vaccinia virus neutralizing antibody levels measured over time in animals treated with intraperitoneal or intracolonically-delivered vvDD-RFP.

<table>
<thead>
<tr>
<th>Days post-infection</th>
<th>Intraperitoneal Titre (% of samples)</th>
<th>Intracolonic Titre (% of samples)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Virus (Mock)</td>
<td>Virus (Mock)</td>
</tr>
<tr>
<td>6</td>
<td>NPR (100%) NPR (100%)</td>
<td>NPR (100%) NPR (100%)</td>
</tr>
<tr>
<td>10</td>
<td>1:10 (66.7%) NPR (100%)</td>
<td>1:10 (66.7%) NPR (100%)</td>
</tr>
<tr>
<td>14</td>
<td>1:50 (83.3%) NPR (100%)</td>
<td>1:10 (66.7%) NPR (100%)</td>
</tr>
<tr>
<td>18</td>
<td>1:500 (83.3%) NPR (100%)</td>
<td>1:100 (83.3%) NPR (100%)</td>
</tr>
<tr>
<td>22</td>
<td>1:500 (83.3%) NPR (100%)</td>
<td>1:500 (83.3%) NPR (100%)</td>
</tr>
<tr>
<td>26</td>
<td>1:500 (83.3%) NPR (100%)</td>
<td>1:500 (100%) NPR (100%)</td>
</tr>
<tr>
<td>30</td>
<td>1:500 (100%) NPR (100%)</td>
<td>1:500 (100%) NPR (100%)</td>
</tr>
</tbody>
</table>

Control samples

<table>
<thead>
<tr>
<th></th>
<th>Virus (Mock)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive control</td>
<td>1:500 (100%)</td>
</tr>
<tr>
<td>Negative control</td>
<td>NPR (100%)</td>
</tr>
</tbody>
</table>

Abbreviation: NPR, no plaque reduction

5.2.4 Multiple dose treatment with vvDD-RFP reduces the number of polyps and surface area of dysplasia in mice bearing azoxymethane and 2% dextran sodium sulfate-induced adenomatous polyps

Mice bearing AOM and 2% DSS-induced adenomatous polyps were treated with three doses of IP or IC-delivered vvDD-RFP (10⁹ pfu) or mock solution, starting on week 8 and thereafter every six days until day 12. Animal distribution was as follows: IP vvDD n=14, IP mock n=14, IC vvDD n=14, and IC mock n=13. The effect of vvDD-RFP was assessed by comparing the number of polyps as well as the SA of residual dysplasia between the treatment groups. To do so, four mice from each treatment group were sacrificed and analyzed 5 days after the last treatment dose. From each mouse six histological sections from each segment of the colon were obtained.
Slides were scanned and analyzed using imaging software that calculated the SA of areas of interest.

**Figure 5.3** summarizes disease burden of each treatment group. With respect to colon lengths, mice with IC-delivered virus had longer colons relative to mock-treated animals (13.7±0.2 cm versus 11.9±0.5 cm, p=0.04) (**Figure 5.3A**). There was no statistically significant difference in the IP treatment group. To determine if treatment with vvDD-RFP reduced the number of polyps, an independent t test was performed between the mean number of polyps between virus- and mock-treated animals. Animals with IC delivery of multiple doses of vvDD had a significantly lower number of polyps relative to mock counterparts in both the middle (3.5±0.6 versus 6.3±0.5, p=0.02) and distal (5.3±1.1 versus 10.3±0.3, p=0.02) segment (**Figure 5.3B**). In contrast, multiple doses of IP-delivered vvDD only significantly reduced the number of polyps in the middle segment (5.2±0.2 versus 10.2±0.5, p<0.001).

The other aim of this experiment was to determine the impact of vvDD-RFP on the surface area of dysplasia after multiple dose treatment. To do so an independent t test was performed between the mean surface area of dysplasia between virus- and mock-treated animals. Multiple doses of vvDD significantly reduced the SA of dysplasia relative to mock-treated animals either when treatment was delivered IP (vvDD 2.6±0.07 mm² versus mock 3.1±0.1 mm², p<0.001) or IC (vvDD 1.9±0.1 mm² versus mock versus 3.2±0.1 mm², p<0.001) (**Figure 5.3C**). Mice that received multiple IP doses of vvDD had significantly lower SA of LGD than mock counterparts (0.4±0.03 mm² versus 1.1±0.04 mm², p<0.0001). Multiple doses of IC-delivered vvDD not only significantly reduced the SA of HGD relative to mock treatment (1.0±0.09 mm² versus 2.3±0.04 mm², p<0.0001), but also significantly decreased that of LGD (0.6±0.06 mm² versus 1.1±0.1 mm², p=0.002).
Figure 5.3. Efficacy of multiple dose treatment as measured by disease burden in mice bearing AOM and 2% DSS-induced adenomatous polyps.

A

B
Eight weeks post-AOM, mice were treated with three doses of IP- or IC-delivered vvDD-RFP (10^9 pfu) or mock solution. To assess the effect of treatment animals were euthanized 5 days after the last dose. Number of animals per treatment group was n=4 for all treatment groups. (A) Comparison of the mean colon length between the different treatment groups. (B) Distribution of mean number of polyps per segment of colon between treatment groups. (C) Comparison of the mean surface area (mm^2) of normal tissue, LGD, HGD, and total dysplasia between different treatment groups. Bars represent results obtained from the middle and distal segment combined. Six sections were obtained from each segment of the colon. Error bars represent ± two SEM. Brackets with asterisks represent an independent t test performed between the data from virus- and mock-treated animals *, p≤0.05, **, p≤0.01, ***, p≤0.001.

5.2.5 Multiple dose treatment with vvDD-RFP prolongs the survival of mice bearing azoxymethane and 2% dextran sodium sulfate-induced adenomatous polyps

Mice bearing AOM and 2% DSS-induced adenomatous polyps were treated with three doses of IP or IC-delivered vvDD-RFP (10^9 pfu) or mock solution, starting on week 8 and thereafter every six days up to day 12. The number of animal that were randomized per treatment group was as follows: IP vvDD n=10, IP mock n=10, IC vvDD n=10, and IC mock n=9. Animals were examined and euthanized using the same parameters for single dose therapy. Figure 5.4 shows the Kaplan-Meier survival curves comparing the different treatment groups. The main indication
to euthanize animals was the development of irreducible rectal prolapse. Median survival dates for IP vvDD, IP mock, IC vvDD, IC mock 77, 70, 97, and 52 days post-treatment, respectively. Mice that received IC-delivered virus survived significantly longer than IC mock (median survival 97 versus 52 days, p=0.002), IP mock (median survival 97 versus 70 days, p=0.005), and IP virus (median survival 97 versus 77 days, p=0.03) treated animals. IP virus treatment improved the survival relative to IP mock-treated controls (median survival 77 versus 70 days, p=0.03).

Figure 5.4 Impact of multiple dose vvDD-RFP treatment on survival of mice bearing AOM and 2% DSS-induced adenomatous polyps.

Kaplan-Meier survival curves of mice bearing AOM and 2% DSS-induced adenomatous polyps treated with three doses of vvDD-RFP (10⁹ pfu) or mock solution (HBSS) every six days as of week 8. Treatment was administered either intraperitoneally or intracolonically. The number of animals per treatment group was as follows: intraperitoneal vvDD n=10, intraperitoneal mock n=10, intracolonic vvDD n=10, and intracolonic mock n=9. Median survival was compared using the log-rank test.
5.2.6 Comparison of single and multiple dose treatment with vvDD-RFP

Efficacy of single and multiple dose treatment was assessed by comparing the mean percentage of polyps and SA of dysplasia relative to mock animals. The efficacy of multiple and single dose treatment was assessed by comparing the mean of the percentage of disease (polyps or SA of dysplasia) relative to mock, which was calculated using the formula: percentage of disease relative to mock = (number of polyps or SA of dysplasia of virus-treated animal)/(mean number of polyps or SA of dysplasia of mock-treated animals) x 100. Figure 5.5A depicts the mean percentage of polyps relative to mock (See Figure 5.1B and 5.3B for absolute values). In general, multiple dose treatment of vvDD-RFP did not have a greater reduction in the number of polyps, except with IP treatment. In the middle segment, the percentage of polyps relative to mock was lower with multiple dose treatment than with single dose treatment (51.2±2.4% versus 67.0±4.6%, p=0.02). Figure 5.5B illustrates the mean percentage of SA of dysplasia relative to mock (See Figure 5.1B and 5.3B for absolute values). Multiple dose IP treatment had a lower percentage of LGD relative to mock than single dose IP treatment (20.8±1.8% versus 26.8±1.6%, p=0.02), but did not have a greater effect on HGD (83.7±2.4% versus 69.4±2.6%, p<0.0001). Overall, multiple dose IP treatment was not associated with a lower percentage of dysplasia relative to mock when compared to single dose IP treatment (81.9±2.4% versus 78.8±2.4%, p=0.4). When compared to single dose treatment, multiple dose IC treatment had a lower percentage of LGD (39.3±2.1% versus 27.9±2.8%, p=0.002), HGD (51.4±0.5% versus 37.6±3.2%, p<0.0001), and dysplasia (74.5±1.7% versus 41.4±3.4%, p<0.0001) relative to mock.
Figure 5.5 Comparison of the effect between single and multiple dose vvDD-RFP treatment as measured by the percentage of disease relative to mock treatment.

The efficacy of single and multiple dose treatment was assessed by comparing the percentage of polyps (A) and SA of dysplasia (B) relative to mock. The percentage of polyps or SA of dysplasia relative to mock animals represented the proportion of disease (number of polyps or surface area of dysplasia) in the virus-treated animal relative to the mean of mock-treated mice. Error bars represent ± two SEM. *, \( p \leq 0.05 \), **, \( p \leq 0.01 \), ***, \( p \leq 0.001 \).
5.3 Summary

Efficacy was evaluated by treating mice bearing AOM and 2% DSS-induced adenomatous polyps with either vvDD (10⁹ pfu) or mock solution and then comparing the number of polyps and SA of dysplasia as well as survival between the treatment groups. Treatment was delivered 8 weeks post-AOM because based on our previous results this time point had sufficient burden of LGD and HGD. Single dose treatment was first evaluated. Post-mortem analysis indicated that at 5 days post-infection, IC-delivered vvDD-RFP significantly reduced the number of polyps both in the middle and distal segments of the colon relative to mock treatment. IP delivery did not induce any changes in the number of polyps. With regards to the number of polyps, a significant reduction was observed both in the middle and distal segment with IC-delivered vvDD-RFP. IP-delivered virus significantly reduced the number of polyps only in the middle segment. On histological analysis, vvDD-treated mice had an overall lower SA of dysplasia than mock counterparts. Stratifying by type of dysplasia revealed that the burden of LGD was significantly decreased only with IP delivery whereas the burden of HGD was reduced only with IC delivery. The impact on number of polyps and surface area of dysplasia translated into significantly prolonged survival of IC vvDD-RFP treated mice relative to controls on Kaplan-Meier survival analysis.

With the aim of setting up a multiple dose therapy experiment, we asked if there was a difference in the immune response between the two routes of delivery. Anti-VV neutralizing antibodies were induced as of day 10 post-virus infection. IP delivery induced a quicker anti-VV response, as antibodies peaked earlier at 18d pvi, while IC delivery reached the same antibody titre at 22d pvi.

Knowing that there was a window of opportunity to administer additional doses, multiple dose therapy was assessed. In this case, IC-delivered virus significantly reduced the number of polyps in both the middle and distal segment, but IP-delivered virus only had an effect in the middle segment. IC-delivered virus not only reduced the SA of HGD, but it also affected LGD. Similar to what was observed with single dose treatment, IP-delivered virus only affected the SA of LGD. In terms of survival, multiple doses of virus significantly improved the survival of animals
relative to mock-treated mice. IC delivery continued to be associated with the most significant improvement of survival relative to mock animals. There was a slight improvement in the survival of IP-treated animals when compared to mock. Comparing single to multiple dose treatment revealed that overall the latter did not have a greater reduction in the number of polyps than single dose treatment. Multiple dose IP treatment was not associated with a lower percentage of residual dysplasia relative to single dose IP treatment. In contrast, multiple dose IC treatment did induce a more significant reduction in LGD and HGD relative to single dose IC treatment.

5.4 Discussion

vvDD-RFP significantly reduced the number of polyps and SA of dysplasia and prolonged the survival rate of mice. This is the first study demonstrating that an OV can infect, successfully replicate in, and treat colonic dysplasia and it is the first study to use VV to target and treat precancerous lesions. Despite these results, treatment with vvDD-RFP was not curative and had limited efficacy as virus-treated animals still had adenomatous polyps, dysplasia, and did not survive. The potential treatment benefits of vvDD-RFP may have been limited by the fact that the virus was outgrown by the rapidly replicating dysplastic tissue. Notably, this extensive disease burden is not typical of sporadic human adenomatous polyps; therefore the efficacy of vvDD-RFP in terms of a clinically-relevant model remains to be determined. Nonetheless our study provides evidence that the virus is specific to colonic dysplasia and that it induces notable reduction in disease burden. Further work will be necessary in order to optimize the efficacy for use in a clinical setting.

Various routes of delivery have been tested for oncolytic virotherapy, including intravenous, IP, intraarterial, intratumoural, intracerebral, intraductal, intrapleural, and intravesical. In this study we chose to compare IP versus IC delivery of vvDD-RFP because each offers particular advantages. Adenomatous polyps are well-vascularized lesions as shown by the high microvessel density (MVD) \(^{362, 369-372}\); therefore with IP delivery, an OV would have several access points to reach target cells. However given that adenomatous polyps grow from the epithelium and protrude towards the lumen, dysplastic cells may also respond to intracavitary delivery. This
route of delivery allows the entire colonic mucosa to receive direct and simultaneous treatment. Additionally, using IC delivery may have the potential advantage of avoiding exposure to the anti-viral immune response, at least for a short period of time while the virus starts to replicate.

When changes in disease burden and outcome were compared between the two treatment groups, IC delivery had a greater impact than IP delivery as shown by the lower SA of dysplasia. These findings are comparable to those observed in other animal studies in which OVs were administered through a cavity. In animal models of bladder cancer, several OVs including HSV (e.g., Oncovex\textsuperscript{GALV/CD}, G207, and Nv1020)\textsuperscript{350, 373, 374}, VSV (e.g., AV3)\textsuperscript{375}, reovirus\textsuperscript{376}, and adenovirus (e.g., CG0070 and AxdAdB-3)\textsuperscript{349, 377} reduced tumour burden by 46-90%, and in some cases were shown to significantly increase tumour-free survival time relative to mock or standard treatment (BCG immunotherapy). The treatment benefits of intracavitary delivery have also translated into humans. In clinical studies, response rates for intracavitary delivery, such as intravesical and as a mouthwash reach 37.0–48.6%\textsuperscript{328, 378} versus the 10% obtained with IV delivery\textsuperscript{379-382}.

The benefit of systemic delivery is that it provides the opportunity to simultaneously treat multiple lesions in different sites at the same time, assuming they are well-vascularized. As a result, systemic delivery is the preferred method to treat patients with advanced or metastatic tumours as well as those with tumours that would have limited viral distribution due to physiological barriers (e.g., blood-brain barrier)\textsuperscript{383}. Based on this we hypothesized that systemic delivery would be ideal for adenomatous polyps as they are in proximity to blood vessels in the submucosa. Nonetheless, reduction in disease burden was only observed in the middle segment and survival was not prolonged relative to mock treatment after a single dose (median survival time: 63 versus 61 days, p=0.2).

The promising results observed with single dose therapy led us to question if multiple doses would improve the treatment efficacy of vvDD-RFP. Before considering this approach, we characterized the rate in which neutralizing anti-VV antibodies were produced. Understanding the kinetics of neutralizing antibody production allowed us to determine if there was a window of opportunity to administer multiple doses.
In our study neutralizing antibodies were detectable as of day 10 pvi, similar to what has been reported in the literature\textsuperscript{384-386}. Neutralizing antibodies increased over time, peaking at day 18 and 22 for IP and IC delivery, respectively. Both of these time points are later than what has been reported in the literature. For example in humans, peak levels of neutralizing antibodies are observed between day 13 and 16 post-vaccination\textsuperscript{384}. The overall delay in the peak of antibodies likely was due to the route of delivery as this is a major determinant of the level of the anti-viral immune response in a virus-based therapy or vaccination setting\textsuperscript{387, 388}. First, delivering VV intraperitoneally typically gives the lowest humoural response relative to other routes, namely intravenous delivery\textsuperscript{389}. Second, delivering VV intracolonically hypothetically slows down the immune response by minimizing systemic viral exposure. Intracavitary delivery of OVs has been shown to induce a minimal humoural response\textsuperscript{328, 378}. For example, despite being a highly immunogenic virus\textsuperscript{390}, ONYX-015 demonstrated minimal increases in antibody titres over the course of treatment when administered in the form of a mouthwash\textsuperscript{328}. Of seven patients, only one demonstrated an increase (two-fold) in titres over the course of the experiment.

Multiple dose treatment is theoretically limited by the fact that VV induces a strong humoural response. The fact that neutralizing anti-VV antibody titers as low as >1:32 show protective immunity against smallpox and also VV\textsuperscript{391, 392, 392}, suggests oncolytic VV may have a minimal anti-tumour effect in previously immunized patients or after multiple dose treatment. Despite this premise, there is evidence indicating that anti-tumour effects can be elicited even in the presence of anti-VV neutralizing antibodies, as long as virus can be delivered to the tumour. In a murine xenograft model of CRC, systemic or intratumoural administration of vvDD demonstrated delivery and treatment efficacy despite the fact that mice had high antibody titres to VV\textsuperscript{393}. A clinical trial demonstrated that intravenously delivered JX-594 infected, replicated, and expressed the transgene GM-CSF specifically in tumours of patients with a known history of childhood smallpox vaccination and detectable levels of neutralizing antibodies\textsuperscript{394}. The explanation for these positive outcomes is that VV produces ‘stealth’ particles in the form of EEV, which are not neutralized by antibodies nor the complement system\textsuperscript{240, 241}. Another theory is that IV delivery may transiently saturate native mechanisms of viral clearance thereby allowing a fraction of the dose to reach the target tissue\textsuperscript{394}.
The SA of LGD and total dysplasia showed improvement relative to single dose treatment with IP and IC delivery, respectively (Figure 5.5B). On survival analysis, these changes manifested with IC-delivered virus continuing to have the longest survival rates and IP-delivered virus to gain a slight, but statistically significantly improvement relative to mock-treated animals (Figure 5.4). This study adds to the body of evidence in the literature that suggests that multiple doses of OVs improve treatment efficacy. For example, in a phase 1 study of patients with non-muscle invasive bladder cancer, multi-dose treatment with CG0070, a serotype-5 oncolytic adenovirus that destroys Rb-defective cells and expresses GM-CSF, demonstrated that response rates were slightly higher than that of single dose treatment (63.6% versus 44.4–61.5%)\textsuperscript{378}.

A t test is used to determine whether the mean of two groups are significantly different or if the difference is due to chance alone. Nevertheless problems arise when more than one t test is performed. The most important problem is that performing multiple t tests with a set of data increases the probability of falsely rejecting the null hypothesis (type I error)\textsuperscript{395}. Every time a t test is conducted the type I error rate is increased by 5%. As a result, instead of falsely declaring significance at the low, acceptable 5% rate, error may occur at an unacceptably high rate\textsuperscript{395}. Based on this, finding only marginal significance with multiple t tests (a significant p value slightly less than 0.05) implies that the difference may be due to random chance alone. Performing multiple t tests is considered to be valid only in two cases\textsuperscript{395}: (1) when none of the t tests are significant and (2) when the significance is extremely high (p<0.001). Multiple comparisons between data were carried out with the same set of data because there were several groups to analyze based on treatment, time point, and type of tissues. Various t tests obtained levels of significance less than 0.001, but the data presented in Figure 5.1B, Figure 5.1C, and Figure 5.3B may have limitation in the data interpretation.

Our study brings has shown for the first time that oncolytic vaccinia can target colonic dysplasia. Here we show for the first time that vvDD–RFP exhibits grade-dependent specificity and oncolytic activity in colonic dysplasia, resulting in improved survival rates in an immunocompetent chemically-induced murine model of adenomatous polyps.
Chapter 6
Discussion and Future Directions

Up to 15% of colorectal polyps are not amenable to standard treatment and require advanced techniques and/or surgery\textsuperscript{125, 129, 130}. Between 2002 and 2008 the overall number of patients who underwent CRC screening increased from 53.8\% to 64.2\%\textsuperscript{396, 397}, and the number of patients in the US that will undergo colonoscopy will likely continue to grow from the \~15 million that are currently performed each year\textsuperscript{398, 399}. Locally, in Ontario, approximately 300,000 colonoscopies are performed annually and volumes are reported to grow at an average rate of 12-13\% per year\textsuperscript{400}. This suggests that the detection rate of adenomatous polyps, the most common pathological finding on colonoscopy\textsuperscript{123}, will also increase. Certainly, “complex” polyps will also continue to be diagnosed. Current treatment options, such as EMR and ESD, are limited in that they are associated with significantly high failure (13.3-23.3\%)\textsuperscript{139-141} and recurrence rates (3.1-46\%)\textsuperscript{136, 142-144}, which require patients to undergo multiple treatment sessions and/or ultimately surgical resection. As a result, developing novel therapeutics is necessary. New treatment options may be more appealing if they can specifically and effectively target precancerous colonic lesions both in a safe and non-invasive manner. Given that 74\% of patients with “complex” polyps have dysplasia\textsuperscript{121}, treatment with oncolytic virotherapy may be of benefit as the molecular features that facilitate OV infection are also present in dysplasia. Our study shows that colonic dysplasia is permissive to infection of vvDD-RFP in a grade-dependent and that it induces a significant effect on disease burden. Nevertheless, treatment was not completely effective as virus-treated animals still had macro- and microscopic disease. Further work should focus on optimizing the efficacy of treatment.

Oncolytic virotherapy is being developed as a potential treatment option for cancer patients and may also be of interest for use in precancerous colonic lesions due to its unique characteristics. OVs specifically target aberrantly proliferative cells through exploitation of the very same cellular defects that promote tumour growth\textsuperscript{401}. As a result, toxicity to normal tissue is kept to a minimum. OVs have multiple and diverse innate mechanisms of anti-tumour action, which include vascular shutdown\textsuperscript{203} and anti-tumour immune responses\textsuperscript{402}. The killing capacity of newer generations of OVs has been enhanced by inserting genes that encode for toxic or
immune-stimulatory products\textsuperscript{403}. Furthermore owing to their ability to self-replicate, the number of cell killing viral particles has the potential to grow exponentially, distinguishing them from conventional therapeutics, which have a finite dose\textsuperscript{401}.

Oncolytic VV offers the potential to destroy precancerous colonic lesions because it specifically replicates in actively dividing cells. Dysplastic cells have down-regulated tumour suppressor genes, such as \textit{APC}, and upregulated oncogenes, such as \textit{KRAS} and \textit{BRAF}\textsuperscript{69, 77} which lead to an overtly proliferative state that is greater in HGD than LGD\textsuperscript{322-324}. At a molecular level, the concentrations of nucleotide synthesizing enzymes, such as \textit{TK}\textsuperscript{319, 320} and nucleotides\textsuperscript{321}, are also upregulated. Dysplastic cells may provide a pro-viral state if like other precancerous lesions they have impaired anti-viral defense mechanisms\textsuperscript{404, 405}. Lastly, dysplastic lesions have upregulated vascular endothelial growth factor (VEGF) and VEGF receptor (VEGFR), which they use to induce angiogenesis\textsuperscript{569}. It has been hypothesized that VEGF–VEGFR intracellular signaling pathways also induce a pro-viral proliferative state similar to that of \textit{RAS}-activated cancer cells\textsuperscript{406}.

Our results are similar to those from previous work describing the use of oncolytic adenovirus as a treatment for oral dysplasia. Rudin \textit{et al} \textsuperscript{328} reported that daily exposure to ONYX-015, in the form of an oral rinse, reduced the histological severity of oral dysplastic lesions in patients. Gaballah \textit{et al} \textsuperscript{326} confirmed through histological analyses that an oncolytic adenovirus (dl922-947) specifically lysed patient-derived oral keratinocytes originating from dysplastic lesions in a dose-dependent manner while leaving normal mucosa unharmed. Taken together these results create a growing body of evidence in favour of using oncolytic agents against dysplastic lesions.

\textit{vvDD} has the ability to kill their targets by multiple mechanisms of action. The proposed mechanisms of action are complementary given the crucial interdependent relationship the virus has with different components of the microenvironment. The principal mechanisms of action of \textit{vvDD}, through which it most likely killed dysplastic cells, decreased disease burden, and ultimately prolonged survival rates in mice bearing adenomatous polyps, is a combination of necrosis and apoptosis. Necrosis results from severely acute cell damage, whereas apoptosis is cell-controlled death of aged, damaged, and virus-infected cells\textsuperscript{407}. Despite being highly immunogenic, VV manages to survive and replicate within a host organism and induce cell death
via necrosis and apoptosis. Typically, necrosis occurs after viral replication as cytotoxic viral proteins and virions accumulate in the cytoplasm and the cell membrane is disrupted after an excessive amount of viral particles bud from it. Apoptosis-induced death is more complicated because VV delays it from taking place. By using virus-encoded proteins that impede apoptosis (e.g.: factors that inhibit cytokine processing and proteolytic activation of caspases, soluble receptors that neutralize cytokines, factors that inactivate IFN-inducible antiviral enzyme activities, and analogues of growth factors and hormones) VV has enough time to replicate. Eventually, VV induces apoptosis as a means to exit the host cell either by directly activating caspases or stimulating the activation of BH3-only proteins mitochondrial mediated apoptosis.

OVs are modified not only to increase treatment efficacy but also to decrease the risk of toxicity to normal tissue. Biosafety and risk management issues are intensely scrutinized given that oncolytic virotherapy uses replication competent viruses that need to infect cells in order to thrive. In the case of VV, virus entry uses ubiquitous cell surface receptors therefore theoretically VV may be able to infect and damage normal tissue. However studies assessing vvDD biodistribution have shown that the attenuation through deletion of the TK and VGF genes limits viral replication to tumours. When viral particles are found in normal organs the viral titre is several log fold lower. The increased viral titres in the ovaries presumably results from the “leaky” vasculature which allows the large viral particle (~300nm) to extravasate. Notably, this appears to be a species-specific phenomenon because infection of ovaries was not observed when vvDD was studied in non-human primates; virus was not detected in the ovaries at 6 days or 6 weeks post-infection.

Our results demonstrate that treatment with vvDD has no toxic effects even when in direct contact with normal mucosa, one of the fastest self-renewing tissue in adult mammals. The infection rate of normal mucosa as shown by IHC was insignificant (0.005±0.001%) as was viral replication. IP-delivered vvDD-RFP was not associated with toxicity of normal mucosa. The lack of infection of the bowel has been previously reported in a study evaluating safety and biodistribution of intravenously delivered vvDD in rhesus macaques. In this study, after 10^8 pfu of vvDD, virus was not detected in the colon at 6 days or 6 weeks post-treatment. Although we did not specifically assess viral toxicity in other organs, animals did not develop any clinically visible sign of toxicity. To date, the safety of administering an OV as the treatment for
precancerous lesions has been the objective of only one study\textsuperscript{328}. In patients with oral dysplasia, ONYX-015 mouthwash was well-tolerated even at doses of up to $10^{11}$ pfu/day; the authors mainly noted grade 1 diarrhoea and minor febrile episodes\textsuperscript{328}.

Oncolytic virotherapy may have applicability in other types of precancerous lesions. SSA/P and TSA harbour dysplasia that resembles the architectural and cytological features of adenomatous polyps\textsuperscript{22}. These types of serrated polyps contain genetic mutations (e.g., \textit{BRAF} and \textit{KRAS} mutations) that could facilitate replication of vvDD\textsuperscript{51}. Additionally, OVs could benefit from the fact that these lesions have decreased apoptosis\textsuperscript{51}, which would further facilitate viral replication. Other gastrointestinal organs that develop dysplasia, such as the oesophagus and stomach, may likely benefit from oncolytic virotherapy because the dysplasia resembles the histopathological and molecular features of colonic dysplasia\textsuperscript{45}. Precancerous lesions of other origins may also be susceptible to oncolytic virotherapy. Several intraepithelial precancerous lesions, such as ductal carcinoma in situ (DCIS), non-muscle invasive bladder cancer, and cervical intraepithelial lesions, share the histopathological and molecular features of colonic dysplasia. These precancerous lesions are immunosuppressed in order to continue with unregulated growth, and therefore provide a pro-viral microenvironment. These precancerous lesions are typically highly proliferative lesion as shown by proliferation markers such as Ki-67\textsuperscript{412-414}. Factors that regulate IFN signaling are significantly down-regulated in different grades of DCIS\textsuperscript{404, 405}. Cervical squamous intraepithelial lesions have decreased cytotoxic lymphocyte response through altered mechanisms of neoantigen presentation in MHC molecule complexes\textsuperscript{415}. With regards to proliferation markers, TK expression levels in patient-derived DCIS is increased at levels similar to that of invasive ductal carcinoma; and a similar phenomenon occurs with superficial bladder cancer\textsuperscript{416} and cervical intraepithelial lesions\textsuperscript{417}. Additionally, organs such as the stomach and bladder could benefit from intracavitary delivery.

This study also brings forth the possibility of using oncolytic virotherapy for IBD-associated dysplasia. OVs may have the same specificity in these lesions because the carcinogenic process associated with IBD is considered to be similar to the adenoma-carcinoma sequence of sporadic CRC in terms of the types of molecular alterations; these molecular alterations include accumulation of gene mutations in tumour suppressor genes, oncogenes, and DNA repair genes, as well as genomic instabilities\textsuperscript{418}. Despite these similarities, there are several differences in the
timing and frequency of the molecular genetic alterations as well as different etiologic factors and disease burden which will need to be accounted for if OVs are to be tested as a treatment modality for these lesions.

Mathematical modeling indicate that effective in vivo oncolytic therapy requires the pattern of infection to be diffuse, the virus to spread quickly, suppression of immune-mediated viral clearance, and a bystander effect deriving either from a virus-induced anti-tumoural immune response or “arming” of the virus with therapeutic genes. In order for oncolytic virotherapy to become a clinically relevant option for colonic dysplasia several of these features will require reassessment and optimization through further experimental work, including (1) evaluating other OVs in order to exploit the heterogenic nature of adenomatous polyps; (2) optimizing intracolonic delivery as it appears to delay the anti-viral immune response; and (3) exploiting additional mechanisms of action that can kill dysplastic cells directly or indirectly.

At a molecular level, adenomatous polyps are heterogenous. Because adenomatous polyps develop through different combinations of host genetic features, mucosal exposures, and crypt interaction these lesions are polyclonal in nature. To compensate for tissue heterogeneity several OVs may be necessary. In this study we used vvDD, the specificity of which is controlled by deletion of viral genes critical for virus replication in healthy cells, but expendable in aberrantly proliferative cells. It would be interesting to study OVs with other types of genetic modifications. An attractive option would be to use oncolytic adenoviruses with CAR-dependent specificity. CAR is the primary adenovirus receptor; however OVs relying solely on this as a means of specificity have been discarded because many cancers, including gastrointestinal, pancreatic, ovarian, and prostate cancer, lack CARs. Nonetheless, there is evidence that favours their use in colonic dysplasia. On IHC analysis, CAR plasma membrane levels are significantly higher in patient-derived samples of adenomatous polyps than CRC (100% versus 49%, p<0.0001). CAR-independent adenovirus serotype vectors also have potential use against colonic dysplasia. For example, Ad35 has been modified to target cancerous cells that overexpress CD46, a membrane cofactor protein that acts as a receptor for the complement components C3b and C4b. Colorectal adenomatous polyps are a potential target for such an OV because they overexpress CD46. Other OVs with CD46-dependent specificity, such as the
Edmonston B strain of measles virus (MV-Edm)\textsuperscript{422}, are also potential candidate agents for colonic dysplasia.

Another option is to use OVs that carry tumour-specific promoters upstream of necessary viral genes. In this case, virus replication will only occur when these promoters are activated. Fuerer \textit{et al} developed E1A-HD2, an oncolytic adenovirus that carries a fusion between the viral transactivator E1A and the BCL9 HD2 domain\textsuperscript{423}. Mutations in \textit{APC} and \(\beta\)-catenin genes stabilize \(\beta\)-catenin protein and activate transcription by fusing TCF/LEF transcription factors and the HD2 domain of the BCL9 coactivator. The E1A-HD2 fusion protein could be advantageous to oncolytic adenovirus activity against dysplastic cells because binding sites for TCF/LEF transcription factors are inserted into the early viral promoters and these factors are upregulated in dysplastic cells. Another tumour-specific promoter element that could be used to regulate OV specificity in adenomatous polyps is the \textit{MUC1} gene, which encodes for a mucin-like glycoprotein (DF3/Muc1) known as epithelial membrane antigen (EMA) and is overexpressed in up to 76\% of dysplastic lesions in a grade-dependent manner\textsuperscript{424-426}. Because \textit{MUC1} is overexpressed in these lesions it suggests that by inserting the \textit{MUC1} promoter in front of early viral promoters, viral gene expression can be redirected towards dysplastic cells. Several OVs which are driven by the \textit{MUC1} promoter have been developed, including adenovirus (Adeno-DF3-E1A/hTERT-E1A and MUD55)\textsuperscript{427,428} and HSV (DF3\(\gamma\)34.5).

In the AOM and 2\% DSS model of colonic dysplasia, IC delivery was feasible and provided significant treatment benefits over IP delivery. We hypothesize that the reason for this is that IC delivery maximizes the amount of virus reaching the target area with minimum distribution to non-affected tissues. In order to further improve treatment outcome, IC delivery will need to be optimized. Studies derived from our work should focus on determining the optimal dwell time (duration of contact time the treatment solution needs to produce the desired effect) in humans. In this study vvDD was delivered via 30-minute retention enemas based on an \textit{ex vivo} pilot study; however it will be important to further explore the dwell time for humans as this may vary.

Systemic delivery of oncolytic virotherapy should not be completely ruled out because it could offer benefits in treating microscopic lesions. Although adenomatous polyps measuring 1–5 mm
grow very little and those with a size between 6–9 mm may regress\textsuperscript{429}, these small lesions must be treated because from a risk assessment point of view, the total number of adenomatous polyps is an important predictor of occurrence of advanced adenomatous polyps\textsuperscript{40}. Accordingly, systemic treatment may be of advantage given that adenomatous polyps are well-vascularized lesions\textsuperscript{362, 369-372}. Systemically delivered OVs would have multiple access points to simultaneously reach target lesions. With the aim of improving systemic delivery, the immune system will certainly need to be modulated. Because the complement system is critical in pathogen clearance and has a central role in poxvirus clearance\textsuperscript{368}, inhibiting it may improve systemic delivery of oncolytic VV. \textit{In vitro} work by Magge \textit{et al} showed that complement inhibition, specifically of C5 by Staphylococcal superantigen-like protein, led to a 90-fold and 150-fold enhancement of VV infectivity in both the presence and absence of anti-VV antibodies, respectively\textsuperscript{450}. A potential clinical scenario would require administering clinically approved C5 inhibitors of the complement system, such as the humanized monoclonal antibody eculizumab, to improve systemic delivery.

Intrapolyp delivery was not assessed in this study due to limitations in the size of animals and the lack of endoscopic equipment. Nonetheless, injecting OVs directly into adenomatous polyps warrants investigation because intratumoural delivery guarantees that a larger fraction of the total dose reaches the affected area and this has become the preferred route of delivery for localized disease\textsuperscript{431}. Intrapolyp injection of an OV would be ideal for polyps that are classified as “complex” solely based on size >2.0 cm\textsuperscript{125, 126, 128} because it could eradicate the lesion altogether. Furthermore, with the aim of having a broader local distribution of the virus, the dose could be divided and administered simultaneously through multiple injections into a polyp. According to mathematical modeling, having a broader distribution of the virus is more effective than administering high viral concentrations because it decreases the amount of uninfected areas that could outrun viral replication\textsuperscript{419}. Optimizing delivery to improve efficacy also has the benefit of permitting dose reductions. For example, in a human prostate cancer xenograft murine model, an 80\% reduction in tumour growth was achieved with either an intravenous dose of $10^{10}$ pfu or an intratumoural dose of $10^{7}$ pfu\textsuperscript{432}. To evaluate intrapolyp delivery for colonic dysplasia, rats could be used for future studies as this species is known to respond to AOM\textsuperscript{146}. Currently, a safe total colonoscopy with submucosal injection rat model exists and could be used to test intrapolyp delivery of OVs\textsuperscript{433}. 
Given the critical role of angiogenesis in disease progression, OVs with anti-vascular properties should be evaluated for a potential use in the “angioprevention” of adenomatous polyps. Originally, it was hypothesized that tumours induced the angiogenic switch, a process in which tissues acquire the ability to independently induce angiogenesis in order to continue growing. However, this actually starts in the precancerous stage of several human cancers including CRC. Adenomatous polyp-associated vasculature overexpresses the angiogenic factor VEGF, specifically VEGF-A/B, and their corresponding receptors (VEGFR-1/-2). The increases in VEGF manifest with a higher MVD as early as the onset of LGD. Consequently, if adenomatous polyp-induced angiogenesis is inhibited then not only will lesion growth be inhibited but the adenoma-carcinoma progression will be blocked. The impact of using small molecules for “angioprevention” on adenomatous polyps has been previously described. For example, using an anti-VEGF monoclonal antibody reduced disease burden by decreasing MVD and induced long-term survival in Min/ΔAPC mice bearing adenomatous polyps. Certainly, OVs with anti-vascular properties could induce similar results. Conditionally replicating oncolytic adenoviruses encode the E1A protein, which interacts with angiogenic cellular proteins, such as p300, to down-regulate VEGF while other OVs including VV have been ‘armed’ to encode antibodies against VEGF and VEGFR. It may be beneficial to evaluate the submucosal injection of OVs with these anti-vascular properties because this would maximize the concentration of viral particles where blood vessels are located.

Another mechanism of action worth exploring is the virus-induced anti-tumour immune response. Virus-induced cell lysis leads to the release of cytokines, host antigens, and other danger signals, such as DAMPs and PAMPs, into the microenvironment, which then induces a cell-mediated immune response against unaffected tumour cells. In order for OVs to achieve their full potential, reaching a balance that favours an anti-dysplasia immune response and minimizes virus clearance will require further investigation. Research is focusing on harnessing the immune response as a therapeutic mechanism of action for oncolytic VVs through strategies such as (1) insertion of immunotherapeutic transgenes (e.g., GM-CSF) to provide a bystander effect; (2) deletion of viral virulence genes that target cytokines critical for the induction of the immune response; and (3) insertion of tumour antigens into viral vectors, which activate
dendritic cells and in turn present it to T cells in regional lymph nodes, with the aim of providing protective immunity against tumours expressing that particular antigen.

Multiple dose treatment was assessed in this study and not only confirmed what was observed with single dose treatment, but it indicated that there were noteworthy improvements in reducing the SA of dysplasia and prolonging survival. Our study adds to the growing body of evidence in the literature supporting multiple dose treatment. In mice bearing orthotopic bladder tumours treatment with five doses of AxdAdB-3, a double-mutated oncolytic adenovirus with mutations of E1A and deletion of E1B 55KD, on days 4, 7, 10, 13, and 16 significantly improved survival relative to single dose treatment (median survival ~55 days versus ~30 days, p=0.0016). Rudin et al described how multiple doses of ONYX-015 in the form of an oral rinse reduced the histological severity of oral dysplasia in patients. Given that multiple dose treatment with OVs has the potential to improve outcomes, it requires further investigation. As long as the virus can be delivered to the target lesion, replication and lysis will ensue; therefore multiple dose treatment should be explored once delivery of OVs has been optimized. Additionally, if intrapolyp injections become technically feasible it would be worth exploring multiple dose treatment as studies have shown that multiple intratumoural injections show active viral replication over time and eventually induce tumour regression. This treatment approach would certainly benefit from multiple doses as the SA of dysplasia decreased with multiple dose treatment.

Another application of oncolytic viruses in the treatment of precancerous colonic lesions is that of diagnostic imaging. With miss rates of up to 20% reported for adenomatous polyps, especially with flat and depressed lesions being the most difficult to identify with standard white light colonoscopy, the potential to improve polyp detection with the objective of preventing CRC is substantial. Given that vvDD has a high degree of biological selectivity towards colonic dysplasia and that it encodes a fluorescent protein as a means to monitor infection in real-time, using this OV as a diagnostic aid for fluorescence-based endoscopy may be worth studying. Our study demonstrated that RFP-positive samples contained VV-infected dysplasia and that RFP was not detected in dysplasia-free regions of the colon. Based on this, a possible clinical scenario would require administering vvDD-RFP through a retention enema 24 hours before an endoscopic procedure. After conventional white light endoscopy, the colonic mucosa could be
examined with fluorescent light in order to guide tissue biopsy of RFP positive areas. Several experimental studies in mice and human samples have already examined the usefulness of fluorescent protein-encoding OVs as diagnostic tools. These studies have illustrated that OVs significantly increase the detection rate of tumour nodules and free floating malignant cells\textsuperscript{442-448}.

Despite the fact that vvDD-RFP induced significant effects on disease burden and improved survival, treatment was not curative. A possible reason why vvDD-RFP was not effective was that the animal model induced extensive disease burden, which potentially may have outgrown the virus. In this model, mice developed multiple adenomatous polyps in the distal two-thirds of the colon, which spatially represented approximately 27\% of the tissue in each segment. In contrast, a “complex” polyp generally presents as a singular lesion rather than a field of polyps. Because the disease extent is smaller in humans there is hypothetically the potential to obtain treatment efficacy in humans. This is further justified by the fact that OVs are more effective in treating well-defined, small tumours rather than those that are large and diffuse\textsuperscript{419}. Nevertheless, only clinical studies will be able to determine if oncolytic vaccinia specifically infects and effectively treats human colonic dysplasia. Further work will also be necessary to understand the mechanism of action the virus is using to kill dysplastic tissue as well as how to improve viral delivery. Studying these issues will be critical to optimize the use of oncolytic virotherapy for colonic dysplasia. Because the safety of oncolytic VV has been well described in multiple clinical trials in patients with advanced cancer\textsuperscript{394, 449-451}, we believe that vvDD-RFP could be tested in patients with “complex” polyps. Performing such a study would not endanger patients as oncolytic VV is safe and patients with “complex” polyps are theoretically healthier than cancer patients enrolled in oncolytic virotherapy clinical trials. Moreover, participating in a clinical trial to test this OV would not preclude standard treatment for the “complex” polyp.

The application of OVs in the fight against cancer is ever expanding, as more creative strategies are developed to maximize their cancer cell killing mechanism of action. Colonic dysplasia is an attractive target for OVs because dysplastic cells express a wide range of genetic mutations that facilitate specific replication. Additionally, viruses can be directly delivered intracolonically thus minimizing systemic dissemination and potentially delaying immune clearance. Delivery will play a key role in achieving effective results once fully optimized. Bringing oncolytic virotherapy into clinical use is challenging and will require further optimization of viral delivery
and viral distribution, but ultimately may lead to a novel treatment for colonic dysplasia and other precancerous lesions.
References


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Appendices

Appendix 1. Definition of TNM staging according to the AJCC Cancer Staging Manual Seventh Edition.

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<table>
<thead>
<tr>
<th>Primary Tumor (T)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>TX</td>
<td>Primary tumor cannot be assessed</td>
</tr>
<tr>
<td>T0</td>
<td>No evidence of primary tumor</td>
</tr>
<tr>
<td>Tis</td>
<td>Carcinoma in situ: intraepithelial or invasion of lamina propria*</td>
</tr>
<tr>
<td>T1</td>
<td>Tumor invades submucosa</td>
</tr>
<tr>
<td>T2</td>
<td>Tumor invades muscularis propria</td>
</tr>
<tr>
<td>T3</td>
<td>Tumor invades through the muscularis propria into pericolorectal tissue</td>
</tr>
<tr>
<td>T4a</td>
<td>Tumor penetrates to the surface of the visceral peritoneum**</td>
</tr>
<tr>
<td>T4b</td>
<td>Tumor directly invades or is adherent to other organs or structures**,<strong>,</strong>,**</td>
</tr>
</tbody>
</table>

* Note: Tis includes cancer cells confined within the glandular basement membrane (intraepithelial) or mucosal lamina propria (intramucosal) with no extension through the muscularis mucosae into the submucosa.

** Note: Direct invasion in T4 includes invasion of other organs or segments of the colorectum as a result of direct extension through the serosa, as confirmed on microscopic examination (for example, invasion of the sigmoid colon by a carcinoma of the cecum) or, for cancers in a retroperitoneal or subperitoneal location, direct invasion of other organs or structures by virtue of extension beyond the muscularis propria (i.e., respectively, a tumor on the posterior wall of the descending colon invading the left kidney or lateral abdominal wall; or a mid or distal rectal cancer with invasion of prostate, seminal vesicles, cervix, or vagina).

*** Note: Tumor that is adherent to other organs or structures, grossly, is classified cT4b. However, if no tumor is present in the adhesion, microscopically, the classification should be pT1-4a depending on the anatomical depth of wall invasion. The V and L classifications should be used to identify the presence or absence of vascular or lymphatic invasion whereas the PN site-specific factor should be used for perineural invasion.

<table>
<thead>
<tr>
<th>Regional Lymph Nodes (N)</th>
<th></th>
</tr>
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<tbody>
<tr>
<td>NX</td>
<td>Regional lymph nodes cannot be assessed</td>
</tr>
<tr>
<td>N0</td>
<td>No regional lymph node metastasis</td>
</tr>
<tr>
<td>N1</td>
<td>Metastasis in 1-3 regional lymph nodes</td>
</tr>
<tr>
<td>N1a</td>
<td>Metastasis in one regional lymph node</td>
</tr>
<tr>
<td>N1b</td>
<td>Metastasis in 2-3 regional lymph nodes</td>
</tr>
<tr>
<td>N1c</td>
<td>Tumor deposit(s) in the subserosa, mesentery, or nonperitonealized pericolic or perirectal tissues without regional nodal metastasis</td>
</tr>
<tr>
<td>N2</td>
<td>Metastasis in four or more regional lymph nodes</td>
</tr>
<tr>
<td>N2a</td>
<td>Metastasis in 4-6 regional lymph nodes</td>
</tr>
<tr>
<td>N2b</td>
<td>Metastasis in seven or more regional lymph nodes</td>
</tr>
</tbody>
</table>

Note: A satellite peritumoral nodule in the pericolorectal adipose tissue of a primary carcinoma without histologic evidence of residual lymph node in the nodule may represent discontinuous spread, venous invasion with extravascular spread (V1/2), or a totally replaced lymph node (N1/2). Replaced nodes should be counted separately as positive nodes in the N category, whereas discontinuous spread or venous invasion should be classified and counted in the Site-Specific Factor category Tumor Deposits (TD).
### Distant Metastasis (M)

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
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<tbody>
<tr>
<td>M0</td>
<td>No distant metastasis</td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>Distant metastasis</td>
<td></td>
</tr>
<tr>
<td>M1a</td>
<td>Metastasis confined to one organ or site (e.g., liver, lung, ovary, nonregional node)</td>
<td></td>
</tr>
<tr>
<td>M1b</td>
<td>Metastases in more than one organ/site or the peritoneum</td>
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</tr>
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### Anatomic Stage/Prognostic Groups

<table>
<thead>
<tr>
<th>Stage</th>
<th>T</th>
<th>N</th>
<th>M</th>
<th>Dukes*</th>
<th>MAC*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Tis</td>
<td>N0</td>
<td>M0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>I</td>
<td>T1</td>
<td>N0</td>
<td>M0</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>N0</td>
<td>M0</td>
<td>A</td>
<td>B1</td>
</tr>
<tr>
<td>IIA</td>
<td>T3</td>
<td>N0</td>
<td>M0</td>
<td>B</td>
<td>B2</td>
</tr>
<tr>
<td>IIB</td>
<td>T4a</td>
<td>N0</td>
<td>M0</td>
<td>B</td>
<td>B2</td>
</tr>
<tr>
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<td>T4b</td>
<td>N0</td>
<td>M0</td>
<td>B</td>
<td>3</td>
</tr>
<tr>
<td>IIIA</td>
<td>T1-T2</td>
<td>N1/N1c</td>
<td>M0</td>
<td>C</td>
<td>C1</td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>N2a</td>
<td>M0</td>
<td>C</td>
<td>C1</td>
</tr>
<tr>
<td>IIIB</td>
<td>T3-T4a</td>
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<td>C2</td>
</tr>
<tr>
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<td>N2a</td>
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<td>C1/C2</td>
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<tr>
<td></td>
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<td>M0</td>
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<tr>
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<td>T4a</td>
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<td>C</td>
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<tr>
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<td>Any T</td>
<td>Any N</td>
<td>M1a</td>
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<tr>
<td>IVb</td>
<td>Any T</td>
<td>Any N</td>
<td>M1b</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Note*: cTNM is the clinical classification, pTNM is the pathologic classification. The y pre fx is used for those cancers that are classified after neoadjuvant pretreatment (e.g., ypTNM). Patients who have a complete pathologic response are ypT0N0cM0 that may be similar to Stage Group 0 or I. The r pre fx is to be used for those cancers that have recurred after a disease-free interval (rTNM).

- Dukes B is a composite of better (T3 N0 M0) and worse (T4 N0 M0) prognostic groups, as is Dukes C (Any TN1 M0 and Any T N2 M0). MAC is the modified Astler-Coller classification.