The role of MCPyV ST in Merkel cell carcinoma metastasis

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ABSTRACT

Merkel cell carcinoma (MCC) is an aggressive skin cancer of neuroendocrine origin with a high propensity for metastasis via the dermal lymphatic system. In 2008, Merkel cell polyomavirus (MCPyV) was discovered monoclonally integrated within the host genome of at least 80% of MCC tumours. MCPyV transforms and maintains MCC tumours via the expression of the large and small tumour (LT and ST) antigens. Unlike other polyomaviruses, MCPyV ST is thought to be the major viral transforming factor required for MCC development. Since the discovery of MCPyV, a number of novel functions for ST have been identified which contribute to tumourigenesis, but to date, little is known about potential links between MCPyV T antigen expression and the highly metastatic nature of MCC.

Previously, the Whitehouse Laboratory have demonstrated the ability of MCPyV ST to enhance cell motility and migration, suggesting a potential role of MCPyV ST in the highly metastatic nature of MCC. In this thesis, the link between MCPyV ST and MCC metastasis is further explored by focusing on the role of MCPyV ST in promoting early events of initiating cell migration and metastatic spread. Results show that MCPyV ST expression disrupts the integrity of cell-to-cell junctions, thereby enhancing cell dissociation and scatter. Moreover, the functional requirement of cellular sheddases is highlighted in this process, specifically the A disintegrin and metalloproteinase (ADAM) 10 and 17 proteins. These findings therefore suggest MCPyV ST-mediated cell surface accumulation of cellular sheddases play a role in the highly metastatic nature of MCC and may also provide novel therapeutic interventions for disseminated MCC.

Furthermore, results explore he potential of MCPyV ST to initiate an Epithelial to mesenchymal transition [EMT]. In addition to disruption of the cell junctions, other hallmarks of EMT are examined including loss of apical to basal polarity, expression of EMT associated Transcription factors and upregulation of Matrix metalloproteinases. Results show that MCPyV ST expression leads to phenotypic changes suggestive of characteristic EMT mechanisms which may further contribute to the metastatic spread associated with MCC.

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ABBREVIATIONS

°c	degrees celcius
%	percentage
4E-BP1	eukaryotic translation initiation factor 4E-binding protein 1
ADAM	A disintegrin and metalloproteinase
Amp	ampicillin
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BKV	BK virus
bp	base pair
BSA	bovine serum albumin
Cdk	cyclin dependent kinase
cDNA	complimentary DNA
CHiP	chromatin immunoprecipitation
CK	cytokeratin
CRE	cyclic AMP response element
CREB	cyclic AMP response element binding
C-terminus	carboxyl-terminus
Cul	cullin
DMEM	dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleoside [5'-] triphosphate
dox	doxycycline hyclate

ds	double stranded
DTT	dithiothreitol
E.Coli	*Escherichia coli*
ECL	enhanced chemiluminescence
EGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
EMT	Epithelial to Mesenchymal Transition
ERK	extracellular signal-regulated kinases
FCS	foetal calf serum
FRT	FLP recombination target
GAG	glycosaminoglycan
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
HAT	histone acetyltransferase
HDF	Human diploid fibroblast
HEK	human embryonic kidney
HPV	human papilloma virus
HPyV	human polyomavirus
Hr[s]	hours
HRP	horseradish peroxidase
hTERT	human telomerase reverse transcriptase
HTLV	Human T-cell leukaemia virus
IκB	Inhibitor of kappa B
IF	immunofluorescence
IFN	interferon
IgG	immunoglobulin G

IKK	IκB kinase enzyme complex
IL	interleukin
IP	immunoprecipitation
IRAK	interleukin receptor associated kinase
IRS I	insulin receptor substrate I
JCV	JC virus
Kan	kanamycin
kb	kilobase
kbp	kilobase pair
kDa	kilodalton
KIV	KI virus
KS	Kaposi's sarcoma
KSHV	Kaposi's sarcoma-associated herpesvirus
LB	luria broth
LC3	microtubule associated 1A/1B light chain 3
LPyV	B-lymphotropic polyomavirus
LT	large T antigen
MCC	Merkel cell carcinoma
MCPyV	Merkel cell polyomavirus
MEK	mitogen activated protein kinase
MAPK	mitogen activated protein kinase
MEK	MAP and ERK kinase
MMP	matrix metalloproteinase
MPyV	Murine polyomavirus
mRNA	messenger RNA
MT	middle T antigen

mTOR	mammalian target of rapamycin
MWPyV	Malawi polyomavirus
NCCR	non coding control region
NDEL1	nuclear distribution protein nudE-like 1
NEMO	NF-κB essential modulator
NF-κB	nuclear factor κB
NLS	nuclear localisation signal
NP40	tergitol-type NP-40
N-terminus	amino terminus
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI3K	phosphatidylinositol 3-kinase
PML	progressive multifocal leukoencephalopathy
PP2A	protein phosphatase 2A
PP4	protein phosphatase 4
PP4C	protein phosphatase 4 catalytic subunit
PP5	protein phosphatase 5
qRT-PCR	quantitative reverse transcriptase PCR
RACE	rapid amplification of cDNA ends
Rb	retinoblastoma protein
rcf	relative centrifugal force
RNA	ribonucleic acid
RNA pol II	RNA polymerase II

RNase	ribonuclease
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RT	reverse transcriptase
SDS	sodium dodecyl sulphate
SILAC	stable isotope labelling with amino acids in cell culture
siRNA	small interfering RNA
ST	small T antigen
SV40	simian virus 40
TANK	TRAF family member-associated NF-kappa-B activator
TBE	tris-borate-EDTA buffer
TBS	tris buffered-saline
TE	tris-EDTA buffer
TEF-1	Thyroid Embryonic Factor-1
TEMED	N-N-N'-N'-tetramethylethylenediamine
TK	tyrosine kinase
TLR	toll-like receptor
TNF	tumour necrosis factor
TPA	TRAF family member-associated NF-kappa-B activator
TRAF	TNF receptor associated factor
Tris	tris [hydroxymethyl]-aminoethane
TSV	trichodysplasia spinulosa-associated polyomavirus
Ub	ubiquitin
UV	ultra-violet
V	volt
v/v	volume per volume

w/v	weight per volume
WCL	whole cell lysate
WUV	WU virus

Bases

A	adenine
T	Thymine
C	cytosine
G	guanine

Amino acids

Glycine	Gly	G
Alanine	Ala	A
Valine	Val	V
Leucine	Leu	L
Isoleucine	Ile	I
Serine	Ser	S
Threonine	Thr	T
Cysteine	Cys	C
Methionine	Met	M
Tyrosine	Tyr	Y
Proline	Pro	P
Aspartate	Asp	D
Glutamate	Glu	E
Asparagine	Asn	N
Glutamine	Gln	Q

Lysine	Lys	K
Arginine	Arg	R
Histidine	Hs	H
Phenylalanine	Phe	F
Tryptophan	Trp	W

Chapter 1
INTRODUCTION

1.1 Viruses and Cancer

Cancer accounts for approximately 13% of worldwide deaths, with mortality rates higher than AIDs, Malaria and Tuberculosis combined. As such, it is a growing cause for public concern resulting in massive economic burden due to prevention management and treatment costs (Mathers and Loncar 2006). In 2010 alone, the associated cost of cancer care in the United states was $124.57 billion, which is estimated to grow to $157.77 billion in 2020 (Mariotto *et al.* 2011). Notably, by 2040, almost a quarter of people aged 65 are predicted to be cancer survivors (de Martel *et al.* 2012).

There are numerous causes of cancer, among them, viruses and other etiological agents. Approximately 10-15% of human cancers worldwide are linked to human tumour virus infection, with majority of the cases [80%] occurring in developed countries (Mesri *et al.* 2014). Thus, there is a clear role for viruses in the global burden of cancer. However, although there is a high incidence rate, suitable antiviral vaccines or other antiviral therapies are limited (zur Hausen 2009). Of the vast majority of new human tumour virus linked cancer cases, approximately 1.9 million, worldwide are due to either Hepatitis B and C viruses or human papillomavirus [HPV] resulting in mainly liver, cervix/uteri or gastric cancers (de Martel *et al.* 2012).

The concept of tumour virology began with Peyton Rous in 1911, upon the discovery of an avian virus that induced tumours in chickens (Young and Rickinson 2004). In a seminal experiment, Rous infected a healthy chicken with filtered cell-free extract form spindle cell tumour cells acquired from a Plymouth Rock Chicken. Results showed that the cancer could be transmitted through cell-free tumour extracts, signifying that cancer can be caused by a small transmissible agent (Padgett *et al.* 1971; Javier and Butel 2008). It is now known that multiple viruses express oncogenes which induce cell growth and hyperproliferation (Moore and Chang 2010). They can also modify and inhibit tumour suppressor genes that inhibit cell growth (Nakamura *et al.* 2001). Both of these mechanisms can lead to unregulated cell growth and cancer development.

To date, seven human tumour viruses have been identified [Table 1.1]. Human DNA tumour viruses belong to the virus families Herpes, Hepadna, and also Papilloma and Polyomaviridae. Specifically, there are five human DNA viruses, two members of the Herpes family, Epstein Barr Virus [EBV or HHV4] and Kaposi's sarcoma-associated herpesvirus [KSHV]. High- risk human papilloma viruses [HPV]

16 and 18, Merkel cell polyomavirus [MCPyV] and Hepatitis B virus [HBV] (Moore and Chang 2010). There are two additional human RNA tumour viruses, Hepatitis C virus [HCV] of the flavivirdae family and Human T-lymphotropic virus-I [HTLV-I] of the Retroviridae family. As previously stated Retroviruses were initially determined to be the tumour inducing agent associated with the first discovered oncogenic virus, Rous Sarcoma Virus [RSV] (Moore and Chang 2010).

Table 1. 1: Summary of Human Tumour Viruses

Full Name	Year of discovery	Family and genome	Oncogenes	Cancer Association
Epstein-Barr Virus [EBV]	1964	Herpesviridae dsDNA	LMP1	Including Burkitt's lymphoma, nasopharyngeal carcinoma, and some other lymphoproliferative disorders
Hepatitis B virus [HBV]	1965	Hepadnaviridae ssDNA and dsDNA	HBx	Some hepatocellular carcinomas
Human T-lymphotropic virus-I [HTLV-I]	1980	Retroviridae ssDNA and dsDNA	Tax	Adult T cell leukaemia
Human papillomaviruses [HPV] 16 and 18	1983-1984	Papillomaviridae dsDNA	E5, E6, E7	Most cervical cancer and penile cancers.
Hepatitis C virus [HCV]	1989	Hepaciviridae [+]ssRNA	NS5A	Some hepatocellular carcinomas and lymphomas
Kaposi's sarcoma herpesvirus [KSHV]	1994	Herpesviridae dsDNA	LANA, vflip, and vBcl-2, among others	Kaposi's sarcoma and primary effusion lymphoma
Merkel Cell Polyomavirus [MCPyV]	2008	Polyomaviridae dsDNA	T antigens	Most Merkel cell carcinomas

ss: single-stranded, ds: double stranded, (+): positive strand

1.2 Polyomaviruses

Polyomaviruses are small non-enveloped, double-stranded DNA viruses which infect a diverse group of hosts, ranging from mammals and birds. They are very species specific in the sense that they only infect the species from which they were isolated. The first isolated polyomavirus was Murine Polyomavirus [MPyV], characterized in 1958, after the observation that filterable extracts from murine

leukaemia could induce tumours in the parotid-gland of neonatal mice (Stewart *et al.* 1958).

This discovery was followed up two years later, by the identification of Simian Virus 40 [SV40], isolated from African green monkey kidney cells, which were used in the production of the polio vaccine (Sweet and Hilleman 1960; Pipas 2009). Murine Polyomavirus and Simian Virus 40 have since served as excellent models for investigating polyomavirus replication strategies and how this family of viruses cause disease (Stewart *et al.* 1958; Sweet and Hilleman 1960). Historically, polyomaviruses were classified together with papillomaviruses into the papova virus family, however, the 7th International Committee on Taxonomy of Viruses [ICTV] report published in 2001 reclassified Polyomavirus into a distinct family, termed polyomaviridae (Imperiale 2001; Gjoerup and Chang 2010).

1.2.1 Classification of Polyomavirus

The family of Polyomaviridae are separated into three distinct genera: *Orthopolyomavirus* and *Wukipolyomavirus* which represent mammalian virus species and *Avipolyomavirus* for bird virus species (Gjoerup and Chang 2010). The phylogenetic relationship among polyomaviruses based on whole genomic nucleotide sequences can be observed in Figure 1.1. The most obvious difference between the genera is the host and cell specificity. Avian polyomaviruses have a broad host range. Whereas, their mammalian polyomavirus counterparts have a narrow host and cell specificity (Johne and Muller 2007; Gjoerup and Chang 2010; Halami *et al.* 2010).

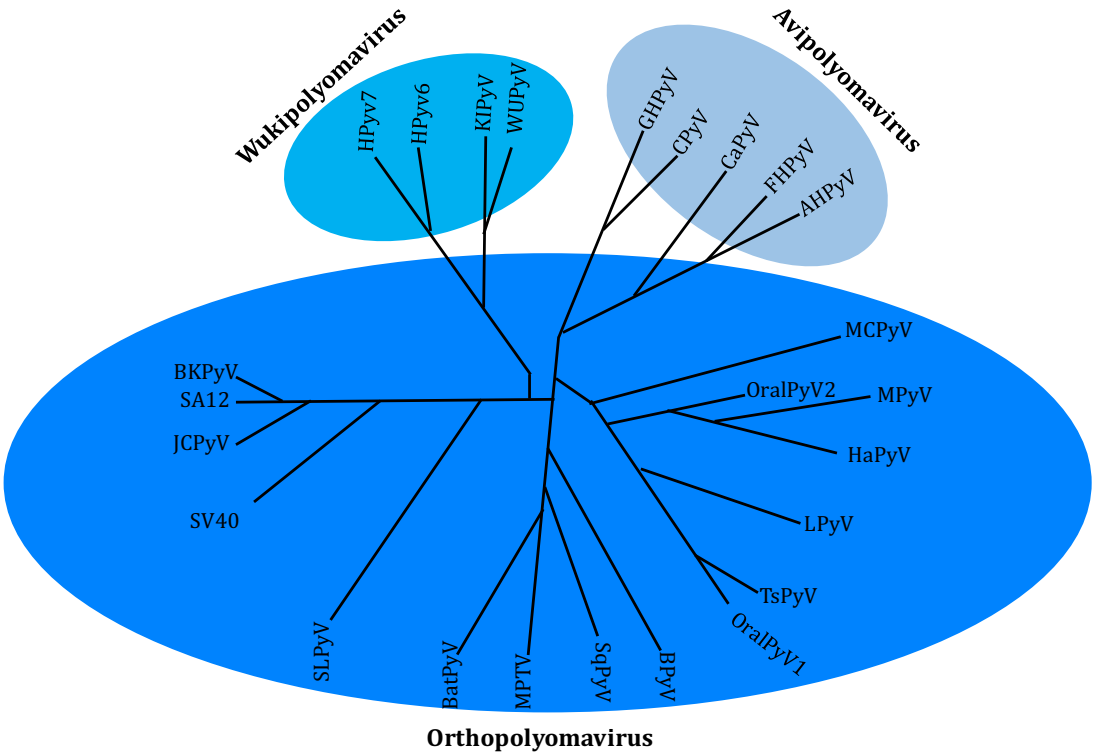

Figure 1. 1: A phylogenetic tree of polyomaviruses developed by analysing whole genome sequences. Three large groups are distinguished: Wukipolyomaviruses, Avipolyomaviruses and Orthopolyomaviruses.

1.2.2 Human Polyomaviruses

Thirteen human polyomaviruses have been discovered to date, eleven since 2011 (White *et al.* 2013). This explosion in identification is mainly due to improved identification technologies such as next generation sequencing and Digital transcriptome subtraction.

1.2.2.1 JCPyV

John Cunningham virus [JCPyV] was initially isolated from the brain of a progressive multifocal leukoencephalopathy [PML] patient in 1971 (Padgett *et al.* 1971). JCPyV is a pervasive virus capable of infecting nerve cells (Bellizzi *et al.* 2013). Approximately 50-90% of adults have been exposed to JCPyV, with a quarter of that subpopulation shedding JCPyV in their urine (L'Honneur and Rozenberg 2016). JCPyV is a ubiquitous polyomavirus that does not maintain exclusive tropism for a single cell type within its host, unlike others. Principally,

JCPyV is found within oligodendrocytes but is also found within tissue of the urinary tract, spleen as well as bone marrow (Kean *et al.* 2009).

JCPyV infects 35-70% of the global population asymptomatically (Egli *et al.* 2009). The virus is predominant in urban sewage systems inferring that the virus is contracted and disseminated through the fecal-oral route (Boothpur and Brennan 2010). The initial site of infection is in the tonsils, while it propagates and replicates within lymphoid cells followed by infection in the gastrointestinal tract and kidneys, where it establishes a latent infection (Maginnis *et al.* 2015). JCPyV has also been shown to induce human and rodent cell transformation *in vitro* and in animal models, however this phenotype has only been observed in neural cells and interestingly neural cells are non-permissive for JCPyV infection (Gjoerup and Chang 2010).

1.2.2.2 BKPyV

BK polyomavirus [BKPyV] was initially identified in 1971 by electron microscopy examination of urine from a renal allograft patient (Gardner *et al.* 1971). Although the transmission route associated with BKPyV infection has not been determined, healthy and diseased individuals infected with BKPyV transiently shed virus that can be detected in various bodily fluids such as urine, blood and faeces. However the most likely route of infection is believed to be via the respiratory tract (Bialasiewicz *et al.* 2009) BKPyV has also been suggested to be sexually transmitted, as it has been detected in 95% of sperm samples, although this theory has been quickly disregarded due to the high rates of infection in children (Shah *et al.* 1973).

As a result of the increase in organ transplantation, as well as a growing dependency on the utilisation of immunosuppressant drugs, the reported incidence of disease caused by BKPyV is increasing. Almost 10% of all renal allografts present symptoms of BK-associated nephropathy [BKVAN], with 50-90% of affected patients reported to eventually experience graft dysfunction or failure (van Doesum *et al.* 2014).

1.2.2.3 Other Human Polyomaviruses

Since the initial discovery of JCPyV and BKPyV, numerous other human polyomaviruses have been readily discovered including Washington University polyomavirus [WUPyV] and Karolinska Institute polyomavirus [KIPyV] in 2007. Notably, due to the advantages of detection technologies, such as next generation sequencing and digital transcript subtraction, more polyomaviruses have been discovered in the last few years. As such the study of human polyomaviruses and its association with human pathology has again become a major research area. Importantly, some human polyomavirus encoded proteins have been linked with transforming capabilities in cell culture and animal models suggest they are tumourigenic. This is typified in the study of Merkel Cell Polyomavirus, which was isolated from patients with Merkel cell carcinoma (Feng *et al.* 2008).

Other diseases associated with human polyomavirus are renal infections and Trichodysplasia spinulosa (Gjoerup & Chang, 2010; Johnson, 2011). To date however, only a subset have been associated with any disease. 13 polyomaviruses found in humans are listed in Table 1.2.

Table 1. 2: The 13 polyomaviridae members that infect humans and their associated clinical complications as a result of their infection. Adapted from DeCaprio & Garcia, 2013.

Full name	Host	Year of discovery	Disease association
John Cunningham virus [JCPyV]	Human	1971	Progressive multifocal leukoencephalopathy [PML]
BK Polyomavirus [BKPyV]	Human	1971	Polyomavirus associated nephropathy [PVAN]/ Renal transplant failure/ Hemorrhagic cystitis
Karolinska Institute Polyomavirus [KIPyV]	Human	2007	None
Washington University Polyomavirus [WUPyV]	Human	2007	None
Merkel Cell Polyomavirus [MCPyV]	Human	2008	Merkel Cell carcinoma
Human Polyomavirus-6 [HPyV6]	Human	2010	None
Human Polyomavirus-7 [HPyV7]	Human	2010	HPyV associated epidermal hyperplasia and pruritus
Trichodysplasia spinulosa-associated Polyomavirus [TSPyV]	Human	2010	Trichodysplasia spinulosa/ Pilomatrix dysplasia
Human Polyomavirus-9 [HPyV9]	Human	2011	None
Malawi Polyomavirus [MWPyV]	Human	2012	None
St. Louis Polyomavirus [STLPyV]	Human	2012	None
New Jersey Polyomavirus [NJPyV]	Human	2013	None
Human Polyomavirus-12 [HPyV12]	Human	2013	None

J. C. and B. K.: patient initials, PML: progressive multifocal leukoencephalopathy, KI: Karolinska Institute, WU: Washington University, St. Luis, TS: trichodysplasia spinulosa, MW: Malawi, STL: St Louis, NJ: New Jersey. Modified from (DeCaprio and Garcea 2013).

1.2.3 SV40 Polyomavirus

SV40 serves as a model for understanding polyomavirus DNA structure, replication and transcription. SV40 naturally infects wild rhesus macaques in a similar method to JCPyV infection in humans (Dang *et al.* 2008). This generally results in an asymptomatic infection, however under immunosuppression, a demyelinating disease manifests, similar to PML (Horvath *et al.* 1992; Axthelm *et al.* 2004; Dang *et al.* 2008).

SV40 Small T and Large T antigens are oncogenic proteins and have transforming properties, which enhances stimulation of cell division and apoptosis prevention. This results in optimisation of the cell cycle driving it into S-phase, which creates an ideal environment for viral replication. The study of the SV40 oncogenic proteins potentially serves as a model for studying the oncogenicity of Merkel cell polyomavirus T antigens. The SV40 Large T [LT] antigen is deduced to be the major oncogenic protein involved in the formation of new and abnormal growth of tissue, while ST is believed to enhance the oncogenic and transformative advancement in SV40 (Noda *et al.* 1987).

1.2.3.1 Genome organization

The Polyomavirus genome is contained within a non-enveloped icosahedral capsid comprising 40-45 nm in size (Gjoerup and Chang 2010). The polyomavirus genome comprises approximately 5,000 base pair of circular double-stranded DNA. The genome can be divided into three functional regions, comprising a non-coding control region [NCCR] bordered by an early gene region and a late gene region. The NCCR contains the early and late promoters, their transcription start-sites as well as the origin of replication. Figure 1.2 shows the generalised polyomavirus genome.

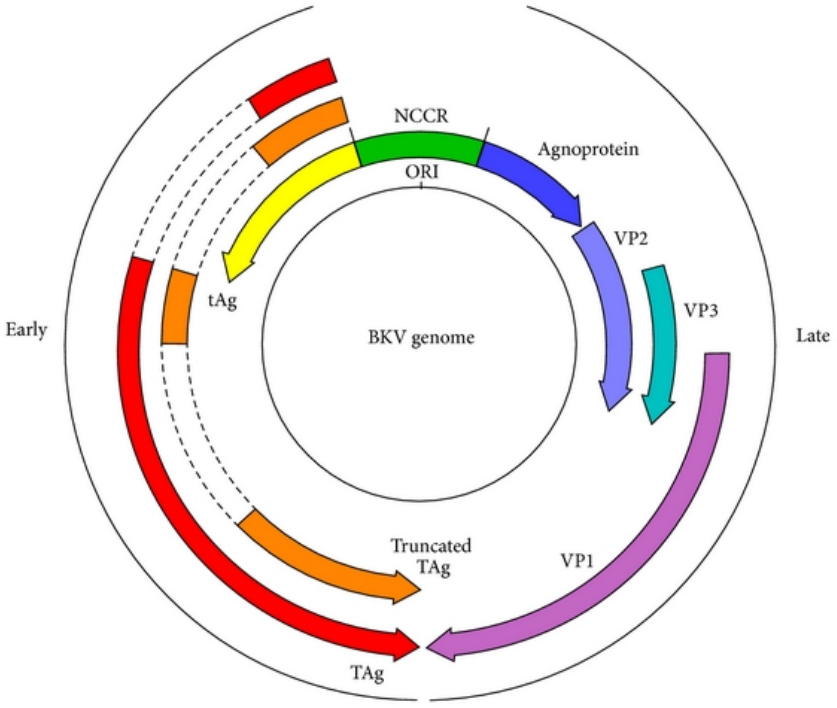

Figure 1. 2: A generalised polyomavirus genome, including the non-coding control region, the early gene region and the late gene region. The early gene region commonly encodes LT, ST, and at least one alternative T antigen product (LT'). The late gene region encodes VP1, VP2 and VP3. Taken from (De Gascun and Carr 2013).

The early region encodes regulatory proteins that are involved in DNA replication and gene expression. It encodes multiple open reading frames from a single transcript, due to alternative splicing events yielding the Small Tumour antigen [ST] and the Large Tumour antigen [LT]. In addition, some polyomaviruses have additional alternatively spliced LT transcripts, referred to as LT' or known as 57kT in MCPyV (Moens *et al.* 2011). Interestingly, the MCPyV early region has also been found to encode an alternative Large T open reading frame [ALTO] transcript (Joseph J. Carter *et al.* 2013).

Moreover, the newly-discovered STLPyV may also express a unique T antigen identified as 229T. This is a third early transcript that shares the first 190 residues of small T antigen with an additional 39 residues from an alternative reading frame of LT (Lim *et al.* 2013). Importantly, human polyomaviruses do not encode a middle T antigen, which is present in other mammalian polyomaviruses, such as MPyV (Noda *et al.* 1987). The late coding region encodes capsid proteins essential for the formation of non-enveloped icosahedral virions. These structural proteins are VP1, VP2 and VP3 (Boothpur and Brennan 2010; Gjoerup and Chang 2010).

SV40 also encodes an additional viroporin, termed VP4 (Raghava *et al.* 2011). The reading frames of VP2, VP3 and VP4 are identical, but translation starts at successive initiating AUG codons (DeCaprio and Garcea 2013). In addition, certain polyomavirus late regions also encode the agno protein, as small protein of unknown function (Safak *et al.* 2001).

1.2.3.2 Polyomavirus life cycle

The life cycle of the polyomaviruses has been well characterized, using SV40 as a model. A generalized life cycle is summarized in Figure 1.3.

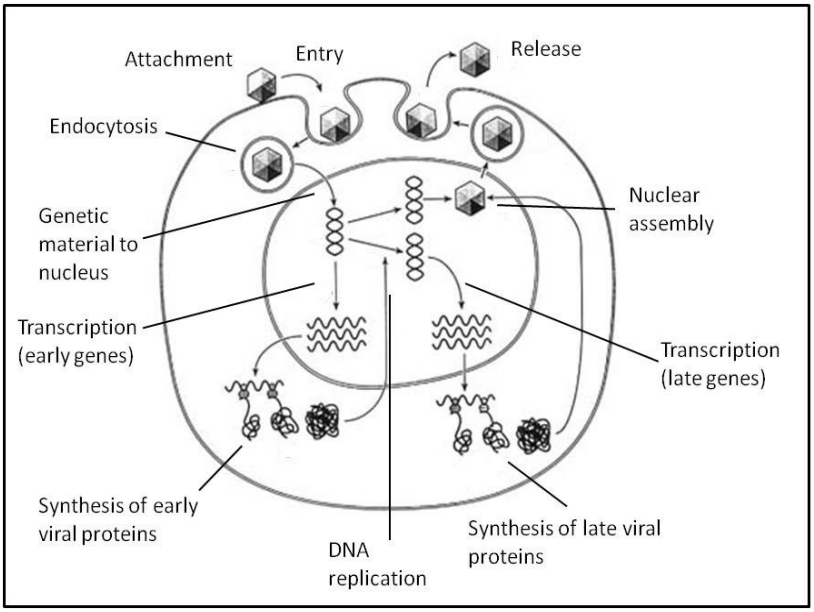

Figure 1. 3: The life cycle of polyomaviruses. This figure shows the essential steps in polyomavirus replication, described in Section 1.2.3.2 Taken from (Knipe, Howley et al. 2006).

To infect an animal cell, a virus initially binds to specific receptor molecules on the cell surface. SV40 entry is mediated by attachment to sialic acid moieties on the cell surface. The ganglioside GM1 is a crucial cell surface receptor essential for SV40 entry however, studies have shown that attachment to the major histocompatibility complex [MHC] can also induce cell attachment and entry (Stakaityte *et al.* 2014). Furthermore, MPyV and BKPyV bind to sialylated glycans, e.g. GT1B while JCPyV is tropic for α-2-6-linked sialic acids (Crandall *et al.* 2006; Moens *et al.* 2007).

Polyomaviruses enter the cell through endocytosis, but this mechanism varies

between polyomaviruses. SV40 entry for example is entirely dependent on caveolin-mediated endocytosis. Subsequent to entry, the virus is transported to the nucleus in order to replicate its DNA. Studies have demonstrated the VP1 structural protein contains a nuclear localisation signal [NLS] which allows for binding of cellular importins and entry into the nucleus via the nuclear pore complex [NPC] (Qu *et al.* 2004).

Polyomavirus DNA is transcribed and replicated within the host cell nucleus. Subsequent to entry into the nucleus and uncoating, the early gene region is transcribed, with SV40 LT functioning to transactivate the early promoter region, as well as binding to the origin of replication to promote the replication of the viral genome (Jat *et al.* 1986). Polyomavirus transcription, translation, and replication is contingent on the host cell machinery, including proteins expressed only during the S-phase and consequently both LT and ST promote entry of the host cell into S-phase (Chowdhury *et al.* 1990).

Once a sufficient concentration of LT is reached, it functions to inhibit the activity of the early promoter and alternatively activates the late promoter to drive the assembly of the structural proteins (Bikel and Loeken 1992). Once synthesis of structural proteins occurs, they are imported back into the nucleus, where virion assembly takes place. Virion assembly occurs by addition and organization of the capsid proteins around the viral minichromosome rather than incorporation of viral DNA into pre-formed capsids. Every VP1 pentamer binds to a molecule of either VP2 or VP3 as a result of strong hydrophobic interactions, suggesting that $VP1_5$ - VP2/3 are the building blocks for SV40 capsids (Chen *et al.* 1998; Gordon-Shaag *et al.* 2002).

1.2.3.4 SV40 early proteins

All polyomaviruses encode the regulatory LT and ST antigens. These T antigens function as transacting factors essential for viral replication. It has also been demonstrated that the T antigens are oncogenic proteins, with the ability to transform cells *in vitro* and in mouse models (Sugano *et al.* 1982; Chang *et al.* 1985; Gordon *et al.* 2000; Hahn *et al.* 2002). Aberrant cell cycle stimulation is the major driving force behind T antigen-mediated cellular transformation. Polyomaviruses do not encode all of the required replicative proteins, as such the

early proteins drive S-phase entry in host cells to promote DNA replication (White and Khalili 2004).

In non-permissive cells, cellular transformation is induced by the viral T antigens as a result of the host cell not providing the essential resources for viral replication. This is deduced by the lack of late gene expression or viral DNA replication in viruses containing mutations in the T antigen (Yang *et al.* 1991). The molecular mechanisms by which polyomavirus early proteins contribute to cellular transformation and anchorage independent growth will be discussed in later sections.

1.2.3.4.1 Small Tumour Antigen

1.2.3.4.1.1 Structure and domains

The coding region of ST comprises 500 base pairs [bp] in length, encoding a 17 kDa protein of 170 amino acids. ST can localize to both the nucleus and cytoplasm, and is highly conserved across Polyomavirus species. For example, JCPyV shares 96% sequence homology with BKPyV ST and 79% identity with SV40 ST (Moens *et al.* 2007).

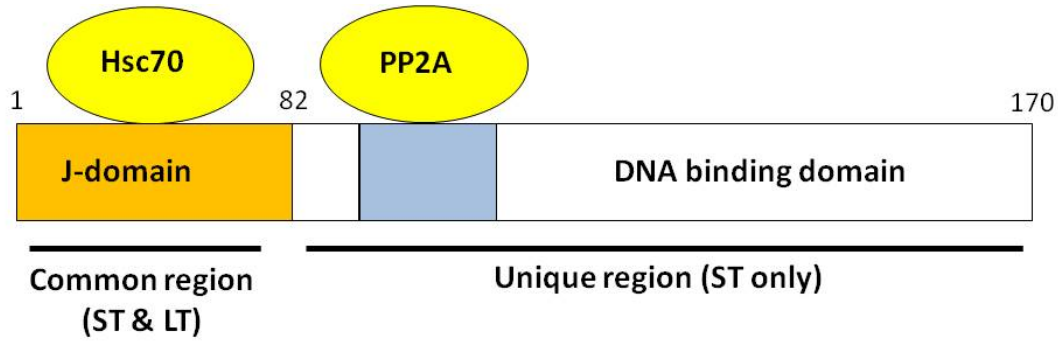

Figure 1. 4: Schematic representation of polyomavirus ST. The DNAJ domain is found in the common region, which runs between amino acids 1-~90. The common region also contains a conserved Cr1 domain. The unique region contains a PP2A binding domain and two cysteine rich clusters which enable Zinc binding. Taken from (Kamel Khalili et al. 2008).

The common region, containing the DNAJ domain is located at the N-terminus of all T antigens and is believed to have similar functions within host cells. The DNAJ domain has a conserved Cr1 domain and binding domain for the cellular heat shock protein, Hsc70. Hsc 70 functions as a chaperone protein to prevent protein aggregation during viral invasion and stress as shown in Figure 1.4 (Liu *et al.*

2012).

The middle and C-terminal portions of each ST mRNA are unique from LT mRNA, produced by alternative splicing. Hence, functions associated with the J domain are ascribed to all T antigens, whereas the remaining C-terminal amino acids form the 'unique' ST regions. Notably, ST unique regions contain a binding domain for the major cellular phosphatase, PP2A and a zinc binding domain. The alignment of MPyV, BKPyV, JCPyV and SV40 ST amino acid sequences has shown the N-terminal region is highly conserved, while the middle and C-terminal portions show more variation (Kamel Khalili *et al.* 2008). Current assumption is that the variation within the middle/C-terminal regions allows for alternative roles for each individual polyomavirus ST, such as mediating virus replication or host cell transformation.

1.2.3.4.1.2 ST interaction with PP2A

Protein Phosphatase 2A, PP2A, is the major cellular serine/threonine phosphatase. The interaction between polyomavirus ST and PP2A is considered to be the major role of the viral ST protein (Axthelm *et al.* 2004). PP2A is a heterotrimeric holoenzyme, composed of three subunits with different functional roles. Subunit A which is approximately 65 kDa functions as a scaffold protein and its C- terminus binds the 32 kDa Subunit C. Subunit C acts as the catalytically active phosphatase and together, with Subunit A, form the core heterodimer (Rodriguez-Viciana *et al.* 2006a). Subunit B allows for the regulatory specificity of this complex and its binding results in the major cellular form of PP2A as a heterotrimeric complex as shown in Figure 1.5. Binding occurs via the Subunit B N-terminus which directly binds Subunit A (Silverstein *et al.* 2002). There are multiple B subunits all with variability within the N-terminal region, allowing for distinct substrate specificity (Janssens and Goris 2001).

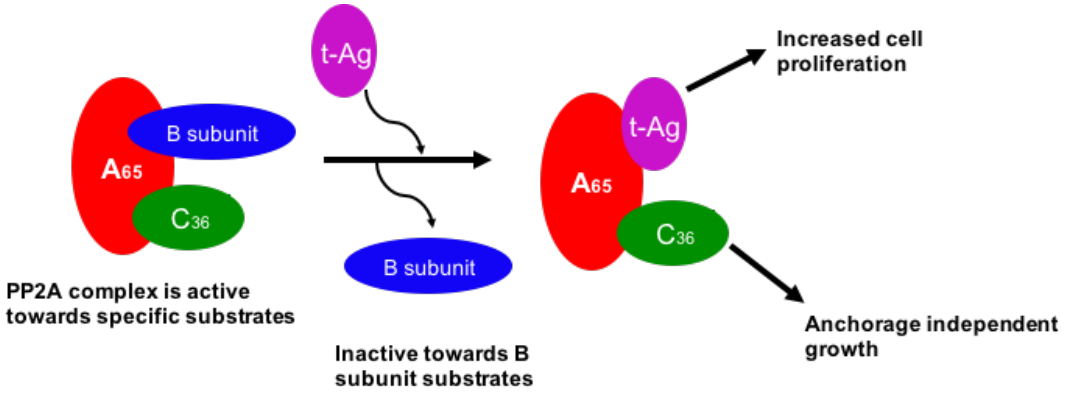

Figure 1. 5: Inactivation of PP2A by ST. ST binds to the A subunit and replaces the B subunit inactivation PP2A function to its substrates. Adapted from (Kamel Khalili et al. 2008).

As a result of the diverse range of possible substrates, PP2A is a vital regulator of multiple downstream signaling pathways and can influence signal transduction, cell cycle regulation, apoptosis and proteolysis pathways (Mumby 2007). Deregulation of this system has been linked to numerous disorders and cancer types, and is an important cellular regulator through which ST can subvert multiple cellular pathways.

The PP2A A subunit is present as two isoforms within the cell – PP2A Aα and PP2A Aβ. Interestingly SV40 ST has only been shown to interact with the Aα subunit not Aβ, whereas other polyomavirus ST proteins, such as MPyV, have been shown to interact with both isoforms (Zhou *et al.* 2003). Crystallisation studies have demonstrated that the SV40 ST Unique region and J domain are essential for ST binding to PP2A as shown in Figure 1.6 (Zhou *et al.* 2003). Both SV40 and JCPyV ST have been revealed to directly interact and bind the Aα subunit and through this interaction ST is able to make contact with the C subunit (Yeh *et al.* 2004).

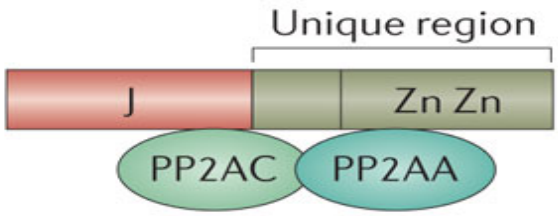

Figure 1. 6: SV40 ST binding of PP2A. Small T antigen binds directly to both the A and C subunit of PP2A Taken from (DeCaprio and Garcea 2013).

Studies have demonstrated that the SV40 ST-PP2A binding site overlaps the B subunit binding site, indicating that ST acts as a competitive inhibitor and competes with B subunits for A subunit binding (Ruediger *et al.* 1994). SV40 ST has been shown to hinder the action of PP2A *in vitro* and *in vivo*, as well as inhibit binding of multiple different substrates to PP2A (Yu *et al.* 2001).

There were currently two distinct theories to explain the manner by which downstream pathways are regulated by the PP2A complex upon destabilization by the ST antigen. The first is that ST may function as a negative inhibitor of the PP2A complex and competitively substitute the B subunit, thus impeding dephosphorylation of specific downstream cellular targets. The second theory is that ST can facilitate the selection of specific substrates and relay PP2A activity to alternative cellular targets. Interestingly, polyomavirus ST proteins has been shown to act by both mechanisms. For example, SV40 ST is capable of increasing PP2A mediated histone H1 dephosphorylation, while inhibiting dephosphorylation by PP2A of various other substrates, like relA (Sugano *et al.* 1982; Yang *et al.* 1991).

1.2.3.4.1.3 ST and Viral Life cycle

MPyV and SV40 ST proteins also have *in vitro* transregulatory activity on promoters transcribed by RNA polymerases II and III (Bikel and Loeken 1992; Cicala *et al.* 1994). This suggests that ST targets and stimulates cellular promoters essential for efficient virus replication. Moreover, mutations which render SV40 ST transregulatory-defective display significantly slower growth in tissue culture compared to wild type viruses (Sugano *et al.* 1982; Huang *et al.* 1999). Consequently, while ST has not been shown to be essential for the viral infection cycle, it is possible that it enhances replication. This theory has been supported by

findings demonstrating that SV40 ST expression increases early viral promoter activity and also has a stimulatory effect on LT-mediated activation of the late viral promoter (Bikel and Loeken 1992; Cicala *et al.* 1994; Campbell *et al.* 1997).

Studies have also demonstrated that MPyV and SV40 DNAJ domains function as chaperones by binding to Hsc70 (Sontag *et al.* 1993; Huang *et al.* 1999). Hsc70 is a cellular heat shock protein important for protein folding and transport, due to its intrinsic ATPase activity (Liu *et al.* 2012). Mutation studies have determined that it plays an essential role in the viral replication cycle (Campbell *et al.* 1997; Rodriguez-Viciana *et al.* 2006b). However, it is uncertain if this is as a result of any direct effects due to Hsc70 binding, or indirect effects that enhance other ST-mediated mechanisms for induction of S-phase entry.

Polyomavirus ST inhibition of PP2A activity has been suggested to contribute to virus replication indirectly. This is due to disruption the regulation of the cell cycle leading to increased G_1-S-phase transition (Sontag *et al.* 1993; Frost *et al.* 1994). The enhanced entry into S-phase enables the recruitment of cellular proteins, as well as co-factors that are essential for DNA replication and subsequent virus replication. MPyV ST has been shown to induce the MAP kinase cascade in mouse cells. This is functionally independent of the DNAJ domain, but completely dependent on its PP2A binding domain (K. Khalili *et al.* 2008). This ST-PP2A interaction increases the expression of AP-1, a transcription factor shown to induce S-phase entry (Frost *et al.* 1994; Polyak *et al.* 1994; Winston *et al.* 1996).

Additionally, it has been established that ST affects cell cycle progression through decreasing cellular levels of p27/kipI (Kamel Khalili *et al.* 2008; Larrea *et al.* 2008). Cyclin dependent kinase [cdk] inhibitors, such as p27/kipI, control cell cycle progression (Sheaff *et al.* 1997). p27/kipI binds cyclin D which in turn regulates the efficient interaction between CDK4 and pRb (Larrea *et al.* 2008). Consequently, elevated levels of p27/kipI result in pRb-mediated cell cycle arrest at the G_1-S checkpoint. Numerous studies have reported that human diploid fibroblast cells [HDFs] expressing SV40 ST show a significant reduction of p27/kipI expression (Kamel Khalili *et al.* 2008). However, a mechanism for ST inhibition of p27/kipI activity has not been established. Although it is possible that ST regulation of PP2A could result in reduced dephosphorylation of cyclin E/cdk2, which can regulate p27/kipI levels (Sheaff *et al.* 1997; Kamel Khalili *et al.* 2008). This provides a plausible mechanism for reduced levels of cellular p27/kipI upon ST expression, resulting in cell cycle regulation and increased S phase entry. Additionally, multiple

proteins associated with cell cycle progression are upregulated upon SV40 ST expression including cyclin D1, cyclin B, dihydrofolate reductase and thymidine kinase (Moreno *et al.* 2004; Sablina and Hahn 2008).

1.2.3.4.1.4 ST and Transformation

Various studies have shown that deletions within the LT and ST DNAJ domain inhibit T antigen-mediated transformation (Kamel Khalili *et al.* 2008). While the exact mechanism of how the DNAJ domain promotes cellular transformation is uncertain, it is likely to be due to an indirect role of the DNAJ domain in promoting S-phase entry in the host cell. This theory is supported by a various studies demonstrating that LT and ST J domain deletion mutants are incapable of causing cell transformation (Pipas 1992).

Additionally, the DNAJ domain contains a conserved Cr1 domain, which functions in a similar manner to the adenovirus E1A protein, in binding p300, the cellular transcriptional co-factor which induces cellular transformation (Peden *et al.* 1990; Pipas 1992). As previously stated, the ST-PP2A interaction is vital for induction of cell entry into S-phase, hence promoting viral replication in permissive cells. In contrast, in non-permissive cells, aberrant stimulation and entry into S-phase can result in cellular transformation. SV40 ST and LT alike are essential for cellular transformation in rodent cells, with ST believed to enhance LT activity (Bikel et al. 1987). Conversely, in human cells, complete transformation requires expression of both SV40 T antigens as well as oncogenic H-ras and hTERT. Interestingly, H-ras and hTERT expression is dependent on ST (Hahn et al. 2002).

The interaction between ST and PP2A has also been shown to play a role in cellular transformation [Figure 1.7]. Specifically, the interaction affects the expression of transcription factors within the host cell. One example is C-myc, an important transcription factor, which is activated by phosphorylation and is involved in regulating the expression of up to 15% of cellular genes (Miller et al. 2012). When activated, it binds numerous promoter/enhancer sequences and recruits Histone acetyltransferases [HATs] to these sites enhancing gene expression (Martinato *et al.* 2008).

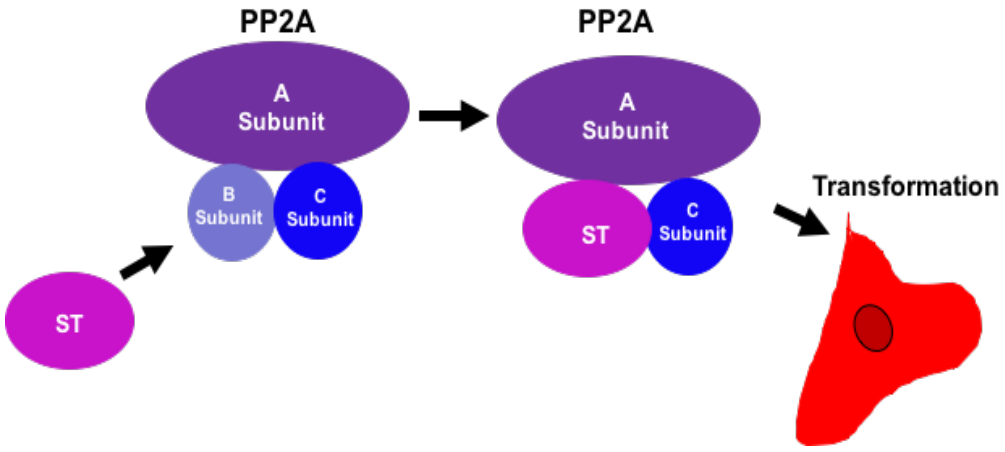

Figure 1. 7: Interaction of ST and PP2A. Polyomavirus ST competes with the PP2A B subunit for binding to the structural A subunit and the catalytic C subunit. In SV40, this interaction between ST and PP2A Aα leads to cell transformation. Although the interaction of MCPyV ST with PP2A Aα may not be necessary for transformation, interaction with PP2A A or the related PP4C may play a role.

PP2A phosphatase activity directly regulates C-myc, and restriction of this process by ST binding results in alteration of C-myc-mediated processes, such as cell proliferation, growth and apoptosis. Yeh et al have demonstrated that expression of a stabilised form of C-myc induces cellular transformation in human cells. Therefore, it is believed SV40 ST expression stabilises C-myc leading to tumourigenesis due to ST inhibiting PP2A-mediated dephosphorylation of C-myc (Yeh *et al.* 2004). As such, inhibition of PP2A-mediated dephosphorylation of C-myc results in extended activation of multiple C-myc targeted transcripts which in turn regulate vital cellular pathways. Interestingly, C-myc has also been shown to be stabilised upon SV40 ST expression in a PP2A dependent manner, however, how this mechanism is regulated is unknown (Zerrahn *et al.* 1993; Tiemann *et al.* 1995; Arnold and Sears 2006).

ST also initiates and activates other cellular transcription factors, such as AP-1, Sp1, CREB and NF-$_K$B. These transcription factors have been shown to play vital roles in cell proliferation and growth and have also been implicated in tumourigenesis (Moens *et al.* 2007). Also, aberrant expression of these transcription factors have been identified in multiple human cancers (Hsu *et al.* 2004; Conkright and Montminy 2005; Piva *et al.* 2006; Ozanne *et al.* 2007).

Modifications in the phosphatidylinositol 3-kinase [PI-3K] pathway have also been associated to human cancer development. SV40 ST has been demonstrated to

initiate aberrant activation of the PI-3K pathway. Interestingly, ST-mediated PI-3K activation results in phosphorylation of cellular targets, such as Akt - a serine/threonine kinase which plays a vital downstream role in regulation of cell survival, angiogenesis and metabolism (Rodriguez-Viciana *et al.* 2006b; Yuan and Cantley 2008). Interestingly, Rodriguez-Viciana et al have shown that SV40 ST promotes Akt phosphorylation in a PP2A-dependent manner (Kang *et al.* 1999). It has been reported that overexpression of PI3K effectors initiate human cell transformation when interchanged with SV40 ST expression (Zhou *et al.* 2002). Notably, inhibition of PI3K activity prevented ST-induced transformation in cells (Hiyama and Hiyama 2004; Rodriguez-Viciana *et al.* 2006a). Additionally, the ST-PP2A interaction has also been associated with promotion of cell immortalization *in vitro* and *in vivo* (Hiyama and Hiyama 2004). Studies have demonstrated that telomerase is upregulated upon SV40 ST expression (Yuan *et al.* 2002). Telomerase activity follows each round of chromosome replication, preserving terminal DNA and constitutive telomerase activity in cells enhances immortalisation (Hiyama and Hiyama 2004). Studies have suggested that impeding Akt desphosphorylation, by ST-mediated binding of PP2A, may induce an increase in telomerase phosphorylation and resulting activity (Kang *et al.* 1999).

Moreover, increase and hyperphosphorylation of the mitotic spindle checkpoint protein Bub1, is believed to cause aneuploidy within host cells (Poulsen 2004; Moens *et al.* 2007). Again, the SV40 ST-PP2A interaction has been linked to hyperphosphorylation of Bub1 via initiation of the MAP kinase signaling pathway, which has been shown to induce tumourigenesis and aneuploidy in transgenic mice (Guo *et al.* 2006).

1.2.3.4.2 Large Tumour antigen

1.2.3.4.2.1 Structure and Domains

The general structure of polyomavirus large T antigens is displayed in Figure 1.8. LT encodes a protein of 708 amino acids in length and comprises multiple domains including ATPase domain, host range (HR) domain, J domain, nuclear localisation signal domain, origin binding domain, Rb-protein binding (LxCxE) motif, and a Zn binding domain (Ahuja *et al.* 2005). As such, SV40 LT antigen targets multiple cellular pathways to elicit cellular transformation and enhance viral genome replication (Sullivan and Pipas 2002).

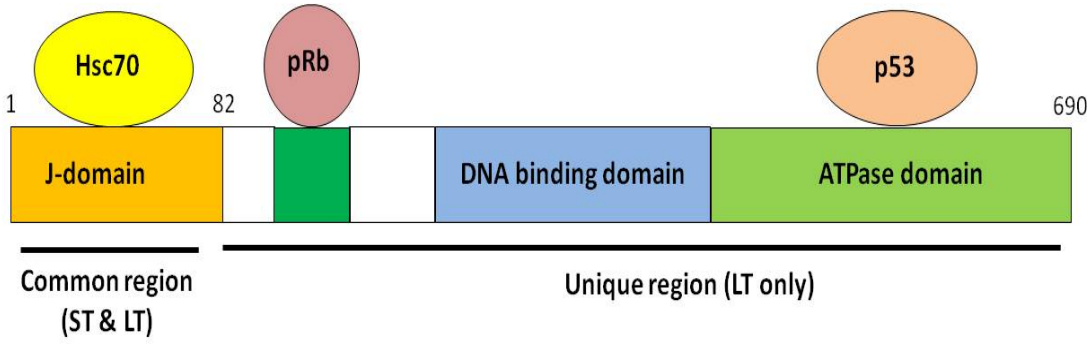

Figure 1. 8: Schematic representation of polyomavirus large T antigens. A J domain is found in the common region, which runs between amino acids 1- ~90. The common region also contains a conserved Cr1 domain. The unique region contains a binding domain for pRb, a DNA binding domain and an ATPase domain which interacts with p53. Taken from (Kamel Khalili et al. 2008).

Large T antigen is a multifunctional regulatory protein and categorized as a member of the helicase super family of proteins. A hallmark of this protein is that it unwinds double stranded DNA and RNA (Stahl *et al.* 1986; Ahuja *et al.* 2005; Sotillo *et al.* 2008). Moreover, SV40 LT targets multiple cellular pathways to elicit cellular transformation and promote transcription of late region genes (Sotillo *et al.* 2008).

1.2.3.4.2.2 LT and Viral Life cycle

1.2.3.4.2.2.1 LT and DNA binding

The Large T antigen induces viral genome replication by binding to the origin of replication in the NCCR, promoting DNA unwinding and then recruiting cellular replication factors. Large T-mediated transformation occurs as a result of its interactions with cellular tumour suppressor proteins (Dean *et al.* 1987). SV40 and JCPyV LT can also promote transcription of the late promoter region, which results in the inhibition of early gene transcription via an origin binding dependent mechanism (Brady *et al.* 1984).

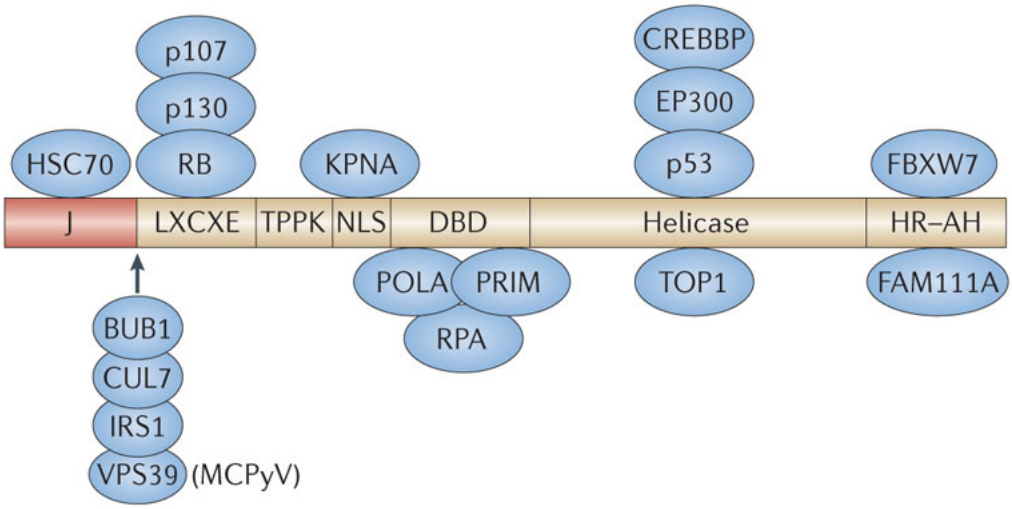

Figure 1. 9: The structure of SV40 LT. SV40 LT contains an N-terminal DnaJ domain that binds Hsc70, and multiple domains which recruit many different binding partners, including a nuclear localisation signal (NLS), a DNA-binding domain (DBD), and an ATPase/helicase domain Taken from (DeCaprio and Garcea 2013).

1.2.3.4.2.2.2 LT and recruitment of cellular proteins

As previously stated, LT has the ability to induce viral DNA replication and it relies on the recruitment of multiple cellular proteins essential for transcription complex formation [Figure 1.9]. Nucleolin and topoisomerase I are recruited by LT, to form components of the holoenzyme required for DNA unwinding (Sachsenmeier and Pipas 2001; Seinsoth *et al.* 2003). The SV40 LT J domain and the ATPase domain are vital for direct binding of LT to the catalytic subunit of DNA polymerase-α *in vivo* (Dornreiter et al. 1990*)*. Furthermore interaction between LT and replication protein A is also essential for DNA polymerase activity and SV40 genome replication (Melendy and Stillman 1993).

1.2.3.4.2.3 LT and Cell Transformation

SV40 LT expression alone is sufficient to initiate cellular transformation in various non-permissive cells, such as rat fibroblasts (Ahuja *et al.* 2005; Moens *et al.* 2007). The key role of LT-mediated transformation is due to interactions with the cellular tumour suppressor proteins, p53 and pRb (Saenz-Robles *et al.* 2001; Sullivan and Pipas 2002). Supporting this theory, are results showing specific mutations in LT regions known to affect p53 or pRb binding can inhibit its transforming capability (Sachsenmeier and Pipas 2001). An alternative role of LT in transformation has also been suggested, as BKPyV and SV40 LT proteins have the ability to induce

mutagenic effects in the host DNA, through SV40 LT binding to Mre11 (Theile and Grabowski 1990). SV40 LT binding to Mre11, results in double stranded break repair (Ahuja *et al.* 2005; Moens *et al.* 2007).

1.2.3.4.2.3.1 LT/pRB

LT binds to and sequesters pRb and pRb family proteins [p107 and p130] via the LT LxCxE motif, as well as the J domain (Sullivan *et al.* 2001; Ahuja *et al.* 2005; Moens *et al.* 2007). LT binding to Rb releases the E2F transcription factor, which then enhances the transcription of E2F-regulated genes, resulting in activation of DNA replication and repair, cell cycle progression and nucleotide metabolism. Importantly, upon LT-mediated Rb sequestration, transcription of E2F-dependent genes allow cell cycle progression into S phase leading to hyper proliferation (Sullivan *et al.* 2001; Ahuja *et al.* 2005; Moens *et al.* 2007).

1.2.3.4.2.3.2 LT/p53

The LT antigen also associates with the p53 tumour suppressor protein. p53 is a crucial regulator of the cell cycle, apoptosis and DNA damage repair mechanisms. Various polyomavirus LT proteins, for example JCPyV and BKPyV, interact with p53 in an ATP-dependent manner (Kierstead and Tevethia 1993; Bollag *et al.* 2000). LT sequestration of p53 prevents activation of p53-responsive genes, resulting in inhibition of apoptosis induction and cellular transformation (Jiang *et al.* 1993; Segawa *et al.* 1993).

1.2.3.4.2.3.3 LT and IRS

It has been established that expression of polyomavirus LT proteins can impede the homologous recombination-direct DNA repair [HRR] response. To this end, SV40 and JCPyV LT antigens are capable of inducing translocation of Insulin receptor substrate I [IRS I] into the nucleus (Lassak *et al.* 2002; Prisco *et al.* 2002; Reya and Clevers 2005). Upon nuclear translocation, IRS I interacts with Rad51, a DNA repair component, resulting in inhibition of homologous recombination-directed DNA repair [HRR] (Urbanska *et al.* 2009). HRR is strongly impeded in JCPyV LT-expressing cells and it is possible this inhibition plays a role in cellular

transformation (Ali *et al.* 2004; Trojanek *et al.* 2006). Supporting this hypothesis is results showing that expression of a dominant negative IRS I mutant negates anchorage independent growth upon JCPyV LT expression (Lassak *et al.* 2002; Prisco *et al.* 2002; Reya and Clevers 2005).

1.2.3.4.2.3.4 LT and β-catenin

β-catenin is a key factor associated with the Wnt signaling pathway. Constitutive activation of the Wnt signalling pathway is a major characteristic of various cancers (Reya and Clevers 2005). Studies have shown that JCPyV LT binds to β-catenin to form complexes with associated transcription factors, resulting in the induction of specific genes, like C-myc and cyclin D1 (Enam *et al.* 2002). The JCPyV LT- β-catenin interaction has been shown to increase β-catenin stability, as well as augment activation of its target genes (Gan and Khalili 2004).

1.2.3.4.2.3.5 LT and Cullin 7

Cullin 7 (cul7) is a major component of the E3 ubiquitin ligase complex, which facilitates protein degradation (Fu *et al.* 2010). Studies have shown that SV40 LT interacts with cul7, and expression of LT mutants incapable of interacting with cul7 negatively affects LT-mediated anchorage-independent growth, as well as cell growth in low serum conditions (Ali *et al.* 2004; Moens *et al.* 2007) . However, the molecular mechanisms associated with this phenotype have not been clearly elucidated, although this interaction clearly plays a major role in LT-mediated tumourigenesis.

1.2.3.4.2.3.6 LT and other Transcription factors

LT proteins associate with numerous other cellular proteins. For example, SV40 LT interacts with Thyroid Embryonic Factor-1 [TEF-1], a cellular transcription factor (Gruda *et al.* 1993). This interaction inhibits TEF-1 repression of the viral late promoter leading to stimulation of viral replication (Ito *et al.* 1977; Berger *et al.* 1996). Interestingly, this interaction may also play a role in cellular transformation, as LT mutants incapable of binding TEF-1 have shown a reduced ability to form foci in cellular transformation assays, in comparison wild type LT (Soeda *et al.* 1979;

Novak and Griffin 1981; Dilworth *et al.* 1986; Zuzarte *et al.* 2000). Additionally, SV40 LT is capable of altering the expression levels of various other transcription factors, including AP-1, C-myc, TFIIC and Sp1 (Moens *et al.* 2007; Fluck and Schaffhausen 2009). These transcription factors control the expression of a wide variety of genes involved in cellular processes, such as angiogenesis, apoptosis, cell proliferation as well as DNA repair (Templeton *et al.* 1986; Freund *et al.* 1992; May *et al.* 2004).

1.2.3.4.3 Middle T

1.2.3.4.3.1 Structure and domains

Alternative splicing mechanisms in rodent polyomaviruses result in the production of the middle T antigen [MT], along with ST and LT antigens. MT is 421 amino acids in length producing a protein of approximately of 55 kDa in size (Ito *et al.* 1977). The structure of MT is very similar to the ST structure. As Figure 1.10 shows, all but the final 4 C-terminal amino acids from ST are present in MT. Consequently, the DNAJ domain and the PP2A binding domains are also expressed in the MT N-terminal common region. The remaining 230 amino acids located at MT C-terminal unique region, contain multiple phosphorylation sites that are essential for MT-mediated recruitment and activation of cellular components. MT also has tight association with the plasma membrane, and this localisation is essential for its putative functions (Soeda *et al.* 1979; Novak and Griffin 1981; Dilworth *et al.* 1986). Its C-terminal transmembrane domain has a long hydrophobic amino acid sequence capable of spanning the lipid bilayer (Fluck and Schaffhausen 2009).

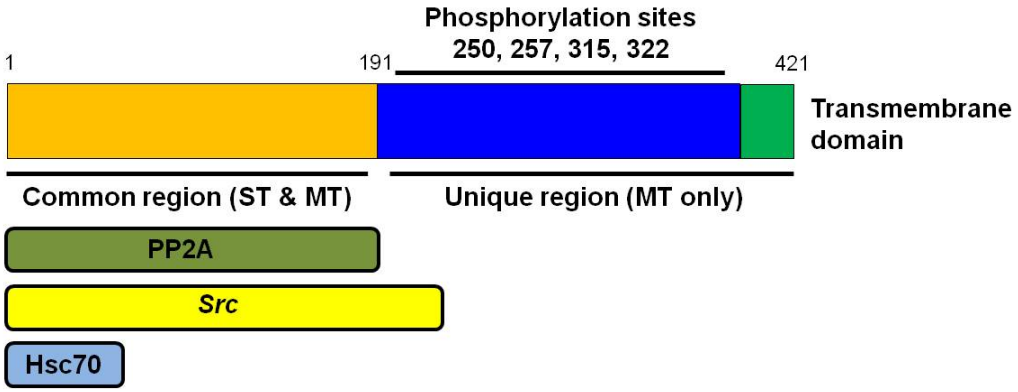

Figure 1. 10: The structure of polyomavirus middle T antigens. The indicated N-terminal amino acids are shared with the small T antigen. This region is involved in binding of Hsc70 and PP2A. The N-terminal 220 amino acids are required for binding of Src family proteins. The unique region contains a number of phosphorylation sites and a transmembrane binding domain.

1.2.3.4.3.2 MT and viral life cycle

Comparable to ST and LT, the MPyV MT antigen can also bind to the viral origin of replication and in turn regulate viral DNA replication. As such, MT is essential for productive infection in murine polyomaviruses (Freund *et al.* 1992). MT also mediates viral replication by activation of the AP-1 transcription factors, Ets protein families and C-jun promoters (Tyndall *et al.* 1981; Veldman *et al.* 1985). Additionally, MT is vital in the maturation of the viral capsid as the absence of MT expression results in aberrant phosphorylation of the VP1, which in turn hinders viral capsid assembly (Garcea and Benjamin 1983; Li and Garcea 1994).

Similar to the human polyomavirus ST and LT oncogenes, MT also sequesters proteins implicated in cell cycle regulation and also activates cell cycle entry to facilitate virus replication. As such, MT expression promotes cell growth and can result in cellular transformation (Li and Garcea 1994). Interestingly, the transcripts that encode MT have not been identified in human specific polyomaviruses, such as MCPyV. Hence human polyomavirus LT and ST may compensate for the absence of MT by encoding alternative proteins, such as ALTO. This protein will be further discussed in a later section [1.4.4.1.2.4].

1.2.3.4.3.3 MT and Transformation

Uniquely, MT is the major transforming protein of rodent polyomaviruses. MT expression alone is sufficient to induce transformation in tissue culture and vital for

tumourigenesis, subsequent to viral infection (Fluck and Schaffhausen 2009). The hydrophobic region, located at the C-terminus, which mediates MT plasma membrane binding is essential for cellular transformation. This was demonstrated by replacing key residues with alternative membrane targeting domains (Templeton and Eckhart 1984; Elliott *et al.* 1998; Zhu *et al.* 1998). Additionally, removal of the six amino acids C-terminal of the hydrophobic domain abolishes MT-mediated transformation (Zhou *et al.* 2011).

1.2.3.4.3.3.1 MT and PP2A

As previously stated, ST and MT have similarities in their N termini coding sequence, including the DNAJ domain and the PP2A binding motif. Consequently, MT is capable of binding PP2A in a similar manner to ST. Not surprisingly, this occurs by MT binding the PP2A A subunit and forming a complex with the C subunit. This again results in disruption of B subunit association and PP2A functioning (Pallas *et al.* 1990; Walter *et al.* 1990). However, alternative to ST, MT can bind both isoforms of PP2A [Aα and Aβ] however the functional significance of this difference has not been elucidated. Mutations in the PP2A Aβ isoform have been associated with numerous cancers (Zhou *et al.* 2003). As a result, this may be associated with MT-mediated transformation. Additionally, MT has been demonstrated to also regulate tyrosine kinases and C-jun functioning in a similar manner to ST, by means of binding PP2A (Cayla *et al.* 1993; Mullane *et al.* 1998).

1.2.3.4.3.3.2 Sequestration of Src family proteins

MT is also capable of binding a number of Src family tyrosine kinases [TKs] at the plasma membrane in a PP2A-dependent manner (Courtneidge and Smith 1983; Kornbluth *et al.* 1987). Once MT is associated with TK proteins, MT is phosphorylated at specific tyrosine residues [namely 250, 315 and 322 residues], resulting in enhanced MT transforming capabilities (Harvey *et al.* 1984; Hunter *et al.* 1984). These post-translational alterations augment and improve the MT transforming ability by enabling it to function as a substrate/mimic in cellular signalling pathways [summarised in Figure 1.11].

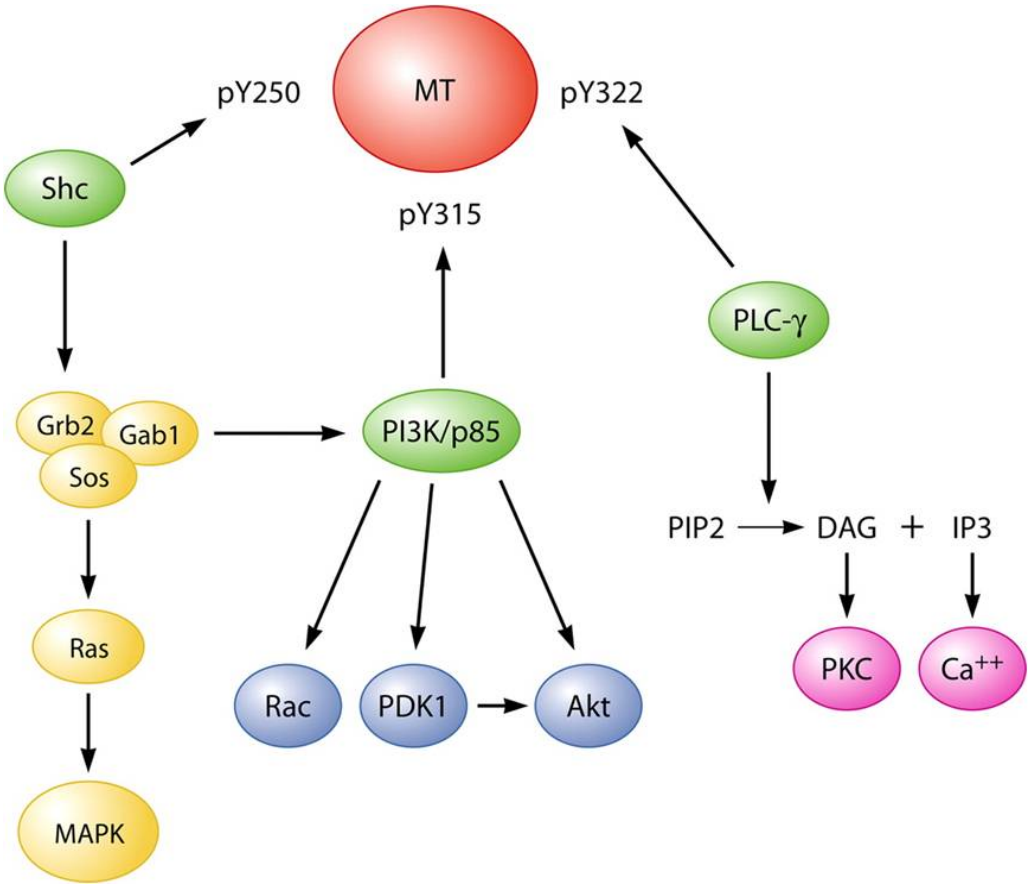

Figure 1. 11. MT phosphorylation influences different cellular signalling pathway. Phosphorylation at MT Y250 confers binding to and phosphorylation of ShcA family proteins, leading to MAP kinase activation downstream of Grb2 complexes. Phosphorylation at Y315 can upregulate PI3 kinase activity (also stimulated by MT-ShcA associations) and Y322 phosphorylation may result in PLC-γ activation. Taken from (Fluck and Schaffhausen 2009).

1.2.3.4.4 Additional alternative spliced early proteins

Additional to the previously described T antigens, SV40 also, as a result of alternative splicing, produces an additional protein known as 17kT. It is comprised of 131 amino acids [from the LT N-terminus], as well as additional A-L-L-T residues at the C-terminus (Zerrahn *et al.* 1993). The function of 17kT in the viral lifecycle is unknown, however, phosphorylation of 17kT has been shown to influence the functions of SV40 LT (Moens *et al.* 2007). Also, expression of 17kT is upregulated in SV40-transformed cells, similarly 17kT can immortalise rat fibroblast cells when is co-expressed with activated H-ras (Zerrahn *et al.* 1993; Bollag *et al.* 2006).

There is however a disparity between polyomaviruses, such as SV40 and JCPyV in expression of alternative T antigens. JCPyV encodes three additional alternatively spliced early proteins- T'_{135}, T'_{136} and T'_{136} (Frisque *et al.* 2003). T'_{135} and T'_{136}

contain the N-terminal 132 amino acids of LT in addition to three or four unique C-terminal residues, respectively. T'_{165} has similar N-terminal residues as T'_{135} and T'_{136} however it also includes the last 32 amino acids from the C-terminus of JCPyV LT. These alternative T' proteins are highly expressed upon JCPyV lytic infection and are consequently essential for virus DNA replication (Frisque *et al.* 2003). It is possible that these proteins play a similar role to SV40 17kT in cellular transformation, as co-expression of the T' proteins with activated H-ras cause immortalisation of rat embryo fibroblasts (Bollag *et al.* 2006). However, their exact role in the JCPyV replication cycle is unknown.

1.3 Merkel cell carcinoma

1.3.1 Merkel cells

Merkel cells are mechanoreceptors, initially described by the German histo-pathologist Friedrich Sigmund Merkel at the end of the 19th century (Moll *et al.* 2005). Merkel cells are clear, oval cells of epidermal lineage. They are found in the skin of all vertebrates, forming Merkel cell-neurite receptor complexes (Morrison *et al.* 2009). These complexes are present in touch-sensitive areas of the skin, including hair follicles as well as epithelial structures known as "touch domes". These touch domes have recently been shown to be a component of the somatosensory system and mediate light-touch responses (Maricich *et al.* 2009).

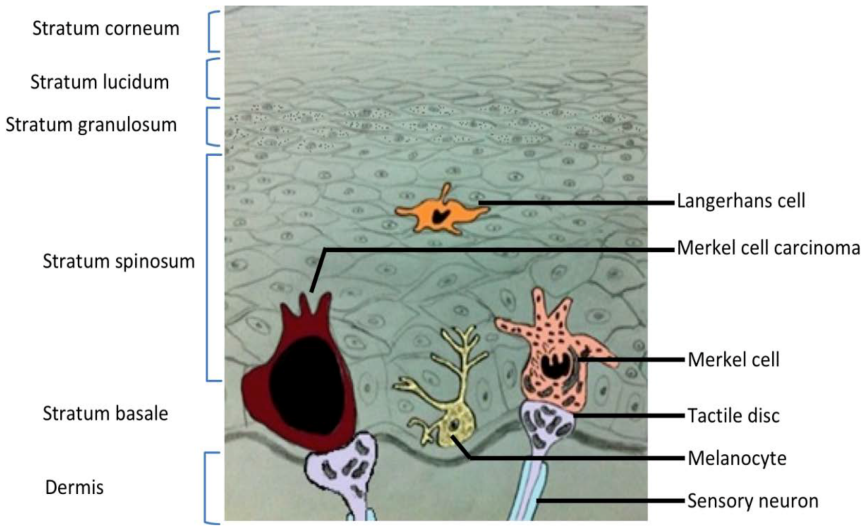

Figure 1. 12: Skin layers in the context of Merkel cells. All skin layers are shown, as well as the Merkel cell-neurite complex at the epidermal/dermal border Taken from (Stakaityte et al. 2014).

1.3.2 Merkel cell carcinoma

Merkel cell carcinoma [MCC] is a rare but highly aggressive skin cancer which affects the elderly and immunocompromised patients (Heath *et al.* 2008). Initially described by Toker in 1972, as a "trabecular cell carcinoma", it has since been characterized as to be a neuroendocrine carcinoma of the skin (Soltani *et al.* 2014).

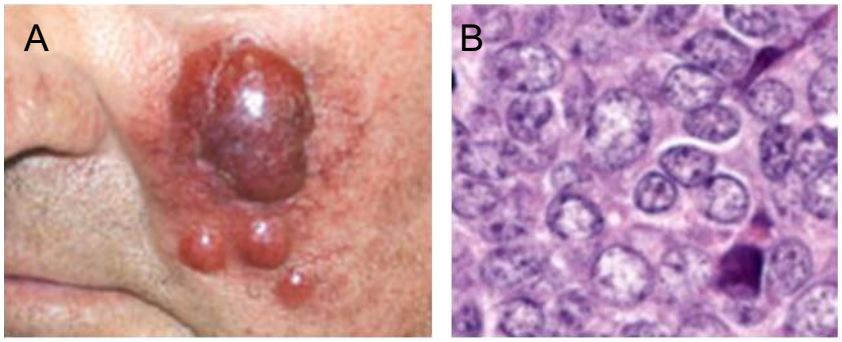

Figure 1. 13:Appearance and histology of MCC. [A] An MCC tumour section (Jouary et al. 2009). [B] MCC tumour cells characterised by large nuclei, a scanty cytoplasm and dispersed, granular chromatin Taken from (Kuwamoto 2011).

In 90% of cases, MCC appears in sun-exposed areas of the head, neck and other extremities as shown in Figure 1.13. This infers that sunlight; specifically, ultraviolet radiation, plays a role in MCC development (Kaae *et al.* 2010; Soltani *et al.* 2014). Current epidemiological data depicts that older, lighter-skinned individuals and the immunocompromised, such as those infected by HIV/AIDS or subjected to organ transplantation, are more susceptible to MCC development (Soltani *et al.* 2014).

1.3.3 Histology and Presentation

MCC tumours present as a pink nodules less than 2 mm in diameter or a mass more than 2 mm in diameter (Goedert and Rockville Merkel Cell Carcinoma 2009). Although primary tumours can be located anywhere on the body, the majority arise on sun-exposed areas of the skin specifically on the head and neck (Calder *et al.* 2007). Additionally, UV exposure is implicated as a strong co-factor for MCC tumourigenesis, with higher rates of MCC directly proportional to UV exposure (Heath *et al.* 2008).

Identification of MCC tumours has often been mistaken for small cell carcinoma of the lungs or Ewings sarcoma which present as similar tumours visually. Under the microscope, MCC tumours are observed as a lesion of stranded or nested small

round cells with a round/oval nucleus, scanty cytoplasm, dispersed granular chromatin as well as inconspicuous nucleoli, among infiltrating cells and vascular invasions (Bobos *et al.* 2006). Recently a detection system has been established based on Cytokeratin [CK] 20 filaments being rearranged in MCC. CK20 is now a specific and sensitive biomarker for MCC, able to clearly distinguish MCC tumours from visibly similar malignancies (Goedert and Rockville Merkel Cell Carcinoma 2009).

While the majority of MCC tumours are characterised as CK20 positive and CK7 negative, there are a few rare cases of CK7+/CK20- negative MCCs reported (Calder *et al.* 2007). Other MCC tumour biomarkers include various neuroendocrine markers, including chromatogranin, somatostatin as well as neuron-specific enolase (Ferringer *et al.* 2005; Goedert and Rockville Merkel Cell Carcinoma 2009). Therefore, MCC tumour diagnosis usually includes reactivity to CK20 and a secondary expressed neuroendocrine marker (Agelli and Clegg 2004).

1.3.4 Epidemiology

Approximately 1500 cases of MCC are diagnosed each year in the United States of America. However, the incidence and MCC specific mortality rates within the United States has significantly increased since 1986 (Schadendorf *et al.* 2017). Similar trends have been observed in the United Kingdom with the age standardized incidence rate of MCC at about 0.9 per 100,000 population. The increase in incidence rate is partly due to the recent advances is MCC diagnostic capabilities. Melanoma and non-melanoma are the most prevalent causes of skin cancer in the United states with approximately 76,000 cases diagnosed annually. However, while MCC has an incidence rate 30 times lower than cases of malignant melanoma, it is twice as lethal (Schadendorf *et al.* 2017). These numbers infer that MCC is as predominant as HTLV-induced cancers and can affect similar numbers as those affected by KSHV-induced cancers which has approximately 4,100 new global cases annually in developed countries (de Martel *et al.* 2012).

The incidence of MCC is highest in white-Caucasians while occurrences are extremely low in black populations. The rate of incidence is intermediate in the remaining ethnic groups (Agelli and Clegg 2004). The higher incidence rate of MCC in patients with European ancestry is believed to be due to a deficiency in melanin, which provides protection against UV radiation.

Although MCC is extremely rare before the age of 50 years, incidence rates increase significantly with age, indicative of accumulation of oncogenic events (Fernandez-Figueras *et al.* 2007). However, with an increase in the aging population, it can be deduced that the incidence of MCC will also increase in the coming years. Also, MCC is more common in males than females, with an incidence ratio of approximately 2:1 white-caucasians and a 5:1 in the other ethnicities (Allen *et al.* 2005).

1.3.5 Staging

Clinical staging of MCC was updated in 2010. Prior to 2010, a four-tiered system was employed. Stage I disease was defined as 'localised tumour, with the tumour smaller than 2 mm in diameter'; stage II disease was defined as 'a tumour still localised but greater than 2 mm'; stage III disease is defined as 'regional metastasis'; while stage IV showed 'distant metastasis' (Feng *et al.* 2008; Schadendorf *et al.* 2017). While this system is still utilized, the 2010 update further differentiates regional metastasis for patients with inconsistencies in pathological and clinical evaluations (Feng *et al.* 2008; Lemos *et al.* 2010).

1.3.6 Prognosis and Treatment

MCC tumours are highly malignant and are also considered to be the most aggressive primary cancer of the skin (Feng *et al.* 2008). MCC overall survival rates are currently poor, 28% of patients die within the first 2 years (Becker *et al.* 2009; Duncavage *et al.* 2009; Andres *et al.* 2010). 45% of MCCs reoccur locally, 70% present with early involvement of local lymph nodes, and half result in distant metastases. The mortality rate for patients with distant metastases is 80%. This high mortality rate for MCC is due to the highly metastatic nature of the tumour (Sarnaik *et al.* 2010). Despite this, there are currently no specific therapeutic treatments available for MCC.

Currently MCC is managed and treated with a wide surgical excision of the primary tumour. This includes a pathological verification of complete tumour removal and is followed by ionizing radiation therapy to decrease the incidence of local recurrence (Goedert and Rockville Merkel Cell Carcinoma 2009; Schadendorf *et al.* 2017). The exact treatment is contingent on several disease characteristics. If the tumour has

metastasized to local lymph nodes, treatment involves surgical removal of tumours, lymphadenectomy, and radiation therapy at the excision sites and lymph nodes. Distant metastasis; however, are treated with chemotherapy regimes including cyclophosphamide, anthracyclines and etoposide. Although half of MCC patients respond to chemotherapy, prognosis is very poor with a median survival of 21.5 months (Schadendorf *et al.* 2017).

1.4 Merkel cell polyomavirus

1.4.1 Discovery

In 2008, a novel human polyomavirus was first isolated from MCC tumours by the Chang and Moore laboratory of University of Pennsylvania (Feng *et al.* 2008). They utilized a novel technique known as digital transcriptome subtraction [DTS] (Feng *et al.* 2008). DTS involves extracting RNA from tumour cells, converting into cDNA libraries and then Pyrosequencing the cDNA. Downstream bioinformatics analysis then remove known human transcripts, co-polymers or human repeat sequences to identify foreign transcripts. Any remaining foreign sequences are then compared to foreign pathogen sequence databases. This DTS approach highlighted novel sequences from MCCs with sequence homology to polyomaviruses. 3' rapid amplification of cDNA ends [3'-RACE], followed by viral genome walking then identified the complete circular genome of Merkel cell polyomavirus (MCPyV) within MCC genomic DNA. Identical RACE products were cloned from the primary tumour and the subsequent lymph node metastasis indicating clonal integration of the virus within the MCC genome occurred prior to local MCC spread. This provided evidence that MCPyV is not a passenger virus, but contributed to MCC development and progression (Feng *et al.* 2008).

Further analysis by other investigators also validated that MCPyV is monoclonally integrated within the genome of MCC tumours (Feng *et al.* 2008; Spurgeon and Lambert 2013). Additionally, a new report using sensitive antibody staining techniques has suggested that MCPyV may be present in 97% or more MCC tumour samples (Agelli and Clegg 2004). This validates the need for numerous MCPyV screening methods and deduces that MCPyV may be abundant among the MCC tumour population. Interestingly, MCPyV is the first human polyomavirus to date that has been irrefutably linked to a human malignancy. However, the role of MCPyV in the pathogenesis of MCC is still poorly understood. This highlights the

importance of elucidating the role of MCPyV in MCC development and understanding the MCPyV life cycle.

1.4.2 Seroprevalence

Serological data detecting the MCPyV viral capsid proteins VP1 and VP2 in patient blood has verified that the majority of the general population is seropositive for MCPyV. An estimated 50% of children under 15 years are infected and approximately 80% of the adult population are seropositive for MCPyV. As such it is believed that MCPyV is a common skin commensal and a common ubiquitous childhood infection of the skin. Additionally, there was no age-related reduction of antibody titre. This is indicative of a persistent MCPyV infection possibly through latent infection (Kean *et al.* 2009; Tolstov *et al.* 2009).

1.4.3 Phylogeny

Multiple phylogenetic studies of human polyomaviruses have been conducted, using VP1, VP2 and LT sequences [Figure 1.14]. It was previously believed that MCPyV is most closely related to the African Green Monkey Lymphotrophic polyomavirus (Dalianis *et al.* 2009). However, recent analysis have suggested that MCPyV is more closely related to MPyV and Chimpanzee polyomavirus (ChPyV), as these viruses share high sequence homology of their VP2 and LT genes. Additionally, the MCPyV VP1 gene is closely related to that of ChPyV and HPyV9. Interestingly, MCPyV LT, VP1, and VP2 have high homology with their counterparts in TSPyV. It can be reasoned that this high degree of homology is due to the fact that they are both common skin commensals (Siebrasse *et al.* 2012). MCPyV is currently classified under the Orthopolyomavirus genus of the *Polyomaviridae* family (Johne and Muller 2007).

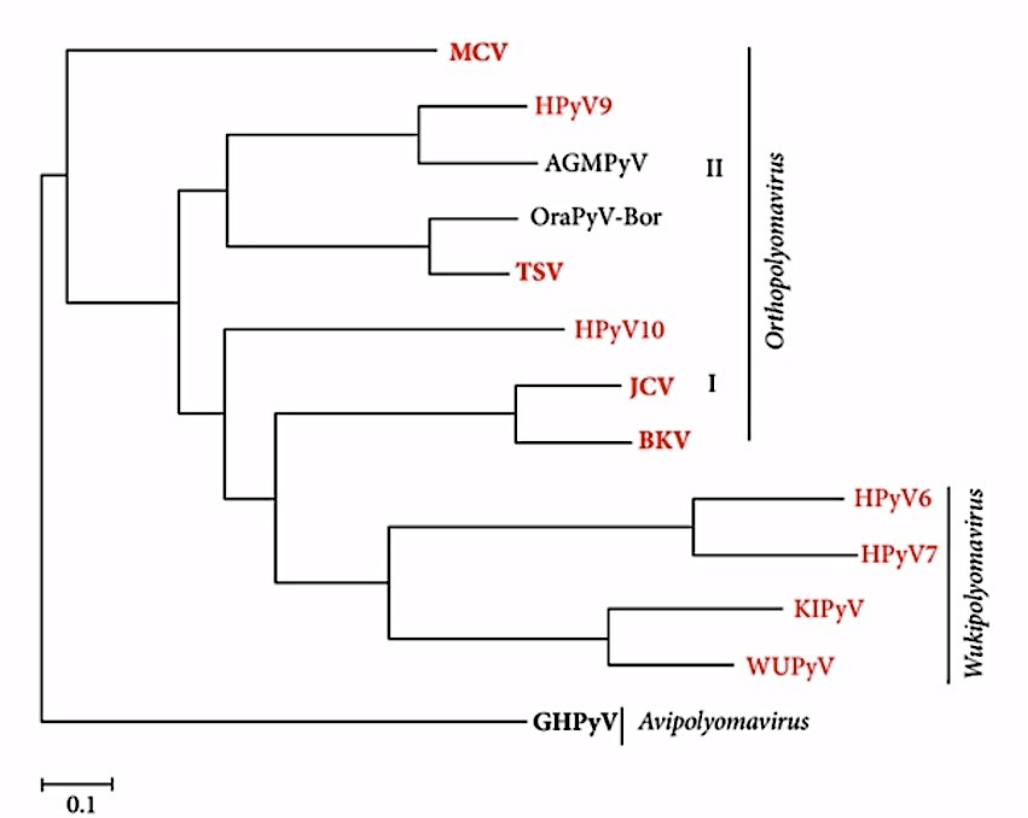

Figure 1. 14: Merkel cell polyomavirus phylogeny. Analysis of whole genome nucleotide sequence analysis. Phylogenetic analysis suggests that among human polyomaviruses MCPyV is most closely related to TSPyV, HPyV9 and HPyV10 Taken from (De Gascun and Carr 2013).

1.4.4 Genome Organization

1.4.4.1 MCPyV Genome

The MCPyV genome comprises 5387 base pairs in length. Like all polyomaviruses, this includes a non-coding control region (NCCR), containing the bidirectional origin of replication, which separates the early and late gene coding regions. This separation allows viral gene expression to be temporal. Figure 1.15 illustrates the MCPyV genome.

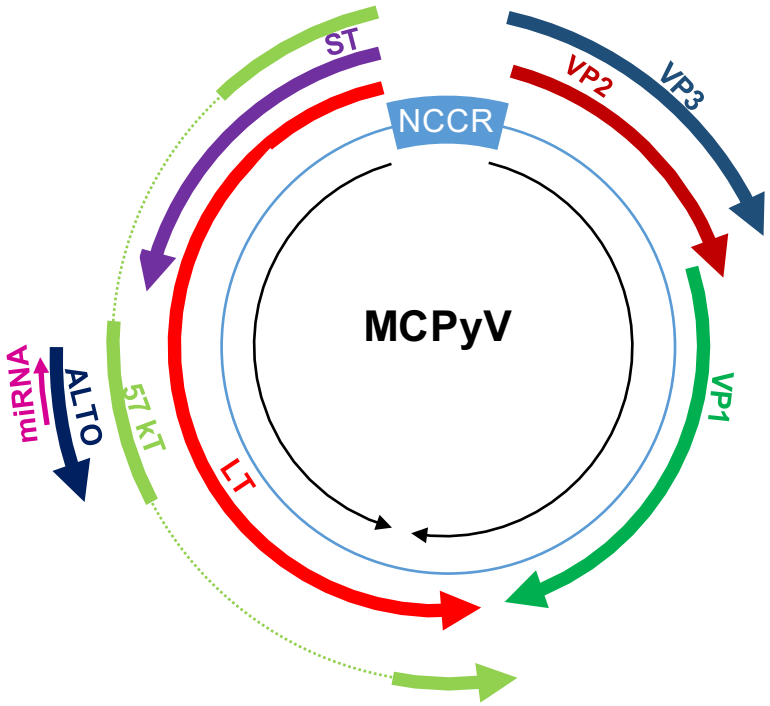

Figure 1. 15: MCPyV genome organisation. Non-coding control region (NCCR): bipartite origin of replication. Early gene region: Large T antigen [LT], small T antigen [ST], 57 kT antigen (57kT), alternative T antigen open reading frame [ALTO], microRNA [miRNA]. Late gene region: capsid proteins [VP1-3].

1.4.4.1.1 Origin of Replication

The MCPyV non-coding control region [NCCR] contains the origin of replication which is 71 base pairs in length. Similar to other polyomaviruses well characterized NCCRs, the origin of replication has an AT rich region, a LT binding domain to initiate viral replication as well as an early enhancer domain. The binding site is comprised of ten repeating guanine-rich pentanucleotide sequences. Eight of the pentanucleotide sequences correspond to the general polyomavirus consensus of 5'-GAGGG-3', while the remaining two are distinct: 5'-GGGGC-3' and 5'-GAGCC-3'. The number of sequences and their proximity to each other are unlike other polyomaviruses (Stakaityte *et al.* 2014).

1.4.4.1.2 MCPyV T antigen locus

The T antigen locus is approximately 3000 base pairs in length and due to alternative splicing, results in multiple transcripts; the small T antigen [ST], the large T antigen [LT], 57 Kilodalton T antigen [57kT] and Alternate frame of the Large T Open reading frame [ALTO]. ST, LT and 57kT all share a short amino-terminal

sequence which includes the J domain. This comprises of the DnaJ [HPDKGG] domain, which is essential for binding the cellular heat shock protein, Hsc70, and a conserved Cr1 epitope. Figure 1.16 illustrates the different T antigen locus-encoded proteins.

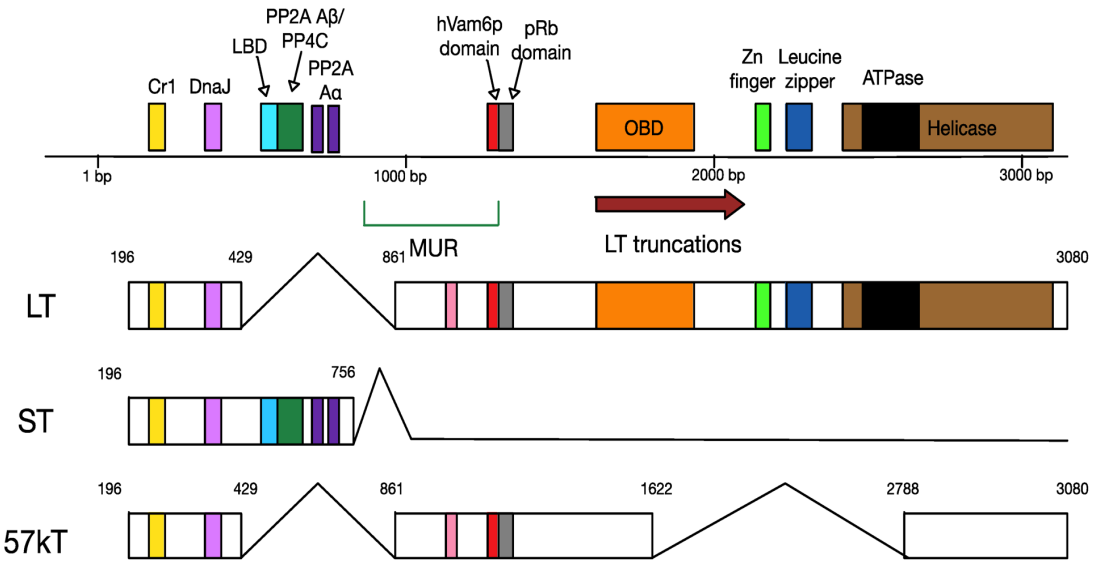

Figure 1. 16: The Merkel cell polyomavirus T antigen locus and its gene products. The schematic shows the relative locations of each gene that encode specific protein binding domains and splicing patterns associated with differential splicing of the primary early transcript. The three T antigens are LT, ST and 57kT. All three encode CR1 [LXXLL] and DnaJ [HPDKGG] domains. ST also contains two PP2A A α binding sites (R7 and L142), a PP2A A /PP4C binding site [amino acids 97–111] and a large T antigen-stabilisation domain [LSD, amino acids 91–95]. LT shares the pRb binding domain with 57kT and has unique origin binding [OBD], zinc finger, leucine zipper, ATPase and helicase domains. The MCPyV-unique region (MUR) of LT contains the hVam6p binding site. Taken from (Stakaityte et al. 2014).

1.4.4.1.2.1 Large Tumour Antigen

MCPyV LT is a spliced transcript comprising of 2 exons producing a protein 816 amino acids in size. Its numerous functions include initiation of viral replication and controlling the host cell cycle. In comparison to other polyomavirus, MCPyV LT antigen contains conserved domains including a nuclear localisation signal [NLS], retinoblastoma protein [pRb] binding domain and the origin-binding domain [OBD] (Bollag *et al.* 2000; Cheng *et al.* 2013). The carboxy-terminal half of MCPyV LT induces domains for viral DNA binding and helicase activity (Kean *et al.* 2009; Viscidi *et al.* 2011; Joseph J. Carter *et al.* 2013). The presence of a nuclear

localisation signal (NLS) results in MCPyV LT localising to the nucleus when expressed in mammalian cells (Bollag *et al.* 2000).

Certain comparisons can be made regarding the interaction of cellular factors with MCPyV LT if structural and functional similarities with SV40 LT are taken under consideration. For example, cellular Chaperone Hsc 70 interacts with the DnaJ domain, while pRb and members of its family p107 and p130 interact with the LXCXCE (Sullivan and Pipas 2002). MCPyV LT OBD and helicase region may also recruit similar proteins for viral replication including, but may not be limited to p53 and CREB binding protein [CBP], a transcription co-activation protein (Lane and Crawford 1979). These assumptions however are not conclusive, as MCPyV LT shares only 34% sequence similarity with SV40 (Topalis *et al.* 2013). Moreover, MCPyV LT contains a unique region, termed the Merkel cell polyomavirus T antigen unique region [MUR], which is 200 amino acids in size. This region serves as a binding site for the host cellular factor, Vam6p which is relocated to the nucleus in the presence of MCPyV LT. Moreover MUR also encodes a viral miRNA (Liu *et al.* 2011).

Studies have also shown that MCPyV LT is truncated in MCC tumour cells, which renders the virus replication disabled (Cheng *et al.* 2013). However, the majority of reported LT mutations involve truncation of the entire C-terminal domain, as opposed to more subtle inactivating mutations. This suggests that there is an additional selective pressure upon transforming cells for removal of this region of LT. Consequently it has been shown that truncated LT is more efficient at inducing cellular proliferation than full length LT (Cheng *et al.* 2013). However, in contrast to SV40, neither full length, nor truncated MCPyV LT can initiate cellular transformation in the absence of ST. This proves to be a major difference between MCPyV and other polyomaviruses (Shuda *et al.* 2011).

1.4.4.1.2.2 Small Tumour Antigen

MCPyV ST, is 186 amino acids in length and has multiple roles in viral replication and cellular transformation. ST has a distinctive carboxy-terminal produced by means of transcriptional read-through of the exon splice site used by both LT and 57kT. ST is localized both to the nucleus and the cytoplasm (Moens *et al.* 2007). Like all polyomaviruses, it contains a protein phosphatase 2A [PP2A] Aα subunit binding site, which is an important region for viral replication and virus-induced

transformation in other polyomaviruses (Pallas *et al.* 1990; Feng *et al.* 2008). It also has a recently has been shown to contain a PP2A Aβ and/or protein phosphatase 4C [PP4C] binding site located near the carboxy terminus which may have a role in protecting MCPyV from the cellular immune response (Griffiths *et al.* 2013). Moreover, it contains a LT-binding domain [LBD] which plays a role in stabilizes LT and assists in the replication of the MCPyV genome. Uniquely the MCPyV ST protein is the major oncogenic protein in MCPyV and its transforming properties will be discussed in more detail in subsequent sections.

1.4.4.1.2.3 57 kiloDalton T antigen

MCPyV 57kT transcript encodes a protein of 432 amino acids in size, resulting from alternative splicing linking three exons. It shares the DnaJ domain and CR1 epitope with ST and LT and it also has a MCPyV unique region as well as a pRB-binding domain found in LT. However, little is known about the function of 57kT although it may play a role in host cell proliferation as it maybe homologous to the SV40 17kt protein (Comerford *et al.* 2012).

1.4.4.1.2.4 Alternative T open reading frame

Aside from the three T antigens, the MCPyV early gene locus also encodes a fourth protein, the alternative T antigen open reading frame [ALTO]. ALTO is transcribed from the 200 amino acid MCPyV unique region within LT. It has been theorized to be evolutionarily related to MT antigen of the murine polyomavirus (J. J. Carter *et al.* 2013). Carter et al 2013 showed that ALTO is encoded by the result of an overprinting event with LT, here Open reading frame is +1 frameshifted relative to the second exon of LT as shown in Figure 1.17 (J. J. Carter *et al.* 2013).

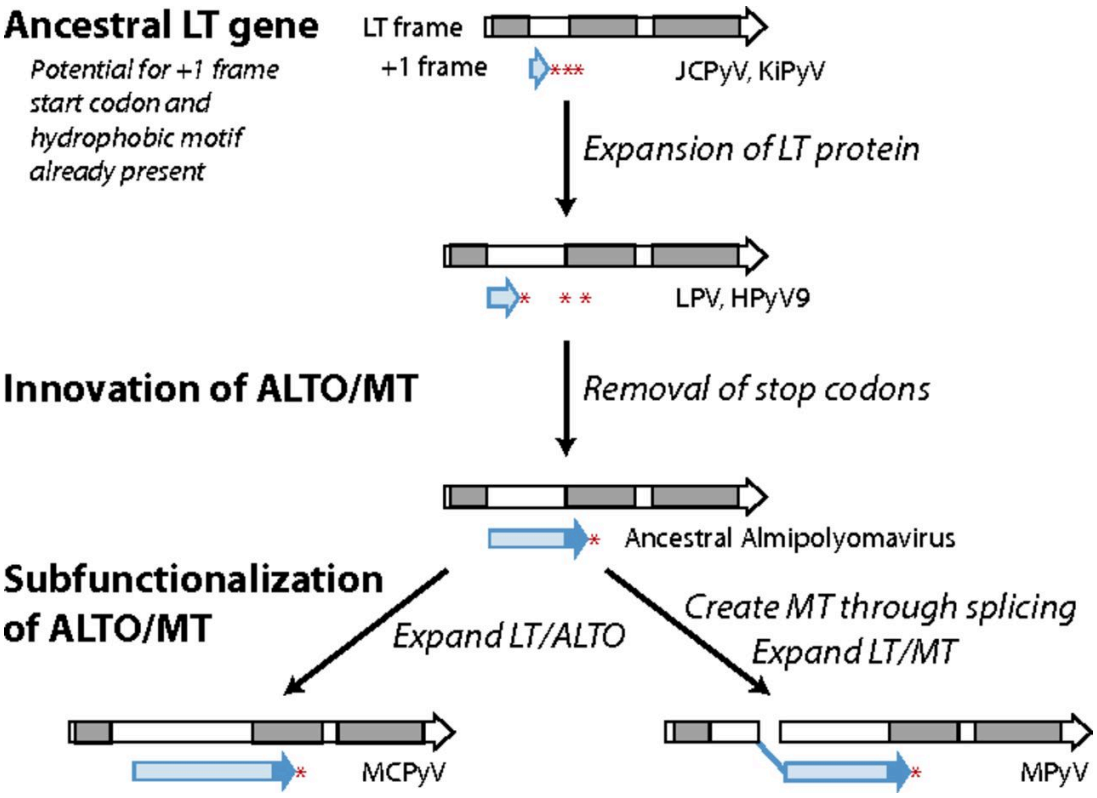

Figure 1. 17: A schematic to represent the de novo gene birth of ALTO by overprinting. ORFs corresponding to the LT and ALTO/MT alternative frame and blue shows ALTO/MT region. Stop codons are represented by asterisks. Taken from (J. J. Carter et al. 2013).

Carter et al demonstrated that the start codon of ALTO overlaps exactly with the YGS/T motif of LT which is located in proximity to pRB binding domain, while the function of the YGS/T motif is unknown, it may be required for the correct and timely folding of the DnaJ domain. Additionally, ALTO has a hydrophobic C-terminus [similar to MT] which is essential for the subcellular localisation of ALTO when present in mammalian cells. While the functional significance of ALTO in relation to MCPyV is yet to be elucidated, data suggests that ALTO may be the twin protein of other polyomavirus MT antigens (J. J. Carter *et al.* 2013).

1.4.4.1.3 Late Proteins

MCPyV, like other polyomavirus encode three structural proteins associated with the virus capsids, encoded from the late region, consisting of viral capsid proteins 1-3 [VP1, VP2 and VP3]. Both the major capsid protein [VP1] and the minor capsid

protein [VP2] have been shown to self-assemble into virus-like particles *in vitro*. However, unlike its contemporaries, VP3 is not conserved and it is unclear whether VP3 is actually expressed at all and part of the capsid. (White and Khalili 2004; Feng *et al.* 2008; Gjoerup and Chang 2010; Schowalter and Buck 2013). The MCPyV capsid is non-enveloped and is icosahedral, composed of 72 pentamers of the major capsid protein, VP1.The overall capsid size is similar to other polyomavirus capsids at approximately 40-55 nm, with a VP1:VP2 ratio of 5:2.

Utilizing crystallography techniques it has been demonstrated that the structure of the VP1 monomer is comprised of two antiparallel β sheets, forming a β-sandwich structure with a jelly-roll topology. The overall shape of the VP1 monomer is a symmetrical, ring-shaped homopentamer organized around a central axis, and variable loops creating distinctive interaction surfaces which may be involved in viral attachment (Stakaityte *et al.* 2014).

The current function of VP2 in the MCPyV lifecycle is relatively unknown, as the protein is not essential for entry mechanisms in most cell lines currently studied. It also does not affect viral DNA packaging into the capsid, trafficking of viral proteins nor is it responsible for binding cellular receptors (Stakaityte *et al.* 2014). A possible function of VP2 may be linked to VP2 myristoylation. This lipidation modification enables cellular membrane disruption, however a specific role has yet to be elucidated. Interestingly, co-expression of VP1 and VP2 result in translocation of VP2 from the cytoplasm to the nucleus, as VP2 alone does not encode a NLS signal, hence VP1 may be crucial for the function of VP2 (Schowalter and Buck 2013).

1.4.4.1.3 MCPyV microRNA

Various polyomaviruses, including SV40 and BKPyV, encode microRNA [miRNAs] which regulate early viral transcript levels (Sullivan *et al.* 2005; Seo *et al.* 2009). The MCPyV early region also encodes a 22-nucleotide-long miRNA, MCPyV-miR-M1-5p, is antisense to the LT gene sequence (Seo *et al.* 2009). The predicted function of the MCPyV miRNA is believed to be associated with downregulating expression of the early genes, as it has complementarity sequences with the LT transcript. Moreover, it may participate in cellular transformation as its expression is observed in about half of MCPyV-positive MCC tumours (Seo *et al.* 2009).

1.4.5 Merkel Cell polyomavirus life cycle

The cellular tropism for MCPyV infection is yet to be fully elucidated, as MCPyV was discovered within a tumour rather than within the environment of its natural host. The origin of MCC, as well as the fact that MCPyV virions are shed from the skin of healthy adults indicate an epidermal source of host cells (Schowalter and Buck 2013). However, recent data suggests human dermal fibroblasts as the natural host cell for MCPyV, which can also support productive MCPyV infection (Liu *et al.* 2016). Additionally, MCPyV pseudovirions can infect human skin-derived primary keratinocytes (HEKa) and transformed melanocytes, but not primary transformed keratinocytes (HeCat) or primary melanocytes, or, indeed, sixty human tumour cell lines investigated (Schowalter *et al.* 2012). Regardless, a MCPyV genome capable of productive viral infection has been established (Neumann *et al.* 2011; Schowalter and Buck 2013).

Results have shown that a full-length wild-type MCPyV genome is capable of being isolated from surface skin swabs of healthy adult volunteers, with the DNA being amplified using random hexamer-primed rolling circle amplification [RCA] (Schowalter and Buck 2013). As an alternative, a synthetic MCPyV genomic clone has been generated from sequence data of MCPyV genomes (Neumann *et al.* 2011). Both of these approaches have produced plasmids, such as pR17b, containing the full recircularised MCPyV genome.

In recent studies, it has been demonstrated that efficient replication of the MCPyV viral genome, both early and late gene expression and viral particle formation, can be observed by transfection of the pR17b plasmid in HEK 293 cells that overexpress the MCPyV LT and ST *in trans*, known as HEK-293-4T cells (Schowalter *et al.* 2011). This viral replication system is key to understanding the MCPyV life cycle and viral-host interactions that contribute to MCC pathology. As mentioned above, human dermal fibroblasts have been suggested as the natural host for MCPyV, allowing a new cell culture model to be developed. MCPyV infection of dermal fibroblasts is facilitated by epithelial and fibroblast growth factors as well as the β-catenin signalling pathway (Liu *et al.* 2016). This cell culture model will be invaluable in future studies, to understand the MCPyV life cycle and MCPyV-induced MCC tumourigenesis.

1.4.5.1 Attachment and entry

The majority of polyomaviruses, such as MPyV, SV40, and BKPyV use sialic acid-containing glycolipids, or gangliosides, to initially attach to cells. MCPyV is an exception, as the initial attachment is a result of association with glycosaminoglycans, in particular heparan sulphate (Feng *et al.* 2008). Binding to gangliosides, specifically GT1B, is also vital for post-attachment entry (Sapp and Day 2009; Schowalter *et al.* 2011).

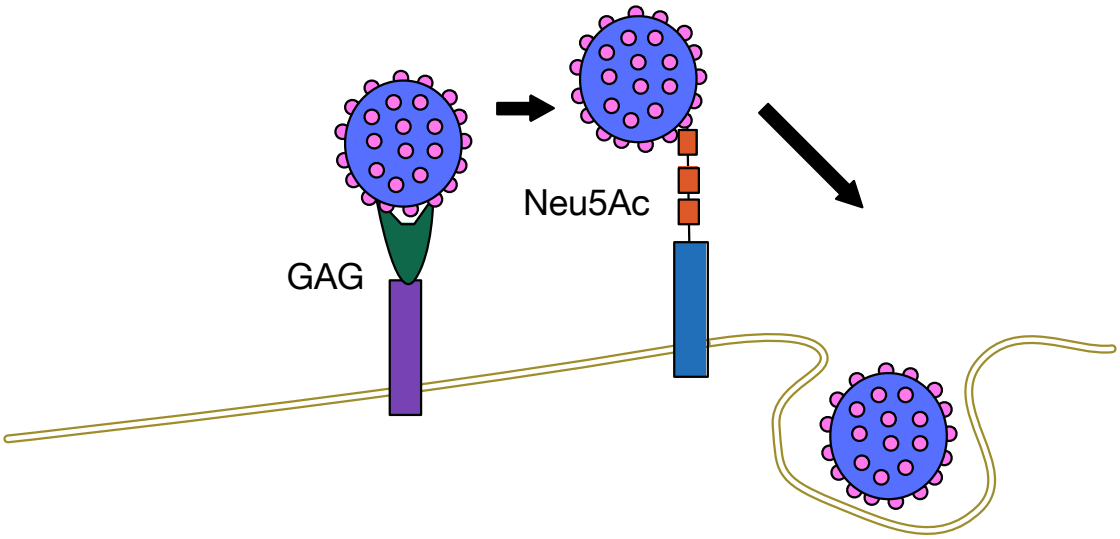

Figure 1. 18: MCPyV attachment and entry. MCPyV entry is a two-step attachment-and-entry process. MCPyV binds glycosaminoglycan (GAG), specifically heparan sulphate as the initial mode of attachment. MCPyV then binds a ganglioside with a Neu5Ac- α2,3-Gal motif facilitate viral entry. Taken from (Stakaityte *et al.* 2014).

X-ray crystallography studies have determined that the MCPyV major capsid protein VP1 has a shallow binding site for cellular glycans containing sialic acid with a Neu5Ac moiety. In particular there is an interaction with carbohydrates which have a linear Neu5Ac-α2,3-Gal motif. Although, mutating this interaction does not affect the initial attachment. As a result, it has been postulated there is a two-step model for the attachment and entry of MCPyV involving two separate types of host cell plasma membrane factors [Figure 1.18] (Neu *et al.* 2012).

1.4.5.2 Merkel cell polyomavirus replication

MCPyV is able to complete its replication cycle and form virions without inducing tumourigenesis in permissive cells. Like other small DNA viruses, MCPyV relies

upon many different host cell factors to replicate its genome. The expression of viral T antigens is essential for this function, and they are transcribed immediately upon virus entry into the nucleus of the host cell. LT and 57kT are transcribed first, with ST shortly afterwards [225]. The T antigens force the host cell to enter S-phase. This alters the cellular environment, making it more hospitable to viral replication (Stahl *et al.* 1986). In later stages of viral replication, it is suggested that further early gene transcription is inhibited by the MCPyV miRNA, likely by causing the degradation of early gene transcripts (Kwun *et al.* 2013). This then shifts the focus to the transcription of the late region and also to the replication of the viral genome itself.

1.4.5.2.1 Large Tumour Antigen and replication

Polyomaviruses require expression of the LT antigen to initiate genome replication. LT oligomerises to form hexameric molecules, two of these hexameric molecules bind to the *ori* in a head to head orientation (Wessel *et al.* 1992). This is followed by LT helicase domain-mediate unwinding of the DNA and resulting replication proceeds in a bidirectional manner, with the two hexamers progressing in opposite directions. The LT OBD interacts with the *ori* by recognising the GAGGC-like motifs. A crystal structure of the *ori* demonstrates that to initiate replication, three of these pentanucleotide sequences are required (Harrison *et al.* 2011). The number and proximity of LT binding sites on the origin likely allows for intermolecular OBD-OBD interactions between molecules of LT (Kwun *et al.* 2009)

MCPyV LT is believed to function in a similar manner to other polyomavirus LT proteins. However, there are several distinctive MCPyV LT-host interactions that affect viral replication. For example, in MCPyV, LT binds to the cytoplasmic vacuolar sorting protein, hVam6p, via its MUR domain (Liu *et al.* 2011). hVam6p is a member of the homotrophic fusion and protein sorting (HOPS) complex, and it is redistributed from the cytoplasm to the nucleus upon MCPyV LT expression. This results in disruption of lysosome clustering. Notably, overexpression of hVam6p induces reduction in MCPyV virus formation by more than 90%, suggesting an inhibitory role (Feng *et al.* 2011). Moreover, abrogating the hVam6p binding domain increases infectious virion production by 4-fold to 6-fold (Feng *et al.* 2011; Liu *et al.* 2011). The inhibitory function of hVam6p may play a role in diminishing viral replication and establishing a persistent infection.

Another factor important for MCPyV LT-mediated viral replication is the chromatin-associated bromodomain containing protein 4 [Brd4]. Brd4 is a member of the BET protein family and plays a role in recruitment of cellular replication factors essential for viral replication. The interaction of MCPyV LT with Brd4 helps recruit replication factor C [RFC] to MCPyV replication (Wang *et al.* 2012). RFC facilitates loading of the PCNA clamp and tethering of the processive DNA polymerase, allowing for the elongation of MCPyV DNA. The importance of the MCPyV LT-Brd4 interaction is highlighted by findings showing that expression of dominant negative forms of Brd4 inhibit MCPyV replication (Feng *et al.* 2011).

Additionally, DNA damage response (DDR) factors are also involved in MCPyV replication, with both DDR pathways, ATM and ATR, being involved. MCPyV LT expression relocalises DDR factors to the nucleus specifically, into the replication foci, where they co-localise with LT (Tsang *et al.* 2014). Interestingly, both DDR and Brd4 activation and recruitment have been observed in HPV replication. These findings stress the importance of the DDR pathways in viral replication across multiple viral families (Gillespie *et al.* 2012; Wang *et al.* 2012).

1.4.5.2.2 Small Tumour Antigen and replication

Although MCPyV LT is the major factor in the replication of MCPyV DNA, it does not facilitate replication efficiently independently. MCPyV ST is essential to enhance replication, as its depletion leads to inhibition of viral replication (Kwun *et al.* 2009). This contrasts with the typical polyomavirus model observed in SV40 ST, as co-expression has minimal effect on SV40 LT-mediated viral replication. Interestingly, the mechanism by which MCPyV ST enhances viral replication does not depend on its ability to bind either PP2A or Hsc70, and the equivalent SV40 ST domains cannot enhance MCPyV LT-mediated viral replication (Shuda *et al.* 2011). This suggests an alternative unique mechanism for MCPyV ST enhancement of viral replication.

One possible mechanism by which MCPyV ST could function in replication enhancement is by promoting the hyperphosphorylation of the translation regulator eIF4E binding protein [4E-BP1]. This leads to increased production of cellular proteins, including host factors necessary for viral replication (Shuda *et al.* 2011). Additionally, MCPyV ST targets the cellular SCF ubiquitin E3 ligase complex, of which Fbw7 is the recognition component (Welcker and Clurman 2008). This

complex targets MCPyV LT for proteasomal degradation, but binding of MCPyV ST to SCF^{Fbw7} , via its LSD region, inhibits MCPyV LT degradation. This prevents the turnover of MCPyV LT, whose half-life without MCPyV ST expression is 3-4 h, going up to 24 h when the two proteins are co-expressed. Interestingly, neither MCPyV ST nor MCPyV 57kT are sufficient for replication in the absence of MCPyV LT. Co-expressing MCPyV 57kT with MCPyV LT shows no increase in replication efficiency (Kwun *et al.* 2013).

1.4.6 Assembly and Egress

Little is known about MCPyV virion assembly and egress, although more research may be forthcoming with the discovery of a potentially permissive cell (Liu *et al.* 2016). MCPyV virions tend to cluster in the nucleus and its periphery before egress (Neumann *et al.* 2011). Unlike other polyomaviruses, including SV40, JCPyV, and BKPyV, MCPyV does not encode an agnoprotein, which is known to be important in virus particle assembly and maturation (Khalili *et al.* 2005). Nor does MCPyV encode an equivalent of the SV40 VP4, which triggers lytic virion release (Daniels *et al.* 2007). Therefore, an alternative mechanism must be utilised by MCPyV. It is possible that redistribution of the lysosomal factor, hVam6p, to the nucleus by MCPyV LT contributes to egress via lysosomal processing during virus replication (Griffiths *et al.* 2013).

Polyomavirus virion release is thought to occur primarily through cell lysis, although shedding of accumulated viral particles in SV40-infected cells has also been observed (Clayson *et al.* 1989). It is possible that if the natural host of MCPyV is a type of skin cell, lysis might not be required for release, as the natural process of keratinocyte desquamation may serve this purpose. The newly developed dermal fibroblast cell culture model may help to fully address these specific questions about the MCPyV life cycle.

1.4.7 Immunity

In order to establish an infection, viruses need to evade the cellular immune response. Host cells use innate immunity as a barrier against invaders, and pathogens of all types have evolved mechanisms to evade or subvert it. MCPyV T antigens appear to play an intriguing role in overcoming the innate immune

response to allow the establishment of viral infection.

MCPyV ST downregulates the innate immune response by interfering with the NF-kB family of transcription factors (Griffiths *et al.* 2013). NF-kB is an important regulator of a number genes involved in both the inflammatory and antiviral response. It is activated in response to various innate immunity signalling, for instance from pattern recognition receptors (PRRs). PRRs detect pathogen associated molecular patterns (PAMPs), e.g. proteins or nucleic acids. PAMP recognition results in the production of antimicrobial peptides, cytokines, and chemokines capable of fighting against and clearing microbial infections (Takeda and Akira 2005).

This recognition also activates a coordinated signalling cascade, leading to the activation of the IKK complex (Karin and Delhase 2000; Takeda and Akira 2005). The IKK complex consists of two catalytic components, IKK IKKα and IKK IKKβ, and a regulator subunit, NF-kB essential modulator (NEMO). NEMO is a molecular scaffold which can recruit upstream signalling (Karin and Delhase 2000; Takeda and Akira 2005). IKK activation causes the phosphorylation and degradation of IkB, which in turn induces the release of NF-kB from the complex. Free NF-kB then translocates to the nucleus, where it stimulates the transcription of proinflammatory cytokine and type 1 interferon genes [Figure 1.19] (Karin and Delhase 2000; Takeda and Akira 2005).

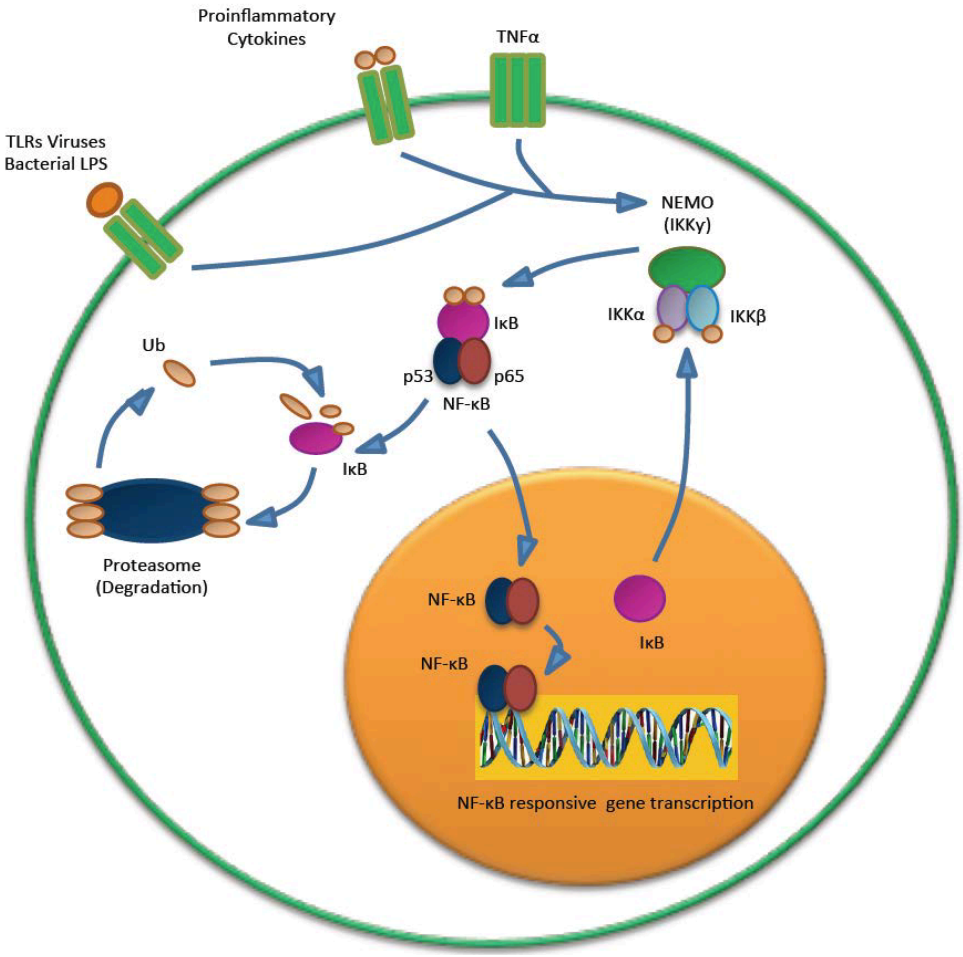

Figure 1. 19: NF-κB mediated gene transcription. Recognition of PAMPS by PRRs facilitates activation of the IKK complex and subsequent degradation of IκB. NF-κB is thereby free to translocate to the nucleus and stimulate transcription of genes involved in mounting an innate immune response. Taken from (Stakaityte et al. 2014).

Many viruses produce proteins that can interfere with NF-kB signalling (Le Negrate 2012), such as HCV core protein and HPV E7 (Joo *et al.* 2005), both of which prevent IkB degradation. Furthermore, murine cytomegalovirus [mCMV] (Fliss *et al.* 2012) and molluscum contagiosum poxvirus (Randall *et al.* 2012)directly target NEMO, disrupting IKK activation. Conversely, SV40 ST upregulates NF-kB activation in a PP2A-dependent manner, while some inflammatory targets like interleukin-8 (IL-8) are downregulated upon SV40 ST expression (Moreno *et al.* 2004). MCPyV ST is able to inhibit IKKα/β phosphorylation via an interaction with NEMO, thus preventing NF-kB release and translocation into the nucleus. This interaction appears to be dependent on protein phosphatases PP2A Aβ and/or PP4C (Griffiths *et al.* 2013). Moreover, a number of NF- kB target genes are

downregulated upon MCPyV ST expression, e.g. CCL20, IL-8, TANK, and CXCL-9 (Griffiths *et al.* 2013).

Viruses also act upstream of NF-kB to inhibit receptors involved in innate immunity, such as Toll-like receptors (TLRs), a subset of PRRs. EBV (Fathallah *et al.* 2010), HPV (Hasan *et al.* 2007), and HBV all downregulate TLR9 expression (Vincent *et al.* 2011). TLR9 detects viral or bacterial double-stranded DNA containing multiple non-methylated CpG motifs (Takeda and Akira 2005). Upon recognition of pathogen DNA, TLR9 activates NF-kB in immune cells, which upregulates the production of inflammatory molecules (Tsujimura *et al.* 2004). MCPyV LT also inhibits TLR9 expression (Shahzad *et al.* 2013) by reducing the mRNA levels of the C/EBP transactivator, a positive regulator of the TLR9 promoter. This leads to a greatly reduced binding of C/EBP to its response element on the TLR9 promoter. Therefore MCPyV LT targeting of C/EBP may be important in establishing viral persistence (Shahzad *et al.* 2013).

It appears that MCPyV has a complex and multifaceted defence against innate immunity, targeting different parts of the various pathways and utilising both major T antigen products. This allows the virus to persist, and may even contribute to tumourigenesis.

1.4.8 MCPyV and pathogenesis of MCC

Since the strong association of MCPyV with MCC tumours was established, causative links for MCPyV in MCC pathogenesis have been reinforced. The monoclonal integration pattern displayed by MCPyV suggests that the viral genome integrates within the host cell genome prior to clonal expansion and metastasis of the tumour cell (Feng *et al.* 2008; Sastre-Garau *et al.* 2009). Moreover, all MCC-derived MCPyV Large T antigen sequences integrated into MCC genomes contain mutations prematurely truncating the helicase domain and also ablating the p53 binding domain. However, the pRb binding domain remains unaffected in every case (Shuda *et al.* 2008). These mutations have been shown to render the virus replication-defective and unable to re-infect MCC tumours, whilst retaining potentially oncogenic ST and LT coding sequences (Shuda *et al.* 2008). Deletions in the VP1 gene of integrated MCC-associated MCPyV have also been reported and are linked with incomplete viral integration into the cellular genome (Kassem *et al.* 2008).

In support of the current evidence, MCPyV positive MCC cell lines have been demonstrated to undergo growth arrest upon siRNA-mediated knockdown of the MCPyV T antigens (Houben *et al.* 2010), indicating that MCPyV positive MCC tumours require expression of these T antigens for tumour growth. Therefore, the current evidence confirms the frequent involvement of MCPyV in tumourigenesis of MCC and dismisses a passenger role for MCPyV within these tumours [Figure 1.20].

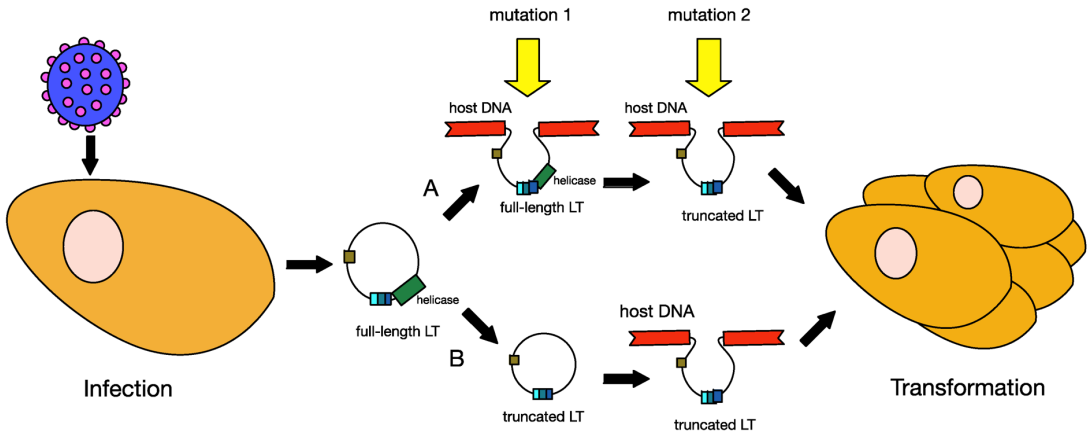

Figure 1. 20: Merkel cell polyomavirus-induced tumourigenesis. MCPyV infection occurs in childhood in most cases. Loss of immunosurveillance leads to proliferation of the virus prior to tumourigenesis. At least two mutations are needed before MCPyV can transform cells. In model A, the first mutation is the integration of the full-length viral genome into host DNA, while the second mutation is the truncation of LT. In model B, truncation of LT occurs prior to integration. These changes in the virus lead to cellular transformation and tumour proliferation. Taken from (Stakaityte et al. 2014).

1.4.8.1 The role of ST in Merkel cell polyomavirus-induced tumourigenesis

Recent studies have shown that unlike most polyomaviruses, such as SV40, where LT is the most important protein for tumourigenesis, MCPyV ST is the major oncogenic protein. Shuda et al demonstrated that MCPyV ST is more commonly expressed in MCC tumour samples than LT, indicative of a significant role in tumourigenesis and tumour cell maintenance. Also, siRNA-mediated depletion of ST in MCPyV-positive MCC cells leads to cell growth inhibition. Moreover, their studies also demonstrated that ST expression leads to a loss of contact inhibition,

anchorage independent, rodent fibroblast transformation and serum independent cell growth (Knight *et al.* 2015). These are all hallmarks of an oncogenic viral protein. Therefore, it can be deduced that MCPyV ST plays a major role in MCC tumourigenesis.

Unlike SV40, where the ST-PP2A Aα interaction is important for SV40-induced transformation and cell proliferation, an alternative mechanism has been described for MCPyV ST. This is due to observations using an MCPyV ST mutant which disrupted the ST-PP2A Aα interaction [R7A mutants] were fully capable of inducing both rodent cell transformation and anchorage-independent colony formation (Shuda *et al.* 2011). One possibility is that SV40 and MCPyV ST proteins affect the PI-3K-Akt-mTOR signaling pathway at different stages. This signaling pathway is an important regulator of translation in tumourigenesis (Buchkovich *et al.* 2008). SV40 ST activates the pathway by preventing the PP2A Aα-mediated dephosphorylation of Akt (Rodriguez-Viciana *et al.* 2006a). However, MCPyV ST has little effect at this stage of the Akt-mTOR pathway, instead acting downstream, at the level of the translation factor 4E-binding protein 1 [4E-BP1] (Shuda *et al.* 2011).

This hinted at another mechanism for MCPyV ST in tumourigenesis. Cap dependent translation requires the eukaryotic translation factor 4E [eIF4E] to bind RNA, which allows ribosome recruitment. This pathway is regulated by 4E-BP1 which controls binding of eIF4E to RNA. Mammalian target of rapamycin [mTOR] phosphorylates 4E-BP1, which releases eIF4E to initiate allowing assembly of eIF4F and cap-dependent translation (Shuda *et al.* 2011). MCPyV ST reduces the turnover of hyperphosphorylated 4E-BP1 which results in increased translation as shown in Figure 1.21 (Gingras *et al.* 1999; Shuda *et al.* 2011). ST also targets the cellular ubiquitin ligase SCF[Fwb7, which stabilises MCP_yV LT and many other cellular oncoproteins, including c-Myc and cyclin E (Shuda *et al.* 2011).

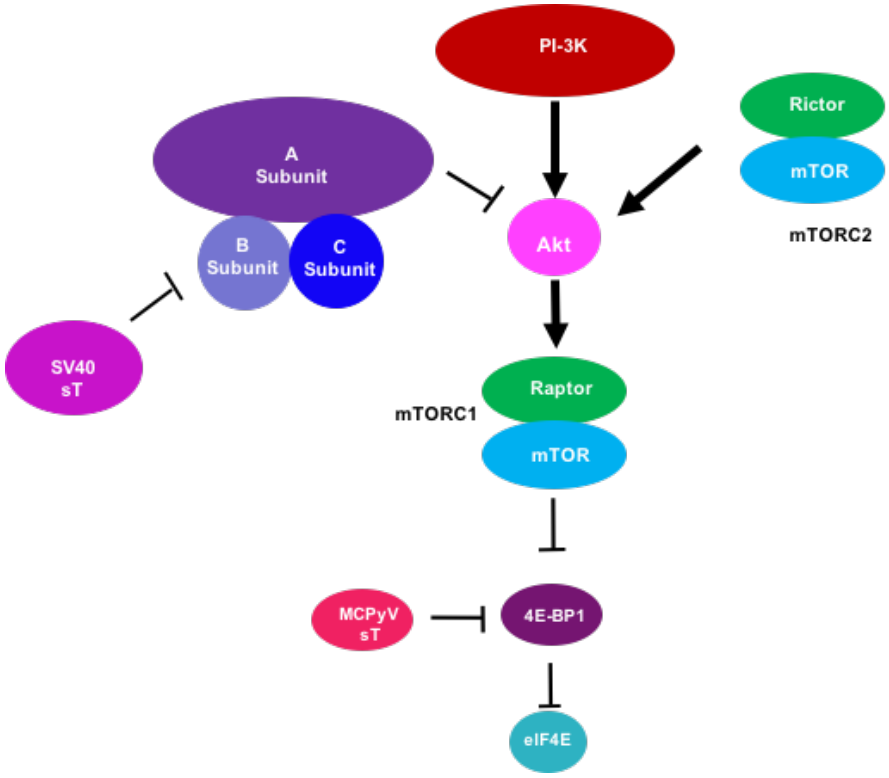

Figure 1. 21: MCPyV ST promotes cell proliferation downstream of the Akt pathway. MCPyV ST targets the translational regulator 4E-BP1, and reduces hyperphosphorylated 4E-BP1 turnover, promoting eIF4E activity and cap-dependent translation. On the other hand, SV40 ST promotes Akt activity by preventing PP2A-mediated Akt dephosphorylation.

1.4.8.2 The role of LT in Merkel cell polyomavirus-induced tumourigenesis

In other oncogenic polyomaviruses, such as SV40, LT has been shown to be the major oncoprotein, with ST playing a lesser role. In SV40, overexpression of LT allows for transformation of mouse fibroblasts. This is due to the inhibition of major tumour suppressor proteins, p53 and Rb (DeCaprio 2009). Like LT from SV40, JCPV and BKPV can also bind to Rb and p53 tumour suppressors, however, MCPyV LT truncated forms in MCC lose the ability to bind p53 (Harris *et al.* 1996; Bollag *et al.* 2000; Poulin *et al.* 2004; Cheng *et al.* 2013). As such, LT alone is not sufficient to induce cell transformation, it however is believed to play a role aiding ST in initiating transformation (Shuda *et al.* 2011). This is supported by the observations that LT and ST proteins are co-expressed in MCC tumour samples.

MCPyV LT expression also increases the levels of the cellular oncoprotein, Survivin. Survivin is a member of the inhibitor-of-apoptosis family of proteins, and it

is elevated in a number of cancers (Arora *et al.* 2012). Survivin mRNA is increased 7-fold in MCPyV-positive MCC tumours, and MCPyV LT expression alone can upregulate survivin, whereas depleting the T antigens decreases both the mRNA and protein levels of survivin, leading to cell death. Survivin may be a viable drug target for MCPyV-positive MCC treatment, as a small molecule inhibitor, YM155 is able to initiate selective MCC cell death (Arora *et al.* 2012).

1.5 Cancer and Metastasis

1.5.1 Metastasis

The dissemination of malignant cells from a primary solid tumour to distant and remote sites within the body is one of the biggest problems facing cancer treatment as it results in over 90% of cancer-associated deaths (Miller *et al.* 2013). Despite the clinical importance of metastasis, the molecular mechanisms by which this phenotype manifests are yet to be fully elucidated (Gueron *et al.* 2011). This poses a significant problem in the analysis of cancer prognosis and treatment of patients. As such, it is an area of cancer biology that is yet to be fully understood and is of major scientific interest.

MCC is regarded as a highly metastatic and aggressive form of skin cancer. Poor patient prognosis has been associated with the quick progression of MCC to distant metastases (Lemos and Nghiem 2007). Novel interactions have been identified that implicate MCPyV in the transformation of Merkel cells, however to date very few studies have addressed the mechanisms by which MCC tumour cells acquire an aggressive metastatic phenotype.

1.5.1.1 The Metastatic cascade

Metastasis is a complex process, with several discrete steps required for the formation of secondary tumour sites: these metastatic hallmarks include loss of cell adhesion, gain of cell motility, dissemination via the vasculature, and colonisation of distant sites [Figure 1.22]. Cell motility is thus a key stage of cancer metastasis, and the metastatic nature of MCC can be potentially linked to the effects of MCPyV ST on enhancing cell motility, first observed by the Whitehouse laboratory (Knight *et al.* 2015).

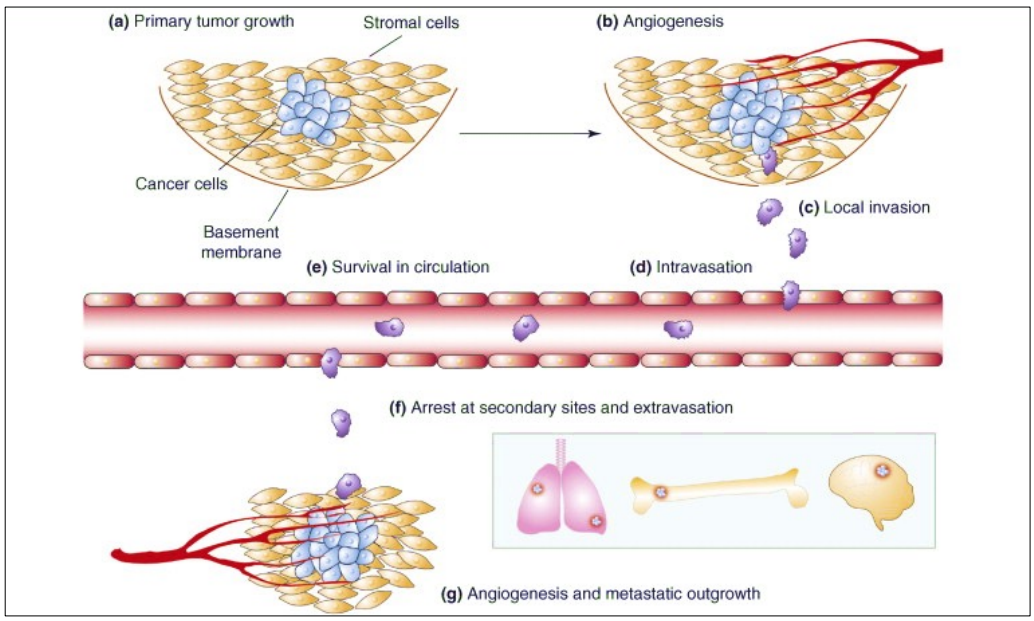

Figure 1. 22: Steps required for escape from the primary tumour site. Upon transformation, some primary carcinoma cells will acquire the capabilities to escape from the primary tumour site into the vasculature. This process involves epithelial-to-mesenchymal transition (EMT) where cells become more motile and express specific integrins and MMPs required to invade local tissue and escape through the basement membrane into blood and/or lymph vessels Taken from (Ma and Weinberg 2008).

1.5.1.1.1 Loss of cell adhesion

Solid tumours are based in the Epithelial tissue as it is composed of cell sheets separated from the basement membrane by stroma. These cell sheets are arranged and organized into highly complex structures that require cell-to-cell and cell-to-Extracellular matrix [ECM] adhesion. Upon initiation of metastasis, cells from the solid tumour needs to detach, which requires the deconstruction of cell adhesion complexes. Unsurprisingly, cancerous cells lose cell adhesive properties (Cavallaro and Christofori 2004). For example, invasive tumours of epithelial origin show the loss of E-cadherin (Beavon 2000). E-cadherin is a member of the tissue-specific cadherin family of transmembrane glycoproteins required to maintain tissue integrity. Cadherins localise to cell junctions called zonula adherens and are responsible for calcium-dependent cell adhesion and signalling (Lewis *et al.* 1994).

A characteristic of many cancers, correlating with increased invasiveness, is a process known as “cadherin switching”, i.e. loss of E-cadherin and increased expression of N-cadherin (Beavon 2000). This process is part of a wider process, known as the epithelial- to-mesenchymal transition (EMT). EMT is characterised by a loss of cell polarity and epithelial proteins such as E-cadherin, occludins, claudins, catenins, and cytokeratin, all of which are responsible for maintaining cell-to-cell

junctions, loss of these proteins result in a gain of a mesenchymal, migratory and invasive phenotype (Yang and Weinberg 2008). These will be discussed in later sections [1.5.3].

Human tumour viruses express various oncoproteins which promote the loss of cell adhesion molecules. For example, HPV E6 expression promotes degradation of epithelial cell tight junctions through an interaction with E6AP, an ubiquitin ligase, which leads to the proteosomal degradation of cell adhesion molecules, in an EMT-like manner (Watson *et al.* 2003). Additionally, the HBV oncoprotein, HBx, downregulates E-cadherin expression, inducing the loss of intracellular adhesion molecules (Lee *et al.* 2005). In addition, SV40 ST promotes, in a PP2A-dependent manner, the redistribution and downregulation of E-cadherin, ZO-1, claudin-1, and occludin, all proteins involved in cell junctions (Nunbhakdi-Craig *et al.* 2003). Notably, MCC tumours also show a pronounced redistribution of E- cadherin from the cell membrane to the nucleus, suggesting MCC possess mesenchymal hallmarks that promote the invasive phenotype of this cancer (Han *et al.* 2000).

1.5.1.1.2 Gain of Cell motility

A critical aspect of tumour cell metastasis is the ability for cells to acquire a migratory phenotype to permit dissemination (Mehlen and Puisieux 2006). Multiple studies have investigated mechanisms to initiate cell motility *in vitro,* using 2D and 3D matrices and advanced *in vitro* imaging techniques (Geiger and Peeper 2009). These motility studies illustrated three broad categories of cell migration: mesenchymal, amoeboid and collective motility. The most characterized of these mechanisms is mesenchymal motility, as 10-40% of carcinomas undergoing EMT use this mechanism (Sahai 2005). Mesenchymal motility is characterised by a polarised, elongated cell body as observed in Figure 1.23 and requires degradation of extracellular matrix components [ECM], generally initiated by activation of receptor tyrosine kinases [RTK], such as c-Met. Together these changes result in directional movement with extended protrusions at the leading edge and retraction at the rear (Sahai 2005).

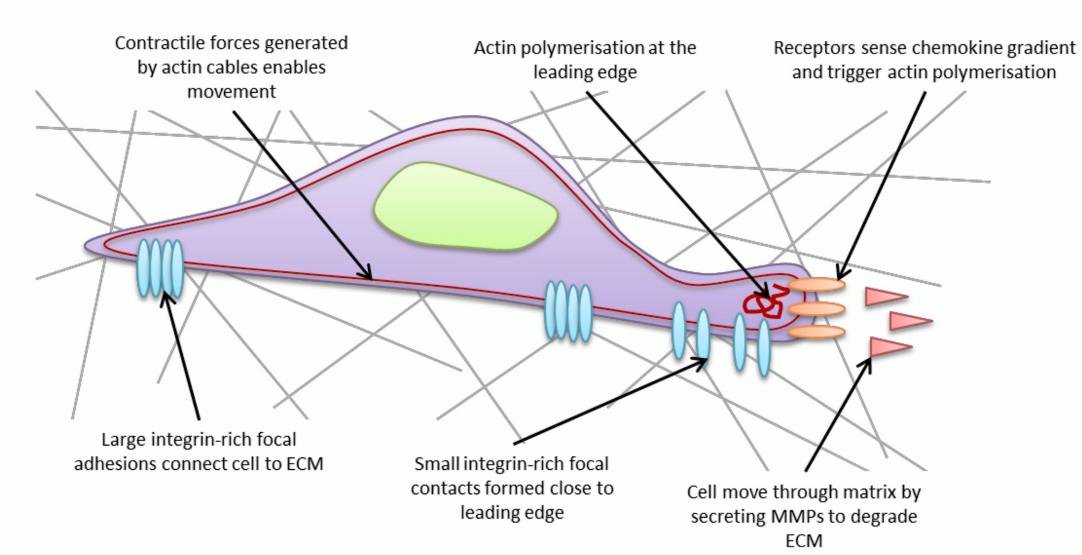

Figure 1. 23: The process of mesenchymal, directional cell motility. Key components of directional, polarised cell motility using the actin cytoskeleton to form protrusions and extracellular matrix degradation to invade local tissue.

Amoeboid motility is similar to leukocyte movement and is generally a feature of malignant cells that do not undergo EMT (Sahai 2005). These receptors on the cell surface sense chemokine gradients, which stimulate the Rho-ROCK signalling pathway. This in turn promotes actin remodelling and cortical actin contraction, allowing the invading cells to 'squeeze' between other cells and change direction rapidly (Geiger and Peeper 2009). In contrast to mesenchymal motility, amoeboid movement does not require degradation of the ECM due to the ability of cells to 'squeeze' through gaps, thus no formation of large actin-based protrusions are observed, characteristic of mesenchymal motility (Sahai 2005).

The final form of motility relies on similar mechanisms to mesenchymal motility and is termed as collective migration, involving whole sheets of cells moving as one. This mechanism has been demonstrated in breast, colon and ovarian carcinomas (Sahai 2005). Collective motility requires the cells at the leading edge of movement to generate proteases that degrade the ECM thereby creating a path for lagging cells to follow (Nabeshima *et al.* 2002). The main difference between mesenchymal and collective forms of motility is that collective motility requires active adhesion complexes, whereas mesenchymal motility requires the loss of these complexes (Friedl and Wolf 2003). This method is less widely understood due to the lack of appropriate *in vitro* techniques capable of modelling this type of movement.

1.5.1.1.3 Epithelial to mesenchymal Transition

Cells vacillate between epithelial and mesenchymal states during embryonic development in a highly dynamic manner (Yang and Weinberg 2008). This shift causes modification of the adhesion molecules expressed by the cell giving the cell the opportunity to adapt to a migratory and invasive phenotype. While initially described as a transformation, this process was ascertained to be reversible due to the plasticity and transition between both states (Nieto *et al.* 2016). The reverse state, not surprisingly referred to as the Mesenchymal-Epithelial transition [MET], is associated with loss of a motile and migratory phenotype and increase in apical to basal cell polarity, as well as cell junction stability. These are known hallmarks of Epithelial tissues (Thiery *et al.* 2009).

1.5.3.1.3.1 Complete EMT

EMT is observed in numerous pathological processes, such as fibrosis and cancer (Lopez-Novoa and Nieto 2009). The majority of signalling pathways and transcription factors used in physiological EMT processes are activated in pathological EMT, such as tumour invasion and its metastatic dissemination (Strizzi *et al.* 2009). The physiological process of EMT presents a sequence of specialized events which occur in succession. This multi-stage process of an EMT includes gradual alteration of epithelial cell architecture and functionality. Here cells lose their epithelial cell–cell junctions and apical-basal cell polarity (Nieto *et al.* 2016).

Next, the underlying basement membrane begins to degrade due to the upregulation of Matrix metalloproteinases (Kaufmann *et al.* 2000). Cell surface proteins like E-cadherin and integrins mediate the connections between neighbouring epithelial cells and with the basement membrane. With their degradation, E-cadherin is replaced with N-cadherin and other groups of integrins. This may aid transitioning of an invasive cell phenotype by mediating contact inhibition of locomotion and also providing the cell with transitory adhesion essential for the mesenchymal phenotype (Stramer and Mayor 2017).

Finally, independent of their neighbours, transitioning cells develop invasive properties and the ability to move across the extracellular matrix. It has been suggested that it is during this process that epithelial cell acquire anoikis resistance. This is due to resulting loss of contact with the basement membrane and the ability to respond to extracellular signals that accurately direct their migration until they

arrive at their distant site (Frisch *et al.* 2013). Once arriving at a distant site, Mesenchymal cells perform the inverse process [MET] in other to colonize their current location (Frisch *et al.* 2013).

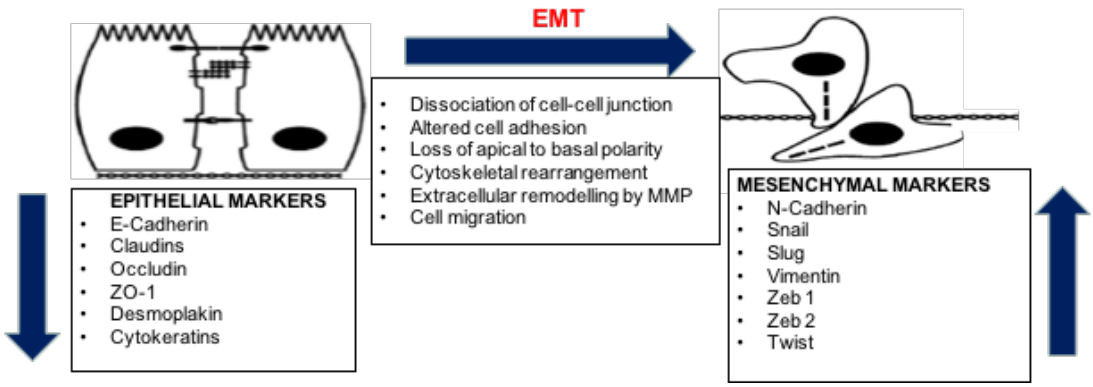

Figure 1. 24: Epithelial mesenchymal transition. Progressive loss of epithelial markers and gain of mesenchymal markers

In cancer development, EMT characteristics have been observed in neoplasms present in breast, ovary, colon or esophagus tumours to name but a few (Larue and Bellacosa 2005). During oncogenic EMT, processes observed include loss of apicobasal polarity, the destabilization of tight junctions and Adherens junctions, reorganization in the cytoskeleton including upregulation of vimentin synthesis and acquisition of a migratory and invasive phenotype [Figure 1.24]. These changes are similar to those observed during physiological EMT process that occurs during development (Geiger and Peeper 2009; Nieto *et al.* 2016). Additionally, multiple initiators of EMT in neoplastic cell lines have been identified, including but not limited to transforming growth factor-β [TGF-β], Wnt, Snail, Slug, Twist, Zeb1 and Zeb2. These initiators are also essential during physiological EMT (Peinado *et al.* 2004; Mercado-Pimentel and Runyan 2007; Peinado *et al.* 2007; Moreno-Bueno *et al.* 2009; Diepenbruck and Christofori 2016)

1.5.3.1.3.1 EMT and human tumour viruses

Known human tumour viruses such as Epstein-Barr virus [EBV], Hepatitis B [HBV], Hepatitis C [HCV], Human papillomaviruses [HPV], and Merkel cell polyomavirus [MCPyV] are defined as etiological agents in various cancers (Moore and Chang 2010; Chen *et al.* 2016). Some of these human tumour viruses have also been

found to play important roles in cancer metastasis, specifically in driving the EMT process. For example, EBV latent membrane protein-1 and -2A, EBV nuclear antigen have both been shown to induce EMT (Chen *et al.* 2016). Studies have shown that LMP1 can downregulate E-cadherin expression by inducing the formation of a transcriptional repression complex, comprised of DNA methyltransferase [DNMT-1] and histone deacetylase. This complex then locates onto the gene promoter of E-cadherin (Tsai *et al.* 2006). LMP1 can also activate cadherin switching of E-cadherin to N-cadherin as well as promote matrix metalloproteinase 9 expression (Shair *et al.* 2009). Additionally, LMP1 regulates EMT transcription factors TWIST and SNAIL although the exact mechanism is yet to be elucidated (Chen *et al.* 2016).

Notably, HPV associated oncoproteins have also been shown to induce EMT. HPV16 E6/E7 has been shown to induce Jagged1, a gene upregulated in human cervical tumours. Jagged1 upregulation correlates with the rapid induction of phosphatidylinositol 3-kinase (PI3-K)-mediated EMT (Veeraraghavalu *et al.* 2005). HPV encoded oncoproteins E6 and E7 induce fibroblast growth factor [FGF] 2 and 4-induced EMT and cervical tumorigenesis (Chen *et al.* 2016). HPV16 E6/E7 expression also been proven to inhibit E-cadherin expression in cervical cancer cells causing FGF ligand stimulation which in turn increases invasive ability (Cheng *et al.* 2012).

HBV encoded X protein [HBx] expression has been linked to decreased E-cadherin expression as well as upregulation of mesenchymal markers N-cadherin and vimentin (Chen *et al.* 2016). Additionally, non-structural HCV protein 5A have been proven to be involved in the regulation of EMT (Chen *et al.* 2016). NS5A induces phosphorylation of Akt and GSK-3β along with binding to integrin αVβ3 and CD44 at the cell surface focal adhesion kinase This initates β-catenin activation, leading to EMT of hepatocytes, inducing downregulation of E-cadherin as well as upregulation of N-cadherin in HCV-infected cells (Iqbal *et al.* 2014).

1.5.1.1.4 Dissemination via the vasculature

The basement membrane is a barrier for invasive tumours of epithelial origin, which must be passed through to access the vasculature. The basement membrane is important for epithelial structure and integrity, and consists of a dense network of proteoglycans and glycoproteins, including laminin and collagen. The basement membrane can be broken down by extracellular matrix proteases, which are normally under strict regulatory control by their localisation, inhibitors and auto-inhibition (Gupta and Massague 2006). Therefore, malignant cells that use the mesenchymal mode of migration need to activate these proteases to degrade the ECM, which can further lead to the production of bioactive peptides able to mediate migration, angiogenesis, and tumour survival (Sahai 2005). One subset of matrix proteases are the calcium-activated, zinc-containing matrix metalloproteinases (MMPs) (Verma and Hansch 2007).

DNA tumour viruses can promote matrix degradation and tumour invasion. For instance, HPV E7 and HBV HBx induce the expression of matrix transmembrane metalloproteinase 1 (MT1-MMP), which is crucial for the degradation of the ECM and activation of additional matrix metalloproteinases 2 and 9 (MMP2 and MMP9) (Zhu *et al.* 2015). MT1-MMP expression also promotes tumour growth and angiogenesis by upregulating expression of vascular endothelial growth factor [VEGF], which correlates with invasiveness (Sounni *et al.* 2002). Interestingly, induction of MMP gene expression by the WNT/ β-catenin signalling pathway and other growth factors stimulates MCPyV infection. These findings further highlight the role of MCC risk factors, such as UV radiation and aging, which are known to stimulate WNT signalling and MMP expression, and this may promote viral infection and pathogenesis (Liu *et al.* 2016).

1.5.1.1.5 Colonisation and secondary tumour growth

In order for tumour cells to access remote tissues and organs, they must access the vasculature. Angiogenesis is the process of growing new blood vessels, and is an important step in cancer malignancy. Angiogenesis prevents hypoxia and necrosis of the growing tumour, by providing nutrients as well as removing waste products from the hyperproliferating cancer cells (Sahai 2005). VEGF is a key factor in angiogenesis, and some virus oncoproteins, e.g. EBV LMP1, can induce hypoxia-inducible factor 1 alpha (HIF-1α) expression, which in turn promotes the expression of VEGF (Wakisaka *et al.* 2004).

Entry into the vasculature is known as intravasation, and is initiated by tumour cells orienting towards the vasculature and migrating towards it (Sahai 2005).The vast majority of tumour cells are either trapped in the capillary bed or rapidly die in the circulatory system. Only a minority of tumour cells that enter the circulation can exit the vasculature and establish metastases (Sahai 2005). Exit from the vasculature is termed, extravasation and is largely dependent on the principles involved in intravasation. Studies have shown that membrane type-4 matrix metalloproteinase [MT4-MMP or MMP17], which is highly expressed on breast cancer cells enable lung metastasis by disrupting vessel integrity surrounding the primary tumour. This in turn facilitates cancer cell intravasation and cell migration (Sohail *et al.* 2011). Another metalloproteinase, ADAM 12, also promotes intravasation however through an alternate mechanism, possibly by disrupting the endothelial junction (Sonoshita *et al.* 2011).

Although intravasation and extravasation of cancer cells both require the deregulation of endothelial junctions for cancer cells to cross the endothelium, known as transendothelial migration [TEM], these mechanisms are essentially different as cancer cells approach the endothelium from alternate sides (Reymond *et al.* 2013). Various integrins on the cell surface of cancer cells have been implicated to play a role in extravasation. Specifically αVβ3 and β1 have been shown to facilitate attachment of cancer cells to the endothelium and transmigration across the endothelium and attachment to the stromal matrix around the blood vessels (Desgrosellier and Cheresh 2010).

Establishment of tumour cells in new sites and eventual secondary tumour growth is not necessarily a random process. For example, breast, lung, and prostate cancers preferentially metastasise to bone tissue (Mundy 2002). This suggests that the organ must be compatible with the metastasising cells, known as the seed and soil hypothesis.

1.5.2 Cell adhesion

1.5.2.1 Cell Junctions

Cell junctions are sites of intercellular adhesion that maintain the structural integrity of epithelial tissue and mediate signalling between cells. In epithelial tissues, cell-to-cell interaction is facilitated by numerous junctional complexes. These complexes are tight junctions (TJs), Adherens junctions (AJs), desmosomes and gap junctions [Figure 1.25], each have a characteristic composition, morphology and function (Dejana 2004).

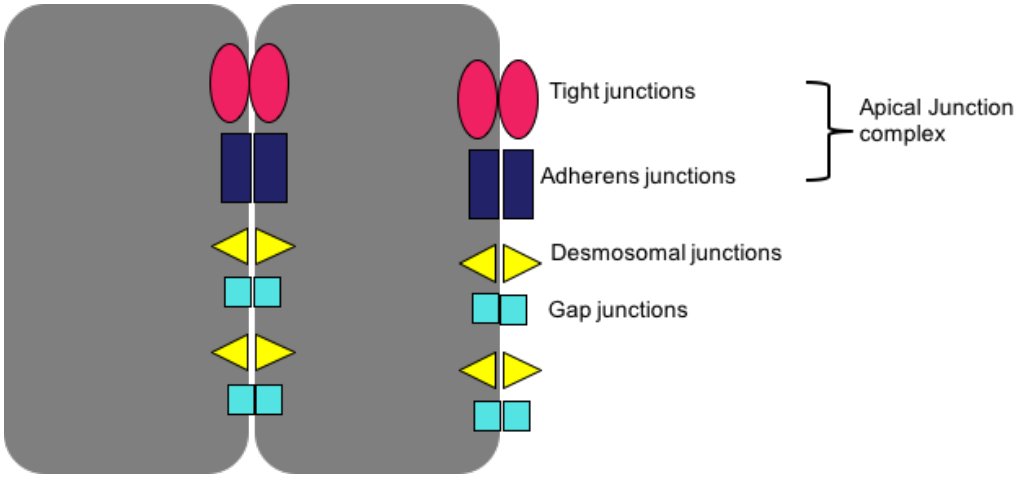

Figure 1. 25: Intercellular junctions form the epithelial barrier. Basic schematic of intercellular junctions including tight junctions, Adherens junctions, desmosomal junctions and gap junctions. Adapted from (Wroblewski and Peek 2011).

1.5.2.1.1 Desmosomes

Desmosomes are a group of anchoring junctions responsible for establishing an intercellular adhesive structure between the cytoskeleton and plasma membrane (Garrod and Chidgey 2008). Desmosomes provide mechanical integrity between cells by creating an anchorage point between intermediate filaments of the cytoskeleton by means of a complex of proteins in the cytoplasmic and extracellular regions of the junction (Yin and Green 2004). Desmogleins (DSGs) and desmocollins (DSCs) are transmembrane proteins that link adjacent cells and are embedded in the cytoplasmic plaques [Figure 1.26]. These proteins have been shown to form the dense midline seen in mature desmosomes. A major role of desmosomes is the anchoring of cytoskeletal keratin intermediates to the cell

membrane (Yin and Green 2004; Garrod and Chidgey 2008; Brooke *et al.* 2012). It also has been suggested that desmosomal genes are regulated by a transcriptional program directed by transcription factors prominent in the EMT process, such as Snail, Slug and Twist (Peinado *et al.* 2007; Chun and Hanahan 2010).

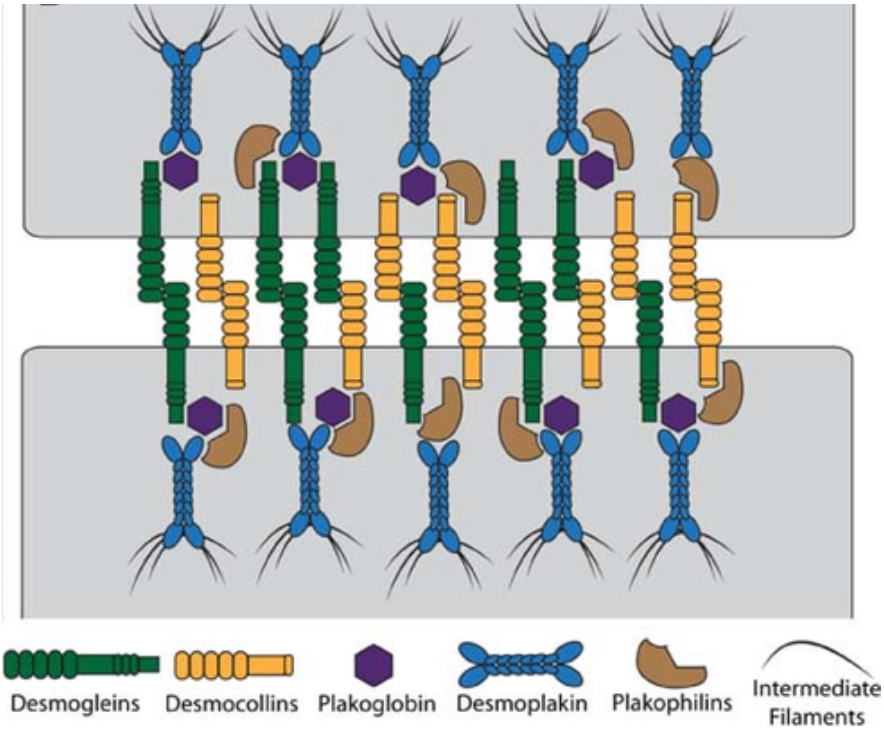

Figure 1. 26: Schematic of Desmosome junction. Desmogleins [DSGs] and desmocollins [DSCs] compose the extracellular link between cells. They form homo- and heterodimers, whose intracellular tails bind the proteins plakoglobin [PG] and the plakophilins [PKPs]. These tether cadherins to the plakin protein desmoplakin [DSP], which in turn connects desmosomes to the intermediate filament network. Taken from (Brooke *et al.* 2012).

1.5.2.1.2 Gap Junctions

Gap junctions are specialized gated intercellular channels found between neighbouring cell plasma membranes. When open, gap junction channels allow metabolite exchange between cells. Each channel is composed of two connexons, one from each communicating cell as seen in Figure 1.27 (Czyz 2008). These connexons float laterally within the plasma membrane until a linkage occurs with a complementary proximal connexon in an adjacent cell. When the linkage occurs, the membranes of the cells are joined at that locus to create a linking channel between the two cells (Defamie *et al.* 2014). These conjoined cells have their metabolic systems partly open to each other and also provide a channel of cell-to-cell communication. This channel is large enough to allow small molecules like

inorganic ions, and other small water soluble molecules <1000 kDa to travel between the cells. However, the channel is not large enough for proteins, nucleic acids or sugars to pass through (Defamie *et al.* 2014). Interestingly, reduction in functional gap junctions has been associated with tumourigenesis and can be linked to the increased growth rate of tumour cells *in vivo* (Eghbali *et al.* 1991).

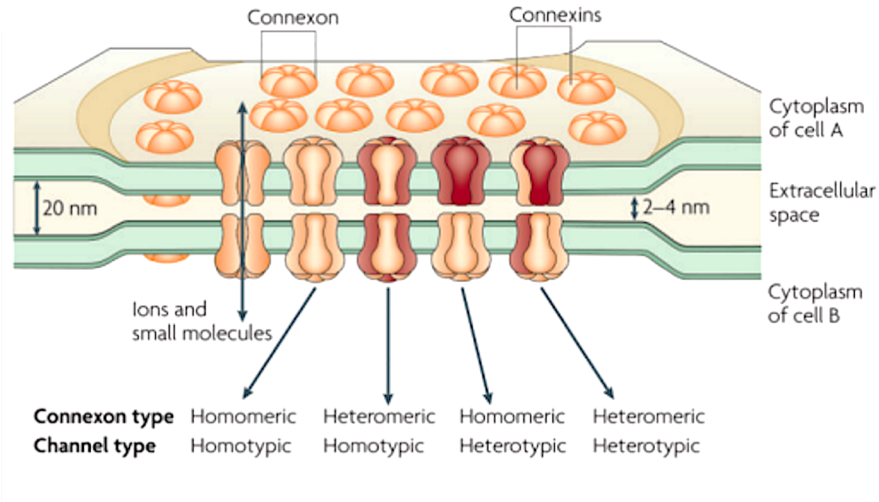

Figure 1. 27: Structure and molecular organization of gap junctions. Gap junctions are formed between the opposing membranes of neighbouring cells. Hemichannels on each side anchor to one another to form conductive channels between the two cells. Each hemichannel or connexon, is comprised of 6 connexin protein subunits that are oriented perpendicular to the cells' membranes to form a central pore. Taken from (Bloomfield and Volgyi 2009).

1.5.2.1.3 Adherens Junction [Zonula adherens]

Adherens junctions are a characteristic feature of all epithelial sheets (Fristrom 1988). Adherens junctions have multifunctional roles, including but not limited to initiation and stabilization of cell-to-cell adhesion, regulation of the actin cytoskeleton as well as transcriptional regulation (Meng and Takeichi 2009). The structural proteins associated with Adherens junction include interacting transmembrane glycoproteins of the classical cadherin superfamily like E-cadherin, and members of the catenin family like p120-catenin, β-catenin and α-E-catenin [Figure 1.28]. The proteins regulate the formation, preservation and function of Adherens junctions (Meng and Takeichi 2009).

Junctional E-cadherin–catenin complexes are essential for precise functioning of the epithelia. Homophilic interactions between the extracellular portions of E-

cadherin molecules provide mechanically strong adhesive connections between cells in the tissue. Adherens junctions and tight junctions comprise two modes of cell-to-cell adhesion that provide different but complimentary functions. Adherens junctions contribute to the definition of an epithelial cell apical–basal axis and function in collaboration with tight junctions to coordinate epithelial apical to basal cell polarity (Desai *et al.* 2009; Yadav *et al.* 2009).

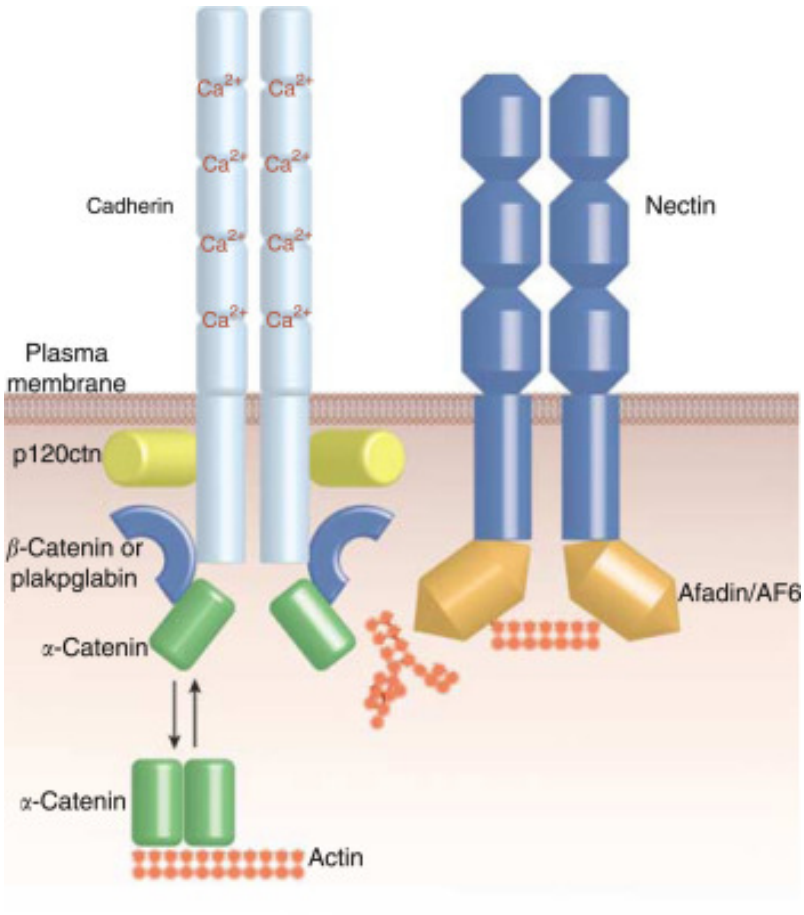

Figure 1. 26 Representation of structural components of the Adherens junction. Interaction between Catenin and Cadherins [Cadherin-Catenin complex] and possible interacting with Actin. Taken from (Niessen 2007).

α-E-catenin which is localized at the Adherens junction also bind cadherins to actin filaments to enable strong cell-to-cell adhesion (Baum and Georgiou 2011). It has also been surmised that ZO-1, which is known to bind α-E-catenin (Muller *et al.* 2005), functions as a scaffolding protein between transmembrane and cytoplasmic proteins, and possibly forms a link between the Adherens and Tight junctions (Hartsock and Nelson 2008). Notably, downregulation of E-cadherin and A-E-

Catenin have been linked to tumour progression in multiple cancers (Rimm *et al.* 1995; Wijnhoven *et al.* 2000; Bajpai *et al.* 2009).

1.5.2.1.4 Tight Junction [Zonula occludens]

Adherens junctions and tight junctions comprise two modes of cell-to-cell adhesion that provide different but complimentary functions. Tight junctions [TJ] are identified as electron microscopically dense protein particles enclosing individual cells and thus sealing the paracellular cleft between two adjacent cells (Anderson *et al.* 1993). TJs are composed of a multi-protein complex of up to 30 transmembrane proteins, as well as cytosolic scaffolding proteins. These proteins comprise transmembrane proteins such as claudins, members of the protein family of Tight junction associated MARVEL [MAL and related proteins] proteins [TAMPs] and also cytosolic membrane-associated guanylate kinases [MAGUKs] (Balda *et al.* 1993). Importantly, tight junctions are located at the most apical end of the junctional complex, forming a physical barrier to free passage of molecules through the paracellular pathway. This barrier prevents systemic contamination by microbes and toxins present in the immediate microenvironment (Tsukita *et al.* 2001).

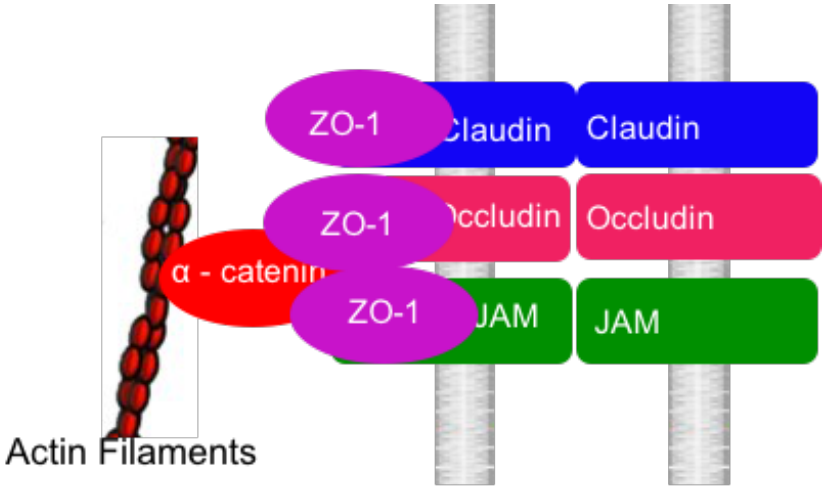

Figure 1. 27: Schematic of tight junction complex. Extracellular domains of claudin, occludin, or junctional adhesion molecule (JAM) interact with their counterparts in a homophilic interaction. Their intracellular domains interact with ZO-1. α-catenin complexes links tight junction with cytoskeleton.

Electron microscopy approaches have confirmed the existence of structures typical of TJ kissing points in the *stratum granulosum* of human epidermis. Results showed

expression of Occludin and ZO-1 in human epidermis and cultured human keratinocytes as seen in Figure 1.29 (Brandner *et al.* 2002). Interestingly, diminished expression of ZO-1 has also been demonstrated to correlate with increased invasiveness in multiple cancers including breast, colorectal, and digestive tract cancers (Wu *et al.* 2017).

1.5.2.2 Sheddases

Sheddases are membrane-bound enzymes that cleave membrane proteins at the cell surface allowing for release of soluble ectodomains causing alteration in their localisation and function. Cellular sheddases belong to either the metalloprotease family [ADAM and MMP] or aspartic protease [BACE] families (Lichtenthaler *et al.* 2011).

1.5.2.2.1 ADAM Proteins

A Disintegrin and metalloproteases [ADAM] proteins, are membrane-anchored glycoproteins and regulatory enzymes that have been associated with cell adhesion and shedding of membrane-bound proteins to soluble forms. ADAM proteins belong to the metzincin zinc-dependent metalloprotease superfamily typified by containing several conserved domains including a prodomain, metalloprotease domain, disintegrin domain, cysteine-rich domain, epidermal growth factor (EGF)-like domain, a transmembrane domain, and a cytoplasmic domain [Figure 1.30] (Wolfsberg *et al.* 1995; Moss and Lambert 2002; Edwards *et al.* 2008). Studies have linked ADAM proteins with various biological functions including, but are not limited to, neurogenesis and angiogenesis. They have also been associated with multiple diseases including cancer (Rocks *et al.* 2008).

Figure 1. 30: ADAM protein. Basic Schematic of ADAM protein including multiple domains – Prodomain, Metalloprotease domain, Disintegrin domain, Cysteine rich region and cytoplasmic tail.

The Protease domain of ADAM proteins is catalytically inactive until this is modified by autocatalysis or removed by a furin-type pro-protein convertase. Notably, a cysteine switch in the protease domain keeps the protease inactive. In contrast, some ADAM proteins contain a HEXXH sequence which have protease activity (Edwards *et al.* 2008). As shown in Figure 1.31, only approximately half of known ADAM proteins contain a catalytic-Zn binding signature for metalloproteases [HExGHxxGxxHD] in their metalloprotease domain and can be catalytically active (Giebeler and Zigrino 2016). The disintegrin domain is located downstream of the protease domain. It is highly conserved, has approximately 90 amino acids and found in all ADAM proteins (Eto *et al.* 2000; Edwards *et al.* 2008).

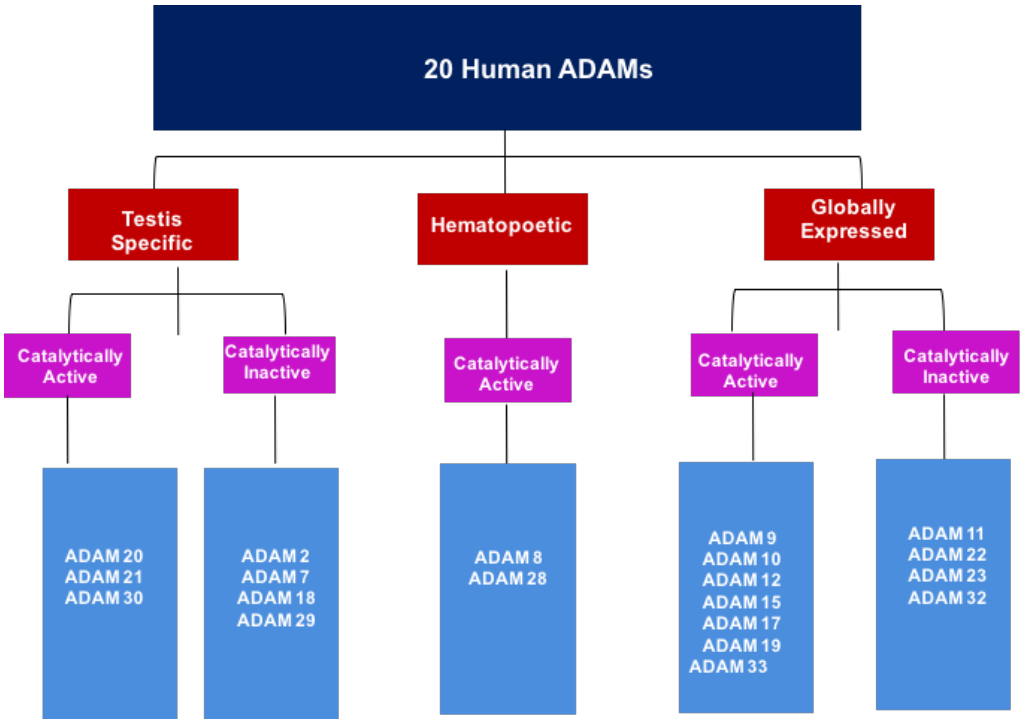

Figure 1. 31: ADAM protein family. The human ADAM proteins grouped by site of expression and catalytic activity.

1.5.2.2.2 ADAM protein sheddase function

ADAM proteins shed the extracellular domains of membrane-bound growth factors, cytokines and their receptors. Some receptors, such as ErbB receptors, play an essential role in cell signalling and may function as a potential target in cancer therapy. ErbB receptors are members of a subclass I of the superfamily of receptor kinases. ErbB receptors are also known as Epidermal Growth Factor (EGF) family of receptor tyrosine kinases (RTKs) have 4 family members; EGFR/ErbB1/HER1,

HER2/ErbB2, HER3/ErbB3, and HER4/ErbB4. Under normal physiological conditions, the ErbB receptors play crucial roles regulating cell growth, cell migration, cell survival, cell migration and invasion.

The ErbB proteins also play a role human tumourigenesis (Kansas 1996). For example, EGFR and HER2 has been linked to the formation and development of multiple cancer types (Keelan *et al.* 1994). Some ligands of the HER family include amphiregulin, β-cellulin, epiregulin, epigen, EGF and TGF (Clark *et al.* 2008). Knockout studies using mouse embryonic cells have demonstrated that ADAM 17 is the major sheddase for amphiregulin, epiregulin and TGF-α. ADAM 10 in contrast is primarily responsible for cleavage of EGF and β-cellulin (Khatib *et al.* 1999). However, as ADAM 10 and ADAM 17 do show functional redundancy, as over expression of both proteins induce EGF release.

It has also been shown that TNF-α is shed by ADAM 17 activity leading to this particular sheddase to be known as TNF-α converting enzyme [TACE]. TNF-α is a pluripotent peptide that is involved in various activities that are important in tumour formation and development. It plays a vital role in cancer initiation and progression due to its ability to induce angiogenic factors and Matrix mellatoproteinase expression, leading to the enhancement of cell migration (Burdick *et al.* 2003). In a breast cancer study, it was shown that overexpression of ADAM 17 increased invasion and proliferation *in vitro*. Whereas, downregulation of ADAM 17 decreased both invasion and proliferation (Sahin *et al.* 2004).

ADAM 10-mediated shedding also plays a role in increasing cell proliferation and migration. Studies have shown that ADAM 10 caused the shedding of E-cadherin, causing β-catenin to translocate to the nucleus and increase proliferation. Shed E-cadherin forms a E-cadherin-HER2-HER3 complex which in turn enhances ERK signalling, increasing proliferation and migration (Gout *et al.* 2006).

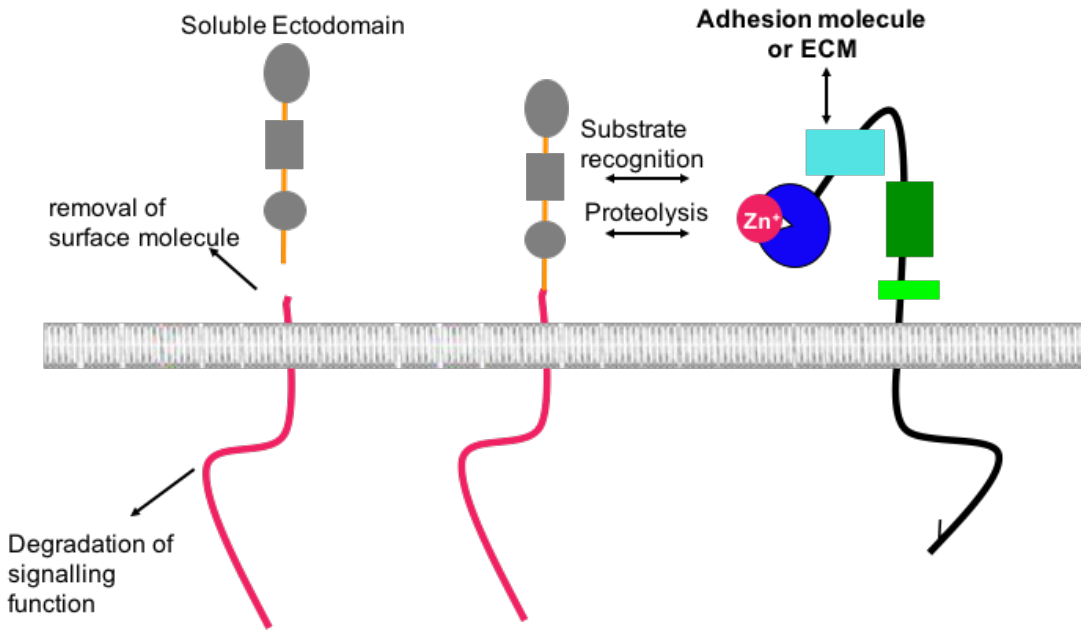

Figure 1.32: The roles of ADAM-mediated sheddase activity. schematic of ADAM protein dual function. The adhesive properties of the disintegrin domain along with the catalytic function of the metalloproteinase domain. Modified from (Dreymueller *et al.* 2015).

Sheddase activity has also been implicated in the Notch signalling pathway. This pathway is an evolutionarily conserved pathway important in regulating cell proliferation, differentiation and death (Murphy 2008). Notch ligands are integral membrane proteins that interact with Notch receptors on adjacent cells. Studies have shown that ADAM 10 alone can cleave the Notch receptor near the membrane. Subsequent to this cleavage event, γ-secretase cleaves the receptor at an intramembrane site. This ADAM 10-mediated cleavage causes the release of the intracellular domain into the cytosol, which then translocates to the nucleus where it functions as a transcription factor (Murphy 2008; Kopan and Ilagan 2009). ADAM10 is essential to the initiation of Notch signalling and is required for stem cell maintenance, as well as normal organismal growth and development. Additionally, ADAM17 has also been shown to be capable of cleaving the Notch receptors (Brou *et al.* 2000).

Importantly, ADAM proteins also function as regulatory enzymes implicated in cell adhesion. ADAM 10 and 17 are upregulated in activated endothelium and studies have shown that they play a role in ectodomain shedding of adhesion molecules during leukocyte recruitment (Boulday *et al.* 2001). Also, CX3CL1 and CXCL16, chemokines which act as adhesion molecules are cleaved by ADAM 10 and 17, yielding a soluble form (sCXCL16). For example, CXCL16 is normally expressed

on macrophages, dendritic cells, monocytes, and B cells and serves as an adhesion molecule for neighbouring cells expressing CXCR6 (Gooden *et al.* 2014). When cleaved by ADAM proteins, CXCL16 yields a soluble form (sCXCL16). sCXCL16 has been detected in the serum of various types of cancer patients (Gooden *et al.* 2014).

There may be a functional role for CXCL16/CXCR6 in cancer however it is yet to be elucidated. Although, they have been reported to have pro-metastatic as well as anti-tumorigenic functions. For example in prostate cancer, high expression of CXCL16 and CXCR6 as well as high serum sCXCL16 levels, were associated with a more aggressive tumour (Lu *et al.* 2008). Furthermore, high serum levels of sCXCL16 in colorectal cancer patients were associated with recurrent liver metastasis and poor prognosis (Matsushita *et al.* 2012). Although, a divergent prognosis has been observed in a murine model of colorectal cancer, whereas expression of CXCL16 appears to inhibit formation of liver metastasis (Kee *et al.* 2013).

CD44, an adhesion molecule associated with inflammatory cells, provides cross-links to endothelial cell hyaluronan, inducing intracellular signalling cascades. This leads to the activation of ADAM 10 and ADAM 17 (Nagano and Saya 2004). Consequently, ADAM protein-induced release of soluble ectodomain fragments from the adhesion molecules such as L-selectin, CXCL16 and CD44, contribute to leukocyte de-adhesion and rolling on activated endothelial cells and their migration to the inter-endothelial junction (Nagano and Saya 2004; Ponnuchamy and Khalil 2008).

1.6 Thesis aims

MCPyV ST antigen has been implicated to play a role cell motility and migration, a complex and multi-step process, which is essential to metastasis. Specifically, when investigating the role of MCPyV ST in the highly metastatic nature of MCC, the Whitehouse laboratory has shown that MCPyV ST affects the microtubule network (Knight *et al.* 2015) and also the actin cytoskeleton to induce filopodia formation (Stakaityte *et al.* 2018), which promotes cell motility.

The aim of this thesis was to further elucidate the role of MCPyV ST in other components of the metastatic pathway including disruption of cell junction stability and also induction of EMT.

Chapter 3 arose from data observed in the a SILAC -based quantitative proteomics dataset. Dataset indicated a down-regulation of cell adhesion-related proteins upon MCPyV ST expression. Following this, the structural integrity of the Adherens and Tight junctions were evaluated. Additionally, possible targets responsible for the disruption of these cell junctions were considered by reviewing members of the ADAM family.

Chapter 4 further explored the role of ADAM 10 and ADAM 17 in cell junction breakdown and increased cell dissociation. Further showing an ADAM 10 dependent disruption of the Adherens and Tight junction. It was established that ADAM 10 is important to MCPyV ST-induced cell dissociation and cell motility using inhibitory peptides and siRNA-mediated depletion studies. Results show that inhibition of ADAM 10 allows for recovery of cell junction stability, reduced cell scatter and cell motility in MCPyV ST-expressing cells.

Chapter 5 then investigates MCPyV ST role in inducing an EMT. Previous studies have inferred that certain markers associated with EMT are enriched upon MCPyV ST expression (Berrios *et al.* 2016). Hallmarks of EMT where assessed in various cell lines and MCPyV-positive MCC tumours. Results, show a downregulation of epithelial markers and upregulation of Mesenchymal markers.

CHAPTER 2
MATERIAL AND METHODS

2.1 Materials

2.1.1 Tissue samples.

MCV tumour sections

Two samples of fresh MCC tumour were supplied by Professor Julia Newton-Bishop at St James' Hospital, Leeds. Upon immediate removal from two patients undergoing surgery, these samples were frozen on dry ice and stored at -80 °C.

Skin Samples

Healthy skin samples were supplied by Professor Alan Melcher. Upon immediate removal from patients undergoing surgery, these samples were frozen on dry ice and stored at -80 °C.

2.1.2 Chemicals

All analytical grade chemicals and solvents were provided by Sigma-Aldrich®, Melford Laboratories Ltd., and Life Technologies™, unless stated otherwise. Solutions were sterilised using 0.22 µm filters (Millex), or by autoclaving (121 C, 30 min, 15 psi). All water used throughout, unless mentioned otherwise, was deionised water sterilised through an ELGA PURELAB ultra machine (ELGA).

2.1.3 Enyzmes

Restriction enzymes were supplied by Invitrogen™, New England BioLabs Inc. or Fermentas™. Other enzymes and their suppliers are listed in table 2.1.

Table 2.1: List of enzymes and their suppliers

Enyzme	Supplier
Platinum R *Pfx* DNA polymerase	Life Technologies™
Superscript R II reverse transcriptase	Life Technologies™
Proteinase K	Life Technologies™
RNase Out	Life Technologies™
DNA-freeTM DNase I treatment kit	Ambion™
2x SensiMixTM SYBR No-ROX Kit	Bioline Reagents Ltd

2.1.4 Antibodies

Primary antibodies were provided by a range of suppliers, detailed in table 2.2. Horseradish peroxidase [HRP] conjugated anti-mouse and anti-rabbit secondary IgG were supplied by Dako and used for western blotting Alexa-fluor conjugated anti-mouse and anti-rabbit Immunoglobulin G [IgG] antibodies were supplied by Life Technologies™ and used for immunofluorescence microscopy at a dilution of 1:500. Additionally, a 2T2 monoclonal antibody which identifies a common leader peptide present on both ST and LT was utilized as a MCPyV T antigen antibody.

Table 2. 2: Primary antibodies, their origins, their working dilutions, and their suppliers

Antibody (supplier catalogue number)	Origin		Working dilution			Supplier
		WB	IF	IHC	FACS	
Anti-FLAG (F7425)	Rabbit	1:1000	1:250	-	-	Sigma
Anti-GAPDH (ab8245)	Mouse	1:5,000	-	-		Abcam
Anti-ADAM10 (ab1997)	Rabbit	1:500	1:250	1:100	1:250	
Anti-ADAM17 (ab2051)	Rabbit	1:750	1:250	1:100	1:250	
Anti-Cytokeratin20 (ab76126)	Rabbit	-	-	1:50	-	
E-Cadherin (24E10)	Mouse	1:250	-	-	1:250	Cell Signaling
N-Cadherin (D4R1H)	Rabbit	-	-	-	1:250	
Claudin-1(D5H1D)	Rabbit	1:1000	-	-	-	
β-Catenin (D10A8)	Rabbit	1:1000	-	-	-	
ZO-1 (D7D12)	Rabbit	1:1000	-	-	1:500	
Snail (C15D3)	Rabbit	1:500	-	-	-	
A-E-Catenin Antibody (#3236)	Rabbit	1:1000	1:250	-	1:250	
CD71 (D7G9X)	Rabbit	1:1000	-	-	1:200	
CMB24	Mouse	-	-	1:50	-	Santa Cruz
anti-TGN46 (Golgi) antibody	Sheep	-	1:100	-	-	Dr Vas Ponnambalam

2.1.5 Mammalian cell culture reagents

All media used was purchased from Lonza. Culture supplements, selection antibiotics and Lipofectamine™ 2000 were provided by Invitrogen™.

2.1.6 Oligonucleotides

Oligonucleotide primers for DNA sequencing and polymerase chain reaction (PCR) were supplied by Sigma-Aldrich®. A full list of primers used is shown in Table 2.3 (Note that primers used for PCR arrays were supplied by SA Biosciences as part of the RT^2 Profiler PCR Array System). In order to perform reverse transcription, $Oligo(dT)_{12-18}$ was obtained from Promega.

Table 2. 3: List of primers used and their applications

Gene name	Use	Sequence (5'-3')
ADAM 10	qRT-PCR	F: TTACGGAACACGAGAAGCTGT R: GGGTCCTTCTCATCAGCAGTT
ADAM 17	qRT-PCR	F: GAGCCGGCCTTTGGTAAC R: CCTAGCCCCTCAATCCTCTTT
ZO-1	qRT-PCR	F: CATGAAGATGGGATTTCTTCG R: GCCAGCTACAAATATTCCAACA
E-CADHERIN	qRT-PCR	F: CCCATCAGCTGCCCAGAAAATGAA R: CTGTCACCTTCAGCCATCCTGTTT
OCCLUDIN	qRT-PCR	F: TGCATGTTCGACCAATGC R: TGCATGTTCGACCAATGC
LGL2	qRT-PCR	F: TTTAACAAGACGGTGGAGCA R:GAGCTTGATGGCTCCAGAAAC
CRB3	qRT-PCR	F: GGCGTTTGGCTTGCCGAT R: CACTGCTGGGCCGGTAG
SCRIBBLE	qRT-PCR	F: AGCTGCCCAAGCCTTTTTTC R: AACCGCTGGATCTCGTTGTC
Snail	qRT-PCR	F: CCAGGAGTGGCCTAACCAG R: GCCAGAGTCAGCCTTAAGAGG
Slug	qRT-PCR	F: CTCACCTCGGGAGCATACAG R: GACTTACACGCCCCAAGGATG
ZEB1	qRT-PCR	F: GCACCTGAAGAGGACCAGAG R: TGCATCTGGTGTTCCATTTT
ZEB2	qRT-PCR	F: TTTCAGGGAGAATTGCTTGA R: CACATGCATACATGCCACTC
PAK1	qRT-PCR	F: AGTTTCAGAAGATGAGGATGATGA R: AATCACAGACCGTGTGTATACAG
MMP3	qRT-PCR	F:AATGCCATCCCCGATAACCT R:AGCCTAGCCAGTCGGATTTGAT
MMP9	qRT-PCR	F: CTTTGAGTCCGGTGGACGA R: TCGCCAGTACTTCCCATCCT
N-CADHERIN	qRT-PCR	F: CACTGCTCAGGACCCAGAT R: TAAGCCGAGTGATGGTCC

2.1.7 Plasmid constructs

Table 2.4 lists plasmid constructs, either purchased or supplied by other laboratories.

Table 2. 4: List plasmid constructs and their sources

Constructs	Source
pEGFP-C1	Clontech
pEGFP-ST	David Griffiths, University of Leeds, UK

2.1.8 siRNA constructs

siRNA constructs to knock down ADAM 10 and ADAM 17 were obtained from Dharmacon. Allstars negative control siRNA was used as the negative control for all siRNA knock down experiments and obtained from QIAGEN.

2.1.9 Inhibitors

Table 2.5 lists small-molecule and peptide inhibitors from a number of different suppliers.

Table 2. 5: Small molecule inhibitors, their working concentrations and suppliers

Inhibitor [Cas number]	Working concentration	Supplier
GI 254023X [Cat. No. 3995]	293 - 100 µM Cos7 - 100 µM MCC - 100 µM WaGa - 50 µM PeTa - 50 µM	Tocris
TAPI-2 acetate salt [SML0420]	293 - 100 µM Cos7 - 100 µM MCC - 100 µM WaGa - 50 µM PeTa - 50 µM	Sigma

2.2 Methods

2.2.1 Plasmid Purification

2.2.1.1 Large scale plasmid purification: Maxi prep

QIAGEN maxiprep kits were utilized for large scale plasmid purification, in accordance to the manufacturer's instructions. Overnight cultures of bacteria were prepared in 250 - 500 ml LB, by shaking incubation at 37 °C for about 18 hours. The cells were pelleted by centrifugation at 6,000 x g for 15 minutes at 4 °C, in a Sorvall Evolution refrigerated centrifuge, using a Sorvall SLA-1500 rotor. The pelleted bacterial cells were resuspended in 10 ml Buffer P1 [50 mM Tris-HCl [pH 8.0], 10 mM EDTA, 100 ug/ml RNase A, Lyse blue]. The resuspension was followed up by addition of 10 ml of Buffer P2 [200 mM NaOH, 1% SDS], with 4-6 vigorous inversions until the solution turned blue and incubated at room temperature for 5 minutes. 10 ml of chilled buffer P3 [3 M potassium acetate, pH 5.5] was added and the mixture immediately mixed by 4-6 vigorous inversions until the solution turned white and incubated for 20 minutes at 4 °C.

Lysates were cleared by centrifugation at 13,000 x g for 30 minutes in a Sorvall SS-34 rotor at 4°C. During this centrifugation step, a QIAGEN-tip 500 column was equilibrated by addition of 10 ml Buffer QBT [750 mM NaCl, 50 mM MOPS [pH 7.0], 15% [v/v] isopropanol, 0.15% [v/v] Triton X-100], the solution was then allowed to drain through by gravity flow. The cleared lysate was applied to the column and allowed to bind the resin by gravity flow. The resin was then washed twice with 30ml Buffer QC [1 M NaCl, 50 mM MOPS [pH 7.0], 15% [v/v] isopropanol]. This was followed by elution of the DNA from the column by addition of 15 ml Buffer QF [1.25 M NaCl, 50 mM Tris-HCl [pH 8.5], 15% [v/v] isopropanol].

10.5 ml isopropanol was then added to the elution to precipitate the DNA and the mixture was centrifuged immediately at 5,000 x g in a chilled Eppendorf 5804 R bench-top centrifuge for 30 minutes. The DNA pellet was washed with 5 ml of 70% [v/v] ethanol and centrifuged at 5,000 x g for 20 minutes at 4 °C. The DNA pellet was air-dried and resuspended in 200 µl dH_2O. Concentration and purify of the DNA were measured by absorbance at 260 nm, using a NanoDrop ND-1000 spectrophotometer [NanoDrop Technologies]. The obtained DNA was diluted to a stock concentration of 1µg/µl and stored at -20°C.

2.2.2 Mammalian cell culture

2.2.2.1 Cell lines

The Human Embryonic Kidney 293 Flp-In™ cell line [HEK-293] were from Life Technologies™. The i293-ST-flag [by Laura Knight], i293-GFP and i293-GFP-ST cell lines [Gabriele Stakaityte] were derived from the HEK-293 cell line. Cos7 cells were obtained from Dr. Andrew Mcdonald. The MCC13 cells were purchased from Health Protection Agency Culture Collection. MCC13 is a MCPyV negative MCC cell line established from a metastatic cervical node which was biopsy confirmed for merkel cell carcinoma.

2.2.2.2 Cell maintenance

All HEK based cell lines [inducible i293-GFP and i293-GFP-ST and i293-ST] along with Cos7 cells were maintained in Dulbecco's modified Eagle's medium [DMEM, Invitrogen™], supplemented with 10% [v/v] foetal calf serum [FCS] and 1% [v/v] penicillin and streptomycin, referred to hereafter as "complete DMEM", at 37°C in the presence of 5% CO_2. 293 FlipIn cells were maintained in complete DMEM containing 100 µg/ml zeocin [Invitrogen™]. MCC13 cells were maintained in Roswell Park Memorial Institute [RPMI] media, supplemented with 10% [v/v] foetal calf serum [FCS] and 1% [v/v] penicillin and streptomycin, referred to hereafter as "complete RPMI", at 37°C in the presence of 5% CO_2.

All cell lines were passaged every 3-4 days when they reached 80% confluence approximately. Confluent cell layers were removed from 75 cm^3 tissue culture vessel [Sigma-Aldrich®] surfaces by kinetic force or by use of Gibco® Cell Dissociation Buffer [Thermofisher] and the cells were split 1:10-1:20 into fresh flasks, containing complete media.

For long term storage, cells were re-suspended at 1 x 10^6 cells/ml in freezing medium [10% [v/v] DMSO, 40% media, 50% [v/v] FCS] and aliquoted into 1.8 ml Cryotubes™ [NUNC™]. The cells were placed in a polystyrene box at -80 °C for 24 hours, before being transferred to liquid nitrogen.

2.2.3 Mammalian cell culture based protocols

2.2.3.1 Mammalian cell transfection

Approximately 5 x 105 HEK 293 Flp In or Cos 7 cells were seeded into each well of a 6-well [35 mm diameter] plate prior to transfection, to achieve approximately 70% confluency at the time of transfection. All transfections were performed using Lipofectamine^{TM} 2000, according to the manufacturer's instructions. Typically, a total of 2 µg total plasmid DNA and 4uL Lipofectamine^{TM} 2000 were added to 100 µl Opti-MEM® [Life Technologies^{TM}] and incubated for 5 minutes at room temperature. After 5 minutes, the Lipofectamine^{TM} solution was mixed with the DNA solution andincubated for 20 minutes at room temperature. After 20 minutes, the complete DMEM on the cells was replaced with 1 ml serum free DMEM and the transfection media was added drop-by-drop onto the cells carefully to avoid cell displacement. The cells were incubated at 37 °C for approximately 6 hours and the media replaced with complete DMEM, followed by incubation at 37 °C for 24-48 hours, unless stated otherwise.

DNA transfections of MCC13 cells was performed by use of TransIT-X2® Dynamic Delivery System [Mirus LLC], in accordance with the manufacturer's protocol. For each well of a 6 well plate 7.5 µl of the transfection reagent was mixed with 2.5 µg of plasmid DNA in 250 µl Opti-MEM®, incubated for 30 min and added drop-wise to cells in growing in 1 ml of serum free RPMI. Cells were incubated at 37^{O}C overnight. Subsequently serum free RPMI was replaced with complete RPMI, followed by incubation at 37 °C for 24-48 hours, unless stated otherwise.

2.2.3.2 Induction of inducible cell line

Approximately 3 x 10^5 cells [i293-ST-flag, i293-GFP or i293-GFP-ST] were seeded per 6-well plate in 2 ml 10% DMEM. 24 h later, 4 µl doxyclycline hyclate [Life Technologies^{TM}] was added to 2ml 10% DMEM and mixed, resulting in a final concentration of 2µg/ml per well. Cells were grown under induction for 24-48 hours other otherwise noted.

2.2.3.3 siRNA knockdown of protein expression

A standard siRNA transfection protocol was followed for all assays unless stated otherwise. Approximately 3 x 10^5 cells were seeded into a 6-well plate 24 hours before transfection. For each reaction, 10 nM of siRNA and 7.5 µl of Lipofectamine 3000 [Life Technologies^{TM}] were mixed in 200 µl Opti-MEM®, incubated for 30 min and added drop-wise to cells in 1 ml per well Serum free DMEM. Cells were incubated at 37^{o}C overnight. Subsequently, serum free DMEM was replaced with complete DMEM and RNAi was allowed to proceed for at least 72 hours.

2.2.3.4 Addition of inhibitors

Approximately 5 x 10^5 cells were seeded into 6-well plates, and transfected and/or induced if appropriate. 24 hours before harvesting, appropriate concentrations of inhibitors dissolved in DMSO were added. 1 µl/ml of DMSO was also added to control cells.

2.2.4 Cell viability assay [MTS assay]

Approximately 5,000 cells per well were seeded into 96-well plates in quintuplicate for each treatment condition. Negative controls of cell-free medium, untreated cells and 1 µl/ml DMSO-treated cells were included. In cases of inhibitor assessment for cell viability, inhibitors were added in a range of concentrations. Cells were grown overnight and appropriately treated the following day. 24 h later, growth medium was replaced with 100 µl fresh complete media per well before 20µl of CellTitre 96®AQueous One Solution Cell Proliferation Assay reagent [Promega] was added to each well. Cells were then incubated for approximately 1 h at 37◦C, and absorbance was read at 490 nm using the Infinite®F50 absorbance microplate reader [Tecan].

2.2.5 Electrophoretic analysis of proteins

2.2.5.1 Mammalian cell lysate preparation

Approximately 2 x 10^6 mammalian cells were harvested from 6 well tissue culture dishes by vigorous pipetting with 1 ml of PBS. Cells were then pelleted by

centrifugation at 3,500 x g at room temperature for 5 minutes. Cell pellets were resuspended in 1ml fresh PBS by mixing and centrifuged as before: three wash steps were performed in a similar method. Cell pellets were lysed in 200 µl lysis buffer [50 mM Tris base ultrapure, 150 mM NaCl, 1% NP40, pH 7.6] plus 1 x Complete®Protease Inhibitor EDTA free (Milla) and incubated on ice for 30 minutes, with intermittent mixing. Cell lysates were centrifuged at 13,200 x g at 4 °C for 20 minutes and the supernatant was then transferred to a fresh eppendorf tube before use.

2.2.5.2 Determination of protein concentration

Protein concentrations were determined by use of the DC Protein Assay Kit (Berx *et al.*), according to the manufacturers' protocol. Initially, a protein standard curve was obtained. Using a microplate [96-well plate], 5 dilutions of a protein standard [BSA] was prepared with concentrations ranging from 0.2 mg/ml to 1.5 mg/ml. The standard was prepared in the same buffer that the test protein samples were prepared in to ensure accuracy of the standard curve.

5 µl of each standard were pipetted into the micro plate in triplicate. Similarly, test samples where measured out at 5 µl in triplicate. Following this 20 µl of Reagent S was added to 1 ml Reagent A in a fresh eppendorf. 25 µl of the mixed solution was then added to each individual well. This was followed by addition of 200 µl Solution B. Samples where then mixed on a Microplate shaker for at least 15 minutes and the absorbance read at 750 nm using the infinite® F50 Robotic microplate reader [Tecan]. Protein concentrations of the test samples were determined by plotting a standard curve using the standard dilution series.

2.2.5.3 Tris-glycine SDS-polyacrylamide gel electrophoresis

Tris-glycine polyacrylamide running gels [Table 2.6] were overlaid with stacking gels [per 1 ml: 170 µl acrylamide/bis acrylamide solution 37.5:1 [Severn Biotech Ltd], 130 µl 0.25M Tris-HCL [pH 6.8], 10 µl 10% [w/v] SDS, 670 µl miliQ water, 10 µl 10% ammonium persulfate, 2 µl TEMED].

Table 2. 5: Reagents and their volumes to prepare a range of trisglycine polyacrylamide running gels

Reagent	6%	8%	10%	12%
MillQ water	2.6 ml	2.3 ml	1.9 ml	1.6 ml
Acrylamide/bisacrylamide (37.5:1)	1 ml	1.3 ml	1.7 ml	2.0 ml
1 M Tris-HCl (pH 8.8)	1.3 ml	1.3ml	1.3 ml	1.3 ml
10% (w/v) SDS	50 µl	50 µl	50 µl	50 µl
10% (w/v) ammonium persuflate	50 µl	50 µl	50 µl	50 µl
TEMED	4 µl	3 µl	2 µl	2 µl

2× protein solubilising buffer [50 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 20% (v/v) glycerol, 50 µg/ml Bromophenol Blue and 10 mM DTT] was added to protein lysates and the samples were denatured by heating at 95◦C for 3–5 min. Heated samples were loaded onto appropriate polyacrylamide gels. Samples were loaded next to pre-stained protein ladder [Bio-Rad Laboratories, Inc.], as an indicator of molecular weight [kDa]. Prior to sample loading, gels were immersed in Trisglycine running buffer [0.25 M Tris-base, 192 mM glycine, 0.1% [w/v] SDS]. Gels were run at 180 V for 45 min or until the solubilising buffer reached the bottom to the gel. All SDS-PAGE was carried out using a Bio-RadTM Mini-PROTEAN 3 cell [Bio-Rad Laboratories, Inc.], set up according to the manufacturer's instructions. Proteins were then analysed by western blot.

2.2.5.4 Western blot analysis

Proteins from SDS-PAGE gels were transferred onto Thermo Scientific™ Pierce™ Nitrocellulose membranes [Thermo Fisher Scientific Inc.], using the Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell [Bio-Rad Laboratories, Inc.], according to the manufacturer's protocol. 4 pieces of Whatman 3 mm filter paper, 1 SDS-PAGE gels and one nitrocellulose membrane were soaked in transfer buffer [25 mM Tris-base, 190 mM glycine, 20% [v/v] methanol] and placed in a cassette in sequential

order. Proteins were transferred from the gel to the membrane at 100 V for 1–2 h, depending upon protein size, followed by incubation of the membrane in 5% TBS-Tween blocking buffer [500 mM NaCl, 20 mM Tris, 0.1% [v/v] Tween-20, 5% non-fat dried milk [Marvel)] at room temperature for 1 h. Subsequently, the membrane was incubated in the corresponding primary antibody [Table 2.2] diluted in 1% TBS-Tween blocking buffer [150 mM NaCl, 20 mM Tris, 0.1% [v/v] Tween-20, 1% non-fat dried milk [Marvel]] overnight at 4◦C. The membranes were then washed 3× for 5 min in 1× TBS-tween buffer [20 mM Tris, 0.1% Tween-20, 150 mM NaCl], and incubated for 1 h at room temperature in the appropriate secondary antibody. The membranes were then washed 3× for 5 min in 1× TBS-tween solution. Protein bands were then visualised by enhanced chemiluminescence using EZ-ECL enhancer solutions A and B kit [Geneflow^{TM}] [1:1 ratio] and exposed to ECLTM hyperfilm [Amersham^{TM}]. Films were developed using a Konica SRX-101A developer. All incubations and washes were done with agitation.

2.2.6 Gene expression analysis by qRT-PCR

2.2.6.1 RNA extraction

Total RNA was isolated from cells using TRIzol (Invitrogen^{TM}), according to the manufacturer's instructions. Cells were seeded at a density of 4 × 10^5 in triplicate per condition [uninduced and induced or EGFP and PEGFP-ST]. Cells were then directly lysed by addition of 1ml TRIzol reagent (Invitrogen^{TM}) per well, mixed by pipetting in an RNase-free microcentrifuge tube, and incubated at room temperature for 5 min. Subsequently, 200 µl chloroform was added per sample, and samples were vortexed for 15 s and incubated at room temperature for 3 min. Samples were then centrifuged at 12,000 × g for 15 min at 4°C. The top aqueous/colourless phase [500 µl] was transferred to a fresh RNase-free microcentrifuge tube. RNA was then precipitated by adding 500 µl isopropanol and incubating samples at room temperature for 10 min before centrifugation at 12,000 × g for 10 min at 4°C. The supernatants were carefully aspirated and the pellets washed in 1 ml 75% ethanol and briefly vortexed, followed by centrifugation at 7,500 × g for 5 min at 4°C. The supernatants were once again aspirated and the pellets air-dried for 5 min. The pellets were resuspended in 16 µl DNase and RNase free H_2O and stored at -80°C or treated immediately with DNase I treated

and reverse transcribed. The RNA concentration was measured using a NanoDrop-1000 spectrophotometer [NanoDrop Technologies] at 280 nm wavelength.

2.2.6.2 DNase treatment

A DNase I kit [Ambion™] was used to remove contaminating DNA from extracted RNA samples, as per the manufacturer's protocol. 0.1 volume of DNase reaction buffer and 0.5µl Amplification Grade DNase I were added to each sample, gently mixed and incubated for 30 min at 37°C. After incubation, 0.1 volume of DNase removal agent was added, samples were mixed thoroughly and incubated at room temperature for 2 minutes and then the DNase removal agent pelleted at 10,000 x g for 2 minutes in a bench top centrifuge.. The supernatant was then taken off and stored in a fresh RNase-free microcentrifuge tube. Purified RNA was stored at -80°C.

2.2.6.3 Reverse transcription

Superscript™ II Reverse Transcriptase [Life Technologies™] was used to synthesize cDNA from the total extracted cellular RNA, according to the manufacturer's protocol. Purified RNA was diluted to 500 µg/ml before reverse transcription was carried out to synthesise cDNA. The initial sample mixture contained 1 µg DNase I treated RNA, 1 µl 10 mM dNTP mix, 1 µl Oligo[dT] 12-18 primer and made up to a total volume of 12 µl. Samples were then mixed and incubated at 65°C for 5 minutes and then quick-chilled on ice. Following this, 4µl 5X First-Strand buffer, 2µl 0.1 M DTT, 1µl RNaseOUT™ and 1 µl Superscript™ II Reverse Transcriptase were added per sample. Samples were mixed and incubated at 42°C for 50 minutes, followed by enzyme inactivation at 70°C for 15 minutes. cDNA was stored at -20°C.

2.2.6.4 Quantitative real-time PCR (qPCR) reaction

RT-qPCR samples were set up in Corbett tubes and a pre-chilled Corbett tube rack [Corbett Life Sciences]. Each primer pair were added in duplicate to RNA samples

extracted at different times [biological replicates]. The PCR master mix contained the following for each reaction: 10 µl SensiMixPlus SYBR™ No-ROX [Bioline], 1 µl primer mix (10 µM of both forward and reverse primers), 5 µl nuclease-free water and 4 µl cDNA [10 ng final amount]. The PCRs were performed using a Rotor-Gene™ 6000 Real-Time PCR machine [Qiagen™] using a 3 Step with Melt program. A typical PCR cycle parameter consisted of 95°C for 10 min and then 35 cycles of: 95°C for 15 s, 60°C for 30 s, and 72°C for 20 s. Quantitative analysis was then performed using the comparative CT method as previously described (Boyne and Whitehouse 2006).

2.2.7 Immunofluorescence microscopy

Coverslips were first briefly immersed in 100% ethanol and air dried, before placing in separate wells of a 6 well tissue culture dish. Surface of the coverslip where then coated with 0.01% poly-L-lysine solution [Life Technologies™] for 15 minutes at room temperature for and washed three times in 2 ml PBS. The coverslips were air dried for a minimum of 2 hours before use.

Approximately 2 x 10^5 cells [HEK 293 FlipIn or Cos 7 cells] were seeded onto the treated coverslips and incubated at 37 °C for around approximately 16 hours. Cells were transfected with appropriate constructs [as Section 2.2.3.1]. After appropriate incubation, cell monolayers were gently washed 3 times with 2 ml PBS, before fixation in 2 ml PBS, 4% [v/v] paraformaldehyde for 10 minutes [all performed at room temperature]. The cells were again washed 3 times in 2 ml PBS. Coverslips were transferred to a humid chamber [a Petri dish lined with parafilm, containing soaked filter paper to prevent cells drying out] and incubated for 1 hour at 37 °C, covered with blocking solution [PBS, 1% [w/v] bovine serum albumin [BSA] and 10% FCS].

After removal of blocking solution, the appropriate primary antibody as seen in Table 2.2 [diluted in blocking solution] was applied to the cells, which were incubated overnight at 4°C. The cell monolayer was carefully washed 5 times in PBS, before an appropriate AlexaFluor conjugated secondary antibody was applied to the cells. After 2 hours incubation at room temperature, the secondary antibody was removed and the cells were carefully washed 5 times in PBS.

Coverslips were mounted onto microscope slides using VECTORSHIELD® with DAPI mounting media [Vector Laboratories] and visualised on Zeiss LSM880 Inverted with Airyscan confocal microscope and Zen 2011 imaging software from Zeiss.

2.2.8 Multicolour Immunohistochemistry

Tissue sections were procured from Origene [CaseID CI0000010366 and SampleID PA15477D3E]. Five-micrometre-thick paraffin tissue sections were deparaffinised with xylene and graded ethanol. Antigen retrieval was performed by heating the sections in 10 mM sodium citrate buffer, pH 6.0, at 95° C for 20 min in a scientific microwave. Samples were then blocked [for non-specific antibody binding] in blocking reagent [10% fetal bovine serum and 3% Bovine serum albumin in 1X PBS., [all serum was heat inactivated]] for 4 hours at 37^{o}C. Samples were incubated with appropriate primary antibodies overnight in a humidified chamber at 4^{o}C, using FitC conjugated anti-CK20 at 1:50 dilution [Abcam] anti-ADAM 10 or ADAM 17 at 1:250 dilution [Abcam] and CM2B4 at 1:50 dilution [Santa Cruz]. Subsequently sections were washed three times for 5 minutes each in PBS and incubated with the appropriate secondary antibody, labelled with fluorochromes. ADAM antibodies where counterstained with Alexa Fluor-546 and CM2B4 antibody was countertstained with Alexa Fluor 643 [Abcam]. Sections were mounted with coverslip using VECTORSHIELD® mounting media [Vector Laboratories] and visualised on Zeiss LSM880 Inverted with Airyscan confocal microscope and Zen 2011 imaging software from Zeiss.

2.2.9 Cell Scatter assay

Prior to seeding cells, coverslips were prepared by washing in 100 % ethanol and coating with 0.01% poly-L-lysine from Sigma-Aldrich™ for 5 min. 20,000 cells, either pEGFP-C1 or pEGFP-ST transfected cells [HEK 293s or Cos7], were seed onto coverslips and allowed to adhere. Following adhesions, cells where serum starved with DMEM with no FCS supplement to allow for aggregation for 12 hours. After the starvation period, cells were induced and coverslips of both EGFP and EGFP-ST expressing cells were fixed at specific timepoints or the duration of the

experiment. Coverslips where then gently washed 3 times with 1x PBS and mounted on microscope slides with DAPI-containing VectaShield™ mounting media from VectaShield™. Cells were imaged utilizing Zeiss LSM880 Inverted with Airyscan confocal microscope and Zen 2011 imaging software from Zeiss.

2.2.10 Analysis of Cell motility

HEK 293, Cos7 were seeded at a density of 1×10^5 cells per well in 6-well plates, grown overnight. Cells were transfected 2.5 µg of plasmid DNA [pEGFP-C1 or pEGFP-ST] per well using the appropriate transfection reagent. Cells were incubated for 24 h at 37°C, and, if applicable, inhibitors were added at appropriate concentrations for another 24 h. Cells were then imaged using the IncuCyte™ Live-Cell Imaging System [Essen BioScience], as per the manufacturer's protocol. Imaging was performed every 30 min over the course of 24 or 48 h and cell motility was then analysed using Image J software.

2.2.11 Flow cytometry

HEK-293 Flp-In ® cells were seeded at 3×10^5 density in 6-well plates, then mock transfected or transfected with 2 µg pEGFP-C1 or pEGFP-ST per well using the standard Lipofectamine 2000 protocol for 6 h. 48 hours post-transfection cells were harvested using Gibco® Cell Dissociation Buffer, a gentle dissociation buffer which is suitable for gentle dissociation when intact cell surface proteins are essential. The harvested cells were washed with ice-cold PBS and resuspended at $1–2 \times 10^6$ cells/ml in freshly made staining buffer [PBS, 10% FCS, 3% BSA]. Cells were incubated in staining solution [PBS, 10% FCS, 3% BSA] Cells were then incubated with appropriate dilutions of primary antibody or staining buffer only for 1 h at room temperature in the dark, washed 3× with staining buffer and incubated with Alexa-Fluor-tagged secondary antibody or staining buffer only for 1 h at room temperature in the dark. Cells were then washed 3× with staining buffer and finally resuspended in 500 µl of staining buffer.

Flow cytometry was performed immediately after staining. During analysis, 100000 events are initially isolated from the sorted population. Upon discarding of unwanted events, 20,000 cells remain in the analysis gate to be quantified using Flow Jo analysis software. All Flow cytometry experiments were performed in triplicate, and each experiment included unstained and secondary-only stained controls. Isotype controls were also performed for all antibodies. Gating was set according to Isotype control for primary antibody staining and untransfected cells for EGFP expression as the gating process required Green cells.

2.2.12 Staining for Apical to basal cell polarity

Cos7 cells were seeded at a density of 5×10^5 in 6 well plates. After 24 hours cells were transfected with appropriate DNA plasmid. 6 h pre-fixation, cells were scratched using a p1000 tip across the center of each well, washed 3× with media and left to move back into the wound gap. Cells were then fixed and stained for the Transgolgi network antibody. cells were visualised visualised using the EVOS AUTO2FL.

2.2.13 Migration Transwell assay

WaGa and PeTa cells were seeded in triplicate (per condition) at a density of 3 x 10^5 cells per 6 well tissue culture plate. Cells were incubated at 37 ^{o}C overnight before incubation with ADAM 10 specific inhibitor in serum free media. Subsequent to cell count using a haemocytometer and seeded in triplicate (per condition) at a density of 1 x 10^5 cell per well, on to pre-coated BD BioCoat^{TM} Angiogenesis System: Endothelial Cell Migration (BD Bioscience) tissue culture plates for the appropriate time allowance per experiment. The cells were then analysed for migration in triplicate, according to the manufacturers protocol. 750 µl FCS containing media was added as a chemoattractant in the bottom wells via the sample ports and the plates incubated for 22 hours at 37 ^{o}C. After incubations, the culture medium was removed from the upper chambers and the insert plates were transferred to 24 well culture plates containing 4 µg/ml Calcein AM^{TM} (BD Bioscience) in 500 µl PBS (per well). The cells were incubated for a further 90

minutes at 37 ^{o}C, 5% Co_2. The fluorescence of invaded and migrated cells was determined using a fluorescence plate reader at excitation wavelengths of between 494-517 nM. Each condition was measured in triplicate and from this the percentage of invasion/migration compared to the control plate could be calculated, as per the manufacturer's instructions.

CHAPTER 3

The relationship between Merkel Cell Polyomavirus Small Tumor antigen [ST] and Cell Adhesion molecules

3.1 Introduction

MCC is a highly metastatic form of skin cancer of neuroendocrine origin. MCC has a predisposition to metastasize through the dermal lymphatic system resulting in a poor 5-year survival rate (Stakaityte *et al.* 2014; Liu *et al.* 2016). It has since been established MCPyV is a causative agent of MCC (Chang and Moore 2012). The MCPyV genome is monoclonally integrated into the host genome in both primary and metastatic cancer forms. This integration has been detected in all MCPyV positive MCC tumours (Griffiths *et al.* 2013). Moreover, assessment of MCPyV LT sequences derived from MCC tumour cells include a specific deletion which renders the virus replication defective upon integration (Martel-Jantin *et al.* 2012). Observation of the monoclonal integration pattern along with deletion in the large tumour antigen provide evidence to dismiss the concept that MCPyV is a passenger virus (Wendzicki *et al.* 2015).

Currently there are limited studies on the mechanisms by which MCC tumour cells acquire there highly metastatic phenotype. Metastasis is the end point of a series of biological processes by which a tumour cell detaches from the primary tumour and disseminates to a distant site through the circulatory system and establishes a secondary tumour (Valastyan and Weinberg 2011). For cancer advancement and metastasis, a vital characteristic is an alteration in cell adhesion properties of cancer cells (Cavallaro and Christofori 2004). This is essential as cell-to-cell and cell-to-matrix adhesion is involved in normal cell processes, such as but not limited to cell communication, cell polarity and maintenance of tissue organization. However, alteration in these processes are associated with pathologies like tumour and cell invasion, as well as metastasis (Wolfsberg *et al.* 1995; Cavallaro and Christofori 2004).

In healthy cells, loss of essential cell-to-cell connection results in a distinct form of apoptosis, termed “Anoikis” (Geiger and Peeper 2009). However, the loss of this control mechanism leads to anchorage-independent growth and tissue independent apoptosis resistance. This is a key element in cancer progression and metastasis (Bogenrieder and Herlyn 2003). The metastatic cascade is a multistep mechanism and loss of cell-to-cell connection helps in disaggregation from the primary tumours and invasion of the primary tumour cells into the circulation (van Zijl *et al.* 2011). By means of the lymphatic system, tumours cells can then be circulated to the secondary site of tumour growth concluding in formation of the secondary tumour (Geiger and Peeper 2009; van Zijl *et al.* 2011).

In healthy cells, specialized molecules known as sheddases function predominantly in cleavage of cell adhesion molecules and the extracellular domain [ECM] (Egeblad and Werb 2002; Moss and Lambert 2002). Cleavage of the ECM allows for creation of space within the extracellular space freeing up the surrounding areas for cell migration (Reinhard *et al.* 2015). Notably, sheddases can modify the interaction between cell adhesion molecules and components of the extracellular matrix. In healthy cells, upon shedding of cell adhesion molecules, cells are disengaged from the ECM and their neighbouring cell allowing for local tissue modelling. However, in cancer cells, there is a marked increase in the cleavage of cell adhesion molecule and once the malignant cell is free, it can migrate, initiating metastasis (Egeblad and Werb 2002; Deryugina and Quigley 2006).

This chapter describes preliminary observations of destabilization in cell junctions and cell adhesion markers along with possible cellular participants in these cleavage processes, upon the expression of the MCPyV ST protein.

3.2 Quantitative proteomic analysis shows MCPyV expression affects cell adhesion-associated proteins

Cell adhesion is essential in regulation of cell communication, development and tissue maintenance (Tsukita 1993). Cell-to-cell adhesion and cell interaction to the extracellular matrix also plays a role in cell migration, cell differentiation and preserving tissue structural integrity (Joyce and Pollard 2009). A SILAC-based quantitative proteomic approach was previously used to determine possible host cell proteome alterations in an inducible cell line, i293-ST, capable of inducible expression of MCPyV ST [previously described in (Knight *et al.* 2015)]. Results from this dataset showed that MCPyV ST expression induced differential expression of proteins associated with the Microtubule Network and Actin cytoskeleton. Specifically, MCPyV ST induced Stathmin, a microtubule associated protein (Knight *et al.* 2015). Similar trends where seen in actin cytoskeletal related proteins such as Cofillin, Cortactin and ARP3 (Stakaityte *et al.* 2018). Interestingly, cell adhesion-related proteins were also down-regulated upon MCPyV ST expression. These proteins are shown in Table 3.1.

Table 3. 1 Quantitative proteomic analysis shows a decrease in cell junction associated proteins levels upon MCPyV ST expression

Protein	Fold decrease in cell adhesion-related proteins	Peptide hits	Function
α -E- Catenin	-5	13	binds E-cadherin and β-catenin in the cell-to-cell adhesion complex and regulates filamentous actin (F-actin) dynamics
Zona occludens [ZO-1]	-3	12	Functions as a scaffold to bind the raft of Tight junction molecules together and provide the link to the actin cytoskeleton and the signaling mechanism of the cell
Protocadherin-7	-10	3	Homophilic cell adhesion through plasma membrane adhesion molecules
Desmoplakin	-3	35	organization of the desmosomal cadherin-plakoglobin complexes into plasma membrane domains. Anchors intermediate filaments to the desmosomes [a form of cell junctions]

To confirm the downregulation observed in these cell adhesion-associated proteins, HEK 293 cells were transfected with pEGFP-C1 control and pEGFP-ST expression plasmids for 48 hours. The parent vector from which the pEGFP-ST was derived from was pEGFP-C1 therefore ST was tagged on the N-terminus. Cell lysates were then evaluated by immunoblotting. Figure 3.1 demonstrates that upon MCPyV ST expression, there is a significant downregulation of A-E-catenin and ZO-1 proteins.

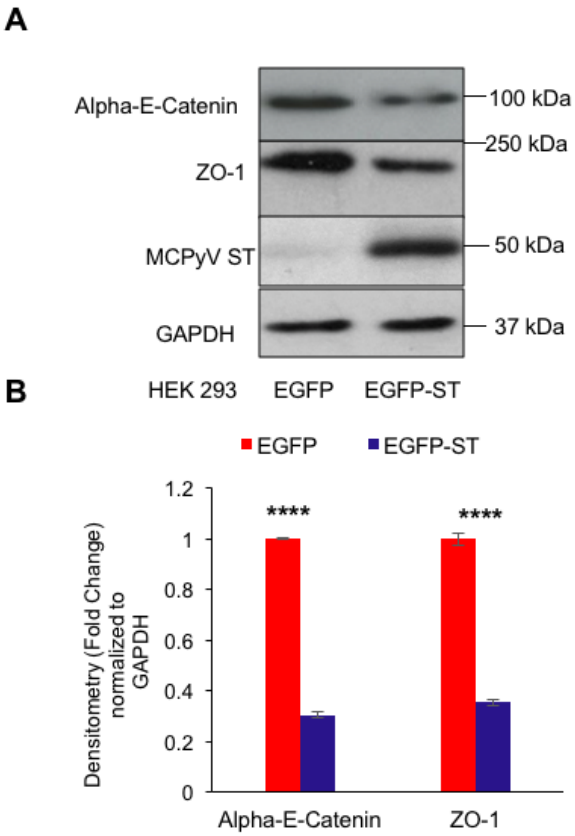

Figure 3. 1: MCPyV ST promotes downregulation of cell junction marker α-E-Catenin and cell adhesion molecule ZO-1 [A] HEK 293 cells were transfected with pEGFP-C1 control and pEGFP-ST expression plasmids for 48 hours. Immunoblot analysis was performed on the cellular lysates and analysed with *α*-E-Catenin and ZO-1 specific antibodies. GAPDH was used as a measure of equal loading, the Tantigen antibody was used to confirm MCPyV ST expression. [B] Densitometry quantification of the western blots was carried out using the Image J software and is shown a percentage of relative densitometry to the loading control, GAPDH. Data analysed using three replicates per experiment, n=3 and statistical analysis using a two-tailed t-test with unequal variance. [ns – P>0.05, ****– P≤ 0.0001].

While the inducible and transfected 293 cell lines are useful and robust tools in for initial investigations, it was important to validate the quantitative proteomic analysis in more relevant cell lines. Therefore, to demonstrate that MCPyV ST expression downregulates cell adhesion related proteins, the MCPyV negative cell line, MCC13, was utilized. Figure 3.2 demonstrates that MCC13 cells expressing MCPyV ST show a similar decrease in α-E-Catenin and ZO-1 expression. Upon quantification by densitometry, MCPyV ST expression results in a significant downregulation of these cell adhesion-related proteins.

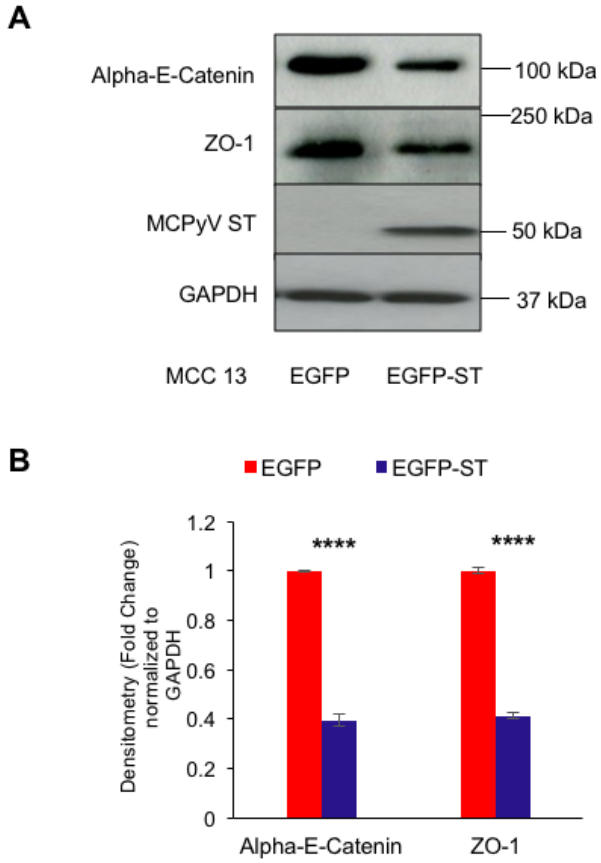

Figure 3. 2: MCPyV ST promotes downregulation of cell junction marker α-E-Catenin and cell adhesion molecule ZO-1in MCC13 cell line. [A] MCC13 cells were transfected with pEGFP-C1 control and pEGFP-ST expression plasmids for 48 hours. Immunoblot analysis was performed on the cellular lysates and analysed with α-E-Catenin and ZO-1 specific antibodies. GAPDH was used as a measure of equal loading, the T antigen antibody was used to confirm MCPyV ST expression. [B] Densitometry quantification of the western blots was carried out using the Image J software and is shown a percentage of relative densitometry to the loading control, GAPDH. Data analysed using three replicates per experiment, n=3 and statistical analysis using a two-tailed t-test with unequal variance comparing MCPyV ST samples to EGFP control samples [****– P≤ 0.0001].

3.3 MCPyV T antigen-positive MCC tumours also show a reduction in cell adhesion-associated proteins

MCPyV has been shown to be monoclonally integrated into MCPyV-Positive tumours and MCPyV ST has been implicated in MCC tumourigenesis (Stakaityte *et al.* 2014). To validate whether cell adhesion-associated proteins are downregulated in MCPyV-positive MCC tumours, two unrelated samples [Tumour 1 and Tumour 2] were compared to a control non-tumour cadaveric skin sample. Samples where homogenized, lysed, sonicated and immunoblotting was performed. Tissue samples where probed for ZO-1 and α-E-Catenin. Results show both ZO-1 and α-

E-Catenin are downregulated in both tumour samples in comparison to the healthy skin control [Figure 3.3].

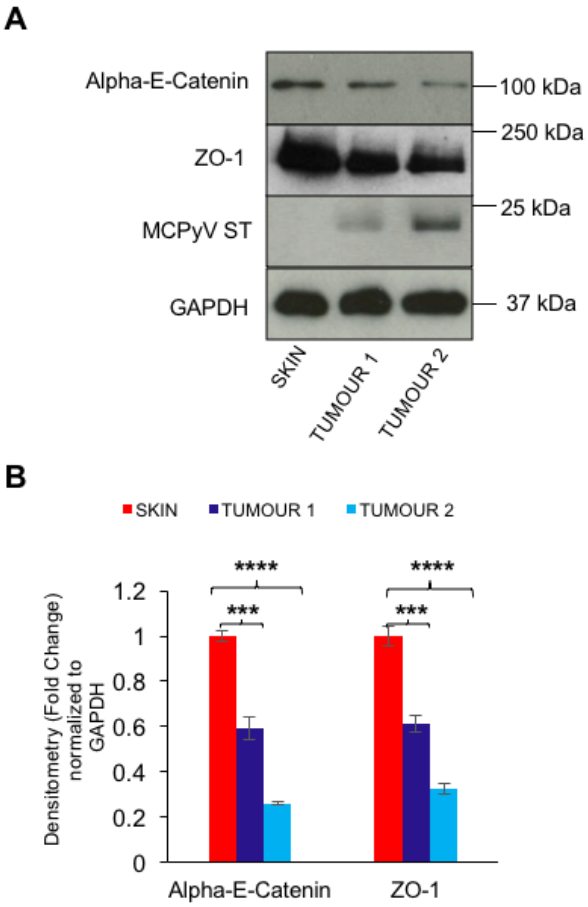

Figure 3. 3: Cell adhesion molecule ZO-1 and cell junction marker α-E-catenin are downregulated in MCPyV-positive MCC tumours. [A] Healthy skin, Tumour 1 and Tumour 2 were crushed using a mortar and a pestle on dry ice, lysed with RIPA buffer for 30 mins and sonicated to further homogenized the samples. Immunoblot analysis was performed on the tissue lysates and analysed with α-E-Catenin and ZO-1 specific antibodies. GAPDH was used as a measure of equal loading, the T antigen antibody was used to confirm MCPyV ST expression. [B] Densitometry quantification of the western blots was carried out using the Image J software and is shown a percentage of relative densitometry to the loading control, GAPDH. Data analysed using three replicates per experiment, n=3 and statistical analysis using a two-tailed t-test with unequal variance comparing MCPyV ST samples to EGFP control samples [*** – P≤ 0.001, ****– P≤ 0.0001].

Interestingly, downregulation of the cell adhesion associated proteins where higher in Tumour 2 sample, which corresponds to a higher degree of MCPyV ST expression. This may suggest that higher levels of MCPyV ST expression may lead to a more metastatic tumour due to lower levels of cell-adhesion molecules. Overall, these results confirm data in the HEK cell line indicating that MCPyV ST expression downregulates cell adhesion molecules which may contribute to the metastatic phenotype of MCC.

3.4 MCPyV ST expression disrupts cell adhesion markers at the cell surface

To determine whether MCPyV ST expression has any effect on the integrity of cell junctions, EGFP and EGFP-ST transfected HEK 293 cells were stained with an α-E-Catenin-specific antibody. α-E-Catenin is a mediator of Adherens junctions, which are predominantly expressed at the cell surface, where it facilitates cell adhesion and its breakdown infers a loss of structural integrity at cell junctions (Kobielak and Fuchs 2004). Results show a complete staining around the surface upon α-E-Catenin staining in control EGFP-expressing cells. In contrast, a reduced and incomplete staining is observed in EGFP-ST-expressing cells, indicative of diminished cell-to-cell adhesion [Figure 3.4].

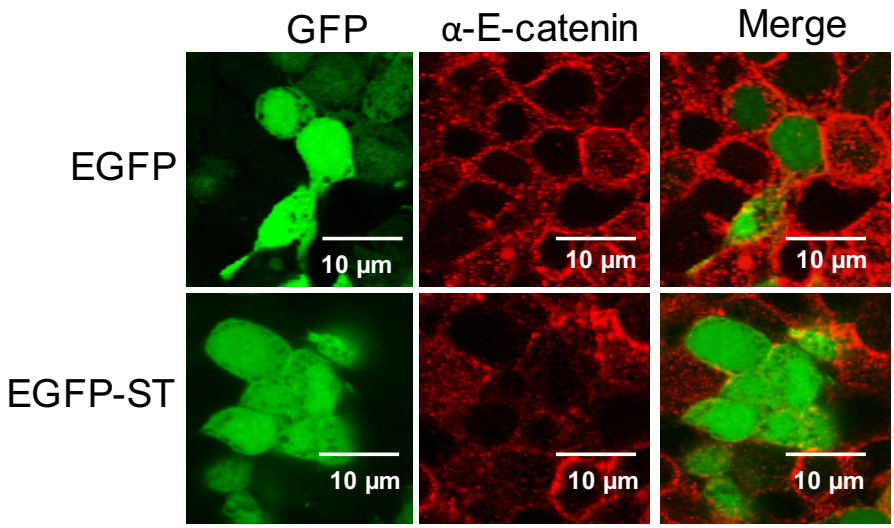

Figure 3. 4: MCPyV ST expression promotes disruption of α-E-Catenin expression at the cell surface. HEK 293 cells where transfected with pEGFP-C1 or pEGFP-ST expression vectors. After 24 hours, cells were fixed and incubated with a specific α-E-Catenin antibody along with a corresponding secondary antibody.

To validate downregulation of α-E-catenin expression at the cell surface upon MCPyV ST expression, Flow cytometry was also used to quantify the levels of α-E-Catenin in EGFP and EGFP-ST-expressing cells. α-E-Catenin expression at the cell surface was then analysed 48 hours post transfection and results demonstrate a reduction of α-E-Catenin expression at the cell surface upon MCPyV ST expression [Figure 3.5].

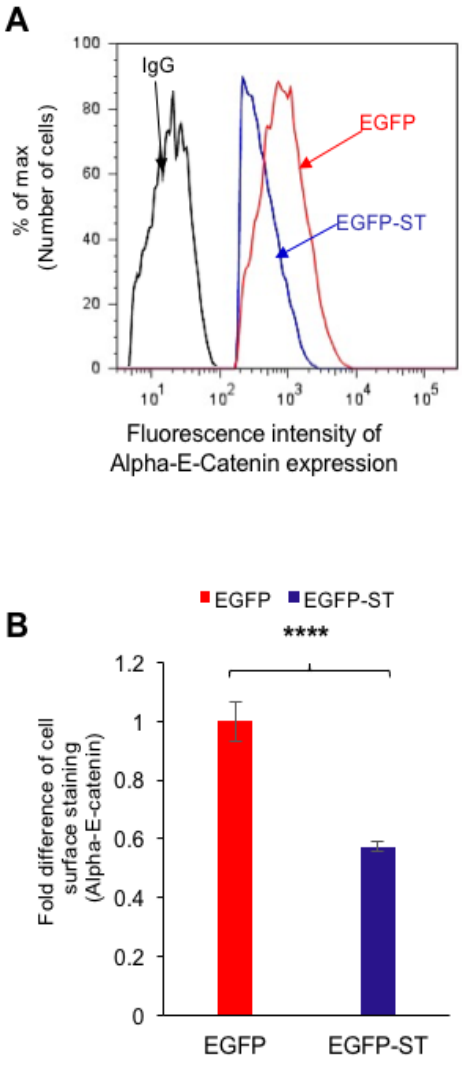

Figure 3. 5: MCPyV ST expression promotes a reduction of α-E-Catenin expression at the cell surface. [A] HEK 293 cells were transfected with pEGFP-C1 control and pEGFP-ST expression plasmids for 48 hours. Cells were harvested and spun down gently, blocked in 3% BSA and stained with a specific α-E-Catenin antibody along with a corresponding secondary antibody. Mean fluorescence intensity of both control EGFP and EGFP-ST cells where plotted as a histogram using Flow Jo analysis software. [B] Fold difference was calculated using Mean fluorescence intensity. Data analysed using three replicates per experiment, n=3 and statistical analysis using a two-tailed t-test with unequal variance comparing MCPyV ST samples to EGFP control samples [****– P≤ 0.0001].

ZO-1 was also used as a secondary cell adhesion marker to confirm disruption of cell junctions (Nunbhakdi-Craig *et al.* 2003). ZO-1 functions as a scaffold to bind the raft of TJ molecules together and is utilized as the link to the actin cytoskeleton and the cell signaling mechanism (Hoover *et al.* 1998). Functioning as a critical regulator of tight junctions, ZO-1 has been shown to be downregulated in highly invasive breast cancer cell lines (Hoover *et al.* 1997). To validate downregulation of

ZO-1 specifically at the cell surface where it is active, Flow cytometry was again used to quantify the levels ZO-1 expression in EGFP and EGFP-ST-expressing cells. Results demonstrated a reduction of ZO-1 expression at the cell surface upon MCPyV ST expression [Figure 3.6].

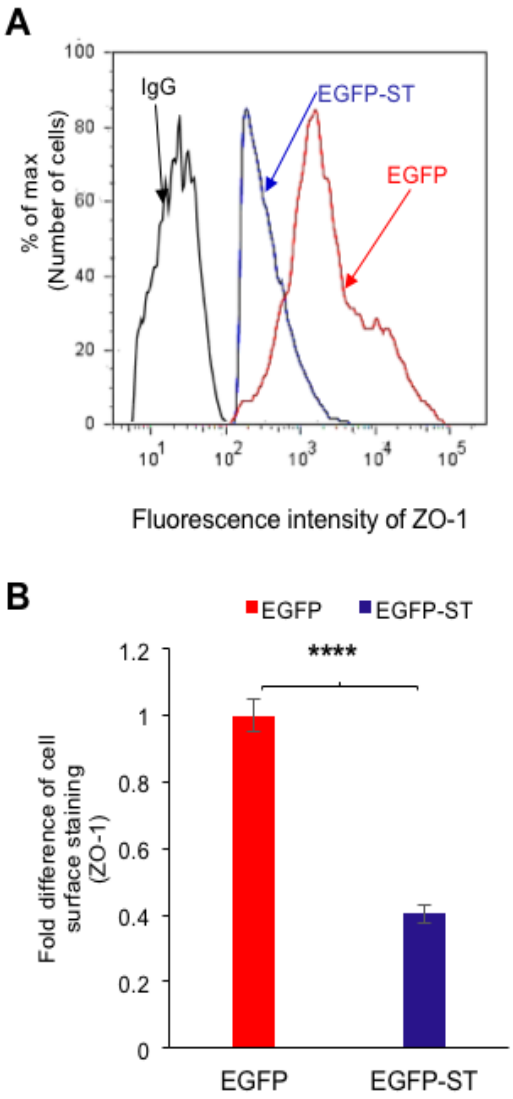

Figure 3. 6: MCPyV ST expression promotes a reduction of ZO-1 expression at the cell surface. [A] HEK 293 cells were transfected with pEGFP-C1 control and pEGFP-ST expression plasmids for 48 hours. Cells were harvested and spun down gently, blocked in 3% BSA and stained with a specific ZO-1 antibody along with a corresponding secondary antibody. Mean fluorescence intensity of both control EGFP and EGFP-ST cells where plotted as a histogram using Flow Jo analysis software. [B] Fold difference was calculated using Mean fluorescence intensity. Data analysed using three replicates per experiment, n=3 and statistical analysis using a two-tailed t-test with unequal variance comparing MCPyV ST samples to EGFP control samples [****– P≤ 0.0001].

3.5 MCPyV ST induces cell scatter, facilitating cell motility and migration

Loss of cell junction integrity enhances the ability of a cell to migrate as it promotes dissociation of cells from its primary site (Tsukamoto and Nigam 1999). The dissolution of cell-to-cell adhesion contributes to cell dissociation, and in turn cell scattering and cell migration (Braga 2000). To determine if MCPyV ST induces cell scatter, facilitating cell motility and migration, a cell scatter assay was performed (Fram *et al.* 2011). Here EGFP and EGFP-ST-expressing HEK 293 cells were incubated in low serum to induce aggregation, upon reintroduction of serum cells were fixed and stained with DAPI at 6 hourly intervals and clusters of cells were imaged to quantify the distance between each cell nucleus [Figure 3.7].

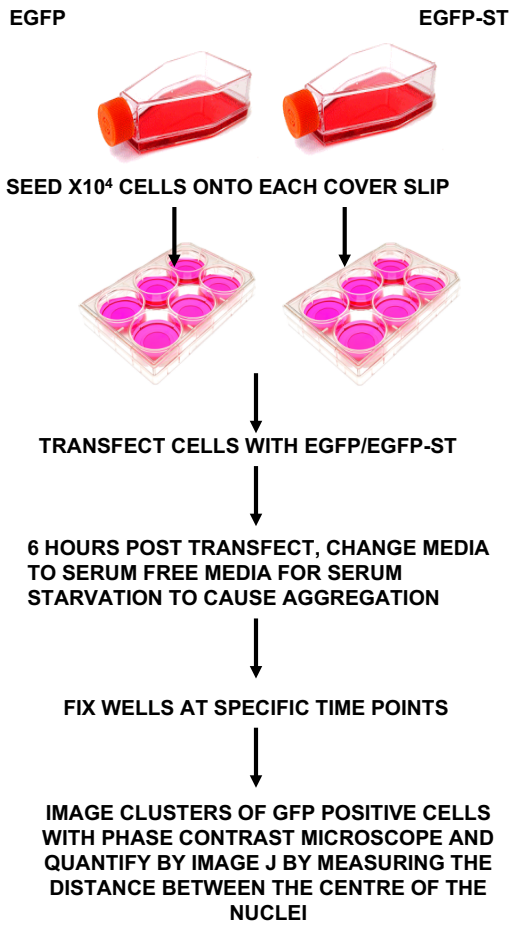

Figure 3. 7: Quantification of cell dissociation. Schematic for cell scatter assay used for the quantification of cell scatter assay.

Results show that EGFP control cells scarcely dissociate overtime, instead remaining in cell clumps. In contrast, MCPyV ST-expressing cells dissociate significantly from their initial cell clusters [Figure 3.8A]. Similar results were also observed in a secondary cell line COS 7 cells [Figure 3.8B]. These results suggest that MCPyV ST expression can lead to the breakdown of cell junctions enhancing cell dissociation and scatter.

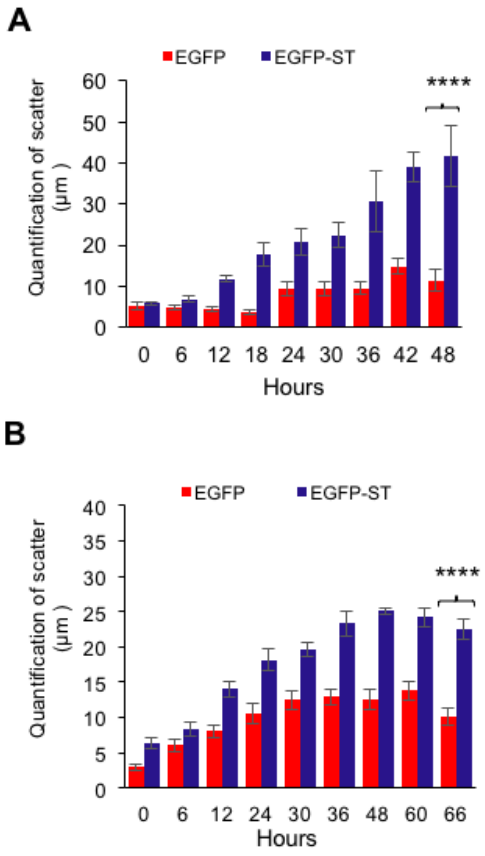

Figure 3. 8: Quantification of cell scatter. [A] HEK 293 and [B] COS 7cells where transfected with pEGFP-C1 or pEGFP-ST expression vectors. Cells where fixed with 4% paraformaldehyde at specific time points and stained with a DAPI solution. EGFP positive cells clusters where imaged using a ZEISS LSM 880 confocal microscope and quantified by measuring distance between the centre of the nucleus of each cell using Image J analysis software. n=20 cells per cluster comparing MCPyV ST samples to EGFP control samples at T = 48 hours in Figure A and T= 66 hours in Figure B [****– P≤ 0.0001].

3.6 MCPyV ST induces upregulation of cellular sheddases in relevant cell lines

Cellular sheddases function predominantly in cleavage of cell adhesion molecules and the extracellular domain. Analysis of a previous proteomic dataset analysing how MCPyV ST expression affects the host cell proteome (Knight *et al.* 2015),

identified that MCPyV ST expression leads to the upregulation of certain cellular sheddases, in particular, ADAM 10 and ADAM 17. To confirm an increase in ADAM 10 and 17 protein levels, expression of a selection of ADAM proteins were evaluated by western blot analysis in the MCPyV ST inducible flag tagged cell line i293-ST, comparing uninduced and induced cell lysates. Results show an increase in the expression levels of ADAM 10 and 17 proteins, in contrast to ADAM TS1 [Figure 3.9A]. Densitometry-based quantification of the immunoblot analysis showed an increase in ADAM 10 and ADAM 17 expression of 6 fold and 4 fold respectively [Figure 3.9B].

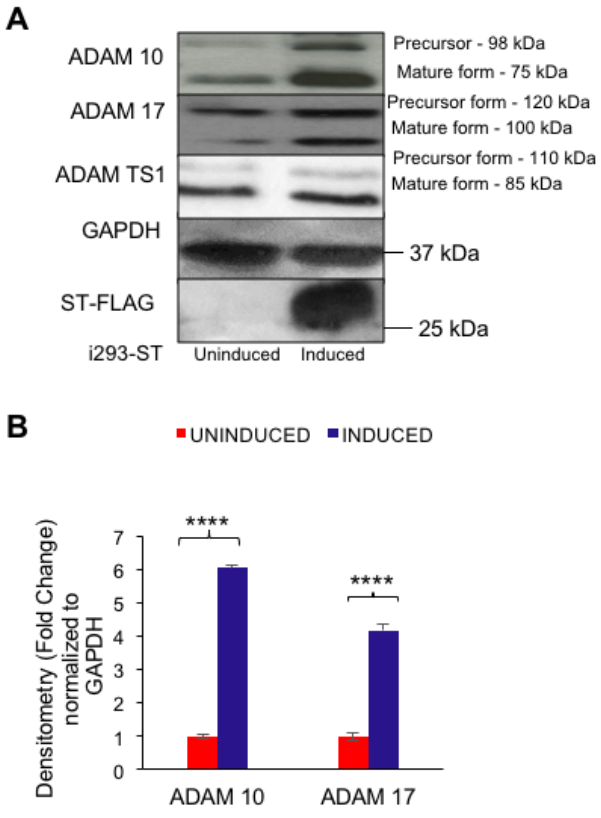

Figure 3. 9: MCPyV ST promotes upregulation of cellular sheddases. [A] i293-ST cells were left uninduced or induced and cell lysates harvested at 48 hours post-induction. Immunoblot analysis was performed on the cellular lysates and analysed with ADAM 10, 17 and TS1 specific antibodies. GAPDH was used as a measure of equal loading, a Flag antibody was used to confirm induction and MCPyV ST expression. [B] Densitometry quantification of the western blots was carried out using the Image J software and is shown a percentage of relative densitometry to the loading control, GAPDH. Data analysed using three replicates per experiment, n=3 and statistical analysis using a two-tailed t-test with unequal variance comparing MCPyV ST samples to EGFP control samples [****– P≤ 0.0001].

Upregulation of ADAM 10 and ADAM 17 was also validated in the MCPyV negative cell line MCC13, transfected with either pEGFP-C1 or pEGFP-ST expression constructs [Figure 3.10]. These results again suggest that MCPyV ST expression leads to the overexpression of specific host ADAM proteins.

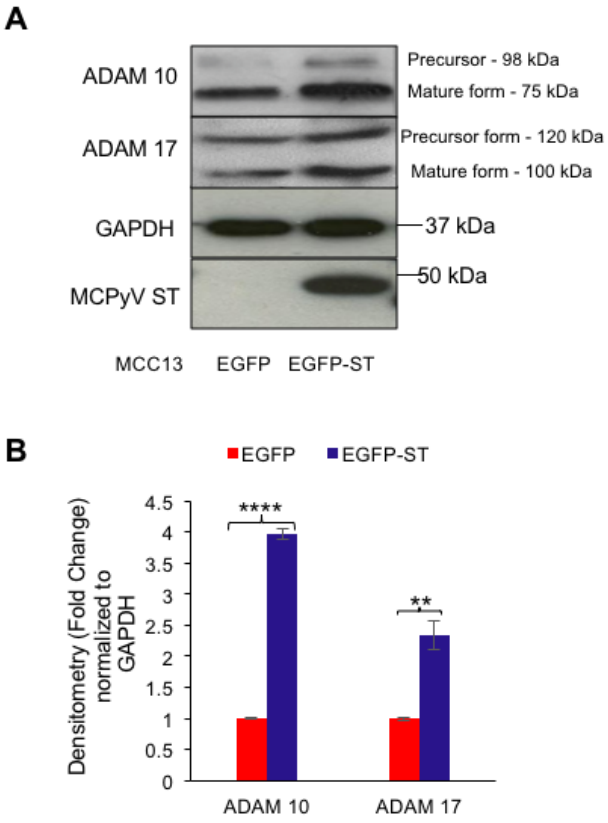

Figure 3. 10: MCPyV ST promotes upregulation of cellular sheddases [A] MCC13 cells were transfected with pEGFP-C1 control and pEGFP-ST expression plasmids for 48 hours. Immunoblot analysis was performed on the cellular lysates and analysed with ADAM 10, 17 and TS1 specific antibodies. GAPDH was used as a measure of equal loading, the T antigen antibody was used to confirm MCPyV ST expression. [B] Densitometry quantification of the western blots was carried out using the Image J software and is shown a percentage of relative densitometry to the loading control, GAPDH. Data analysed using three replicates per experiment, n=3 and statistical analysis using a two-tailed t-test with unequal variance comparing MCPyV ST samples to EGFP control samples [** – P≤ 0.01, ****– P≤ 0.0001].

3.7 Upregulation of ADAM 10 and ADAM 17 protein in MCC tumour samples

To further investigate the expression of ADAM 10 and ADAM 17 in the context of MCC tumours, multicolour immunohistochemistry analysis was performed using formalin-fixed, paraffin-embedded [FFPE] sections of primary MCC tumours. Tumour sections and isotype-matched negative controls were incubated with the

MCC tumour marker, cytokeratin 20 (Bobos *et al.*), CM2B4 [MCPyV LT specific antibody] and ADAM-specific antibodies. Figure 3.11 demonstrated noticeably higher levels of ADAM 10 and ADAM 17 expression correspondent with CK20 positive stained regions of the MCC tumour.

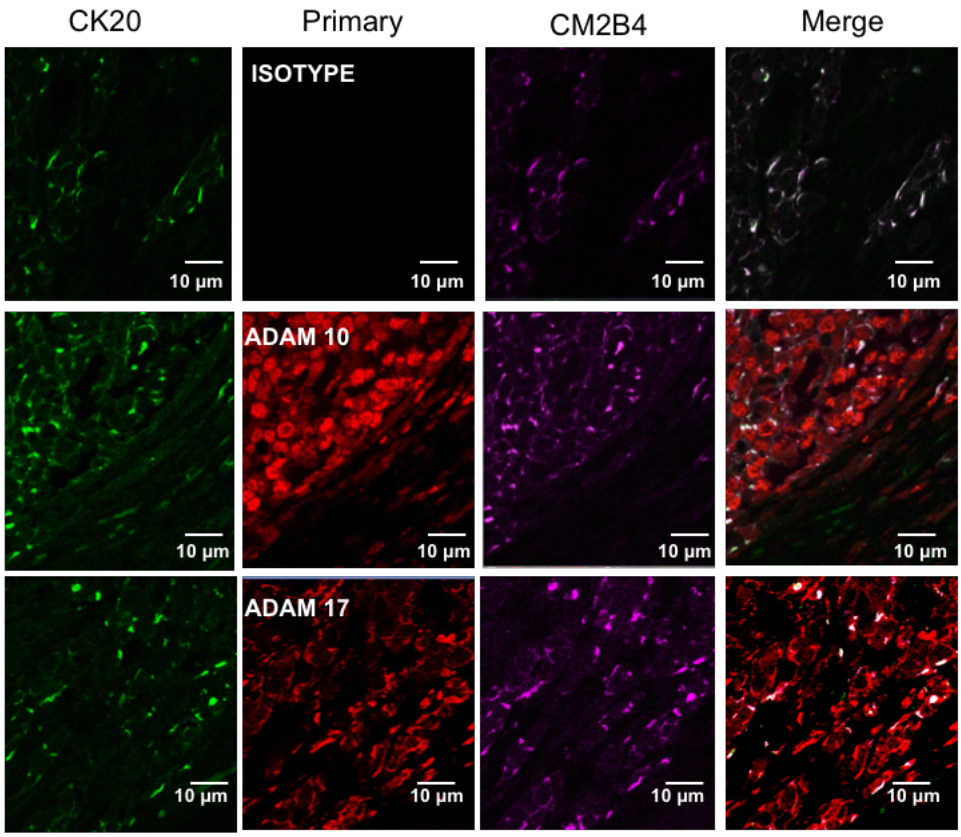

Figure 3. 11: FFPE sections of primary MCC tumours were stained with CK20, MCPyV LT and ADAM 10 and ADAM 17-specific antibodies or an isotype negative control. Sections were then incubated with Alexa Fluor-labelled secondary antibodies and analysed using a Zeiss LSM 800 confocal laser scanning microscope.

Furthermore, immunoblot analysis was performed on the cellular lysates of two unrelated MCC tumour samples comparing protein levels against a negative control non-tumour cadaveric skin sample. Results again demonstrate a similar increase in both ADAM 10 and ADAM 17 protein levels in MCC tumour samples compared to control [Figure 3.12]. Together, this provides *in vivo* evidence that ADAM 10 and ADAM 17 levels are increased in MCC tumour cells and supports the previous *in vitro* quantitative proteomic analysis conducted using i293-ST cells.

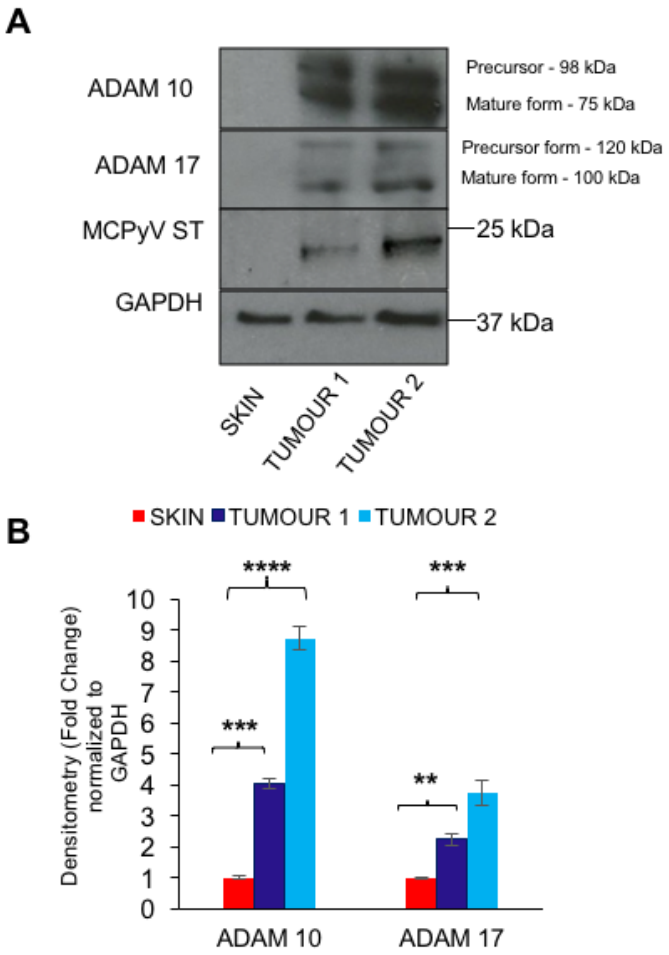

Figure 3. 12: Cellular Sheddases are expressed in MCPyV-positive MCC tumours. [A] Healthy skin, Tumour 1 and Tumour 2 were crushed using a mortar and a pestle on dry ice, lysed with RIPA buffer for 30 mins and sonicated to further homogenized the samples. Immunoblot analysis was performed on the tissue lysates and analysed with ADAM 10 and 17 specific antibodies. GAPDH was used as a measure of equal loading, the T antigen antibody was used to confirm MCPyV ST expression. [B] Densitometry quantification of the western blots was carried out using the Image J software and is shown a percentage of relative densitometry to the loading control, GAPDH. Data analysed using three replicates per experiment, n=3 and statistical analysis using a two-tailed t-test with unequal variance comparing control cadaveric skin sample to each individual tumour [** – P≤ 0.01, *** – P≤ 0.001, ****– P≤ 0.0001].

3.8 MCPyV ST increases ADAM 10 and ADAM 17 levels at a transcriptional level

Previous immunoblot analysis shows that MCPyV ST increases ADAM 10 and ADAM 17 at the protein level. To examine whether MCPyV ST increases ADAM 10 and ADAM 17 expression at a transcriptional or translational level, Real-Time Quantitative Reverse Transcription PCR [qRT-PCR] analysis was performed using complementary DNA that was reverse transcribed from Total RNA isolated from

cells expressing either EGFP or EGFP-ST. Experiments were performed in a variety of cell lines – 293, COS 7 and MCC13. In all cases, MCPyV ST expression resulted in an increase in ADAM 10 and ADAM 17 mRNA levels [Figure 3.13]. Inferring MCPyV ST induces ADAM 10 and ADAM 17 at a transcriptional level.

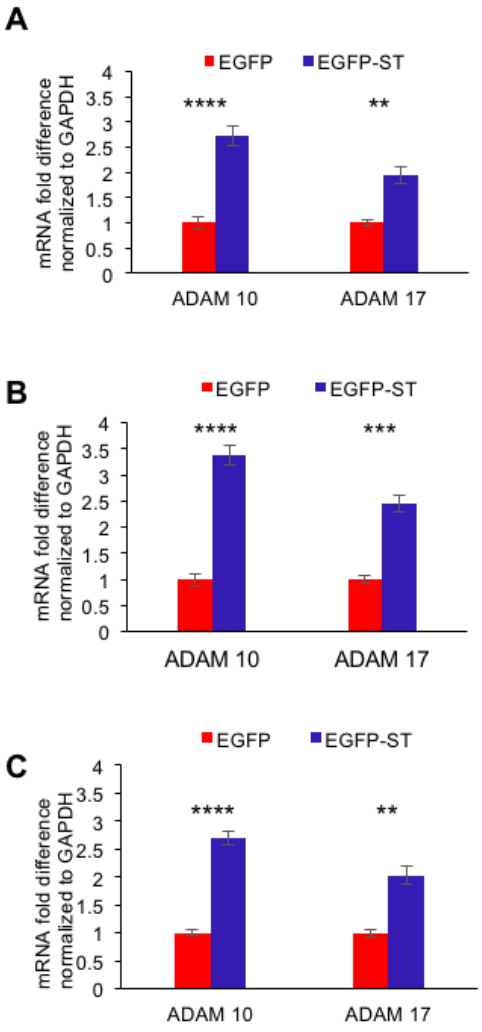

Figure 3. 13: Sheddases are upregulated at the transcript level. [A] HEK 293 cells [B] COS 7 cells [C] MCC13 cells were transfected for 48 hours with pEGFP-C1 and pEGFP-ST expression plasmids. Cellular RNA was extracted using Trizol reagent, reverse transcribed and RT-qPCR was performed. Transcript levels were analyzed using the comparative CT method [n = 3]. Data analysed using three replicates per experiment, n=3 and statistical analysis using a two-tailed t-test with unequal variance comparing MCPyV ST samples to EGFP control samples [** – P≤ 0.01, *** – P≤ 0.001, ****– P≤ 0.0001].

3.9 MCPyV ST induces upregulation of ADAM 10 and ADAM 17 proteins at the cell surface

Western blot analysis validated the upregulation of ADAM 10 and ADAM 17 upon MCPyV ST expression. However, in order for active ADAM proteins to cleave their substrate of interest, they are required to be present at the same subcellular location as their substrate (Murphy 2008). As adhesion molecule receptors are localized at the cell surface, it was important to determine whether MCPyV ST enhancement of ADAM 10 and ADAM 17 protein levels also led to their accumulation at the cell surface (Gumbiner 1996). To this end, immunofluorescence studies were performed to examine the effect of MCPyV ST expression on cell surface localisation of ADAM 10 and ADAM 17 proteins.

For immunofluorescent staining, COS 7 cells were utilized as opposed to HEK cells as they have a larger cell surface to HEK cells, hence allowing for more distinct visualization at the cell surface (Biederer and Scheiffele 2007). COS 7 cells were transfected with plasmids expressing either EGFP or EGFP-ST. Non-permeabilised cells were then stained for endogenous ADAM 10 or ADAM 17 protein localisation and distribution. Results show that endogenous ADAM 10 and ADAM 17 levels are increased at the cell surface in MCPyV ST-expressing cells in comparison to the EGFP control cells [Figure 3.14].

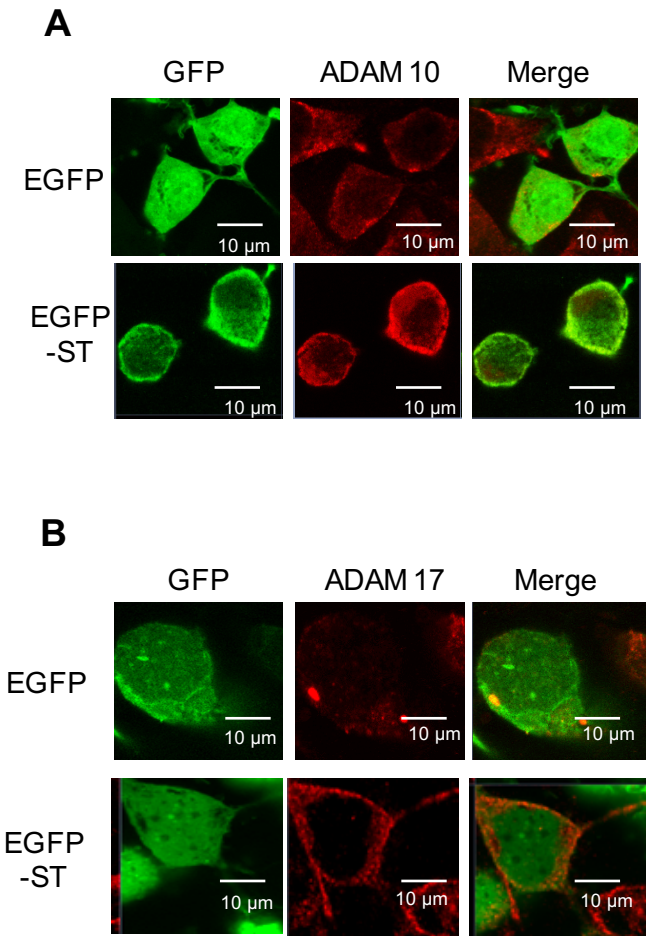

Figure 3. 14: MCPyV ST upregulates ADAM 10 and ADAM 17 at the cell surface. COS 7 cells were transfected with either pEGFP-C1 or pEGFP-ST expression vectors. After 24 h, cells were fixed and GFP fluorescence was analysed by direct visualization whereas staining of endogenous ADAM 10 was performed using specific ADAM 10 antibody. [A] ADAM 10 [B] ADAM 17.

Further validation of MCPyV ST induction of cellular sheddase cell surface accumulation was performed using flow cytometry with ADAM 10- and ADAM 17-specific antibodies (Figure 3.15). Results again show a similar accumulation of ADAM protein at the cell surface. Notably, these multiple assays have highlighted a greater accumulation of ADAM 10 compared to ADAM 17 at the cell surface. Together, these results suggest that MCPyV ST expression results in the accumulation of specific cellular sheddases, primarily ADAM 10, at the cell surface.

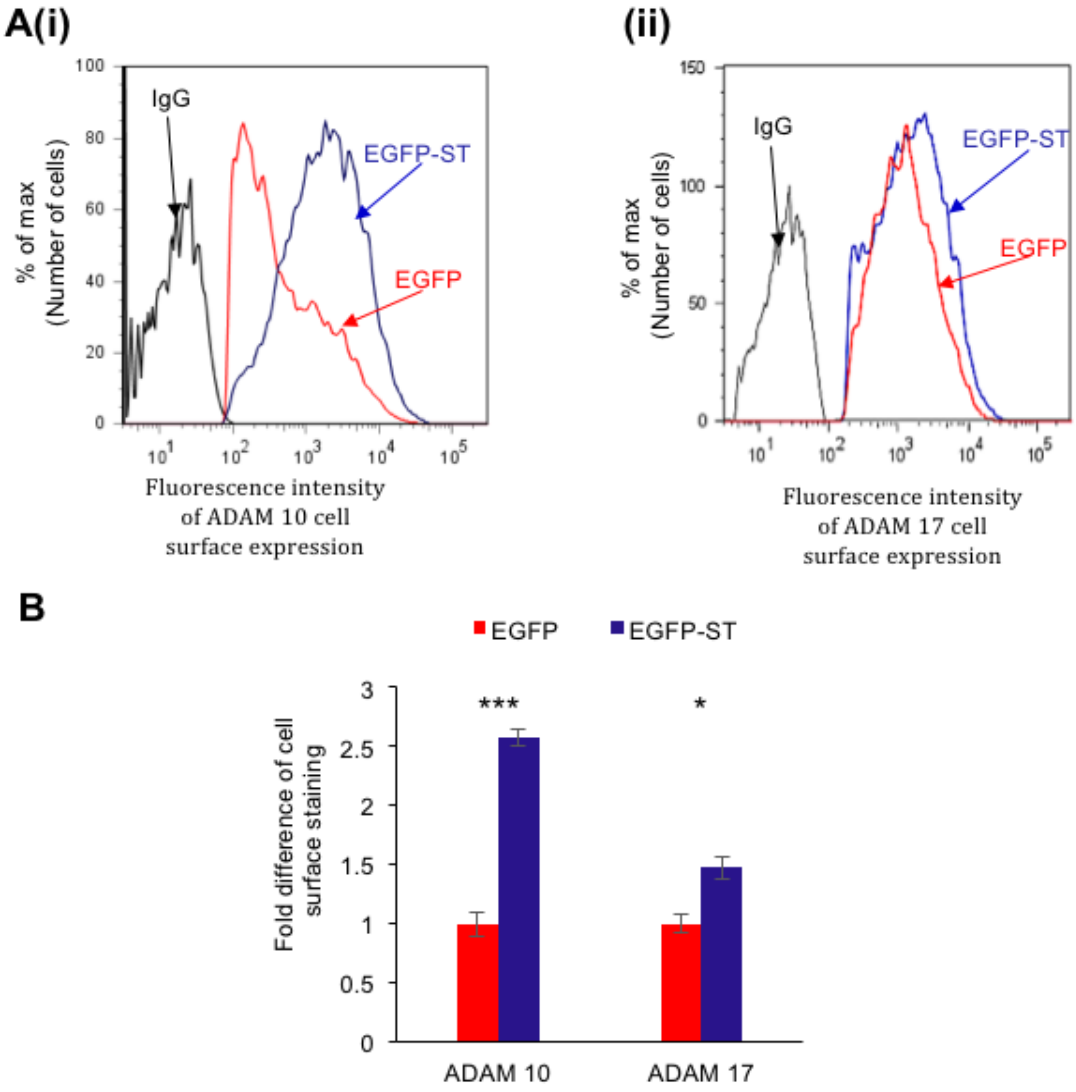

Figure 3. 15: Flow cytometry analysis of ADAM 10 and ADAM 17 expression at the cell surface [A] HEK 293 cells were transfected with pEGFP-C1 control and pEGFP-ST expression plasmids for 48 hours. Cells were harvested and spun down gently, blocked in 3% BSA and stained with a specific ADAM 10 and 17 antibodies along with a corresponding secondary antibody. Mean fluorescence intensity of both control EGFP and EGFP-ST cells where plotted as a histogram using Flow Jo analysis software. [B] Fold difference was calculated using Mean fluorescence intensity. Data analysed using three replicates per experiment, n=3 and statistical analysis using a two-tailed t-test with unequal variance comparing MCPyV ST samples to EGFP control samples [* – P≤ 0.05, *** – P≤ 0.001].

3.10 Discussion

Previous studies have shown that MCPyV ST can promote microtubule destabilization due to ST-mediated activation of Stathmin (Knight *et al.* 2015). MCPyV ST has also been shown to induce filopodia formation (Stakaityte *et al.* 2018). These observed effects on host cell microtubule and cytoskeleton components results in an increase in cell motility of MCPyV ST-expressing cells which may contribute to MCC metastatic potential. Dissociation of cell-to-cell adhesion has also been shown to contribute to cancer metastasis and tumour invasion (van Zijl *et al.* 2011).

Data presented in this chapter demonstrates that cell-to-cell junctions undergo alterations upon MCPyV ST expression. This results in increased levels of cell scatter, due to a reduction in α-E-Catenin and ZO-1 at a protein level at the cell surface. These changes were detected in multiple cell lines [HEK293, COS 7 and MCC13] as well as in MCPyV positive MCC tumour samples.

Concurrent with these observations, is the identification of an upregulation of specific cellular sheddases in the MCPyV ST quantitative proteomics dataset. Specifically, we see an increase in ADAM 10 and ADAM 17 expression, which are associated with cleavage of cell adhesion molecules and the Extracellular domain (Gooden *et al.* 2014). Results validated the upregulation ADAM 10 and ADAM 17, specifically at the cell surface where they can be catalytically active and cleave cell adhesion molecules. This may be particularly relevant for the pathogenic and metastatic potential of MCC, as cell adhesion requires links between Adherens junctions at the plasma membrane and the cytoskeleton (Sheikh *et al.* 2006).

α-E-catenin is a subtype of α-catenin proteins and in epithelial cells is concentrated at the Adherens junction where it binds cadherins to actin filaments to facilitate strong cell-to-cell adhesion (Baum and Georgiou 2011). The interaction of cadherins to the catenins is crucial for cadherin function (Aberle *et al.* 1996). Members of the α catenin family function as invasion suppressors. As such, loss of catenins in many carcinomas have been linked with increased invasiveness and metastatic potential, and reduced expression of α-E-catenin can be related to poor tumour differentiation, as well as lymph node metastasis (Tanaka *et al.* 2003). Interestingly, previous studies assessing the status of cell adhesion molecules showed downregulation of α catenin in MCC (Tanaka *et al.* 2004).

Tight junctions are a multifunctional entity. While they contribute to cell permeability and polarity, in epithelial cells, they also play a role in preventing cell dissociation by demonstrating adhesive capabilities (Martin and Jiang 2009). Studies have linked losses in cell-to-cell interaction, loss of cell adhesion as well as cell membrane degradation to changes in expression of relevant tight junction proteins (Martin 2014). ZO-1 is a critical regulator of epithelial tight junctions and loss of ZO-1 or its dysfunction has been linked to cancer metastasis (Lee *et al.* 2015). Interestingly, studies have highlighted a notable interaction with ZO-1 and α catenin where ZO-1 binding to α-catenin is essential in connecting tight junction assembly to Adherens junction formation providing a strong adhesion state (Imamura *et al.* 1999; Maiers *et al.* 2013).

Sheddases such as ADAM 10 and ADAM 17 control cell adhesion and release of cytokines growth factors in various diseases (Chow and Fernandez-Patron 2007). Specifically ADAM 10 has been shown to localize at the Adherens junction and Tight Junction in epithelial cells, which in turn promotes cell migration (Wild-Bode *et al.* 2006). As MCPyV ST has been shown to upregulate ADAM 10 to a greater extent, as well as the functionally redundant ADAM 17, at the cell surface, it can be inferred that these cellular sheddases access these junctions and promote the shedding of these specific adhesion molecules.

CHAPTER 4

Merkel Cell Polyomavirus Small T Antigen induces ADAM proteins to enhance cell dissociation and motility

4.1 Introduction

A Disintegrin and metalloproteases [ADAM] family of proteins, are membrane-anchored glycoproteins and regulatory enzymes associated with controlling cell adhesion and shedding of membrane-bound proteins to soluble forms (Wolfsberg *et al.* 1995). The superfamily of these metzincin zinc-dependent metalloprotease proteins have similar prodomains, comprising the metalloprotease domain, disintegrin domain, cysteine-rich domain, epidermal growth factor [EGF]-like domain, a transmembrane domain, and a cytoplasmic domain (Moss and Lambert 2002; Edwards *et al.* 2008).

Regulation of ADAM catalytic activity by the metalloprotease domain is controlled by a cysteine-switch mechanism (Wolfsberg *et al.* 1995). In this mechanism, a prodomain cysteine ligand binds zinc in the active site maintaining it in an inactive state. However, disruption of the cysteine-rich domain and zinc binding in the active site allows for an active ADAM protein which can then participate in sheddase activities (Wolfsberg *et al.* 1995; Moss and Lambert 2002; Milla 2011).

The primary substrates cleaved by ADAM proteins are the ectodomain of transmembrane proteins (Edwards *et al.* 2008). Substrate specificity is provided by the disintegrin domain positioned downstream of the protease domain. It is highly conserved, comprising approximately 90 amino acids and is found in all ADAM proteins (Eto *et al.* 2000). As ADAM proteins are detected on cell membranes, it is thought that they are primarily involved in the cleavage of membrane-anchored cell surface adhesion molecules, such as Cadherins, Selectins and Integrins (Katto and Mahlknecht 2011).

Studies have linked ADAM proteins with various biological functions including but not limited to neurogenesis, angiogenesis and cancer (Rocks *et al.* 2008). Of note, various studies have implicated over-expression of various ADAM proteins correlating to the aggressiveness and progression of tumour development (Duffy *et al.* 2009). This infers that these sheddases could be functionally significant in the process of cancer development and the dissemination of metastatic tumour cells (Rocks *et al.* 2008).

Here we elucidate a potential role of MCPyV ST-induced ADAM 10 and 17 proteins in cell junction breakdown and increased cell dissociation. This suggests that MCPyV ST can enhance cellular migration and invasion by increasing ADAM protein expression. This may provide a novel biomarker of MCC prognosis as

vairious ADAMs are emerging as possible cancer biomarkers for aiding cancer diagnosis as well as possibly predicting patient outcome. This is as a result of various ADAM proteins being detected in the fluids of Cancer patients. (Duffy *et al.* 2011). Moreover, linking sheddases to MCPyV-positive MCC metastasis may provide novel therapeutic interventions.

4.2 ADAM 10 is required for MCPyV ST-induced cell junction disruption

Cell-to-cell adhesion and cell interaction to the extracellular matrix is essential for tissue integrity (Joyce and Pollard 2009). Interference of cell-to-cell adhesion consequently enhances cell scattering and is vital for the initiation of cell migration (de Rooij *et al.* 2005). In chapter 3, results showed that MCPyV ST expression led to enhancement of cell-to-cell dissociation and cell scatter. This increase in cell-to-cell dissociation was a result of MCPyV ST-mediated dysregulation of cell-to-cell junctions.

Furthermore, MCPyV ST induced ADAM 10 and 17 upregulation, specifically at the cell surface where these cellular sheddases can function in deregulating cell-to-cell junctions. Therefore, it was pertinent to analyse the overall structure of cell junctions in MCPyV ST-expressing cells with limited sheddases function. In order to determine whether ADAM 10 and/or 17 are involved in MCPyV ST-induced cell-to-cell dissociation, cell scatter and cell motility, two commercially available inhibitors were utilized. GI254023X is a small molecule which contains hydroxamate moieties which inhibits ADAM 10 by binding zinc at the active site, maintaining it in an inactive state (Yiotakis and Dive 2008). Similarly, TAPI-2, is also a hydroxamate-based broad spectrum inhibitor (Arribas *et al.* 1996). Initially, non-cytotoxic concentrations of these inhibitor were determined by MTS assay in various cell lines [Figure 4.1].

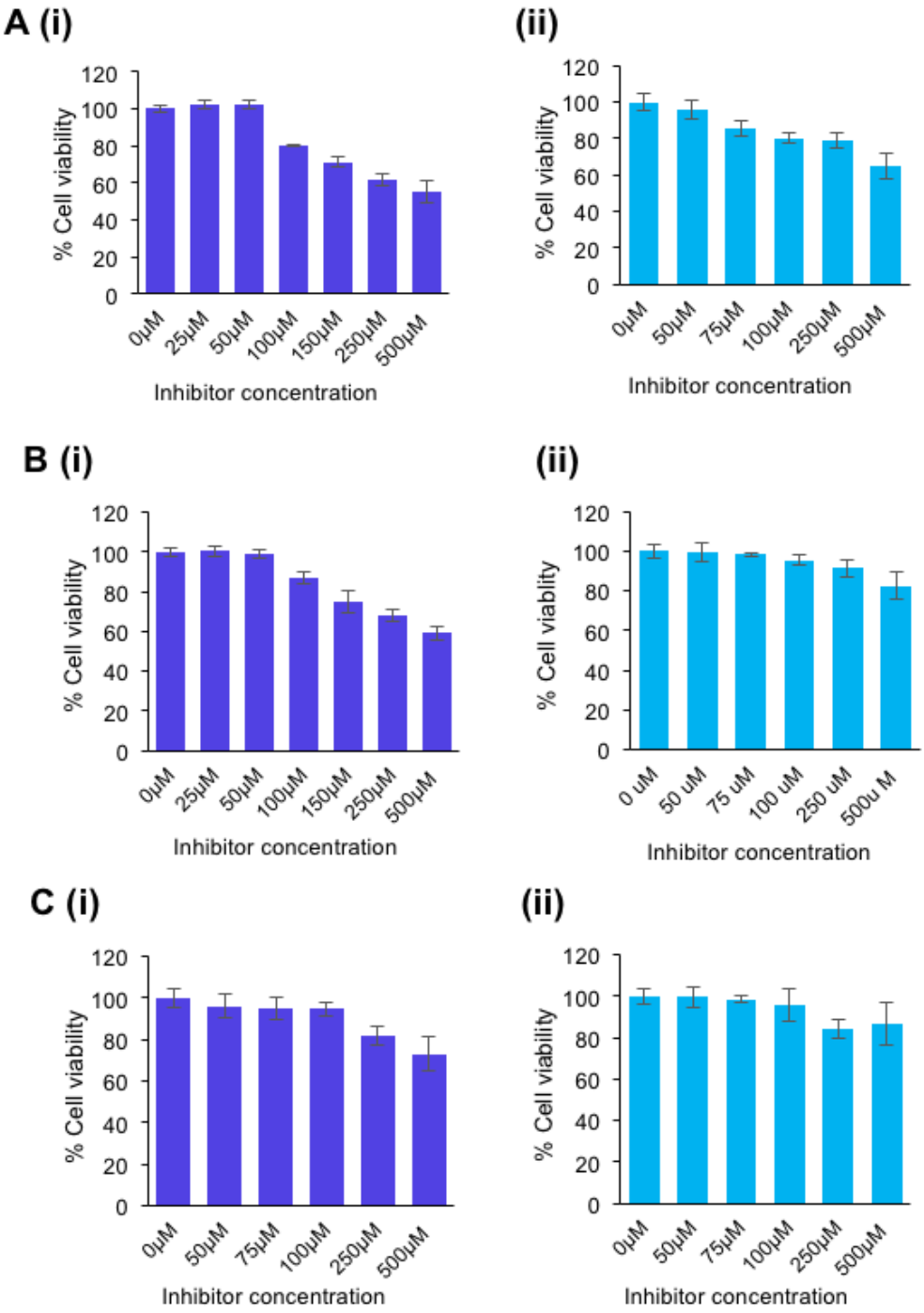

Figure 4. 1: Cell viability assays using Sheddase inhibitors. MTS assays were performed on [A]i293-EGFP-ST [B]COS 7 [C]MCC13 cell lines were incubated with various concentrations of [i] GI254023X, and [ii]TAPI-2 for 24 hours.

To determine whether ADAM 10 and 17 were required for the previously observed MCPyV ST-induced breakdown of cell-to-cell junctions, EGFP and EGFP-ST-expressing cells were incubated for 24 hours in the absence or presence of each ADAM inhibitor at non-cytotoxic concentration, 100μM. Interestingly similar concentrations have been shown to inhibit ADAM 10 sheddase activity (Gooden *et al.* 2014; Mullooly *et al.* 2015). Subsequent to the incubation period, cells were fixed, and stained with an α-E-catenin specific antibody, whereas EGFP expression was visualised by direct fluorescence. Previous findings in chapter 3 had demonstrated that MCPyV ST expression leads to an incomplete staining of the cell junctions signifying a decrease in cell-to-cell adhesion, in contrast EGFP expressing control cells demonstrated a complete staining on the cell surface. Notably, MCPyV ST-expressing cells incubated with both GI254023X and TAPI-2 showed a complete staining of the cell surface inferring that ADAM 10 inhibition and possibly ADAM 17 prevents MCPyV ST-mediated breakdown of cell-to-cell adhesion [Figure 4.2]. There was no observed alteration at the cell junction of EGFP control cells after incubation with either inhibitors.

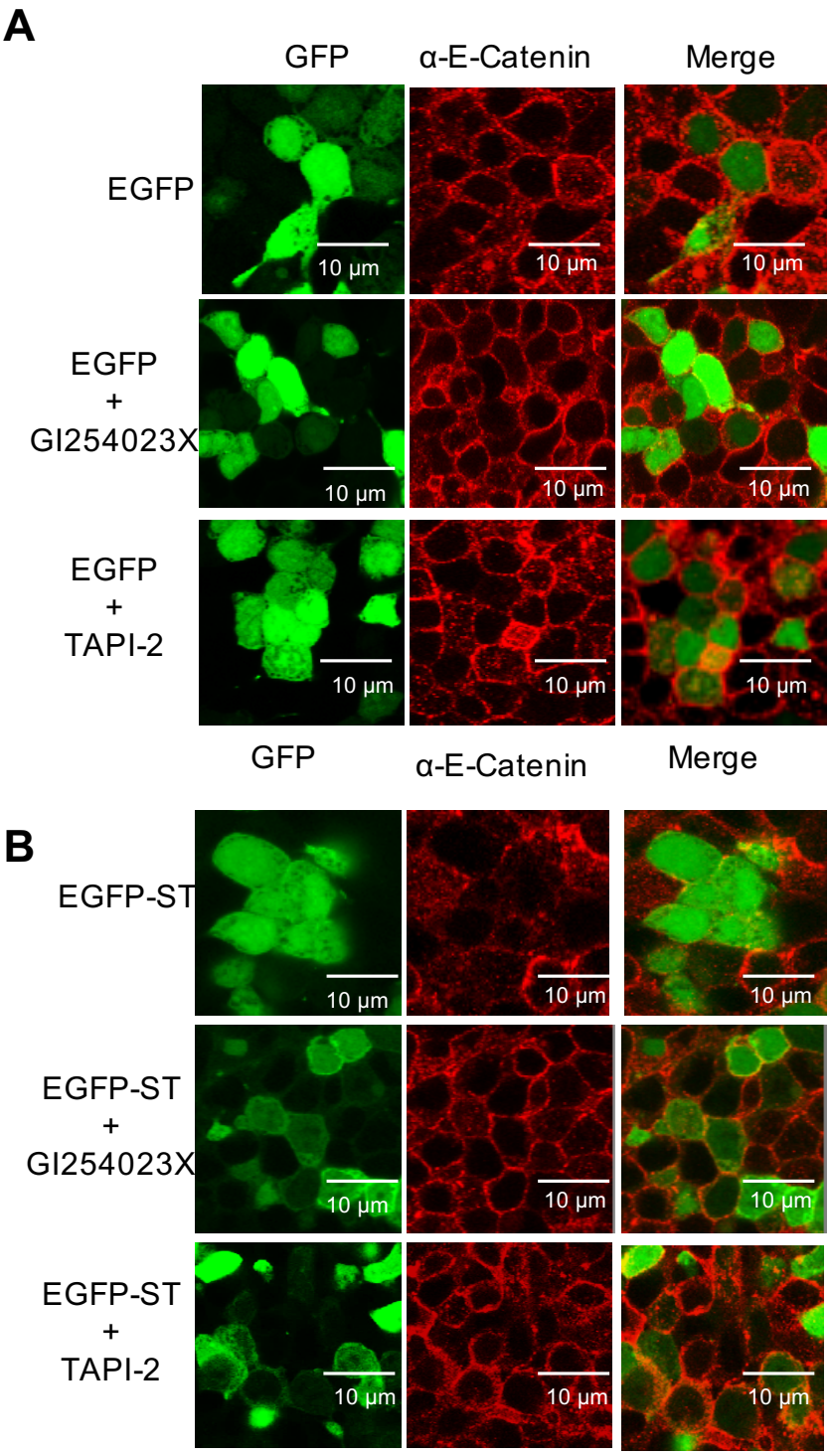

Figure 4. 2: ADAM protein activity is essential for MCPyV ST-induced cell junction disruption. Cell junction staining using a specific α-E-catenin specific antibody, for EGFP control and EGFP-ST-expressing cells in the presence of an ADAM protein inhibitors. [A] Upper panel shows HEK 293 cells transfected with pEGFP-C1. Second panel shows HEK 293 cells transfected with pEGFP-C1 incubated in 100μM GI254023X and Third panel shows HEK 293 cells transfected with pEGFP-C1 incubated in 100μM TAPI-2. [B] Upper panel shows HEK 293 cells transfected with pEGFP-ST. Second panel shows HEK 293 cells transfected with pEGFP-ST incubated in 100μM GI254023X and Third panel shows HEK 293 cells transfected with pEGFP-ST incubated in 100μM TAPI-2.

It was also important to validate and quantify recovery of structural integrity by assessment of α-E-catenin expression at the cell surface upon MCPyV ST expression. Flow cytometry was therefore utilized to quantify the levels of α-E-catenin in EGFP and EGFP-ST-expressing cells in the presence and absence of each ADAM protein inhibitor. Results demonstrated a recovery of MCPyV ST-mediated downregulation of α-E-catenin at the cell surface in the presence of GI254023X and TAPI-2 [Figure 4.3]. Together, these results suggest that ADAM proteins are required for the breakdown of cell-to-cell junction upon MCPyV ST expression.

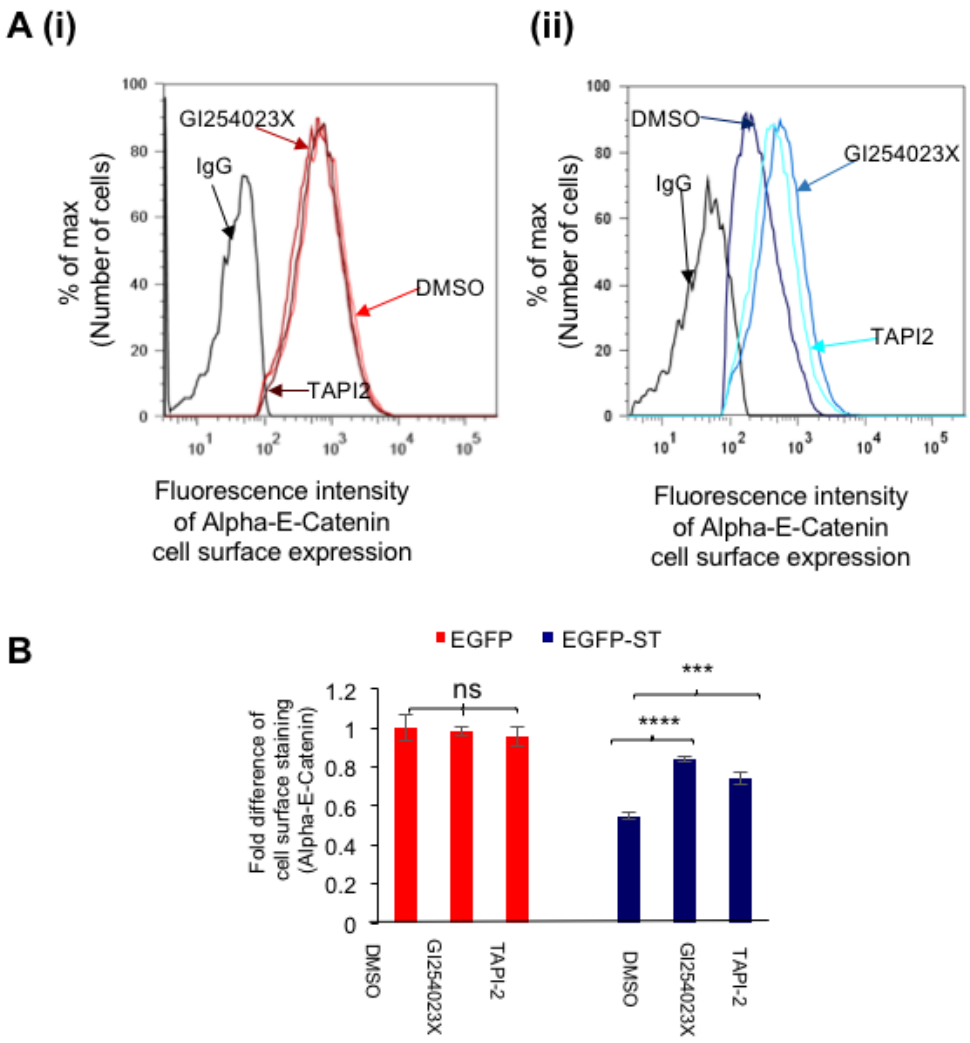

Figure 4. 3: Sheddase expression is required for MCPyV ST-induced cell junction disruption. Flow cytometry was used to quantify recovery of α-E-catenin expression at the cell surface upon incubation of Sheddase inhibitors [Ai] α-E-catenin expression – HEK 293 cells transfected with pEGFP-C1, HEK 293 cells transfected with pEGFP-C1 incubated in 100 μM GI254023X and HEK 293 cells transfected with pEGFP-C1 incubated in 100uM TAPI-2. [Aii] HEK 293 cells transfected with pEGFP-ST, HEK 293 cells transfected with pEGFP-ST incubated in 100uM GI254023X and HEK 293 cells transfected with pEGFP–ST incubated in 100 μM TAPI-2. Mean fluorescence intensity of both control EGFP and EGFP-ST cells where plotted as a histogram using Flow Jo analysis software. [B] Fold difference was calculated using Mean fluorescence intensity. Data analysed using three replicates per experiment, n=3 and statistical analysis using a two-tailed t-test with unequal variance comparing incubated inhibitor samples to their designated DMSO incubated control [*** – P≤ 0.001, ****– P≤ 0.0001].

4.3 Sheddases are essential for MCPyV ST-induced cell dissociation

To confirm that ADAM 10 and/or 17 were required for the enhanced cell dissociation observed in MCPyV ST-expressing cells, a cell scatter assay was repeated in EGFP control cells and MCPyV ST-expressing cells, in the absence

and presence of each inhibitor. Addition of the ADAM 10 specific inhibitor resulted in little change in control cells. However, a significant reduction in cell dissociation, over the course of 48 hours, was observed when EGFP-ST-expressing cells where incubated in GI254023X compared to DMSO-treated MCPyV ST-expressing cells [Figure 4.4]

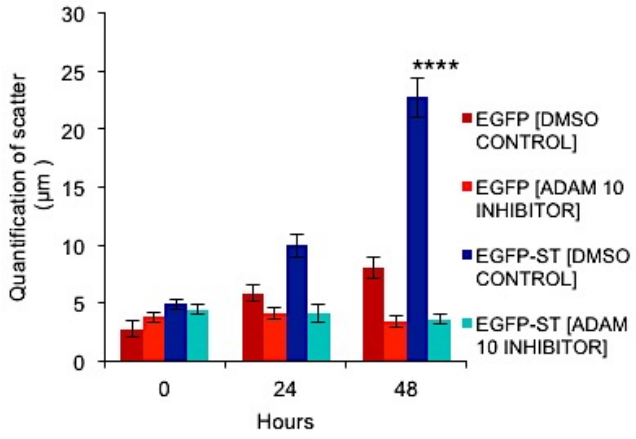

Figure 4. 4: Quantification of cell dissociation of EGFP and EGFP-ST cells in the absence or presence of GI254023X – ADAM 10 specific inhibitor. Cell scatter assay in EGFP and EGFP-ST cells with cells incubated with ADAM 10 inhibitor GI254023x from T=0hr to T=48 hours. Statistical analysis using a two-tailed t-test with unequal variance comparing MCPyV ST control samples to MCPyV ST inhibitor incubated sample at T = 48 hours [****– P≤ 0.0001].

A similar experiment was also performed with the dual ADAM 10/17 inhibitor, TAPI-2. A comparable level of cell dissociation inhibition was observed using the ADAM10/17 dual inhibitor, TAPI-2 as with the ADAM 10 inhibitor alone [Figure 4.5]. This infers that no enhancement of inhibition is seen by simultaneously targeting both ADAM 10 and 17, which may suggest that the prominent sheddase in MCPyV ST functioning is ADAM 10.

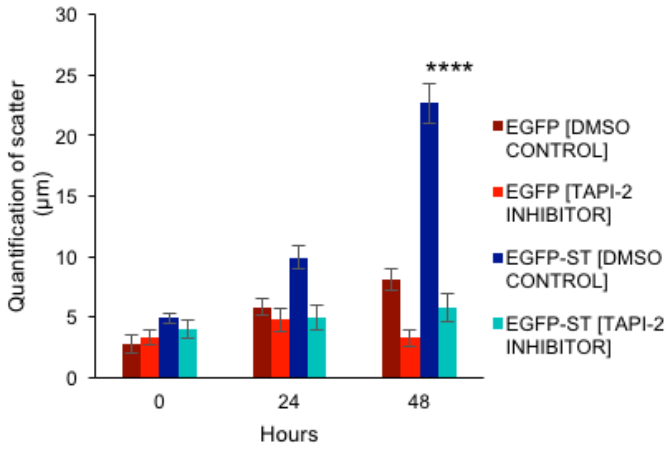

Figure 4. 5: Quantification of cell dissociation of EGFP and EGFP-ST cells in the absence or presence of TAPI-2– Dual ADAM 10/17 inhibitor. Cell scatter assay in EGFP and EGFP-ST cells with cells incubated in dual ADAM 10/17 inhibitor TAPI-2 from T=0hr to T=48 hours. Statistical analysis using a two-tailed t-test with unequal variance comparing MCPyV ST control samples to MCPyV ST inhibitor incubated sample at T = 48 hours [****– P≤ 0.0001].

4.4 ADAM 10 is required for MCPyV ST-induced cell dissociation

To confirm the precise role of ADAM 10 in MCPyV ST-induced cell dissociation, specific siRNA-mediated depletion of ADAM 10 was performed in EGFP and EGFP-ST-expressing HEK 293 cells. ADAM 10 depletion was confirmed by immunoblotting, showing a significant reduction in the MCPyV ST-induced levels of ADAM 10 protein [Figure 4.6].

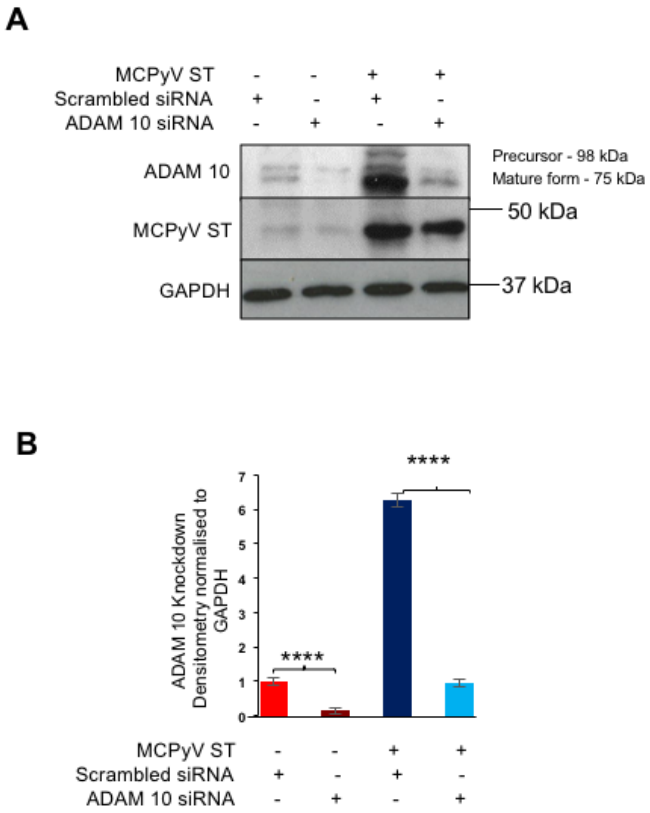

Figure 4. 6: siRNA mediated knockdown of ADAM 10. [A] HEK 293 cells were transfected ADAM 10 specific siRNA and scramble negative control. 48 hours post initial transfection, pEGFP-C1 and pEGFP-ST where transfected in the knockdown negative and positive cells. 48 hours subsequent to transfection, cells were harvested and lysed. Cell lysates were then probed for successful knockdown with a specific ADAM 10 antibody GAPDH was used as loading control and T antigen antibody was used to show MCPyV ST expression. [B] Densitometry quantification of the western blots was carried out using the Image J software and is shown a percentage of relative densitometry to the loading control, GAPDH. Data analysed using three replicates per experiment, n=3 and statistical analysis using a two-tailed t-test with unequal variance comparing siRNA knockdown to their designated siRNA scrambled control [****– P≤ 0.0001].

To assess the effect of ADAM 10 depletion on the levels of the Adherens junction marker α-E-catenin and Tight Junction marker ZO-1, immunoblotting was performed in EGFP and MCPyV EGFP-ST-expressing cells in the presence of scrambled or ADAM 10 specific siRNA. α-E-catenin and ZO-1 protein levels were previously shown to be reduced upon MCPyV ST expression [Chapter 3]. However, here ADAM 10 depletion restored both α-E-catenin and ZO-1 proteins levels to control levels in MCPyV ST-expressing cells [Figure 4.7].

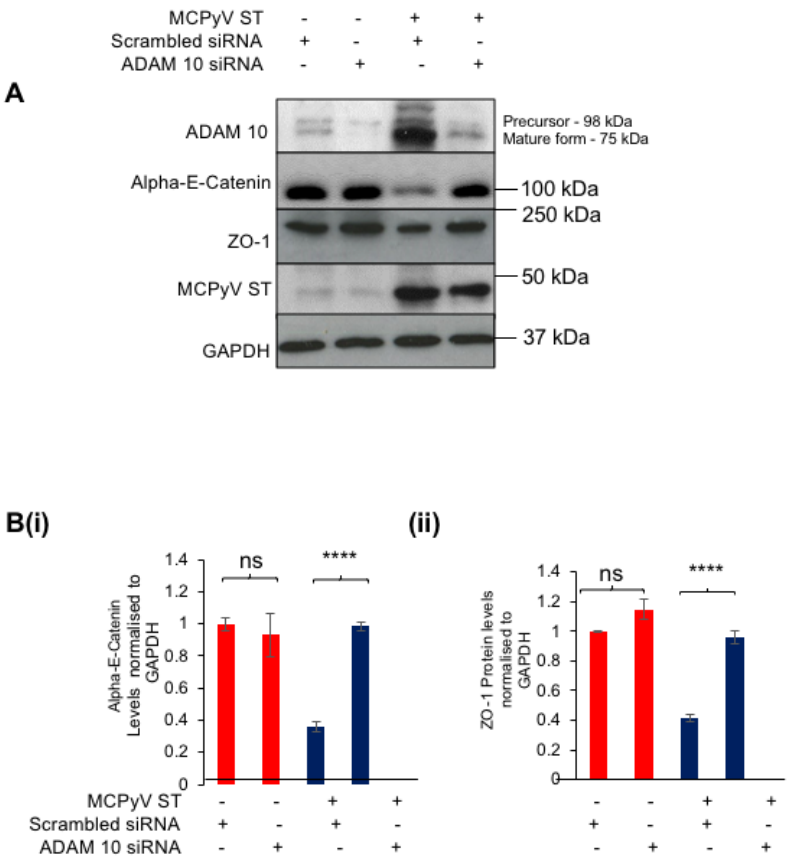

Figure 4. 7: siRNA specific knockdown of ADAM 10 leads to recovery of α-E-catenin and ZO-1 expression. [A] Cell lysates were probed for successful knockdown with specific ADAM 10 antibody as well as ZO-1 and α-E-catenin expression. GAPDH was used as loading control and T antigen antibody was used to show MCPyV ST expression. [B] GAPDH was used as a measure of equal loading and Densitometry quantification of the western blots was carried out using the Image J software and is shown a percentage of relative densitometry to the loading control, GAPDH. Data analysed using three replicates per experiment, n=3 and statistical analysis using a two-tailed t-test with unequal variance comparing siRNA knockdown to their designated siRNA scrambled control [ns – $P>0.05$, ****– $P\leq 0.0001$].

To determine if the observed rescue of cell junction-associated proteins upon ADAM 10 depletion affects MCPyV ST-induced cell dissociation, a cell scatter assay was performed as previously described in EGFP control cells or MCPyV ST-expressing cells, in the presence of either scramble or ADAM 10-specific siRNAs. Depletion of ADAM 10 resulted in a similar reduction in cell dissociation levels, as observed using the ADAM 10 specific inhibitor, GI254023X [Figure 4.8]. These results suggest that ADAM 10 is required for MCPyV ST-mediated cell dissociation.

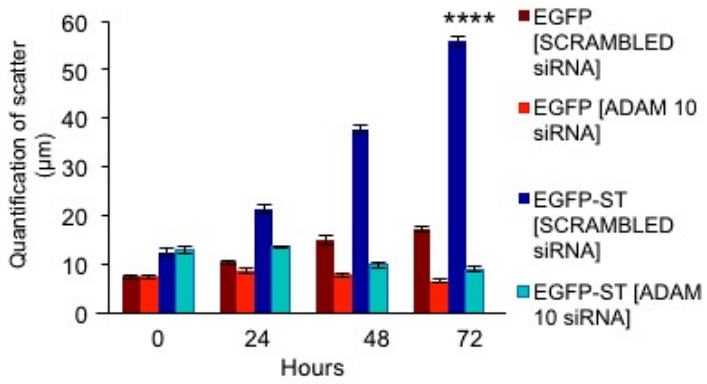

Figure 4. 8 : Quantification of cell dissociation of EGFP and EGFP-ST cells in the absence or presence of ADAM 10 specific siRNA. Cell scatter assay in EGFP and EGFP-ST cells comparing scramble and ADAM 10 depletion from T=0hr to T=72 hours. [****– P≤ 0.0001] Statistical analysis using a two-tailed t-test with unequal variance comparing MCPyV ST ADAM 10 siRNA knockdown to designated siRNA scramble control at T = 72 hours [****– P≤ 0.0001].

4.5 MCPyV ST induces cell motility in a variety of cell lines

MCPyV ST-expressing cells have been shown to increase cell motility (Knight *et al.* 2015). Using IncuCyte live cell imaging, it has been shown that EGFP-ST-expressing cells are significantly more motile than EGFP control cells [P<0.0001]. Therefore, in order to assess the role of ADAM proteins in cell motility it was initially important to set up a reproducible cell motility assay. HEK 293 cells were therefore transfected with either pEGFP-C1 or pEGFP-ST expression plasmids. The cells were then incubated and imaged in the IncuCyte zoom for 24 hours and cells were tracked with the Fiji manual tracking program.

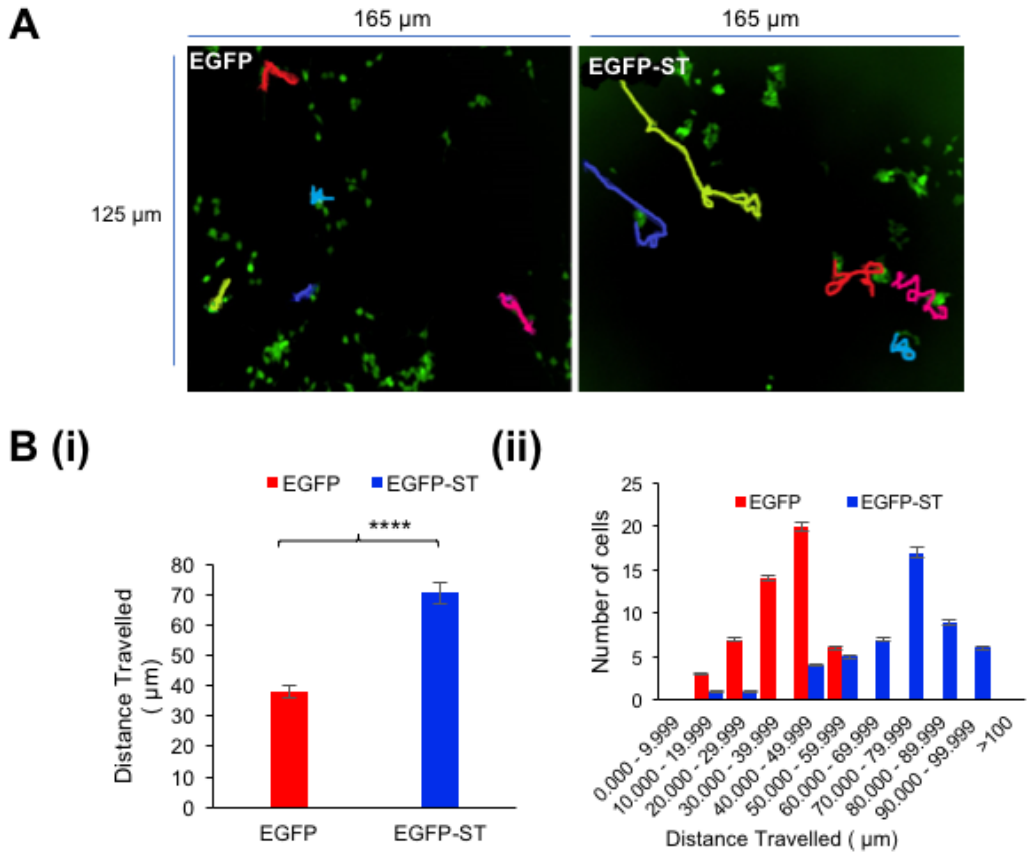

Figure 4. 9: MCPyV ST expression promotes cell motility in HEK 293 cells. [A] Cells were transfected with either pEGFP-C1 or pEGFP-ST and 24 hours after cell motility was analysed using an IncuCyte kinetic live cell imaging system. Images were taken over the course of 24 hours every 30 minutes and cell movement tracked using Image J software. Red, blue, cyan and green line tracks depict the path of cell movement over the time course. [Bi] The average distance moved by transfected cells was measured in µm [n=50]. [Bii] Quantification of distance travelled for EGP and EGFP-ST-expressing cells 10 µm ranges. Data analysed using three replicates per experiment, n=3 and statistical analysis using a two-tailed t-test with unequal variance comparing MCPyV ST samples to EGFP control samples [****– P≤ 0.0001].

Figure 4.9 shows that MCPyV ST-expressing HEK 293 cells travel significantly further than EGFP control cells Similar results were observed in COS7 and MCC13 transfected cells [Figure 4.10].

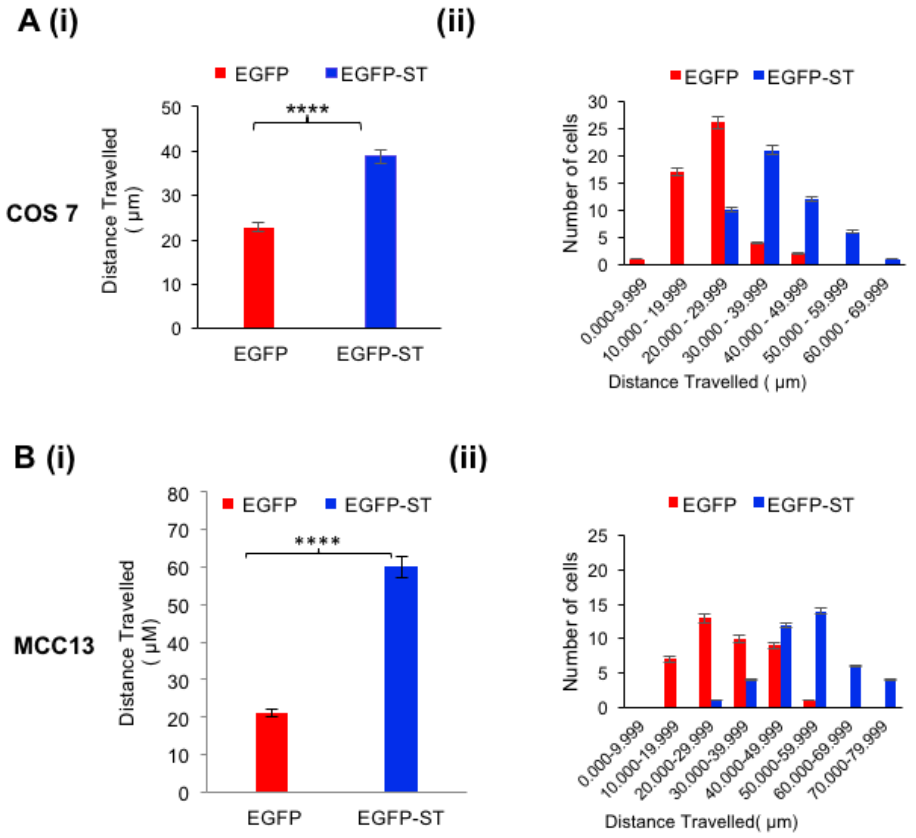

Figure 4. 10: MCPyV ST expression promotes cell motility in alternative cell lines cells. Cells were transfected with either pEGFP-C1 or pEGFP-ST and 24 hours after cell motility was analysed using an IncuCyte kinetic live cell imaging system. Images were taken over the course of 24 hours every 30 minutes and cell movement tracked using Image J software [A] COS 7 [B] MCC13 [i] The average distance moved by transfected cells was measured in µm [n=50]. [ii] Quantification of distance travelled for EGP and EGFP-ST-expressing cells 10 µm ranges. Data analysed using three replicates per experiment, n=3 and statistical analysis using a two-tailed t-test with unequal variance comparing MCPyV ST samples to EGFP control samples [ns – P>0.05, ****– P≤ 0.0001].

4.6 Sheddase inhibitors impedes the ability of MCPyV ST-mediated cell motility

Preceding results have demonstrated the crucial role of ADAM proteins in enhancing cell junction breakdown and cell dissociation in MCPyV ST-expressing cells. As cell junction integrity has been linked to the initiation of cell migration, it was important to investigate if ADAM proteins have any downstream influence on the motility and migratory potential of MCPyV ST-expressing cells. Therefore, the migratory potential of EGFP control and EGFP-ST HEK 293 expressing cells were assessed using IncuCyte kinetic live cell imaging, in the absence or presence of non-cytotoxic concentrations of the ADAM 10-specific inhibitor [GI254023X], as previously described.

Incubation of the ADAM 10 [GI254023X] inhibitor show a minimal, but insignificant decrease in the motility of EGFP control cells. In comparison, ADAM 10 inhibition resulted in a substantial decrease in the distance travelled of MCPyV ST-expressing cells, similar to EGFP control cell migration [Figure 4.11].

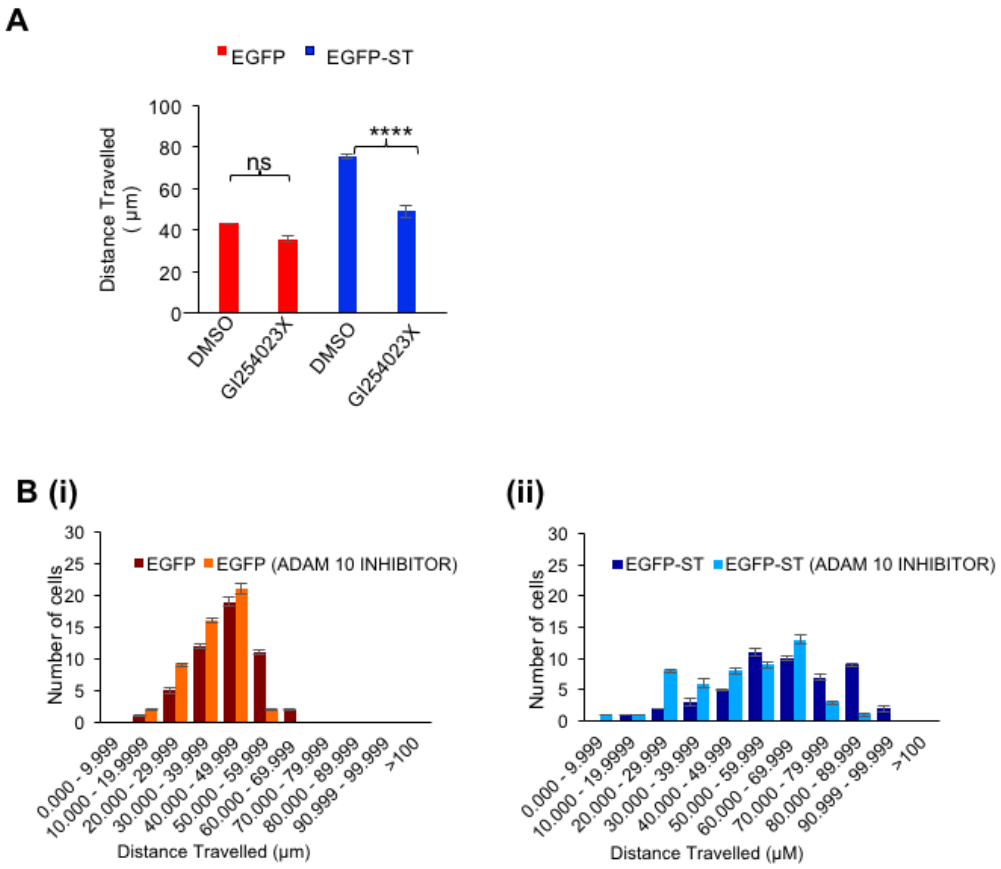

Figure 4. 11: Live cell imaging shows MCPyV ST-induced cell motility is dependent ADAM 10. [A] The average distance travelled by EGFP and EGFP-ST cells in the absence or presence of the ADAM 10 inhibitor [n=50]. [B] Quantification of distance travelled for [i] EGFP control cells and EGFP cells incubated with ADAM10-specific inhibitor GI254023X and [ii] EGFP-ST control cells and EGFP-ST cells incubated with ADAM10-specific inhibitor GI254023X. Motility was compared to each other in 10 µm ranges [n=50]. Data analysed using three replicates per experiment, n=3 and statistical analysis using a two-tailed t-test with unequal variance comparing incubated inhibitor samples to their designated DMSO incubated control [ns – P>0.05, ****– P≤ 0.0001].

A similar trend was also observed with the dual ADAM 10/17 inhibitor [TAPI-2] [Figure 4.12]. Again, this suggests that inhibition of ADAM 10 alone is sufficient to repress the MCPyV ST-induced cell migratory phenotype.

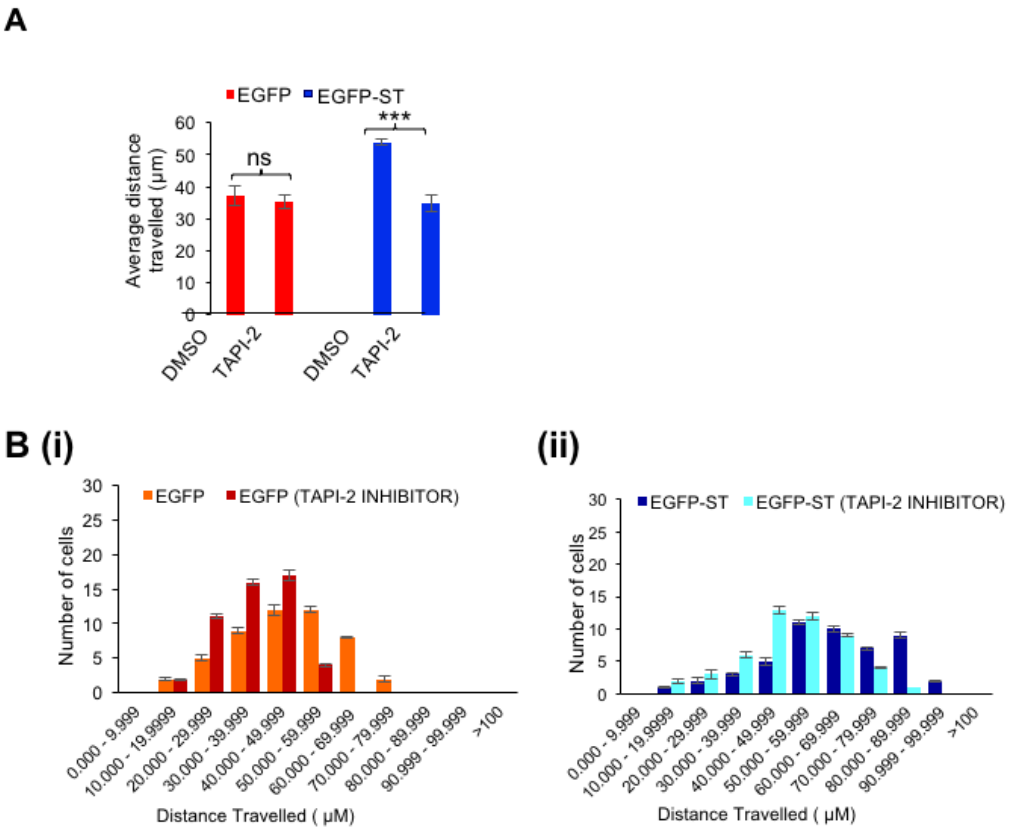

Figure 4. 12: Live cell imaging shows a dependence of MCPyV ST-induced cell motility on sheddases. [A] The average distance travelled by EGFP and EGFP-ST cells in the absence or presence of the dual ADAM 10/17 inhibitor TAPI-2 [n=50]. [B] Quantification of distance travelled for [i] EGFP control cells and EGFP cells incubated with dual ADAM 10/17 inhibitor TAPI-2 and [ii] EGFP-ST control cells and EGFP-ST cells incubated with the dual ADAM 10/17 inhibitor TAPI-2. Motility was compared to each other in 10 µm ranges [n=50]. Data analysed using three replicates per experiment, n=3 and statistical analysis using a two-tailed t-test with unequal variance comparing incubated inhibitor samples to their designated DMSO incubated control [ns – P>0.05, *** – P≤ 0.001].

To validate that the observed downregulation of MCPyV ST-mediated cell migration in HEK transfected cells is dependent on ADAM 10 inhibition similar live cell imaging experiments were carried in additional cell lines. Live cell imaging was carried out in COS 7 and MCC13 pEGFP-C1 and pEGFP-ST transfected cells in the absence or presence of either ADAM 10 specific or Dual ADAM 10/17 inhibitors. Results validated previous observations in HEK 293 cells showing a decrease in MCPyV ST-mediated cell motility upon addition of ADAM protein inhibitors [Figure 4.13].

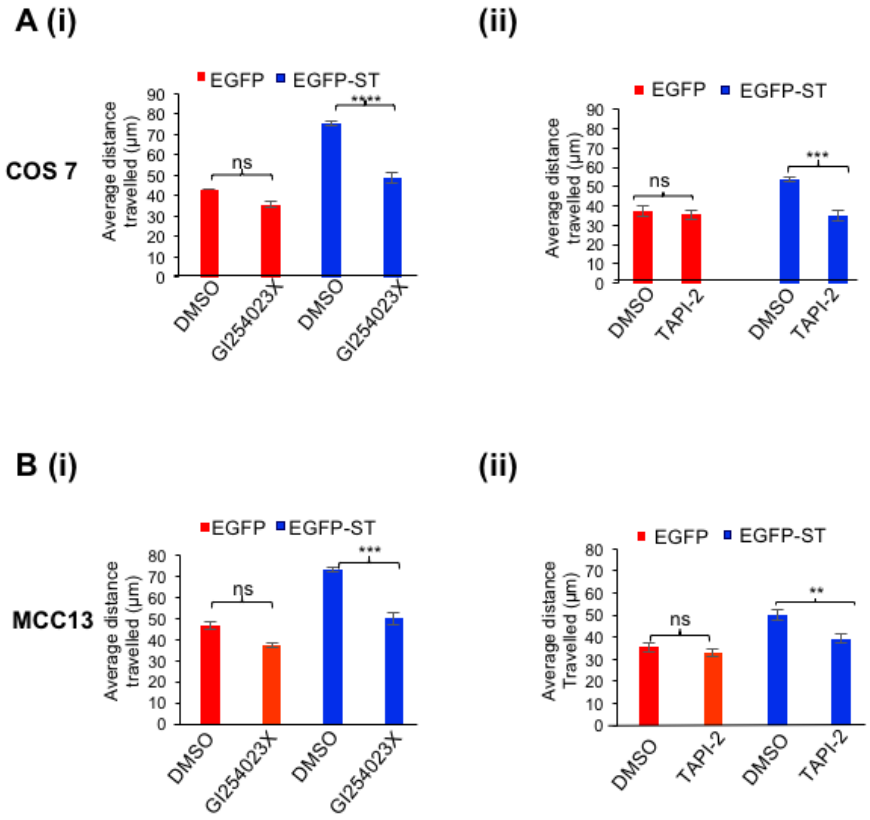

Figure 4. 13: Live cell imaging shows a dependence of MCPyV ST-induced cell motility on ADAM proteins. [A] COS7 [B] MCC13 [i]The average distance travelled by EGFP and EGFP-ST cells in the absence or presence of the ADAM 10 inhibitor [n=50]. [ii] The average distance travelled by EGFP and EGFP-ST cells in the absence or presence of TAPI-2 inhibitor [n=50]. Data analysed using three replicates per experiment, n=3 and statistical analysis using a two-tailed t-test with unequal variance comparing incubated inhibitor samples to their designated DMSO incubated control [ns – P>0.05, ** – P≤ 0.01, *** – P≤ 0.001, ****– P≤ 0.0001].

4.7 Depletion of ADAM 10 reduces MCPyV ST-induced cell motility

Previous results have shown that various ADAM 10 inhibitors are sufficient to decrease the migration potential of MCPyV ST-expressing cells. It was therefore important to corroborate the use of ADAM-specific inhibitors using similar live cell imaging motility assays in siRNA-mediated ADAM 10 depleted EGFP and EGFP-ST-expressing cells. HEK cells were therefore transfected with scrambled or ADAM 10 specific siRNA and pEGFP-C1 or pEGFP-ST expression plasmids. Subsequent to successful knockdown of ADAM 10 [Figure 4.6], cells were then imaged using the IncuCyte kinetic imaging system, every 30 minutes for 24 hours. Similar to the inhibitor studies, ADAM 10 depletion again resulted in a reduction in the motility of MCPyV ST-expressing cells, similar to levels of control EGFP-expressing cells [Figure 4.14]. Importantly, ADAM 10 depletion had no effect on the motility of EGFP

expressing cells. Taken together, these results suggest that ADAM 10 dysregulation of the cell junction is required for MCPyV ST-mediated enhanced cell motility.

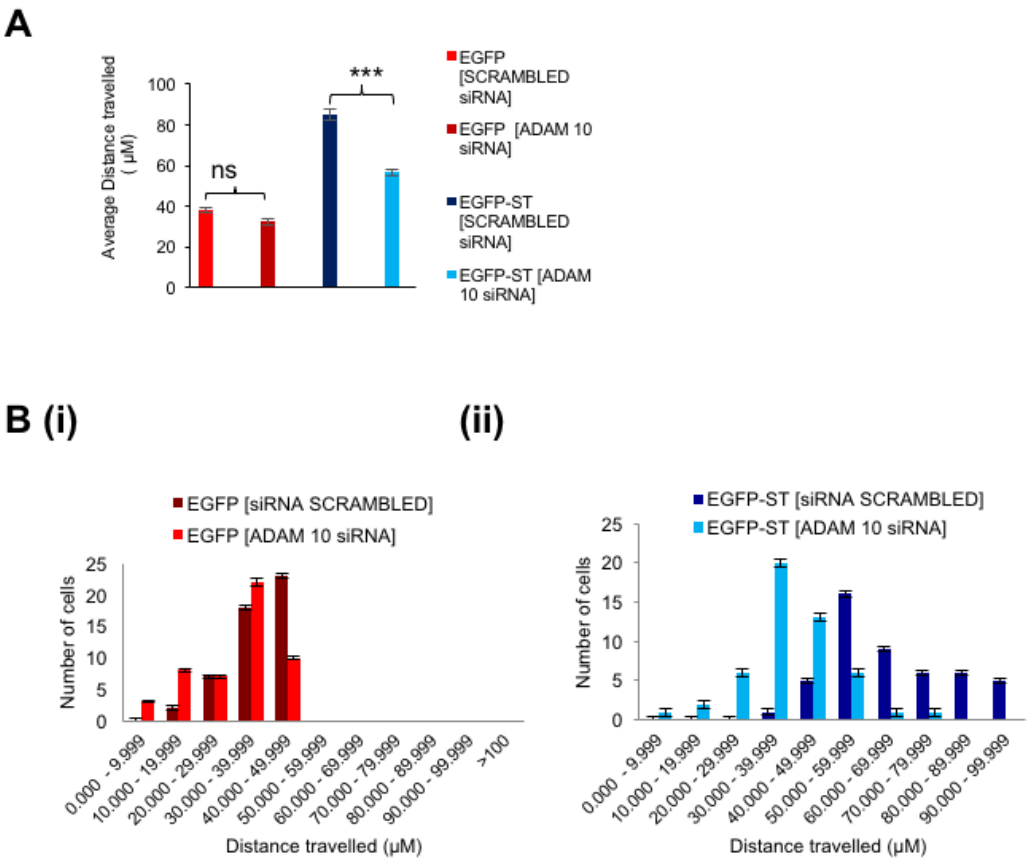

Figure 4. 14: siRNA knockdown of ADAM 10 decreases MCPyV ST-mediated cell migration. HEK 293 cells were transfected with ADAM 10 specific siRNA and scramble negative control. 48 hours post initial transfection, pEGFP-C1 and pEGFP-ST where transfected in the knockdown negative and positive cells. [A] The average distance travelled by EGFP and EGFP-ST cells upon ADAM 10 Knockdown [n=50]. [Bii] Quantification of distance travelled for scramble EGFP control cells and ADAM 10 Knockdown EGFP cells are compared to each other in 10 µm ranges [n=50] [Bii] Quantification of distance travelled for scramble EGFP-ST control cells and ADAM 10 knockdown EGFP-ST cells are compared to each other in 10 µm ranges [n=50]. [ns – P>0.05, *** – P≤ 0.001].

To further investigate the role MCPyV ST plays in promoting cell motility and migration, cell growth characteristics were analysed using matrigel- and fibronectin-based transwell migration assays using MCPyV positive MCC cell lines. A transwell assay was utilized as known MCPyV positive MCC cell lines are non-adherent cells. Subsequent analysis of ADAM 10 inhibition in the context of matrigel-based migration assays was conducted, in order to validate the potential of ADAM 10 upregulation on MCPyV ST-enhanced cell motility and migration.

Here, MCPyV positive MCC cell lines PeTa and WAGA-1 cells were assessed in the absence or presence of ADAM 10 specific inhibitor GI254023X. Initially, non-cytotoxic concentrations of these inhibitor were determined by MTS assay in PeTa and WAGA cell lines [Figure 4.15A]. Cells incubated in the absence or presence of non-cytotoxic concentration of ADAM 10 inhibitor for 24 hours. Following the initial incubation period, cells were seeded onto matrigel-coated transwells in the absence or presence of the inhibitors. Cells were then allowed to migrate over the course of 22 hours. This was followed by cell staining and the number of cells that migrated was calculated as a fold change of the untreated samples. Figure 4.15B clearly demonstrates that ADAM 10 inhibition in cells positive for MCPyV show a statistically significant decrease in motility and migration through the trans-wells. This confirms that ADAM 10 is a crucial component for MCPyV ST-mediated cell motility and migration.

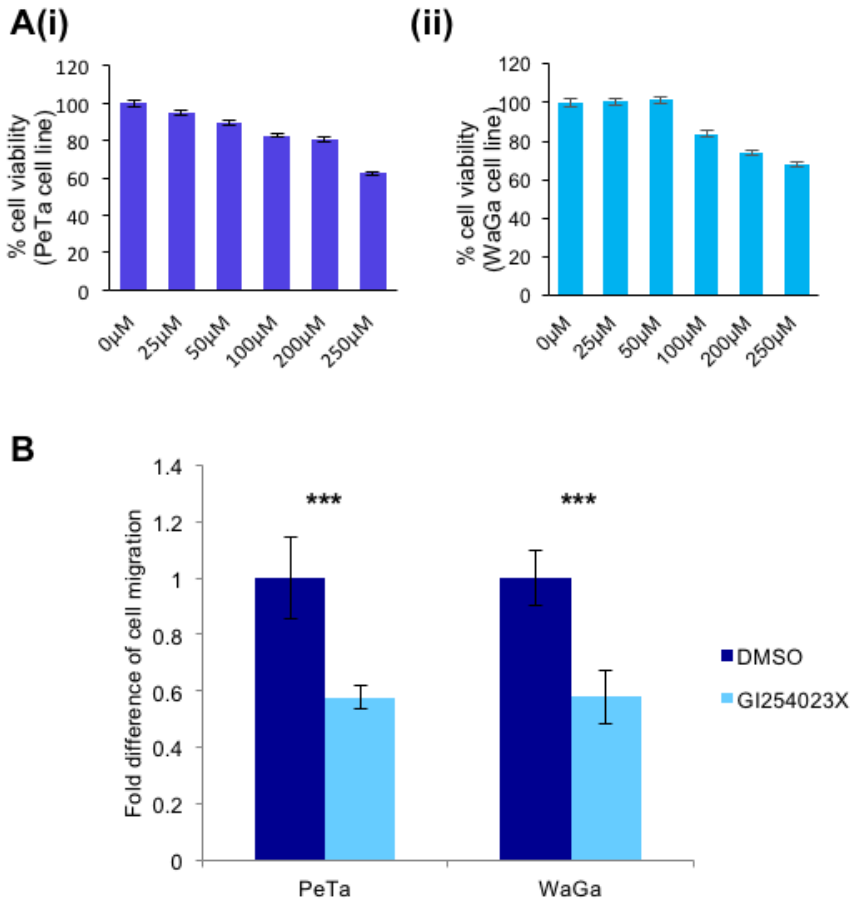

Figure 4. 15: ADAM 10 is required for MCPyV ST-mediated cell migration in transwell assays. [A] Cell vialbility with ADAM 10 inhibitors GI254023x. Cells were grown for 24 h, then treated with inhibitors [i] PeTa cells [ii] WaGa cells. [B] PeTa and WaGa cells were seeded in the absence or presence of ADAM 10 specific inhibitor. Cells were incubated for 22 hours and then labelled for 90 minutes with Calcein AM fluorescent dye. The fluorescence was measured between 494-517 nM and the relative percentage of cells moved through the trans-well was calculated as a fold difference of the untreated cells [set at 1]. Data analysed using three replicates per experiment, n=3 and statistical analysis using a two-tailed t-test with unequal variance comparing incubated inhibitor samples to their designated DMSO incubated control [*** – P≤ 0.001].

4.8 Discussion

Multiple lines of inquiry and evidence have proven that specific ADAM proteins are expressed in malignant tumors and play a role in cancer pathogenesis (Arima *et al.* 2007; Mochizuki and Okada 2007). Data herein, demonstrates that MCPyV ST-mediated upregulation of ADAM proteins promote cell-to-cell dissociation, cell scatter and motility in a range of cell lines such as HEK, COS7 and MCC13 cell lines.

Interestingly, in chapter 3 we observed that ADAM 10 and ADAM 17 were found upregulated at the cell surface by immunofluorescence studies Flow cytometry in MCPyV ST-expressing cells. To validate the role of MCPyV ST in promotion of ADAM proteins in cell dissociation, scatter and migration, 2 commercially available inhibitors an ADAM 10 specific inhibitor and a dual ADAM 10/17 inhibitor were utilized. These inhibitors are utilized to assess the role of these proteins in MCPyV-related metastasis studies as the ADAM10-specific inhibitor GI254023X has been widely used and shown to inhibit cell migration in various breast cancer cell lines BT20, MDA-MB-231 and MDA-MB-453 (Mullooly *et al.* 2015).

Results in chapter 4 using live cell imaging assay showed that incubation of the ADAM 10 inhibitor, GI254023X reduced ST-mediated cell migration. This was validated by use of TAPI-2 inhibitor, a dual ADAM 10/17 inhibitor and further corroborated by siRNA mediated-depletion of ADAM 10. Surface expression of ADAM10 and ADAM17 have been analysed in previous studies in a variety of human T cell and tumour cell lines. These studies have shown that ADAM10 is constitutively present at significantly higher levels on the majority of the tested cell types. In comparison, in all assessed cell lines, ADAM 17 showed a significantly lower constitutive expression (Ebsen *et al.* 2013). This data verifies the hypothesis that ADAM proteases induces ST-mediated cell migration. As ADAM 17 does share some substrate redundancies with ADAM 10, it would be important to specifically categorize ADAM 17 sheddase activity in MCC cells (Le Gall *et al.* 2009).

ADAM10 has previously been shown to correlate with outcome in basal-type breast cancer (Mullooly *et al.* 2015). In a previous study, an alternative ADAM 10 inhibitor, INCN3619, has been shown inhibit synergistic growth when combined with dual EGFR/HER2 tyrosine kinase inhibitor, GW2974 [Sigma Aldrich] in MCF-7 breast cancer cells (Duffy *et al.* 2011). ADAM 10 has also been shown to be a potential therapeutic target for HER2 positive breast cancers. Newton et al. [2010] have reported use of INCB7839, a dual ADAM10/17 inhibitor, in combination with

trastuzumab in drug trials. Along with a high tolerance for the new drug, overall response rate in patients with advanced breast cancer were between 40% and 55% (Newton *et al.* 2010).

The results in this study suggest that ADAM10 may be a new prognostic biomarker for MCPyV ST-mediated metastasis. If validated, ADAM 10 selective inhibitors such as the one used in this investigation, GI254023X, can be used as a potential therapeutic treatment for MCC, once assessed for efficacy and toxicity in animal models.

Interestingly, a similar inhibitor INCB7839 is currently undergoing initial clinical trials in HER2-positive advanced breast cancer patients. Preliminary results from these clinical trials suggest that this inhibitor is currently tolerated with no negative effects for participants. Additionally, administration of Trastuzumab along with INCB7839 to over 50 patients with advanced HER2-positive breast cancer induced response in 13/26 (50%) evaluable patients. Addition of INCB7839 to Trastuzumab therapy, increased efficacy (Duffy *et al.* 2011).

CHAPTER 5

MCPyV ST is Sufficient to Drive an Epithelial to Mesenchymal Transition

5.1 Introduction

The epithelial to mesenchymal transition [EMT] is a biological process that occurs when polarized epithelial cells which customarily interact with basement membrane by means of their basal surface, undertake various biochemical alterations and modifications leading to a phenotype associated with a mesenchymal cell (Al-Azayzih *et al.* 2015; Chen *et al.* 2016). The hallmarks of EMT include enhancement in migratory potential, invasiveness, resistance to apoptosis and degradation of the basement membrane (Kalluri and Weinberg 2009).

The mechanism for epithelial to mesenchymal transition provides mobility to cancer cells, allowing cancer cells to invade neighbouring tissues and organs, as well as entry to the circulatory system (Gao and Mittal 2012). This is essential for metastatic spread as upon intravasation, cancer cells are transported through the circulatory system and later exit the blood stream at secondary locations to establish metastatic tumours (Geiger and Peeper 2009; Hanahan and Weinberg 2011).

Recent studies have shown that that oncoviruses and their associated oncoproteins play a role in metastasis and EMT-related mechanisms. For example, HPV16 E6 and E7 oncoproteins have been shown to repress the expression of the E-cadherin protein in cervical cancer cells, inducing FGF ligand stimulation resulting in increased invasiveness (Cheng *et al.* 2012) . EBV oncoprotein, LMP1, has also been proven to induce cadherin switching (Shair *et al.* 2009), as well as regulate transcription factors such as TWIST and Snail thereby increasing a migratory phenotype (Horikawa *et al.* 2011; Chen *et al.* 2016).

Although transcriptome analysis has suggested that certain markers associated with EMT are increased upon MCPyV ST expression (Berrios *et al.* 2016), there lacks any detailed analysis to confirm if MCPyV ST induces a complete EMT. It is important to assess differential expression of EMT markers at the RNA level, as well as the protein level, as changes in RNA levels regulate EMT progression due to differential splicing and microRNA-regulated mechanisms of EMT markers (Lamouille *et al.* 2014).

This chapter investigates whether MCPyV ST is sufficient to drive an epithelial to mesenchymal transition. Specific steps in the EMT have been investigated herein such as: 1) loss of cell-to-cell adhesion mediated by E-cadherin at Adherens junctions and ZO-1 and Occludin at tight junctions 2) loss of apical to basal cell

polarity, 3) cytoskeletal reorganization 4) degradation of the basement membrane, 5) The state of Cadherin switching.

5.2 MCPyV ST expression downregulates of Cell adhesion markers

The epithelial cell phenotype is characterized by strong cell-to-cell interaction at the Adherens and tight junctions, observed by the cobblestone morphology (Moreno-Bueno *et al.* 2009). In chapter 3, a downregulation of α-E-catenin and ZO-1 was observed upon MCPyV ST expression at the protein level as well as specifically at the cell surface suggesting a reduction in cell-to-cell adhesion. In epithelial cells, E-Cadherin has been identified as a major structural protein of the Adherens junction (Furness and Speight 1998). As a known tumour suppressor protein, downregulation of E-cadherin is considered to be one of the first essential steps of EMT, in both healthy and cancerous cells (Baum and Georgiou 2011). Therefore, it was important to assess the transcript and protein level of this cell adhesion associated marker. Interestingly however, HEK 293 cells do not express E-Cadherin, which was confirmed by flow cytometry [Figure 5.1]. HEK 293 cells are transformed by sheared adenovirus 5 DNA which may contribute to loss of E-cadherin expression. Therefore, experiments were performed in COS 7 and MCC13 along with HEK 293.

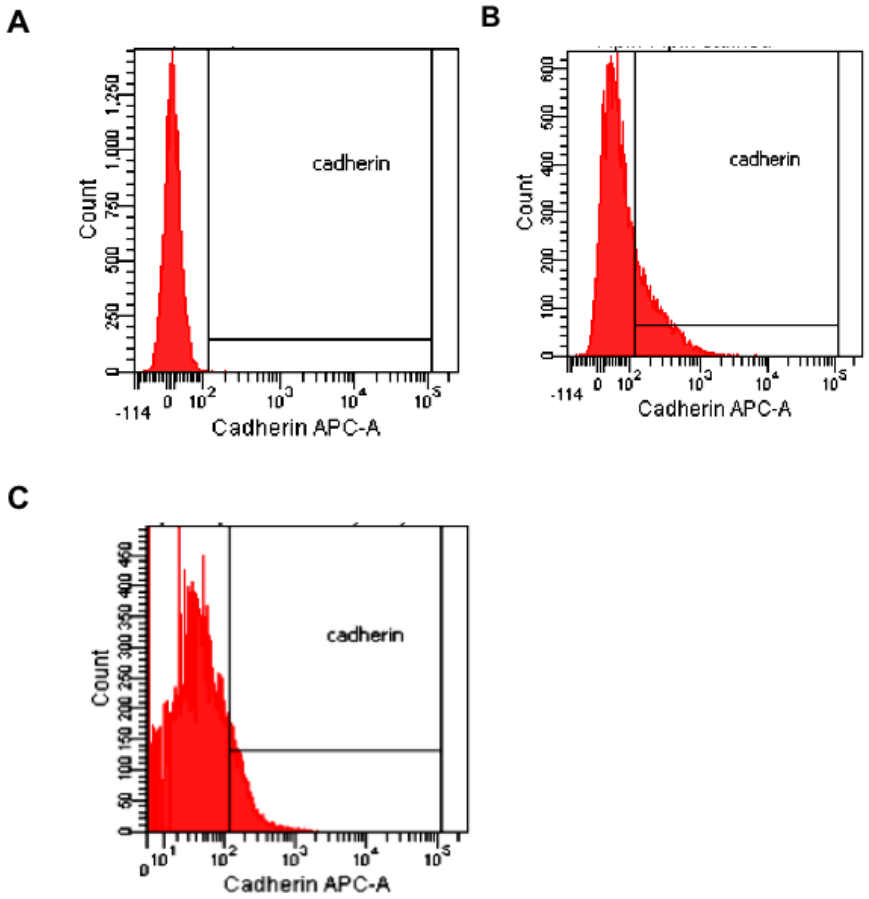

Figure 5. 1: HEK 293 cells do express E-cadherin. [A] Unstained HEK 293 cells function as a negative control for E-cadherin staining. [B] Stained HEK 293 cells illustrating absence of staining [C] Secondary only staining to confirm absence of unspecific binding and staining.

To assess the levels of cell adhesion-associated proteins upon MCPyV ST expression, mRNA levels were evaluated 48 hours post transfection. We assessed E-Cadherin, ZO-1 as well as Occludin, an integral plasma membrane protein which is specifically localized at the tight junction (Feldman *et al.* 2005). RT-qPCR indicated significant downregulation in the mRNA levels of E-cadherin, ZO-1 and Occludin upon MCPyV ST expression in 293, COS7 and MCC13 cells [Figure 5.2].

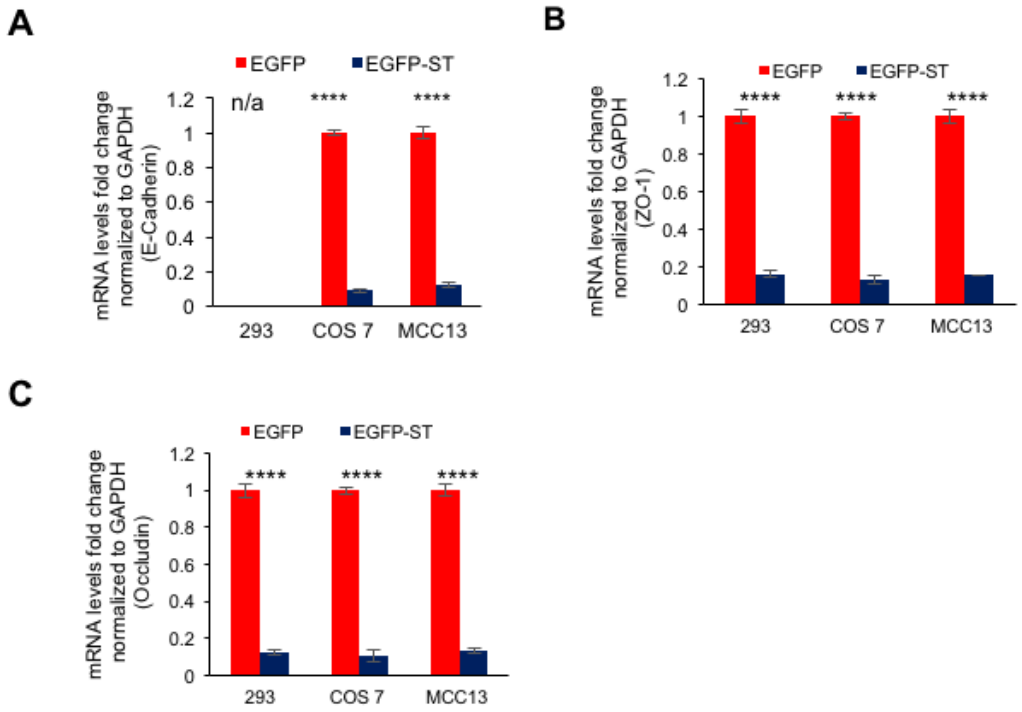

Figure 5. 2: Cell adhesion molecules are downregulated at the transcript level. Various cell lines [293, COS7 and MCC13] were transfected for 48 hours with pEGFP-C1 and pEGFP-ST expression plasmids. Cellular RNA was extracted using Trizol reagent, reverse transcribed and RT-qPCR was performed. Transcript levels were analysed using the comparative CT method [n = 3]. [A] E-cadherin [B] ZO-1 [C] Occludin. Data analysed using three replicates per experiment, n=3 and statistical analysis using a two-tailed t-test with unequal variance comparing MCPyV ST samples to EGFP control samples [P-≤ 0.0001].

To mediate cell-to-cell adhesion, newly synthesized E-cadherin proteins are packaged in the Golgi. The trans-Golgi network is the first main site of E-cadherin sorting and differential regulation. E-cadherin is essential for maintaining epithelial cell polarity, hence it must be sorted and delivered to the lateral cell surface in a polarized manner (Bryant and Stow 2004). It is at the cell surface where they function in cell adhesion (Peng *et al.* 2010). Therefore, it was important to assess whether MCPyV ST affects levels of E-Cadherin at the cell surface. Flow cytometry analysis was used to determine levels of E-Cadherin in COS 7 cells at the cell surface 48 hours post transfection of either pEGFP-C1 or pEGFP-ST expression vectors. Results demonstrated a reduction E-Cadherin expression at the cell surface upon MCPyV ST expression [Figure 5.3].

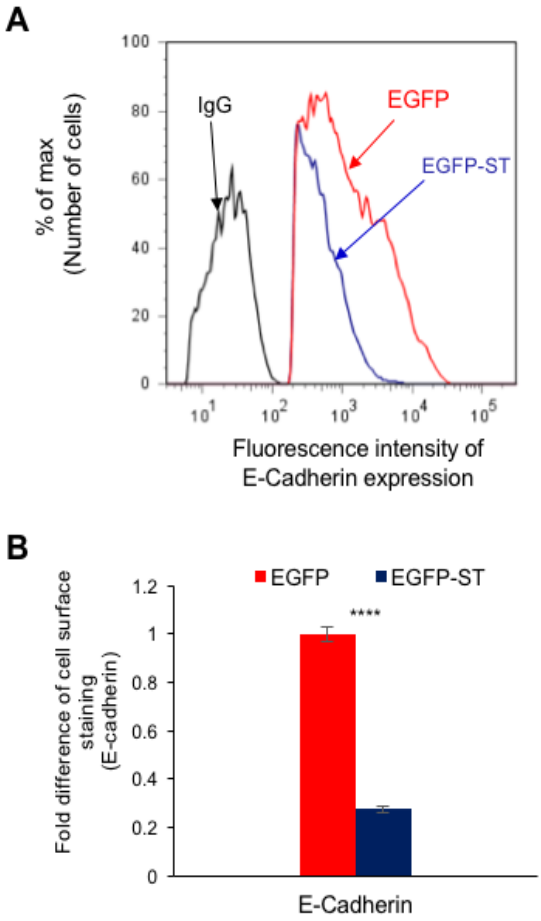

Figure 5. 3: Flow cytometry shows E-cadherin downregulation at the cell surface. [A] COS7 cells were transfected with pEGFP-C1 control and pEGFP-ST expression plasmids for 48 hours. Cells were harvested and spun down gently, blocked in 3% BSA and stained with a specific E-cadherin antibody along with a corresponding secondary antibody. Mean fluorescence intensity of both control EGFP and EGFP-ST cells where plotted as a histogram using Flow Jo analysis software. [B] Fold difference was calculated using Mean fluorescence intensity. Data analysed using three replicates per experiment, n=3 and statistical analysis using a two-tailed t-test with unequal variance comparing MCPyV ST samples to EGFP control samples [****– P≤ 0.0001].

De novo formation of epithelial markers were also assessed by immunoblotting to determine their expression levels upon MCPyV ST expression. Immunoblotting of EGFP or EGFP-ST cell lysates confirmed downregulation of a variety cell adhesion-associated proteins, namely E-cadherin, β-catenin and Claudin upon expression of MCPyV ST, in both COS7 and in MCC13 cells [Figure 5.4].

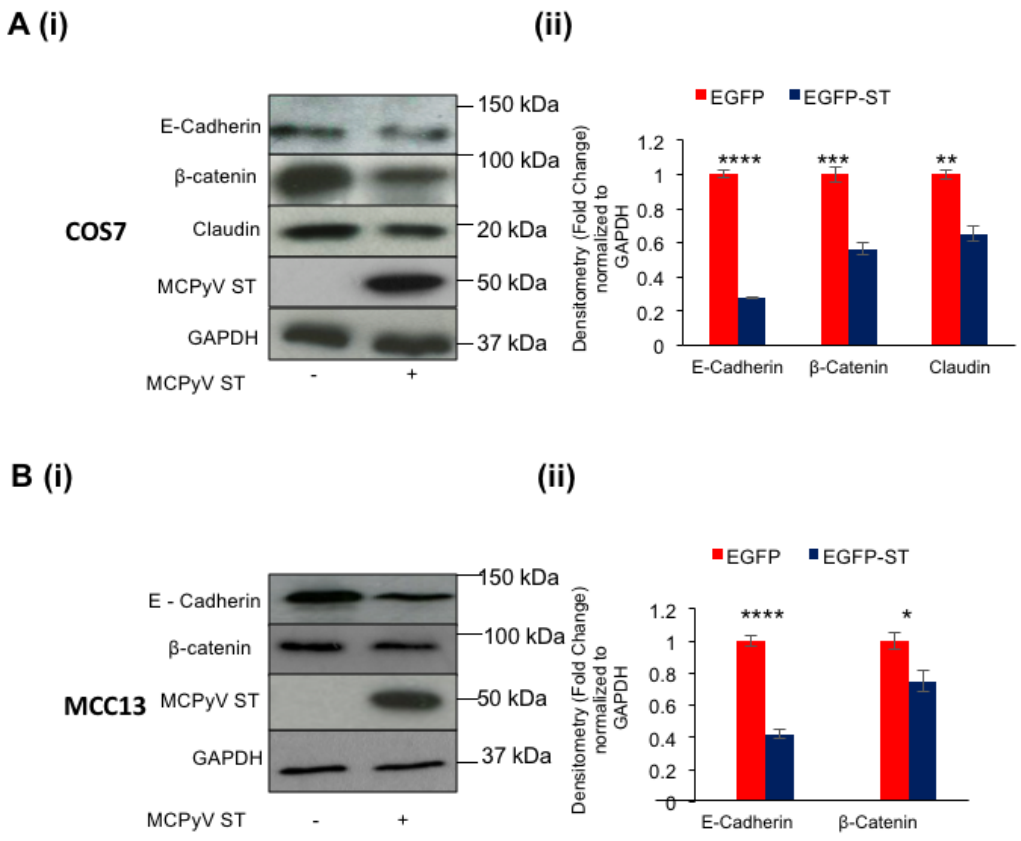

Figure 5. 4: MCPyV ST promotes downregulation of cell adhesion molecules. [Ai] COS7 and [Bi] MCC13 cells were transfected with pEGFP-C1 and pEGFP-ST expression plasmids for 48 hours. Cell lysates were then probed with specific antibodies. GAPDH was used as loading control and T antigen antibody was used to show MCPyV ST expression. [Aii and Bii] Densitometry quantification of the western blots was carried out using the Image J software and is shown as fold change of relative densitometry to the loading control, GAPDH. Data analysed using three replicates per experiment, n=3 and statistical analysis using a two-tailed t-test with unequal variance comparing MCPyV ST samples to EGFP control samples. [* – P≤ 0.05, ** – P≤ 0.01, *** – P≤ 0.001, ****– P≤ 0.0001].

Additionally, to confirm downregulation of E-cadherin and other cell-adhesion-associated proteins in the context of MCC tumours, immunoblot analysis was performed on the cellular lysates of two unrelated MCC tumour samples along with a negative control, non-tumour cadaveric skin sample. Results again demonstrate a decrease in E-cadherin and β-Catenin protein levels in MCC tumours compared to control [Figure 5.5]. ZO-1 protein levels were shown to be downregulated in MCC tumours [Figure 3.3]. Notably as previously shown in Figure 3.3, higher levels of MCPyV ST expression in Tumour 2 resulted in a more significant downregulation of cell adhesion molecules compared to Tumour 1.

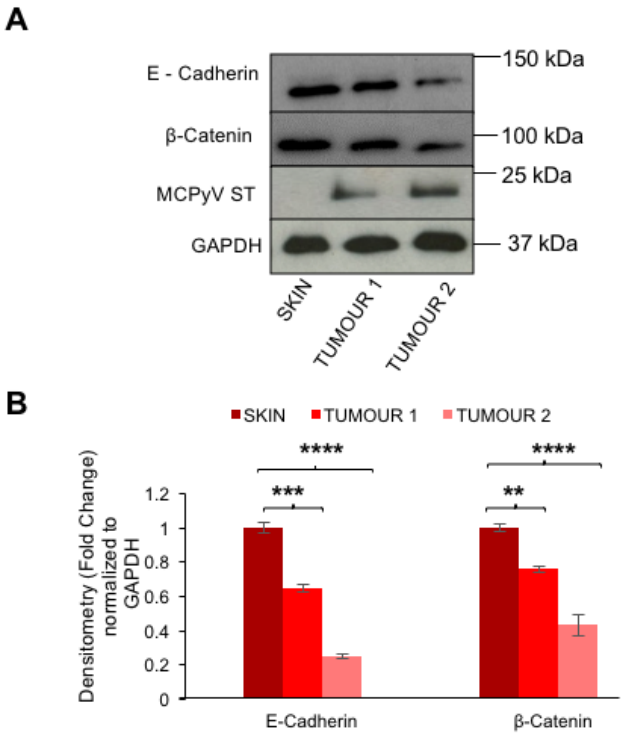

Figure 5. 5: Cell adhesion molecules are downregulated in MCPyV-positive MCC tumours. [A] Healthy skin, Tumour 1 and Tumour 2 were crushed using a mortar and a pestle on dry ice, lysed with RIPA buffer for 30 mins and sonicated to further homogenized the samples. Immunoblot analysis was performed on the tissue lysates and analysed with E-cadherin and β-catenin specific antibodies. GAPDH was used as a measure of equal loading, the T antigen antibody was used to confirm MCPyV ST expression. [B] Densitometry quantification of the western blots was carried out using the Image J software and is shown as fold change of relative densitometry to the loading control, GAPDH. Data analysed using three replicates per experiment, n=3 and statistical analysis using a two-tailed t-test with unequal variance comparing control cadaveric skin sample to each individual tumour [** – P≤ 0.01, *** – P≤ 0.001, ****– P≤ 0.0001].

5.3 Cadherin Switching

During EMT, E-cadherin expression is decreased while N-cadherin is upregulated (Kalluri & Weinberg 2009). This interchange is commonly known as the E- to N-cadherin switch. E-cadherin functions in maintenance of epithelial integrity and N-cadherin has been shown to be involved in invasion (Bremmer *et al.* 2015). As such, the cadherin switch from E-cadherin to N-cadherin, has been identified as a hallmark of EMT (Ye and Weinberg 2015; Chen *et al.* 2016). As downregulation of E-cadherin, had been observed [Figures 5.2 – 5.5], it was important to assess the state of the cadherin switch upon MCPyV ST expression.

Initially, the transcript levels of N-Cadherin were assessed comparing control and MCPyV ST-expressing cells. Surprisingly however, no detectable change in N-

Cadherin expression was observed upon MCPyV ST expression [Figure 5.6A]. Additionally, localisation N cadherin was also assessed upon MCPyV ST expression. While there was a significant downregulation of E-cadherin expression at the cell surface upon MCPyV ST expression [Figure 5.3], there was no detected change of N-cadherin expression at the cell surface [Figure 5.6B] inferring an absence of E-N cadherin switching upon MCPyV ST expression.

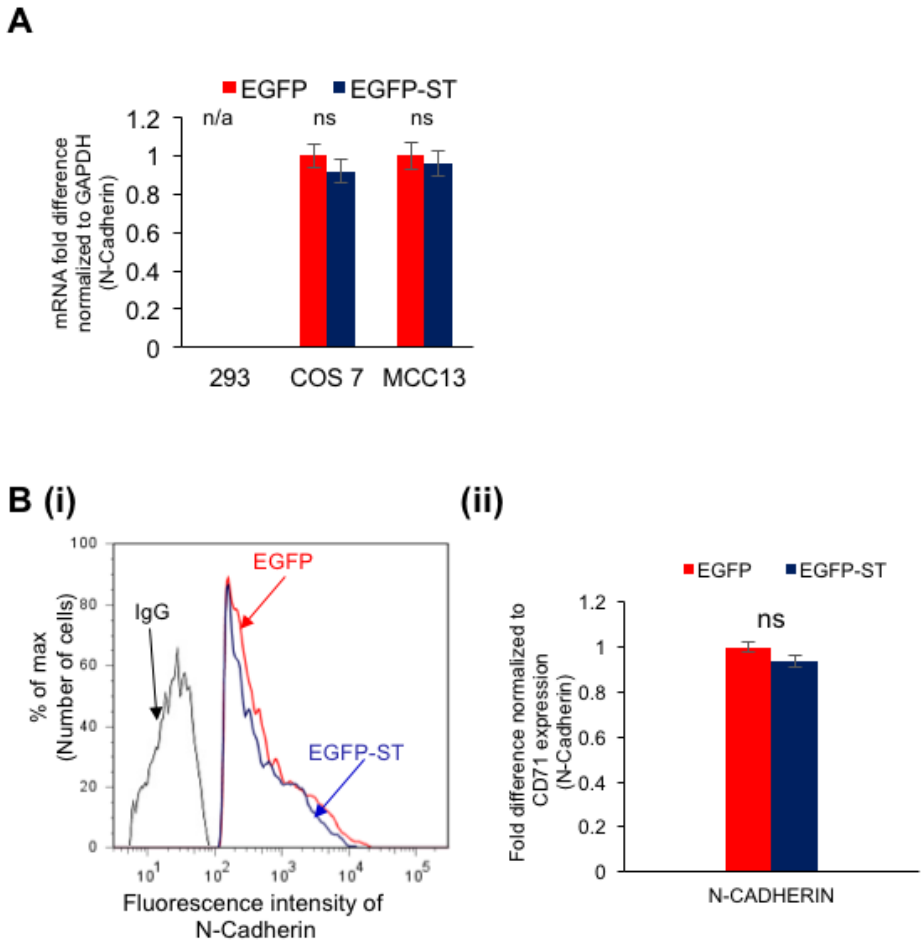

Figure 5. 6: MCPyV ST expression does not induce E-N Cadherin Switching. [A] Various cell lines [293, COS7 and MCC13] were transfected for 48 hours with pEGFP-C1 and pEGFP-ST expression plasmids. Cellular RNA was extracted using a Trizol reagent, reverse transcribed and RT-qPCR was performed. Transcript levels were analysed using the comparative CT method [n = 3]. [B] COS7 cells were transfected with pEGFP-C1 control and pEGFP-ST expression plasmids for 48 hours. [Bi] Cells were harvested and spun down gently, blocked in 3% BSA and stained with a specific E-cadherin antibody along with a corresponding secondary antibody. Mean fluorescence intensity of both control EGFP and EGFP-ST cells where plotted as a histogram using Flow Jo analysis software. [Bii] Fold difference was calculated using Mean fluorescence intensity. Data analysed using three replicates per experiment, n=3 and statistical analysis using a two-tailed t-test with unequal variance comparing MCPyV ST samples to EGFP control samples [ns – P>0.05].

5.4 MCPyV ST expression leads to a loss of apical to basal cell polarity

Epithelial cells are known to display apical-basal polarity that is essential for maintenance of tissue integrity. The epithelial cell polarity is based on the relationship between Adherens junctions, tight junctions and cell polarity complexes. This relationship contributes to the formation and the maintenance of apical and basolateral domain (Campbell *et al.* 2011; Lamouille *et al.* 2014). This linkage contributes to the structural integrity of epithelial cell sheets (Ye and Weinberg 2015).

The loss of epithelial junctions during EMT results in the loss of apical-basal polarity, tissue integrity and importantly, is a hallmark of malignant tumours (Ohara *et al.* 2012). In polarized epithelial cells, the Golgi complex is typically oriented toward the apical plasma membrane domain (Pu *et al.* 2015). Thus, loss of apical to basal cell polarity can be observed by loss of the Trans-Golgi network at the leading edge of a scratch assay.

To examine if MCPyV ST leads to loss of apical to basal cell polarity, COS 7 cells were transfected with pEGFP-C1 and pEGFP-ST expression plasmids. When the transfected cells were confluent approximately 48 hours post transfection, a scratch wound was created in the centre of the well. 6 hours post scratch the cells were fixed and stained with a TGN specific antibody. Results show that MCPyV ST expression leads to a reduction of the Sheet-like structure identified by TGN localisation at the leading edge [Figure 5.6], suggesting the loss of apical-basal polarity.

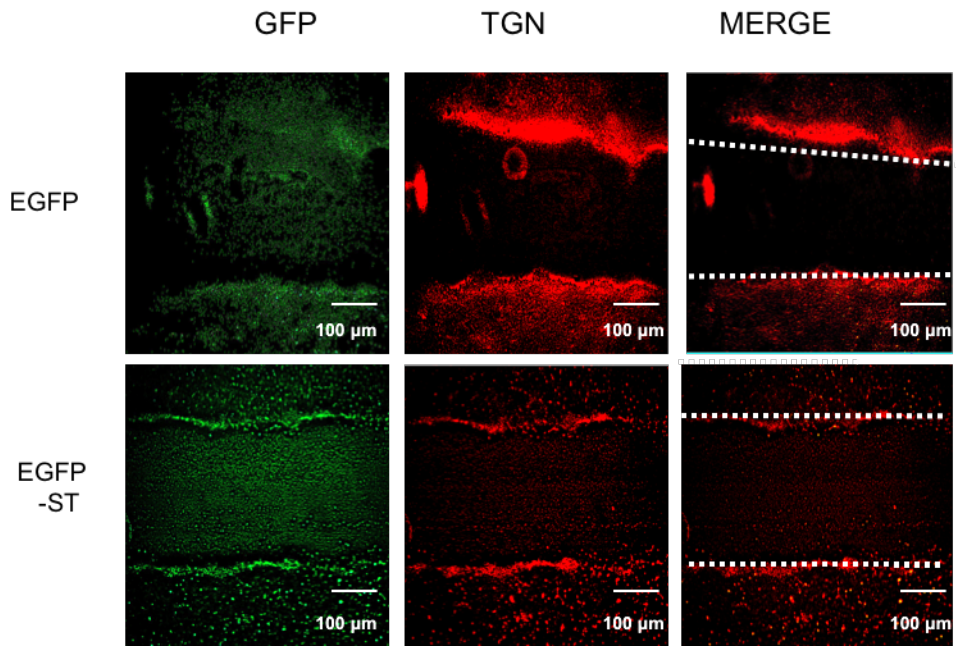

Figure 5. 7: Loss of apical to basal cell polarity in MCPyV ST-expressing cell. COS7 cells were transfected for 48 hours with pEGFP-C1 and pEGFP-ST-expressing vectors for 48 hours. Scratches were made across the surface of the well and cells were then left to move into the gap for 6 hours. Cells were then fixed, stained for the Trans-Golgi network and imaged using an EVOS Auto2FL. TGN staining was observed at the scratch line detecting either solid staining at the scratch line or punctuation at the leading edge which infers a loss of apical to basal cell polarity.

Upon this initial observation of the loss of apical to basal cell polarity due to MCPyV ST expression, it was next important to assess the expression of specific apical to basal cell polarity proteins. Apical-basal cell polarity is controlled by three evolutionarily conserved protein complexes - the Crumbs complex, the PAR complex and the Scribble complex. The mRNA levels CRB3, a Crumb complex marker in addition to SCRIB and LGL2 of the Scribble complex were therefore assessed in EGFP control and EGFP-ST-expressing cells. Upon MCPyV ST expression, a significant downregulation in SCRIB, LGL2 and CRB3 expression was observed validating the initial observation of the loss of apical to basal cell polarity [Figure 5.8].

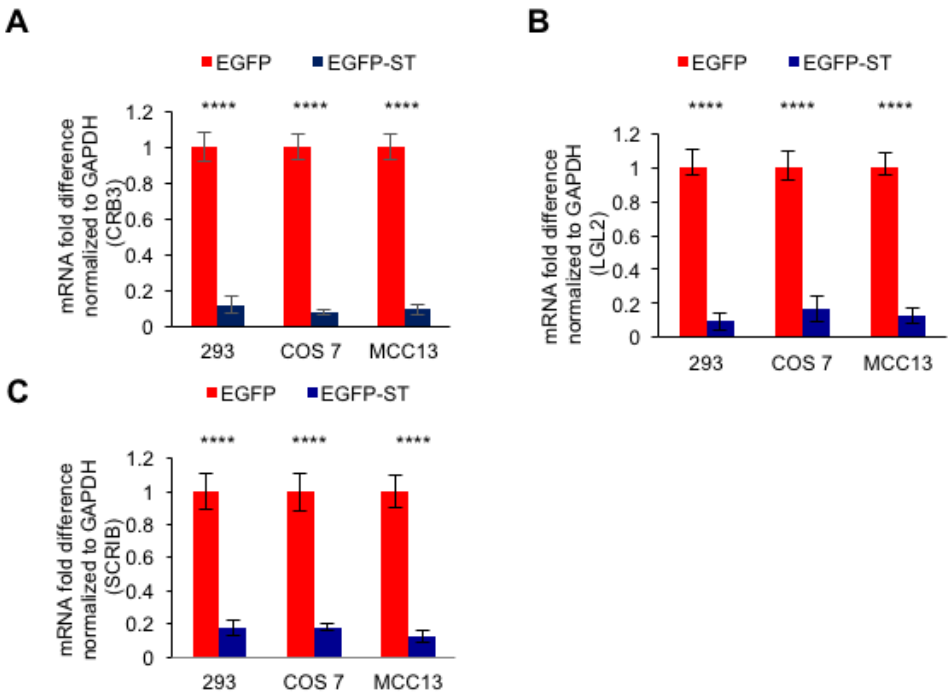

Figure 5. 8: Apical to basal cell polarity regulators are downregulated at the transcript level. Various cell lines [293, COS7 and MCC13] were transfected for 48 hours with pEGFP-C1 and pEGFP-ST expression plasmids. Cellular RNA was extracted using a Trizol reagent, reverse transcribed and RT-qPCR was performed. Transcript levels were analysed using the comparative CT method. Data analysed using three replicates per experiment, n=3 and statistical analysis using a two-tailed t-test with unequal variance comparing MCPyV ST samples to EGFP control samples [****– P≤ 0.0001]. [A] CRB3 [B] LGL2 [C] SCRIB.

5.5 MCPyV ST upregulates cellular transcription factors associated with EMT

In carcinomas, various extracellular signals, such as HGF, EGF, PDGF, and TGF-β, which induce EMT in numerous cell types have been identified (Kalluri and Weinberg 2009). These extracellular signals induce EMT associated-transcription factors such as ZEB1, ZEB2, TWIST, Snail and Slug, which function pleiotropically to coordinate EMT (Thiery *et al.* 2009). It is believed these transcription factors act as the key molecular switches, responding to various signalling pathways to initiate EMT. Therefore, the expression of EMT-associated transcription factors was assessed at the transcript level in control and MCPyV ST-expressing cells in multiple cell lines. Results show that MCPyV ST expression induces upregulation of ZEB 1, ZEB 2, as well as Snail and Slug compared to EGFP control cells [Figure 5.9].

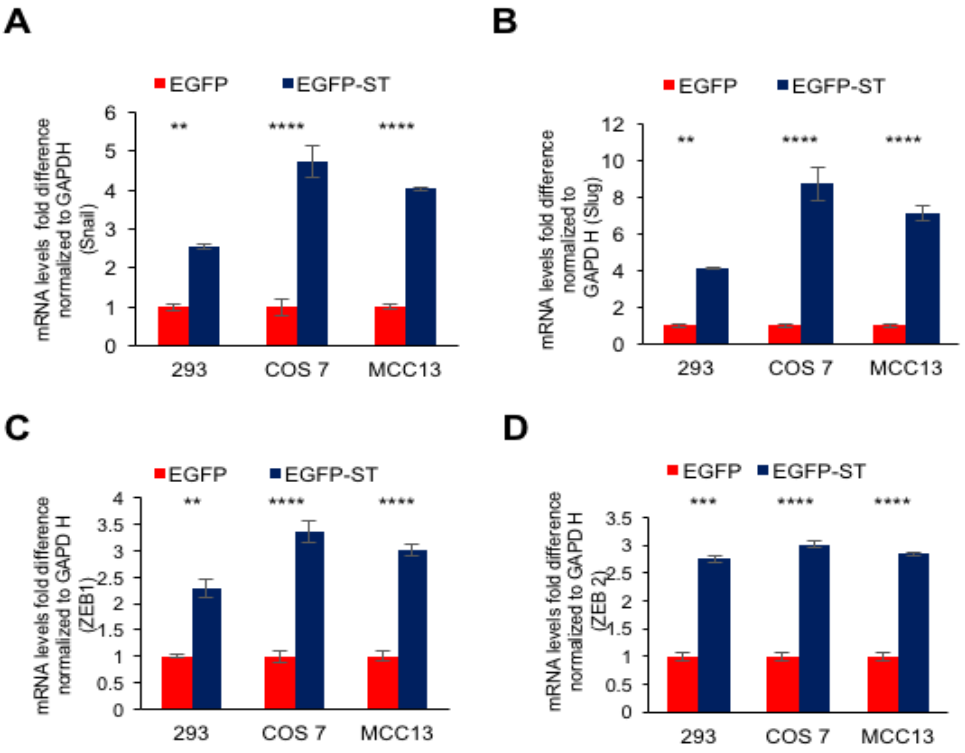

Figure 5. 9: EMT Transcription factors are upregulated at the transcript level. Various cell lines [293, COS7 and MCC13] were transfected for 48 hours with pEGFP-C1 and pEGFP-ST expression plasmids. Cellular RNA was extracted using a Trizol reagent, reverse transcribed and RT-qPCR was performed. Transcript levels were analysed using the comparative CT method [n = 3. [A] Snail [B] Slug [C] ZEB1 [D] ZEB2. Data analysed using three replicates per experiment, n=3 and statistical analysis using a two-tailed t-test with unequal variance comparing MCPyV ST samples to EGFP control samples [** – P≤ 0.01, *** – P≤ 0.001, ****– P≤ 0.0001].

To confirm upregulation of these EMT-associated transcription factors at a protein level, as well as the observed transcript induction, Snail protein levels were further assessed in EGFP versus EGFP-ST-expressing COS7 and MCC13 lines and in 2 unrelated MCC tumour lysates in comparison to healthy cadaveric skin as a negative control. Snail protein levels were upregulated upon MCPyV ST expression in both MCPyV ST-expressing cell lines, as well as MCC tumour samples in comparison to the healthy skin sample [Figure 5.10].

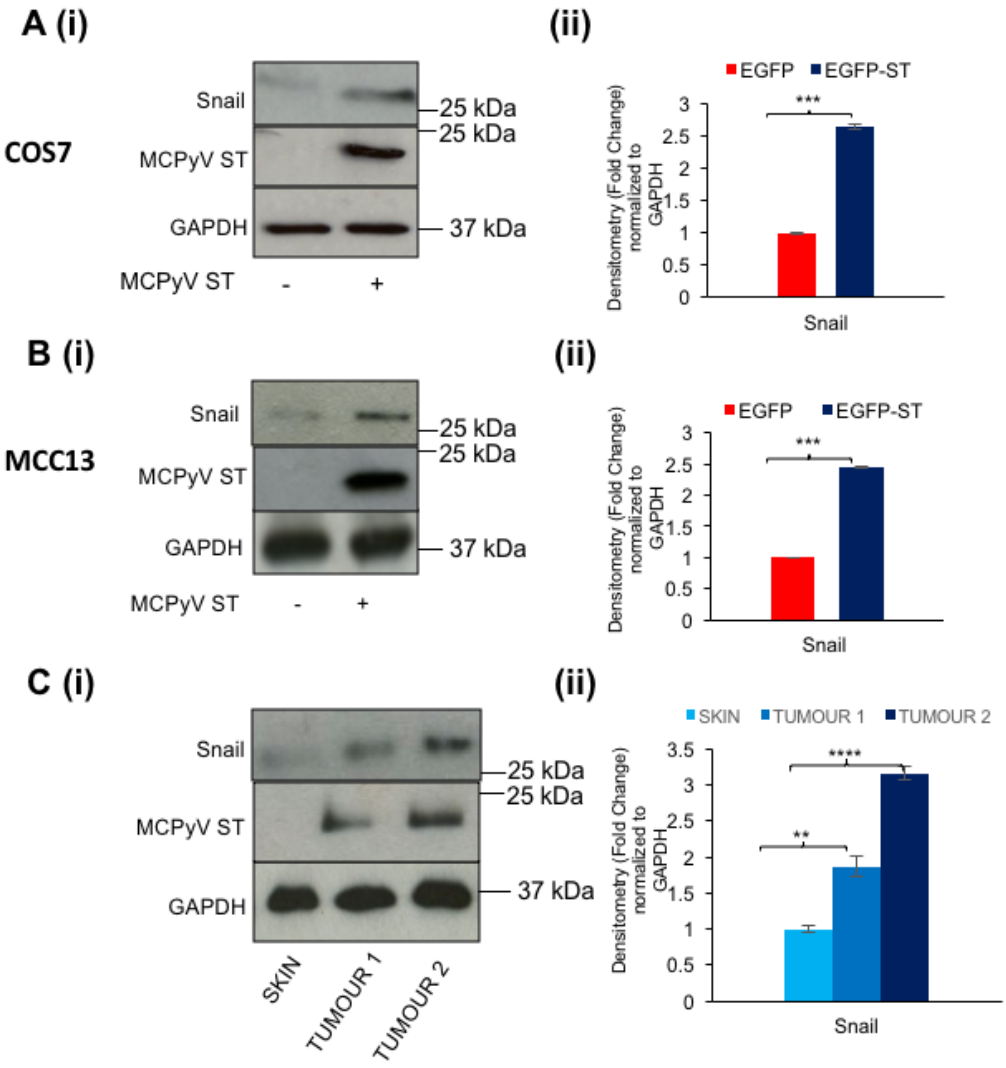

Figure 5. 10: Snail expression is induced in MCPyV ST-expressing cells and MCC tumour samples. [A] COS7 cells and [B] MCC13 were transfected with pEGFP-C1 and pEGFP-ST expression plasmids for 48 hours. Cell lysates were then probed with a Snail specific antibody. GAPDH was used as loading control and T antigen antibody was used to show MCPyV ST expression. [ii] Densitometry quantification of the western blots was carried out using the Image J software and is shown as fold change of relative densitometry to the loading control, GAPDH. Data analysed using three replicates per experiment, n=3 and statistical analysis using a two-tailed t-test with unequal variance. [C] Healthy skin, Tumour 1 and Tumour 2 were crushed using a mortar and a pestle on dry ice, lysed with RIPA buffer for 30 mins and sonicated to further homogenized the samples. Immunoblot analysis was performed on the tissue lysates and analysed with a Snail specific antibody. GAPDH was used as a measure of equal loading, the T antigen antibody was used to confirm MCPyV ST expression. [ii] Densitometry quantification of the western blots was carried out using the Image J software and is shown as fold change of relative densitometry to the loading control, GAPDH. Data analysed using three replicates per experiment, n=3 and statistical analysis using a two-tailed t-test with unequal variance comparing MCPyV ST samples to EGFP control samples or comparing control cadaveric skin sample to each individual tumour [** – P≤ 0.01, *** – P≤ 0.001, ****– P≤ 0.0001].

Snail is a prominent inducer of EMT. It functions as a key transcriptional repressor protein, for example inhibiting E-cadherin expression. It is believed to function by recruiting repressive chromatin remodelling factors, such as the Sin3A-HRAC1/2 complex and PRC2 to repress E-cadherin expression (Herranz *et al.* 2008). This repressive activity requires Snail to be localized in the nucleus, which is regulated by PAK-1 mediated phosphorylation of Serine residue 246 on Snail. To determine if induction of EMT by MCPyV ST as result of Snail relocalization into the nucleus is possible, PAK-1 expression at the transcript levels were assessed in EGFP versus EGFP-ST-expressing HEK 293, COS7 and MCC13 cell lines [Figure 5.11].

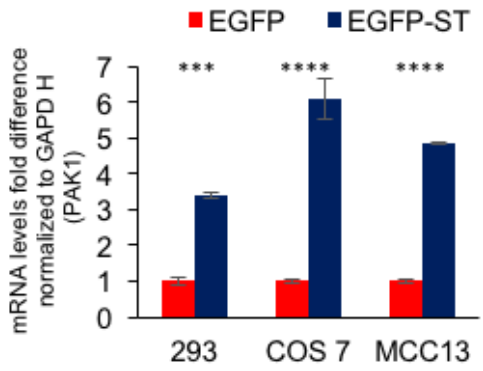

Figure 5. 11: PAK1 is upregulated at the transcript level upon MCPyV ST expression. Various cell lines [293, COS7 and MCC13] were transfected for 48 hours with pEGFP-C1 and pEGFP-ST expression plasmids. Cellular RNA was extracted using a Trizol reagent, reverse transcribed and RT-qPCR was performed. Transcript levels were analysed using the comparative CT method [n = 3. [A] Snail [B] Slug [C] ZEB1 [D] ZEB2. Data analysed using three replicates per experiment, n=3 and statistical analysis using a two-tailed t-test with unequal variance comparing MCPyV ST samples to EGFP control samples [*** – P≤ 0.001, ****– P≤ 0.0001].

PAK1 expression was also assessed at the protein level in COS 7, MCC13 cells as well as 2 unrelated MCC tumour lysates in comparison to healthy skin as a negative control. PAK1 was upregulated upon MCPyV ST expression in MCPyV ST transfected cells. Similarly, PAK1 was also upregulated in MCC tumour samples in comparison to negative control, healthy skin [Figure 5.12].

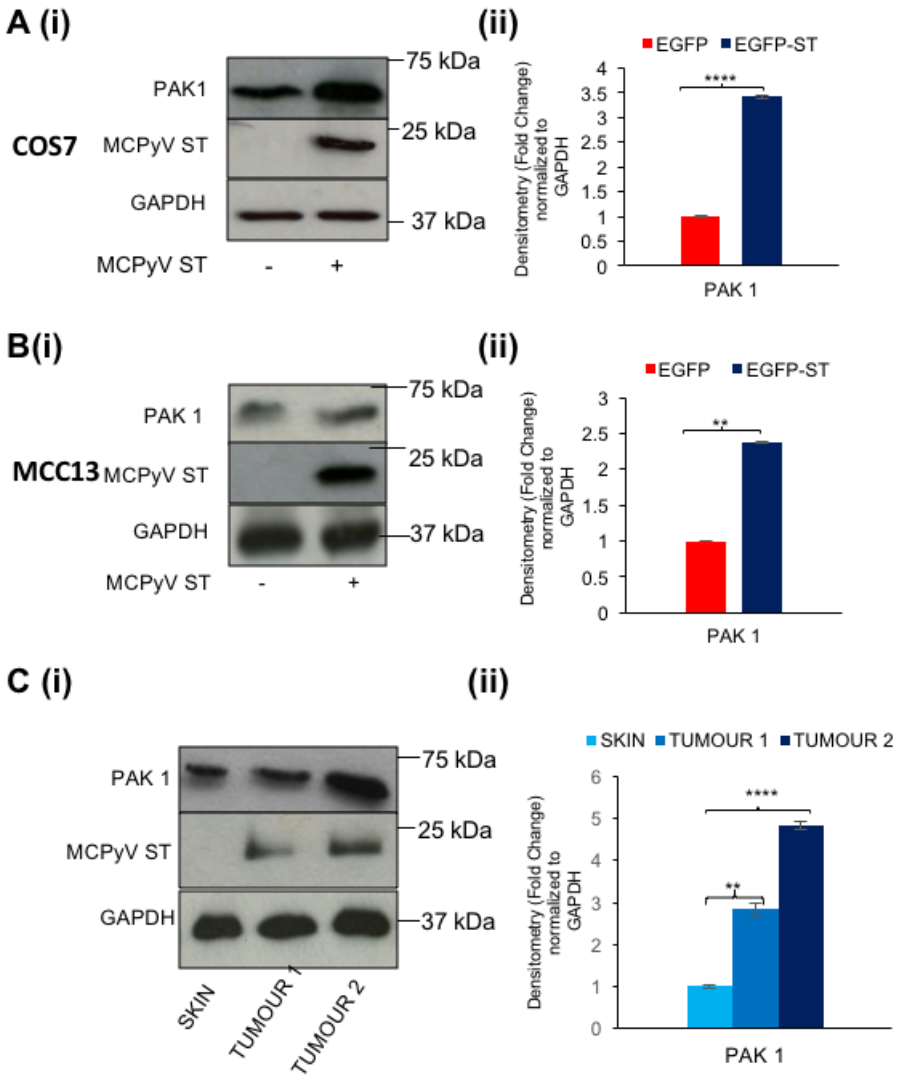

Figure 5. 12: PAK1 Expression in MCPyV ST-expressing cells and MCC tumour samples. [A] COS7 cells and [B] MCC13 were transfected with pEGFP-C1 and pEGFP-ST expression plasmids for 48 hours. Cell lysates were then probed with a PAK-1 specific antibody. GAPDH was used as loading control and T antigen antibody was used to show MCPyV ST expression. [ii] Densitometry quantification of the western blots was carried out using the Image J software and is shown as fold change of relative densitometry to the loading control, GAPDH. Data analysed using three replicates per experiment, n=3 and statistical analysis using a two-tailed t-test with unequal variance. [** – P≤ 0.01, *** – P≤ 0.001, ****– P≤ 0.0001]. [C] Healthy skin, Tumour 1 and Tumour 2 were crushed using a mortar and a pestle on dry ice, lysed with RIPA buffer for 30 mins and sonicated to further homogenized the samples. Immunoblot analysis was performed on the tissue lysates and analysed with a PAK-1 specific antibody. GAPDH was used as a measure of equal loading, the T antigen antibody was used to confirm MCPyV ST expression. [ii] Densitometry quantification of the western blots was carried out using the Image J software and is shown as fold change of relative densitometry to the loading control, GAPDH. Data analysed using three replicates per experiment, n=3 and statistical analysis using a two-tailed t-test with unequal variance comparing MCPyV ST samples to EGFP control samples or comparing control cadaveric skin sample to each individual tumour [** – P≤ 0.01, *** – P≤ 0.001, ****– P≤ 0.0001].

5.6 MCPyV ST induces breakdown in host cell basement membrane

The basement membrane is a specialized form of extracellular matrix [ECM], essential for epithelial structural integrity. It is comprised of a network of glycoproteins and proteoglycans, such as Type IV collagen and Laminin and provides a barrier from invasion by tumour cells. Therefore, the basement membrane must be degraded in order for invasion of tumour cells into the circulatory system for metastasis to occur (Horejs 2016).

Epithelial cells interact with basement membrane via integrins which are located on the basal epithelial surface. These integrins bind to various ECM components such as Collagens and Laminins (Huang *et al.* 2012). Studies have shown that when inactive, integrins are unable to bind to ECM (Kim *et al.* 2011) and interestingly MCPyV ST has been shown to induce downregulation of Integrin β1 (Stakaityte *et al.* 2018). Various extracellular matrix proteases are employed by malignant cancers to degrade the ECM and various components. This plays a key role in the migration potential of Mesenchymal cells. Notably, changes in Integrin expression have been correlated with expression of proteases during EMT (Deryugina and Quigley 2006).

Matrix Metalloproteinases such as MMP2, MMP3 and MMP9 have been shown to enhance ECM degradation and invasion upon expression (Egeblad and Werb 2002; Reinhard *et al.* 2015). Therefore, it was important to determine whether levels of metalloproteinases were altered upon MCPyV ST expression. MMP3 and MMP9 mRNA expression where assessed in a variety of cell lines transfected with pEGFP-C1 and pEGFP-ST expression vectors. Results shown that MCPyV ST-expressing cells showed an increased level of MMP3 and MMP 9 expression [Figure 5.13].

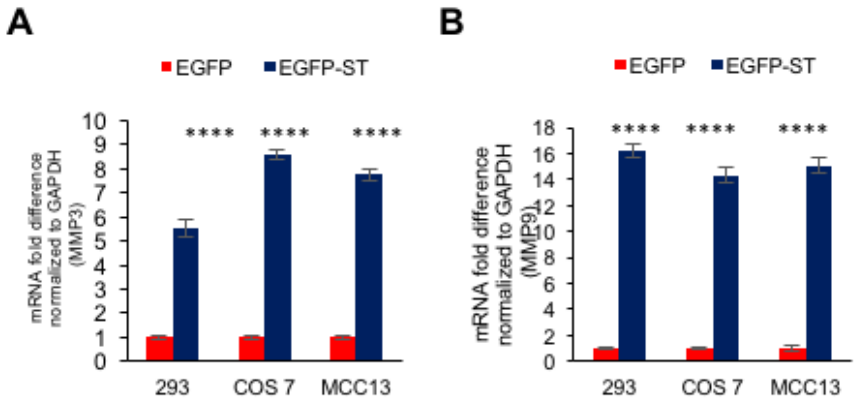

Figure 5. 13: Matrix Metalloproteinases [MMP3 and MMP9] are upregulated at the transcript level in MCPyV ST-expressing cells. Various cell lines [293, COS7 and MCC13] were transfected for 48 hours with pEGFP-C1 and pEGFP-ST-expressing plasmids. Cellular RNA was extracted using a Trizol reagent, reverse transcribed and RT-qPCR was performed. Transcript levels were analysed using the comparative CT method [n = 3. [A] MMP3 [B] MMP9. Data analysed using three replicates per experiment, n=3 and statistical analysis using a two-tailed t-test with unequal variance comparing MCPyV ST samples to EGFP control samples [****– P≤ 0.0001].

5.7 MCPyV ST enhances alteration in the host cell cytoskeleton

Cell motility is dependent on activity of integrin receptors and Rho-family GTPases resulting in the remodelling of the actin cytoskeleton, forming various cellular protrusions: such as lamellipodia, filopodia and invadopodia. Current work in the Whitehouse laboratory has shown that the MCPyV ST expression can induce the formation of filopodia-like structures in a variety of cell lines. MCPyV ST induced filopodia formation was assessed in Primary Epidermal Keratinocytes.

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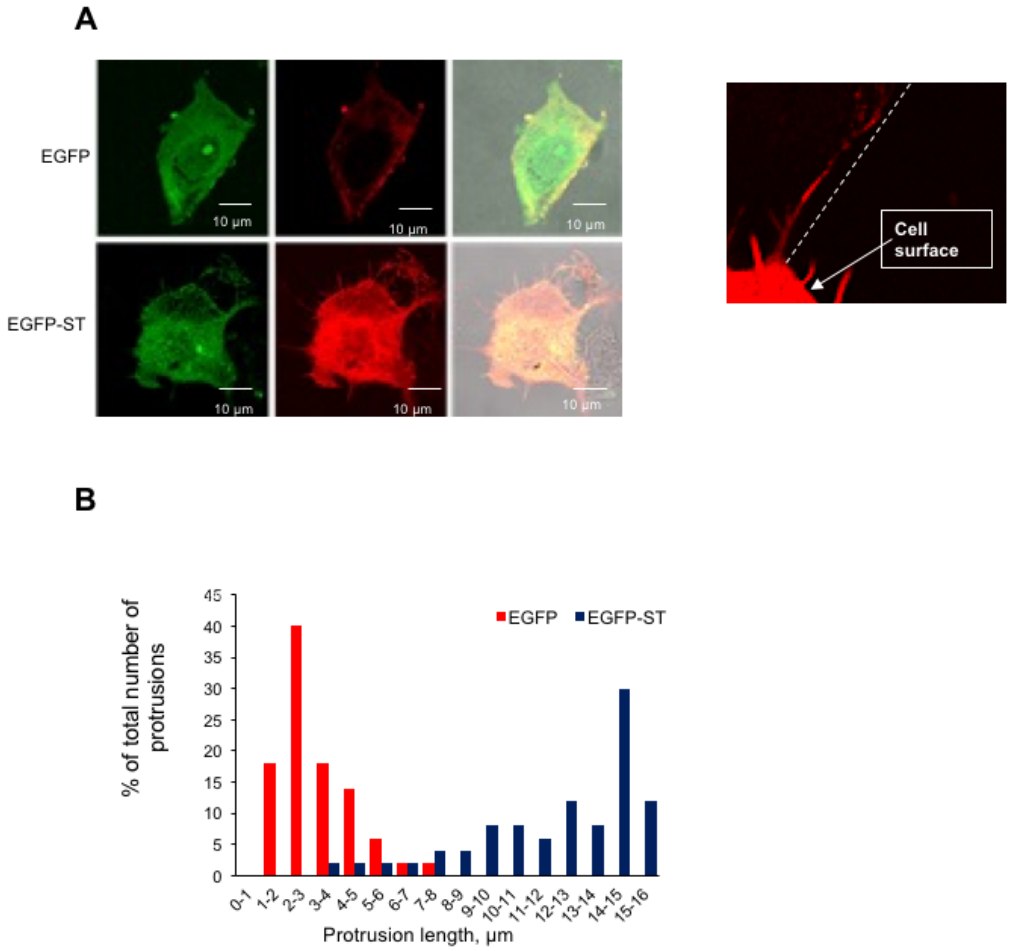

Figure 5. 14: MCPyV ST expression results in an increase in the numbers and length of actin-based protrusions in Primary Epidermal Keratinocytes. [A] Cells were transfected with 1-5 mg of either pEGFP-C1 or pEGFP-ST expression vectors. Cells were fixed after 24 h and stained with rhodamine-phalloidin. Slides were then analysed using a Zeiss LSM 8800 confocal laser scanning microscope [B] The number and length of actin-based protrusions in each cell line were analysed for 100 cells per condition using ImageJ software. Length of protusions were quantified by measuring the from the surface of the cell to the end of the protusion (**As observed in the zoomed in image**) Protrusion length is presented as a percentage of total number of protrusions.

To validate the change in phenotype due to reorganization of actin cytoskeleton similar experiments were performed primary human dermal fibroblast cells. Results also demonstrated an increase in the number and length of actin -based protrusions upon MCPyV ST expression.

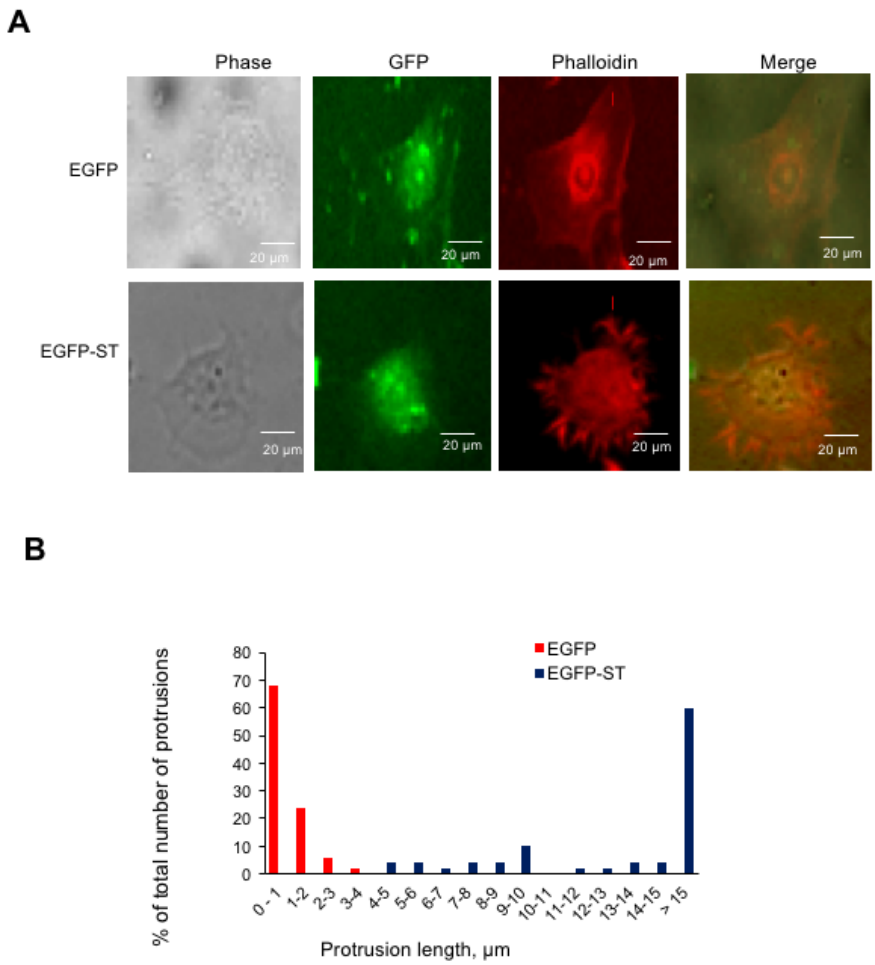

Figure 5. 15: MCPyV ST expression results in an increase in the numbers and length of actin-based protrusions in Human Dermal Fibroblasts. [A] Cells were transfected with 1-5 mg of either pEGFP-C1 or pEGFP-ST expression vectors. Cells were fixed after 24 h and stained with rhodamine-phalloidin. Slides were then analysed using a Zeiss LSM 8800 confocal laser scanning microscope [B] The number and length of actin-based protrusions in each cell line were analysed for 100 cells per condition using ImageJ software. Protrusion length is presented as a percentage of total number of protrusions.

Further work has shown that Rho-family GTPases are essential for MCPyV ST-induced filopodia formation and cell motility. The GTPase member of the Rho family are a superfamily of signalling molecules and have been implicated the metastatic and migratory potential of various cancers. Specifically, the Whitehouse Laboratory has shown that MCPyV ST-induced filopodia formation is reduced upon expression of Cdc42 or RhoA transdominant mutants (Stakaityte *et al.* 2018). This is analogous to other oncogenic viruses such as SV40, where SV40 ST is involved in the rearrangement of filamentous actin, due to loss of RhoA-dependent stress fibres, Cdc42-induced filopodia formation and Rac-induced lamellipodia formation (Nunbhakdi-Craig *et al.* 2003).

Therefore, to determine if the activity of Cdc42 and RhoA are altered in MCPyV ST-expressing cells, an affinity precipitation assay was employed to measure the amount of RhoA-GTP or Cdc42-GTP forms. Transfected EGFP or EGFP-ST HEK-293 cell lysates were incubated with either PAK1 PBD or Rhotekin RBD agarose beads. PAK1 PBD Agarose Beads are designed to pull down only the active form of Cdc42 while Rhotekin RBD Agarose beads are designed to pull down only the active form of RhoA (Stakaityte *et al.* 2018).These Agarose beads selectively bind to the GTP-bound, but not GDP-bound, forms of Cdc42 and RhoA. Upon MCPyV ST expression, elevated levels of active Cdc42 and RhoA are observed in comparison to the EGFP control cells [Figure 5.16]. This increase in active forms of Rho-GTPases enhances actin rearrangement leading to enhanced cell motility.

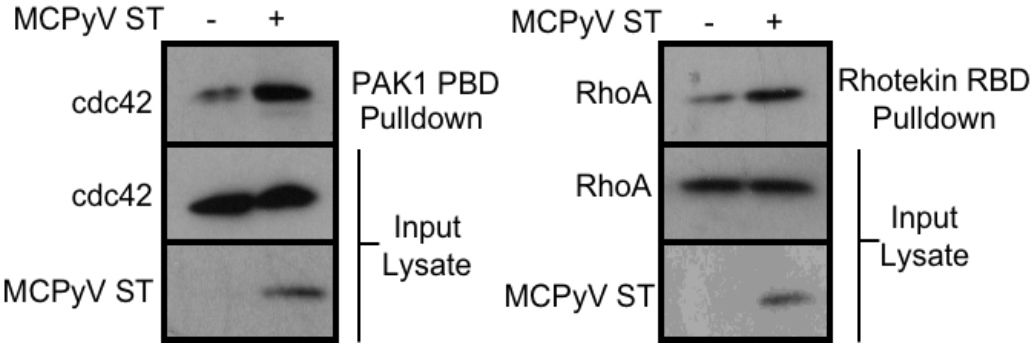

Figure 5. 16: MCPyV ST expression enhances the levels of active Rho-family GTPases. HEK-293 cells were transfected with 1 µg pEGFP-C1 or pEGFP-ST expression plasmids and after 24 hours cell lysates were incubated with either PAK1 PBD or Rhotekin RBD Agarose beads. Pulldowns were then immunoblotted with Cdc42 and RhoA-specific antibodies and the T antigen antibody was used to confirm MCPyV ST expression.

5.8 Vimentin expression is increased upon MCPyV ST expression

Vimentin is a type III intermediate filament cytoskeletal protein expressed in mesenchymal cells. It regulates cellular shape, adhesion, migration and signalling. As such Vimentin has become an important biomarker for EMT. As an important participant in the mesenchymal cytoskeleton, its expression has been shown to be upregulated in many different cancers upon the onset of EMT (Harner-Foreman *et al.* 2017). While the mechanism has yet to be elucidated, upon the transition from an epithelial phenotype to a mesenchymal phenotype, intermediate filaments comprised of Vimentin are formed in place of cytokeratin

intermediate filaments (Liu *et al.* 2015). Therefore, if MCPyV ST expression is sufficient to induce an EMT, it was important to assess Vimentin expression.

To confirm upregulation of this key mesenchymal marker, Vimentin protein levels were assessed in EGFP versus EGFP-ST-expressing COS7 and MCC13 cells and in 2 unrelated MCC tumour lysates in comparison to healthy cadaveric skin as a negative control. Vimentin protein levels were upregulated upon MCPyV ST expression in both MCPyV ST-expressing cell lines as well as MCC tumour samples in comparison to the healthy skin sample [Figure 5.17].

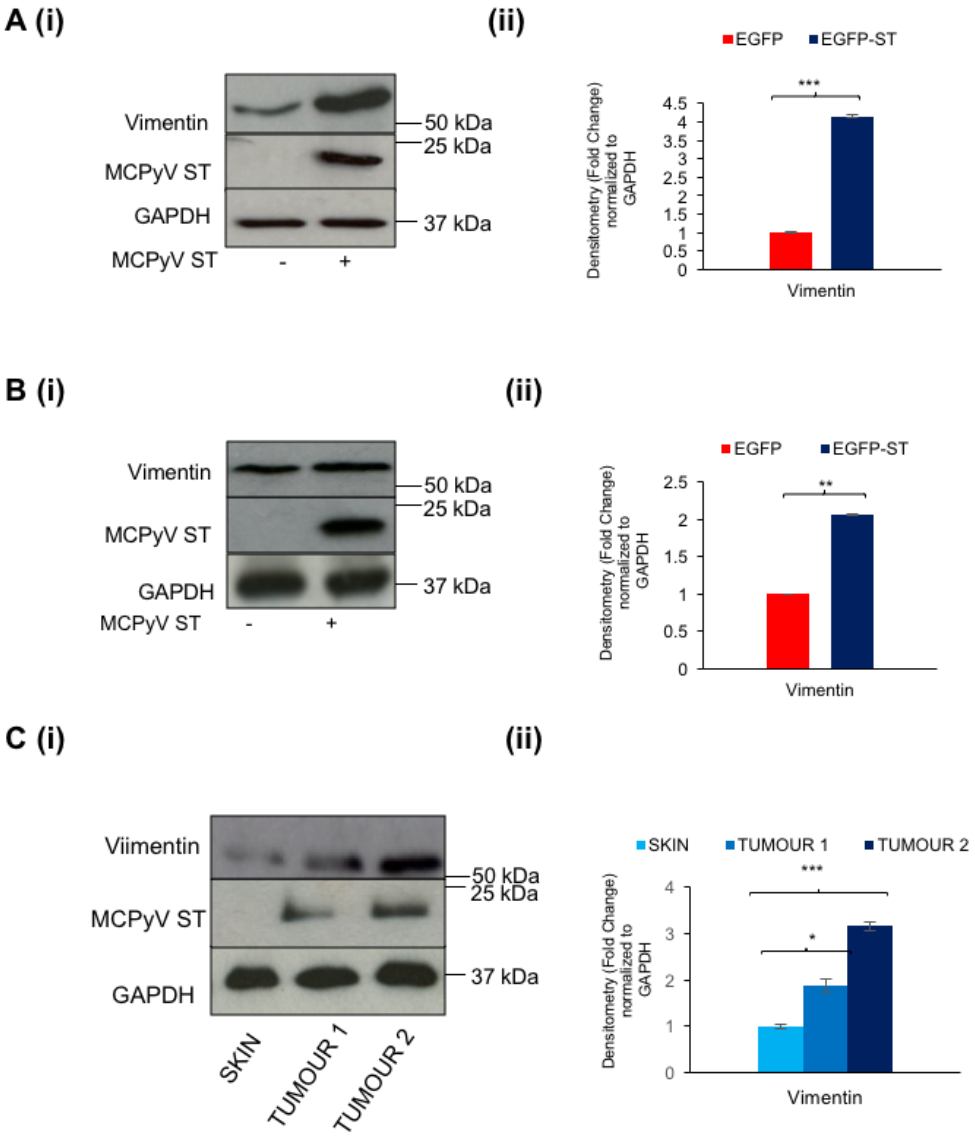

Figure 5. 17: Vimentin expression in MCPyV ST positive cells and tumour samples. [A] COS7 cells and [B] MCC13 were transfected with pEGFP-C1 and pEGFP-ST expression plasmids for 48 hours. Cell lysates were then probed with a Vimentin specific antibody. GAPDH was used as loading control and T antigen antibody was used to show MCPyV ST expression. [ii] Densitometry quantification of the western blots was carried out using the Image J software and is shown as fold change of relative densitometry to the loading control, GAPDH. Data analysed using three replicates per experiment, n=3 and statistical analysis using a two-tailed t-test with unequal variance. [** – P≤ 0.01, *** – P≤ 0.001]. [C] Healthy skin, Tumour 1 and Tumour 2 were crushed using a mortar and a pestle on dry ice, lysed with RIPA buffer for 30 mins and sonicated to further homogenized the samples. Immunoblot analysis was performed on the tissue lysates and analysed with a Vimentin specific antibody. GAPDH was used as a measure of equal loading, the T antigen antibody was used to confirm MCPyV ST expression. [ii] Densitometry quantification of the western blots was carried out using the Image J software and is shown as fold change of relative densitometry to the loading control, GAPDH. Data analysed using three replicates per experiment, n=3 and statistical analysis using a two-tailed t-test with unequal variance comparing MCPyV ST samples to EGFP control samples or comparing control cadaveric skin sample to each individual tumour [* – P≤ 0.05 *** – P≤ 0.001].

Although Vimentin is a cytoskeletol protein and should be restricted to the cytosol, expression of Vimentin has also been observed a the cell surface (Satelli and Li 2011). For example, cell surface biotinylation experiments, have shown Vimentin is detected on the cell surface of cardiomyocytes and vascular smooth muscle (Ise *et al.* 2010). Although, extracellular staining of vimentin shows a punctuate like appearance, suggesting that only a small portion of the vimentin is expressed on the cell surface.

Moisan *et al* suggest that β3-integrin-mediated recruitment of vimentin to the cell surface mediates the adhesion strength of cells binding to the substrate (Moisan and Girard 2005). Vimentin depletion is also linked to reduced alterations in the cytoskeleton organization, as well as in focal adhesions (Weidle 2011; Liu *et al.* 2015). Interestingly, although the majority of epithelial cancers do express vimentin during EMT, the extracellular location of vimentin is yet to be elucidated.

With the previously observed decrease in cell-to-cell interaction upon MCPyV ST expression, Vimentin expression was analysed at the cell surface using flow cytometry analysis to determine if extracellular vimentin expression was altered upon MCPyV ST expression. Results show that MCPyV ST expression leads to a decrease in expression of Vimentin at the cell surface, which may suggest a possible decrease in Focal adhesion [Figure 5.18].

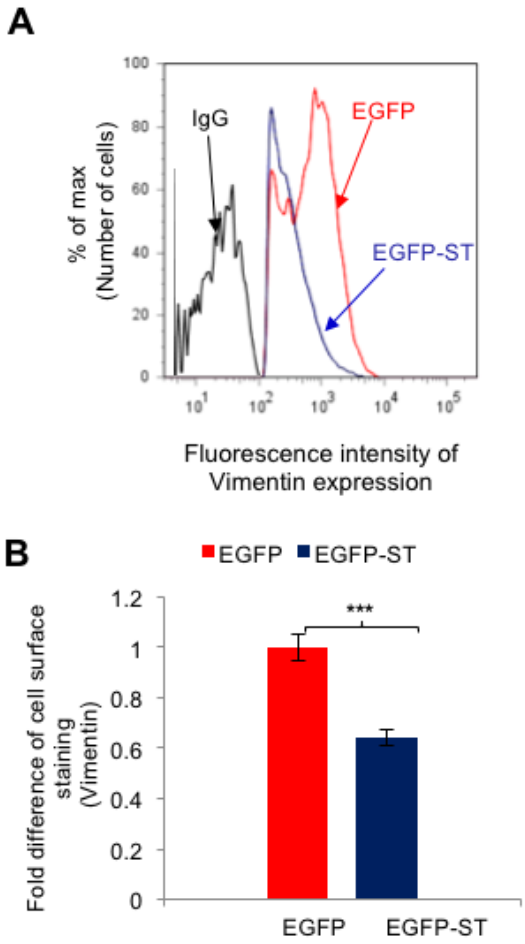

Figure 5. 18: MCPyV ST expression promotes downregulation of Vimentin at the cell surface. COS7 cells were transfected with pEGFP-C1 control and pEGFP-ST expression plasmids for 48 hours. Cells were harvested and spun down gently, blocked in 3% BSA and stained with a specific Vimentin antibody along with a corresponding secondary antibody. Mean fluorescence intensity of both control EGFP and EGFP-ST cells where plotted as a histogram using Flow Jo analysis software. [B] Fold difference was calculated using Mean fluorescence intensity. Data analysed using three replicates per experiment, n=3 and statistical analysis using a two-tailed t-test with unequal variance comparing MCPyV ST samples to EGFP control samples [*** – P≤ 0.001].

5.9 Discussion

The underlying mechanism for the high propensity of MCC tumours to metastasize is yet to be elucidated. Tumour viruses have been shown to express oncogenic proteins that induce an EMT. This resulting EMT provides cells with migratory and invasive capabilities and also inhibits apoptosis-related mechanisms (Kyprianou 2010).

Results in this chapter suggest that MCPyV ST induces an EMT which contributes to the metastatic potential of MCPyV-associated MCC. Several lines of evidence support this finding with several EMT hallmarks induced by MCPyV ST expression. Firstly, MCPyV ST expression contributes to the dissolution of tight junctions observed by decreased expression of Claudin, Occludin and Zo-1. Moreover, Adherens junctions are also destabilised by MCPyV ST, as typified by a reduction in E-cadherin.

Secondly, a major mediator of cell polarity is the formation of new cell-to-extracellular-matrix adhesion. These extracellular cues contribute to cell polarity, as studies have shown that isolated cells with no cell-to-cell contacts do not experience cell polarity (Desai *et al.* 2009). Studies have shown that loss of E-cadherin contributes to depolarization by preventing contact between SCRIB and the lateral membrane (Navarro *et al.* 2005). With the observed decrease cell adhesion markers E-cadherin, ZO-1, Claudin, Occludin and α-E-catenin, a decrease in apical to basal cell polarity was also observed upon MCPyV ST expression. This was supported by a marked downregulation of SCRIB, LGL2 and CRB3. This mechanism is important for cell motility as studies have shown that the deregulation or redistribution of major polarity complex components, such as CRB3 and LGL2, causes cells to lose their polarity and progressively assume a fibroblastic-like morphology to engage in locomotion (Thiery 2002). Additionally, loss of human Scrib has been shown to assist Ras in promoting cell invasion, by deregulating MAPK signalling (Dow *et al.* 2008).

Thirdly, MMPs are also known to target transmembrane proteins, like E-cadherin, contributing to the destabilisation of Adherens junction. Furthermore, another pathway by which MMPs contribute to EMT is inducing the expression of RAC1B, a splice variant of RAC 1, which results in upregulation of cellular reactive oxygen species. This in turn induces an increase in SNAIL expression, which has been observed upon MCPyV ST expression. While the matrix metalloproteinase family are the primary proteases involved in ECM degradation, studies have also shown

that ADAM families also have the ability to degrade components of the ECM (Wolfsberg *et al.* 1995). ADAM 10 has been shown to degrade the Type IV collagen, and MCPyV ST has been shown to upregulate ADAM 10 [Chapter 3 and Chapter 4].

Moreover, as cancer cells undergo EMT, there is a marked change in cell morphology which necessitates rearrangement of the cytoskeleton. Along with the reorganization of the actin architecture, cells obtain migratory capabilities by forming protrusions such as filopodia, invadopodia and lamelliopodia. MCPyV ST has recently been shown to induce filopodia formation (Stakaityte *et al.* 2018). Vimentin, another important component of the cytoskeleton, has also been shown to be upregulated in various cancers during EMT, and again is shown to be upregulated upon MCPyV ST expression. Studies have shown that Vimentin mediates expression of the EMT transcription factor, slug, which augments EMT phenotypes and cancer malignancy (Liu *et al.* 2015). Ras-induced transition to a mesenchymal phenotype has also been were shown to be determined on the upregulation of vimentin (Satelli and Li 2011).

However, one classic EMT hallmark, namely the E-N cadherin switch, was not observed upon MCPyV ST expression. While E-Cadherin, an identified tumour suppressor was downregulated, expression of N-cadherin, which contributes to invasion, was not altered. Therefore, an intriguing question is why does MCPyV ST expression lead to all the hallmarks of EMT apart from N-cadherin upregulation. One explanation may be the multifunctional nature of MCPyV ST. Studies have shown that neutralizing N-Cadherin expression using a specific inhibitor, GC-4 mAb induced a reduction in filopodia numbers, suggesting that N-Cadherin plays a role in filopodia formation. Studies in the Whitehouse laboratory have shown that MCPyV ST induces filopodia formation possibly by an alternative mechanism. Specifically, MCPyV ST interaction with PP4C, which leads to dephosphorylation of Integrin β1. Therefore, it may be the case that MCPyV ST does not need to increase N-cadherin levels to induce cell motility. In summary, the results suggest that MCPyV ST expression leads to an EMT inducing the majority of classical EMT hallmarks.

CHAPTER 6
FINAL DISCUSSION AND FUTURE WORK

6.1 Introduction

MCC is a highly aggressive form of skin cancer of neuroendocrine origin, which arises from Merkel cells located within the basal layer of the epidermis (Hodgson 2005). Merkel cells are touch simulated mechanoreceptor cells forming the Merkel-cell neurite complex (Haeberle and Lumpkin 2008). As a highly metastatic cancer, MCC has a propensity to disperse and spread through the dermal lymphatic system and early establishment to distant metastasis. As such, MCC has a poor 5 year survival rate (Kaae *et al.* 2010). MCPyV has been established as the causative agent of MCC tumour development. It has been found monoclonally integrated in 80-90% of both primary and metastatic MCC tumours (Goedert and Rockville Merkel Cell Carcinoma 2009).

MCPyV expresses two oncogenes from its T antigen locus: LT and ST. MCPyV LT sequences derived from MCPyV positive tumour samples have been found to contain specific LT truncation mutations that render the virus replicate defective (Angermeyer *et al.* 2013). As a result of the observed monoclonal integration along with the specific truncation mutation, MCPyV was determined to be a direct tumourigenic agent as opposed to a passenger virus (Feng *et al.* 2008).

Several oncogenic viruses have been shown to induce metastasis in infected tumour cells. For example, HPV encoded oncogenic proteins E6 and E7 [E6/E7], EBV latent membrane protein-1 and -2A, EBV nuclear antigen, HBV-encoded X antigen, as well as nonstructural HCV protein 5A are known to promote cancer metastasis (Mesri *et al.* 2014; Chen *et al.* 2016). While the overall mechanisms differ, they can include modification of cellular adhesion complexes, breakdown of the extracellular matrix, changes in gene expression and cytoskeletal remodeling.

Since the discovery of MCPyV, multiple studies have highlighted a variety of functions of the MCPyV T antigens, in viral replication and tumour formation (Liu *et al.* 2011; Demetriou *et al.* 2012). Studies have shown that MCPyV ST expression alone is sufficient to induce a loss of contact inhibition, anchorage independent, rodent fibroblast transformation and serum independent growth (Shuda *et al.* 2011). Currently multiple studies addressing the underlying molecular mechanisms that promote MCC metastasis and the potential role of the MCPyV T antigens are ongoing.

Previous work in the Whitehouse laboratory utilized high-throughput quantitative SILAC-based proteomic analysis to showed that MCPyV ST expression promotes

the differential expression of a number of proteins involved in cytoskeletal reorganization and cell motility, associated with the microtubule network (Knight *et al.* 2015) and the actin cytoskeleton (Stakaityte *et al.* 2018). Additionally, MCPyV ST expression was found to induce differential expression of cell adhesion and cell junction-related proteins. Differential expression of proteins involved in Epithelial to Mesenchymal Transition [EMT] were also observed. Together, this suggests that MCPyV ST expression may contribute to the highly metastatic nature of MCC tumours. This is also supported by recent studies showing that engraftment of MCC cell lines into SCID mice results in circulatory tumour cells and metastasis formation (Knips *et al.* 2017).

6.2 MCPyV ST deregulation of cell junctions

Cell-to-cell adhesion is essential in epithelial cells, playing multiple roles in a variety of cellular processes (Hale *et al.* 2012). One of the major cell-to-cell adhesion complexes found in epithelial cells are the Adherens junctions comprised of cadherins and catenins. As part of the catenin component, α-catenin is critical for the formation of the Adherens junctions (Hirohashi and Kanai 2003). α-catenin has three different subtypes present in humans; [α-T-catenin, testis], [α-N-catenin, Neural] and [α-E-catenin, epithelial] (Gumbiner 1996). Initial findings, in multiple cell lines, showed that MCPyV ST expression downregulates the expression of cell Adherens junction adhesion molecule, specifically α-E-catenin.

To function in cell adhesion α-catenin has to be localized at the plasma membrane. Upon MCPyV ST expression, there is an observed downregulation of α-E-catenin at the cell surface, which suggests a breakdown in the structural integrity of the Adherens junction, due to the key functional role of α-E-catenin as a mediator of structural integrity within the junction.

Various metastatic cancers, including breast, colorectal and lung cancers, have been found to have downregulated levels of α-catenin (Benjamin and Nelso 2008). Cells that lack α-catenin adhere poorly to one another even when expressing high levels of E-cadherin and β-catenin (Jeanes *et al.* 2008). While an exact mechanism is yet to be elucidated, what is clear is that α-catenin plays a major role in cadherin-based cell–cell interactions. As such it is an ideal target for MCPyV ST.

Metastasis is associated with disruption in cell-to-cell communication as numerous tumours have been observed to contain defects in multiple cell junctions such as

tight junctions (Martin and Jiang 2009). The relationship between Adherens and tight junctions is demonstrated in biochemical interactions between major components of each junction. Specifically, α-catenin and ZO-1 form a complex on the initiation of junction formation. This complex is recruited to forming junctions, thereby linking the assembly of tight and Adherens junctions (Zihni *et al.* 2016).

In epithelial cells the tight junction functions in an adhesive manner and can prevent cell dissociation. It has been inferred that the tight junction may be lost with the loss of tumour differentiation. This was validated in a mouse model which demonstrated the absence of the tight junction in breast adenocarcinomas (Weinstein *et al.* 2016). Maintenance of tight junctions is sustained by anchorage of the transmembrane proteins such as ZO-1. ZO-1 functions as a scaffold to bind the raft of tight junction molecules and link the actin cytoskeleton an signalling mechanism of the cell (Martin and Jiang 2009).

MCPyV ST expression was shown to downregulate ZO-1 expression in multiple cell lines. Additionally, upon MCPyV ST expression, there is a marked decrease of ZO-1 expression at the cell surface, which infers a loss of structural integrity at the tight junction, perhaps in collaboration with MCPyV ST effect on the Adherens junction. Interestingly, decreased expression of ZO-1 has been shown to correlate with increased invasiveness in breast, colorectal, and digestive tract cancers (Kaihara *et al.* 2003). As a major tight junction protein, ZO-1 has also been found to be involved in tumour invasion-associated EMT (Polette *et al.* 2007).

Additionally, diminished expression of α-E-catenin and ZO-1 proteins was observed in MCC tumours when compared to MCC-negative healthy skin. Interestingly, these tumour samples also exhibited differential expression of MCPyV ST and notably, lower expression of cell adhesion molecules α-E-catenin and ZO-1 were correlated with higher expression of MCPyV ST. This shows that multiple junctions may be targeted by MCPyV ST.

Results also demonstrate that this MCPyV ST-mediated disruption of cell-to-cell adhesion leads to enhanced cell dissociation. This was observed using a 72 hour scatter assay in 293 transfected cells. Results showed that upon MCPyV ST expression, cells significantly dissociated from each other, presumably as a result of disruption of cell-to-cell junctions. Interestingly, previous studies of Simian Virus 40 have also shown that expression of ST alone prompts intracellular adhesion and tight junction breakdown. This observed deregulation of epithelial cell adhesion may also contribute to tumour invasiveness (Nunbhakdi-Craig *et al.* 2003). It would be

interesting to perform this scatter assay in MCC cell lines which have depleted MCPyV ST, using siRNA lentivirus-based systems. This would confirm a direct role of MCPyV ST in cell-to-cell dissociations. However, MCPyV-positive MCC cell lines that are currently available are all suspension cultures, therefore there is a limitation in the experimental design. Additionally, desmosomes and gap junctions play a role in intercellular communication, anchorage and signalling. These types of junctions are comprised of proteins and associated molecules that also participate in maintaining the integrity of normal cell adhesion. With the observed MCPyV ST-induced breakdown of the Adherens and tight junction, it will be interesting to analyse the state of the gap junction and desmosomes. Recent studies have showed that gap junction intercellular communication [GJIC] between tumour cells and normal cells can facilitate metastasis and host colonization (Aasen *et al.* 2017). Additionally, low expression of multiple desmosome-associated cell adhesion proteins, including but not limited to Dsg2, Dsg3 and Dsc2 have been linked to various cancers including gastric and colorectal cancer (Chidgey and Dawson 2007).

Results also show that MCPyV ST leads to upregulation of the cellular sheddase ADAM 10, which induces cell-to-cell dissociation (Weber and Saftig 2012). MCC is a highly aggressive and metastatic cancer, hence upregulation of a cellular protein that induces loss of tissue integrity could contribute to the metastatic potential of MCC (Tsukita 1993; Geiger and Peeper 2009). ADAM 10 has been shown to be overexpressed in various cancer types (Mullooly *et al.* 2015; You *et al.* 2015), however the mechanism by which it contributes to MCC metastasis is unknown.

Interestingly, bioinformatics analysis has shown that ADAM10 transcripts are significantly increased in MCPyV-positive MCC compared with MCPyV-negative MCC [Collaboration between the Whitehouse and Boyne laboratories]. Here, gene expression profiles for a total of ninety-four patients were obtained from a publicly available data-set [accession number GSE39612 (Huber *et al.* 2015)]. Expression profiles were pre-processed including background correction, normalisation and summation of the intensities for each sample using R/Bioconductor (Ritchie *et al.* 2015) and the R limma package used to call differentially expressed genes. Analysis identified a significant increase (2.5-fold, p=0.03) in ADAM10 expression in MCPyV-positive MCC compared with MCPyV-negative MCC control samples. These data support our *in vitro* observations.

6.3 ADAM proteins enhance cell dissociation and cell motility

In chapter 4, the relationship between ADAM 10 and cell-to-cell dissociation as well as cell migration, was investigated using two different inhibitors, an ADAM 10 specific inhibitor and a dual ADAM 10/17 inhibitor. Previously, the ADAM10-specific inhibitor GI254023X has been shown to inhibit cell migration in various breast cancer cell lines BT20, MDA-MB-231 and MDA-MB-453 (Mullooly *et al.* 2015). Results herein using a Live cell imaging assay showed that incubation of GI254023X reduced ST-mediated cell migration. This was validated using TAPI-2, a dual ADAM 10/17 inhibitor. As ADAM 17 does share some substrate redundancies with ADAM 10, it would be important to specifically categorize ADAM 17 sheddase activity in MCC cells using direct siRNA-mediated approaches (Le Gall *et al.* 2009).

Interestingly, we observed that ADAM 10 was found to be upregulated at the cell surface by immunofluorescence studies and Flow cytometry. Sheddases, such as ADAM 10 and ADAM 17, control cell adhesion and release of cytokines growth factors in various diseases (Chow and Fernandez-Patron 2007). It is possible that the MCPyV ST-mediated upregulation of ADAM 10 at the cell surface is pertinent for the shedding of E-cadherin (Tsukita 1993; Cavallaro and Christofori 2004; Scholz *et al.* 2005). This dissociation of cell-to-cell adhesion could contribute to MCC metastatic potential and tumour invasiveness. To confirm that ADAM 10 sheddase activity is required for cell-to-cell dissociation, we repeated our scatter assay in the presence of ADAM inhibitors. Results again confirmed cell junction breakdown, which was reversed by inhibition of ADAM protein in MCPyV ST-expressing cells.

As previously discussed in chapter 4, there is ongoing therapeutic research into development of small-molecule inhibitors which target metalloproteinases for the treatment of cancer. Alternative options can also involve activation of endogenous protease inhibitors that can limit the function of tumour enhancing proteases. Active metalloproteinases are inhibited by mammalian tissue inhibitors of metalloproteinases [TIMPs]. While TIMPs inhibit all matrix metalloproteinases [MMPs], they have a high level of specificity and selectivity for ADAM proteins (Jackson *et al.* 2017). Specifically, TIMP1 has been shown to reduce ADAM10 activity shedding of the MET receptor, to maintain MET RTK signalling in models of liver metastasis (Jackson *et al.* 2017). As a natural inhibitor of ADAM 10, TIMP1 overexpression has been proven to inhibit NOTCH signalling (Amour *et al.* 2000).

However, it is important to be aware of balanced expression of proteases and their inhibitors as this a major criterion of homeostasis. While TIMP is a natural inhibitor of several endo-metalloproteinases, expression of TIMP in cancer patients is negatively correlated with their survival. Kopitz et al have demonstrated that overexpression of TIMP-1 induces a pro-metastatic function including scattered invasion in liver metastasis (Kopitz *et al.* 2007). Hence will be important to further elucidate the protease and protease inhibitor networks [endogenous metalloproteinases and TIMPs], prior to application in therapeutic studies.

6.4 MCPyV ST induces EMT

It has been firmly established that EMT supports an aggressive phenotype in tumour behaviour, specifically in cancer metastasis. This observation is strongly reinforced by studies showing that EMT increases cell migration *in vitro* and drives characteristics of tumour-initiating cells (Yang and Weinberg 2008). Also, a mesenchymal phenotype has been observed in tumour cells that are present in the circulatory system of cancer patients (Friedl and Wolf 2003). Moreover, EMT has been found to play a role in carcinoma progression (Aigner *et al.* 2007). Mesenchymal cells are able to establish metastasis and are significantly harder to target by means of chemotherapy. Hence it was important to assess the state of EMT in MCC by characterizing the phenotype of MCPyV ST-expressing cells as either Epithelial or Mesenchymal. Specific hallmarks of EMT where assessed, such as dissociation of the cell junction, loss of apical to basal cell polarity, expression of EMT-TF, breakdown of the basement membrane, changes in the cytoskeleton as well as cadherin switching.

EMT is detected upon examination of cell morphology and assessing the absence or presence of epithelial or mesenchymal markers. Known Epithelial markers include cell-to-cell contact proteins such as ZO-1 and E-cadherin. In contrast, mesenchymal markers include N-Cadherin, Vimentin, and various transcription factors like ZEB1, ZEB2, Twist, Snail, and Slug which function as transcriptional activators of EMT (Roth *et al.* 2017).

6.4.1 MCPyV ST downregulates epithelial cell junction proteins

Interestingly, MCPyV-ST-mediated deregulation of the cell junction was observed in our preliminary proteomic studies. MCPyV ST deregulates components of the

Adherens and the tight junction, as highlighted by the marked reduction of α-E-catenin and ZO-1, respectively at the protein level. However, as EMT is regulated by changes at the transcript level, further assessment was performed showing downregulation of multiple cell adhesion molecules, including previously discussed α-E-catenin and ZO-1 as well as E-Cadherin and Occludin levels upon MCPyV ST expression. This change in cell junction integrity was also shown to contribute to deregulation of Apical to basal cell polarity which together with disruption of cell junction integrity, has been shown to enhance cell migration (Ye and Weinberg 2015; Chen *et al.* 2016).

The decrease in expression of E-cadherin, a known tumour suppressor, by MCPyV ST is acknowledged as a vital event in EMT(Bae *et al.* 2013). Repressors of E-cadherin are known as EMT transcription factors [EMT- TFs]. Notably, the exogenous expression of an individual EMT-TF can pleiotropically initiate EMT. Not surprisingly, MCPyV ST induce expression of EMT-related transcription factors – Snail, Slug, ZEB1 and ZEB 2.

In vivo models of prostate cancer have demonstrated that cells with low expression of E-cadherin along with high Snail expression have a significantly increased rate of metastasis (Deep *et al.* 2014). Snail can also regulate its own expression by binding to its promoter through recognition of an E-box motif. This results in downregulation of Snail mRNA (Peiro *et al.* 2006). Altogether, interaction of the EMT-TFs with the promoter region of the E-cadherin gene results in epigenetic silencing by various histone modifications which result in DNA hypermethylation. Specifically, upon Snail expression, the SNAG domain associates with histone deacetylases [HDAC] 1 and 2, forming a repressor complex with Sin3A, which targets the E-box regions of the E-cadherin promoter, resulting in histone H3 and H4 deacetylation (Peinado *et al.* 2004). Promoter deacetylation mediates recruitment of the polycomb repressor complex 2 [PRC2]. Altogether, this functions to repress E-cadherin expression (Herranz *et al.* 2008). After initial repression of E-cadherin transcript levels, Snail induces the expression of ZEB1, which contributes to further inhibition of E-cadherin, through PRC2-independent mechanisms (Herranz *et al.* 2008). Future work will involve analyzing repressed promoters by ChIP assays and identifying which repressor proteins are bound to the repressed epithelial promoters upon MCPyV ST expression.

6.4.2 MCPyV ST represses Apical-basal cell polarity

The disruption of the intracellular contacts causes dissociation of cells from the basement membrane. As a result, it induces a loss of cell polarity. Definitively, loss of apical-basal polarity corresponds with cells adopting a front-rear polarity, which is mediated by actin cytoskeleton reorganization and facilitated by localized induction of Rho GTPases. One major consequence of the destabilization of intercellular junctions is loss of cell polarity.

MCPyV ST induced a marked downregulation of SCRIB, LGL2 and CRB3. Interestingly, in Drosophila, polarity proteins such as Crumbs, Scribble, Dlg and Lgl function as tumour suppressor genes (Bilder 2004). Additionally, research in mouse tumour model systems demonstrate a similar role wherein loss of expression of either CRB3 or SCRIB promotes tumour progression (Karp *et al.* 2008; Pearson *et al.* 2011). Defect in the actin cytoskeleton and Tight junction disruption have been linked to loss of cell polarity. In polarized epithelial cells, expression of SV40 ST alone is sufficient to induce deregulation of actin cytoskeleton and intercellular adhesion as a result of interaction of ST and PP2A. Hence, it has been suggested that viral oncoproteins have various mechanisms which can alter cell polarity pathways (Nunbhakdi-Craig *et al.* 2003; Javier 2008).

Interestingly, other components of the epithelial cell junction and cell polarity factor genes are directly repressed by EMT-TFs (Vandewalle *et al.* 2005). During EMT, EMT-TFs, specifically Snail and ZEB family members, are upregulated to alter intercellular contacts and also cell polarity, signalling loops, both feedback and feedforward, have been established in the regulation of EMT. Studies have shown that the apical polarity Crumbs complex is destabilized via Snail-regulated repression of CRB3 (Whiteman 2008) and basolateral polarity is disrupted by loss of Scribble complexes. Additionally, LGL2 is believed to be a direct target of ZEB1 transcriptional repression (Aigner 2007).

6.4.3 MCPyV ST induces basement membrane breakdown

EMT progression is also linked to upregulation of matrix metalloproteinases [MMPs] and cytoskeletal reorganization. Together, both mechanisms enable cell migration and invasion across the basement membrane (Aresu *et al.* 2011). For cells to travel through their environment, upregulation multiple matrix metalloproteinases [MMPs] is essential, to facilitate degradation of the basement membrane and

numerous ECM components (Paterson *et al.* 2013). Specifically, secreted MMPs -2,-3 -9, as well as membrane-bound MT1-MMP have important roles in the degradation of the ECM as well as disruption of the intercellular junction in order to initiate EMT (Bae *et al.* 2013; Yang *et al.* 2013).

MCPyV ST was shown to induce expression of MMP3 and MMP9 as well as ADAM proteins (Horejs 2016). Interestingly, Matrigel™ assays utilize a solubilized basement membrane preparation to quantify the ability of cells to attach to the matrix, invade into and through the matrix, and migrate towards a chemoattractant (Hall and Brooks 2014). In chapter 4 [Figure 4.15] ADAM 10 inhibition in cells positive for MCPyV showed a statistically significant decrease in motility and migration through the trans-well Matrigel™ based assay. This showed that ADAM 10 is a crucial component for MCPyV ST-mediated cell motility, migration and possible, basement membrane breakdown. Future work will involve analysing basement membrane associated proteins, like Laminin and Collagen IV upon MCPyV ST expression and possible ramification of inhibiting EMT related endo-metalloproteinases.

Induction of EMT-TFs expression consequently regulates the expression of MMPs, which in turn leads to cell invasion. Slug has been shown to control and regulate pancreatic cancer cell migration and invasion, through induction of MMP9 expression and in turn and rearrangement of F-actin filaments (Zhang *et al.* 2012). Studies have also shown that activation of EMT-TF Snail results in pro-invasive cell phenotype through upregulation of MMP14 (MT1-MMP) and MMP15 (Ota *et al.*) and this phenotype can be reversed upon knockdown of MMP14 or 15 (Ota *et al.* 2009). It will also be of interest to determine if MMP14 and/or 15 are also upregulated upon MCPyV ST expression.

6.4.4 MCPyV ST-mediated cytoskeleton reorganization

Transcriptional repression of E-cadherin promotes depolarization by hindering contact between SCRIB and the lateral membrane (Navarro 2005). Altogether, loss of apical-basal polarity corresponds with cellular adoption of front-rear polarity, facilitated by actin cytoskeleton reorganization and mediated by localized induction of Rho GTPases. Specifically, CDC42 and RAC1 are activated in the front of the cell, resulting in Arp2/3 complex-mediated actin assembly (Rotty 2013), and microtubule stabilization by DIAPH1, APC and EB1 complex formation (Wen 2004).

In the rear of the cell, RHOA is activated and participates in regulating contractile actomyosin filaments which support cell detachment and motility (Ridley 2003).

Bioinformatics analysis showed that MCPyV ST expression promotes the differential expression of a number of proteins involved in the regulation of the cytoskeleton and cell motility, both associated with the microtubule network (Knight *et al.* 2015) and with the actin cytoskeleton (Stakaityte *et al.* 2018). Results from multiple cell lines showed that MCPyV ST expression upregulates actin-associated proteins filopodia formation. Additionally, expression of actin-associated proteins was observed in MCC tumours when compared to MCC-negative healthy skin (Stakaityte *et al.* 2018. Intermediate filament marker Vimentin was also upregulated in MCPyV ST-expressing cells as well as in MCPyV positive MCC tumour cells. Correlative studies have confirmed that Vimentin expression contributes to cancer cell invasion (Wei *et al.* 2008; Liu *et al.* 2015). Vimentin intermediate filaments are also essential for invadopodia elongation and loss of Vimentin expressing causes truncation of invadopodia extension (Schoumacher *et al.* 2010). Although we have shown an increase in Vimentin expression, the ramifications of this induction on the host cell need to be further investigated.

6.4.5 Expression of EMT transcription factors

EMT-TFs can regulate their own expression as well as expression of other EMT-TFs. Simultaneously, they also facilitate upstream signalling pathways and downstream alterations including loss of E-cadherin and upregulation of MMPs which contribute to differential regulation and function of the EMT-TFs also (Onder *et al.* 2008). Interestingly, posttranscriptional regulation via microRNAs [miRNA] contribute to EMT progression. Studies have shown that the miR-200 family, targets multiple sequences in the 3' UTR of ZEB2, leading to downstream regulation of E-cadherin expression (Christoffersen *et al.* 2007). Additionally, it has been demonstrated that ZEB1 is also a target of this miRNA family, and that inhibition of miR-200 results in reduced E-cadherin, and increased Vimentin expression (Park *et al.* 2008). It would also be interesting to determine whether MCPyV ST expression also affects miRNA expression. Here is a global analysis assay, miRseq, could be utilized. Alternatively, a more focused analysis of the miR-200 family could be performed.

6.4.6 MCPyV ST-mediated Cadherin switching

The E to N-cadherin switch can be a part of EMT in some carcinomas like melanoma and pancreatic carcinoma. MCPyV ST expression did not induce E to N-cadherin switching. With other hallmarks of EMT being observed, it was important to assess what could cause the absence of E to N-cadherin switching. Interestingly, studies have shown exceptions to the E- to N-cadherin switch. In metastatic cells, there are other cadherin switches that occur and have been linked to invasion and metastasis. Examples of other cadherin switches are E- to T-cadherin and E- cadherin to cadherin 11 or E- to P-cadherin switching (Wheelock *et al.* 2008). Also E- and N-cadherin are not always mutually exclusive as observed in colon carcinoma and various endodermal epithelium derived cells (Straub *et al.* 2011). As such, these various cadherin switches need to be analysed further in MCC cells.

6.5 Final Thoughts

Additional work is ongoing to further identify mechanisms by which ST induces MCPyV-positive MCC metastasis. Previous publications have suggested that the JNK signalling cascade is activated, but there is an absence of ERK or p38 MAPK cascade activation (Houben *et al.* 2006; Wu *et al.* 2016). In contrast, preliminary data has shown that MCPyV ST expression induces both ERK and p38 phosphorylation. The mechanism through which these pathways are activated is yet to be elucidated, however the downstream targets of p38, MSK1 and MK2 have both been shown to be phosphorylated. Additionally, the use of p38 chemical inhibitors are not only able to abrogate phosphorylation, but prevent the motile phenotype typically associated MCPyV ST-expressing cells. This could therefore provide new potential targets, in addition to those identified previously, to inhibit the metastatic nature of MCPyV-positive MCCs [Personal communication].

In summary, this thesis explores the role of MCPyV ST in cell junction dissociation and the Epithelial to Mesenchymal Transition in MCC metastasis. Results presented herein implicate MCPyV ST in cell junction destabilization at the Adherens and tight junctions which promote an increase in cell motility. In addition, expression of MCPyV ST induces Epithelial to Mesenchymal transition contributing to a highly metastatic phenotype. These findings elaborate our current understanding of the function of MCPyV ST as a major oncogenic protein in MCC

and propose new potential therapeutic targets, with ADAM 10 providing a potential drug target to disrupt the metastatic nature of MCC.

CHAPTER 7
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