Merkel Cell Carcinoma

Therapeutic and Diagnostic Strategies Using CRISPR

A Thesis

Submitted to

Indian Institute of Science Education and Research Pune

in partial fulfillment of the requirements for the

BS-MS Dual Degree Programme

by

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April, 2019

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CERTIFICATE

This is to certify that this dissertation entitled “Merkel Cell Carcinoma – Therapeutic and Diagnostic Strategies Using CRISPR” towards the partial fulfillment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Komal Gupta at National Centre for Biological Sciences, Bangalore under the supervision of Prof. Sudhir Krishna and Dr. Reety Arora during the academic year 2018 – 2019.

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This dissertation is dedicated to my beloved parents and brother.

Thanks for always being there for me!

DECLARATION

I hereby declare that the matter embodied in the report entitled “Merkel Cell Carcinoma – Therapeutic and Diagnostic Strategies Using CRISPR” are the results of the work carried out by me at the Department of Biology, National Centre for Biological Sciences, Bangalore, under the supervision of Prof. Sudhir Krishna and Dr. Reety Arora and the same has not been submitted elsewhere for any other degree.

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ACKNOWLEDGMENTS

Past 10 months of my dissertation project at National Centre for Biological Sciences, Bangalore has been a fantastic journey. I have learnt a lot, developed as a scientist and as a person. I have several people to thank for this.

Firstly, I am immensely grateful to Dr. Reety Arora for being such an amazing mentor. Her problem solving skills, approach towards experiments and positive attitude are just a few qualities of gazillions that I have tried to learn and wish to inculcate in my future research. Her emphasis on understanding the details and learning something new from every failed experiment has taught me lot about conducting research and the spirit of science.

I would like to thank Prof. Sudhir Krishna for sharing his valuable advice and critical review of the work that has helped immensely for conducting this work. I would like to acknowledge all the Lab7 members for their suggestions and constant encouragement. Special mention to Lamiya Dohadwala, Anjali Vijaykumar and Bhavana Nayer for their assistance in conducting experiments and enthusiastic encouragement during failures!

I would like to extend my gratitude to Prof. Sanjeev Galande, my project advisor, for sparing his valuable suggestions and his critical review of the work. Also,

I thank Dr. Debojyoti Chakraborty and Mohammed Azhar from IGIB, Delhi for providing some plasmids and advice on CRISPR related experiments. I also thank Aurelie Jory, Mohamed Haroon and Dr Praveen Vemula for providing purified Cas proteins for pilot experiments.

Lastly, but most importantly, I thank my family for their constant motivation and support throughout this journey. I am immensely grateful for their confidence in me and for always boosting me to achieve higher!

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ABSTRACT

Merkel cell carcinoma (MCC) is a rare, aggressive skin cancer caused either by Merkel cell polyomavirus (MCV) T antigen gene expression, post integration (~80% cases), or by UV mediated DNA damage. Viral-positive Merkel tumors are not only caused by but also oncogenically addicted to tumor antigen expression. In this study we used CRISPR-based gene-editing to develop therapeutic and diagnostic tools for MCV positive MCC.

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas system is a genome editing technology whereby a guide RNA (gRNA) molecule, targets a Cas endonuclease to a specific genomic site, using sequence homology, and induces a double strand break. To target MCV T antigens, we designed 13 gRNAs targeting the T antigen genomic region. We validated the MCV TAg targeting efficiency of our gRNAs by *in vitro* cleavage assays. To translate this finding, we delivered this CRISPR system in patient-derived MCC cell lines as well. Our proof-of-concept study shows that 2 MCV targeting CRISPR/Cas gRNAs in combination can knock out MCV T antigen, thus, being of therapeutic importance. This CRISPR system can be potentially delivered *in vivo* using Adenovirus associated vectors (AAV) for advancing MCV positive MCC treatment in the future.

To a pathologist both MCV positive and negative tumors are identical. However, overall survival of patients suffering from MCV positive Merkel cell carcinoma is better, making this knowledge of important diagnostic and prognostic value. Hence, we used the DETECTR method, pioneered by Chen *et al*., 2018, to create an *in vitro* Cas12a based molecular diagnostic test for MCV. Briefly, when RNA-guided Cas12a binds target MCV dsDNA it also leads to indiscriminate single-stranded DNA reporter cleavage. DETECTR couples recombinase polymerase based amplification of target MCV DNA with Cas12a mediated detection. We show that DETECTR system can detect MCV integrated in Merkel tumor rapidly, specifically and efficiently. This rapid MCV DNA detecting system is promising and can be coupled with histopathological and immunohistochemical studies to diagnose the viral status in MCC.

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Chapter 1

INTRODUCTION

1.1 Viruses causing cancer

Viruses cause 10-15% of human cancers worldwide, substantiating a significant burden of disease (Parkin 2006). These oncogenic viruses may induce tumors by altering DNA repair mechanisms, epigenetic modifications, cell cycle dysregulation which leads to genomic instability. In certain cancers the virus integrates its DNA within the host genome thereby altering signal transduction pathways and gene expression which plays a role in cancer metastasis and relapse (Moore and Chang 2013). Currently, a total of seven viruses have been identified as Group 1 carcinogens by the International Agency for Research on Cancer (Table 1) (Blackadar 2016). Merkel cell polyomavirus (MCV) is the most recent addition to this set of 7 known human tumor viruses (Feng, 2008).

Virus	Year identified	Family	Genome	Size	Cancer association	Reference
Epstein-Barr virus (EBV)	1964	*Herpesviridae*	dsRNA	~170kb	Most Burkitt's lymphoma and nasopharangeal carcinoma	Epstein et al., Lancet, 1964
Hepatitis B virus (HBV)	1965	*Hepadnaviridae*	ssDNA and dsDNA	~3.2kb	Some hepatocellular carcinoma	Blumberg et al., JAMA, 1965
Human T-lymphotrophic virus-I (HTLV)	1980	*Retroviridae*	Positive-strand, ssRNA	5.5kb	Adult T cell leukemia	Poiesz et al., PNAS, 1980
Human papillomaviruses (HPV) 16 and HPV 18	1983-1984	*Papillomaviridae*	dsDNA	~7.8kb	Most cervical and penile cancers	Durst et al., PNAS, 1983 Boshart et al., EMBO J,1983
Hepatitis C virus (HCV)	1989	*Flaviviridae*	Positive-strand, ssRNA	9.6kb	Some hepatocellular carcinoma and some lymphomas	Choo et al., Science, 1989
Kaposi's sarcoma herpesvirus (KHSV)	1994	*Herpesviridae*	dsDNA	~165kb	Kaposi's sarcoma, primary effusion lymphoma	Chang et al., Science, 1994
Merkel cell polyomavirus (MCV)	2008	*Polyomaviridae*	Double-stranded DNA	~5.4kb	Merkel cell carcinoma	Feng et al., 2008

Table 1: Viruses causing cancer

1.2 Merkel cell carcinoma

Merkel cell carcinoma (MCC) is a rare, lethal, primary skin cancer frequently having a severe outcome (Figure 1) (Becker et al. 2017). MCC was first described in 1972 as Trabecular carcinoma of the skin (Toker C, 1972). However, later it was observed that the tumour cells resembled Merkel cells, thus, the name Merkel cell carcinoma was adopted. Though controversial, it is thought that MCC derives from mechanoreceptor Merkel cells, located in the basal layer of the epidermis and form an integral part of the sensory system of the skin. These cells express both endocrine and neural markers (Maricich et al. 2009). Though rare, this cancer is highly prevalent in immunosuppressed patients or elder patients of age above 70 years (Engels et al. 2002). MCC is frequently metastatic and has an estimated 33–46% disease specific mortality (Agelli M 2010). The incidence of MCC is between 0.13 – 1.6 per 100,000 people (Harms et al. 2018).

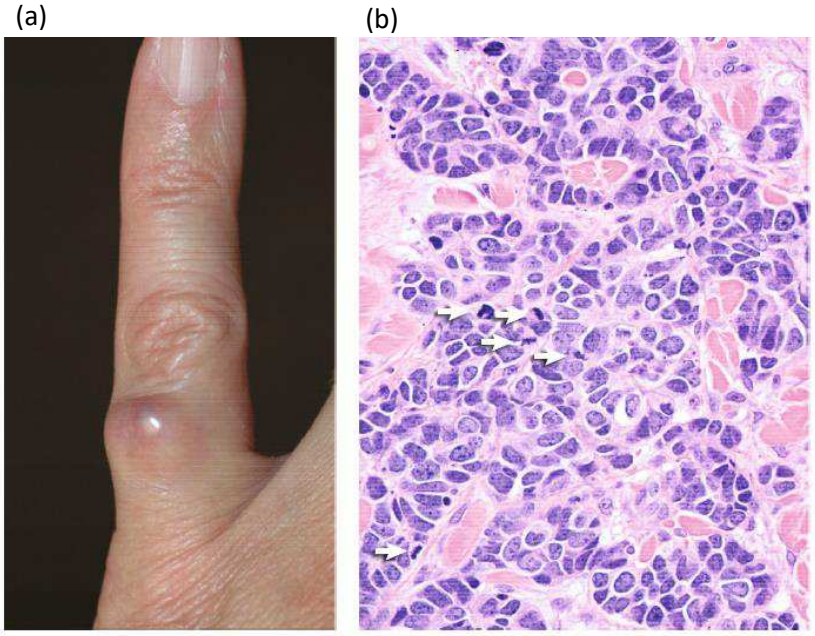

Figure 1: Clinical and histological appearance of MCC.

(a) Picture showing the clinical appearance of Merkel cell carcinoma (MCC). Such nodules and lesions are also commonly observed on a patient's head or neck.

(b) Haematoxylin and eosin staining of a sample showing the histological appearance of Merkel Cell carcinoma. Image depicts round cells with minimal cytoplasm and arrows indicate several mitotic figures. (Image from Isaac Brownell *et al*., Nature Rev Clinical Oncology, 2018)

MCC carcinogenesis either arises due to clonal integration, followed by T antigen truncation-mutation, of Merkel cell polyomavirus (MCV) (Feng, 2008) or chronic exposure to ultraviolet light (Goh et al. 2015) (Figure 2). In MCV positive MCC (60 – 80% of the cases), MCV is the main driver of cancer and the tumour genome has lesser mutations. On the other hand, MCV negative MCC has high frequency of mutations caused by UV damage and high degree of aneuploidy (Goh et al. 2015; Madore et al. 2015) .

Interestingly, in a majority of the human population MCV is a harmless, asymptomatic and life-long infection. However, MCV initiates an aggressive cancer if it is integrated into the susceptible host genome and acquires viral mutations that lead to replication incompetence as described in later sections.

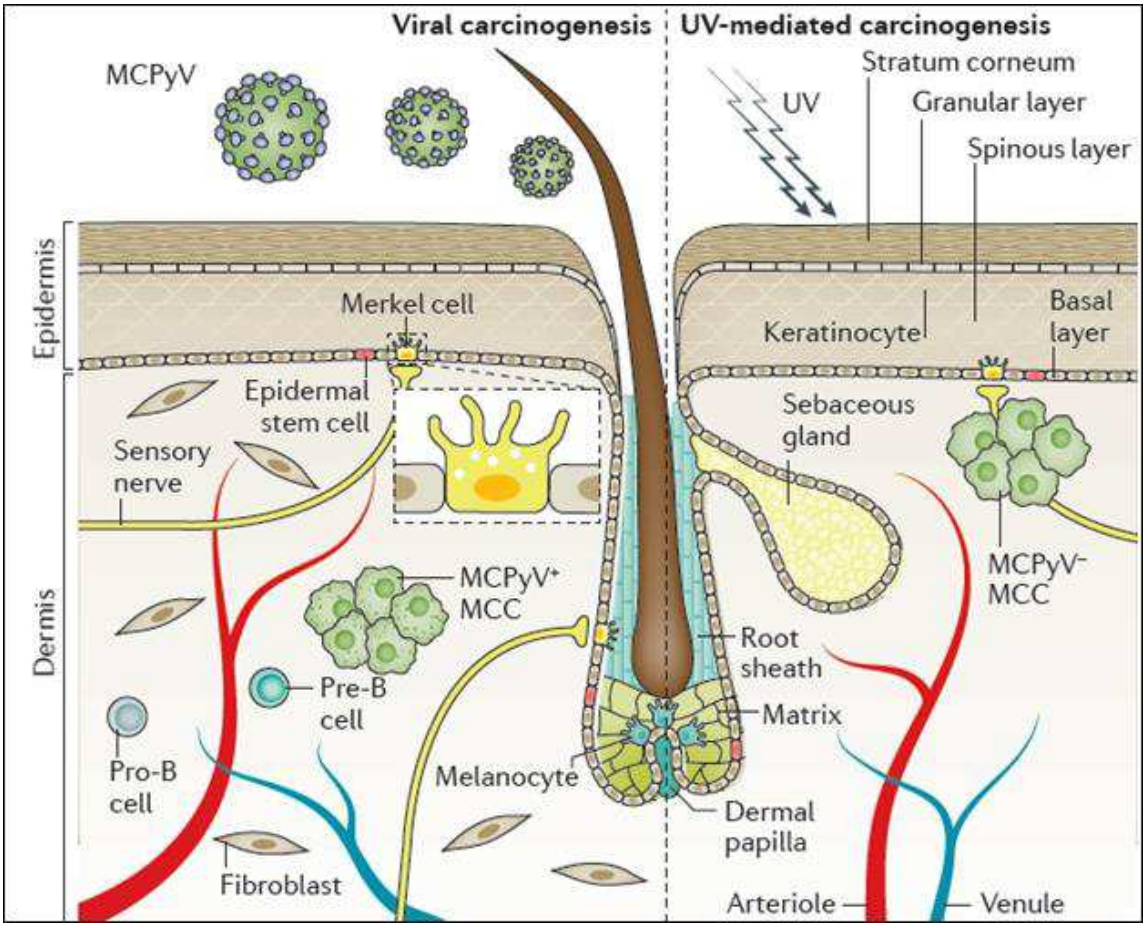

Figure 2: MCC cell origin and location.
A schematic showing location of Merkel cell on the basal layer of epidermis connected to a sensory neuron, near a hair follicle. Left panel shows the MCV driven MCC, which is more common in countries with low UV exposure. The right panel shows UV mediated MCC which is prevalent in high UV exposure countries and is characterized by DNA mutations. (Image from Becker *et al*; Nature reviews, 2017)

1.3 Merkel Cell Polyomavirus

Merkel cell polyomavirus belongs to the Polyomaviridae family of viruses. Polyomaviruses are non-enveloped dsDNA based viruses with approximately 5.2 kb genome and 45nm particle size. This family comprises of 14 human polyomaviruses including BK virus (BKV) and JC virus (JCV). Merkel Cell Polyomavirus (MCV) was discovered by Feng, *et al.* in 2008 by a method known as Digital Transcriptome Subtraction (DTS). In this method, whole mRNA was extracted from the Merkel cell genome, complementary DNA (cDNA) was synthesized followed by sequencing. These sequences were screened for non-human sequences using bioinformatics. The sequence candidates obtained from the DTS were then aligned against known viral and bacterial database using BLAST. One candidate was similar yet distinct from the known Lymphotrophic polyomavirus. Further analysis discovered that the MCV genome was integrated in the cellular genome.

1.3.1 MCV genome

MCV is a double-stranded DNA virus with a genome size of ~5.3 kb. It contains the early region which encodes the small T-antigen (ST), large T-antigen (LT) and spliced variants of LT namely 57kT (LT') and ALTO (Alternate frame of LT Open reading frame). The late region codes for the capsid proteins VP1 and VP2. The two transcriptional units are separated by the Non Coding Control Region (NCCR) region, containing the bidirectional promoter, enhancer and an origin of replication (Figure 3).

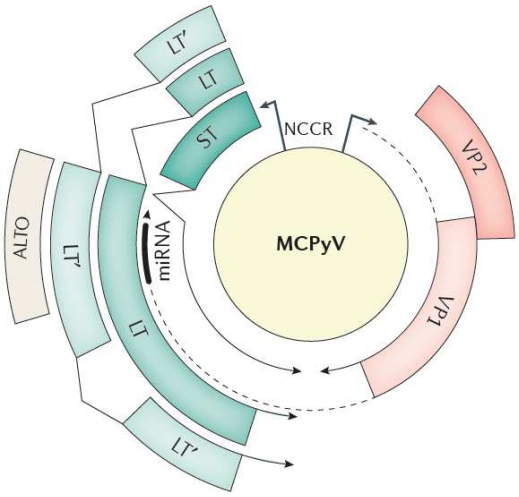

Figure 3: Schematic of MCV genome map.
Merkel cell polyomavirus consists of 5,387 bp circular double- stranded DNA genome having two transcriptional units, the early and late regions. The transcription of early region leads to four spliced mRNAs encoding four proteins: two alternatively spliced isoforms of the large T antigen, LT and 57kT, the small T antigen (ST) and ALTO (alternate frame of the LT open reading frame). The late region encodes viral coat proteins, VP1 and VP2.

1.3.2 Large Tumour antigen (LT)

Large tumour antigen is a splice variant of the T antigen locus comprising of Exon 1 and Exon 2. LT has several common domains, which are required for facilitating viral life cycle (Figure 4). The N-terminal of LT has DnaJ domain consisting of Conserved Region 1 (CR1) motif and Hsc70 binding motif which are essential for MCV replication (Wendzicki, Moore, and Chang 2015). In addition to these domains there are the OBD and helicase/ATPase domains required for viral DNA replication. The Nuclear Localization Signal (NLS) is another important domain that helps the viral genome to enter into the host nucleus. The LT also consists of motifs, which can disable the tumour suppressor pathway of the host cell. For example, the highly conserved Retinoblastoma-associated protein (RB) - binding (LXCXE) motif leads to dysregulation of E2F-mediated transcription which in turn results in cells getting in S-phase (Houben et al. 2012). Thus, LT, primarily by its Rb binding activity contributes to oncogenesis.

Interestingly, in MCV positive MCC cells the LT is truncated due to premature stop codon mutation or deletions. These truncations result in a way that the Rb binding LXCXE domain is preserved but the helicase/ATPase domain required for replication of MCV DNA is eliminated (Masahiro Shuda, 2008). Thus, the virus is replication-incompetent in Merkel tumour cells.

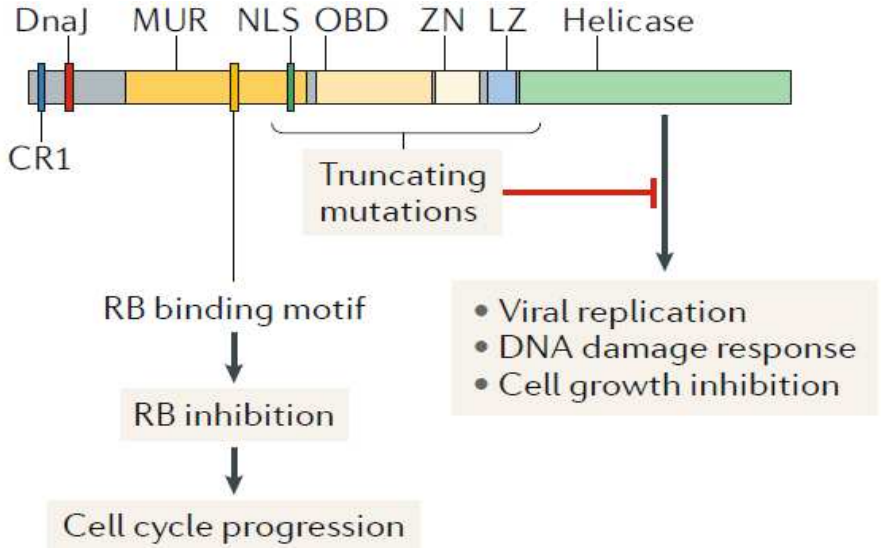

Figure 4: Large Tumor Antigen: Domains and their functions.
LT has a N terminal J domain, MCV unique region (MUR)-1 and MUR2, LXCXE motif, which binds to the retinoblastoma-associated protein (RB1), nuclear localization signal (NLS), DNA or origin binding domain (OBD) and helicase domain. In Merkel cell carcinoma (MCC), mutations in MCV DNA result in truncated LTs that retain the LXCXE motif.

1.3.3 Small Tumour Antigen

ST protein is composed of exon 1 and intron 1 of the T antigen locus. The functional domains of ST extend into the spliced site of LT generating a unique carboxy terminal which harbors the protein phosphatase 2A (PP2A) binding site and the LT stabilizing domain (LSD).

ST inhibits PP2A, a serine threonine phosphatase via the PP2A binding domain. This interaction leads to inhibition of NFkB pathway and also increases cell motility (Rodriguez-viciana, Collins, and Fried 2006). However, PP2A binding domain is not necessary for tumorigenesis. The second important feature of ST, LT-Stabilization Domain (LSD), plays a role in inhibiting SCFFbw7 E3 ubiquitin ligase (Hyun Jin Kwun, 2014). One of the targets of Fbw7 is LT, thus, sT stops the degradation of LT. Fbw7 has several other targets as well which are involved in carcinogenesis such as c-Myc and cyclin E. Thus, LSD also prevents the degradation of these oncogenes resulting in initiation and progression of tumorigenesis. The LSD domain also plays a role in hyper-phosphorylating 4E-BP1 which is an important regulator of eukaryotic cap-dependent translation (Masahiro Shuda, 2011). This hyper-phosphorylation of 4E-BP1 at serine 65 makes it insensitive to mTOR pathway inhibitors. This causes uncontrolled cell proliferation leading to carcinogenesis.

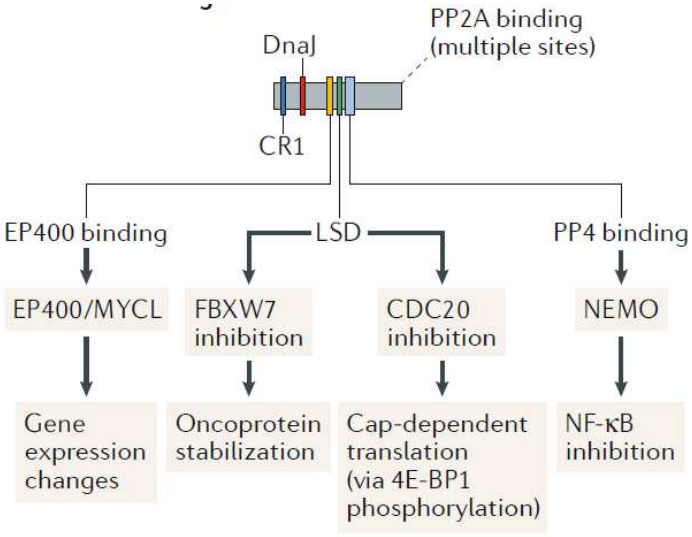

Figure 5: Small Tumor Antigen: Domains and their functions.
ST contains an N terminal J domain and a unique domain absent in LT. ST can bind to regulatory and catalytic subunits of protein phosphatase 2A (PP2A). ST also has LT stabilization domain (LSD), which inhibits SCFFbw7 E3 ubiquitin ligase.

1.4 CRISPR/Cas system

The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas9 system is a programmable gene editing system which was derived from the bacterial immune system (Boyaval et al. 2007). This system enables the bacteria to remember the DNA inserted by a phage and destroy it in any subsequent infection by that phage. The system consists of a site-specific guide RNA (gRNA) and a Cas endonuclease. The Cas protein is guided to its target with the help of gRNA. gRNA consists of two components, the crRNA, which has the sequence complementary to the target DNA sequence, and the tracrRNA, which is the scaffold that links crRNA to the Cas9 as shown in Figure 6 (Swarts et al. 2017). Another requirement for Cas9 endonuclease activity is the presence of a 3 bp sequence called as Protospacer Adjacent motif (PAM) immediately downstream of the target sequence. Different Cas homologs have different PAM sequence requirement, for example, PAM site for Cas9 is NGG whereas for Cas12a is TTTN.

When the Cas9 with the help of gRNA binds the target, a conformational change takes place which leads to proper positioning of its two endonuclease domains and opposite strands of the target DNA are cleaved leading to a Double-Strand Break (DSB) 3-4 nucleotides upstream of PAM. These DSBs are mostly repaired via the mutagenic non-homologous end joining (NHEJ) repair pathway leading to the disruption of the targeted gene (Figure 6).

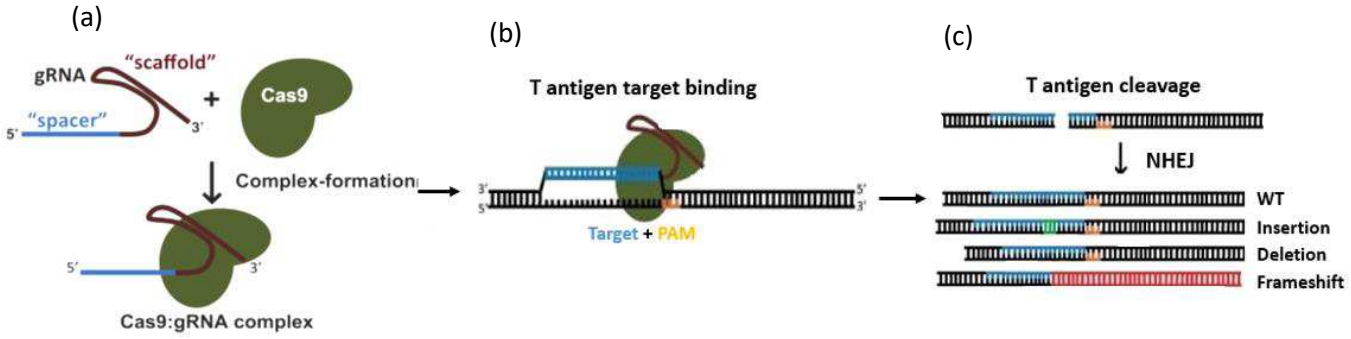

Figure 6: CRISPR/Cas mediated gene editing.

(a) Schematic shows two components of gRNA, spacer and scaffold. Binding of scaffold and the Cas9 protein makes the Cas9-gRNA complex.
(b) This complex then searches and binds to the target DNA provided that target is upstream (5') of a Protospacer Adjacent Motif (PAM)
(c) Cleaves of the target DNA leads to mutagenic repair via NHEJ pathway.

1.5 OBJECTIVE 1: Developing diagnostic strategy for MCV in MCC

Merkel cell carcinoma has 2 causes – either by MCV or by UV-linked mutations. A recent study showed that out of 282 MCC tumors 20% were MCV negative using multimodal qPCR and immunohistochemistry analysis (Moshiri et al. 2017). Interestingly, the study indicated that as compared to MCV negative MCC patients, MCV positive MCC patients had significantly better progression-free survival, MCC-specific survival, and overall survival from MCC (Figure 7). Hence identifying viral status is important for prognostic predictability as well. In this study we develop a CRISPR/Cas12a based *in vitro* molecular diagnostic system for MCV.

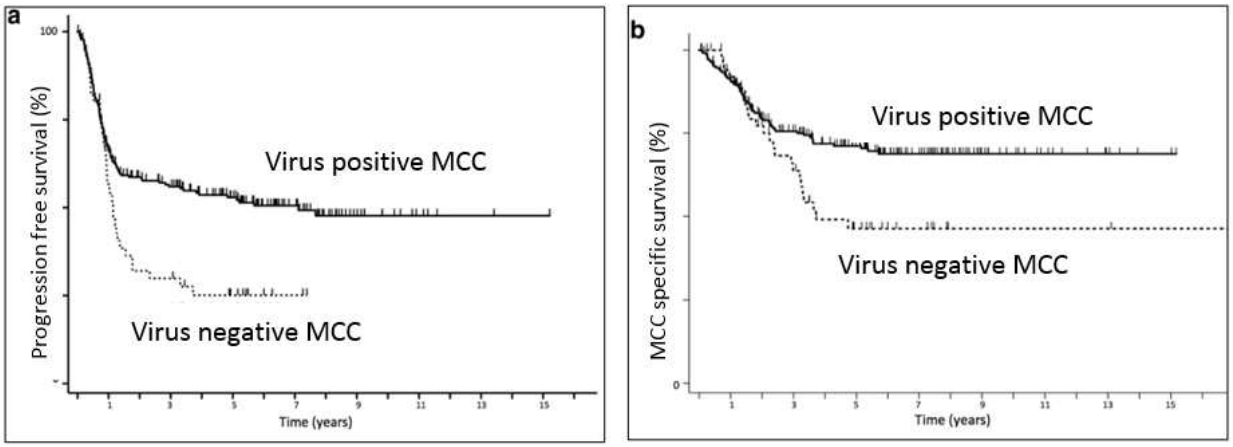

Figure 7: MCV positive MCC vs MCV negative MCC disease prognosis
Graphs representing (a) Progression free survival (n = 247) and (b) MCC specific survival (n = 281) for MCV positive vs MCV negative MCC patients. MCV positive MCC patients have better progression-free survival, MCC-specific survival, and overall survival from MCV negative MCC patients.

1.5.1 Cas12a – A homolog of Cas9

Cas12a (Also known as Cpf1) is a type V CRISPR protein having various properties distinct from Cas9 (Dong et al. 2016). Cas12a enzymes recognize a T nucleotide–rich protospacer-adjacent motif (PAM) i.e. TTN (Figure 8). These enzymes also catalyze their own guide CRISPR RNA (crRNA) maturation (Zetsche et al. 2015). They generate a PAM-distal dsDNA break with staggered 5′ and 3′ ends whereas Cas9 generates blunt end dsDNA cut.

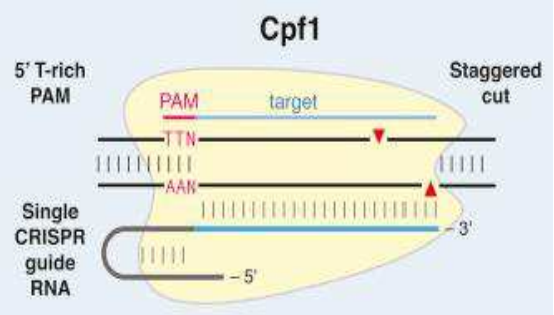

Figure 8: Cas12a cleavage activity. Cas12a, with the help of the gRNA, cleaves the target DNA in a staggered manner, creating a 5 nucleotide 5 overhang 18-23 bases away from the PAM sequence.

1.5.2 DNA endonuclease-targeted CRISPR trans reporter (DETECTR)

Interestingly, after dsDNA target binding, the Cas12a enzyme leads to indiscriminate trans-ssDNA cleavage activity. This trans ssDNA cleavage activity is conserved among the Class V Cas proteins. Chen *et al.* used this property to develop a DNA detection tool called DNA Endonuclease-TargEted CRISPR Trans Reporter (DETECTR) system (Chen et al. 2018). DETECTR couples Recombinase Polymerase Amplification (RPA) based isothermal amplification of target DNA with Cas12a mediated target DNA detection.

We adapted the DETECTR system to detect the presence of MCV DNA integration in Merkel cell tumor genome. We used AsCas12a derived from *Acidaminococcus* sp. in comparison to the *Lb sp* used by Chen et al. AsCas12a also shares the trans ssDNA shredding activity (Figure 9).

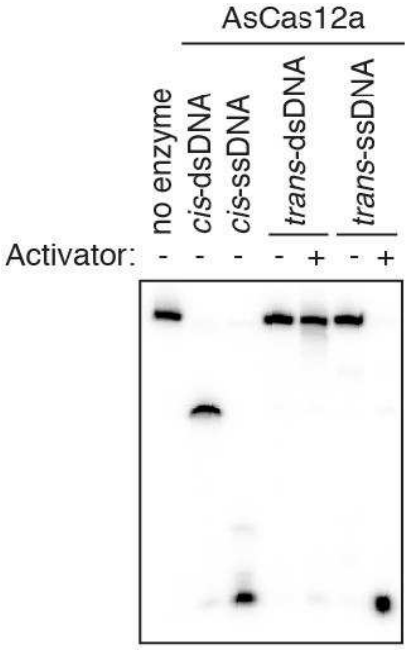

Figure 9: Cas12a target binding leads to indiscriminate single stranded DNase activity PAGE depicting AsCas12a, in the presence of cis dsDNA activator also shreds the non-complimentary ssDNA but not dsDNA.

Briefly, we assemble a reaction mix containing AsCas12a, MCV specific gRNA, test genomic DNA sample and fluorophore-quencher (FQ) ssDNA substrate. In the presence of MCV DNA, Cas12a binds and cleaves the cis target DNA followed by trans cleavage of fluorescently labelled ssDNA (Figure 10). This Cas12a mediated DNase activity leads to fluorescence based detection of MCV. We designed gRNAs targeting MCV genome and validated their target cleaving efficiency using *in vitro* cleavage assay. The trans ssDNA cleavage activity of AsCas12a was assessed using ssDNA M13 and FQ reporters. We show that DETECTR system can detect MCV in MCV positive MCC cells. This MCV DNA detecting system can be coupled with histopathological and immunohistochemical studies to diagnose the viral status in MCC.

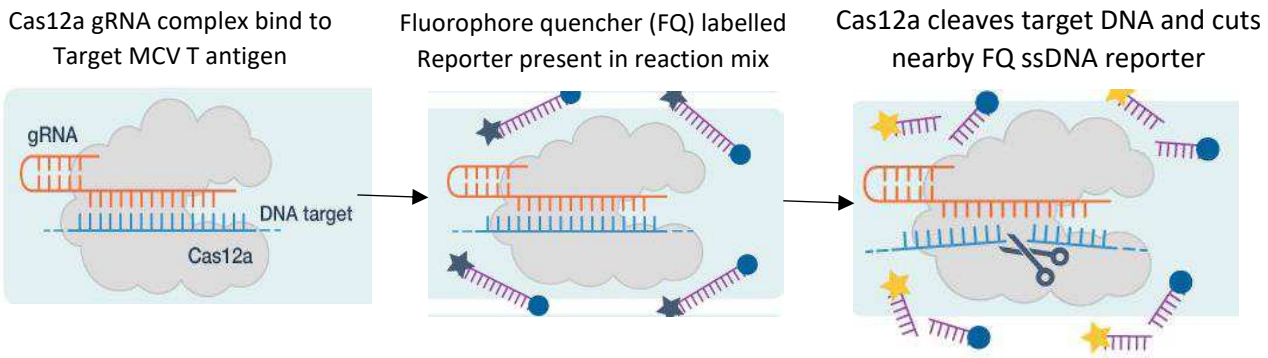

Figure 10: DETECTR diagnoses target dsDNA via labelled ssDNA reporter shredding

Schematic showing DETECTR system. First, the Cas12a and MCV targeting gRNA complex bind the complimentary dsDNA. After sis MCV target cleavage, the trans FQ labelled ssDNA is also cleaved, thus, reporting the presence of MCV DNA.

1.6 OBJECTIVE 2: Developing therapeutic strategy for MCV positive MCC

1.6.1 MCV positive MCC cells are oncogenically addicted to T antigen expression

According to Shuda M, *et al*. expression of MCV sT alone leads to *in vitro* transformation of rodent fibroblasts in soft agar and focus formation assays. Also, ST is required for continued cell proliferation in MCC (Figure 11). Further, when MCV ST is expressed in mice it results in *in vivo* proliferative activity. These studies show that MCV T antigens drive carcinogenesis in MCV positive Merkel cells.

According to Houben, R. *et al.,* when these viral oncogenes are silenced in MCV positive MCC, the cells die. Thus, these unique, external, viral oncoproteins can be used as important therapeutic and diagnostic targets for MCC. Thus, in this study, we developed therapeutic and diagnostic strategies for MCV positive MCC, targeting the T antigens using CRISPR/Cas.

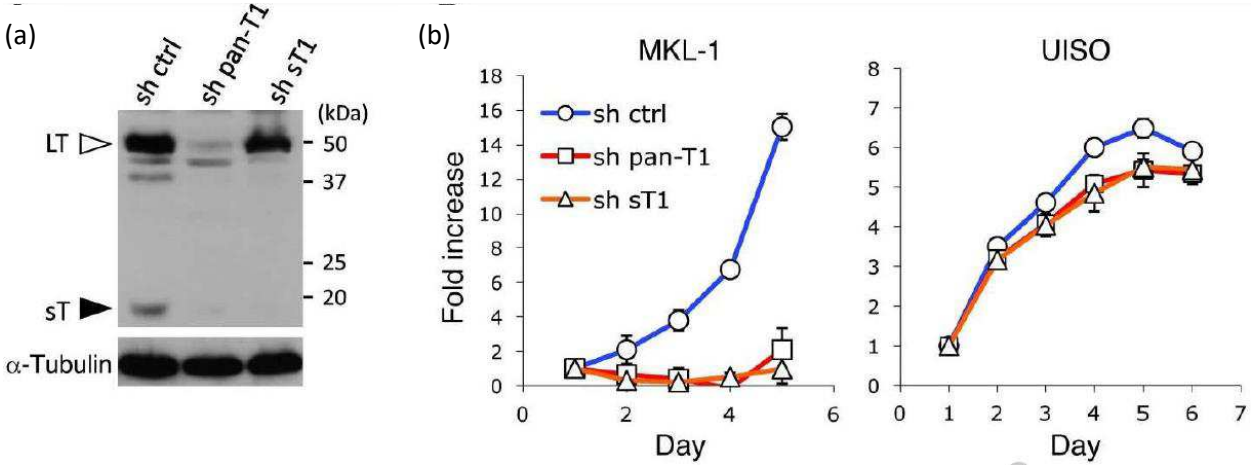

Figure 11: MCV positive MCC cells are oncogenically addicted to Tag expression

(a) Immunoblot showing shRNA mediated knock down MCV T antigens. Sh Pan-T1 targets both sT and LT whereas sh sT1 specifically targets sT.

(b) WST assay showing effect of knocking down T antigens in MKL-1 (MCV positive) and UISO (MCV negative) cells. Knocking down T antigens in MKL-1 cells leads to cell death, whereas there is no effect in UISO cells. Thus, MCV positive MCC cells are addicted to T antigen expression for survival

1.6.2 T antigen gene editing using the CRISPR/Cas system

Using transfection or lentiviral transduction, it is possible to induce the cleavage of specific sequences in the human genome by the expression of SpCas9 and a gRNA molecule together. Gene inactivation using this system is highly efficient, and SpCas9 has been previously used to target various genes including HIV proteins and HPV E6/E7 oncoproteins in cervical carcinoma (Hsu et al. 2015).

In this study, we used the CRISPR/Cas system to target the T antigen oncogenes in MCV positive MCC cell lines. We first designed several gRNAs and tested their ability to target T antigens in cells. We then selected 2 gRNAs and validated their efficiency using an *in vitro* cleavage assay. Further, we used two gRNAs together to target the T antigen loci. This results in DSBs at two sites leading to excision of the DNA sequence flanking between the two target sites. This strategy is more robust as compared to targeting via single gRNA as the excision of DNA sequence leads to knock out of target gene (Figure 12). Our study provides a proof of principle for the use of CRISPR technology to edit out MCV T antigen from the Merkel tumor genome. Removal of MCV T antigens by using SpCas9/gRNA combinations system has significant clinical potential in the treatment of MCV positive MCC.

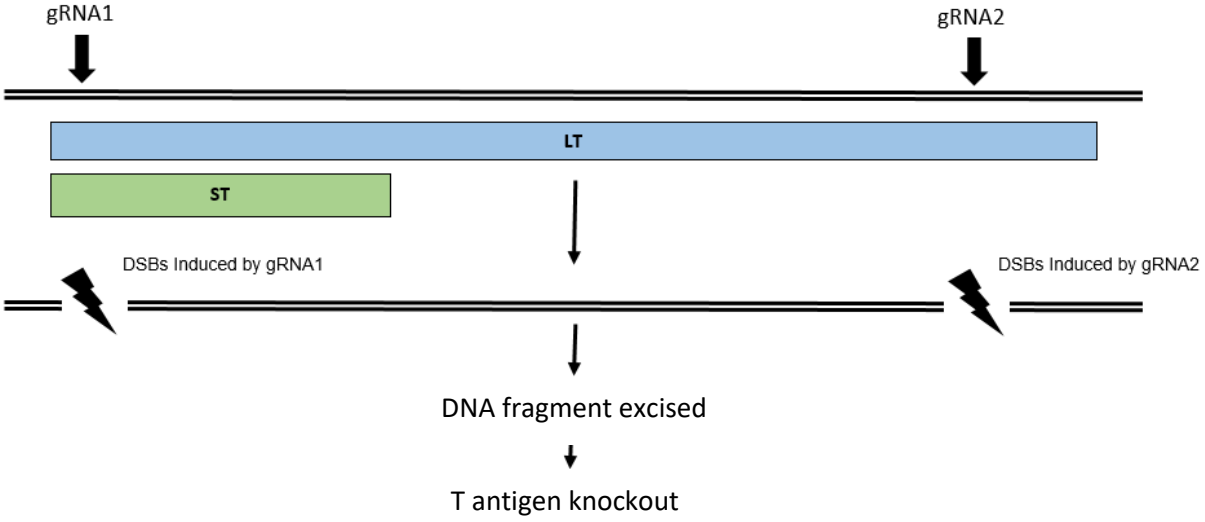

Figure 12: Schematic of T antigen gene editing using the CRISPR/Cas system.
The CRISPR/Cas system could induce double strand breaks in the T antigen oncogene. Using two gRNAs in combination leads to excision of the DNA sequence flanking between the two gRNA target sites. This excision leads to knock out of MCV T antigen. Black arrows represent Cas9 enzyme-mediated DSB breaking sites upon gRNA recognition.

1.7 OBJECTIVE 3: Investigating reprogramming capacity of MCV Small T antigen

1.7.1 ST binds to MYCL

Cheng *et al*. demonstrated that ST binds to the promoter of MYCL, a reprogramming transcription factor and a known oncogene, leading to its transcriptional activation (Cheng et al. 2017). They further showed that elevated levels of MYCL in turn bind to the ST-EP400-MAX complex using immunoprecipitation. The EP400 histone acetyltransferase complex contributes to transcriptional activation of genes by acetylating histone proteins while MAX is involved in sequence-specific DNA binding. The ChIP-Seq data revealed that ST-MYCL-EP400-MAX complex binds to the promoter region of several genes to activate gene expression in MCV positive MCC cell lines. Also, disruption of the MYCL-ST-p400 complex led to decreased MCC cell proliferation and increased levels of differentiation-associated genes.

1.7.2 Generation of iPSCs with MYCL, OCT4, SOX2 and KLF4 expression

MYCL along with OCT4, SOX2 and KLF4 (OSK) expression leads to induced pluripotent stem cells generation (Takahashi et al. 2006). As MCV ST binds to MYCL, Cheng *et al*. investigated the reprogramming capability of ST. To test this they developed stable keratinocyte cell line expressing MYCL or ST. They introduced the other reprogramming factors using an inducible OSK expressing plasmid. OSK expression when coupled with ST led to iPSC generation. Thus, ST binding to MYCL and EP400 complex leads to reprogramming of keratinocytes to induced pluripotent stem cells.

The Retinoblastoma (pRb) pathway poses a roadblock from reprogramming human fibroblasts to iPSCs, implying that the inactivation of pRb could promote stemness and cellular plasticity. Since MCV LT, interacts with pRB and sequesters it we wanted to investigate if the presence of MCV large T accelerates this iPSC formation, in the presence of small T antigen. In this study, we attempted to investigate the reprogramming capacity of ST along with LT in fibroblasts. This may imply that MCV T-antigen expression in Merkel cells can induce dedifferentiation to a carcinogenic state.

Chapter 2

MATERIALS AND METHODS

2.1 sgRNA design.

For CRISPR/Cas9 mediated MCV TAg knock out experiments, 13 gRNAs were designed to target the small and large Tumor antigen genes. Three CRISPR design tools: Broad institute software, CRISPR-MIT (http://crispr.mit.edu) and CRISPOR were used. gRNAs with highest specificity score and with lowest possible off targets were selected. Synthetic gRNA oligonucleotides were cloned into pLentiCRISPRv2 (Addgene #52961) at BsmBI (# R0580S, NEB) restriction sites. Plasmid DNA was prepared using Stbl3 competent cells (#C7373-03, Invitrogen) and Qiagen midi prep kits (#12143).

For CRISPR/Cas12a mediated MCV detection project, 10 AsCas12a gRNAs were designed to target the Non coding Control Region (NCCR), small and large Tumor (sT and LT, respectively) antigen of MCV. Two CRISPR gRNA design tools: Benchling (https://benchling.com/pub/cpf1) and RGEN (www.rgenome.net/cas-designer/) were used. MKL-1. MCV gRNA target region sequence conservation was analysed using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

For both Cas9 and Cas12a gRNAs, MKL-1 (Genbank Accession #: FJ173815.1) and MS-1 (Genbank Accession #: JX045709.1) MCV sequences were used as target DNA sequences. gRNAs with highest specificity score and with lowest possible off targets were selected.

2.2 Synthesis of sgRNAs using IVT

Two oligonucleotides were designed as DNA templates, per gRNA, for *in vitro* transcription (IVT). For CRIPSR/Cas9 gRNAs, forward oligo consisted of the T7 promoter (TAATACGACTCACTATAGG), guide RNA, and start of Cas9 gRNA scaffold. Reverse oligo consisted of complete gRNA scaffold with complementarity to 15nt in the forward oligo (shown below). Three GGG's were added at the gRNA start for efficient T7 transcription.

Target substrate for C3 gRNA *in vitro* cleavage (TARGET / pam):

5' GATGAAAGCTGCTTTCAAAAGAAGCTGCTTAAAGCATCACCCTGATAAAGgggGAAATCCTGTTAT3'

crRNA oligo for C3 gRNA *in vitro* transcription (**T7** / ADDED G / GUIDE / *SCAFFOLD*):

Forward primer **TAATACGACTCACTATA**GGGAAAGCATCACCCTGATAAAG*GTTTTAGAGCTAGAA*

Reverse primer: *AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATT TTAACTTGCTATTTCTAGCTCTAAAAC*

Target substrate for C13 *in vitro* cleavage (TARGET / pam):

5' CCAGTGTACCTAGAAATTCTTCCAGAACGGATGGCACCTgggAGGATCTCTTCTGCGATGAATC 3'

crRNA oligo for C13 gRNA *in vitro* transcription (**T7** / ADDED G / GUIDE / *SCAFFOLD*):

Forward primer: **TAATACGACTCACTATA**GGGTTCCAGAACGGATGGCACCT*GTTTTAGAGCTAGA*

Reverse primer: *AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTT AACTTGCTATTTCTAGCTCTAAAAC*

Templates for IVT were amplified by the following PCR reaction: Denaturation at 95 o C for 3 mins followed by 30 cycles of 95^{o}C – 30s, 45^{o}C – 30s and 72^{o}C for 30s and final annealing at 72^{o}C for 7 min. The PCR products were cleaned up using PCR clean up kit (# A9282, Promega).

For CRISPR/Cas12a gRNAs, forward oligonucleotide consisted of the T7 promoter (TAATACGACTCACTATAGG) followed by AsCas12a sgRNA scaffold and 20 nucleotide long guide RNA. Three G's were added after T7 promoter sequence for efficient T7 transcription.

Target substrate for AsCas12a NCCR gRNA1 *in vitro* cleavage (pam/TARGET):

5' GGCCTCTCTCTTTTtttcCAGAGGCCTCGGAGGCTAGGAGCCCCAAGCCTCTG 3'

crRNA oligo for *in vitro* transcription (**T7** / ADDED G / *SCAFFOLD* / GUIDE*):*

Forward oligo

TAATACGACTCACTATAGGG*TAATTTCTACTCTTGTAGAT*CAGAGGCCTCGGAGGCTAGG

Reverse oligo:

CCTAGCCTCCGAGGCCTCTG*ATCTACAAGAGTAGAAATTA*CCC**TATAGTGAGTCGTATTA**

Forward and reverse oligonucleotides were annealed by heating to 95^{o}C for 5 minutes and slow cooling on bench top.

gRNAs were synthesized using the MEGAShortScript TM Kit (# AM1354, Invitrogen) following the manufacturer's protocol with 2ug DNA template over night at 37^0C. The RNA was treated with 1μl of Dnase TURBO at 55ºCfor 15 minutes followed by purification using the MEGAclear Transcription Clean-Up Kit (# AM1908, Invitrogen).

2.3 In vitro cleavage (IVC) assay.

In vitro cleavage reaction was performed with purified SpCas9 protein (Kind gift from Dr. Praveen Vemula's Laboratory) or AsCas12a (#1081068, IDT) at 37ºC in cleavage buffer consisting of 20 mM HEPES (pH 7.5), 150 mM KCl, 10 mM MgCl2, 1% glycerol and 0.5mM DTT for 1 hour. For Cas9 IVC, 300 ng of sgRNA and 200 ng of the target DNA were used for the reaction. For AsCas12a IVC, 30nM AsCas12a (#1081068, IDT) and 36nM gRNA were pre-assembled in the cleavage buffer at 37ºC for 10 minutes. 18.5nM dsDNA target was added to the reaction (20μL). PCR amplicons of sT and LT gene regions from plasmid RAZ1 (Addgene #114381) were used as target DNA templates (Refer Table 2 for primers). The PCR amplicon was purified using Ampure beads (#A63881, Beckman Coulter) as per manufacturer's protocol.

For M13 cleavage assays, 30nM AsCas12 and 36nM gRNA were preassembled at 37ºC for 10 minutes in cleavage buffer. 40nM ssDNA activator and 10nM single stranded M13mp18 phage (#N4040S, NEB) were added to initiate the reaction (3μl). The reaction was incubated at 37ºC for 60 minutes. The IVC reactions were stopped by treatment with 2ul of Proteinase K (10mg/ml) at 55ºC for 10 minutes. 1x gel loading dye was added to the reactions and samples were run on 2% agarose gels (# RM273, HIMEDIA).

2.4 Gibson Cloning the gRNAs in pDECKO mCherry.

For expressing the gRNAs in MCV positive cell lines, C3 and C13 were cloned into pDECKO mCherry lentiviral plasmid using Gibson assembly. The backbone plasmid (kind gift of Dr. Debjyoti Chakroborty, IGIB) was digested using BsmBI at 55ºC for 2 hours. The cut plasmid was run on a 0.8% agarose gel and the linear band was gel extracted. The oligonucleotides for insert 1 consisting of the gRNAs, T7 and part of H1 promoter and gRNA scaffold were designed using http://crispeta.crg.eu. Insert 1 was

cloned into digested vector and was transformed into Stbl 3 competent cells. Positive clones were selected using colony PCR followed by plasmid isolation. This intermediate plasmid was again digested by BsmB1. The constant insert 2 consisting of remaining part of gRNA 1 scaffold and H1 promoter was PCR amplified from the complete pDECKO plasmid and was cloned into the intermediate plasmid. The reaction was transformed in Stbl 3 competent cells. Positive clones were validated using colony PCR and sequencing.

2.5 Cell culture conditions.

HEK 293T cells were obtained from ATCC and grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum, 1X Penicillin/Streptomycin (Pen Strep, catalog # 15140122). Cells were trypsinized using 0.5% trypsin and split every 3-4 days as per cell confluency. Merkel Cell Carcinoma cell line, MKL-1 was obtained from ECACC (# 09111801) and cultured in Roswell Park Memorial Institute (RPMI) Media with 10% FBS and 1X Penicillin/Streptomycin. The cells were grown at 37^{o}C with 5% CO_2. These cells were split according to cell growth.

2.6 Lentiviral vector construction and transduction.

Lentiviral vectors were constructed by co-transfecting HEK 293 T cells with plasmid of interest, the Gag-Pol packaging plasmid psPAX2 (Addgene #12259), and pVSVG (Addgene #8454) expressing plasmid in a ratio of 5:3.75:1.25. Cells were transfected at 60-70% confluency. Lipofectamine® LTX (Invitrogen #15338100) was used in 3:1 ratio with the total amount of DNA to be transfected. DNA, Lipofectamine LTX (Invitrogen #15338500) and OptiMEM mix were incubated at RT for 20 minutes and added to cells in antibiotics free media. Viral media was collected 48h and 72h post transfection. The viral media was filtered through 0.45 um filter followed by concentration through Amicon column and addition of 1/3rd volume of LentiX Concentrator (Takara #631231). Viral pellet was obtained by spin down at 1500g at 4 o C, resuspended in RPMI media and added to the MKL-1 cells to be transduced. Screening of gRNAs was done by transducing pLentiCRISPRv2 plasmid containing the gRNAs in MKL-1 cells followed by puromycin (1ug/mL) selection. pLentiCRISPRv2 (Addgene #52961) without gRNA was

used for mock transduction. For constructing MKL-1 pDECKO mCherry C3+C13 cell line, MKL-1 cells were transduced with pDECKO mCherry C3+C13 plasmid. pDECKO mCherry empty vector (EV; without gRNAs) was used as an empty vector for mock transduction. MKL-1 Cas9 cell line was developed using pCW Cas9 Blast (Addgene #) with Blasticidin (10ug/mL) selection for 10 days post transduction. Cas9 was expressed by 1ug/ml of Doxycycline induction.

2.7 Western blot analysis.

Cells were lysed in EBC buffer (50 mM Tris pH-8, 150 mM NaCl, 0.5mM EDTA, β mercaptoethanol-1:10,000, 0.5% NP-40, 1 mM PMSF supplemented with protease inhibitor cocktail (Roche, Cat #118575). Protein lysate was quantitated using Pierce BCA protein assay kit. The lysate was denatured in 1x Laemmli buffer by boiling at 99^0C for 5 minutes. The proteins were separated using SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (#88518, Biorad). The PVDF membrane was blocked with 5% BLOTTO (SC-2325, Santacruz) in 1X TBS-T. Immunostaining The blot was developed was performed with primary antibodies overnight at 4^0C as follows: anti-MCV Tumor antigen monoclonal antibody (1:5000; Ab3 and Ab5 were used), anti-Cas9 monoclonal antibody (1:1000; Cell signaling #14697), and anti-Vinculin monoclonal antibody (1:10000; Sigma #V9131). The PVDF membrane was incubated in peroxidase-labeled secondary antibodies. The ECL™ Prime Western Blotting System (#RPN2232, GE Healthcare) was used for the detection of chemiluminescence according to the manufacturer's protocol. The chemiluminescence was detected using ImageQuant™ Las 4000 (GE Healthcare).

2.8 Isolation of Cellular DNA.

Cells were lysed in 0.1M NaCl, 10mM Tris HCl pH 8, 25mM EDTA pH 8, 0.5% SDS and 200ug/mL Proteinase K at 55ºC for 3 hours – overnight. Genomic DNA was extracted using TE saturated Phenol: Chloroform: Isoamyl alcohol (25:24:1) (Invitrogen; Cat#15593-031) as per manufacturer's protocol.

2.9 Fluorophore quencher (FQ)-labeled reporter assays

200nM AsCas12a, 250nM gRNA and 4nM ssDNA/dsDNA target template were pre-assembled in a 5μl reaction for 30 minutes at 37ºC. Reaction was initiated by diluting these complexes to AsCas12a: gRNA: dsDNA template to 50nM : 62.5nM: 1nM in 1x Binding buffer (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 5 mM MgCl2, 1 mM DTT, 5% glycerol, 50 μg ml^{-1} heparin) and 50nM FQ probe (#4331182*,* Thermo). The 20ul reactions were incubated at 37ºC for 1 hour in a 384 well microplate format. The fluorescence was measured using Tecan Infinite pro (Excitation: 485nm, Emission: 535nm).

2.10 Recombinase Polymerase Amplification (RPA) reaction

RPA reaction mix was prepared by adding 0.48uM forward Primer and reverse primer, 29.5 μl primer Free Rehydration buffer, 1μL of extracted genomic DNA and water to 13.2 μl (Total volume 47.5 μl). This was mixed by vortexing and spinning down. The reaction mix was added to one TwistAmp® Basic (TwistDx) reaction tube. 2.5μl of 280mM Magnesium Acetate (MgOAc) was added and mixed well to start the reaction. The reaction was incubated at 37ºC for 10 minutes. 18μL of this reaction mix was assembled with 2μL of 50nM AsCas12a, 62.5nM gRNA and 50nM FQ substrate. Reaction was incubated at 37ºC for 1hour and fluorescence was measured every minute.

Primer No.	Description	Sequence (5'-3')
Oligonucleotides for C3 and C13 in vitro transcription		
1	C3 IVT OLIGO F	TAATACGACTCACTATAGGGAAAGCATCACCCTGATAAAGGTTTTAGAGCTAGAA
2	C13 IVT OLIGO F	TAATACGACTCACTATAGGGTTCCAGAACGGATGGCACCTGTTTTAGAGCTAGAA
3	gRNA scaffold IVT oligo R	AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTG CTATTTCTAGCTCTAAAAC
Primers for PCR amplifying Cas9 MCV IVC template		
4	IVC RAZ1 target F	TTGGCTGCCTAGGTGACTTT
5	IVC RAZ1 target R	GTGGATGTGGAATGTGTGCG
Oligonucleotides for Gibson cloning of C3 and C13 in pDECKO mCherry		
6	C3/C13.1R_Gib	TCAGGGTGATGCTTTCCGGTTTCGTCCTTTCCACAAGAT
7	C3/C13.2F_Gib	ACCGGAAAGCATCACCCTGATAAAGGTTTTAGAGCTAGAAGAGAC
8	C3/C13.3R_Gib	GAGACGGGATCCTAGGAATTCCGTCTCTTCTAGCTCTAAAAC

9	C3/C13.4F_Gib	TTCCTAGGATCCCGTCTCTCTGTATGAGACCACTCTTTCCC
10	C3/C13.5R_Gib	AACAGGTGCCATCCGTTCTGGAAGGGAAAGAGTGGTCTCAT
11	C3/C13.6F_Gib	GAACGGATGGCACCTGTTTTAGAGCTAGAAATAGCAAGTT
12	Scaffold F	GTTTTAGAGCTAGAAATAGCAAG
13	H1_R	GTGGTCTCATACAGAACTTATAAG
Primers for Cas9 target region PCR from MKL-1 genomic DNA		
14	C3 target F	TTGGCTGCCTAGGTGACTTT
15	C3 target R	CCAGGACCTCTGCAAAATCT
16	C13 target F	TGCTTACTGCATCTGCACCT
17	C13 target R	AAATGGCAAAACAACTTACTGTT
Oligonucleotides for AsCas12a gRNA in vitro synthesis		
18	HPV L1 gRNA F	TAATACGACTCACTATAGGGTAATTTCTACTCTTGTAGATCTACATTACAGGCTAACAAA
19	HPV L1 gRNA R	TTTGTTAGCCTGTAATGTAGATCTACAAGAGTAGAAATTACCCTATAGTGAGTCGTATTA
20	MCV.NCCR1.F	TAATACGACTCACTATAGGGTAATTTCTACTCTTGTAGATAACAAGGGAGGCCCGGAGGC
21	MCV.NCCR1.R	GCCTCCGGGCCTCCCTTGTTATCTACAAGAGTAGAAATTACCCTATAGTGAGTCGTATTA
22	MCV.NCCR2.F	TAATACGACTCACTATAGGGTAATTTCTACTCTTGTAGATCTGGAGAGGCGGAGTTTGAC
23	MCV.NCCR2.R	GTCAAACTCCGCCTCTCCAGATCTACAAGAGTAGAAATTACCCTATAGTGAGTCGTATTA
24	MCV.NCCR3.F	TAATACGACTCACTATAGGGTAATTTCTACTCTTGTAGATCAGAGGCCTCGGAGGCTAGG
25	MCV.NCCR.3.R	CCTAGCCTCCGAGGCCTCTGATCTACAAGAGTAGAAATTACCCTATAGTGAGTCGTATTA
26	MCV.Exon 1.1 F	TAATACGACTCACTATAGGGTAATTTCTACTCTTGTAGATGGACTAAATCCATCTTGTCT
27	Exon 1.1 R	AGACAAGATGGATTTAGTCCATCTACAAGAGTAGAAATTACCCTATAGTGAGTCGTATTA
28	MCV.Exon 1.2 F	TAATACGACTCACTATAGGGTAATTTCTACTCTTGTAGATGAGATTGCTCCTAATTGTTA
29	MCV.Exon 1.2 R	TAACAATTAGGAGCAATCTCATCTACAAGAGTAGAAATTACCCTATAGTGAGTCGTATTA
30	MCV.Exon 2.1 F	TAATACGACTCACTATAGGGTAATTTCTACTCTTGTAGATCCATCTAGGTTGACGAGGCC
31	MCV.Exon 2.1 R	GGCCTCGTCAACCTAGATGGATCTACAAGAGTAGAAATTACCCTATAGTGAGTCGTATTA
32	MCV.Exon 2.2 F	TAATACGACTCACTATAGGGTAATTTCTACTCTTGTAGATTGGATCTTGAGTTGGTCCCG
33	MCV.Exon 2.2 R	CGGGACCAACTCAAGATCCAATCTACAAGAGTAGAAATTACCCTATAGTGAGTCGTATTA
Primers for RPA of MCV target		
34	NCCR3_RPA_FP	GCAGCAATAAAAGTTCAATCATGTAACCACAA
35	NCCR3_RPA_RP	CTTGGGATCTGCCCTTAGATACTGCCTTTTT
36	Exon1.1_RPA_FP	TAGTGAGGTAGCTCATTTGCTCCTCTGCTCTT
37	Exon1.1_RPA_RP	TTGCCATAACAATTAGGAGCAATCTCTAAAAG
39	Exon1.2_RPA_FP	CTCCTTCTGCATATAGACAAGATGGATTTAGTC
40	Exon1.2_RPA_RP	ATTCCATCATTATAACAGGATTTCCCCCTTTAT
41	Exon 2.1_RPA_FP	GTAAGTATTAGATATGGAAAAGTCTATAAGGCAA
42	Exon 2.1_RPA_RP	GATTCAGCTTCGGGAAGGCATACGAATATGG
43	Exon 2.2_RPA_FP	GGGACCACTAAATTCAAAGAATGGTGGAGATC
44	Exon 2.2_RPA_RP	ACGCTGAGAAGGACCCATACCCAGAGGAAGAG
45	HPV16_RPA_FP	TTGTTGGGGTAACCAACTATTTGTTACTGTT
46	HPV16_RPA_RP	CCTCCCCATGTCGTAGGTACTCTTTAAAG

Table 2: Primers used in the study.

Chapter 3

RESULTS

3.1 Diagnostic strategy for MCV in MCC

3.1.1 Conserved region across MCVs were used for gRNA design.

To detect the presence of integrated MCV DNA in MCC cells, we designed 10 AsCas12a gRNAs. These gRNAs were complementary in region consisting Non Coding Control Region (NCCR), small T and large T antigen of MKL-1 genomic sequence (Figure 13a). 3 gRNAs targeted NCCR, 5 gRNAs targeted the Exon 1 and rest 2 targeted Exon 2 till the conserved Retinoblastoma (Rb) binding domain (Figure 13b). The gRNAs were designed in conserved sequences across MCV genomes. To test this we first did CLUSTAL omega analysis of 54 MCV sequences and designed gRNAs only in conserved regions.

gRNA	gRNA name	Strand	Sequence	PAM	Benchling Score	RGEN score
MCV NCCR gRNA 1	NCCR1	-1	AACAAGGGAGGCCCGGAGGC	TTTC	98.01	66.3
MCV NCCR gRNA 2	NCCR2	-1	CTGGAGAGGCGGAGTTTGAC	TTTC	98.24	74.2
MCV NCCR gRNA 3	NCCR3	-1	CAGAGGCCTCGGAGGCTAGG	TTTC	95.67	69.1
MCV exon 1 gRNA 1	Exon1.1	-1	GGACTAAATCCATCTTGTCT	TTTA	92.81	60.1
MCV exon 1 gRNA 2	Exon1.2	1	GAGATTGCTCCTAATTGTTA	TTTA	93.40	62.4
MCV exon 1 gRNA 3	Exon1.3	1	GTCCTAAATAGGAAAGAAAGAGA	TTTA	79.1	73.9
MCV exon 1 gRNA 4	Exon1.4	-1	CCCCTTTATCAGGGTGATGCTTT	TTTC	93.34	79.4
MCV exon 1 gRNA 5	Exon1.5	-1	CTCCAAAGGGTGTTCAATTCCAT	TTTG	95.56	75.2
MCV Exon 2 gRNA 1	Exon2.1	1	CCATCTAGGTTGACGAGGCC	TTTC	99.32	75
MCV Exon 2 gRNA 2	Exon2.2	-1	TGGATCTTGAGTTGGTCCCG	TTTC	98.04	69.3

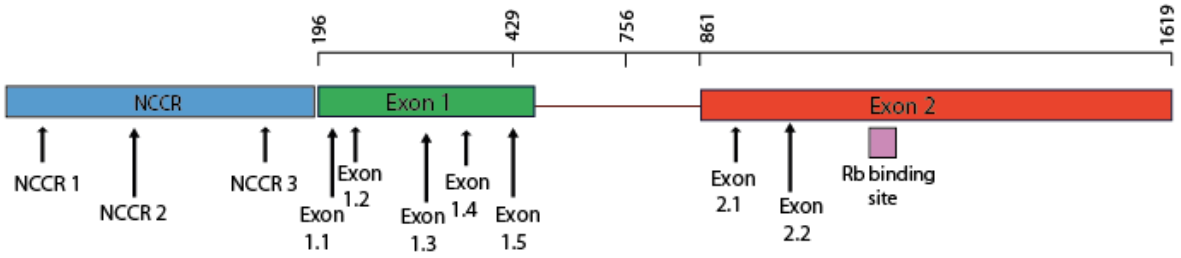

Figure 13: 10 gRNAs were designed in the conserved region of MCV for DETECTR

(a) Table showing all the designed gRNAs, sequences, target strand, PAM sequences, Benchling scores and RGEN scores.
(b) gRNAs annotated on MCV DNA. 3 gRNAs targeted the NCCR, 5 gRNAs targeted the ST antigen and rest 2 gRNAs targeted the LT antigen.

3.1.2 Optimizing the AsCas12a mediated *in vitro* cleavage assay

To test the cleavage activity of Cas12a we first performed *in vitro* cleavage (IVC) assay using LbCas12a (NEB) and 3 MCV Exon 1 targeting gRNAs. However, none of the MCV gRNAs led to the target cleavage (Figure 14a). We tried the same experiment by varying the amounts of Cas12a and gRNA, which was not successful either. Next in order to test if our gRNAs were not able to target and cleave we used tested and reported HPV L1 gRNAs and target (Chen, et al 2018). Surprisingly, there was no cleavage with these gRNAs as well indicating that our LbCas12a was ineffective. We tried to purify the LbCas12a from plasmid containing LbCas12a, however, we were not able to induce the expression of LbCas12a in BL21 cells.

Thus we switched to AsCas12a (IDT) and performed the IVC using 2 HPV L1 targeting gRNAs. Interestingly the IVC was successful this time as indicated by the cleaved bands (Figure 14b).

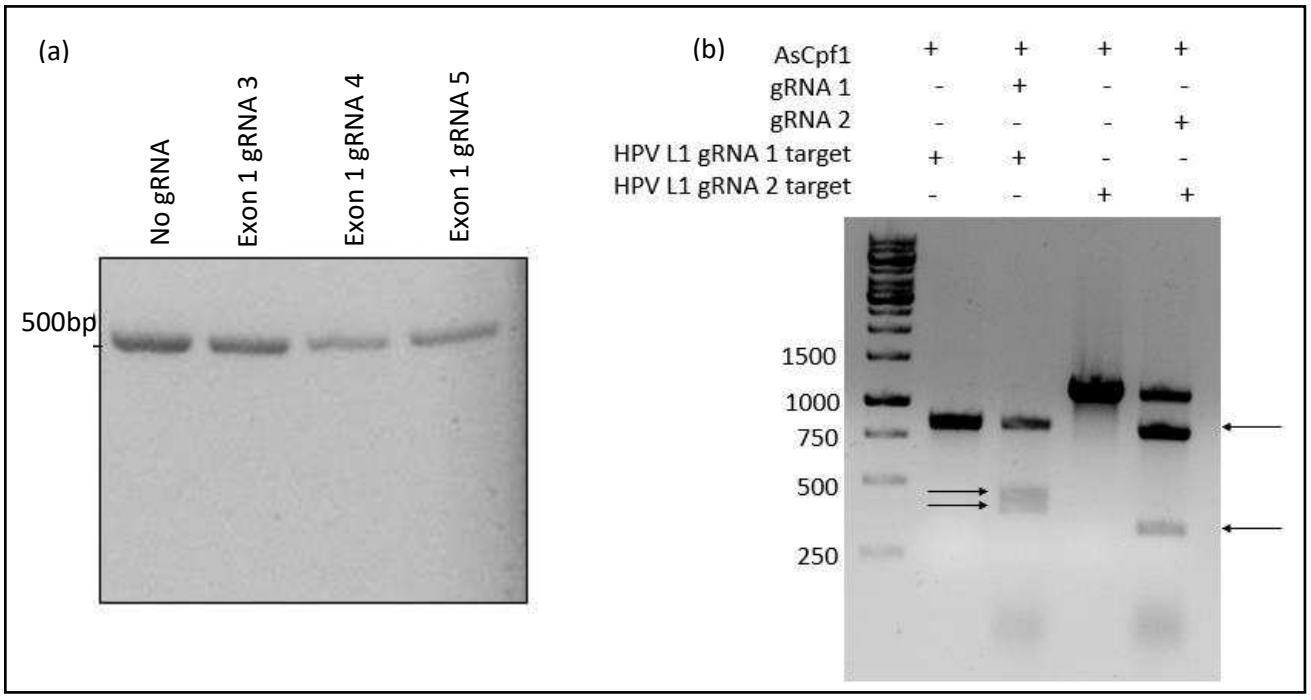

Figure 14: Optimizing Cas12a mediated *in vitro* cleavage assay

(a) LbCas12a (NEB) with 3 MCV targeting gRNAs did not lead to target cleavage.

(b) AsCas12a (IDT) with reported HPV L1 targeting gRNAs led to HPV dsDNA target cleavage as shown by the arrows.

3.1.3 Screening and validation of gRNAs targeting MCV genome

To investigate the cleavage efficiency of MCV targeting gRNAs, we performed *in vitro* cleavage assay. We cloned the NCCR and T antigen derived from MKL-1 into a pLentiCMV backbone vector (RAZ2) and used the target region PCR amplicon as the DNA substrate for our assays. 6 out of 10 gRNAs efficiently cleaved the target substrate as shown by the cut bands (Figure 15a). We also performed an *in vitro* cleavage experiment with these selected gRNAs with RAZ2 plasmid as target DNA. All the 5 gRNAs cleaved the circular plasmid as shown by the linearized plasmid band (Figure 15b).

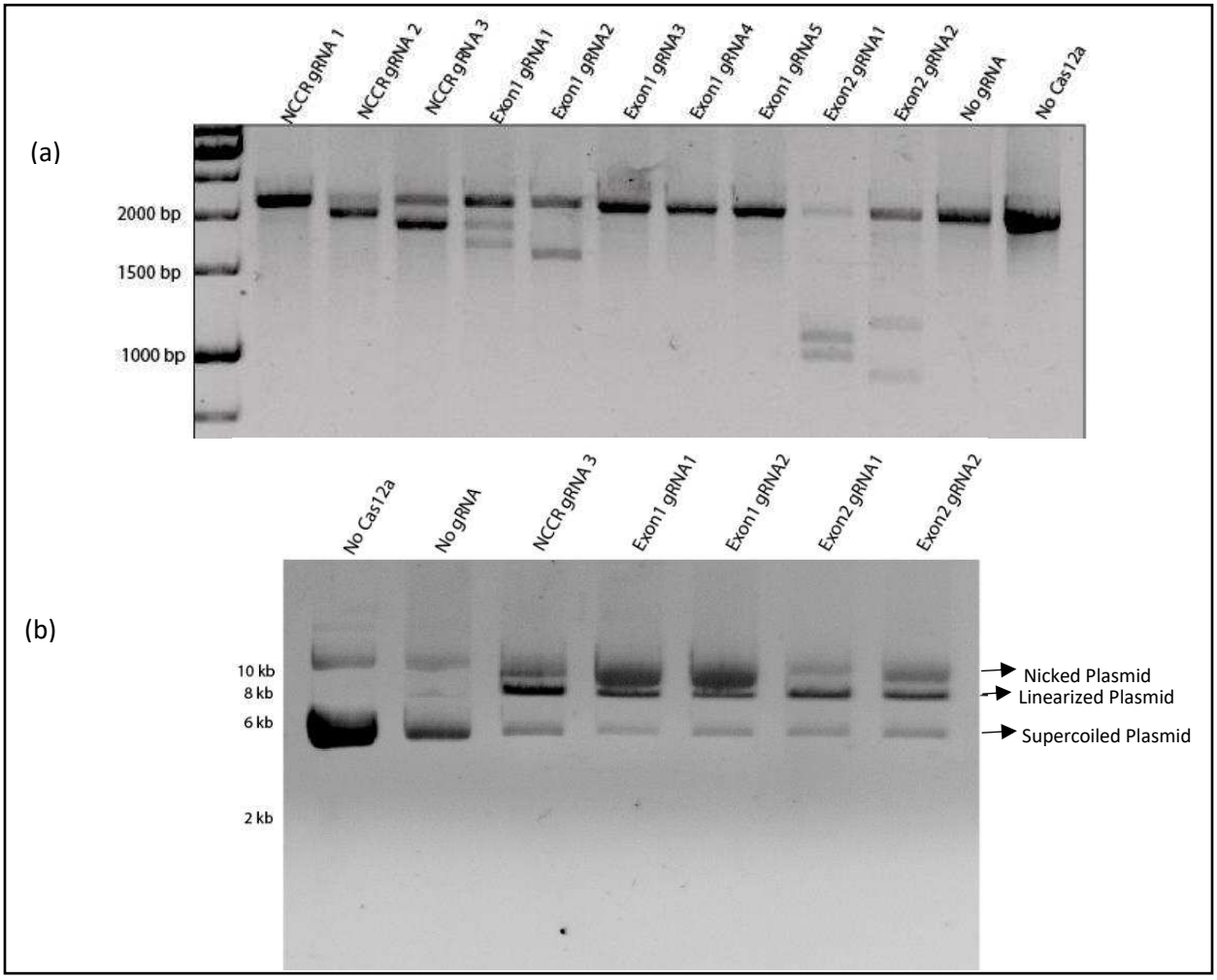

Figure 15: Screening all the AsCas12a MCV gRNAs using *in vitro* cleavage assay

(a) All the 10 MCV gRNAs were assembled with AsCas12a and MCV dsDNA target template. 6 out of 10 gRNAs showed target cleavage as indicated by the cleaved bands.
(b) 5 gRNAs were selected and used for IVC using MCV NCCR, ST and LT containing plasmid as target template. All the selected gRNAs were able to cleave the plasmid as shown by the linearized plasmid band.

3.1.4 AsCas12a cleaves nonspecific trans-ssDNA after cis target DNA binding

To validate the non-specific trans-ssDNA shredding activity of AsCas12a, we used a non-complementary, circular, single stranded M13 DNA phage reporter. In the presence of MCV gRNA and complementary ssDNA target (cis - activator), AsCas12a led to indiscriminate shredding of single stranded M13 phage (Figure 16). Thus MCV gRNAs NCCR 1, Exon 1.1, Exon 1.2, Exon 2.1 and Exon 2.2 were selected for further studies for detecting MCV using fluorescence quencher (FQ) labelled ssDNA reporter. HPV L1 gRNA was used as a positive control.

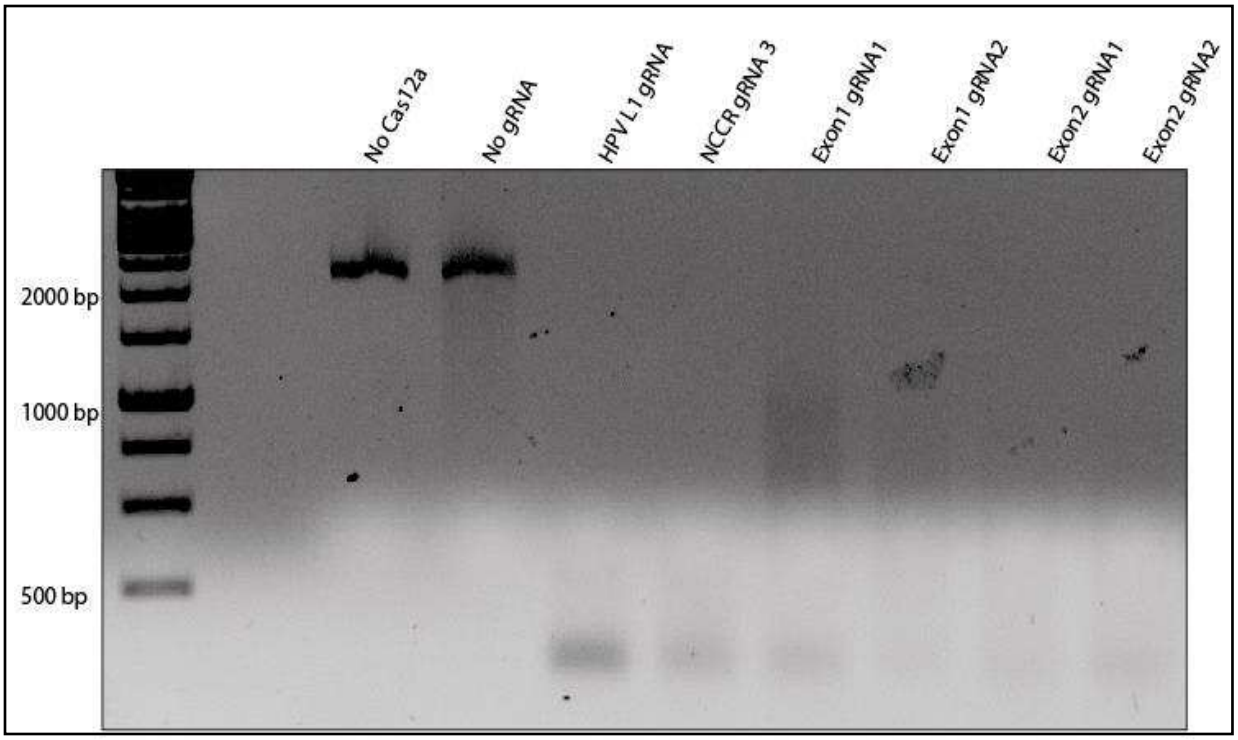

Figure 16: AsCas12a leads to non-specific trans ssDNA shredding

AsCas12a, in the presence of MCV targeting gRNA and complimentary dsDNA target leads to trans-single stranded M13 phage DNA shredding as shown by the smeared M13 DNA. AsCas12a without the gRNA does not lead to shredding of M13.

3.1.5 Cas12a mediated Fluorophore Quencher (FQ) assay detects MCV

To develop a MCV DNA detection system, we used a fluorophore quencher (FQ)–labeled reporter assay. To test the FQ based DNA detection system, we first used the reported HPV gRNA targeting HPV 16 L1 gene. We assembled AsCas12a with its HPV gRNA and a complementary dsDNA cis HPV target. We introduced a non-specific ssDNA FQ reporter to this reaction. In the presence of HPV gRNA and complementary target DNA, AsCas12a cleaved the ssDNA FQ reporter as observed through the emitted fluorescence (Figure 17a).

Next, we tested our 5 selected MCV targeting gRNAs with the FQ assay to detect MCV DNA. We found that MCV NCCR3 gRNA cleaved the target DNA most efficiently among the selected 5 gRNAs as indicated by highest emitted fluorescence (Figure 17b). DNase was used as a positive control for cleaving ssDNA FQ reporter.

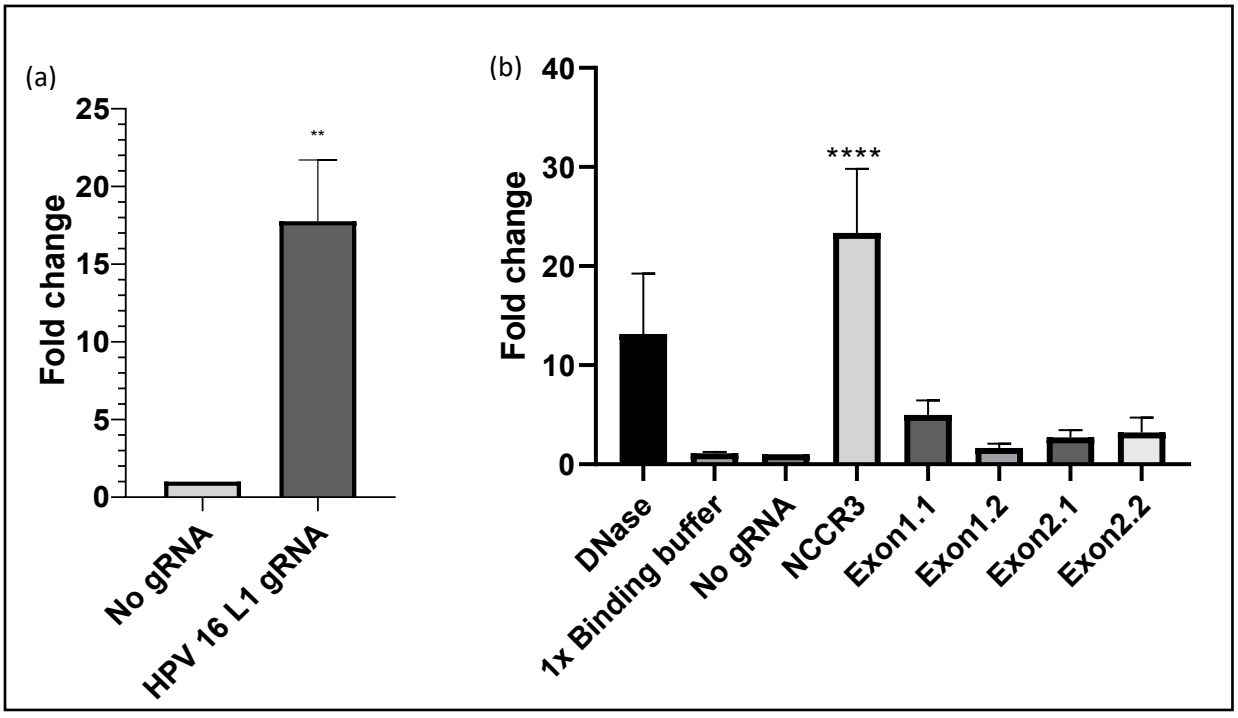

Figure 17: AsCas12a mediated trans ssDNA FQ assay detects MCV

(a) AsCas12a was assembled with or without HPV L1 targeting gRNA and HPV L1 dsDNA target. AsCas12a in the presence of HPV gRNA binds to complementary cis dsDNA target and further shreds the FQ labelled ssDNA reporter. Fluorescence values were normalized to no gRNA sample.

(b) MCV gRNAs MCV detection activity was tested using FQ ssDNA reporter assay. DNase was used as positive control for ssDNA cleavage. Fluorescence values were normalized to no gRNA sample. Error bar represents mean +/- SD where ** - $p<0.01$ and *** - $p<0.0001$. One way ANOVA was used to compare the variance.

3.1.6 NCCR3 gRNA detects MCV target specifically

Since NCCR3 gRNA consistently emitted high level of fluorescence in our DETECTR system we used this for further analysis. To test the specificity of MCV gRNA NCCR3, we assembled AsCas12a and NCCR3 gRNA with complimentary MCV dsDNA or non-complimentary HPV dsDNA target. ssDNA FQ assay showed that Cas12a and NCCR3 does not lead to cleavage of ssDNA FQ reporter in the presence of non-complimentary HPV target, thus is specific to the MCV target (Figure 18a). To test if the MCV NCCR3 gRNA can detect a target plasmid DNA we assembled AsCas12a and NCCR3 gRNA with complimentary target RAZ2 plasmid or a non-specific plasmid. We found that AsCas12a only leads to FQ reporter shredding in the presence of target RAZ2 plasmid.

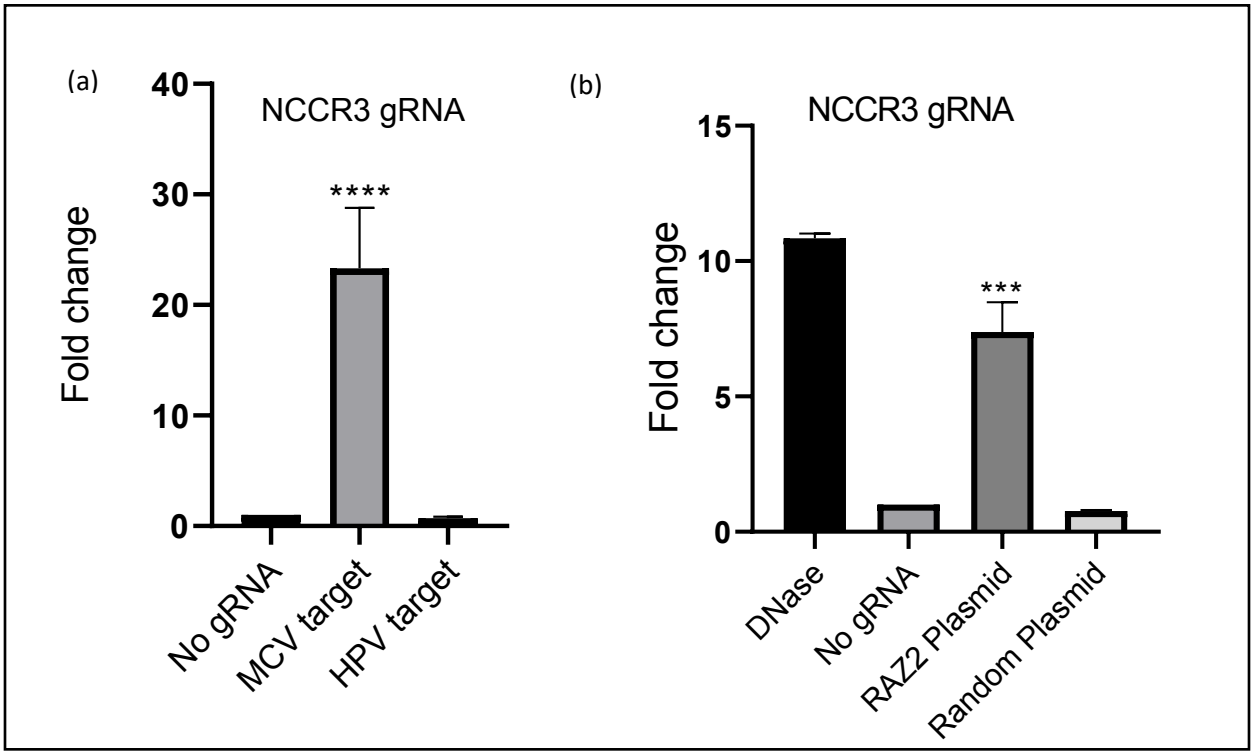

Figure 18: MCV NCCR3 gRNA detects MCV target DNA specifically

Cas12a and MCV NCCR3 gRNA lead to ssDNA FQ reporter shredding only in the presence of complimentary MCV (a) dsDNA target or (b) plasmid target. AsCas12a does not lead to fluorescence in the presence of non-complimentary HPV dsDNA or random plasmid target. Fluorescence values were normalized to no gRNA sample. Error bar represents mean +/- SD where ** - $p<0.01$ and *** - $p<0.0001$. One way ANOVA was used to compare the variance.

3.1.7 DETECTR can diagnose MCV in MCC genomic DNA

Next we wanted to investigate if DETECTR can diagnose MCV DNA integrated in MCV positive MCC cells. For this we extracted genomic DNA from MKL-1 cells. We amplified 100 – 200 bp long gRNA target regions using RPA. We introduced AsCas12a, respective MCV gRNA and ssDNA FQ reporter to this target MCV amplicon. We used genomic DNA from HEK293T cells as negative control. We found that DETECTR can detect the presence of MCV DNA only in MKL-1 cell lysate and not in non-specific *HEK293T cell lysate. Also, Cas12a alone does not lead to MCV detection in MKL-1 cells (Figure 19).

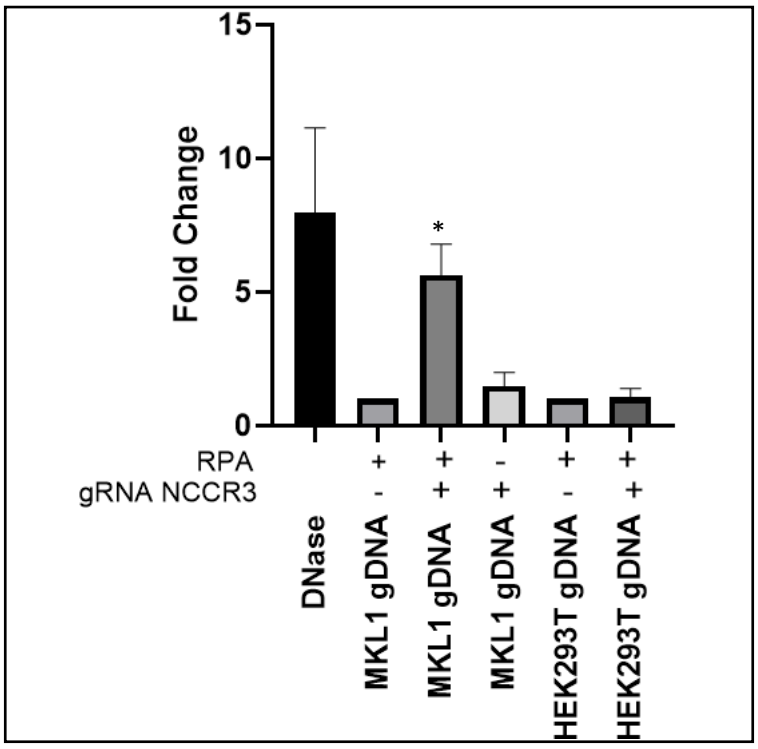

Figure 19: DETECTR can diagnose MCV in MCC genomic DNA

DETECTR assay to detect MCV DNA in MKL-1 genomic DNA with or without RPA. DETECTR in the presence of gRNA NCCR3 specifically can diagnose MCV target in MCC genomic DNA when amplified via RPA.

3.1.8 Investigating the MCV DNA diagnosing sensitivity of DETECTR

DETECTR couples Recombinase Polymerase Amplification (RPA) with Cas12a mediated DNA detection to increase the sensitivity. RPA is an isothermal DNA amplification reaction, which can amplify DNA quickly, efficiently and specifically. To investigate the sensitivity of this Cas12a mediated MCV detection, we will use dsDNA and RAZ2 plasmid target with varying concentrations with or without RPA.

3.2 Therapeutic strategy for MCV positive MCC.

3.2.1 Screening and validation of gRNAs targeting MCV Tumour antigens

In order to target and disrupt the integrated MCV Tumour antigens present in MCV positive MCC cells, we designed 13 gRNAs. These gRNAs were complementary in region between 247 to 1254 nucleotides of MKL-1 genomic sequence that encompassed both LT and sT (Table 3). 5 gRNAs (C1 – C5) targeted exon1, common region between sT and LT, 4 gRNAs (C6 – C9) targeted only sT and rest 4 (C10 – C13) targeted only LT (Figure 20).

gRNA	Sequence	Location	Strand	MIT-CRISPR Score	Broad-Software Score	CRISPOR Specificity score	CRISPOR Out of frame score
Target both large and small T							
C1	GAGATTGCTCCTAATTGTTA	Exon 1 (+247 to +266)	sense	70	0.3268	73	57
C2	GGGATGTTGCCATAACAATT	Exon 1 (+259 to +278)	antisense	74	0.3962	81	62
C3	AAAGCATCACCCTGATAAAG	Exon 1 (+312 to +331)	sense	69	0.6128	71	65
C4	CAGGATTTCCCCCTTTATCA	Exon 1 (+324 to + 343)	antisense	65	0.5483	49	67
C5	TGATGGAATTGAACACCCTT	Exon 1 (+350 to +369)	sense	76	0.4187	79	63
Target only small T							
C6	GCTAGATTTTGCAGAGGTCC	Exon 1 (+496 to +515)	sense	71	0.3757	77	65
C7	AGTTGCTTAAGCATGCACCC	Exon 1 (+517 to +536)	antisense	83	0.7104	86	66
C8	CAAAAAAACTGTCTGACGTG	Exon 1 (+607 to +626)	sense	73	0.6609	84	61
C9	GTTTGGATTTCCTCCTACTT	Exon 1 (+663 to +682)	sense	66	0.3988	72	69
Target only Large T							
C10	TTGACGAGGCCCCTATATAT	Exon 2 (+862 to +884)	sense	92	0.5301	95	73
C11	AATATGGGCCCAATCCACAC	Exon 2 (+943 to +965)	sense	82	0.6633	82	70
C12	TATATCGGGTCCTCTGGACT	Exon 2 (+1093 to +1115)	antisense	84	0.5193	91	66
C13	TTCCAGAACGGATGGCACCT	Exon 2 (+1232 to + 1254	sense	76	0.5995	80	69

Table 3: CRISPR/Cas9 gRNAs targeting MCV T antigens

The table shows MCV TAg targeting gRNAs, their sequences, target sites and scores from MIT CRISPR, Broad and CRISPOR softwares.

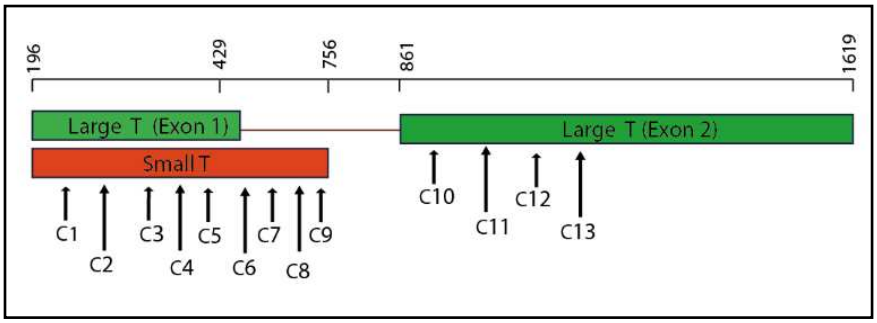

Figure 20: MCV Tumor antigen targeting CRISPR/Cas9 gRNAs

CRISPR/Cas9 gRNAs C1 to C13 annotation on MCV T antigen genes. C1 – C5 target Exon1, C5 – C9 target intron1 and C10 – C13 target Exon2.

These gRNAs were cloned into pLentiCRISPRv2 plasmid, under the control of the U6 promoter. This plasmid also expressed SpCas9 protein under the control of the EF1a promoter (Figure 21a). To investigate the cleavage activity of these 13 gRNAs, we transduced MKL-1 cells with these 13 constructs followed by puromycin selection for 4 days. Immunoblotting showed significant reduction in the expression of both ST and LT by 2 gRNAs – C3 and C13 (Figure 21b).

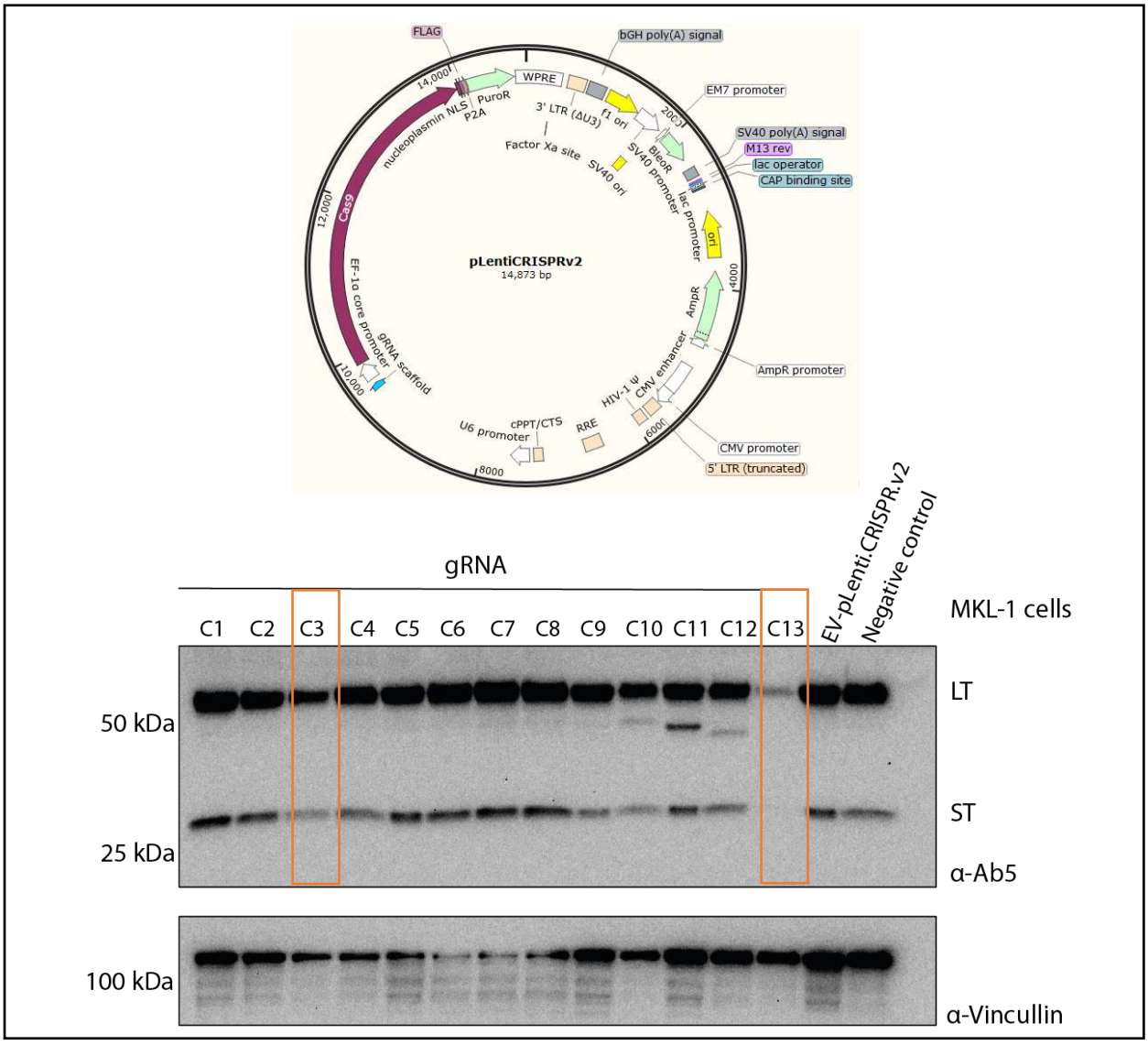

Figure 21: Screening MCV TAg targeting gRNAs.

(a) Plasmid map showing pLentiCRISPRV2 vector expressing Cas9 under T7 promoter and gRNA under U6 promoter.

(b) Immunoblot depicting MKL-1 cells transduced with vectors containing 13 gRNA constructs. TAg expression has reduced upon transduction of with pLentiCRISPRV2 vectors containing C3 and C13 gRNAs, respectively.

3.2.2. *In vitro* cleavage assays validate gRNA targeting efficiency

To further validate the cleavage efficiency of C3 and C13, we performed *in vitro* cleavage assays. We cloned the NCCR and T antigen derived from MKL-1 into a pLentiCMV backbone vector and used the target region PCR amplicon as the DNA substrate for our assays. The two gRNAs were efficiently cleaving the target substrate as shown by the cut bands of sizes 562bp and 367bp for C3 and 512bp and 400bp for C13 respectively (Figure 22a). To address the time taken for this cleavage, we performed a time course assay. Both the gRNAs were able to cleave the target within 10 minutes (Figure 22b). Therefore, C3 and C13 can effectively target and cleave MCV T antigen genomic sequence.

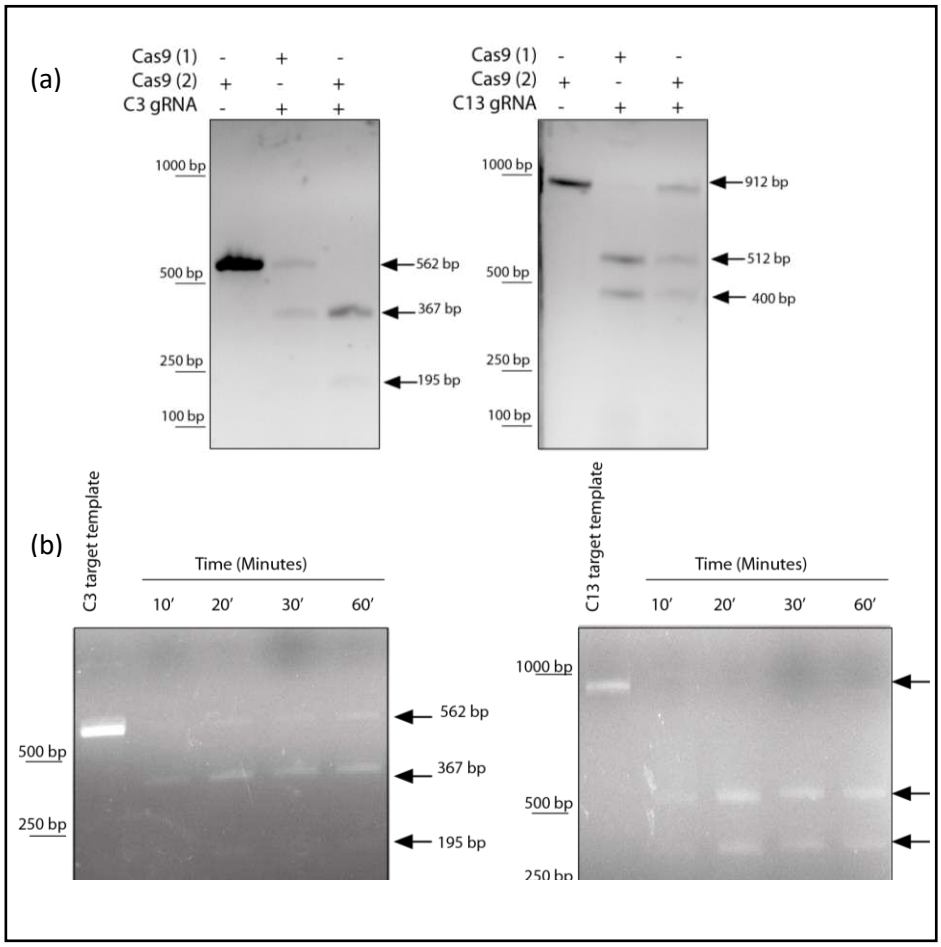

Figure 22: *In vitro* cleavage assay validated C3 and C13 gRNAs targeting efficiency

(a) MCV C3 and C13 gRNA target region template was amplified from RAZ2 plasmid. *In vitro* cleavage reaction shows SpCas9 mediated MCV target cleavage in the presence of gRNAs C3 and C13 as indicated by arrows.
(b) Time course IVC assay shows SpCas9 can cleave the MCV target template within 10 minutes.

3.2.3 Targeting MCV TAg using gRNAs combination leads to TAg knock down

Next, we targeted MCV T antigen locus in MKL-1 cells using C3 and C13 gRNAs individually using lentiviral-vector delivery. We transduced MKL-1 cells and harvested cells at different time points post selection. Interestingly, though the T antigen expression initially reduced, it recovered upon further culturing (Figure 23).

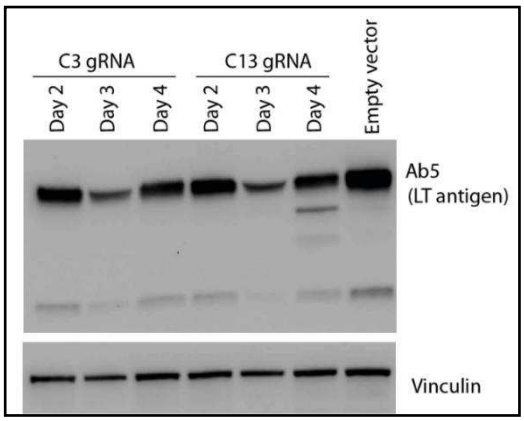

Figure 23: TAg expression recovered after Cas9 with single gRNA delivery in MKL-1 cells
MKL-1 cells were transduced with C3 and C13 pLentiCRISPRv2 lentiviral constructs. Immunoblotting showing TAg expression initially reduced but later recovered upon further culturing.

We speculate that this could be an effect of random targeting and due to repairing of the T antigen locus in the cells via NHEJ. Thus, we decided to switch to the use of gRNAs in combination. Since the use of dual targeting leads to excision of DNA region between the 2 target sites, it serves as a more robust strategy (Figure 24).

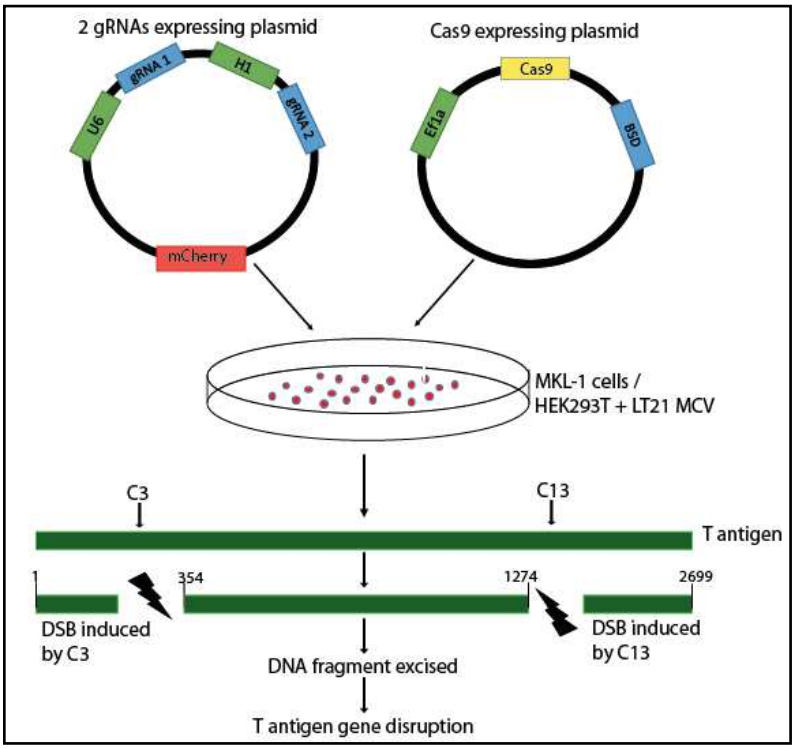

Figure 24: Targeting MCV TAg using 2 gRNA combination strategy
Both gRNAs C3 and C13 were cloned into pDECKO mCherry vector. Cas9 was expressed using pLentiCas9 vector. Both these vectors were delivered in HEK293T cells or MKL-1 cells via transfection or transduction. Use of dual targeting leads to excision of DNA region between the 2 target sites.

Using *in vitro* cleavage assay, we validated the combinatorial target cleavage by gRNAs C3 and C13. The effective cleavage activity is shown by the 3 cleaved bands at 1131 bp, 940 bp and 374 bp as indicated by the arrows (Figure 25a).

To investigate the effects of these CRISPR/Cas9 constructs in combination, we cloned C3 and C13 into pDECKO mCherry vector. This vector expresses 2 gRNAs together under U6 and H1 promoters respectively (Figure 24). To test the efficiency of this construct we co-transfected HEK293T cells with Merkel cell polyomavirus (MCC LT162) early region expressing plasmid, Cas9-expressing plasmid and pDECKO mCherry empty vector (EV) or pDECKO mCherry C3+C13. Figure 25b shows western blot data revealing a loss of LT expression upon transfection of pDECKO C3 + C13 relative to pDECKO mCherry empty vector.

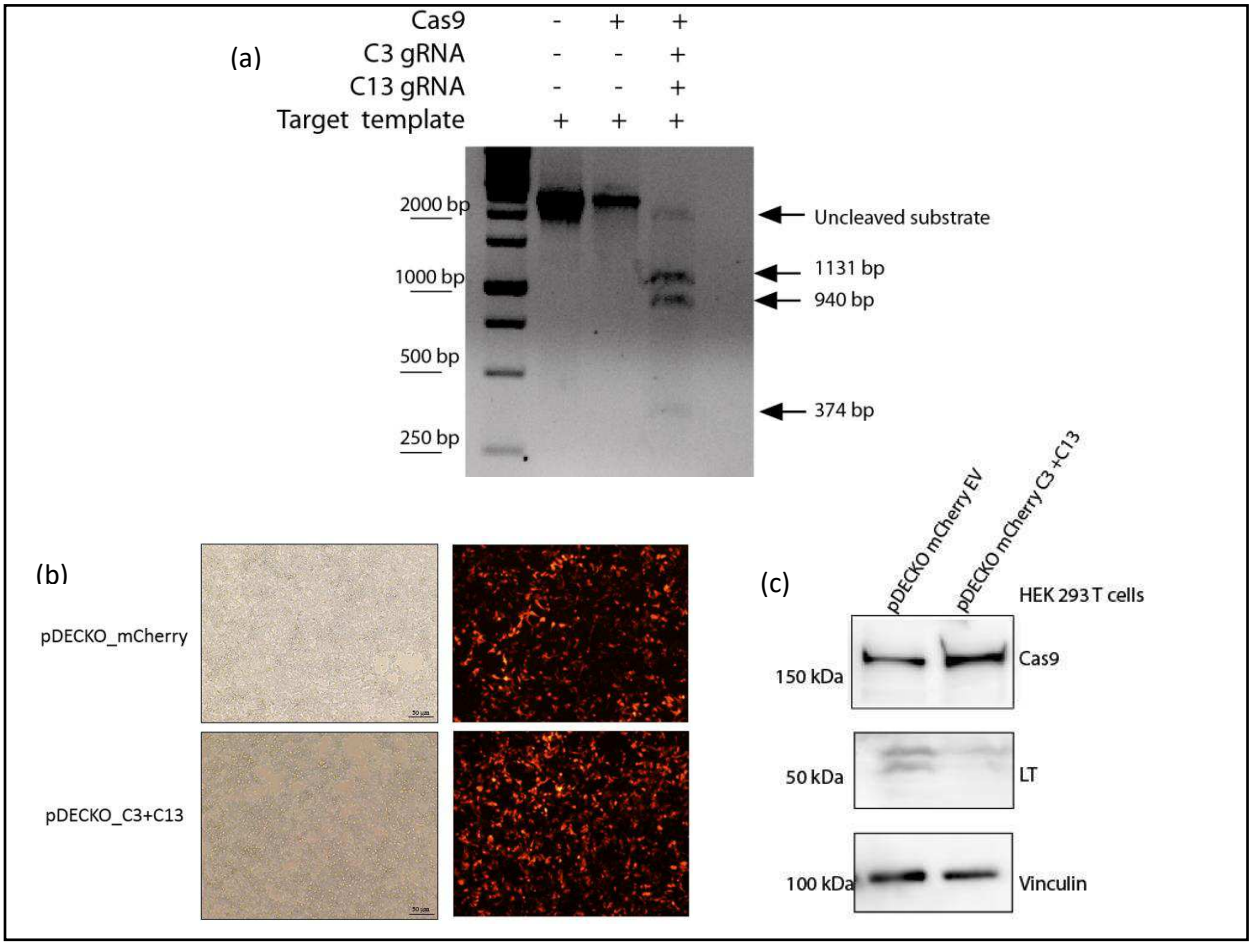

Figure 25: gRNAs C3 and C13 combination leads to MCV T antigen knock down

(a) IVC assay showing C3 and C13 gRNAs cleave the MCV target efficiently in combination. A 3445 bp long MCV target was amplified from RAZ2 plasmid. Cas9 in the presence of C3 and C13 gRNAs led to combinatorial target cleavage.

HEK293 T cells were co-transfected with Merkel cell polyomavirus (MCC LT162) early region expressing plasmid, Cas9-expressing plasmid and pDECKO mCherry empty vector (EV) or pDECKO mCherry C3+C13. (b) Transfection efficiency in HEK293T was measured using mCherry fluorescence post transfection. (c) LT expression was reduced upon transfection of pDECKO C3 + C13 relative to pDECKO mCherry empty vector.

3.2.4 MCV TAg targeting CRISPR/Cas9 system in MKL-1 cells

Next we wanted to analyze the effects of this CRISPR/Cas9 gRNAs combination on MCV T antigens in MCV positive MCC cells. We transduced MKL-1 cells with Cas9 expressing and pDECKO mCherry EV / C3 + C13 expressing vectors simultaneously. The cells were selected using both Blasticidin as well as puromycin for 4 days post transduction. The experiment is being performed currently and results will be analysed using immunoblot and WST assays.

We also developed stable pDECKO mCherry EV and pDECKO mCherry C3+C13 expressing cell lines by infecting MKL-1 cells with pDECKO lentiviral vectors followed by Puro selection. Further we created a Tet- inducible Cas9 expressing MKL-1 stable cell line using Blasticidin selection. We will use both systems to introduce the other construct. Infection followed by selection of MKL-1 cells with a combination of the 2 gRNAs and Cas9 will be used to knock out MCV T antigen in the near future.

3.3 Reprogramming capacity of MCV ST

3.3.1 iPSC generation by ST and LT expression with OSK

Human dermal Fibroblast (HDF) cell line, expressing combinations of MCV T antigens (sT + LT, sT only and LT only) were made using lentiviral transduction followed by puromycin selection. These cells were then transduced using Sendai virus expressing Oct4, Sox2 and Klf4 (OSK) (Figure 26a). HDF cells were also treated with all 4 factors (OSKM) as positive control for comparative analysis. These cells were re-seeded into standard matrigel with mTeSR media 2 days post transduction. Media was changed until the emergence of iPSC colonies (Figure 26b).

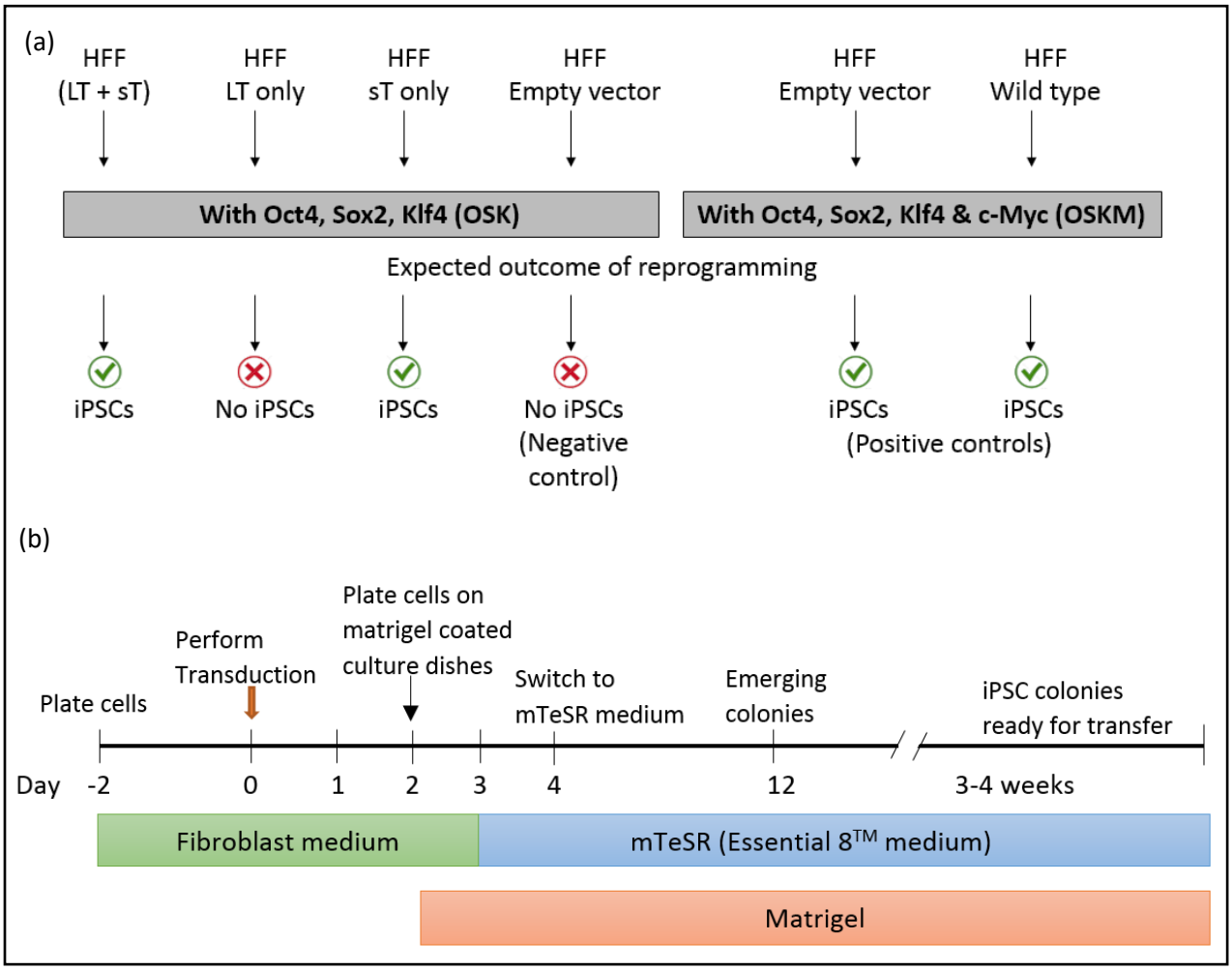

Figure 26: Strategy and work flow for iPSC generation by ST and LT expression

(a) Schematic showing the strategy for investigating the reprogramming capacity of MCV sT and LT. sT, LT or both sT or LT expressing HDFF stable cell lines were developed using lentiviral transduction. OSK factors were introduced into these cell lines using Sendai virus. HDFF cells transduced with all 4 factors (OSKM) were used as positive controls for iPSC generation.

(b) Schematic depicting the work flow for iPSC generation.

The positive control cells with all 4 factors generated iPSC colonies (Figure 27). We expected that the HDF cells expressing both T antigens to be reprogrammed in the presence of OSK. We observed that HDF cells expressing ST and LT along with OSK were very closely packed as compared to cells expressing only sT or LT with OSK. However, ST and LT were not able to cooperate with OSK to generate iPSCs even after one month. Next we made a Doxycycline inducible HDFF ER21 cell line using Lentiviral transduction. We repeated the iPSC generation experiment using different titers of Sendai virus and Lentivirus. However, we observed a lot of cell death and no iPSC generation even in the positive control set-up (Data not shown). These experiments will be repeated in the laboratory.

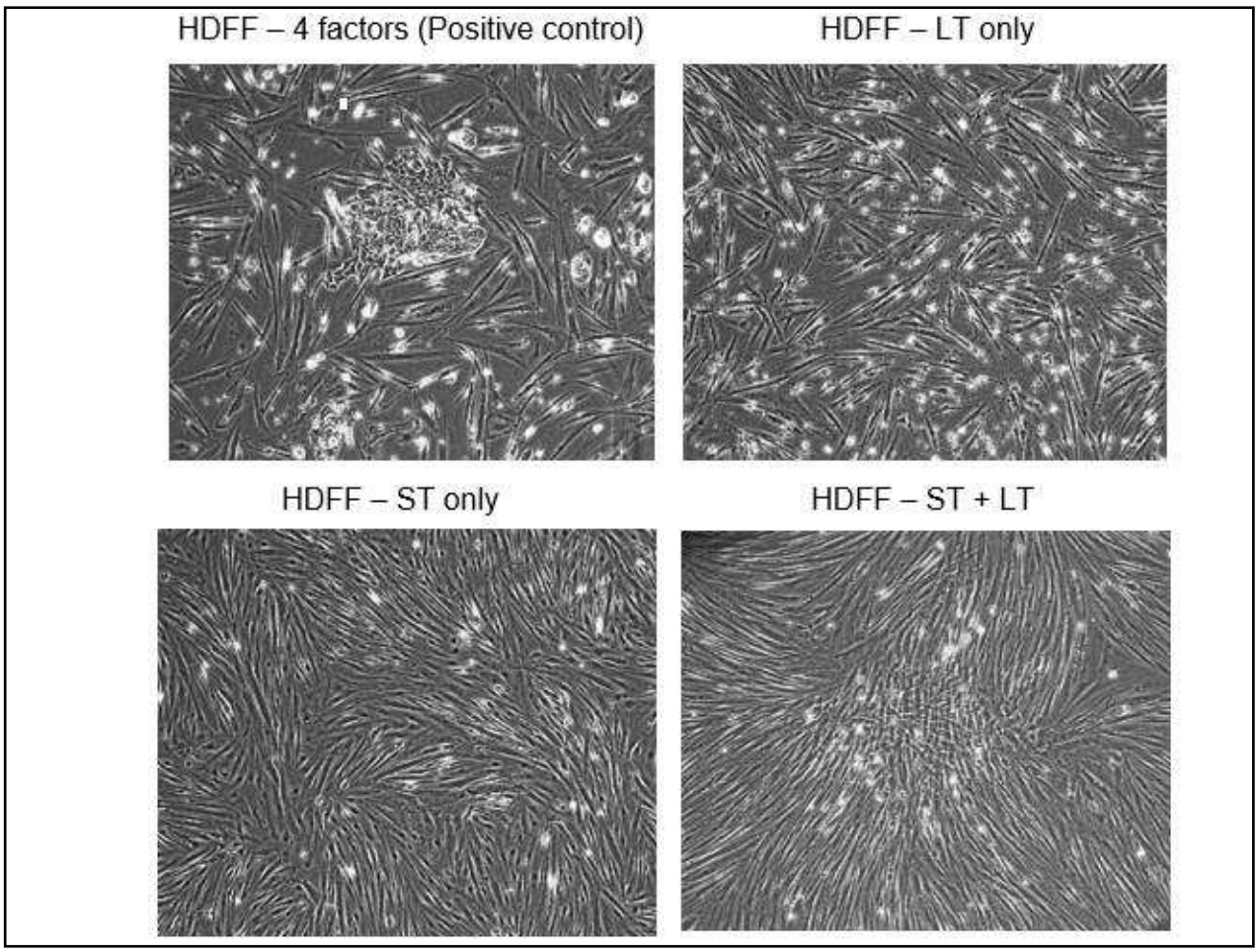

Figure 27: MCV T antigens did not cooperate with OSK to generate iPSCs.
HDFF cell lines expressing sT, LT or both were transduced with OSK expressing Sendai viruses. Positive control cells (HDFF cells transduced will all 4 factors (OSKM)) generated iPSC colonies. HDFF cells expressing sT and LT only or both were closely packed, however, did not produce iPSC colonies.

Chapter 4
DISCUSSION

Merkel cell polyomavirus (MCV) is one of the newer members of the DNA tumor virus family and also one of the seven viruses known to cause tumors in humans. Merkel cell carcinoma (MCC) is an uncommon but lethal, primary skin neoplasm frequently having a poor prognosis. Similar to other skin cancers prolonged UV exposure, advanced age and loss of immune competence are risk factors for MCC. MCV T antigens contain conserved domains present across different polyomaviruses, such as DnaJ and LXCXE retinoblastoma protein binding motifs, which are important for various tumor suppressor-targeting functions of the virus. MCV wild type T antigens however, are distinct from MCV tumor derived T antigens. In tumor derived MCVs, mutations truncate the T antigens, removing the C terminal helicase and origin binding domains thereby rendering the virus replication-incompetent in the tumors.

MCV sT and LT are necessary for MCV positive MCC cell survival and proliferation. Knock down of T antigens leads to MCC cell death. As MCV positive MCC cells are addicted to Tumour antigen expression, they become attractive therapeutic targets. Even though MCV T antigen targeting small interfering RNAs (siRNAs) or drugs are potential therapeutic approaches to kill the MCC cells, they cannot eliminate the MCV T antigen completely. Thus, in the first part of this study we combine Cas9 and MCV TAg targeting gRNAs to knock out TAg genes, integrated in the tumour genome. CRISPR/Cas system leads to DSB at the target site, which is repaired by mutagenic NHEJ pathway, thus, knocking out the target gene.

We screened various gRNAs by transducing MKL-1 cells with Cas9 and gRNA expressing lentiviral vectors. Two gRNAs out of 13 showed significant decrease in TAg expression and were selected for further analysis. We validated the cleavage efficiency of these selected gRNAs using *in vitro* cleavage assay. We found that single gRNAs were not able to reduce the TAg expression effectively, thus, we used a gRNA combination strategy. This strategy is more robust as compared to targeting via single gRNA as the excision of DNA sequence leads to knock out of target gene. To translate these findings into MCV positive MCC cell lines, we delivered Cas9/sgRNA expression

vectors in MKL-1 cells using lentiviral transduction. We will test CRISPR/Cas mediated TAg knockout in MKL-1 cells using immunoblotting, WST assay and SURVEYOR assay. Thus, this study is a proof-of-principle for CRISPR system as a therapeutic tool for MCV positive MCC. We think that this tool can be delivered *in vivo* using Adeno associated virus (AAV) vectors. These vectors are smaller and safer than lentivirus or retrovirus vectors and can infect with high titers. As AAV vectors have also been employed previously for gene therapy they could be potential vectors for *in vivo* delivery of Cas9 and gRNAs.

60-80% of MCC cases are caused due to MCV DNA integration. Also, MCV positive MCC patients have a better prognosis and higher chance of survival than MCV negative MCC patients. Thus, it becomes important to detect the viral status of the disease. Currently, PCR, immunostaining, immunohistochemistry techniques are used to detect the presence of MCV. Thus, the second part of this project involved developing a rapid and sensitive CRISPR/Cas12a based MCV DNA detecting tool.

Cas12a, a class V CRISPR enzyme, upon dsDNA target binding leads to indiscriminate trans-ssDNA shredding. We applied this property of Cas12a to develop a MCV DNA detecting tool. We designed various AsCas12a gRNAs targeting the MCV NCCR, Exon1 and Exon2. In the presence of complementary MCV DNA, Cas12a cleaves the target and further leads to shredding of fluorescently labelled ssDNA reporter. We found that NCCR3 gRNA led to efficient detection of MCV DNA using dsDNA amplicon targets. To detect MCV DNA from cellular genomic DNA we adapted the (DNA Endonuclease Targeted CRISPR *Trans* Reporter (DETECTR) system. DETECTR combines isothermal amplification target using Recombinase Polymerase Amplification (RPA) followed by Cas12a mediated detection. This made the Cas12a based MCV detection more sensitive. This system is a rapid, highly sensitive and specific tool for diagnosing MCV DNA in MCC cellular genomic DNA.

This Cas12a based MCV detection tool can be potentially converted into a portable kit. Previously a Cas13a based RNA detection system, SHERLOCK, has been developed into a paper spotting and lyophilized tool (Jonathan S Gootenberg et al. 2017). Similar approach can be used to develop a Cas12a based MCV diagnosing kit by providing lyophilized Cas12a-crRNA complexes which can be used after rehydration. Similarly

RPA can be supplied as a lyophilized reagent as well and FQ reporter can be turned into a lateral flow readout based system (J S Gootenberg 2018), thus, making the whole kit easy to use and portable. This DNA detection tool can be combined with other diagnosing techniques like immunohistochemistry (IHC) or immunostaining for efficient MCV detection.

Lastly, we tried to investigate the reprogramming capacity of sT and LT antigens in human fibroblasts. Previous study has shown that sT can reprogram keratinocytes into induced pluripotent stem cells in the presence of Oct4, Sox2 and Klf4 (OSK). We used sT and LT in combination to investigate if LT enhances the reprogramming capacity of sT. As LT sequesters Retinoblastoma (pRb) protein, thus could have promoted proliferation and stemness thereby accelerating the iPSC generation process. However, in our study sT and LT were not able to cooperate with OSK to generate iPSCs.

There could be several reasons for this observation. Firstly, there is a possibility that sT and LT expressing HDFF cells with OSK were incompletely reprogrammed**,** as seen by the closely packed phenotype. This could be due to insufficient L-myc expression at the time when transduced Oct4, Sox2, Klf4 expression peaked. Secondly, we used fibroblasts whereas Cheng *et al.* had used keratinocytes, which are known to have higher reprogramming efficiency. This is because of lower levels of p53 and p21 proteins in keratinocytes. This difference might have played an important role at cellular level for reprograming. Lastly, Cheng *et al.* had immortalized the human keratinocytes via hTERT lentiviral transduction. This could play a role in the reprogramming ability of the cells as well.

All in all, this study looked at various aspects of MCV positive Merkel cell carcinoma, ranging from developing CRISPR based therapeutic and diagnostic applications to understanding the causes for carcinogenesis. Our study has reached an exciting and promising stage for treating and diagnosing MCV positive MCC.

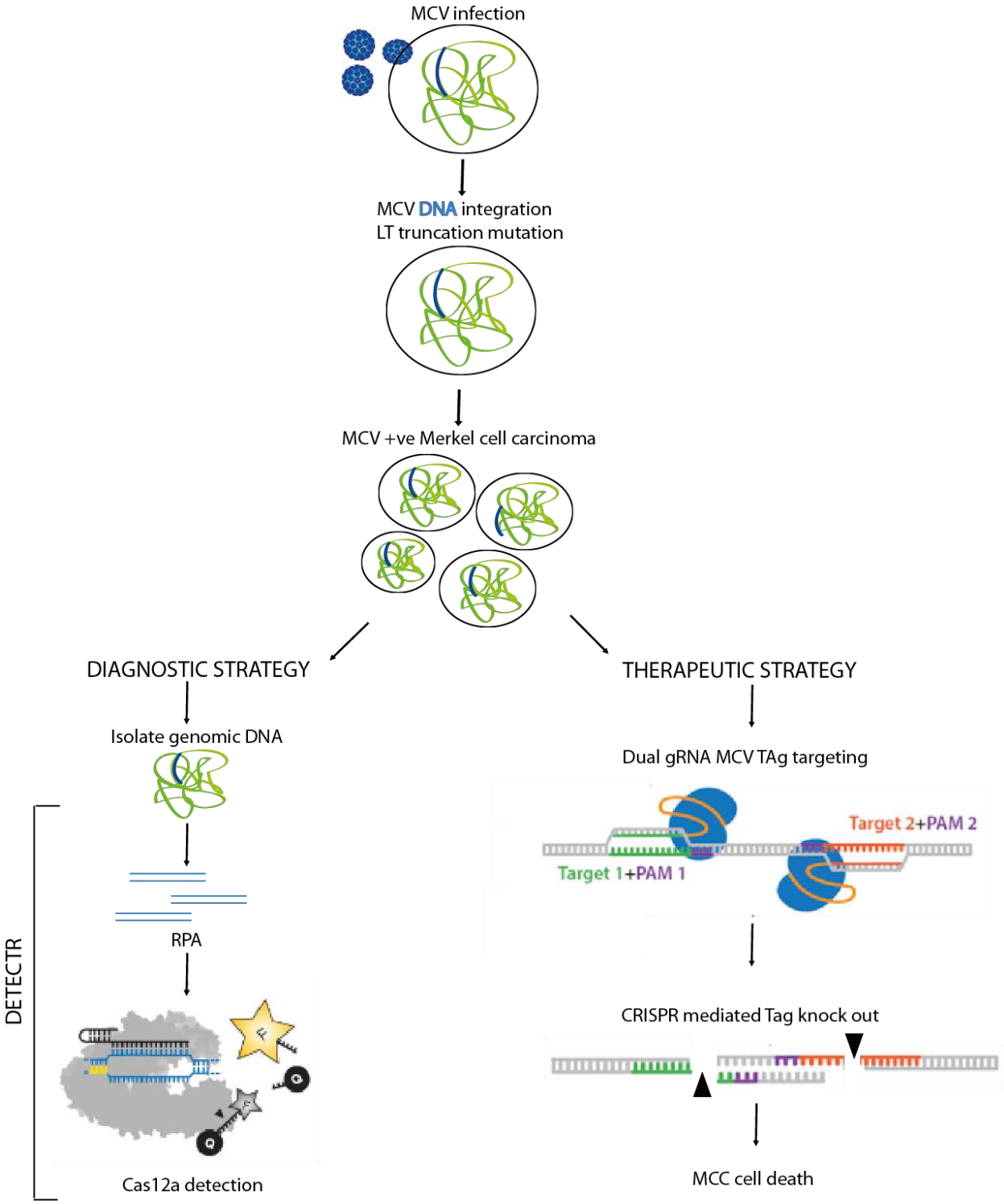

Figure 28: Schematic summary

Schematic depicts diagnostic and therapeutic strategies for MCV positive MCV using CRISPR.

Chapter 5

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