Respiratory syncytial virus and human metapneumovirus : the morphological and transmission studies

Nguyen, Huong Tra

2017

Nguyen, H. T. (2017). Network intrusion detection via distributed machine learning on smart gateway network. Master's thesis, Nanyang Technological University, Singapore.

<http://hdl.handle.net/10356/71463>

<https://doi.org/10.32657/10356/71463>

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**RESPIRATORY SYNCYTIAL VIRUS AND HUMAN
METAPNEUMOVIRUS: THE MORPHOLOGICAL AND
TRANSMISSION STUDIES**

NGUYEN HUONG TRA

SCHOOL OF BIOLOGICAL SCIENCES

2017

**RESPIRATORY SYNCYTIAL VIRUS AND HUMAN METAPNEUMOVIRUS:
THE MORPHOLOGICAL AND TRANSMISSION STUDIES**

NGUYEN HUONG TRA

NGUYEN HUONG TRA

School of Biological Sciences

A thesis submitted to the Nanyang Technological University
in fulfillment of the requirement for the degree of
Master of Science

2017

ACKNOWLEDGEMENT

First of all, I would like to acknowledge my gratitude to my supervisor Associate Professor Richard Sugrue. Without his mentoring and guidance, I could not have achieved what I have, which is to be able to pursue science with an enthusiastic heart. I am thankful to him for always providing support, motivation and a stable study situation which allowed me to finish this Master Program.

Second, I am grateful for Dr Tan Boon Huan from DSO, she and Prof. Sugrue had given me a chance to work and experience a researcher's life in NTU and DSO where my scientific vision is greatly broadened by being able to join in a collaborative research environment.

Third, I would like to thank my colleagues in NTU (Raihan, Laxmi, and Patricia) and DSO (Puisan, Dennis, Daniel, Dawn, and Guy) who provided mentoring and a great deal of help, academically and emotionally, while I have been working and studying.

I am grateful for my dear husband, who has been constantly supportive and by my side from the very beginning of this recent decision to study for my Masters. He has motivated me with love and helped me celebrate every milestone of this course. I also want to dedicate this thesis to my family back in Vietnam, who have also been supportive by enduring the long separation while I have been both working and studying. Nevertheless, their support has been unwavering.

I am thankful for being able to be in SBS where my knowledge has been enhanced through useful courses which help me to be more efficient in coping with my research duties. Here, I have also the opportunity to meet wonderful collaborators and friends who share common research interests and have helped me improve my perspective with regard to my future career.

Last but not least, no amount of words can be enough for me to thank God; without His grace, I am sure I would not have been able to come to Singapore, work, study, overcome the struggles grow into the person I am today.

TABLE OF CONTENTS

CHAPTER 1. Introduction and background.....	1
1.1 Epidemiology of respiratory viruses	1
1.1.1 RSV.....	1
1.1.2 HMPV.....	3
1.2 Classification	5
1.2.1 RSV.....	5
1.2.2 HMPV.....	5
1.3 Virus structure and replication	6
1.3.1 RSV.....	9
1.3.1.1 Genomic arrangement	9
1.3.1.2 Replication process	13
1.3.1.3 Genomic arrangement	17
1.3.1.4 The HMPV replication	20
1.4 Host-virus interactions responses from respiratory virus infection	21
1.4.1 Interactions with host factors.....	21
1.4.2 Host immune responses to viral infection	22
1.4.2.1 Host immune responses to RSV infection.....	22
1.4.2.2 Host responses to HMPV infection	24
1.5 Aims of research	25
CHAPTER 2. Materials and Methods.....	26
2.1 Reagents in use	26
2.1.1 Tissue culture reagents.....	26
2.1.2 Cell lines and viruses.....	26
2.1.3 Immunofluorescence and immune-staining reagents.....	27
2.1.4 Protein analysis by SDS-PAGE electrophoresis	27

2.1.5	Western blotting reagents.....	28
2.1.6	Antibodies.....	29
2.1.7	Commercial kits in use.....	30
2.2	Methods	30
2.2.1	Mammalian cells maintenance.....	30
2.2.2	RSV propagation	30
2.2.3	HMPV propagation- HMPV isolation from clinical specimens.....	31
2.2.4	Plaque assay for virus titration	31
2.2.5	SDS-PAGE and Western blot analysis	32
2.2.6	Biotin labeling, chemical crosslinking and immunoprecipitation	33
2.2.6.1	Biotinylation of the surface glycoproteins	33
2.2.6.2	Chemical crosslinking.....	34
2.2.6.3	Immunoprecipitation	34
2.2.7	Continuous sucrose ultracentrifugation of proteins.....	34
2.2.8	RSV purification	35
2.2.9	RSV morphology analysis in HAE cells	36
2.2.10	Immunofluorescence assay and immunofluorescent microscopy.....	36
2.2.11	Light microscopy	37
2.2.12	Confocal microscopy	37
CHAPTER 3.	Morphology and Transmission in mammalian cell lines.....	38
3.1	RSV morphology and transmission in mammalian cell lines.....	39
3.1.1	RSV morphology and transmission in non-polarized cells- HEP-2, A549 and MDCK	39
3.1.1.1	RSV morphology in HEP-2 cells	39
3.1.1.2	RSV transmission in mammalian cell lines	42
3.1.2	Role of G protein in RSV transmission	53

3.1.3	RSV infection and host cell responses	60
3.1.3.1	RSV infection and host responses in a single infection cycle (MOI 0.5).....	60
3.1.3.2	RSV infection and host responses in multiple infection cycles (low MOI)	63
CHAPTER 4. RSV morphology and transmission in human NASAL epithelial cells		68
4.1	Viral morphological studies in NE cell model	68
4.2	Transmission of RSV on NE cells	70
4.3	NE cell host response from RSV infection	75
CHAPTER 5. HMPV morphological and transmission studies.....		79
5.1	HMPV morphology in mammalian cells.....	79
5.1.1	Lab-adapted HMPV morphology	79
5.1.2	Morphology of HMPV isolated from clinical samples.....	83
5.2	HMPV transmission studies using LLC-MK2 cell line.....	88
5.3	Observation of M protein- translocation into host cell's nucleus at early infection and its progression during infection course	93
5.4	HMPV infection and host response in LLC-MK2 cell line	94
CHAPTER 6. Conclusions.....		98
CHAPTER 7. Future works.....		100
7.1	RSV works	100
7.1.1	Develop EM method to study RSV morphology	100
7.1.2	RSV transmission in polarized MDCK cells.....	100
7.1.3	Host factors knock out in NE cells in correlation to RSV infection and host interaction	100
7.2	HMPV works	101
7.2.1	Comparing replication and host responses caused by lab- adapted strain HMPV with some clinical isolates.	101

7.2.2	Detailed studies of M protein function at early infection are needed.	101
7.2.3	Validate HMPV infection in correlation with JAK/STAT signaling pathway	101
7.2.4	HMPV study on NE cells.....	102
7.2.5	Transfect NS1 and NS2 of RSV in HMPV-infected cells to see if these non- structural proteins that antagonize type I interferon can enhance in vitro HMPV cultivation.	102
7.2.6	HMPV/RSV morphology and transmission studies under the effects of Ribavirin or Synagis.....	102
CHAPTER 8.	Publications	104
CHAPTER 9.	Bibliography	106

LIST OF FIGURES

<i>Figure 1</i> Etiology of acute respiratory infections in children.....	3
<i>Figure 2</i> Classification of Paramyxoviridae.....	5
<i>Figure 3</i> General depiction of a Paramyxovirus particle with its major structural proteins.....	7
<i>Figure 4</i> (a) Schematic presentations of RSV genome and its proteins' brief descriptions and functions. Schematic model of a RSV particle with its components arrangement.....	10
<i>Figure 5</i> (A) Depiction of a F protein. (B) Conformation changes of F protein through states in order to fuse its viral membrane to host cell membrane.	11
<i>Figure 6</i> Schematic presentation of RSV fusion to Airway Epithelial cell.	14
<i>Figure 7</i> RSV replication model in airway epithelial cells.....	16
<i>Figure 8</i> Schematic models of (a) Structure of HMPV viral particle. (b) Genome map of HMPV.....	17
<i>Figure 9</i> Schematic representation of the cleaved HMPV fusion protein.	19
<i>Figure 10</i> Illustration of HMPV replication.	21
<i>Figure 11</i> Schematic presentation of airway epithelial cells immune responses to RSV infection.	23
<i>Figure 12</i> Distribution of the F, G and SH virus glycoproteins in the infected cell clusters.	40
<i>Figure 13</i> Study of RSV morphology in a single infection cycle at time points of 4 hpi, 8 hpi, 12 hpi, 16 hpi.	41
<i>Figure 14</i> Localized RSV transmission occurs within the HEp2 cell monolayers.....	44
<i>Figure 15</i> Localized RSV transmission occurs within A549 cell monolayer which is similar to that in HEp-2 cell monolayer.	45
<i>Figure 16</i> Localized virus transmission occurs within RSV-infected MDCK cell monolayers.....	46
<i>Figure 17</i> RSV-infection induces localized changes in the F-actin distribution in HEp-2 cell monolayers.	47
<i>Figure 18</i> The rac-1 and rho A kinases are required for localised cell-to-cell transmission in the HEp-2 cell monolayer.....	50
<i>Figure 19</i> The presence of cell-free virus particles occurs late in infection and correlates with increased cell permeability and cytotoxicity.....	52
<i>Figure 20</i> Trypan blue staining is detected in the HEp-2 cell monolayers.	53
<i>Figure 21</i> Interaction of the F and G proteins on the surface of virus filaments.....	54

<i>Figure 22</i> Interactions of RSV G and F proteins on HEp-2 cell surface by recombinant protein expression.	56
<i>Figure 23</i> HEp-2 cells were co-transfected by RSV pCAGGs- G and pCAGGs- F flag followed by biotinylation and immunoprecipitation using anti F (1,2) or anti G (3,4).	57
<i>Figure 24</i> LLC-MK2 cells were co- transfected with HMPV pCAGGs- G flag and pCAGGs- F myc ..	58
<i>Figure 25</i> The presence of the G protein is required for efficient cell-to-cell transmission.	59
<i>Figure 26</i> Infected HEP-2 cells by RSV MOI of 0.5, UV- inactivated RSV and respective Mock-Infected cells were harvested at 4 hpi, 8 hpi, 12 hpi, 18 hpi.....	62
<i>Figure 27</i> Virus infection induces cell signaling pathways associated with cell death at 3 dpi.....	63
<i>Figure 28</i> Distribution of STAT1, pSTAT1, pMAPKp38 and pJNK in the RSV-infected monolayers..	66
<i>Figure 29</i> TUNEL assay of RSV-infected HEp-2 cell monolayers.	67
<i>Figure 30</i> NE cells were infected with RSV at MOI of 3, at 5 dpi, cells were fixed and stained with (A) anti-N, anti-M, (B) anti-N, anti-G, (C) anti-SH and anti-F.....	69
<i>Figure 31</i> RSV low multiplicity infection in nasal epithelial cells with densely packed ciliated cells....	71
<i>Figure 32</i> RSV infection progress on NE cells examined by immunofluorescence microscopy over 13 days infection period.....	72
<i>Figure 33</i> Z-stack analysis of advanced infected fused NE cells at 13 dpi.	73
<i>Figure 34</i> Titer of cell-free RSV from mucous wash.	74
<i>Figure 35</i> RSV-induced interferon signaling is associated with RSV particle formation.....	76
<i>Figure 36</i> RSV-induced Viperin expression and apoptosis occurs in RSV-infected ciliated cells.....	78
<i>Figure 37</i> Trypsin supplement effect on HMPV culture.	80
<i>Figure 38.</i> HMPV viral proteins (F, M, G, P) were characterized by IF.....	81
<i>Figure 39</i> HMPV-infected cells exhibit two specific sub-populations of the M protein.	82
<i>Figure 40</i> Variations of M protein morphology visualized by IF examined by fluorescent microscopy.	86
<i>Figure 41</i> Second passage of clinical isolates coded 20131195 and 20131384.....	87
<i>Figure 42</i> Observation of HMPV infection at MOI 0.001 from 1 dpi- 10 dpi.....	89
<i>Figure 43</i> HMPV cell-associated fraction (CA) and cell-free fraction (CF) analysis.	90
<i>Figure 44</i> LDH assays indicates cell damage over HMPV infection period of 10 days.	91
<i>Figure 45.</i> Titers of cell-associated and cell-free HMPV in correlation to cultivation time at either 33°C or 37°C.....	92

<i>Figure 46</i> Observation of M protein expression in a single infection cycle.....	93
<i>Figure 47</i> Lysates of low MOI infected were immunoblotted with either viral antibodies (G, M) to confirm infectivity or host antibodies (JAK/STAT1), apoptotic antibodies.	95
<i>Figure 48</i> Low moi HMPV infection on LLC-MK2 was conducted to evaluate correlation of viral spread (M protein) to STAT-1 protein level presence within cell monolayer over period of 1dpi to 6 dpi.	96

LIST OF TABLES

<i>Table 1</i> Proteins of RSV (Genus Pneumovirus) and HMPV (Genus Metapneumovirus).....	8
<i>Table 2</i> Amino Acid sequence relatedness (% identity) between the proteins of HRSV Subgroup A (HRSV-A) or HMPV Subgroup A (HMPV-A) and the indicated viruses ^a	8
<i>Table 3</i> Formulas for reagents used in SDS-PAGE gel preparation and sample lysis.	28
<i>Table 4</i> Formulas for buffers used in SDS-PAGE and Western Blot.....	29
<i>Table 5</i> Summary results of screened SAF clinical isolates collected from 2009 to 2013 by immunofluorescence assay using anti-M HMPV.	85

ABBREVIATIONS

A549	Adenocarcinomic human Alveolar basal epithelial cells
AEC	Airway epithelial cells
APS	Ammonium Persulphate
ARI	Acute respiratory infection
BSA	Bovine serum albumin
CA	Cell-Associated fraction
CF	Cell-Free fraction
Da	Dalton
DSP	Dithiobis succinimidyl propionate
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
F	Fusion protein
FBS	Foetal Bovine Serum
FITC	Fluorescein isothiocyanate
G	Attachment protein
GFP	Green Fluorescent Protein
HBSS	Hanks balanced salt solution
HCl	Hydrochloric acid
HEp-2	Human Epidermoid laryngeal carcinoma 2
HMPV	Human Metapneumovirus
Hpi	Hour post infection
Hpt	Hour post transfection
HRP	Horse radish peroxidase
IFA	Immunofluorescence assay

IF	Immunofluorescence
IP	Immunoprecipitation
kDa	Kilo Dalton
L	Large polymerase subunit
LDH	Lactate Dehydrogenase
M	Matrix protein
M2	Matrix protein 2
M2-1	Matrix-like protein (anti-termination factor)
M2-2	Matrix-like protein (transcription regulation)
Mab	Monoclonal antibody
MDCK	Madin-Darby canine kidney cells
ml	Milliliter
mM	Millimolar
moi	Multiplicity of infection
mRNA	Messenger RNA (ribonucleic acid)
N	Nucleocapsid protein
NE	Nasal Epithelial cells
nM	Nano Molar
NP	Nucleoprotein
NP-40	Nonyl Phenoxypolyethoxylethanol 40
NS1	Non-structural protein 1
NS2	Non-structural protein 2
NT	Non-treated
PBS	Phosphate Buffered Saline
pCAGGS	Expression plasmid with chicken beta-actin promoter

pfu	Plaque forming unit
PEG	Polyethylene glycol
PFA	Paraformaldehyde
PLA	Proximity Ligation Assay
PMSF	Phenyl Methyl Sulphonyl Fluoride
RAF	Ras-activated factor
RhoA	Ras homolog A
RNA	Ribonucleic Acid
RNP	Ribonucleoparticle
RSV	Respiratory Syncytial Virus
SDS-PAGE	Sodium Dodecylsulphate Polyacrylamide gel electrophoresis
SH	Small Hydrophobic protein
TEMED	Tetramethylethylenediamine
UTR	Untranslated region
VF	Virus filament
vRNA	Viral Ribonucleic Acid
vRNP	Viral Ribonucleoprotein
WHO	World Health Organization
hNESPCs	human Nasal Epithelial Stem/Progenitor Cells
%	Percentage
μl	Microliter
μM	Micro molar

ABSTRACT

Respiratory syncytial virus (RSV) and human metapneumovirus (HMPV) are the most important viral agents which cause upper and lower respiratory tract infections commonly present in children under the age of five. These infections are also prevalent in the elderly and persons with compromised immune systems. Currently, available drugs for RSV prevention and treatment are not cost-effective (e.g. Palivizumab, ribavirin whose mechanism of action is unproven and has toxic side effects), which makes the treatment of these infections difficult, especially for infections in young children in low in-come families. In the case of HMPV, there is no established prevention and treatment.

In this thesis, we studied the transmission pathway of RSV in several permissive cell lines utilizing low multiplicity of infection (moi). We discovered a bi-phasic mode of RSV transmission. At the early infection time from 1 day post infection (dpi) to 3 dpi, there was localized transmission from cell-to-cell, no cell free virus was detected in the culture media up to 2 dpi. At a later infection time (4 dpi, 5 dpi), there was massive cell disruption and detachment releasing a small amount of cell-free virus into the culture supernatant. Cell free virus correlated with changes in cell physiology and increased cytotoxicity. Further, we came to understand RSV transmission using an *in vitro* system of primary human nasal epithelium cells (hNESPCs) with regard to host responses. Interestingly, NE cells mostly exhibited the mode of cell-to-cell transmission, and event of NE host cell response of p38MAPK activation correlated with the increase in viral particle assembly and changes in host cell physiology. RSV G gene deficiency caused changes in the viral filament morphology, reduced viral transmission efficiency *in vitro*, suggesting that G protein plays an important role in the virus transmission pathway.

By successfully adapting a HMPV strain in culture, we characterized its morphology by imaging analyses and conducted a detailed transmission study. Data suggested the virus likely transmits from cell-to-cell. Host responses arising from HMPV infection showed evidence that the virus triggered the cellular pathway to “turn off” STAT-1 protein level as a strategy to hijack LLC-MK2 cells’ defense pathway in order to penetrate, replicate and thrive, which may be useful in designing future protocols to support HMPV cultivation *in vitro*.

CHAPTER 1. INTRODUCTION AND BACKGROUND

1.1 Epidemiology of respiratory viruses

Respiratory diseases are one of the top causes for human morbidity and mortality worldwide [18, 24]. In previous studies, paramyxoviruses including respiratory virus (RSV) and human metapneumovirus (HMPV) are reported to infect almost all children below the age of two [18, 24] and five [24, 88, 93], respectively. RSV and HMPV account for 23.3% [47] and 5% [93], respectively, of hospitalized infants who have problems with respiratory tract diseases [85]. Children under two, prematurely born children whose immunity is relatively weaker and who display a tendency to be susceptible to respiratory viruses are the ones that have high risk of serious respiratory viral infection. Even after recovery, there are studies reporting high potential of wheezing and asthma development among these children at later ages [65, 67, 83]. Fatal cases caused by untreated RSV and HMPV were reported in [39] and [93], respectively. Besides, RSV and HMPV are also the leading causes of severe lower respiratory tract disease in the elderly and immunosuppressed individuals [18].

1.1.1 RSV

RSV was discovered in 1956 from a laboratory chimpanzee with upper respiratory tract diseases [18]. Later, RSV was also found to be common among young children with respiratory diseases [15]. RSV is now widely regarded as the most important viral agent as it causes about 63% of reported hospitalized cases by respiratory viral and bacterial infection among children [71]. RSV is responsible for more than 80% of lower respiratory tract infections (LRTIs) in infants younger than one year during the peak of viral season. Children aged from six weeks to nine months are reported to have a tendency to develop bronchiolitis and pneumonia which can be explained by the drop of the maternal antibody titers in the same age period, causing them to be more susceptible to respiratory viral infection during this age [24]. Approximately two-thirds of infants are infected with RSV during the first year of life, and 90% have been infected one or more times by the age of two [23]. RSV also is a significant cause of morbidity and mortality in the elderly, children born prematurely or having chronic lung diseases, and immunocompromised individuals, especially allogeneic bone marrow transplant

recipients [21, 68]. In the United States, it is estimated that RSV causes 73,400 to 126,300 cases annually of hospitalized children under one year of age who have bronchiolitis and pneumonia [90]. Globally, the World Health Organization estimates that RSV causes 64 million infections and 160,000 deaths annually [18]. Although RSV has a single serotype with two antigenic subgroups of A and B, reinfection can occur throughout life [18]. Morbidity and mortality from RSV is highest in infants with pre-existing illness e.g. chronic lung disease. In the 1970s, RSV infected infants with congenital cardiac or pulmonary disease with a mortality rate that reached 40%, but this has recently declined to 3 to 4% due to the improvement of supportive treatment [25]. In a recent report, a high mortality rate of 80% to 100% associated with pneumonia-developed by RSV was reported in adults having leukemia or hematopoietic stem cell transplant [102].

RSV distributes worldwide and is a seasonal illness with a peak in winter/spring in countries with temperate climates and during summer in tropical countries [45]. Hence, in Singapore, RSV circulation is found year round which contributes to a large portion in respiratory diseases in young children.

As RSV is considered one of the leading cause of lower respiratory tract infection [38], intensive studies for RSV prevention and treatment have been attempted since 1961 as the first successful isolation of the virus by the National Institute for Allergy and Infectious Disease. Formalin inactivated virus is the first produced RSV vaccine. However, children treated by formalin-inactivated RSV still experienced reinfection and hospitalization and there were fatal cases caused by consumption of this product proving this method of vaccination was not adequate [64]. Currently, several approaches have been developing to tackle RSV e.g. anti-viral therapy by using nucleoside analogs (Ribavirin) [76]. Besides ribavirin, Palivizumab (Synagis) is in clinical use for the prevention of serious RSV disease in high-risk infants. It is a RSV-neutralizing monoclonal antibody targeting the F protein. However, the high cost of Palivizumab made RSV prevention by the drug is not cost-effective [18, 24]. Several studies focused on the antigenic properties of F to design small molecules targeting F protein which had been used in animal models to block fusion (JNJ-2408068) [94]. Anti-inflammatory agents of corticosteroids (LTC₄, LTD₄, LTE₄) are usually used to reduce respiratory tract disease and were considered to be advantageous in RSV infection

treatment. In fact, these treatments may prolong virus shedding which was observed in previous studies on cotton rats [72]. Virus like particles (VLPs) were considered as potential vaccine candidates as they resemble the virus particle structure and also display viral antigens (F and G) which can trigger host immune responses. After 50 years under intensive research, there is still no specific therapeutics cure or vaccines found to be cost effective to combat RSV. Nonspecific therapies mentioned above (ribavirin and intravenous immunoglobulin) are found to be limited in efficacy and costly [1, 2] which poses a strong demand for more studies about RSV in order to thoroughly understand the virus and discover efficient treatment solutions.

Figure 1 Etiology of acute respiratory infections in children. Figure is taken from [71].

1.1.2 HMPV

HMPV was first discovered in 2001 upon inoculation of tertiary kidney cells with respiratory specimens from children with RTIs conducted by van den Hoogen's group [87]. HMPV also has a single serotype with two antigenic subgroups like RSV. Serological evidence showed that the existence of the virus dates back more than four decades ago from its discovery in the 2000s. Numerous HMPV surveillance studies which have been conducted all over the world suggested HMPV infection is a global incident which demands intensive research [29, 73]. Previous studies showed that most infants are infected by this virus by the age of five and reinfections frequently occur [30]. Patients infected by HMPV also exhibit similar symptoms to those infected by

RSV for examples mild cold-like, wheezing, running nose, even bronchiolitis, pneumonia, and febrile seizures and can potentially lead to death [24]. Approximately 12% of outpatients with lower respiratory tract illness associated with HMPV infection. In a previous study, 3.9% of 668 hospitalized cases in Nashville and Rochester associated with HMPV infection [63]. In Singapore, the first surveillance project conducted from 2005 to 2007 reported HMPV was responsible for 5.3% of 400 samples collected from pediatric patients with lower respiratory tract infection (LRTI) and upper respiratory tract infection (URTI) at KK Women's and Children's Hospital. Among these results, 67% of HMPV isolates belong to lineage A; 11 of 21 children (52%) with HMPV infections had lower respiratory tract infections (LRTI) [93]. Data suggested the recurrent of HMPV infection is common, especially in immunocompromised patients, patient with a history of asthma. Besides infecting young children, HMPV also causes infection among adults, especially ones with hematologic malignancies or those in critical health conditions [31, 33]. A previous study showed HMPV caused 9% of 251 respiratory infection cases in adults, in which 16 cases occurred in patients with hematopoietic stem cell transplant, some of whom died [103]. HMPV is reported to be the second or third most common cause of severe acute upper and lower respiratory tract disease in children, just after RSV.

HMPV outbreaks occur during late winter and early spring, especially the second half of winter. Overlapping of RSV and HMPV outbreaks lead to co-infection of the two viruses in young children which cause a more severe disease [28]. Similarly to RSV, reinfection caused by HMPV is frequently observed.

After more than 10 years under extensive research, knowledge of HMPV is still limited due to the difficulties of slow growth rate and delayed cytopathic effect of HMPV isolates *in vitro*. These facts influence a poor outcome of HMPV prevention and treatment. Similar to RSV treatment, corticosteroids as anti-inflammatory agents have also been used to ease infection. Montelukast was one of those, however it was reported to improve wheezing after bronchiolitis [6]. Candidates of live vaccine for HMPV are being developed based on the same principals as RSV. Recombinant HMPVs lack of M2-2 ORF or G gene showed their attenuated features in rodents [4, 5, 10, 78]. A chimera of HMPV whose P gene was replaced by its AMPV counterpart also showed its attenuation in African green monkeys [70].

1.2 Classification

Figure 2 Classification of Paramyxoviridae. Figure is taken from [71].

Paramyxoviridae family is divided into two subfamilies named Paramyxovirinae and Pneumovirinae. RSV belongs to the sub-family Pneumovirinae, under genus Pneumovirus. HMPV also belongs to the sub-family Pneumovirinae but under genus Metapneumovirus. More details of Paramyxoviridae family's classification is listed in [Figure 2]. Hence, RSV and HMPV share some common features in their genome structures as well as viral morphologies. The differences lie on the size of the viral particle and the shape of the nucleocapsid structures [24]. Both are enveloped viruses which possess a single-stranded negative sense RNA genome.

1.2.1 RSV

HRSV was divided into 2 main subgroups, A (A1, A2) and B, based on the ability of the reaction of the virus glycoproteins (majorly G), nucleoprotein and matrix protein to antibodies used to test against them. Subtype A strains are considered to be more prevalent and virulent than B, and both strains co-circulate during outbreak.

1.2.2 HMPV

HMPV viruses can be divided into 4 subgroups A1, A2, B1 and B2 [93]. In particular, subgroup A2b is considered to be the one that has most widely circulated and is associated with the most severe HMPV infections and has caused most of the HMPV epidemics worldwide [50].

1.3 Virus structure and replication

In [Figure 3], a typical Paramyxovirus schematic presentation is depicted. Fusion proteins (F) work as fusion factors and Hemagglutinin-neuraminidase (HN) proteins work as attachment elements and are marked all over the surface of the particle. Both of them have transmembrane domains. These proteins are observed to localize together on the surface of Paramyxovirus infected host cells, they form filamentous structures called virus filaments which are the specific indicators of either RSV or HMPV infection. Multiple studies have been done using either RSV or HMPV infection or recombinant expression plasmids encoding the two proteins proving F and HN tightly interact together in forming these virus particles or virus-like-particles, respectively [55, 56, 66, 92]. It was reported that purified Paramyxoviruses particles containing actin or tubulin as their major components. [11, 22, 37, 42]. This suggested the filamentous structures caused by F and HN could interfere with host cell cytoskeleton network and the viruses utilize actin and/or tubulin network in their transmission by interfering with the host cell signaling. Besides F and HN, the third glycoprotein named SH which also has a transmembrane domain, the function of SH is debatable.

M protein is found to layer underneath cell membrane and it has an important function in defining the virus particles' shape as well as assisting virus assembly during the budding process. P (Phosphoprotein), N (Nucleocapsid), L (Large polymerase) together with vRNA form a ribonucleoprotein (RNP) complex which is characteristic of Paramyxovirus and plays an important role in genetic material storage by providing protection to vRNA and it is involved in the replication process of the vRNA. RNP is usually observed in the inclusion bodies structures of the Paramyxoviruses infected cells. In RSV and HMPV, RNP also contains M2-1 protein.

Figure 3 General depiction of a Paramyxovirus particle with its major structural proteins. Figure is taken from [1].

Besides these structural proteins present in all Paramyxovirus, RSV also has 2 nonstructural proteins of NS1 and NS2 whose functions are related to host factor modulation. In contrast to RSV, the HMPV genome lacks these non structural proteins.

Proteins of RSV and HMPV and their functions are listed in [Table 1].

Protein	M_a^r ($\times 10^{-3}$, HRSV)	Comments
Nucleoprotein (N) ^b	45	Major nucleocapsid protein; tightly binds along the entire length of genomic and antigenomic RNA
Phosphoprotein (P) ^b	33	Nucleocapsid associated; polymerase cofactor; forms a soluble complex with free monomeric N protein
M2 ORF 1 protein (M2-1) ^b	22	Nucleocapsid associated; essential transcription elongation/antitermination factor for HRSV; nonessential and function unknown for HMPV
Large (L) ^b	250	Nucleocapsid associated; major polymerase component; contains polymerase catalytic domains
Matrix (M) ^b	25	Inner surface of envelope; important in virion morphogenesis
Fusion (F) ₀ ^b	70	Transmembrane envelope surface protein; N-linked sugars; mediates fusion in viral penetration and syncytium formation; F ₀ precursor activated by cleavage into disulphide-linked F ₁ (48 kD) and F ₂ (26 kD) subunits
Glycoprotein (G) ^b	90	Transmembrane envelope surface protein; N- and extensive O-linked sugars; major

		attachment protein, although not essential; gene deletion virus is viable but attenuated
Small hydrophobic (SH) ^b	7.5-60	Transmembrane envelope surface protein; N-linked sugars and polylectosaminoglycan; protein function unknown; gene deletion virus is viable and slightly attenuated
M2 ORF2 protein (M2-2)	11	Status as structural/non-structural unknown; low abundance; shifts balance of RNA synthesis from transcription to replication; gene deletion virus is viable but attenuated
Nonstructural-1 (NS1) (<i>Pneumovirus</i> only)	13.8	Putative non-structural protein; interferes primarily with interferon- α/β induction and secondarily with signalling; gene deletion virus is viable but attenuated
Nonstructural-2 (NS2) (<i>Pneumovirus</i> only)	14.5	Putative non-structural protein; interferes primarily with interferon- α/β induction and secondarily with signalling; M _r anomalously larger than that of NS1; gene deletion virus is viable but attenuated

Table 1 Proteins of RSV (Genus *Pneumovirus*) and HMPV (Genus *Metapneumovirus*). Table is taken from [24].

^a Apparent molecular weight estimated by polyacrylamide gel electrophoresis under denaturing and reducing conditions, shown only for HRSV

^b Virion structural proteins

		% Amino Acid Sequence Identity for the Indicated Protein										
Viruses Compared		NS1	NS2	N	P	M	SH	G	F	M2-1	M2-2	L
HRSV-A versus	HRSV-B	87	92	96	91	91	76	53	89	92	72	93
	BRSV	69	84	93	81	89	38	30	81	80	42	84
	PVM	16	20	60	33	42	23	12	43	43	10	53
	HMPV-A	^b —	^b —	42	35	38	23	15	33	36	17	45
	AMPV-A	^b —	^b —	41	32	38	19	16	35	37	12	43
HMPV-A versus	HMPV-B	^b —	^b —	96	85	97	59	37	95	96	89	94
	AMPV-C	^b —	^b —	88	68	87	24	23	81	83	56	80
	AMPV-A	^b —	^b —	70	58	77	20	12	68	73	25	64
	AMPV-B	^b —	^b —	69	53	76	20	13	67	71	27	NA ^c

Table 2 Amino Acid sequence relatedness (% identity) between the proteins of HRSV Subgroup A (HRSV-A) or HMPV Subgroup A (HMPV-A) and the indicated viruses^a. Table is taken From [24]

^a Viruses are listed in order of decreasing relatedness to HRSV-A and HMPV-A

^b Does not encode NS1 or NS2

^c NA, sequence not available

In [Table 2], Collins et al. [18] described the relatedness (%) of all viral component proteins between RSV-A and HMPV-A with various other viruses from the same family

or genus. Considering this data, we focused specifically on comparison of the amino acid sequence of different proteins belonging to HRSV-A and HMPV-A (bolded line). The data showed their low similarity in all aspects mentioned for viral proteins from N, P, M, SH, G, F, M2-1, M2-2 and L (HMPV lacks NS1 and NS2), with a common protein sequence similarity lower than 50% (42%, 35%, 38%, 23%, 15%, 33%, 36%, 17%, 45%, respectively). These results were consistent with other studies confirming the differences between the two viruses' morphologies.

1.3.1 RSV

RSV is negative-sense single-stranded RNA virus. The viral envelope is lipid bilayer enriched with cholesterol, filled with viral fusion (F), attachment (G) and small hydrophobic (SH) proteins. A layer of matrix (M) protein is underneath the envelope staying closely in contact with the viral RNPs. Viral RNP contains nucleocapsid (N) protein, the phosphoprotein (P), M2-1 protein and the large (L) protein. The RNP complex wraps around and provides protection for the viral RNA. The viral particle's diameter varies in size is from 150 nm to 10 μ m. Native staining of viral particle shows an outer spikes layer represents the glycoproteins, and "herring-bone" like helical ribonucleo protein capsid [3].

1.3.1.1 Genomic arrangement

RSV possess a non-segmented genome which consists of 10 genes in the order of 3'-NS1-NS2-N-P-M-SH-G-F-M2-L-5'. It should be noted that gene M2 encodes two overlapped open reading frame for M2-1 and M2-2 proteins while other genes, each encodes for only one single viral protein. The whole genome length is about 15,222 nucleotides (RSV A2) and 15,225 nucleotides (RSV B1). To analyze RSV viral proteins, groups of them are addressed in a specific order from the outside to the core of the viral particle [Figure 4].

Figure 4 (a) Schematic presentations of RSV genome and its proteins' brief descriptions and functions. Schematic model of a RSV particle with its components arrangement. Figure taken from [27].

a. Structural proteins

Structural proteins are divided into two groups: proteins belonging to the glycoprotein group and proteins belonging to the RNP structure (N, M2, L, P). It is reported that M2-1 and P interaction is important for the viral replication [17]. Glycoproteins in RSV include fusion (F) protein, attachment (G) protein and small hydrophobic (SH) protein. These proteins are decorated on the outside of the particle and have important functions during the attachment and fusion phases of the viral membrane to the host membrane.

The F protein plays an important role during membrane fusion process between the viral particle and the host cell. F is synthesized as a precursor, F₀, and contains furin-like cleavage sequences which are digested by intracellular host protease. Cleavage occurs at two sites (amino acids 109/110 and 136/137) to produce 3 progeny proteins of F₂ (109 amino acids), p27 (27 amino acids), and F₁ (438 amino acids). F₂ and F₁ remain linked by a disulfide bond and represent the active form. Even though the F protein has two cleavage sites, activation appears to be readily achieved by ubiquitous cellular proteases. F is also considered as a famous target for studying drugs that target RSV.

Figure 5 (A) Depiction of a F protein. (B) Conformation changes of F protein through states in order to fuse its viral membrane to host cell membrane. Figure is taken from [59].

In [Figure 5], a schematic presentation of F protein is depicted in (A). F0 protein (from position 1 to 574) with size of 67 kDa containing SP (signal peptide). This inactive F0 contains F1 (from position 136/137 to 574) and F2 (from position 22 to 109/110) with pep27 which is a 27 amino acid peptide lying in between the two subunits. During furin cleavage, pep27 is released, F0 is processed into F1 and F2. The F2 subunit contains Heptad repeat C domain (HRC), F1 contains fusion peptide (FP), Heptad repeat A (HRA) and B (HRB) domains, domain I and II, transmembrane domain (TM).

In the pre-fusion state, F protein in 3D arrangement is as depicted where fusion peptide is buried within the complex. In the intermediate state, when G protein binds to the cell membrane at its receptor, conformation changes in F are induced resulting in 3 domains of HRA of F (red) which elongates exposing the fusion protein (blue) and which is then inserted into the host membrane. 3 domains of HRA are undergoing trimerization to form a coiled structure, when HRB comes in contact with HRA trimer to form 6-HB complex as a hairpin this enables fusion of the viral membrane to the host membrane by mixing lipids. After the event, or in the post-fusion state, a pore formation is formed where cytoplasm of the virus and host cell are connected enabling transportation of the viral genomic material into host cell's cytoplasm to occur [59].

G protein's length is around 290 amino acids however, the size of mature G protein is 84-90 kDa due to extensive sugar modification. It contains several N-linked carbohydrate side chains and an estimated 24 to 25 O-linked side chains on the ectodomain. In our study using the NE cells, size of 150 kDa G protein was observed (unpublished data). G protein is essential for the attachment of the viral particle to the host. Deletion of G from the RSV genome caused the virus to replicate poorly *in vitro* and lose its replicative function *in vivo* [96].

SH small hydrophobic protein (21-30 kDa) was proposed to play a role as an ion channel or have an effect on host cell membrane's permeability but to date, there are no specific conclusions with persuasive data to confirm these hypotheses. There was also an research opinion suggesting SH was not essential for RSV infection because the SH gene deleted RSV showed no reduction of RSV activity both *in vitro* and *vivo* [96]. A molecular modeling study suggested that the SH protein may be an ion channel forming viroporins. Viroporins have been identified in a number of viruses and play roles in virus assembly and release as well as in pathogenesis and cytotoxicity. Rixon's group reported the observation of SH accumulation within lipid-raft structures in Golgi during the infection process suggesting SH might play a role in viral component packaging and targeting to the host cell surface [81]. In our previous studies, we showed evidence of correlation and interaction of RSV glycoproteins, in detail: F and G, G and SH are found to co-localise in immunostaining experiments and co-precipitate in immunoprecipitation experiments.

M (matrix) protein with a molecular weight of 28 kDa forms a layer underneath the viral membrane, plays an important role in virion morphogenesis [51].

L (large) protein (~200 kDa) is the largest protein in the genome, and functions as a catalytic component of RNP complex, it is in charge of enzymatic activities for viral replication and transcription. The co-expression of RSV P, L, N produced a protein complex which is necessary for RNA replication [13].

N (Nucleoprotein) with a molecular weight of 44 kDa encapsulates genomic RNA and its intermediate positive-sense, hence, it protects the structures from degradation by the host cell toll-like receptors (TLRs) or innate immune responses.

P (Phosphoprotein) a 34 kDa protein is a cofactor in viral RNA synthesis.

M2 proteins including M2-1 (22 kDa) and M2-2 (11 kDa) are involved in transcription and replication modulation. Immunofluorescent staining data suggests that M2-1 is accumulated mostly in inclusion bodies in the infected cells. While M2-2 seems to be a transiently expressed protein, however the protein existence form due to infection has not been discovered and studied.

b. Non-structural proteins NS1 (22 kDa) and NS2 (18 kDa)

These two nonstructural proteins are believed to have regulation functions during the infection process, and may be involved in host response modulation. The proteins are packed into the virion but not in abundance. The two protein species are reported to target STAT2 and play role in blocking signaling from type I IFN [54].

1.3.1.2 Replication process

The replication process starts with the attachment and fusion of the viral membrane to the host cell membrane.

a. Attachment and Fusion process.

Figure 6 Schematic presentation of RSV fusion to Airway Epithelial cell. Figure is taken from [49].

In vitro, infection starts by attachment to the host cell membrane which is performed by the association of F and G proteins to specific receptor like sulfate glycosaminoglycans (GAGs) for examples heparin sulfate and chondroitin sulfate B through a putative heparin-binding domain in the G protein.

After attachment, F0 (70 kDa) precursors are cleaved by furin- like enzymes presenting in all cell types to produce 2 subunits of F1 (~50 kDa) and F2 (20 kDa). This process is pH independent. F2 is the active form of the protein causing the membrane fusion of viral particle and host cell membrane. Recently, Tayyari et al. proposed a potential RSV receptor on Airway Epithelial Cell's apical surface (AEC) called nucleolin whose molecular weight is 100 kDa [95]. Nucleolin was found to be co-immunoprecipitated with F1 but not with G, suggesting that the RSV F protein binds to nucleolin. This finding also contributed to the understanding of RSV entry which occurred specifically on an apical cell surface. Other studies were found to be consistent to this study by addressing experiments where nucleolins of AECs were blocked by antibodies significantly having impact on lowering RSV infection compared to the controls [Figure 6].

The event of RSV uptake by membrane fusion (hemifusion) was believed to be done by either clathrin- mediated endocytosis nor caveolar- mediated endocytosis where host cell factors were much related to e.g dynamin, flotillin, caveolin, actin [37]. However, drugs treatment targeting these factors did not seem to prevent or reduce RSV infection.

In another report, San-Juan-Vergara et al. suggested cholesterol-rich microdomains (lipid rafts) in the plasma membrane are utilized as docking platforms for RSV [58], [9] to enter the normal human bronchial epithelial cells (NHBE) by providing evidence of RSV virions association with ganglioside-1 (GM-1) at the hemifusion stage [84]. This contributed to a hypothesis of RSV F protein binds to nucleolin, and is embed in cholesterol-rich lipid microdomains containing GM-1 [8] at the host cell surface. Once this event happens, it triggers Pak-1 to activate and reorganize the actin network in order to prepare for RSV hemifusion to take place.

Recent research of recombinant RSVs which lack either G or SH proteins suggested these viruses still retain some of their RSV classical characteristics *in vitro* for examples: cell-to-cell transmission, syncytium formation, filamentous viral particles [96]. However, they do show virulent attenuation in *in vivo* studies using either mice or *in vitro* studies using human airway cells [32]. In the case of G gene deleted virus, virus replication showed a significantly slower rate in comparison to that of the wildtype (unpublished data). This presents an intriguing the question with regard to G function: besides being involved in attachment, what else does G do during RSV infection. And if lacking G, the virus is still able to survive and replicate within host cell, this would imply further functions of F rather than just playing a role in fusion during infection progress.

b. Viral replication

Once viral membrane and host membrane fusion happens, the RNP complex containing negative- single- stranded vRNA is transported into the host cell's cytoplasm, L protein initiates the viral transcription [Figure 7].

vRNA is transcribed into 5'-capped and 3'-polyadenylated mRNAs under a catalytic function of viral RNA-dependent polymerase complex. These mRNAs are used

to synthesize the viral structure and the non-structure proteins which are later used to produce the viral progeny particles and then modulate the process within the host cell. Paparelli, anti-genomic RNA is synthesized moderately and used as the template to produce more vRNAs. Compare to genomic RNA, anti-genomic RNA is always less abundant from 10 to 20 folds. It was reported that M2-2 may play a role in regulating transcription and replication. If the level of M2-2 level is lower, the transcriptional process is more favored and vice versa, if the level of M2-2 is higher, the replication process is more favored [17].

c. Particle assembly and budding process

Figure 7 RSV replication model in airway epithelial cells. Figure is taken from [49].

It is observed that, RNP (N, M2-1, P, M, L) materials are accumulated in structures called inclusion bodies in the host cell cytoplasm. Hence, inclusion body structures' appearance is used as one of indication for RSV infection *in vitro*. RNP associates with genomic vRNA together with glycoproteins and nonstructural proteins targets the cell surface membrane, favorably lipid raft structures or lipid-enriched domains for particle assembly. During viral morphogenesis, M proteins are layered directly beneath the plasma membrane, recruiting glycoprotein outward and

making contact with RNP vRNA complex inward. Possibly M proteins also assist in the virus budding process by forming membrane curvature. The final step of RSV replication is virus maturation in which, progeny virus particles bud from the host cell to infect nearby cells or to be released into culture medium.

1.3.1.3 Genomic arrangement

a. Model structure of hMPV

b. Proteins encoded by hMPV genome

Figure 8 Schematic models of (a) Structure of HMPV viral particle. (b) Genome map of HMPV. Figure is taken from [16].

[Figure 8a] The virus virion has a lipid membrane envelope where the Matrix protein forms a layer underneath. Three glycoproteins of F (Fusion), G (Glycoprotein), SH (Small Hydrophobic) are transmembrane proteins with complexes densely marked with glycans. These play important roles in viral attachment and fusion. The ribonucleoprotein (RNP) complex consists of N (Nucleoprotein), P (Phosphoprotein) and L (Large polymerase protein) and a segmented single-stranded negative sense RNA

genome which is from 13,280 to 13,378 nucleotides in length, having coding for 8 genes with 9 opening reading frames. The HMPV genome is similar to that of RSV in the M2 gene which encodes for M2-1 and M2-2 proteins [Figure 8b]. The differences between the 2 genomes are HMPV lacks 2 nonstructural proteins (NS1 and NS2) and the order of the genes between M and L is not the same (in RSV, it is SH-G-F-M2 while in HMPV, it is F-M2-SH-G). The genome of HMPV contains noncoding regions between each ORF which range in size from 23 to 209 nucleotides and contain gene end signals, intergenic regions, and gene start signals. The role of these noncoding regions is likely the same as that for other paramyxoviruses, where the gene end and gene start sequences control transcription termination and re-initiation.

F (Fusion) protein [Figure 9] The mature HMPV F protein has a full length of 522 amino acids (non-cleaved F called F0). F0 contains F2 subunit (82 residues) and F1 subunit (440 residues). F1 contains an extracellular domain; a 23 amino acid transmembrane domain which harbors the protein into cell membrane, which forms a cytoplasmic tail; heptad repeat A and B and a fusion peptide. Based on this organization of protein fusion structural components, HMPV F is classified into viral class I fusion proteins which is synthesized at full length F0 then is proteolytically cleaved into an active form of F1 and F2 by exogenous protease or low pH [85]. However, there are publications from some laboratories where adapted HMPV strains have been successfully cultured without exogenous protease supplements, likely due to a change in the cleavage site of (RQPR2F) [4]. Recently, it was shown that the HMPV F protein can bind to host cells via an integrin 1 via Arg-Gly-Asp (RGD) motif, supporting the hypothesis that F also plays a role in attachment to host cell while serving a membrane fusion function. Membrane fusion promoted by paramyxovirus F proteins generally occurs at the plasma membrane of the host cell at neutral pH, which contrasts with the case for viruses gaining entry via a pH-dependent endocytic route [20]. However, the trigger that leads to the membrane fusion event remains poorly understood.

Figure 9 Schematic representation of the cleaved HMPV fusion protein. Figure is taken from [20].

Attachment protein of HMPV is a type II integral membrane protein called either HN, H, or G [85]. G protein is encoded by 219 amino acids with a predicted molecular weight of 24 kDa. However, intensive glycosylation of N-linked and O-linked glycans caused an increase of the protein size up to 90-100 kDa. It is reported that both RSV and HMPV viruses lacking the G and SH maintain their infectivity *in vitro* which indicates that their F proteins are capable in complementing both attachment and fusion functions for the viruses' entry. However, the G protein has been proved to be essential for the viruses *in vivo* infection [4]. G proteins from RSV and HMPV are different in homologies and sizes but both are thought to serve attachment function of the viruses by strengthening viral particles' adhesion to the host cell membrane surface via binding to receptors e.g. heparans sulfate or glycosamineoglycans (GAGs) [20]. However, in [14], Chang et al., it was reported that the HMPV F protein was able to bind to heparan sulfate in order to engage the host cell membrane. Bao et al. reported HMPV G has the ability to disrupt mitochondrial signaling in AECs which suggested G not only played a role in attachment but was also involved in modulating host cell factor [3]. G-deleted mutants showed potential to increase the host's interferons and NF- κ B related genes expression levels [4].

Besides the F and G proteins, HMPV has another glycoprotein harboring to the membrane is **SH** encoded by 179 amino acids with predicted molecular weight of 20 kDa. Similar to RSV, SH of HMPV is believed to associate with F and G to form a complex in the viral envelope. A recent study reported SH has functions similar to viroporin and may be involved in fusion activity to the host cells [14]. Recombinant HMPVs lacking only the SH gene showed only marginal attenuation *in vitro* and in animal models while recombinant HMPVs lacking both SH and G genes were greatly attenuated which made them potential vaccine candidates [4].

M protein contains 254 amino acids with a predicted weight of 28 kDa and is believed to form a layer underneath the viral membrane which is important for virus morphogenesis. In our studies, we noticed that M of HMPV also exhibit a pattern of viral filaments which is different compared to RSV-M. Moreover, the M structure showed there are two different sub-populations of the tip (round shape) and long (rod) filaments can both form. In some clinical isolates, the ratio between the tip and the rod may indicate a different replication rate of a different sub-type virus. Further investigations are needed to confirm this observation [4].

M2-1 and M2-2 are both encoded by the M2 gene with weights of 21 kDa and 71 kDa, respectively. There are speculations about their functions: M2-1 could be a transcriptional factor which is similar to M2-1 of RSV; M2-2 has the ability to control mutation rate of the viral RNA polymerase or M2-2 could inhibit the transcriptional and replication process of HMPV. Recently, Ren et al. reported that M2-2's has the ability to inhibit innate response in monocyte-derived dendritic cells and cellular signaling [78, 79]. HMPV M2-1 function of zinc binding was reported to be indispensable for viral replication and pathogenesis in vivo [12].

N (Nucleoprotein) gene encoded for its product of 394 amino acids weighed 43kDa. **P (Phosphoprotein)** gene encoded for its product of 294 amino acids weighed 33 kDa. **L (Large) subunit** of RNA-dependent RNA polymerase gene encoded for its product of 539 amino acids weighed 230kDa. L protein plays a role in transcription of viral mRNA and viral replication cycle. L, N, P together form a viral RNP complex which is essential for the virus genome function and storage. The level of understanding with regard to L, N, P and HMPV RNP functions is less clear compared to RSV.

1.3.1.4 The HMPV replication

Up to now, the details of HMPV replication activities have not been studied thoroughly. Most of the understandings were inferred from RSV and other paramyxoviruses. Generally, infection starts when G proteins attach to a host cell's specific receptors. This process may or may not occur simultaneously with the fusion event when the viral membrane fuses with the host membrane which is mediated by F protein. Following membrane fusion, the viral RNP containing the viral RNA (vRNA) genome is released into the cytoplasm. Replication and translation of vRNA occur by

utilizing the host machineries. In this case, the HMPV genome is a negative sense RNA. This negative sense is used to produce the positive senses which will be used as the template to translate into viral structural proteins and produce viral components. Positive senses are also used to make new copies of vRNA negative senses. Details in the steps of the HMPV replication cycle after the synthesis of RNA and viral proteins are not fully understood [Figure 10]. Components for progeny viruses newly synthesized using host resources including viral structural and nonstructural proteins as well as vRNA are transported toward the host apical membrane surface to assemble into new viruses followed by budding out of the host cell to be released into culture environment or transmitted into adjacent cells. These process are also less known.

Figure 10 Illustration of HMPV replication. Figure is taken from [85].

1.4 Host-virus interactions responses from respiratory virus infection

1.4.1 Interactions with host factors

As soon as either RSV or HMPV enters the host cells, their viral RNAs trigger pathogen recognition receptors (PRRs) as these PRRs' role is sensing foreign RNAs and

their biological intermediate components which are generated within the host cells once the viral replicative process is initiated [62].

PRRs are divided into 2 groups: PRRs associated with endosomes and PRRs in cellular cytosol. The first group includes Toll-like receptors (TLRs) of 3, 7, 8 and 9. Endosomal TLRs use IRF3 and IRF7. TLRs 3 induces interferon- β for IRF3 for activation, TLR7 (TLR8 and TLR9) activates IRF7 using myeloid differentiation primary response gene (88) and adaptor protein (MyD88) [99]. Once high levels of IRF7 are expressed, it also leads to induction of IFN- α/β which contains inducible cytokines in reaction to viral, bacteria or parasite infection with many viruses having evolved strategies to overcome this antiviral effects. IFNs are classified into Type I, II and III IFNs. Type I IFNs or IFNs- α/β are primary cytokines involving in innate immune responses and present in many cell types in response to viral infection, regulated at transcriptional level [69]. The latter group included factors e.g. RNA-helicases-like receptors (RLRs), retinoic acid inducible gene I (RIG-I). Melanoma differentiation-associated gene 5 (Mda5). RIG-I and Mda5 are known to interact with viral RNA through their helicase domains [46]. On the other hand, these two factors also interact with caspase recruitment domains which have the ability to regulate adaptor molecule interferon (IFN)- β promoter simulator I's (IPS-1) activity. IPS-1 is a mitochondrial antiviral-signaling protein/virus-induced signaling adapter. Signal via IPS-1 once generated, activates various other downstream factors e.g. the transcription regulatory factor 3 (IRF3) (leads to upregulation of type I interferons (IFNs)); NF- κ B (primarily mediates induction of inflammatory cytokines) [26, 36].

1.4.2 Host immune responses to viral infection

1.4.2.1 Host immune responses to RSV infection

In [Figure 11] Lay et al. [49] presented series of host responses of airway epithelial cells to RSV infection.

Figure 11 Schematic presentation of airway epithelial cells immune responses to RSV infection. Figure is taken from [49].

Upon recognition of vRNA via PRRs, host responses follow the patterns described above where RIG-1, TLR3 and NF- κ B are translocated to the host nucleus. Pro-inflammatory genes are upregulated e.g. IFN- α/β ; CC (i.e. RANTES, MIP-1- α and -1- β), CXC (i.e. the host nucleus, IL-8), and CX(3)C (fractalkine) chemokines. IL-8 attracts inflammatory cells such as neutrophils, which can mediate cell damage. RSV NS1 and NS2 presence inhibits host type I IFN [54]. Monocytes/macrophages, memory T cells, memory T lymphocytes, and NK cells are recruited via CCL5, CCRs, CCL2 along with CCR2, respectively. A high level of CCL2 also increases Th2 responses when CXCL10 recruit monocytes, NK cells, T cells (Th1 cells express CXCR3 receptor). There were studies addressing the balance between the response of Th1 and Th2 CD4+ lymphocytes and its relatedness to pathogenic response to HRSV infection and reinfection [2, 82] [Figure 11]. Studies in mice suggested that interferon- γ secreted by NK cells and HRSV-specific CD8+CTLs during infection plays an important role in enhancing Th1 and inhibiting Th2 responses to HRSV antigens [90]. Interferon- α/β induced by infection also appeared to enhance Th1 and suppress Th2 responses (10). However, it is reported that RSV infection actually did not cause the elevation of interferon- α/β expression [54]. Interferon β was also reported to be inhibited by RSV

replication in human lung epithelial cells [61]. RSV NS2 is reported to play a role in STAT-2 degradation, leads to inhibit JAK/STAT signaling a response to the binding of interferon- α , - β and - γ to their receptors [106].

Several studies in mice suggested CD8⁺ CTLs are important in RSV elimination, especially in the upper respiratory tract. In the BALB/c mouse, the presence of natural killer (NK) cells were observed as early as two days, followed by pulmonary CD8⁺ cytotoxic T lymphocytes (CTLs) and systemic antibodies. Clearance of the virus was associated temporally with the increase in CD8⁺ CTLs (9). In another study, primed T cells showed the effects in clearing the infection in nude mice. In mice with CD4⁺ and CD8⁺ T-lymphocyte subsets depletion individually or together showed that both are important in clearing a primary infection [31].

1.4.2.2 Host responses to HMPV infection

Despite studies of HMPV have been conducted for more than 10 years, host response mechanisms to the infection have yet to be clearly understood. Most of understanding with regard to HMPV and host interaction have been inferred from common knowledge of other host-virus interactions which emphasized the important role of pattern recognition receptors (PRRs) in sensing vRNA and its replication activities and components.

Recently, Spann et al. in [91] examined the critical roles of both IRF3 and IRF7, and also signal transduction via IPS-1, in regulation of the IFN and inflammatory responses to HMPV infections, and viral clearance. HMPV CAN-97-83 infected neonatal mice with IRF3, IRF7, IR3/7 and IPS-1 gene deficiency showed that the infection level was increased in the absence of IPS-1 > IRF3/7 > IRF3, and associated with a reduction in IFN- β . This suggested that in different knockouts, IFN regulation by these factors were greatly affected under the impact of the infection. In mice with IRF3/7 absence, neutrophilic inflammation was observed (by IRF7 deficiency), there were exaggerated type 1 helper T-cell (Th1)/Th17, natural killer (NK) T cells a (by IRF3 deficiency). In mice without IPS-1, no inflammatory responses were observed. Although, elevated IFN levels were present, in many cases, were they unable to clear viral infection. Hence, IFN- β , but not IFN- λ 2/3, plays key role in HMPV clearance.

1.5 Aims of research

The aims of this research include:

- a. To understand RSV morphology and transmission in permissive cell lines *in vitro*. To evaluate G protein function in RSV morphology and transmission in comparison of wildtype RSV to G-deleted mutant RSV.
- b. To understand transmission of RSV in the NE cells which are human-derived well-differentiated primary cells that mimic the human host response under infection. This knowledge serves as a closer step to illuminate RSV transmission and host response in humans and aid the future drug development against RSV transmission.
- c. To have better understanding of HMPV infection at the molecular level by studying the virus morphology and transmission in correlation to LLC-MK2 host response. It is possible to purify HMPV particles and analyze them by mass spectrometry in order to understand the protein components of the particles and their origins (e.g. some host proteins can be incorporated in to viral particles). This data can be used later to better explain HMPV infection pathway and morphogenesis *in vitro*.

CHAPTER 2. MATERIALS AND METHODS

2.1 Reagents in use

2.1.1 Tissue culture reagents

DMEM + GlutaMAX	Invitrogen, USA #10569
Heat- Inactivated Foetal Bovine serum	Invitrogen, USA #10500
Phosphate buffered saline pH 7.2, 1X	Invitrogen, USA #20012
Penicilin-Streptomycin 100X (10,000 IU Penicillin and 10,000 µg Streptomycin) PAA	Laboratories #P11-010
Trypsin-EDTA 0.25%	nvitrogen, USA #25200
Trypsin-TPCK (L-1-tosylamido-2-phenylethyl chloromethyl ketone)-treated	
Worthington, USA	

2.1.2 Cell lines and viruses

Cell lines were maintained in DMEM media containing 10% FBS (Gibco), 1% Penicilin/Streptomycin 100µg/ml (Gibco), using bio-incubator at 37°C with 5%CO₂.

- HEp-2 cells ATCC CCL23, USA
- A549 ATCC CCL185
- MDCK ATCC CCL-34
- LLC-MK2 ATCC CCL7.1, USA
- hNESPCs

Well-differentiated Human nasal epithelial stem/progenitor cells (hNESPCs) were obtained from the Department of Otolaryngology, Yong Loo Lin School of Medicine, Singapore. These in vitro passaged hNESPCs retain the potential of full differentiated, stratified, multilayered, mucociliary airway epithelium containing both ciliated columnar cells and goblets cells through air-liquid interface (ALI) culture. Detailed culture methods were described previously [104]. Approval for the hNESPCs was obtained (DSRB Ref: D/11/228). The fully differentiated cells showed visible cilia beating and mucus production and were obtained from 32 to 35 days in ALI culture and were characterized by either IF staining method using ciliated and goblet protein markers (b4 tubulin, mouse anti human, clone ONS.1A6, ab11315; Abcam,

Cambridge, Mass) and rabbit anti human mucin 5AC (sc-20118; Santa Cruz Biotechnology, Dallas, Texas); or using scanning electron microscopy (SEM) to reveal distinct ciliated structure and goblet cells.

f. RSV A2 (from professor Sugrue, School of Biological Sciences, NTU)

g. HMPV clinical strain The HMPV A2 strain NCL03-4/174 was isolated from nasal secretions of children with respiratory infection, and the virus was cultured as described previously [35].

2.1.3 Immunofluorescence and immune-staining reagents

Paraformaldehyde	ICN Biochemicals Inc.
Saponin	Sigma-Aldrich
Triton-X100	Chemicon
Methanol:acetone (1:1)	Fisher scientific

2.1.4 Protein analysis by SDS-PAGE electrophoresis

15% Resolving Gel	1980 µl 30% Acrylamide/Bis solution (37.5:1, 2.6% C) (BioRad, USA #161-0158) 960 µl water 1000 µl Tris-HCl pH 8.8 (Promega, USA #H5135) 40 µl 10% SDS (sodium dodecyl sulphate) (BioRad, USA #161-0302) 40 µl 10% APS (ammonium persulfate) (Promega, USA #V3131) 4 µl TEMED (N,N,N,N-tetra methyl ethylenediamine) (BioRad, USA #161-0800)
12% Resolving Gel	1584 µl 30% Bis-Acrylamide 1356 µl water 1000 µl Tris-HCl pH 8.8 40 µl 10% SDS

	40 µl 10% APS 4 µl TEMED
10% Resolving Gel	1600 µl 30% Bis-Acrylamide 1400 µl water 1000 µl Tris-HCl pH 8.8 40 µl 10% SDS 40 µl 10% APS 4 µl TEMED
4% Stacking Gel	198 µl 30% Bis-Acrylamide 900 µl water 378 µl Tris-HCl pH 6.8 15 µl 10% SDS 15 µl 10% APS 1.5 µl TEMED
5X Laemmli buffer	31.25 ml of 1M Tris-HCl pH 6.8 10 gm of SDS 25 ml of Glycerol 750 µl Bromophenol Blue (2%) in ethanol 5 ml of β-mercaptoethanol (Merck, USA #8.05740.0250) Add ddH ₂ O and top up to 100 ml

Table 3 Formulas for reagents used in SDS-PAGE gel preparation and sample lysis.

2.1.5 Western blotting reagents

SDS-PAGE running buffer (1X)	57.6 gm Glycine 12 gm Tris base 4 gm SDS ddH ₂ O top up to 4 L total
Transfer buffer (1X)	3.03 gm Tris base 14.41 gm Glycine 200 ml methanol

	ddH ₂ O top up to 1 L total
5% (w/v) milk blocking solution	1 gm of skimmed milk powder in 20 ml of PBS-Tween 20
Precision plus protein standards unstained ladder	Bio-rad, USA #161-0363

Table 4 Formulas for buffers used in SDS-PAGE and Western Blot.

2.1.6 Antibodies

Mouse anti-Filamin-1 (E-3) from Santa Cruz/ sc17749 used 1:100 (IF) and 1:1000 (WB)

Mouse anti-G RSV mab8582 used 1:100 (IF) and 1:3000 (WB)

Mouse anti-STAT1 from BD biosciences used 1:100 (IF) and 1:3000 (WB)

Mouse anti-pSTAT1 pY701 from BD biosciences used 1:1000 (IF) and 1:1000 (WB)

Rabbit anti-JNK used 1:1000 (IF) and 1:1000 (WB)

Rabbit anti-pJNK used 1:1000 (IF) and 1:1000 (WB)

Rabbit anti-p38MAPK used 1:1000 (IF) and 1:1000 (WB)

Rabbit anti-pp38MAPK used 1:1000 (IF) and 1:1000 (WB)

Mouse anti-actin from Sigma Aldrich used 1:100 (IF) and 1:3000 (WB)

Phalloidin-FITC from Sigma Aldrich used 1:150 (IF)

Mouse anti-RSV from Novacastra used 1:300 (IF)

Rabbit anti-N RSV, home made, used 1:200 (IF) and 1:5000 (WB)

Rabbit anti-F RSV, home made, used 1:200 (IF) and 1:5000 (WB)

Mouse anti-M70 RSV, home made, used 1:100 (IF), and undiluted (WB)

Mouse anti M2-1 RSV, home made, used undiluted for both IF and WB

Mouse anti-M HMPV, from Hybridoma clone #8A11, #3F8, used undiluted for both IF and WB

Mouse anti-F HMPV, from G.Toms, #F58, used undiluted for both IF and WB

Mouse anti-G HMPV, commercial source, used undiluted for both IF and WB

Goat anti-mouse IgG peroxidase conjugate from Sigma Aldrich, USA #A4416, used 1:10000 for WB

Goat anti-rabbit IgG peroxidase conjugate from Sigma Aldrich, USA #A0545, used 1:10000 for WB

Goat anti-mouse IgG Alexa Fluor 555 conjugate, Invitrogen, USA #A21428 used 1:2000 (IF)

Goat anti-rabbit IgG Alexa Fluor 488 conjugate, Invitrogen, USA #A11001 used 1:200 (IF)

2.1.7 Commercial kits in use

Proximity Ligation Assay	Sigma Aldrich
DeadEnd™ Fluorometric TUNEL system	Promega
Lactate Dehydrogenase assay	Roche
Annexin V	BD Bioscience

2.2 Methods

2.2.1 Mammalian cells maintenance

Mammalian cells (HEp-2, LLC-MK2, MDCK, A549) were maintained in DMEM (Gibco) with 10% FBS and 1X Pen/Strep within cell incubator of 37°C, supplemented with 5% CO₂. When the cells reached confluence of 90%, they were split into new culture flasks. First, the cell monolayer was washed with warm sterile 1X PBS once, detach agent of 0.25% trypsin-EDTA in a small volume was added onto the monolayer and incubated for a short time in 37°C. Cell detachment was assisted by flask tapping. New growth media of 10% FBS DMEM Pen/Strep was added to deactivate trypsin activity and a small volume of split cells was transferred into new culture flasks with a sufficient volume of growth media. Flasks were put into the cell incubator to enable the cells to grow. When the flasks of cell confluence were at 90%, they were used for virus propagation. The virus kept at -80 was thawed quickly, spun at 400 × g for 10 minutes to pellet cell debris.

2.2.2 RSV propagation

Propagation was done in T175 culture flasks. For each T175 flask, 1 ml of supernatant from the virus inoculum was diluted in pre-warmed 2% FBS DMEM 1X Pen/Strep to reach total volume of 10 ml. Growth media was removed and virus solution was added onto cell monolayer. Input virus for the propagation was estimated at MOI of 0.01. The flask was then put into a cell incubator of 33°C, supplemented with 5% CO₂. After 2 hours of incubation, fresh 2% FBS DMEM 1X Pen/Strep media were topped up into the infected flasks to achieve a proper volume of 20 ml. 48 hpi, obvious

CPE will be observed, syncytia forming as well as cell floating were indicators for a good infection. Infected cells together with the tissue culture supernatant were harvested using acid washed 3 mm diameter lime glass beads. The harvested virus solution was homogenized and aliquoted into small volumes and kept frozen at -80°C with proper label.

2.2.3 HMPV propagation- HMPV isolation from clinical specimens

Maintenance media used in the HMPV propagation contained DMEM, 2.5% BSA, 1X Pen/Strep, 2 mg/ml trypsin TPCK. Propagation was done in T75 tissue culture flasks. For each flask, 1 ml of clarified virus inoculum was diluted in 4 ml pre-warmed maintenance media. The growth media was removed; the cell monolayer was washed once with sterile PBS 1X. The diluted virus solution was then introduced into the cell monolayer at MOI of 0.01. The culture flask was returned to the incubator at 37°C, 5% CO₂. After 4 hours, 5 ml of fresh maintenance media was topped up into each flask to arrive at a total volume of 10 ml in T75. Flasks were then observed over time, and the image recording viral infection progress were taken, around 10 dpi, when about 70% of the monolayer started floating with intense CPE, the cells were ready to be harvested using the scraping method. The virus solution was homogenized, vortexed at high speed for 1 minute, sonicated briefly in water bath sonicator and then aliquoted into appropriate volume.

2.2.4 Plaque assay for virus titration

The harvested virus was frozen in -80 freezer previously. The virus was snap thawed, spun at 400 × g for 10 minutes, and then diluted serially (1; 1:10; 1:100; 1:1000; 1:10000, etc.) in maintenance media 2% FBS DMEM 1X Pen/Strep, and applied on a 90% confluent monolayer of HEp-2 cell (in case of RSV) or LLC-MK2 (in case of HMPV). The infection procedure (for each virus) was as mentioned above. After 48 hpi, cells are fixed with either 4% Paraformaldehyde in PBS for 20 minutes or an ice cold methanol: acetone (1:1) for 10 minutes. Primary antibody against a viral antigen of choice was incubated with the cell monolayer to detect the plaque formation by virus infection, followed by a secondary antibody conjugated with fluorescent signal (eg. FITC, alexa-488, alexa-555). Samples then were examined using fluorescent microscope, plaque numbers were counted and used to calculate the virus titer according

to its dilution factor. The formula to determine the virus titer used a format of 24 well plate with coverslip is listed as below:

$$\frac{N}{10^{-X}} \times \frac{1000}{\text{vol of virus}} \times \frac{\text{radius 24 well}^2}{\text{radius coverslip}^2} = \text{titer}$$

Where N is numbers of plaque counted, X is dilution factor.

The virus titer value (at form of plaque forming unit per ml or pfu/ml) gives the known of effective infectious particles within 1 ml of the inoculum. The titer of the virus inoculum is used to calculate for MOI used in future experiments.

MOI is calculated based on the estimated infectious virus particle to apply onto a certain monolayer with the known cell number in order to achieve certain infection conditions.

2.2.5 SDS-PAGE and Western blot analysis

Cells were seeded into multiple well tissue culture plate to achieve 90% of confluence. Virus infection was achieved according to the steps outlined above. At certain time points in infection process, the cell monolayer was harvested. First, culture supernatant was removed, then the cells were washed twice with sterile PBS 1X. 1X Laemli buffer (Boiling Mixture) was added to the well. Cells were scraped thoroughly within the buffer and collected into properly labeled eppendorf tubes. Samples were boiled in a dry bath heat block at 95°C for 5 minutes and then chilled on ice. To further lyse the cells and extract expressed proteins, boiled samples were sonicated using a Sonicator with power of 20 mAh, pulse 2 seconds. Samples were boiled again at 98°C for 10 minutes, followed by centrifugation at 10,000 × g for 1 minute using a tabletop centrifugation machine. Finally, samples were chilled on ice, ready to be loaded onto SDS-PAGE.

Gels for the SDS-PAGE were casted as formulas and PAGE cassettes of BioRad was utilized. A protein ladder of BioRad was used as a marker. Processed samples were loaded into wells. The power pack for the SDS- PAGE was set at 200 V, 2 mAh, time for running varied from 45- 50 minutes depending on the percentage of gels were in use.

After the SDS-PAGE, run gels were used for Western Blot transfer. Protein bands from the gels were transferred onto methanol activated PVDF membrane using BioRad Western Blot transfer cassette with the transfer buffer formulated as listed. The power pack was set at 100 V, 2 mAh, 60 minutes. After transfer, the protein side of the membrane was marked and blocked using 5% skim milk in 0.25% Tween 20 PBS overnight at 4°C.

Probing of the membrane started with washing off the milk blocking solution with 0.25% Tween 20 PBS. Primary antibodies at appropriate dilution were applied on the protein side of the membrane for 1 hour, followed by secondary antibodies conjugated with HRP for 1 hour. Finally, the membrane was washed 3 times by 0.25% Tween 20 PBS and was developed using ECL system in dark condition. X-ray film was placed on top of the membrane within the dark cassette and then developed using Kodak film developer.

2.2.6 Biotin labeling, chemical crosslinking and immunoprecipitation

2.2.6.1 Biotinylation of the surface glycoproteins

Either HEp-2 cells were infected by RSV for 20 hours or transfected with suitable plasmids for 18 hours or LLC-MK2 were infected by HMPV for 6 days. Media was removed, the cell monolayer was washed three times with PBS pH 8.0, followed by incubation with 0.5 mg/ml EZ- Link Sulfo-NHS-LC-LC-Biotin (Pierce Biotechnology, USA) solution in PBS pH 8.0 for 1 hour at room temperature. Later, the excess biotin was removed, and the cell monolayer was washed three times in PBS pH 8.0.

Biotin labels the surface proteins by binding to their amine groups, e.g. lysine groups. The cells then either went through chemical crosslinking step if needed or straight away were lysed using ice cold RIPA buffer (1% NP40, 1 mM EDTA, 0.1% SDS, 2 mM PMSF) in PBS at 4°C for 20 minutes, occasionally rocking. Cell lysate was collected, spun for 10 minutes at 4 °C with the speed of $10,000 \times g$. Clear supernatant was collected and used for immunoprecipitation of the proteins of interest.

2.2.6.2 Chemical crosslinking

After the step of Biotinylation step, the cell monolayer was wash three times with PBS pH 8.0. A crosslinking chemical of Dithiobis [Disuccinimidyl propionate] (DSP, Pierce Biotechnology, USA) was made up into DMSO to form a solution with a concentration of 1 mM. The cell monolayer was incubated with a proper DSP concentration for 1 hour at room temperature. After the incubation, excess DSP solution was removed and the cell monolayer was washed with PBS pH 8.0.

2.2.6.3 Immunoprecipitation

Lysate was incubated with the appropriate antibodies overnight at 4°C in a binding buffer (0.5% NP-40, 1 mM EDTA, 0.25% BSA, 2 mM lysine). Later, 30 µl of protein A- Sepharose (Sigma, USA) was added into each reaction and incubated for 90 minutes with slow rotation (70 rpm) at 4°C. Protein A- Sepharose pulled down the antibody- protein complexes of interest. Samples were then centrifuged at $10,000 \times g$ for 10 minutes until a pellet of Protein A- Sepharose- antibody- respective protein was visualized. Pellets were washed three times using a low salt buffer (1X PBS, 1 mM EDTA, 1% Triton X-100) and suspended in 1X Laemmli buffer, then heated at 95°C for 5 minutes. Samples were loaded onto a SDS-PAGE gel followed by the Western Blot transfer step onto PDVF membrane. The membrane was probed with Streptavidin-HRP conjugate (Invitrogen. USA) followed by visualization by using ECL system (Amersham, UK).

2.2.7 Continuous sucrose ultracentrifugation of proteins.

The infected cell monolayer was biotinylated, chemical crosslinked and extracted by RIPA, 2 mM PMSF for 20 minutes at 4°C.

A continuous sucrose gradient was comprised by fractions of 5- 40% sucrose (w/v) in 0.1% Triton-X in PBS. After loading fractions into ultracentrifugation tubes, the gradient was kept at room temperature for 3- 4 hours until interfaces between fractions were merged. Then the gradient was chilled at 4°C until ready for ultracentrifugation step. The ultracentrifugation machine and rotor (P40ST) was pre-chilled at 4°C before the run started. Right before the ultracentrifuge ran, extracted

soluble protein lysate was loaded onto the continuous gradient, the tubes within ultracentrifuge buckets were well balanced by weight adjustment. The tubes were properly inserted into the buckets, the buckets were balanced within the rotor inside the machine, the machine vacuum was set at proper level before actual run started. The ultracentrifugation was set at $165,000 \times g$ for 20 hours, temperature at 4°C .

After the ultracentrifugation, protein fractions were harvested by 1 ml each from the top of the tube to the bottom. These samples can be used in the Western Blot and the Immunoprecipitation to study the proteins' migration and protein in interaction complexes throughout the continuous gradient.

2.2.8 RSV purification

Infected materials were kept at -80 (from at least 5 T175 flasks) and then thawed quickly and clarified twice by centrifugation at $5,000 \times g$ for 15 minutes in order to refrain any cell debris macro elements. Clarified supernatant was transferred into a sterile conical flask with a magnetic stirrer. 50% PEG-6000 (w/v) was added drop by drop into the supernatant until the total mixture had a concentration of PEG-6000 at 10%. The mixture was stirred at a low speed in 4°C for 1 hour. After the precipitation by PEG-6000, the mixture was poured into conical tubes and centrifuged at $5,000 \times g$ for 15 minutes. A white pellet was observed and this was well resuspended in 20% sucrose (w/v) in Hank's buffer saline (HBSS). The solution was then load onto a sucrose cushion comprised of 30% (w/v) sucrose in HBSS using P28S tubes and P28ST rotor for ultracentrifugation at a speed of $51,000 \times g$ for 1 hour. After the sucrose cushion step, a pellet was expected which was resuspended in 20% (w/v) sucrose in HBSS. The solution was loaded onto a discontinuous gradient comprised of 20, 35, 45 and 60% (w/v) sucrose in HBSS. The tube in use was P40 in utilization of P40ST ultracentrifugation rotor. The machine was set at $165,000 \times g$ for 3 hours. After this step, under a focus light, 3 visible bands of virus were detected. The opalescent band between fractions of 35% and 45% was the active virus band that was collected for further activity of the Western Blot analysis or virus activity recovery by diluting out the sucrose.

2.2.9 RSV morphology analysis in HAE cells

HAE cells are primary cells derived from the upper airway epithelial cells. By culturing these in 3D using a transwell supporter, HAE cells are able to polarize and show their activity *in vitro*.

Well-differentiated HAE cells with fully ciliated structures in the insert part of the transwell are washed gently with warm sterile PBS once. Growth media in the basal part was used without any chemicals to assist differentiation. The virus inoculum volume of 100 μ l was added onto the insert part of the transwell. The virus was incubated within the insert for 3 hours at 33°C. After that, the virus inoculum was removed, and the cells were washed once again with sterile PBS. The transwell was put back into the media containing differentiate chemicals at basal part. At the preferred time points, the cells were fixed at both the apical and the basal parts with 4% PFA in PBS for IF work or harvested within 1X Laemli buffer for Western blot analysis.

2.2.10 Immunofluorescence assay and immunofluorescent microscopy

Cells were seeded onto glass coverslips (12 mm diameter) in 24 well plate overnight. The infection with the appropriate MOI were performed as described above. At certain time points of interest, the cells were washed once with PBS then fixed either with 4% PFA in PBS for 20 minutes or with ice cold Methanol: Acetone (1:1) for 10 minutes. Cells were then permeabilized by 0.1% Saponin in PBS for 20 minutes or 0.1% Triton-X100 in PBS for 10 minutes at room temperature. Cells were washed three times afterwards and ready for antibodies labeling. The appropriate primary antibodies were incubated with the cells for 1 hour followed by the secondary antibodies for another 1 hour at room temperature. Cells were washed with PBS, and excess PBS was removed by tipping on tissue and mounted on glass slide using glycerol-based mounting media (Dakocytomation Fluorescence Mounting Media, Dako, USA). The edges were sealed by transparent nail polish. Samples then were examined using fluorescent microscope using objectives of 20X, 40X or 100X (Nikon Eclipse 80i, Japan). QCapture Pro software (QImaging, USA) was used to capture images.

2.2.11 Light microscopy

Cell cultures were observed and analyzed using a standard inverted light microscope (Leica, Germany). An attached digital camera (Olympus, Japan) was used to take pictures through 10X or 20X objective.

2.2.12 Confocal microscopy

Samples prepared for the immunofluorescent microscopy were further analyzed by using a Zeiss 510 laser scanning confocal microscope (Carl Zeiss GmbH, Germany). Images of different focal planes were captured based on observation using objective of 63X.

CHAPTER 3. MORPHOLOGY AND TRANSMISSION IN MAMMALIAN CELL LINES

Permissive cell lines (HEp-2, A549 and MDCK) are suitable for RSV propagation as they help produce high titer and reproducible infectious progenitor RSVs. However, there have been no detailed studies of RSV transmission to answer questions about the nature of the virus transmission e.g. how progeny viruses transmit from one cell to another, either by a direct cell-to-cell mode or by virus release into the culture environment; and how RSV infection impacts on host cells; cell death by RSV infection is triggered by which cellular pathways, etc. In this chapter, we address these questions to study RSV with regards to the aspects of viral morphological and transmission. This is done comprehensively, by using a very low MOI infection of 0.0002 which enabled us to observe multiple infection cycles of the virus from 1 dpi to 5 dpi. Low MOI infection allowed us to start the infection with fewer Defect Interfering particles (DI) presenting within the virus inoculum, hence the effects on the viral morphogenesis and transmission of these DIs would be minimized. In a clinical context, it is observed that viruses transmitted by direct cell-to-cell contact would have advantages over a predominantly cell-free virus state for example evasion of the host immune response by HIV-1 during cell-to-cell transmission via the immune synapse [34]. Applying low MOI infection helps us mimic better the infection transmission events *in vitro*. RSV transmission can be divided into 2 phases: the first phase of cell-to-cell transmission (1-3 dpi) starting with singular host cells invaded by the virus and transmitted into surrounding cells forming syncytia, and the second phase (the late infection 4-5dpi) of infected syncytia exhibiting serious cell membrane integrity damage and leading to a massive release of progeny viruses into the culture media. Previous studies supported the interaction of viral glycoproteins, for examples: G and F protein, G and SH [9, 56]. To further analyze these interactions, we conducted experiments involving recombinant viruses (g224RSV-FSG - wildtype green RSV; g224RSV-FS - G gene deleted virus) to understand how F or SH expression levels would be affected and how RSV viral morphology and transmission would be altered in scenarios of G gene deletion.

3.1 RSV morphology and transmission in mammalian cell lines

3.1.1 RSV morphology and transmission in non-polarized cells- HEP-2, A549 and MDCK

HEp-2 is a known permissive cell line to RSV [57]. Previous studies indicated RSV morphology as filamentous particles which bud on the surface of the infected cells. However, the detailed mechanism of RSV transmission is less well known. In this study, we monitored the RSV infection progress from a very low starting MOI of 0.0002 through a period of time from 1 day post infection to 5 days post infection using analytical methods of bright-field microscopy, immunofluorescent microscopy, cytotoxicity assay to examine host cellular membrane integrity and DNA fragmentation during the infection course. The results observed in HEp-2 had also been used to compare with that of the experiments with similar settings on A549 and MDCK cells.

3.1.1.1 RSV morphology in HEp-2 cells

There is evidence suggesting the virus filament formation is essential for transmission of the virus [11]. This process tightly correlates to lipid-raft microdomains and actin network of the host cell [7-9]. Our study suggested that infectious viral particles mostly associate with host cells, there is just a small fraction of virus particles presented as cell-free form which exhibit pleomorphic morphologies. These particles' sizes range from 0.1 μm to 1 μm in diameter.

HEp-2 cell monolayers were infected with RSV low MOI of 0.01, at 30 hpi, cells were fixed, stained and the distribution of individual viral structural proteins (G, F, M and SH) were examined. This enabled us to visualize the filamentous structures of F and G as viral filaments [Figure 12A]. Although we observed the prominent anti-SH Golgi-type staining pattern we also noticed a filamentous anti-F and anti-SH co-staining pattern in [Figure 12B]. This confirmed the recruitment of the SH protein into the virus filaments.

Figure 12 Distribution of the F, G and SH virus glycoproteins in the infected cell clusters. HEp2 cell monolayers were infected with RSV using infection moi of 0.01 and 30 hpi the cells were fixed and stained using either (A) anti-G and anti-F or (B) anti-SH and anti-F. B(ii) is an enlarged image from (B) (i). The stained cells were viewed using confocal microscopy at an optical plane that allowed imaging of the virus filaments. The virus filaments (white arrow) and the typical Golgi staining in the SH stained cells (*) are indicated. Figure and figure legends taken from publication [34].

In another setting, RSV morphological studies utilizing single infection cycle (from 4 hpi to 16 hpi with time interval of 4 hours) were performed. HEp-2 cells on coverslips were infected by RSV at MOI 0.5, at 4 hpi, 8 hpi, 12 hpi, 16 hpi and the cells were fixed and co-stained by anti G and anti P and examined by fluorescent microscopy as shown in [Figure 13]. As early as 4 hpi, no staining patterns were observed at either anti G or anti P stained channels. At 8 hpi, the whole infected cell was detected by antibody against P, and tiny inclusion bodies were shown. This observation is consistent with previous studies confirming that as soon as 8 hpi, inclusion bodies were formed as an indicator of RSV infection [74].

Figure 13 Study of RSV morphology in a single infection cycle at time points of 4 hpi, 8 hpi, 12 hpi, 16 hpi. The RSV single infection cycle on HEP-2 cells was observed at 4 hpi, 8 hpi, 12 hpi and 16 hpi by immunofluorescent co-staining using anti G to detect viral filaments with anti P to detect major staining in clusion bodies. **A.** Mock-infected as negative control showed no staining pattern for both P and G indicating healthy non infected cells. **B.** Infected cells were fixed at 4 hpi and co-stained by P and G, no specific staining were observe. **C.** At 8 hpi, anti P staining showed mild staining of the whole infected cell with small inclusion bodies (long white arrows) while anti G staining remained undetected. **D.** At 12 hpi, infected cells were strongly stained by anti P showing dominant larger inclusion bodies (long white arrows) comparing to earlier time points while anti G stained midly the whole infected cells with no specific filamentous structure. **E, F.** At 16 hpi, infected cells were visualized in both anti G and anti P staining. Virus filaments were observed from G staining (short white arrows). In P staining, beside strongly detected feature of inclusion bodies (long white arrows), small and short filaments also were detected.

However, with anti G, at either 8 hpi or 12 hpi, we failed to detect any specific filamentous staining pattern. This suggests that during early infection, despise the sensitivity of the antibodies, G protein could be expressed later than P protein. At 12 hpi, the P staining pattern was easier to recognize comparing to earlier time points, the inclusion body structures were larger. From 16 hpi, filamentous staining pattern of G was detected, at later time points of 18 and 24 hpi, we also noticed the elongation of these filamentous structures. At time point of 16 hpi, beside strongly detected feature of inclusion bodies, small and short filaments also were detected in P staining, which implied the export of P from inclusion bodies into viral filaments as a mode of virus assembly in preparation of virus export into either the outer environment or transmit into the surrounding host cells.

3.1.1.2 RSV transmission in mammalian cell lines

In this study, a low MOI infection of RSV at 0.0002 was carried out on 90% confluent HEp-2 monolayer. Infection was observed over a period of 1- 5 days. At 1 day post infection, under bright-field microscope, no visible change of the infected monolayer was observed, immunostaining with mouse anti-RSV followed by anti-mouse Alexa 488 showed individual infected cells (from 5 to 8 cells) suggesting efficient primary infection. It was noted that, no agarose was overlaid on the monolayer, hence the virus was allowed to spread and this spread resulted in syncytium formation which were observed as distinct individual plaques from 2 days post infection to 3 days post infection. By 2 days post infection, using bright- field visualized small plaques were observed in [Figure 14a], and this was confirmed by immunofluorescent staining data. There were similar numbers of plaque to the single infected cells observed on 1 dpi. Each plaque contained approximately 25 ± 5 cells. By 3 days post infection, bigger sized plaques (> 100 cells) but fewer numbers of them compared to 2 days post infection were noted. Cytopathic effects presented suggesting morphological changes within the cell monolayer. These observations in [Figure 14b] highlighted the event of viral localized transmission from 1 day to 3 days post infection. This suggested strictly cell-to-cell transmission of the virus during this period, otherwise we would expect the virus to be released and freely spread among the monolayer which could result in larger numbers of infected, randomly distributed cells. At 4 and 5 days post infection, the infection had spread across the monolayer, in some areas the dead cells detached leaving gaps within the cell monolayer.

In a similar analysis, A549 cells were infected by RSV. As indicated in [Figure 15], at time points of 1, 2 and 3 dpi the cells were stained with anti-RSV and examined using immunofluorescence microscopy. At 1 dpi, single infect cells were observed similar to what we observed in HEp-2. At 2 and 3 dpi, infected clusters in A549 were observed but the size of plaques tended to be smaller than what we observed in HEp-2. This suggested localized transmission not only occurred in HEp-2 but consistently showed in A549 cells.

MDCK cells were grown on either glass coverslip or on Transwell™ membranes. Low multiplicity of infection of RSV was used to infect cells on the glass and

transmembrane. At time points of 1-5 dpi, the cells were fixed and IF staining were conducted using anti-RSV to detect infection progress. We also noticed from 1-3 dpi, in both cases, the transmission was tended to be localized within the infected cluster, at 4 dpi and 5 dpi, especially on the coverslip, these cells were extensively infected. Cells on the transmembrane showed a smaller size of infected plaques compared to the ones grown on coverslips as shown in [Figure 16].

Using different cell lines (HEp-2, A549, MDCK), the microscopic examination described above was consistent with the occurrence of two phases of virus infection in the low MOI infection mode. At an early stage (up to 3 dpi) the formation of the infected cell clusters was observed that was consistent with localised cell-to-cell virus transmission. The presence of the virus filaments suggested that this localized virus transmission occurs via these virus-induced structures. Interestingly, this localized virus transmission was not associated with the obvious signs of RSV-induced CPE in the cell monolayer. Slight morphological changes in the cell monolayer was observed at 2 dpi and became apparent at 3 dpi, which were consistent with the start of virus-induced CPE. These changes in the cell monolayer became more extensive at 4 and 5 dpi, with extensive CPE and cell detachment being evident. These observations suggested that although the process of virus transmission eventually gives rise to syncytial formation, the processes of virus transmission and syncytial formation are two distinct processes.

Figure 14 Localized RSV transmission occurs within the HEP2 cell monolayers. HEP2 cell monolayers were either mock-infected or RSV-infected using a multiplicity of infection (moi) of 0.0002. a At between 1 and 5 days post-infection (dpi) the monolayers in the tissue culture dish were fixed using 3 % glutaraldehyde and viewed using an inverted light microscope (objective x4 magnification). The virus-induced morphological changes in the monolayer are highlighted (black arrows). b In a parallel analysis the virus-infected cells were stained using anti-RSV and anti-mouse IgG conjugated to Alexa 488 at (i) 1 dpi, (ii) 2 dpi, (iii) 3 dpi and (iv) 4 dpi. The stained cells were then viewed using fluorescence microscopy (IF) and by bright-field (BF) microscopy (objective x20 magnification). The infected cell cluster (open white box), virus-induced morphological changes in the monolayer (open black box) and region of the monolayer cleared of cells (*) are highlighted. Figure and figure legends are taken from publication [34].

Figure 15 Localized RSV transmission occurs within A549 cell monolayer which is similar to that in HEp-2 cell monolayer. A549 and HEp-2 cell monolayers were infected with RSV using a multiplicity of infection (moi) of 0.0002 and at between 1 and 3 days post-infection (dpi) the virus-infected cells were stained using anti-RSV and anti-mouse IgG conjugated to Alexa 488 and viewed using fluorescence microscopy (objective x20 magnification). The infected cell clusters in the A549 (white arrows) and HEp-2 (open white box) cell monolayers are highlighted. Figure and figure legends are taken from publication [34].

Figure 16 Localized virus transmission occurs within RSV-infected MDCK cell monolayers. MDCK cell monolayers were prepared either on **(A)** glass substrate or **(B)** on Transwell™ inserts and infected with RSV using a multiplicity of infection (moi) of 0.0002. At between 1 and 5 days post-infection (dpi) the virus-infected cells were stained using anti- RSV and anti- mouse IgG conjugated to Alexa 488 and viewed using fluorescence microscopy (IF) (objective x20 magnification). The infected cell clusters (open white box), syncytia (black asterisk) and zones of clearing in the cell monolayer (white asterisk) are highlighted. Figure and figure legends are taken from publication [34].

Figure 17 RSV-infection induces localized changes in the F-actin distribution in HEp-2 cell monolayers. A HEp-2 cell monolayers were infected with RSV using a multiplicity of infection of 0.0002 and at between 1 and 5 days post-infection (dpi) the cells were co- stained using anti- G and Phalloidin-FITC (to detect F-actin). The mock-infected sample was stained at 5 dpi. The stained cell monolayers were viewed by immunofluorescence microscopy (objective X20 magnification). Individual infected cells at 1 dpi (white arrows), infected cell clusters (open white box), cell detachment (*) and syncytia (**) are indicated. b Virus-induced increased Phalloidin-FITC staining at the intercellular junctions. Mock and virus-infected cells at 2 dpi were stained with anti-G and Phalloidin-FITC. The increased Phalloidin-FITC staining in the virus infected cells is highlighted (white arrows). The stained cell monolayers were viewed by immunofluorescence microscopy (objective 100X magnification). Figure and figure legends are taken from publication [34].

Previous studies suggested that F-actin is involved in RSV filament formation and is a component itself in RSV particles [11, 37, 44]. To confirm that, we conducted an experiment of low MOI RSV infection as described above, at time points of 1-5 dpi, cell monolayers were fixed and co-stained with anti-G and Phalloidin-FITC (F-actin). At magnification of 20X, the infected cell clusters were visible from 2 dpi, we noted that boundaries of these cell clusters or inter-cellular contacts of the infected cells to non-infected cells were in close proximity as shown in [Figure 17b]. This suggested changes had occurred at the cell junction points during infection. From 3 dpi, cells within the same infected clustered seemed to merge to form a bigger infected cell clusters, and at 4 dpi, 5 dpi, the whole cell monolayer had large Phalloidin-FITC stained

syncytia indicating further intercellular membrane fusion and syncytia formation at the later stages of infection in [Figure 17a]. This suggested intercellular membrane fusion between the individual cells had occurred, which was consistent with the start of syncytia formation. However, the same IF procedures applied on mock-infected cells at 5 dpi clearly showed defined stained intercellular junctions stating no changes in the F-actin network.

Cellular actin is believed to be regulated via rho GTPase proteins [11, 37, 80]. Rac-1 kinase was reported to play role in RSV morphogenesis, hence we examined this to see if rac-1 also plays a role in localized transmission of RSV in this context. Cells were infected with RSV using a moi of 0.1, and at 24 hpi the cells were stained using anti- rac-1 and anti-F and examined using confocal microscopy as shown in [Figure 18a, b]. Rac-1 was identified to be co-localised within the virus filament structure. To further confirm the presence of Rac-1 in RSV viral particle, we proceeded with purification of the RSV particle as described previously [75]. Basically, virus particle was released from the host cells and run onto a continuous sucrose gradient centrifugation comprised of (20% (w/v) sucrose, 35% (w/v) sucrose, 45% (w/v) sucrose and 60% (w/v) sucrose). The material at the 20-35% (w/v) sucrose (band-1 fraction), 35-45% (w/v) sucrose (band-2 fraction) and 45-60% (w/v) sucrose (band-3 fraction) interfaces were harvested. The band-2 was identified as containing the infectious RSV particles once was used to challenge the healthy HEp-2 cells. Immunoblotting this fraction showed the presence of Rac-1. [Figure 18c] again confirmed the presence of Rac-1 protein within viral filament.

It was reported previously that NSC23766 has a function in inhibiting virus filament formation [37]. In this study, we again used NSC23766 to determine if it played a role in localized transmission of RSV. RSV infected HEp-2 cell monolayers were treated with NSC23766 at 5 hpi. To have a control for the experiment, we also conducted an infection with NSC23766 non treated cells. At 30 hpi, cells were fixed and stained with anti- RSV to determine infection state. Non-treated cells showed a larger cluster size of (14 ± 3 cells per cluster) shown in [Figure 18e, d] comparing to treated cells only indicated singularly infected cells. The data provided evidence of NSC23766's ability to disrupt localized transmission of RSV on HEp-2.

Figure 18 The rac-1 and rho A kinases are required for localised cell-to-cell transmission in the HEp-2 cell monolayer. **a** Mock and RSV-infected cells (multiplicity of infection (moi) of 0.1) were stained using anti- F and anti- rac-1 at 24 hpi and imaged by confocal microscopy. Only the merged image is shown in the mock- infected cells. **b** Co-stained cells showing virus filaments bridging two cells. In all cases the virus filaments are highlighted (white arrows). **c** Virus particles were dissociated from infected cells and infectious virus particles isolated using discontinuous sucrose gradient centrifugation. **c** (i) A representative gradient showing the locations of the fractions at the 20-35 % sucrose (band-1), the 35-45 % sucrose (band-2) and 45- 55% sucrose (band-3) interfaces. (ii) Each fraction was examined by immunoblotting using anti- rac-1. A protein band corresponding in size to the rac-1 protein is highlighted (black arrow). **d** and **e** The rac-1 inhibitor NSC23766 prevents virus filament formation and virus transmission. **d** RSV-infected HEp-2 cells (moi of 0.1) were either non-treated or NSC23766-treated and the cells stained using anti-G. The virus filaments in non-treated (white arrows) and punctuate staining pattern in NSC23766-treated cells (*) are highlighted. In each case (ii) is an enlarged image of the region highlighted by the white box in (i). **e** rac-1 activity is required for cell-to-cell transmission. HEp2 cell monolayer were infected using a moi of 0.0002 and at 5 hpi the cells were either non-treated or NSC23766 treated. At 18 hpi the inhibitor was either removed (washout) or maintained. At 36 hpi the cells were fixed and stained using anti-RSV. The cells were imaged using fluorescence microscopy (objective x20 magnification). The infected cell clusters in the NT cells (open white box) and smaller clusters in the NSC23766- treated cells (white arrows) are indicated. **f** HEp-2 cell monolayers were infected with RSV using a moi of 0.0002 and at 18 hpi the cells were either non- treated (NT) or treated with Y-27632 (YT). At 48 hpi the cells were fixed and stained using anti- RSV and imaged using immunofluorescence microscopy (objective x20 magnification). The infected cell clusters in the NT cells (open white box) and smaller clusters in the YT cells (white arrows) are indicated. Figure and figure legends are taken from publication [34].

Based on the observation of the 2 phases mode in RSV transmission: from 1-3 dpi, transmission was mostly via cell-to-cell mode while at 4 dpi, and 5 dpi, the infection progress escalated quickly and destroyed the cell monolayer which leads us to question how efficient these transmission modes are and how to quantify infectious viral particles produced by the two modes. Hence, we decided to harvest 2 different fractions of virus from the infected monolayer over time (1- 5 dpi). The supernatants during culture (1- 5 dpi) (called Cell Free fraction- CF) were collected independently with the infected cell monolayer (called Cell Associated fraction- CA, was scraped thoroughly within fresh media). These fractions were used to infect a new HEp-2 cell monolayer to determine the titer of RSV secreted to media and if the RSV remained within the cell monolayer over different time points. In [Figure 19a], results showed that CF- virus was only present from 3 dpi, meaning there was no virus particles were released from the monolayer at an earlier infection time point. Meanwhile, presence of RSV within the cell monolayer was detected from 1 dpi and increased consistently over time. This was in agreement with the microscopic observation above.

In the same setting, we further used lactate dehydrogenase (LDH) release cytotoxicity assay to measure the levels of LDH released from cells in correlation to the infection progress [Figure 19b]. Supernatants from both Mock-infected and infected cells from 1 dpi to 5 dpi were collected to accommodate this experiment. At an early infection time of 1 dpi and 2 dpi, we observed the similar LDH levels in the tissue culture supernatant of both mock-infected and RSV-infected cells. From 3 dpi we noticed an increase of 10% in LDH levels in virus-infected cell materials in comparison to mock-infected cell materials. At later time points of 4 dpi and 5 dpi, LDH levels in the tissue culture medium of virus-infected cells showed strikingly higher to that in the mock-infected cells.

Figure 19 The presence of cell-free virus particles occurs late in infection and correlates with increased cell permeability and cytotoxicity. HEP2 cell monolayers were infected with RSV using a multiplicity of infection of 0.0002 and (a) between 1 and 5 days post-infection (dpi) the cell- associated virus infectivity (solid line) and the cell- free virus infectivity (broken line) in the tissue culture supernatant was measured by plaque assay. Average values (duplicates) for the infectivity measurements are shown and for all times the standard error for each of the infectivity measurements was $\leq 10\%$. Representative data obtained from one experiment is shown. b Lactate dehydrogenase (LDH) release from RSV-infected HEP-2 cells. HEP-2 cell monolayers were either mock- infected (broken line) or RSV- infected (solid line) using a multiplicity of infection of 0.0002 and at between 1 and 5 dpi the levels of LDH was measured in the tissue culture supernatant. The values are average values of triplicate measurements at each time point. Representative data from one experiment is shown. Figure and figure legends are taken from publication [34].

Trypan Blue (TB) is a vital stain which can mark out dead cells, where the cell membrane becomes permeable to TB uptake. Hence, we utilized TB to visualize damaged cell membrane areas due to infection [Figure 20]. At 1 and 2 dpi, data showed

no significant staining of the cell monolayer by TB. At 3 dpi, we noticed that infected plaques were visualized. At 4 dpi, 5 dpi, the same findings were noted. Interestingly, these plaques had their centers (or syncytia) were more intensively TB-colored than the periphery of infected plaques. The same procedures were done on Mock-infected cell monolayer and we failed to detect any specific staining pattern by TB. This data together with the LDH assay data, and the appearance of CF-virus at 3 dpi indicated cell membrane changes correlates with the RSV infection progress and transmission over time.

Figure 20 Trypan blue staining is detected in the HEP-2 cell monolayers. HEP-2 cell monolayers were either mock- infected or RSV- infected using an multiplicity of infection of 0.0002 and (a- f) at between 1 and 5 days post- infection (dpi) the monolayers were stained using trypan blue and imaged using an inverted light microscope (objective x10 magnification). The regions of the cell monolayer showing trypan blue staining is highlighted (black arrows). In plate (c) selected regions in the monolayer (indicated by open white box) are enlarged in the inset. Plate (e) shows enlarged images from the region indicated in plate (d) (indicated by open white box). Figure and figure are legends taken from publication [34].

3.1.2 Role of G protein in RSV transmission

Currently, there are works studying the importance of RSV glycoproteins, including G, F and SH, in the virus binding, replication *in vitro* and *in vivo*. Recent findings suggested that in recombinant RSV, if F gene is deleted, RSV loses its ability to infect and replicate in both *vitro* and *vivo*, on the other hand, RSV with G gene deleted or SH deleted still can replicate *in vitro* but its ability to replicate *in vivo* is reduced [96]. In our previous works, we observed that the protein complex of the F and G protein consistently exist on the surface of infected HEp2 cells and on ciliated epithelial cells

[34, 44]; which raised questions of whether G and F protein form a single complex in the virus envelope and if they do, how do G and F interact in mediating virus entry. To address this hypothesis, we extend our previous work to examine whether the protein complex involving F and G proteins exists within the envelope of the mature virus particles and how this complex facilitates F protein in binding process of the RSV particle to host cell membrane.

Figure 21 Interaction of the F and G proteins on the surface of virus filaments. Hep-2 cells were infected by a multiplicity of infection of 0.0001 and the cells examined at 48 hpi. (A) Cells co-stained with anti-F and anti-G and the cells were examined by confocal microscopy (i) is a low magnification image and (ii) is an enlarged image taken from the region demarcated (open white square) in (i). (B) The cells were co-stained with anti-F and anti-G and the cells stained using anti-G and the proximity ligation assay (PLA) performed (i) an image of the cell top and (ii) cell periphery is shown. In each case the PLA signal is shown as multiple intense bright spots. (C) Cells were stained using either anti-G or anti-F and the PLA performed. While staining with each antibody can be seen no PLA signal is recorded (D) and (E). The cells were co-stained with anti-F and anti-G and the cells stained using anti-G and the PLA performed. The co-stained cells were examined by confocal microscopy at a focal plane that allowed the (D) cell periphery and (E) virus filaments to be visualized. In each case (ii) is an enlarged image from the region demarcated (open white square) in (i).

From previous studies, mature RSV particles were observed at apical cell surface as filamentous structures with a size ranging from 150- 250 nm in diameter of spherical form to 10 μ m long in filamentous form [89]. These observations were confirmed using not only various mammalian cell lines (HEp-2, LLC-MK2, A549, MDCK) but also in human derived primary nasal epithelial cells (NE).

To examine F and G proteins co-existence on the surface of virus filaments, HEp-2 cells were infected with MOI 0.0001, at 48 hpi, cells were co-stained with anti-F and anti-G and [Figure 21A, B] showed co-localisation of the filamentous stained F and G on the surface of the RSV infected cell, Proximity ligation assay (PLA) was utilized to detect F and G interaction in situ. In the same experimental setting, infected HEp-2 cells at 48 hpi were incubated with both primary antibodies against F and G, and PLA probes conjugated with DNA probes were used as secondary antibodies. After the incubation steps, PLA probes in close proximity with DNA strands were amplified by a polymerase reaction to produce bright red dots which can be observed at Cy3 channel of fluorescent microscope. In [Figure 21 E, G] a staining pattern together with PLA result of bright red dots distributed where both antigens presented indicating strong interaction levels between the two proteins; while [Figure 21C] showed negative control results of infected cells where only either anti F or anti G was applied. The visualization of [Figure 21D] the cell periphery and [Figure 21E] cell top of the stained cells by confocal microscopy at optical planes showed the presence of the anti-G stained virus filaments on the surface of infected cells and the PLA staining on the virus filaments. Hence, the PLA assay indicated an interaction between the F and G proteins on the surface of the virus filaments.

We also conducted transfection experiments in order to examine the expression of recombinant RSV F, G proteins and HMPV F, G proteins. HEp-2 cells at confluence of 60% were transfected singularly with RSV pCAGGs- G or RSV pCAGGs- F-flag to examine the expression by Fluorescent microscopy and Confocal microscopy. [Figure 22A, B] showed that RSV G recombinant protein was expressed as long filamentous structures which is similar to the G-stained feature in the RSV infected cells shown at [Figure 22A (i)]. Recombinant F however was detected as the smaller finer filamentous structures compared to G [Figure 22B]. At [Figure 22C], co-transfected RSV F and G cells were stained revealing F and G protein expression at close proximity as distinct

filamentous structures at the host cell surface. This also confirmed the co-existence of F and G in these filament structures at the host cell surface. To further validate F and G co-localisation and interaction, we conducted confocal microscopy analysis with these samples. In [Figure 22C], F and G co-expression was observed abundantly on the host cell surface as long filamentous structures while in [Figure 22D], we observed within the cell and found that F and G co-localisation only can be found at the host cell peripheries. These observations of RSV F, G recombinant protein expression again confirmed F and G were expressed and targeted to the same location at host cell membrane.

Figure 22 Interactions of RSV G and F proteins on HEp-2 cell surface by recombinant protein expression. HEp-2 cells at confluence of 60% were transfected singularly with either RSV pCAGGs- G plasmid (A (i)) or (A (ii)) RSV pCAGGs- F Flag plasmid; in the same set up, HEp-2 cells were also co-transfected with RSV (pCAGGs- G and pCAGGs- F flag). At 14 hpt, cells were fixed and stained with anti G to detect G expression and/or anti-Flag to detect F expression. (B) co- transfection of G and F was examined using fluorescent microscopy where both G and F were expressed as distinguish filamentous structures. (C) co- expression of F and G on host cell surface was examined by confocal microscopy. White arrow heads highlighted areas where have filamentous structures stained by either anti Flag or G indicating co- localisation of F and G on filamentous structures. Scale bar of 10 μm is included.

To examine RSV F and G recombinant protein interaction, pCAGGs- G and pCAGGs- F flag were co-transfected in HEp-2, at 18 hpt, and cells were treated with biotin in pH 8.0 PBS to surface label them. Cells were lysed using ice cold RIPA to extract the protein content. The lysates were pulled down with either anti-F or anti-G,

the immunoprecipitated products were then used to run a Western Blot and probed by Streptavidin-HRP. Results are indicated in [Figure 23] showing anti-F pulled down G protein, and anti-G pulled down F protein indicating the interaction of the two proteins or the two proteins existed at same location in filamentous structures on surface of the host cells. However, anti-G pulled down more F protein than anti-F does with G protein which could imply that most of the recombinant F protein is associated with the recombinant G protein but not the other way around. This data supported our previous observation of F and G proteins, in either recombinant expression or infection studies, are targeted into the same structure of filaments on the host cell surface indicating tight interaction of the two proteins during viral particles assembly.

Recombinant expression studies of HMPV F, G proteins were also conducted in LLC-MK2 cells, where co-transfection (G-flag and F-myc) was conducted for 14 hpt. Cells then were fixed and stained to detect the proteins' expression. In [Figure 24], we observed expression of G with F in close proximity at the filamentous structures on the surface of the host cells which suggested that these proteins also co-localized and interacted closely in structure and function of HMPV-like-particles.

Figure 23 HEp-2 cells were co-transfected by RSV pCAGGs- G and pCAGGs- F flag followed by biotinylation and immunoprecipitation using anti F (1,2) or anti G (3,4). 1 and 3 using mock cell lysate (transfected with blank pCAGGs); 2 and 4 using cell lysate containing F and G co-transfected. In the cell lysate where F antibody was used to pull down, G was observed (black arrow). In the cell lysate where G antibody was used to pull down, F protein was observed (white arrow). This result confirmed recombinant expressed biotin labeled F and G interacted on HEp-2 cells surface.

Figure 24 LLC-MK2 cells were co- transfected with HMPV pCAGGs- G flag and pCAGGs- F myc At 14 hpt, cells were fixed and stained with appropriate antibodies to detect recombinant protein expression. Fluorescent microscopy (A) and confocal microscopy (B) imaging analysis was conducted to examine co- expression of the two proteins. (i) was stained by anti- flag to detect G expression; (ii) were stained by anti- myc to detect F expression. (iii) super- imposed by confocal imaging analysis software showing co-localisation of F and G in filamentous HMPV virus-like-particle. White arrow heads indicated small stained filamentous structures where these recombinant proteins were both found in co- transfection experiments. Scale bar of 10 μ m is indicated.

Based on the above results, F and G exists in close proximity, with co-localisation and interaction of the two proteins are confirmed by various imaging approaches. This raised an interesting question of how G- deletion would affect F's functions, hence how RSV morphology and transmission would be altered. To examine these issues, we conducted recombinant RSV (including green wildtype RSV, green- G gene deleted RSV kindly provide by Mark Peeples) to study and gain knowledge of RSV infection in the scenario where G is absent. In this sector, we characterized and compared the two different viruses in different aspects e.g. viral morphology, growth rate, individual viral proteins expression.

Figure 25 The presence of the G protein is required for efficient cell-to-cell transmission. (A) Hep2 cells were infected with either g224RSV-FSG or g224RSV-FS using a multiplicity of infection of 0.0001 and at between 1 and 5 days post infection the cells were stained with anti-F and the anti-F and EGFP staining patterns imaged using immunofluorescence microscopy. The infected cell clusters are indicated (open white boxes). (B) The cells were harvested at between 1 and 4 dpi and examined by immunoblotting using anti-G, anti-F and anti-M2. Protein bands corresponding in size to the respective virus proteins are indicated (C) and (D). The anti-F stained Hep-2 cells were infected with either (C) g224RSV-FSG or (D) g224RSV-FS examined by confocal microscopy to detect the presence of virus filaments. (C.ii) is an enlarged image from the region demarcated (open white square) in (C.i). (D.i) is an image of g224RSV-FS infected cells taken at the same camera settings as in (C.i) and (D.ii) is the same image in (D.i) but was recorded using a reduced camera setting that allows the virus filaments to be more clearly imaged. (D.iii) is an enlarged image from the region demarcated (open white square) in (D.ii). The virus filaments are highlighted (white arrows).

Hep-2 cells were infected and cell lysates were harvested between 1 dpi to 4 dpi, the western blot analyses were done using anti F, anti G, anti SH and anti M2 [Figure 25B]. Protein bands corresponding in size to the respective viral proteins were indicated. Immunoblotting with anti-G confirmed the absence of the G protein in g224RSV-FS infected cells, while similar levels of M2-1 and F protein were present in infected materials with both viruses. In G deleted virus infected lysate, we observe a difference

in SH protein level expression, in details: less SHg is shown at 3 dpi and 4 dpi in RSV-FS infected cell lysate compared to RSV-FSG infected lysate at the similar infection time points. In contrast, more SH0 is found in the 4 dpi RSV-FS infected lysate in the RSV-FSG infected lysate at 4 dpi. More studies need to be performed to understand G and SH interaction better. Collectively, this suggested that although the F protein could mediate cell-to-cell transmission, the G protein plays an important role in mediating cell-to-cell the transmission via the virus filaments. We hypothesized that the absence of the G protein could affect the formation of the virus filaments. Therefore, the anti-F stained cells were examined by confocal microscopy to determine if the virus filaments formed in the absence of the G protein. On g224RSV-FSG infected cells the presence of anti-F staining virus filaments was detected, which is similar in appearance to that the RSV A2 wildtype. In contrast fewer but longer and more intensely anti-F stained filamentous structures were detected on RSV-FS infected cells [Figure 25C]. Although there appeared to be subtle changes in the virus filaments in the RSV-FS infected cells, these observations suggested that the presence of the G protein was not required for virus filament formation. To detect the virus filaments clearly on RSV-FS infected cells the sensitivity on the camera setting had to be reduced [Figure 25C ii; iii]. This revealed an accumulation which was consistent with that of the F protein within these virus filaments. After 5 dpi we noted that cells infected with g224RSV-FSG showed extensive cytopathic effects and cell detachment was apparent. We therefore examined the virus titer to determine the level of released virus in cells infected with both recombinant viruses. Cells were infected with g224RSV-FSG or g224RSV-FS using a MOI of 0.0001 and at 5 dpi the virus titer was measured in the tissue culture supernatant. The g224RSV-FSG infected cells showed a virus titer of 4.1×10^4 pfu/ml while the g224RSV-FS infected cells showed a virus titer of 3.2×10^2 pfu/ml. This indicated a 100 fold reduction in levels of infectious virus in cells infected with the g224RSV-FS virus, and this was consistent with the impaired virus transmission by the g224RSV-FS virus.

3.1.3 RSV infection and host cell responses

3.1.3.1 RSV infection and host responses in a single infection cycle (MOI 0.5)

A single infection cycle is defined as follows: from the time the virus is added into the cell culture to around 18 hpi. During this infection time, RSV is able to replicate once, in which its attachment, fusion, and replication activity has certain impacts on the

host cells. In order to evaluate the host cell responses to RSV, we conducted RSV infection at MOI of 0.5 on HEp-2 cell monolayer. Cell lysates were harvested at 4 hpi, 8 hpi, 12 hpi and 18 hpi. A Mock-infected cell monolayer and HEp-2 monolayer infected by UV inactivated RSV were both used as negative controls were harvested at same time points to the infected ones. Each provided advantages over judgement of the host responses in comparison with the RSV-infected cell monolayer, in details: Mock-infected cells that had no interact with RSV particles or RSV viral proteins produced a clean signal from elements which existed within a healthy host cell monolayer while the HEp-2 monolayer infected by UV inactivated RSV (has all RSV viral proteins present e.g. F, G, etc. but only has no vRNA which was destroyed by UV, hence this UV-inactivated RSV lost its replication ability) still had host responses to viral protein attachment to receptors on the membrane surface, and even viral particle fusion into the host cell. Having two negative controls provided baselines to compare host responses to RSV infection to a non-infected healthy host baseline and to a “false” RSV infection where viral and host protein-protein interactions were available but no replication of the viral genome was presented. Cell lysates were immunoblotted with anti- M2-1, [Figure 26A] confirmed successful RSV infection at all time points. We noticed a distinguishably higher level of M2-1 in RSV-infected compared to UV inactivated RSV. The signal caused by UV inactivated RSV could be the product of input virus stuck onto the surface of host cell HEp-2. We continued to examined the lysates with antibodies against STAT-1, JNK, p38MAPK and their phosphorylated forms; I κ B and lastly actin as a loading control [Figure 26B]. STAT-1 levels at all time points regardless of whether they were mock-infected or infected or UV inactivated infected remained at a similar level, however when the blots were probed with anti-pSTAT1, only RSV-infected materials showed an activation at as early as 4 hpi, which peaked at 8 hpi and gradually decreased from 12 hpi. Blots examined by p38MAPK and pp38MAPK probing both showed activations of these protein as soon as 4 hpi and these gradually decreased over time at RSV-infected materials. Interestingly, the materials from UV inactivated RSV infection also showed a transient activation of p38MAPK and pp38MAPK only at 4 hpi and quickly diminished at later infection time points. This combined data suggested the initial interaction of the host cells with viral proteins serving in virus attachment and fusion function at early infection (whether it was RSV particle or UV-inactivated RSV particle) produced an activation upon p38MAPK and pp38MAPK. However, in the case

of the replicative RSV particle invaded HEp-2 host cells, it is possible that vRNA and its activity prolonged the presence of p38MAPK and pp38MAPK up to 12 hpi; in contrast with the case of non-replicative UV inactivated RSV particle where the host cells quickly recognized no activity of vRNA and levels of p38MAPK and pp38MAPK were decreased back to that in the mock-infected cells. Using the same lysate sets, immunoblotting with JNK and pJNK showed no obvious expression of these two proteins under RSV infection at such early infection time points.

Figure 26 Infected HEP-2 cells by RSV MOI of 0.5, UV- inactivated RSV and respective Mock-Infected cells were harvested at 4 hpi, 8 hpi, 12 hpi, 18 hpi. Cell lysates were immunoblotted using M2-1 as a viral marker to confirm infectivity of samples, result showed an increase trend of M2-1 from 4 hpi to 18 hpi indicating virus replication in RSV infected materials while in UV-inactivated infected materials, M2-1 levels remained similar at all time points. Antibodies of STAT-1, JNK, P38MAPK (and their phosphorylated forms), IKB were used to determine host cell responses at such infection time points showing a slight dip in infected lysates comparing to mock lysates and UV-inactivated infected materials. Anti-actin was used as loading control showing levels of sample loaded in each reaction are of similar.

3.1.3.2 RSV infection and host responses in multiple infection cycles (low MOI)

Unlike single infection cycle, in multiple infection cycles, a small amount of virus is put into the cell culture. Over time (one day interval), we can observe the infection progress from a single infected cells to adjacent cells, forming syncytial clusters and virus transmission within the cell monolayer, which cannot be done using a single infection cycle mode.

Figure 27 Virus infection induces cell signaling pathways associated with cell death at 3 dpi. A HEP-2 cell monolayers were either mock- infected (M) or infected with RSV using a multiplicity of infection of 0.0002. At between 1 and 4 days post-infection (dpi) cell lysates were prepared and the presence of the N and M2-1 proteins detected by immunoblotting. Actin is the loading control. b The presence of (i) STAT1 and pSTAT1, (ii) JNK and pJNK and (iii) MAPKp38 and pMAPKp38 were detected in the RSV-infected cell lysates by immunoblotting with the relevant antibody at 1, 2 and 3 dpi. In this case the infected cells were compared with the mock-infected cell lysate harvested at 3 dpi. Figure and figure legends are taken from publication [34].

Immunoblotting experiments were performed using RSV infected HEP-2 cell lysates harvested from 1- 4 dpi. To confirm successful infection, we blotted them with some viral proteins (N and M2-1) [Figure 27a], and the results showed that there was a steady increase of virus materials over time, which peaked at 3 dpi and reduced somewhat at 4 dpi. We continued to examine the lysate with anti-actin as a loading control, and the result revealed a consistent level of cell materials at both Mock-infected cells and Infected ones from 1 to 3 days of infection, but there was a slight dip in the cell material at 4 dpi which can be explained by the cell mass lost due to advanced infection which leads to cell float (this is consistent with observation of gaps in monolayer using bright-field microscopy) and could not be recovered during the harvest process. Moreover, in [Figure 27b] we noted an increase level of STAT1 over infection time (1- 3 dpi), which is the same trend that happened to pSTAT-1 which showed its

activation first at 2 dpi and peaked at 3 dpi. With anti JNK and p38MAPK, we did not see any changes during the infection course, but their phosphorylated form (p-JNK and p-p38MAPK, respectively) only showed their presence at 3 dpi. These observations proved that RSV infection leads to induction of type 1 interferon expression on HEp-2 host cells. Previous studies demonstrated type 1 interferon signaling (JAK/STAT pathway) links to cellular kinase (p38MAPK and JNK signaling), which ultimately leads to cell death. We also conducted infected monolayer IF staining at 2 dpi with STAT-1, JNK, p38MAPK and their phosphorylated form to validate our above mentioned immunoblotting observation. IF results showed at [Figure 28] were consistent with our immunoblotting findings. In Infected cell monolayers, we found STAT-1, pSTAT-1, pp38MAPK, pJNK staining structures co-localized with the viral N structure while there was no staining pattern indicated in IF experiments using p38MAPK and JNK. We also noted no staining features of all antibodies (STAT-1, pSTAT-1, JNK, pJNK, p38MAPK and pp38MAPK) in mock-infected cells.

Based on data from the host responses to an RSV single infection cycle, we noticed a transient activation of p38MAPK and its phosphorylated form pp38MAPK as early as 4 hpi, where the increased levels of p38MAPK and pp38MAPK compared to that in mock-infected cells were maintained until 12 hpi, and then found to decrease back to baseline as in the mock-infected cells at 18 hpi. While our data collected from multiple infection cycles (span from 1 dpi to 3 dpi) showed that p38MAPK levels stayed similar to that of the mock-infected cells and pp38MAPK level was only detectable at 3 dpi. Similarly, pSTAT1 was found to be transiently upregulated as early as 4 hpi, and this level of pSTAT1 was maintained until 12 hpi, followed by a slight dip over time, while in the setting of multiple infection cycles, pSTAT1 level increased between 2 dpi and 3 dpi where RSV replication and transmission are known to thrive within the HEp-2 monolayer based on our IF data in [Figure 14b]. Examining HEp-2 cell host responses due to RSV infection with consideration for both single infection and multiple infection cycle, these research methods enabled us to have insightful information of such transient activation and upregulation of pSTAT1, p38MAPK and their phosphorylated forms according to the infection progress over time. Further experiments are required to help clarify a bigger picture of cellular pathways under RSV infection.

DNA fragmentation in the cell monolayers occurs at the latter stages of RSV infection. By carefully examining the whole infection process from 1- 5 dpi, we were able to correlate the host cell death, host cell membrane integrity to the infection progress over time. Our above immunoblotting data also suggested that the infection triggered cellular signaling pathways (JNK, MAPK) which lead to cell damage and death. Hence, how does the host react to such damage? Would cellular factors also contribute to this death once the damage is irreversible? In other words, is there any correlation of apoptosis to the fate of RSV infected HEp-2 monolayers?

To answer these questions, we used the TUNEL staining assay to detect the presence of DNA fragmentation which is classified as a signature apoptotic feature in the infected cell's nucleus. In the same setting of low MOI RSV infection, from 1 dpi to 5 dpi, TUNEL with anti G co-staining on either mock-infected or infected HEp-2 cell monolayers was applied. At 1-3 dpi, no fragmentation of DNA was detected in the infected monolayer [Figure 29a, b, c] which indicated at early and mediocre infection, no apoptotic events were noticed, in consistency with previous reports of the ability of RSV preventing apoptosis in early infection by a PI3K-dependent signaling pathway.

At 4 and 5 dpi [Figure 29d, e, f] TUNEL signal was detected and accompanied within syncytia structures caused by intensive infection, and co-localized to anti-G staining pattern. We performed the same procedures on mock-infected monolayer and failed to detect any specific TUNEL signal. This data suggested cell death was induced by the virus only happen at late infection.

Figure 28 Distribution of STAT1, pSTAT1, pMAPKp38 and pJNK in the RSV-infected monolayers. HEP2 cell monolayers were mock-infected or infected with RSV using a multiplicity of infection of 0.0002. a and b At 2 days post-infection (dpi) the cells were stained using anti- N and either (a) anti- STAT1 or (b) anti- pSTAT1. The cells were viewed by IF microscopy (objective x20 magnification). The STAT1 and pSTAT1 regions in the cell monolayer are highlighted (white arrow), and the region of the monolayer containing non-infected cells is indicated (*) (c and d) HEP-2 cell monolayers were mock-infected or infected with RSV using a multiplicity of infection of 0.0002. At 3 dpi the cells were stained using anti-N and either (c) anti- pMAPKp38 or (d) anti- pJNK. The cells were viewed by fluorescence microscopy (objective x20 magnification). The pMAPKp38 and pJNK regions in the cell monolayer are indicated (white arrow). The region of pMAPKp38 and pJNK staining in each image is demarcated (white outline). Figure and figure legends are taken from publication [34].

Figure 29 TUNEL assay of RSV-infected HEp-2 cell monolayers. HEp-2 cell monolayers were either mock- infected or infected with RSV using a multiplicity of infection of 0.0002. At (a) 1 day post infection (dpi), (b) 2 dpi, (c) 3 dpi, (d) 4 dpi and (e) 5 dpi the monolayers were co- stained using anti- G and TUNEL. The stained cells were then viewed using fluorescence microscopy. The anti- G infected cell clusters (white arrows) and the increased TUNEL staining (*) are indicated (objective x20 magnification). (f) Enlarged image showing virus- induced syncytia viewed using bight field (BF), TUNEL staining, and anti-G staining at 5 dpi. The syncytia (*), TUNEL staining (**) and anti-G staining (white arrow) are highlighted. Mock infected monolayers are shown at 1, 4 and 5 dpi. Figure and figure legends are taken from publication [34].

CHAPTER 4. RSV MORPHOLOGY AND TRANSMISSION IN HUMAN NASAL EPITHELIAL CELLS

Nasal epithelium is considered the first line of mechanical and immunological defense of the upper respiratory tract [100]. In our previous study, the influence of RSV infection on the lower respiratory tract at the level of dendritic and macrophage studies had been addressed [104]. However, the understandings of the viral transmission, replication dynamics in regards to host immune response of the upper respiratory tract have not been studied in detail. In this chapter, we used human nasal epithelial stem/progenitor cells (hNESPCs) or nasal cells (NE cells) *in vitro* to explore RSV infection at this level. These cells provided an easy, clean system to control viral infection in comparison to any available animal models. Furthermore, the virus caused human disease like RSV can be easily adapted to hNESPCs system and expose true intrinsic biological properties without having to adapt to new host systems such as to animal models [105]. The system showed advantages to study not only RSV replication, innate immunity, apoptotic events but also many other viruses that infect the human respiratory epithelium.

4.1 Viral morphological studies in NE cell model

In our previous study [44], using the hNESPCs, we observed RSV morphogenesis during the infection period from 1 dpi- 5 dpi. Between 1 dpi to 2 dpi, the trafficking of viral antigens of F and G was noticed on the cell surface of ciliated structures. At 3 dpi, alteration of the β 4-tubulin network distribution along cilia shaft as well as co-localization of β 4-tubulin with G and F proteins were observed by immunostaining and confirmed by western blot data. By 5 dpi which was considered the late phase of the infection, there was a reduction of 90% of the ciliated structures on the surface of the nasal cells which is directly related to cilia impairment while viral filament structures extensively budded on the areas where the ciliated structures were cleared. This suggested the correlation of RSV activities to the cilia's impairment and dysfunction once RSV infection occurred.

In this chapter, we continued to expand the study of the infection for a longer term, of up to 13 days post infection to assess the transmission of RSV with regards to hNESPCs host response.

Figure 30 NE cells were infected with RSV at MOI of 3, at 5 dpi, cells were fixed and stained with (A) anti-N, anti-M, (B) anti-N, anti-G, (C) anti-SH and anti-F. (ii) white box highlighted image, (i) images from confocal microscopy at focal planes that allowed visualization of the cell top. White arrows indicate co-stained virus filaments.

RSV infection at MOI of 3 was conducted on NE cells. At 5 dpi cells were fixed and co-stained using anti-M and anti-N or anti-F and anti-SH [Figure 30]. Staining patterns were examined by confocal microscopy. Staining with either anti-M or anti-N showed the filamentous patterns which confirmed the viral filament formation had taken place. NE cells were co-stained with anti-SH and anti-F. The result showed that the two viral proteins were both presented on the ciliated structures of the infected cells, implicating beside F and G, SH protein had also been trafficked onto the filamentous structures during the infection.

4.2 Transmission of RSV on NE cells

RSV at MOI of 0.01 was used to infect the well-differentiated NE cells. At 5 dpi and 10 dpi, the cells were co-stained using anti β 4-tubulin and anti-N to examine infection status. At 5 dpi, approximately 10% of the cells were singularly infected. At 10 dpi, we did not observe an increase of the number of infected cells in comparison to that at 5 dpi, however, the infected clusters exhibited a larger size (from 2 to 5 cells) rather than the singularly infected cells as at 5 dpi implicating that RSV transmitted in NE cells via a cell-to-cell route which is similar to what we observed in mammalian cell lines in the previous chapter [Figure 31]. The β 4-tubulin staining confirmed the presence of the ciliated structures of well-differentiated NE cells. We also noticed RSV only infected cells having these cilia, suggesting RSV receptors may only present on the ciliated structures' surface.

In another set up, NE cells were infected by RSV at MOI of 0.5, at 9 dpi and 13 dpi, cells were stained with anti- β 4-tubulin and anti-N, images were captured by confocal microscopy [Figure 32]. We noticed at 9 dpi, most cells were singularly infected with cilia loss due to the primary infection, the β 4-actin network dramatically changed while at 13 dpi, no ciliated structures were found, the β 4-actin antibody here labeled on the bare surface of the infected cells, where there were syncytial-alike forming clusters which were approximately 100 μ m in length, at least 4-5 times bigger than a single NE cell's size. Z-stack imaging was recorded, the data suggested N-antigen was distributed mostly on the apical surface of the cells but not at the basolateral one [Figure 33]. This analysis suggested that the virus may spread on the apical parts of the ciliated cells in close proximity and there may be no spread at the base part of the cells. If the effective transmission mode was via cell-free virus particles, we would not observe the bigger infected cell clusters over time but more in numbers of the singularly infected cells across the membrane. Hence, our observation suggested localized cell-to-cell transmission is a primary route of RSV in NE cell model.

Figure 31 RSV low multiplicity infection in nasal epithelial cells with densely packed ciliated cells. Cells were either mock- infected or RSV- infected with RSV at MOI 0.05. and 5 or 10 days post infection, cells were co-stained using anti- β4-tubulin (green) and anti- N (red) and the cell surface was examined using immunofluorescence microscopy (objective 20X magnification). The RSV infected cells are indicated as white arrow heads. Infected clusters are indicated as (*).

Figure 32 RSV infection progress on NE cells examined by immunofluorescence microscopy over 13 days infection period. In Mock-infected cells, we noticed more abundant of healthy cilated structure, coincide with no N stained pattern detected. While in Infected cells, we observed clearly single infected cells by N staining from 2 dpi to 9 dpi, (the white arrows). By 13 dpi, (*) highlighted fewer number but bigger size of infected cells comparing to previous time point. This suggested (*) may be the result of fusion occurred to (white arrows) cells. We also noted lower number of cells had cilated structures over time, this might be coincide with the progression of infection.

Figure 33 Z-stack analysis of advanced infected fused NE cells at 13 dpi. NE cells were infected with MOI of 0.5, at 13 dpi, we observed large fused cells by IF using anti N (red) co-staining with anti β4-tubulin (green). By confocal analysis, we noticed all ciliated structure were gone, only the general structure of cell surface was detected by anti β4-tubulin. (A) Pictures at apical plane showed anti β4-tubulin distribution was co-localised with N protein distribution. (B) When we focused into inside of the cell, we saw the scheme of the nucleus (white arrow), around that N and β4-tubulin were found co-localised within the cells, noted that size of the infected cell was around 4 to 5 single infected cells observed at earlier time points. (C) Z-stack imaging data suggested N- antigen distributed mostly on apical surface of the cells but not at basolateral one (white arrows). This analysis implied that virus may spread among ciliated structures of cell in close proximity and there may be no spread at base part of the cells. Scale bar of 100 μm is indicated.

A number of previous studies suggested that in human, the nasal mucous plays a role as a protection barrier for the respiratory tract by trapping the foreign objects physically. The mucous also contains anti bacteria compounds contributing to the protection of the human respiratory tract from viral infection spread [44, 104, 105]. In healthy well-differentiated NE cells (~30 days in culture), a thin layer of mucous is observed. Before conducting RSV infection, the cell monolayers were washed gently with sterile warm PBS to remove this mucous layer in order to achieve good infection. However, in low MOI experiments, after 5 to 10 days, we were able to observe that the mucous layer had formed again, from which we can speculate that it may have a function to trap free RSV particles as a control mode of virus spreading. We decided to examine the level of infectious virus trapped within the mucous. Low MOI infection of 0.1 was

done on NE cells, at each time point of 1, 2, 3, 5 and 10 dpi, the mucous layer was washed using warm media of 2% FBS DMEM containing Pen/Strep for 10 minutes at 37°C, and then was collected and titrated to determine the titer of the secreted cell-free RSV particles. Titration results are indicated in [Figure 34] showing an increase trend of virus titer from 1 day to 5 days post infection. This indicated the increased export of RSV free viral particles over time. In details, at 6 hpi, the first mucous wash was collected and its titer served as a control baseline of log(2) which represented the input virus particle bounding to the host cells' surface when the infection first started. A mucous wash fraction collected at 1 dpi had its titer of log(4) which showed a sharp increase from our baseline of 6 hpi. However, from 1dpi to 5 dpi, the virus titer of each time point increased slowly and was stable at around log(5) [Figure 34]. An approximate 400-fold increase of virus titer was detected between 3 dpi and 5 dpi, but there was not an apparent further increase at 10 dpi. This indicated the maximum virus production was at 5 dpi. In other words, the titer of RSV free particles which were trapped in the mucous did not exponentially develop over infection time but rather hit a plateau around 3 dpi and dropped slightly toward the end time point of the experiment of 10 dpi. This suggested that releasing virus free particle to the culture environment was not a favored transmission pathway of RSV on human NE cells.

Figure 34 Titer of cell-free RSV from mucous wash. Low MOI infection of 0.1 was done on NE cells, at each time point of 1, 2, 3, 5 and 10 dpi, mucous wash with warm media of 2% FBS DMEM containing Pen/Strep for 10 minutes at 37°C were collected. These samples were titrated using HEp-2 cells.

4.3 NE cell host response from RSV infection

Our observation of RSV transmission on NE cells suggested that the secondary RSV infection in NE cells was not as successful as in *in vitro* permissive mammalian cell lines, which led to speculation that RSV may trigger NE host responses against the secondary infection by turning on the host cellular pathways. Hence, we decided to conduct RSV infection at MOI of 3 on NE cells, the infected cell lysates from Mock, 1 dpi, 2 dpi, 3 dpi, 5 dpi, 10 dpi were harvested to evaluate host cell responses in corresponding infection levels. Various antibodies against viral proteins (M2-1, G, N), proteins belonging to JAK/STAT pathway (STAT1, JNK, P38MAPK and their phosphorylated forms) as well as apoptotic related proteins (caspase 3, 7, 9 and their cleaved forms), especially RSAD2 (Viperin) which was reported in previous publications to have antiviral function in human cells, were used in this study [43, 60].

Immunoblotting using anti M2-1 and anti-N showed a gradual increase of the viral proteins' levels between 3 and 5 dpi. With anti-STAT1, we observed a concrete increase during the infection process from 1 to 5 dpi, which was observed previously in our RSV study using permissive HEp-2 cell line. With anti-pSTAT1, we recorded a peak of its expression at 2 dpi. A minor increase of p38MAPK level at 1 dpi was correlated to the presence of pp38MAPK at 2 dpi and 3 dpi. JNK was detected from 1 dpi to 3 dpi followed by a sharp decrease at 5 dpi. However, we did not observe any expression of pJNK at any time points [Figure 35] Among the examined proteins, the p38MAPK expression level was found to increase between 2 dpi and 3 dpi suggesting a temporal activation in NE cells which could be correlated to the increase of virus particle assembly process. A similar observation of p38MAPK expression was noticed in the RSV infected permissive cell line HEp-2 [34]. This led us to a hypothesis that p38MAPK phosphorylation during RSV infection could induce changes in the cell physiology which are important for the virus transmission on NE cells. Anti-actin was used as a loading control in all our immunoblotting experiments. Probing the membrane with anti-actin showed the similar levels of actin in all samples at different harvested time points indicating no major difference of cell monolayer materials.

Figure 35 RSV-induced interferon signaling is associated with RSV particle formation. Nasal epithelial (NE) cells were infected with RSV using a multiplicity of infection (moi) of 3 and at 1, 2, 3 and 5 days post-infection (dpi), the cells were analysed by immunoblotting using anti- M2-1, anti- N, anti-STAT1, anti-pSTAT1, anti-p38MAPK, anti-pp38MAPK, anti-JNK, anti-pJNK and anti- actin. M is mock-infected cell lysate.

In another set up, the NE cells were infected using RSV at MOI 3 and harvested at 2 dpi, 5 dpi, 10 dpi in which 2 dpi represented an early infected time point, with 5 dpi as a mediated time point and 10 dpi as a late time point. The lysates were immunoblotted and probed with different antibodies. With viral antibodies, e.g. anti- G, anti M2-1 and anti M, we noticed the highest amount of viral antigens occurred at 5 dpi indicating this as the peak of virus replication, which is consistent with previous experiments. At the late time point of 10 dpi, we noted a sharp decrease of all viral protein levels which suggested the release of the virus into the culture environment. Immunoblotting with anti G showed 3 different glycosylated forms of G (150 kDa, 90 kDa and 80 kDa) existed in RSV infected NE cell lysate. At 2 dpi, the G population of 90 kDa were the abundant in comparison to the 150 kDa form of G which were expressed more abundantly at 5 dpi. [Figure 36A], both 90 kDa and 150 kDa populations of G are shown to be decreased

which could be due to the reduction of the viral material by the shedding of the infectious virus particles as mentioned above. With anti-NS2 which is a host antiviral factor, we noticed at 2 dpi, the cells produced the maximum amount of the protein which may indicate a trigger of some signaling pathways involved. RSV is able to target IFN-signaling by blocking the function of the cytoplasmic RIG-I sensor through NS2 protein and inhibiting STAT-2 activation via NS1 and NS2 proteins [52]. We detected a low levels of NS2 and Viperin expression which suggested that these defense mechanisms may not engage in RSV-infected NE cells but only play a role in restricting the RSV infection within the nasal cavity. These antiviral responses also contributed to preventing the increase of cell-free virus after 5 dpi which is consistent with the cell-free virus titer drop that we observed in [Figure 34]. We also observed a gradual increase expression level of Viperin during the span of infection which peaked at the latest time point of 10 dpi [Figure 36A] showed the increase of the Viperin expression level over RSV infection time in hNESPCs cells which is similar to what we observed in the RSV infected permissive HEp-2 cells in our previous study [43]. Interestingly, Viperin is a known antiviral protein which is involved in fighting viral disease. To confirm this, we conducted IF co-staining NE cells with anti-Viperin (RSAD2) and anti-N and recorded an increasing expression of RSAD2 over the infection period. Between 2 dpi and 5 dpi, Viperin was only detected in the infected cells. However, at the late time point of 10 dpi, not only the infected cells but also non-infected cells surrounding the infected ones were labeled with Viperin. We applied the same staining and analysis in mock infected cells and detected none of N, G or Viperin staining patterns. This indicated the findings are only exclusive in RSV infected cells [Figure 36B,C,D,E].

The interferon responses can lead to the expression of several different interferon stimulated genes (ISG) [86]. In addition, other cellular pathways e.g. apoptosis can be induced by interferon α [97]. To examine if apoptosis is induced by virus assembly, cell lysates were immunoblotted with different caspases antibodies such as caspase-3 (C3), caspase-7 (C7), caspase-9 (C9) and their cleaved forms c-C3, c-C7, c-C9. While the full forms of C3, C7, C9 presented at all time points, the cleaved forms of these proteins only presented at between 2 dpi and 5 dpi, and diminished at 10 dpi, which indicated their short term existence suggesting a transient induction of RSV in apoptotic activation within RSV infected NE cells [Figure 36F].

Figure 36 RSV-induced Viperin expression and apoptosis occurs in RSV-infected ciliated cells. Cells were infected with RSV using moi of 3, at 2, 5 and 10 days post infection (dpi) the cells were analysed by immunoblotting using (A) anti- G, anti-M2-1, anti- M, anti- NS2, anti- RSAD2, anti- STAT1 and anti-actin. (B) RSV- induced viperin expression is confined to virus infected ciliated cells. Cells were mock-infected or infected with RSV using a moi of 3 and at 5 dpi the cells were stained using (B, C) anti- viperin and anti- N and (D,E) anti- viperin and anti- G. (B and D) objective 20X, (C and E) objective 100X magnification. The co- stained cells (white arrows) and anti- viperin stained inclusion bodies are indicated. (F) RSV- infection induces apoptosis in NE cells. Cells were infected with RSV using a moi of 3 and at 2, 5, 10 days post infection, cells lysates were harvested for immunoblotting analysis using anti- caspase 3 (C3), anti- cleaved caspase 3 (cC3), anti- caspase 7 (C7), anti- cleaved caspase 7 (cC7), anti- caspase 9 (C9), anti- cleaved caspase 9 (cC9) with anti- actin as loading control. M is mock- infected cells lysate.

CHAPTER 5. HMPV MORPHOLOGICAL AND TRANSMISSION STUDIES

5.1 HMPV morphology in mammalian cells

HMPV is known to be difficult to be cultivated *in vitro*. Although the virus is stable to multiple free-thaw cycles from -80 to 4°C, it becomes unstable at 37°C during a long cultivation time which could be up to 2 to 3 weeks. Most HMPV studies were conducted based on RT-PCR methods [19, 40, 41] or recombinant HMPV proteins expression [55]. There have only been few studies conducted with systematic methods of HMPV cultivation, which has led to a lack of knowledge of the virus replication, assembly and budding. We set up our methods to cultivate HMPV *in vitro* and successfully adapted an HMPV strain which can be used to further study HMPV morphology and transmission. In this chapter, we report the results involving the HMPV morphology in different cell lines, the viral protein characterization (M, F, G, P), and the HMPV transmission in correlation with host response utilizing a low multiplicity infection mode. We also noticed an interesting event where M-protein is involved in host cell modulation at early infection.

5.1.1 Lab-adapted HMPV morphology

Previous HMPV cultivation studies emphasized exogenous trypsin's function in assisting the HMPV replication *in vitro* based on the knowledge that F protein contains a furin-like cutting site [77]. In constructing a method to improve HMPV growth *in vitro*, there is a common practice of adding trypsin to the maintenance media in order to promote F protein processing and boost HMPV replication, even though it was reported that some HMPV clinical strains may replicate well without the presence of trypsin [14, 22, 101].

In this set of experiments, to test the function of trypsin on HMPV replication in different cell lines (A549, HEp-2, LLC-MK2, 293T and MDCK), we supplemented the growth medium with a low concentration of trypsin-TPCK of 0.3 mg/ml to study our lab-adapted strain of HMPV A2 NCL03-4/174. We observed that the HMPV infected clusters were larger in cells with the trypsin supplement compared to the ones without

trypsin in medium [Figure 37]. This confirmed that trypsin assists our HMPV strain to grow better *in vitro*. Among cell lines in use, LLC-MK2 and 293T showed to be effectively infected by HMPV. Between LLC-MK2 and 293T, 293T also showed good HMPV infection but the cell size is smaller with the thinner cell membrane in comparison to that of LLC-MK2, which leads to the worse display of HMPV infected features e.g. the filamentous structures or inclusion bodies. Based on these observations, we decided to use LLC-MK2 for our further experiments.

Figure 37 Trypsin supplement effect on HMPV culture. Trypsin of concentration 0.3 mg/ml was used in HMPV culture on various cell lines (A549, HEp-2, LLC-MK2, 293T and MDCK). (-) indicated where trypsin was absent in culture media, (+) indicated where trypsin was supplemented in culture media. By 3 dpi, cells were fixed and stained by anti- M HMPV. HMPV infected clusters with trypsin supplement were larger compared to ones without trypsin in medium.

Acknowledging the function of trypsin in our HMPV strain replication, we started supplementing trypsin-TPCK with a concentration of 2 mg/ml in HMPV maintenance medium to propagate new virus. This resulted in progeny virus which can replicate faster than its earlier passages with the higher titer and this does not affect the host cell LLC-MK2's growth. Using our lab adapted strain HMPV, we characterized the

virus features using antibodies to label the viral structural components for examples: M (Matrix protein); F (Fusion protein), G (Glycoprotein) and P (Phosphoprotein).

Figure 38. HMPV viral proteins (F, M, G, P) were characterized by IF using (A) anti-F. (B) anti-M. (C) anti-G by confocal microscopy. (D) using anti-P by fluorescent microscopy. Mock lysate (M) was used as control to compare with Infected cell lysate (I). (E) Infected cell lysates at 10 dpi were harvested from either infected cell pellet (IP) or infected supernatant (IS). Immunoblotting was performed using anti G resulting in 2 bands of 80 kDa and 125 kDa which were 2 glycosylated form of G protein. Blotting using anti-P showed correct band of the protein at 37 kDa. In membrane probing with anti-M, band of 28 kDa were visualized at both infected cell pellet and supernatant suggesting besides M protein within cell monolayer, the protein was also exported into the infected supernatant which is consistent with previous studies.

The M protein was detected with major prominent filamentous structure that expressed dominantly from the nuclear peripheries to the surface of the host cell membrane. IF samples examined by either fluorescent microscopy or confocal microscopy showed 2 distinct components of the filament structure formed by M proteins: the rod-shaped portions started from within the host cell and the round-shaped portions at the very end of the ectodomain of the filament. This observation confirmed that the morphology of HMPV M protein is different from that of RSV M protein. How these structures of M relate to its function is not yet understood [Figure 38B]. F and G proteins are exhibited as the filamentous structures that bud on the surface of the infected host cells [Figure 38A, C]. IF results indicated that P protein location was coincided within the inclusion bodies which were underneath the cell membrane [Figure 38D]. HMPV infected LLC-MK2 cell lysate were harvested at 10 dpi and western blot analysis was performed. The membrane was probed with anti M, anti G and anti P showing the

corresponding size of the viral proteins at 28 kDA, 80 and 125 kDA (2 glycosylated forms of G) and 37 kDA, respectively [Figure 38E]. These results confirmed the HMPV proteins' presence in our infected lysate.

Figure 39 HMPV-infected cells exhibit two specific sub-populations of the M protein. Human metapneumovirus (HMPV)- infected LLC-MK2 cells were stained using anti- M, and imaged by confocal microscopy. **(A)** The virus filaments (white arrows) and **(B)** spherical M protein staining pattern (arrow heads) are highlighted. **(ii)** is an enlarged image taken from the area highlighted by the white box in **(i)**. Figure taken from [42].

To further analyze the M protein staining pattern, we infected LLC-MK2 with our lab-adapted HMPV. At 3 dpi, the cells were fixed and stained with anti-M. The staining revealed 2 specific sub-populations of M protein: a filament pattern whose length was around 1 µm to 5 µm and a spherical pattern whose diameter was around 0.5 µm as indicated in [Figure 39]. This marked an interesting and unique observation of HMPV M morphology, which was strikingly different from its closely related M protein from RSV. Due to the very small size of 2 sub-populations of M, especially spherical ones, they were not yet characterized and the functions related to their structures were not been known.

5.1.2 Morphology of HMPV isolated from clinical samples

The nasal wash samples of SAF were kindly provided by DSO. The samples were categorized based on the year they were collected (ranging from 2009 to 2013) and preserved frozen carefully at -80 to guarantee good quality storage. Using these clinical isolates, our first aim was to use IF to determine which among the sample pool contained HMPV. The second aim was to select from among the HMPV positive clinical samples a portion to further adapt into *in vitro* cultivation. If this succeeds, it could result in several HMPV lab-adapted strains to further study HMPV infection mechanism, transmission pathway in relations to host responses in a laboratory scale. The third aim was to sequence a whole genome of the sample pool to gain knowledge of HMPV strains circulated in SAF from 2009 to 2013. In this sector, we show results from our first and second aims listed above. The presence of HMPV was confirmed by IF using antibody against M protein. It was noted that the nasal wash samples could contain more than one virus infecting the host patients. The summary results of IFA screened HMPV clinical isolates from 2009 to 2013 by using antibody against HMPV-M were presented in [Table 5].

Year 2009- 2010				
Sample	Start CPE	Duration of isolation	IFA result	Note
20090259	-	21 dpi	-	
20090299	-	21 dpi	+++	
20091196	-	21 dpi	-	
20091369	-	21 dpi	-	
20091116	11 dpi	21 dpi	-	
20090581	-	21 dpi	-	
20090168	-	21 dpi	-	
20090144	-	21 dpi	+	
20091743	11 dpi	21 dpi	++	
20090613	-	21 dpi	-	
20100503	-	21 dpi	-	
20102308	-	21 dpi	+	
20100737	-	21 dpi	+	

20100668	-	21 dpi	-	
20101795	-	21 dpi	-	
20100712	11 dpi	21 dpi	-	
20102583	14 dpi	21 dpi	-	
20100456	1 dpi	21 dpi	-	
20102914	-	21 dpi	-	
20102826	14 dpi	21 dpi	-	
<u>Year 2011</u>				
20110468	8 dpi	9 dpi	+	F _c
20112081	8 dpi	9 dpi	+	F _c
20110652	5 dpi	5 dpi	+	F _c +Y
20110921	8 dpi	9 dpi	+	F _c
20110632	8 dpi	9 dpi	-	F _c
20110244	8 dpi	9 dpi	+	F _c
20111860	8 dpi	9 dpi	+	F _c
20111912	8 dpi	9 dpi	-	F _c
20110188		21 dpi	-	(-) CPE
20111881	8 dpi	9 dpi	+	F _c
20111147	8 dpi	10 dpi	+	
20111815	8 dpi	9 dpi	+	
20112159	-	21 dpi	-	
20111704		16 dpi	+	
20111154		10 dpi	+	Y
20111255		10 dpi	+	
20111260	-	21 dpi	-	
20111916	-	21 dpi	-	
20111799	-	21 dpi	+	
<u>Year 2012</u>				
20121325	12 dpi	20 dpi	-	
20120577	12 dpi	14 dpi	+++	
20121179	12 dpi	14 dpi	++	
20120503	12 dpi	14 dpi	-	
20121331	12 dpi	20 dpi	-	
20120699	13 dpi	15 dpi	+++	

20120781	12 dpi	14 dpi	+++	
20120696	12 dpi	14 dpi	+++	
20121447	9 dpi	12 dpi	-	
20120973	9 dpi	12 dpi	+++	
20121525		21 dpi	-	
20120998	15 dpi	20 dpi	+	
<u>Year 2013</u>				
20130814	18 dpi	21 dpi		
20130848	12 dpi	13 dpi	+++	
20131141	11 dpi	13 dpi	+++ (F)	
20131158	8 dpi	12 dpi	+++ (F)	
20131168	11 dpi	13 dpi	+++ (F)	
20131191	12 dpi	13 dpi	+++ (F+S)	
20131192	-	21 dpi		
20131195	11 dpi	12 dpi	+++ (F)	
20131200	13 dpi	21 dpi	+	
20131216		12 dpi	+++ (F)	
20131230	12 dpi	14 dpi	+++ (F+S)	
20131316	15 dpi	19 dpi		
20131325	11 dpi	13 dpi	+++ (F)	
20131336	11 dpi	13 dpi	+++ (F)	
20131349	12 dpi	14 dpi	+++ (F+S)	
20131384	11 dpi	13 dpi	+++ (F)	
20131385	18 dpi	21 dpi		
20131386	-	21 dpi		
20131395	12 dpi	13 dpi	+++ (F)	
20131419	-	21 dpi		

Table 5 Summary results of screened SAF clinical isolates collected from 2009 to 2013 by immunofluorescence assay using anti-M HMPV. (-) indicated negative result; (+) indicated less than 30% cells were stained; (++) indicated 30% to 50% cells were stained; (+++) indicated over 50% cells were stained. F indicated sample with Filamentous Mprotein morphology, S indicated Spherical M morphology. F_c indicated Fungi contamination presence in culture, Y indicated Yeast presence in culture.

Variations of M staining patterns were observed and classified into 3 categories: Long filamentous, short filamentous and Spherical pattern. The level of infectivity was categorized into (-) means negative; (+) positive with less than 30% cells infected; (++)

positive with 30-50% cells infected; (+++) positive with above 50% cells infected. Being able to differentiate the level of infection caused by the different clinical isolates gave us a better opportunity when selecting the better grown isolates to study HMPV morphology and infectivity potentials in the future.

Figure 40 Variations of M protein morphology visualized by IF examined by fluorescent microscopy. Clinical samples were isolated using LLC-MK2 during 21 day of virus cultivation with trypsin- TPCK supplemented every 7 day. Cells were fixed and stained with anti- M. **(A)** Sample coded 20120781 under objective 100x showed M protein as small spherical particles without any filament pattern. **(B)** Sample coded 20120696 under objective 20X with dominant short filamentous formed of M protein. **(C)** 20121179 under objective 100X exhibited both long filamentous filament rod with a rounded tip structure at the end of filament. **(D)** 20131349 under objective 40X with M protein as major spherical form. **(E)** 20131325 under objective 40X had M protein as dominant long filamentous, rounded tip feature was minor. **(F)** 20131200 only can be visualized under objective 100X with minor rounded tip M form. Arrows in each figure indicate specific the M shape of the related clinical isolate.

We observed common features of M staining patterns using our lab-adapted HMPV strain and the clinical isolates, e.g. the M protein staining pattern comprised of 2 distinguished sub-populations of the filamentous part and the spherical-shaped tips. Especially in the clinical samples, the variations of the length of filament part were observed which can be categorized into: Long filamentous, short filamentous. Some samples, for example sample coded 20120781, showed a very short filamentous part while its spherical tip was more noticeable [Figure 40A]. Variations in filament length and ratio between 2 sub-populations can be observed through some of representative M-staining at different clinical isolates in [Figure 40]. More studies are needed to understand the causes of these differences (e.g. sequencing to examine genome variation)

as well as how these morphological differences affect the function of M proteins during HMPV infection and transmission.

Figure 41 Second passage of clinical isolates coded 20131195 and 20131384. The two isolates were chosen based on their M protein morphology where 20131195 has long dominant filamentous with minor round tipped M morphology while 20131384 has medium filamentous with larger rounded tip structure. **(A)** indicated IF result of passage 1 viruses (20131195 and 20131384) using anti- M to confirm their infectivity and M morphologies. **(B)** Pictures of the viruses from passage 1 to passage 2 in culture condition at 21 dpi prior to harvest, both isolates showed intensive CPE due to HMPV infection. **(C)** The harvested passage 2 viruses were used to infect new LLC-MK2 cell monolayers, IF using anti-M was performed on 9 dpi samples to examine the two HMPV isolates' infectivity and titer.

To further determine how the differences in morphology of M protein correlate to its HMPV isolate growth *in vitro*, we chose two representative samples of 20131195 (long dominant filamentous with minor round tipped M morphology) and 20131384 (medium filamentous with visible round tip structure) to further passage during the cultivation period of 21 days at 37°C, trypsin was supplemented every seven days. CPE over the infection time were observed, confirming that successful infection was in progress. At 21 days post infection, the infected cell monolayers were scraped

thoroughly within the culture supernatant, homogenized, aliquoted into 1 ml vials and kept at -80°C. Titration of these p2 viruses was done on LLC-MK2 cells as mentioned above. At 9 dpi, the cells were fixed and IF-stained using antibody against M protein was performed. In 20131195 p2, a better titer level was observed, with bigger infected plaques shown, in comparison to that of 20131384 p2 [Figure 41]. This can be explained by various reasons: even though the cultivation conditions for the two clinical isolates samples were similar, the variations of the viral growth always can occur. Some virus strains may be prone to *in vitro* cultivation than others. Most of the primary isolations showed virus growth, however, the further the virus gets passaged, the titers would drop over time and the underlying reasons are not well-known. In this case, a better grown 20131195 p2 compared to 20131384, it can be speculated based on the shape of M protein: that the longer, more filamentous filaments may contribute to the better transmission ability of the virus.

Based on the previous studies of RSV [48, 98], the small short or spherical filaments may be the sign of the defective interfering (DI) viral particles. This leads to a hypothesis of correlation of the M protein's shape to HMPV growth and transmission potential. To address this, we can approach in two different directions of 1) Morphological studies done by selecting HMPV clinical isolates of choice and adapting them to culture on a larger scale of sample numbers and further passage numbers in order to correlate a M protein profile to the virus growth potential. 2) Genome studies by sequencing the whole genome of HMPV isolates of choice and correlating their (M, F, G) genes to the virus growth potential in the culture.

5.2 HMPV transmission studies using LLC-MK2 cell line

In these experiments, 90% confluent LLC-MK2 cell monolayer was infected by HMPV with a low multiplicity of 0.001. The infection was observed from 1 to 10 dpi. We noticed a gradual increase of the viral antigen M by IF and bright-field microscopy. The increasing level of infection was closely related to the CPE exhibited in the cell monolayer [Figure 42].

Figure 42 Observation of HMPV infection at MOI 0.001 from 1 dpi- 10 dpi. In this experiment, 90% confluent monolayer of LLC-MK2 was infected by HMPV at MOI 0.001. Changes of monolayer were observed by both bright-field (BF) microscopy and Immunofluorescent staining (IF) with anti-M HMPV. Data also suggested infection progress according to increase level of the M protein expression over time.

From 1 dpi to 3 dpi, even though there was no visible change of CPE, IF data revealed infection first occurred as single cell infection throughout the monolayer at 1 dpi, at 3 dpi we started seeing the infected clusters containing 18 ± 3 cells which was similar to what we observed previously at RSV low MOI experiments on HEP-2 cells. At 5 dpi, we still noticed large clusters of HMPV infected cells. It was noted that HMPV infection seemed to happen at a slower rate compared to RSV and besides the defined clusters of HMPV infected cells, we also observed many single infected cells outside the plaques at 3 dpi and 5 dpi. This indicated that HMPV might follow cell-to-cell transmission pathway which is similar to RSV at early infection, however the differences in viral particle size and temperature of cultivation might affect the transmission, making it “tight” cell-to-cell or “loose” cell-to-cell which means the particle release mechanism of HMPV may differ compared to that of RSV. At the later time points of 7 dpi and 10 dpi, basically most of the monolayer was infected and gaps started appearing at 10 dpi indicating cell loss due to severe infection.

Figure 43 HMPV cell-associated fraction (CA) and cell-free fraction (CF) analysis. HMPV infected LLC-MK2 supernatant and cell monolayer were harvested separately to form CA and CF samples, respectively at 1 dpi, 3 dpi, 5 dpi, 7 dpi and 10 dpi. Each fraction was used to challenge new LLC-MK2 cell monolayer overnight, after that cells were fixed and IF stained with anti-M to determine infectivity of each fraction.

From the experiment above, we observed HMPV infection progress from 1-10 dpi with an increase in numbers of cell infection and the bigger syncytia appearing over time. We decided to harvest CA and CF fractions from each time point and use them to challenge new cell monolayers of LLC-MK2. After 24 hours, the monolayers were fixed and infectivity by HMPV was examined by IF using anti-M HMPV. From 1 dpi to 10 dpi, we observed a gradual increase in the infected cell numbers caused by both CF and CA fractions. However, infection caused by CF fractions resulted in fewer infected cells compared to that by CA fractions [Figure 43]. The results indicated that most of the

infectious HMPV virus was associated with the host cell while there was very little amount of virus was released into the culture supernatant. This result confirmed that the HMPV virus primarily associates with the host cells.

LDH assays were used to examine LLC-MK2 membrane permeability changes under the HMPV infection effect. We observed a steady increase of the membrane permeability which was an indicator for the cell monolayer damage. Comparing this to the supernatant collected from Mock-infected cells, which only indicated a minor increase in cell damage (by cell aging during 10 dpi of observation), HMPV infected materials exhibited a steady increase of cell damage over time, at 10 dpi, the data suggested approximately 70% of the cell monolayer was damaged [Figure 44].

	1dpi	3dpi	5dpi	7dpi	10dpi
Mock	1.21%	2.62%	4.42%	6.37%	8.51%
Infected	0.72%	6.21%	17.52%	34.54%	69.98%
STDEV Mock	0.006523	0.001095	0.003364	0.000736	0.003626
STDEV Infected	0.001863	0.005	0.00478	0.012069	0.020772

Figure 44 LDH assays indicates cell damage over HMPV infection period of 10 days. Supernatant from both mock-infected monolayer at same time points as infected ones were recovered for this purpose. Results showed that there was a consistent increase in cell damage caused by infection over time. Comparing to that of mock- infected cell, there was only a marginal increase due over confluent of cell monolayer over time. At last time point of 10 dpi, infected monolayer showed around 8 times more damaged comparing to the mock-infected. Standard deviation (STDEV) values are shown by the error bars.

Sample	Dilution	Count	Titer	Sample	Dilution	Count	Titer	Sample	Dilution	Count	Titer	Sample	Dilution	Count	Titer
33 CF 1dpi	1	0	0	33 CA 1dpi	100	14	9415.929	37 CF 1dpi	1	0	0	37 CA 1dpi	1	12	80.70796
33 CF 3dpi	1	7	47.07965	33 CA 3dpi	100	25	16814.16	37 CF 3dpi	1	8	53.80531	37 CA 3dpi	10	44	2959.292
33 CF 5dpi	1	46	309.3805	33 CA 5dpi	100	121	81380.53	37 CF 5dpi	10	19	1277.876	37 CA 5dpi	100	215	144601.8
33 CF 7dpi	10	20	1345.133	33 CA 7dpi	100	350	235398.2	37 CF 7dpi	100	22	14796.46	37 CA 7dpi	100	540	363185.8
33 CF 10dpi	100	24	16141.59	33 CA 10dpi	100	450	302654.9	37 CF 10dpi	100	350	235398.2	37 CA 10dpi	100	600	403539.8

Figure 45. Titers of cell-associated and cell-free HMPV in correlation to cultivation time at either 33°C or 37°C. The graph showed an increase trend of both CA or CF cultivated at either 33 or 37°C. The table listed in details data collected from titration experiments with average titers in numbers of each parameter at each time point.

In this experiment, HMPV cultivation was observed during a period of 10 days at either 33°C or 37°C with 5% CO₂ supplement, the samples of CA and CF were harvested at 2, 3, 5, 7 and 10 dpi. CA and CF samples of each time point were titrated to determine the levels of released virus (CF) or the host cell associated virus (CA). We noticed a gradual increase of the virus titer at both CA and CF over time, at any time points, the CF values tend to be around 1% of the CA values which suggested most HMPV exist in association to the host cell rather than being freely released into the media. (Except CF value at 10 dpi 37 °C culture almost reach 50% of CA 10 dpi 37°C culture, this can be explained due to the extensively damaged cell monolayer when referring back to contrast image taken at the same time samples were harvested [Figure 45]. The virus titers from the HMPV cultivation set done at 33°C revealed very low

value of CF over time compared to the related CA sample of the same set further confirmed the conclusion. Additionally, temperature was proven to be an important factor that facilitates the HMPV growth rate. At 10 dpi, titer of the CA grown at 37°C was 25% higher than that grown at 33°C, while the titer of CF at 37°C was a log higher than that grown at 33°C.

5.3 Observation of M protein- translocation into host cell's nucleus at early infection and its progression during infection course

Figure 46 Observation of M protein expression in a single infection cycle. In this experiment, 70% confluent LLC-MK2 monolayer was infected by HMPV at MOI 0.5. We observed the infection from period of 6 hpi to 16 hpi with interval of 2 hours by IF using anti- M HMPV. (A) indicated infection progress observed in objective 20X. It showed that infection can be detected as soon as 6 hpi (2- 3 cells within 1 view), along with time, more infected cells were observed, until 14hpi, as many as 50% of the monolayer showed effective infection. (B) In the same experiment, infection was observed in higher magnification of 40X. At 6 hpi, we detected infected cells by general stained cell pattern, no filaments were produced. From as early as 8 hpi, arrow head highlighted small structures within nucleus, still no filaments were produced which lead to the question of M protein's function in modulating host cellular factor prior to utilizing the cell replication and regulation machinery in serving HMPV replication. At 10 hpi, small dots were not any more observed but small thin filaments started showing their appearance. Along with time, we observed filament structures stained by anti-M elongated and became more dominant by 12 hpi, 14 hpi. At 16 hpi, protrusion of filaments toward outside cell surface was noticed.

LLC-MK2 were infected by HMPV MOI 0.5, the infection was observed from 6 hpi to 16 hpi to detect early infection events. As early as 6 hpi, infected cells in single forms were detected by IF using antibody against M protein. No filaments were shown at this time point. At 8 hpi [Figure 46B], we noted an interesting event: M protein was translocated into the nucleus structure, which suggested a M modulation function to the

host cell. At the later time points of 10 hpi, 12 hpi, 14 hpi, 16 hpi, less M was detected within the infected host cell nucleus, instead, M was found as filamentous structures which elongated over infection time. This observation is consistent with the knowledge from M of RSV in previous studies that suggest a transient regulatory function of M protein at early infection [53]. In this case, possibly, HMPV infection required a longer time to thrive in LLC-MK2 resulting in a longer period is needed for M to play its regulatory role at early infection compared to RSV, hence we can capture data showing this M activity within the cells' nucleus.

5.4 HMPV infection and host response in LLC-MK2 cell line

LLC-MK2 cell monolayer was infected by HMPV at MOI 0.05 as described above. At certain time points of 1 dpi, 2 dpi, 4 dpi and 6 dpi, the cell lysates were harvested in Laemmli buffer, and used in immunoblotting experiments. The mock-infected cell lysate was also harvested and used as a negative control. Firstly, to confirm the infectivity of the lysates, we probed the membrane using some viral antibodies (anti-G and anti-M). The results showed a gradual increase of the infected materials over time, confirming the effective infection. Anti-actin was used as a loading control, showing that the level of materials at each time point was relatively similar [Figure 47A]. Secondly, we examined the lysates with some interferon signaling antibodies belonging to JAK/STAT1 pathway to find out if HMPV infection have effects on cellular immunity, proliferation, differentiation, apoptosis or oncogenesis. With JNK, pJNK, p38MAPK and pp38MAPK, we observed an immediate drop of the proteins' expression at 1 dpi compared to the Mock-infected. This was followed by an increasing trend and finally a temporal peak at 4 dpi. While with STAT1, there was a gradual decrease of the protein expression over time, which was totally diminished at 6 dpi. This suggested HMPV virus may be able to turn off STAT1 signaling to create an advantageous condition for its replication within the host. With pSTAT1, the protein was found to express after 1 dpi and peaked at 2 dpi [Figure 47B]. Infection of HMPV on LLC-MK2 finally lead to death of the cell monolayer. We decided to examine the lysates further using some apoptotic antibodies (C3, cleaved- C3, C7, cleaved-C7, C9, cleaved- C9, PARP, cleaved-PARP) to find out if HMPV triggered the death through apoptotic signaling pathways. Among these factors, C7 and cleaved forms of C7, C3 and C-PARP only appeared due to the infection and peaked temporally at 4 dpi, which was consistent with

the JNK and p38MAPK data. C3 were detected at all time points and also at the Mock. Besides, we also noted an immediate dip of C7, PARP and C9 at 1 dpi [Figure 47C].

Figure 47 Lysates of low MOI infected were immunoblotted with either viral antibodies (G, M) to confirm infectivity or host antibodies (JAK/STAT1), apoptotic antibodies. HMPV MOI at 0.005 were used to infect LLC-MK2 monolayer. (A) A gradual increase of viral materials was confirmed along infection period of 6 dpi. No major cell lost was observed based on actin profile of each sample. (B) JAK/STAT1 antibodies were used to test host responses to HMPV infection. A STAT-1 de-activation trend was observed by decreased level of STAT-1 protein started 1 dpi, by 6 dpi, level of STAT-1 reduced to almost nothing comparing to high expression of STAT-1 at Mock-infected cells. JNK, p38MAPK and their phosphorylated forms showed a slight dip at 1 dpi and temporal peaks consistently at 4 dpi. (C) An array of apoptotic antibodies was used to test the lysates. Cleaved- form of C7, C3 and PARP were only found from 4 dpi in HMPV-infected lysates.

Figure 48 Low moi HMPV infection on LLC-MK2 was conducted to evaluate correlation of viral spread (M protein) to STAT-1 protein level presence within cell monolayer over period of 1dpi to 6 dpi. In Mock infected cells, no M antigen was detected while STAT-1 expressed abundantly across the monolayer. From 1 dpi to 6 dpi, M antigen expression increase gradually correlated to progress of viral infection and transmission. In contrast to the increase of M antigen, the STAT-1 expression level was gradually diminished over time.

To confirm our observation of the decrease of STAT1 level over infection time, we conducted IF analysis on the same setting. LLC-MK2 cells were seeded on coverslips, infected by HMPV at MOI of 0.1, at the different time points of 1 dpi, 2 dpi, 4 dpi and 6 dpi, the cells were fixed by Methanol/Acetone and stained with either anti-M to confirm level of the infection over time or with anti-STAT1 to detect the level of the protein within the cell monolayer using an imaging analysis approach. Starting with the Mock-infected sample, we detected no infection by anti-M while anti-STAT1 stained most of the cells within the monolayer. With samples stained by anti-M we noticed a gradual increase of level of infection from 1 dpi to 6 dpi while the opposite

trend of STAT-1 protein was presented where STAT-1 diminished over time under the impact of HMPV infection. This observation is consistent with the western blot result from [Figure 48]. It suggested that HMPV could be able to turn off STAT-1 expression and/or alter JAK/STAT1 pathway in order to fence off host cell defense mechanism to support effective viral spread. However, further studies are needed thoroughly understand this mechanism.

CHAPTER 6. CONCLUSIONS

In low multiplicity RSV infection on HEp-2 cells, we identified a bi-phasic mode of RSV transmission. At the early infection, efficient localized cell-to-cell transmission was observed to be the main transmission mode with the absence of cell-free virus particles. This correlated closely with F-actin network changes, especially at cell junctions between the infected cells and the non-infected cells. At the later phase of the infection (3 dpi), the virus induced changes and formed large fused structures called syncytia with the presence of the cell-free virus. With increased incubation time, cell death, and increased levels of cell-free virus (less than 1% of total virus infectivity) were observed. The presence of cell-free virus infectivity correlates with increased cell membrane permeability, cell cytotoxicity and other signaling events.

By performing similar experiments in NE cells, we noticed a similar manner of RSV cell-to-cell transmission. From 2 dpi, NE cells exhibited only a “singular” form of infected cells, but these were different from what was observed with HEp-2 cells. This pattern of single-infected cell lasted longer (up to 9 dpi), with a very minor increase of cell free virus released into the mucus layer. Until 13 dpi, i.e. very late stage of infection, we started to notice fused cells formed by 4- 5 single cells merging together. Again, very low cell-free virus was detected suggesting cell-to-cell (well-differentiated cells with defined cilia structures) transmission as the likely main mode of transmission in NE cells. Mucus appears to play a role as barrier to trap cell-free viruses, and to prevent them from causing secondary infection.

HMPV knowledge is mostly inferred from RSV and other paramyxoviruses due to difficulties in virus cultivation. We had developed a lab-adapted HMPV strain which is very useful in studying the virus replication for correlation with host cell responses. HMPV M protein appears to play a modulation function in host cells at early infection, although further validation is needed. Hence, M may be a good target to consider in designing an antiviral strategy. We also have evidence of HMPV de-activating STAT-1 signaling molecule which may suggest a novel direction for understanding HMPV interactions with host cells.

We also discover that *in vitro*, RSV and HMPV both initially transmit from cell-to-cell rather than by free viral particles, which may be considered for future drug development.

CHAPTER 7. FUTURE WORKS

7.1 RSV works

7.1.1 Develop EM method to study RSV morphology

EM method is a useful tool to study any virus structure with the better resolution compared to immunofluorescent imaging, and we aim to apply it in RSV morphology studies. Some viral structural proteins are well known (F, G) but the remainders are not, for example M2-1, there are only a few reports stating M2-1's function and location.

7.1.2 RSV transmission in polarized MDCK cells

We conducted detailed RSV morphological and transmission studies in different non-polarized cells in vitro as reported in chapter 3. Among those results, non-polarized MDCK cells showed a transmission pattern similar to either HEp-2 or A549. Preliminary observation of RSV infection and transmission on polarized MDCK during the time course of 1- 5 dpi revealed interesting information of cells buckle up as round large structures on the surface of the monolayer which can be interpreted as an indicator of homogenous monolayer disruption. This observation proposes a need for further research to understand whether RSV would be able to transmit through polarized MDCK cell tight junctions or bud on the surface of the cell then spread utilizing a liquid environment in culture. To do this we will conduct low MOI RSV infection on MDCK with a tight junction marker attached to a fluorescent signal. Imaging analysis will be applied to examine whether virus transmission co-localized with tight junctions.

7.1.3 Host factors knock out in NE cells in correlation to RSV infection and host interaction

Our recent studies of RSV morphology and transmission studies using NE cells which are shown to be an efficient model for further host and viral infection interaction. In our previous work [74], we detected the presence heat shock proteins (HSP70, HSC70 and HSP90) in purified RSV particles, and especially HSP90 was revealed to have an

important function in RSV particle assembly. In the future, we would want to use either CRISPR or Lentivirus to knock out these host factors, e.g. HSP90, and examine how that would affect RSV infection and host responses.

7.2 HMPV works

7.2.1 Comparing replication and host responses caused by lab- adapted strain HMPV with some clinical isolates.

As we possess numbers of successful isolated HMPV from clinical samples, it is possible to adapt several of them to in vitro cultivation. Being able to do that will give us a pool of research objects which can be applied to compare host responses onto host cells of LLC-MK2. The results obtained, if possible, will enlarge the picture of HMPV and host interaction in the context of clinical research.

7.2.2 Detailed studies of M protein function at early infection are needed.

We observed the transportation of the M protein into the host cell nucleus at early infection and this might indicate the protein interferes with host transcription and translation machinery. More single cycle infection should be done, followed by M staining with host nucleus markers to confirm the presence of M within the host cell nucleus at early infection points (4- 6 hpi). Transfection with M- HMPV plasmid on LLC-MK2 are favored to observe the expression of the recombinant protein over time from as early as 4 hpi to late expression of 16 hpi. If the results are consistent with recent data by infection experiment, we can speculate about M's regulation function at early infection. Hence, infection or M-HMPV transfection experiments can be done with or without the presence of drugs which can stop nucleus material transportation to see if there is any effect on the infection/expression profile.

7.2.3 Validate HMPV infection in correlation with JAK/STAT signaling pathway

We observed an interesting event: HMPV infection turn off STAT1 in host cells which suggests by what method the virus hijacks the host response for better replication. As we are able to produce a better titer HMPV, it is possible to conduct biochemical analysis of purified viral particles to visualize by EM to have a better understanding of

the viral particle's morphology. We also can apply mass spectrometry to study purified HMPV particles' protein contents which will provide a better understanding of which host components are recruited into the virus particles. This data would be useful to elucidate about HMPV mechanism of infection, assembly and budding.

7.2.4 HMPV study on NE cells.

The human nasal epithelial stem/progenitor cells (hNESPCs) provided an easy, clean *in vitro* model to study human respiratory viruses. In this thesis, we reported a successful RSV morphological and transmission study utilizing hNESPCs. In the future, we also could conduct HMPV works using this model to obtain insightful information of HMPV morphology and transmission.

7.2.5 Transfect NS1 and NS2 of RSV in HMPV-infected cells to see if these non-structural proteins that antagonize type I interferon can enhance in vitro HMPV cultivation.

NS1 and NS2 of RSV inhibit the induction of interferon α/β in response to viral infection. In a minireplicon system complemented by plasmid-supplied support proteins, expression of NS1 was strongly inhibitory to transcription and RNA replication, affecting both genomic and antigenomic promoters. NS2 also showed inhibitory properties to both processes but only at relatively high levels of expressed protein. This suggested unidentified roles of NS1 and NS2 in RNA synthesis. Deletion of NS1 and NS2 from recombinant RSV attenuates virus growth *in vitro* [24]. HMPV lacking both NS1 and NS2 proteins has less growth potential compared to RSV *in vitro*. Hence, we can transfect NS1 or/and NS2 plasmids of RSV in HMPV-infected cells to test whether the presence of these non-structural proteins can enhance HMPV cultivation *in vitro*.

7.2.6 HMPV/RSV morphology and transmission studies under the effects of Ribavirin or Synagis.

Due to broad viral specificities of both ribavirin and Palivizumab (Synagis), studies were conducted to compare the ability of these two agents to inhibit the replication of RSV and HMPV in tissue culture-based assays. However, these studies only take into account the two drugs with the aspects of efficacy in treatment, not with

the morphological and transmission of RSV and/or HMPV aspects. Using our low MOI infection settings, both of the drugs can be used to study in detail, how HMPV or RSV morphology and transmission would be affected under the drugs' treatment. However, Synagis is a MAb which was developed specifically against RSV F protein and the usage of Synagis results in blocking RSV infection so it is expected that RSV viral particles would be blocked and RSV infection would not occur in cases where Synagis is in use, hence, Synagis can be useful for RSV or HMPV transmission studies but not the morphological studies. On the other hand, Ribavirin has antiviral activity in cell culture and in cotton rats. However its mode of action is unclear, hence, it would be interesting to observe how ribavirin would affect HMPV or RSV viral morphogenesis and transmission *in vitro*.

CHAPTER 8. PUBLICATIONS

Book Chapters

Tra Nguyen Huong, Boon Huan Tan, Richard J. Sugrue: *A Proteomic-Based Workflow Using Purified Respiratory Syncytial Virus Particles to Identify Cellular Factors as Drug Targets*. Human Respiratory Syncytial Virus, 01/2016; , ISBN: 978-1-4939-3685-4

Journal Publications

Tra Nguyen Huong, Laxmi Iyer Ravi, Boon Huan Tan, Richard J. Sugrue: *Evidence for a biphasic mode of respiratory syncytial virus transmission in permissive HEp2 cell monolayers*. Virology Journal 12/2016; 13(1). DOI:10.1186/s12985-016-0467-9

Muhammad Raihan Jumat, Yan Yan, Laxmi Iyer Ravi, Pusan Wong, Tra Nguyen Huong, Chunwei Li, Boon Huan Tan, De Yun Wang, Richard J Sugrue: *Morphogenesis of respiratory syncytial virus in human primary nasal ciliated epithelial cells occurs at surface membrane microdomains that are distinct from cilia*. Virology 07/2015; 484. DOI:10.1016/j.virol.2015.05.014

Muhammad Raihan Jumat, Tra Ngyen Huong, Laxmi Iyer Ravi, Rebecca Stanford, Boon Huan Tan, Richard J Sugrue: *Viperin protein expression inhibits the late stage of respiratory syncytial virus morphogenesis*. Antiviral Research 11/2014; 114. DOI:10.1016/j.antiviral.2014.11.007

Muhammad Raihan Jumat, Tra Nguyen Huong, Pusan Wong, Liat Hui Loo, Boon Huan Tan, Fiona Fenwick, Geoffrey L Toms, Richard J Sugrue: *Imaging analysis of*

human metapneumovirus-infected cells provides evidence for the involvement of F-actin and the raft-lipid microdomains in virus morphogenesis. Virology Journal 11/2014; 11(1). DOI:10.1186/s12985-014-0198-8

CHAPTER 9. BIBLIOGRAPHY

1. http://viralzone.expasy.org/all_by_species/556.html.
2. Adkins, B., C. Leclerc, and S. Marshall-Clarke, *Neonatal adaptive immunity comes of age*. Nat Rev Immunol, 2004. **4**(7): p. 553-64.
3. Bao, X., et al., *Human metapneumovirus glycoprotein G disrupts mitochondrial signaling in airway epithelial cells*. PLoS One, 2013. **8**(4): p. e62568.
4. Biacchesi, S., et al., *Recombinant human Metapneumovirus lacking the small hydrophobic SH and/or attachment G glycoprotein: deletion of G yields a promising vaccine candidate*. J Virol, 2004. **78**(23): p. 12877-87.
5. Biacchesi, S., et al., *Infection of nonhuman primates with recombinant human metapneumovirus lacking the SH, G, or M2-2 protein categorizes each as a nonessential accessory protein and identifies vaccine candidates*. J Virol, 2005. **79**(19): p. 12608-13.
6. Bisgaard, H., M. Study Group on, and V. Respiratory Syncytial, *A randomized trial of montelukast in respiratory syncytial virus postbronchiolitis*. Am J Respir Crit Care Med, 2003. **167**(3): p. 379-83.
7. Brown, G., et al., *Caveolin-1 is incorporated into mature respiratory syncytial virus particles during virus assembly on the surface of virus-infected cells*. J Gen Virol, 2002. **83**(Pt 3): p. 611-21.
8. Brown, G., H.W. Rixon, and R.J. Sugrue, *Respiratory syncytial virus assembly occurs in GM1-rich regions of the host-cell membrane and alters the cellular distribution of tyrosine phosphorylated caveolin-1*. J Gen Virol, 2002. **83**(Pt 8): p. 1841-50.
9. Brown, G., et al., *Analysis of the interaction between respiratory syncytial virus and lipid-rafts in Hep2 cells during infection*. Virology, 2004. **327**(2): p. 175-85.
10. Buchholz, U.J., et al., *Deletion of M2 gene open reading frames 1 and 2 of human metapneumovirus: effects on RNA synthesis, attenuation, and immunogenicity*. J Virol, 2005. **79**(11): p. 6588-97.
11. Burke, E., et al., *Role of cellular actin in the gene expression and morphogenesis of human respiratory syncytial virus*. Virology, 1998. **252**(1): p. 137-48.
12. Cai, H., et al., *Zinc binding activity of human metapneumovirus M2-1 protein is indispensable for viral replication and pathogenesis in vivo*. J Virol, 2015. **89**(12): p. 6391-405.
13. Carromeu, C., et al., *Intracellular localization of human respiratory syncytial virus L protein*. Arch Virol, 2007. **152**(12): p. 2259-63.
14. Chang, A., et al., *Human Metapneumovirus (HMPV) Binding and Infection Are Mediated by Interactions between the HMPV Fusion Protein and Heparan Sulfate*. Journal of Virology, March 2012: p. 3230-3243.
15. Chanock, R.M., et al., *Acute respiratory diseases of viral etiology. IV. Respiratory syncytial virus*. Am J Public Health Nations Health, 1962. **52**(6): p. 918-25.

16. Cheemarla, N.R. and A. Guerrero-Plata, *Immune Response to Human Metapneumovirus Infection: What We Have Learned from the Mouse Model*. Pathogens, 2015. **4**(3): p. 682-96.
17. Cheng, X., et al., *Overexpression of the M2-2 protein of respiratory syncytial virus inhibits viral replication*. J Virol, 2005. **79**(22): p. 13943-52.
18. Collins, P.L. and B.S. Graham, *Viral and host factors in human respiratory syncytial virus pathogenesis*. J Virol, 2008. **82**(5): p. 2040-55.
19. Cote, S., Y. Abed, and G. Boivin, *Comparative evaluation of real-time PCR assays for detection of the human metapneumovirus*. J Clin Microbiol, 2003. **41**(8): p. 3631-5.
20. Cox, R.G. and J.V. Williams, *Breaking in: human metapneumovirus fusion and entry*. Viruses, 2013. **5**(1): p. 192-210.
21. Dowell, S.F., et al., *Respiratory syncytial virus is an important cause of community-acquired lower respiratory infection among hospitalized adults*. J Infect Dis, 1996. **174**(3): p. 456-62.
22. El Najjar, F., et al., *Human metapneumovirus Induces Reorganization of the Actin Cytoskeleton for Direct Cell-to-Cell Spread*. PLoS Pathog, 2016. **12**(9): p. e1005922.
23. Ferkol, T. and D. Schraufnagel, *The global burden of respiratory disease*. Ann Am Thorac Soc, 2014. **11**(3): p. 404-6.
24. Fields, B.N., D.M. Knipe, and P.M. Howley, *Fields Virology*. 2007, Philadelphia : Wolters Kluwer Health/Lippincott Williams & Wilkins. p. 1601-1646.
25. Fixler, D.E., *Respiratory syncytial virus infection in children with congenital heart disease: a review*. Pediatr Cardiol, 1996. **17**(3): p. 163-8.
26. Gantke, T., et al., *IkappaB kinase regulation of the TPL-2/ERK MAPK pathway*. Immunol Rev, 2012. **246**(1): p. 168-82.
27. Ghildyal, R., A. Ho, and D.A. Jans, *Central role of the respiratory syncytial virus matrix protein in infection*. FEMS Microbiol Rev, 2006. **30**(5): p. 692-705.
28. Greensill, J., et al., *Human metapneumovirus in severe respiratory syncytial virus bronchiolitis*. Emerg Infect Dis, 2003. **9**(3): p. 372-5.
29. Gulen, F., et al., *Ten year retrospective evaluation of the seasonal distribution of agent viruses in childhood respiratory tract infections*. Turk Pediatri Ars, 2014. **49**(1): p. 42-6.
30. Haas, L.E., N.X. de Rijk, and S.F. Thijsen, *Human metapneumovirus infections on the ICU: a report of three cases*. Ann Intensive Care, 2012. **2**(1): p. 30.
31. Haas, L.E., et al., *Human metapneumovirus in adults*. Viruses, 2013. **5**(1): p. 87-110.
32. Hallak, L.K., et al., *Glycosaminoglycan sulfation requirements for respiratory syncytial virus infection*. J Virol, 2000. **74**(22): p. 10508-13.
33. Hasvold, J., et al., *The role of human metapneumovirus in the critically ill adult patient*. J Crit Care, 2016. **31**(1): p. 233-7.
34. Huong, T.N., et al., *Evidence for a biphasic mode of respiratory syncytial virus transmission in permissive HEp2 cell monolayers*. Virol J, 2016. **13**(1): p. 12.
35. Ingram, R.E., et al., *Detection of human metapneumovirus in respiratory secretions by reverse-transcriptase polymerase chain reaction, indirect immunofluorescence, and virus isolation in human bronchial epithelial cells*. J Med Virol, 2006. **78**(9): p. 1223-31.

36. Ivashkiv, L.B. and L.T. Donlin, *Regulation of type I interferon responses*. Nat Rev Immunol, 2014. **14**(1): p. 36-49.
37. Jeffree, C.E., et al., *Ultrastructural analysis of the interaction between F-actin and respiratory syncytial virus during virus assembly*. Virology, 2007. **369**(2): p. 309-23.
38. Johani, S.A. and J. Akhter, *Virology and Molecular Epidemiology of Respiratory Syncytial Virus (RSV)*, in *Respiratory Disease and Infection - A New Insight*. February 6, 2013, InTech. p. 33-46.
39. Johnson, J.E., et al., *The histopathology of fatal untreated human respiratory syncytial virus infection*. Mod Pathol, 2007. **20**(1): p. 108-19.
40. Jokela, P., et al., *Detection of human metapneumovirus and respiratory syncytial virus by duplex real-time RT-PCR assay in comparison with direct fluorescent assay*. Clin Microbiol Infect, 2010. **16**(10): p. 1568-73.
41. Jolly, C., et al., *HIV-1 cell to cell transfer across an Env-induced, actin-dependent synapse*. J Exp Med, 2004. **199**(2): p. 283-93.
42. Jumat, M.R., et al., *Imaging analysis of human metapneumovirus-infected cells provides evidence for the involvement of F-actin and the raft-lipid microdomains in virus morphogenesis*. Virol J, 2014. **11**: p. 198.
43. Jumat, M.R., et al., *Viperin protein expression inhibits the late stage of respiratory syncytial virus morphogenesis*. Antiviral Res, 2015. **114**: p. 11-20.
44. Jumat, M.R., et al., *Morphogenesis of respiratory syncytial virus in human primary nasal ciliated epithelial cells occurs at surface membrane microdomains that are distinct from cilia*. Virology, 2015. **484**: p. 395-411.
45. Karron, R.A., et al., *Severe respiratory syncytial virus disease in Alaska native children. RSV Alaska Study Group*. J Infect Dis, 1999. **180**(1): p. 41-49.
46. Kell, A.M. and M. Gale, Jr., *RIG-I in RNA virus recognition*. Virology, 2015. **479-480**: p. 110-121.
47. Kim, H.W., et al., *Epidemiology of respiratory syncytial virus infection in Washington, D.C. I. Importance of the virus in different respiratory tract disease syndromes and temporal distribution of infection*. Am J Epidemiol, 1973. **98**(3): p. 216-25.
48. Kiss, G., et al., *Structural analysis of respiratory syncytial virus reveals the position of M2-1 between the matrix protein and the ribonucleoprotein complex*. J Virol, 2014. **88**(13): p. 7602-17.
49. Lay, M.K., et al., *Advances in understanding respiratory syncytial virus infection in airway epithelial cells and consequential effects on the immune response*. Microbes Infect, 2013. **15**(3): p. 230-42.
50. Li, J., et al., *Evolutionary dynamics analysis of human metapneumovirus subtype A2: genetic evidence for its dominant epidemic*. PLoS One, 2012. **7**(3): p. e34544.
51. Liljeroos, L., et al., *Architecture of respiratory syncytial virus revealed by electron cryotomography*. Proc Natl Acad Sci U S A, 2013. **110**(27): p. 11133-8.
52. Ling, Z., K.C. Tran, and M.N. Teng, *Human respiratory syncytial virus nonstructural protein NS2 antagonizes the activation of beta interferon transcription by interacting with RIG-I*. J Virol, 2009. **83**(8): p. 3734-42.
53. Liu, J., et al., *Characterization of respiratory syncytial virus M- and M2-specific CD4 T cells in a murine model*. J Virol, 2009. **83**(10): p. 4934-41.

54. Lo, M.S., R.M. Brazas, and M.J. Holtzman, *Respiratory syncytial virus nonstructural proteins NS1 and NS2 mediate inhibition of Stat2 expression and alpha/beta interferon responsiveness*. J Virol, 2005. **79**(14): p. 9315-9.
55. Loo, L.H., et al., *Evidence for the interaction of the human metapneumovirus G and F proteins during virus-like particle formation*. Virol J, 2013. **10**: p. 294.
56. Low, K.W., et al., *The RSV F and G glycoproteins interact to form a complex on the surface of infected cells*. Biochem Biophys Res Commun, 2008. **366**(2): p. 308-13.
57. Martinez, I., et al., *Cultures of HEp-2 cells persistently infected by human respiratory syncytial virus differ in chemokine expression and resistance to apoptosis as compared to lytic infections of the same cell type*. Virology, 2009. **388**(1): p. 31-41.
58. McCurdy, L.H. and B.S. Graham, *Role of plasma membrane lipid microdomains in respiratory syncytial virus filament formation*. J Virol, 2003. **77**(3): p. 1747-56.
59. McDonald, T.P., et al., *Evidence that the respiratory syncytial virus polymerase complex associates with lipid rafts in virus-infected cells: a proteomic analysis*. Virology, 2004. **330**(1): p. 147-57.
60. McGillivray, G., et al., *Replication of respiratory syncytial virus is inhibited by the host defense molecule viperin*. J Innate Immun, 2013. **5**(1): p. 60-71.
61. Merolla, R., et al., *Respiratory syncytial virus replication in human lung epithelial cells: inhibition by tumor necrosis factor alpha and interferon beta*. Am J Respir Crit Care Med, 1995. **152**(4 Pt 1): p. 1358-66.
62. Mogensen, T.H., *Pathogen recognition and inflammatory signaling in innate immune defenses*. Clin Microbiol Rev, 2009. **22**(2): p. 240-273.
63. Mullins, J.A., et al., *Human metapneumovirus infection among children hospitalized with acute respiratory illness*. Emerg Infect Dis, 2004. **10**(4): p. 700-5.
64. Murphy, B.R. and E.E. Walsh, *Formalin-inactivated respiratory syncytial virus vaccine induces antibodies to the fusion glycoprotein that are deficient in fusion-inhibiting activity*. J Clin Microbiol, 1988. **26**(8): p. 1595-7.
65. Nair, H., et al., *Global burden of acute lower respiratory infections due to respiratory syncytial virus in young children: a systematic review and meta-analysis*. The Lancet, 2010. **375**(9725): p. 1545-1555.
66. Oshansky, C.M., et al., *Respiratory syncytial virus F and G proteins induce interleukin 1alpha, CC, and CXC chemokine responses by normal human bronchoepithelial cells*. J Infect Dis, 2010. **201**(8): p. 1201-7.
67. Panda, S., et al., *Human metapneumovirus: review of an important respiratory pathogen*. Int J Infect Dis, 2014. **25**: p. 45-52.
68. Parham, D.M., et al., *Cytologic diagnosis of respiratory syncytial virus infection in a bronchoalveolar lavage specimen from a bone marrow transplant recipient*. Am J Clin Pathol, 1993. **99**(5): p. 588-92.
69. Perry, A.K., et al., *The host type I interferon response to viral and bacterial infections*. Cell Res, 2005. **15**(6): p. 407-22.
70. Pham, Q.N., et al., *Chimeric recombinant human metapneumoviruses with the nucleoprotein or phosphoprotein open reading frame replaced by that of avian metapneumovirus exhibit improved growth in vitro and attenuation in vivo*. J Virol, 2005. **79**(24): p. 15114-22.

71. Piedimonte, G. and M.K. Perez, *Respiratory syncytial virus infection and bronchiolitis*. *Pediatr Rev*, 2014. **35**(12): p. 519-30.
72. Prince, G.A., et al., *Vaccine-enhanced respiratory syncytial virus disease in cotton rats following immunization with Lot 100 or a newly prepared reference vaccine*. *J Gen Virol*, 2001. **82**(Pt 12): p. 2881-8.
73. Principi, N. and S. Esposito, *Paediatric human metapneumovirus infection: epidemiology, prevention and therapy*. *J Clin Virol*, 2014. **59**(3): p. 141-7.
74. Radhakrishnan, A., et al., *Protein analysis of purified respiratory syncytial virus particles reveals an important role for heat shock protein 90 in virus particle assembly*. *Mol Cell Proteomics*, 2010. **9**(9): p. 1829-48.
75. Radhakrishnan, A., et al., *Protein Analysis of Purified Respiratory Syncytial Virus Particles Reveals an Important Role for Heat Shock Protein 90 in Virus Particle Assembly*. *Molecular Cell Proteomics*, 2010 Sep: p. 9(9): 1829–1848.
76. Ravi, L.I., et al., *Increased hydroxymethylglutaryl coenzyme A reductase activity during respiratory syncytial virus infection mediates actin dependent inter-cellular virus transmission*. *Antiviral Res*, 2013. **100**(1): p. 259-68.
77. Rawling, J., B. Garcia-Barreno, and J.A. Melero, *Insertion of the two cleavage sites of the respiratory syncytial virus fusion protein in Sendai virus fusion protein leads to enhanced cell-cell fusion and a decreased dependency on the HN attachment protein for activity*. *J Virol*, 2008. **82**(12): p. 5986-98.
78. Ren, J., et al., *Human metapneumovirus M2-2 protein inhibits innate cellular signaling by targeting MAVS*. *J Virol*, 2012. **86**(23): p. 13049-61.
79. Ren, J., et al., *Human metapneumovirus M2-2 protein inhibits innate immune response in monocyte-derived dendritic cells*. *PLoS One*, 2014. **9**(3): p. e91865.
80. Ridley, A.J., *Rho GTPases and actin dynamics in membrane protrusions and vesicle trafficking*. *Trends Cell Biol*, 2006. **16**(10): p. 522-9.
81. Rixon, H.W., et al., *The small hydrophobic (SH) protein accumulates within lipid-raft structures of the Golgi complex during respiratory syncytial virus infection*. *J Gen Virol*, 2004. **85**(Pt 5): p. 1153-65.
82. Roman, M., et al., *Respiratory syncytial virus infection in infants is associated with predominant Th-2-like response*. *Am J Respir Crit Care Med*, 1997. **156**(1): p. 190-5.
83. Rossi, G.A. and A.A. Colin, *Infantile respiratory syncytial virus and human rhinovirus infections: respective role in inception and persistence of wheezing*. *Eur Respir J*, 2015. **45**(3): p. 774-89.
84. San-Juan-Vergara, H., et al., *Cholesterol-rich microdomains as docking platforms for respiratory syncytial virus in normal human bronchial epithelial cells*. *J Virol*, 2012. **86**(3): p. 1832-43.
85. Schildgen, V., et al., *Human Metapneumovirus: lessons learned over the first decade*. *Clin Microbiol Rev*, 2011. **24**(4): p. 734-54.
86. Schoggins, J.W. and C.M. Rice, *Interferon-stimulated genes and their antiviral effector functions*. *Curr Opin Virol*, 2011. **1**(6): p. 519-25.
87. Schuster, J.E. and J.V. Williams, *Human Metapneumovirus*. *Microbiol Spectr*, 2014. **2**(5).
88. Shahda, S., et al., *The human metapneumovirus: a case series and review of the literature*. *Transpl Infect Dis*, 2011. **13**(3): p. 324-8.
89. Shaikh, F.Y., et al., *Respiratory syncytial virus assembles into structured filamentous virion particles independently of host cytoskeleton and related proteins*. *PLoS One*, 2012. **7**(7): p. e40826.

90. Shay, D.K., et al., *Bronchiolitis-associated mortality and estimates of respiratory syncytial virus-associated deaths among US children, 1979-1997*. J Infect Dis, 2001. **183**(1): p. 16-22.
91. Spann, K.M., et al., *IRF-3, IRF-7, and IPS-1 promote host defense against acute human metapneumovirus infection in neonatal mice*. Am J Pathol, 2014. **184**(6): p. 1795-806.
92. Stokes, K.L., et al., *The respiratory syncytial virus fusion protein and neutrophils mediate the airway mucin response to pathogenic respiratory syncytial virus infection*. J Virol, 2013. **87**(18): p. 10070-82.
93. Sugrue, R.J., B.-H. Tan, and L.-H. Loo, *The emergence of human metapneumovirus*. Future Virology, 2008. **3**(4): p. 363-371.
94. Sun, Z., et al., *Respiratory syncytial virus entry inhibitors targeting the F protein*. Viruses, 2013. **5**(1): p. 211-25.
95. Tayyari, F., et al., *Identification of nucleolin as a cellular receptor for human respiratory syncytial virus*. Nat Med, 2011. **17**(9): p. 1132-5.
96. Techaarpornkul, S., N. Barretto, and M.E. Peeples, *Functional analysis of recombinant respiratory syncytial virus deletion mutants lacking the small hydrophobic and/or attachment glycoprotein gene*. J Virol, 2001. **75**(15): p. 6825-34.
97. Thyrell, L., et al., *Mechanisms of Interferon-alpha induced apoptosis in malignant cells*. Oncogene, 2002. **21**(8): p. 1251-62.
98. Treuhart, M.W. and M.O. Beem, *Defective interfering particles of respiratory syncytial virus*. Infect Immun, 1982. **37**(2): p. 439-44.
99. Troutman, T.D., J.F. Bazan, and C. Pasare, *Toll-like receptors, signaling adapters and regulation of the pro-inflammatory response by PI3K*. Cell Cycle, 2012. **11**(19): p. 3559-67.
100. Vareille, M., et al., *The airway epithelium: soldier in the fight against respiratory viruses*. Clin Microbiol Rev, 2011. **24**(1): p. 210-29.
101. Wei, Y., et al., *Roles of the putative integrin-binding motif of the human metapneumovirus fusion (f) protein in cell-cell fusion, viral infectivity, and pathogenesis*. J Virol, 2014. **88**(8): p. 4338-52.
102. Whimbey, E., et al., *Community respiratory virus infections among hospitalized adult bone marrow transplant recipients*. Clin Infect Dis, 1996. **22**(5): p. 778-82.
103. Williams, J.V., et al., *Human metapneumovirus infection in children hospitalized for wheezing*. J Allergy Clin Immunol, 2005. **115**(6): p. 1311-2.
104. Yan, Y., et al., *Human nasal epithelial cells derived from multiple subjects exhibit differential responses to H3N2 influenza virus infection in vitro*. J Allergy Clin Immunol, 2016. **138**(1): p. 276-281 e15.
105. Zhao, X., et al., *The use of nasal epithelial stem/progenitor cells to produce functioning ciliated cells in vitro*. Am J Rhinol Allergy, 2012. **26**(5): p. 345-50.
106. Zheng, J., et al., *Respiratory Syncytial Virus Nonstructural Proteins Upregulate SOCS1 and SOCS3 in the Different Manner from Endogenous IFN Signaling*. J Immunol Res, 2015. **2015**: p. 738547.