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Comments

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Novel methods for detecting glycan receptors for influenza A virus and exploration of the function of the sialyltransferases on influenza viral infection

By

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Sialic acids (Sias) are receptors for influenza A viruses (IAVs). The influence of individual sialyltransferases on the Sias expressed is not understood. Also, sensitive methods to distinguish Sias on the cells are lacking. Our goals were to establish a method to detect Sias on the cell and to evaluate the importance of CMP-sialic acid transporter (SLC35A1) and ST6 beta-galactoside alpha-2,6-sialyltransferase 1 (ST6GAL1) for IAV infection. A proximity ligation assay was established to detect and quantify Sias. Knockout mutants ΔSLC35A1 and ΔST6GAL1 of A549 cells were generated. Compared to the wild type, neither α2,3-linked Sias (SA2,3Gal) nor α2,6-linked Sias (SA2,6Gal) were detected on ΔSLC35A1, and SA2,6Gal was reduced on ΔST6GAL1. Expression of SA2,3Gal and SA2,6Gal associated sialyltransferase genes changed in the mutants. Most of the tested IAVs did not replicate on ΔSLC35A1, but six of the H3N2 human seasonal IAVs did. This suggests that certain IAVs may not require Sia-dependent attachment.

DEDICATION

I would like to dedicate this thesis to my family: my wife, Lei Zhong and my baby. Thank you for all of your support, encouragement and steadfast love throughout this journey. Without you, I can't imagine how helpless and lonely I would be. Let's look forward to the next chapter of our life. I love you all.

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CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE

Sialic acids (Sias), have been recognized as the receptors for Influenza A Viruses (IAV). The aim of this thesis is to evaluate the role specific enzymes that regulate glycosylation have on the expression of IAV receptors and the effects on IAV infection. To do accomplish study, we need to understand the basis of glycosylation, develop an effective detection method to distinguish glycans and generate mutant cell lines. The methods that we used in this study include proximity ligation assays and CRISPR-Cas9.

1.1 Glycans

It is well documented that the exterior surface of cells is coated by the glycans, termed as "glycocalyx" (Solís et al. 2015)(Gabius 2015). The existence of this "coat" is associated with the biological functions, such as protein stability and function (Tran and Ten Hagen 2013), cell communication (Bassagañas et al. 2014), host-pathogen interaction (Feng and Elson 2011)(Hajishengallis et al. 2012), and so on. There are six glycan types in the eukaryotes, including *O*-linked glycans (abbreviated as *O*-glycans), *N*-linked glycans (abbreviated as *N*-glycans), glycosaminoglycans, glycosylphosphatidylinositol (GPI) anchors, glycosphingolipids (GSLs) and sialic acids (Sias)(Corfield and Berry 2015).

1. *O*-glycans: α-linkage of *N*-acetyl-D-galactosamine to the side-arm hydroxyl groups of serine and threonine residues (Corfield and Berry 2015). No consensus

sequence has been identified in proteins with *O*-glycans.

2. *N*-glycans: β-N-glycosidic linkage of an *N*-acetyl-D-glucosamine residue with a peptide or protein asparagine. The protein has been recognized with a tri-amino acid sequon: Asparagine-X-serine/threonine, X might be any amino acid, except proline (Kelleher and Gilmore 2006).
3. Glycosaminoglycans: repeated disaccharide units that consist of either sulfated or non-sulfated monosaccharides. Diversity of glycosaminoglycans depends on the tissue distribution (Afratis et al. 2012).
4. GPI anchors: ethanolamine-phosphate-6Manα1-2Manα1-6Manα1-4GlcNα1-6myo-inositol-1-phosphate-lipid. Proteins are attached to the amino group of the ethanolamine through their C-terminal carboxyl groups (Menon 2013).
5. Glycosphingolipids (GSLs): a membrane lipid core covalently linked to glycan motifs. The variety of glycan structure relies on the process of precursor synthesis and sugar transporting (D'Angelo et al. 2013).

Sias are a group of the *O*- or *O*-substituted derivatives of 2-keto-3-deoxynononic-acid (Kdn) or neuraminic acid (Neu), which are negatively charged acidic sugars and six membered-ring monosaccharides with C7 to C9 glycerol chain attaching C6. And there is a carboxyl group on C2 and an amino group on C5 with acetyl or glycolyl, which serves as the parents for derivatives with modification. Specifically, *O*-acetylation usually occurs at position 4, 7, 8, and 9, *O*-methylation and *O*-sulfatation at position 8, and *O*-lactylation, phosphatation, and *O*-sulfatation at position 9. In the α/β configuration, the glycosidic hydroxyl at C2 forms linkages to other sugars. Thus,

the linkages of α2,3, α2,6, and α2,8 (to other Sias) are frequently observed. Anhydro forms exist through the linkage between C2 and C3, for example, in Neu2en5Ac (Verheijen 2008). There is only a difference at C5 amino group substitution with acetyl and glycolyl (Angata and Varki 2002)(Verheijen 2008). To date, more than 50 Sias have been identified. *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc) are the two common Neu derivatives in mammalian cells (Angata and Varki 2002). However, humans are lack of Neu5Gc, due to the inactivation of hydroxylase in humans (Varki 2007). The plants, prokaryotes, arthropod and most invertebrates are without Sias (Verheijen 2008).

Biosynthesis process of the Sias initiates *de novo* from UDP-GlcNAc via the function of the UDP-GlcNAc epimerase/kinase complex and culminates with the CMP-β-*O*-Neu5Ac formation. When Neu5Gc is produced from the diet, CMP-β-*O*-Neu5Gc is generated through the activation of salvage pathways. As the substrates for the sialyltransferase (ST), CMP-β-O-Sias are found to form the huge diversity of sialoglycoconjugates (Corfield and Berry 2015). Due to the wide range of potential acceptors, the process of biosynthesis is mediated by STs in the Golgi. Some STs show specificity to acceptors, i.e., glycoproteins, glycolipids or gangliosides. A special peptide sequences exist in the STs, termed 'sialylmotifs', which are found in most eukaryote but not in bacteria, suggesting distinct evolutionary routes (Harduin-Lepers et al. 2005).

Kdn, a Sia-related monosaccharide, is found in lower level eukaryotes. In the protozoa, trypanosome recognizes and then cleaves cell surface-bound Sias. After that, the trypanosome keeps transferring Sias to protozoa cell surface acceptors for immune

escape. The trypanosome is defined as a novel transferase, but more than a transferase for its function (Schenkman et al. 1994).

A variety of sialoglycoconjugates to Sias have been identified in the eukaryote (Büll et al. 2016). Selectin, sialic acid-binding immunoglobulin-like lectin (Siglec) and factor H are two mainly binding receptors for Sias (Varki 2007)(Blaum et al. 2015), and Siaglecs are the most abundant one in humans. Siaglecs are mainly secreted by immune cells and separated into two groups: the conserved Siglecs (1, 2, 4, and 15) and the CD33-related Siglecs (3, 5, 6, 7, 8, 9, 10, 11, 14, and 16) (Pillai et al. 2012). Of note, Siglec12 lost the ability to bind Sias and Siglec-13/17, which are inactivated during human evolution, are not in these two groups (Wang et al. 2012). Most Siglecs have an intracellular immunoreceptor tyrosine-based inhibition motif (ITIM), mediating inhibition of binding Sias (Powell and Varki 1995)(MacAuley et al. 2014).

Sia was the first viral receptor discovered by Hirst and McClelland (Hirst 1941). The influenza virus was discovered to have the hemagglutination ability. Also, the attached virus could be eluted from erythrocytes at 37°C, indicating the an enzymatic destruction of a receptor substance on the cells (Hirst 1941). Later, a similar action was shown in *Vibrio cholerae* cultures, and then the term ‘receptor-destroying enzyme was introduced (Burnet and Stone 2007). The substance was first characterized as a carbohydrate with low molecular weight (Gottschalk and Lind 1949) and then identified as *N*-acetyl-D-neuraminic acid (Tischer 1959). Therefore, it was clear that the sialic acid was the receptor determinant and neuraminidase was the viral enzyme.

1.2 Sialyltransferase (ST)

In mammals, Sias are often linked with glycoconjugates sugar chains at non-

reducing terminal position, α2,3-linked to Gal residue, α2,6-linked to Gal/GalNAc/GlcNAc residue. Sias are also found to be α2,8-linked to gangliosides and polysialic acid (PSA), a linear α 2,8-homopolymer (Harduin-Lepers et al. 1995)(Dall'Olio and Chiricolo 2001). Sialylation process initiates in the Golgi and is catalyzed by a transferase family, named sialyltransferase (Li and Chen 2012)(Bork et al. 2017). To date, 20 human STs have been characterized since 2001 (Harduin-Lepers et al. 2001)(Cohen and Varki 2010). Based on the linkage types, the STs are divided into four groups: β-Gal α2,3-STs (n= 6, ST3Gal1-6), β-Gal α2,6-STs (n=2, ST6GAL1 and 2), GalNAc α2,6-STs (n=6, ST6GALNAc1-6), and α2,8-STs (n=6, ST8Sia1-6)(Bork et al. 2017). Such an abundant number of STs in mammals suggests the variety of Sias derivatives. The STs of the same group may have some overlapping functions but different substrate specificities (Cazet et al. 2010). Mammalian STs require specific acceptors but have relatively relaxing donor specificity (Datta 2009). CMP-Neu5Gc, CMP-Neu5,9Ac2 (Shelton and Epps 2016), and CMP Kdn (Angata et al. 1998) have shown to be acceptable donor substrates by some mammalian STs (GalNAc alpha 2,6 sialyltransferase / Galbeta1-->4GlcNAc alpha2, 6-sialyltransferase)(Li and Chen 2012). The donor specificities have been less explored.

Like other glycosyltransferases in vertebrates, mammalian STs are Type II membrane proteins, settled in the Golgi. The structure has been dissected: an *N*-terminal cytoplasmic domain, a single transmembrane domain (16–20 amino acid residues), a size variable (20–300 amino acid residues) stem region, and a soluble relatively well-conserved C-terminal catalytic domain of 300 (± 20) amino acid residues in the Golgi lumen (Audry et al. 2011)(Chen and Varki 2010). The mammalian STs have four

conserved sialylmotifs of the catalytic domains, including long (L), short (S), very small (VS) motifs, and motif III (Datta and Paulson 1995)(Datta et al. 1998)(Jeanneau et al. 2004). The functions of these motifs include disulfide bonds formation, substrates recognition (Datta and Paulson 1995)(Datta et al. 1998), and catalytic action (Jeanneau et al. 2004). The sialylmotifs are helpful to identify eukaryotic ST genes (Harduin-Lepers et al. 2001). A disulfide bond between L and S keeps conserved among STs (Datta et al. 2001). The mutation of ST3Gal1 showed L functions in the binding to the donor whereas motif III and VS are involved in the binding to the acceptor and S in the binding to both the donor and the acceptor (Datta 2009)(Audry et al. 2011)(Rao et al. 2009)(Paulson and Rademacher 2009). Based on the X-ray crystal structures, VS has a conserved histidine residue as the catalytic base in a single Rossmann domain (Rao et al. 2009). Among the four group STs in mammals, several STs being involved in the *N*-glycan modification including ST6GAL1, ST6GAL2, ST3GAL4, ST3GAL6, ST8Sia2, and ST8Sia4, have been studied more than others.

1.2.1 α2,6 STs

In 1970s, STs activities were first identified in mammalian tissues (Bartholomew et al. 1973)(Vena and Versace 2011). Following that, researchers isolated and characterized an α2,6-ST from bovine in 1977 (Paulson et al. 1977a)(Paulson et al. 1977b). This enzyme was termed later as ST6GAL1. Of interests, the soluble ST6GAL1 in body fluids are different types of those in the cell (Weinstein et al. 1987). Subsequently, this ST is found to be cleaved in a late Golgi or post-Golgi compartment (Kitazume-Kawaguchi et al. 1999)(Ma et al. 1997) and several proteases, including cathepsin D and BACE, could be involved (Kitazume et al. 2001)(Lammers and

Jamieson 1989). Only until 2002, another ST, ST6GAL2, was identified and characterized via cloning and sequencing (Takashima et al. 2002).

ST6GAL1 expresses with tissue specificity, and the expression is modulated by multiple promoters (Svensson et al. 1992)(Wang et al. 1993)(Wen et al. 1992). One promoter keeps its expression in the liver at a low but steady level. The other promoter, an inducible promoter P1 only in the liver, drives high ST6GAL1 expression and increases soluble ST6GAL1 in the blood during inflammation (Appenheimer et al. 2003). This inducible pool is quite important to regulate myelopoiesis/hematopoiesis (Jones et al. 2010)(Nasirikenari et al. 2014). A recent study showed that CMP-Sias are released by activated platelets to serve as the donor to be used by circulating ST6GAL1 (Lee et al. 2014). This process results in the remodeling of the glycans on hematopoietic progenitor cells. Another study further showed that IgGs expressed by ST6GAL1 deficient B cells are sialylated by circulating ST6GAL1 (Davison 2011).

1.2.2 α2,3 STs

These STs function by adding a single Sia with α2,3-linkage to Gal residues of glycoconjugates. It is reported Galβ1,4(3)GlcNAc on *N*-glycans , along with core-2, core-3 or core-4 *O*-glycans, is sialylated by ST3Gal4 (Yang et al. 2012), and so is the Galβ1,3GalNAc terminated structures in glycoconjugates (Kitagawa and Paulson 1994)(Sasaki et al. 1993). The ST3Gal4 recognizes Galβ1,4GlcNAc as acceptors on glycoconjugates, but has an additional preference for the glycolipid moieties. Since both enzymes catalyze Galβ1,4GlcNAc, they synthesize determinant of sialyl Lewis X (sLeX) on leukocyte E, L and P-selectin ligands (Kitagawa and Paulson 1994). These ligands are essential for binding endothelial cells, and their slow rolling and tethering

prior to extravasation (Mayer et al. 2017).

1.2.3 α2,8 STs

The polySia plays a very important role during the cell migration and plasticity in the brain (Werten et al. 1996; Rutishauser 2008; Colley et al. 2014). ST8Sia2 and ST8Sia4 catalyze the polymerization of long chains extending both *N*-linked and *O*-linked glycans from 8 to over 400 residues (Sato and Kitajima 2013). Through structural analysis of the polysialylation of neural cell adhesion molecule (NCAM), the polySias are shown to be added to tetra-antennary complex glycans with different numbers (von Der Ohe et al. 2002)(Wuhrer et al. 2003). ST8Sia4 polysialylates neuropilin-2 (NRP-2) in COS-7 cells, and core-1 and core-2 O-glycans are sialylated (Rollenhagen et al. 2013). It is the acceptor specificity that differs polysialylation from the glycosylation catalyzed by α2,6- and α2,6-STs, which makes very few glycoprotein substrates be recognized and then polysialylated by polySTs (Colley et al. 2014). Interestingly, the two polySTs prefer different substrates. For instance, while whereas the NCAM can be polysialylated by both ST8Sia2 and ST8Sia4, the polysialylation of NRP-2 is exclusively catalyzed by ST8Sia4, and the polysialylation of SynCAM-1 is exclusively catalyzed by ST8Sia2 (Galuska et al. 2010; Rollenhagen et al. 2012, 2013). In mammals, polySias on *N*- and *O*-linked glycans are essential for nervous system development and to support the conduction process in the adult brain (Werten et al. 1996; Rutishauser 2008; Colley et al. 2014). The regeneration function of polySia has been revealed in damaged neurons and liver development (Bonfanti 2006; El Maarouf et al. 2006; Zhang et al. 2007a, 2007b). In addition, tumor cells can upregulate polySia expression, which is related to increased cell invasion and metastasis (Colley et al. 2014)(.A. Falconer et

al. 2012). The polySia is used as a biodegradable substitute to maintain the stabilization and prolong half-life of therapeutic materials (Bader and Wardwell 2014), and as a vector component for drug delivery (Zhang et al. 2014)(Zhang et al. 2016).

1.3 CRISPR

The bacteria and archaea have an immune system that is used to protect themselves from the invaders, like virus (phages) and some plasmids (Mohanraju et al. 2016). The immune system has evolved in several ways to defend these "non-self" particles with DNA interference, abortive infection and the modification of the receptors, which belongs to the innate immunity (Makarova et al. 2013a). Meanwhile, there is another way present in the all archaea and approximately half of bacteria, named CRISPR-Cas (Clustered Regularly Interspaced short palindromic Repeats)-(CRIPSR associated proteins) system (Mojica et al. 2005). This system is responsible for the adaptive immunity, which can provide a rapid adaption and effective elimination of the evolving virus. It was in 1987, the first time when the researchers realized CRISPR loci existing through the sequencing. At that time, the conclusion was "the biological significance of these sequences is not known" (Ishino et al. 1987). Until the end of last century, a relatively comprehensive document appeared, which benefited from the sequence accumulation of the prokaryotic genome (Mojica et al. 2000). After that, the related gene and transcripts were figured out in 2002 (Tang et al. 2002). A milestone was the findings if there was homology between the spacers and the sequence in phages or foreign plasmids, there was a lower possibility that they would be infected by the phages.

Based on the discoveries above, an idea came that CRISPR- Cas systems can

defend the foreign elements. The following experiments and tests have been performed to demonstrate the features of the system. At the beginning of infection the system was found to integrate the spacers specific for the virus into the array, while later during the infection the spacers are used to eliminate the foreign nucleic acid (Bolotin et al. 2005). These observations demonstrate that the system is adaptive and evolutional. In addition, mutation of the catalytic residues of the RNase which is responsible for the generation of mature CRISPR RNAs, abolished CRISPR defense (Barrangou et al. 2007). Furthermore, another study by Marraffini and Sontheimer showed that CRISPR immunity limits horizontal gene transfer in *S. epidermidis* (Marraffini and Sontheimer 2010). A spacer in the *loci* of *Staphylococcus epidermidis*, matches part of the nickase gene of staphylococcal plasmids, which prevented the conjugative transfer. Moreover, CRISPR-Cas defense targets DNA, rather than RNA (Marraffini and Sontheimer 2010). CRISPR interference is blocked when the nickase gene is inserted into a self-splicing intron (Pennisi 2013). These studies indicate CRISPR-Cas system may be possible for the establishment of DNA-based manipulation tools.

Considering the similarity between Cas proteins and the locus structure, the CRISPR-Cas system has been identified as two classes. Class 1 systems are recognized as the evolutionary ancestral system and Class 2 systems, which are used for gene edition, with different nucleases are the development of Class 1 systems (Mohanraju et al. 2016). The two classes are temporarily further separated into six types and then 19 subtypes (Makarova et al. 2015)(Shmakov et al. 2015).

1.3.1 Class 1 systems

Class 1 systems exist in all archaea and most if bacteria and are grouped further

into types I, III, and IV. The effector complexes are composed of 4 to 7 protein subunits, containing variable RRM (RNA recognition motif) domains (Makarova et al. 2011a)(Makarova et al. 2013b). Among these three types, type I and III are more studied. The CRISPR-associated complex (Cascade) and Cas 3 nuclease are the core units for type I systems (Brouns et al. 1993). Cascade cuts at each position of the stem-loop region in the precursor crRNA and crRNA guides are then generated. The target DNA is scanned by the Cascade-crRNA complex for sequence matching. An R-loop forms when annealing between the crRNA and the target region. From the 5' end of the crRNA, the first 8 nucleotides play an important role for target binding (Haurwitz et al. 2010)(Jore et al. 2011)(Wiedenheft et al. 2011). The mutated phages in this region are able to escape type I systems (Semenova et al. 2011). However, mutant on the sixth position is not helpful. The interaction between a mature crRNA and its target DNA is uncovered through the crystal structure analysis, showing that ribbon structure is formed, not engaged in the base pair formation(Jackson et al. 2014)(Mulepati et al. 2014)(Zhao et al. 2014). Finally, Cas3 nuclease cleaves the target and also degrades the complementary strand (Hochstrasser et al. 2014)(Huo et al. 2014)(Mulepati and Bailey 2013)(Sinkunas et al. 2011).

Type III systems have the Cas 6 complex and Cas 10 complex(Carte et al. 2008)(Sokolowski et al. 2014). The crRNA tag, a repeat sequence with the length of 8 nucleotides flanks at the 5' end of the spacer. With a repeat-specific endoribonuclease named Cas6, the generated small crRNAs combine with a larger complex, the Cas10-Csm for type III-A systems or Cas10-Cmr complex for III-B systems (Carte et al. 2008)(Marraffini and Sontheimer 2010). The complex trims the 3' end at 6-nucleotide

intervals (Hale et al. 2008)(Hatoum-Aslan et al. 2011)(Hatoum-Aslan et al. 2013). Different from type I and II systems, both the target DNA (Deng et al. 2013)(Goldberg et al. 2014)(Samai et al. 2015) and its transcripts (Kawecki and Ebert 2004)(Staals et al. 2014)(Tamulaitis et al. 2014)(Zebec et al. 2014)(Zhang et al. 2012) are cleaved by type III systems. Cas10 cleaves of the non-template DNA strand, Csm3 in type III-A(Staals et al. 2014) or Cmr4 in type III-B (Tamulaitis et al. 2014) is required for cleavage of the transcripts (Samai et al. 2015). Up to now, no PAM request appears during type III systems targeting (Marraffini and Sontheimer 2010). The binding of crRNA tag and the flanking sequence helps avoid self-targeting. To prevent DNA targeting resulting in autoimmunity, exact match between the tag and the repeat sequence is required (Marraffini and Sontheimer 2010)(Samai et al. 2015)(Zebec et al. 2014).

The similarity of the sequence between each subunit of type I and type III effector complexes is low, although the overall architectures of the complex are strikingly similar, suggesting a common origin of these two effector complexes (Shmakov et al. 2015)(Makarova et al. 2011a)(Rouillon et al. 2013)(Spilman et al. 2013). The ancestral CRISPR-Cas systems tend to resemble the extant type III complexes, indicated by the presence of the large Cas10 subunit with an active DNA polymerase when appearing (Shmakov et al. 2015)(Makarova et al. 2011a)(Makarova et al. 2013b).

1.3.2 Class 2 systems

Class 2 systems, which are less common than Class 1 systems, include types II, V and VI systems. Compared with Class 1 systems, the effector complex has a single multidomain protein (Makarova et al. 2015). Cas9 in type II systems is the best

characterized Class 2 effector protein, containing two unrelated nuclease domains HNH and RuvC, which are used for the cleavage of the target and the displaced strand, respectively (Savell and Day 2017). At the same time, two RNAs are required: the crRNA and trans-acting CRISPR RNA (tracrRNA). The tracrRNAs are essential for pre-crRNA generating and target recognition (Deltcheva et al. 2011)(Chylinski et al. 2014). The sequence analysis of amino acid showed only one traceable nuclease domain (RuvC-like) in type V effector Cpf1 (Zetsche et al. 2015). Interestingly, the second nuclease domain has been revealed with the combination of Cpf1 and crRNA, whereas the structure of this domain is different from HNH in Cas9 but functionally analogous to the HNH domain (Schaber 2016).

There are several differences between Cas9 and Cpf1. The most important one is that Cas9 is crRNA-dependent endonuclease, but Cpf1 doesn't need tracrRNA. Moreover, without the RNase III, the Cpf1 can process pre-crRNA generation. The cleavage pattern and PAM also differ in Cpf1 and Cas9 (Chylinski et al. 2014). Furthermore, the distant relationship between Cas9 and Cpf1, indicates that other distinct variants could exist. Type VI systems have the effector protein containing two conserved higher eukaryotes and prokaryotes nucleotide-binding (HEPN) domains with RNase activity (Shmakov et al. 2015)(Shmakov et al. 2017). C2c1 and C2c2 (endoribonuclease of HEPN superfamily) are expressed and able to operate interference (Shmakov et al. 2015)(Abudayyeh et al. 2016).

The computational pipeline has been used to analyze the genome of CRISPR-Cas systems (Shmakov et al. 2015), revealing an evident trend that the complexes of adaptation and effector modules have evolved, as well as a vital contribution of MGEs

to the origin of the CRISPR-Cas systems and the diversification of the effector complexes (Takeuchi et al. 2012)(Koonin and Wolf 2015). The universal existence of Class 1 systems, along with the development of the RRM domain in Class 1 effector complex, suggesting the ancestral systems belonged to Class 1 (Mohanraju et al. 2016). Then through several substitutions of the Class 1 locus with nuclease genes, the Class 2 variants evolved. Particularly, it seems that effector variants of Type V originated from the TnpB, a widespread transposase (Shmakov et al. 2015), while the Cas9 (Type II) is likely to evolve from IscB, a unique transposon family (Kapitonov et al. 2016). Remarkably, Class 2 systems appears to be the derivatives of different MGEs: Cas1 from a casposon, Cas2 from a toxin-antitoxin module, and other effector proteins from respective transposable elements (Shmakov et al. 2015).

1.4 Techniques used in this study

1.4.1 Proximity Ligation Assay (PLA) and polyclonal antibody-based proximity ligation assays (polyPLA)

The proximity assay is a method based on the combination of protein-protein interactions and quantitative PCR (qPCR). Generally, the oliognucleotides (‘proximity arms’) are attached to specific protein-binding reagents. When two such proximity probes recognize and bind to the target, the ends of their conjugated oligonucleotides come close to be ligated. Then the ligation product can be detected and quantified through qPCR. In 2009, PLA was used to detect the AIV antigens (Schlingemann et al. 2010). And it was more sensitive than the “sandwich” enzyme-linked immunosorbent assay (ELISA) (Schlingemann et al. 2010)(Belák et al. 2009).]

Recently by using polyclonal antibody, a new method, called polyPLA, was

developed to detect the antigenic variants (Martin et al. 2015). This method was used to detect cytokines for proteome analyses (Gullberg et al. 2004). Also, the foot-and-mouth disease virus was detected rapidly and sensitively by PLA (Nordengrahn et al. 2008).

In our project, the probes are formed between the STV-oligonucleotide and biotinylated lectin through the hydrogen bonds. The lectin is used to recognize and bind the Sias on the cell surface. Then the pair of oligonucleotides ligates with each other to form the template for the qPCR reaction. The more templates form, the stronger signal produces. The ΔCt was determined by the quantity of the targets.

1.4.2 Proximity Ligation Assay (*in situ* PLA)

Similar to PLA, *in situ* PLA use a pair of oligonucleotide-labeled specific protein-binding reagent (PLA probes). The hybridizing connector oligos join the PLA probes only if they are in close proximity to each other and ligase forms a closed, circle DNA template that is required for rolling-circle amplification (RCA). The PLA probe then acts as a primer for a DNA polymerase, which generates concatemeric sequences during RCA. This allows up to 1,000-fold amplified signal that is still tethered to the PLA probe, allowing localization of the signal. Lastly, labeled oligos hybridize to the complementary sequences within the amplicon, which are then visualized and quantified as discrete spots (PLA signals) by microscopy image analysis.

Examples of the use of *in situ* PLA include the demonstration of protein- protein interactions. Emerin-lamins interactions showed by *in situ* PLA are important for the maintenance of nuclear integrity (Sakaki et al. 2001). Also, the E-cadherin and the beta-catenin interactions were confirmed by *in situ* PLA (De Wever et al. 2008). These interactions play an important role in cellular adhesion in the epithelial cells (Stemmer et

al. 2008).Based on the different situation, the signals cane be acquired from IHC or IFA.

In our project, the probes are formed between the oligonucleotide and non-labeled lectin through the SANH and aldehyde. The lectin is used to recognize and bind the Sias on the tissue surface. Then the pair of oligonucleotides ligates with each other to form the template for rolling circle amplification (RCA). The detection probes with different fluorescent signals will be available for fluorescent microscope for observation.

1.4.3 CRISPR-Cas9 gene edition

To maximize the successes of CRISPR-Cas9 gene edition, it is commonly to target the first exon or at least the first one third part of the target gene. There are several online tools being available (Broad Institute, CloneTech, MIT) for design single guide RNA (sgRNA), which contains a short crRNA sequence fused to the scaffold tracrRNA sequence. Then, the sgRNA was inserted into a plasmid vector, which will be transfected into bacteria with antibiotics (or other methods) for selection. Then, Surveyor Nuclease, a member of the CEL family of mismatch-specific nucleases, recognizes and cleaves mismatches due to the presence of single nucleotide polymorphisms (SNPs) or small insertions or deletions. The electrophoresis analysis tells the results and then single cell clone need to be picked for culture. After the cell growths well, the genotyping and phenotyping test need to be figured out. If possible, the deep sequencing is necessary for determining off-target effect.

Currently, several researchers have found that CRISPR to generate knock-out (KO) cell lines are very useful for evaluating the function of the host genes in the infection process. In IAV infection studies using KO cell lines, a CMP-sialic acid transporter, SLC35A1 was shown to be essential for IAV receptor expression(Han et al. 2018). In

another study, the MHC class II proteins were shown to mediate cross-species entry of bat influenza virus(Karakus et al. 2019).

1.4.4 Hemagglutination assay (HA)

Hemagglutination assays are a classical method routinely used in influenza research to determine virus' ability to agglutinate red blood cells (RBCs). The interaction is formed between hemagglutination (HA) protein and RBCs. The sialic acid-binding site is a shallow pocket located on the globular head of the HA. The hemagglutination ability of a virus depends on the HA affinity and the structure of the underlying oligosaccharide and protein or lipid moieties of the receptors. The hosts of RBCs commonly used influenza research include those from chicken, turkey, guinea pig, horse, and sheep.

Based on the flow cytometry, chicken , turkey and guinea pig RBCs display both α2,3- and α2,6- Gal linkages, while horse and sheep RBCs display almost α2,3- Gal linkages(Trombetta et al. 2018). Therefore, to select an appropriate RBCs to perform HA has a significant effect on the titers.

1.5 Overall challenges and the objectives of this study

Sias, especially SA2,3Gal and SA2,6Gal, have been recognized as the receptors for IAVs. However, prior studies through glycan array suggested that influenza viruses could bind to non-sias glycans. It is unclear what glycan receptors other than sias can be used by influenza viruses. In addition, due to the limitation of the current detection method, it has been challenging to quantify the distribution of sias on the surface of cells and tissues and understand the impacts of the distributions of different types of sias on cell, tissue, and host tropisms of influenza viruses.

In the study, the overall goals are to develop and validate a sensitive and robust method for detecting sias on the surface of cells and tissues and to study the function of sias transporter gene and STs on sias expression and in influenza virus infection. Three specific aims will be proposed: 1) to develop and validate a novel PLA based method, PLA-glycan, for detecting and quantifying the Sias receptors on the surface of cells and tissues; 2) to explore the function of sias transporter gene and STs in sias expression and influenza virus infection. This project can help us understand molecular mechanism for influenza cell, tissue, and host tropisms.

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CHAPTER II

NOVEL METHODS FOR DETECTING GLYCAN RECEPTORS FOR INFLUENZA A VIRUS

2.1 Abstract

Glycans on the cell surface, such as the sialic acids (Sia), have been well documented as a receptor to initiate virus attachment to the cells during influenza virus infection. Rapid identification and quantification of glycan species on the cell surface and their distributions on host tissues are critical to understanding the molecular mechanisms underlying the cell, tissue, and host tropisms of influenza viruses. The conventional methods to detect glycan species on cell surface include indirect fluorescent assay (IFA) and immunohistochemistry (IHC), however, both of which have limitations of limited sensitivities and low specificity. In addition, neither IFA nor IHC are effective in quantifying the distributions of glycans on cell surface. In this study, two novel methods were developed based on the proximity ligation assays (PLA), PLA-glycan, to identify and quantify glycan receptors on cell and tissue surface respectively. PLA-glycan detects glycans using glycan binding lectins, and the abundance of a glycan is quantified directly through *ex situ* PLA-glycan with quantitative PCR or PLA *in situ* assays the fluorescent signals. The *ex situ* PLA-glycan was validated to efficiently detect α2,6-linked sialic acids (SA2,6Gal) on cell surface. The PLA *in situ* was confirmed to efficiently detect α2,3-linked sialic acids (SA2,3Gal) and SA2,6Gal on tissue surface. We found that the *ex situ*

PLA-glycan could be applied to samples of at least 3.5*10^3 cells/ml and it could clearly quantitate (SA2,6Gal) on tRBCs surface. In summary, with its high sensitivity and specificity, PLA-glycan can be further developed to determine glycan distributions on cells and tissues.

2.2 Introduction

Attachment to the cell surface is the first step of the virus life cycle during virus infection. The receptor is a molecule on the cell that the virus initially bind to , and co-receptors sometimes are needed for the entry process through post-attachment events (Matrosovich et al. 2015). Receptor molecules are components of the cell membrane, and the virus receptor could be a protein or a carbohydrate of glycoconjugates (Hirst 1941). In addition, soluble proteins, containing certain types of carbohydrates in body fluids and mucus of respiratory and enteric epithelia can interfere with viral binding (Matrosovich et al. 2015).

Sias were identified firstly as the receptor for influenza viruses (Hirst 1941). Influenza virus was found to have the ability to hemagglutinate, and then the red blood cells absorbed influenza A virus (IAV) can be eluted from red blood cells at 37 °C, suggesting an enzyme associated destruction of the receptor (Hirst 1941). Subsequently a similar process of the enzymatic activity was found to occur in *Vibrio cholerae* cultures, and the concept "receptor-destroying enzyme" was then introduced (Fisher and Ginsberg 1956)(Styk 1973). The products released by IAV from soluble proteins as hemagglutination inhibitors were identified as a carbohydrate with low molecule weight and characterized as *N*-acetyl-*D*-neuraminic acid on crystalline form (Gottschalk and Lind 1949). Therefore, the receptor determinant were Sias and the viral enzyme was neuraminidase. Sias was identified as a receptor first for influenza viruses and also recognized as the receptor by large numbers of other viruses.

There is a large variation in sequence and structural diversity of Sias. Different subtypes of IAV may prefer different Sias receptors. For example, the human influenza

A virus prefers Neu5Acα2,6Gal, whereas avian influenza A virus prefers Neu5Acα2,3Gal (Connor et al. 1994)(Matrosovich et al. 2000)(Rogers and Paulson 1983). These preferences are consistent with the predominant expression of Neu5Acα2,6Gal in human airway epithelial cells and Neu5Acα2,3Gal in epithelial cells of the birds' intestinal and respiratory tract, respectively (Yin et al. 2004)(Gambaryan et al. 2002)(Ito et al. 1998)(Kuchipudi et al. 2009)(Pillai and Lee 2010)(Shinya et al. 2006). Infection studies using human and avian IAVs in differentiated human airway epithelium cells indicated that epithelial cells at lower the human airway tract are covered abundant Neu5Acα2,3Gal, a receptor preferred by for avian IAV.

During the early stage of virus infection, human IAVs prefer non-ciliated cells, whereas avian IAVs primarily infect ciliated cells (Matrosovich et al. 2004). The studies on the distribution of the receptors in human fresh or fixed tissues with lectin *Sambucus nigra agglutinmin* (SNA), *Maackia amurensis agglutimins* (MAA) I and II, and even influenza viruses (HA protein) as the probe suggested that lack of Neu5Acα2,3Gal in the human upper respiratory tract (Self and Hill 2003; Van Riel et al. 2006, 2007; Nicholls et al. 2007) could contribute to limited transmission of avian influenza A viruses from human to human (Herfst et al. 2012)(Imai et al. 2012). In pigs, the lectin staining of tracheal epithelium showed both Neu5Acα2,3Gal and Neu5Acα2,6Gal are widely distributed across the pig upper respiratory tract (Ito et al. 1998). Due to its potential in infecting both human and avian influenza A viruses, pigs are regarded as the "mixing vessel" to generate reassortants posing threats for public health (Scholtissek 1990).

Various detecting methods have been used to determine the profiling of the Sias in cells, tissues, and hosts. There are two kinds of methods. One is based on the antigen and antibody binding, including indirect fluorescent assay (IFA) and immunohistochemistry (IHC). The other one is mass spectrometry (MS). The digoxigenin (DIG) labeled lectins were used to detect the Sias in duck intestine (colon) and pig trachea, and the fluorescein isothiocyanate-labeled anti-DIG antibody and rhodamine-labeled anti-DIG antibody were used for fluorescent signals (Ito et al. 1998). A study on the receptor differences between chickens and ducks by IFA, showed SA2,6Gal was dominant in chickens' trachea while SA2,3Gal was abundant in ducks' trachea. (Kuchipudi et al. 2009). H5N1 influenza virus can transmit from avian to human, but rarely from human to human. Nicholls et al 2007 determined the receptor distributions by IFA and the viral infection distribution by IHC and showed that avian IAVs do not infect the upper respiratory tract of humans because of a lack of the preferred receptor thus limiting the shedding by the first human host and uptake by the second human host providing a rational explanation for this transmission barrier (Nicholls et al. 2007). The glycomics analysis of the human respiratory tract tissues by IFA showed why H5N1 viruses at present rarely infect and spread between humans although they can replicate efficiently in the lungs (Shinya et al. 2006). As an animal model for most influenza virus infection, glycomics characterization of respiratory tract tissue in ferrets was studied by MS. The respiratory tissues of ferret heterogeneously express both SA2,3Gal and SA2,6Gal SAs (Jia et al. 2014). The porcine, a "mixed vessel" for influenza, has the trachea and lung to be analyzed for the N-glycans by MS. The SA2,6Gal is preferable from trachea to lung tissues (Sriwilaijaroen et al. 2011)(Nelli et al. 2010). While these methods were proved useful for identifying the presence of the glycan receptors and

the distribution at the tissue level, they can't be used quantify the glycans on cell surface and determine their quantitative distributions across cells, tissues, and hosts.

The goal of this study was to develop a sensitive and robust method, PLA-glycan, to quantify glycans on cell surfaces that is applicable in tissues (i.e. through PLA-glycan *in situ*) and cells (i.e. through PLA-glycan *ex situ*).

2.3 Materials and Methods

2.3.1 Cells

The turkey red blood cells (tRBCs) (Lampire Biological Laboratories, Pipersville, PA, USA) were prepared with 0.5% concentration for use.

The wild type A549 cells, and two knock out cells (i.e. ΔSLC35A1 (provided by Dr. Boon) and ΔST6GAL1; also see Chapter III) were propagated in DMEM with high glucose (Cat# 10313-039, Life Technologies) plus 10% FBS.

2.3.2 Tissues for swine respiratory system

The turbinate, trachea and lung tissues from swine were obtained from influenza seronegative pigs. The tissues were embedded into paraffin blocks, supporting the tissue structure and enabling thin sections to be cut, and then mounted onto microscope slides for analyses. Tissues were deparaffinized and retrieved in Antigen Retrieval Solution pH 6.0 (Dako, Carpinteria, CA), then blocked with 10% normal goat serum (Invitrogen, Carlsbad, CA) for 1 hr.

2.3.3 Hemagglutination assay (HA)

Hemagglutination assays are a conventional method used to determine influenza

viral titers based on the virus' ability to agglutinate RBCs. The interaction between virus and cells is formed primarily between hemagglutination (HA) protein and RBCs. The sialic acid receptor binding site is a pocket located on the globular head of the HA. Virus-RBC binding affinities depends on the structure of HA receptor binding site and the structure of the glycan receptors on the RBCs.

The HA assay was carried out as described elsewhere (Masurel et al. 1981). Briefly, 50µl volumes of the culture supernatants were serially diluted 2-fold in phosphate-buffered saline (PBS) in round bottom plates. Subsequently, 50µl of a 0.5% suspension of tRBCs were added to each well. The plates were incubated at 37°C for 30 min before recording the HA titers.

2.3.4 Lectins and probe preparation

For PLA-glycan *ex situ* assays, the biotinylated *Sambucus Nigra* Lectin (SNA) , *Maackia Amurensis* Lectin I (MAA I) and *Maackia Amurensis* Lectin II (MAA II) (Vector Laboratories, Burlingame, CA, USA) were used to form the probes with 3' and 5' TaqMan Prox-Oligos (Thermo Scientific Pierce, Rockford, IL, USA). With the equal volume, the diluted (200nM) biotinylated lectin was mixed with of either 200nM 3' prox-Oligo or 200nM 5' prox-Oligo, and incubated at room temperature for 1hr. Assay Probe Storage Buffer (Thermo Scientific Pierce, Rockford, IL, USA) was added with nine times the volume of the combination. Then briefly centrifuged, and the solution were incubated at room temperature for another 20mins. The probes were stored at -20°C until they are used.

For PLA-glycan *in situ* assays, the non-labeled lectins SNA and MAA II were used to form the probes. To remove the carrier and preservative, the lectins were

dialysed 4°C in cold 1 × PBS (pH 7.4) by Slide-A-Lyzer Dialysis Cassettes (Thermo Scientific Piece, Rockford, IL, USA) and the buffer was changed 5 times each 2hr. The concentration of the collected lectin was determined spectrophotometrically and adjusted to an optical density of 1.4 which is approximately 1 mg/ml. Then they were conjugating with The Duolink® In Situ Probemakers (Sigma-Aldrich, USA) using the manufacturer's protocol. Basically, 2µL of conjugation buffer is added to the 20µL lectin and then mixed by gently pipetting. The mixture was transferred to the vial of the lyophilized oligonucleotides (PLUS or MINUS) and mixed by gently pipetting, and then incubated the mixture at room temperature overnight. 2µL Stop Reagent was added and incubated for 30 min at room temperature. Then 24µL of Storage Solution was added and the probe was stored at 4°C until it was used.

2.3.5 Forced proximity test

An aliquot of biotinylated lectin was diluted to 200nM (30µg/mL); 2µL of diluted biotinylated lectin was added to 2µL of equal volume mixture of 200nM 3' and 5' TaqMan Prox-Oligo, designated probe A and probe B (Life Technologies, Carlsbad, CA), and incubated at room temperature for 1 hr. A control was made by replacing diluted biotinylated lectin with 2µL antibody dilution buffer. After incubation, 396µL of assay probe dilution buffer was added and incubated for another 30 min at room temperature. Then 96µL of ligation solution (0.1µL of diluted [1:500] ligase, 5µL of 20 × ligation reaction buffer, and 90.9µL of dH2O) was added and incubated at 37ºC for 10 min, and then cooled at 4ºC for 10 min. After incubation, 9µL was transferred to a new 0.2mL microcentrifuge tube with 11µL qPCR mix [10µL of TaqMan Protein Assays Fast Master Mix (2×) (Thermo Fisher Scientific, Waltham, MA, USA) and 1µL

20X Universal PCR Assay (Thermo Fisher Scientific, Waltham, MA, USA)] and was briefly centrifuged. The qPCR cycling was set as follows: 95ºC for 2 min, 40 cycles of 95ºC for 15 sec, and 60 ºC for 1 min.

The change in threshold cycle (ΔCt) values was calculated for each testing probe: Average Ct (control) – Average Ct (forced proximity probe). If the $\Delta Ct \geq 8.5$, the testing probe was considered qualified for use in the PLA. With the threshold set at 0.2, the $\Delta CT \geq 3.0$, can be determined as the positive samples, otherwise, the samples are negative (Schlingemann et al. 2010).

2.3.6 Detecting Sias on cell surface using PLA-glycan *ex situ* assays

The 5′- and 3′- probes prepared above were equally mixed, and then the mixture was diluted (1:10) in assay probe dilution buffer. A 2μL of the diluted probes was added to 2μL of cell samples/1× PBS (negative control) and incubated at 37ºC for 1 hr. The 96μL of ligation mixture (0.1μL of diluted [1:500] ligase, 5μL of 20X ligation reaction buffer, and 90.9μL of ddH2O) was added to each incubation product, incubated at 37ºC for 10 min, and then put on ice. Diluted protease was then added to the ligation products and incubated at 37ºC for 10 min and at 95ºC for 5 min and then put on ice. Lastly, 4.5μL of protease products was added to 5μL of TaqMan Protein Assays Fast Master Mix (2×) (Thermo Fisher Scientific, Waltham, MA, USA) and 0.5μL 20× Universal PCR Assay (Thermo Fisher Scientific, Waltham, MA, USA), and qPCR was performed as follows: 95ºC for 20 sec, 40 cycles at 95ºC for 1 sec, and 60ºC for 20 sec. The threshold was set at 0.2, and the change in the cycle threshold (ΔCT) were calculated by [average CT (control) – average CT (sample)]; quantitative real time PCR was performed on each sample in triplicate.

2.3.7 Detecting Sias on tissues using PLA-glycan *in situ* assays

Formalin-fixed, paraffin-embedded swine respiratory tissues were used for in-situ assays. The slides were labeled with permanent marker and pre-processed as the follows. The slides are incubated in the oven at 65°C for 20 min, and deparaffinized and hydrated. The antigen retrieval was conducted for each slide in the retrieval solution 1× DAKO (Agilent, Santa Clara, CA, USA) with the steamer for 20 min. The slides were cooled to the room temperature and then rinsed the slides with the TBST twice, each for 5 min.

For the PLA-glycan *in situ* assay, three drops of Blocking Solution were added to each slide, and the slide was then incubated at 37°C for 30 min in a humidity chamber. The PLA-glycan *in situ* assay specific probes were diluted 1:5 in Antibody Diluent (8 µL PLUS, 8µL MINUS and 24µL Antibody Diluent). The diluted probes are then added to the slide and then subjected to incubation at 37°C for 1 hr. The Ligation Stock is diluted in purified water (8µL Ligation Stock and 31µL water). The slides are washed with Buffer A twice (5 min each) with gentle agitation. Ligase was added to the diluted ligation stock (1µL ligase and 38µL ligation stock). Then the ligation solution was dropped on the slides and incubated at 37°C for 30 min in a humidity chamber. The Amplification Stock was diluted as 1:5 in high purity water (8µL Amplification Stock and 31.5µL water). Slides were washed with Buffer A twice (5 min each) with gentle agitation. Polymerase was added to the diluted amplification stock (0.5µL ligase and 39.5µL ligation stock). Then the slides were incubated with the amplification solution at 37°C for 100 min in a humidity chamber. After the incubation, slides were washed with Buffer B twice (10 min each) and then 0.01X Buffer B for 1 min. Slides get dry at

room temperature in the dark.

The slide processed above was mounted with a cover slip using a minimal volume of the Mounting Medium with DAPI, ensuring no air bubbles. The slides were sealed 15 min before analyzing in a fluoresce or confocal microscope, using at least a 20X objective. The slides were stored at –20°C in the dark.

2.3.8 Removal and addition of SA2,6Gal to tRBCs

To remove Sias, the tRBCs were treated with 10mU *Vibrio cholerae* neuraminidase for 30 min at 37°C. The PBS buffer with PH 7.0 was used for the reaction.

To add back the Sias, the neuraminidase treated tRBCs was pretreated with 2µL CMP sialic acid synthase (NmCSS) and 10µL α2,6-ST from *Photobacterium damsel* (Pd26ST) in PBS (pH 8.0) at 4°C overnight.

2.3.9 Evaluation of PLA-glycan *ex situ* using tRBCs

To evaluate the effectiveness of PLA-glycan, we performed three experiments: 1) to quantify SA2,6Gal in tRBCs, which was reported with positive results; 2) to quantify SA2,6Gal in tRBCs from which SA2,6Gal was removed by neuraminidase and in tRBCs from which SA2,6Gal was removed by neuraminidase but then added SA2,6Gal by 2,6 STs: 3) to quantify SA2,6Gal in different number of tRBCs ranging from 0.01% to 10% tRBCs to determine the minimal number of cells required for PLA-glycan ex situ assays.

2.3.10 Image analyses

PLA-glycan *in situ* assays were evaluated with Image J software (https://imagej.nih.gov/ij/) to obtain the objective quantification of the signals from PLA-glycan *in situ* assays using the protocols from the online

tutorial(https://imagej.nih.gov/ij/docs/examples/index.html). In our experiment, we normalized the signal by the cell numbers (counted through the DAPI) in each region.

2.4 Results

2.4.1 Forced proximity test

A forced proximity test was performed to ensure the amount of free biotin in the solution will not affect the detection process. SNA is specific for SA2,6Gal and MAA I, along with MAA II is specific for SA2,3Gal. Results from forced proximity tests showed that the SNA probe had a ΔCt of 12.529, which is above the minimum criteria of ΔCt > 8.5 (Table 2.1).

Table 2.1 The Forced proximity test for lectins

Probe	Average Ct value (+SD)	ΔCt Value**
Control	35.185 (+0.142)	
SNA	22.656 (+0.028)	12.529
MAA-II	21.423 (+0.087)	13.762
MAA-I	28.456 (+0.131)	6.729

*experiments were performed in triplicate, and SD denotes standard deviation; ** (ΔCt) = Average Ct (control) – Average Ct (forced proximity probe), and if the ΔCt ≥ 8.5, the test ing probe was considered qualified for use in the PLA-glycan.

2.4.2 Detecting Sias on cells by PLA-glycan

By using 0.5% tRBCs, PLA-glycan successful detected SA2,6Gal on the tRBC using SNA probe with a ΔCt of 3.931 (± 0.170) (Table 2.2). To demonstrate that this binding was specific, SA2,6Gal was removed from tRBCs using neuraminidase. In this case, no SA2,6Gal was detected, with a ΔCt of 2.118 (± 0.162). Furthermore, after adding

Sias back to tRBCs through α2,6-ST from *Photobacterium damsel* (Pd26ST), ΔCt was increased to 6.022 (± 0.041) (Table 2.3). For confirmation, we used HA testing to show SA2,6Gal removal and addition (Table 2.3 and Figure 2.1).

Table 2.2 Sias detection in intact tRBCs using PLA-glycan with SNA probe

Sample	Average Ct value (± SD)	ΔCt value
Control	24.085 (± 0.191)	
0.5%tRBCs	20.154 (± 0.170)	3.931

Table 2.3 Sias detection in tRBCs with and without SA2,6Gal using PLA-glycan with SNA probe

Sample	Average Ct value (±SD)	ΔCt Value
Control	32.541 (±0.118)	
original tRBCs*	29.147 (±0.005)	3.394
Treated tRBCs**	30.423 (±0.162)	2.118
Treated tRBC***	26.593 (±0.041)	6.022

Note: *0.5% intact tRBCs; **0.5% tRBCs were treated with neuraminidase; 0.5% tRBCs were treated with neuraminidase+Pd26ST.

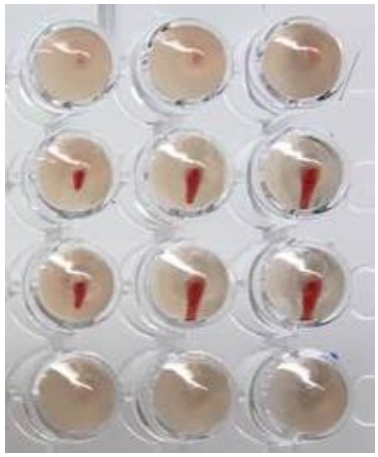

Figure 2.1 Results for hemagglutination assays using tRBCs demonstrating the presence of SA2,6Gal, removal by neuraminidase and Pd26ST adding SA2,6Gal back.

Lane 1: hemagglutination of original tRBCs (0.5%), Lane 2: failure in hemagglutination of tRBCs (0.5%) treated by 0.02U neuraminidase; Lane 3: failure in hemagglutination of tRBCs (0.5%) treated by 0.04U neuraminidase; Lane 4: hemagglutination of tRBCs (0.5%) treated by 0.02U neuraminidase and then Pd26ST.

To determine the minimal number of tRBC cells required for PLA-glycan assays, a concentration of 10% to 0.01% were used. Each reaction needed 2 ul cell solution. The PLA-glycan could detect glycan at 0.1% concentration (about 3.5*10^3 cells/ml), with a ΔCt of 3.743 (± 0.457) (Table 2.4).

Table 2.4 Sensitivity of PLA-glycan with SNA probe in detecting SA2,6Gal in tRBCs.

Sample	Average Ct value (±SD)	ΔCt Value
Control	25.299 (±0.235)	
10%tRBCs	17.659 (±0.113)	7.570
1%tRBCs	18.653 (±0.170)	6.567
0.1%tRBCs	21.486 (±0.457)	3.743
0.01%tRBCs	23.168 (±0.139)	2.131

2.4.3 Detecting SAα2,6Gal by PLA-glycan *in situ* assays

The swine respiratory tissues are abundant with both SA2,3Gal and SA,26Gal(Sriwilaijaroen et al. 2011). However, SA2,3Gal and SA2,6Gal are not evenly distributed across respiratory swine tissues (Thongratsakul et al. 2010). PLA-glycan *in situ* assay was developed to detect and quantify the distribution of SA2,3Gal and SA2,6Gal.

Results from SA2,6Gal specific SNA probe show that the SA2,6Gal are widely distributed across the respiratory tissues, including turbinate, trachea, and lung tissues (Figure 2.2). To identify SA2,3Gal MAL-II probe was used. Each slide labeled with SNA probe was further evaluated with MA-II probe (SNA/MAL-II). Results suggested there were very few SA2,3Gal in turbinate apical surface but abundant in the basal membrane of turbinate epithelial cells and in the alveoli in the lung tissue (Figure 2.3).

To examine whether the order of incubation between SNA and MAL-II probes could interfere the detection of Sias species on tissues, we switched the order of probes in the dual detection experiment. Specifically, SA2,3Gal specific MAL-II probe was used, followed by SA2,6Gal specific SNA probe (MAL-II/SNA). Results showed that the presence of SA2,6Gal and SA2,3Gal across swine respiratory tissues by MAL-II/SNA (Figure 2.3) were consistent with SNA/MAL-II (Figure 2.4).

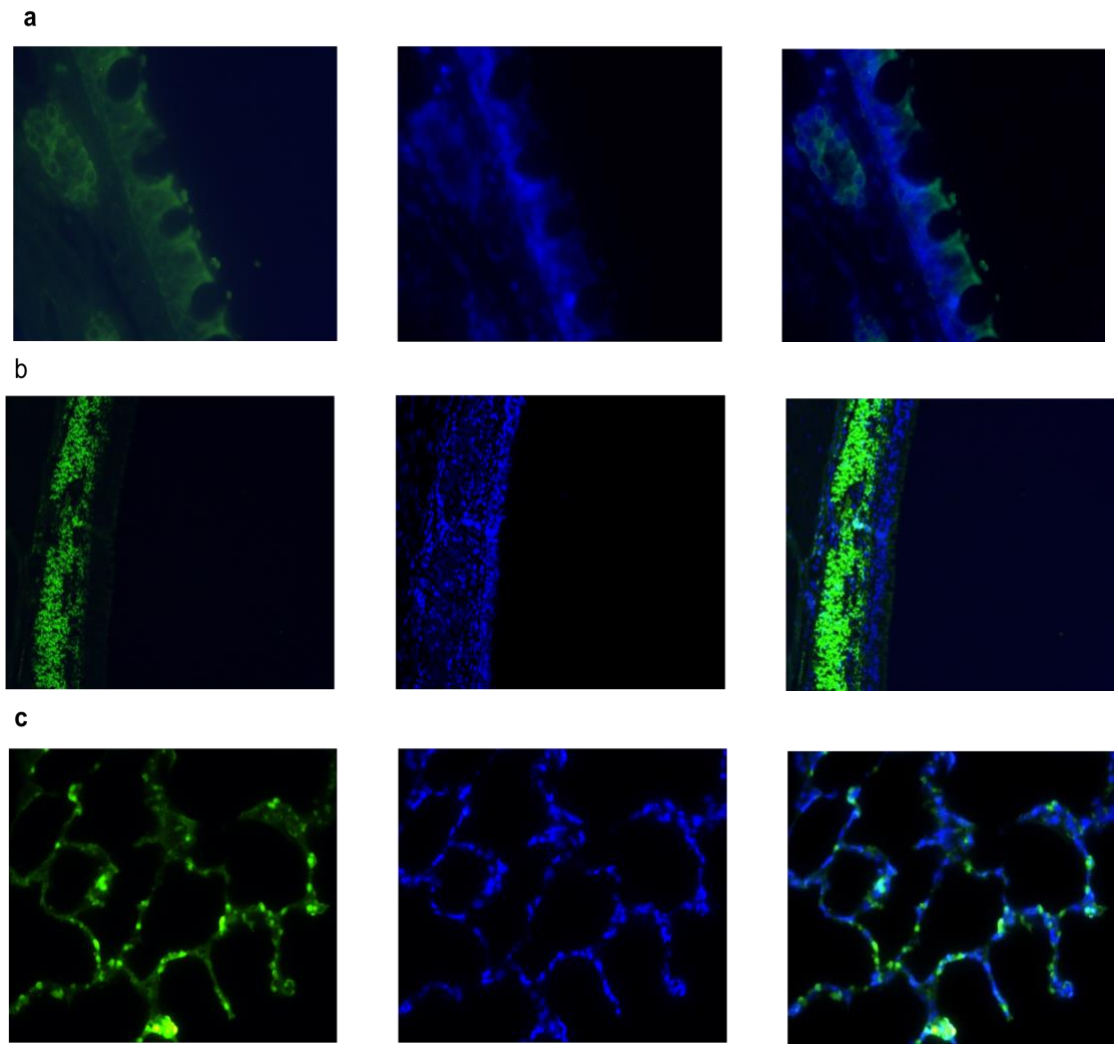

Figure 2.2 Detection of SA2,6Gal in turbinate (a), trachea (b) and alveoli (c) using PLA-glycan *in situ* assays with SNA probe. Left image is fluorescein label due to PLA *in situ*, center is DAPI counter stain and right is the combined image.

All tested turbinate, trachea and lung tissues were detected with SA2,6Gal. SA2,6Gal was found to be distributed on apical surface of turbinate (a) whereas on the basal membrane of trachea tissues (b).

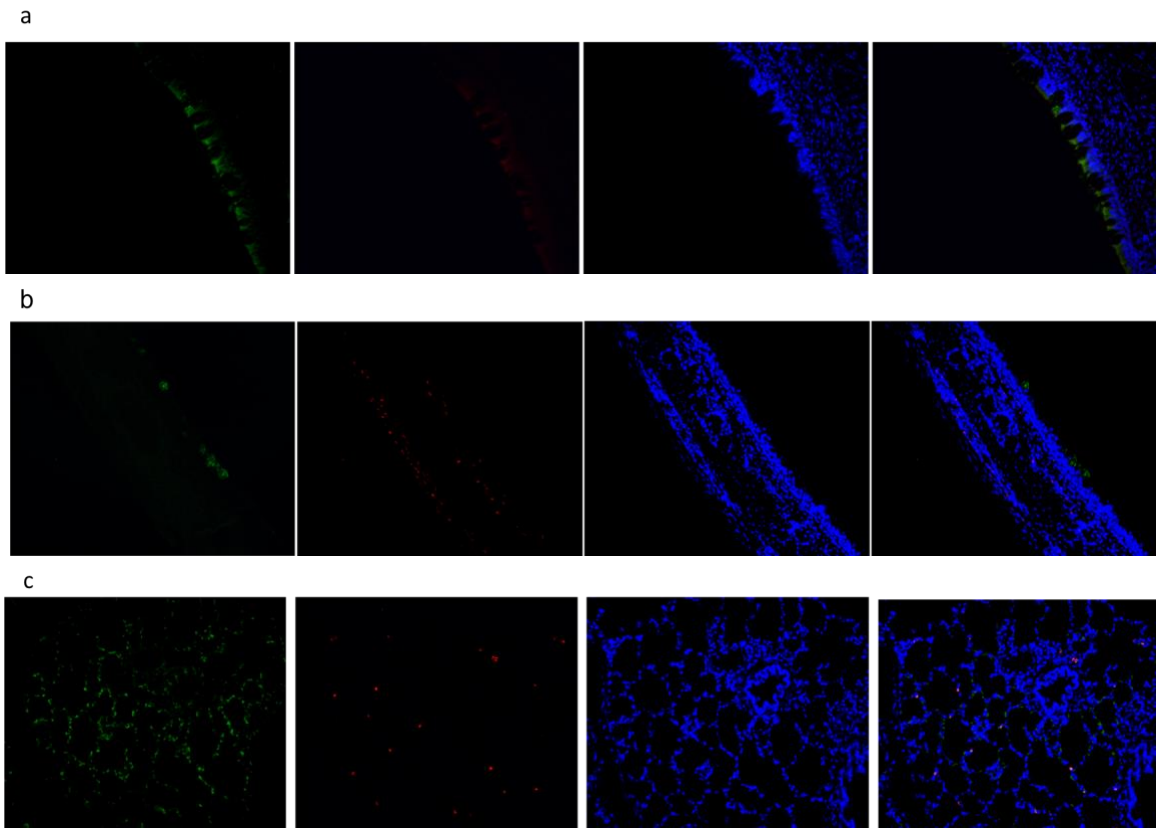

Figure 2.3 Detection of SA2,6Gal and SA2,3Gal in turbinate (a), trachea (b) and alveoli (c) using PLA-glycan *in situ* assays with SNA probe followed by MAA II probe. Left image shows green SNA (SA2,6Gal) label, center left shown red MAL-II (SA2,3Gal) label, center right show DAPI counter stain and right shows combined images.

The turbinate showed more SA2,6Gal distribution on the tissue surface; The SA2,3Gal in lung tissue is more abundant than those in turbinate and trachea, but quantity of SA2,3Gal is much less than SA2,6Gal.

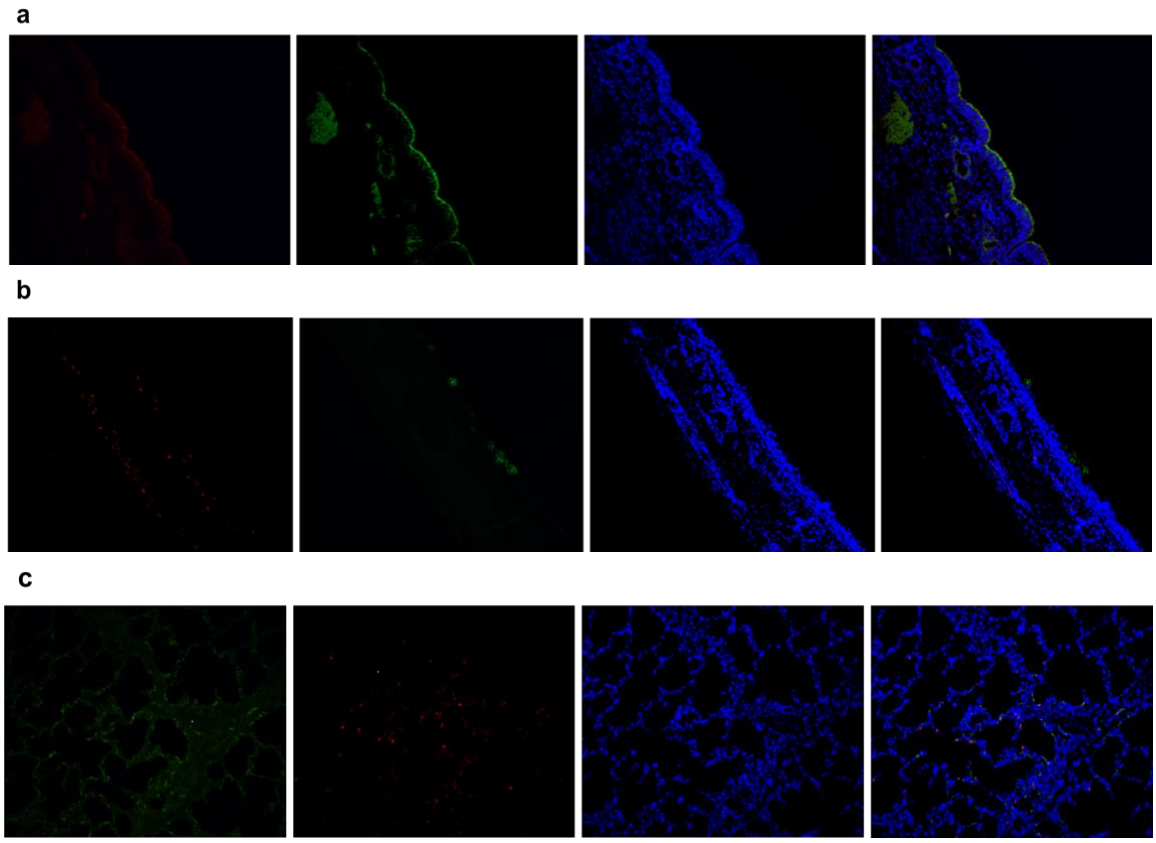

Figure 2.4 Detection of SA2,3Gal and SA2,3Gal in turbinate (a), trachea (b) and alveoli (c) using PLA-glycan *in situ* assays with MAA II probe followed by SNA probe.

the turbinate showed more α2,6-SA distribution on the surface; The SA2,3Gal in lung tissue is abundant than those in turbinate and trachea, but quantity of SA2,3Gal is much less than SA2,6Gal.

We quantified the SA2,3Gal and SA2,6Gal specific staining in turbinate, trachea, and lung tissues using image analysis. Based on the different colors, the image was separated into three channels for analyses. The DAPI staining was used to normalize the signals (Figure 2.5). For each tissue, three slides were used. Results showed that SA2,3Gal is the most abundant in swine lung tissues whereas the least in swine turbinate tissues (Figure 2.6). However, SA2,6Gal was widely distributed across various tissues

(Figure 2.6).

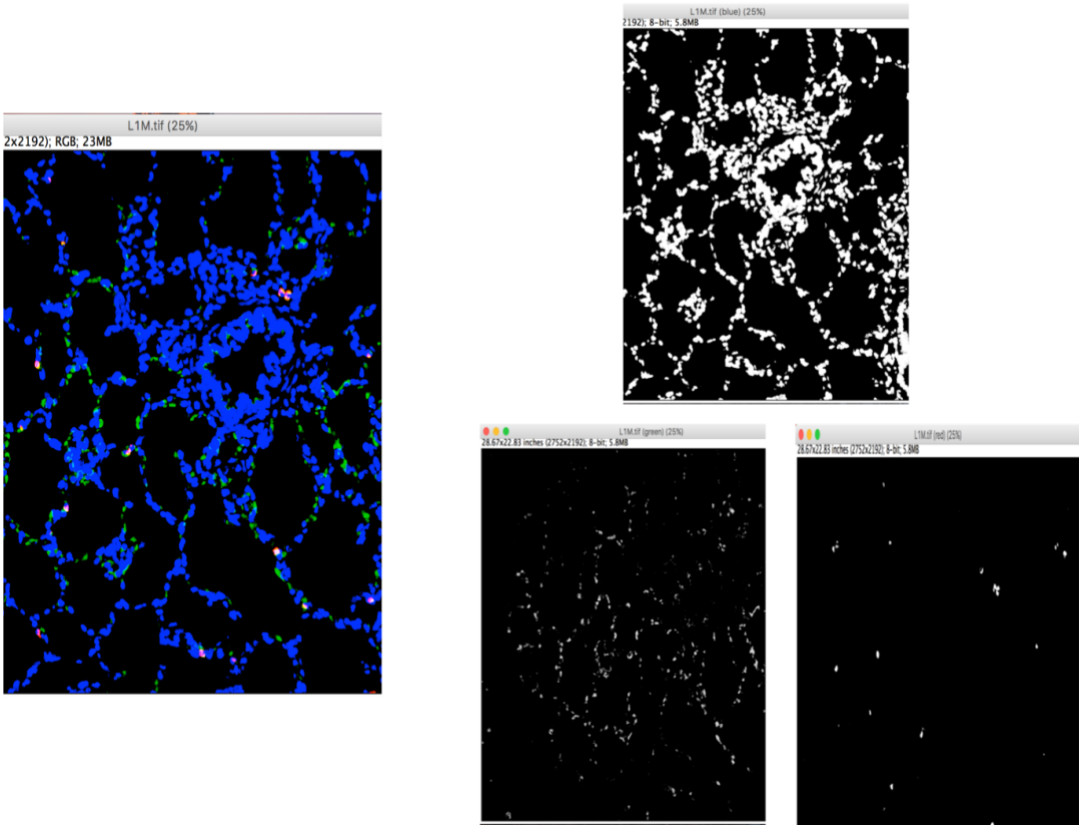

Figure 2.5 Illustration of the method of image analysis of the results by Image J

based on the different colors. The original image was separated into three images for analyses: the blue was DAPI stain used to normalize the section, the green was SNA probe and the red was MAL-II probe.

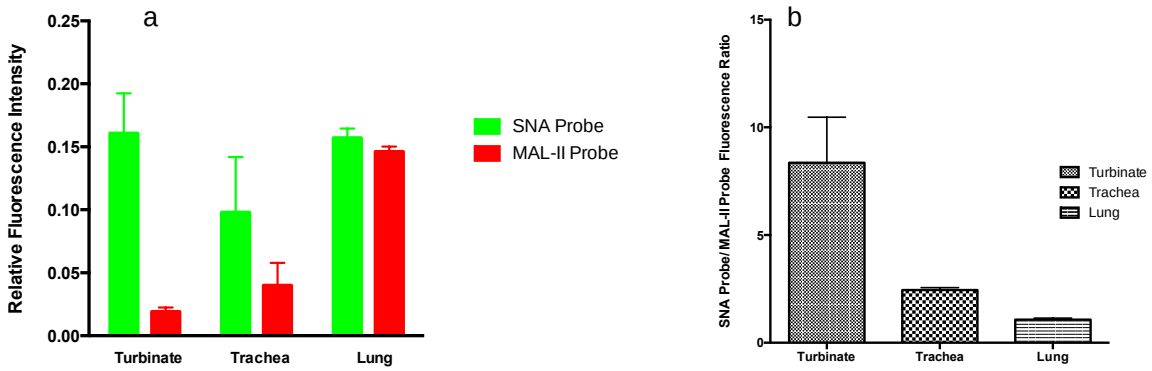

Figure 2.6 Quantitative analysis of SA2,3Gal (SNA probe) and SA2,6Gal (MAL-II probe) in swine respiratory system using image analysis.

a. quantification of the receptors; b. the receptor distribution and their ratio from upper to lower respiratory tract

2.5 Discussion

This study demonstrated the utility of the PLA-glycan method to detect and quantify the Sias on cell surface. We generated the SNA and MAL-II probe to detect the α2,6- and α2,3-SA respectively. The SNA and MAL-II probe passed the Forced test. The SNA probe can detect the α2,6-SA well. And the treated tRBCs were detected and distinguished by SNA probe. The results were then confirmed by the HA with the treated tRBCs. The detecting limitation of the SNA probe is around one thousand cells in the solution. The PLA-glycan *in situ* method was effective detecting the location and quantifying the Sias in the tissue. The unlabeled SNA and MAL-II lectin were used to generate the probe for detection. These two pairs of probes can be used to detect the tissues at the same time. The fluorescent signal produced by the probes can be quantified by the analyses of the images. The original PLA methods were based on the binding of

specific antibodies and detection using PCR. It first depends on the antibody and antigen binding and then the oligos can be ligated to produce the quantified signals. This PLA method to identify the location of the targets in the tissues and cells. The quantified signals will be helpful to understand the distribution of the targets. The quantified signals can demonstrate the distribution of the targets on the tissue surface.

The unique characteristics of the PLA methods we used in this study is that we can get the quantified signals of the targets, and then we can understand the distribution of the targets with in the tissue, even do the comparison of different targets on the same section. After the oligos ligate with each other, the template can be amplified by the polymerase. The signal can be magnified significantly and detected by the different labels. The sensitivity of the PLA-glycan is to detect signal from around 3.5*10^3 cells/ml. It is still far away from the single cell detection. Both MAL-I and Mal-II bind α2,3-SA. MAL-I prefers α2-3Galβ1-4GlcNAc, while MAL-II prefers α2-3Galβ1-3GalNAc. Due to the Forced test results, we selected MAL-II for PLA-glycan experiments. For the consistency, we selected MAL-II also for the PLA *in situ* experiments. The MAL-I probe may be useful for *in situ* PLA method as well.

There are two limitations for developing probes for the PLA method. The first one is the protein to be used for the PLA must bind specifically to the target. For PLA-glycan, there are limited proteins found to bind the glycan with specific structures. The second limitation is the oligonucleotides must be attached to the probes. All these proteins have the potential to form the probes if they can pass the Forced ligation test, which can be used for diverse detections. I our study we found substantial differences in the utility of the biotinylate MAL-II probe, given this we suggest that each preparation must be validated

for specificity. Also, the conjugation methods to form the probe have limitations. For PLA, the biotin label may change the structure of the binding reagent, which may cause the failure of the binding. For PLA *in situ*, some proteins are recalcitrant to attaching the PLA oligonucleotides.

There are several methods used for detecting different glycans in tissues. A comparison of the practical considerations is presented in Table 2.5. The IFA, IHC and Flow cytometry (FC) are the methods based on binding with a lectin or a specific antibody and the MS method is the physical methods to detect the target. They have their own advantages and disadvantages.

Using the PLA-glycan *in situ*, we were able to detect the distribution and relative density of SA2,6Gal and SA2,3Gal in swine respiratory tract, demonstrating a higher density of SA2,6Gal in all tissues but the SA2,3Gal was predominantly in the lower portion of the respiratory tract. Furthermore, the strong SA2,6Gal staining was on the respiratory surface of the turbinate tissue but on the basal membrane in the trachea. This suggests the process of viral spread may differ in these tissues. The low number of receptors on the apical surface may result in a low number of cells being initially infected in the trachea but the high concentration of receptors in the basal membrane of the trachea may facilitate cell to cell spread once the tissue is infected.

Table 2.5 Comparison of the methods for detecting glycans of cells and tissues.

	Method	Detection	Quantification	Sensitivity	Specificity	High throughput	Time	Economy
Cell	IHC	-	-	-	-	-	-	-
	IFA	√	√	5-20ug/ml lectin	Non-specific dot	x	1 day	cheap
	FC	√	√/-	single cell	√	x	Relative long	expensive
	MS	√	x	10^6 cells	-	x	four days	expensive
	PLA	√	√	3.5*10^3 cells/ml	√	√	2-3 hr	cheap
Tissue	IHC	√	-	10ug/ml lectin	Non-specific dot	x	Two days	Cheap
	IFA	√	√	10ug/ml lectin	Non-specific dot	x	Two days	Cheap
	FC	-	-	-	√	-	-	-
	MS	√	x	-	-	x	Four days	expensive
	PLA	√	√	1 mg/ml	√	x	6 hr	cheap

√ indicates the method has this feature
X indicates the method doesn't have this feature
- indicates not applicable

2.6 Reference

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CHAPTER III

THE EXPLORATION OF THE FUNCTION OF SIALYLTRANSFERASE ON INFLUENZA VIRAL INFECTION

3.1 Abstract

In the process of Sias metabolism, a protein in the solute carrier family 35, SLC35A1 is responsible for CMP-sialic acid transferring to the medial- and trans-Golgi apparatus, the place for the sialylation by divergent sialyltransferases (STs). Among these, there are totally eight α2,6-SA associated STs existing in humans. ST6GAL1 is an α2,6-SA associated STs with high gene expression in cells and involved into the *N*-glycan modification. Previous studies suggested that decreased expression of ST6GAL1 could impair the replication of the human influenza virus whereas overexpressed SLC35A1 may enhance the replication of human influenza virus. However, the functions of SLC35A1 and ST6GAL1 in influenza infection are not fully understood. In this project, using the A549 cells as the template. two knock-out mutants, ΔSLC35A1 and ΔSLC35A1, were generated using CRISPR/Cas9 gene editing tool, and the impacts of depletion of SLC35A1 and SLC35A1 on the expression of Sias on cell surface, the expression of other STs, and influenza infections were determined. Results showed that, compared to the wild type A549 cells, neither α2,3-linked sialic acids (SA2,3Gal) nor α2,3-linked sialic acids (SA2,6Gal) was detected on ΔSLC35A1, and the expression level of SA2,6Gal was reduced approximately 38% on ΔST6GAL1. Quantitative

analyses showed that, among 14 testing SA2,3Gal and SA2,6Gal associated sialyltransferase genes, compared with that of the wild type A549 cells, the gene expression level of ST6GALNAC1 increased significantly whereas ST6GAL2 decreased significantly in both mutants. Growth kinetics analyses showed the testing IAVs have a large extent of variation in their replication abilities on both mutants. Of interest, whereas most of the tested IAVs did not replicate on ΔSLC35A1, a few H3N2 human seasonal IAVs replicated on ΔSLC35A1. In summary, two mutants, ΔSLC35A1 and ΔST6GAL1, were generated, and our phenotyping analyses data suggested that certain IAVs may not be Sias dependent in virus attachment.

3.2 Introduction

To date, at least 17 genes in humans belong to the solute carrier family 35 (SLC35), encoding nucleotide sugar transporters. These transporters are localized at the Golgi apparatus/the endoplasmic reticulum (ER), transporting nucleotide sugars from the cytosol into the lumen for glycoconjugates synthesis. Among these transporters, SLC35A1 is characterized as the CMP-Sia transporter (Ishida and Kawakita 2004). This transporter was shown to move CMP-Sia across the cytoplasmic membrane of hepatocytes. Expression of human SLC35A1 in CHO cells revealed its localization in the Golgi membranes by IFA (Carey et al. 1980), this is consistent with the known site of a high level of CMP-Sia transporting activity(Ishida et al. 1998). SLC35A1 is a type-III membrane protein with both *N*- and *C*-termini exposed to the cytosolic surface (Bolivar et al. 2010). A mutation Gly189Glu in the well-conserved residue accounts for the loss of its transport activity (Eckhardt et al. 1998). Inclusion body myopathy 2 (IBM2) is a disease in adults due to muscular dystrophy, and it has been recently found IBM2 was

caused by the mutations in UDP-N- acetylglucosamine 2-epimerase for biosynthesis of CMP-Sia (Eisenberg et al. 2001). Malfunction of SLC35A1 can also sporadically result in the IBM (Eisenberg et al. 2001). Sias biosynthesis occurs in the cytosol. This involves three enzymes during four steps. A bifunctional enzyme GNE catalyzes within the first two steps, both UDP-GlcNAc 2-epimerase and ManNAc kinase activities. The epimerase converts UDPGlcNAc to ManNAc and the kinase then phosphorylates ManNAc to produce Neu5Ac. Then Neu5Ac 9-phosphate synthase (NANS) and Neu5Ac-9-phosphate phosphatase (NANP) does the condensation and dephosphorylation, respectively (Li and Chen 2012). The Neu5Ac is transferred to the nucleus for activation by cytosine 5'-monophosphate N-acetylneuraminic acid (CMPNeu5Ac) synthetase. CMP-Sia is transported to the Golgi by SLC35A1 for the formation of glycoconjugates by STs (Wang et al. 2016). Using a genome-wide CRISPR/Cas9 screen, the sialic acid transporter SLC35A1, is found to be an essential for IAV entry (Han et al. 2018). H1N1 and H3N2 showed a significant reduction in viral titer.

STs catalyze the addition of Sias to non-reducing termini of different acceptors on proteins and lipids with different linkages (Crespo et al. 2013)(Harduin-Lepers et al. 2001)(Li and Chen 2012). Most of STs are integral membrane proteins, and these STs are located at the Golgi apparatus and add Sias to glycoconjugates during the syntheses. It is also reported that STs modify the immunoglobulin G (IgG) at plasma membrane (Jones et al. 2010)(Cabral et al. 2010). Each ST presents a preference toward its acceptor to generate α2,3-, α2,6-, or α2,8-linkages, respectively.

ST6GAL1 expresses with tissue specificity, and the expression is modulated by multiple promoters (Svensson et al. 1992)(Wang et al. 1993)(Wen et al. 1992). One

promoter keeps its expression in the hepatocytes at a low but steady level. The other promoter, an inducible promoter P1 only in the liver, drives high ST6GAL1 expression and increases soluble ST6GAL1 in the blood during inflammation(Appenheimer et al. 2003). This inducible pool is quite important to regulate myelopoiesis/hematopoiesis (Jones et al. 2010)(Nasirikenari et al. 2014). A recent study found CMP-Sias are released by activated platelets and these serve as the donor for circulating ST6GAL1; the, which makes hematopoietic progenitor cells remodel the glycans (Lee et al. 2014). A further study showed that IgGs expressed by ST6GAL1 deficient B cells are sialylated by circulating ST6GAL1 (Davison 2011).

Engineering biological systems provides endless potential applications for basic science and medicine (Arowolo et al. 2011). The programmable endonucleases for specific sequences make this possible, which facilitates precise cutting in genome (Ding et al. 2013)(Soldner et al. 2011). This helps to systematically interrogate genetic elements and variations. Several genome editing techniques have developed in the past years, like zinc-finger nucleases (ZFNs)(Porteus and Baltimore 2003)(Miller et al. 2007)(Wood et al. 2011), transcription activator-like effector nucleases (TALENs)(Christian et al. 2010)(Reyon et al. 2012) and the RNA-guided CRISPR-Cas nuclease system(Marraffini and Sontheimer 2010)(Makarova et al. 2011b)(Cho et al. 2013). The ZFNs and TALENs tether endonuclease catalytic domains to DNA binding proteins for generation of DNA double-stranded breaks (DSBs) at target position. However, with the CRISPR-Cas nuclease system Cas9 nuclease is guided by small RNAs pairing with target DNA, which is incredibly easier to design (Garneau et al. 2010)(Savell and Day 2017)(Gasiunas et al. 2012). Due to the existence of guide RNAs, the system is highly specific and efficient,

meanwhile the system is also well-suited for high-throughput and multiplexed gene editing at the same time.

Cas9 generates a DSB at a target region and the position then undergoes DNA damage repair: the non-homologous ending joining (NHEJ) pathway or homology directed repair (HDR) pathway. When absent from a repair template, DSBs are repaired through the NHEJ pathway, which induces insertion/deletion (indel) mutations (Urnov et al. 2010)(Hsu and Zhang 2012). This pathway can be used to generate gene knockouts, if the indels are in a coding exon, resulting in frameshift or stop codons (Perez et al. 2008)(Chen et al. 2011).

The functions of SLC35A1 and ST6GAL1 in influenza infection are not fully understood. In this project, using the A549 cells as the template, two knock-out mutants, ΔSLC35A1 and ΔSLC35A1, were generated using CRISPR/Cas9 gene editing tool, and the impacts of depletion of SLC35A1 and SLC35A1 on the expression of Sias on cell surface, the expression of other STs, and influenza infections were determined.

3.3 Materials and Methods

3.3.1 Cells and culture reagents

The A549 cell line and the knock-out cell line ΔSLC35A1 were provided by Dr. Boon from Washington University in St. Louis. DMEM, high glucose (Cat# 10313-039, Life Technologies) plus 10% FBS, qualified and heat inactivated (Cat# 10438-034, Life Technologies) is used to culture the wild type and knockout cell lines.

3.3.2 Viruses

The influenza A virus isolates used for growth kinetics are listed as follows: A/California/04/2009 (H1N1), A/Singapore/INFIMH-16-0019/2016 (H3N2), A/Hong

Kong/4801/2014(H3N2), A/Perth/16/2009 (H3N2), A/Vietnam/1203/2004 (H5N1), A/Sichuan/26221/2014 (H5N6), A/Shanghai/2/2013 (H7N9), A/Swine/Texas/A01104013/2012 (H3N2), A/swine/South Dakota/A01349306/2013 (H1N1); A/BCM/2/1968 (H3N2), A/BCM/1/1974 (H3N2), A/BCM/1/1982 (H3N2), A/Memphis/7/1994 (H3N2), A/BCM/1/2001 (H3N2), and A/Oklahoma/309/2006 (H3N2).

3.3.3 TCID50

We plated MDCK cells into wells (of 96 well plate) with 10^4 cell numbers/ 100µl in Optim-MEM. Then we added 100µl serially diluted virus into the wells (generally a series of virus dilutions from 10E-1 to 10E-12). After 72 hr, the HA was performed to test the positive wells for calculation using 0.5% tRBC as described in chapter 2.

3.3.4 Target selection and sgRNA design

We searched the ST6GAL1 gene in the website: https://useast.ensembl.org/index.html and identified the protein coding region, the 5'-UTR, exons, introns and the 3'-UTR. After that, we selected the first exon or the first one third of the Consensus Coding Sequence (CCDS) region for gRNA design. The length for target is 20nt and it is necessary to have NGG, the PAM sequence for recognition for *S. pyogenes* Cas9. An online design tool sgRNA Designer: CRISPRko was used (https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design). Due the on-target efficiency and the off-target effect, the top three candidates were selected for experiment based on their ranks. It is noticed when expressing the sgRNA, the U6 promoter prefers a guanine (G) nucleotide as the first base of its transcript, G is replaced at the 5' of the sgRNA where guide RNA begin without G.

3.3.5 sgRNA construction

The vector selected for construction was pSpCas9(BB)-2A-Puro (PX459) V2.0 (Addgene, Cambridge, MA, USA). When synthesizing the selected guide RNAs and its complementary oligos, it was necessary to add the nucleotides for enzyme cut site BbsI. The vector carries the GFP tag and the Puro antibiotic resistance gene, which allows screen for selection after transfection.

BbsI (Cat# R3595, NEB) was used to digest the PX459 at 37℃ for 30 min, and then the digested plasmid was purified by GeneJET PCR Purification Kit (Cat# K0702, Thermo Fisher). The oligos (100μM each) were annealed with T4 PNK (Cat# M0201, NEB) under the gradient temperature-decreasing condition. The diluted annealed oligos (1:200) were ligated with digested plasmid using quick ligase (Cat# M2200, NEB) at room temperature for 10 min.

The constructed plasmid was transformed into the Stbl3 competent *E. coli* (Cat# C737303, Thermo Fisher). The component cells were Incubated on ice for 10 mins, heat-shocked at 42℃ for 30 sec and returned to the ice for 2 min. 100μl SOC medium was added and the mixture was distributed on the 100μg/ml Amp+ LB plate. The colonies were picked for sequencing the insertion with the U6 promotor primer. The positive plasmids were amplified and extracted by QIAprep spin miniprep kit (Cat# 27106, Qiagen). The plasmids were store at -20℃ for use.

3.3.6 Transfection and selection

The A549 cells were dissociated from a tissue culture flask and transferred into the 24 well-plate 16h-24h before transfection. The cell density is around 70% of the well. Cells were transfected using Lipofectamine 2000 (Cat# 11668027, Life

Technologies). The plasmid (100ng) and liposome (10µl) were added gently in the Opti-MEM I reduced-serum medium onto the cells of each well (Cat# 11058-021, Life Technologies). The concentration of liposome complex and medium ranged from 10µl per 1.0 to 3.0 ml of medium. After 24h, the supernatant was discarded and cell culture medium with the diluted Puromycin dihydrochloride (Cat# A11138-03, Life Technologies) was added for selection. 4µg/ml Puro was used in the DMEM with 10% FBS at the 1st day, 2µg/ml Puro at the 2nd day and the 1µg/ml at the 3rd day. After that, DMEM with 10% FBS was added into the well and let the rest cells grow.

3.3.7 Mutation detection and isolation the clonal cell

When the cells in the wells grew to approximately 80% confluence, they were digested with trypsin and separated into three aliquots: the first one for screening cloning (50%), the second for limiting dilution assay (20-25%), and the third as seed cells to be propagated and stored in a liquid nitrogen freezer.

The cell candidates were screened for target mutants. DNA was extracted from the first aliquot of cells mentioned above using by QuickExtract DNA extraction solution (Cat# QE09050, Epicentre). The extracted DNA was prepared to a final concentration of 100-200 ng/µl with ddH_2O. The targeted region was amplified, purified and diluted to 20ng/µl. Then evaluated for heteroduplex formation using the SURVEYOR mutation detection kit for standard gel electrophoresis (Cat# 706025, Transgenomic) according to the manufacturer's protocols. Because SURVEYOR cleavage can detect naturally occurring single nucleotide polymorphisms (SNPs), it is important to run negative control samples of un-transfected cells.

The cells for Limiting dilution cloning (LDC) are well-dissociated and diluted

in the 96 well plate for a number around 100 cells in one well. Approximately 100 cells were collected and added into the 10ml medium and then aliquoted with 100µl volume to each well. The plate incubated for about 10 days then cell colonies were further evaluated if they were derived from genetically modified samples identified by SURVEYOR screening.

3.3.8 Genotyping and phenotyping test

The cells from candidate colonies were selected and grown in a T-25 flask. Then genomic DNA was extracted and amplified (Forward primer: ATGATTCACACCAACCTGAAGAAAAAGTTC; Reverse primer: CGATTTCTCTGCCTAGTTGGGAGGACTTCA) for genotyping. This was done by Sanger sequencing (sequencing primer: GAGGGCCTATTTCCCATGATTC) by Eurofins. Meanwhile, the protein expression in one T-25 flask of cells analyzed by Western blotting. Briefly, the cells of the flask were washed three times with cold PBS. The lysis buffer (Cat# 89901, Thermo Fisher) was used to collect the cell sample. The sample was centrifuged 14,000 rpm for 15 min. During this process the samples were held on ice. The supernatant was stored in -80°C until it and one aliquot was mixed with Laemmli Sample Buffer (Cat# 161-0747, Bio-Rad) and held in a boiling water for 10 min and 20µl of each sample was separated by SDS-polyacrylamide gel electrophoresis and electro-transferred to a nitrocellulose membrane. Specific protein bands were detected as follows- the anti-ST6GAL1 antibody (1:1000) was used (Cat# AF5924, R&D Systems) in conjunction with HRP labeled anti-goat Ig antibody (Cat# A4171, Sigma-Aldrich). The beta-actin specific antibody (Cat# A5441, Sigma-Aldrich) was used with HRP labeled anti-mouse Ig antibody (Cat# 32723, Thermo Fisher) to detect Beta actin as the internal control. We used XRS

from Bio-Rad to image the membrane. Please see the instruction from the company (https://www.rndsystems.com/products/human-st6-gal-sialyltransferase-1-st6gal1-antibody_af5924#product-details).

3.3.9 Gene expression by qPCR

We used the RNeasy Plus Mini Kit for total RNA extraction. The cells were directly lysed in the T-25 flask, using 350µL lysis buffer RLT, after all medium was aspirated. Then, we added 1 volume 70% ethanol and mixed well. The mixture was transferred to RNeasy spin column with a collection tube and centrifuged for 15 sec at ≥ 8000g. The membrane was washed by 700µL RW1 and then 500µL RFE for twice. We placed the spin column into a new 1.5 ml collection tube and added 50µL RNase-free water for elution. The total RNA is stored at -20°C. We detected the STs gene expression with the Taqman probes (Table 3.1). The TaqMan® RNA-to-CT™ 1-Step Kit was used for qPCR with 10µL volume for each reaction (5µL Mix, 0.25µL Enzyme Mix). We combined the required volumes of reaction components in tubes on ice (900nM primer and 250nM probe), then inverted the tubes to mix and centrifuged the tubes. Finally, we sealed the tubes and ran the qPCR.

Table 3.1 Primers and probes of ST genes for qPCR

STs gene	Primers and Probes	
ST6GAL1	Forward	CCTCAAGTTGCTACGGTTCA
	Reverse	AAGCCCATCTCTCTCCAAATC
	Probe:	ACAGGCAGGTAGGATTCAGTGTGC
ST6GAL2	Forward	GTTCATTCTGCCACCCATCT
	Reverse	CATTCGTTGTCTCCATTGCTTC
	Probe:	AGAGACCTGCCATGAAACCACACT
ST6GALNAC1	Forward	CTCCAGACACTTCAACCAGAG
	Reverse	CTTCTGCACCAAGGAGTAGTT
	Probe:	TTTGCACCACCCTTTGGCTTCATG
ST6GALNAC2	Forward	CTACTGGAATCTGGGCTTCAC
	Reverse	CCGATCTCAGCATCACATAGTC
	Probe:	CCACAAGGACAGGACCTGCAGTAT
ST6GALNAC3	Forward	CCAGAAGGTGGGAAATGAGATAG
	Reverse	GTATGGGACACAACTCGAATCA
	Probe:	AGGTTATGAAGAAGATGTCGGCCGC
ST6GALNAC4	Forward	TGTGAGGAGATCGTGGTCTAT
	Reverse	CCCTTCTCAAAGTAGTGGTAAGG
	Probe:	TGCAGTAGCTGTCGCTGACCATC
ST6GALNAC5	Forward	CTATCAGAAGGCCACGTCAAA
	Reverse	CACTCTCACCTCTTTCCATTCC
	Probe:	AGGTGTTAGCACATGCCAGAAGGT

Table 3.1 (continued)

ST6GALNAC6	Forward	GATGTATCCTCCAGCCAATCAG
	Reverse	CCCTGACTGCACAAGAAAGA
	Probe:	TTGGCGAATCAGGGATTTGGGAGT
ST3GAL1	Forward	GCTTGGATGGTTTGTGGAATG
	Reverse	GGAGACTCCTAGCCTGAGAATA
	Probe:	CCCAACCCAACCTTCCTTCTTCCT
ST3GAL2	Forward	GAGTAGGGAGTAGCTGGGATTA
	Reverse	CAAGACTAGCCTGACCAACAT
	Probe:	ATGCACCACCACTCCCGGATAATT
ST3GAL3	Forward	TGGTTGGGTTCTGCCTTTAG
	Reverse	GGCTCAGGGAAGAGCTTTATT
	Probe:	AAAGGGAAGTGAGGCCCAAGGAG
ST3GAL4	Forward	AAACAACCCAGACACACTCC
	Reverse	TTCGCACCCGCTTCTTATC
	Probe:	TCGTCCTGGTAGCTTTCAAGGCAA
ST3GAL5	Forward	CAGATCACGCTCAAGTCCAT
	Reverse	GGTTCTTGATAGCTCCCATCTC
	Probe:	AGGCCATAATGTCTCCCAAGAGGC
ST3GAL6	Forward	GGACATAAAGATGGGAACGATAGA
	Reverse	ATCATCCAGGTTGTGAGCATAG
	Probe:	AGGGCAAGGGCTGAAAGACTATGT

3.3.10 Detection Sias distribution by PLA-glycan

PLA-glycan was used to detect Sias distribution on the three cell lines. The 5′- and 3′- probes prepared above were equally mixed, and then the mixture was diluted (1:10) in assay probe dilution buffer. A 2 μL of the diluted probes was added to 2μL of cell samples/1× PBS (negative control) and incubated at 37ºC for 1 hr. The 96μL of ligation mixture (0.1μL of diluted [1:500] ligase, 5μL of 20X ligation reaction buffer, and 90.9μL of ddH2O) was added to each incubation product, incubated at 37ºC for 10 min, and then put on ice. Diluted protease was then added to the ligation products and incubated at 37ºC for 10 min and at 95ºC for 5 min and then put on ice. Lastly, 4.5μL of protease products was added to 5μL of TaqMan Protein Assays Fast Master Mix (2×) (Thermo Fisher Scientific, Waltham, MA, USA) and 0.5μL 20× Universal PCR Assay (Thermo Fisher Scientific, Waltham, MA, USA), and qPCR was performed as follows: 95ºC for 20 sec, 40 cycles at 95ºC for 1 sec, and 60ºC for 20 sec. The threshold was set at 0.2, and the change in the cycle threshold (ΔCT) were calculated by [average CT (control) – average CT (sample)]; quantitative real time PCR was performed on each sample in triplicate.

3.3.11 Growth kinetics

Each testing influenza virus were used to infect the wild type A549, ΔSLC35A1 and ΔST6GAL1 knockout with 0.01 multiplicity of infection (MOI). We collected the supernatant at 12h, 24h, 48h and 72h post infection (triplicate), and their TCID50 titers were determined. HA was used to indicate positive wells in the TCID50 as described above. The experiments were repeated three times.

3.4 Results

3.4.1 Generating a ST6GAL1 deletion cell line

We evaluated the ST6GAL1 gene for optimum location for the CRISPR-Cas9 mutation. Based on the structure of the ST6GAL1 gene, the first exon was selected as the target region for gRNA design. Top 3 ranking gRNAs were selected (Figure 3.1).

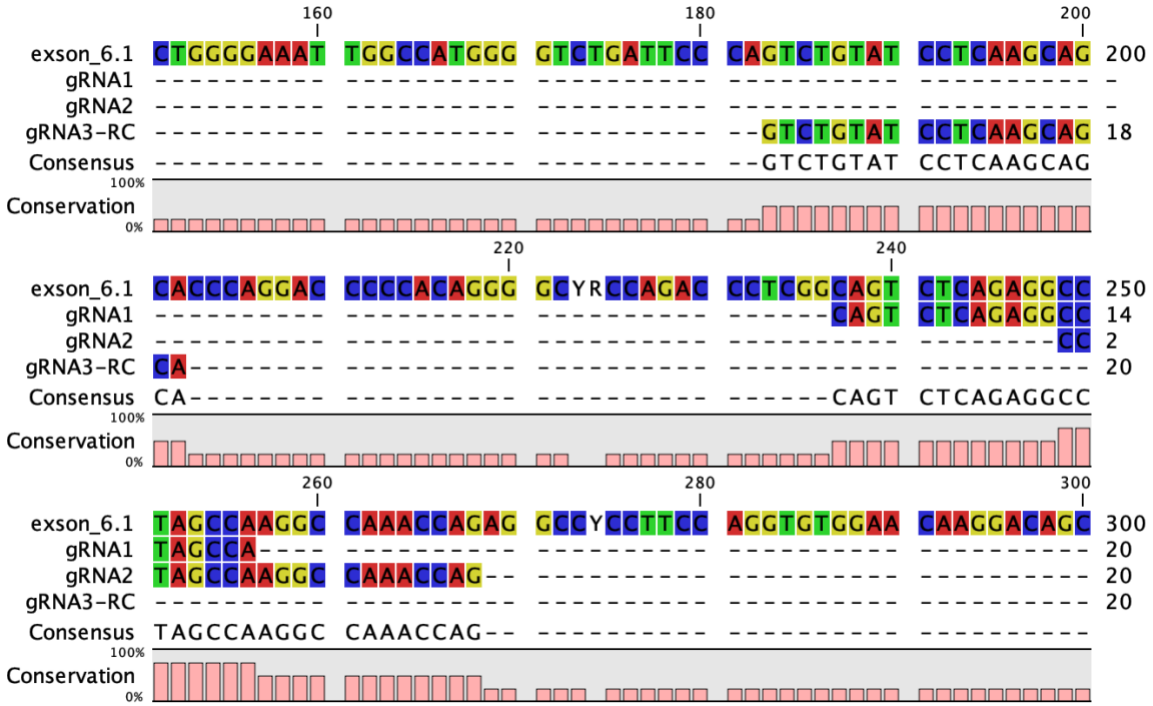

Figure 3.1 Nucleotide sequence of the first coding exon of the ST6GAL1 gene and the location of the selected gRNA targets. gRNA1: CAGTCTCAGAGGCCTAGCCA; gRNA2: CCTAGCCAAGGCCAAACC; gRNA3: CCTAGCCAAGGCCAAACC.

3.4.2 Plasmid construction

The digested PX459 was ligated with the annealed oligos, the transformation was completed, and the plasmids were extracted for sequencing. An example of our analyses based on the sequencing results is shown in Figure 3.2, the gRNA1 was inserted

successfully in clones 2 and 3 but not clone 1 (Figure 3.2).

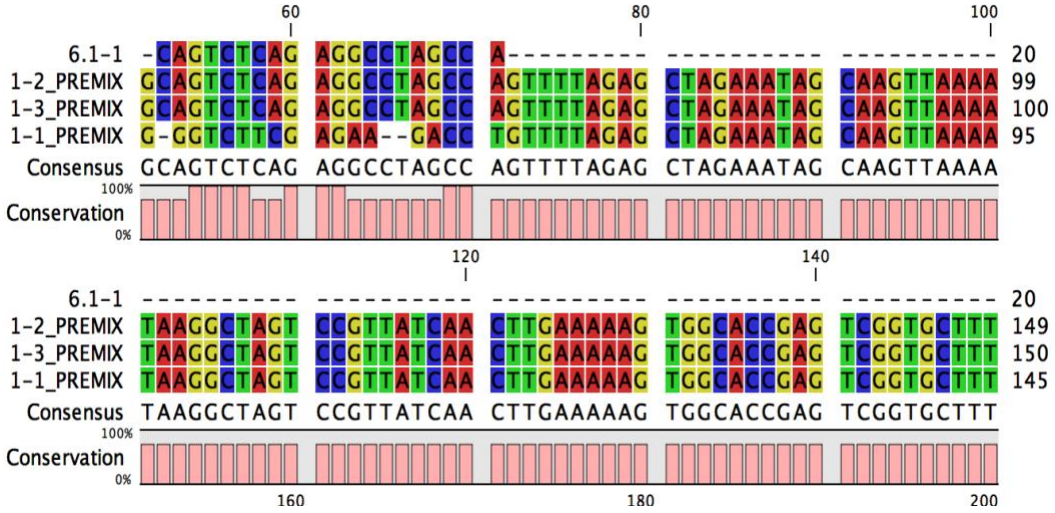

Figure 3.2 The sequence alignment of the insertion of the gRNA1 into the PX459 vector

3.4.3 Transfection and mutation detection

The three plasmids were transfected into the cells and the gradient concentration of Puro was used for selecting the cells that were transfected. The DNA of the target region of the transfected cells was extracted and amplified and evaluated for the heteroduplex assay. The image showed three gRNAs worked successfully for generation of the mutations (Figure 3.3).

1 2 3 4 5 6

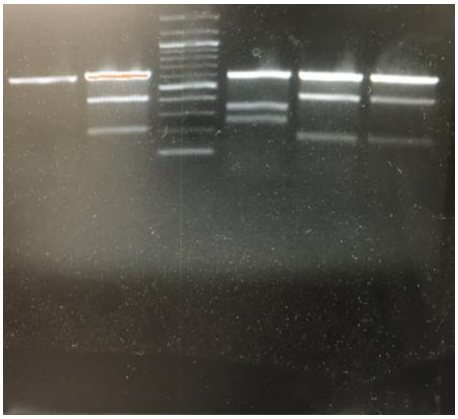

Figure 3.3 Gel Electrophoresis image of heteroduplex assay for mutation detection of DNA extracted from CRISPR-Cas 9 treated cells using gRNA1, 2 and 3.

Lane 1: Wild type A549, lane 2: Positive control, lane 3: DNA ladder (DL5000), lane 4: gRNA1 treatment, lane 5: gRNA2 treatment, 6 gRNA3 treatment. The three gRNAs work well for the mutation generation. The results showed three bands from the reannealed PCR product (heteroduplex formation) and digested with endonuclease-S indicating mutations have occurred in the cell pool.

3.4.4 Genotyping and phenotyping test

The single cell colony was selected through LDC (12 clones), Sanger sequencing was used for genotyping test and Western blot was used to evaluate loss of ST6GAL1 expression. The sequencing results show four of these single cell colonies had indel mutations in the genome. Figure 3.4 demonstrates the clones from gRNA3 experiment (Figure 3.4.4a & b). Of the 4 candidates one clone, D11-F11, showed consistent loss of ST6GAL1 expression indicating that the ST6GAL1 gene of clone was knocked out successfully (Figure 3.4c).

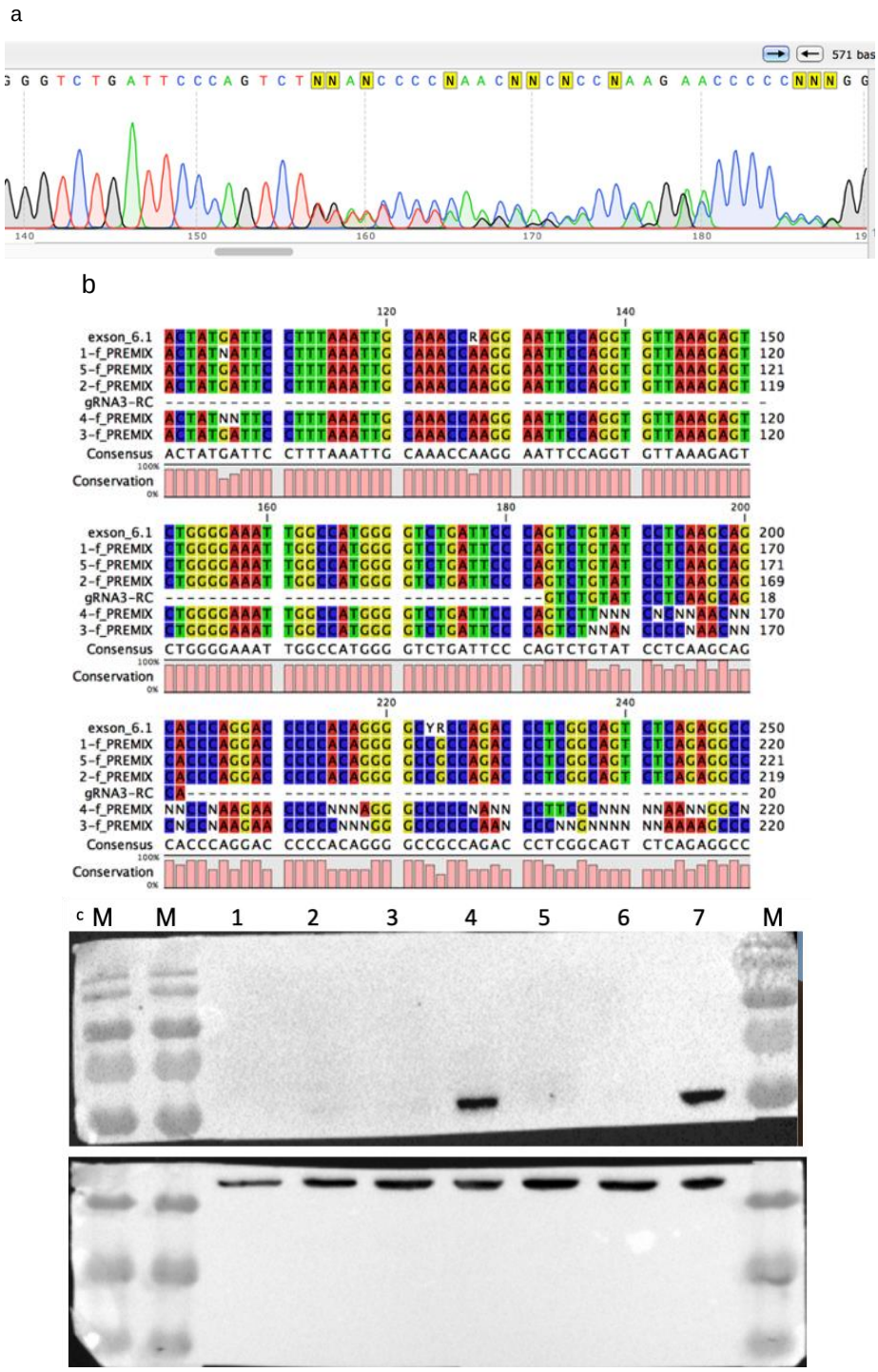

Figure 3.4 Genotyping (Sanger sequencing) and phenotyping (Western Blot) confirmation of the mutant

a. the mutation shows the double peak after the cut site;
b. the sequencing results showed after the cut site random nucleotides added,
c. Lane 1 D11-F11 3rd passage, Lane 2 D11-F11 8th passage, Lane 3 D11-F11 13th passage, Lane 4 Scramble, Lane 5 D11-F11 1st frozen seed, Lane 6 D11-F11 2rd frozen seeds, Lane 7 Wild type A549; the two bands above were the target ST6GAL1 and the seven bands below were the internal control beta-actin

3.4.5 Detection of α2,6-SA by PLA-glycan

Three cell lines were digested and diluted to 3*10^3 cell number for PLA-glycan detection. The results show the SLC35A1 has no (or no detectable) α2,6-SA on the cell surface. The D11-F11 shows a 38% decreased signal, meaning the α2,6-SA reduces on the cell surface. (Table 3.2).

Table 3.2 The use of PAL-glycan with the SNA probe to indicate relative expression of α2,6-SA on surface of the 2 mutant and the parent cell lines.

Sample	Average Ct value (±SD)	ΔCt Value
Control	27.979 (±0.072)	-
A549 (wild type)	20.620 (±0.068)	7.359 (100%)
A549 (ΔSLC35A1)	27.011 (±0.110)	0.968 (0%)
A549 (ΔST6GAL1)	21.090 (±0.083)	6.889 (62%)

3.4.6 Gene expression of STs in A549 wild type and mutant cells

Gene expression analysis using ST specific qPCR for all known 2,3 and 2,6 ST of the human genome demonstrated substantial changes in the D11-F11 clone (ΔST6GAL1). Compared with the wildtype A549, in the ST6GAL1 knockout cell line, the ST6GAL2 and ST3GAL2 gene expression decreased with almost 6.5-fold change. While the ST6GALNAC1 gene expression increased sharply with approximately 140-fold change. The ST3GAL6 has no gene expression detected in the mutant cell line while ST6GALNAC6 gene expression was similar to the wild type cells.

In the SLC35A1 knockout cell line, ST6GAL2 gene expression decreased by almost 9-fold change and the ST3GAL6 decreased by about 16-fold change. While the

ST6GALNAC1 gene expression increased by about 10-fold change and the ST6GALNAC2 gene expression increased by about 2.5-fold change.

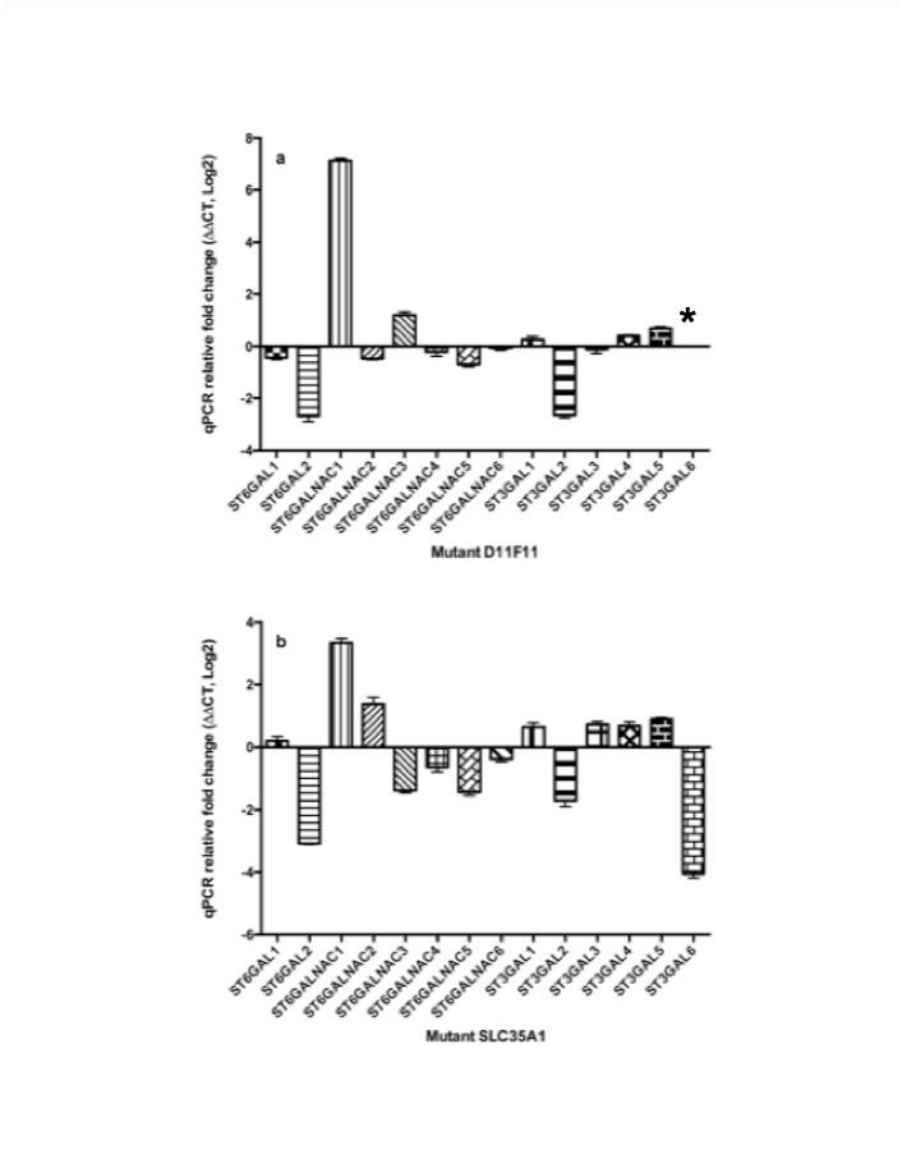

Figure 3.5 Relative gene expression of 2,3 and 2,6 STs in the D11-F11 cell mutant (a) and the SLC35A1 cell mutant (b) compared to the expression levels in the parent cell line A549 as determined by qPCR. Asterisk indicated no expression was detected in the mutant cell.

3.4.7 Growth curve test

When the mutant cell lines were used in growth curve experiments using different influenza virus isolates, most isolates had poor replication. The initial screening of nine isolates showed that results show except the human H3N2 influenza virus, all other tested influenza viruses did not grow well in the SLC35A1 knockout A549 cell line. This knockout cell line can't express Sias on the cell surface, which means Sias transporter plays a very important role during the viral infection for most viruses. However, without the transporter gene, the replication for human H3N2 viruses still existed. When it comes to D11-F11 mutant, the replication of human H1N1 and H3N2 don't show the significant difference with wild type. However, the two human H5 subtype viruses showed a better replication in the D11-F11 cells than in the wild type A549 cells, especially the H5N6 virus strain (Sichuan). H5N6 can't replicate in the wild type cells, but it can replicate in the ST6GAL1-KO cell line, even though the titer is 2 ($log_{10}TCID_{50}$/mL). Compared with the wild type, H5N1 shows a significant increase at 24 and 48 h. The human H7N9 shows a decreased replication ability on D11-F11 mutant, which indicates the glycan receptors catalyzed by ST6GAL1 are helpful to H7N9 replication. For the two swine influenza viruses, the swine H3N2 shows a better replication ability in the mutant than wild type, while the swine H1N1 shows a similar replication trend in the wild type and D11-F11, but better at 72h in the D11-F11 mutant (Figure 3.6).

The replication ability of H3N2 on SLC35A1 is different from other subtype viruses. Therefore, we selected six additional historic human H3N2 viruses for growth curve. We found the virus isolated in 1974 showed a different replication ability on SLC35A1 knockout cell lines. Compared with other H3N2 viruses, the one in 1974

has no virus replication (Figure 3.7).

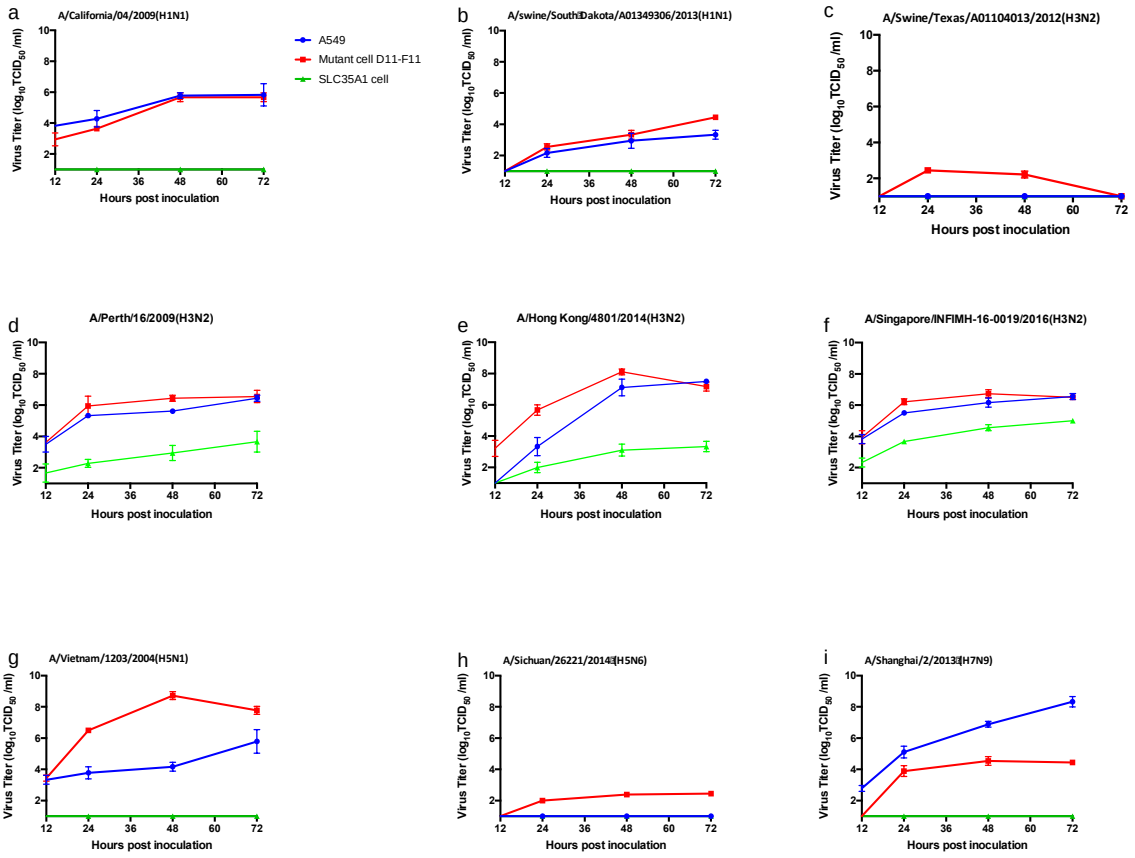

Figure 3.6 The growth curve test for 9 influenza viruses comparing the ability of the virus to replicated in the mutants and the wild type parent cells.

The red: D11-F11; the green: SLC35A1; the blue: wild type
(a) pdmH1N1; (b) swine H1N1; (c) swine H3N2; (d)-(f) human H3N2; (g)&(h) human H5; (i) human H7N9

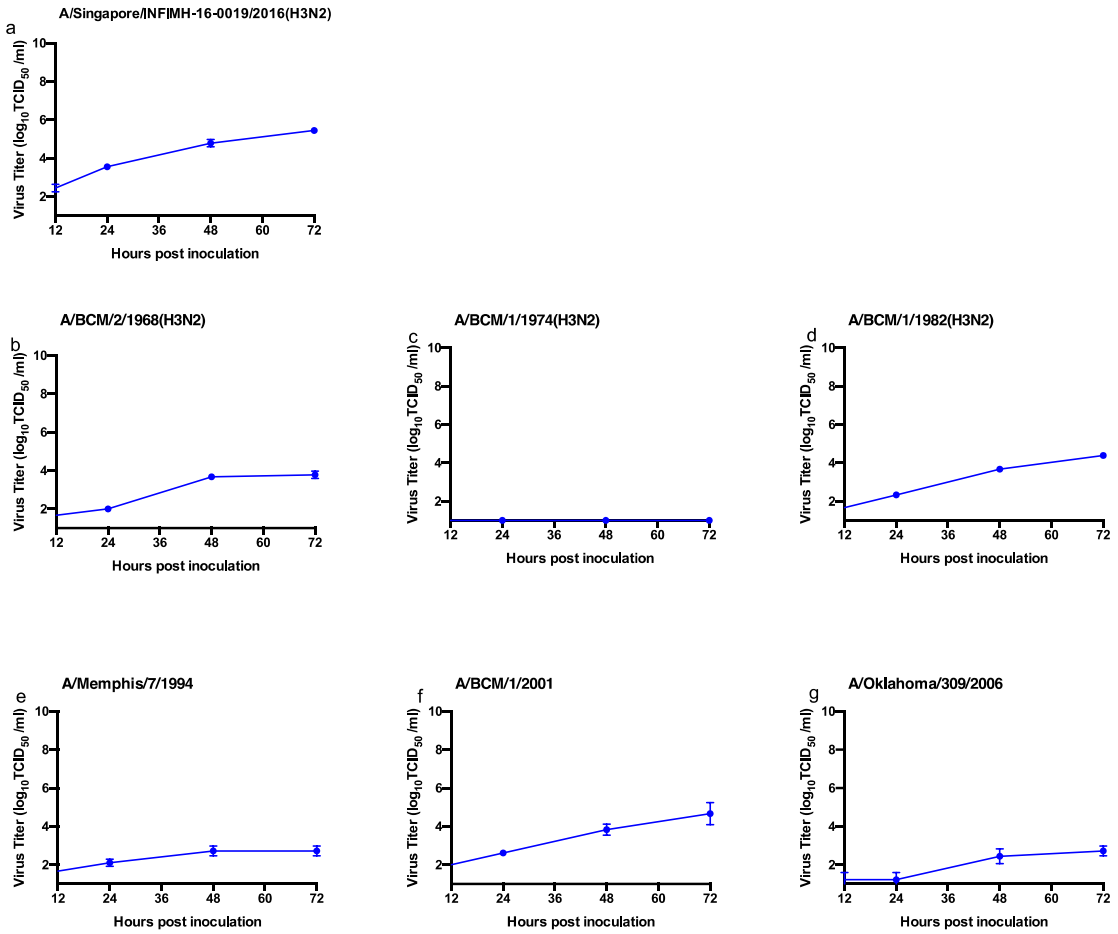

Figure 3.7 The growth curve test on SLC35A1 knockout cell line for 7 seasonal H3N2 human influenza viruses.

(a) A/Singapore/INFIMH-16-0019/2016; (b) A/BCM/2/1968 (c) A/BCM/1/1974; (d) A/BCM/1/1982; (e) A/Memphis/7/1994; (f) A/BCM/1/2001; (g) A/Oklahoma/309/2006

3.5 Discussion

Based on the genotyping and phenotyping test, an interesting phenomenon happened that the genotyping test picked totally 12 candidate mutant clones and the western blot results just revealed one mutant without protein expression. The genome did have the mutation generated and the target site was repaired, however, the repaired

consequence might differ among these mutants. The results show that the host may have certain mechanism to homologous repair the genome without template, like the spatial limitation or the energy conservation. Also, some are likely mutated in one of the copies in the diploid genome but not in the other copy(Hsu and Zhang 2012). These would appear as a mutant but retain the wild-type phenotype. We did not evaluate the mutants that retained some protein expression. The one mutant that we evaluated further had lost expression ST6GAL1 and it was stable in this phenotype.

The biological test of PLA-glycan just showed a 38% α2,6-SA reduced on the D11-F11, when compared to the wild type. However, the growth curve test showed significant differences among the tested viruses. These human viruses with the preference of α2,6-SA showed two situations: the H1N1 and H3N2 don't show the significant differences, whereas the H5 (H5N1 and H5N6) and H7N9 are with significant differences. The H7N9 showed decreased replication on the D11-F11, whereas the H5N1 and H5N6 had increased replication. This contrast also happened when looking into the replication of the swine isolates we evaluated: H3N2 had increased replication and H1N1 had no effect. Therefore, it may be reasonable to hypothesize that the ST6GAL1 is responsible for transferring the Sias to specific substrates and these glycoconjugates may be more critical for the attachment of some virus isolates than others. The PLA-glycan results also demonstrated that the ST6GAL1 knockouts don't lose all the α2,6-SA on the cell surface, which could explain the remaining sensitivity to most human IAVs displayed from the growth curve test.

Lastly, two more human H3N2 virus have been tested, they show the same results that they all can replicate on SCL35A1 cell lines, which means some viruses

require no Sias to attach for entry, which enter the cells through endocytosis or other receptors. However, when we tested the historic human H3N2 viruses, we found the H3N2 isolated in 1974 has no viral replication in SLC35A1. The recent study about the receptor binding specificity mentioned there is a remarkable change of receptor binding among the virus from 1968 to 2011(Peng et al. 2017). Before, the H3N2 viruses preferred short and branched sialylated glycans, while recent virus binds better to long polylactosamine chains terminating in sialic acid.

In the study of Han et al (2018), the SLC35A1 substantially reduced H3N2 virus replication (over 5 log reduction in titer)(Han et al. 2018). We also saw a reduction in virus replication efficiency. But unlike the other strains, some replication did occur in 8 of the 9 H3N2 isolates tested.

During the sgRNA design process, it is very important to minimize the chances of off-target mutations. When we chose the sgRNA from the online tool we selected candidates with low off-target efficiency as much as possible. There are limitations to the selection process because there is a short target region which provides a limited sgRNA library. When we designed the sgRNAs, we used several online tools (CRISPR-Cas9 gRNA Checker/ Quick Design of CRISPR gRNAs/ Guide Design Resources) for prediction and then we compared the results to arrive with the common design. They can confirm with each other. With the development of sequencing technology and the deciphering of the genomics, the online tools will be more efficient for sgRNA designing. Furthermore, it is helpful to do the deep sequencing after the knockouts to ensure only the target of interest is mutated.

When doing the transfection, it is necessary to control the cell density. The

recommended number is around 70% in the 24 well plate. Based on the adherent ability of the cell, we can adjust the number of the cells, like 293T cells (80%) and MDCK cells (60%). In addition, when using the strategy of the antibiotic selection, the sensitivity of the cells to the antibiotic needs to be evaluated in advance. During the antibiotic selection, the medium must be changed every day and the cell debris needs to be removed. When selecting clones of cells, it is important to seed back up cultures. For the heteroduplex analyses, using the high-fidelity polymerase is crucial because of the Nuclease S from celery efficiently cleaves even single base mutations. Besides Herculase II fusion polymerase that we used, the PfuUltra (Agilent) or Kapa HiFi (Kapa Biosystems) can be used as the substitute. In addition, due to SNPs, the negative control or non-transfected need to be included in the evaluations. The other way is to sequence the target region from the parent cells at the beginning before designing the sgRNA.

The ST6GAL1 and 2 are responsible for *N*-glycan transferring in the host. However, the ST6GAL1 knockout cell line showed the decrease expression of ST6GAL2, whereas the ST6GALNAC1 gene expression increased sharply with 180-fold change. It indicated that the ST6GAL2 gene expression might rely on the ST6GAL1 gene expression, which means the ST6GAL1 expression can regulate the ST6GAL2 gene expression. The ST6GALNAC1 is responsible for *O*-glycan (Harduin-Lepers et al. 2001). It is possible that the *N*-glycan decrease caused a up regulation of ST6GALNAC1 gene expression to produce more *O*-glycan but the regulation process of ST genes is poorly understood. Other *O*-glycan related STs gene expression changed a little. Our PLA-glycan cannot distinguish between *O*- and *N*- glycosylation. This distinction may help elucidate the specific changes on the cell surface and the associated differences in

virus susceptibility with this mutant. Most studies on STs evaluate changes in gene expression associated with cancer and the pathogenesis of the specific tumors. At this point little is known about their specific roles in modifying the glycans on specific acceptors. In the SLC35A1 knockout cell mutant, the STs showed various changes in expression, but there is no Sia for them to transfer to the membrane. Therefore, the importance of these expression changes is likely not relevant. We confirmed that there is no sialic acid on the cell surface of SLC35A1 knockout cell mutant. To clearly understand the ST profiles of this mutant we need to evaluate the protein expression. The further study for generation of other STs mutants need to be carried on for more information and the glycomics study of the cells will be helpful to explain the biological experiments.

This study demonstrated that crispr-cas9 mutation of specific cell lines is useful for evaluating the specific glycans that may play a role in influenza virus attachment. In our limited study we were able to distinguish attachment mechanisms were different in four different subtype human influenza isolates. These studies offer the opportunity to characterize the specific differences in the glycans and identify how these are related to virus attachment.

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CHAPTER IV

CONCLUSIONS

It is important to investigate the Sias distributions on cells and tissues to further understand influenza virus attachment for entry the host. The conventional methods are neither sensitive enough nor qualitative to satisfy the goals. In our projects, the methods based on proximity ligation assay (PLA), PLA-glycan, are stablished for Sias detection not only on the cell surface, but also on the tissues surface. We use a classical method HA test to validate the *ex situ* PLA-glycan method and use the traditional method IFA to support the *in situ* PLA-glycan method. Our results demonstrated these two methods are established successfully. Due to the good specificity and sensitivity, the methods can be used to quantify the Sias, which can help us further figure out the Sias distribution.

When looking into the sialyltransferases (STs) for glycoconjugates, we wanted to specify the function of each ST, which is helpful in understanding receptor formation and distribution. This project aimed to generate the cell mutants by gene editing tool CRISPR-Cas9 system. We generated the ΔST6GAL1 A549 cell line successfully based on the genotyping and phenotyping tests. The biological experiment, growth curve test, showed the different replication modes of the influenza viruses, which suggests this ST does have an impact on the Sias distribution. We also evaluated the ΔSLC35A1 A549 cell line. This transporter is indispensable for the CMP-Sia delivery to Golgi apparatus. Without this transporter, most influenza viruses tested were unable to infect the cells,

however, the human H3N2 still infected the cells. This exception demonstrated that Sias attachment might not be the only way for IAVs to infect the host.

Our projects can not only help us further understand the influenza virus infection, they also give some clues for medical treatment targets and vaccine production. In addition to the influenza virus, many other viruses bind Sias to facilitate entry into hosts, which means our projects can also be helpful to study the other viruses.