VECTOR ENGINEERING FOR CELL AND GENE THERAPY

A Dissertation

by

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ABSTRACT

Progress in gene therapy has been hampered by the absence of a suitable delivery vector that is both easy to produce and delivers genetic payloads efficiently and specifically to the targeted disease cells. Cell-targeting proteins, primarily monoclonal antibodies, already exist in abundance but there is currently no robust and reproducibly effective way to functionalize viral vectors with these proteins. In the case of antibodies, non-covalent approaches to incorporate antibody onto a lentivirus surface leaves the linkage vulnerable to interference from serum immunoglobulins in immune-competent individuals.

This dissertation focuses on enabling facile reprogramming of lentiviral vectors to deliver genetic payloads to specific cell types through in vitro covalent functionalization with cell-binding proteins. Two covalent-bond forming protein-protein pairs were explored to conjugate a HER2-binding protein to lentivirus pseudotyped with a binding-deficient, fusion-competent Sindbis virus envelope protein. Both the strategies resulted in functionalization of lentivirus and the titers were significantly higher compared to the naked virus. A receptor dependent retargeting was observed with functionalized virions. Lastly, the covalent bond was observed to be stable during prolonged dialysis and in presence of serum complement. A chemical conjugation approach was successfully exploited to functionalize the virions with an antibody fragment.

In another project, we aim to develop a new platform technology, building upon the antibody-guided chicken vaccine technology and the single emulsion technology, for

the discovery of chicken IgY antibodies against target cancer antigens. The antibody-guided chicken vaccine technology approach was successfully developed to generate an immune response in chickens against the target cancer antigen.

DEDICATION

To my family and all the teachers in my life

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Contributors

Part 1, faculty committee recognition

This work was supervised by the dissertation committee consisting of Dr. Zhilei Chen, Dr. Carolyn Cannon and Dr. Julian Leibowitz of the Department of Microbial Pathogenesis and Immunology and Dr. Luc R. Berghman of the Departments of Poultry Science and Veterinary Pathobiology.

Part 2, Student/collaborator contributions

All the work conducted for this dissertation was completed by Nagarjun Kasaraneni independently, under the guidance of Dr. Zhiliei Chen of the Department of Microbial Pathogenesis and Immunology.

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NOMENCLATURE

LV	Lentiviral Vector
MLV	Mouse Leukemia Virus
HIV-1	Human Immunodeficiency Virus (HIV-1)
PIC	Pre-integration Complex
CMV	Cytomegalovirus
RSV	Rous Sarcoma Virus
VSV	Vesicular Stomatitis Virus
MHC	Major Histocompatibity Complex
ENV	Envelope
SIND	Sindbis Virus
HER2/neu	Receptor Tyrosine Protein Kinase erbB-2
InaD	Inactivation no after potential A
NorpA	No-receptor potential A
DARPin	Designed Ankyrin Repeat Protein
CnaB2	Collagen Adhesion Domain
FbaB	Fibronectin Binding Protein
CHO	Chinese Hamster Ovary Cells
Fab	Fragment Antigen Binding
Fc	Fragment Crystallization Region
SUMO	Small Ubiquitin Like Modifier
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Ides	IgG Degrading Enzyme of *Streptococcus Pyrogenes*
DBCO	Dibenzocyclootyne
scFv	Single Chain Variable Fragment
AzF	*p*-acetyl phenylalanine
TCEP	Tris-(2-carboxyl ethyl) phosphine
BCA	Bicinchoninic Acid
CDR	Complementarity Determining Regions
HA	Hemagglutinin

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction to gene therapy

Gene therapy was an idea developed in the early 1980s; however, its application was riddled with misses and delays (Cavazzana-Calvo et al. 2000; Friedmann 1992; Scollay 2001). These obstacles were overcome due to the notion that gene therapy could present a real therapeutic alternative to pharmaceuticals. The initial goal for gene therapy was to treat monogenic diseases by supplying patients with a wild type gene to compensate for the defective gene (Kaufmann et al. 2013; Naldini 2015; Scollay 2001; Sheridan 2011). A major setback in gene therapy came during the clinical trials in France were introduction of gamma-retroviruses to patients suffering from X-linked severe combined immunodeficiency (X-SCID) resulted in leukemia development (Hacein-Bey-Abina et al. 2003), halting the use of retroviruses in gene therapy. The mechanism of oncogenesis in these patients was due to insertion and activation of LMO2 T-cell oncogene by the gamma-retroviral vector used (Hacein-Bey-Abina et al. 2003). The risk of insertional mutagenesis is associated with the preferred integration of gamma-retroviruses near the promoter regions and CpG islands of DNA (Cattoglio et al. 2010).

In order to generate an effective and safe vector for gene therapy, both non-viral and viral vectors have been extensively studied and used in clinical trials. There are limitations in each of the vectors, including the fact that non-viral and some viral vectors can efficiently transfect/transduce cells but do not integrate their genetic payload into the genome of targeted cells, resulting in transient expression (Ramamoorth and Narvekar

2015; Yin et al. 2014). On the other side, gamma-retroviruses, lentiviruses, adenovirus (St George 2003) and adeno-associated viruses (Daya and Berns 2008) are able to integrate their transgenes into targeting cells ensuring long-term expression albeit with different degrees of efficiency.

Among the integrating viral vectors, lentiviral vectors have the largest payload capacity for packaging (8-10 kb-long) (Vigna and Naldini 2000), compared to 8 and 5 kb size limits in gamma-retroviral vectors and adeno-associated viral vectors, respectively. A major limitation of retroviruses is that they can only transduce actively dividing cells because the integration step requires the breakdown of the nuclear membrane during infection (Miller et al. 1990; Roe et al. 1993).

1.2 Lentivirus

Vectors derived from retroviruses such as lentivirus and onco-retroviruses are among the most suitable vectors to achieve long-term gene transfer, since they allow stable integration of a transgene and its propagation in next generations. Lentiviral vectors (LV) were selected over onco-retroviruses as the gene delivery vector due to their ability to infect non-dividing target cells (Cockrell and Kafri 2007). Lentivirus belongs to the family *retroviridae* and the genome of lentivirus, such as human immunodeficiency virus (HIV-1) and oncoretroviruses, such as mouse leukemia virus (MLV), have been extensively studied in the past three decades because of their clinical relevance and their potential role as gene therapy vectors.

1.2.1 Lentiviral genome and structure

Lentivirus include primate and non-primate retroviruses. Examples of the former are HIV, SIV (simian immunodeficiency virus) and of the latter FIV (feline immunodeficiency virus) (Poeschla et al. 1998), BIV (bovine immunodeficiency virus) (Molina et al. 2002), CaEV (caprine arthritis-encephalitis virus) (Mselli-Lakhal et al. 2006), EIAV (equine infectious anemia virus) and visnavirus.

Each lentiviral particle genome has two identical copies of a single positive 9 kb RNA strand, and each RNA strand contains 9 open reading frames (ORFs), which encode for 15 viral proteins. The *gag* gene encodes for capsid, matrix, and nucleocaspid protein. The *pol* gene encodes for viral polymerase, which is comprised of proteases, reverse transcriptase and integrase. The *env* gene encodes for a glycosylated envelope protein and transmembrane domain. These are viral capsid proteins that are viral enzymes necessary for the reverse transcription and integration steps, and envelope glycoproteins to form new viral particles. The remaining six unique lentiviral proteins are accessory and regulatory proteins: Vif, Vpr, Vpu, Nef, Tat and Rev (Frankel and Young 1998).

The following table 1.1 illustrates the function of each of these accessory proteins, and each one has been shown to affect the production of new lentiviral particles and their virulence.

Table 1.1. Functional activity of different accessory proteins

Gene	Name	Function	Reference
Vif	Viral infectivity factor	Viral infectivity and identified to interact with APOBEC3G, which can impair the infectivity of virions	(Mariani et al. 2003; Subbramanian and Göttlinger 1996)
Vpr	Viral protein R	Nuclear targeting – contains nuclear localization signal (NLS) and directs the preintegration complex (PIC) to the nucleus without the breakdown of nuclear membrane, helping in infecting non-dividing cells. Responsible to induce cell cycle arrest in G2 phase to increase viral protein production	(Emerman 1996; Goh et al. 1998)
Vpu	Viral protein unique	Help in virus budding	(Nomaguchi et al. 2008)
Nef	Negative regulatory factor	Promotes Virus infectivity by down regulating the host immune response	(Piguet and Trono 1999)
Tat	Transactivator of transcription	Promotes Virus gene expression – binds to the 5'end of all nascent viral mRNA and enhances transcription	(Giacca 2004)
Rev	Regulator of expression of virion proteins	Promotes Structural gene expression – binds to Rev responsive element (RRE) of the RNA to transport viral mRNA out of nucleus for translating viral proteins	(Pollard and Malim 1998)

Within a mature lentiviral particle, two copies of viral genomic RNA surrounded by nucleocapsid proteins are enclosed in a conical shell formed by capsid proteins along with several viral enzymes (protease, reverse transcriptase, integrase), and the accessory proteins, Vif, Vpr and Nef. The viral capsid is wrapped inside a lipid membrane in which viral matrix proteins cover the inner side of the lipid membrane and viral envelope proteins localize across and outside of it (Briggs et al. 2004).

1.2.2 Lentiviral lifecycle

The lentiviral life cycle can be broken down into several steps (Tang et al. 1999):

- The lentiviral particle binds to a cognate receptor on the target cells; the viral surface protein undergoes a conformational change to reveal secondary binding sites.
- After binding, further conformational changes trigger fusion of the viral and cell membranes, which causes the release of the viral core into the target cells.
- After the viral core gains entry into the cell, it uncoats itself and forms the reverse transcription complex. The single stranded RNA molecule is reverse transcribed into double stranded cDNA, which can then be integrated into the host genome.
- Reverse transcription process is initiated by the binding of tRNA to the primer binding site on the 5' end of the viral genome (Abbink and Berkhout 2008).
- A small fragment of cDNA is synthesized.
- This cDNA dissociates from the RNA-DNA complex and undergoes first strand transfer, in which the cDNA binds to the 3' end of the viral genome and acts as the primer for the negative strand cDNA synthesis.
- The viral genomic RNA is degraded by RNase H, except at two polypurine tracts (PPT) – central region (cPPT) and 3' end.
- The fragment of positive strand cDNA beginning from the 3' PPT undergoes second strand transfer, in which the cDNA fragment binds to the primer binding site region on the negative strand cDNA.
- The complete synthesis of the both strands results in the final desired product of double stranded cDNA.

- The central 99-nucleotide overlap sequence of the positive strand guides the nuclear import the pre-integration complex (PIC) in non-dividing cells, which is eventually removed by cellular endonucleases (Charneau et al. 1994).
- Once the PIC enters the nucleus, the provirus is integrated into the cellular genome by viral integrase (McDonald et al. 2002).
- Both spliced viral mRNAs and unspliced viral genomic RNA are transferred to cytoplasm by the Rev protein.
- The viral proteins are synthesized, along with the new viral RNA genome; new viral particles are generated and released from the cell surface by budding (Stevenson et al. 1990).

1.3 Basic engineering of lentiviral vectors

The retroviral gene delivery vectors were introduced in the early 1980s (Friedmann 1992). The most commonly used retroviral vectors were based on Moloney Mouse Leukemia Virus (MLV). The major advantages of retroviral vectors are: (i) their lack of viral proteins, which renders them replication deficient and less immunogenic, and (ii) their ability to integrate into the host genome, establishing a stable gene expression. However, there are some prominent limitations, such as: (i) instability of the viral particles (Andreadis et al. 1997), (ii) low viral titers (Andreadis et al. 1999), (iii) inability to transduce non-dividing cells, and (iv) insertional mutagenesis. To overcome these drawbacks, vectors based on lentiviruses have gained prominence as viral vectors. They are capable of transducing quiescent cells and display lower frequencies of insertional mutagenesis (Korin and Zack 1998). The general strategy for a safe lentiviral vector is

similar to that followed for retroviral vector design, namely, separating *cis*-acting sequences (viral noncoding elements necessary for RNA synthesis, packaging, reverse transcription and integration) and *trans*-acting sequences (viral enzymes, along with structural and accessory proteins).

1.3.1 Lentiviral vector packaging system

1.3.1.1 Different generation of lentiviral vectors

To avoid the generation of replication-competent lentiviruses (RCL), trans-acting elements are put on separate plasmids. The lentiviral particles are divided into "generations" according to the packaging plasmid used for production. The first generation packaging plasmid includes the entire gag and pol sequences, as well as all of the viral accessory genes and regulatory genes. To ensure that it only expresses viral proteins and enzymes for viral packaging, the viral long terminal repeat (LTR) promoter was replaced with cytomeglovirus (CMV) or rous sarcoma virus (RSV) promoter (Lai and Brady 2002). Poly A tail was added to the 3' end of packaging plasmid (Zaiss et al. 2002). In the second-generation packaging system, the four accessory genes (vif, vpr, vpu and nef) were removed without affecting the viral infectivity and titer (Zufferey et al. 1997). The third generation packaging system put the regulatory gene, rev, on another separate plasmid to increase biosafety, and removed tat by replacing the 5' LTR with a constitutively active promoter in the transfer vector (Dull et al. 1998). The combination with deletion in U3 region from 3'LTR in self inactivating (SIN) vector, viral LTR can be eliminated, thus further reducing the genotoxicity of viral LTR (Sarkis et al. 2008; Zufferey et al. 1998).

1.3.1.2 Design and improvement of lentiviral transfer vector

Long-term stable transgene expression due to lentiviral vector integration is very useful for the treatment of diseases in which permanent cell correction is required. To avoid the possibility of genetic recombination with wild-type lentiviruses, the new generation vector should include only the necessary viral proteins. A major breakthrough in lentiviral vector design was developed at the Salk Institute in 1998, (Miyoshi et al. 1998) by removing the enhancer/promoter sequence in U3 region, thus making the LTR inactive during transgene expression. Interestingly, the deletion did not reduce viral titer, but and importantly, minimized RCL generation. It also decreases the chances of host gene activation around the insertional site. The transduction activity in non-dividing cells was enhanced by incorporation of the cPPT element and CTS *pol* gene, that facilitate PIC entry to the nucleus (Follenzi et al. 2000; Zennou et al. 2000). Another improvement in the transfer vector was the addition of the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE). WPRE is added to the 3' end of the transgene, and has shown to enhance mRNA transcript stability and increase the overall transgene expression (Popa et al. 2002).

One of the goals for lentiviral gene therapy is to express the transgene only in the target cells while avoiding nonspecific infection. Three different strategies were developed to achieve the stringent regulation of transgene expression in desired cells. The first strategy is called transductional targeting and relies on the modification of the vector surface either by the incorporation of foreign envelope glycoproteins that have restricted tropism, or by the insertion of specific ligands often fused to the envelope proteins that will determine

the affinity of the vector for a given target cell. In this strategy, the vector specificity is determined at entry state and therefore, only the cells that carry the specific receptor will be transduced.

The second strategy is transcriptional targeting which is often achieved by the insertion of a tissue-specific promoter, or a fragment of this promoter, upstream of the therapeutic transgene. This strategy was very promising but still non-specific targeting occurs due to the broad tropism of the envelope for non-target cells. The third strategy takes advantage of the microRNA (miRNA) post-transcriptional regulation to increase tissue specificity of gene expression. Several studies have shown effective suppression of gene expression in certain cells with the lentiviral vector containing sequences matching endogenous miRNA. When transduced cells express endogenous miRNA, transgene expression is repressed.

1.4 Surface targeting of lentiviral vectors

When lentiviral particles exit infected cells, they are surrounded by a lipid membrane, termed the envelope (Env), derived from the infected cells. The envelope contains both virus-derived and cellular proteins (CP), which may perform distinct functions for the virus. The displayed proteins play a role in virus-cell and virus-medium interactions to complete their life cycle. Apart from the virus encoded Env proteins, CP are also incorporated into the viral envelopes through three processes: (i) interaction of host proteins with viral proteins (type 1 incorporation), (ii) incorporation due to directed colocalisation (type 2 incorporation) and (iii) random incorporation (type 3 incorporation). The proteins associated with the Gag proteins are subsequently incorporated with similar

efficiency, the incorporation of protein cyclophilin A to HIV-1 particles is an example of type 1 incorporation (Hammarstedt and Garoff 2004). The type 2 incorporation primarily happens at the membrane microdomains like lipid rafts. The glycosylphosphatidylinositol-anchored CD55 (decay accelerating factor) and CD50 (protectin) were incorporated into viral envelopes through the type 2 incorporation (Breun et al. 1999; Saifuddin et al. 1997). This incorporated complement regulatory factors had offered sufficient high enough protection from the human complement system, a part of the innate immune system for the virion particles. Interestingly, it has been demonstrated that most of the pseudotyping events form through the co-localization of molecules at membrane microdomains (Briggs et al. 2003; Metzner et al. 2008a; Metzner et al. 2008b). It is hard to distinguish between type 2 and type 3 incorporation events as most of these events happen passively, i.e concentration of proteins is not increased compared to normal membrane composition. The proteomic approaches have identified host proteins found in viral envelopes, have concluded a range of molecules involving in cellular adhesion. The viruses may profit from these molecules as they provide additional initial anchoring before the specific interaction between the envelope and the cognate viral receptor (Chertova et al. 2006; Segura et al. 2008).

1.4.1 Surface modification of lentiviral particles

Surface modification of lenvitiral particles can be broadly separated into five categories:

- Pseudotyping
- Generation of fusion proteins

- Post translational modification of proteins with lipophilic residues
- Utilization of adapter molecules
- Direct chemical modifications

1.4.1.1 Pseudotyping

The phenomenon that surface proteins (glycoproteins) of one viral species can be displayed on the surface of another viral species is termed "pseudotyping". The primary role of viral surface glycoproteins is to mediate binding and entry of host cells. The replacement of Env molecules in most cases changes the tropism of the vector. This strategy has been exploited in gene therapy, as it allows the broadening or redirecting of the virus to a broad range of cells. For example, HIV-1 based LV vectors, can be redirected from CD4+ cells by replacing the Env protein with that of the vesicular stomatitis virus (VSV), thus generating a vector that targets cells encoding the LDL-R (Bischof and Cornetta 2010). VSV-G, the surface glycoprotein of VSV, is probably the most often used molecule for pseudotyping applications. Recently, lentiviral vectors have been pseudotyped with envelope proteins from Sindbis (Morizono et al. 2010; Yang et al. 2006), Influenza (Hay et al. 2001) or measles virus (Frecha et al. 2011), as the binding activity of these viruses is independent from the fusion activity. In such cases, the native binding activity may be abrogated and replaced with a binding function of choice.

1.4.1.2 Fusion proteins

Fusion of retroviral envelope proteins with molecules of interest allows for modification of viral surfaces. The advantage of this method is that theoretically, no limit is placed on the type of amino acid sequence introduced and that incorporation to the viral

particles is in most cases efficient (Ryu et al. 2008). The fused parts may be ligands (Kasahara et al. 1994), peptides (Gollan and Green 2002) or single-chain antibodies (Anliker et al. 2010a). However, a major limitation is the loss of infectivity due to the disturbance in structural or functional elements in the vector. This issue became apparent when insertion of a CD33 specific single-chain antibody to the envelope protein of MLV, hindered fusion of the virus and cell membranes during infection (Zhao et al. 1999), likely due to the inability of the chimeric protein to undergo a mandatory conformational change, which activates the fusion activity. In some cases, the virion-targeting functions of chimeric proteins can also be provided by mixed modifications using fusion proteins of other glycoproteins, such as Sindbis virus, Influenza or measles virus with engineered novel binding properties. In these cases, the wild type binding specificity was destroyed and replaced with molecules conferring specific targeting to molecules such as integrin (Morizono et al. 2009a) or the B lymphocyte marker CD20 (Anliker et al. 2010a).

1.4.1.3 Post translational modification of proteins with lipophilic residues

Glycosylphosphatidylinositol (GPI) modification is a type of post-translational modification occurring in eukaryotic cells to attach proteins to lipid membranes. GPI-linked proteins are targeted to the outer surface of the cell membrane and are frequently associated with dynamic membrane microdomains also known as lipid rafts (Legler et al. 2005). Due to their hypermobility, these can re-integrate to lipid membranes of the cells and viruses, a process termed cellular or viral painting, respectively.

A range of recombinant GPI-anchored proteins have been produced including glypiated GFP and CD4 (Legler et al. 2005). The GPI-anchored proteins can be employed

for the modification of lentiviral vectors by: (i) transfection of virus producing cells, where their integration in the viral particle is facilitated by the co-localization of glypiated proteins at the site of viral budding and (ii) by viral painting, re-introducing purified GPI-anchored proteins to mature viral particles. In the first approach, viral particles displaying GPI-anchored molecules on their envelopes were produced by co-transfection of cells with constructs expressing the GPI-anchored proteins and the necessary lentiviral plasmids. In one study, the transfection of the murine retroviral producer cell line PALSG/S with the human GPI-anchored protein CD59, yielded viral particles that are resistant to the activity of complement in human serum (Breun et al. 1999). In other studies, the recombinant GPI-anchored cytokines such as interleukin-2 (IL-2), and granuolocyte-marcophage colony stimulating factor (GM-CSF) were observed to be functional to elicit the cellular responses such as differentiation and proliferation (Kueng et al. 2007). In the second approach, GPI-anchored proteins that have been extracted and purified from cells are inserted into lentiviral vectors after incubation with enveloped viruses. This appraoch was first described for the GPI-linked model protein CD59his, which associates with MLV and HIV-1 viral vectors (Metzner et al. 2008b).

The major advantage of this approach is that stable transfection of LV producer cell lines co-transfected GPI-anchored proteins can provide a reproducible long–term source of modified viral particles. The merit of the viral painting approach is flexibility; however, as this process is carried out as a post-exit surface modification; the fully formed viral particles may lose viral infectivity.

1.4.1.4 Adaptor structures

Another strategy to modify viral particles is to introduce an adaptor molecule onto the viral particle, which can mediate association with other molecules. These adaptors can be soluble, non-covalently attached molecules or membrane bound factors. The bispecific molecules or assemblies were used, specifically contacting a molecule present on the viral surface and another on the cells about to become infected. For example, two different antibodies, modified with biotin, were linked via avidin or streptavidin (Roux et al. 1989), thus providing specificity for viral surface proteins and the target molecule on the cell. This highly flexible system was applied to infect major histocompatibity complex (MHC) I and II expressing cells with murine retroviruses (Roux et al. 1989). Alternatively, a receptor ligand chimeric protein may be used, in which the binding partner of the adaptor protein incorporated in the viral vector is coupled to a targeting moiety. This strategy was implemented with vectors pseudotyped with avian sarcoma and leucosis virus (ASLV). The chimeric bridge protein consisted of the extracellular domains of the cellular receptor for ASLV, fused to the ligands such as epidermal growth factor (EGF) or vascular endothelial growth factor (VEGF), thus targeting cells expressing the respective receptors (Snitkovsky et al. 2000; Snitkovsky et al. 2001; Snitkovsky and Young 2002).

The main advantage of adaptor structures is their flexibility; however, sometimes post-exit modification steps may be necessary, which could potentially reduce infectivity of lentiviral vectors. In some studies, binding of biotin to vectors is achieved by chemical modification or after incorporation of a biotin-adaptor peptide (BAP) (Morizono et al. 2009b; Nesbeth et al. 2006). Mixing of different strategies like pseudotyping of an LV

vector with chimeric envelope molecules containing an adaptor element can and have been used to modify LV vectors. One strategy is to incorporate antibody binding adaptor molecules to modify viral surfaces. For examples, insertion of immunoglobin G-binding domains (the ZZ-domain of staphylococcal protein A) into the Env protein of MLV vectors allowed for the binding of specific HER2 specific antibodies thereby redirecting the vectors to the receptor positive target cells (Morizono et al. 2001; Pariente et al. 2008). In a similar approach, the same antibody-binding domain was fused to the sindbis envelope glycoprotein. The main disadvantage of adaptor systems is that an additional, separate element is necessary for the system to work, thus introducing a new level of complexity. Additionally, adaptors may dissociate from their binding partner, due to a non-covalent interaction or competition from serum antibodies *in vivo* (Morizono et al. 2009a). The adaptor association in most cases requires post-exit procedures, which can contribute to a loss of infectivity.

1.4.1.5 Direct chemical modification

The direct chemical linkage of substrates to viral surfaces has been successfully applied by incorporation or fusion of polymers and polypeptides to adenoviruses and adeno-associated viruses (Croyle et al. 2002; Croyle et al. 2000). While limited, this approach has proven successful in lentiviral vectors. One successful example is the attachment of monomethoxy-poly(ethylene)glycol (PEG) to VSV-G pseudotyped LV vectors (Croyle et al. 2004). The activated form of PEG is covalently attached to lysine residues on the proteins displayed on the virus. The PEGylated virions were observed to be stable when exposed to human and murine complement. In another study, the viral

tropism was changed by the chemical addition of carbohydrate (galactose) moieties on the MLV viral surface (Neda et al. 1991). This study allowed transduction of human cells with ecotropic MLV vectors, which normally cannot infect human cells. The chemical display of biotin through a metabolic engineering approach has also been exploited in many studies. The biotin-adaptor peptides were introduced into the viral surface glycoproteins allowing for the specific biotinylation of these proteins by the secreted biotin ligase, conferring the possibility for avidin, streptavidin or neutravidin linkage. These adaptors can also be attached with secondary biotinylated compounds. More recently, usage of biorthogonal chemistry has been explored for different approaches. Bioorthogonal chemistry allows chemical reactions to occur in a controlled and specific manner. Cell surfaces can be modified by oxidation of sialic acids present on glycosylated surface proteins by periodate, generating reactive aldehyde groups, which in turn, can be modified by conjugation of aminooxy-functionalized compounds (Zeng et al. 2009). The chemical conjugation between VSV-G pseudotyped MoMLV, carrying the aminooxy-biotin was subsequently associated with streptavidin magnetic beads, further facilitating purification and concentration of virus preparations (Wong and Kwon 2011). Direct chemical modification of herpesvirus particles with radioactive labels has also been demonstrated in biodistribution studies (Schellingerhout and Bogdanov 2002). The major advantage of chemical modification is lower non-specific targeting, which would contribute to the safety of gene therapy.

1.5 Transductional targeting

A key element of successful and efficient gene therapy is the ability to target only the subset of cells requiring treatment after systemic administration. The ideal viral vector for *in vivo* gene therapy approaches is that which shows no non-specific infection and less risk of insertional mutagenesis. In the case of LV vectors, the viral glycoproteins located in the envelope function as recognition and entry devices to allow access to target cells. The Env protein consist of two subunits, the surface (SU) and transmembrane (TM), both with distinct functions. SU mediates the first contact with the host cells by engaging the viral receptor. The binding specificity of the SU subunit therefore determines the host cell range of the virus. Upon this first contact, TM fusogenic properties are activated, which allow viral and cellular membranes to fuse, resulting in viral entry. Modifications introduced in the Env proteins are sometimes not tolerated, and may lead to severe reduction in infectivity. However, these modifications are crucial to achieve transductional targeting, with specificity being the most important parameter.

A range of different strategies have been tested to change the infection tropism including the use of glycoproteins from heterologous viral species (pseudotyping) or chimeric envelope glycoproteins (Env fusion proteins), as well as bridging molecules (adaptors). The application of pseudotyping for transduction targeting is limited by the available glycoproteins (King and Daly 2014). An efficient strategy is the use of chimeric envelope proteins in which the protein responsible for receptor binding is replaced with peptides, ligands or single chain antibodies, thereby redirecting the virus to desired cells.

Additionally, adaptors have the capability to associate with both the viral glycoprotein and the cellular receptor mediating the linkage between the virus and the target cells.

Transduction targeting progress has been made by exploiting envelope proteins whose binding and fusion properties are independent (Morizono et al. 2006). Among the most promising candidates are genetically engineered variants of the Sindbis virus (Sind) glycoproteins, the influenza virus hemagglutinin and measles virus glycoprotein. It was found that envelope of alphavirus Sindbis virus was able to pseudotype retroviruses and lentiviruses. The two integral membrane glycoproteins, E1 and E2, form a heterodimer and function as a unit. E2 binds to the host cell receptor. E1 mediates the membrane fusion in a low pH-dependent manner (Li et al. 2010). The primary lentiviral-gene targeting system was based on the modified Sindbis virus envelope (Sind-ZZ) that encoded the ZZ domain of protein A. Monoclonal antibodies were able to functionalize the virions and direct them to cell-specific-antigens (Ohno et al. 1997). Although the targeting was efficient, the functionalized Sind-ZZ resulted in high levels of infectivity in liver and spleen cells during *in-vivo* targeting, due to the high affinity towards laminin and heparin sulfate receptors (Wang et al. 1992). Several E2 mutants with reduced levels of non-specificity combined with other mutations in other domains R1, R2 and R4 respectively, resulted in a pseudotyped virus, termed m168 (Morizono et al. 2005). This pseudotyped virus showed enhanced selectivity with high viral titer. This engineered Sindbis envelope was used to pseudotype lentivirus to enhance specific targeting through the cell-binding moiety. By incorporating adaptor molecules, the same basic viral particle can be modified with a range of binding properties to suit the specific needs of the targeting application. In

summary, combination of LV viral surface glycoproteins capable of inducing virus/cell fusion independent of binding with a flexible adaptor system represents the most promising candidate for targeting applications.

1.6 Research objectives

The focus of this research project is to exploit different covalent bond formation approaches to create a reprogrammable lentiviral vector to deliver genetic payloads to specific cell types. The following chapters contain a detailed description of the experimental techniques used to characterize each retargeting approach, and the conclusions drawn from the obtained results.

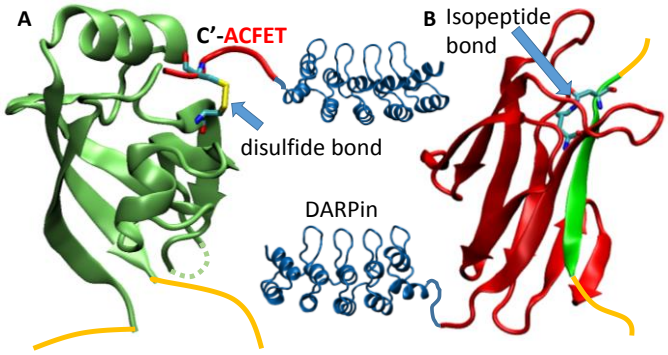

Figure 1.1: Depiction of InaD PDZ1 complex with DARPin-TEFCA (A) and SpyTag complex with DARPin-SpyCatcher (B). Green: InaD PDZ1 (pdb: 1IJH) or SpyTag (pdb: 2x5p), to be incorporated into viral Env; red: TEFCA or SpyCatcher, to be fused to cell-attachment protein; blue: DARPin (pdb: 4J7W), a model cell-binding protein; orange line: Sindbis Env E2.

Chapter II focuses on development of a facile retargeting lentiviral vector, using a disulfide bond formation protein-peptide pair from the *Drosophila* visual system. The N-terminal PDZ1 domain of InaD protein (PDZ1) and its pentapeptide ligand (TEFCA) from NorpA, were exploited to functionalize pseudotyped lentiviruses. Chapter III explores an isopeptide bond forming protein-peptide pair from *Streptococcus pyogenes* to create a retargeting lentiviral vector. The N-terminal fragment (SpyCatcher) and C-terminal fragment (SpyTag) of the collagen adhesion domain (CnaB2) from the fibronectin binding protein (FbaB), were applied to functionalize lentiviruses. The functionalization of virions with the cell targeting protein displayed receptor dependent targeting. The bond was observed to very stable compared to the previous non-covalent approaches. These encouraging results provide a new platform to develop an *in-vivo* targeting system for gene therapy applications (Figure 1.1).

Chapter IV describes the characterization of the cell surface display of the agonistic monoclonal anti-chicken CD-40 (mAb 2C5). The displayed mAb 2C5 was active to interact with the purified chicken CD-40 ectodomain. The developed DNA constructs will be used to carry out the immunization process in chickens to develop antibodies against tumor antigens.

CHAPTER II

RETARGETING LENTIVIRAL VECTORS THROUGH COVALENT FUNCTIONALIZATION USING PDZ1-TEFCA PROTEIN-PEPTIDE PAIR

2.1. Overview

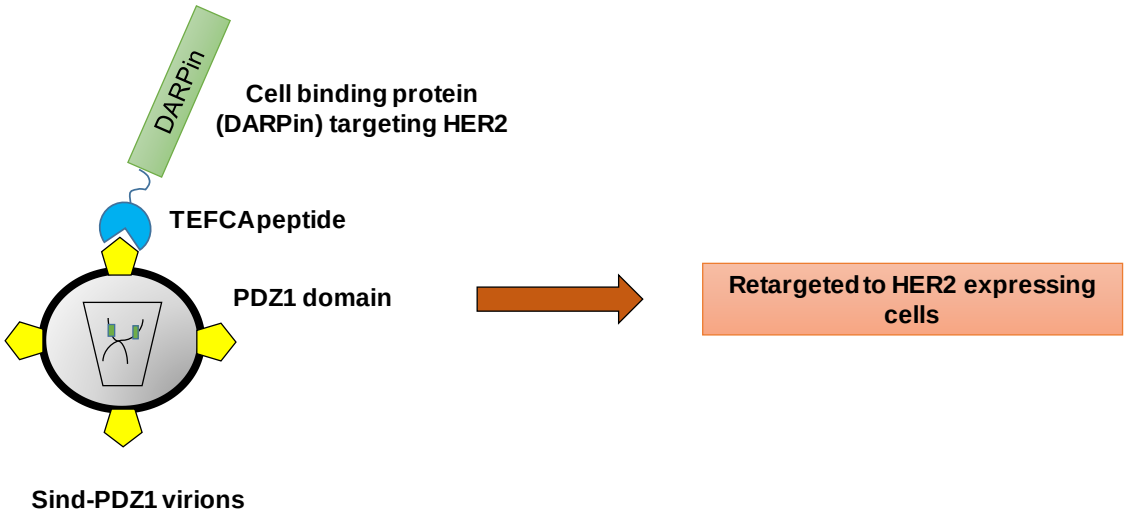

Figure 2.1: Schematic illustration of the retargeting strategy for PDZ1-TEFCA protein-peptide pair. TEFCA peptide is genetically fused to the C-terminus of DARPin to form DARPin-TEFCA. The PDZ1 was inserted into an extracellular loop of E2 on a binding-deficient, fusion-competent Sindbis virus envelope protein (Sind, (Morizono et al. 2010)) to form Sind-PDZ1. Lentiviruses pseudotyped with Sind-PDZ1 (Sind-PDZ1-pp) was incubated with the TEFCA-functionalized DARPin for a period, and was then used to transduce HER2-positive cells.

Selectivity/specificity is a crucial factor for the safety and efficacy of gene therapy vectors. The production of gene therapy vectors programmed to deliver genetic payloads efficiently and specifically to cells of interest remains a non-trivial task. In this study, we developed a facile strategy to reprogram pseudotyped lentiviral vectors to desired cell

types through *in vitro* covalent functionalization with cell-binding proteins. A disulfide bond-forming protein-peptide pair from the *Drosophilia* visual system, the N-terminal PDZ domain of the Inactivation no after potential D (InaD) protein (PDZ1) and its pentapeptide ligand (TEFCA) from no-receptor potential A (NorpA), were exploited to functionalize pseudotyped lentiviruses incorporating a binding-deficient, fusion-competent Sindbis virus envelope protein with a HER2/neu-binding designed ankyrin repeat protein (DARPin) (Figure 2.1). Functionalized lentiviruses transduced $HER2^+$ cells >100-fold more efficiently than the unfunctionalized virions (8.9×10^6 vs 7.4×10^4 IU/mL). The association of the cell binding protein with pseudotyped lentivirus was stable under non-reducing conditions. Finally, the transduction efficiency of DARPin-functionalized lentiviruses was not compromised in the presence of pooled human serum, pointing to a high potential for their *in vivo* application in human gene therapy.

2.2. Introduction

Gene therapy has the potential of treating any genetically caused diseases including monogenetic disorders and cancers. A significant barrier to gene therapy is achieving delivery of the genetic material in sufficient quantities to the correct target cells to provide the desired level of therapeutic effect. Viruses are natural gene delivery machines and have been extensively exploited as gene therapy vectors (Kotterman et al. 2015). In particular, lentiviral vectors engineered from human immunodeficiency virus (HIV) are capable of efficient gene delivery to both mitotic and nondividing cells (Naldini et al. 1996), and have emerged as a promising and apparently safe vehicle for clinical gene therapy. Importantly, lentiviral vectors integrate into the host cell genome and thus, are duplicated along with

the host DNA during mitosis, enabling long-term transgene expression. However, most current lentiviral vector-based gene therapies involve *ex vivo* gene delivery as lentiviruses pseudotyped with VSV-G, the most commonly used envelope protein, were found to be rapidly neutralized by serum complement (DePolo et al. 2000). In addition, an ideal *in vivo* gene therapy vector should exhibit minimal toxicity to otherwise healthy tissue, while efficiently and selectively delivering the therapeutic gene into the desired cells (Escors and Breckpot 2010; Naldini 2015).

A number of strategies has been developed to create cell-targeted lentiviral vectors. One strategy used to modulate lentiviral tropism is to incorporate envelope glycoproteins derived from different viruses (Dropulic 2011). However, most clinically relevant cell-types cannot be specifically targeted by natural viruses directly, and efficient pseudotyping often requires extensive protein engineering of the foreign glycoprotein cytoplasmic region (Funke et al. 2008; Funke et al. 2009; Girard-Gagnepain et al. 2014; Palomares et al. 2013). Another strategy is to incorporate new cell binding proteins onto the virus. Entry of enveloped viruses into cells involves two major steps: 1) virus-cell attachment and 2) fusion of viral and cellular membrane. Fortunately, for many viruses, abolishment of viral attachment through mutation/deletion of the attachment function does not impair the fusion function. For viruses with an abolished wild type attachment function (blinded-envelope protein), incorporation of a new cell-targeting protein can retarget the virus. A prominent strategy to incorporate new cell-targeting proteins onto viruses is by fusing the cell-targeting protein directly to the viral envelope protein (Anliker et al. 2010b; Bender et al. 2016a; Engelstadter et al. 2000; Munch et al. 2011a). This approach proved

successful for Buchholz and co-workers where a panel lentiviral vectors targeting different cell types was created by fusing different cell-targeting proteins to a binding deficient Nipah virus envelope protein and pseudotyping the virus with the new chimeric protein (Bender et al. 2016a). However, some cell-binding proteins cannot be genetically incorporated onto viruses using recombinant approaches, limiting the types of cells accessible for gene therapy (Friedel et al. 2015).

In this study, we developed a facile "plug-and-play" strategy that enables lentiviruses to be reprogrammed, thereby delivering genetic payloads to specific cell types. This strategy exploited a disulfide bond forming protein-peptide pair from the *Drosophilia* visual system, the N-terminal PDZ domain (PDZ1) of InaD protein and its pentapeptide ligand (TEFCA) from NorpA (Kimple et al. 2001; Shieh et al. 1997). The PDZ1 was inserted into a previously engineered binding-deficient, fusion-competent Sindbis virus envelope protein (Morizono et al. 2005) to form Sind-PDZ1, and the TEFCA tag was fused to the C-terminus of a model HER2/neu binding DARPin.X (Zahnd et al. 2007) to form DARPin.X-TEFCA. Lentiviruses pseudotyped with Sind-PDZ1 (Sind-PDZ1-pp) were efficiently functionalized with DARPin.X-TEFCA and the resulting lentiviral vectors efficiently transduced $HER2^+$ human ovaraian carcinoma SKOV3 cells ($8.9x10^6$ IU/mL) >100-fold more efficiently than "naked" Sind-PDZ1-pp ($7.4x10^4$ IU/mL). The association of DARPin.X-TEFCA and Sind-PDZ1-pp appears to be non-reversible under non-reducing conditions. Importantly, the functionalized virions retained full infectivity in the presence of human serum, indicating that our engineered lentiviral vectors are not

neutralized by human serum complement and pointing to a high potential for their *in vivo* application in human gene therapy.

2.3. Material and methods

2.3.1. Cells and chemicals

HEK 293T cells were purchased from Invitrogen (Carlsbad, CA). Human ovaraian carcinoma SKOV3 cells were kindly provided by Christian Buchholz (Paul-Ehrlich Institut; Langen, Germany) (Munch et al. 2011b). All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) containing 4,500 mg/liter glucose, 4.0 mM glutamine, and 110 mg/liter sodium pyruvate (Thermo Scientific HyClone, Logan, UT) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 1X non-essential amino acids (Thermo Scientific HyClone, Logan, UT). Dulbecco's phosphate-buffered saline (DPBS) was purchased from Thermo Scientific HyClone (Logan, UT).

2.3.2. Plasmids

Plasmid encoding the N-terminal PDZ domain of *Drosophila* InaD protein (PDZ1) was kindly provided by John Sondek (University of North Carolina, Chapell Hill). Plasmids encoding HIV Gag-Pol and vesicular stomatitis virus (VSV) envelope protein were kindly provided by Charles Rice (Rockefeller University, NY) (Evans et al. 2007b). The pTRIP-eGFP plasmid was constructed as previously described (Chamoun-Emanuelli et al. 2015). Sind-PDZ1 was constructed by replacing the NpuC domain in Sind-C* (Chamoun-Emanuelli et al. 2015) with PDZ1. HER2-binding DARPins were provided by Andreas Plückthun (University of Zurich; Zurich, Switzerland). A TEFCA or TEFSA

penta-peptide tag was fused to the C-terminus of DARPin.X to form DARPin.X-TEFCA/TEFSA and inserted into pET15b vector between the NdeI and XhoI restriction sites.

2.3.3. Pseudoparticle production

Lentiviral pseudoparticles were generated by co-transfecting 293T cells with plasmids encoding 1) HIV gag-pol (Evans et al. 2007b), 2) pTRIP-eGFP (Chamoun-Emanuelli et al. 2015) and 3) the appropriate envelope protein at a 1:1:4 weight ratio using the TransIT reagent (Mirus Bio LLC, Madison, WI). The supernatants containing the pseudoparticles were collected 48 h later, filtered (0.22 µm pore size) and stored at -80 °C in aliquots.

2.3.4. Cell surface expression

To confirm cell surface expression of chimeric envelope proteins, 1.6×10^6 HEK 293T cells were transfected with 960 ng of the appropriate plasmid using Trans IT (Mirus Bio LLC; Madison, WI) as per manufacturer's protocol. Forty-eight hours post transfection, cells were harvested, washed and stained with a 1:1000 dilution of mouse anti-Flag (Genscript; Piscataway, NJ) in DPBS supplemented with 1% bovine serum albumin (BSA) for 1 h. These cells were then washed and stained with a 1:500 dilution of Dylight 488 goat anti-mouse (Jackson ImmunoResearch Laboratories, Inc; West Grove, PA) diluted in DPBS/1%BSA for 30 min. After removal of excess antibody, samples were resuspended in DPBS containing 1% paraformaldehyde (PFA) and analyzed using a BD FACScan flow cytometer (BD Biosciences; San Jose, CA).

2.3.5. Viral incorporation of chimeric envelope proteins

Lentiviruses pseudotyped with the indicated envelope proteins were harvested, concentrated by ultracentrifugation (90 min; 40000xg; 4°) and resuspended in DPBS prior to mixing with 2X SDS loading buffer (0.5 M Tris-HCl, pH 6.8, 20% glycerol, 10% w/v SDS, 0.1% w/v bromophenol blue, 2% β-mercaptoethanol). Samples were boiled for 5 min at 95 °C, resolved by SDS-PAGE and electrotransferred onto a polyvinylidene fluoride transfer membrane (Pall Corporation; Pensacola, FL). Immunoblot analysis was performed with mouse anti HIV-1 p24 (NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: Monoclonal Antibody to HIV-1 p24 (No. 71-31) from Dr. Susan Zolla-Pazner) (Gorny et al. 1989) or mouse anti-Flag (Genscript; Piscataway, NJ) and horseradish peroxidase-conjugated goat anti-mouse (Jackson ImmunoResearch; West Grove, PA). Protein bands were visualized by chemiluminescence using a ChemiDoc-It imager (UVP, LLC; Upland, CA).

2.3.6. Protein expression and purification

All DARPin constructs were expressed in *Escherichia coli* BL21 (DE3) cells. Briefly, BL21 DE3 cells were transformed with the appropriate plasmid and plated on agar plates containing 50 µg/ml of kanamycin. The next day, 25-50 colonies from each plate were harvested and inoculated in 500-1000 ml of 2x LB media supplemented with the same antibiotics. The cells were grown at 37°C to an OD600 of ~0.6. Protein expression was induced by the addition of 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and the cells were grown at 18°C for 15h. Following expression, cells were harvested by centrifugation at 6,000 x g, 4°C for 20 minutes, and stored at -80°C until use. For protein

purification, cell pellets were resuspended in lysis buffer (50mM Tris-base, 500mM NaCl, pH 8.0) at 10 ml per gram of wet pellet, disrupted by sonication and centrifuged at 14,000 xg for 20 min. The soluble lysate was loaded onto a gravity column containing 0.5-1 ml Ni-NTA slurry (Qiagen, Valencia, CA). The column was washed once with 5 ml lysis buffer followed by a second wash with 5 ml lysis buffer containing 10 mM imidazole. Protein was eluted in 3-5 ml lysis buffer supplemented with 150 mM imidazole. Purified protein was concentrated to ~20-30 mg/ml using ultra-filtration spin columns (MWCO 10 kDa, Amicon Ultra, Millipore; Billerica, MA), dialyzed overnight against lysis buffer and stored at -80°C until use. For gel analysis, the proteins were mixed with equal volume of SDS sample buffer supplemented with 0.5 M β-mercaptoethanol (β-ME) and boiled for 5 min prior to resolution on 12% SDS-PAGE gels and visualized by molecular imager gel doc XR system (BioRad; Hercules, CA).

2.3.7. Protein sequences

Amino acid sequences corresponding to DARPin-TEFCA and PDZ1 constructs

6his-DARPin.9.16-*L*-**TEFCA**:

MGSSHHHHHHSSGLVPRGSHMGSDLGKKLLEAARAGQDDEVRILMANGAD
VNAHDFHGLTPLHLAAGMGHLEIVEVLLKNGADVNAVDTDGITLLHLAAYYG
HLEIVEVLLKHGADVNAHDYAGSTPLHLAANTGHLEIVEVLLKNGADVNAQDK
FGKTAFDISIDNGNEDLAEILQ*GGGG***TEFCA**

6his -DARPin.9.26-*L*-**TEFCA**:

MGSSHHHHHHSSGLVPRGSHMGSDLGKKLLEAARAGQDDEVRILMANGAD
VNAKDFYGITPLHLAAAYGHLEIVEVLLKHGADVNAHDWNGWTPLHLAAKYG

HLEIVEVLLKHGADVNAIDNAGKTPLHLAAAHGHLEIVEVLLKYGADVNAQDK

FGKTAFDISIDNGNEDLAEILQ*GGGG***TEFCA**

6his -DARPin.9.29-*L*-**TEFCA**:

MGSSHHHHHHSSGLVPRGSHMGSDLGKKLLEAARAGQDDEVRILMANGAD

VNAHDFYGITPLHLAANFGHLEIVEVLLKHGADVNAFDYDNTPLHLAADAGHL

EIVEVLLKYGADVNASDRDGHTPLHLAAREGHLEIVEVLLKNGADVNAQDKFG

KTAFDISIDNGNEDLAEILQ*GGGG***TEFCA**

6his -DARPin.H14R-*L*-**TEFCA**:

MGSSHHHHHHSSGLVPRGSHMGSDLGKKLLEAARAGQDDEVRILMANGAD

VNATDIHGHTPLHLAAAMGHLEIVEVLLKNGADVNANDWRGFTPLHLAALNG

HLEIVEVLLKNGADVNATDTAGNTPLHLAAWFGHLEIVEVLLKNGADVNAQD

KFGKTAFDISIDNGNEDLAEILQ*GGGG***TEFCA**

6his -DARPin.9.26-*L*-**TEFSA**:

MGSSHHHHHHSSGLVPRGSHMGSDLGKKLLEAARAGQDDEVRILMANGAD

VNAKDFYGITPLHLAAAYGHLEIVEVLLKHGADVNAHDWNGWTPLHLAAKYG

HLEIVEVLLKHGADVNAIDNAGKTPLHLAAAHGHLEIVEVLLKYGADVNAQDK

FGKTAFDISIDNGNEDLAEILQ*GGGG***TEFSA**

InaD-PDZ1 (11 to 107 Amino Acids)

AGELIHMVTLDKTGKKSFGICIVRGEVKDSPNTKTTGIFIKGIVPDSPAHLCGR

LKVGDRILSLNGKDVRNSTEQAVIDLIKEADFKIELEIQTFDK

Sind_env–*Flag*–**PDZ1**–Sind_env

DTTTSGAASANKYRYMAAAAVT*DYKDDDDK***AGELIHMVTLDKTGKKSFG**
ICIVRGEVKDSPNTKTTGIFIKGIVPDSPAHLCGRLKVGDRILSLNGKDVRNS
TEQAVIDLIKEADFKIELEIQTFDKGVTTVKEGTMDDIKISTSGPCR

2.3.8. In vitro conjugation assay

Purified DARPin.X-TEFCA/TEFSA and InaD-PDZ1 proteins were incubated at a 1:1 molar ratio for 4 h at 25°C. For SDS-PAGE analysis, the proteins were mixed with an equal volume of 2x SDS sample buffer in the presence or absence 0.5 M β-mercaptoethanol (β-ME) and boiled for 5 min prior to resolution by 12% SDS-PAGE gel. The percentage of reacted PDZ1 with the different DARPin.X-TEFCA constructs was quantified using the Trace Quantity module in Quantity One Software (BioRad, Hercules, CA, USA).

2.3.9. Infection assays

Unconcentrated supernatant harboring lentivirus pseudotyped Sind-PDZ1 (Sind-PDZ1-pp) were incubated with the appropriate concentrations of DARPin.X-TEFCA or DARPin.9.26-TEFSA at room temperature (RT) for 4 h. The mixture was then diluted 50-fold in OptiMEM medium and used to transduce SKOV3 cells via spinoculation (300xg for 1 h @ RT + 2 h at 37 °C). The cells were washed to remove unbound viruses and incubated at 37 °C / 5% CO_2. Forty eight hours later, these cells were harvested and the percentage of transduced cells (GFP^+) was analyzed via flow cytometry. To determine viral titers, functionalized virions were serially diluted and used for transduction. The

infectious units per milliliter (IU/mL) were calculated from at least three dilutions with linear correlation between the dilution factor and the percentage of GFP^+ cells.

2.3.10. Stability assay

Undiluted lentiviruses pseudotyped with Sind-PDZ1 (Sind-PDZ1-pp) were incubated with 2.5 µM DARPin 9.26-TEFCA for 4 h at RT. Functionalized virions were diluted 10-fold in dialysis buffer (1x PBS supplemented with 10mM L-glutathione and 0.05% sodium azide) and divided in two aliquots. One aliquot was kept at 4°C and the second aliquot was subjected to continuous dialysis against dialysis buffer at 4°C. At each time point, the appropriate volume of virus from each aliquot was removed, supplemented with BSA (2.7mg/ml), and stored at -80°C. Later, equivalent aliquot volumes were used to infect SKOV3 cells (4×10^4 cells/well in 48-well plate) seeded the previous day via spinoculation as described above.

2.3.11. Serum complement assay

Undiluted Sind-PDZ1-pp were incubated with DARPin.9.26-TEFCA (2.5 µM) for 4 h at 25°C, and mixed with an equal volume of untreated human AB serum (Corning; Corning, NY) or heat inactivated human serum for 1 h at 37°C. The preps were diluted 50-fold in OptiMEM media and used to transduce SKOV3 cells (4×10^4 cells/well; 48-well plate) via spinoculation.

2.4. Results

2.4.1. In vitro conjugation of DARPin.X.TEFCA and PDZ1

Using a splicing deficient variant of the natural split intein *Nostoc punctiforme* (Ramirez et al. 2013; Zettler et al. 2009), our laboratory previously developed an approach to non-covalently append a cell binding protein to the surface of lentiviruses *in vitro*. Specifically, one half of the split intein – NpuN – was fused to a cell-binding protein, while the other half – NpuC*– was displayed on lentiviral vectors as fusion to a binding-deficient, fusion-competent Sindbis virus envelope protein (Morizono et al. 2005) (Sind-C*). The split intein functioned as a molecular Velcro linking the cell-binding protein to the pseudotyped lentivirus. However, despite low nanomolar affinity between the two halves of the split intein (Shah et al. 2011), the cell-targeting protein gradually dissociated from the virus during extended periods of dialysis due to the non-covalent intein association, reducing the specific transduction efficiency of the virus over time.

In this study, we replaced the intein system with a covalent-bond-forming protein-peptide pair – the N-terminal PDZ domain (PDZ1) of InaD protein and its pentapeptide ligand (TEFCA) from NorpA (Kimple et al. 2001; Shieh et al. 1997) – to conjugate a cell-binding protein to the lentiviral vector. Proteins containing a C-terminal TEFCA tag can be selectively pulled-down by PDZ1-functionalized resin with a K_{on} >500 $M^{-1}S^{-1}$ under non-reducing conditions (there is no K_{off} due to the covalent linkage) (Kimple and Sondek 2002).

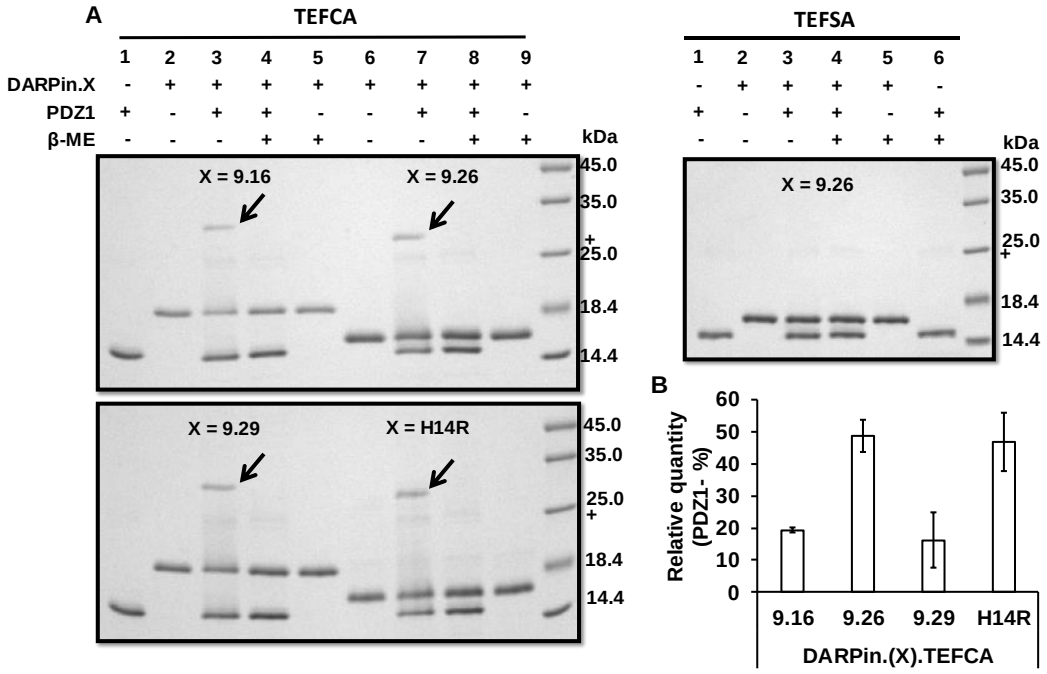

Figure 2.2: In vitro conjugation of DARPin.X-TEFCA and PDZ1. (A) PDZ1 (100 μM) was incubated with various DARPin.X-TEFCA or control DARPin.9.26-TEFSA (100 μM) at room temperature for 4 h. The mixture was then loaded on 12% SDS-PAGE gel under reducing or non-reducing conditions. The conjugation product (indicated by black arrow) can only be seen under non-reducing condition (lane 3). '+' denotes unidentified band. **(B)** The percentage of PDZ1 reacted with different DARPin.X.TEFCA was calculated by the ratio of the PDZ1 band intensity in presence (lane 3 and 7) and absence of DARPin.X-TEFCA (lane 1), with respect to the specific DARPin-TEFCA constructs. Values and error bars represent the average and standard deviation, respectively, of at least two independent experiments.

We first fused the TEFCA tag to a panel of HER2/neu-binding designed ankyrin repeat protein (DARPin.X) (Munch et al. 2011a), and confirmed the ability of these fusion proteins to form a disulfide bond with purified PDZ1 *in vitro* (Figure 2.2 A). As negative control, we mutated the Cys in TEFCA to Ser to form TEFSA penta-peptide, and fused it to DARPin.9.26. Surprisingly, despite sharing the same overall structure, the efficiency of disulfide bond formation was quite different for the different DARPins (Figure 2.2 B). For

DARPin.9.26 and .H14R, ~50% of the input protein formed disulfide complex with PDZ1 after 4 h incubation at room temperature, while <20% of DARPin.9.16 and .9.29 formed disulfide bonds under the same condition. It is unclear what caused the different disulfide bond formation efficiency, as the TEFCA tag was fused to the C-terminus of each DARPin via the same short GGGG linker.

2.4.2. Cell surface expression and virion incorportation of PDZ1

Next, we replaced the NpuC* in Sind-C* with PDZ1 to form Sind-PDZ1, and confirmed the high cell surface expression of the new chimeric Sind-PDZ1 and its ability to be incorporated onto lentivirus, it is worth nothing that both Sind-C* and Sind-PDZ1 transduced cells contain a small subpopulation exhibiting a very high surface expression level of the chimera protein. The significance of this subpopulation is unknown (Figure 2.3 B). Significant amount of PDZ1 was detected on the lentiviral virions, albiet at a much lower band intensity than that of Sind-C* (Figure 2.3 C). The reduced intensity of Sind-PDZ1 band than Sind-C* may be an artifact due to the different antibody binding efficiency between the 3xFlag-tag and the Flag-tag present on Sind-C* and SinD-PDZ1, respectively. However, it is also possible that less Sind-PDZ1 is incorporated into the virions due to its reduced cell surface display efficiency. Never theless, a significant amount of PDZ1 can be detected on lentiviral virion, confirming the ability of lentiviruses to be pseudotypes with Sind-PDZ1.

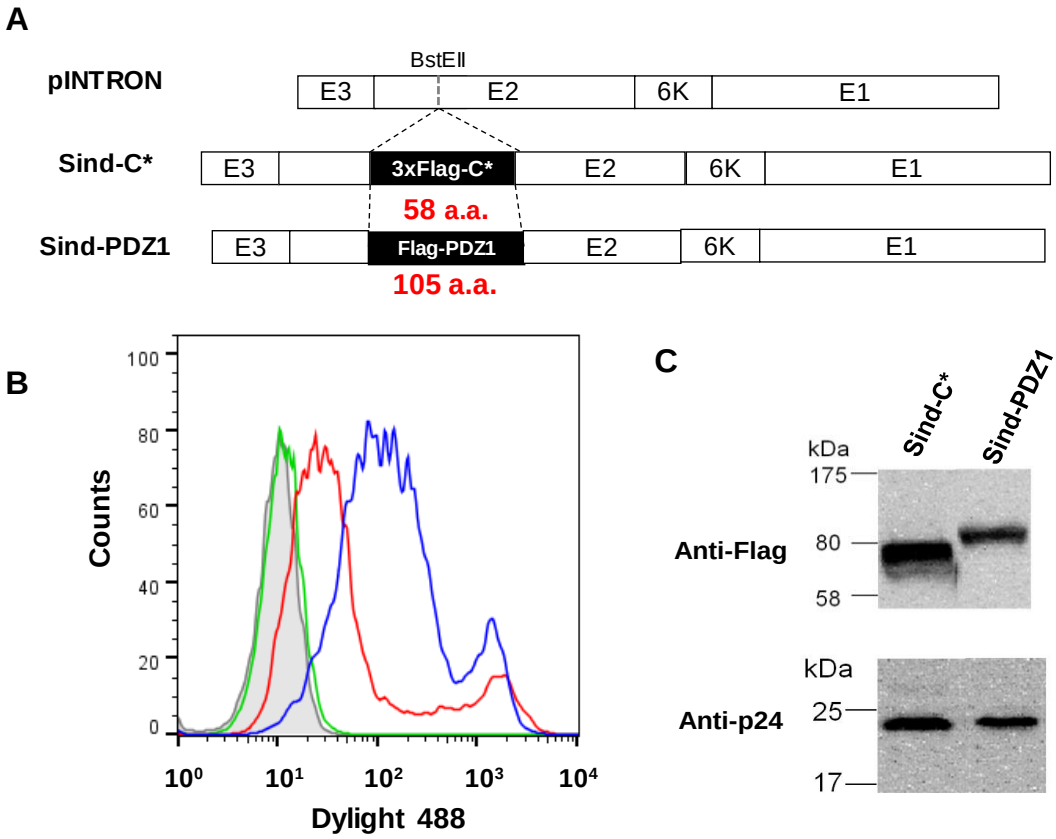

Figure 2.3: (A) Schematic representation of different Sindbis envelope proteins. Sind-C* contains the C-intein from a splicing deficient DnaE intein from Nostoc punctiforme inserted between amino acids 71 and 74 of the Sindbis E2 protein. In Sind-PDZ1, the PDZ1 domain of Drosophila InaD protein was inserted by replacing the C*. **(B)** Cell surface expression of Sind-PDZ1. HEK 293T cells were transfected with plasmids encoding Sind-C* (Blue) or Sind-PDZ1 (Red), unstained 293T cells (Gray) and 293T stained with primary and secondary antibody (Green) served as negative controls. **(C)** Virion incorporation of Sind-PDZ1-pp. Virus containing supernatants were harvested, concentrated 100-fold by ultracenfrifugation, and loaded onto a 12% SDS-PAGE gel. After separation, the protein bands were transferred to a PVDF membrane and detected using mouse anti-HIV p24 (1:250) or mouse anti-Flag (1:1000) and goat anti-mouse-HRP secondary antibody (1:1000).

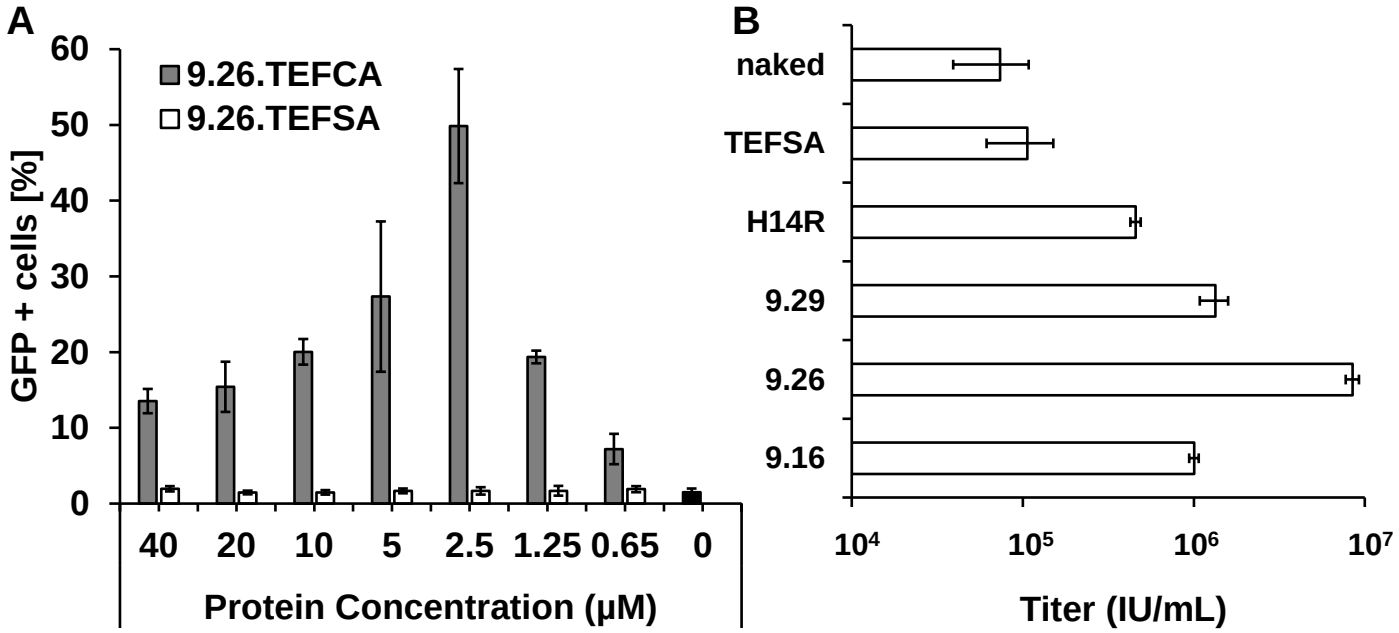

Figure 2.4: Transduction of HER2$^+$ SKOV3 cells by DARPin-functionalized Sind-PDZ1-pp. (A) Bar diagram of the percentage of cells transduced with Sind-PDZ1-pp functionalized with different concentrations of DARPin.9.26-TEFCA or –TEFSA. The presence of intracellular GFP indicates successful transduction. **(B)** Infectious titer of Sind-PDZ1-pp functionalized with the different DARPins (2.5 µM) in SKOV3 cells. Values and error bars represent the average and standard deviation, respectively, of three independent experiments.

2.4.3. Retargeting Sind-PDZ1-pp with DARPin.X-TEFCA

Since DARPin.9.26 exhibited the highest disulfide-bond forming efficiency with PDZ1, and was able to most efficiently retarget lentivirus pseudotyped with Sind-C* in our previous study (Chamoun-Emanuelli et al. 2015), we first carried out a dose-response experiment to determine the ability of DARPin.9.26-TEFCA to retarget Sind-PDZ1-pseodutyped lentivirus (Sind-PDZ1-pp) harboring a GFP reporter gene. Sind-PDZ1-pp was incubated with increasing concentrations of DARPin.9.26-TEFCA at room temperature for 4 h, diluted 50-fold in OptiMEM medium and used to transduce HER2/neu$^+$ ovarian cancer cell line SKOV3. DARPin.9.26-TEFSA was used as negative control. As shown in Figure 2.4 A, DARPin.9.26-TEFCA was able to efficiently and dose-dependently retarget Sind-PDZ1-pp, with an optimal concentration of 2.5 μM (achieved >50% GFP$^+$ cells). In contrast, only background transduction (~2% GFP$^+$ cells) was observed for Sind-PDZ1-pp incubated with DARPin.9.26-TEFSA, confirming that the specific interaction between TEFCA penta-peptide and PDZ1 is responsible for the virus retargeting. However, the lower transduction observed at higher protein concentrations is likely attributed to the competition of excess unconjugated DARPin with the functionalized virions for the HER/neu receptor.

To determine the infectious titers (IU/mL) of the retargeted lentivirus, Sind-PDZ1-pp was incubated with 2.5 μM of different DARPin.X-TEFCA at room temperature for 4 h, serially diluted and used to transduce SKOV3 cells. The average infectious titer for each DARPin construct was calculated from at least three dilutions that showed linear correlation between the dilution factor and the percentage of GFP$^+$ cells. Unfunctionalized

and DARPin.9.26-TEFSA (TEFSA) functionalized Sind-PDZ1 pp were included as controls. As shown in Figure 2.4 B, virions functionalized with DARPin.9.26-TEFCA ($8.9x10^6$ IU/mL) displayed a~100-fold higher transduction efficiency, compared to unfunctionalized naked ($7.4x10^4$ IU/mL) or DARPin.9.26-TEFSA functionalized Sind-PDZ1-pp ($1.1x10^5$ IU/mL). Lentiviruses functionalized with DARPin.9.16 and DARPin.9.29 achieved intermediate infectious titer of 10^6 and $1.3x10^6$ IU/mL, respectively, possibly in part due to the less efficient disulfide bond formation between these DARPins and PDZ1 (Figure 2.2 B). Conversely, despite high disulfide bond forming efficiency, Sind-PDZ1-pp loaded with DARPin.H14R was least infectious against SKOV3 cells with a titer of $4.8x10^5$ IU/mL.

Recently, Buchholz and coworkers fused the same set of DARPins to Nipah virus (NiV) envelope protein and used them to retarget NiV-pseudotyped lentivirus (NiV-pp) (Bender et al. 2016b). Contrary to our findings, they showed that NiV-pp displaying DARPin.H14R exhibited the highest transduction efficiency, ~100-fold higher than those displaying DARPin.9.26. As proposed by the authors, fusion between viral and host membrane requires that these membranes be brought sufficiently close to each other by the viral envelope protein and the cell surface receptor. The threshold distance required for viral fusion is expected to be viral envelope protein dependent, and it is <100 Å for NiV. Unlike the other DARPins used in this study, which all bind to domains I-III of HER2/*neu* receptor, DARPin.H14R associates with domain IV (Steiner et al. 2008), which is located in close proximity to the cell membrane. HER2/*neu* receptor Domain I and II are located at the tip of the receptor, farthest away from the cell membrane, while Domain

III is in between domain IV and domain I/II. DARPin.H14R, through interaction with domain IV, likely was able to reduce the distance between the viral and host membrane to below a threshold value, resulting in high transduction efficiency of DARPin.H14R-displaying NiV-pp.

On the other hand, DARPin.9.26, which interacts with a more distal domain on HER2, was unable to do so, resulting in lower transduction efficiency of NiV-pseudotyped lentiviruses displaying DARPin.9.26 than those displaying DARPin.H14R. The close proximity of domain IV also means that, to interact with domain IV, the viral protein needs to reach pass domains I-III. In the NiV study, different DARPins are tethered to the C-terminus of NiV-G protein via a flexible linker ($(G_4S)_3$), enabling these DARPins to freely reach out to the cell receptor. In our study, the DARPins are fused to TEFCA ligand via a short linker (G_4) and are anchored onto the lentiviruses through interaction with PDZ1, which was inserted into a surface-exposed loop on the E2 glycoprotein of the Sindbis virus. Thus, our DARPins likely exhibit much reduced flexible on the virions, and are likely unable to efficiently interact with Domain IV due to steric hindrances, resulting in reduced transduction efficiency of DARPin.H14R-functionalized Sind-PDZ1-pp.

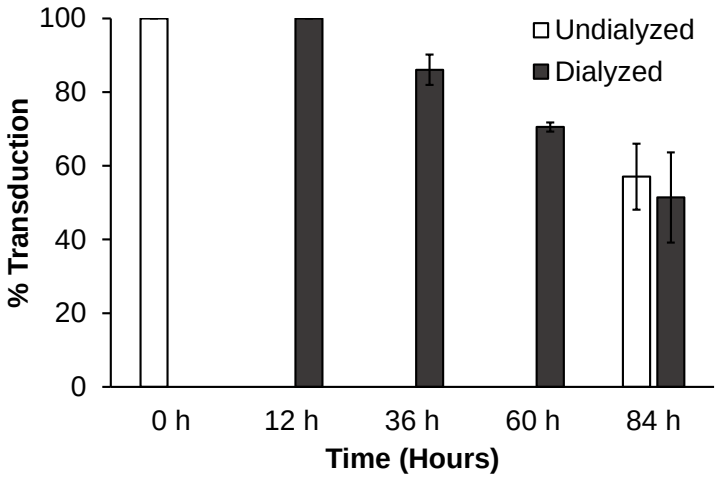

Figure 2.5: PDZ1/TEFCA-mediated functionalization of Sind-PDZ1-pp is stable. Sind-PDZ1-pp was incubated with DARPin.9.26-TEFCA (2.5 μM) at RT for 4h, divided into two groups. Group 1 was continuously dialyzed in dialysis buffer (1x PBS supplemented with 10mM L-glutathione and 0.05% sodium azide) at 4 °C while group 2 was stored at 4 °C without dialysis. The infectivity of these samples at indicated time points was quantified in SKOV3 cells, and normalized to the corresponding value at time 0. Values and error bars represent the average and standard deviation, respectively, of two independent experiments.

2.4.4. Stability of DARPin.X-TEFCA-functionalized Sind-PDZ1-pp

Since PDZ1 forms a covalent intermolecular disulfide bond with the TEFCA peptide (Kimple et al. 2001; Kimple and Sondek 2002; Lu et al. 2014), the complex of PDZ1-TEFCA should be stable under non-reducing conditions, such as human serum (Paulson 1996). To assess the stability of the retargeted lentivirus, Sind-PDZ1-pp was loaded with DARPin.9.26-TEFCA and then dialyzed in >1,000-fold of dialysis buffer (1x PBS supplemented with 10mM L-glutathione and 0.05% sodium azide) at 4 °C for >3 days. We reasoned that, if the functionalization is stable, we should observe minimum infectivity change between the dialyzed and undialyzed viruses. On the other hand, if the

conjugation is not stable (reversible), any DARPin.9.26-TEFCA molecules that dissociate from the Sind-PDZ1-pp should be removed during the dialysis, leading to reduced virus infectivity of the dialyzed virus compared to undialyzed virus overtime. In our previous study, the reversible interaction between the two halves of the split intein, despite of a low nanomolar K_d, led to significantly reduced infectivity of the functionalized virion after 24 h of dialysis (Chamoun-Emanuelli et al. 2015). Sind-PDZ1-pp was conjugated with DARPin.9.26-TEFCA (2.5 μM) at RT for 4 h, diluted 10-fold in dialysis buffer and divide into two samples; sample A was kept intact at 4 °C while sample B was extensively dialyzed at 4 °C. Aliquots from both samples were harvested at different times and used to infect SKOV3 cells. As shown in Figure 2.5, similar infectivity was observed between the dialyzed and undialyzed virus sample even after 84 h of continuous dialysis, confirming that the PDZ1-TEFCA complex can be used to stably functionalize pseudotyped lentivirus. The reduced infectivity seen in both virus samples is likely due to intrinsic virion inactivation at 4 °C.

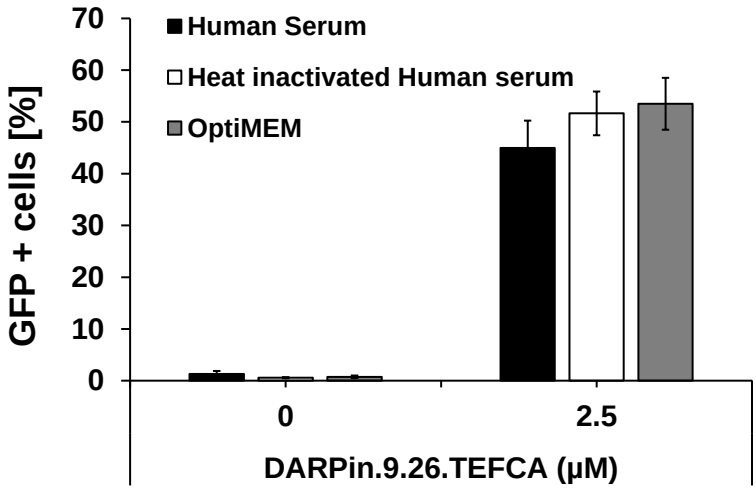

Figure 2.6: DARPin-functionalized Sind-PDZ1-pp is not inactivated by human complement. Sind-PDZ1-pp functionalized with DARPin.9.26-TEFCA were incubated with an equal volume of untreated, heat-inactivated human serum or OptiMEM medium at 37°C for 1 hr. Each virus was diluted 50-fold with OptiMEM medium and used to infect SKOV3 cells. Values and error bars represent the average and standard deviation, respectively, of at least three independent experiments.

Finally, for *in vivo* application in humans, the gene therapy vector should not be inactivated by serum complements, which attack pathogens by several mechanisms such as lectin, classical and alternative pathways (Roozendaal and Carroll 2006; Schauber-Plewa et al. 2005). To investigate this, DARPin.9.26-TEFCA-functionalized Sind-PDZ1-pp was mixed with an equal volume of normal or complement-inactivated (heat inactivated) human serum and incubated at 37 °C for 1 h. The mixture was diluted 50-fold in OptiMEM medium and used to transduce SKOV3 cells. As shown in Figure 2.6, similar infectivity was observed for virions incubated with normal and complement-inactivated human serum, indicating that our virus retargeting strategy is compatible with immunocompetent hosts. This result is expected, as our envelope protein (Sind) was

derived from 2.2, which was previously found to exhibit reduced sensitivity toward human serum (Morizono et al. 2010), and the functionalization moiety – PDZ1 – is a *Drosophila* protein.

2.5. Discussion

In summary, we developed a new, modular platform for retargeting lentivirus through functionalization of a cell-binding protein. A pair of disulfide-bond forming protein-peptide pair, the N-terminal PDZ domain (PDZ1) of InaD protein and its pentapeptide ligand (TEFCA) from NorpA, was exploited as a molecular Velcro to retarget pseudotyped lentivirus to a desired cell type. Her2/neu-specific DARPins were fused to the N-terminus of TEFCA, and loaded onto lentiviruses pseudotyped with a receptor-blinded Sindbis virus envelope protein harboring the PDZ1 in an exposed extracellular loop. Chimeric envelope protein Sind-PDZ1 can be efficiently displayed on the cell surface and be incorporated onto the lentivirus. Pseudotyped lentivirus functionalized with HER2/neu-specific DARPin.9.26-TEFCA was highly infectious and specific to $HER2^+$ cells achieving an infectious titer of $8.9x10^6$ IU/mL in SKOV3 cells, a >100-fold higher than that of the unfunctionalized naked lentivirus ($7.4x10^4$ IU/mL). Functionalization of lentivirus with DARPins targeting different domains of HER2/neu resulted in varying degrees of infectivity. The highest infectivity was observed with DARPin.9.26, which targets a distal domain on HER2/neu, while functionalization with DARPin.H14R, which binds to a domain closest to the cell membrane (domain IV) displayed the lowest infectivity ($4.8x10^5$ IU/mL). This result is in disagreement to that previously published where DARPin.H14R-displaying Nipah virus envelope protein pseudotyped lentivirus

(NiV-pp) achieved a much higher infectivity than the same virus displaying the DARPin.9.26 (Bender et al. 2016b). This discrepancy likely stems from the poor ability of DARPin.H14R on Sind-PDZ1-pp to interact with the HER2/neu domain IV due to steric hindrance. It is important to note that the interaction between PDZ1 and TEFCA penta-peptide is covalent and stable under non-reducing condition, leading to low infectivity loss of functionalized virions during prolonged dialysis (Figure 2.5), confirming that Sind-PDZ1-pp can be stably functionalized with a TEFCA-tagged cell-targeting protein. Finally, unlike lentivirus pseudotyped with VSV-G (DePolo et al. 2000) or full-length antibody (Morizono et al. 2010), our retargeted lentivirus was insensitive to serum complement, pointing to the potential of these viral vectors to be applied *in vivo*. Taken together, our work establishes a new facile lentivirus retargeting strategy that avoids the dependence of "surface compatibility" of intracellularly expressed fusion constructs and allows any cell-binding protein to be appended onto pseudotyped lentivirus via a facile "plug-and-play" fashion. We envision that this technology should provide a convenient and powerful tool for the engineering of a new generation of cell-specific lentiviral vectors for *in vivo* gene therapy.

CHAPTER III

VERSATILE RETARGETING LENTIVIRAL SYSTEM BEARING THE ISOPEPTIDE BOND PAIR SPYTAG-SPYCATCHER

3.1. Overview

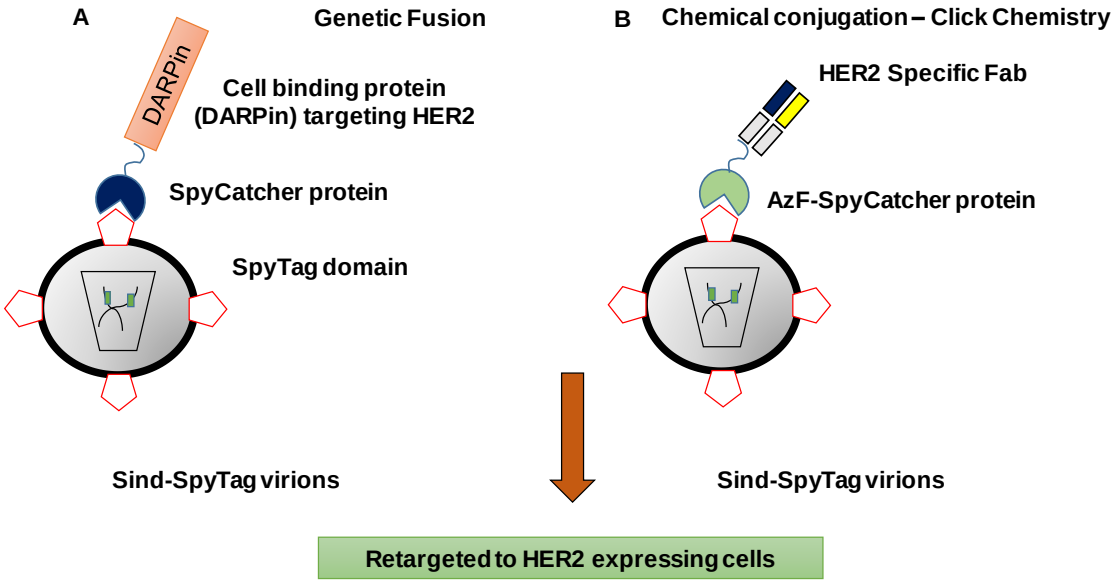

Figure 3.1: Schematic representation of the retargeting strategy for SpyTag-SpyCatcher protein-peptide pair. (A) DARPin was genetically fused to SpyCatcher protein on the N-terminal, **(B)** SpyCatcher is chemically conjugated to HER-specific fragment antigen binding (Fab) through click chemistry reaction to form Fab-SpyCatcher. The fused protein were allowed to interact with lentivirus pseudotyped with chimeric Sindbis envelope protein with the c-terminal fragment of collagen adhesion domain (CnaB2) protein (SpyTag). The functionalized virions were allowed to transduce HER2+ cells.

Specific transduction of desired cells represents the holy grail of gene therapy. We report a versatile and broadly applicable lentiviral retargeting system, exploiting the

isopeptide bond forming protein-peptide pair, the N-terminal fragment (SpyCatcher) and C-terminal fragment (SpyTag) of the collagen adhesion domain (CnaB2) from the fibronectin binding protein (FbaB) in *Streptococcus pyogenes*. The SpyTag protein was genetically incorporated into the extracellular loop of a binding deficient, fusion competent Sindbis envelope protein and the SpyCatcher was genetically and chemically fused to a HER2 targeting protein or fragment antigen binding (Fab) (Figure 3.1). Functionalized Sind-SpyTag virions were able to target and transduce the HER2+ SKOV3 cells (8×10^6 IU/mL) with a 100-fold higher efficiency compared to the naked SpyTag virions (5.8×10^4 IU/mL), and exhibited higher HER2-receptor-specific selectivity index in a mixed cell population. The isopeptide bond was observed to be stable during prolonged dialysis and in presence of serum complement, supporting its potential use in *in vivo* applications. Finally, the chemically conjugated HER2 specific Fab functionalized virions were able to target HER2+ cells in a dose dependent manner, allowing this plug and play retargeting system to accommodate the plethora of commercially available antibodies.

3.2. Introduction

Lentiviral vectors can infect both dividing and non-dividing cells and integrate their transgene into the host cell chromosome for sustained gene expression, which is favorable for therapy of chronic and malignant diseases (Gunzburg et al. 1996; Kotterman et al. 2015). The gene therapy approaches through *ex vivo* transduction have obtained success in hematopoietic diseases, such as X-linked SCID, but the same approach is not suitable for gene transduction of solid organs or most body tissues. However, these limitations can

be overcome by intravenous administration of gene therapy vectors that specifically home in on and transduce desired cells and tissues *in vivo* (Kaufmann et al. 2013; Kotterman et al. 2015; Naldini 2015).

Development of robust targeting vectors remains a major drawback in gene therapy. To date, different approaches have been adopted including pesudotyping lentiviral vectors with chimeric envelope proteins, where targeting molecules are genetically fused to the envelope protein (Boeckle and Wagner 2006; Waehler et al. 2007) and conjugating viruses with adaptor molecules that function as bridges between the targeting moiety and the viral vector (Larochelle et al. 2002). However, each approach possesses its own limitations in the first approach, as the targeting molecule is genetically fused to the envelope protein, it is necessary to generate a new chimeric envelope for each new target, which sometimes may destroy the function and structure of the chimeric protein. In the second approach, however, the adaptor molecule should display high affinity and stability to maintain the conjugation between the viral vector and the targeting moiety.

Previously, Morizono *et.al*, developed targeting lenitiviral vectors using the second approach. These targeting vectors were conjugated with monocloncal antibodies via the interaction between the Fc-binding region of protein A (ZZ domain) inserted into the envelope protein and the Fc region of the antibodies (Morizono et al. 2001; Morizono et al. 2005; Pariente et al. 2008). However, its use was limited to immunocompromised species. Using a similar principal, our group developed another targeting lentiviral vector using a splicing deficient DnaE intein from *Nostoc puntiforme* (Npu). The splicing deficient variant C-Intein, NpuC*, was inserted into the extracellular loop region of an

attachment-deficient Sindbis E2 envelope protein. The other intein fragment, N-intein (NpuN), was fused to a cell targeting protein. The electrostatic interaction between inteins mediated the conjugation of the targeting moiety to the virus thereby redirecting the vector to the intended cell (Chamoun-Emanuelli et al. 2015; Ramirez et al. 2013; Zettler et al. 2009). However, in both approaches, the conjugation between the virus and the cell targeting protein was observed to be unstable due to the non-covalent interaction.

To stabilize the conjugation between the targeting molecule and the vector, we employed an isopeptide bond forming pair, the N-terminal fragment (SpyCatcher) and C-terminal fragment (SpyTag) of the collagen adhesion domain (CnaB2) from the fibronectin binding protein (FbaB) in *Streptococcus pyogenes* (Kang et al. 2007; Li et al. 2014; Zakeri et al. 2012; Zakeri and Howarth 2010). First, the ZZ domain in the envelope protein was replaced with the SpyTag peptide, and its partner, SpyCatcher, was genetically fused to a cell targeting protein. Through the isopeptide bond, the SpyTag virions were conjugated with the SpyCatcher construct, producing a viral vector displaying a cell targeting protein. To accommodate the use of commercially available antibodies as targeting moieties in our system, a non-natural amino acid was introduced upstream of the SpyCatcher protein allowing chemical conjugation of cell targeting proteins via click chemistry. Through this approach, a HER2 specific Fab fragment was chemically conjugated to SpyCatcher, and used to functionalize the virions. Virions functionalized with targeting moieties fused or chemically conjugated to SpyCatcher displayed a receptor specific targeted gene transduction.

3.3. Materials and methods

3.3.1. Cells and chemicals

HEK 293T cells were purchased from Invitrogen (Carlsbad, CA). SKOV3, chinese hamster ovary cells (CHO-K1) cells and selected clone was kindly provided by Christian Buchholz (Paul-Ehrlich Institut; Langen, Germany) (Munch et al. 2011b). Unless otherwise stated, all cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) containing 4,500 mg/liter glucose, 4.0mML-glutamine, and 110 mg/liter sodium pyruvate (Thermo Scientific HyClone, Logan, UT) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 1X non-essential amino acids (Thermo Scientific HyClone, Logan, UT). For the selected clone, CHO-HER2-K6, growth media was supplemented with 1.2mg/mL of the antibiotic G418. Dulbecco's phosphate-buffered saline (DPBS) was purchased from Thermo Scientific HyClone (Logan, UT).

3.3.2. Plasmids

Plasmids encoding HIV Gag-Pol and vesicular stomatitis virus (VSV) envelop protein were kindly provided by Charles Rice (Rockefeller University, NY) (Evans et al. 2007a). The pTRIP-eGFP plasmid was constructed by replacing the Gaussia luciferase gene in pTRIP-Gluc (Chockalingam et al. 2010) with eGFP (Chamoun-Emanuelli et al. 2015). The plasmid encoding the 2.2 envelope protein was purchased from Addgene (Addgene plasmid 34885) (Pariente et al. 2007). Sind-SpyTag was constructed by overlap extension PCR, using the primers ST1 and ST2 (Table 2). The amplified insert was digested with the BstEII restriction enzyme and cloned into the BstEII digested 2.2 plasmid. pET28-

SUMO-SpyTag (Fierer et al. 2014) and pDEST14-SpyCatcher (Addgene plasmid # 35044) (Zakeri et al. 2012) constructs were kindly provided by Mark Howarth (Oxford university, UK). The truncated version SpyCatcher2 was constructed by removing the 23 N-terminal and 9 C-terminal residues from the wild-type SpyCatcher. The truncated version has comparable efficiency as the full length SpyCatcher to react with SpyTag (Zhang et al. 2013). pI-SpyCatcher2 was generated by PCR amplification of the insert from the construct pDEST14-SpyCatcher, using the primers SC1 and SC2. To construct pI-DARPin.9.26-SpyCatcher2 and pI-AzF-SpyCatcher2, inserts were PCR amplified from the construct pDEST14-SpyCatcher, using the primers DSC1 and DSC2, and AS1 and AS2, respectively (Table 2). The amplified inserts were digested by NdeI and XhoI restriction enzymes and inserted into the pI vector digested with the same enzymes.

Table 3.1 List of primers used for cloning SpyTag-SpyCatcher constructs

Label	Primer sequence
ST1	5'-GGTAACCGACTACAAAGACCATGACGGTGATTATAAAGATCATGACATCGACTAC-3'
ST2	5'-ACGATGTGGGCACCGCCCTTGTCGTCGTCGTCTTTGTAGTCGATGTCATGATCTTTATAA-3'
SC1	5'- CATATGAGCGGCGATAGTGCTACCCATATTAAATTCTCAAAAC-3'
SC2	5'- CTCGAGGCCATTTACAGTAACCTGACCT-3'
DSC 1	5'- GCTAGC GATTACGACATCCCAACGACC-3'
DSC 2	5'-CTCGAGTTAAATATGAGC GTCACCTTTAGTTGC-3'
AS1	5'-ATT ATA CAT ATG TGG GAA CTG CAG CAG AGC TAG GGT GGC AGC GGC GAT AGT GCT ACC-3'
AS2	5'-ATT ATA CTC GAG TTA GCC ATT TAC AGT AAC CTG-3'

3.3.3. Protein expression and purification

All the SpyCatcher2 and SpyTag constructs were expressed in *Escherichia coli* BL21 (DE3) cells. Briefly, BL21 DE3 cells were transformed with the appropriate plasmid and plated on LB-agar plates containing 50 µg/ml of kanamycin. The next day, 25-50 colonies from each plate were used to inoculate 500-1000 ml of 2x LB media supplemented with the same antibiotics. The cells were grown at 37°C to an OD_{600} of ~0.6. Protein expression was induced by the addition of 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and the cells were grown at 18°C for 15h. Following expression, cells were harvested by centrifugation at 6,000x g, 4°C for 20 minutes, and stored at -80°C until use. For protein purification, cell pellets were resuspended in lysis buffer (50mM Tris-base, 500mM NaCl, pH 8.0) at 10 ml per gram of wet pellet, disrupted by sonication and centrifuged at 14,000xg for 20 min. The soluble lysate was loaded onto a gravity column containing 0.5-1 ml Ni-NTA slurry (Qiagen, Valencia, CA). The column was washed once with 5 mL lysis buffer followed by a second wash with 5 mL lysis buffer containing 10 mM imidazole. Protein was eluted in 3-5 mL of lysis buffer supplemented with 150 mM imidazole. Purified protein was concentrated to ~10-20 mg/ml using ultra-filtration spin columns (MWCO 10 kDa, Amicon Ultra, Millipore; Billerica, MA), dialyzed overnight against lysis buffer and stored at -80°C until use. For SDS-PAGE analysis, the proteins were mixed with an equal volume of SDS sample buffer supplemented with 0.5 M β-mercaptoethanol (β-ME) and boiled for 5 min prior to resolution on 12% SDS-PAGE gels and visualized by molecular imager gel doc XR system (BioRad; Hercules, CA).

3.3.4. Protein sequences

Amino acid sequences corresponding to the SpyTag and SpyCatcher2 constructs

Myc-tag-DARPin.9.26-*L*-**SpyCatcher2**-6His

MEQKLISEEDLGSDLGKKLLEAARAGQDDEVRILMANGADVNAKDFYGITP
LHLAAAYGHLEIVEVLLKHGADVNAHDWNGWTPLHLAAKYGHLEIVEVLLKH
GADVNAIDNAGKTPLHLAAAHGHLEIVEVLLKYGADVNAQDKFGKTAFDISID
NGNEDLAEILQEAC*GGGGSGGGGS*AS**SGDSATHIKFSKRDEDGKELAGATME**
LRDSSGKTISTWISDGQVKDFYLYPGKYTFVETAAPDGYEVATAITFTVNEQ
GQVTVNGLEHHHHHH

6His-SUMO-*L*-**SpyTag**

MGSSHHHHHHGSDSEVNQEAKPEVKPEVKPETHINLKVSDGSSEIFFKIKKTT
PLRRLMEAFAKRQGKEMDSLRFLYDGIRIQADQTPEDLDMEDNDIIEAHREQI*G*
*GG***AHIVMVDAYKPTK**GY

SpyCatcher2-6His

MSGDSATHIKFSKRDEDGKELAGATMELRDSSGKTISTWISDGQVKDFYLYP
GKYTFVETAAPDGYEVATAITFTVNEQGQVTVNGLEHHHHHH

6His-WELQ-**AzF**-*L*-SpyCatcher2

MGSSHHHHHHSSGLVPRGSHNYHMWELQQS**azF***GG*SGDSATHIKFSKRDED
GKELAGATMELRDSSGKTISTWISDGQVKDFYLYPGKYTFVETAAPDGYEVAT
AITFTVNEQGQVTVNG

3.3.5. Protein expression, purification and activity of AzF-SpyCatcher2

For the AzF-SpyCatcher2 construct, the BL21 (DE3) cells were co-transformed with pEVOL-AzFRS (Chin et al. 2002; Guan et al. 2015) expression construct for $tRNA_{CUA}$(Tyr) and tyrosyl-tRNA synthetase specific for azido-L-Phenylalanine from *Methanococcus jannaschii* (which incorporate unnatural amino acid azido-L-Phenylalanine (AzF) at the amber codon site), and plated on LB-agar plates containing 100 µg/ml of ampicillin and 34 µg/ml of chloramphenicol. The next day, 5-10 colonies from each plate were used to inoculate 50 ml of 2x LB media supplemented with the same antibiotics. The cells were grown at 37°C to an OD_{600} of ~0.6. Protein expression was induced by the addition of 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), 0.02% arabinose and 5 mM 4-azido-L-phenylalanine (VWR, Radnor, PA). The cells were grown at 18°C for 15 h and protein purification was carried out as described above. Purified protein was concentrated and dialyzed against PBS buffer pH 7.4 using ultra-filtration spin columns (MWCO 10 kDa, Amicon Ultra, Millipore; Billerica, MA), stored at -80°C until use. For SDS-PAGE analysis, the proteins were mixed with an equal volume of SDS sample buffer supplemented with 0.5 M β-mercaptoethanol (β-ME) and boiled for 5 min prior to resolution on 12% SDS-PAGE gels and visualized by molecular imager gel doc XR system (BioRad; Hercules, CA).

The activity of incorporated AzF was determined by interacting the purified AzF-SpyCatcher2 with DBCO-PEG4-TAMRA dye (Sigma-Aldrich, St.Louis, MO) at a 1:50 molar ratio for 1 h at 22°C. The product was resolved by SDS-PAGE and visualized by UV light using the molecular imager gel doc XR system (BioRad; Hercules, CA).

3.3.6. Cell surface expression

To confirm cell surface expression of chimeric envelope proteins, 1.6×10^6 HEK 293T cells were transfected with 960 ng of the appropriate plasmid using Trans IT (Mirus Bio LLC; Madison, WI) as per manufacturer's protocol. Forty-eight hours post transfection; cells were harvested, washed and stained with a 1:1000 dilution of mouse anti-Flag (Genscript; Piscataway, NJ) in DPBS supplemented with 1% bovine serum albumin (BSA) for 1 h. These cells were then washed and stained with a 1:500 dilution of Dylight 488 goat anti-mouse (Jackson ImmunoResearch Laboratories, Inc; West Grove, PA) diluted in DPBS/1%BSA for 30 min. After removal of excess antibody, samples were resuspended in DPBS containing 1% paraformaldehyde (PFA) and analyzed using a BD FACScan flow cytometer (BD Biosciences; San Jose, CA).

3.3.7. Viral incorporation of chimeric envelope proteins

Lentiviruses pseudotyped with the indicated envelope proteins were harvested, concentrated by ultracentrifugation (90 min; 40000 xg; 4°) and resuspended in DPBS prior to mixing with 2X SDS loading buffer (0.5 M Tris-HCl, pH 6.8, 20% glycerol, 10% w/v SDS, 0.1% w/v bromophenol blue, 2% β-mercaptoethanol). Samples were boiled for 5 min at 95 °C, resolved by SDS-PAGE and electrotransferred onto a polyvinylidene fluoride transfer membrane (Pall Corporation; Pensacola, FL). Immunoblot analysis was performed with mouse anti HIV-1 p24 (NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: Monoclonal Antibody to HIV-1 p24 (No. 71-31) from Dr. Susan Zolla-Pazner) (Gorny et al. 1989) or mouse anti-Flag (Genscript; Piscataway, NJ) and horseradish peroxidase-conjugated goat anti-mouse (Jackson ImmunoResearch; West

Grove, PA). Protein bands were visualized by chemiluminescence using a ChemiDoc-It imager (UVP, LLC; Upland, CA).

3.3.8. In vitro conjugation

Purified DARPin.9.26-SpyCatcher2/SpyCatcher2 and SUMO-SpyTag proteins were incubated at a 1:1 molar ratio for different time-periods at 25°C. For SDS-PAGE analysis, the proteins were mixed with an equal volume of SDS sample buffer supplemented with 0.5 M β-mercaptoethanol (β-ME) and boiled for 5 min prior to resolution on 12% SDS-PAGE gels.

3.3.9. Infection assays

Undiluted Sind-SpyTag pseudotyped lentiviruses were incubated with the indicated concentrations of DARPin.9.26-SpyCatcher2/SpyCatcher2 for 1 h at 25°C. Preps were diluted 50-fold and used to spin-transduced naïve SKOV3 cells (4×10^4 cells/well in 48-well plates) for 3 h (1 h for 300xg at 25°C and 2 h at 37°C). Percent of transduced cells (GFP+) was determined 48 h later using a BD FACScan flow cytometer (BD Biosciences; San Jose, CA).

For titer determination, undiluted lentivirus pseudotyped with Sind-SpyTag were incubated with 10 µM DARPin.9.26-SpyCatcher2 for 1 h at 25°C. Preps were diluted 50-fold in OptiMEM media and a serial amount of functionalized virions were used to spin-transduced naïve SKOV3 cells (4×10^4; 48-well plate) for 3 h (1 h for 300xg at 25°C and 2 h at 37°C). Percent of transduced cells was determined 48 h later as described above. The infection units per milliliter (IU/ml) were calculated by selecting the dilutions

showing linear correlation between the dilution factor and the number of GFP-positive cells.

For selective transduction assays, five-fold diluted lentiviruses pseudotyped with Sind-SpyTag were incubated with 20 µM DARPin.9.26-SpyCatcher2 for 1 h at 25°C. Preps were used to infect target cells (4×10^4 cells/well; 48-well plate) seeded the previous day for 3 h at 37°C. Forty-eight hours post infection; cells were harvested and analyzed using a BD FACScan flow cytometer (BD Biosciences; San Jose, CA).

For mixed cell population studies, CHO-K1 and CHO-HER2-K6 cells were mixed at the indicated ratios and 4×10^4 cells were seeded in each well of a 48-well plate. The next day, cells were infected with five-fold diluted lentiviruses pseudotyped with Sind-SpyTag conjugated to 20 µM DARPin.9.26-SpyCatcher2 for 3 h at 37 °C. Forty-eight hours post transduction, cells harvested from each well were stained with anti-HER-2/neu PE (BD Biosciences; San Jose, CA) as per manufacturer's protocol. Samples were analyzed using BD FACScan flow cytometer (BD Biosciences; San Jose, CA).

3.3.10. Stability assay

For the SpyTag-SpyCatcher2 stability assay, five-fold diluted lentiviruses pseudotyped with Sind-SpyTag were incubated with 10 µM DARPin.9.26-SpyCatcher2 for 2 h at RT. Functionalized Sind-SpyTag virions were divide in two aliquots. The first aliquot (undialyzed) was kept at 4°C for the indicated amount of time. The second aliquot (dialyzed) was subjected to continuous dialysis against dialysis buffer (1x PBS buffer pH 7.4) for 12, 36, 60 or 84 h at 4°C. At each time point, the appropriate volume of virus from each aliquot was removed, supplemented with BSA (2.7mg/ml), and stored at -80°C.

Later, the aliquots were used to infect SKOV3 cells (4 x 10^4 cells/well; 48-well plate) seeded the previous day for 1 h with spinoculation@300 xg at 25°C followed by a 2 h incubation at 37°C. Forty eight hours post infection, cells were harvested and analyzed using BD FACScan flow cytometer (BD Biosciences; San Jose, CA).

3.3.11. Serum complement assay

For the serum complement assay, undiluted lentivirus pseudotyped with Sind-SpyTag were incubated with 10 µM DARPin.9.26-SpyCatcher2 for 1 h at 25°C. Functionalized Sind-SpyTag were incubated with an equal volume of human AB serum (Corning; Corning, NY) or heat inactivated human serum for 1 h at 37°C. The preps were diluted 50-fold in OptiMEM media and functionalized virions were used to spin-transduced naïve SKOV3 cells (4 x 10^4 ; 48-well plate) for 3 h (1 h at 300xg and 25°C and 2 h at 37°C). Percent of transduced cells was determined 48 h later using BD FACScan flow cytometer (BD Biosciences; San Jose, CA).

3.3.12. Conjugation of Trastuzumab Fab to AzF-SpyCatcher2

The HER2 full-length antibody (Trastuzumab) was digested by IgG degrading enzyme of streptococcus pyogenes (Ides) in 1% (wt/wt) ratio for 1 h at 37°C to generate Fc and Fab fragments. The Fc fragments were removed by interacting the mixture with magnetic protein A binding beads (Pierce, Waltham, MA). The purified Fab fragment was mildly reduced by Tris (2-carboxyethyl) phosphine (TCEP) (Sigma-Aldrich, St. Louis, MO) for 30 min at 37°C. The reduced Fab fragments containing reactive thiols were allowed to interact with Sulfo-dibenzocyclooctyne (DBCO)-PEG4-maleimide (DBCO-maleimide) (Click Chemistry tools, Scottsdale, AZ) at a 1:5 molar ratio for 14 h at 4°C to create DBCO modified-Fab. The unreacted DBCO-maleimide was removed by size exclusion chromatography. Later, the azide-functionalized SpyCatcher2 (AzF-SpyCatcher2) was reacted with DBCO modified-Fab, at a 1:8 molar ratio for 16 h at 22°C to produce Fab-SpyCatcher2. The unreacted AzF-SpyCatcher2 was removed through size exclusion chromatography. The concentration of the conjugate Fab-SpyCatcher2 was determined using the bicinchoninic acid (BCA) assay kit (Thermo Fisher Scientific, Waltham, MA).

3.4. Results

3.4.1. Construction and cell surface expression of Sind SpyTag

Previously, our lab developed a split-intein-mediated approach to non-covalently append a cell-binding proteins to the surface of lentiviruses *in vitro* (Chamoun-Emanuelli et al. 2015). This approach exploited a splicing-deficient variant of the naturally split intein from *Nostoc punctiforme* (Ramirez et al. 2013). One half of the split intein – NpuN – was fused to a cell-binding protein, while the other half – NpuC*– was displayed on

lentiviral vectors as fusion to a binding-deficient, fusion-competent Sindbis virus envelope protein (Morizono et al. 2005) to form Sind-C*. The split intein functioned as molecular Velcro and links the cell-binding protein to the pseudotyped lentivirus. However, despite the low nanomolar affinity between the two halves of the split intein (Shah et al. 2011), the complex of cell-targeting protein and the virus gradually dissociates during extended periods of dialysis due to the non-covalent intein association, reducing the selective transduction efficiency of the virus over time.

In the current study, we exploit an isopeptide bond forming protein–peptide pair to retarget lentiviruses. The N-terminal fragment (SpyCatcher) and C-terminal fragment (SpyTag) of the collagen adhesion domain (CnaB2) from the fibronectin binding protein (FbaB) in *Streptococcus pyogenes* were used to covalently functionalize pseudotyped lentiviruses with a targeting protein (Zakeri et al. 2012). The SpyTag peptide (**AHIVMVDAYKPTK**) was inserted into the binding deficient, fusion-competent Sindbis virus envelope protein and the SpyCatcher was genetically or chemically fused to a cell binding protein (e.g. DARPin, antibody).

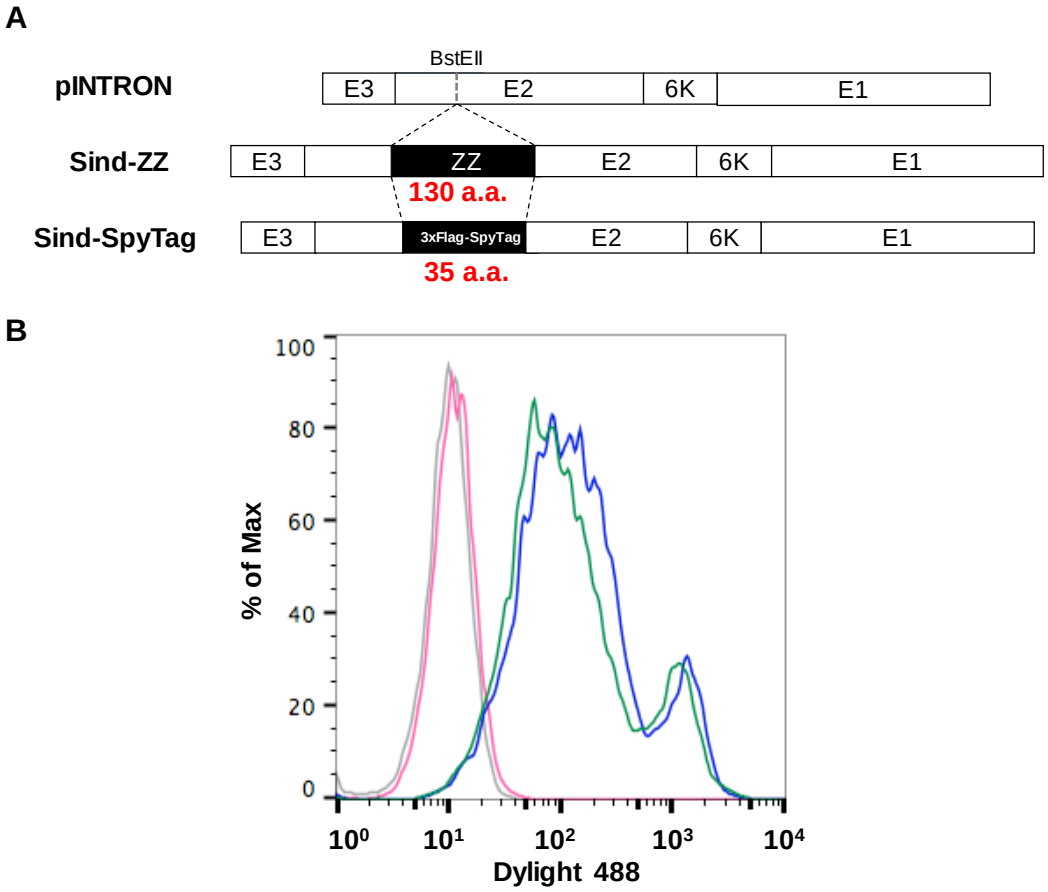

Figure 3.2: (A) Schematic representation of Sindbis envelope protein (pINTRON) and different chimeric sindbis envelope proteins created by inserting the following proteins, IgG binding domain of protein A (Sind-ZZ) (Morizono et al. 2001) and c-terminal fragment of collagen adhesion domain (CnaB2) protein (Sind-SpyTag) (Zakeri et al. 2012). The chimeric proteins were inserted between the amino acids 71 and 74 of E2 envelope protein. **(B) Sind-SpyTag can be efficiently expressed on the cell surface.** Plasmids expressing sindbis envelope proteins (Sind-C* (Blue) and SpyTag (Green)) were transfected into HEK 293 T cells. Surface expression was monitored after 48 h using flag-specific antibody followed by Dylight 488 anti-mouse. Samples were analyzed using flow cytometry. Unstained naïve cells (Gray) and naïve cells stained with primary and secondary antibody (Pink) served as negative controls and staining for Sind-C* served as positive control (Chamoun-Emanuelli et al. 2015).

We replaced the ZZ domain in Sind-ZZ (Morizono et al. 2001) with the SpyTag peptide to form Sind-SpyTag (Figure 3.2 A). A 3xFlag tag was inserted to the N-terminus

of SpyTag to facilitate downstream analysis. We first evaluated the cell surface expression level of Sind-SpyTag as it is critical for the efficient pseudotyping. HEK 293T cells were transiently transfected with a plasmid encoding Sind-SpyTag or Sind-C* (positive control). The cell surface protein expression level was assesed 48 h later by immunostaining with mouse anti-Flag antibody and Dylight 488 goat anti-mouse secondary antibody using flow cytometry (Chamoun-Emanuelli et al. 2015). High expression of Sind-SpyTag was detected on the cell surface, similar to that attained for Sind-C*, suggesting that incorporation of SpyTag into the envelope protein does not hinder its cell surface expression (Figure 3.2 B).

3.4.2. Virion incorporation of SpyTag

Next, we assayed the ability of Sind-SpyTag to be incorporated into the lentivirus. Lentiviruses pseudotyped with Sind-C* or Sind-SpyTag were subjected to western blotting and probed using the anti-flag antibody. As shown in Figure 3.3, SpyTag was efficiently incorporated into the lentiviruses, albeit at a lower efficiency than Sind-C*.

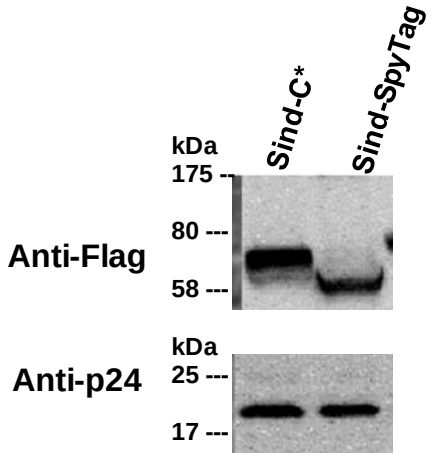

Figure 3.3: Sind-SpyTag is efficiently incorporated into the virion. HEK 293T cells were transiently co-transfected with 1) Sind-SpyTag/NpuC*, 2) HIV-gag-pol, 3) a reporter pTRIP-eGFP plasmids. Virus containing supernatants were harvested 48 h later. Western blot analysis was carried out with 100 fold concentrated samples by ultracentrifugation (90 min; 40000 x g; 4°) of the indicated LVs. After separation on a 12% SDS gel and transferred to a PVDF membrane, proteins NpuC* (~57kDa) and SpyTag (~54kDa) were detected using mouse anti-Flag and horseradish peroxidase-conjugated secondary goat anti-mouse antibody and mouse anti HIV-1 p24 (Gorny et al. 1989).

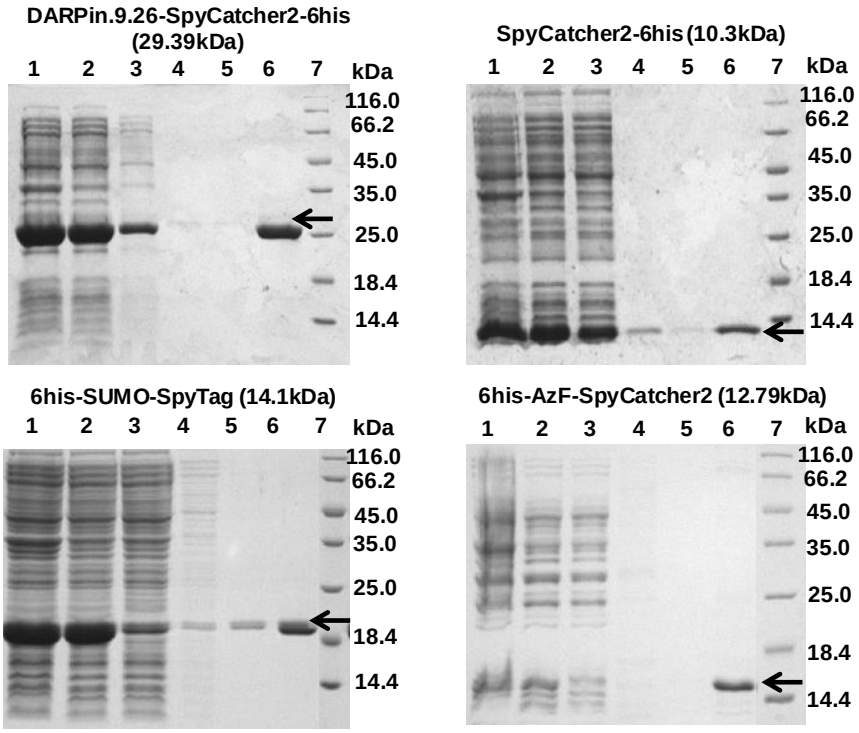

Figure 3.4: Protein expression and IMAC purification of DARPin-SpyCatcher2, SpyCatcher2, SUMO-SpyTag and AzF-SpyCatcher2. The target protein is indicated in black arrow. The collected samples during the purification process were mixed with equal volume of 2x SDS sample buffer + 0.5 M β-mercaptoethanol (β-ME) and boiled for 5 min prior to resolution on 12% sodium dodecyl sulphate-polycarylamide gel (SDS-PAGE).

3.4.3. In vitro conjugation of SpyTag with SpyCatcher2 and DARPin.9.26–SpyCatcher2 protein

For the current retargeting strategy, we used the truncated version of SpyCatcher (SpyCatcher2), lacking the first 23 aa of the N-terminal and the last 9 C-terminal residues (Zhang et al. 2013). We choose to use this truncated version of SpyCatcher, because the full-length SpyCatcher showed significant non-specific interaction with an unknown cell surface receptor(s), leading to strong background transduction of SpyCatcher-alone

functionalized lentiviruses (data not shown). Since DARPin.9.26 exhibited the highest retargeting efficiency in our previous study (Chamoun-Emanuelli et al. 2015), we genetically fused the SpyCatcher2 protein to the C-terminal of this designed ankyrin repeat protein (DARPin.9.26) previously engineered to bind the HER2 receptor with nanomolar affinity to create DARPin.9.26- SpyCatcher2 (Steiner et al. 2008). As control, we used the SpyCatcher2 protein lacking any targeting moiety. We also expressed and purified the fusion protein as well as a SpyTag containing protein (6xHis-SUMO-SpyTag) by IMAC. All proteins exhibited high protein expression levels (Figure 3.4).

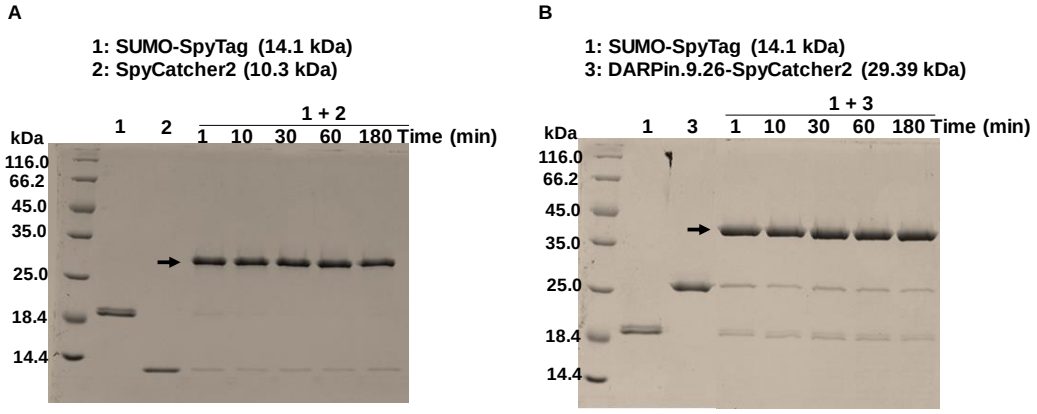

Figure 3.5: SpyTag and SpyCatcher2 associate spontaneously through an isopeptide bond. (A) SpyCatcher2 (2) or **(B)** DARPin.9.26-SpyCatcher2 (3) were mixed with SUMO-SpyTag (1) at a 1:1 ratio (10 μM) for the indicated amount of time (4-8 in A and B) and the amount of product at each time point was assessed via SDS-PAGE. The conjugated product is indicated by the black arrow.

We then confirmed the ability of DARPin-SpyCatcher2 to form an isopeptide bond with SUMO-SpyTag. Purified SpyCatcher2 or DARPin.9.26-SpyCatcher2 was incubated

with SUMO-SpyTag at a 1:1 molar ratio for the indicated amount of time (1-180 min) and the resulting products was analyzed by SDS-PAGE under reducing conditions. As shown in Figure 3.5, more than 90% reaction completion was achieved within the first minute for both constructs, suggesting that fusion of a DARPin to the N-terminal of SpyCatcher2 does not hinder its interaction with SpyTag.

3.4.4. Retargeting Sind-SpyTag via DARPin.9.26- SpyCatcher2

To assess the ability of DARPin.9.26-SpyCatcher2 to functionalize Sind-SpyTag-pp and retarget the virions to HER2+ SKOV3 cells, undiluted Sind-SpyTag-pp was interacted with increasing concentrations of DARPin.9.26- SpyCatcher2 or SpyCatcher2 (0 - 40μM) for 1 h at room temperature, diluted 50-fold in OptiMEM media and used to transduce HER2+ SKOV3 cells. The DARPin-loaded Sind-SpyTag-pp displayed a dose dependent transduction of HER2/neu+ SKOV3 cells (Figure 3.6 A). A similar transduction efficiency was observed for virions functionalized with either 10 or 20μM DARPin 9.26-SpyCatcher, suggesting complete saturation of the available SpyTag in the virions. The lower transduction observed at 40μM is likely attributed to the competition of excess unconjugated DARPin with the functionalized virions for the HER2/neu receptor. In contrast, virions loaded with SpyCatcher2 lacking any cell attachment protein exhibited only background levels of transduction (SpyCatcher2) at all tested concentrations. This was comparable to that of unconjugated Sind-SpyTag-pp (0 μM). Since virions conjugated with 10 and 20 μM DARPin 9.26-SpyCatcher2 displayed similar transduction efficiency, these concentrations were selected for subsequent experiments.

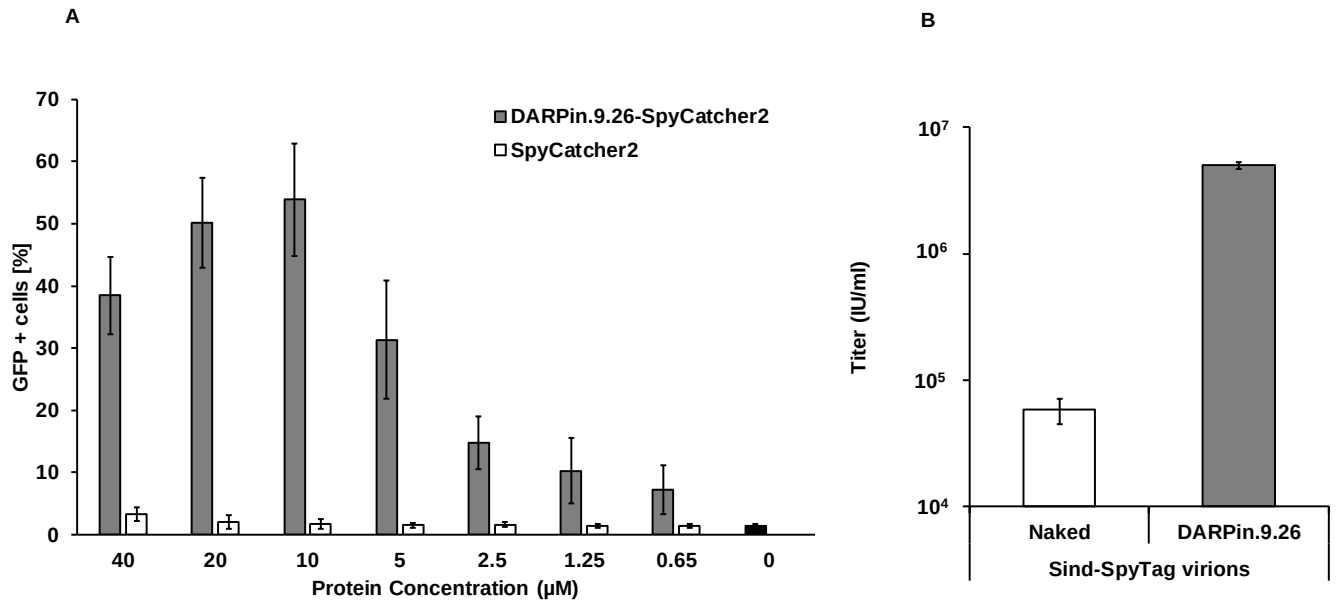

Figure 3.6: DARPin functionalized Sind-SpyTag-pp transduce HER2+ SKOV3 cells in dose dependent manner. (A) Undiluted Sind-SpyTag-pp was conjugated with increasing concentrations of DARPin.9.26-SpyCatcher2 or SpyCatcher2 (0 - 40 µM) for 1 h at room temperature. The functionalized virions were diluted 50 fold in OptiMEM media and used to spin-transduced HER2+SKOV3 cells. The percentage of transduced cells (GFP+) was measured 48 h later by flow cytometry. Values and error bars represent the average and standard deviation, respectively of two independent experiments. **(B) Unconcentrated screening titers of DARPin.9.26-SpyCatcher2 functionalized Sind-SpyTag-pp.** Viral infectivities were determined by titration of at least five serial dilutions of DARPin-functionalized Sind-SpyTag-pp on HER2+SKOV3 cells. After 48 h, the percentage of GFP-positive cells was determined by flow cytometry and the infectious units per milliliter (IU/mL) were calculated by selecting the dilutions showing a linear correlation between the dilution factor and the number of GFP-positive cells. Values and error bars represent the average and standard deviation, respectively of at least three independent experiments.

To determine the infectious titers (IU/mL), unconcentrated Sind-SpyTag-pp was incubated with 10 μM of DARPin.9.26-SpyCatcher2 at room temperature for 1 h, serially diluted and used to transduce SKOV3 cells. The average infectious titer for DARPin construct was calculated from at least three dilutions that showed linear correlation between the dilution factor and the percentage of GFP+ cells. As shown in Figure 3.6 B, functionalization with DARPin.9.26-SpyCatcher2 resulted in an infectious titer of $8x10^6$ IU/mL, ~ 100-fold higher than that of the control naked Sind-SpyTag-pp ($5.8x10^4$ IU/mL).

3.4.5. HER2/neu receptor dependent transduction

Next, we determined if the transduction ability of DARPin functionalized Sind-SpyTag-pp is mediated by the HER2 receptor. The functionalized virions were used to transduce $HER2^-$ (CHO-K1) and $HER2^+$ (SKOV3 and CHO-K6) cells. As anticipated, the transduction efficiency of DARPin loaded virions appeared to be proportional to the levels of cell surface HER2 receptor, displaying a 5 to 6 fold higher transduction in HER2+ (SKOV3 and CHO-K6) cells compared to the HER2- CHO-K1 cells (Figure 3.7). In contrast, control VSV-Gpp, whose entry is mediated by the LDL receptor, exhibited similar transduction efficiency in all the cell lines. Only background transduction levels were attained for non-functionalized virions (0 μM).

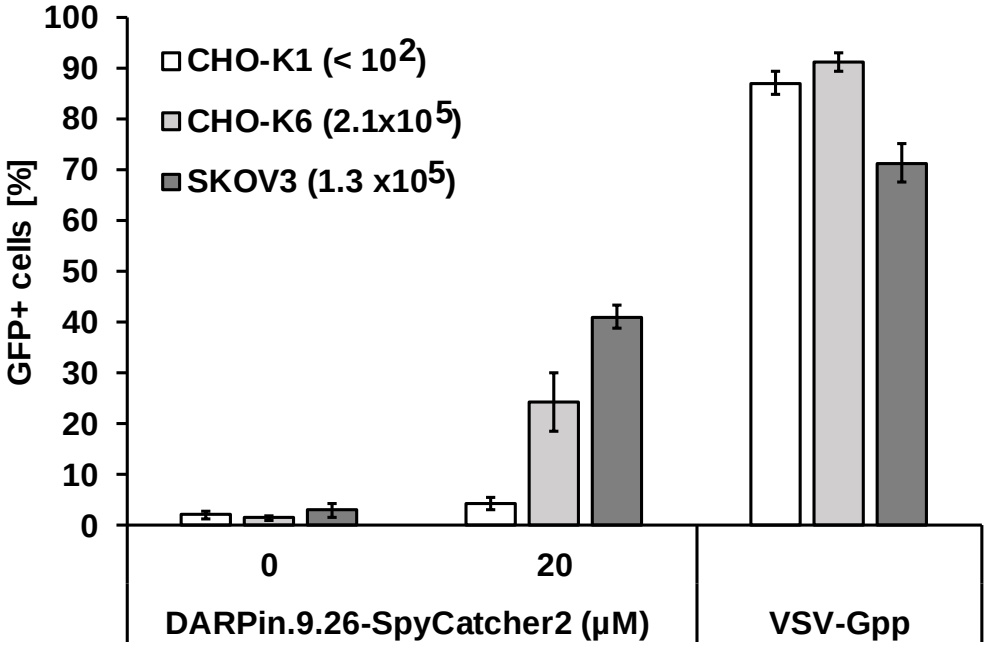

Figure 3.7: DARPin functionalized Sind-SpyTag selectively transduce HER2/neu+ cells. Five-fold diluted Sind-SpyTag virions were conjugated with 20 µM DARPin.9.26-SpyCatcher2 at room temperature for 1 h. Mixture was used to transduce the indicated cell for 3 h at 37ºC. Flow cytometry analysis was performed 48 h later to assess transduction efficiency (GFP+). VSV-Gpp and naked Sind-SpyTag-pp were used as control. Values and error bars represent the average and standard deviation, respectively, of at least 3 independent experiments.

Next, we investigated whether the DARPin functionalized Sind-SpyTag-pp could specifically target HER2+ cells in a mixed cell population experiment. CHO-K1 and CHO-HER2-K6 cells mixed at different ratios 0:1, 1:1 and 1:0 were transduced with functionalized Sind-SpyTag virions. To distinguish the CHO-HER2-K6 cells from CHO-K1 cells, the infected cells were stained with an anti-HER2 antibody. At 1:1 ratio of the cells, DARPin functionalized Sind-SpyTag virions displayed higher transduction selectivity towards HER2/neu+ cells (Figure 3.8, middle panels). Furthermore, across the cell ratios, the calculated HER2 receptor specific selectivity index for DARPin functionalized SpyTag-pp for CHO-K6 cells was observed to be significantly higher in mixed cell culture (6.79) compared to individual cell culture (2.3) (Figure 3.9). On the other hand, VSV-Gpp transduced both HER2+ and HER2- cells, indicating their relative lack of specificity. In sum, our data suggest that DARPin functionalized SpyTag virions can selectively target HER+ cells in mixed culture.

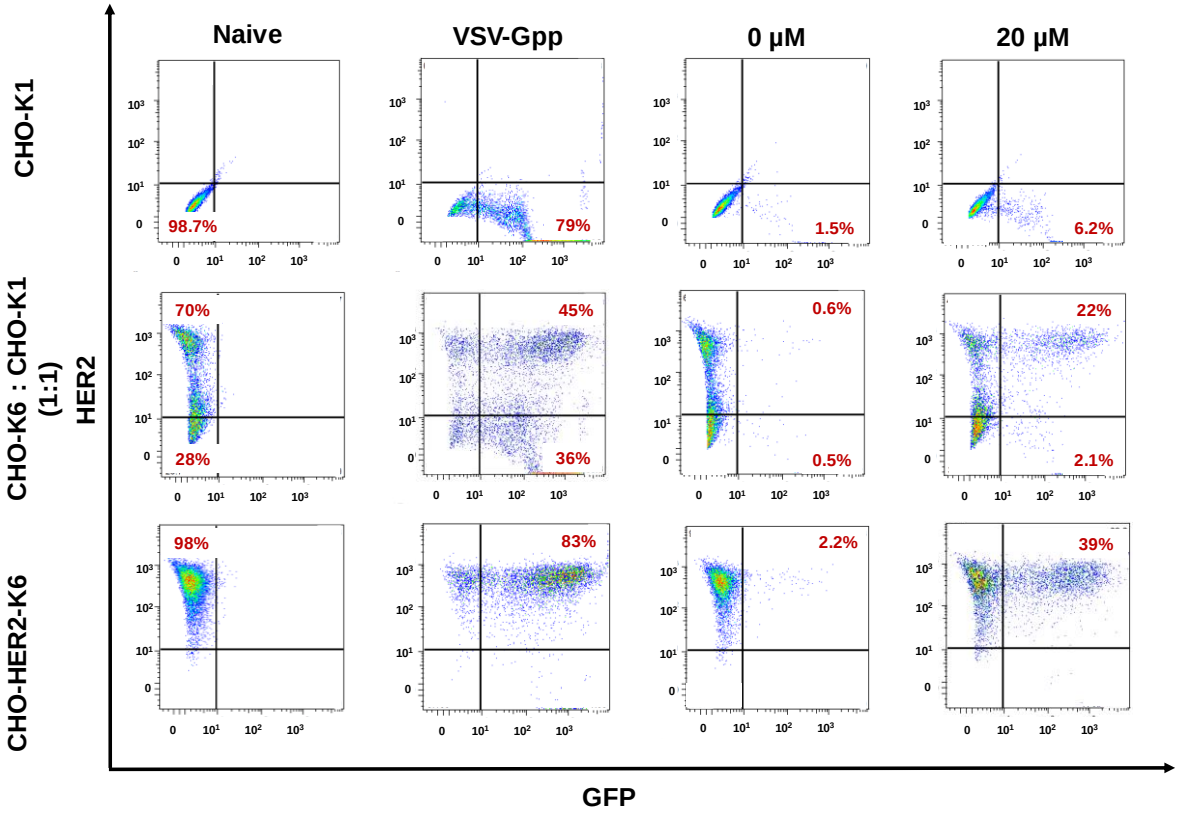

Figure 3.8: Functionalized SpyTag virions display higher selectivity for HER2 expressing cells. Functionalized Sind-SpyTag virions selectively transduce HER2+ cells in co-culture. CHO-K1 (HER2 –low) and CHO-HER2-K6 (HER2 – High) cells, either in isolation or at a 1:1 ratio, were transduced with functionalized Sind-SpyTag-pp, Sind-SpyTag-pp or VSV-Gpp at 37°C for 3 h. Forty-eight hours post transduction, cells were analyzed for GFP and HER2 expression by flow cytometry. Representative flow cytometry plots of four independent experiments are shown.

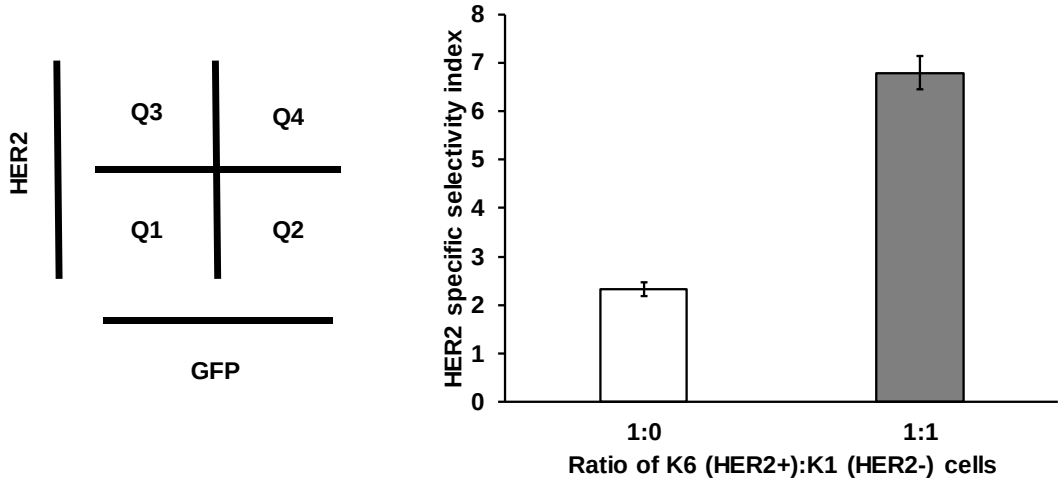

Figure 3.9: HER2 receptor-specific selectivity index. The "cell specific selectivity index" for mixed population of CHO cells was calculated as follows [(SI) = Q4/ (Q3+Q4) / Q2 / (Q2+ Q1)], where Q4 and Q2 represent the % infected cells (+GFP) cells and Q3 and Q1 represent the % uninfected cells. Later, we calculated the HER2 receptor specific selectivity index by Sind Env CHO-K6 SI / VSV-G CHO-K6 SI for mixed population and isolated cells. This formulation allowed us to evaluate the selectivity of functionalized Sind-SpyTag-pp for infecting HER2+ CHO-K6 cells relative to that of VSV-Gpp. Values and error bars represent the average and standard deviation, respectively, of at least 3 independent experiments.

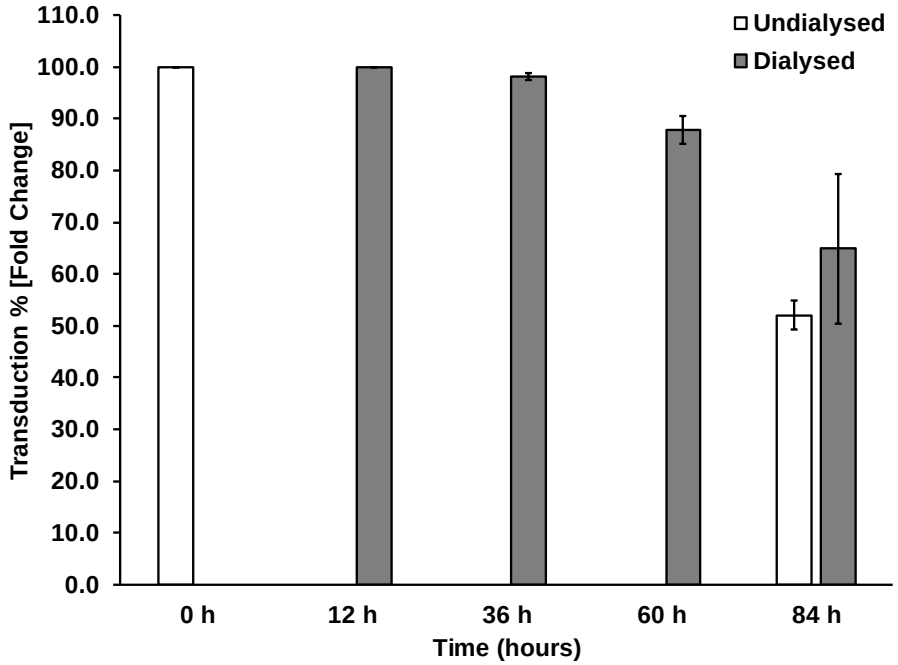

Figure 3.10: The association of SpyTag with SpyCatcher2 is irreversible. Sind-SpyTag-pp (5-fold diluted) was incubated with DARPin.9.26-SpyCatcher2 (20 µM) at RT for 2 h in dialysis buffer (1x PBS buffer, pH 7.4). The mixture was divided into two aliquots. Aliquot 1 (Undialyzed) was kept intact at 4°C while aliquot 2 (Dialyzed) was continuously dialyzed against dialysis buffer at 4°C. An aliquot was collect from sample 2 at the indicated time points, mixed with BSA (2.7 mg/ml) and stored at -80°C. Later, these aliquots were used to infect SKOV3 cells and the percentages of transduced cells were quantified by flow cytometry 48 h later, and normalized to the corresponding values taken on day 0.Values and error bars represent the average and standard deviation, respectively of at least two independent experiments.

3.4.6. Stability of SpyTag – SpyCatcher2 complex

SpyTag has been shown to form a spontaneous irreversible isopeptide bond with SpyCatcher2 (Li et al. 2014; Zakeri et al. 2012). Thus, we hypothesize that interaction of Sind-SpyTag-pp with DARPin-SpyCatcher2 will result in an irreversible isopeptide bond and thus a stable conjugation. To test whether this association is reversible, we determined

the infectivity of DARPin-functionalized Sind-SpyTag-pp subjected to extensive dialysis in 1000-fold excess dialysis buffer. If the conjugation is reversible, all the DARPin.9.26-SpyCatcher2 that dissociates from Sind-SpyTag-pp would be removed during dialysis, leading to reduced infectivity. On the other hand, the ability of the functionalized virions to retain infectivity after dialysis would confirm the irreversibility of the interaction.

Sind-SpyTag-pp were diluted 5-fold and incubated with 20 μM DARPin.9.26-SpyCatcher2 for 1 h at 22˚C. The mixture was then divided in two aliquots. Aliquot 1 was kept at 4ºC without dialysis (undialyzed sample). Aliquot 2 was extensively dialyzed in a Spectra/Por Float-A-Lyzer dialysis device (300kDa cut off, spectrum lab) against 1000-fold excess dialysis buffer (1x PBS buffer, pH 7.4) at 4ºC. An aliquot from each sample was collected at 12, 36, 60 and 84 h after the initiation of dialysis, mixed with BSA (2.7 mg/ml) and stored at -80˚C. The infectivity of these viruses was later quantitated in SKOV3 cells. The isopeptide bond stability was confirmed by the retained infectivity of the conjugated virions in the dialyzed sample (Sample 2) at different time periods. The dialyzed sample retained significant infectivity during the first 60 h of dialysis but exhibited infectivity loss at 84 h (Figure 3.10). The loss of infectivity was comparable to the undialyzed sample (Sample 1) saved at 4˚C; suggesting viral inactivation. Thus, the lack of infectivity loss during the first 60 h of dialysis supports the irreversible association of the SpyTag-SpyCatcher isopeptide bond.

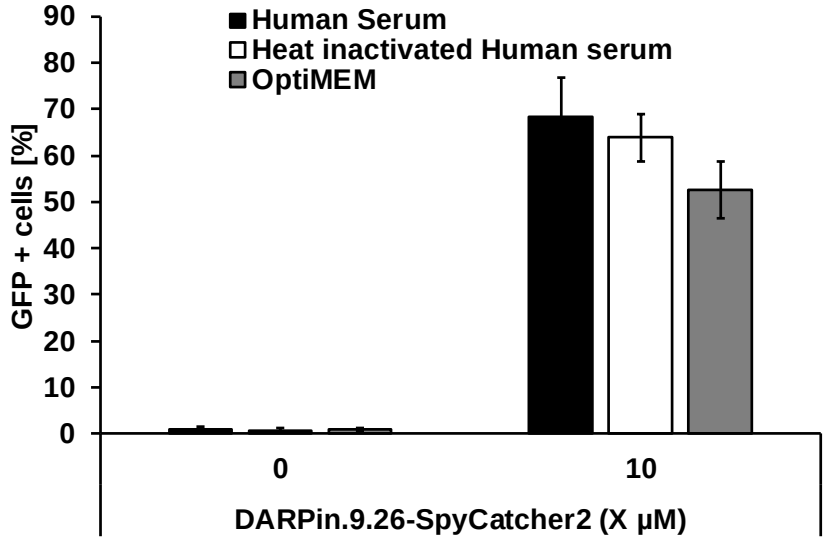

Figure 3.11: Functionalized SpyTag virions retain infectivity in the presence of human serum. Functionalized (10 µM) and naked (0 µM) SpyTag virions were incubated with an equal volume of serum or heat-inactivated serum at 37°C for 1 h, OptiMEM media was used as control. Each virus was diluted 50-fold with OptiMEM media and used to spin-transduced HER2+SKOV3 cells. EGFP expression was assayed by flow cytometry 48 h post transduction. Values and error bars represent the average and standard deviation, respectively of at least three independent experiments.

We further examined whether the DARPin functionalized Sind-SpyTag-pp became inactivated in the presence of human serum complement. The DARPin functionalized and naked Sind-SpyTag-pp were incubated with heat-inactivated human serum or normal human serum at a 1:1 ratio for 60 min prior to infection. OptiMEM media incubation was used as control. As shown in Figure 3.11, DARPin functionalized Sind-SpyTag virions incubated in human serum displayed similar transduction efficiency as those incubated in OptiMEM. Thus, the functionalized virions were not inactivated by human serum complement and can be potentially applied as an *in vivo* delivery vector in immunocompetent hosts.

3.4.7. Retargeting Sind-SpyTag-pp through chemical conjugation

Antibodies, most commonly IgGs, have been widely used as targeting moieties in research and therapeutic applications due to their high specificity and efficacy (Hui et al. 2014). An intrinsic property of the full-length IgG format is it's binding capacity to Fc receptors on immune cells, thereby increasing its circulation half-life and hence antigen targeting (Mitragotri et al. 2014). The disadvantage of full-length antibody IgG is the lack of deep penetration into the tumor environment, which can be solved by using smaller protein scaffolds. Fab and single chain variable fragment (scFv) have been engineered for drug conjugation and several IgG fragments are currently under preclinical evaluation for targeting different diseases (Deonarain et al. 2015). Introduction of non-natural amino-acids is a strategy currently used to link drug conjugates to the antibody. Many applications require antibodies to be conjugated onto surfaces (e.g. nanoparticles, beads and microplates); however, most conventional bioconjugation techniques exhibit several limitations including low crosslinking (Wagner et al. 2014), reduced functionality due to non-site-specific labeling and random surface orientation (Wagner et al. 2014). In order to overcome these limitations and to favorably expand our lentiviral retargeting system, we have taken advantage of the recently developed azide-alkyne copper-free click reactions. These reactions form covalent bonds, are highly efficient, and are biorthogonal, as they do not react with endogenous molecules (Baskin et al. 2007; Chang et al. 2010). Axup et.al recently generated a highly potent Fab-auristatin through the incorporation of an unnatural amino *p*-acetyl phenylalanine (AzF) into an anti-Her2 antibody Fab fragment and full length IgG and oxime ligation for drug conjugation (Axup et al. 2012). To

incorporate the chemical conjugation approach into our system, the non-natural amino acid AzF was introduced upstream of the SpyCatcher2 protein. Briefly, the SpyCatcher2 protein was genetically modified to incorporate an azide-containing unnatural amino acid AzF into the amber codon encoded in the linker region using an orthogonal amber suppressor tRNA/aaRS pair, derived from the corresponding tyrosyl *Methanococcus jannaschii* pair (Chin et al. 2002; Guan et al. 2015; Wan et al. 2010). The activity of the incorporated AzF was confirmed by the reaction with DBCO-PEG4-TAMRA dye (Sigma-Aldrich, St.Louis, MO) and visualized under UV light (Figure 3.12 B). Later, the anti-HER2 antibody Fab was conjugated with dibenzocyclooctyne (DBCO)-maleimide to form Fab-DBCO through the thiol-maleimide coupling reaction. Briefly, the HER2 full-length antibody (Trastuzumab) was digested by IgG degrading enzyme of streptococcus pyogenes (Ides) at 1% (wt/wt) ratio for 1 h at 37°C to generate Fc and Fab fragments (von Pawel-Rammingen et al. 2002). The Fc fragments were removed by interacting with magnetic protein A binding beads. The purified Fab fragment was mildly reduced with Tris (2-carboxyethyl) phosphine (TCEP) for 30 min at 37°C. The reduced Fab fragments, containing reactive thiols were allowed to interact with Sulfo-DBCO-PEG4-maleimide (DBCO-maleimide) at a 1:5 molar ratio for 14 h at 4°C to create DBCO modified-Fab fragment (Humphreys et al. 2007). The unreacted DBCO-maleimide was removed by size exclusion chromatography. Later, the azide-functionalized SpyCatcher2 (AzF-SpyCatcher2) was reacted with DBCO modified-Fab, at a 1:8 molar ratio for 16 h at 22°C via copper-free click chemistry to produce Fab-SpyCatcher2. The unreacted AzF-SpyCatcher2 was removed through size exclusion chromatography.

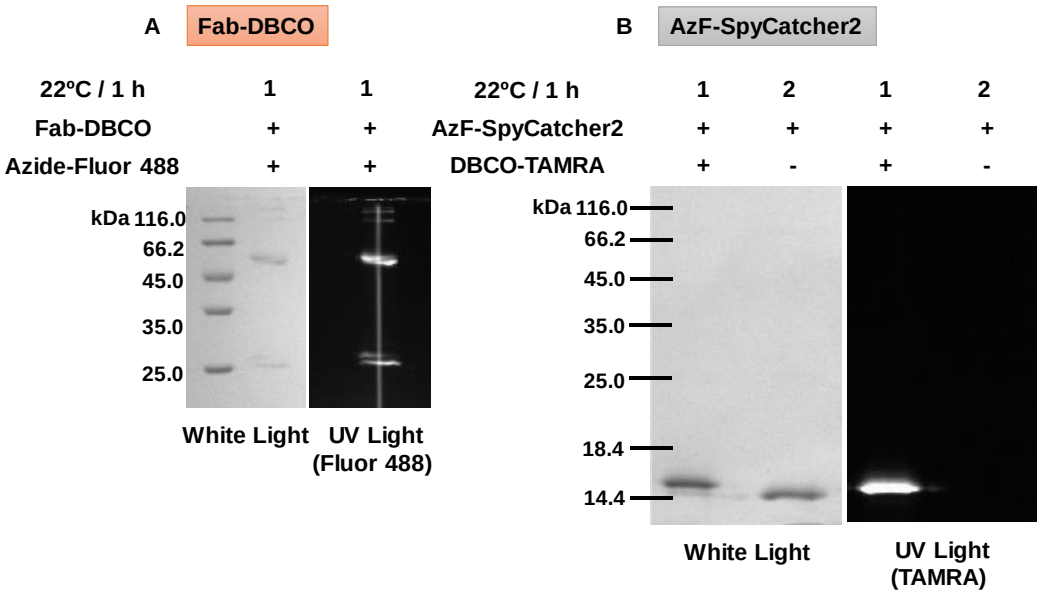

Figure 3.12: The functionalized groups were active to form conjugated product. (A) The conjugated DBCO to the Fab was active to interact with the azide group. To confirm the conjugation of Fab-DBCO through the thioester reaction, conjugated Fab-DBCO (10 µM) was incubated with azide-fluoro 488 (500 µM) in PBS buffer (pH 7.4) for 1 h at room temperature. The bands were first visualized under UV light, and then stained with Coomassie Blue and analyzed again under white light. **(B)** The incorporated AzF was active to interact with DBCO. To confirm the formation of DBCO-SpyCatcher2, purified AzF-SpyCatcher2 (10 µM) was incubated with DBCO-TAMRA (500 µM) in DPBS buffer (pH 7.4) for 1 h at room temperature and resolved by 12% SDS-PAGE. Untreated Azf-SpyCatcher2 was used as the negative control. .The samples were resolved and visualized as previously described in (A).

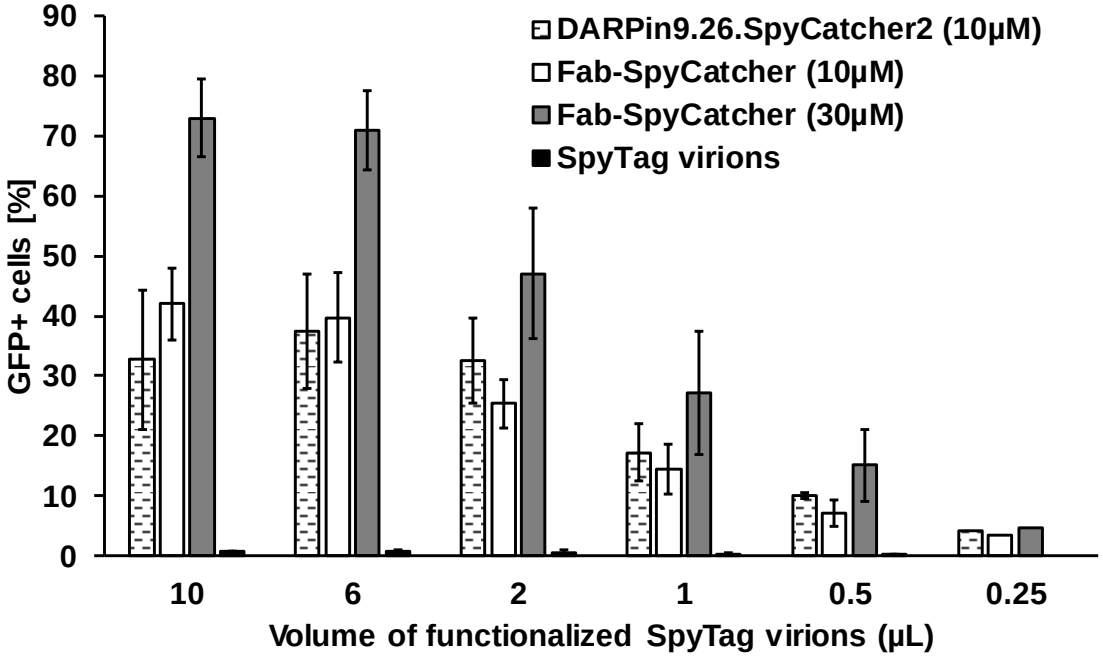

Figure 3.13: SpyTag-pp can be functionalized with a HER2 specific fragment antibody (Fab) SpyCatcher2 and efficiently transduce HER2+ cells. Transduction of Fab-SpyCatcher2- functionalized Sind-SpyTag-pp. Sind-SpyTag-pp was incubated with increasing concentrations of Fab-SpyCatcher2 in OptiMEM at 22˚C for 1 h. The functionalized virions were diluted 50-fold in OptiMEM media and used to spin-transduce HER2+SKOV3 cells. The cells were washed 3 h later and the percentages of transduced cells (GFP+) were analyzed by flow cytometry 48 h later. Sind-SpyTag-pp functionalized with fusion protein DARPin.9.26-SpyCatcher2 (10 µM) was used as the positive control. Values and error bars represent the average and standard deviation, respectively of at least three independent experiments.

We next determined the ability of Sind-SpyTag-pp to transduce HER2+ SKOV3 cells upon functionalization with Fab-SpyCatcher2. Undiluted Sind-SpyTag-pp harboring a pTRIP-eGFP expression cassette were incubated with different concentrations of Fab-SpyCatcher2 at 22°C for 1 h, diluted 50-fold in OptiMEM media and used to spin-transduce HER2+ SKVO3 cells for 3 h. Forty-eight hours post infection, cells were harvested, resuspended in DPBS/1% PFA and the percentage of transduced cells (GFP+)

was determined using a BD FACScan flow cytometer. For comparison, the transduction efficiency of DARPin functionalized to the Fab-functionalized SpyTag-pp was assessed. As shown in Figure 3.13, similar transduction efficiencies were observed for both constructs at a concentration of 10μM. Conjugation of SpyTag-pp with increasing concentrations of Fab-SpyCatcher resulted in a dose dependent transduction, suggesting the efficient interaction of Fab-SpyCatcher2 with incorporated SpyTag on the virion surface. In summary, the HER2-specific Fab-SpyCatcher2 was able to efficiently functionalize SpyTag-pp and retarget the functionalized virions to HER2+ SKOV3 cells.

3.5. Discussion

Modification of the lentivirus surface for each gene therapy application can be laborious and time-consuming, requiring cloning, production and evaluation of the new virus. Thus, developing a standard virus platform that could be applicable for many different purposes would represent an advantage. To address this challenge, we have developed a versatile receptor targeting lentivirus platform that can be coupled to different cell targeting proteins through a "plug and play" fashion. Our design takes advantage of a previously engineered sindbis virus envelope protein, mutated to abolish its natural tropism as well as to reduce non-specific transduction (Froelich et al. 2010; Morizono et al. 2010; Morizono et al. 2005; Yang et al. 2008), and an isopeptide bond forming protein-peptide pair, SpyTag-SpyCatcher from the fibronectin binding protein (FbaB) in *Streptococcus pyogenes*. The SpyTag peptide was introduced into the engineered sinbis envelope protein by replacing the ZZ domain. Incorporation of the SpyTag peptide into the E2 envelop protein did not hinder the expression and cell surface translocation of the

chimeric protein or its incorporation into mature virions (Figure 3.2 B and 3.3) (Munch et al. 2011a). Fusion of targeting moieties to the SpyCatcher protein did not obstruct its interaction with the SpyTag peptide, with more than 90% of the product formed within the first minute (Figure 3.5).

Conjugation of SpyTag-pp with SpyCatcher2 fused to a HER2 targeting protein (DARPin 9.26) granted its entry into HER2+ cells, with the highest transduction attained for virions decorated with 10 and 20 μM DARPin 9.26-SpyCatcher2 (Figure 3.6 A). Transduction of functionalized virions was mediated by the conjugated DARPin as naked or virions conjugated to SpyCatcher2-only displayed a low transduction efficiency. The calculated titer of functionalized SpyTag-pp ($8x10^6$ IU/mL) was observed to be 100 fold higher than that of naked SpyTag-pp ($5.8x10^4$ IU/mL) (Figure 3.6 B).

To assess the influence of cell surface HER2/neu density on vector targeting, the transduction efficiency of functionalized SpyTag-pp against a panel of cell lines expressing different levels of HER2/neu was evaluated. The functionalized SpyTag-pp exhibited a gene transfer efficiency relative to the level of HER2/neu expression, with a five-fold higher transduction in CHO-HER2-K6 and SKOV3 cells compared to CHO-K1 cells (Figure 3.7). Thus, targeting specificity of the functionalized SpyTag-pp was critically dependent on the receptor density. Importantly, in a mixed cell population assay, functionalized SpyTag-pp efficiently discriminated between receptor positive and negative cells, displaying higher selectivity for high HER2 expressing cells (Figure 3.8). The calculated HER2 receptor specific selectivity index for CHO-HER2-K6 cells in mixed and individual demonstrated a higher selective targeting of functionalized SpyTag-pp in

mixed culture setting (Figure 3.9). Our data, suggests the applicability of functionalized virions in mixed cell cultures can exhibit receptor specific targeting.

The success of the targeting vector depends on its stable conjugation to the targeting moiety until transduction of the targeted cells for gene delivery is achieved. The rapid degradation of the targeting vector or detachment of the targeting moiety due to a non-covalent conjugation are among the major obstacles encountered in virus-based gene therapy. We found that our functionalized SpyTag-pp exhibited stable conjugation under continues dialysis for 5 days representing the stability of the isopeptide bond (Figure 3.10). Interestingly, the little loss in the infectivity of the functionalized SpyTag-pp observed after extended dialysis was primarily due to the inactivation of SpyTag-pp in both the dialyzed and undialyzed samples. Previously, it was shown that virions pseudotyped with VSV-G were cleared from the circulation within an 8 h period due to the activation of innate immunity (Geraerts et al. 2006; Schauber-Plewa et al. 2005). Our current system takes advantage of a previously engineered sindbis envelope demonstrated to retain activity in presence of human serum complement (Morizono et al. 2010). In line with previous studies, functionalized SpyTag-pp retained a comparable infectivity to the media control in the presence of human serum. Altogether, the exhibited long-term stability and uncompromised nature in presence of serum complement makes the functionalized SpyTag-pp highly attractive for *in vivo* targeting applications.

One novel application of this targeting vector is the ability to accommodate commercially available antibodies as targeting moieties (Sanz et al. 2005). In the current study, we successfully conjugated a HER2 specific Fab derived from the Trastuzumab

antibody onto the Sind-SpyTag virions by exploiting the azide-alkyne cycloaddition reaction. The covalent bond formed between the AzF incorporated upstream of the SpyCatcher2 protein and the dibenzocyclooctyne (DBCO) group conjugated to the reduced cysteine on the HER2 specific Fab fragment helped to generate the Fab-SpyCatcher2 conjugate. Strikingly, the chemically coupled Fab-SpyCatcher2 efficiently functionalized SpyTag-pp, with the resulting virions displaying receptor specific retargeting (Figure 3.13). Previous successful work of several groups showed that the azide-alkyne click chemistry for virus envelope modification with different ligands did not alter the functionality of the virus. In one study, surface functionalization of virus like particles with antibody fragment and granulocyte-macrophage colony stimulating factor (GM-CSF) through azide-alkyne chemistry resulted in stimulation and proliferation of immune cells (Patel and Swartz 2011). In another study, the vaccinia virus and avian influenza A virus envelopes were labeled through the cycloaddition reaction with quantum dots to identify the infectivity and single virion tracking (Hao et al. 2012). One more advantage of the cycloaddition approach is the lack of laborious molecular cloning to create fusion constructs.

In conclusion, this work provides proof of principle of the utility of an isopeptide bond forming protein-peptide pair (SpyTag-SpyCatcher) as a versatile tool for targeting of lentivirus vectors. This peptide tagging based strategy is readily adaptable to different targets. Consequently, the facile "plug and play" model will further enhance the potential of lentiviral vectors as widely used gene delivery vehicles for functional genomics and gene therapy purposes.

CHAPTER IV

DEVELOPMENT OF CHICKEN ANTIBODIES AGAINST TUMOR ANTIGENS

4.1. Overview

In this section, we propose to develop a novel platform technology that enables isolation of antibodies targeting highly conserved regions of cancer-specific-receptors. Antibodies are considered important therapeutic candidates against cancer due to their ability to bind the target with high affinity and specificity. As most of the cancer antigens are considered to be self-antigens, they are not immunogenic for the host. The need to develop new antibodies against these self-antigens has increased. Chickens are considered better immune hosts because of their evolutionary distance from mammals. The proposed new technology platform will facilitate the engineering of chicken monoclonal antibodies (IgY) against human cancer receptors. In this preliminary work, we constructed a plasmid that expresses an agonistic anti-chicken CD40 single chain variable fragment (2C5 scFv) as the N-terminal fragment of the eB7 display domain, which consists of transmembrane and cytosolic tail of murine CD80. The cell surface expression was confirmed through immunostaining. The activity of the displayed 2C5 scFv was identified by the interaction between purified the 2C5 scFv and chicken CD-40 ectodomain. This DNA vaccine will be utilized to carry out chicken immunization experiments to generate IgY antibodies against the model human cancer receptor hCD20.

4.2. Introduction

Monoclonal antibodies (mAbs) are widely used for the treatment of cancer, inflammatory diseases and other disorders. Their ability to target antigens with high

affinity and specificity has rendered them particularly useful in precision cancer therapy. Currently, most of the monoclonal antibodies are generated from mice using the hydridoma technology, which immortalizes mouse B-cell clones producing specific mAbs. However, both humans and mice are mammals and we share many conserved regions in our proteins. These homologous regions are likely preserved through evolution due to their important cellular functions. However, since self-antigens are not immunogenic for the host, it is difficult to obtain murine antibodies against homologous regions from human proteins.

It is a well-known concept that the further the evolutionary distance between the antigen source and the immune host, the more potent is the immune response. Therefore, to obtain antibodies against a human protein, chickens are far better immune host than any mammal (Camenisch et al. 1999; Schade et al. 2005; Tini et al. 2002; Williams et al. 2001). Chickens have been shown to develop robust immune responses and produce antibodies against different antigens (Murata et al. 1996). In addition, chicken IgY antibodies are sufficiently similar to human IgG antibodies, making it possible to be easily humanized via complementarity-determining regions (CDR) grafting (Chen et al. 2010; Chen et al. 2012). However, it is currently difficult to obtain monoclonal chicken antibodies due to the lack of a hybridoma-equivalent technology for chickens. Although the Gel Encapsulated Microenvironment (GEM) technology, developed by Crystal Bioscience, has been successfully used to isolate chicken B cells producing a desired antibody, it requires highly specialized tools and is proprietary, and cannot be easily used by the general research community.

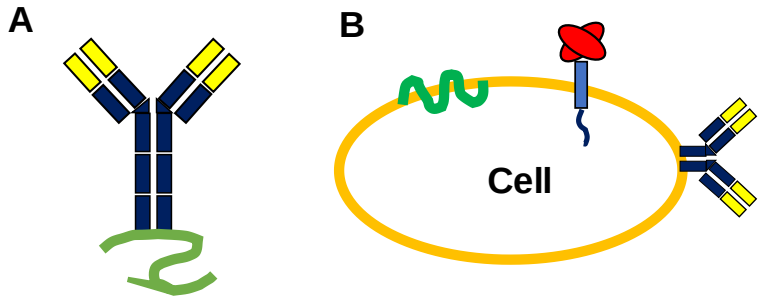

Figure 4.1: Antibody-guided vaccine technology. (A) Antigen protein (*green line*) functionalized with a CD40 mAb (*blue*) is able to elicit much stronger immune response than antigen mixed with adjuvant. **(B)** In this study, chicken cells will display the membrane localized antigen protein (*green line*) and CD40 scFv (*red*) or full length CD40 mAb (*blue-yellow*).

In this study, we will develop a new technology to facilitate the engineering of chicken monoclonal antibodies (IgY) against a human cancer receptor. We will first elicit chicken polyclonal antibodies against the ectodomains of a receptor protein using the antibody-guided vaccine technology developed by collaborator Dr. Luc Berghman, in which a target antigen is chemically conjugated to the agonistic anti-chicken CD40 mAb (mAb 2C5) to be targeted to CD40-rich chicken dendritic cells (DCs) for antigen presentation (Chen et al. 2010). mAb 2C5-functionalized antigens were found to elicit significantly stronger and much more rapid (4 days) immune responses than unconjugated antigens mixed with adjuvant. Since most of the cancer receptors are membrane proteins and are difficult to purify and functionalize with an antibody directly, in the current study, we will expand

this antibody-guided vaccine technology to cells displaying a desired membrane protein antigen (Figure 4.1).

DNA encoding the target antigen and CD40 single chain antibody (scFv) or CD40 mAb will be delivered to chicken muscle cells via electroporation. DNA vaccination has emerged as a promising alternative to conventional protein-based vaccination and was found to induce both humoral and cellular immune responses (Meunier et al. 2016). In one study, a single injection of plasmids expressing the hemagglutinin (HA) protein from avian influenza virus (AIV) protected chickens from severe subsequent challenge of AIV (Ogunremi et al. 2013). In another study, immunization with a DNA vaccine successfully induced neutralizing antibodies and protected the chickens against chicken anemia virus (CAV) (Moeini et al. 2011). We choose to use a DNA vaccine to induce the expression of the desired antigen in chicken muscle cells. We hypothesized that the co-expression of CD40 scFv or CD40 mAb should enhance the immune response through specific recruitment and/or activation of dendritic cells.

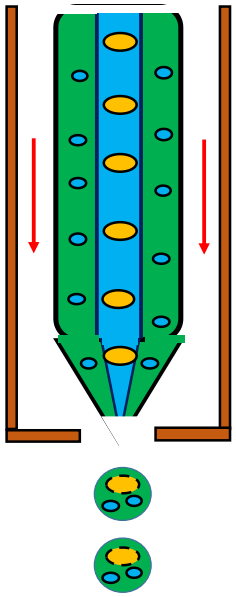

Figure 4.2: Single cell emulsion technology. An axisymmetric flow-focusing nozzle will isolate single cells and poly(dT) magnetic beads into emulsions of predictable size distributions. An aqueous solution of cells (orange circles) in PBS (blue) and cell lysis buffer (green) with poly(dT) beads (blue circles) exit an inner and outer needle and this surrounded by a rapidly moving annular oil phase (red arrows). Aqueous streams focus into a thin jet, which coalesce the emulsion droplet into predictable sizes. Cells are in contact with the lysis buffer only at the point of droplet formation.

Upon development of a polyclonal antibody response, a mammalian cell-based screen will be developed and used to isolate chicken monoclonal antibodies against the desired membrane protein antigen. Mammalian cell display is superior to microbial display for antibody engineering, because antibodies displayed on mammalian cells exhibit reduced aggregation, correct glycosylation patterns, and are more amenable to future large-scale antibody production in mammalian cells (Doerner et al. 2014). However, a significant shortcoming of mammalian cell display technology is the poor transfection efficiency of mammalian cells, limiting its application in single chain antibody (scFv) library screening, which typically requires the screening of a large library due to the low probability of a

heavy chain to be paired with a light chain derived from the same B-cells. To overcome this limitation, a simple and efficient single-cell emulsification technology (Figure 4.2), developed by Georgiou and co-workers in early 2016, will be used to enable the construction of correctly paired heavy-light chain (VH-VL) libraries from chicken B-cells (DeKosky et al. 2013; DeKosky et al. 2015). This approach will significantly reduce the number of clonal variants that need to be screened for scFv selection and will be used to construct a paired VH-VL cDNA library from immunized chickens and display this library on mammalian cells.

In the current study, human CD20 (hCD20) will be used as a model membrane protein antigen (Stashenko et al. 1980). hCD20 plays an important role in B-cell activation and proliferation, is highly expressed on normal and malignant B-cells (Cragg et al. 2005), and has been extensively explored to treat B-cell lymphomas (Link and Friedberg 2008). The anti-CD20 mAb, rituximab, a chimeric mouse-human antibody, was able to deplete B-cells mainly through complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) (Scott 1998). However, about 30% of the B-cells in non-Hodgkin lymphoma patients are refractory to rituximab (Stolz and Schuler 2009). Recently, a few newer anti-CD20 antibodies have been developed. Ofatumumab a fully human antibody generated from transgenic mice that binds a distinct epitope on CD20 from rituximab and induce killing via CDC (Cheson 2010).

Figure 4.3: Sequence alignment of CD20 of human (h) and mouse (m) origin. Included for comparison is the protein (cS2536) that most closely resembles CD20 from chickens. Ectodomains are shaded in gray. The regions that interact with the antibody are boxed (Binder et al. 2006; Cheson 2010)**.**

A high sequence homology is shared between human and mouse CD20 (Figure 4.3). Since all the current therapeutic anti-CD20 antibodies were derived from mouse, only antibodies targeting non-identical regions on the protein have been developed (Binder et al. 2006; Klein et al. 2013). Given the potentially important role of highly conserved/identical regions, and the tendency of antibodies that bind different epitopes to induce different cell-killing mechanisms, the discovery of mAb binding to new epitopes on hCD20, including highly conserved protein regions, would be highly desirable for continued therapeutic development.

In this study, we aimed to develop a new platform technology, building upon the antibody-guided chicken vaccine technology and the single cell emulsion technology, for the discovery of chicken scFv targeting conserved ectodomains of hCD20 protein. This

technology should facilitate the identification of new chicken antibodies targeting other human cancer receptors, creating an opportunity for the development of new anti-cancer mAbs and a new anti-hCD20 mAb to treat lymphoma.

4.3. Material and methods

4.3.1. Cell and chemicals

Cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2. HEK 293T cells were purchased from Invitrogen (Carlsbad, CA) and were grown in Dulbecco's modified Eagle's medium (DMEM) containing 4,500 mg/liter glucose, 4.0 mM glutamine, and 110 mg/liter sodium pyruvate (Thermo Scientific HyClone, Logan, UT) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 1X non-essential amino acids (Thermo Scientific HyClone, Logan, UT). Chicken HD11 macrophages cells (Crippen et al. 2003) and were kindly provided by Luc R. Berghman (Texas A&M University; College Station, TX), were cultured in DMEM medium containing 8% fetal bovine serum and 5% chicken serum (Sigma, St.Louis, MO). Dulbecco's phosphate-buffered saline (DPBS) was purchased from Thermo Scientific HyClone (Logan, UT).

4.3.2. Plasmids

The plasmid encoding the single chain variable fragment (scFv) of agonistic anti-chicken CD-40 (2C5) was custom synthesized by Addgene (Cambridge, MA). The pLPns vector contains the eB7 display domain, which consists of the IgG like C-type domain, transmembrane domain and the cytoplasmic tail of murine CD80. The insert was digested

with sfiI restriction enzyme and cloned into the SfiI digested pLPns backbone. The 2C5 was inserted at the N-terminal of the eB7 display domain.

4.3.3. Cell surface display

To confirm cell surface expression of 2C5-scFv display proteins, 5 x 10^5 HEK 293T cells were transfected with 1000 ng of the appropriate plasmid using Trans IT (Mirus Bio LLC; Madison, WI) as per the manufacturer's protocol. Forty-eight hours post transfection, cells were harvested, washed and treated with 0.5 % sodium azide (Sigma, St.Louis, MO) for 30 min. The fixed cells were stained with a 1:10 dilution of anti-HA-biotin (Miltenyi Biotec, Bergisch Gladbash, Germany) in DPBS supplemented with 1% bovine serum albumin (BSA) for 1 h. The cells were then washed and stained with a 1:200 dilution of FITC-streptavidin (BD Biosciences, San Jose, CA) and diluted in DPBS/1%BSA for 30 min. After removal of excess antibody, samples were resuspended in DPBS containing 1% paraformaldehyde (PFA) and analyzed using a BD FACScan flow cytometer (BD Biosciences; San Jose, CA).

4.3.4. Activity assay for expressed 2C5 scFv

To confirm the activity of cell surface expressed 2C5-scFv display proteins, 5 x 10^5 HEK 293T cells were transfected with 1000 ng of the appropriate plasmid using Trans IT (Mirus Bio LLC; Madison, WI) as per the manufacturer's protocol. Forty-eight hours post transfection, cells were harvested, washed and treated with 0.5 % sodium azide (Sigma, St.Louis, MO) for 30 min. The fixed cells were incubated with a 1 µg/ml of purified chicken CD-40 ectodomain for 1 h. These cells were washed and stained with a 1:1000

dilution of mouse anti-his (R&D systems, Inc, Minneapolis, MN) in DPBS supplemented with 1% bovine serum albumin (BSA) for 1 h. These cells were then washed and stained with a 1:500 dilution of Dylight 488 goat anti-mouse (Jackson ImmunoResearch Laboratories, Inc; West Grove, PA) diluted in DPBS/1%BSA for 30 min. After removal of excess antibody, samples were fixed in DPBS containing 1% paraformaldehyde (PFA) and analyzed using a BD FACScan flow cytometer (BD Biosciences; San Jose, CA).

4.4. Results

4.4.1. Cell surface display of 2C5

In the current study, we initially planned to display the anti-CD-40 antibody 2C5 as scFv on the N-terminus of the eB7 display domain. A HA-tag was inserted to the N-terminus of the 2C5 to facilitate downstream analysis. We evaluated the cell surface expression level of 2C5 on 293T cells, as it is critical for the interaction with the chicken CD-40 ectodomain for immune activation against the antigen. The 2C5 scFv was displayed on the cell surface, which was confirmed using flow cytometry. The presence of positive cells in the 2C5 transfected sample after immunostaining with anti-HA-biotin antibody and FITC-streptavidin secondary antibody (Figure 4.4).

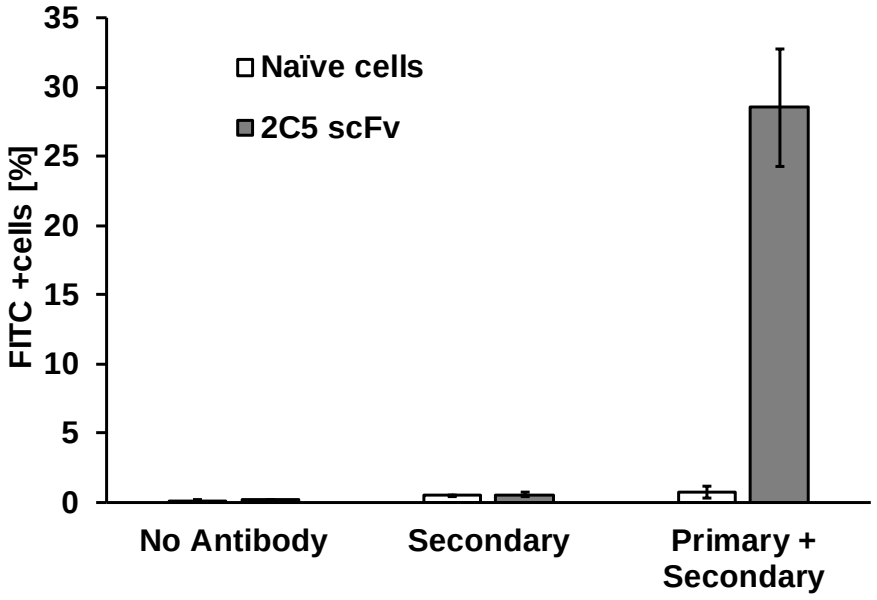

Figure 4.4: The 2C5-scFv was efficiently displayed on the cell surface. Plasmids expressing the 2C5-eB7 display were transfected into HEK 293T cells. Surface display was monitored after 48h using anti-HA biotin antibody followed by FITC-streptavidin. Samples were analyzed using flow cytometry.

4.4.2. Activity of display 2C5-scFv

To confirm the ability of cell surface displayed 2C5-scFv to activate the immune response through CD-40 rich chicken dendritic cells, the transfected cells were incubated with 1μg/ml of native chicken CD-40 ectodomain protein. As shown in figure 4.5, the cells transfected with 2C5-scFv had slightly higher positive staining compared to the naïve cells in the presence of primary and secondary antibodies. Surprisingly, the untransfected cells also had positive staining. The staining may be due to non-specific interaction with the primary anti-his antibody or the native chicken CD-40 ectodomain protein.

Optimization experiments have to be conducted, but overall, the slightly higher staining in transfected cells support the presence of the cell surface expressed 2C5-scFv.

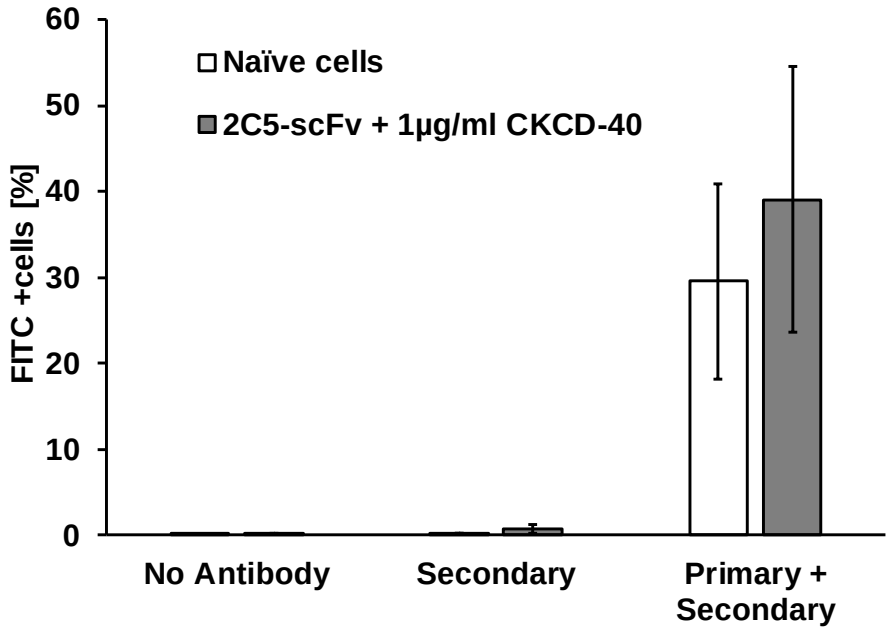

Figure 4.5: The cell surface displayed 2C5-scFv was active to interact with native chicken CD-40 protein. Plasmids expressing the 2C5-eB7 display were transfected into HEK 293T cells. After 48h, the fixed transfected cells were interacted with 1µg/ml of native chicken CD-40 protein for 1h. The activity was determined using a mouse anti-His antibody followed by goat anti-mouse 488-dylight. The interaction was determined using flow cytometry.

4.5. Discussion

In this section, we report the preliminary results of experiments aimed at developing a new platform technology, building upon the antibody-guided chicken vaccine technology (Chen et al. 2010) and the single cell emulsion technology (DeKosky et al. 2015), for discovery of new antibodies in chickens targeting human tumor antigens. A DNA vaccine

strategy was adopted to develop the polyclonal antibodies against the tumor antigen hCD20. Initially, we constructed a plasmid encoding the single chain variable fragment (scFv) of agonistic anti-chicken CD-40 at the N-terminal of the eB7 display domain (Liao et al. 2003). A higher surface expression of scFv was achieved on eB7 than on other commercial vectors, such as human platelet-derived growth factor receptor (PDGFR) and the GPI signal peptide from decay accelerating factor (DAG) (Cheng and Roffler 2008; Lin et al. 2013). A higher 293T cell surface expression was confirmed by immunostaining (figure 4.4). The important advantage of this strategy is that it required only one plasmid to display 2C5-scFv. Further, the activity of the displayed 2C5-scFv was confirmed to interact with native chicken CD-40 ectodomain protein. Surprisingly, positive staining was observed in both the control and 2C5 transfected cells, but transfected cells had slightly higher staining (figure 4.5). The lack of definitive evidence from the activity assay can be due to the non-specific interaction of the mouse anti-his antibody or the native chicken CD-40 ectodomain protein.

In conclusion, in these preliminary results, we were able to confirm the cell surface expression, but we were unable to determine the activity of the displayed 2C5-scFv. As the lack of activity of displayed 2C5-scFv can result in weak immune response, we plan to develop an engineered cell line of chicken fibroblasts, which will display both the 2C5-scFv and the tumor antigen. Later, these cells will be used to immunize chickens to develop polyclonal antibodies against the tumor antigen. As another strategy, we will clone and display the full-length mAb 2C5 through the associated proteins – human Igα and Igβ. The variable regions of 2C5-scFv will be grafted onto the constant heavy and

light chain to form 2C5-mAb to be displayed on the chicken macrophages. We anticipate that 2C5-mAb will retain full activity to provide the required immune response.

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

Retargeting of lentiviral vectors entry to cell types of interest is a key factor in improving the safety and efficacy of gene transfer. Lentiviral vectors allow stable long-term transgene expression in non-dividing cells and in tissues. These properties have made them ideal gene therapy vectors for research and clinical applications (Cockrell and Kafri 2007). A lot of attention has been provided for vector design for improving the safety and efficacy of lentiviral vector-mediated gene therapy (Vigna and Naldini 2000). Restricted gene therapy has gained interest by using tissue-specific promoters (transcriptional targeting) and detargeting of irrelevant cell types from gene types for gene expression by inserting target sequences from tissue specific miRNAs (Frecha et al. 2008). The ideal way to restrict gene transfer would be at the cell entry itself (transductional targeting), Lentiviral vectors are usually pseudotyped with glycoprotein (G) of vesicular stomatitis virus (VSV) and other retroviruses and non-lentivirial envelope proteins (Levy et al. 2015). Several previous attempts to engineer receptor usage have been unsuccessful due to the close activity of receptor binding and membrane fusion function, such as the VSV-G. Recently, lentiviral vectors have been pesudotyped with engineered Sindbis virus glycoproteins without the ability to recognize their natural receptor and have been modified to either noncovalently bind to monoclonal antibody directed against a surface antigen (Morizono et al. 2005). Further mutations in the Sindbis glycoprotein made a binding-deficient and fusion competent molecule. For the current study, the mutated Sindbis glycoprotein was engineered to incorporate one of the protein partner obtained

from naturally occurring covalent bond protein pairs. Later the other protein partner was fused to a cell-targeting moiety.

In the first approach, we evaluated the disulfide bond forming protein-peptide pair PDZ1 -TEFCA from the *Drosophila* visual system (Kimple and Sondek 2002). Our results show the development of a covalent bond lentiviral vector that can be applied for gene therapy applications. The retargeting of the functionalized virions was observed to be dependent on the protein concentration and receptor of entry. The titer of functionalized virions was observed to be higher compared to the unfunctionalized and mutant functionalized virions. The disulfide bond was observed to be stable in prolonged dialysis and in presence of serum complement.

In the second approach, we explored the isopeptide bond forming protein-protein pair SpyTag-SpyCatcher from the *Streptococcus pyogenes* (Zakeri et al. 2012). We successfully demonstrated building up another covalent bond retargeting lentiviral vector. The functionalized SpyTag virions exhibited a nice protein and HER2 dependent transductional acitivity compared to unfunctionalized virions. The high target versus non-target cell discrimination was demonstrated in mixed cell populations by the functionalized virions. The isopeptide bond was observed to stable during prolonged dialysis and in presence of serum complement. The application of different antibodies for retargeting lentiviral vectors was made possible through click chemistry approaches. The chemically conjugated Fab-SpyCatcher was able to functionalize SpyTag virions and was able to transduce the specific cells in a dose dependent manner.

In comparison between both the developed retargeting approaches, both the strategies can be optimized to develop novel lentiviral vectors for clinical consideration. There are several reasons the SpyTag-SpyCatcher approach will be ideal for optimization to apply in clinical settings. These reasons include, the spontaneous bond forming capability of SpyTag-SpyCatcher, the observed low immunogenicity against SpyTag and SpyCatcher in *in vivo* setting (Brune et al. 2016) and the optimized application of antibody fragments with SpyCatcher through click chemistry approaches. Although my work primarily focused on developing covalent retargeting methodologies, the other hurdles include the vector production for clinical use, other immune system barriers and questions of systemic application and dosage are still to be addressed through future work. However, instead of these hurdles, our results demonstrate the development of plug and play lentiviral vector retargeting models through covalent functionalization for gene therapy applications.

Lastly, we have built up the strategy to develop antibodies against human tumor antigens in chickens. Through our preliminary work, we demonstrated the surface expression of mouse anti-chicken CD40 scFv (2C5). This cell surface expression plays role in activation of adaptive immune response. The ultimate goal of this research will be to utilize the potential of antibody guided vaccine technology and single cell emulsion technology to isolate and optimize the selected antibodies against hCD20 antigen.

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