DEVELOPMENT OF NOVEL EXOSOME-BASED DELIVERY SYSTEM FOR PROTEINS

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2016

DEVELOPMENT OF NOVEL EXOSOME-BASED DELIVERY SYSTEM FOR PROTEINS

By

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Thesis submitted in fulfilment of the requirements for the degree of Master of Science

March 2016

ACKNOWLEDGEMENTS

I thank to my supervisor Dr.Syed Ali who had guided me throughout the project. I would like to thank Prof. Narazah Mohd Yusoff for being an excellent advisor who helped me throughout the study.

I would also like to thank all my friends and colleagues especially Siti Aisyah Mualif, Teow Sin Yeang, Tasyriq Che Omar, Alif and Nurdianah Harif Fadzilah.

Last but not least, I wish to thank my family especially my parents Chew Keng Lee and Lee Siew Ying who have always given me a full support and encouraged to reach my goal. Without their guidance, training and support I would not be able to complete my studies.

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LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency symptoms
Amp	Ampicillin
APS	Ammonium persulfate
bp	Base pair
CFU	Colony-forming unit
cm	Centimeter
Da	Dalton
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulfoxide
DNA	Deoxy ribonucleic acid
FBS	Fetal bovine serum
HEK293	Human embroyonic kidney 293 cells
His	Histidine
HIV	Human immunodeficiency virus
h	Hour
IMAC	Immobilized metal affinity chromatography
IPTG	Isopropylthio-β-galactoside
kb	Kilo base
kDa	Kilo Dalton
LB	Luria Bertani media
mL	Mililiter
mM	Milimolar
MW	Molecular weight
Nef	Negative factor
OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction

PBS	Phosphate buffered saline
pH	Potential hydrogeni
RE	Restriction enzyme
RNA	Ribonucleic acid
RT	Room temperature (22°C-25°C)
SDS	Sodium dedecyl sulfate
SDS-PAGE	Sodium dedecyl sulfate polyarylamide gel electrophoresis
TAE	Tris-Acetate-EDTA
TEMED	N,N,N,N'-tetramethylethylenediamine
UV	Ultraviolet
Vif	Virus infectivity factor
VSV-G	Vesicular stomatitis virus-G protein
μm	Micrometer
μM	Micromolar
μL	Microliter
°C	Degree Celsius

PEMBANGUAN SISTEM PENYAMPAIAN BAHARU BERASASKAN EXOSOME BAGI PROTEIN

ABSTRAK

Sel mempunyai beribu-ribu protein yang menyumbang kepada laluan isyarat penting terhadap fungsi sel normal. Kebanyakan penyakit terjadi akibat daripada kepincangan tugas salah satu atau lebih daripada protein berkenaan. Untuk meredakan disfungsi berkenaan, terapi protein yang menggunakan protein terapeutik untuk membetulkan fungsi sel dianggap pendekatan yang paling selamat untuk merawat penyakit. Terdapat lebih daripada 1,500 sasaran rawatan penyakit yang berpotensi di dalam sel tanpa laluan reseptor yang jelas sebagai sasaran khusus mereka. Keperluan untuk penghantaran intra sel yang mensasarkan sel perumah tertentu merupakan satu halangan yang sukar dalam pembangunan intra sel baharu yang berasaskan protein. Exosomes yang bersaiz 40-120nm, merupakan vesikel membran yang dirembeskan oleh sel dan telah dikenalpasti sebagai pengangkutan semulajadi untuk penghantaran makromolekul. Exosomes berinteraksi dengan sel dan turut memberi kesan terhadap fungsi sel lain yang berdekatan melalui pemindahan inter sel mRNA, microRNA, reseptor dan enzim, dan didapati memainkan peranan penting dalam kawalselia imun. Beberapa protein virus seperti protein HIV-1 Nef, Vif dan VSV-G dilaporkan meransang pengeluaran exosome. Pemerhatian ini membawa kepada hipotesis bahawa expresi berlebihan yang serentak daripada protein perangsang exosome/ exosome inducing protein (ExIP) dan protein heterologous dari vektor plasmid yang sama akan menyebabkan pengeluaran exosome yang mengandungi protein yang dikehendaki. Untuk menguji hipotesis ini, kami telah membangunkan vektor mamalia bicistronic dalam expresi pelbagai ExIP dan GFP secara serentak sebagai protein reporter. Kami telah mendapati bahawa kedua-dua protein HIV-1 Nef dan VSV-G berupaya meransang induksi exosome yang mengandungi GFP. Pemerhatian ini berupaya membawa kepada kemungkinan baharu untuk penghantaran

protein rekombinan (seperti antibodi) secara cekap dan merintis jalan baharu dari segi pembangunan ubat biologi berasaskan protein intra sel.

DEVELOPMENT OF NOVEL EXOSOME-BASED DELIVERY SYSTEM FOR PROTEINS

ABSTRACT

A cell contains thousands of proteins that contribute to critical signalling pathways in normal cellular functions. Most diseases somehow result from the malfunction of one or more of such proteins. To palliate such dysfunction, protein therapy that makes use of therapeutic proteins to correct the cellular functions is considered the most direct and safe approach for treating diseases. There are more than 1,500 potential disease treatment targets that reside inside the cell, without a clear receptor pathway for their specific targeting. The need for intracellular delivery and targeting select host cell compartment represents a formidable hurdle to the development of novel prospective intracellular targeting protein-based biologics. Exosomes, 40-120 nm membrane vesicles secreted by cells, have recently emerged as promising natural vehicles for the delivery of macromolecules. Exosomes interact with cells and affect the functions of neighbouring cells through intercellular transfer of mRNA, microRNA, receptors and enzymes, and found to play important roles in immune dysregulation. A number of viral proteins such as HIV-1 Nef, Vif and VSV-G protein are reported to induce exosome production. These observations lead us to hypothesize that simultaneous overexpression of exosome inducing proteins (ExIP) and a heterologous protein from the same plasmid vector will result in the production of exosomes that contain the desired protein. To test our hypothesis we have constructed bicistronic mammalian expression vectors that concomitantly express various ExIPs and GFP as a reporter protein. We have found that both HIV-1 Nef and VSV-G proteins induce the induction of GFP-containing exosomes. These observations may lead to novel possibilities for the efficient delivery of recombinant proteins (such as antibodies) and opens up new avenues in terms of the development of intracellular protein biologic drugs.

CHAPTER 1. INTRODUCTION

1.1. Background

The word "Protein" was proposed by Jöns Jakob Berzelius in 1838. He claimed that substances such as egg white, blood serum albumin, fibrin and wheat gluten are a combination of simple ratios of sulphur and phosphorus that are found in nature. He called these substances as "protein". The word "protein" was coined from the Greek word π ρ ω τ ε ι˜ ο ς (Vickery, 1950). In general, a protein has a 3D structure formed from a long chain of amino acids and is involved in major functions within living organisms, including metabolism, DNA replication, response to stimuli, and movement. Some of the common diseases are a result of the failure of one or more proteins. To palliate such dysfunction, compensatory therapy using therapeutic proteins to correct the cellular functions is considered the most direct approach for treating such diseases (Dimitrov, 2012; Carter, 2011; Yan *et al.*, 2010).

Since the early 1980s, therapeutic proteins have been proposed as candidate therapy. Advances in protein engineering allow for the systemic dissection of protein structure-function relationship, and the subsequent generation of novel proteins with enhanced activities or entirely new properties. Human insulin (Humulin®), the first human therapeutic protein commercialized in 1982, is derived from recombinant DNA technology (Goeddel *et al*., 1979).

Therapeutic proteins are divided into five major groups based on their designated pharmacological activities: (a) replacing a protein that is deficient or abnormal; (b) supplementing an existing pathway; (c) providing a novel function or activity; (d) interfering with a molecule or organism; and (e) delivering other compounds or proteins such as radionuclide, cytotoxic drug or effectors proteins (Dimitrov 2012). Therapeutic proteins include antibody-based drugs, anticoagulants, blood factors, bone morphogenetic proteins, engineered protein scaffolds, enzymes, Fc fusion proteins, growth factors, hormones,

interferon, interleukins and thrombolytics. To date, hundreds of therapeutic proteins are in various phases of clinical trials for cancer therapy, immune disorders, infection, and other illnesses. For example, Belimumab for systemic lupus erthematosus by Benlysta *et al.,* 2011; Ipilimumab for metastatic melanoma by Yervoy *et al.,* 2011.It is believed that protein-based therapy will become the treatment modality of choice for numerous diseases for next 10-20 years (Carter, 2011).

Protein-based therapeutics is developing to be one of the largest class of new chemical entities in the drug industry (Gerngross, 2004). In European Union (EU) and the USA have approved about 100 genuine unmodified therapeutic proteins have been approved, for clinical purpose by July 2011 (Dimitrov, 2012). A large number of popular drugs are protein-based , conjuring more than 60 billion USD in sales annually (Dimitrov, 2012).

In earlier applications, most of the therapeutic proteins were derived from natural resources, and their production is impeded by complicated and costly manufacturing procedures (Lu *et al.,* 2006). The next generation of protein-based drugs were largely restricted to extracellular targets, or by targeting the receptors to transduce signals indirectly to cellular effectors (Gerngross, 2004). The size and electrical charges of these therapeutic proteins would prevent them from passing through the plasma membrane and directly interact with intracellular targets. However, there are more than 1,500 potential targets for disease treatment inside the cells without a clear specific receptor pathway to modulate their activities. The need for intracellular delivery and targeting to select host cell compartments represents a formidable hurdle to the development of prospective protein-based biologics (Pisal *et al.,* 2010). The various delivery methods currently used or being actively developed are further described in the following.

1.2. Delivery systems for therapeutic protein and peptides

In the past 20 years, there has been a dramatic increase in the development of new large therapeutic molecules such as proteins, peptides and nucleic acids, aided by advances in

recombinant DNA technology and solid-phase synthesis. However, the safety and efficiency of these initial generations of therapeutic proteins were limited by high molecular weight, short half-lives, instability, immunogenicity, poor oral bioavailability, and poor penetration across biological membrane (Lu *et al.*, 2006; Pisal *et al.*, 2010). In the next generation of therapeutic proteins, there was a focus on either a change in the bioactive agent itself or by an improvement in drug formulation. The common concerns in developing therapeutic proteins include maintaining the delivery efficiency in different and challenging cells, ensuring rapid endosomal release, the ability to reach the target, drug activity at low doses, reducing cellular toxicity and improving facilities for therapeutic application (Heitz *et al.*, 2009). For example, early therapeutic proteins may be taken up into cells by endocytosis and gets degraded in the lysosomes, rather than being released into the cytoplasm where their targets are present. New approaches for drug formulation like liposomes-encapsulated proteins, cell-penetrating peptides, single-protein-core nanocapsules and thin polymer shell are now being employed (Lu *et al.*, 2006; Yan *et al.*, 2010; Carter, 2011).

1.2.1. Liposome-encapsulated proteins

The basic function of a cell membrane is to separate the cytoplasm from the extracellular environment, which also keep naked hydrophillic therapeutic proteins away from their intended intracellular targets. Liposomes are useful in drug-delivery because of their suitability in living bodies and adaptability to various types of drugs (Kaneda 2000; Hirai *et al.*, 2015). A liposome-encapsulated protein is protected from pretease-mediated that results in improved delivery. The major reasons behind low efficiency in cargo delivery to target tissues by liposomes are the formation of aggregates with negatively-charged serum proteins, decreased macrophage uptake and insufficient drug entrapment (Kaneda, 2000; Mufamadi *et al.*, 2011).

1.2.2. Cell-penetrating peptides

Large therapeutic biomolecules such as plasmid DNA, oligonucleotides, siRNAs, peptide-nucleic acids (PNA), proteins, and peptides may not pass through the cell membrane readily.

This problem can be overcome with the conjugation of protein transduction domain (PTDs) also referred to as cell-penetrating peptides (CPPs) to the biomolecules (Heitz *et al.,* 2009). In 1988, Frankel and Pabo discovered that the transactivator of transcription (TAT) protein of HIV can cross cell membranes and be efficiently internalized by cells, resulting in the transactivation of viral promoter *in vitro*. Most CPPs are short peptides composed 30-40 amino acid residues. CPP-conjugated molecules readily cross the plasma membrane, in energy-dependent and/or independent mechanism and without the necessity of chiral recognition by specific receptors. Based on their origin, CPPs can be classified either as peptides derived directly from proteins, chimeric peptides formed by the fusion of two natural sequences, and synthetic CPPs rationally designed based on structure activity studies (Francesca Milletti, 2012).

Nonetheless, CPPs may lack selectivity and CPP-conjugated proteins can be delivered into non-target cells as well, thereby increasing the risk of drug-induced toxic effect in normal tissues. In addition, there is a risk of proteolytic cleavage of CPPs in blood, thus reducing the therapeutic effect of the molecules that actually reach their targets *in vivo* (Koren and Torchilin, 2012; Bechara and Sagan, 2013).

The current research on CPPs is focussed on three areas: (i) their use as a vector to transport various macromolecules for targeted cellular therapies *in vitro* and *in vivo*; (ii) to define the structural basis of the internalization capacities in order to engineer new CPP with optimum activity; (iii) to clarify the mechanisms of cell entry and final localization (Heitz *at al.,* 2009; Bechara and Sagan, 2013; Copolovici *et al.,* 2014).

1.2.3. Nanocapsules composed of single-protein core and thin polymer shell

Recent developments suggest that nanocapsules composed of single-protein core and thin polymer shell may be efficient means of delivery for therapeutic proteins. The single-protein core nanocapsules have been shown to be effective and low-toxicity intracellular protein delivery system. Moreover, nanocapsules conjugated with a protein core and a thin

permeable polymeric shell is able to remain stable at different pHs. However, this technology is still at the early experimental stages(Yan *et al.,* 2010).

1.3. Exosome-like nanovesicles as novel delivery vehicles

Drug delivery system is under constant improvement to overcome the limitations of biological therapeutics, most notably the susceptibility to degradation, inability to cross biological membranes and eliciting immune responses. Extracellular vesicles (EVs) is a promising strategy being actively explored to deliver biological therapeutics to the cytosol of target cells (Kooijmans *et al.,* 2012). James Rothman, Randy Schekman and Thomas Südhof shared the Nobel Prize in Physiology or Medicine 2013 for their work on the machineries regulating vesicle traffic, as one of the fundamental processes in cell physiology (Admin, 2015). Their work laid down the groundwork on how vesicles are delivered with timing and precision intra- and extracellularly.

Exosomes were first described in maturing sheep reticulocytes, involved in the externalisation of a receptor (Pan and Johnstone, 1983). Exosomes are 40-120 nm in diameter that are secreted by dendritic cells (DC), B cells, T cells, mast cells, epithelial cells and tumour cells. Exosomes are composed of lipids (Ramachandran and Palanisamy, 2012), proteins (Bellingham *et al.,* 2012; Gonda *et al.,* 2013) and nucleic acids (El Andaloussi *et al.,* 2013). Exosomes are cup-shaped or round well-delimited vesicles when observed under transmission and cryo-electron microscopy (Simons and Raposo, 2009; Orozco and Lewis 2010; Lakhal and Wood, 2011; Bellingham *et al.,* 2012). The density of exosomes range between 1.13 and 1.19 g/mL, and can be separated from multivesicular late endosomes by using sucrose-density gradient centrifugation (Simons and Raposo, 2009).

Exosomes are produced from endocytic/exocytic pathways. Early endosome is formed from endocytic vesicles during cell membrane invagination. The inward budding of the endosomal membrane then forms intraluminal vesicles within the late endosomal compartments, which are also known as the Multivesicular Bodies (MVBs). The intraluminal vesicles are the

exosomes that are released into the extracellular space upon fusion of MVBs with the plasma membrane. Ceramides and phosphatidic acid are reported to be involved in inducing exosome formation and subsequent release. Proteins involved in gene silencing, Ago2 and GW182 associate with MVB, and are thought to facilitate the packaging of microRNAs in exosomes **(Figure 1.1)** (Marsh and van Meer, 2008; Laulagnier *et al.,* 2004; Trajkovic *et al.,* 2008). Exosomes is believed to be a unique method of cell-to-cell communication. Exosomes affect the neighbouring cells through intercellular transfer of mRNA, microRNA, receptors and enzymes and are found to play important roles in immune dysregulation (O'Loughlin *et al.,* 2012; El Andaloussi *et al.,* 2013). The advantage of exosomes as mediator in extracellular communication is that they can be targeted to multiple locations. Exosomes have been shown to be promising as natural vehicles for the delivery of macromolecules. Wood and colleagues published a proof-of-concept study for the delivery of exogeneous siRNA using exosomes. Five other independent studies confirmed the potential of membrane vesicles in RNA transportation (Alvarez-Erviti *et al.,* 2011).

At the same time, exosomes are also investigated in their value as molecular diagnostic markers. Exosomes from tumour cells are found to express specific antigens which are enriched in heat shock proteins and activate antigen-presenting cell through delivery of danger signals (Sadallah *et al.,* 2011). In addition, the surface markers on exosomes and their internal cargo are often reflective of the physiological status of the cells and organs. For example, exosomes found in urine may be developed as HIV and hepatitis C virus diagnostic tools as virus transmission has been shown to be facilitated through exosomes (Ramachandran and Palanisamy, 2012).

The exact mechanism by which macromolecules are packaged into exosomes is not known. Thus extrapolating knowledge obtained from in vitro models to the more directly applicable area of translation research is a major focus of future research in exosomes (Ramachandran and Palanisamy, 2012).

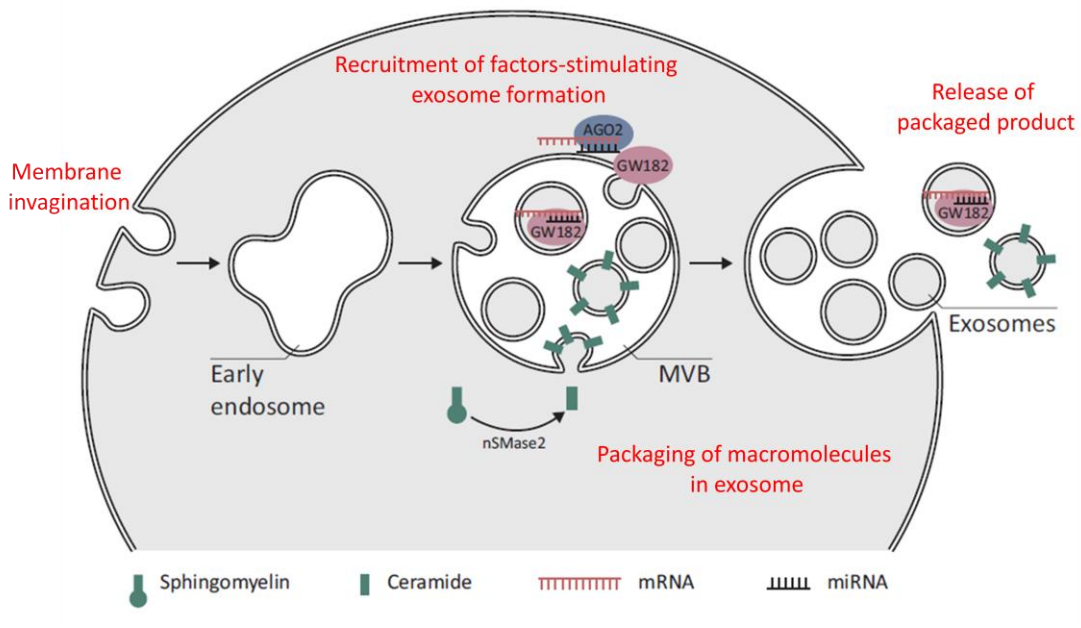

Figure 1.1. Schematic representation of exosome production. Exosomes are intraluminal vesicles of endosomal origin. Recruitment of stimulating factors induces exosome formation, and the packaging of macromolecules. Exosomes are then released extracellularly when the multivesicular body (MVB) fused with plasma membrane. Figure is adapted from Lakhal and Wood, 2011.

1.4. Bicistronic expression vector

Although less conventional, two or more exogenous genes could be expressed simultaneously in host cells by using a single plasmid under single promoter. Several strategies have been used such as fusing promoters to each open reading frame (ORF),or inserting splicing signals, proteolytic cleavage sites, Internal Ribosome Entry Site (IRES), or P2A peptides between two genes(Ghattas *et al.,* 1991; Mizuguchi *et al.,* 2000; Hellen and Sarnow, 2001).

1.4.1. Internal ribosome entry site (IRES)

Canonical translation initiation is dependent on ribosome scanning from the 5'-capped end of a mature eukaryotic mRNA to the initiator AUG codon (Borman *et al.,* 1997). However, Pelletier and Sonenberg (1998) described an IRES element in 1988 which allows translation initiation by cap-independent manner in 5' non-coding region of poliovirus RNA **(Figure 1.2)**. IRES sequence can be found in the RNA of all family members of *Picornaviridae*. The IRES DNA sequence is 450-500 nucleotides long and allows the expression of multiple genes on one mRNA. IRES DNA sequence can be inserted in between two genes for the co-expression of both exogenous proteins under a single promoter (Jang *et al.,* 1989; Ghattas *et al.,* 1991; Mountford and Smith 1995).

The IRES elements from picornaviruses can be classified into three distinct groups based on their activities under different salt conditions and the requirement of other co-factors. Type I IRES element is found in enterovirus and rhinovirus, while Type II IRES element is identified in the genome of cardiovirus and aphthovirus. Lastly, Type III IRES element is found in hepatitis virus (Liebig *et al.,* 1993; Borman *et al.,* 1995; Borman *et al.,* 1997)

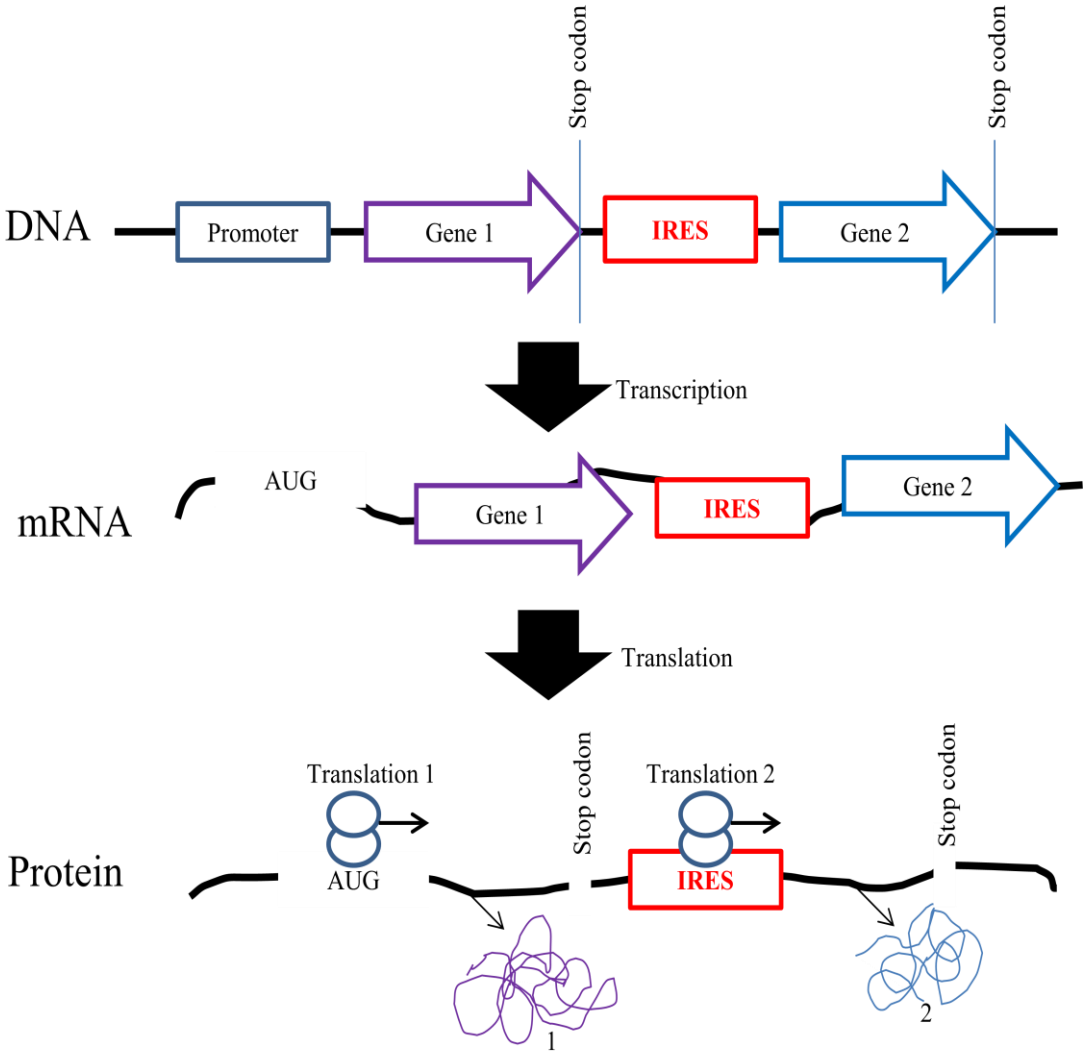

Figure 1.2. IRES-mediated translation- bicsictronic constructs for the heterlogous co-expression of two hypothetical proteins. IRES sequence is inserted between the coding sequences of protein 1 and protein 2 to drive a cap-independent, internal initiation of protein 2 translations, in parallel to Protein 1 cap-dependent translation initiated at the bicistron transcript 5' end.

1.4.2. Foot-and-mouth disease virus (FMDV)-2A peptide

Ryan and colleagues first identified a "self cleaving" peptide at the 2A region of foot-and-mouth disease virus (FMDV). This observation is subsequently observed in all other members of *Picornaviridae* that have a conserved peptide sequence of around 18-22 amino acids. For example, the P2A peptide sequence is A T N F S L L K Q A G D V E E N P G P, and the "cleavage" occurs between the Glycine and Proline residues at the C-terminus of the P2A peptide. These 2A-like CHYSEL (*cis*-acting hydrolase elements) peptide sequences lead to ribosome "skipping" during translation whereby the single peptide bond in between Glycine and Proline is not synthesised. The 2A peptide-mediated cleaving property is subsequently used in co-expression of exogenous proteins in cell lines, embryonic stem cells and neurons (Lengler *et al*,. 2005; Kim *et al.,* 2011) shown at **Figure 1.3**.

A functional model of 2A-mediated "cleavage" strategy was proposed as such: (i) Ribosome initiates translation at the start codon of the ORF upstream of 2A peptide sequence; (ii) elongation of peptide continues until the C-terminus of FMDV 2A sequence; (iii) the nascent peptide chain is released at a C-terminus of the FMDV 2A sequence; (iv) the ribosome subsequently re-initiate translation of downstream sequences and terminates normally at the stop codon of the entire ORF (Brown and Ryan, 2010).

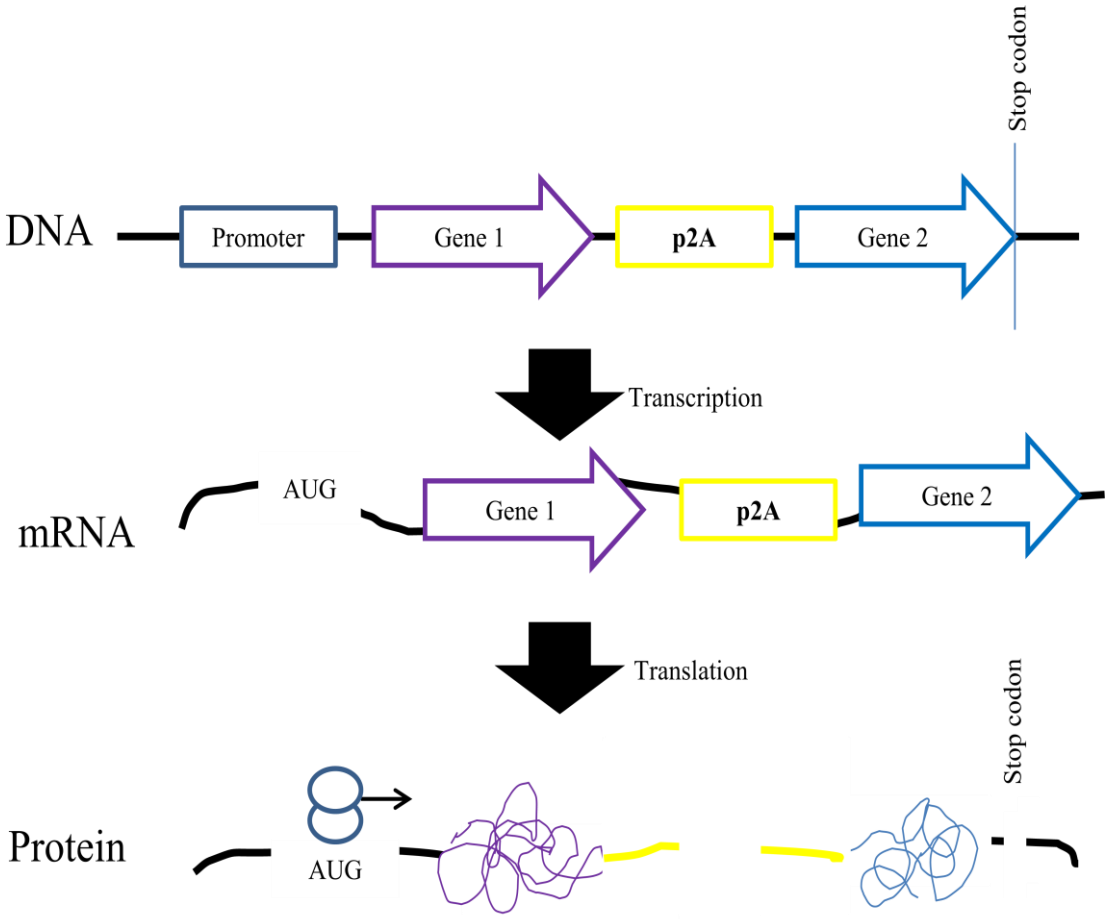

Figure 1.3. 2A peptide-mediated translation - bicsictronic constructs for the heterlogous co-expression of two hypothetical proteins. 2A sequence is inserted between the coding sequences of protein 1 and protein 2 to induce 'ribosomal skipping' during translation leading to the co-translational release of the two proteins.

1.5. Literature review for model protein

Previous studies suggested that exosomes formation can be induced by overexpression of certain virus proteins such as HIV-1 Nef protein (Ali *et al.,* 2010; Shelton *et al.,* 2012; Campbell *et al.,* 2012), Viral infectivity factor (Vif) (Columba and Federico 2013), and Vesicular Stomatitis Virus - G protein (VSV-G) (Mangeot *et al.,* 2011) .

1.5.1. HIV-1 Nef protein

Human immunodeficiency virus type-1 (HIV-1) accessory protein Nef is 27 kDa in size and is produced early during HIV infection of cells. Nef protein plays a major role in the pathogenesis of HIV and simian immunodeficiency virus (SIV) infections (S. Y. Kim *et al.,* 1989). It affects viral infectivity and the down-regulation of cell surface CD4 on T cells through interaction with host cell plasma membrane and proteins (Sanfridson *et al.,* 1997). Most of the studies are focused on intracellular functions of Nef in infected cells. However, Nef protein is also secreted from Nef-transfected and HIV-1 infected cells, and can be detected in the serum of clinical samples from AIDS patients. Protein markers for endosomes or lysosomes, such ascathepsin D and LAMP2 are present in Nef-induced intracellular vesicles, indicating the origin of these vesicles. This is in line with the observation where expression of Nef induces the intracellular accumulation of MVBs (Sanfridson *et al.,*1997; Stumptner-Cuvelette *et al.,* 2003). These Nef-induced exosome-like vesicles were also found to contain acetylcholinesterase (AChE) and CD45 (Ali *et al.,* 2010). Thus, AchE and CD45 can be used as specific markers to detect Nef-induced exosome-like vesicles. The secreted exosomal Nef protein could be important for the HIV-1 pathogenesis by modulating the behaviour of non-infected neighbouring cells and inducing apoptosis in other non-infected $CD4^+$ T cells (Campbell *et al.,* 2008; Shelton *et al.,* 2012).

1.5.2. HIV-1 Vif protein

Human immunodeficiency virus type-1 (HIV-1) protein, virion infectivity factor (Vif) is a 23 kDa cytoplasmic protein. Vif is expressed from one of the accessory genes during the late phase of HIV-1 replication. There are several known regulatory roles of Vif, including the

modulation of proviral DNA synthesis, viral core structure in released virions, and the efficient incorporation of envelop protein into release virion. In addition, Vif protein is secreted in small exosome-like vesicles , which is detected using RT-PCR method (Navid Madani and David Kabat, 1998; Hui Zhang *et al.,* 1998; Columba Cabezas and Federico, 2013). As such, Vif is chosen as one of the proteins to be tested regarding the role in exosome formation.

1.5.3. Vesicular stomatitis virus - G protein (VSV-G)

Vesicular stomatitis virus (VSV) is a type of rhabdovirus first characterized in 1982 (Lefrancois and Lyles, 1982). The G protein of VSV (VSV-G) is found on the cell surface of the infected host cells and is important for the direct budding of virus from the plasma membrane. VSV-G is synthesized on membrane bound polyribosomes (Rose and Gallione, 1981). The presence of VSV-G can increase the stability of vector particles during purification process (Burns *et al.,* 1993). In a recent study, Mangeot and colleagues reported a novel method using VSV-G induced vesicles (named as gesicles) as a tool to deliver proteins in human cells. The expression of VSV-G is sufficient to induce the budding of pseudovirion, transmitting the replicons to neighbour cells. Therefore, we also tested VSV-G on its ability to induce exosomes when co-expressed in bicistronic transfection vector.

1.6. Aim of this study

Previous study has shown that the N-terminal portion (amino acid 1-70) of HIV-1 Nef protein is sufficient to induced exosome secretion when expressed in a variety of cells such as THP1 monocytes, Jurkat T cells and HEK 293 (Ali *et al.*, 2010; Campbell *et al.*, 2012; Shelton *et al.*, 2012). We have named it exosome-inducing peptide (ExIP). Interestingly, the exosomes secreted from induce exosome secrrtion expressing cells contained exosomes and its mRNA, and also proteins and RNA from the host cell (US Patent 20100317566). This indicates that the exosomes can package proteins and mRNA from the cytoplasm. Following up on that, we are interested in examining the efficiency of various other viral proteins in inducing exosomes formation and the potential for use in packaging therapeutic proteins. A

bicistronic vector model will be used for simultaneous expression of GFP and different proteins of viral origin (Nef, Vif, and VSV-G) in 293-T cells. Various bicistronic vectors using internal ribosomal entry site (IRES) and 2A peptide (from FMD virus) will be tested. From this, we aim to complete a proof-of-concept study to show that recombinant protein (GFP) can be packaged and released in induced exosomes **(Figure 1.4)**. This study will serve as a platform for further research in developing a novel exosome-based autologous production, packaging, and delivery system of recombinant therapeutics to target cells. Since exosomes are naturally produced by a variety of cell types, they do not elicit immune response thus are promising vehicles to deliver biologically active macromolecules.

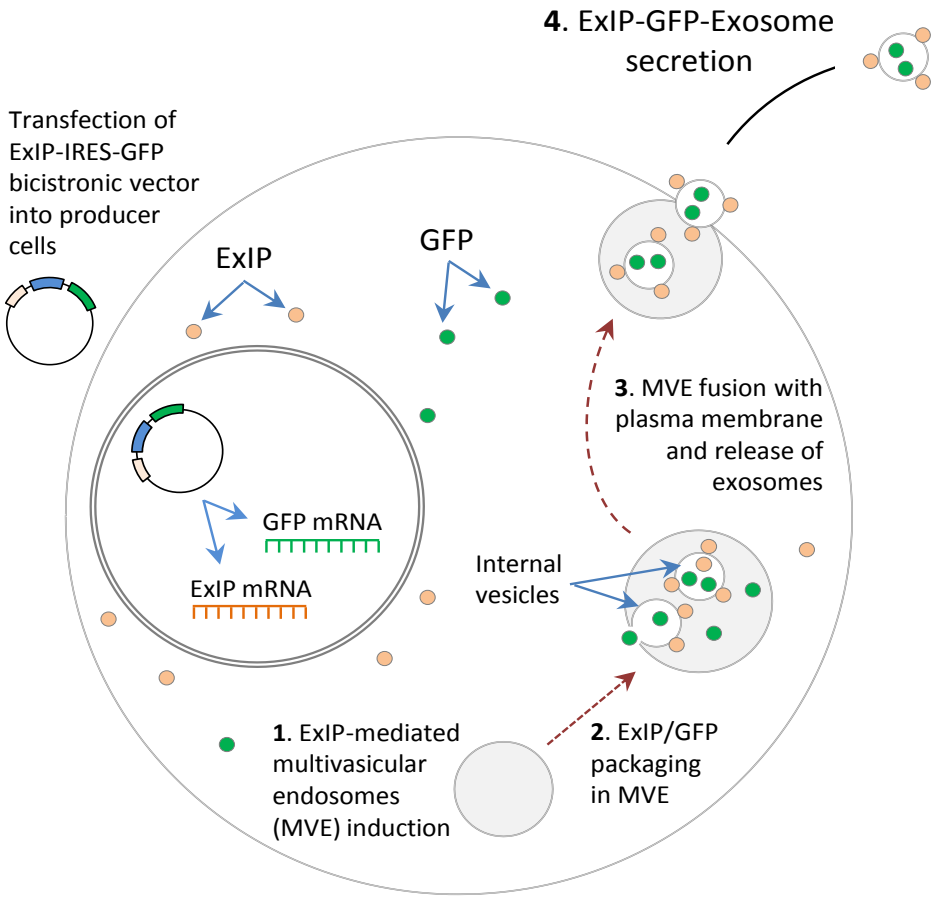

Figure 1.4. Pathway of recombinant proteins packaging and release in Nef-induced exosome.

1.6. Main goal of the research

Exosomes are nano-sized membranous vesicles, produced naturally by cells as a means of cell-to cell communication. We aim to engineer cells to produce therapeutic protein-containing exosomes. The main goal of this work is to construct novel mammalian expression vectors that can simultaneously induce exosome production and packaging of recombinant protein into produced vesicles.

1.6.1. Specific objectives:

1. To construct bicistronic vectors for simultaneous expression of exosome-inducing protein(s) and GFP (a model protein).
2. To evaluate various viruses -derived ExIPs (Nef, Vif, and VSV-G) for their ability to induce exosome biogenesis.
3. To demonstrate that the released exosome contain GFP.

CHAPTER 2. MATERIAL AND METHODS

2.1. Materials

2.1.1. Culture media

See Appendix A for culture media used in this study (refer to page 148).

2.1.2. General buffers, stock solutions, and antibiotics

See Appendix A for general buffers, stock solutions, and antibiotic used in this study (refer to page 150).

2.2. Experimental strategy

PCR amplification was used to propagate the gene sequences used for the construction of various encoding plasmid vectors. *Nef1-70* (1-70 amino acid of HIV-1 Nef protein), *nef*206 (encoding HIV-1 Nef full length protein), *gfp*NS (encoding GFP but without stop codon) and *gfp* (encoding full length green fluorescent protein) were PCR amplified from pQBI-Nef1-70GFP (Ali *et al*. 2010; Shelton *et al.*, 2012). The *vif* gene was PCR amplified from pNL4.3 plasmid. The VSV-G gene was PCR amplified from pCMV-VSV-G plasmid. For cloning purpose, the genes were PCR amplified using either KAPA Hifi DNA polymerase (KAPA Biosystem) or *Pfu* DNA polymerase (Thermo Scientific, #EP0571). The number of PCR amplification cycles was kept below 25 to minimize the chances of PCR-mediated mutations. PCR-amplified DNA was resolved on either 0.7% (vector) or 1.5% (insert) agarose gel using TAE as running buffer and then purified using PCR Clean-up kit (Macherey-Nagel, #740609). The purified DNA (insert or vector) was treated with restriction enzymes for optimal time and temperature. The linearised vector DNA was further dephosphorylated using 1 µL of shrimp alkaline phosphatase (rSAP, New England BioLabs (NEB)) at 37°C for 1 hour to prevent self-ligation. The restricted insert and vector DNA were purified using NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, #740609). Then the DNA concentration was quantified using Qubit® dsDNA HS Assay Kit (Life technologies Q32851). The vector and insert DNA were mixed in 1:3 ratios and ligated using 1µL of T4 ligase (NEB). The ligation mixture was incubated at 16°C for 8-12 hours.

The parent vector pEF1α-IRES contained beta lactamase (*bla*) gene for the selection of positively transformed bacterial colonies on LB agar plate containing 100 µg/mL Ampicillin. Ligation products were transformed into DH5α competent cells by heat shock. The DH5α cells were spread on LB agar plates containing 100 µg/mL Ampicillin and incubated at 30°C overnight (16-18 hours). Ten positively-transformed bacterial colonies were selected for colony PCR to verify the presence of correct insert. The vector and insert-specific primer were used for colony PCR. The bacterial colonies positive for expected amplicon were

grown in LB broth containing Amplicillin at 30°C overnight. The plasmid was isolated using NucleoSpin® Plasmid Extraction Kit (Macherey-Nagel, #740588.250) and verified by appropriate restriction enzymes. Upon verification, the DH5α cells transformed with desired plasmid were grown in 100 mL of LB broth containing Amplicillin at 30°C overnight. The expression plasmid was purified using Endotoxin-free Plasmid Extraction Kit (Macherey-Nagel, #740420). The expression plasmid was concentrated using phenol chloroform extraction and ethanol precipitation, adjusted to 1 μg/μL in dH_2O and stored at -20°C.

Endotoxin-free plasmid was transient transfect into HEK293 or 293T cells. The supernatant (which contained exosome) was collected at 24 hours and 72 hours. The transfected cells were harvested at 72 hours to analyze transcription or replication of the transfected gene. The culture medium was collected and centrifuged by differential ultracentrifugation to isolate and purify the exosome.

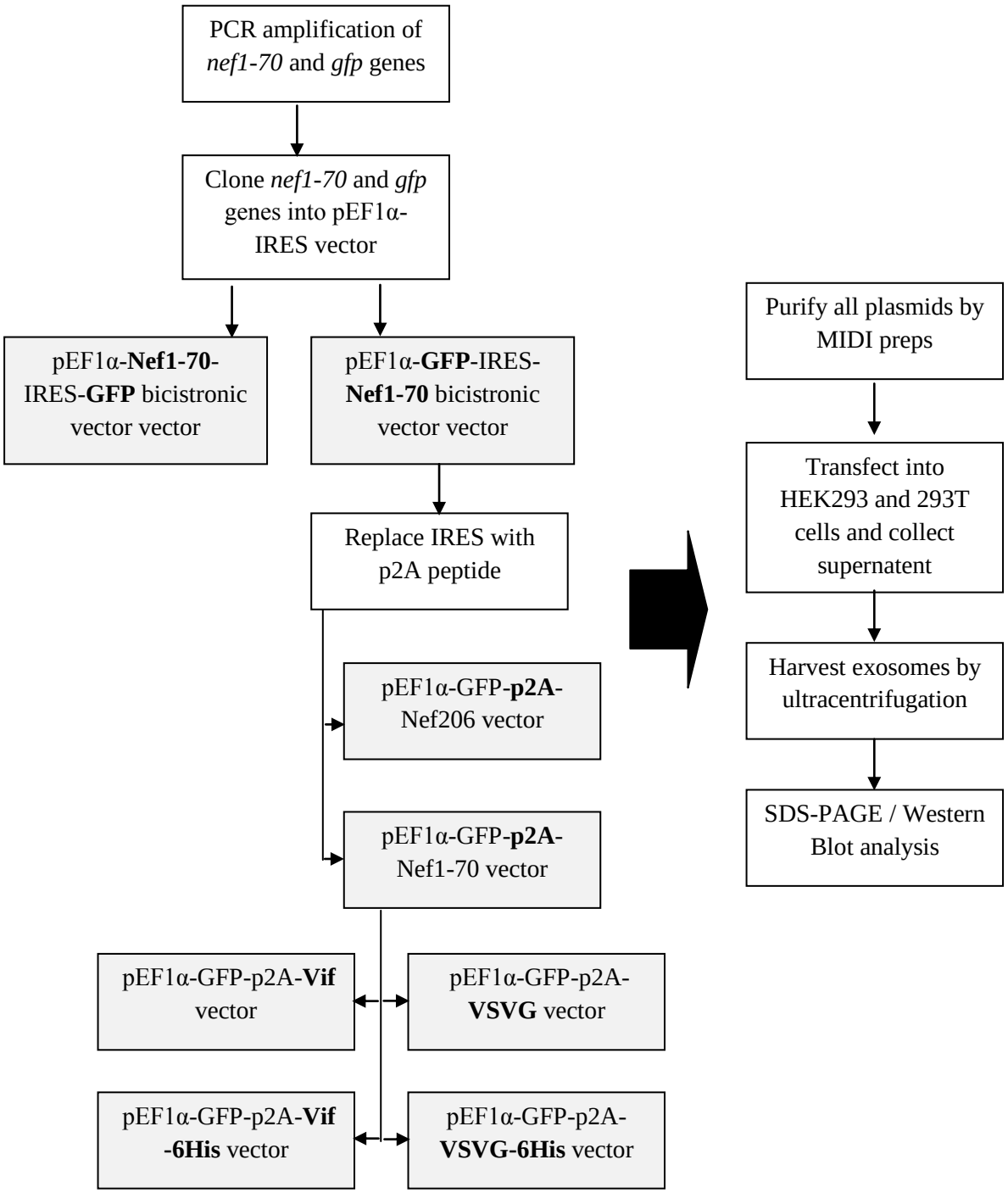

Figure 2.1. Experimental overview of this study

2.3. Methods

2.3.1. Bacterial strains and culture conditions

The DH5α *E.coli* (NEB, #C2987H) strain was used to transform plasmid vectors. *E.coli* was maintained on Luria Bertani (LB) agar or broth without any antibiotic. The competent *E.coli* DH5α were prepared as follows:

2.3.1.1. Preparation of competent cells

First, 0.5 M piperazine-1,2-bis[2-ethanesulfonic acid] (PIPES) was prepared by adding 15.1 g of PIPES in 80 mL of ddH_2O water. Then, the pH was adjusted to pH 6.7 using 5M KOH. The volume was made to 100 mL using ddH_2O water. The buffer was sterilized using syringe filters (0.45 µm, Sartorius Stedim Minisart® Syringe Filters). PIPES buffer was aliquoted in 20 mL and stored at -35°C. The inoue transformation buffer was prepared by mixing the following chemicals: 10.88 g of $MnCl_2.4H_2O$, 2.2 g of $CaCl_2.2H_2O$, 18.65 g of KCL and 20 mL of PIPES (0.5 M, pH 6.7) in 800 mL of ddH_2O water. The final volume was adjusted to 1 L with ddH_2O water. The Inoue transformation buffer was filter sterilized using 0.45 µm and stored in 50 mL aliquots at -80 °C.

A single colony of DH5α bacterial was used initiate to a starter culture in 10 mL of SOB medium in a 100 mL Schott bottle. The culture was incubating for 8 hours at 37°C while shaking at 250 rpm. The starter culture was then transferred into three separate 1 L flasks, each containing 100 mL of SOB medium. The first flask received 4 mL of starter culture, the second flask received 1.6 mL and the third flask received 0.8 mL. Those flasks were incubated at room temperature (22°C - 25°C) with moderate shaking (80 rpm) overnight. The optical density culture in three flasks was at 600 nm by using spectrophotometer. The culture with 0.4-0.5 OD_{600} was transferred to an ice-water bath and incubated for 10 minutes. The other two cultures were discarded. The bacterial cells were harvested by centrifugation at 2500X g for 15 minutes at 4°C. The SOB medium was decanted and the pellet was dried for 5 minutes by inverting the tubes on a tissue paper. The cells were gently resuspended in 32 mL of ice-cold Inoue transformation buffer and centrifuged at 2,500X g for 15 minutes at

4°C. The supernatant was removed completely using vacuum aspirator. Then, 8 mL of ice-cold Inoue transformation buffer was used to resuspend the cells gently. Then, 0.6 mL of DMSO was added and mixed by swirling. The cells were than incubated in wet ice for 10 minutes. The cells were the added into autoclaved and UV-sterilized 0.5 mL pre-chilled microcentrifuge tubes. Three hundred microlitter competent cells were frozen in liquid nitrogen and stored at -80°C.

2.3.1.2. Short-term storage of bacterial strains

Sixteen to eighteen hour old agar plates were wrapped with parafilm to prevent dehydration and contamination and stored at 4°C for up to 1 month.

To recover the *E.coli*, bacteria colonies were inoculated in 2-5 mL LB broth (with or without selection antibiotic). Bacteria were then cultured at 30°C for 12-16 hours with shaking (250 rpm).

2.3.1.3 Long-term storage of bacterial strains

For storage, 1 mL of 8-12 hours old bacteria culture was added into 500 µL of LB broth / 60% glycerol. The total culture was split into two 1.5 mL micro-centrifuge tubes and stored at -80°C for up to 1 year.

For recovery, the bacterial glycerol stock was placed on wet ice immediately after removal from -80°C freezer. A sterile wire loop was used to transfer frozen chips from the glycerol stock and spread on a LB agar plate (with or without selection antibiotics). The agar plate was then incubated at 30°C for 18 hours until colonies were visible.

2.3.2. Primer Design

All oligonucleotides were obtained from Integrated DNA Technologies (IDT). Primers were designed using the Vector NTI Advance® Sequence Analysis software. The lyophilised oligonucleotides were dossolved in Tris - HCl (pH 8.0, 10 mM) to a final concentration of 10 µM. working stock were prepared by adding 100 µM stock to 10 µM stock.

Several factors were considered during primer design. Primers lengths were kept within 18-30 nucleotides long. The melting temperatures (T_m) of forward and reverse primers were adjusted to within 5°C of each other. The GC contents of the primer were kept between 40-60%. Besides that, the 3'- ends of primers were preferentially C or G to avoid formation of secondary intermolecular structures that affect the amplification step during PCR (Frey *et al*. 2008). Three to four nucleotides were also added 5' to the restriction site as that would increase the restriction enzyme efficiency. Finally, extra care was taken to avoid intra-primer homology or inter-primer that could lead to self-dimers and primer-dimers.

Table 2.1. Oligonucleotides for plasmid construction

Primer Name	Sequence (5' → 3')	Tm (°C)
Nef1-70-*Nhe*I-F	TGAC**GCTAGC**ATGGGTGGCAAGTGGTCAA	60.7
Nef1-70-*Mlu*I-R	GCGC**ACGCGT**TCAATGGTGATGGTGGTGATGAGCTGCGACTG GAAAACCCACCTCTTC	58.5
GFP-*Xba*I-F	GTCC**TCTAGA**ATGGCAAGCAAAGGAGAAGAACTCTTCAC	56.9
GFP-*Not*I-R	GGAA**GCGGCCGC**TCAGTTGTACAGTTCATCCATGC	52.2
GFP-*Nhe*I-F	GTCC**GCTAGC**ATGGCAAGCAAAGGAGAAGAACTCTTCAC	56.9
GFP-*Mlu*I-R	GGAA**ACGCGT**TCAGTTGTACAGTTCATCCATGC	52.2
Nef1-70-*Xba*I-F	TGAC**TCTAGA**ATGGGTGGCAAGTGGTCAA	60.7
Nef1-70-*Not*I-R	GCGC**GCGGCCGC**TCA**ATGGTGATGGTGGTGATG**AGCTGCGAC TGGAAAACCCACCTCTTC	58.4
GFP-*Nhe*I-F	TAG**GCTAGCATG**GCAAGCAAAGGAGAAGAAC	54.2
GFPNS-*Mlu*I-R	ACC**ACGCGT**GTTGTACAGTTCATCCATGCCATG	55.8
Nef1-70-*Xba*I-F	TGAC**TCTAGA**ATGGGTGGCAAGTGGTCAA	60.7
Nef206-*Not*I-R	GGAA**GCGGCCGC**TCAGTGATGGTGATGGTGATGAGCTGCG CAGTTCTTGAAGTACTCCGG	50.8
P2A-F	CGCGTGGTAGTGGTGCTACTAATTTTTCTCTTCTTAAACA AGCTGGTGATGTTGAAGAAAATCCTGGTCCTT	-
P2A-R	CTAGAAGGACCAGGATTTTCTTCAACATCACCAGCTTGTT TAAGAAGAGAAAAATTAGTAGCACCACTACCA	-
*Mlu*I-P2A-F	CACGCGTGGTAGTGGTGCTAC	55.5
pEF1-R	GCATTAACCCTCACTAAAGGG	50