การแก้ไขการกลายพันธุ์ของยีนที่เกี่ยวข้องกับการเกิดโรค Wiskott-Aldrich syndrome โดยวิธี zinc finger nucleases

นายประมุข อัมรินทร์นุเคราะห์

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TARGET EDITING OF THE WISKOTT-ALDRICH SYNDROME (WAS) GENE USING ZINC FINGER NUCLEASES

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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ประมุข อัมรินทร์นุเคราะห์ : การแก้ไขการกลายพันธุ์ของยีนที่เกี่ยวข้องกับการเกิดโรค Wiskott-Aldrich syndrome โดยวิธี zinc finger nucleases. (TARGET EDITING OF THE WISKOTT-ALDRICH SYNDROME (WAS) GENE USING ZINC FINGER NUCLEASES) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. พญ. ดร. กัญญา ศุภปีติพร, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. นพ. วรศักดิ์ โชติเลอศักดิ์, 73 หน้า.

Wiskott -Aldrich syndrome (WAS) เป็นโรคทางพันธุกรรมที่ถ่ายทอดแบบยีนด้อยบนโครโมโซม X (X-linked recessive) ซึ่งมีลักษณะอาการประกอบด้วยภาวะเกล็ดเลือดต่ำ เกล็ดเลือดมีขนาดเล็ก ติดเชื้อบ่อยครั้ง ผื่นบริเวณผิวหนัง และมีความเสี่ยงต่อการเกิดภาวะภูมิแพ้ตนเองและโรคมะเร็งในระบบโลหิต โดยพบว่ามีสาเหตุจากการกลายพันธุ์ของยีนที่สร้างโปรตีน WAS (WASP) ซึ่งทำหน้าที่ควบคุมโครงสร้าง actin และ chromatin โปรตีน WASP มีการแสดงออกในเซลล์ต่างๆ ของระบบโลหิต ผู้ป่วยโรค WAS ชนิดรุนแรงมักเสียชีวิตภายในช่วงอายุ 10 ปีแรกจากภาวะติดเชื้อหรือมีเลือดออกมากจากการมีเกล็ดเลือดต่ำ การปลูกถ่ายไขกระดูก เป็นการรักษาวิธีเดียวเท่านั้นที่จะทำให้ผู้ป่วยโรค WAS หายขาดจากโรคได้ อย่างไรก็ตาม จากความยากลำบากในการหาผู้บริจาคไขกระดูกที่มี HLA เข้ากันได้กับผู้ป่วย และภาวะแทรกซ้อนที่เกิดขึ้นจากการปลูกถ่ายไขกระดูก ได้กระตุ้นให้เกิดการพัฒนาการรักษารูปแบบใหม่ในระดับยีน (gene therapy) ด้วยการปรับปรุงและพัฒนาเทคนิคการสร้างเซลล์ต้นกำเนิดเม็ดเลือดจากเซลล์ต้นกำเนิดชนิด induced pluripotent stem cells (iPS) ซึ่งสร้างมาจากเซลล์ร่างกายของผู้ป่วย และความก้าวหน้าในวิธีการแก้ไขพันธุกรรม (gene targeting) ทำให้การใช้เซลล์ต้นกำเนิดเม็ดเลือดที่สร้างมาจากเซลล์ต้นกำเนิดชนิด iPS จากผู้ป่วยซึ่งได้รับการแก้ไขการกลายพันธุ์แล้วอาจจะเป็นอีกทางเลือกหนึ่งในการรักษาโรคที่มีประสิทธิภาพ การวิจัยครั้งนี้ได้นำเสนอความสำเร็จในการแก้ไขการกลายพันธุ์ในเซลล์ iPS ของผู้ป่วยโรค WAS โดยใช้วิธี zinc finger nuclease ซึ่งผลการทดลองได้เซลล์ iPS จำนวน 8 โคลนที่ได้รับการแก้ไขการกลายพันธุ์และพบว่าไม่มีการเกิด random integration และเซลล์ที่ได้รับการแก้ไขการกลายพันธุ์เหล่านี้สามารถนำไปทดสอบเพิ่มเติมก่อนการนำไปใช้ในจริงในทางคลินิก การศึกษาครั้งนี้เป็นก้าวหนึ่งที่สำคัญในการพัฒนาการรักษาโรค WAS โดยใช้หลักการ gene targeting เพื่อแก้ไขการกลายพันธุ์ในยีน WASP ในเซลล์ iPS ของผู้ป่วย

สาขาวิชา ชีวเวชศาสตร์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

5287859820 : MAJOR BIOMEDICAL SCIENCES
KEYWORDS: WISKOTT-ALDRICH SYNDROME / ZINC FINGER NUCLEASES / GENE TARGETING / HOMOLOGOUS RECOMBINATION / INDUCED PLURIPOTENT STEM CELLS

PRAMUK AMARINTHNUKROWH: TARGET EDITING OF THE WISKOTT-ALDRICH SYNDROME (WAS) GENE USING ZINC FINGER NUCLEASES. ADVISOR: ASSOC. PROF. KANYA SUPHAPEETIPORN, M.D. Ph.D.,PROF. VORASUK SHOTELERSUK, M.D., 73 pp.

Wiskott-Aldrich syndrome (WAS) is an X-linked recessive disorder characterized by thrombocytopenia with small-sized platelets, recurrent infections, eczema, and increased susceptibility to autoimmune diseases and hematologic malignancies. It is caused by mutations in the gene encoding the WAS protein (WASP), a regulator of actin cytoskeleton and chromatin structure. The WASP is expressed in all hematopoietic lineages. Patients with severe WAS mostly die from infection or bleeding within the first decades of life. Hematopoietic stem cell transplantation (HSCT) remains the only curative therapy for WAS. The difficulty in finding HLA-matched donors and complications related to transplantation urge the development of gene therapy strategies for WAS. With the improvement in techniques for generating clinical-grade hematopoietic stem cells from induced pluripotent stem cells (iPSCs) and advances in gene targeting strategies, gene-corrected hematopoietic stem/progenitor cells derived from the patient iPSCs could be an effective alternative for treatment of severe immunodeficiency disorders. Here we reported on the successful generation of the genetically-corrected patient-specific WAS-iPSCs using zinc finger nuclease-based strategies. A total of eight corrected WAS-iPSC clones were obtained and revealed no random integration. These corrected WAS-iPSCs could be further validated prior to use in a clinical setting. This study is one of the most important steps in using gene targeting strategies to precisely correct the WASP mutation in patient-derived iPSCs.

Field of Study: Biomedical Sciences
Academic Year: 2014

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

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CHAPTER I
INTRODUCTION

Background and rationale

Wiskott-Aldrich syndrome (WAS) is a rare X-linked recessive disease. The prevalence of Wiskott-Aldrich syndrome is approximately 1-10 in 1 million live births.[1] It was first found to be inherited in an X-linked recessive manner in a Dutch-American family in 1954 by Dr. Robert Anderson Aldrich.[2] In 1994, Derry et al. found that WAS was caused by mutations in the *WASP* gene which was located on the short arm of the X chromosome.[3]

The *WASP* gene is located on chromosome Xp11.23. It contains 12 exons and encodes 502 amino acids. The Wiskott-Aldrich syndrome protein (WASP) is mainly expressed in hematopoietic stem cells (HSCs) and HSC lineages.[4] Its exact function remains unknown and is under investigation. Previous studies have suggested that WASP is a key regulator of the actin cytoskeleton that controls several immune cell function, such as migration, activation, proliferation, and antigen uptake.[5] Mutations in the *WAS* gene therefore lead to defective function of WASP resulting in complex immunodeficiency.

Clinical manifestations of WAS include thrombocytopenia (low platelet count), immune deficiency, recurrent infections, and malignancies (lymphoma and leukemia). The patients can sometimes develop eczema, bleeding, and autoimmune disorders.[6] The disease onset occurs early in childhood, during the first decade and usually before the age of 3 years. Without appropriate treatment, they will die at an early age. Around 55-60% of patients die from infection, 24-27% die from bleeding, and 5-10% die from malignancy.[7]

WAS can be diagnosed on the basis of clinical parameters. Some patients have low levels of immunoglobulin which may cause recurrent infections. Some patients may develop autoimmune diseases. Skin immunologic testing (allergy testing) may reveal hyposensitivity. Currently, diagnosis of WAS can be confirmed by mutation analysis of the *WASP* gene.[8]

For therapeutic approaches, allogeneic hematopoietic stem cell transplantation (HSC) has been the only curative therapy for WAS. However, this therapeutic strategy has obvious obstacles including complications related to transplantation and difficulty of finding HLA-matched donors.[9] From these obstacles, the patient's own cells will be the best cell source for transplantation.

Currently, autologous stem cell transplantation for treatment of several genetic diseases become possible as the patient's own cells can be induced to induced pluripotent stem (iPS) cells. This cell source can eliminate the obstacles of using allogeneic stem cell transplantation. The patients' fibroblasts can be reprogrammed to pluripotent stem cell-like stage by transfection of the cells using the reprogramming genes such as *Oct4*, *Sox2*, *Klf4*, and *C-Myc*.[10]

Gene therapy has been established and developed for treatment of several genetic diseases including WAS. In 2010, Boztug et al. successfully performed hematopoietic stem cell gene therapy for WAS by using a retroviral vector. In additional, Aiuti et al. also recently performed WAS gene therapy using hematopoietic stem cell with lentiviral vector.[11] After gene therapy, the patients' immune cells were functionally corrected and the clinical condition significantly improved. However, the limitation of this approach is vector integration which may disrupt some endogenous genes and cause unpredictable phenotype such as cancers.[12] Therefore, the issues of random integration, appropriate viral vector design, optimal levels of WASP expression in each hematopoietic lineage, and prevention of transgene silencing need to be solved before it could become a safer and more effective therapy.

Zinc finger nucleases (ZFNs) can be used for efficient genetic modifications in several organisms including human cells. There have been several studies demonstrating that diseases with causative mutations can be successfully corrected by ZFNs including sickle cell anemia[13], X-linked SCID[14], α-antitrypsin deficiency[15], hemophilia B[16]. Moreover, HIV clinical trials using the ZFN-based approach are also underway.[17]

This study is the first time in Thailand to use the ZFN strategy in iPS cells of a patient with WAS. This study is one of the important milestones towards the application of ZFNs in correction of disease phenotype and in stem cell-based therapy.

Key word

Wiskott-Aldrich syndrome, zinc finger nucleases, gene targeting, homologous recombination, induced pluripotent stem cells

Abbreviation

WAS, Wiskott-Aldrich syndrome; ZFNs, zinc finger nucleases; HR, homologous recombination; iPS cells, induced pluripotent stem cells

Research question

Can the zinc finger nuclease-based strategy be used for mutation correction of the *WASP* gene in iPS cells of a patient with WAS?

Hypothesis

The ZFN-based strategy can be used to correct a *WASP* gene mutation in a patient with WAS.

Objective

1. To correct the identified mutation in iPS cells obtained from the WAS patient by the ZFN-based strategy

2. To maintain and prepare the corrected iPS cells for hematopoietic stem cell therapy

Research design

Descriptive and *in vitro* studies

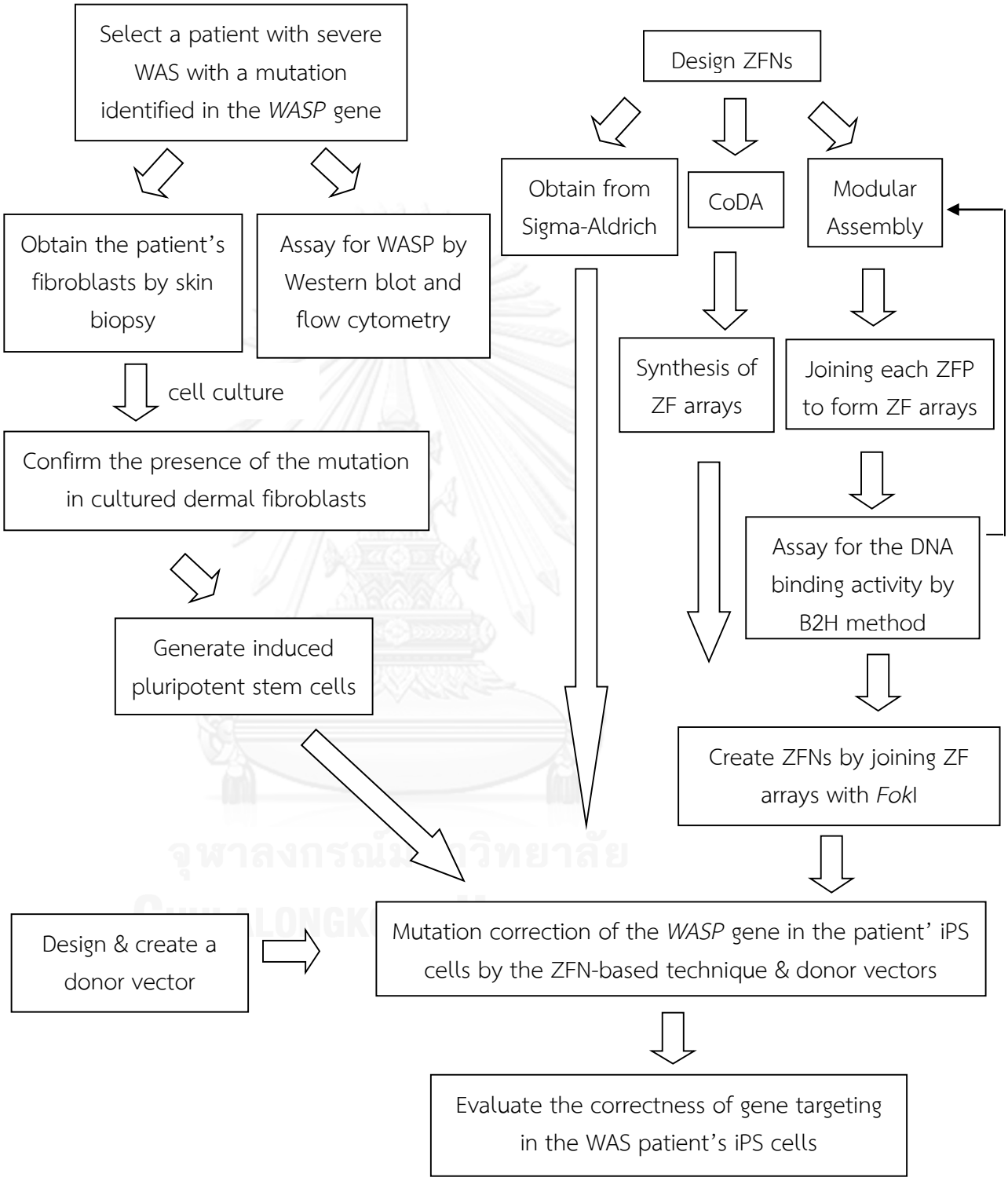
Ethical consideration

This study has been approved by the local Ethics Committee. Written informed consent was obtained from the parents of a patient who participated in the study.

Expected benefit and application

This study will generate ZFNs that result in mutation correction of the *WASP* gene in a patient's iPS cells. It is one of the important steps towards the application of ZFNs in correction of disease phenotype and in stem cell-based therapy.

Conceptual Framework



CHAPTER II

BACKGROUND AND LITERATURE REVIEW

Hematopoietic stem cells and induced pluripotent stem cells

Autologous stem cell transplantation is an alternative strategy that has been developed for patient-specific therapy. There are two potential sources for autologous stem cell transplantation.

The first is hematopoietic stem cells (HSCs). HSCs are multipotent stem cells that can differentiate into all blood cell types. HSCs are generally obtained from the bone marrow. A smaller numbers of HSCs can be also collected from peripheral blood. However, this cell source is still difficult to maintain in a pluripotent stage for a long term. In addition, these cells are difficult to be isolated as a single clone because they usually differentiate into other cell types.

The second is induced pluripotent stem (iPS) cells. Based on the success of reprogramming cells, the first report of generating iPS cells from somatic cells was published by Takahashi and Yamanaka in 2006. They introduced twenty four candidates, pluripotency-associated genes into mouse embryonic fibroblasts (MEFs). After culture on feeder layers in ES medium, they found that the MEFs had ESC-like gene expression, proliferation and morphology, suggesting that the MEFs were transformed into ESC-like stage. After narrowing down the essential associated gene factors, they identified four factors, namely Oct4, Sox2, Klf4, c-Myc which were important for reprogramming. These four essential factors are often referred to "Yamanaka factors".[10, 18-20]

The iPS cells can be derived from somatic cells, such as adult dermal fibroblasts. The adult cells can be reprogrammed by transfection of certain stem cell-associated genes such as Klf4, Oct4, c-Myc, Sox2. After 3–4 weeks, small numbers of transfected cells begin to become morphologically and biochemically similar to pluripotent stem cells. These iPS cells can be maintained for a long term and isolated as a single clone.

Currently, the iPS cells can be differentiated into many cell types including neurons[21, 22], cardiomyocytes[23-25], hepatocytes[26-28], hematopoietic cells[29, 30] (figure 1). From the ability similar to ESCs, iPSCs have been developed and therefore become new tools for conducting rapid high-throughput drug screening[31, 32], toxicity testing[32, 33] developing effective gene targeting strategies, and addressing the species-specific features[34, 35].

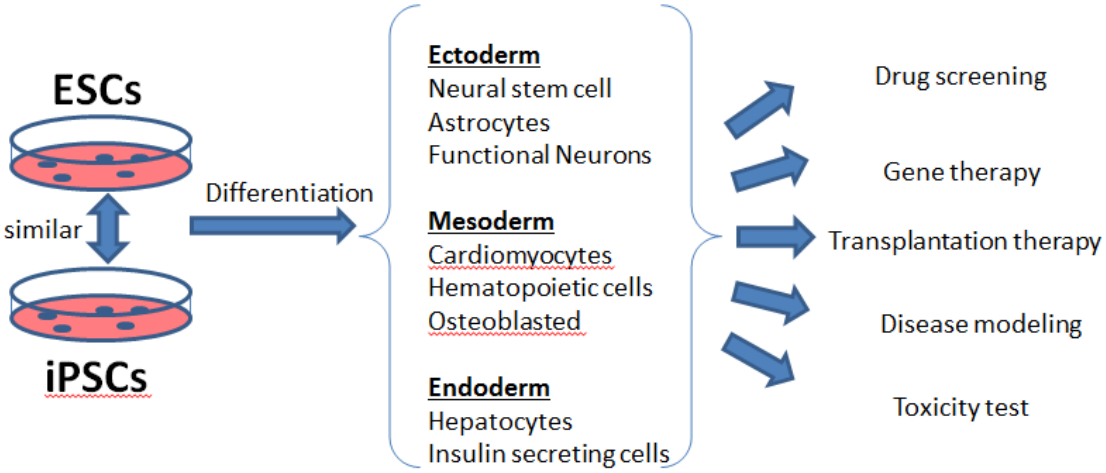


Figure 1 Differentiation and Application of embryonic stem cells and induced pluripotent stem cells.

Hematopoietic stem cell transplantation & gene therapy for WAS

A recent report in 2010 demonstrated that hematopoietic stem cell gene transfer for WAS could be used successfully in correction of early hematopoietic progenitor cells. However, preferential targeting of genes including potential oncogenes associated with the use of retrovirus-based stem-cell gene transfer still existed.[36] In addition, a recent report provided evidence supporting the risk of using retroviral vectors. The outcome of gene therapy in nine patients with X-linked severe combined immunodeficiency was revealed. After almost 10 years of follow-up, the study found that one patient (11%) died from leukemia in 60 months after gene therapy, and three patients (33%) developed acute leukemia and were treated with chemotherapy.[37]

With all the limitations associated with gene therapy using viral gene transfer, several approaches have been developed for targeted gene editing. The Zinc finger nucleases (ZFNs) strategy has been used successfully in genome editing of human cells

by Porteus M.H.P and Baltimore. They used ZFNs to stimulate gene targeting and convert mutant GFP reporter genes back to normal GFP in human embryonic kidney cell lines (HEK293).[38] Subsequently there were several studies showing that ZFN-based strategies could be used to modify the genome. For example, Urnov FD, et al. used ZFNs to create a novel restriction enzyme recognition site in exon 5 of IL2Rγ in K562 cells. They found that 18-21% of K562 cells showed conversion in exon 5 of IL2Rγ and this conversion in genome remained stable more than one month after transfection. Moreover, misintegration was not found in these cells.[14] Currently, there are several reports showing that diseases with causative mutations can be successfully corrected by ZFNs such as sickle cell anemia[13], α-antitrypsin deficiency[15], and hemophilia B[16]. In addition, these studies demonstrated that ZFN-based strategies could be used for specific editing of the human genome with more safety comparing to virus-based strategies.

Zinc finger nucleases

ZFNs are artificial restriction enzymes that are generated by fusing zinc finger proteins (DNA-binding domain) to an endonuclease (DNA-cleavage domain).

For specificity of the zinc finger, it can be engineered to target a desired DNA sequence which enables zinc-finger nucleases to target unique sequence within a complex genome.

Individual zinc finger protein can be called “module”. It contains a DNA-binding domain. Each zinc finger array is composed of three to six modules and can recognize between 9-18 base pairs of DNA sequences in a mammalian genome. Various strategies have been developed to engineer and select optimal zinc fingers to bind the desired sequences.[39] These include both "modular assembly" and “Context–Dependent assembly (CoDA)” algorithms. Each individual zinc finger is optimized against the target DNA triplet sequence, and linked together to form three or four ZFP-arrays against 9 or 12-bp sequence.[40]

The DNA-cleavage domain of ZFNs is from non-specific cleavage restriction endonuclease (*Fok*I). The two individual ZFNs must bind to opposite strands of DNA on each half-site in order to allow endonucleases to dimerize and cleave specific DNA

sequence at the spacer region (figure 2B).[41] The composition of ZFNs can be summarized in figures 2A and B.

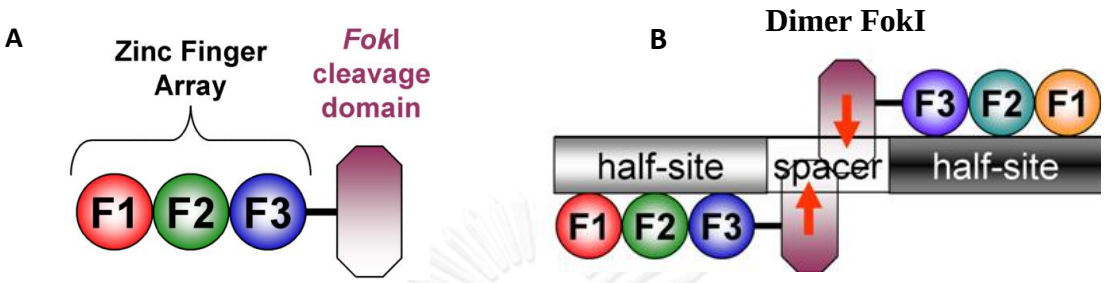


Figure 2 (A) A zinc finger nuclease is composed of three zinc finger proteins joining together with endonuclease (FokI). Each zinc finger array can recognize 9 bp in specific genome (half-site). (B) A pair of zinc finger nucleases bind to their target site. [42]

Mechanism of ZFNs and DNA repairing process

ZFNs can have 3 to 6 individual zinc-finger domains arranged to recognize a target site of 9 to 18 base-pair long. Additionally, the *Fok*I nuclease domain functions as a dimer. Therefore, a pair of ZFNs must be engineered to bind the target site in a way that permits the nuclease domains forming a dimer (figure 1B) and create the double-strand breaks (DSBs). Then DSBs will be repaired by cell repairing processes such as non-homologous end-joining (NHEJ), and homologous recombination (HR). Repairing of ZFN-induced DSBs by non-homologous end-joining occurs immediately after DNA is damaged. This process can repair the break without homologous template. Therefore it can lead to the introduction of mutagenic insertions or deletions (indels) with high frequency that can be used to create knockout or deletion in some genes. In addition, DSBs created by ZFNs also stimulate homologous recombination-mediated repair that is important for gene therapy. Therefore, ZFNs can be used to induce high frequency gene targeting by introducing a homologous "donor DNA template" harboring investigator-specified mutations or insertions into cells (figure 3).[43]

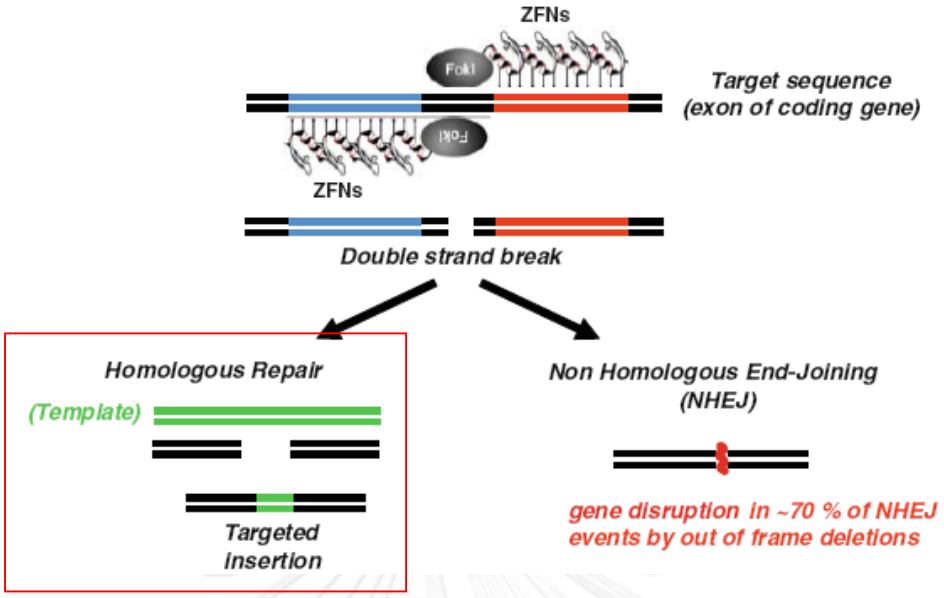


Figure 3 ZFNs can create DSBs in a specific target site and induce DNA repairing process such as NHEJ and HR.[43]

CHAPTER III

METHODOLOGY

Experimental Procedure

Subjects and sample collection

The study subject was a patient with the most severe form, classic Wiskott Aldrich Syndrome (WAS). He presented with recurrent mucous bloody diarrhea, chronic eczema and thrombocytopenia. The patient was hemizygous for a mutation in exon 12 of the *WASP* gene. It changed T to A at nucleotide 1507 (c.1507T>A) resulting in changing the termination codon to arginine (p.X503R). This mutation is expected to result in elongation of mRNA and protein degradation.[44]

Skin fibroblast collection

After informed consent was obtained, skin fibroblasts were collected from the WAS patient by skin biopsy (figure 4), and were maintained by the standard protocol of cell culture.

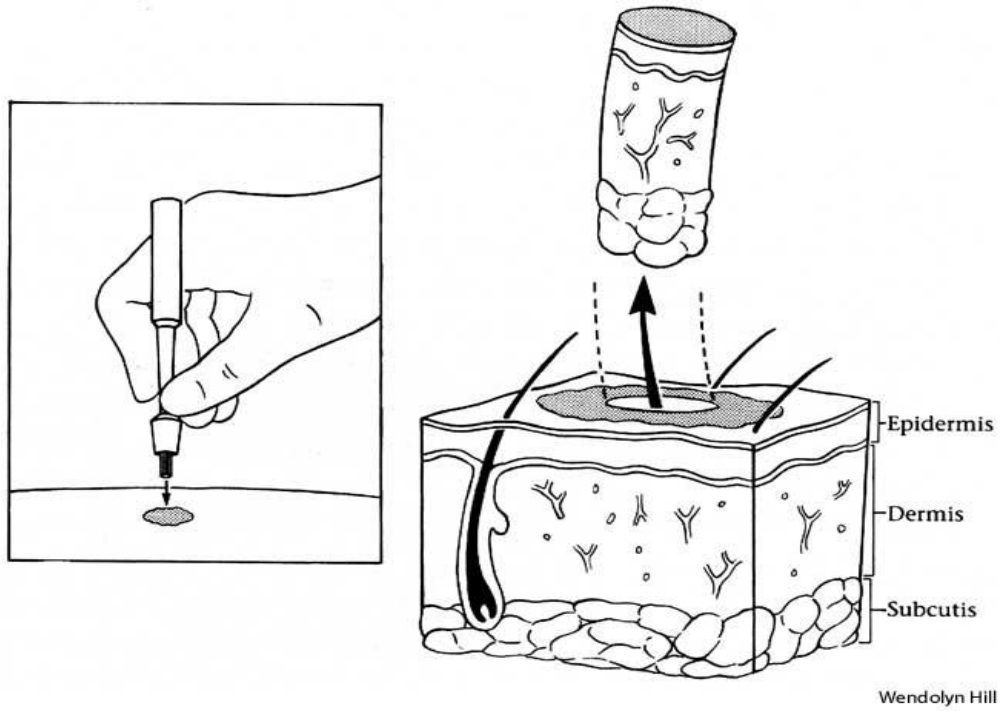


Figure 4 Punch biopsy, a core of tissue is obtained by using a sharp and circular instrument.[45]

WASP mutation analysis

DNA extraction

Genomic DNA was extracted from the patient's fibroblasts using QIAamp®DNA Blood Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instruction.

DNA amplification and sequencing

WASP exon 12 was PCR-amplified and sequenced as previously described to confirm the presence of c.1507T>A (p.X503R) in the patient's cells.[44] Sequence data were analyzed using Sequencher version 5.0 (Gene Codes Corporation, Ann Arbor, MI).

Protein expression analysis

Western blot

PBMCs were isolated from the patient's blood by Ficoll–Hypaque density gradient centrifugation and were washed with phosphate-buffered saline (PBS). Protein in PBMCs was extracted at 4°C by sonication in RIPA buffer. The protein concentration was determined by Micro BCA™ Protein Assay kit (Thermo Scientific Bremen, Germany) to the manufacturer's instruction. 40 micrograms of total protein lysates were mixed with 2X Laemmli buffer pH 6.8 (loading dye) and were boiled at 95°C for 5 minutes. The protein lysates were loaded onto 10% sodium dodecyl-sulfate polyacrylamide gel and were run at 110 volts for 2 hours. The samples in the gel were transferred to polyvinylidenedifluoride (PVDF) membrane by iBlot® Dry Blotting System (Invitrogen Corporation, Carlsbad, CA). The PVDF membrane was incubated 1 hour with 5% non-fat milk in TBST for blocking. After that, it was incubated overnight with 1:200 anti-WASP monoclonal antibody raised against a recombinant protein corresponding to the N-terminal region of human WASP (B-9; Santa Cruz Biotechnology, Santa Cruz, CA). Then, PVDF was washed in TBST for three times, and incubated with 1:1000 secondary goat anti-mouse IgG2a-HRP (sc-2005; Santa Cruz Biotechnology) at least 2 hours. The protein in PVDF could be detected by SuperSignal West Pico Chemiluminescent Substrate kit (Thermo scientific). GAPDH was used as a control for protein loading.

Generation of zinc finger nucleases

Construction of zinc finger protein

ZFNs can be designed efficiently to target specific sequences using algorithm programs such as Modular Assembly, OPEN, and CoDA. They are available in the ZiFiT online-software on the website: www.zincfingers.org. For this experiment, ZFNs were designed from Modular Assembly and CoDA algorithms.

Modular Assembly

This is the original algorithm to design ZFNs. This algorithm can select individual zinc finger protein (ZFP) from a zinc finger pool which is optimized against the target triplet DNA sequences. Then each ZFP can be linked together to form three- or four-ZFP array against 9- or 12-bp sequences as shown in figure 5.[43]

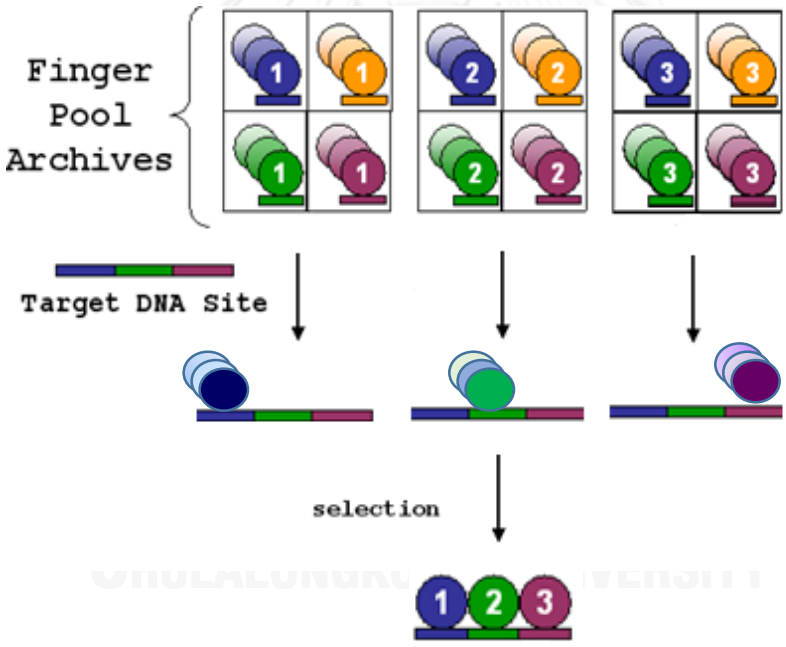


Figure 5. The modular assembly method for designing an artificial zinc finger through the sequential panning of a phage library for each of the three fingers.

For assembly, the desired *WASP* sequences (~1000 bp) were submitted online and the modular assembly algorithm of the ZiFiT software was used. This algorithm can search possible target sites on the submitted sequences and ZFP candidates from

the zinc finger pool. ZFPs that had high specificity recognition and located near the target position (<100 bp) were selected. The ZFPs were assembled together to generate a zinc finger array.

ZF1 (module one), ZF2 (module two), and ZF3 (module three) can be assembled respectively. The ZF vector module one and two were assembled first by double digestion with restriction enzyme *Age*I, *Xma*I and *Bam*HI. Both ZF modules were subsequently ligated by ligase. Finally, ZF module three was assembled with the module one and two by the same method.[42] This was summarized in figure 6.

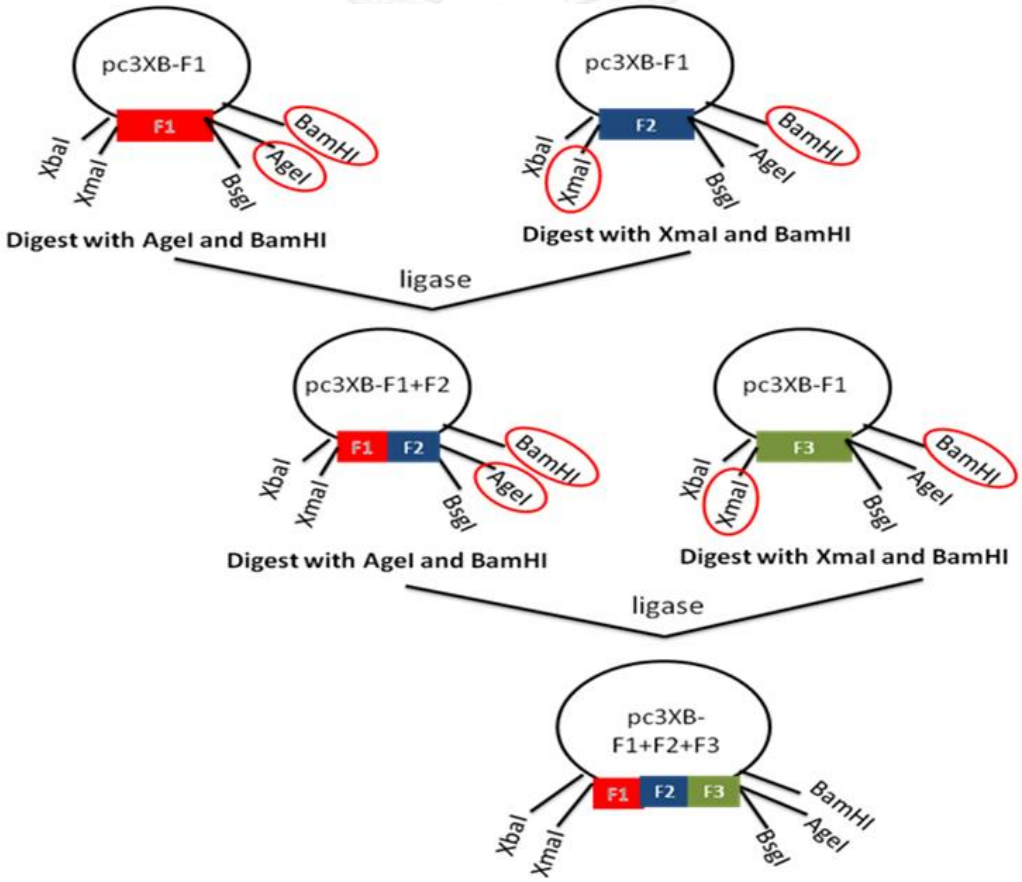


Figure 6. The process of creating a zinc finger array construct from individual zinc finger vectors by using restriction enzyme and ligase.[42]

After ligation, the ZFP array vectors were transformed into *E.coli* XL1-Blue strain and incubated 16-18 hours at 37°C for growing. The transformed bacteria were selected by ampicillin resistance. After 18 hours, some bacterial colonies were randomly picked to determine the correctness of ZFP assembly by PCR with specific primers and

sequencing (ZF primer-F and R). The primers and PCR condition were performed as shown in table1. The assembly of each zinc finger was determined from the size of PCR products.

Context –Dependent assembly (CoDA)

This algorithm is developed further from modular assembly to eliminate the context-dependent effect between adjacent fingers. The CoDA does not evaluate zinc fingers as independent modules, but it includes evaluation of the adjacent fingers as context dependent. Three zinc finger modules are therefore predicted by selection of F1 and F3 units from other validated arrays that share the same common middle finger (F2 unit). These three modules are then combined together to form a CoDA ZF-array. This approach is described in figure7.

This approach has been used with high success rate to generating active zinc finger nucleases. With this method, the B2H assay step can be skipped and the CoDA ZF array can be directly tested in the cells.[46]

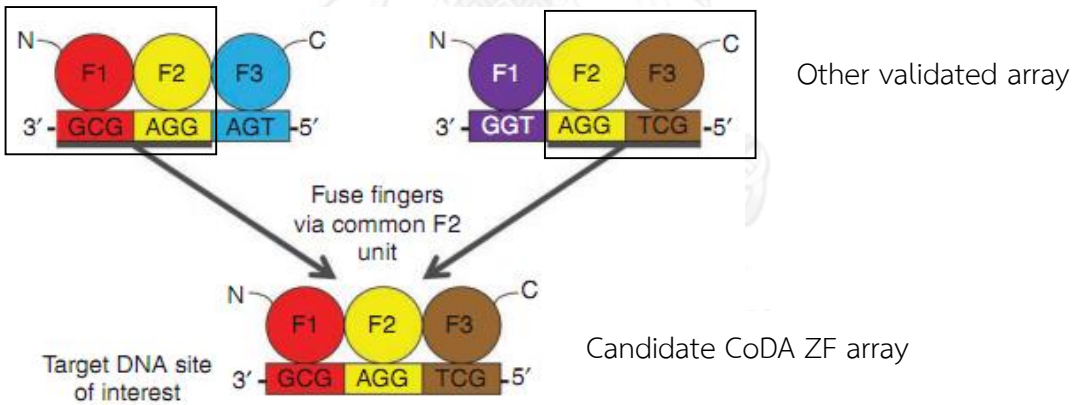


Figure 7. The process of generating CoDA through selection of other validated arrays containing the same middle finger. [46]

For construction, the desired *WAS* sequence data (~1000 bp) were submitted to the CoDA algorithm of the ZiFiT online-software. After CoDA evaluation, the candidate CoDA sequences were synthesized by commercial DNA synthesis (1st BASE).

CompoZr® Custom Zinc Finger Nuclease (ZFN)

The active ZFNs are also available for targeting experiments. The ready-to-use ZFNs are designed and generated by Sigma-Aldrich Corporation.

DNA binding activity of zinc finger arrays

The ZFPs from modular assembly design were tested for DNA-binding ability prior to testing in the cells. The bacterial cell-based two-hybrid (B2H) reporter assay was used. The B2H is a rapid assay and does not require transfection, and protein purification.

In the B2H reporter system, binding of a candidate zinc finger array to its target site leads to interaction of transcriptional activation Gal11P with Gal4 and leads to RNA polymerase induced *lacZ* gene transcription (figure 8). The *lacZ* encodes enzyme β-galactosidase that can be detected by β-galactosidase assay.

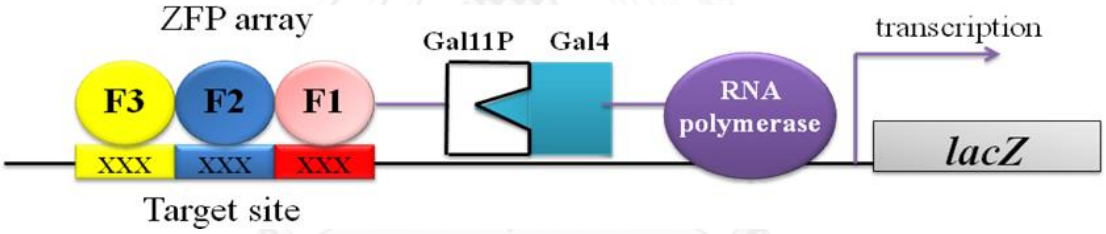


Figure 8.Schematic of the bacterial two-hybrid reporter system.

Construction of ZFP array-Gal11P vectors

The candidate ZFP array construct and Gal11P vector were double digested by restriction enzyme *Bsg*I and *Xba*I, and were then subjected to ligation using ligase to generate the ZF array-Gal11P construct. The ZF array-Gal11P construct was transformed into *E.coli* XL1-Blue strain and incubated for 16-18 hours at 37°C. They were selected by ampicillin resistance. These bacterial clones were randomly picked to determine the correction of ZF array-Gal11P assembly by PCR with specific primers and sequencing (PGP-FF primers-F and R). The primers and PCR condition were performed as shown in the table 1.

Construction of target site-*lacZ* reporter vectors

For generating of the reporter construct, a pair of target site was synthesized as oligonucleotides. Annealing was performed using 10 uM of each oligonucleotide in annealing buffer at 95°C for 2 minutes. The samples were cool down slowly to 25°C to form a short double-strand DNA fragment with overhang.

The *lacZ* reporter vector backbone was digested by restriction enzyme *BsaI*-HF, and was generated overhang by incubation with pfu polymerase and dCTP at 72°C for 10 minutes.

The short double strand target site fragments was inserted into the lacZ backbone overhang to generate target site-lacZ reporter constructs by ligation, and were transformed into the E.coli EPI max300 strain and incubated 16-18 hours at 37°C. The transformed bacteria were selected by chloramphenicol resistance. They were picked to determine the target site insertion by PCR-RFLP and sequencing. OK163 primers-F & R were used for amplification and sequencing (table1), and the *BsaI*-HF was used for RFLP. When the target site was inserted into the *lacZ* backbone, the *BsaI* site of the backbone was obliterated. The result of RFLP was shown in figure9.

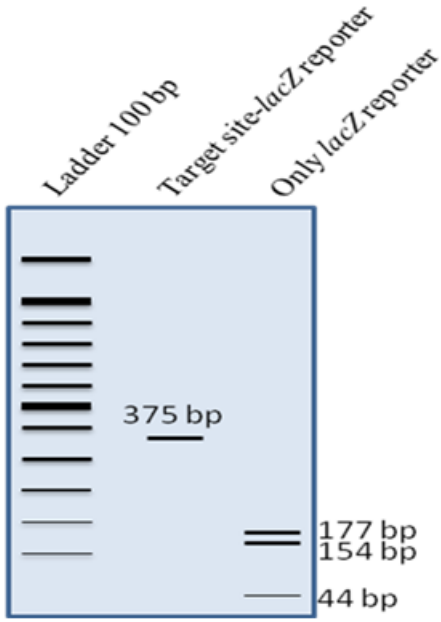


Figure 9.The PCR-RFLP of target site-lacZ reporter vector.

Bacterial cell-based two-hybrid (B2H) reporter

For B2H assay, the target site-lacZ reporter construct was transformed into the *E.coli* KJBAC1 strain and the reporter competent cells were prepared according to the standard protocol. The reporter competent cells were then doubly transformed with ZFP array-Gal11P and Gal4-RNA polymerase constructs to determine the DNA binding ability of ZFP array using *lacZ* expression (figure10). The *lacZ* encodes β-galactosidase that can be detected by the β-galactosidase assay.

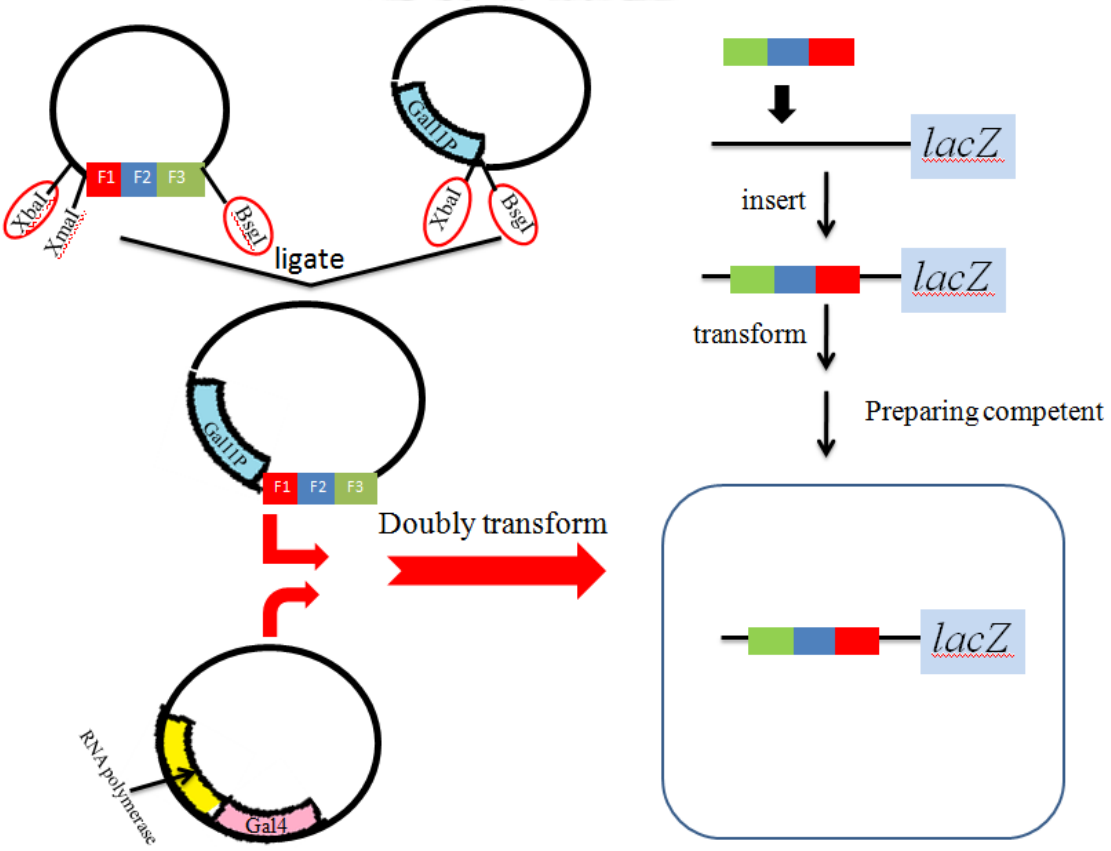


Figure 10. The process of bacterial cell-based two-hybrid (B2H) reporter.

β-galactosidase assay

The single clones that were doubly transformed were incubated in 5 ml of LB broth at 37°C for overnight. 100 ul of the culture was lysed by adding the lysis

master mix (popculture and lysozyme) to release the β-galactosidase from cells. Then 15 ul of lysates were added into Z buffer + ONPG (substrate) into 96 well plates, and incubated at 28°C. The β-galactosidase activity was detected by serial measurements of OD_{420} (measurements every 10 sec.) The results were plotted on the graph between A420 and time. The velocity of ONPG cleavage (*V*) was calculated from the slope of a line. Finally, the unit of β-galactosidase for each assay was calculated using the following formula.

$$\text{Unit of } \beta\text{-galactosidase} = V \times 1000 / (OD_{600})$$

The ZFP-arrays with β-galactosidase activity more than three-fold comparing with the negative control were selected for generating ZFNs. The schematic of β-galactosidase assay was summarized in figure 11.

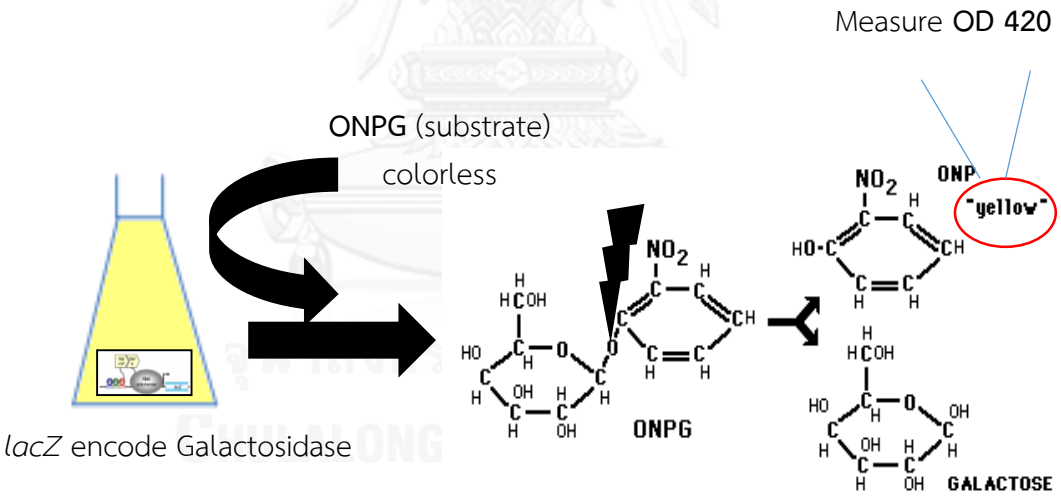


Figure 11. The schematic diagram of β-galactosidase assay. The β-galactosidase can cleave the ONPG (substrate) into galactose and ONP. β-galactosidase activity can be followed by measuring for yellow color of ONP at OD_{420}.

Construction of ZFN expression vectors

The selected modular and CoDA ZFP-arrays, and *Fok*I backbone vector were doubly digested by restriction enzyme *Xba*I and *BamH*I. Then ZFP-arrays and *Fok*I backbone were joined together to form ZFN expression vectors by ligase (figure12).[42] The insertion of zinc finger into the backbone was detected by PCR with specific primers (pST1374-F and R) and sequencing. The primers and PCR condition were shown in table1.

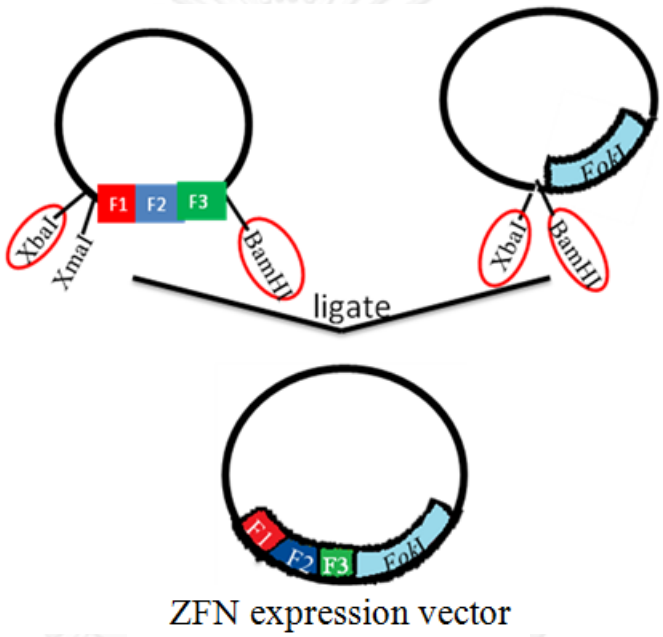


Figure 12. The process of generating a zinc finger nuclease construct from the zinc finger array and *Fok*I by using restriction enzyme and ligase.

***In vitro* DNA cleavage assay**

B2H assay can test only the binding activity of each ZFP array, but it cannot detect the ability of ZFN pair in inducing DNA double strand breaks (DSBs). When a pair of ZFNs bind and create DSBs at the target site, The DSBs can usually be repaired by non-homologous end joining (NHEJ) DNA repairing process that results in producing small insertions or deletions (indel mutations) at the DSB site. The ability of ZFNs pair in causing indel mutations was tested by T7 endonuclease I assay (T7EI assay).

10^6 of HEK293 cells were transfected with the modular ZFNs, CoDA ZFNs, and ZFNs from Sigma expression vectors by the nucleofector kit V (Amaxa) according to the manufacturer's instruction, and cultured with the culture media (DMEM+10% FBS and 1:100 penstrep) in a 6-well plate. After 72 hours of transfection, the genomic DNA of the transfected cells was extracted by the high molecular weight DNA extraction kit (5 PRIME). To assess the DSBs caused by ZFNs in the genome, the target site segments were PCR-amplified with specific primers (table2) and expand high fidelity tag polymerase (Roche). The PCR products were incubated for 5 minutes at 95°C, and stayed at room temperature at least 2 hours for random re-annealing. One unit of T7EI was then added in the PCR products, and incubated at 37°C for 20 minutes to allow the mismatched molecule (bubble) to be cleaved. Finally, the samples were subjected to gel electrophoresis to detect DSBs in the genome as shown in figure13, 14.

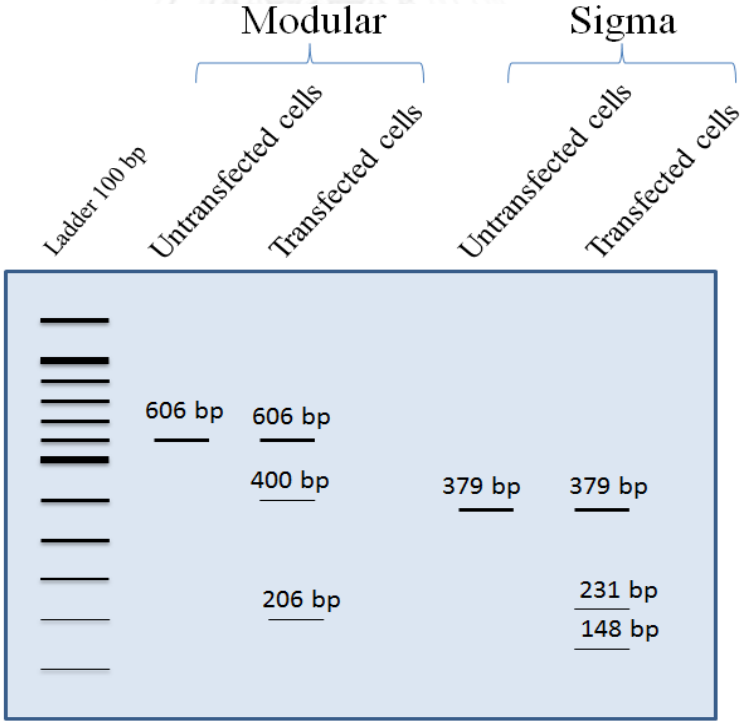


Figure 13. Gel electrophoresis showing the PCR products after digestion with T7EI.

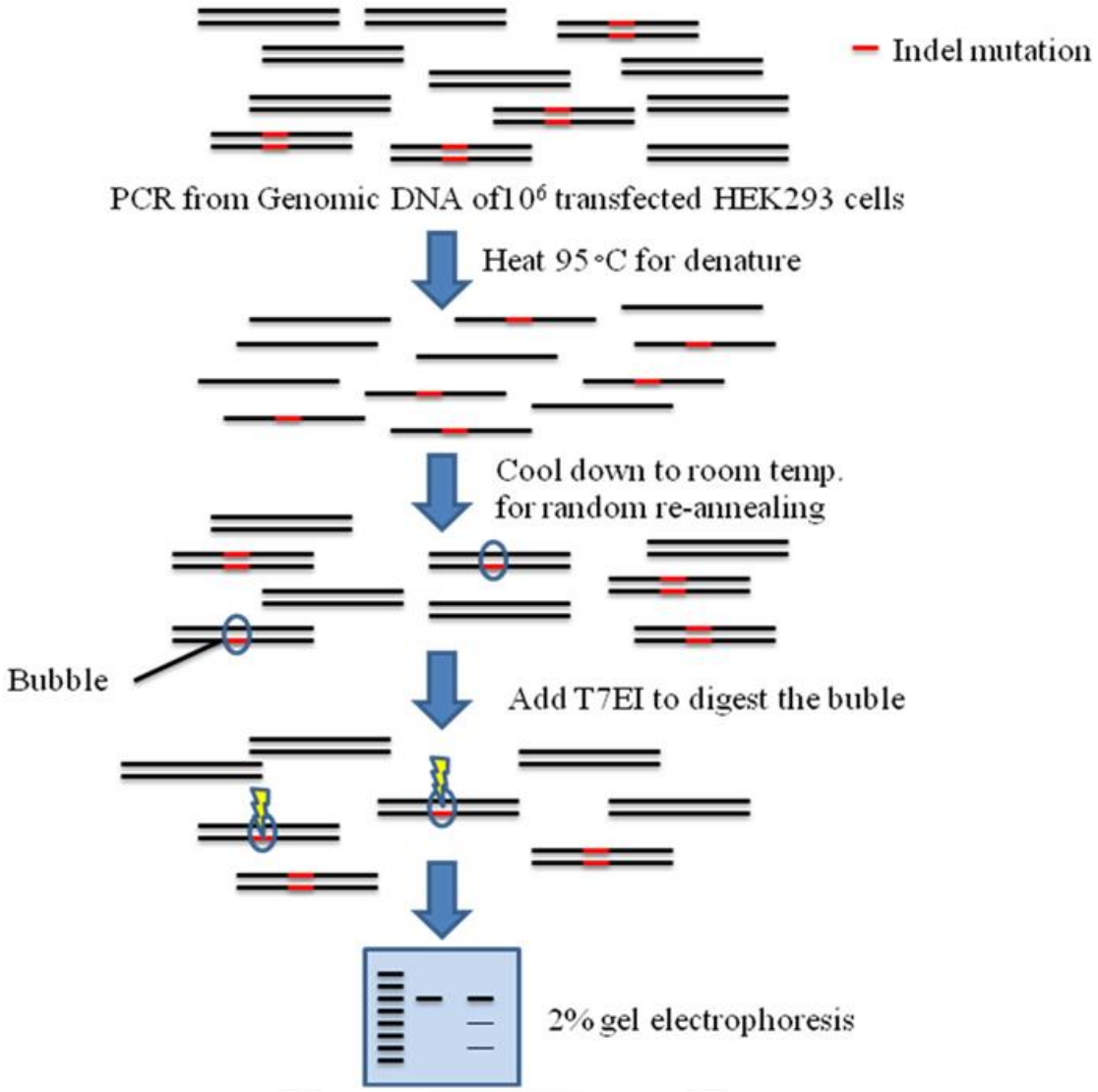


Figure 14. Detection of DSBs using T7 endonuclease I assay

Generation of a donor vector

The donor vector is important for inducing homologous recombination. The donor vector of this experiment has three important domains: 1. The 757-bp *WAS* homology left arm that contains intron 11 and exon 12 including the mutation, 2. The 736-bp right *WAS* homology arm that contains 3'UTR and intron 12, 3. The *loxp*-flanked PGK- hygromycin (Hyg) cassette for drug selection (figure 15).

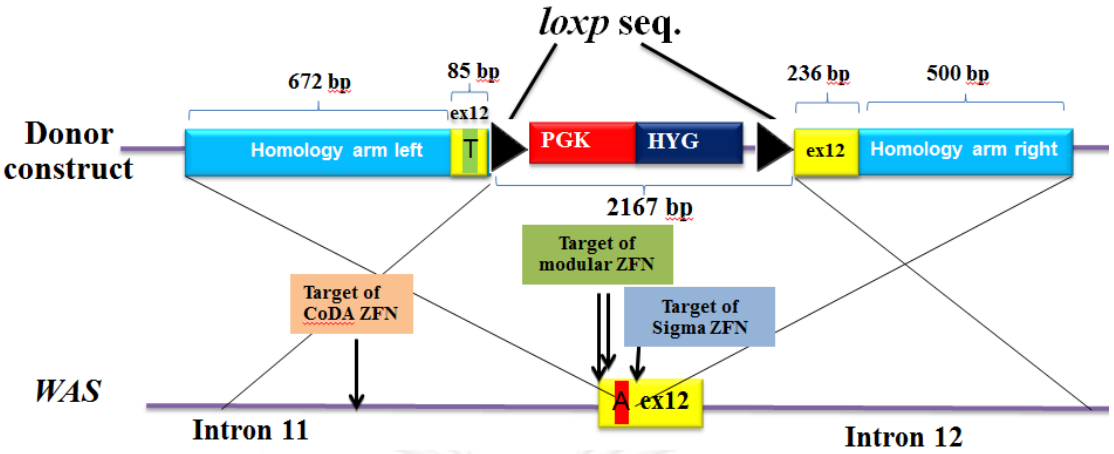


Figure 15. The *WASP* donor construct consists of 757-bp homology left arm containing 672 bp of intron 11 and 85 bp of exon 12 including the wild-type stop codon (TGA), 736-bp homology right arm containing 236 bp of exon12 which includes the 3'UTR region and 500 bp of intron 12, and 2167 bp of the selection domain containing loxp-flanked and hygromycin that is controlled by the PGK promoter.

The left and right *WASP* homology arms were amplified from genomic DNA of the unaffected control with specific primers and Phusion Hot Start II High-Fidelity DNA polymerase (Thermo Scientific). The primer sets, PCR product size, and PCR condition were shown in table 3.

After amplification of both homology arms, the homology left arm was inserted into the pCR-Blunt vector (Invitrogen) according to the manufacturer's instructions. Then the right homology arm and back bone (pCR-Blunt-left homology arm) were digested with *Nhe*I and *Xba*I, and were ligated together. Finally, the Hyg selection vector (BD2 vector) and pCR-Blunt with both homology arms were digested with *Nhe*I. The Hyg fragments were kept from BD2 vector, and ligated into the pCR-Blunt-right and left homology arms at 3'UTR of the WAS exon 12 to form the complete *WAS* donor construct. The process was shown in figure16.

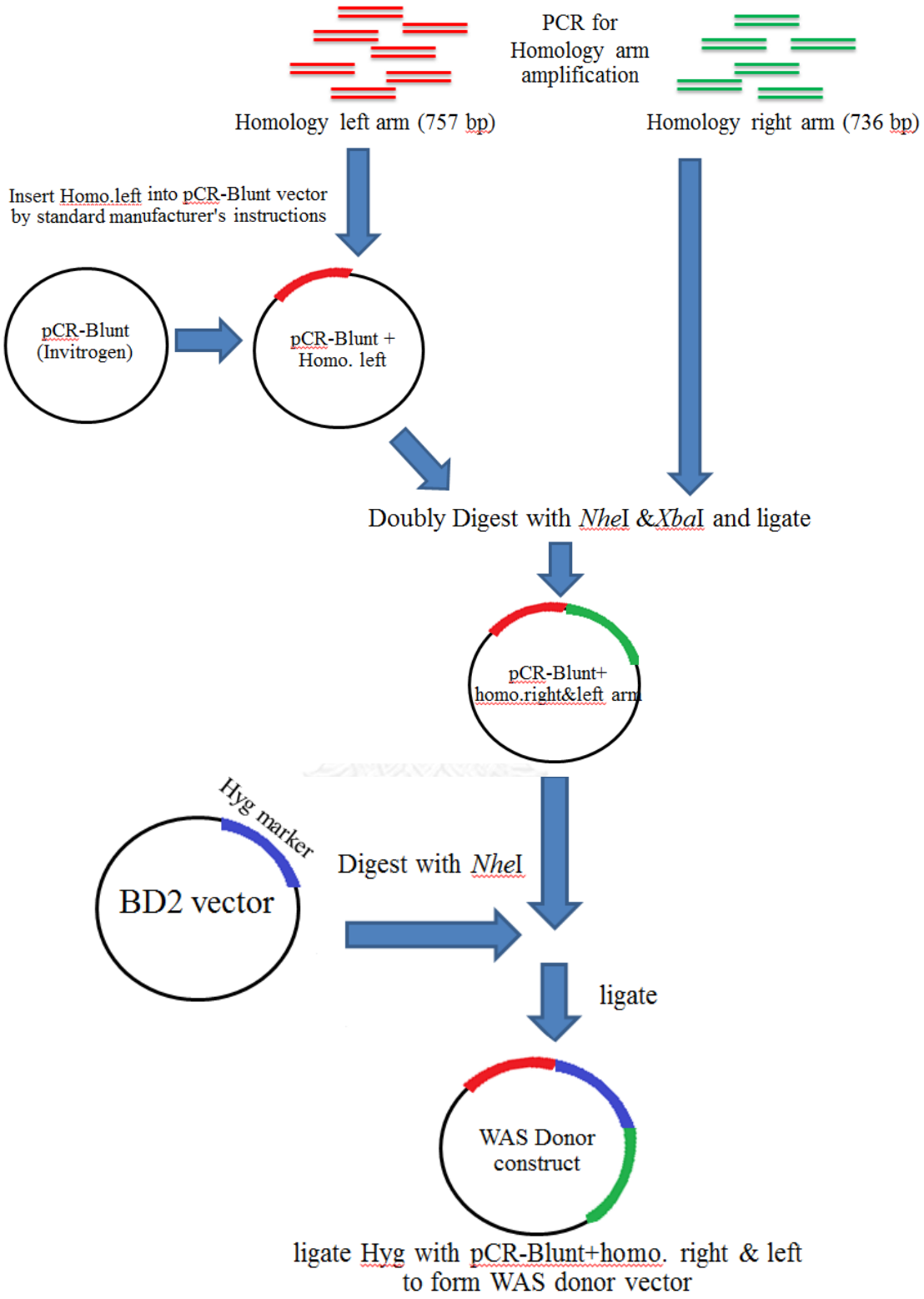


Figure 16. The process of making the *WASP* donor vector.

In vivo homologous recombination assay

When ZFNs create DSBs at the target site, the homologous sequence of donor vector would align and induce homologous recombination (HR) DNA repairing process for repairing of DSBs. The donor vector containing the PGK-Hyg can be integrated into the genome at the 3'UTR of *WAS* exon 12 after repairing (figure17). Therefore detection of homologous recombination activity can be performed by testing for hygromycin resistance ability.

10^6 of HEK293 cells were triply-nucleofected by a pair of ZFNs and the donor vector according to the manufacturer's instruction of Cell line nucleofector kit (Amaxa), and cultured with culture media (DMEM+10%FBS+1:100 penstrep) in a 6-well plate. Three days after transfection, the transfected cells were plated into a 100-mm tissue culture dish with 250 ug/ml of hygromycin B selection media, and continually cultured. Five days after transfection, all of colonies in the 100-mm dish were harvested. Genomic DNA (gDNA) was extracted from these cells by DNeasy blood & Tissue kit (Qiagen) and tested for PGK-Hyg by PCR using 5' junction and 3' junction primers. The primers, PCR product size, and PCR condition were shown in table4.

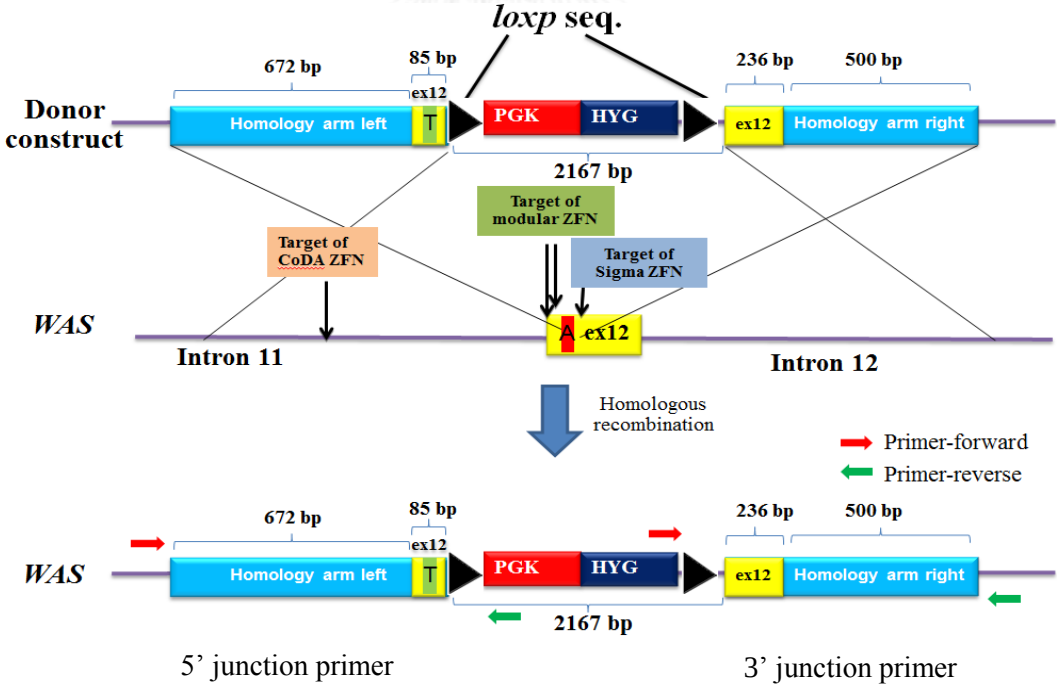


Figure 17. A scheme of DNA recombination in HEK293 cells.

Mutation correction

Culture of WAS-iPSCs

These WAS-iPS cells were cultured by feeder-free system and E8 medium. The first 6-well plate was prepared by coating the plate with Matrigel^{TM} (Basement membrane matrix). The Matrigel^{TM} (BD biosciences) was always thawed on ice and was dissolved in 12 ml of cool DMEM/F12. Then 1ml of Matrigel in DMEM/F12 was immediately coated in each well of 6-well plate and let it stand for one hour at room temperature before use. The plating medium was then changed to 1.5 ml E8 medium (Invitrogen). 0.5 ml of the WAS-iPS cell suspension was slowly added into the prepared well, and was incubated at 37°C/ 5% CO_2.

Gene targeting and selection

For transfection, the iPS cells were cultured with feeder because feeder could support growing of transfected iPS cells, and inhibit iPS cell differentiation in the selection medium.

The Mouse Embryonic Fibroblast (MEF) feeder was plated into the gelatin coated 6-well plate and was incubated overnight at 37°C/ 5% CO_2. Then $5x10^6$ of WAS-iPS cells (~3wells of the 6-well plate) were harvested with accutase, and were triply-nucleofected by a pair of ZFNs and the donor vector using cell line nucleofector kit (Amaxa) according to the manufacturer's instruction. The transfected WAS-iPS cells were cultured with E8 medium in the 6-well plate with MEF feeder. 72 hours after transfection, the E8 medium was changed to selection medium (E8 plus 20 ug/ml of hygromycin B). With hygromycin selection, each single colony of transfected WAS-iPS cells was picked and cultured without feeder. To detect for the presence of PGK-Hyg, gDNA was extracted from each clone by DNeasy blood & tissue kit (Qiagen). PCR was performed using primers and condition as shown in table4.

Correction of a WASP mutation in WAS-iPS cells

There were two approaches in evaluation for mutation correction. The first was to identify the presence of homologous recombination in transfected WAS-iPS cells by PCR using 5' and 3' junction primers. The second was to evaluate for the locus specific

targeting in transfected WAS-iPS cells by long-range PCR using 5' junction forward primer and 3' junction reverse primer. The PCR condition and list of primers were shown in table4.

Random integration assay

To detect for random integration of the donor vector in the genome, Southern blot was performed. Hygromycin probe was synthesized from the BD2 vector using Roche's PCR DIG Probe Synthesis kit according to the manufacturer's instruction. The probe primers, PCR condition, and the product size were showed in table5. For this experiment, *Sca*I was selected to digest the gDNA, and expected to give a fragment of 3,308 bp (figure 18).

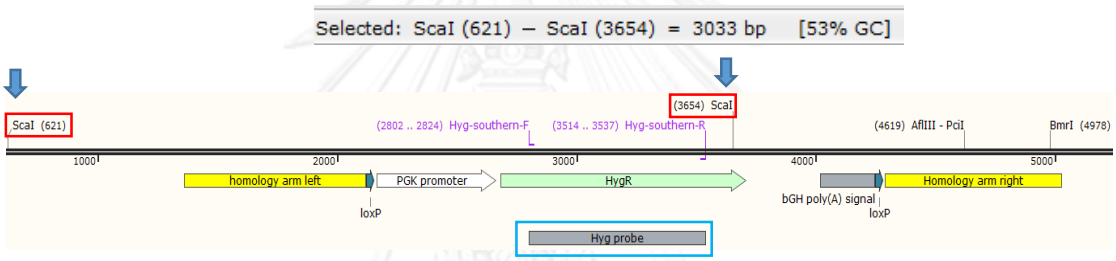


Figure 18. The gDNA map obtained from SnapGene program was used to select the enzyme and design the Hyg probe for Southern blot analysis.

Then 7 ug of gDNA of transfected WAS-iPS cells were digested with *Sca*I at 37°C for overnight. The digested gDNA was loaded and run in 0.7% gel at 120 volts for one and a half hours. The gDNA in the gel was denatured and neutralized by the denaturing buffer and neutralizing buffer before the "sandwich" assembly (figure19).

The digested gDNA was transferred to the nylon membrane in 20x saline sodium citrate (SSC) buffer overnight. Then the nylon membrane was taken out to expose with ultraviolet light for crosslinking. The nylon membrane was hybridized with the Hyg probe in the DIG Easy Hybridize solution at 44°C overnight.

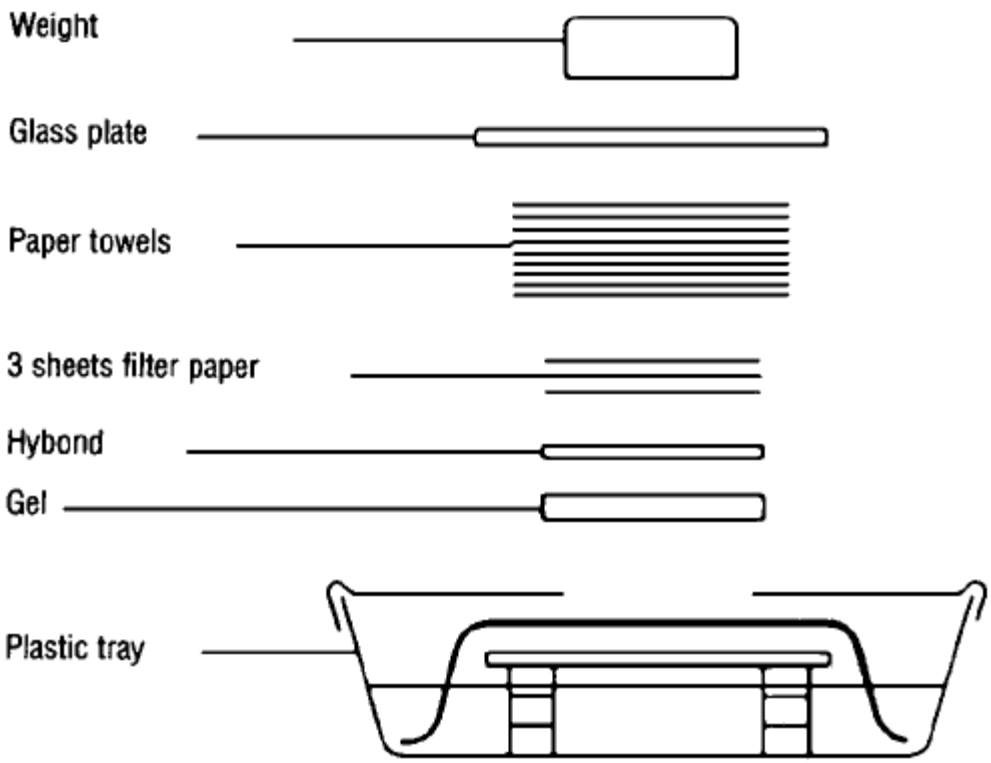


Figure 19. The sandwich assembly to transfer digested gDNA from gel to nylon membrane.

The hybridized nylon membrane was washed stringently at 65° C with 2xSSC+0.1% SDS, 0.5xSSC+0.1% SDS, and 0.1x SSC+0.1% SDS, respectively. After the washing step, the hybridized nylon membrane was blocked with 1xDIG blocking solution at room temperature for 30 minutes. Then, the membrane was incubated with anti-digoxigenin-AP, Fab fragment (1:10,000) in the blocking solution at room temperature for 30 minutes. The hybridized nylon membrane was washed twice with 1xDIG washing buffer at room temperature for 15 minutes, and equilibrated in 1XDIG detection buffer at room temperature for 3 minutes. Next, the hybridized nylon membrane was incubated with chemiluminescent alkaline phosphatase substrate (CSPD) working solution at 37° C for 10 minutes. Finally, it was exposed with a luminescent imager for 5-30 minutes.

.

Table 1 The primer list and PCR condition for ZFN construction

Primer	5'-Sequence-3'	Annealing (°c)	Size (bp)
ZF primer-F	CGCAAATGGGCGGTAGGCGTG	58	365 (Only ZF 1) 465 (ZF 1+2) 565 (ZF 1+2+3)
ZF primer-R	CCTTCCAGGGTCAAGGAAGGC		
PGP-FF primer -F	GGGTAGTACGATGACGGAACC	60	236 (only Gal11P) ~ 536 (ZF + Gal11P)
PGP-FF primer-R	CCTCTTCGCTATTACGCCAG		
OK163primer-F	GCAGAAGGCCATCCTGACGG	64	352
OK163primer-R	CGCCAGGGTTTTCCCAGTCACGAC		
pST1374-F	GAGAACCCACTGCTTACTGG	64	354 (only FokI) ~ 654 (ZF + FokI)
pST1374-R	TCCGGTTTCCTTGATCCACC		

Table 2. The primer list and PCR condition for T7EI assay.

Primer	5'-sequence-3'	Annealing (oc)	Size (bp)
Modular(WAS)primer-F	GACTACAGGCACGCCACTACG	66	606
Modular(WAS)primer-R	GACCAGGCAGGGATAACAGC		
Sigma (WAS) primer-F	TCCCTTTCTTGTCCCAAATG	56	379
Sigma (WAS) primer-R	CTTGGGATTGTTGGGTGAGT		

Table 3. The primer list and PCR condition for left and right homology arm amplification.

Primer	5'-sequence-3'	Annealing (°c)	Size (bp)
Donor WAS homo arm L-F	ACCAGGCCTGGCTGTTAACC	68	757
Donor WAS homo arm L-R	CGGCTAGCAGCACAGGGCAGCAAGTAACTC		
Donor WAS homo arm R-F	CGGCTAGCCTCCCCGCAGGACATGGCT	72	736
Donor WAS homo arm R-R	GCTCTAGATGCTCCCTCAGCCTGGAACC		

Table 5. The primer list and PCR condition for homologous recombination assay and mutation correction

Primer	5’-sequence-3’	Annealing (°c)	Size (bp)
5’WAS-HR-F	TGATGAGCTCTCACTACCAAGCTC	70	977
5’WAS-HR-R	CTAAAGCGCATGCTCCAGACT		
3’WAS-HR-F	GATGCGGTGGGCTCTATGG	70	844
3’WAS-HR-R	CTCATGCCAAGCCTTTTCTC		
5’WAS-HR-F	TGATGAGCTCTCACTACCAAGCTC	72	3,831
3’WAS-HR-R	CTCATGCCAAGCCTTTTCTC		

Table 4. The primer list and PCR condition for Hyg probe synthesis.

Primer	5’-sequence-3’	Annealing (°c)	Size (bp)
HygPb-F	ATGTAGGAGGGCGTGGATATGTC	60	736
HygPb-R	TCATCGAAATTGCCGTCAACCAAG		

CHAPTER IV

RESULTS

WASP mutation analysis

Mutation analysis by PCR-sequencing confirmed the presence of c.1507 T>A (p.X503R) in the skin fibroblasts obtained from our WAS patient. (figure 20).

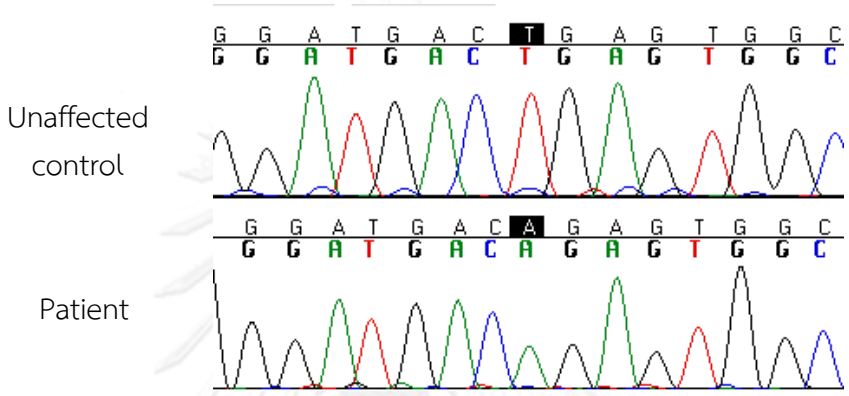


Figure 20 Electropherogram of *WASP* exon 12 showing the c.1507 T>A mutation expected to result in changing the stop codon to arginine (p.X503R).

WAS protein expression analysis

Western blot analysis revealed a 60 kD band in both PBMC's unaffected controls. However, it was absent in the PBMCs from the WAS patient (figure 21).

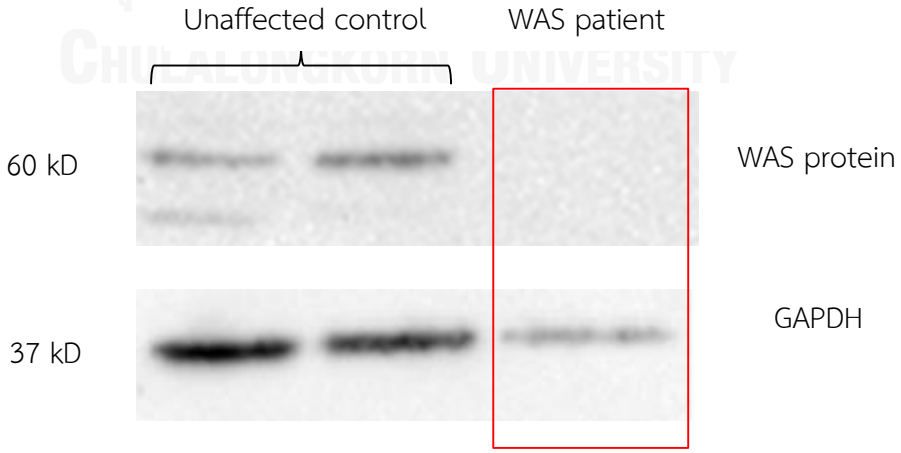


Figure 21 Western blot analysis for WAS protein expression

Selection of the ZFN recognition sites and generation of zinc finger nucleases

The ZFN recognition sites and each ZF-module were identified by Modular Assembly and CoDA algorithms using the ZiFit program. The previous study has demonstrated that the HR efficiency dramatically decreases when the cleavage site locates more than 100 bp from the target site.[47] The ZFNs that were expected to recognize the sites within the 100 bp range of the mutation were therefore selected.

Selection of ZFN recognition sites

Modular assembly algorithm

There were only two predicted ZFN recognition sites that were located within the 100-bp range around the mutation. One was located at 60 bp and the other at 54 bp upstream of the mutation (figure 22).

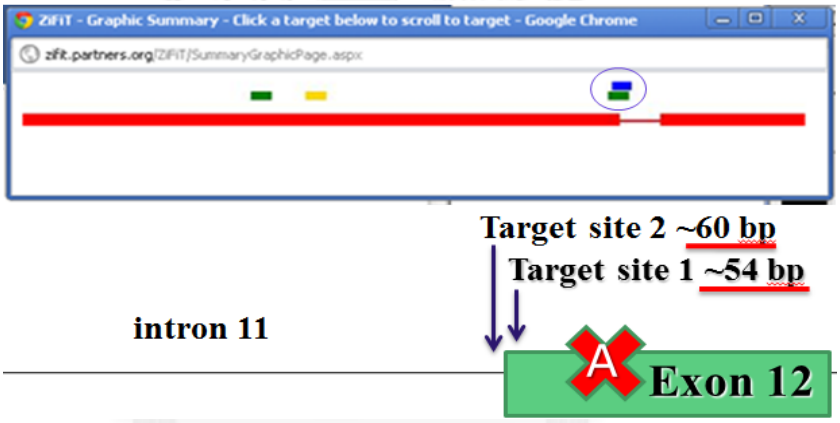


Figure 22 The predicted target sites obtained from the modular assembly algorithm.

CoDA algorithm

There was only one predicted ZFN recognition site that was located 465 bp upstream of the mutation (figure 23). This recognition site was located more than 100 bp far from the mutation site. The CoDA-ZFNs were therefore not used for gene targeting.

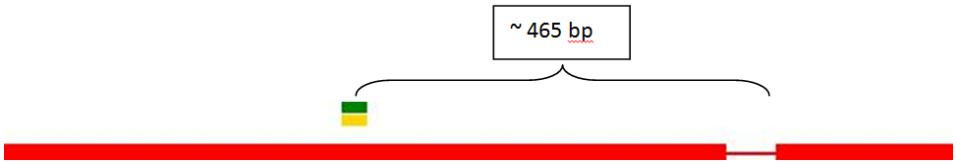


Figure 23 The predicted target site obtained from CoDA algorithm.

ZFNs from Sigma-Aldrich

ZFNs from Sigma were designed to recognize the target site that was located 23 bp downstream of the mutation (figure 24).

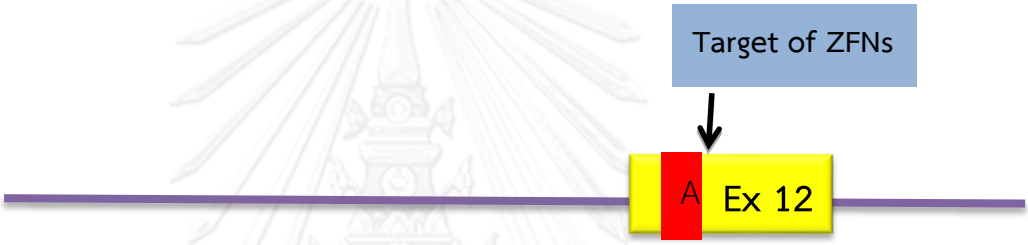


Figure 24 The target site of ZFNs obtained from Sigma.

Assay for DNA binding ability of zinc finger arrays

For modular assembly, twenty-two modular ZFP arrays were selected from the result of the ZiFit program, and were generated by joining the ZF modules 1, 2, and 3 together. They were tested for DNA binding ability before using in iPS cells. The seventeen selected modular ZFP arrays were shown in the appendix.

Bacterial two hybrid (B2H) assay

The two individual ZFNs must bind to opposite strands of DNA on each half-site in order to allow endonucleases to dimerize and cleave specific DNA sequence at the spacer region. The ZFN pair therefore contains the left and right ZFNs. Four left ZFNs and one right ZFN that were predicted to bind at the 54-bp DNA sequence upstream of the mutation site had β-galactosidase activity more than three-folds comparing with the negative control. The four left ZFNs included 114-20-55, 117-20-55, 118-20-55, and 129-20-55. The right ZFN was 2-20-51. There were four pairs of modular ZFNs that could be used for further experiments. The results were shown in Figure 25 and 26, and table 6 and 7.

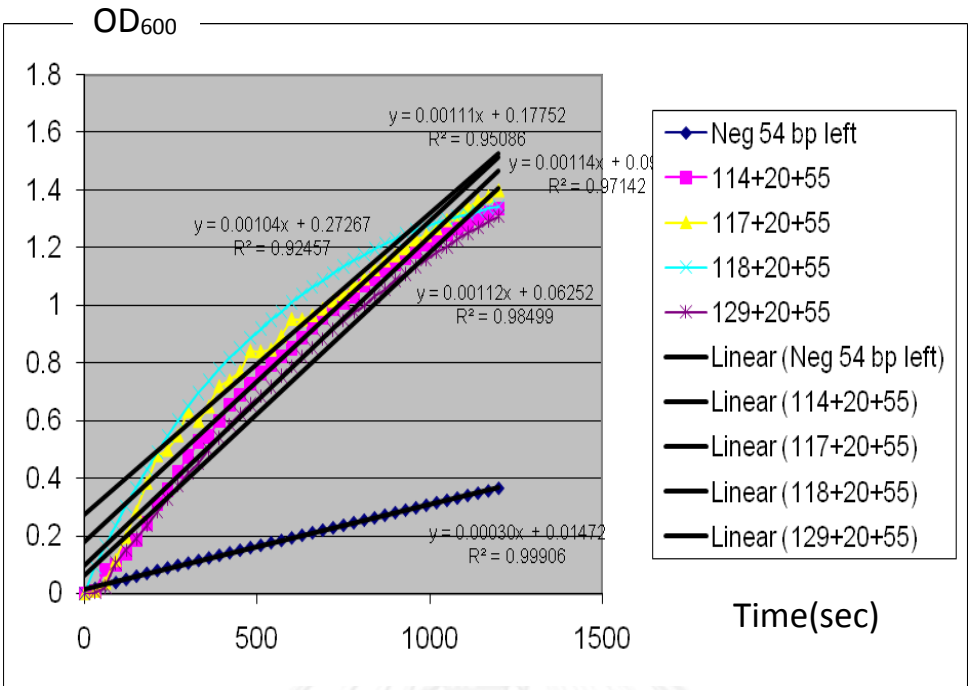


Figure 25 The β-galactosidase activity from the B2H assay of the left ZFNs. The blue line represents the negative control. The pink line represents the ZFN 114-20-55. The yellow line represents ZFN 117-20-55. The cyan line represents ZFN 118-20-55. The purple line represents ZFN 129-20-55.

Table 6 The B2H result of the left ZFNs that were predicted to bind at 54-bp DNA sequence upstream from the mutation site.

ZFP-array	*V*	OD600	Unit	Fold
Negative	0.0003	0.42	0.714286	1
114-20-55	0.00114	0.438	2.60274	3.643836
117-20-55	0.00111	0.421	2.63658	3.691211
118-20-55	0.00104	0.393	2.64631	3.704835
129-20-55	0.00112	0.437	2.56293	3.588101

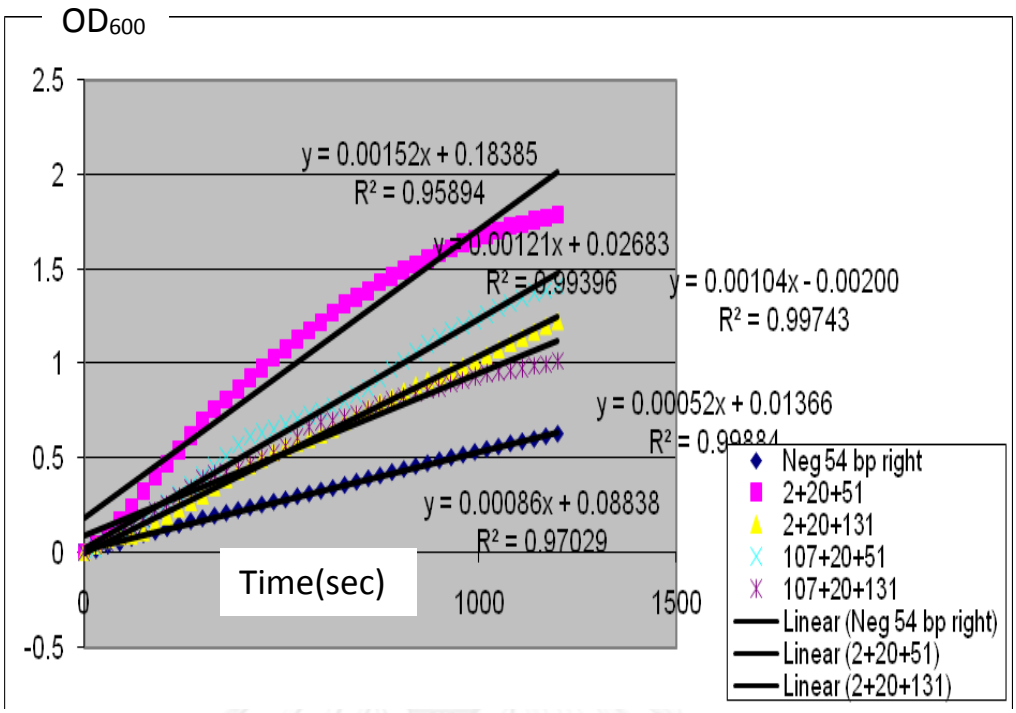


Figure 26 The β-galactosidase activity from the B2H assay of the right ZFN. The blue line represents the negative control. The pink line represents the ZFN 2-20-51. The yellow line represents the ZFN 2-20-131. The cyan line represents the ZFN 107-20-51. The purple line represents the ZFN 107-20-131.

Table 7 The B2H result of the right ZFNs that were predicted to bind at 54-bp DNA sequence upstream from the mutation site.

ZFP-array	*V*	OD600	Unit	Fold
Negative	0.00052	0.442	1.176471	1
2-20-51	0.00152	0.351	4.330484	3.680912
2-20-131	0.00104	0.475	2.189474	1.861053
107-20-51	0.00121	0.404	2.99505	2.545792
107-20-131	0.00086	0.486	1.769547	1.504115

***In vitro* DNA cleavage assay (T7EI assay)**

Prior to using the candidate ZFNs in iPS cells, testing for DSBs in HEK 293 cells was required. The genomic DNA was extracted from HEK293 cells transfected with each ZFN pair and was PCR-amplified with specific primers. The PCR products were subsequently digested with T7 Endonuclease I (T7EI).

For ZFNs obtained from Sigma, genomic DNA from transfected and non-transfected HEK 293 cells was amplified with specific primers. After T7EI digestion and gel electrophoresis, the expected bands (231- and 148-bp) could be detected in only ZFN-transfected cells. The bands were absent from untransfected cells with/without T7EI (figure 27). This ZFN pair was selected for further experiments in iPS cells.

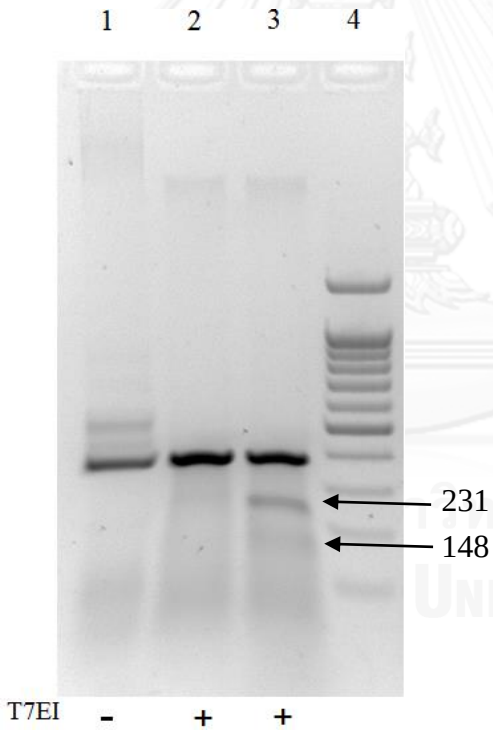


Figure 27 The T7EI digestion result of Sigma ZFNs. The first lane represents nontransfected cells without T7EI. The second lane represents untransfected cells with T7EI, and the third lane represents ZFN-transfected cells with T7EI.

For Modular ZFNs, four modular-ZFN pairs were tested in HEK293 cells. After T7EI digestion and gel electrophoresis, the expected bands (400-and 206-bp) could be detected in transfected cells with either of three Modular ZFNs (114, 117, 118) and untransfected cells with T7EI. However, these expected bands could not be detected in untransfected cells without T7EI (figure 28).

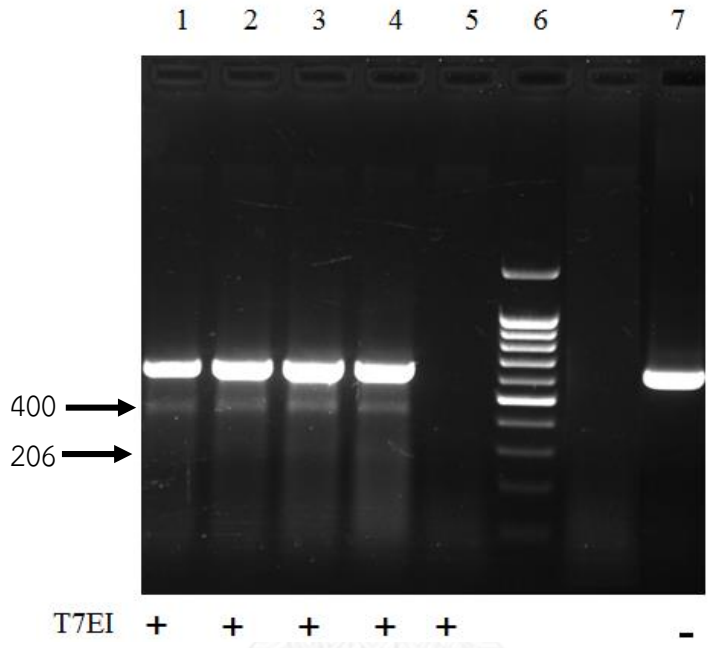


Figure 28 T7EI result of Modular ZFNs. The first lane represents untransfected cells with T7EI. The second lane represents cells transfected with Modular ZFN 114&2 with T7EI. The third represents cells transfected with Modular ZFN 117&2 with T7EI. The fourth lane represents cells transfected with Modular ZFN 118&2 with T7EI. The fifth lane represents cells transfected with Modular ZFN 129&2 with T7EI. The sixth lane represents the 100-bp ladder, and the last lane represents untransfected cells without T7EI.

In vivo homologous recombination assay

After HEK293 cells were transfected with Sigma-ZFNs and the donor vector, the genomic DNA was extracted from these transfected cells. They were subsequently tested for homologous recombination by PCR amplification with the 5’ junction and 3’ junction primers.

For 5'junction amplification, the result showed an expected band of 977 bp in transfected HEK293 cells. There was also a light band of similar size in non-transfected HEK293 cells (figure 29A).

For 3'junction amplification, an expected 844-bp band was detected in transfected HEK293 cells, not in untransfected cells (figure 29B).

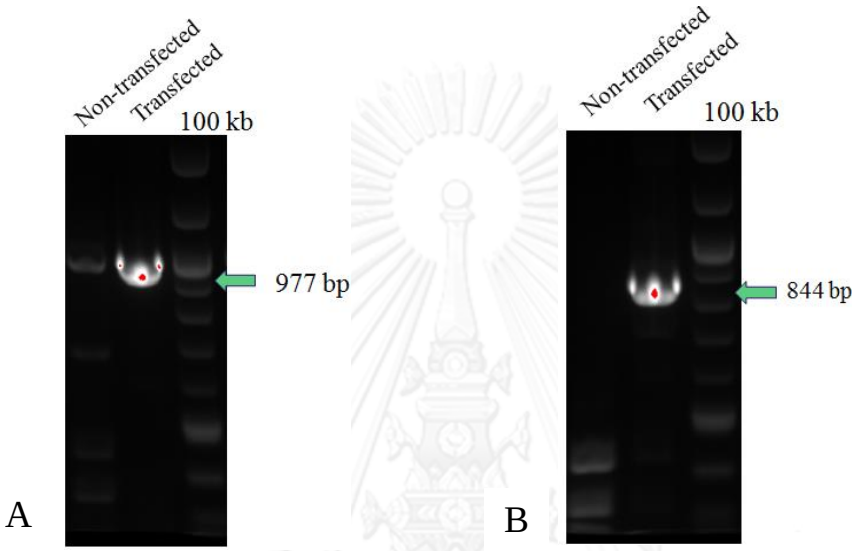


Figure 29 Homologous recombination assay. Gel electrophoresis showing the expected PCR products. (A) PCR with 5' junction primers and (B) PCR with 3' junction primers

For confirmation, the 977-bp and 844-bp bands from 5' and 3' junction amplification, respectively were extracted with the QIAquick Gel Extraction kit and sent for sequencing. The sequencing results revealed an insertion of the hygromycin marker (figure 30) indicating the presence of homologous recombination at the expected site.

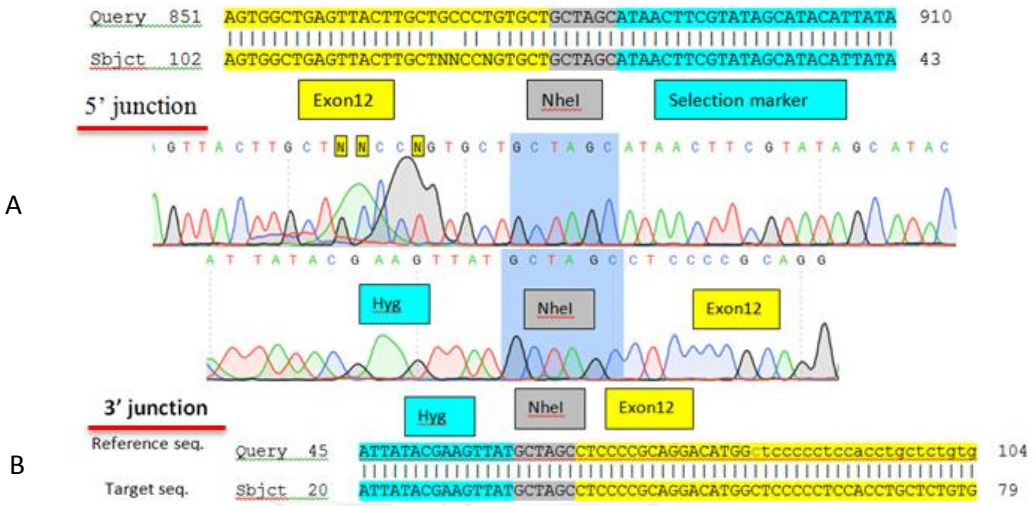


Figure 30 The electropherogram showing an insertion of the hygromycin marker in HEK293 cells transfected with the ZFNs and donor vector. (A) DNA sequence at 5' junction and (B) DNA sequence at 3' junction.

Mutation correction in WAS-iPS cells

After transfection for 3 days, transfected WAS-iPS cells were cultured in the selection medium. The iPS cells with the hygromycin marker resulted from homologous recombination were able to survive and grow as a single clone in this medium (figure31). Four clones were obtained on the first set and seven clones on the second. There were a total of 11 clones available for further evaluation.

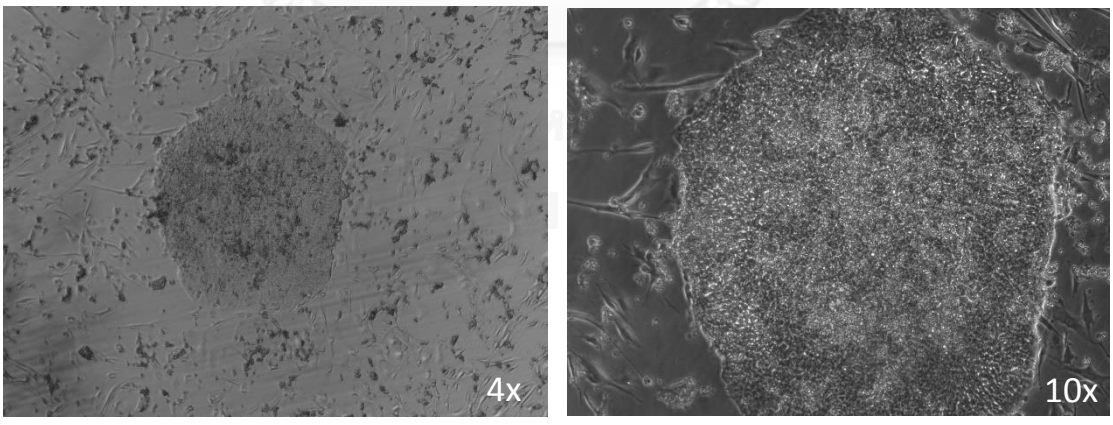


Figure 31 The WAS-iPS cell clone in the selection medium.

Evaluation of homologous recombination

For the first evaluation, these single clones were harvested and subjected to DNA extraction. The genomic DNA was PCR-amplified using the 5'&3' junction primers. The results were shown in figure 32 and 33.

The expected 977-bp band was detected in all clones except the clone 11 (figure 32).

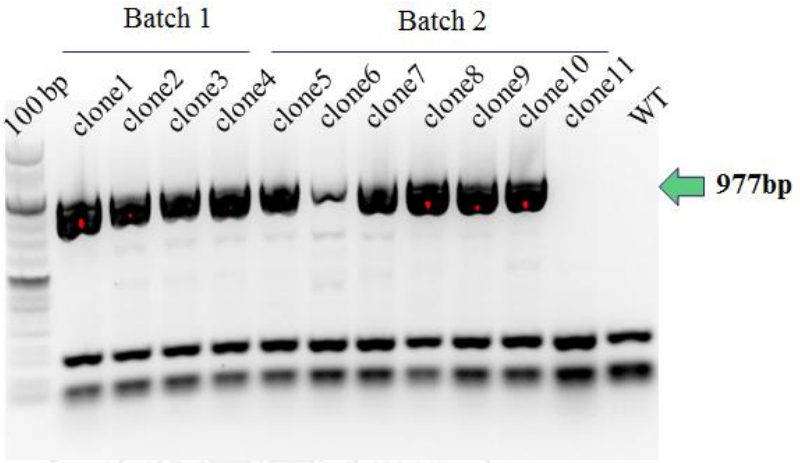


Figure 32 The gel electrophoresis showing the 977-bp band from PCR amplification of the transfected WAS-iPS cells (all except clone 11) using the 5' junction primers.

Using 3'junction primers, an 844-bp band was detected in eight clones (figure 33).

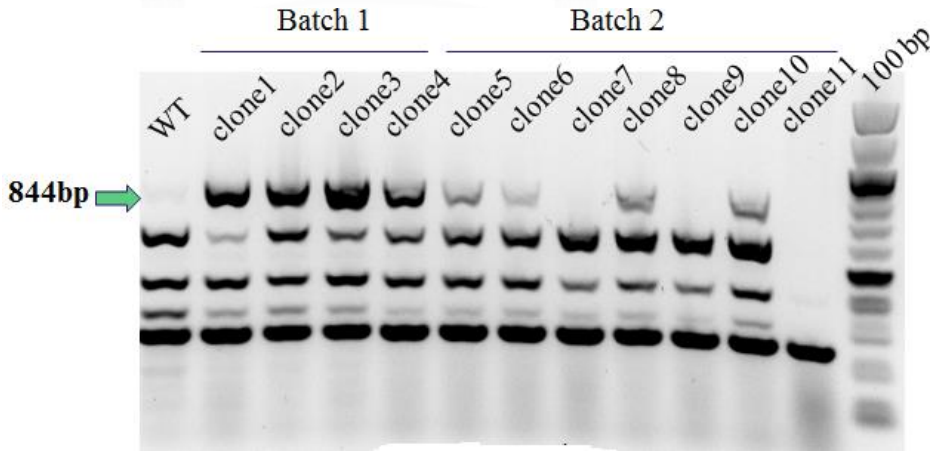


Figure 33 The gel electrophoresis showing the 844-bp band from PCR amplification of the transfected WAS iPS cells (clone 1, 2, 3, 4, 5, 6, 8, 10) using 3' junction primers.

Evaluation of locus specific targeting

For the second evaluation, these single clones were tested for the locus specific targeting by long- range PCR. If there was an insertion of the hygromycin marker into the target site, the PCR product size would be 3.8 kb. On the other hand, if there was no insertion of the hygromycin marker into the target site, the PCR product size would be 1.6 kb (figure 34).

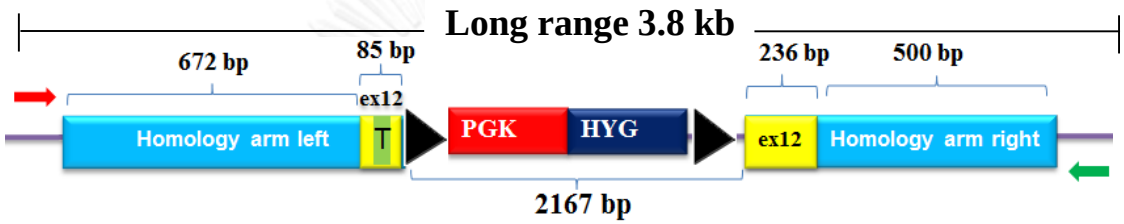


Figure 34 Schematic diagram showing the predicted size from long-range PCR to detect the presence of locus specific targeting in corrected WAS-iPS cells.

The result showed that there was an expected band of 3.8 kb in nine single corrected WAS-iPS clones (figure 35).

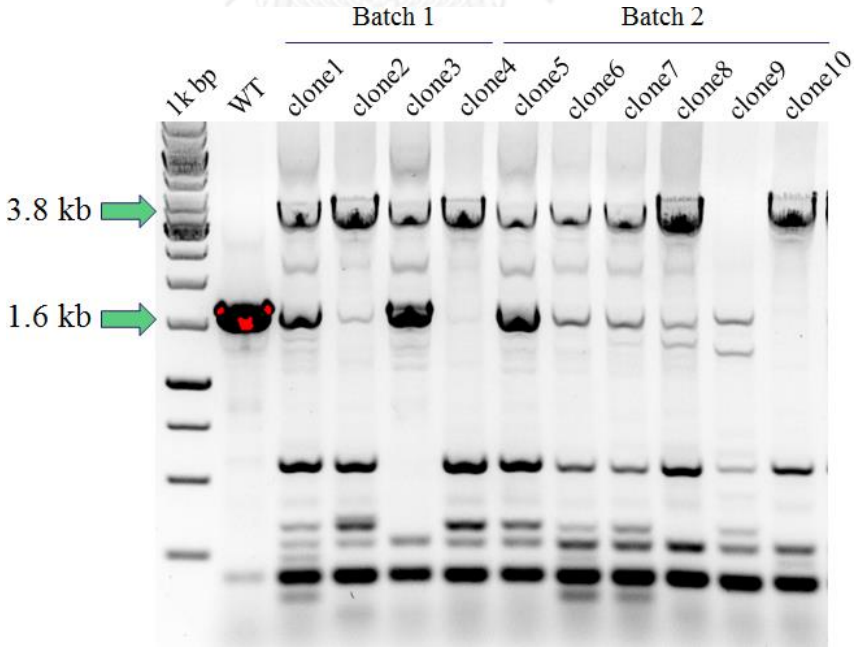


Figure 35 The gel electrophoresis showing the 3.8-kb band in nine of the corrected WAS-iPSC clones.

From these results, there were a total of eight clones (clone 1,2,3,4,5,6,8, and 10) with homologous recombination at the correct target site.

Mutation correction analysis

PCR-sequencing was performed in all eight clones to see if the mutation was corrected. The result revealed the normal sequence. The c.1507 T>A mutation identified in the *WASP* gene of all clones was corrected. The insertion of hygromycin marker was also detected (figure 36).

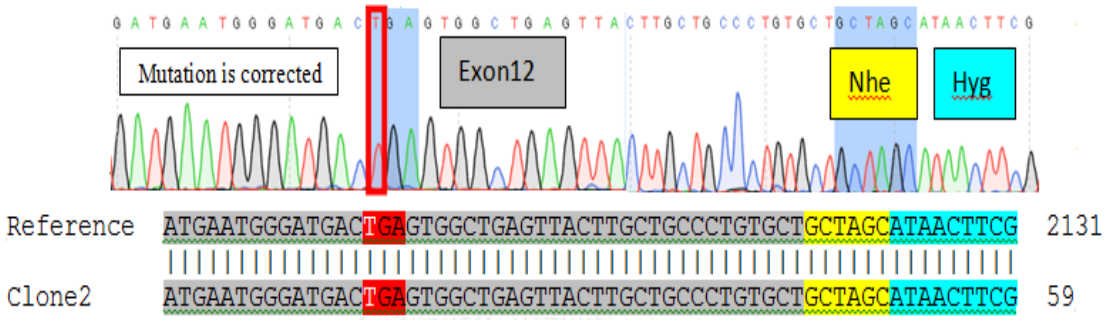


Figure 36 The electropherogram showing the normal sequence of the corrected WAS-iPSC clone 2.

Assay for random integration

After evaluation of mutation correction, these eight corrected WAS-iPSC clones were tested for random integration by Southern blot analysis.

The hygromycin probe was amplified by Roche's PCR DIG Probe Synthesis Kit. The best way to check the success of probe amplification is gel elecreophoresis. The probe fragment (labeled DNA) will migrate slower in the gel than the unlabeled DNA. With Hyg probe (labeled DNA), the expected size was about 800 bp while the actual size of hygromycin fragment (unlabeled DNA) was 736 bp (figure 37).

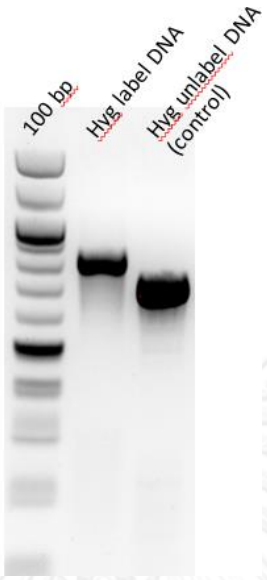


Figure 37 The gel electrophoresis showing the band obtained from synthesis of hygromycin probe.

The probe was used to detect the hygromycin marker in the genome. If there was a random integration of hygromycin marker into the genome, the unexpected band would be detected in the Southern blot.

The Southern blot showed an expected band of 3.3 kb in each clone. The band was absent in the WAS-iPS cells (WT control) (figure 38).

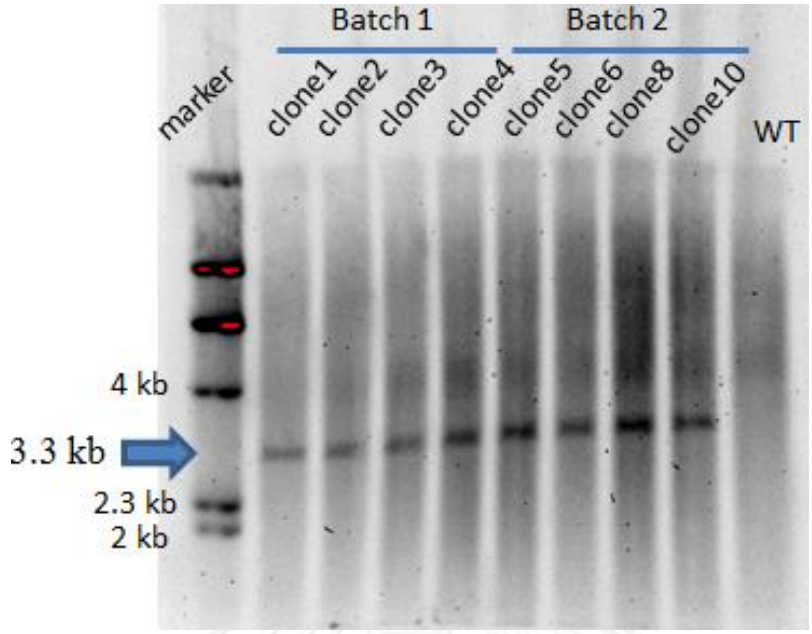


Figure 38 Southern blot analysis using Hyg probe to detect random integration of the donor vector containing the hygromycin marker.

All these eight WAS-iPSC clones were corrected at the specific site and had no random integration.

CHAPTER V
DISCUSSION

This study has successfully generated genetically-corrected patient-specific WAS-iPS cells. ZFN-based strategies were applied to specifically correct the mutation of the WASP gene in the patient's iPS cells without random integration.

Patients with severe WAS mostly die from infection or bleeding within the first decades of life. Restoring immune cell functions and platelets normally requires allogeneic hematopoietic stem cell transplantation, the only curative therapy for WAS. The difficulty in finding HLA-matched donors and complications related to transplantation urge the development of gene therapy strategies for WAS. Hematopoietic stem cell gene therapy using retroviral and lentiviral vectors has shown to restore natural killer, T and B cell functions both in mouse models [48, 49] and in two patients with WAS [50]. Nevertheless, one of the patients from the clinical trials subsequently developed T cell leukemia, potentially caused by vector integration at the T-cell oncogene, LMO2 site.[51] Therefore, the issues of random integration, appropriate viral vector design, optimal levels of WASP expression in each hematopoietic lineage, and prevention of transgene silencing need to be solved before it could become a safer and more effective therapy.

The ability to generate iPSCs has provided potentially unlimited sources for studying disease processes and generating disease-relevant cell types for autologous cell therapy. The proof of concept study conducted in a mouse model by Hanna et al. showed that genetically corrected mouse iPSCs could be used to cure genetic diseases such as sickle cell anemia.[52] Subsequent studies in human iPSCs demonstrated that transgene-corrected Fanconi anemia iPSCs can generate phenotypically normal myeloid and erythroid progenitors.[53]

A gene targeting approach using artificial restriction enzyme has been established. There have been several reports demonstrating the potential of gene targeting that could be applied for therapy in several disorders especially

immunodeficiency diseases including AIDS (17), X-SCID(54) , and hematologic disorders including thalassemia(55).

The zinc finger nuclease technique has become one of the most effective approaches to correct specific mutations identified in human cells.[38] Designing the ZFNs can be performed with different algorithms. Three algorithms including modular assembly, CoDA, and Sigma-Aldrich CompoZr were used to design the ZFNs for this experiment. Each has different efficiency and specificity (table 8).

For the modular assembly, although this algorithm is cheaper, has many alternative recognition sites and provides a positive result for in vitro testing, the success rate to cut the target site in a cell-based system remains low comparing with other algorithms.[46, 56, 57] A study about gene targeting in CCR5 using modular assembly ZFNs was performed in 2009. 208 modular-ZFN arrays were synthesized and 315 ZFN pairs were tested for targeting a total of 33 sites in CCR5.The result revealed that 44% of modular-ZFNs were able to cleave the target DNA efficiently in vitro digestion assay, but only 7.3% were able to function efficiently in the cell-based system.[58] So, ZFNs that have been tested positive in vitro might not be able to cleave at the specific sites in the cell-based system. It is consistent with our experiment. We synthesized 17 modular-ZFN arrays to cut two specific sites in the WASP gene. There were only 5 arrays (29.4%) that could bind the target sites when tested in vitro using B2H and none were found to cleave the DNA at the specific site when transfected in human cells (HEK293). This results suggest that the cell-based system, but not the in vitro assay (B2H), is a reliable method with which to assess the genome editing potential of ZFNs.

For CoDA algorithm, it has been found to be easy to perform, and in vitro testing for a binding ability is not required. The success rate for making functional ZFN pairs in plants, zebrafish, and human cells were reported to be as high as 18%.[46] However, it was shown that CoDA could not target some sites where modular assembly was able to cleave.[46] In our experiment, we found only one CoDA predicted target site which located more than 100 bp far from the mutation. Therefore, it was not used for further analysis.

ZFNs from Sigma-Aldrich CompoZr have been generated and validated through partnership with Sangamo Biosciences Inc. It has been shown that the success rate of using this ZFNs in cleaving the DNA at specific sites for our experiment is 9.4%. Sigma ZFN pairs and the donor vector were used to transfect the WAS-iPS cells. Out of 11 hygromycin-resistant iPS clones, eight WAS-iPS clones were found to be corrected and had no random integration. These corrected WAS-iPSCs will be selected for further analysis of WASP expression and disease phenotype.

Table 8 A comparison between different algorithms for ZFN design.

ZFN design	Advantage	Disadvantage
Modular assembly	• Cheap (~25,600 baht: 1 modular library set) • Can be designed more than 3 finger subunits in one array • Many alternative target sites in the genome[46, 58]	• Low success rate for making functional ZFN pairs (< 6%)[57] • labor intensive
CoDA	• Cheap (~ ฿32,000 baht : ZFN 1 pair) • less labor intensive • High success rate for making functional ZFN pairs (up to 18%)[46]	• Limit only 3 finger subunits in one array • Limit of target sites in the genome[46]
Sigma-Aldrich CompoZr	• Ready to use • Already validate	• Very expensive (320,000 – 640,000 baht: 1 pair).

In addition to ZFNs, other nucleases have been developed to provide more rapid and efficient approaches in targeted genome modification. The very recent approach is the use of RNA nucleases (CRISPR/Cas9). The CRISPR/Cas9 system has expanded the possibility of genome editing in model organisms. We have used the CRISPR/Cas9 to target at the specific DNA sequences of the four hematopoietic stem cell marker genes for future experiments (see appendix).

In conclusion, this study has successfully applied ZFN-based strategies in mediating gene correction by homologous recombination in the WASP gene. With the improvement in techniques for generating clinical-grade hematopoietic stem cells from pluripotent stem cells and in gene targeting strategies, hematopoietic stem/progenitor cells derived from the corrected patient-specific iPSCs may become a viable alternative for treatment of severe immune deficiency diseases.

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APPENDIX

The seventeen modular assembly zinc finger nucleases for this experiment

Table 9. The modular assembly zinc finger nuclease arrays (left side).

Zinc finger nuclease number (subunit 1)	Zinc finger nuclease number (subunit 2)	Zinc finger nuclease number (subunit 3)
116	119	116
116	119	122
116	119	123
122	119	116
122	119	122
122	119	123
123	119	116
123	119	122
123	119	123
9	20	55

Table 10. The modular assembly zinc finger nuclease arrays (right side).

Zinc finger nuclease number (subunit 1)	Zinc finger nuclease number (subunit 2)	Zinc finger nuclease number (subunit 3)
2	20	51
2	20	131
107	20	51
107	20	131
131	116	107
131	122	107
131	123	107

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR-Cas9)

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR-Cas9) functions as immune system found in bacteria and archaea. It uses short RNA to bind and cleave foreign nucleic acid directly. From this ability, it has been developed, and become a new genome editing tool. CRISPR-Cas9 consists of four important domains including noncoding RNAs or guide RNA (crRNAs), trans-activating crRNA (tracrRNA), Cas 9 nuclease, and protospacer adjacent motif (PAM) (figure39).

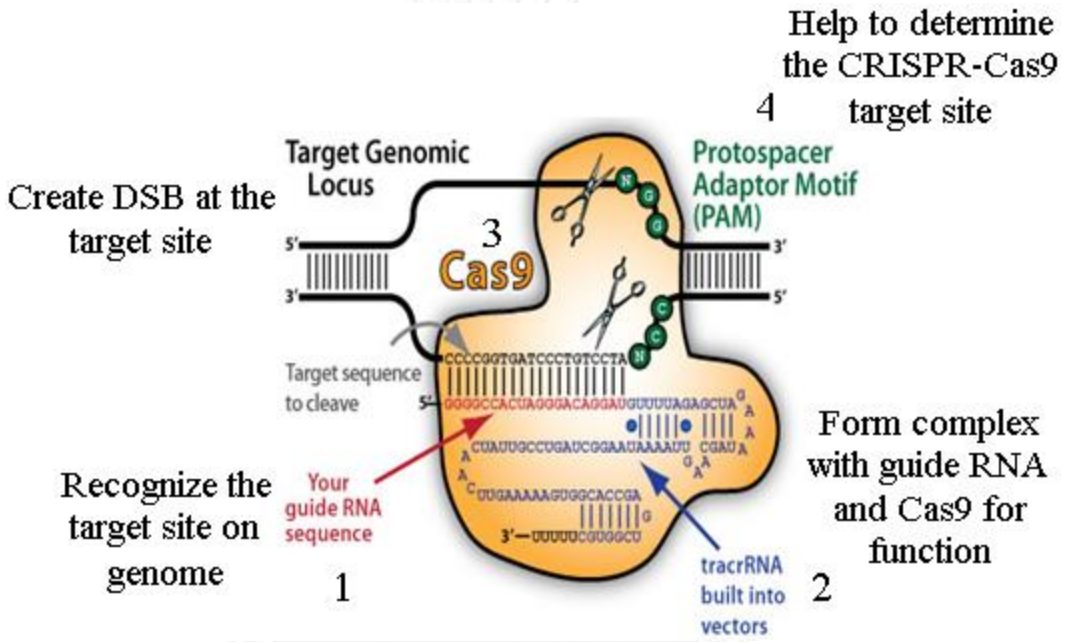

Figure 39. A schematic picture of the pre-crRNA:tracrRNA complex. The target sequence of CRISPR-Cas9 in the genome is determined by PAM. The guide RNA (red) will complement with tracrRNA (blue) to form complex with Cas9 protein. Then guide RNA will recognize and induce Cas9 protein to cleave the target sequence.

We have used CRISPR-Cas9 to generate DSBs at the specific sites of the four HSC marker genes including *RUNX1*, *MYB*, *CD41*, and *TAL1*.

The objective of this work is knock in the double selection marker using antibiotic (Puromycin) and Green fluorescent (GFP) marker into the HSC marker genes to determine HSC gene expression and select for the HSCs. The HSC marker genes were selected for this experiment including *RUNX1*, *MYB*, *CD41*, and *TAL1*.

The process started with CRISPR-Cas9s generation. The CRISPR-Cas9s were made in two days according to Zhang's lab protocol. Then the working CRISPR-Cas9s

were transfected into HEK293 cells to measure the DSB efficiency. After 72 hours of transfection, the transfected HEK293 cells were harvested. The gDNA was extracted and analyzed by the T7EI assay.

From T7EI digestion result, the CRISPR-Cas9 could be used to generate the DSBs in these four genes (figure 40A to D).

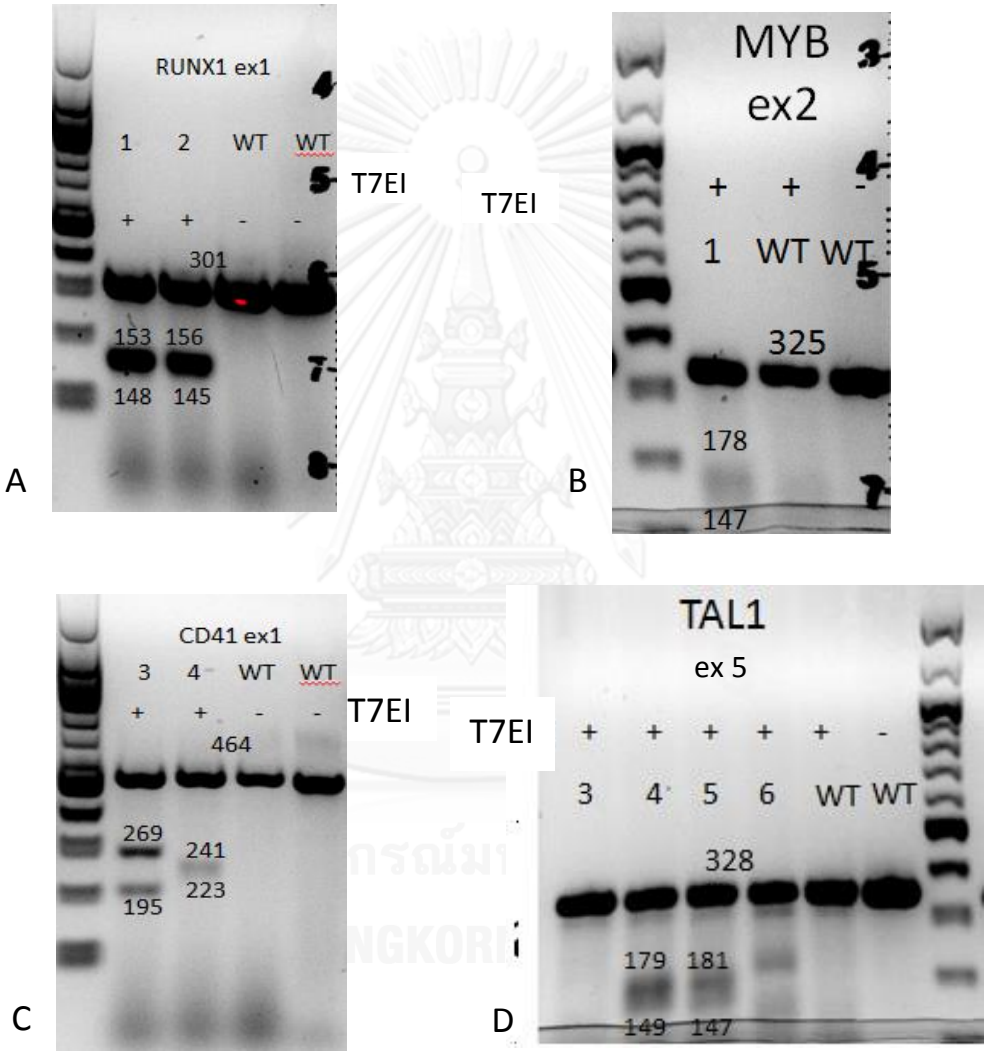

Figure 40. The T7EI digestion result of CRISPR-Cas9 in four HSC marker genes. (A) T7EI digestion result of *RUNX1* exon 1 (B) T7EI digestion result of *MYB* exon 2. (C) T7EI digestion result of *CD41* exon 1. (D) T7EI digestion result of TAL 1 exon5 (last exon).

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Poster presentation

- Amarinthnukrowh P, Suphapeetiporn K and Shotelersuk V. Clinical and mutational analysis of the TBX5 gene in patients with Holt-Oram Syndrome.16th Genetic Society of Thailand Meeting. Bangkok .2009

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Well knowledge about

Molecular genetics: DNA, Protein and plasmid study such as plasmid construction, DNA Sequencing, Restriction enzyme digestion, gene targeting, southern blot, Western blot, Bacterial two hybridge assay, T7EI assay, and both Cell line and bacteria culture