博士論文

Elucidation of Pathophysiological Mechanisms in Wiskott Aldrich Syndrome Using Patient-Derived Induced Pluripotent Stem Cells

（患者由来人工多能性幹細胞を用いたウィスコットオルドリッチ症候群における病態解明研究）

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Contents

Abbreviations:

Arp 2/3: actin related protein 2/3

APC: allophycocyanin

bFGF: basic fibroblast growth factor

BFU-E: burst forming unit-erythroid

BM: bone marrow

CD: cluster of differentiation

CGD: chronic granulomatous disease

CFU-E: colony forming unit-erythroid

Cy7: cyanin7

DAPI: 4, 6-diamidino-2-phenylindole dihydrochloride

DMEM: dulbecco's modified eagle's medium

E: erythroid

EVH1: ena/vasp homology proteins

FACS: fluorescence-activated cell sorter

FBS: fetal bovine serum

FITC: fluorescein isothiocyanate

FLT3: fms-related tyrosine kinase 3 ligand

G: granulocyte

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

GM: granulocyte-monocyte

HIV: human immunodeficiency virus

HPC: hematopoietic progenitor cell

HSC: hematopoietic stem cell

IMDM: iscove's modified dulbecco's medium

iPSC: induced pluripotent stem cell

KLF: kruppel-like factor

M: macrophage

MEF: mouse embryonic fibroblast

MK: megakaryocyte

NK: natural killer cell

NOD/SCID: non-obese diabetic/severe combined immunodeficiency

N-WASp: neuronal – wiskott aldrich syndrome

OCT: octamer-binding transcription factor

PBS: phosphate-buffered saline

PE: phycoerythrin

PFA: paraformaldehyde

PCR: polymerase chain reaction

SCAR/WAVE: suppressor of cAR /WASP family verprolin-homologous complex

SCF: stem cell factor

SIN: self -inactivation

TCR: T cell receptor

TPO: thrombopoietin

VEGF: vascular endothelial growth factor

WAS: wiskott aldrich syndrome

WASp: wiskott aldrich syndrome protein

WH1: WAS homology 1

WIP: WAS Interacting Protein

XLT: X-linked thrombocytopenia

Abstract

Wiskott Aldrich Syndrome (WAS) is an X-linked disease, which is caused by mutations in the gene encoding the WAS protein (WASp). As thrombocytopenia is the most typical feature of this disease, often exposing patients to a significant risk of life-threatening hemorrhage, it has been the main subject of study for many researchers. Because of limitations in disease modeling, precise mechanisms of the platelet abnormality remain to be elucidated. In this study, I established induced pluripotent stem cell (iPSC) lines from two XLT (X-linked thrombocytopenia) patients and one WAS patient as disease models to address the issues. I first confirmed that these disease-specific iPSCs retained gene mutations derived from each patient. Using our differentiation culture system, I demonstrated that numbers of both megakaryocytes (MK) and platelets obtainable from both XLT- and WAS-iPSCs were significantly smaller than those from healthy iPSCs. Detailed analysis revealed that the observed defects were mainly due to insufficient production of proplatelet-bearing cells, but not to impaired platelet production per MK. Further, I demonstrated that less numbers of proplatelet-bearing cells are attributed by the defective TPO signaling pathway in WASp deficient progenitor cells. Lentiviral-mediated gene transfer led to appearance of WASp expression in patient iPSC-derived MKs. The expression of WASp, however, did not reach the normal level that was seen in control-iPSC-MKs; yields of platelets showed significant increase after gene transfer. These results indicate the utility of iPSC-based disease modeling for WAS. More detailed analysis is required to understand the mechanism underlying insufficient WASp expression in megakaryocytes after gene transfer and consequently discern how critically expression levels of WASp will affect the efficacy in platelet number recovery after gene transfer.

Introduction

1- Wiskott Aldrich Syndrome

In 1930's, two physicians described patients showing some clinical phenotypes such as bloody diarrhea, eczema and microthrombocytopenia [1]. Later on the disease with those symptoms were named Wiskott Aldrich Syndrome (WAS). The gene encoding Wiskott Aldrich Syndrome protein (WASp) was subsequently identified on the X chromosome by positional cloning as the responsible for WAS patients [2]. Depending on the type of mutation and expression level of WASp, patients are categorized to 5 classes. The first scoring system was virtually based on the severity of disease [3], but later on Mahalaoui *et al*. developed the modified one by categorizing patients with platelet number of $\leq 10x10^9$ /L as score 5 [4]. XLT (X-Linked thrombocytopenia) is the milder phenotype of the disease in which the WASp is not completely absent. XLT patients are generally characterized by thrombocytopenia alone, but some patients may suffer from immunodeficiency and eczema [4], [5]. In the severe form of this disease, which is called WAS, WASp is completely absent or severely defected, leading to microthrombocytopenia, eczema, immunodeficiency and increased risk of autoimmunity and malignancy [6], [7]. The incidence of XLT/WAS is estimated to be 10 in 1 million new born [8].

2- Wiskott Aldrich Syndrome protein: structure and function

WAS gene has 12 exons, which are responsible for coding 502 amino acids. At the N terminal of WASp, it has an Ena-VASP homology domain (EVH1)/WASp homology

domain 1(WH1), which allows this protein to recognize and bind to the proline-rich region of WIP molecule (WASp Interacting Protein). WIP is responsible for maintenance and stability of WASp, by mediating formation of a WASp-WIP complex as well as localization of WASp [9]. It has been identified that most of missense mutations in the patients happen within WH1 domain [10]. Mutations in WH1 domain of WASp, have been shown to interfere WASp/WIP complex formation leading to degradation of WASP [11], [12]. After WH1 domain, WASp possesses a basic region (BR) and a GTPase binding domain (GBD) followed by a proline-rich region (PPP). The C terminal of WASp contains of verpolin homology domain (V), the confilin homology domain (C) and the acidic region (A) [2][13]. WASp exists in the form of an auto-inhibited inactive form in the cytoplasm of cell which is derived by intramolecular interaction of VCA domain and BR/GBD [14]. Binding of Rho family GTPase cell division cycle 42 (CDC42) to the WASp at GBD domain activates WASp which allows binding of VCA domain to the actin related protein 2/3 (Arp 2/3) complex [15]**(Figure 1).**

WASp is a member of family of proteins which are involved in signaling and cytoskeletal organization. Two other members of this family including N-WASp and Scar/WAVE were subsequently identified following WASp discovery [16]–[19]. WASp has significant role in actin nucleation through its binding to the Arp 2/3 via the C-terminal acidic region [20], [21]. Tyrosine phosphorylation of WASp is known to enhance actin polymerization and filopodia formation through Arp 2/3 [22]–[24].

3- Role of WASp in hematopoietic cells

WASp expression is restricted to the hematopoietic cell lineages [1], [25], [26] and has been shown to play significant roles in a variety of hematopoietic cells such as T cells, B cells, dendritic cells, monocytes and platelets, which is known to mediate certain signaling events through surface receptors leading to the actin reorganization [27]. WASp mutations are associated with low numbers of T cells in the early life [26], defective T cell proliferation in response to TCR stimulation, and disturbed clonal distribution of TCR beta-chain families [6], [27], [28]. It has been demonstrated that WASp has a critical role in development and function of regulatory T cells (Treg) [31], [32].

Our understanding of B cell function in WAS patients has been only limited for many years, but some studies have recently demonstrated that WASp deficiency perturbs B cell homeostasis characterized by depletion of circulating mature B cells, marginal zone B cells and splenic marginal zone precursors [32], [33]. In addition, WAS patients are known to have defects in immune synapse formation in NK cells [34]–[36] with their numbers ranging from normal to elevated ones. Depending on the type of mutation, iNKT (Invariant NKT) cells are decreased or absent in XLT/WAS patients [37] and recently some studies have demonstrated that WASp has non-redundant role in iNKT cell function and homeostasis [37], [38].

4- Platelet defects in WAS

One of the interesting topics in the field of hematology is thrombopoiesis. It has been accepted that megakaryocytes are the origin source of platelets for many years [39]. Before the idea obtained consensus, there had been discussion regarding a leukocyte or red blood cell origin for platelets. In 1910, a model, which is based on microscopic images suggested that megakaryocytes extend their pseudopodial processes to the blood vessels from bone marrow cavity and release platelets [39]. Later on, release of platelets from megakaryocyte pseudopodial extensions was fully described as a proplatelet formation model by Italiano *et al*. on 1999 [40]. Using a video microscopy they demonstrated extension of large pseudopodia from mouse megakaryocytes in which platelet-sized swellings are connected by cytoplasmic bridges and ends to platelet-sized particles [40].

One of key features for the disease is thrombocytopenia, which is defined as decreased platelet numbers in peripheral blood to less than 150×10^9/L. Decrease in platelet numbers can be as a result of reduced platelet production from the megakaryocytes in the bone marrow or increased platelet destruction and sequestration in the spleen. There are variety of diseases with genetic defects which cause thrombocytopenia whit different degrees ranging from mild to severe [41].

The thrombocytopenia in XLT/WAS is regarded as unique, because of small platelet size, which is called microthrombocytopenia. In contrast, other genetic disorders compatible with thrombocytopenia are reported to be characterized by the size of platelets being normal or large (macrothrombocytopenia). Small platelet size has been consistently observed in all reported cases having the defects in WASp so far, except for some patients

(XLT patients) recently reported as having normal platelet size (MPV 7.5-7.6). In this clinical examination, two novel gene mutations has been demonstrated; one is an insertion which leads to frame-shift making premature stop codon in exon 9 and another is two base pair deletion, which causes premature stop codon at exon 7 [42]. Both patients are categorized as XLT score 1, which is the mildest phenotype of the disease.

Life-threating bleeding has been reported to affect ~30% of patients due to the thrombocytopenia [6]. Despite the adverse effect of microthrombocytopenia on the life of patients, which is correlated with significant risk of haemorrhages, not much has been shown about the cause of this phenomena. Many groups have addressed the question whether increased platelet consumption or impaired platelet production is responsible for thrombocytopenia in XLT/WAS patients. Some clinical trials have shown rapid platelet turn over in WAS patients [43]–[49]. On the other hand some reports favor the concept of abnormal platelet production in WAS/XLT patients [12], [47], [50], [51]. Furthermore, Haddad *et al*. have been suggesting a peripheral mechanism for platelet defect [52]. Overall, I need more clear answer to this important issue, for development of the measures to cure the patients.

5- iPSC as a disease model

Approaches to elucidate the mechanisms underlying WAS/XLT disease has been limited to certain range of experimental models including the one that uses knockout mice, patient primary samples, and clinical examination. However, since there are some inherent limitations within each of them, I need the new type of modeling system that can

phenocopy human diseases more properly. For example, there have been reported several WASp-knockout mouse models, showing moderate thrombocytopenia and lacking the feature of small-sized platelets. Besides, there are several concerns regarding performance of scientific examinations on patient samples in such a rare disease especially that WAS/XLT patients are usually in young ages (incidence of death is high and in severe cases patients doesn't grow old without HSCT or gene therapy [53]) that affect the availability of primary samples (Peripheral Blood or Bone Marrow) . In addition, decreased number of platelets in patients is another obstacle for using the patient samples for the studies with the purpose of understanding the functional abnormality in platelets.

Recently, human pluripotent stem cells (including human embryonic stem cells [hESC] and induced pluripotent stem cells [iPSC]) have become available as a tool for disease modeling, giving a unique opportunity to scientists to work on understanding the pathophysiology of genetic diseases [54][55][56][57]. iPSCs are patient-derived cells, which have potential to reproduce the cellular defects unique to patient cells and to enable infinitely proliferation *in vitro*. I therefore thought that they could be used as a useful model providing important information about microthrombocytopenia in WAS/XLT patients. Thereby, I aimed at using iPSCs as a disease model for better understanding of mechanisms underlying thrombocytopenia in WAS patients.

6- Gene therapy

The treatments, which are currently available for WAS/XLT patients include splenectonomy (for thrombocytopenia) and the use of antibiotics (for immune deficiency). The potentially curative treatments are allogeneic BM transplantation (BMT) and gene therapy. Even though BMT is known to be a successful treatment for WAS, lack of HLA-matched donors can be a limitation for some patients. One should also be aware of the risk of some complications, which may lead to mortality and morbidity especially for the cases of mismatched transplantations [58]–[60]. Gene therapy is one of the promising available option for patients with genetic disorders. Virus vector-mediated therapeutic gene transfer has been successfully used in HSC gene therapy trials for some inherited diseases such as X-linked adrenoleukodystrophy (X-ALD) [61], adenosine deaminase deficiency (ADA-SCID) [62], [63], X-linked severe combined immunodeficiency (SCID-X1) [64], [65], X-linked chronic granulomatous disease (X-CGD) [66], [67] and Metachromatic leukodystrophy (MLD) [68].

In the context of WAS-gene therapy, preclinical proofs have been obtained by demonstrating the advantage of successful gene therapy in reconstitution of hematopoietic cell functions *in vitro* and *in vivo* [69]–[71]. On 2006, the first clinical trial was performed on 10 patients in Germany using the gamma-retrovirus vector. Even though the results showed increase in number of platelets and normalization of T cell counts and function, 7 patients developed T cell leukemia due to vector-mediated activation of the protoconcogene *LMO2* [69]. In contrast, clinical trials by Italian groups using HIV-based gene transfer did not induce *in vivo* selection of clones carrying integrations near oncogenes [72]. In

addition, it has been shown that HIV (human immunodeficiency virus)-based vectors are considered to be safer than non–SIN murine leukemia virus (MLV)-based oncoretroviral vectors [73], [74]. With aim of improvement of gene therapy for patients, several groups have worked on different vectors (MLV-RV, HIV-LV) or even in the concept of comparing different promoters [70], [73]–[79]. Unfortunately, all of these groups have targeted mainly T and B cells or CD34+ progenitor cells. Considering that thrombocytopenia is the main feature of the disease, which affects all patients regardless of the type of mutations, I thought it to be highly desirable to investigate the underlying mechanisms. Of note is that in all clinical trials the number of platelets has not reportedly reached the normal range after gene therapy.

Objectives

As it was discussed in the introduction section, because of limitations in disease modeling, precise mechanisms of the platelet abnormality in XLT/WAS patients remain to be elucidated. To address the issue, I established induced pluripotent stem cell (iPSC) lines from peripheral blood $CD34^+$ cells obtained from two XLT and one WAS patients (each harboring different mutations) for disease modeling. By utilizing the megakaryocyte and platelet differentiation protocols, which has previously been established in our laboratory, the first series of experiments were to compare the megakaryocyte/ platelet production processes in WAS/XLT patient samples *in vitro* with those in healthy individual counterparts **(Figure 2, A)**. Also, the possible difference in proliferation/differentiation properties due to distinct WASp mutations was examined in hematopoietic progenitors. To gain insights into gene therapy, gene transfer experiments were carried out to investigate how efficiently WASp expression restored by lentiviral vectors would lead to improvement in the functional defects observed in patients' samples **(Figure 2, B)**.

Material and methods

Patient samples

In this study I performed our experiments on two XLT patients categorized as score 2 and one WAS patient with score 5, each having different mutations. All these three patients are currently under hospital cares in the Tokyo University of Medical and Dental Science. Patient number 1 (Pt1) was diagnosed as an XLT patient with 168 C>T mutation (exon2). A double C deletion in exon 10 (1068 del GG) was identified in patient number 2 (Pt2) as the cause of XLT. Patient number 3 (Pt3) showing the most severe phenotype, was found to have 29 C deletion, which causes a frame shift and premature stop codon. All of these patients are suffering from microthrombocytopenia. More detailed patient characteresitics are shown in **Table 1.**

Ethical Review

All experiments were performed under institutional guidelines. Collection of PB from healthy volunteers and patients was approved by the ethics committee of the Institute of Medical Science at the University of Tokyo. Animal experiments were performed with approval of Institutional Animal Care and Use Committee of the Institute of Medical Science, University of Tokyo.

Establishment of iPSC

Peripheral blood was collected from either XLT or WAS patients as well as healthy individuals after written informed consent was obtained for each case. CD34+ cells were isolated using the Micro Beads (Miltenyi Biotec, Bergisch Gladbach, Germany) from mononuclear cells of peripheral blood. These cells were pre-stimulated in IMDM medium (Invitrogen, Grand Island, NY, USA), containing 15% Fetal Bovine Serum (FBS; Invitrogen) , 50 ng/ml human Stem Cell Factor (SCF), 20 ng/ml Granulocyte-colony stimulating factor (G-CSF), 10 ng/ml Interleukin-3 (IL-3) , 10 ng/ml Interleukin-6 (IL-6), 50 ng/ml human Thrombopoietin (TPO; all from R&D System, Minneapolis, MN), and 50 ng/ml human FMS-related tyrosine kinase 3 ligand (FLT3-L; PeproTech, Rocky Hill, NJ, USA) for overnight at incubator (37^0C, 5% co2 CO_2, 20% O_2). On the following day, cells were transduced by Sendai virus vector with four Yamanaka factors (SOX2, KLF4, OCT3/4 and c-MYC). Three to 5 days later, cells were transferred onto feeder layers of mouse embryonic fibroblasts (MEF), which were irradiated at 50 Gy and medium was replaced from the one described above to the one capable of maintaining ES/iPSC culture (Dulbecco's modified Eagle's medium/F-12 medium [DMEM/F-12; Sigma, St. Louis, MO] supplemented with 0.1 mM nonessential amino acid [Invitrogen], 1x penicillin streptomycin glutamine [Sigma], 20% knockout serum replacement [KSR; Invitrogen], 0.1 mM 2-mercaptoethanol [Invitrogen] and 5 ng/ml recombinant human basic fibroblast growth factor [bFGF; WakoPure Chem., Osaka, Japan]. iPSC like colonies were picked around day 25 and transferred to fresh MEF feeders. Maintenance of iPSCs were performed either using feeder-free culture condition based on Essential 8^{TM} medium (Life

Technologies, Grand Island, NY, USA) or by on-feeder culture with irradiated MEF feeders in DMEM supplemented with 20% KSR (Invitrogen) and 5 ng/ml basic fibroblast growth factor (bFGF; WakoPure) as previously described [80].

A special screening step of patient number 3-iPSCs

The status of Pt3 was post-bone marrow transplantation, showing that his peripheral blood did not show 100% donor cell chimerism. This meant that in order to obtain patient-own iPSCs I needed to exclude those derived from transplantation-donor cells. I primarily picked up 15 iPSC clones and checked the patient-specific gene mutation in them. Out of those clones, 11 clones were demonstrated to harbor the mutant gene. To further exclude any contamination of donor-cells, three primary iPSC clones were subjected to single cell sorting and cultured to obtain sub-clones. Finally, established sub-clones were also checked for the mutation. After confirmation of existence of patient –specific mutation in sub-clones, one sub-clone used for experiments.

Single cell sorting of iPSCs

To obtain subclones derived from single-cells, iPSCs (cultured in E8 medium) were washed with PBS, trypsinized (trypsin containing 0.25mM EDTA) for 3 minutes, and quickly collected in a tube containing medium with ROCK inhibitor. Single cells were sorted using FACSAria^{TM} II (BD Biosciences, San Jose, CA, USA) directly onto feeder layers of MEF

prepared in 96-well dishes. Clones capable of showing stable growth in this condition were selected and used in subsequent experiments.

Immunohistochemistry

iPSCs were washed with PBS and fixed by 4% paraformaldehyde. After two times washes with rinse buffer (20 mM Tris-HCl pH=7.4, 0.05% tween 20 and 0.15 M NaCl), cells were permeabilized with 0.1% triton X-100 (Sigma) for 10 minutes. After washing with rinse buffer, cells were incubated with 4% goat serum for overnight in 4^0C. Antibodies against human SSEA4 (abcam, Cambridge, UK), Tra1-60, and Tra1-81 (both from Millipore, Temecula, CA, USA) were used as primary antibodies with one-hour incubation at room temperature. Cells were washed with rinse buffer followed by one-hour incubation with Alexa 546-anti-mouse IgG antibody (invitrogen) at room temperature. After staining with DAPI (Sigma), cell images were analyzed using epiflurecence microscope (Zeiss, Oberkochen, Germany).

Alkaline phosphatase (AP) staining

AP assay was conducted using Vector Red Alkaline Phosphatase Substrate Kit (Vector Laboratories, Burlingame, CA, USA) according to manufacturer's instructions.

Karyotyping

Karyotyping of iPSCs was performed by Nihon Gene Research Laboratories.

Teratoma formation

For teratoma formation assay, iPSCs were trypsinized and resuspended in PBS. Aliquots of cells (3 x 10^6) were injected into the testes of anesthetized male NOD/SCID mice. Mice were sacrificed after ~8 weeks and the dissected tumor samples were fixed in 4% paraformaldehyde and proceeded for hematoxylin & eosin (HE) staining.

Sequencing for patient's specific mutations

Genomic DNA samples were extracted from MEF-free iPSCs using NucleoSpin Tissue XS kit (MACHEREY-NAGEL, Duren, Germany) from 1x10^5 cells and used to as template in PCR reactions.

PCR products extracted from the gel using QIAquick gel extraction kit (QIAGEN, Hilden, Germany) were subjected for sequencing analysis. List of primers used for the procedures is shown in **Table 2**.

Western blot analysis

For Western Blotting, cells were collected on day 24 of differentiation. Cell lysate was prepared by adding lysis buffer (50 mM Tris-HCL, 300 mM NaCl, 2 mM EDTA and 0.5% Triton-X 100) to the cell pellet. Protein concentration was measured using Pierce® BCA

Protein Assay Kit (Thermo Scientific, Rockford, IL USA). Lysate was dissolved in Sample Buffer (containing 2ME with final concentration of 10%) and heated for 3 minutes at 97ºC before use. Each sample was separated by SDS-PAGE, and electro blotted onto a polyvinylidene difluorid (PVDF) transfer membrane (Millopore). After blocking the membrane for one hour at room temperature with blocking solution (0.5% Skim Milk in TBST [10 mM Tris-HCl, 150 mM NaCl and 0.1% Tween 20]), it was probed with primary antibody against WASp (abcam, Cambridge, UK) at 4ºC for overnight. Membrane was washed with TBST and incubated with horseradish peroxidase-conjugated secondary antibody (GE Healthcare UK, Little Chalfont, UK) in the following day and after washing with TBST, labeled proteins were detected using Super Signal West Pico Chemiluminescent Substrate (Thermo). The probes were stripped with Blot Restore Membrane Rejuvenation Kit (Chemicon International, Temecula, CA, USA) and prepared for the detection of GAPDH.

Differentiation of iPSCs

Differentiation of iPSCs to megakaryocytes was performed as previously described [80]. C3H10T1/2 cells were irradiated by 50 Gy or treated with mytomycin C for two hours before use, and seeded into 100-mm dishes at 8 x 10^5 cells. iPSCs were transferred onto treated C3H10T1/2 cells and cultured in differentiation medium containing VEGF (20 ng/ml; R & D). Culture was fed with fresh medium every 3 days until sac-like structures (SAC) appeared by containing hematopoietic progenitor cell (HPC)s within each. On day 14 or 15, HPCs were harvested and seeded onto C3H10T1/2 cell feeder layers freshly

prepared in 6-well plates. HPCs were cultured for another 9 days in the presence of 100 ng/ml TPO (R & D), 50 ng/ml SCF (R & D) and 25 unit/ml heparin (Ajinomoto, Tokyo, Japan), and megakaryocytes and platelets were collected for analysis.

Quantification of Megakaryocyte and Platelets

On day 14 of SAC formation, HPCs were harvested and passed through a 40 μm cell-strainer (BD Bioscience). These cells were stained with APC-anti-CD34 antibody (Biolegand, San Diego, California) and FITC-anti-CD41 antibody (Biolegand) for 20 minutes on ice and used for sorting after washing with staining medium. Fixed number of sorted cells were seeded onto C3H10T1/2 cells in 6-well dishes (3×10^4 cells/well) containing the differentiation medium with 100 ng/ml TPO, 50 ng/ml SCF and 25 unit/ml heparin. The culture was fed by fresh medium on days 17 and 20, to keep active cytokines. Quantification of megakaryocytes and platelets was performed on day 23 of differentiation. Cells were gently collected by the use of Anticoagulant Citrate Dextrose solution (ACD), followed by staining with antibodies against human CD41a (FITC; Biolegand) and CD42a (PE; BD Bioscience) antigens. For quantification, megakaryocytes and platelets were respectively counted using Flow-Count™ Fluorospheres beads (Beckman Coulter, Brea, CA, USA), and TruCount™ tubes (BD).

Proplatelet formation assay

Hematopoietic progenitor cells were harvested from SACs on day 14 of differentiation, then seeded at 5 x 10^4 cells onto irradiated C3H10T1/2 cells in grid dishes (CORNING. NY, USA). The same protocol and cytokine combination as described in megakaryocyte differentiation were applied to the cells. Proplatelet-bearing cells were counted on day 23 of culture.

Hematopoietic colony-forming assay

Hematopoietic colony-forming assay was performed using MethoCult H4434 semisolid medium (STEMCELL Technologies Inc. Vancouver, Canada). iPSCs (HD-iPSC, Pt1-iPSC, Pt2-iPSC and Pt3-iPSC) were seeded onto a layer of C3H10T1/2 cells. After 14 days, SAC were harvested and stained with antibodies against CD 34 (PECy 7), CD 43 (APC), CD 45 (APC Cy7), CD 235a and CD41 (FITC) (all from Biolegands) for 30 minutes on ice. Then cells were washed whit staining medium and used for cell sorting. 4x10^3 hematopoietic progenitor cells ($CD41^-$, $CD235a^-$, CD 34^+, $CD45^+$, and $CD43^+$) were sorted and seeded on MethoCult medium. Cells were incubated in 37^o C in 5%CO2. After 14 days colonies were counted.

Phosphorylation of signaling molecules downstream of MPL

Hematopoietic progenitor cells harvested at day 14 of SAC formation from iPSCs were cytokine-starved for three hours in IMDM medium containing 0.05 % FBS. At first cells were stained with Zombie Aqua Fixable Viability Kit (Biolegend) to mark dead/dying cells,

then cells were washed with IMDM and stained with CD34-APC mAb (eBioscience, San Diego, CA, USA) for 30 minutes at 37ºC. Stained cells were stimulated with 100 ng/ml TPO for 10 minutes at 37 ºC and immediately fixed with Lys/Fix buffer (BD) for 10 minutes at 37 ºC. Permeabilization was done using Perm buffer III (BD) for 30 minutes on ice. Samples washed with staining medium for three times were then proceeded for immunostaining with pSTAT5, pAKT and mAbs (BD) in room temperature for one hour. Finally samples were washed with staining medium and used for flow cytometry analysis.

Puromycin-resistant C3H10T1/2 cell line

Parental C3H10T1/2 cells were used to generate a puromycin-resistant cell line. On day 1, cells were seeded into 6-well plates ($5x10^5$ cell/well) and incubated overnight. On the following day (day 2), cells were transduced with the lentivirus (MOI = 5) harboring puromycin resistance gene downstream of the Ubc (Ubiquitin-C) promoter. On day 3, cells were passaged to 100-mm dishes, followed by the selection culture started on day 4 in the presence of 1 μg/ml puromycin (Invitrogen). After 1 week, viable cells were obtained and considered to be puromycin-resistant. These cells were used in subsequent experiments for differentiation of transduced iPSCs.

Stable transduction of iPSCs

The iPSC clone from Pt3 was used for gene transfer studies. iPSCs were seeded at $1x10^5$/well in 24 well plates, which were coated with matrigel (BD) for one hour at room temperature, then maintained for overnight in the presence of ROCK inhibitor.

Transduction of iPSCs were performed on the following day by adding lentiviral supernatant at MOI = 20. Cells were incubated for 6 hours in 5% CO_2, followed by medium change. On the next day, cells were passaged to 12 well plates and maintained for another two days. Transduced cells were then subjected to selection pressure in the presence of 1 μg/ml of puromycin (Invitrogen). Thereafter, established puromycin-resistant iPSCs were maintained in the presence of 1μg/ml of puromycin, and subjected to differentiation culture also under the selection pressure.

Intracellular FACS analysis of WASp expression

Megakaryocytes were collected on day 24 of culture and used for surface staining as described above. After washing with staining medium, cells were fixed for 20 minutes in 4^0 C using Perm/Fix Solution, followed by washing with 1x Perm/Wash solution (both from BD). Permeabilized cells were then stained with anti-WASp antibody (abcam) for 30 minutes on ice. After washing with Perm/Wash solution, cells were stained with Alexa 647-conjugated anti-rabbit IgG antibody (Invitrogen) for 20 minutes, washed with Perm/Wash solution, and subjected to flow cytometry analysis.

Statistics

Statistical difference in the values between samples was estimated by using Student's *t* test.

Results

Successful reprograming of patient-derived hematopoietic cells having defects in WASp to iPSCs

For modeling XLT/WAS disorders, three patients were available each having distinct mutations in *WAS* gene (Table 1). I were successful in obtaining colonies showing ES cell-like morphologies from all these patients, by our established method using Sendai virus-mediated Yamanaka factor-transduction into peripheral blood hematopoietic progenitor cells. Characterization of iPSC lines revealed that all iPSC clones retained typical characterization of pluripotent stem cells. As shown, established iPSCs expressed pluripotency markers such as SSEA-4, Tra1-60, and Tra1-81 **(Figure 3, A)**. In addition, Alkaline phosphatase (AP) staining of iPSC clones were all positive **(Figure 3, B)**. Karyotyping analysis of iPSC clones confirmed normal chromosome numbers in all samples **(Figure 3, C)**. As a more rigorous assay for pluripotency, I assessed teratoma formation ability of these iPSCs. As a result, I confirmed teratoma formation in NOD/SCID mouse tissues **(Figure 4, A)**, and observed three germ layers in those teratoma tissues by the immunohistochemistry analysis **(Figure 4, B)**. I thus concluded that WASp defects would not negatively impact on the reprogramming process despite its known importance in many cellular functions such as actin cytoskeleton regulation, and that iPSC-based modeling could be applicable to XLT/WAS patients.

XLT/WAS-iPSCs phenocopy WASp deficiency at the molecular levels

As first as a disease modeling study, I analyzed patient-specific mutations by sequencing genomic DNA samples obtained from each iPSC line derived from three patients. As shown, I identified patient-own mutations in two XLT patient samples (168 C>T in exon 2 of Pt1-iPSCs and 1066 del CC in exon 10 of Pt2-iPSCs) **(Figure 5, A)**. Patient-specific mutation was also confirmed for a WAS patient (29 del G) **(Pt 3, Figure 5, A)**. To confirm further the patient characteristics, I utilized mature megakaryocytes differentiated from each iPSC line to test the WASp expression level. As shown in **Figure 5 B,** Western blot analysis detected WASp expression in healthy donor-derived cells differentiated from iPSCs on day 24, despite with the level lower than that of Jurkat cells, a control T-cell leukemic line. In contrary, WASp was not detectable for both XLT and WAS patient samples. The disease modeling using iPSCs thus revealed successful so far by recapturing both genetic defects and defective protein expression in samples derived from patients with both XLT and WAS.

Impaired ability of patient-derived iPSCs to produce megakaryocytes and platelets

I then tested if I could recapitulate the platelet abnormality characteristic to XLT/WAS patients by using established iPSC lines. For comparison, similarly established iPSCs from healthy individuals (HD) were used. Differentiation protocol of iPSCs to the megakaryocytes and platelets with a high yield has been established in our laboratory

previously [80]. A schematic diagram of the protocol is shown in **Figure 6, A.** When I performed the differentiation culture following this protocol, SAC structures appeared at day 14 in which emergence of hematopoietic progenitor cells (HPC) was visible **(Figure 6, B)**. After harvest, these cells were shown to contain megakaryocyte/erythrocyte progenitor cells (MEP), which were distinguished as a fraction of cells displaying a $CD34^{+}CD41^{+}$ immunophenotype **(Figure 6, C).** To allow comparative analysis, this MEP fraction was sorted at a fixed number into megakaryocyte/platelet differentiation culture, followed by further cultivation for 9 days. As shown, megakaryocytes/platelets were clearly identifiable as the events positive for both CD41 and CD42a **(Figure 6, D)**, and were, quantifiable.

Having this comparative measure available, the ability to produce megakaryocytes/platelets was compared between each patient sample and HD cells. To minimize the effect of clonal variation, randomly picked 5 clones from each sample were simultaneously subjected to the analysis (for Pt 3, one sub-clone iPSC was used for the analysis). As consequence, both XLT patients (Pt1 and Pt2) as well as WAS patient (Pt3) samples produced significantly smaller numbers of megakaryocytes than those from the healthy counterpart **(Figure 7, top)**. At the same time, absolute counts of platelets obtained from patient samples were significantly less than those from HD samples **(Figure 7, bottom)**.

Sustained defect in platelet production

Reduced numbers of platelets observed in XLT/WAS samples could be as a result of premature platelet release from megakaryocyte in culture. Alternatively, platelet production could be totally impaired all through the differentiation culture by the defects in

WASp molecule. To know more precisely the kinetics of platelet production, I counted platelets at different times of differentiation culture. In our culture system, proplatelet formation begins as early as day 19 in the iPSC differentiation protocol as a primary step of platelet production (data not shown). I thus counted platelets starting from day 20, then every the other days until day 26, by comparing XLT patients' samples along with the HD control sample. The decrease in platelet numbers was already evident for XLT samples at as early as day 20, and was consistently observed during the observation period up to day 26 **(Figure 8, A)**. The data therefore support the idea that reduced numbers of platelets due to WASp defects were attributable primarily to impaired overall production, not to altered kinetics of differentiation/maturation of megakaryocytes.

Reduced proplatelet-forming cells in Mk differentiation culture system

It is well known that one sign of maturation of megakaryocytes is elongation of their demarcation membrane system accompanied by production of tubulin, leading to generation of extension structures, which are called proplatelet-formation. Platelets are then produced from these cell extensions. Therefore I next compared XLT/WAS megakaryocyte progenitor cells with HD counterparts for their capability of forming proplatelet-bearing cells, the efficiency of which would faithfully reflect platelet production efficacy. On day 14 of SAC formation, fixed numbers of sorted MEP cells ($CD41^+CD34^+$) were applied for this estimation, and the cells with proplatelet-like structures **(Figure 8, B)** were counted on day 23. As shown in **Figure 8, C**, significantly reduced numbers of proplatelet-bearing

cells were observed for both XLT and WAS samples comparing with the healthy samples. Together with the results shown in **Figure 7**, these results indicate that the defects in WASp significantly impair differentiation/maturation processes of megakaryocyte-lineage cells.

Unaltered platelet production ability of WASp-defective megakaryocytes

The overall platelet production process may be divided into two phases. The first phase represents the process of megakaryocyte differentiation and maturation accompanied by proplatelet elongation. The second phase includes proplatelet maturation and platelet release.

So far our data indicate that both XLT and WAS patient samples have abnormal megakaryocytogenesis. I next sought to test the ability to produce platelets in each individual iPSC-derived megakaryocyte. At first, megakaryocytes on day 24 did not show significant morphological difference between the ones obtained from XLT/WAS-iPSCs and the other from healthy-iPSCs **(Figure 9, A)**. Interestingly, when the same number of megakaryocytes from each sample were allowed to produce platelets, no significant difference was observed between HD control and patient cells **(Figure 9, B)**. The data suggest that the machinery to produce platelets may not be severely compromised in megakaryocytes by WASp deficiency.

Possible role of WASp for maintenance of multipotency or fate decision upon differentiation in hematopoietic progenitor cells

Our data so far suggest that hematopoietic progenitor cells rendered WASp-defective have defects in the process of differentiation/maturation to become proplatelet-bearing cells in the presence of cytokines (SCF and TPO). Of note is that it has been proposed that intact WASp is critical for maintenance of hematopoietic stem and progenitor cells by the observation of non-random inactivation of X-chromosome in those cells of heterozygous female carriers [81], [82]. WASp is also implicated to have role in hematopoietic cell proliferation and differentiation *in vitro* [50]. I thus next examined how WASp deficiency would affect colony formation ability of HPCs using our iPSC samples. To start with, I sorted HPC populations (CD 41^-, $CD235^-$, $CD34^+$, $CD43^+$, $CD45^+$ cells) that formed within SAC on day 14 of culture from either patients' or healthy samples. These cells were then allowed to form hematopoietic colonies in methylcellulose medium in the presence of SCF, GM-CSF, IL-3, and erythropoietin. Interestingly, test HPCs significantly differed in the capability to form hematopoietic colonies and their cellular composition, depending on the types of WASp mutation **(Figure 10)**. Regarding the GM/M colonies, all the patient samples showed modest, but significant reduction in numbers comparing with the healthy control (blue histograms): Pt1, 46.6 ± 2.6; Pt2, 31.6 ± 5.4; Pt3, 46.3 ± 5.4; HD, 58.3 ± 2.4 (# of colonies per 4 x 10^3 HPCs, mean ± SD). As for erythroid colonies including BFU-E and CFU-E, the results varied considerably among three patients. HPCs of Pt1 having an amino acid substitution in its EvH1 domain of WASp (see Table 1) showed comparable numbers of BFU-E/CFU-E colonies (11.6 ± 3.8) to those formed in HD culture (13.6 ± 3.0). In sharp contrast, Pt2 HPCs having a 2-bp deletion compatible with C-terminal truncation in WASp

(Table 1) produced significantly more erythroid-lineage colonies (49.3 ± 3.3) than the above two samples (orange histograms). It was remarkable to see that HPCs derived from Pt3 iPSCs, which were compatible with complete absence of WASp (Table 1) formed only marginal numbers of BFU-E/CFU-E colonies (0.6 ± 0.4). Far more strikingly, Pt3 HPCs also failed to yield GEMM colonies (gray histograms), indicating that in the near total absence of WASp multipotent primitive cells did not develop well or were severely impaired in their survival/proliferation potential. In contrast, both XLT HPCs produced GEMM colonies at similar numbers (14.6 ± 2.8 and 17.6 ± 7.9 for Pt1 and Pt2, respectively) as those yielded by HD sample (9 ± 2.1). All together, these results suggest that WASp has critical role in HPCs, affecting their differentiation properties and/or multilineage potential.

Involvement of WASp in TPO/MPL signaling

A recent study implies that WASp plays an important role in Kit function and biology [83]. Although TPO is only the cytokine except for SCF, which I used as the main stimulator of megakaryocyte differentiation, whether WASp plays role in TPO/MPL signaling remains unknown. I thus sought for that possibility. At first I examined the expression of MPL on the surface of megakaryocytes ($CD41^{+}CD42a^{+}$) derived from patient and HD cells. As shown in **Figure 11, A**, there was no alteration in the expression level of the receptor in patient samples comparing with the healthy counterpart.

I then tested if WASp-deficiency affected the phosphorylation of signaling molecules downstream of MPL upon stimulation. HPCs harvested from SAC on day 14 of differentiation were stimulated for 10 min with 100 ng/ml TPO after 3-hour starvation and staining with CD34 mAb. Among the signaling molecules tested, flow cytometry analysis revealed blunted phosphorylation kinetics in STAT5, AKT for the Pt3 samples **(Figure 11, B)**. Therefore, these data suggest that the alteration of TPO/MPL signaling due to WASp-deficiency partly explain impaired megakaryocyte differentiation/maturation processes leading to formation of proplatelet-bearing cells.

Lentiviral vector-mediated transfer of WASp partially reverses megakaryocyte/platelet abnormalities in differentiation culture modeling WAS.

To test how efficiently add-back expression of WASp could rescue the defects in megakaryocyte/platelet production, I performed lentivirus-mediated transduction of Pt3-iPSCs, representing a "WASp-null" model. To this aim, I used the SIN-LV backbone vector containing Ubiquitin-C promoter (Ubq) and the puromycin-resistant gene (Puro) for drug-selection of transduced cells; this was used as control vector (Ubq-Puro) **(Figure 12, A).** Therapeutic vector contained additionally *WASp* cDNA under the Ubq promoter, followed by 2A peptide sequence and Puro (Ubq-WAS-Puro). After LV transduction, Pt3-iPSCs were selected by puromycin. Of note is that I continued to add the selection pressure for the entire period of megakaryocyte/platelet differentiation. As shown in **Figure 12, B**, the cells transduced with the therapeutic vector (Ubc-WAS-Puro) showed modest, but

statistically significant levels of increase in both megakaryocyte and platelet numbers, in comparison with those treated with the control vector (Ubc-Puro)($p < 0.05$). I next examined intracellular expression of WASp in megakaryocytes by using multi-color flow cytometry analysis. As shown in **Figure 12, C**, Pt3-megakaryocytes treated with the control vector showed virtually no expression of WASp (Ubq-Puro), whereas healthy control exhibited WASp expression at a remarkable level (HD). Expression of WASp became clearly detectable in Pt3 samples when transduced with the therapeutic vector (Ubq-WAS-Puro). However, the expression level of WASp in these samples did not reach the one observed in HD cells, likely consistent with the marginal level of improvement in megakaryocyte/platelet production by these gene transfer procedures. These results indicate that iPSC-based WAS modeling is useful in studies aiming at elucidation of pathophysiology, and may also be used in optimizing therapeutic modalities to improve the clinical gene therapy by maximizing its efficacy while by minimizing the inherent risks.

Discussion

I showed that using iPSCs disease modeling of Wiskott Aldrich Syndrome was feasible. By utilizing this measure, I could add another layer of understanding for the mechanisms underlying thrombocytopenia in XLT/WAS patients. In our system, the major defect caused by WASp-deficiency was identified in the process of HPC differentiation leading to production of proplatelet-bearing cells, rather than in the ability of each megakaryocyte to produce platelets. Our results so far support the idea that the process of platelet production is primarily defective in XLT/WAS patients, consistent with other group's studies [12], [47], [50], [51], although I cannot completely reject the theory of exacerbated platelet destruction in the patients as suggested in some other studies [39]-[45]. In relation to the latter, mechanisms of rapid platelet consumption was addressed by Marathe *et al* [84]. This study suggested two possible mechanisms inducing rapid platelet turnover using a murine WAS model; an antibody-dependent mechanism (WASp deficiency results in increased incidence of antiplatelet antibodies) and an antibody-independent mechanism (increased sensitivity to antibody-induced platelet clearance). Noteworthy, it has been shown that splenectomy leads to increase in platelet numbers in patients as well as murine models [49], but the counts usually doesn't get normalized. So it seems that sequestration of platelets in the spleen is not merely the cause of thrombocytopenia in XLT/WAS patients.

In the past, pathophysiology studies for WAS/XLT have been conducted mainly using patient primary samples. As our results indicated the defects existing in the process of megakaryocyte production, one may speculate that XLT/WAS patients should have a decrease in megakaryocytes *in vivo*. However, it seems not the case because Ochs *et al*.

[47] originally reported an increase in megakaryocytes in one WAS patient, and normal counts for two other patients. Bone marrow samples taken by aspiration would represent just a limited area within the total marrow in the body, thus may lead to inaccurate estimation. Besides, there may be compensatory mechanisms operating *in vivo* to prevent excess loss of these exceptionally important cells for human lives in the absence of functional WASp expression. As for *in vitro* studies, variable conclusions have been made for the effects of WASp deficiency on megakaryopoiesis. Haddad *et al*. reported that XLT/WAS patient CD 34 $^{+}$ cells (from BM, with some exception of PB) showed no gross abnormality in megakaryocyte differentiation and proplatelet formation. As shown in a different setting reported by Luthi *et al*. [12], there was a case that XLT patient $CD34^{+}$ cells (though these were from PB) produced more CFU-MKs than control. Regarding the relationship between genotypes and phenotypes, Kajiwara *et al*. [50] reported difference between XLT and WAS patients using their BM $CD34^{+}$ cells as test sources. Overall, WAS cells showed severer defects than XLT counterparts, with significant decrease observed in production of megakaryocyte colonies, GM colonies, erythroid colonies, and proplatelet-forming cells. XLT progenitors exhibited apparent defects only in proplatelet-forming cell production.

So far, no reports have been published describing a modeling hematopoietic abnormality in XLT/WAS patients using iPSCs. The fact may raise a question how relevant the iPSC-based modeling can be for this setting. I concluded that our data were mostly consistent with those reported by Kajiwara *et al*., thus supporting utility of this method for XLT/WAS modeling. Taking two results into consideration, it seems reasonable to state that there is

significant difference in biological consequences between XLT-compatible WASp mutations and the ones causing WAS phenotypes. The rationale explanation for the difference may be that WASp mutations causing XLT only affect the late stage in megakaryocyte differentiation (proplatelet-bearing cell production), but not the early stage including HPC proliferation (primitive colony formation) and initial differentiation into megakaryocyte lineages. In contrast, WAS-compatible mutations in WASp should negatively impact cells at both stages. Although there remain some inconsistency between our results and Kajiwara's, the variability in initial test populations for each assay may constitute the major reason. They used $CD34^+$ cells isolated from bone marrow all through the experiments. In contrast, I used progenitor populations with a $CD34^+CD43^+CD45^+$ phenotype but not expressing lineage-markers (CD41 , CD235a) for colony-forming cell assays, and $CD34^+CD41^+$ megakaryocyte progenitors for quantification of the final yields in megakaryocytes and platelets. Of note, similar sorting technology for the first time allowed us to test the ability to produce platelets in each megakaryocytic cell. Our data demonstrated that megakaryocytes of patients were immunophenotypically normal and were capable of producing comparable numbers of platelets per cell comparing to the healthy donor samples. I believe that by using iPSC-based disease modeling, further dissection will become possible for the mechanisms at what processes WASp-deficiency affects hematopoiesis leading to megakaryocyte/thrombocyte genesis.

One unique finding obtained in this study is highly variable results depending on the mutation types of WASp observed in a hematopoietic colony assay. HPCs derived from Pt3 iPSCs failed to form the most primitive colonies, consistent with the severe type of WASp

mutation. Interestingly, Pt2 HPCs produced increased numbers of erythroid colonies comparing with the others, including healthy counterparts. This XLT patient has a mutation in C-terminus of *WASp* gene, whereas another XLT one (Pt1) has a mutation in its N-terminus that has been associated with normal erythroid colonies. These results indicate that WASp may act on the fate decision of cells during differentiation, directly as a transcriptional modifier, or indirectly through other mechanisms such as alteration of signaling events.

Regarding signal transduction, there is a report showing involvement of WASp in KIT signaling [83]. Although it has been suggested that TPO signaling may also be affected by WASp [50], definitive evidence remains yet to be demonstrated. To date, various studies have demonstrated the important role of TPO in megakaryopoiesis as the major physiological regulator [85]–[88]. TPO/MPL signals are thought also to play a critical role in function of hematopoietic stem cells. On the other hand, a role of WASp in maintenance of hematopoietic stem cells has been suggested by a growth disadvantage for stem/progenitor cells harboring *WASp*-deficient gene observed in carrier female [81], [82]. In addition, Miki *et al*. have shown that WASp participates in megakaryocyte differentiation through tyrosine kinase signaling using a megakaryoblastic cell line [89]. Using our system, I were able to obtain some of evidence that WASp was involved in TPO signaling in hematopoietic progenitor cells. Having shown blunted phosphorylation of signaling molecules downstream of MPL in WASp-null progenitor cells, I concluded that WASp-deficiency could broadly affect signaling events that followed TPO stimulation. There is recombinant human TPO (rhTPO) available, which has been shown effective in

recovering thrombocytopenia for the patients with lung cancer [90], gynecological cancer [91] and ITP [92]. As the recent report showed less production of new platelets with eltrombopag (an oral small molecule as a TPO mimetic) treatment in XLT/WAS than ITP (55^{th} ASH Annual Meeting, 2013), our data may partially explain the difference by the blunted response in MPL signaling due to WASp-deficiency.

Finally I showed feasibility of modeling gene therapy by using disease-specific iPSCs. Currently, clinical trials of stem cell gene therapy (SCGT) for WAS patients are underway, showing so far good outcomes [72]. Although shown beneficial, however, the platelet count recovery remains not complete even in this one of the most successful cases of SCGT. In addition, the pioneering studies, in which SCGT was performed for WAS patients using the gammaretroviral vector resulted in occurrence of leukemias in several patients due to insertional mutagenesis [93]. These observations would prompt us for development of ideal SCGT protocols with maximum efficacy and minimum toxicity. By using the lentiviral vector harboring *WASp* cDNA under the Ubq promoter, I showed that the expression level of WASp after gene transfer in mature megakaryocytes did not reach the normal level, but was sufficient to increase the yields of megakaryocytes and platelets significantly. As mentioned, in a recent successful SCGT trial platelet recovery was incomplete. Interestingly, they reported that the WASp expression level after gene therapy varied in different hematopoietic lineages; it reached normal in T cells whereas it did not for platelets [68]. There is remarkable difference between two occasions, *i.e.*, our *in vitro* experiments and the above-mentioned clinical trial, in the use of promoter. Ours used the constitutive Ubq promoter, while the latter used a WAS 1.6 kb promoter. Despite this difference,

insufficient recovery in both WASp expression level and the platelet numbers was common observation. It remains unknown, which is necessary to achieve normalization of platelet yields by gene transfer, the more powerful constitutive promoter, or the promoter capable of perfect mimicry in endogenous WASp expression. As shown in this study, disease modeling for WAS using iPSCs is also useful for modeling gene therapy, giving us a precious platform to test various approaches to develop ultimate treatment protocols.

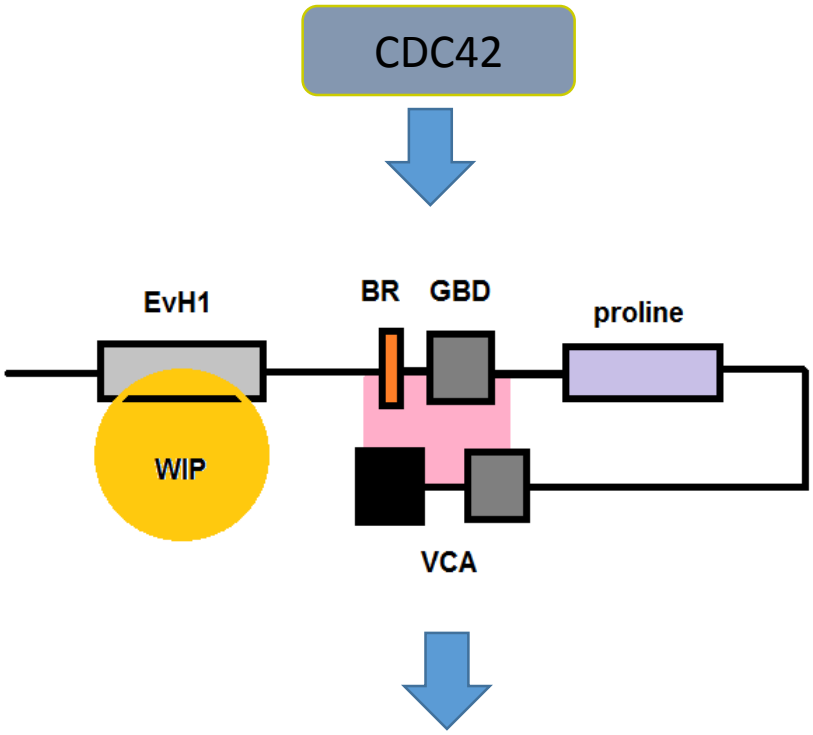

Figure 1. Schematic representation of WASp.

WIP protein biding to N-terminal is crucial for the stability of WASp. Affinity of VCA domain with BR/GBD forms an auto- inhibited state for WASp that conceals the binding sites for Arp 2/3 complex. Binding of CDC42 to the GBD domain release auto-inhibition structure leading to binding of VCA domain to Arp 2/3.

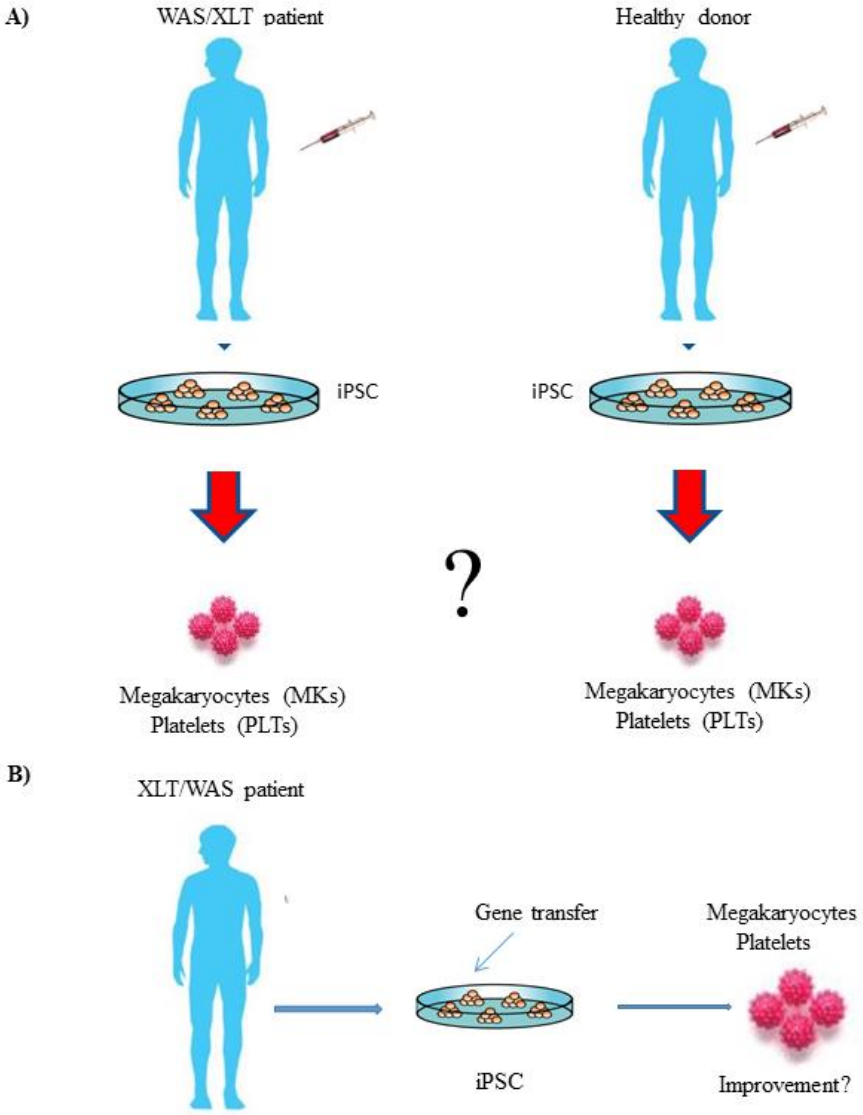

Figure 2. Schematic diagrams of experimental designs
A) Experimental design part one. *In vitro* Comparison of patient sample with healthy individual. Establishment of iPSC from XLT/WAS patients as well as healthy individual and differentiation to megakaryocytes and platelets with aim of investigation for any quantitative and qualitative difference.
B) Add- back of defected gene to the patient sample using lentivirus and seek for any improvement.

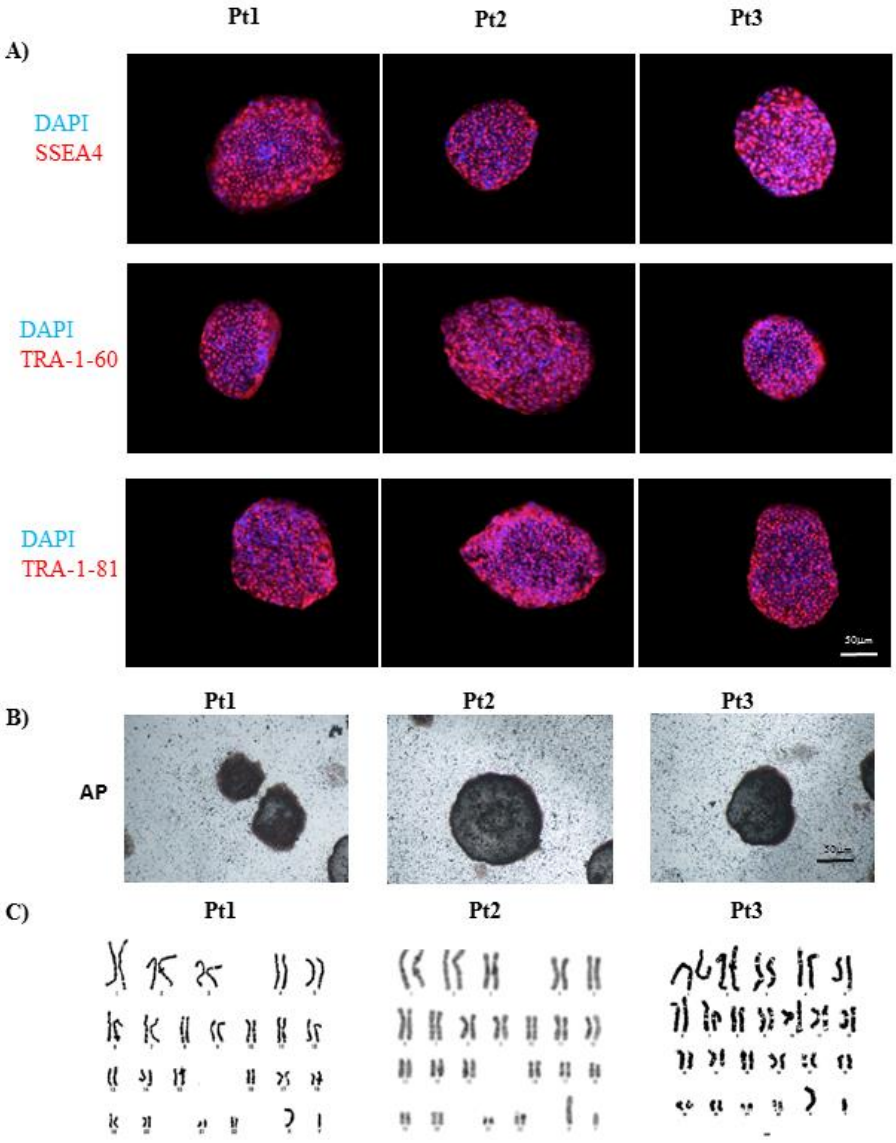

Figure 3. Generation of patient specific iPSCs.

A) Immunofluorescence staining of pluripotency markers (upper panel:SSEA-4, middle panel: Tra-1-60, lower panel: Tra-1-81) for XLT/WAS- iPSC clones. Pluripotency markers are retained in patient samples. Scale bar represents 50 µ m.

B) Alkaline phosphatase (AP) staining of XLT/WAS- iPSC clones. Scale bar represents 50 µ m.

C) Karyotyping analysis of XLT/WAS- iPSCs showing normal karyotype of patient samples after reprograming.

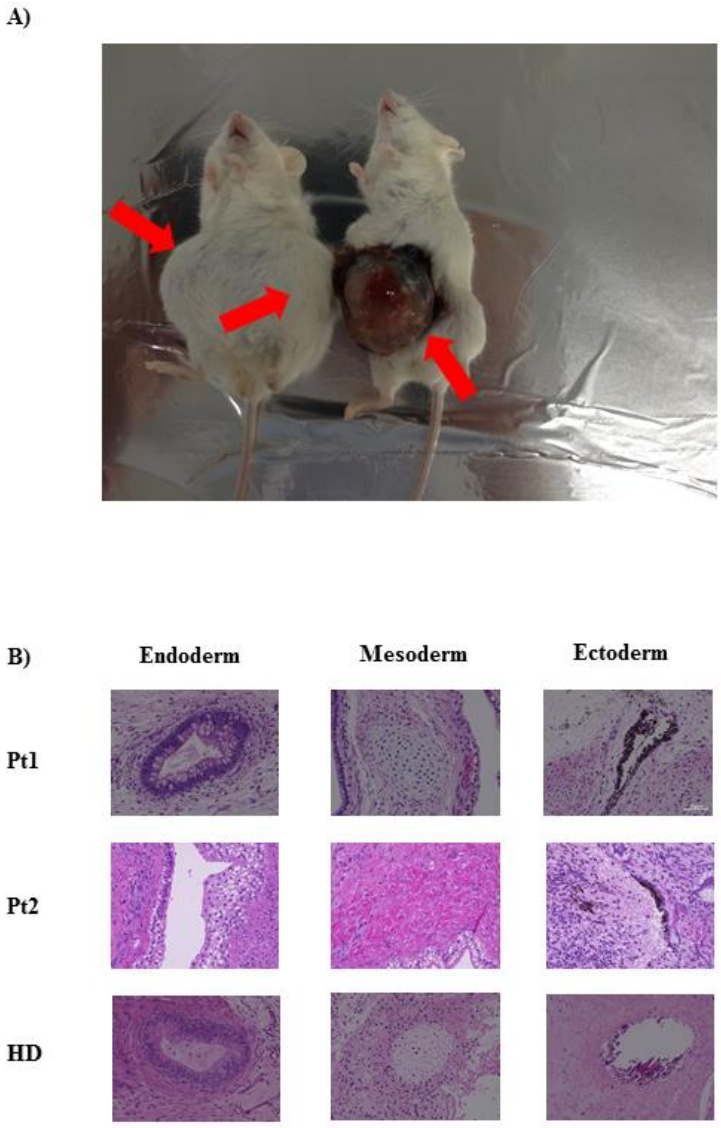

Figure 4. Teratoma formation by iPSCs.
A) Representative testis tumors (arrows) due to the injection of iPSCs.
B) Immunohistochemistry of three germ layers originating from teratoma tissues formed via XLT-iPSCs (Pt1 and Pt2) and healthy counterpart (HD). Scale bar represents 50 μ m.

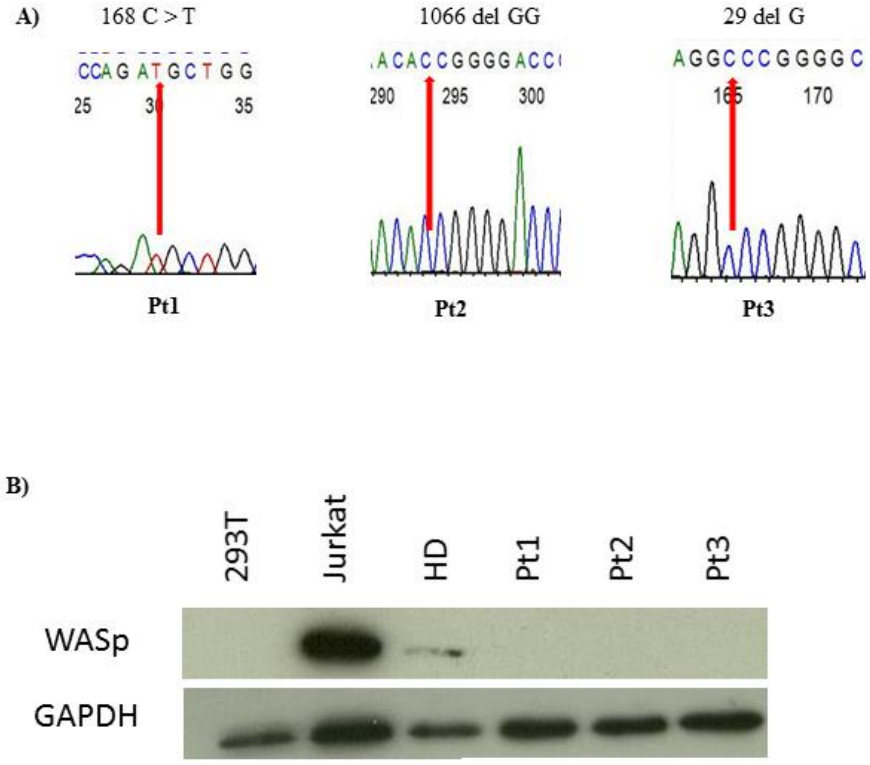

Figure 5. iPSC and iPSC derived megakaryocytes imitate patient characteristic
A) Patient specific mutations in iPSCs. Three patients have different mutation which is shown by DNA sequencing using genomic DNA of patient- derived iPSCs. Arrows point the site of mutation.
B) WASp expression level in differentiated XLT/WAS- iPSCs. At day 24 of differentiation of iPSCs to megakaryocytes, cells were collected and objected to western blot analysis. GAPDH antibody used as internal control. (Jurkat cells: positive control, 293 T cells: negative control)

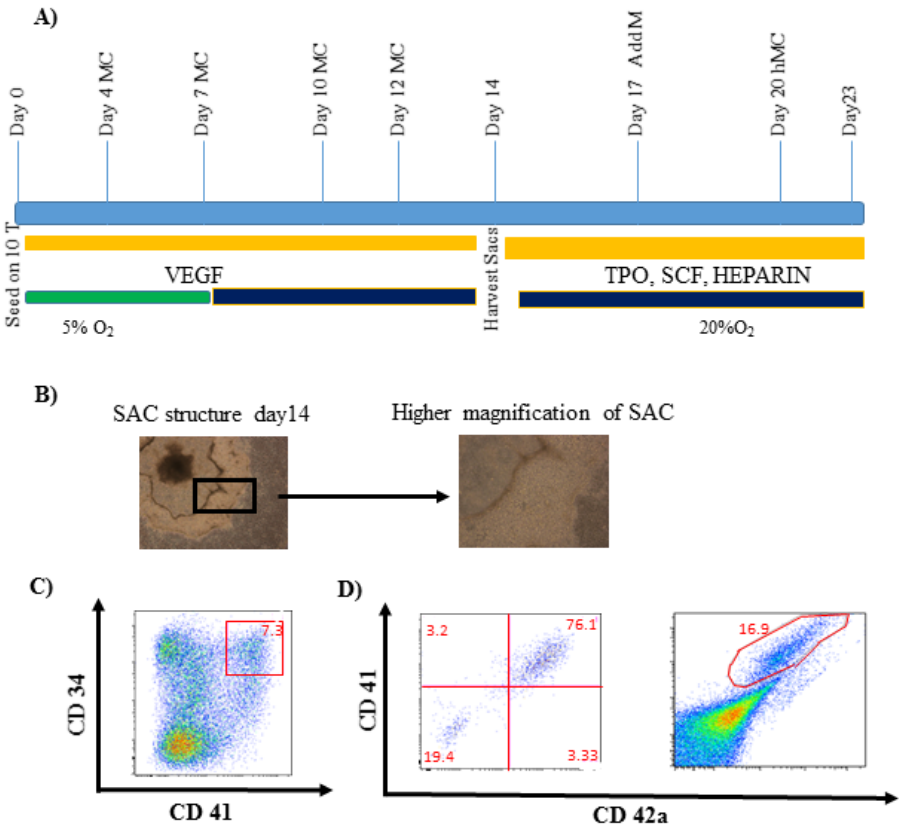

Figure 6. Differentiation of iPSCs to megakaryocytes and platelets

A) Schematic diagram of the experimental procedure for megakaryocyte and platelet differentiation from iPSCs. First iPSCs were seeded on CH310T1/2 cells and stimulated by VEGF for 14 days. Hematopoietic progenitor cells were harvested on day 14 and stimulated with various cytokines. Megakaryocytes and platelets analyed on day 23. MC: medium change, hMC: half medium change

B) Representative photomicrograph showing differentiated iPSC (SAC structure; Day14) in the presence of VEGF. A higher magnification view displaying SAC containing numerous bright, spherical HPCs (Hematopoietic Progenitor Cells).

C) Expression of hematopoietic progenitor cell markers, CD34 and CD 41. Double positive cell population (CD34+ CD 41+) on day 14 were sorted for quantification analysis of megakaryocytes and platelets.

D) Representative FACS pattern of megakaryocytes (left panel) and platelets (right panel) on day 23, expressing cell surface markers CD 41 and CD 42a.

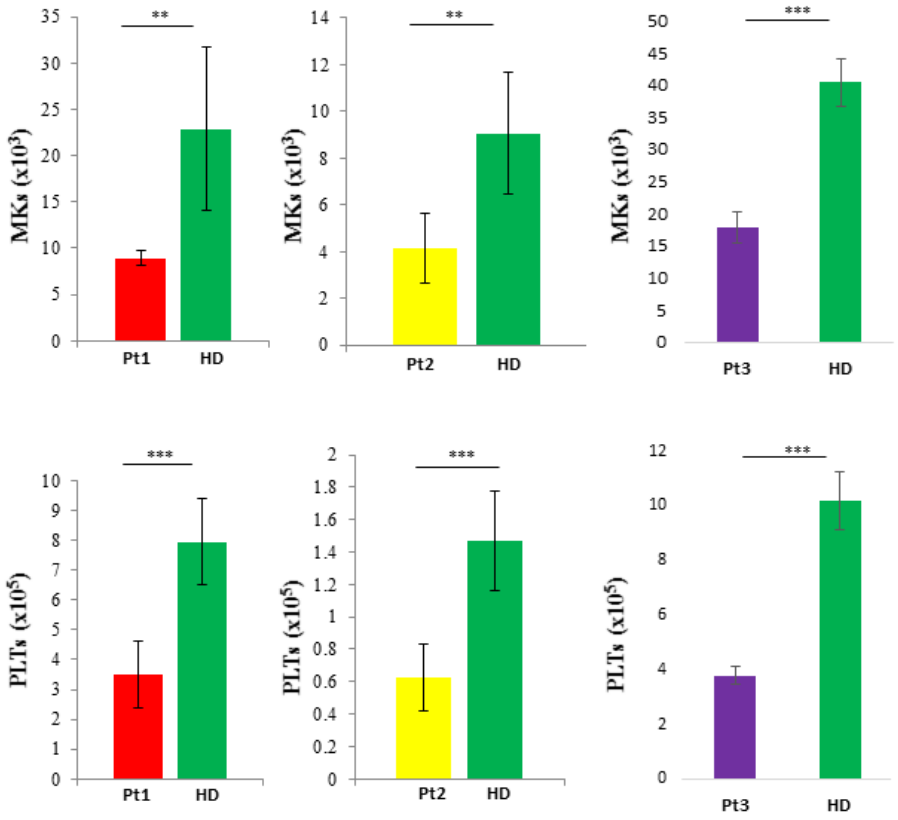

Figure 7. Decreased megakaryocyte and platelet counts in WAS/XLT patients.
Upper panel: Absolute numbers of megakaryocytes in XLT-Pt samples and WAS-Pt samples comparing to healthy counterpart (HD). Both XLT patients as well as WAS patient showed significantly reduced numbers of megakaryocyte comparing to HD.
$0.001 < P \leq 0.01$ for both Pt1 and Pt2, $0.0001 < P \leq 0.001$ for Pt3 samples (comparing to HD).
Lower panel: Absolute numbers of platelets in XLT-Pt samples and WAS-Pt samples comparing to healthy counterpart (HD). Both XLT patients as well as WAS patient showed significantly reduced numbers of platelets. $0.0001 < P \leq 0.001$ for all Pt samples comparing to HD.
Yields were quantified by FACS analysis with fluorescent beads. The results are represented as the mean absolute count ± SD. (Data are from 5 clones of each HD, Pt1 and Pt2. data related to WAS- iPSC are obtained from three independent experiments using one sub- clone)

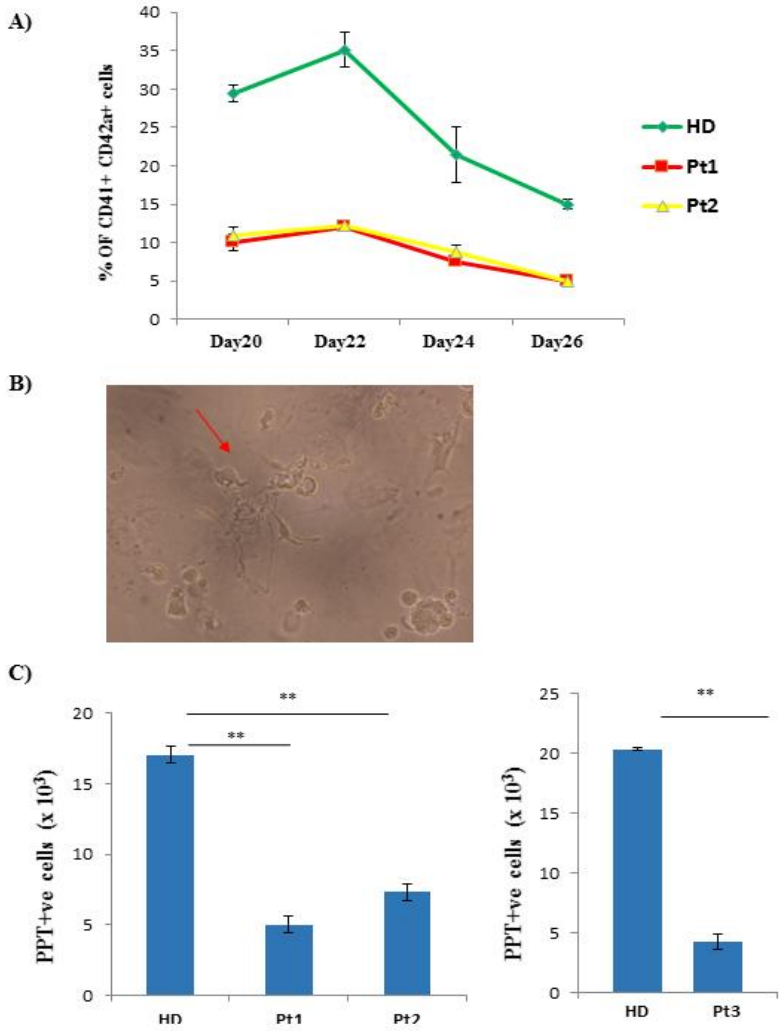

Figure 8. Reduced platelet count in patients is due to decreased proplatelet-bearing cell number.

A) Decrease in platelet numbers observed for XLT patient samples throughout the culture period. Quantification of platelets performed in different time points by FACS analysis. Results are represented as the mean platelet count ± SD (n=3).

B) Representative proplatelet-bearing megakaryocyte at day of counting (Day24) . Data is from HD.

C) Declined numbers of proplatelet-bearing cells (PPT+ve cells) in patient samples comparing to healthy sample. Proplatelet-bearing cells obtained from fixed initiate number of progenitor cells were counted using grid dishes. Both XLT patients as well as WAS patient showed significantly reduced number of PPT+ve cell. $0.001 < P \leq 0.01$ for all Pt samples comparing to HD. Results are represented as the mean PPT+ve cell counts ± SD (n=3).

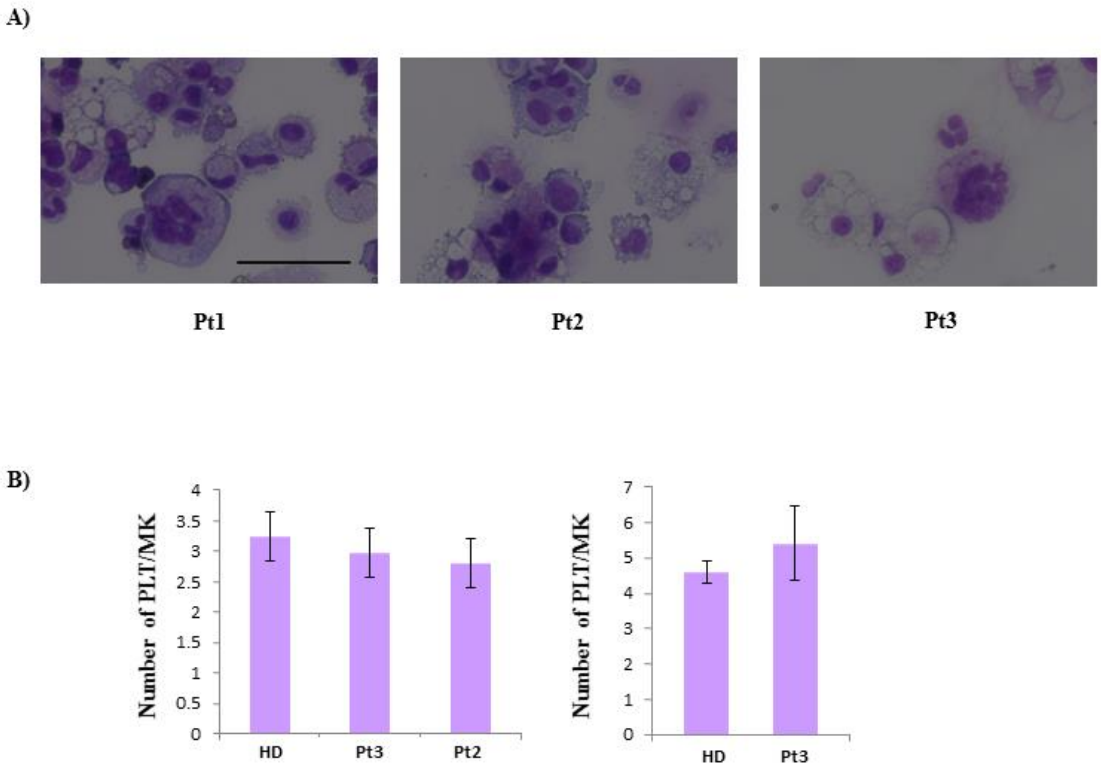

Figure 9. Normal megakaryocytes' ability to produce platelets in patient samples
A) Normal phenotype of patient-iPSC derived megakaryocytes on day 24 of differentiation. Scale Bar shows 50 μm.
B) Comparable numbers of platelets produced from XLT/WAS-Pt megakaryocytes and HD megakaryocytes. Same numbers of megakaryocytes for each group were used to estimate platelet number produced by each megakaryocyte. Results are represented as the mean number of platelets produced by one megakaryocyte ± SD (n=3).

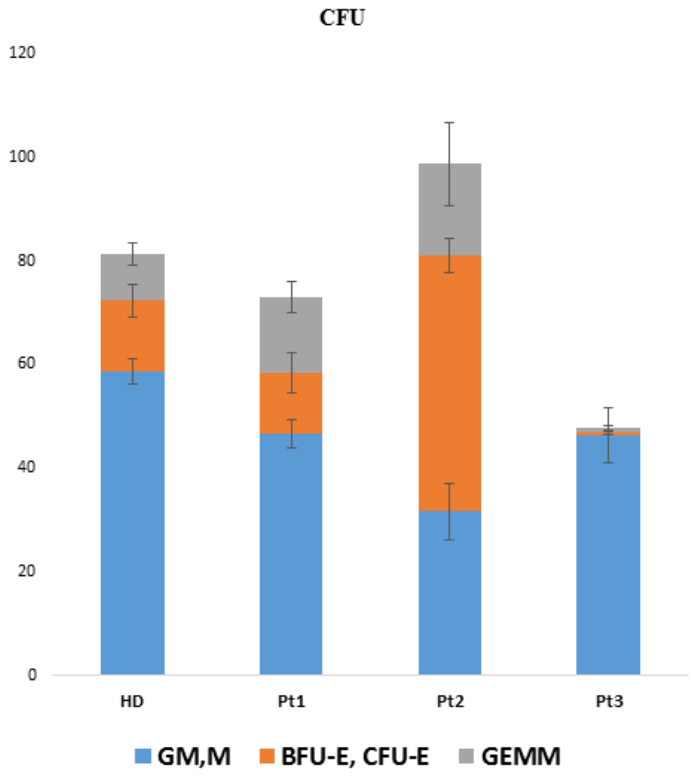

Figure 10. Defected hematopoietic lineage differentiation ability in XLT/WAS samples

Variable abilities in hematopoietic lineage differentiation depending on distinct genetic mutations in *WAS* gene shown in colony-formation assay. CFU: colony-forming units. Results are represented as the mean number of colonies ± SD (n=3).

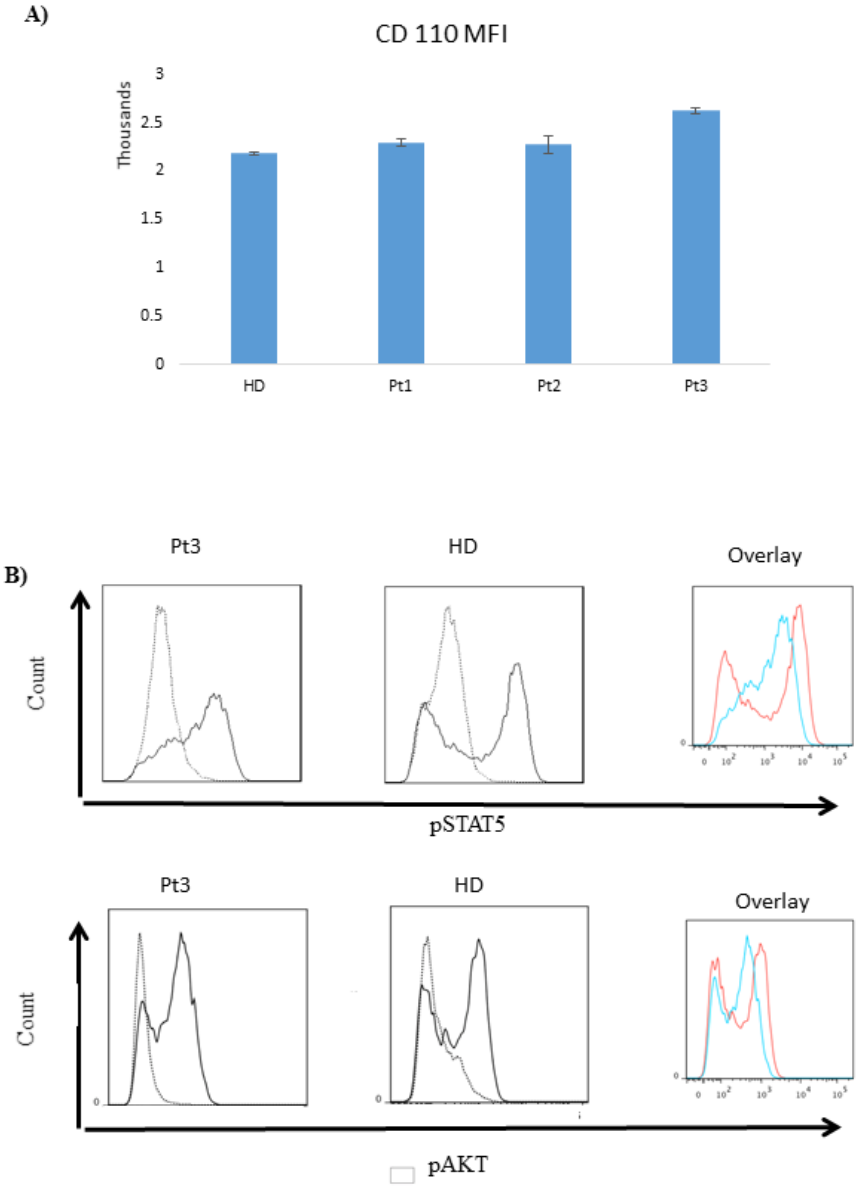

Figure 11. Phosphorylation of signaling molecules downstream of MPL is affected by WASp deficiency

A) MPL (CD110) expression on Megakaryocytes were analysed by FACS on day 23 of differentiation of iPSCs to megakaryocytes. MFI calculation showed no significant difference between MPL expression levels on megakaryocytes (CD41+, CD42+) in patient samples comparing to the healthy counterpart. MFI: Mean Florescence Intensity. Results are represented as the mean MFI ± SD (n=3).

B) Blunted phosphorylation kinetics of STAT5 and AKT in CD34+ cells upon stimulation with TPO. Left panel: Pt3, Middle panel: HD (Dots line: vehicle, Black line: TPO stimulated)

Right panel: overlay of histograms of TPO stimulated samples. (Blue line: Pt3, Red line: HD)

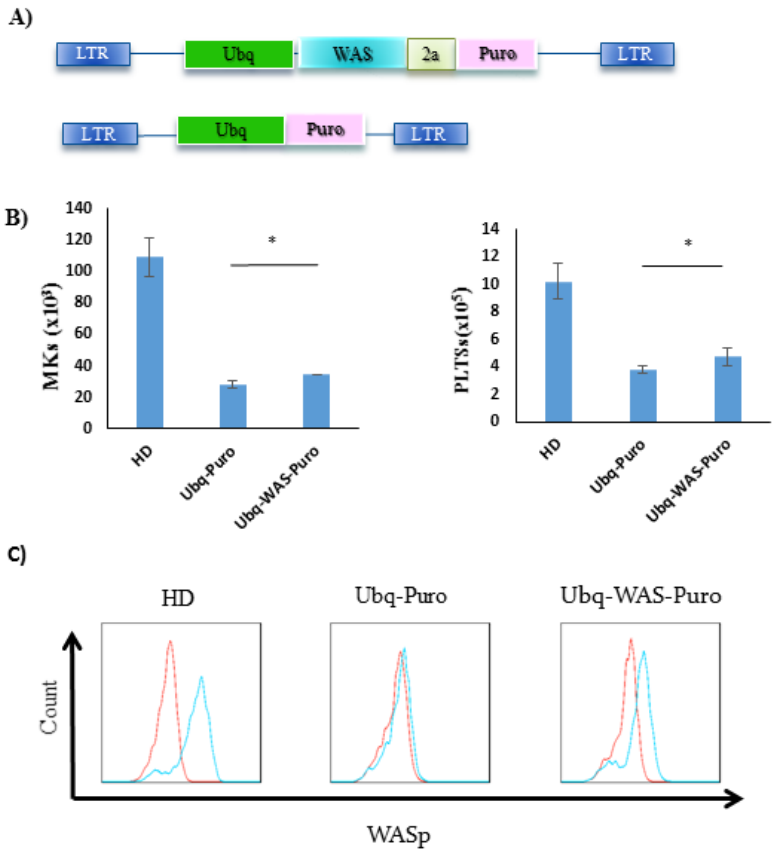

Figure 12. Increased platelet and megakaryocyte count after gene therapy.
A) Diagram of lentivirus vector design. Therapeutic vector with Ubq promoter harboring *WAS* gene is shown in upper panel and control vector in lower panel. Both vectors harbor Puromycin as selection marker. Ubq: ubiquitin promoter; WAS: *WAS* gene; Puro: Puromycin.
B) Megakaryocyte and platelet counts after gene therapy (Pt3). Values showed statistical difference between groups ($p < 0.05$). Results are represented as the mean megakaryocyte/platelet count ± SD (n=3). Ubq-puro: control vector; Ubq-WAS-Puro: therapeutic vector.
C) Intracellular FACS analysis of WASp expression in megakaryocytes (Pt3) after gene therapy comparing to HD. (Red: Isotype control, Blue: WASp)

	Patient no. 1 (Pt1)	Patient no. 2 (Pt2)	Patient no. 3 (Pt3)
Mutation	168C>T	1066del (cc)	29del(G)
Affected AA	Thr45Met	Pro356fsx138	35, Stop codon
Platelet count	$10^4/mm^3$	$2x10^4/mm^3$	-
Clinical score	2	2	5
WASp	Negative	Negative	Negative
Status	Post- splenectomy	_	Post- BMT

Table 1. Characteristic of patient samples
Three patients with distinct mutations were used in this study. Two XLT patients with score 2 and one WAS patient with score 5. All patients are suffering from thrombocytopenia.

Primer Name	Expected band	Primer Sequence	Used for sequence
WAS exon2-F	293 bp	5'-TCCAAGACCTTGTGGCTACC-3'	*
WAS exon2-R		5'-TGAGGTCTTGAAGCTATGGACAC-3'	
WAS exon10-F	745 bp	5'-ACGCTCCCTTCTCTAGCCCAAGCAG-3'	*
WAS exon10-R		5'-TCAAGGCTTCCCACCAACCTTTCAAC-3'	*
01-18 WAS-PCR1-F1	473 bp	5'-AAGCTCAGCCTAACGAGGAG-3'	*
01-19 WAS-PCR-R1		5'-GGCAAGTAGACATTGAGATCTTCC-3'	

Table 2: Primers used in this study

Pairs of primers used for Pt1, Pt2, and Pt3 respectively. F: Forward primer, R: Reverse primer

Some primers used for the sequencing as well which are shown by *.

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