

Efficient retrovirus-mediated *PIG-A* gene transfer and stable restoration of GPI-anchored protein expression in cells with the PNH phenotype

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Paroxysmal nocturnal hemoglobinuria (PNH) is a clonal hematopoietic stem cell disorder characterized by complement-mediated hemolysis due to deficiencies of glycosylphosphatidylinositol-anchored proteins (GPI-APs) in subpopulations of blood cells. Acquired mutations in the X-linked phosphatidylinositol glycan-class A (*PIG-A*) gene appear to be the characteristic and pathogenetic cause of PNH. To develop a gene therapy approach for PNH, a retroviral vector construct, termed MPIN, was made containing the *PIG-A* complementary DNA along with an internal ribosome entry site and the nerve growth factor receptor (NGFR)

as a selectable marker. MPIN transduction led to efficient and stable *PIG-A* and NGFR gene expression in a *PIG-A*-deficient B-cell line (JY5), a *PIG-A*-deficient K562 cell line, an Epstein-Barr virus-transformed B-cell line (TK-14⁻) established from a patient with PNH, as well as peripheral blood (PB) mononuclear cells from a patient with PNH. *PIG-A* expression in these cell lines stably restored GPI-AP expression. MPIN was transduced into bone marrow mononuclear cells from a patient with PNH, and myeloid/erythroid colonies and erythroid cells were derived. These transduced erythroid cells restored surface expression of GPI-APs and resis-

tance to hemolysis. These results indicate that MPIN is capable of efficient and stable functional restoration of GPI-APs in a variety of *PIG-A*-deficient hematopoietic cell types. Furthermore, MPIN also transduced into PB CD34⁺ cells from a normal donor, indicating that MPIN can transduce primitive human progenitors. These findings set the stage for determining whether MPIN can restore *PIG-A* function in multipotential stem cells, thereby providing a potential new therapeutic option in PNH. (Blood. 2001;97:3004-3010)

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Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired clonal hematopoietic stem cell disorder characterized by intravascular hemolytic anemia.^{1,2} Abnormal blood cells are deficient in glycosylphosphatidylinositol-anchored proteins (GPI-APs).³⁻⁶ In the affected hematopoietic cells from patients with PNH, the first step in biosynthesis of the GPI-anchor is defective.^{7,8} At least 5 genes are involved in this reaction step,⁹ and one of them, an X-linked gene termed phosphatidylinositol glycan-class A (*PIG-A*),¹⁰ is mutated in affected cells.^{11,12} The *PIG-A* gene is mutated in every patient with PNH reported to date, and deficiency of GPI in PNH has thus been considered to be due solely to the *PIG-A* mutation(s).³⁻⁶

The clinical manifestation of PNH is complex, involving primarily 3 sets of symptoms: hemolysis with acute exacerbation, cytopenia of varying severity, and a tendency for thrombosis. PNH derives its name from the episodes of brownish urine (hemoglobinuria) that frequently appear in the morning¹³ and that are due to intravascular hemolysis because of deficiencies of decay accelerating factor (DAF) and CD59 in erythroid cells.^{4,5} However, the mechanism of thrombosis and its relation to defects in PNH are not entirely clear. Many patients with PNH have evidence of deficient hematopoiesis, and the degree of bone marrow (BM) failure varies from subclinical cytopenia to the development of severe aplastic

anemia (AA). Also, patients with AA have an increased risk of developing PNH.¹⁴⁻¹⁷ Episodes of infection occur frequently and may be attributable in part to leukopenia or to functional defects in leukocytes, which may be due to impaired migration of neutrophils^{18,19} and/or defective T-cell activation in PNH.^{20,21} GPI-APs may thus function not only as complement regulatory proteins, but also as receptors, or adhesion molecules, and they may be involved in signal transduction.

Although PNH generally lasts for many years,^{22,23} thrombotic disease and hematopoietic failure are the major risk factors affecting survival.^{23,24} For example, Socie et al²³ reported that patients with thrombosis at presentation had only a 40% survival rate at 4 years. Currently, allogeneic bone marrow transplantation (BMT) is the only available cure for PNH; however, this is associated with high levels of morbidity and mortality. Gene therapy involving the transduction of the *PIG-A* gene into autologous pluripotent stem cells is a potential alternative strategy for treating PNH. In order to develop a gene therapy approach for PNH, we have made a retroviral vector construct, termed MPIN, containing the *PIG-A* complementary DNA (cDNA) along with internal ribosome entry sites (IRES) and the nerve growth factor receptor (NGFR) as a selectable marker. In the current study, we

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Submitted October 10, 2000; accepted January 18, 2001.

Partly supported by a grant from the Japan Intractable Diseases Research

Foundation and The Osaka Medical Research Foundation for Incurable Diseases.

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evaluated whether MPIN could restore PIG-A function in various hematopoietic cells expressing the PNH phenotype.

Materials and methods

Vector construction

The plasmid p Δ LNGFR²⁵⁻²⁷ (generously provided by Fulvio Mavilio, Istituto Scientifico H. San Raffaele, Milan, Italy) was digested by *EagI* (5') and *BamHI* (3') to isolate a DNA fragment containing a truncated, non-ligand-binding version of the LNGFR cDNA (termed Δ LNGFR). The resulting fragment combined 5' with an *NcoI*-to-*EagI* linker, encoding the first 18 base pairs (bp) of the Δ LNGFR cDNA. The MN vector plasmid was generated by cloning this fragment between the *NcoI* and *BamHI* sites of the retroviral vector plasmid MFG (generously provided by S. Kaye Spratt, Somatix Therapy, Alameda, CA) as previously described²⁶ (Figure 1). The functional *PIG-A* cDNA was amplified from pEBPIG-A¹⁰ as a template by polymerase chain reaction (PCR) by means of the primer set of *Nco*-PIGA (5'-GCGGCCCATGGCCTGTAGAGGAGGA) and *Bam*-PIGA (5'-GCGGCGGATCCCTACCTGGTTTCAGA) to generate the unique *NcoI* (5') and *BamHI* (3') sites and was then digested by these enzymes. The MP vector plasmid was generated by cloning this fragment between the *NcoI* and *BamHI* sites of the retroviral vector plasmid MFG after the sequence to confirm without mutation by PCR.

The plasmid pCITE-1 (Novagen, Madison, WI), containing the encephalomyocarditis virus-IRES sequence,²⁸ was digested by *BalI* (5') and *XbaI* (3'), and then a *BalI*/*EagI* oligonucleotide and an *EagI* (5')/*XbaI* (3') fragment from p Δ LNGFR containing the Δ LNGFR cDNA were ligated into the modified pCITE vector so that the reading frame of the IRES start codon was preserved. The plasmid was digested by *EcoRI* (5') and *XbaI* (3') to isolate a DNA fragment containing the IRES-LNGFR cDNA. Finally, the MPIN vector plasmid was constructed by blunt insertion of this fragment into the unique *BamHI* site of the MP vector plasmid (Figure 1).

The MN and MPIN amphotropic vector packaging line was generated by transfection of the ecotropic retroviral vector producer line E86, followed by infection of the amphotropic producer AM12. LNGFR-expressing AM12 cells (termed AM12/MN or MPIN) were then isolated by fluorescence-activated cell sorting (FACS), and supernatant was collected by growing AM12/MN or MPIN packaging cells to 80% confluence and replacing the media with fresh media. For both supernatants, the medium was Dulbecco's modified Eagle's medium (DMEM) (Gibco, Gaithersburg, MD) containing 10% fetal calf serum (FCS) (Gibco), and flasks with fresh media were incubated at 37°C for 16 hours, and the supernatant was collected and centrifuged to remove cell debris. All supernatants were aliquoted and frozen at -70°C.^{26,29}

Retroviral vector gene transfer

To characterize the titer and the expression of the Δ LNGFR vectors, 1×10^5 NIH3T3 cells were grown overnight in a 60-mm dish and then exposed to 1 mL of a 1:25 dilution of vector containing supernatant in the presence of 8 μ g/mL polybrene (Sigma, St Louis, MO). After 2 hours, 3 mL

additional medium was added. The near-confluent infected cells were harvested by trypsinization 72 hours later and stained for expression of Δ LNGFR. The titer was determined by multiplying the proportion of cells expressing Δ LNGFR by 25×10^5 .²⁹

To introduce the *PIG-A* gene into various hematopoietic cells expressing the PNH phenotype, generally 2×10^6 cells were transduced by MN or MPIN supernatant, with the use of polybrene at 8 μ g/mL or fibronectin (Takara, Ohtsu, Japan) at 100 μ g/mL, with or without centrifugation. In some experiments, the transduction procedure was repeated 1 to 3 additional times. After transduction, cells were immunophenotyped with various monoclonal antibodies.

Isolation and culture conditions of cells expressing the PNH phenotype

The *PIG-A*-deficient B cell line (JY5) and a *PIG-A*-deficient K562 cell line³⁰ (generously provided by Dr Shinichi Hirose, Fukuoka Medical College, Japan) were maintained in DMEM with 10% FCS. The Epstein-Barr virus (EBV)-transformed B-cell line (TK-14⁻) established from a patient with PNH was grown in DMEM/Ham's F12 (Gibco) with 10% FCS.³¹ Peripheral blood mononuclear cells (PBMCs) were obtained from a patient with PNH,²⁴ having 33.1% of CD59⁻ cells in CD2⁺ MNC fraction, by Ficoll-Hypaque centrifugation (Lymphocyte Separation Solution) (American Red Cross, Rockville, MD) after informed consent. To enrich for T lymphocytes, PBMCs were cultured in RPMI 1640 (Gibco) with 10% FCS in the presence of interleukin (IL) 2 (Chiron, Emeryville, CA) at 100 U/mL, anti-CD3 (OKT3) (Ortho, Raritan, NJ) at 50 ng/mL, and anti-CD28 (Becton Dickinson, Bedford, MA) at 50 U/mL for 2 days.²⁹ After transduction, PBMCs were maintained in RPMI 1640 with 10% FCS and 10% T-Stim (Becton Dickinson) in the presence of IL-2 at 50 μ g/mL. Bone marrow mononuclear cells (BMMCs) having 49.9% of CD59⁻ cells in the BMMC fraction were obtained from a patient with PNH²² by Ficoll-Hypaque centrifugation after informed consent. After transduction, BMMCs were maintained in Iscove's modified Dulbecco's medium (IMDM) (Gibco) with 20% BIT 9500 (Stemcell Technologies, Vancouver, British Columbia, Canada) including flt-3 ligand (Genzyme, Cambridge, MA) at 50 ng/mL, erythropoietin (Epo) (generously provided by Chugai Pharmaceutical, Tokyo, Japan) at 1000 U/L, and IL-3 (R&D Systems, Minneapolis, MN) at 2.5 ng/mL for 10 days.³³ BMMCs were also cultured in MethoCult (Stemcell Technologies) containing 0.9% methylcellulose in IMDM, 30% fetal bovine serum, 1% bovine serum albumin, 3000 U/L Epo, and optimized concentrations of stem cell factor (SCF), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-3, and granulocyte CSF (G-CSF), to generate burst-forming unit-erythroid (BFU-E), as well as colony-forming unit-granulocyte-macrophage (CFU-GM) and CFU-mix colonies as described.³⁴ The culture dishes were incubated at 37°C, and the numbers of colonies/bursts were scored on day 12 after 3 days' transduction. PB CD34⁺ cells were isolated from a G-CSF-primed normal donor undergoing leukapheresis for autologous stem cell transplant after informed consent; to increase the number of CD34⁺ cells available for analysis, the sample was depleted of lineage-committed cells by using StemSep antibody cocktail for CD34⁺CD38⁻ cells (Stemcell Technologies) including anti-CD2, anti-CD3, anti-CD14, anti-CD16, anti-CD19, anti-CD24, anti-CD56, anti-CD66b, anti-CD45RA, anti-CD36, anti-CD38, and glycophorin A. After transduction, hematopoietic progenitor cells containing CD34⁺ cells were maintained in IMDM with 20% BIT 9500 for one more day.

FACS analysis and flow cytometric sorting

Monoclonal antibodies (mAbs) included anti-CD59-fluorescein isothiocyanate (FITC), anti-CD55-phycoerythrin (PE), and anti-CD48-FITC (Pharmingen, San Diego, CA) for the detection of GPI-APs, anti-NGFR-PE (Chromoprobe, Mountain View, CA), anti-CD3-FITC (Pharmingen), anti-CD34-FITC (Pharmingen), and anti-CD45-peridinin chlorophyll protein (Pharmingen). For the detection of erythroid cells, E6,³⁵ an antibody to red cell surface protein band 3 (generously provided by Dr Marilyn Telen, Duke University Medical Center, Durham, NC) as a first antibody and a secondary antibody conjugated to FITC (Jackson ImmunoResearch Lab,

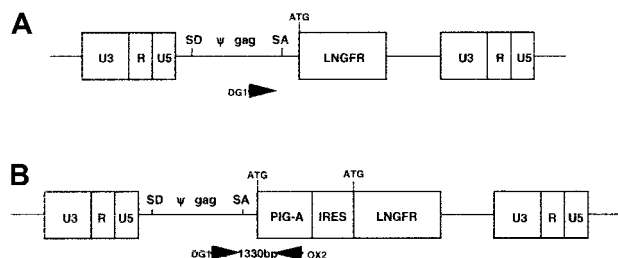


Figure 1. Schematic representation of the MN (upper) and MPIN (lower) retroviral vectors. Primers used for polymerase chain reaction are indicated at the corresponding positions by arrowheads. U3, R, U5 = Moloney long terminal repeat elements; SD = splice donor site; SA = splice acceptor site; ψ = vector packaging sequence; ATG = start codon.

West Grove, PA) were used for staining. Antihuman IgG2a-FITC, antihuman IgG2a-PE and antihuman IgG1-PE (Pharmingen) were used for negative isotype controls. After staining, cells were analyzed with a FacsCalibur (Becton Dickinson). To separate CD59⁺, CD59⁻, NGFR⁺, and/or NGFR⁻ cells, the sample was sorted on a FACStar Plus (Becton Dickinson) or a FACS Vantage (Becton Dickinson) after staining.

Molecular analysis by PCR for *PIG-A* integration

DNA was isolated from transduced (MN or MPIN) or untransduced (mock) NIH3T3 cell lines (JY5, K562 mutant, and TK-14⁻), BMMCs (primary erythroid cells), and colonies/bursts (mixture of CFU-GM, CFU-mix, and BFU-E). A 1330-bp region including vector plus 5' *PIG-A* was amplified by PCR with the use of the primer set of DG1 (5'-TCTCTCCCCTTGAACC-3') and OX2 (5'-GCTCCAAAAGACGCAC-3') as shown in Figure 1. For positive control, the MPIN vector plasmid was used as a template, and Marker 6 (Takara) was loaded as a molecular marker, in each experiment.

Small-scaled Ham test

Serum was separated from whole blood of a blood-group-matched healthy volunteer. Serum was acidified with a 10% volume of 0.2 N HCl. Washed samples suspended in saline ($3 \times 10^6/0.01$ mL) were incubated with 0.1 mL of the serum for 1 hour at 37°C. For 100% lysis, water was used in place of serum, and for 0% lysis, heat-inactivated serum was used. After incubation, each sample was centrifuged, and the optical density of hemoglobin in the supernatant was measured at 412 nm.³⁶

Aerolysin assay

Cells (1×10^6) were incubated for 90 minutes at 37°C with aerolysin (1.5×10^{-8} M), which is a toxin secreted by *Aeromonas hydrophila* and is capable of killing target cells by binding to GPI-anchored receptors, produced by trypsin activation of pro-aerolysin (Protox Biotech, Victoria, British Columbia, Canada).^{37,38} For 100% lysis, water was used in place of aerolysin, and for 0% lysis, PBS was used. After incubation, each sample was centrifuged, and the optical density of hemoglobin in the supernatant was measured at 412 nm.³⁶

Results

Retroviral vector MPIN restores *PIG-A* function in hematopoietic cell lines bearing PNH phenotype

The MPIN vector was initially transduced into NIH3T3 cells. As shown in Figure 2A, 97.0% and 68.9% of NIH3T3 cells expressed

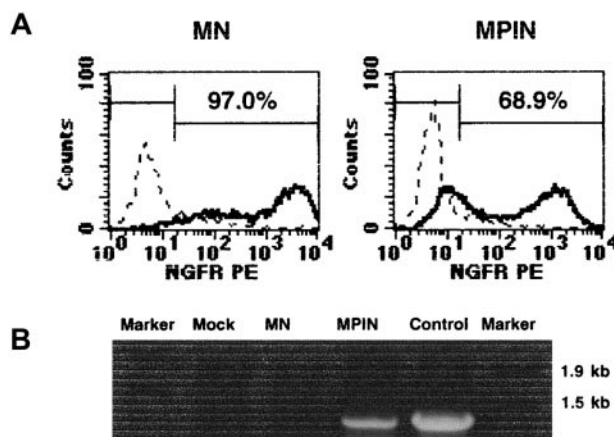


Figure 2. Characterization of MN and MPIN vectors. (A) NGFR surface expression in the transduced NIH3T3 cells with 1:25 dilution of MN or MPIN vector supernatant. (B) Detection of the region including *PIG-A* in MPIN-transduced NIH3T3 cells by PCR. DNA was isolated from transduced (MN or MPIN) or untransduced (mock) NIH3T3. A region including vector plus 5' *PIG-A* was amplified by PCR. For positive control, the MPIN vector plasmid was used as a template, and Marker 6 was loaded as a marker.

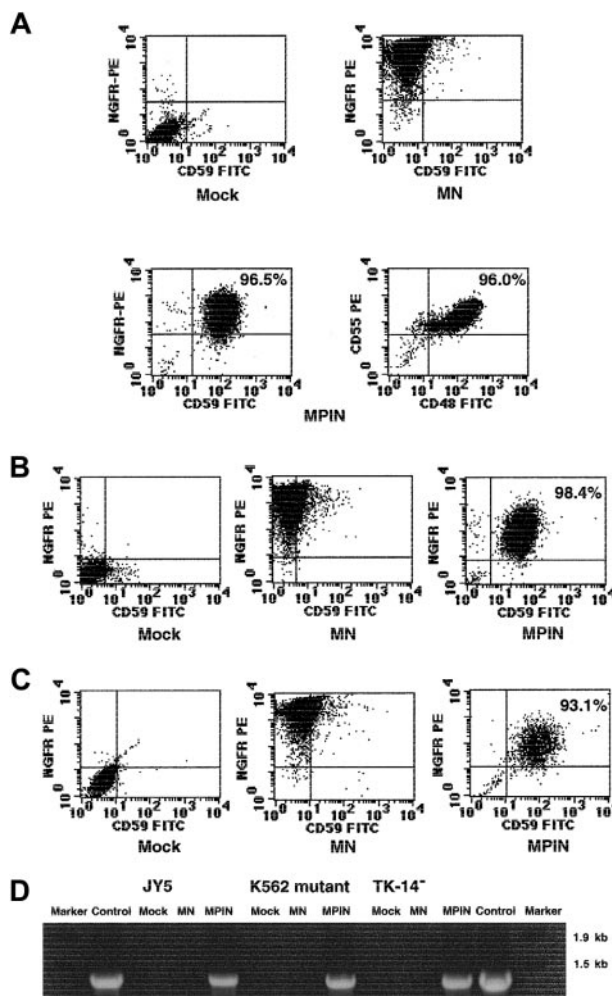


Figure 3. Gene transfer by MPIN into PNH-phenotype hematopoietic cell lines restores *PIG-A* function. (A) GPI-APs (CD55, CD59, CD48) and NGFR surface expression of *PIG-A*-deficient B-lymphoblastoid cell line JY5 transduced with MN or MPIN on day 39. (B) (C) GPI-AP (CD59) and NGFR surface expression of *PIG-A*-deficient K562 mutant cell line (panel B) and *PIG-A*-deficient B-lymphoblastoid cell line (TK-14⁻) established from a patient with PNH (panel C), transduced with MN or MPIN on day 21. (D) Detection of the region including *PIG-A* in MPIN-transduced cell lines (JY5, K562 mutant, and TK-14⁻) by PCR. DNA was isolated from transduced (MN or MPIN) or untransduced (mock) cell lines. A region including vector plus 5' *PIG-A* was amplified by PCR. For positive control, the MPIN vector plasmid was used as a template, and Marker 6 was loaded as a marker.

LNGFR following transduction of MN and MPIN, respectively, yielding a titer of 4.8×10^6 infectious U/mL for MN and 3.4×10^6 infectious U/mL for MPIN. High levels of NGFR were expressed from MPIN, indicating that this could be a useful marker gene for analyzing gene transfer into hematopoietic cells. PCR analysis detected the region including *PIG-A* only in MPIN-transduced NIH3T3 cells (Figure 2B).

To evaluate whether MPIN would restore *PIG-A* function in hematopoietic cells, we transduced it into several *PIG-A*-deficient cell lines. MPIN was transduced into the *PIG-A*-deficient B-lymphoblastoid cell line JY5, and then on days 10 and 20, NGFR⁺CD59⁺ cells were sorted to be enriched. On day 39, transduced cells were stained with mAbs for NGFR and GPI-APs. In the selected JY5 cells, MPIN expressed NGFR at high levels and restored stable and efficient expression (greater than 95%) of GPI-APs including CD55, CD59, and CD48 (Figure 3A). MPIN was also transduced into a *PIG-A*-deficient K562 mutant cell line, and an EBV-derived B cell line (TK-14⁻) established from a patient

with PNH. After 21 days in cultures, CD59⁺NGFR⁺ cells were sorted for enrichment, and on day 27, transduced cells were stained with mAbs for NGFR and CD59. Again, MPIN expressed NGFR at high levels in these cell lines and restored stable and efficient expression of GPI-AP (greater than 93%) (Figure 3B-C). These restored expressions were still stable after repeated freezing and thawing (data not shown). These observations indicate that MPIN can restore PIG-A function in multiple hematopoietic cell types. The presence of DNA including the *PIG-A* sequence was also confirmed only in MPIN-transduced cell lines (Figure 3D).

Retroviral vector gene transfer by MPIN restores PIG-A function in PBMCs with PNH phenotype

To test MPIN in primary peripheral blood cells, CD59⁻ PBMCs were isolated from a patient with PNH and transduced with MPIN after stimulation with IL-2, anti-CD3, and anti-CD28 as previously described.²⁹ On day 19 in culture, 93.0% were positive for CD3, a T-cell marker, and negative for CD19, a B-cell marker, and the gene-transfer efficiency was 16.9% (16.8 ± 0.7%; range, 15.0% to approximately 18.4%; n = 4) as measured as a percentage of NGFR⁺ cells (Figure 4). MPIN restored expression of GPI-APs at 8.8% (9.7 ± 0.4%; range, 8.8% to approximately 10.6%; n = 4) as measured by NGFR⁺CD59⁺ cells (Figure 4). Even though we confirmed that the sorted PBMCs were mostly of the CD59⁻ phenotype, 11.0% of cells in mock control expressed CD59. The most likely explanation for this paradox is that contaminated CD59^{dim} PBMCs brightly expressed after the culture with stimulation. The CD59⁻NGFR⁺ cells may also have been observed because initiation or completion of translation did not occur in the *PIG-A* sequence but, for currently unknown reasons, did occur in the downstream NGFR sequence. Expression of both NGFR and GPI-APs was stable for the duration of the cell cultures (about a month). There was no significant difference in growth rates between mock, MN-infected, and MPIN-infected cells, suggesting that there was no toxicity from the virus.

Retroviral vector MPIN restores PIG-A function and confers resistance to hemolysis in PNH-phenotype primary erythroid cells from a patient with PNH

One of the primary clinical manifestations of PNH is increased sensitivity of red cells (RBCs) to hemolysis by complement. To test whether transduction with MPIN would restore resistance to hemolysis in PNH RBCs, CD59⁻ BMNCs were isolated from a patient with PNH and transduced with MPIN. Cells were then cultured with flt-3 ligand, Epo, and IL-3, as previously described, to generate erythroid progeny.³³ Multiparameter FACS analysis performed on day 12 of culture demonstrated that 16.1% (19.2% ± 2.0%; range, 16.1% to approximately 24.9%; n = 4) of transduced cells were CD59⁺NGFR⁺; 90.9% were positive for E6,

an erythroid marker; and most of the cells were negative for CD45, a leukocyte marker (Figure 5A). The integrated region including *PIG-A* was detected only in MPIN-infected erythroid progeny (Figure 5B).

After the separation of CD59⁺NGFR⁺ and CD59⁻NGFR⁻ cells, their sensitivity to hemolytic action was measured by means of the Ham test. An average of 79.0% ± 1.7% (range; 75.7% to approximately 81.5%; n = 3) of CD59⁺NGFR⁺ cells were lysed, whereas only 11.9% ± 5.5% (range, 6.1% to approximately 22.9%; n = 3) of CD59⁺NGFR⁺ cells with restored expression of GPI-AP were lysed ($P = .0003$) (Figure 5C). Their sensitivity to aerolysin, a toxin capable of killing target cells by forming channels after binding to GPI-APs, was also measured. Only 2.2% ± 1.9% (range, 0.3% to approximately 4.1%; n = 2) of CD59⁻NGFR⁻ cells were lysed, whereas 80.3% ± 1.5% (range, 78.8% to approximately 81.8%; n = 2) of CD59⁺NGFR⁺ cells with restored expression of GPI-AP were lysed ($P = .001$) (Figure 5D). Thus, MPIN restored resistance to hemolysis and the cytotoxic effectiveness of aerolysin, as well as expression of GPI-APs.

Retroviral vector gene transfer by MPIN into PNH-phenotype and normal BMNCs

BMNCs were also isolated from a patient with PNH and transduced with MPIN. Cells were then cultured in methylcellulose with Epo, SCF, GM-CSF, IL-3, and G-CSF to generate colonies and bursts. The numbers of colonies/bursts were scored on day 15 in culture, and the ratio of BFU-E to CFU-GM to CFU-mix was about 1:1:1 (data not shown). FACS analysis performed on day 15 of culture demonstrated that 13.6% of transduced cells were CD59⁺NGFR⁺ (Figure 6A). The integrated region including *PIG-A* was also detected only in MPIN-infected colonies/bursts (Figure 6B).

PB CD34⁺ cells were enriched from a G-CSF primed donor after mobilization by means of StemSep and were transduced with MPIN. Gene-transfer efficiency in CD34⁺ cells was about 6% as measured by NGFR⁺ cells on day 5 in culture (Figure 7).

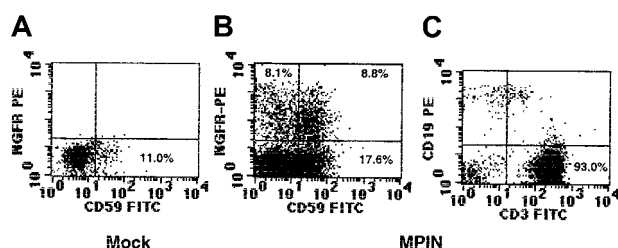


Figure 4. Retroviral vector MPIN restores PIG-A function in PNH-phenotype PBMCs. GPI-AP (CD59), NGFR, CD3, and CD19 surface expression of MPIN-transduced MNCs from a patient with PNH on day 19.

Discussion

BMT is currently the only available cure for PNH. Syngeneic and allogeneic BMTs, including eradication of the PNH clone, have been successful in several patients with PNH.³⁹⁻⁴¹ In most, the indication for BMT was BM failure, and guidelines similar to those for AA have been proposed for the use of BMT in these patients. BMT was performed on one patient who had portal and hepatic vein thrombosis, with resolution of much of the thrombosis.⁴² Socie et al²³ reported that thrombosis and hematopoietic failure were the major risk factors affecting survival. For example, patients with thrombosis at presentation have only a 40% survival rate at 4 years.²³ Therefore, these patients are also good candidates for early and aggressive therapies.

Autologous BMT, with or without depletion of GPI⁻ hematopoietic stem cells, has been proposed as a possible cure for PNH. However, the low number of hematopoietic stem cells in the BM of a patient with PNH may pose a problem, particularly in those with BM failure. In many patients, one cannot easily isolate the normal cells and repopulate the BM with them, because the patients have few normal cells remaining and/or have BM hypoplasia, including AA. Indeed, our preliminary data show that the mean fraction of circulating polymorphonuclear cells (PMNs) deficient in GPI-APs

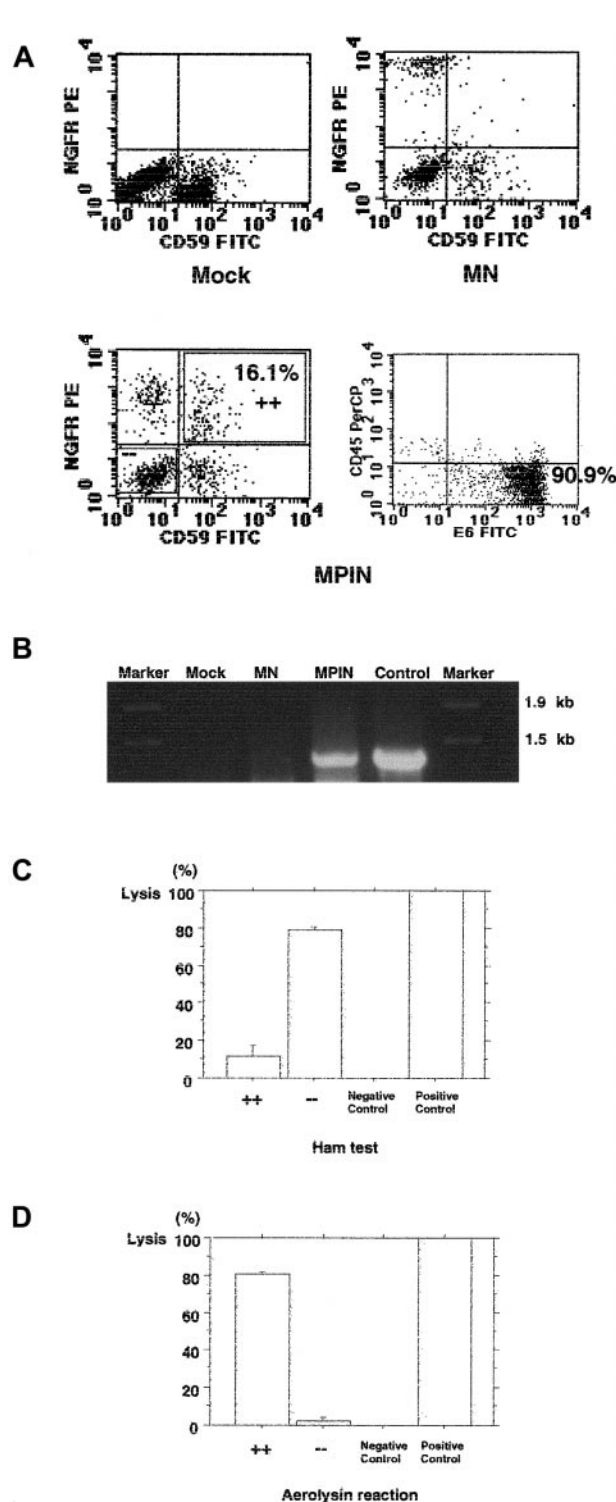


Figure 5. Gene transfer by MPIN into PNH-phenotype primary erythroid cells from a patient with PNH restores *PIG-A* function and resistance to hemolysis. (A) GPI-AP (CD59), NGFR, E6, and CD45 surface expression of MPIN-transduced erythroid cells generated from GPI-AP⁻ (CD59⁻) BMNCs on day 12. (B) Detection of the region including *PIG-A* in MPIN-transduced BMNCs by PCR. DNA was isolated from transduced (MN or MPIN) or untransduced (mock) BMNCs. A region including vector plus 5' *PIG-A* was amplified by PCR. For positive control, the MPIN vector plasmid was used as a template, and Marker 6 was loaded as a marker. (C) (D) Small-scaled Ham test (panel C), and aerolysin assay (panel D). The percentage of lysis of CD59⁺NGFR⁺ (++) and CD59⁻NGFR⁻ (--) cells from MPIN-transduced BMNCs were compared. For 100% lysis, water was used in place of serum, and for 0% lysis, heat-inactivated serum was used. Results are shown as mean \pm SE.

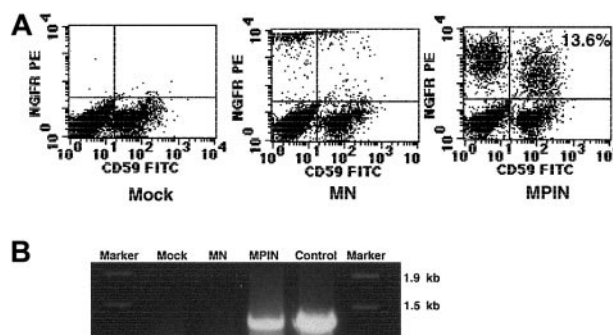


Figure 6. Retroviral vector MPIN restores *PIG-A* function in PNH-phenotype BMNCs. (A) GPI-AP (CD59) and NGFR surface expression of the pooled colonies/bursts generated from PNH-phenotype BMNCs transduced with MN or MPIN. (B) Detection of the region including *PIG-A* in MPIN-transduced colonies/bursts generated from PNH-phenotype BMNCs by PCR. DNA was isolated from transduced (MN or MPIN) or untransduced (mock) colonies/bursts. A region including vector plus 5' *PIG-A* was amplified by PCR. For positive control, the MPIN vector plasmid was used as a template, and Marker 6 was loaded as a marker.

at the time of diagnosis was 70% and that 50% of patients have fewer than 20% normal PMNs. The fraction of deficient PMNs directly reflects the fraction of *PIG-A* mutant stem cells.

An alternative to BMT would be therapy designed to restore the surface expression of GPI-APs, such as infusion of GPI-APs. Kooyman et al⁴³ demonstrated that GPI-APs expressed on the surface of transgenic mouse red cells were transferred in a functional form to endothelial cells in vivo. In addition, Dunn et al⁴⁴ created a murine knock-out embryonic stem cell via targeted disruption of the *Pig-a* gene and demonstrated transfer in situ of GPI-APs from normal to knock-out cells. Subsequently, Sloand et al⁴⁵ demonstrated that enriched DAF and CD59 reincorporated into the membranes of PNH erythrocytes by means of high-density lipoprotein were functional. At this time, however, strategies to transfer therapeutic proteins to the GPI⁻ PNH clone remain exploratory.

The discovery of *PIG-A* allows a new approach to therapy for patients with PNH, and molecular therapy to introduce a normal *PIG-A* gene into *PIG-A* mutant hematopoietic stem cells appears attractive. Autologous BMT after ex vivo transduction of a normal *PIG-A* gene into mutant hematopoietic stem cells could avoid both graft failure and graft-versus-host disease seen in allogeneic BMT. However, there remains the risk of recurrent PNH, caused by the re-establishment of clonal dominance by any residual *PIG-A* mutant stem cells. The mechanisms of clonal dominance of mutant stem cells in PNH are yet to be clarified. A possible mechanism is selection for *PIG-A* mutant cells, such as an immunological selection. For example, if cytotoxic T cells are involved in the autoimmunity, stem cells defective in the surface expression of GPI-APs may be less sensitive to the cytotoxic cells because the

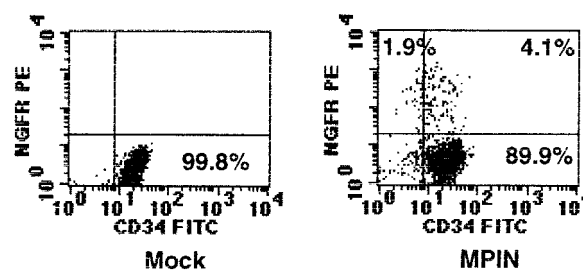


Figure 7. Retroviral vector gene transfer by MPIN into CD34⁺ cells from a normal donor. GPI-AP (CD59) and NGFR surface expression of the transduced CD34⁺ MNCs from the peripheral blood of a normal donor on day 5.

effector-target interaction may be inefficient owing to a lack of certain GPI-anchored types of adhesion molecules. In any case, selective killing of normal stem cells would result in selective survival and expansion of the GPI-deficient stem cell clone. Indeed, syngeneic BMT without conditioning in patients with PNH has not been universally successful because of an apparent survival advantage of mutant stem cells.^{46,47} If pretransplantation BM conditioning is effective in eliminating such selective conditions, then residual mutant stem cells would be eliminated or might not become dominant. The other possibility is that expanded clonal cells have a genetic abnormality in addition to *PIG-A* mutation and that the 2 in combination or the putative second abnormality may impart an ability to expand autonomously even in a normal bone marrow environment. In this case, the *PIG-A*-restored PNH stem cell clone might still maintain the ability to expand autonomously. More complete understanding of the pathogenesis of PNH is required as a scientific foundation for the rational design of further molecular therapeutic approaches. At this time, autologous BMT after the transduction of the normal *PIG-A* gene into mutant hematopoietic stem cells, along with depletion of residual mutant hematopoietic cells, seems the best molecular therapeutic approach to PNH.

As proof of the principle of gene therapy for PNH, a functional recombinant transmembrane form of CD59 was expressed in GPI-deficient PNH B cells by means of retroviral gene transfer.⁴⁸ This restored surface expression of CD59 and resistance to complement. In this study, we demonstrated that MPIN, a retroviral vector containing the full coding sequence of *PIG-A*, was able to restore GPI-AP expression and was capable of long-term stable gene transfer into various cell lines expressing the PNH phenotype.

Subsequently, we demonstrated that MPIN was capable of efficient and stable gene transfer into primary peripheral T cells and progenitor cells from a patient with PNH. In addition, MPIN was capable of efficient functional restoration of GPI-APs that confer resistance to hemolytic action, indicating that MPIN can transduce erythroid progenitors. Finally, we also demonstrated that MPIN can transduce CD34⁺ primitive human progenitors.

Taken together, our observations provide convincing evidence that stable and functional *PIG-A* gene transfer into human hematopoietic cells is possible. These findings set the stage for determining whether MPIN can restore *PIG-A* function in multipotential stem cells from patients with PNH. An additional advantage of MPIN vector is that the NGFR marker gene also permits easy determination of gene transfer efficiencies, as well as rapid isolation of transduced cells by means of a flow cytometric technique. Since we recently developed a PNH mouse model in which only the hematopoietic system has *Pig-a*-deficient cells,⁴⁹ we could initiate the study for determining the restoration of the *PIG-A* function in multipotential stem cells using this mice model system, thereby providing a potential new therapeutic option in PNH.

Acknowledgments

We thank Dr Yasuhiko Horiguchi for advice on use of aerolysin; and Toshiyuki Hirota, Yvonne Ellis, Yuki Murakami, Keiko Kinoshita, and Reiko Fukuyama for their excellent technical assistance.

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