이학석사학위논문

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부 위 원 장 김 진 수 (인)
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Abstract

Targeted genomic inversion using zinc finger nucleases : Toward gene therapy for Haemophilia A

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As whole genome sequencing technology develops rapidly, there are many reports about structural variation in genome. Structural variations, which include insertions, deletions, duplications, translocations and inversions, occur in more wide region of genome than single-nucleotide polymorphisms. These structural variations are found in normal populations of human in some cases, but these are the cause of many genetic diseases. Especially, Haemophilia A and Hunter syndrome are

caused by genomic inversion.

For this reasons, structural variations are highly regarded in the fields of therapy of genetic disorders. Despite the importance of structural variations, it is difficult to investigate the mechanism of these, because there are no molecular tools to induce structural variations in genome.

Here in this study, inducements of genomic inversions in human genomes are attempted using zinc finger nuclease technology. Zinc finger nucleases are made to induce genomic inversions in the region of Intron 1 of *F8* gene-cause of severe Haemophilia A-and validated by various methods. Theses results hold new promises for the gene therapy of Haemophilia A and it is hoped that zinc finger nuclease technologies are widely used in the fields of gene therapy, based on this study.

Keywords : zinc finger nuclease (ZFN), double-strand break (DSB), structural variation(SVs), genomic inversion, Haemophilia A, *F8* gene.

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I. Introduction

Zinc finger nucleases (ZFNs) are artificial restriction enzymes which can induce sequence-specific double-strand breaks (DSBs) in targeted genomic region. From this point of view, ZFNs are used as powerful tools for gene therapy.

Haemophilia A is genetic disorders that caused by deficiency of blood coagulation factor VIII (*F8*). The reasons of *F8* deficiency are various and intron1 of *F8* gene-related inversion accounts for about 5% of them.(Bagnall et al. 2002) Many researchers focus on Haemophilia A as good models of gene therapy. First, Haemophilia A is genetic disorders which cause by defect of single gene, *F8* gene. Second, only few amounts of compensation of coagulation factor VIII are sufficient to moderate the bleeding phenotype. In murine models, Xu et al. shown that transplantation of iPSCs which express the *F8* to the haemophilia A mice is effective to moderate bleeding phenotype and prolong the life of mice.(Xu et al. 2009)

As the murine models, this approach will be applied in human. Recently, it is possible to produce patient-specific induced pluripotent stem cells. For cell therapy, ZFN-mediated gene corrections in patients-derived cells are performed and the gene corrected cells are transplanted to patients again. This method has benefits that the gene-corrected cells are originated from patients himself, so there are

few or no risk of immune rejection.

This ZFN-mediated cell therapy has benefit compare with current Haemophilia A therapy. The protein replacements therapy has some problems related to contamination of other proteins and the cost of that therapy is very expensive. (Youjin and Jun 2009) Viral vector-mediated gene transfer therapy also has the risk of genome integration, immune rejection.

In this study, ZFN-mediated genomic inversions in intron1 of *F8* gene are performed in cultured human cells and primary human blood cells. This results open up the possibilities that ZFN can be used as powerful tools for gene therapy.

II. Materials and Methods

1. Plasmid construction

(a) Zinc finger nucleases.

Plasmid encoding ZFNs targeting intron 1 of *F8* gene region were constructed by modular assembly as described previously.(Kim et al. 2009; Kim et al. 2011b) Various FokI domains were used in this study.(Miller et al. 2007; Szczepek et al. 2007; Doyon et al. 2011; Guo et al. 2010)

2. Cell culture and transfection method

Human embryonic kidney 293T/17 (ATCC, CRL-11268TM) cells were maintained in Dulbecco's modified Eagle medium (Welgene Biotech) supplemented with fetal bovine serum (10%), penicillin (100 units/ml), and streptomycin (100ug/ml) at 37 °C and 5% CO_2. $2x10^5$Cell were seeded at 24-well plate, incubated for 20~24 hours, and then transfected with plasmid DNA using polyethylenimine. Genomic DNA are isolated using the G-DEX^{TM} IIc Genomic DNA Extraction Kit (iNtRON, cat#17231) as described by the manufacturer's protocol.

3. T7 endonuclease I assay.

T7 endonuclease I assay protocols were based on previous study.(Kim et al. 2009) The genomic region encompassing the ZFN target site was amplified using appropriated primer sets, usually hemi nested or nested PCR method. The PCR products were melted and annealed to form hetero-duplex DNA. The melting/annealing were done using PCR machine.(95℃ 2min, -2℃/s to 85℃, -0.1℃/s to 25℃) The annealed DNA was treated with T7 endonuclease 1 for 20 min at 37 ℃ and analyzed 2% agarose gel assay.

4. PCR analysis of genomic inversion and sequencing of breakpoint junctions.

Genomic DNA were quantified by Nanodrop and usually 100ng were used to PCR analysis. Nested PCR was performed to efficient amplification. For sequencing analysis, PCR products were purified and cloned into the T-Blunt vector using the T-Blunt PCR Cloning Kit (SolGent). The colonies were analyzed by colony PCR and appropriate-sized colonies were inoculated and plasmid DNA were extracted. Then the plasmid DNA were sequenced using appropriated

primers.

5. Estimation of inversion frequencies.

Digital PCR analysis was performed based on our previous study(Lee et al. 2010). Genomic DNA samples were quantified using Nanodrop and serially diluted with Tris-EDTA buffer. The serially diluted genomic DNA were amplified using appropriate primer sets. The frequencies of inversion were calculated using Extreme Limiting Dilution Analysis software(Hu and Smyth 2009).

6. Isolation of single clones.

ZFNs encoding plasmids were transfected using Lipofectamine 2000 in HEK 293T/17. After 3days, cells were seeded in 96-well plates at a density of 30 cells per well. When each wells reached appropriated confluency, each wells were split to two population, one of which was used PCR analysis to select inversion positive pools. The inversion positive pools were liming dilution in 96-well plates at a density of 0.4cells per well on average. After about 2 weeks, there were colonies in some wells and the colonies were expanded in large wells and a

portion of cells were harvested to PCR analysis to confirm inversion positive single clones.

7. Genomic DNA preparation from Haemophilia A patients.

Seoul National University Institutional Review Board(SNUIRB) approval was obtained for the analysis performed in this study. Blood sample of Haemophilia A patients was obtained at Korea Haemophilia Foundation Clinic and genomic DNA was obtained using QIAamp DNA blood Maxi kit.

III. Results and Discussion

1. Construction and validation of ZFNs

As discussed above, there are two type of inversion in severe Haemophilia A patients. One is intron22 of *F8* gene-related inversion and the other is intron1 of *F8* gene-related inversion. Intron22-related inversion cases are more common but in this cases genomic inversion events occur between about 600kb distant region. Intron1-related inversion occur between about 140kb distant region, which is shorter than intron22-related inversion, is focused in this study.

To induce genomic inversion, there must be two DSBs each in the two homologous region, homolog1 and homolog 2.(Figure 1) The two homologous regions are near identical in respect of nucleotide sequences, only one pairs of ZFNs are necessary to induce two DSBs in that region. To construct ZFNs, a computer algorithm(Toolgen, Inc.) is used to find potential target sites of ZFNs in the 1041bp homologous sequences. The parameters to construct ZFNs are listed below. (i) 3finger or 4 finger in half-target site, (ii) spacer lengths, between the two half-target sites, are 5bp or 6bp. Under this parameters, 53 pairs of ZFNs were designed in designated the 1041bp homologous region. All of them were synthesis by modular assembly

method.(Kim et al. 2011b)

To validate the ability of ZFNs to induce genomic inversion in targeted locus, PCR method which is specific to intron1-related inversion is used.(Bagnall et al. 2002) As listed in Figure 2, There are four combination of primer sets. Before inversion evens occur, F1+R1 and F2+R2 primer pairs are used to amplify normal genomic regions. When ZFN-mediated inversion events occur, F1+R1 and F2+R2 primer pairs cannot amplify any genomic regions but F1+F2, R1+R2 primer pairs really can amplify inverted genomic regions.

To identify which ZFN pairs can induce inversion between the homologous region, All of 53 pairs of ZFNs were trasnfected in HEK 293 T/17 cells according the protocol discussed in Materials and Methods. Each 53 kinds of genomic DNAs are isolated and used to PCR templates. The templates of negative control are isolated from empty vector transfected cells.

The genomic DNAs from 6 pairs of ZFNs were amplified by the two inversion-specific PCR primer sets.(Figure 3) As well as inversion-specific PCR primer sets, F1+R1 and F2+R2 primer sets, which can amplify normal genomic DNA region, also can amplify all that 6 types of genomic DNAs.(data not shown) This means that inverted and normal gemonic DNA are mixed in ZFN-treated cells. The inverted portion of that are depend on the efficiency of ZFNs. As well as efficiency of ZFNs, the number of chromosomes affects the frequency of inversion. According to previous study(Bylund et al.

2004), there are hyper-copies of chromosomes in HEK 293 cells. The *F8* gene locates in the X chromosome and there are three copies of X chromosomes in HEK 293 cells.

To identify cellular toxicity of ZFNs which can induce inversion, each ZFNs treated-cells were maintained and harvested in time course. As listed Figure 4., the genomic DNAs were isolated at 2day, 4day, 6day after transfection. The genomic DNAs from each time points were used template and inversion-specific PCR were performed.

At the time point of 2day-post-transfection, All of 6 types of genomic DNAs are positive to inversion-specific PCR screening.(Figure 4) The PCR products were analyzed by agarose gel eletrophoresis. The two sets of inversion-specific PCR primer pairs were used and only one sets of that were shown in Figure 4. All PCR analysis were performed with 100ng of genomic DNA and with same cycles, so the band intensity of each PCR products indicated the amounts of inverted genomic DNA indirectly. As see in Figure 4., ZFN 4, ZFN 10 and ZFN 53 treated genomic DNA shown relatively high intensity and ZFN 5 treated genomic DNA shown weak intensity compare with others.

At the time point of 4day-post-transfection, ZFN 5, ZFN 13 and ZFN 45 treated genomic DNA shown very weak intensity compare with their intensity of 2day-post-transfection. This means that these ZFNs has cellular toxicity, specially cased by off-target.(Kim et al. 2009)

At the time point of 6day-post-transfection, genomic DNA from ZFN 5 treated cells had no more inversion-specific bands. According to the

results, ZFN 4 and ZFN 10 has less cellular toxicity than others.

2. Improvements of ZFN specificity and activity.

As discussed above, ZFNs had cellular toxicity and the desired events were disappeared within few days if the ZFN had high cellular toxicity. To overcome that problems, many researchers made an effort.(Miller et al. 2007; Szczepek et al. 2007; Guo et al. 2010; Doyon et al. 2011) Each groups engineered FokI nucleases domains to reduce homo-dimerization. These improved forms of FokI nucleases domains were usually named to "Obligate hetero-dimer" forms. In this study among many obligate hetero-dimer forms, the "sharkey" variants, which made by Guo et al., were used to improve the activity and specificity of ZFN. The "sharkey" variants were made by directed evolution of FokI cleavage domains to improve activity of ZFNs and the cellular toxicity of that variants were measured by well-established assay, phosphorylated histone H2AX staining, and there were no cost effects of off-target cleavages.

The amino acid sequences of Fok1 variants are listed in Table 2. Theses FokI variants were cloned by site-directed mutagenesis method. Among 6 pairs of above ZFNs, ZFN 10 was selected to test FokI variants.

As listed in Figure 5., Left-sharkey RR and Right-sharkey DAS combination (donated RD in Fgure 5.) shown improved activity and less cellular toxicity than FokI wild type. The designated 30 degree menas that ZFN treated cells were incubated 1day in 37 degree followed 2day in 30 degree.(Doyon et al. 2010) The DS variants shown ~4% of local mutation frequencies according to T7 endonucleases assay.

Next, the junction sequences that indicated in Figure 1 were obtained according to the protocols in Materials and Methods. The inversion-specific PCR products were cloned into T-blunt vector and the plasmid DNAs were isolated from each colonies. Sequencing analysis of each colonies shown that many of them were perfect joined after inverted and some of them had indels, which were the common patterns of ZFN-mediated mutation.(Figure 6)

3. Frequency of ZFN-induced inversion and clonal assay.

The frequency of ZFN-induced inversion was measured by digital PCR method. As described in Figure 7., 3day-post transfected and 17 day-post transfected genomic DNA were used to identify frequencies of inversion. The estimated frequencies of inversion were 0.1~0.3% in 3day-post transfected genomic DNA and that frequencies were 10 times

more higher than in 17 day-post transfected genomic DNA. The exact frequencies of inversion were calculated using ELDA software.(Hu and Smyth 2009)

As described in Materials and Methods, inversion positive single clones were isolated. Three independent clones were obtained and each clones were confirmed by PCR and sequencing analysis.(Figure 8.) The two clones out of three shown perfect joint junction sequences in the two breakpoint junctions, and one clone had two base pair deleted sequences in junction 1. As there are three copies of X chromosome in HEK 293 cells, all of three clones contained wild type allele.

4. Inducing inversion in primary human blood cells.

As described above, it was confirmed that ZFN-mediated genomic inversion were possible in cultured human cells. In theory, this same ZFN pair could be used to revert the inverted genomic region of haemophilia A patient. That is, cells from haemophilia A patient are treated with ZFN so as to induce reversion the inverted genomic region. And then, the gene corrected cells are transferred to the patient again, especially through iPSCs (induced-pluripopent stem cell) technology, to produce deficient *F8* clotting factors.

To approach that theory, ZFN-mediated genomic inversion was

performed in primary human blood cells. Human blood cells were good sources to make iPSCs, so ZFN-mediated genomic inversion was performed in that cells. Blood cells from Haemophilia A patients are best source of this study, as gene corrected cells were used directly to make iPSCs and used in ex vivo cell therapy. There are only one cases in Korea that the intron1-related inversion is cause of Haemophilia A. The blood samples of Haemophilia A patient are obtained at Korea Haemophilia Foundation Clinic. The genomic DNA is isolated from that samples according to the protocols in Materials and Methods and, using inversion-specific PCR, genomic inversion events were confirmed as in Figure 10.

But it is very difficult to isolate transfectable cells from haemophilia A patient blood samples. As the second best plan, primary blood cells from normal male were used to perform ZFN-mediated genomic inversion. As in Figure 9., there are cellular toxicity according to the doses of ZFNs. ZFN-treated cells were analyzed by PCR to confirm inversion events. As shown in Figure 9., it is possible to induce genomic inversion in primary human blood cells.

ZFN technology is good tools for gene therapy. Here in this study, It is demonstrated that ZFN can induce targeted genomic inversion in intron 1 of *F8* gene, cause of severe haemophilia A. The frequencies of inversion in cultured human cells are 0.25%. The same ZFN pair are used to induce genomic inversion in primary human blood cells and can induce genomic inversion. This results holds new promisea for gene

and cell therapy. As discussed above, ZFN technology with iPSCs technology is powerful tools for ex vivo cell therapy.

IV. Figures and Tables

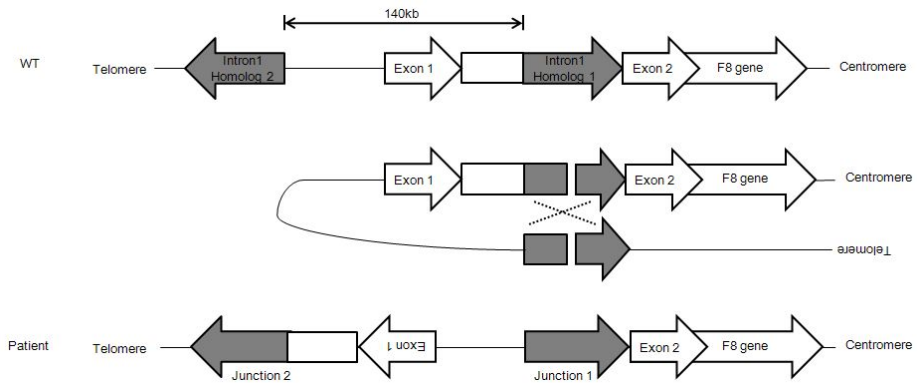


Figure 1. Schematic representation of a intron 1-related chromosomal inversion in severe Haemophilia A patient.

NAHR between two homologous regions, one in intron 1 of the *F8* gene (here named homolog 1) and the other located 140-kbp upstream (homolog 2), gives rise to an inversion found in severe hemophilia A. The two homologous regions are oriented in opposite directions

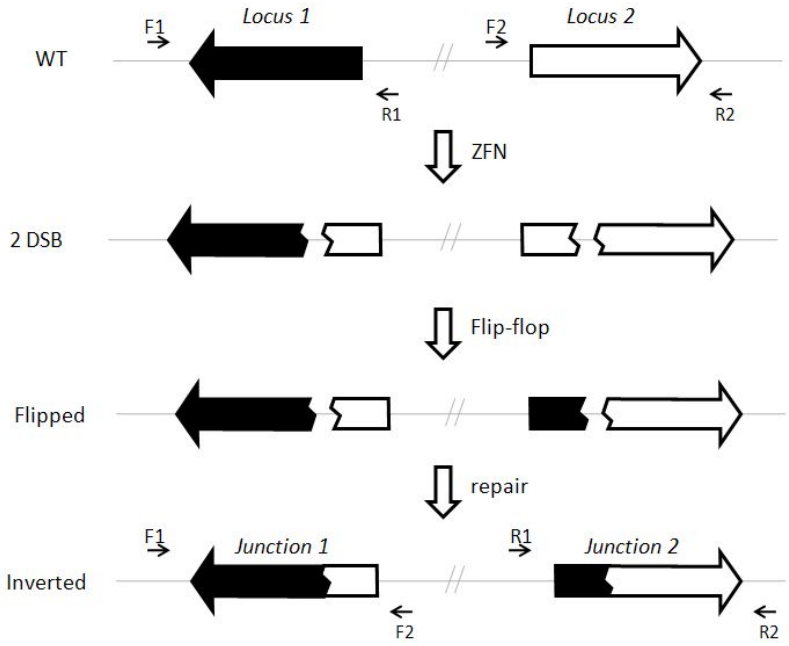


Figure 2. Schematic overview of PCR-based inversion detection method.

PCR primers (F1, F2, R1 and R2) used to detect the inversion are shown. Inversion events are detected using F1+F2 primer pairs or R1+R2 primer pairs.

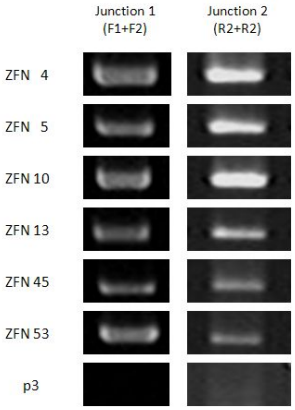


Figure 3. Screening of ZFNs which can induce inversion in genome.

Each ZFNs are trasnfected in human cells and their genomic DNA are analyzed by inversion specific PCR method.

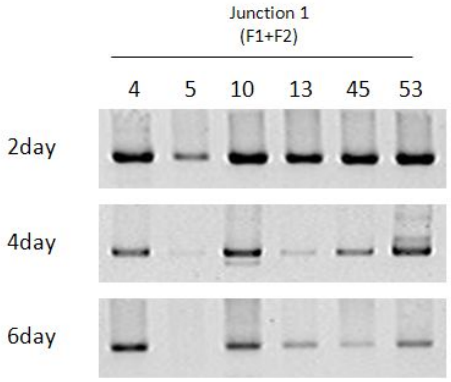


Figure 4. Cellular toxicity of each ZFNs.

ZFNs-transfected genomic DNA are obtained in time course and genomic Inversion events are detected using inversion-specific PCR.

Target site	ZFN name	F1	F2	F3	F4	Target sequence (5'to 3')
Intron1	4L	RDHT	mQSNR1	QSSR1	rdnq	AAGGCAGAAAGG
	4R	RDHT	DSAR2	RDER2	DSNRa	GACGTGGTCTGG
	5L	qntq	mQSNR1	QSHV	RDHT	TGGTGAGAAATA
	5R	sadr	QSNV3	DSAR2	QSSR1	GCAGTCCAAACA
	10L	RSHR	RDER2	rdnq	QSSR1	GCAAAGGTGGGG
	10R	DSAR2	RDHT	rdne		CAGTGGGTC
	13L	mQSNR1	QSSR1	rdnq	mDSCR	GCCAAGGCAGAA
	13R	RDHT	DSAR2	RDER2		GTGGTCTGG
	45L	RDHT	DSNRa	mQTHQ	RDHT	AGGAGAGACTGG
	45R	rdne	QSNV3	DSAR2		GTCCAACAG
	53L	RDHT	RDER2	mQTHQ		AGAGTGTGG
	53R	RSHR	thse	VSTR	mQTHQ	AGAGCTCCAGGG

Table 1. List of ZFNs which can induce inversion.

FokI type	Amino acid sequences.
Wild type	LVKSELEEKKSELRHKLKYVPHEYIELIEIARNSTQDRILEMKVMEFFMKVYGYRGKHLG GSRKPDGAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQADEMQRYVEENQTRNKHINPNEW WKVYPSSVTEFKFLFVSGHFKGNYKAQLTRLNHITNCNGAVLSVEELLIGGEMIKAGTLT LEEVRRKFNNGEINFSISSSSSLGPX
Sharkey WT	LVKSELEEKKSELRHKLKYVPHEYIELIEIARNPTQDRILEMKVMEFFMKVYGYRGEHLG GSRKPDGAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQADEMQRYVEENQTRNKHINPNEW WKVYPSSVTEFKFLFVSGHFKGNYKAQLTRLNHITNCNGAVLSVEELLIGGEMIKAGTLT LEEVRRKFNNGEINFSISSSSSLGPX
Sharkey DS	LVKSELEEKKSELRHKLKYVPHEYIELIEIARNPTQDRILEMKVMEFFMKVYGYRGEHLG GSRKPDGAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQADAMQSYVEENQTRNKHINPNEW WKVYPSSVTEFKFLFVSGHFKGNYKAQLTRLNHITNCNGAVLSVEELLIGGEMIKAGTLT LEEVRRKFNNGEINFSISSSSSLGPX
Sharkey RR	LVKSELEEKKSELRHKLKYVPHEYIELIEIARNPTQDRILEMKVMEFFMKVYGYRGEHLG GSRKPDGAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQAREMQRYVEENQTRNKHINPNEW WKVYPSSVTEFKFLFVSGHFKGNYKAQLTRLNHITNCNGAVLSVEELLIGGEMIKAGTLT LEEVRRKFNNGEINFSISSSSSLGPX

Table 2. Amino acid sequences of FokI variants.

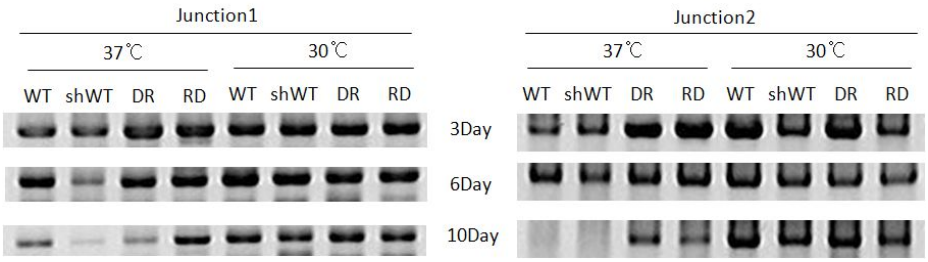


Figure 5. Improvements of Z10 specificity and activity.

Various Fok1 variants are linked to Z10 and their activity and toxicity are measured in time course using inversion-specific PCR method. DR means sharkey DS-RR combination and RD means sharkey RR-DS combination.

Figure 6. DNA sequences of breakpoint junctions of inversion events.

Two Z10 target sites(in homolog1 and homolog2 as Figure 1) are shown in italic.

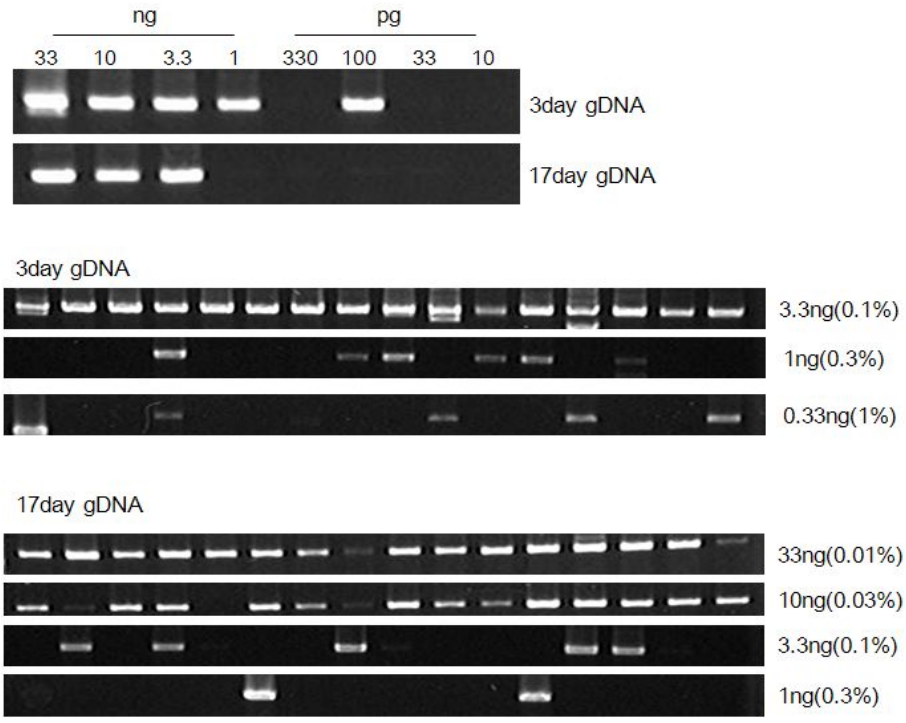
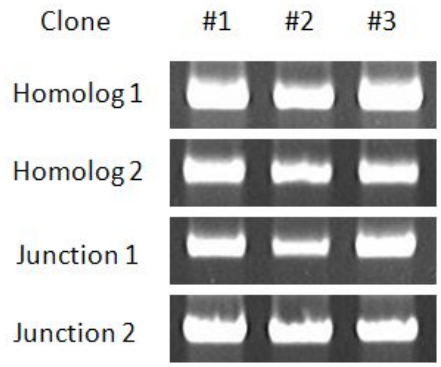


Figure 7. Frequency of Z10-induced genomic inversion.

Genomic DNA of two time points, 3dpt(day post transfection) and 17dpt, are quantified and digital PCR method are used to detect frequency of inversion.

ZFN	Length (kbp)	Amount of genomic DNA (Copy number per half genome)										Frequency (%)		p value (Fit)
		100 ng (30000)	30 ng (10000)	10 ng (3000)	3 ng (1000)	1 ng (300)	300 pg (100)	100 pg (30)	30 pg (10)	10 pg (3)	3 pg (1)	Estimate	Upper and Lower Limits	
Inversion														
ZFN-224	15	1/1	5/5	5/5	9/9	17/17	17/17	12/17	7/17			5	(3-7)	0.997
Z10	150			16/16	16/16	6/16	4/16	0/1	0/1	0/1		0.25	(0.2-0.4)	0.25

Table 3. Frequencies of ZFN-induced genomic inversion.



Homolog 1

```
WT        ..CAAGGAGACCCACTGAGTTGGGCAAAGGTGGGGCCGAC..
CLONE #1  ..CAAGGAGACCCACTGAGTTGGGCAAAGGTGGGGCCGAC..
CLONE #2  ..CAAGGAGACCCACTGAGTTGGGCAAAGGTGGGGCCGAC..
CLONE #3  ..CAAGGAGACCCACTGAGTTGGGCAAAGGTGGGGCCGAC..
```

Homolog 2

```
WT        ..GTCGGCCCCACCTTTGCCCAACTCAGTGGGTCTCCTTG..
CLONE #1  ..GTCGGCCCCACCTTTGCCCAACTCAGTGGGTCTCCTTG..
CLONE #2  ..GTCGGCCCCACCTTTGCCCAACTCAGTGGGTCTCCTTG..
CLONE #3  ..GTCGGCCCCACCTTTGCCCAACTCAGTGGGTCTCCTTG..
```

Junction 1

```
WT        ..CAAGGAGACCCACTGAGTTGGGCAAAGGTGGGGCCGAC..
CLONE #1  ..CAAGGAGACCCACTGAGT--GGCAAAGGTGGGGCCGAC..
CLONE #2  ..CAAGGAGACCCACTGAGTTGGGCAAAGGTGGGGCCGAC..
CLONE #3  ..CAAGGAGACCCACTGAGTTGGGCAAAGGTGGGGCCGAC..
```

Junction 2

```
WT        ..GTCGGCCCCACCTTTGCCCAACTCAGTGGGTCTCCTTG..
CLONE #1  ..GTCGGCCCCACCTTTGCCCAACTCAGTGGGTCTCCTTG..
CLONE #2  ..GTCGGCCCCACCTTTGCCCAACTCAGTGGGTCTCCTTG..
CLONE #3  ..GTCGGCCCCACCTTTGCCCAACTCAGTGGGTCTCCTTG..
```

Figure 8. Single clonal assay of inversion positive pools and validation of each clones

Three independent clones are obtained and validated by PCR. Each PCR products are used to sequencing analysis.

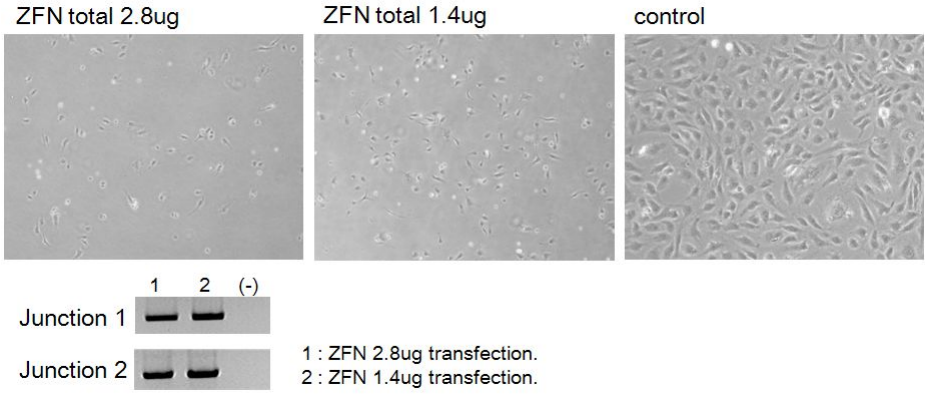


Figure 9. Z10-mediated inversion in primary human blood cells.

Z10 are treated in primary human blood cells by eletroporation. Inversion events are detected by PCR method using primer sets in Figure 2.

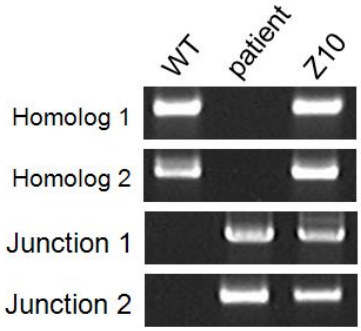


Figure 10. Haemophilia A patient.

The genomic DNA are isolated from Haemophilia A patient and PCR confirmation of genomic inversion is performed.

V. Reference

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국 문 초 록

유전체 상의 염기 서열 분석 방법이 급속도로 발달하면서, 최근 유전체 상의 구조 변이에 대한 연구가 활발히 진행되고 있다. 일반적으로 구조 변이는 단일 유전자 변이(SNP) 보다 광범위 하게 일어나는 기작으로 유전체 상의 삽입, 결실, 중복, 전좌, 그리고 역위 등이 있다. 이러한 구조적 변이는 평범한 사람들의 유전체 상에서 일부 보여 지고 있지만, 많은 경우에는 실제로 유전병을 발병시키는 원인이 되기도 한다. 특히 유전체 상의 역위에 의해서, 혈우병을 비롯한 헌터 증후군 등이 발병된다고 보고되어 있다.

이러한 이유에서 유전병 치료 분야에서 구조 변이가 주목받고 있지만, 실제 구조 변이에 대한 기작 연구에는 어려움이 있었다. 실제 유전병을 가지고 있는 동일한 개체 또는 세포 수준에서 손쉽게 구조 변이를 유도할 수 있는 도구가 없었기 때문이다.

본 연구에서는, 인간 세포에서 징크 핑거 뉴클레아제 기술을 이용하여 유전체 상에 역위를 일으킬 수 있음을 보이고자 하였다. 특히 혈우병 A 유형의 발병 원인이 되는, 혈액 응고 인자 8번 유전자 상의 역위를 인위적으로 유도할 수 있는 징크 핑거 뉴클레아제를 만들고 검증해 보았다. 이를 통하여 이와 동일한 징크 핑거 뉴클레아제가 혈우병 A 환자의 세포 치료에 활용 될 수 있는 가능성을 열었다는데 의미가 있다. 본 연구를 바탕으로 하여 징크 핑거 뉴클레아제 기술이, 유전병 치료 분야에서 널리 이용되고 연구되기를 바라는 바이다.

주요어 : 징크핑거뉴클레아제(ZFN), 이중가닥손상(DSB), 구조 변이(SVs), 유전체 역위, 혈우병 A, *F8* 유전자.

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