様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

科研費
KAKENHI

科学研究費助成事業　　研究成果報告書

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研究課題名（和文）Evaluating the role of cis-regulatory tandem DNA repeats in human disease and evolution

研究課題名（英文）Evaluating the role of cis-regulatory tandem DNA repeats in human disease and evolution

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交付決定額（研究期間全体）：（直接経費）　13,400,000円

研究成果の概要（和文）：私たちはヒトiPS細胞でVNTR（反復配列多型）のコピー数を制御する遺伝子編集に着目しました。まず、22株のiPS細胞株における5座位のVNTRのコピー数を探索しました。このプロファイルを参考に、CRISPR-Cas9を用いた実験を行い、コピー数を1つまで減らすことに成功しました。さらに、一部の反復配列を切断せずに保護することで、複数のコピー数のパターンを作り出す技術を確立しました。現在は本手法により様々なコピー数のVNTRを持つiPS細胞を作製し、ヒトおよび霊長類におけるVNTRの意義を探索する研究を進めています。

研究成果の学術的意義や社会的意義
VNTR copy-number editing is a new gene editing technique that allows scientists to study causal relationships between repetitive DNA and human disease. Achieving this in human iPS cells enables disease modeling of a new variety of genetic disorders in virtually all cell types of the body

研究成果の概要（英文）：Repetitive sequences are poorly understood regions of the human genome. Variable Number Tandem Repeats (VNTRs) are strings of DNA repeats that change in number between individuals. These changes can affect gene expression and may cause disease. To understand these relationships, we developed a novel gene editing technique to change VNTR copy numbers in human cells. After characterizing the copy number differences of 5 naturally occurring VNTRs across 22 human iPS cell lines, we used CRISPR-Cas9 to cut each repeat, triggering a cellular DNA repair that reduces repeat number to one. We repeated this process but protecting some repeats from being cut, enabling the generation of cells with intermediate repeat numbers, something which has never been previously achieved. We used this method to generate iPS cells with VNTRs of various copy numbers to model human disease. With these novel tools, we have begun to study VNTRs more broadly across the human genome and in non-human primate models.

研究分野：ゲノム生物学

キーワード：ゲノム編集　ゲノム解析　DNAリピート　ヒトiPS細胞

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様　式　Ｃ－１９、Ｆ－１９－１（共通）

１．研究開始当初の背景

Variable Number Tandem Repeats (VNTRs) are genomic regions with consecutive repeats that are polymorphic across the human population. VNTRs occur at coding and non-coding regions and have been associated with changes in gene expression and human disease. However, the connection between VNTR copy-number and phenotype is weak and would benefit from biological models that functionally link them to disease.

Induced pluripotent stem (iPS) cells retain the genome of their donor and can be differentiated into any cell type in the body. The generation of VNTR polymorphisms in an isogenic platform such as an iPS cell line will enable to experimentally link variants to functional outcomes or disease states.

２．研究の目的

In this study, we aimed (1) to explore VNTR polymorphisms already present in iPS cell biobanks and cell lines commonly used for research, and (2) to develop a technique to reliably generate a series of VNTR polymorphisms in iPS cells while retaining an isogenic background.

３．研究の方法

(1) iPS cell culture and VNTR genotyping

iPS cells were cultured under feeder-free conditions and passaged every 7 days. VNTR polymorphisms were genotyped by amplifying iPS cell genomic DNA by polymerase chain reaction (PCR) and repeat copy numbers were estimated by TapeStation capillary electrophoresis of the PCR product, followed by verification with Sanger sequencing. To establish VNTR edited monoclonal cell lines, colonies derived from single cells were picked following low-density plating of edited iPS cells, and culture in 96-well format. The genotypes were estimated by PCR and TapeStation capillary electrophoresis and verified by Sanger sequencing.

(2) gRNA design and electroporation

CRISPR gRNAs for each VNTR were designed using the software MENTHU (Ata et al., 2018). An equimolar amount of synthetic crRNA and tracrRNA sequences (IDT) was hybridized to form functional crRNA:tracrRNA duplexes (gRNA). gRNA was incubated with Cas9 nuclease in a 1:1 molar ratio to form RNP complexes, and RNP complexes were electroporated into iPS cells using a NEPA21 Electroporator. For dCas9 and Cas9 administration experiments, various ratios of dCas9:Cas9 (0:100, 10:90, 25:75, 50:50, 75:25, 90:10, and 100:0) were mixed, and a 1:1 molar ratio of gRNA was added to form the RNP complexes.

(3) Nanopore sequencing and analysis

Long-read sequencing of edited iPS cells was performed on an Oxford Nanopore Technologies MinION Mk1B according to the manufacturer's instructions for the Ligation Sequencing Kit for amplicons (SQK-LSK109) with PCR barcoding (EXP-PBC001). Basecalling and demultiplexing was performed using the dorado super accuracy model and read quality checks were performed using NanoPlot for visualization, minimap2 for read mapping, and samtools and chopper for read filtering. VNTR editing genotyping was performed using the software DAJIN2 (Kuno et al., 2022) using default parameters.

４．研究成果

(1) VNTR polymorphisms are represented in iPS cells from various donors

From the literature, we selected VNTR loci neighbouring 4 different genes (TRIB3, MAOA, IL1RN, and SLC6A3) associated with conditions such as antisocial behaviour, stress, ADHD, and breast cancer. Twenty-two iPS cell lines including five commonly used iPS cell lines for research and seventeen others obtained from the CiRA Healthy and Rare Disease biobanks (Saito, et al. 2023) were selected for VNTR genotyping. We observed VNTR polymorphisms (both homozygous and heterozygous) across all iPS cell lines tested (**Fig. 1**).

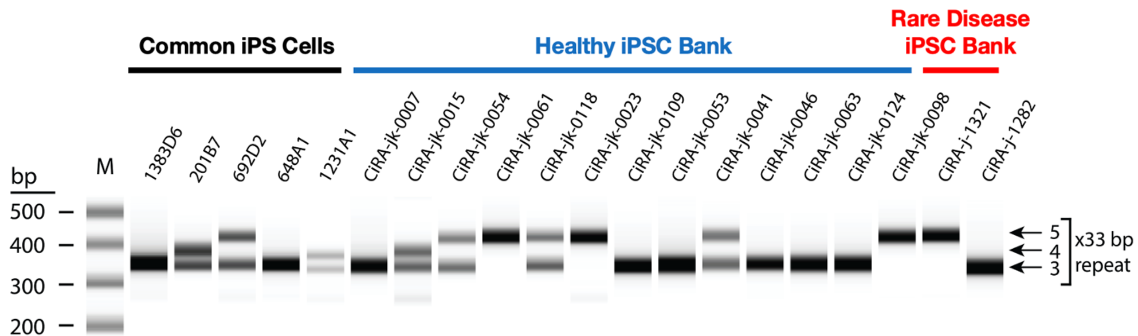


Figure 1. Analysis of VNTR polymorphisms within human iPS cell lines using genomic PCR and capillary electrophoresis.

(2) gRNA placement within VNTRs influences the efficiency and fidelity of repeat contractions

We used MENTHU, a software tool that aims to increase the chance of repeat contractions following a DNA double strand break (Ata et al., 2018), to design CRISPR gRNAs targeting different locations within a repeat of the TRIB3 VNTR (**Fig. 2A**). When delivered into homozygous 3-copy iPS cells (1383D6), each gRNA resulted in TRIB3 VNTR contractions at different efficiencies, as estimated by electrophoresis. While VNTRs tended to contract into a single copy, double strand break location within each repeat affected the relative amount of each predicted VNTR length, as evidenced by electrophoresis (**Fig. 2B**). We performed a similar gRNA design and selected the top three gRNAs for VNTR contraction across the other 3 loci, and the results were reproduced in 2 different iPS cell lines.

To assess the fidelity of the VNTR contractions and maintenance of the repeat structure, we performed long-read sequencing of PCR amplicons using Oxford Nanopore Technology. Long-read sequencing data was analyzed using DAJIN2, a tool that specializes in Nanopore read classification from a mixed genetic pool following gene editing. DAJIN2 confirmed the presence of intermediate intact VNTR alleles using some gRNAs, albeit with different levels of indel formation (**Fig. 2C**). For the TRIB3 VNTR, gRNAs 1, 3, and 4 resulted in the highest proportion of reads representing contraction to a single copy. However, many of the single-copy alleles generated by gRNA 4 contained additional indels, disrupting the natural sequence of the TRIB3 repeat. Typically, for each VNTR, gRNAs with cut sites closest to the repeat period that displayed the highest fidelity and were selected for later experiments.

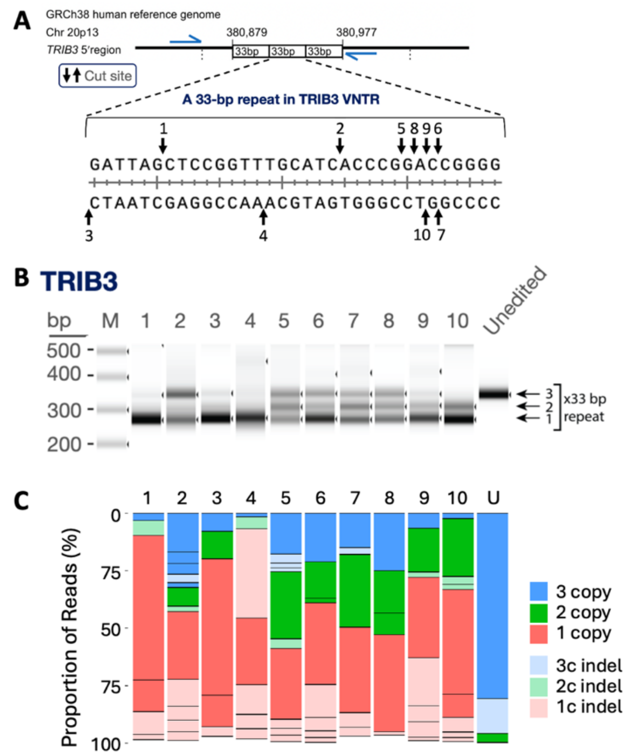


Figure 2. Efficiency and fidelity of repeat editing. A.) TRIB3 33-bp repeat sequence and the positions of DSB sites for each gRNA. B.) Genomic PCR and electrophoresis of the polyclonal populations after VNTR editing. C.) DAJIN2 analysis of Nanopore sequence data for each population. Each copy number is represented by color. Opaque sections represent contracted VNTRs with indels.

<u>(3) Competition for repeat binding with catalytically dead Cas9 results in intermediate VNTR copy numbers</u>

We hypothesized that co-administration of catalytically inactive Cas9 (dCas9) to compete with Cas9 for VNTR binding would increase the yield of intermediate VNTR copy numbers in CRISPR-Cas9 VNTR contraction experiments using the gRNAs selected in (2). As expected, co-administration of dCas9 and Cas9 complexed with the same gRNA targeting the TRIB3 repeat influenced the distribution of intermediate VNTR lengths in iPS cells (**Fig. 3A**). A titration of dCas9:Cas9 ratios demonstrated that increased levels of dCas9 improve the yield of intermediate VNTR copy-numbers. Moreover, we demonstrated that the result is not a consequence of reduced Cas9 concentration alone (**Fig. 3B**). These results were replicated the other 3 VNTR loci.

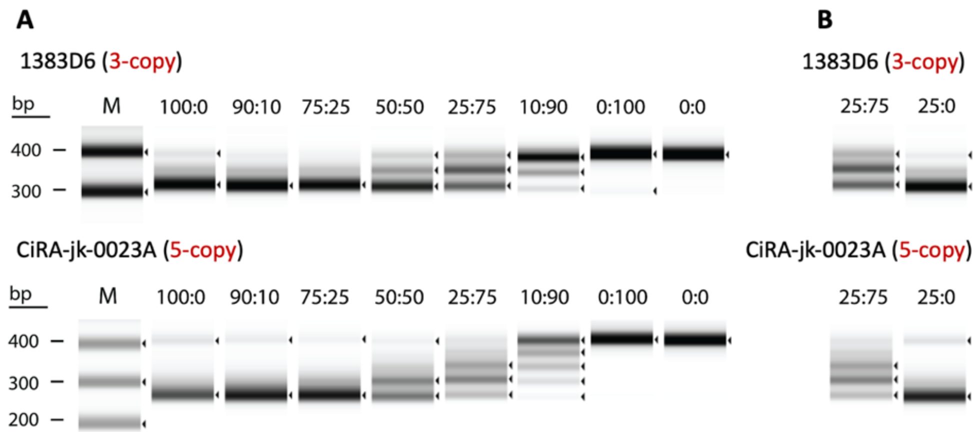
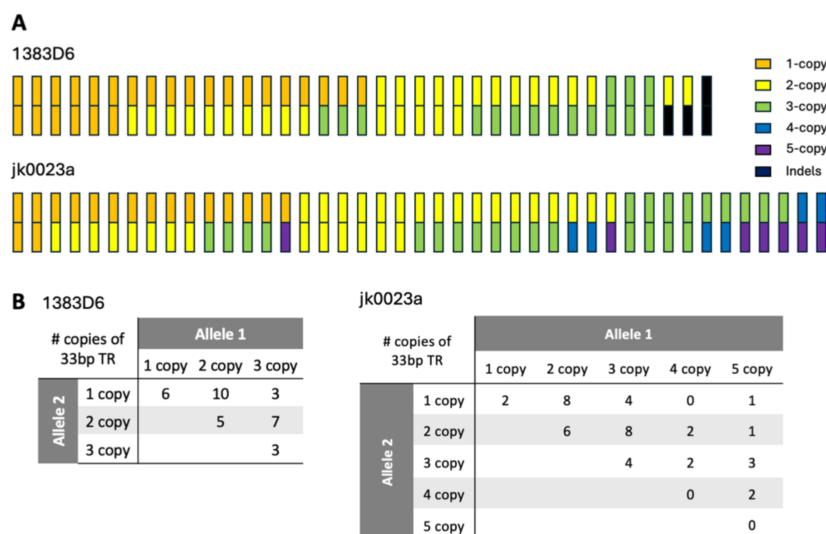


Figure 3. Control of VNTR contraction using dCas9. A.) Analysis of genomic PCR from polyclonal populations following gene editing with various ratios of dCas9:Cas9. B.) Analysis of genomic PCR from polyclonal populations following gene editing with reduced Cas9 concentrations.

(4) Isolation of clonal iPS cell lines with polymorphic VNTRs

We hypothesized that controlled VNTR contraction in an iPS cell line homozygous for a given VNTR length should result in all possible diploid combinations of reduced VNTR copy-number variants, given enough monoclonal colonies are sampled. To test this, we electroporated two different iPS cell lines with RNPs made from gRNA 3 for TRIB3 VNTR editing and the 90:10 dCas9:Cas9 mixture, which together displayed the most homogeneous distribution of intermediate VNTR alleles. For the TRIB3 VNTR, 1383D6 is a 3-copy homozygous cell line while CiRA-jk-0023a is a 5-copy homozygous cell line (**Fig. 1**). Electrophoresis was used to estimate the genotypes of the picked clones (**Fig. 4A**). We were able to isolate all 6 possible genotype combinations in the 1383D6 background and 12/15 combinations in the CiRA-jk-0023a background, from only 48 clones per cell line (**Fig. 4B**).



# copies of 33bp TR (Allele 2)	Allele 1: 1 copy	2 copy	3 copy
1 copy	6	10	3
2 copy		5	7
3 copy			3

# copies of 33bp TR (Allele 2)	Allele 1: 1 copy	2 copy	3 copy	4 copy	5 copy
1 copy	2	8	4	0	1
2 copy		6	8	2	1
3 copy			4	2	3
4 copy				0	2
5 copy					0

Figure 4. Genotypes on monoclonal iPS cell lines. A.) Diploid genotypes of the monoclonal cell lines determined by electrophoresis and Sanger sequencing. B.) Enumeration of the distribution of diploid clones with various combinations of VNTR alleles.

５．主な発表論文等

〔雑誌論文〕　計1件（うち査読付論文　0件／うち国際共著　0件／うちオープンアクセス　0件）

１．著者名	４．巻
Martinez-Galvez Gabriel、Lee Suji、Niwa Ryo、Woltjen Knut	7
２．論文標題	５．発行年
On the edge of deletion: Using natural and engineered microhomology to edit the human genome	2024年
３．雑誌名	６．最初と最後の頁
Gene and Genome Editing	100033～100033
掲載論文のDOI（デジタルオブジェクト識別子）	査読の有無
10.1016/j.ggedit.2024.100033	無
オープンアクセス	国際共著
オープンアクセスではない、又はオープンアクセスが困難	－

〔学会発表〕　計12件（うち招待講演　4件／うち国際学会　1件）

１．発表者名
Gabriel Felipe Martinez-Galvez
２．発表標題
A comparative analysis of DNA double strand break repair predictors for template-less precision gene editing
３．学会等名
日本ゲノム編集学会第7回大会
４．発表年
2022年

１．発表者名
Gabriel Felipe Martinez-Galvez
２．発表標題
Deploying MENdel MMEJ prediction for the efficient generation of frameshift deletions and disease modeling in human induced pluripotent stem cells
３．学会等名
CiRA Retreat 2022
４．発表年
2022年

１．発表者名
Knut Woltjen
２．発表標題
Modeling genetic and infectious disease using Gene Editing and iPS Cell technologies
３．学会等名
International Conference on Trends and Challenges in Health Sciences, Lahore, Pakistan（招待講演）
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1．発表者名
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2．発表標題
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3．学会等名
School of Biomedical Engineering Seminar Series, UBC, Canada（招待講演）
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2022年

1．発表者名
Knut Woltjen
2．発表標題
ヒトiPS細胞における精密なゲノム編集技術
3．学会等名
第21 回日本再生医療学会総会（招待講演）
4．発表年
2022年

1．発表者名
Xiaoyan Ren, Gabriel Felipe Martinez-Galvez, Knut Woltjen
2．発表標題
Discovery and generation of polymorphic copy number variants in human iPS cells
3．学会等名
第3回研究交流サロン
4．発表年
2023年

1．発表者名
Xiaoyan Ren, Gabriel Felipe Martinez-Galvez, Knut Woltjen
2．発表標題
Discovery and generation of polymorphic copy number variants in human iPS cells
3．学会等名
CiRA International Symposium 2023
4．発表年
2023年

１．発表者名
Xiaoyan Ren, Gabriel Felipe Martinez-Galvez, Knut Woltjen

２．発表標題
Discovery and generation of polymorphic copy number variants in human iPS cells

３．学会等名
日本分子生物学会第46回大会

４．発表年
2023年

１．発表者名
Xiaoyan Ren, Gabriel Felipe Martinez-Galvez, Knut Woltjen

２．発表標題
Discovery and generation of polymorphic copy number variants in human iPS cells

３．学会等名
CiRA Retreat 2023

４．発表年
2024年

１．発表者名
Knut Woltjen

２．発表標題
Genome Editing in Human iPS Cells for Modeling Infectious and Genetic Disease

３．学会等名
2023 Till & McCulloch Meetings（招待講演）（国際学会）

４．発表年
2023年

１．発表者名
Knut Woltjen

２．発表標題
Genome Editing in Human iPS Cells for Modeling Infectious and Genetic Disease

３．学会等名
国際キャリア基礎と実践

４．発表年
2023年

1．発表者名 Knut Woltjen
2．発表標題 Genome Editing in Human iPS Cells for Modeling Infectious and Genetic Disease
3．学会等名 ゲノム編集医療概論
4．発表年 2024年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

－

6．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考
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7．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

8．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関