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

令和　２ 年　 ６ 月 ２３ 日現在

機関番号：１４３０１
研究種目：基盤研究(C)（一般）
研究期間：2017～2019
課題番号：１７Ｋ０７２５６

研究課題名（和文）再生医療の実現に向けたHLA発現制御可能ユニバーサルiPS細胞の樹立

研究課題名（英文）Establishment of universal iPS cells for regenerative medicine applications through regulated HLA expression

研究代表者
ウォルツェン　クヌート（Woltjen, Knut）

京都大学・iPS細胞研究所・准教授

研究者番号：５０５８９４８９

交付決定額（研究期間全体）：（直接経費）　　3,800,000円

研究成果の概要（和文）：ヒト白血球抗原（Human Leukocyte Antigen）は、細胞移植の際、拒絶反応を引き起こす細胞表面の糖タンパク質であり、個人が固有のパターンを持つ。個別化iPS細胞は自家移植を可能にするため、細胞療法として大きな可能性を持つ。しかし、全ての治療においてのオンデマンド作製やバンキングは技術的にも経済的にも実現性が低い。一方で、固有のHLAを持たない「ユニバーサル」iPS細胞は、免疫拒絶反応の回避ができると考えられる。我々は、再生医療に広く適用可能で安全なiPS細胞の提供を目標として、HLAの発現と提示を制御するためのエピジェネティック制御システムを開発し、その可逆性を調べた。

研究成果の学術的意義や社会的意義
We created a reversible gene regulation system which is sensitive and could be applied in vivo. We made a new finding that epigenetic changes caused by an RNA-programmable repressor may persist through differentiation. Our system should be useful for the study of gene function and immunoregulation.

研究成果の概要（英文）：Human leukocyte antigens (HLA) are highly polymorphic gene loci that encode cell-surface glycoproteins which are the strongest transplant antigens leading to T-cell activation, antibody production, and allograft rejection. Personalized iPS cells hold great promise as cellular therapies by autologous transplantation. However, on-demand generation or banking of individualized iPS cells to treat all patients is neither technically nor economically feasible. On the other hand, ‘Universal’ iPS cells which bear no intrinsic HLA signature, would avoid immune rejection. We developed an epigenetic regulation system to control HLA gene expression and presentation. We explored the reversibility of the system with the goal of providing widely applicable and safe iPS cells for regenerative medicine.

研究分野：Medical genome science

キーワード：Human leukocyte antigen　HLA　ゲノム編集　iPS細胞　CRISPR Cas9　KRAB　B2M　allograft

※科研費による研究は、研究者の自覚と責任において実施するものです。そのため、研究の実施や研究成果の公表等については、国の要請等に基づくものではなく、その研究成果に関する見解や責任は、研究者個人に帰属されます。

１．研究開始当初の背景

Human leukocyte antigens (HLA) are highly polymorphic gene loci that encode cell-surface glycoproteins which are the strongest transplant antigens leading to allograft rejection. While personalized iPS cells hold great promise as cellular therapies by autologous transplantation, on-demand generation or banking of individualized iPS cells to treat all patients is neither technically nor economically feasible, and proposed HLA-homozygous iPS cell stocks present certain limitations.

２．研究の目的

'Universal' iPS cells, which bear no intrinsic HLA signature, would avoid immune rejection and have unrestricted application. However, the inability to intervene in the case of a potential danger brings significant risk. We proposed a reversible approach to immune evasion in order to provide widely applicable and safe iPS cells for regenerative medicine.

３．研究の方法

To generate 'Universal' iPS cells we aimed to develop a drug-regulated CRISPRi system for reversible knockdown of target genes responsible for HLA presentation and "cloak" (hide) cells from the immune system. By reversibly regulating HLA presentation, we could "reveal" transplanted cells to the host immune system as a safety net in the prevention of graft malfunction or tumorigenesis.

４．研究成果

For reversible B2M knockdown, we used an iPS cell line containing a tet-ON KRAB-dCas9 transgene targeted to the AAVS1 (PPP1R12C) safe-harbor locus. We selected and tested 6 gRNAs targeting the transcription start site of B2M, and found 2 gRNAs that achieved > 95 % knockdown, even when B2M expression was boosted by IFNγ treatment (**Figure 1**). The specificity of KRAB-dCas9 for B2M knockdown was verified by qPCR for B2M, TAP1, Nanog, and GAPDH. We performed a detailed analysis of the dynamics of KRAB-Cas9 expression as well as B2M repression and de-repression over time. We reproduced these results in a second iPS cell line known to have a different HLA haplotype.

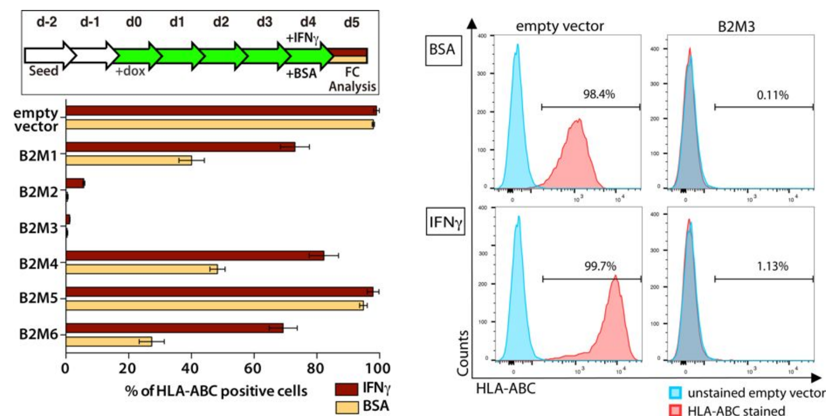

Figure 1. Preliminary screening of six CRISPRi B2M gRNAs (B2M1-6) by random integration in CRISPRi iPSCs. With 5 days dox, gRNAs B2M2 and B2M3 achieve >90% knockdown, even in the presence of IFN-γ (left, red bars). Representative FACS data of the empty vector control and B2M gRNA3 in the presence of dox with or without IFN-γ (right).

As chronic, high-level dox treatment is unfavorable for iPS cell growth, differentiation, and eventual in vivo application, we developed an AAVS1-targeted tet-OFF CRISPRi system. We found the tet-OFF system to be 10,000-fold more sensitive to dox than Tet-ON. We targeted the most effective gRNA in single-copy to the second AAVS1 allele, and re-analyzed for B2M knockdown. We found one gRNA copy sufficient to repress B2M expression, even when challenged with IFNγ treatment. Using qPCR we noted minimal pleiotropy on neighbouring gene expression.

We evaluated Tet-OFF iPS cell differentiation. We found that endoderm and ectoderm maintained the CRISPRi system, but mesoderm showed a loss of expression. We hypothesized that mesodermal cells would be unable to maintain B2M repression, and would become HLA-ABC+. We differentiated Tet-OFF iPS cells to CD45+ hematopoietic cells and found they were HLA-ABC negative. Although the B2M promoter is not normally methylated in iPS cells or CD45+ cells, we found that, unlike de-repressed (+ dox) iPS cells, the B2M promoter of CD45+ cells remained highly methylated.

Finally, we compared B2M repression using dCas9 with and without the KRAB domain (**Figure 2**). Interestingly, dCas9 alone was sufficient to interfere with basal-level B2M

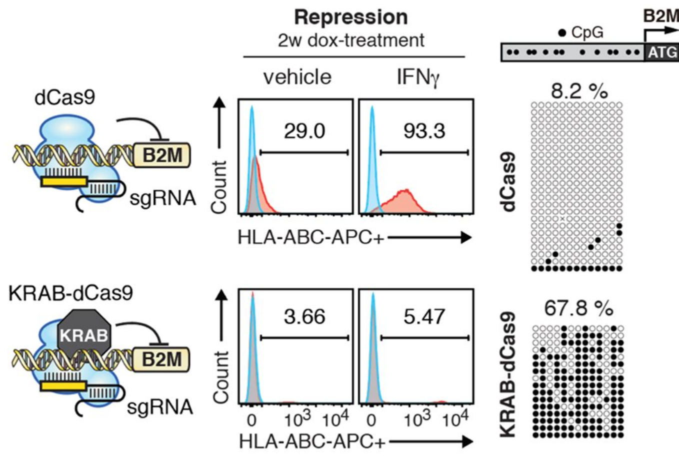

Figure 2. Perturbation of an endogenous gene (B2M) can be achieved using dCas9 (top) or KRAB-dCas9 (bottom). Steric hinderance by dCas9 can be overcome by stimulatory signals (IFNɣ). On the other hand, the KRAB-domain induces local epigenetic modifications affecting DNA and histones, resulting in a strong knockdown which is resistant to IFNɣ stimulation.

expression, but could not overcome stimulation with IFNɣ. We found that even in iPSCs, the B2M promoter was methylated by KRAB. Therefore, an attenuated KRAB transcriptional repressor domain, or the dCas9 system alone, may be effective to achieve reversible repression of HLA as outlined in Aim2 of the grant.

In conclusion, we created a novel CRISPRi system which is reversible and sensitive, avoiding the need for chronic, high-dosage dox treatment, making it suitable for in vivo applications. We made a new finding that epigenetic changes caused by KRAB-dCas9 may persist through differentiation, and suggest a potentially valuable approach to permanently regulate gene expression without genetic mutation. We hope to refine and apply this system to additional immunoregulatory gene targets in future studies.

５．主な発表論文等

〔雑誌論文〕　計3件（うち査読付論文　2件／うち国際共著　2件／うちオープンアクセス　2件）

１．著者名 Woltjen Knut、Bortvin Alex	４．巻 52
２．論文標題 Editorial overview: Embroidering the canvas of life	５．発行年 2018年
３．雑誌名 Current Opinion in Genetics & Development	６．最初と最後の頁 ii～iv
掲載論文のDOI（デジタルオブジェクト識別子） 10.1016/j.gde.2018.11.002	査読の有無 無
オープンアクセス 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	国際共著 －

１．著者名 Kim Shin-Il、Matsumoto Tomoko、Kagawa Harunobu、Nakamura Michiko、Hirohata Ryoko、Ueno Ayano、Ohishi Maki、Sakuma Tetsushi、Soga Tomoyoshi、Yamamoto Takashi、Woltjen Knut	４．巻 9
２．論文標題 Microhomology-assisted scarless genome editing in human iPSCs	５．発行年 2018年
３．雑誌名 Nature Communications	６．最初と最後の頁 939
掲載論文のDOI（デジタルオブジェクト識別子） 10.1038/s41467-018-03044-y	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 該当する

１．著者名 Kieffer Timothy J、Woltjen Knut、Osafune Kenji、Yabe Daisuke、Inagaki Nobuya	４．巻 9
２．論文標題 Beta-cell replacement strategies for diabetes	５．発行年 2017年
３．雑誌名 Journal of Diabetes Investigation	６．最初と最後の頁 457～463
掲載論文のDOI（デジタルオブジェクト識別子） 10.1111/jdi.12758	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 該当する

〔学会発表〕　計10件（うち招待講演　7件／うち国際学会　3件）

１．発表者名 Knut Woltjen
２．発表標題 Japan's experience in regenerative medicine and superdonor cells
３．学会等名 NRC's HEALTH CHALLENGE PROGRAM WORKSHOP : 'Transforming Health Outcomes through Engineered Cell and Gene Therapies'（招待講演）
４．発表年 2018年

１．発表者名 Knut Woltjen
２．発表標題 Precise human disease allele creation and correction through microhomology-mediated end joining
３．学会等名 IREM Seminar, Zurich, Switzerland（招待講演）
４．発表年 2018年

１．発表者名 Knut Woltjen
２．発表標題 Genetic engineering of pluripotent stem cells by CRISPR/Cas
３．学会等名 糖尿病ミニシンポジウム Kyoto Diabetes Mini-Symposium: Beta-Cell Replacement Strategies（招待講演）
４．発表年 2017年

１．発表者名 Knut Woltjen
２．発表標題 iPS細胞におけるゲノム編集技術を活用した遺伝性皮膚炎のモデル化
３．学会等名 第38回日本炎症・再生医学会（大阪国際会議場）（招待講演）
４．発表年 2017年

１．発表者名 Knut Woltjen
２．発表標題 Endogenous DNA repair pathways for gene editing in human induced pluripotent stem cells
３．学会等名 Celllular & Physiological Sciences Departmental Seminar（招待講演）
４．発表年 2017年

１．発表者名 Knut Woltjen
２．発表標題 The development of induced pluripotent stem cells and emerging tools for genome editing
３．学会等名 "Stem Cells and Regenerative Medicine: What does the Future Hold?" University of British Columbia（招待講演）
４．発表年 2017年

１．発表者名 Anika Reinhardt, Mitchell J. S. Braam, Suji Lee, Yanjun Lan, Shin-Il Kim, Timothy J. Kieffer, Knut Woltjen
２．発表標題 CRISPRi regulates HLA presentation on human cells
３．学会等名 Keystone Symposium
４．発表年 2019年

１．発表者名 Suji Lee, Anika Reinhardt, Michiko Nakamura, Tomoko Matsumoto, Knut Woltjen
２．発表標題 Characterization and application of a new CRISPR interference system for reversible gene knockdown
３．学会等名 日本ゲノム編集学会（国際学会）
４．発表年 2019年

１．発表者名 Anika Reinhardt, Suji Lee, Mitchell J. S. Braam, Timothy J. Kieffer, Knut Woltjen
２．発表標題 REVERSIBLE GENE KNOCKDOWN IN PLURIPOTENT AND DIFFERENTIATED CELLS
３．学会等名 ISSCR（国際学会）
４．発表年 2019年

１．発表者名 Knut Woltjen and Suji Lee
２．発表標題 Gene correction and immune evasion strategies for autologous and allogenic cell therapies
３．学会等名 EASD-Hagedorn Oxford Workshop（招待講演）（国際学会）
４．発表年 2019年

〔図書〕　計1件

１．著者名 Suji Lee, 晴信 香川, 智子 松本, Fabian Oceguera-Yanez, and Knut Woltjen	４．発行年 2019年
２．出版社 羊土社 実験医学別冊	５．総ページ数 386
３．書名 完全版　ゲノム編集実験スタンダード; ヒトiPS細胞のAAVS1遺伝子座への遺伝子組込み	

〔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