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研究課題名（和文）Assessment of a potential application of endogenous stem cells to treat congenital disorders

研究課題名（英文）Assessment of a potential application of endogenous stem cells to treat congenital disorders

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交付決定額（研究期間全体）：（直接経費）　　2,200,000円

研究成果の概要（和文）：先天性疾患では、毒性の遺伝子変異による幹細胞の機能障害により、器官の発達が不完全となることがあります。手術が一般的ですが、その効果は十分ではありません。ゲノム編集の進歩により、幹細胞の遺伝子変異を修正する希望が広がっています。ヒルシュスプルング病をモデルとし、腸の神経細胞が不足する状態において、内因性幹細胞が自己修復する可能性を探るために、病気を持つマウスモデルを生成しました。これにより、幹細胞の修復が病気の改善と消化管機能の向上につながることが示されました。この研究は、内因性幹細胞の他の先天性疾患への利用の可能性を示しています。

研究成果の学術的意義や社会的意義
科学的には、私たちの研究は先天性疾患に影響を受ける組織を再生するための内因性幹細胞の潜在能力を明らかにしました。私たちは、Hirschsprung病の画期的なマウスモデルを開発し、標的変異修正を実証しました。社会的には、これにより先天性疾患に対する治療成果の向上が期待され、特に乳幼児におけるアガングリオノーシスなどの状態の予防が進んだ幹細胞療法が提供されます。

研究成果の概要（英文）：Many congenital diseases display incomplete organ development due to impaired function of stem cells caused by toxic effects of gene mutations. While surgery is a common treatment, their effects remain unsatisfactory. Advances in genome editing offer hope for correcting these mutations in stem cells, potentially aiding organogenesis. We used Hirschsprung disease as a model for congenital diseases, a condition where intestinal nerve cells are absent, often requiring surgery. To investigate if endogenous stem cells that reside within the affected body can restore themselves, we generated a mouse model with the disease in which the causative mutation can be conditionally removed or corrected. Correcting these stem cells resulted in increased cell proliferation and ameliorate the disease severity and also enhancing the gastrointestinal motility function. This research sheds light on potential utilization of endogenous stem cells for other congenital disorders.

研究分野：developmental biology and regenerative medicine

キーワード：tissue regeneration　enteric nervous system　endogenous stem cell　Hirschsprung disease

１．研究開始当初の背景/ Background at the beginning of research

Many congenital diseases display incomplete organogenesis due to impaired function of stem cells caused by toxic effects of gene mutations. Current treatment of such organ defects relies mostly on surgical interventions, but their effects remain unsatisfactory. With the advancement of genome editing technology, it will become possible to correct gene mutations in stem cells in vivo. However, **it is currently unknown whether such gene correction can make those intrinsic stem cells regain their function and contribute to organogenesis.** To address this issue, I will use Hirschsprung disease (HSCR) as a model for congenital stem cell disease. HSCR is a congenital disorder characterized by the absence of the enteric ganglia (intestinal aganglionosis), which is caused by incomplete colonization of enteric nervous system (ENS) stem cells in the developing gut 1. Current treatment for HSCR involves surgical removal of the aganglionic colon segment. However, HSCR patients with aganglionic segments extending to small bowels are difficult to treat with current surgical interventions (intractable HSCR, hereafter referred to iHSCR). To unravel the capacity of ENS stem cells to regenerate the ENS, I will generate a new mouse model of iHSCR in which pathogenic mutations can be removed by genetic recombination

２．研究の目的/ Purpose of research

The purpose of this proposed study is **to assess the potential use of intrinsic ENS stem cells for regenerating the ENS in a mouse model of HSCR**. This proposed study is the first attempt to assess the potential use of intrinsic stem cells for treating congenital tissue defects. Until now, regenerative medicine has been performed on cells in which defective or damaged tissues are induced to differentiate from embryonic stem (ES) cells or iPS cells. It has evolved with the concept of supplementing. In this study, tissue regeneration is performed by deploying stem cells that already exist in the living body. The content is highly novel and original in terms of life. Endogenous stem cells exist in a physiological environment. Therefore, it is possible to avoid the problems of cell transplantation and engraftment, and there is also concern about tumorigenesis, which is a concern for ES cells and iPS cells. This concept also has the advantage of being less.

３．研究の方法/ Research method

Based on my original discovery that heterozygous Ret(S812F) mutation can cause HSCR-like intestinal aganglionosis in mice via its dominant-negative action, I will generate a new mouse model of HSCR. I will modify the Ret locus such that the pathogenetic mutation can be removed by the Cre-loxP-mediated gene recombination. The ENS stem cells in this HSCR model will be subjected to the removal of the pathogenetic mutation at different developmental time periods and with different degrees. The ability of those ‘fixed’ ENS stem cells will be assessed by histological examination of the ENS. Finally, if significant regeneration is observed, I will perform virus-mediated removal of the pathogenetic mutation in ENS stem cells in utero. More detailed experimental plans follow.

1. **Generation of a mouse model of HSCR**: I will introduce the pathogenetic mutation into the mouse Ret gene to induce HSCR. The Ret locus will be engineered such that the causative

mutation can be corrected by Cre-loxP-mediated recombination. To this end, a gene cassette composed of floxed RET cDNA harboring point mutation RET S811F (human counterpart of mouse Ret S812F) followed by mCherry cDNA will be inserted under the Ret promoter (Figure 2).3 RET(S811F) is a dominant-negative (dn) mutant, and expression of this single dnRET allele is known to induce HSCR in mice (the manuscript is under revision). By utilizing Cre-mediated recombination, dnRET cDNA can be removed, and the recombined cells will be detected by mCherry expression.

2. **Removal of the dnRET allele and examination of the ENS:** To investigate the role of ENS stem cells in HSCR mice, two different Cre mouse lines will be utilized. Firstly, utilizing Phox2B Cre mouse line (Phox2BCreERT2/+) that is useful for labelling vagal neural crest-derived ENS stem cells. Secondly, utilizing DhhCre mouse line (DhhCre/+) that is useful for labelling Schwann cell-derived ENS stem cells. Each of those Cre mouse line harbors either CreERT2 or Cre transgene, respectively. In CreERT2 driver mouse line, Cre recombination will be activated after administering Hydroxytamoxifen (4-OHT). By administering 4-OHT into iHSCR mice, the causative mutations (dnRET) located between loxP sites will be removed. Removal of dnRET in iHSCR results in specific labelling of the ENS stem cells in vagal neural crest or Schwann cell progenitors. In this condition, recombination can be detected by mCherry expression.

To examine the ENS regeneration, the dnRET in iHSCR will be removed at various developmental time periods. The labelled-fixed ENS stem cells in vagal neural crest will be analyzed by immunohistochemical detection of mCherry expression. The proliferation, migration, and differentiation assay will be examined by immunostaining of 5-Ethynyl-2′-deoxyuridine (EdU) and immunostaining of several markers (Protein gene product 9.5 (PGP9.5), pan neuronal; nitric oxidase synthase (nNOS), inhibitory motor neuron; Calretinin, excitatory motor neuron), respectively. The information obtained by this experiment will provide crucial information for the optimal timing and amount of the ENS stem cells to sufficiently restore their function. To assess their functional regeneration, the gut motility and postnatal survival will be assessed.

3. **Virus-mediated removal of the dnRET allele in utero:** CRISPR/Cas9 delivered by Adeno-associated virus (AAV) vectors has shown promise in treating various diseases in preclinical models. AAV has a good safety profile in animal models and human trials, making it suitable for gene therapy. If significant regeneration is seen in iHSCR mice, we will use an AAV-Cre system to correct the dnRET mutation in utero. This involves injecting AAV with Cre recombinase to trigger recombination in ENS stem cells, mimicking *in utero* gene correction in a clinical setting. Corrected ENS stem cells should restore their function and generate a healthy ENS, potentially rescuing intestinal aganglionosis. These experiments aim to validate this approach.

4. **Gene expression analysis of 'fixed' ENS stem cells**: In this experiment, I plan to exploit RNAsequencing (RNA seq) analysis to examine differential expression genes (DEGs) of 'fixed' ENS stem cells. Isolated ENS stem cells obtained from the gut tissues of mutant (iHSCR) are subjected for cell sorting based on mCherry expression using Fluorescence-activated cell sorting (FACS) analysis. Total RNA samples will be prepared for RNA-seq analysis and quantitative real-time polymerase chain reaction (qRT-PCR). qRT-PCR will be used to confirm those identified genes in the RNA-seq analysis. The information obtained from these experiments will be important to identify genes involved in ENS stem cells regeneration.

4．研究成果/ Research results

Conditional RET(S811F) mutant mice serve as a novel model of HSCR

Based on my initial discovery, I have demonstrated that a natural mutation of RET S811F that occurred in HSCR patient is a dominant-negative (dn) mutation, and the expression of this single dnRET allele is sufficient to induce HSCR in mice.[3] I have successfully engineered the conditional RET(S811F) allele, a gene cassette composed of floxed RET cDNA[4] harboring point mutation RET S811F (human counterpart of mouse Ret S812F), followed by mCherry cDNA that was inserted under the *Ret* promoter **(Figure 1A).** Mice carrying this allele, termed *Ret*$^{RET51(S811F)\text{-}flox(mCherry)/+}$, exhibited ENS deficits, like what was observed in *Ret*$^{S812F/+}$ mice **(Figure 1B).**

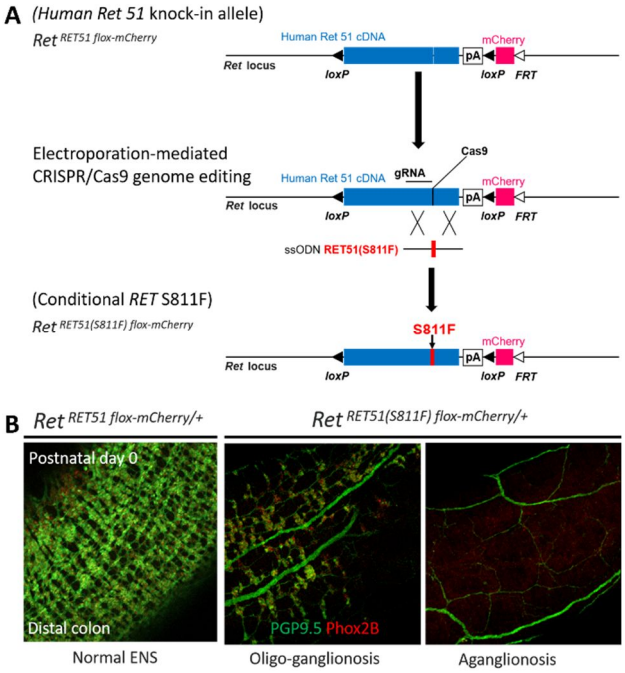

Figure 1. A Novel HSCR mouse model carrying a conditional dominant-negative mutant allele. A) Generation of conditional allele inducing HSCR by inserting the dominant-negative mutation using CRISPR-Cas9 genome editing. B) Heterozygous mice carrying the conditional allele exhibit ENS deficits, either oligo-ganglionosis or aganglionosis.

Rescued endogenous ENS stem cells significantly contributes to ENS regeneration to prevent the aganglionosis

To investigate the role of endogenous ENS stem cells in ENS regeneration in HSCR mice, I have utilized two different Cre mouse lines that useful for labelling Vagal neural crest-derived and Schwann cell precursor (SCP)-derived ENS stem cells. By employing Phox2B-CreERT2 and Dhh-Cre mouse lines as reporters, the removal of the RET(S811F) pathogenic mutation in Vagal neural crest-derived and Schwann cell precursor (SCP)-derived ENS stem cells, respectively, resulted in significant amelioration of the severity of the aganglionosis phenotype **(Figure 2A and B)**. Moreover, ratio of newly formed enteric neurons (mCherry) to enteric neuronal marker (Phox2b) was significantly increase in the distal colon of animal with corrected-pathogenic mutation in both Vagal neural crest-derived and Schwann cell precursor (SCP)-derived ENS stem cells.

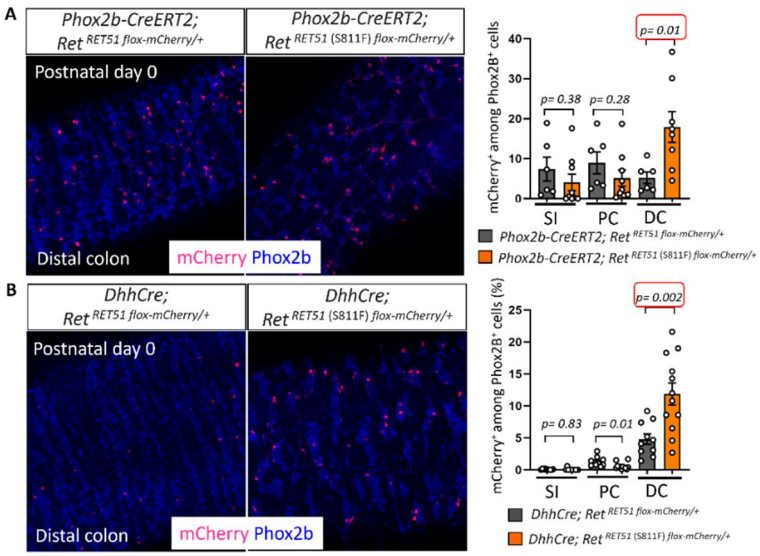

Figure 2. Removal of the pathogenic mutation in endogenous ENS stem cells significantly prevents aganglionosis. A) in Vagal neural crest-derived B) Schwann cell precursor (SCP)-derived ENS stem cells. Note that the ratio of rescued cells labeled by mCherry to total Phox2b-positive cells is significantly increased in the distal colon of mutant compared to control mice.

Role of endogenous ENS stem cells in regeneration

Rescued endogenous ENS stem cells (mCherry-positive cells) were able proliferate, migrated, and accumulated at the distal end colon, and differentiated into various enteric neuron subtypes **(Figure 3)**. These findings suggest a crucial role for endogenous ENS stem cells in the regeneration process. Further supporting this, we observed an accumulation of mCherry-positive enteric neurons in the distal colon and functional restoration of gastrointestinal motility in adult mice, as evidenced by comparable colon transit time analysis **(Figure 4)**.

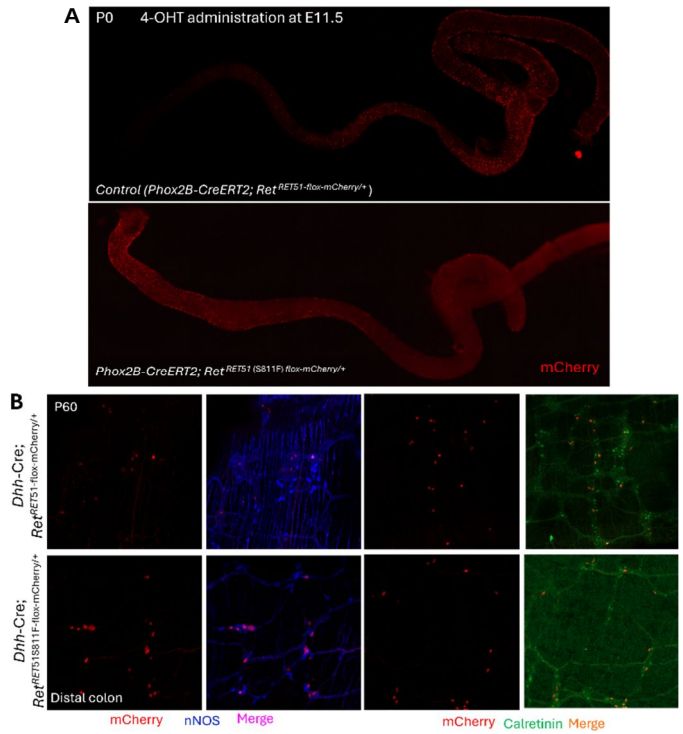

Figure 3. Rescued endogenous ENS stem cells are migrated and differentiate into various enteric neuron subtypes. A) (top) in control animal without pathogenic mutation, mCherry positive cells are evenly distributed throughout colon, while in corrected pathogenic mutant animal (bottom), mCherry positive cells are accumulated at the distal end of the colon. B) Newly formed enteric neurons are differentiated into nNOS and calretinin neuron subtypes.

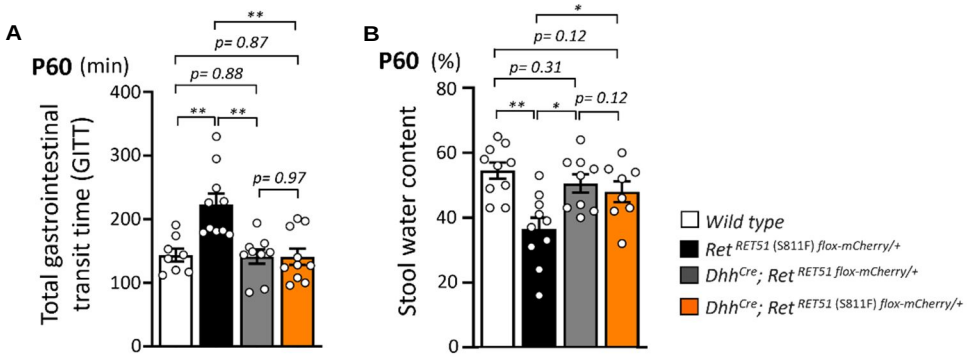

Figure 4. Gastrointestinal function was restored in the HSCR mouse model with the corrected pathogenic mutation at P60. A) Gastrointestinal transit assay. B) Stool water content assay.

Gene expression analysis of corrected ENS stem cells: We are currently preparing to isolate mCherry-positive cells from gut samples in our mouse models for RNA sequencing (RNA-seq) analysis. This will allow us to conduct a detailed gene expression analysis of the corrected ENS stem cells.

Virus-Mediated Removal of the dnRET allele in Utero: We are optimizing the appropriate AAV serotype for our study.

５．主な発表論文等

〔雑誌論文〕　計0件

〔学会発表〕　計2件（うち招待講演　2件／うち国際学会　1件）

１．発表者名 Mukhamad Sunardi
２．発表標題 Potential application of endogenous stem cells for the treatment of congenital diseases
３．学会等名 Kobe University-University of Washington Joint Symposium（招待講演）
４．発表年 2023年

１．発表者名 Mukhamad Sunardi
２．発表標題 Assessment of the potential use of endogenous stem cells for congenital organ deficits
３．学会等名 The 7th International Enteric Nervous System (ENS) meeting 2024（招待講演）（国際学会）
４．発表年 2024年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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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

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