# Pcgf1 gene disruption reveals primary involvement of epigenetic mechanism in neuronal subtype specification in the enteric nervous system

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学 位 論 文 の 内 容 要 旨

# Pcgf1 gene disruption reveals primary involvement of epigenetic mechanism in neuronal subtype specification in the enteric nervous system

ポリコーム群遺伝子 Pcgf1 は腸管神経系のニューロンサブタイプ決定に寄与している

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## SUMMARY

### Introduction

The enteric nervous system (ENS) regulates gut functions independently from the central nervous system (CNS) by its highly autonomic neural circuit that integrates diverse neuronal subtypes. Although each neuronal subtype has its differentiation timeline, varying from as early as embryonic day (E) 8 to postnatal periods, the molecular mechanism underlying the generation of neuronal diversity in the ENS remains largely unknown. In the CNS, the fate of a given neuronal subtype is determined by actions of specific transcription factors induced by the concentration gradient of BMPs (bone morphogenetic proteins) and Shh (sonic hedgehog) in the neural tube. Transcriptional control of neuronal subtype specification was also demonstrated in the ENS by several mouse studies in which genetic disruption of transcription factors, *Pbx3, Sox6,* and *Tbx3,* showed impaired differentiation of some enteric neuronal subtypes. However, the overall mechanisms underlying neuronal subtype specification in the ENS have yet to be elucidated.

Polycomb group proteins are the epigenetic modifiers that regulate cell differentiation via chromatin compaction and modification, which are grouped into two multiprotein complexes, Polycomb repressive complexes 1 and 2 (PRC1 and PRC2). In cell lineage determination, it is generally accepted that transcription factors activate or silence gene expression, thereby determining the cell fate, and epigenetic mechanisms successively stabilize the gene expression status. A recent study demonstrated that the epigenetic mechanism could primarily regulate cell fate determination as shown by

disruption of the components of PRC1 which led to cell fate conversion in the hematopoietic lineage.

PCGF1 (Polycomb group RING finger protein 1), a member of the PRC1, is involved in the neural induction stage, stem cell renewal, and differentiation. During mid-gestation, *Pcgf1* is highly expressed in the peripheral nervous system, including the dorsal root ganglion, sympathetic and parasympathetic ganglia, and ENS. PCGF1-PRC1 complex is essential to shape Polycomb chromatin domains and initiate gene repression during differentiation along with PRC2 recruitment.

In this study, we examined the biological function of PCGF1 in the development and differentiation of the ENS by using a $Pcgf1^{fl/fl}$; $Phox2b^{Cre/+}$ (*Pcgf1* cKO), which is *Pcgf1* gene was selectively deleted in the autonomic-lineage of $Phox2b^{+}$ cells. *Phox2b* is expressed immediately after the specification of the autonomic lineage, including the enteric, sympathetic, and parasympathetic ganglia in the developing peripheral nervous system. We provide evidence on the possible epigenetic regulation of neuronal subtype specification in the ENS.

**Methods**

*Pcgf1* cKO was obtained by first generating *Phox2b*-Cre knockin mice ($Phox2b^{Cre/+}$) using gene targeting followed by CRISPR/Cas9 genome editing. We crossed *Pcgf1*-floxed mice with $Phox2b^{Cre/+}$ mice to obtain *Pcgf1* cKO mice. To confirm *Pcgf1* ablation, we performed *in-situ* hybridization (ISH) in the embryonic mice (E13.5) and BaseScope analysis in the myenteric plexus from the proximal colon of three weeks old wild-type (WT) and *Pcgf1* cKO mice.

We analyzed the mice phenotype by survival rate and body weight measurement analysis of WT, *Phox2b*$^{Cre/+}$, and *Pcgf1* cKO mice. In addition, we performed gastrointestinal transit time and fecal water content analyses to examine gut motility between *Phox2b*$^{Cre/+}$ and *Pcgf1* cKO mice. We then performed immunohistochemistry analysis on embryonic (E14.5), newborn (P0), and postnatal mice (three weeks old) to investigate the basic formation of ENS, such as the gut colonization by ENS precursors and total neuron numbers, along with enteric neuronal subtype differentiation by using several subtype-specific antibodies (calretinin, NOS, calbindin, and somatostatin).

**Results**

To confirm whether the *Pcgf1* gene was successfully disrupted in the autonomic lineage of the *Pcgf1* cKO mice, we conducted ISH analysis on E13.5 embryos using riboprobes that hybridize the floxed genomic region of the *Pcgf1* gene. This analysis revealed the absence of *Pcgf1* expression in the ENS and the sympathetic chain but not in the dorsal root ganglion (DRG) in *Pcgf1*$^{fl/fl}$*; Phox2b*$^{Cre/+}$ embryos, confirming the disruption of the *Pcgf1* gene in the autonomic nervous system. In the postnatal ENS, *Pcgf1* is almost ubiquitously expressed in enteric neurons. We also validated the absence of *Pcgf1* transcripts in the myenteric plexus of *Pcgf1*$^{fl/fl}$*; Phox2b*$^{Cre/+}$ mice (3-week-old), as shown by the BaseScope *in-situ* assay.

*Pcgf1*$^{fl/fl}$*; Phox2b*$^{Cre/+}$ mice displayed small body size at the postnatal stage 3-week-old. Therefore, we compared growth and mortality rates between *Pcgf1*$^{fl/fl}$*; Phox2b*$^{Cre/+}$, WT, and Phox2b$^{Cre/+}$ mice. Although both WT and *Phox2b*$^{Cre/+}$ had similar survival rates, we observed slight growth retardation in Phox2b$^{Cre/+}$ mice compared to WT at 3 ($p = 0.035$) and 8 weeks ($p = < 0.0001$). This observation led us to use *Phox2b*$^{Cre/+}$

as the control for the following experiments. *Pcgf1*$^{fl/fl}$*; Phox2b*$^{Cre/+}$ mice displayed significantly lower body weight than *Phox2b*$^{Cre/+}$ mice at 3 weeks ($p$ = 0.002), although they could finally catch up with *Phox2b*$^{Cre/+}$ mice's growth at eight weeks. Only half of *Pcgf1*$^{fl/fl}$*; Phox2b*$^{Cre/+}$ mice ($p$ = 0.0002) survived to adulthood. These data suggest that the absence of PCGF1 impacts growth and survival rate.

Macroscopic examination of the gut showed no difference between 3-week-old *Phox2b*$^{Cre/+}$ and *Pcgf1*$^{fl/fl}$*; Phox2b*$^{Cre/+}$ mice, including gut length. Furthermore, we evaluated the effect of *Pcgf1* deletion on gut functions, such as motility and water secretion. GITT was significantly longer in the *Pcgf1*$^{fl/fl}$*; Phox2b*$^{Cre/+}$ than *Phox2b*$^{Cre/+}$ mice ($p$ = 0.005). Slower transit was associated with lighter fecal weights ($p$ = 0.0395) and drier feces in *Pcgf1*$^{fl/fl}$*; Phox2b*$^{Cre/+}$ mice ($p$ = 0.0271). These observations indicate that the absence of PCGF1 impairs gut motility at the early postnatal stage.

Impaired gut motility in *Pcgf1*$^{fl/fl}$*; Phox2b*$^{Cre/+}$ mice suggested the presence of anatomical deficits of the ENS. Therefore, we analyzed ENS development and differentiation from embryonic to postnatal stages. The basic structure of the ENS is formed by colonization of the entire gut by migrating ENS precursors, a process known to occur from E9 to E14. Examination of the embryonic gut at E14.5 revealed that ENS precursors fully colonized the colon in *Phox2b*$^{Cre/+}$ and *Pcgf1*$^{fl/fl}$*; Phox2b*$^{Cre/+}$ mice. Moreover, the numbers of ENS precursors, revealed by Phox2B staining, were comparable in these mice. These data suggest that *Pcgf1* deficiency does not influence migration and proliferation of ENS precursors. In newborn mice (P0), the numbers of myenteric neurons in the lower small intestine and colon were comparable between *Phox2b*$^{Cre/+}$ and *Pcgf1*$^{fl/fl}$*; Phox2b*$^{Cre/+}$ mice. These results suggest that PCGF1 deficiency does not impact ENS development before birth.

At a postnatal stage (3-week-old), we examined the total number of neurons and several neuronal subtypes in the myenteric and submucosal plexus of the *Phox2b*$^{Cre/+}$ and *Pcgf1*$^{fl/fl}$*; Phox2b*$^{Cre/+}$ mice. The staining of myenteric neurons with HuC/D, a pan-neuronal marker, showed no difference in all gut regions. Examination of calretinin- and NOS1-expressing neurons, two major neuronal subtypes, also revealed no significant difference between *Pcgf1*$^{fl/fl}$*; Phox2b*$^{Cre/+}$and *Phox2b*$^{Cre/+}$ mice. However, we found that the numbers of somatostatin-expressing (Sst$^{+}$) neurons (descending/inhibitory neurons) were significantly decreased in the ileum ($p$ = 0.016) and proximal colon ($p$ = 0.032*)* of *Pcgf1*$^{fl/fl}$*; Phox2b*$^{Cre/+}$ mice as compared to *Phox2b*$^{Cre/+}$ mice. In contrast, the numbers of calbindin$^{+}$ neurons (ascending/excitatory neurons) were increased ($p$ = 0.008) in the proximal colon of *Pcgf1*$^{fl/fl}$*; Phox2b*$^{Cre/+}$ mice. These findings reveal the opposing effects of PCGF1 in determining the numbers of Sst$^{+}$ and calbindin$^{+}$ neurons.

Examination of the total numbers of neurons in the submucosal plexus with pan-neuronal marker PGP9.5$^{+}$ revealed no significant difference in all gut regions between *Pcgf1*$^{fl/fl}$*; Phox2b*$^{Cre/+}$ and *Phox2b*$^{Cre/+}$ mice. Although we did not observe the difference in the calretinin$^{+}$ neurons, we detected a significant decrease in the numbers of Sst$^{+}$ neurons in the jejunum ($p$ = 0.032), ileum ($p$ = 0.032) and proximal colon ($p$ = 0.04) in *Pcgf1*$^{fl/fl}$*; Phox2b*$^{Cre/+}$ mice. These analyses reveal that the Sst$^{+}$ neurons are commonly affected by *Pcgf1* ablation in both the myenteric and submucosal plexus. Taken together, the data suggest that PCGF1 plays a crucial role in neuronal subtype specification in the ENS, especially Sst$^{+}$ and calbindin$^{+}$ neuron determinations, without affecting the total neuronal numbers.

## Conclusion

Although ENS precursor migration and enteric neurogenesis were largely unaffected, neuronal differentiation was impaired in the *Pcgf1*-deficient mice, with the number of $Sst^+$ neurons were decreased in multiple gut regions. Notably, the decrease in $Sst^+$ neurons was associated with the corresponding increase in $calbindin^+$ neurons in the proximal colon. These findings suggest that neuronal subtype conversion may occur in the absence of PCGF1 and that epigenetic mechanism is primarily involved in specification of some enteric neuron subtypes.

神戸大学大学院医学(系)研究科（博士課程）

# 論文審査の結果の要旨

| 受付番号 | 甲 第3320号 | 氏名 | BAYU PRATAMA PUTRA |
|---|---|---|---|
| 論文題目 Title of Dissertation | Pcgf1 gene disruption reveals primary involvement of epigenetic mechanism in neuronal subtype specification in the enteric nervous system<br><br>ポリコーム群遺伝子 Pcgf1 は腸管神経系のニューロンサブタイプ決定に寄与している | | |
| 審査委員 Examiner | 主査 Chief Examiner 古屋敷智之<br>副査 Vice-examiner 児玉裕三<br>副査 Vice-examiner 森 隆史 | | |

（要旨は１，０００字～２，０００字程度）

**Introduction**

The enteric nervous system (ENS) regulates gut functions independently from the central nervous system (CNS) by its highly autonomic neural circuit that integrates diverse neuronal subtypes. Although several transcription factors are shown to be necessary for the generation of some enteric neuron subtypes, the mechanisms underlying neuronal subtype specification in the ENS remain elusive. A recent study demonstrated that the epigenetic mechanism could primarily regulate cell fate determination. The disruption of the components of Polycomb repressive complex 1 (PRC1) led to cell fate conversion in the hematopoietic lineage. PCGF1 (Polycomb group RING finger protein 1), a member of PRC1, is involved in neural differentiation and highly expressed in the peripheral nervous system, including the dorsal root ganglion, sympathetic and parasympathetic ganglia, and ENS. In this study, we examined the biological function of PCGF1 in the development and differentiation of the ENS by disrupting the *Pcgf1* gene selectively in the autnomomic-lineage cells (*Pcgf1* cKO mice). We provide evidence on the possible epigenetic regulation of neuronal subtype specification in the ENS.

**Methods**

*Pcgf1* cKO was obtained by crossing *Pcgf1*-floxed mice with Phox2b-Cre knockin mice. To confirm *Pcgf1* ablation, we performed in-situ hybridization in the embryonic mice (E13.5) and BaseScope analysis in the myenteric plexus from the proximal colon of three weeks old wild-type (WT) and *Pcgf1* cKO mice. We analyzed the mice phenotypes, such as survival rate and body weight analysis of WT, $Phox2b^{Cre/+}$, and *Pcgf1* cKO mice. Gut functions were assessed by gastrointestinal transit time and fecal water content analyses. Immunohistochemistry analysis was performed on embryonic (E14.5), newborn (P0), and postnatal mice (3 weeks old) to investigate gut colonization by ENS precursors and total neuron numbers. Enteric neuron differentiation was assessed by immunohistochemistry using enteric neuronal subtype-specific markers including calretinin, NOS, calbindin, and somatostatin.

**Results and Discussion**

*Pcgf1* cKO mice displayed increased lethality and growth retardation during the early postnatal period (~3 weeks old). Interestingly, the mutant mice showed delayed gastrointestinal transit time, suggesting that some deficits in the ENS impaired gut motility. Despite the ubiquitous and early onset of expression of the *Pcgf1* gene during ENS development (as early as E13.5), *Pcgf1* deficiency did not affect neural precursor migration and neurogenesis, and no deficit was found in the ENS at birth. Although the total gut neuron numbers were unchanged in 3-week-old *Pcgf1* cKO mice, a detailed histochemical examination of the ENS revealed that the numbers of somatostatin-expressing ($Sst^+$) neurons were decreased in multiple gut locations of the mutant mice. Notably, in the proximal colon, we observed that the decrease in $Sst^+$ neurons was associated with increased $calbindin^+$ neurons, suggesting that neuronal subtype conversion (from $Sst^+$ to $calbindin^+$ neurons) occurs in the absence of PCGF1.

**Conclusion and Discussion**

Although *Pcgf1*-deficient ENS displayed largely normal development, it displayed impaired differentiation in restricted neuronal subtypes. In particular, in the proximal colon, neuronal subtype conversion-like phenotype was observed. Although overall mechanisms underlying this phenotype have yet to be elucidated, this study provides evidence, for the first time to our knowledge, that the epigenetic mechanism may primarily regulate specification of some enteric neuron subtypes.

The candidate, having completed studies on enteric neuron subtype specification, with a specialty in primary involvement of PCGF1 in neuronal subtype specification in the ENS, and having advanced the field of knowledge in the area of Physiology and Cell Biology, is hereby recognized as having qualified for the degree of Ph.D. (Medicine).