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東 洋 医 学 研 究 所 紀 要
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巻 頭 言

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東洋医学研究所 所長・教授
武田 卓

東洋医学研究所は、近畿大学病院の開設と同時に大学直属の漢方専門研究所として発足し、日本で最初の漢方の臨床・基礎を研究する研究所として、多くの実績をあげてまいりました。私は、平成24年4月1日より、新所長・新教授として東北大学医学部先進漢方治療医学講座より赴任いたしました。

漢方診療は、西洋医学だけでは改善しにくいような難治性疾患や西洋医学では病気として相手にされないような不定愁訴に対して有効な場合がしばしば認められます。また、最近ではがん治療に伴う様々な副作用対策にも漢方が用いられるようになってきました。このように、従来からの東洋医学だけではなく、西洋医学の確かな専門性を持ったうえでの漢方治療も必要です。そこで、私の専門性を生かした女性漢方外来、冷え症外来を開設しています。さらに、最近のフェムテックへの感心の高まりを背景に、月経前症候群・月経前不快気分障害への新規治療薬開発や、アプリを用いた認知行動療法開発、生薬成分によるサプリメント開発といった、東洋医学の枠組みを超えた女性ヘルスケア領域全般を対象とした臨床研究も実施中です。これらにも関連して、最近ではAMED研究や厚生労働省研究斑において女性特有疾患に関する指針策定にも関与しております。

また、大阪大学医学部より、脳神経科学研究をリードする遠山正彌名誉教授を客員教授にお迎えし、宮田信吾教授を中心とした基礎研究部門も同時にスタートしました。漢方薬の作用メカニズムを最先端の分子生物学的手法によって科学的に解明します。現在、「抑肝散」という漢方薬を中心に、漢方薬の神経機能に対する効果についての科学的解析を行っており、有効成分の同定、作用機序の解明から新規創薬への展開を目指しています。

古代中国からの長い歴史をもつ漢方治療ですが、これからは21世紀の現代医療にマッチした診療・研究、さらにはトランスレーショナルリサーチを展開していきたいと考えております。

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東洋医学研究所紀要
(2025年度)

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ORIGINAL ARTICLE

Awareness of premenstrual dysphoric disorder, attitudes toward premenstrual symptoms, and probability of suspected premenstrual dysphoric disorder among Japanese university students

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Abstract

Aim: Premenstrual dysphoric disorder (PMDD) interferes with patients' work, school, social activities, or relationships with others. For female students with PMDD to be active in society, both the patients and the surrounding population should be familiar with PMDD, be positive for treatment, and be aware that its disruptions can negatively impact society. Hence, this study aimed to determine PMDD awareness and attitudes toward premenstrual symptoms among male and female university students in Japan and to investigate the probability of suspected PMDD.

Methods: Through a survey, we asked all participants about their awareness of PMDD and their attitudes toward premenstrual symptoms. Female participants were also screened for premenstrual symptoms using the Premenstrual Symptoms Questionnaire (PSQ). Those who met certain criteria were classified into the "PMDD" group.

Results: A total of 1846 students (949 females, 892 males, and 5 unspecified) completed the survey on awareness and attitude. Only 20.6% of them were familiar with PMDD. Some believed that premenstrual symptoms should be endured. Only 36.2% had the idea that the disruptions caused by these symptoms were a loss to society. The PSQ was completed by 920 females. We found that 3.7% were classified into the "PMDD" group. Some participants in the "PMDD" group were not familiar with PMDD, while some perceived that premenstrual symptoms should be endured.

Conclusion: Despite having suspected PMDD, a certain percentage of women remain unaware of their condition and endure their symptoms. PMDD awareness through education is crucial to achieving an active life for females of menstruating age.

KEYWORDS

awareness survey, premenstrual dysphoric disorder, premenstrual symptoms, Premenstrual Symptoms Questionnaire

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INTRODUCTION

Premenstrual dysphoric disorder (PMDD) is a mood disorder characterized by severe psychiatric symptoms, such as depressive mood, anxiety, instability, and irritability, and physical symptoms, such as breast tenderness and feeling of bloating, before menstruation. It interferes with patients' work, school, social activities, or relationships with others.¹ Generally, its symptoms emerge during the luteal phase or before menstruation and then improve or disappear once menstruation starts. According to the *Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition, Text Revision*,¹ the 12-month prevalence of PMDD is 5.8%. However, its prevalence in previous reports varies widely.^{2,3}

The loss of disability-adjusted life years due to PMDD is estimated at 1400 days,² while the reduction in quality-adjusted life years is approximately 0.14 years or longer,⁴ that cannot be underestimated. According to previous studies, a certain percentage of university and college students suffer from PMDD.^{5–9} For female students with PMDD to be active in their home, school, and social lives, at least the following conditions should be met. First, female students and the population surrounding them at home, school, and in the workplace should be familiar with PMDD regardless of sex because awareness is the first step in managing and caring of PMDD. However, according to previous reports targeting university students in Pakistan¹⁰ and Japan,¹¹ PMDD awareness was very low. Second, they should be positive for treating this disorder. Selective serotonin reuptake inhibitors^{12–16} and combined oral contraceptives^{17,18} effectively improve PMDD symptoms to some extent. However, university and college students are reportedly reluctant to seek treatment for it.¹⁰ Third, they should understand the home, social, and economic loss caused by PMDD.^{2,4}

While meeting these conditions is beneficial for all women of reproductive age, it is particularly advantageous for younger individuals as it can significantly reduce the long-term burden of disease.

In this study, we aimed to determine PMDD awareness and attitudes toward premenstrual symptoms among university students through a survey. One of the characteristics and strengths of this study is that we included not only females but also males. Unless both women and men understand premenstrual symptoms, PMDD, and the accompanying disturbances, women with PMDD will not be able to be fully active. We also investigated the probability of suspected PMDD using a self-administered questionnaire.

We recognize that premenstrual syndrome (PMS) also causes physical and mental discomfort before menstruation, but this study focused on PMDD because PMS is more well-known, and PMDD causes more severe psychiatric symptoms.

SUBJECTS AND METHODS

This study was approved by the Ethics Committee of Ibaraki University (approval no.: 230600). We conducted an online survey targeting the students at four campuses of two national universities in

Japan (three campuses of Ibaraki University and Shimane University Matsue Campus) in April 2024. Ibaraki University is located in eastern Japan, whereas Shimane University is located in western Japan. Both universities have faculties in the sciences and humanities. Approximately 3% of the students in these campuses belonged to psychology departments, but none were medical students.

The questionnaire was created using Microsoft Forms. Written information about the study was provided on the first page of the form to inform all potential respondents that their participation was voluntary and that their responses would remain anonymous. Potential respondents who read and agreed to participate in the survey proceeded to the questionnaire. All the participants were asked about their awareness of PMDD and their attitudes toward premenstrual symptoms. We also asked the students about their awareness of PMS to help them distinguish between PMS and PMDD.

Furthermore, female participants were screened for premenstrual symptoms by using the Premenstrual Symptoms Questionnaire (PSQ): a screening tool for both PMS and PMDD.¹⁹ PSQ translates the DSM-IV criteria into a rating scale with the following degrees of severity: "not at all," "mild," "moderate," and "severe." These degrees have been described in Japanese. One question was "Within the last three months, have you experienced the following premenstrual symptoms starting during the week before menstruation and remitting or disappearing a few days after the onset of menstruation?" The premenstrual symptoms listed on the PSQ include 11 symptoms, 4 core symptoms, namely, "Depressed mood," "Anxiety or tension," "Tearfulness," and "Anger or irritability," and seven other symptoms such as "Decreased interest in work, home, or social activities," "Difficulty in concentrating," "Fatigue or lack of energy," "Overeating or food cravings," "Insomnia or hypersomnia," "Feeling of being overwhelmed," and "Physical symptoms such as breast tenderness, feeling of bloating, headache, joint or muscle pain, and weight gain." The PSQ also asked whether such premenstrual symptoms interfered with the following three domains: "Work efficiency or productivity and home responsibility," "Social life activities," and "Relationships with coworkers or family." In this study, females were classified into the "PMDD" group if they met all of the following conditions: their symptoms remitted or disappeared within a few days after the onset of menses; they reported at least one of the four core symptoms as severe and ≥ 5 out of the 11 symptoms as moderate to severe; and they reported that their symptoms severely interfered with their function in at least one of the three domains. The group of females not classified as the "PMDD" group was defined as the "Other" group. The PSQ has strong evidence of reliability and validity.²⁰ Female participants were also asked whether their menstruation was regular or irregular.

Groups were compared using the chi-square test. A p-value less than 0.05 was considered to be statistically significant, and effect sizes (ϕ and Cramer's V) of 0.1 and 0.3 were considered to be small and medium, respectively. The Statistical Package for the Social Sciences software version 28 (IBM) was employed for the data analysis.

RESULTS

Participants

Out of 1868 participants (952 females, 911 males, and 5 unspecified), 1846 (949 females, 892 males, and 5 unspecified) completed the survey on PMDD awareness and attitude toward menstrual symptoms. The response rate was 19.9% (1868/9375), and the completion rate was 98.8% (1846/1868). The age range was 18–47 years. The mode, median, first quartile (Q1), and third quartile (Q3) values for age were 18, 19, 18, and 21 years, respectively. Furthermore, 920 (96.6%) female participants completed the PSQ, with 18, 19, 18, and 21 years as the mode, median, Q1, and Q3 age values, respectively. The distribution of the participants' ages and grades is summarized in Table 1.

Awareness of PMDD

Table 2 shows participants' awareness of PMDD and their sex differences. Sex differences were observed in the awareness of PMDD. Moreover, a higher proportion of female students was familiar with PMDD and had seen or heard about it but was not familiar with it; meanwhile, a higher proportion of male students did not know about it.

Attitudes toward premenstrual symptoms

Table 3 lists the data on the participants' attitudes toward premenstrual symptoms and their sex differences. We found that some

participants thought that "premenstrual symptoms should be endured even if they are severe." Additionally, more than half of the participants agreed with the idea that "the disruptions to daily life and studies caused by symptoms are a loss for the person." Moreover, approximately one-third of the participants agreed with the idea that "the disruptions to daily life and studies caused by symptoms are a loss not only for the individual but also for society."

More females than males thought that "premenstrual symptoms should be endured even if they are severe." More males than females agreed with the idea that "the disruptions to daily life and studies caused by symptoms are a loss not only for the individual but also for society."

Prevalence of PMDD by screening

Table 4 presents the prevalence of the premenstrual symptoms and interference caused by them according to severity. Out of 920 participants who completed the PSQ, 34 (3.7%) were classified into the "PMDD" group according to the same criteria used in a previous study.¹⁹

Awareness and attitude of participants in the "PMDD" group

Among the 34 participants in the "PMDD" group, 17 (50.0%) of them were familiar with PMDD, 8 (23.5%) had seen or heard of it but were not familiar with it, and 9 (26.5%) did not know about it. Table 5 shows the awareness of PMDD among the students in the "PMDD" group and the

TABLE 1 Distribution of age and grade of the participants.

		Sex			Total
		Male	Female	Unspecified	
Age (years)	18	256	303		559 (30.3%)
	19	153	212	2	367 (19.9%)
	20	119	147		266 (14.4%)
	21	151	163	1	315 (17.1%)
	22	82	57		139 (7.5%)
	23	86	40	2	128 (6.9%)
	24–29	41	20		61 (3.3%)
	30–29	2	4		6 (0.3%)
	40–47	2	3		5 (0.3%)
Grade	First-year	294	348		642 (34.8%)
	Second-year	171	209	2	382 (20.7%)
	Third-year	117	159		276 (15.0%)
	Fourth-year	171	156	1	328 (17.8%)
	Graduate students	136	77	2	215 (11.6%)
	Unspecified	3			3 (0.2%)

TABLE 2 Awareness of premenstrual dysphoric disorder (PMDD) and its sex difference.

Sex	Familiar with it	Have seen or heard about it	Do not know about it	Chi-square	Freedom	p-Value	Cramer's V
Male	168 (18.8%)	208 (23.3%)	516 (57.8%)	30.6	2	<0.001	0.13
Female	213 (22.4%)	307 (32.3%)	429 (45.2%)				

TABLE 3 Attitudes toward premenstrual symptoms and its sex difference.

	Sex	Agree	Disagree	Chi-square	Freedom	p-Value	ϕ
Symptoms should be endured even if they are severe.	Female	67 (7.1%)	882 (92.9%)	46.9	1	<0.001	0.16
	Male	7 (0.8%)	885 (99.2%)				
	Unspecified	0	5				
The disruptions to daily life and studies caused by symptoms are loss for the person.	Female	539 (56.8%)	410 (43.2%)	1.41	1	0.235	
	Male	531 (59.5%)	361 (40.5%)				
	Unspecified	3	2				
The disruptions to daily life and studies caused by symptoms are a loss not only for the individual but also for society.	Female	297 (31.3%)	652 (68.7%)	20.6	1	<0.001	0.11
	Male	370 (41.5%)	522 (58.5%)				
	Unspecified	3	2				

TABLE 4 Prevalence rates of premenstrual symptoms and interference induced by them.

	Not at all	Mild	Moderate	Severe
<i>Symptoms</i>				
1. Depressed mood	346 (37.6%)	294 (32.0%)	206 (22.4%)	74 (8.0%)
2. Anxiety or tension	205 (22.3%)	349 (37.9%)	283 (30.8%)	83 (9.0%)
3. Tearfulness	370 (40.2%)	239 (26.0%)	212 (23.0%)	99 (10.8%)
4. Anger or irritability	313 (34.0%)	294 (32.0%)	241 (26.2%)	72 (7.8%)
5. Decreased interest in work, home, or social activities	521 (56.6%)	249 (27.1%)	121 (13.2%)	29 (3.2%)
6. Difficulty in concentrating	401 (43.6%)	314 (34.1%)	156 (17.0%)	49 (5.3%)
7. Fatigue or lack of energy	319 (34.7%)	320 (34.8%)	206 (22.4%)	75 (8.2%)
8. Overeating or food cravings	305 (33.2%)	286 (31.1%)	235 (25.5%)	94 (10.2%)
9. Insomnia or hypersomnia	343 (37.3%)	241 (26.2%)	214 (23.3%)	122 (13.3%)
10. Feeling of being overwhelmed	586 (63.7%)	193 (21.0%)	98 (10.7%)	43 (4.7%)
11. Physical symptoms	346 (37.6%)	296 (32.2%)	212 (23.0%)	66 (7.2%)
<i>Interference</i>				
1. Work efficiency or productivity and home responsibility	364 (39.6%)	360 (39.1%)	154 (16.7%)	42 (4.6%)
2. Social life activities	634 (68.9%)	204 (22.2%)	58 (6.3%)	24 (2.6%)
3. Relationships with coworkers or family	701 (76.2%)	157 (17.1%)	47 (5.1%)	15 (1.6%)

TABLE 5 Awareness of premenstrual dysphoric disorder (PMDD) and attitude toward the idea that “premenstrual symptoms should be endured even if they are severe” among female students in the “PMDD” group and the “Other” group.

	The “PMDD” group	The “Other” group	Chi-square	Freedom	p-Value	Cramer's V
Awareness of PMDD						
Familiar with it	17 (50.0%)	188 (21.2%)	15.8		<0.001	0.13
Have seen or heard about it	8 (23.5%)	293 (33.1%)		2		
Do not know about it	9 (26.5%)	405 (45.7%)				
Premenstrual symptoms should be endured even if they are severe						
Agree	4 (6.1%)	62 (93.9%)	1.1	1	0.47	
Disagree	30 (3.5%)	824 (96.5%)				

“Other” group. A higher proportion of students in the “PMDD” group was familiar with PMDD. Four (11.8%) of those in the “PMDD” group agreed that “premenstrual symptoms should be endured even if they are severe.”

DISCUSSION

Awareness of PMDD

This study showed that only approximately one-fifth of the participants were familiar with PMDD. In addition, the female participants were more familiar with it than the male participants. PMDD awareness was higher in this study than in a previous study targeting female university students studying physical therapy in 2017¹¹ despite the fact that our participants included males and were non-medical students who could have been less familiar with PMDD than those students who were involved in the medical field. The 7-year difference in the survey could be one of the causes of this discrepancy. These results indicate that, although slowly, people have become increasingly aware of PMDD.

Attitudes toward premenstrual symptoms

Some participants believed that premenstrual symptoms should be endured even if they are severe. Previous studies both in Japan and overseas have pointed out that menstruation is surrounded by many stigmas including that it is normal to endure the pain and other symptoms.^{21–23} More than half of the participants believed that “the disruptions to daily life and studies caused by symptoms are a loss for the person,” but only about one-third believed that “the disruptions to daily life and studies caused by symptoms are a loss not only for the individual but also for society.” These results suggest that even if the females suffer from premenstrual symptoms, they are not in an environment where they can receive support from the surrounding population.

Fewer males perceived that “premenstrual symptoms should be endured” than females, and more males than females thought that

“the disruptions to daily life and studies caused by symptoms are a loss not only for the individual but also for society.” The possible reasons for these sex differences are that traditionally, in Japan and some other countries, girls are told at home that menstruation is unclean, that it should be endured, and that it is not something to talk about openly. Furthermore, males could be rather neutral or unbiased, which could be a positive trend. On the contrary, the results that some females had negative attitudes toward premenstrual symptoms is concerning, because such females may be harsh not only on themselves but also on other females who suffer from premenstrual symptoms.

Prevalence of PMDD

Several previous studies have reported the prevalence of PMDD among university and college students in Japan^{5,6} and other countries,^{7–9} using self-administered questionnaires. The probability of suspected PMDD obtained in our study was lower than that in some of the previous studies, but similar to or higher than that in others. The causes of the differences are not clear, but ethnicity might be a possible factor, as was reported in a previous study.²⁴ Whether the same degree of pain or discomfort is perceived as normal or abnormal may vary among different ethnic groups.

Awareness and attitudes of participants in the “PMDD” group

One distinct characteristic of this study is that we investigated the awareness and prevalence of PMDD, and attitudes toward premenstrual symptoms concurrently. Although the students in the “PMDD” group were more familiar with PMDD than other students, some participants in the “PMDD” group did not know about PMDD or just heard or seen it but were not familiar with it, and some believed that premenstrual symptoms should be endured even if they are severe. Owing to their lack of health literacy regarding

premenstrual symptoms, patients with PMDD are losing their opportunity to be treated. If they had a better understanding of PMDD and were more positive about seeking treatment, their social activities could have been better. Enlightenment in society, including higher education, is necessary for a better home, school, work, and social life among females of menstruating age.

Strength and limitations

The strength of this study is that we asked about awareness of PMDD and attitude toward premenstrual symptoms to both sexes. To the best of our knowledge, studies on male awareness and attitudes toward PMDD are scarce.²⁵ This study also has limitations. First, the participants were students from two national universities; thus, this was not a nationwide study. Second, we did not ask the participants about their department. Thus, we cannot tell if there were any differences in awareness of PMDD or attitudes toward premenstrual symptoms among students' departments. Third, we used a retrospective self-administered questionnaire for screening to investigate the prevalence of PMDD but did not use a prospective evaluation for diagnosis. Fourth, even though we asked whether their premenstrual symptoms remitted or disappeared within a few days after the onset of menses, we admit that some patients with premenstrual exaggeration of mental disorders could not be excluded. Fifth, we did not ask the participants about their history of mental disorders. Finally, we have no data on how many of the participants with PMDD were receiving treatment, including hormone treatment. It is important to ask about previous medical history when targeting clinical populations. However, since the participants of our survey were general students, it was necessary to make the questionnaire as short as possible to reduce the respondents' burden and to have a high completion rate.

CONCLUSION

Ideally, all people should be aware of PMDD regardless of sex, and appropriate treatment and accommodation should be provided so that females with PMDD can be more active at home, school, and the workplace. The disruptions to life caused by premenstrual symptoms are a loss not only for the individual but also for society. However, the current situation of Japanese university students is far from ideal. Enlightenment in society, including higher education, is therefore necessary. In future research, we will produce educational materials for university students and examine whether the learning experience will change their awareness of PMDD and attitude toward premenstrual symptoms.

AUTHOR CONTRIBUTIONS

Yasuko Fuse-Nagase: Writing—original draft; methodology; funding acquisition; conceptualization; data curation; investigation. **Yoshie Kono:** Methodology; funding acquisition; conceptualization; investigation. **Takashi Takeda:** Methodology; resources.

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CONFLICT OF INTEREST STATEMENT

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS APPROVAL STATEMENT

This study was approved by the Ethics Committee of Ibaraki University (approval no. 230600).

PATIENT CONSENT STATEMENT

N/A.

CLINICAL TRIAL REGISTRATION

N/A.

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Menopause Education Programs for Healthcare Professionals Based on the Japanese Society of Psychosomatic Obstetrics and Gynecology

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Objectives: Healthcare professionals are poorly educated about menopausal disorders. To address this issue, we launched an online Menopause Instructor Course, which is certified by the Japanese Society of Psychosomatic Obstetrics and Gynecology (JSPOG).

Methods: Courses covering general remarks, hormone replacement therapy (HRT), non-HRT treatments, mental health, decision-making, and healthcare support were held in 2023 and 2024. Cognitive behavioral therapy was included in 2024. JSPOG-member healthcare professionals who completed all on-demand lectures and passed a post-test received certification as Menopause Instructors. In 2024, a questionnaire survey assessed participant feedback and compared Obstetricians and Gynecologists (OB/GYNs) to other healthcare professionals.

Results: A total of 198 and 143 professionals were certified in 2023 and 2024, respectively, with most of them being OB/GYNs. Certified Menopause Instructors were located across Japan. A survey conducted in 2024 showed that 93.7% of respondents were satisfied with the course. OB/GYNs found general remarks, HRT, and non-HRT treatments to be significantly easier. The overall self-assessed understanding was high (8.04 ± 1.01), with OB/GYNs scoring significantly higher than other healthcare professionals (8.29 ± 0.95 vs. 7.87 ± 1.02 , $P < 0.05$).

Conclusions: The course successfully increased the number of trained professionals in menopause in Japan. Enhancing the course content to better serve OB/GYNs and other healthcare professionals is essential.

Key Words: Delivery of health care, Education, Gynecologists, Menopause, Psychosocial intervention

INTRODUCTION

Menopause is the irreversible termination of menstrual cycles due to the decline in ovarian follicular function [1]. Perimenopause, the period around the time of menopause, refers to the period from when the menstrual cycle is > 7 days longer than usual to one

year before menopause [2]. The period around menopause is also often called “konenki” in Japan [3]. Many women experience unpleasant symptoms around the time of menopause.

In Japan, where the population is aging rapidly. In 2023, approximately 91 million women were equivalent to the menopausal age (45–54 years), accounting

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for 7.3% of the total population [4]. Various symptoms of menopause can affect women's daily lives, including their careers. Indeed, Japan's Ministry of Economy, Trade and Industry estimates that the economic loss due to menopausal symptoms is 1.9 trillion yen per year [5].

Many women with menopausal disorders visit unspecialized departments, consequently delaying the necessary therapy needed to treat the symptoms [6]. A biopsychosocial approach to the care of menopausal women is also essential [7]. To provide adequate care for menopausal women, there remains an urgent need for comprehensive menopause education for healthcare professionals [8].

The Japanese Society of Psychosomatic Obstetrics and Gynecology (JSPOG) is an academic organization that aims to deepen understanding of women's specific disorders, focusing on mental health. Menopausal disorders are one of the primary conditions that members manage. In 2023, with the support of Astellas Pharma Inc., we launched an educational program called the Menopause Instructor Course. We hypothesized that hosting this course would allow JSPOG to address the lack of education on menopausal disorders among Japanese healthcare professionals and increase the number of professionals across Japan who can confidently manage these disorders, including those related to mental health. We aimed to investigate the regional distribution, specialization, understanding, and satisfaction of participants who completed the course and obtained certification as Menopause Instructors. Since Obstetricians and Gynecologists (OB/GYNs) and non-OB/GYNs have significantly different educational backgrounds regarding menopause, comparisons were made between these two groups in terms of course understanding and other outcomes.

MATERIALS AND METHODS

Education program

A working group for Menopause Instructor Course consisting of JSPOG directors and secretaries was formed. All matters relating to the program were determined at the working group meetings.

We distributed pamphlets at various academic conferences to encourage medical professionals to participate in the course. The first course was held in September 2023, and the second in October 2024. The eligibility requirement for the course was a healthcare profession with a national qualification in the medical field. Participants watched the lectures on demand for one month after applying in advance. The contents of the lectures and the time are shown in Table 1. Traditional Chinese medicine is commonly used to treat menopausal disorders in Japan. Therefore, the course devotes one lecture to treatments other than hormone replacement therapy (HRT). Additionally, to highlight the unique characteristics of JSPOG, which specializes in psychosomatic medicine and is composed of professionals from various fields, one lecture is devoted to mental health. Also, a lecture on cognitive behavioral therapy was added to the program in 2024.

The lectures were delivered by experts in their respective fields and videos were created using Microsoft PowerPoint (Microsoft Corporation). In the 2024 program, the PDF (Adobe) of the lecture materials was made available for download by participants. All participants took a pre-test before the lecture, which consisted of three questions for each lecture. In addition, a post-test was administered after all lectures were completed, which consisted of five questions for each lecture and included the same questions as the pre-test. Those who passed the post-test with a score of $\geq 80\%$ were considered to have completed the course. The

Table 1. Program and lecture times

	2023	2024	Time (min)
1	Health problems in women during and after menopause (general remarks)	Health problems in women during and after menopause (general remarks)	40
2	Basics of hormone replacement therapy	Basics of hormone replacement therapy	30
3	Treatment options for menopausal disorders other than hormone replacement therapy	Treatment options for menopausal disorders other than hormone replacement therapy	30
4	The impact of menopause on mental health and management	The impact of menopause on mental health and management	30
5	Supporting patient decision-making and healthcare	Supporting patient decision-making and healthcare	30
6		Cognitive and behavioral approach to menopausal disorders	30

post-test could be taken as many times as necessary. Those who completed all lectures, passed the post-test, and are members of JSPOG were certified as Menopause Instructors and received a certificate.

The e-learning system was constructed by KYOWA KIKAKU Ltd. In 2024, participants were asked to complete a questionnaire at the end of the course. The questionnaire asked about backgrounds, overall and individual lecture satisfaction, time, and difficulty. Participants were also asked to rate their overall understanding on a scale of 1 to 10 ([Supplementary](#), available online).

Ethics

This study was conducted in accordance with the principles outlined in the Declaration of Helsinki. The participants' backgrounds and survey results were obtained anonymously. The Ethics Committee of Fukushima Medical University determined that an ethical review was not required for this study.

Statistical analysis

Data on participant characteristics were calculated using Microsoft Excel 2025 (Microsoft Corporation) and reported as numbers and proportions. OB/GYNs are specialists in menopausal medicine in Japan, and their level of understanding and satisfaction may differ from

Table 2. Attendee specializations in 2023 and 2024 and percentage of attendees certified as Menopause Instructors

	Applicants	Post-test passers	Post-test pass rate	Certified as a Menopause Instructor	Certification rate
2023					
Total	233	214	91.8	198	85.0
Doctor	129 (55.4)	121 (56.5)	93.8	116 (58.6)	89.9
Midwife	43 (18.5)	38 (17.8)	88.4	31 (15.7)	72.1
Nurse	21 (9.0)	20 (9.3)	95.2	18 (9.1)	85.7
Pharmacist	14 (6.0)	13 (6.1)	92.9	12 (6.1)	85.7
Acupuncturist	9 (3.9)	7 (3.3)	77.8	7 (3.5)	77.8
Physiotherapist	5 (2.1)	5 (2.3)	100.0	5 (2.5)	100.0
Certified public psychologist	3 (1.3)	3 (1.4)	100.0	3 (1.5)	100.0
Registered dietitian	3 (1.3)	2 (0.9)	66.7	2 (1.0)	66.7
Public health nurse	2 (0.9)	2 (0.9)	100.0	1 (0.5)	50.0
Dentist	1 (0.4)	1 (0.5)	100.0	1 (0.5)	100.0
Others	3 (1.3)	2 (0.9)	66.7	2 (1.0)	66.7
2024					
Total	177	168	94.9	143	80.8
Doctor	91 (51.4)	87 (51.8)	95.6	79 (55.2)	86.8
Midwife	32 (18.1)	30 (17.9)	93.8	25 (17.5)	78.1
Pharmacist	14 (7.9)	13 (7.7)	92.9	8 (5.6)	57.1
Nurse	13 (7.3)	13 (7.7)	100.0	10 (7.0)	76.9
Acupuncturist	6 (3.4)	6 (3.6)	100.0	6 (4.2)	100.0
Certified public psychologist	6 (3.4)	6 (3.6)	100.0	5 (3.5)	83.3
Dentist	3 (1.7)	3 (1.8)	100.0	3 (2.1)	100.0
Physiotherapist	3 (1.7)	2 (1.2)	66.7	1 (0.7)	33.3
Public health nurse	2 (1.1)	2 (1.2)	100.0	1 (0.7)	50.0
Registered dietitian	1 (0.6)	1 (0.6)	100.0	1 (0.7)	100.0
Others	6 (3.4)	5 (3.0)	83.3	4 (2.8)	66.7

Data are presented as number only, number (%), or %.

that of other healthcare professionals. For this reason, we compared the results of the 2024 survey between OB/GYNs and other healthcare professionals. Categorical variables were analyzed using chi-squared tests or Fisher's exact tests to compare proportions. To account for multiple comparisons, Bonferroni correction was applied. Overall comprehension was compared using the independent samples *t* test as a continuous variable. *P* value of < 0.05 was considered statistical significance. All data were analyzed using SPSS Statistics 29.0 (IBM Co.).

RESULTS

Table 2 lists the number of participants, the number of individuals who passed the post-test, and the number of certified Menopause Instructors for each medical specialty qualification in 2023 and 2024. In 2023, 233 attended and 198 (85.0%) were certified as Menopause Instructors, while in 2024, 177 attended, and 143 (80.8%) were certified. In both years, more than 50% of all participants were doctors.

The regional distribution of certified Menopause Instructors each year is illustrated in Figure 1. Certified Menopause Instructors were located throughout Japan. There were many certified Menopause Instructors in the Tokyo metropolitan area, including Tokyo, Saitama, and Kanagawa. Meanwhile, there are currently no certified Menopause Instructors in Fukui, Shimane, Tokushima, or Kochi.

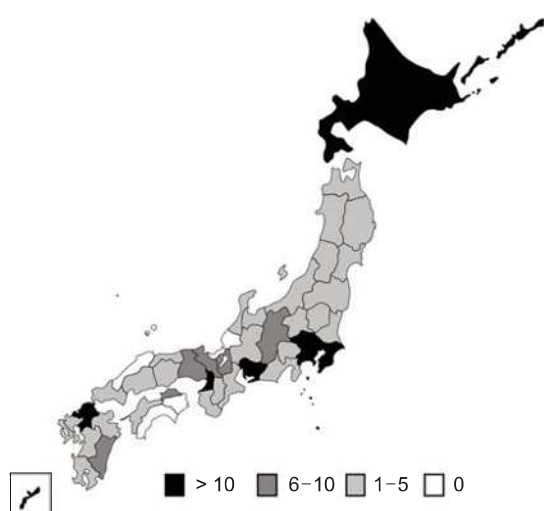


Fig. 1. Number in each prefecture of certified Menopause Instructors.

Tables 3 and 4 show the results of a questionnaire given to participants at the end of the course in 2024. The most common profession of the participants, accounting for 40.9%, was OB/GYNs. A total of 36.5% of participants joined JSPOG when they enrolled in this course. Compared with other healthcare professionals, many OB/GYNs were already JSPOG members before the course was held. Participants were mainly in their 40s and 50s, accounting for ~70%. Overall satisfaction was very high, with 93.7% of participants reporting either very satisfied or satisfied; 86.6% indicated that the course was just the right length; 98.1% indicated they could use the course in their future work.

Many participants thought the level of difficulty of each course was just right; however, more OB/GYNs felt that general remarks and HRT programs were easier than other healthcare professionals. Although the difficulty ratings for non-HRT lectures showed significant group differences in the unadjusted analysis, these differences were not significant after applying the Bonferroni correction. The overall understanding of the course was high, at 8.04 ± 1.01 (mean $\pm$ standard error). In addition, OB/GYNs scored 8.29 ± 0.95 , while other healthcare professionals scored 7.87 ± 1.02 , indicating that OB/GYNs had a significantly higher level of understanding ($P = 0.009$).

DISCUSSION

Education on menopausal disorders is inadequate in medical schools. A survey of internal medicine residents in the United States found menopause management to be one of the most important topics, but residents indicated inadequate clinical exposure to it [9].

Research suggests differences in beliefs and attitudes about menopause among general practitioners, which may contribute to negative attitudes toward prescribing treatments for menopausal symptoms [10-12]. One reason healthcare providers struggle to deliver the biopsychosocial care essential for managing menopausal women is the insufficient education on menopausal disorders [13]. Therefore, standardized training in the management of menopausal women remains warranted.

In the present study, compared to other professionals, OB/GYNs reported that lectures on general remarks and HRT were easier for them. In Japanese medical education, menopausal disorders are part of the obstetrics and gynecology curriculum. OB/GYNs learn

Table 3. Participants' backgrounds based on responses to 2024 questionnaire (n = 159)

	Total (n = 159)	OB/GYNs (n = 65)	Other than OB/GYNs (n = 94)	<i>P</i> value
Are you a member of the Japanese Society of Psychosomatic Obstetrics and Gynecology?				
Already a member before taking the course	58 (36.5)	30 (46.2)	28 (29.8) ^a	0.013
Joined in order to attend this course	77 (48.4)	31 (47.7)	46 (48.9)	
Non-member	24 (15.1)	4 (6.2)	20 (21.3) ^a	
If you are a doctor: tell us your specialty (n = 84)				
Obstetrics and Gynecology	65 (40.9)	-	-	0.147
Internal Medicine	8 (5.0)	-	-	
Psychiatry	4 (2.5)	-	-	
Psychosomatic Medicine	3 (1.9)	-	-	
Other than above	4 (2.5)	-	-	
Age (y)				
20–29	3 (1.9)	2 (3.1)	1 (1.1)	0.147
30–39	18 (11.3)	10 (15.4)	8 (8.5)	
40–49	57 (35.8)	28 (43.1)	29 (30.9)	
50–59	58 (36.5)	17 (26.2)	41 (43.6)	
60–69	18 (11.3)	7 (10.8)	11 (11.7)	
≥ 70	5 (3.1)	1 (1.5)	4 (4.3)	
Overall course satisfaction				
Very satisfied	63 (39.6)	24 (36.9)	39 (41.5)	0.684
Satisfied	86 (54.1)	38 (58.5)	48 (51.1)	
Average	9 (5.7)	3 (4.6)	6 (6.4)	
Dissatisfied	0 (0.0)	0 (0.0)	0 (0.0)	
Very dissatisfied	1 (0.6)	0 (0.0)	1 (1.1)	
Overall course volume				
Too much	0 (0.0)	0 (0.0)	0 (0.0)	0.515
A little too much	5 (3.1)	1 (1.5)	4 (4.3)	
Just right	133 (83.6)	54 (83.1)	79 (84.0)	
A little too little	20 (12.6)	10 (15.4)	10 (10.6)	
Too little	1 (0.6)	0 (0.0)	1 (1.1)	
Do you think this course will be useful for your future activities?				
Very useful	101 (63.5)	39 (60.0)	62 (66.0)	0.545
Fairly useful	55 (34.6)	24 (36.9)	31 (33.0)	
Can't say either way	3 (1.9)	2 (3.1)	1 (1.1)	
Not very useful	0 (0.0)	0 (0.0)	0 (0.0)	
Not at all useful	0 (0.0)	0 (0.0)	0 (0.0)	

Data are presented as number (%).

OB/GYNs: Obstetricians and Gynecologists; -: not applicable.

^aStatistically significant compared to OB/GYNs.

more about menopausal disorders even after graduation. However, those in other specialties have few opportunities to learn about menopausal disorders after

graduating from school. In addition, the importance of menopause education for non-physician health-care professionals is increasingly recognized. Indeed,

Table 4. Impressions of each lecture and overall understanding of the course, based on responses to 2024 questionnaire (n = 159)

	Total (n = 159)	OB/GYNs (n = 65)	Other than OB/GYNs (n = 94)	P value
Health problems in women during and after menopause (general remarks)				
Lecture satisfaction				
Very satisfied	72 (45.3)	25 (38.5)	47 (50.0)	0.379
Satisfied	74 (46.5)	35 (53.8)	39 (41.5)	
Average	12 (7.5)	5 (7.7)	7 (7.4)	
Dissatisfied	0 (0.0)	0 (0.0)	0 (0.0)	
Very dissatisfied	1 (0.6)	0 (0.0)	1 (1.1)	
Lecture time				
Too long	0 (0.0)	0 (0.0)	0 (0.0)	0.237
A little too long	9 (5.7)	5 (7.7)	4 (4.3)	
Just right	147 (92.5)	60 (92.3)	87 (92.6)	
A little too short	3 (1.9)	0 (0.0)	3 (3.2)	
Too short	0 (0.0)	0 (0.0)	0 (0.0)	
Difficulties with the lecture				
Too easy	3 (1.9)	3 (4.6)	0 (0.0) ^a	0.002
Somewhat easy	25 (15.7)	17 (26.2)	8 (8.5) ^a	
Just right	126 (79.2)	44 (67.7)	82 (87.2) ^a	
Somewhat difficult	5 (3.1)	1 (1.5)	4 (4.3)	
Too difficult	0 (0.0)	0 (0.0)	0 (0.0)	
Basics of hormone replacement therapy				
Lecture satisfaction				
Very satisfied	70 (44.0)	23 (35.4)	47 (50.0)	0.234
Satisfied	69 (43.4)	33 (50.8)	36 (38.3)	
Average	19 (11.9)	9 (13.8)	10 (10.6)	
Dissatisfied	0 (0.0)	0 (0.0)	0 (0.0)	
Very dissatisfied	1 (0.6)	0 (0.0)	1 (1.1)	
Lecture time				
Too long	0 (0.0)	0 (0.0)	0 (0.0)	0.809
A little too long	6 (3.8)	3 (4.6)	3 (3.2)	
Just right	149 (93.7)	61 (93.8)	88 (93.6)	
A little too short	3 (1.9)	1 (1.5)	2 (2.1)	
Too short	1 (0.6)	0 (0.0)	1 (1.1)	
Difficulties with the lecture				
Too easy	4 (2.5)	4 (6.2)	0 (0.0) ^a	< 0.001 ^a
Somewhat easy	26 (16.4)	21 (32.3)	5 (5.3) ^a	
Just right	117 (73.6)	40 (61.3)	77 (81.9) ^a	
Somewhat difficult	12 (7.5)	0 (0.0)	12 (12.8) ^a	
Too difficult	0 (0.0)	0 (0.0)	0 (0.0)	

Table 4. Continued

	Total (n = 159)	OB/GYNs (n = 65)	Other than OB/GYNs (n = 94)	P value
Treatment options for menopausal disorders other than hormone replacement therapy				
Lecture satisfaction				
Very satisfied	68 (42.8)	24 (36.9)	44 (46.8)	0.455
Satisfied	75 (47.2)	35 (53.8)	40 (42.6)	
Average	15 (9.4)	6 (9.2)	9 (9.6)	
Dissatisfied	1 (0.6)	0 (0.0)	1 (1.1)	
Very dissatisfied	0 (0.0)	0 (0.0)	0 (0.0)	
Lecture time				
Too long	0 (0.0)	0 (0.0)	0 (0.0)	0.682
A little too long	7 (4.4)	4 (6.2)	3 (3.2)	
Just right	143 (89.9)	58 (89.2)	85 (90.4)	
A little too short	8 (5.0)	3 (4.6)	5 (5.3)	
Too short	1 (0.6)	0 (0.0)	1 (1.1)	
Difficulties with the lecture				
Too easy	2 (1.3)	2 (3.1)	0 (0.0)	0.042
Somewhat easy	18 (11.3)	11 (16.9)	7 (7.4)	
Just right	122 (76.7)	48 (73.8)	74 (78.7)	
Somewhat difficult	17 (10.7)	4 (6.2)	13 (13.8)	
Too difficult	0 (0.0)	0 (0.0)	0 (0.0)	
The impact of menopause on mental health and management				
Lecture satisfaction				
Very satisfied	59 (37.1)	25 (38.5)	34 (36.2)	0.247
Satisfied	73 (45.9)	31 (47.7)	42 (44.7)	
Average	23 (14.5)	6 (9.2)	17 (18.1)	
Dissatisfied	4 (2.5)	3 (4.6)	1 (1.1)	
Very dissatisfied	0 (0.0)	0 (0.0)	0 (0.0)	
Lecture time				
Too long	0 (0.0)	0 (0.0)	0 (0.0)	0.598
A little too long	11 (6.9)	6 (9.2)	5 (5.3)	
Just right	141 (88.7)	55 (84.6)	86 (91.5)	
A little too short	5 (3.1)	3 (4.6)	2 (2.1)	
Too short	2 (1.3)	1 (1.5)	1 (1.1)	
Difficulties with the lecture				
Too easy	0 (0.0)	0 (0.0)	0 (0.0)	0.884
Somewhat easy	12 (7.5)	5 (7.7)	7 (7.4)	
Just right	130 (81.8)	54 (83.1)	76 (80.9)	
Somewhat difficult	17 (10.7)	6 (9.2)	11 (11.7)	
Too difficult	0 (0.0)	0 (0.0)	0 (0.0)	

Table 4. Continued

	Total (n = 159)	OB/GYNs (n = 65)	Other than OB/GYNs (n = 94)	P value
Supporting patient decision-making and healthcare				
Lecture satisfaction				
Very satisfied	63 (39.6)	26 (40.0)	37 (39.4)	0.727
Satisfied	69 (43.4)	30 (46.2)	39 (41.5)	
Average	26 (16.4)	9 (13.8)	17 (18.1)	
Dissatisfied	1 (0.6)	0 (0.0)	1 (1.1)	
Very dissatisfied	0 (0.0)	0 (0.0)	0 (0.0)	
Lecture time				
Too long	0 (0.0)	0 (0.0)	0 (0.0)	0.416
A little too long	13 (8.2)	6 (9.2)	7 (7.4)	
Just right	141 (88.7)	56 (86.2)	85 (90.4)	
A little too short	4 (2.5)	3 (4.6)	1 (1.1)	
Too short	1 (0.6)	0 (0.0)	1 (1.1)	
Difficulties with the lecture				
Too easy	0 (0.0)	0 (0.0)	0 (0.0)	0.220
Somewhat easy	10 (6.3)	2 (3.1)	8 (8.5)	
Just right	126 (79.2)	51 (78.5)	75 (79.8)	
Somewhat difficult	23 (14.5)	12 (18.5)	11 (11.7)	
Too difficult	0 (0.0)	0 (0.0)	0 (0.0)	
Cognitive and behavioral approach to menopausal disorders				
Lecture satisfaction				
Very satisfied	75 (47.2)	31 (47.7)	44 (46.8)	0.832
Satisfied	64 (40.3)	27 (41.5)	37 (39.4)	
Average	19 (11.9)	7 (10.8)	12 (12.8)	
Dissatisfied	1 (0.6)	0 (0.0)	1 (1.1)	
Very dissatisfied	0 (0.0)	0 (0.0)	0 (0.0)	
Lecture time				
Too long	3 (1.9)	1 (1.5)	2 (2.1)	0.254
A little too long	13 (8.2)	7 (10.8)	6 (6.4)	
Just right	136 (85.5)	52 (80.0)	84 (89.4)	
A little too short	7 (4.4)	5 (7.7)	2 (2.1)	
Too short	0 (0.0)	0 (0.0)	0 (0.0)	
Difficulties with the lecture				
Too easy	0 (0.0)	0 (0.0)	0 (0.0)	0.196
Somewhat easy	6 (3.8)	1 (1.5)	5 (5.3)	
Just right	119 (74.8)	46 (70.8)	73 (77.7)	
Somewhat difficult	33 (20.8)	17 (26.2)	16 (17.0)	
Too difficult	1 (0.6)	1 (1.5)	0 (0.0)	
Overall understanding of the course (1: I didn't understand at all – 10: I understood perfectly)				
	8.04 ± 1.01	8.29 ± 0.95	7.87 ± 1.02 ^a	0.009

Data are presented as number (%) or mean ± SD.

OB/GYNs: Obstetricians and Gynecologists, SD: standard deviation.

^aStatistically significant compared to OB/GYNs.

numerous nurses and midwives expressed interest in learning more about providing care for menopausal women [14]. Our course responded to the interest in menopausal disorders in various healthcare professionals; those who watched all the lectures and fulfilled the conditions were awarded a certificate of Menopause Instructor. Our establishment of the qualification of Menopause Instructor might have increased the motivation of participants to complete the course.

Since the course is online and easily accessible from everywhere, it certifies Menopause Instructors all over Japan. The British Menopause Society has launched an online course for healthcare professionals in the United Kingdom since 2024 [15], which, like our course, allows participants to gain certification by completing the course. The initiatives to increase the number of healthcare professionals who manage menopausal disorders through online courses may expand further. Conversely, some prefectures still lack certified Menopause Instructors. The reason for this is unclear, but these prefectures are all rural areas. Since we primarily promoted the course at academic events, medical professionals in rural areas who have difficulty attending such events may not have received the information. Therefore, it is necessary to provide information through channels other than academic societies, such as social media, in the near future.

During the course, we delivered five lectures in 2023 and six in 2024. The International Menopause Society offers International Menopause Society Professional Activity for Refresher Training (IMPART) as a training course for healthcare professionals regarding menopausal disorders [16]. IMPART is divided into two levels, each consisting of eight modules. IMPART provides more detail about HRT and lectures on osteoporosis, heart disease, and cancer. In addition, the European Menopause and Andropause Society recommended a curriculum for menopause education programs for health professionals in 2022 [17], which includes terminologies, menopause symptoms, assessment and screening, staying healthy, treatment options, long-term health, and delivering menopause health care. In this study, OB/GYNs demonstrated a higher level of understanding compared to other professionals. OB/GYNs typically have more opportunities to learn about menopause disorders during their residency and at medical conferences. However, they do not have sufficient opportunities to learn about internal medicine topics relevant to women's health. To make the course

more satisfying for both OB/GYNs and non-OB/GYNs, we may add more detailed lectures on heart disease and osteoporosis, as referenced in the two aforementioned programs.

Helping health professionals retain knowledge about menopausal symptoms is challenging. A study of menopause education for OB/GYNs and family medicine residents found that specialized menopause curricula produced short-term increases in menopause-related knowledge but did not improve overall knowledge retention at 3 months [18]. In our course, we delivered lectures on menopausal symptoms at annual conferences to update participants' knowledge. Furthermore, we are also considering setting up a web platform on our website where course participants can watch videos of the lectures. We must evaluate how effectively participants retain the knowledge gained from our course in the future.

This study has some limitations. First, this course was held by a medical society specializing in women's psychosomatic disorders; therefore, the participants may be biased toward healthcare professionals with an interest in psychosomatic medicine. Second, the 2024 questionnaire analyzed in this study was responded to only by those who completed the course; therefore, it does not reflect the opinions of those who dropped out for any reason. We will reconsider the survey method and ask those who dropped out for the reasons why. Third, the system prevented us from evaluating participants' knowledge improvement. We are considering rebuilding the system to compare knowledge improvement between OB/GYNs and non-OB/GYNs, which would help us plan future lectures. Additionally, two years is too short to evaluate the course's effectiveness. We will continue this course and reevaluate the pre- and post-tests, as well as the questionnaire surveys. We will also further investigate the activities of those who have obtained Menopause Instructor certification. This will allow us to continue evaluating the effectiveness of the certification program. Our findings will be reported.

As the strength of this research, to the best of our knowledge, this is the first educational program in Japan specifically for healthcare professionals specializing in menopausal disorders. The results of this study provide insights into the attitudes of healthcare professionals toward menopausal disorders in Japan.

In conclusion, we held the Menopause Instructor Course as an educational course for healthcare professionals on menopausal disorders. Over 2023 and 2024, we certified

more than 300 healthcare professionals from all over Japan as Menopause Instructors. Although overall satisfaction with the course was high, there were differences in the perceived level of difficulty and achievement between OB/GYNs and other healthcare professionals. Retention of knowledge by participants and refinement of content to benefit OB/GYNs and other healthcare professionals should be explored.

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CONFLICT OF INTEREST

Mariko Ogawa received speaker fees from Fuji Pharma Co., Ltd., Otsuka Pharmaceutical Co., Ltd., Bayer Yakuhin Ltd., and ASKA Pharmaceutical Co., Ltd.; Masakazu Terauchi received speaker fees and/or honoraria from Fuji Pharma Co., Ltd., Otsuka Pharmaceutical Co., Ltd., and Astellas Pharma Inc.; Masahiro Hashizume received speaker fees from Daiichi Sankyo Co., Ltd.; Kiyoshi Takamatsu received speaker fees from Fuji Pharma Co., Ltd., Otsuka Pharmaceutical Co. Ltd., Bayer Yakuhin Ltd.; Takashi Takeda received speaker fees from TSUMURA Co., Kuracie Pharmaceutical Ltd. The remaining authors declare no potential conflict of interest relevant to this article was reported.

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Original Paper

eHealth Literacy and Adolescent Health in Japanese Female High School Students in Sendai: Cross-Sectional Study

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Abstract

Background: In the digital age, adolescents increasingly rely on online sources for health-related information. eHealth literacy—defined as the ability to find, evaluate, and apply online health information—plays a crucial role in health outcomes. However, limited research exists on eHealth literacy among Japanese high school students, particularly on its association with menstrual health and psychological well-being.

Objective: This study aimed to assess the eHealth literacy of Japanese female high school students and examine its association with premenstrual symptoms, psychological distress, loneliness, and self-esteem.

Methods: A cross-sectional, web-based survey was conducted in December 2024 among 1607 female students from 2 public high schools in Sendai, Japan. A total of 909 students with regular menstrual cycles completed all survey items. The survey included the eHealth Literacy Scale (eHEALS), Premenstrual Symptoms Questionnaire, Kessler Psychological Distress Scale (K6), Revised UCLA Loneliness Scale, Rosenberg Self-Esteem Scale, and a numerical rating scale for menstrual pain. Statistical analyses, including Student t tests, chi-square tests, correlation analyses, and logistic regression analyses, were used to examine the relationships between eHealth literacy and various health outcomes.

Results: The mean eHEALS score was 22.8 (SD 7.3), with 32.1% (292/909) of participants classified as having high eHealth literacy (eHEALS $\geq$ 26). Students with higher eHealth literacy reported significantly lower levels of loneliness and higher self-esteem. The severity of premenstrual symptoms, particularly feeling overwhelmed, was significantly lower in the high eHealth literacy group. Additionally, interpersonal difficulties related to premenstrual symptoms were less prevalent among students with high eHealth literacy. Pearson correlation analysis indicated negative associations between the eHEALS score and psychological distress (K6) and loneliness, whereas a positive association was observed with self-esteem. Logistic regression analysis showed that high self-esteem was significantly associated with high eHealth literacy.

Conclusions: This study highlights the importance of eHealth literacy in adolescent health care. Higher eHealth literacy is linked to lower levels of loneliness, higher self-esteem, and reduced premenstrual symptom severity, particularly feeling overwhelmed. Although the cross-sectional design limits causal conclusions, these findings suggest that higher eHealth literacy is associated with better mental and reproductive health in adolescents. Future research should adopt longitudinal designs, include more diverse populations—such as male adolescents—and explore additional contributing factors to better elucidate these associations.

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KEYWORDS

female adolescents; mental health; premenstrual symptoms; self-esteem; eHealth

Introduction

Growing Role of eHealth in Adolescent Health

In recent years, many people have begun to use eHealth information to manage their own health [1]. As internet accessibility continues to expand globally, more individuals are obtaining health-related information online. Internet use is also high in Japan, standing at 86.2% in 2023, according to data from the Ministry of Internal Affairs and Communications [2]. The usage rate is particularly high among adolescents aged 13 years to 19 years (98.7%). Adolescence is an important transition period from childhood to adulthood, during which adolescents undergo many physical, cognitive, and psychosocial changes; establish their ego; build their own world; and develop healthy lifestyle habits. During this period, adolescents seek information on topics such as sexual health, nutrition, beauty, and infectious diseases through websites and social media [3].

Concept and Importance of eHealth Literacy

Health literacy is the ability to find, understand, and use health information to make informed decisions. These include reading medical instructions, understanding prescriptions, and evaluating health information from various sources. Good health literacy helps individuals manage diseases, correctly follow treatment, and effectively communicate with health care providers. It also involves numeracy skills such as understanding dosages and medical test results. Low health literacy can lead to poor health outcomes and medication errors. Therefore, promoting health literacy empowers individuals to take charge of their well-being and make healthier choices. Among digitally native adolescents, the internet has replaced traditional sources such as books for gathering health information [4,5].

Assessment of eHealth Literacy Among Adolescents

Given this shift, eHealth literacy—the ability to find, evaluate, and use health information from the internet to solve one's health problems—has become increasingly important [6]. The 6-item Lily Model of eHealth literacy, comprising traditional, health, information, scientific, media, and computer literacy, was previously proposed [6]. An 8-item rating scale called the eHealth Literacy Scale (eHEALS), based on this model, has been developed [7], translated into many languages, and used worldwide [8–11]. As adolescents continue to rely on the internet for health-related information, assessing their eHealth literacy is essential. However, despite increasing reliance on digital health information, research on eHealth literacy among adolescents remains scarce. Most studies have focused on university students or adults, leaving a significant knowledge gap regarding younger adolescents [4,12]. Moreover, global studies on adolescent eHealth literacy are sparse, with only a few investigations conducted in specific regions such as Turkey, Serbia, and Brazil [13–15]. These studies suggest variations in eHealth literacy levels among different populations, further highlighting the need for region-specific studies.

Premenstrual Symptoms and Psychological Well-Being

Premenstrual symptoms are characterized by unpleasant psychosomatic symptoms that impair the quality of life of many women from adolescence to adulthood [16–18]. Premenstrual

syndrome (PMS) is characterized by various premenstrual symptoms and is classified as premenstrual dysphoric disorder when psychological symptoms become particularly severe [19]. Recently, the concept of premenstrual disorders was introduced, considering both conditions as part of a continuum [20]. Menstrual pain and premenstrual symptoms are correlated and, as menstrual symptoms, have a significant impact on performance [21,22]. In Japan, a study with working women found that those with higher health literacy reported lower presenteeism and better performance at managing menstrual symptoms [22]. However, this study did not examine premenstrual symptoms or social dysfunction in detail. In addition, few studies have examined the relationship between health literacy, including eHealth literacy, and menstrual symptoms in adolescents.

Psychological Distress, Loneliness, and Self-Esteem in Adolescents

High school students are at an increased risk of psychological distress, depression, anxiety, loneliness, and trauma [23]. Loneliness, the subjective feeling of isolation, has gained attention as a major public health concern owing to its strong association with mental health conditions such as depression and anxiety. It is also a key risk factor for poor mental health outcomes, particularly during adolescence [24–26]. A survey of Japanese high school students conducted in 2021 found that psychological distress was correlated with the severity of premenstrual symptoms, whereas loneliness was independently linked to both conditions. Self-esteem, which reflects an individual's overall sense of self-worth [27], is another critical factor affecting the mental health of adolescents. Low self-esteem is linked to depression, eating disorders, risky behaviors, and academic decline [28]. Additionally, it has a strong reciprocal relationship with loneliness [27,28]. Low self-esteem and loneliness are particularly concerning during adolescence, which is the formative period for developing self-identity [29]. As adolescents increasingly rely on online health information, eHealth literacy may play a role in their psychological well-being by influencing their access to and interpretation of health-related content.

Study Objectives

The purpose of this study was to assess the current level of eHealth literacy among Japanese female high school students, with a particular focus on examining its association with premenstrual symptoms. In addition, we investigated how eHealth literacy is related to other key aspects of adolescent health, including psychological distress, loneliness, and self-esteem. We hypothesized that students with higher eHealth literacy would report fewer premenstrual symptoms, lower levels of psychological distress and loneliness, and higher levels of self-esteem. These associations were examined to better understand the potential role of digital health literacy in promoting reproductive and mental well-being among adolescents.

Methods

Ethical Considerations

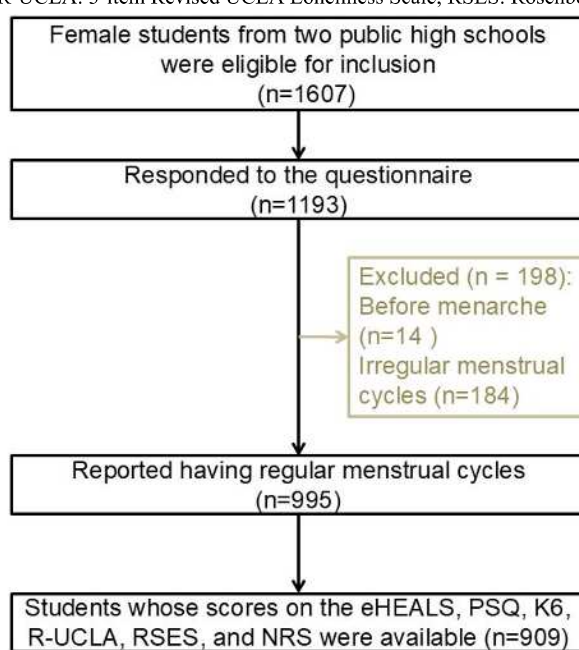
This study was conducted in accordance with the principles of the Declaration of Helsinki, and the Kindai University Ethics Committee approved the research protocol (approval number: R06-131; approval date: October 2, 2024). Students who participated in the study provided informed consent.

The Kindai University Ethics Committee approved the waiver of parental informed consent, and the students' consent and willingness to participate were considered sufficient. The omission of parental informed consent was in accordance with the Ethical Guidelines for Medical and Health Sciences Research Involving Human Subjects issued by the Ministry of Education, Culture, Sports, Science, and Technology, and the Ministry of Health, Labour and Welfare. The data were anonymized and did not contain identifiable information regarding the participants. Participants were not compensated.

Settings and Participants

In mid-December 2024, a school survey was conducted among 1607 female students attending 2 public high schools in Sendai, Japan. We conducted a web survey using Google Forms. The link to the self-administered survey form was distributed through school classes, and the survey was administered over the internet with consent. Given the sensitive nature of the questions, students were instructed to complete the survey independently at home after school hours, rather than during class time. A total of 1193 students responded to the survey. Of these, 995 had regular menstrual cycles of 25 days to 38 days (Figure 1). Because premenstrual symptoms appear solely during ovulatory cycles, only those with regular menstrual cycles were selected. Furthermore, 909 students who completed all items from the following measures were selected: eHEALS, Premenstrual Symptoms Questionnaire (PSQ), 6-item Kessler Psychological Distress Scale (K6), 3-item Revised UCLA Loneliness Scale (R-UCLA), Rosenberg Self-Esteem Scale (RSES), and numerical rating scale (NRS) for menstrual pain intensity.

Figure 1. Study flowchart. eHEALS: eHealth Literacy Scale; K6: 6-item Kessler Psychological Distress Scale; NRS: numerical rating scale; PSQ: Premenstrual Symptoms Questionnaire; R-UCLA: 3-item Revised UCLA Loneliness Scale; RSES: Rosenberg Self-Esteem Scale.



Questionnaires

eHEALS

The eHEALS is the only questionnaire that examines online health literacy [7]. In this study, we used the Japanese version, which has demonstrated validity and reliability [10]. The eHEALS is composed of 8 items and is scored on a 5-point scale (1=strongly disagree to 5=strongly agree). The total eHEALS score ranges from 8 to 40: the higher the score, the better the eHealth literacy. In this study, the Cronbach α coefficient for the scale was 0.935. Participants were divided into 2 groups—a high-scoring group (eHEALS $\geq$ 26; n=292) and a low-scoring group (eHEALS<26; n=617)—according to criteria reported previously [29].

PSQ

The PSQ, previously used in research, was chosen for this study as a simple screening tool for premenstrual symptoms [18,30]. Its reliability and validity are well established [31]. The PSQ starts with: “In the past three months, have you experienced any premenstrual symptoms that start a week before menstruation and stop a few days after it begins?” It includes 11 items based on the Diagnostic and Statistical Manual of Mental Disorders—premenstrual dysphoric disorder criteria and assesses the impact of symptoms on (1) work and family responsibilities, (2) social activities, and (3) relationships. Severity and impact are rated on a 4-point scale (1=not at all to 4=severe). The total score (range: 14-56) indicates symptom severity. The Cronbach α was 0.936.

K6

The K6 was chosen for this study because it is a widely used screening tool for psychological distress in the general population [32,33]. Psychological distress was measured using the Japanese version of K6 [34], with established reliability and validity. The K6 consists of 6 items rated on a 5-point scale (0=not at all to 4=all). The total score ranges from 0 to 24, with higher scores indicating greater distress. The Cronbach α was 0.957.

R-UCLA

The R-UCLA was chosen for this study because it is a widely used and simple assessment tool for evaluating loneliness [35]. The Japanese version of the 3-item R-UCLA was used, and its reliability and validity have been well established [36]. This scale consists of 3 items rated on a 3-point scale (1=hardly ever, 2=sometimes, and 3=often), yielding a total score between 3 and 9. Higher scores indicate greater loneliness. The scale demonstrated strong internal consistency, with a Cronbach α of 0.913.

RSES

The RSES is a widely used self-esteem assessment tool that was selected for this study owing to its broad applicability [27]. The Japanese version of RSES (RSES-J) was used to measure self-esteem, and its reliability and validity have been confirmed [37]. The RSES-J consists of 10 items: 5 positive and 5 negative. Negative items are rated on a 4-point scale (1=strongly agree, 2=agree, 3=disagree, and 4=strongly disagree), whereas positive items are scored inversely. The total score ranges from 10 to 40, with higher scores indicating greater self-esteem. In this study, the Cronbach α for the RSES was 0.873.

NRS

Menstrual pain is closely linked to premenstrual symptoms [21,31]. In this study, pain severity was assessed using the NRS, a standard tool for measuring pain perception. Participants rate their pain on an 11-point scale ranging from 0 (no pain) to 10 (worst imaginable pain).

Participant data included age, school grade, weight, height, age at menarche, menstrual cycle length, internet usage time, and

sleep duration. Menarche marks the onset of puberty and is linked to health issues such as early pregnancy, sexually transmitted diseases, depression, and anxiety [38,39]. It has been identified as a key factor in adolescent health. A regular cycle was defined as 25 days to 38 days. BMI was calculated by dividing weight by height squared (kg/m^2). As obesity is associated with PMS, low self-esteem, and loneliness, BMI was included in the analysis [40,41].

Statistical Analysis

The Cronbach α coefficient was used to assess the reliability of each scale (eHEALS, PSQ, K6, 3-item R-UCLA, and RSES). We calculated the means and SDs for continuous variables and proportions for categorical variables. Differences in the background characteristics of the high and low eHealth literacy groups were compared using Student *t* tests and Pearson chi-square tests. The Steel-Dwass test was used to compare differences in eHEALS scores between school years. The exact Wilcoxon signed-rank test was used to compare the severity of 11 premenstrual symptoms and 3 social disability items on the PSQ between the high and low eHealth literacy groups. Correlations among the 5 psychological questionnaires (total eHEALS, PSQ, K6, 3-item R-UCLA, and RSES) were examined using Pearson correlation coefficients. The factors significantly associated with high eHealth literacy were determined using multivariable logistic regression analysis. A total of 10 items—age, BMI, age at menarche, menstrual pain intensity, duration of internet use, sleep duration, total PSQ score, total K6 score, R-UCLA, and RSES—were included in the model.

Statistical analyses were performed using JMP Pro 18.1.0 (SAS). A *P* value<.05 was considered statistically significant.

Results

Participant Characteristics

The characteristics of all participants are presented in Table 1. The mean eHEALS score was 22.8 (SD 7.3). Approximately one-third (292/909, 32.1%) of the participants belonged to the high eHealth literacy group. The high eHealth literacy group had lower R-UCLA scores and higher RSES scores than the low eHealth literacy group.

Table 1. Characteristics of the study participants.

Characteristic	Overall (N=909)	Low eHealth literacy group (eHEALS ^a <26; n=617)	High eHealth literacy group (eHEALS ^a ≥26; n=292)	<i>P</i> value
Age (years), mean (SD)	16.8 (0.9)	16.8 (0.9)	16.8 (1.0)	.88 ^b
School year, n (%)				.58 ^c
First year ^d	270 (29.7)	188 (69.6)	82 (30.3)	
Second year ^e	297 (32.7)	195 (65.7)	102 (34.3)	
Third year ^f	342 (37.6)	234 (68.4)	108 (31.6)	
BMI (kg/m ²), mean (SD)	20.5 (2.6)	20.5 (0.1)	20.3 (0.15)	.29 ^b
Age at menarche (years), mean (SD) ^g	12.1 (1.3)	12.1 (0.1)	12.0 (0.1)	.12 ^b
Menstrual pain intensity, mean (SD)	4.8 (2.6)	4.9 (0.1)	4.8 (0.2)	.52 ^b
Duration of internet use (hours), mean (SD) ^h	3.1 (1.9)	3.2 (2.1)	3.1 (1.6)	.89 ^b
Sleep duration (hours), mean (SD) ⁱ	6.1 (1.0)	6.0 (1.0)	6.1 (1.0)	.12 ^b
Total PSQ ^j score, mean (SD)	26.8 (9.5)	27.0 (0.4)	26.3 (0.6)	.24 ^b
K6 ^k , mean (SD)	7.4 (7.4)	7.5 (0.3)	7.0 (0.4)	.27 ^b
R-UCLA ^l , mean (SD)	5.8 (2.7)	6.0 (0.1)	5.5 (0.2)	.02 ^b
RSES ^m , mean (SD)	25.1 (6.1)	24.3 (0.2)	26.8 (0.4)	<.001 ^b
eHEALS, mean (SD)	22.8 (7.3)	19.0 (5.2)	30.8 (4.1)	<.001 ^b

^aeHEALS: eHealth Literacy Scale.^bStudent *t* test.^cPearson chi-square test.^dMedian age: 16 (IQR 15-16) years.^eMedian age: 17 (IQR 16-17) years.^fMedian age: 18 (IQR 17-18) years.^gMissing data: 7 (0.8%).^hMissing data: 24 (2.6%).ⁱMissing data: 4 (0.4%).^jPSQ: Premenstrual Symptoms Questionnaire.^kK6: 6-item Kessler Psychological Distress Scale.^lR-UCLA: 3-item Revised UCLA Loneliness Scale.^mRSES: Rosenberg Self-Esteem Scale.

Comparison of eHealth Literacy Between School Years

Differences in eHEALS scores between school years were analyzed in terms of the total score and each of the 8 items (Table 2). We found no differences in total eHEALS scores between school years. Of the 8 items, second-year students' scores were significantly higher than those for first-year students

for Q6 (I find on the Internet, I know how to use the Internet to answer my questions about health) and Q7 (I can distinguish high-quality health resources from low-quality health resources on the Internet). This suggests that some aspects of eHealth literacy may improve with age or school experience, but the overall literacy level remains consistent across the years.

Table 2. Study participants' eHealth Literacy Scale (eHEALS) scores by school year level.

Question and total scores	First year, median (IQR)	Second year, median (IQR)	Third year, median (IQR)	<i>P</i> value (second vs first) ^a	<i>P</i> value (third vs first) ^a	<i>P</i> value (third vs second) ^a
Q1 ^b	3 (2-3)	3 (2-4)	3 (2-4)	.48	.30	.90
Q2 ^c	3 (2-3)	3 (2-3)	3 (2-3)	.35	.71	.82
Q3 ^d	3 (2-4)	3 (2-4)	3 (2-4)	.41	.57	.97
Q4 ^e	3 (2-4)	3 (2-4)	3 (2-4)	.54	.59	≥.99
Q5 ^f	3 (2-4)	3 (2-4)	3 (2-4)	.40	.51	.98
Q6 ^g	3 (2-3)	3 (2-3.5)	3 (2-4)	.02	.47	.33
Q7 ^h	3 (2-3)	3 (2-4)	3 (2-4)	.02	.11	.82
Q8 ⁱ	3 (2-3)	3 (2-4)	3 (2-4)	.08	.10	≥.99
Total eHEALS score	23 (16-26)	24 (19-28)	24 (18-28)	.10	.27	.88

^aSteel-Dwass test.^b"I know what health resources are available on the Internet."^c"I know where to find helpful health resources on the Internet."^d"I know how to use the health information I find on the Internet to help me."^e"I know how to find helpful health resources on the Internet."^f"I have the skills I need to evaluate health resources."^g"I know how to navigate the Internet and use it to answer my questions about health."^h"I can distinguish high-quality health resources from low-quality health resources on the Internet."ⁱ"I feel confident in using information from the Internet to make health decisions."

eHealth Literacy and Premenstrual Symptoms

Next, the differences in premenstrual symptoms and level of social impairment were examined according to the level of eHealth literacy (high and low literacy; Table 3). Of the 11 premenstrual symptoms listed in the PSQ, only feeling overwhelmed was significantly lower in the high eHealth

literacy group than in the low eHealth literacy group. In terms of social life impairment, only interpersonal impairment was significantly lower in the high eHealth literacy group than in the low eHealth literacy group. However, there were no significant differences in work productivity nor participation in social activities between the 2 groups.

Table 3. Differences by eHealth literacy level (high [n=292] and low [n=617] literacy) in terms of premenstrual symptoms and level of social impairment.

Characteristic	Responses to questions				<i>P</i> value ^a
	Not at all, n (%)	Mild, n (%)	Moderate, n (%)	Severe, n (%)	
Premenstrual symptoms					
Depressed mood					.96
Low eHealth literacy ^b	231 (37.4)	189 (30.6)	149 (24.2)	48 (7.8)	
High eHealth literacy ^c	106 (36.3)	97 (33.2)	68 (23.3)	21 (7.2)	
Anxiety or tension					.27
Low eHealth literacy	166 (26.9)	211 (34.2)	183 (29.7)	57 (9.2)	
High eHealth literacy	93 (31.9)	89 (30.5)	87 (29.8)	23 (7.9)	
Tearful					.49
Low eHealth literacy	244 (39.6)	159 (25.8)	151 (24.5)	63 (10.2)	
High eHealth literacy	125 (42.8)	72 (24.7)	61 (20.9)	34 (11.6)	
Anger or irritability					.10
Low eHealth literacy	177 (28.7)	201 (32.6)	192 (31.1)	47 (7.6)	
High eHealth literacy	99 (33.9)	95 (32.5)	75 (25.7)	23 (7.9)	
Decreased interest in work, home, or social activities					.10
Low eHealth literacy	333 (54)	164 (26.6)	95 (15.4)	25 (4.1)	
High eHealth literacy	176 (60.3)	67 (23)	35 (12)	14 (4.8)	
Difficulty concentrating					.41
Low eHealth literacy	230 (37.3)	215 (34.9)	126 (20.4)	46 (7.5)	
High eHealth literacy	118 (40.4)	96 (32.9)	59 (20.2)	19 (6.5)	
Fatigue or lack of energy					.39
Low eHealth literacy	159 (25.8)	198 (32.1)	183 (29.7)	77 (12.5)	
High eHealth literacy	87 (29.8)	86 (29.5)	84 (28.8)	35 (12)	
Overeating or food cravings					.32
Low eHealth literacy	149 (24.2)	194 (31.4)	197(31.9)	77 (12.5)	
High eHealth literacy	84 (28.8)	85 (29.1)	86 (29.5)	37 (12.7)	
Insomnia or hypersomnia					.40
Low eHealth literacy	233 (37.8)	157 (25.5)	148 (24)	79 (12.8)	
High eHealth literacy	119(40.8)	72 (24.7)	66 (22.6)	35(12)	
Feeling overwhelmed					.03
Low eHealth literacy	343 (55.6)	172 (27.9)	78 (12.6)	24 (3.9)	
High eHealth literacy	189 (64.7)	58 (19.9)	33 (11.3)	12 (4.1)	
Physical symptoms					.86
Low eHealth literacy	298 (48.3)	190 (30.8)	95 (15.4)	34 (5.5)	
High eHealth literacy	144 (49.3)	77 (26.4)	56 (19.2)	15 (5.1)	
Interference with work, usual activities, or relationships					
Work efficiency, productivity, home responsibilities					.55
Low eHealth literacy	249 (40.4)	210 (34)	130 (21.1)	28 (4.5)	
High eHealth literacy	128 (43.8)	90 (30.8)	56 (19.2)	18 (6.2)	
Social activities					.15
Low eHealth literacy	422 (68.4)	127 (20.6)	52 (8.4)	16 (2.6)	

Characteristic	Responses to questions				<i>P</i> value ^a
	Not at all, n (%)	Mild, n (%)	Moderate, n (%)	Severe, n (%)	
High eHealth literacy	214 (73.3)	50 (17.1)	22 (7.5)	6 (2.1)	.045
Relationships with coworkers and family					
Low eHealth literacy	431 (69.9)	142 (23)	33 (5.4)	11 (1.8)	
High eHealth literacy	224 (76.7)	48 (16.4)	15 (5.1)	5 (1.7)	

^aExact Wilcoxon signed-rank test.

^beHealth Literacy Scale (eHEALS)<26.

^ceHEALS≥26.

Correlations Between eHealth Literacy and Psychological Measures

Subsequently, we examined the correlations between the eHEALS and the 4 psychological questionnaires used in this

study (Table 4). The Pearson correlation coefficient analyses showed that the eHEALS score was negatively associated with the K6 total score ($r=-0.071$) and the R-UCLA score ($r=-0.086$) and positively associated with the RSES score ($r=0.170$).

Table 4. Correlations among eHealth literacy, premenstrual symptoms, psychological distress, loneliness, and self-esteem.

Characteristics	eHEALS ^a	Total PSQ ^b	Total K6 ^c	R-UCLA ^d	RSES ^e
eHEALS					
<i>r</i>	— ^f	−0.023	−0.071	−0.086	0.170
<i>P</i> value	—	.49	.03	.01	<.001
Total PSQ					
<i>r</i>	−0.023	—	0.447	0.401	−0.358
<i>P</i> value	.49	—	<.001	<.001	<.001
Total K6					
<i>r</i>	−0.071	0.447	—	0.212	−0.285
<i>P</i> value	.03	<.001	—	<.001	<.001
R-UCLA					
<i>r</i>	−0.086	0.401	0.212	—	−0.468
<i>P</i> value	.01	<.001	<.001	—	<.001
RSES					
<i>r</i>	0.170	−0.358	−0.285	−0.468	—
<i>P</i> value	<.001	<.001	<.001	<.001	—

^aeHEALS: eHealth Literacy Scale.

^bPSQ: Premenstrual Symptoms Questionnaire.

^cK6: 6-item Kessler Psychological Distress Scale.

^dR-UCLA: 3-item Revised UCLA Loneliness Scale.

^eRSES: Rosenberg Self-Esteem Scale.

^fNot applicable.

Factors Associated With High eHealth Literacy

We performed multivariable logistic regression analysis to identify the factors significantly associated with high eHealth

literacy (Table 5) and found that high self-esteem was significantly associated with high eHealth literacy.

Table 5. Factors associated with high eHealth literacy (eHealth Literacy Scale $\geq$ 26; R²=0.04).

Factors	Odds ratio (95% CI)	P value
Age (years)	1.026 (0.880-1.197)	.74
BMI (kg/m ²)	0.954 (0.899-1.010)	.11
Age at menarche (years)	0.900 (0.801-1.091)	.07
Menstrual pain intensity	0.976 (0.917-1.040)	.46
Duration of internet use (hours)	1.015 (0.942-1.091)	.70
Sleeping duration (hours)	1.066 (0.929-1.224)	.36
Total PSQ ^a	1.010 (0.980-1.023)	.34
Total K6 ^b	1.001 (0.979-1.023)	.94
R-UCLA ^c	0.996 (0.933-1.062)	.90
RSES ^d	1.076 (1.047-1.107)	<.001

^aPSQ: Premenstrual Symptoms Questionnaire.

^bK6: 6-item Kessler Psychological Distress Scale.

^cR-UCLA: 3-item Revised UCLA Loneliness Scale.

^dRSES: Rosenberg Self-Esteem Scale.

Discussion

Principal Findings

This study examined the eHealth literacy levels of female Japanese high school students and their associations with premenstrual symptoms, psychological distress, loneliness, and self-esteem. The findings highlight the importance of eHealth literacy in adolescent health and suggest that students with higher eHealth literacy tend to experience better mental and reproductive health outcomes. Additionally, this study contributes to the growing body of evidence indicating that digital health literacy plays a significant role in adolescent development and well-being—particularly as reflected in their health-related decision-making processes.

eHealth Literacy Level

The results showed that the mean eHEALS score among participants was 22.8, with 32.1% classified as having high eHealth literacy. Compared with eHEALS scores of 23.6 and 23.5 for university students and the general population in Japan, respectively [10,12], the values obtained in this study appear to be slightly lower. As the data on adolescents overseas in the same age group are limited, performing comparisons becomes challenging. However, among 702 high school students in Serbia (mean age: 16.5 years), the average eHEALS score was 26.0 [14], and among 260 high school students in Brazil (mean age: 15.6 years), the average eHEALS score was 28.1 [15]. Based on these values, Japanese students appear to have a lower eHEALS score. The eHealth literacy of Japanese high school students obtained in this study was lower than that of Japanese university students, adults, and overseas high school students, suggesting a need for further improvement. The results of this study showed that eHEALS scores increased for some items in the second year compared with those in the first year, but there was no difference in scores between the years when assessed as a whole. Therefore, it is difficult to expect an improvement

in eHealth literacy during the natural course of events, and educational interventions may be necessary.

eHealth Literacy, Psychological Well-Being, and Social Connectedness

The negative association between eHealth literacy and loneliness suggests a potential link between digital health literacy and social connectivity. Adolescents with higher eHealth literacy may be more likely to engage with credible online health communities, which could help alleviate feelings of isolation. Additionally, the positive correlation between eHealth literacy and self-esteem indicates that adolescents with stronger digital health literacy may feel more empowered to make informed health decisions, potentially contributing to greater confidence and a more positive self-image.

Association Between eHealth Literacy and Menstrual Symptom Severity

The intensity of menstrual pain and premenstrual symptoms, as assessed using the total PSQ score, did not differ according to the level of eHealth literacy. In terms of premenstrual symptoms, the high eHealth literacy group reported significantly lower levels of “feeling overwhelmed” than the low eHealth literacy group. However, other PMS symptoms, such as mood swings, fatigue, and irritability, were not significantly different between the groups. This suggests that, although higher eHealth literacy may be related to better psychological resilience, its direct relationship with overall premenstrual symptom severity remains unclear. Further investigation is needed to determine whether increased eHealth literacy leads to improved symptom management or whether other factors, such as coping strategies and social support, mediate this relationship. It is possible that individuals with high eHealth literacy possess more accurate knowledge of premenstrual symptoms, which might help them manage symptoms more calmly and avoid feeling overwhelmed, potentially reducing interpersonal difficulties. These findings suggest that higher eHealth literacy may be associated with

improved psychological resilience and coping strategies for menstruation-related issues. Moreover, our results are consistent with those of prior research linking higher health literacy to improved management of menstrual symptoms among working women [22]. However, this study extends these findings by focusing on younger adolescents and exploring the relationship between eHealth literacy and premenstrual symptoms in relation to more detailed symptoms and details of social dysfunction, an area that has received less attention in previous studies.

Correlation Between eHealth Literacy and Psychological Measures

The Pearson correlation analysis revealed a negative association between eHEALS scores and psychological distress (K6: $r=-0.071$; $P=.03$), as well as loneliness (R-UCLA: $r=-0.086$; $P=.01$), whereas a positive association was observed between the eHEALS score and self-esteem (RSES: $r=0.170$; $P<.001$). Although these associations were statistically significant, the effect sizes were small, suggesting that eHealth literacy alone may not be a strong predictor of psychological well-being. Additional factors, such as social relationships and coping mechanisms, should be considered in future research. These results suggest that adolescents with higher eHealth literacy are better equipped to access and interpret mental health information, which may help explain the lower psychological distress and higher self-worth observed in this study. Future research should investigate the causal relationships between eHealth literacy and mental well-being using longitudinal data.

Factors Associated With High eHealth Literacy

The multivariate logistic regression analysis showed that high self-esteem was significantly associated with high eHealth literacy. These results align with previous findings among university students in the United Kingdom [42]. This finding highlights a strong link between self-esteem and eHealth literacy, suggesting that positive self-perception may be an important factor to consider in adolescent eHealth literacy programs.

Limitations and Future Directions

Despite its strengths, this study has some limitations. First, the cross-sectional design precludes causal inference; therefore, longitudinal studies are needed to establish temporal relationships between eHealth literacy and health outcomes. Second, the study sample was limited to female students from 2 public high schools in Sendai, which may limit the generalizability of the findings to other regions and populations. Future studies should include more diverse samples, including students from different geographic and socioeconomic backgrounds. Expanding this research to include male adolescents and individuals from rural areas would provide a more comprehensive understanding of how eHealth literacy affects various demographics. Third, this study used self-administered online questionnaires completed by participants in their home environments. Although this approach offers convenience and accessibility, it has the following limitations: (1) potential response bias, including social desirability bias and misinterpretation of questions resulting from lack of direct supervision; (2) the home setting, which

may have allowed outside influences or noncompliance with instructions, potentially affecting the authenticity of responses; and (3) collecting sensitive psychological and health-related information in an uncontrolled environment, which raises concerns about data integrity. However, discussing reproductive health is often taboo worldwide, especially in Asia, including Japan, with its conservative cultural and religious background [43]. Openly discussing menstrual issues in Japanese school classrooms is very difficult, and online after-school surveys are more likely to reveal the true status of students. Fourth, the study did not evaluate other participant-related factors that may influence eHealth literacy, such as parental education level, family economic status, and participants' medical history [13,44-46]. Although the survey was administered online and completed by students after school, these questions were considered sensitive and difficult to ask. Therefore, it was challenging to include them in a study conducted as part of regular high school activities. Moreover, we acknowledge that other factors—such as access to internet resources, support from family and the community, and a family or maternal history of symptoms or gynecological or endocrine conditions—may also influence eHealth literacy and its related outcomes. Although these variables were not assessed in this study, their potential impact on health-related factors, such as self-esteem, should not be overlooked. These factors may interact with the psychosocial and reproductive health outcomes examined here. We recommend that future studies incorporate these elements to enhance the understanding of adolescent health in relation to eHealth literacy. Fifth, the logistic regression analysis in this study included 10 variables but did not account for potential confounders such as socioeconomic status, academic stress, home environment, and previous digital health education. As mentioned previously, items related to socioeconomic background and home environment were excluded because of ethical and practical limitations inherent in this school-based survey. Although past health education could have been included as a question, there are very few systematic digital health education programs for high school students in Japan. We judged that there was little variation in this factor and thus did not consider it meaningful as an analytical variable. Nevertheless, the omission of these factors may limit the comprehensiveness of the regression analysis, and future studies should include these variables as much as possible, depending on the circumstances.

Furthermore, this study focused only on specific health outcomes—namely, premenstrual symptoms, psychological distress, loneliness, and self-esteem. Future research should investigate additional physical and mental health outcomes to develop a more comprehensive understanding of how eHealth literacy influences adolescent health. Further research should explore potential interventions to enhance eHealth literacy and examine their long-term impact on adolescent health. In fact, a study with secondary school students in Spain demonstrated the educational impact of training sessions on eHealth literacy [47].

Conclusions

In conclusion, this study highlights the importance of eHealth literacy for adolescent well-being. We found that higher eHealth

literacy was associated with lower loneliness, higher self-esteem, and a reduced premenstrual symptom burden, particularly regarding feelings of being overwhelmed. These findings suggest that integrating eHealth literacy education into school programs could potentially benefit adolescents' mental and reproductive health, although this should be confirmed in future studies. Future studies should aim to include more diverse populations—such as male adolescents and students from various socioeconomic backgrounds—and adopt longitudinal

approaches to better understand the causal relationships between eHealth literacy and adolescent health outcomes. Future research should also focus on developing and evaluating interventions aimed at improving eHealth literacy and its long-term impact on adolescent health outcomes. By prioritizing digital health literacy initiatives, educators, health care professionals, and policymakers may help cultivate a more informed and health-conscious future generation.

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Data Availability

The data obtained in this study are available from the corresponding author upon request.

Authors' Contributions

TT, KY, SK, and FI designed the study. TT and FI collected the data. TT analyzed and interpreted the data and wrote the manuscript. KY, SK, and FI revised the manuscript. KY and SK supervised the study and provided conceptual assistance. All the authors read and approved the final version of the manuscript.

Conflicts of Interest

None declared.

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Abbreviations

eHEALS: eHealth Literacy Scale
K6: 6-item Kessler Psychological Distress Scale
NRS: numerical rating scale
OR: odds ratio
PMS: premenstrual syndrome
PSQ: Premenstrual Symptoms Questionnaire
R-UCLA: 3-item Revised UCLA Loneliness Scale
RSES: Rosenberg Self-Esteem Scale
RSES-J: Japanese version of the RSES

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Note

Dietary Patterns and Premenstrual Syndrome: A Cross-Sectional Study

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Summary This study investigated the cross-sectional association between major dietary patterns and premenstrual syndrome (PMS). Participants were 427 women aged 22–50 y who responded to a mail survey in 2022–2023. Dietary patterns were derived using principal component analysis based on consumption of 52 food and beverage items ascertained by a validated self-administered diet history questionnaire. PMS were assessed using the Premenstrual Symptoms Questionnaire. Logistic regression analysis was used to estimate odds ratios of PMS according to tertiles of dietary pattern scores. The prevalence of moderate to severe PMS was 9.6% (41 women). We identified four dietary patterns: vegetable, tomato and fish, Japanese, and alcohol dietary patterns. No dietary pattern was significantly associated with PMS. However, the odds ratios of PMS in the highest tertile of the vegetable dietary pattern (characterized by high intake of vegetables, mushrooms, potatoes, seaweeds, and chicken) tended to be lower compared to the lowest tertile. The multivariable-adjusted odds ratio of PMS for the highest versus lowest tertile of the vegetable dietary pattern score was 0.69 (95% confidence interval 0.30–1.59). None of the dietary patterns were appreciably associated with PMS. The finding of lower odds of PMS among women with a high score for the vegetable dietary pattern deserves further investigation.

Key Words cross-sectional studies, dietary patterns, Japanese, premenstrual syndrome, principal component analysis

Globally, premenstrual syndrome (PMS) was estimated to affect 956 million women in 2019 (1). PMS interferes with personal and social relationships, and work, and reduces quality of life in women due to its physical and psychological symptoms (2). Therefore, it is important to identify the determinants of PMS in preventing or reducing its symptoms and improving quality of life in women. Diet is suggested to be one of the factors contributing to PMS symptoms. For instance, higher intakes of vitamin B1, vitamin B2, calcium, and nonheme iron have been associated with a decreased risk of PMS (3–5). In addition, some studies have reported dietary patterns associated with PMS; however, the evidence remains limited, and findings regarding the association between specific dietary patterns—such as a healthy dietary pattern characterized by a high intake of foods rich in these nutrients—and PMS are inconsistent (6–10). Moreover, dietary patterns vary across countries and ethnic groups (11), and to date, only one Japanese study has examined this association, and it was conducted among university students (10). Therefore, the association between dietary pat-

terns and PMS among Japanese working adult women remains unclear. Here, we examined the cross-sectional association between major dietary patterns and PMS in Japanese women.

Materials and Methods

The study was conducted under a cross-sectional design by a mail survey in 2022–2023 among graduates of Fukuoka Women's University, Fukuoka, Japan. We mailed two types of survey questionnaire (diet and health-related lifestyle questionnaires) which we asked participants to complete and return. The study was approved by the Ethics Committee of Fukuoka Women's University (2021-29). Written informed consent was obtained from all participants.

Of 3,256 graduates who received the questionnaires, 623 participated in the survey (response rate 19%). We excluded participants who did not complete the questionnaire; were pregnant or breast feeding; reported a history of cancer, myocardial infarction, or stroke; or reported extreme total energy intake (exceeding mean $\pm$ 3 standard deviations), leaving 551 participants

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Table 1. Factor loading matrix for major dietary patterns identified by principal component analysis.¹

	Vegetable dietary pattern	Tomato and fish dietary pattern	Japanese dietary pattern	Alcohol dietary pattern
Other root vegetables	0.73			
Cabbage/Chinese cabbage	0.70			
Carrots/pumpkin	0.66	0.34		−0.17
Mushrooms	0.62		0.19	
Green leaves vegetables	0.60	0.26		
Japanese radish/turnip	0.53		0.32	
Potatoes	0.44			
Seaweeds	0.41		0.28	
Chicken	0.41			
Lettuces/cabbage (raw)	0.38	0.34		0.15
Pork/beef	0.33	−0.23	−0.25	
Egg	0.17			
Cola drink/soft drink	−0.27			
100% fruit and vegetable juice	−0.27			
Tomatoes		0.54		
Dried fish/salted fish		0.52		
Japanese confectioneries	−0.21	0.44		
Coffee		0.43		0.22
Other fruit		0.41		
Green tea		0.39		
Lean fish	0.21	0.36	0.24	
Citrus fruit		0.21	0.16	
Persimmons/strawberries/kiwifruit		0.19		
Pickled green leaves vegetables	0.16	0.17		
Rice	−0.17	−0.49	0.57	−0.23
Miso soup			0.51	
Small fish with bones		0.18	0.38	
Natto ²			0.37	0.22
Tofu/atsuage ³	0.18		0.25	
Oily fish	0.15	0.23	0.24	0.21
Liver			0.23	
Other pickles			0.21	
Japanese wheat noodles			−0.21	0.20
Spaghetti and macaroni			−0.26	
Chinese noodles	−0.16	−0.15	−0.31	0.19
Mayonnaise/dressing	0.21	0.15	−0.32	
Bread	−0.20	0.16	−0.34	
Ham/sausage/bacon	0.16	−0.18	−0.34	
Ice cream			−0.38	
Beer				0.72
Shochu		−0.18		0.61
Wine				0.47
Whisky				0.46
Sake				0.41
Squid/octopus/shrimp/shellfish				0.29
Rice crackers/rice cake/okonomiyaki ⁴				−0.20
Western-type confectioneries	−0.30	0.23	−0.19	−0.30

¹ Factor loading $< \pm 0.15$ were not listed for simplicity. Omitted in the table were food items with factor loadings $< \pm 0.15$ for all dietary patterns (canned tuna, milk and yogurt, black tea/oolong tea, buckwheat noodles, and low-fat milk and yogurt).

² Fermented soybeans.

³ Deep-fried tofu.

⁴ Japanese-style pancakes.

for inclusion in the analysis of dietary patterns. For the present analysis, we further excluded participants who had not experienced menstruation for 3 mo or who had reached menopause; reported a history of PMS; took the contraceptive pill or hormonal therapy; had diabe-

tes, hypertension, or dyslipidemia; or had missing data on the variables used. Finally, 427 participants aged 22–50 y were enrolled.

Dietary habits were assessed using a validated brief self-administered diet history questionnaire (BDHQ)

Table 2. Characteristics of participants according to tertiles (T) of dietary pattern scores.

	Vegetable dietary pattern			Tomato and fish dietary pattern			Japanese dietary pattern			Alcohol dietary pattern		
	T1 (low)	T3 (high)	Trend p^1	T1 (low)	T3 (high)	Trend p^1	T1 (low)	T3 (high)	Trend p^1	T1 (low)	T3 (high)	Trend p^1
No. of participants	142	142		142	142		142	142		142	142	
Age (mean $\pm$ SD, y)	31.1 $\pm$ 7.8	32.5 $\pm$ 8.7	0.18	30.8 $\pm$ 7.8	34.3 $\pm$ 8.7	<0.001	32.1 $\pm$ 8.2	32.4 $\pm$ 8.5	0.71	32.9 $\pm$ 8.9	31.6 $\pm$ 7.9	0.21
BMI (mean $\pm$ SD, kg/m ²)	20.3 $\pm$ 2.2	20.2 $\pm$ 2.4	0.85	20.4 $\pm$ 2.6	20.5 $\pm$ 2.4	0.63	20.7 $\pm$ 2.5	20.2 $\pm$ 2.4	0.12	20.1 $\pm$ 2.1	20.6 $\pm$ 2.6	0.060
Employment status (yes, %)	90.1	87.3	0.45	91.5	82.4	0.013	89.4	88.7	0.87	88.0	90.8	0.42
Overtime work ($\geq$ 11 h/mo, %) ²	44.5	37.9	0.31	43.8	38.5	0.39	39.4	34.1	0.32	40.8	41.9	0.80
Age at menarche (<15 y old, %)	90.1	93.0	0.40	93.7	91.5	0.55	95.1	90.1	0.16	88.7	92.3	0.35
Pregnancy or childbirth experience (%)	16.2	32.4	0.004	26.8	32.4	0.27	29.6	23.9	0.27	35.9	19.0	0.002
Leisure-time physical activity (high, %) ³	23.2	30.3	0.14	23.2	31.7	0.09	16.9	31.0	0.007	16.9	29.6	0.019
Sedentary time at work (high, %) ⁴	25.4	29.6	0.41	23.9	24.6	0.94	30.3	19.7	0.036	28.2	23.9	0.39
Supplement use (yes, %)	32.4	29.6	0.56	31.5	35.1	0.51	25.4	30.3	0.50	26.1	31.7	0.43
Total energy intake (kcal/d)	1,454 $\pm$ 445	1,515 $\pm$ 373	0.20	1,491 $\pm$ 370	1,448 $\pm$ 430	0.34	1,481 $\pm$ 411	1,503 $\pm$ 385	0.61	1,562 $\pm$ 417	1,429 $\pm$ 407	0.006

¹ Based on the Mantel-Haenszel chi-squared test for categorical variables and linear regression analysis for continuous variables, assigning median score to the tertiles of each dietary pattern as a continuous variable.

² Denominator was the number of participants who reported yes to employment status.

³ Moderate to vigorous physical activity in leisure time of $\geq$ 12.0 min/d.

⁴ Sedentary time during work and housework or while commuting of $\geq$ 9 h/d.

(12). Dietary intake of 58 food and beverage items, energy and selected nutrients was estimated using an ad hoc computer algorithm for the BDHQ. We performed principal component analysis based on energy-adjusted intakes using the density method for 52 food and beverage items (excluding 6 items that overlapped with others) to identify dietary patterns. The factors were rotated by orthogonal transformation (varimax rotation) to maintain uncorrelated factors and greater interpretability. We determined four factors with eigenvalues, the scree plot, and the interpretability of the factors. Dietary patterns were named based on the food items with the highest loadings on each of the four factors. The scores for each dietary pattern were calculated by summing intakes of food items weighted by their factor loadings.

PMS was assessed using the Premenstrual Symptoms Questionnaire (PSQ) (13). The PSQ enquires about 11 premenstrual symptoms and 3 items to evaluate the degree of social disability caused by these symptoms. We divided participants with premenstrual symptoms into three groups: "no/mild PMS," "moderate to severe PMS," and "premenstrual dysphoric disorder (PMDD)" according to criteria reported previously (13). Since few participants were identified as having PMDD ($n=9$), we combined them together with those having moderate to severe PMS. The PSQ is useful, and its reliability and validity have been fully evaluated (14).

Participants were divided into tertiles of each dietary pattern score. Characteristics of participants across each dietary pattern score were expressed as mean (standard deviation) or percentage. Trend associations were assessed using linear regression analysis, treating the median score in each tertile of dietary pattern score as a continuous variable, and the Mantel-Haenszel chi-squared test. We performed multiple logistic regression to estimate odds ratios of moderate to severe PMS for each dietary pattern, taking the lowest tertile as reference. The first model was adjusted for age and survey month, and the second model was further adjusted for age at menarche, body mass index, pregnancy or childbirth experience, employment status and overtime work, moderate to vigorous physical activity in leisure time, sedentary time during work and housework or while commuting, supplement use, and total energy intake. We did not adjust for smoking status due to the small number of smokers ($n=2$). Trend associations were assessed by assigning a median score to each tertile of each dietary pattern score as a continuous variable. Two-sided p values less than 0.05 were regarded as statistically significant. All analyses were performed using SAS version 9.4 (SAS Institute, Cary, NC, USA).

Results

We identified four dietary patterns by principal component analysis and designated them as follows: 1) a vegetable dietary pattern, characterized by high intake of vegetables, mushrooms, potatoes, seaweeds, and chicken; 2) a tomato and fish dietary pattern, characterized by high intake of tomatoes, dried fish/salted fish, Japanese confectioneries, coffee, and fruits and low in-

Table 3. Odds ratios (95% confidence intervals) of moderate to severe premenstrual syndrome according to tertiles (T) of dietary pattern scores.

	T1 (low)	T2	T3 (high)	<i>p</i> for trend ¹
No. of participants	142	143	142	
Vegetable dietary pattern				
No. of cases	17	13	11	
Adjusted OR (95% CI) ²	1.00 (ref)	0.85 (0.39–1.87)	0.70 (0.31–1.59)	0.39
Adjusted OR (95% CI) ³	1.00 (ref)	0.92 (0.41–2.04)	0.69 (0.30–1.59)	0.38
Tomato and fish dietary pattern				
No. of cases	15	14	12	
Adjusted OR (95% CI) ²	1.00 (ref)	0.93 (0.42–2.05)	1.00 (0.44–2.28)	0.99
Adjusted OR (95% CI) ³	1.00 (ref)	1.00 (0.45–2.24)	0.98 (0.42–2.31)	0.96
Japanese dietary pattern				
No. of cases	17	10	14	
Adjusted OR (95% CI) ²	1.00 (ref)	0.51 (0.22–1.18)	0.82 (0.38–1.77)	0.67
Adjusted OR (95% CI) ³	1.00 (ref)	0.50 (0.21–1.19)	0.85 (0.38–1.90)	0.74
Alcohol dietary pattern				
No. of cases	13	10	18	
Adjusted OR (95% CI) ²	1.00 (ref)	0.63 (0.26–1.52)	1.18 (0.54–2.59)	0.50
Adjusted OR (95% CI) ³	1.00 (ref)	0.59 (0.24–1.47)	1.07 (0.47–2.45)	0.66

CI, confidence interval; OR, odds ratio.

¹ Based on multiple logistic regression analysis, assigning median score to the tertiles of each dietary pattern as a continuous variable.

² Adjusted for age (y) and survey month (March in 2022, November in 2022, or July in 2023).

³ Additionally adjusted for age at menarche (<15 or ≥15 y old), body mass index (kg/m²), pregnancy or childbirth experience (yes or no), employment status and overtime work (unemployed or employed and overtime work <11 or ≥11 h/mo), moderate to vigorous physical activity in leisure time (min/d, tertile), sedentary time during work and housework or while commuting (<5, 5–<9, or ≥9 h/d), supplement use (yes or no), and total energy intake (kcal/d).

take of rice; 3) a Japanese dietary pattern, characterized by high intake of rice, miso soup, small fish with bones, natto, and tofu/atsuage and low intake of ice cream, processed meat, and bread; and 4) an alcohol dietary pattern, characterized by high intake of alcohol and squid/octopus/shrimp/shellfish (Table 1). The proportion of total variance explained by the four dietary patterns was 23.0%.

Characteristics of participants according to tertiles of dietary pattern scores are shown in Table 2. Participants with a higher score for the vegetable dietary pattern were more likely to have experienced pregnancy or childbirth than those with a lower score. Women with a higher score for the tomato and fish dietary pattern were older and had a lower proportion of people in employment compared to those with a lower score. The Japanese dietary pattern score was positively associated with leisure-time physical activity, but inversely associated with sedentary time in work.

We identified 41 women (9.6%) as having moderate to severe PMS. The odds ratios of PMS according to dietary pattern scores are shown in Table 3. The odds ratio of PMS in the highest tertile of the vegetable dietary pattern score was 31% lower than that of the lowest tertile (odds ratio 0.69, 95% confidence interval 0.30–1.59), albeit that the difference was not statistically significant. None of the other dietary patterns were associated with PMS.

Discussion

In this cross-sectional study of Japanese women, we found no significant association between any dietary pattern and PMS. Nevertheless, women with the highest score for the vegetable dietary pattern had lower, albeit not statistically significant, odds of PMS.

Some previous cross-sectional studies of the association between posteriori dietary patterns and PMS have identified dietary patterns similar to the vegetable dietary pattern observed in the present study. In an Iranian study (8), a “healthy dietary pattern” (high intake of dried fruits, nuts, canned fruits, legumes, and vegetables) was inversely associated with PMS among 559 women aged 20–46 y. In contrast, a “lacto-ovo vegetarian diet” (high intake of fresh vegetables, fruits, and dairy products) in China (9), “traditional dietary pattern” (high intake of fish/seafood, vegetables, kimchi, seaweed, potatoes, and fruits) in Korea (7), and “healthy dietary pattern” (high intake of vegetables, fruits, natural juice, olive, tea and coffee, fish, low-fat dairy products, and legumes) in Iran (6) were not associated with PMS. The only study in Japanese women, in 172 university students, identified four dietary patterns, none of which were associated with score of menstrual symptoms during the premenstrual period (10). The extracted dietary patterns vary among countries or study populations. In addition, even for dietary patterns similar to the vegetable dietary pattern in our study, the foods contributing to the pattern differed among region, eth-

nicity, and study populations. These differences might explain the inconsistent findings among studies, at least in part. The present study observed a suggestive decrease in the prevalence of PMS associated with the highest score for the vegetable dietary pattern, showing that a dietary pattern associated with high intake of vegetables, mushrooms, potatoes, seaweeds, and chicken might reduce the risk of PMS.

Previously, B vitamins and minerals such as iron and magnesium have been shown to be associated with depression (15–17), which is one of the symptoms of PMS. Homocysteine, which is involved in the onset of depression, is metabolized through pathways involving vitamin B6, vitamin B12, and folate (18); therefore, deficiencies in these vitamins may be linked to depression. In addition, vitamins and minerals are involved in neurotransmitter synthesis, antioxidant activity, and anti-inflammatory processes (19, 20). Accordingly, the intake of vitamins and minerals is suggested to help prevent PMS and alleviate its symptoms. In fact, the Nurses' Health Study reported that high intakes of vitamin B1, vitamin B2, nonheme iron, and calcium were associated with a decreased risk of PMS (3–5). In the present study, participants in the highest tertile of the vegetable dietary pattern score consumed greater amounts of these nutrients compared to those in the lowest tertile (highest vs. lowest tertile: 2.1 vs. 1.3 mg/1,000 kcal for vitamin B6; 11.8 vs. 8.5 μ g/1,000 kcal for vitamin B12; 567 vs. 348 μ g/1,000 kcal for folate; 768 vs. 563 mg/1,000 kcal for calcium; 12.3 vs. 8.7 mg/1,000 kcal for iron). The inverse association observed between the vegetable dietary pattern and PMS in this study may be explained by the combined effects of these nutrients.

This study had some limitations. First, associations derived from a cross-sectional study do not necessarily indicate causality. Second, the low participation rate raises the possibility of selection bias. Third, as the study participants were graduates of a university, the findings may not be applicable to populations with a different background. Finally, principal component analysis requires subjective decisions in determining the number of factors to retain, in choosing the method of rotation of the initial factors, and in labeling the dietary patterns.

In conclusion, we did not find significant associations between major dietary patterns and PMS among Japanese women. The study might not be large enough to detect the observed association between the vegetable dietary pattern and PMS with statistical significance. Confirmation of the present findings of a suggestive association between the vegetable dietary pattern and PMS warrants larger scaled investigation.

Authorship

Research conception and design: AN; investigation: AN, MN, and MO; the data curation: AN and MN; statistical analysis of the data: AN and MN; interpretation of the data: AN, MN, TT, and MO; manuscript drafting: AN. All authors have read and approved the final man-

uscript.

Disclosure of state of COI

No conflicts of interest to be declared.

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オリゴデンドロサイト発達における Mbp mRNA 輸送の分子機構解明

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Protocol

An Efficient Electroporation Protocol Supporting In Vitro Studies of Oligodendrocyte Biology

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Abstract: Oligodendrocytes form myelin in the central nervous system, and their dysfunction can cause severe neurological symptoms, as large-scale analyses have highlighted numerous gene expression alterations in pathological conditions. Although in vivo functional gene analyses are preferable, they have several limitations, especially in large-scale studies. Therefore, standardized in vitro systems are needed to facilitate efficient and reliable functional analyses of genes identified in such studies. Here, we describe a practical and efficient method for oligodendrocyte precursor cell (OPC) isolation from mouse brains on postnatal day 6–8 and a gene delivery method for the isolated OPCs. By modifying the magnetic-activated cell sorting (MACS) procedure with reduced processing volumes, we simplified OPC isolation, allowing simultaneous handling of multiple samples and improving workflow efficiency. We also optimized electroporation parameters to achieve robust transfection efficiency with minimal cell death. Transfected OPCs are suitable for both monoculture-based differentiation assays and co-culture with dorsal root ganglion (DRG) explants, in which they reliably differentiate into mature oligodendrocytes and myelinate along the axons. This system enables stable and reproducible in vitro analysis of oligodendrocyte function, supports investigations into both intrinsic differentiation and neuron–glia interactions, and provides a powerful platform for oligodendrocyte research with efficient and timely gene manipulation.

Keywords: OPC isolation; magnetic-activated cell sorting (MACS); OPC gene delivery; electroporation; dorsal root ganglion (DRG) explant; co-culture; myelination; neuron–glia interaction



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1. Introduction

Oligodendrocytes (OLs) generate myelin sheaths around neuronal axons in the central nervous system (CNS) and enable fast and accurate signal propagation across neuronal networks. Moreover, evidence has demonstrated that OLs actively contribute to neuronal functions by supplying energy, releasing trophic factors, and regulating action potential conduction through dynamic myelin remodeling [1–3]. Recent studies have suggested that OL activity affects circuit synchronization [4,5], highlighting its broader role in shaping neuronal communication beyond structural support. Accordingly, OL dysfunction and altered gene expression have been increasingly reported in the pathogenesis of various neurodegenerative and neuropsychiatric disorders, including Alzheimer’s disease (AD) [6], Parkinson’s disease (PD) [7,8], and schizophrenia (SZ) [9], suggesting that OL malfunction plays a pivotal role in CNS pathology. While in vivo analyses provide critical insights into OL biology [10–13], they are unsuitable for rapid and systematic evaluation of gene expression data. In this context, efficient and reproducible in vitro systems remain indispensable as they offer a practical

platform to gain initial functional insights into candidate genes for further *in vivo* validation. Thus, the importance of *in vitro* analyses highlights the need to standardize key procedures, such as cell isolation and gene delivery, which make *in vitro* OL analysis more accessible to a broader range of researchers.

For *in vitro* studies of OLs, oligodendrocyte precursor cells (OPCs) are commonly obtained from neonatal mouse brains or generated from pluripotent stem cells, such as iPSCs or NSCs [14–16]. However, these traditional isolation methods are technically demanding and often require highly specialized protocols or extended culture times, making them unsuitable for handling multiple samples in parallel. One of the remaining challenges for *in vitro* OPC studies is the lack of a reliable and easy method for introducing exogenous genes. Although electroporation-based and virus-based approaches have been employed previously, they generally require large input cell numbers or sophisticated handling skills [17–20]. Taken together, these challenges highlight the need to establish more accessible and efficient methods for investigating OL biology, especially in the context of gene function and disease modeling.

To address these issues, we established a scaled-down OPC isolation protocol using a magnetic-activated cell sorting (MACS) strategy targeting platelet-derived growth factor receptor alpha (PDGFR α), a well-characterized OPC-specific cell surface antigen [21,22]. This procedure reduces the buffer usage and simplifies the process, allowing the efficient processing of multiple samples simultaneously. Furthermore, we presented an optimized electroporation-based method that enables efficient gene delivery into primary OPCs, even at low cell densities, while preserving their viability and differentiation capacities. Moreover, this approach is compatible with previously reported neuron–OPC co-culture systems [23], enabling gene manipulation and functional studies of genes of interest involved in axon–glial interactions and myelination. We anticipate that this protocol will serve as a valuable tool for accelerating OL research, particularly in the context of genetically modified models and limited sample availability.

2. Experimental Design

This protocol outlines a method for isolating OPCs from the brains of mice on postnatal days 6–8, followed by efficient gene transfection using an electroporation-based approach. In addition, we describe a co-culture system of OPCs with dorsal root ganglion (DRG) explants, enabling the investigation of neuron–glial interactions, particularly the formation of myelin sheaths along the DRG axons. The experimental flow and estimated time for each procedure are presented in schematic drawings: preparation of a single-cell mixture from postnatal brains (Figure 1), magnetic separation (Figure 2), electroporation steps (Figure 3), and DRG–OPC co-culture procedures (Figure 4).

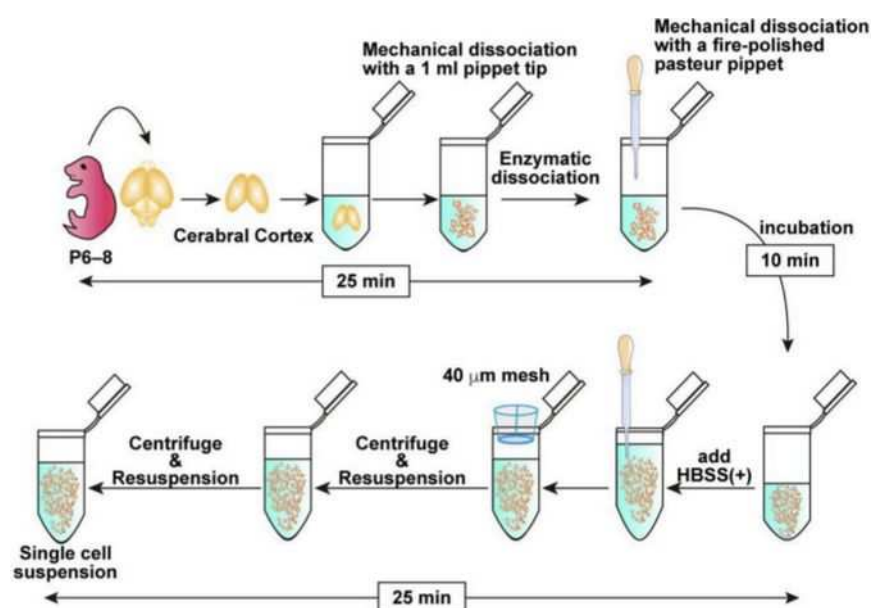


Figure 1. Schematic representation of the workflow from brain dissection to mechanical and enzymatic dissociation, followed by single-cell suspension preparation using a mesh filter with approximate times indicated at each major step, demonstrating that the entire procedure takes approximately 60 min.

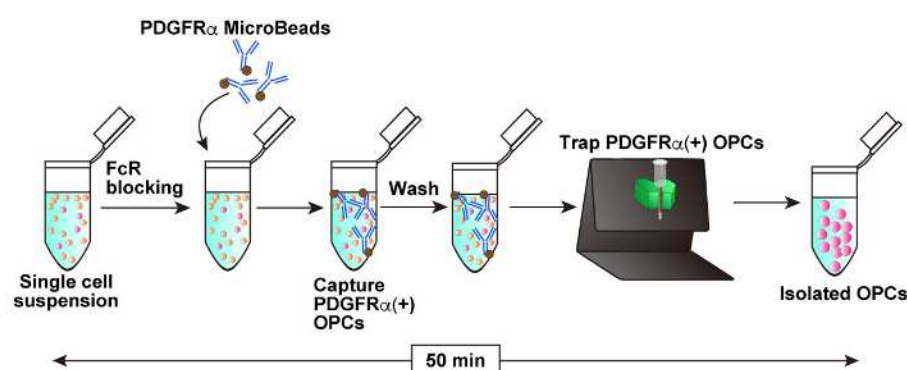


Figure 2. Schematic representation of PDGFR α -expressing OPC isolation following single-cell suspension preparation, illustrating the blocking step, antibody-based OPC capture, and elution of magnetically retained cells. The entire procedure takes approximately 50 min.

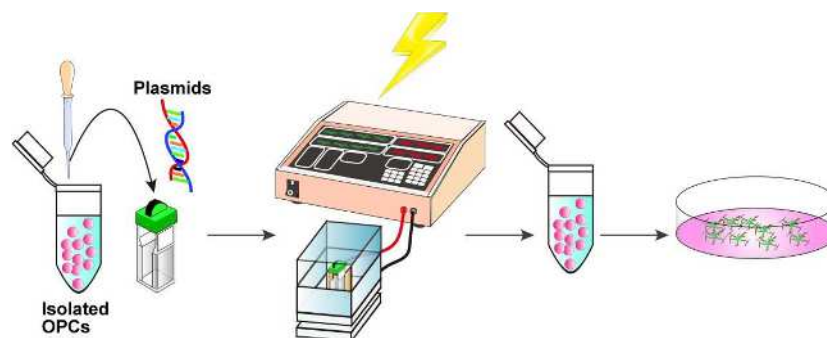


Figure 3. Schematic representation of the electroporation procedure. Isolated OPCs are gently mixed with 10 µg plasmid DNA, and the cell-DNA suspension is transferred into an electroporation cuvette. The suspension is then subjected to electrical pulses. Electroporated cells are immediately transferred to a culture medium.

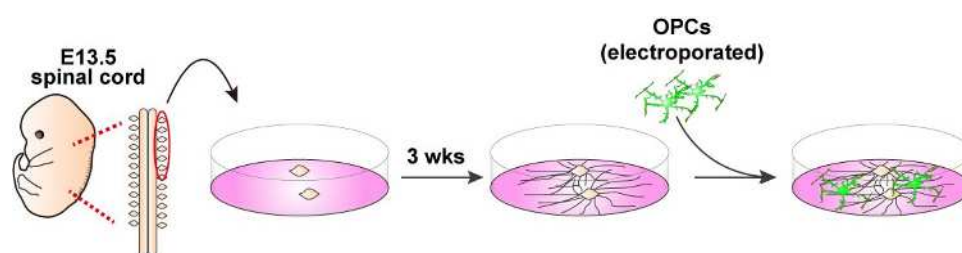


Figure 4. Schematic representation of embryonic DRG explant culture and co-culture with OPCs. DRGs are collected from E13.5 embryonic spinal cords and 3–4 explants are plated on PLO/MG-GFR-coated cover glasses. After 3 weeks of axonal outgrowth, OPCs are added for co-culture. Myelin formation is evaluated by immunostaining after 10 days of differentiation.

2.1. Materials

- DMEM/F12 (ThermoFisher Scientific, Waltham, MA, USA, Cat. no.: 10565018)
- Neurobasal medium (ThermoFisher Scientific, MA, USA, Cat. no.: 12348017)
- Opti-MEM I Reduced Serum Medium (ThermoFisher Scientific, MA, USA, Cat. no.: 31985062)
- Hanks' Balanced Salt Solution without Calcium and Magnesium (HBSS(−)) (ThermoFisher Scientific, MA, USA, Cat. no.: 14175053)
- HBSS with Calcium and Magnesium (HBSS(+)) (ThermoFisher Scientific, MA, USA, Cat. no.: 14025092)
- Poly-L-Ornithine (PLO) (Sigma, St. Louis, MO, USA, Cat. no.: A-004-M)
- Matrigel growth factor reduced (MG-GFR) (Corning, NY, USA, Cat. no.: 354230)
- Trace Elements B (1000×) (Corning, NY, USA, Cat. no.: 25-022-CI)
- B27 supplement (50×) (ThermoFisher Scientific, MA, USA, Cat. no.: 17504044)
- Platelet-derived growth factor (PDGF-AA) (Funakoshi, Tokyo, Japan, Cat. no.: 100-13A)
- Ciliary neurotrophic factor (CNTF) (Funakoshi, Tokyo, Japan, Cat. no.: 450-13)
- Neurotrophin-3 (NT3) (Funakoshi, Tokyo, Japan, Cat. no.: 450-03)
- Triiodothyronine (T3) (Sigma, MO, USA, Cat. no.: T6397)
- Nerve growth factor (NGF) (Merck, Darmstadt, Germany, Cat. no.: 01-125)
- 5-Fluoro-5'-deoxyuridine (FdU) (Sigma, MO, USA, Cat. no.: F8791)
- N-Acetyl-L-cysteine (NAC) (Sigma, MO, USA, Cat. no.: A9165)
- Isoflurane (FUJIFILM, Osaka, Japan, Cat. no.: 099-06571)
- 4% Paraformaldehyde Phosphate Buffer Solution (4% PFA) (FUJIFILM, Osaka, Japan, Cat. no.: 161-20141)
- Neural Tissue Dissociation Kit (P) (NTDK (P)) (Miltenyi Biotec, Bergisch Gladbach, Germany, Cat. no.: 130-092-628)
- CD140a (PDGFR α) MicroBead kit (Miltenyi Biotec, Bergisch Gladbach, Germany, Cat. no.: 130-101-502)
- Dead Cell Makeup Deep Red (DOJINDO, Kumamoto, Japan, Cat. no.: C556)
- AA3-PLP/DM20 (PLP) antibody [24]
- β III-Tubulin antibody (Sigma, MO, USA, Cat. no.: T8578)
- GFP antibody (Abcam, Cambridge, UK, Cat. no.: ab13970)
- Olig2 antibody (Merck, Darmstadt, Germany, Cat. no.: MABN50)
- PiggyBac Transposon Vector (Funakoshi, Tokyo, Japan, Cat. no.: PB530A-2)
- Super PiggyBac Transposase Expression Vector (Funakoshi, Tokyo, Japan, Cat. no.: PB210PA-1)

2.2. Equipment

- MS columns (Miltenyi Biotec, Bergisch Gladbach, Germany, Cat. no.: 130-042-201)

- MiniMACS Separator (Miltenyi Biotec, Bergisch Gladbach, Germany, Cat. no.: 130-042-102)
- MACS MultiStand (Miltenyi Biotec, Bergisch Gladbach, Germany, Cat. no.: 130-042-303)
- Super Electroporator NEPA21 Type II (NEPA21) (NEPAGENE, Chiba, Japan)
- Nepa Electroporation Cuvettes 2 mm Gap (NEPAGENE, Chiba, Japan, Cat. no.: EC-002)
- Cuvette chamber (NEPAGENE, Chiba, Japan, Cat. no.: CU500)
- 13 mm cover glass (MATSUNAMI, Osaka, Japan, Cat. no.: C013001)
- 4 well multi-dish (ThermoFisher Scientific, MA, USA, Cat. no.: 176740)
- Pasteur pipette (AS ONE, Osaka, Japan, Cat. no.: 2-2045-01)
- Mini Cell Strainer II 40 μ m (Funakoshi, Tokyo, Japan, Cat. no.: HT-AMS-04002)
- All-in-one fluorescence microscopes (KEYENCE, Osaka, Japan, Cat. no.: BZ-X810)

3. Procedure

3.1. Preparation of Cover Glasses and NTDK (P) Reagents

1. Place 13 mm cover glasses in each well of 4-wells chamber.
2. Coat with 350 μ L of PLO at 37 °C for overnight.
3. Remove the PLO, rinse with distilled water twice, and dry the cover glasses.
4. Coat the cover glasses with Matrigel GFR (MG-GFR) and incubate in a CO₂ incubator for $\geq$ 30 min.
5. Prepare Enzyme Mix 1 and Enzyme Mix 2 of NTDK (P) as described in the manual (Table 1).
6. Warm 500 μ L Enzyme Mix 1 in a 1.5 mL tube at 37 °C, and keep Enzyme Mix 2 on ice until use.

Table 1. Recipe for enzyme mix of NTDK (P).

Name	Recipe
Enzyme Mix 1	12.5 μ L Enzyme P and 487.5 μ L Buffer X
Enzyme Mix 2	20 μ L Buffer Y and 10 μ L Enzyme A

3.2. Brain Collection and Cell Dissociation (See Figure 1; Estimated Time: 60 min)

1. Anesthetize a postnatal day 6–8 (P6–8) mouse with isoflurane and sterilize its body using 70% ethanol.
Note: P6–8 mice are commonly used in this protocol because their tissue is easier to handle and typically yields sufficient numbers of OPCs. While younger mice can also be used, efficiency tends to decrease in older animals beyond this age.
2. Decapitate and collect the brain in cold HBSS (–).
3. Carefully dissect and collect the cortex. If possible, remove the meninges.
Note: Meningeal removal is optional. Retained meninges do not affect the OPC purification efficiency. While removal of the meninges is preferable when feasible, prolonged efforts to remove them may reduce the overall cell yield. Therefore, if meningeal removal is perceived to be time-consuming, it is advisable to proceed promptly with tissue dissociation to preserve cell viability.
4. Transfer the collected brain tissue into 500 μ L of pre-warmed Enzyme Mix 1 prepared in a 1.5 mL tube. Pipette up and down with a 1 mL pipette tip 7–10 times to mince the tissue into small pieces approximately 1 mm in size.
5. Incubate in the tube for 10 min at 37 °C.
Note: Although rotation is recommended by the manufacturer’s protocol, it is not required under the present conditions.
6. Add 7.5 μ L Enzyme Mix 2, tap the tube to briefly mix the solution, dissociate the tissue mechanically with a fire-polished Pasteur pipette and incubate at 37 °C for an

additional 10 min.

Note: Enzyme Mix 2 must be mixed before mechanical dissociation as the tissue solution tends to be very sticky because of the release of genomic DNA from dead cells. Complete dissociation is not required in this step. Remaining Enzyme Mix 2 can be stored at 4 °C and retains enzymatic activity for at least 1 month.

7. Add 750 µL HBSS (+). Then, further dissociate the tissue into smaller pieces by gently pipetting up and down into a single-cell suspension using a new fire-polished Pasteur pipette.
8. Pass half of the cell suspension through a 40 µm cell strainer into a new 1.5 mL tube to obtain a single-cell suspension. Subsequently, apply 700 µL of HBSS (+) to the strainer to collect any remaining cells on the mesh. Repeat the same procedure with the remaining half of the cell suspension, collecting in a separate 1.5 mL tube.
Note: If approximately 1.0×10^5 cells are sufficient for downstream applications, the second filtration step may be omitted.
9. Centrifuge the cell suspension at $400 \times g$ for 10 min at 4 °C and remove the supernatant carefully.
10. Resuspend the cell pellet in 1 mL of HBSS (+), then repeat the centrifugation under the same condition, and remove the supernatant carefully.

3.3. Magnetic Sorting with CD140a (PDGFR α) Microbead Kit (See Figure 2; Estimated Time: 50 min)

1. Resuspend the cell pellet in 80 µL of PBS containing 0.5% BSA (0.5% BSA/PBS) and add 7 µL of FcR Blocking Reagent supplied in the CD140a (PDGFR α) Microbead kit.
2. Incubate for 10 min at 4 °C.
3. Add 7 µL of CD140a (PDGFR α) MicroBeads and mix gently by pipetting.
4. Incubate for 10 min at 4 °C.
5. Wash the cell suspension by adding 1.2 mL of 0.5% BSA/PBS and centrifuge at $500 \times g$ for 10 min.
6. Remove the supernatant carefully and resuspend the cell pellet in 500 µL of 0.5% BSA/PBS.
7. Proceed to the following magnetic isolation procedure.
8. Place an MS column onto a MACS separator settled on a MACS Multistand.
Note: MS columns are suitable for processing small volumes of cell suspensions. Prepare one MS column for one 1.5 mL cell suspension.
9. Apply 500 µL of 0.5% BSA/PBS to the column and allow the buffer to pass through completely.
10. Apply the whole cell suspensions to the column.
11. Wash the column with 500 µL of 0.5% BSA/PBS 3 times.
12. Remove the column from the MACS separator, apply 1 mL of Opti-MEM to the column, and collect the magnetically retained PDGFR α -positive cells by firmly plunging it into a 1.5 mL tube.
Note: If the isolated cells are cultured directly without electroporation, use the culture medium for cell elution.
13. Count the collected cells. Typically, $1.0\text{--}2.0 \times 10^5$ cells in total would be collected.
Note: This cell number is from one MS column, meaning that $2.0\text{--}4.0 \times 10^5$ cells would be collected from one mouse brain since cell suspensions were split into two 1.5 mL tubes. However, applying cell suspensions from the two split tubes to one MS column does not increase the total cell number for recovery.

- 3.4. *In Vitro Electroporation with a NEPA21 Electroporator (See Figure 3; Estimated Time: 10 min)*
1. Centrifuge the isolated cells at $500\times g$ for 10 min at 4 °C. Discard the supernatant and gently resuspend the cell pellet in Opti-MEM at a density of 5×10^4 cells per 90 μ L.
Critical: At this step, the Opti-MEM should be kept at room temperature because the chilled medium may cause condensation on the cuvette surface, which can reduce the electroporation efficiency.
 2. Add 10 μ g of DNA solution to 90 μ L cell suspension, adjust the total volume to 100 μ L with Opti-MEM, mix gently with a fire-polished Pasteur pipette, and transfer the entire cell–DNA mixture into an electroporation cuvette.
Critical: Ensure that no bubbles are present in the cuvette. If bubbles are present, gently tap the cuvette until they disappear.
 3. Firmly insert the cuvette into the cuvette chamber.
 4. Perform electroporation using the NEPA21 system under the following conditions:
Poring pulse, 150 V; pulse length, 1 ms; interval, 50 ms; number of pulses, 2; decay rate, 10%; pulse direction switch, none.
Transfer pulse, 30 V; pulse length, 50 ms; interval, 50 ms; number of pulses, 5; decay rate, 50%; pulse direction switch, yes.
 5. Immediately transfer the transfected cells into 500 μ L of OPC proliferation medium (Table 2).
Optional: Debris can be removed by passing the electroporated cell solution through a 40 μ m mesh. However, a significant number of cells may be lost due to their retention on the mesh.
 6. Plate the cells onto PLO/MG-GFR-coated 13 mm cover glasses placed in a 4-well multi-dish and culture in the OPC proliferation medium.
 7. For OPC differentiation, replace the OPC proliferation medium with OPC differentiation medium (Table 2) on day 2. Replace half of the medium every other day until analysis.

Table 2. Medium composition for OPC culture.

Name	Composition
OPC proliferation medium	DMEM/F12, B27 supplement, 10 ng/mL PDGF-AA, 10 ng/mL CNTF, 1 ng/mL NT3
OPC differentiation medium	DMEM/F12, B27 supplement, 10 ng/mL CNTF, 30 ng/mL T3, 5 μ g/mL NAC

- 3.5. *DRG Explants and Transfected OPCs Co-Culture (See Figure 4)*
1. Collect embryonic day 13 (E13.5) embryos from a time pregnant female mouse and collect DRGs from cervical and thoracic regions of the spinal cord.
 2. Plate 3–4 explants onto a 13 mm cover glass coated with PLO/MG-GFR in DRG medium (Table 3). Replace half of the medium every other day. During the first week, add FdU at a final concentration of 10 μ M to eliminate proliferating cells, such as glial cells.
 3. Allow neuronal axons to extend radially. Axons are typically ready for co-culture with OPCs after 3 weeks of culture.
 4. For the neuron–glia co-culture, isolate OPCs as described above, transfect them with EGFP-expressing plasmids using electroporation, and plate the cells onto the established DRG explant culture.
Note: For long-term co-culture, OPCs were electroporated with 5 μ g of a PiggyBac Transposon vector encoding EGFP and 5 μ g of a Super PiggyBac Transposase expression vector.

Optional: Debris can be removed by passing the electroporated cell solution through a 40 µm mesh. However, a significant number of cells may be lost due to their retention on the mesh.

5. Culture DRGs and OPCs in co-culture growth medium (Table 3) for 2 days.
6. On day 2, replace the medium with co-culture differentiation medium (Table 3). Continue replacing half of the medium every other day until analysis.

Table 3. Medium composition for DRG culture and co-culture.

Name	Composition
DRG medium	Neurobasal medium, B27supplement, 10 ng/mL NGF
Co-culture growth medium	Neurobasal medium:DMEM/F12 = 1:1 B27 supplement, N2 supplement, 10 ng/mL NGF, 10 ng/mL PDGF-AA, 10 ng/mL CNTF, 1 ng/mL NT3
Co-culture differentiation medium	Neurobasal medium:DMEM/F12 = 1:1 B27 supplement, N2 supplement, 10 ng/mL NGF, 10 ng/mL CNTF, 30 ng/mL T3, 5 µg/mL NAC

3.6. Immunocytochemical Staining and Dead Cell Labeling

3.6.1. Dead Cell Labeling

This step may be omitted if cell viability assessment is not required for subsequent analyses.

1. Prepare 1000× Dead Cell Makeup stock solution according to the product manual.
2. Dilute the 1000× stock solution with the culture medium to prepare a 1× working solution.
3. Replace the culture medium with the 1× Dead Cell Makeup working solution and incubate in a CO₂ incubator for 15 min at 37 °C.
4. Rinse the cells twice with PBS and proceed to the following immunostaining steps.

3.6.2. Immunocytochemical Staining

1. Fix the cells with 4% PFA solution for 20 min at room temperature.
2. Wash the cells 3 times with PBS and permeabilize cells with PBS containing 0.5% Triton X-100 (PBT) for 10 min at room temperature.
3. Proceed to a blocking step by incubating PBT containing 10% normal donkey serum (blocking buffer) for 1 h.
4. Remove the blocking buffer and apply first antibodies against targets such as GFP, PLP, and βIII-tubulin. Incubate overnight at 4 °C.
5. Wash 3 times with PBS and apply fluorophore-conjugated secondary antibodies. Incubate the mixture for 2 h at room temperature.
6. Wash 3 times with PBS and mount the coverslips for microscopic observation.

4. Expected Results

4.1. Efficiency of OPC Isolation and Their Viability

The MACS cell isolation technique, which utilizes cell type specific antigens expressed on cell surfaces, has been widely used to isolate several neural cell types [25–28]. We modified the manufacturer’s protocol to adjust the buffer volume to be suitable for small tissue sizes derived from only one mouse brain, scaling down to approximately one-fourth of the recommended volume. The modified procedure typically yielded approximately 4×10^5 total cells with two MS columns from a postnatal days 6–8 mouse brain (Figure 5A) within 2 h. These isolation efficiencies are comparable to those of a previous study in which an immunopanning strategy was adopted [15]. To confirm purification efficiencies for OPC isolation, collected cells were cultured in the OPC proliferation medium for

2 days, immunostained for PDGFR α , and combined with dead cell staining to assess cell viability. Despite the modest proportion of non-viable cells observed during processing and culturing, this protocol enabled highly efficient OPC enrichment, as $85 \pm 4.5\%$ of the surviving population demonstrates PDGFR α expression (Figure 5B). It is worth noting that PDGFR α expression may have partially decreased below the detection level during the 2-day culture. To further validate the enrichment of oligodendrocyte lineage cells, immunostaining for Olig2, a pan-oligodendrocyte marker, was performed. The results showed that nearly all viable cells ($97.2 \pm 0.98\%$) were Olig2-positive (Figure S1), confirming that MACS isolation using PDGFR α -conjugated beads is highly effective for obtaining an OPC-enriched population.

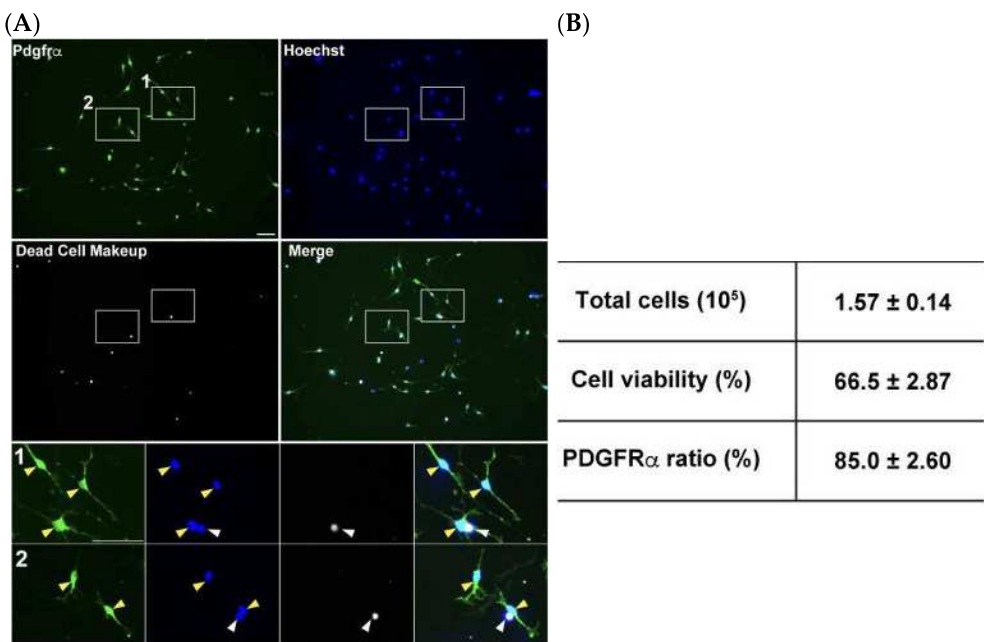


Figure 5. Efficient OPC isolation from the postnatal brain using a single MS column. (A) Collected OPCs were cultured for 2 days in the OPC proliferation medium and stained for PDGFR α (green), Dead cells (gray) and total cells with Hoechst (blue). Magnified images of boxed area are displayed in the small panels. White arrowheads indicate dead cells stained with Dead Cell Makeup stain, and yellow arrowheads indicate PDGFR α -positive cells. (B) Quantification data of total cells collected per MS column, cell viability, and the ratio of PDGFR α -positive cells among viable cells. Scale bars: 50 μ m. Data are presented as the mean $\pm$ SD ($n = 4$).

4.2. Gene Delivery into Isolated OPCs with an Electroporation Strategy

Primary cultured OPCs are one of the most challenging cell types for gene delivery. Owing to the significant advancements made in previous studies, virus-mediated gene delivery has become a reliable method, yielding favorable results in many cases [19,20]. However, this approach has certain limitations, including the time-consuming process of viral vector production and delayed gene expression following infection. Notably, some genes exert their functions exclusively during the early stages of OL differentiation [29]. In such cases, a system that enables rapid gene manipulation is essential to accurately assess gene function. For this purpose, we adopted an electroporation strategy using the NEPA21 electroporator, which allows researchers to manually adjust the electroporation parameters.

To achieve efficient DNA introduction into isolated OPCs, we systematically evaluated electroporation parameters using the NEPA21 apparatus. For optimization, we tested four distinct voltages, such as 200, 150, 125, and 100 V, as the poring pulse and found that 150V yielded a high transfection efficiency, with approximately 40% of viable cells expressing

EGFP after two days in culture (Figure 6A). Both 150 V and 125 V conditions resulted in approximately 50% of total cells being viable after 2 days in culture (Figure 6B). As shown in Figure 5, isolated OPCs exhibited about 66% viability after 2 days without electroporation, indicating that 150 V and 125 V maintained roughly 75% viability relative to the non-electroporated controls. During the optimization steps, we found that although 200 V of the poring pulse yielded a transfection efficiency comparable to that of 150 V among viable cells, it was associated with a substantial number of dead cells across multiple experiments (Figure 6B). In some cases, more than 70% of the cells were dead in 2 days of culture and the reproducibility was poor, as evidenced by the large standard deviation observed across replicates (Figure 6C). These findings indicate that, despite the high efficiency in some trials, 200 V is not a reliable condition. In contrast, electroporation at 100 V resulted in minimal cell death. However, few or no EGFP-positive cells were observed (Figure 6C). Considering the balance between the transfection efficiency and cell viability, we concluded that 150 V was the optimal poring pulse condition for OPC electroporation.

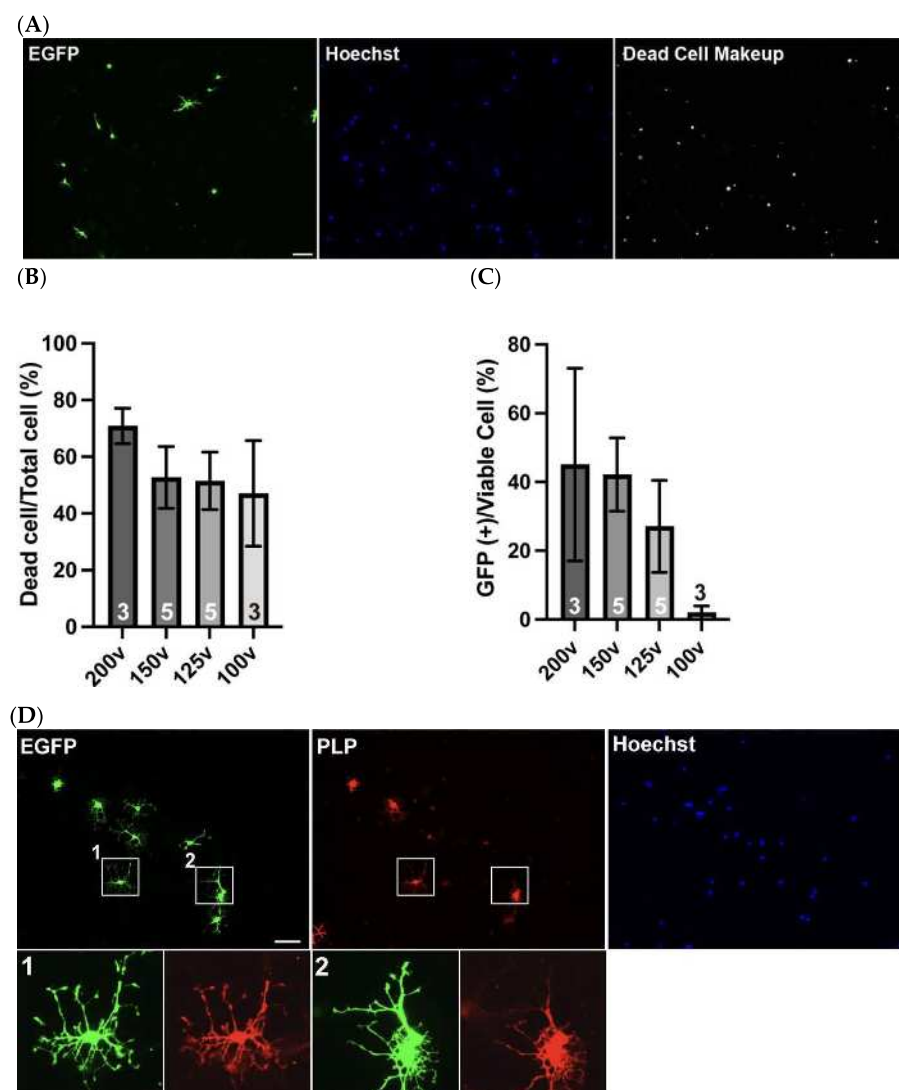


Figure 6. Electroporated cells retain their differentiation potential. **(A)** Transfected cells were cultured for 2 days and immunostained for EGFP (green) and dead cells (gray), with total nuclei labeled by Hoechst (blue). **(B)** Quantification of dead cells among all Hoechst-labeled cells. Voltage values indicate the poring pulse conditions. Higher voltages are associated with reduced cell viability. **(C)** Quantification of EGFP-positive cells among viable cells. Voltage values indicate the poring pulse

conditions. (D) Differentiated OPCs are immunostained for EGFP (green) and PLP (red) after 4 days of differentiation. $36.5 \pm 10.6\%$ of EGFP-expressing cells differentiate into PLP-expressing mature oligodendrocytes. Magnified images of boxed area are displayed in the small panels. Scale bar: 50 μm . Data are presented as mean $\pm$ SD, and n values are shown in columns.

For subsequent analysis, electroporated OPCs were cultured in the OPC differentiation medium for 4 days. OPC differentiation was assessed by immunostaining for PLP, a mature OL marker. The result demonstrated that a substantial number ($36.5 \pm 10.6\%$) of EGFP-positive cells were also positive for PLP, clearly indicating that electroporated cells retained their full capacity for OL differentiation (Figure 6D).

4.3. DRG Neuron–OPC Co-Culture for Neuron–Glial Interaction

Since OPCs differentiate into myelin-forming OLs, their capacity is typically assessed not only using monoculture differentiation assays, but also with co-culture systems with neurons [30–32]. To enable this evaluation, we employed a co-culture system using isolated and electroporated OPCs together with DRG neurons based on a previously established DRG explant culture model [23].

Three to four DRG explants derived from E13.5 spinal cords were first cultured for 3 weeks to allow neurite extension. Subsequently, isolated and electroporated OPCs were plated onto the established DRG explants to initiate co-culture (Figure 7A). Immunostaining analyses were conducted 4 and 10 days after the induction of OPC differentiation to detect EGFP-expressing cells. Although many EGFP-positive cells were observed on day 4, their number was markedly reduced by day 10 (Figure 7B,C). Nevertheless, a substantial number of PLP-positive signals were still detected at this later time point, indicating the presence of differentiated OLs that were no longer expressing detectable levels of EGFP. This suggests that the initially observed EGFP fluorescence may have diminished over time, potentially due to reduced transgene expression or degradation. As such, the reduced number of EGFP-positive cells on day 10 did not allow for statistically meaningful quantifications.

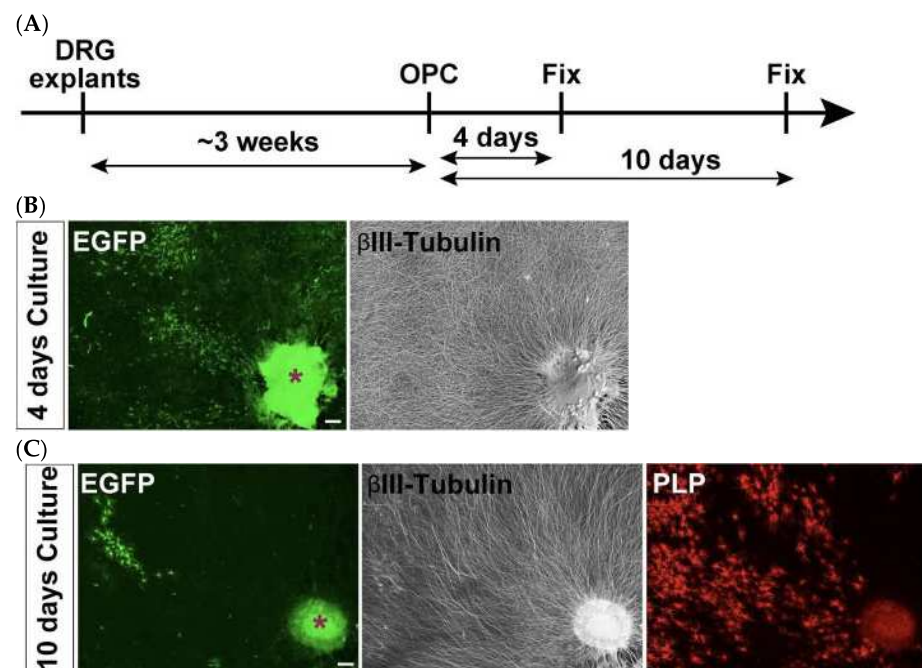


Figure 7. Transient EGFP expression supports short-term co-culture but limits long-term myelination assessment. (A) Schematic representation of the co-culture timeline. (B) Co-cultures were immunostained for EGFP (green) and β III-tubulin (gray) after 4 days of differentiation. A substantial

number of EGFP-positive cells are observed. (C) Co-cultures were immunostained for EGFP (green), β III-tubulin (gray), and PLP (red) after 10 days of differentiation. Very few EGFP-positive cells remain. Note that, despite the loss of EGFP-positive cells, PLP-positive mature OLs are abundant. Asterisks indicate DRG explants. Scale bars: 200 μ m.

To overcome this limitation, we adopted a permanent labeling strategy, using the PiggyBac Transposon system [33,34] (Figure 8A), which enables efficient genomic integration of the transfected DNA and stable expression of the inserted genes. For electroporation, 5 μ g each of the PiggyBac Transposon vector encoding EGFP and Super PiggyBac Transposase expression vectors was used. The OPCs were then cultured for 10 days under the differentiation condition. This approach led to a marked improvement, as a substantial number of EGFP-expressing cells remained even after 10 days of differentiation (Figure 8B). Furthermore, immunostaining for the OL differentiation marker PLP revealed that a substantial number ($31.0 \pm 2.73\%$) of EGFP-expressing cells are positive for PLP and exhibit close association with axons of DRG explants (Figure 8B), demonstrating that the PiggyBac Transposon system is a powerful tool for long-term gene manipulation, enabling efficient investigation of OL myelination in this co-culture.

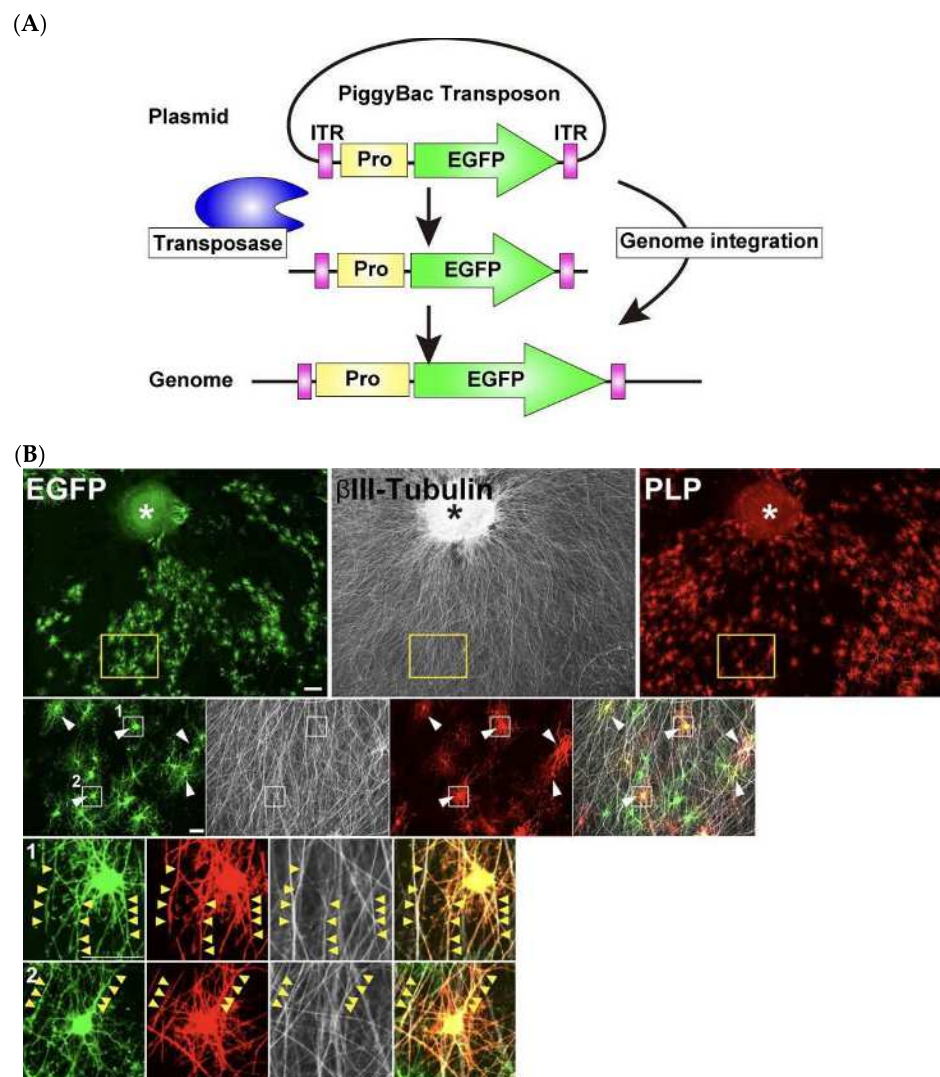


Figure 8. PiggyBac Transposon system enables long-term observation of exogenous gene expression in OLs within DRG–OPC co-cultures. (A) Schematic representation of the PiggyBac transposon system.

DNA sequences flanked by two ITRs are excised from plasmids and integrated into the mouse genome using PiggyBac Transposase. (B) Co-cultures were immunostained for EGFP (green), β III-tubulin (gray), and PLP (red) after 10 days of differentiation. Magnified images of the yellow boxed area are displayed in the small panel. A significant number of EGFP-expressing cells were also observed. Among them, $31.0 \pm 2.73\%$ of EGFP-positive cells are PLP positive, extending their processes and contacting with neuronal axons (white arrowheads). Furthermore, magnified images of the white boxed area are displayed in the smaller panels. Yellow arrowheads indicate EGFP-PLP double positive OL processes extending along DRG neuronal axons. Asterisks indicate the DRG explants. Scale bars: large panels, 200 μ m; small panels, 50 μ m. Abbreviations: ITR, inverted terminal repeat; Pro, promoter.

5. Discussion and Conclusions

We described a protocol for the efficient isolation and genetic manipulation of oligodendrocyte precursor cells (OPCs), which overcomes several limitations of existing methods and provides a flexible platform for studying oligodendrocyte biology. This protocol is composed of three core components that collectively support a wide range of experimental applications.

First, we modified a MACS-based OPC isolation method that uses a reduced buffer volume, enabling the simultaneous processing of multiple samples derived from individual mouse brains. This approach allows researchers to isolate OPCs from single postnatal mice, facilitating parallel comparison across multiple experimental conditions. For instance, different pharmacological compounds can be administered to individual animals and analyzed simultaneously, for example, using omics approaches such as transcriptomics or proteomics. This feature is particularly advantageous in developmental studies, where it is often difficult to acquire a cohort of age-matched animals at the same time. Furthermore, the ability to process multiple samples in parallel makes it feasible to isolate and analyze OPCs from different genotypes of genetically modified mice under identical conditions, which is an important advantage for genotype-phenotype comparisons that were previously constrained by throughput limitations.

Second, we established an electroporation-based gene delivery protocol designed for freshly isolated OPCs. Previous protocols often relied on preset or undisclosed electrical parameters and required proprietary buffers [18], making it difficult to reproduce or customize electroporation conditions. In contrast, our method offers transparent and adjustable pulse settings using a commonly available system, allowing researchers to optimize electroporation conditions for their specific needs. Notably, our protocol includes newly established parameters that are effective even with limited cell numbers, which is particularly advantageous when working with primary cultures where large quantities of cells are difficult to obtain. This flexibility enhances reproducibility and accessibility across laboratories. Importantly, the detailed information provided here enables researchers to introduce gene constructs without the need for viral vectors, thereby reducing biosafety concerns and technical complexity.

Third, by integrating the DRG–OPC co-culture system with a PiggyBac transposon-based gene delivery system [33], we demonstrate that long-term gene expression can be maintained in differentiating oligodendrocytes. This feature is particularly beneficial for studying late-stage events such as myelin sheath formation or axon–glia interactions, which are often missed when transient gene expression systems are used. Furthermore, our system is expected to be compatible with inducible gene expression methods, such as the tetracycline-inducible (Tet-on/off) system [35]. This would enable researchers to activate gene expression at specific time points, such as during the late phase of differentiation or after myelination has occurred. Such temporal control is critical for dissecting gene functions involved in demyelination or axonal support. Moreover, because gene delivery

occurs at the time of OPC isolation, gene expression is restricted to the OL lineage, without affecting neurons in the co-culture. This enhances the specificity and interpretability of downstream analyses.

Taken together, this protocol offers a robust and versatile platform for investigating gene functions in OL development and myelination. We believe this method will be a valuable resource for researchers seeking to dissect the molecular underpinnings of OL biology.

6. Troubleshooting

The most common pitfalls and their recommended solutions are summarized as follows:

- **Low cell recovery after tissue dissociation:**
This often results from excessive pipetting force when trying to obtain a single-cell suspension. Other contributing factors include the generation of bubbles and the use of pipette tips with sharp edges. To address this, gentle pipetting is recommended, and fire-polished glass pipettes should be used to minimize shear stress and mechanical damage.
- **Clogging of the MS column:**
Failure to properly resuspend the cell pellet after centrifugation can lead to column clogging. Ensure the suspension is homogeneous with no visible clumps before loading onto the MS column.
- **Low transfection efficiency during electroporation:**
A common cause is the presence of air bubbles in the electroporation cuvette. Use a glass pipette to slowly and carefully apply the cell suspension to avoid introducing bubbles. If bubbles are present, they should be removed either by gently tapping the cuvette or by using a glass pipette to aspirate them.
- **High cell mortality after electroporation:**
Cells should be promptly transferred to culture medium after electroporation to improve viability. Moreover, plasmid DNA should be purified using endotoxin-free kits to minimize toxicity.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/mps8030064/s1>, Figure S1. Most of the isolated cells are positive for Olig2. (A) OPCs collected by MACS were cultured for 2 days in the OPC proliferation medium and stained for Olig2 (green), Dead cells (gray), and total cells with Hoechst (blue). Magnified images of boxed area are displayed in the small panels. White arrowheads indicate dead cells stained with Dead Cell Makeup stain and yellow arrowheads indicate Olig2-positive cells. Quantification of Olig2-positive cells among viable cells is displayed within the Olig2 panel. Scale bars: 50 μ m. Data are presented as the mean $\pm$ SD ($n = 4$).

Author Contributions: Y.I. designed and performed experiments, analyzed and interpreted the data, and wrote the manuscript. S.S. and S.M. provided comments and edited the manuscript. S.M. supervised the project. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data that support the results are available from the corresponding author upon reasonable request.

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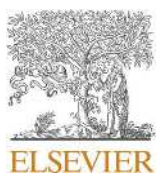
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Research paper

Nuclear distribution element 1 is involved in cell proliferation in the ventricular–subventricular zone during early postnatal brain development

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ABSTRACT

The ventricular–subventricular zone (V-SVZ) is a specialized neurogenic niche localized on the walls of the lateral ventricles in the postnatal mammalian brain. In the V-SVZ, neural stem cells (NSCs) generate transit-amplifying neural progenitor cells, which produce neuroblasts. V-SVZ-derived neuroblasts have proliferative potential and migrate through the rostral migratory stream (RMS) toward the olfactory bulb (OB), where they differentiate into OB interneurons. However, the manner in which V-SVZ cell proliferation and OB neurogenesis are regulated during early postnatal brain development, a transition period from embryonic to adult stage, remains unclear. We focused on the role of the dynein cofactor nuclear distribution element 1 (NDE1) in early postnatal brain development. We found that *Nde1* mRNA is expressed in NSCs, transit-amplifying neural progenitor cells, and neuroblasts in the V-SVZ in mice at early postnatal stage, and this expression was detected in migrating neuroblasts in the RMS and OB. *Nde1* knockdown via the *in vivo* postnatal electroporation of *Nde1* short hairpin RNA (shRNA) in a neonatal mouse brain decreased V-SVZ cell proliferation, which was rescued by overexpression of wild-type NDE1-shRNA resistant but not by overexpression of dynein-binding region-deficient NDE1-shRNA resistant. The V-SVZ cell proliferation defect was followed by impaired proliferation of V-SVZ-derived neuroblasts, thus leading to decreased generation of neuroblasts, which resulted in the reduction of newly generated OB neurons. Therefore, these results indicate that NDE1 is involved in the proliferation of NSCs and neural progenitors in the developing V-SVZ, suggesting that NDE1 plays an important role in early postnatal V-SVZ-derived neurogenesis.

1. Introduction

The ventricular–subventricular zone (V-SVZ) is the largest neurogenic regions of the postnatal brain. V-SVZ is located on the wall of the lateral ventricles and harbors neural stem cells (NSCs) which are multipotent cells that self-renew and produce neurons and glia (Fuentesalba et al., 2012). In the V-SVZ, NSCs generate transit-amplifying neural progenitor cells, which produce new immature neurons, referred to as neuroblasts (Lim and Alvarez-Buylla, 2016). These neuroblasts have proliferative potential and migrate along the rostral migratory stream (RMS) toward the olfactory bulb (OB), where they differentiate into OB interneurons (Lim and Alvarez-Buylla, 2016). Developmentally, postnatal NSCs in the V-SVZ are derived from the embryonic NSCs, radial glial cells (Merkle et al., 2004). During embryogenesis, radial glial cells actively contribute to neurogenesis; however, a subpopulation of radial

glial cells transiently slows cell division, enters a quiescent state, and remains quiescent until reactivation in the postnatal brain (Fuentesalba et al., 2015; Furutachi et al., 2015). In the first few weeks after birth, the embryonic ventricular zone, containing radial glial cells, converts into the postnatal V-SVZ niche (Tramontin et al., 2003). During this process, radial glial cells disappear, retracting their long radial processes and transforming into the postnatal NSCs, ependymal cells and parenchymal astrocytes (Merkle et al., 2004; Fuentesalba et al., 2015; Furutachi et al., 2015; Ortiz-Álvarez et al., 2019). Thereafter, the more quiescent adult V-SVZ is established, where adult NSCs, which usually maintain a non-proliferative state with potential to repopulate as necessary, and ependymal cells organize into a pinwheel architecture (Mirzadeh et al., 2008; Chaker et al., 2016). The V-SVZ has received considerable attention because of its role as a source of neuronal progenitors for repair, particularly following injury and disease in adulthood. However,

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little is known about the regulation of V-SVZ cell proliferation and neurogenesis during early postnatal stage, a transition period between the embryonic and adult stages. Studies have reported that the human neonatal V-SVZ harbors remarkable neurogenic activity (Sanai et al., 2011; Paredes et al., 2016), suggesting that the neonatal V-SVZ has the potential for neuronal replenishment and endogenous regeneration after neonatal brain injury. Therefore, further investigations should be conducted on the regulatory mechanisms of V-SVZ cell proliferation and neurogenesis during early postnatal stage to understand the pathological conditions and design treatment strategies for neonatal brain injury, such as hypoxia–ischemia.

Nuclear distribution element 1 (NDE1) is an evolutionarily conserved protein that is essential for cortical neurodevelopment (Kitagawa et al., 2000; Sasaki et al., 2000; Feng et al., 2000). *Nde1* and *NDE1* have been identified as causal genes for microcephaly in rodents and in clinical case studies of human patients with *NDE1* mutations, respectively (Feng and Walsh, 2004; Pawlusz et al., 2008; Alkuraya et al., 2011; Bakircioglu et al., 2011; Guven et al., 2012; Paciorkowski et al., 2013; Lipka et al., 2013; Doobin et al., 2016). NDE1 is involved in the regulation of cytoplasmic dynein through its direct physical interaction with dynein and the dynein cofactor LIS1, which is encoded by the lissencephaly-associated gene *LIS1* (Stehman et al., 2007; McKenney et al., 2010; Bradshaw et al., 2013; Monda and Cheeseman, 2018; Garrott et al., 2022; Zhao et al., 2023). *Nde1* is highly expressed by cortical progenitors in the cortical ventricular zone during early embryonic development (Feng et al., 2000), and *Nde1* knockout mice display microcephaly-related malformations during cortical development (Feng and Walsh, 2004). They exhibit neurogenesis impairment, which is attributed to dysregulated mitosis in the proliferating of cortical progenitor cells accompanied by impaired neuronal fate specification and neuronal migration (Feng and Walsh, 2004). Studies on NDE1 have focused primarily on embryonic brain development. Although NDE1 was reported to be expressed in the V-SVZ of the perinatal and postnatal brain (Feng and Walsh, 2004; Pei et al., 2014), little attention has been paid to NDE1 function during postnatal brain development. In this study, using *in vivo* postnatal electroporation to introduce *Nde1* short hairpin RNA (shRNA) into neonatal mouse brains, we investigated the role of NDE1 in regulating cell proliferation within the V-SVZ during early postnatal brain development.

2. Materials and methods

2.1. Animals

Jcl:ICR mice of both sexes were purchased from CLEA Japan, Inc. (Tokyo, Japan) and used for *in situ* hybridization (ISH), combined ISH and immunohistochemistry (ISH-IHC), and *in vivo* postnatal electroporation studies. Mice were allowed to mate; the morning after plug detection was defined as embryonic day 0.5 (E0.5). Mouse pups at each postnatal developmental stage were used in the experiments. All animal experiments were performed in accordance with the protocols approved by the Animal Ethics Committee of Kindai University.

2.2. Antibodies

The primary antibodies used in this study were rabbit anti-Sox2 (1:500; Cat# AB5603; MilliporeSigma, Burlington, MA, USA), rabbit anti-Mash1 (1:300; Cat# ab211327; Abcam, Cambridge, UK), rabbit anti-NDE1 (1:1000; Cat# 10233-1-AP; Proteintech, Rosemont, IL, USA), rabbit anti-BLBP (1:100; Cat# ABN14; MilliporeSigma), rabbit anti-DCX (1:100; Cat# ab18723; Abcam), chicken anti-GFP (1:1000; Cat# ab13970; Abcam), rat anti-BrdU (1:700; Cat# ab6326; Abcam), rabbit anti-PH3 (1:1000; Cat# 06-570; MilliporeSigma), mouse anti-NeuN (1:400; Cat# MAB377; MilliporeSigma), and mouse anti-GAPDH (1:1000; Cat# sc-32233; Santa Cruz Biotechnology, Dallas, TX, USA). For immunohistochemistry, the following goat secondary antibodies

were used: AlexaFluor Plus 488-conjugated anti-chicken IgY (1:1000; Cat# A32931; Thermo Fisher Scientific, Waltham, MA, USA), AlexaFluor Plus 594-conjugated anti-rabbit IgG (1:1000; Cat# A32740; Thermo Fisher Scientific), AlexaFluor Plus 594-conjugated anti-mouse IgG (1:1000; Cat# A32742; Thermo Fisher Scientific), and AlexaFluor 568-conjugated anti-rat IgG (1:1000; Cat# A-11077; Thermo Fisher Scientific). The nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (1:5000; Cat# 62248; Thermo Fisher Scientific). For western blotting, horseradish peroxidase-conjugated anti-rabbit IgG (1:5000; Cat# 7074; Cell Signaling Technology, Danvers, MA, USA) or anti-mouse IgG (1:5000; Cat# 7076; Cell Signaling Technology) was used as the secondary antibody.

2.3. Plasmids

For *Nde1* knockdown (KD) experiments, we constructed a plasmid vector expressing shRNA targeting *Nde1*. The target sequence for mouse *Nde1* was 5'-GCGTTTGAATCAAGCCATTGA-3', as previously reported (Doobin et al., 2016). The control shRNA sequence was 5'-GCCAGCCGAATTAGCGCAGCTAC-3'. Annealed shRNAs were ligated into a pBasi-mU6 Pur vector (Takara Bio Inc., Shiga, Japan). For GFP or NDE1 wild-type (WT) expression vectors, GFP or full-length WT mouse *Nde1* was amplified from pEGFP-C3 or pGEM-T/*Nde1*, respectively, and inserted into the pCAGGS vector using In-Fusion cloning (Takara Bio Inc.) (Shimizu et al., 2018). NDE1 shRNA-resistant (SR) or dynein-binding region deficient-NDE1 SR (NDE1 SR Δ BR) expression vectors were generated using the KOD Plus Mutagenesis Kit (Cat# SMK-101; Toyobo Co., Ltd., Osaka, Japan) with the following forward/reverse primers: 5'-CAGGCTATCGAAAGAAATGCCTTCCTAGAGAGT-3'/5'-GTTTGTCTTTGCTCAAAGTCTTCAGGGACAT-3' or 5'-GCGCA-GACGAAAGCCATTAAAGACCAACTG-3'/5'-CATGGTGGATCGGATCCAGATCTATCGATG-3' using pCAGGS/NDE1 WT or pCAGGS/NDE1 SR as a template, respectively, according to the manufacturer's instructions (Shimizu et al., 2018).

2.4. Cell culture and transfection

HEK293T and N2a cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. HEK293T and N2a cells were transfected using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's instructions.

2.5. Western blot

To analyze NDE1 expression, brains were obtained from P1, P7, P14, and P21 mice, and the V-SVZ at each stage was isolated as previously described (Mirzadeh et al., 2010). To analyze NDE1 expression *in vitro*, HEK293T and N2a cells were collected two days after transfection. Proteins were extracted in TNE + 1% NP40 buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, and 1% NP-40) in the presence of protease inhibitors (Roche, Basel, Switzerland) using sonication. Lysates were boiled for 5 min in sodium dodecyl sulfate sample buffer, and the samples were subjected to western blot using anti-NDE1 and anti-GAPDH antibodies as described previously (Shimizu et al., 2014). Immunoreactivity was detected by chemiluminescence using an ECL Prime Kit (GE Healthcare, Chicago, IL, USA). Densitometric quantification was performed using ImageJ software with GAPDH as a loading control.

2.6. RNA ISH and double fluorescence ISH-IHC analysis

Nde1 cDNA fragments containing the 3'-UTR sequence of mouse *Nde1* were obtained via polymerase chain reaction using the primers 5'-TCCTTACTTTATAGTCGGCTTTTGTGCGCT-3'/5'-AGGACGCA-CATCTATTGAGCAGGGATTCT-3'. These fragments were used as templates for probe synthesis. Digoxigenin-labeled RNA probe synthesis and hybridization were performed as previously described (Shimizu et al.,

2014). V-SVZ *Nde1* messenger RNA (mRNA) intensities were quantified using ImageJ software. To detect *Nde1* mRNA, double-fluorescence ISH-IHC was performed as previously reported (Zhao et al., 2013). The probe signals of *Nde1* were amplified using the TSA Plus Cyanine 3 detection kit (Akoya Biosciences, Marlborough, MA, USA).

2.7. *In vivo* postnatal electroporation and BrdU labeling

In vivo postnatal electroporation was performed as previously described (Takahashi et al., 2015) with some modifications. In brief, P1 mouse pups were anesthetized on ice for 2 min before the injection procedure. Approximately 1 μ L of plasmid solution at 3–4.5 μ g/ μ L was injected manually into the right lateral ventricle. Five electrical pulses at 95 V for 50 ms at 950 ms intervals were then applied, with 5-mm tweezer electrodes positioned on each side of the pup's head using an electroporator (Cat# CUY-21SC; Nepa Gene Co., Chiba, Japan). After electroporation, pups were recovered on a heating pad and returned to their mothers. After electroporation, P3 or P5 mice were intraperitoneally injected with BrdU (Sigma-Aldrich, St. Louis, MO, USA; 50 mg/kg) and then sacrificed 1 h after BrdU injection.

2.8. Immunohistochemistry

At P3, P5, P8, and P29 mice were transcardially perfused with 4% paraformaldehyde. Their brains were removed and immersion-fixed in 4% paraformaldehyde at 4 °C overnight. After post-fixation, brains were suspended in 30% sucrose overnight at 4 °C. After the brains were frozen by dry ice powder, 30–50- μ m-thick coronal or sagittal sections were prepared at –20 °C using a cryostat (CM3050 S; Leica Biosystems, Wetzlar, Germany). All free-floating sections were washed with phosphate-buffered saline (PBS) at room temperature and then incubated in blocking solution (5% bovine serum albumin and 0.3% Triton X-100/PBS) for 1 h before overnight incubation with blocking solution containing primary antibodies at 4 °C. For GFP and BrdU double immunostaining, 30–50- μ m coronal or sagittal free-floating tissue sections were incubated with chicken anti-GFP antibodies at 4 °C overnight before BrdU staining. After washing in PBS, tissue sections were fixed with 4% paraformaldehyde for 10 min. After washing in PBS, tissue sections were treated with 2 N HCl for 15 min at 37 °C and incubated in a 0.1 M boric acid solution for 10 min at room temperature. After incubating with blocking solution for 1 h, sections were incubated with blocking solution containing anti-BrdU antibody diluted at a 1:700 at 4 °C overnight. After washing in PBS, sections were incubated with secondary antibodies (Thermo Fisher Scientific) in blocking solution applied at a 1:1000 dilution for 2 h at room temperature. For nuclear DNA visualization, the sections were stained with 1 μ g/mL DAPI.

2.9. Quantitative analysis

To quantify GFP staining in the V-SVZ, RMS, and OB, these regions were manually identified via DAPI counterstaining patterns. For imaging of whole sagittal brain sections, a fluorescence microscope (BZ-X700/BZ-X810; Keyence, Osaka, Japan) equipped with an advanced observation module (BZH4XD; Keyence), a Plan Apochromat 10 $\times$ objective (NA0.45, BZ-PA10; Keyence) and a Plan Apochromat 20 $\times$ objective (NA 0.75, BZ-PA20; Keyence) was used to capture tiled images. The number of GFP-positive (GFP+) cells was counted for every second section in the brain region encompassing the V-SVZ, RMS, and OB using ImageJ software (Figs. 3E, 4C, E, and F). Z-stack images were captured using a confocal microscope (Nikon, AX R with NSPARC) equipped with a Plan Apochromat Lambda D 40 $\times$ objective lens (NA 0.95; Nikon, Tokyo). The reconstructions were created from z-stack images taken at 2- μ m intervals. The numbers of GFP+ cells, GFP + BrdU + cells, and GFP + PH3+ cells were counted in every second section in the region encompassing the V-SVZ (Fig. 2G and H), and the V-SVZ, RMS, and OB (Fig. 3F and G) using ImageJ software.

2.10. Statistical analyses

All statistical analysis was done using GraphPad Prism software (version 10; GraphPad Software, Boston, MA, USA). All data are expressed as mean $\pm$ standard deviation. For multiple comparisons, one-way or two-way analysis of variance tests were performed followed by Tukey's post-hoc test for evaluation of pairwise group differences. The threshold for statistical significance was set at $P < 0.05$.

3. Results

3.1. *Nde1* was expressed by NSCs, transit-amplifying neural progenitors, and neuroblasts in the V-SVZ during early postnatal brain development

Studies have reported that NDE1 is expressed in the V-SVZ in perinatal and postnatal mouse brains (Feng and Walsh, 2004; Pei et al., 2014). However, a detailed analysis of NDE1 expression has not been performed in the early postnatal brain. To examine the expression of *Nde1* mRNA during the early postnatal stages, we performed ISH using an antisense probe against *Nde1* mRNA in P1, P7, P14, and P21 mice. *Nde1* mRNA was expressed in the V-SVZ of mice between P0 and P21, and its expression was detected throughout the V-SVZ, including the dorsal, lateral, and ventral regions (Fig. 1A). Developmentally, *Nde1* mRNA expression in the V-SVZ peaked at P1 (Fig. 1A and B). Thereafter, the expression of *Nde1* in the V-SVZ decreased, and the number of cells expressing *Nde1* mRNA in the V-SVZ decreased (Fig. 1A and B). We investigated whether *Nde1* mRNA was expressed in the V-SVZ–RMS–OB pathway, by which newborn neuroblasts from the V-SVZ migrate tangentially to the OB, using the sagittal sections of the brains harvested at P1, P7, P14, and P21. At P1, many cells expressing *Nde1* mRNA were found in the RMS and OB (Fig. 1C). Thereafter, cells expressing *Nde1* in the RMS and OB decreased (Fig. 1C and D). To identify *Nde1* mRNA expression, we performed double fluorescence ISH-IHC analysis using the NSC marker BLBP, the NSC/transit-amplifying neural progenitor marker Sox2, the transit-amplifying neural progenitor marker Mash1, and the neuroblast marker DCX. At P5, *Nde1* is expressed by BLBP-, Sox2-, Mash1-, and DCX- immunopositive cells in the V-SVZ (Fig. 1E) and also expressed by DCX-immunopositive cells in the RMS and OB (Fig. 1E). Furthermore, western blot showed that endogenous NDE1 protein expression in the V-SVZ was gradually downregulated from P1 to P21, consistent with the results of the ISH analysis (Fig. 1F and G). *Nde1* is expressed in NSCs/transit-amplifying neural progenitor and neuroblasts during the early development of the V-SVZ, thus suggesting that NDE1 plays a crucial role as a dynein cofactor in V-SVZ cell proliferation and V-SVZ-derived neurogenesis in the early postnatal brain.

3.2. *Nde1* KD decreased cell proliferation in the early postnatal V-SVZ

On the basis of the above findings, we investigated the effects of *Nde1* shRNA KD on cell proliferation in the neonatal V-SVZ. To conduct *in vivo* KD and rescue experiments, we constructed plasmid vectors expressing shRNA targeting *Nde1* from the U6 promoter as previously reported (Doobin et al., 2016) and plasmid vectors expressing NDE1 SR and dynein-binding region deficient-NDE1 SR (NDE1 SR Δ BR) (Shimizu et al., 2018). Overexpression of NDE1 WT was downregulated by shRNA, whereas the overexpression of NDE1 SR and NDE1 SR Δ BR were not (Fig. 2A). Endogenous NDE1 was efficiently downregulated by shRNA *in vitro* (Fig. 2B). To knock down *Nde1* in the neonatal V-SVZ, we used *in vivo* postnatal electroporation of mouse brains. We introduced the *Nde1* KD plasmid in combination with a GFP-expressing plasmid into the lateral ventricles of P1 mice and transduced them into cells in the V-SVZ via electroporation (Fig. 2C and D). Two days after electroporation, we performed as *in vivo* BrdU labeling assay at P3 to test the effect of *Nde1* KD on cell proliferation in the V-SVZ (Fig. 2C). BrdU was injected into post-electroporated mouse pups intraperitoneally, and brains were harvested 1 h after BrdU incorporation (Fig. 2C). *Nde1* KD resulted in a

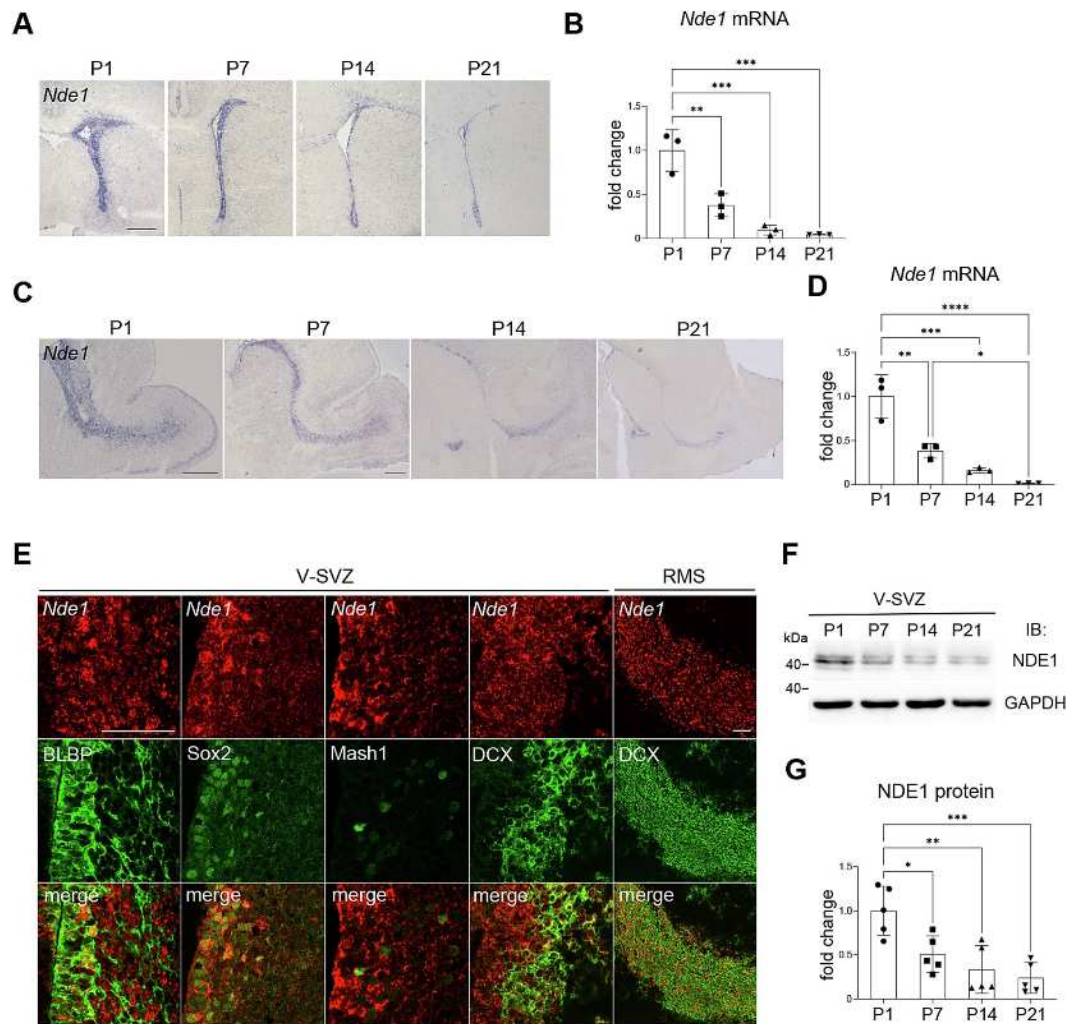


Fig. 1. Expression of *Nde1* in early postnatal brain development. (A, C) In situ hybridization (ISH) analyses of coronal sections (A) and sagittal sections (C) from P1, P7, P14, and P21 mouse brains using antisense RNA probes for *Nde1* mRNA. (B, D) Quantification of *Nde1* mRNA signals in the V-SVZ (B) and the RMS and OB (D). *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$ by Turkey's test following one-way ANOVA, $n = 3$. (E) double fluorescence ISH-IHC analyses of brain sections from P5 mice using antisense RNA probes for *Nde1* mRNA and antibodies against BLBP, Sox2, Mash1 and DCX in the V-SVZ and RMS. (F) Western blot analysis of NDE1 and GAPDH protein expression in the V-SVZ at different postnatal stages. (G) Densitometric quantification of western blot analysis. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ by Turkey's test following one-way ANOVA, $n = 5$. Scale bar: (A and C) 500 μm , (E) 50 μm .

significant decrease in the 1h BrdU-labeling index relative to that of the control (Fig. 2E and G). IHC against the mitotic marker PH3 was performed on sections obtained 2 days after electroporation, showing a significant reduction in the mitotic index under the *Nde1* KD condition (Fig. 2F and H). Conversely, NDE1 SR overexpression significantly rescued cell-proliferation and mitotic defects in the V-SVZ under *Nde1* KD condition, but NDE1 SR ΔBR did not (Fig. 2D–H). These *in vivo* data illustrate that NDE1 is involved in V-SVZ cell proliferation as dynein cofactor during the early postnatal stage.

3.3. *Nde1* KD in neonatal V-SVZ impaired the proliferation of V-SVZ-derived neuroblasts

To determine whether *Nde1* KD in the V-SVZ affects the generation of V-SVZ-derived neuroblasts, we assessed the proliferation of V-SVZ-derived neuroblasts, which are characterized by their unique ability to proliferate during migration toward the OB (Luskin et al., 1997). Four days after electroporation, we conducted *in vivo* BrdU labeling assay at P5 to examine the effect of *Nde1* KD on cell proliferation in V-SVZ-

derived migrating neuroblasts (Fig. 3A). At P5, BrdU was injected into post-electroporated mouse pups via intraperitoneal injection, and brains were collected after 1 h BrdU pulse (Fig. 3A). In sagittal brain sections, *Nde1* KD led to significantly less BrdU incorporation into GFP-positive cells than in the control group (Fig. 3C and F). Furthermore, the proportion of PH3-expressing cells in the GFP-positive population at P5 was significantly lower in *Nde1* KD conditions (Fig. 3D and G). Conversely, NDE1 SR overexpression significantly restored cell-proliferation and mitotic defects under *Nde1* KD conditions (Fig. 3C, D, F, G). Furthermore, we examined the effects of *Nde1* KD in the V-SVZ on the localization of V-SVZ-derived neuroblasts migrating toward the OB at P5. In the sagittal brain sections, we counted the number of GFP-labeled cells in the V-SVZ, RMS, and OB (Fig. 3B). The percentage of GFP-labeled cells localized to the OB was significantly lower in the *Nde1* KD condition than in the control condition (Fig. 3B and E). Conversely, NDE1 SR overexpression significantly rescued the localization defects observed with *Nde1* KD (Fig. 3B and E). These results indicate that *Nde1* KD in the V-SVZ caused localization defects that impaired the proliferation of V-SVZ-derived neuroblasts, thus suggesting that NDE1 plays a crucial role

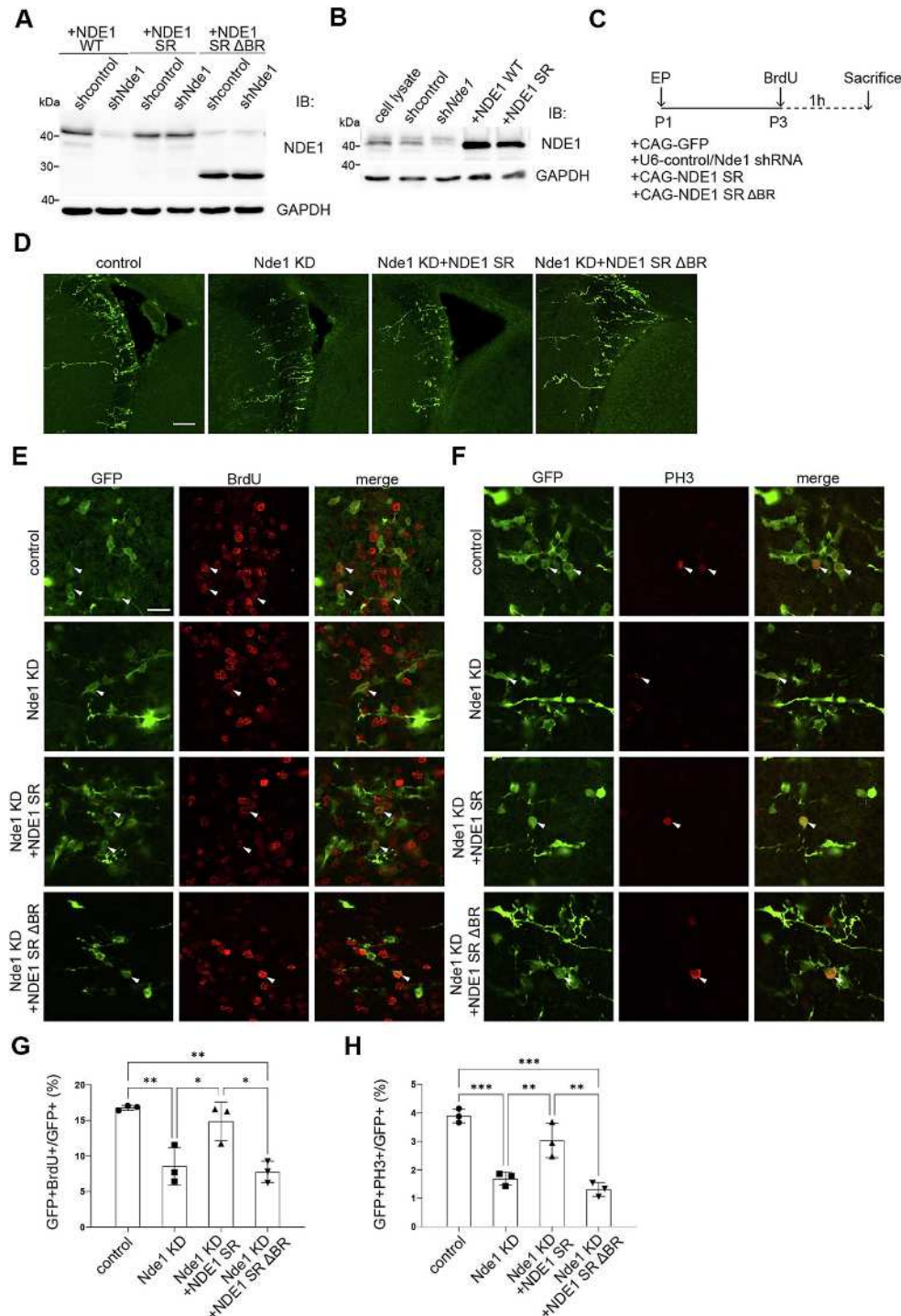


Fig. 2. *Nde1* knockdown decreases cell proliferation in the early postnatal developing V-SVZ. (A and B) Western blot analysis of NDE1 expression. Lysates were prepared from HEK293T cells harvested 48 h after transfection with plasmid vectors expressing either control shRNA (shcontrol) or *Nde1* shRNA (sh*Nde1*) and either NDE1 wild-type (NDE1 WT), NDE1 shRNA-resistant (NDE1 SR) or dynein-binding region deficient-NDE1 SR (NDE1 SR ΔBR) (A). Lysates were prepared from N2a cells harvested 48 h after transfection with plasmid vectors expressing either shcontrol or sh*Nde1* (B). NDE1 expression was assessed by western blotting using antibody against NDE1. (C) Experimental scheme. (D) Low-magnification images of the V-SVZ of mice at P3, 48 h after postnatal electroporation with GFP (green, electroporated) and shcontrol (control); GFP and sh*Nde1* (*Nde1* KD); GFP, sh*Nde1*, and NDE1 SR (*Nde1* KD + NDE1 SR); and GFP, sh*Nde1*, and NDE1 SRΔBR (*Nde1* KD + NDE1 SRΔBR) expression vectors. (E, F) High-magnification images of the V-SVZ of mice at P3 for GFP and BrdU (E) or GFP and PH3 (F). A 1 h BrdU pulse was performed before sample collection. Arrowheads indicate colocalization. (G, H) The proportion of GFP+BrdU+/GFP+ cells (G) and GFP+PH3+/GFP+ cells (H) in the V-SVZ at P3 in the control, *Nde1* KD, *Nde1* KD + NDE1 SR, and *Nde1* KD + NDE1 SR ΔBR expression groups. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ by Turkey's test following one-way ANOVA, $n = 3$; 440–880 cells from three animals for each condition. Scale bar: (D) 100 μ m, (E) 20 μ m.

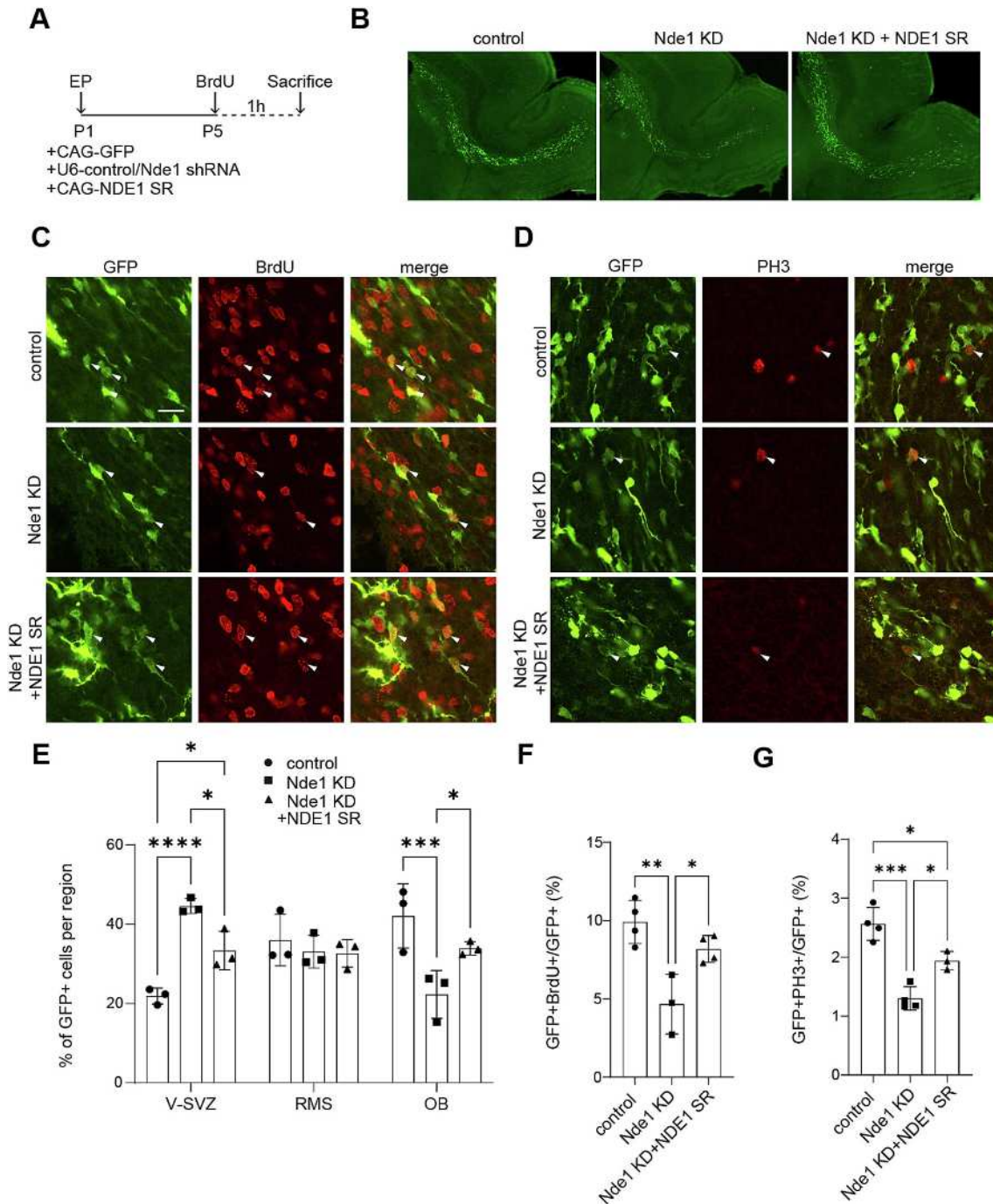


Fig. 3. *Nde1* knockdown inhibits cell proliferation of the V-SVZ-derived neuroblasts in early postnatal brain development. (A) Experimental scheme. (B) Low-magnification images of sagittal brain sections at P5, 4 days after postnatal electroporation. (C and D) High-magnification immunohistochemical images of the RMS of mice at P5 for GFP and BrdU (C) or GFP and PH3 (D). A 1 h BrdU pulse was performed before sample collection. Arrowheads indicate colocalization. (E) Quantification of the distribution of GFP + transfected cells in the V-SVZ–RMS–OB pathway in the brains of P5 mice. *: $p < 0.05$, ***: $p < 0.001$, ****: $p < 0.0001$ by Turkey's test following two-way ANOVA, $n = 3$; 981–2369 cells from three animals for each condition. (F, G) The proportion of GFP + BrdU+/GFP+ cells (F) and GFP+PH3+/GFP+ cells (G) in the V-SVZ–RMS–OB pathway in the control, *Nde1* KD and *Nde1* KD + NDE1 SR expression groups. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ by Turkey's test following one-way ANOVA, $n = 3, 4$; 1057–1870 cells from three or four animals for each condition. Scale bar: (B) 200 μm , (C) 20 μm .

in the generation of V-SVZ-derived neuroblasts during early postnatal development.

3.4. *Nde1* KD in the neonatal V-SVZ decreased the production of V-SVZ-derived neuroblasts, resulting in a reduction in newly generated OB neurons

Finally, we examined the effects of *Nde1* KD on the generation of V-SVZ-derived neuroblasts at P8. V-SVZ-derived neuroblasts take approximately 1 week to reach the OB from the V-SVZ (Lois and Alvarez-Buylla, 1994; Doetsch and Alvarez-Buylla, 1996), differentiate into several types of interneurons, and integrate into the OB circuitry (Lim and Alvarez-Buylla, 2016). Seven days after electroporation, we counted the number of GFP+ cells in the V-SVZ, RMS, and OB (Fig. 4A). The ratio of GFP+ cells in the OB to the total number of GFP+ cells was significantly reduced in the *Nde1* KD condition, suggesting that *Nde1* KD in the V-SVZ impaired the overall production of V-SVZ-derived neuroblasts (Fig. 4B and C). However, NDE1 SR overexpression significantly recovered the impaired generation observed under *Nde1* KD (Fig. 4B and C). Furthermore, we examined the effects of *Nde1* KD on the generation of OB neurons using neuronal marker NeuN at P29, because neuronal differentiation and maturation of OB interneurons take approximately 4 weeks (Lemasson et al., 2005) (Fig. 4A). There was no difference in the percentage of GFP-NeuN double-positive OB cells of the total number of GFP-labeled OB cells (Fig. 4D and E), but the proportion of GFP-NeuN double-positive OB cells of the total number of GFP-labeled cells in the V-SVZ was significantly lower than in the control condition, and NDE1 SR overexpression significantly restored the decrease observed with *Nde1* KD (Fig. 4D and F). These results indicate that *Nde1* KD in the neonatal V-SVZ decreased V-SVZ cell proliferation, followed by impaired proliferation and generation of V-SVZ-derived neuroblasts, resulting in a reduced number of newly-generated OB neurons during early postnatal development. These results suggest that NDE1 plays an important role in early V-SVZ-derived neurogenesis.

4. Discussion

NDE1 is highly expressed in cortical progenitors within the ventricular zone of developing cerebral cortex at embryonic stage, and essential for normal cortical progenitor proliferation (Feng et al., 2000; Feng and Walsh, 2004). This study revealed that NDE1 expression persisted during the early postnatal stage in the developing V-SVZ and that NDE1 was involved in the regulation of V-SVZ cell proliferation, which is essential for the proper progression of OB neurogenesis.

Nde1 is expressed in NSCs, transit amplifying neural progenitor cells, and neuroblasts in the V-SVZ-RMS-OB pathway during the early postnatal period, with its peak at the neonate stage. The early postnatal V-SVZ is characterized by a high level of proliferation of NSCs and neural progenitor cells, and V-SVZ-derived neurogenesis peaks in this stage (Kim et al., 2020). Previous studies have revealed that the number of NSCs in the V-SVZ decreases after birth (Obernier et al., 2018; Cebrian-Silla et al., 2025). Taken together, it can be inferred that a decrease in *Nde1*+ cells in the V-SVZ during postnatal development is related to the developmental decrease in NSC lineages in the V-SVZ, suggesting that the function of NDE1 in the V-SVZ persists during early postnatal brain development. However, it remains unknown whether NSCs in the V-SVZ homogeneously or heterogeneously express NDE1. Studies have shown V-SVZ NSC heterogeneity, including in the proliferation states and regional identities of NSCs (Obernier and Alvarez-Buylla, 2019). In the V-SVZ, multiple subpopulations of NSCs generate diverse subsets of neurons and glia, depending on their location (Obernier and Alvarez-Buylla, 2019). Future studies should examine NDE1 expression levels in individual NSC populations and NSC proliferation states in the postnatal brain to investigate whether NDE1 can be used as a proliferation marker in specific NSC populations.

The present data demonstrated that *Nde1* KD in the neonate V-SVZ decreased V-SVZ cell proliferation. Thereafter, the reduced V-SVZ cell proliferation was followed by impaired proliferation of V-SVZ-derived neuroblasts, thus leading to decreased neuroblast generation.

Eventually, *Nde1* KD in the V-SVZ resulted in a decrease in V-SVZ-derived newly generated OB neurons. However, the underlying molecular mechanisms behind NDE1's role in this are unclear. One study reported that *Nde1* knockout mice displayed microcephaly with thin superficial cortical layers showing significantly fewer neurons than controls (Feng and Walsh, 2004). The reduction in superficial cortical neurons in these mice was attributed to defective cell division and precocious neurogenesis of cortical progenitor cells (Feng and Walsh, 2004). Taking these findings into account, it can be inferred that *Nde1* KD might impair mitosis in NSCs and neural progenitor cells in the V-SVZ, producing a progressive reduction in proliferative cells in the V-SVZ and, accordingly, leading to premature differentiation of these cells by due to early exit from the proliferation cycle and ultimately resulting in a reduced total number of OB neurons. Further, it has been reported that *Nde1*-deficient cortical progenitor cells showed altered spindle orientations and mispositioned chromosomes during mitosis (Feng and Walsh, 2004). Functional analysis of NDE1 during mitosis in *in vitro* studies has revealed that NDE1 is required for spindle alignment and chromosome segregation during mitosis (Yan et al., 2003; Stehman et al., 2007; Vergnolle and Taylor, 2007). Therefore, it is highly possible that NDE1 may be involved in these functions as a dynein cofactor during mitosis in proliferating cells in the V-SVZ. In addition, another study showed that NDE1 is essential for cell-cycle processes preceding mitosis, as *NDE1* KD caused abnormalities in interkinetic nuclear migration (INM) prior to mitotic entry in embryonic cortical progenitors (Doobin et al., 2016). INM is a cell cycle-dependent oscillatory nuclear movement observed in the proliferative ventricular zone of embryonic brains (Spear and Erickson, 2012) and has been reported to persist postnatally in the developing V-SVZ (Tramontin et al., 2003). Therefore, the present findings suggest that NDE1 may be involved in INM and mitosis in proliferating cells in the early developing V-SVZ. Furthermore, NDE1 has been reported to function in primary cilium biogenesis. V-SVZ NSCs contain a cellular protrusion of the primary cilium, which likely regulates cell proliferation by functioning as a sensory antenna for detecting morphogens or growth factors in the cerebrospinal fluid (Han et al., 2008; Tong et al., 2014). The primary cilium is a dynamic organelle whose assembly and disassembly are tightly regulated in coordination with the cell cycle and are essential for cell cycle progression (Anvarian et al., 2019). Studies have reported that NDE1 negatively regulates the primary cilium length, with *NDE1* KD promoting overelongated primary cilia *in vitro* (Kim et al., 2011; Maskey et al., 2015) and *in vivo* (Doobin et al., 2016). This indicates that NDE1 is involved in the disassembly of the primary cilium. Taken together, we predicted that *Nde1* KD in the V-SVZ would lead to defects in NSC cell cycle progression by dysregulating ciliary disassembly. Furthermore, a recent study reported that V-SVZ-derived neuroblasts possess a primary cilium and that primary cilium dynamics are linked to the proliferation and saltatory movement of neuroblasts (Matsumoto et al., 2019). Thus, we inferred that NDE1 function may also be related to the regulation of the proliferation and migration of V-SVZ-derived neuroblasts via the disassembly of the primary cilium.

During the first few weeks after birth, the embryonic ventricular zone transforms into the postnatal V-SVZ niche (Tramontin et al., 2003). During this period, rapidly dividing embryonic radial glial cells differentiate into neurons and glia, and quiescent embryonic radial glial cells transform into postnatal NSCs and other cell types to establish postnatal V-SVZ niche, resulting in disappearance of radial glial cells in the V-SVZ (Merkle et al., 2004; Fuentealba et al., 2015; Furutachi et al., 2015; Ortiz-Álvarez et al., 2019). However, the time at which radial glial cells enter quiescence varies depending on their location in the V-SVZ. Radial glial cells in the lateral wall enter quiescence embryonically (Fuentealba et al., 2015; Furutachi et al., 2015), while those in the dorsal wall enter quiescence in the early postnatal period (Borrett et al., 2020; Marcy et al., 2023). Therefore, the neonatal V-SVZ harbors several populations of embryonic radial glial cells, namely rapidly dividing radial glial cells and quiescent/slow-dividing radial glial cells. One related limitation of

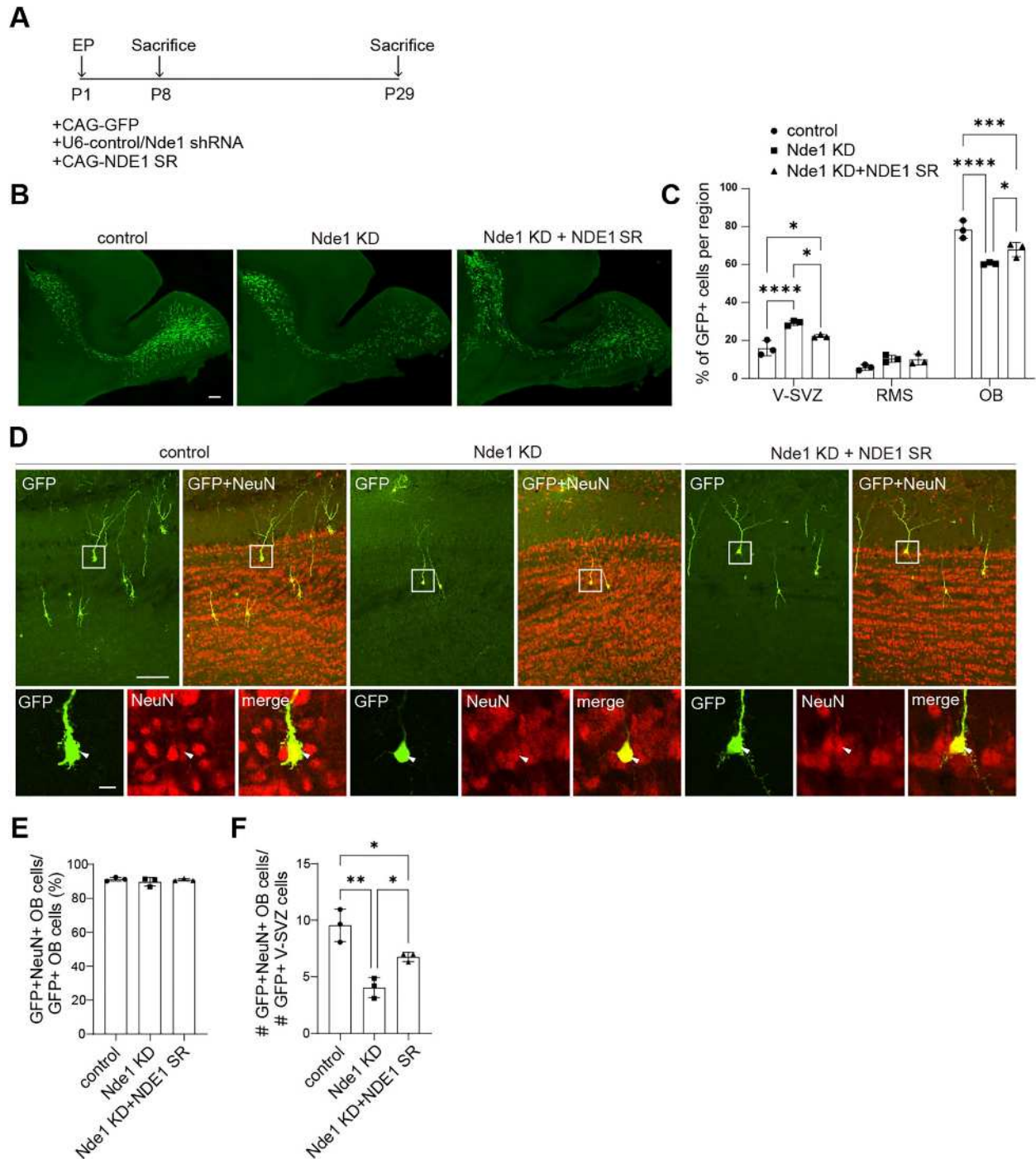


Fig. 4. *Nde1* knockdown decreases the generation of V-SVZ-derived neuroblasts in early postnatal brain development. (A) Experimental scheme. (B) Low-magnification images of sagittal brain sections at P8, 7 days after postnatal electroporation. (C) Quantification of the distribution of GFP + transfected cells in the V-SVZ–RMS–OB pathway in the brains of P8 mice. *: $p < 0.05$, ***: $p < 0.001$, ****: $p < 0.0001$ by Turkey's test following two-way ANOVA, $n = 3$; 1,314–3545 cells from three animals for each condition. (D) Immunohistochemical images of the OB of mice at P29 for GFP and NeuN. Magnified images of boxed areas in lower panels. Arrowheads indicate colocalization. (E, F) The proportion of GFP+NeuN+ OB cells of the total number of GFP+ cells in the V-SVZ (F) in the control, *Nde1* KD and *Nde1* KD + NDE1 SR expression groups. *: $p < 0.05$, **: $p < 0.01$ by Turkey's test following one-way ANOVA, $n = 3$; 356–623 cells from three animals for each condition. Scale bar: (B) 200 μ m, (D) 100 μ m (upper panels) and 10 μ m (lower panels).

the present study is that the short-term labeling induced using *in vivo* postnatal electroporation could not be used to distinguish between these populations; therefore, it remains unclear whether the regulatory function of NDE1 in cell proliferation differs between these populations. To examine the function of NDE1 in quiescent/slow-dividing radial glial cells destined to establish the postnatal V-SVZ niche, further analysis using long-term labeling experiments are needed.

In summary, we found that NDE1 plays an important role as a dynein cofactor in the regulation of V-SVZ cell proliferation during early postnatal brain development. Our study provides novel insight into molecular mechanisms involved in dynein regulation in V-SVZ proliferation during early postnatal stage. Further understanding of the dynein regulatory mechanisms including other dynein cofactors underlying neonatal V-SVZ cell proliferation in physiological and pathological conditions may contribute to the development of regenerative therapies in treating injury and disease.

CRedit authorship contribution statement

Shoko Shimizu: Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation. **Yugo Ishino:** Writing – review & editing, Methodology. **Shingo Miyata:** Writing – review & editing, Supervision.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

The datasets generated in this study are available from the corresponding author on reasonable request.

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A variety of cortical inhibitory interneuron (IN) subtypes differentially but coordinately operate in local circuits to process neuronal signals. The subcellular arrangement of IN synaptic outputs contributes to subtype-specific functional specialization. However, it remains poorly understood what intercellular molecular interactions enable the matched synapse formation between IN subtypes and specific subcellular domains. Using mice of both sexes, we demonstrate that Neurofascin-186 (NF186), a cell adhesion molecule specifically expressed in the axon initial segments (AISs) of pyramidal neurons, is necessary for chandelier cells (ChCs) to develop a string of synaptic boutons along the AIS (an axon cartridge). Furthermore, we

discovered that Gliomedin, a known receptor for NF186 in the nodes of Ranvier, is preferentially expressed in ChCs and mediates ChC axon cartridge development by acting as a major receptor for NF186. Thus, the intercellular interaction through subcellularly restricted ligands and cell type-specific receptors ensures a high degree of IN subcellular synapse specificity.

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