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ORIGINAL ARTICLE

FGF2-responsive genes in human dental pulp cells assessed using a rat spinal cord injury model

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Abstract

The central nervous system in adult mammals does not heal spontaneously after spinal cord injury (SCI). However, SCI treatment has been improved recently following the development of cell transplantation therapy. We recently reported that fibroblast growth factor (FGF) 2-pretreated human dental pulp cells (hDPCs) can improve recovery in a rat model of SCI. This study aimed to investigate mechanisms underlying the curative effect of SCI enhanced via FGF2 pretreatment; we selected three hDPC lines upon screening for the presence of mesenchymal stem cell markers and of their functionality in a rat model of SCI, as assessed using the Basso, Beattie, and Bresnahan score of locomotor functional scale, electrophysiological tests, and morphological analyses. We identified FGF2-responsive genes via gene expression analyses in these lines. FGF2 treatment upregulated *GABRB1*, *MMP1*, and *DRD2*, which suggested to contribute to SCI or central the nervous system. In an expanded screening of additional lines, *GABRB1* displayed rather unique and interesting behavior; two lines with the lowest sensitivity of *GABRB1* to FGF2 treatment displayed an extremely minor effect in the SCI model. These findings provide insights into the role of FGF2-responsive genes, especially *GABRB1*, in recovery from SCI, using hDPCs treated with FGF2.

Keywords Human dental pulp cells · Spinal cord injury · Fibroblast growth factor 2 · GABRB1

Introduction

Over the past few decades, numerous studies have reported the use of stem cells for regenerative medicine. Mesenchymal stem cells are commonly used for such cell-based therapy [1–3]. Human dental pulp cells (hDPCs) are a type of mesenchymal cells originating from the neural crest [4–6]. They can be easily obtained from teeth that have been discarded as medical waste. We have recently established approximately 300 hDPC lines, wherein we have analyzed the following characteristics: (1) high proliferation potency and low contamination rate, which is required for hDPC banking [7], (2) their behavior under hypoxic and chemically defined conditions to develop methods for efficient and safe preparation of cultures of these cells [8–10], and (3) derivation of human leukocyte antigen haplotype homo-induced pluripotent stem cells (iPSCs) for allogenic transplantation therapies [9–12]. In these studies, a homeotic gene, *DLX4*, was predominantly expressed in hDPCs obtained from younger donors, which resulted in higher iPSC derivation rates [13]. We hypothesized that this donor-dependent

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difference in hDPCs may underlie other complex biological process, and we evaluated hDPC lines obtained from differently aged donors, using a rat model of spinal cord injury (SCI), one of the targets of cell transplantation therapy [14].

Previously, Sakai et al. [15] and Matsubara et al. [16] reported that hDPCs obtained from deciduous teeth had improved functional recovery after SCI and that conditioned serum-free medium from human deciduous dental pulp induced anti-inflammatory M2-like macrophages after SCI. More recently, we reported that FGF2-pretreated hDPCs improved locomotor function in a rat model of SCI [17], and that FGF2 priming protected hDPCs from hydrogen peroxide-induced cell death in vitro. We also have measured FGF2 protein levels in cell lysates of hDPCs with (DPC-FS) or without (DPC-S) FGF2 pretreatment, using a growth factor antibody array; however, neither accumulation nor increase in FGF2 production was observed in cell lysates, suggesting that FGF2 protein trapped in extracellular matrices or cell surface did not contribute to the recovery from SCI in vivo. To better understand the mechanisms underlying these therapeutic effects of hDPCs pretreated with FGF2 and to evaluate donor-dependence, we attempted to identify genes differentially upregulated by FGF2 in hDPC lines in this study.

Materials and methods

Ethics statement

The protocols for human dental pulp tissue collection, transplantation into animals, and genome/gene analyses were carried out in accordance with the guidelines for the care in human studies adopted by the Ethics Committee of the Gifu University Graduate School of Medicine (approval number: 26-116). Informed consent was obtained from all individuals participating in the study. All animal experiments in this study strictly followed protocols approved by the Gifu University Institutional Animal Care and Use Committee (approval number: 25-68, 27-83).

Isolation and culture of hDPCs

Human dental pulp cells were isolated and cultured in accordance with previously reported techniques [7]. We cultured hDPCs at 37 °C in a humidified atmosphere (21% O_2) with 5% CO_2 in Mesenchymal Stem Cell Growth Medium (MSCGM; Lonza, Ltd., Basel, Switzerland). Among 14 lines listed in Supplementary Table 1, four lines (DP184, DP185, DP193, and DP213) were obtained from older patients [8]. Owing to a low proliferation rate, these cells were cultured in 3% O_2 during the first 4–5 passages. Before transplantation, hDPCs were cultured in alpha minimum essential medium (α-MEM, Sigma-Aldrich Co. LLC, MO, USA) supplemented with 10% fetal bovine serum (FBS), 3% L-glutamine, and 100 IU/mL benzylpenicillin (Meiji Co., Ltd, Tokyo, Japan) for at least five passages. For FGF2 treatment of hDPCs, FGF2 (10 ng/mL; R&D SYSTEMS, Inc., MN, USA) was added to the medium daily for at least three passages. Control cells were cultured simultaneously in the same manner; however, FGF2 was not supplemented. Both FGF2-treated and untreated hDPCs were used at passages 10–13 (counted from primary culture) in all experiments.

Transplantation surgeries and postoperative management

Animals were anesthetized using a combination of three anesthetics (medetomidine hydrochloride, 0.375 mg/kg, Kyoritsu Seiyaku, Tokyo, Japan; midazolam, 2.0 mg/kg, Meiji Seika Pharma Co., Ltd, Tokyo, Japan; butorphanol tartrate, 2.5 mg/kg, Sandoz Ltd., Basel, Switzerland) in accordance with previous reports [17]. We conducted a laminectomy at the tenth thoracic vertebral level. The spinal cord was transected gently, at least twice for complete transection, using a sharp surgical blade (stainless steel no. 11 mini, Kai Medical, Tokyo, Japan), as described previously [17, 18]. Hemostasis treatment was provided. Thereafter, we slowly grafted approximately 1×10^6 cells in 10 µL of α-MEM containing 10% FBS into the epicenter of the lesion, using a Hamilton syringe (Sigma-Aldrich). In the control group, the rats were injected with 10 µL of phosphate-buffered saline (PBS). The animals were left undisturbed for 10 min before suturing the muscle and skin around the incision. The surgery lasted approximately 30 min for each rat. The rats were then placed in warmed chambers until they awoke from the anesthesia. The rats received a daily injection of antibiotics (sulbactam/ampicillin, SANDOZ, Tokyo, Japan, 10 mg/kg body weight) for 1 week. To minimize suffering and distress in all animals, all rats were weighed and administered an appropriate infusion volume of antibiotics each day. Bladder evacuation was applied daily until the rats were euthanized. All rats were intraperitoneally administered an immunosuppressive agent (cyclosporine, NEORAL, Novartis, Basel, Switzerland; dose, 10 mg/kg body weight) daily from the day before transplantation to the day before euthanasia. An immunosuppressive agent was used in accordance with our previously reported protocols [17, 18]. We monitored animal health on the basis of weight, appetite, and mobility every day. When animals showed signs of infection, they were administered antibiotics (sulbactam/ampicillin, 10 mg/kg body weight). Specific criteria for humane endpoints were determined to immediately euthanize rats via carbon dioxide gas to prevent prolonged suffering, when they showed severe decubitus ulcer, inappetence, immobility, or weight loss. The duration of the experiment to evaluate locomotor function

after surgery was 8 weeks. Animals died before completion of Basso, Beattie, and Bresnahan (BBB) scoring; these were as follows: 27 rats within 2 weeks, 25 rats within 3–4 weeks, and 12 rats within 5–8 weeks. Among these, 9 rats were euthanized because of severe decubitus ulcer. The cause of death for the rest of animals was urinary tract infections confirmed by the presence of purulent urine. The number of animals euthanized after completion of all the planned experiments was 93.

Electrophysiology

After the final BBB scoring, rats were anesthetized via inhalation of 1.0–2.0% isoflurane. Descending transmission of electrical activity through the grafts was assessed using previously described techniques [19]. The stimulation-induced increase in potential was recorded using an analog-to-digital converter (PowerLab; AD instruments, Dunedin, New Zealand) at a sampling rate of 40 kHz. The electrodes for spinal cord recording were connected to an amplifier (VC-11; Nihon Kohden Co., Tokyo, Japan). We used an ordered bipolar electrode comprising tungsten microelectrodes (φ 0.2 mm; Unique Medical Co., Ltd, Tokyo, Japan) spaced 1 mm apart. The stimulating site was at the 8th thoracic vertebral level. The recording site was at the 13th thoracic vertebral level. The electrodes were inserted into the spinal cord at 0.5–0.75 mm lateral to the midline 1.0–1.5 mm below the surface. The rats were immobilized in a vertebra-fixing apparatus (ST-7R-HT; NARISHIGE, Tokyo, Japan), the microelectrodes were adjusted with a manipulator, and the position of the microelectrodes was confirmed microscopically; brief, square wave pulses (amplitude, 0.6 mA; duration, 0.2 ms; interval, 1 s) were delivered. Electrophysiology experiments lasted approximately 60–90 min in total. Thereafter, 10 rats were subjected to transcardial perfusion for immunohistochemical analysis. The remaining rats were immediately euthanized with CO_2 gas.

Results

Characterization of hDPCs and FGF2-pretreated hDPCs

We selected hDPC lines obtained from differently aged donors to investigate the presence of donor-related differences in the curative effect of FGF2-pretreated hDPCs in a rat mode of SCI (Supplementary Table 1). First, DP1, DP31, DP165, and DP296 were selected and characterized by the expression of mesenchymal stem cell markers (CD73, CD90, and CD105) and the absence of hematopoietic markers (CD34 and CD45), via flow cytometry (Supplementary Fig. 1, Supplementary Table 2). These cell lines expressed markers for dental pulp stem cells, such as nestin, βIII-tubulin, and glial fibrillary acidic protein (GFAP), but not myelin basic protein (MBP), as assessed via immunocytochemistry (Supplementary Fig. 2). No significant difference was detected among the four cell lines in these analyses. Similarly, no significant effect of pretreatment with FGF2 was observed on the short-term growth of hDPCs (Supplementary Fig. 3a). Genes associated with osteo-/odontoblastic lineages; *RUNX2* was significantly down-regulated upon FGF2 treatment in all four lines. *COL1A1* was also down-regulated upon FGF2 treatment in DP31, DP165, and DP296; however, such effects were not significant in DP1. Similarly, *BMP2* was significantly upregulated upon FGF2 treatment in DP31, DP165, and DP296, but not in DP1 (Supplementary Fig. 3b).

Curative effects of three FGF2-pretreated hDPC lines on SCI

We compared the four FGF2-pretreated hDPC lines (DP1, DP31, DP165, and DP296) using our SCI model. The FGF2-pretreated DP1, DP31, and DP165 cell lines induced similar and significant improvement in locomotor function, compared with the control group, as assessed via calculation of the BBB score (Fig. 1). Interestingly, the experimental group transplanted with DP296 cells did not show this effect (Fig. 1).

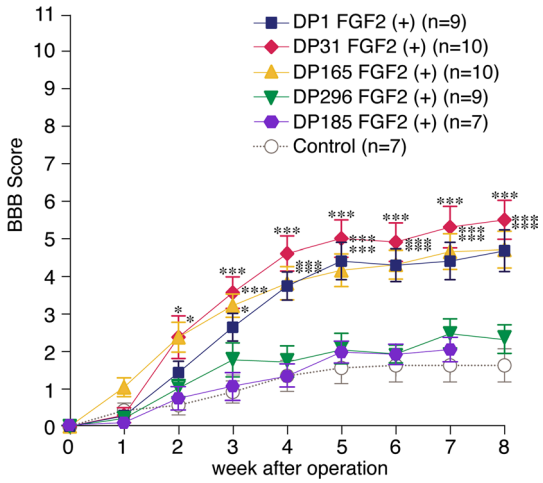


Fig. 1 Locomotor functional analysis after transplantation of fibroblast growth factor (FGF) 2-pretreated human dental pulp cells (hDPCs) to a rat model of spinal cord injury (SCI). Functional analysis of open-field locomotor activity, based on the BBB score, after transplantation of FGF2-treated hDPCs. Data were tested using two-way analysis of variance (ANOVA), followed by Bonferroni's multiple comparisons test (F [48, 395] = 2.250, $P = 0.0001$). Asterisks indicate statistical significance: $^{*}P < 0.05$ and $^{***}P < 0.001$, compared with the control group (injected with PBS). Error bars indicate ± standard error of the mean values

Recovery of locomotor function correlates with electrophysiological measurements [19], and to evaluate the effects of FGF2-pretreated hDPCs in SCI more quantitatively, we conducted an electrophysiological test. Consistent with BBB scores, the latency in DP296 was significantly extended (6.14 ± 1.4 ms) compared with that of the other hDPC groups (DP1, 4.24 ± 0.38 ms; DP31, 3.67 ± 0.34 ms; DP165, 3.72 ± 0.32 ms) and intact animals without surgery (3.21 ± 0.1 ms) at 0.6 mA electric stimulation (Fig. 2). In the PBS-treated control group, evoked potentials were not observed (Fig. 2a).

In addition to the PBS group, two hDPC lines (DP1, DP31) without pretreatment with FGF2 did not exhibit any significant recovery of locomotor function in BBB score (Supplementary Fig. 4a), as we observed previously [17]. In addition, we observed that only 1 out of 6 animals transplanted with these non-pretreated hDPCs demonstrated electric stimulation-induced evoked potential across the transection site at 8 weeks after SCI, and its latency time was greater than those showing significant recovery in accordance with BBB scoring (Supplementary Fig. 4b). These facts suggest that DP296 treated with FGF2 displays differential behavior than the other three lines, DP1, DP31, and DP165, rather similar to DP1 and DP31 without pretreatment with FGF2.

Immunohistochemical analyses of the spinal cord at 8 weeks after complete transection revealed that in rats that received FGF2-treated DP1, DP31, or DP165, massive GAP-43-positive axons regenerated along with GFAP-positive resident astrocytes (Fig. 3a–f). In addition, the regenerated axons were associated with mature MBP-positive oligodendrocytes (Fig. 3g). However, poor axon regeneration was observed in rats that received FGF2-treated DP296 cells (Fig. 3h–j).

Hence, we concluded that DP296 pretreated with FGF2 uniquely displayed poor performance in the present SCI model in comparison with the other three lines.

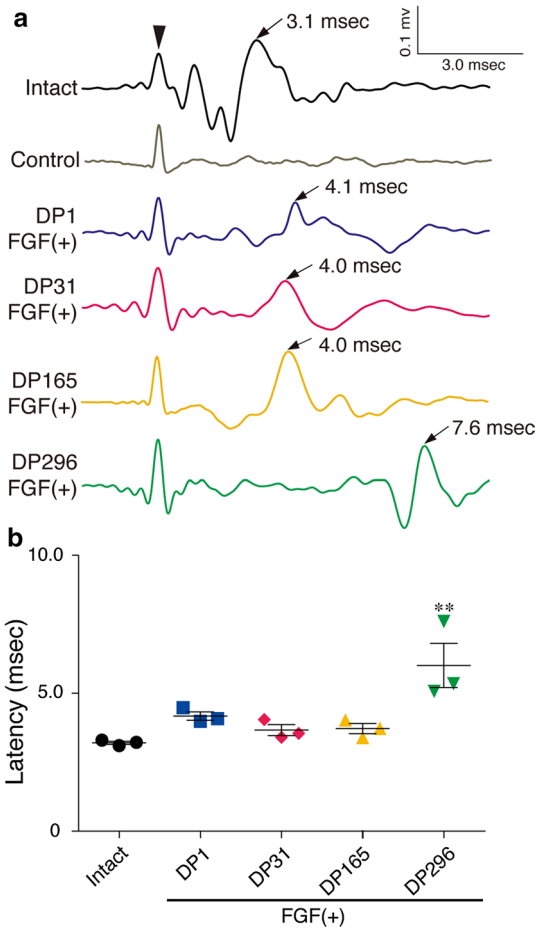


Fig. 2 Electrophysiological analysis at 8 weeks after transplantation of fibroblast growth factor (FGF) 2-pretreated dental pulp cells (DPCs) in a rat model of spinal cord injury (SCI). **a** Stimulation-evoked potentials across the transection site upon electrical stimulation (0.6 mA). Stimulation at the 8th thoracic vertebral level in intact rats evoked a short latency (3.1 ms) at the 13th thoracic vertebral level. The evoked potentials were completely obliterated in the control group. Recovery of an evoked response of prolonged latency in groups grafted with DP1, DP31, DP165, and DP296 cells was observed; however, the latency in the DP296 group was markedly extended compared with that in the other hDPC groups. **b** Latency by electrical stimulation (0.6 mA) for each experimental group. Data were tested using one-way ANOVA, followed by Tukey's multiple comparisons test (F [4, 10] = 8.035, P = 0.0036). Asterisks indicate statistical significance: *P < 0.05, compared with all the groups. Error bars indicate ± standard error of the mean values (n = 3)

FGF2-responsive genes in hDPCs

In our previous study [17], FGF2 pretreatment improved the curative effect of hDPCs; therefore, we attempted to identify the genes upregulated upon FGF2 treatment. We performed microarray analysis in the DP1, DP31, and DP165 cells, which were effective for functional repair after SCI (Supplementary Table 3). We selected the eight most upregulated genes showing a greater than 40-fold increase, and we conducted real-time PCR to evaluate expression levels after FGF2-treatment in four cell lines, including DP296. *MMP1*, *DRD2*, *ABCA6*, *TMEM100*, *THBD*, *NTSR1*, and *SCG2* were similarly upregulated upon FGF2 treatment in the four hDPCs lines (Fig. 4). However, gamma-aminobutyric acid A ($GABA_A$) receptor beta1 subunit (*GABRB1*) was strongly upregulated in DP1, DP31, and DP165 cells, but not in DP296 cells ($P > 0.05$) upon FGF2 administration (Fig. 4). Baseline expression of *GABRB1* in DP296 without FGF2 treatment was greater than that in DP1; however, it was lower than that in DP31 or DP165. In contrast, expression levels in DP296 upon FGF2 treatment and upregulation ratio (FGF2(+)/FGF2(−)) was much lower than that in the other three lines, DP1, DP31, and DP165 (Supplementary Fig. 5a–c).

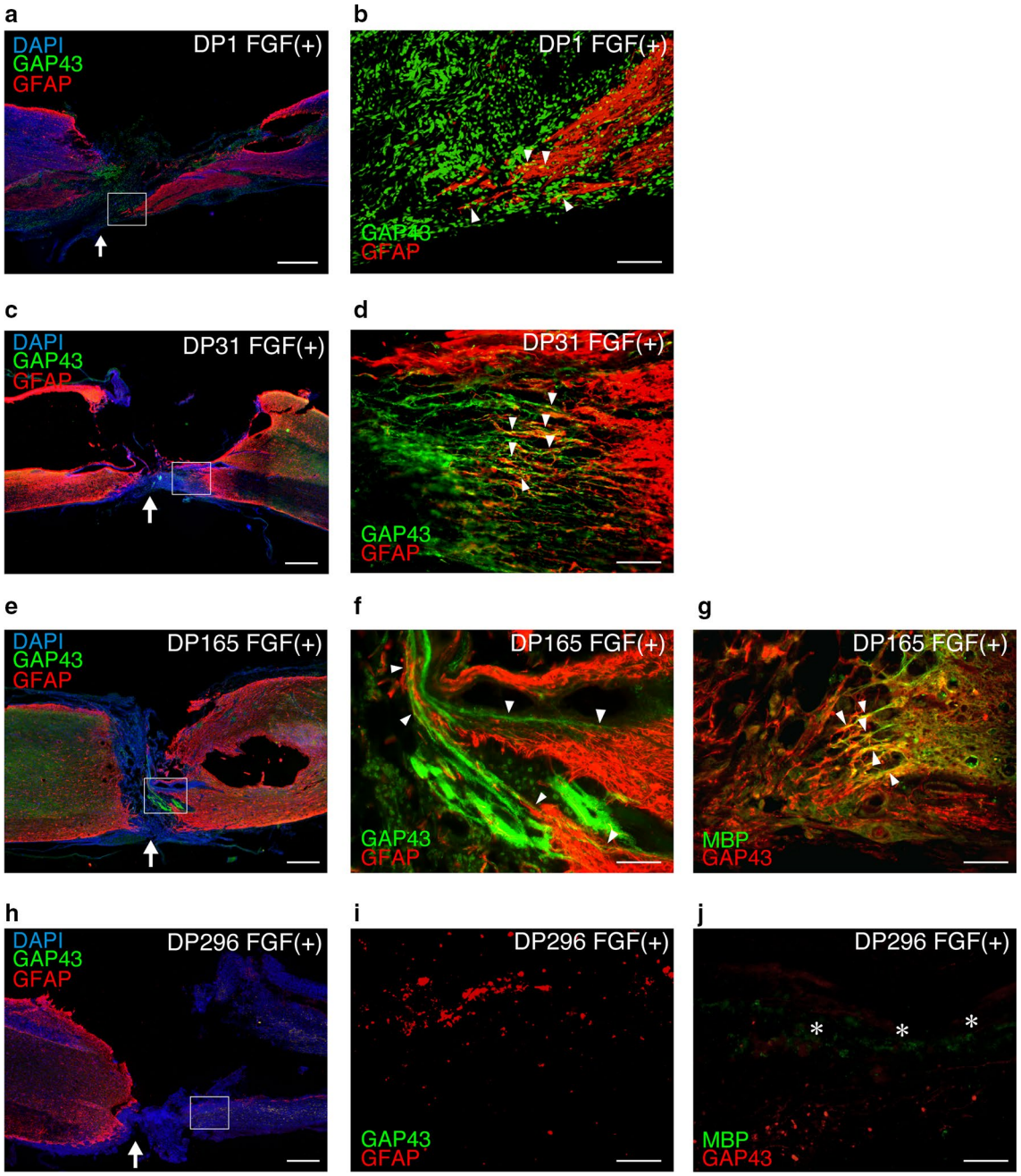
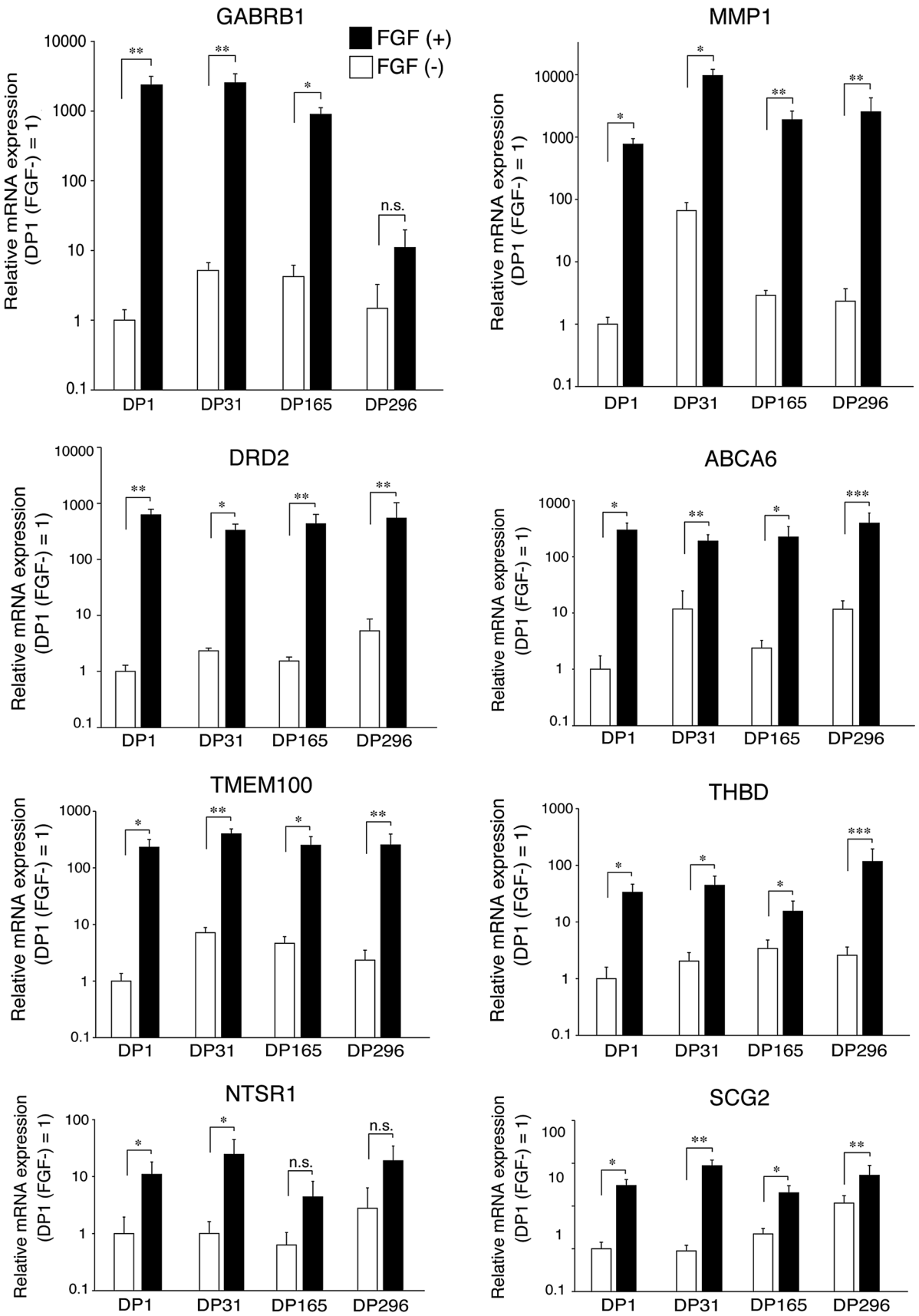


Fig. 3 Fibroblast growth factor (FGF) 2-pretreated human dental pulp cell (hDPC)-supported axonal regeneration associated with mature oligodendrocytes after spinal cord injury (SCI) in rats. Images of the sagittal section of the spinal cord at 8 weeks after transplantation with DP1 (**a**, **b**), DP31 (**c**, **d**), DP165 (**e**–**g**) and DP296 (**h**–**j**) cells immunohistochemically stained for GAP-43 and GFAP. **a**, **c**, **e** GAP-43-positive growth cones (green) and GFAP-positive astrocytes (red) were detected downstream of the epicenter of the lesion (white arrow). **b**, **d**, **f** High-magnification view of the boxed area in **a**, **c**, and **e**. White arrowheads indicate GAP-43-positive growth cones (green) associated with GFAP-positive astrocytes (red). **g** White arrowheads show GAP-43-positive growth cones (red) merged with MBP-positive mature oligodendrocytes (green) at the ventral portion caudal to the epicenter. **h** GAP-43-positive cells (green) were scarcely observed around the epicenter of the lesion (white arrow) in the animals transplanted with DP296 cells. **i** High-magnification view of the boxed area in **h**. **j** Some MBP-positive cells (asterisks) were observed; however, these were not associated with GAP-43-positive axons (red). Resident scale bar: 500 μm in **a**, **c**, **e**, and **h**; 100 μm in **b**, **d**, **f**, **g**, **i**, and **j**

To identify other cell lines wherein *GABRB1* was relatively insensitive to FGF2 treatment, we analyzed effect of FGF2 treatment on *GABRB1* expression in another 10 hDPC lines (Supplementary Fig. 5a–c). We selected DP185, wherein the baseline *GABRB1* expression levels were the lowest and the response was relatively insensitive to FGF2


GABRB1
FGF (+)
FGF (-)
Relative mRNA expression (DP1 (FGF-) = 1)
10000
1000
100
10
1
0.1
**
**
*
n.s.
DP1
DP31
DP165
DP296
MMP1
*
*
**
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DRD2
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ABCA6
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TMEM100
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THBD
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NTSR1
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n.s.
n.s.
SCG2
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◂**Fig. 4** Expression levels of genes upregulated by fibroblast growth factor (FGF) 2. The expression levels of *GABRB1, MMP1*, *DRD2*, *ABCA6*, *TMEM100*, *THBD*, *NTSR1*, and *SCG2* were quantified using real-time polymerase chain reaction. In particular, *GABRB1* was significantly upregulated in the DP1, DP31, and DP165 groups, but not in the DP296 group. Error bars indicate ± standard error of the mean values ($n=3$). Asterisks indicate statistical significance: $*P<0.05$ and $**P<0.01$, compared with the non-treated group

treatment and similar to that of DP296 and evaluated it in the rat model of SCI (Fig. 1). Interestingly, the FGF2-treated DP185 cell line also induced limited recovery of locomotor function in the rat model of SCI. These results suggest that relative insensitivity of *GABRB1* to FGF2 treatment is correlated with the degree of recovery from SCI after hDPC transplantation.

To measure protein expression levels of GABRB1, we performed Western blotting using specific antibodies (Supplementary Fig. 6). Recombinant GABRB1 protein (38 kDa) was used as a positive control. Consequently, we could not detect expression of native GABRB1 protein (52 kDa) both in the presence and absence of FGF2, suggesting that the functional $GABA_A$ receptor may not be expressed in DPCs in vitro.

Discussion

Sakai et al. [15] reported that hDPC transplantation induces recovery after SCI. In our previous study [17], we also noted that FGF2-pretreated hDPCs displayed further improvements in locomotor function when transplanted into rats after SCI. Thereafter, in this report, we reproduced this effect using FGF2-treated hDPCs in three of four hDPC lines (DP1, DP31, and DP165); however, one line, DP296, did not induce a significant improvement even upon pretreatment with FGF2.

Donor-dependent variation in autologous cell transplantation therapy to SCI patients has been documented [12, 20]. The use of allogenic cells evaluated for performance and the associated risk of side effects before transplantation may overcome this issue [21, 22]. Previously, we reported that a homeobox gene, *DLX4*, was expressed predominantly in younger donors and facilitates the derivation of iPSCs [13]. In this study, we identified eight genes strongly upregulated upon FGF2 treatment. Among these, only *GABRB1* showed unique reactivity to FGF2 treatment in the DP296 cells (Fig. 4; Supplementary Fig. 5), suggesting a potential association with the loss of functional recovery after transplantation. This possibility was validated via an additional screening of human DPC lines for responsiveness of *GABRB1* expression upon FGF2 treatment; we identified another cell line, DP185, which displayed a gene expression pattern and therapeutic effect similar to that of DP296 (Fig. 1; Supplementary Fig. 5). In the absence of FGF2, analyses of *GABRB1* mRNA expression levels did not provide an obvious threshold to predict the effectiveness in SCI. However, *GABRB1* mRNA expression levels were markedly higher in DP1, DP31, and DP165 than in DP296 in the presence of FGF2, suggesting that an induction ratio upon FGF2 treatment may be used to predict the effectiveness of the cells in SCI (Supplementary Fig. 5b, c). An additional investigation to identify a set of transcription factors binding to the regulatory region of *GABRB1* gene and changes in the epigenetic state may help elucidate the mechanisms underlying this phenomenon.

Increased production of reactive oxygen species after SCI induces the death of motor neurons in the spinal cord in mice [23]. Recently, Ohashi et al. [24] reported that activation of extra-synaptic $GABA_A$ receptors could attenuate H_2O_2-induced acute neuronal damage. In this study, *GABRB1* mRNA expression was induced by FGF2; however, GABRB1 protein was not detected in hDPCs. However, functional $GABA_A$ hetero-receptors are suggested to be expressed in human dental pulp in vivo [25]; therefore, we still hypothesize that FGF2-treated hDPCs survive in conditions of elevated oxidative stress and prevent cellular loss in damaged tissue via an unknown mechanism involving $GABA_A$ receptors.

In summary, several genes were remarkably upregulated upon FGF2 treatment in hDPC lines, which seemed to correlate with the curative effects of these cells after SCI. Moreover, we observed two cell lines that showed poor performance in inducing recovery from SCI and low responsiveness of *GABRB1* after FGF2 treatment, suggesting that donor-wise differences in the effectiveness of cell transplantation might be assessed on the basis of gene regulation patterns. Upon further analyzing such variation in gene expression patterns in an increasing number of cell lines in therapeutic cell banks, we hope to gain greater insights into the potential of each cell line to develop a safer and more reliable therapy for SCI.

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Additional information Accession codes: the microarray data were submitted to the NCBI GEO database under the accession number (GSE83902).

Compliance with ethical standards

Ethical approval All procedures involving human participants were performed in accordance with the ethical standards of the institutional and with the 1964 Helsinki Declaration and its later amendments or

comparable ethical standards. All applicable institutional and national guidelines for the care and use of animals were followed.

Conflict of interest This manuscript was partly developed with support from Daiichi Sankyo Company Ltd.

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