Blockade of PD-L1/PD-1 signaling promotes osteo-/odontogenic differentiation through Ras activation

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INTRODUCTION

Human dental pulp stem cells (hDPSCs) are rich in human dental pulp tissues. Recently, hDPSCs have been substantially considered for several applications in the regenerative medicine and tissue engineering fields due to their benefits over the other sources. First, they are easily accessible and are easily obtained during routine dental procedures without ethical concerns, and can retain their stem cell properties even after long cryopreservation. Second, hDPSCs have a great potential with multilineage differentiation; they can differentiate into odontogenic lineage or whether PD-1 positively regulates hDPSCs osteo-/odontogenic differentiation. Treatment with nivolumab (a human anti-PD-1 monoclonal antibody), which targets PD-1 to prevent PD-L1 binding, successfully enhanced osteo-/odontogenic differentiation of hDPSCs through enhanced Ras activity-mediated phosphorylation of ERK and AKT. Our findings underscore that downregulation of PD-L1 expression accompanies during osteo-/odontogenic differentiation, and hDPSCs-intrinsic PD-1 signaling inhibits osteo-/odontogenic differentiation. These findings provide a significant basis that PD-1 blockade could be effective immunotherapeutic strategies in hDPSCs-mediated dental pulp regeneration.

The programmed cell death ligand 1 (PD-L1) and its receptor programmed cell death 1 (PD-1) deliver inhibitory signals to regulate immunological tolerance during immune-mediated diseases. However, the role of PD-1 signaling and its blockade effect on human dental pulp stem cells (hDPSCs) differentiation into the osteo-/odontogenic lineage remain unknown. We show here that PD-L1 expression, but not PD-1, is downregulated during osteo-/odontogenic differentiation of hDPSCs. Importantly, PD-L1/PD-1 signaling has been shown to negatively regulate the osteo-/odontogenic differentiation of hDPSCs. Mechanistically, depletion of either PD-L1 or PD-1 expression increased ERK and AKT phosphorylation levels through the upregulation of Ras enzyme activity, which plays a pivotal role during hDPSCs osteo-/odontogenic differentiation. Treatment with nivolumab (a human anti-PD-1 monoclonal antibody), which targets PD-1 to prevent PD-L1 binding, successfully enhanced osteo-/odontogenic differentiation of hDPSCs through enhanced Ras activity-mediated phosphorylation of ERK and AKT. Our findings underscore that downregulation of PD-L1 expression accompanies during osteo-/odontogenic differentiation, and hDPSCs-intrinsic PD-1 signaling inhibits osteo-/odontogenic differentiation. These findings provide a significant basis that PD-1 blockade could be effective immunotherapeutic strategies in hDPSCs-mediated dental pulp regeneration.

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PD-L1 inhibits hDPSCs differentiation into the osteo-/odontogenic lineage. As shown in Fig. 2e, PD-L1 knockdown (Fig. S1). Therefore, we checked whether these signals are affected by PD-L1 expression. As shown in Fig. 2e, PD-L1 knockdown markedly increased basal and ODM-induced phosphorylation levels of ERK and AKT, but not p38 (data not shown), in hDPSCs. Moreover, treatment of ERK inhibitor U0126 or PD98059 or Akt inhibitor MK-2206 abolished the PD-L1 silencing-induced expression levels of differentiation markers (Fig. 2f). Together, these results demonstrate that PD-L1 expression negatively regulates the hDPSCs differentiation into the osteo-/odontogenic lineage via downregulation of the ERK and AKT signals.
Fig. 1  PD-L1 expression is downregulated during hDPSCs differentiation into the osteo-/odontogenic lineage. a hDPSCs were harvested for the isolation of membrane and cytosolic fractions. Immunoblotting analyses were carried out and representative band intensity was quantified. b and c hDPSCs were cultured with or without ODM for the indicated days. Immunoblotting analyses were carried out and representative band intensity was quantified (b). Real-time PCR data for CD274, PDCD1, RUNX2, and DSPP (c). *P < 0.05; **P < 0.01; ***P < 0.001, Student’s t test. d and e hDPSCs were treated with or without A.A (L-ascorbic acid 2-phosphate), β-gly (β-glycerol phosphate), Dex (dexamethasone), or ODM for one day. Immunoblotting analyses were carried out and representative band intensity was quantified (d). Real-time PCR data for CD274 and PDCD1 (e). *P < 0.05; **P < 0.001, Student’s t test.
Based on our findings that PD-1 and PD-L1 had similar effects on Ras activity and its-dependent downstream signals and osteo-/odontogenic differentiation, we hypothesized that hDPSCs-intrinsic PD-1 signaling is engaged by PD-L1 to regulate osteo-/odontogenic differentiation and the corresponding signaling pathways. To confirm this, we used the FDA-approved
nivolumab (Opdivo), to block PD-L1-mediated intrinsic PD-1 signaling.13 We treated hDPSCs with nivolumab or isotype control antibody in the presence of ODM, and revealed that nivolumab-treated hDPSCs had higher activity and expression of Ras (Fig. 5a), phosphorylation levels of ERK and AKT (Fig. 5b), and expression levels of differentiation markers (Fig. 5c, d) compared to the IgG4 control antibody-treated cells. As a result, the nivolumab-treated cells exhibited increased ALP activities.

**Fig. 3** PD-1 inhibits hDPSCs differentiation into the osteo-/odontogenic lineage. a and b The control siRNA or PD-1 siRNA-transfected hDPSCs were cultured with or without ODM for 3 days (for RUNX2) or 6 days (for DSPP). Immunoblotting analyses were carried out and representative band intensity was quantified (a). Real-time PCR data for RUNX2 and DSPP (b). **P < 0.01, two-way ANOVA test. c and d The control siRNA or PD-1 siRNA-transfected hDPSCs were cultured with or without ODM for 6 days (for ALP staining and activity assay) or 21 days (for Alizarin red S staining and quantification). ALP staining and activity assay were performed (c). Alizarin red S staining was performed and quantified (d). *P < 0.05; **P < 0.01, two-way ANOVA test. e The control siRNA or PD-1 siRNA-transfected hDPSCs were cultured with ODM for one day. Immunoblotting analyses were carried out and representative band intensity was quantified. f The control siRNA or PD-1 siRNA-transfected hDPSCs were cultured with ODM or the indicated inhibitors (U0126, 10 μmol·L⁻¹; PD98059, 10 μmol·L⁻¹; or MK-2206, 5 μmol·L⁻¹) for 3 days (for RUNX2) or 6 days (for DSPP). Immunoblotting analyses were carried out and representative band intensity was quantified.
Silencing of PD-L1/PD-1 promotes hDPSCs differentiation into the osteo-/odontogenic lineage via Ras activation. a hDPSCs were cultured with or without ODM for the indicated days. RBD pull-down assay and immunoblotting analyses were carried out, and representative band intensity was quantified. b hDPSCs were cultured with or without ODM for the indicated days. Immunoblotting analyses were carried out and representative band intensity was quantified. c hDPSCs were cultured with or without ODM or Abd-7 (20 μmol·L⁻¹) for one day. Immunoblotting analyses were carried out and representative band intensity was quantified. d and e hDPSCs were cultured with or without ODM or Abd-7 (20 μmol·L⁻¹) for 3 days (for RUNX2) or 6 days (for DSPP). Real-time PCR data for RUNX2 and DSPP (d). Immunoblotting analyses were carried out and representative band intensity was quantified (e). *P < 0.05; **P < 0.001, two-way ANOVA test. f hDPSCs were cultured with or without ODM or Abd-7 (20 μmol·L⁻¹) for 2 days. Immunoblotting analyses were carried out and representative band intensity was quantified. g and h hDPSCs were cultured with or without ODM or Abd-7 (20 μmol·L⁻¹) for 6 days (for ALP staining and activity assay) or 21 days (for Alizarin red S staining and quantification). ALP staining and activity assay were performed (g). Alizarin red S staining was performed and quantified (h). **P < 0.01, two-way ANOVA test. The control siRNA or PD-L1 siRNA-transfected hDPSCs were cultured with or without ODM for one day. RBD pull-down assay and immunoblotting analyses were carried out, and representative band intensity was quantified. The control siRNA, PD-L1 siRNA, or PD-1 siRNA-transfected hDPSCs were cultured with or without ODM or Abd-7 (20 μmol·L⁻¹) for 3 days (for RUNX2) or 6 days (for DSPP). Immunoblotting analyses were carried out and representative band intensity was quantified.
Fig. 5  PD-L1/PD-1 signaling blockade nivolumab promotes hDPSCs differentiation into the osteo-/odontogenic lineage. a hDPSCs were cultured with or without ODM for one day in the presence of IgG4 or Nivolumab (10 μg/mL). RBD pull-down assay and immunoblotting analyses were carried out, and representative band intensity was quantified. b hDPSCs were cultured with or without ODM for one day in the presence of IgG4 or Nivolumab (10 μg/mL). Immunoblotting analyses were carried out and representative band intensity was quantified. c and d hDPSCs were cultured with or without ODM for 3 days (for RUNX2) or 6 days (for DSPP) in the presence of IgG4 or Nivolumab (1 or 10 μg·mL⁻¹). Immunoblotting analyses were carried out and representative band intensity was quantified (c). Real-time PCR data for RUNX2 and DSPP (d). **P < 0.01, Student’s t test. e and f hDPSCs were cultured with or without ODM for 6 days (for ALP staining and activity assay) or 21 days (for Alizarin red S staining and quantification) in the presence of IgG4 or Nivolumab (10 μg/mL). ALP staining and activity assay were performed (e). Alizarin red S staining was performed and quantified (f). ***P < 0.001, Student’s t test. g Schematic diagram of the proposed mechanism.
DISCUSSION

Many types of dental stem cells have been identified. These stem cells include the dental pulp stem cells (DPSCs),6 stem cells from exfoliated deciduous teeth (SHED),8 periodontal ligament stem cells (PDLSCs),9 gingiva-derived MSCs (GMSCs),10 apical papilla stem cells (APSCs),11 and stem cells from dental follicles (DFSCs).12 DPSCs are ectoderm-derived stem cells, which are originated from migrating neural crest cells.13,14 DPSCs share many biological characteristics, such as a fibroblast-like morphology, surface marker expression, differentiation, proliferation, and colony-forming behavior similar to those of MSCs, including bone marrow MSCs (BM-MSCs) and adipose tissue-derived stem cells (ADSCs).15,16 However, their proliferation potential and differentiation potential varies; DPSCs have a higher proliferation rate and clonogenic potential than MSCs 1,27 DPSCs exhibit stronger odontogenesis and neurogenesis capabilities, but relatively low potential to produce osteogenic, adipogenic, and chondrogenic tissues than BM-MSCs.12,17 In addition to their potential for proliferation and multilineage differentiation capacities, DPSCs have been shown to possess potent immunosuppressive activities28–30 that are found in BM-MSCs.29,30

PD-1-mediated inhibitory signals play a critical role in immune tolerance and homeostasis. PD-1 signaling has been intensively studied with a focus on the PD-1–expressed immune cells, including activated T cells.8 Recently, functional roles of PD-1 signaling have been extended to non-immune cell types, such as tumor cells,31–33 retinal ganglion cells,34 and stem cells.35 In particular, Shi group demonstrated that SHED express PD-1, which regulates cell proliferation and differentiation.36 However, the potential functions and expression patterns of PD-L1 and PD-1 during the differentiation of hDPSCs into the odontogenic lineage are unknown.

In this study, we found out that hDPSCs constitutively express both PD-L1 and PD-1 in the cytomembrane. However, only PD-L1 expression was lost upon initiation of osteo-/odontogenic differentiation, indicating that PD-L1 expression may be unnecessary and could exert an inhibitory role in the hDPSCs differentiation into the osteo-/odontogenic lineage; when we depleted PD-L1 expression in hDPSCs, more hDPSCs differentiated into the osteo-/odontogenic lineage, demonstrating that PD-L1 expression negatively regulates the hDPSCs differentiation into the osteo-/odontogenic lineage. Although PD-1 expression was not altered during the hDPSCs differentiation into the osteo-/odontogenic lineage, PD-1 expression exhibited the same function as its ligand, PD-L1. Notably, nivolumab treatment, which prevents the binding of PD-L1 to PD-1, enhanced hDPSCs differentiation into osteo-/odontogenic lineage. These data suggest that the hDPSCs–intrinsic function of the PD-L1/PD-1 axis plays an inhibitory role in the osteo-/odontogenic differentiation.

Most defined PD-1 signaling has been demonstrated by studies on acutely activated T cells. In T-cell receptor (TCR)-stimulated T cells, ligands-engaged PD-1 becomes phosphorylated at two tyrosine residues in its cytoplasmic domain, leading to binding of protein tyrosine phosphatases (PTPs), such as SHP2.2 These PTPs directly dephosphorylate proximal TCR signaling molecules and subsequently antagonize downstream signaling of the TCR, such as PI3K/AKT and ERK.28 Notably, the inhibitory roles of PD-1 in the AKT and ERK signals were also observed in some specific tumor cells.39 Consistently, our study revealed that PD-1 signaling suppresses the pro-oste/o-odontogenic differentiating pathways, AKT and ERK, in hDPSCs. Interestingly, we found that Ras enzyme activity is induced; this is required for its own expression, the activation of both ERK and AKT pathways, and hDPSCs differentiation into osteo-/odontogenic lineage. To the best of our knowledge, this is the first study to show the role of Ras protein in hDPSCs differentiation into the odontogenic lineage. Nivolumab-mediated hDPSCs–PD-1 blockade and hDPSCs-specific PD-L1 or PD-1 knockdown studies showed that hDPSCs–intrinsic PD-1 signaling negatively regulates Ras enzyme activity and its dependent Ras expression. On the other hand, hDPSCs–intrinsic PD-1 silencing-induced osteo-/odontogenic differentiation was reversed by pharmacologic inhibition of Ras, suggesting that PD-1 silencing inhibits osteo-/odontogenic differentiation through deactivation of Ras enzyme activity. The divergent effects of PD-1 signaling on Ras activity have been reported in different cell types. For instance, PD-1 inhibits TCR-mediated activation of Ras through an unknown mechanism in T cells,36 in contrast; PD-1 signaling enhances Ras activation through phosphorylation of Ras GRP2 in tumor cells.37,38 In addition to SHP2 phosphorylation activity, it functions as an adapter that binds to receptor tyrosine kinases (RTKs) and recruits the GRB/SOS complex to the plasma membrane, enhancing GTPase-activating protein SOS-mediated Ras activation.39 SHP2 is also expressed by hDPSCs.40 However, there is a need for further investigations on whether SHP2 is directly or indirectly involved in the Ras activation for hDPSCs differentiation into osteo-/odontogenic lineage. In addition, further studies are needed for clear elucidation of the precise underlying mechanism of PD-1–mediated Ras inactivation in hDPSCs.

hDPSCs is from human adult permanent dental pulp tissue1 while SHED are isolated from exfoliated deciduous teeth.20 These cells have abilities in multilineage differentiation, self-renewal, and immunomodulatory functions.40 Hence, both hDPSCs and SHED have potential advantages in regenerative treatments and immunotherapy. Compared with hDPSCs, SHED show a higher differentiation potential, proliferation, and ability to form mineralized nodules in vivo.40–42 Liu et al. showed that PD-1 negatively regulates the SHED differentiation into osteo-/odontogenic lineage through suppressing β-catenin signal pathway,35 which is a distinct mechanism from our findings, although the role of PD-1 in the differentiation is the same in hDPSCs and SHED.

We showed different expression patterns of PD-L1 and PD-1 during the hDPSCs differentiation into osteo-/odontogenic lineage. In our differentiation system using general differentiation medium, only PD-L1 expression was downregulated during osteo-/odontogenic differentiation. It was revealed that dexamethasone inhibits CD274 transcriptional expression, but has no effect on PDCD1, in hDPSCs. Consistent with our results, dexamethasone has been shown to mediate transcriptional suppression of CD274 depending on the GR/STAT3 complex.43 These results suggest that the regulatory molecular mechanisms in the PD-L1/PD-1 expression seem to be different at the transcriptional levels.

Nivolumab is the most representative PD-1 inhibitor for the clinical treatment of advanced tumors.16–18 Besides cancer immunotherapy, as the first study demonstrating the effect of nivolumab in promoting hDPSCs differentiation into the osteo-/odontogenic lineage, the present study findings have strong clinical positive implications in the field of dental pulp therapy and provides a foundation for future clinical trials in dental pulp immunotherapy; our in vitro findings provide a basis for further research on the nivolumab effects with preclinical animal models.

In conclusion, we showed that inhibition of hDPSCs–intrinsic PD-L1/PD-1 signaling promotes osteo-/odontogenic differentiation via Ras activation (Fig. 5g). Moreover, for the first time, we showed that FDA-approved PD-1 blockade nivolumab exerted a
pro-osteo-/odontogenic differentiating effect on hDPSCs. Therefore, a combination of hDPSCs transplantation and PD-1 blockade or Ras activation could be a potential new therapeutic method for the regeneration or repairment of the dentin-pulp tissue.

**MATERIALS AND METHODS**

**Chemicals and reagents**

Abd-7 (Cat. # HY-122862) was purchased from MedChem Express (Princeton, NJ). l-ascorbic acid (Cat. # A4544), β-glycerophosphate (Cat. # 50020), dexamethasone (Cat. # D1756), ALP buffer (Cat. # A9226), p-Nitrophenyl phospahte tablets (Cat. # P5744), Alizarin Red (Cat. # TMS-008), hexadecylpyridinium chloride monohydrate (Cat. # C9002), and U0126 (Cat. # 662005) were acquired from Sigma-Aldrich (St. Louis, MO). hDPSCs (Cat. # PT-5025; Switzerland) were maintained in α-MEM (Welgene, South Korea), supplemented with 10% FBS (MERCK; Kenilworth, NJ). The passage numbers of the cells used in this study were from ReproCell (Beltsville, MD).

Culture of hDPSCs

hDPSCs (Cat. # PT-5025; Switzerland) were maintained in α-MEM (Welgene, South Korea), supplemented with 10% FBS (MERCK; Kenilworth, NJ). The passage numbers of the cells used in this study were limited to 2–3. To induce the hDPSCs differentiation into the osteo-/odontogenic lineage, the cells were cultured with osteo-/odontogenic differentiation medium (ODM) containing l-ascorbic acid (50 µg·mL⁻¹), β-glycerophosphate (10 mmol·L⁻¹), and dexamethasone (10 µmol·L⁻¹).

Real-time PCR analysis

Real-time PCR was performed as described in our previous study. For a ALP activity assay, hDPSCs were dissolved with ALP buffer, and then an ALP activity was determined in cell supernatant. An ALP staining was performed as described in our previous study. For a ALP activity assay, hDPSCs were dissolved with ALP buffer, and then an ALP activity was determined in cell supernatant.

Immunoblot analysis

Immunoblot analysis was performed in accordance to our previous study. The used antibodies were: rabbit anti-PD-L1 (1:1000), rabbit anti-ERK (pT202/pY204, 1:1000), rabbit anti-ERK (1:1000), rabbit anti-AKT (pS473, 1:1000), rabbit anti-AKT (1:1000), mouse anti-E-cadherin (1:500), mouse anti-PD-L1 (1:1000), rabbit anti-ERK (pT202/pY204, 1:1000), rabbit anti-AKT (pS473, 1:1000), mouse anti-ERK (1:1000), rabbit anti-AKT (pS473, 1:1000), rabbit anti-PD-1 (1:1000), mouse anti-E-cadherin (1:500), mouse anti-PD-L1 (1:1000), rabbit anti-ERK (pT202/pY204, 1:1000), rabbit anti-AKT (pS473, 1:1000), mouse anti-ERK (1:1000), rabbit anti-AKT (pS473, 1:1000), rabbit anti-PD-1 (1:1000), rabbit anti-ERK (pT202/pY204, 1:1000), rabbit anti-AKT (pS473, 1:1000), mouse anti-E-cadherin (1:500), mouse anti-PD-L1 (1:1000), rabbit anti-ERK (pT202/pY204, 1:1000), rabbit anti-AKT (pS473, 1:1000).

Data analysis

All the quantitative results are shown as means ± standard deviation (SD) of at least three independent experiments with duplicates or triplicates. A two-group comparison or a simultaneous comparison of more than two groups was conducted using a two-sided, two-sample Student's t-test or two-way ANOVA with Sidak's multiple comparisons test, respectively. P < 0.05 was considered statistically significant.

**DATA AVAILABILITY**

All data are included in the manuscript.

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AUTHOR CONTRIBUTIONS
S.M.J. contributed to the conception, design, and data acquisition; J.S.L., S.H.P., H.J.K. contributed to data acquisition; H.-R.K. and J.-H.L., contributed to conception, design, data acquisition, interpretation and analysis, drafted and critically revised the manuscript.

ADDITIONAL INFORMATION
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