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Original Article

Injectable platelet-rich fibrin promotes proliferation and trichogenic inductivity of dermal papilla cells through activating TGF-β/Smad signaling pathway

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Abstract

Dermal papilla cell (DPC) belongs to a specialized mesenchymal stem cell for hair follicle regeneration. Maintaining the ability of DPCs to stimulate hair in vitro culture is important for hair follicle morphogenesis and regeneration. As the third generation of platelet concentrate, injectable platelet-rich fibrin (i-PRF) is a novel biomaterial containing many growth factors and showing promising effects on tissue reconstruction. We aimed to explore the influences of i-PRF on the proliferative, migratory, as well as trichogenic ability of DPCs and compared the effects of i-PRF and platelet-rich plasma (PRP), the first generation of platelet concentrate. Both PRP and i-PRF facilitated DPCs proliferation, and migration, along with trichogenic inductivity as well as stimulated the TGF-β/Smad pathway, while the impacts of i-PRF were more significant than PRP. A small molecule inhibitor of TGF-beta receptor I, Galunisertib, was also applied to treat DPCs, and it rescued the impacts of i-PRF on the proliferative, migratory, trichogenic inductivity, and proteins-associated with TGF-β/Smad pathway in DPCs. These findings revealed that i-PRF had better effects than PRP in enhancing the proliferative, migratory, and hair-inducing abilities of DPCs by the TGF-β/Smad pathway, which indicated the beneficial role of i-PRF in hair follicle regeneration.

Keywords: Injectable platelet-rich fibrin, Platelet-rich plasma, Dermal papilla cell, Proliferation, Hair inducing ability, Hair follicle regeneration, TGF-β/Smad signaling pathway.

1. Introduction

Alopecia is featured by shorter anagen together with longer telogen phases in the hair cycle and is a psychological challenge affecting social communication of the patients. Suitable seed cells, enough seed cells with biological functions when cultured *in vitro*, and a suitable microenvironment are essential for hair follicle reconstruction [1]. Dermal papilla cells (DPCs) belong to specialized mesenchymal stem cells in hair follicles and have potential to differentiate into many types of cells, which makes them beneficial for the repair of hair formation or growth. DPCs are deemed to have the capacity to treat alopecia because of their hair inductivity [2, 3]. Some studies revealed DPCs as a therapeutic target of alopecia [4-6]. However, the replicative senescence of DPCs causes changes in cellular phenotypes and reduces the hair-inducing ability. Therefore, keeping the hair inductivity of DPCs is essential for the therapy of alopecia.

Platelet concentrate can release various growth factors like platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) along with vascular endothelial growth factor (VEGF), playing a very crucial role in enhancing cell recruitment, proliferation, as well as differentiation of tissue regeneration [7]. It has been validated that platelet-rich plasma (PRP), the first generation of platelet concentrate, can effectively promote hair regeneration [8, 9]. However, PRP needs to be activated by exogenous activators before application, is rapidly degraded *in vivo*, and rapidly releases growth factor, which restricts the clinical use of PRP. Platelet-rich fibrin (PRF) belongs to a second-generation platelet concentrate that does not contain anticoagulants. However, due to the fibrous gel shape of PRF: PRF is not easy to combine with other biomaterials, which limits its clinical application [10]. Injectable platelet-rich fibrin (i-PRF) is the third generation of platelet concentrates [11]. It has the advantages combined with the first and second-generation platelet concentrate. i-PRF not only releases abundant cytokines and growth factors stably and continuously, but also promotes tissue regeneration and improves the survival rate of tissue transplantation. Moreover, freshly prepared i-PRF can combine with various biological materials and will exist in the form of fibrin gel about 15 minutes after preparation. Moreover, compared with PRP and PRF, i-PRF has a simpler prepara-

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tion process and does not need to add any exogenous activator, thus avoiding related ethical problems. As an ideal three-dimensional scaffold in tissue engineering, i-PRF has been extensively used in clinical practice [12, 13].

In this study, influences of i-PRF on the proliferative, migrative, and hair-inducing abilities of human and mouse DPCs were explored, which is beneficial for the efficient preparation of tissue-engineered hair follicles *in vitro* and is meaningful for future clinical study on alopecia.

2. Materials and methods

2.1. Participants

Human intact scalp skin was obtained from patients with benign skin tumors or scalp injury. Whole venous blood samples were donated by healthy volunteers without smoking history or abnormality of biochemical tests. Assays using human samples were used with the approval of the Ethical Committee of the Affiliated Taizhou Second People's Hospital of Yangzhou University after obtaining the written informed consent from all patients.

2.2. Animals

C57BL/6J mice aged at 4-6 weeks of both sexes were acquired from the Vital River company (Beijing, China). Under the approval of the Institutional Animal Care and Use Committee of the Affiliated Taizhou Second People's Hospital of Yangzhou University, animal experiments were conducted.

2.3. Isolation and culture of DPCs from mouse (mDPCs) and human (hDPCs)

Anagen vibrissae follicles of mice were dissected with fine forceps. Excessive hair shafts outside the skin and the subcutaneous fat tissue were removed with microscissors. Samples were rinsed with Phosphate Buffer Saline that contains 1% penicillin-streptomycin three times, and 2.5 mg/ml collagenase IV was used to digest the surrounding collagen capsules in the follicles at 37°C for 2 h. A large proportion of DPCs were exposed at the bulbs of follicle. Next, isolation of DPCs was taken under a microscope. Isolated DPCs were taken to culture in Dulbecco's modified Eagle's medium (DMEM) which included 10% fetal bovine serum together with 1% penicillin-streptomycin at 37°C in a humidified atmosphere with 5% CO_2. Every three days, the cell culture medium was taken to replace. When DPCs reached 80% confluence, cells were added with 0.25% trypsin-ethylene Diamine Tetraacetic Acid and shifted to new culture flasks with a split ratio of 1:2. hDPCs were prepared the same as mDPCs. DPCs in passage 2 were adopted for the subsequent experiments. For each assay, there were three primary cell cultures with three independent technical replicates. DPCs were characterized according to a previous study [14].

2.4. Preparation of i-PRF and PRP

Whole venous blood (10 mL) was drawn from the right median cubital vein. After transfer into a coagulant-free plastic test tube, the samples were centrifuged with a Sorvall ST1 Plus desk centrifuge (75009740; ThermoFisher Scientific) [15]. The upper liquid layer was taken to collect as i-PRF and placed in a 6-well plate for 1.5 h to form a complete clot followed by cultivation with 5 mL DMEM (Gibco) with no other supplements in an incubator with 90% humidity and 5% CO_2 for 3 d. Next, the medium was aspirated and served as 100% i-PRF conditioned medium, which was diluted with standard culture medium with serum and antibiotics to create 20% (v/v) i-PRF conditioned medium since 20% concentration is the best for regenerating hair follicle [16].

PRP was produced using a Pro health care system Prosys PRP Kit (KMedicins, Korea) and a concentration kit according to a previous study [17]. 10% PRF-conditioned medium was used to culture DPCs [18].

2.5. Evaluation of cell proliferation

DPCs (4×10^3 cells/well) were planted in 96-well plates. After 12, 24, 72, as well as 120 h, each well was taken to treat with 10 μL CCK-8 reagent, and cells were taken to incubate at 37°C for 2 h. A Microplate Absorbance Reader (HBS-1096A; Beyotime, Shanghai, China) was used to assess optical density (OD) by testing cell absorbance at a wavelength of 450 nm. For clone formation assay, DPCs were treated with i-PRF: PRP, or i-PRF + Galunisertib and planted into 6-well plates at the concentration of 1000 cells per wall. Every 3 days, cell culture medium was replaced. Fourteen days later, cells were taken to fix and dye with crystal violet, and the colonies were counted.

2.6. Cell migration assay

Using a Boyden chamber, hDPCs (6×10^4 cells/well) were cultured in the serum-free medium in the upper chamber of the polyethylene terephthalate cell culture inserts (pore size: 8 μm; Corning). The PRP or i-PRF-conditioned mediums were added into the lower chamber. Followed by 24 h, cells were taken to fix with 4% paraformaldehyde (P1110, Solarbio) for 20 min and stain with crystal violet dye (E607309-0100, Sangon Biotech, Shanghai, China) for 15 min. Cells remaining on the upper chamber were considered as non-migrated cells and were removed. Migrated cells were photographed from five random fields via an inverted microscope (Zeiss, Germany). The ImageJ software was used for cell counting quantification.

2.7. Reverse transcription-quantitative PCR

Total RNAs were taken to isolate from hDPCs and mDPCs after culture with the PRP or i-PRF conditioned medium for 14 days via a Total RNA Extraction Kit (R1200, Solarbio, China). cDNA was immediately synthesized from a total of 1 μg RNA via a Universal RT-PCR Kit (M-MLV) (RP1100, Solarbio). Relevant primer sequences were listed as follows (orientation: 5'→3'): ALP (human), F: CATCCTGTATGGCAATGGG and R: TGTTGTGAGCATAGTCCAC; β-catenin (human), F: CCAAGTCCTGTATGAGTGGG and R: GCATACTGTCCATCAATATCAGC; Versican (human), F: AGTGGATAGGCCTCAATGAC and R: TCCAATTCTCGTATTGCAGTG; TGFBR1 (human), F: AGCTGTGAAGCCTTGAGAG and R: CAATGCTGTAAGCCTAGCTG; TGFBR2 (human), F: AATCCTGCATGAGCAACTG and R: CATTCTTTCTCCATACAGCCA; GAPDH (human), F: TCATTTCCTGGTATGACAACGA and R: GTCTTACTCCTTGGAGGCC; ALP (mouse), F: TTCCTGGGAGATGGTATGG and R: AATTTGTCCATCTCCAGCC; β-catenin (mouse), F: CGCCTTCATTATGGACTGC and R: TCCAACAGTTGCCTTTATCAG; Versican (mouse), F: ACTGTCAGATATCCCATACGG and R: AATCCATAGGTCCGAACCC; TGFBR1 (mouse), F: GCTGACATCTATGCAATGGG and R: CTGATAGTCTTCATGGATTCCAC;

TGFBR2 (mouse), F: CAAGTCGGTTAACAGTGATGTC and R: AGTGGACAGTCTCACATCG; GAPDH (mouse), F: ACTCTTCCACCTTCGATGC and R: CCGTATTCATTGTCATACCAGG. PCR was implemented using BeyoFusion™ PCR Master Mix (2X) (D7250-5ml, Beyotime) on an ABI 7500 real-time PCR system (4351106, Applied Biosystems). Relative RNA expression was calculated on the grounds of the $2^{-\Delta\Delta Ct}$ method [19] and was normalized to GAPDH.

2.8. Western blotting

The proteins were taken to extract from DPCs using RIPA Lysis Buffer, separated using a 12% SDS-PAGE gel, and shifted to a nitrocellulose membrane. After sealing in 5% skimmed milk and washing with a TBST buffer three times, samples were incubated with primary antibodies including ALP, β-catenin, Versican, TGFBR1, TGFBR2, SMAD2, SMAD3, p-SMAD2, p-SMAD3, and β-actin at 4ºC overnight. Subsequently, the blots were detected by chemiluminescence using the corresponding secondary antibodies. All the antibodies were purchased from Abcam (Shanghai, China). A ChemiDoc MP Imaging System (17001402, BIO-RAD) was adopted for band detection.

2.9. Immunofluorescence staining

DPCs (6×10^4 cells/well) were plated on glass slides, fixed with 4% paraformaldehyde, washed with PBS, as well as permeabilized with 1% Triton X-100 (Beyotime, China). Next, cells were taken to block with 5% BSA for 1 h and incubated with primary antibodies against ALP, Versican, and β-catenin overnight at 4ºC. On the second day, cells were taken to incubate with relevant secondary antibodies for 2 h at room temperature. All the antibodies were purchased from Abcam. DAPI (C1002, Beyotime, China) was used for staining the nuclei. An inverted fluorescence microscope (Zeiss, Germany) was used to observe the fluorescence signals.

2.10. Statistical analysis

Data were derived from three replicates in three primary cultures and expressed as mean ± standard deviation (SD). P values ≤ 0.05 were deemed to be significant and were calculated by one-way ANOVA followed by Tukey's or Dunnett's *post hoc* tests and two-way ANOVA using the GraphPad Prism 9.0 software.

3. Results

3.1. i-PRF promotes the proliferative and migrative capacities of DPCs

As shown in Figure 1A-B, i-PRF and PRP increased the viability and proliferative ability of DPCs. Figure 1C revealed that i-PRF and PRP increased number of migrated DPCs. i-PRF had a more significant effect on promoting the viability, proliferative and migrative capacities of DPCs than PRP.

3.2. i-PRF promotes ALP, Versican, along with β-catenin expression in DPCs

PCR, western blotting, and immunofluorescence staining assays were conducted to assess ALP, Versican, along with β-catenin expression in DPCs by treating PRP and i-PRF. The results revealed that either PRP or i-PRF can enhance ALP, Versican, and β-catenin expression, while i-PRF caused more significantly higher expression of these proteins (Figure 2A-C).

3.3. The TGF-β/Smad pathway is activated by i-PRF

Expression of TGFBR1 together with TGFBR2 was increased by PRP and i-PRF in DPCs, as revealed by PCR analysis (Figure 3A). Western blotting was conduc-

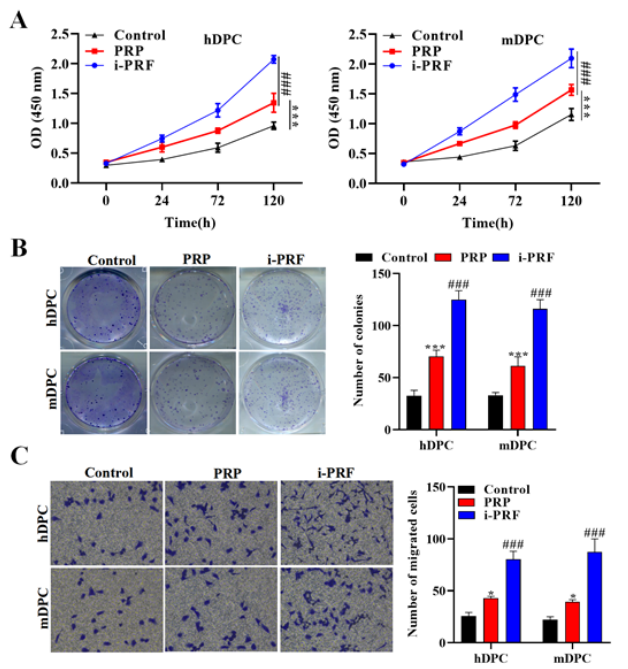

Fig. 1. i-PRF promotes the proliferation and migration of DPCs. (A) A CCK-8 assay was done to evaluate cell viability of DPCs by treatment of i-PRF and PRP 0 h, 24 h, 72 h, and 120 h. (B) Number of colonies formed by DPCs after treatment of i-PRF and PRP was counted. (C) A Transwell assay was performed to reveal the number of migrated DPCs after treatment of i-PRF and PRP. **p < 0.01.

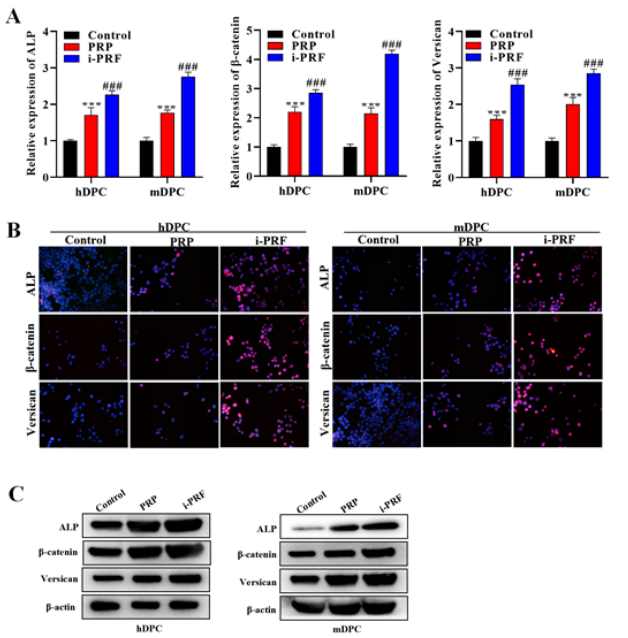

Fig. 2. i-PRF promotes ALP, Versican, and β-catenin expression in DPCs. (A) Relative ALP, Versican, and β-catenin expression in DPCs after treatment of i-PRF and PRP was assessed by PCR. (B-C) Immunofluorescence staining assays and western blotting were conducted to reveal expression of ALP, Versican, and β-catenin proteins in DPCs after treatment of i-PRF and PRP. **p < 0.01.

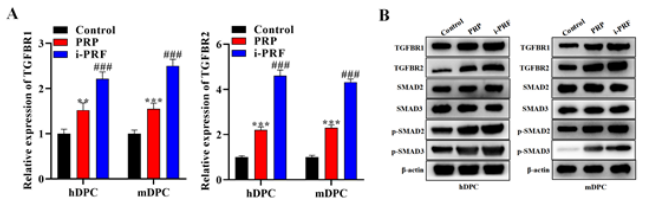

Fig. 3. The TGF-β/Smad pathway is activated by i-PRF. (A) Relative ALP, Versican, and β-catenin expression in DPCs after treatment of i-PRF and PRP was assessed by PCR. (B-C) Immunofluorescence staining assays and western blotting were conducted to reveal expression of ALP, Versican, and β-catenin proteins in DPCs after treatment of i-PRF and PRP. **p < 0.01.

ted to further assess the TGF-β/Smad pathway-associated proteins. PRP and i-PRF enhanced TGFBR1 and TGFBR2 proteins and increased the ratios of p-SMAD2/3 to SMAD2/3 (Figure 3B).

3.4. Galunisertib rescues the influences of i-PRF on the viability, proliferative and migrative capacities of DPCs

A small molecule inhibitor of TGF-beta receptor I, Galunisertib, was used to treat DPCs to explore whether the influences of i-PRF on DPCs were dependent on the TGF-β/Smad pathway. Galunisertib rescues the influences of i-PRF on viability (Figure 4A), proliferation (Figure 4B), and migration of DPCs (Figure 4C).

3.5. Galunisertib rescues the influences of i-PRF on ALP, Versican, along with β-catenin expression in DPCs

The positive effects of i-PRF on ALP, Versican, as well as β-catenin expression at mRNA and protein levels in DPCs were rescued by Galunisertib (Figure 5), suggesting that i-PRF promotes the trichogenic ability of DPCs by the TGF-β/Smad pathway.

4. Discussion

Hair follicles go through complicated epithelial-mesenchymal interactions in embryonic and birth stages. DPCs facilitate epithelial cell proliferation and induce epithelial stem cell differentiation to regulate the cycle of hair follicles [20]. The transplantation of DPCs can induce hair follicles in nude mice [21]. However, DPCs lose hair follicle inductivity after several passages during the *in vitro* culture, which is a major problem in its clinical application [21, 22]. As a purely autologous 3D living biomaterial, i-PRF possesses better biological properties compared with PRP and PRF, which makes it a more promising tool for tissue regeneration [15]. In this research, the functions of i-PRF on the viability, proliferation, and migration, together with trichogenic ability of DPCs were assessed and the influences of i-PRF and PRP were compared. The results revealed that i-PRF had the better effects than PRP in promoting the proliferative, migrative, and hair-inducing abilities of DPCs.

i-PRF contains more cytokines and growth factors than PRP and PRF, which may explain its better functions than PRP in DPCs in our findings. It contains all components of blood except plasma and red blood cells, namely platelets, white blood cells, fibrin, growth factors including PDGF,

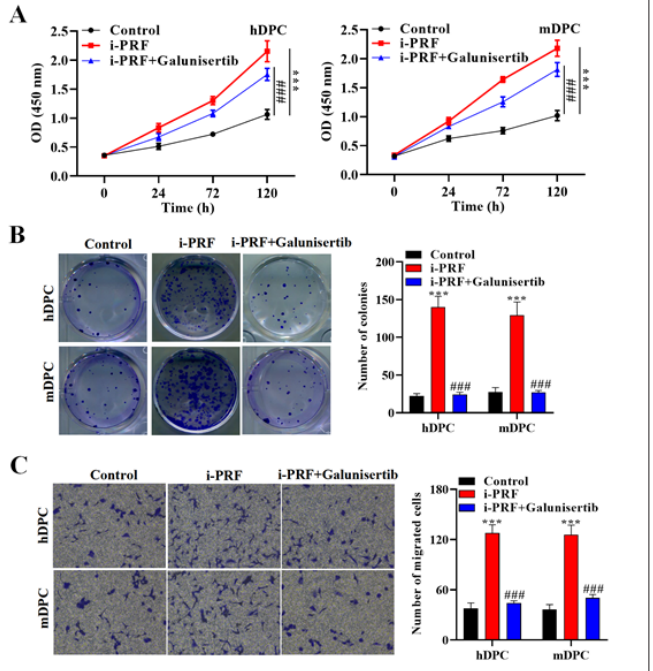

Fig. 4. Galunisertib rescues the effects of i-PRF on the viability, proliferation, and migration of DPCs. Cell viability of DPCs by treatment of i-PRF or i-PRF + Galunisertib (10 μM) after incubation with CCK-8 reagent was evaluated. (B-C) After treatment of i-PRF or i-PRF + Galunisertib, colonies formed by DPCs (B) and migrated DPCs (C) were photographed and counted. **p < 0.01.

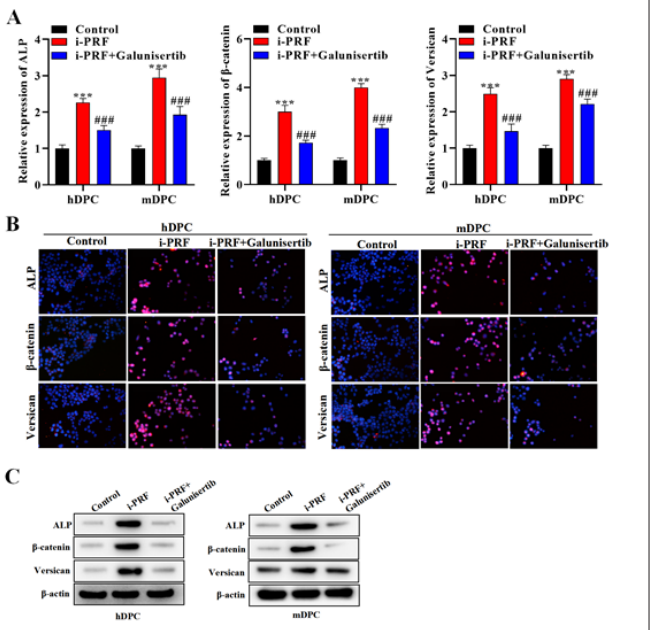

Fig. 5. Galunisertib rescues the effects of i-PRF on ALP, Versican, and β-catenin expression in DPCs. (A) Relative mRNA expression of ALP, Versican, and β-catenin in DPCs after treatment of i-PRF or i-PRF + Galunisertib (10 μM) was detected by PCR. (B-C) Immunofluorescence staining and western blotting of ALP, Versican, and β-catenin proteins in DPCs after treatment of i-PRF or i-PRF + Galunisertib. **p < 0.01.

basic FGF, epidermal growth factor, VEGF, transforming growth factor-β, as well as insulin-like growth factor-1 [23] with relevant biomolecular activities. For example, it has been shown that FGF2 can enhance the proliferation of DPCs and maintain its hair induction ability [24]. PDGF is necessary for inducing and maintaining hair follicles in

the growth phase *in vivo* [25]. PDGF and FGF2 have been confirmed to synergistically promote proliferative capacity and hair induction activity of DPCs [26].

The TGF-β/Smad as well as Wnt/β-catenin pathways can influence the development of hair follicles [27-29]. The mechanisms of the TGF-β signaling cascade in hair regeneration were associated with BMPs [30, 31]. BMP2 [32], BMP4 [33], BMP6 [28], and BMP7 [34] were candidate genes that regulate hair follicle growth. In this study, i-PRF caused a higher expression of TGFBR1, and TGFBR2, led to a higher ratio of p-SMAD2/3 to SMAD2/3 than PRP, suggesting that i-PRF had better effects in stimulating the TGF-β/Smad pathway than PRP, and that the better impacts of i-PRF than PRP in promoting the proliferative, migrative, and hair-inducing abilities of DPCs were associated with the TGF-β/Smad pathway.

However, our *in vitro* study based on cell culture only mimics a portion of the complex architecture of native organs. *In vivo* hair growth experiments in animals were needed to further compare the functions of i-PRF and PRP in hair follicle regeneration. Our future study will also compare the influences of i-PRF and PRF in hair follicle reconstruction to further support the advantages of i-PRF in tissue regeneration.

In conclusion, our study suggests that i-PRF, the third generation of platelet concentrates, enhances the proliferative, migrative, and hair-inducing abilities of DPCs by the TGF-β/Smad pathway, indicating it as an ideal biocompatible material for hair restoration. Moreover, i-PRF had a better promotive effect than PRP in trichogenic ability of DPCs, confirming the strength of i-PRF as a promising platelet concentrate in hair follicle regeneration.

Informed Consent
The authors report no conflict of interest.

Availability of data and material
We declared that we embedded all data in the manuscript.

Authors' contributions
XK, DX and YM conducted the experiments and wrote the paper; CB, ZL and DC analyzed and organized the data; HT conceived, designed the study and revised the manuscript.

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