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Supplemental Information

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Photobiomodulation therapy for hair regeneration: A synergetic activation of β-CATENIN in hair follicle stem cells by ROS and paracrine WNTs

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Histology, immunostaining and western blot

Mice skin tissues were fixed in 4% paraformaldehyde at 4℃ overnight and then dehydrated to be embedded in the OCT compound for frozen tissue preparations, 10 μm frozen sections were performed on a Leica CM 1850 Cryostat for hematoxylin/eosin staining and immunohistochemical. Sections were incubated in corresponding primary antibodies at 4℃ overnight. Primary antibodies were conjugated to Alexa Fluor 488/555/647 secondary antibodies. The antibodies used for immunofluorescence are displayed on Supplementary Table 2. Two skin tissues from different regions were observed in each mouse. At least 20 sections which included more than 3 complete HFs in each skin tissues were analyzed. The average fluorescence intensity of immunohistochemical staining and HF length were analyzed by ImageJ software. For immunohistochemical quantification, we select pictures of equal area and use Image J software to analyze the average fluorescence intensity of each picture. For animal experiments, we averaged the average fluorescence intensity of all sections of each animal as the quantitative result of per mouse. For cell experiments *in vitro*, we averaged the average fluorescence intensity of each experimental picture (n = 10), and each experiment was repeated at least 3 times, and finally quantitative analysis was performed.

Mouse skin tissue was excised and floated on Dispase II (1 U/mL) overnight at 4℃, the epidermis was separated from dermis, and HF tissue was scraped from the epidermis. HF tissue and cell lysates were prepared in a lysis buffer containing 50

mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 100 μg/mL PMSF with protease inhibitor cocktail set I for 60 min on ice and quantified by the Bradford assay. Protein samples were separated on a 10% SDS-PAGE gel and transferred to a PVDF membrane (Millipore, Billerica, MA, USA), and then detected with different primary antibodies as shown in Supplementary Table 2.

HFSC, SKP and Fibroblast isolation and culture

HFSC isolation: whole dorsal mouse skin was harvested from C57BL/6 mice within 24 h after birth. The skin tissue was excised and floated on Dispase II (1 U/mL) at 37°C for 60 min in 10 cm cell culture dish. The epidermis was manually separated from dermis. To cut up the epidermal tissue, and then tissue was incubated in a 0.3% collagenase I (Sigma) solution in Dulbecco's modified Eagle's medium/F12, 3:1 (DMEM/F12) (Gibco) for 30 min at 37°C. Next, to centrifuge at 350 g for 5 min, and then it was resuspended with 1-2 ml 0.25% trypsin and digested at 37°C for 5 min, DMEM containing 2% FBS was added to stop the digestion. The digested epidermal tissue was filtered through a 40 μm cell strainer, centrifuged, the cell suspension was resuspended and seeded in cell culture dishes coated with 10 μg/cm^2 collagen IV (Corning, USA), and cultured in DMEM supplemented with 10% FBS (Gibco) for 60 min. The non-adherent cells were removed, and adherent cells were incubated in CnT-07 PCT Epidermal Keratinocyte Medium to obtain HFSCs. To maintain the basic characteristics of primary HFSCs, primary HFSCs not passaged cells were used in all experiments.

SKP isolation: whole dorsal mouse skin was harvested from C57BL/6 mice within 24 h after birth. The skin tissue was excised and floated on Dispase II (1 U/mL) at 37°C for 60 min in 10 cm cell culture dish. The dermis was manually separated from epidermis. To cut up the dermis tissue to be incubated in a 0.1% collagenase I (Sigma) solution in DMEM/F12 for 30-60 min at 37℃. Next, DMEM containing 2% FBS was added to stop the digestion. The digested dermal tissue was filtered through a 40 μm cell strainer, centrifuged, resuspended. Single-cell suspensions were seeded in 10 cm cell culture dishes coated with 0.05% F127 (P-3000MP, Gibco), and cultured in DMEM/F12 supplement with B27, 20 ng/mL epidermal growth factor (EGF) (Gibco, PHG0311) and 20 ng/mL basal fibroblast growth factor (bFGF) (Gibco, 13256029) for 12 h. The cell culture dish is gently shaken, then the non-adherent cells in the supernatant are aspirated and centrifuged at 350 g for 5 min, wash it with PBS, centrifuge again at 350 g for 5 min. Single-cell suspensions were seeded in 10 cm cell culture dishes coated with 0.05% F127, and cultured in DMEM/F12 supplement with B27, 20 ng/mL EGF and 20 ng/mLbFGF to isolate SKPs.

Fibroblast isolation: the separation of skin fibroblasts refereed to the previous method (Lichti et al., 2008)

Flow cytometer and colony-forming assays

Cells were stained with K15 and SOX9 antibodies in a solution of 1% BSA in PBS

for 30 min at room temperature. Ten thousand cells were analyzed by the FACS Canto flow cytometer (Becton Dickinson, Mountain View, CA, USA) using FlowJo VX10 software. 20000 cells per well were seeded using a six-well plate (Corning-Costar, USA). The cells were grown in CnT-07 PCT Epidermal Keratinocyte Medium, and PBM was treated every other day and maintained for 10 d. To visualize the HFSC colonies, cells were fixed with 4% paraformaldehyde and stained with 0.1% Crystal Violet Staining Solution. Three independent experiments were conducted.

Monitoring ROS production

Fluorescent probes 2′, 7-Dichlorodihydrofluorescein diacetate (DCF-DA, Beyotime Biotechnology) were diluted in serum-free DMEM medium to a final concentration of 10 μM. HFSCs were treated with PBM, followed by incubated with DHF-DA for 40 min, the HFSCs were washed three times with PBS, then detection by flow cytometer and confocal microscope (LSM 880; Carl Zeiss MicroImaging, Jenna, Germany).

Real-time PCR

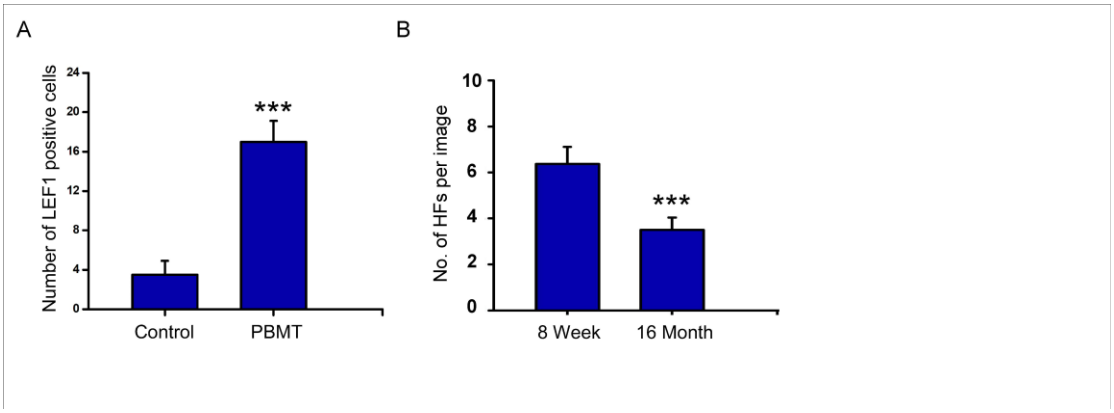
Total RNA was extracted with RNAiso Plus (TaKaRa, D9108A) following the manufacturer's instructions. The first-strand cDNA was synthesized with a ReverTra Ace qPCR (Quantitative Real-time PCR) RT Kit (TOYOBO, FSQ-301). Supplementary Table 1 provides primers for amplification of murine genes used in experiments. Real-time PCR was performed using SYBR Green PCR mix on a CFX Connect^{TM} Real-Time System normalized against β-actin, and fold changes were

calculated based on the $2^{-\triangle\triangle Ct}$ algorithm.

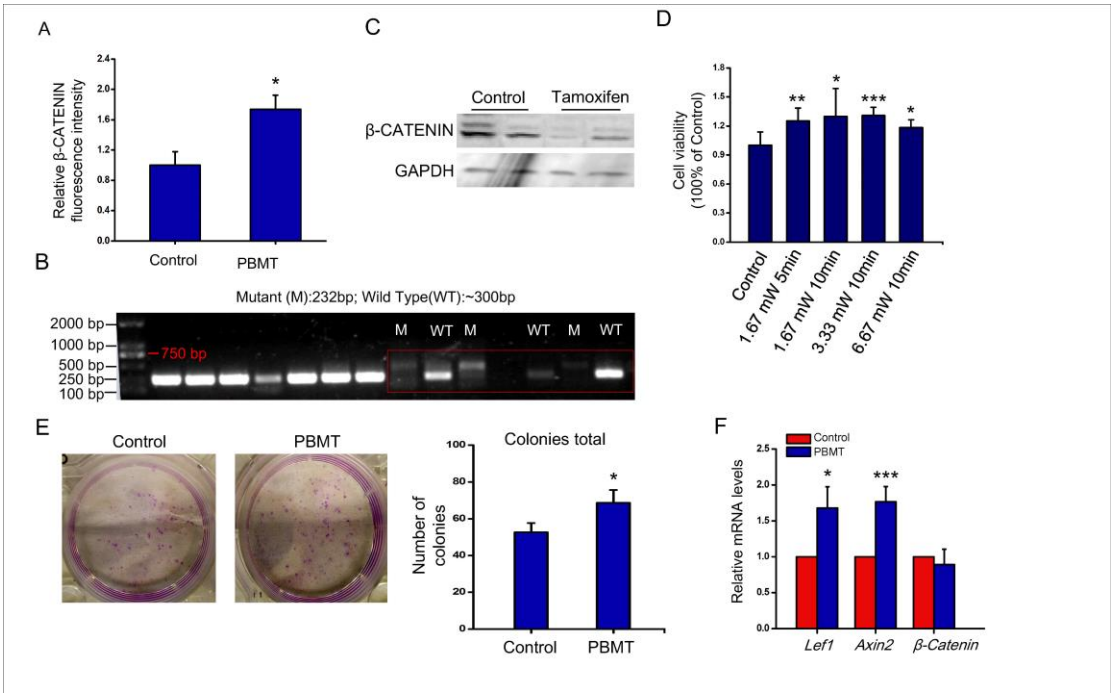
Cell Proliferation Assay

The cell-counting kit-8 (CCK-8) was evaluated HFSC proliferation, 5000 cells were seeded in 96-well plates, starved with DMEM serum-free medium for 8 h, and treated with PBMT, then incubated at 37℃ in 5% CO_2 for 24 h, followed added to 10 μL of the CCK-8 solution for another 3 h. Microplate reader (TECAN, infinite M200, Austria) was used to measure the absorbance at 450 nm.

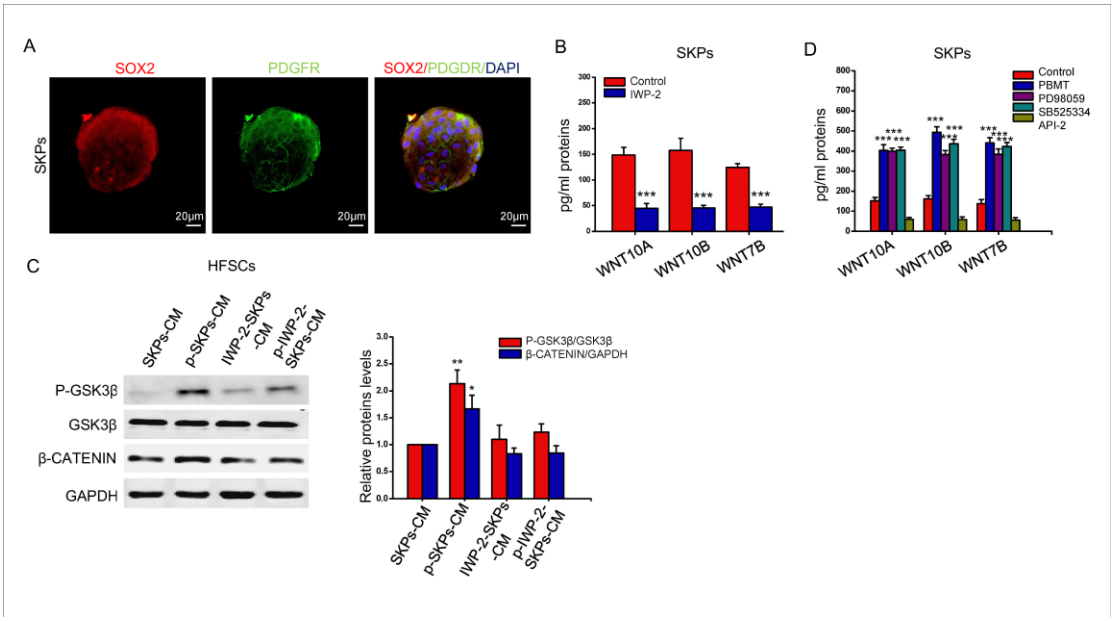
SUPPLEMENTARY FIGURE 1. PBMT induces hair regeneration



SUPPLEMENTARY FIGURE 2. PBMT drives HFSCs activation *in vitro*



SUPPLEMENTARY FIGURE 3. PBMT promotes HFSCs proliferation by activating paracrine WNT signals in SKPs



Supplementary Information

FIGURE LEGENDS:

SUPPLEMENTARY FIGURE 1. PBMT induces hair regeneration

(A) Immunofluorescence detects the positive number of LEF1 in HFs, n = 8 mice per group and > 60 HFs per mouse.

(B) The number of HFs in young and old mice, n = 8 mice per group and > 60 HFs per mouse. ANOVA was used for significance test. *P < 0.05; **P < 0.01; ***P < 0.001.

SUPPLEMENTARY FIGURE 2. PBMT drives HFSCs activation *in vitro*

(A) Immunofluorescence analysis of β-CATENIN levels after continuous PBMT treatment in mouse HF for 4 days. n = 8 mice per group and >60 HFs per mouse. ANOVA was used for significance test. *P < 0.05; **P < 0.01; ***P < 0.001.

(B) The genotype of *β-CATENIN*$^{flox/flox}$ mice. M: mutant; W: wild type.

(C) Western blot analysis of β-CATENIN protein levels in mice after tamoxifen injection for 10 consecutive days.

(D) CCK-8 analysis was used to evaluate the viability of HFSCs under different light dose conditions. The data represented mean ± S.D., n = 3 independent replicates. *P < 0.05; **P < 0.01; ***P < 0.001.

(E) PBMT promotes colony forming ability of HFSCs cultured *in vitro*. The data represented mean ± S.D., n = 3 independent replicates. *P < 0.05.

(F) qPCR analysis of *β-Catenin*, *Lef1* and *Axin2* of HFSCs after PBMT. The data represented mean ± S.D., n = 3 independent replicates. *P < 0.05; ***P < 0.001.

SUPPLEMENTARY FIGURE 3. PBMT promotes HFSCs proliferation by activating paracrine Wnt signals in SKPs

(A) The expression of SOX2 and PDGFR in SKPs was detected by immunofluorescence.

(B) ELISA analysis of WNT10A, WNT10B and WNT7B levels after pre-treated with IWP-2 (5 μM).

(C) Representative western blot of β-CATENIN and P-GKS-3β in HFSCs after HFSC treatment with SKPs conditional medium in the presence of IWP-2 (5 μM).

(D) ELISA was used to detect the secretion of WNT10A, WNT10B and WNT7B of SKPs after adding PD98059 (5 μM,), SB525334 (200 nM) and API-2 (2 μM).

The data represented mean ± S.D. of at least three times biological replicates. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Supplementary Table S1. Primers for Real-Time PCR detection.

Genes	Forward	Reverse
Cd133	CATTTGCCTCTACCCTGGAAGC	ATGCTGGTGGATGGCTCTTATATTC
Sox2	TCCATGGGCTCTGTGGTCAAG	TGATCATGTCCCGGAGGTCC
Alp1	CCCAAAGGCTTCTTCTTGC	GCCTGGTAGTTGTTGTGAG
Hey1	GCCGACGAGACCGAATCAAT	GCTGGGATGCGTAGTTGTTG
Fgf7	AGCGGAGGGGAAATGTTCG	TCCAGCCCTTTCTTGGTTACTGAGA
Fgf10	ATTTCCCCCTGTATGCATCCTAAC	TTCCCACGGAGGCAGAACTC
Lef1	TCCTGAAATCCCCACCTTCT	TGGGATAAACAGGCTGACCT
Wnt7b	GCGTCCTCTACGTGAAGCTC	TCTTGTTGCAGATGATGTTGG
Wnt10b	CTGCGGATGGAAGGGTAGTGG	GGCGTCCCACCCTGTTGTTG
β-Catenin	CCTCCCAAGTCCTTTATGAATGG	CCGTCAATATCAGCTACTTGCTCTT
β-Actin	GTGACGTTGACATCCGTAAAGA	GCCGGACTCATCGTACTCC

Supplementary Table S2 Specific information about the antibodies used in our experiments.

Antibodies	Source	Catalog number
Rabbit anti-LEF1	Cell Signalling Technology	Cat#2230S
Rabbit anti-SOX9	Cell Signalling Technology	Cat#82630S
Rabbit anti-β-CATENIN	Cell Signalling Technology	Cat#8480S
Mouse anti-β-CATENIN	Cell Signalling Technology	Cat#37447S
Rabbit anti-phospho AKT	Cell Signalling Technology	Cat#4060S
Rabbit anti-Phospho-AKT	Cell Signalling Technology	5315
Rabbit anti-AKT	Cell Signalling Technology	Cat#4685S
Rabbit-anti-phospho-GSK-3β	Cell Signalling Technology	Cat#5558S
Rabbit-anti-GSK-3β	Cell Signalling Technology	Cat#12456S
Mouse anti-PCNA	Cell Signalling Technology	Cat#2586S
Rabbit-anti-non-phospho (Active) β-CATENIN (Ser45)	Cell Signalling Technology	Cat#19807S
anti-phospho-β-CATENIN	Cell Signalling Technology	Cat#9566S

(Ser552)		
Mouse anti-BrdU	Cell Signalling Technology	Cat#5292
Rabbit anti-WNT10B	Abcam	Cat#ab70816
Rabbit anti-WNT10A	Abcam	Cat#ab106522
Rabbit Anti-WNT7A	Abcam	Cat#ab100792
Rabbit Anti-PDGFR alpha	Abcam	Cat# ab93531
Rabbit Anti-Cytokeratin 15	Abcam	Cat# ab214393
Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488)	Abcam	Cat#ab150077
Goat Anti-Mouse IgG H&L (Alexa Fluor® 488)	Abcam	Cat#ab150113
Goat Anti-Rabbit IgG H&L (Alexa Fluor® 555)	Abcam	Cat#ab150078
Goat Anti-Mouse IgG H&L (Alexa Fluor® 555)	Abcam	Cat#ab150118
Goat Anti-Rabbit IgG H&L (Alexa Fluor® 647)	Abcam	Cat#ab150079
Mouse anti-WNT10A	Santa Cruz	Cat#sc-376028
Mouse anti-WNT7A/B	Santa Cruz	Cat#sc-365459
Mouse anti-SOX2	Santa Cruz	Cat#sc-365823
Mouse anti-GAPDH	Santa Cruz	Cat#sc-32233
Mouse anti-β-ACTIN	Santa Cruz	Cat#sc-47778
BV510 Mouse anti-BrdU	BD	Cat# 563445

REFERENCES

Lichti, U., Anders, J., and Yuspa, S. (2008). Isolation and short-term culture of primary keratinocytes, hair follicle populations and dermal cells from newborn mice and keratinocytes from adult mice for in vitro analysis and for grafting to immunodeficient mice. Nature protocols *3*, 799-810.