Temperature activated cryogel-PRP for long-term osteogenesis of adipose-derived stem cells to promote bone repair

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Materials and Methods

Materials

Gelatin, dopamine, N,N′-methylene-bisacrylamide (MBA), poly (ethylene glycol) diacrylate (PEG-MA, Mw = 700), tetramethylethylenediamine (TEMED) were all purchased from Sigma (St Louis, USA). The Live/Dead cell staining kit was from Molecular Probes (Life Technologies). The Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies (Japan). The primary antibodies of α-actinin, osteopontin (OPN), and alkaline phosphatase (ALP) were acquired from Abcam. F4/80 antibody was purchased from Ebioscience (USA). Alkaline phosphatase kit was ordered from Boster Biological Technology (Wuhan, China).

Preparation of DOPA-MBA cross-linker

DOPA-MBA cross-linker was prepared according to the previous report.[2] In briefly, 500 mg of MBA (3 mmol) was dissolved in deionized water/ethanol (v/v = 4:3, pH = 6) solution to reach 70.1 mg/ml final concentration. And then, 475 mg dopamine was added into the solution under nitrogen protection. This mixture was stirred at 45 ℃ for 3 days in darkness and then the DOPA-MBA cross-linker was obtained. The DOPA-MBA cross-linker solution was lyophilized and stored at -20 ℃.

Preparation of Gelatin-MA

Gelatin with double bond (Gelatin-MA) was also prepared according to previous reports.[3] In briefly, 1g gelatin and 0.5 ml methacrylic anhydride (MA) were dissolved in 10 ml PBS (pH = 7.4) at 50 ℃ separately. After being strongly stirred for 1 hour, the reaction was then stopped by adding another 10 ml PBS. After being dialyzed against deionized water and lyophilized, the Gelatin-MA was obtained.

Preparation of Platelet-rich plasma (PRP)

PRP was prepared according to the previous studies.[1] A 1.2 ml intracardiac full blood sample was drawn using a syringe preloaded with 120 μl of anticoagulant natrium citrate buffer. It was centrifuged at 200g for 30 min at 4 ℃ to separate the plasma in the superior layer from the red blood cells in the inferior layer, and the white blood cells in the intermediate layer. In a second step, the plasma was centrifuged at 2000g for 10 min at 4 ℃. Platelets were resuspended in 120 ml of supernatant to obtain PRP. Some unactivated PRP was applied for the preparation of the PRP-cryogel. The other PRP was activated in the form of a PRP-gel using bovine thrombin (10 U/ml) (Sigma, Germany) and 2.5 mM CaCl2, and applied to PRP release experiment as a control group. Sprague-Dawley rats (180–220 g, male) were purchased from the Experimental Animal Center in Southern Medical University. All animal experiments were permitted by the Institutional Animal Care and Use Committee (IACUC) of the Southern Medical University.

PRP composation analysis

The platelet count was detected with an SYSMEX Blood analyzer. 100 μl intracardiac full blood sample or PRP sample was added into the detect tube with 10 μl anticoagulant

natrium citrate buffer. Then the platelets, erythrocytes, and leukocytes were counted with an animal blood analyzer (XN-1000V SYSMEX). Five rats were used for blood or PRP analysis.

SEM of the PRP-cryogels

The micro-structures of the cryogels were observed using Scanning Electron Microscopy (SEM, S-3000N, Hitachi, Japan). For the mechanical properties of different cryogels, all samples were synthesized with 4~6 mm height and 10 mm diameter. The cycling compressive test was executed up to 60% sample deformation at a compressive speed of 10 mm/min for 100 cycles by LS1 materials testing system (AMETEK, America). The stress-strain curves and modulus of different cryogels were also measured in single continued compressive at the same speed.

Physical characterization of hydrogel

The swelling ratio and mechanical properties were all tested after the equilibrium of water contents. The swelling ratio of PRP-cryogels and cryogel were calculated based on the weight of wet to dry hydrogel at different time points. Hydrogels were incubated in PBS at 37 °C and at different time intervals, the hydrogels were removed, excess of water was wiped with a tissue, and weighed (Ww). The hydrogels were dried in oven at 37 °C overnight to obtain the dry weight (Wd). The swelling ratio is expressed as the ratio of wet to dry weight (Ww/Wd). The hydrogels were made in a mold with 20 mm diameter and height of 3 mm. Hydrogels were subsequently put in PBS and incubated at 37 °C overnight. The resulting gels were placed at the ground plate and the upper plate was lowered to a measuring gap size of approximately 3 mm to an initial force of

0.5 N/m2. The storage (G0) modulus was recorded using a strain of 1% and a frequency of 1 Hz. Infrared spectra were performed by using an FTIR spectrometer (ThermoFisher Nicolet is50). The specimens were ground and compressed into KBr pellets. All data were presented as transmittance %.

PRP activity detection

The activation of platelets in as prepared PRP, inactivated PRP-cryogel, and temperature-activated PRP-cryogel were detected by measuring the percentage of CD63-positive platelet with the Flow Cytometry. Briefly, the as prepared PRR, inactivate PRP-cryogels and temperature-activated PRP-cryogels were ground carefully and then suspended and well mixed with cold PBS. After 200g centrifugation for 10 min, the supernatants were collected and further centrifuged at 2000g for 10 mins. The precipitates (platelets) were fixed with paraformaldehyde and used for PRP activity detection. The collected platelets weres permeabilized with 0.1% Triton X-100 for 10 min in tubes. Further, the FITC labeled anti-CD63 (Abcam ab108949 1:200) were added in EP tubes as manufacturer's instructions and incubated for 1 h. After 2000g centrifuged and rasing with PBS, the flow cytometry was performed for detecting the percentage of positive platelets.

Degradation of gels

The hydrogels were made in a mold (10 mm*4 mm, diameter*height). Hydrogels were subsequently put in PBS and incubated at 37 °C. Every 2 days, the hydrogels were taken out, wiped and weighed to get the remaining mass. The solution was refreshed every 2–3 days. The results are shown as the mass fraction of the original gel weight. The

degradation time was defined as the time required to completely dissolve the hydrogel (n = 5).

PRP release and growth factor quantification.

All frozen PRP-cryogels were simply thawed in room temperature and then immersed in 10 ml of PBS solution (pH = 7.4) at 37 ℃. At predetermined time points of 1, 2, 3, 4, 5, 6, 7, 8 and 10, 12, 14, 16, 18, 20, 22, 24, 26, 28 and 30 days, 100 μl of solution was removed and replaced with fresh PBS, and the growth factors could be released from platelets by deep temperature activation. The amount of released PDGF (#DHD00C)and TGF-β (#DB100B) was quantified with PDGF and TGF-β Immunoassay kit (R&D Quantikine #DBP200, USA) using a microplate reader (Bio-Rad, Hercules, CA, USA).

The Adipose tissue mesenchymal stem cells (ADSCs) isolation and culture

In all experiments, we used 28-day-old male SD rats weighing 250 g, which were maintained in the animal facility with constant temperature (22 ±2 °C), free access to water and food, and a standard light/ dark cycle of 12-h light followed by 12-h dark. All the procedures were accepted by the Institution of Animal Care (IACUC) at the Southern Medical University Animal Ethics Committee. Cells were harvested as previously described. The ADSCs were isolated from the inguinal adipose tissue using 0.075% type II collagenase (Gibco-Invitrogen) at 37 °C for 40 min. The cells were centrifuged, the foating adipocytes were removed, and the pellet was resuspended in a growth medium. The ADSCs were cultured in the growth medium in 75 cm2 fasks for 10 days. During the entire culture time, the cells were maintained at 37 °C in a

humidified atmosphere of 95% air and 5% CO_2, and the medium was changed every other day.[4]

The ADSCs were cultured with Dulbecco's modified Eagle's Medium/F12 (DMEM/F12, GIBCO) supplemented with 10% fetal bovine serum (FBS, GIBCO), streptomycin (100 μg/mL) and penicillin (100 U/ml) at 37 °C in a 5% CO_2 incubator. The PRP-Cryogel was thoroughly washed with sterilized PBS solution several times. Hydrogels were then activated at 37 °C in a sterilized incubator for 30 min for the release of growth factors. 1×105 ADSCs cells suspension (1 mL) were seeded onto the surface of the hydrogel. 1×10^5 ADSCs cells suspension (1 mL) were seeded onto the surface of the hydrogel. The culture media was changed every 2 days.

Sprague-Dawley rats (180–220 g, male) were purchased from the Experimental Animal Center in Southern Medical University. All animal experiments were permitted by the Institutional Animal Care and Use Committee (IACUC) of the Southern Medical University.

Biocompatibility evaluation for PRP-cryogel

Cyto-compatibility and cell viability of PRP-Cryogels were analyzed utilizing Live/Dead cell staining assay. After cultured for 1, 3, 7 days, respectively. ADSCs on scaffolds were rinsed with PBS three times, followed by incubating in staining solution at 37 °C for 30 min in the dark. The stained samples were transferred onto a glass slide and then photos concerning stained samples were obtained applying a laser scanning confocal microscope (Olympus IX 51 fluorescence microscope). The quantifications of cell viability were the same as above.

The CCK-8 assay was performed as follows. Briefly, the ADSCs were seeded in different cryogels in a 96-well culture plate, six duplicate wells for each group. At day 3 and day 7 of culture, the culture medium was replaced with 100 μl working solution (CCK-8 solution/serum-free medium: 10 μl /90 μl). Then after the samples were incubated for 2 hours, the working solutions of all wells were transferred into a new 96-well culture plate for absorbance detection. The OD value was measured at 450 nm using a microplate reader.

Morphology and ultrastructure observation

The morphology of gels with ADSCs was examined using SEM. All samples were coated with gold (thickness < 10 nm) to make them conductive for SEM examination. After culturing for 3 days, ADSCs on cryogels were washed with PBS and fixed with 2.5% of glutaraldehyde overnight. The fixed cells were then dehydrated with graded ethanol (50%, 70%, 90%, and 100%) and freeze-dried. Finally, cells were stained with dioxin and lead citrate. As for the SEM analysis, the samples were washed three times with PBS and fixed with 2.5% glutaraldehyde and 1.0% osmium tetroxide. Next, washed in PBS at 4℃ overnight, then the samples were dehydrated with graded ethanol solutions and embedded with Eponate 812. All samples were sliced into ultrathin sections (about 50 nm), and the micrographs were observed under a SEM.

ALP Activity

DMEM/F12 medium containing 10% FBS was prepared. Ascorbic acid, β-glycerol phosphate and dexamethasone were then added to the prepared medium to serve as osteogenic inducer, the final concentrations were 50 μM, 10 mM, and 100 nM,

respectively. ADSCs cells were seeded onto the surface of different hydrogels (Cryogel, PRP-1-Cryogel, PRP-2-Cryogel) in the six-well plate, DMEM/F12 medium was added into the wells to cover the gels. The ADSCs cells were then cultured until the cells grow to a basic fusion. DMEM/F12 medium containing osteogenic inducer was then used to replace the culture solution for osteoinduction. The medium containing osteogenic inducer was changed every two days.

To analyze the intracellular ALP activity, 800 μL cold cell lysis solution containing 0.1% Triton X-100 and 5 mM $MgCl_2$ was added to each well after PBS washing. After incubating at 4 ℃ for 10 min to lysis cells, the solution was centrifuged at 13,000× g for 10 min and the supernatant was examined for ALP activity using pnitrophenyl phosphate as a substrate for the enzymatic hydrolysis reaction. Briefly, 50 μL supernatant was reacted with 50 μL p-nitrophenyl phosphate (5 mM) for 60 min at 37 ◦C before adding 50 μL 0.2 M NaOH as a stop solution to denature ALP. The ALP activity was determined from the optical density value of the solution at 405 nm (OD_{405}) using an enzyme-linked immunosorbent assay (ELISA) reader.[5]

The qPCR analysis for osteogenesis in the PRP-cryogel in vitro

ADSCs were isolated from SD rats. The cells were induced in 10% FBS-supplemented DMEM/F12 containing a combination of osteogenic chemical supplements (osteogenic inducer including: 50 μg/ml L-ascorbic acid 1-phosphate, 10 mM β-glycerophosphate and 100 nM examethasone). The osteogenic medium was changed for every 2–3 days. At day 3, 7 and 14 after the osteogenic induction, the samples were washed with D-PBS (Dulbecco's Phosphate Buffered Saline). Then, cells on hydrogels were treated

with liquid nitrogen and smashed. In order to validate the gene expression of osteogenic differentiation in all samples, total RNA isolation and cDNA synthesis were conducted using TRIzol and Oligo dT (Invitrogen, USA), according to the standard procedures. Then quantitative real-time PCR (qPCR) was performed by SYBER Green assays kit (Applied Biosystems, USA). Amplification conditions were as follows: hold at 95 °C for 10 min, followed by 40 cycles at 15 s at 95 °C and 1 min in 60 °C. Thermal cycling and fluorescence detection were done using the StepOnePlus™ real-time PCR System (Applied Biosystems, USA) to analyze the expression of the osteogenesis related gene ALP and OPN. GAPDH was used as an internal reference gene. The mRNA expression levels were determined relative to the GAPDH by the ΔCt method. The sequences of PCR primers (forward and backward, 5' to 3') were as follows:

ALP, 5'- TGCAGGATCGGAACGTCAAT -3' and 5'-GAGTTGGTAAGGCAGGGTCC-3';

OPN, 5'-CCAGCCAAGGACCAACTACA -3' and 5'-AGTGTTTGCTGTAATGCGCC-3'.

Alizarin red staining

The Alizarin red staining was applied to evaluate the adipogenic ability of samples. After cultured for 14, 21 days, the samples were stained with 40 mmol/L alizarin red (pH = 4.2) for 30 min respectively, and then imaged and photographed under light microscopy.

Oil red O staining

The oil red O staining was applied to evaluate the osteogenic ability of samples. After ADSCs were seeded in Cryogel or PRP-Cryogel and cultured for 7, 14 days, the samples were prepared and incubated slide in propylene glycol for 2 min, then incubated slide in oil red O solution for 6 min, differentiated section in 85% propylene glycol for 1 min, rinsed slide twice in water, incubated in hematoxylin for 1-2 min, rinsed slide three times in water, coverslip with an aqueous mounting medium, and then imaged and photographed under light microscopy.

Immunofluorescence analysis for ADSCs on the PRP-cryogels

CLSM was employed to examine the protein expression of OPN after 7 days and 14 days in culture. The ADSCs scaffolds were washed three times with cold PBS, and the cells were fixed with 4% paraformaldehyde in PBS at room temperature for 15 min. After fixation, the cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min, and the scaffolds were rinsed three times with PBS. The cells were incubated in a 10 μM phallacidin, 1% (w/v) BSA solution for 30 min, followed by three PBS rinses. The samples were incubated with primary antibody against OPG (Tianjin Sungene Biotech Co, Ltd. Tianjing China) overnight at 4 °C, followed by horseradish peroxidase conjugated anti-rabbit secondary antibody for 1 h at room temperature, and three washes in PBS. The samples examined by CLSM. At least 5 fields of each sample were quantified.

Histology Analysis

After euthanasia at predetermined time points, calvaria were excised and fixed in 4% paraformaldehyde for 48 h and transferred to a 70% ethanol solution. Calvaria were

partially decalcified for ~4 h by using a rapid decalcifying formic acid/ hydrochloric acid mixture (Decalcifying Solution; VWR). The defect area was cut in cross-section with a razor blade and embedded in paraffin wax. Sections (5 μm) of the cross-section were stained with Masson's trichrome stain and imaged by using bright-field microscopy.

Statistical analysis

All results were analyzed with the SPSS22.0 and GraphPad prism 5 software. The data were expressed as means ± standard deviations (SD). Statistical analyses were performed using one-way analysis of variance (ANOVA). Tukey HSD post hoc testing was used as the post hoc correction to compare multiple groups. To compare the differences between the two groups, two-tailed unpaired Student's t-tests were used. Differences were considered significant at $p < 0.05$.

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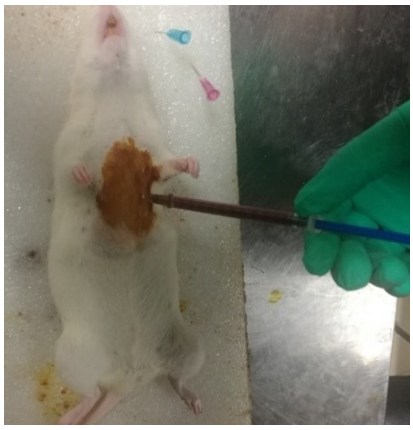

Figure S1. The Rats heart puncture for intracardiac full blood.

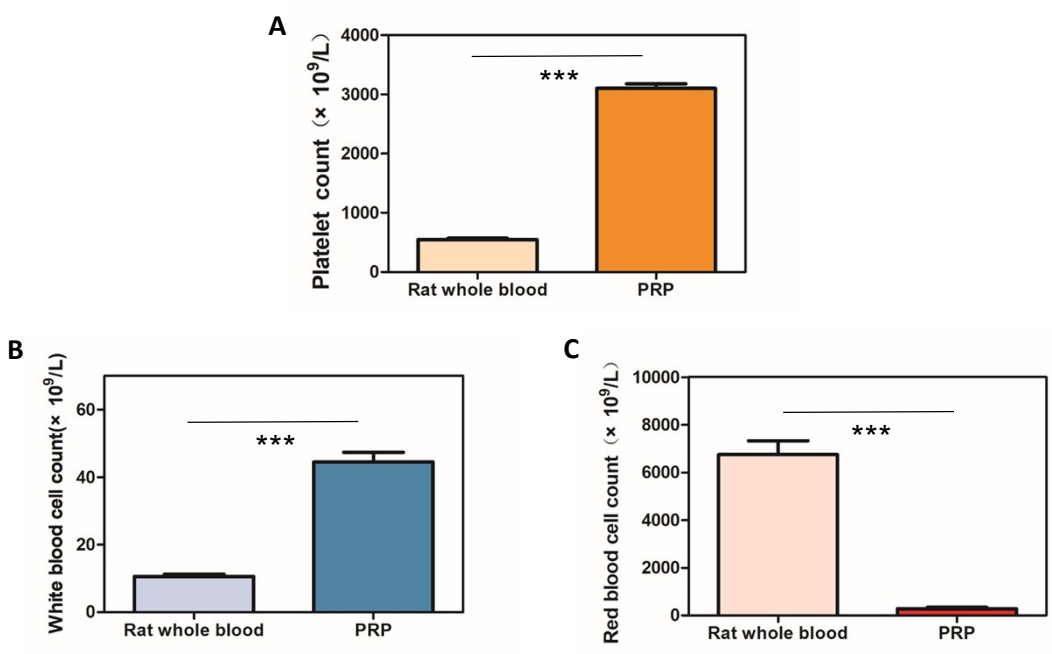

Figure S2. Platelet (A), white blood cell (B) and red blood cell (c) count of the rat whole blood and the as prepared PRP. n = 5 ,*$P < 0.05$, **$P < 0.01$, ***$p<0.001$

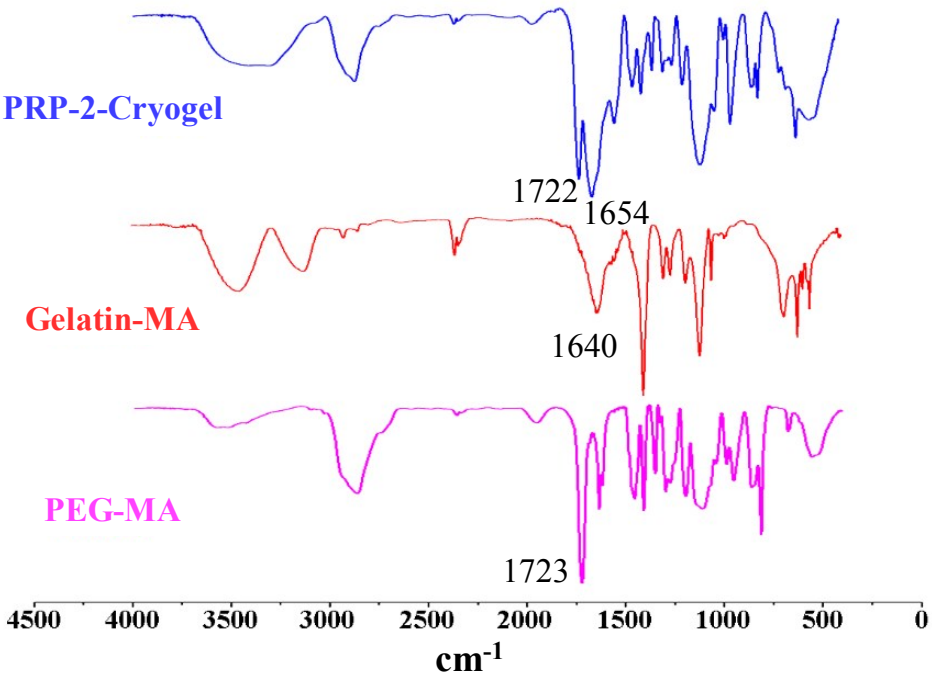

Figure S3. FTIR analysis of PEG-MA, Gelatin-MA and PRP-2-Cryogel.

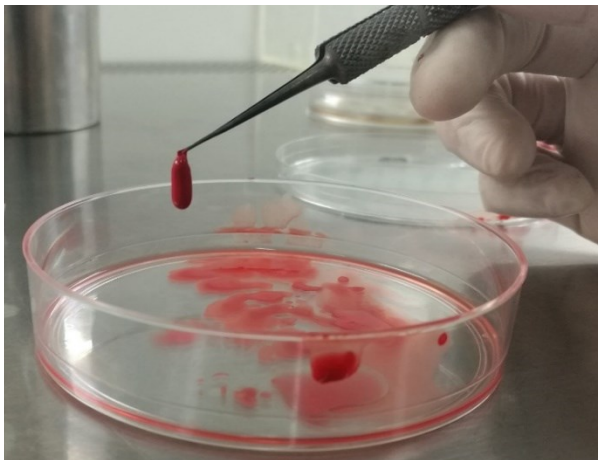

Figure S4. PRP was activated in the form of a gel using bovine thrombin and $CaCl_2$.

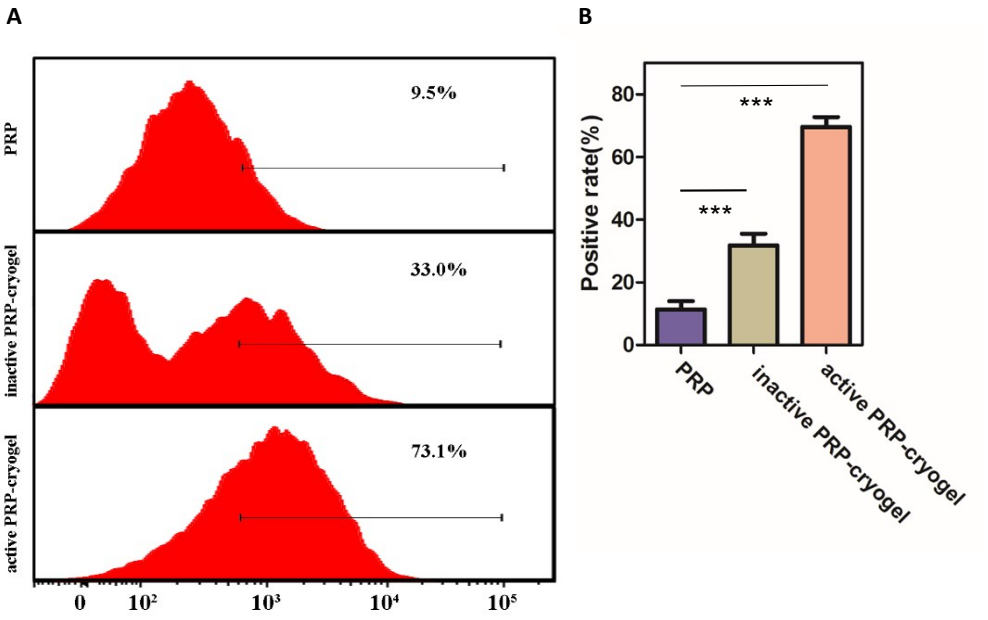

Figure S5. Flow cytometry analysis (A) and positive rate (B) of CD63 expression of platelets in as prepared PRP, inactive PRP-2-Cryogel and active PRP-2-Cryogel.

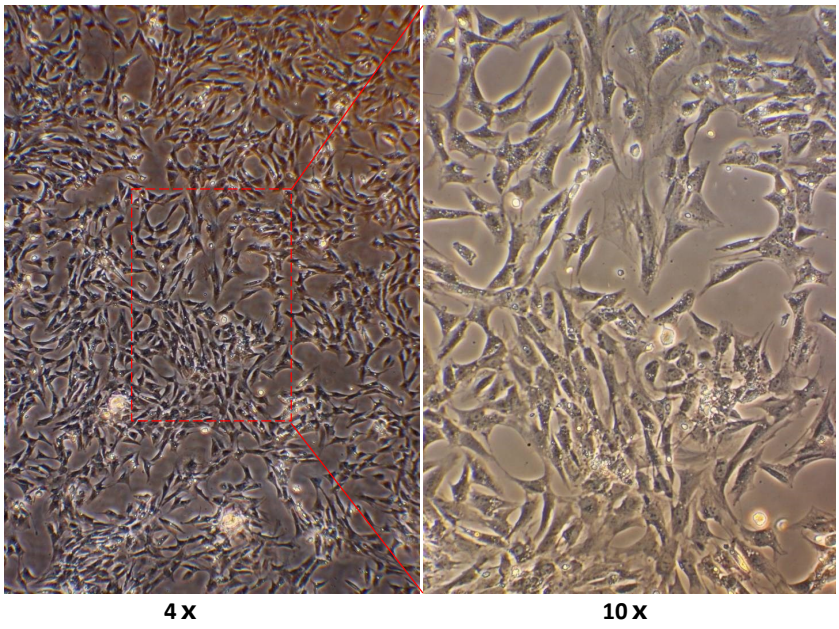

Figure S6. The isolated ADSCs from adult rats was cultured in flasks for 10 days.

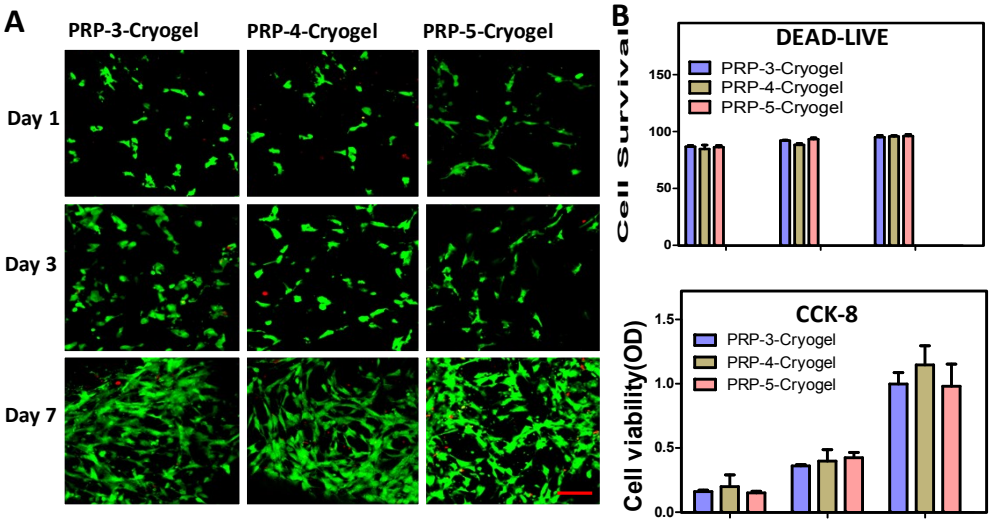

Figure S7. Cytotoxicity assay of PRP-3-Cryogel, PRP-4-Cryogel and PRP-5-Cryogel. (A) Cell viability of ADSCs cultured on the different scaffolds was analyzed using Live/Dead staining at day 1, day 3 and day 7 (Scar bar = 100 μm).The live cells are green and the dead cells are red. (B) Quantitative cell viability of ADSCs based on the live-dead staining. n = 3. **p<0.01, ***p<0.001. (C) ADSCs proliferation on different scaffolds detected by the CCK-8 assay at 1, 3 and 7 days. A linear relationship exists between OD value of cell proliferation and culture time. n =6. *p<0.05, **p<0.01, ***p<0.001

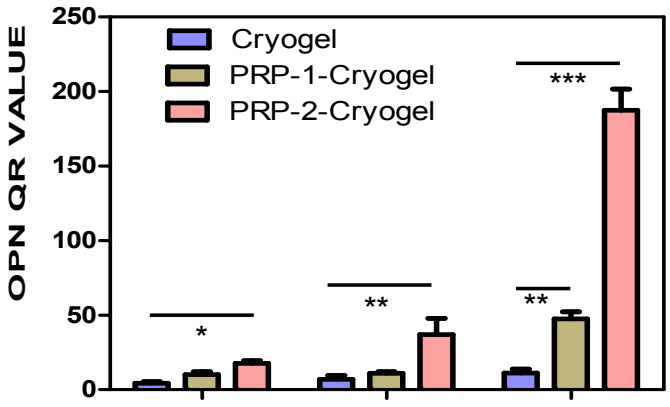

Figure S8. Osteogenic gene expression levels of ADSCs after cultured in different scaffolds. Osteopontin (OPN) gene expression was quantified using quantitative real-time PCR.* $p < 0.05$, **$p<0.01$, ***$p<0.001$

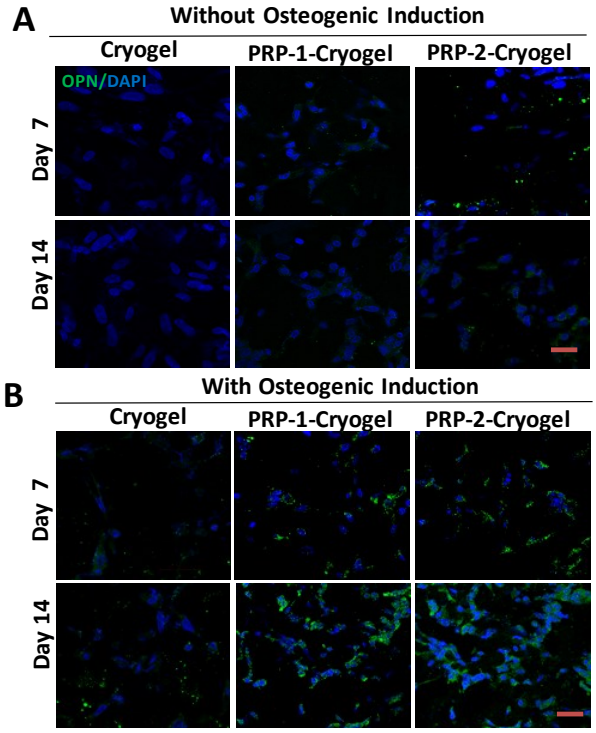

Figure S9. Immunofluorescence analysis for ADSCs on the PRP-cryogels without (A) and with (B) osteogenic inducer at day 7 and 14 days. The expression level of OPN was significantly higher in the PRP-cryogel group than cryogel group（All scale bars = 50 μm).

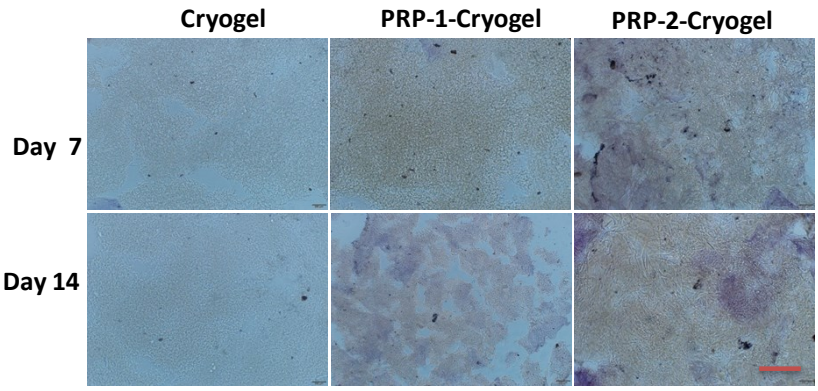

Figure S10. Oil red O staining of ADSCs cells cultured on the different cryogels for 7 and 14 days without adipogenic inducer.(4×magnification, bar = 1mm).

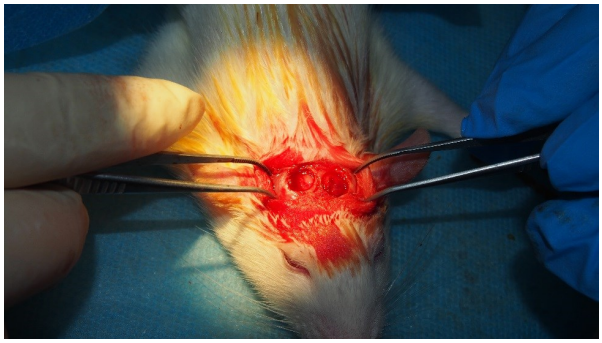

Figure S11. Bone defect experimental in vivo, two circular critical size defects (8.0 mm diameter) were created.

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