A simple method for deriving functional MSCs and applied for osteogenesis in 3D scaffolds

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Supplementary Materials and Methods

1. Production of mRNA reprogramming mRNA cocktail

The transcript templates for reprogramming factors Oct4, Sox2, Klf4, c-Myc, Lin28 were generated from the plasmids (from Addgene) by adding 120 T through tail PCR. Synthetic mRNA was generated in IVT reactions using a 4:1 ratio of ARCA cap analog to GTP to give a high percentage of capped transcripts. Complete substitution of 5m-CTP for CTP and Pseudo-UTP for UTP in the nucleotide triphosphate (NTP) mix was employed to reduce the immunogenicity of the RNA products. Cap analog and modified NTPs were purchased from Trilink Biotechnologies. A 2.5X NTP mix was prepared (ARCA:ATP:5m-CTP:GTP:Pseudo-UTP at 15:15:3.75:3.75:3.75 mM) to replace the standard NTPs provided with the MEGAscript T7 Kit (Ambion) used to perform IVT reactions. Each 40 uL IVT reaction comprised 16 uL NTP mix, 4 uL 10X T7 Buffer, 16 uL DNA template and 4 uL T7 polymerase. Reactions were incubated 4–6 hours at 37°C and treated with 2 uL TURBO DNase for a further 15 minutes at 37°C before being purified on MEGAclear (Ambion) spin columns; the RNA products being eluted in a volume of 100 uL. To remove immunogenic 5′ triphosphate moieties from uncapped transcripts, 10 uL of Antarctic Phosphatase

reaction buffer and 3 uL of Antarctic Phosphatase (NEB) were added to each prep. Phosphatase reactions were incubated for 30 minutes at 37°C and the IVT products were repurified. RNA yield was quantified by Nanodrop technology (Thermo Scientific), and the preps were subsequently adjusted to a standardized working concentration of 100 ng/uL by addition of TE pH 7.0 (Ambion). IVT product quality was checked with an RNA bioanalyzer. Cocktails were assembled by pooling preps representing the various RFs in the desired stoichiometric ratios. All reprogramming factors being equimolar except for Oct4 (3X molar concentration).

2. Molecular Characterization of iPSCs

Cells were fixed in 4% paraformaldehyde for 20 min. Washed cells were treated with 0.2% Triton X (Sigma) in PBS for 30 min. Cells were blocked with 3% BSA (Invitrogen) and 5% donkey serum (Sigma) for 2 hr at RT. Cells were stained in blocking buffer with primary antibodies at 4°C overnight. Cells were washed and stained with secondary antibodies) in blocking buffer for 3 hr at 4°C or for 1 hr at RT, protected from light. The images were taken with a LA confocal microscope.

3. Teratoma formation

Adult SCID mice were anesthesized with avertin, 0.5-1.0 ml IP (20 mg/ml) ~ 125-240 mg/kg. 50 ul of iPS cell suspension (1 $\times 10^6$ cells) were injected intramuscularly into the flanks (hamstring muscles) of the mice. After awaking from anesthesia, the mice were returned to their cage. After 8 weeks, the mice

were sacrificed by CO_2 euthanisia and the tumors were histologically analyzed.

4. Long-term maintenance of iPSCs

iPSCs were routinely cultivated in standard hESC medium on Matrigel (BD Biosciences) -coated 6-well plates containing irradiated human feeder cells (ATCC CRL 2429). The hESC medium comprised KO DMEM/F12 supplemented with 20% Knockout Serum Replacement (Invitrogen), 10 ng/mL of bFGF (Invitrogen), 1x nonessential amino acids (Invitrogen), 0.1 mM β-Mercaptoethanol (Sigma), 1 mM L-glutamine (Gibco), plus 1% penicillin/streptomycin(Sigma). Cells were passaged every 7-9 days using 1 mg/ml collagenase IV (Invitrogen).

5. Karyotyping

2×10^5 cells were plated and grown in a slide flask (Thermo Scientific Nunc) for 24 hours. Cells were treated with 10ng/ml colcemid (Sigma-Aldrich) for 50 min. Cells were then washed twice with PBS (Ca^{2+} and Mg^{2+} free), followed by a serial gradient fixation at 17%, 38%, 58%, and 100% fixation solution (1:3 of acetic acid:ethanol) for 5, 5, 5, and 40 min, respectively. Slides were dried on wet filter papers at room temperature. Karyotyping was performed by mounting the slide with DAPI-antifate (0.2 μg/ul) solution followed by imaging in an Axioplan 2 microscope (Leica microsystem). Images were captured with IPlab Spectrum and analyzed with Quips CGH/Karyotyper (Vysis).

6. Analysis of pluripotent markers in hiPS-MSCs by flow cytometry

The hiPS-MSCs (passage 8 and 12) were harvested by trypsinization. Cells were

washed with PBS/2%FBS twice, followed by fixing with 1X FACSlysing solution for 10 min at room temperature. Fixed cells were permeabilized with FACSPerm solution for 10min at 4 °C and washed with PBS/2%FBS twice. Then cells ($1X10^6$ per group) were stained with antibodies against OCT3/4 (BD Pharmingen™, Cat# 560253), NANOG (BD Pharmingen™, Cat# 560791) or TRA-1-81 (BD Pharmingen™, Cat# 560793). For isotope controls, fluorochrome and concentration matched isotopes were used (IgG1 for OCT3/4 and NANOG, and IgM forTRA-1-81, BD Biosciences). Following two washes in PBS/2%FBS, cells were resuspended in 1% formaldehyde in PBS while vortex mixing. Cells were analyzed using a FACSAria™ III (BD Biosciences) equipped with a 375 nm, 488 nm, 561 nm, and 633 nm laser. For each sample 30,000 cells were collected and subsequently analyzed using FlowJo software (v. 9.3.3, TreeStar Inc., Ashland, OR).

7. PCL-Hyaluronan-TCP scaffold fabrication and cell seeding

PCL (MW = 50 kDa, Perstorp, UK) scaffolds were made by fused deposition modeling at a processing temperature of 100 ^{0}C with a BioScaffolder (SYS + ENG GmbH, Germany). Cylindrical scaffolds with a diameter of 4 mm were punched out from a 2 mm thick porous PCL mat using a sterile biopsy punch (Acuderm, Florida). To increase surface hydrophilicity, the scaffolds were etched in 5 M sodium hydroxide for 3 hours, rinsed in sterile water multiple times, and then in 70% ethanol for sterilization. For fabrication of the PCL/Hyaluronan/TCP hybrids *(PHT)*, PCL scaffolds were immersed in a 4.0 mg ml-1 hyaluronic acid solution (780 kDa, Lifecore Biomedical Inc., Lot P9805-9A) with β-TCP (Berkeley

advanced biomaterials Inc., Lot: TCPCH01) overnight at room temperature followed by freeze-drying (FTS, NJ, US). The weight ratio of hyaluronic acid to β-TCP is 10:1.

PHT scaffolds were placed in 1% agarose-coated 24-well culture plates (one scaffold well^{-1}). 10 µl of an iPS-MSCs suspension (1.25×10^7 cells / well) was dispensed onto scaffolds, giving a seeding number of 1.25×10^5 cells per scaffold. After 2 hours incubation, 1 ml basic medium consisting of DMEM-high glucose (Invitrogen), 2 mM L-Glutamine, 1% penicillin/streptomycin, and 10% FBS (Gibco), was added to each well and scaffolds were incubated overnight to allow the cells to adhere. In the control group, naked PCL scaffolds were seeded with 1.25×10^5 cells per scaffold. For in vitro experiments, cell-seeded scaffolds were either stimulated with osteogenic medium or cultured in basal medium throughout the study for 3 weeks. Cell/scaffold constructs were collected on days 7, 14 and 21 for DNA, ALP, calcium assay, and gene expression analysis. Cell viability on PHT scaffolds were assessed by CellTracker^{TM} green staining on day 2 and day 7. Cellular distribution and morphology in scaffolds on day 21 were visualized in a scanning electron microscope (SEM). For in vivo experiments, cell-seeded scaffolds were pre-differentiated in osteogenic differentiation medium for 2 weeks before implantation. Media in all cultures were changed every 3-4 days.

8. ALP/DNA/Calcium assay

Four cellular-scaffold constructs from each group (PCL and PHT, basic and osteogenic medium) were transferred to 2 ml tubes with 400µl DMEM w/o Phenol

Red (Gibco 21063) and sonicated at intervals of 1 s on and 5 s off with amplitude of 50% (0.046 KJ) in a Bandelin Sonopuls sonicator (Buch & Holm, Denmark) for a total of 1 min. Aliquots of 60 µl were used for ALP activity assay. The remaining volume was supplemented with 10µl/tube Collagenase (Sigma C8176, 100mg/ml) and incubated in a 37 ˚C water bath for 3 hours. Seventeen micro liter Proteinase K (Sigma P2308, 20mg/ml) and 3µl Pen/Strep were then added and the samples were incubated at 45 ˚C for 20 hrs. Scaffold debris was spun down at 10.000 x g for 5 min. Aliquots of 90 µl were used for DNA quantification. The remaining volume was mixed with an equal volume of 1 M Acetic acid and left on a shaker overnight to dissolve the calcium deposition. The calcium amount was measured by Arsenazo III calcium assay.

ALP activity was determined using a colorimetric endpoint assay measuring the enzymatic conversion of P-nitrophenyl phosphate (Sigma) to the yellowish product P-nitrophenol in the presence of ALP. Absorbance of P-nitrophenol was measured in a microplate reader (Victor3 1420 Multilabel Counter, PerkinElmer Life Sciences, Denmark) at wavelengths of 405 and 600 nm. Standards were prepared from P-nitrophenol (concentration range: 0-0.2 mM). Technical duplicates were used for each biological sample. ALP activity was expressed as nmol Nitrophenol/min/µgDNA.

DNA contents were estimated by the Quant-iT™ PicoGreen® dsDNA assay (Invitrogen). Samples were diluted 1:10 in Tris–EDTA buffer. From each sample, 2 x 50 µl were drawn and suspended with 50 µl of PicoGreen (diluted 1:200 in TE buffer). The mixtures were incubated for 5 min in the dark, and measured in a

96-well plate using a microplate reader (Victor3 1420 Multilabel Counter, PerkinElmer Life Sciences, Denmark). Samples were excited at 480 nm, and the fluorescence emission intensity was measured at 520 nm. Standards were prepared according to the manufacturer's instruction (lambda DNA, concentration range: 0–1μg ml^{-1}).

Calcium contents were quantified by a colorimetric endpoint assay based on the complexformation of one Ca^{2+} ion with two Arsenazo III molecules to a blue-purple product (Diagnostic Chemicals Limited, Charlottetown, PE, Canada). The samples were diluted (1:2 on day 7 and 1:5 on day 14 and 1:10 on day 21) with double distilled water and aliquots of 20 μl were transferred to a 96-well plate. Arsenazo III solution (280 μl) was added and incubated for 10 min at room temperature. A standard dilution series of calcium ranging from 0 to 50 μg ml^{-1} was prepared and Ca^{2+} concentration was quantified spectrophotometrically at 650 nm. Calcium content was expressed as micrograms of Ca^{2+} per scaffold.

9. Scanning electron microscope (SEM) and confocal imaging

For SEM analysis, samples were fixed in 2.5% glutaraldehyde containing 0.1 M sodium cacodylate buffer (pH 7.4) and dehydrated in a graded series of ethanol (50–99%) before being transferred to an excicator for air-drying. Constructs were analysed using a low vacuum secondary electron detector (Nova NanoSEM 600, FEI Company) and element component of cell/scaffold construct was analyzed by energy dispersive X-ray spectrometer (EDX).

To assess cell viability, the cell/scaffold constructs were incubated for 30 min in

DMEM containing 10 μM CellTracker™ Green CMFDA (Invitrogen). The staining medium was replaced with fresh DMEM/10%FBS and the constructs were incubated for another 30 min at 37˚C. The cell/scaffold constructs were then rinsed in prewarmed PBS, fixed in 10% formalin for 5 min at room temperature, and stained with 1 μg/ml Hoechst 33258 (Sigma-Aldrich) in PBS for 20 min. Living cells were labelled green. Nuclei of the cells were stained with Hoechst, labelled red. Images were acquired using a laser scanning confocal microscope, 510 Meta (Zeiss Microimaging GmbH, Germany). The confocal settings (excitation, laser power, detector gain, and pinhole size) were same during all cell imaging. Separate channels and filters were used. Excitation/emission wavelengths were 488 nm / 505-530 nm for CellTracker™ Green and 405 nm / 420 nm for Hoechst.

10. Q-PCR

Total RNA were isolated from cell/scaffold constructs with an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions, followed by quantification with a Nanodrop spectrophotometer and qualification by 1% agarose gel electrophoresis. Complete removal of genomic DNA from the RNA samples was achieved by treatment with DNase I (Invitrogen). For first-strand DNA synthesis, 2μg of total RNA were reverse transcribed using a cDNA synthesis kit (Bio-Rad, Hercules, CA) in a final volume of 20 μl. Q-PCR primers for *ALPL*, *COL1A1*, *OC*, *RUNX2*, *GAPDH*, and *BACT* are listed in **Supplementary Table 1**. One μl (five times diluted) cDNA was used for Q-PCR analysis. Q-PCR was performed using the LightCycle 480 SYBR Green I Mater kit on LightCycler 480 (Roche) with the

following PCR program: 1 hold at 95 °C for 5 min; 50 cycles at 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 15 s (signal acquisition once). Relative gene expression was calculated using the $2^{-\Delta\Delta ct}$ method after normalization to the reference gene GAPDH.

11. Micro-CT analysis

The implants from each mouse were scanned with a high resolution micro-CT system (vivaCT 40, Scanco Medical AG, Brüttisellen, Switzerland), resulting in three-dimensional (3D) reconstruction of cubic voxel sizes 10.5 × 10.5 × 10.5 μm3. Micro-CT images were segmented using techniques described in detail previously [1] to obtain accurate 3D datasets of bone tissues. A mean optimal global threshold was applied to segment bone image datasets. Three-dimensional microarchitectural properties of implant bone tissue were analyzed.

12. Histology analysis

After micro-CT scanning, the scaffolds from each group were embedded in methylmethacrylate (MMA) and sectioned at 25-μm thickness with a cryostat. Sections were stained with Goldener's Trichrom and Alizarin Red to visualize tissue morphology and evidence of new bone formation.

13. Statistical analyses

Results are presented as mean ± standard deviation (SD). Statistic analyses were performed using Stata software. The data of ALP/DNA/calcium content and gene

expression were analyzed by the paired sample test. Micro CT data were analyzed with one sample mean test. Significance was set at $P<0.05$.

Reference:

1. Ding, M., Odgaard, A. & Hvid, I. Accuracy of cancellous bone volume fraction measured by micro-CT scanning. *Journal of biomechanics* **32**, 323-326 (1999).

Supplementary Figure Legends

Supplementary Fig. 1. Immunostaining of OCT4, SOX2, and KLF4 expression in fibroblasts at 16 hours after modified mRNA transfection. OCT4, SOX2, and KLF4 were stained in green. Nuclei were stained with DAPI (blue). Scale bar: 32 μm.

Supplementary Fig. 2. ALP staining of iPS-MSCs after 7-days culturing in proliferation medium (**a**) or osteogenic medium (b).

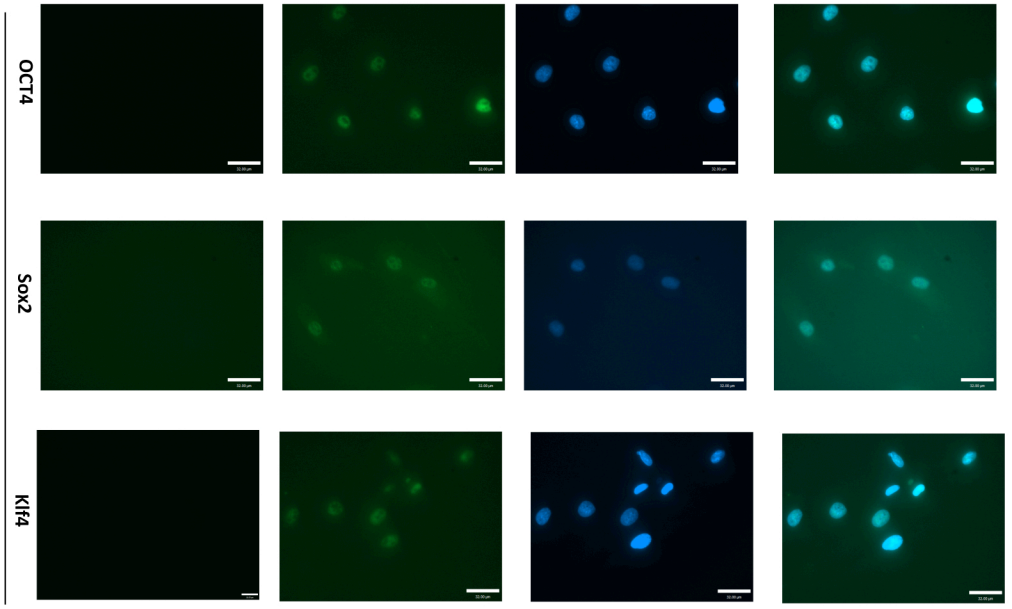
Supplementary Table 1. List of primers used for Q-PCR

Supplementary Table 2. Quantitative comparison of microarchitectures between iPS-MSCs seeded scaffolds (PCL-MSC or PHT-MSC) and scaffolds without cells after implantation in nude mice for 12 weeks. BS/BV, bone surface to bone volume ratio (mm^{-1}); BS/TV, bone surface to total volume ratio (mm^{-1}); BV/TV, bone volume to total volume ration; SMI, structure model index; P-ratio, degree of

anisotropy; CD, connective density (mm^{-3}); TbTh, trabecular thickness; TBSP, trabecular separation (mm); TbN, trabecular number. Results are given as Mean difference between iPS-MSCs seeded scaffolds (PCL-MSC or PHT-MSC) and scaffolds without cells (PCL or PHT), respectively, ± standard deviation. The P value represents one sample mean test against zero. Significant level $p < 0.05$.

S Fig. 1

YL. top


OCT4
Sox2
Klf4
Modified mRNA
0 hour
16 hour
Time Post-transfection

S Fig. 2

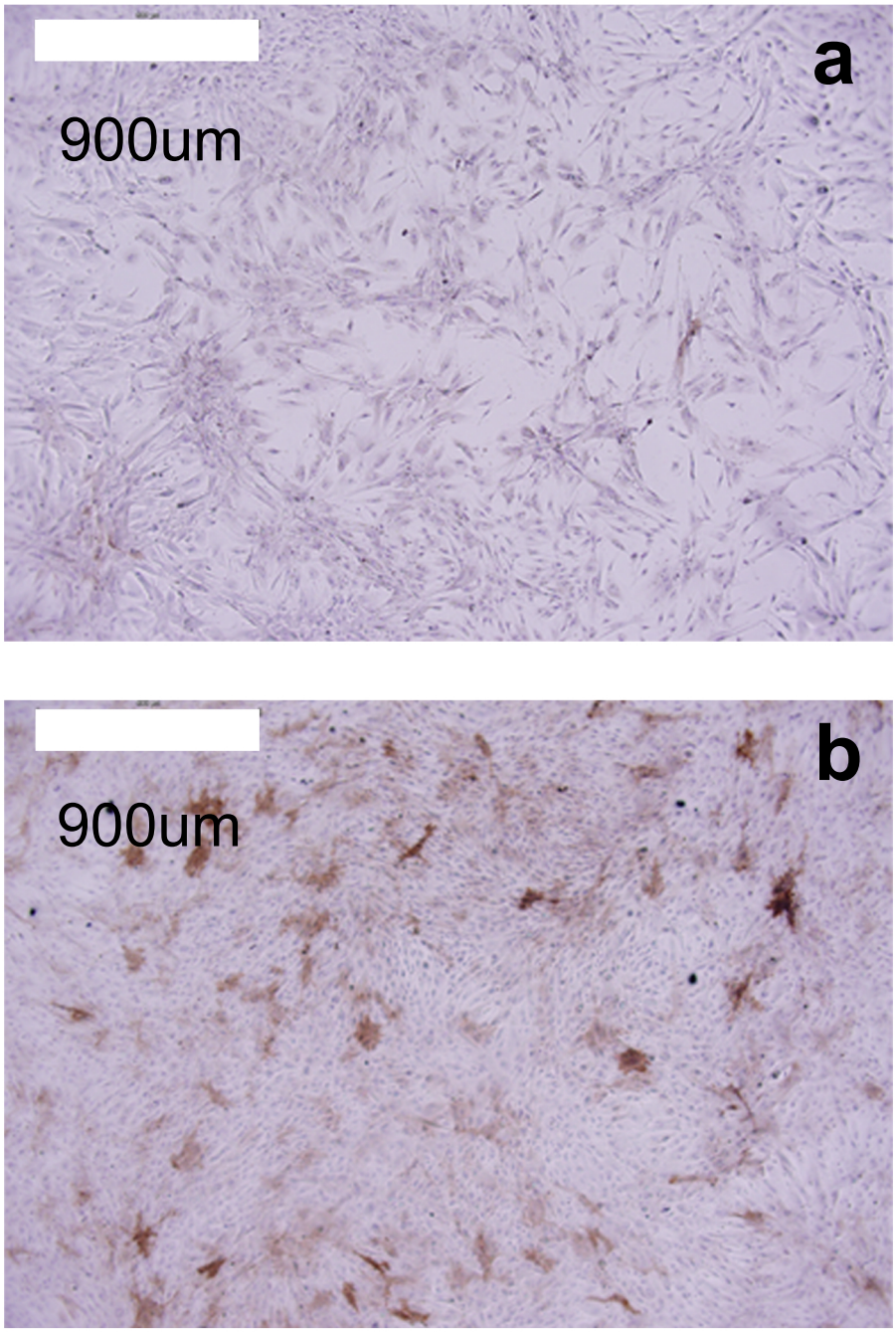


Table S1

Gene	Sequences (5'→3')	exons	Amplicon (bp)
hALPL-F	ATCAGGGACATTGACGTGATC	6-7	137
hALPL-R	TTCCAGGTGTCAACGAGGTC		
hCol1a1-F	AGGGCCAAGACGAAGACATC	1-2	138
hCol1a1-R	AGATCACGTCATCGCACAAC		
hOC-F	AGTCCAGCAAAGGTGCAGCC	2-4	169
hOC-R	TCAGCCAACTCGTCACAGTC		
hRunx2-F	CAGTAGATGGACCTCGGGAA	5-6	188
hRunx2-R	CCTAAATCACTGAGGCGGTC		
hGAPDH-F	TGGTATCGTGGAAGGACTCATGAC	7-8	189
hGAPDH-R	ATGCCAGTGAGCTTCCCGTTCAGC		

Table S2

Parameters	Obs.	MEAN±SD	Pvalue
(OP=102)			
BS/BV			
PCL-MSC vs. PCL	4	13.335±24.725	0.359
PHT-MSC vs. PHT	4	-4.072±14.323	0.609
BS/TV			
PCL-MSC vs. PCL	4	0.689±0.673	0.133
PHT-MSC vs. PHT	4	0.751±1.158	0.285
BV/TV			
PCL-MSC vs. PCL	4	0.007±0.009	0.201
PHT-MSC vs. PHT	4	0.01±0.012	0.183
SMI			
PCL-MSC vs. PCL	4	0.125±0.193	0.285
PHT-MSC vs. PHT	4	0.49±0.839	0.326
P-ratio			
PCL-MSC vs. PCL	4	0.035±0.05	0.267
PHT-MSC vs. PHT	4	0.026±0.085	0.589
CD			
PCL-MSC vs. PCL	4	-38.221±53.435	0.247
PHT-MSC vs. PHT	4	-47.132±74.107	0.293
TbTh			
PCL-MSC vs. PCL	4	-0.001±0.003	0.603
PHT-MSC vs. PHT	4	-0.001±0.007	0.717
TBSP			
PCL-MSC vs. PCL *	4	-0.037±0.003	<0.001
PHT-MSC vs. PHT	4	-0.05±0.053	0.163
TbN			
PCL-MSC vs. PCL *	4	1.178±0.253	0.002
PHT-MSC vs. PHT	4	1.375±1.404	0.145