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Appendix - I

Scaffold fabrication and characterisation:

Hydroxyapatite was synthesized by wet precipitation involving ammoniated calcium nitrate solution and ammoniated ammonium dihydrogen orthophosphate solution [1]. Green body for cylindrical blocks of HA was fabricated from the HA powder by gel-casting technique. After drying the green body was machined to form hollow cylinder and heated to remove the binders and other additives. The cylinder then soaked in silica sols [2]and sintered at 1150- 1250°C to cause solid phase reaction to form the triphasic system and for strengthening. The scaffold was then ultrasonically cleaned, dried and sterilized by autoclaving before use. The whole process was carried out in a clean environmental condition. The scaffold was characterized for phase purity and crystallinity by powder X-ray Diffraction, morphology by scanning electron microscope (SEM) analysis, porosity by mercury intrusion porosimetry and for the presence of trace level heavy elements by inductively coupled optical emission spectroscopy (ICP-OES).

The final product was mounted on a stainless steel (SS) wire with loops in place at top and bottom. This was mounted in a glass bottle and triple bagged in sterilizable covers (Westfield Medical) and then steam sterilized. Quality of sterilization was validated by microbial culture method.

Isolation and characterization of bone marrow-derived mesenchymal stem cells (BM-MSC):

Under general anesthesia, (40 ml) bone marrow was harvested from the right posterior iliac crest and mononuclear cells were harvested, cultured and characterized for MSC cell surface markers. A total of 121 $x10^6$ mononuclear cells were obtained from bone marrow after Ficoll-Pacque density gradient separation. The cells were characterised by flowcytometry (BD FACSCalibur™, BD Biosciences, C.A) for cell surface markers CD45 (87.5%), CD34 (2.19%), CD14 (4.24%), CD73 (1.62%), CD90 (1.20%) and CD105 (2.96%). The primary cells were cultured (11.25 $x10^6$ cells per T75 cm^2) at 37°C incubator with 5% CO_2 for eleven days and expanded till passage one (5 $x10^5$ cells per T75 cm^2) up to nine days in alpha Minimum Essential Medium (α-MEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin/streptomycin 10,000 U/ml). Mesenchymal stem cells were identified by their a) ability to adhere to plastic, b) spindle-shaped fibroblast-like morphology and c) lacked expression hematopoietic cell surface markers CD45 (1.93%), CD34 (3.22%), CD14 (1.89%), and expressed CD73 (98.50%), CD90 (99.10%) and CD105 (99.56%) prior to cell seeding on scaffolds (Fig.2a, b) as recommended by the International Society of Cellular Therapy (ISCT) [3]. Further, i*n vitro* multilineage differentiation potential was demonstrated by adipogenic (Oil-O-Red) and osteogenic differentiation (alizarin Red-S) staining (Fig. 2c).

Preparation of the tissue-engineered graft:

The customized scaffolds (4 cm long x2 cm wide) were allowed to prime in culture medium for 24 hours and further allowed to dry for another 24 hours. For cell seeding, the scaffold was placed on an aluminium stand as shown (Fig. S.1a). Mesenchymal stem cells at a density of 1x 10^5 per sq. cm was seeded on the scaffold with a surface area of 37.2cm^2 (curved surface, proximal and distal sites, and within the inner hollow surface to ensure uniform seeding). The cells were allowed to attach for 30-45 minutes inside CO_2 incubator prior to the addition of culture medium. The osteogenic differentiation medium composed of α-MEM with 15% FBS, 10mM β-glycerophosphate, 10^{-8}M dexamethasone and 0.05 mg/mL L-ascorbic acid was added twenty four hours later and cultured) upto five days prior to transplantation (Fig. S.1b). The cell-seeded scaffold passed the release criteria for transplantation

after being negative for microbial culture (BacT/ALERT, Biomerieux, Durham, NC), mycoplasma (ATCC, Manassas, Virginia) and endotoxin (<0.5 EU/ml) (Charles River, Wilmington, MA) [4].

Additional analysis on scanning electron microscopy (FETQuanta 200, FEI, Eindhoven, Netherlands) revealed the presence of cell sheet structures and good fibroblast spread morphology on scaffold surface (Fig. 2d, e); viability (Fig.2 f) of cells on the scaffold was assessed by live dead assay (Molecular Probes, Invitrogen, USA) where we can observe microscopically that the live cells have taken up Calein (green) and dead cells have taken up Ethidium homodimer (red).

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1. Varma, H., S.P. Vijayan, and S.S. Babu, *Transparent Hydroxyapatite Ceramics through Gelcasting and Low-Temperature Sintering.* Journal of the American Ceramic Society, 2002. 85(2): p. 493-495.
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4. Mizukami, A. and K. Swiech, *Mesenchymal Stromal Cells: From Discovery to Manufacturing and Commercialization.* Stem Cells International, 2018. 2018.

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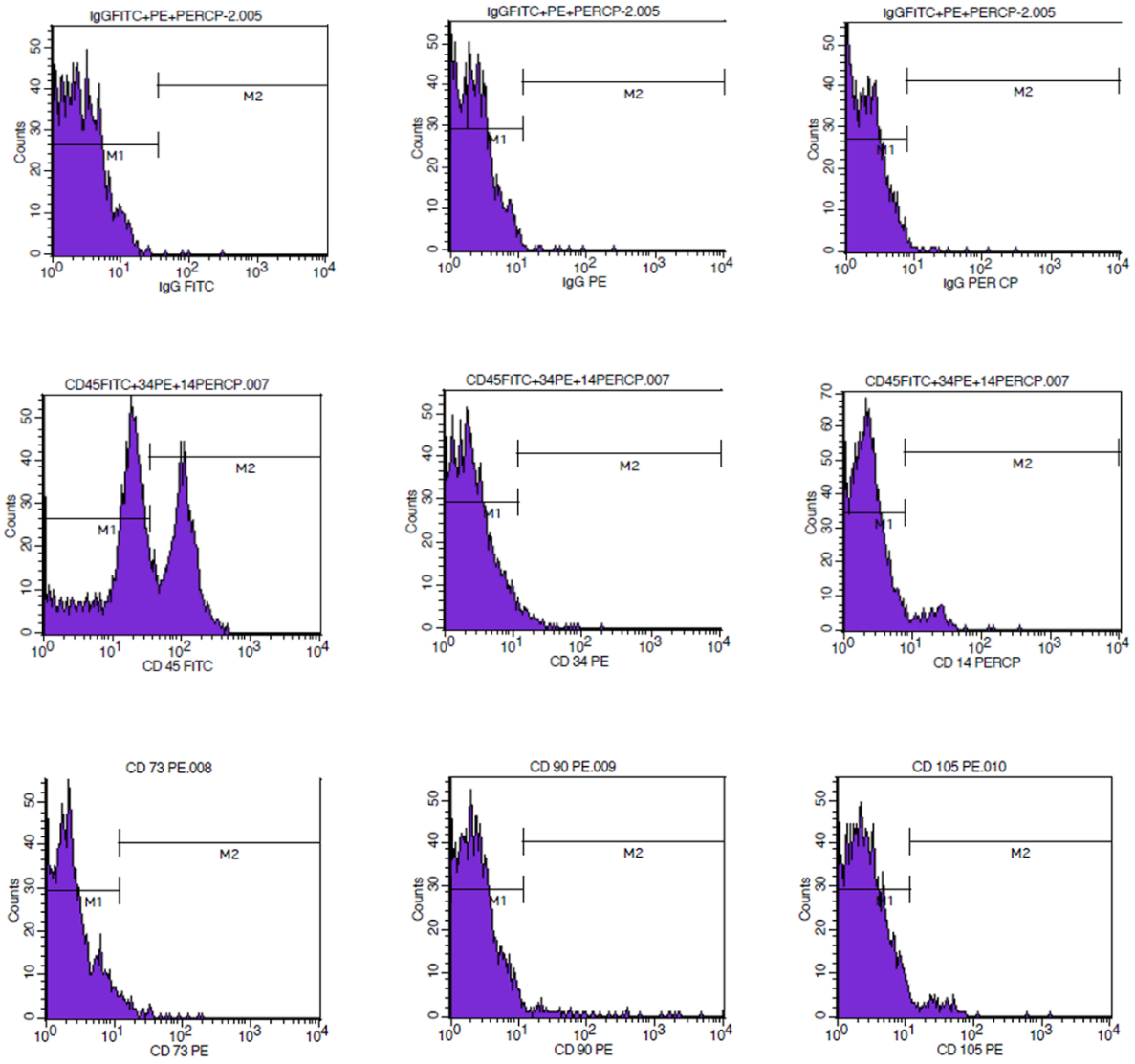

Figure S1

Flow cytometry analysis of harvested bone marrow mononuclear cells at harvest and showing the percentage of cells positive for human MSC cell surface markers. Isotype controls (<5%); negative markers: CD45 (87.47%), CD34 (2.19%), CD14 (4.24%); positive markers: CD73 (1.62%), CD 90 (1.20) and CD105 (2.96%).

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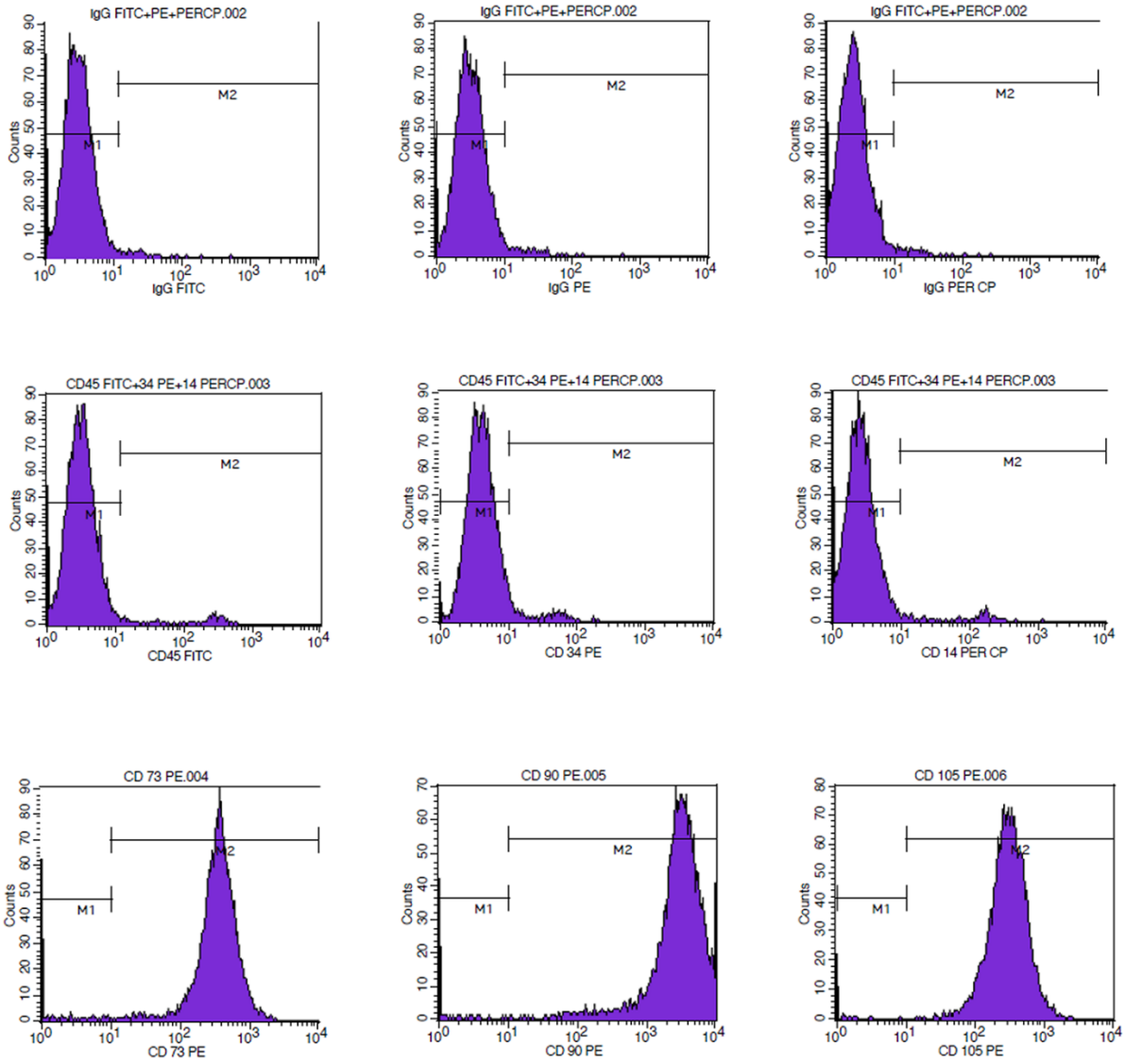

Figure S2

Flow cytometry analysis of mesenchymal stem cells at passage one. Isotype controls (<2%); negative markers: CD45 (1.93%), CD34 (3.22%), CD14 (1.89%); positive markers: CD73 (98.5%), CD 90 (99.19%) and CD105 (99.56%).

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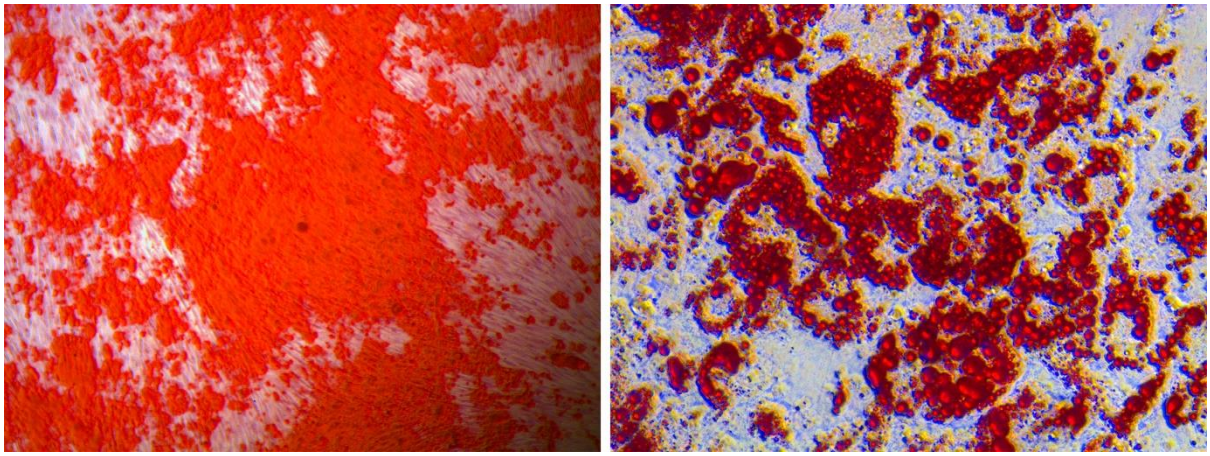

Figure S3
Multilineage differentiation of MSC into osteogenic (left) and adipogenic (right) lineage assessed by Alizarin Red S and oil –O red staining, respectively.

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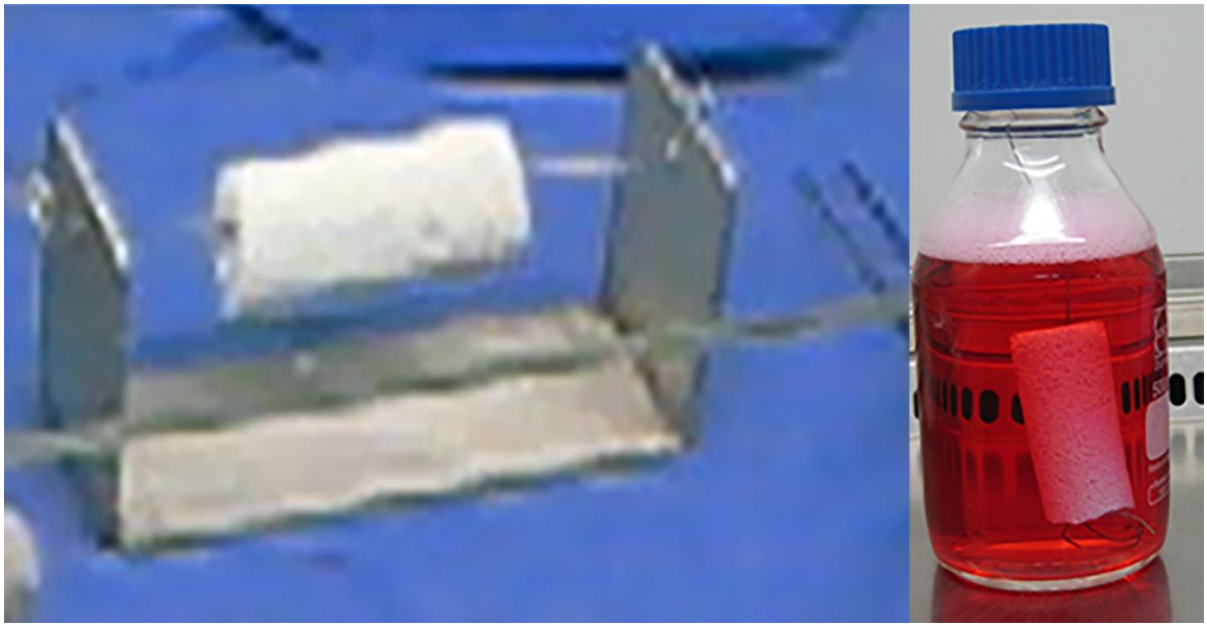

Figure S4

The process of seeding mesenchymal stem cells on hydroxyapatite scaffold (left); b. the process of culturing cell-seeded construct under static conditions (right).

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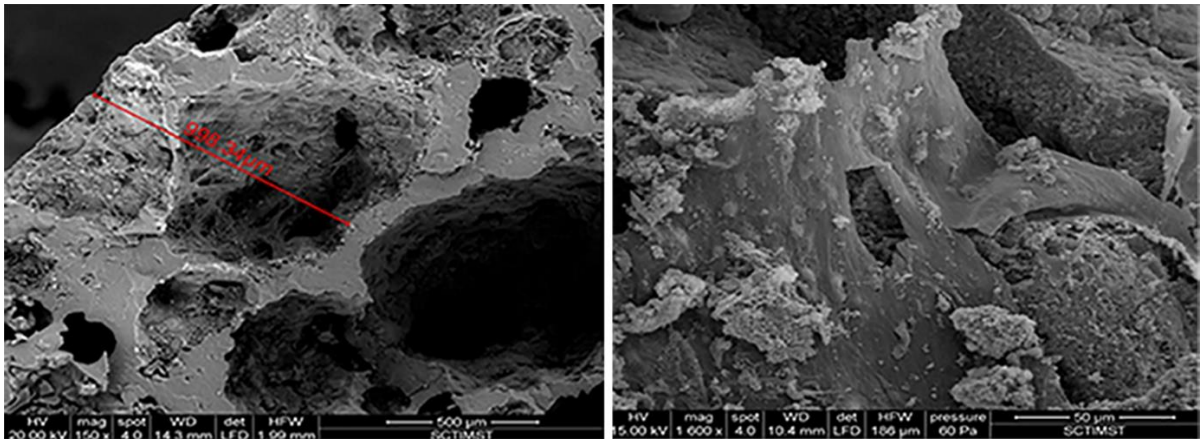

Figure S5

Scanning electron micrographs of hydroxyapatite tricalcium silicate (HASi) showing the porous structure of the scaffold (left, scale 500 µm); and right showing cell-seeded scaffold with excellent cell coverage and cell sheet across the scaffold on day 7 after osteogenic differentiation (scale 50 µm).

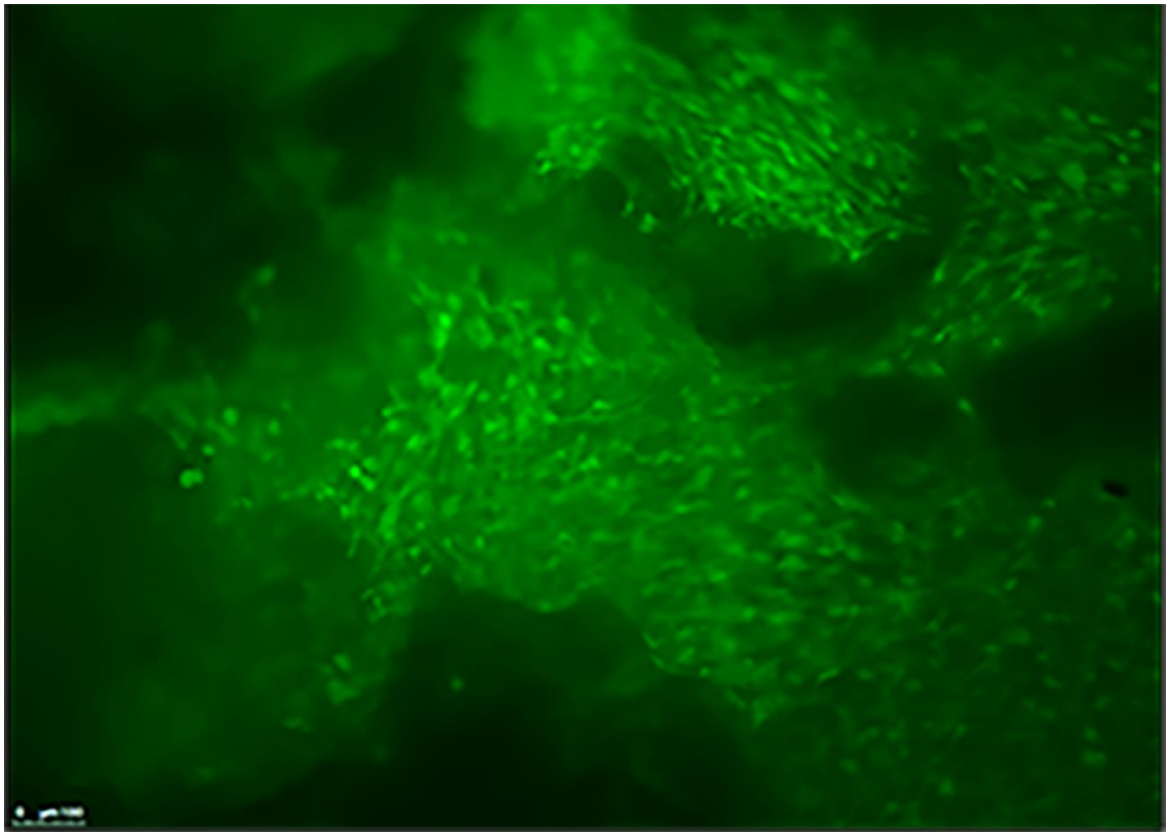

Figure S6

A fluorescent microscopic image at day 7 of cell-seeded construct. Live-dead assay showing over 98% viability (green, calcein dye) of human MSC seeded on hydroxyapatite scaffold (scale 100 μm).