MATERIALS AND METHODS

Sample Preparation

Pure tricalcium phosphate powder was prepared through solid state synthesis, by milling two moles calcium hydrogen phosphate anhydrous ($CaHPO_4$, Alfa Aesar, >99.0%) and one mole calcium carbonate ($CaCO_3$, Alfa Aesar, >98.0%) at a powder: milling media ratio of 1:4 for two hours, and then calcined at 1050°C for 24 hours in a muffle furnace. The calcined powder was naturally cooled to room temperature. Copper (II) oxide (CuO) was added to $CaHPO_4$ and $CaCO_3$ precursors at 0.25, 0.5 and 1.0 wt. % concentrations to prepare the doped TCP, and the mixture was processed similar to undoped TCP. The powder was then crushed and mixed with ethanol for 6 hours at a powder: ethanol: milling media ratio of 1:1.5:5. The mixture was then dried at 65 °C for 3-4 days, until all ethanol was evaporated. Powder was poured into a 13mm diameter die set, which was held at 25.5 kN for two minutes by a uniaxial press. Samples were then transferred to a porous zirconia substrate and sintered at 1050 °C and 1150°C for 2 hrs using a muffle furnace, followed by cooling to room temperature.

Phase Composition, Surface Morphology and Density

Phase composition of samples was analysed by Rigaku D/MAX diffractometer using CuKα radiation, equipped with Ni filter at step size of 0.04° and count time of 1.5 seconds per step. The relative α-TCP phase intensity and correspondent α-TCP phase amount was calculated by:

$$\frac{Intensity\ of\ \alpha\ phase}{\Sigma\ Intensity\ of\ \alpha\ and\ \beta\ phases}$$

After applying a thin layer coating of Pd/Au, microstructure of top surface of samples was investigated using field emission scanning electron microscope (FESEM, Hitachi model S4500).

Relative Bulk and apparent densities were measured by specimen dimension and Archimedes' method. Samples were submerged in boiling water for two minutes to ensure all trapped air was removed from internal pores, and then immediately submerged in room temperature water to obtain the wet weight.

Osteoblast Cell Preparation

The human fetal osteoblast cell line (hFOB 1.19, ATCC, Manassas, VA) was cultured in filter sterilized Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F12, Sigma, St. Louis, MO), supplemented with 300 mg/L G418 (Sigma), 10% fetal bovine serum (FBS, Sigma) and antibiotic/antimycotic. Upon 85% confluency, cells were detached and seeded on autoclave-sterilized samples. Disks were maintained in 5% CO_2/ 95% air at temperature of 34ºC and cell culture medium was replenished every 2-3 days during the culture experiment.

Assessment of Cell Growth using XTT assay

Cells were suspended in medium at concentration of 50×10^3 cells /20 µl and seeded on samples as mentioned above. After 5 or 10 days, media was carefully removed from wells and samples were washed gently with 1 ml DPBS (ThermoFisher Scientific, Waltham, MA). Samples were then removed and placed into a new 24-well plate followed by addition of 400 µl of DPBS and 100 µl of XTT working solution (Trevegin, Gaithersburg, MD) and incubated at 34℃ for 3 hours then analyzed on an Epoch plate reader (Biotek, Winooski, VT).

Assessment of Gene Expression

For gene expression studies, 2×10^6 cells in 20 µl were seeded on top of each TCP disk in triplicate wells of 24-well plates, followed by addition of 400 µl cell culture medium. After 1 or 5 day incubation, media was removed and disks were washed gently with 1 ml DPBS. Samples were placed into new 24-well plates and cells were immediately lysed using 1 ml TRIzol reagent (ThermoFisher Scientific) and total RNA was isolated following manufacturer's protocol as previously published [1,2]. Reverse transcription reactions were performed using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, Madison, WI). Quantitative real-time PCR (qPCR) was conducted using the ViiA7 real-time PCR

instrument (Life Technologies, Grand Island, NY). Gene expression was calculated by subtracting the threshold cycle (C_T) for the house keeping gene (GAPDH) from the gene of interest and relative gene expression was compared to cells grown on pure TCP disks. The following primers were used in this study: GAPDH, 5'-CTCGACTTCAACAGCGACA- 3' (forward) and 5'-GTAGCCAAATTCGTTGTCATACC-3' (reverse); OPG, 5'-GTCTTTGGTCTCCTGCTAACTC-3' (forward) and 5'-CCTCACACAGGGTAACATCTATTC-3' (reverse); COLA1, 5'-CGATGGATTCCAGTTCGAGTATG-3' (forward) and 5'-CTTGCAGTGGTAGGTGATGTT-3' (reverse); ALP-1, 5'-CCTACCAGCTCATGCATAACA-3' (forward) and 5'-GGCTTTCTCGTCACTCTCATAC-3' (reverse); OCN, 5'-CAGGCGCTACCTGTATCAAT-3' (forward) and 5'-CGATGTGGTCAGCCAACT-3' (reverse); VEGF, 5'-GATGAGCTTCCTACAGCACAA-3' (forward) and 5'-CTTTCCCTTTCCTCGAACTGAT-3' (reverse); IL-6, 5'-TCCAAAGATGTAGCCGCCC-3' (forward) and 5'-CAGTGCCTCTTTGCTGCTTTC-3' (reverse); IL-17 5' -TAAGATAATGGCCCTGAGGAATG- 3' (forward) and 5' -GACAATGTCTCCTCCCAGAAC- 3' (reverse); TNF-α, 5'-CCAGGGACCTCTCTCTAATCA-3' (forward) and 5'-TCAGCTTGAGGGTTTGCTAC-3' (reverse); and RANKL, 5'-AGCACATCAGAGCAGAGAAAG-3' (forward) and 5'-TGTCGGTGGCATTAATAGTGAG-3' (reverse).

Cell Imaging

After 1, 5 or 10 days, media were carefully removed and disks were gently washed with 1 ml DPBS and then placed into new 24-well plate. 600 µl 2% glutaraldehyde/2% formaldehyde in 0.1 M phosphate buffer was then added to the side of the wells and covered the top of each TCP disk. Plates were sealed with parafilm and incubated at 4℃ overnight. Disks were rinsed with 0.1 M phosphate buffer three times followed by addition of 100 µl 2% osmium tetroxide directly on top of each disk for 2 hours at room temperature to fix cells. Cells were rinsed with 0.1 M phosphate buffer again three times, then dehydrated with 1 ml of a series ethanol (30%-50%-75%-95%-100%). Finally, 600 µl of hexamethyldisilane (HDMS) were added to each disk for 10 minutes, then another 1 ml HDMS was added and samples were dried under the fume hood overnight. Samples were then coated with Au/Pd and morphology of cells was investigated using SEM (Lyra3 GMU FIB SEM, Tescan).

RESULTS AND DISCUSSION

Surface Microstructure of Samples

Microstructure of pure and Cu-doped samples are presented in figure 1S.

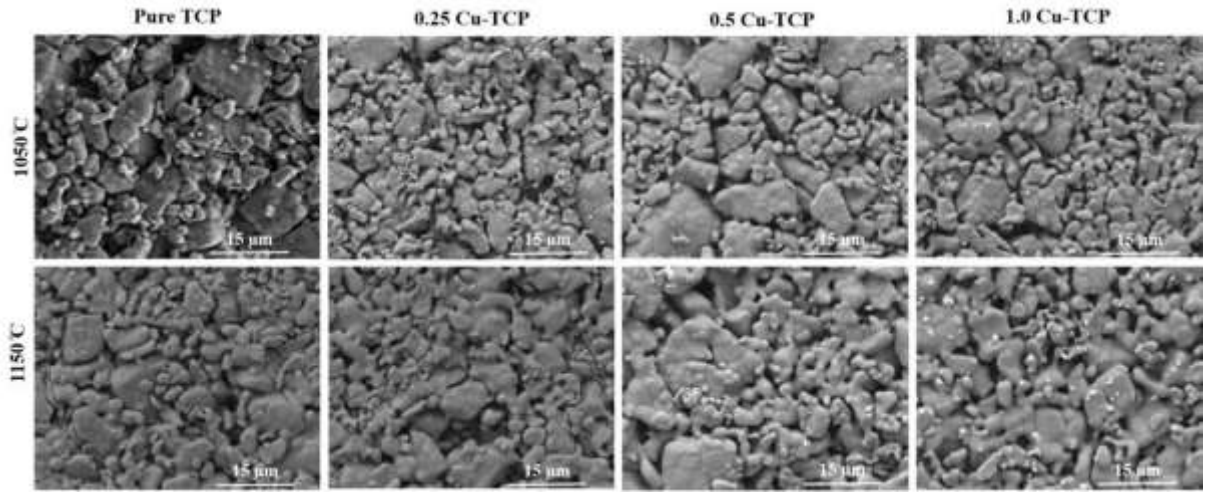

Figure 1S. Microstructure of pure and Cu-doped TCP sintered at 1050 ºC and 1150 ºC.

Density

Table 1S presents the total and open porosity values for pure and Cu-doped TCP samples, sintered at two different temperatures of 1050 ºC and 1150 ºC.

Table 1S. Total and open porosity of pure and Cu-doped TCP samples sintered at 1050 ºC and 1150 ºC.

Composition	Sintering Temperature	Total Porosity (%)	Open Porosity (%)
Pure TCP	1050 ºC	33.74 ± 0.51	29.6 ± 0.95
	1150 ºC	31.26 ± 0.61	27.25 ± 0.67
0.25 Cu-TCP	1050 ºC	32.45 ± 0.14	29.84 ± 0.03
	1150 ºC	37.45 ± 0.39	30.3 ± 0.98
0.5 Cu-TCP	1050 ºC	33.87 ± 0.66	31.29 ± 0.67
	1150 ºC	33.35 ± 0.44	28.31 ± 0.50
1.0 Cu-TCP	1050 ºC	31.87 ±0.16	29.29 ± 0.32
	1150 ºC	31.89 ± 0.67	28.97 ± 0.64

Cellular Morphology

Morphology of cells on samples after 1, 5, and 10 days.

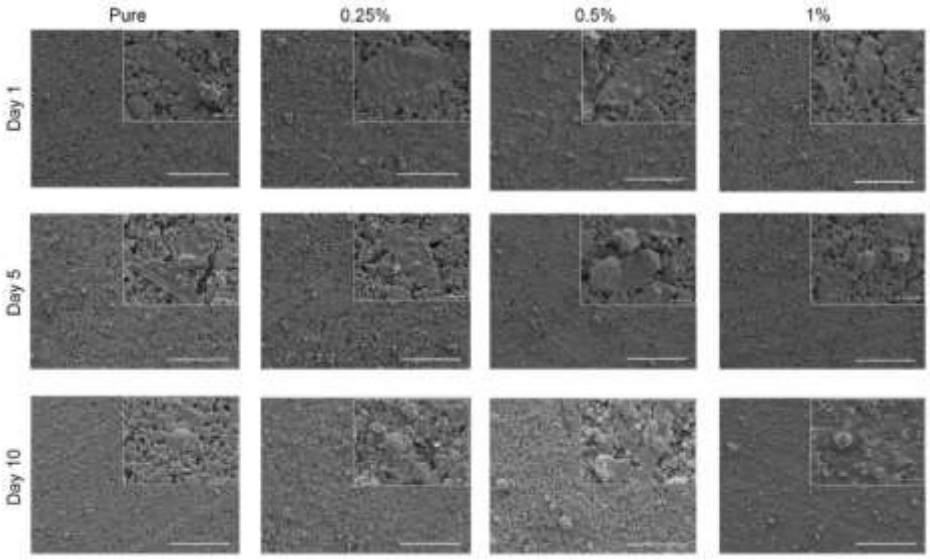

Figure 2S. SEM micrographs of osteoblasts cultured for 1, 5 and 10 days on pure TCP (Pure), or Cu-doped TCP . Scales for each image are represented by white lines and are 100 µm and 10 µm for lower and higher magnification, respectively

[1] H. Cummings, W. Han, S. Vahabzadeh, and S.F. Elsawa, JOM **69**, 1348 (2017).

[2] D.A. Jackson, T.D. Smith, N. Amarsaikhan, W. Han, M.S. Neil, S.K. Boi, A.M. Vrabel, E.J. Tolosa, L.L. Almada, M.E. Fernandez-Zapico, and S.F. Elsawa, J. Immunol. Baltim. Md 1950 **195**, 2908 (2015)