Supplementary Information

Biomineralization Guided by Paper Templates

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Supplementary Information

Structure of bone

Bone is a dynamic structure that continuously remodels throughout its lifetime [1]. Remodeling takes place in response to changes in biomechanical forces, mechanical injury, or to adapt the strength of the bone [2]. An injury to the bone initiates a cascade of complex processes that regenerate the damaged areas. The process of spontaneous healing begins with the formation of a hematoma (blood clot), and elicits an inflammatory response. The hematoma attracts immune cells via signaling molecules [3]. Fibroblasts subsequently migrate towards the site of the injury and lay down extracellular matrix (ECM), which is primarily composed of collagenous proteins (mainly collagen type I) and proteoglycans [4]. Deposition of matrix leads to the formation of a fibrous cartilage (callus, rich in collagen type I), which stabilizes the healing tissue mechanically. As the repair progresses, the callus progressively vascularizes and mineralizes into woven bone, which is eventually replaced by compact bone [3].

Bone is composed—in addition to its solid hydroxyapatite structural elements—of four types of cells: osteoclasts, bone-lining cells (also known as osteoprogenitor cells), osteoblasts, and osteocytes [4]. Osteoclasts are multinucleated cells that are derived from macrophages. The primary function of osteoclasts is to digest bone by secreting acidic proteins and enzymes [2]. The bone-lining cells are of mesenchymal origin from the bone marrow and remain quiescent unless there is an external stimulus (mechanical, hormonal, and/or nutritional) [2]. The bone-lining cells turn into osteoblasts when the signaling molecules direct them to deposit bone minerals in response to an external factor (mechanical stimulation, microdamage, and/or injury) [1]. Osteoblasts are responsible for formation of bone by laying down collagenous matrix, which is subsequently mineralized by precipitation of calcium and phosphate [5]. During the process of mineralization, some of the osteoblasts are trapped and become buried inside the matrix; there, they terminally differentiate into osteocytes [2]. The osteocytes provide a structural network for

the bone. In this work, we studied osteoblasts, and their deposition of minerals in structured scaffolds.

Scaffolds for bone

The ideal scaffold for bone must be porous, resilient, biodegradable, biocompatible, osteoinductive (enabling differentiation of cells into the bone lineage), and osteoconductive (enabling bone to grow on a surface) [6]. The most common scaffolds of bone include polymeric materials (e.g., poly (caprolactone) (PCL), poly(lactic-co-glycolic acid) (PLGA), poly(propylene fumarate) (PPF), polyurethane (PU), polyethylene glycol (PEG), gelatin, collagen, alginate, chitosan, silk, and starch), metals (e.g., stainless steel, platinum, titanium, and cobalt), and inorganic materials (e.g., hydroxyapatite and β-tricalcium phosphate (β-TCP)) [7]. Composite materials have also been used to overcome limitations and improve the characteristics of single-material scaffolds [8]. Different materials, which can complement the features of each other, can be combined to control the properties of composite scaffolds such as degradation, biocompatibility, and osteointegration.

Paper-based cell culture platforms

Although they have been explored for other aspects of cell biology [9-13], the simple and flexible features of paper-based materials have not been used to guide deposition of hydroxyapatite by osteoblasts. We previously reported a technique for culturing cells in gel-impregnated paper scaffolds [9]. We refer to this approach as cells-in-gels-in-paper (CiGiP). It provides an experimentally simple method to generate 3D tissues. The CiGiP approach forms multi-layered 3D models of tissues by stacking layers containing cells in the hydrogel slabs supported mechanically on paper. We used CiGiP for studying the migration of mammalian cells [12], developing invasion assays [10, 11], generating parallel arrays of 3D cell cultures, high-throughput testing of drugs for cancer cells [13], and controlling diffusion of oxygen and nutrients to cells that were cultured in multi-layered stacks [9]. In addition to cancer cells, we

also cultured cardiac myocytes, fibroblasts, and primary lung tumor cells in paper [12, 14]. The cell cultures in paper allowed high cell viability and metabolic activity, as well as biocompatibility upon subcutaneous implantation in mice [9]. The scaffolds made of paper combine the simplicity of a biocompatible material with the ability to form free-standing structures.

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Supplementary Figures

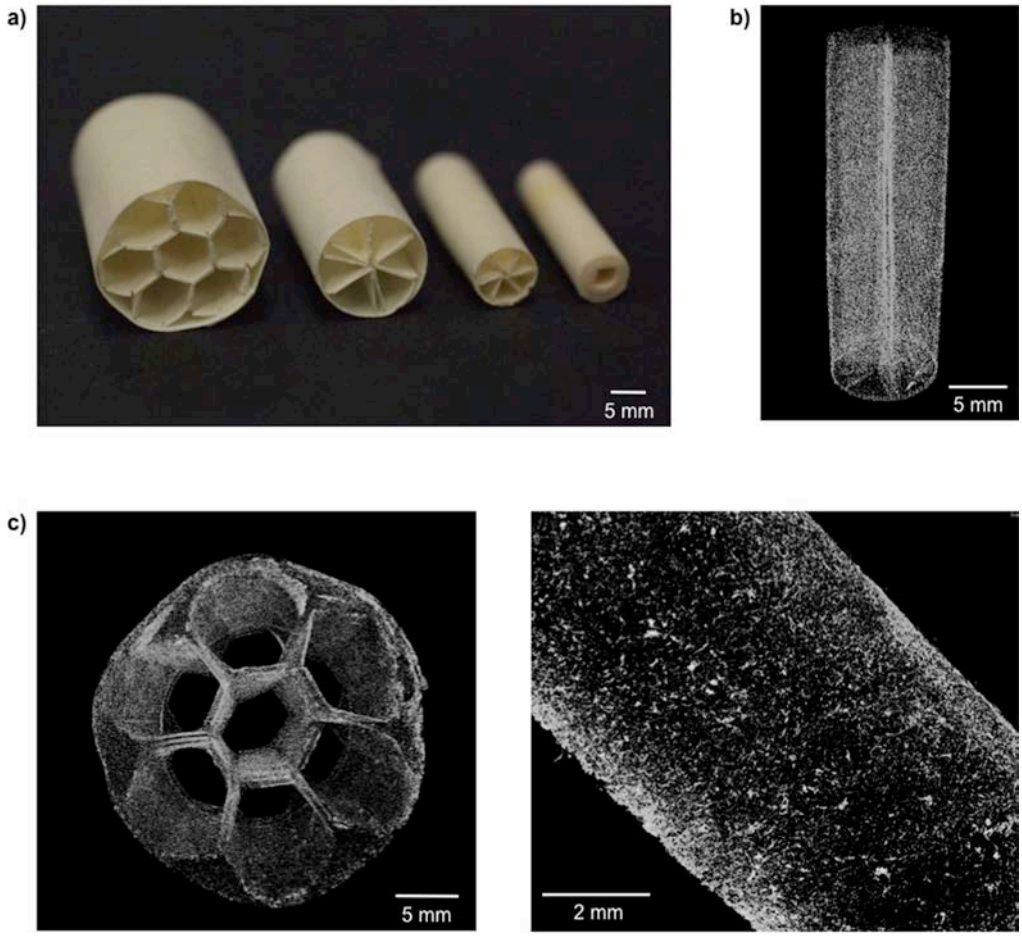

Figure S1. Biomineralized origami-inspired paper scaffolds. a) The cells were seeded in the paper scaffolds and cultured for 21 days. b-c) The micro-CT X-Ray scans illustrated the mineralized areas in the paper constructs in bright white color.

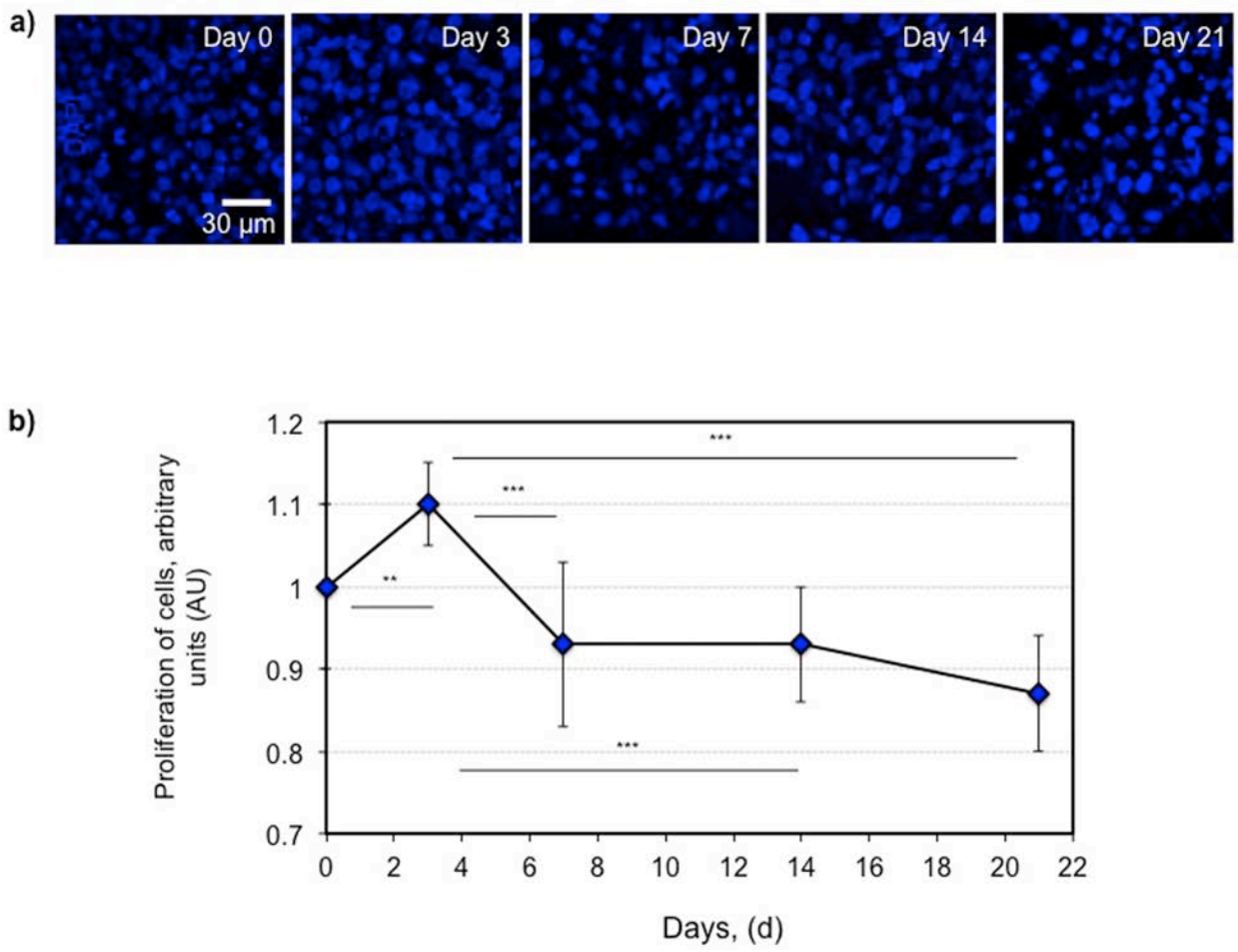

Figure S2. Proliferation of cells in the collagen matrix in paper scaffolds at different time points. We stained the nuclei of the cells to image the distribution and proliferation of the cells on days 0, 3, 7, 14, and 21. The initial seeding density was 1.6×10^6 cells/sample. We stained the samples with DAPI (blue), and obtained the images by confocal microscopy. The results indicated that proliferation increased until day 3 and then decreased after day 7. Because proliferation slows down at the onset of mineralization, this result is expected. The scale bar represents 30 µm. We used GraphPad Prism (Version 4.02, La Jolla, CA) to carry out one-way ANOVA analyses, and determined the statistical differences between different conditions (n=10, Error bars: ± SD). The *p*-values < 0.05 were considered as statistically significant (**$p < 0.01$, and ***$p < 0.001$).

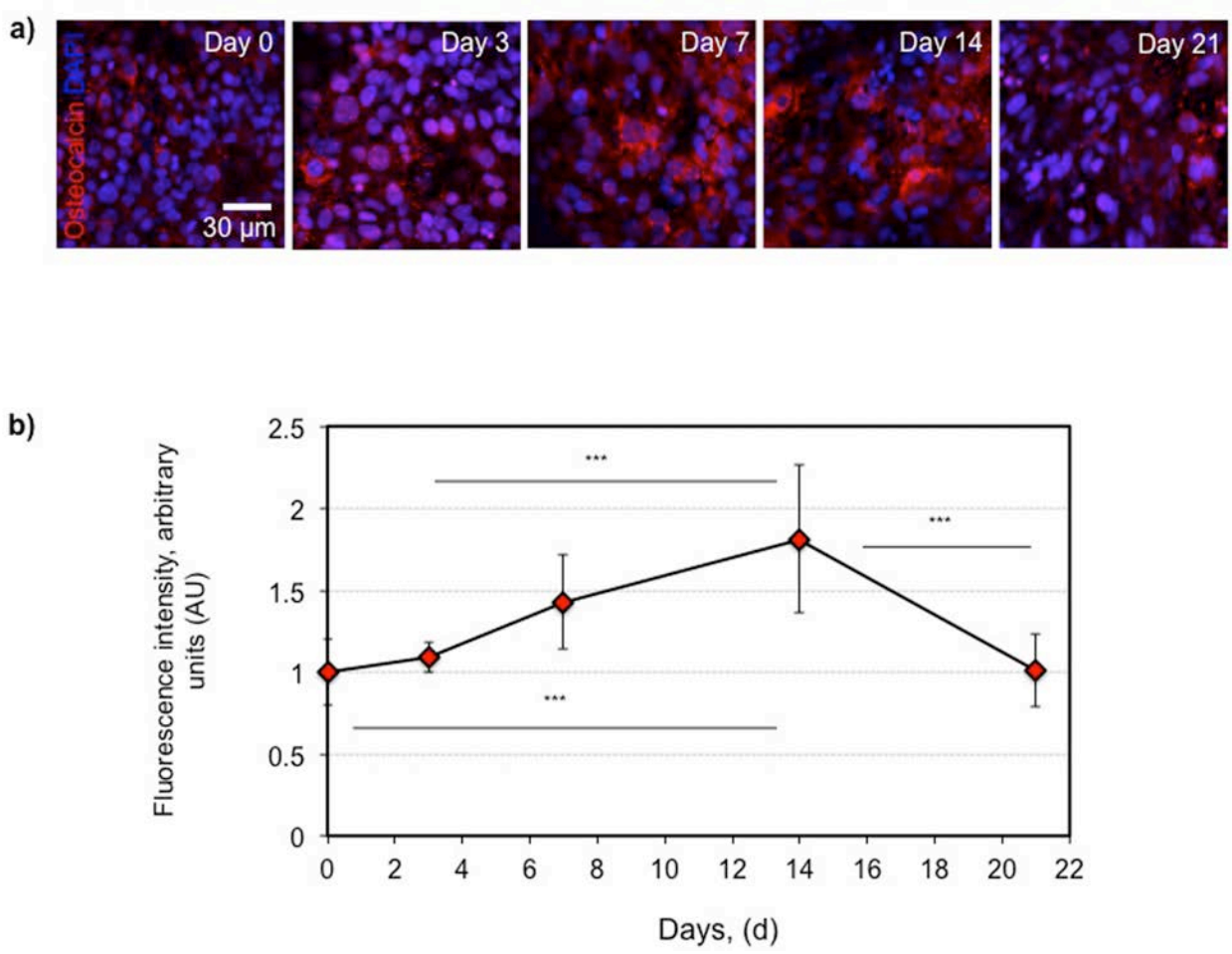

Figure S3. Expression of a bone-specific marker, osteocalcin, was determined by immunocytochemistry in the paper scaffolds. The initial cell density was $1.6x10^6$ cells/sample. We carried out immunostaining for osteocalcin (red) on days 0, 3, 7, 14, and 21, and acquired the fluorescent images by confocal microscopy. We counter-stained the cells with DAPI (blue) to visualize the nuclei of the cells. The expression of osteocalcin increased until day 14 and then decreased. This result could be due to increasing mineralization after day 14. The scale bar represents 30 μm. We used GraphPad Prism (Version 4.02, La Jolla, CA) to perform one-way ANOVA, and determined the statistical differences between different conditions (n=10, Error bars: ± SD). We considered *p*-values < 0.05 as statistically significant (***$p < 0.001$).