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**Freestanding stacked mesh-like hydrogel sheets enable
the creation of complex macroscale cellular scaffolds**

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Cell culture

The human hepatocellular carcinoma cell line (HepG2) was maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% v/v fetal bovine serum and 1% v/v penicillin–streptomycin in a 5% CO₂ incubator at 37°C. The HepG2 cells were split every 3–4 days, and the culture medium was exchanged every other day after washing the cells with phosphate-buffered saline (PBS; Gibco/Life Technologies, Carlsbad, CA). The NIH3T3 mouse embryo fibroblast cell line was maintained under the same conditions as the HepG2 cells.

Cell proliferation assay

Cell proliferation was assessed to confirm viability, as well as for normalization of albumin and urea secretion using tetrazolium salt metabolic activity (CCK-8; Dojindo, Rockville, MD). Metabolic activity was assessed with a 4-h incubation period followed by measurement of the absorbance at 450 nm using a microtiter plate reader (SPECTRAMax250; Molecular Devices, Sunnyvale, CA).

Gene expression analysis

HepG2 cells were cultured in stacked and detached structures using each of four hydrogel sheets for 7 days. Following removal of the media, total RNA was extracted using TRIzol reagent (Invitrogen, Eugene, OR). To remove hydrogel debris from the extract, a solution of each sample was centrifuged in a 1.5-mL tube at 4°C and 12,000 rpm (or $13,400 \times g$ relative centrifugal force) for 15 min, and the supernatant containing the RNA was used. Biotinylated cRNA was prepared from 0.55 µg of total RNA using an Illumina TotalPrep RNA Amplification Kit (Ambion, Austin, TX). Following fragmentation, 0.75 µg of cRNA was hybridized with an Illumina Human HT-12 v4 Expression BeadChip (Illumina, Inc., San Diego, CA) according to the protocols provided by the manufacturer. Arrays were scanned using the Illumina Bead Array Reader Confocal Scanner. The data were exported, processed and analyzed using Illumina GenomeStudio v2011.1 (Gene Expression Module v1.9.0).

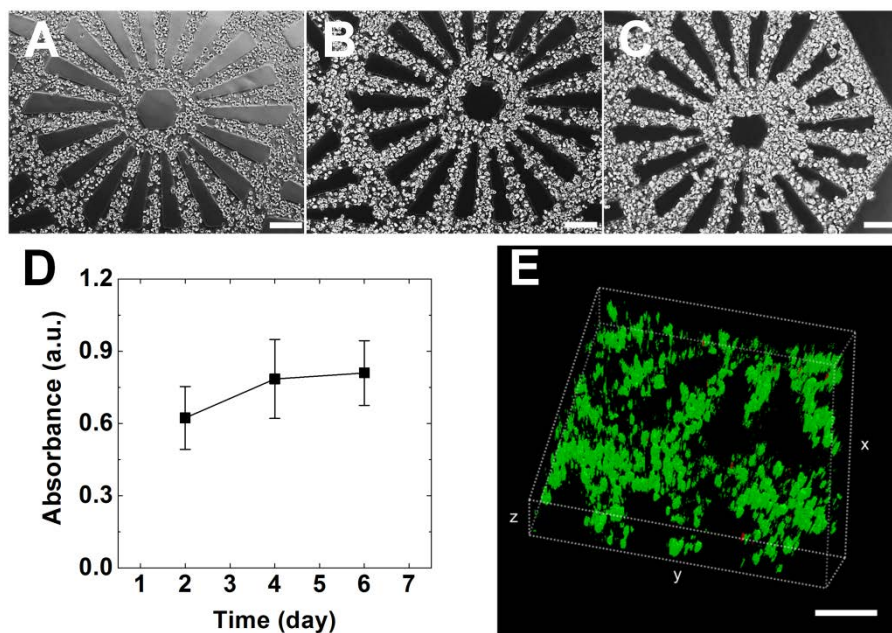


Figure S1. Cell culture in a single hydrogel sheet. (A–C) HepG2 cell culture images on (A) day 0, (B) day 2, and (C) day 6. The cells aggregated and proliferated continuously. (D) The cell proliferation assay of the three hydrogel sheet groups on days 2, 4, and 6 via tetrazolium salt cell metabolism. Data are shown as means \pm standard error. (E) Live/dead stained cells patterned on a hydrogel sheet after 8 days via 3D confocal imaging. Live HepG2 cells were stained with calcein-AM, and dead cells were stained red with ethidium homodimer-1. Scale bars represent 200 μm ; $n = 3$.

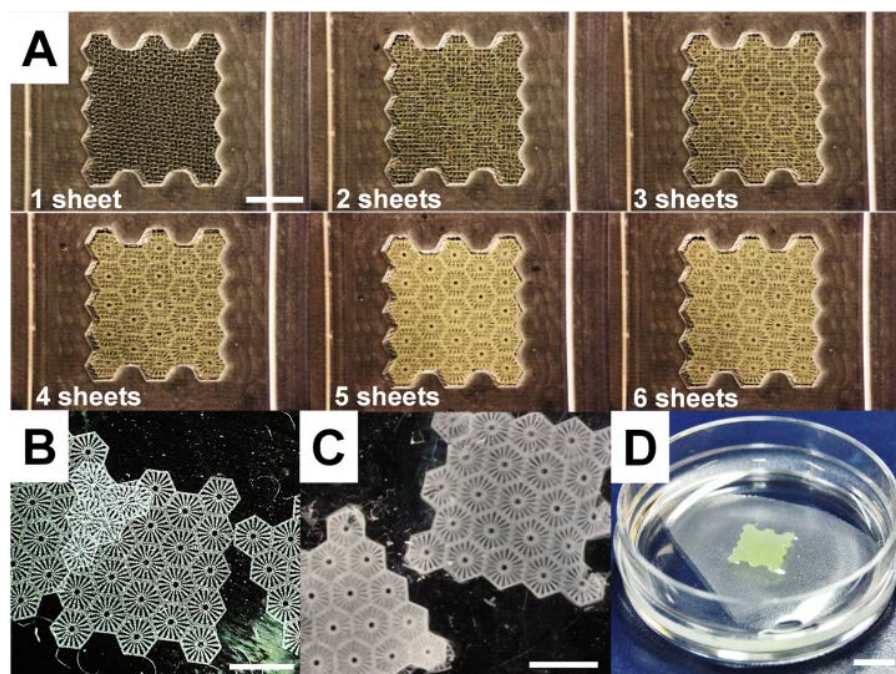


Figure S2. Demonstration of hydrogel sheet stacking with microbeads. Six hydrogel sheets were placed and aligned in the drainage well. The aligned and connected openings in the stacked hydrogel sheets provided a large diffusion area for supply of culture medium and oxygen. Both (B) a single hydrogel sheet and (C) stacked hydrogel sheets were freestanding. (D) Fabricated stacked hydrogel sheets were manipulated by filter paper and used for multicellular culture in culture dish or well plate. Scale bars represent 3 mm (A–C) and 5 mm (D).

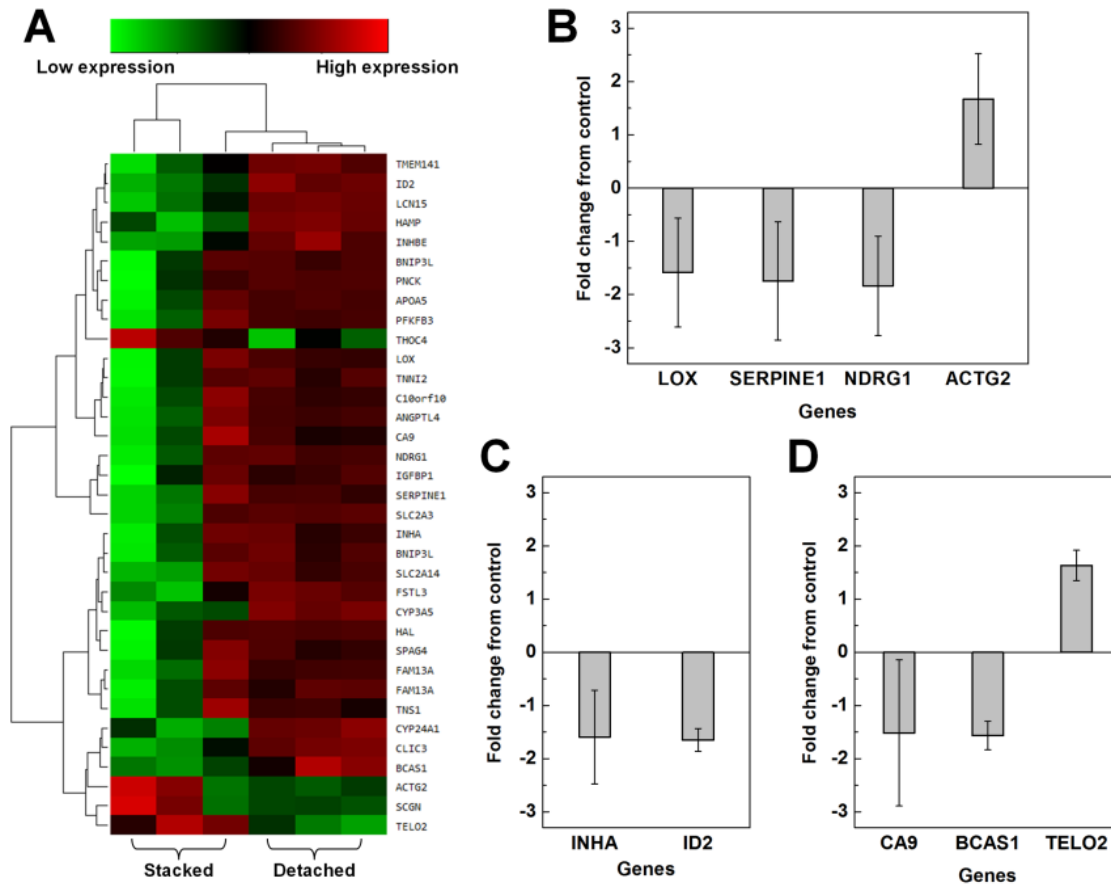
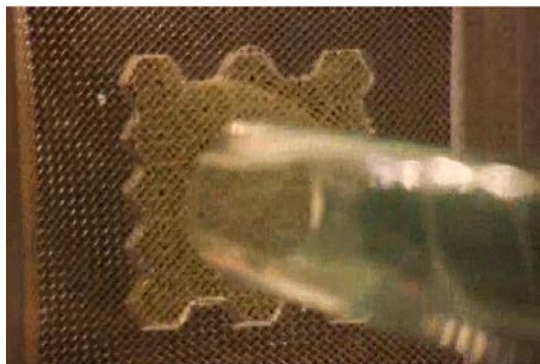


Figure S3. Gene expression analysis between stacked hydrogel sheets (Stacked) and detached hydrogel sheets (Detached). (A) A hierarchical clustering heatmap of 35 genes significantly expressed in microarray analysis of mRNA expression from Stacked and Detached. Each column shows the mRNA expression in Stacked ($n = 3$) and Detached ($n = 3$) samples. The samples of Stacked and Detached have each four HepG2 cell-embedded hydrogel sheets. (B–D) Mean fold expression changes of Stacked compared to Detached (control) for (B) metastasis-, (C) proliferation/differentiation- and (D) oncogene-associated gene set. Increased expression of Lox gene and SERPINE1 gene, also called plasminogen activator inhibitor-1 (PAI-1), is associated with hypoxia-induced metastasis [1–4]. Also, overexpression of NDRG1 gene, related with aggressive invasion and metastasis, has been observed in human liver cancer [5]. However,

ACTG2 gene, which acts on with E-cadherin via β -catenin [6] correlated with a low risk of metastasis [7]. (B) In stacked hydrogel sheets, Lox, SERPINE1 and NDRG1 gene were down-regulated, but ACTG2 gene was up-regulated. INHA gene, which controls cell proliferation and differentiation, was strongly expressed in most hepatic tumor samples [8]. Especially, down-regulated ID2 gene induced inhibition of proliferation and differentiation of HepG2 cells [9]. (C) In stacked hydrogel sheets, INHA and ID2 gene were down-regulated. CA9 gene encodes CA9, which is a tumor-associated protein and is a strong hypoxia-inducible gene. The gene was highly expressed in several tumors including hepatic carcinoma although it is less expressed in normal tissues [10]. BCAS1 gene, implicated in cancer progression, was up-regulated in HepG2 cells [11,12]. TELO2 gene associated with the cell cycle was less expressed in hepatic tumor [13]. (D) However, CA9 and BCAS1 gene were down-regulated and TELO2 gene was up-regulated in stacked hydrogel sheets. Plus and minus fold change expressions indicate up- and down-regulation, respectively. $*p < 0.01$ and $**p < 0.001$ compared with the control. INHA, inhibin alpha; ID2, DNA-binding protein inhibitor 2; CA9, carbonic anhydrase IX; BCAS1, breast carcinoma amplified sequence 1; TELO2, telomere maintenance 2; LOX, lysyl oxidase; SERPINE1, serpin peptidase inhibitor, clade E (same as PAI-1, plasminogen activator inhibitor-1); NDRG1, N-myc downstream-regulated gene 1; ACTG2, actin gamma 2.



Supporting Video 1. The movie shows real-time stacking and aligning of a hydrogel sheet in a PDMS drainage well. The hydrogel sheet was transferred via an amputated micropipette and aligned by a tweezer or micropipette. When the hydrogel sheet was loaded, the hydrogel sheets, already stacked, did not float up to the top of the drainage well because the liquid containing the loaded hydrogel sheet was drained through micropillars of the drainage well.



Supporting Video 2. The movie shows simple manipulation of stacked hydrogel sheets on a filter paper by a tweezer. The stacked structure, with six hydrogel sheets, was fixed to the filter paper. It could be detached from the filter paper and also be manipulated by the filter paper.

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