Supporting Information

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SI Materials and Methods

Preparation of Switching Hydrogels. Sodium alginate (100,000–200,000 MW; 65–70% G content; Sigma) was dissolved in Ca^{2+} ($CaCl_2$)-free Dulbecco's modified Eagle medium (DMEM; Invitrogen) at a concentration of 3% (wt/vol) overnight at 37 °C and stored at 4 °C thereafter. Calcium carbonate ($CaCO_3$) in combination with D-glucono-δ-lactone (GDL) was used as a source of calcium ions (Ca^{2+}) to initiate gelation of alginate solutions (Sigma). A molar ratio of 1 Ca^{2+} ion:1.23 GDL molecules was used to achieve a pH-7.2 final solution. A 3% (wt/vol) sodium alginate solution was syringed into tubes and diluted to the correct concentration (wt/vol) with Ca^{2+}-free DMEM (mixed by vortex and stirring). The correct volume of prevortexed 1-M $CaCO_3$ suspension was added and immediately mixed for 1 min. A 1-M GDL solution was then added to the suspension and mixed for 1 min. Cross-linking alginate solutions were injected by using an 18-gauge needle into molds or onto non-tissue culture-treated plasticware. After loading, solutions were transferred to high-humidity cell-culture incubators [37 °C with 5% (vol/vol) CO_2] for 30 min of gelation. The indicated alginate gel concentrations are final wt/vol concentrations. Type-I collagen (5 mg/mL) from rat tail (stored in acetic acid solution; Invitrogen) was neutralized with NaOH (25 mM final concentration) on ice before gelation at 37 °C. Matrigel (BD Biosciences) was thawed on ice and used directly with gelation at 37 °C. Optimized alginate gels consisting of 1.2% (wt/vol) alginate containing 34 mM $CaCO_3$ and 42 mM GDL were used as a base to generate composite hydrogels. Ice-chilled 3% (wt/vol) alginate solution was syringed into tubes [final concentration: 1.2% (wt/vol)] on ice. Type-I collagen and/or Matrigel was added on ice to the correct concentration and stir-mixed for 1 min. Poly(vinyl alcohol) (PVA) was added at this stage if required. The correct volume of prevortexed 1 M $CaCO_3$ suspension (final concentration: 34 mM) was added and immediately stir-mixed for 1 min. If type-I collagen was added, then 0.025× collagen volumes of 1 M NaOH was added and mixed on ice. If cells were then to be encapsulated, then 50 μL of cell suspension in Ca^{2+}-free DMEM was added and stir-mixed on ice. A 1-M GDL solution was then added (final concentration: 42 mM) to the suspension and immediately stir-mixed for 1 min on ice. Composite hydrogel solutions were then injected by using an 18-gauge needle into molds or onto non-tissue culture-treated plastic ware. After transfer solutions were transferred to high-humidity cell-culture incubators [37 °C; 5% (vol/vol) CO_2] for 30 min of gelation. If gels were cultured, then $CaCl_2$ concentration was adjusted from 1.8 to 3 mM to prevent hydrogel swelling/de-cross-linking.

Switching of Hydrogels. Directly after ionic cross-linking or after culturing, composite hydrogels were de-cross-linked to liquefy alginate by Ca^{2+} chelation. This process was carried out at room temperature. Hydrogels in 24-well plates were washed for 1 min in 1 mL of prep buffer [1.1% (wt/vol) $CaCl_2$, 0.1% 2-(*N*-cyclohexylamino)ethane-sulfuric acid (CHES), pH 7.2] and then washed quickly and incubated for 10 min in 1 mL of fresh stabilization buffer [0.15 M NaCl, 1.1% (wt/vol) CHES, 0.05% L-lysine, pH 7.2]. Ca^{2+} was chelated, and alginate was removed by washing hydrogels for 10 min in 1 mL of de-cross-linking buffer (200 mM sodium citrate, 30 mM EDTA, 0.15 M NaCl, pH 8.2). Hydrogels were then washed for 1 min in 1 mL of wash buffer [0.15 M NaCl, 1.1% (wt/vol) CHES, pH 7.2], followed by 1 min in 1 mL of PBS and 1 min in 1 mL of culture medium. Hydrogels were then placed in fresh culture medium at 37 °C with 5% (vol/vol) CO_2 to allow full collagen/Matrigel reconstitution and for subsequent culture. Tubes were processed for cross-linking as described above but were submerged in the relevant solution with 5 mL of the same solution repetitively flowed through the tube lumen using a syringe for the time indicated. Full reconstitution was assessed after 2 h of culturing.

Cell Culture, Viability, and Proliferation Analyses. HUES7 human embryonic stem cell (HESC) culture, embryoid body (EB) formation, and differentiation-inducing medium (DIFF medium) were as described (1). For transcription-factor-driven differentiation, lentiviruses were created and transduced as described (2). For growth-factor-driven differentiation, reagents and preparation was as described (3). When encapsulating cells in hydrogels, cells were trysinized, resuspended in Ca^{2+}-free DMEM, and added before initiation of gelation at a concentration of 2×10^6 per milliliter. Cell viability was assessed by using the AlamarBlue Assay (Invitrogen), using manufacturer's guidelines, and cell numbers were calculated by comparing fluorescence values to known cell number standards. For each measurement constructs were incubated for 50 or 20 min with 1 mL of stem cell maintenance media BGK containing 10% (vol/vol) AlamarBlue substrate for newly created constructs (containing 0–0.5 × 10^6 or 0.5–3 × 10^6 cells, respectively) or for 20 min with 2 mL of BGK medium, containing 10% (vol/vol) AlamarBlue for mature constructs (containing 3–20 × 10^6 cells). For cell encapsulation in hydrogels, non-tissue culture-treated plastic was used to minimize the adhesion of cells to culture vessel surfaces. Live/Dead assay (Invitrogen) was used to determine cell viability according to the manufacturer's instructions.

Microscopy. Bright-field and fluorescent images were taken by using a Nikon Eclipse TS100 Microscope. The Nikon SMZ1500 microscope with a SPOT insight camera (Diagnostic instruments) was used to image the whole hydrogel area. For 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) staining, constructs were fixed overnight in 4% (wt/vol) paraformaldehyde (PFA), and infused with sucrose–PBS [30% (wt/vol) sucrose in PBS with 5 mM $CaCl_2$]. DiI (Invitrogen; 5 μL/mL) in sucrose–PBS was used to stain cells for 30 min at 37 °C. Constructs were destained for 30 min in sucrose–PBS and a Macro confocal (TCS LSI; Leica) was used to image z-stacks throughout vibrotome sections. For SEM, constructs were fixed overnight in 2% (vol/vol) glutaraldehyde containing 3 mM $CaCl_2$ to prevent swelling and dehydrated with sequential washes with 50%, 75%, 95%, and 100% (vol/vol) ethanol. Constructs were air-dried and sputter-coated with Au for observation. For environmental scanning electron microscopy (E-SEM), constructs were air-dried and sputter-coated with platinum for 90 s (2.2 kV; 15 mA). Samples were imaged by using an FEI XL30 FEG-ESEM using an accelerating voltage of 15 kV.

Gene Expression Analyses. Hydrogels were de-cross-linked as described by using 1 mL of de-cross-linking solution. Material was centrifuged at 100 × *g* for 5 min, washed three times in PBS, and then digested with 1 mL of collagen digestion solution (0.5 mg/mL collagenase I and 5 μg/mL dispase) at 37 °C for 10 min with regular agitation. Recovered cells were homogenized by vortexing in RNA extraction buffer (RLT buffer) and passed through a QiaShedder column (Qiagen). RNA was then prepared and reverse-transcribed as described in Dixon et al. (2). Pluripotency and differentiation-associated gene expression was assessed as described (2).

Alkaline Phosphatase Activity Assessment. The alkaline phosphatase (AP) (86R-1kit; based on Naphthol AS-BI and fast red violet

LB; Sigma) was used to semiquantitatively demonstrate AP activity in human pluripotent stem cells (HPSCs) or differentiated cultures/hydrogels. Monolayers were washed twice with PBS, fixed in citrate/acetone/formaldehyde fixative, washed twice with dH_2O, and incubated with staining solution for 20 min at 37 °C. Monolayers were washed twice with water before microscopy. Hydrogel constructs were processed as for monolayers except for longer 5-min washes with dH_2O postfixation. Stained constructs were placed in PBS after staining to avoid gel swelling. The StemTAG AP activity colorimetric assay kit (CBA-301; Cell Biolabs) was used to quantitatively measure AP activity in HPSCs or differentiated cultures/hydrogels. Monolayer cells were recovered by conventional trypsinization. Hydrogels were reverse-cross-linked and digested with collagen digestion solution. Recovered cells were lysed with 0.25 mL of lysis buffer (Cell Biolabs) and incubated for 10 min at 4 °C. Lysates were cleared by centrifuging at 12,000 × *g* for 10 min, and the supernatant was transferred. Bradford assay to determine protein concentration was undertaken to normalize AP activity levels to protein concentration. Triplicates of 100-μL reactions were initiated by addition of 50 μL of lysate with 50 μL of substrate in clear polystyrene 96-well plates (Nunc). Reactions were placed for 30 min at 37 °C and stopped with the addition of 50 μL of stop solution and mixing for 30 s on a plate shaker. The absorbance was measured at 405 nm on an Infiniti plate reader (Techan), and data were normalized to protein yield from 1×10^6 cells grown in monolayer and expressed as nmol pNitrophenol (pNP) by using the kit calibration curve generated by reaction products from serial dilution of pNP.

Homogeneity Analyses of Hydrogels. Homogeneity of cylindrical hydrogels was characterized with dry- to wet-weight ratios. Gels were extracted from non-tissue culture plastic 12-well plates and cut into four sections across the circular disk parallel to each other. The wet weight of the gel was measured in tubes, and slices were dried at 40 °C for 24 h. Dry slices were weighed again after drying, and their dry/wet ratios were calculated. Data display average and SDs of triplicates.

Analyses of Switching of Hydrogels by Wet/Dry Weights. The effectiveness of alginate removal from cylindrical composite gels was characterized with wet weight ratios pre- and postchelation. Gels were extracted from non-tissue culture plastic 12-well plates and cut into four sections across the circular disk parallel to each other. The wet weight of the gel was measured in tubes, and Ca^{2+} ions were chelated. Wet slices postchelation were weighed again, and their pre/post wet ratios were calculated. Data display average and SDs of triplicates.

Analyses of Switching of Hydrogels by Alginate/Collagen Composition. Composition of hydrogels was assessed with assays for collagen and alginate before and after switching. A total of 100 μL of hydrogels were processed by initially de-cross-linking using 10 μL of de-cross-linking solution at 37 °C for 30 min. Insoluble material was collected by centrifugation (15,000 × *g*; 10 min), supernatant was collected, and pellets were digested with 100 μL of 1:10 collagen digestion solution (0.05 mg/mL collagenase I and 500 ng/mL dispase) at 37 °C for 2 h with regular agitation. De-cross-linked supernatants and collagen digests were pooled to give 200 μL of solubilized hydrogel. Alginate-only gels were used to zero collagen quantitation, and collagen-only gels were used to zero alginate quantitation.

Collagen was quantified in hydrogels by using protein assays (BioRad). We used un-cross-linked collagen [diluted in 1:1 de-cross-linking:collagen digestion (1:10) buffer] as a standard and ratioed to collagen-only gels as 100% starting concentration. Alginate was quantified by complex formation with a cationic dye, 1,9-dimethyl methylene blue (DMMB) (Sigma). We measured the absorbance ratio of complexes at 520:650 nm, which are the absorbance maxima of bound and unbound DMMB, respectively. A total of 50 μL of 0.8 M NaOH was mixed with 50 μL of solubilized hydrogel solution and neutralized after 5 min with 23 μL of 2.25 M citric acid. To assay alginate, 2 μL of DMMB (62.6 mM; final concentration: 1 mM) was then added to the sample, which was vortex-mixed and incubated at room temperature for 1 h. Absorbance was measured at 520 and 650 nm, and the 520:650-nm absorbance ratio was calculated. We used un-cross-linked alginate [diluted in 1:1 de-cross-linking:collagen digestion (1:10) buffer] as a standard (0.1–5 mg/mL) and ratioed to alginate-only gels as 100% starting concentration.

Tube Fabrication and Perfusion. Cells were suspended in hydrogel before gelation and injected into polytetrafluoroethylene cylinder molds (to create tubes of 1-mm wall thickness, 2-mm lumen diameter, 16-mm length). Molds were mounted onto 2-mm steel needle tubing with a carbon fiber mandrel and clamped and incubated for 30 min at 37 °C for gelation. Molds and mandrels were removed, and the tubes were connected to a perfusion bath system. Tubes were subject to unidirectional perfusion at 0.5 mL/min.

Mechanical Testing and Ultrasound Analyses. To determine the elastic modulus of the hydrogels we used uniaxial tensile testing with a Dynamic mechanical analyzer (Stable Micro Systems). Ultrasound analyses were performed by using an automated microscope stage-mounted ultrasound transducer at a frequency of 50 MHz. The acoustic impedance of the hydrogels is a function of the density (ρ) and bulk modulus (K) of a material:

$$Z = \sqrt{\rho K}.$$

Statistical Analysis. Statistical comparisons were carried out by using the GraphPad Prism software package. Comparisons were made by using Tukey–Kramer ANOVA. Results were considered significant if $P < 0.05$.

1. Burridge PW, et al. (2007) Improved human embryonic stem cell embryoid body homogeneity and cardiomyocyte differentiation from a novel V-96 plate aggregation system highlights interline variability. *Stem Cells* 25(4):929–938.
2. Dixon JE, Dick E, Rajamohan D, Shakesheff KM, Denning C (2011) Directed differentiation of human embryonic stem cells to interrogate the cardiac gene regulatory network. *Mol Ther* 19(9):1695–1703.
3. Burridge PW, et al. (2011) A universal system for highly efficient cardiac differentiation of human induced pluripotent stem cells that eliminates interline variability. *PLoS ONE* 6(4):e18293.

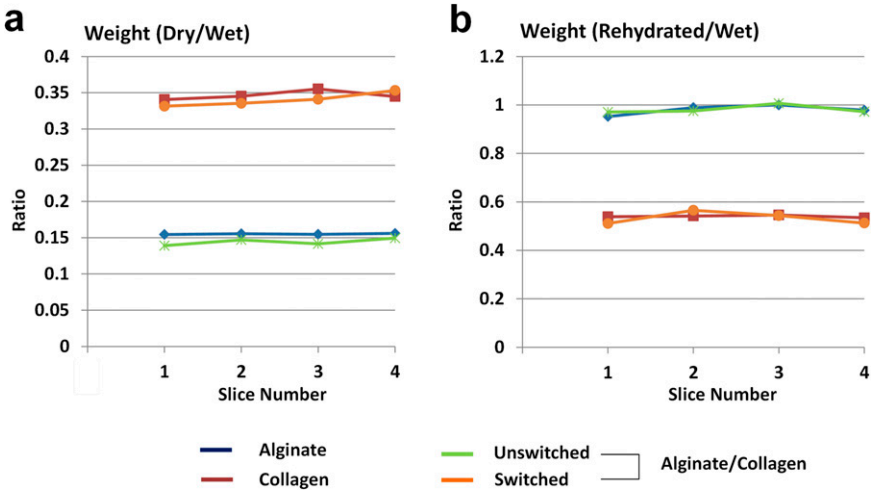

Fig. S1. Homogeneity analyses of switching in combined hydrogels. To test homogeneous material properties of hydrogels, alginate, collagen, and combined hydrogels were cross-linked, sliced into four equal portions, and weighed as wet, dry (*A*), or rehydrated material (*B*). Unswitched combined hydrogels behave similarly to alginate-only hydrogels. Switched combined hydrogels behave similarly to collagen only hydrogels. The four slices retained consistently similar properties demonstrating homogeneity across all compositions of gels ($n = 3$).

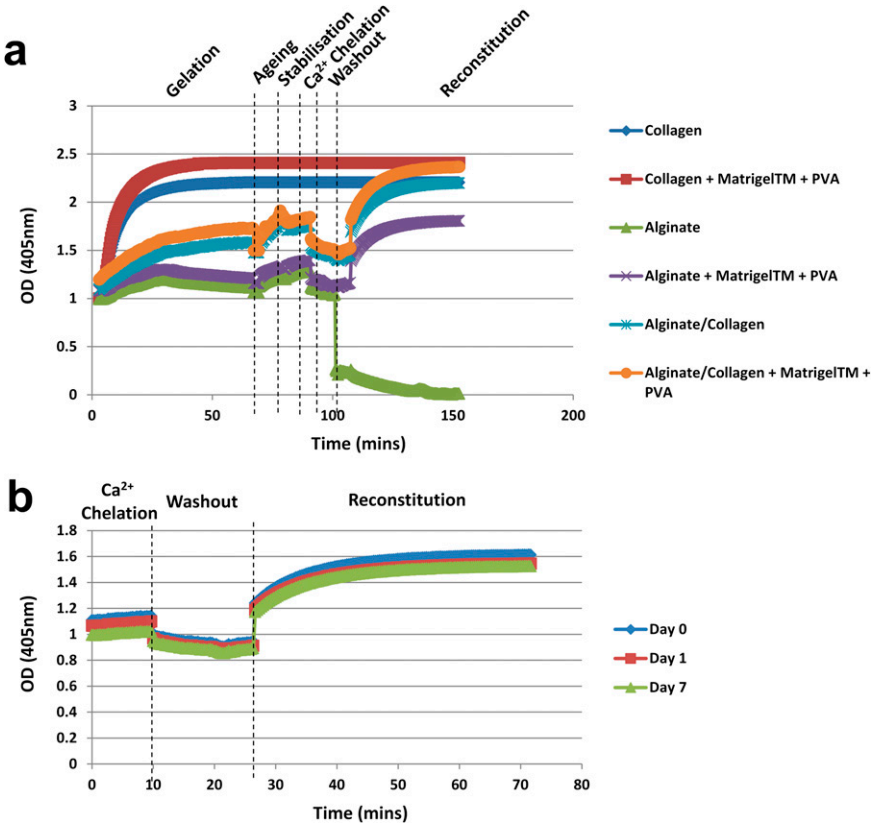

Fig. S2. Spectroscopy of optimized combined hydrogels and delayed switching. (*A*) Spectroscopy measuring collagen fiber character during cross-linking and switching of optimized hydrogels containing Matrigel and PVA. Optical density at 405 nm was used to compare alginate-only, collagen-only, and alginate/collagen-composite hydrogels with and without Matrigel or PVA during processing. Alginate prevents complete collagen-network formation even with Matrigel and PVA until chelation and washout. The switching process (40 min) and the gelation/reconstitution processes are highlighted ($n = 3$). (*B*) Spectroscopy measuring collagen fiber character during and after switching of optimized hydrogels 0, 1, or 7 d postfabrication. Collagen fibrillogenesis was initiated upon switching at all days postfabrication tested ($n = 3$).

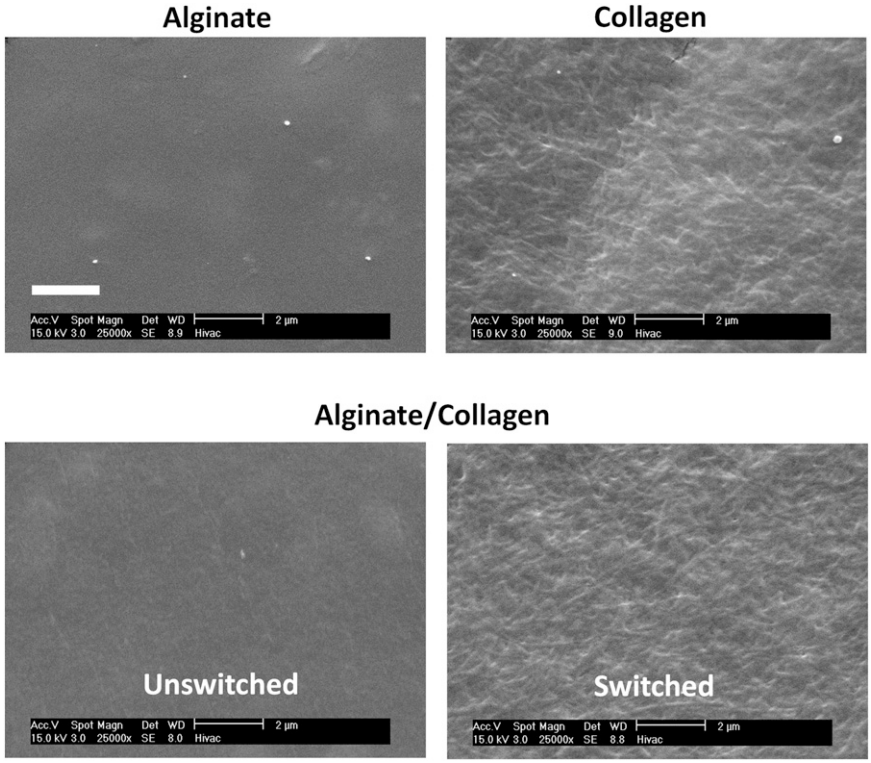

Fig. S3. E-SEM analyses of combined hydrogels. Images demonstrate that in the presence of alginate that collagen fiber formation is significantly inhibited. (Bar: 2 μm.) When alginate is removed by switching, the gel resembled a collagen-only fibrous gel.

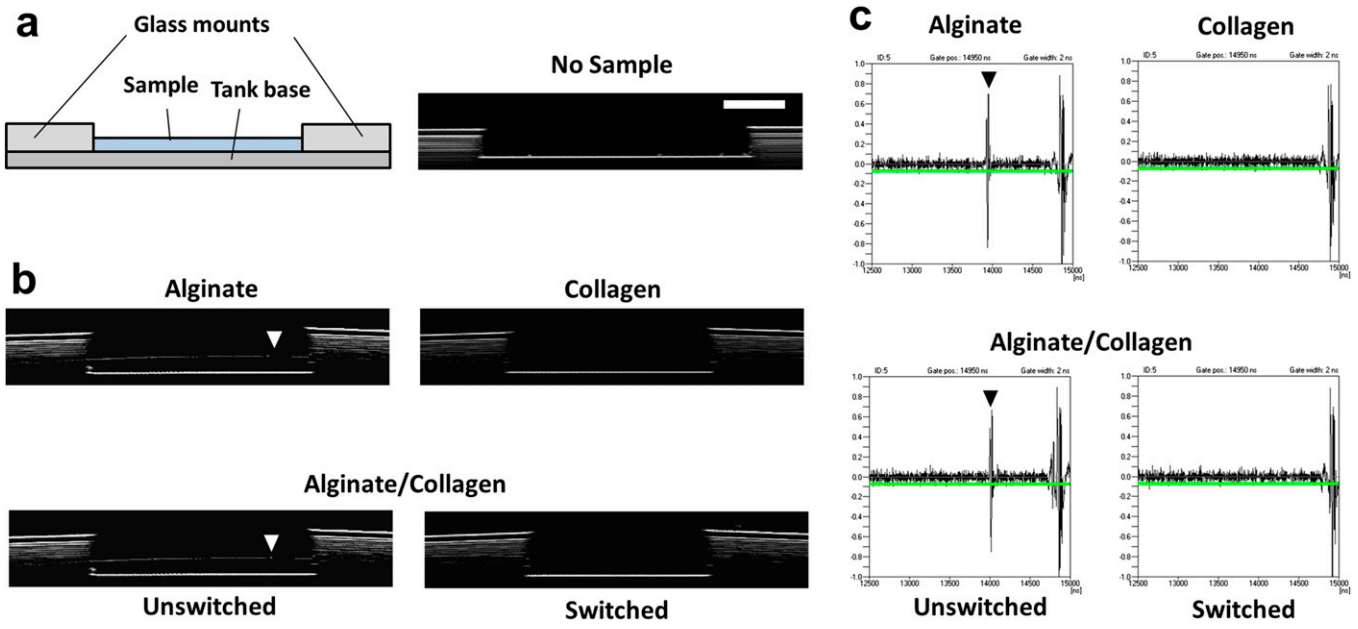

Fig. S4. Acoustic impedance assessment of combined hydrogels using ultrasound. (*A*) Schematic of ultrasound system. (*Left*) Hydrogels (1-mm thickness) were placed in a tank of distilled water held by glass mounts. (*Right*) An ultrasound of a no sample control is shown. (Bar: 5 mm.) (*B*) Ultrasound images of samples. Alginate-containing hydrogels can be visualized by ultrasound, but collagen-only or switched combined hydrogels are not visible. Collagen or switched combined gels are not visible due to their acoustic impedance being close to that of water. Arrowheads show the top of the gel surface. (*C*) Frequency plots of the acoustic properties of the hydrogels showing the characteristic peak for alginate (black arrow), which is absent in collagen-only or switched combined hydrogels.

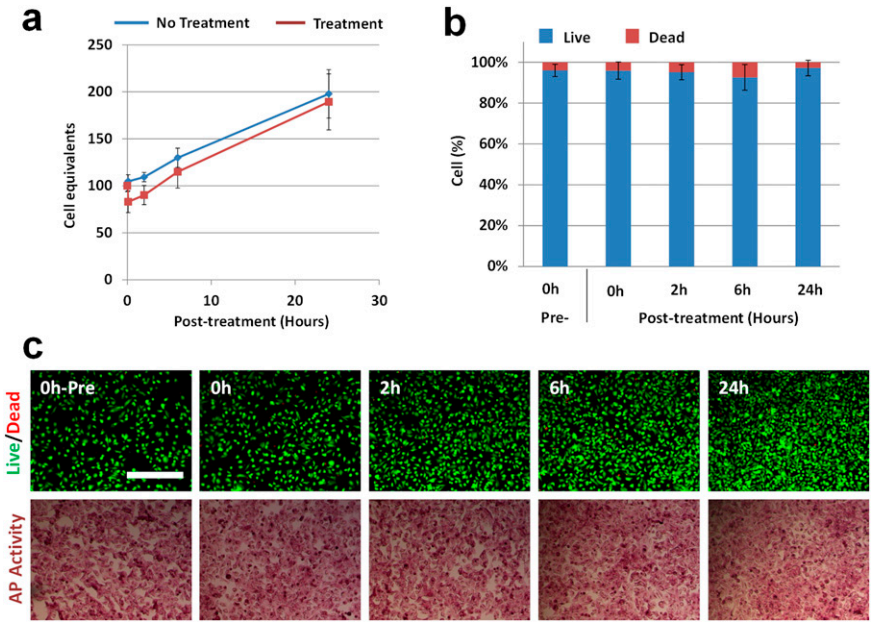

Fig. S5. Nontoxic control of HPSC interaction with switching hydrogels. (*A*) Switching using divalent ion chelation (sodium citrate/EDTA) does not affect HPSC proliferation/metabolism. Alamarblue assays were used to assess any metabolic effect on monolayers of HUES7 HESCs over a 24-h period. (Bars are SD; $n = 6$.) (*B* and *C*) Switching does not affect HPSC viability or pluripotency. Live/Dead staining was used to determine viability of monolayer cultures of HUES7 HESCs over a 24-h period (*B*, quantification; *C*, microscopy) ($n = 6$). HUES7 HESCs were also assessed for pluripotency by AP activity, which remained unaffected after treatment with switching chemicals. (Bar: 100 μm.) ($n = 3$.)

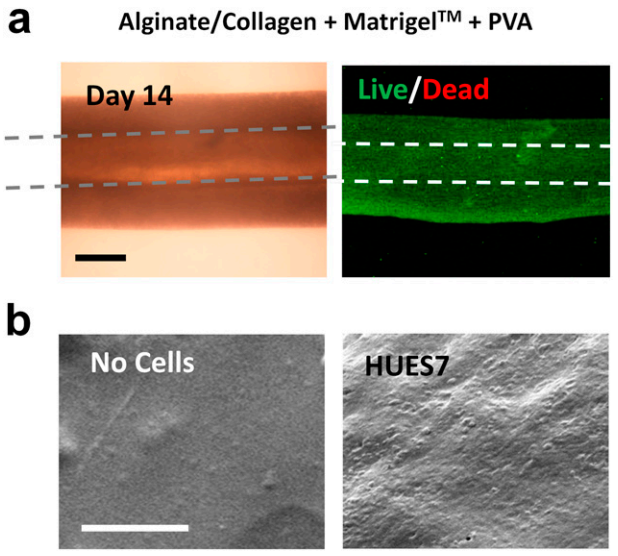

Fig. S6. Tubular constructs from composite hydrogels and HPSCs. (*A*) Example perfused HUES7-supporting tubular geometry (created from alginate/collagen hydrogel optimized with Matrigel and PVA). (*Left*) Tube lumen can be seen (gray dashed lines), which is fully populated with cells. (Bar: 1 mm.) (*Right*) Cells remain viable throughout the tube walls as indicated by Live/Dead staining. (*B*) SEM imaging of acellular tubes (*Left*) and a HPSC-containing construct (*Right*) showing full population (including the surface) with HUES7 HESCs (exhibiting classic rounded morphology of HPSCs). (Bar: 50 μm.)

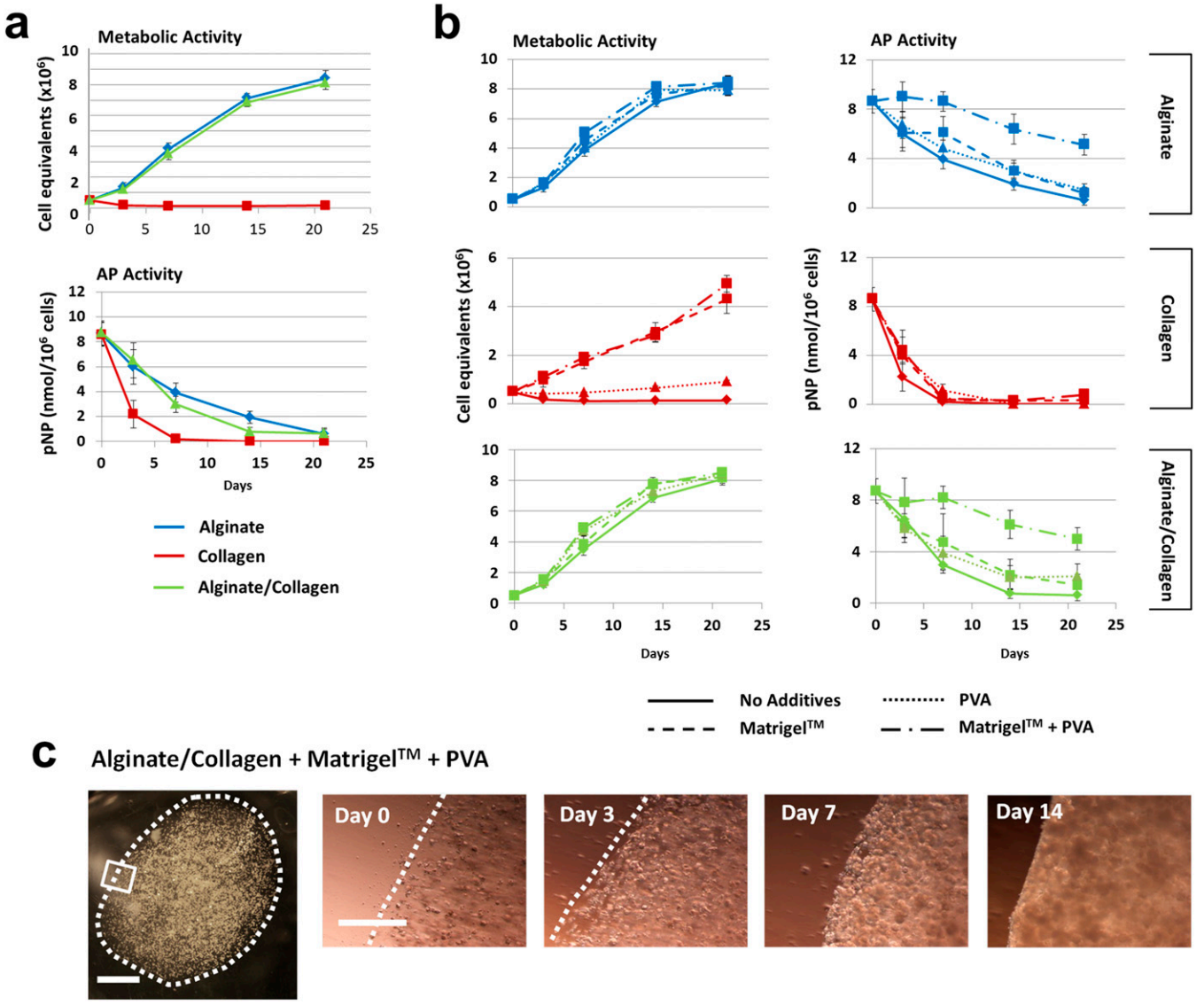

Fig. S7. Tailoring of switching hydrogels to achieve self-renewal of HPSCs. (*A*) HUES7 HESCs were assessed for metabolic activity (using AlamarBlue assay) and pluripotency (using AP activity assessment) in alginate-only, collagen-only, or alginate/collagen composite gels. (Bars are SD; $n = 3$.) (*B*) Gels were optimized to achieve efficient HPSC self-renewal by the addition of Matrigel and PVA. (*C*) Light microscopy of HUES7 HESCs cultured in composite hydrogels (containing Matrigel and PVA) over 14 d. Cells proliferated, forming EB-like aggregates until the entire volume of the gel was confluent by 14 d. (Bars: 120 μm, *Left*; 40 μm, *Right*.) Borders of the hydrogels are highlighted with dashed lines.

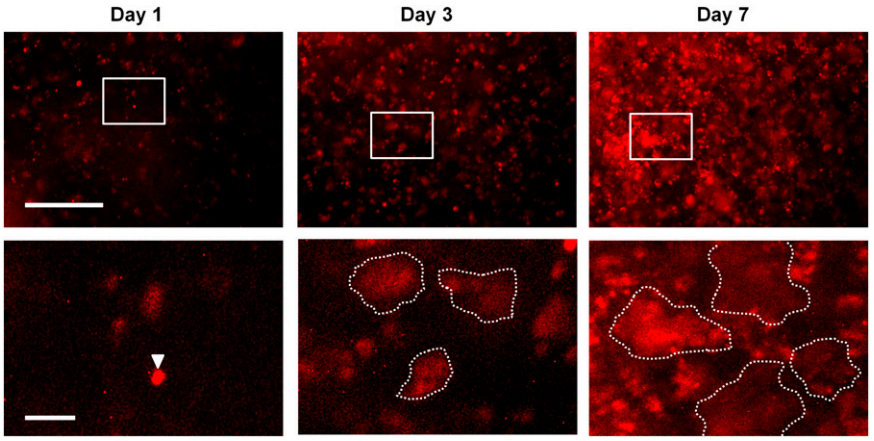

Fig. S8. Analyses of HPSC growth in combined hydrogels with DiI staining. HUES7 HESCs were cultured within optimized combined hydrogels (as described in Fig. 2). Hydrogels were fixed with PFA, vibrotome sectioned, and stained with DiI to visualize cells. On day 1, cultures were mainly single-celled, small aggregates were visible by day 3, and large networks of interconnected aggregates were observed by day 7. (Bars: 160 μm, *Upper*; 40 μm, *Lower*.) Aggregates are outlined with dashed lines.

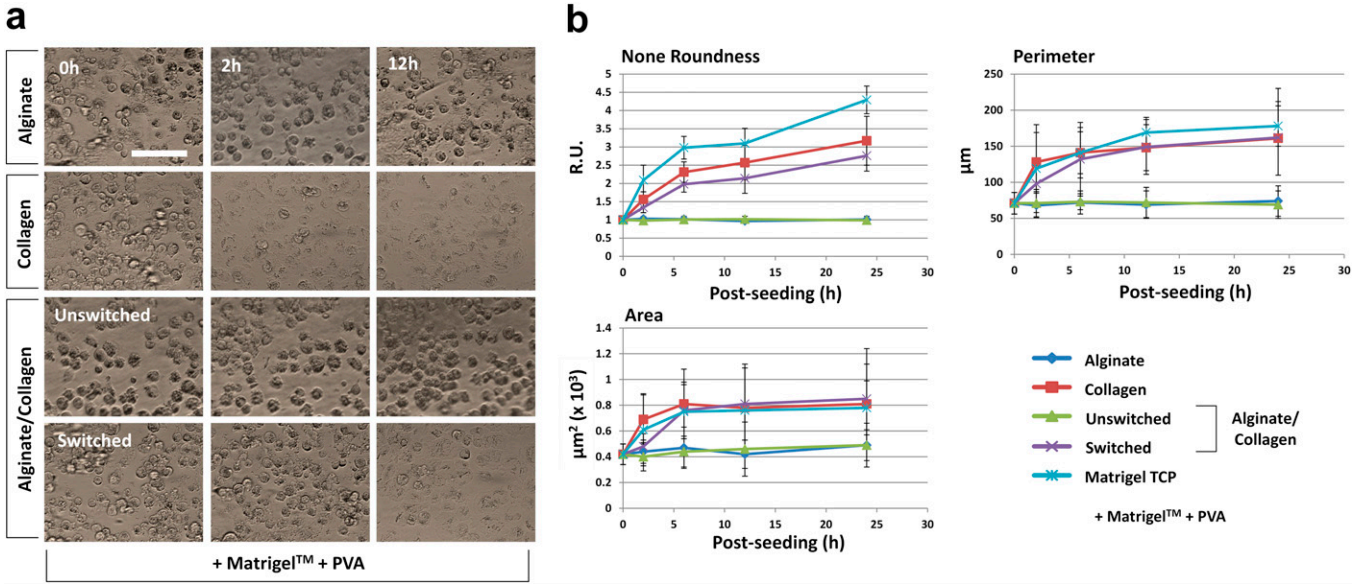

Fig. S9. Interaction of HPSCs and composite hydrogels with or without switching. (*A*) Switching hydrogel microenvironment alters the interaction of HUES7 HESCs to alginate/collagen hydrogels. HUES7 were seeded onto hydrogels, and the morphological character of cells was assessed (switching was carried out preseeding). After switching alginate/collagen hydrogels interact with HUES7 HESCs similarly to collagen-only gels (all hydrogels also contained Matrigel and PVA). (Bar: 50 μm.) (*B*) HPSC/hydrogel interaction influences cell roundness ($p^2/[4\pi A]$), perimeter (p), and area (a), which can be controlled by switching ($n = 6$).

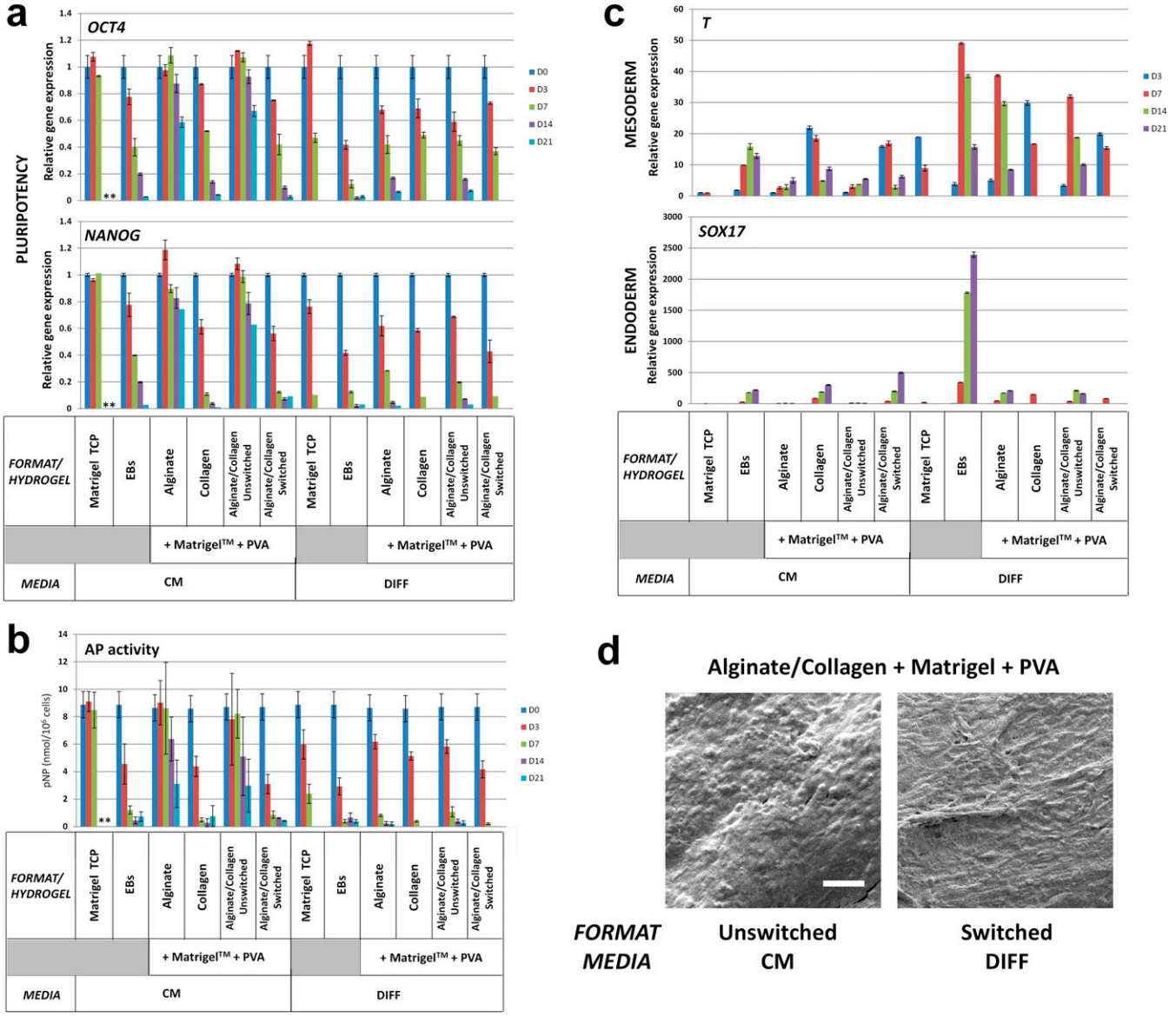

Fig. S10. Media conditions influence HPSC self-renewal and differentiation in switching hydrogels. (*A*) Quantitative gene-expression analyses of HESCs cultured in CM or DIFF medium with or without switching. HESCs cultured in CM within alginate or unswitched alginate/collagen hydrogels retained pluripotent gene expression over the 21-d period. Those cultured in DIFF medium and within collagen or switched alginate/collagen hydrogels rapidly down-regulated both *OCT4* and *NANOG* markers consistent with loss of self-renewal and differentiation comparable with EB-differentiated cells. (Bar is SE; $n = 6$.) (*B*) AP activity of HUES7 HESCs in these hydrogel conditions is consistent with loss of pluripotency within collagen or switched alginate/collagen hydrogels and retention of pluripotency in those cultured in CM within alginate or unswitched alginate/collagen hydrogels. (Bar is SD; $n = 6$.) (*C*) Differentiation markers *T* (*BRACHYURY*) for mesodermal and *SOX17* for endodermal differentiation were assessed by quantitative gene-expression analyses. Consistent with analyses of pluripotency markers, HUES7 HPSCs cultured in CM within alginate or unswitched alginate/collagen hydrogels did not up-regulate differentiation markers as significantly as EBs or those cultured in DIFF medium and within collagen or switched alginate/collagen hydrogels. (Bar is SE; $n = 6$.) (*D*) SEM imaging of HUES7 HESCs cultured within alginate/collagen (with Matrigel and PVA) constructs in pluripotency-conferring (unswitched in CM) or differentiation-conferring (switched in DIFF medium) environments. Pluripotency-condition-cultured HUES7 HESCs appear rounded with classical morphology; differentiation-condition-cultured constructs appear elongated with multiple morphologies indicative of loss of self-renewal and a differentiated phenotype. (Bar: 10 μm.)

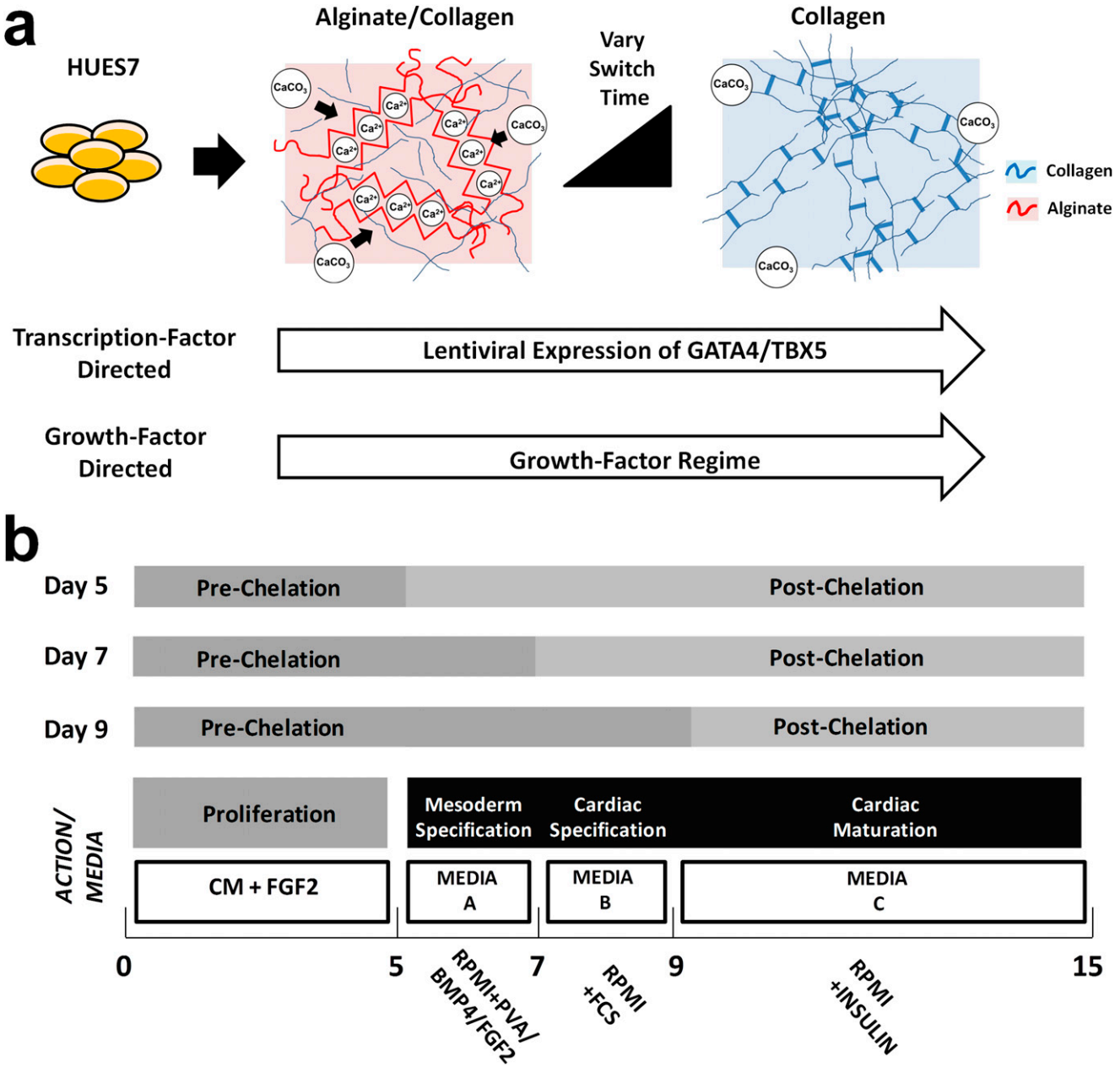

Fig. S11. Schematic of the use of switching hydrogels to direct differentiation of HPSCs by transcription or growth factors into cardiomyocytes. (*A*) For transcription factor-directed experiments HUES7 HESCs were transduced at high-efficiency with lentiviruses constitutively expressing *GATA4* and *TBX5* (*GT*) to induce cardiac specification (2). Twenty-four hours after transduction, cells were transferred to switching hydrogels. For growth-factor-directed differentiation, a published regime for EB cardiac differentiation was used (3). Switching time of alginate/collagen hydrogels was varied to determine the optimal micro-environmental conditions for cardiac specification and differentiation by both methods. (*B*) Schematic of the growth-factor regime used to direct cardiac differentiation in switching hydrogels. The treatment of HUES7 HESCs with the regime medium was kept consistent with a variation in switching time (day 5, 7, or 9 or no switch postseeding).

Table S1. Efficient removal of alginate during switching

Composition	Hydrogel weight, mg			Dry/wet Ratio	Rehyd/wet ratio	Wet (switched)/ wet ratio
	Wet	Dry	Rehydrated			
1.2% alginate	178.9 ± 5.2	27.5 ± 1.2	177.4 ± 4.7	0.15	0.99	—
2 mg/mL collagen	70.5 ± 1.7	24.0 ± 0.5	37.7 ± 0.6	0.34*	0.53*	—
1.2% alginate: 2 mg/mL collagen	183.6 ± 1.9	26.6 ± 0.4	181.8 ± 2.3	0.14	0.99	—
1.2% alginate: 2 mg/mL collagen (switched)	70.7 ± 1.1	24.6 ± 0.6	37.9 ± 1.5	0.35*	0.54*	0.39

We assessed switching by hydrogel weight and hydration properties. Alginate, collagen, and combined hydrogels were cross-linked in tubular geometries (16-mm length, 2-mm outer diameter, 0.5-mm wall thickness) and weighed (wet weight). Tubes were dried and weighed (dry weight) to determine extent of hydration as a gel. Dried material was then rehydrated and further weighed (rehydrated weight) to determine extent of rehydration from dry. This process was also repeated for switched combined hydrogel. These values were ratioed to assess bulk material properties. Switching generated significant weight loss indicative of alginates efficient removal. Also switching converted composite gels from alginate- to collagen-dominated characteristics. Alginate gels are more hydrated and can be fully rehydrated. Collagen gels are less hydrated and cannot be fully rehydrated ($n = 6$). Rehyd, rehydrated. * indicates values showing collagen dominated character. Values are ± SD.