Supporting Information

Przybyla and Voldman 10.1073/pnas.1103100109

SI Materials and Methods

Perfusion Device Fabrication. The microfluidic perfusion device was molded from polydimethylsiloxane (PDMS; Corning), after mixing in a ratio of 10:1 base to curing agent and degassing in a vacuum chamber for 30 min. The mold was fabricated from an Autocad file using a stereolithography foundry (FineLine Prototyping). The fluidic and vacuum layers were cured separately and bonded after exposure to oxygen plasma. Input and output fluid holes, as well as vacuum line holes, were punched using a 0.072-inch-diameter hypo tube. The device layout consists of two sets of triplicate millimeter-sized enclosed culture chambers with individual addressability to enable two experimental conditions side by side. These chambers are connected to external syringe pumps that provide continuous pulse-free perfusion, and the chip also includes microvalves to direct liquid flow and cells to the desired locations.

Culture Media Additives. The following concentrations of culture media additives were used for all experiments: Activin 30 ng/mL (Peprotech), FGF2 12 ng/mL (Peprotech), PD0325901 1 μM (Stemgent), CHIR99021 3 μM (Stemgent), sodium chlorate 20 mM (Sigma-Aldrich), heparin 1 μg/mL (Sigma), collagenase 1 ng/mL (Sigma #C9722), JAK inhibitor I 1 μM (Calbiochem), and Ro 32-3555 50 μM (Tocris). Conditioned media was concentrated from a 10-cm static culture dish using spin filter concentrator columns with a 3-kDa molecular mass cutoff (Millipore).

Perfusion Culture Conditions. For all perfusion experiments, mouse embryonic stem cells (mESCs) at a density of 1×10^6 cells/mL were loaded into the device, and perfusion was initiated once the cells were firmly attached to the surface. For pulse perfusion, 50 μL of media per side of a device (three chambers) was perfused in a span of 30 min once every 8 h to completely replenish the media in all three chambers (≈4-μL chamber volume). For recirculating perfusion, a peristaltic pump (Rainin Dynamax) was used at a flow rate of 0.1 mL/h with a media reservoir of 500 μL in an Eppendorf tube to create a total recirculating volume of approximately 1 mL.

Quantitative RT-PCR. Cells were harvested using TrypLE Express trypsin replacement (Invitrogen), and total RNA was isolated using the RNeasy Mini Kit (Qiagen), according to manufacturer's instructions. RNA was converted to cDNA using the DyNAmo cDNA synthesis kit with Oligo(dT) primer, and quantitative PCR reactions were set up using the DyNAmo SYBR Green qPCR kit (New England Biolabs), according to the manufacturer's instructions. Reactions were run on an MJ Opticon DNA Engine thermal cycler. Primers are listed in Table S1. Quantitative RT-PCR array analysis was performed using an mESC-specific PCR array (PAMM-081; SABiosciences).

Flow Cytometry. After harvesting cells, direct intracellular immunostaining was performed with an Alexa Fluor 647-linked anti-mouse Nanog antibody (eBioscience). Internal fluorescent intensity was measured on a FACSCaliber flow cytometer (BD Biosciences).

Immunofluorescence. For phospho-Stat3 and phospho-ERK1/2 staining, cells were incubated overnight with phosphorylated Stat3 antibody (Cell Signaling Technology) or phosphorylated ERK1/2 antibody (BD Biosciences), both at 1:100, and secondary antibody (anti-rabbit GFP and anti-mouse AF546, respectively; Invitrogen) was added for 2 h at 1:1,000. For HepSS, cells were blocked with endogenous biotin blocking kit (Invitrogen) and incubated overnight with HepSS antibody at 1:100 (Lifespan Biosciences), and secondary antibody (TMR NeutrAvidin; Invitrogen) was added at 1:500 for 1 h. For Oct4, cells were incubated overnight with primary Oct4 antibody (Abcam) at 5 μg/mL, and secondary antibody (anti-goat Cy3; Abcam) was added for 1 h at 1:250. Staining along the chamber was quantified using an automated MATLAB script, and differences in staining intensity in any area within the chamber was not found to be statistically significant. All cells were counterstained with 1:100,000 Hoechst (Sigma).

Embryoid Body Formation. ESCs were harvested from culture and replated at 4×10^5 cells in a 60-mm ultralow attachment culture dish (Corning). Cells were grown in ESC medium with no leukemia inhibitory factor (LIF), and medium was replenished every 2 d.

ELISA. ELISA was performed on samples from static conditioned medium or from the medium collected from the perfusion output. Cells were either perfused for 30 h or remained in static culture for 30 h, and medium was collected. Results were normalized by the average cell density (using an exponential growth model and the initial/final cell numbers) and duration to determine a secretion in grams per cell per hour under both conditions. Because of the discrepancy in volume between these two types of samples, perfusion output medium was spun down using an Amicon 3-kDa cutoff filter spin column and reconstituted to the same volume as the static conditioned medium. VEGF ELISA was purchased from R&D Systems, and assay was performed according to the manufacturer's instructions. Matrix metalloproteinase (MMP) 2 ELISA was purchased from RayBiotech, and assay was performed according to the manufacturer's instructions.

Cell Recovery. To capture and count cells recovered from the perfusion device, cells were collected into 4% formaldehyde and transferred to 4 °C twice daily. All recovered cells were combined and stained with Hoechst (Sigma), then transferred to a black-walled 96-well plate, where they were automatically scanned and counted using a MATLAB script that was previously calibrated using known quantities of cells collected, treated, and counted in the same manner as the cells recovered from the device.

Statistical Analysis. All results were analyzed by Student's t test, and the resulting pairwise P values are reported. Significance was established at $P < 0.05$ and was evaluated up to the level of $P < 0.001$.

SI Discussion

Fluid Transport Qualitative Model. Pe is given by vh/D, where v is a characteristic fluid velocity in the system (in our case, the average velocity, ≈0.0296 mm/s), h is a characteristic length (in our case, half the chamber height, 125 μm), and D is the diffusivity of a relevant molecules (for a ≈20-kDa cytokine, $D \approx 10^{-6}$ cm^2/s). This results in $Pe \approx 37$, where $Pe > 1$ indicates a convection-dominated regime. The ratio of the Peclet number and the Damkohler number Da is given by $v/k_{on}R_s$, where k_{on} is the ligand binding on-rate ($\approx 10^6$ M^{-1}s^{-1} for a strong interaction) and R_s is the receptor density (which we take to be ≈12 receptors/μm^2 for an 8-μm radius cell with ≈10,000 receptors). This results in Pe/Da of ≈1,500, indicating that convection dominates over reaction.

As medium flows by the autocrine-secreting cells, the axial convection and transverse mass transport will induce a concentration boundary layer above the cells, increasing in thickness along the length of the chamber (1). The boundary layer in general will decrease flux to and from the surface, and thus the concentration of secreted factor at the cell surface will be higher at the cell outlet than the inlet. The thickness of the boundary layer in microsystems such as ours generally scales as $1/Pe^{1/3}$ and thus will get thinner at higher Pe, whereas the flux through the boundary layer increases as $Pe^{1/3}$, therefore motivating operation at high Pe and use of short chambers, along with experiments assessing axial heterogeneity (Fig. S3 *A* and *B*).

Effect of Colonies on Fluid Flow. To account for any flow rate differences in the chambers that could result from the presence of 3D cell colonies, we use a model described by Gaver and Kute (2). In general, for a cell or colony whose height is <30% of the chamber height (for our 250-μm-high chambers, this corresponds to a 75-μm-high colony), there is only a minor effect (0.05%) on flow rate realized in the system, owing to increasing flow resistance from the decreased gap size between the colony and the chamber walls. Cells or colonies present in the chambers will also affect the shear stress, specifically increasing the surface shear stress compared with the shear stress on a flat surface. However, Gaver and Kute (2) demonstrated that the cells/colonies increase shear by a maximum of threefold with respect to a flat surface when the cell/colony height is <25% of the channel height (corresponding to 62 μm for our chambers). We measured colony heights in our chamber using optical microscopy to be 25–55 μm (average 40 μm), smaller than the 62-μm or 75-μm thresholds. Thus, for our chamber geometry, any cells or colonies smaller than 62 μm in height would negligibly affect the flow and would have surface shear stresses well below shear stresses that have been shown to negatively affect cells.

1. Squires TM, Messinger RJ, Manalis SR (2008) Making it stick: cCnvection, reaction and diffusion in surface-based biosensors. *Nat Biotech* 26:417–426.

2. Gaver DP, III, Kute SM (1998) A theoretical model study of the influence of fluid stresses on a cell adhering to a microchannel wall. *Biophys J* 75:721–733.

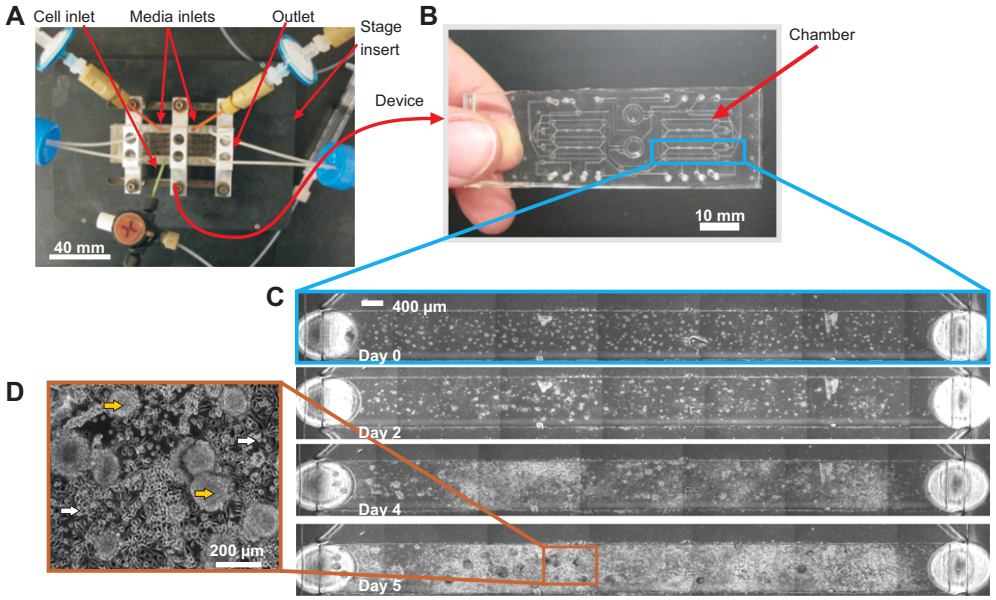

Fig. S1. Microfluidic device. (*A*) Image of the device setup, showing the device, inlets, and outlets clamped to an insert for imaging on the microscope. (*B*) Image of the PDMS device, showing the culture chambers. (*C*) Images of cells growing for the indicated number of days in one chamber of a device. (*D*) Close-up of a portion of the chamber in *C*, where yellow arrows represent colonies with ESC-like morphology, and white arrows represent surrounding differentiated-looking cells.

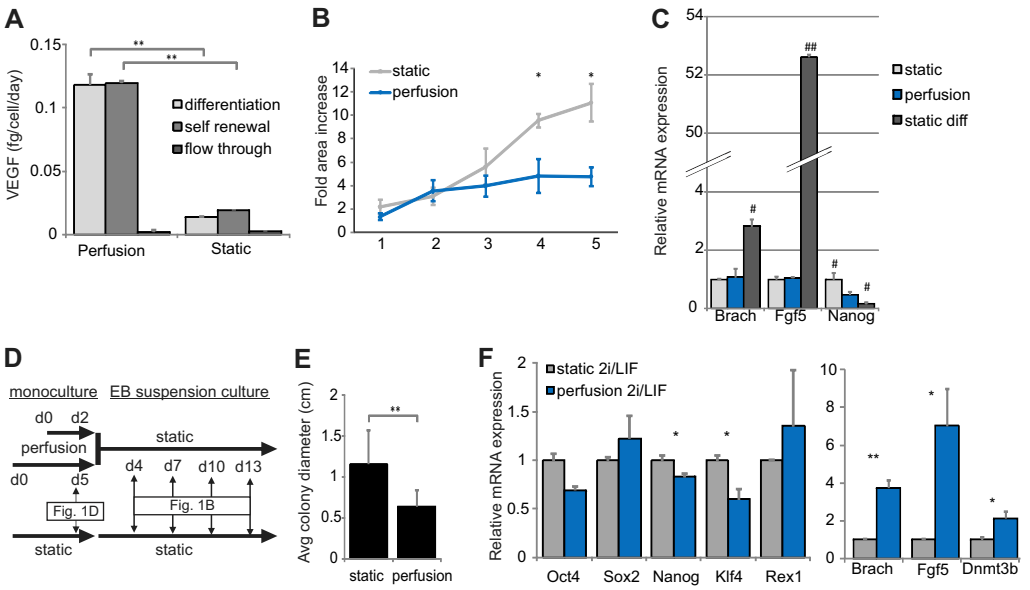

Fig. S2. Soluble factor removal and cell characteristics under perfusion. (*A*) ELISA measurements of VEGF collected from mESCs cultured in static and perfused systems, either in self-renewal (N2B27+LIF+BMP4) or differentiation (N2B27) environments. Also shown are the VEGF levels measured in systems without cells ("flow through"). (*B*) Fold increase in area of static or perfusion culture surfaces covered by cells over time. A 50-mm^2 area was analyzed at each time point under each condition. (*C*) mRNA expression levels of key markers after 3 d of growth in static or perfusion self-renewal culture or in static differentiation culture. (*D*) Schematic depicting the experimental progression used to culture cells in static or perfusion before subjecting them to embryoid body (EB) suspension culture, the results of which are depicted in Fig. 1*B* (main text). (*E*) Day-5 average colony diameter. (*F*) mRNA expression levels of self-renewal (*Left*) or differentiation (*Right*) markers after 5 d in static or perfusion culture in N2B27+2*i*/LIF media. $^{**}P < 0.001$, $^{*}P < 0.05$; $^{\#}P < 0.05$, $^{\#\#}P < 0.001$ for all pairwise comparisons; error bars represent SD.

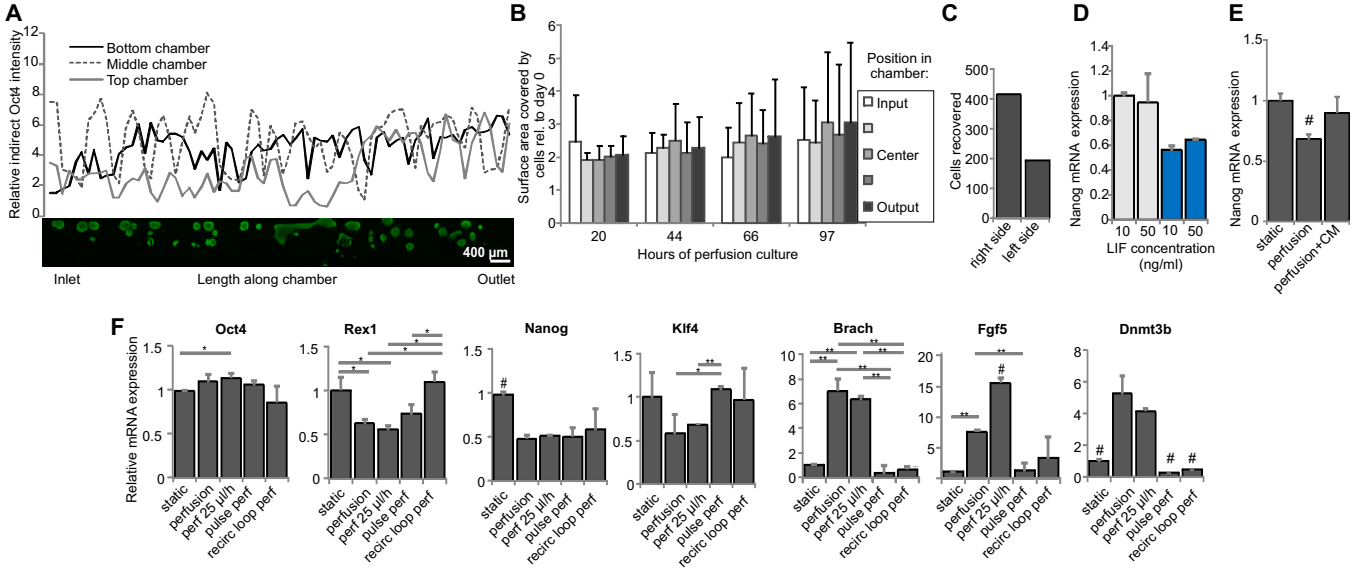

Fig. S3. Spatial analysis and physical manipulations of cells grown under perfusion. (*A*) Quantification and image of Oct4 immunofluorescent staining of day-5 perfusion culture to assess the relative abundance of marker expression along the length of the chamber. No statistically significantly trend was apparent ($P = 0.53$). (*B*) Quantification of surface area covered by cells over time on average between six chambers along the length of the chambers (light to dark gray). (*C*) Quantification of cells recovered from the perfused microdevice output over 5 d. Each bar represents the total number of cells recovered per chamber on either side of the device (three chambers per side). (*D*) Nanog mRNA expression levels in static and perfusion with either 10 ng/mL LIF or 50 ng/mL LIF. (*E*) Nanog mRNA expression levels in static and perfusion in the presence of serum+LIF media, with or without the addition of soluble cell-secreted factors from static conditioned media (CM). (*F*) mRNA expression levels of self-renewal and differentiation markers in normal static and perfusion cultures compared with levels in cells grown under perfusion with fourfold lower volume flow rate than normal (perf 25 μl/h), cells grown in a perfusion device with feeding intervals akin to those in static (pulse perf), and cells grown under recirculating perfusion in a total volume of 1 mL (recirc loop perf). $^{**}P < 0.001$, $^{*}P < 0.05$; $^{\#}P < 0.05$ for all pairwise comparisons; error bars represent SD.

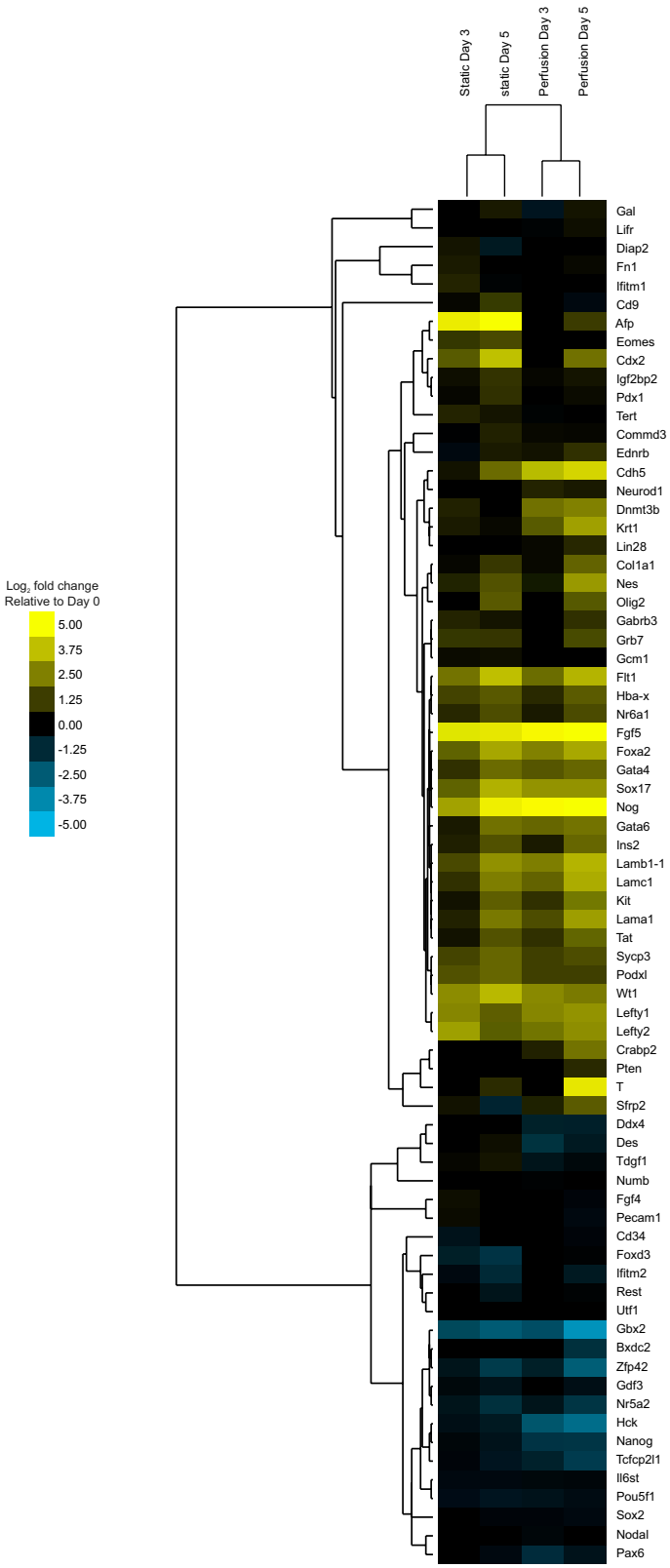

Fig. S4. Heatmap comparing expression levels of relevant mESC genes. Quantitative RT-PCR was performed on samples isolated from static and perfusion cultures after 3 and 5 d of culture, and expression levels were compared with those on day 0 (1 d after plating). Each data point represents the average of triplicate runs. Quantitative data are listed in Table S2.

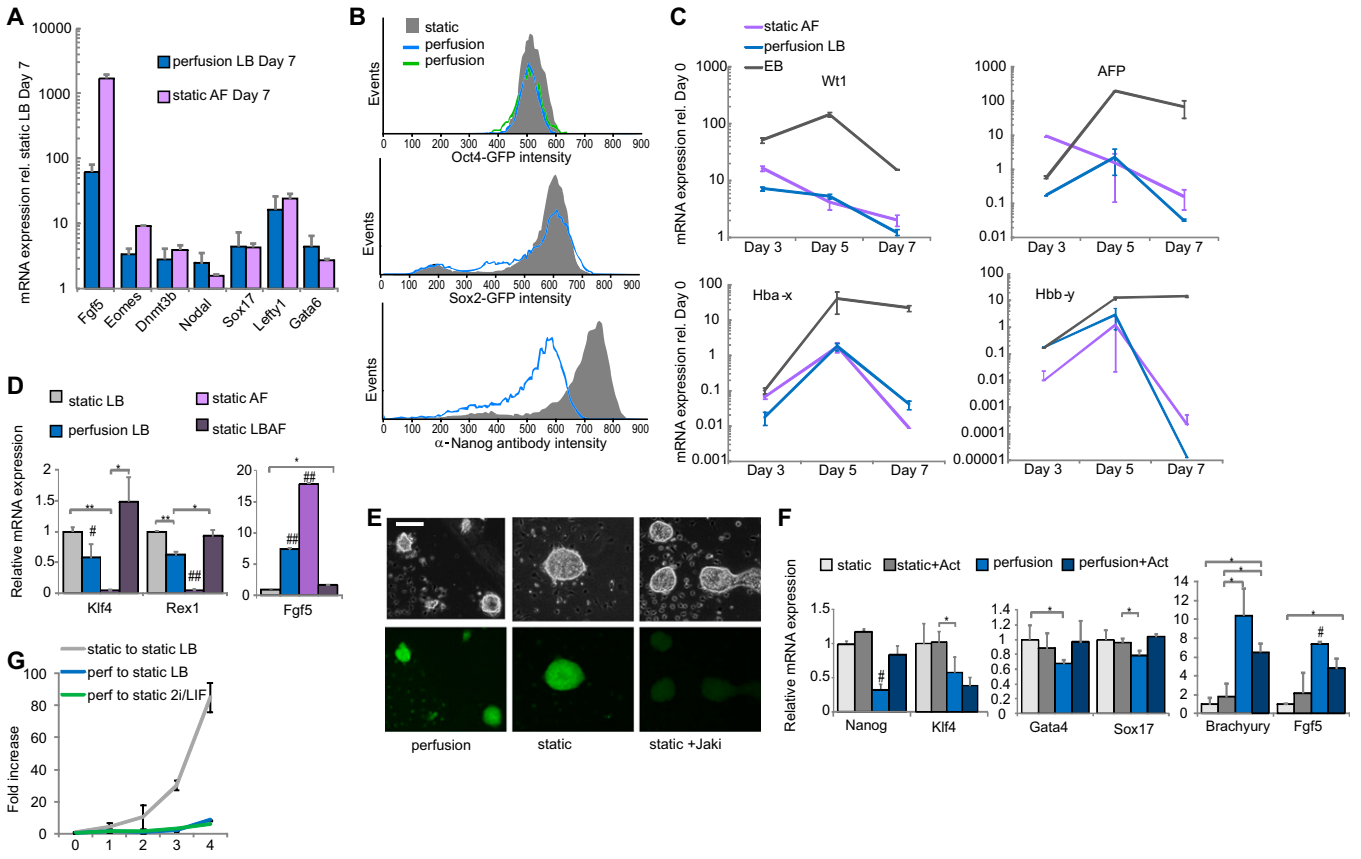

Fig. S5. Additional epiblast-like characteristics of cells grown under perfusion. (*A*) mRNA expression levels of epiblast-specific markers after 7 d of growth in self-renewal media under perfusion (N2B27+LIF+BMP4, perfusion LB) or in epiblast stem cell (EpiSC) media in static culture (N2B27+Activin+FGF2, static AF), compared with levels at day 7 in self-renewal media in static culture. (*B*) Flow cytometry histograms showing levels of Oct4 in an mESC Oct4-GFP cell line (*Top*), levels of Sox2 in a Sox2-GFP cell line (*Middle*), and levels of Nanog using direct immunofluorescence staining (*Bottom*), all in static and perfusion day-5 populations. (*C*) mRNA expression levels of differentiation markers at 3, 5, and 7 d in either static AF, perfusion LB, or floating embryoid body culture (EB). (*D*) mRNA expression levels of EpiSC up- and down-regulated markers in cells grown in static or perfusion LB, or in static AF, or static with all four additions (LBAF). (*E*) Phase and fluorescence images of cells grown in perfusion culture or in static culture with or without a Jak inhibitor (added 6 h before staining) and stained for phosphorylated (Y705) Stat3. (*F*) Relative levels of mRNA expression for self-renewal (*Left*), early endoderm (*Center*), or other differentiation lineage (*Right*) genes in the presence and absence of Activin under static and perfusion conditions. (*G*) Fold increase in growth of cells that were replated into indicated conditions from growth in static or perfusion. (Scale bar, 200 μm.) $^{**}P < 0.001$, $^{*}P < 0.05$; $^{\#}P < 0.05$, $^{\#\#}P < 0.001$ for all pairwise comparisons; error bars represent SD.

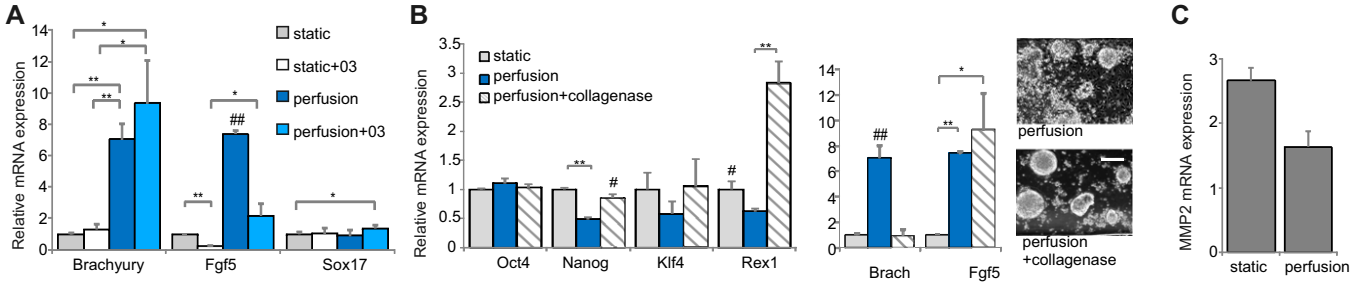

Fig. S6. Effects of ECM-related exogenous additions in static and perfusion cultures. (*A*) Relative levels of mRNA expression for differentiation genes in the presence and absence of PD03 under static and perfusion self-renewal conditions. (*B*) mRNA expression levels of self-renewal (*Left*) and differentiation (*Right*) markers for cells grown in static and perfusion compared with levels in cells grown in perfusion in the presence of 1 ng/mL collagenase. Representative images of perfusion cells in the presence or absence of collagenase are shown. (Scale bar, 200 μm.) (*C*) Relative levels of MMP2 mRNA expression in static and perfusion cultures. $^{**}P < 0.001$, $^{*}P < 0.05$; $^{\#}P < 0.05$, $^{\#\#}P < 0.001$ for all pairwise comparisons; error bars represent SD.

Table S1. Quantitative RT-PCR primers

Gene	Forward primer	Reverse primer
FGF5	GAAAAGACAGGCCGAGAGTG	GAAGTGGGTGGAGACGTGTT
Nanog	CTGCTCCGCTCCATAACTTC	TTTCCCTAGTGGCTTCCAAA
Gapdh	CACTGAGCATCTCCCTCACA	GTGGGTGCAGCGAACTTTAT
T	CAGCCCACCTACTGGCTCTA	GAGCCTGGGGTGATGGTA
Dnmt3b	GCATGAAGGCCAGATCAAAT	GCTTCCACCAATCACCAAGT
AFP	CCTGTGAACTCTGGTATCAG	GCTCACACCAAAGCGTCAAC
Gata4	TCTCACTATGGGCACAGCAG	GGGACAGCTTCAGAGCAGAC
Sox17	CTTTATGGTGTGGGCCAAAG	GCTTCTCTGCCAAGGTCAAC
Klf4	CAGGCTGTGGCAAAACCTAT	CGTCCCAGTCACAGTGGTAA
Sox1	CCTGAAAATGATGCTGCTGA	GGAGTAGCTGTGGGTGTGGT
Nestin	GATCGCTCAGATCCTGGAAG	AGTTCTCAGCCTCCAGCAGA
Sox2	AACGCCTTCATGGTATGGTC	TCTCGGTCTCGGACAAAAGT
Oct4	CACGAGTGGAAAGCAACTCA	TTCATGTCCTGGGACTCCTC
Rex1	CCCCCTGGAAGTGAGTCATA	CCACTTGTCTTTGCCGTTTT
Eomes	TTCCGGGACAACTACGATTC	GACCTCCAGGGACAATCTGA
Nodal	ACCATGCCTACATCCAGAGC	ATGCTCAGTGGCTTGGTCTT
Lefty1	TATGTGGCCCTGCTACAACA	GGAGGTCTCTGACACCAGGA
Gata6	CAAAAGCTTGCTCCGGTAAC	TGAGGTGGTCGCTTGTGTAG
Wt1	ATCCGCAACCAAGGATACAG	GGTCCTCGTGTTTGAAGGAA
Hbb-y	GGCCTGTGGAGTAAGGTCAA	GCAGAGGACAAGTTCCCAAA
Hba-x	ATGCGGTTAAGAGCATCGAC	GGGACAGGAGCTTGAAGTTG

Table S2. Quantification of expression levels of genes in a stem cell marker panel in static and perfusion cultures at days 3 and 5 of culture, compared with expression levels on day 0

Gene	Static day 3	Perfusion day 3	Static day 5	Perfusion day 5
Afp	25.42	0.824	115.4	2.312
Bxdc2	0.673	0.775	0.580	0.372
Cd34	0.614	0.922	0.767	0.777
Cd9	1.231	0.990	2.259	0.705
Cdh5	1.406	12.95	4.356	18.68
Cdx2	3.449	0.961	13.89	4.588
Col1a1	1.223	1.304	2.205	3.925
Commd3	0.895	1.271	1.678	1.252
Crabp2	0.796	1.671	0.934	4.878
Ddx4	0.964	0.491	1.160	0.501
Des	1.109	0.361	1.379	0.562
Diap2	1.458	0.984	0.568	1.009
Dnmt3b	1.651	4.684	1.122	5.875
Ednrb	0.713	1.401	1.593	2.027
Eomes	2.195	1.158	2.715	1.058
Fgf4	1.366	1.003	0.976	0.767
Fgf5	22.72	30.72	23.93	115.6
Flt1	4.886	4.410	13.82	11.56
Fn1	1.600	1.120	1.060	1.296
Foxa2	3.852	5.949	9.652	10.03
Foxd3	0.518	0.827	0.356	0.867
Gabrb3	1.780	1.177	1.473	2.019
Gal	0.784	0.578	1.511	1.464
Gata4	2.021	3.251	4.336	4.038
Gata6	1.522	3.964	4.649	4.752
Gbx2	0.243	0.219	0.174	0.059
Gcg	ND	ND	ND	ND
Gcm1	1.307	ND	1.361	ND
Gdf3	0.725	0.808	0.623	0.640
Grb7	2.194	1.168	2.143	2.811
Hba-x	2.570	1.884	3.436	3.568
Hbb-y	ND	ND	ND	ND
Hck	0.641	0.193	0.557	0.123
Iapp	ND	ND	ND	ND
Ifitm1	1.743	1.163	0.831	1.067
Ifitm2	0.696	0.857	0.422	0.564
Igf2bp2	1.372	1.239	2.085	1.438
Il6st	0.712	0.726	0.721	0.752
Ins2	1.638	1.583	3.016	4.097
Kit	1.427	2.001	3.662	5.039
Krt1	1.546	3.450	1.278	8.982
Lama1	1.688	2.910	5.201	8.675
Lamb1-1	2.763	5.559	7.287	11.54
Lamc1	2.025	3.903	5.544	10.43
Lefty1	6.377	6.341	3.640	7.515
Lefty2	8.879	4.911	3.595	7.003
Lifr	0.814	0.828	1.056	1.391
Lin28	1.088	1.267	0.981	1.804
Myf5	ND	ND	ND	ND
Myod1	ND	ND	ND	ND
Nanog	0.751	0.356	0.604	0.340
Nes	1.715	1.496	3.188	8.222
Neurod1	0.950	1.723	1.190	1.505
Nodal	0.887	0.750	0.909	0.909
Nog	9.297	31.35	26.47	57.41
Nr5a2	0.597	0.595	0.375	0.343
Nr6a1	1.830	1.518	2.960	2.825
Numb	1.047	0.870	1.024	1.019
Olig2	ND	ND	3.428	3.307
Pax4	ND	ND	ND	ND
Pax6	0.928	0.401	0.715	0.616

Table S2. Cont.

Gene	Static day 3	Perfusion day 3	Static day 5	Perfusion day 5
Pdx1	1.242	1.196	1.999	1.320
Pecam1	1.311	0.896	0.954	0.708
Podxl	3.014	2.508	4.055	2.474
Pou5f1	0.668	0.607	0.580	0.672
Pten	0.909	1.183	0.860	1.899
Ptf1a	ND	ND	ND	ND
Rest	1.005	0.999	0.601	0.832
Runx2	ND	ND	ND	ND
Sema3a	ND	ND	ND	ND
Serpina1a	ND	ND	ND	ND
Sfrp2	1.432	1.648	0.466	3.535
Sox17	3.847	7.482	10.93	7.607
Sox2	1.143	0.761	0.760	0.699
Sst	ND	ND	ND	ND
Sycp3	2.686	2.454	4.031	2.902
T	0.979	1.167	1.953	23.96
Tat	1.427	2.004	3.127	3.958
Tcfcp2l1	0.777	0.477	0.592	0.308
Tdgf1	1.234	0.600	1.454	0.728
Tert	1.746	0.842	1.463	1.075
Utf1	0.910	0.968	0.704	0.930
Wt1	6.734	6.560	12.69	5.379
Zfp42	0.601	0.510	0.312	0.169

ND, not detected, indicating expression levels below the quantitative PCR detection limit of 35 cycles.