Electronic Supplementary Material (ESI) for Chemical Communications

A simplified culture system to examine soluble factor interactions between mammalian cells

Electronic Supplementary Information

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Materials and Methods

Device Fabrication

Devices were fabricated by constructing a negative mould and then casting PDMS into the mould to form the three-dimensional ring that separates the two culture surfaces. Using an Epilog Mini18 40 Watt Laser Cutter (Epilog Laser Golden, CO), negatives of the PDMS rings were cut 250 µm or 500 µm deep into a sheet of acrylic. Subsequent negatives of pillars were engraved into the rings at a height of 50 µm. The spacing between pillars was optimized to provide a 'stop' to maintain the cover slip culture in place over the culture space within the PDMS ring (Suppl. Fig. 1). PDMS base and curing agent was mixed at a ratio of 10:1, degassed for 20 minutes, and cured in the moulds for 60 minutes at 80°C. Devices were removed and sterilized in 70% ethanol overnight. Devices could be stored for at least a month in this solution. Coverslips were acid washed in 1M HCl overnight, followed by 4 washes in ultrapure water for 10 minutes each. Coverslips were then soaked in ethanol for one hour and exposed to a UV lamp for 30 minutes for sterilization.

Cell Lines and Reagents

Unless noted otherwise, all reagents were purchased from Sigma-Aldrich (St. Louis, MO). The ovarian cancer cell line OVCA433 was obtained from Dr. R. Bast (MD Anderson Cancer Center, Houston, TX).[1] The peripheral blood acute monocytic line THP-1 was purchased from ATCC (Manassas, VA).[2] OVCA433 were cultured in Medium 199/MCDB 105 supplemented with 10% Fetal Bovine Serum (Invitrogen; Carlsbad, CA) and 1% Penicillin-Streptomycin. THP-1 were cultured in RPMI (CellGro; Manassas, VA) supplemented with 10% Fetal Bovine Serum (Invitrogen), 1% Penicillin-Streptomycin, and 50 µM 2-mercaptoethanol.

Co-culture Setup

THP-1 were differentiated to a macrophage-like phenotype according to previously described protocols.[3] In brief, 9 mm coverslips were placed in individual wells of a 24 well plate and THP-1 were seeded at 150,000 or 300,000 cells per well. THP-1 were then treated with 30 ng/mL of PMA for 6 hours followed by additional treatment with 25 ng/mL IL-4 and 25 ng/mL IL-13 (PeproTech; Rocky Hill, NJ) for 66 hours. Cells were then washed with PBS and changed to OVCA433 serum-free media for 24 hours in preparation for co-culture. Control coverslips for monoculture conditions were prepared by omitting the THP-1 cells, but exposing the coverslip to the same differentiation protocol. PDMS devices were placed in individual wells of a separate 24 well plate and coated with a 100 μL layer of 2% gelatin solution that was aspirated after a 30 minute incubation at 37°C. 25,000 OVCA433 cells were seeded per device and allowed to attach for 24 hours. OVCA433 were then serum starved for 24 hours prior to the start of co-culture. To initiate co-culture, THP-1 cultured coverslips and control-treated coverslips were added to the devices for 4 hours for phosphoprotein assays and 48 hours for proliferation, qRT-PCR and ELISA assays. After 24 hours, media was supplemented with 3.6 or 7.2 μL (10% of initial media volume for 250 μm or 500 μm devices respectively; depending on environmental conditions, 20% supplementation may be necessary to account for evaporation).

Viability Assay

After 48 hours of co-culture, media was removed from the devices and cells were washed once with PBS before adding a solution of 5 μM ethidium homodimer, 1 μM calcein-AM (Live/Dead, Invitrogen) solution in PBS. Following 30 minutes of staining at 37°C, cells were washed once with PBS and imaged on a 3i / Olympus Deconvolution System with Spherical Aberration Correction (fluorescence and phase contrast capabilities), Orca ER camera, and SlideBook imaging software.

RNA Extraction and qRT-PCR

RNA was collected using RLT Buffer and Micro-RNeasy Extraction kit according to manufacturer's instructions from co-culture conditions with 300,000 THP-1 in the 250 μm devices (Qiagen, Valencia, CA). cDNA was synthesized at 70 ng/20 μL using SuperScript III First-Strand Synthesis System (Invitrogen). qRT-PCR was performed on 7 ng of cDNA per reaction using primers for *FCGR1A* and *GAPDH* (Qiagen), SYBR Green RT-PCR Master Mix (Invitrogen), and an Applied Biosystems StepOne Plus (Applied Biosystems; Foster City, CA).

Proliferation Assay

Cell proliferation was quantified after 48 hours of co-culture using Click-iT Imaging Assay (Invitrogen) according to manufacturer's instructions. Cell nuclei were counter-stained with 5 μg/mL Hoechst 33258 (Invitrogen). Four biological replicates per condition were imaged on a 3i/ Olympus Deconvolution System with Spherical Aberration Correction (fluorescence and phase contrast capabilities), Orca ER camera, and SlideBook imaging software, taking four fields of view per replicate. Using ImageJ software (NIH), all cell nuclei and EdU-positive nuclei were counted in order to calculate percentage of proliferating cells.

Western Blot Analysis

OVCA433 were lysed following 4 hours in monoculture or co-culture using phospho-protein lysis buffer composed of 50 mM β-glycerophosphate (Boston Bioproducts; Boston, MA), 10 mM NaPP (Boston Bioproducts), 30 mM NaF (Boston Bioproducts), 50 mM Tris (Fisher Scientific, Waltham, MA), 2% Triton X-100, 150 mM NaCl, 1 mM benzamidine, 500 mM EGTA (Boston Bioproducts), 100 μM sodium orthovanadate, 1 mM DTT, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 μg/mL pepstatin, 1 μg/mL Microcystin-LR. Protein concentration was determined through a BCA assay (Thermo Scientific), and 1 μg of protein was loaded per lane in a 4-12%

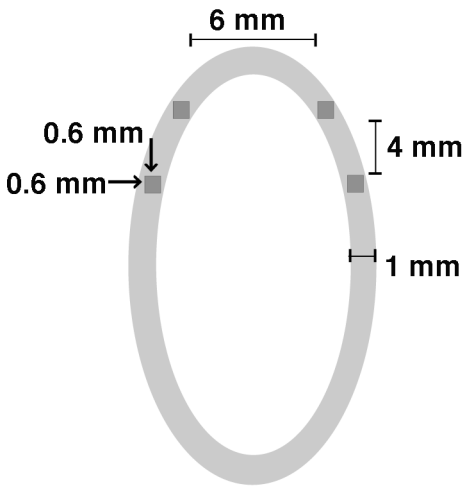
Bis-Tris Gel (Invitrogen). Following transfer to a nitrocellulose membrane (BioRad Laboratories; Hercules, CA), the membrane was blocked with 5% BSA in Tween 20 Tris-Buffered Saline Solution (TBS-TB) and incubated with rabbit-anti-pERK1/2 (1:1000, Cell Signaling; Boston, MA) and rabbit-anti-β-tubulin (1:2500, Cell Signaling) in TBS-TB overnight at 4^{O}C. Goat-anti-rabbit HRP (5 ng/mL in TBS-TB, Invitrogen) was incubated with the membrane for 1 hour at room temperature. Detection was performed using Clarity Western ECL Substrate and a BioRad ChemiDoc MP (BioRad).

Quantification of Secreted IL-8 Levels

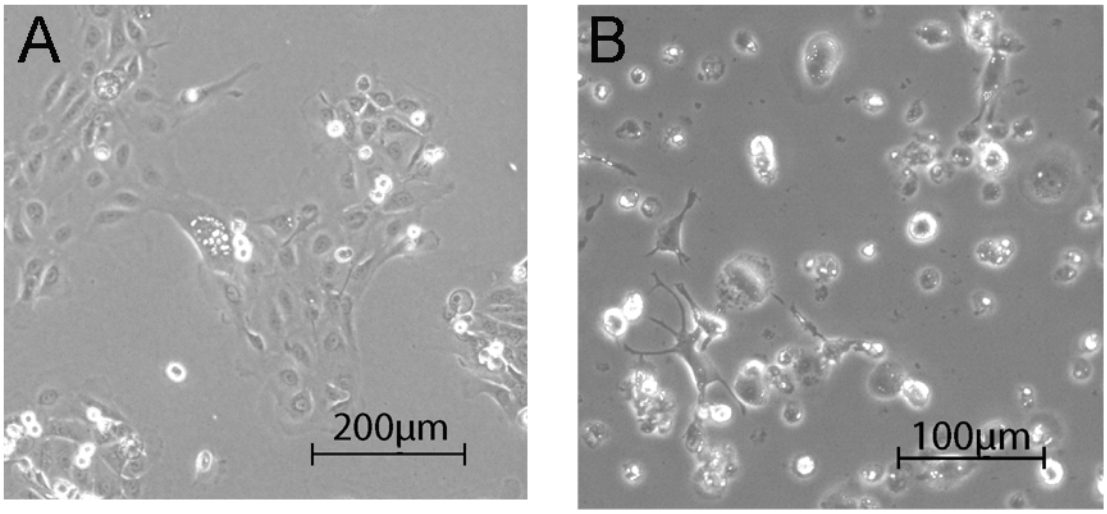
Media was collected from monoculture and co-cultures in order to determine the levels of cell-secreted factors. IL-8 levels were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R&D Systems; Minneapolis, MN). IL-8 concentration was determined from the standard curve using a four-point logistic curve fit.

Statistical Analysis

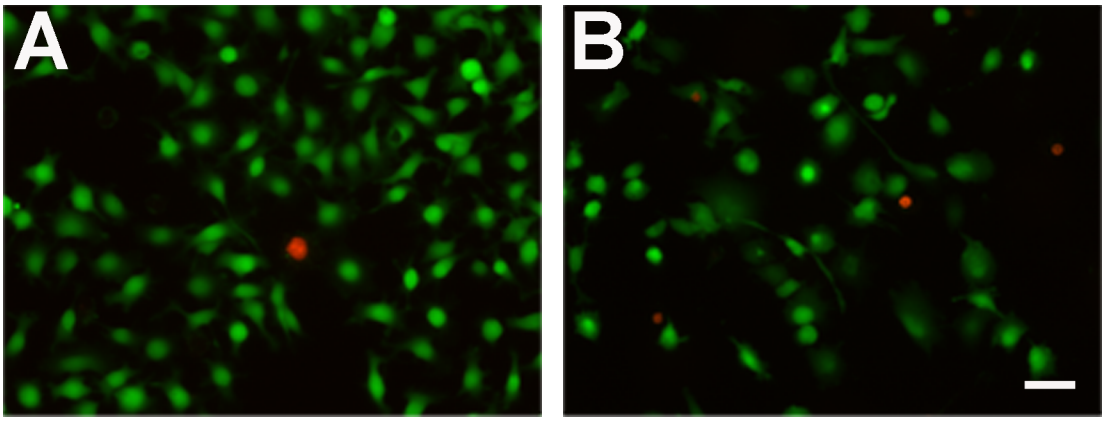
Proliferation and IL-8 levels were analysed using one-way ANOVA followed by Tukey-HSD for each device size. All statistical calculations were done with the software package JMP 4.1 (SAS Institute; Cary, NC).



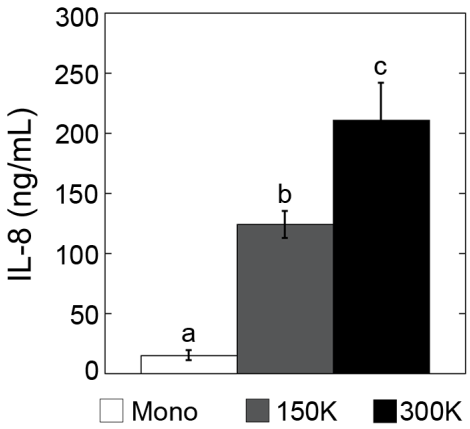
SI Figure 1. Detailed dimensions of PDMS ring portion of co-culture system.



SI Figure 2. Phase images of OVCA433 **(A)** and differentiated THP-1 **(B)** in the co-culture device after 24 hours. OVCA433 cultured in these devices maintained an epithelial morphology similar to that seen in standard culture setups. In response to differentiation media, THP-1 adhered to the culture surface.



SI Figure 3. Live (green) and dead (red) cells for **(A)** OVCA433 and **(B)** 300,000 THP-1 after 48 hours in co-culture. Scale bar represents 50 μm.



SI Figure 4. IL-8 concentration was significantly higher when THP-1 were included in co-culture devices compared to OVCA433 monoculture (Mono). IL-8 levels were further increased when THP-1 density was increased from 150,000 (150K) to 300,000 (300K). Media was collected from 250 μm co-culture devices after 48 hours of co-culture. Data plotted as average ± SEM, different letters indicate significantly different than other conditions, $p<0.05$ (n=4).

SI Table 1. Expression of *FCGR1A* in Co-culture Populations

Cell Type	Culture Condition	ΔCt (Avg ± SD)	ΔΔCt (Avg ± SD)
OVCA433	Monoculture	17.9 ± 1.9	-0.9 ± 2.1
	Co-culture	16.9 ± 1.0	
THP-1	Monoculture	4.6 ± 0.3	2.4 ± 0.5
	Co-culture	7.0 ± 0.4	

Analysis by qRT-PCR indicated that the neither cell population crosses into the other cell population. The level of F_c Type IA Receptor (*FCGR1A*) was quantified and compared between monoculture and co-culture in 250 μm devices after 48 hours. THP-1 in monoculture expressed high levels of *FCGR1A*, and this level was slightly increased in co-culture. Conversely, OVCA433 expressed low levels of *FCGR1A* in monoculture, and this level was slightly decreased in co-culture. Combined, these trends indicate that the high-expressing THP-1 did not migrate to the OVCA433 surface and vice versa. ΔCt was calculated relative to *GAPDH* levels and ΔΔCt relative to that cell type in monoculture, (n=3).

Supplementary References

1. R. C. Bast, Jr., M. Feeney, H. Lazarus, L. M. Nadler, R. B. Colvin and R. C. Knapp, *J Clin Invest*, 1981, 68, 1331-1337.

2. S. Tsuchiya, Y. Kobayashi, Y. Goto, H. Okumura, S. Nakae, T. Konno and K. Tada, *Cancer Res*, 1982, 42, 1530-1536.

3. I. Caras, C. Tucureanu, L. Lerescu, R. Pitica, L. Mielinceanu, S. Neagu and A. Salageanu, *Tumori*, 2011, 97, 647-654.