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Cellular observations enabled by microculture: paracrine signaling and population demographics[†]

Maribella Domenech^{†,a}, Hongmei Yu^{†,a}, Jay Warrick^a, Nisha M. Badders^b, Ivar Meyvantsson^a, Caroline M. Alexander^b, and David J. Beebe^{*,a}

^a University of Wisconsin-Madison, 3144 Engineering Centers Building, 1550 Engineering Drive, Madison, WI 53706, USA.

^b University of Wisconsin-Madison, Department of Oncology, Madison, WI, USA

Abstract

The cellular microenvironment plays a critical role in shaping and directing the process of communication between the cells. Soluble signals are responsible for many cellular behaviors such as cell survival, proliferation and differentiation. Despite the importance of soluble signals, canonical methods are not well suited to the study of soluble factor interactions between multiple cell types. Macro-scale technology often puts cells into a convective environment that can wash away and dilute soluble signals from their targets, minimizing local concentrations of important factors. In addition, current methods such as transwells, require large numbers of cells and are limited to studying just two cell types. Here, we present data supporting the use of microchannels to study soluble factor signaling providing improved sensitivity as well as the ability to move beyond existing co-culture and conditioned medium paradigms. In addition, we present data suggesting that microculture can be used to unmask effects of population demographics. In this example the data support the hypothesis that a growth promoting subpopulation of cells exists in the mouse mammary gland.

Introduction

One goal of cell biology research is to understand the basic mechanisms of cell regulation. A plethora of tools are available to interrogate cell behavior including direct physical methods (*e.g.* patch clamp electrophysiology, fluorescent microscopy), animal models (*e.g.* transgenic mice), and molecular analysis (*e.g.* PCR/microarrays, mass spectrometry) to name just a few. Each tool provides a unique perspective or window through which we can observe cell behavior and thus, study cell regulation. Micro- and nano-scale technology are beginning to provide additional tools for cell biology. Surface patterning of cell adhesion ligands has been used to study factors determining the fate of cell division axis orientation, geometric control of cell fate and progenitor cell fate.^{1–3} Akin *et al.* utilized bacterial-mediated nanoparticle delivery to transport reporter molecules into living cells.⁴ Microfluidics has the potential to be one of these new windows that will allow us to peer at cell biology from new perspectives.⁵ Indeed, Sawano *et al.* have used microfluidics and the inherent property of laminar flow to examine intracellular signaling in ways not possible with canonical tools.⁶ Here we show that microchannel culture without flow improves our ability to study soluble factor signaling

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djbeebe@wisc.edu; Fax: 608/265-9239; Tel: 608/262-2260.

*Contributed equally.

providing new functionality, increased sensitivity and insights into the mechanisms of growth regulation.

While cell communication is complex and occurs *via* many different mechanisms it is clear that secreted factors play a major role both locally *via* autocrine/paracrine signaling and systemically *via* endocrine signaling. Current methods for exploring paracrine signaling include conditioned media experiments and the use of co-culture systems (*e.g.* transwell devices). Conditioned media experiments are used to test whether a particular behavior is dependent on secreted factors. Media from one culture is removed and added to a second culture to see if the effect under study is dependent on a soluble factor from the other cell type. Due to practical considerations, conditioned media is often stored before it is used limiting its ability to test for soluble factors with short half lives—thus, a negative result can be inconclusive. In addition, the use of conditioned media does not allow reciprocal signaling between cells such as those implicated between tumor cells and the stroma during cancer progression.⁷

Insight, innovation, integration

First, the data suggests that the inherent properties of microchannel culture (*e.g.* transport by diffusion, lack of convection, reduced dilution) lead to an improved ability to study soluble factor signaling (*e.g.* paracrine/autocrine signaling). Second, microchannel culture allows for an exploration of the stochastic effects of cell heterogeneity via studies that utilize hundreds of cells (instead of single cell analyses or traditional population-based studies that use thousands to millions of cells). Specifically, the data (using primary mouse mammary gland cells) suggests that a subpopulation of cells has a growth promoting effect on the majority. Microchannel cell culture is still an emerging area that has not been thoroughly characterized. Here we utilize both simple straight and curved channels as well as introduce a multichamber device design to facilitate co-culture between multiple cell types. The work integrates microchannel culture (both cell lines and primary cells), numerical simulation and new device designs to examine the potential of microculture for studying soluble factor effects and population demographics. This integration made possible experiments that are not possible using traditional methods and led to the insights described above.

The transwell co-culture system (Fig. 1C) provides a means to culture two cell types, one above the other, separated by a porous membrane. While retaining reciprocal signaling, each cell type is placed on a different surface (*e.g.* tissue culture plastic/polyester porous membrane) potentially leading to the activation of cellular responses due to differences in surface interaction rather than or in addition to paracrine signaling. Transwell assays are expensive and secreted factors are diluted into large volumes (Fig. 1C).

Here we present the results of microchannel culture experiments that suggest that the inherently diffusion dominant and low volume microenvironment of a microchannel (Fig. 1A) provides a tool to observe and manipulate cell behavior. Microchannels allow for the culture of two or more cell types that are spatially segregated but sharing the same substrate and aqueous environment (medium). Our results suggest that microchannel culture may reveal soluble factor signaling effects that are hidden in canonical culture systems.

The ability of a soluble factor to cause a cellular response is dependent on the activity and concentration of the factor. The activity of a given soluble factor varies with a number of parameters including pH, aggregation, presence of appropriate partners to form complexes and temperature. The concentration of a given soluble factor increases with the cellular production rate; the half-life; and, in *in-vitro* culture, the initial concentration in the media. The physical geometry of the container that cells are cultured in plays an important role in how much of a soluble factor is available in a particular location (Fig. 1). The volumes and air/liquid interfaces

present in open wells (*e.g.* Petri dish or multi-well plate) (Fig. 1B–D) allow cell secreted factors to become diluted and mixed *via* both diffusion and convection diminishing their concentration and reducing the possibility of reaching their targets in sufficient concentration before being degraded or denatured. By comparison, in microchannels under no flow conditions, diffusive transport is the primary mode of transport, and dilution is reduced as compared to canonical vessels.^{8,9} Thus, both the volumes and geometries of the culture vessel should be considered to understand the influence of the culture system on cell behavior.¹⁰ As suggested previously^{11,12} and expanded upon by the results presented here, such a cellular scale microenvironment can enhance our ability to observe cell behavior.

Microchannel culture has the potential to enable the study of soluble factor effects in several specific ways. First, microchannels provide an environment where important threshold concentrations can be reached faster than in traditional well-based plates, including Terasaki wells (10 μ l).¹¹ Second, microchannels allow for the testing of the effects of population demographics. Low volume well-plates (*e.g.* Terasaki plates) can be used to culture small numbers of cells, however microchannels have the advantage of being able to control for effects of cell density (by maintaining a constant cells mm^{-2} between experiments with different numbers of cells). Third, microchannels can be used to create individually addressable co-culture chambers containing different cell types that are fluidically connected. These microchambers can be designed to be in close proximity to one another for studying the effects of secreted factors (autocrine/paracrine) without *cell–cell* contact. Direct comparisons between microscale culture and macroscale culture are sometimes difficult. For example, transwells introduce additional variables such as different surfaces (porous vs. flat surfaces) and the long distance between the two compartments may influence the kinetics of cellular responses.

Previous work in embryo culture¹³ and insect cell culture in microchannels¹⁴ gave rise to our hypothesis that the convection-free environment within a microchannel under no conditions might provide a culture platform that would enhance cell-cell communication *via* secreted factors.¹² We have demonstrated that microchannels can provide a convection-free environment under appropriate experimental conditions.⁸ Recently, we have performed a direct comparison of microchannel vs. open-well culture examining the effects of varying media change frequency to further test the hypothesis.¹¹ The growth of mouse mammary epithelial cells (NMuMGs) was increased in microchannels as compared to 96-well plates, and improved in both platforms when the time between media changes was lengthened (*e.g.* every 4 h vs. every hour). In addition, the difference in growth rates between microchannels and open wells is enhanced at lower cell densities. These previous data are all consistent with the hypothesis that microchannels provide a unique environment with which to observe cell behavior—an environment in which the influence of secreted factor is enhanced.

Here we present additional experimental data that support the use of microchannels as a platform for studying soluble factor signaling and suggests microchannel culture can be used to screen for cell behaviors previously unobservable. This is done in two parts. In part A, we used a convection-free, multi-chambered microchannel device to compare interactions between epithelial and stromal cells *via* secreted factors in micro- and macroscale culture. In part B, we screened for the effects of population size (while maintaining a constant surface cell density) on growth kinetics in primary mammary epithelial cells.

Results and discussion

Part A. Co-culture in microchambers magnifies the effect of secreted factors

The multichamber co-culture device (shown in Fig. 2) consists of one to four side chambers (or lobes) connected to a center chamber. Tubeless microfluidics utilizing fixed volume patterning¹⁵ and passive pumping¹⁰ was used to pattern each cell population a chamber (Fig.

S1, ESI[†]). The side chambers are connected with the center chamber through diffusion ports (Fig. 2) allowing secreted soluble factors to diffuse between the chambers. Simulation results (Fig. 3) show that the device functions by facilitating accumulation, mitigating depletion, and allowing diffusion-based communication between the lobes and center chamber within the relevant experimental times (see Methods section and ESI[†] for details). A transwell system was used as a macroscale comparison. Transwells consist of a hanging insert placed about a millimeter above a tissue culture treated polystyrene well of a 24-well plate. The insert contains a transparent polyester membrane (PET) with a pore size of 0.4 μm (Corning, Lowell, MA).

As a model system to explore the relative co-culture responses in microchambers and transwell assays, we used the previously reported induction of osteoclastogenesis in the murine monocyte cell line RAW 264.7 through soluble secreted factors (RANK-L, MCP-1 and IL-8) from human prostate cancer cell lines.^{16,17} In this study, the human prostate cancer cell line PC3-MM2 was co-cultured with RAW 264.7 in a device with one side chamber (Fig. 2—see inset of two-chamber device) and in transwells to determine whether or not both platforms produce a comparable response (*i.e.* number of differentiating osteoclasts per unit area). PC3-MM2 is a more aggressive subline of PC-3 (human prostate cancer cells), derived from a mouse bone metastasis.^{18,19} Briefly, RAW 264.7 and PC3-MM2 cells were seeded at equal surface cell densities (200 cells mm^{-2}) in transwells (top insert and bottom well respectively) and microchambers (Fig. 5A) in reduced serum (2%) conditions for a total of 4 days. Media was replaced every other day in both platforms. After 96 h, cells were fixed and stained for tartrate resistant acid phosphatase (TRAP, an early marker of osteoclast differentiation). As shown in Fig. 4, the formation of differentiating cells occurred in both platforms and had similar morphology. TRAP (+) multinucleated (nucleus >2) cells were manually counted as differentiating osteoclasts (Fig. 5B) in each platform.²⁰ Because transwell inserts and microchambers have different surface areas (~ 33 and ~ 9 mm^2 , respectively), the number of differentiating cells per mm^2 was used to compare between both systems. While, a significant number of differentiating osteoclasts per unit area were detected in both platforms (Fig. 5B), a significantly higher fold increase ($P < 0.0005$) was observed in microchambers as compared to transwells (Fig. 5C).

The data provide insights into the potential implications of the inherent differences between the experimental platforms. The microchannel platform led to an increased response as compared to transwells in co-culture experiments. Given that the relative cell culture areas were different while the cell densities were the same, there was less volume per cell in a microchamber. Reduced volume per cell can increase rates of factor accumulation. Accumulation rates can be important if there are threshold concentrations to be reached in order to induce differentiation. This is supported by previous work, where microchannels were found to provide an environment where threshold concentrations can be reached faster than in 96-well plate format.¹¹ However, other factors may also be important in producing the observed increase in cell differentiation. The increased response could also be due to differences in endogenous factor concentrations, reciprocal signaling, decay/absorption/adsorption of labile factors or changes in productions/consumption. The difference in differentiation is likely the result of a combination of these factors. Yet, it is difficult to make a direct comparison between the platforms. The transwell platform introduces additional variables such as the different material used in the transwell insert to enable visualization of the cells. Different surfaces (porous *vs.* flat) and the increased distance between the two compartments may significantly influence cell behavior.

[†]Electronic supplementary information (ESI) available: Materials and methods: Device model – geometry validation. Fig. S1–4. See DOI: 10.1039/b823059e

While direct comparisons are difficult, the efficiency of stromal-derived soluble factors to induce osteoclastogenesis (*i.e.* differentiated cell density in mono-culture *vs.* co-culture) was enhanced in the microchannel platform. This enhanced response suggests that microchannels provide an environment in which cells are more sensitive to paracrine signaling, and therefore may reveal cell responses that are masked at the macro scale due to convection/dilution effects or other platform artifacts (*e.g.* material differences).

Part B. Micro-culture of small numbers of cells enable unmask effects of population demographics

While the experiments in Part A help us to understand the microchannel platform, we sought also to utilize micro-culture to study the interactions of subpopulations of primary mammary epithelial cell populations and gain insights into their role in growth regulation. In mammary gland development *in-vivo*, it is well known that there is a coordinated conversation between multiple cell types *via* soluble factors, cell-cell contact, and cell-matrix interactions. We explored the relative importance of various subpopulation on growth promotion using primary cells in no-flow microchannel culture.

Initially, we characterized the density dependence of both primary mouse mammary epithelial cells (MECs) and a nontransformed mammary epithelial cell line (NMuMG cells) in microchannel culture (Fig. S3A-B, ESI[†]). The growth of NMuMG cells and MECs were both density dependent. Specifically, NMuMG cells seeded at low density increased in number by 1.5 times in 24 h (Day2/Day1 or D2/D1), whereas cells seeded at 120 cells mm⁻² had divided ~2 times. Primary MECs showed similar trends but with a significantly higher variance. To explore the source of this variance, we proposed that it reflected the heterogeneity of the primary population plated and could be masked or unmasked when culturing a large or small number of cells. To test the idea that there could exist a minor population that influences the majority, we first measured the proliferation of MECs plated at equal surface and volume density but different cell numbers and second, the population effects on MEC growth was studied to understand the properties of potential subpopulations (*i.e.* relative frequencies) that may play a role in promoting growth within the MEC microenvironment.

Although the device used in part A can be used to study interactions between cell populations, a different device was needed to perform the next sets of experiments in order to facilitate manipulation of culture surface area. By changing the amount of culture surface area available to cells, cell number can be changed without changing cell surface density. This is important due to the previously described density dependant growth characteristics of the cell types of interest. The microchannel designs are shown in Fig. 6A. These microchannel designs also facilitate high-throughput measurements of cell number, which was validated in previous work.²¹

MECs and NMuMG cells were cultured at a surface density of 75 cells mm⁻² over a range of cell numbers (350, 700, 1500, 3000). As shown in Fig. 6B–C the cell number plated affected the growth of MECs, while NMuMG cells exhibited no cell number-related effects. Specifically, microchannel cultures with 350 MECs had lower growth rates with small variances (1.35 ± 0.15 , $n = 6$), and cultures with 3000 MECs had high growth rates, also with small variances (2.0 ± 0.20 , $n = 6$). However, microchannel cultures with 700 or 1500 MECs had either large or slow growth rates with larger variances (1.7 ± 0.60 , $n = 6$). Non-parametric analysis revealed two groups, one with a high growth rate and one with a low growth rate (Fig. 6B) existed ($P < 0.05$), suggesting a rare event mechanism could be responsible $\sim 1/2500$ (Poisson statistics).

To determine if mammary gland subpopulations confer growth promotion to the majority, we performed recombination experiments. In these experiments the “reporter population” is

defined as 300 MECs (from unsorted preparations)—a small population which would have a low probability of containing cells from rare populations. Using methods similar to Stingl,²² we sorted MECs into four subpopulations: mammary repopulating units (“MRU”, CD45⁺Ter119⁺CD31⁺CD49f^{hi}CD24^{med}), myoepithelial population (“MYO”, CD45⁺Ter119⁺CD31⁺CD49f⁺CD24^{low/-}), mammary colony forming cells (“CFC”, CD45⁺Ter119⁺CD31⁺CD49f^{low}CD24^{high}) and “DN” (double negative- subpopulations of all stromal cells, CD49f⁺ or CD24⁺), and recombined them with the reporter population (unsorted MECs 300 cells) to test for growth promotion (Fig. S4, ESI[†]). As shown in Fig. 7, the growth rate of the reporter cells increased 2.7-fold after recombination with the MRU subpopulation suggesting a growth promotion role for the MRU subpopulation. A similar increase was not observed upon doubling of the heterogeneous MEC population (MEC 600 cells) or from recombination of other subpopulations (CFC, MYO, DN). The unsorted (reporter) and sorted (MRU, CFC, MYO and DN) cell populations could be differentiated *via* staining for hPaP (human placenta alkaline phosphatase, see Materials and Methods for details). Only reporter cells were counted for determining fold-increase in Fig. 7. Although the sorted and unsorted cells were cultured in the same channel, observed cell–cell contact was minimal due to low cell densities. Conditioned media experiments were also performed (data not shown), but results were inconclusive, suggesting that the mechanism responsible for growth promotion may depend upon reciprocal signaling or short half-life molecules.

Taken together, the results from Parts A and B illustrate important capabilities of microchannel platforms for *in vitro* cell culture studies. Micro-devices can provide a low-volume and diffusion-dominant environment that may enhance the study of paracrine signaling. Such environments may reveal stochastic effects by giving researchers the flexibility to control and vary the heterogeneity of cell populations while controlling for cell density.

Conclusions

The ability to study soluble factor effects has been limited by existing methods. Canonical methods utilize convective environments and large volumes that may mask important cellular responses. Microchannel co-cultures differ from macro-scale analogs in several important ways including decreased volume-per-cell ratios (emphasizing intercellular signaling processes) and the ability to position cell populations in close proximity (but without direct cell–cell contact) with soluble factor transport governed by diffusion. Our studies suggest that microchannels provide an environment in which cells are more sensitive to paracrine signaling, and therefore may reveal cell responses that are masked at the macro-scale due to convection/dilution effects or other platform artifacts (*e.g.* material differences). Specifically, osteoclastogenesis was enhanced in co-cultures, and stochastic effects of cell heterogeneity were observed.

It is important to note that the use of microfluidic devices for cell culture is still in its infancy and additional work is needed to further validate microchannel cell culture before it will be widely adopted. One potential bias is the use of PDMS. PDMS is commonly used in microscale cell culture applications due to its gas permeability and ease of fabrication. However, small hydrophobic molecules are readily absorbed into the bulk polymer potentially causing experimental artifacts.^{23,24} Still, the results presented here as well as by others suggest unique and fundamental differences between macro- and micro-scale culture systems that could significantly improve our ability to study soluble factor effects. Importantly, the tubeless microfluidic platform used in these studies is not dependent on elastomers and can be seamlessly integrated with existing biological methods and infrastructure (pipetting and standard microscopy) reducing barriers to use in the life sciences.

Experimental

Materials and methods

Microchannels—The microchannels were fabricated from PDMS using well established micromolding methods.^{25,26}

Microchamber-transwell comparison experiment

Cell culture—Human prostate cancerous cells (PC3-MM2-MM2) were obtained from Dr C. Pettaway, MD Anderson Cancer Centre, TX, USA). Mouse monocytes (RAW 264.7) were purchase from the American Type Culture Collection (ATCC, Rockville, MD, USA). All cells were maintained in RPMI 1640 media (Invitrogen), supplemented with 10% fetal bovine serum (Biomed) and 1% penicillin/streptomycin at 37 °C in 5% CO₂ and passaged with 0.05% trypsin-EDTA solution (Invitrogen) when grown near 75% confluency. Viable cells were counted using a hemocytometer (0.4% Trypan Blue solution), diluted to the desired cell densities and seeded in microchambers or wells.

The array of microchannels was made out of poly-(dimethylsiloxane) (PDMS), sterilized (autoclave) and mounted in a flat-bottom tissue-culture treated plate (Omnitray, Nunc). The side chambers and the center chamber have a height of 250 µm and a volume of about 2 µl each. Microchambers were vacuum filled and cells were loaded using the passive pumping method.¹⁰ Briefly, a 5 µl drop of cell culture medium was placed at the output port and a 2.0 µl drop of cell suspension was delivered to the input port of each chamber. RAW 264.7 cells (center chamber, 9.6 mm²) and PC3-MM2-MM2 cells (side chamber, 6.8 mm²) were seeded at a cell density of 200 cells mm⁻². The cells were successfully patterned in the chamber, no cells passing through the diffusion ports (Fig. 4A). RAW 264.7 were seeded at the top (inserts, ~ 33 mm²) and PC3 MM2 were seeded at the bottom (well, ~ 191 mm²) of the transwell system. During the experiment, cells were cultured in serum reduced media (RPMI 1640, 2% FBS, 1% Penn/Strep) for at total four days.

TRAP assay—At day four, RAW 264.7 cells were fixed and stained with TRAP staining kit (387-A, Sigma Aldrich). After stained, cells were washed with PBS and led dry overnight. Cells were count manually and photos were obtained using brightfield microscope (Olympus IX70).

Device design and validation—Diffusion simulations were used to determine the ability of the device to facilitate autocrine and paracrine signaling for co-culture. The use of simulations to predict diffusion in tubeless microfluidic devices such as the co-culture devices used here has been validated in previous work by Abhyankar *et al.*²⁷ Simulations were performed in 2-D using COMSOL Multiphysics v3.4 (Palo Alto, CA). A 2-D model was chosen in order to facilitate computation and, as such, the 2D geometry was altered compared to the top view of the actual 3-D device. The alteration was needed to reflect the narrow thickness of the soluble factor exchange region between the lobes and the center. Details and validation of this alteration are included in ESI.[†]

The 2-D geometry was then used to model diffusion of a ~ 10 kDa molecule (diffusion coefficient of 100 µm² s⁻¹) for two different conditions. The first condition (condition A) simulates a treatment of a lobe (*i.e.* the use of a pipette to replace the fluid contents) with a solution containing the 10 kDa factor at a nominal concentration of 1 [au]. The factor is then allowed to diffuse throughout the device. In the second condition (condition B), the lobe concentration is maintained at 1 [au] throughout the simulation. Condition B was included as it might be more indicative of a situation where cells are able to maintain a specific concentration of the factor. The goal of simulating these two conditions is to determine: (1)

the general time-scale of diffusive signaling in the device, (2) whether the device allows significant exchange between the lobes and center, and (3) whether depletion of factors is sufficiently slow to facilitate accumulation of autocrine factors. The simulations were performed for devices with one lobe and with two, denoted with a suffix of 1 and 2, respectively, creating four different scenarios, A.1, A.2, B.1 and B.2 (see Fig. 3).

Fig. 3 depicts the exchange and depletion of factors between the lobes in two ways. The graph in the top of the figure plots the nominal or normalized concentration with time. The lower portion of Fig. 3 shows a table of concentration maps for specific times of each simulation. The plot shows that for all conditions, the center chamber can begin to sense the factor emanating from the lobe(s) in about an hour. The plot also shows that depletion of factor into the sink (the channel that forms a ring around the device) is slow relative to the culture times and fluid replacement protocols used in this study. This is evidenced by the fact that in condition A, factor concentrations in the lobes have only dropped by $\sim 50\%$ over 48 h. Similarly, if depletion were more significant than the accumulation in the center chamber, concentration levels of the factor would not accumulate to the extent shown in the plot at 48 h.

It is important to note that the results are all relative and any biological effects due to concentration levels are all subject to production rates of the cells emitting the factor of interest and the sensitivity of the effector cells to the given factor. However, simulation results do suggest that the device functions as intended, by facilitating accumulation, mitigating depletion, and allowing diffusion-based communication between the lobes and center chamber within the relevant experimental times.

Statistical analysis—Data was normalized to monoculture conditions to obtain the fold change. Results were compared using the Wilcoxon two-sided test.

Growth property studies

Microchannels preparation—PDMS microchannels were fabricated by using well-established methods and by attaching to positive charged glass slides. Growth factor reduced Matrigel® (BD, #40230A) was diluted in ice-cold DMEM to a final concentration 0.6 mg mL^{-1} to coat the microchannel (Fig. 6A). The microchannels were incubated at 37°C for more than 30 min. Microchannels were washed once by flowing culture medium before cell culture.

Cell growth and isolation—Normal murine mammary gland cells (NMuMG, ATCC) were regularly maintained in DMEM (Gibco-Invitrogen) with 5% FBS (Hyclone) and insulin (10 mg mL^{-1} ; Sigma) in 50 mm tissue culture plates before microchannel experiments. The unsorted primary mammary epithelial cells (MECs) were isolated from the mammary glands of 12–16 week old Balb/c virgin mice (Jackson) as previously described.²⁸ For microchannel experiments, MECs and NMuMGs were cultured in DMEM supplied with 20 ng mL^{-1} EGF (Sigma) and $10 \text{ }\mu\text{g mL}^{-1}$ insulin. The sorted populations of MECs were isolated from FVB-hPAP²⁹ transgenic mice (courtesy of Erik Sandgren's lab, School of Veterinary Medicine, University of Wisconsin-Madison). In order to obtain MRU, CFC, MYO and DN mammary epithelial subpopulations, we followed the same protocol as described by Stingl.²² Cell sorting was performed using BD FACSVantage in the Flow Cytometry Facility at UW-Madison.

Microchannel cell growth assay—In order to characterize the effects of seeding density on the growth of MECs and NMuMGs in microchannels, both cell types were seeded into microchannels of the same dimensions (Supplemental Fig. 3) to get 15, 30, 60, 120, 240 and $480 \text{ cells mm}^{-2}$ (day1, D1) The samples were fixed and stained with Hoechst 33342 at D1 and D2 to obtain population expansion from D1 to D2 (ratio of the total cells at D2 and D1) and the confluency at D2 (ratio of the total cell area to the culture area) ($n = 4$). In order to examine

if small cell number (population composition) affect MEC growth, a total of 350, 700 or 850, 1500 and 3000 of MECs and NMuMGs were seeded in microchannels to achieve equal surface density 75 cells mm⁻² at day 1. The D2/D1 ratio and confluency at D2 for MEC cultures were obtained ($n = 6$).

Subpopulation specific growth promotion activity—The total number and viability of sorted cells were evaluated by using microchannel cell counting device as previously described³⁰ and adjusted to the concentrations required for recombination test. All the experiments on sorted populations were performed by using the straight 2 μ l microchannels except a 3000 MEC control group (12 μ l microchannels). Each of the sorted MEC subpopulations were diluted in SFM to 2000, 1000, 500 cells μ l⁻¹ and 1 : 1 mixed with 300 unsorted MEC (as reporters of growth promotion) and seeded into the 2 μ l microchannels. Unsorted MECs were also seeded into the 2 μ l and 12 μ l microchannels as controls. Replicates in each experiment varied from three to six depending on FACS collection efficiency. Cells were cultured as above. The culture medium in the microchannels was left undisturbed from the start to the end of recombination culture (48 h).

Statistics—Statistics were conducted as an ANOVA, $P < 0.05$. Poisson statistics were used to test the frequency of growth promotion units.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Comparison of volume ratios between macro- and microscale cell culture devices.

Measurements represent the height of the volume of the liquid. Volumes used were chosen based on most commonly used working volumes as follows: (A) 2–5 μ L for microchannels (channel volume without ports), (B) 2–3 ml for tissue culture flask (24 mm²), (C) 600 μ L for transwells (24 well plate with inserts) and (D) 200 μ L for 96-well plate. Devices were filled with red food color dye for visualization purposes.

Fig. 2.

Schematic view of the multiwell co-culture microchannel. The microchannel design has a central chamber with channels running radially to the output track. The side-chambers are connected with the center chamber through 12–17 μm tall diffusion ports. All the chambers, channels and output tracks are 250 μm tall. Gray and black colors represent solid wall (PDMS) and white represents empty space. The diagram at the bottom correlates the number of chambers with the microchannel design. The ports (inlets and outlets) and ceiling are not shown.

Fig. 3.

Plot of concentration with time and table of concentration maps for the conditions listed in the table. The color map on the right side of the table correlates color with observed nominal or normalized concentrations. The simulations are for a ~ 10 kDa molecule by using a diffusion coefficient of $100 \mu\text{m}^2 \text{s}^{-1}$.

Fig. 4. Representative phase images of RAW 264.7 cells at 24 h, 96 h and after TRAP stain in mono-culture and co-culture conditions. Mono-culture for transwells (not shown) was similar to microchambers (shown). Circles are showing a representation of differentiating cells. Scale bar = 20 μ m.

Fig. 5. (A) Representative image of cells in a two-chamber micro-device. (B) Average of total number of differentiating osteoclasts per chamber or well. $P < 0.005$ for transwells and $P < 0.0005$ for microchambers compared to mono-culture control. (C) Average number of differentiated cells per mm^2 . Data was normalized to its own monoculture control. $P < 0.0005$ for microchambers compared to transwells co-culture. Columns are mean of triplicates; error bars represent SD. Data are representative of three independent experiments.

Fig. 6. Bimodal cell growth. (A) Design of microchannel plates. The microchannels from column 1 through column 6 in this design have identical width (0.5 mm) and height (0.25 mm) but decreasing length (60, 30, 15 and 7.5 mm) such that various sample volumes (10, 5, 2.5 and 1.25 μ l) with identical density can be tested. Columns 7–12 are duplicates of columns 1–6. The largest channel occupies columns 1 and 2, each half covering the equivalent of one well area of a 96 well plate, such that larger numbers/volumes of cells can be analyzed in experiments. (B–C) Growth patterns of MECs with different cell numbers but same surface density and volume density. Microchannel plates (A) were coated with matrigel and seeded with MECs (B) and NMuMGs (C) to get 350, 700, 1500 and 3000 cells in each channel with 75 cells mm^{-2} at day 1 (D1). The growth rates of day 2 over day 1 (D2/D1) were plotted against the initial cell numbers. The growth of MECs showed a bimodal distributed growth rates. Non-

parameter statistics showed two groups of samples with high growth rate and with low growth rate existed ($p < 0.05$, $n = 6$).

Fig. 7.

The growth promoting activity of mammary epithelial cell subpopulations. The population expansion rates of the four subpopulations were compared using the population size at day 2 (48 h) normalized by that day 1 (24 h). It showed that there was a 3.6-fold increase of the population in MRU cultures, and a 3.2-fold increase in the CFC but little or no growth in the other cultures ($n = 3$, $p < 0.05$).