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

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Abstract

Interactions between different cell types play critical roles in normal development and disease, but remain challenging to analyse. Here, a co-culture system is described that overcomes many of the limitations of existing methods, is simple to construct and modify, and is compatible with standard cellular and molecular assays.

Multiple different cell types interact through paracrine signalling to direct tissue development and homeostasis. These interactions also play key roles in disease progression; for example, in cancer it is now recognized that the interactions between different cell types in the tumour microenvironment are a key component of disease progression and as such could hold potential therapeutic targets.¹ Specifically, macrophage interactions with tumour cells have been proposed to support tumour growth and metastasis.² Clinically, pro-inflammatory macrophage infiltration into tumors has been correlated with a worse prognosis in ovarian cancer.³ These macrophages are a source of pro-tumorigenic factors such as VEGF, IL-8, and IL-6, which have been shown to initiate angiogenesis, proliferation, and chemoresistance, respectively.^{4, 5} Therefore, in order to identify therapeutic targets that slow or stop tumour progression, it will be necessary to develop *in vitro* models that allow for dynamic cell-cell interactions to be examined.

Current methods to study cell-cell interactions include conditioned media and transwell co-cultures; however, these methods have substantial technical limitations. In conditioned media stimulation, media is collected from one cell type and applied to a second cell type, allowing the researcher to investigate the impact of the soluble factors secreted by cell-type one on cell-type two.⁶ While widely used in biological studies, this method only allows for study of oneway interactions. Additionally, the finite pool of secreted factors in the conditioned media may degrade or be depleted over time, leading to an underestimation of the impact of the interaction. In transwell experiments, cells are seeded on a removeable transwell insert that is then placed inside of a well plate where a second cell type has been

seeded. The transwell insert is porous, allowing for dynamic interactions between the cells via secreted factors; however, transwell cultures require large media volumes (approximately 500 μ L), substantially diluting the secreted factors and potentially muting weak interactions. In addition, transwells require large populations of cells, which is an importation consideration for researchers using isolated primary cells.

To overcome the limitations of these standard techniques, researchers have turned to microfluidics to develop new systems to study cell-cell interactions. Many of these devices are constructed through soft lithography, where micron-sized channels of various geometries are fabricated in polydimethylsulfoxide (PDMS) and cells are cultured within these devices. Devices have been fabricated to study cell-cell interactions between individual cells⁷ and between populations of cells cultured in parallel channels connected via diffusion channels that allow for exchange of secreted factors between the two cell populations.⁸ While each device design addresses at least one key limitation of standard co-culture methods, these devices have not yet gained wide acceptance by biologists due to other limitations. Perhaps most importantly, fabrication of microfluidic devices requires specialized skills and equipment to generate photolithography masks. Additionally, many devices utilize complex injection pumps to seed and treat cells. Current device designs can also increase the variability observed in cell responses; for example, many co-culture devices place cells in parallel channels in the same plane. As a result, the distance between cell types varies across the channel width, setting up gradients of secreted factors. Finally, by design these devices utilize very small numbers of cells. While this is ideal for use with primary cells, the small number of cells available limits the biological assays a researcher is able to easily use.

Given the limitations of the current methods for examining cell-cell interactions *in vitro*, we sought to develop a co-culture device that is fabricated using simple methods (*i.e.*, methods that are accessible to non-specialists), can be modified to investigate various parameters that impact the strength of the cell-cell interaction, maintains uniform cell-cell interactions, and is compatible with standard cellular and molecular assays such as ELISAs, immunofluorescence imaging, western blots, and qRT-PCR.

The co-culture device is composed of three main components, a PDMS barrier ring, a tissue culture substrate and a separate glass coverslip (Fig. 1). Prior to initiating the co-culture, one cell type is cultured within the confines of the PDMS ring placed on a tissue culture substrate, while the second cell type is cultured on the square glass coverslip. In this way, each cell population can be exposed to any pre-treatments of interest in isolation. In addition to the general culture setup on tissue culture plastic/glass, the system can be modified to include 3D gels or adsorbed extracellular matrix proteins on the different components, allowing for flexibility in co-culture substrates. To initiate co-culture, the coverslip containing the second cell-type is simply inverted and placed on top of the first cell-type contained within the PDMS ring. The PDMS ring maintains the two cell populations at a fixed distance, improving the uniformity of secreted factor distribution compared to two cell populations cultured in parallel on the same plane; additionally, PDMS is sufficiently permeable to allow for gas diffusion between the media and surrounding air.⁹ Importantly, by simply removing the coverslip, cells can be separated for individual analysis through assays such as qRT-PCR or western blot.

With this device, we have the capability to test the impact of physical parameters such as cell separation distance and cell density on signalling and phenotypic effects in co-culture. In addition, the device was scaled to fit within a 24 well dish, allowing for multiple conditions to be run in parallel. Using this device, outlined in Fig. 1, Fig. S1, and Supplemental Methods (ESI), we examined interactions between OVCA433 (an ovarian cancer cell line) and differentiated THP-1 (a macrophage-like cell line), demonstrating that the device is compatible with a variety of standard biological assays.

Cells cultured in the co-culture devices had normal morphologies and were viable, with OVCA433 forming monolayers of clustered cells and THP-1 adhering in response to differentiation stimuli (Fig. S2 and S3, ESI). Importantly, cells maintained their physical separation on the two culture substrates, with qRT-PCR results demonstrating the expected high levels of *FCGIRA* in THP-1, but not in OVCA433 (Table S1, ESI). As a result of this physical separation, all cell-cell interactions observed in the device will be mediated by cell-secreted factors. The design parameters of the device concentrate secreted factors by 8-fold compared to a typical 24-well transwell assay, which may allow for identification of analytes that are secreted at low levels. To determine if these soluble factors could be characterized, media was collected from monoculture and co-culture conditions and analysed for the levels of IL-8. IL-8 is a chemokine secreted by both tumour cells and macrophages, and has been shown to influence proliferation and other cellular behaviours.^{10, 11} IL-8 was detected in both monoculture and co-culture conditions, with significantly higher levels in co-culture devices as would be expected for two cell types that both produce the examined factor (Fig. S4, ESI). Importantly, cells can secrete multiple factors simultaneously. To characterize a broader spectrum of these factors, co-cultures could be analysed by multiplex technologies such as the Luminex platform to measure cytokines, chemokines, and growth factors.¹²

We next examined if OVCA433 cells in the co-culture system could sense and respond to the factors secreted by THP-1. We first examined whether this communication could be observed at early times in the form of changes in the signalling network within the tumour cell fraction. Cells were lysed after 4 hours in monoculture or co-culture and examined by western blot for phosphorylation of ERK (Fig. 2). The results demonstrated that OVCA433 cells sense and respond to factors secreted by THP-1, with increased levels of pERK in co-culture. In future studies, multiple pathways could be analysed simultaneously using multiplex methods such as the Luminex platform.¹²

Finally, we determined if the stimuli provided by co-culture with THP-1 was sufficient to influence OVCA433 proliferation and if this response was impacted by the design variables of device separation distance and cell density. After 48 hours in culture, OVCA433 were stained with Click-iT EdU and imaged by microscopy to determine the percentage of proliferating cells (Fig. 3). In both 250 μ m and 500 μ m devices, OVCA433 had significantly higher proliferation when co-cultured with THP-1 compared to when maintained in monoculture.

Interestingly, cells cultured in 500 μ m devices showed a dosedependent response with respect to THP-1 number, while cells cultured in 250 μ m devices were insensitive to this

difference. Increasing the device height also increases the volume of media in the device, which will change the amount of nutrients available and the concentration of secreted factors. Proliferation in monoculture conditions was unaffected by device size, indicating that the change in media volume did not impact baseline cell behaviour. Additionally, cells in both devices achieved the same maximum level of proliferation, further indicating nutrient levels did not constrain this cell behaviour. The lack of a density-dependent response in the 250 μm device may indicate that the secreted factors were concentrated enough by the small volume of the device that even the lower cell density was able to induce the maximum proliferation. In the larger 500 μm device, the increased volume resulted in a dilution of secreted factors and the lower density of THP-1 cells was unable to induce the maximum level of proliferation. By increasing the THP-1 density, the level of secreted factors was sufficiently elevated and the maximal proliferation response in OVCA433 was observed. These results indicate that by varying the device design it may be possible to use fewer cells while still maintaining a strong cell-cell interaction, which will be important when using limited primary cell samples.

In conclusion, the device described here provides a useful tool to characterize cell-cell interactions, as it can be easily adapted to examine these interactions in a variety of biological contexts. Our results demonstrated that the device is compatible with standard cellular and molecular assays including qRT-PCR, western blot, ELISA, and imaging, and we expect that the samples generated from these co-cultures will be compatible with high-throughput assays that can simultaneously analyse multiple targets in the same sample. Importantly, the device could be easily modified to incorporate additional inputs. For example, in this study OVCA433 were cultured on adsorbed extracellular matrix, but by increasing the height of the PDMS ring to account for the increase in substrate height, cells could be cultured in or on top of three-dimensional extracellular matrix gels while maintaining a fixed distance to cells cultured on the other substrate. While this modification would lead to less concentration of the soluble factors, co-cultures with gels that were 100 μm thick or less would still be 5X more concentrated than transwell cultures. Alternatively, soluble stimuli such as growth factors and cytokines can be easily spiked into the co-culture media to add another layer of complexity. While this study focused on signalling between two adherent cell types, the platform could be modified to study the interactions between adherent cells and non-adherent cells, with the adherent population cultured on the coverslip and the suspension cells occupying the space within the PDMS ring. Additionally, by combining and separating the two culture surfaces at defined times, researchers could easily examine the dynamics of cell-cell interactions. For example, using this device it would be possible to determine if tumour cell response to macrophages is influenced by prior exposure to other cells in the tumour microenvironment such as fibroblasts.¹³ Finally, by adapting the design parameters, the device can be used with smaller samples such as primary cells isolated from patients in order to better understand the unique interactions within a particular tumour. Through the study of cell-cell interactions in simple, but flexible, co-culture devices such as this, it may be possible to identify new therapeutic targets to treat complex multi-cellular diseases such as cancer.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1.

Co-culture device design. Shown are the (A) top profile and (B) cross-sectional views. By controlling the height of the PDMS ring, cell separation was varied from 250 μm to 500 μm . In our experimental design, teal cells represent ovarian cancer cells (OVCA433) grown on gelatin adsorbed onto tissue culture plastic, and white cells represent the monocytic cell line (THP-1) differentiated on coverslips.

Fig. 2.

The OVCA433 cell signalling network was activated by co-culture with THP-1 compared to OVCA433 monoculture (Mono). Increasing the density of THP-1 macrophages from 150,000 to 300,000 THP-1 (150K, 300K respectively) increased the level of phosphorylated ERK. Shown is a representative blot for the 500 μ m device. Bands were quantified by densitometry and normalized to tubulin; quantified data normalized to concurrently analysed monoculture condition (avg $\pm$ SEM, n=3).

Fig. 3.

OVCA433 proliferation was elevated following 48 hours of co-culture with THP-1. OVCA433 were cultured in monoculture (Mono) or with 150,000 or 300,000 THP-1 (150K, 300K respectively). Data plotted as average $\pm$ SEM, different letters indicate significantly different than other conditions for the same device size, $p < 0.05$ ($n=4$).