A high-throughput platform for stem cell niche co-cultures and downstream gene expression analysis

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Stem cells reside in ‘niches’, where support cells provide critical signalling for tissue renewal. Culture methods mimic niche conditions and support the growth of stem cells in vitro. However, current functional assays preclude statistically meaningful studies of clonal stem cells, stem cell–niche interactions, and genetic analysis of single cells and their organoid progeny. Here, we describe a ‘microraft array’ (MRA) that facilitates high-throughput clonogenic culture and computational identification of single intestinal stem cells (ISCs) and niche cells. We use MRAs to demonstrate that Paneth cells, a known ISC niche component, enhance organoid formation in a contact-dependent manner. MRAs facilitate retrieval of early enteroids for quantitative PCR to correlate functional properties, such as enteroid morphology, with differences in gene expression. MRAs have broad applicability to assaying stem cell–niche interactions and organoid development, and serve as a high-throughput culture platform to interrogate gene expression at early stages of stem cell fate choices.

Understanding how somatic stem cells self-renew and differentiate to produce the functional cells of their resident tissue is essential for determining the mechanisms underlying a broad range of issues related to human health and disease. The intestinal epithelium undergoes one of the most rapid rates of renewal of any mammalian tissue, making it an excellent model system for understanding stem-cell-driven physiological renewal. Although in vivo lineage tracing remains an important technique for the analysis of stem cell behaviour, developments in primary stem cell culture have expanded the stem cell biologist’s toolkit to include powerful, complementary in vitro assays¹.

Lgr5/high or Sox9/low ISCs are capable of forming ‘enteroid’ structures in vitro, demonstrating multipotency and self-renewal²–⁴. In vivo, ISCs are closely associated with Paneth cells (PCs), which function as niche cells and express soluble and insoluble factors associated with stemness, such as Wnt and Notch ligands⁵,⁶. PCs have been shown to increase the efficiency of enteroid formation by ISCs in vitro⁶. However, these studies relied on the co-culture of hundreds of ISCs with hundreds of PCs, and may not reflect physiological normal conditions in single intestinal crypts, where much smaller numbers of ISCs (~15) and PCs (~8) interact⁷–⁸.

Technical limitations hinder efficient functional in vitro studies of ISC–niche interactions. Furthermore, the field lacks a robust assay to study ISCs at the clonal level, a tool that has driven the understanding of stem cell niches in the haematopoietic system and mammary glands⁹,¹⁰. Array-based technologies are emerging as a powerful method to study the functional characteristics of single and/or small numbers of stem cells in the haematopoietic system, and hold similar promise for epithelial tissues such as the intestine¹¹. In the present study, we describe a platform to study large numbers of single ISCs simultaneously, either at the clonal level or in the presence of niche cells. Microfabricated culture arrays modified for long-term three-dimensional culture are used to capture and functionally assay clonal ISCs and ISC–niche cell co-cultures, effectively providing a platform for high-throughput niche reconstruction using primary stem and niche cells. Finally, the platform allows for efficient retrieval of single ISCs and developed enteroids for downstream gene expression analysis at different time points.

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Figure 1  Modified MRAs are compatible with long-term culture of primary ISCs. (a) MRAs consist of polystyrene ‘raft’-lined PDMS microwells mounted to a glass slide with a thin layer of PAA, and attached to a cassette containing media chambers. (b) Cassettes can be scaled to divide a single MRA into 2 or 4 separate media reservoirs. (c) Microwells are 200 µm², arranged in a grid, with the physical addresses stamped into the PDMS at 5-microwell intervals. Scale bar represents 600 µm. (d) ISCs are isolated from Sox9EGFP: CAGDsRed transgenic mice, which express DsRed throughout the intestinal epithelium. Scale bars represent 50 µm. (e) Isolated cells are seeded into microwells through centrifugation in media, and then overlaid with Matrigel. (f–h) ISCs are randomly distributed across arrays immediately after plating (f), with some microwells containing single ISCs (g, arrow), and others containing multiple ISCs (h, arrowhead). (i–k) Imaging of the same array at 48 h reveals widespread enteroid formation (i), with typical cystic growth of early structures (j,k). Scale bars for f–k represent 600 µm. (l,m) Long-term culture experiments demonstrate that developed ISCs grow out from their original microwells over the course of 4 weeks (l; black arrowhead indicates well address corresponding with MRA position; white arrowhead indicates enteroid at 120 h), and can be sustained in the array format for 8 weeks or longer (m; upper two wells are empty in this image). Scale bars represent 200 µm.

RESULTS

MRAs are adaptable to cell culture and imaging

We reasoned that previously described polydimethylsiloxane (PDMS)/polystyrene MRAs could be used to isolate and culture single ISCs in three-dimensional extracellular matrices12 (Fig. 1a–c). As ISCs require several days to develop into enteroids, MRAs had to be amenable to media changes3,4. To meet these requirements, polycarbonate cassettes, with dividers to create multiple media reservoirs, were bonded to MRAs (Fig. 1a,b and Supplementary Fig. 1H). Cassettes were fabricated with two or four culture chambers (~2,500 or 5,000 microwells per culture chamber, respectively, Fig. 1b). Physical well addresses, stamped into PDMS at 5-microwell intervals, were included in the array design to allow for tracking of single cells and enteroids across many time points (Fig. 1c).

Tile-scanning microscopy produced high-resolution images of whole MRAs for downstream analysis (Fig. 1f,i and Supplementary Fig. 2).

MRA support long-term, clonal ISC culture

To facilitate tracking of isolated cells in MRAs, Sox9EGFP mice were crossed to CAGDsRed mice, which express the DsRed fluorescent transgene ubiquitously across all cell and tissue types3,13 (Fig. 1d). Sox9EGFP:CAGDsRed ISCs were plated in a single culture chamber of a two-chamber MRA, randomly seeded into microwells by centrifugation, and overlaid with Matrigel and ISC-supporting growth factors (Fig. 1e)6,14. This resulted in a random distribution of ISCs across MRAs, with microwells containing one, multiple or no ISCs (Fig. 1f–h). For biocompatibility experiments, we used high-efficiency ISC culture methods that drive high Wnt and Notch signalling14.
To rapidly assess the cellular contents in each of the microwells, we developed an image analysis computational pipeline that identifies objects and applies filters based on the size and shape profiles of known ISCs is used to analyse segmented microwell images. If the number of objects initially identified by CellProfiler is equal to those that pass filtering parameters, the microwell passes inclusion criteria (g, top microwell), otherwise it is excluded as debris containing or empty (g, middle and bottom microwells). Scale bars represent 50 µm. (d–f) To distinguish between true ISCs and debris/noise, visually scored wells were analysed by CellProfiler to determine the distribution profiles of target events (ISC, blue) and debris (red) for the variables compactness, form factor and eccentricity. (g) A new CellProfiler pipeline that identifies objects and applies filters based on the size and shape profiles of known ISCs is used to analyse segmented microwell images. If the number of objects initially identified by CellProfiler is equal to those that pass filtering parameters, the microwell passes inclusion criteria (g, top microwell), otherwise it is excluded as debris containing or empty (g, middle and bottom microwells). Scale bars represent 50 µm. (h,i) Application of the optimized pipeline to randomly selected microwell images reveals an overall accuracy of 99.87%, 71.53%, 46.51% and 29.03% for wells containing 1, 2, 3 and 4 ISCs, respectively (n=2, 2,544 randomly selected microwells).

Figure 2 Software-assisted post hoc analysis identifies initial well contents of MRA culture. (a) A defined workflow facilitates post hoc image analysis to identify well contents in MRA cultures. (b) Tile-scanned images are stitched together to form a single composite image, which is then segmented into individual microwell images, each with a unique address corresponding to its physical position in the array. Scale bars represent 100 µm. (c) Visual examination of individual microwells reveals the presence of debris and fluorescent glare as well as ISCs. Scale bars represent 50 µm. (d–f) To distinguish between true ISCs and debris/noise, visually scored wells were analysed by CellProfiler to determine the distribution profiles of target events (ISC, blue) and debris (red) for the variables compactness, form factor and eccentricity. (g) A new CellProfiler pipeline that identifies objects and applies filters based on the size and shape profiles of known ISCs is used to analyse segmented microwell images. If the number of objects initially identified by CellProfiler is equal to those that pass filtering parameters, the microwell passes inclusion criteria (g, top microwell), otherwise it is excluded as debris containing or empty (g, middle and bottom microwells). Scale bars represent 50 µm. (h,i) Application of the optimized pipeline to randomly selected microwell images reveals an overall accuracy of 99.87%, 71.53%, 46.51% and 29.03% for wells containing 1, 2, 3 and 4 ISCs, respectively (n=2, 2,544 randomly selected microwells).

To provide proof-of-concept for stem cell niche experiments using MRAs, we sought to co-culture ISCs and PCs to assess clonal and...
Figure 3  Single-cell qPCR confirms PC purity. To assess non-PC contamination in CD24\textsuperscript{High}:SSC\textsuperscript{High}:Sox9\textsuperscript{EGFP\_neg} populations at the single-cell level, we isolated, lysed and processed 96 individual PCs for cDNA using the Fluidigm C1 microfluidics-based platform. (a) Single cells ‘captured’ on the microfluidics chip contained granules and were morphologically consistent with PCs before cell lysis. Scale bars represent 10 µm. (b) Gene expression analyses indicate that single cells are enriched for PC markers and do not express ISC or enteroendocrine markers. (c) All cells expressed the PC-specific transcript Defcr-rs. Furthermore, isolated cells variably express delta/serrate/lag-2 ligands associated with ISC niche function (n=96 PCs).

PC-influenced enteroid formation \textit{in vitro}. Previous studies have isolated PCs by FACS of CD24\textsuperscript{High}:SSC\textsuperscript{High} populations\textsuperscript{5}. However, CD24 is also expressed on ISCs, transit-amplifying progenitors and enteroendocrine cells\textsuperscript{3,14,16–18}. As the experimental approach of MRA cultures examines events on a ‘per well’ basis, it was critical to refine isolation procedures for PCs to meet purity requirements of clonal and microscale co-cultures and avoid artefactual results due to contaminating non-PC cell types. Previous characterization of the Sox9\textsuperscript{EGFP} mouse model demonstrated that Sox9 is expressed at different levels in ISCs, progenitors and enteroendocrine cells, but that the Sox9\textsuperscript{EGFP} transgene is preferentially silenced in PCs (ref. 7).

We exploited this property to isolate a highly pure population of PCs by FACS exclusion of Sox9\textsuperscript{EGFP} populations. PCs were FACS-isolated using CD24\textsuperscript{High}:SSC\textsuperscript{High} parameters, and the additional exclusion of all Sox9\textsuperscript{EGFP}--positive cells (Supplementary Fig. 3B,C). Gene expression analysis revealed a twofold increase in lysozyme expression, a fivefold decrease in Lgr5 expression, and a greater than tenfold decrease in Chga using Sox9\textsuperscript{EGFP} exclusion, indicating de-enrichment of ISCs and enteroendocrine cells (Supplementary Fig. 4).

PC purity was further examined by single-cell qPCR on 96 Sox9\textsuperscript{EGFP\_neg}–excluded PCs. Sox9\textsuperscript{EGFP\_neg}:CD24\textsuperscript{High}:SSC\textsuperscript{High} cells were morphologically consistent with PCs, exhibiting typical granulation
and large cell size (Fig. 3a). Importantly, all isolated cells expressed the PC-related gene *Defcr-rs* (Fig. 3b,c). Lgr5 was detected in some cells, consistent with recent reports on Lgr5 expression in a subset of PCs in *in vivo* (Fig. 3c). Similarly, although all cells were negative for the enteroendocrine transcript Tac1, expression of Chga was observed in a single PC, consistent with reports of PC progenitors expressing this enteroendocrine marker (Fig. 3c).

Emerging evidence demonstrates that multiple intestinal progenitor cell populations are capable of dedifferentiating and functioning as ISCs *in vitro* and *in vivo*.19 – 21 To address the possibility that enteroids might form from early progenitors in the PC population, 2,810 individual PCs were examined in subsequent *in vitro* experiments. We only once observed enteroid production by a cell isolated using Sox9EGFP<sup>−/−</sup>:CD24<sup>+</sup>:SSC<sup>−/−</sup> parameters (0.04%). Together, these data indicate that Sox9<sup>−/−</sup>:CD24<sup>+</sup>:SSC<sup>−/−</sup> populations are genotypically and phenotypically consistent with mature PCs.

**PC–ISC contact is required for increased enteroid formation *in vitro***

As previous studies have speculated that PC-secreted Wnts are responsible for enhancing ISC growth *in vitro*, ISC–PC co-culture experiments were carried out in the absence of exogenous Wnt, to avoid ‘masking’ the potential impact of PCs on enteroid formation.14– 16 The GSK3β inhibitor CHIR99021, a Wnt agonist, was also excluded from co-culture experiments. To address the possibility that a PC in
one microwell might affect the growth of an ISC in an adjacent, but separate microwell, we modelled diffusion dynamics of cell-secreted molecules in MRAs. Diffusion between microwells was deemed negligible under models relying on liberal rates of diffusion and decay (Supplementary Note 1).

We reasoned that increased numbers of PCs would result in increased Wnt secretion and ISC growth, and examined microwells with initial contents consisting of any combination of 1–5 ISCs and 0–2 PCs. ISCs were isolated from Sox9<sup>EGFP</sup> or Lgr5<sup>EGFP</sup> mice and PCs from Sox9<sup>EGFP</sup>:Lgr5<sup>DsRed</sup> mice (Fig. 4a). DsRed fluorescence was used as a readout of ISC contamination in PC populations. Replicate experiments were conducted for each ISC biomarker to increase sample size per ISC–PC combination, and data were analysed from four total MRA experiments, consisting of 4,830 data points, each corresponding to an individual microwell containing any combination of ISCs and PCs (Supplementary Table 1). Surprisingly, examination of survival percentages across all combinations of ISCs and PCs revealed no statistically significant trends, regardless of whether ISCs were isolated using Sox9<sup>EGFP</sup> or Lgr5<sup>EGFP</sup> (Fig. 4b,c). To investigate the overall effect of PCs on enteroid survival, we next analysed the percentage of enteroids formed in microwells containing any number of ISCs or any number of ISCs with any number of PCs (Fig. 4d). These analyses also failed to produce statistically significant differences between ISC-alone and ISC–PC microwells, suggesting that PC presence alone is insufficient to increase enteroid formation in vitro at physiologically relevant numbers.

Previous studies have suggested that cell-to-cell contact between ISCs and PCs may influence enteroid formation, but this has not been formally tested by comparison between touching and non-touching ISCs and PCs (ref. 6). Using the same data generated in our ISC–PC co-culture experiments, we reanalysed initial MRA contents to classify microwells by cell-to-cell contacts at t = 0 h and correlated this status with enteroid formation outcome (Fig. 4e). This comparison yielded results with overall statistical significance (conditional logistic regression, \( P = 0.0282 \)), indicating that ISCs in direct contact with PCs are more likely to form enteroids than ISCs alone, or ISC–PC wells that are not in direct contact. Interestingly, ISC–ISC contact events were also more likely to form enteroids than non-touching ISCs, suggesting that cell-to-cell contact between two or more ISCs may also positively influence enteroid formation. As expected, PC–PC contact did not
result in the formation of enteroids, consistent with the post-mitotic status of PCs in vivo and the high level of purity observed in PC populations (Fig. 4g).

**Single cells do not form de novo contacts after plating**
The contact-dependent effects on enteroid formation prompted us to examine whether de novo cell–cell contacts occurred after initial cell–cell contacts after plating. High-throughput qPCR was used to interrogate gene expression from the contents in each raft. (b) A heat map of dCT values demonstrates high levels of active ISC biomarkers at t=0. Later time points demonstrate an increase in secretory progenitor markers followed by differentiated cell markers consistent with a progression towards fully mature enteroids at 240 h post-plating. (c) Violin plots of Ct values show distribution of gene expression levels during each day of enteroid development. (d) Early enteroids adopt a cystic or columnar morphology; 48 h shown here. (e) Quantification of cell width in enteroid monolayers validates the morphological difference between cystic and columnar enteroids (n=20 cystic and 17 columnar enteroids at 24 h; 15 cystic and 15 columnar at 48 h; unpaired t-test; *P=8.1×10^{-3}; **P=0.0003). The boxes represent the upper and lower quartiles and the whiskers represent the upper and lower extremes. (f) PCA demonstrates that cystic and columnar enteroids become increasingly genotypically distinct at 48 h. (g) Cystic enteroids express significantly higher levels of cyclins Ccnb1, Ccnb1, and Ccne1, as well as higher levels of Lgr5, Ascl2, and Wnt receptor Fzd6, suggesting that cystic morphology is associated with active proliferation (n=13 cystic and 14 columnar enteroids at 24 h; 13 cystic and 12 columnar at 48 h; different letters represent statistical significance; one-way ANOVA, P < 0.05. Exact P values are presented in Supplementary Table 3). All scale bars represent 50 μm.

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**Figure 6** Microraft retrieval facilitates gene expression analysis of enteroid development and morphology. (a) Lgr5<sup>Cre</sup> cells were seeded in MRAs containing magnetic polystyrene rafts. Individual rafts with either single cells or developing enteroids were collected every 24 h for 4 days and again at day 10 (240 h) post-plating. High-throughput qPCR was used to examine whether de novo contacts after initial cell–cell contacts occurred after plating. (b) A heat map of dCT values demonstrates high levels of active ISC biomarkers at t=0. Later time points demonstrate an increase in secretory progenitor markers followed by differentiated cell markers consistent with a progression towards fully mature enteroids at 240 h post-plating. (c) Violin plots of Ct values show distribution of gene expression levels during each day of enteroid development. (d) Early enteroids adopt a cystic or columnar morphology; 48 h shown here. (e) Quantification of cell width in enteroid monolayers validates the morphological difference between cystic and columnar enteroids (n=20 cystic and 17 columnar enteroids at 24 h; 15 cystic and 15 columnar at 48 h; unpaired t-test; *P=8.1×10^{-3}; **P=0.0003). The boxes represent the upper and lower quartiles and the whiskers represent the upper and lower extremes. (f) PCA demonstrates that cystic and columnar enteroids become increasingly genotypically distinct at 48 h. (g) Cystic enteroids express significantly higher levels of cyclins Ccnb1, Ccnb1, and Ccne1, as well as higher levels of Lgr5, Ascl2, and Wnt receptor Fzd6, suggesting that cystic morphology is associated with active proliferation (n=13 cystic and 14 columnar enteroids at 24 h; 13 cystic and 12 columnar at 48 h; different letters represent statistical significance; one-way ANOVA, P < 0.05. Exact P values are presented in Supplementary Table 3). All scale bars represent 50 μm.
Figure 7 Culture conditions drive transcriptional changes in single Lgr5* cells. Single Lgr5* ISCs were sorted directly into lysis buffer, or plated in MRAs with and without growth factors (EGF, Noggin, Jagged-1, CHIR99021, LY2157299, and Thiazovivin) for 2 h. (a) Single Lgr5* cells clustered according to their treatment group by PCA. (b) Exposure to growth factors resulted in a significant upregulation of Lgr5 as well as a significant downregulation of Bmi1 and Lrig. No significant changes were detected for Hopx or Tert, regardless of treatment, suggesting that co-expression of Lgr5 with these markers is variable at the single-cell level, both in vivo and in very early Matrigel cultures. Interestingly, despite elevated expression of Lgr5 in the presence of growth factors, these conditions also elicited a downregulation of Olfm4 in a subset of single ISCs. We also noted that elevated Lyz2 expression was driven by growth factor conditions, and may indicate an early culture-dependent divergence from ISC-associated transcriptional programs in a subset of Lgr5* cells (n = 36 cells per condition, different letters represent statistical significance; one-way ANOVA, P < 0.05; exact P values presented in Supplementary Table 3).

Previous studies demonstrate significant enteroid movement and merging in vitro. To further examine cell movement in the MRA platform, we performed time-lapse imaging of microwells. Single cells demonstrated appreciable movement as they developed into enteroids over the first 24 h of culture (Supplementary Video 1). Larger clumps of cells merged into single enteroids, suggesting that the movement and merging of ISCs in vitro may be influenced by cell number or heterogeneity within populations (Supplementary Video 2).

**Modified MRAs facilitate genetic analysis of enteroid development**

Microscale culture systems for primary tissues are potentially powerful tools for high-throughput screening, drug discovery and personalized medicine. The power of such platforms is enhanced when differences in phenotype, such as organoid size or morphology, can be correlated with differences in gene expression. We reasoned that MRAs could be used to examine genetic heterogeneity in single ISCs, early enteroids (2–3 cell) and developed enteroids. To facilitate single ISC and enteroid retrieval for downstream analysis, MRAs were modified so that standard polystyrene rafts at the bottom of each microwell were replaced with magnetized rafts, as recently described22 (Supplementary Fig. 5A). A raft release device was fitted to a ×10 objective to liberate the rafts from the PDMS wells, and a magnetic wand was used to retrieve rafts for transfer to RNA lysis buffer (Supplementary Fig. 5A–D). Rafts containing single Lgr5* ISCs or enteroids were retrieved and complementary DNA was generated from a total of 192 rafts, representing single ISCs, early enteroids and developed enteroids, across 6 time points (Fig. 6a and Supplementary Fig. 5G). High-throughput qPCR was then used to assess the expression of 20 genes associated with ISCs, progenitors and post-mitotic lineages (Fig. 6b,c). Representative analysis of Sox9, Hopx and Ccd1 in empty rafts (n = 12) produced a only a single Ct value for Sox9 (Ct = 36.97 of 40 cycles), but did not produce a visible band by gel electrophoresis, demonstrating negligible background in retrieved rafts.

As expected, Lgr5 messenger RNA was highly expressed by single ISCs, demonstrating population purity (Fig. 6c). Although other crypt-base columnar ISC markers Olfm4 and Smoc2 were strongly detected in single ISCs, a number of cells were negative for putative ‘+4’ ISC markers Bmi1, Hopx and Tert, contrary to studies conducted on populations of Lgr5* cells. To determine whether this finding...
TECHNICAL REPORT

was reflective of a transcriptional response to ISC culture conditions, we compared gene expression profiles of single Lgr5\textsuperscript{shb} cells sorted directly into lysis buffer with those exposed to Matrigel culture with and without growth factors/small molecules (EGF, Noggin, Jagged-1, CHIR99021, LY2157299 and Thiazovivin). Although growth factors specifically elicited upregulation of Lgr5, Olfm4 and Lyz2 in single cells, Lgr5\textsuperscript{shb} ISCs exhibited heterogeneity in terms of Bmi1, Hopx and Tert regardless of exposure to culture conditions (Fig. 7). Together, these data demonstrate that ISC culture conditions upregulate ISC-associated genes and Lyz2 in single cells and suggest transcriptional heterogeneity for Bmi1, Hopx and Tert in Lgr5\textsuperscript{shb} cells.

Gene expression changes in developing enteroids were consistent with cellular differentiation (Fig. 6c). Hes1 was enriched at 24 h post-plating, suggesting initiation of Notch signalling, which was recently shown to be important for progenitor fate decisions\textsuperscript{27}. Subsequently, Dll1 expression initiates in most enteroids at 48 h, consistent with the emergence of secretory progenitor populations\textsuperscript{21,28}. Early expression of Sis seems to be coincident with upregulation of Hes1, a known driver of enterocyte fate, and Muc2 and Chga are upregulated at later time points, coincident with increased Atoh1 expression\textsuperscript{29,30}. By 10 days in culture, enteroids are enriched for the expression of transcripts associated with absorptive enterocytes (Sis), goblet cells (Muc2), PCs (Lyz, Defr-rs1) and endocrine cells (Chga, Chgb), consistent with a fully developed organoid phenotype\textsuperscript{6} (Fig. 6b,c).

MRAs reveal genetic differences associated with enteroid phenotype

Although enteroid development demonstrated an expected pattern of gene expression associated with differentiation, we noted bimodal distribution of several genes across the developmental timeline, suggesting heterogeneity in enteroid populations (Fig. 6c). To examine this, we chose two previously observed morphologies, cystic and columnar, and collected enteroids for gene expression analysis at 24 h and 48 h, based on these morphologies\textsuperscript{5} (Fig. 6d). The cellular ‘monolayer’ of cystic enteroids was determined to be significantly thinner than that of columnar enteroids, confirming the morphological difference (Fig. 6c). As cystic enteroid morphology is observed coincident with increased Wnt signalling, we expanded our analysis to include genes associated with Wnt and Notch ligands, including Wnt and Notch ligands\textsuperscript{5,6}. In the present study, we examine the impact of PC presence and contact with ISCs on enteroid formation. Interestingly, we find that PC presence alone is not predictive of enteroid formation. Rather, direct cell-to-cell contact between ISCs and PCs is required for enhanced ISC growth, suggesting that insoluble or very short-range soluble signals support ISCs in vitro. These results support \textit{in vivo} findings that stemness is most strongly correlated in cells that exist in intimate contact with PCs, and provide insight into the functional role of PCs in maintaining stemness\textsuperscript{6}. Importantly, the MRA platform was critical in testing dose and contact dependency of PCs in a microscale format.

In addition to providing a robust platform for studying functional outcomes of clonal stem cells or stem cell niche cultures, MRAs allow for the retrieval of single cells and organoids for downstream analysis. This ability enhances the power of MRAs by integrating high-throughput functional and genetic/genomic data. We used magnetic MRAs in combination with microfluidic qPCR technologies to assess gene expression in single cells and small populations, such as 2–3-cell enteroids. This allowed for observations of gene expression changes over the course of enteroid development, as well as proof-of-principle analysis of morphological differences in early enteroids. These data experimentally reinforce a previously observed correlation between cystic enteroid morphology and increased Wnt signalling, by demonstrating that cystic enteroids express higher levels of genes associated with stemness and active proliferation\textsuperscript{5}. Together with methodology for low-input RNA-seq, MRAs potentiate screening of genetic mutants and drugs/small molecules at the genomic level\textsuperscript{32}.

Array-based stem cell culture platforms are growing in use and present an efficient and cost-effective alternative to conventional cell culture\textsuperscript{33,34}. However, most platforms are not amenable to long-term cultures, such as required for the development of ISC-derived enteroids and other self-assembled, stem-cell-derived organoids\textsuperscript{35–38}. MRAs facilitate the culture of thousands of primary stem cells over many days and weeks as well as high-throughput reconstitution of the stem cell niche at physiologically relevant cell numbers. The power of the MRA platform is further highlighted by the ability to retrospectively ‘mine’ existing high-throughput MRA data sets to test new hypotheses, such as niche cell dose dependency, cell–cell contact and cellular movement within microwells.

MRAs provide robust methodology for screening candidate mitogens and morphogens for their effect on enteroid formation and development, and the study of other ISC niche cells, such as pericryptal myofibroblasts, endothelial cells and neurons. Furthermore, MRAs have broad potential for \textit{in vitro} reconstruction of stem cell niches across a range of cell and tissue types, especially those that require three-dimensional extracellular matrices. The ability to easily retrieve cell–niche interactions \textit{in vivo}. As an alternative approach, \textit{in vitro} techniques that rely on the co-culture of isolated stem and niche cell populations have recently been used to assess the impact of individual niche components on stem cell behaviour\textsuperscript{6,31}. However, these methods commonly rely on large numbers of cells, which may not reflect physiologically relevant niche behaviour, and are not amenable to high-throughput studies. Here, we present an array-based platform that facilitates the study of thousands of isolated stem and niche cells.

PCs express soluble and insoluble ISC niche signalling components, including Wnt and Notch ligands\textsuperscript{5,6}. In the present study, we examine the impact of PC presence and contact with ISCs on enteroid formation. Interestingly, we find that PC presence alone is not predictive of enteroid formation. Rather, direct cell-to-cell contact between ISCs and PCs is required for enhanced ISC growth, suggesting that insoluble or very short-range soluble signals support ISCs in vitro. These results support \textit{in vivo} findings that stemness is most strongly correlated in cells that exist in intimate contact with PCs, and provide insight into the functional role of PCs in maintaining stemness\textsuperscript{6}. Importantly, the MRA platform was critical in testing dose and contact dependency of PCs in a microscale format.

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MRAs provide robust methodology for screening candidate mitogens and morphogens for their effect on enteroid formation and development, and the study of other ISC niche cells, such as pericryptal myofibroblasts, endothelial cells and neurons. Furthermore, MRAs have broad potential for \textit{in vitro} reconstruction of stem cell niches across a range of cell and tissue types, especially those that require three-dimensional extracellular matrices. The ability to easily retrieve
a high replicate number of organoids early in their development allows investigators to associate functional observations with dynamic changes at the genetic and transcriptomic level, facilitating next-generation forward genetic screens in primary stem cells.

**METHODS**

Methods and any associated references are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

A.D.G. and S.T.M. conceived and designed experiments. I.A.W. developed and optimized image analysis algorithms and analysed data with M.J.J., X.F.L., R.J.L. and T.G. P. conducted statistical analysis of enteroid formation and modelling with N.L.A. F.W. and L.L. developed and provided methodology for highly efficient enteroid culture. J.A.G. conducted statistical analysis of enteroid formation assays. A.D.G. and S.T.M. wrote the manuscript with critical insight and commentary regarding array fabrication and analysis; D. Trotier for technical assistance with enteroid retrieval and graphics support; P. K. Lund, S. Henning (SH) and C. Dekaney for useful discussions and critical review of the manuscript. A.D.G. received partial salary support from U01 DK085541 (SH). This work was financially supported by the National Institutes of Health R01 DK091427 (S.T.M.), R03 EB013803 (Y.W/S.T.M.), R01 EB012549 (N.L.A.), Small Business Innovation Research R44 GM106421 (S.T.M./Y.W.), U01 DK085507-01 (L.L.), University Cancer Research Fund of the University of North Carolina (S.T.M./N.L.A.), and the Center for Gastrointestinal Biology and Disease P30 DK034987 (S.T.M., Y.W., I.A.G.). LL is a member of the Intestinal Stem Cell Consortium, supported by NIDDK and NIAID. A.D.G. was supported by a UNC Graduate School Dissertation Completion Fellowship.

**COMPETING FINANCIAL INTERESTS**

N.L.A., C.E.S., Y.W. and S.T.M. disclose a financial interest in Cell Microsystems. From all co-authors. S.T.M. initiated and supervised the project.

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METHODS

Mice/phenotyping. Sox9CreERT transgenic mice were originally generated by the GENSAT Brain Atlas Project, and are maintained on an outbred CD-1 background. Lgr5 CreERT male and female mice were used for all other experiments requiring constitutive expression of DsRed in isolated ISCs, heterozygous Sox9CreERT mice were bred to homozygous CAG-CAG-tdTomato mice to produce Sox9CreERT::CAG-CAG-tdTomato offspring heterozygous for both alleles. Sox9CreERT::CAG-CAG-tdTomato were phenotyped by examining tail snips taken at ~10 days postnatal for EGFP and DsRed fluorescence by epithelium fluorescent microscopy. Lgr5CreERT::tdTomato mice were genotyped by previously described PCR protocols. All experiments were conducted on adult mice between 8 and 16 weeks of age. All protocols for animal use were reviewed and approved by the University of North Carolina Institutional Animal Care and Use Committee.

Plating ISCs in MRAs. Isolated ISCs and PCs were plated in MRAs (Cell Microsystems) at ratios of ~1:5 cells per microwell for ISCs and ~1 cell per 2 microwells for PCs. Sorted cells were added to array reservoirs in ISC Sort/Culture Media and cells were seeded into microwells by centrifugation at 51g for 5 min at 4°C. After centrifugation, medium was gently aspirated and arrays were overlaid with 600 µl (2 reservoir array) or 200 µl (4 reservoir array) Matrigel and growth factors. Arrays were centrifuged a second time at 51g for 5 min at 4°C, to recapture any cells displaced by the addition of Matrigel. Matrigel was allowed to polymerize for 30 min at 37°C before being overlaid with 1 ml (2 reservoir array) or 0.5 ml (4 reservoir array) ISC Sort/Culture Media. Matrigel and growth factors were overlaid with 600 µl (2 reservoir array) or 200 µl (4 reservoir array) ISC Sort/Culture Media. Matrigel and growth factors were overlaid with 600 µl (2 reservoir array) or 200 µl (4 reservoir array) ISC Sort/Culture Media. Matrigel and growth factors were overlaid with 600 µl (2 reservoir array) or 200 µl (4 reservoir array) ISC Sort/Culture Media. Matrigel and growth factors were overlaid with 600 µl (2 reservoir array) or 200 µl (4 reservoir array) ISC Sort/Culture Media. Matrigel and growth factors were overlaid with 600 µl (2 reservoir array) or 200 µl (4 reservoir array) ISC Sort/Culture Media for ISC/PC co-culture experiments, the following growth factors were used: 1 µM Jagged-1 peptide, 50 ng ml⁻¹ RSPO1 (AnaSpec), 750 ng ml⁻¹ Noggin and 1 µg ml⁻¹ Rspo1 (R&D). Medium was changed every four days, and no CHIR99021 or Thiazovivin was used past initial plating. For ISC/PC co-culture experiments, growth factors, minus Jagged-1 peptide and Y27632, were supplemented every two days, and medium was changed every four days, as previously described[43].

Image acquisition, stitching and segmenting. Microwell arrays were tile-scanned in bright-field, GFP and DsRed wavebands using an automated stage and the Scan Slide function in the Metamorph Imaging Suite (Molecular Devices) immediately after plating and overlaying media (Supplementary Fig. 3B). Arrays were housed in a physiological chamber mounted on a fluorescent microscope during imaging, to prevent cell death due to the imaging procedure. Scanned images were stitched into a single composite image using the open source image analysis suite Fiji[44] and then segmented into address-associated individual well images using an algorithm, 'Segmenter', designed in MATLAB (MathWorks). CellProfiler-based image analysis is described in detail in Supplementary Information. Source files for 'Segmenter.m', CellProfiler 2.0 pipeline 'SCPipeline.cp' and 'WellContents.xls' are available here as Supplementary Software and for download at http://www. magneslab.org/#/vitae1-page-1/vitesc-official-software-downloads.

Raft retrieval and generation of cDNA from ISCs and enteroids. FACS-isolated Lgr5CreERT ISCs were applied to the MRA device at a density that favored single cells (22,500 Lgr5CreERT cells for 15,000 wells). 24–36 rafts containing either single or double Lgr5CreERT ISCs, developing enterospheres or fully developed enteroids were collected at the time points described. Raft retrieval was conducted on an Olympus IX-81 microscope fitted with a physiologic chamber to maintain humidity and temperature. Microwells containing an ISC or enteroid were identified and imaged before release of the raft. Rafts were released using the commercially available IsoRaft release and retrieval system (Cell Microsystems), consisting of a mechanically actuated needle fitted to a standard objective lens. Following liberation of the raft from the PDMS well, the magnetic microwand (Cell Microsystems) was used to collect the raft, which was then transferred to a single well of a U-bottomed 96-well plate containing 5 µl of RNA lysis buffer (CellsDirect, Life Technologies). Rafts were retrieved in 44.8 ± 14.3 s with a retrieval success rate of 93.3% (n = 30 rafts). When accounting for time spent locating and imaging rafts of interest, up to 36 rafts could be collected in approximately 1 h. DNA removal and cDNA synthesis was conducted according to manufacturer’s protocols with the exception that volumes were scaled down to work with 5 µl (5 × 1 µl) of cDNA. Specific target amplification (STA) was conducted according to manufacturer’s protocols using Taqman probes (Supplementary Table 2). The STA cDNA was applied to a 192.24 Integrated Fluidic Chip (IFC; Fluidigm) and assayed for gene expression using the Biomark HD platform (Fluidigm). Ct values were normalized to 18S signal and ΔCt values were analysed using Fluidigm Real-time PCR analysis software to generate heat maps and Singular 2.0 (based on R) to generate violin plots.

Fabrication of glass-mounted microwell arrays. SU-8 photoresist was purchased from MicroChem. The Sylgard 184 silicone elastomer kit was purchased from Dow Corning. y-Butyrolactone (GBL), ooctyltrichlorosilane and propylene glycol monomethyl ether acetate were obtained from Sigma-Aldrich. Poly(acrylic) acid (molecular mass ~ 5,000 Da) was obtained from Polysciences. Falcon Petri dishes were obtained from BD Biosciences. Polycarbonate plates (12 inch × 12 inch × 0.5 inch) were purchased from McMaster-Carr and glass slides (75 mm × 50 mm × 1 mm) were purchased from Corning.

An SU-8 master mould with an array of microposts was fabricated using standard photolithography with 100-µm-thick SU-8 as described previously[45]. The SU-8 master was coated by octyltrifluorosilane using vapour deposition to render the surface of the master non-sticky to PDMS (ref. 12). Clean glass slides (75 mm × 50 mm × 1 mm) were spin-coated with a 15-µm-thick layer of poly(acrylic) acid (PAA) by using 50 wt% solution and a spin speed of 2,000 r.p.m., followed by a 1 h bake on a 100°C hotplate to remove the water. The slide coated in PAA was treated in a plasma cleaner for 10 min (Harrick Plasma). PDMS prepolymer (10:1 mixture of basecuring agent of Sylgard 184 kit) was spread on the SU-8 master mould and degassed under vacuum to remove trapped air bubbles. To control the thickness of the PDMS mould, 300-µm PDMS spacers were placed between the SU-8 master mould and glass slides. PDMS was spin-coated and cured by spin-coating glass with PDMS prepolymer at 200 r.p.m. for 30 s and cured on a 120°C hotplate for 30 min. The plasma-treated PAA-coated glass slide was then placed on the master mould, flattening the PDMS prepolymer between the master mould and the glass slide (Supplementary Methods and Supplementary Fig. 1A). The ends of the master–glass slide assembly were secured by paper clips to prevent movement during curing of the PDMS. The PDMS was cured in a 95°C oven for 1 h (Supplementary Methods and Supplementary Fig. 1B). The glass-backed PDMS microwell array was then obtained by separating the array from the silanized master mould (Supplementary Methods and Supplementary Fig. 1C). The resulting microwell array has an array area of 25.4 mm × 25.4 mm, and each microwell has a dimension of 200 µm × 200 µm × 100 µm spaced 30 µm apart. Glass-bound MRAs were reproducibly imaged in a single Z-plane without any noticeable out-of-focus sagging (n = 50; Supplementary Fig. 2).

A polystyrene solution was prepared by dissolving polystyrene Falcon Petri dishes in GBL at 20 wt% concentration. The polystyrene solution was spread onto the PDMS microwell array and degassed under vacuum to remove trapped air bubbles (Supplementary Methods and Supplementary Fig. 1D). The glass-backed PDMS array was then immersed in the polystyrene solution and withdrawn vertically at a speed of 0.83 mm min⁻¹ by a stepper motor controlled by a custom controller (Supplementary Methods and Supplementary Fig. 1E). Owing to the surface tension difference between the PDMS and the polystyrene solution, the polystyrene solution dewetted from the array, resulting in isolated pockets of polystyrene solution in the microwells (Supplementary Methods and Supplementary Fig. 1F). This phenomenon is called discontinuous dewetting[46]. The array was then placed in a 95°C oven overnight to evaporate the GBL solvent, and pockets of polystyrene solution shrank into solid polystyrene micrafts with a concave geometry (Supplementary Methods and Supplementary Fig. 1G).

The array was attached to a rigid 2-chamber or 4-chamber polycarbonate cassette, which was fabricated by a computer numerical control machine (Supplementary Methods and Supplementary Fig. 1H). Before attachment, the cassette was cleaned by sonication in a solution of 1 wt% detergent in water for 1 h, followed by a 1 h sonication in a 75% ethanol solution. The cassette was rinsed thoroughly in deionized water and baked for 15 min in a 95°C oven to remove any remaining solvent. Both the array and the cassette were treated in plasma cleaner for 10 min before being glued together with PDMS (cured in a 95°C oven for 1 h). The arrays were treated with air plasma for 5 min, sterilized with 75% ethanol, and stored in sterile 1 x PBS before use.

Epithelial isolation and FACS. Epithelial cells were isolated from whole murine intestines, as previously described, with some modifications[47]. Briefly, intestines were opened longitudinally, rinsed in DPBS (Life Technologies), minced and incubated in 3 mM EDTA (Sigma) in DPBS for 45 min at 4°C with gentle agitation. Intestinal fragments were transferred to fresh DPBS and shaken by hand for 2 min to release epithelium. Remnant submucosa was discarded, epithelium was rinsed twice in DPBS, and then dissociated to single cells by incubation in 0.3 U ml⁻¹ dispase (Life Technologies) in 10 ml HBSS (Life Technologies) at 37°C for 8–10 min with shaking every 2 min. Single epithelial cells were filtered through 100, 70 and 40 µm filters before being resuspended in ISC Sort/Culture Media (Advanced DMEM/F12

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METHODS

Autofluorescence and reflections off microwell walls distort the processed microwell images and can reduce the accuracy of stem cell identification. Raw images loaded into CellProfiler undergo thresholding using the ‘MoFlo Global’ method, which separates objects from background on the basis of an estimate of the amount of the image occupied by objects. The image occupation of a single cell was estimated by CellProfiler 2.0 analysis.

CellProfiler 2.0 analysis. Computational analysis of initial microwell contents was carried out in CellProfiler 2.0 (Broad Institute) in two steps: segmented microwell images were loaded in sequence into CellProfiler to determine the contents of each microwell of the array; (2) raw data generated by CellProfiler were converted into object delineated data using ‘WellContents.xls’, which compiled data on the objects’ shape and location characteristics. We focused on three specific parameters to develop an analytical pipeline to identify ISC activity, namely size, abundance and borders. The detailed method, including code availability, is provided in the Supplementary Note 2.

Quantification of cell movement in microwells. For cell movement experiments, pairs of PCs, ISC, and ISC–PC pairs were identified in microwell images at t = 0. Distance between each cell in a pair was measured at t = 0 and t = 24. Relative change in distance (d/d₀) was calculated by subtracting distance at t = 24h (d₂₄) from distance at t = 0 (d₀) and normalizing to d₀ (Supplementary Fig. 5A). Pairs of cells were included in the experiment only if they were alive at t = 24h, based on the presence of EGFP or DsRed fluorescence. Distances were measured from the centre of each cell to account for changes in cell size. Statistics were analysed in Prism 6 (GraphPad) by one-way ANOVA with Tukey’s multiple comparisons test. To control for measurement error, primary intestinal epithelial cells were isolated and fixed in 4% PFA for 30 min at room temperature, rinsed three times in PBS, and plated in MRA. Distance between fixed cell pairs (n = 50) was calculated as per live cells, above. Results from the fixed cell experiment were compared with each group of live cell pairs by unpaired Student’s t-test to determine statistically significant movement.

Single-cell isolation and qPCR. For single-PC gene expression analysis, PCs were sorted as described earlier and applied to a 10–17 μm C1 Autoprep microfluidics chip ( Fluidigm) to generate a targeted cDNA library. Specific targets were pre-amplified using the following Taqman (Life Technologies) probe sets against genes of interest (Supplementary Methods and Supplementary Table 2). All other operations were conducted according to manufacturers’ protocols. The STA libraries were applied to the Biomark HD microfluidics chip ( Fluidigm) to conduct qPCR. The same probes and conditions were used for the STA and qPCR. Figure 6a shows representative images of retrieved rafts/enteroids from 192 total rafts. Figure 6d shows morphological changes of cystic and columnar enteroids, from a total of 35 and 32 examined, respectively. Supplementary Figure 2C shows representative images of retrieved enteroids/rafts from 905 pairs of non-touching cells quantified in subsequent panels of the same figure. Figure 6a shows representative data from MRA culture experiments, which have been independently replicated at least 70 times at the time of publication, and are ongoing in our laboratory. Figure 6a shows representative data from MRA culture experiments, which have been independently replicated at least 70 times at the time of publication, and are ongoing in our laboratory.
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43. Gracz, A. D., Puthoff, B. J. & Magness, S. T. Identification, isolation, and culture of intestinal epithelial stem cells from murine intestine. *Methods Mol. Biol.* **879**, 89–107 (2012).
Supplementary Figure 1 Fabrication of glass-mounted microwell arrays. (A) PDMS is liquid molded onto a PAA-coated glass slide on a SU-8 master mold. (B,C) PDMS/SU-8 mold assemblies are cured, and cured, solid PDMS is gently removed from the mold. (D) Polystyrene solution is added to the array and degassed. (E) Dip coating with a programmed stepper motor removes excess polystyrene. (F) Subsequent discontinuous dewetting generates isolated polystyrene pockets inside PDMS microwells. (G) Arrays are baked at 95°C to remove solvent, resulting in solid polystyrene microrafsts embedded in PDMS microwells. (H) Micraft arrays are mounted to polycarbonate cassettes to complete the fabrication process.
Supplementary Figure 2 Glass-mounting reduces PDMS sagging in microwell arrays. (A,C) The elastic properties of PDMS cause standard microwell arrays to sag, preventing tile-scanned imaging in a single Z-plane. (B,D) Mounting arrays to glass slides prevents sagging and facilitates imaging. Scale bars represent 300μm.
Supplementary Figure 3 Sox9EGFP transgenic mice facilitate high purity FACS isolation of Paneth cells. (A) To increase population purity, we developed novel FACS criteria for Paneth cell sorting. Standard size, double, and live-dead exclusion criteria were applied to all FACS isolations. (B) We compared putative Paneth cell populations isolated using previously described methods, which define Paneth cells as CD24\textsuperscript{hi}:SSC\textsuperscript{hi}, and (C) our newly developed method, which applies the same parameters, but excludes all Sox9EGFP expressing cells.
Supplementary Figure 4  Sox9EGFP\(^{\text{neg}}\).CD24\(^{\text{high}}\).SSC\(^{\text{high}}\) enriches for highly pure Paneth cell populations. Gene expression analysis demonstrates upregulation of Paneth cell marker Lyz2, and downregulation of ISC marker Lgr5, as well as EE cell marker Chga in Paneth cell populations isolated with Sox9EGFP exclusion, when compared to populations isolated with CD24 and SSC alone (values represent three technical replicates carried out on one biological replicate per sorting strategy).
Supplementary Figure 5 Retrieval of magnetic rafts for downstream gene expression analysis. (A) Raft retrieval. A device containing a fine needle positioned in the center of a clear plexiglass window was fitted onto a 10X objective lens. Z-plane focus was used to puncture the bottom of the PDMS liberating the raft. A magnetic wand was used to collect the magnetic raft. (B) The magnetic wand facilitates efficient retrieval of magnetic rafts (note red magnetic raft on tip of wand). (C) The raft is liberated from the magnetic wand when placed in a 96-well format dish that is positioned over a stronger magnet place on ice. (D) Time lapse image of raft retrieval (frame 1-4). The raft with enteroid depicted in frame 1 was captured by magnetic wand and placed in PBS (frame 5). Enteroid was efficiently lysed in RNA lysis buffer prior to cDNA synthesis (frame 6). (E) A large well-developed enteroid was identified in the MrA and retrieved using the magnetic wand. (F) Matrigel anchors the large enteroid to the magnetic raft for efficient capture. (G) Rafts and associated images can be ordered in a conventional 96-well format for indexing and retrospective analysis. Scale bars represent 100μm.
Supplementary Figure 6 Enteroid morphologies are correlated with gene expression analysis. Cystic ("Cyst") and columnar ("Col") enteroids are analyzed at (A) 24hr and (B) 48hr by microfluidic qPCR against 33 genes. Image capture immediately prior to raft retrieval allows for matching phenotypic characteristics, such as morphology, with gene expression results. Heat map represents Ct values.
Supplementary Figure 7 dCt values reveal differences in enteroid morphology at the genetic level. Gene expression changes in cystic and columnar enteroids at 24hr and 48hr, for all genes assayed. Violin plots represent dCt values normalized to 18s signal; n = 13 cystic and 14 columnar enteroids at 24hr; 13 cystic and 12 columnar at 48hr; different letters represent statistical significance, one-way ANOVA, p < 0.05. Exact p values presented in Supplementary Table 3. Graphs without letters do not have statistically significant differences between groups.
### Supplementary Table 1

Number of individual microwells examined for each combination of initial contents.

| ISC number | PC number | Sox9EGFP<sup>hi</sup> replicates | ISC number | PC number | Lgr5EGFP<sup>high</sup> replicates |
|------------|-----------|-----------------------------------|------------|-----------|------------------------------------|
| 1          | 0         | 825                               | 1          | 0         | 851                                |
| 1          | 1         | 321                               | 1          | 1         | 216                                |
| 1          | 2         | 75                                | 1          | 2         | 42                                 |
| 2          | 0         | 645                               | 2          | 0         | 328                                |
| 2          | 1         | 248                               | 2          | 1         | 77                                 |
| 2          | 2         | 45                                | 2          | 2         | 17                                 |
| 3          | 0         | 358                               | 3          | 1         | 103                                |
| 3          | 1         | 145                               | 3          | 2         | 22                                 |
| 3          | 2         | 50                                | 3          | 3         | 8                                  |
| 4          | 0         | 189                               | 4          | 0         | 25                                 |
| 4          | 1         | 74                                | 4          | 1         | 7                                  |
| 4          | 2         | 18                                | 4          | 2         | 4                                  |
| 5          | 0         | 88                                | 5          | 0         | 15                                 |
| 5          | 1         | 41                                | 5          | 1         | 1                                  |
| 5          | 2         | 8                                 | 5          | 2         | 0                                  |

**Supplementary Table 1** Number of individual microwells examined for each combination of initial contents.
### Supplementary Table 2: Taqman probes used for qPCR

| gene name | probe ID          |
|-----------|-------------------|
| 18S       | Hs99999901_s1     |
| Ascl2     | Mm01268891_g1     |
| Atoh1     | Mm00476035_s1     |
| Bmi1      | Mm00776122_g1     |
| Ccn1      | Mm00432337_m1     |
| Ccnb1     | Mm03053893_gH     |
| Ccnd1     | Mm00432360_m1     |
| Ccne1     | Mm01266311_m1     |
| Cd24      | Mm00782538_sH     |
| Chga      | Mm00514341_m1     |
| Chgb      | Mm00483287_m1     |
| Defcr-rs1 | Mm00655850_m1     |
| Dil1      | Mm01279269_m1     |
| Dll4      | Mm00444619_m1     |
| Fzd2      | Mm02524776_s1     |
| Fzd5      | Mm00445623_s1     |
| Fzd6      | Mm00433387_m1     |
| Fzd7      | Mm00433409_s1     |
| Gapdh     | Mm99999915_g1     |
| Hes1      | Mm00486891_m1     |
| Hopx      | Mm00558630_m1     |
| Jag1      | Mm00496902_m1     |
| Lgr5      | Mm00438890_m1     |
| Lrig1     | Mm00456116_m1     |
| Lyz2      | Mm01612741_m1     |
| Muc2      | Mm00458299_m1     |
| Myc       | Mm00487804_m1     |
| Olfm4     | Mm01320260_m1     |
| Sis       | Mm01210305_m1     |
| Smoc2     | Mm00491553_m1     |
| Sox4      | Mm00486317_s1     |
| Sox9      | Mm00448840_m1     |
| Tac1      | Mm00436880_m1     |
| Tert      | Mm00436931_m1     |
| Tgfb1     | Mm00436964_m1     |
| Tgfb2     | Mm00436977_m1     |
| Wnt3a     | Mm00437337_m1     |
| Wnt5a     | Mm00437347_m1     |
| Yap1      | Mm01143263_m1     |
**Supplementary Table 3** p values for Figures 5C, 6G, 7B and Supplementary Figure 7.

**Supplementary Video Legends**

**Supplementary Video 1** The movie shows an enteroid developing from two touching ISCs (Sox9EGFP+) and migrating away from a Paneth cell (DsRed+) that dies early in culture, demonstrating movement of cells within microwells. The microwell was imaged every 30min for the first 22hrs of culture.

**Supplementary Video 2** The movie shows several small multimers of Sox9EGFP+ ISCs merging to form an enteroid in the lower left corner of the microwell. Images were taken every 30min for the first 22hrs of culture.
Supplementary Note 1

Modeling of protein diffusion between microwells in MRAs

To determine if secreted proteins from a cell in one microwell of a MRA could impact behavior of a cell in an adjacent microwell, the concentration of a known Paneth cell niche protein, WNT3, produced by a single Paneth cell was simulated assuming diffusive transport (COMSOL Multiphysics 4.4, Transport of Dilute Species Module). The MRA was modeled as a 3-dimensional, 3×3 microwell array with 200µm² square microwells and 30µm spacing between the microwells. The shape of the microwell bottom corresponded to that of the upper surface of a microraft. The microwells were 55µm deep in the center and 30µm deep in the corners. The intervening surface of the microraft (microwell bottom) was curved with a radius of curvature of 30µm. Liquid medium extended 3mm above the array (equivalent to the liquid height of 2mL medium on a 25.4mm² microraft array). Convective mixing was not permitted in the liquid and diffusion was assumed to be isotropic throughout the liquid solution. A diffusion coefficient $7 \times 10^{-11} \text{ m}^2/\text{s}$ was used for WNT3 (M.W. = 39.7 kDa) and was based on the experimentally measured diffusion coefficient of VEGF (M.W. = 40.0 kDa), which possesses a similar molecular weight to that of WNT3. To experimentally determine a liberal rate of WNT3 secretion for our model, we cultured L-cells overexpressing WNT3A (ATCC, CRL2647, Manassas, VA) and based our assumptions on the results of an ELISA assay (SEP155Hu, USCN Life Sciences Inc., Wuhan, Hubei, China). $1.6 \times 10^7$ WNT3A-expressing L-cells cultured for 16h produced supernatant with a WNT3A concentration of 120ng/mL. Applying this same rate of secretion to Paneth cells and assuming a constant secretion over time, a single Paneth cell in our model secreted WNT3 at a rate of 9 fg/h. For simulations of [WNT3], a Paneth cell was represented as a hemisphere with a radius of 5 µm located in the center of the central microraft. A uniform and constant secretion was assumed across the entire surface area with a flux of $4.01 \times 10^{-13} \text{ mol/m}^2 \text{ s}$. WNT3 was assumed to be degraded after its production with a half-life of 2, 15, 60 or 120 min. The [WNT3] in the region surrounding the Paneth cell and adjacent microwells was simulated after varying time spans (12, 24, 48 and 96 h).

In all scenarios, the steady-state [WNT3] was attained rapidly (<12 h). The highest [WNT3] occurred adjacent to the surface of the Paneth cell reaching maximal concentrations of 30pM (Table 1, Figure 1A, location i). The [WNT3] dropped rapidly with the distance from the Paneth cell falling to ≤1.9pM at the edges (Location ii, Figure 1A) of the well in which the Paneth cell was located. The [WNT3] continued to decline as the distance increased diminishing to ≤1.3pM and ≤1.0pM at the edge (Location iii, Figure 1A) and center (Location iv, Figure 1A), respectively, of the adjacent well. These predictions are overestimates of the [WNT3] in the adjacent wells since convective mixing in the overlying solution is expected to further reduce the [WNT3] within the microwells.

Culture of isolated intestinal stem cells or crypts to form organoids typically requires ≥10 ng/mL (1.5nM) recombinant WNT3A. Thus under the culture conditions used, the concentration of WNT3 is well below that need to sustain cell growth in a
conventional *in vitro* culture format, even under the liberal assumptions of our modeling. Importantly, our modeling suggests that a single Paneth cell may be insufficient to drive enteroid formation through diffusible Wnt signaling *in vitro*, even in the same microwell. However, it is critical to note that post-translation modifications affecting ligand stability could also influence Wnt signaling and that concentration thresholds required to induce a phenotypic outcome likely differ between ligand-receptor pairs. In context of the present study, these simulations further support the experimental data suggesting that direct cell-to-cell contact is required for optimal Paneth-cell-support of stem cells *in vitro*.

### Supplementary Note Table 1- Steady-state [WNT3] (pM)

| Half-life (min) | Sampling Location (Figure 1A) |
|----------------|-------------------------------|
|                | i                | ii               | iii              | iv               |
| 2              | 27.6             | 0.5              | 0.2              | 0                |
| 15             | 29.0             | 1.2              | 0.6              | 0.3              |
| 60             | 29.7             | 1.6              | 1.0              | 0.7              |
| 120            | 30.0             | 1.9              | 1.3              | 1.0              |

**Supplementary Note Figure 1** - COMSOL simulation of WNT3A diffusion after secretion from a Paneth cell on the central microrhaft. (A) 3-Dimensional model of a 3×3 MRA. The upper panel is a top view while the lower panel is a cross-sectional view along the red dashed line in the upper panel. Locations *i*-iv are the points where [WNT3] was simulated. The Paneth cell is located at *i*. Steady state [WNT3] assuming WNT3 has a half-life of (B) 2 min, (C) 15 min, (D) 60 min and (E) 120 min. Scale bar = 100 µm.
References

1. Chen, R.R., Silva, E.A., Yuen, W.W. & Mooney, D.J. Spatio-temporal VEGF and PDGF delivery patterns blood vessel formation and maturation. *Pharmaceutical research* **24**, 258-264 (2007).

2. Sato, T. *et al.* Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. *Nature* **469**, 415-418 (2011).
Supplementary Note 2

Software guide

Microwell-based segmentation of MRA images

To facilitate high-throughput quantification of microwell contents, large mosaic images of the entire MRA surface are segmented into individual images displaying a single microwell. Single microwell images are generated using the image processing script “Segmenter.m” (available at magnesslab.org) created in the MATLAB computing environment (http://www.mathworks.com/products/matlab/).

1. Upon opening the MATLAB environment, users are prompted with a command window displaying the contents of the current working folder and command history.
2. Load the Segmenter.m script by selecting “Open” from the “Home” toolbar and navigating to the file location.
3. An editor window automatically opens once the Segmenter.m script is loaded, displaying the code used to segment and name the microwell images.
   ○ NOTE: Avoid editing the script within this window to ensure continued function
4. Select “Run” from the “Editor” toolbar to begin the Segmenter.m script
   ○ NOTE: If Segmenter.m file is not located in the MATLAB current folder the user will be prompted with a window asking how to continue. Select “Add to Path” from the choices available in the window.
5. In the command window input the full path to the folder containing the stitched images you wish to segment and press the return key
6. When prompted, input the full filename of the first stitched image to be segmented and press the return key.
7. Next input the full filename of the second stitched image to be segmented and press the return key.
   ○ NOTE: If only one image is desired to be segmented the same image name can be entered for both stitched images
8. Users are prompted to open one of the stitched images in FIJI (http://fiji.sc) to determine the reference coordinates. Once the image is displayed in FIJI, press the return key.
9. Determine the pixel coordinates of the center each of the corner wells by hovering over the location with the cursor in FIJI.
10. Input the x-axis pixel coordinate of the center of the top-left well when prompted for the X1-value and press the return key.
11. Input the y-axis pixel coordinate of the center of the top-left well when prompted for the Y1-value and press the return key.
12. Input the x-axis pixel coordinate of the center of the top-right well when prompted for the X2-value and press the return key.
13. Input the y-axis pixel coordinate of the center of the top-right well when prompted for the Y2-value and press the return key.
14. Input the x-axis pixel coordinate of the center of the bottom-right well when prompted for the X3-value and press the return key.
15. Input the y-axis pixel coordinate of the center of the bottom-right well when prompted for the Y3-value and press the return key.
16. Input the x-axis pixel coordinate of the center of the bottom-left well when prompted for the X4-value and press the return key.
17. Input the y-axis pixel coordinate of the center of the bottom-left well when prompted for the Y4-value and press the return key.
18. Input the number of horizontal rows of microwells contained in the MRA image and press the return key.
19. Input the number of vertical columns of microwells contained in the MRA image and press the return key.
20. In FIJI, measure the length of one microwell using the line tool available in the FIJI tool bar
   ○ Select the line tool from the toolbar and construct a line with and angle=0 (shown in Fiji toolbar) that spans the width of a microwell.
   ○ Use the length of this line as the width of one microwell
21. Input the measured microwell width and press the return key.
22. Segmenter.m then displays all of the dimension values input by the user and asks for confirmation
   ○ Input y and press the return key if displayed values are accurate
   ○ Input n and press the return key if displayed values are not accurate
23. Segmenter.m is now ready to segment the stitched images into individual microwell images and prompts users to close all unnecessary programs in order to reduce processing time
   ○ Once other programs are closed and the user is ready to proceed with segmentation input y and press the return key
24. Stitched images are loaded and segmented over the course of several minutes with the message “Done!!!” displayed in the command window when the script is completed.

CellProfiler image analysis
Initial microwell contents of the MRA are determined through automated analysis using CellProfiler 2.0.1170 (http://www.cellprofiler.org). Analysis is performed using pipeline “SCPipeline.cp” (available at magnesslab.org).

1. Upon opening the CellProfiler Automated Analysis suite users are prompted with a window displaying the current analysis settings. First, select the folder containing the image series to be analyzed (from “Segmenter.m”, see above) in the menu titled “Default Input Folder:”
2. Select the folder where CellProfiler analyzed data will be stored in the menu titled “Default Output Folder:”
3. Name the data file that CellProfiler will generate at the conclusion of the analysis in the menu titled “Output Filename:”
4. To load the pipeline select “Load Pipeline…” under the menu option “File” and navigate to the location containing the SCPipeline.cp file.
5. Opening the Pipeline will automatically load 6 modules into the analysis window dictating the functions that CellProfiler will perform. Select the first module titled “LoadImages”, causing CellProfiler to display the module settings in the main panel of the CellProfiler window.
6. In the “LoadImages” module panel, edit the menu titled “Text that these image have in common (case-sensitive)” to match the base name of the image series.
7. Edit the menu titled “Regular expression that finds metadata in the file name” replacing the text “BaseName” with the base name of the image series.
8. Analyze the image series by selecting “Analyze Images” in the bottom right-hand corner of the CellProfiler window.
   o NOTE: CellProfiler allows users to directly observe the analysis of the input image series in real-time
   o To observe any step of the analysis pipeline open the eyeball graphic next to the corresponding module in the pipeline window by selecting it with the cursor.
   o NOTE: Direct observation of the SCPipeline significantly increases the time required to analyze an image series.

Data analysis of CellProfiler results

After the well contents are determined by automated analysis, “cell per microwell” data is extracted from the .csv file generated in “SCPipeline.cp” using the “WellContents.xls” file (available at magnesslab.org).

IMPORTANT!: Since per-microwell analysis of CellProfiler data in the WellContents file depends on formulas embedded in the spreadsheet, we recommend opening “WellContents.xls” as a read-only file to prevent changes to the original file.
1. Open the “SCPipeline.cp” generated .csv file (this file has a name generated by
the user, see “CellProfiler image analysis”, step 3) and the WellContents.xls file
2. Copy all of the entries contained in the “SCPipeline.cp” generated data file
(column A to column AA) into the WellContents file starting at cell A1 on the
sheet titled “csv”.
   o Only select and transfer data in column A to column AA to avoid editing
     formatting formulas in the WellContents file.
3. Per-microwell contents of the MRA are automatically extracted through
   formatting expressions built into the WellContents file and displayed on the sheet
   titled “WellDelineated”.
4. Microwell addresses are displayed in the sheet titled “target wells”, and grouped
   by microwell content (intact cells only; microwells with debris are excluded) as
   follows:

   | column A | 1 cell |
   |---------|--------|
   | column B | 2 cell |
   | column C | 3 cell |
   | column D | 4 cell |

5. Data summary, showing the distribution of cells within the MRA are displayed in
   the sheet titled “Distribution”.

References

1. Carpenter, A.E. et al. CellProfiler: image analysis software for identifying and
   quantifying cell phenotypes. Genome Biol 7, R100 (2006).