Immortalized Intestinal Telocytes - A Stem Cell Niche In Vitro

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Summary

Telocytes, a distinct type of mesenchymal cell, have recently emerged as crucial components of the intestinal stem cell niche, primarily by providing essential Wnt proteins that drive stem cell function. However, their detailed biology and the specific mechanisms by which they regulate stem cell activity remain largely unexplored. Here, we established an immortalized intestinal telocyte cell line that retains the key cellular and molecular characteristics of native telocytes. Remarkably, this cell line, unlike telocyte-depleted immortalized mesenchyme, supports the growth of both mouse and human organoids in co-culture without the need for external factor supplementation. These findings indicate that telocytes are not only essential but are also sufficient to maintain stem cell activity. This immortalized telocyte line provides a valuable source of factors that support stem cell activity in vitro, offering a powerful tool for advancing our understanding of telocyte biology and the reciprocal communication between stem cells and their niche.

Introduction

Telocytes, unique mesenchymal cells characterized by the expression of the transcription factor Foxl1, have recently been recognized as crucial for maintaining intestinal homeostasis[1-4]. Historically, telocytes were described primarily through electron microscopy as special interstitial cells present in the stroma of many organs[5-11]. They are distinct from fibroblasts due to their exceptionally long cellular extensions, known as telopodes, which can extend hundreds of microns from the cell body. These telopodes vary in width, and feature dilated regions called podoms, which contain mitochondria, caveolae, Ca^{2+} -releasing units, and elements of endoplasmic reticulum (ER).Through these extensions, telocytes form intricate 3D networks with other telocytes and diverse cell types, including epithelial cells, blood and lymphatic vessels, immune cells, fibroblasts, myofibroblasts and nerve bundles[8,10-12]. While they are present in many organs, the essential role of telocytes in maintaining homeostasis was first explored in the intestine[2].

In the intestine, telocytes establish an extensive 3D network along the basal side of the crypt-villus epithelium, playing a critical role as the stem cell niche by supplying Wnt

proteins essential for stem cell function. Additionally, telocytes facilitate enterocyte differentiation[3]. They serve as signaling hubs, expressing a wide array of signaling molecules from key pathways, including Wnt, Rspo, Bmp, Tgfβ and Shh. Notably, telocytes exhibit heterogeneity in their signaling molecule expression based on their position along the crypt-villus axis, correlating with active signaling pathways in the epithelium[2,3].

The crucial role of telocytes in maintaining intestinal homeostasis highlights the need for effective strategies to establish in vitro sources of these cells while preserving their in vivo features and functions. This is essential for advancing our understanding of their biology and functional mechanisms. However, several challenges complicate the maintenance of primary telocyte cultures. Telocytes are present in very low numbers, constituting about 0.5% of the intestinal stromal population, as determined by FACS[2]. Their unique characteristics and long cellular extensions render them particularly vulnerable to damage during dissociation and sorting procedures. Although current protocols for isolating mesenchymal cells from mouse intestines can yield a relatively high proportion of viable telocytes[13], their low abundance and non-proliferative nature present significant hurdles.

Our goal was to develop a cell line system of telocytes for in vitro studies. Immortalization involves manipulating cells to bypass normal limits on cell division, allowing for ‘infinite’ proliferation in vitro. Immortalized cell lines provide a consistent supply of cells and facilitate genetic manipulations[14]. One widely used method for generating immortalized cell lines involves introducing viral oncogenes. Among these, infection with SV40 (simian vacuolating virus) proteins, particularly Large T-antigen[15], is one of the most successful immortalizing genes. Immortalization by Large T-antigen is achieved through its (i) binding to the Retinoblastoma protein (Rb)-E2F complex, which relieves cell proliferation restrictions by activating of E2F-mediated transcription[16-20], and (ii) binding and inhibition of p53 activity[21-30]. These mechanisms prevent growth arrest and senescence, resulting in continuous cell proliferation.

In this study, we utilized Foxl1Cre-driven reporter mice to isolate GFP$^+$ telocytes from the mouse jejunum, and employed the SV40 Large T-antigen immortalization protocol to create an immortalized intestinal telocyte cell line. Our results demonstrate that this cell line retains key telocyte features, including the ability to form extensive networks via telopodes, cover large surface areas, and express factors known to be essential for supporting stem cell activity. Furthermore, the immortalized telocytes preserve molecular characteristics typical of native telocytes, showing high enrichment for PDGFRα, CD34, and Mcam. Remarkably, these immortalized cells support growth of organoids-derived from both mouse and human tissues, without requiring additional factor supplementation. These findings indicate that teloytes are not only essential but also sufficient to promote stem cell function. We propose that this immortalized telocyte cell line can be used as a resource for niche factors to support stem cell activity in vitro. Moreover, this cell line represents a valuable tool for advancing our understanding of telocyte biology and their interactions with stem cells in health and disease.

Results

Immortalized telocytes retain structural characteristics

We developed an immortalized telocyte cell line by crossing Foxl1-promoter-driven Cre mice with Rosa-mTmG reporter mice, which express a plasma membrane-bound green fluorescence protein (GFP)[1] (**Figure 1A** scheme). This approach allowed us to label telocytes with a membrane-tagged GFP (green), while Foxl1-negative cells were labeled with membrane-tagged tdTomato (red). We dissected the mouse jejunum and isolated the mesenchyme[13], which was then cultured for two weeks to recover from potential dissociation damage. Following recovery, we dissociated the mesenchymal cells, FACS-sorted and plated GFP$^+$ telocytes and tdTomato$^+$ (telocyte-depleted) mesenchymal cells separately.

To achieve immortalization, we employed the Simian Virus 40 (SV40) Large T-antigen protocol, a well-established method known for its simplicity and reliability in immortalizing various cell types[34-36]. We infected GFP$^+$ telocytes and tdTomato$^+$ telocyte-depleted mesenchymal cells with viral particles expressing the SV40 Large T-antigen. Viral

particles were produced by transfecting a lentiviral vector encoding the protein into HEK293T cells. The immortalized cells were expanded through 7-9 passages before being frozen for further analysis.

Immortalization can alter cellular phenotypes; therefore, we assessed the characteristics of our immortalized telocyte cell line. The immortalized telocyte cells demonstrated high proliferation rates and varying levels of GFP expression. The cells appeared flat and thin, with long cellular projections extending hundreds of microns from the cell body (**Figure 1B**). Dilated regions along these extensions showed high GFP signals, likely corresponding to the podom regions of the telopodes. During proliferation, cells appeared condensed and rounded extending multiple cellular protrusions, but after division, they spread out thin and flat over a large surface area (**Video 1, Figure 1C**). Remarkably, dividing cells maintained contact with each other and extended protrusions to form networks (**Video 1)**. The cells were either mono- or multi-nucleated, a phenomenon that remains unclear - whether it is an artifact of immortalization or represents an intermediate state that is not normally observed in vivo.

The cells displayed pronounced polarization, with cytoskeletal elements concentrated in specific regions. Actin filaments, stained with phalloidin, were distributed asymmetrically beneath the plasma membrane (**Figure 1D**) and arranged in a regular striped pattern, suggesting contractility[37,38]. Cellular extensions were rich in actin, featuring bundles of parallel filaments, lamellipodia or membrane-ruffles (**Figure 1D** boxed regions), sometimes continuous with stress fibers (**Figure 1D** arrowhead). Microtubule distribution, stained for α-tubulin (**Figure 1E** red**)** revealed a distinct pattern: thick, wide bundles along broader extensions, and thin, branched fibers at the base of thinner extensions and specific sites within the cytoplasm (**Figure 1E** boxed regions). This suggests that thick bundles of microtubules may represent stable structures, while thin, branched fibers indicate dynamic microtubules[39,40].

Thus, immortalized telocytes retain their highly polarized structure and the distinctive long cellular extensions that interconnect to from a network, preserving the key cellular features of native telocytes.

Immortalized telocytes preserve molecular expression profile

In vivo, intestinal telocytes are a distinct mesenchymal population, characterized by their expression of PDGFRα, CD34 and Foxl1. The diverse expression of signaling molecules in these cells highlights the heterogeneity within this population, which likely arises from complex interactions with surrounding cells. However, it was unclear whether telocytes that are immortalized and cultured outside their native environment, isolated from other cell types, would retain their molecular markers. To evaluate the molecular profile of these immortalized cells, we first validated Foxl1 expression, which drives our reporter mice, using single-molecule RNA fluorescence in situ hybridization (smFISH). Our analysis confirmed that Foxl1 mRNA was present in GFP^+ telocytes, but absent in the $tdTomato^+$ mesenchymal cells lacking telocytes (**Figure 2A-B**). This result confirms that, despite immortalization, the cell populations can still be distinguished by their Foxl1 expression.

Next, we assessed two additional molecular hallmarks of intestinal telocytes, PDGFRα and CD34, using FACS analysis and immunofluorescence staining (**Figure 2C-F**). The majority of the immortalized telocytes expressed both PDGFRα and CD34. We observed two distinct populations based on PDGFRα expression levels: a $PDGFR\alpha^{high}$ population and a $PDGFR\alpha^{low}$ population (**Figure 2C-D**).

To further characterize the differential expression profiles between GFP^+ telocytes and $tdTomato^+$ telocyte-depleted mesenchymal cells, we conducted FACS analysis using additional mesenchymal markers. We included markers of epithelial cells ($EpCAM^+$), immune cells ($CD45^+$) and endothelial cells ($CD31^+$) to confirm the culture's purity. We then stained for various mesenchymal markers, including the mucin-type glycoprotein Podoplanin (Pdpn), melanoma cell adhesion protein (Mcam), and the cell surface glycoproteins CD81, CD90 and CD201(**Figure 2G-H**). Our analysis revealed significant differences in marker expression: Mcam was found in over 65% of GFP^+ telocytes compared to just 14% of $tdTomato^+$ cells, while CD81 was present in 28% of GFP^+ cells but 54% of $tdTomato^+$ cells.

To examine the expression patterns of these markers and their relationship to native intestinal telocytes, we performed immunofluorescence staining on jejunum sections from Foxl1Cre; Rosa-mTmG mice, where telocytes are labeled with membrane-bound GFP

(green) (**Figure 2I-K**). We found that Mcam was co-expressed in most of the GFP$^+$ telocytes (**Figure 2J**). In contrast, CD81 showed minimal co-localization, being expressed in only a subset of telocytes located along the crypt epithelium (**Figure 2K** arrowhead). These findings demonstrate that immortalized telocytes maintain the original molecular profile of a heterogenous mesenchymal population, showing high enrichment for Foxl1, PDGFRα, CD34 and Mcam expression, effectively preserving their key characteristics and markers.

Immortalized telocytes exhibit matrix remodeling and contraction skills

We next aimed to explore functional characteristics of telocytes that are difficult to study in vivo but can be effectively examined using in vitro cultures. Given the polarized distribution of cytoskeletal elements in GFP$^+$ immortalized telocytes, we focused on their potential to contract and remodel the extracellular matrix (ECM). We stained GFP$^+$ immortalized telocytes for matrix metalloproteinase 10 (Mmp10), known for its role in ECM degradation and clinical relevance in modulating intestinal bowel disease (IBD)[41] and α-smooth muscle actin (αSMA), a marker linked to contractile activity[42]. Both Mmp10 and αSMA were positively stained in a subset of the GFP$^+$ immortalized telocytes (**Figure 3A**), highlighting that even when detached from their native environment, these immortalized telocytes retain significant heterogeneity.

Next, we utilized Matrigel-based contraction assay to evaluate the telocytes' ECM remodeling capabilities. This assay measures the contraction of the Matrigel dome to reflect cell-extracellular matrix interactions and cellular 3D contractility. Immortalized telocytes were embedded in Matrigel at a density of 10,000 cells per 30μl of Matrigel. Forty-two hours after seeding, the telocytes extended their processes and spread across the Matrigel dome (**Figure 3B-C**). We observed a significant contraction of the gel, indicating the telocytes' active participation in ECM manipulation. This contraction, along with the morphological rearrangement of telocytes, suggests their potential to influence tissue architecture through mechanical forces, underscoring their role in the matrix environment.

Immortalized telocytes retain expression of stem cell niche signaling molecules

In vivo, telocytes play a crucial role as a signaling hub maintaining intestinal homeostasis by supporting both stem cell function and enterocyte differentiation[1,2]. To determine if immortalized telocytes retain these niche functions, we assessed their expression of key signaling molecules.

Immunofluorescence staining revealed that a subset of immortalized telocytes expressed Porcupine (Porcn), an O-acyltrasferase localized in the endoplasmic reticulum, crucial for Wnt secretion (**Figure 4A**). This observation suggests that telocyte may exhibit heterogeneity in their ability to secrete Wnt proteins. Additionally, Vangl2, a receptor involved in the non-canonical Wnt-signaling pathway, displayed variable expression levels and a polarized distribution along the telocytes' extensions (**Figure 4B**). This pattern suggests that telocytes not only support signaling but also have the capability to sense and respond to these signals.

Quantitative PCR analysis further confirmed that immortalized telocytes retain key functions of the stem cell niche. Compared to mesenchymal telocyte-depleted population, immortalized telocytes showed significant enrichment of the canonical Wnt2b ligand (**Figure 4C).** Wnt2b, which is expressed in native telocytes along the crypt epithelium[2], is known to regulate intestinal inflammatory cytokines[43], and mutations in this gene are linked to neonatal-onset chronic diarrhea in humans[44].

In addition to Wnt2b, immortalized telocytes were enriched for the non-canonical Wnt5a, a ligand highly expressed in native villus-tip telocytes[3]. This enrichment further indicates that our cell line encompasses diverse populations of telocytes. Furthermore, Rspo2, a crucial Wnt inducer, and Noggin, a Bmp inhibitor - both vital for the stem cell niche - were also enriched in telocytes compared to mesenchymal telocyte-depleted population.

Interestingly, although Rspo3 was expressed in telocytes, its expression was higher in the telocyte-depleted population compared to telocytes, reflecting the in vivo situation [2]. smFISH for Wn5a validated the presence of mRNA molecules within telocytes, showing close proximity to the expression of mRNAs of the AP-1 transcription factor component Fos (**Figure 4D)**. This finding further supports the role of teloytes in both supporting and responding to signaling cues. Thus, immortalized telocytes maintain their heterogeneity,

encompassing diverse populations of telocytes that express stem cell niche factors in vitro.

Immortalized telocytes support organoid growth without additional growth factor supplements

Finally, we investigated whether immortalized telocytes retain their ability to maintain stem cell niche activity in vitro. Stem cells are characterized by their capacity to self-renew and differentiate into specialized progeny. Organoids - 3D tissue models formed by the proliferation and differentiation of either pluripotent or adult stem cells within a matrix scaffold - serve as a powerful tool in both fundamental and biomedical research (45-50). These models have been successfully established for various organs and tissues in both mice and humans.

The generation of organoids from adult or induced pluripotent human stem cells has opened up opportunities for modeling human development, disease, drug discovery and personalized medicine [45,46]. In these models, stem cell-containing crypts are isolated and embedded in Matrigel as an ex vivo replacement for the ECM. Crucially, the establishment and maintenance of organoids require supplementation with niche factors that support the formation of organized and functional tissue[45-49].

To assess the potential of immortalized telocytes to support organoid growth without additional niche factors, we co-cultured these cells with mouse small intestinal-derived organoids embedded in Matrigel. This setup was compared with tdTomato$^+$ mesenchymal telocyte-depleted cells, as well as organoids supplemented with or without external niche factors: EGF, Noggin, Rspondin and FGF (ENRF) (**Figure 5A** experimental design). By day 1 of culture, organoids in co-culture with GFP$^+$ immortalized telocytes exhibited the largest sphere morphology compared to all other conditions (**Figure 5B-C**).

We monitored organoid growth over four days. By day 2, organoids in all conditions - except those co-cultured with immortalized telocytes or supplemented with external niche factors - began to deteriorate (**Figure 5B**). In contrast, organoids co-cultured with GFP$^+$

immortalized telocytes not only maintained large sphere structures on days 1 and 2 but also began budding, differentiating, and forming crypt-like structures more prominently by day 3. Unlike tdTomato^{+} mesenchymal cells, which remained within the Matrigel, GFP^{+} immortalized telocytes extended and established close contact with the basal side of the epithelial organoids (**Figure 5D**), mimicking the positioning of telocytes in the intestine.

To evaluate stem cell activity, we compared organoids co-cultured with immortalized telocytes to those supplemented with external niche factors on day 4. Organoids were stained for Ki67, a proliferation marker and Sox9, a Wnt target gene expressed in intestinal stem and transit amplifying cells in vivo[50] (**Figure 5E**). Interestingly, organoids co-cultured with immortalized telocytes showed restricted expression of Ki67 and Sox9 at the base of the budding epithelium, unlike the broader expression across the entire budding segment observed in organoids supplemented with external factors (+ENRF). This suggests that immortalized telocytes promote a more controlled stem cell activity and differentiation, more closely reflecting the in vivo environment.

The ability of immortalized telocytes to support organoid growth without the supplementation of any other factors demonstrates that telocytes are not only essential but also sufficient for sustaining stem cell activity. This underscores their significant potential as a key source of stem cell niche factors in vitro. Consequently, this cell line could be pivotal for investigating stem cell and niche interactions under both normal and pathological conditions.

Immortalized telocytes support human organoid growth

To evaluate the clinical relevance of our immortalized telocyte cell line as a component of the stem cell niche, we tested its ability to support human organoids derived from normal colon samples without the addition of external niche factors. Remarkably, while human organoids cultured without external niche factors began to die by Day 2, those co-cultured with immortalized telocytes formed close associations with the telocytes and gradually developed cystic-like structures, similar to colon organoids grown in commercially enriched media (**Figure 6A-B**).

Notably, despite the telocyte cell line originating from the mouse small intestine and the organoids being derived from human colon tissue, the immortalized telocytes were still able to support organoid growth and preserve the original colon features. This suggests that telocytes may possess flexibility in their secretion profiles and niche-supporting factors, likely influenced by their interactions with the epithelium.

In conclusion, our findings highlight the potential of immortalized telocytes as a valuable resource for studying stem cell-niche interactions across both mouse and human models. This emphasizes the relevance of this cell line for applications in clinical research.

Discussion

Telocytes have recently been recognized as crucial components of the intestinal stem cell niche, yet their detailed biology and the specific mechanisms by which they sustain stem cell function and intestinal homeostasis remain largely unexplored. In this study, we developed an immortalized telocyte cell line that retains the cellular and molecular characteristics of native intestinal telocytes. These immortalized telocytes continue to express key niche factors and support the stem cell activity of both mouse- and human-derived organoids without the need for external supplementation.

Our research underscores the unique role of telocytes within the stem cell niche, as we demonstrated that mesenchyme lacking telocytes cannot support organoid growth. This finding suggests that immortalized telocytes retain critical stem cell niche functions in vitro, emphasizing their importance in maintaining stem cell niche activity.

Stem cells hold significant potential for applications ranging from regenerative medicine to patient-specific disease modeling, toxicology screening, and cultivated food production. However, directing stem cells to exhibit desired behaviors, such as expanding in a naïve state or differentiating into specific mature lineages, remains a challenge. The heterogenous networks of telocytes maintained in vitro, which express diverse signaling molecules that support both stem cell maintenance and differentiation, hold promise for more accurately recapitulating the natural differentiation process of stem cells. This approach provides a more native environment for controlling stem cell activity compared to the external addition of factor cocktails.

Co-culturing telocytes with organoids, rather than relying solely on niche factors, offers a valuable approach for studying the molecular and cellular mechanisms involved in stem cell growth and differentiation within their niche. This method enables the exploration of reciprocal communication and bidirectional signaling between stem cells and their niche-dynamic that cannot fully captured through external factors alone.

The establishment of an immortalized telocyte cell line will significantly enhance our understanding of basic telocyte biology, such as RNA localization along cellular extensions and the mechanisms they use to communicate with and signal to the epithelium. Advances in genome editing and CRISPR technology further enable the manipulation of both telocytes and organoids, offering new insights into stem cell-niche interactions in both homeostasis and disease.

Moreover, the ability of immortalized telocytes to support human organoid growth opens up opportunities to explore clinically relevant questions about telocyte interactions in both healthy and diseased states.

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Author Contributions

M.C wrote the manuscript, conceived, carried out experiments, analyzed and interpreted the data. A.G carried out smFISH experiments and FACS analysis. D.D carried out Matrigel contraction assay and human organoid culture experiments; L.B.-S wrote the Helsinki protocol. Y.B-N, I.B-P, M.G, and M.S.-C designed and supervised the study. M.S.-C wrote the manuscript and directed the study.

Declaration of Interests

The authors declare no competing interests.

Figure legends

Figure 1. Immortalized telocytes retain structural characteristics

(**A**) Schematic illustrating the experimental strategy for creating the immortalized telocyte cell line. The jejunum from Foxl1-Cre; Rosa26-mTmG mouse model was dissected, and the mesenchyme was isolated and sorted into GFP^+ telocytes and $tdTomato^+$ mesenchymal cells (depleted of telocytes) and cultured separately. Each cell population was then infected with SV40 T-antigen viral particles and selected with hygromycin to obtain the infected cells. (**B**) Live imaging of immortalized telocyte cell culture reveals key cellular characteristics of these cells. Note the length of telopodes (194 µm), the branching, and the presence of dilated regions, podoms (indicated by asterisk). Additionally, immortalized telocytes form a network by interconnecting through several telopodes and cellular protrusions. (**C**) Immunofluorescence of immortalized telocytes stained for GFP (green) and DAPI (blue) reveals the thin flat structure of telocytes and their interconnected network. (**D**) Immunofluorescence of immortalized telocytes stained for phalloidin (red) reveals asymmetrical distribution of actin filaments beneath the cell membrane and along cellular extensions. (**E**) Immunofluorescence of immortalized telocytes stained for α-tubulin (red) reveals the distribution of both thick and thin microtubule fibers, including branched structures across various cell compartments. Scale bar 100 µm, 10 µm.

Figure 2. Immortalized telocytes preserve molecular expression profile

(**A–B**) Single molecule RNA in situ fluorescence hybridization (smFISH) for Foxl1 reveals that Foxl1 mRNA (white) is expressed in GFP^+ immortalized telocytes but not $tdTomato^+$ immortalized mesenchymal cells. Scale bar 10 µm. (**C–F**) FACS analysis and immunofluorescence of GFP^+ immortalized telocytes stained for PDGFRα (C-D) and CD34 (F-E) identifies two populations: $PDGFR\alpha^{High}$ and $PDGFR\alpha^{Low}$. The majority of GFP^+ immortalized telocytes are $CD34^{Low}$ (**G–H**) FACS analysis of immortalized GFP^+ telocytes and $tdTomato^+$ mesenchymal cells for mesenchymal markers shows that GFP^+ telocytes are enriched for Mcam expression compared to $tdTomato^+$ mesenchymal cells. Conversely, Cd81 expression is enriched in $tdTomato^+$ mesenchymal cells relatived to

GFP$^+$ telocytes. (**I-K**) Immunofluorescence of Foxl1Cre; Rosa-mTmG mouse jejunum sections stained for Mcam (J) and Cd81(K) reveals that Mcam is predominantly expressed in GFP+ telocytes and Cd81 is expressed in GFP+ telocytes located along the crypt epithelium (K arrowhead).

Figure 3. Immortalized telocytes exhibit matrix remodeling and contraction skills

(A) Immunofluorescence of GFP$^+$ immortalized telocytes for matrix metalloproteinase10 and alpha smooth muscle actin (αSMA) (red) reveals expression in a subset of GFP$^+$ immortalized telocytes (green). (**B**) Confocal images of Matrigel domes with and without 10,000 GFP$^+$ immortalized telocytes. Images were acquired 0, 18, 42 and 92hours post-seeding. Scale bar 1 mm. (**C**) Quantification of Matrigel area with and without GFP$^+$ immortalized telocytes.

Figure 4. Immortalized telocytes retain expression of stem cell niche signaling molecules

(**A–B**) Immunofluorescence of GFP$^+$ immortalized telocytes for the Wnt modifier enzyme, Porcupine and the non-canonical Wnt receptor Vangolin2 (red) reveals their expression in a subset of GFP$^+$ immortalized telocytes. Scale bar 100 µm. (**C**) RT-qPCR analysis of GFP$^+$ immortalized telocytes reveals the expression of stem cell niche signaling molecules in GFP$^+$ immortalized telocytes. (**D**) smFISH for Wnt5a (white) and Fos (red) reveals the expression of the signaling molecules in GFP$^+$ immortalized telocytes. Scale bar 10 µm, 1 µm.

Figure 5. Immortalized telocytes support organoid growth without additional growth factor supplements

(**A**) Schematic illustration of organoid co-culture strategy. Mouse small intestinal organoids were cultured under four conditions: in co-cultured with tdTomato+ immortalized mesenchymal cells, in co-culture with GFP$^+$ immortalized telocytes, supplemented with Egf, Noggin, Rspo1 and FGF or organoids only (**B**) Photographs of cultured organoids, taken daily over four days, show that organoids die within 24 hours under all conditions except when co-cultured with GFP$^+$ immortalized telocytes or

supplemented with external factors. (**C**) Quantification of organoid diameter 24 hours after seeding reveals that organoids co-cultured with GFP^+ immortalized telocytes formed significantly larger spheroids compared to those in all other conditions ($p < 0.005$). (**D**) Confocal images reveal that GFP^+ immortalized telocytes establish close contact with organoids, whereas tdTomato+ immortalized mesenchymal cells are embedded in the Matrigel alongside dying organoids (**E**) Immunofluorescence of organoids co-cultured with GFP^+ immortalized telocytes or supplemented with external growth factors, stained for Ki67 and Sox9 on Day 4, shows that in co-cultured organoids, expression is restricted to the base of the budding region. In contrast, supplemented organoids exhibit broad expression along the entire budding region. Asterisk- staining of immortalized telocytes. Scale bar 100 µm.

Figure 6. Immortalized telocytes support human organoid growth

(**A**) Photographs of human colon organoids show that organoids without GFP^+ immortalized telocytes dye, whereas organoids co-cultured with GFP^+ immortalized telocytes grow gradually similar to colon organoids cultured in enriched commercial media. Note the close contact between organoids and telocytes. (**B**) Quantification of organoid diameter reveals gradual growth in organoids co-cultured with GFP^+ immortalized telocytes similar to organoids cultured in enriched media.

Methods

Mouse models

Foxl1-Cre mice[31] were crossed with Rosa-membrane-targeted dimer tomato protein (mT) or membrane targeted green fluorescent protein (mG) (Rosa-mTmG)[32] (Jackson Laboratories, Bar Harbor, ME #007676).
All animal experiments were approved by the Animal Care and Use Committee of the Hebrew University of Jerusalem.

Intestinal mesenchyme isolation and culture

Foxl1-Cre; Rosa-mTmG mouse jejunum was dissected and mesenchyme was isolated as previously described[13]. Briefly, the intestine was cut into 0.5 cm pieces. The epithelium

was removed using 1 mM DL-dithiothreitol (DTT) and 2 mM EDTA. The remaining mesenchyme was then digested with 100 U/ml collagenase type VIII and 75 mg/ml DNase I in CM1640. Both primary and immortalized mesenchymal cells were cultured in DMEM medium containing 15% Fetal Bovine Serum and 1% Penicillin-Streptomycin.

Immortalization by SV-40 Large T-antigen infection

FACS-sorted GFP^+ telocytes and $tdTomato^+$, mesenchymal telocyte-depleted cells were infected with viral particles expressing the SV40 Large T antigen (Addgene #170255), two weeks after sorting. Virus production followed standard procedures, involving transfection of a lentiviral vector encoding the protein, pHR-ΔR8.2 packaging vector, and the pCMV-VSV-G envelope plasmid into HEK293T cells. After transfection, supernatants containing viral particles were collected and concentrated by centrifugation. Intestinal mesenchymal cells were infected with 50µl of concentrated viral particles in 5-cm dishes, and selection was performed with Hygromycin (Thermo Fisher Scientific).

FACS analysis

For single-cell suspension preparation, both primary and immortalized mesenchymal cells were incubated for 7 minutes with 0.25% Trypsin/1mM EDTA, then centrifuged at 1800 rpm for 5 minutes at 4°C. Single cells were resuspended in FACS buffer (5% FBS / PBS without Ca^{2+} and Mg^{2+}). To optimize fluorescence compensation settings, anti-mouse (BD #552843) and anti-rat (BD #552845) beads were used. Cells were sorted based on GFP and tdTomato signals. For FACS analysis GFP^+ immortalized Telocytes and $tdTomato^+$ telocyte-depleted mesenchymal cells, were incubated for 30 minutes with the following antibodies:

Antibody	Company	Catalogue number	Fluorophore
CD45	BD	563792	BUV395
CD31	Biolegend	102526	PECF594
GP38	Biolegend	127418	APC-CY7
CD34	Biolegend	119321	BV421
CD201	Biolegend	141506	APC
CD90	Biolegend	140318	BV605

CD81	Biolegend	104912	Percpcy5.5
CD138	Biolegend	142514	PE-CY7
CD146	Biolegend	361022	BV510
CD321	BD	744657	BV786
CD249	BD	741743	BUV737
CD326	Biolegend	118245	BV786

Immunofluorescence

Cultured cells and organoids were fixed in 4% PFA for 20 minutes at room temperature. Fixed cells were incubated with primary and secondary antibodies diluted in CAS-Block (Invitrogen 008120) and imaged using a Nikon Eclipse Ti2 Confocal System (Nikon, Japan). Image processing was performed using Fiji or NIS Elements software packages.

RNA isolation and RT-qPCR

Passage 7 GFP^+ immortalized telocytes and $tdTomato^+$ mesenchymal telocyte-depleted cells were seeded on a 10cm Petri dishes and cultured. RNA was extracted using TRIzol reagent (Thermo Fisher,15596018) according to the manufacturer's instructions. Briefly, approximately 5x10^6 cells per dish were incubated with 1ml of TRIzol. The cell lysate was then treated with chloroform and isopropanol to extract the RNA. RNA quantity and quality were assessed using NanoDrop™ and Qubit ™ (Thermo Fisher, A38189). Up to 1µg RNA was converted to cDNA using the iScript cDNA synthesis kit (Bio-Rad, 1725037), and qRT-PCR was performed in duplicate reactions using the LunaScript® RT SuperMix Kit (NEB, E3010). Expression values were normalized to HRPT-1 and GAPDH levels for GFP^+ and $tdTomato^+$ cells. mRNA levels were measured by TaqMan, and primer list is provided in the following table:

Gene name	Direction	Sequence
Wnt2b	Forward Reverse	CCATTACGGTGTTCGCTTTGCC CAGCTTCAGGAATCTCCGAACAG
Wnt5a	Forward Reverse	GGAACGAATCCACGCTAAGGGT AGCACGTCTTGAGGCTACAGGA
GFP	Forward	CTGCTGCCCGACAACCAC

	Reverse	TCACGAACTCCAGCAGGA
Noggin	Forward Reverse	GCCAGCACTATCTACACATCCG CGTCTCGTTCAGATCCTTCTCC
Wnt4	Forward Reverse	GAGAACTGGAGAAGTGTGGCTG CTGTGAGAAGGCTACGCCATAG
Rspo3	Forward Reverse	CCAAGTGGATATTACGGAACTCG CGCAACTGTCAAGGCACTTTCC
Rspo2	Forward Reverse	ACCGATGGAGACGCAATAAGCG GCACTCTCCATACTGACGCATTC

Single-molecule RNA Fluorescence in-situ hybridization (smFISH)

Cells were fixed in 3.7% Formaldehyde for 20 minutes at room temperature. Custom probe libraries, designed using the Stellaris FISH Probe Designer software (Biosearch Technologies, Inc., Petaluma, CA) and coupled to Cy5 or TMR were used for hybridization with the target coding RNA.

For hybridization preparation, cells were permeabilized with 10 µg/ml proteinase K (Ambion AM2546) for 3 minutes, followed by two washes in 2×SSC (Ambion AM9765). Cells were then incubated in wash buffer (20% formamide, Ambion AM9342, 2× SSC) for 5 minutes before hybridization. The hybridization was carried out overnight at 30°C with smFISH probes diluted 1:3000 in hybridization buffer (10% dextran sulfate, Sigma D8906, 20% formamide, 1 mg/ml E. coli tRNA, Sigma R1753, 2× SSC, 0.02% BSA, Ambion AM2616, 2 mM vanadyl-ribonucleoside complex, NEB S1402S).

After hybridization, cells were washed with wash buffer containing 100 ng/ml DAPI at 30°C for 30 minutes. For antibody staining, the GFP antibody was diluted in hybridization buffer, and the Alexa 488 secondary antibody was diluted in GLOX buffer and incubated for 20 minutes. DAPI staining was performed using Sigma-Aldrich, D9542.

Matrigel contraction assay

GFP^+ Immortalized telocytes were seeded at a density of 10,000 cells in 30µl Matrigel (BD Biosciences, 356230). Images of Matrigel domes were captured at 1,18, 42 and 92hours post-seeding. The circumference of Matrigel domes was measured using Fiji, and area quantification was analyzed and plotted in GraphPad Prism 9.5.1.

Organoid-telocytes co-culture assay

Crypts were isolated from the jejunum of 10 to 15-week-old wild-type mice as previously described[33]. Briefly, the jejunum was flushed with ice-cold PBS, cut into pieces smaller than 5mm, and crypts were isolated by mechanical dissociation in PBS with 2mM EDTA on ice. Crypts were embedded onto Matrigel (BD Biosciences, 356230), 30μl per well in 24-well-plate. The culture medium was DMEM/F12 (Gibco) containing GlutaMax (1:100, Gibco) and Penicillin-Streptomycin (1:100 Biological Industries) supplemented with B-27 (1:50 Gibco), mouse Noggin (100 ng/ml Peprotech 250-38), mouse Rspo1 (500 ng/ml Peprotech, 315-32), mouse EGF (20 ng/ml Peprotech, 315-09), and human basic Fgf (10 ng/ml Peprotech, 450-33). The medium was changed every 2 days until co-culture.

To establish co-culture of murine organoids with GFP^+ immortalized telocytes or $tdTomato^+$ mesenchymal telocyte-depleted cells, organoids were dissociated, and mesenchymal cells were trypsinized (incubated 7 minutes in 0.25% Trypsin-1mM EDTA, then centrifuged at 1800 rpm for 5 minutes at 4°C). 10,000 mesenchymal cells were embedded onto 30μl Matrigel. The four different conditions were as follow: organoids, organoids+ supplements (ENRF), organoids+ immortalized telocytes, organoids+ immortalized mesenchymal telocyte-depleted cells. Images were acquired once a day for four consecutive days, and organoid diameters were measured using Fiji and analyzed with GraphPad Prism 9.5.1.

Human healthy colon organoids were obtained from the Organoid Center of Hadassah-Hebrew University Medical Organization, Jerusalem, under the Helsinki protocol HMO-20-921 and were co-cultured using the same protocol as for the mouse organoids. Organoids, prior co-culture, were grown in Intesticult Organoid growth Medium (Human) from StemCell technologies.

Supplemental Information

Video 1. Live imaging of GFP^+ immortalized telocyte cell line (6 minutes, 331 frames, 6fps) reveals the dynamic structure of telocytes before and after cell division. The video highlights how telocytes establish and maintain interconnected networks. Scale bar 50 µm.

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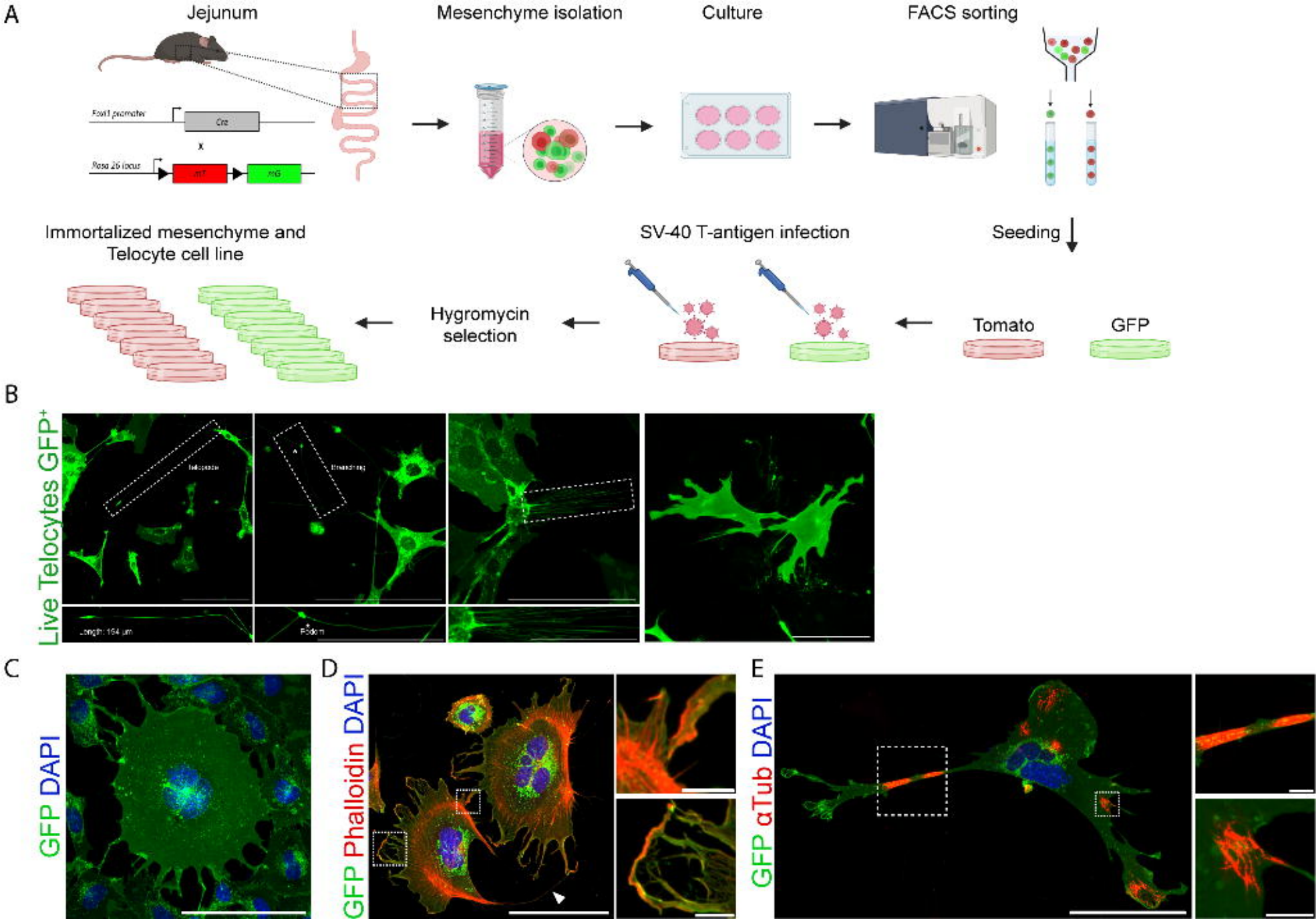
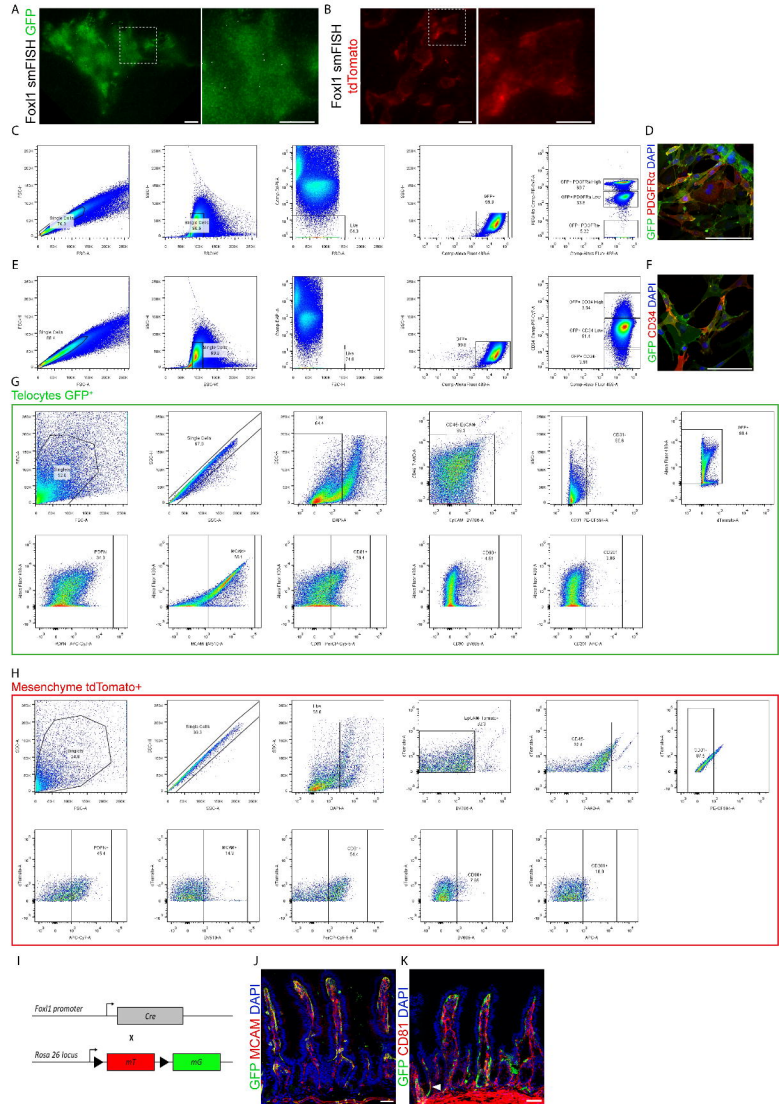

A
Jejunum
Mesenchyme isolation
Culture
FACS sorting
Foxl1 promoter
Cre
x
Rosa 26 locus
mT
mG
Immortalized mesenchyme and
Telocyte cell line
SV-40 T-antigen infection
Seeding
Hygromycin
selection
Tomato
GFP
B
Live Telocytes GFP+
C
GFP DAPI
D
GFP Phalloidin DAPI
E
GFP αTub DAPI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Foxl1 smFISH GFP
B
Foxl1 smFISH tdTomato
C
D
GFP PDGFRα DAPI
E
F
GFP CD34 DAPI
G
Telocytes GFP+
H
Mesenchyme tdTomato+
I
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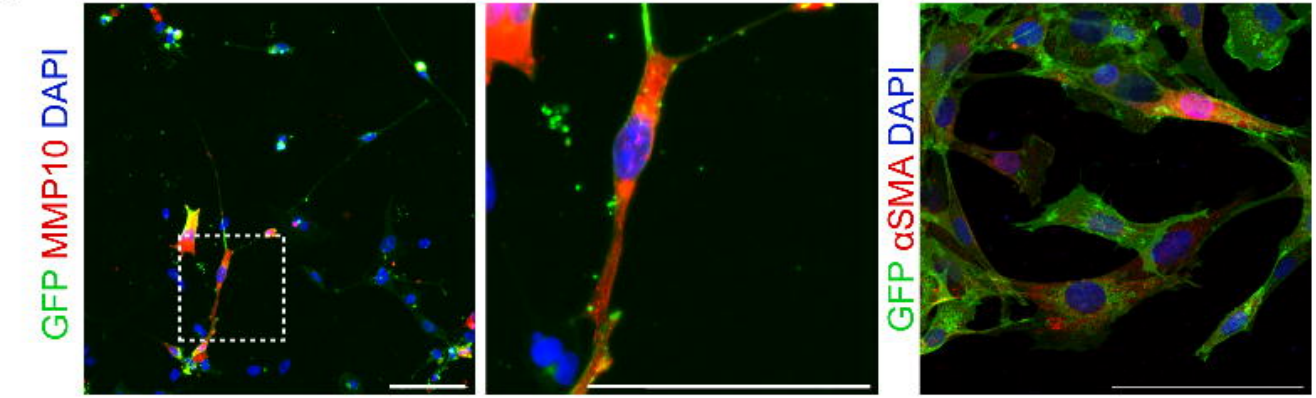
Cre
x
Rosa 26 locus
mT
mG
J
GFP MCAM DAPI
K
GFP CD81 DAPI

A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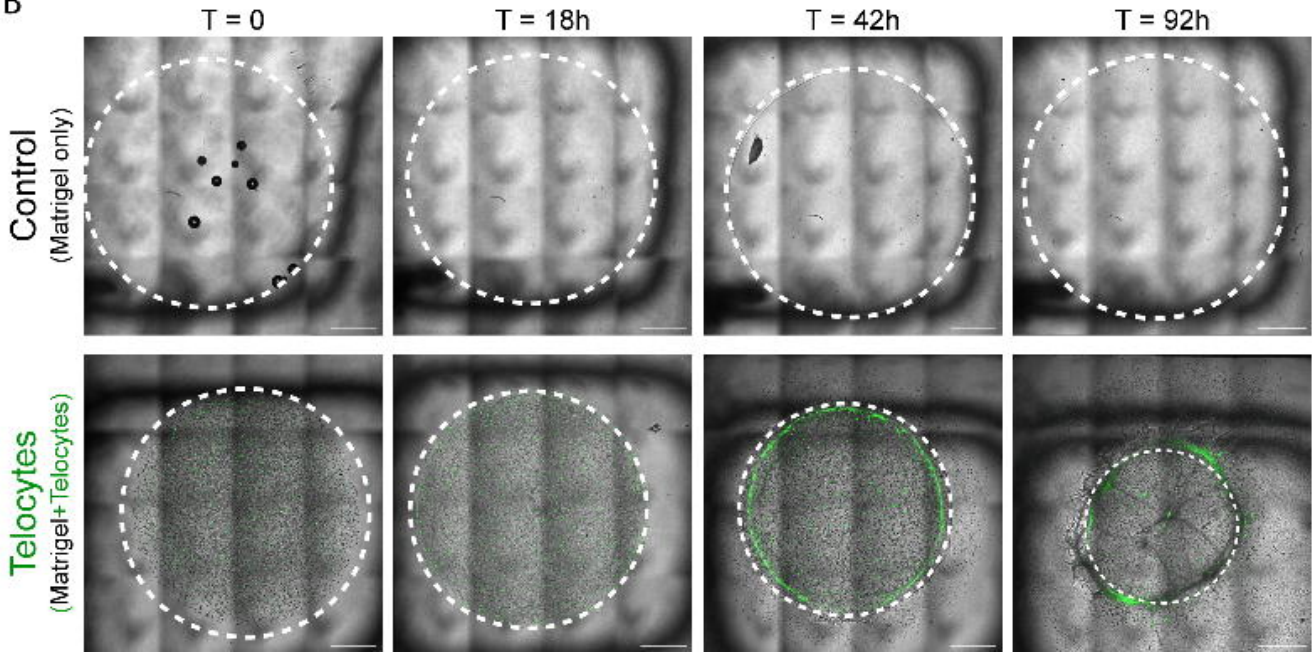
GFP αSMA DAPI

B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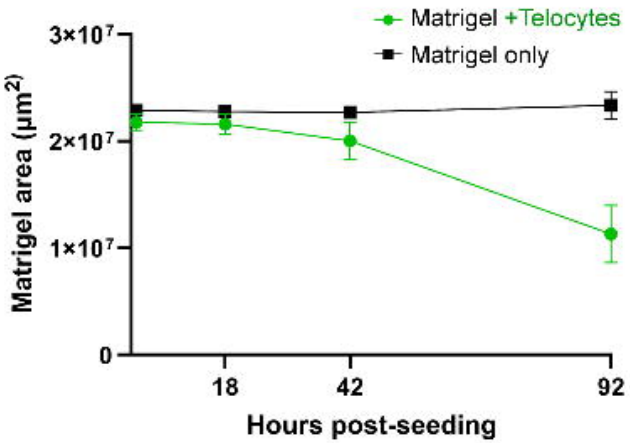
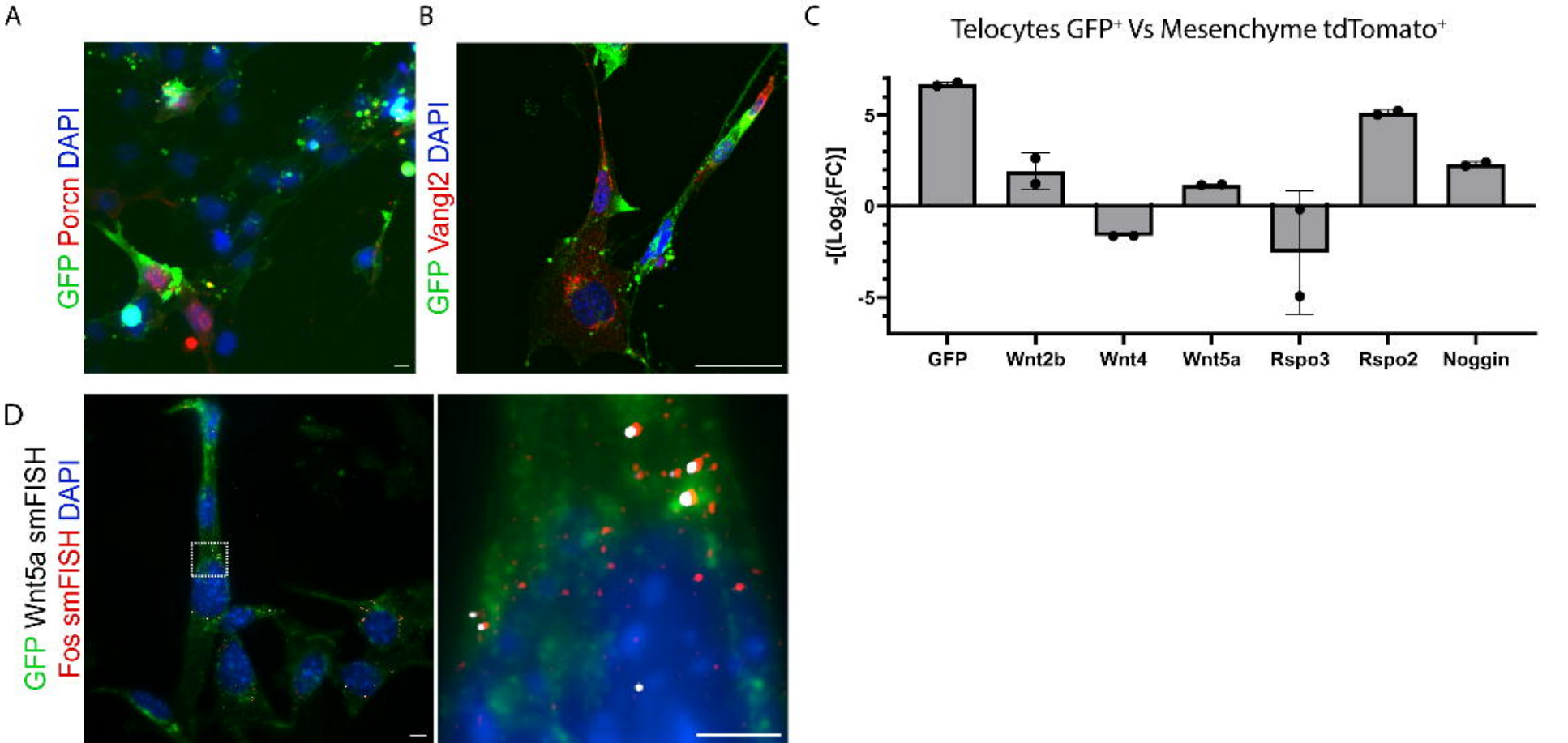
T = 18h
T = 42h
T = 92h
Control
(Matrigel only)
Telocytes
(Matrigel+Telocytes)

C


Matrigel +Telocytes
Matrigel only
Matrigel area (μm²)
3×10⁷
2×10⁷
1×10⁷
0
18
42
92
Hours post-seeding


A
GFP Porcn DAPI
B
GFP Vangl2 DAPI
C
Telocytes GFP+ Vs Mesenchyme tdTomato+
-[(Log2(FC)]
5
0
-5
GFP
Wnt2b
Wnt4
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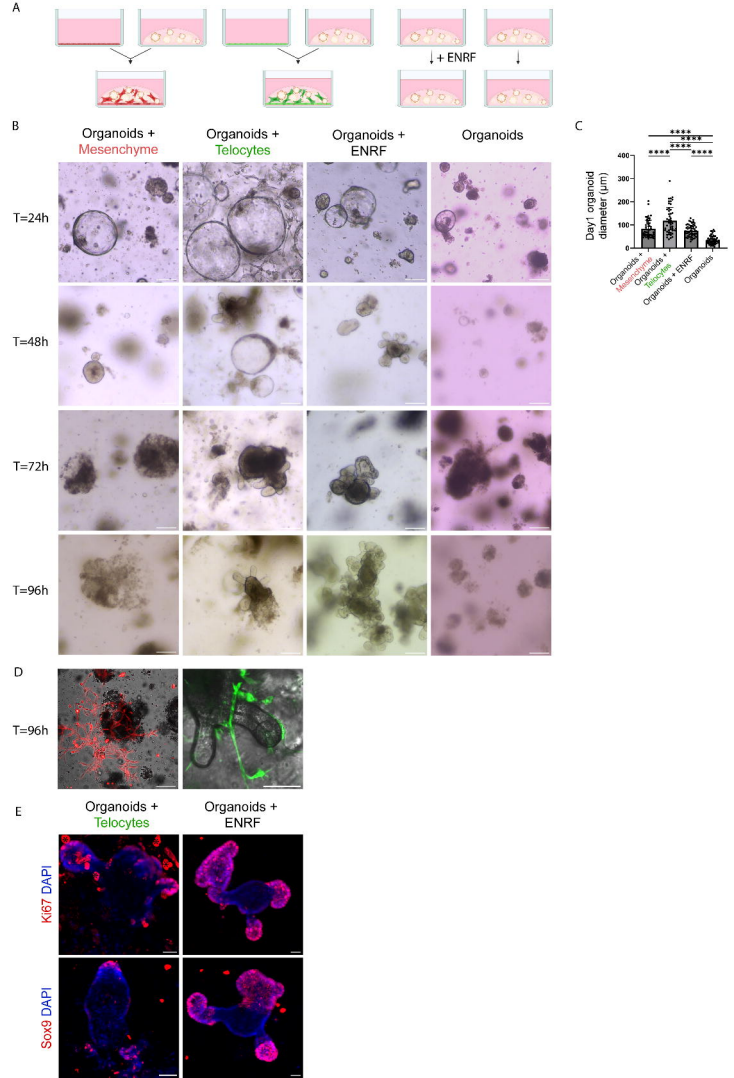
Rspo3
Rspo2
Noggin
D
GFP Wnt5a smFISH
Fos smFISH DAPI


A
+ ENRF
B
Organoids + Mesenchyme
Organoids + Telocytes
Organoids + ENRF
Organoids
T=24h
T=48h
T=72h
T=96h
C
Day1 organoid diameter (µm)
400
300
200
100
0
****
****
****
****
ns
Organoids + Mesenchyme
Organoids + Telocytes
Organoids + ENRF
Organoids
D
T=96h
E
Organoids + Telocytes
Organoids + ENRF
Ki67 DAPI
Sox9 DAPI

A

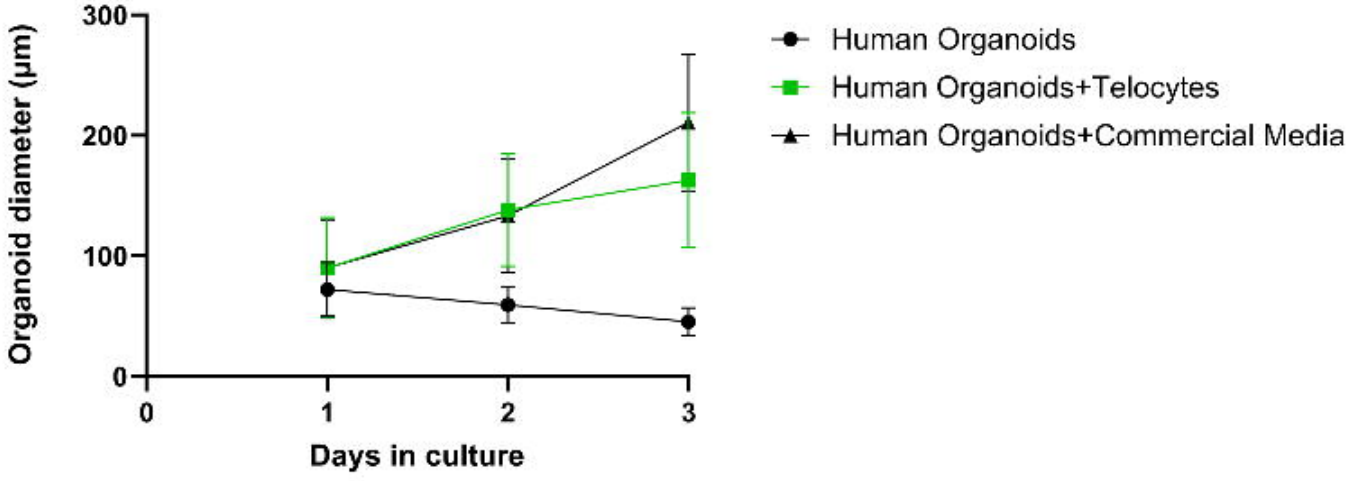
Human organoids

Human organoids+ Telocytes

Human organoids+ Commercial media

T=24h

T=48h

T=72h

B