

Supplemental Information

L-WRN conditioned medium for gastrointestinal epithelial stem cell culture shows replicable batch-to-batch activity levels across multiple research teams

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Table S1. Oligonucleotide sequences used for quantitative reverse transcriptase PCR.

Species	Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
Mouse	Axin2	TGACTCTCCTTCCAGATCCCA	TGCCCACACTAGGCTGACA
Mouse	Car1	ACAGTAGCAACCAATCTGTTCTG	AGGCCATCAGCCTTGGAGA
Mouse	Gapdh	TCAAGAAGGTGGTGAAGCAGG	TATTATGGGGGTCTGGGATGG
Mouse	Lgr5	CCTACTCGAAGACTTACCCAGT	GCATTGGGGTGAATGATAGCA
Mouse	MKi67	ATCATTGACCGCTCCTTTAGGT	GCTCGCCTTGATGGTTCCT
Human	AXIN2	CATGACGGACAGCAGTGTAGA	AACTCCAGCTTCAGCTTTTCC
Human	CAR1	ACAACGATAACCGATCAGTGC	CTTTGCAGAATTCCAGTGAGC
Human	GAPDH	GACCTGCCGTCTAGAAAAACC	GCTGTAGCCAAATTCGTTGTC
Human	LGR5	CCTTCATAAGAAAGATGCTGGAAT	GTTTAATGGGGGAAATGTACAGAG
Human	MKI67	AAGAGAGTGTCTATCAGCCGAAGT	GTGGCCTGTACTAAATTGACTGTG

Table S2. Publications citing use of L-WRN CM for gastrointestinal stem cell culture*.

General subject category	Citation	PMID
Epithelial development & physiology (n=15)	Beyaz S, Mana MD, Roper J, Kedrin D, Saadatpour A, Hong SJ, Bauer-Rowe KE, Xifaras ME, Akkad A, Arias E, Pinello L, Katz Y, Shinagare S, Abu-Remaileh M, Mihaylova MM, Lamming DW, Dogum R, Guo G, Bell GW, Selig M et al. (2016) High-fat diet enhances stemness and tumorigenicity of intestinal progenitors. <i>Nature</i> 531: 53-8	26935695
	Curtis VF, Cartwright IM, Lee JS, Wang RX, Kao DJ, Lanis JM, Burney KM, Welch N, Hall CHT, Goldberg MS, Campbell EL, Colgan SP (2018) Neutrophils as sources of dinucleotide polyphosphates and metabolism by epithelial ENPP1 to influence barrier function via adenosine signaling. <i>Mol Biol Cell</i> 29: 2687-2699	30188771
	Demitrack ES, Gifford GB, Keeley TM, Carulli AJ, VanDussen KL, Thomas D, Giordano TJ, Liu Z, Kopan R, Samuelson LC (2015) Notch signaling regulates gastric antral LGR5 stem cell function. <i>EMBO J</i> 34: 2522-36	26271103
	Gifford GB, Demitrack ES, Keeley TM, Tam A, La Cunza N, Dedhia PH, Spence JR, Simeone DM, Saotome I, Louvi A, Siebel CW, Samuelson LC (2017) Notch1 and Notch2 receptors regulate mouse and human gastric antral epithelial cell homeostasis. <i>Gut</i> 66: 1001-1011	26933171
	Miyoshi H, VanDussen KL, Malvin NP, Ryu SH, Wang Y, Sonnek NM, Lai CW, Stappenbeck TS (2017) Prostaglandin E2 promotes intestinal repair through an adaptive cellular response of the epithelium. <i>EMBO J</i> 36: 5-24	27797821
	Moon C, VanDussen KL, Miyoshi H, Stappenbeck TS (2014) Development of a primary mouse intestinal epithelial cell monolayer culture system to evaluate factors that modulate IgA transcytosis. <i>Mucosal Immunol</i> 7: 818-28	24220295
	Patel KK, Miyoshi H, Beatty WL, Head RD, Malvin NP, Cadwell K, Guan JL, Saitoh T, Akira S, Seglen PO, Dinanuer MC, Virgin HW, Stappenbeck TS (2013) Autophagy proteins control goblet cell function by potentiating reactive oxygen species production. <i>EMBO J</i> 32: 3130-44	24185898
	Powell RH, Behnke MS (2017) WRN conditioned media is sufficient for in vitro propagation of intestinal organoids from large farm and small companion animals. <i>Biol Open</i> 6: 698-705	28347989
	Puzan M, Hosic S, Ghio C, Koppes A (2018) Enteric Nervous System Regulation of Intestinal Stem Cell Differentiation and Epithelial Monolayer Function. <i>Sci Rep</i> 8: 6313	29679034
	Rauch I, Deets KA, Ji DX, von Moltke J, Tenthorey JL, Lee AY, Philip NH, Ayres JS, Brodsky IE, Gronert K, Vance RE (2017) NAIP-NLRC4 Inflammasomes Coordinate Intestinal Epithelial Cell Expulsion with Eicosanoid and IL-18 Release via Activation of Caspase-1 and -8. <i>Immunity</i> 46: 649-659	28410991

	Riehl TE, Santhanam S, Foster L, Ciorba M, Stenson WF (2015) CD44 and TLR4 mediate hyaluronic acid regulation of Lgr5+ stem cell proliferation, crypt fission, and intestinal growth in postnatal and adult mice. <i>Am J Physiol Gastrointest Liver Physiol</i> 309: G874-87	26505972
	Saito-Diaz K, Benchabane H, Tiwari A, Tian A, Li B, Thompson JJ, Hyde AS, Sawyer LM, Jodoin JN, Santos E, Lee LA, Coffey RJ, Beauchamp RD, Williams CS, Kenworthy AK, Robbins DJ, Ahmed Y, Lee E (2018) APC Inhibits Ligand-Independent Wnt Signaling by the Clathrin Endocytic Pathway. <i>Dev Cell</i> 44: 566-581 e8	29533772
	Sumigray KD, Terwilliger M, Lechler T (2018) Morphogenesis and Compartmentalization of the Intestinal Crypt. <i>Dev Cell</i> 45: 183-197 e5	29689194
	Szabady RL, Louissaint C, Lubben A, Xie B, Reeksting S, Tuohy C, Demma Z, Foley SE, Faherty CS, Llanos-Chea A, Olive AJ, Mrsny RJ, McCormick BA (2018) Intestinal P-glycoprotein exports endocannabinoids to prevent inflammation and maintain homeostasis. <i>J Clin Invest</i> 128: 4044-4056	30102254
	Tsuruta T, Saito S, Osaki Y, Hamada A, Aoki-Yoshida A, Sonoyama K (2016) Organoids as an ex vivo model for studying the serotonin system in the murine small intestine and colon epithelium. <i>Biochem Biophys Res Commun</i> 474: 161-167	27105910
Regenerative medicine & tissue engineering (n=3)	Kasendra M, Tovaglieri A, Sontheimer-Phelps A, Jalili-Firoozinezhad S, Bein A, Chalkiadaki A, Scholl W, Zhang C, Rickner H, Richmond CA, Li H, Breault DT, Ingber DE (2018) Development of a primary human Small Intestine-on-a-Chip using biopsy-derived organoids. <i>Sci Rep</i> 8: 2871	29440725
	Tsai YH, Czerwinski M, Wu A, Dame MK, Attili D, Hill E, Colacino JA, Nowacki LM, Shroyer NF, Higgins PDR, Kao JY, Spence JR (2018) A Method for Cryogenic Preservation of Human Biopsy Specimens and Subsequent Organoid Culture. <i>Cell Mol Gastroenterol Hepatol</i> 6: 218-222 e7	30105282
	Wang Y, Gunasekara DB, Reed MI, DiSalvo M, Bultman SJ, Sims CE, Magness ST, Allbritton NL (2017) A microengineered collagen scaffold for generating a polarized crypt-villus architecture of human small intestinal epithelium. <i>Biomaterials</i> 128: 44-55	28288348
Host-microbiome & host-pathogen interactions (n=14)	den Hartog G, Chattopadhyay R, Ablack A, Hall EH, Butcher LD, Bhattacharyya A, Eckmann L, Harris PR, Das S, Ernst PB, Crowe SE (2016) Regulation of Rac1 and Reactive Oxygen Species Production in Response to Infection of Gastrointestinal Epithelia. <i>PLoS Pathog</i> 12: e1005382	26761793
	Han X, Lee A, Huang S, Gao J, Spence JR, Owyang C (2018) Lactobacillus rhamnosus GG prevents epithelial barrier dysfunction induced by interferon-gamma and fecal supernatants from irritable bowel syndrome patients in human intestinal enteroids and colonoids. <i>Gut Microbes</i> : 1-18	30040527
	Hill DR, Huang S, Nagy MS, Yadagiri VK, Fields C, Mukherjee D, Bons B, Dedhia PH, Chin AM, Tsai YH, Thodla S, Schmidt TM, Walk S, Young VB, Spence JR (2017) Bacterial colonization stimulates a complex physiological response in the immature human intestinal epithelium. <i>Elife</i> 6	29110754

	Howitt MR, Lavoie S, Michaud M, Blum AM, Tran SV, Weinstock JV, Gallini CA, Redding K, Margolskee RF, Osborne LC, Artis D, Garrett WS (2016) Tuft cells, taste-chemosensory cells, orchestrate parasite type 2 immunity in the gut. <i>Science</i> 351: 1329-33	26847546
	Kaiko GE, Ryu SH, Koues OI, Collins PL, Solnica-Krezel L, Pearce EJ, Pearce EL, Oltz EM, Stappenbeck TS (2016) The Colonic Crypt Protects Stem Cells from Microbiota-Derived Metabolites. <i>Cell</i> 165: 1708-1720	27814510
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	Moon C, Baldridge MT, Wallace MA, D CA, Burnham, Virgin HW, Stappenbeck TS (2015) Vertically transmitted faecal IgA levels determine extra-chromosomal phenotypic variation. <i>Nature</i> 521: 90-93	25686606
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	Pan J, Zhang L, Odenwald MA, Shen L, Turner JR, Bergelson JM (2015) Expression of human decay-accelerating factor on intestinal epithelium of transgenic mice does not facilitate infection by the enteral route. <i>J Virol</i> 89: 4311-8	25653430
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	Verma MS, Fink MJ, Salmon GL, Fornelos N, Ohara TE, Ryu SH, Vlamakis H, Xavier RJ, Stappenbeck TS, Whitesides GM (2018) A Common Mechanism Links Activities of Butyrate in the Colon. <i>ACS Chem Biol</i> 13: 1291-1298	29584955
	Wilke G, Ravindran S, Funkhouser-Jones L, Barks J, Wang Q, VanDussen KL, Stappenbeck TS, Kuhlenschmidt TB, Kuhlenschmidt MS, Sibley LD (2018) Monoclonal Antibodies to Intracellular Stages of Cryptosporidium parvum Define Life Cycle Progression In Vitro. <i>mSphere</i> 3	29848759
	Williamson IA, Arnold JW, Samsa LA, Gaynor L, DiSalvo M, Cocchiari JL, Carroll I, Azcarate-Peril MA, Rawls JF, Allbritton NL, Magness ST (2018) A High-Throughput Organoid Microinjection Platform to Study Gastrointestinal Microbiota and Luminal Physiology. <i>Cell Mol Gastroenterol Hepatol</i> 6: 301-319	30123820
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Disease pathogenesis (n=8)	Bradford EM, Ryu SH, Singh AP, Lee G, Goretsky T, Sinh P, Williams DB, Cloud AL, Gounaris E, Patel V, Lamping OF, Lynch EB, Moyer MP, De Plaen IG, Shealy DJ, Yang GY, Barrett TA (2017) Epithelial TNF Receptor Signaling Promotes Mucosal Repair in Inflammatory Bowel Disease. <i>J Immunol</i> 199: 1886-1897	28747340
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	Garber JJ, Mallick EM, Scanlon KM, Turner JR, Donnenberg MS, Leong JM, Snapper SB (2018) Attaching-and-Effacing Pathogens Exploit Junction Regulatory Activities of N-WASP and SNX9 to Disrupt the Intestinal Barrier. <i>Cell Mol Gastroenterol Hepatol</i> 5: 273-288	29675452
	Graham DB, Lefkovith A, Deelen P, de Klein N, Varma M, Boroughs A, Desch AN, Ng ACY, Guzman G, Schenone M, Petersen CP, Bhan AK, Rivas MA, Daly MJ, Carr SA, Wijmenga C, Xavier RJ (2016) TMEM258 Is a Component of the Oligosaccharyltransferase Complex Controlling ER Stress and Intestinal Inflammation. <i>Cell Rep</i> 17: 2955-2965	27974209
	Sarvestani SK, Signs SA, Lefebvre V, Mack S, Ni Y, Morton A, Chan ER, Li X, Fox P, Ting A, Kalady MF, Cruise M, Ashburn J, Stiene J, Lai W, Liska D, Xiang S, Huang EH (2018) Cancer-predicting transcriptomic and epigenetic signatures revealed for ulcerative colitis in patient-derived epithelial organoids. <i>Oncotarget</i> 9: 28717-28730	29983891
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	Tao L, Zhang J, Meraner P, Tovaglieri A, Wu X, Gerhard R, Zhang X, Stallcup WB, Miao J, He X, Hurdle JG, Breault DT, Brass AL, Dong M (2016) Frizzled proteins are colonic epithelial receptors for C. difficile toxin B. <i>Nature</i> 538: 350-355	27680706
	Tinkum KL, Stemler KM, White LS, Loza AJ, Jeter-Jones S, Michalski BM, Kuzmicki C, Pless R, Stappenbeck TS, Piwnica-Worms D, Piwnica-Worms H (2015) Fasting protects mice from lethal DNA damage by promoting small intestinal epithelial stem cell survival. <i>Proc Natl Acad Sci U S A</i> 112: E7148-54	26644583
Tumorigenesis (n=8)	Dame MK, Attili D, McClintock SD, Dedhia PH, Ouillet P, Hardt O, Chin AM, Xue X, Laliberte J, Katz EL, Newsome GM, Hill DR, Miller AJ, Tsai YH, Agorku D, Altheim CH, Bosio A, Simon B, Samuelson LC, Stoerker JA et al. (2018) Identification, isolation and characterization of human LGR5-positive colon adenoma cells. <i>Development</i> 145	29467240

	Goto N, Ueo T, Fukuda A, Kawada K, Sakai Y, Miyoshi H, Taketo MM, Chiba T, Seno H (2017) Distinct Roles of HES1 in Normal Stem Cells and Tumor Stem-like Cells of the Intestine. <i>Cancer Res</i> 77: 3442-3454	28536281
	Hennenberg EM, Eyking A, Reis H, Cario E (2017) MDR1A deficiency restrains tumor growth in murine colitis-associated carcinogenesis. <i>PLoS One</i> 12: e0180834	28686677
	Li B, Orton D, Neitzel LR, Astudillo L, Shen C, Long J, Chen X, Kirkbride KC, Doundoulakis T, Guerra ML, Zaias J, Fei DL, Rodriguez-Blanco J, Thorne C, Wang Z, Jin K, Nguyen DM, Sands LR, Marchetti F, Abreu MT et al. (2017) Differential abundance of CK1alpha provides selectivity for pharmacological CK1alpha activators to target WNT-dependent tumors. <i>Sci Signal</i> 10	28655862
	Miyoshi H, Maekawa H, Kakizaki F, Yamaura T, Kawada K, Sakai Y, Taketo MM (2018) An improved method for culturing patient-derived colorectal cancer spheroids. <i>Oncotarget</i> 9: 21950-21964	29774115
	Okuchi Y, Imajo M, Mizuno R, Kamioka Y, Miyoshi H, Taketo MM, Nagayama S, Sakai Y, Matsuda M (2016) Identification of Aging-Associated Gene Expression Signatures That Precede Intestinal Tumorigenesis. <i>PLoS One</i> 11: e0162300	27589228
	Toden S, Ravindranathan P, Gu J, Cardenas J, Yuchang M, Goel A (2018) Oligomeric proanthocyanidins (OPCs) target cancer stem-like cells and suppress tumor organoid formation in colorectal cancer. <i>Sci Rep</i> 8: 3335	29463813
	Xu H, Li J, Chen H, Ghishan FK (2019) NHE8 Deficiency Promotes Colitis-Associated Cancer in Mice via Expansion of Lgr5-Expressing Cells. <i>Cell Mol Gastroenterol Hepatol</i> 7: 19-31	30465020
Culture of non-gastro-intestinal cells (n=1)	Aly H, Rohatgi N, Marshall CA, Grossenheider TC, Miyoshi H, Stappenbeck TS, Matkovich SJ, McDaniel ML (2013) A novel strategy to increase the proliferative potential of adult human beta-cells while maintaining their differentiated phenotype. <i>PLoS One</i> 8: e66131	23776620

*Selection method: Publications written in English and accessible online were selected for further review if they were listed in Scopus (on or before January 22, 2019) as citing one of the following reports from the Stappenbeck laboratory: Miyoshi H, *et al.* (2012) *Science* 338: 108-13; Miyoshi H, *et al.* (2013) *Nat Protoc* 8: 2471-82; VanDussen KL, *et al.* (2015) *Gut* 64: 911-20. A publication was included in the above table only if its Materials and Methods section specifically stated the use of L-WRN CM for the culture of epithelial stem cells.

Table S3. Summary of expected results for L-WRN CM quality control testing procedures using colonic spheroids.

Assay	Expected result*
Visual inspection	<p><u>Proliferating spheroids</u> (cultured in L-WRN CM): Spherical shape, clear lumen, outer walls with thin, smooth edges, budding structures absent, spheroid size should continue to increase over time (see Fig. 3)</p> <p><u>Differentiated spheroids</u> (cultured in DM+EP4i): Roughly spherical shape although some budding protrusions may form over time, outer walls with thick, smooth edges and smaller lumen make spheroid appear darker, spheroid size will no longer increase over time (see Fig. 3)</p> <p><i>Approximate spheroid diameter at Day 3 post-seeding</i> <u>Mouse</u>: Mean = 123 μm (25% percentile, 68 μm; 75% percentile, 131 μm), n = 2,415 spheroids measured from CM1-13 in this study <u>Human</u>: Mean 88 μm (25% percentile, 51 μm; 75% percentile, 93 μm), n = 2,427 spheroids measured from CM1-13 in this study</p>
Cell Titer Glo	<p><u>Assay run on Day 3 post-seeding with mouse spheroids</u> (see Fig. 4): An average 16-fold increase in luciferase activity was observed comparing L-WRN CM cultured mouse spheroids to the negative control (CHX+TNF). DM+EP4i spheroids will exhibit similar luciferase levels as proliferating spheroids if the assay is run at this time point.</p> <p><u>Assay run on Day 3 post-seeding with human spheroids</u> (see Fig. 4): An average 2-fold increase in luciferase activity was observed comparing L-WRN CM cultured mouse spheroids to the negative control (CHX+TNF). DM+EP4i spheroids will exhibit similar luciferase levels as proliferating spheroids if the assay is run at this time point.</p> <p><u>Assay run on Day 2 post-seeding with mouse spheroids</u> (see Fig. 2): An average 78-fold increase in luciferase activity was observed comparing L-WRN CM cultured mouse spheroids to the negative control (CHX+TNF). An average 20-fold increase in luciferase activity was observed comparing DM+EP4i cultured mouse spheroids to the negative control (CHX+TNF).</p>
LDH	<p>LDH levels should be low in L-WRN CM alone and in supernatants of spheroids cultured in L-WRN CM. We typically observed LDH levels that were 8% and 3% of the LDH positive control included in the kit for L-WRN CM and DM (both media alone), respectively.</p>

Assay	Expected result*
Cdc25-luc colonic spheroids	<p>A 3- to 6-fold increase in luciferase activity is typically observed comparing the 24-hr to 0-hr time point, depending on whether the assay is run on Day 2 or Day 3 post-seeding (see Fig. 2 and Fig.6)</p> <p><i>Technical considerations for this assay</i></p> <p><u>Benefits:</u> Direct, sensitive measure of spheroid proliferation</p> <p><u>Disadvantages:</u> Requires specialized spheroid line and a luminometer with enhanced sensitivity; assay is technically challenging and requires skilled personnel because initial seeding density and uniform spheroid size critically affect assay reproducibility</p>
qPCR for key marker genes	<p>Lgr5, Axin2 and MKi67 mRNA expression should be readily detectable and relatively high in L-WRN CM vs. DM+EP4i cultured spheroids. In contrast, Car1 mRNA expression should be readily detectable and relatively high in DM+EP4i vs. L-WRN CM cultured spheroids (see Fig. 5)</p> <p><i>Approximate fold changes for spheroids cultured in L-WRN vs. DM+EP4i:</i></p> <p><u>Mouse:</u> Lgr5 = 1,849-fold; Axin2 = 40-fold; MKi67 = 49-fold; Car1 = -4-fold</p> <p><u>Human:</u> Lgr5 = 312-fold; Axin2 = 38-fold; MKi67 = 14-fold; Car1 = -2-fold</p>
Wnt reporter	<p>With this reporter, 1 µg/mL of rWnt3a yielded an average 27-fold induction and 5% L-WRN CM yielded an average 17-fold induction relative to baseline (reporter cells in HEK/primary culture medium) luminescence (see Fig. 7)</p>

*Spheroid density and size can affect all spheroid-based assays, in particular, mouse colonic spheroids due to their relatively fast growth rate. When mouse colonic spheroids are plated too densely, a growth plateau and decreased proliferation can occur.

Table S4. Trouble-shooting procedures related to L-WRN CM production.*

Observation	Potential cause(s)	Potential solution(s)
L-WRN cells growing very slowly, lacking spindle morphology following thaw	Seeded too sparsely; harsh handling during thaw; contamination with other cell line	<p>1) Do not apply the selective antibiotics hygromycin and G418 to freshly thawed L-WRN cells. Wait until after the initial passage, once L-WRN cells have recovered from the freeze-thaw process.</p> <p>2) Wait for small colonies to form, which will support better L-cell growth (if colonies do form, then cells will reach confluency. Proceed with the L-WRN CM production protocol only upon); or</p> <p>3) Transfer cells to a smaller vessel, wait for cells to reach confluency and then passage into the appropriate culture vessel to proceed with L-WRN CM production; or</p> <p>4) Start over, thaw a different vial of L-WRN cells, potentially seeding in a smaller vessel initially, and then passage into the appropriate culture vessel to proceed with L-WRN CM production.</p> <p>5) If the above steps do not yield cells that display the characteristic fibroblast morphology and growth characteristics of the L-WRN cell like, consider checking your cells for potential contamination with another cell type.</p> <p>6) Consider whether frozen stocks of the L-WRN cells were properly prepared or properly thawed, i.e. were a large proportion of the L-WRN cells dead in the culture vessel? Was the cell concentration of the frozen L-WRN stocks appropriate for the chosen culture vessel?</p>

Observation	Potential cause(s)	Potential solution(s)
Lack of robust spheroid growth in intestinal epithelial cells cultured with L-WRN CM	<p>A. Technical error during L-WRN CM production affecting L-WRN CM activity (most common)</p> <p>B. Technical error in spheroid passaging, unrelated to L-WRN CM activity (less common)</p> <p>C. Mishandling of L-WRN cell line during preparation of L-WRN cell frozen stocks affecting L-WRN CM activity (rare)</p>	<p>A.1) Ensure that L-WRN cells have reached post-confluency prior to CM collection.</p> <p>A.2) Ensure that L-WRN selective antibiotics, hygromycin and G418, are not carried over into the CM. Add selective antibiotics only when indicated in the protocol. If L-WRN cells are cultured in L-cell medium containing selective antibiotics and in the same culture vessel as L-WRN CM production (as occurs in the small-scale L-WRN CM production protocol), be sure to carefully perform wash steps removing L-cell medium containing selective antibiotics to remove all traces of the selective antibiotics prior to the addition of primary culture medium for L-WRN CM production.</p> <p>A.3) Ensure the correct composition and/or handling of all reagents used for L-WRN CM production (e.g. the DMEM composition is essential, etc.).</p> <p>A.4) Serum concentration: This protocol yields L-WRN CM with 20% serum. Other organoid culture protocols use lower serum concentrations or omit serum completely. L-WRN CM lacking serum can be produced, but the use of serum-free L-WRN CM will greatly diminish the spheroid growth rate. If reduced serum or serum-free L-WRN CM is needed, one recommendation would be to expand spheroids in L-WRN CM containing 20% serum and then switch to L-WRN CM lacking serum for sensitive experiments.</p> <p>A.5) Ensure that the correct media volume was used for the surface area of the culture vessel (i.e. be aware of potential surface area differences between brands and catalog numbers of culture vessels).</p>

Observation	Potential cause(s)	Potential solution(s)
<p>Continued...</p> <p>Lack of robust spheroid growth in intestinal epithelial cells cultured with L-WRN CM</p>	<p>Continued...</p> <p>A. Technical error during L-WRN CM production affecting L-WRN CM activity (most common)</p> <p>B. Technical error in spheroid passaging, unrelated to L-WRN CM activity (less common)</p> <p>C. Mishandling of L-WRN cell line during preparation of L-WRN cell frozen stocks affecting L-WRN CM activity (rare)</p>	<p>Continued...</p> <p>A.6) Ensure complete mixing of the “100%” L-WRN CM with fresh primary culture medium to achieve “50%” L-WRN CM. L-WRN CM optimally supports long-term spheroid growth at 50% concentration. Long- and/or short-term spheroid growth is negatively affected by L-WRN CM concentrations that are higher or lower.</p> <p>A.7) Although two freeze-thaw cycles with L-WRN CM did not affect short-term spheroid growth, additional freeze-thaw cycles would be predicted to have a negative impact on protein stability and spheroid growth.</p> <p>B.1) Ensure the correct composition and/or handling of all reagents used for spheroid passaging. In particular, trypsin is the critical reagent to review. Variations in trypsin strength between brands and catalog numbers can have major effects on spheroid survival and size post-passage. Use of growth factor-reduced Matrigel or other basement membrane matrix products can also affect spheroid growth.</p> <p>B.2) Ensure the correct technical execution of the spheroid passaging protocol. In particular, the technique used for mechanical disruption of the spheroids following exposure to trypsin is critical. Too much or too little mechanical disruption can have major effects on spheroid survival and size post-passage.</p>

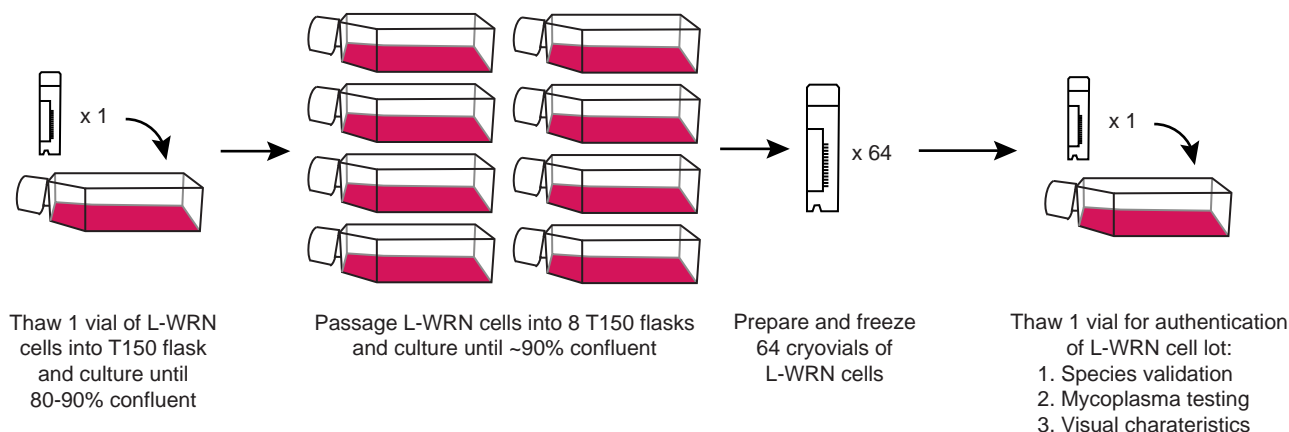
Observation	Potential cause(s)	Potential solution(s)
Continued... Lack of robust spheroid growth in intestinal epithelial cells cultured with L-WRN CM	Continued... A. Technical error during L-WRN CM production affecting L-WRN CM activity (most common) B. Technical error in spheroid passaging, unrelated to L-WRN CM activity (less common) C. Mishandling of L-WRN cell line during preparation of L-WRN cell frozen stocks affecting L-WRN CM activity (rare)	Continued... B.3) Spheroid size and density must be taken into account when passaging to expand or maintain cultures. As necessary, modify the time exposed to trypsin or mechanical disruption to maintain spheroids at a consistent size (e.g. see Fig. 3C-E). If spheroid density is sparse, use a lower passage split ratio and/or wait up to 7 days to passage (especially beneficial for establishing new human biopsy-derived lines with low crypt isolation yields). In particular with mouse colonic spheroids, seeding too densely may result in a growth plateau and early stages of differentiation. B.4) It is beneficial to include ROCK inhibitor in the L-WRN CM provided to spheroids immediately following passage. Inclusion of a TGFBR inhibitor is also required for human spheroids, and can also be provided to mouse spheroids if spheroids are very small or sparse to aid recovery. C.1) To minimize variations in L-WRN cell line behavior and resulting L-WRN CM activity levels, we highly recommend preparing relatively large lots of L-WRN cell frozen stocks upon receipt of this cell line. Once the L-WRN cell line has recovered from freeze-thaw, use the selective antibiotics hygromycin and G418 to maintain the R spondin 3 and Noggin dual expression cassette present in this cell line. Improper handling of the L-WRN cell line can have detrimental effects on L-WRN CM activity and spheroid growth.

Observation	Potential cause(s)	Potential solution(s)
Spheroid cultures are overrun by live, rapidly growing L-WRN cells	Did not sufficiently remove live L-WRN cells from the L-WRN CM prior to use in spheroid culture; did not freeze L-WRN CM prior to use in spheroid culture	<p>1) Be sure to freeze-thaw the L-WRN CM prior to use in spheroid culture. Centrifugation is the primary method used in this protocol to remove L-WRN cells carried over into the L-WRN CM. This alone does not fully eliminate L-WRN cell carryover into the CM. However, the initial freeze-thaw of the stored L-WRN CM does destroy any transferred, live L-WRN cells.</p> <p>2) Consider applying an additional filtration step to remove L-WRN cells from the CM. Be sure to select a filter that will not bind and remove/reduce levels of the essential growth factor proteins from the L-WRN CM.</p>

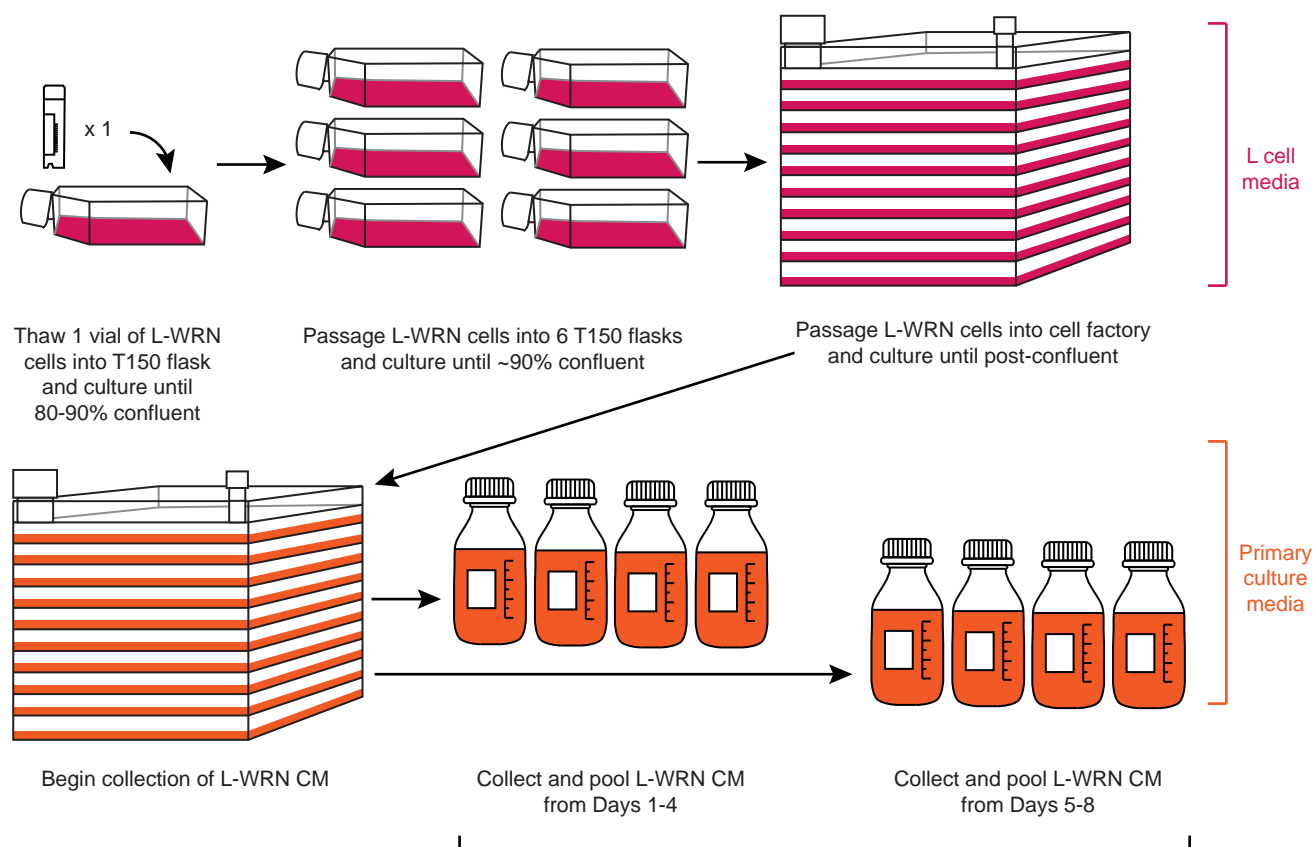
*These observations, causes, and solutions are based on personal observations of Stappenbeck laboratory members and of other research groups who reached out to our laboratory for assistance. To our knowledge, all groups achieved optimal L-WRN CM activity and spheroid growth after following these trouble-shooting procedures.

Figure S1

A PREPARATION AND AUTHENTICATION OF L-WRN CELLS



B PRODUCTION & QUALITY CONTROL TESTING OF L-WRN CM



Perform quality control testing of L-WRN CM from each collection period
If possible, include previous batches of L-WRN CM in testing procedures to ensure similar batch activities between production periods

Minimum procedures recommended for L-WRN CM quality control testing:

1. Visual characteristics
2. Spheroid growth assay (e.g. CellTiter-Glo)
3. Gene expression analysis of key marker genes

Figure S1. Authentication and quality control of L-WRN cells and L-WRN CM. (A) Schematic of workflow for preparation of relatively large lots of L-WRN cells for cryogenic storage to maintain low passage cells with similar behavior. Authentication procedures 1-3 are performed with at least one vial per lot. (B) Schematic of the large-scale L-WRN CM production workflow. L-WRN cells are expanded and seeded into a Cell Factory and then cultured until post-confluent in L cell media. Once L-WRN cells reach post-confluency (2-3 days), the L cell media is removed from the Cell Factory and replaced with primary culture media. The CM is collected from the Cell Factory daily for 8 days, with pooling of the medium collected over two 4-day periods, Days 1-4 and Days 5-8, to minimize any potential day-to-day variations in CM activity. The CM is diluted 50/50 with fresh primary culture medium for use in intestinal spheroid culture, such that 16 L of 50% L-WRN CM are collected during each L-WRN CM production period. The minimal quality control procedures 1-3 are recommended for each batch of L-WRN CM (i.e. Days 1-4 & Days 5-8, including those of previously collected batches if available).

Supplementary Figure S2

A

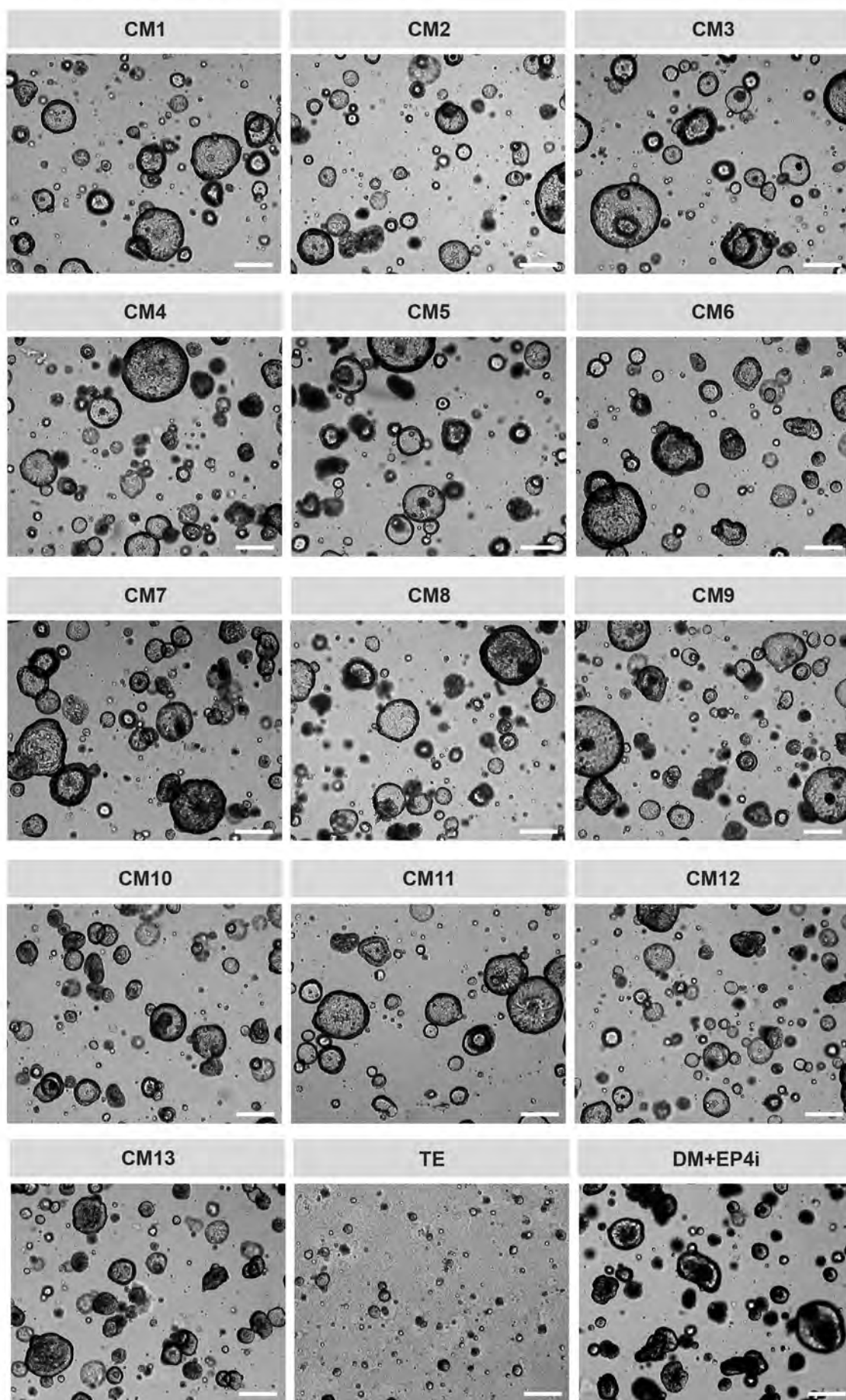


Figure S2. Additional images of cultured mouse spheroids. Representative images of mouse colonic spheroids cultured in L-WRN CM batches CM1-13, the TE medium batch, or in colonocyte differentiation medium (DM+EP4i). Images shown are from a single experiment, with spheroids from the same line, passage, and seeding. Scale bars, 200 μm .

Supplementary Figure S3

A

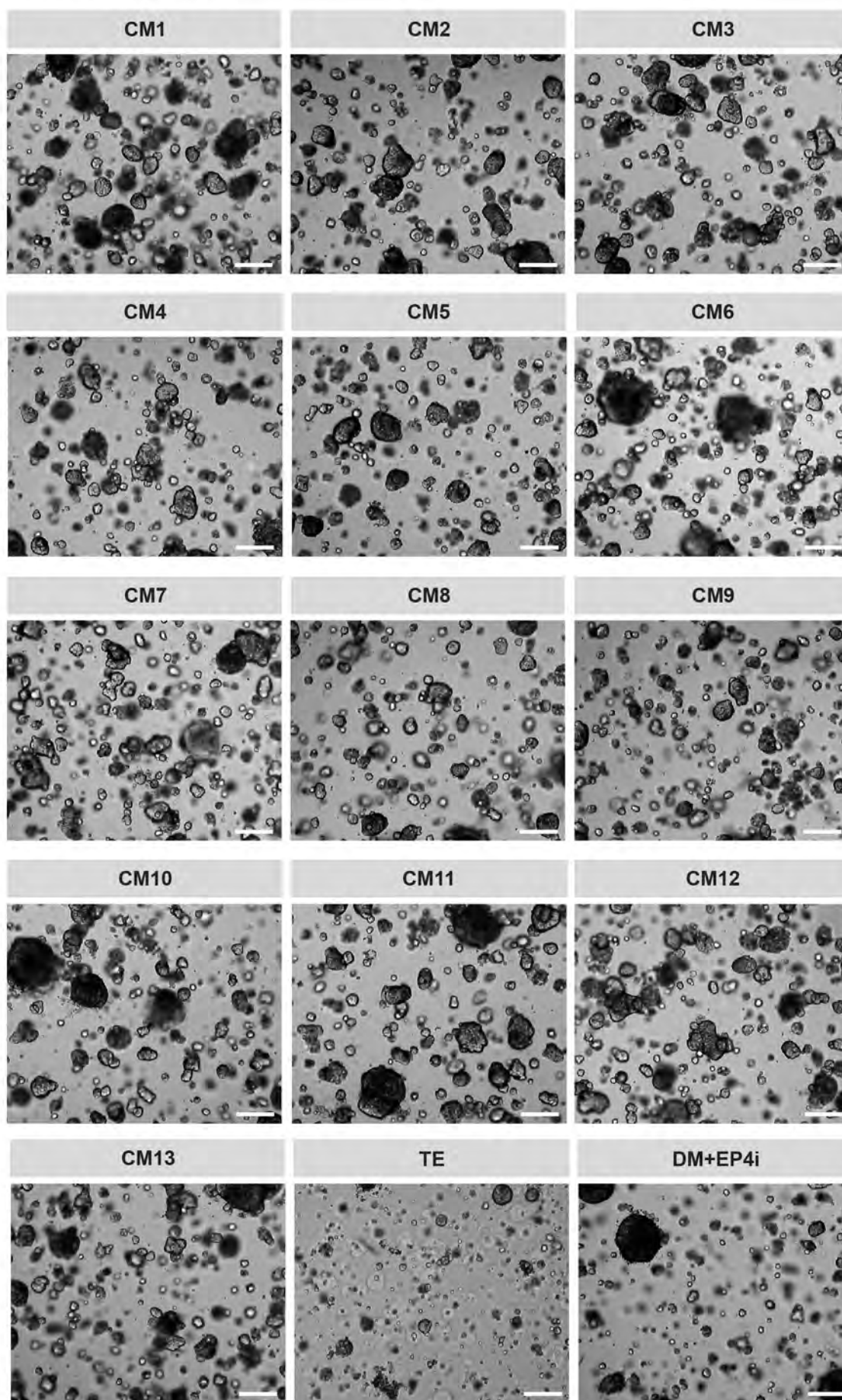


Figure S3. Additional images of cultured human spheroids. Representative images of human colonic spheroids cultured in L-WRN CM batches CM1-13, the TE medium batch, or in colonocyte differentiation medium (DM+EP4i). Images shown are from a single experiment, with spheroids from the same line, passage, and seeding. Scale bars, 200 μm .

Figure S4

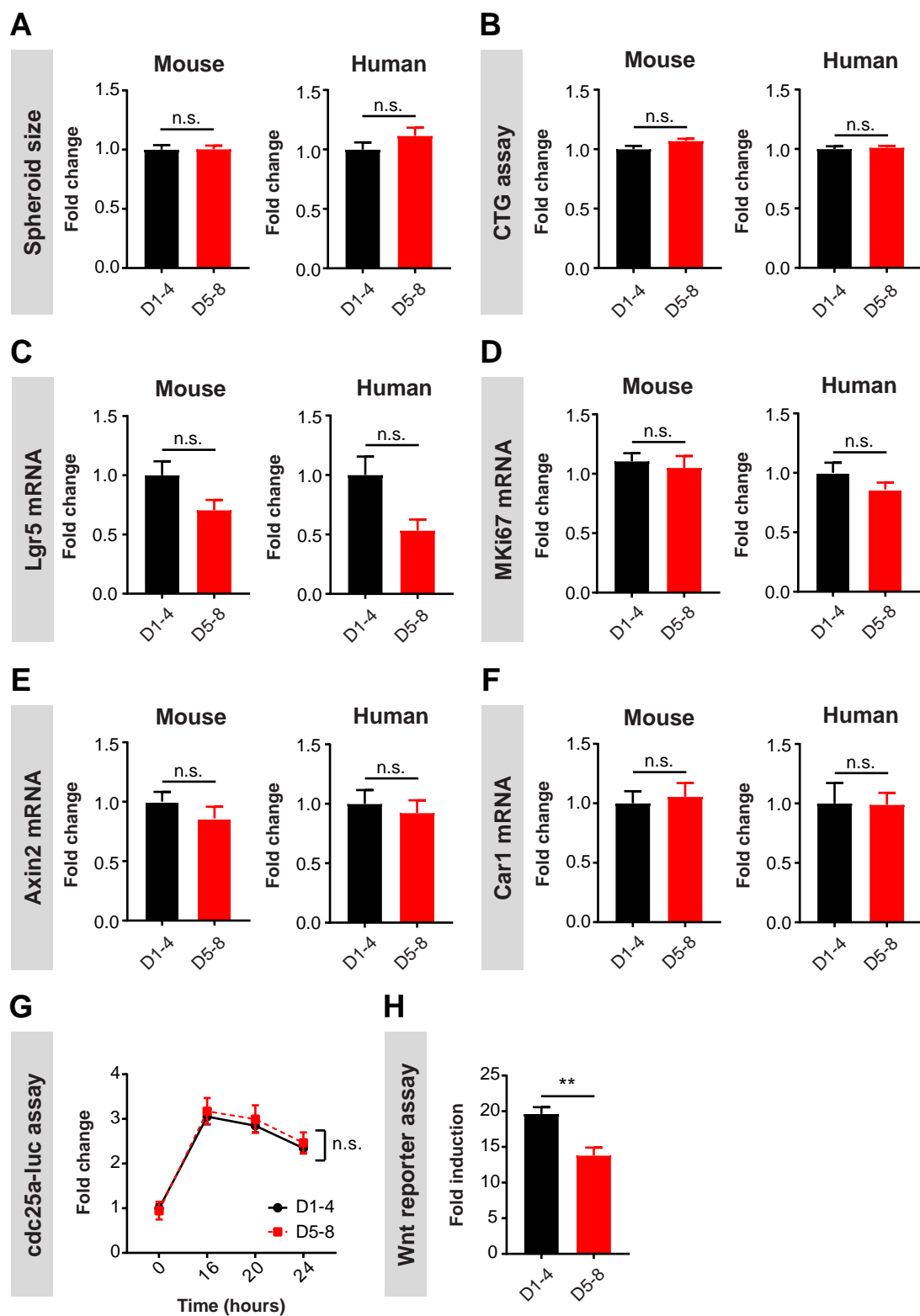


Figure S4. Grouped comparisons of L-WRN CM batches based on collection period. (A-H)

Data for spheroid size (A), spheroid growth with CellTiter-Glo assay (B), qPCR gene expression analysis of Lgr5 (C), MKi67 (D), Axin2 (E), and Car1 (F), spheroid proliferation by Cdc25A-CBR-luc assay (G), and the HEK293 Wnt reporter assay (H) were generated as previously described. Here, data points were grouped according to the collection period of the L-WRN CM batches CM1-13, either Days 1-4 (D1-4) or Days 5-8 (D5-8), to specifically test for potential effects on L-WRN CM activity due to this variable. Data are presented as fold change (mean \pm s.e.m.) relative to the average Days 1-4 value, with additional normalization to time 0 hr for the Cdc25A-CBRluc assay; n=8 for D1-4; n=5 for D5-8. ** P <0.01 or n.s. (not significant) by unpaired two-tailed t test (A-F, H) or 2-way repeated measures ANOVA (G).

Figure S5

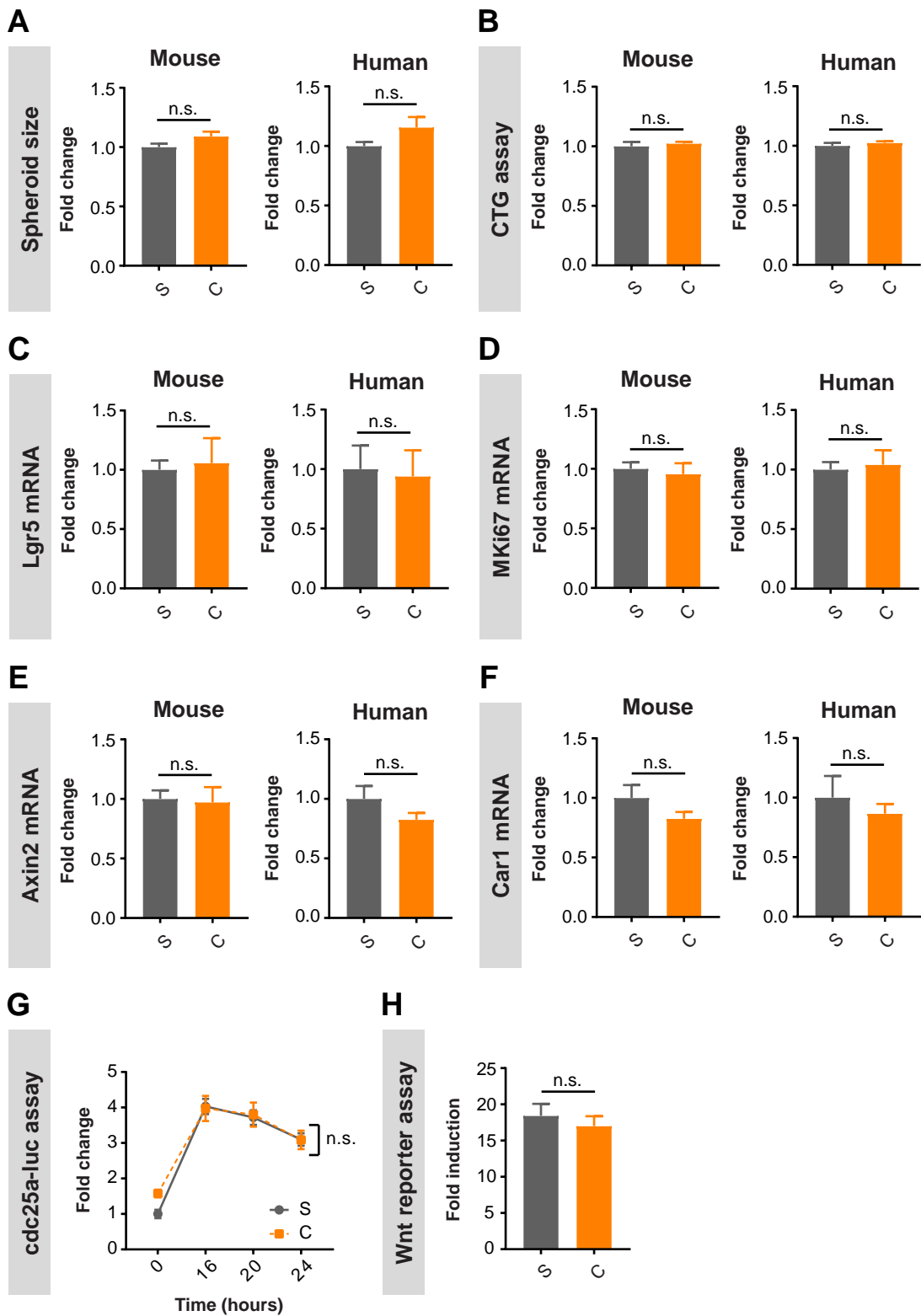


Figure S5. Grouped comparisons of L-WRN CM batches based on production laboratory.

(A-H) Data for spheroid size (A), spheroid growth with CellTiter-Glo assay (B), qPCR gene expression analysis of Lgr5 (C), MKi67 (D), Axin2 (E), and Car1 (F), spheroid proliferation by Cdc25A-CBR-luc assay (G), and the HEK293 Wnt reporter assay (H) were generated as previously described. Here, data points were grouped according to the production laboratory of the L-WRN CM batches CM1-13, either the Stappenbeck laboratory (S) or collaborating laboratories (C), to specifically test for potential effects on L-WRN CM activity due to this variable. Data are presented as fold change (mean \pm s.e.m.) relative to the average Stappenbeck laboratory value, with additional normalization to time 0 hr for the Cdc25A-CBRluc assay; n=7 for S; n=6 for C. n.s. (not significant) by unpaired two-tailed t test (A-F, H) or 2-way repeated measures ANOVA (G).

Figure S6

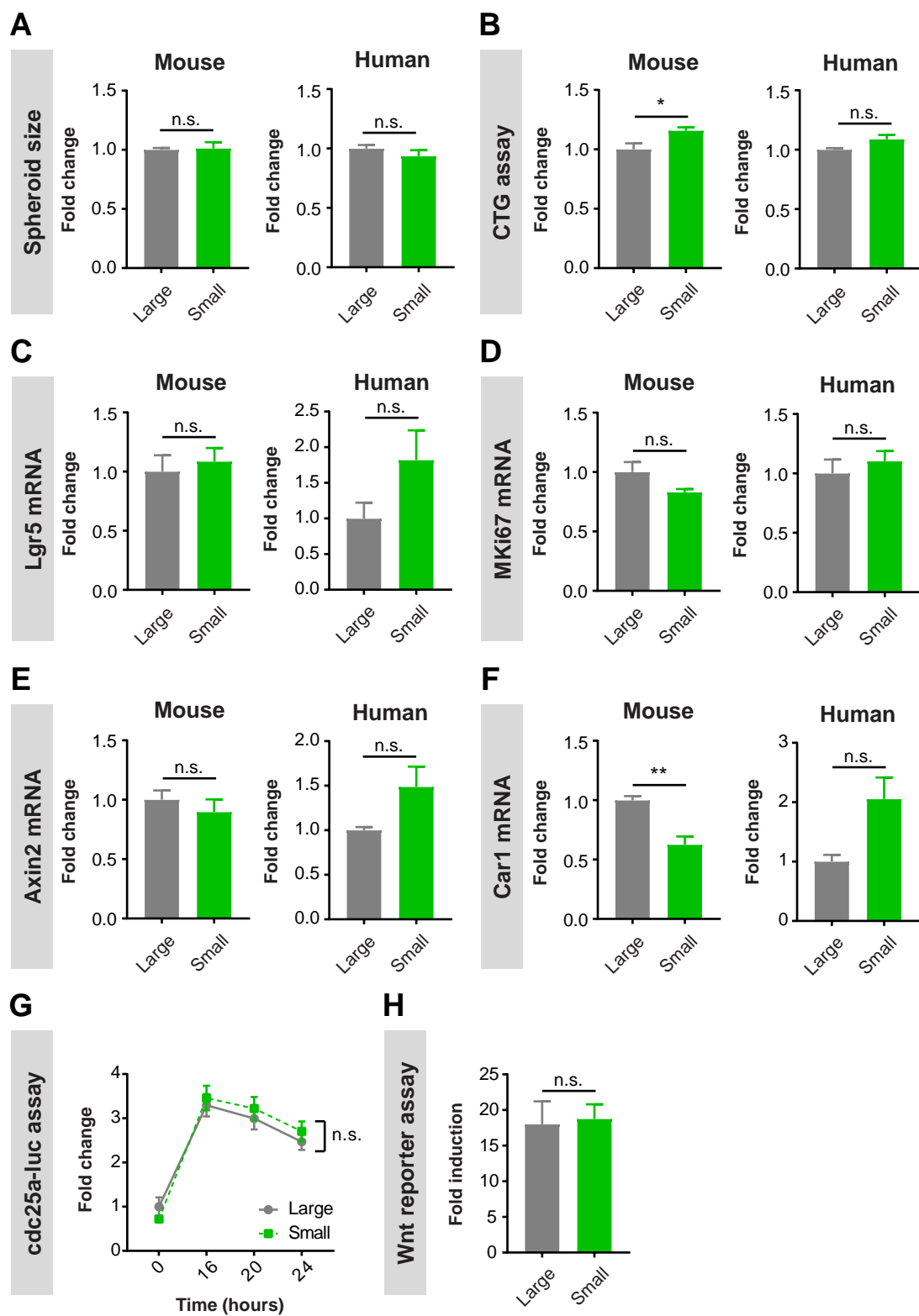


Figure S6. Grouped comparisons of L-WRN CM batches based on production team size.

(A-H) Data for spheroid size (A), spheroid growth with CellTiter-Glo assay (B), qPCR gene expression analysis of Lgr5 (C), MKi67 (D), Axin2 (E), and Car1 (F), spheroid proliferation by Cdc25A-CBR-luc assay (G), and the HEK293 Wnt reporter assay (H) were generated as previously described. Here, data points were grouped according to the Stappenbeck laboratory production team size of the L-WRN CM batches CM1-7, either Large or Small, to specifically test for potential effects on L-WRN CM activity due to collection period. Data are presented as fold change (mean \pm s.e.m.) relative to the average Large value, with additional normalization to time 0 hr for the Cdc25A-CBRluc assay; n=3 for Large; n=4 for Small. * P <0.05, ** P <0.01 or n.s. (not significant) by unpaired two-tailed t test (A-F, H) or 2-way repeated measures ANOVA (G).