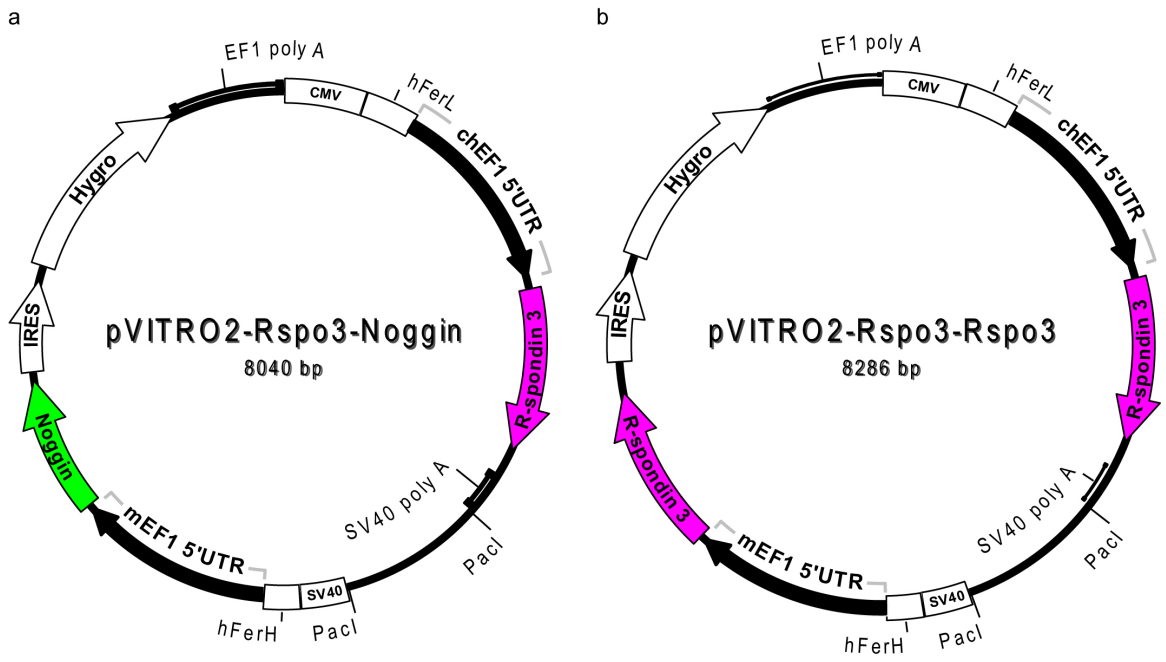
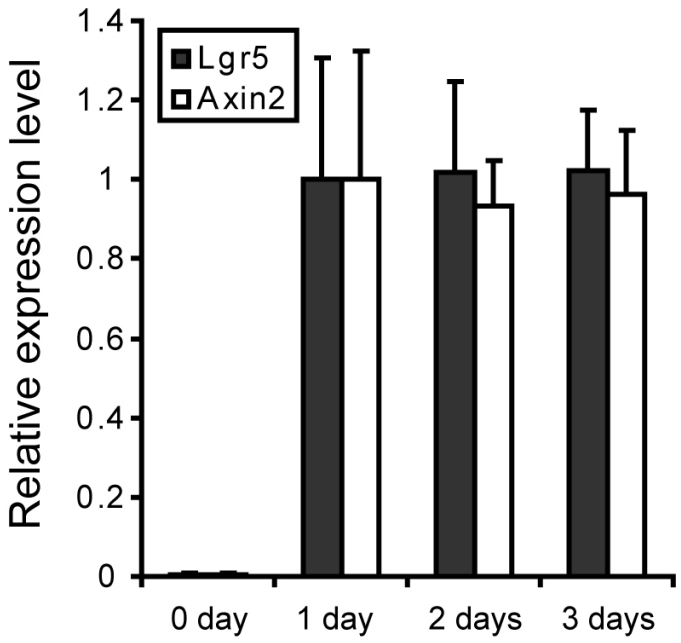
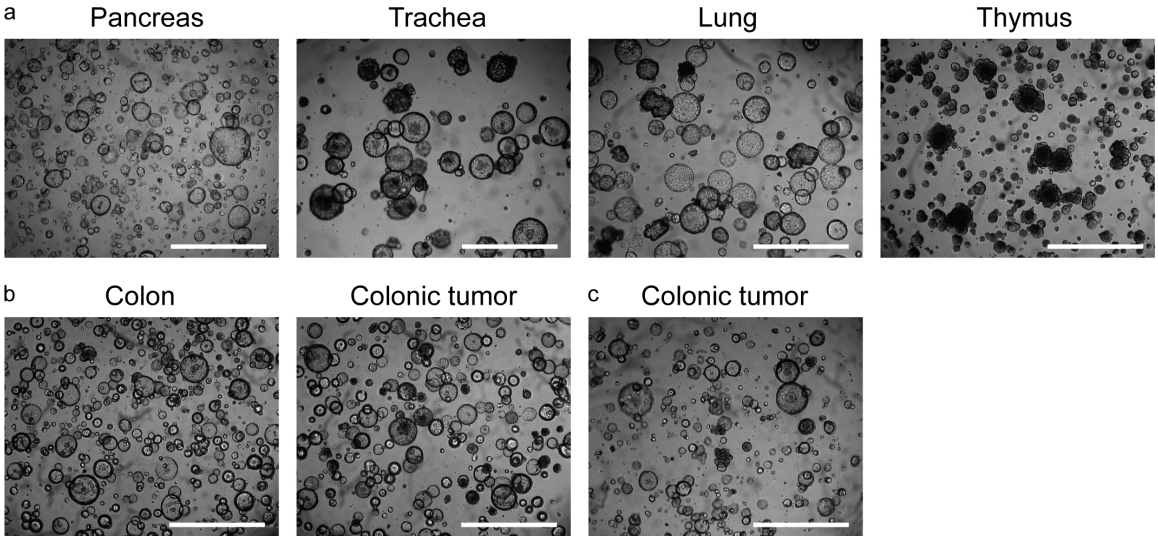


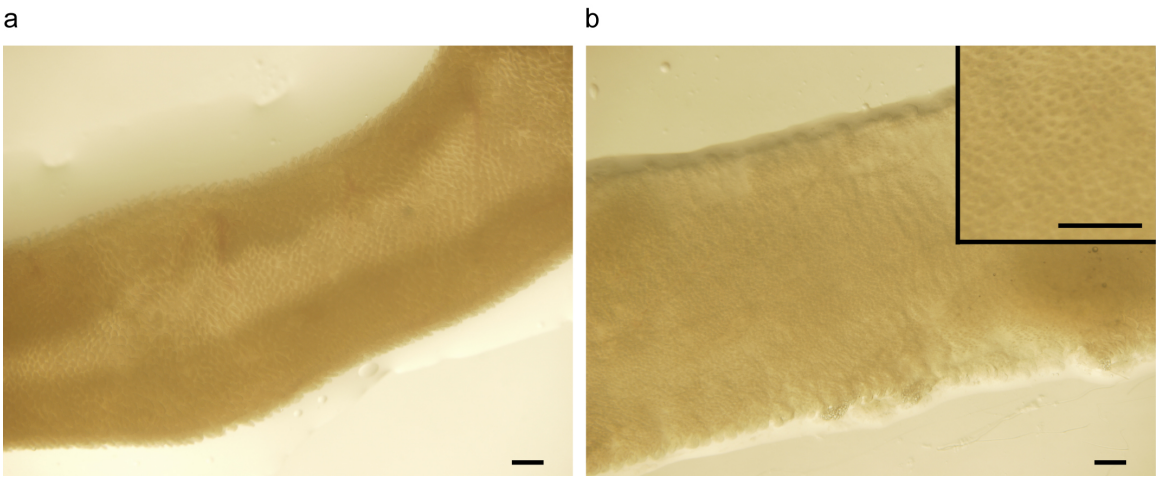
Supplementary Figure 1| Schematic representation of vectors for transfection into L-Wnt3a cells. (a) A dual expression vector for R-spondin 3 and noggin. (b) An expression vector for R-spondin 3. Both vectors were constructed based on pVITRO2-hygro-mcs (Invivogen). PacI: enzyme digestion sites for linearization. More information about the original vector is available from manufacturer's website (http://www.invivogen.com).



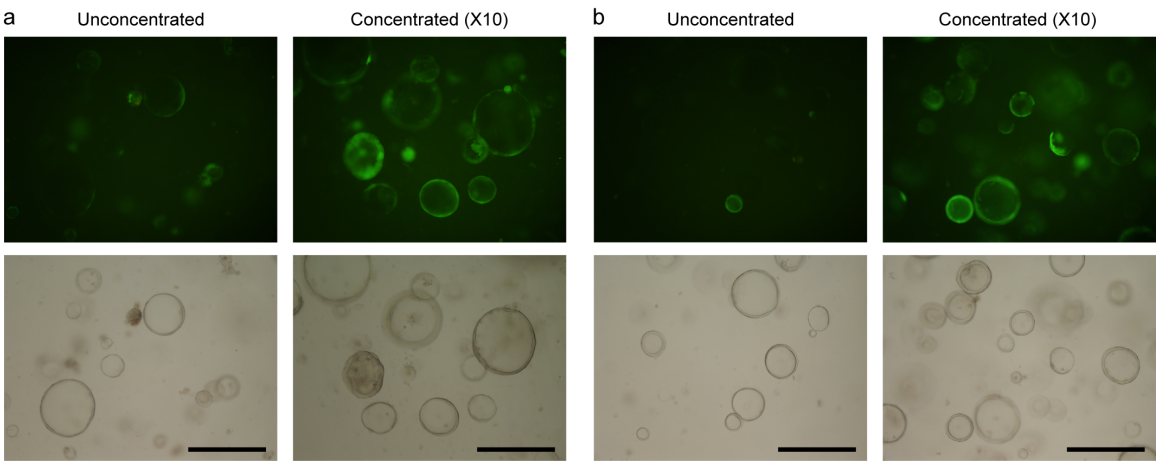
Supplementary Figure 2| The effect of harvest timing on activity of conditioned media. Primary culture media was incubated with L-WRN cells for the indicated number of days. Colonic organoids were incubated with each conditioned media and relative expression levels of Axin2 (a target of canonical Wnt signaling) and Lgr5 (a stem cell marker) were determined by quantitative RT-PCR. Plots of mean (+SD) relative mRNA expression levels were determined by quantitative RT-PCR analysis (n=3 samples/group). Data were analyzed using one-way ANOVA followed by Tukey's post-test. P=0.0005 (Axin2), P=0.0006 (Lgr5). There was no significant difference between the activities of 1-, 2-, and 3-day media.



Supplementary Figure 3| Application of the culture protocol to non-gastrointestinal stem cells and tumor cells. (a) Typical morphology of pancreatic, tracheal, lung, and thymic organoids. Bars=1 mm. (b) Typical morphology of colonic tumor spheroids and colonic organoids derived from an ApcMin mouse. (c) Typical morphology of colonic tumor spheroids derived from an AOM/DSS-treated mouse. Organoids derived from normal tissues were cultured in 50% L-WRN conditioned media containing 10 μM Y27632 and 10 μM SB431542, whereas tumor spheroids were cultured in the basal media (0% conditioned media) containing 10 μM Y27632 and 10 μM SB431542. Bars=1 mm.



Supplementary Figure 4| Preparation of the mouse small intestine. A piece of dissected small intestine (ileum) before (a) and after (b) scraping villi was shown. Individual crypts are visible after scraping (inset). Bars=0.5 mm.



Supplementary Figure 5| Efficient gene transduction of intestinal organoids with concentrated lentiviruses (a, b). Small intestinal (a) and colonic (b) organoids were infected with unconcentrated (1 ml) or concentrated (100 μl) lentiviruses. Representative fluorescence (upper) and bright field images (lower) are shown. Bars=0.5 mm.

Supplementary Methods

Mice

All animal studies were performed according to protocols approved by the Washington University School of Medicine Animal Studies Committee.

Cell lines

L-Wnt3a cells (CRL-2647) [1] were obtained from American Type Culture Collection. HEK293FT cells were purchased from Invitrogen (Carlsbad, CA).

Generation of L-WRN and L-WR cells

Full-length R-spondin 3 (Rspo3) and Noggin (Nog) cDNA fragments with a KOZAK consensus sequence were amplified from mouse embryonic cDNA using pairs of primers (5'- CCACCATGCACTTGCGACTGATTTCTTG - 3'and 5'-GAGTGGTAACTGATGTGTTGTCC - 3' for Rspo3, 5'-CCACCATGGAGCGCTGCCCCAGC - 3'and 5'-CTAGCAGGAACACTTACACTCGG - 3' for Nog) and cloned into pCRII-TOPO vector (Invitrogen), respectively. Sequences of both cDNAs were verified using M13 forward and M13 reverse primers. To generate the pVITRO2-Rspo3-Nog vector for L-WRN cells **(Supplementary Fig. 1a)**, a Noggin cDNA fragment was separated from pCRII-TOPO vector with *Eco* RI, blunted and inserted into the *Eco* RV site on pVITRO2-hygro-mcs dual expression vector (InvivoGen: pvitro2-mcs), then an Rspo3 cDNA fragment was separated from pCRII-TOPO vector with the restriction enzyme *Eco* RI and inserted into the *Eco* RI site. To generate the pVITRO2-Rspo3-Rspo3 vector for

L-WR cells **(Supplementary Fig. 1b)**, an Rspo3 cDNA fragment was separated from pCRII-TOPO vector with *Eco* RI, blunted and cloned into the *Eco* RV site on pVITRO2-hygro-mcs vector, then another Rspo3 fragment that was cut with *Eco* RI was cloned into the *Eco* RI site. To maximize expression of recombinant factors, L-Wnt3a cells were transfected with the concatemeric DNA fragments containing multiple expression units. Briefly, 10 μg pVITRO2-Rspo3-Nog or pVITRO2-Rspo3-Rspo3 vector plasmids were linearized with *Pac*I (Thermo Scientific), extracted with phenol-chloroform-isoamyl alcohol (25:24:1) solution, precipitated with ethanol and dissolved in 5 μl Tris-EDTA (pH 8.0). Then DNA fragments were polymerized using DNA Ligation Kit (Takara), extracted with phenol-chloroform-isoamyl alcohol (25:24:1) solution, precipitated with ethanol and gently dissolved in 20 μl Tris-EDTA (pH 8.0). ~200,000 L-Wnt3a cells were plated in a well of the 24-well plate one day before transfection and transfected with 0.8 μg polymerized DNA using Lipofectamine 2000 (Invitrogen). Cells were passaged in a 9 cm cell culture dish one day after transfection and independent colonies were generated by treating cells with 500 μg/ml hygromycin (InvivoGen) and 500 μg/ml G418 (SIGMA). Total 24 independent clones were picked up and conditioned media was taken from each clone to select the most active media by monitoring Lgr5 induction in colonic organoids. Plasmid sequences and maps were constructed using SeqBuilder software (DNASTAR, Madison, WI).

RNA isolation and RT-PCR

Total RNA from each culture well was purified using the NucleoSpin RNA II Kit (Machery-Nagel, Duren, Germany). cDNA was synthesized with SuperScript III reverse

transcriptase (Invitrogen), and quantitative PCR analysis was performed using SYBR Green reagents (Clontech, Palo Alto, CA) and an Eppendorf Mastercycler. Normalization to B2m (β-2 microglobulin) was used to determine relative expression levels. Primer pairs for mouse B2m (5'- TTTCAGTGGCTGCTACTCGGC - 3' and 5'- GCAGTTCAGTATGTTCGGCTTCCC - 3'), Axin2 (5'- TGACTCTCCTTCCAGATCCCA - 3' and 5'- TGCCCACACTAGGCTGACA - 3'), and Lgr5 (5'- CCTACTCGAAGACTTACCCAGT - 3' and 5'- GCATTGGGGTGAATGATAGCA - 3') were used.

Statistical analysis

All statistical analyses were performed using GraphPad Prism software.

References

1. Willert, K. et al. Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature* **423,** 448-452 (2003).
2. Miyoshi, H. Ajima, R. Luo, C. T. Yamaguchi, T. P. & Stappenbeck, T. S. Wnt5a potentiates TGF-β signaling to promote colonic crypt regeneration after tissue injury. *Science* **338,** 108-113 (2012).