Defined Matrix and Wnt Agonists for Human Intestinal Organoid Culture

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Introduction

Epithelial organoid culture typically relies on Wnt signaling stimulated by exogenous growth factors such as Wnt-3a and R-Spondin as well as a physical matrix such as a hydrogel to promote self-renewal and expansion[1]. Thermal instability and uneven availability of Wnt-3a could lead to heterogeneous organoid growth and behavior[2]. Furthermore, the most commonly used hydrogel to support organoid growth is basement membrane extract (e.g. Cultrex™ and Matrigel®). These animal-derived matrices are composed of thousands of different proteins including peptides and growth factors[3]. Recombinant fusion proteins that can act upon Wnt receptors such as LGR-5, Frizzled, and LRP-5/6 have been previously described[4]. These fusion proteins, also known as Wnt surrogates, have increased solubility and could potentially replace Wnt and R-Spondin in cell media. In this study, these Wnt surrogates were tested with a synthetic matrix to assess their capability to support human intestinal organoid (enteroid) expansion and growth. This synthetic matrix is a defined animal-free matrix functionalized with key extracellular matrix protein mimics, such as collagen, and is designed to support 3D organoid models.

Method

Organoid Culture: Human enteroids were expanded in Cultrex UltiMatrix domes (Cat # BME001). Cell density was 10,000 cells per 10 µl of dome. Expansion media composed of Advanced DMEM/F12, GlutaMAX (1X), penicillin-streptomycin (1X), HEPES (1X), N21-MAX (1X, Cat # AR008), Nicotinamide (10 mM, Cat # 4106), NAC (1.25 mM, Cat # 7874), Noggin (100 ng/mL, Cat # 6057-NG), EGF (50 ng/mL, Cat # 236-GMP), Prostaglandin E_2 (1 µM, Cat # 2296), A 83-01 (500 nM, Cat # 2939), SB 202190 (10 µM, Cat # 1264), and either Wnt-3a (100 ng/mL, Cat # 5036-GMP) with R-Spondin 1-3 (1000 ng/mL, Cat # 4645-RS, 3266-RS, 3500-RS, respectively) or fusion proteins Wnt/RSPO1, Wnt/RSPO2, or Wnt/RSPO3 (Cat # BT-WRSP1, BT-WRSP2, BT-WRSP3, respectively) at various concentrations. Differentiation medium consisted of Advanced DMEM/F12, GlutaMAX (1X), penicillin-streptomycin (1X), HEPES (1X), N21-MAX (1X), Nicotinamide (10 mM), NAC (1.25 mM), Noggin (20 ng/mL), EGF (10 ng/mL), R-Spondin 1 (200 ng/mL).

Affinity Measurements, Binding Kinetics, and Bioactivity: Affinity measurements and binding kinetics of Wnt/R-Spondin agonists against Frizzled-7 (Cat # 6178-FZ), LRP-6 (Cat # 1505-LR), and Lgr5/GPR49 (Cat # 8078-GP) were performed using Surface Plasmon Resonance. Recombinant receptors were immobilized on a Biacore™ Sensor Chip CM5 and bound to agonists at various concentrations. The Sensorgram was fitted to a 1:1 binding model to assess binding kinetics and affinity. Bioactivities (Figure 1, bottom row) of the Recombinant Human Wnt/R-Spondin 1-3 (blue) Agonist proteins were assessed by measuring the abilities of the proteins to induce Wnt pathway activation using a HEK293 TCF9-SEAP Wnt reporter cell line.

Defined Matrix: Human duodenal intestinal stem cells were cultured in Cultrex UltiMatrix or Cultrex Synthetic Matrix domes with media described previously. Cell density was 10,000 cells per 10 µl of dome for UltiMatrix and 15,000 cells per 10 µl of dome for Synthetic Matrix. Organoid growth was tracked using Incucyte® SX5. Total organoid area was measured on day 5 using Incucyte's analytical software.

Immunocytochemistry: Organoids in domes were fixed for 30 minutes overnight with 2% PFA at 4 °C, washed with 1X PBS, blocked with 0.2% Triton X-100 and donkey serum. Primary antibodies (Villin, Cat # NBP2-75707) were applied to organoids overnight at 4 °C. Organoids were then washed, incubated with secondary antibodies for 48 hours, washed with PBS, and counterstained with DAPI (Cat # 5748). Organoids were imaged using Leica Thunder.

Results

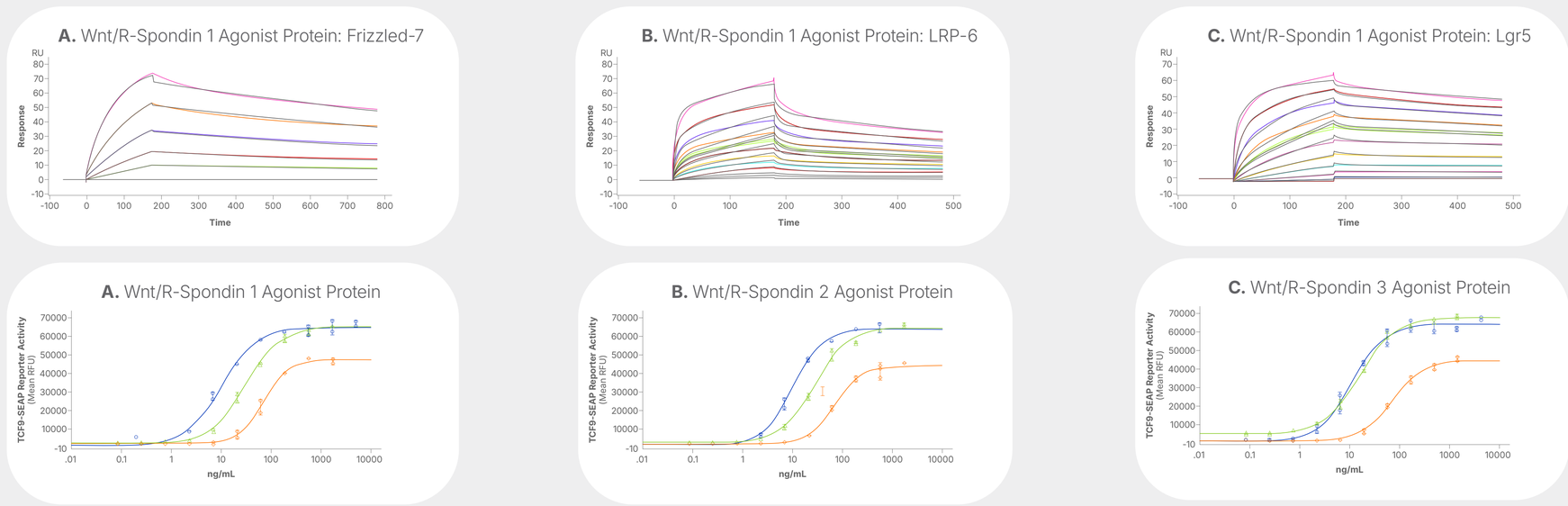

FIGURE 01:

Top Row. Affinity Measurements and Binding Kinetics of the Wnt/R-Spondin 1 Agonist Protein by SPR. Recombinant Human Frizzled-7 **(A)**, LRP-6 **(B)**, or Lgr5/GPR49 **(C)** Fc binding to the Recombinant Human Wnt/R-Spondin 1 Agonist (0.024 nM to 500 nM **(A)**, 0.244 nM to 500 nM **(B)**, and 0.244 to 500 nM **(C)**). KD was determined to be 2.178 nM for Frizzled, 34 nM for LRP-6, and 6.68 nM for Lgr5. **Bottom Row.** Bioactivities of the Recombinant Human Wnt/R-Spondin 1-3 (blue) Agonist Protein compared to Recombinant Human Wnt-3a (orange) alone or Recombinant Human Wnt-3a and R-Spondin 1-3 (green) using the same assay. Wnt/R-Spondin Agonist Proteins exhibit better activity than the Wnt-3a protein alone and similar or better activity than Wnt-3a and the respective R-Spondin proteins added together in this Wnt reporter assay.

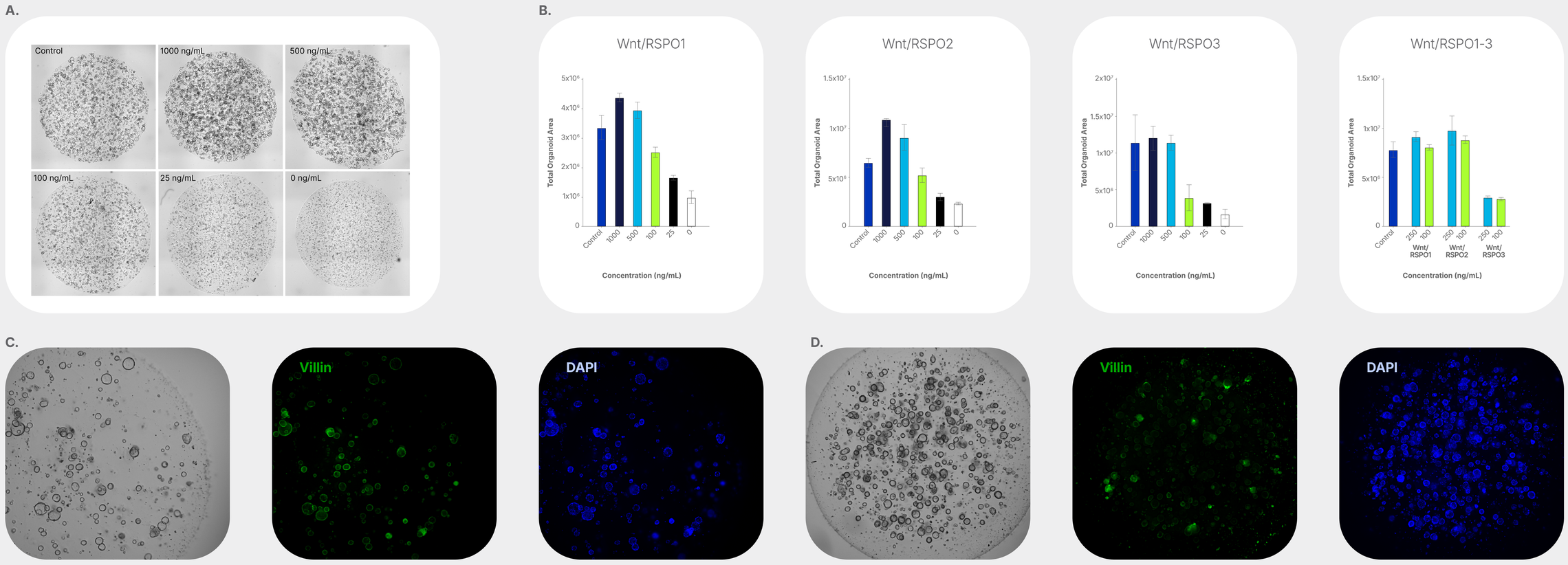

FIGURE 02:

A. Brightfield images of human intestinal organoids expanded with various concentrations of Wnt/RSPO2 or control (Wnt-3A and R-Spondin 2) in Cultrex UltiMatrix domes. **B.** Quantification of total organoid area, which is the area in brightfield image with organoids with Wnt/RSPO1-3. Wnt/RSPO1 and Wnt/RSPO2 showed a dose dependent organoid growth rate. At higher concentrations (i.e. 500 and 1000 ng/mL), Wnt/RSPO1 and Wnt/RSPO2 organoids expanded significantly faster than the control. Wnt/RSPO1 and Wnt/RSPO2 groups matched the control group's growth rate at 250-100 ng/mL. While a dose dependent response was observed for Wnt/RSPO3, the higher concentrations matched the growth of the control. **C.** Enteroid stained with Villin and DAPI in Cultrex UltiMatrix and in **D.** Cultrex Synthetic matrix in media containing Wnt-3a and human R-Spondin 2. Synthetic matrix supported the growth of enteroids without the need to alter expansion media formulation. Cell plating density was slightly adjusted (15,000 in Synthetic Matrix vs. 10,000 in UltiMatrix) to achieve comparable expansion rate.

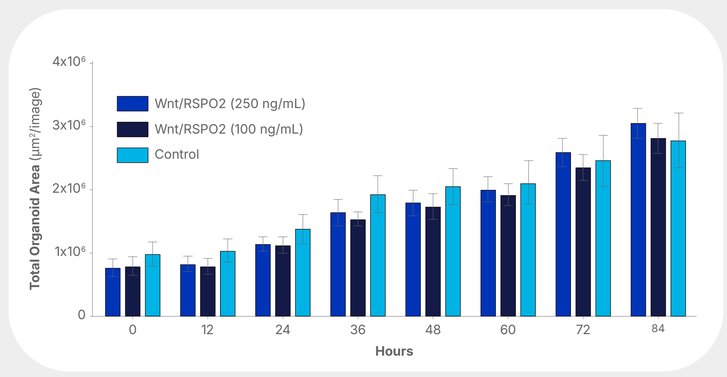

FIGURE 03:

Dose study demonstrated that at 250 and 100 ng/mL of Wnt/RSPO2, similar rate of enteroid expansion in synthetic matrix was achieved compared to control (Wnt-3a and R-Spondin 2). This growth rate is similar as to what was observed in enteroids cultured in Cultrex UltiMatrix.

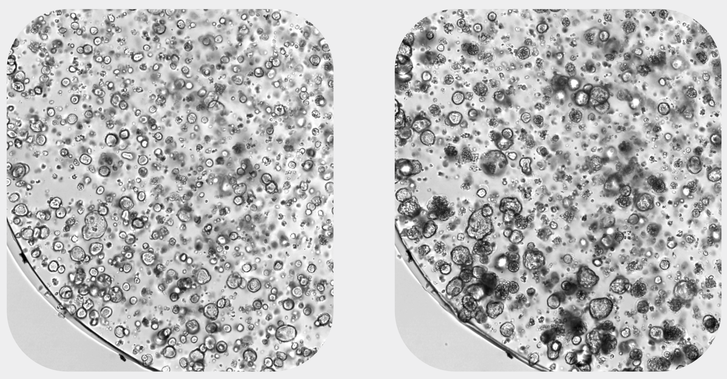

FIGURE 04:

Brightfield images of enteroids cultured in the synthetic matrix under expansion conditions **(A)** and then differentiated to intestinal organoids 3 days after switching to differentiation media **(B)**. Organoid darkening is clearly observed with the majority of organoids showing cystic morphology.

Conclusion

- **Wnt/RSPO1-3 agonists supports enteroid organoid culture.**
- **Wnt/RSPO1-2 agonists demonstrate higher bioactivity and drives greater organoid growth at lower concentrations.**
- **Cultrex Synthetic Matrix supports robust enteroid organoids expansion and differentiation with Wnt/RSPO2 agonist.**

References

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3. *Hughes et al. Proteomics. 2010.*
4. *Janda et al. Nature 2017.*

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