bio-techne® | R&D SYSTEMS

Application Note

Characterization of Potent Wnt/R-Spondin Agonist Proteins for Research and Therapeutic Applications

Introduction

Wnt/β-Catenin signaling is an evolutionarily conserved pathway that plays a critical role in embryonic development, stem cell self-renewal and proliferation, and the maintenance of adult tissue homeostasis. The Wnt family consists of 19 mammalian proteins that promiscuously interact with one of ten Frizzled (FZD) family receptors and the co-receptors, low density lipoprotein receptor-related proteins-5/6 (LRP-5/6), to transduce the Wnt signal. Formation of the Wnt-receptor complex inhibits the activity of the Axin-GSK3-CK1-APC β-Catenin destruction complex that is formed in the absence of Wnt ligands, resulting in the stabilization of β-Catenin, which then translocates to the nucleus, where it activates gene expression through its association with the TCF/LEF family transcription factors.

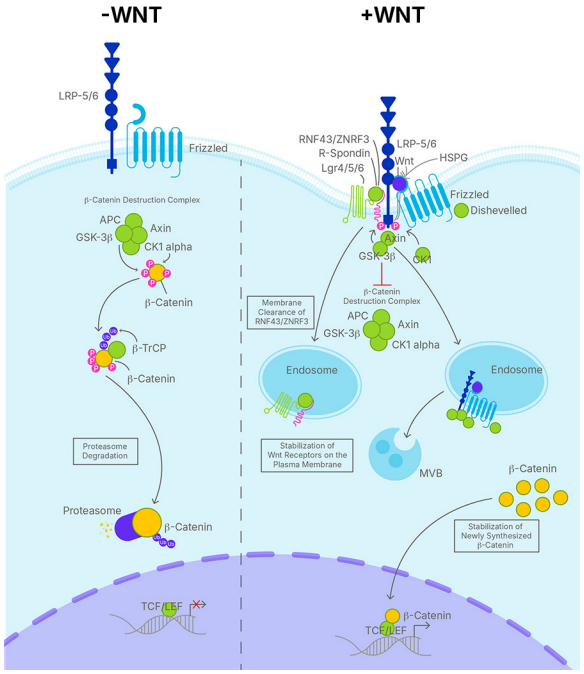

Figure 1. Overview of the Canonical Wnt/β-Catenin-dependent Signaling Pathway. In the absence of Wnt (-Wnt; left-hand side of the graphic), cytoplasmic β-Catenin is phosphorylated by GSK-3β and CK1α in the Axin-GSK3-CK1-APC β-Catenin destruction complex. Phosphorylation of β-Catenin creates a docking site for the β-TrCP E3 ubiquitin ligase on the β-Catenin protein, leading to its ubiquitination and proteasomal degradation. In the presence of Wnt (+Wnt; right-hand side of the graphic), Wnt binds to the Frizzled receptors and LRP-5/6 co-receptors, leading to Dishevelled activation and recruitment of the Axin-GSK3β-CK1 protein complex to the receptor. GSK-3β and CK1 phosphorylate LRP-5/6 and the entire protein complex is subsequently internalized in endosomes that give rise to multivesicular bodies (MVB). Formation of MVBs containing the Wnt-Frizzled-LRP-5/6-Axin protein complexes compromises the ability of CK1 and GSK-3β to phosphorylate newly synthesized β-Catenin, allowing the unphosphorylated protein to accumulate and translocate to the nucleus where it associates with TCF/LEF family transcription factors and co-activators to promote target gene expression.

Wnt proteins undergo extensive post-translational modifications, including glycosylaton and palmitoylation, a lipid modification that is necessary for both Wnt secretion and function. As a result, Wnt proteins are highly hydrophobic and are typically associated with the cell membrane or extracellular matrix. This has made it difficult to purify Wnt proteins in an active state and has hindered the exploration of Wnts as potential therapeutic agents for tissue repair and regeneration.

To overcome these challenges, we have developed and characterized Wnt/R-Spondin agonist proteins that are designed to mimic canonical Wnt signaling, without Wnt protein. These water-soluble Wnt/R-Spondin agonist proteins are structurally unrelated to the Wnt family proteins but are capable of promoting Wnt receptor activation and canonical Wnt signaling. As shown in this application note, the Wnt/R-Spondin agonist proteins bind to Frizzled-7, LRP-5/6, and the R-Spondin receptor, Lgr5 to induce activation of Wnt signaling. The Wnt/R-Spondin agonist proteins display better activity in a Wnt/β-Catenin reporter assay than the Wnt-3a protein alone and similar or better activity than Wnt-3a and the individual R-Spondin proteins together. Additionally, the Wnt/R-Spondin agonist proteins support the culture of intestinal organoids at lower concentrations than when the Wnt-3a and R-Spondin 1 proteins are added together.

Key Takeaways

- The Wnt/R-Spondin agonist proteins are structurally unrelated to Wnt proteins, but they are capable of promoting Wnt receptor activation and canonical Wnt signaling.
- The Wnt/R-Spondin agonist proteins bind to the FZD-7 Wnt receptor, the LRP-6 Wnt co-receptor, and the Lgr5, R-Spondin receptor.
- The Wnt/R-Spondin agonist proteins exhibit better activity than the Wnt-3a protein alone in a Wnt reporter assay, and either similar or better activity than Wnt-3a and the individual R-Spondin proteins together.
- The Wnt/R-Spondin agonist proteins support intestinal organoid culture at lower concentrations than when the Wnt-3a and R-Spondin 1 proteins are added together.

Results

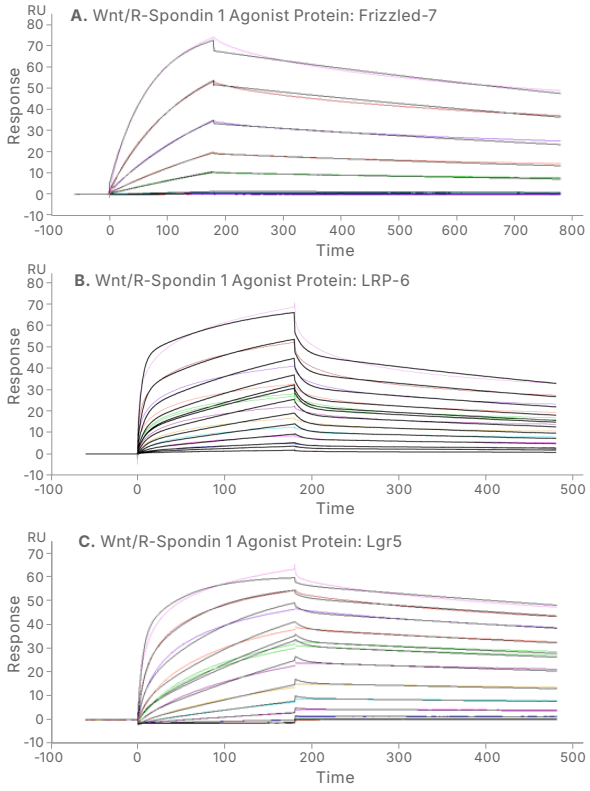

Figure 2. Affinity Measurements and Binding Kinetics of the Wnt/R-Spondin 1 Agonist Protein with Frizzled-7, LRP-6, and Lgr5 by Surface Plasmon Resonance (SPR). (A) Recombinant Human Frizzled-7 Fc Protein (R&D Systems, Catalog # 6178-FZ) was immobilized on a Biacore Sensor Chip CM5, and binding to the Recombinant Human Wnt/R-Spondin 1 Agonist Protein (R&D Systems, Catalog # BT-WRSP1) was measured at a concentration range between 0.024 nM and 50 nM. The double-referenced sensorgram was fit to a 1:1 binding model to determine the binding kinetics and affinity, with an affinity constant of KD = 2.178 nM. **(B)** Recombinant Human LRP-6 Fc Protein (R&D Systems, Catalog # 1505-LR) was immobilized on a Biacore Sensor Chip CM5, and binding to the Recombinant Human Wnt/R-Spondin 1 Agonist Protein (R&D Systems, Catalog # BT-WRSP1) was measured at a concentration range between 0.244 nM and 500 nM. The double-referenced sensorgram was fit to a 1:1 binding model to determine the binding kinetics and affinity, with an affinity constant of KD = 34 nM. **(C)** Recombinant Human Lgr5/GPR49 Fc Protein (R&D Systems, Catalog # 8078-GP) was immobilized on a Biacore Sensor Chip CM5, and binding to the Recombinant Human Wnt/R-Spondin 1 Agonist Protein (R&D Systems, Catalog # BT-WRSP1) was measured at a concentration range between 0.244 nM and 500 nM. The double-referenced sensorgram was fit to a 1:1 binding model to determine the binding kinetics and affinity, with an affinity constant of KD = 6.68 nM.

TABLE // 01

	Lgr5 (aa 1-560)	LRP-6 (aa 1-1368)	Frizzled-7 (aa 1-185)
Wnt/RSPO1 Agonist Protein	KD = 6.68 nM	KD = 34.0 nM	KD = 2.178 nM
Wnt/RSPO2 Agonist Protein	KD = 1.60 nM	KD = 48.3 nM	KD = 0.435 nM
Wnt/RSPO3 Agonist Protein	KD = 21.4 nM	KD = 28.4 nM	KD = 1.248 nM

Table 1. Summary of the Affinity Measurements and Binding Kinetics of the Wnt/R-Spondin Agonist Proteins with Lgr5, LRP-6, and Frizzled-7 by SPR. Recombinant Human Lgr5/GPR49 Fc Protein (R&D Systems, Catalog # 8078-GP), Recombinant Human LRP-6 Fc Protein (R&D Systems, Catalog # 1505-LR), or Recombinant Human Frizzled-7 Fc Protein (R&D Systems, Catalog # 6178-FZ) was immobilized on a Biacore Sensor Chip CM5 and binding of the Recombinant Human Wnt/R-Spondin 1 Agonist Protein (R&D Systems, Catalog # BT-WRSP1), the Recombinant Human Wnt/R-Spondin 2 Agonist Protein (R&D Systems, Catalog # BT-WRSP2), or the Recombinant Human Wnt/R-Spondin 3 Agonist Protein (R&D Systems, Catalog # BT-WRSP3), was measured at a concentration range between 0.244 nM and 500 nM for the immobilized Lgr5/GPR49 and LRP-6 proteins, and at a concentration range between 0.024 nM and 50 nM for the immobilized Frizzled-7 protein. Double-referenced sensorgrams were fit to a 1:1 binding model to determine the binding kinetics and affinity constants shown in the table.

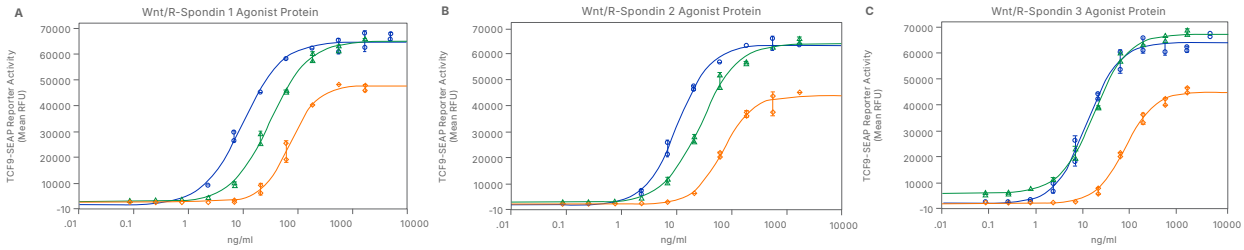

Figure 3. The Wnt/R-Spondin Agonist Proteins Display Better Activity than the Wnt-3a Protein Alone and Similar or Better Activity than the Individual Wnt-3a and R-Spondin Proteins Added Together in a Wnt Reporter Assay. The bioactivities of the **(A)** Recombinant Human Wnt/R-Spondin 1 Agonist Protein (R&D Systems, Catalog # BT-WRSP1; blue line), **(B)** Recombinant Human Wnt/R-Spondin 2 Agonist Protein (R&D Systems, Catalog # BT-WRSP2; blue line) and **(C)** Recombinant Human Wnt/R-Spondin 3 Agonist Protein (R&D Systems, Catalog # BT-WRSP3; blue line) were assessed by measuring the abilities of the proteins to induce Wnt pathway activation using a HEK293 TCF9-SEAP Wnt reporter cell line. The activities of the proteins were compared to Recombinant Human Wnt-3a (R&D Systems, Catalog # 5036-WN; orange lines) alone or Recombinant Human Wnt-3a (R&D Systems, Catalog # 5036-WN) added with either Recombinant Human R-Spondin 1 (R&D Systems, Catalog # 4645-RS; part A, green line), Recombinant Human R-Spondin 2 (R&D Systems, Catalog # 3266-RS; part B, green line), or Recombinant Human R-Spondin 3 (R&D Systems, Catalog # 3500-RS; part C, green line) using the same assay. The results demonstrate that the 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.

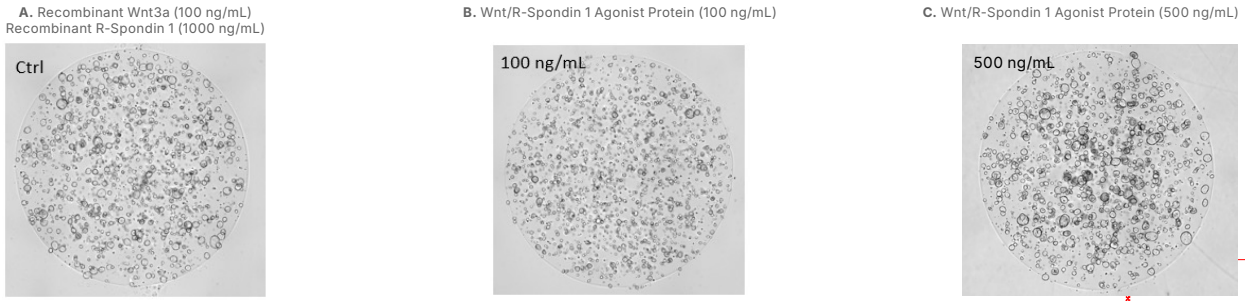

Figure 4. The Wnt/R-Spondin 1 Agonist Protein Supports Intestinal Organoid Culture at Lower Concentrations than the Wnt-3a and R-Spondin 1 Proteins Added Together. Human adult intestinal organoids were cultured using Cultrex™ UltiMatrix RGF Basement Membrane Extract (R&D Systems, Catalog # BME001-05) and intestinal organoid culture medium including **(A)** 100 ng/mL Recombinant Human Wnt-3a (R&D Systems, Catalog # 5036-WN) and 1 ug/mL Recombinant Human R-Spondin 1 (R&D Systems, Catalog # 4645-RS) as a control (Ctrl), **(B)** 100 ng/mL Recombinant Human Wnt/R-Spondin 1 Agonist Protein (R&D Systems, Catalog # BT-WRSP1) or **(C)** 500 ng/mL Recombinant Human Wnt/R-Spondin 1 Agonist Protein (R&D Systems, Catalog # BT-WRSP1), along with the other reagents listed in the intestinal organoid culture medium recipe. Brightfield images of the organoids taken at 5 days of culture demonstrate that the Wnt/R-Spondin 1 Agonist Protein supports intestinal organoid culture at lower concentrations than when the individual Wnt-3a and R-Spondin 1 proteins are added together to the cultures.

Conclusions

In this study, we describe the characterization of three recombinant Wnt/R-Spondin agonist proteins that promote canonical Wnt signaling but are structurally unrelated to the Wnt family proteins. Using SPR, we demonstrate that the Recombinant Wnt/R-Spondin agonist proteins bind to Frizzled-7, LRP-6, and Lgr5, and they exhibit higher activity than the Wnt-3a protein alone, and either higher or similar activity as Wnt-3a and the individual R-Spondin proteins together in a Wnt/β-Catenin reporter assay. Finally, we show that the Wnt/R-Spondin agonist proteins support robust intestinal organoid culture at lower concentrations than when the Wnt-3a and R-Spondin 1 proteins are added together. As the Wnt/R-Spondin agonist proteins are water-soluble and are significantly easier to purify than the Wnt family proteins, they offer new opportunities for investigating Wnt signaling, exploring the functional significance of different Frizzled receptors, and determining the efficacy of using Wnt activators as therapeutic agents for tissue repair and regeneration.