

Review

Design principles and therapeutic applications of novel synthetic WNT signaling agonists

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SUMMARY

Wingless-related integration site or Wingless and Int-1 or Wingless-Int (WNT) signaling is crucial for embryonic development, and adult tissue homeostasis and regeneration, through its essential roles in cell fate, patterning, and stem cell regulation. The biophysical characteristics of WNT ligands have hindered efforts to interrogate ligand activity *in vivo* and prevented their development as therapeutics. Recent breakthroughs have enabled the generation of synthetic WNT signaling molecules that possess characteristics of natural ligands and potently activate the pathway, while also providing distinct advantages for therapeutic development and manufacturing. This review provides a detailed discussion of the protein engineering of these molecular platforms for WNT signaling agonism. We discuss the importance of WNT signaling in several organs and share insights from the initial application of these new classes of molecules *in vitro* and *in vivo*. These molecules offer a unique opportunity to enhance our understanding of how WNT signaling agonism promotes tissue repair, enabling targeted development of tailored therapeutics.

INTRODUCTION

WNT (“Wingless-related integration site” or “Wingless and Int-1” or “Wingless-Int”) signaling plays key roles in controlling development, tissue homeostasis, and the regeneration of many organs and tissues.¹ Although modulation of WNT signaling pathways has the potential to treat many degenerative diseases and tissue injuries, developing research tools and therapeutics that modulate specific aspects of WNT signaling has been challenging. Beyond the complexity of the signaling components and the number of WNT ligands and receptor systems, the biology of the WNT signaling in each adult tissue remains to be fully elucidated. The ability to design appropriate molecules endowed with drug-like properties will be critical to address unanswered research questions and to develop novel therapeutics. In this review, we describe efforts by our group and others in developing novel synthetic WNT agonists. These extensive efforts have enabled researchers to directly modulate the WNT signaling pathway, thereby promoting repair and regeneration in specific tissues.

WNT ligands activate diverse downstream signaling cascades. One widely studied WNT signaling pathway involves the Frizzled (FZD) family of receptors, low-density lipoprotein receptor-related protein (LRP) coreceptors, and the transcription factor β -catenin. After WNT binding, FZD and LRP play important roles in recruiting molecules such as disheveled (DVL) and AXIN to the cell membrane.^{2,3} Subsequently, the β -catenin destruction complex containing AXIN, APC, GSK-3, and CK1 in the cytoplasm disassembles, thereby stabilizing β -catenin. Finally, β -catenin enters the nucleus and with TCF/LEF transcription factors activates downstream target genes of WNT/ β -catenin signaling, resulting in cell proliferation and cell fate determination. The complex WNT/ β -catenin signaling is regulated by multiple mechanisms including self-limiting negative feedback regulation (Figure 1). A total of 19 WNT ligands have been identified in mammals, and ten FZDs (FZD_{1–10}) and two LRPs (LRP5 and 6) have been found to play roles in the WNT signaling system.⁴ WNT ligands are promiscuous in that each WNT can bind multiple FZDs and LRPs. WNTs are also post-translationally modified; for example, long-chain fatty acid modification is required for receptor binding and signaling. Consequently, endogenous WNT proteins pose challenges in drug development, and this pathway was long believed to be undruggable. Four FZD subfamilies, FZD_{1,2,7}, FZD_{5,8}, FZD₄, and FZD_{9,10}, mediate WNT/ β -catenin (canonical) signaling, whereas FZD_{3,6} is involved primarily in non-canonical signaling independent of β -catenin.^{5,6} Overlap in FZD expression often exists; however, distinct expression patterns are also observed.^{7,8} WNT ligands have undesirable biophysical properties and a lack of specificity and consequently are unsuitable for answering specific biological questions or developing therapeutics. On the other hand, directly targeting specific FZD and LRP receptors with molecules that mimic natural

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<https://doi.org/10.1016/j.isci.2024.109938>



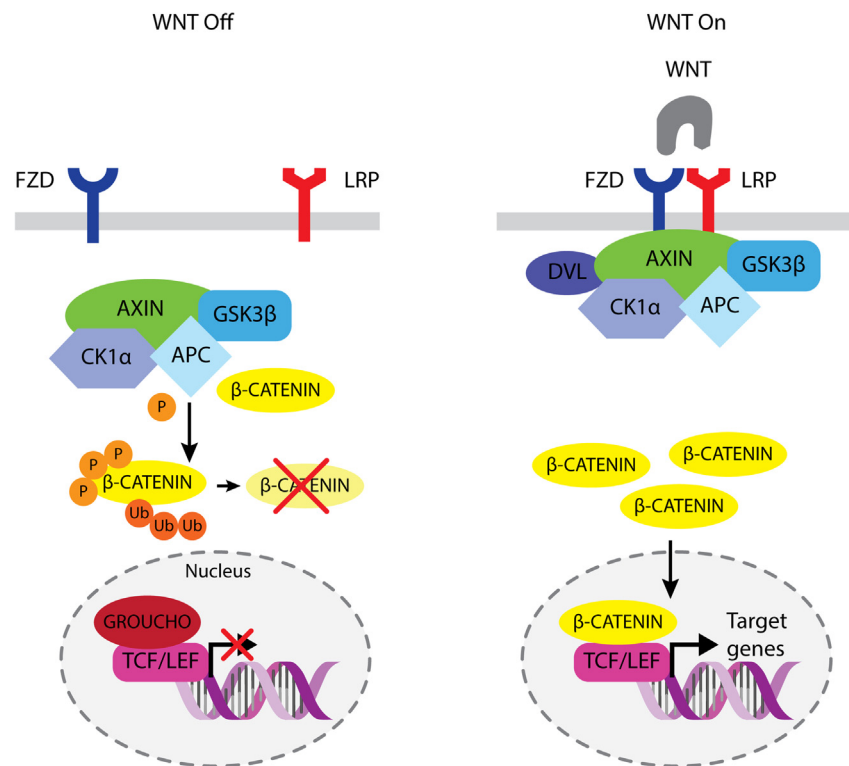


Figure 1. The canonical WNT signaling pathway



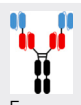


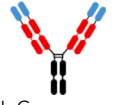
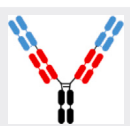
WNT off: in the absence of WNT binding to FZD and LRP, the destruction complex, consisting of AXIN, APC, CK1α, and GSK3β, targets cytoplasmic β-catenin for degradation. Consequently, GROUCHO binds TCF/LEF in the nucleus and blocks transcription of target genes. WNT on: after binding of WNT to FZD and LRP, the destruction complex is recruited to, and anchored at, the membrane via DVL. β-catenin is free to accumulate in the cytoplasm and translocates to the nucleus, where it binds TCF/LEF and initiates transcription of target genes.

ligands has promise in research and therapeutic activation of WNT/β-catenin signaling in specific tissues/cell types. However, how to engage the receptors to mimic the natural ligand actions and induce efficient downstream signaling was determined only recently. Platforms of WNT ligand mimetics have been developed that assemble FZDs and LRPs in proximity, with the desired geometry and valency.

Cell surface-bound E3 ubiquitin ligases, zinc and ring finger 3 (ZNF3), and ring finger protein 43 (RNF43) are induced after WNT/β-catenin pathway activation.^{6,9} ZNF3 and RNF43 target FZD (and LRP) for degradation and turn off WNT/β-catenin signaling. R-spondins (RSPO1–4) are a family of ligands that bind the receptor complex containing ZNF3/RNF43 and co-receptor leucine-rich repeat-containing G-protein-coupled receptors (LGR4, 5, or 6). RSPO binding causes clearance or sequestration of the ternary receptor complex, thereby removing ZNF3 and RNF43 and limiting their effects on FZD, stabilizing WNT receptors, and amplifying WNT/β-catenin signaling. This endogenous WNT/β-catenin signaling enhancing system plays important roles in tissue homeostasis and injury repair, particularly when WNT ligands are abundant, but FZD receptor-mediated signals are limiting. However, LGRs are not tissue or cell type specific, and exogenous RSPO treatment has the potential to amplify WNT/β-catenin signaling in multiple tissues/organs. Therefore, tissue- and cell type-specific RSPO mimetics are desirable. Details of novel targeted RSPO mimetics are described in the following. Molecules with a combination of WNT and RSPO ligand characteristics (“superagonists”) may also be considered in certain contexts.

Cell-to-cell signaling is integral to the complex orchestration of growth, fate choice, and movements of cells that ultimately lead to tissue and organ formation during embryogenesis, homeostasis, and injury-induced repair in adults. WNT/β-catenin signaling is a major developmental pathway that plays crucial roles in many embryonic contexts. In adults, it provides critical niche signals for stem cell renewal and differentiation, and, in some tissues, WNT/β-catenin can imbue stem cells with a self-organizing ability, orchestrating regeneration and restoration of tissue structure and function.¹⁰ In this review, we discuss efforts regarding the design principles of various tools for modulating WNT signaling. We additionally describe the biological insights learned from the initial application of these tools, including WNT mimetics, RSPO mimetics, and superagonists with various receptor and cell specificity (collectively called “WNT agonists”), to promote cell expansion *in vitro* and tissue repair *in vivo*. These tools will further advance understanding of the mechanistic interactions of WNT signaling with inflammation, fibrosis, tissue regeneration, and other signaling pathways culminating in physiological tissue repair. With this enhanced understanding, WNT signaling agonists can be designed to promote tissue regeneration in disease or after injury, and to tailor therapeutics to benefit patients.

Table 1. Partial list of representative WNT mimetic formats

Format	Molecule specificity summary		Ref.	Date filed or published
	FZD specificity	LRP specificity		
 scFv-DKK1c	1,2,5,7,8	5,6	WO2016/040895	09/2014
	5,8		Janda et al. ¹⁴	05/2017
Helix bundle-DKK1c	1,2,7		WO2020/018445	07/2018
DARPin-DKK1c	4		Miao et al. ¹⁵	08/2020
 VHH-IgG	1,2,5,7,8	5,6	WO2019/126398	12/2017
	1,2,7		WO2020/010308	07/2018
	5,8		Chen et al. ¹⁶	03/2020
	4		Fowler et al. ¹⁹	05/2021
	9,10		Xie et al. ⁷	05/2022
	Other mono or poly specificities		Nguyen et al. ²⁴	09/2022
 scFvs-Fc	1,2,5,7,8	5,6	WO2019/126398	12/2017
	1,2,7		WO2020/010308	07/2018
	5,8		Chen et al. ¹⁶	03/2020
	7		Gumber et al. ²³	12/2020
 Diabody-Fc-diabody	1,2,5,7,8	5,6	WO2019/126398	12/2017
	2		WO2019/159084	02/2018
	4		Tao et al. ¹⁸	08/2019
	5		Chidiac et al. ²⁵	06/2021
	7			
 VHHs-Fc	8 and various specificities	5,6	WO2019/126398	12/2017
 Fv-IgG	1,2,5,7,8	5,6	WO2019/126398	12/2017
	1,2,7		WO2020/010308	07/2018
	5,8		WO2021/173726	02/2020
	4		Post et al. ²⁰	07/2023
	4,9		Ding et al. ⁸⁷	06/2023
	10			
 Fab-IgG	1,2,5,7,8	5,6	WO2019/126398	12/2017
			WO2020/010308	07/2018
			WO2021/173726	02/2020
			Post et al. ²⁰	07/2023

Only representative formats and molecules are listed. Additional information on other combinations of different binding domains, linker lengths, orientations, geometries, combinations of different receptor specificities, stoichiometry, and symmetrical vs. nonsymmetrical designs, with or without the Fc domain, etc. can be found in the references.

NOVEL WNT-ACTIVATING APPROACHES

Discovery and development of WNT mimetics activating WNT/ β -catenin signaling

Elucidating the individual ligand or receptor functions of the 19 mammalian WNT ligands, ten FZD receptors (FZD₁₋₁₀) and two LRP coreceptors (LRP5 and LRP6) in tissues or cell types of interest have been hindered by several challenges. First, WNT ligands undergo extensive post-translational modifications. In particular, palmitoylation on a conserved serine residue, a modification critical for secretion and FZD binding, makes the ligands insoluble and difficult to produce.¹¹ Second, WNT and receptor interactions are promiscuous, such that each ligand can bind and activate multiple FZD/LRP pairs.^{12,13} These challenges pose major obstacles in basic research aimed at understanding the roles and functions of an individual ligand or receptor, as well as in the development of potential regenerative therapeutics targeting this pathway. Recent advances in WNT mimetic (or surrogate) development have offered exciting opportunities to overcome these challenges that have hampered the field for decades.

In the seminal work by Janda et al., the first WNT surrogate was constructed by linking an FZD binder and an LRP binder unrelated to WNTs into a single polypeptide chain, thereby creating a bispecific molecule capable of heterodimerizing these two receptors (Janda et al.¹⁴; Table 1; Figure 2B). This strategy was favored over re-engineering natural ligands, to avoid the inherent challenges of acylation and the

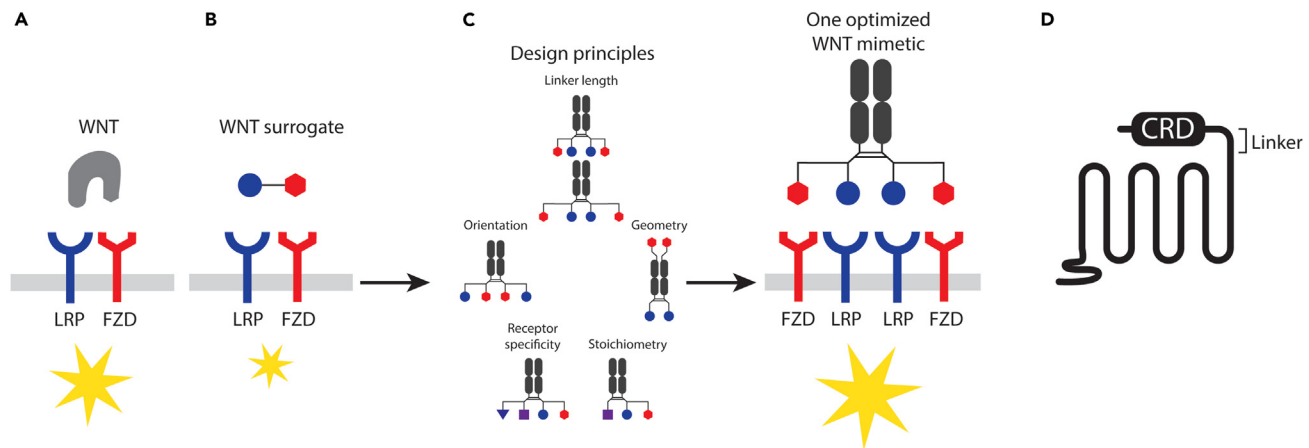


Figure 2. Discovery and development of WNT mimetics

(A) Endogenous WNT ligand binding FZD and LRP, resulting in downstream signaling events.
 (B) WNT surrogates consisting of an FZD binder and an LRP binder elicit similar signaling cascades.
 (C) Design principles and one optimized WNT mimetic.
 (D) Linker region after the CRD of FZD. Yellow stars denote activation of the WNT signaling pathway.

extensive receptor binding site homology.¹² Two FZD binders were selected for the initial surrogate construction: one was the single-chain variable fragment (scFv) of an antagonistic antibody, vantictumab (18R5), with FZD_{1,2,5,7,8} specificity; the other was a *de novo*-designed four-helix bundle protein, B12, which binds FZD_{5,8}. The LRP binder was the C-terminal domain of the human WNT antagonist DKK1. These two WNT surrogates, 18R5scFv-DKK1c and B12-DKK1c (Table 1), have been found to induce WNT/ β -catenin signaling in an FZD-selective fashion *in vitro*, thus replacing WNT3A in supporting growth of a range of human organoid cultures, and to exhibit WNT activity *in vivo* in the mouse liver after adenovirus-mediated expression.¹⁴ Alternative FZD-binding domains based on ankyrin repeat proteins (designed repeat protein binder, DRPB) were subsequently used to improve the affinity, FZD specificity, and potency of the early WNT surrogates (Miao et al.¹⁵; Table 1). Beyond organoid studies, these new molecules, when delivered via adenovirus expression *in vivo*, revealed FZD subfamily-specific effects in the intestine and liver. These studies demonstrated that WNT/ β -catenin signaling can be activated by heterodimerization of receptors, thus laying the groundwork for further development of these tools for research and therapeutics.

The development of WNT mimetics has also focused on antibody-based scaffolds. However, in contrast to the WNT mimetics described in the preceding paragraph, in which one FZD binder was linked to one LRP binder (a bivalent bispecific format denoted 1:1 design herein), WNT mimetics using antibody fragments for both FZD and LRP binding in the 1:1 format resulted in little to no activity (WO2019/126398,^{16,17} WO2019/159084;¹⁸). In one such study of bivalent bispecific antibody-based molecules, WNT mimetic activity was present in crudely purified protein preparations but was lost after the final size exclusion chromatography. Analysis of all size exclusion chromatography fractions indicated recovery of activity in fractions containing species with molecular weights of two or more times the expected molecular weights of the monomers (WO2019/126398,^{16,17}). This unexpected finding suggested that WNT mimetic generation based on antibody fragments may require clustering of receptor complexes of more than one FZD and one LRP for efficient signaling. Early examples of such multivalent molecules used a tetravalent bispecific format, or 2:2 design, with broad or subfamily FZD specificity (WO2019/126398). Given that these tetravalent molecules have two FZD binding arms, each of which can bind the same or different FZDs, or can bind one or two copies of FZDs at the same time, and that the same binding variations can occur on the LRP side, many different permutations could potentially occur in the receptor complexes assembled on the cell surface for signaling. For example, the assembled complex could contain two of the same FZDs and two of the same LRPs, two different FZDs and two of the same LRPs, two of the same or different FZDs and one or two LRPs, etc. Therefore, different design parameters, including valency, linker lengths, relative orientation of binders, geometry, stoichiometry, epitope, and receptor specificity, have been systematically evaluated to further understand signaling requirements (WO2019/126398,¹⁶ WO2020/010308,¹⁹ WO2019/159084¹⁸; Figure 2C; Table 1). Although many parameters appear to be binder specific, multivalent binding to receptors with a ratio of two FZDs to two LRP binders (tetravalent or 2:2 design) appeared optimal in all cases tested (WO2019/126398,¹⁶ WO2019/159084^{18,20}; Figure 2C; Table 1). A ratio of two FZDs and one LRP binder (trivalent or 2:1 design) has also been found to be highly effective in some reports (WO2019/126398,¹⁶ WO2020/010308), although such findings have not been observed in other studies (WO2019/159084¹⁸). This stoichiometry requirement with the WNT mimetics is consistent with findings from structural studies revealing FZD dimerization after natural ligand binding as either a 2:1²¹ or 2:2 complex with LRP.²² These systematic analyses have also revealed that receptor complexes consisting of multimers of different FZD or LRP isotypes produce efficient signaling^{16,18} (Figure 2C). These observations may be biologically relevant. Given the promiscuous binding of WNT/FZD/LRP interactions and the ability of FZD to dimerize after ligand binding, natural ligand/receptor complexes might potentially contain different stoichiometries and various permutations.

Such design concepts have been applied to several antibody-based formats. Different antibody formats may vary in the distances and geometries between various binding arms. In a direct comparison of tandem scFv-Fc, Fv-IgG, and Fab-IgG formats with the same set of

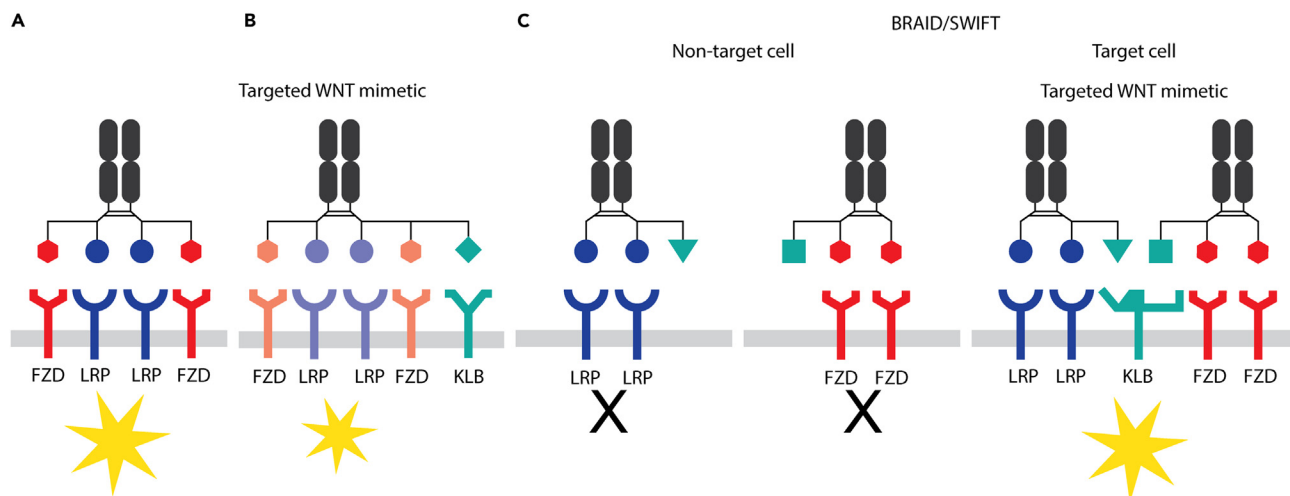


Figure 3. Cell-targeted WNT mimetics

(A) A bispecific tetravalent WNT mimetic.

(B) A cell targeting receptor fused to a WNT mimetic.

(C) A WNT mimetic split into two inactive molecules: one with two FZD binding arms and the other with two LRP binding arms. Each molecule is connected to a binder that binds a different epitope of the common bridging receptor β Klotho (KLB). Yellow stars denote activation of WNT signaling.

FZD and LRP binders, Fv-IgG format has been found to produce the most active molecules²⁰ (Table 1). Further studies are necessary to determine whether this preference is specific to the FZD/LRP binder combination tested in the study or a general preference of this particular format for WNT mimetic construction. A set of tool molecules covering all WNT/ β -catenin signaling FZDs with subfamily or monospecificity has been developed²⁰ and may aid in the study of the effects of different FZDs in tissues and cell types. Other formats described to date include tandem scFv-Fc,^{16,23} VHH-immunoglobulin G (IgG),^{7,19,24} VHH-VHH-Fc or VHH-Fc-VHH (WO2019/126398), and diabody-Fc-diabody^{18,25} (Table 1). The WNT mimetics from the Surrozen group have been referred to as Surrozen WNT signal activating proteins (SWAPs),^{7,16,19,20,24} and the molecules from the Sidhu and Angers groups have been called FZD/LRP agonists (FLAGs).¹⁸

Because WNTs bind the cysteine-rich domain (CRD) of FZDs,¹² most efforts in antibody binder discovery and WNT mimetic construction have focused on the FZD CRD.^{14,16,18} Two antibodies specific to the FZD₇ linker region after the CRD (1291 and 1791, WO2016/205551 and WO2016/205566, Figure 2D), when assembled in a tetravalent bispecific format (2:2) with either an LRP5 binder in the VHH-IgG format (1291-3 and 1791-3, WO2020/010308) or an LRP6 binder in the tandem scFv-Fc format (F7L6, F7 is 1791²³), have also shown WNT mimetic activity with monospecificity toward FZD₇. These studies have expanded the potential effective epitopes on FZD suitable for WNT mimetic construction, further supporting that WNT/ β -catenin signaling can be activated by heteromultimerization of receptors.

Beyond antibody-based modalities, small-molecule WNT mimetics capable of clustering FZD and LRP have also been explored. A small molecule (MFH) targeting site 2 of FZD₈ CRD, when fused to a peptide derived from the N-terminal region of human DKK1 (MFH-ND), has been found to weakly induce WNT/ β -catenin signaling alone and significantly augment WNT3A activity.²⁶ Other modalities, such as peptides, cyclic peptides, or aptamers, may also be suitable for WNT mimetic construction. Similar principles may be applied to the study of WNT/ β -catenin-independent signaling pathways. Overall, the WNT mimetics based on non-WNT scaffolds, particularly antibody-based molecules, offer advantages of improved biophysical properties, such as solubility and stability, ease of production, and ability to target specific FZD/LRP pairs. This approach is expected to advance basic research and regenerative therapeutic development.

Cell-targeted WNT mimetics

Although FZD and LRP specificity can now be achieved with the WNT mimetic agonistic antibodies described earlier, because each of the ten FZDs and two LRP coreceptors is broadly expressed on many cell types in different tissues, tissue/cell targeting cannot be achieved with WNT mimetics alone. Given the important roles of WNT in many tissues during development, and in adult tissue homeostasis and injury repair, often with opposing roles in parenchymal vs. nonparenchymal cells, being able to target WNT or WNT mimetic action to a specific cell type is critical for research and development of therapeutics.

Cell-targeted WNT mimetics can be generated by fusing a binding domain to a cell-targeting receptor to a WNT mimetic (Figure 3B). This approach, sometimes referred to as chimeric activators, has been explored in other growth factor or cytokine systems.²⁷ Another novel cell targeting approach called bridged activation by intra/intermolecular division (BRAID) has been developed, in which an active drug molecule is divided into inactive parts that are assembled via a bridging receptor specific to the target cell.²⁷ The application of this concept to WNT mimetics is called splitting of WNT to induce functional targeting (SWIFT). In a proof-of-concept study, taking advantage of active WNT mimetics requiring multivalent binding to FZD and LRP in the same molecule, an active tetravalent bispecific WNT mimetic (Figure 3A) has been

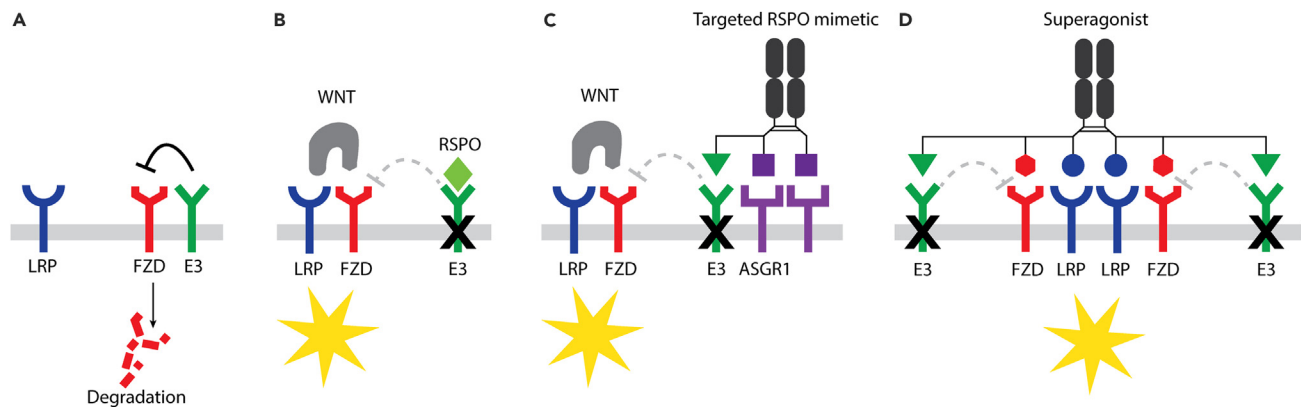


Figure 4. Cell-targeted RSPO mimetics and WNT superagonists

(A) An E3 ligase targets FZD for receptor degradation, thus inhibiting the cellular response to WNT.

(B) RSPO enhances the cellular response to WNT by interfering with E3 ligase-mediated FZD degradation. RSPO alone does not activate WNT signaling in the absence of endogenous WNTs.

(C) An ASGR1-targeted RSPO molecule specifically enhances WNT signaling in ASGR1-expressing hepatocytes.

(D) A WNT superagonist consisting of a WNT mimetic and an RSPO mimetic can replace both WNTs and RSPOs. Yellow stars denote activation of WNT signaling.

split into two inactive molecules, one consisting of two FZD binding arms and the other having two LRP binding arms. These two inactive components were then linked to two different binders capable of binding to two different epitopes on a common bridging receptor, β Klotho (KLB) (Figure 3C). The mixture of the two tethered inactive components has been found to specifically induce WNT signaling in primary hepatocytes or a hepatoma cell line expressing β Klotho, but not HEK293 or intestinal organoids in which β Klotho is not expressed.²⁷ This novel approach now enables specific targeting of WNT activation in desired cell types, to enhance the responses of target cells in a microenvironment and decrease undesirable off-target effects.

Development of cell/tissue-targeted RSPO mimetics

WNT signaling is fine-tuned by both positive and negative regulators. One such control system involves RSPO proteins, which work synergistically with WNTs to enhance signaling levels but do not activate in the absence of WNTs. This pathway consists of two membrane E3 ubiquitin ligases, secreted RSPO1–4, and their receptors, LGR4–6 or heparin sulfate proteoglycans (HSPGs).²⁸ The two E3 ligases, ZNRF3 and RNF43, are WNT target genes that ubiquitylate FZD and LRP and consequently lead to receptor degradation and downregulation on the cell surface, thereby inhibiting the cellular response to endogenous WNTs (^{9,29}; Figure 4A). RSPOs counter E3 actions by binding both E3 ligases and either LGRs or HSPGs, thus leading to cell membrane clearance of the E3 ligases, increasing FZD and LRP levels, increasing cellular sensitivity to endogenous WNTs, and thereby amplifying WNT signaling³⁰ (Figures 4A and 4B). Therefore, RSPOs also play important roles in stem cell renewal, tissue homeostasis, and injury repair.³¹ Given that RSPOs do not have an intrinsic ability to activate WNT signaling, RSPO selectively boosts WNT signaling only in cells in which endogenous WNT ligands are present. However, the potential applications of RSPOs in regenerative medicine are limited, because of the broad distribution of their receptors and their pleiotropic effects on many tissues.

Tissue-selective RSPO mimetics have been developed by redirecting E3 ligase sequestration or degradation away from LGRs and HSPGs, and toward cell-specific cell surface proteins. Two studies have successfully deployed a bispecific molecule consisting of a fusion between specific E3 binders and a cell surface receptor binder of interest. The E3 binders described to date are either specific antibodies that bind RNF43 or ZNRF3,³² or mutant RSPOs that retain the ability to bind E3 but are unable to bind LGRs and HSPGs³³ (Figure 4C). The cell surface receptor binders explored include a cytokine interleukin (IL)-2 for immune cell targeting via binding CD25³² and an anti-ASGR1 antibody for hepatocyte targeting.³³ ASGR1 is a receptor localized on the surfaces of hepatocytes. The ASGR1-targeted RSPO mimetic molecules (termed Surrozen WNT signal enhancers engineered for tissue specificity; SWEETS) show specific signaling in hepatocytes and avoid RSPO-sensitive intestinal tissue (Figure 4C). ASGR1 SWEETS works by enhancing local endogenous WNT signaling in the liver. Both *in vitro* and *in vivo*, these molecules have been found to lead to increased hepatocyte proliferation and improved liver function in a mouse model of liver injury.³³ Additional examples of SWEETS targeting other cell surface receptors, such as the transferrin receptor (TFRC), Ly6/PLAUR domain-containing protein 3 (LYPD3), and Desmoglein-3 (DSG3), have also been reported.³³ Interestingly, these RSPO mimetic molecules act as protein degraders. Antibody-based targeting of E3 ligases and ASGR1 have been found to facilitate proteasomal degradation via ubiquitylation or lysosomal pathway degradation.^{32,34–41} These studies have demonstrated the feasibility of cell-targeted RSPO mimetics and enabled new opportunities for the development of RSPO mimetic WNT modulators for regenerative medicine.

Development of WNT superagonists

For the generation of potent antagonist molecules to inhibit WNT signaling, bispecific molecules that bind the two E3 ligases (ZNRF3 and RNF43) and FZD have been constructed to enhance this natural negative regulatory mechanism in FZD receptor ubiquitylation and

degradation²⁰ (WO2021/173726). Removal of WNT receptors from the cell surface would be expected to inhibit pathway activation. This concept is similar to that underlying other targeted protein degrader systems, such as PROTAC (proteolysis targeting chimera⁴³). In contrast to the expected results of enhanced FZD degradation, these bispecific E3-FZD engagers have shown the opposite activity, increasing FZD levels and enhancing cellular sensitivity to WNTs, similarly to RSPO mimetic treatment.²⁰ Although the mechanisms leading to these unexpected results remain to be determined, these observations have provided an opportunity to combine an RSPO mimetic and a WNT mimetic into a single molecule, thus creating a WNT “superagonist”²⁰ (Figure 4D). The WNT superagonist platform combines WNT and RSPO mimetics’ effects into one trispecific hexavalent molecule by binding FZD, LRP, and E3 ligase. This platform can be fine-tuned as a set of tool molecules activating all WNT/ β -catenin FZD receptors with subfamily or monospecificity replacing both WNTs and RSPOs. For example, in organoid cultures dependent on both WNT and RSPO, a single superagonist can replace both factors in the medium. This set of WNT agonist combination molecules is easily expressed and purified and supports better expansion of various human and mouse organoid systems. These WNT-modulating platforms can also be broadly applied to organoids, pluripotent stem cells (PSCs), and *in vivo* research and may serve as a basis for future regenerative therapeutic development.

APPLICATIONS OF WNT AGONISTS

Genetic manipulation of the components of the WNT pathway has made enormous progress toward the mechanistic understanding of this pathway in development and adult tissue function. However, mutations in signal transduction components, such as APC, RNF43, or β -catenin, may have fundamentally different effects from endogenous ligands; moreover, genetic manipulations of ligands and receptors do not recapitulate the transient nature of the signaling and are complicated by the promiscuous interactions between WNTs and their receptors, and the functional redundancies in the system. Therefore, additional approaches are needed to elucidate this complex signaling pathway. The development of receptor-specific and cell type-specific modulators, as described in the preceding section, has offered unprecedented ability to probe this pathway. In this section, we discuss several applications of these tools reported to date. Further refinement of these tools is expected to enable additional biological insights and the expansion of studies into an even broader set of tissues.

Liver

Hepatocytes and non-parenchymal cells of the adult liver are organized as anatomical and functional units termed liver lobules.⁴³ Within each lobule, the expression of metabolic genes and their associated functions display a zonal distribution along the radial axis between the hepatic portal and central veins—a phenomenon described as metabolic zonation. WNT/ β -catenin signaling activity near the central vein is a master regulator of liver zonation. Pathway inactivation by liver-specific deletion of receptors, ligands, or β -catenin abolishes pericentral gene expression signatures.⁴⁴ Pathway overactivation leads to expansion of the pericentral gene profile across the liver lobule.^{45,46} In addition to its role in patterning the adult liver lobule, WNT/ β -catenin activity acts as a critical instructive signal for hepatocyte proliferation and regeneration, inducing the expression of key cell-cycle regulators such as cyclin D1.^{44,47–49} Genetic pathway inactivation in hepatocytes decreases liver size in mice and significantly delays hepatocyte proliferation and liver regeneration after partial hepatectomy (PH).⁴⁴ Conditional deletion of *Apc*, in contrast, causes hepatocyte hyperproliferation and increased liver size.⁴⁵ Hence, given the crucial roles of WNT and RSPO in liver regeneration and metabolic zonation, stimulating WNT/ β -catenin activity to treat various liver diseases has great therapeutic promise.

The original WNT surrogate 18R5scFv-DKK1c¹⁴ and the next-generation surrogate WNTs¹⁵ have both been shown to induce signaling, zonation, and proliferation in the liver. Based on *Fzd* expression and the comparison of WNT agonists with different FZD specificities, WNT signaling in the liver is induced by different FZD subtypes. When expressed together with RSPO2 in mice, surrogate WNTs specific for FZD_{1,2,7} or FZD₄ expand pericentral WNT target gene expression while repressing expression of periportal genes; in contrast, an FZD_{5,8}-specific surrogate WNT has not demonstrated such effects in the mouse liver.¹⁵ In another study using antibody-based WNT mimetics, Post et al. have shown that pan-FZD-specific, FZD_{1,2,7}, or FZD₄-specific agonists induce liver growth.²⁰ Hu et al. have investigated the treatment effects of an FZD_{1,2,5,7,8} WNT agonist (FL6.13) in liver injury models. Similar to findings from other studies, FL6.13 has shown signaling, zonation, and proliferation effects in naive mouse liver.⁵⁰ After PH, FL6.13 has been found to rescue metabolic zonation and liver regeneration delay. In a high-dose APAP mouse liver injury model, treatment of FL6.13 at 32 h post-APAP injection has been found to promote liver repair by activating WNT/ β -catenin signaling and inducing hepatocyte proliferation, whereas early treatment of FL6.13 increases mortality.⁵⁰ This work demonstrates the regenerative potential of WNT agonists for treating liver diseases but also highlights potential challenges in developing WNT mimetics for liver regeneration. Of note, FL6.13 not only lacks FZD specificity but also may activate WNT signaling in non-parenchymal liver cells and other non-targeting tissues. Development of hepatocyte-targeted WNT mimetics (Section [Cell-targeted WNT mimetics](#)) may facilitate investigation of the roles of specific FZD effects on hepatocytes.

Various studies have shown that the effects of WNT mimetics in the liver are enhanced by simultaneous RSPO treatment^{15,20}; consequently, receptor expression level might be a limiting factor in mediating WNT stimulatory effects. This conclusion is further supported by the WNT superagonist described by Post et al.,²⁰ which has more potent effects on primary hepatocytes than WNT mimetics alone. The presence of endogenous WNT ligands in the liver may render treatment with RSPO alone sufficient to elicit pro-proliferative responses. Treating mice with recombinant RSPO1 alone induces hepatocyte proliferation and accelerates liver regeneration after PH.⁴⁶ However, the strong proliferation-promoting effect of RSPOs in other tissues, including the intestines, prevents direct application of RSPO for therapeutic purposes. As described in the section “[Development of cell/tissue-targeted RSPO mimetics](#),” Zhang et al.³³ first described a hepatocyte-targeted RSPO2 mimetic for liver regeneration. A mutant RSPO2 fragment fused with an antibody to ASGR1 (α ASGR1-RSPO2-RA) was found to specifically stimulate WNT signaling in ASGR1-expressing hepatocytes *in vitro* and *in vivo*. α ASGR1-RSPO2-RA also demonstrated liver-specific

proliferation effects but did not affect another RSPO-responsive tissue, the intestine.³³ In a TAA liver injury study, α ASGR1-RSPO2-RA also improved liver coagulation function, as indicated by accelerated INR recovery.³³ Further protein engineering to improve biophysical properties and achieve higher stability and more drug-like properties has yielded the clinical candidate compound SZN-043 (www.surrozen.com; Fisher et al.⁵¹).

SZN-043 has shown robust efficacy in preclinical animal models of liver injury, by stimulating hepatocyte proliferation, improving synthetic function, and decreasing fibrosis. SZN-043 is the first cell type-specific RSPO mimetic to enter clinical studies and is currently being evaluated for severe liver diseases that might benefit from rapid hepatocyte regeneration, including severe alcoholic hepatitis (March 2022, ANZCTR registry ACTRN12622000392763).

Intestine

Cells in the intestinal epithelium are continually renewed by intestinal stem cells (ISCs), to maintain a healthy and functional epithelial barrier against mechanical, chemical, and biological insults from the intestinal lumen.⁵² Stem cell niche signals consist of WNTs expressed by epithelial cells, neighboring mesenchymal cells, and enteric neuronal cells, as well as RSPOs secreted by mesenchymal cells and muscle cells in the muscularis mucosa layer beneath the intestinal crypts; the generated WNT/ β -catenin signal gradient is crucial to ISC maintenance, proliferation, and differentiation.⁵³ Severe injuries may compromise or deplete the LGR5⁺ ISCs located at the crypt base. In these injury scenarios, progenitors and fate-defined epithelial cells of absorptive, enteroendocrine, tuft, and Paneth cell lineages also display plasticity and can de-differentiate and subsequently repopulate the ISCs for epithelial repair.⁵⁴ WNT is also among the most investigated signaling pathways for damage-induced intestinal epithelial repair mechanisms.^{55–58} For instance, in response to DSS-induced colitis damage, WNT-producing stromal cells significantly increase in number and express higher levels of RSPO3. In contrast, when WNT ligand secretion is blocked in mice, radiation-induced intestinal epithelial damage cannot be repaired.⁵⁹

Treating chemotherapy-induced intestine mucositis and chemically induced colitis mouse models with RSPO1 has further demonstrated the possibility of improving epithelial repair with WNT agonism.^{31,60} However, although direct application of RSPO is effective in protecting cells against colitis damage or accelerating repair process, its strong mitogenic activity in the intestine, stimulating intestine hyperplasia, has prevented its further development for therapeutic use.^{60,61} WNT mimetics in various formats have been tested on regeneration and repair of intestinal epithelium *in vitro* and *in vivo*. The original WNT surrogate 18R5scFv-DKK1c stimulates intestinal organoid growth *in vitro*.¹⁴ The tetravalent WNT agonizing antibodies engineered by Tao et al. are also active toward intestinal organoids and maintain LGR5⁺ stem cells when cotreated with a porcupine inhibitor in mice.¹⁸ Yan et al. have shown that 18R5scFv-DKK1c alone can maintain LGR5⁺ ISCs in the absence of endogenous WNT signals in the mouse small intestine and can stimulate epithelium hyperproliferation when combined with RSPO1. These results suggest non-equivalent roles of WNT mimetic and RSPO in maintaining ISC and stimulating epithelial regeneration.⁶¹

Fzds display a differential expression pattern along the crypt-villus axis in the intestinal epithelium.¹⁶ The identification of the specific FZD subfamily essential for intestinal regeneration continues to be of great interest. Ongoing efforts can be divided into FZD-specific agonists, antagonists, or genetic approaches. Chen et al.¹⁶ have shown that WNT mimetics tailored toward FZD₅ specificity demonstrate better regenerative potential in intestinal organoids. Both Chen et al.¹⁶ and Miao et al.¹⁵ have found that WNT mimetics with FZD_{1,2,7} specificity are not as potent in stimulating organoid growth, or in stimulating mouse intestinal epithelial cell proliferation *in vivo*, as FZD_{5,8}-specific WNT mimetics in the presence of exogenous RSPO.^{15,16} Consistently, an inhibitory study has shown that an FZD_{5,8}-specific binder induces catastrophic intestinal crypt and villus loss in intestinal organoids and in mice, whereas binders targeting other FZDs do not.⁶² Interestingly, other genetic depletion and inhibitory studies appear to suggest that FZD₇ is the key WNT receptor in ISCs mediating WNT signaling and epithelial regeneration. Conditionally knocking out FZD₇ in the adult intestinal epithelium or inhibiting its function causes ISC loss and impaired epithelial regeneration after damage.^{63,64} Further investigations may be warranted to comprehensively understand the differential roles of all FZDs expressed in the intestinal epithelium. However, studies to date suggest overlapping roles of FZD₅ and FZD₇ in ISC regeneration and pathway stimulation, although FZD₅ may have greater therapeutic potential for intestinal diseases such as inflammatory bowel disease.

WNT mimetics with FZD_{5,8} specificity, compared with FZD_{1,2,7} specificity, have shown superior efficacy in eliciting colon epithelium repair and regeneration in a mouse colitis model, thus achieving better histology and disease activity improvement.⁷ In agreement with previous reports, WNT mimetics alone effectively repair damaged epithelium but do not cause hyperplasia of the intestinal epithelium.⁷ Using single-cell RNA sequencing, Xie et al.⁷ have observed epithelial-specific WNT target gene expression and a transient expansion of progenitor cell proliferation after FZD_{5,8} mimetic treatment, but no alterations in epithelial cell differentiation potential after WNT mimetic treatment.⁷ The observed decrease in inflammation and amelioration of animal disease appear to be secondary to healing of the epithelium and barrier. SZN-1326, an optimized version of the FZD_{5,8}-specific mimetic described by Xie et al.,⁷ is the first tetravalent WNT mimetic molecule to enter clinical studies (March 2022, ANZCTR registry ACTRN12622000392763). SZN-1326's ability to directly induce epithelial repair and healing differentiates it from all current standard-of-care medicines targeting inflammatory components of the disease. This new mechanism offers great promise in directly improving histological remission and achieving better long-term outcomes in ulcerative colitis.

Vascular blood-retinal and blood-brain barriers

The major roles of WNT/ β -catenin signaling in vascular biology, particularly in the retina, and in brain vasculature development and function, have been well documented. Norrin, an atypical WNT ligand, and its receptor complex, FZD₄/LRP5/TSPAN12, have been recognized for their importance in the development of retinal vascular structure and the maintenance of the blood-retinal barrier function.^{65–67} Genetic perturbations in these four proteins cause vascular defects and visual impairments in humans and rodents.^{1,68,69} Dysregulation of WNT/ β -catenin

signaling in the eyes has also been observed in human ocular diseases such as diabetic retinopathy (DR). Abnormally elevated β -catenin levels have been reported in patients with non-proliferative DR⁷⁰; however, DKK1 levels are also significantly elevated in the vitreous in patients with DR.⁷¹

Given its essential roles in the retinal vessels, WNT/ β -catenin signaling modulation was explored as a therapeutic approach for retinopathies. SZN-413, a tetravalent bispecific WNT mimetic antibody targeting FZD₄ and LRP5, is highly potent in inducing WNT/ β -catenin signaling in vascular endothelial cells *in vitro*, as compared with the natural ligands Norrin or WNT3A.²⁴ In an oxygen-induced retinopathy (OIR) mouse model, SZN-413 significantly decreased pathological neovascular tuft formation to a similar extent as the current standard-of-care anti-VEGF drug aflibercept, while unexpectedly achieving superior regeneration of normal vessels in this model. SZN-413 also upregulated the tight junction proteins CLDN5 and ZO1 in retinal vascular cells and inhibited VEGF-driven retinal vascular leakage in a rabbit retinopathy model.²⁴ It is remarkable that this novel FZD₄/LRP5-targeting WNT mimetic molecule can simultaneously correct both vascular non-perfusion and leakage, thus addressing the underlying pathophysiology of retinopathies. Another tetravalent FZD₄/LRP5-targeting WNT mimetic molecule, F4L5.13, has been independently developed.²⁵ Similarly to SZN-413, F4L5.13 has been demonstrated to decrease neovascular tufts in OIR mice and restore blood-retinal barrier function in *Tspan12* knockout (KO) mice.^{25,72} However, F4L5.13 does not restore the vessels in the ischemic area in the developing eyes of OIR mice, in contrast to SZN-413.²⁵ Further experiments are necessary to clarify the reasons for the differences between F4L5.13 and SZN-413 in the vascular regeneration observed in the OIR model. Nevertheless, these results suggest that FZD₄-targeting WNT mimetics have great promise for treating patients with retinal vascular diseases, such as DR.⁶⁹ An optimized version of F4L5.13, called Restoret (EYE103), is the second tetravalent WNT mimetic molecule to achieve clinical applications; this therapy is currently being evaluated for diabetic macular edema and advanced neovascular age-related macular degeneration (June 2023,⁷³ clinicaltrials.gov NCT05919693).

FZD₄-targeting WNT mimetics may also modulate blood-brain barrier (BBB) function. Mice with genetic mutations in any components of the Norrin/FZD₄/LRP5/TSPAN12 axis show phenotypically similar defects in the retina and the brain vasculature.^{65,67,74,75} The BBB defects in the KO mice are observed in the cerebellum and are caused by downregulation of tight junction proteins and/or upregulation of plasma-lemma vesicle-associated protein in vascular endothelial cells.^{76–79} BBB defects have been suggested as a cause of the cognitive/psychosocial impairments observed in a subpopulation of patients with Norrie disease.⁸⁰

Beyond the Norrin/FZD₄/LRP5/TSPAN12 signaling axis, the WNT7/FZD/GPR124/RECK signaling axis is another critical WNT/ β -catenin-dependent signaling pathway for angiogenesis in the central nervous system (CNS) and BBB formation/maintenance.^{81–84} Genetic ablation of *Wnt7*, *Gpr124*, or *Reck* in vascular endothelial cells impairs CNS angiogenesis and BBB formation.^{79,82,85} The Norrin/FZD₄/LRP5/TSPAN12 and WNT7/FZD/GPR124/RECK signaling axes both regulate CNS angiogenesis and maintain BBB function, thus demonstrating the critical function of WNT/ β -catenin signaling in CNS vascular development and function, though exerting their effects in different regions of the CNS.⁷⁷ A BBB-specific WNT agonist targeting GPR124/RECK has been shown to protect against ischemic stroke infarction and glioblastoma expansion.⁸⁶ Moreover, a bioengineered FZD₄-selective WNT mimetic, L6-F4-2, also significantly restores the function of BBB in aberrant brain vessels under ischemic stroke conditions and rescues stroke phenotypes.⁸⁷ Therefore, the collective scientific evidence suggests that the agonistic approaches to FZD₄ regulation have therapeutic potential not only for retinal vascular diseases but also for brain diseases caused by BBB dysfunction such as stroke, traumatic brain injury, epilepsy, and neurodegenerative diseases.

Bone

WNT/ β -catenin signaling is critical for skeletal development, bone mass regulation in adults, and injury repair.^{88–92} WNT/ β -catenin signaling activation promotes osteogenic differentiation, induces bone formation,^{89–92} and decreases bone resorption.⁹³ Human mutations in multiple WNT pathway genes result in bone disorders. For instance, gain-of-function or loss-of-function mutations in the human *LRP5* gene induce an autosomal dominant high-bone-mass trait or diminished bone mass with osteoporosis-pseudoglioma syndrome, respectively.^{94,95} Mutations in the *WNT16* gene influence bone strength and increase osteoporotic fracture risk,⁹⁶ and mutations in *WNT1* induce osteogenesis imperfecta.⁹⁷ Sclerostin, a glycoprotein produced primarily by osteocytes, is a key WNT pathway inhibitor that competes with endogenous ligands for binding LRP5/6.^{98,99} Genetic abnormalities in the *SOST* gene, encoding sclerostin protein, cause high bone mass in human diseases such as sclerosteosis and Van Buchem's disease.^{100,101} A neutralizing monoclonal antibody against sclerostin, romosozumab, activates WNT/ β -catenin signaling, increases bone formation, and protects patients with osteoporosis against high fracture risk.¹⁰⁰ Thus, activation of WNT signaling has promise in enhancing bone regeneration and healing in conditions such as osteoporosis, fractures, and bone defects.

The importance of WNT/ β -catenin signaling in bone regeneration has led to the evaluation of WNT mimetics in various bone studies. The original WNT surrogate, 18R5scFv-DKK1c, described in the section "Discovery and development of WNT mimetics activating WNT/ β -catenin signaling," has been shown to upregulate the early osteogenic marker alkaline phosphatase in mouse mesenchymal stem cells *in vitro*.¹⁴ FZDs are broadly expressed in osteoblasts and osteoblast precursors; notably, expression of FZD₁, FZD₂, and FZD₇ has been observed in both mouse and human bones.¹⁹ The new generation of bispecific antibody-based WNT mimetics targeting FZD_{1,2,7} or FZD_{1,2,5,7,8} shows rapid and potent osteogenic effects in various bone disease models, including osteoporosis, aging, and bone fractures, thus demonstrating the remarkable potential of WNT mimetics in achieving effective bone regeneration,¹⁹ and serving as therapeutic agents for bone diseases.

In addition to FZD_{1,2,7} subtypes, other FZDs have also been reported to regulate bone homeostasis. For example, loss of *Fzd8* induces osteoclastogenesis, thereby leading to osteopenia.¹⁰² *Fzd9* is induced during osteoblast differentiation, and *Fzd9* KO mice exhibit diminished bone mass due to impaired bone formation.¹⁰³ Additionally, *Fzd4* is expressed in osteoblasts and is crucial for normal bone acquisition.¹⁰⁴ Interestingly, loss of *Fzd4* is compensated for by the upregulation of *Fzd8*; consequently, these two FZDs may have redundant function in

osteoblasts.¹⁰⁴ The availability of FZD-selective WNT mimetics and the potential to develop cell-specific modulators described in the section “[Novel WNT-activating approaches](#)” are expected to advance understanding of the roles of various FZDs in different bone cell types and facilitate the development of WNT mimetics as therapeutics for bone diseases.

Cornea

The corneal epithelium, the outermost layer of the eye, functions to refract light and forms the primary barrier of the eye against external insults.¹⁰⁵ Once injured, the corneal epithelium must be quickly and properly repaired to restore optical clarity and to maintain vision. WNT/ β -catenin signaling is active under normal physiology and is necessary to maintain the homeostasis of the corneal epithelium.^{106,107} In the corneal limbus, where limbal stem cells reside, the WNT/ β -catenin pathway is an important niche factor in the control of proliferation and cell fate.¹⁰⁸ Exogenous activation of WNT/ β -catenin signaling by small molecules or recombinant WNT proteins induces the proliferation and colony-forming efficiency of human corneal epithelial stem cells *in vitro*.^{109–112} The small molecule WNT mimetic MFH-ND (described in the section “[Discovery and development of WNT mimetics activating WNT/ \$\beta\$ -catenin signaling](#)”) also enhances limbal epithelial cell stemness and proliferation in culture.²⁶ In some corneal disorders, such as diabetic cornea, WNT/ β -catenin signaling-associated factors are downregulated. In a mouse model of diabetic corneal alterations, treatment with the pathway activator lithium chloride promotes accelerated healing.¹¹³ Together, these results suggest that WNT mimetics may be used to stimulate corneal epithelial healing in disease or injury. Among the ten FZDs, *Fzd7* is differentially expressed in the limbal stem cell population^{113,114}; therefore, testing an FZD₇-specific WNT mimetic for treatment of corneal epithelial diseases such as limbal stem cell deficiency and neurotrophic keratopathy would be of particular interest.

The corneal endothelium is a single layer of specialized cells on the inner surface of the cornea. This layer is a critical structure for the cornea, which maintains corneal clarity through its pump function. Endothelial cells are lost over time with aging but can also be damaged through chemical and mechanical exposures or genetic dystrophies.¹¹⁵ Although human corneal endothelial cells have limited proliferation capability, WNT/ β -catenin signaling activation is of interest in tissue-regeneration approaches.¹¹⁶ Corneal endothelial cell density has been found to significantly increase *in vitro* after RSPO1 treatment in rabbit and human cornea organ cultures.^{117,118} In addition, WNT10B promotes corneal endothelial cell proliferation.¹¹⁹ The latest generation of WNT mimetics will be of interest in further exploration of therapeutic approaches to corneal endothelial cell dysfunction. Future investigations may include deciphering specific WNT receptor expression profiles in endothelial cells and the proliferative ability of human cells *in vivo*.

Although both cell types of the cornea respond to WNT/ β -catenin signaling, an approach tailored to each distinct cell layer is likely to be necessary to explore the full therapeutic potential of pathway activation.

Salivary glands

Salivary glands are exocrine glands that produce saliva through a system of acini and ducts. The three major glands produce serous, mucinous, or seromucous secretions, which facilitate digestion and lubrication. WNT signaling has been implicated in salivary gland development and adult stem cell maintenance and is activated after injury. Components of the WNT pathway, such as WNT protein and receptors, are expressed in adult salivary glands in both mice and humans.^{120,121} Using a transgenic WNT reporter, Hai and colleagues have demonstrated that WNT signaling is active in developing mice and is reactivated in adult submandibular glands during regeneration.¹²² In another study, transient *Wnt1* overexpression has been found to protect tissue against radiation-induced chronic salivary gland dysfunction.¹²³ Furthermore, *in vitro*, WNT3A is a key driver of salivary gland stem cell expansion as organoids.¹²⁴ More recently a novel platform of FZD-specific WNT mimetics has elucidated the role of FZD_{1,2,7} in this pro-proliferative salivary gland phenotype.²⁰ In a comparison of subfamily-specific WNT mimetics targeting FZD_{1,2,5,7,8}, FZD_{1,2,7}, FZD_{5,8}, FZD₄, FZD_{4,9}, or FZD₁₀, FZD_{1,2,7}-targeting WNT activators had the most potent effects *in vitro* and *in vivo*. With systemic treatment, WNT activation through FZD_{1,2,7} resulted in salivary gland tissue expansion, as measured by acinar cell proliferation and organ weight increase. In addition, murine organoid expansion was enhanced in a dose-dependent manner.²⁰ The salivary gland is one of the first studied tissues with a strong biased response to a single FZD subfamily. Many other tissue cell types respond to both FZD_{1,2,7} and FZD_{5,8} activation, despite having differential receptor mRNA expression levels. The exact mechanisms underlying salivary gland FZD_{1,2,7} receptor preference, and whether these rules apply to other exocrine glandular tissues such as the lacrimal gland or pancreas, remain unknown. In addition, the FZD-specific effects on human salivary gland cells remain to be determined. Thus, further exploration of WNT signaling in salivary gland regeneration is encouraged. The tissues’ easy accessibility is expected to open avenues for local administration of WNT mimetics or cell therapy approaches.

Lungs

The lungs are a complex organ with myriad epithelial, stromal, and immune cell types¹²⁵ and a highly regionalized architecture. The proximal and distal airways and the alveoli have regenerative ability after injury.¹²⁶ In the alveoli, a subset of AT2 cells are stem cells that can self-renew and differentiate into AT1 cells.^{127–130} WNT/ β -catenin signaling in AT2 cells is critical for their renewal and alveolar regeneration.^{129,130} During normal homeostasis and after injury, PDGFRA+ fibroblasts secrete WNT ligands, which signal to AT2 cells^{129,131,132}; AT2 cells also use autocrine WNT signals to regulate expansion after injury.¹²⁹ In contrast, WNT/ β -catenin signaling must be diminished for AT2 cells to differentiate into AT1 cells.^{129,130} The association of active WNT/ β -catenin signaling with epithelial stem/progenitor cell renewal and expansion, and downregulation of the pathway to differentiation are shared features among the proximal and distal airway compartments, and other adult stem cell-maintained tissues such as the intestinal epithelium.

A fine balance of WNT signaling in stromal and epithelial cells in the lungs underlies normal renewal and repair after injury, and changes in the levels of WNT/ β -catenin signaling are associated with lung diseases such as chronic obstructive pulmonary disease (COPD) and idiopathic pulmonary fibrosis (IPF). The loss of alveoli (emphysema) in COPD is associated with decreased WNT/ β -catenin signaling,^{133–135} whereas activation of WNT/ β -catenin signaling leads to less airspace expansion in elastase and smoking models of COPD.^{133,134,136,137} In hypercapnia (elevated blood CO₂ level) injury models, similarly to COPD, loss of alveoli is associated with decreased WNT/ β -catenin signaling, and pathway activation rescues AT2 cell proliferation.¹³² On the basis of these studies, further studies may investigate whether targeting WNT and RSPO mimetics to AT2 cells and/or disease-specific distal epithelial cells facilitates alveolar regeneration.

IPF is characterized by myofibroblast-mediated deposition of extracellular matrix.^{138,139} In addition to the loss of alveoli, aberrant disease-associated epithelial cells contribute to driving fibrosis.^{140,141} Critically, in the bleomycin model, loss of WNT/ β -catenin signaling in AT2 cells leads to enhanced pulmonary fibrosis¹⁴²; therefore, defects in AT2 cell renewal and alveolar regeneration may enhance fibrotic damage, and alveolar regeneration might have anti-fibrotic benefits. A WNT mimetic that targets FZD₅, which is enriched in alveolar cells, has been shown to expand AT2 organoids; however, in the bleomycin model, treatment with the FZD₅-specific WNT mimetic leads to AT2 cell expansion into the airways but does not restore alveoli or decrease fibrosis.¹⁴³ Although the dosing in this experiment limited the relevance of the findings to repairing established fibrosis, one interpretation may be that activating WNT/ β -catenin signaling only on AT2 cells may be insufficient to repair the lungs after severe injury such as fibrosis.

An additional complication in developing WNT/ β -catenin signaling modulators to treat IPF is that many studies have reported a connection between active WNT/ β -catenin signaling and the formation of pulmonary fibrosis.^{136,144,145} These results suggest that a WNT/ β -catenin signaling antagonist targeted to fibroblasts might limit the formation of fibrosis, whereas a pathway activator should avoid this cell type. However, perhaps surprisingly, a broad-spectrum FZD-binding WNT mimetic that engages FZD_{1,2,5,7,8} and LRP6 has been found to activate the pathway in multiple lung cell types, decrease inflammation and fibrosis in the lungs, and restore lung function in an acute bleomycin model.¹⁴⁶ In line with these results, broader, non-targeted activation of the pathway via an antibody to DKK1, an LRP antagonist, has been found to decrease fibrosis in the acute bleomycin model.¹⁴⁷ Potentially relevant to these findings, WNT/ β -catenin signaling may also play key roles in other cells including immune cells relevant in IPF. For example, in several pulmonary injury models, macrophages have been reported to respond to RSPO3 by shifting from a pro- to an anti-inflammatory phenotype.¹⁴⁸

Because WNT/ β -catenin signaling plays multiple roles in lung injury, disease, and repair, and in light of the recent finding that pathway activation in multiple cell types is beneficial in an animal model of pulmonary fibrosis, several questions must be answered to engineer targeted WNT/ β -catenin signaling modulators for repairing damaged/diseased lung tissue. Will focused activation of WNT/ β -catenin signaling (via WNT/RSPO mimetics) in one cell type be sufficient to promote alveolar regeneration and limit or decrease fibrosis, or must multiple cell types be targeted to orchestrate regeneration? Can targeted WNT/ β -catenin signaling agonists revert aberrant, disease-specific epithelial cells to a more normal phenotype *in vivo*? Is there a time and place where pathway antagonism could be beneficial? Even though there are many unresolved questions, the strong rationale and recent data make it an exciting time to explore the development of regenerative therapeutics for lung disease.

Ex vivo stem cell expansion

The discovery of WNT signaling as an essential cellular signaling pathway for stem cell maintenance *in vivo* has further enabled researchers to expand and maintain stem cells in culture. Three-dimensional grown structures derived from PSCs or adult stem cells are known as organoids. By recapitulating the stem cell niche, with factors such as WNT, organoids self-organize into organ-specific structures with differentiated cell types. In most organoid culture systems, WNT signaling activation is essential to maintain stemness and proliferation. One earlier well-known example is the derivation of small intestinal epithelial organoids from single LGR5⁺ stem cells.¹⁴⁹ In the presence of RSPO1 and other growth factors, crypt stem cells proliferate and form crypt-like budding structures. In murine intestinal organoids, WNT3A is secreted from Paneth cells, and the addition of RSPO1 to the culture medium importantly further enhances WNT signaling pathway activity. However, most organoid cultures described to date have insufficient endogenous secretion of WNT protein and must be supplemented with exogenous ligands. The establishment of other WNT-dependent organoid cultures, such as human colon cultures, has built on the addition of recombinant WNT3A protein or WNT3A-conditioned medium.¹⁵⁰ Other examples include human liver organoids,¹⁵¹ human pancreatic organoids,¹⁵² human stomach organoids,¹⁵³ and human salivary gland organoids.¹²⁴ In addition, embryonic stem cells and induced PSCs (iPSCs) are responsive to WNT signaling activation. Supplementation with WNT3A protein has been found to reprogram differentiated cells to pluripotent state and promote the generation of iPSCs.¹⁵⁴ Moreover, activation of WNT signaling through inhibition of GSK-3 has been reported to maintain pluripotency in human and mouse embryonic stem cells.¹⁵⁵ The GSK-3 inhibitor CHIR99021 has also been used to expand adult stem cell-derived organoids of liver hepatocytes and lung alveolar type 2 cells.^{156–158}

The discovery and increased availability of WNT mimetics have also transformed the field of organoid research. Antibody-based WNT mimetics enable potent induction of proliferation and stemness, as well as deciphering FZD/LRP-specific biology *in vitro*. Surrogate WNT molecules are superior, or comparable, to recombinant WNT3A protein or WNT3A-conditioned medium in promoting continuous growth and single-cell outgrowth efficiency in several organoid types.^{14–16,20} The newest generation of WNT mimetics is tailored to activate the pathway through a subfamily or a single FZD receptor. Application of these new tools is beginning to elucidate some of the FZD subtype-specific functions in stem cell biology. Activation through FZD_{1,2,7} or FZD_{5,8}, for example, supports mouse intestinal organoid growth,¹⁶ whereas WNT activation through FZD_{1,2,7} appears to be the most potent stimulant in murine salivary gland organoid expansion.²⁰ An FZD₇- and LRP6-selective WNT activator has demonstrated that activation through FZD₇ alone is sufficient to promote mesodermal

differentiation of hPSCs.²³ Using lung alveolar epithelial cell cultures, Nabhan et al. have shown that FZD₅- and FZD₆-specific agonists activate canonical WNT signaling in stem cells.¹⁴³ These WNT mimetics can replace CHIR99021 and have been found to outperform recombinant WNT3A in alveolar cell expansion *in vitro*.¹⁴³ More recently, cell-specific WNT mimetics or WNT superagonists that combine both WNT and RSPO activity have shown ongoing improvement in WNT mimetics and ready applicability in organoid research.^{20,27}

The ability of WNT mimetics to improve the expansion of stem cells and progenitor cells will be of interest in cell therapy. Epithelial cell cultures such as organoids retain the ability to rebuild their tissue of origin after *in vivo* transplantation. Successful transplantation of organoids for regenerative purposes has been demonstrated in mouse models of intestinal and salivary gland injury.^{159–161} Transplantation of organoids from these two tissues is currently in human clinical trials to test for functional organ recovery after cell therapy. WNT mimetics may potentially provide an abundant and sustainable source of cells for therapeutic purposes.

DISCUSSION, CONCLUSION, AND FUTURE DIRECTIONS

More than four decades after the discovery of WNT and many seminal studies on this fundamental developmental signaling pathway, we are in the dawn of an exciting era of understanding WNT biology in adult tissue repair. Through the efforts of many researchers, new tools including receptor-specific WNT mimetics, enhancers of WNT signaling including cell-specific RSPO mimetics, superagonists, and other tissue-specific engineered tools have been created. In this review, we highlighted the recent advances in these molecules and tools, collectively referred to as WNT agonists. We then summarized WNT agonists' early applications and the rationale for their use in liver, intestine, retina vessel, BBB, cornea, salivary gland, lung, and *ex vivo* stem cells. We also discussed functional studies applying WNT mimetics, and the effects of specific FZDs and FZD subfamilies on tissues of interest. Beyond the list of tissues described earlier, we and others in the field have investigated the effects of WNT agonists on many more tissues/organs and certain specific cell types within those tissues. These ongoing efforts are likely to improve understanding of the similarities and distinct features of regenerative effects of WNT signaling in various tissues, thus culminating in a pipeline of knowledge, and supporting a rationale for therapeutic design for specific tissues and diseases.

Among WNT agonists, the ideal context for engineered WNT mimetics might be diseases involving endogenous WNT ligand deficiency or a loss of connection between WNT producer cells and target stem or precursor cells because of tissue damage. If abundant WNT ligands are present in diseased tissues, but the signaling toward critical parenchymal cells is insufficient, RSPO mimetics, tissue/cell-specific WNT enhancers, might be desirable to channel WNT signaling toward parenchymal cells and away from other cells, such as inflammatory cells and fibroblasts. In situations in which both WNT ligands and receptors are deficient, a superagonist approach combining WNT and RSPO mimetics might be considered. These theoretical assumptions could be tested in disease models of different tissues, and the effects of WNT or RSPO mimetics or superagonists could be compared.

Several lines of evidence suggest that WNT or RSPO mimetics, in the context of acute treatment, benefit tissue healing in a self-limited manner: (1) these molecules are designed to retain key negative feedback inhibitors of WNT signaling, such as AXIN2 and NOTUM; (2) from liver, intestine, and retina vascular studies, for example, WNT or RSPO mimetics have been found to have effective but transient effects on cell proliferation; (3) tissue functions are properly restored by newly regenerated healthy parenchymal cells; (4) tissue structures are restored with normal organization and restoration of all key cell types (from liver, intestine, and retina vessel disease models). These results further suggest the tremendous therapeutic potential of WNT and RSPO mimetics and indicate that the effects of these molecules fundamentally differ from those caused by mutations in genes such as APC, RNF43, or CTNNB1, which result in constitutive activation of the pathway.

The effects of these new WNT agonist tools appear to mimic the phenotype of endogenous WNT or RSPO ligands on the basis of comparison of these new studies and previous genetic studies. Furthermore, these new tools—such as FZD subfamily- or individual FZD-specific WNT mimetics or superagonists, and tissue-specific RSPO mimetics—may provide critical insights into specific receptor and tissue biology. In contrast, when feasible, antagonists inhibiting specific FZD receptors may be invaluable tools to complement agonist studies, and provide validation, contrast, or additional insights into the WNT signaling biology of specific tissues.

The WNT mimetic agonists described herein are in therapeutically developable molecular formats, and many demonstrate excellent drug-like properties. These molecules work effectively in various *in vitro* and *in vivo* preclinical models and may confer potential benefits in multiple therapeutic areas, such as those detailed in this review and beyond. Moreover, a wealth of literature in many disease areas has implicated WNT signaling in tissue repair and regeneration (Figure 5). For example, strong evidence from human genetics indicates that WNT signaling is required for healthy bone density, and the anti-sclerostin antibody romosozumab has been approved by the Food and Drug Administration (FDA) for the treatment of osteoporosis. Together, these findings validate the potential benefits of activating WNT signaling in adult tissues and suggest the feasibility of achieving safe and effective treatment with WNT signaling activation to promote adult tissue repair. Future directions will include incorporating knowledge of disease-specific characteristics, such as WNT receptor expression, expression of other targeting receptors, and dysregulation of WNT signaling, to enable the design of targeted, fit-for-purpose therapeutics.

ACKNOWLEDGMENTS

The authors thank all staff at Surrozen, Inc., for their dedication and contributions to the development and study of various WNT modulating platforms. We thank our scientific founders Christopher Garcia, Roeland Nusse, Clavin Kuo, and Hans Clevers and our current and former Scientific Advisory Board members and Board of Directors for their guidance and support. We thank Craig Parker for helpful discussions and critical reading of this manuscript. This work was funded by Surrozen, Inc.

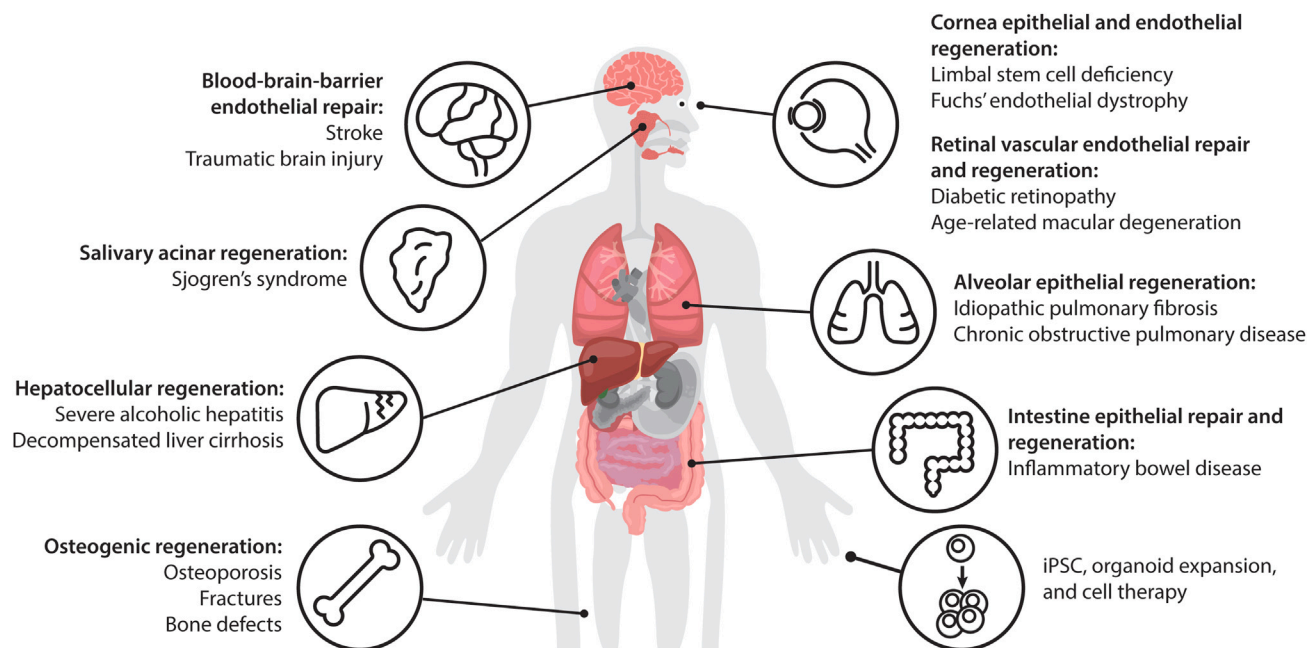


Figure 5. Applications of WNT mimetics

Different WNT mimetics targeting different FZDs and cell types have been applied and have shown regenerative potential in bone, liver, cornea, intestine, lung, retina vessel, BBB, and salivary gland. WNT mimetics can also be applied to enhance patient-derived or allogeneic iPSC or organoid expansion *ex vivo* that can be used as cell therapy.

AUTHOR CONTRIBUTIONS

Y.P., C.L., R.B.F., W.-C.Y., H.N., and S.-J.L., conceptualization, formal analysis, resources, investigation, visualization, methodology, writing – original draft, writing – review and editing; Y.L., conceptualization, formal analysis, resources, investigation, visualization, supervision, funding acquisition, methodology, writing – original draft, writing – review and editing.

DECLARATION OF INTERESTS

All authors are current or former full-time employees and shareholders of Surrozen, Inc. Y.L. is Executive Vice President of Research at Surrozen, Inc. Patent applications are pending for the work described in this manuscript.

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