Uncoupling the BMP receptor antagonist function from the WNT agonist function of R-spondin 2 using the inhibitory peptide dendrimer RW\textsuperscript{d}

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Signaling by bone morphogenetic proteins (BMPs) plays pivotal roles in embryogenesis, adult tissue homeostasis, and disease. Recent studies revealed that the well-established WNT agonist R-spondin 2 (RSPO2) is also a BMP receptor (BMP receptor type 1A) antagonist, with roles in early Xenopus embryogenesis and human acute myeloid leukemia (AML). To uncouple the BMP antagonist function from the WNT agonist function and to promote development of AML therapeutics, here we identified a 10-mer peptide (RW) derived from the thrombospondin 1 domain of RSPO2, which specifically prevents binding between RSPO2 and BMP receptor type 1A without altering WNT signaling. We also show that a corresponding RW dendrimer (RW\textsuperscript{d}) exhibiting improved half-life relieves inhibition of BMP receptor signaling by RSPO2 in human AML cells, reduces cell growth, and induces differentiation. Moreover, microinjection of RW\textsuperscript{d} in Xenopus embryos ventralizes the dorsoventral embryonic patterning by upregulating BMP signaling without affecting WNT signaling. Our study corroborates the function of RSPO2 as a BMP receptor antagonist and provides a proof of concept for pharmacologically uncoupling BMP antagonist from WNT agonist functions of RSPO2 using the inhibitor peptide RW\textsuperscript{d} with enhanced target selectivity and limited side effects.

Bone morphogenetic proteins (BMPs) are transforming growth factor beta family of growth factors with diverse roles in embryonic development, adult tissue homeostasis, and disease. Their misregulation is implicated in various diseases, including cancer (1–5). Thus, clinical therapies targeting BMP signaling attract great interest (6).

BMPs initiate signaling from a tetrameric receptor kinase complex consisting of type I (BMP receptor type 1A (BMPR1A), BMP receptor type 1B, activin A receptor type I (ACVR1), or activin A receptor–like type I) and type II receptors (BMPR2, ACVR2A, and ACVR2B) (7) in a combinatorial manner (8). Activated BMP receptors phosphorylate small mothers against decapentaplegic 1 (SMAD1), SMAD5, and SMAD8, which enter the nucleus in cooperation with SMAD4 to regulate gene expression (9, 10) and activate Erk, c-Jun N-terminal kinase/P38, or PI3K/Akt mitogen-activated protein (MAP) kinases (MAPKs) (11, 12). A number of extracellular antagonists of BMP signaling are known, which target ligand or receptor activation to modulate amplitude of the signaling (13). Recently, we found that the well-characterized WNT agonists of the R-spondin (RSPO) class have a dual role as BMP receptor antagonist (14).

RSPO1–4 are a family of secreted stem cell growth factors involved in embryonic development, stem cell maintenance, and cancer (15–21). Mechanistically, RSPOs activate WNT signaling by preventing frizzled/lipoprotein receptor–related protein (LRP)/5/6 WNT receptor ubiquitination and degradation mediated via the transmembrane E3 ubiquitin ligases ring finger 43 (RNF43) and zinc and ring finger 3 (ZNRF3) (17, 22–24). To do so, RSPOs bind to ZNRF3/RNF43 in conjunction with the stem cell marker leucine-rich-repeat-rich repeat containing G protein–coupled receptor 5 (LGR5), or two related proteins, LGR4 and LGR6, leading to the internalization of the RSPO–LGR–ZNRF3/RNF43 complex and lysosomal degradation (16, 17, 25). We showed that among the four RSPOs, only RSPO2 and RSPO3 are bifunctional, activating WNT signaling and inhibiting BMP signaling. Mechanistically, RSPO2 specifically interacts with BMPR1A and tethers it to ZNRF3 in an LGR-independent manner to trigger endocytosis and degradation of BMPR1A (14, 26).

In vivo support for a role of RSPO2 as selective BMP antagonist comes from early Xenopus embryogenesis, where RSPO2 cooperates with other BMP antagonists of the Spemann organizer to inhibit BMP signaling during embryonic axis formation (14). RSPO2 functions as BMP antagonist rather than WNT agonist also in acute myeloid leukemia (AML) cells, where RSPO2 promotes self-renewal (26). Moreover, in AML patients, elevated RSPO2 expression is a prognostic marker that strongly correlates with poor disease progression. Thus, RSPO2 is a promising therapeutic target in AML, the more so as it is accessible to extracellularly acting drugs. Indeed, monoclonal antibody antagonists of RSPO family members are effective in inhibiting tumor growth in various solid tumor xenograft models (15). However, a caveat of bulky antibody antagonists is that they are likely to inhibit...
both WNT agonist and BMP antagonist function of RSPO2 and RSPO3, thus compromising their specificity.

We therefore sought to identify an inhibitor that would specifically target the BMP antagonist (RSPO2\textsuperscript{BMP}) but not WNT agonist (RSPO2\textsuperscript{WNT}) function of RSPO2. RSPOs harbor two furin-like repeat (FU1 and FU2) domains that engage ZNRF3/RNF43 and LGRs, respectively (16, 27, 28). C-terminally, they contain a thrombospondin 1 (TSP1) domain that mediates binding to heparan sulfate proteoglycans and promotes WNT5A/planar cell polarity signaling (29), but that is largely dispensable for WNT/LRP6 signaling (30–32). The key insight toward a BMP-specific inhibitor was our observation that RSPOs bind BMPR1A via its TSP1 domain (14). Building on this observation, we identified a 10-mer peptide (RW) derived from the RSPO2 TSP1 domain, which binds BMPR1A and competes RSPO2 binding. A RW peptide dendrimer (RW\textsuperscript{d}) neutralizes the RSPO2-mediated decrease of BMP4 signaling without affecting WNT/β-catenin signaling. In THP-1 AML cells, RW\textsuperscript{d} upregulates BMP signaling, induces cell differentiation, and inhibits cell growth independent of WNT signaling. Finally, administration of RW\textsuperscript{d} to Xenopus embryos increases BMP signaling and induces ventralization during dorsoventral axis formation without affecting WNT signaling. Our study corroborates the RSPO2–BMP4 antagonism and provides a proof of concept that BMP antagonism and WNT agonism can be uncoupled by specific pharmacological intervention with the TSP1 domain of RSPO2 (RSPO2\textsuperscript{TSP1})–BMPR1A binding.

**Results**

**Identification of a RSPO2\textsuperscript{TSP1} domain–derived peptide targeting RSPO2–BMPR1A**

Building on the specific interaction of RSPO2\textsuperscript{TSP1} with BMPR1A, we wondered whether a peptide derived from RSPO2\textsuperscript{TSP1} may compete with RSPO2 for BMPR1A binding (Fig. 1A). To this end, we scanned the TSP1 domain designing 11 overlapping 10 to 13 mer peptides derived from human RSPO2\textsuperscript{TSP1} (Fig. 1B). We set up a solid-phase binding assay with immobilized RSPO2\textsuperscript{TSP1} to which was added a fusion protein of alkaline phosphatase (AP) and the BMPR1A\textsuperscript{ECD} (extracellular domain). We then tested the TSP1-derived peptides as competitors, monitoring inhibition of AP-BMPR1A\textsuperscript{ECD} binding to RSPO2 in the solid-phase binding assay. At 100 μM, treatment with peptides RW (RNNRTSGFKW), DE (DTILSP-TIAE), and SG (SRRSKMTMRHSPG) reduced the RSPO2\textsuperscript{TSP1}–BMPR1A\textsuperscript{ECD} interaction greater than 50% (Fig. 1, C and D). Since RW was the most potent peptide, we focused on it and confirmed that it inhibited RSPO2\textsuperscript{TSP1}–BMPR1A\textsuperscript{ECD} binding with an IC\textsubscript{50} = 40 μM (Fig. 1E). The RW sequence is only partially conserved in other RSPOs (Fig. 1F). An in vitro binding assay with biotinylated RW peptide confirmed that it binds BMPR1A\textsuperscript{ECD} directly (Fig. 1, G and H). We conclude that RW peptide derived from RSPO2\textsuperscript{TSP1} is a pseudoligand of BMPR1A that competes with RSPO2 for BMPR1A binding.

**Monomeric RW peptide has low stability**

Low storage stability and short half-life *in vivo* are known disadvantages of monomeric peptides (33–35). Indeed, we found that storage of reconstituted RW monomer reduces its ability to inhibit RSPO2–BMPR1A binding (Fig. S1A). To retain peptide stability, we tested covalent attachment of PEG to both N and C terminus of the peptide (PEG-RW) (36). TK peptide, which showed no effect on RSPO2–BMPR1A interaction (Fig. 1, D and G), was also PEGylated and utilized as a control (PEG-TK). However, in cell surface–binding assays (Fig. S1, B–D), PEGylation of monomeric RW proved ineffective, since PEG-RW inhibited RSPO2–BMPR1A interaction only upon short-term (3 h) but not during long-term (16 h) treatment (Fig. S1, C and D).

**Dendrimerized RW peptide retains stability and disrupts RSPO2–BMPR1A interaction**

As alternative to PEGylation, we considered peptide oligomerization in peptide dendrimers, known to strengthen target binding by enhancing avidity (37) and to provide improved resistance to proteases (38, 39). Therefore, we tested RW dendrimers where individual peptide monomers are assembled on an octavalent lysine core matrix, forming an 8-mer complex with expected size of ~12 kDa. MiniPEG was either added to the N terminus of the RW dendrimer (N-PEG-RW\textsuperscript{d}) or inserted as a linker between peptide monomer and core matrix (C-PEG-RW\textsuperscript{d}) (Fig. 2A). As controls, we employed corresponding KR peptide derivatives, which did not affect RSPO2–BMPR1A interaction *in vitro* (Fig. S2A). To test whether RW dendrimer retains stability and ability to disrupt the RSPO2–BMPR1A interaction, we performed cell surface–binding assays in cells transfected with BMPR1A or with LGR4 that binds to the FU2 instead of the TSP1 domain (16) (Fig. 2B). Three-hour exposure to 20 μM RW\textsuperscript{d} and N-PEG RW\textsuperscript{d} abolished RSPO2 binding to BMPR1A transfected cells, whereas KR\textsuperscript{d} and N-PEG KR\textsuperscript{d} showed no effect (Fig. 2, C–F). RW\textsuperscript{d} and N-PEG RW\textsuperscript{d} were equally effective, indicating that PEGylation does not affect the efficacy of dendrimerized RW. Unlike monomeric RW peptide, 16 h of RW\textsuperscript{d} and N-PEG RW\textsuperscript{d} treatment was still able to completely disrupt RSPO2–BMPR1A binding (Fig. S2, B and C). Interestingly, 20 μM RW\textsuperscript{d} was unable to abolish RSPO3 binding to BMPR1A transfected cells (Fig. 2, G and H). Altogether, these results suggest that RW peptide dendrimer is stable and potently competes the RSPO2–BMPR1A interaction without affecting LGR4 binding, which is required for WNT signal activation.

**RW dendrimer derepresses BMP4–BMPR1A signaling**

Given that RSPO2 antagonizes BMP4 signaling (14, 26), we expected that RW\textsuperscript{d} derepresses RSPO2-mediated inhibition of BMP4 signaling. To test this prediction, we employed human hepatocellular carcinoma (HepG2) cells, which harbor very low endogenous RSPO2 expression (14). BMP4 induced Smad1 phosphorylation (pSmad1), a hallmark of BMP signaling activation, and RSPO2 treatment inhibited this
activation, as previously described (Fig. 3A) (14). Importantly, RWd but not KRd rescued the RSPO2-mediated but not the RSPO3-mediated decrease of pSmad1 levels, suggesting that RWd specifically impairs the BMP antagonist function of RSPO2 (Fig. 3, A and B). Administration of RWd without simultaneous treatment of RSPO2 showed no effect on pSmad1 level in HEPG2 cells (Fig. 3C). This result supports that RWd acts via RSPO2 to modulate BMP signaling. To corroborate this finding, we tested RWd on human lung carcinoma (H1581) cells, which express elevated levels of RSPO2 (14). Strikingly, RWd increased phosphorylation of Smad1 similar to RSPO2 deficiency (14, 26) (Fig. 3D). BMP signaling activation is also known to induce phosphorylation of MAPKs, referred as a non-Smad1 BMP signaling (11, 12, 40, 41). Therefore, we analyzed whether RWd derepresses RSPO2-mediated inhibition of MAPK phosphorylation. BMP4 induced P38 MAPK and P44/42 MAPK phosphorylation (pP38 and pP44/42), and RSPO2 treatment disrupted this activation. Similar to Smad1-dependent BMP4 signaling, RWd but not KRd rescued the RSPO2-mediated decrease of pP38 and
RSPO2–BMPR1A intervening peptide derepresses BMP4 signaling

Figure 2. RW dendrimer blocks RSPO2–BMPR1A interaction at the cell membrane. A, cartoons illustrating three different RW dendrimers used in the study. B, scheme for cell surface–binding assay in (C–H). Cells were transfected with BMPR1A or LGR4 DNA and treated with RSPO2/3-AP with or without 20 μM dendrimers for 3 h as indicated. Binding was detected as purple stain on cell surface by chromogenic AP assay. C, images of cells transfected with DNA and treated with RSPO2-AP and 20 μM non-PEGylated dendrimers as indicated. Data show a representative from three independent experiments. The scale bar represents 1 mm. D, quantification of (C). n = 3 biologically independent samples. Data are displayed as means ± SD. E, images of cells transfected with DNA and treated with RSPO2-AP and 20 μM N-PEGylated dendrimers as indicated. Data show a representative from three independent experiments. The scale bar represents 1 mm. F, quantification of (E). n = 3 biologically independent samples. Data are displayed as means ± SD. G, images of cells transfected with DNA and treated with RSPO3-AP and 20 μM non-PEGylated dendrimers as indicated. Data show a representative from three independent experiments. The scale bar represents 0.5 mm. H, quantification of (G). n = 3 biologically independent samples. Data are displayed as means ± SD.

AP, alkaline phosphatase; BMPR1A, BMP receptor type 1A; LGR4, leucine-rich repeat containing G protein–coupled receptor 4; PEG, PEGylation; RSPO2, R-spondin 2.
pP44/42 levels (Fig. 3E), confirming that RWd diminishes the BMP antagonist function of RSPO2.

In contrast to both Smad1 and non-Smad1 BMP signaling, RWd had no effect on WNT-mediated activation of β-catenin (Fig. 3F), corroborating that RW modulates BMP signaling specifically and confirming that the dual function of RSPO2 in BMP and WNT signaling can be uncoupled.

Since RSPO2 antagonizes BMP signaling by inducing membrane clearance of BMPR1A (14), we hypothesized that RWd stabilizes BMPR1A at the plasma membrane. We monitored localization of overexpressed BMPR1A (BMPR1A-HA) by immunofluorescence and found indeed upon RWd but not KRd treatment an increase (approximately threefold) in the number of cells showing BMPR1A-HA membrane staining, similar to siRNA knockdown of RSPO2 (Fig. 3, G and H), consistent with our model of RW blocking the RSPO2–BMPR1A interaction (Fig. 1A). Collectively, we conclude that RWd derepresses BMP4–BMPR1A signaling by specifically neutralizing RSPO2 as a BMPR1A antagonist.

**Figure 3. RW dendrimer augments BMP4–BMPR1A signaling.**

A, Western blot analysis of phosphorylated Smad1 (pSmad1) and total Smad1 (tSmad1) in HEPG2 cells stimulated by BMP4, treated with or without RSPO2 and 0.5 μM dendrimers for 1 h as indicated. Cells were starved 3 h before the stimulation. B, Western blot analysis of pSmad1 and tSmad1 in HEPG2 cells stimulated by BMP4, treated with or without RSPO3 and 0.5 μM dendrimers for 1 h as indicated. Cells were starved 3 h before the stimulation. C, Western blot analysis of pSmad1 and tSmad1 in H1581 cells treated with or without 0.5 μM dendrimers for 3 days as indicated. E, Western blot analysis of phosphorylated P38 (pP38), phosphorylated P44/42 (pP44/42), total P38 (tP38), and total P44/42 (tP44/42) MAP kinases in HEPG2 cells stimulated by BMP4, treated with or without RSPO2 and 0.5 μM dendrimers for 1 h as indicated. Cells were starved overnight before the stimulation. F, Western blot analysis of activated β-catenin in H1581 cells treated with WNT3A and 0.5 μM dendrimers as indicated. Tubulin was used as a control. G, immunofluorescence in H1581 cells transfected with BMPR1A-HA upon 0.5 μM RW and KR dendrimer treatment or siRSPO2 treatment for 3 days. BMPR1A (red) was stained against HA antibody. The scale bar represents 20 μm. H, quantification of cells harboring membrane-localized BMPR1A from (G). BMP4, bone morphogenetic protein 4; BMPR1A, BMP receptor type 1A; HA, hemagglutinin; HEPG2, human hepatocellular carcinoma cell line; MAP, mitogen-activated protein; RSPO2, R-spondin 2; Smad, small mothers against decapentaplegic.

**RW dendrimer induces THP-1 differentiation and inhibits cell growth**

We recently showed that RSPO2 inhibits BMP signaling in AML cells to maintain self-renewal and prevent differentiation independent of WNT signaling (26). We therefore asked whether RWd could reduce endogenous RSPO2 activity of THP-1 AML cells to upregulate BMP signaling and thereby induce monocyte to macrophage (CD11B+) differentiation. Such an effect would mimic cell differentiation therapy of leukemic cells. Indeed, RWd but not KRd treatment induced pSmad1 (Fig. 4, A–C). Elevated pSmad1 levels were detected in nonmodified-treated cells, N-PEG-treated cells, and C-PEG RWd-treated cells, suggesting that PEGylation does not
interfere with the functionality of RW<sup>d</sup>. Moreover, Dox-inducible shRSPO2 knockdown abolished the ability of RW<sup>d</sup> to induce pSMAD1, supporting that RW<sup>d</sup> functions via RSPO2 (Fig. S3, A and B). Importantly, by flow cytometric analysis, RW<sup>d</sup> treatment induced the number of CD11B<sup>+</sup> cells up to ~10-fold, indicating effective monocyte to macrophage differentiation (Fig. 4, D–F). Consistently, treatment with BMPR inhibitor LDN-193189 reverted induction of CD11B<sup>+</sup> cell by RW<sup>d</sup> (Fig. 4G).

To confirm that RW<sup>d</sup> increases BMP signaling and induces THP-1 differentiation independently of WNT signaling, we stimulated THP-1 cells with WNT3A, analyzed active β-catenin levels, and found them unaffected by RW<sup>d</sup> treatment (Fig. S3, C and D). We conclude that RW<sup>d</sup> specifically relieves BMP inhibition by RSPO2 in THP-1 cells and thereby induces their monocyte to macrophage differentiation.

In AML, RSPO2 acts as an autocrine growth factor to maintain self-renewal and cell proliferation (26). Hence, inhibition of RSPO2 by RW<sup>d</sup> may provide a therapeutic benefit in AML by derepressing BMP signaling and reducing cancer cell viability. Consistently, RW<sup>d</sup> efficiently reduced viability of THP-1 cells in a dose-dependent manner, with the IC<sub>50</sub> of 1.8, 3.6, and 6.5 μM for nonmodified dendrimer, N-PEG dendrimer, and C-PEG dendrimer, respectively (Fig. 4H–J). In contrast, immortalized human adult fibroblast cell line (iHAFs) as control cells were less affected by RW<sup>d</sup>, tolerating 10-fold higher dendrimer treatment. Moreover, unlike RW<sup>d</sup>, KR<sup>d</sup> did not affect the viability of either THP-1 or iHAF cells. The results raise the possibility of a therapeutic window of higher sensitivity in AML cells to RSPO2-BMP inhibition.

**RW dendrimer increases BMP signaling in Xenopus embryonic axis development**

To analyze the efficacy and specificity of RW<sup>d</sup> in vivo, we employed Xenopus embryos, where an activity gradient of BMP signaling regulates dorsoventral axis formation (42–44), and Rspo2 acts as a negative feedback regulator (14). The RW peptide sequence is completely conserved between human and Xenopus (Fig. 5A), suggesting that administration of RW<sup>d</sup> might impact Xenopus dorsoventral axis formation by upregulating BMP signaling. *bmp4* overexpression characteristically induces
ventralized embryos, featuring small heads and enlarged ventral structures (Fig. 5, C and D) (14). Hence, we injected RW\textsuperscript{d} or KR\textsuperscript{d} into the blastocoel cavity of \textit{Xenopus} blastulae (Fig. 5 B). Interestingly, injection of RW\textsuperscript{d} but not KR\textsuperscript{d}, closely phenocopied \textit{bmp4} overexpression (Fig. 5, C and D) and expanded BMP target gene expression (\textit{sizzled}) toward the dorsal side (Fig. 5, E and F) indicative of ventralization. Of note, both dendrimers induced only a minor growth retardation during gastrulation that may be due to blastocoel swelling after injection but showed no embryotoxicity. To confirm that RW\textsuperscript{d}-mediated defects were due to upregulated BMP signaling but not reduced WNT signaling, we performed BMP-responsive Vent\textsubscript{2} reporter assays (Fig. 5 G) and WNT-responsive Top-Flash reporter assays (Fig. 5 H) using RW\textsuperscript{d} injected embryos or KR\textsuperscript{d} injected embryos. While RW\textsuperscript{d} expectedly enhanced endogenous BMP signaling (Fig. 5 G), it had no effect on WNT signaling, unlike antisense morpholino inhibition of the WNT receptor Lrp6 (Fig. 5 H). The results indicate that RW\textsuperscript{d} is stable \textit{in vivo} and hyperactivates endogenous BMP signaling independently of WNT signaling.
RSPO2–BMPR1A intervening peptide derepresses BMP4 signaling

Discussion

The dual function of RSPO2 as BMP antagonist and WNT agonist raises important questions about its mode of action in the many biological processes where RSPO2 is implicated, ranging from development to cancer (14, 18, 21, 26, 31, 45–48). Our study provides an important step toward addressing these questions by corroborating RSPO2 as BMP receptor antagonist and providing a proof of concept and a research tool for pharmacologically uncoupling BMP antagonist from WNT agonist function of RSPO2 with the inhibitor RWd.

Uncoupling BMP antagonist from WNT agonist function of RSPO2 with the inhibitor RWd

Building on the fact that RSPOs binds BMPR1A specifically via its TSP1 domain (11), we identified a TSP1-derived 10-mer peptide "RW" and improved its efficacy in a dendrimer, which competes for binding of RSPO2 to BMPR1A. RSPOs consist of FU1, FU2, and TSP1 domains. While both the FU1 domain and the TSP1 domain of RSPO2 are important to destabilize cell surface BMPR1A (14), utilizing a FU1 domain–derived peptide to target BMPR1A signaling would be inadequate since the FU1 domain engages the E3 ubiquitin ligase ZNRF3/RNF43 that is also crucial to potentiate WNT signaling. Instead, we took advantage of the specificity of the TSP1 domain for antagonizing BMP4 signaling, which is dispensable for WNT signaling (14). We confirmed that RWd neither affects LGR4 binding nor impairs WNT/β-catenin signaling in H1581 and THP-1 cells or Xenopus embryos.

RW directly binds to the BMPR1A extracellular domain and likely does so at the RSPO2 interaction site in BMPR1A, which is unknown, though. Hence, RWd presumably functions as a BMPR1A binder that prevents RSPO2 docking. RWd binding to BMPR1A does not change BMP signaling directly, since in the absence of RSPO2, as in HEPG2 cells or in shRSPO2–treated THP-1 cells, the dendrimer does not affect BMP signaling (Figs. 3B and S3B). Consistent with the mode of action whereby a RWPO2–ZNF3 complex ultimately internalizes BMPR1A (11), RWd counteracts BMPR1A internalization and stabilizes the receptor at the plasma membrane (Fig. 3, G and H). Our previous study showed that RSPO3 can also inhibit BMP signaling, and it likely does so by a similar mechanism (14). It is therefore surprising that RWd does not also prevent RSPO3 from BMP signaling inhibition (Fig. 3B), suggesting that the slight amino acid differences between the RSPO2 and RSPO3 TSP1 domains manifest in distinct binding modes that can be discerned with a competitor peptide.

Altogether, the results corroborate RSPO2-mediated inhibition of BMP4–BMPR1A signaling by showing that RWd functions as competitor against RSPO2 to access BMPR1A, stabilizes BMPR1A at the plasma membrane, and promotes BMP4 signaling (Fig. 5J). Moreover, they demonstrate pharmacological uncoupling of BMP antagonist from WNT agonist function of RSPO2 with the inhibitor RWd.

Projecting from the RWd approach, it may be possible to specifically interfere with RSPO2/LGR/WNT signaling via dendrimer analogs of FU2, the domain that engages LGR4/5/6 but that is not required for antagonizing BMP signaling (11). Along similar lines, RSPO mutants were recently described to target LGRs (49, 50).

Targeting RSPO2–BMPR1A interaction by RWd as a potential therapeutic approach for AML

AML is an aggressive disease with rapid progression of undifferentiated myeloid blasts, and there is the need of additional therapeutic targets and reagents (51, 52). RSPOs, in particular RSPO2 and RSPO3, maintain the growth and differentiation—block of AML cells and high RSPO2 expression correlate with adverse disease status (26, 48). By targeting BMPR1A, autocrine RSPO2 acts as a BMP antagonist to promote AML stem cell self-renewal, and loss of RSPO2 delays AML progression (26). Phenocopying RSPO2 deficiency in THP-1 cells (26), RWd disrupts the interaction between RSPO2 and BMPR1A, increases BMP signaling, inhibits cell growth, and induces monocyte to macrophage differentiation. These observations not only corroborate the relevance of RSPO2 in AML but also suggest that harnessing the RSPO2–BMPR1A interaction may be beneficial in AML therapy.

A number of studies have identified BMP2 and BMP7-mimetic peptides that globally augment BMP signaling (53–58). These mimetics typically show strong affinity to BMPRs and activate signaling ligand independently. Given the many roles of BMP signaling in normal cell homeostasis, administration of these mimetics may result in severe side effects (59). In contrast, RWd specifically impairs the RSPO2–BMPR1A interaction, restricting upregulated BMP signaling to cells where BMPR1A is predominant in transmitting BMP signaling and is subject to RSPO inhibition. This specificity limits on the one hand the applicability of a therapeutic approach to fewer cell lineages; on the other hand, it avoids generalized BMP signaling upregulation and reduces potential side effects.

Modulating protein–protein interaction by inhibitory peptides attracts increasing attention since they may target protein–protein interactions with high specificity and affinity. However, peptide-based reagents frequently suffer from poor pharmacokinetics because of their short half-life (60–62). Emerging strategies enhance the stability of therapeutic peptides by introducing chemical modifications, assembling monomers into highly ordered complexes, or by designing for peptide analogs (63). Different from RW monomer, which has poor in vivo inhibitory function on RSPO2–BMPR1A, RW dendrimer features enhanced peptide stability and avidity and robustly inhibits RSPO2 in the micromolar range. A large therapeutic window is a key criterion for drug development as it limits potential side effects in normal cell (64). We observed no Xenopus embryotoxicity of RWd at concentrations that robustly induced BMP signaling, and human adult fibroblasts tolerate at least 10-fold higher RWd concentrations than THP-1 cells. However, therapeutic application requires a reagent of higher affinity than that of RWd, which acts in the micromolar range. Computational modeling and medicinal chemistry may
derive small molecules mimicking RWd with improved pharmacological properties.

Looking beyond AML, RSPO2 is implicated in various other cancer entities, notably colon cancer (45, 46, 48, 65). The availability of RWd invites to explore both the potential role and therapeutic opportunities of RSPO–BMP inhibition in these contexts in the future.

**Experimental procedures**

**Synthetic peptides**

All peptides used in the study were synthesized and obtained from ProteoGenix, Inc. For overlapping peptide screening, monomeric peptides derived from the TSP1 domain of human RSPO2 (14) were designed with exchange of internal Cys to Ser residue. Peptides were synthesized and obtained of >70% purity, without modifications. Lyophilized peptides were reconstituted in Dulbecco’s PBS (DPBS), adjusted with pH 7.4 (GW, HR, GC, RW, GV, TK, KC, DE, PK, and SG) or in DPBS with 0.1% dimethyl sulfoxide, adjusted with pH 7.4 (CR). For PEGylated monomeric peptide, RW and TK peptides were mini-PEGylated at both N and C termini and reconstituted in DPBS, pH 7.4. For dendrimerized RW and KR, individual peptides were synthesized and assembled on an octavalent Lys core matrix, forming an 8-mer complex with purity of >80%. The sequences for the RW and KR dendrimers are (RNRNRTSGFW/8)-Lys4-Lys2-Lys-β-Ala (RW dendrimer) and (KMTMR)-8-Lys4-Lys2-Lys-β-Ala (KR dendrimer), respectively. For PEGylated dendrimers, mini-PEGylation of individual peptides within the dendrimers was performed at the N- or C-terminus, followed by attachment to the core matrix (designated as N-PEG dendrimer or C-PEG dendrimer, respectively). The sequences of N- and C-PEG RW dendrimers are (PEG-RNRNRTSGFW/8)-Lys4-Lys2-Lys-β-Ala and (RNRNRTSGFW-PEG)8-Lys4-Lys2-Lys-β-Ala. The sequences of N- and C-PEG KR dendrimers are (PEG-KMTMR)-8-Lys4-Lys2-Lys-β-Ala and (KMTMR-PEG)-8-Lys4-Lys2-Lys-β-Ala. The type of dendrimer used for each experiment is indicated in the figure legends. For biotinylated RW and TK peptides, peptides were synthesized and terminally modified by the addition of Biotin with a 6-aminohexanoic acid or ethyl-enediamine spacer at the N- or C-terminus, respectively.

**Cell lines and growth conditions**

Human embryonic kidney 293T cells (HEK293T) and HEPG2 cells (American Type Culture Collection) were maintained in Dulbecco’s modified Eagle’s medium-high glucose (catalog no.: 11960; Gibco) with 10% fetal bovine serum (FBS) (catalog no.: FBS-12A; Capicorn), 1% penicillin–streptomycin (catalog no.: P0781; Sigma), and 2 mM L-glutamine (catalog no.: G7513; Sigma). H1581 cells (gift from Dr R. Thomas) were maintained in RPMI (catalog no.: 21875; Gibco) with 10% FBS, 1% penicillin–streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate (catalog no.: S8636; Sigma). THP-1 cells were maintained in RPMI with 10% FBS, 1% penicillin–streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate. iHAFs were derived from primary human adult fibroblasts immortalized using large T antigen and kindly provided by Prof Jochen S. Utikal, DKFZ. All cell lines were cultured at 37 °C and 5% CO2 in a humidity-controlled incubator. Mycoplasma contamination was negative in all cell lines used.

**Production of conditioned medium**

Conditioned medium was generated as previously described (14). In brief, HEK293T cells were seeded in 10 or 15 cm culture dishes and transiently transfected with RSPO2-AP, RSPO3-AP, RSPO2TSP1-HA, or BMPR1AEC-AP plasmids, followed by harvesting three times every 2 days. Produced medium was validated with Western blot analyses and AP activity analyses.

**In vitro competitive binding assay**

High-binding 96-well plates (catalog no.: M5811; Greiner) were coated with 1 to 2 μg of anti-HA antibody (catalog no.: 11867423001; Roche) overnight at 4 °C. Coated wells were blocked with 5% bovine serum albumin in TBS with Tween-20 (TBST) for 1 h, followed by washing with TBST. Wells were treated overnight with HA-tagged RSPO2TSP1 conditioned media, followed by treatment of AP-tagged BMPR1AEC in combination with 100 μM of monomeric peptides or with increasing concentration of monomeric RW peptide for 3 h. Wells were washed with TBST, and bound AP activity was measured by the chemiluminescent AquaSpark AP substrate (catalog no.: 42593.01; Serva). Validation of IC50 was executed using GraphPad (GraphPad Software, Inc).

**In vitro direct binding assay**

Streptavidin-coated 96-well plates (catalog no.: 15218; Thermo Fisher Scientific) were incubated with biotinylated RW and TK peptides (50 μM) for 3 h, followed by washing with TBST. Wells were treated overnight with AP-tagged BMPR1AEC. Wells were washed with TBST, and bound AP activity was measured by the chemiluminescent AquaSpark AP substrate (catalog no.: 42593.01; Serva).

**Cell surface–binding assay**

Cell surface–binding assays were executed as previously described (14). In brief, HEK293T cells were transfected with BMPR1A-HA and LGR4 DNA and incubated with conditioned media in combination with 50 μM monomeric peptides or 20 μM dendrimers for 3 h or 16 h. Surface binding was detected by development with BM-Purple (catalog no.: 11442074001; Sigma). Images were obtained with DMIL microscope/Canon DS126311 camera (LEICA).

**Western blot analysis**

Cultured HEPG2, H1581, and THP-1 cells were lysed in Triton lysis buffer (14) or radioimmunoprecipitation buffer with cOmplete Protease Inhibitor Cocktail (catalog no.: 11697498001; Roche). SDS-PAGE samples were prepared with lysates mixed with Laemmli buffer containing β-mercaptoethanol, followed by boiling at 95 °C for 5 min. Western blot
images were acquired with SuperSignal West pico ECL (catalog no.: 34580; Thermo Fisher Scientific) or Clarity Western ECL (catalog no.: 1705061; Bio-Rad) using LAS-3000 system (Fujifilm).

**Immunofluorescence**

H1581 cells were transfected with BMPR1A-HA using Lipofectamine 3000 (catalog no.: L3000; Invitrogen). After 48 h, cells were treated with 0.5 μM RW or KR dendrimers and fixed in 4% paraformaldehyde for 10 min. Cells were treated with primary antibodies (1:250) overnight at 4 °C, and secondary antibodies (1:500) and Hoechst dye (1:500) were applied for 2 h at room temperature. Images were obtained using an LSM 700 microscope (Zeiss). Quantification was executed using ImageJ, version 1.51k software (https://imagej.nih.gov/ij/).

**Fluorescence-activated cell sorting analysis**

For the differentiation analysis of THP-1, cells were harvested, pelleted, and resuspended in ice-cold blocking buffer (PBS supplemented with 1% bovine serum albumin and 0.1% NaN3). Cells were treated with Fc receptor binding inhibitor as recommended by the manufacturer (catalog no.: 14916173; eBioscience) and stained directly with FITC-conjugated antibodies diluted in blocking buffer. Isotype-matched antibodies were used as control. Dead cells were excluded by counterstaining with propidium iodide. Samples were analyzed with FACScanto (fluorescence-activated cell sorting (FACS); BD Biosciences). About 10,000 events per samples were acquired, and results were processed with FACSDiva software (BD Biosciences) or FlowJo (FlowJo, LLC).

**Cell viability assay**

Cells were seeded into 96-well plate in high density with increasing amounts of dendrimers. After incubation of 48 h, cells were harvested, and viability was measured with a CellTiter-Glo Luminescent Cell Viability Assay kit (catalog no.: G7570; Promega) according to the manufacturer’s instructions.

**Xenopus tropicalis**

*Xenopus tropicalis* male and female frogs were obtained from Nasco, National Xenopus Resource and European Xenopus Resource Centre. All *X. tropicalis* experiments were approved by the state review board of Baden-Württemberg, Germany (permit number: G-141-18) and executed according to federal and institutional guidelines and regulations. Developmental stages of the embryos were determined according to Nieuwkoop and Faber (Xenbase; https://www.xenbase.org).

**Xenopus microinjection**

In *vitro* fertilization, microinjection, and culture of *X. tropicalis* embryos were executed following the protocol from Xenbase (https://www.xenbase.org). *X. tropicalis* embryos were microinjected using Harvard Apparatus microinjection system. About 20 nl of 50 μM reconstituted dendrimers in PBS were microinjected into the blastocoel of stage 9 embryos. Microinjected embryos were cultured until desired stages. For phenotype analysis, scoring of phenotypes was executed with binning, and data are representative images from two independent experiments. *Xenopus* embryos were obtained using AxioCam MRc 5 microscope (Zeiss). Embryos in each image were selected using Magnetic Lasso tool or Magic Wand tool of Adobe Photoshop CS6 software and pasted into the uniform background color for presentation.

**Xenopus reporter assays**

*Xenopus* embryos were microinjected with 200 pg of *Vent-Luc* or *Topflash* along with 100 pg of *Renilla-TK* plasmid DNA at 2-cell stage. At stage 9, 20 nl of 50 μM reconstituted dendrimers in PBS was microinjected into the blastocoel of embryos. Three pools of four embryos each at stage 14 to 15 were lysed with passive lysis buffer, and luciferase activity was measured with the dual luciferase reporter assay system (catalog no.: E1960; Promega). Firefly luminescence (Vent2/TopFlash) was normalized to Renilla. Data are displayed as average of biological replicates with SD. Statistical analyses were made with the PRISM7 software (GraphPad Software, Inc) using unpaired *t* test or one-way ANOVA test. Not significant (ns) **p < 0.01** and ***p < 0.001.

**Antibodies**

Phospho-Smad1/5 (Ser463/465) (catalog no.: 9516; Cell Signaling), Smad1 (catalog no.: 9743; Cell Signaling), active-β-catenin (catalog no.: 05-665; Millipore), α-tubulin (catalog no.: T5168; Sigma), HA (catalog no.: E1960; Promega). Firefly luminescence (Vent2/Top-Flash) was normalized to Renilla. Data are displayed as average of biological replicates with SD. Statistical analyses were made with the PRISM7 software (GraphPad Software, Inc) using unpaired *t* test or one-way ANOVA test. Not significant (ns) **p < 0.01** and ***p < 0.001.

**Data availability**

All raw data are available upon reasonable request.

**Supporting information**—This article contains supporting information.

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Abbreviations—The abbreviations used are: ACVR1, activin A receptor type I; AML, acute myeloid leukemia; AP, alkaline phosphatase; BMP, bone morphogenetic protein; BMPR1A, BMP receptor type 1A; DPBS, Dulbecco’s PBS; ECD, extracellular domain; FBS, fetal bovine serum; FU, furin-like repeat; HA, hemagglutinin; HEK293T, human embryonic kidney 293T cell line; iHAF, immobilizing human adult hepatocellular carcinoma cell line; iHAF, immobilizing human adult fibroblast cell line; LGR, leucine-rich repeat containing G protein–coupled receptor; LRP, lipoprotein receptor-related protein; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; pSmad1, Smad1 phosphorylation; RNF43, ring finger 43; RSPO2, R-spondin 2; RSPO2FIP1, TSP1 domain of RSPO2; RW6, RW dendraim; SMAD, small mothers against decapentaplegic; TBST, TBS with Tween-20; TSP1, thrombospondin 1; ZNRF3, zinc and ring finger 3.

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