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R-Spondin2 Is a Secreted Activator of Wnt/β-Catenin Signaling and Is Required for *Xenopus* Myogenesis

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Summary

We have carried out a small pool expression screen for modulators of the Wnt/β-catenin pathway and identified *Xenopus R-spondin2* (*Rspo2*) as a secreted activator of this cascade. *Rspo2* is coexpressed with and positively regulated by Wnt signals and synergizes with Wnts to activate β-catenin. Analyses of functional interaction with components of the Wnt/β-catenin pathway suggest that Rspo2 functions extracellularly at the level of receptor ligand interaction. In addition to activating the Wnt/β-catenin pathway, *Rspo2* overexpression blocks Activin, Nodal, and BMP4 signaling in *Xenopus*, raising the possibility that it may negatively regulate the TGF-β pathway. Antisense Morpholino experiments in *Xenopus* embryos and RNAi experiments in HeLa cells reveal that *Rspo2* is required for Wnt/β-catenin signaling. In *Xenopus* embryos depleted of *Rspo2*, the muscle markers *myoD* and *myf5* fail to be activated and later muscle development is impaired. Thus, *Rspo2* functions in a positive feedback loop to stimulate the Wnt/β-catenin cascade.

Introduction

Wnt/β-catenin signaling is implicated in diverse processes during embryonic patterning in all metazoa tested (Moon et al., 1997; Wodarz and Nusse, 1998). During early *Xenopus* embryogenesis, the Wnt/β-catenin pathway is involved in axial patterning at two stages, before and after midblastula transition (MBT), the onset of zygotic transcription (Moon et al., 1997). Zygotic activation of the pathway functions in antagonizing Spemann's head organizer (Niehrs, 2004) and in activating myogenesis. In chick and mammals, the role of Wnt signaling in muscle development is well established (Cossu and Borello, 1999; Borycki and Emerson, 2000). In *Xenopus*, injection of plasmid DNA encoding *Xwnt8* under the control of a CSKA promoter induces ectopic *myoD* expression. Inhibition of Wnt signaling, e.g., by dominant-negative *Xwnt8* or *frzb1*, inhibits expression of the myogenic genes *myf5* and *myoD* and blocks muscle formation (Hoppler et al., 1996; Leyns et al., 1997).

Wnt/β-catenin signaling engages two transmembrane receptor classes, Frizzled (Fz) type 7-transmembrane proteins and lipoprotein receptor-related proteins 5 and 6 (LRP5/6), which are members of the LDL receptor superfamily (Wodarz and Nusse, 1998; He et al., 2004). Wnts are not the only secreted factors able to activate the β-catenin pathway. Secreted Frizzled-related protein 1 can activate Wnt/β-catenin signaling at low dose while it inhibits at high dose (Uren et al., 2000). Dkk2, a member of the Dickkopf family of secreted Wnt antagonists, binds and activates LRP6 (Wu et al., 2000; Mao and Niehrs, 2003). However, in the presence of its coreceptor Kremen, Dkk2 inhibits Wnt/β-catenin signaling (Mao and Niehrs, 2003). Another LRP6 ligand, Wise, can also activate or inhibit Wnt signaling in a context-dependent manner (Itasaki et al., 2003). Recently, a novel Frizzled ligand, Norrin, has been identified, which activates β-catenin signaling specifically through Frizzled 4 (Xu et al., 2004).

In this study, we show that *R-spondin2* (*Rspo2*), a gene of previously unknown function, is regulated by Wnts and directly activates Wnt/β-catenin signaling. *Rspo2* is required for Wnt/β-catenin signaling in HeLa cells and specifically for Wnt-dependent myogenesis in *Xenopus*. The data indicate that Rspo2 represents a secreted effector of the Wnt/β-catenin pathway.

Results

Isolation of *Xenopus R-spondin2*

To isolate novel genes modulating Wnt/β-catenin signaling, we carried out an expression screen in HEK293T cells by transfecting the Wnt-responsive reporter TOPFLASH (Korinek et al., 1997) together with cDNA pools from a *Xenopus* eye library. One pool was identified which stimulated the reporter 7-fold (Figure 1A). A cDNA harboring the activity was isolated from this pool by sib selection. Sequence analysis revealed that this gene represents the *Xenopus* homolog of *R-spondin2* (Supplemental Figures S1A–S1C at http://www.developmentalcell.com/cgi/content/full/7/4/525/DC1/), member of a recently identified gene family of unknown function (Chen et al., 2002; Kamata et al., 2004). There are four related members in this family, which we term *R-spondin1-4*, respectively (Supplemental Figures S1A–S1C). While there are a number of EST sequences showing homology to *R-spondin* in other vertebrates, no homologs were found in *Drosophila* and *C. elegans*.

Xenopus Rspo2 is predicted to encode a secreted protein with 243 amino acids (mature protein) and an isoelectric point of 9.8. All R-spondins contain an N-terminal signal peptide (SP), two furin-like domains (FU), one thrombospondin type1 domain (TSP1), and a C-terminal low complexity region enriched with positively charged amino acids (C) (Figure 1F).

While *Xenopus* Rspo2 contains a predicted N-terminal signal peptide, secreted protein is almost undetectable in the medium of transiently transfected 293T cells. Since the C terminus is enriched with basic amino acids, which promotes cell surface retention (LaRochelle et al., 1991; Houck et al., 1992), we tested a C-terminally

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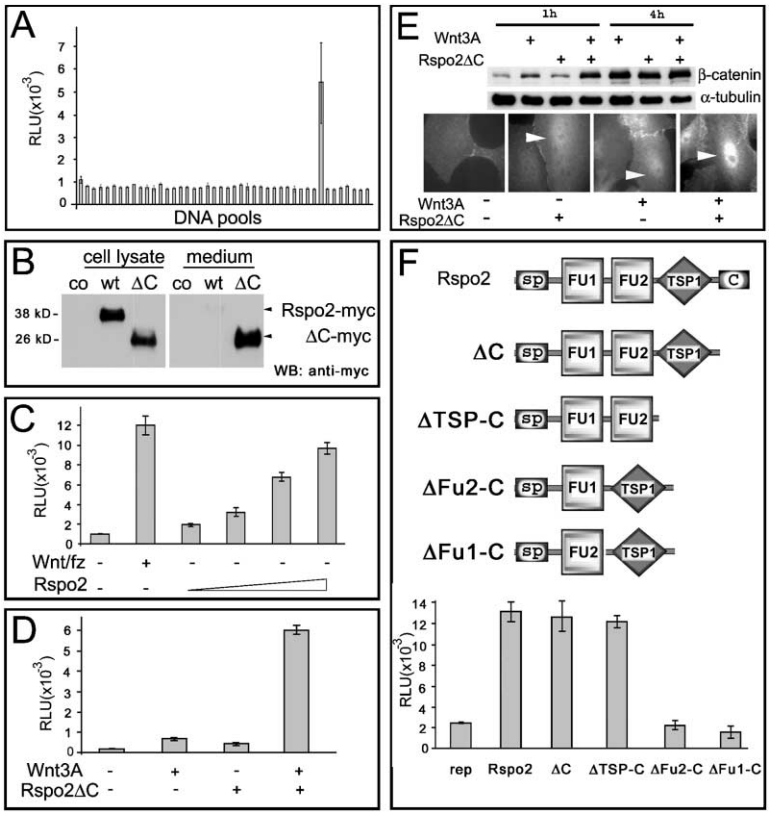

Figure 1. *Rspo2* Promotes Wnt/β-Catenin Signaling

(A) Identification of *Xenopus Rspo2* by expression screening. RLU, relative light units.

(B) The C terminus of Rspo2 mediates cell surface retention. Myc-tagged *Rspo2* (wt) or *Rspo2*ΔC (ΔC) were transfected in 293T cells, and cell lysate and medium were analyzed by Western blot. Co, untransfected cells.

(C) *Rspo2* activates Wnt/β-catenin signaling. TOPFLASH luciferase reporter assays were carried out in 293T cells with the following transfected DNAs: mouse *Wnt1* (Wnt), 5 ng; mouse *frizzled8* (fz), 1 ng; *Xenopus Rspo2* (Rspo2), 0.1, 0.3, 0.9, and 2.7 ng.

(D) Rspo2 enhances Wnt3A signaling. Mouse Wnt3A, *Xenopus* Rspo2ΔC (Rspo2ΔC), or mock-conditioned media were added to 293T cells followed by TOPFLASH luciferase reporter assays.

(E) Rspo2 stabilizes β-catenin. Top: 293T cells were treated with Rspo2ΔC, Wnt3A, or mock-conditioned media for 1 or 4 hr as indicated. Cytosolic fractions were subjected to Western blot and probed for β-catenin and α-tubulin (loading control). Bottom: Immunohistochemical staining of β-catenin in SHEP cells after 3 hr treatment with the conditioned media indicated. Arrowheads indicate nuclear β-catenin. The percentage of cells showing the represented staining is 90% (Co), 85% (Rspo2), 80% (Wnt3A), and 90% (Wnt3A + Rspo2).

(F) Domain analysis of Rspo2. Top: schematic drawing of *Xenopus Rspo2* and deletion constructs. sp, signal peptide; FU1, 2, furin-like domains; TSP1, thrombospondin type1 domain; C, positively charged C terminus. Bottom: TOPFLASH luciferase reporter assays were carried out in 293T cells with the indicated constructs. Equal protein production was confirmed by Western blot (data not shown).

truncated protein. Rspo2ΔC is effectively secreted into the medium from 293T cells (Figure 1B) and is functionally active (Figure 1F). Since all R-spondins share the basic C terminus and since there are no obvious ER or Golgi retention signals, this suggests that the proteins are normally associated with the cell surface.

R-spondins Promote Wnt/β-Catenin Signaling in 293T Cells

Transfected *Xenopus Rspo2* stimulates the TOPFLASH reporter in a dose-dependent manner in 293T cells (Figure 1C) but not the mutant reporter TOPFLASH (Korinek et al., 1997; data not shown). All tested members of the *Rspo* family (e.g., murine *Rspo1-3*, human *Rspo2, 3*) show equivalent effects (Supplemental Figure S2A and data not shown).

Rspo2 signaling is sensitive to the Wnt/β-catenin pathway inhibitors dominant-negative *TCF* (Molenaar et al., 1996), *Axin* (Zeng et al., 1997), and *dickkopf1* (Glinka et al., 1998) (Supplemental Figure S2B). A synergistic signaling effect is observed when *Rspo2* is cotransfected with extracellular but not with intracellular components of the Wnt/β-catenin pathway (Supplemental Figure S2C). The greatest cooperation is reproducibly seen between Rspo2 and Wnts, either using conditioned media (Figure 1D) or following cotransfection (Supplemental Figure S2C).

A hallmark of Wnt/β-catenin signaling activation is the cytosolic accumulation of β-catenin due to its stabilization (Moon et al., 1997; Wodarz and Nusse, 1998). Treatment of 293T cells with Wnt3A conditioned medium induces cytosolic β-catenin after 1 hr, and while recombinant Rspo2ΔC alone is not able to stabilize β-catenin during this interval, it strongly enhances activity of Wnt3A to do so (Figure 1E, top). After 4 hr treatment, both Wnt3A and Rspo2ΔC conditioned media are able to induce β-catenin accumulation to similar levels (Figure 1E, top). β-catenin is known to enter the nuclei in response to Wnt stimulation and activate gene expression together with Lef/Tcf transcription factors (Wodarz and Nusse, 1998). Treatment of SHEP cells by either Wnt3A or Rspo2ΔC conditioned media weakly induces β-catenin nuclear localization, while cotreatment with Wnt3A and Rspo2ΔC strongly enhances nuclear accumulation (Figure 1E, bottom).

We conclude that R-spondins represent a family of secreted proteins capable promoting Wnt/β-catenin signaling.

To functionally study its signaling domains, we created serial deletions of *Xenopus Rspo2* (Figure 1F). As discussed, the basic C terminus can be removed without loss of activity and this is also true for the TSP1 domain (Figure 1F). However, the furin-like domains are necessary for Wnt/β-catenin signaling since deletion of either the furin-1 or -2 domains abolishes the activity in reporter assays (Figure 1F).

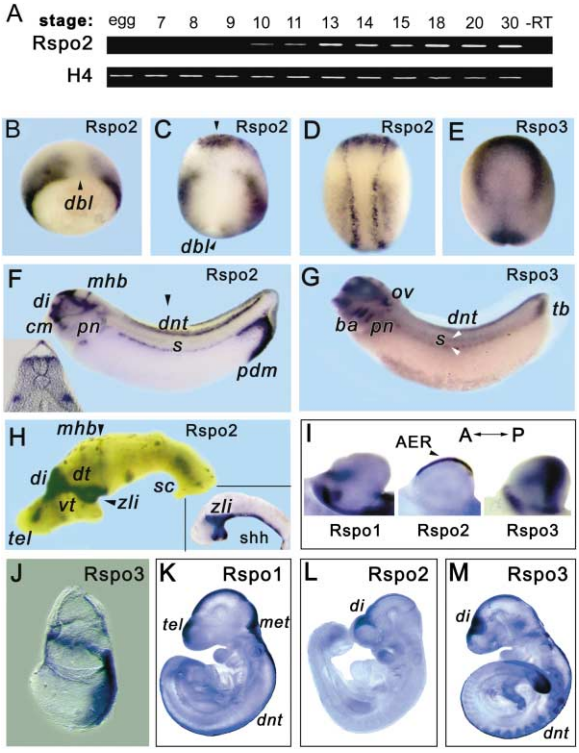

Figure 2. Expression Analysis of *Xenopus* and Mouse *R-spondins*

(A) *Rspo2* expression during *Xenopus* development at the indicated embryonic stages analyzed by RT-PCR. Histone H4 was used for normalization. −RT, minus reverse transcription control.

(B–H) *Xenopus* whole-mount in situ hybridizations of the indicated genes.

(B) Stage 11 embryo, dorso-vegetal view; dbl, dorsal blastoporal lip.

(C) Stage 12 embryo, dorsal view with anterior up. An anterior neural expression domain is indicated by arrowhead.

(D) Stage 15 embryo, dorsal view with anterior up.

(E) Stage 14 embryo, dorsal view with anterior up.

(F and G) Tailbud stage embryos; ba, branchial arches; cm, cranial musculature; di, diencephalon; dnt, dorsal neural tube; mhb, midbrain-hindbrain boundary; ov, otic vesicle; pn, pronephros; pdm, proctodeum; s, somites; tb, tailbud mesoderm. Inset in (F) shows a transverse section at the level indicated by arrowhead, showing expression in dorsal neural tube and in the dorsal- and ventral-most parts of the somites.

(H) Dissected *Xenopus* tadpole brain (lateral view) showing expression in diencephalons (di) and zona limitans intrathalamica (zli), where *sonic hedgehog* is expressed (inset). dt, vt, dorsal and ventral thalamus, respectively; sc, spinal cord; tel, telencephalon.

(I–M) Mouse whole-mount in situ hybridizations of the indicated genes.

(I) Limb buds of day 12.5 mouse embryos. AER, apical ectodermal ridge.

(J) Day 7.5 mouse embryo showing *Rspo3* expression in the primitive streak.

(K–M) Day 9.5 mouse embryos. di, diencephalon; dnt, dorsal neural tube; met, metencephalon; tel, telencephalon.

Expression of *R-spondin* Genes in *Xenopus* and Mouse Embryos

In *Xenopus* embryos, no maternal *Rspo2* RNA is detected by RT-PCR. Its zygotic expression starts at early gastrula stage and remains constant throughout neurulation and organogenesis (Figure 2A). By whole-mount in situ hybridization, weak expression of *Rspo2* is observed throughout the ectoderm of early gastrulae (not shown). During gastrulation, strong expression is detected in the marginal zone in both deep and superficial layers but is excluded from the Spemann organizer (Figure 2B). At late gastrula stage, *Rspo2* expression persists in lateral plate mesoderm and becomes detectable in the anterior neural plate (Figure 2C). At stage 15, expression is seen in two longitudinal stripes along the neural plate (future roof plate), in the anterior neural plate, and in lateral and posterior mesoderm (Figure 2D). Expression of *Rspo2* at tailbud stage (Figure 2F) is restricted to several regions of the brain, including diencephalon and midbrain-hindbrain boundary, pronephros, and dorsal neural tube. Expression is also detected in the dorsal- and ventral-most portions of somites, the dorsal fin and the proctodeum. *Rspo2* expression in the brain of late tadpoles is mainly restricted to diencephalon, including the zona limitans intrathalamica (zli) (Figure 2H).

Xenopus Rspo3 expression is related to that of *Rspo2*. It is first detected at gastrula stage (not shown) and in neurulae is expressed in the anterior border of the neural plate and posterior mesoderm (Figure 2E). At tailbud stage, it is coexpressed with *Rspo2* in the central nervous system but shows additional expression in branchial arches and the tailbud (Figure 2G).

In mouse, *Rspo3* transcripts are detected by in situ hybridization at day 7.5 in the primitive streak (Figure 2J) while expression of *Rspo1* and *2* is not detectable. At day 9.5, *Rspo1-3* show differential expression in various neural and mesodermal derivatives (Figures 2K–2M), mainly along dorsal neural tube (*Rspo1* and *3*), diencephalon (*Rspo1, 2, 3*), somites *(Rspo3*), and tailbud mesoderm (*Rspo3*). In limb buds all three genes show prominent differential expression (Figure 2I), particularly in the morphogenetically active region such as the apical ectodermal ridge (AER) (*Rspo2*).

R-spondins Are Coexpressed with and Regulated by *Wnts*

R-spondins not only show functional interaction with Wnt signaling, but also coexpression with *Wnt* genes in many regions during *Xenopus* and mouse embryonic development. In gastrula mesoderm of both *Xenopus* and mouse, *Rspo2* and *3* are coexpressed with *XWnt8* and *mWnt3*, respectively. Similarly, at later stages, *R-spondin* family members are widely coexpressed with a number of *Wnt* genes, e.g., in midbrain-hindbrain boundary, dorsal neural tube, and limb bud and tail bud (Parr et al., 1993; Liu et al., 1999). A direct comparison between the expression patterns of *Xenopus Rspo2* and *Wnt8* and *Wnt3A* shows a large overlap (Figure 3A).

Indeed, Wnts are able to induce *Rspo* expression since *Xenopus* embryos injected with pCS-*Wnt8* or pCS-β-*catenin* DNA upregulate both *Rspo2* and *Rspo3* by RT-PCR (Figure 3B). Likewise, embryos injected with pCS-*Wnt8* or pCS-*Wnt3A* DNA show ectopic *Rspo2* expression by in situ hybridization (Figures 3C–3E). The results indicate that the observed coexpression is due to regulation of *R-spondins* by *Wnts*. This is consistent with the observation that *Rspo1* expression is reduced in dorsal spinal cord of *Wnt1* or *Wnt3A* knockout mouse embryos (Kamata et al., 2004).

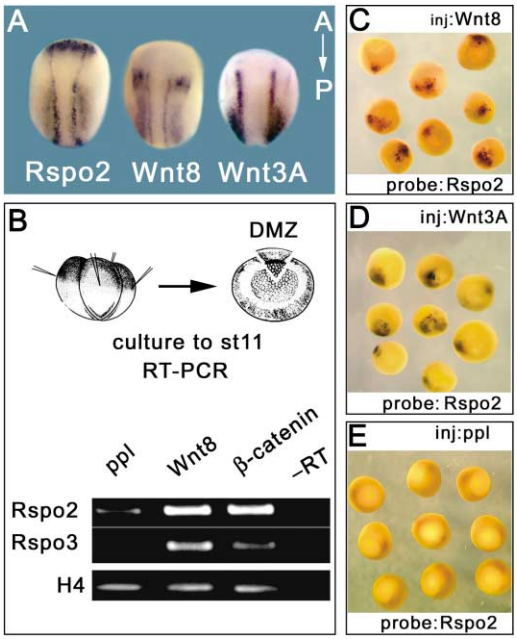

Figure 3. Regulation of *Xenopus R-spondins* by Wnt Signaling

(A) Comparison of *XRspo2*, *XWnt8*, and *XWnt3A* expression pattern in early neurula embryos by whole-mount in situ hybridization. Dorsal view, anterior up.

(B) Top: Diagram of experiment. 4-cell stage embryos were injected with 50 pg pCS-*ppl* (preprolactin), pCS-X*Wnt8*, or pCS-β-*catenin* into each blastomere, and DMZs were dissected, cultured until stage 11 equivalent, and analyzed by RT-PCR. Bottom: RT-PCR analysis of the indicated genes. −RT, minus reverse transcription control.

(C–E) 4-cell stage embryos were injected with 50 pg pCS-*Wnt8*, pCS-*Wnt3A*, or pCS-*ppl* into one blastomere and fixed at stage 11 for in situ hybridization with *XRspo2*.

R-spondin2 Activates Neural Markers and Promotes Muscle Formation

When the Wnt/β-catenin pathway is overactivated in *Xenopus* embryos, a variety of responses are observed: (1) mRNA injection of pathway activators typically induces secondary embryonic axes in whole embryos and anterior neural markers in animal caps and whole embryos (Baker et al., 1999); and (2) DNA injection of pathway activators driving expression after MBT posteriorizes the central nervous system (CNS) (Niehrs, 2004). To test if *Rspo2* is able to mimic any of these effects, we microinjected synthetic mRNA in *Xenopus* embryos. However, *Rspo2* mRNA injection does not induce secondary axes in whole embryos, and injection of *pCS-Rspo2* DNA does not posteriorize CNS, since heads are normal sized, *Otx2* expression is expanded, and *en2* is unaffected (Figures 4D and 4H).

In animal caps, *Rspo2* induces the panneural markers *NCAM* and *N-tubulin* and the anterior neural marker, *Otx2*, as do *Xwnt8* and β-*catenin* (Figure 4A). Neural marker induction occurs in the absence of muscle actin, indicating that the neuralizing effect is direct. Similarly, in whole embryos injected with *Rspo2* mRNA, ectopic cement glands and lateral expansion of the neural plate are observed at the injected side, as shown by expression of neural markers *Sox3*, *Otx2*, *not2*, *en2*, and *Rx1* (Figures 4B–4H). Ectopic *Otx2* expression is observed already in gastrula stage embryos (Figures 4I and 4J).

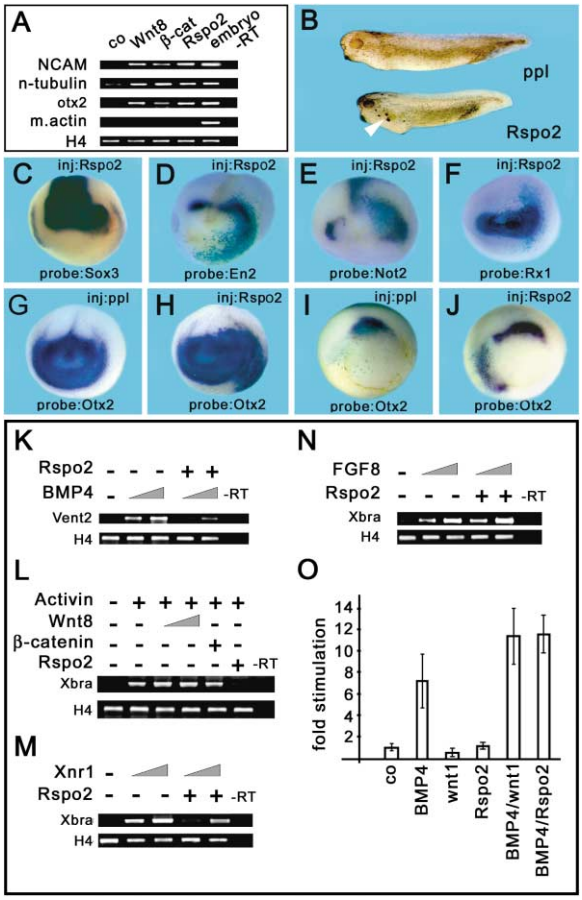

Figure 4. *Rspo2* Inhibits TGF-β Signaling in *Xenopus*

(A) 4-cell stage embryos were injected animally with 100 pg *Rspo2*, 100 pg *Xwnt8*, or 200 pg β-*catenin* mRNA in each blastomere. At stage 8, animal caps were dissected, cultured until stage 18 equivalent, and analyzed for expression of the indicated marker genes. −RT, minus reverse transcription control.

(B) 2-cell stage embryos were injected with 100 pg *Rspo2* or *preprolactin* (*ppl*) mRNA in each blastomere. Note ectopic cement glands (arrowhead) and shortened body axis in *Rspo2*-injected embryo.

(C–J) Whole-mount in situ hybridizations of the indicated genes. 8-cell stage embryos were injected with 100 pg of *Rspo2* or *ppl* mRNAs as indicated into one animal blastomere. LacZ mRNA was coinjected as lineage tracer in all panels except (C), (G), and (H).

(C–H) Stage 15 neurulae in anterior view.

(I and J) Stage 11 gastrulae in vegetal view, dorsal up.

(K–N) 4-cell stage embryos were injected animally with indicated RNAs. At stage 8, animal caps were dissected, cultured until stage 10 equivalent, and analyzed for expression of *Xvent2* (K) or *Xbra* (L–N). −RT, minus reverse transcription control. Embryos injected with 100 pg *preprolactin* (*ppl*) were used as control. Amounts of mRNAs used: 100 pg *Rspo2*, 50 pg or 250 pg of *BMP-4*, 50 pg of *activin,* 25 or 100 pg of *Wnt8,* 200 pg of β-*catenin*, 50 or 100 pg of *Xnr1*, 2 or 20 pg of *FGF*8.

(O) BMP responsive luciferase reporter assay in 293T cells. Concentrations of plasmids were: BRE4-luc, 10 ng, *BMP4*, 10 ng, *Wnt1*, 5 ng, *Rspo2*, 5 ng.

To further analyze the mRNA overexpression effect of Rspo2 on BMP4, Activin, Nodal, and FGF signaling, we carried out animal cap assays and tested for the induction of target genes by these growth factor signals (Figures 4K–4N). *Rspo2* expectedly blocks BMP4-mediated *Xvent2* expression but surprisingly also inhibits

Activin- and Nodal-mediated *Xbra* induction (Figures 4K–4M). This is unlike *XWnt8* and β-*catenin* mRNA injections, which do not affect *Xbra* induction by Activin (Figure 4L). In contrast to signaling by these TGF-β type growth factors, FGF8-induced *Xbra* expression is unaffected by overexpressed *Rspo2* (Figure 4N). To test if BMP signaling is also affected in 293T cells, luciferase reporter assays were carried out using BRE4-luc (Hata et al., 2000). Neither Wnt1 nor Rspo2 have an effect on BMP4-stimulated luciferase activity (Figure 4O). We conclude that *Rspo2* overexpression in addition to activating the Wnt/β-catenin pathway is also able, in *Xenopus*, to interfere with signaling by three TGF-β family growth factors.

Another well-known effect of zygotic Wnt/β-catenin signaling is its ability to promote myogenesis (Cossu and Borello, 1999). For example, *XWnt8* can induce muscle formation in ventral mesodermal cells (Hoppler et al., 1996). When ventral marginal zones (VMZs) from *Rspo2*-injected embryos are dissected and cultured until stage 40, they elongate, form tail-like structures, and are contractile. This phenotype is indistinguishable from control lateral marginal zone explants (LMZs), which typically differentiate muscle (Figures 5A and 5B). In situ hybridization confirmed induction of *muscle actin* in both *Wnt8*- and *Rspo2*-injected VMZs but not in control (preprolactin-injected) VMZs (Figure 5C). Since *R-spondins* are able to enhance Wnt signaling in 293T cells, we tested their cooperation in *Xenopus* myogenesis. *Myf5* is a myogenic marker characteristically expressed in lateral mesoderm (Dosch et al., 1997). However, it is excluded from dorsal mesoderm, and dorsal marginal zone (DMZ) explants express only little *myf5* as determined by RT-PCR (Figure 5D). In DMZs from embryos injected with DNA constructs driving post MBT expression, *myf5* is weakly induced by *Rspo2*, moderately by *Wnt8*, and strongly by their combination (Figure 5D, top). The myogenesis-promoting effect of *Rspo2* is repressed by dominant-negative *dishevelled* (*Xdd1*), *dkk1*, and *GSK-3*β, which all block Wnt signaling (Figure 5D, bottom). In summary, the results suggest that *Rspo2* can promote myogenesis via the Wnt/β-catenin pathway.

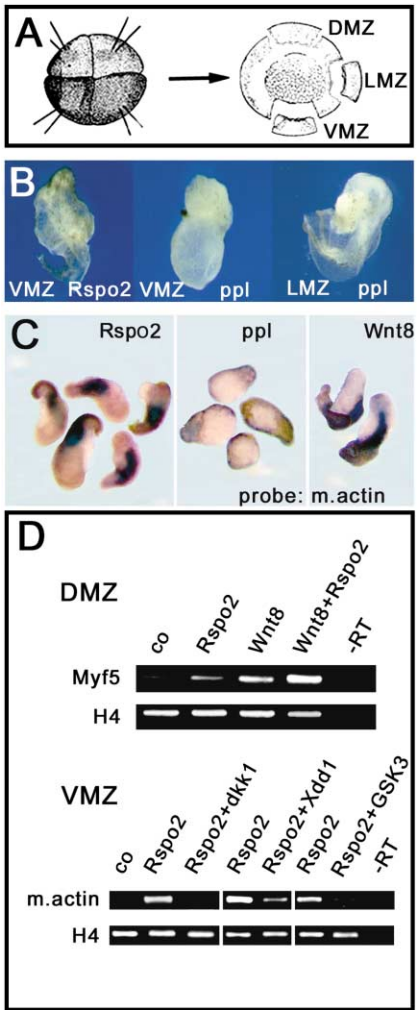

Figure 5. *Rspo2* Promotes Myogenesis in *Xenopus* Embryos

(A) Diagram of the experiments. 4-cell stage embryos were injected with 50 pg plasmid DNA constructs in all blastomeres, and the indicated fragments were explanted at stage 10.5, cultured, and processed for whole-mount in situ hybridization (C) or RT-PCR (D).

(B) Stage 40 equivalent VMZ or LMZ explants. Note tail-like structures in VMZs from *Rspo2*-injected embryos.

(C) In situ hybridization of stage 25 VMZs for *muscle actin*.

(D) RT-PCR analysis for the indicated genes in stage 11 equivalent DMZ and stage 25 equivalent VMZ explants. Xdd1, dominant-negative *Xenopus dishevelled*, Co, *preprolactin*. −RT, minus reverse transcription control.

R-spondin2 Is Required for Wnt/β-Catenin-Mediated Myogenesis

To investigate the physiological role of *Rspo2* during *Xenopus* embryogenesis, we injected antisense morpholino oligonucleotides (Rspo2Mo). The ability of Rspo2Mo to block Rspo2 protein production is demonstrated by Western blot (Figure 6A). Injection of this Morpholino into one dorso-animal blastomere at 8-cell stage results in eye defects, although the expression of early eye *(Rx1*, *Pax6*), anterior neural (*Otx2*), and panneural markers (*Sox3*) is not obviously affected (not shown).

Equatorial injection of Rspo2Mo in one blastomere at 8-cell stage leads to muscle defects at the injected side (Figure 6B). In situ hybridization for *muscle actin* and transverse trunk sections show that Rspo2Mo injection causes disorganized somites (panels a and b) and reduced myotomes (panels c and d). Lineage tracing experiments showed that Rspo2Mo-injected cells contribute to lateral plate mesoderm instead of somites or undergo cell death (data not shown). At gastrula stage, expression of the myogenic markers *myf5* and *myoD* is strongly downregulated at the Rspo2Mo-injected side (panels e–h), while the panmesodermal marker *Xbra* and the organizer marker *Xnot2* are unaffected (panels i–l).

To test the specificity of Rspo2Mo, rescue experiments were performed by coinjecting Rspo2Mo together with an *Rspo2* RNA, in which six noncoding nucleotides were mutated so that it would not be targeted by this morpholino. Expression of *myf5* (Figure 6C) and *myoD* (data not shown) is effectively rescued by this mutant *Rspo2* RNA, indicating that the effect of Rspo2Mo on myogenesis is specific.

We used Rspo2Mo as a tool to examine the epistatic

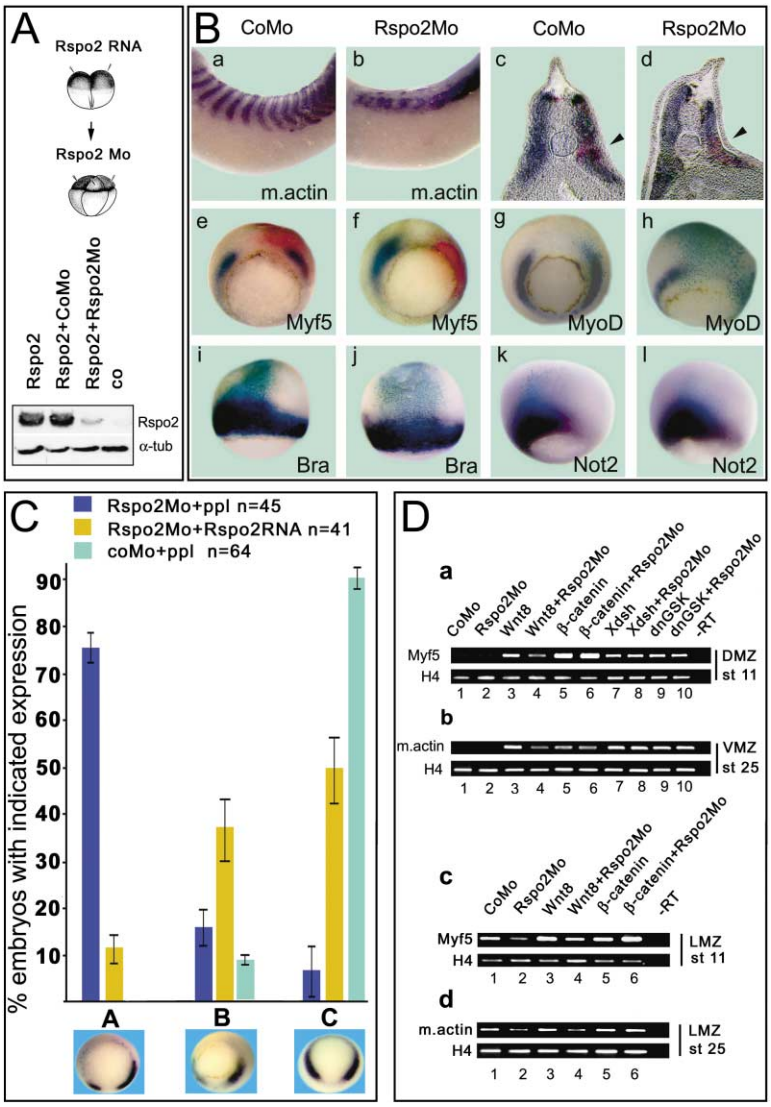

Figure 6. *Xenopus Rspo2* Is Required for Muscle Formation

(A) Rspo2Mo specifically inhibits translation of its cognate DNA. Top: Diagram of the experiment. 2-cell stage *Xenopus* embryos were injected with 100 pg Myc-tagged *Rspo2* mRNA in the animal region, and at 8-cell stage, the same embryos were then injected with 5 ng of Rspo2Mo or CoMo in all animal blastomeres, harvested at stage 11, and processed for Western blot analysis of Myc-tagged Rspo2 and α-tubulin.

(B) Depletion of Rspo2 protein causes muscle defects and downregulation of myogeneic markers. 4-cell stage embryos were injected equatorially into one blastomere with 5 ng control morpholino oligonucleotides (CoMo) or Rspo2Mo as indicated together with 50 pg ppl mRNA or 50 pg LacZ RNA as lineage tracer and analyzed at tailbud or gastrula stage by in situ hybridization for the indicated genes. In (a)–(f), double in situ hybridization for gene of interest (dark blue) and for ppl (red) was used. (a–d) Stage 25 embryos. (a and b) Myotomes, visualized by *muscle actin* expression, show malformations on Rspo2Mo-injected side (b). (c and d) Transversal section at the trunk level showing reduced muscle volume in (d). (e–h) *myf5* and *myoD* expression (dark blue) is downregulated in the Rspo2Mo-injected region (red in [e], [f] or light blue in [g], [h]). (i–l) *Xbra* and *Xnot2* expression (dark blue) is not affected in the region of Rspo2Mo injections (light blue).

(C) Rspo2Mo act specifically. Rescue of *myf5* reduction by coinjected *Rspo2* mRNA. 4-cell stage embryos were injected in one blastomere with 5 ng of control morpholino (CoMo), Rspo2Mo, 50 pg ppl mRNA, or 50 pg *Rspo2* mRNA containing mismatches to Rspo2Mo. Expression of *myf5* was analyzed by in situ hybridization at gastrula stage. The percentage of embryos with strongly affected (A), moderately reduced (B), and normal (C) *myf5* expression as displayed in the representative embryos is indicated. Standard deviation was calculated from three independent experiments.

(D) Rspo2Mo blocks Wnt signaling upstream of *dishevelled* during muscle formation. 4-cell stage embryos were radially injected with 5 ng of Rspo2Mo or CoMo, 50 pg pCS-X*Wnt8*, pCS-*dishevelled* (Xdsh), pCS-dominant-negative *GSK-3*β (dnGSK), or pCS-β-*catenin*. At stage 10.5 DMZs (a) or VMZs (b) or LMZs (c and d) were explanted and cultured until stage 11 (a, c) or 25 (b, d) for RT-PCR analysis of the genes indicated. −RT: minus reverse transcription control.

position of *Rspo* in the Wnt/β-catenin pathway. As read out for Wnt/β-catenin signaling, we used the expression of *myf5* and *muscle actin* in marginal zone explants (Figure 6D). In DMZ and VMZ explants, *Wnt8* DNA induces *myf5* and *muscle actin*, and this induction is significantly blocked by Rspo2Mo (Figure 6D, panels a and b, lanes 3 and 4). However, *myf5* and *muscle actin* induction by intracellular activators of the Wnt pathway like *disheveled*, *dnGSK-3*β, and β-*catenin* is unaffected by Rspo2Mo (panels a and b, lanes 5–10). In LMZ explants, Rspo2Mo injection downregulates endogenous *myf5* and *muscle actin* expression (Figure 6D, panels c and d, lanes 1 and 2). In DNA coinjections, this effect is rescued effectively by β-*catenin* but only poorly by *XWnt8* (lanes 3–6). The residual effect of *XWnt8* is likely due to its non-cell-autonomous action on cells that did not receive Rspo2Mo. Taken together, these results indicate that *Rspo2* affects the Wnt/β-catenin pathway at the level or upstream of *dishevelled*.

R-spondins Are Required for Wnt/β-Catenin Signaling in HeLa Cells

We next investigated the requirement of *Rspo2* for Wnt signaling in mammalian cells using siRNA. Since there are four *R-spondins* with apparently redundant function, we selected HeLa cells, which only express *Rspo3* and very weakly *Rspo2* (Figure 7A). Various other cell lines, such as 293T cells, express all four genes, complicating a siRNA approach.

siRNA mediated gene knockdown was carried out by transfecting pSUPER constructs (Brummelkamp et al., 2002) to produce siRNAs targeted against human *Rspo2*

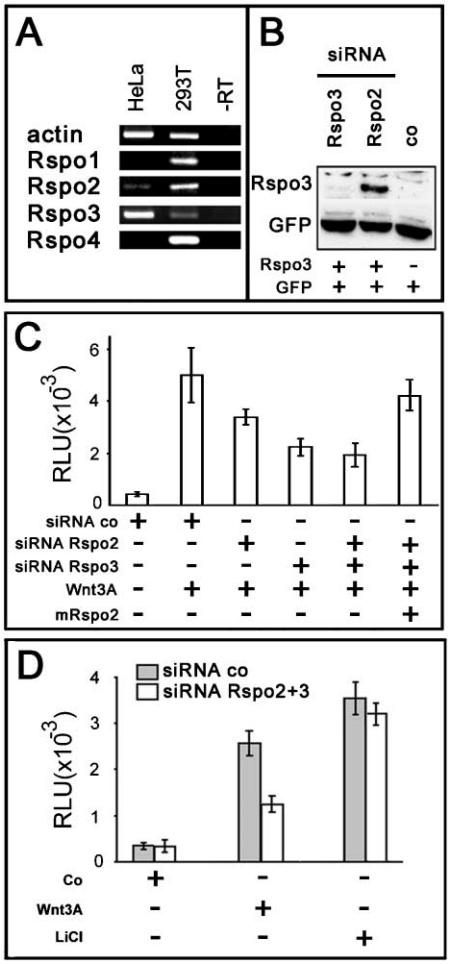

Figure 7. *R-spondins* Are Required for Wnt Signaling in HeLa Cells

(A) RT-PCR analysis showing differential expression of *human Rspo1-4* in HeLa and 293T cell lines. *Actin* was used for normalization.

(B) Specificity of siRNAs. 293T cells were cotransfected with pSUPER-Rspo2 or 3 (siRNA), FLAG-tagged Rspo3, and GFP (transfection control). Expression of Rspo3 and GFP were analyzed by Western blot.

(C and D) R-spondins are required for Wnt/β-catenin signaling in HeLa cells. Wnt luciferase reporter assay in HeLa cells cotransfected with the indicated constructs. Wnt3A was added as conditioned medium. RLU, relative light units.

and *3* (siRNA *Rspo2,3*). As control, a nonsense siRNA was used. To test the efficiency of siRNA, FLAG-tagged human *Rspo3* was cotransfected with siRNAs and its production was repressed by siRNA *Rspo3* but not siRNA *Rspo2* (Figure 7B).

In Wnt-reporter assays, both siRNA *Rspo2* and *3* decreased Wnt3A-induced luciferase activity compared to control siRNA (Figure 7C). When siRNA *Rspo2* and *3* are cotransfected, reporter activity drops to 40%. This effect is specific since the decreased Wnt signaling can be rescued by cotransfected mouse *Rspo2*, which is not targeted by siRNAs against human *R-spondins* (Figure 7C). Furthermore, unlike Wnt3A-induced reporter activity, siRNA *Rspo2+3* do not affect Li^+-induced Wnt/β-catenin signaling (Figure 7D). Since Li^+ acts by inhibiting GSK-3β activity (Klein and Melton, 1996), this is again consistent with *Rspo2* acting at the level or upstream of *dishevelled*.

We conclude that in HeLa cells R-spondins are required for full Wnt/β-catenin signaling.

Expression of *R-spondin* Genes in Tumors

Misregulation of Wnt/β-catenin signaling is implicated in tumorigenesis, e.g., colon cancer, breast cancer, and melanoma (Bienz and Clevers, 2000). Since *R-spondins* promote Wnt/β-catenin signaling, they may also play a role in tumorigenesis. We therefore analyzed expression of *Rspo1-4* in human tumor and corresponding normal tissue from individual patients (Supplemental Figure S3). *HRspo1* is weakly expressed in adult organs. It shows upregulation in ovary tumors from two patients and in one stomach tumor sample. *HRspo2* is expressed in organs of endodermal origin, including colon, rectum, small intestine, and lung. Its expression decreased in corresponding tumor samples. *HRspo3* is expressed widely and decreases in many tumor samples. In general, the expression of *hRspo1-3* is deregulated in a number of tumors, while *hRspo4* expression is very weak both in human adult organs and tumors (data not shown).

Discussion

Rspo2 Is an Extracellular Activator of Wnt/β-Catenin Signaling and Is Required for Myogenesis

We have identified *Rspo2* as an activator of the Wnt/β-catenin pathway. To our knowledge, the only other secreted activators of the pathway are Wnts themselves as well as the recently described Fz4 ligand Norrin (Xu et al., 2004). While Dkk2, sFrp1, and Wise are able to activate the pathway as well when overexpressed, they also function as inhibitors and there is no evidence that the activation observed in in vitro assays is of physiological relevance (Uren et al., 2000; Wu et al., 2000; Itasaki et al., 2003). Our results show that the activation of Wnt/β-catenin signaling by *Rspo2* is of physiological relevance during myogenesis and is also required for Wnt signaling in HeLa cells. Expression of the myogenic genes *myf5* and *myoD* depends on Wnt/β-catenin signaling in *Xenopus* as well as in other vertebrates (Cossu and Borello, 1999). In mouse, signals from the neural tube and dorsal ectoderm, which induce *myf5* and *myoD*, respectively, can be mimicked by *Wnt7a*-expressing cells. Likewise, paraxial mesodermal cells cultivated in vitro express *myf5* and *myoD* in response to Wnt1 and Wnt7a, respectively (Tajbakhsh et al., 1998). Furthermore, *myf5* is repressed in *Wnt1/Wnt3A* double mutant mice (Ikeya and Takada, 1998). In *Xenopus*, Wnt/β-catenin signaling is required much earlier for myogenic gene expression than in mouse, since the expression of *myf5* and *myoD* is repressed/activated already at gastrula stage by various Wnt inhibitors/activators (Hoppler et al., 1996; Leyns et al., 1997; Marom et al., 1999). Indeed, *myf5* is an immediate-early response gene for zygotic Wnt/β-catenin signaling at gastrula stage (Shi et al., 2002). Consistent with this, we observe that Rspo2Mo depletion blocks myogenic gene expression at early gastrula stage.

Rspo2 is coexpressed in a variety of tissues with Wnts and can be ectopically activated by Wnt signaling. Likewise, mouse *Rspo1* expression is downregulated in mouse *Wnt1* and *Wnt3A* mutants (Kamata et al., 2004). Therefore, *Rspo2* may play a role in a positive feedback loop of Wnt/β-catenin signaling, similar to components of other growth factor signaling pathways, e.g., BMP4, Delta-Notch, and FGF8, which form synexpression groups (Gawantka et al., 1998; Niehrs and Pollet, 1999). Rspo2 protein is closely associated with the cell surface, suggesting that it acts at short range, while vertebrate Wnt proteins can act at long range (Kiecker and Niehrs, 2001). This raises the possibility that Rspo2 plays a role in a local feedback to promote high levels of Wnt/β-catenin signaling in Wnt-expressing cells.

In mouse, the expression of *Rspo3* is very similar to that of *Xenopus Rspo2*, suggesting that the two genes play equivalent roles. However, *mRspo1* and *2* also show prominent expression in regions with active Wnt signaling, including the midbrain-hindbrain boundary and roof plate, and *Rspo2* is coexpressed with *Wnt3* in the apical ectodermal ridge (Barrow et al., 2003). Although we have focused on the functional analysis of *Rspo2*, we note that the other members of the R-spondin family also activate the Wnt-luciferase reporter (Supplemental Figure S2A and data not shown), suggesting that the whole family acts as Wnt modulator.

Mechanism of Rspo2 Action

The evidence that *Rspo2* is an effector of Wnt/β-catenin signaling rests on the following findings: (1) *Rspo2* is coexpressed with and induced by *Wnts*; (2) *Rspo2* induces Wnt/β-catenin signaling and strongly synergizes with *Wnt* in this respect; (3) *Rspo2* signaling is blocked by Wnt inhibitors; and (4) *Rspo2* is required for Wnt/β-catenin signaling in HeLa cells and in *Xenopus*. Finally, recombinant Rspo2+Wnt3A synergistically stabilize β-catenin within 1 hr, suggesting that its effects on the pathway are direct.

The results indicate that Rspo2 functions extracellularly at the level of receptor-ligand interaction during Wnt/β-catenin signaling: (1) Rspo2 is a secreted protein; (2) it strongly synergizes with extracellular components of the Wnt pathway, including Wnts, Fz, and LRP5, 6, but not with the intracellular transducers, e.g., Dishevelled or β-catenin; (3) Rspo2 signaling is blocked by *dkk1*, *Xdd1*, and *GSK-3*β; and (4) Wnt8/Wnt3A but not Dishevelled or Li^+ signaling is dependent on Rspo2. Rspo2 could either directly activate or derepress Wnt/β-catenin signaling. Obvious nodes of direct interaction for Rspo2 in the Wnt pathway are Wnts, Dkks, Fz, and LRPs. However, we failed to observe direct binding between any of these proteins (Dkk1-3, Fz1-8, LRP5, 6) and recombinant Rspo2. This suggests that its mechanism of interaction with the Wnt/β-catenin pathway is more complex and may involve an unknown cofactor, which renders cells competent to react to Rspo2. Indirect evidence for such a cofactor is that *Rspo2* is not generally required for Wnt/β-catenin signaling, it neither promotes maternal Wnt/β-catenin signaling to induce secondary axes nor zygotic signaling to posteriorize embryos (data not shown). This may be explained by differential expression of such a Rspo2 cofactor. Another possibility is that Rspo2 interacts with a very specific Frizzled receptor we have not tested, similar to the Norrin-Fz4 interaction (Xu et al., 2004).

Supporting a complex mechanism of action is the ability of Rspo2 to block signaling of Activin, Nodal, and BMP4. While inhibition of BMP4 signaling and neural induction in animal caps may be accounted for by activation of the Wnt/β-catenin pathway (Baker et al., 1999), the effect on Activin, Nodal, and BMP suggests a general TGF-β inhibiting effect of *Rspo2*. In this respect, Rspo2 somewhat resembles CTGF and Cerberus, which affect both the TGF-β as well as the Wnt/β-catenin pathway (Piccolo et al., 1999; Abreu et al., 2002; Mercurio et al., 2004). However, these overexpression effects of BMP and Activin inhibition by Rspo2 may not be physiologically relevant: (1) BMP signaling in 293T cells is unaffected by Rspo2; (2) *Rspo2* is coexpressed with both *Xbra* (an Activin target) and *Otx2* and *Rx1* (negative BMP4 targets); and (3) our Morpholino data show neither expansion of *Xbra* nor reduction of *Otx2* or *Rx1* (Figure 6B and data not shown), as would be expected if these signals were under negative control of *Rspo2*.

Experimental Procedures

Isolation of *R-spondins* and Constructs

A *Xenopus* adult eye cDNA library in pCS2+ was used to prepare pools of about 250 colonies. Plasmid DNA from each pool was transiently transfected into 293T cells together with the Wnt receptor *frizzled8*, the Wnt reporter TOPFLASH (Korinek et al., 1997) and pRL-TK (Promega) using FuGENE6 (Roche) transfection reagent. Luciferase assay was carried out 24 hr after transfection. A positive clone was isolated from the pool by sib selection. Human *Rspo2* and *3* cDNAs were obtained from RZPD. Fragments of *hRspo1* and *4* were RT-PCR amplified from mRNA of 293T cells and used as hybridization probes. Full-length mouse *Rspo1* and *2* were isolated from a mouse embryonic day 13.5 cDNA library. The sequence of *X. tropicalis Rspo3* was obtained from Sanger Institute database and a cDNA fragment was cloned by RT-PCR from *X. tropicalis* embryos. C-terminally Myc- or FLAG-tagged constructs and all deletion constructs were created by PCR. *Xenopus Rspo2*ΔC was cloned by deleting the last 37 amino acids. The *Rspo* cDNAs were cloned in pCS2+ and Bluescript vectors for use in gene expression and as probes, respectively.

Cell Culture, Recombinant Proteins, and Luciferase Reporter Assays

HEK293T, SHEP, and HeLa cell lines were maintained in DMEM, 10% FCS, and 10% CO_2. *Xenopus* Rspo2ΔC conditioned medium was produced by transient transfection in 293T cells. Mouse Wnt3A conditioned medium was produced from mouse L cells stably transfected with Wnt3A (ATCC#CRL-2647). Luciferase reporter assays in 293T cells were carried out in 96-well plates as described (Wu et al., 2000). Luciferase reporter assays in HeLa cells were carried out in 24-well plates in triplicates using Lipofectamine Plus transfection reagent (Invitrogen). Per well a total of 400 ng DNA were transfected, including 80 ng *7lef-fos-Luc* (Novak et al., 1998), 10 ng *pRL-TK*, 10 ng mouse *frizzled8*, 2 ng mouse *lef1*, and 300 ng pSuper plasmid DNAs. 3 days after transfection, either mouse Wnt3A conditioned medium or medium containing 30 mM LiCl was added to stimulate Wnt signaling. 24 hr later, luciferase activity was determined using the Dual luciferase system (Promega).

Embryos, Explants, In Situ Hybridization, and RNA Synthesis

In vitro fertilization, embryo culture, staging, microinjection, and culture of *Xenopus* embryo explants were carried out as described (Gawantka et al., 1995). Double- and single-labeling whole-mount in situ hybridization was carried out according to Bradley et al. (1996). A PCR fragment of *tropicalis Rspo3* cDNA was used for in situ hybridization on *Xenopus laevis* embryos. Whole-mount in situ

hybridization of mouse embryos was performed according to previously described procedures (Koop et al., 1996).

Morpholino Antisense Oligonucleotides and siRNA Constructs

The 5′ nucleotide sequence of an additional (pseudo-) allele for *Xenopus Rspo2* gene was obtained using 5′ RACE (GeneRacer kit, Invitrogen). Based on these sequences, an antisense morpholino oligonucleotide targeting both pseudoalleles around the ATG start codon was designed (Rspo2Mo): GCCGTCCAAATGCAGTTTCAAC. pSuper constructs producing siRNA against human *Rspo2,3* or a nonsense control were made according to Brummelkamp et al. (2002). The sequences are: human *Rspo2*, TCCCATTTGCAAGGGT TGT; human *Rspo3*, AGCTGACTGTGATACCTGT; nonsense control, ACTACCGTTGTTATAGGTG.

Immunohistochemistry, Western Blot, and Dot Blot Analysis

Immunohistochemistry to detect β-catenin in SHEP cells was carried out according to Scheiffele et al. (1998) using anti-β-catenin antibody (Transduction Laboratories). For detection of tagged Rspo proteins or loading controls on Western blot, anti-Myc (clone 9E10), anti-FLAG (M2, SIGMA) monoclonal antibodies, chick anti-GFP (Chemicon), and mouse anti-α-tubulin (SIGMA) antibodies were used. Chemiluminescence detection (SuperSignal solution, Pierce) was performed using anti-mouse IgG-HRP (Pierce). For *Rspo* expression analysis in tumor samples, the Cancer Profiling Array II (Clontech) was used.

RT-PCR

RT-PCR assays were carried out as described (Dosch et al., 1997; Glinka et al., 1997); additional primers were: *Xenopus Rspo2* (forward, GAATGCCCAGAAGGATTTGC; reverse, GGGATGGTGTCTT TTGCTGG); *Xenopus Rspo3* (forward, GAAGCAAATTGGAGTCTG TCG; reverse, GATTGTTCTCAAACCCTTCAGG); human *Rspo1* (forward, ACAGACACAAGACACACACGC; reverse, TGTCTTCTGGTGG CCTCAG); human *Rspo2* (forward, CCGAGCCCCAGATATGAAC; reverse, TGACCAACTTCACATCCTTCC); human *Rspo3* (forward, AGG GACTGAAACACGGGTC; reverse, TGTCTTCTGGTGGCCTCAG); human *Rspo4* (forward, AAGCTGGGACACAGCACAG; reverse, GAAG CCTTGGAGCCTTGTC).

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Accession Numbers

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