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Wnt/β-catenin signaling regulates expression of PRDC, an antagonist of the BMP-4 signaling pathway

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Abstract

To identify novel target genes regulated by Wnt/β-catenin signaling, microarray analysis was performed with L929 fibroblast cells. In this report, we show that the expression of *PRDC* (Protein Related to DAN and Cerberus), previously known as an antagonist of Nodal, BMP (bone morphogenetic protein), and Wnt signals, is specifically induced by Wnt/β-catenin signaling, and also show that the transcriptional activation is mediated in a Tcf/LEF-independent manner. The PRDC induced by Wnt/β-catenin does not inhibit Wnt signaling, but does inhibit BMP-4 signaling. The inhibition of BMP-4-induced reporter activity by the treatment of conditioned media from β-catenin(S37A)-expressing cells suggests that the PRDC induced by β-catenin is secreted outside of cells and antagonizes BMP-4 signaling. We propose that PRDC might serve as a mediator to antagonize BMP-4 signaling by Wnt.

Keywords: Wnt; β-Catenin; Target genes; BMP; Tcf/LEF

Wnt signaling has pivotal roles in development, and perturbations in that signaling are known to function in the pathogenesis of diverse human diseases such as cancers and osteoporosis [1,2]. Wnts are a family of secreted glycoproteins and can transduce signals via two functionally separated pathways. One is a canonical pathway that is mediated by a key effector molecule, β-catenin, and the other is a non-canonical pathway that is conducted via Ca^{2+} signaling or small G-protein Rho/Rac [3].

Canonical Wnt signaling is initiated upon the binding of Wnts to transmembrane receptor, Frizzled, and co-receptor, LRP5/6, which activates a cytoplasmic protein, Dishevelled (Dvl), and inactivates the β-catenin degradation complex, leading to the accumulation of β-catenin in cytoplasm. The accumulated β-catenin enters into the nucleus and interacts with the Tcf/LEF (T cell-specific transcription factor/lymphoid enhancer-binding factor). In the absence of Wnt signaling, Tcf/LEF acts as a repressor of Wnt target genes by forming a complex with Groucho [4]. Once in the nucleus, β-catenin converts the Tcf/LEF repressor complex into a transcriptional activator complex and activated downstream target genes such as *c-myc* and *cyclin D1* [5,6].

To date, a number of target genes have been identified in several cancers or loss-of-function analyses, especially in vertebrates. The expressions of *cyclin D1* and *c-myc* are highly induced in cancer cells; the expressions of these genes are consistent with uncontrolled proliferation of tumors [5,6]. Loss of a single Wnt gene during development causes various phenotypes, ranging from embryonic lethality to diverse abnormalities in organogenesis of the brain and kidney [7,8]. It is also known that these diverse biological phenomena result from mis-regulation in the expression of target genes [9,10]. An interesting feature of the target genes of Wnt signaling is that some of the differentially expressed target genes are involved in feedback regulation. For example, Dickkopf and Axin2, which are induced by Wnt/β-catenin signaling, inhibit signaling by blocking the interaction between Wnt and LRP and by down-regulating the level of cytoplasmic β-catenin, respectively [11–13].

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The current list of Wnt-responsive genes has been found primarily in epithelial cells, with an aim at isolating specifically regulated genes during cancer formation. We thought that we could identify additional downstream target genes of Wnt signaling that are not involved in tumorigenesis, but may have other roles upon activation of Wnt/β-catenin signaling in fibroblast cells. With that aim, we searched for genes regulated by inducible expression of Dvl and β-catenin in L929 fibroblast cells by using microarray analysis. As a result, we were able to find up-regulated genes, and in the present study, we focused on a novel target gene *PRDC* (Protein Related to DAN and Cerberus), which belongs to the same gene family as Cerberus, previously known as an antagonist of Nodal, BMP, and Wnt signaling in the *Xenopus* embryo [14,15].

Materials and methods

Generation of stable cell lines and microarray analysis. Mouse L929 stable cell lines expressing CMV-rtTA/pBI-EGFP, CMV-rtTA/pBI-EGFP-Dvl, or CMV-rtTA/pBI-EGFP-β-catenin(S37A) were established (Supplementary Fig. 1A). Cells were transfected with both CMV-rtTA-neo vector [16], which includes a neomycine resistance gene as a selection marker, and a fourfold dose of pBI vector expressing EGFP, EGFP/Dvl, or EGFP/β-catenin(S37A), using Superfect (Qiagen). Transfected cells were selected under media containing 1.0 mg/ml G418 (Life Technologies, Gibco, BRL) and then maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). The stable cell lines were treated with 1 μg/ml doxycycline for 24 h, and total RNA was isolated using TRIZOL (Invitrogen) according to the protocol of the manufacturer. RNA samples that produced two distinct bands, 18S and 28S rRNAs, on 1% agarose gel were used for microarray analysis. Microarray analysis was carried out using a mouse 11K DNA chip produced by Macrogen Inc. (Seoul, Republic of Korea). Two-independent sets of experiments were performed.

Immunofluorescent analysis. L929 stable cell lines expressing EGFP, Dvl, or β-catenin by treatment with doxycycline for 24 h were fixed and incubated with anti-β-catenin monoclonal antibody as previously described [16]. The nuclei stained with 4,6-diamidino-2-phenylindole (DAPI) were incubated with secondary antibody and examined via fluorescence microscopy (LEICA).

Reverse transcriptase-PCR. Total RNAs isolated from the stable cell lines were reverse-transcribed using random primers (5′-NNNNNN-3′) and ImProme II reverse transcriptase (Promega), and amplification was carried out at 94 °C for 2 min, followed by 30 cycles at 94 °C for 1 min, at appropriated annealing temperatures for each primer for 1 min, and at 72 °C for 50 s. The following primers were used for PCR: for *mPRDC*, 5′-CTCTGTCATCGTAGAGCTCGAAT-3′ and 5′-AAGCTTCACTT GTGGTCCTCATT-3′; for *hPRDC*, 5′-CAACAACTCGGAGAG ATGG-3′ and 5′-TTCTGGATTTTCTTGAGTCG-3′; for *mGastrin*, 5′-T GCTGGTCTTAGTGCTGG-3′ and 5′-AGTCCATCCATCCGTAGG-3′; for *mNecdin*, 5′-TGTGGTACGTGTTGGTGAAG-3′ and 5′-CACC CTGTCTAGCTCCTCTG-3′; for *Axin*, 5′-CAGGGTTTCCCCTTGG ACC-3′ and 5′-GGTCAAACATGGCAGGAT-3′; for *Axin2*, 5′-ACTT TCCTGGAGAGGGAG-3′ and 5′-GCTGGTGCAAAGACATAGCC-3′; for *β-actin*, 5′-AGGCCAACCGCGAAGATGACC-3′ and 5′-GA AGTCCAGGGCGACGTAGCAC-3′; for *GAPDH*: 5′-AAGAAGGT GGTGAAGCAG-3′ and 5′-TCATACCAGGAAATGAGC-3′.

Construction of plasmids. To isolate putative promoter for *PRDC*, PCR was performed with the following primers using total genomic DNA isolated from L929 cells: 5′-GTCGACGTTGGACAACTGAGTCCCTG-3′ and 5′-CCATGGCTGCAATGACGAGATGGAAG-3′. An amplified fragment of approximately 1.8 kb, a region that contains three conserved TCF-binding sequences (WWCAAAG: W = A or T), was cloned into pGL3-Basic vector (Promega). The promoter sequence was confirmed by sequencing, and this construct was named PRDC-p-Luc. Deletions and introduction of point mutation in the promoter were performed using basic molecular biology techniques. To obtain mPRDC cDNA, RT-PCR was performed with mouse postnatal total RNA with pfu polymerase (Promega) using the following primers: 5′-AATTGAATTCGATGT TCTGGAAGCTCTCG-3′ and 5′-AATTTCTAGATCACTGCTTGTC GGAGTC-3′. The amplified DNA was cloned and confirmed through sequencing analysis. Untagged PRDC was cloned into pCDNA3 and N-, C-terminal myc epitopes or HA-tagged PRDC were cloned into pCS2 vector.

Luciferase reporter assay. 293T cells were seeded in 24-well plates and transiently co-transfected with the indicated plasmids. Luciferase activity was measured by using a Dual Luciferase assay kit (Promega) as previously described [13]. pSuperTOP-FLASH and SBE-Luc (kindly provided by Dr. Randall Moon and Dr. Carl-Henrik Heldin, respectively) were used to measure Wnt and BMP signaling, respectively.

Results and discussion

PRDC levels are up-regulated by both Dvl and β-catenin(S37A)

While the mechanism of action of Dvl is not clearly known, Dvl functions upstream of β-catenin and GSK3β, resulting in the accumulation of β-catenin in cytoplasm as a positive regulator of Wnt signaling. A mutated form of β-catenin with alanine instead of serine at the 37th residue (β-catenin(S37A)) is resistant to proteasomal degradation and behaves as a potent activator of canonical Wnt signaling [17]. These two Wnt signaling component genes were expressed using a Tet-On inducible system to activate canonical Wnt signaling [18]. In order to identify new downstream target genes of the canonical Wnt signaling pathway, microarray analysis was performed by comparing the gene expression profile of mouse fibroblast L929 cells expressing CMV-rtTA/pBI-EGFP, CMV-rtTA/pBI-EGFP-Dvl, or CMV-rtTA/pBI-EGFP-β-catenin(S37A).

Localization of β-catenin to the nuclei upon inducible expression of Dvl and β-catenin(S37A) and up-regulation of pSuperTOP-FLASH reporter activity, which can measure the activity of canonical Wnt signaling, suggest that Dvl and β-catenin(S37A) work as activators of Wnt signaling in L929 fibroblast cells (Supplementary Fig. 1B and data not shown). We found a number of genes that were up-regulated by both Dvl and β-catenin(S37A), including previously identified target genes regulated by Wnt signaling such as *Axin2*, *CD44* antigen, *Follistatin*, *Gastrin*, *Matrix metalloproteinase 7*, and *Drosophila twist* homolog (data not shown).

In most cases, the expression of target genes of Wnt/β-catenin signaling is mediated via binding of Tcf/LEF transcription factor to the conserved consensus TCF binding sites (WWCAAAG: W = A or T) in their promoter region [6,13]. To select target genes for further study, we assessed the presence of conserved TCF binding sites in both mouse and human sequences at about 2 kb upstream of their putative transcription start of candidate genes that were identified by microarray analyses. After selection of several

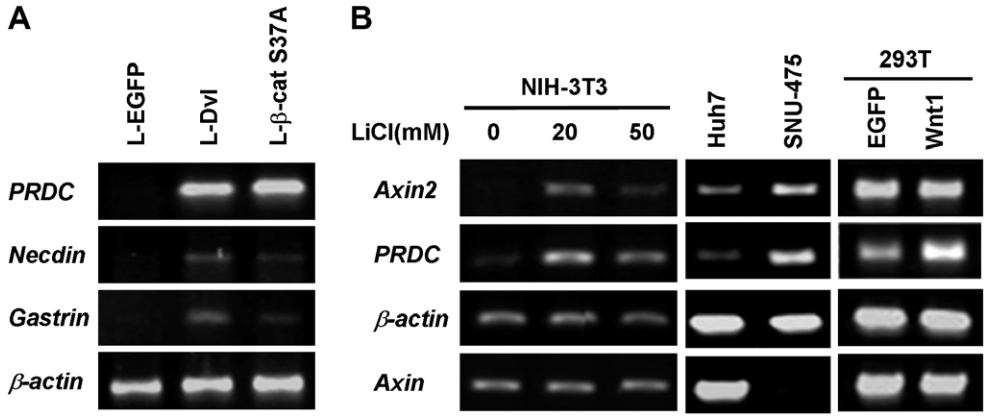

Fig. 1. Ectopic expression of Dvl/β-catenin induces the expression of PRDC. (A) RT-PCR analysis shows that the expressions of *PRDC*, *Necdin*, and *Gastrin* were induced by inducible expression of Dvl or β-catenin. (B) RT-PCR analysis shows that the level of PRDC mRNA is induced in diverse cell lines by activation of Wnt signal.

putative target genes, RT-PCR with specific primers of the candidate genes was performed. As shown in Fig. 1A, *PRDC* and *Necdin* are specifically increased by inducible expression of both Dvl and β-catenin(S37A) in L929 cells and real-time PCR analysis showed consistent result (data not shown). In this study, we focused on *PRDC*, which is suggested to block BMP, and Wnt signaling [14,19,20]. To further confirm that the expression of *PRDC* is induced by Wnt signaling, NIH-3T3 cells were treated with Li^+, a specific inhibitor of GSK3 β that behaves as an activator of Wnt/β-catenin signaling, and RT-PCR was performed. The levels of *PRDC* and *Axin2*, a well-known target gene, were increased, while Axin and β-actin were not changed upon LiCl treatment (Fig. 1B, left panel). Since the SNU475 hepatocellular carcinoma (HCC) cell line does not express Axin due to mutations, Wnt/β-catenin signaling is up-regulated in that cell line, while another HCC cell line, Huh7, has normal signaling [21]. Higher levels of both *Axin2* and *PRDC* were present in SNU475 cells compared to Huh7 cells (Fig. 1B, middle panel). Transient transfection of Wnt1 into HEK 293T cells also led to an increase of *PRDC*, while the levels of *Axin2*, *Axin*, and *β-actin* were not changed (Fig. 1B, right panel). Overall, the above data strongly suggest that *PRDC* is a novel target gene induced by Wnt/β-catenin signaling.

Induction of PRDC is mediated in a Tcf/LEF-independent manner

Promoter analysis was performed to examine which transcription factors are involved in the regulation of *PRDC*. Induction of luciferase activity by co-transfection of β-catenin(S37A) into HEK 293T cells suggests that the *PRDC* promoter responds to Wnt/β-catenin signaling (Fig. 2, right panel). To examine whether the activation of *PRDC* promoter is mediated by Tcf, wild-type Tcf-4E

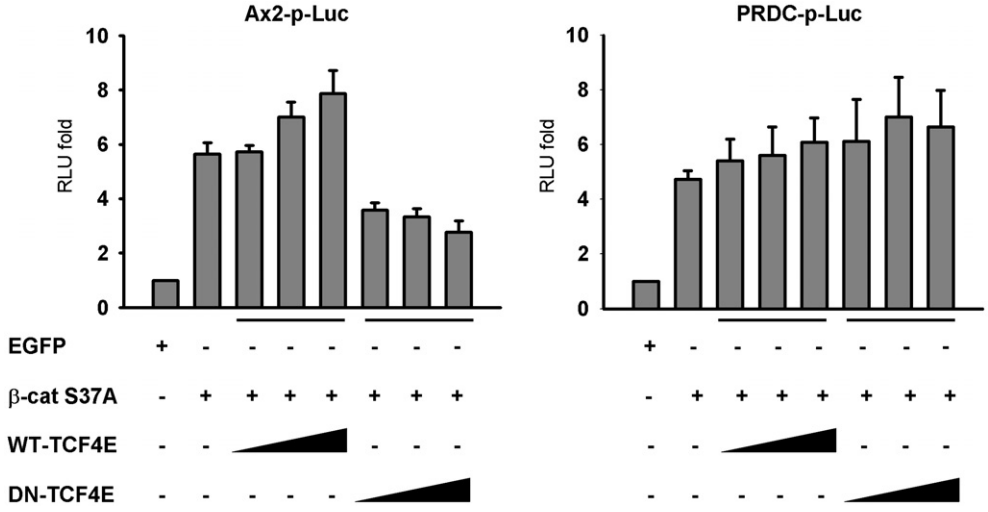

Fig. 2. Regulation of PRDC promoter is mediated in a Tcf/LEF-independent manner. The activity of *PRDC* and *Axin2* promoter was induced by the co-transfection of β-catenin(S37A) in HEK 293T cells. Dominant-negative (DN) Tcf4E abolishes β-catenin-mediated induction of luciferase activity driven by *Axin2* promoter (left panel), while it does not block luciferase activity from PRDC promoter (right panel). The error bars indicate the standard deviations on the basis of triplicate luciferase assays.

or the dominant negative form of Tcf-4E (DN-Tcf4E), which cannot activate transcription of target genes due to deletion of the β-catenin interacting domain, was co-transfected with β-catenin(S37A) [13]. Unexpectedly, DN-Tcf-4E did not inhibit the induced luciferase activity (Fig. 2, right panel). The Axin2 promoter-driven luciferase activity induced by the transfection of β-catenin(S37A) could be blocked by co-transfection of DN-Tcf-4E, as reported (Fig. 2, left panel; [13]). Luciferase activity with deletion constructs, which lack the regions containing conserved Tcf/LEF sites, showed only slight reduction (Supplementary Fig. 2), and this result is consistent with data using DN-Tcf-4E as shown in Fig. 2. Overall, these data suggest Tcf/LEF does not play a major role in the activation of the *PRDC* promoter by β-catenin.

It has been reported that WISP-1 is a Wnt1- and β-catenin-responsive oncogene, and the CREB site in that promoter is important for the transcriptional activation [22]. To test the possibility that CREB has some involvement in the transcriptional activation of *PRDC*, a conserved CREB binding site (CGTCA) found in Exon 1 of *PRDC* was mutated to AGATC (Supplementary Fig. 2). However, this mutation did not have any effect on the luciferase activity, which suggested that CREB may not be involved in the β-catenin-mediated activation of *PRDC* promoter.

mPRDC inhibits BMP4 signaling but not Wnt signaling

Sequence analysis has revealed that Cerberus and Dan are members of a family of secreted proteins, referred to as the CAN family, of which there are at least six family members: Cerberus, Cerl, Drm, PRDC, Dan, and Dte in the mouse [19]. Pearce et al. [19] suggested that the CAN family members play important roles in patterning the early mouse embryo by inhibiting the actions of specific TGFβ superfamily members, especially signaling of BMP4. Additionally, it has been reported that both *Xenopus* Cerberus (xCerberus) and *Coco*, belonging to the *Dan* and *Cerberus* gene family, respectively, antagonize BMP, TGFβ, and Wnt signals [15,23]. The function of PRDC *in vivo* not yet been clarified, but the expression pattern and its primary structure suggested that the gene product functions as a signaling molecule in various aspects of embryonic development, especially in neural development [14].

Since we identified *PRDC* as a novel target gene of Wnt signaling and xCerberus is known to function as an antagonist of Wnt signaling [15], we hypothesized that PRDC might serve as a negative feedback regulator of Wnt signaling. Full-length cDNA of mouse PRDC (mPRDC) was cloned by RT-PCR using a postnatal mouse RNA, which was then inserted into the pCS2MT vector to attach myc epitope tag at the N-terminus (myc-mPRDC). To avoid any potential hindrance of interaction with receptors due to the epitope tag, untagged mPRDC (mPRDC), the myc epitope tag at the C-terminus (mPRDC-myc), and the HA epitope tag at the C-terminus (mPRDC-HA) of mPRDC were cloned (Supplementary Fig. 3A). Western blot analysis showed a band of expected size with some higher molecular weight bands resulting from different degrees of *N*-glycosylation, as shown previously (Supplementary Fig. 3B, [19]).

To test whether PRDC functions as a Wnt antagonist, the effect of mPRDC on the Wnt-induced transcriptional activity was measured by using pSuperTOP-Flash plasmid (Fig. 3A). As a positive control for inhibition of Wnt signaling, Dickkopf, which is a secreted protein and is known as an antagonist of the Wnt signal by blocking interaction between Wnt and LRP, was used [11]. Co-transfection of

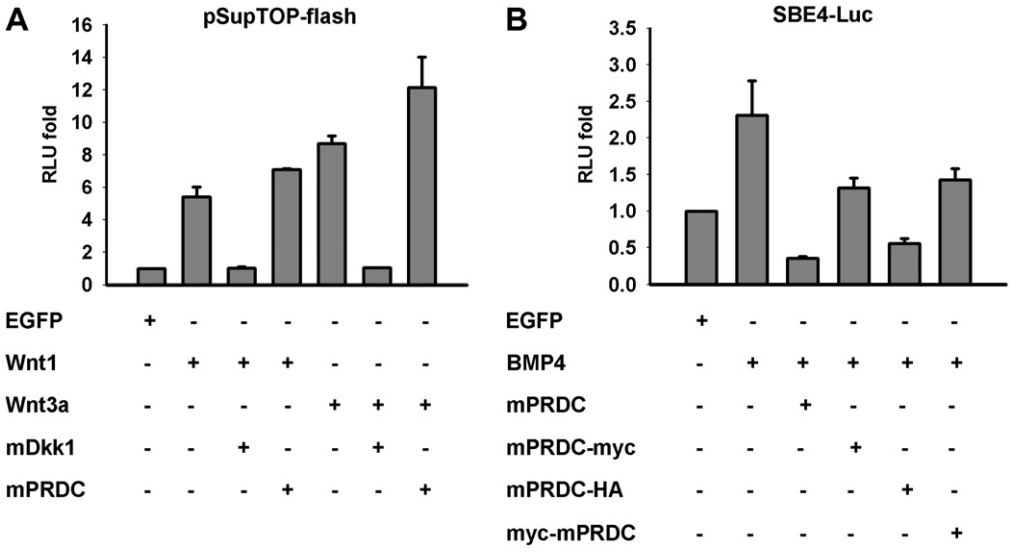

Fig. 3. mPRDC inhibits BMP4 signaling but not Wnt signaling. (A) To measure effects on the Wnt signaling-induced pSuperTOP-Flash reporter activity, the constructs depicted in the figure were transfected into HEK 293T cells. While mDKK1 inhibits Wnt-induced pSuperTOP-Flash reporter activity, mPRDC does not (left panel). (B) BMP4-mediated SBE4-Luc activity is blocked by the co-transfection of mPRDC or other epitope-tagged PRDCs. Error bars indicate standard deviations.

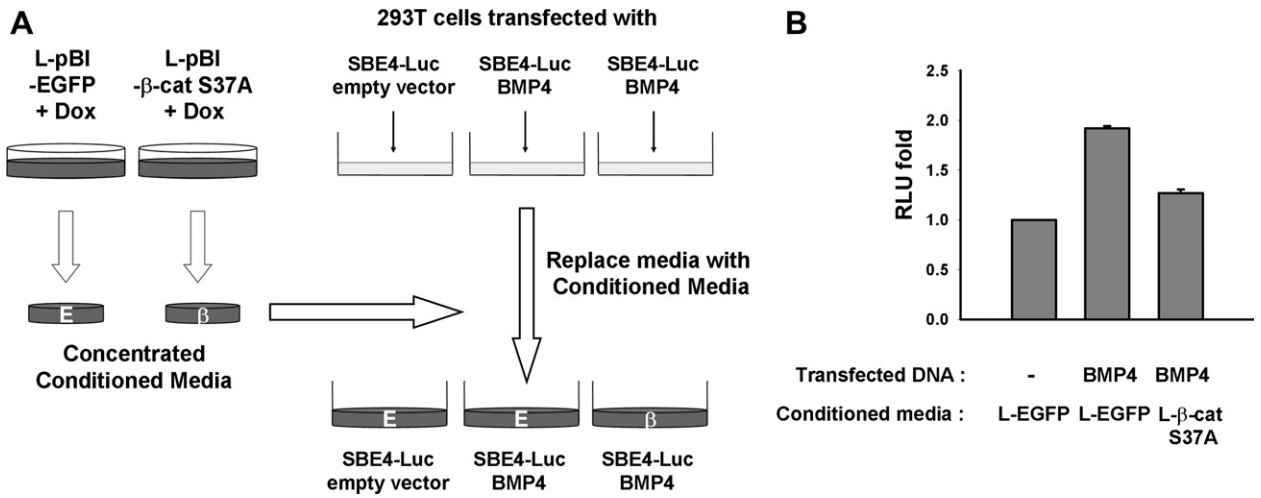

Fig. 4. mPRDC induced by Wnt/β-catenin signaling is secreted outside of cells and antagonizes BMP4 signaling. (A) Diagram for experimental procedures. HEK 293T cells transfected with SBE4-Luc or SBE4-Luc/BMP-4 were incubated with conditioned media from either EGFP- or β-catenin(S37A)-expressing L929 cells. (B) Reporter analysis. The cells incubated with conditioned media from β-catenin(S37A)-expressing cells showed inhibition of luciferase activity induced by BMP-4. The data shown are representative of three-independent experiments and error bars indicate standard deviations.

mouse Dickkopf (mDKK1) clearly blocked either Wnt1- or Wnt3a-mediated activation of pSuperTOP-Flash as expected, but mPRDC did not (Fig. 3A). These data suggest that PRDC does not behave as an antagonist of Wnt signaling.

Recently, it was shown that PRDC is a potent antagonist for BMP4 signaling [20]. Consistent with the report, mPRDC and other forms of epitope-tagged mPRDC inhibits BMP4-mediated SBE4-Luc activity (Fig. 3B). These data suggest that mPRDC inhibits BMP4 signaling, but not Wnt signaling.

mPRDC induced by Wnt/β-catenin signaling is secreted outside of cells and antagonizes BMP4 signaling

Since we could not detect endogenous mPRDC using commercially available antibodies (data not shown), we indirectly tested the hypothesis that the mPRDC, which is induced by Wnt/β-catenin signaling, is secreted outside of cells and inhibits BMP4 signaling (Fig. 4). SBE4-Luc or SBE4-Luc with BMP4-containing plasmids were transfected into 293T cells, and the transfected cells were incubated with conditioned media from Dox-treated mouse fibroblast L929 cells expressing CMV-rtTA/pBI-EGFP or CMV-rtTA/pBI-EGFP-β-catenin(S37A) (Fig. 4A). As shown in the reporter assay in Fig. 4B, conditioned media from CMV-rtTA/pBI-EGFP-β-catenin(S37A) reproducibly blocked BMP4-induced luciferase activity. This result suggests that the Wnt/β-catenin-induced PRDC is secreted to the outside of cells and may antagonize BMP4 signaling. However, we could not eliminate the possibility that other unknown factor(s) secreted from CMV-rtTA/pBI-EGFP-β-catenin(S37A) blocks BMP4-mediated SEB4-Luc activity. To show that PRDC is a unique and specific factor that can be secreted to block BMP-4 signaling, we could have utilized siRNA for mPRDC in the assay used in Fig. 4. However, due to a lack of appropriate antibodies to detect endogenous levels of PRDC, this experiment was not performed.

In summary, in this report, we showed that *PRDC* is a novel target gene of Wnt/β-catenin signaling, and the transcriptional activation is mediated in a Tcf/LEF-independent manner. Analyses with reporter constructs suggest that PRDC does not inhibit Wnt signaling, but does inhibit BMP-4 signaling. The inhibition of BMP-4-induced reporter activity with conditioned media from β-catenin(S37A)-expressing cells suggests that the PRDC induced by β-catenin signaling is secreted outside of cells and antagonizes BMP-4 signaling. It is known that cross-regulation between Wnt and BMP is critical in determination of dorso-ventral patterning of the *Xenopus* embryo [24,25]. We propose that PRDC might serve as a mediator to antagonize BMP-4 signaling by Wnt.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.12.205.

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