Repulsive Guidance Molecule RGMa Alters Utilization of Bone Morphogenetic Protein (BMP) Type II Receptors by BMP2 and BMP4*

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Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β superfamily of multifunctional ligands that transduce their signals through type I and II serine/threonine kinase receptors and intracellular Smad proteins. Recently, we identified the glycosylphosphatidylinositol-anchored repulsive guidance molecules RGMa, DRAGON (RGMb), and hemojuvelin (RGMc) as coreceptors for BMP signaling (Babbit, J. L., Huang, F. W., Wrighting, D. W., Xia, Y., Sidis, Y., Samad, T. A., Campagna, J. A., Chung, R., Schneyer, A., Woolf, C. J., Andrews, N. C., and Lin, H. Y. (2006) Nat. Genet. 38, 531–539; Babbit, J. L., Zhang, Y., Samad, T. A., Xia, Y., Tang, J., Schneyer, A., Woolf, C. J., and Lin, H. Y. (2005) J. Biol. Chem. 280, 29820–29827; Samad, T. A., Rebbapragada, A., Bell, E., Zhang, Y., Sidis, Y., Jeong, S. J., Campagna, J. A., Perusini, S., Fabrizio, D. A., Schneyer, A. L., Lin, H. Y., Brivanlou, A. H., Attisano, L., and Woolf, C. J. (2005) J. Biol. Chem. 280, 14122–14129). However, the mechanism by which RGM family members enhance BMP signaling remains unknown. Here, we report that RGMa binds to radiolabeled BMP2 and BMP4 with $K_d$ values of 2.4 ± 0.2 and 1.4 ± 0.1 nm, respectively. In KGN human ovarian granulosa cells and mouse pulmonary artery smooth muscle cells, BMP2 and BMP4 signaling required BMP receptor type II (BMPRII), but not activin receptor type IIA (ActRIIA) or ActRIIB, based on changes in BMP signaling by small interfering RNA inhibition of receptor expression. In contrast, cells transfected with RGMa utilized both BMPRII and ActRIIA for BMP2 or BMP4 signaling. Furthermore, in BMPRII-null pulmonary artery smooth muscle cells, BMP2 and BMP4 signaling was reduced by inhibition of endogenous RGMa expression, and RGMa-mediated BMP signaling required ActRIIA expression. These findings suggest that RGMa facilitates the use of ActRIIA by endogenous BMP2 and BMP4 ligands that otherwise prefer signaling via BMPRII and that increased utilization of ActRIIA leads to generation of an enhanced BMP signal.

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Bone morphogenetic proteins (BMPs) are a large subfamily of the transforming growth factor-β (TGF-β) superfamily of multifunctional ligands that regulate cell proliferation and differentiation, chemotaxis, and apoptosis. Members of the TGF-β superfamily, including BMPs, transduce their signals through binding to type I and II serine/threonine kinase receptors. Upon ligand binding, constitutively active type II receptors phosphorylate type I receptors, which then phosphorylate receptor-activated Smad proteins. Activated receptor-activated Smad proteins complex with the common partner Smad4 and subsequently translocate to the nucleus to regulate gene transcription (reviewed in Refs. 1–3). There are only five type II receptors: BMP receptor type II (BMPRII); activin receptor type II (ActRIIA and ActRIIB); TGF-β receptor type II; anti-müllerian hormone receptor type II; and seven type I receptors designated as activin receptor-like kinases (ALKs), including ALK1–ALK7. For the BMP ligands, three type II receptors (BMPRII, ActRIIA, and ActRIIB) and three type I receptors (ALK3, ALK6, and ALK2) have been identified, and these receptors transduce BMP signals through receptor-activated Smad1, Smad5, and Smad8.

Signaling by TGF-β superfamily members, including BMPs, is modulated by extracellular secreted soluble binding proteins such as noggin, chordin, gremlin, and follistatin (3–6). Noggin is the best characterized BMP antagonist. It binds to BMP2 and BMP4 with an apparent $K_d$ of ~20 pm and also binds to BMP5–BMP7, GDF5, and GDF6, but with lesser affinity (4–6). Recently, kielin/chordin-like protein has been described as a paracrine enhancer of BMP7 signaling (7). Another key regulatory mechanism for many TGF-β superfamily members is mediated through membrane-anchored proteins that assist with ligand binding to receptor or that alter receptor specificity. For example, betaglycan (TGF-β receptor type III) mediates TGF-β2 binding to TGF-β receptor type II (8) and also increases the affinity of inhibin for the activin and BMP type II receptors (9–11). Cripto, an epidermal growth factor-CFC (Cripto/ERL-1/Cryptic) motif-containing glycosylphosphatidylinositol-anchored membrane protein, mediates the binding of nodal and GDF1 to activin receptors (12).

The abbreviations used are: BMPs, bone morphogenetic proteins; TGF-β, transforming growth factor-β; BMPRII, bone morphogenetic protein receptor type II; ActRII, activin receptor type II; ALKs, activin receptor-like kinases; RGM, repulsive guidance molecule; siRNA, small interfering RNA; PASMCs, pulmonary artery smooth muscle cells; RT, reverse transcription.
We have shown recently that the repulsive guidance molecule (RGM) family members RGMa, DRAGON (RGMb), and hemojuvelin (RGMc) are coreceptors that enhance BMP signaling (13–15). RGM family members share 50–60% protein sequence homology and have similar structural features, including a signal sequence, conserved proteolytic cleavage site, partial von Willebrand factor type D domain, and glycosylphosphatidylinositol anchor site (16–19). RGMa and DRAGON are expressed in a complementary manner in the central nervous system, where DRAGON is involved in neuronal cell adhesion through homophilic interactions, whereas RGMa binds to the receptor neogenin and mediates retinotectal projections as well as neural tube closure (16, 17, 20). RGMa and DRAGON are also expressed in many other organs, including the kidney, testis, and ovary (14, 19, 21). Our recent work has shown that DRAGON is localized in gonadal germ cells as well as in epithelial cells of the reproductive tract, including the epididymis and uterus, and that DRAGON is localized in lipid rafts as predicted from the presence of a glycosylphosphatidylinositol anchor site (21). Hemojuvelin is expressed at high levels in the skeletal muscle cells of the reproductive tract, including the epididymis and ovary (14, 19, 21). Our recent work has shown that DRAGON is localized in gonadal germ cells as well as in epithelial cells of the reproductive tract, including the epididymis and uterus, and that DRAGON is localized in lipid rafts as predicted from the presence of a glycosylphosphatidylinositol anchor site (21). Hemojuvelin is expressed at high levels in the skeletal muscle, heart, and liver (22). It is mutated in juvenile hemochromatosis, a disorder of iron overload (23). Interestingly, hemojuvelin mutants associated with hemochromatosis have impaired BMP signaling ability. BMP-induced expression of hepcidin, a key regulator of systemic iron homeostasis, is enhanced by hemojuvelin and blunted in hemojuvelin-null hepatocytes (13).

The three RGM proteins bind directly to radiolabeled BMP2 and BMP4, but not to BMP7, activin, TGF-β1, TGF-β2, or TGF-β3. RGM proteins enhance BMP signaling even in the absence of exogenous BMP ligands. Although we have obtained data suggesting that RGM proteins appear to mediate BMP signaling through the classical BMP pathway (13–15), the molecular mechanisms by which RGM family members enhance BMP signaling remain largely unknown. In this study, we investigated the mechanism of action of RGMa using specific siRNA-mediated inhibition of endogenous BMP ligands and receptors. We found that RGMa has \( K_v \) values of 2.4 ± 0.2 nM for BMP2 and 1.4 ± 0.1 nM for BMP4 and that RGMa enhances BMP signaling by allowing BMP2 and BMP4 to increase their utilization of ActRIIA.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—KGN human ovarian granulosa cells were cultured in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 2 mM L-glutamine, and antibiotics. Pulmonary artery smooth muscle cells (PASMCs) were isolated from mice that were homozygous for a conditional \( BmpRII \) allele, and the \( BmpRII \) gene was disrupted as described previously (24). PASMCs were cultured in RPMI 1640 medium containing 10% fetal bovine serum, L-glutamine, and antibiotics. All transfections were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

**Reverse Transcription (RT)-PCR**—Total RNA was isolated from KGN cells and PASMCs using an RNeasy mini kit (Qiagen Inc.) including DNase by column digestion with an RNase-free DNase set (Qiagen Inc.) according to the manufacturer’s instructions. First-strand cDNA synthesis was performed using an iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer’s instructions. Transcripts of \( BMP2 \), \( BMP4 \), \( BMP7 \), \( BMPRII \), \( BmpRII \), and \( Rgma \) were amplified using the primers summarized in Table 1. Transcripts of \( ActRIIA \) and \( ActRIIB \) were amplified using the primers previously described (25).

**siRNA Targeting**—siRNA duplexes in annealed and purified form were obtained from Ambion, Inc. (Austin, TX). Sense sequences of duplexes used for gene targeting are summarized in Table 2 for \( BMP2 \), \( BMP4 \), \( BMP7 \), \( BMPRII \), \( BmpRII \), and \( Rgma \). Mouse \( BmpRII \), \( ActRIIA \), \( ActRIIB \), \( Rgma \), and \( Rgmb \) Mouse \( BmpRII \), \( ActRIIA \), \( ActRIIB \) siRNA sequences were described previously (24). siRNA duplexes were added at the concentrations indicated along with plasmids to subconfluent KGN cells or PASMCs in a mixture of Lipofectamine 2000 and Opti-MEM I (Invitrogen). Negative control siRNA (scrambled sequences) was used to balance siRNA where necessary. Assays to measure target mRNA levels, luciferase activity, or BMP-mediated Smad1/5/8 phosphorylation were performed ~48 h after transfection.

**Measurement of Gene Expression**—Real-time quantification of mRNA transcripts was performed using an AB 7300 real-time system (Applied Biosystems). First-strand cDNA was amplified with the primers shown in Table 3 and detected using a SYBR-green dye-based detection method.
TABLE 3

Sequences, expected product sizes, and GenBank™ accession numbers for the primers used in real-time PCR

| Gene     | Forward primer (5’ to 3’)                                      | Reverse primer (5’ to 3’)                                      | Size (bp) | Accession no.     |
|----------|-----------------------------------------------------------------|----------------------------------------------------------------|-----------|------------------|
| hBMP2    | CGCAGCTCTACGCCATAAGTGAAGAA                                      | CCTGAGAAGCTGCGAAGTTGATA                                         | 116       | NM_001200        |
| hBMP4    | AGGCTAGTCCACCTGGATAGGAC                                          | TGGAGGAGGGGAGGAAAGAGATG                                         | 175       | NM_001202        |
| hBMP6    | TGGAAGAAGGCTGCGAAGTTGATA                                         | AGCTGAGAAGCTGCGAAGTTGATA                                         | 214       | NM_001718        |
| hBMPRII  | TGGAGAAGGCTGCGAAGTTGATA                                         | GCCTGCTGCTGCGAAGTTGATA                                          | 176       | NM_001204        |
| hActRIIA | CGATACCTACGGAGGCACTGAGGS                                         | AGCTGAGAAGCTGCGAAGTTGATA                                         | 211       | NM_001616        |
| hActRIIB | GCCTGCTGCTGCGAAGTTGATA                                          | AGCTGAGAAGCTGCGAAGTTGATA                                         | 206       | NM_001106        |
| mActRIIA | CGAGAGGCTGCGAAGTTGATA                                           | AGCTGAGAAGCTGCGAAGTTGATA                                         | 176       | NM_007396        |
| mActRIIB | CAAGAGGCTGCGAAGTTGATA                                           | AGCTGAGAAGCTGCGAAGTTGATA                                         | 160       | NM_007397        |
| mrgma    | AGGAAGAGAAGCTGCGAAGTTGATA                                       | AGCTGAGAAGCTGCGAAGTTGATA                                         | 166       | NM_177740        |
| mrgmb, hBMPb | AGCGAGCTACGGAGGCACTGAGGS                                       | AGCTGAGAAGCTGCGAAGTTGATA                                         | 107a      | NM_178615, NM_001012761 |
| hRPL19   | AGCTGAGAAGCTGCGAAGTTGATA                                         | AGCTGAGAAGCTGCGAAGTTGATA                                         | 159       | NM_000981        |
| mRpl19   | AGCAGTCACCTGGGGATACGGGAGAG                                       | TGGAGAAGGCTGCGAAGTTGATA                                          | 164       | BC089549         |

SYBR® Green PCR Master Mix (Applied Biosystems) according to the manufacturer’s instructions. In parallel, RPL19 (ribosomal protein-like 19) or Rpl19 transcripts were amplified and detected in a similar manner to serve as an internal control (26). Standard curves were generated from accurately determined dilutions of plasmid cDNAs or purified PCR fragments as templates. Results are expressed as a ratio of the gene of interest to RPL19 or Rpl19.

Luciferase Assay—KGN cells or PASMCs were transiently transfected with a BMP-responsive firefly luciferase reporter (BRE-Luc) (27) or an activin-responsive firefly luciferase reporter [(CAGA)₁₂ MPL-Luc] (28) construct in combination with pTK-Renilla (Promega Corp., Madison, WI) at a ratio of 10:1 to control for transfection efficiency with or without cotransfection with siRNAs in the absence or presence of RGMa cDNA. Approximately 24 h after transfection, the medium was replaced with serum-free medium supplemented with 0.1% bovine serum albumin with or without BMP or activin ligands (R&D Systems, Minneapolis, MN). After treatment for 16 h, the cells were lysed, and luciferase activity was determined with the Dual-Reporter assay kits (Promega Corp.). Experiments were performed in triplicate wells. Relative light units were calculated as the ratios of firefly (reporter) and Renilla (transfection control) luciferase values.

Measurement of Smad1/5/8 Phosphorylation—KGN cells plated to 80% confluence were transiently transfected with BMPRII, ActRIIA, ActRIIB, or control siRNA; with RGMa cDNA; or with empty vector. 24 h after transfection, cells were replaced with serum-free medium supplemented with 0.1% bovine serum albumin. After starvation for 16 h, cells without RGMa cDNA transfection were incubated with 10 ng/ml BMP2 for 1 h at 37 °C. Cells were lysed in 20 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, and 0.1% Nonidet P-40 containing protease inhibitor mixture (Pierce) and phosphatase inhibitor mixture (Pierce) for 30 min on ice. After centrifugation for 10 min at 4 °C, the supernatant was assayed for protein concentration by colorimetric assay (BCA kit, Pierce). 30 μg of protein was separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were probed with rabbit anti-phospho-Smad1/5/8 polyclonal antibody (1:1000 dilution; Cell Signaling Technology, Beverly, MA). Membranes were stripped in 0.2 M glycine (pH 2.5) and 0.5% Tween 20 for 10 min and reprobed with rabbit anti-Smad1 polyclonal antibody (1:1000 dilution; Upstate Biotechnology, Lake Placid, NY), followed by horseradish peroxidase-conjugated secondary antibody. Antibody binding was detected with chemiluminescence reagent (PerkinElmer Life Sciences) and exposed to X-Omat film (Eastman Kodak Co., Rochester, NY). Densitometry was performed using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Ligand Iodination—Carrier-free human BMP2 and BMP4 were purchased from R&D Systems. Ligand (2 μg/reaction) was iodinated with 125I by the modified chloramine-T method as described previously (29).

Binding Assays—The RGMa-Fc fusion protein was produced by fusing the soluble extracellular domain of RGMa to the Fc portion of human IgG and was purified from the medium of stably transfected cells via one-step protein A affinity chromatography using HiTrap rProtein A FF columns (Amersham Biosciences) as described previously (14). Purified RGMa-Fc (20 ng) mixed in 1× Tris-buffered saline/casein blocking buffer (BioFX Laboratories, Owings Mills, MD) was incubated overnight at 4 °C with 125I-BMP2 or 125I-BMP4 and increasing doses of unlabeled BMP2 or BMP4 (R&D Systems). The reaction mixture was then incubated for 1.5 h at 4 °C on protein A-coated plates (Pierce); the plates were washed with wash solution (KPL, Inc., Gaithersburg, MD); and individual wells were counted with a standard γ-counter. For mixing studies, buffer alone; purified RGMa-Fc alone (1 ng); ActRIIA-Fc alone (2 ng); a combination of ActRIIA-Fc and ALK3-Fc (R&D Systems); or a combination of RGMa-Fc, ActRIIA-Fc, and ALK3-Fc was incubated with 125I-BMP2, followed by incubation on protein A-coated plates and determination of radioactivity as described above.

Data Analysis—Results from luciferase assay experiments are expressed as the means ± S.E. of at least three replicates. For homologous binding assays, dissociation constants were determined by Scatchard analysis using Prism (GraphPad Software, San Diego, CA). The Western blots shown in Fig. 6 are representative experiments, and the densitometry data represent the means ± S.E. of two replicates. Differences were assessed by Student’s t test with p < 0.05 used to indicate significance.

RESULTS

BMP2 and BMP4 Are Both Endogenous Ligands for RGMa in KGN Cells—We demonstrated previously that RGMa-Fc binds BMP2 and BMP4, but not BMP7 and TGF-β1, and that RGMa-
mediated BMP signaling can be inhibited by an antibody against both BMP2 and BMP4 (14). Therefore, it is possible that BMP2, BMP4, or both are endogenous ligands for RGMa. To further investigate ligands for the RGMa coreceptor, we screened KGN cells, which do not express RGMa (RT-PCR data not shown), by RT-PCR for expression of BMP2 and BMP4 and the closely related members BMP5–BMP7 (Fig. 1A). Among these ligands, only BMP2, BMP4, and BMP6 were detected in KGN cells. We then tested whether RGMa-induced BMP signaling is affected by siRNA-mediated specific inhibition of BMP2, BMP4, or BMP6. As shown in Fig. 1B, BMP2, BMP4, and BMP6 expression was specifically inhibited by the respective gene-specific siRNA duplexes (60 nM), with minimal effect on the expression of the other ligands. The siRNAs reduced BMP2, BMP4, and BMP6 mRNA expression levels by 80, 60, and 75%, respectively. KGN cells were transfected with BRE-Luc in combination with control, BMP2, BMP4, or BMP6 siRNA (60 nM) (Fig. 2A). As expected, transfection of KGN cells with RGMa increased BRE-Luc activity in a dose-dependent manner in the absence of exogenous ligands. Inhibition of BMP2 or BMP4 expression dramatically reduced RGMa-mediated BMP signaling. In contrast, inhibition of BMP6 expression did not change BRE-Luc activity induced by RGMa compared with control siRNA (Fig. 2A). RGMa-mediated BMP signaling was completely inhibited to below the basal levels by double inhibition of both BMP2 and BMP4 expression (Fig. 2B). These results demonstrate that BMP2 and BMP4 are both endogenous
The mean affinity of RGMa-Fc for BMP4 was 1.4 ± 0.1 nM, slightly higher than that for BMP2 (2.4 ± 0.2 nM) (Fig. 3, A and B). The relative potencies of BMP2 and BMP4 were also investigated for

### TABLE 4

| Radioligand/competitor | EC<sub>50</sub> (nM) | Relative potency |
|------------------------|----------------------|------------------|
| **BMP2**              |                      |                  |
| BMP2                   | 9.90 ± 0.61          | 1                |
| BMP4                   | 2.26 ± 0.06          | 0.23             |
| **BMP4**              |                      |                  |
| BMP2                   | 3.63 ± 0.19          | 0.94             |
| BMP4                   | 3.63 ± 0.19          | 0.94             |

RGMa-Fc (Fig. 3, C and D; and Table 4). When BMP2 was the radioligand (Fig. 3C and Table 4), unlabeled BMP4 was ~5-fold more potent than unlabeled BMP2. When radiolabeled BMP4 was examined in this system (Fig. 3D and Table 4), unlabeled BMP4 was ~3-fold more potent than unlabeled BMP2.

**RGMa-mediated BMP Signaling Is Reduced by Inhibition of Either ActRIIA or BMPRII Expression**—Our previous study demonstrated that RGMa-mediated BMP signaling occurs via the BMP type I receptors ALK3 and ALK6 (14). To investigate the utilization of BMP type II receptors by RGMa, we first screened KGN cells for expression of BMPRII, ActRIIA, and ActRIIB by RT-PCR. As shown in Fig. 4A, all three type II receptors were expressed. The expression of the three receptors was selectively reduced by 80–90% with the introduction of specific siRNAs (40 nM) (Fig. 4B). We then examined whether or not inhibition of endogenous type II receptors affects RGMa-mediated stimulation of BRE-Luc activity. As expected, treatment of KGN cells with BMP2 (10 ng/ml) increased BRE-Luc activity to 5.8-fold above the base line (Fig. 5A). This stimulation was dramatically reduced by BMPRII-specific siRNA to only 1.5-fold above the base line (p < 0.01), but was not reduced by ActRIIA- or ActRIIB-specific siRNA. Similar data were observed when cells were treated with BMP4 (Fig. 5B). These results suggest that, in KGN cells, BMP2 and BMP4 signaling is primarily transduced by BMPRII and not by ActRIIA or ActRIIB.

When KGN cells were transfected with RGMa cDNA, BRE-Luc activity increased to 3.4-fold above the base line (Fig. 5C). This stimulation was reduced to 1.7- and 1.2-fold above the base line by BMPRII- and ActRIIA-specific siRNAs, respectively (p < 0.05 for both). RGMa-mediated BRE-Luc activity was not altered by inhibition of ActRIIB expression. As a control, activin A-induced CAGA-Luc activity was diminished by either ActRIIA or ActRIIB inhibition, but not by BMPRII inhibition (Fig. 5D), indicating that the ActRIIA and ActRIIB siRNAs were effective. These results suggest that BMPRII and ActRIIA are both utilized to transduce BMP2 and BMP4 signaling in the presence of the RGMa coreceptor, indicating that RGMa enhances the utilization of ActRIIA by BMP2 and BMP4.

We also examined the effects of inhibition of BMPRII, ActRIIA, and ActRIIB on RGMa-induced Smad1/5/8 activation (Fig. 6). BMP2 treatment increased phospho-Smad1/5/8 levels compared with unstimulated cells (Fig. 6, A and B). BMP2-
induced phospho-Smad1/5/8 levels were dramatically reduced by BMPRII inhibition, but were not altered by ActRIIA or ActRIIB inhibition. Transfection with RGMa cDNA increased phospho-Smad1/5/8 levels compared with transfection with control siRNA alone (Fig. 6, A and B). Phospho-Smad1/5/8 levels were reduced by BMPRII- or ActRIIA-specific siRNA. ActRIIB siRNA transfection did not affect RGMa-induced increases in phospho-Smad1/5/8 levels. Thus, activation of Smad1/5/8 corroborated results obtained with luciferase reporter assays.

Inhibition of Endogenous Rgma Expression Attenuates BMP2 Signaling in BmprII-null Mouse PASMCs—To further examine the role of RGMa in the utilization of ActRIIA by BMP2 and BMP4, we used BmpRII-null PASMCs derived from Bmpr2<sup>−/−/−</sup> mice as described previously (24). As expected, BmpRII mRNA was easily detected by RT-PCR in wild-type PASMCs, but in BmpRII knock-out cells (Fig. 7A). Consist-

**FIGURE 4.** Expression of BMP type II receptors in KGN cells and specificity and efficacy of siRNAs targeting human BMPRII, ActRIIA, and ActRIIB expression. A, total RNA from KGN cells was extracted for RT-PCR to determine the expression of human BMPRII, ActRIIA, and ActRIIB. β-Actin was used as a control for cDNA quality, and reactions without cDNA were used as negative controls. B, siRNA-mediated specific inhibition of human BMPRII, ActRIIA, and ActRIIB expression. siRNAs (40 nm) derived from sequences of BMPRII, ActRIIA, and ActRIIB were employed to decrease their expression in KGN cells. mRNA levels were measured 46 h after cells were transfected with specific siRNA by quantitative real-time PCR, were normalized to RPL19 mRNA levels, and are expressed as a fraction of values from cells treated with control siRNA. The values shown are the means ± S.E. of triplicate measurements. BMPRII (si-BMPRII), ActRIIA (si-ActRIIA), and ActRIIB (si-ActRIIB)-specific siRNAs reduced the expression of those genes by 80–90%.

**FIGURE 5.** Impact of siRNA targeting of BMPRII, ActRIIA, and ActRIIB on BMP2 and BMP4 signaling and RGMa-mediated BMP signaling in KGN cells. A and B, impact of siRNA targeting of BMPRII, ActRIIA, and ActRIIB on BMP2 and BMP4 signaling, respectively. KGN cells were transfected with the BRE-Luc reporter and the pRL-TK Renilla luciferase vector in combination with control siRNA or siRNA specific for BMPRII (si-BMPRII), ActRIIA (si-ActRIIA), or ActRIIB (si-ActRIIB) (40 nm). Transfected cells were then incubated in the absence or presence of 10 ng/ml BMP2 (A) or 10 ng/ml BMP4 (B). Luciferase activity was measured in cell extracts and normalized for transfection efficiency relative to Renilla activity. The values shown are the means ± S.E. of triplicate experiments. C, impact of siRNA targeting of BMPRII, ActRIIA, and ActRIIB on RGMa-mediated BMP signaling. KGN cells were transfected with BRE-Luc and pRL-TK in combination with control siRNA or siRNA specific for BMPRII, ActRIIA, or ActRIIB (40 nm) and in the absence or presence of RGMa cDNA (4 ng) for 46 h prior to measurement of luciferase activity. D, impact of siRNA targeting of BMPRII, ActRIIA, and ActRIIB on activin A signaling. KGN cells were transfected with CAGA-Luc and pRL-TK in combination with control siRNA or siRNA specific for BMPRII, ActRIIA, or ActRIIB (40 nm). Transfected cells were then incubated in the absence or presence of 20 ng/ml activin A. *, p < 0.05; **, p < 0.01 versus controls with treatments.
ent with previous findings (24), BMP2 signaling was diminished in BmpRII-null cells compared with wild-type PASMCs (Fig. 7B).

We then examined the utilization of type II receptors by BMP2 ligand and the RGMa coreceptor in BmpRII-null PASMCs using previously validated siRNA (24) specific for mouse BmpRII, ActRIIA, or ActRIIB (20 nM). Exogenous BMP2 increased BRE-Luc activity to 4.7-fold above the base line (Fig. 7C). As predicted from the lack of BmpRII, BmpRII siRNA transfection did not affect BMP2-induced luciferase activity. The stimulation in BRE-Luc activity by BMP2 was reduced by inhibition of ActRIIA expression to below the basal levels. Inhi-
bition of ActRIIB expression did not affect activation of the BRE-Luc reporter by BMP2. These results confirm the previous finding that ActRIIA is the type II receptor transducing BMP2 or BMP4 signals in BmpRII knock-out PASMCs (24). Transfection of BmpRII-null PASMCs with RGMa cDNA increased BRE-Luc activity to 3.9-fold above the base line (Fig. 7D). This stimulation was completely blocked by inhibition of ActRIIA-specific siRNA. Transfection of BMPRII or ActRIIB siRNA had no effect. These results suggest that RGMa exclusively uses ActRIIA in BmpRII-null PASMCs to mediate BMP2 or BMP4 signaling.

By RT-PCR, we found that Rgma mRNA was expressed in both wild-type and BmpRII-null PASMCs (Fig. 8A). The
expression levels of Rgma mRNA were similar between the wild-type and BmpRII-null cells as determined by quantitative real-time PCR (data not shown). We then tested whether BMP2 signaling is affected by siRNA-mediated inhibition of endogenous Rgma in BmpRII-null cells. As shown in Fig. 8E, the two siRNA sequences (40 nM) reduced Rgma mRNA expression levels by 70 and 80%, respectively. Treatment of BmpRII-null PASMCs with BMP2 increased BRE-Luc activity by 4.9-fold above the base line (Fig. 8C). This stimulation was significantly reduced to 3.3- and 2.8-fold above the base line by the two Rgma siRNAs, respectively (p < 0.05 for both) (Fig. 8C). As a control, BMP2 signaling in BmpRII-null cells was completed blocked by ActRIIA siRNA (Fig. 8C). Similar results were seen when cells were treated with BMP4 (data not shown). BRE-Luc activity induced by BMP7, a ligand that does not bind Rgma (14), was not affected by the two Rgma siRNAs, although it was abolished by ActRIIA siRNA (Fig. 8D). In wild-type PASMCs (Fig. 8E), BMP2 signaling was not significantly affected by inhibition of Rgma using the two siRNA sequences. As a control, BMP2 signaling was dramatically reduced by BmpRII siRNA as reported previously (24). These results suggest that ActRIIA is used by endogenous Rgma to enhance BMP2 or BMP4 signaling in the absence of BMPRII and that this function is less critical in the presence of BMPRII.

In addition to Rgma mRNA, Rgmb mRNA was also expressed in wild-type and BmpRII-null PASMCs, whereas Rgmc mRNA was not detected by RT-PCR (data not shown). The two siRNA sequences (40 nM) reduced Rgma mRNA expression by 82 and 85%, respectively (Fig. 9A). In BmpRII-null cells (Fig. 9B), transfection of the two Rgmb siRNAs did not change BMP2 signaling, and cotransfection of Rgma and Rgmb siRNAs did not further reduce BMP2 signaling compared with the reduction in BMP2 signaling by Rgma siRNA alone. In wild-type PASMCs (Fig. 9C), transfection of Rgma siRNA, Rgmb siRNAs, or both did not change BMP2 signaling. These results suggest that endogenous Rgmb does not play a significant role in regulating BMP2 signaling in PASMCs.

BMP Signaling Mediated by Overexpressed RGMa Is Reduced by Inhibition of Either ActRIIA or BmpRII Expression in Wild-type Mouse PASMCs—Our previous study showed that BMP2 or BMP4 uses BMPRII (but not ActRIIA) in wild-type PASMCs (24). To examine whether Rgma can enhance the utilization of ActRIIA in wild-type PASMCs, in which endogenous Rgma plays an undetectable role in BMP2 signaling as shown in Fig. 8E, we overexpressed RGMa in these cells. Treatment of wild-type PASMCs with BMP2 increased BRE-Luc activity to 6.6-fold above the base line (Fig. 10A). This stimulation was reduced by inhibition of BmpRII expression to 1.8-fold above the base line (p < 0.01). Inhibition of ActRIIA or ActRIIB expression did not affect the activation of the BRE-Luc reporter by BMP2. These results confirm the previously published finding that BMP2 uses BMPRII in wild-type PASMCs (24). Transfection of PASMCs with Rgma cDNA increased BRE-Luc activity to 4.2-fold above the base line (Fig. 10B). This stimulation was significantly reduced to 2.2- and 1.6-fold above the base line by BmpRII- and ActRIIA-specific siRNAs, respectively (p < 0.01 for both). RGMa-induced BRE-Luc activity was not affected by inhibition of ActRIIB expression. These results suggest that both BMPRII and ActRIIA in wild-type PASMCs are used to transduce BMP signals when Rgma is overexpressed, indicating that Rgma can enhance the utilization of ActRIIA by BMP2 and BMP4 in wild-type PASMCs.
RGMa Increases Binding of BMP2 to ActRIIA and ALK3—Some TGF-β superfamily coreceptors such as betaglycan enhance ligand binding to the signaling receptors (8–11). We therefore tested whether RGMa-Fc increases binding of BMP2 to ActRIIA and ALK3 (Fig. 11). Purified RGMa-Fc, ActRIIA-Fc, ALK3-Fc, or their combinations were incubated in solution with 125I-BMP2, followed by incubation on protein A-coated plates and determination of radioactivity. RGMa-Fc significantly bound 125I-BMP2. As expected, ActRIIA-Fc was unable to bind, and the combination of ActRIIA-Fc and ALK3-Fc significantly bound 125I-BMP2. Addition of RGMa-Fc to ActRIIA-Fc and ALK3-Fc increased binding to 125I-BMP2 compared with the combination of ActRIIA-Fc and ALK3-Fc (Fig. 11).

**DISCUSSION**

Although originally discovered as glycosylphosphatidylinositol-anchored cell-surface proteins involved in neuronal differentiation and cell-cell contact in the developing nervous system (16–18), it is now clear that RGM family members are also coreceptors for BMP signaling (13–15). Emerging evidence suggests that RGM family members increase the sensitivity of cells to BMP stimulation and thus allow these cells to respond to low endogenous levels of BMP ligands (13–15). However, the mechanisms by which RGM family members facilitate the interactions between components of the BMP pathway are not fully understood.

In this study, we first examined the expression of BMP2 and BMP4–BMP7 ligands in KGN cells, which respond to BMP stimulation but do not express RGM family members (data not shown). We found that only BMP2, BMP4, and BMP6 were expressed. We then used siRNAs for these ligands to specifically inhibit their expression. Inhibition of BMP2 or BMP4 dramatically reduced RGMa-mediated BMP signaling, whereas inhibition of BMP6 expression had no effect. Inhibition of expression of both BMP2 and BMP4 completely abolished RGMa-induced BMP signaling. In combination with our previously published findings (14), the results suggest that BMP2 and BMP4 are both endogenous ligands and that they are likely the sole endogenous ligands for the RGMa coreceptor to induce BMP signaling in KGN cells.

It has been documented that both BMPRII and ActRIIA can mediate BMP2 and BMP4 signaling, but in most cell lines studied so far, BMPRII is the principal type II receptor (24, 30–33). Mazzerbourg et al. (33) showed that treatment of the pre-osteoblast MC3T3 cell line with Bmprii or Actriia short hairpin RNA suppresses BRE promoter activity stimulated by BMP2 in a dose-dependent manner. The inhibitory effects of Bmprii short hairpin RNA are more pronounced compared with those of Actriia short hairpin RNA, indicating that BMPRII is the preferential type II receptor for BMP2 signaling in MC3T3 cells. We have shown previously that BMP4 signaling is impaired by inhibition of Bmprii (but not Actriia or Actriib) expression in wild-type PASMCs, whereas in Bmprii-null cells, BMP4 signaling is inhibited by Actriia siRNA (24). These data suggest that wild-type PASMCs transduce BMP signals via BMPRII, whereas Bmprii-deficient cells transduce BMP signals via Actriia. In this study, we found that either BMP2- or
BMP4-induced signaling in KGN cells (as determined by the activity of BRE-Luc) was reduced by 70–75% upon inhibition of BMPRII expression, but was not significantly reduced upon inhibition of ActRIIA or ActRIIB expression (Fig. 5, A and B). These data suggest that BMP2 or BMP4 signal is also primarily transduced through BMPRII in KGN cells. The ability of transfected RGMa to increase BRE-Luc activity was reduced by transduction through BMPRII in KGN cells. The ability of transduction through BMPRII by showing that exogenous BMP2 or BMP4 signaling or BMP signaling mediated by transfected RGMa was abolished by ActRIIA inhibition, but was not affected by ActRIIB inhibition (Fig. 7, C and D). We then examined whether these cells express RGMa endogenously. Unlike KGN cells, both wild-type and BmprII-null PASMCs express Rgma (Fig. 8A). In BmprII-null PASMCs, we found that BMP2 or BMP4 signaling was significantly diminished by inhibition of Rgma expression (Fig. 8C). The reduction in BMP signaling was specific for BMP2 and BMP4, whereas signaling by BMP7 was not affected by the two Rgma siRNAs (Fig. 8D). These results suggest that, although RGMa is not necessary for BMP2 or BMP4 to use ActRIIA, there is an increased utilization of ActRIIA by BMP2 and BMP4 in the presence of RGMa. RGMa may serve to augment sensitivity to BMP2 and BMP4 ligands that otherwise would have a limited effect in BmprII-null, BmprII-low, or mutant BmprII cell populations via the activation of ActRIIA.

In wild-type PASMCs, inhibition of endogenous RGMa did not change BMP2 signaling (Fig. 8E), a result that is consistent with the preference of BMP2 or BMP4 to use BMPRII (but not ActRIIA and ActRIIB) in wild-type PASMCs cell (Fig. 9) (24). However, when RGMa expression was elevated by the transfection of Rgma cDNA, both ActRIIA and BMPRII could be used for BMP signaling (Fig. 10). These results are consistent with the data from KGN cells (compare Figs. 5 and 10).

Inhibition of Rgmb expression in BmprII-null or wild-type PASMCs failed to inhibit BMP2 signaling. This indicates that the protein expression level of RGMb is likely to be too low to assess its function. Because these cells do not express RGMc, our results argue that the inability of RGMa inhibition to reduce BMP2 signaling in wild-type PASMCs is not due to redundancy between RGMa and other RGM members in these cells. However, it is possible that redundancy may occur in other cell lines and cell types.

A number of TGF-β superfamily coreceptors, including betaglycan, endoglin, and Cripto, have been described to promote ligand binding to the signaling receptors (8–12). This results from this study revealed an increase in the binding of BMP2 to ALK3 and ActRIIA in the presence of RGMa-Fc in our cell-free binding assays. Whether RGMa can increase the binding of BMP2 and BMP4 to ActRIIA and type I receptors on the cell surface remains to be investigated.

Taken together, our findings suggest a mechanism of RGMa action in sensitizing cells to low endogenous levels of BMP2 and BMP4 ligands. In cells expressing both BMPRII and ActRIIA, ActRIIA is not significantly used by BMP2 and BMP4 in the absence of RGMa or in the presence of RGMa at a low level, BMP2 or BMP4 signal is transduced through BMPRII, but not through ActRIIA and ActRIIB. BMP2 or BMP4 binds to BMP type I receptors (R-I) and BMPRII to generate an active signaling complex. Upon formation of the complex, BMPRII phosphorylates type 1 receptors, which then activate the intracellular cascade. β, in the presence of RGMa at a high level, BMP2 or BMP4 signals through ActRIIA in addition to BMPRII. RGMa interacts with BMP2 or BMP4, BMP type I receptors, and ActRIIA to generate an active signaling complex. Within the complex, ActRIIA phosphorylates type 1 receptors, which then activate the intracellular signaling cascade. The utilization of both BMPRII and ActRIIA leads to an increased signal in response to BMP2 or BMP4.

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FIGURE 12. Schematic diagram depicting the role of RGMa in enhancing the utilization of ActRIIA by BMP2 and BMP4 in cells with expression of BMP2 or BMP4. A, in the absence of RGMa or in the presence of RGMa at a low level, BMP2 or BMP4 signal is transduced through BMPRII, but not through ActRIIA and ActRIIB. BMP2 or BMP4 binds to BMP type I receptors (R-I) and BMPRII to generate an active signaling complex. Upon formation of the complex, BMPRII phosphorylates type I receptors, which then activate the intracellular cascade. β, in the presence of RGMa at a high level, BMP2 or BMP4 signals through ActRIIA in addition to BMPRII. RGMa interacts with BMP2 or BMP4, BMP type I receptors, and ActRIIA to generate an active signaling complex. Within the complex, ActRIIA phosphorylates type 1 receptors, which then activate the intracellular signaling cascade. The utilization of both BMPRII and ActRIIA leads to an increased signal in response to BMP2 or BMP4.
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