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THE RGM/DRAGON FAMILY OF BMP CO-RECEPTORS

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Abstract

The BMP signaling pathway controls a number of cell processes during development and in adult tissues. At the cellular level, ligands of the BMP family act by binding a hetero-tetrameric signaling complex, composed of two type I and two type II receptors. BMP ligands make use of a limited number of receptors, which in turn activate a common signal transduction cascade at the intracellular level. A complex regulatory network is required in order to activate the signaling cascade at proper times and locations, and to generate specific downstream effects in the appropriate cellular context.

One such regulatory mechanism is the repulsive guidance molecule (RGM) family of BMP co-receptors. This article reviews the current knowledge regarding the structure, regulation, and function of RGMs, focusing on known and potential roles of RGMs in physiology and pathophysiology.

Keywords

BMP; receptors; RGM; DRAGON; HJV

1. Introduction

Bone morphogenetic proteins (BMPs) are a large subfamily that belong to the Transforming Growth Factor-beta (TGF- β) superfamily of ligands, which also includes TGF- β 1, - β 2, and - β 3, Activins, Inhibins, Nodal, Growth and Differentiation Factors (GDFs), Muellerian Inhibiting Substance or anti-Muellerian hormone (MIS or AMH), Vg1, and Myostatin. Altogether, there are approximately 40 ligand members (1 and 2). Broadly conserved across the animal kingdom, BMPs and other ligands of the TGF- β superfamily play a crucial role in both embryonic development and postnatal life by mediating fundamental processes such as cellular proliferation, differentiation, apoptosis, migration, body patterning, organogenesis, tissue homeostasis and repair, reproduction, inflammation, and host immunity (1–4). The importance of BMPs during development is evidenced by the fact that functional loss of many of the components of this signaling pathway are embryonic lethal and/or cause major malformations of many tissues and organ systems including bone and cartilage, heart, lung, kidney, eyes, reproductive tissue, nervous system, placenta and placental connections, vasculogenesis and angiogenesis (Reviewed in 5 and 6). Postnatally,

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BMPs continue have an important role in many areas, including in the induction of bone and cartilage formation (7 and 8) and vascular homeostasis (9). More recently, BMPs have been shown to be instrumental in iron metabolism (10,11) (see below), and BMPs have been suggested to play a role in glucose metabolism (12,13). In addition to their important physiologic roles, BMPs are also involved in several pathological conditions, such as cancer, fibrosis, inflammatory diseases, and reproductive dysfunctions (1–4, 14–16).

Because of its involvement in such a diverse array of cellular and systemic functions during both embryonic and adult life, the BMP signaling pathway must be tightly regulated. This review focuses on the recently identified roles of the repulsive guidance molecule (RGM) family of BMP co-receptors in the regulation of BMP signaling in health and disease.

2. BMPs signaling pathway

All TGF- β superfamily members share common structural features and a common model of signaling transduction. The active form of these ligands is a disulfide-linked dimeric protein, which is cleaved from a larger precursor protein and secreted. Once secreted, usually as homodimers or rarely as heterodimers, TGF- β superfamily members act by binding to two distinct receptor types, type I and type II. Seven type I (ALK1, ALK2/ACTR-I, ALK3/BMPR-IA, ALK4/ACTR-IB, ALK5/T β R-I, ALK6/BMPR-IB, ALK7) and five type II receptors (T β R-II, ACTR-IIA, ACTR-IIB, BMPRII, AMHRII) have been described in mammals, of which only ALK1, ALK2, ALK3, ALK6, ACTRIIA, ACTRIIB, and BMPRII are receptors for the BMP subfamily. Both types of receptors have an N-terminal extracellular ligand binding domain, a transmembrane region, and a C-terminal intracellular domain containing serine/threonine kinases. The structure of the dimeric ligands suggests the formation of a hetero-tetramer made by two type I and two type II receptors. Upon complex formation, the constitutively active type II receptors phosphorylate type I receptors, causing the activation of specific SMAD proteins. SMAD proteins are intracellular signaling molecules, which can be classified in three categories: receptor-regulated SMADs (R-SMADs), common-partner SMADs (Co-SMADs), and inhibitory SMADs (I-SMADs). The activated type I receptor phosphorylates R-SMADs, which in turn bind Co-SMADs, and the SMAD complex translocates to the nucleus. In the nucleus, these R-SMADs/Co-SMAD oligomers interact with different DNA-binding proteins and bind to the transcription promoter regions of the target genes (1,2,17). In addition to the main canonical SMAD pathway, other signaling cascades such as the MAP kinase pathway, can be activated by BMPs in certain cell types (18–20).

On the basis of the activation of specific R-SMADs, TGF- β superfamily members can be divided into two branches: the TGF- β /Activin/Nodal branch, which activates SMAD2 and 3, and the BMP/GDF/ MIS-AMH branch which activates SMAD1, 5, and 8. In mammals, both intracellular signaling branches share SMAD 4 as a Co-SMAD. These two groups also have two distinct ligand-receptor interactions. The TGF- β /Activin/Nodal subfamily members show high affinity for the extracellular binding domain of type II receptors, and only after this interaction do they interact with type I receptors. The BMP/GDF/MIS-AMH subfamily members have high affinity for type I receptors and low affinity for type II receptors, with the ligand/type I receptor complex subsequently exhibiting a higher affinity for binding to the type II receptor. Although most TGF- β superfamily members either activate Smad 2 and 3, or SMAD 1, 5, and 8, in certain cell types such as endothelial cells, TGF- β ligand is able to activate both type of SMAD signaling (1,2,17,21).

3. BMP signaling modulators

The BMP signaling pathway requires extensive regulation at multiple levels to ensure appropriate spatio-temporal activation and specific downstream responses. The role of

BMPs modulators has been extensively reviewed (2, 17, 20, 22–26). Extracellular, membrane, and intracellular regulatory mechanisms have all been described (Table 1).

3.1 Extracellular modulators

Several secreted proteins have been described to bind and sequester BMPs and subsequently prevent their interaction with BMP receptors: Noggin, Cerberus, Chordin family (Cordin and Crim1), Follistatin, Dan family (Gremlin, Usag1, Sclerostin, Coco, PRDC), Tsg, and CCN family. Besides their role as antagonists, some of these molecules have additional modulating actions. For example, Gremlin not only binds and inhibits extracellular BMP ligands, but it also interacts intracellularly with BMP4 precursor protein, causing an additional inhibition of BMP activity. Noggin and Sclerostin can bind to each other, removing the BMP inhibitory effect of each and allowing BMP signaling pathway activation. Another group of secreted proteins act by enhancing BMP signaling. For example, KCP/Kielin increases the binding of BMP7 to its receptor and simultaneously inhibits the transduction through the SMAD 2 and 3 pathway. Cv2 in mammals also seems to function as a BMP signal enhancer, but may also function as an inhibitor in some contexts. Other TGF- β superfamily ligands can modulate the BMP signaling pathway. For example, Inhibin, in concert with its co-receptor betaglycan (see below,) can act as a BMP antagonist by competing with BMPs for type II receptors. The extracellular matrix, via the binding to BMPs and BMP-antagonists, likely also contributes to the regulation of BMP signals.

3.2 Intracellular modulators

Inside the cell, a multitude of molecules are able to finely modulate BMP signals. Because of the low affinity and low specificity of SMADs for DNA binding, SMADs must cooperate not only with each other, but also with other DNA-binding proteins which function as transcription factor partners of the R-SMAD/Co-SMAD complex. These SMAD-partner proteins can bind directly or indirectly to SMADs, and they can be either ubiquitous or cell specific, ensuring cell-dependent transcriptional responses. For example, murine Shn2 can interact with DNA-bound SMAD1/SMAD4 and with the intermediate protein C/EBP α (which in turn binds a DNA motif), activating the transcription of *Pparg2* in response to BMP signaling (27). R-SMAD/Co-SMAD transcriptional co-activators and co-repressors can also regulate transcription by inducing acetylation and de-acetylation of histones respectively. Several different cytoplasmic and nuclear proteins act through modulating the phosphorylation, dephosphorylation, shuttling, and degradation of SMADs and BMP receptors. For example, Smurf1 targets SMAD1 and 5 for destruction in unstimulated cells (28). Inhibitory SMADs (I-SMADs), SMAD6 and SMAD7 also play an important role in this process. SMAD6 and SMAD7 interact with type I receptors to block R-SMAD phosphorylation or to promote receptor degradation or dephosphorylation. SMAD6 is able to interfere with the formation of the SMAD1/SMAD4 complex formation. While SMAD7 expression is activated by both branches of the TGF- β superfamily signaling pathway and its inhibitory action affects both branches, SMAD6 is induced only by BMPs and acts preferentially on the BMPs intracellular branch.

Recently *micro-RNAs* have been described as BMP signaling modulators. For example, in cultured murine mesenchymal pluripotent stem cells, miR-199a was shown to act as a BMP-2 responsive micro-RNA to adversely regulate early chondrocyte differentiation via direct targeting of SMAD1 (29). In fetal liver cell lines the Mir-23b cluster of microRNAs target SMAD 3, 4, and 5, contributing to the regulation of the differentiation fate of these cells (30). The BMP signaling pathway is also modulated by cross-talk between the canonical SMAD signaling pathway and other pathways such as the Mitogen Activated Protein Kinase (MAPK), PI3K/Akt, Notch, and STAT pathways. The signaling cross-talk

can have either synergistic or antagonistic effects, depending on the cellular context (18–20).

3.3 Membrane modulators

Pseudoreceptors such as BAMBI are one mechanism of membrane modulation of BMP signaling. With an extracellular domain homologous to type I receptors, but lacking an intracellular serine/threonine-kinase domain, BAMBI interacts with type I receptors and interferes with the proper formation of the heterotetrameric receptor complex and the signal transduction to R-SMADs (31). *More* recently, the repulsive guidance molecule (RGM) family of proteins has been described as a co-receptors for the BMP pathway, acting as facilitators and enhancers of BMP signaling transduction. These BMP Co-receptors are the object of the present review.

4. The RGM family of BMP Co-Receptors

4.1 Overview

Before the identification of BMPs co-receptors, other co-receptors for the TGF- β /activin/nodal subfamily had been described. Endoglin, a transmembrane glycoprotein expressed on vascular endothelial cells, is required for efficient TGF- β transduction via the type I receptor ALK1, indirectly inhibiting TGF- β /ALK5 signaling. Thus, endoglin plays a pivotal role in the balance of ALK1 and ALK5 signaling to regulate endothelial cell proliferation (32,33). EGF-CFC proteins are GPI anchored membrane proteins that function as essential co-receptors required for Nodal, Vg1, and GDF1 signaling. The EGF-CFC protein Cripto has a complex role. Cripto acts as a true signal enhancer by independently binding Nodal and its receptor ALK 4 to promote signal transduction (34), in this context perhaps functioning also as a secreted co-ligand factor (35). Cripto also inhibits Activin-dependant signaling activation by interfering with the binding of the Activin/type II receptor complex to type I receptors (36).

Betaglycan (also known as TGF- β type III receptor) is a promiscuous transmembrane proteoglycan co-receptor for the TGF- β superfamily. Betaglycan can bind and present TGF- β ligands to the type II receptor, T β R-II, forming a high affinity ternary complex which increases the receptor/ligand affinity and the cell responsiveness (37). Betaglycan is also a receptor for inhibin (38), and appears to bind inhibin at a site that is distinct from the two TGF- β binding sites (39). More recently, betaglycan has been shown to bind several BMPs with relatively low affinities ($K_d \sim 10 \mu M$) (40).

RGM proteins are the first known family of high-affinity co-receptors that are specific for BMPs ($K_d \sim 1$ to 5 nM) (10, 41,42). The first description of an RGM family member was made in chick embryos in 2002 (43). To date, four members of the RGM family has been described in vertebrates: RGMa (also known as RGM), RGMb (also known as DRAGON), RGMc (also known as hemojuvelin or HJV), and the recently identified RGMd, which is expressed only in fish. RGM proteins are also found in nonvertebrates, which generally have only a single RGM gene (44). RGMa, DRAGON, and HJV paralogues have approximately 50–60% amino acid identity while RGMd shares approximately 30–40% identity. All RGMs share some common structural features: an N-terminal signal peptide, a partial von Willebrand factor type D domain (vWF-type D), which includes a highly conserved proteolytic cleavage site, a hydrophobic domain of unknown function, and a C-terminal GPI-anchor (43,45). Studies suggest that RGM proteins can exist both in a single chain uncleaved form and as a two chain form after cleavage of a conserved acid-labile aspartic acid-proline proteolytic cleavage site (46,47). Like many GPI anchored proteins, at least some RGMs have been demonstrated to be expressed in lipid rafts (48) Unlike DRAGON, RGMa and HJV also possess an RGD (Arg-Gly-Asp) motif, which may be involved in cell-

cell adhesion (49). RGMs have been implicated in mouse models of diseases and in some human pathologic states (Table 2).

4.2 RGMa

In 2002, Repulsive Guidance Molecule (RGM), a GPI-anchored protein expressed in chick embryonic optic tectum, was cloned and functionally characterized as a chemorepulsive axon guidance cue. Expressed in a high posterior, low anterior gradient, RGM inhibits temporal retinal axons from entering the posterior region of the optic tectum, instead steering them toward their correct targets in the anterior tectum (43). Subsequently, neogenin, a homologue of the netrin receptor DCC (deleted in colorectal cancer), was discovered to be a receptor for RGM (50). Co-immunoprecipitation and biochemical studies show a high affinity binding between neogenin and RGM with a K_D of 230 pM. Neogenin is expressed in temporal retinal axons, and the RGM-neogenin interaction was found to be responsible for RGM-mediated chemorepulsion of temporal retinal axons (50). Subsequently, mammalian genes homologous to chick RGM were described: RGMa, RGMb/DRAGON, and RGMc/HJV, of which RGMa is the most closely related to chick RGM (51,52). Subsequent work has shown a role for RGMa in mediating axonal guidance in *Xenopus* embryo forebrain supraoptic tract (53) and in the developing mouse hippocampus (54), although interestingly RGMa does not appear to play a role in retinal axonal patterning in developing mice (52). RGMa has also been implicated as an inhibitor of axonal regeneration after injury in the adult mammalian central nervous system (55). RGMa expression is increased around the lesion site in rats with spinal cord injury (55, 56) and humans with focal cerebral ischemia or traumatic brain injury (57), and intrathecal administration of anti-RGMa antibody is able to promote the regeneration of corticospinal tract axons after thoracic spinal cord injury in rats (55).

RGMa has a wide range of tissue expression, in different species, both during development and in the adult, suggesting that the potential functions of RGMa are not confined to axonal guidance. During mouse development, RGMa is widely expressed in the central nervous system, mostly non-overlapping with RGMb. These separate expression domains in the central nervous system persist after birth in several brain areas (51,58). RGMa is also expressed in the developing mouse cochlea, lung, limb primordia(83), and gut (59). In adult murine tissues, RGMa expression has been found in the heart, brain, lung, liver, skin, kidney, testis (41), and gut (59). In addition to its function as a repulsive axon guidance molecule, RGMa has also been shown to play a role in neural tube closure. The RGMa knockout mouse model exhibits defects in neural tube closure: approximately 50% of the RGMa knockout embryos have an exencephalic phenotype (52). RGMa also has a role as a cell survival factor, by inhibiting the proapoptotic activity of neogenin (60). RGMa has been shown to promote neuronal differentiation in the embryonic chick mid- and hindbrain through its receptor neogenin (61)

A role for RGMa in the BMP signaling pathway was investigated after the homologous DRAGON/RGMb was shown to function as a BMP co-receptor *in vitro* (42) (see below). Transfection of RGMa cDNA into BMP responsive cells enhances signaling by endogenous BMP ligands *in vitro* as measured by BMP-responsive promoter luciferase reporter assays (BRE-Luc ref. 41). TGF- β signaling was unaffected (62). The BMP-signal enhancing activity is dependent on the presence of BMP ligands because it could be inhibited by Noggin (41) or by siRNA-mediated knockdown of endogenous BMP-2 and BMP-4 ligand expression (62). Transfection of RGMa cDNA into cells also increases BRE-Luc activity induced by exogenously added BMP-2 ligand (41,63). siRNA-mediated knockdown of endogenous RGMa expression reduces BRE-Luc activity induced by exogenous BMP-2 (63). In a cell-free system, purified human RGMa.Fc (the extracellular domain of RGMa fused to the Fc portion of human IgG) binds directly to 125 I-BMP-2 and 125 I-BMP-4 (41)

with a K_D of 2.4 and 1.4 nM respectively (62). A similar binding affinity of RGMa.Fc for BMP-2 has been determined by BIAcore assay (63). The binding of RGMa.Fc to ^{125}I BMP-2 in a cell-free binding assay is not competed by excess cold BMP-7 or TGF- β 1 ligand, suggesting that RGMa.Fc selectively binds to BMP-2 and BMP-4, but not BMP-7 or TGF- β 1 (41). Further studies are needed to determine the binding affinity of RGMa to the full range BMP ligands.

RGMa mediates BMP signaling via an interaction with type I receptors (ALK3 and ALK6) and type II receptors (ACTRIIA and BMPRII) thereby activating the canonical intracellular SMAD1/5/8 cascade (41,62; Figure 1). This has been shown by demonstrating that dominant negative type I receptors ALK3 and ALK6 inhibit RGMa induced BMP signaling in cell culture (41). Furthermore, siRNA-mediated inhibition of the type II receptors ACTRIIA and BMPRII, but not ACTRIIB, inhibits RGMa induced BMP signaling in cell culture (62). Evidence of a physical interaction between RGMa, BMP type I receptors, and BMP type II receptors has been shown by demonstrating that RGMa.Fc forms a complex with ALK6 in the absence or presence of BMP-2 in solution (41), and RGMa.Fc increases binding of ^{125}I -BMP2 to ALK3 and ACTRIIA in a cell-free binding assay (62). Finally, transfection of RGMa cDNA into cells increases phosphorylation of SMAD1/5/8 proteins (62), and co-transfection of dominant negative SMAD1 with RGMa inhibits RGMa induced BMP-responsive promoter activity (41).

How does RGMa enhance BMP-2 and BMP-4 signaling? One mechanism is by allowing BMP-2 and BMP-4 ligands, which normally prefer to signal via BMPRII, to signal via ACTRIIA in addition to BMPRII (41) (see Figure 1). This increased utilization of ACTRIIA may lead to the generation of an enhanced BMP signal. The precise molecular mechanism by which RGMa enhances BMP2 and BMP4 signaling and the structure of the active BMP ligand/RGMa/type I receptor/type II receptor complex at the cell surface are not known. Other questions that remain unanswered are: What is the physiologic role of the BMP signaling function of RGMa? Does the BMP signaling function of RGMa have a role in the other known functions of RGMa in mediating axonal guidance, neural tube closure, cell survival, neuronal differentiation? One study suggests that, at least for axon guidance, the RGMa-neogenin signal transduction pathway appears to involve the small GTPase RhoA and its downstream effector Rho kinase, but not the BMP signaling pathway (64).

4.3 RGMb/DRAGON

The gene encoding RGMb/DRAGON was identified by using a genomic DNA-binding strategy to identify genes regulated by DRG11, a homeobox transcription factor expressed in embryonic dorsal root ganglion (DRG) and dorsal horn neurons (49). The gene was named DRAGON, reflecting that it was turned “on” in the “DRG”. Independently, the same gene was cloned as a mammalian homolog of the chick RGM (51,52) and was named RGMb. DRAGON expression is transcriptionally regulated by DRG11 and overlaps with DRG11 in embryonic dorsal root ganglion and spinal cord. However, DRAGON expression starts earlier than DRG11 during development, and DRAGON is also expressed in areas where DRG11 is not found, suggesting that DRAGON expression is also regulated by other mechanisms. DRAGON is strongly expressed in several area of the murine embryonic central nervous system, essentially non-overlapping with RGMa, and DRAGON expression persists after birth in some areas, albeit at reduced levels. (49,51,58). DRAGON is also expressed in several other tissues in the adult rodent, including the bone, heart, lung, liver, kidney, the reproductive axis, including the testis, epididymis, ovary, uterus, and pituitary (41,62 and unpublished data). DRAGON expression has also been found in the embryonic gut, the ganglia cells of the adult small intestine and colon, and in the crypt compartment (exclusive of Paneth cells) (59).

As a homolog of RGMa, DRAGON was tested for its ability to mediate axon repulsion. DRAGON does not have any detectable repulsive role in embryonic and neonatal DRG neurites (49). Instead, adhesion of DRG neurons to HEK293 cells was increased after transfection of HEK293 cells with DRAGON cDNA, indicating a potential role for DRAGON in cell-cell adhesion (49). Since DRAGON is also expressed in DRG neurons themselves, this adhesion may be due to homophilic interactions. Indeed, co-immunoprecipitation experiments show that DRAGON can interact homophilically (49). Notably, DRG axons do not express endogenous neogenin (at least in the chick) and are unresponsive to RGMa, but are converted to responsiveness by neogenin expression (50). Although no published studies have shown an interaction between DRAGON and neogenin, this may be a common property of all RGMs, since RGMc/hemojuvelin has also been shown to bind neogenin (46). Whether DRAGON can bind neogenin to mediate repulsion of axons that express neogenin has not yet been reported.

In 2005, DRAGON was the first member of the RGM family to be identified as a BMP co-receptor that potentiates BMP signaling (42). A role for DRAGON in the BMP signaling pathway was investigated because the expression pattern of DRAGON during development in the mouse and *Xenopus* was reminiscent of the expression of BMP receptors (42). Similar to RGMa, transfection of DRAGON cDNA into cells enhances transcription of a BMP-responsive luciferase reporter (BRE-Luc), but not a TGF- β responsive luciferase reporter (CAGA-Luc), and DRAGON sensitizes cells to respond more robustly to low levels of BMP-2 ligand (42, 48). Inhibition of DRAGON expression by siRNA inhibits BMP-2 signaling as measured by both BRE-Luc and alkaline phosphatase activity assays in C2C12 murine myoblast cells (63). Although transfection of DRAGON cDNA alone is sufficient to enhance BMP signaling, this action is BMP ligand-dependent since it is blocked by administration of Noggin, which sequesters the endogenously expressed BMP ligands (42). Purified soluble DRAGON.Fc (the extracellular domain of DRAGON fused to the Fc portion of human IgG) binds directly to 125 I-BMP-2 with a K_D of 1.5 nM as measured by a cell free binding assay (42). BIAcore assay also confirmed that DRAGON.Fc binds directly to BMP-2 with a similar binding affinity (K_D 5.43 nM) (63). Excess cold BMP-4 was able to compete for 125 I-BMP-2 binding to DRAGON.Fc in a cell-free binding assay, but not BMP-7, Activin A, TGF- β 1, TGF- β 2, or TGF- β 3 (42). While cell-surface, GPI-anchored DRAGON enhances BMP signaling, soluble DRAGON.Fc inhibits BMP signaling, presumably by binding to BMP ligands and preventing their access to cell surface type I and type II receptors in a similar manner to Noggin (42, 65). DRAGON.Fc is selective in its ability to inhibit BMP ligands. DRAGON.Fc is most potent as an inhibitor of BMP-2 and BMP-4 and does not inhibit BMP-9. DRAGON.Fc does have some inhibitory activity against BMP-5, BMP-6, and BMP-7 at higher concentrations, but significantly less compared with BMP-2 and BMP-4 (65). Together, these data suggest that DRAGON preferentially binds to BMP-2 and BMP-4, with a lower affinity for other BMP ligands. Binding affinities of DRAGON for the full range of BMP ligands remains to be determined directly.

Similar to RGMa, DRAGON interacts directly with BMP type I and type II receptors, and enhances signal transduction through the canonical intracellular SMAD1/5/8 pathway (42). Additionally, co-immunoprecipitation assays in transfected HEK293 cells show that DRAGON can form a complex with tagged BMP type I receptors ALK2, ALK3, and ALK6 and BMP type II receptors ACTRIIA, BMPRII, and ACTRIIB (42).

More recent data suggests that DRAGON shares the ability of RGMa to alter type II receptor utilization by BMP-4 ligand. Whereas BMP-4 signals via the type II receptor BMPRII alone in a kidney inner medullary collecting duct cell line in the absence of

DRAGON, BMP-4 signals through ACTRIIA in addition to BMPRII in these cells in the presence of DRAGON (unpublished data, see Fig. 1).

One published study reports that the BMP-induced osteoblastic differentiation of the muscle cell line C2C12, was inhibited by both GPI-anchored and soluble DRAGON protein in a co-transfection model with constitutively activated forms of BMP receptors or constitutively active SMAD1 and SMAD4 (66). The authors suggested the possible presence of a novel cell surface molecule(s) which can bind to DRAGON to transduce an inhibitory signals that interferes with SMAD transcriptional activity in a cell-type dependent manner. However, the mechanism of this inhibitory effect is unknown and its relevance in physiology needs to be tested (66).

What is the physiologic role of DRAGON *in vivo*? The widespread tissue distribution of DRAGON expression suggests that DRAGON may have a role in many tissues, either as a BMP co-receptor, an adhesion molecule, or with another as yet undetermined function. The ability of DRAGON to enhance BMP signaling *in vivo* is demonstrated by the fact that injection of DRAGON mRNA into *Xenopus* embryos enhances the ability of SMAD1 to induce endodermal and mesodermal markers, and also promotes a neuronal phenotype and inhibits neural crest differentiation (42). The finding that DRAGON is expressed and dynamically regulated throughout the reproductive tracts, overlapping the expression sites of the BMP signaling system, suggests that DRAGON may play a role in mammalian reproduction (48). Recently, DRAGON has been found to be upregulated in the retinas of glaucoma-affected mice, together with RGMa and neogenin (67). DRAGON has also been shown to be up-regulated around the lesion site of spinal cord injury in rats (68). *In vitro*, a soluble form DRAGON inhibits neurite outgrowth in rat cerebellar granule neuron cultures (68). These findings suggest that DRAGON may have a role in the response to injury of the nervous system (68). In 2004, mice DRAGON knockout were described in a poster presentation at the Society for Neuroscience: they die three weeks post-natally without evident defect in sensory motor functions or nervous system development (45). Further studies are ongoing in order to understand the cause of this premature death which will likely provide important insights into the physiologic roles of DRAGON *in vivo*.

4.4 RGMc /HFE2/HJV

In 2003, a gene was identified in humans by a positional cloning strategy for the locus associated with the iron overload disorder juvenile hemochromatosis; it was named *HFE2* and its protein product was called hemojuvelin (69). The name *HFE2* was given because *HFE* is the name of the gene that is most commonly mutated in adult forms of hereditary hemochromatosis. It was later proposed that the hemojuvelin gene be given a new designation of *HJV* since the designation of this gene as *HFE2* is contrary to established convention because it is not a member of the *HFE* family (70). Independently, the same gene known as RGMc had been identified earlier as a homologue of RGMa and DRAGON as discussed above (49,52).

Juvenile hemochromatosis is an autosomal-recessive disease characterized by early-onset systemic iron overload, with deposition of iron in the heart, liver and endocrine glands, joint, and skin. The heart and endocrine glands, more susceptible to iron toxicity, succumb to its effect earlier and cardiomyopathy and hypogonadism will dominate the clinical picture. If untreated, this disease can be lethal before the fourth decade of life (71). So far, more than thirty *HJV* mutations in more than sixty pedigrees have been described worldwide, the most prevalent being the point mutation G230V (70,71). Hemojuvelin is expressed predominantly in the skeletal muscle and heart, as well as in the liver (49,52,69,72,73). The significance of *HJV* expression in the skeletal muscle and heart is unknown.

Patients with mutations in the HJV gene share the same phenotype as that of patients with mutations that disrupt the gene encoding hepcidin (74). A defensin-like small peptide synthesized predominantly by the liver, hepcidin is the master regulator of iron metabolism. Hepcidin acts to downregulate the sole iron exporter ferroportin on the surface of duodenal enterocytes, macrophages, and hepatocytes, thereby inhibiting iron release into the bloodstream from the diet and from body iron stores in reticuloendothelial macrophages and the liver (75). Hepcidin expression is up-regulated by iron, thereby serving as a negative feedback inhibitor to limit further iron absorption. Hepcidin expression is down-regulated by anemia and hypoxia, thereby increasing iron availability when need for red blood cell production (76). Hepcidin expression is also increased by inflammatory cytokines. This is presumably a protective mechanism to sequester iron from invading pathogens; however, this also is likely the pathogenic mechanism for the anemia of inflammation, characterized by low serum iron even in the face of normal or elevated body iron stores. Interestingly, patients affected by juvenile hemochromatosis due to HJV mutations and HJV null mice with a similar iron overload phenotype showed depressed levels of the hepcidin protein (69,77,78). These data suggest that hepcidin deficiency is the underlying cause of iron overload in juvenile hemochromatosis due to HJV mutations and that HJV acts upstream of hepcidin, positively modulating its levels (69,77,78). Indeed, transfection with HJV cDNA into hepatoma-derived cells increases hepcidin mRNA expression by quantitative real-time RT-PCR and increases hepcidin promoter activity in a luciferase assay (10).

The underlying molecular mechanism by which HJV regulates hepcidin levels was unknown until 2006, when it was shown that HJV, like other RGM family members, is also a BMP co-receptor, and that BMP signaling positively regulates hepcidin transcription in liver cells *in vitro* (10). Like other RGM family members, transfection of HJV into hepatoma-derived cells enhances BMP, but not TGF- β signaling as measured by BRE-Luc and CAGA-Luc assays (10, 63, 79). siRNA-mediated inhibition of endogenous HJV inhibits BMP-2 signaling as measured by both BRE-Luc and alkaline phosphatase activity assays in C2C12 murine myoblast cells (63).

Like other RGM family members, HJV-mediated BMP signaling is dependent on BMP ligands, and HJV can bind directly to BMP ligands. BRE-Luc induction by HJV is inhibited by Noggin, BMP-2/4 neutralizing antibody, and siRNA inhibition of BMP-2, BMP-4, or BMP-6 ligands, suggesting that BMP-2, BMP-4, and BMP-6 can all function as ligands for HJV in hepatoma-derived cells *in vitro* (10,79). Indeed, purified soluble HJV.Fc fusion protein (analogous to RGMa.Fc and DRAGON.Fc) binds to 125 I-BMP-2 and 125 I-BMP-4 and this binding can be competed by excess unlabeled BMP-2 and BMP-4, but not BMP-7 or TGF- β 1 in a cell free binding assay. Soluble HJV.Fc can also bind directly to BMP-6, forming a complex with BMP-6 in solution in a pull-down assay (65). The ability of HJV to bind BMP-2 has subsequently been confirmed by other groups by several other methods (63,80,81). The binding affinity of HJV.Fc for BMP-2 by BIAcore assay was found to be similar to RGMa.Fc and DRAGON.Fc with a K_D of 4.22 nM. Notably, another group found a ~30–50-fold lower binding affinity of soluble HJV to BMP-2 with a K_D of 140 nM when a monomeric form of soluble HJV (no Fc tail) was used rather than the dimeric HJV.Fc form (81). The difference in binding affinities are attributed to avidity effects of dimeric HJV.Fc (81). Interestingly, HJV appears to have a different ligand selectivity profile compared to other RGM family members based on the bioinhibition properties of soluble HJV.Fc protein (analogous to RGMa.Fc and DRAGON.Fc) for inhibiting signaling by various BMP ligands in a reporter assay. Whereas DRAGON.Fc is most potent as an inhibitor of BMP-2 and BMP-4, weakly inhibits BMP-5, BMP-6, and BMP-7, and does not inhibit BMP-9; HJV.Fc is most potent as an inhibitor of BMP-6 ligands, with a less strong inhibition of BMP-2, BMP-4, and BMP-5, and little or no inhibition of BMP-7 or BMP-9 (11,65). In a head-to-head comparison, DRAGON.Fc is a much more potent inhibitor of BMP-2 and BMP-4

compared with HJV.Fc, while HJV.Fc is a more potent inhibitor of BMP-6 compared with DRAGON.Fc (65). RGMa.Fc has a bioinhibition profile that is more similar to DRAGON.Fc (unpublished data). The exact binding affinity of HJV for the full range of BMP ligands has yet to be determined. Why DRAGON.Fc and HJV.Fc appear to have similar binding affinities by BIAcore assay, but different abilities to inhibit BMP-2 activation by a BMP responsive promoter assay remains to be determined.

Similar to other RGM family members, HJV signals via BMP type I and type II receptors and the SMAD intracellular signaling pathway (10,79). HJV-mediated BMP signaling is inhibited by siRNA-mediated inhibition of endogenous type I receptors ALK2, ALK3, and ALK6, suggesting that HJV can signal via all three of these BMP type I receptors (79). HJV can also form a complex with ALK6 in the presence of BMP-2 in a co-immunoprecipitation assay (10). Notably, human liver (the main site of HJV action in the regulation of hepcidin), expresses only ALK2 and ALK3. HJV-mediated BMP signaling is inhibited by siRNA-mediated inhibition of endogenous type II receptors BMPRII and ACTRIIA, but not ACTRIIB, in hepatoma-derived cells, suggesting that HJV signals via BMPRII and ACTRIIA, but not ACTRIIB (79). Interestingly, HJV allows BMP-2 and BMP-4, which normally prefer to signal via BMPRII alone, to signal via ACTRIIA. This ability to alter utilization of BMP type II receptors by BMP-2 and BMP-4 ligands appears to be a common property of all RGM family members (Figure 1). For HJV, this may be particularly important in its role as a co-receptor to enhance BMP signaling in the liver because ACTRIIA is the predominant BMP type II receptor expressed in human liver, and BMPRII is not expressed in human liver (79; see Fig 2).

Since HJV increases hepcidin expression and HJV is a BMP co-receptor, it was investigated whether the BMP signaling function of HJV was important for its ability to increase hepcidin expression. Mutant HJV associated with juvenile hemochromatosis has impaired ability to generate BMP signals as measured by BRE-Luc assay (10). Furthermore, livers from *Hjv* null mice exhibit decreased BMP signaling, as measured by phosphorylated SMAD 1/5/8 protein expression (10). Importantly, BMP ligands robustly increase hepcidin expression at the transcriptional level (10,11,82), and HJV enhances hepcidin induction by BMP ligands (10). Two BMP-responsive elements have been identified on the hepcidin promoter which appear to be important for basal and HJV/BMP-mediated hepcidin expression (83–86).

The importance of the BMP signaling pathway, mediated by HJV, in hepcidin regulation and iron metabolism *in vivo* has been confirmed by multiple lines of evidence. BMP-2 or BMP-6 administration into mice increases liver hepcidin expression and decreases serum iron (11,65). Administration of BMP inhibitors, including HJV.Fc (11) and the small molecule BMP type I receptor kinase inhibitor Dorsomorphin (102), inhibit hepcidin expression, mobilize reticuloendothelial cell iron stores, and increase serum iron levels *in vivo*. Mice with a conditional knockout of co-SMAD4 in the liver have low hepcidin levels and iron overload (87). More recently, *Bmp6* null mice have also been shown to have hepcidin deficiency and an iron overload phenotype resembling *Hjv* null mice (65, 88). Although BMP-2, BMP-4, and BMP-6 all can act as ligands for HJV *in vitro*, these data suggest that BMP-6 is the main endogenous ligand for HJV in the regulation of hepcidin expression and iron metabolism *in vivo*, at least in mice (see Figure 2) These data also suggest that activators of the BMP signaling pathway may have a role in treating iron overload disorders due to hepcidin deficiency, while BMP inhibitors may have a role as hepcidin lowering agents in the treatment of anemia of inflammation.

Among the RGMs, HJV has been best characterized in regards to its production and processing. HJV is transcribed from a gene of 4265 bp. Immunoblotting of isolated muscle

membrane proteins show three immunoreactive species of ~50, 35 and 20 KDa under reducing condition, and only the 50 KDa band under non-reducing conditions. These three bands have been identified as the full-length intact molecule of 50KDa, and a disulfide linked heterodimer composed of 35 and 20 KDa fragments, after autoproteolytic cleavage between aspartic acid and proline at amino acid 172 (in human HJV), joined together by disulfide bonds. (46,47,52). In addition to its cell associated form, soluble forms of HJV ~40 and 50 kDa have been detected in the conditioned media of transfected cells (47,89–91) These soluble forms have been identified as full-length HJV and HJV which has been cleaved by the proprotein convertase furin (89–91)

In vitro, full-length HJV, independent of the proteolytic cleavage, is released from the cell surface and accumulates in the extracellular medium with a half-life exceeding 24 hours, while the predominant membrane associated form (the disulfide-linked heterodimer), is not found in the extracellular fluid and it disappears rapidly from the cell surface after interruption of protein synthesis (47). In addition, cell transfected with the murine equivalent of the most common human mutation HJV-G320V appeared to have a radically different protein maturation process (47,92), and are less efficient (92) or fail to reach the plasma membrane (46). It is still unclear whether soluble HJV is independently secreted by the cell (90,92) or is a cleavage product of the membrane associated protein (89,91). Both pathways are potentially active. Although, there is some evidence of the existence of soluble HJV protein in the serum of humans and animal models (47,89,93,94) the quantity and physiologic relevance of soluble HJV protein is unknown. One hypothesis is that while the membrane form of HJV acts as a co-receptor to enhance the BMP signaling pathway (10), the soluble form of HJV antagonizes the BMP signaling pathway, either solely by removing the enhancing effects of the membrane form, or with the additive effect of binding to BMP ligands and preventing their interaction with cell surface signaling receptors (11,65,93). Consistent with this hypothesis, iron overload has been shown to reduce soluble HJV production *in vitro* (92,93), while iron deficiency increases soluble HJV levels both *in vitro* and *in vivo* (94). Hypoxia may also upregulate production of the soluble HJV *in vitro* (90).

In 2005, similarly to RGMa, HJV was found to interact with Neogenin by co-immunoprecipitation in embryonic human kidney 293 cells (46). Later, this interaction was shown to be necessary for the shedding of HJV, suggesting a role for Neogenin in limiting the effect of membrane HJV by promoting its release in the extracellular space as a soluble form (95). It has been demonstrated that Neogenin binds preferentially to the membrane associated heterodimeric form of HJV, while BMP-2 interacts primarily with the 40 kDa soluble species. Both Neogenin and BMP-2 appear able to bind the 50 kDa full-length membrane form, suggesting that different isoforms of HJV may have selective interactions with other proteins and play unique physiological roles (80). Recently it was shown that HJV-induced BMP signaling and hepcidin expression were not altered by neogenin overexpression or by inhibition of endogenous neogenin expression, indicating that HJV is able to mediate the BMP signaling independently of Neogenin (79). However, a contradictory report suggests that Neogenin is required for BMP-4 signaling by HJV (96). Further studies are needed in order to clarify the role of Neogenin, if any, in the BMP signaling pathway and in hepcidin expression and iron metabolism.

In addition, another protein, TMPRSS6 or Serine Protease Matriptase-2, has been shown involved in HJV processing (95). In humans and mice, TMPRSS6 mutations cause an iron-deficient anemia poorly responsive to enteral iron administration, associated with inappropriately high levels of hepcidin (97–99). Recently, in an overexpression system *in vitro*, TMPRSS6 has been shown to bind to and cleave membrane HJV, suggesting that HJV cleavage molecular mechanism by which TMPRSS6 downregulates hepcidin, although this awaits further confirmation in an *in vivo* system (95).

4.5 RGMd

Based on the phylogenetic trees, a novel gene belonging to the RGM family, named RGMd, has been identified only in fish (44). RGMd sequence shows the most similarity to RGMb (42). Since fish, and mainly *Zebrafish*, are fairly extensively used as research tools in the studies of RGMs and iron metabolism, this finding may contribute additional complexity to the zebrafish system.

Conclusions

Repulsive guidance molecules (RGMs) were first described in 2002, and were initially defined by their roles as adhesion proteins that guided developing neurons. RGM proteins have now been shown to be BMP co-receptors and to participate in BMP signaling in a variety of cell types. RGM mRNA and proteins have been found in many different tissues and organs and these molecules have been demonstrated to play a role in the development and healing of the central nervous system, in iron metabolism, and in the reproductive system. RGM proteins can bind BMP ligands in a selective and specific manner with high affinities ($K_d \sim 1$ to 5 nM). RGM proteins appear to alter the ability of BMP ligands to utilize BMP type II receptors, thus increasing the sensitivity of cells to low levels of endogenous BMP ligands. The exact role of RGM proteins, either as adhesion molecules or as BMP co-receptors, remain unknown for most tissues and organs in which they are expressed. Future research in this emerging field of study will undoubtedly prove to be as informative and as surprising as in the past several years.

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Biographies

Elena Corradini received her M.D. degree at the University of Modena and Reggio Emilia in Italy in 2001. From 2001 to 2006 she trained as an Internal Medicine Specialist and Hepatologist in the Internal Medicine Department and in the Center for Hemochromatosis and Hereditary Liver Diseases at the University Hospital of Modena, Italy. In 2008, she obtained a junior faculty member position at the University of Modena and Reggio Emilia.

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Herbert Y. Lin obtained his PhD from the Massachusetts Institute of Technology and the Whitehead Institute in 1993, and his MD degree from Harvard Medical School in 1995 from the combined Harvard/MIT Division of Health Science and Technology program. He worked briefly as a post-doctoral research fellow at the Hubrecht Laboratory in the Netherlands. Dr. Lin then completed his Internship, Residency and Clinical Fellowship in Nephrology at the Massachusetts General Hospital and Harvard Medical School. At present, he is an Associate Professor in Medicine and a practicing nephrologist at Harvard Medical School and the Massachusetts General Hospital, Division of Nephrology, Program in Membrane Biology, and Center for Systems Biology in Boston, Massachusetts, USA. His research interest focuses on the TGF- β /BMP signaling pathway in the kidney and in iron homeostasis.

Jodie L. Babitt received her M.D. degree from the combined Harvard Medical School/Massachusetts Institute of Technology, Division of Health Sciences and Technology. She completed her residency training at Beth Israel Deaconess Medical Center and a postdoctoral fellowship in Nephrology at the Massachusetts General Hospital. At present, she is an Instructor in Medicine and a practicing nephrologist at Harvard Medical School and the Massachusetts General Hospital, Division of Nephrology, Program in Membrane Biology, and Center for Systems Biology in Boston, Massachusetts, USA. She is a winner of the 2009 Marcel Simon Award for outstanding contributions to hemochromatosis given by the International BioIron Society. Her research interest focuses on the BMP signaling pathway and iron homeostasis.

Figure 1. Schematic diagram depicting the role of RGMs in enhancing the utilization of ActRIIA by BMP2 and BMP4 (modified from Xia Y. JBC 2007, ref. 62)

(*Top left*) In absence of an RGM, BMP2 and BMP4 ligands normally prefer to signal via the type II receptor BMPRII. BMP ligands bind to BMP type I receptors (Type I-R) and with type II receptors (BMPRII) to generate an active signaling complex. (*Top right*) In the presence of an RGM, BMP2 and BMP4 ligands signal through ActRIIA in addition to BMPRII, leading to an increased activation of the signaling cascade in cells. (*Top middle*) Upon formation of the receptor complex, type II receptors phosphorylate type I receptors, which then phosphorylate SMAD1, SMAD5, SMAD8 (R-SMAD). (*Bottom*) Phosphorylated R-SMADs form a complex with the common mediator SMAD4 (Co-SMAD), and then the SMAD complex translocates into the nucleus activating transcription of target genes.

Figure 2. Schematic diagram depicting the role of HJV/RGMc in the BMP signaling regulation of hepcidin in the hepatocytes (modified from Babitt J. L. Nature Genetics 2006, ref. 10 and Xia Y., ref 79)

On the hepatocyte plasma membrane, HJV interacts with BMP6 ligand and with BMP type I receptors (ALK2 and ALK3) and type II receptors (ActRIIA) to generate an active signaling complex to increase transcription of hepcidin mRNA.

Table 1

BMP signaling modulators.

<i>Extracellular modulators</i>	<ul style="list-style-type: none"> ■ BMP Antagonists ■ BMP Agonists ■ Extracellular Matrix Components
<i>Membrane modulators</i>	<ul style="list-style-type: none"> ■ Pseudoreceptors ■ BMP Co-Receptors (e.g. RGMs)
<i>Intracellular modulators</i>	<ul style="list-style-type: none"> ■ DNA-binding proteins ■ R-SMAD/Co-SMAD transcriptional co-activators and corepressors ■ Inhibitory SMADs (SMAD6 and SMAD7) and other partner proteins which modulate phosphorylation, dephosphorylation, shuttling, and degradation of the BMP receptors and SMADs ■ MicroRNAs ■ Cross-talks with other signaling pathways

Table 2

RGMs in physiopathology.

	RGMa	RGMb/DRAGON	RGMc/HFE2/HJV
Knockout mouse models	<i>Defect in cephalic neural tube closure</i> (52)	<i>Early post-natal death</i> , without evident defect in sensory motor functions or nervous system development (45)	<i>Hemochromatosis</i> (77,78)
Rodent pathologic models	<ul style="list-style-type: none"> <i>Spinal cord injury</i> model in rat: lesional, perilesional, and scar expression of RGMa (56) <i>Glaucoma</i> model in mouse: retinal m-RNA upregulation (67) 	<ul style="list-style-type: none"> <i>Spinal cord injury</i> model in rat: lesional, perilesional, and scar expression of RGMa (68) <i>Glaucoma</i> model in mouse: retinal m-RNA upregulation (67) 	<i>Acute inflammation</i> model in mice (<i>LPS</i> , <i>IL-6</i> , <i>TNF-α</i>): hepatic mRNA downregulation (78,100)
Human pathologic conditions	<ul style="list-style-type: none"> <i>Focal cerebral ischemia</i> and <i>Brain traumatic injury</i>: lesional, perilesional, and scar expression of RGMa (57). 	Component of the signature expression profiles correlating with disease activity in <i>rheumatoid arthritis</i> (101)	<i>Juvenile form of hemochromatosis</i> associated with HJV mutations (69)