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Repulsive Guidance Molecules (RGMs) and Neogenin in Bone Morphogenetic Protein (BMP) signaling

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Summary

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor-beta (TGFβ) superfamily. BMPs mediate a highly conserved signal transduction cascade through the type I and type II serine/threonine kinase receptors and intracellular Smad proteins. The BMP pathway regulates multiple developmental and homeostatic processes. Mutations in this pathway can cause various diseases in humans, such as skeletal disorders, cardiovascular diseases and various cancers. Multiple levels of regulation, including extracellular regulation, help to ensure proper spatiotemporal control of BMP signaling in the right cellular context. The family of repulsive guidance molecules (RGMs) and the type I trans-membrane protein neogenin, a paralog of DCC (Deleted in Colorectal Cancer), have been implicated in modulating the BMP pathway. In this review, we discuss the properties and functions of RGM proteins and neogenin, focusing on their roles in the modulation of BMP signal transduction.

Keywords

RGM; neogenin; BMP; HJV; DRAG-1; TGFβ

The BMP signal transduction pathway

Bone morphogenetic proteins (BMPs) were first discovered in 1965, when Marshall Urist found that dead bone matrix fragments have bone-inducing activity (Urist. 1965). It's not until the late 1980s when the proteins responsible for bone induction were purified and their genes cloned (Wozney et al. 1988) using an ectopic bone induction assay developed in 1981 (Sampath and Reddi. 1981). The identified molecules, called BMP2 through BMP7, belong to the transforming growth factor β (TGFβ) superfamily (Wozney. 1992). The BMP subfamily is known to contain over 20 members (Bragdon et al. 2011). BMPs have been shown to regulate a wide variety of cellular processes including cell fate specification, cell proliferation, cell migration, and cell death during the development of multi-cellular organisms (Wu and Hill. 2009). Malfunction of the pathway causes many somatic and hereditary disorders in humans, including skeletal abnormalities, cardiovascular diseases and various types of cancers (Gordon and Blobe. 2008,Massague. 2012,Cai et al. 2012).

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The BMP signal transduction pathway has been well studied. The ligand is generated as a pre-protein containing a N-terminal prodomain and a C-terminal mature domain that is bioactive. The prodomain is then cleaved off by serine endoproteases such as furin in the secretory pathway. The resulting active forms of ligands form dimers that are stabilized by hydrophobic interactions and an inter-subunit disulfide bond (Shi and Massague. 2003). The BMP receptors belong to the receptor serine/threonine kinase family that is divided into two classes: type I and type II (Attisano et al. 1994,Massague. 2012,Xu et al. 2012). Both types of receptors are type I trans-membrane proteins, comprised of an extracellular domain, a trans-membrane region, and a C-terminal serine/threonine kinase domain. The extracellular domains of type I and type II receptors are capable of binding to the ligands. Unlike the TGFβ subfamily of ligands that display a higher affinity for the type II receptors than the type I receptors, BMP ligands, such as BMP-2, exhibit a higher affinity for the type I receptors and a lower affinity for the type II receptors (Groppe et al. 2002,Allendorph et al. 2006). So far, four type I receptors (ALK3/BMPR1A, ALK6/BMPRIB, ALK1/Acvrl1 and ALK2/ActRI,) and three type II receptors (BMPRII, ActRIIA and ActRIIB) have been described to function in the BMP pathway. The type I, but not the type II, receptors contain a characteristic SGSGSG sequence, also known as the GS domain, which is located N-terminal to the kinase domain. Once the ligand binding brings the type I and type II receptors to close proximity, the constitutively active type II receptor phosphorylates type I receptor at its GS domain (Shi and Massague. 2003).

The activated type I receptor is poised to relay the BMP signal from the cytoplasm to the nucleus through the Smad proteins (Feng and Derynck. 2005,Heldin and Moustakas. 2012). Two classes of Smad proteins are required for signal transduction: receptor regulated Smads (R-Smads) and common mediator Smads (co-Smads). R-Smads are directly phosphorylated at the C-terminal Ser-X-Ser (SXS) motif by the type I receptors. The phosphorylated R-Smads then complex with the constitutively phosphorylated Co-Smad (Nakao et al. 1997,Roelen et al. 2003) and enter the nucleus, where the complex incorporates different DNA-binding cofactors to regulate downstream gene expression (Feng and Derynck. 2005, Massague. 2012). A third class of Smads, the inhibitory Smads, can associate with type I receptors and competitively block R-Smad recruitment and phosphorylation (Feng and Derynck. 2005). Among the R-Smads, the BMP subfamily utilizes Smads 1, 5 and 8, while the TGFβ subfamily utilizes Smad2 and Smad3. In addition to the Smad pathway, BMP signals can also activate other “non-canonical” or non-Smad signaling pathways, such as the P38 MAPK pathway, that complement Smad function (Massague. 2012).

Modulation of BMP signaling at the ligand-receptor level

Because of its various roles in development and homeostasis, the BMP pathway is modulated at multiple levels, including receptor activation outside the cell, Smad access to the receptors in the cytoplasm, Smad nucleocytoplasmic shuttling, and Smad-dependent transcription in the nucleus (Balemans and Van Hul. 2002,Umulis et al. 2009,Huang and Chen. 2012,Massague. 2012,Ramel and Hill. 2012).

A number of proteins bind to the ligand, receptors and/or ligand-receptor complexes to regulate BMP signaling at the level of receptor activation. One group of them is the

extracellular secreted proteins that can function as ligand binding traps (Balemans and Van Hul. 2002,Shi and Massague. 2003). These include Noggin, Follistatin, Chordin/Short gastrulation (Sog), members of the Gremlin/Cerberus/DAN/Coco family and matrix GLA protein (MGP) (Balemans and Van Hul. 2002,Zakin and De Robertis. 2010,Cai et al. 2012). Noggin and Follistatin are probably the best known BMP antagonists. They sequester the ligands and block the ligands from binding to the type I and II receptors (Groppe et al. 2002). MGP appears to function in a similar way (Zebboudj et al. 2002). Chordin and its Drosophila homolog Sog can form a complex with Tsg (Twisted gastrulation). This complex sequesters BMPs to attenuate BMP signaling locally but enhances long range BMP signaling by transporting BMPs through tissues (O'Connor et al. 2006). Crossveinless 2 (CV2), which is also referred to as BMPER (BMP endothelial cell precursor derived regulator), can activate or inhibit BMP signaling by directly binding to the BMP ligands, Chordin or the Chordin-BMP complex (Ambrosio et al. 2008,Serpe et al. 2008,Zhang et al. 2008). Members of the Gremlin/Cerberus/DAN/Coco family of proteins are multivalent BMP inhibitors that also affect Nodal and Wnt signaling pathways (Hsu et al. 1998,Avsian-Kretchmer and Hsueh. 2004).

A number of membrane or membrane-associated proteins are also important modulators of BMP signaling. The extracellular matrix (ECM) protein, Type IV Collagen, has been shown to bind to the Drosophila BMP homolog Dpp (Decapentaplegic) and affect the formation of the Dpp morphogenetic gradient (Wang et al. 2008). In both Drosophila and *C. elegans*, Dally and LON-2, members of glycosylphosphatidylinositol (GPI)-anchored proteoglycan family, can bind to the BMP ligands and modulate BMP signaling in vivo. In particular, Dally positively regulates the distribution of the Dpp morphogen in Drosophila (Grisaru et al. 2001,Han et al. 2005,Takeo et al. 2005), while LON-2 functions as a negative regulator of the BMP-like Sma/Mab pathway in *C. elegans* (Gumienny et al. 2007,Taneja-Bageshwar and Gumienny. 2012). Betaglycan, also known as the TGFβ type III receptor, not only binds to TGFβ ligands and helps present them to the type II receptors, but also binds to BMP molecules and increases BMP-mediated signaling output (Kirkbride et al. 2008,Bilandzic and Stenvers. 2011). Similarly, endoglin, another glycoprotein that acts as a TGFβ co-receptor, can potentiate BMP signaling (Scherner et al. 2007). BMPs can also interact with the decoy receptor BAMBI (BMP and activin membrane-bound inhibitor), which resembles the type I receptors but lacks an active kinase domain. BMP-BAMBI interaction leads to inhibition of BMP signaling by sequestering the BMP ligands from the active receptors (Onichtchouk et al. 1999). More recently, RGM (Repulsive Guidance Molecule) proteins and a RGM interaction protein neogenin have been implicated in the modulation of BMP signaling. RGMs and neogenin will be the focus of the reminder of this article.

The roles of RGM proteins in modulating BMP signaling

Introduction

RGM proteins are named after the discovery of RGMa, which is expressed in a gradient in the optic tectum in chick embryos and functions as a repulsive guidance molecule for the temporal retinal axons (Monnier et al. 2002). It is now known that the RGM family members in vertebrates include RGMa, RGMb and RGMc/Hemojuvelin (HJV), while

invertebrates, such as *C. elegans* and sea urchin, only have a single RGM protein encoded in each genome (Camus and Lambert. 2007). The three vertebrate RGMs share approximately 40-50% identity in primary amino acid sequences. They share similarities in predicted protein domains and overall structure, as inferred by *ab initio* molecular modeling (Severyn et al. 2009). Specifically, all RGMs have an N-terminal signal peptide, a partial von Willebrand type D domain (vWF-type D), which includes a highly conserved autoproteolysis site, and a C-terminal GPI (Glycosylphosphatidylinositol)-anchor. RGMa and RGMc/HJV, but not RGMb, have a RGD motif. RGM proteins also contain a number of highly conserved cysteine residues (Figure 1). RGMa and RGMc have been shown to go through autoproteolytic cleavage at a conserved FGDPH site to generate two fragments that are linked by disulfide bonds (Zhang et al. 2005,Tassew et al. 2012). RGMa and RGMc/HJV also undergo additional processing (more discussion later). Recent studies have shown that RGM proteins function as BMP co-receptors and may allow cells to selectively respond to low levels of BMP ligands (Corradini et al. 2009,Severyn et al. 2009).

RGMa

RGMa was the first discovered RGM family member (Monnier et al. 2002) and it has a broad expression pattern during mouse development (Corradini et al. 2009). RGMa is expressed in the central nervous system in a mostly non-overlapping fashion with RGMb (Oldekamp et al. 2004,Schmidtmer and Engelkamp. 2004,van den Heuvel et al. 2013). It is also expressed in the developing mouse cochlea, lung, limb, and gut (Oldekamp et al. 2004,Metzger et al. 2005). In the adult mouse, RGMa expression is found in the brain, skin, heart, liver, lung, kidney, testis and gut (Babitt et al. 2005,Metzger et al. 2005,Corradini et al. 2009,van den Heuvel et al. 2013). The broad expression pattern of RGMa in the developing mouse embryo suggests that it likely plays important roles in development, especially in central nervous system development. In fact, a large fraction of RGMa knockout mice die due to failure of neural tube closure (Niederkofler et al. 2004). RGMa has also been found to function in various other processes, such as axon guidance in the developing visual system and in axon tract formation in the embryonic brain *in vivo* (Monnier et al. 2002,Matsunaga et al. 2004,Tassew et al. 2008), in neural tube closure (Kee et al. 2008), neuronal differentiation (Matsunaga et al. 2006), cell survival (Koeberle et al. 2010), as well as cell migration and adhesion (Lah and Key. 2012). Neogenin, as a receptor for RGMa that functions in cells abutting those expressing RGMa, is involved in all the processes described above (see more on neogenin below). Recently, RGMa has also been implicated in colorectal, prostate, and breast cancer (Li et al. 2011,Li et al. 2012,Zhao et al. 2012), and in the immune response (Nohra et al. 2010,Muramatsu et al. 2011,Mirakaj et al. 2011).

RGMa was found to be a BMP co-receptor *in vitro* only after its homolog RGMb was found to be so (Samad et al. 2005). Both overexpression in LLC-PK1 porcine kidney epithelial cells (Babitt et al. 2005) and siRNA knockdown in mouse myoblast C2C12 cells (Halbrooks et al. 2007) showed that RGMa specifically enhances BMP signaling activity through the canonical BMP signaling pathway, but not the TGFβ signaling pathway. The enhancement depends on BMP ligands, because it was blocked by co-transfection of Noggin, a BMP inhibitor, or BMP2 and BMP4 neutralizing antibodies (Babitt et al. 2005). Besides the

requirement of the ligand by RGMa, BMP type I receptors ALK3 and ALK6 as well as Smad1, 5, and 8 are also involved, because dominant negative forms of ALK3 and ALK6 blocked the RGMa-induced enhancement of both BMP-responsive reporter expression and Smad1/5/8 phosphorylation (Babitt et al. 2005).

Purified RGMa.Fc (the extracellular domain of RGMa fused to the Fc portion of human IgG) protein binds directly and selectively to ^{125}I-BMP2 and ^{125}I-BMP4 with a K_D of 2.4 nM and 1.4nM, respectively, but not to BMP7 or TGFβ1 ligands (Babitt et al. 2005,Halbrooks et al. 2007,Xia et al. 2007). Results from two Surface Plasmon Resonance Biacore assays also supported direct binding between RGMa and different BMP molecules, the binding of RGMa.Fc to BMP2 has a K_D ~2.46nM (Babitt et al. 2005,Halbrooks et al. 2007) and the binding between RGMa and BMP2 has a K_D ~22nM (Wu et al. 2012). The different results obtained from the two Biocore assays are likely due to the fact that RGMa.Fc fusion proteins form dimers, while the free RGMa proteins act as monomers (Wu et al. 2012). In addition to the BMP ligands, RGMa has also been found to bind to the BMP type I receptor ALK6 (Babitt et al. 2005). RGMa.Fc binds to ALK6.Fc with or without the presence of BMP2, and the binding between RGMa and ALK6 increased the binding of ^{125}I-BMP2 to ALK6 (Babitt et al. 2005). However, there is no evidence that RGMa binds to any of the type II receptors in BMP signaling.

RGMa may function to allow cells to respond to low levels of BMP ligands by changing the utilization of type II receptors from BMPRII alone to both BMPRII and ActRIIA (Xia et al. 2007). In both human ovarian granulosa KGN cells and mouse pulmonary artery smooth muscle cells, where BMP2 and BMP4 signaling is primarily transduced by BMPRII but not ActRIIA or ActRIIB, both BMPRII and ActRIIA are being used to transduce BMP2 and BMP4 signaling when these cells are transfected with RGMa. Furthermore, the addition of RGMa not only increased the binding of ^{125}I-BMP2 to the BMP type I receptor ALK3.Fc, but also increased the binding of ^{125}I-BMP2 to the type II receptor ActRIIA.Fc in the presence of ALK3.Fc (Xia et al. 2007).

Despite the above in vitro studies, there is no *in vivo* evidence that RGMa functions as a BMP co-receptor. As described earlier, RGMa has been implicated in a diverse array of functions during development. However, it is not know which, if any, of these RGMa-mediated processes is due to the potentiation of BMP signaling by RGMa. For example, BMP signaling occurs in neurons of the mouse adult spinal cord that expresses RGMa (Babitt et al. 2005), however, it is not known if RGMa functions by mediating BMP signaling there. Many of the processes that RGMa is involved in require the function of the RGM receptor neogenin (discussed more later). However, it is not known whether both proteins function together via BMP signaling to regulate these processes in vivo.

RGMb

RGMb is currently less well characterized than RGMa. RGMb, also known as DRAGON, was first identified by using a genomic DNA-binding array to identify downstream genes controlled by DRG11, a homobox transcription factor expressed in embryonic dorsal root ganglion (DRG) (Samad et al. 2004). In the developing brain, RGMa and RGMb exhibit non-overlapping expression patterns. Unlike RGMa, RGMb does not have any detectable

repulsive guidance role in embryonic and neonatal DRG neuritis (Samad et al. 2004). RGMb expression is also found in the brain, bone, heart, lung, liver, kidney, testis, ovary, uterus, epididymis and pituitary in adult mouse (Corradini et al. 2009). Because of this wide expression of RGMb, it is not surprising that RGMb knockout mice die 2-3 weeks after birth (Xia et al. 2011). Even though the exact cause of this lethality has not been determined, several studies have provided in vitro and in vivo evidence supporting the functions of RGMb in the nervous system and the immune system: RGMb controls aggregation and migration of neogenin-positive dentate precursor cells (Conrad et al. 2010), promotes neurite outgrowth and peripheral nerve regeneration (Liu et al. 2009,Ma et al. 2011), and negatively regulates IL-6 expression in macrophage cells (Xia et al. 2011). Furthermore, RGMb appears to be a negative regulator of breast cancer proliferation, adhesion, and migration *in vitro* (Li et al. 2012).

RGMb was the first RGM family member shown to be a BMP co-receptor. The reason to test its involvement in BMP signaling is due to the shared expression pattern between RGMb and the BMP type I and type II receptors in the developing mouse and *Xenopus* embryos (Samad et al. 2005,Babitt et al. 2005). Similar to RGMa, RGMb enhances BMP signaling but not TGFβ signaling in multiple cell lines, including kidney epithelial LLC-PK1 cells, 10 T1/2 cells, and human liver-derived HepG2 cells, and in *Xenopus* embryos (Samad et al. 2005), while siRNA treatment of RGMb inhibits BMP signaling in C2C12 cells (Halbrooks et al. 2007). Like RGMa, RGMb enhancement of BMP signaling, as shown in kidney epithelial LLC-PK1 cells, is ligand-dependent and through the canonical BMP signaling pathway (Samad et al. 2005). Also like RGMa, RGMb.Fc binds directly to ^{125}I-BMP2 ($K_D \sim 1.5$nM) and this binding can be competed by the addition of unlabeled BMP2 and BMP4, but not BMP7 or TGFβ (Samad et al. 2005). Biacore assays confirmed that both RGMb.Fc and RGMb bind directly to BMP2 and BMP4 with similar binding affinities ($K_D \sim 5.5$ nM for BMP2 and $K_D \sim 2.6$nM for BMP4 (Wu et al. 2012). RGMb also physically associates with BMP type I receptors (ALK2, ALK3 and ALK6) and BMP type II receptors (ActRII and ActRIIB) as indicated by co-immunoprecipitation (co-IP) experiments when these components are expressed in HEK293T cells (Samad et al. 2005). The function of RGMb to promote BMP signaling requires its membrane association, as RGMb with the C-terminal GPI anchor deleted, failed to increase BMP signaling (Samad et al. 2005).

While most of the studies have shown that RGMb positively promotes BMP signaling, one study showed that RGMb inhibits constitutively active BMP receptor-induced, or constitutively activated Smad1-induced, BMP signaling in C2C12 myoblasts (Kanomata et al. 2009). This inhibitory effect of RGMb on BMP signaling seems to be via a novel mechanism. How RGMb mediates this inhibition and the functional significance of this inhibition need to be further investigated.

The *in vivo* relevance of RGMb as a BMP co-receptor has just started to be discovered. As described earlier, RGMb negatively regulates the expression of IL-6 expression specifically in macrophage cells in vitro and in vivo. RGMb knockout mice have increased IL-6 expression in the lungs and liver (Xia et al. 2011). Interestingly, this BMP-dependent regulation of IL-6 expression by RGMb appears to be through the non-canonical P38 MAPK and Erk1/2 pathway, rather than the canonical Smad1/5/8 pathway (Xia et al. 2011). Further

studies are needed to determine the mechanistic basis of this regulation. RGMb also promotes neurite outgrowth and peripheral nerve regeneration by modulating BMP signaling (Ma et al. 2011). DRG (dorsal root ganglion) explants from RGMb knockout mice exhibit reduced neurite outgrowth, which can be rescued by BMP2. In addition, the BMP inhibitor Noggin inhibits neurite outgrowth in wild-type DRG explants. Reduced BMP signaling and axonal regeneration have also been observed in vivo in RGMb knockout mice or by Noggin treatment in wild-type mice (Ma et al. 2011). Whether this function is mediated via the canonical Smad1/5/8 pathway or the non-canonical P38 MAPK pathway is not clear. Like RGMa, RGMb also physically associates with neogenin (Conrad et al. 2010). It is not known if neogenin functions together with RGMb in these processes to mediate BMP signaling.

RGMc/HJV

RGMc was identified by positional cloning of the locus that is associated with juvenile hemochromatosis (JH) (Papanikolaou et al. 2004). JH is a rare autosomal recessive disease characterized by high penetrance, early-onset systemic iron overload that affects young patients and leads to severe clinical complications typically in the first and second decade of life (De Gobbi et al. 2002,Camaschella et al. 2002,De Domenico et al. 2008a). JH is also caused by the lack of functional hepcidin (Roetto et al. 2003). Hepcidin is a defensin-like small peptide secreted predominantly from hepatocytes that is essential for iron homeostasis (Park et al. 2001,Pigeon et al. 2001). It acts by binding to and degrading Ferroportin, a rate-limiting sole iron exporter (De Domenico et al. 2008b). Hepcidin expression is up-regulated by high iron level in the body; therefore it is a feedback regulator of iron absorption (Pigeon et al. 2001,Kautz et al. 2008).

HJV is both required and sufficient for hepcidin expression. JH patients with HJV mutations exhibit a reduction of hepatic hepcidin expression, which results in severe iron accumulation in liver, heart and pancreas (Papanikolaou et al. 2004). *Hjv-/-* mice exhibit a similarly low hepcidin mRNA expression and iron deposition in liver, heart and pancreas (Huang et al. 2005). Transfection of hepatoma-derived Hep3B cells with *HJV* cDNA increases hepcidin mRNA expression and hepcidin promoter activity in a luciferase assay (Babitt et al. 2006). Introducing *HJV* into the hepatocytes of *Hjv-/-* mice using adeno-associated virus 2/8 as a vector is sufficient to completely restore the decreased hepatic hepcidin expression and to lower the high serum iron level to the wild type level (Zhang et al. 2010). Consistent with HJV regulating hepcidin expression in the liver, *HJV* is expressed predominantly in the liver, skeletal muscle and heart (Niederkofler et al. 2004,Papanikolaou et al. 2004,Samad et al. 2004,Rodriguez et al. 2007). The functional significance for skeletal muscle and heart-expressed HJV is currently not fully understood. Skeletal muscle-expressed HJV has been suggested to be a reservoir for soluble HJV (Zhang et al. 2010; see section on HJV processing). However, skeletal muscle specific knockout of HJV led to no change in hepcidin expression or systemic iron homeostasis (Gkouvatsos et al. 2011,Chen et al. 2011), suggesting that muscle HJV is dispensable for iron metabolism.

Like other RGM family members, HJV functions as a co-receptor in BMP signaling. Transfection of HJV into hepatoma-derived HepG2 and Hep3B cells enhances BMP, but not

TGFβ, signaling activity (Babitt et al. 2005,Halbrooks et al. 2007). siRNA knockdown of HJV in C2C12 myoblast reduces BMP2 signaling (Babitt et al. 2005,Halbrooks et al. 2007). Similar to RGMa and RGMb, HJV's role in potentiating BMP signaling is dependent on the BMP ligand, because HJV-induced BMP signaling is abolished by the BMP inhibitor Noggin, and by BMP2 and BMP4 neutralizing antibodies (Babitt et al. 2006). Like RGMa and RGMb, purified HJV.Fc directly binds to ^{125}I-BMP2 and ^{125}I-BMP4, and this binding can be competed away by excess of unlabeled BMP2 and BMP4, but not by BMP7 or TGFβ1 (Babitt et al. 2006). HJV.Fc also directly binds to BMP6 (Andriopoulos et al. 2009). Several Surface Plasmon Resonance Biocore assays have shown that HJV can bind to multiple BMP ligands with high but differential affinity and that the dimeric form of HJV appears to have a stronger affinity to BMP than the monomeric form (Halbrooks et al. 2007,Yang et al. 2008,Wu et al. 2012). In particular, HJV exhibits the highest affinity for BMP6 ($K_D \sim 8.1$nM), and this binding affinity is much higher than those of RGMb-BMP6 binding ($K_D \sim 28$nM) and of RGMa-BMP6 binding ($K_D \sim 55$nM) (Wu et al. 2012). HJV also binds BMP5 ($K_D \sim 17$nM) and BMP7 ($K_D \sim 20$nM) with higher affinities higher than those of RGMb and RGMa ($K_D \sim 33$nM for RGMb-BMP5, ∼83nM for RGMa-BMP5, 166nM for RGMb-BMP7, and 63nM for RGMa-BMP7) (Wu et al. 2012). In addition to binding to the ligand, HJV can form a complex with the type I receptor ALK6 in the presence of BMP2, as indicated by a co-IP assay in HEK293 cells (Babitt et al. 2006). By using siRNA knockdown of different BMP ligands, type I or type II receptors in various cell lines, including Hep3B, Huh-7 and HepG2 cells, and examining the expression of these different ligands and receptors in human liver, Xia and colleagues suggested that HJV likely uses the ligands BMP2, BMP4 and BMP6, the type I receptors ALK2 and ALK3, and the type II receptor ActRIIA in vivo (Xia et al. 2008).

Results from multiple studies have convincingly shown that the underlying mechanism of HJV's role in regulating hepcidin expression is through BMP signaling. Multiple BMP ligands are able to increase hepcidin expression through phosphorylated Smad1/5/8 signaling in hepatocytes in vitro (Babitt et al. 2006,Truksa et al. 2006,Andriopoulos et al. 2009). Furthermore, BMP2-induced hepcidin expression is enhanced by HJV (Babitt et al. 2006). Babitt and colleagues also showed that while wild-type HJV can increase hepcidin expression, HJV carrying JH disease-causing mutations failed to increase hepcidin expression, in liver cells (Babitt et al. 2006). Consistent with these *in vitro* data, *Hjv-/-* mice have reduced hepatic hepcidin expression and deceased level of liver pSmad, an indication of reduced BMP signaling activity. Hepatic expression of HJV in the liver of *Hjv-/-* mice leads to increased hepcidin expression and pSmad1/5/8 level (Zhang et al. 2010). The critical role of BMP signaling in regulating hepcidin expression and iron homeostasis is further supported by the reports that mutations in the genes encoding the ligand BMP6, the type I BMP receptors ALK2 and ALK3, and the co-Smad SMAD4, all result in hepcidin deficiency and iron overload (Wang et al. 2005,Andriopoulos et al. 2009,Meynard et al. 2009,Steinbicker et al. 2011).

In addition to binding to the BMP ligands and receptors, RGMc/HJV also binds to neogenin. The relationship between RGM, neogenin and BMP signaling will be discussed in more detail in the “neogenin” section of this article.

HJV post-translational processing and significance

Because of the role of HJV in regulating iron homeostasis and the fact that mutations in HJV cause JH, HJV has been intensively studied since its discovery. Much of the analysis has been based on HJV proteins over produced in different mammalian cell lines. These studies showed that the HJV protein undergoes complex post-translational modification and processing. It has three predicted N-glycosylation sites. The muscle-expressed and liver-expressed HJV proteins are differentially glycosylated (Fujikura et al. 2011). However, the functional significance for this differential glycosylation is unknown.

Under reducing conditions, cell membrane localized HJV proteins that are overexpressed in HEK293 cells have three major species, with apparent molecular weight of 50, 35, and 20kD respectively. Under non-reducing conditions, HJV only runs as one 50kD band, which corresponds to the full-length form. The 35kD and 20kD species are from an intracellular autoproteolytic cleavage event that happens in a mildly acidic environment. The cleavage site is located in a conserved FGDPH sequence motif in the vWF type D domain of HJV between amino acid 172D (aspartic acid) and 173P (proline), and the resulting two polypeptides are joined together by disulfide bonds to form a two-chain form (Zhang et al. 2005,Kuninger et al. 2006). The membrane-bound two-chain species of HJV is the predominant isoform (Kuninger et al. 2006,Silvestri et al. 2007). Several JH disease-causing mutations appear to affect this autocleavage event. These mutations include those that lie in or near the cleavage site (D172E, F170C and W191C), as well as one predominant disease causing mutation G320V (Silvestri et al. 2007,Pagani et al. 2008). It has been found that these mutant forms of HJV are retained in the endoplasmic reticulum and have low signaling activities, while other disease causing mutations, such as G99V and C119F, that have no effect on HJV autocleavage but have reduced signaling activities do reach the plasma membrane (Silvestri et al. 2007). These studies suggest that HJV cleavage is essential for their export to the plasma membrane.

HJV also undergoes active cleavage by the furin proprotein convertase, resulting in a soluble form of cleaved HJV (s-HJV) that has a molecular mass of ∼40kD (Kuninger et al. 2008,Lin et al. 2008,Silvestri et al. 2008). The cleavage site has been mapped to a RNRR sequence that is not conserved in RGMa and RGMb (Lin et al. 2008,Silvestri et al. 2008). Furin is predominantly localized in the trans-Golgi network (TGN) compartment, with only a small portion of it being on the plasma membrane (Thomas. 2002). Consistent with this, it has been shown that HJV cleavage occurs in the TGN after full-length HJV is being endocytosed from the plasma membrane (Maxson et al. 2009). Furthermore, the binding of neogenin to HJV on the plasma membrane triggers the retrograde trafficking and subsequent release of soluble HJV, s-HJV (Zhang et al. 2008). s-HJV is present not only in transfected cell lines and cell lines that endogenously express HJV, but also in human and rat serum (Kuninger et al. 2004,Lin et al. 2005,Silvestri et al. 2007,Zhang et al. 2007). However, concerns have been raised regarding a commercial assay used to measure s-HJV in humans (Gutiérrez et al. 2012).

s-HJV is an inhibitor of BMP signaling by binding to BMP2, BMP4, and BMP6 to suppress hepcidin expression *in vitro* (Lin et al. 2007,Kuns-Hashimoto et al. 2008,Andriopoulos et al.

2009,Nili et al. 2010). Injection of s-HJV decreases hepatic hepcidin mRNA level *in vivo* (Babitt et al. 2007). s-HJV is hypothesized to compete with hepatocyte membrane-bound HJV for BMP ligands, acting as a negative regulator of the BMP-mediated hepcidin expression. Because hepcidin expression is positively regulated by body iron load, it is reasonable to hypothesize that body iron load may negatively regulate the amount of s-HJV. In fact, s-HJV release is negatively regulated by iron level *in vitro* (Lin et al. 2005,Silvestri et al. 2007,Zhang et al. 2007). In acutely iron-deficient rats, decreased serum Transferrin and Ferritin saturation is associated with decreased hepcidin expression and increased serum s-HJV level (Zhang et al. 2007). Therefore, s-HJV is a negative regulator of hepcidin expression in response to body iron status.

A type II transmembrane serine protease matriptase-2 (MT2), encoded by the gene *TMPRSS6*, is also involved in regulating body iron load (Silvestri et al. 2008). In mice and zebrafish, MT2 mutations cause hepatic hepcidin overexpression and iron-deficient anemia (Du et al. 2008,Folgueras et al. 2008,Silvestri et al. 2008,Ramsay et al. 2009). In humans, mutations in *TMPRSS6* cause iron-refractory iron deficiency anemia (IRIDA), a familial anemia disorder (Finberg et al. 2008). MT2 and HJV are co-expressed in the liver (Velasco et al. 2002). Co-IP experiments in HeLa cells suggest that they physically interact with each other (Silvestri et al. 2008). This interaction results in the cleavage of cellular HJV into many fragments that are released to the media (Silvestri et al. 2008). Analysis of double mutants between *Hjv-/-* and *Tmprss6-/-* or a catalytic mutation $Tmprss6^{mask}$ showed that the double mutants exhibit the *Hjv-/-* single mutant phenotypes, suggesting that MT2 acts through HJV in regulating hepcidin expression and iron homeostasis (Truksa et al. 2009,Finberg et al. 2010). Consistent with the genetic analysis, over-expression of MT2 can inhibit HJV- and BMP-induced hepcidin expression (Du et al. 2008,Silvestri et al. 2008). However, the *Hjv-/-*; *Tmprss6-/-* double mutant phenotype is also consistent with the hypothesis that *Tmprss6* functions in a separate pathway from, but is also dependent upon, the HJV pathway for full induction of hepcidin. Consistent with this hypothesis, *Tmprss6* mutations that have lost their protease activity can still down regulate hepcidin expression when expressed in HeLa cells and the hepatocyte cell line Huh7, suggesting that the serine protease activity of MT2 may not be important for the regulation of *Tmprss6* on hepcidin (Guillem et al. 2012). Moreover, liver membrane HJV level is decreased rather than increased in *Tmprss6-/-* mutant mice, suggesting that the relationship between HJV and MT2 is more complex *in vivo* (Krijt et al. 2011). *Tmprss6* mRNA expression is stimulated by iron loading treatment or BMP6 protein injection and is blocked by injection of neutralizing antibody against BMP6 in mice (Meynard et al. 2011). Since iron induces hepcidin expression, the up-regulation of MT2, a negative regulator of hepcidin, by iron indicates that MT2 likely represents a negative feedback regulating mechanism to balance hepcidin overexpression induced by iron to maintain systemic iron homeostasis. Further experiments are needed to determine whether MT2 is required for cleaving HJV in vivo, and what the precise relationships among MT2, HJV, BMP signaling and hepcidin expression are.

In addition to RGMc/HJV, processing of RGMa has also been observed (Tassew et al. 2012). Tassew and colleagues showed that RGMa is N-glycosylated, processed by

autocleavage and by proprotein convertases SKI-1 (Subtilisin Kexon Isozyme-1) and Furin. These cleavages together with the formation of a disulfide bridge result in the production of 4 membrane-bound and 3 soluble RGMa species, which are all capable of inhibiting neurite outgrowth via neogenin in vitro. The in vivo significance of these cleavages and their relationship with BMP signaling are not known. It's also unclear whether RGMb undergoes similar complicated post-translational processing.

RGM protein function in invertebrates: the sole C. elegans RGM protein is a positive modulator of a BMP-like signaling pathway

RGM proteins are not only found in vertebrates, but also found in invertebrates, such as mollusks, echinoderms and nematodes, which contain one RGM homolog in each corresponding genome (Camus and Lambert. 2007). Surprisingly, no RGM sequence is found in the *Drosophila* genome (Camus and Lambert. 2007). *C. elegans* RGM protein DRAG-1 share high degree of amino acid sequence conservation with human RGMs, including conserved vWF type D domain and cysteine residues (Figure 1A,B). Moreover, most of the JH-disease-associated hypomorphic mutations lie in residues that are conserved in DRAG-1 (Figure 1B). We have shown that the sole *C. elegans* RGM protein DRAG-1 positively modulates a BMP-like signaling pathway, called the Sma/Mab pathway (Tian et al. 2010).

There are two TGFβ related signaling pathways in *C. elegans*: the Sma/Mab pathway that regulates body size, mail tail patterning (Patterson and Padgett. 2000,Savage-Dunn. 2005), and postembryonic mesoderm development (Foehr et al. 2006); and the dauer pathway that controls formation of dauer larvae in response to environmental stresses (Ren et al. 1996). The Sma/Mab pathway utilizes the BMP-like molecule DBL-1 as the ligand, SMA-6 as the type I receptor, DAF-4 as the type II receptor (shared with the dauer pathway), SMA-2 and SMA-3 as the R-Smads, and SMA-4 as the co-Smad. Loss-of-function mutations in each of the above components cause a small body size and male tail patterning defects (Suzuki et al. 1999,Suzuki et al. 1999,Savage-Dunn et al. 2003). They also suppress the postembryonic mesoderm dorsoventral patterning defect of *sma-9* mutants (Foehr et al. 2006). *sma-9* encodes a zinc finger transcription factor that is the *C. elegans* homolog of the *Drosophila* and vertebrate Schnurri (SHN) proteins (Liang et al. 2003).

drag-1 was identified in a *sma-9* suppressor screen (Tian et al. 2010). In addition to suppressing the *sma-9* mesoderm patterning defects, *drag-1(0)* mutants are also small. However, they do not exhibit dauer formation defects. These mutant phenotypes and results from genetic epistasis analysis between *drag-1(0)* and null mutants of the Sma/Mab pathway demonstrate that *drag-1* functions exclusively in the Sma/Mab pathway at the ligand/receptor level to positively modulate Sma/Mab signaling. DRAG-1 appears to function in a cell type-specific manner because *drag-1* is not expressed in the male tail and *drag-1(0)* mutants do not have male tail patterning defects. Consistent with the vertebrate RGM proteins acting as a BMP co-receptor, DRAG-1 is membrane-associated, and is expressed and functions in the signal-receiving cells to modulate Sma/Mab signaling. Despite the lack of biochemical evidence of interactions between DRAG-1 and the Sma/Mab pathway members, this study establishes a direct link between RGM proteins and BMP signaling in

vivo, and provides a simple genetic system for further mechanistic studies on RGM protein regulation of BMP signaling in vivo.

The roles of neogenin in modulating BMP signaling

Introduction

Neogenin encodes a type I transmembrane protein that is homologous to the tumor suppressor gene DCC (Deleted in Colorectal Cancer) (Fearon et al. 1990,Vielmetter et al. 1994). Neogenin and DCC proteins share about 50% sequence identity and similar secondary structures. Neogenin, like DCC, has an extracellular domain, a transmembrane domain and an intracellular domain (Figure 2A). The extracellular domains of neogenin and DCC are closely related and both contain four immunoglobuin (IG) domains and six fibronectin type III (FNIII) domains. The intracellular regions of the two proteins share little homology except for three conserved motifs: P1, P2, and P3. Vertebrates such as zebrafish, *Xenopus* and mammals contain both neogenin and DCC (Cole et al. 2007,Wilson and Key. 2007,Yamashita et al. 2007). However, in invertebrates such as *Drosophila* and *C. elegans*, only a single protein that resembles both DCC and neogenin is present in each organism, namely, Frazzled in Drosophila and UNC-40 in *C. elegans* (Chan et al. 1996,Kolodziej et al. 1996).

Unlike DCC, whose expression in vertebrates is primarily restricted to the developing nervous system (Gad et al. 1997), neogenin has a much broader expression pattern. Neogenin is found in the dividing neurogenic and gliogenic progenitors as well as maturing neurons throughout the embryonic and adult central nervous system (Vielmetter et al. 1994,Keeling et al. 1997,Fitzgerald et al. 2006a,Fitzgerald et al. 2006b,van den Heuvel et al. 2013,). Outside of the nervous system, neogenin is found in many developing and adult tissues, including gut, heart, kidney, lung, liver, skeletal muscle and bone cells (Gad et al. 1997,Rodriguez et al. 2007). The broad presence of neogenin outside of the nervous system suggests that it may have non-neuronal functions. Recent research is beginning to shed light on these functions of neogenin outside of the nervous system.

Neogenin as a netrin receptor

DCC has been well established to be a receptor for netrin in axon guidance and migration (Kennedy et al. 1994,Colavita and Culotti. 1998,Hong et al. 1999,Wang et al. 1999,Qin et al. 2007). Because of the sequence similarity shared by DCC and neogenin, earlier work on neogenin focused on its interaction with netrins. Although less well studied compared to DCC, neogenin has a netrin-dependent role in mediating chemoattractant responses to netrin in supraoptic axons in the *Xenopus* forebrain (Wilson and Key. 2006). Neogenin also regulates cell-cell adhesion and tissue organization through interacting with netrins (Srinivasan et al. 2003,Kang et al. 2004,Park et al. 2004,Jarjour et al. 2008,Lejmi et al. 2008). The netrin-binding site in neogenin has not been mapped. But because DCC binds to netrin via the fourth and fifth FNIII domains (FNIII-4, FNIII-5) (Geisbrecht et al. 2003,Kruger et al. 2004), and DCC and neogenin share high degree of sequence similarity, neogenin is expected to bind netrin via similar domains. Consistently, the binding affinity

between netrin and neogenin appears similar to the DCC-netrin binding affinity, which is around 2 nM (Keino-Masu et al. 1996,Wang et al. 1999).

The signal transduction cascades initiated upon neogenin-netrin interaction are not well understood. While DCC-netrin interaction has been found to activate signal transduction cascades that include intracellular tyrosine kinases, second messengers, and Rho GTPases (Gomez and Zheng. 2006,Lin and Holt. 2007,Moore et al. 2007,Round and Stein. 2007,Rajasekharan and Kennedy. 2009,Lai Wing Sun et al. 2011), limited evidence suggests neogenin may have differential downstream effects and interaction partners than DCC (Li et al. 2004,Liu et al. 2004,Xie et al. 2005,Ren et al. 2004, 2008). For example, while both DCC and neogenin become tyrosine phosphorylated in response to netrin-1 in cortical neurons, tyrosine phosphorylated DCC and neogenin exhibit differential binding to effector proteins, such as SHIP-1 (Src Homology 2-containing Inositol 5-Phosphatase 1, Ren et al. 2008). Similarly, in mouse migratory GnRH neuron-derived NLT cells, DCC and neogenin both interact with myosin X (Myo X), an unconventional actin-based motor protein important for filopodium formation, but they exert differential regulatory roles on myosin X activity (Zhu et al. 2007,Liu et al. 2012). Much research is needed to further illustrate the downstream signal transduction events of neogenin-netrin interaction.

Neogenin interaction with RGM proteins

In addition to netrins, neogenin also binds to RGM proteins. Neogenin-RGMa interaction is first identified to be required for the chemorepulsion of temporal retinal axons in the anterior tectum of the chick (Rajagopalan et al. 2004). Later on, neogenin-RGMa interaction has been recognized in many of the processes that RGMa regulates, including axon tract formation in the embryonic brain (Monnier et al. 2002,Matsunaga et al. 2004,Tassew et al. 2008), neural tube closure (Kee et al. 2008), neuronal differentiation (Matsunaga et al. 2006), cell survival (Koeberle et al. 2010), cell migration, and cell-cell adhesion (Lah and Key. 2012). The expression patterns of neogenin and RGMa have been documented in some of these studies, mostly by using mRNA in situ hybridization. Baring the resolution of this technique, neogenin and RGMa appear to exhibit complementary or abutting expression domains and appear to function *in trans* as a ligand-receptor interaction (Monnier et al. 2002,Rajagopalan et al. 2004,Kee et al. 2008,Tassew et al. 2012).

The interaction between RGMa and neogenin has been dissected in more detail. By performing AP (Alkaline phosphatase) binding assays in monkey COS-7 transfected cells and co-immunoprecipitation experiments in HEK293 cells, Rajagopalan and colleagues (Rajagopalan et al. 2004) showed that the binding affinity of chick RGMa to mouse neogenin is about 10-fold higher than that of netrin to neogenin, suggesting that RGMa may be the primary ligand for neogenin in the CNS (Rajagopalan et al. 2004). They further showed that the binding site for chick RGMa resides within the FNIII domains of mouse neogenin; and that the binding of RGMa to neogenin is unaffected by netrin-1 binding, suggesting that neogenin likely binds to these two ligands via distinct binding sites (Rajagopalan et al. 2004). Surprisingly, both the N- and C-terminal chick RGMa fragments, which are the protease cleavage products of full length RGMa and share no sequence similarity to each other, bind to the same FNIII-(3-4) region of neogenin (Tassew et al.

2012). Whether these regions mediate neogenin-RGMa interaction in vivo will need to be further determined.

Besides RGMa, neogenin also directly binds to RGMb and RGMc/HJV. RGMb-neogenin interaction controls aggregation and migration of a population of dentate precursor cells *in vitro* and in mice (Conrad et al. 2010). But the interaction between neogenin and HJV has been investigated in much more depth because of the involvement of HJV in JH. Neogenin was first shown to interact with HJV via immunoprecipitation in human embryonic kidney 293 (HEK293) cells transfected with both HJV and neogenin (Zhang et al. 2005). Zhang and colleagues further showed that co-expression of HJV and neogenin in HEK293 cells increases iron accumulation, whereas HJV carrying a disease-causing G320V mutation fails to interact with neogenin (Zhang et al. 2005). Yang and colleagues (Yang et al. 2008,Yang et al. 2011) subsequently purified the ectodomain of human neogenin and showed that the HJV-binding site is localized to FNIII-5, FNIII-6 and the proximal juxtamembrane region of neogenin, with FNIII-6 mediating most of neogenin-HJV interaction. They further solved the crystal structure of FNIII-5 and FNIII-6 of neogenin, and proposed, by comparing neogenin FNIII-6 domain (that binds to HJV) to neogenin FNIII-5 domain and DCC FNIII-6 domain (that do not bind to HJV), that two loops (C-C′ loop and E-F loop) and the C′ strand in the FNIII-6 domain of neogenin may be involved in mediating HJV-neogenin interaction (Yang et al. 2011). However, these regions have not been directly tested for roles in mediating HJV-neogenin interaction. Notably, the HJV binding site (FNIII-5, 6) in neogenin differs from the RGMa binding site (FNIII-3, 4) in neogenin (Rajagopalan et al. 2004,Tassew et al. 2012). Further studies are needed to determine whether this difference is due to intrinsic differences between the RGMa and HJV/RGMc proteins, or differences between the specific assay conditions used. It is intriguing though that HJV/RGMc and neogenin are co-expressed in hepatocytes (Zhang et al. 2009), thus likely function *in cis* in regulating iron homeostasis, while RGMa and neogenin are expressed in adjacent cells, and thus likely function *in trans*, in regulating axon growth (Rajagopalan et al. 2004,Tassew et al. 2012). These different modes of action may be another reason underlying the differences between the RGM binding domains in neogenin described above.

Discrepancy also exists regarding the neogenin-binding site in RGM proteins. As described above, Tassew and colleagues showed that both the N- and C-terminal chick RGMa fragments, which are the protease cleavage products of full length chick RGMa and share no sequence similarity to each other, bind to the same FNIII-(3-4) region of mouse neogenin (Tassew et al. 2012). In another study, Itokazu and colleagues (Itokazu et al. 2012) showed, via co-immunoprecipitation assays in HEK293T cells, that amino acids 259-295, which are located in between the vWF type D motif and the hydrophobic region of human RGMa, directly bind to the ectodomain of neogenin. While the discrepancy between the two studies could be due to the specific cell lines and assay conditions used, an additional variable may be the sources of the RGMa and neogenin sequences: chick, mouse vs. human cDNAs being used. Future work should also address in vivo the functional importance of RGM-neogenin interaction and the interaction motifs in each protein.

Modulation of BMP signaling by Neogenin

Earlier work using tissue culture cells has suggested conflicting roles of neogenin in BMP signaling. Zhang and colleagues showed that HJV and neogenin are co-expressed in hepatocytes and that knocking down of neogenin in human hepatoma-derived HepG2 cells leads to decreased BMP signaling and reduced hepcidin expression, suggesting a positive role of neogenin in modulating BMP signaling (Zhang et al. 2009). In a separate study using Hep3B cells, also hepatoma-derived, Xia and colleagues showed that HJV-mediated BMP signaling and hepcidin regulation could occur independently of neogenin (Xia et al. 2008). The use of different cell lines in these studies may have contributed to the conflicting results. More recently, the analysis of the neogenin knockout mice provides convincing evidence supporting a role of neogenin in promoting BMP signaling. Homozygous mice of a hypomorphic gene-trap line of neogenin, which exhibit ∼90% reduction of the neogenin protein level, die within a month of birth and share many defects resembling those caused by reduced BMP signaling (Bae et al. 2009,Lee et al. 2010). In particular, these neogenin-hypomorphic mice show impaired digit/limb development and endochondral ossification, apparently due to reduced Smad1/5/8 phosphorylation by BMP2 (Zhou et al. 2010). Importantly, defective BMP signaling and chondrogenesis in the neogenin-hypomorphic chondrocytes can be rescued by high dose of BMP2, supporting a role of neogenin in modulating BMP signaling and function (Zhou et al. 2010). Similarly, neogenin-hypomorphic mice have reduced hepatic hepcidin expression, severe iron overload, and reduced BMP signaling (Lee et al. 2010), which are similar, although not identical, phenotypes to those seen in *Hjv*-/- mice (Niederkofler et al. 2005,Huang et al. 2005). Major differences include the level of reduction of hepcidin expression and the severity and onset of iron overload (Lee et al. 2010,Niederkofler et al. 2005,Huang et al. 2005). These differences may partly be due to the hypomorphic nature of the neogenin mutant mice. Alternatively, neogenin and HJV may have overlapping as well as distinct functions in regulating iron homeostasis. It is important to note that neogenin and HJV share similar expression patterns in the liver, while neogenin and RGMa, RGMb and RGMc are all expressed in chondrocytes, suggesting that neogenin and RGM proteins may function in the same cells *in cis* to modulate BMP signaling (Lee et al. 2010, Zhou et al. 2010).

The mechanism of how neogenin modulates BMP signaling is controversial and poorly understood. Neogenin has been implicated in the processing and secretion of HJV. As described earlier, cellular HJV can be cleaved by furin, matriptase-2 (MT2) and via auto-cleavage to release soluble HJV (s-HJV), which can bind BMP molecules and suppress BMP-induced hepcidin expression (Zhang et al. 2010). On the one hand, neogenin appears to promote the secretion of s-HJV: neogenin can bind to both MT2 and HJV to facilitate the cleavage of HJV by MT2 in HepG2 and HEK293 cells; and knockdown of neogenin in HepG2 cells abolished HJV secretion (Zhang et al. 2005,Zhang et al. 2007,Enns et al. 2012). On the other hand, Lee and colleagues reported that neogenin inhibits HJV secretion in HEK293 cells, which is consistent with their in vivo observations that neogenin positively modulates hepcidin expression and BMP signaling (Lee et al. 2010). The discrepancy might be due to different cell lines being used. It is also worth noting that little is known about whether neogenin-MT2 interaction is also involved in regulating the cleavage of RGMa and

RGMb proteins and how neogenin-MT2 interaction intersects with the BMP pathway in vivo.

Zhou and colleagues (Zhou et al. 2010) showed that in mouse chondrocytes upon BMP stimulation, both neogenin and BMP receptors become associated with the lipid raft microdomains and the association of BMP receptors to these membrane microdomains requires neogenin. Interestingly, RGM proteins appear to bridge neogenin with BMP receptors in these lipid raft microdomains as neogenin does not directly associate with the BMP receptors. Based on these findings, the authors proposed a model in which neogenin positively modulates BMP signaling by promoting the formation of BMP-induced super receptor complexes, including RGMs, neogenin and BMP receptors, in membrane microdomains (Zhou et al. 2010). However, results from a different study showed that neogenin negatively regulates BMP2-induced osteoblastic differentiation of C2C12 cells and phosphorylation of Smads1/5/8, apparently by activating RhoA independently of the BMP receptors (Hagihara et al. 2011). Because of these results and the finding that neogenin directly associates with recombinant human BMP1, BMP4, BMP6 and BMP7, the authors proposed that neogenin acts as a receptor for BMPs to negatively regulate BMP function (Hagihara et al. 2011). This negative role of neogenin in BMP signaling may be context dependent and requires further support from *in vivo* analysis.

Conclusions and perspective

In summary, RGM proteins clearly play important roles in modulating the BMP signaling pathway. Neogenin, the RGM receptor, also appears to be required for BMP signaling. However, much work is still needed, especially in the case of neogenin, to dissect the mechanistic basis on how RGM and neogenin proteins function in modulating BMP signaling. In the case of RGM proteins, further studies are needed to provide in vivo evidence for the involvement of RGMa in BMP signaling. RGM proteins, in particular, RGMc/HJV, appear to undergo complex post-translational modifications. However, except for the processing of HJV by matriptase-2, the in vivo significance of these cleavages in BMP signal transduction is not known. Nor is it known about how the activities of the different processing enzymes are regulated to ensure tight control of proper processing and function of the substrates. All three RGM proteins have been found to bind to the ligand and BMP receptors in vitro, the specific motifs in each of the proteins that are involved in each pair-wise interaction have not been identified, let alone the in vivo significance of these interactions. Finally, the impact of various JH-disease causing mutations on BMP signal transduction should also be further investigated. These natural mutations affect many highly conserved residues among RGM proteins and may provide powerful entry points to uncovering new molecular interactions or functions for RGMs in general.

Compared to RGM proteins, our understanding of the function of neogenin in BMP signal transduction is much more fragmented, and in some cases contradictory. It will be worth investigating whether the reported conflicting roles of neogenin in BMP signaling are due to differences of the various cell culture systems used, or reflect the in vivo diversity of cell type-specific functions of neogenin in modulating BMP signaling. It will also be important to determine the functional significance of RGM-neogenin interaction in modulating BMP

signaling in vivo. For example, the neogenin hypomorphic mutant mice exhibit BMP loss of function phenotypes in bone development and iron homeostasis (Zhou et al. 2010). Do any of the RGM protein knockout models exhibit similar phenotypes? Does disruption of RGM-neogenin interaction affect BMP signaling in vivo? Zhou and colleagues proposed a model (Figure 2B) in which RGM and neogenin form a super complex with the ligand and receptors of the BMP pathway in lipid raft membrane microdomains. Is RGM-neogenin interaction required for the formation of this complex in vivo? How does the complex get recruited to these membrane microdomains? What other components are also located and function in these membrane microdomains in mediating BMP signaling? How do we reconcile this model with the model in which neogenin is involved in matriptase-2-mediated processing of HJV?

Neogenin is involved in both netrin-mediated signaling and RGM-mediated signaling. Are these two independent processes or does neogenin represent a point of crosstalk between the two signaling pathways? Unlike RGM proteins, which are GPI-anchored proteins, neogenin has a large cytoplasmic domain (CTD) containing three conserved motifs. While the CTD is clearly required for neogenin to mediate netrin signaling, it is not known whether CTD is also required for neogenin's role in mediating BMP signaling, and if it is required, what the downstream effectors are in BMP signal transduction. Okamura and colleagues reported that neogenin can be cleaved by the tumor necrosis factor-alpha converting enzyme TACE, which in turn desensitizes cortical neurons to RGMa-induced repulsive behavior (Okamura et al. 2011). While the importance of this cleavage in RGMa function in vivo has not been determined, in light of the roles of neogenin in modulating the BMP pathway, one wonders whether the cleavage of neogenin also plays a role in BMP signaling.

In addition to the research on these proteins in vertebrate systems, studies of RGM proteins and neogenin in invertebrate model systems such as *C. elegans* and *Drosophila* may also help shed light on whether and how these proteins function in modulating BMP signaling in vivo. *Drosophila* does not contain a RGM homology but encodes a single protein Frazzled, which is a homolog of both neogenin and DCC (Kolodziej et al. 1996). In *C. elegans*, we have previously shown that the single RGM protein DRAG-1 positively modulates BMP signaling (Tian et al. 2010). *C. elegans* also contains one protein UNC-40, which is homologous to both DCC and neogenin (Chan et al. 1996). The functions of UNC-40 in axon guidance and cell migration have been well established (Quinn and Wadsworth. 2008). In light of the interaction between vertebrate RGM proteins and neogenin, it will be interesting to determine whether UNC-40 also plays a role in RGM mediated BMP signaling. If it does, the power of genetics in *C. elegans* will allow for functional dissection of the relevance of RGM-neogenin interaction in modulating BMP signaling in vivo, and allow us to determine at single cell resolution whether RGM and neogenin function *in cis* or *in trans* to modulate BMP signaling. From an evolutionary perspective, it will also be interesting to determine whether the Drosophila DCC/neogenin protein Frazzled can function in modulating BMP signaling, and if yes, how it functions independent of a RGM protein.

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Abbreviations

BMP	bone morphogenetic protein
TGFβ	transforming growth factor-beta
RGM	repulsive guidance molecule
DCC	deleted in colorectal cancer
R-Smad	receptor regulated Smad
co-Smad	common mediator Smad
MAPK	mitogen activated protein kinase
Sog	short gastrulation
MGP	matrix GLA protein
Tsg	twisted gastrulation
CV2	crossveinless 2
ECM	extracellular matrix
GPI	glycosylphosphatidylinositol

Sma/Mab	small/male tail abnormal
BAMBI	BMP and activin membrane-bound inhibitor
HJV	hemojuvelin
vWF	von Willebrand factor
DRG	dorsal root ganglion
co-IP	co-immunoprecipitation
JH	juvenile hemochromatosis
s-HJV	soluble HJV
TGN	trans-Golgi network
MT2	matriptase-2
IRIDA	iron-refractory iron deficiency anemia
IG	immunoglobulin
FNIII	fibronectin type III
CTD	cytoplasmic domain
TACE	tumor necrosis factor-alpha converting enzyme

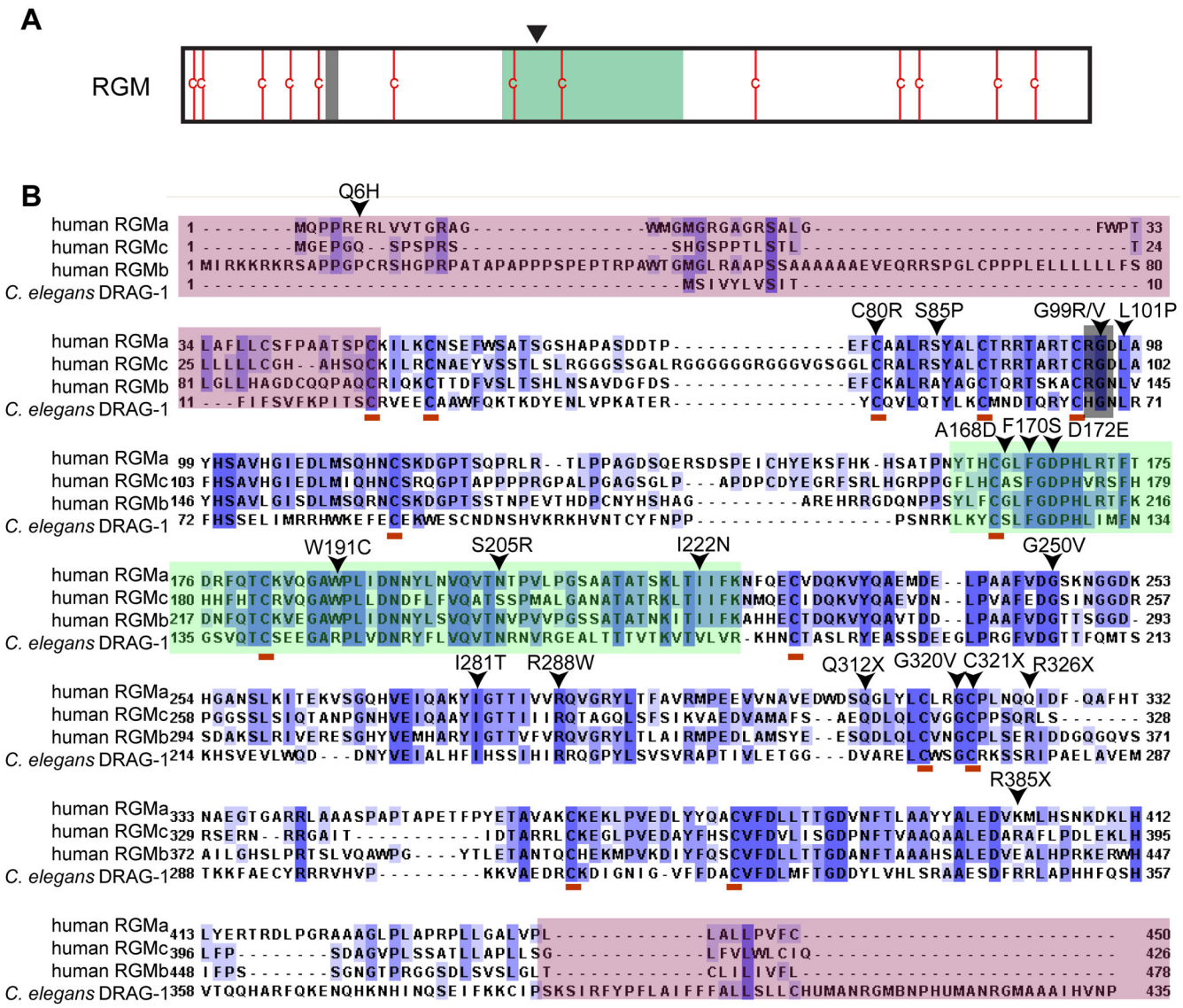

Figure 1. Conserved features of RGM proteins

A. A schematic highlighting features of the mature RGM proteins, including an RGD motif (grey line, only in RGMa and RGMc), a partial vWF type D domain (shaded in green) with an autocleavage site (arrowhead), and conserved cysteine residues (red lines). B. Sequence comparison between human RGMa, RGMb, RGMc, and *C. elegans* DRAG-1, highlighting conserved residues (blue), the RGD motif (grey), partial vWF type D domain (green), conserved cysteine residues (red underline). The JH (juvenile hemochromatosis) disease-associated hypomorphic mutations in RGMc/HJV are shown (De Gobbi et al. 2002,Lee et al. 2004,Lanzara et al. 2004). Notice that many of them lie in the conserved residues between *C. elegans* DRAG-1 and human RGM proteins. Sequences shaded in pink are highly divergent between DRAG-1 and its vertebrate counterparts.

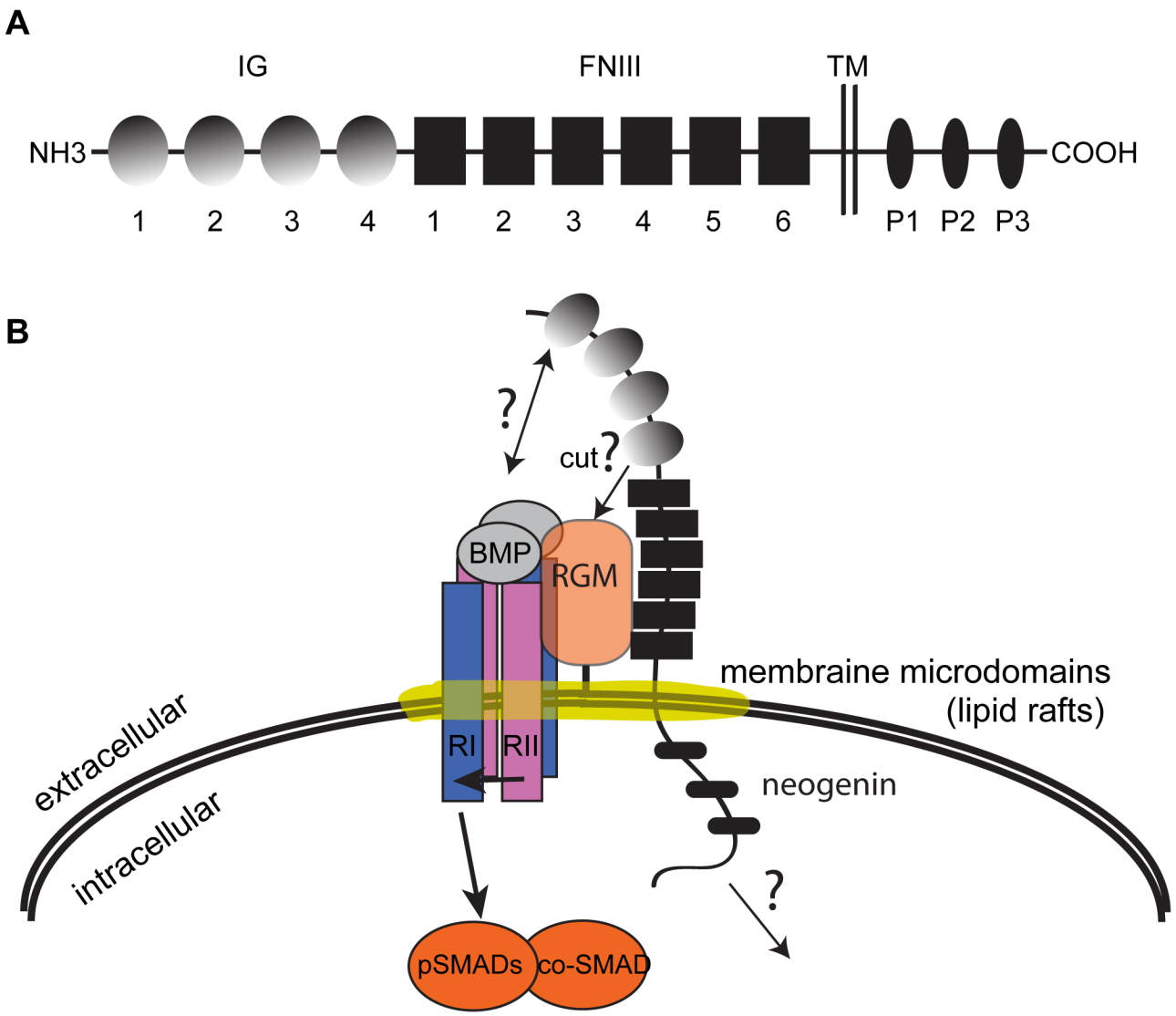

Figure 2. A model on RGM and neogenin functioning in modulating BMP signaling

A. A schematic of the neogenin protein, which has four immunoglobulin (IG) repeats and six fibronectin type III (FNIII) repeats located in the extracellular domain, a transmembrane (TM) domain, and three conserved P1, P2 and P3 motifs located in the intracellular domain. B. A model based on one proposed by Zhou et al. (2010) on how RGM and neogenin modulate BMP signaling. RGM and neogenin may form a super complex with the ligand and receptors of the BMP pathway in lipid raft membrane microdomains to mediate signaling. However, many questions remain as to how neogenin may modulate BMP signaling, which include, but are not limited to those marked by the question marks. For example, does neogenin play a role in the processing of RGM? What other proteins interact with neogenin? What is the role of the intracellular domain of neogenin in BMP signaling? See text for more discussions.