Dragon Enhances BMP Signaling and Increases Transepithelial Resistance in Kidney Epithelial Cells

Yin Xia,* Jodie L. Babitt,* Richard Bouley,* Ying Zhang,* Nicolas Da Silva,* Shanzhuo Chen,* Zhenjie Zhuang,* Tarek A. Samad,† Gary J. Brenner,† Jennifer L. Anderson,* Charles C. Hong,‡ Alan L. Schneyer,§ Dennis Brown,* and Herbert Y. Lin*

*Center for Systems Biology, Program in Membrane Biology and Division of Nephrology, Department of Medicine and †Neural Plasticity Research Unit, Department of Anesthesia, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts; ‡Division of Cardiovascular Medicine, Vanderbilt University Medical Center, Nashville, Tennessee; and §Reproductive Endocrine Unit, Department of Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts

ABSTRACT

The neuronal adhesion protein Dragon acts as a bone morphogenetic protein (BMP) coreceptor that enhances BMP signaling. Given the importance of BMP signaling in nephrogenesis and its putative role in the response to injury in the adult kidney, we studied the localization and function of Dragon in the kidney. We observed that Dragon localized predominantly to the apical surfaces of tubular epithelial cells in the thick ascending limbs, distal convoluted tubules, and collecting ducts of mice. Dragon expression was weak in the proximal tubules and glomeruli. In mouse inner medullary collecting duct (mIMCD3) cells, Dragon generated BMP signals in a ligand-dependent manner, and BMP4 is the predominant endogenous ligand for the Dragon coreceptor. In mIMCD3 cells, BMP4 normally signaled through BMPRII, but Dragon enhanced its signaling through the BMP type II receptor ActRIIA. Dragon and BMP4 increased transepithelial resistance (TER) through the Smad1/5/8 pathway. In epithelial cells isolated from the proximal tubule and intercalated cells of collecting ducts, we observed coexpression of ActRIIA, Dragon, and BMP4 but not BMPRII. Taken together, these results suggest that Dragon may enhance BMP signaling in renal tubular epithelial cells and maintain normal renal physiology.


Bone morphogenetic proteins (BMPs) represent a large subfamily of the transforming growth factor β (TGF-β) superfamily of ligands that play roles in numerous physiologic and pathologic processes including cell proliferation, differentiation, apoptosis, and specification of developmental fate during embryogenesis and in adult tissues.1 In the kidney, BMPs play an important role in nephrogenesis. During normal development, BMPs are expressed in the metanephric mesenchyme and the ureteric bud and play a key role in the epithelialization of the metanephric mesenchyme and reciprocal induction of collecting duct differentiation. Loss of BMP expression has profound effects on kidney development.2–11 The role of BMPs in the adult kidney is less well understood. The expression of multiple BMP ligands, including BMP4, BMP6, and BMP7, and BMP receptors persists in the adult kidney,12 supporting the notion that the adult kidney can respond to BMP stimulation.13 Response to injury and repair frequently recapitulates development,
and given the function of BMPs during nephrogenesis, it has been hypothesized that BMPs may have a similar role in the adult kidney as epithelial differentiation and survival factors that protect against damage and promote recovery in response to injury.14,15

Functional tight junctions are essential for the establishment and maintenance of the polarized architecture of the epithelial cells,16–18 a process that occurs during kidney development and in response to injury and repair.19–22 Tight junctions also provide a barrier that is involved in regulation of paracellular transport of small molecules.23 Trans-epithelial resistance (TER) reflects paracellular ionic conductance and it is a measure of tight junction complexity and function.24,25 In the kidney, TER varies dramatically across different nephron tubules and changes in response to physiologic and pathologic conditions.26 Interestingly, it has been shown that BMP signals enhance TER in some epithelial cells,27 suggesting that this could be one function of BMP signaling in the kidney.

BMP signaling is initiated by ligand binding to combinations of two type II and two type I serine/threonine kinase receptors. Upon ligand binding, the type I receptor is phosphorylated by the type II receptor. Type I receptors then act downstream, determining the specificity of the signal via phosphorylation of the receptor-activated Smads (R-Smads). The BMP subfamily signals via one set of R-Smads (Smad1, Smad5, and Smad8), whereas the TGF-β subfamily signals via another set of R-Smads (Smad2, Smad3). All R-Smads then form heteromeric complexes with the common mediator (co-Smad), Smad4. The activated Smad complexes then move from the cytoplasm to the nucleus where they act as transcriptional regulators to modulate gene expression.1

Recently, we identified the three repulsive guidance molecule (RGM) proteins including RGMa, RGMb (Dragon), and RGMc (hemojuvelin) as coreceptors for BMP signaling.28–30 RGM proteins share 50% to 60% sequence homology and have similar structural features including a signal sequence, conserved proteolytic cleavage site, partial von Willebrand factor type D domain, and glycoprophatidylinositol (GPI) anchor. RGM proteins are retained on the outer layer of the plasma membrane through the GPI anchor motif,31 although they can be shed from the cell membrane through cleavage at the GPI anchor by phospholipases.31 We have shown that all three RGM proteins physically interact with BMP receptors and specific BMP ligands and increase intracellular Smad phosphorylation in response to BMP ligands.28–30,33 We have also revealed a mechanism shared by RGMa and hemojuvelin in increasing BMP signaling (i.e., facilitating the use of ActRIIA by endogenous BMP-2 and BMP-4 ligands that normally prefer signaling through BMPRIIA).33,34 However, the precise molecular mechanisms of Dragon’s action in regulating BMP signaling remain to be investigated.

Dragon is expressed in the central nervous system, where it is involved in neuronal cell adhesion through homophilic interactions.31 However, a detailed examination of Dragon expression in other tissues and a physiologic role for the BMP signaling function of Dragon has yet to be determined.

Here, we show that Dragon is expressed in the kidney epithelial cells in the thick ascending limbs, distal tubules, and collecting ducts. We demonstrate that Dragon can act in a mouse inner medullary collecting duct (mIMCD3) cell line to generate BMP signals as a ligand-dependent coreceptor. Furthermore, Dragon enhances utilization of a BMP type II receptor, ActRIIA, by BMP4 in mIMCD3 cells. Finally, we show that Dragon and BMP4 increase TER in mIMCD3 cells, and that the small-molecule BMP inhibitor LDN-193189 can block this increase. Thus Dragon could potentially generate BMP signals in tubule cells in the kidney and may play a role in regulating the functions of tight junctions in the epithelia of kidney tubules.

RESULTS

Dragon mRNA and Protein Are Expressed in the Kidney

To investigate the presence of Dragon mRNA in mouse tissues, Northern blots containing 10 μg of total RNAs prepared from various mouse tissues (whole embryos, kidney, heart, liver, muscle, and brain) were hybridized with a specific probe for Dragon. As shown in Supplemental Figure 1A, there are two message sizes for Dragon—a major band at approximately 4 kb in size and a smaller band at 2.4 kb. Both are expressed in the kidney and in other tissues such as brain, heart, liver, muscle, and whole embryo.

To further confirm Dragon expression in the kidney, we performed Western blot analyses of proteins from adult mouse whole kidney lysates separated by reducing SDS polyacrylamide gel electrophoresis and probed with a previously characterized anti-Dragon antibody.31 A band of approximately 55 kD was seen in the kidney lysates (Supplemental Figure 1B, lane 2). This correlates well with the previously described molecular weight of Dragon in Western blots of protein extracts from neonatal and adult dorsal root ganglia.31 The 55-kD band was eliminated when Dragon antibody was preincubated with competing immunizing peptide (Supplemental Figure 1B, lane 1). A band of approximately the same size was detected by Western blot analysis of lysates generated from HEK293 cells transfected with Dragon cDNA but not in untransfected cells (Supplemental Figure 1C); thus, Dragon protein is expressed in the kidney.

Cellular Localization of Dragon in the Kidney

We then investigated the localization of Dragon protein in the adult mouse kidney by indirect immunofluorescence. As shown in the photograph at low magnification (Figure 1A), Dragon is expressed in various tubules. In photographs at high magnification, Dragon protein was weakly expressed in the glomeruli and Bowman’s capsules (Figure 1B) compared with Dragon expression in collecting ducts (Figure 1B) identified by
V-ATPase staining (Figure 1C). Weak staining was also detected in the brush border of proximal tubules (Figure 1B). The staining appears to be specific for Dragon, because it could be completely blocked by preincubation of the Dragon antibody with the competing immunizing peptide (Figure 1D) whereas the V-ATPase staining was not affected by the presence of the Dragon peptide (Figure 1, C and E). The expression of Dragon in proximal tubules is supported by the detection of Dragon mRNA in isolated epithelial cells of proximal tubules from mice obtained through BSA-Alexa555 labeling of proximal tubule cells followed by laser capture microdissection (Figure 7A).

To further characterize the cellular distribution of Dragon in collecting ducts, we performed dual staining of Dragon protein with aquaporin 2 (AQP2), a marker for principal cells,35 or V-ATPase, a proton pump that is localized to intercalated cells.36 Dragon (red) was expressed in the AQP2-containing cells (green, inset, Figure 2B) and V-ATPase-containing cells (Figures 1 and 3). These results suggest that Dragon is expressed in principal and intercalated cells in collecting ducts. Of note, Dragon expression was polarized in collecting ducts in the cortex (Figures 1B and 2A) and in the outer stripe (Figure 3A), with Dragon staining being concentrated toward the apical pole of the cells, although weak staining was observed throughout the cytoplasm and at the basal membrane. In contrast, Dragon staining appears to be diffuse in collecting ducts in the inner stripe (Figure 3D) and papilla (Figure 3F).

Apical staining of Dragon was also seen in the epithelial cells positive for calbindin 28, a calcium-binding protein specifically expressed in the mouse distal convoluted tubules and connecting segments37 (Figure 2, C and D), and in epithelial cells positive for Tamm-Horsfall glycoprotein, a marker for thick ascending limbs38 (Figure 2, E and F). Dragon was also detected in thin limbs of Henle’s loop in the papilla (Figure 3F). Taken together, the data show that Dragon protein is expressed in the glomerulus and in epithelial cell types in various tubules in the kidney.

**Dragon Mediates BMP4 Signaling in the Kidney Collecting Duct Cell Line mIMCD3**

Because Dragon is highly expressed in several kidney tubule epithelial cell types, including collecting duct cells, we tested if Dragon can mediate BMP signaling in mIMCD3 cells, a well established kidney inner medullary collecting duct cell line known to respond to BMP ligands. mIMCD3 cells were transfected with a BMP-responsive luciferase reporter gene construct (BRE-Luc)39 (Figure 4A) or a TGF-β-responsive luciferase reporter gene construct [(CAGA)$_{12}$MLP-Luc]40 (Figure 4B) alone or in combination with Dragon cDNA. Transfected cells were then incubated with or without 40 ng/ml BMP4 or 2 ng/ml TGF-β1. In the absence of Dragon, stimulation with BMP4 and TGF-β increased the relative luciferase activity for their respective reporters compared with unstimulated cells (Figure 4, A and B). Transfection with Dragon increased BRE luciferase activity even in the absence of BMP stimulation (Figure 4A) but did not alter (CAGA)$_{12}$ MLP-Luc activity compared with the baseline (Figure 4B). The increase of Dragon signaling is dose-dependent, and Dragon signaling reached peak at 200 ng (Figure 4C). These results demonstrate that Dragon can stimulate BMP signaling but not TGF-β signaling in mIMCD3 cells.
To determine whether Dragon and BMP ligands are additive in their BMP signaling effects, mIMCD3 cells were transfected with the BRE-Luc construct in the absence or presence of Dragon cDNA. Cells were then incubated in the absence or presence of BMP4. The presence of Dragon augmented BMP4 stimulation of BRE-Luc activity (Figure 4D). Similar results were obtained when BMP4 was replaced with BMP2 (Supplemental Figure 2). Thus, Dragon behaves in a manner consistent with a role as an accessory receptor for BMPs.

We then explored whether Dragon expression in vivo correlates with its hypothesized role as a coreceptor for BMP signaling. We therefore determined whether Dragon-expressing cells in the kidney showed evidence of BMP signaling (i.e., nuclear accumulation of p-Smad1/5/8). As shown in Supplemental Figure 3, nuclear staining of phospho-Smad1/5/8 was detected in glomeruli, principal and intercalated cells of collecting ducts, distal convoluted tubules and connecting segments, and thick ascending limbs. Thus, BMP signaling occurs in Dragon-expressing cells in the kidney, consistent with a role for Dragon as a BMP coreceptor in vivo.

**BMP4 Is the Endogenous Ligand for Dragon in mIMCD3 Cells**

Dragon has been shown to signal in a ligand-dependent manner. We have previously shown that Dragon binds BMP2 and BMP4 but not BMP7 and TGF-β1. Therefore, it is possible that BMP2, BMP4, or both are endogenous ligands for Dragon. To investigate endogenous ligands for the Dragon coreceptor in mIMCD3 cells, we screened these cells by reverse-transcriptase PCR (RT-PCR) for expression of mRNAs for BMP2; BMP4; and the closely related members BMP5, BMP6, and BMP7 (Figure 5A). Among these ligands, BMP4 and BMP6 mRNA were readily detected in mIMCD3 cells, whereas BMP2 and BMP7 were barely detectable and BMP5 was not detected, although all of these ligands were expressed in the native mouse kidney (Figure 5A), which contains many different cell types. We then tested whether Dragon-induced BMP signaling is affected by small interfering RNA (siRNA)-mediated specific inhibition of BMP2, BMP4, BMP6, or BMP7. As shown in Supplemental Figure 4, BMP2, BMP4, BMP6, or BMP7 expression was specifically inhibited by 60% to 70% by the respective gene-specific siRNA duplexes (60 nM), with minimal effect on the expression of the other ligands. mIMCD3 cells were transfected with BRE-Luc in combination with control, BMP2, BMP4, BMP6, or BMP7 siRNA (60 nM) (Figure 5B). Inhibition of BMP4 expression abolished Dragon-mediated BMP signaling. In contrast, inhibition of BMP2, BMP6, and BMP7 expression did not change BRE-Luc activity induced by Dragon compared with control siRNA (Figure 5B). These results demonstrate that BMP4 is the predominant endogenous ligand for Dragon in mIMCD3 cells.
Dragon Enhances Utilization of ActRIIA by BMP4 in mIMCD3 Cells

We previously demonstrated that RGMa and RGMc/hemojuvelin increase BMP signaling by increased utilization of BMP type II receptor ActRIIA by BMP2 and BMP4, which normally signal through BMPRII. To test whether Dragon also alters utilization of BMP type II receptors, we examined the effect of siRNA-mediated specific inhibition of BMP type II receptors on Dragon-induced BMP signaling in mIMCD3 cells. To this end, we screened mIMCD3 cells for expression of BMPRII, ActRIIA, and ActRIIB mRNA by RT-PCR. As shown in Supplemental Figure 5A, all three type II receptors were expressed. The expression of the three receptors was selectively reduced by more than 50% with the introduction of specific siRNAs (60 nM) (Supplemental Figure 5B). We examined whether or not inhibition of endogenous type II receptors affects Dragon-mediated stimulation of BRE-Luc activity. As expected, treatment of mIMCD3 cells with BMP4 (20 ng/ml) increased BRE-Luc activity to 7.5-fold above the baseline (Figure 6A). This stimulation was dramatically reduced by BMPRII-specific siRNA to 3.7-fold above the baseline \((P < 0.05)\) but was not reduced by ActRIIA- or ActRIIB-specific siRNA. These results suggest that in mIMCD3 cells BMP4 signaling is primarily transduced by BMPRII but not by ActRIIA or ActRIIB.

Transfection of mIMCD3 cells with Dragon cDNA increased BRE-Luc activity to 6.1-fold above the baseline (Figure 6B). This stimulation was reduced to 3.9- and 4.5-fold above the baseline by BMPRII and ActRIIA-specific siRNAs, respectively \((P < 0.05\) for both). Dragon-mediated BRE-Luc activity was not altered by inhibition of ActRIIB expression. These results suggest that BMPRII and ActRIIA are both utilized to transduce endogenous BMP4 signal in the presence of the Dragon coreceptor, indicating that Dragon enhances the utilization of ActRIIA by BMP4 in mIMCD3 cells.

ActRIIA is Coexpressed with Dragon in Proximal Tubule Cells and Intercalated Cells

To examine whether BMP4/Dragon/ActRIIA signaling occurs in the kidney, we analyzed expression of these genes along with BMPRII by RT-PCR in proximal tubule cells and intercalated cells isolated from adult kidneys (Figure 7). Consistent with the Dragon immunostaining, Dragon mRNA was detected in proximal tubule cells (Figure 7A) and intercalated cells (Figure 7B). BMP4 and ActRIIA mRNAs were also detected in both cell types, whereas BMPRII mRNA was not detected in either cell type. BMP2 was detected in proximal tubule cells but not in intercalated cells. These results suggest that Dragon may form a functional signaling unit with BMP4 and ActRIIA in vivo to enhance BMP signaling.
Dragon Increases TER

It has been shown that BMP ligands enhance TER in colonic epithelial cells. We therefore examined whether BMP4 and Dragon played a role in the establishment of TER of kidney epithelial cells. mIMCD3 cells were plated on Transwells (25,000 cells/well) in 12-well plates. Three days later TER was measured daily. As shown in Figure 8A, untreated mIMCD3 cells showed a small increase in TER with time. The addition of BMP4 (5 ng/ml) to these cells significantly increased TER on days 2 and 3 compared with untreated control cells. Increased concentration of BMP4 (50 ng/ml) caused greater increases in TER on days 1, 2, and 3. This increase in TER was not due to an increase in cell proliferation because cell numbers decreased in a dose-dependent manner after a 3-day exposure to BMP4.

To test whether the effect of BMP4 on TER is mediated through the Smad1/5/8 pathway or through the mitogen-activated protein kinase (MAPK) pathway, we incubated mIMCD3 cells with BMP4 in the presence or absence of LDN-193189 (DM-3189), a small-molecule derivative of the BMP inhibitor Dorsomorphin. Consistent with previous findings, LDN-193189 inhibited Smad1/5/8 phosphorylation induced by BMP4 but did not affect p38 MAPK phosphorylation induced by BMP4 (Supplemental Figure 6B). As shown in Figure 8B, LDN-193189 (40 nM) completely abolished stimulation of TER by BMP4. These results suggest that BMP4 signals through the Smad1/5/8 pathway but not through the MAPK pathway to regulate TER.

To examine the effect of Dragon on TER, mIMCD3 cells at 90% confluency in Transwells were transfected with Dragon cDNA, and TER was measured daily. Dragon overexpression

**Figure 5.** BMP4 is the endogenous ligand for Dragon in mIMCD3 cells. (A) Expression of BMP ligands in mIMCD3 cells. Total RNA from IMCD3 cells was extracted for RT-PCR to determine the expression of BMP2 and BMP4 through BMP7. Total RNA from mouse kidney was used in PCR analyses as positive controls. BMP4 and BMP6 mRNAs were readily detected, BMP2 and BMP7 mRNAs were weak, and BMP5 mRNA was undetectable in mIMCD3 cells. (B) Effect of siRNA targeting of BMP2, BMP4, BMP6, and BMP7 on Dragon-mediated BMP signaling. mIMCD3 cells were transfected with BRE luciferase reporter alone or with increasing amounts of Dragon cDNA (0, 2, 20, 200, and 500 ng). Relative luciferase activity was measured from cell extracts. *P < 0.05 and ***P < 0.001 versus the control. (C) BMP4 and Dragon signals are additive. mIMCD3 cells were transfected with BRE-Luc construct in the absence or presence of BMP4 (40 ng/ml). Cells were then incubated without (open bars) or with BMP4 (filled bars) and relative luciferase activity was measured from cell extracts. ***P < 0.001, bar 3 versus bar 1; **P < 0.05, bar 4 versus bar 2.
significantly increased TER on days 2 and 3 after transfection (Figure 8C), whereas cell numbers were significantly reduced as counted on day 3 after transfection (Supplemental Figure 6C). Transfection of Dragon in the presence of BMP4 further increased TER measured on day 3 compared with Dragon transfection or BMP4 treatment alone (Figure 8D). These results suggest that BMP and Dragon stimulate TER in mIMCD3 cells.

To determine the potential mechanism of BMP4 and Dragon action, we examined the mRNA expression levels of tight junction proteins claudins 1 to 16; occludin; ZO-1, ZO-2, and ZO-3; MUPP-1; JAM-A, JAM-B, and JAM-C; and cingulin in mIMCD3 cells treated with BMP4. We found that MUPP-1 and JAM-A were significantly upregulated by BMP4. The expression of MUPP-1 and JAM-A was also increased by transfection of Dragon (Figure 9). MUPP-1 and JAM-A were expressed in intercalated cells, and JAM-A but not MUPP-1 were detected in proximal tubule cells (Figure 7). These results suggest that Dragon may regulate expression of tight junction components such as JAM-A and MUPP-1 in the kidney.

DISCUSSION

The RGMs RGMa, RGMb (Dragon), and RGMc (hemojuvelin) are GPI-anchored membrane proteins. Our studies have shown that the three proteins are BMP coreceptors that enhance BMP signaling. Although RGMc/hemojuvelin has been shown to regulate hepcidin expression and iron homeostasis through the BMP pathway, the biologic roles of RGMa and Dragon as BMP coreceptors remain largely unknown.

To investigate the potential biologic functions of Dragon in the kidney, we examined the cellular distribution of Dragon in this organ. We found that Dragon is expressed in epithelial cells of various tubules, including thick ascending limbs, distal tubules, and collecting ducts in the kidney. Similar to other GPI-anchored proteins, Dragon is concentrated most heavily on the apical surfaces of tubular epithelial cells in the kidney, although basolateral staining is also present. These results suggest that Dragon may play a role in transport or signaling in kidney tubular epithelial cells.

BMPs play important roles in numerous physiologic and pathologic processes during kidney development and in response to injury and repair. Here we found that Dragon can mediate BMP signaling but not TGF-β signaling in mIMCD3 cells. Interestingly, Dragon-expressing cells in the kidney (e.g., intercalated cells and principal cells of collecting ducts, epithelial...
and BMP4 are very likely to be the key endogenous ligands used by the Dragon coreceptor in the kidney. In mIMCD3 cells, Dragon-induced BMP signaling was blocked by siRNA inhibition of BMP4 expression but was not affected by siRNA inhibition of BMP2, BMP6, and BMP7. This suggests that BMP4 is likely the sole endogenous ligand for the Dragon BMP coreceptor in mIMCD3 cells. The failure of BMP2 inhibition to affect Dragon-mediated BMP signaling in mIMCD3 cells was unexpected, but it is most likely due to the low levels of endogenous BMP2 expression. However, this result does not rule out the possibility that BMP2 is also an endogenous ligand for the Dragon coreceptor in the native kidney. The failure of BMP6 inhibition to affect Dragon-mediated BMP signaling suggests that endogenous BMP6 did not play a significant role in Dragon action in this system, presumably because of the lower affinity of Dragon for BMP6 compared with that for BMP4. These data are consistent with our bioinhibition data showing that Dragon.Fc was significantly less potent at inhibiting BMP6 compared with BMP4. Interestingly, this contrasts to hemojuvelin, which uses BMP6 as a ligand and appears to have higher affinity for BMP6 compared with BMP4.34,46

We also tested the utilization of BMP type II receptors used by the BMP4 ligand in mIMCD3 cells in the presence or absence of transfected Dragon. siRNA-mediated inhibition of BMPRII or ActRIIA expression significantly reduced BMP4 signaling mediated by Dragon. This indicates that, in the presence of transfected Dragon, endogenous BMP4 ligand signals through BMPRII and ActRIIA. This is consistent with prior data showing that Dragon physically interacts with BMPRII and ActRIIA when overexpressed in HEK293 cells.28 In contrast, in the absence of Dragon overexpression, exogenous BMP4 signals were inhibited by BMPRII siRNA but not ActRIIA siRNA, suggesting that BMP4 normally signals through BMPRII in mIMCD3 cells. These results suggest that Dragon enhances utilization of ActRIIA by BMP4 in mIMCD3 cells, similar to our previously reported finding for RGMa33 and hemojuvelin.34 This ability to enhance utilization of ActRIIA by BMP ligands appears to be a common feature of the entire RGM family and may be one mechanism by which these coreceptors enhance cellular responses to BMP ligands.

In the isolated proximal tubule cells and intercalated cells, mRNAs for Dragon and ActRIIA were both detected, whereas BMPRII was not detected. These results are consistent with our
general hypothesis that Dragon or other RGM family members may play an important role in regulating BMP4 (or BMP2) signaling in BMPRII-null, BMPRII-low, or BMPRII-deficient cells,33 which would not respond well to BMP4 or BMP2 in the absence of the RGM family of coreceptors. Indeed, several other cell types have been identified where RGM family members are expressed, and ActRIIA is the predominant BMP type II receptor whereas BMPRII is not or is barely detectable, including epithelial cells of ureteric branches in embryonic kidneys,47,48 human liver cells,34 and oocytes in the ovary.49,50 Although it remains to be investigated whether ActRIIA is expressed in other Dragon-expressing cells within the kidney, the expression of the BMP4/Dragon/ActRIIA system in the proximal tubule cells and intercalated cells supports our hypothesis that Dragon plays a role in adult kidney function.

A study by Peiris et al. showed that BMP2 modulated epithelial barrier maturation as assessed by the increases in TER in colonic epithelial cells.27 Consistent with this result, we found that BMP4 dramatically increased TER in mIMCD3 cells cultured in Transwell inserts through the Smad1/5/8 pathway, and Dragon further enhanced the BMP4 effect on TER. Furthermore, Dragon and BMP4 significantly increased the mRNA expression of tight junction components MUPP-1 and JAM-A, providing a potential mechanism by which Dragon and BMP increase TER.

Functional tight junctions are essential for the establishment and maintenance of the polarized architecture of the epithelial cells.16–18 Therefore, modulation of tight junction functions by BMP signaling may be a mechanism by which BMPs regulate kidney recovery in response to injury, and Dragon may play an important role in these processes by sensitizing renal epithelial cells to BMP signals. In addition, tight junctions provide a barrier that regulates paracellular transport. In renal tubules, the paracellular pathway is recognized to play an important role in the epithelial permeability of ions (e.g., sodium, chloride, calcium, and magnesium), and variations in TER among different nephron segments may be involved in regulation of selectivity for these transported ions.26,51–53 Thus, our results raise the possibility that BMPs/Dragon may play a role in paracellular transport in tubular epithelia in the kidney.

In summary, we found that Dragon is strongly expressed in epithelial cells of thick ascending limbs, distal tubules, and collecting ducts of the kidney and is weakly expressed in proximal tubules, glomeruli, and Bowman’s capsules. In mIMCD3 cells, BMP4 is the predominant endogenous ligand used by Dragon to mediate BMP signaling. Similar to RGMa and RGMc/hemojuvelin, Dragon enhances utilization of ActRIIA by BMP4, which normally signals through BMPRII. ActRIIA is expressed in proximal tubule cells and intercalated cells, which also express Dragon. Dragon and BMP4 increase tight junction protein expression and TER of cultured mIMCD3 cells. These results suggest that Dragon may play an important role in kidney function by enhancing BMP signaling.

**CONCISE METHODS**

**Western Blot Analysis**

Extracts from mIMCD3 cells treated with BMP4 in the presence or absence of LDN-193189 were subjected to Western blotting with anti-phospho-SMAD1/5/8 (Cell Signaling), anti-total-SMAD1 (Cell Signaling), anti-phospho-MAPK p38 (Cell Signaling), or anti-total MAPK p38 (Cell Signaling).

**Immunohistochemistry Analysis**

Sixty-day-old mice were perfused with paraformaldehyde-lysine-perioptate fixative (4% paraformaldehyde, 75 mM lysine-HCl, 10 mM sodium periodate, and 0.15 M sucrose in 37.5 mM sodium phosphate). Frozen sections (5 μm) were collected onto Superfrost Plus precleaned charged microscope slides (Fisher Scientific, Pittsburgh, PA).

Sections were treated with 1% SDS for 4 minutes for retrieval of antigenic sites.34 The sections were incubated with a previously characterized rabbit anti-Dragon antibody31,32 diluted at 1:5000 in Dako antibody diluent (Dako, Carpinteria, CA) in combination with chicken anti-V-ATPase E1 subunit (1:10),35 goat anti-AQP2 (0.4 μg/ml, Santa Cruz Biotechnology), mouse anti-calbindin 28 (1:1600, Sigma-Aldrich, St. Louis, MO), or mouse anti-Tamm Horsfall glycoprotein (1 μg/ml, Cappel Laboratories, Inc., Cochranville, PA). The Dragon staining specificity was assessed by preincubating the Dragon antibody with immunizing peptide (200 ng/μl) before applying to tissue sections. For phospho-Smad1/5/8 staining, immersion-fixed kidneys were used; the sections were treated with 1% SDS for 12 minutes.

**Luciferase Assays**

mIMCD3 cells (ATCC CRL-2123) were cultured in DME medium (Cellgro, Mediatech., VA) supplemented with 10% FBS. All transfect-
tions were performed with LipoFectamine-2000 (Invitrogen Life Technologies, Carlsbad, CA).

To test the effect of Dragon on BMP signaling, mIMCD3 cells were transiently transfected with Bre-Luc or (CAGA)12MLP-Luc alone or in combination with cDNA encoding full-length Dragon. A control pRl-TK Renilla luciferase reporter (Promega, Madison, NY) was included to control for transfection efficiency. Cells were then serum starved for 6 hours before treatment with varying amounts of BMP2, BMP4, or TGF-β1 ligands (R&D systems, Minneapolis, MN) for 16 hours. Cells were lysed and luciferase activity was determined with the dual reporter assay (Promega, Madison, NY). Relative light units were calculated as ratios of Firefly (reporter) and Renilla (transfection control) values. Results from luciferase assay experiments are expressed as mean ± SD of triplicates from representative experiments. Two to three independent experiments were performed in each experimental setting.

Isolation of Proximal Tubule Cells and Intercalated Cells

The epithelial cells of proximal tubules were isolated as described previously. Briefly, mice were injected with BSA-Alexa555 via tail veins to label proximal tubules and 15 minutes later kidneys were collected and frozen. Sections (5 μm) were used to isolate proximal tubule cells using laser capture microdissection.

To isolate intercalated cells, we used ATP6V1B1-EGFP mice, which express enhanced green fluorescent protein (EGFP) specifically in intercalated cells within the kidney. Populations of EGFP-positive cells from kidney preparations were isolated by FACS on the basis of their green fluorescence intensity. A fraction of each sample was reanalyzed by flow cytometry to estimate the purity (>95%). EGFP-positive cell samples were collected in nuclease-free PBS (N. Da Silva, unpublished material).

RT-PCR

Total RNA was isolated from mIMCD3 cells, intercalated cells, and mouse kidneys using an RNaseasy mini kit (Qiagen Inc.) or PicoPure RNA isolation kit (Molecular Devices) according to the manufacturer’s instructions. First-strand cDNA synthesis was performed using an iScript cDNA synthesis kit (Bio-Rad). For samples from isolated proximal tubule cells captured by laser microdissection, total RNAs were amplified using RiboAmp Plus KIT0521 (Molecular Devices) before reverse transcription. Transcripts of mouse BMP2, BMP4, BMP5, BMP6, and BMP7 were purchased from Dharmacon. siRNA duplexes were added at the concentrations indicated along with plasmids to subconfluent mIMCD3 cells using Lipofectamine-2000 (Invitrogen). Assays to measure target mRNA levels or luciferase activity were performed 46 hours after transfection.

Measurement of Gene Expression

Real-time quantification of mRNA transcripts was performed as described previously. First-strand cDNA was amplified with the primers as in Supplemental Tables 1 and 2 (mouse BMP2, BMP4, BMP5, BMP6, and BMP7; JAM-A, JAM-B, and JAM-C; ZO-1, ZO-2 and ZO-3) or as described previously (mouse BMPRII, ActRIIA, ActRIIB, and RPL19); claudins 1 to 16; occludin; cingulin; and MUPP-1). Results are expressed as a ratio of the gene of interest to RPL19.

Acknowledgments

H.Y.L. was supported by National Institutes of Health (NIH) grant RO1 DK-071837 and RO1 DK-069533. D.B. was supported by NIH grant DK-42956. Y.X. was supported by NIH grant R03HD60641. J.L.B. was supported by NIH grant K08 DK-075846. The authors thank David A. Fabizio and Jason Campagna for technical assistance.

Disclosure

None.

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