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Renin-Angiotensin System-Growth Factor Cross-Talk: a Novel Mechanism for Ureteric Bud Morphogenesis

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Abstract

The renin-angiotensin system (RAS) plays a critical role in kidney development. Mutations in the genes encoding components of the RAS cause a spectrum of congenital abnormalities of the kidney and renal collecting system ranging from hypoplasia of the renal medulla and hydronephrosis in mice to renal tubular dysgenesis in humans. However, the mechanisms by which an intact RAS controls proper renal system development and how aberrations in the RAS result in abnormal kidney and renal collecting system development are poorly understood. The renal collecting system originates from the ureteric bud (UB). A number of transcription and growth factors regulate UB branching morphogenesis to ultimately form the ureter, pelvis, calyces, medullary and cortical collecting ducts. Importantly, UB morphogenesis is a key developmental process that controls organogenesis of the entire metanephros. This review emphasizes emerging insights into the role for the RAS in UB morphogenesis and explores the mechanisms whereby RAS regulates this important process. A conceptual framework derived from recent work indicates that cooperation of angiotensin II AT₁ receptor and receptor tyrosine kinase signaling performs essential functions during renal collecting system development *via* control of UB branching morphogenesis.

Keywords

kidney development; ureteric bud; renin-angiotensin; GDNF; c-Ret; EGF receptor

INTRODUCTION

Branching morphogenesis of the ureteric bud (UB) is a key developmental process that controls not only formation of the renal collecting system, but organogenesis of the entire metanephros. Derangements in UB morphogenesis cause a spectrum of congenital abnormalities of the kidney and urinary tract (CAKUT). Many instances of CAKUT have a genetic basis and are associated with hereditary human syndromes (1). The inheritance pattern of some nonsyndromic cases of CAKUT (eg., autosomal-dominant or recessive polycystic kidney disease) is well known (2). In other nonsyndromic forms of CAKUT (eg., obstructive uropathy, vesico-ureteral reflux, kidney hypodysplasia, duplex kidneys), the hereditary and molecular mechanisms remain to be determined.

Mutations in the genes encoding components of the renin-angiotensin system (RAS) or pharmacological inhibition of RAS in animals or humans cause diverse malformations of the renal excretory system that include papillary and medullary hypodysplasia, hydronephrosis, collapsed collecting ducts, aberrant UB budding, duplicated collecting system, and urinary

concentrating defect (3–10). Since CAKUT are the major cause of renal failure in childhood (11), identification of the molecular mechanisms that lead to diverse forms of CAKUT under conditions of disrupted RAS has important clinical implications.

OVERVIEW OF METANEPHRIC KIDNEY DEVELOPMENT

The development of the metanephric kidney begins when the nephric duct (ND) gives rise to ureteric bud (UB) on embryonic (E) day E10.5 in mice and E28 in humans (12). UB outgrowth from the ND is followed by its repetitive branching, growth and remodeling, a process called branching morphogenesis (Fig. 1), to eventually form the renal collecting system (13,14). Initial generations of UB branches will be remodeled into the ureter, renal pelvis and calyces, whereas subsequent generations of UB branches will give rise to collecting ducts. The ureter will translocate from the ND to fuse with the bladder (15,16). Collecting ducts will subsequently undergo patterning to contribute importantly to the renal papilla and medulla. Each UB tip is capable of inducing the adjacent metanephric mesenchyme to undergo mesenchymal-to-epithelial transition (MET) and form nephrons (from the glomerulus through the distal tubule) (13). Even subtle defects in the efficiency of UB branching result in a significant decrease in nephron endowment (17). In turn, decreased nephron endowment is linked to renal hypodysplasia, CAKUT, hypertension and eventual progression to chronic renal failure (11,18,19).

GROWTH FACTORS IN UB MORPHOGENESIS

UB initiation, branching and patterning is regulated by reciprocal interactions of transcription and growth factors expressed in metanephric mesenchyme, stroma, angioblasts and UB itself (20) (Fig. 2). Signals from the metanephric mesenchyme, stroma and angioblasts induce the UB to originate from the ND, invade the mesenchyme and undergo branching morphogenesis. Metanephric mesenchymal cells secrete glial-derived neurotrophic factor (*GDNF*) which interacts with the *c-Ret* tyrosine kinase receptor expressed in the UB tip cells to induce branching (21). *GDNF* or *c-Ret* expression is activated by paired box 2 (*Pax-2*), a transcription factor present in the metanephric mesenchyme (22,23). Stromal cells provide critical signals to regulate UB branching via forkhead/winged helix transcription factor d1 (*Foxd1*) or retinoic acid. *Foxd1* or combined retinoic acid receptor (*RAR*) α/β 2 deficiency in mice compromises UB branching (24,25). Interestingly, UB tip cells in *RAR* α/β 2-deficient mice express less *c-Ret* and genetic overexpression of *c-Ret* in these mice rescues UB branching and stromal patterning. Thus, not only stroma-derived signals regulate UB morphogenesis, but *c-Ret*-dependent UB output is important in stromal development. In addition, UB signaling is critical for the induction and maintenance of nephrogenesis. For example, conditional inactivation of *Wnt9b*, normally expressed in the UB epithelium, leads to defects in formation of renal vesicles (26). This is accompanied by the absence of the early nephron markers such as *Wnt4*, *Fgf8* and *Pax8*. UB branching in *Wnt9b*-deficient mice is also disrupted possibly due to decreased levels of *GDNF* in the mesenchyme and of *Wnt11* in the UB tip cell domain (26).

Wnt11 cooperates with *GDNF/c-Ret* pathway to regulate UB morphogenesis. Metanephroi of *Wnt11*-null mice express less *GDNF* and have fewer nephrons and UB branches compared to wild-type littermates (27). Moreover, *c-Ret* and *Wnt11* interact genetically to induce UB branching. Thus, *Wnt11* acts as a critical component of *GDNF/c-Ret/Wnt11* signaling loop to stimulate UB branching (28). Augmenting effects of *GDNF/c-Ret/Wnt11* pathway on UB development are antagonized by *Spry1*. *Spry1* acts to inhibit *c-Ret* receptor tyrosine kinase (RTK) activity, prevent expansion of *GDNF* and *Wnt11* domains thus decreasing UB branching (28). Recent studies demonstrated that *Wnt*-dependent UB branching is mediated via the canonical β -catenin pathway (29). Targeted inactivation of β -catenin in UB cell lineage causes decreased expression of transcription factors *Emx2*, *Pax2*, *Lim1* followed by downregulation

of *GDNF/c-Ret/Wnt11* and results in disrupted branching morphogenesis (29). Stem cell factor (*SCF*) represents yet another signaling molecule released by the UB. *SCF* interacts with RTK *c-kit* located in *Foxd1*-negative interstitial cells to induce UB branching (30). Interestingly, a subset of *c-kit*-positive cells express angioblast markers *Flk-1* and *Podx1*. Antagonism of *c-kit* RTK activity inhibits UB branching, reduces the number of angioblasts and downregulates *Flk-1* gene expression. Angioblasts can also modulate UB branching by regulating *GDNF* expression in the metanephric mesenchyme (31).

In addition to *GDNF*, a number of other RTK-stimulating growth factors have been shown to regulate UB morphogenesis. While fibroblast growth factors (*FGF*) 7 and 10 or insulin-like growth factor (*IGF-1*) promote, *TGF-β* and activin A inhibit UB budding (32,33,34,35). Genetic inactivation of *FGF7* or *FGF10* in mice results in a reduced number of UB branches, medullary collecting ducts and leads to papillary hypodysplasia (32,34). Both *FGF7* and *FGF10* are present in the stroma and bind *FGF* RTK2 which is expressed exclusively in the UB (36,37,38). UB-specific inactivation of the *FGF* RTK2, but not *FGF* RTK1, in mice reduces UB branching and nephron number (37). Epidermal growth factor receptor (EGFR) is expressed in renal collecting ducts (39). Stimulation of the EGFR by its ligand enhances branching tubule formation in mouse inner medullary collecting duct (IMCD-3) cells grown in three-dimensional collagen matrix gels *in vitro* (40). *In vivo* studies demonstrate that EGFR-mutant mice have abnormal collecting ducts (41). As RTK-stimulating growth factors, *GDNF*, *FGF7/FGF10* or *EGF* may signal *via* shared downstream pathways to stimulate UB branching. Moreover, *GDNF/c-Ret*-dependent pathway can be bypassed by other morphogenetic growth factors. In this regard, addition of *FGF7* along with activin A, an inhibitor of *TGF-β* superfamily, induces branching in isolated nephric ducts *in vitro* (42). Therefore, there is a functional redundancy between growth factor signaling during UB morphogenesis.

Bone morphogenetic proteins (*BMP*), members of *TGF-β* superfamily, play an important role in UB morphogenesis. *BMP* growth factors signal *via* activin-like kinase (ALK) type I serine/threonine kinase receptors coupled to downstream cytoplasmic signal-transducing Smad proteins (43). *BMP2*, *BMP4* and *BMP7* are most clearly involved in regulating UB branching. Treatment of embryonic kidney explants or isolated UBs with exogenous *BMP2*, *BMP4* or high doses of *BMP7* inhibits UB branching *in vitro* (44,45,46,47). *BMP4* is expressed exclusively in the stroma. *BMP4*^{+/-} embryos exhibit duplicated UBs suggesting that *BMP4* acts to suppress ectopic UB budding (46). UB-specific inactivation of ALK3 receptor subtype in mice causes aberrant UB and collecting duct morphogenesis resulting in hypoplasia of the renal medulla (48). Since these mice exhibit a decrease in the expression of *Pax2* in the UB and metanephric mesenchyme, initial decrease in UB branching may be due, in part, to a reduced *Pax2*-dependent signaling.

RENIN-ANGIOTENSIN SYSTEM-GROWTH FACTOR CROSS-TALK IN UB MORPHOGENESIS

Angiotensin (Ang) II [Ang-(1–8)] is the principal effector peptide growth factor of the renin-angiotensin system (RAS) which acts *via* two major types of G protein-coupled receptors (GPCR): AT₁R and AT₂R. AT₁R is a mitogen for renal vascular, mesangial and tubular cells (49). In contrast, AT₂R inhibits growth and proliferation in mesangial cells (50). Ang-(1–7) is a newly discovered member of the RAS. Ang-(1–7) is formed from Ang II by angiotensin-converting enzyme 2 (ACE2) and acts *via* GPCR Mas to oppose Ang II-AT₁R-mediated effects (51,52). Genetic inactivation of the AT₁R, AT₂R and other RAS components in mice or pharmacologic antagonism of the RAS cause a spectrum of abnormalities in the development of the renal collecting system (3–10). Angiotensinogen, angiotensin-converting enzyme (ACE) or AT₁R-deficient mice manifest hydronephrosis, hypoplastic medulla and papilla. Studies from Dr. Ichikawa's laboratory have suggested that medullary hypoplasia and hydronephrosis

in AT₁R-deficient mice may be due to a hypoplastic ureteral smooth muscle layer which exhibits impaired peristalsis (53). Functionally, ACE- and AT₁R-null animals have a reduced ability to concentrate urine (6,7). Use of ACE inhibitors and AT₁R blockers in humans cause oligohydramnios and anuria (54,55). Therefore, these drugs should not be used by pregnant women. AT₂R-mutant mice demonstrate a decrease in the rate of apoptosis of mesenchymal cells around the nascent UB (56) and increased incidence of duplex ureters and vesicoureteral reflux (57). Thus, it is conceivable that absence of timely apoptosis of mesenchymal cells may cause an aberrant UB budding and lead to duplicated ureters. In addition, AT₂R may act as restricting signal to limit anterior expansion of the *GDNF* domain (Fig. 2). The role of ACE2-Ang-(1-7)-Mas axis in kidney development and UB morphogenesis remains to be determined. However, based on the findings that ACE2 homolog collectrin is expressed in the UB branches as early as on E13 in the mouse (58) and that Ang-(1-7), acting *via* Mas, inhibits Ang II-induced phosphorylation of MAP kinase in kidney proximal tubular cells (52), such role is likely.

Mutations in the genes encoding for angiotensinogen, renin, ACE or AT₁R in humans lead to renal tubular dysgenesis (RTD) (10,59). In RTD, renal cortex exhibits a paucity of proximal tubules. In the medulla, collapsed collecting ducts and abundant interstitial fibrosis are observed (59). Importantly, RTD is characterized by perinatal death due to anuria combined with pulmonary hypoplasia. The more severe outcome in humans than in mice without the functional RAS may be due to the temporal difference in completion of nephrogenesis. In mice, nephrogenesis is completed 2 weeks after birth, whereas in humans- at 38 weeks of gestation. Collectively, the observed abnormalities in UB/medullary development imply that UB-derived structures are targets for Ang II actions during metanephric development in both mice and humans.

We recently demonstrated that angiotensinogen and Ang II AT₁R are present in both UBs and stroma on E12 in the mouse and that their expression increases progressively from E12 to E16 (60). Importantly, AT₁R immunoreactivity is present on both luminal and basolateral aspects of UB branches. Since renin-producing cells originate from the mesenchyme on E11-E12, at a time when UB branching is just beginning (61), Ang II may be generated locally in the mesenchyme to act in a paracrine fashion on the adjacent AT₁R-expressing UBs to regulate branching (Fig. 2). In accord with this hypothesis, we further demonstrated that Ang II, acting *via* the AT₁R, stimulates UB growth and branching in the intact whole embryonic (E) day E11.5 metanephroi grown *ex vivo* and studied at 24 and 48 hours after Ang II treatment (62). These findings indicate that Ang II promotes morphogenesis of the renal collecting system. Ang II can stimulate UB branching directly or through interaction with other transcription and growth factors present in the metanephric mesenchyme, stroma, angioblasts or the UB that are known to regulate UB morphogenesis (Fig. 2). For example, Ang II may stimulate *Pax2* present in the UB or mesenchyme and thereby enhance UB branching. This possibility is supported by the ability of Ang II to increase *Pax2* gene expression in metanephric kidney (63). Since such RTK-stimulating growth factors as *FGF7* or *EGF* enhance the ability of *GDNF* to elicit ectopic UB budding (42), Ang II may induce UB branching by enhancing sensitivity of the UB to *GDNF*, *EGF*, or *FGF7*. In addition, Ang II may alter the expression of competence growth factors in favor of UB branching. In keeping with this hypothesis, renal papilla of angiotensinogen- and AT₁R-deficient mice exhibits reduced *EGF* mRNA levels (4). Moreover, ACE inhibition during neonatal nephrogenesis in the rat decreases *IGF-1* mRNA and protein expression in the renal medulla (64). Both AT₁R and *FGF7* are expressed in the stromal mesenchyme, and Ang II increases *FGF7* mRNA levels in luteal cells (65). These observations indicate that RAS interacts with *EGF* and *IGF-1* and may induce *FGF7* during medullary development.

Given that *GDNF/c-Ret* signaling pathway is a major positive regulator of UB branching morphogenesis program (22), we recently tested whether Ang II enhances *GDNF* and *c-Ret*

expression during active UB branching. Treatment with Ang II induced expression of *GDNF* mRNA in the metanephric mesenchyme (66). This was accompanied by increased expression of *c-Ret*, *GDNF* RTK, and of *Wnt11*, a downstream target of *c-Ret* signaling, in the UB tip cells. Moreover, Ang II caused preferential proliferation of UB tip cells, whereas inhibition of endogenous AT₁R signaling inhibited UB tip cell apoptosis (66). Therefore, Ang II not only induced *GDNF/c-Ret* expression during critical period of UB morphogenesis, but induced mitogenic and prosurvival effects in the UB tip cells leading to UB growth and branching. To determine the mechanism of Ang II-induced upregulation of *GDNF/c-Ret* signaling, we next examined the effect of Ang II and its AT₁R on the expression of *Spry1*, an endogenous inhibitor of *c-Ret* RTK signaling (28). Ang II decreased *Spry1* in an AT₁R-dependent manner (66). These findings indicate that AT₁R-*GDNF/c-Ret* cross-talk involves downregulation of *Spry1*. This in turn facilitates *c-Ret* RTK signaling leading to activation of the *GDNF/Ret/Wnt11* positive feedback loop. It is conceivable that Ang II induces focal bursts of proliferation of UB tip cells, and together with decreased apoptosis, plays an important role in the expansion of the ampulla, subsequent branching and directional bud elongation.

Alternatively or additionally, stimulation of the AT₁R by its ligand can transactivate RTK to enhance morphogenetic signaling without altering the expression levels of a given positive growth factor or its cognate RTK. To test this hypothesis, we recently investigated the ability of Ang II to transactivate EGFR in UB cells and examined the contribution of EGFR activation to Ang II-induced UB branching. Ang II increased tyrosine phosphorylation of EGFR in UB cells (56). Moreover, inhibition of EGFR tyrosine kinase activity abrogated Ang II-induced UB branching in the intact metanephros (62). These findings indicate that cross-talk between the AT₁R and EGFR is crucial in UB morphogenesis and that cooperation of AT₁R and EGFR signaling promotes the development of the renal collecting system.

MECHANISMS OF ANGIOTENSIN II-GROWTH FACTOR INTERACTIONS DURING UB MORPHOGENESIS

Cross-talk may be defined as a functional interaction between different signaling pathways. These interactions enhance the ability of the cell to integrate, process and respond to the information received from its microenvironment. The consequences of cross-talks are that the expression of a given set of target genes is regulated by multiple signaling pathways. Thus, the expression of the UB morphogenetic program genes and UB growth depend on complex network of regulatory pathways and signaling effectors. Ang II may cross-talk with RTK and their cognate ligands *via* intracellular signaling pathways, induction of cleavage of membrane-bound RTK proligand, facilitation of physical interaction between AT₁R/AT₂R and RTK or other mechanisms. For example, inhibition of PI3K blocks *GDNF*-dependent UB branching and migration of *c-Ret*-transfected MDCK cells (67,68). Stimulation of Ang II AT₁R activates PKC (69) leading to MAP kinase (MAPK)-dependent upregulation of transcription of cell cycle progression genes, such as cyclin D1, through activation of the transcription factor AP-1 (70). Thus, one of the possible mechanisms facilitating Ang II-EGFR/*c-Ret* interactions and UB morphogenesis may involve EGFR- or *c-Ret*-mediated stimulation of MAPK and PI3K/Akt pathways. Whether effects of AT₁R and EGFR/*c-Ret* result in synergistic stimulation of MAPK/PI3K and UB branching remains to be determined. AT₂R-dependent mesenchymal apoptosis (35) may result from activation of MAP kinase phosphatase 1 leading to inactivation of ERK1/2 (50). AT₂R-dependent activation of *Pax2* in whole metanephroi and mesenchymal cells is mediated by JAK2/STAT pathway (63).

Another mechanism by which Ang II may activate EGFR is by stimulating the proteolytic cleavage of pro-heparin-binding *EGF* at the cell membrane (71). Coupling of Ang II with AT₁R activates extracellular metalloproteinase which cleaves transmembrane *EGF* precursor and releases mature *EGF* (71). Mature *EGF* interacts with the ectodomain of the EGFR and

activates intracellular signal. Facilitation of direct physical interaction between Ang II receptors and RTK may involve clustering in lipid rafts. For example, caveolae/lipid rafts are essential for Ang II-induced transactivation of EGFR (72). Interestingly, *EGF* induces phosphorylation of AT₁R and leads to formation of a multireceptor complex containing AT₁R and activated EGFR (73). These findings suggest that cross-talk between the RAS and RTK can be bi-directional. One mechanism whereby Ang II may transactivate RTK during UB branching is the balance between AT₁R- and AT₂R-mediated actions. In this regard, recent reports suggest that AT₂R offsets the growth-promoting effects of the AT₁R (74) and that part of the antagonism between the two receptor types might be due to the formation of AT₁R/AT₂R heterodimers whereby the AT₂R directly inhibits AT₁R (75). Since AT₂R expression in the metanephros progressively decreases and of AT₁R increases with gestation (76), it is conceivable that increased levels of AT₁R not complexed with AT₂R may facilitate AT₁R-growth factor/RTK cross-talk to stimulate UB morphogenesis.

Future studies investigating the effect of AT₁R and AT₂R null mutations on the expression of the critical growth factors and signaling networks known to regulate UB branching will provide essential information regarding the nature of events culminating in the formation of the mature metanephros.

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Figure 1.

Ureteric bud branching morphogenesis in the mouse. Ureteric bud (UB) emerges from the nephric duct on embryonic (E) day E10.5. Metanephros isolated on E11.5 from Hoxb7-GFP transgenic mouse embryo and grown *ex vivo* for 48 hours. Hoxb7 promoter directs expression of green fluorescent protein (GFP) to the UB. The number of branching structures increases progressively with time. Adapted from (62) (with permission from the American Society of Nephrology).

Figure 2.

Schematic representation of the concept of angiotensin II-growth factor cross-talk in ureteric bud morphogenesis. Metanephric mesenchyme and stroma interact reciprocally with the ureteric bud (UB) and angioblasts to form the metanephros. Multiple gene regulatory networks have been shown to regulate UB elongation, branching and nephron formation. Signaling molecule Slit2 and its receptor Robo2, forkhead/winged helix transcription factors C1 and C2 (FoxC1/C2), bone morphogenetic protein (BMP) 4 and angiotensin (Ang) II AT₂R act to restrict glial derived neurotrophic factor (GDNF) expression anteriorly and specify correct site of UB outgrowth from the nephric duct (46,57). GDNF/c-Ret/Wnt11 pathway is the major positive regulator of UB development (22). Stromal factors Foxd1, retinoic acid receptor (Rar) β2, BMP4, fibroblast growth factors (FGF) 7 and 10 play an essential role in UB morphogenesis (77). FGF7/10 act via FGF receptor tyrosine kinase 2 (RTK2). Angiotensinogen (AGT) and Ang II are emerging stromal factors that regulate UB morphogenesis (60). Since renin-expressing cells are present in the stroma on E12 (61), co-localization of AGT (60) and renin in the stroma suggests that Ang II is generated around the UB and acts in a paracrine manner on the AT₁R and AT₂R expressed in the UB to regulate branching. Ang II AT₁R cross-talks with GDNF/c-Ret/Wnt11 pathway and EGFR. Stimulation of the AT₁R by Ang II inhibits *Spry1* gene expression and thereby releases inhibition of signaling via the GDNF/c-Ret/Wnt11 pathway (66). In addition, Ang II enhances tyrosine phosphorylation of EGFR in UB cells (62). Inhibition of EGFR tyrosine kinase activity abrogates Ang II-induced UB

morphogenesis (62). Coupling of Ang II with the AT₂R upregulates paired box 2 (*Pax2*) and stimulates UB branching (63). Stem cell factor (SCF) is expressed in the UB and acts *via* its receptor c-kit to induce *GDNF* expression in the metanephric mesenchyme and thereby modulate UB branching (31).

Figure 3.

Proposed mechanisms of angiotensin II-growth factor cross-talk in ureteric bud branching morphogenesis. Heterodimerization of AT₁R and AT₂R leads to inhibition of AT₁R signaling by AT₂R (75). AT₁R- and AT₂R-mediated activation of multiple signaling pathways involving Jak2/STAT (63), Ras/ERK1/2 (69,70), PI3K/Akt (67,68), epidermal growth factor receptor (EGFR) transactivation (62) may stimulate UB cells proliferation, survival, migration, and promote UB morphogenesis. Ang II may stimulate cleavage of EGFR proligand (71). Facilitation of a direct physical interaction between Ang II receptors and RTK (EGFR, c-Ret) may involve clustering in lipid rafts (72).