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Angiotensin II stimulates *in vitro* branching morphogenesis of the isolated ureteric bud

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Abstract

Mutations in the renin-angiotensin system (RAS) genes are associated with congenital anomalies of the kidney and urinary tract (CAKUT). As angiotensin (Ang) II, the principal effector peptide growth factor of the RAS, stimulates ureteric bud (UB) branching in whole intact embryonic (E) metanephroi, defects in UB morphogenesis may be causally linked to CAKUT observed under conditions of disrupted RAS. In the present study, using the isolated intact UB (iUB) assay, we tested the hypothesis that Ang II stimulates UB morphogenesis by directly acting on the UB, identified Ang II target genes in the iUB by microarray and examined the effect of Ang II on UB cell migration *in vitro*. We show that isolated E11.5 mouse iUBs express Ang II AT_1 and AT_2 receptor mRNA. Treatment of E11.5 iUBs grown in collagen matrix gels with Ang II (10^{-5} M) increases the number of iUB tips after 48 hours of culture compared to control (4.8±0.4 vs. 2.4±0.2, $p<0.01$). A number of genes required for UB branching as well as novel genes whose role in UB development is currently unknown are targets of Ang II signaling in the iUB. In addition, Ang II increases UB cell migration (346±5.1 vs. 275±4.4, $p<0.01$) *in vitro*. In summary, Ang II stimulates UB cell migration and directly induces morphogenetic response in the iUB. We conclude that Ang II-regulated genes in the iUB may be important mediators of Ang II-induced UB branching. We hypothesize that Ang II-dependent cell movements play an important role in UB branching morphogenesis.

Keywords

kidney development; ureteric bud; angiotensin; Etv 4; Etv5

1. Introduction

Metanephric development begins when the nephric duct (ND) gives rise to the ureteric bud (UB) on embryonic (E) day E10.5 in mice and E28 in humans (Grobstein, 1953; Saxen, 1987; Ekblom, 1989; Costantini and Kopan, 2010). UB outgrowth from the ND is followed by its repetitive branching, a process called branching morphogenesis. Each UB tip can induce the adjacent metanephric mesenchyme (MM) to undergo mesenchymal-to-epithelial transition and form nephrons (from the glomerulus through the distal tubule) (Grobstein, 1953; Saxen, 1987; Ekblom, 1989; Costantini and Kopan, 2010). In turn, signals emanating

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from the MM control UB morphogenesis. Initial generations of UB branches ultimately undergo patterning to form the ureter, renal pelvis and calyces, whereas subsequent generations of UB branches will give rise to collecting ducts. Notably, defects in UB morphogenesis cause a spectrum of congenital anomalies of the kidney and urinary tract (CAKUT), the single most common cause of renal failure in children (NAPRTCS Annual Report, 2010).

The critical role of the renin-angiotensin system (RAS), a major regulator of the blood pressure and fluid/electrolyte homeostasis (Kobori et al., 2006), in kidney and renal collecting system morphogenesis is evident from occurrence of diverse forms of CAKUT in animals or humans as a result of RAS gene mutations. These forms of CAKUT include papillary and medullary hypodysplasia, hydronephrosis, renal tubular dysgenesis, collapsed collecting ducts, aberrant UB budding, duplicated collecting system and urinary concentrating defect (Niimura et al., 1995; Esther et al., 1996; Nagata et al., 1996; Oliverio et al., 1998; Nishimura et al., 1999; Oshima et al., 2001; Gribouval et al., 2005; Song and Yosypiv, 2011). Collectively, these findings indicate that UB and its derivatives, the collecting ducts, are principal target for the RAS.

Our previous work demonstrates that lack of endogenous Ang II in angiotensinogen (AGT)-deficient mice leads to defects in UB branching and that both Ang II AT_1 (AT_1R) and AT_2 (AT_2R) receptor signaling promotes UB branching morphogenesis in intact whole embryonic kidneys (Yosypiv et al., 2008; Yosypiv et al., 2008 Song et al., 2010a, 2010b, 2010c).

In this work, we tested the hypothesis that Ang II can directly regulate branching morphogenesis in the intact isolated UB (iUB) independent of confounding effects of the mesenchyme. Given the important role of cell movement in UB morphogenesis (Tang et al., 2002; Chi et al., 2009), we next examined the effect of Ang II on UB cell migration *in vitro*. In addition, we conducted a global analysis of mRNA expression in iUBs cultured with or without Ang II. The results demonstrate that Ang II, when applied directly to the iUB organ culture, stimulates UB branching and induces directional migration of cultured UB cells *in vitro*. Finally, we identified a UB cell-autonomous transcriptome downstream of Ang II signaling.

2. Results

2.1. Effect of Ang II on ureteric bud (UB) branching morphogenesis in iUB culture

In a previous study, we demonstrated that Ang II can induce a branching morphogenesis-like program in immortalized ureteric bud-derived cells cultured in collagen matrix gels *in vitro* (Iosipiv and Schroeder, 2003). In this study, we investigated the direct effects of Ang II on UB branching in a physiologically more relevant system which allows to isolate the effects of the mesenchyme and examine the specific effects of Ang II on division and elongation of ampullary structures, such as iUB culture (Qiao et al., 1999). Absense of mesenchymal contamination of the iUB preparation was confirmed by the lack of mesenchyme-specific marker, *GDNF*, expression in the iUB (Fig. 1A). Notably, iUBs express UB tip cell-specific marker, Ret, as well as Ang II AT_1R and AT_2R mRNA (Fig. 1A-C). Treatment of E11.5 iUBs grown *ex vivo* with Ang II (10^{-5} M) increased the number of UB tips compared to control after 48 hours of culture compared to control (Mean±SEM: 4.8±0.4 vs. 2.4±0.2, $p<0.01$) (Fig. 1D, E. Supplemental Fig. 4 and 5). These data demonstrate that Ang II can induce morphogenetic response in UB epithelia in UB cell-autonomous manner.

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2.2. Effect of Ang II on UB cell migration *in vitro*

To gain further insight into the cellular mechanisms by which Ang II stimulates UB branching, we examined the effect of Ang II on UB cell movement *in vitro* using transwell migration assay. Ang II increased the number of cells that migrated through the filter compared with control (media) (Mean±SEM: 346±5.1 vs. 275±4.4, p=0.003) (Fig. 2A). These results demonstrate a direct stimulatory effect of Ang II on directional migration of UB cells *in vitro*.

2.3. A screen for Ang II-regulated genes in the UB

Given that only a few genes regulated by Ang II during UB morphogenesis were identified (Yosypiv et al., 2008), we conducted a global analysis of gene expression in iUBs cultured with or without Ang II. RNA was extracted from two pools of iUBs grown with Ang II and two pools of iUBs grown in the absence of Ang II, and surveyed with Agilent mouse GE4X44K chips. Of the ~44,000 transcripts represented on the GE4X44K array, 1057 were upregulated by two-fold or more with Ang II, 24 by 4-fold or more, whereas 18 were downregulated by 4-fold or more. Table 1 lists select genes most highly upregulated or downregulated by Ang II ($p<0.001$ to <0.05 vs. without Ang II). The genes upregulated by Ang II encode secreted and growth factors (*Wnt11*), adhesion molecules (*Icam1*), receptors (*Grik2*, *Crlf1*, *Fgfr1*), transcription factors (*Zfp42*, *Etv4*, *Etv5*), chemotactic factors (*Ccl20*), phosphatases (*Dusp8*), signaling molecules (*Vsnl1*, *Rhof*), regulators of signal transduction (*Spry1*) and cell cycle (*Ect2, Myb*), consistent with a role of Ang II in proliferation of UB tip cells (Yosypiv et al., 2008). Genes downregulated by Ang II include matrix metalloproteases (*Mmp* 7 and 9), receptors (*Gpr123*), ion transporters (*Clc9a2*) and secreted and growth factors (*Wnt9b, Fgf12*). We hypothesize that these genes together mediate the effects of Ang II on UB branching and kidney development.

Given that GDNF is essential positive regulator of UB branching (Costantini and Kopan, 2010) and that treatment of intact embryonic metanephroi *ex vivo* with exogenous Ang II induces *GDNF* expression in the mesenchyme (Yosypiv et al., 2008), we next determined direct (GDNF/Ret-independent) and indirect (GDNF/Ret-dependent) targets of Ang II in the ureteric cell lineage. To do so, we compared transcriptomes of Ang II- and GDNF-stimulated iUBs (Lu et al., 2009) (Table 1). While Ang II or GDNF caused similar changes in the expression of a number of genes, a subset of genes were differentially regulated by Ang II or GDNF. The list of genes upregulated by Ang II contained a number of GDNF targets (*Etv4*, *Etv5*, *Vsnl1*, *Crlf1*). Genes induced by Ang II, but not by GDNF, include *Zfp42*, *Rhof* and *Ccl20* (Table 1). These finding indicate that a part of iUB transcriptome is regulated by Ang II independent of GDNF. We validated the results of our global gene expression analysis using quantitative real time PCR (qPCR) and *in situ* hybridization. qPCR demonstrated changes in mRNA expression consistent with results obtained by microarray (Fig. 2B). Even though our array assays did not reveal significant changes in *Ret* expression, the more sensitive qPCR assays indicated upregulation of *Ret* expression by Ang II (Fig. 2B).

Because members of the Pea3 family of ETS transcription factors, ETS variant (Etv) 4 and Etv5 are key transcription factors that mediate the effects of GDNF/Ret on UB morphogensis (Lu et al., 2009), we examined the spatial expression levels of Etv4/Etv5 by *in situ* hybridization. Consistent with qPCR findings, expression of Etv4 and Etv5 was increased in the UB tips in E12.5 metanephroi treated with Ang II (Fig. 2C). Together, these results demonstrate that Ang II regulates the expression of a number of genes required for UB branching as well as of novel genes whose role in UB development is currently unknown independent of the mesenchyme.

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3. Discussion

The present study demonstrates that angiotensin (Ang) II stimulates *in vitro* branching morphogenesis of the isolated intact ureteric bud (iUB) and induces migration of UB cells. Expression of a number of known UB morphogenetic program genes as well as novel genes whose role in UB development is currently unknown is altered by Ang II signaling independent of the influence of the mesenchyme.

The embryonic kidney expresses all the components of the renin-angiotensin system (RAS). Angiotensinogen (AGT), Ang II AT_1R and AT_2R are expressed in the UB and the mesenchyme as early as on E12 in the mouse (Iosipiv and Schroeder, 2003; Song et al., 2010a). Renin-expressing cells are present in the stroma on E12 (Lopez et al., 2001). Angiotensin-converting enzyme (ACE) is detected in the embryonic kidney in rodents and humans (Jung et al., 1993; Yosypiv et al., 1994). Collectively, the fetal metanephros has the capacity to both generate Ang II and to transmit its actions.

We recently reported that Ang II, acting *via* the AT_1R or AT_2R, stimulates UB branching morphogenesis in the whole intact metanephric kidneys cultured *ex vivo* and induces tubulogenesis in immortalized UB cells grown in collagen matrix gels *in vitro* (Iosipiv and Schroeder, 2003; Yosypiv et al., 2006). In the present study, using the iUB model, we tested the hypothesis that Ang II can stimulate UB morphogenesis by directly acting on the UB, examined the role of cell movement in Ang II-induced UB branching and identified genes that mediate the effects of Ang II on UB morphogenesis independent of the mesenchyme. Consistent with our results in immortalized UB cells (Iosipiv and Schroeder, 2003), Ang II stimulated UB branching in the iUB. In light of the presence of AT_1R/AT_2R mRNA in the iUB, these results support the hypothesis that Ang II, generated locally in the mesenchyme, acts in a paracrine fashion on the adjacent AT_1R/AT_2R-expressing UBs to regulate branching *in vivo*.

One mechanism by which Ang II can stimulate UB branching is by inducing migration of the UB cells. In this regard, treatment with glial cell-derived neurotrophic factor (GDNF), a ligand for Ret, stimulates directional migration of *Ret9*-transfected Madin-Darby canine kidney *(MDCK)* cells towards GDNF source *in vitro* (Tang et al., 2002). Recent *in vivo* studies demonstrate that movement of *Ret*-expressing Wolffian duct (WD) cells accounts for the UB outgrowth from the WD (Chi et al., 2009). The present study demonstrates that Ang II induces migration of cultured UB tip cells *in vitro*. We speculate that Ang II-induced UB cell movement may contribute to directional bud elongation and UB branching.

Expression of a variety of novel genes was altered by Ang II in the iUB culture. Most highly upregulated genes include *Dusp8*, *Grik2*, *Zfp42, Ccl20, Rhof*, *Crlf1*, *Etv4*, *Etv5* and *Vsnl1*. Dusp8 is a phosphatase which preferentially inactivates p38 and JNK MAP kinases. Although JNK is not detected in the developing kidney, inhibition of p38 retards growth of embryonic metanephroi grown *ex vivo* (Awazu et al, 2002). Grik2 is a member of glutamate receptor ion channels which mediate excitatory transmission in the mammalian brain (Traynelis et al., 2010). The *Zfp42* (*Rex-1*) gene encodes a zinc finger family transcription factor which plays an important role in stem cell self-renewal and differentiation (Thompson and Gudas, 2002). Ccl20 is a chemokine which mediates renal recruitment of T lymphocytes in glomerulonephritis and renal cell carcinoma (Turner et al., 2010; Middel et al., 2010). Rhof is a member of the Rho-GTPase family which controls the formation of actin stress fibers, fundamental components of the actin cytoskeleton that produce contractile force, in MDCK cells (Fan et al., 2010). Since contraction at the apical cell surface is important during outpouching of the prospective UB tips (Meyer et al., 2004), Ang II-induced upregulation of *Rhof1* expression in the UB may act to promote expansion of the ampulla.

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Interestingly, Ang II increased expression of ETS transcription factors *Etv4* and *Etv5* compared with control. Etv4/Etv5 are key transcription factors which function downstream of Ret to regulate UB morphogenesis. Specifically, $Etv4^{-/-}$;$Etv5^{+/-}$ WD cells have limited ability to contribute to the UB tip domain *in vivo* (Kuure et al., 2011). Thus, it is conceivable that Ang II may stimulate UB cell movement, in part, *via* upregulation of *Etv4*/*Etv5*. *Vsnl1*, which encodes a calcium-sensing protein and is expressed exclusively in the UB tip domain, is upregulated by both Ang II and GDNF (Ola et al., 2011). Because UB tip cells contain elevated intracellular calcium levels (Ola et al., 2011) and activation of calcium signaling induces actin cytoskeleton reorganization in MDCK cells (Miranda et al., 2010), Ang II-induced upregulation of Vsnl1 expression in the UB may act to promote expansion of the ampulla by creating wedge-shaped cells through an apical purse-string mechanism (Meyer et al., 2004).

Genes downregulated by Ang II in iUB culture include matrix metalloproteases (MMP) *MMP7* and *MMP9*. MMPs promote UB branching by degrading components of the extracellular matrix and releasing growh factors. Given that MMP9 stimulates UB branching (Arnould et al., 2009), the significance of Ang II-induced downregulation of MMP9 mRNA expression in the iUB model is not clear. Since *MMP9*-null metanephroi exhibit decreased levels of activated form of the stem cell factor (SCF) receptor, c-kit, expressed in the mesenchyme (Arnould et al., 2009), it is conceivable that observed decline in MMP9 expression in iUB culture may result from disrupted cross-talk between the mesenchyme and the UB. The role of MMP7 in kidney development is unknown. Of interest, MMP7 expression can be induced in the adult kidney by noncanonical WNT signaling (Jovanovic et al., 2008). Because noncanonical Wnt9b signaling is important in oriented cell division and elongation of UB-derived collecting ducts (Karner et al., 2009), decreased MMP7 levels may reflect reduced ratio of noncanonical to canonical Wnt signaling to promote UB branching (Bridgewater et al., 2008). Ingenuity Pathway Analysis of Ang II targets in the iUB revealed upregulation of the p85 and p110 subunits of the phosphatidylinositol 3-kinase (PI3K) pathway (Fig. 3). Given that pharmacologic inhibition of PI3K pathway abrogates Ang II-induced UB branching in whole embryonic metanephroi grown *ex vivo* (Song et al., 2010c), it is conceivable that Ang II-induced UB cell migration depends on activation of PI3K signaling.

In summary, the present study demonstrates that Ang II stimulates *in vitro* branching morphogenesis of the isolated ureteric bud and directional migration of UB cells. Expression of the number of genes in the UB lineage is altered by Ang II signaling independent of the influence of the mesenchyme. A part of iUB transcriptome is regulated by Ang II independent of GDNF. We hypothesize that these genes together mediate the effects of Ang II on UB branching. As *RAS* gene mutations in humans result in CAKUT, understanding the molecular mechanisms behind these renal birth defects would aid in developing tools that may direct preventional strategies, predict functional outcomes or guide interventions for these children.

4. Experimental Procedures

4.1. Intact isolated UB culture

Intact UBs (iUBs) were isolated from embryonic (E) day E11.5 metanephroi of *Hoxb7*-GFP^{+} mice which express green fluorescent protein (GFP) exclusively in the UB. The day when the vaginal plug was observed was considered to be E0.5. Metanephroi were incubated with trypsin/DNAse-I for 15 min at 37° C and iUBs were then mechanically dissected from the loosened mesenchyme. Absence of mesenchymal contamination was determined by the lack of GDNF expression in the iUBs. iUBs were suspended in 100 μl of Matrigel (BD Biosciences) and grown for 48 hours at 37° C on transwell filters (Corning Costar, 0.5 μm)

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located on top of DMEM/F12 medium (Gibco BRL) containing medium conditioned by cells derived from metanephric mesenchyme (BSN) (Shah et al., 2009), GDNF (120 ng/ml, R&D Systems, Minneapolis, MN) and FGF1 (100 ng/ml) (control, n=8) or BSN/GDNF/FGF1 and Ang II (10^{-5} M, Sigma, n=8). Addition of GDNF and FGF1 was required to maintain viability of iUBs grown in Matrigel. The media were changed every 24 hours. Images were acquired at time of iUB isolation and after 48 hours of culture directly from the plates *via* an Olympus IX70 inverted phase-contrast microscope and Olympus MagnaFire FW camera, processed with Adobe PhotoShop 7.0, and the number of UB tips was counted. All samples were blind-coded for counting.

4.2. Transwell Migration Assay

UB cells were initially obtained from microdissected ureteric buds of an E11.5 mouse transgenic for simian virus 40 (SV40) large T antigen (Immorto-mouse, Charles River) (Barasch et al., 1996). UB cells were used at passage numbers 10 to 15. Quiescent UB cells were trypsinized, resuspended in serum-free DMEM/F12 medium, plated at 1×10^5 cells/ml on top of transwell filters (24 well plates, 8-μm-pore size, Corning, Acton, MA) the bottom side of which was precoated with 2 μg/ml fibronectin, and incubated overnight at 37° C and 5% CO_2. Ang II (10^{-5} M) or media (control) were added to the bottom of the well. Following overnight culture, the cells on the upper surface of the filter were removed with a cotton swab. Cells on the bottom of the well were fixed with paraformaldehyde (PFA 4% in PBS; Electron Microscopy Sciences, Hatfield, PA) for 15 min at room temperature and stained with hematoxylin/eosin, and the membrane was mounted on a glass slide. The mean number of cells that migrated through each membrane was determined by counting the number of cells in 5 high-power fields (x400) in each well (n=4 wells/treatment group) utilizing an Olympus IX70 inverted phase-contrast microscope. Experiment was repeated twice. All samples were blind-coded for counting.

4.3. Reverse-transcription polymerase chain reaction (RT-PCR)

Semiquantitative RT-PCR was utilized to assess potential contamination of iUB preparation by the mesenchyme and to determine whether iUBs express AT_1R and AT_2R mRNA. RNA was extracted using the TRIzol reagent (Invitrogen). 3 μg RNA was reverse-transcribed. cDNA was amplified using the Perkin Elmer Gene Amplification system 2400 (Cetus Instruments, Norwalk, CT) from 25% of RT mixture using gene-specific primers: AT_1R: sense- 5′-GCATCATCTTTGTGGTGGG-3′; antisense- 5′-GAAGAAAAGCACAATCGCC-3′; AT_2R: sense-5′-ATTCCTGTTCTCTACTAC-3′; antisense- 5′-GTAACACGTTGCTATGAA-3′; Ret: sense-5′-GGCATTAAAGCAGGCTACGGCA-3; antisense- 5′GAGGAATAACTGATTGGGAA-3′; GDNF: sense- 5′-GCCCTTCGCGCT-GACCAGTGAC-3′; antisense-5′-GATGG-TGGCGATAGTGGGATA-3′.

4.4. Agilent microarray and pathway analysis

iUBs were isolated from E11.5 CD1 mice metanephroi (Charles River Laboratories, New York, NY) and grown on air-fluid interface on polycarbonate transwell filters (Corning Costar, 0.5 μm) inserted into 6-well plates containing DMEM/F12 medium (Gibco BRL) alone or in the presence of Ang II (10^{-5} M) for 6 hours at 37° C and 5% CO_2. At this concentration, Ang II stimulates UB branching in whole intact E11.5 metanephroi grown *ex vivo* and in UB cells grown in collagen matrix gels *in vitro* (Iosipiv and Schroeder et al., 2003; Yosypiv et al., 2006). Following incubation, iUBs were homogenized in TRIzol and stored at −80 °C. RNA was isolated from batches of 30–40 pooled ureteric buds, quantified and checked for integrity using a NanoDrop 2000 bioanalyzer (Thermo scientific). Two pools of iUBs cultured with Ang II (10^{-5} M) and two pools without Ang II were used for hybridization to Agilent mouse GE4X44K gene expression microarray. Hybridization,

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scanning and analysis were done by a core facility of the Tulane Cancer Center. Microarray data analysis was performed in R (versions 2.9-2.10) (R Development Core Team, 2009) using independently developed quality-control scripts to identify array artifacts and normalize for spatial variation. Loess and intensity quantile normalization routines from the Bioconductor limma package (Smyth et al., 2005) were applied to adjust for dye bias and variation among arrays, respectively. A linear model was then fit to the normalized data using the "lmFit" function in the limma package to estimate the average log2 ratios and associated standard errors for each probe. The Benjamini-Hochberg correction for multiple testing was applied to the set of p-values generated for the probe coefficients, and probes with adjusted p-values of <0.05 were identified as being significantly differentially expressed. Molecular pathway analyses were performed by Ingenuity Pathway Analysis version 7.1 (Redwood City, CA). All experiments involving mice were approved by Tulane Institutional Animal Care and Use Committee.

4.5. Quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR)

SYBR Green quantitative real-time RT-PCR was conducted on RNA obtained from E11.5 iUBs treated or not with Ang II for 6 hours as described above for microarray analysis in the Mx3000P equipment (Stratagene, La Jolla, CA) using MxPro QPCR software (Stratagene) (Yosypiv et al., 2008). The quantity of each target mRNA expression was normalized by that of GAPDH mRNA expression. Three RNA samples per treatment group were analyzed in triplicates in each run. PCR reaction was performed twice.

4.6. In situ hybridization

Wild-type CD1 mice embryos were dissected aseptically from the surrounding tissues on E12.5 and grown on air-fluid interface on polycarbonate transwell filters (Corning Costar, 0.5 μm) inserted into 6-well plates containing DMEM/F12 medium (Gibco BRL) alone, in the presence of Ang II (10^{-5} M; Sigma) for 24 hours at 37° C and 5% CO_2 as previously described (Yosypiv et al., 2008) and then processed for *in situ* hybridization. Preparation of RNA probes and whole-mount in situ hybridization were performed according to protocols (http://www.hhmi.ucla.edu/-derobertis/protocol_page/-mouse.PDF) established in the De Robertis laboratory. 5 embryonic kidneys per treatment group per probe were examined. The metanephroi were photographed using an Olympus model SC35 camera mounted on an Olympus model BH-2 microscope, and digital images were captured using Adobe Photoshop software.

4.7. Statistical analysis

Data are presented as Mean±SEM. Differences among the treatment groups in the number of UB tips and mRNA levels in media vs. Ang II were analyzed by Student's t test. A p value of <0.05 was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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HIGHLIGHTS

- Ang II stimulates *in vitro* branching morphogenesis of the isolated intact ureteric bud (iUB) and directional migration of UB cells.
- Expression of the number of genes in the iUB is altered by Ang II signaling independent of the influence of the mesenchyme.
- A part of iUB transcriptome is regulated by Ang II independent of GDNF.

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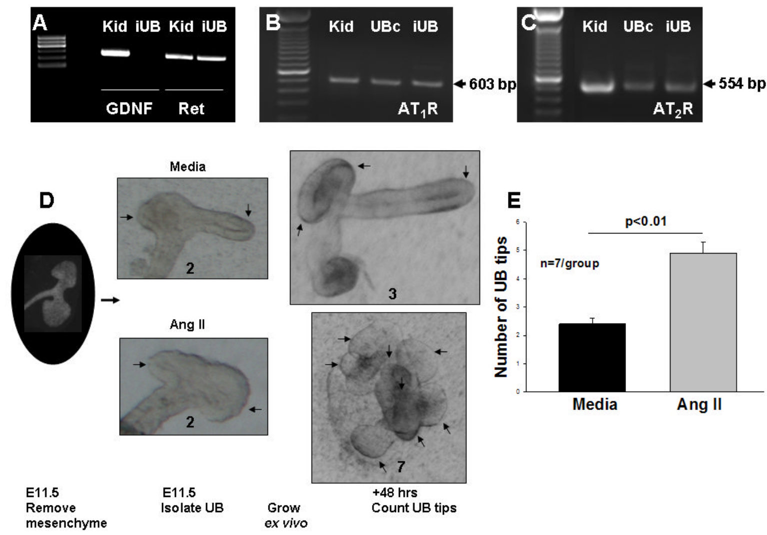

Figure 1.

A: Isolated intact E11.5 ureteric buds (iUB) express Ret (300 bp), but not GDNF (291 bp), mRNA. E12.5 mouse kidneys (Kid) express both Ret and GDNF mRNA. B and C: iUB, immortalized UB cells (UBc) and E12.5 mouse kidneys express angiotensin (Ang) II AT_1R and AT_2R mRNA. D: Effect of Ang II on branching of iUBs. iUBs were dissected on E11.5 and grown in collagen matrix gels in the presence of GDNF, FGF1 and media (control) or GDNF, FGF1 and Ang II (10^{-5} M) for 48 hours. Numbers below each iUB image show number of UB tips (marked with arrows) at each time point. E: Bar graph shows the effect of treatments on the number of UB tips after 48 hours of culture. The number of iUB tips in higher in Ang II-treated iUBs compared with media control (Mean±SEM).

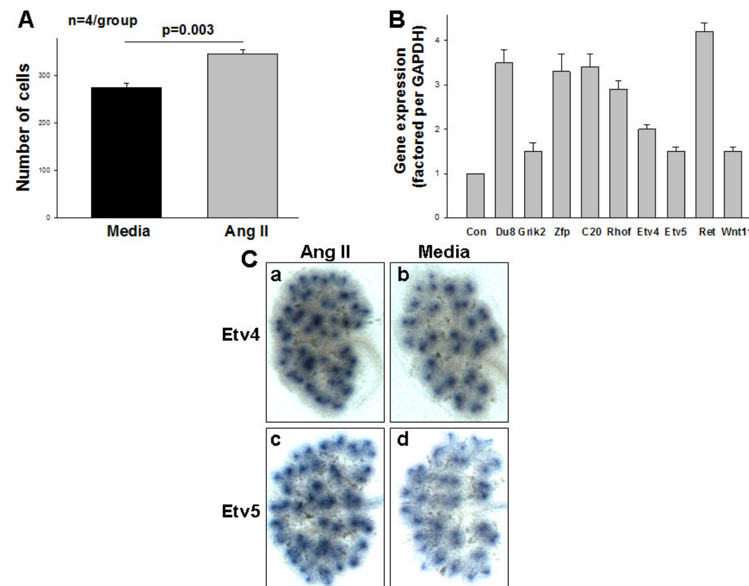

Figure 2.

A: Bar graph shows that the number of ureteric bud-derived cells (UB cells) that migrated across the filter is higher in the presence of angiotensin (Ang) II compared to control (media) (Mean±SEM). B: Quantitative real-time reverse transcription-polymerase chain reaction confirming the changes in gene expression observed in the microarray analysis in isolated E11.5 ureteric buds treated with Ang II or media (control). Media values are normalized to 1 (Con- control) and data are presented as relative-fold difference. Du- Dusp8, Zfp- Zfp42, C20- Ccl20. C: Ang II upregulates Etv4 and Etv5 mRNA expression in E12.5 mouse metanephroi that were grown *ex vivo* for 24 h in the presence of Ang II (10^{-5} M) or media (control). After 24 h in culture, kidney explants were processed for whole-mount *in situ* hybridization. Representative images demonstrate that treatment with Ang II increases Etv4 (a) and Etv5 (c) mRNA expression in the UB compared to control (media, b, d).

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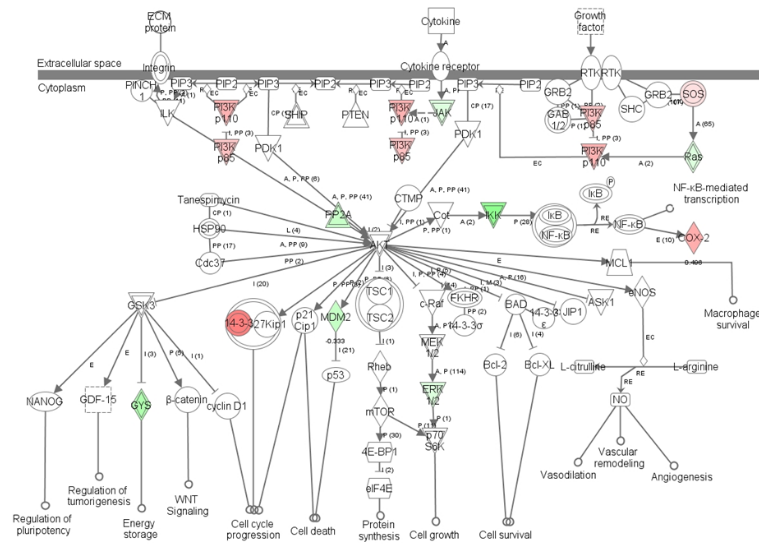

Figure 3.

Ingenuity pathway analysis of phosphatidylinositol 3-kinase (PI3K) signaling pathway affected by angiotensin II. Pink color indicates upregulated genes; green color indicates downregulated genes.

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Table 1

Change in gene expression patterns in isolated intact ureteric buds cultured with angiotensin (Ang) II or GDNF.

Gene symbol	Gene name	Fold change	
		Ang II	GDNF
Upregulated:			
Dusp8	Dual specificity phosphatase 8	6.5	-
Dusp6	Dual specificity phosphatase 6	-	2.9
Grik2	Glutamate receptor, ionotropic, kainate 2	6.1	-
Zfp42	Zinc finger protein 42	5.4	-
Ccl20	Chemokine (C-C motif) ligand 20	5.1	-
Rhof	Ras homolog gene family, member f	4.7	-
Icam1	Intercellular adhesion molecule	-	4.1
Crlf1	Cytokine receptor-like factor 1	3.0	4.4
Fgfr1	Fibroblast growth factor receptor 1	2.5	-
Etv4	Ets variant gene 4	2.4	2.9
Etv5	Ets variant gene 5	2.0	5.5
Wnt11	Wingless-related MMTV integration site 11	2.0	-
Ect2	Ect2 oncogene	2.0	-
Myb	*Myeloblastosis oncogene*	2.0	3.8
Vsnl1	Visinin like 1	2.0	-
Spry1	Sprouty 1	1.6	3.3
Ret	Rearranged during transfection	-	3.3
Cxcr4	Chemokine receptor 4	-	10.0
Ccnd1	Cyclin D1	-	2.9
Ccnb1	Cyclin B1	-	2.6
Arg2	Arginase type 2	-	3.5
Downregulated:			
Mmp9	Matrix metalloprotease 9	6.1	-
Gpr123	G protein-coupled receptor 123	5.2	-
Mmp7	Matrix metalloprotease 7	5.1	-
Clc9a2	Solute-carrier 9a	4.7	-
Fgf12	Fibroblast growth factor 12	4.2	-
Wnt9b	Wingless-type MMTV integration site 9B	2.0	-
Dkk1	Dickkopf homolog 1 (Xenopus laevis)	-	1.9
Vamp2	Vesicle-associated membrane protein 2	-	1.9
Igfbp7	Insulin-like growth factor binding protein 7	-	2.0
Slc40a1	Solute carrier family 40 member 1	-	2.1
Pth1r	Parathyroid hormone 1 receptor	-	2.1
Hspb1	Heat shock protein 1	-	2.2

- No change. Data on the effect of GDNF are from Lu et al., 2009