UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Role of PKA Activation in the Branching Morphogenesis of the Ureteric Bud

A thesis submitted in partial satisfaction of the
requirements for the degree Master of Science

in

Bioengineering

by

Rongshu Wang

Committee in charge:

Sanjay Nigam, Chair
Robert Sah, Co-Chair
Shyni Varghese

2011

The Thesis of Rongshu Wang is approved and it is acceptable in quality and form for publication on microfilm and electronically:

_____

_____

Co-Chair

_____

Chair

University of California, San Diego

2011

Dedication

This thesis is dedicated to my parents and grandparents, whose unconditional love led me to become who I am today.

Table of Contents

List of Figures

List of Tables

Acknowledgements

I would like to acknowledge and thank Dr. Sanjay Nigam for his advice and continuous support throughout my graduate studies. Without his insights and expertise, my research would not have been elevated to the level it is at today.

I would also like to thank especially Dr. Xing Zhang, who introduced me into the lab and has always been great help from the beginning. He is not only a great mentor in research, but also a great mentor and friend in life, offering me advices on how to become a better person and live a better life.

Additionally, I would like to thank everyone else in my lab. They are always so accommodating, supportive, and willing to help. I would like to thank Dr. Kevin Bush, whose expert knowledge was like a lighthouse in the dark, guiding me back on course from being astray. I would like to thank Dr. Ankur Dnyanmote and for always giving me a good laugh and keeping me company; Dr. Thomas Gallegos for always answering my stupid questions and foreseeing my experimental results; Dr. Derina Sweeney for being helpful despite her being in Ireland; Dr. Wei Wu for teaching me life lessons and letting me know the difficulties of being a graduate student and a researcher.

Lastly, I would like to thank Dr. Dnyanmote and Dr. Thomas Gallegos again for taking their times out and proofreading my thesis. I would also like to thank Ms. Katrina Carrion for teaching me many lab techniques and thoroughly editing my thesis.

ABSTRACT OF THE THESIS

The Role of PKA Activation in the Branching Morphogenesis of the Ureteric Bud

by

Rongshu Wang

Master of Science in Bioengineering

University of California, San Diego, 2011

Professor Sanjay Nigam, Chair

Professor Robert Sah, Co-Chair

Development of the embryonic kidney initiates with the formation of the ureteric bud (UB) from the Wolffian duct (WD) and proceeds as the UB and the MM reciprocally induce the growth and differentiation of one another. Branching morphogenesis of the UB is crucial for the development of the overall structure of the

kidney, and defects in this process are correlated to many renal diseases. Studies over the past two decades have identified numerous molecules involved in the process of UB branching. However, the influence that these molecular interactions have on branching still remains unclear. Among these branching signaling molecules, the activation of protein kinase A (PKA) has been implicated in epithelial cell branching and UB outgrowth from the WD (Gupta et al., 1999; Santos et al., 1993; Tee et al., 2010). Here, an approach combining biological network analysis and compound screen was utilized to investigate the mechanisms by which PKA interacts with other molecules to regulate UB branching. Activation of PKA by dibutyryl cyclic adenosine monophosphate (dbcAMP) was shown to inhibit UB branching in *in vitro* systems, including 3D UB cell culture, isolated UB culture, and whole embryonic kidney (WEK) culture. Using network analysis and compound screen, it was hypothesized that the inhibitory effect of PKA activation on UB branching via TGF-β1 or cyclic AMP is mediated through p38 MAPK and NF-κB. The proposed pathway involving TGF-β1, PKA, p38 MAPK, and NF-κB was subsequently validated in 3D UB cell culture, with TGF-β1 being an upstream activator of PKA, and p38 MAPK and NF-κB being downstream effectors of PKA.

Chapter 1 Introduction

The branching morphogenesis of the ureteric bud (UB) is an essential process in the overall development of embryonic kidney. For over two decades, *in vitro* 3-dimensional cultures of kidney epithelial cells in extracellular matrix (ECM) gels have been used extensively to identify many growth factors, membrane receptors, ECM proteins, transcription factors, and intracellular signaling molecules involved in the developmental program of the UB. However, many molecules involved in this process still remain to be uncovered. Furthermore, the process by which this set of molecules interacts to bring about the branching process is still largely unknown.

PKA activation has been shown to inhibit the branching morphogenesis of cultured renal epithelial cells (Gupta et al., 1999; Santos et al., 1993). In addition, a recent study showed that PKA activity mediates the UB outgrowth from the Wolffian duct (WD) (Tee et al., 2010), one of the key events in early embryonic kidney development that is closely related to the branching morphogenesis of the UB. However, the exact effects of PKA activation on UB branching and the specific role it plays in this process remain to be uncovered. Here, an approach combining biological network analysis and compound screening was utilized to address this issue. Such an approach can be potentially applied on other models such as cancer cell transformation and epithelial tubulogenesis.

1.1 Kidney development

Kidneys have evolved to conserve water, excrete waste, and maintain electrolyte balance, each of which is essential to the maintenance of homeostasis and a crucial survival mechanism. The mammalian kidney is one of the most complex organs. On average, each adult human kidney contains more than one million filtration units called nephrons, and more than 25 distinct cell types, whose coordinated spatiotemporal development is tightly controlled to give rise to the mature adult kidney (Little et al., 2010). The developmental process of the kidney, on both the cellular and molecular level, has been investigated for several decades. With the advancement of bio-molecular technologies, many questions regarding kidney development have been answered, although many more remain unsolved. As a good model system for studying organogenesis, kidney possesses a developmental process containing the distinctive features of epithelial-mesenchymal interactions, epithelial cell polarization, and the branching morphogenesis of sub-structures, all of which are shared by many other organs such as lung, pancreas, and salivary glands (Costantini, 2010).

The development of a mature kidney begins as a hollow epithelial structure, known as the WD, which forms from the intermediate mesoderm and progressively forms the pronephros and the mesonephros. Metanephric kidney development is initiated when the caudal parts of the WD interact with an aggregate of mesenchymal cells, called the metanephric mesenchyme (MM), and lead to the outgrowth of the UB, one of the two main components of the metanephros and a major progenitor of a mature kidney. The extending UB invades into the surrounding MM and begins to

branch repeatedly in a dichotomous manner under the inductive signals from the MM. Induction of the MM by the UB in turn causes some of the mesenchymal cells to aggregate near the tips of the newly formed branches and undergo mesenchyme-to-epithelium transition (MET), while other mesenchymal cells form the interstitial stroma. The polarization of some mesenchymal cells as they undergo MET leads to the generation of renal vesicles, which transform into an s-shaped bodies and fuse with adjacent UBs to generate a continuous epithelial tube. The reciprocal inductions between the UB and MM are central to the proper development of both components within the early kidney (Figure 1) (Sampogna and Nigam, 2004). The iterative branching of the UB eventually gives rise to the urinary collecting system, a complex tubular structure consisting of ureter, calyces, and medullary and cortical collecting ducts, while the MM-derived structures include glomeruli, proximal and distal tubules, loops of Henle, and connecting tubules (Costantini and Shakya, 2006). Investigations into the mechanisms of kidney development is not only an important problem in organogenesis, but also has great clinical implications: kidney and urinary tract malformations, such as failure to form the ureter and kidney or incorrect positioning of the UB, account for almost 1% of all birth defects in human (Mendelsohn, 2009; Pohl et al., 2002). Moreover, more subtle defects in UB branching is correlated with reduced nephron number, which can lead to renal diseases later in life (Pohl et al., 2002).

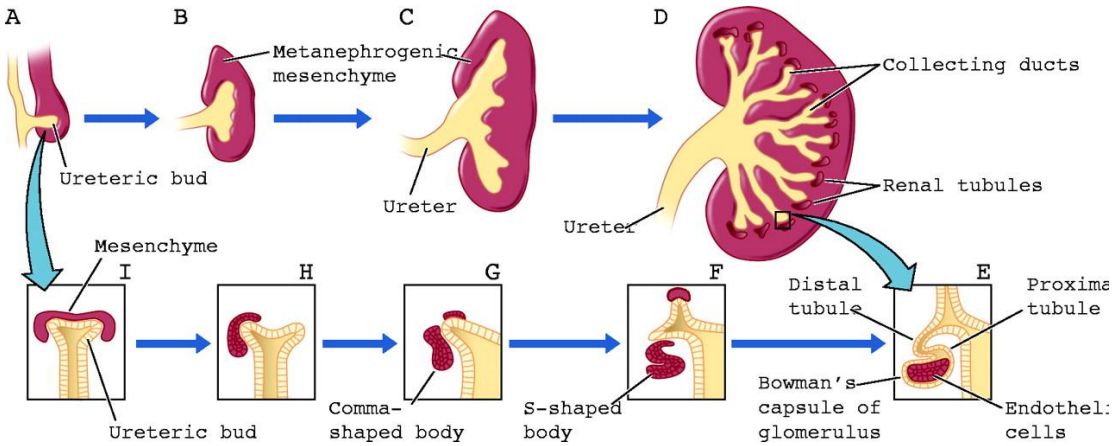

Figure 1. Process of renal branching morphogenesis and nephron formation. UB buds from the WD under the induction from the MM (A, B), and branches out iteratively to form the ureter and collecting duct system (C, D). Around the UB tips, UB also stimulates the development of the MM by inducing it to undergo MET (I, H) and form the nephrons and tubular system (F, E) through the formation of comma- and S-shaped bodies (G, F). (Sampogna and Nigam, 2004).

1.1.1 Branching morphogenesis of the Ureteric Bud

The proper outgrowth and branching morphogenesis of the UB is a crucial step in the development of the urogenital system. The failure in the initial outgrowth of the UB results in renal agenesis, a condition with undeveloped kidney; and the improper branching morphogenesis of the UB leads to severe reduction in kidney size in mouse embryos and renal hypoplasia with reduced nephron number, which could potentially lead to hypertension and kidney failure (Hoy et al., 2008; Ichikawa et al., 2002; Moore et al., 1996; Poladia et al., 2006). After budding from the WD and invading into the MM, the UB branches repeatedly, mostly by bifurcation at the tips, for about 10 rounds, followed by elongation of the UB trunks and final rounds of terminal branching (Cebrian et al., 2004). As stated earlier, the proper branching morphogenesis is guided by the surrounding MM and autocrine factors from within

the epithelium. A multitude of growth factors are expressed by the MM or the UB itself, including fibroblast growth factors (FGFs), transforming growth factor beta (TGF-β) family proteins, hepatocyte growth factor (HGF) and glial-cell-line-derived neurotrophic factor (GDNF) (Costantini and Shakya, 2006; Moore et al., 1996; Pichel et al., 1996; Qiao et al., 1999; Santos et al., 1993). A selective list of cell surface and extracellular matrix proteins, such as integrins, proteoglycans, matrix metalloproteinases, and proteinase-inhibitors are also involved in this process (Falk et al., 1996; Kispert et al., 1996; Lelongt et al., 1997; Muller et al., 1997; Ota et al., 1998).

The branching morphogenesis of the UB is controlled by epithelial-mesenchymal signaling interactions, the most important set of which is the GDNF/Ret signaling pathway (Costantini and Shakya, 2006). In *Gdnf*$^{-/-}$ and *Ret*$^{-/-}$ transgenic mice, mutants for the receptor tyrosine kinase (RTK) for GDNF and its co-receptor *Gfra1*, the UB fails to develop (Costantini and Shakya, 2006). As the UB first buds from the WD, there is an organized movement of cells within the WD (Chi et al., 2009). It has been shown that WD cells have varied levels of Ret activity before the induction by the MM. Upon localized stimulation by GDNF from the MM, WD cells with high Ret activity will migrate past the cells with low Ret activity and move toward the region that will eventually give rise to the budded UB (Chi et al., 2009). Although the outgrowth of a single UB is an event coordinated by many proteins, such as BMP4 (a TGF-β superfamily protein) and Sprouty1, both of which inhibit the outgrowth of UB (Bush et al., 2004; Challa et al., 2004), and Gremlin1, a BMP

antagonist (Michos et al., 2007), the interplay between GDNF and Ret is critical to WD budding.

As UB buds from WD, Ret positive cells migrate towards the GDNF source, soon the UB quickly divides into distinct tip and trunk regions. Furthermore, branching events happen mostly in the periphery of the UB, where most tip cells are located and going through rapid proliferation (Michael and Davies, 2004). Obviously, such an observation indicates the different characteristics of the tip and trunk cells. In fact, there are many genes expressed only by tip cells but not in the trunk cells – such as *Wnt11*, *Crif1*, and *Cxcl14* (Costantini and Shakya, 2006; Schmidt-Ott et al., 2005). As the tips proliferate and branch out, many tip cells are pushed into the trunk region, indicating that the tip cells are potent in acting as trunk cells, which signifies a rapid switch in the set of genes that is activated. Although it is certain that the GDNF/Ret signaling pathway is essential for the branching morphogenesis of UB, its role in the spatiotemporal branching pattern of UB is rather unclear. It has been long thought that GDNF acts as a chemo-attractant to give positional cues to the UB, but a study using a transgenic mouse line expressing GDNF only in the WD and UB showed no significant impairment in the development of kidney (Shakya et al., 2005). Therefore, the expression of Ret and Wnt11 at the tips of the UB could have significant implications in the spatio-branching pattern of the UB. In addition, two other major factors in determining the spatiotemporal branching pattern of the UB are BMP4 and Gremlin1, as *Bmp4* is expressed along the UB stalks and *Gremlin1* in the surrounding MM (Michos et al., 2007). Furthermore, the addition of BMP4 inhibits the branching

morphogenesis *in vitro* (Bush et al., 2004), while the addition of Gremlin1 results in abnormal extra budding and branching from the WD and UB (Michos et al., 2007).

1.1.2 Nephron development

The development of a kidney essentially consists of two parts: the development of the UB and that of the MM. Although both structures arise from the intermediate mesoderm (IM), cells in the dorsal IM form the WD, while the mesenchymal cells in the ventral IM later develop into the MM. The MM gives rise to all the nephrons of the metanephric kidney and also induces the outgrowth and branching morphogenesis of the UB. After the UB buds from the WD, the MM surrounds the UB; according to gene expression studies, the surrounding MM could be separated into two populations– the capping mesenchyme (*Six2*$^{+}$, *Cited1*$^{+}$, *Wnt4*$^{-}$) and the induced mesenchyme (*Six2*$^{+}$, *Cited1*$^{-}$, *Wnt4*$^{+}$) (Mugford et al., 2009). It is also to be noted that the capping mesenchyme cells increase about 16 fold to generate about 13,000 nephrons in the mouse and 1,500,000 nephrons in human (Costantini and Kopan, 2010).

As the UB branches, the MM is in turn induced at each UB tip and undergoes MET. MET leads to the formation of a sphere of cells called the renal vesicle underneath each UB tip. After MET, the renal vesicle becomes proximal-distal-defined, where the surface near the UB tip is distal and the opposite end is proximal. The renal vesicle becomes more structurally defined as a single cleft in the vesicle transforms it into a comma-shaped body; and later, an s-shaped body, resulting from

the formation of a second cleft. The distal end of the comma-shaped body invades the UB tip and fuses with it to form a continuous lumen. With the formation of s-shaped body, the structural pattern is already formed. The proximal end of the s-shaped body differentiates into glomerular epithelial cells (podocytes), and the invasion of endothelial cells into the proximal cleft leads to the formation of the glomerular basement membrane, which along with the endothelial cells forms the glomerulus. The proximal segment connected to the most proximal end gives rise to the proximal tubule. On the distal end, the loop of Henle and the distal tubule, that joins with the collecting duct, are formed.

1.1.3 Molecular basis of kidney development

Kidney development is a complex process involving a vast number of genes and signaling pathways. As noted in the earlier sections, the kidney originates from the WD. For normal WD development from the mesoderm, expression of transcription factors *Pax2*, *Pax8*, and *Lhx1* are required to initiate the expression of *Gata3* (Bouchard et al., 2002; Grote et al., 2006). Along with β-catenin, *Gata3* is needed for the expression of *Ret*, which is crucial to the later outgrowth and branching events of the UB (Bridgewater et al., 2008). Initially a mesenchymal cord, the WD transforms into an epithelial tube under the influence of signals, such as BMP4, most likely from the ectoderm (Torres et al., 1995). After the maturation of the WD, the UB buds as a pseudostratified epithelium. This progression requires proper *RET* expression in the budding UB and induction of GDNF from the

surrounding MM (Costantini and Shakya, 2006). Transcription factors such as *Sall1*, *Eya1*, *Pax2*, and *Hox11* paralogs, or upstream proteins, such as nephronectin, integrin alpha 8 beta 1, and Gdf-11, are required for the normal transduction in the GDNF/Ret pathway (Bouchard, 2004; Boyle and de Caestecker, 2006). *Slit2*, *Robo2*, *Foxc2*, *Spry1*, BMP4, and Gremlin1 are also implicated in the outgrowth of a single UB, among which *Slit2*, *Robo2*, and *Foxc2* limit the expression of GDNF to a specific region; *Spry1* negatively regulate the response to GDNF and the balance between BMP4 and Gremlin1 directs the outgrowth of UB (Costantini and Shakya, 2006; Michos et al., 2007). In addition, neuropeptide Y (NPY) has been found to act through the budding pathway and to be reciprocally regulated by GDNF and BMP4 (Choi et al., 2009). Another type of RTK ligands, FGFs, including FGF7, is able to bypass the GDNF/Ret signaling pathway and induce the budding event of UB (Maeshima et al., 2007). More importantly, most of these signaling molecules modulating the outgrowth of the UB are also highly involved in the branching morphogenesis of the UB under the effect from MM.

As the WD develops and the UB subsequently buds from the WD, the MM first differentiates from the ventral IM. The IM progenitor population expresses *Rarb*, *Osr1*, and *Hox11* paralogs (Costantini and Kopan, 2010), and the *Osr1*- expressing cells differentiate to both epithelial cells and interstitial cells later during MM development (Mugford et al., 2008). Also, *Osr1* is required for the expression of *Eya1* and *Pax2*, both of which combine to form a complex with *Hox11* to initiate transcription of *Six2* and *Gdnf* (Gong et al., 2007). Also derived from the MM progenitors, *Foxd1* is expressed in the stroma and the renal capsule, and the deletion

of *Foxd1* results in abnormal formation of the renal capsule and kidney (Levinson et al., 2005). To form tubular renal vesicles, Wnt9b initiates the MET process in MM cells, while the capping mesenchyme avoids such a process by stabilizing β-catenin with *Six2* expression. After the formation of the renal vesicle, the MM-derived structure becomes patterned with proximal-distal polarity by altering gene expression. The distal domain becomes defined by elevated expressions of *Ccnd1*, *Jag1*, *Ccdc86*, *Cdh6*, and *Wnt4*, and restricting expressions of *Dkk1*, *Papss2*, *Greb1*, *Dll1*, *Pcsk9*, *Lhx1*, *Bmp2*, and *Pou3f3*; the proximal region is associated with strongly expressed *Tmem100* and *Wt1*(Georgas et al., 2009). Additionally, *Lhx1-/-* cells is only expressed in the most proximal region in the nephron, or Bowman's capsule and podocytes (Kobayashi et al., 2005), indicating a potential role of *Lhx1* in determining the fate of the central region. Further studies revealed that downstream of *Wnt* signaling, *Lhx1* leads to the activation of *Dll1*, a Notch ligand, and *Brn1* (Cheng et al., 2007; Nakai et al., 2003).

Along with MM maturation, the UB branches out and becomes patterned mainly through the GDNF/Ret signaling pathway. Many downstream effectors of Ret, such as the PI3K-AKT signaling pathway, have been proven to be important for UB branching (Costantini and Kopan, 2010; Tang et al., 2002). Two ETS transcription factors, *Etv4* and *Etv5*, are important mediators of RTK signals in the development of many organ systems, including the kidney. They are also up-regulated by the GDNF/Ret signaling pathway via PI3K in both the UB and the MM (Lu et al., 2009). Many important genes for proper kidney development, including *Myb*, *Cxcr4*, *Met*, and *Mmp14*, are identified to be downstream targets of *Etv4* and *Etv5* (Lu et al., 2009).

When the UB branches, tip and trunk domains become distinct. One factor that promotes such differentiation is heregulin α (HRGα), which results in the downregulation of tip markers and upregulation of trunk markers (Sakurai et al., 2005). Many genes and signaling pathways are turned on to promote differentiation of α, β intercalated cells and principal cells in mature collecting ducts from the UB. Foxo1 is necessary for the development of intercalated cells (Blomqvist et al., 2004). In a mouse model, inactivation of Mind-bomb1, a protein involved in activation of Notch, led to increased intercalated cells and decreased principal cells (Costantini and Kopan, 2010). However, many pathways still remain to be identified.

1.1.4 Kidney diseases with developmental origins

Kidney development is a tightly regulated process, and any small perturbation in this process could potentially lead to a variety of renal diseases. One of the most common renal diseases with developmental origins is renal agenesis, which occurs 0.48 to 0.58 times for every 1000 live births (Pohl et al., 2002). As stated earlier, the GDNF/Ret signaling pathway is critical to the proper branching morphogenesis of the UB. Humans with renal agenesis usually have *Ret* mutations (Costantini and Kopan, 2010). Many other causes of renal agenesis are mutations of single genes encoding transcriptions factors or upstream proteins of the GDNF/Ret pathway (Costantini and Kopan, 2010). Besides renal agenesis, collecting system duplication, indicated by bifid ureter and duplex kidney, is another common renal abnormality, affecting approximately 1% of the population (Shah et al., 2004). However, the mechanisms have not yet been identified, but it is suggested that the UB either splits into two or

two UBs grow out from the WD. Other defects in early branching process of the UB can lead to dysplastic kidneys that can eventually involute (Hiraoka et al., 2002).

Aside from defects in UB branching, abnormal development of the MM, which is in turn affected by the branching morphogenesis of the UB, can also predispose an individual to renal disorders. Individuals with reduced nephron numbers are highly susceptible to hypertension and renal failure later in their lives. Reduced nephron number, which is also equivalent to reduced filtration surface area, causes increased glomerular volume and mean arterial pressure, thus leading to hypertension (Shah et al., 2004).

Other common forms of renal disease are polycystic kidney diseases, among which autosomal dominant polycystic kidney disease (ADPKD) is most commonly observed. Many causes can lead to polycystic diseases. General causes for ADPKD include mutations in *Pkd1* and *Pkd2*, genes that code for cilia on the apical surface of epithelial cells on the lumen; while a mutation in *Ofd1*, a gene regulating cilia generation and structure, is associated with X-linked dominant polycystic kidney disease (Romio et al., 2003). In addition, abnormally high levels of TGFα and EGFR, factors that promote branching, are linked with ADPKD and associated with cystic phenotypes in mice (Lee et al., 1998; Shah et al., 2004; Takayama et al., 1997).

1.1.5 Regenerative medicine and the kidney

As the United States Renal Data System indicates, the prevalence of end stage renal disease (ESRD) increases every year, putting more and more patients on the non-ideal treatment of hemodialysis. Although organ transplantation represents the

best long-term option for patients with ESRD, the main limitation lies in the limited number of organ donors (Perin et al., 2010). Therefore, advancement in the field of kidney regeneration is imminent so as to develop alternatives to hemodialysis, such as bio-artificial kidneys or stem cell therapies. The goal of *de novo* kidney construction is that an *in vitro*-grown kidney remains functional with low immunogenicity when transplanted *in vivo*. Although this goal is seemingly far out of reach today, there are several approaches that researchers are exploring.

Some research groups have successfully induced embryonic stem cells (ESC) and induced pluripotent stem cells (iPS) to differentiate into renal cells under the stimulation of specific differentiation cocktails (Morizane et al., 2009; Perin et al., 2010). In addition, Mesenchymal stem cells (MSC) have been shown to have the ability to differentiate into renal tubular cells when co-cultured with injured kidney cells *in vitro* (Singaravelu and Padanilam, 2009). These results show promise for using stem cells towards renal regeneration. Indeed, the use of exogenous stem cells for kidney regeneration is widely researched. The injection of bone marrow, amniotic fluid stem cells, and MSCs have been demonstrated to aid kidney recovery from injury. However, the mechanism by which stem cells assist in kidney repair is rather unclear, as the frequency of stem cell engraftment only ranges 3% to 22% (Perin et al., 2010). Thus, it is likely that the protective effect of stem cells is rather from the cytokines or hormones that they produce. A typical study by Lee et al. showed that the repeated administration of MSCs into nephrectomized rats significantly improved the protective effect on remnant kidney injuries mainly through modulating the expression of interleukin-6, a pro- and anti-inflammatory cytokine; and increasing the

expression of interleukin-10, an anti-inflammatory cytokine; and not by differentiation or engraftment. The use of bone marrow-derived cells (BMDC) is also explored by many studies and it has been shown that BMDC could replace, although to a low fraction, tubular cells, mesangial cells, podocytes, interstitial cells, and endothelial cells (Dankers et al., 2011). The secreted paracrine factors by BMDC, such as VEGF, FGF, HGF, TGF-β, and insulin-like growth factor (IGF), can also improve recovery (Dankers et al., 2011). Nevertheless, the long-term side effects of stem cell or BMDC treatments still need to be investigated.

Tissues from embryonic stages were also used to form kidney-like structures that can be transplanted and maintained *in vivo*. When isolated WD and isolated MM are cultured together, the WD develops a branched collecting duct duct system and induces MM to differentiate into a metanephro-like structure, and glomeruli develop (Rosines et al., 2010). When aggregates of IMCD cells were cultured in a hanging drop manner, they formed buds with lumens similar to that of WD budding, and when cultured with isolated MM, formation of small comma-shaped bodies were evident; the same method with UB cell aggregates induced MM to form nascent nephrons (Rosines et al., 2010). Moreover, kidney-like tissues resulting from *in vitro* co-culture of UB and MM were able to survive for up to 5 weeks upon implantation into an immunocompromised host nude mouse (Rosines et al., 2010). Many other studies also showed that developing embryonic metanephroi were able to survive, develop and even function to a certain extent when transplanted into an *in vivo* model (Perin et al., 2010). However, such an approach is limited by materials and size of the final product.

Another approach is the usage of scaffolds to aid in the regeneration of kidney *in vivo*. It has been shown that rat renal segments were able to form new glomeruli and tubules when seeded onto polymer scaffolds and implanted in nude mice (Kim et al., 2003). Nakayama et al. were able to produce a decellularized kidney scaffold with structural, mechanical, and physiological properties for the support of renal tissues. The decellularized kidney demonstrated ability to support the recellularization of the scaffold by *Pax2*$^{+}$/*vimentin*$^{+}$ cells (Nakayama et al., 2010).

1.1.6 Model systems for studying kidney development

It is difficult to correlate the effect of an individual molecule or gene to particular regions of the developing kidney – it is more likely that interactions between the UB, MM, stromal cells, and extracellular matrix surrounding these tissues are all affected in some way. The inhibition of a specific molecule or gene leading to an easily observable effect on the UB does not necessarily point to that molecule or gene as the direct cause. Rather, such an occurrence could also be explained to be the direct effect of the molecule or gene on the MM. The altered expression pattern of the MM is likely to also manifest an effect on the UB. Therefore, while *in vivo* studies on kidneys with perturbed genetic or molecular expressions and *in vitro* whole organ culture will always be important in uncovering whole organ-wide effects – model systems that enable us to reveal specific roles of particular molecules, genes, and pathways in isolation are extremely useful (Pohl et al., 2000b).

The first model system started when Grobstein and colleagues developed *in vitro* whole embryonic kidney culture model with metanephroi harvested from mouse embryos (Saxen L. Organogenesis of the Kidney). The developing kidney is then cultured over medium for several days, making the visualization of the developmental process of nephron formation and UB branching possible. Another widely used tool to test for the study of the systemic mechanism of kidney development is genetically-engineered mice, such as mice with specific knockout genes (Pohl et al., 2000b). Such a method provides strong evidence for the role of an individual gene in the embryonic development of the kidney. However, as stated earlier, both organ culture and genetically-engineered mice cannot isolate the effect of a specific gene or molecule to either the MM or the UB, thus the definite role of these genes and molecules are difficult to deduce.

Two very useful models to distinguish between UB and MM effects are cell culture and isolated UB (iUB) culture. Cell models of Madin-Darby canine kidney (MDCK) cells and murine inner medullary collecting duct (mIMCD) cells, both derived from mature renal epithelia; and UB cells, which are isolated from developing UBs of SV40-transgenic mouse on embryonic day 11.5 (Sakurai et al., 1997a), are used to resolve specific cellular processes involved in branching morphogenesis. All three types of cells are able to branch into tubular structures in response to various soluble factors, but UB cells are especially useful in studying the developmental process of the UB, as they are isolated from the developing tissue itself and thus likely contains almost the same genetic expression pattern and capabilities as the UB does *in vivo*. Results obtained from the knockout models and

the UB cell culture model have been mostly consistent. Additionally, many molecules found to be modulating branching morphogenesis of UB cells, such as EGF, MMP2, MMP9, and integrin beta1, were also confirmed in the knockout models.(Lee et al., 1998; Lelongt et al., 1997; Pohl et al., 2000a; Sakurai and Nigam, 1997; Zent et al., 2001; Zhang et al., 2009). Also, the establishment of cell models enables the testing of genes or molecules that could not be tested in knockout models, as many genes are essential for the fundamental development of the embryo. The Isolated UB culture model is similar to the UB cell model, with isolated E13 rat UBs grown in the same medium (BSN-CM, a MM cell-derived conditioned medium), and in similar extracellular matrix conditions. Together these various models are useful in identifying and understanding the molecular mechanisms of kidney development.

1.2 Epithelial branching morphogenesis

Epithelial branching morphogenesis is a recurrent theme in the developmental process of many organs, including the kidney, lung, pancreas, vascular system and salivary glands (19778532). However, the actual tubulogenesis process can vary dramatically among different organs. For example, tubules in the salivary glands do not require the complex branching structures involved in the branching morphogenesis process of organs such as vertebrate lung, vasculature, or kidney, where large surface area is essential for optimal exchange of oxygen, metabolites, and nutrients (Andrew and Ewald, 2010). In addition, the progenitor cells of branching organs are not all from the same germ layer – the tracheal system arises from

ectoderm, precursors cells of the lung originate from the endoderm, while the kidney initially develops from the mesoderm (Horowitz and Simons, 2008). Although the specific branching program in each organ remains unique, there is one general branching pattern – the tip cells receive inductive signals and are at the forefront of the branching process, while the stalk cells elongate and are inhibited to branch through a negative feedback loop, and branching typically occurs through bifurcations. This pattern ensures an efficient tubular system with maximum functional surface area.

The best known branching system is the *Drosophila* trachea, mostly because its branching pattern is relatively simple. The branching process of *Drosophila* trachea eventually leads to six primary branches that are followed by secondary branches at the ends, forming the long tracheoles (Kerman et al., 2006; Takahashi et al., 2000). The main driving force for the branching morphogenesis of *Drosophila* trachea is FGF, while other signaling molecules such as TGF-β, BMP, and Wnt are also required for more defined outgrowth of the branches (Andrew and Ewald, 2010; Chihara and Hayashi, 2000; Sutherland et al., 1996). Similar to its role in *Drosophila* trachea development, FGF signaling plays important roles in the branching morphogenesis of many organs, including lung, kidney, mammary gland, and salivary gland. Conversely, different organs also have their own key set of signaling pathways, such as GDNF/Ret signaling in kidney; nerve growth factor (NGF) for neurons; vascular endothelial growth factor (VEGF) during angiogenesis (Carmeliet and Tessier-Lavigne, 2005); and TGF-β superfamily, endothelial growth factor (EGF), and sonic hedgehog (shh) signaling in lung, salivary, and mammary glands (Andrew

and Ewald, 2010). One thing to note is that most of these key signaling molecules that induce branching morphogenesis in the mammalian systems are ligands of the receptor tyrosine kinase (RTK) family.

Even though epithelial cells in organs have the inherent potential to branch, they could not do so without the mesenchymal and stromal interactions. An important observation is that skin epithelium can be induced to form mammary branches under influence from embryonic mammary mesenchyme, and that salivary gland mesenchyme can stimulate embryonic mammary epithelium to form salivary gland-like branches (Lu and Werb, 2008), suggesting that regulatory cues from mesenchyme are crucial to the proper branching morphogenesis and differentiation of epithelial cells. Stroma resides close to the epithelial tissue and maintain a population of stem cells that can differentiate into the epithelial tissue (Lu and Werb, 2008).

1.3 PKA signaling

Protein kinase A (PKA), also known as the cAMP-dependent protein kinase, was discovered more than half a decade ago, and has been studied thoroughly in a number of cell types and organ systems. In canonical PKA signaling pathway, a G protein-coupled receptor (GPCR) undergoes a conformational change upon binding to its extracellular ligand and activates the associated heterotrimetric G-protein. The subsequent dissociation of one of the stimulatory G-proteins from the transmembrane complex of GPCR and its binding to adenylyl cyclase result in the catalysis of ATP to Cyclic-adenosine monophosphate (cAMP). PKA, a rare protein enzyme with two

separate subunits, is the major downstream effector of cAMP. At low levels of cAMP, PKA is inactive due to the sequestration effect of the regulatory subunits on the catalytic subunits. With increased levels of cAMP, PKA becomes activated as cAMP binds to the two binding sites on the regulatory subunits, resulting in the release of the catalytic subunits. Two classes of PKA have been discovered – type I and type II, distinguished by the differences in their R subunits. Generally, type I PKA has a higher affinity for cAMP than type II PKA. The subcellular distribution and localization of PKA is dictated by A-kinase anchoring proteins (AKAPs), which allow for enhanced spatiotemporal specificity. There are more than 50 AKAP members identified to date, each with different affinity for PKA and targeting specific subcellular compartments.

PKA activity has been implicated in many physiological processes, such as regulation of cell cycle, cardiovascular function, steroid biosynthesis, immune function, and metabolism in adipocytes (Tasken and Aandahl, 2004). Moreover, abnormalities in the PKA signaling pathway are associated with a variety of diseases, including McCune-Albright syndrome (mutations in the *GNAS1* gene leads to elevated PKA activity), the inherited multiple endocrine neoplasia syndrome Carney Complex (mutations in the PKA regulatory *PRKAR1A* gene), adrenocortical neoplasms (degradation of cAMP) (Kirschner et al., 2009). PKA activation is also linked with hyperproliferation and tumorigenesis.

To uncover the specific roles of PKA, many gene knockout mouse models have been used. In *Prkaca*$^{-/-}$ mice, which are void of one of the PKA catalytic subunit genes, severe delayed growth and malformation in sperm were observed (Skalhegg et

al., 2002). PKA is also involved in learning and memory, as mice with *Prkacb*$^{-/-}$, another PKA catalytic subunit gene, have impaired hippocampal synapse potentiation and learning defects (Howe et al., 2002). In addition to the defects resulting from knockouts of the catalytic subunits, deletion of regulatory subunits can also have significant impact, such as failure of normal mesodermal development, defective hippocampal function, impaired motor function, and predisposition to tumorigenesis (Kirschner et al., 2009). Tissue-specific knockout models in heart, pituitary gland, and neural crest also demonstrated involvement of PKA in heart development, myxomagenesis, which is the most common primary tumor of the heart, pituitary tumorigenesis, and Schwann cell tumorigenesis (Kirschner et al., 2009). Altogether, PKA is essential for the normal development of many organs and the defect of which is a major cause for tumorigenesis.

PKA exerts its effects through interactions with a wide range of proteins. The most well-known downstream effector of PKA is cAMP response element-binding protein (CREB), a nuclear transcription factor that controls the transcription of many downstream genes. After dissociation, the free catalytic subunit of PKA can enter the cell nucleus and phosphorylate transcription factors such as the aforementioned CREB, as well as CREM, NF-κB, and other nuclear receptors (Daniel et al., 1998). It has been demonstrated in a variety of cells that PKA can block Ras-dependent signals to ERKs by blocking Raf-1 activation, and such a process might be mediated by the activation of Rap1 by PKA, as activated Rap1 by PKA antagonizes Ras activation of Raf-1 and Erks by moving Raf-1 away from Ras (11340161). On the contrary, there has also been evidence of PKA activation of ERK through members of the Src family

and Rap1, or even through Ras (Iida et al., 2001; Lindquist et al., 2000; Stork and Schmitt, 2002). In mouse Y1 adrenocortical tumor cells ACTH39, PKA mediates the rapid dephosphorylation of Akt/PKB and a posttranscriptional downregulation of the c-Myc protein, which leads to an anti-mitogenic effect (Pinedo and Duursma, 1975). Type I PKA has also been shown to be associated with EFGR, the overexpression of which is correlated with several types of human cancers through the binding of the RI regulatory subunit of PKA to the Grb2 adaptor protein upstream of the mitogen-activated protein kinase (MAPK) pathway (Ciardiello and Tortora, 1998). Possibly upstream of PKA, PKC has been speculated to be involved in the stimulation and inhibition of the two main PKA regulatory subunits (Stork and Schmitt, 2002). In summary, PKA possesses the ability to interact with a broad spectrum of proteins ranging protein kinases to transcription factors, and can respond to mechanical stimuli. Additionally, it has great physiological significance. However, the particular manner of how PKA interacts with other proteins is unique to the type of cells or tissues, and the aforesaid interactions involving PKA are limited samples that represent only the tip of the iceberg.

1.4 Biological network analysis

Biological systems, like all things in nature, are extremely complex, with components such as DNA, RNA, and proteins all interacting with one another. In recent years, researchers have realized the importance of utilizing a systems level approach to study biological systems, as a simple molecular perturbation might only

lead to changes in a few genes and proteins initially, but could ultimately result in a network-wide effect across the whole biological system. In addition, advances of many high-throughput technologies, such as automated DNA sequencing, global gene expression measurement techniques, and high throughput screening, can generate large amounts of data. The accumulated database on gene or protein interactions necessitate analytical methods on a systems level, such as pathway and gene set analysis (Ekins et al., 2007). Such analytical methods have proven to be important to a wide range of researches, from drug discovery to identification of novel biological interactions and to predicting metabolic responses in lower organisms. However, computationally derived predictive gene or molecular networks that employ the knowledge of known gene and protein interactions should be experimental validated (Ganter and Giroux, 2008). Also, it should be noted that although biological network analysis and the systems biology approach are very similar, the former relies more heavily on systemic network analysis and data collecting techniques, while the latter emphasizes computational and statistical learning (Kwoh and Ng, 2007).

According to Ekins et al., pathways are consecutive reaction steps of biochemical interactions or signaling events that are already predetermined. In contrast, networks are dynamic, as they are constructed from building blocks in different pathways (Ekins et al., 2007). In a network, proteins are usually represented as nodes and protein-protein or protein-gene interactions are represented as links between each two nodes. The core information in a pathway lies in these edges, as it is through these edges that different building blocks can be connected into a network. Therefore, it is important to have a pathway database with high-confidence. Currently,

there are many structured databases that contain different types of information. For example, for protein-protein interactions, there are Database of Interacting Proteins (DIP), Alliance for Cellular Signaling Molecule Pages Database (AfCS), and Human Protein Reference Database (HPRD); for metabolic pathways, there are Kyoto Encyclopedia of Genes and Genomes (KEGG), Comprehensive Enzyme Information System (BRENDA); for signaling pathways, there are Cytokines Online Pathfinder Encyclopedia (COPE) and Cell Signaling Technology Pathway Database Pathway Diagrams (CST); for gene regulatory networks, there are Transcription Factor Database (TRANSFAC), Object Oriented Transcription Factors Database (ooTFD), and Prokaryotic database of gene regulation (PRODORIC). After the construction of a network, in order to interpret or predict the network based on a specific disease, cellular function or processes, network analysis is conducted to reduce the information to a more manageable and interpretable state. In order to achieve such a concerted and relevant subnetwork, the generated biological networks need to be scrutinized for its relevance to input data, biological processes, diseases, or pathways through statistical calculations of p-value, z-score, or g-score (Ekins et al., 2007). To date, many network analysis platforms are being widely used; the better known ones include MetaCore, Ingenuity Pathway Analysis, PathArt, and Pathway Studio (Ekins et al., 2007).

1.5 High-throughput screening

High-throughput screening (HTS) is the scientific experimentation method that enables the examination of thousands of biochemical, genetic, or pharmacological tests in a relatively short amount of time and a few experiments. Throughout the last decade, HTS has been extensively utilized to identify therapeutic compounds, pathways, cell functions, and chemical probes in pharmaceutical and biotechnology industries as well as in basic and applied research in academia (Soh et al., 2010). Moreover, HTS methods have contributed significantly to drug discovery.

In the traditional target-based HTS approach that is generally used in the pharmaceutical industry, screenable targets such as enzymes, receptors, or ion channels are first identified and selected from either previously known pharmaceutically relevant targets, *in silico* simulations, or genetic studies. After target identification and selection, a target-based HTS assay is conducted in an *in vitro* setting with purified proteins or in cells, bacteria, or yeasts with the addition of compound libraries constituting one compound per condition. In order to reduce false negative results, compounds are generally dissolved in an H_2O/DMSO mixture to reduce the effect of DMSO on biological targets and screening is usually conducted at low concentrations of compounds to minimize nonspecific interactions (Landro et al., 2000). A good HTS assay should also employ an efficient detection method with high consistency and high signal-to-noise ratio. There are two general detection strategies – radiometric and non-radiometric. Radiometric strategy includes measuring filtration scintillation proximity, while non-radiometric methods include measuring absorbance, fluorescence and luminescence (Landro et al., 2000). In the next stage, compounds

that are initially identified to be active against the target need to be re-tested in the same condition, in a different condition, in a cellular environment, and at different concentrations to confirm the target hit. Lead optimization phase happens after confirmation of target hits. The purpose of lead optimization is to produce lead compounds from the original hits with improved potency, selectivity, and pharmacokinetic parameters of the original hit through chemical modification of the hit structure. The last step involves validation of the lead compounds in mammals before moving onto preclinical and clinical studies (Butcher, 2005; Soh et al., 2010).

However, with the traditional HTS approach, there is a high failure rate in developing actual useful compounds, as there are several limitations in this approach (Giacomotto and Segalat, 2010). The first limitation is that the traditional HTS approach depends on target availability, as it is difficult to identify targets that are deeply involved in diseases and the interaction or inhibition of which would reverse the disease phenotype. The second limitation is that many disease conditions cannot be produced *in vitro*, and thus cannot be tested in a traditional HTS assay. The last limitation is the reliability of the target hits, as most do not behave the same under physiological conditions, such as in preclinical trials in mammals. Therefore, several new approaches have been proposed and tested. In the traditional assay, in each screen, compounds could only be tested against one target. A proposed high-throughput kinase profiling approach enables the testing of compounds against hundreds of targets in a single screen, providing a number of targets to pursue, as opposed to the traditional assay in which compounds could only be tested against one target in each screen (Goldstein et al., 2008). Another modification to the traditional

approach involves pooling, in which mixtures of compounds are examined in each well. The pooling methods are strategically designed to minimize the number of tests needed to generate meaningful hits and reduce false positives and negatives (Kainkaryam and Woolf, 2009). Another novel approach suggesting screening on whole animals such as zebrafish and *Caenorhabditis elegans* worm instead of screening against targets has been proposed and has proven to be efficient, as there is no specific target required and compounds can be tested in a whole-animal context (Lieschke and Currie, 2007; Segalat, 2006). However, systems using small organisms also have major drawbacks as some diseases cannot be reproduced because of the genetic and biological differences between model organisms and humans. Therefore, Butcher recommended a similar approach that utilizes complex human-cell systems, so that compound efficacy and safety in human biological models of disease can be directly monitored (Butcher, 2005). Such an approach is also aided by the rapid advances in cellular biosensors that are compatible with miniaturized plate formats (Snowden and Green, 2008).

Chapter 2 Materials and Methods

2.1 Network building in Ingenuity Pathway Analysis (IPA)

Network consisting of the molecules affecting the branching morphogenesis of the UB was constructed using IPA (Version 8.6, Ingenuity System Inc.). Molecules known to affect the UB branching and that are under investigation were input into IPA. Direct and indirect interactions among these molecules were added through the network building function in IPA according to the Ingenuity Knowledge Base.

2.2 UB cell line

The UB cell line was isolated from micro-dissected UB of a day-11.5 mouse embryo transgenic for simian virus 40 (SV40) large T antigen (Immorto mouse, Charles River) as previously described (Sakurai et al., 1997a). The UB cells were cultured in DMEM/F-12 media suppletmented with 5% fetal bovine serum (FBS) in a 5% CO_2 incubator at 32℃. The UB cells were grown to confluency and passaged every 6 days. Only passage number 4 to 14 of the UB cells were used for the experiments.

2.3 Generation of conditioned media

BSN-conditioned media (BSN-CM) were collected from confluent monolayers of BSN cells grown on 10 cm culture dishes. BSN cells were incubated with DMEM/F-12 suppletmented with 10% FBS in a 5% CO_2 incubator at 37℃.

After reaching confluency, cells were incubated in fresh DMEM/F-12 without FBS for an additional 2 days before collection. The conditioned media was then collected, centrifuged to remove cell debris, and further concentrated with Amicon Ultra-15 Centrigal Filter Units (Millopore, Billerica, MA) in DMEM/F-12.

2.4 Three-dimensional gel culture

Three-dimensional gel culture with the UB cells were performed by suspending the cells in a 4:1 mixture of type I collagen gels at pH 7 and growth factor reduced Matrigel (BD Biosciences, Franklin Lakes, NJ) in at 5×10^4 cells/ml. Type I collagen gel was prepared by mixing 8 parts of type I rat tail collagen (BD Biosciences, Franklin Lakes, NJ), 1 part of 10× DMEM/F-12, and 1 part of *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES) (pH 7.8), and the final pH was adjusted to 7.4 with 1 M NaOH. For 384-well culture plates, a coating layer consisting of 15 μl of the gel without any cells was added to the bottom of each well and incubated at 37℃ for 20 minutes for gelation. 30 μl of gel containing appropriate amount of cells were then added to each well as aliquots. After gelation, 40 μl of culture media containing 3 parts of 50× BSN-CM, 2 parts of DMEM/F-12 supplemented with 5% FBS, and additional compounds if desired, were added on top of each well. For 24-well transwell plates, 400 μl of gel containing cells were added to the top of each Transwell filter (CoStar, Cambridge, MA), and 700 μl of culture media were added to each well in the 24-well culture dish. The cells were then incubated in a 5% CO2 incubator at 37℃ for 5 to 10 days. For quantification, a

randomly selected field of vision was selected under the 10× view of a phase contrast microscope (Nikon) and number of branched colonies and cystic colonies were counted.

2.5 Compound screen

UB cells were cultured in 384-well plates for the branching morphogenesis assay as described earlier, with the exception that 200 μM of dbcAMP was added in the media of each of the experimental conditions. Out of the 384 wells, 12 wells were used as positive controls and 8 wells were used for negative controls. 242 kinase inhibitors were added into the culture media at a uniform concentration of 5 μM. All kinase inhibitors were from EMD Biosciences, USA. Three replicates were conducted for each condition. For the purpose of screening, cells were scored on days 5 and 10 for the extent of their branching. A totaled score was assigned to each condition from the summation of two scoring days (day 5 and 10) and the three replicates. Conditions with top scores were picked out for further analysis and validation.

2.6 [^{3}H]thymidine incorporation assay

The UB cells were suspended in a gel mixture as described in a 96 well plate (100 μl gel, 100 μl medium per well) with or without the presence of dbcAMP. After incubation for 48 hours, [^{3}H]thymidine was added to each well at 1 μCi/well. The medium was discarded and the gels were dialyzed in PBS for 24 hours to remove the unbound [^{3}H]thymidine after incubation for another 24 hours following the addition

of [^{3}H]thymidine. The gels were then digested with 10% SDS, and the radioactivities of lysates were measured with a liquid scintillation counter.

2.7 Western blot analysis

Cells in gels were lysed with T-PER Tissue Protein Extraction Reagent (Thermo Scientific, Rockford, IL) and homogenized with a syringe. Samples were then centrifuged at 10,000x g and supernatants were collected and saved. Protein concentration was determined with Bradford assay by measuring absorbance at a wavelength of 595 nm. Twelve micrograms of protein from each sample were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with NuPAGE Novex Bis-Tris Gels (10%) (Invitrogen, Carlsbad, CA) and transferred to a Nitrocellulose membrane (Hybond^{TM}-C, Amersham Biosciences, Piscataway, NJ). The membrane was blocked with 1% Blotto (1 L TBST, 50 g nonfat dry milk, 10 g BSA) to reduce nonspecific interaction of the membrane with the antibodies. Nitrocellulose membrane containing transferred proteins were incubated with primary antibodies against either phosphor-p38 MAPK (1:1000; Cell Signaling, Danvers, MA) or p38 MAPK (1:1000; Biolegend, San Diego, CA) overnight and subsequently washed with TBST. After overnight incubation with horseradish peroxidase (HRP) conjugated secondary antibody (1:1000), the membrane was washed with TBST and visualized by exposure to X-ray film using Supersignal West Pico Chemiluminescent Substrate (PIERCE, Rockford, IL).

2.8 Immunofluorescence

To determine the nuclear translocation of NF-κB, UB cells were suspended in 3D collagen I/Matrigel mixture at a density of 2×10^4 cells/ml in 96 wells (40 µl gel, 60 µl media). After 5 or 10 days of growth, cells were fixed in 4% paraformaldehyde (PFA) for 1 hour, blocked with 0.05% Triton X-100, 0.7% fish gelatin in PBS, and gel was quenched with 75 mM NH_4Cl, 20 mM Glycine in PBS (pH adjusted to 6). Cells were subsequently incubated with NF-κB antibody (1:1000) overnight at 4℃, then washed in PBS three times over 24 hours and incubated with the appropriate secondary antibody (1:1000) and DAPI overnight at 4℃.

2.9 Culture of isolated whole embryonic kidneys

Embryonic kidneys from gestational day 14 were dissected from Sprague-Dawley rat embryos as previously described (Qiao et al., 1999), and cultured on the top of Transwell filters (CoStar, Cambridge, MA) within wells of a 24-well tissue culture dish containing 700 µl of DMEM/F-12 media. For the inhibitor experiments, inhibitors were added to the media at desired concentrations.

2.10 Isolated UB culture

UBs were dissected from kidneys of embryonic day 13 Harlan rats and suspended in a gel mixture containg 1:1 growth factor-reduced Matrigel (BD Biosciences, Franklin Lakes, NJ) and DMEM/F12 as described previously (Bush et al., 2004; Pohl et al., 2000a; Qiao et al., 1999). The isolated UBs were grown in media suppletmented with

10% FBS, 1% antibiotics, 125 ng/ml FGF1, and 125 ng/ml GDNF with or without the addition of dbcAMP for 5 days.

2.11 Statistical analysis

The differences between averages were compared using Student's t-test, with a p-value less than 0.05 being considered statistically significant.

Chapter 3 Results

3.1 Effect of PKA activation on UB branching

3.1.1 UB cells under cAMP-PKA activation

The embryonic murine immortalized UB cell line has been studied extensively to model the cellular branching mechanism of the UB. When cultured in ECM gel composed of collagen I and Matrigel, UB cells grow to form complex multicellular tubular structures with the right supplement of nutrients and growth factors from BSN conditioned media. PKA activation has been proven to hinder the budding of the UB from the WD, and the branching morphogenesis of mIMCD and MDCK cells (Gupta et al., 1999; Santos et al., 1993; Tee et al., 2010). To investigate the effect of PKA on UB branching, dbcAMP, an analog of cAMP and known activator of PKA, was added into the media of UB cells in 3D cultures. With cAMP-PKA signaling activated, branching morphogenesis of UB cells were inhibited in a concentration-dependent manner. For positive control, UB cells were treated with media containing only 30× BSN-CM supplemented with 2% FBS. Three replicates were used for each condition, and each condition was normalized to the positive control. Generally, without normalizing, 80% to 90% of UB cells form branched colonies under the branching condition. Increasing dbcAMP concentration resulted in statistically significant decrease in the percentage of branched colonies, evidenced by less branched colonies and more cyst presence (Figure 2A-E). 200 μM of dbcAMP treated UB cells had 74.59% of the amount of branched colonies observed in the positive control, while

UB cells treated with 500 μM and 1 mM only had 56.59% and 32.29%, respectively, of the positive control.

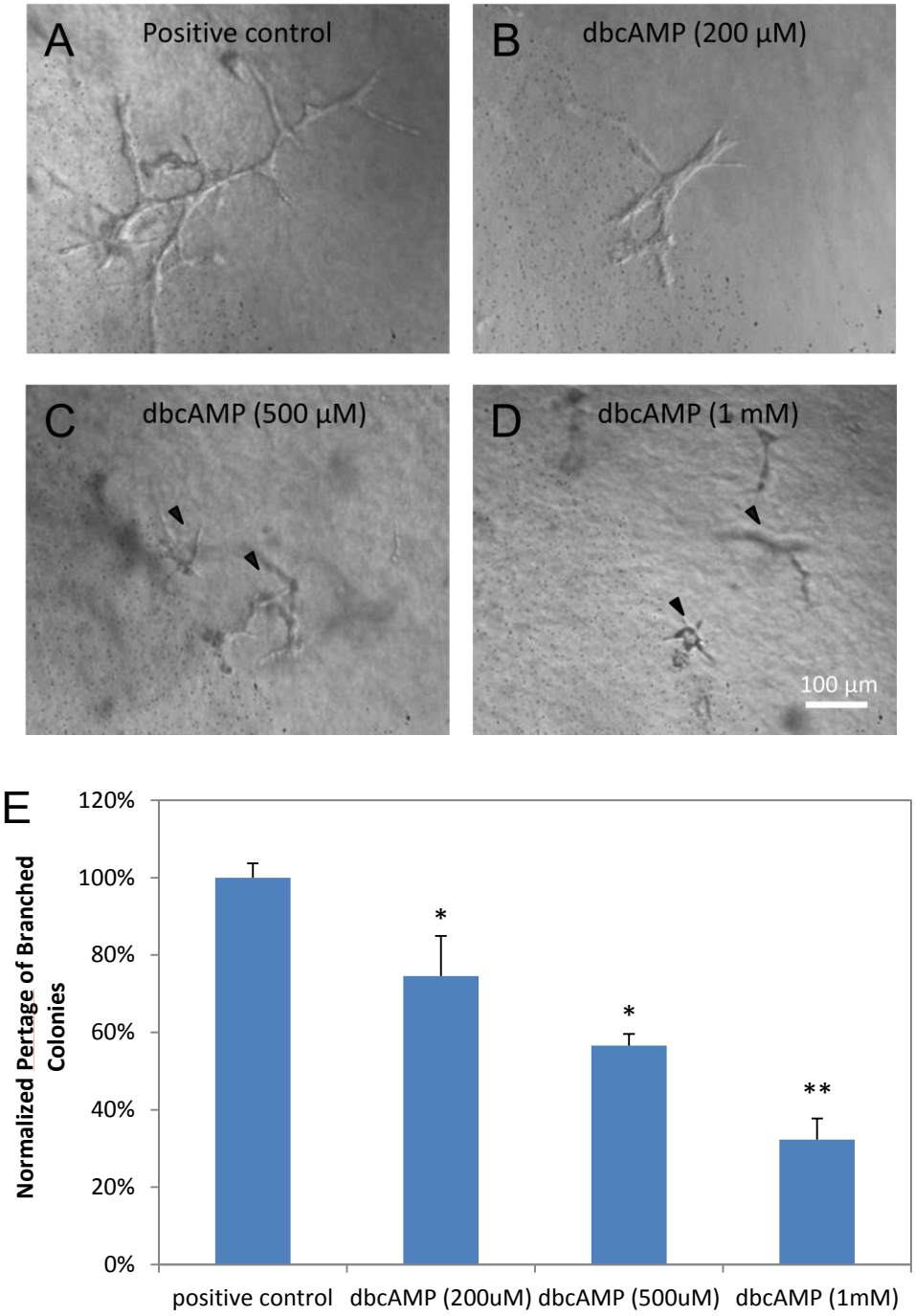

Figure 2. UB cell branching under different concentrations of dbcAMP. UB cells were grown in 3D culture for 10 days. (A) Positive control with media consisting of BSN-CM and 2% FBS. UB cells were also cultured under increasing concentrations of dbcAMP at increasing concentrations of dbcAMP at 200 µM (B), 500 µM (C), and 1 mM (D). (E) Quantification analysis of percentage branched colonies shows statistical significance between treatments of 200 µM, 500 µM, and the positive control ($p < 0.05$). Treatment of 1 mM dbcAMP also returned statistically significant result compared to treatment of 500 µM dbcAMP. Black arrows point to the branched colonies. *$P < 0.05$, experimental condition compared to positive control. **$P < 0.01$, treatment of 1 mM dbcAMP compared to positive control. Scale bar = 200 µm.

3.1.2 Isolated UB under cAMP-PKA activation

To further verify the effect of cAMP-PKA activation on branching morphogenesis of the UB, isolated UB was cultured *in vitro* with dbcAMP at increasing concentrations. Similar to UB cells, isolated UB had reduced branching morphogenesis at increasing dbcAMP concentrations (Figure 3, provided by Chiharu Ito). With cAMP-PKA activation, there was a decrease in the number of buds branching out from the iUB. At 175 μM of dbcAMP, there were only around 3 buds on each iUB, comparing to about 20 branched buds in positive control treated with only 125 ng/ml FGF1 and 125 ng/ml GDNF.

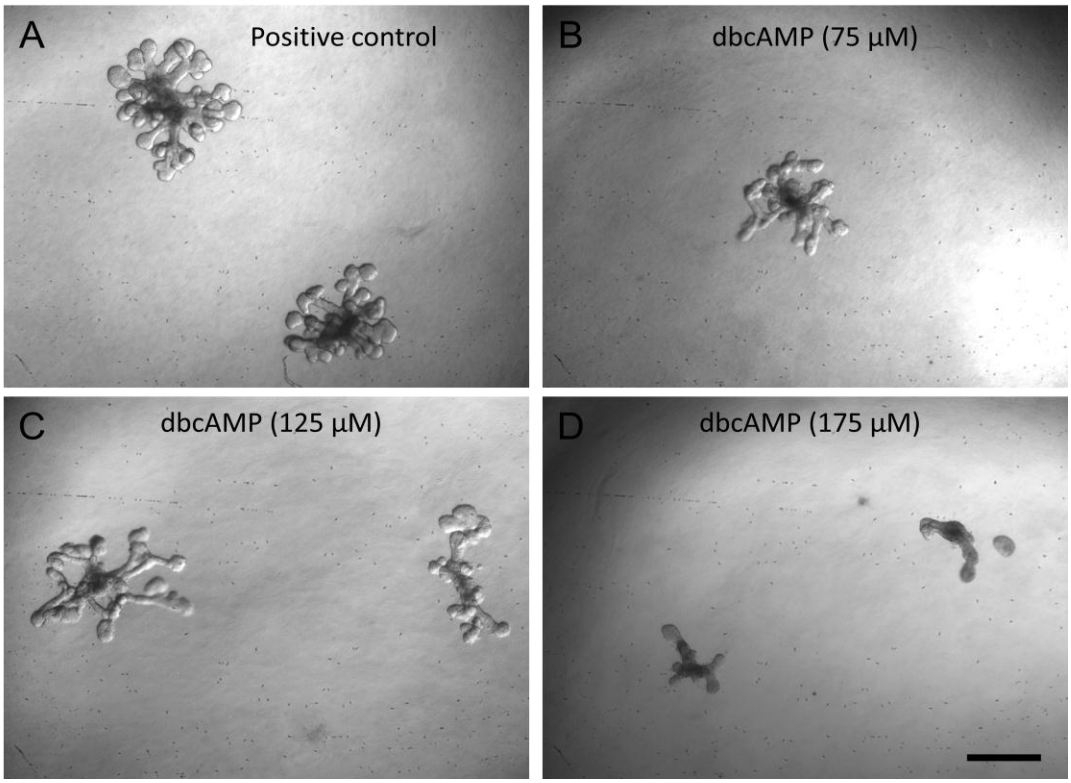

Figure 3. Effect of dbcAMP on isolate UB branching in vitro. Isolated UBs were suspended in media of 125 ng/ml FGF1 and 125 ng/ml GDNF without (A) or with 75 μM dbcAMP (B), 125 μM dbcaMP (C), or 175 μM dbcAMP (D) and cultured for 5 days. Scale bar = 200 μm.

3.1.2 cAMP-PKA activation and cell proliferation rate

To examine whether the inhibition of branching morphogenesis of UB cells by cAMP-PKA activation was a result of altered cell proliferation, a [^{3}H]thymidine incorporation assay was conducted in 3D culture conditions. The thymidine incorporation assay is one of the most common methods used to study cell proliferation. The assay operates on the principle that [^{3}H]thymidine, a radioactive nucleoside, is incorporated into newly synthesized DNA during DNA replication, a step necessary for cell division; thus the rate of cell proliferation is correlated with the amount of [^{3}H]thymidine incorporated into the cells. Among the positive control and two conditions with increasing dbcAMP concentrations (200 μM and 500 μM), no significant difference in proliferation rate was observed (Figure 4). By counting radioactivity, conditions with 200 μM and 500 μM had 98.77% and 96.92% of normalized positive control's counts per minute, respectively. The results indicate that activation of cMAP-PKA inhibits UB branching through a mechanism independent of altering cell proliferation rate.

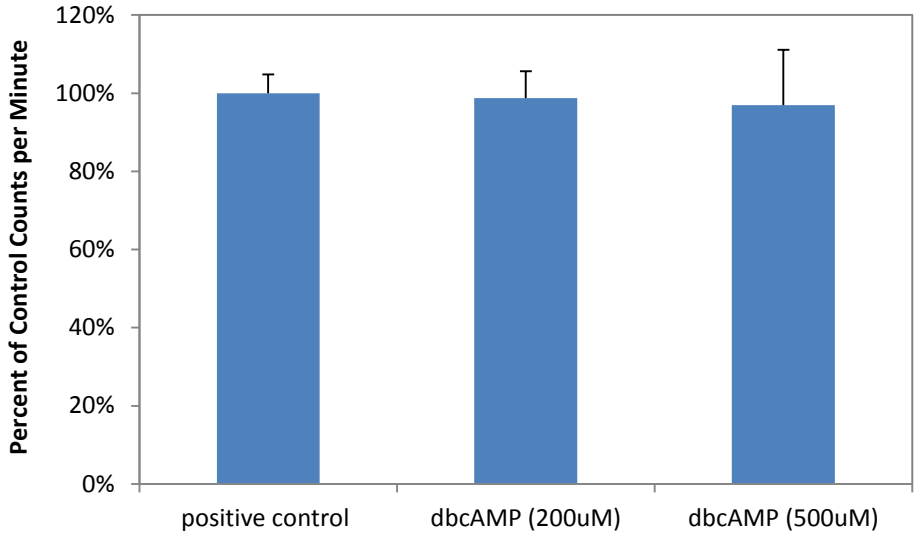

Figure 4. [3H]thymidine incorporation assay for 3D UB cell culture. [^{3}H]thymidine incorporation assay was conducted for UB cells in 3D culture to examine the effect of PKA activation on cell proliferation rate. Measuring radioactivity of incorporated [^{3}H]thymidine using a scintillation counter revealed no statistically significant difference among the positive control, treatment of 200 μM, and 500 μM.

3.2 Compound screen

3.2.1 Target hits

Here, a compound screen with a compound library consisting of 242 kinase inhibitors were used to quickly identify potential molecules that could reverse the effect of cAMP-PKA activation on UB branching and might be interacting with PKA in the context of UB branching. UB cells were seeded in 3D gel in three replicates of 384 well plates, and hundreds of kinase inhibitors, such as that of ERK, PI-3K, PKC, and JAK, were injected into the wells (for a complete list of the compounds please see appendix 1). The plates included positive and negative controls for direct comparison with the experimental conditions. At day 5 and day 10, each well was scored according to the extent of branching of the colonies as compared to the

positive control. 19 compounds, ranging from inhibitors of common kinases such as PKC, p38 MAPK, and ERK, to inhibitor of CDK4/D1, a cyclin complex that is essential for the regulation of cell cycle, were identified to rescue branching morphogenesis, either fully or partially, from cAMP-PKA activation (Table 1).

Table 1. Hits from compound screening.

Compound Name	Primary Target	Secondary Target(s)
BPIQ-I	EGFR	
Protein tyrosine kinase inhibitor	EGFR tyrosine kinase	
Gö6983	PKCα, PKCβ	PKCs
IKK-2 Inhibitor VIII	IKK-2	
SB 203580, Sulfone	P38K	IL-1 (IC_{50} = 200 nM)
p38 MAP Kinase Inhibitor VII, SD-169	p38α MAP Kinase	p38 beta (122nM)
RSK Inhibitor, SL0101	RSK	
Aurora Kinase Inhibitor III	Aurora Kinase Inhibitor III	Lck, Bmx, IGF-1R, and Syk (IC_{50} = 131, 386, 591, and 887 nM, respectively)
Cdk4 Inhibitor	CDK4/D1	IC_{50} = 520 nM and 2.1 μM for Cdk2/E and Cdk1/B, respectively
Flt-3 Inhibitor	Flt-3	22 other kinases (IC_{50} ≥3 μM)
Syk Inhibitor III	Syk Inhibitor III	inhibits Src only at much higher concentrations (IC_{50} = 29.3 μM)
TGF-b RI Kinase Inhibitor	TGF-β receptor I kinase	p38a MAP kinase (IC_{50} = 740 nM)
PD 169316	p38 MAP kinase	
TGF-b RI Inhibitor III	TGF-β RI Kinase Inhibitor III	activin receptor-like kinase 4 (IC_{50} = 129 nM), 5 (IC_{50} = 47 nM), and 7 (same as TGF-B)
SB 220025	P38MAPK	Exhibits over 2000-fold greater selectivity for p38 MAPK over ERK (p42/p44 MAP kinase), 500-fold over PKA, 50-fold over PKC, and >1000-fold over EGFR. Also acts as a potent inhibitor of angiogenesis and as an inhibitor of LPS-induced TNF-α production.
Polo-like Kinase Inhibitor I	plk1	
SKF-86002	(LPS)-stimulated IL-1, TNF-α production	cyclooxygenases and 5-lipoxygenase, and osmotic stress. SKF-86002 also acts as a specific p38 MAP kinase inhibitor.
ERK Inhibitor II, FR180204	ERK1 and ERK2	Exhibits ~20-fold greater selectivity over p38α (IC_{50} = 10 μM)
SB 203580	P38MAPK	Inhibits IL-1 and TNF-α production from LPS-stimulated human monocytes and the human monocyte cell line THP-1 (IC_{50} = 50-100 nM).

3.2.2 P38 MAPK and NF-κB related inhibitors in the screen

Out of the 19 compounds, five were direct inhibitors of p38 MAPK. Inhibition of p38 MAPK resulted in recovery of branching morphogenesis in UB cells for all five p38 MAPK inhibitors screened (Figure 5A-G), and recovery of all five inhibitors were statistically significant (Figure 6). Besides p38 MAPK inhibitors, three compounds that either directly or indirectly inhibit NF-κB also showed protective effects from dbcAMP treatment. Aurora kinase is known to activate NF-κB through IκBα phosphorylation, and the inhibition of Aurora kinase has been shown to downregulate NF-κB (Briassouli et al., 2007; Sun et al., 2007). Ribosomal s6 kinase (RSK) regulates transcription factors such as CREB, NF-κB, and it has been demonstrated to be actively involved in the activation of NF-κB (Pierrat et al., 1998; Wang et al., 2006). SL0101, a RSK inhibitor, was able to counter the branching inhibition effect from cAMP-PKA activation. IκB kinase (IKK) is a well-known protein that is part of the NF-κB signaling transduction cascade and is essential for the activation of NF-κB. In the screening, three compounds that modulate NF-κB activation, including Aurora kinase inhibitor III, SL0101, and IKK-2 inhibitor VIII, were identified to effectively rescue the branching morphogenesis of UB cells (Figure 7A-E). However, among the three compounds, although SL0101 led to distinct recovery in morphology, quantification of percentage of branched colonies did not yield a statistically significant result, while IKK-2 inhibitor VIII and Aurora kinase inhibitor III both caused statistically significant rescue effects (Figure 8).

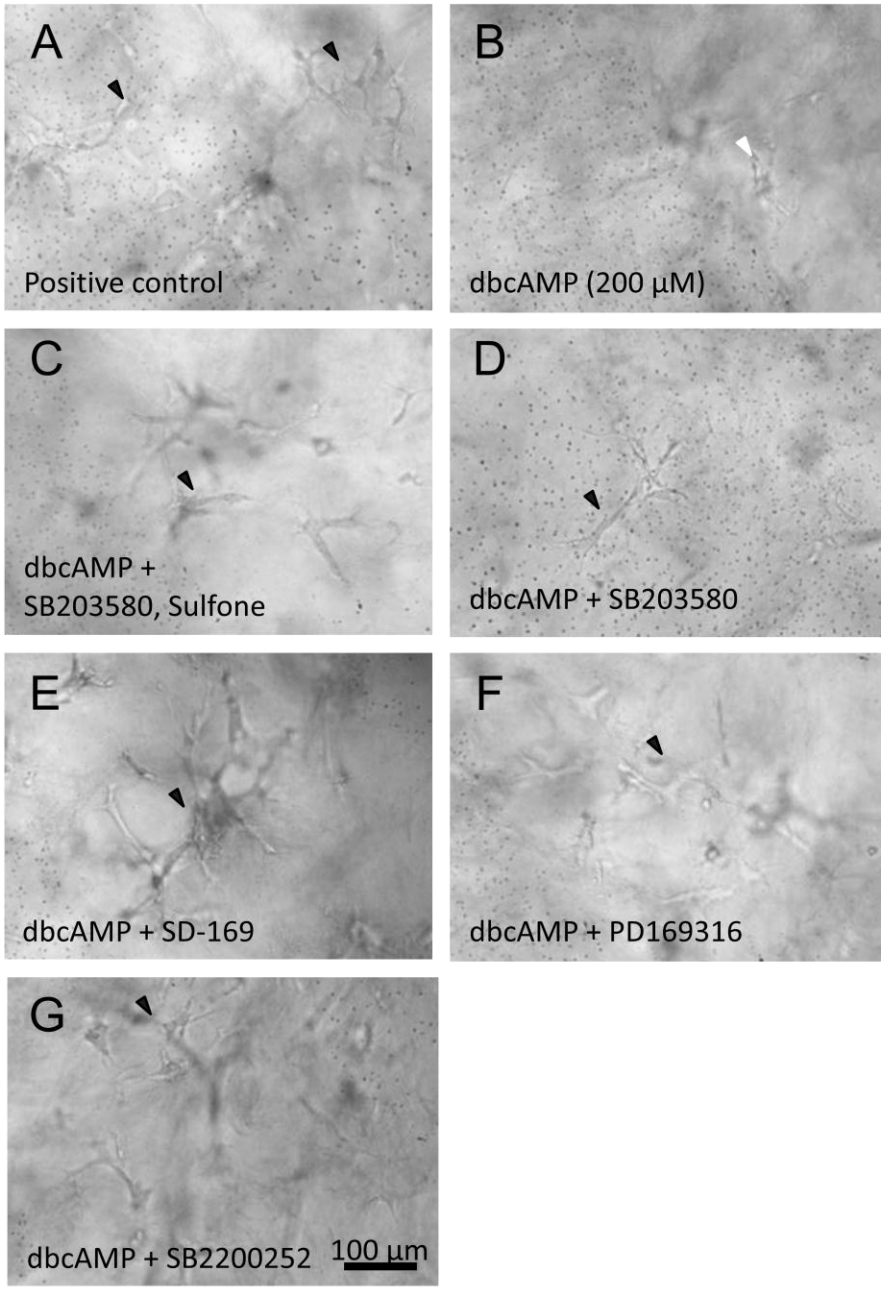

Figure 5. P38 MAPK inhibitors in the compound screen. Aside from the positive control (A), and negative control (B), in which UB cells were treated with 200 µM dbcAMP, the screen also injected p38 MAPK inhibitors, such as Sulfone SB203580 (C), SB203580 (D), SD-169 (E), PD169316 (F), SB220025 (G), into the wells with media already containing 200 µM dbcAMP. For all five conditions with p38 MAPK inhibitors, there are obvious tubular structures. Black arrows point to branched structures. White arrows point to cystic structures. Micrographs were taken at day 10 of the culture. Scale bar = 100 µm.

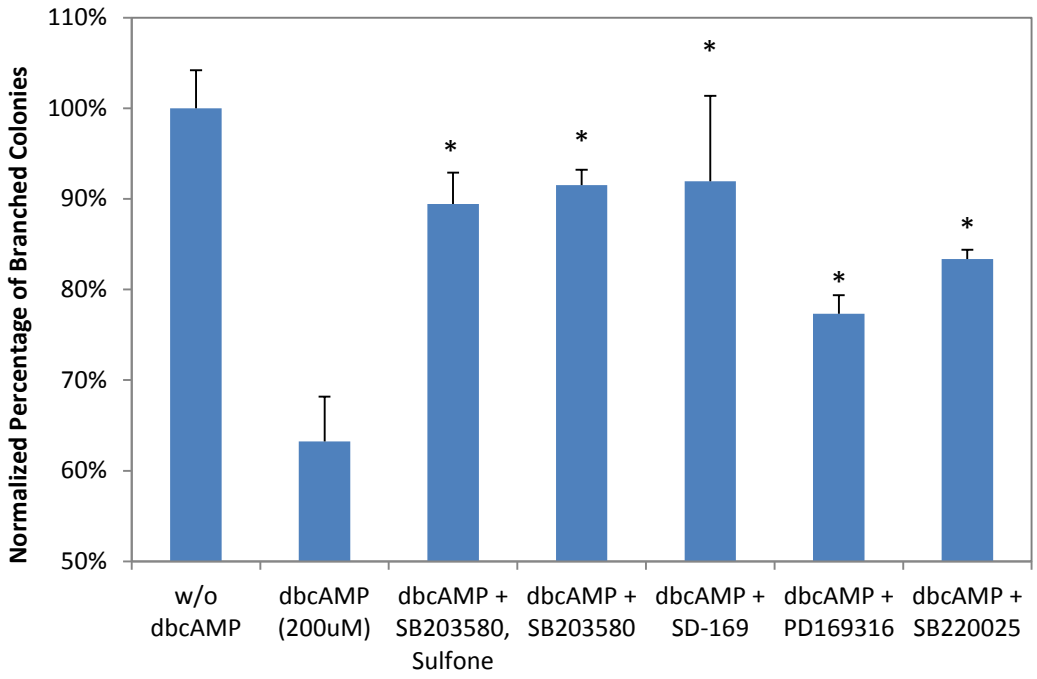

Figure 6. Quantification of p38 MAPK inhibitors in the compound screen. Quantitative analysis of percentage of branched colonies showed that all p38 MAPK inhibitors significantly recovered branching morphogenesis from treatment of dbcAMP. *$p < 0.05$, comparison of experimental conditions and dbcAMP treated condition.

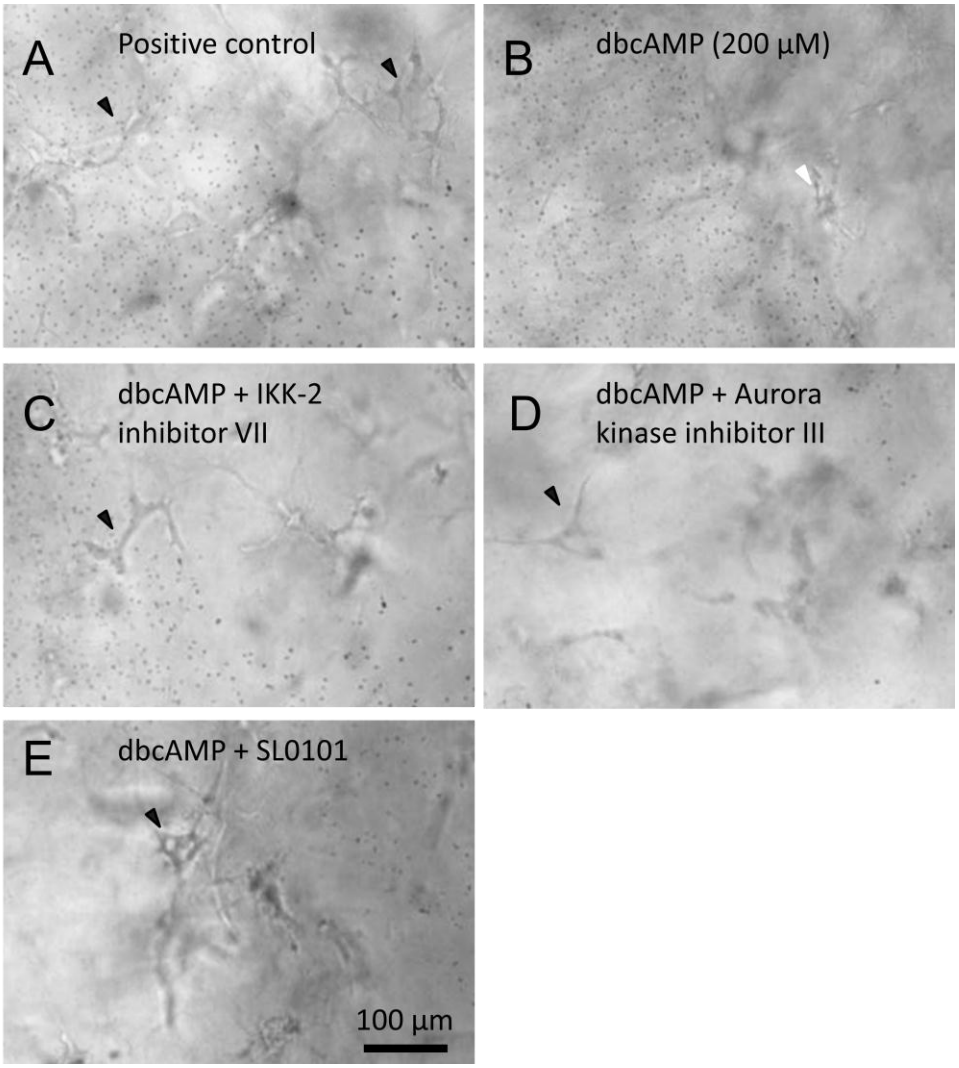

Figure 7. NF-κB related inhibitors in the compound screen. Aside from the positive control (A), and negative control (B), in which UB cells were treated with 200 µM dbcAMP, the screen also injected NF-κB related inhibitors, such as IKK-2 inhibitor VIII (C), Aurora kinase inhibitor III (D), and SL0101 (E), into the wells with media already containing 200 µM dbcAMP. Black arrows point to branched structures. White arrows point to cystic structures. Micrographs were taken at day 10 of the culture. There are tubular structures in (A and C-E). Scale bar = 100 µm.

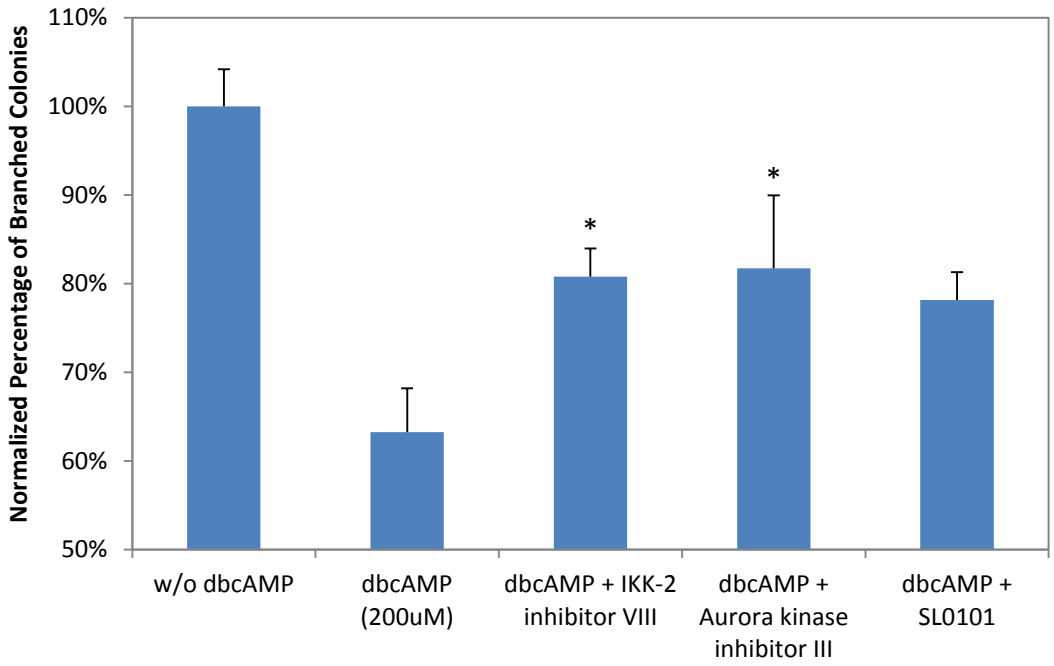

Figure 8. Quantification of NF-κB related inhibitors in the compound screen. Quantitative analysis of percentage of branched colonies showed that IKK-2 inhibitor VIII and Aurora kinase inhibitor III both significantly recovered branching morphogenesis from treatment of dbcAMP. SL0101 partially rescued branching, but the result was not statistically significant ($p = 0.077$). *$p < 0.05$, comparison of experimental conditions and dbcAMP treated condition.

3.3 Network analysis

To further analyze the hypothesis that cAMP-PKA activation inhibits branching morphogenesis of the UB through interactions with p38 MAPK and NF-κB, a network consisting of 54 known molecules modulating UB or IMCD cell tubulogenesis, such as HGF, EGF, integrins, BMP2, was constructed using IPA based on existing literature data. Interactions among these molecules were added according to the IPA knowledge database. The new molecules identified to be involved in the branching morphogenesis of UB, such as NF-κB, p38 MAPK, IKK, and Aurora kinase, were then added to the network and were shaded in pink (Figure 9). Connections were made among the newly added molecules and the original molecules through the IPA knowledge database, and represented as green lines. The molecules without connections to the newly added molecules were excluded from the network for a clearer representation. From the network, it can be seen that cyclic AMP activates PKA, which then indirectly activates p38 MAPK, resulting in the activation of NF-κB further down; in addition, Aurora kinase regulates NF-κB/RelA through interaction with IKBKB, also known as IKK-2. Furthermore, network analysis shows that TGF-β1, TGF-β2, and TGF-β3 are potential activators of this pathway.

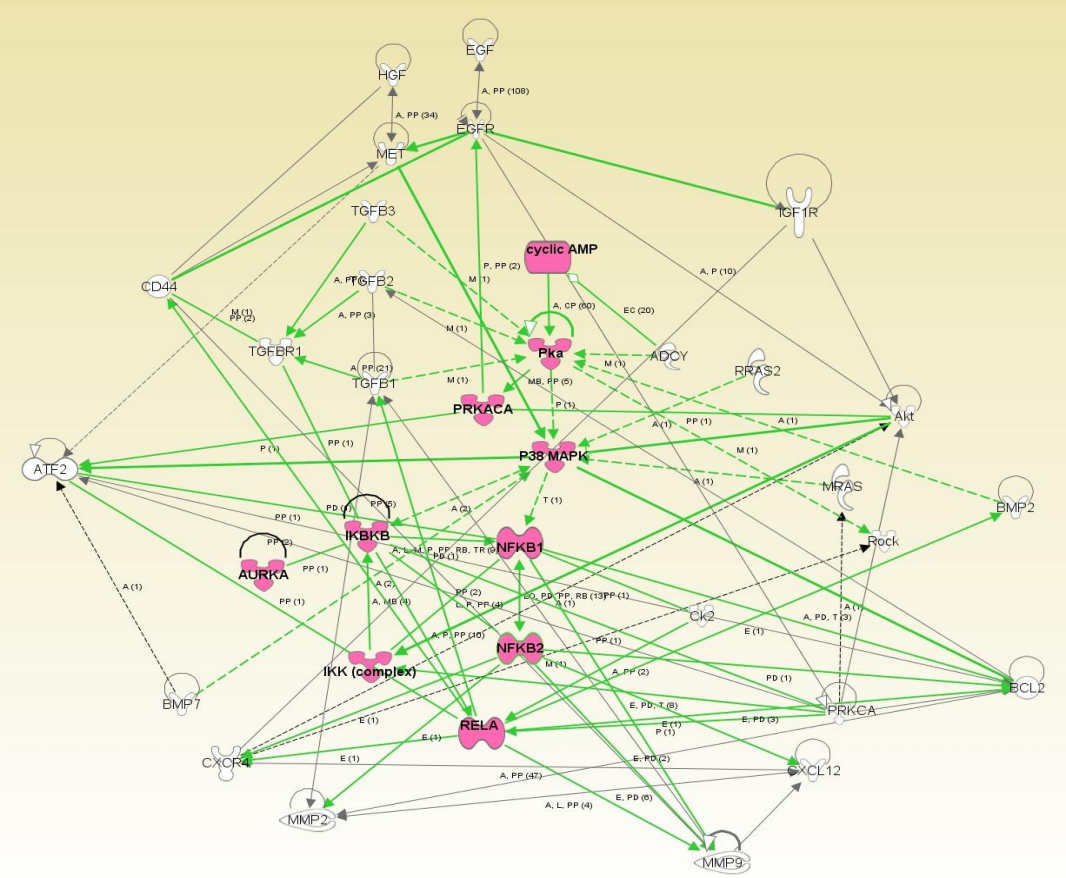

Figure 9. Molecular network with hits from the compound screen. Ingenuity Pathway Analysis of hits from Kinase244 compound screen revealed extensive interactions with an existing branching network, and a potential pathway involving TGF-β1, PKA, p38 MAPK, and NF-κB.

3.4 Effect of p38 MAPK or NF-κB inhibition on UB branching

3.4.1 3D UB cell culture

The results obtained with the compound screen could not be used directly as proof that p38 MAPK and NF-κB are able to reverse the effect of cAMP-PKA activation in UB cells, as all the compounds were added at a uniform concentration of 5 μM, which was higher than the IC_{50} values of the secondary targets for some

compounds. In addition, the interactions proposed through network building also needed to be experimentally validated. Therefore, further experimentation was needed to fully verify the preliminary results from the compound screen. SB203580, an inhibitor of p38 MAPK, was used at concentrations of 2 μM, 10 μM, and 50 μM along with dbcAMP. At 2 μM, SB203580 fully rescued branching morphogenesis of UB cells, as more branched colonies and fewer cysts were observed than the samples treated with only dbcAMP and the result was also statistically significant comparing to the dbcAMP treated samples ($p < 0.05$). Nevertheless, when concentration of SB203580 increased, the percentage of branched colonies decreased, indicating that while moderate inhibition of p38 MAPK is able to reverse the effect of cAMP-PKA activation, excessive inhibition abolishes the essential branching conditions of UB cells (Figure 10D, 11). A broad spectrum IKK inhibitor that inhibits IKK-1, IKK-2, and IKK complex also showed statistically significant recovery from the PKA activation effect ($p < 0.05$; Figure 10E, 11), suggesting that when NF- κB activation is blocked by IKK inhibition, cAMP-PKA activation effect could be eliminated. These observed results are consistent with the results given by the compound screen. Hence, inhibition of p38 MAPK and NF- κB were proven to be able to reverse the effect of cAMP-PKA activation and rescue branching morphogenesis in UB cells in 3D culture. In addition, to show that the dbcAMP effect observed is mediated through PKA, a second PKA activator was used, and the same branching inhibition effects were observed with the second PKA activator and dbcAMP (Figure 10B, C, 11).

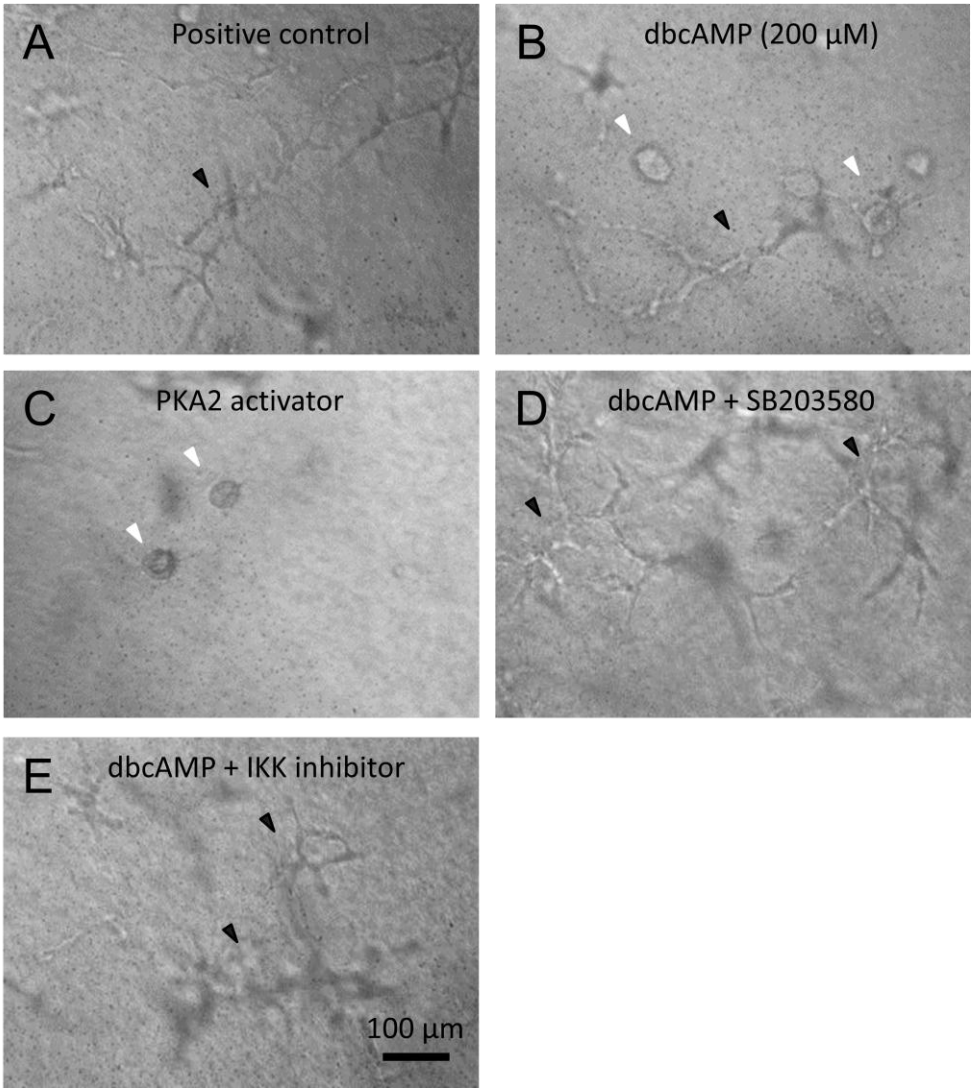

Figure 10. Effect of PKA activation, p38 MAPK, and IKK inhibition on UB cell branching. Treatment of UB cells in 3D culture with 2 µM SB203580 (D) and 1 µM IKK inhibitor (E) in addition to 200 µM of dbcAMP protected the branching inhibition effect by PKA activation as seen in treatments with 200 µM dbcAMP (B) and a PKA2 activator (C). Positive control (A) shows tubular structures. Black arrows point to branched structures. White arrows point to cystic structures. Micrographs were taken at day 10 of the culture. Scale bar = 100 µm.

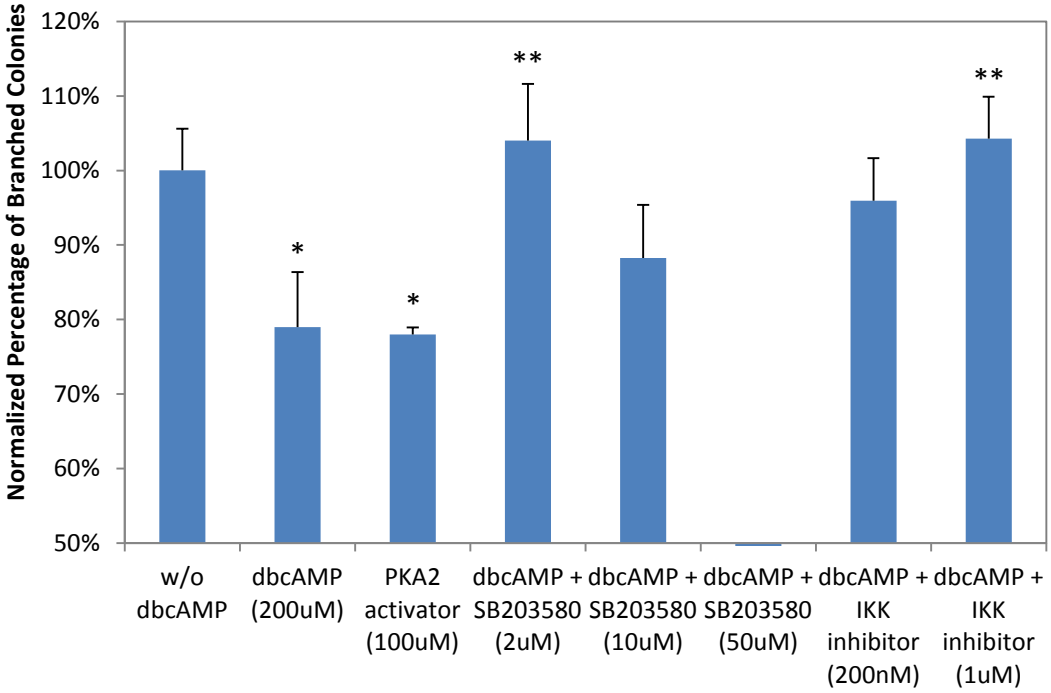

Figure 11. Quantification of branching with p38 MAPK and IKK inhibitors in the presence of PKA activators. Quantitative analysis of branched colonies showed that PKA2 is just as effective as dbcAMP in inhibiting branching. Also, 2 μM of SB203580 and 1 μM IKK inhibitor were both able to significantly reverse the branching inhibition from PKA activation. *$p < 0.05$, comparison of positive control and dbcAMP treated condition. **$p < 0.05$, comparison of experimental conditions and dbcAMP treated condition.

3.4.2 Whole embryonic kidney culture

To confirm the consistency of the results across different culture models and to evaluate the effect of PKA on UB branching in a more physiological condition, organ culture of whole embryonic kidney (WEK) was conducted. E14 kidneys were explanted from rat embryos and cultured in the presence of serum and desired compounds. At day 6, no apparent size and appearance disparities were observed among four samples of positive control, dbcAMP, dbcAMP with SB203580, and dbcAMP with IKK inhibitor (Figure 12A-D). Staining the samples with Dolichos

bifluorus lectin, which binds to UB-derived structures, returned results that were consistent with the results obtained in 3D cultures of UB cells. Treatment of WEK with dbcAMP resulted in dilation of UB-derived tubules, especially in the central regions that would give rise to the ureters, and a reduction in peripheral branching points, indicating defects in the branching program of the UB (Figure 13 A, B, 14). Upon addition of SB203580 and IKK inhibitor to the samples already containing dbcAMP, the dilation effect and reduction in peripheral branching points were partially recovered (Figure 13C, D). In addition, samples treated with SB203580 showed more prominent signs of rescue than the ones treated with IKK inhibitor, as indicated by the quantification of branching points, although both showed reversal of the branching inhibition effect by cAMP-PKA activation (Figure 14). Furthermore, while the dilation effect was partially mitigated by the addition of p38 MAPK and IKK inhibitors, the UB-derived tubules did not branch as smoothly as the positive control, which meant that the effect due to cAMP-PKA activation was not completely counteracted.

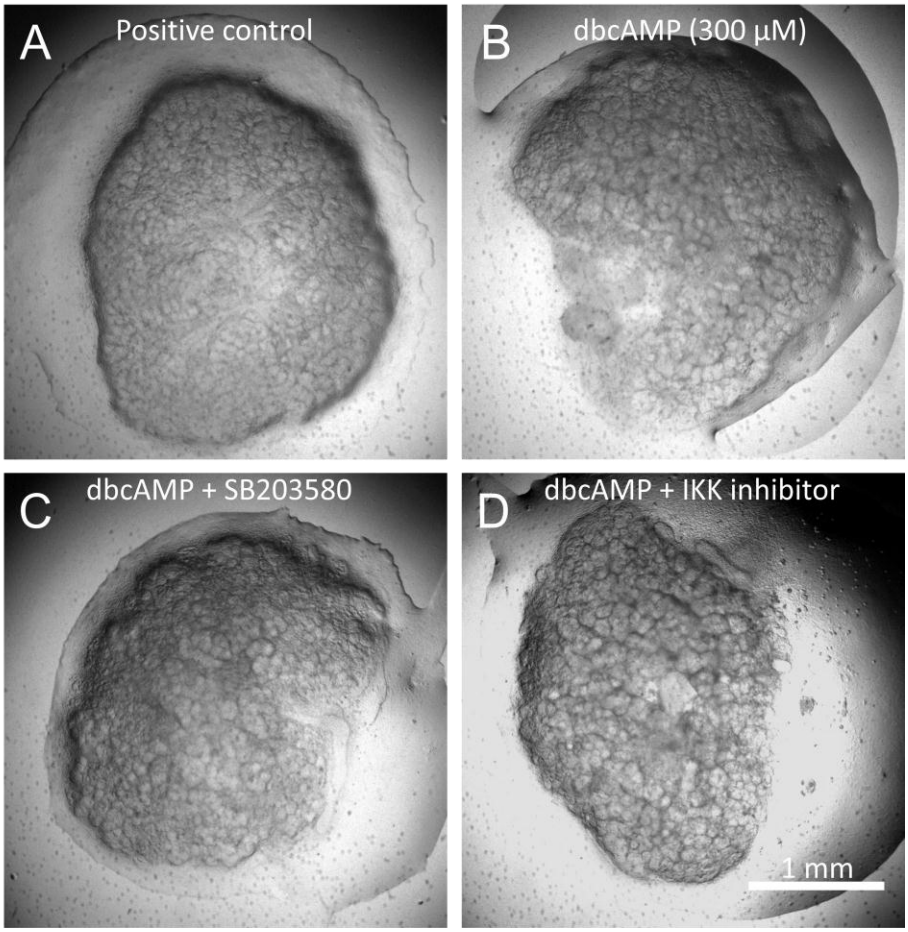

Figure 12. Embryonic kidney culture with dbcAMP, SB203580, or IKK inhibitor. Phase contrast micrographs of embryonic day 14 kidneys isolated and cultured *in vitro* for 6 days. Positive control (A), WEK treated with 300 μM dbcAMP (B), 300 μM dbcAMP plus 2 μM SB203580 (C), and 300 μM dbcAMP plus 1 μM IKK inhibitor (D) all grew to relatively the same size. No significant disparities could be seen among the four conditions. Scale bar = 1 mm.

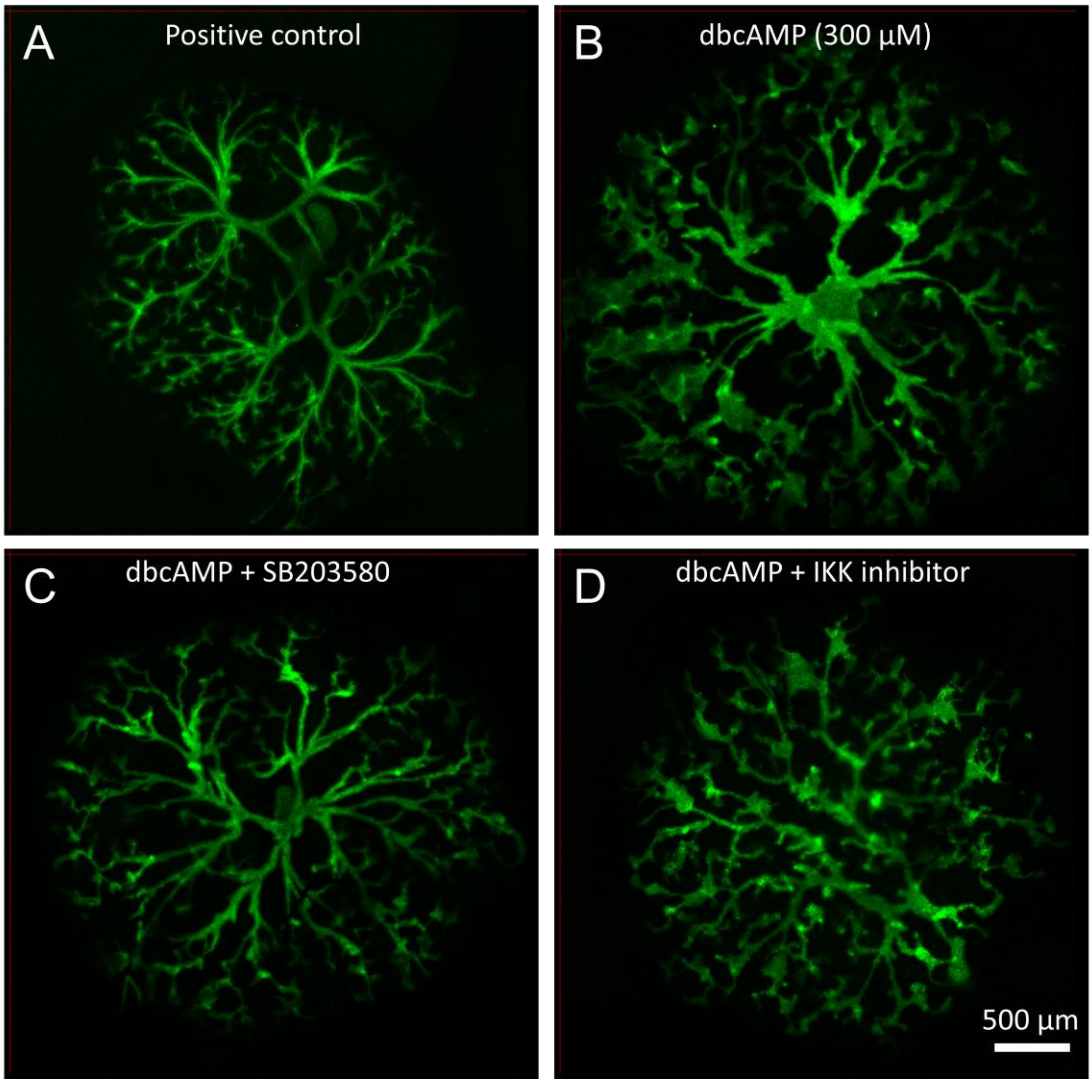

Figure 13. Whole embryonic kidney with dbcAMP, SB203580, or IKK inhibitor. WEK treated with 300 µM dbcAMP (B) had tubule dilation comparing to the positive control (A). 300 µM dbcAMP plus 2 µM SB203580 (C), and 300 µM dbcAMP plus 1 µM IKK inhibitor (D) both partially recovered the dilation effect by dbcAMP. Green = DB lectin, UB-derived tissues. Scale bar = 500 µm.

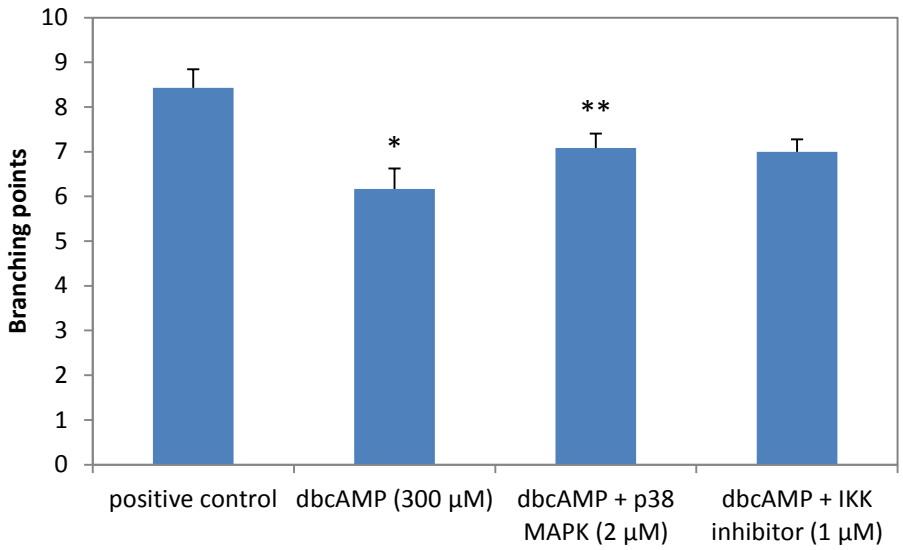

Figure 14. Quantification of branching points in WEKs. Extent of branching was quantified by counting the number of branching points from the tip of a tubule to its origin. Treatment of WEK with dbcAMP significantly reduced number of branching points, while the additional treatment with p38 MAPK had a significant rescue effect. Although the additional treatment with IKK inhibitor did not significantly rescue branching, it yielded higher number of branching points than treatment with dbcAMP alone. *$p < 0.05$, comparison of positive control and dbcAMP treated condition. **$p < 0.05$, comparison of experimental conditions and dbcAMP treated condition.

3.5 Activation of p38 MAPK and NF-κB by cAMP-PKA

From the network analysis, p38 MAPK was seen to be activated by PKA. Such an interaction would explain the observation that p38 MAPK inhibition reverses the effect of PKA activation on branching morphogenesis of the UB. Western blot showed that p38 MAPK was phosphorylated in the sample treated with dbcAMP, while it was not activated in the positive control (Figure 15). Examining the Western blot result, it is intuitive that inhibition of p38 MAPK would block the effect of PKA, since PKA exerts its branching inhibition effect through the activation of p38 MAPK.

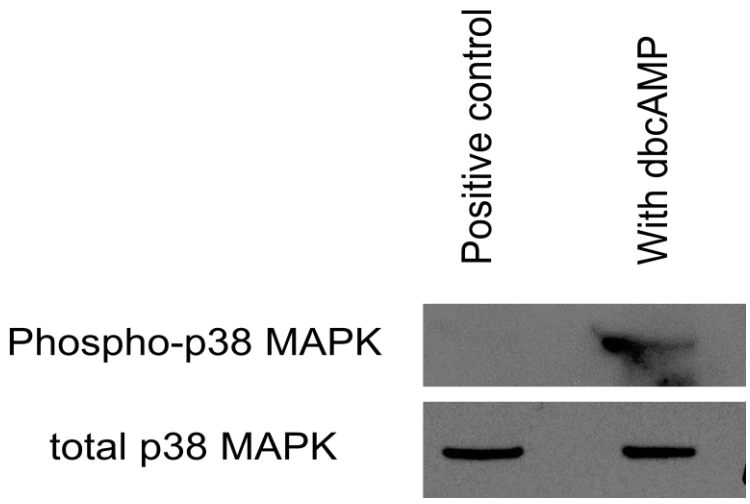

Figure 15. Activation of p38 MAPK by PKA during UB cell branching morphogenesis. Proteins collected from UB cells grown in 3D culture for 10 days were collected and analyzed with Western blot. Under the treatment of 200 μM dbcAMP, p38 MAPK was activated (shown as phospho-p38 MAPK), while the amount of total p38 MAPK remains the same in UB cells treated with or without dbcAMP (positive control).

Similarly, the network analysis pointed to the indirect activation of NF-κB by PKA. Thus, NF-κB activation was also investigated here. The activation of NF-κB is signified by the nuclear translocation of RelA, or NF-κB p65 subunit. Therefore, immunofluorescence was conducted with antibodies against NF-κB p65. Translocation of p65 is displayed as an overlap between p65 (red fluorescence) and a nuclei-staining agent DAPI (blue fluorescence), which is shown as pink. With increasing dbcAMP concentration, there appeared to be a greater extent of nuclear translocation of NF-κB p65 (Figure 16A-C). It can be seen that at relatively low dbcAMP concentration at 200 μM, cells lost their ability to branch; at higher dbcAMP concentration of 2 mM, cells lost the ability to form aggregates altogether. However, the difference in nuclear translocation of NF-κB p65 between the positive

control and treatment with 200 μM dbcAMP is rather challenging to distinguish, as there was not complete overlap between red and blue fluorescence, indicating the nuclear translocation of only a fraction of the NF-κB p65.

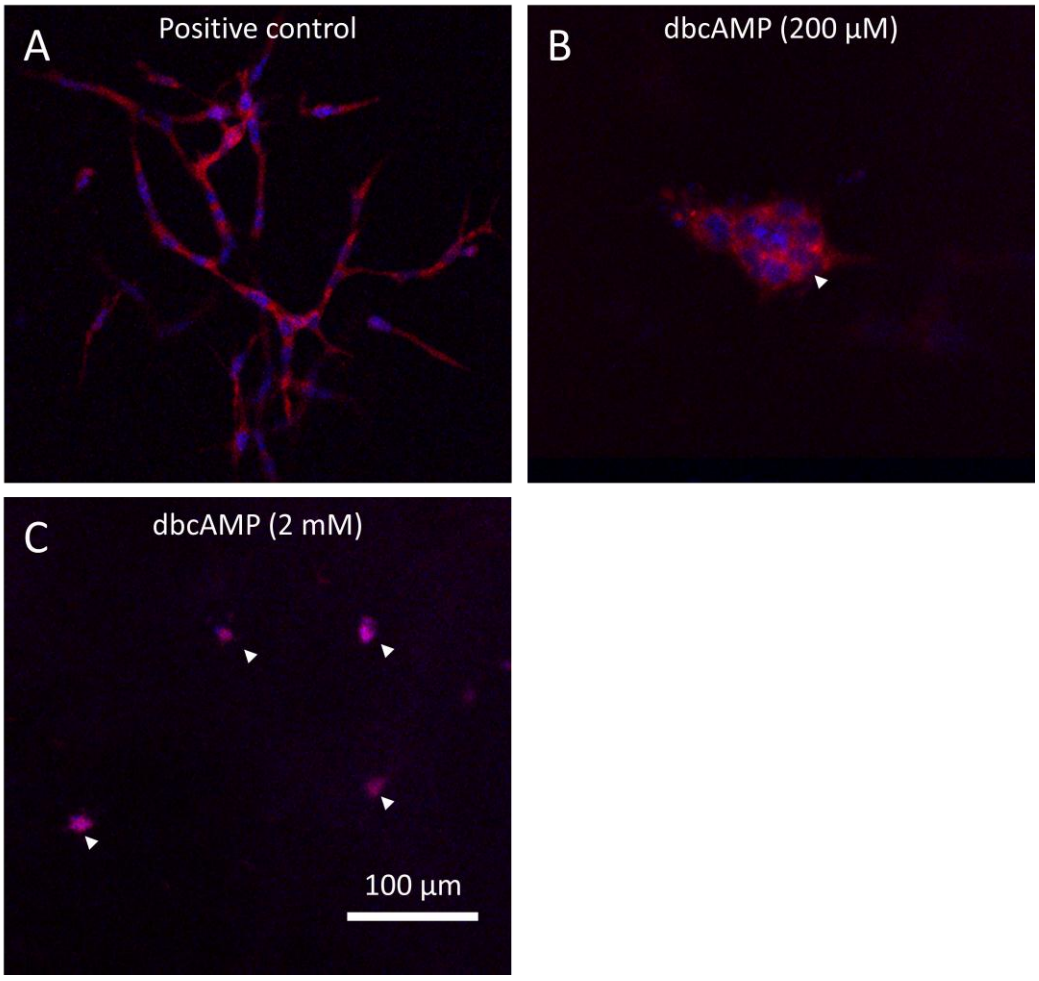

Figure 16. Effect of PKA activation on NF-κB nuclear translocation. Increasing dbcAMP concentrations at 200 μM (B) and 2 mM (C) showed progressively more overlap between blue and red, indicating a greater extent of nuclear translocation of NF-κB p65. There were few overlaps between blue and red in the positive control (A). UB cells were grown for 10 days before staining. White arrows point to regions where p65 subunits and nuclei overlap. Blue = DAPI; red = NF-κB p65 subunit. Scale bar = 100 μm.

Furthermore, the addition of either SB203580 or IKK inhibitor alone to UB cells in 3D culture does not increase the percentage of branched colonies (Figure 17). Also considering the observation that the addition of SB203580 or IKK inhibition was able to reverse the effect of PKA activation, it shows that inhibition of p38 MAPK or NF-κB does not have the innate ability to stimulate branching morphogenesis, but rather, they are downstream effectors of PKA.

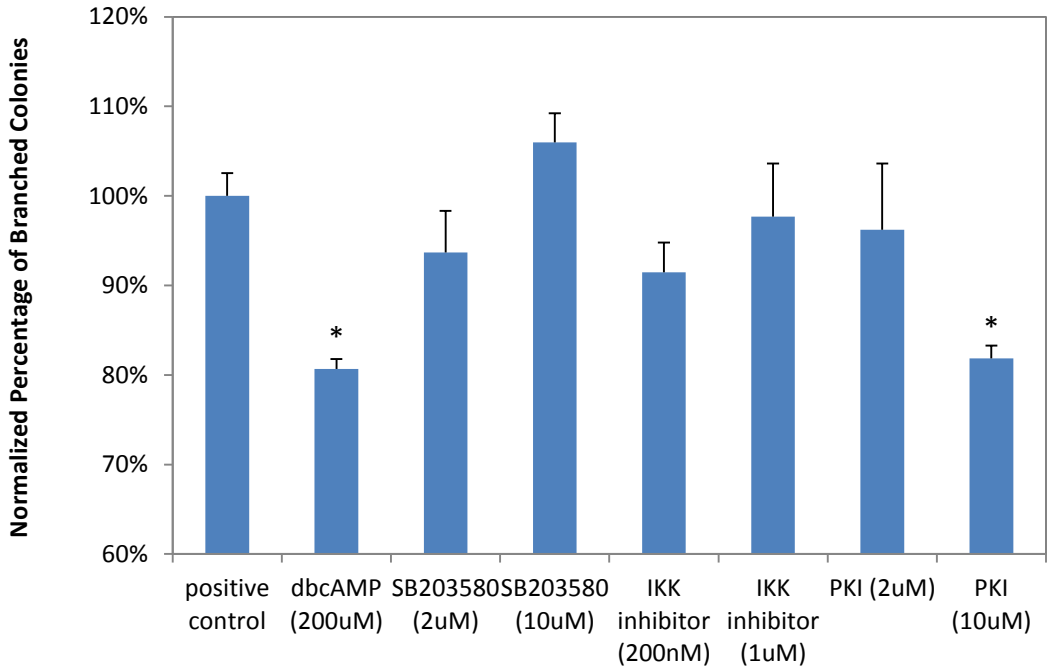

Figure 17. Effect of various inhibitors on UB cell branching. UB cells were treated with various inhibitors including SB203580, IKK inhibitor, PKA inhibitor (PKI), but quantitative analysis yielded no significant difference between these samples and the positive control, other than the samples treated with 10 μM PKI. *$p < 0.05$, comparison of positive control and experimental conditions.

3.6 Involvement of TGF-β1 in the network

3.6.1 TGF-β1 as an activator of PKA

TGF-β1 was proven to have an inhibitory effect on UB branching (Sakurai and Nigam, 1997). As seen from the network analysis, TGF-β1, TGF-β2, and TGF-β3 are potential activators of PKA. To demonstrate the validity of this interaction in the context of UB branching morphogenesis, the effect of PKA, p38 MAPK, or IKK inhibition was tested in UB cells under the stimulation of TGF-β1. With the addition of PKA inhibitor 5-24 at 1 μM, there was statistically significant recovery from the branching inhibition effect by TGF-β1, confirming the potential link established by the network analysis (Figure 18A-E).

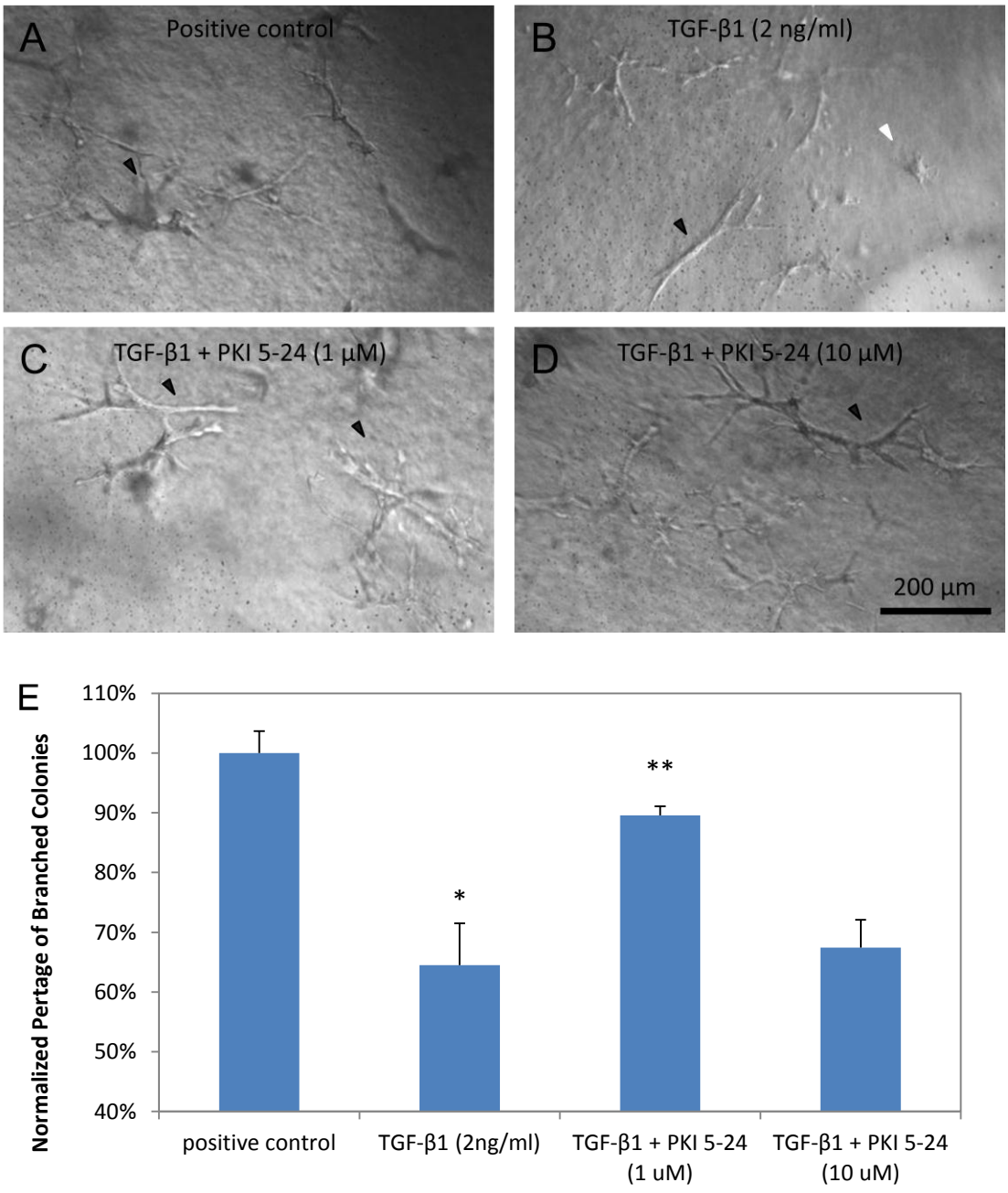

Figure 18. Effect of PKA inhibition on TGF-β1 in UB cells. Phase contrast micrographs showed that UB cells treated with TGF-β1 (B) had significantly fewer well branched structures than the positive control (A), but the addition of 1 μM PKI 5-24 in TGF-β1 treated UB cells (C) showed significant recovery. Addition of 10 μM PKI 5-24 in TGF-β1 treated UB cells (D) returned similar level of branching to UB cells treated with only TGF-β1. Quantitative analysis confirmed the statistical significant levels of the aforementioned findings. Black arrows point to branched structures. White arrows point to cystic structures. *$p < 0.05$, comparison of positive control and TGF-β1 treated condition. **$p < 0.05$, comparison of experimental conditions and TGF-β1 treated condition.

To further confirm that PKA is activated by TGF-β1, proteins were collected from isolated UBs treated in different conditions, including TGF-β1, Activin A (a member of the TGF-β superfamily), and dbcAMP. Western blot showed that with the treatment of TGF-β1 and Activin A, CREB (cAMP response element-binding), a downstream transcription factor regulated by PKA, and ATF-1 (cyclic AMP-dependent transcription factor), were phosphorylated and activated identical to the treatment of iUB with dbcAMP, indicating the activation of PKA with the addition of TGF-β1 or Activin A (Figure 19, provided by Chiharu Ito).

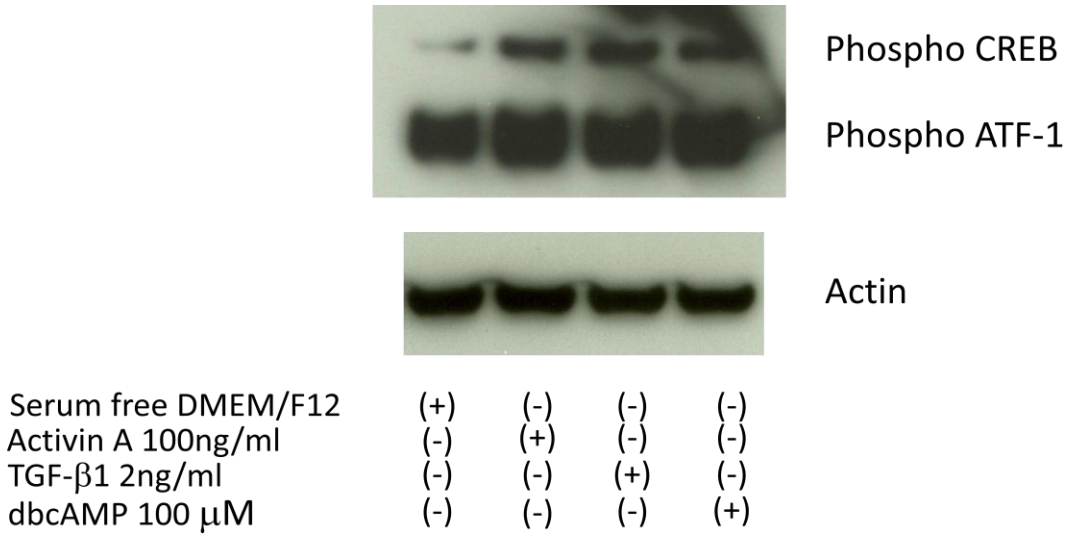

Figure 19. Activation of PKA by TGF-β1 in iUB branching. Western blot showed the phosphorylation of CREB upon treatment with Activin A, TGF-β1, and dbcAMP, indicating the activating of PKA by these three proteins.

3.6.2 P38 MAPK or NF-κB inhibition on TGF-β1 mediated branching inhibition

Furthermore, since PKA exerts its inhibitory effect through p38 MAPK and NF-κB, and TGF-β1 activates PKA to inhibit UB branching, the inhibition of p38 MAPK and NF-κB should also abolish the effects by TGF-β1. Such a hypothesis was verified in an experiment in which SB203580 and IKK inhibitor were used to block the effect of TGF-β1. The addition of 2 μM of SB203580 rescued branching morphogenesis of UB cells treated with 2 ng/ml TGF-β1 (Figure 20C, 21). The addition of 200 nM of IKK inhibition partially rescued branching, but the result was statistically indistinguishable ($p = 0.067$) (Figure 19D, 20). The outcomes show that PKA is a downstream effector of TGF-β1, and p38 MAPK and NF-κB mediate PKA's inhibitory effect on UB branching, and are possible downstream effectors of PKA.

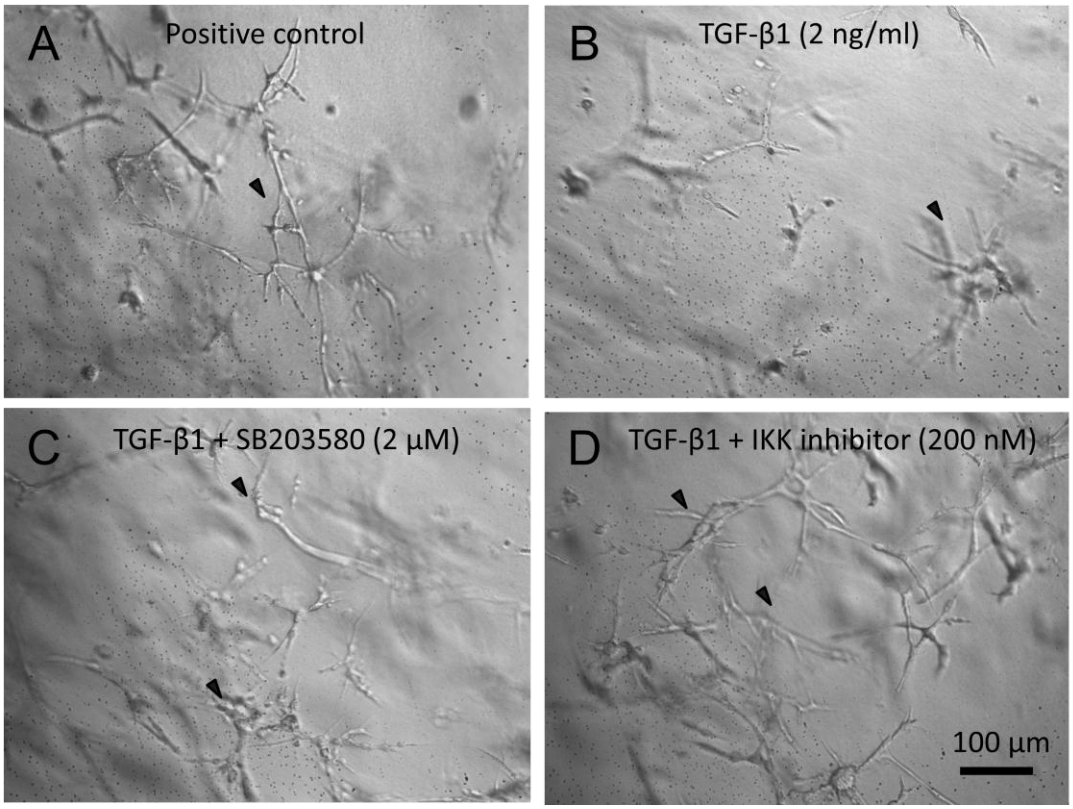

Figure 20. Effect of p38 MAPK and IKK inhibition on TGF-β1 in UB cells. Although TGF-β1 inhibits the branching morphogenesis of UB cells, the addition of 2 μM SB203580 to TGF-β1 treated cells (C) recovered branching from treatment of 2 ng/ml TGF-β1 alone (B). The addition of 200 nM IKK inhibitor to TGF-β1 treated cells (D) also generated many branched colonies. Black arrows point to branched structures. Positive control (A) showed well branched structures. Scale bar = 200 μm.

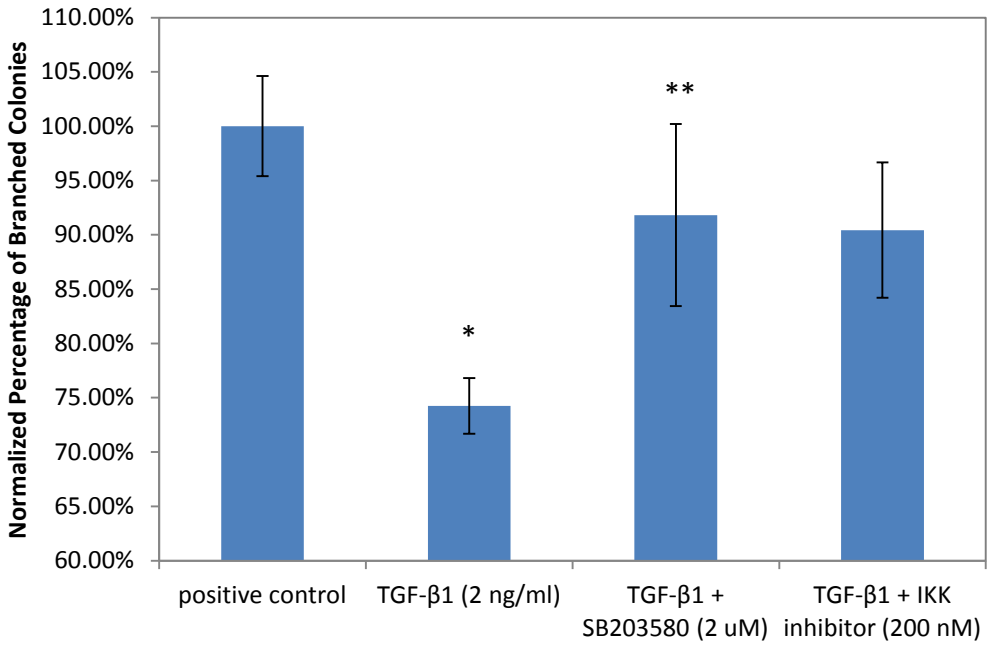

Figure 21. Quantification of p38 MAPK and IKK inhibition on TGF-β1 in UB cells. Quantification of branched colonies demonstrated statistically significant recovery of SB203580 to TGF-β1. *$p < 0.05$, comparison of positive control and TGF-β1 treated condition. **$p < 0.05$, comparison of experimental conditions and TGF-β1 treated condition.

3.7 Summary of the Pathway

It was shown that p38 MAPK and NF-κB inhibition were able to reverse the branching inhibition effect by PKA and that they were downstream effectors of PKA. In addition, TGF-β1 was identified to be an upstream activator of PKA. A summary of the largely validated pathway is shown in Figure 22. From the figure it can be seen that TGF-β1 and cAMP both activate PKA, albeit TGF-β1 activates PKA indirectly. Downstream of TGF-β1 and cAMP, PKA phosphorylates p38 MAPK, which in turn activates NF-κB. The activation of NF-κB is also dependent on the activity of IKK and its nuclear translocation.

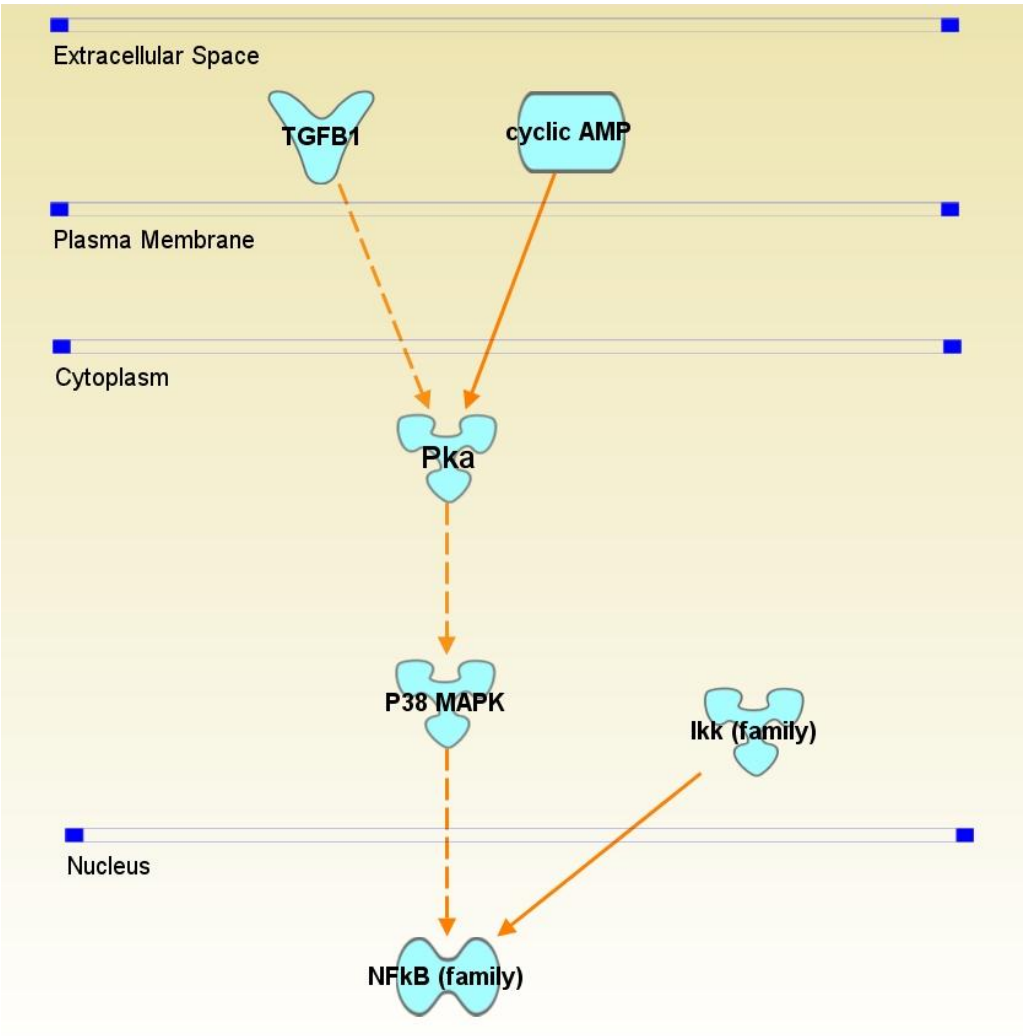

Figure 22. Summary of the pathway centered around PKA. A simple pathway was created to summarize the findings regarding the interactions of the molecules that are centered around PKA. Dashed lines indicate indirect relationships and solid lines indicate direct interactions.

Chapter 4 Discussion

4.1 cAMP-PKA and UB branching

Multiple cell culture models, including MDCK and mIMCD, have been utilized to show that PKA is one of the key regulators in renal epithelial branching morphogenesis (Santos et al., 1993), and mediates the branching inhibition effect exerted by BMP2 (Gupta et al., 1999). Even though both MDCK and mIMCD cells are of kidney epithelial tissue origin, they might not fully capture the complex mechanism and signaling events taking place in a developing embryonic kidney or branching UB, since MDCK is not from the UB lineage, and mIMCD is derived from mature collecting ducts, which, while ultimately of UB origin, could have altered genetic patterning. PKA has also been demonstrated to negatively regulate the outgrowth of the UB from the WD (Tee et al., 2010). Although WD budding is similar to the branching morphogenesis of the UB in that they are both generally guided by the GDNF/Ret signaling cascade and both involve tubule formation from epithelial tissue, it is fundamentally distinct from UB branching morphogenically and genetically. The budding of UB is mainly due to the rearrangement of cells along the WD, while the branching of the UB involves the alteration of the genetic pattern of the cells, such as the transformation of tip cells to stalk cells (Nigam and Shah, 2009). In addition, the outgrowth of the UB is tightly restricted spatiotemporally, while UB branching morphogenesis is rather spatiotemporally dynamic. Furthermore, with the exception of GDNF/Ret signaling, the signaling pathway employed by each process involves a unique set of modulators and regulators. For example, while *Spry1*

negatively regulates the outgrowth of UB from the WD, its absence during UB branching has a rather minor effect (Costantini, 2010). Additionally, it has been suggested that cAMP elevation is the key pathogenesis event for autosomal-dominant and recessive polycystic kidney diseases (Belibi et al., 2004); and it is widely accepted that cysts in autosomal-recessive polycystic kidney diseases arise from the collecting ducts derived from the UB. Despite significant evidence hinting at the importance of PKA in the branching morphogenesis of the UB, this has yet to be verified in the developing UB itself.

It was shown here that the addition of dbcAMP significantly inhibited the branching morphogenesis of both UB cells and the isolated UB in 3D culture in a concentration dependent manner. Examining the proliferation rate of UB cells upon induction with PKA showed that PKA does not inhibit branching through the alteration of cell proliferation rates. Out of the three processes most important to epithelial branching morphogenesis – mitogenesis, motogenesis, and morpohgenesis – mitogenesis is already eliminated as the mechanism upon which PKA exerts its branching inhibition effect. Many studies have shown the involvement of PKA in cellular migration. In the context of vascular sprouting, which is a similar process to epithelial branching morphogenesis, activated PKA stimulated cell-cell adhesion while suppressing endothelial cell polarization, motility, angiogenesis, and vascular permeability (Jin et al., 2010). PKA activation has also been implicated in inhibiting fetal lung fibroblast chemotaxis, perhaps through modulating migration (Kohyama et al., 2001; Kohyama et al., 2009). Additionally, a cellular permeable cAMP analog has also been shown to inhibit the migration of human gastric cancer cells (Chen et al.,

2005). On the contrary, many studies also point to the stimulatory effect on cellular migration by PKA. In damaged airway epithelium, bronchial epithelial cells migrate in response to PKA stimulation (Allen-Gipson et al., 2007; Spurzem et al., 2002; Wyatt et al., 2002). In early neural crest development, EMT and migration are enhanced by PKA signaling (Sakai et al., 2006). With varied migratory effects for different cells under different situations, the exact consequence of PKA activation on UB cell migration needs to be investigated. However, it would not be surprising if the branching inhibition effect is due to altered cellular migration properties, since PKA is known to control signaling events that are critical for polarized actin cytoskeletal dynamics, which is essential to motogenesis (Paulucci-Holthauzen et al., 2009).

Another possible explanation for PKA's branching inhibition effect would be based on cellular morphogenesis. A crucial process in the morphogenesis would be partial EMT (p-EMT) that leads to the initiation of branching by conferring increased motility and invasiveness to a subset of cells. It has even been speculated that the invasive behavior resulted from p-EMT could be the driving force behind branching morphogenesis, because in physiological conditions, in order for epithelium to branch out, the newly generated branches need to enter the surrounding tissues (Affolter et al., 2003). A typical example of p-EMT is the branching process of MDCK cells. MDCK cells branch by first growing into a cyst, which then branches out under the stimulation of HGF. During the shift from polarized cyst to processes extending out of the cyst, the cells that give rise to the processes undergo a temporary rearrangement in polarity and specialization of plasma membrane subdomains, signifying the progression of p-EMT (Pollack et al., 1998). Such a procedure

involving p-EMT was also observed in mammary epithelial cells (Simian et al., 2001). Similar to the p-EMT inductive effect of HGF to MDCK cells, some growth factors or molecules in BSN-CM likely stimulate p-EMT in the UB in order for proper branching morphogenesis to occur. The activation of PKA might negatively modulate the signaling cascade induced by these growth factors or change the genetic pattern of UB cells so that they lose the ability to undergo p-EMT; or on the other extreme, it could constitutively stimulate p-EMT so that cells lose their adhesiveness to each other. In MDCK cells, cAMP-elevating agents inhibited TGF-β1-induced EMT (Zhang et al., 2006). On the contrary, another study showed that TGF-β1 induces apoptosis and EMT, and this process is likely mediated by PKA (Yang et al., 2006). Also, PKA has been characterized as one of the main kinases responsible for the phosphorylation of Snail1, a transcription factor closely associated with EMT. It is also interesting to note that in primary mouse embryonic fibroblasts that display constitutive PKA signaling, MET is significantly enhanced (Nadella et al., 2008). In PKA activated UB cells, follow-up experiments demonstrated that the expression of E-cadherin and ZO-1, two membrane adhesion molecules that are expressed in the epithelial cells, increased more than two-fold (data not shown). However, as stated earlier, the exact mechanism by which PKA inhibits UB branching needs to be inspected closely.

The defect in UB branching resulting from PKA activation might be clinically significant as it could lead to reduced nephron number, which predisposes one to hypertension, and abnormalities of the ureters (Shah et al., 2004). Since UB branching is closely tied to nephrogenesis from the MM, the abnormalities in UB

development could translate to abnormalities in glomeruli and MM development related diseases such as glomerulocystic disease. Moreover, a study by Magenheimer et al. showed that treatment of E13.5 to 15.5 mice metanephric kidneys with cAMP agonists resulted in the formation of dilated tubules that enlarge over several days and eventually give rise to cyst-like structures of proximal tubule and collecting duct origin (Magenheimer et al., 2006). Kidneys treated with cAMP agonists and control samples grew similar in size, although there were obvious tubule dilations in the cAMP stimulated samples, a result consistent with the findings presented in Figures 12 and 13 in this paper. In kidneys diagnosed with polycystic kidney disease, the cystic structures were six-fold larger than those of wild-type kidneys (Magenheimer et al., 2006), signifying the greater danger of over-expression of cAMP in polycystic kidney disease conditions.

4.2 Compound screen

The compound library of the compound screen contains a wide variety of common kinase inhibitors, such as inhibitors for ERK, PI-3K, PKC, PKA, and JAK. Therefore, it is a useful screen to check for interactions specific proteins of interest. In this case, since UB cells were treated with dbcAMP before the injection of compounds, the screen was used to examine the possible interactions of PKA with other kinases or proteins in the context of UB branching. The compound screen was also extremely effective because it was directly targeting PKA under the context of UB branching morphogenesis, instead of examining general interactions that

molecules might have with PKA. The possible interactions include direct and indirect relationships. In the compound screen, if an inhibitor for protein "X" is able to reverse the effect by PKA, protein "X" could possibly be a direct downstream effector of PKA; an indirect downstream effector of PKA via the activation of downstream effectors of PKA; or an essential subunit in PKA itself, such as the catalytic or regulatory subunit. Another possibility would be that protein "X" has its own independent pathway that modulates the transduction of the PKA pathway, such that the activation of the pathway with protein "X" is necessary for the normal functioning of PKA or its downstream effectors. A good example, although not in the context of UB branching, would be the direct control of protein tyrosine phosphatase (PTP) by PKA, resulting in PTP negatively regulating the pathway of ERK and p38 MAPK (Nika et al., 2004). The last possibility, which is not desired, is that protein "X" does not interact with the target at all, and the reversal of target's effect due to the innate ability of protein "X" inhibition to generate a counter-effect. However, such hits could be likely identified and eliminated by referencing the most current knowledge database about the model used in the screen. In the case of UB branching, many proteins that are implicated in the branching morphogenesis are known and their effects on branching have been thoroughly studied. Overall, the compound screen is good for screening potential direct or indirect relationships, but does not shed light on the specific interactions there are between the target hits and the target.

Although an efficient system in checking potential interactions, the system has limitations. An obvious drawback is in the scoring system of the target hits, the manual scoring process is labor intensive, and ultimately, the scoring is qualitative,

not quantitative. However, precautions were taken to minimize error and scoring bias by implementing two rounds of scoring – on day five and day ten of the screen – and having three replicates for each condition. Another limitation is that every compound was used at 5 μM, which is much higher than the IC_{50} values for some of the compounds. Therefore, there is the possibility that nonspecific binding occurred between the compounds and undesired proteins. If a conclusion was to be deduced from one of these compounds, thorough experimentation needs to be conducted. From the screen, 5 hits of p38 MAPK inhibitors were identified, although one of them, Sulfone SB203580, has a secondary target of IL-1 with IC_{50} at 200 nM. The overwhelming evidence from the screen pointed to an interaction between p38 MAPK and PKA. Similarly, three NF-κB related inhibitors were identified as target hits. However, of the three inhibitors, two, except IKK-2 inhibitor VIII, were not direct inhibitors of NF-κB. Consequently, further experiments were needed to verify the finding by the compound screen.

It is important to note that other than the 8 inhibitors that were later validated, there were 11 other target hits. These target hits were not investigated further in the work presented here not because they were not valid, but rather because of the time limitation and desired efficiency for the purpose of this research. TGF-β receptor kinase and EGF receptor each had two hits from the screen. From the current knowledge, it is known that both are involved enormously in the branching morphogenesis of the UB and development of the kidney (Lee et al., 1998; Sakurai and Nigam, 1997; Sakurai et al., 1997b). It would not be surprising if these two kinases interact with PKA to modulate the branching inhibition effect.

4.3 Network analysis

A commercially available software package, IPA, was used to analyze the potential interactions among the molecules implicated in the branching process of the UB. The original network (not shown) was built from the a list of literature based genes, termed legacy data, containing 54 molecules involved in the branching morphogenesis of IMCD and UB cell culture systems. Interactions among these molecules were curated based on their relationships from the IPA knowledge database, which integrates existing knowledge on drugs, chemicals, genes, proteins, biological processes, and pathways. IPA is useful in a way that it is able to further the understanding of the specific interactions among molecules and genes by building on the basic finding that these molecules or genes interact. A recent study effectively utilized IPA to disclose important implications of PDGF-BB and TGF-β1 in the interaction between endothelial cells and vascular smooth muscle cells by constructing networks from only a list of differentially expressed proteins (Qi et al., 2011).

The network generated for the prediction of interactions that molecules have with PKA did not only encompass the molecules that were identified through the screen, but rather, it also integrated the legacy data containing current knowledge regarding the branching morphogenesis of UB and IMCD cells for the prediction interactions with not only p38 MAPK, IKK, NF-κB, but also the known branching molecules. With such an approach, aside from the pathway of PKA to p38 MAPK to NF-κB, much more information could be extracted from the network. As seen in this paper, TGF-β1, a known inhibitory regulator for UB branching, was predicted to be

upstream of PKA, and was validated by experimentation. Another point of interest would be the activation of NF-κB by PKC (PRKCA). One would suspect the modulatory effect of PKC on the PKA pathway through acting on NF-κB. This hypothesis was supported by a pilot study which showed that the proper inhibition of PKC was able to reverse the branching inhibition effect by PKA (data not shown).

4.4 PKA signaling in UB branching

From the combination of compound screen and network analysis, it was speculated that PKA exerts its effects on branching through p38 MAPK and NF-κB, with TGF-β1 as an upstream activator of PKA.

In the study presented here, the inhibition of p38 MAPK and NF-κB was shown to reverse the effect of PKA inhibition in both the UB cell and WEK model, demonstrating their involvement in the branching morphogenesis of the UB. Additionally, the inhibition of p38 MAPK and NF-κB alone did not increase the extent of branching, indicating that they were merely mediating the effect by PKA. It was identified through Western blot that p38 MAPK was phosphorylated upon induction of PKA, identifying it as a downstream effector of PKA in the branching inhibition pathway of PKA, thus elucidating how the inhibition of p38 MAPK could abolish the branching inhibition effect by PKA activation. By examining the nuclear translocation of NF-κB p65 by immunofluorescence, it was observed that the extent of NF-κB p65 nuclear translocation rises with increasing PKA activation. It is interesting to note that under the induction of 2 mM dbcAMP, UB cells were

scattered as single cells. Such an observation could be explained by a couple of possibilities. The first is that high level of PKA activation led to the loss of epithelial characteristics so that cells lose their adhesive abilities to form aggregates. However, as stated earlier, an increase in expression of epithelial markers were observed. The second possibility is that cells cease to proliferate at such a high dbcAMP concentration, which could be non-physiologically relevant. Therefore, it would be desirable to check the activation of NF-κB with a secondary method such as Western blot or immunoprecipitation. On the other hand, the mRNA expression level of NF-κB increased dramatically with the induction of 200 μM dbcAMP, mostly highlighted by the 16-fold increase in *Ikk2* and 4-fold increase in *Nfkb2* (data not shown). The expression profile of NF-κB related genes confirms that NF-κB was likely to be constitutively activated with the stimulation from PKA, supporting the immunofluorescence result.

The findings presented above that p38 MAPK and NF-κB are downstream effectors of PKA are supported by numerous literature evidences. The p38 MAPK signaling cascade is known to participate mainly in response to environmental stress, but also has important roles in the regulation of cell survival and differentiation (Cuadrado and Nebreda, 2010). Many mechanisms of activation have been found for p38 MAPK, including induction by stress factors through ligands that bind to different receptors, and even physical stresses that change membrane fluidity or other specialized signaling systems that operate independently of membrane receptors (Keshet and Seger, 2010). There have been many studies showing that p38 MAPK activation to be PKA-dependent. In NIH 3T3 cells, CREB is activated by p38 MAPK

through a time-delayed PKA-dependent manner (Delghandi et al., 2005). In B lymphocytes, it was demonstrated that p38 MAPK phosphorylation was regulated by the G-stimulatory (Gs)/cAMP/PKA pathway, and via the inactivation of hematopoietic protein tyrosine phosphatase (HePTP) (McAlees and Sanders, 2009). The expression of uncoupling protein 1 (UCP1) was also shown to be activated by the p38α isoform of MAPK through a PKA-dependent manner (Asherson and Cervera, 1992).

Another downstream effector of PKA that is demonstrated here – NF-κB, is not only involved in UB branching, but has also been implicated in renal injuries or as a response to inflammatory stimuli. TNF superfamily cytokines and angiotensin II have been documented to be involved in inflammatory responses to renal diseases through NF-κB activation (Esteban et al., 2004; Sanz et al., 2008), in addition to many inflammatory response genes whose expression levels are also controlled by NF-κB (Sanz et al., 2010). Moreover, NF-κB activation is involved in renal diseases such as diabetic nephropathy, glomerular disease, and acute kidney injury through its activation in macrophages, glomerular and tubular parenchymal cells (Loverre et al., 2004; Mezzano et al., 2003; Mezzano et al., 2001; Schmid et al., 2006; Zheng et al., 2006). More importantly, Zhong et al. have proven with extensive data that PKA was able to enhance NF-κB-dependent transcription by regulating the association of NF-κB p65 with CREB-binding protein (CBP) (Zhong et al., 1998).

Aside from the validation of p38 MAPK and NF-κB activation to be PKA dependent, the relationship between p38 MAPK and NF-κB was not inspected, although the network analysis suggests that NF-κB activation is in a p38 MAPK

dependent manner. There has also been evidence that activated p38 MAPK could activate NF-κB to regulate the expression of various cytokines, chemokines, and adhesion molecules (Goebeler et al., 2001; Tamura et al., 1998), putting forward the validity of the interaction hypothesized by IPA.

The last connection made in this paper was from TGF-β1 to PKA. TGF-β family members are pleiotropic factors that are implicated in various biological processes, such as embryonic development, tumorigenesis, inflammation, and fibrosis (Akhurst, 2004; Derynck et al., 2001). They are able to induce many biological responses such as EMT, growth inhibition, and apoptosis (Spagnoli et al., 2000; Valdes et al., 2002). During the developmental process of the kidney, TGF-β superfamily, including TGF-βs, BMPs, and Activin A, inhibit the outgrowth of the UB from the UB or the branching morphogenesis in the stalk region of the UB either by secretion from the MM surrounding the UB or by the appropriate spatiotemporal expression of its receptors (Bush et al., 2004; Maeshima et al., 2006; Michos, 2009). The expression of inhibitory signals such as TGF-βs coordinates with stimulatory signals such as GDNF, HGF, FGFs to guide the correct outgrowth and branching of the UB. However, as a well-known inhibitor of UB branching, the mechanism through which TGF-β1 regulates branching morphogenesis has been unclear. Through Western blot analysis, it was shown that TGF-β1 led to the phosphorylation of CREB and ATF-1, downstream effectors of PKA. Furthermore, the inhibition of PKA was able to reverse the effect of TGF-β1, proving the interaction between the two. Such a finding is also supported by the evidence that TGF-β1 has been reported to activate PKA in a variety of cell types (Giannouli and Kletsas, 2006; Wang et al.,

1998; Zhang et al., 2004). Given that p38 MAPK and NF-κB are downstream of PKA, and TGF-β1 is upstream of PKA, it was also shown that p38 MAPK and NF-κB inhibition were able to partially rescue the branching phenotype. Therefore, it is likely that the TGF-β superfamily inhibit the branching process in the UB by stimulating the PKA signaling pathway that is mediated by p38 MAPK and NF-κB.

Chapter 5 Conclusion and future work

This research presents an approach combining biological network analysis and compound screen to identify potential interactions among molecules and pathways that are involved in the branching morphogenesis of the UB. Here, PKA was shown to inhibit UB branching morphogenesis in three model systems including the UB cell, isolated UB, and WEK culture. Utilizing a medium-throughput screen with a compound library containing inhibitors of a wide range of kinases, p38 MAPK and NF-κB were discovered to be able to reverse the inhibitory effect mediated by PKA and could have potential interactions with PKA. Biological network analysis using a literature based branching network identified p38 MAPK and NF-κB as potential downstream effectors of PKA, and TGF-β1 as an upstream activator of PKA. Further experiments verified this finding as p38 MAPK and NF-κB inhibitors rescued branching morphogenesis in UB cells and WEK that are stimulated with dbcAMP, and were identified to be downstream of PKA. Treatment with TGF- β1 significantly inhibited the branching morphogenesis of UB cells, but the inhibitory effect was abolished with the additional treatment of PKA, p38 MAPK, or NF-κB inhibitors. PKA was also demonstrated to be activated by TGF-β1, thus supporting a pathway of TGF- β1 – PKA – p38 MAPK – NF-κB.

Future work should include the full validation of the pathway, in which PKA, p38 MAPK, NF-κB is shown to be activated in a step-wise fashion upon the induction of TGF-β1. Tissue specific knockout of PKA in kidney could be generated to examine the specific phenotype under physiological conditions. Microarray of

tissue with over-activation of PKA could be examined to pull out more potential interactions and construct a global network centered around PKA.

The approach presented here is an efficient method for the identification of the interplay of molecules in the branching morphogenesis of the UB, an area of kidney development research with many outstanding questions regarding molecular controls and mechanisms. With a screen targeting molecules other than PKA or employing a different compound library, the method utilized here could efficiently identify other interactions and pathways involved in UB branching morphogenesis. Such an approach enables the discovery of novel interactions under specific contexts without having to resort to drawing lessons from research results in parallel fields. Moreover, this approach should not be limited to studying only the branching mechanism of the UB or epithelial branching processes. Rather, it could be useful when applied to research on other developmental processes, studies in cancer mechanisms and stem cell differentiation.

Appendix

Table 2. Compound library for compound screening

EMD Biosciences Catalog Number	Primary Target	ID	Plate ID
528114	PI 3-Kγ Inhibitor VII	23049	L11
328009	ERK Inhibitor III	49037	E22
371963	PKA	50128	M18
559388	p38β	50161	M22
220285	PKC	50229	C07
217696	Cdk1/cyclin B	50279	C14
428205	Lck64-509 Y 394	50435	G15
420119	JNK 1, JNK 2, JNK 3	50458	I16
402085	Gsk-3β	50601	C06
475880	Mlck	50621	H19
422000	Gsk-3β	50627	K10
440202	specific phosphatidylinositol 3-kinase inhibitor	50688	G17
371958	MLCK	50799	F07
420121	JAK-3	50820	G11
219479	mClk4, mClk1, mClk2, mClk3	50869	E10
572635	Src	50952	I04
400090	CK1d	50990	I10
513000	MAP kinase	51000	M08
557360	p34cdk1/cyclin B	51071	N13
676487	VEGF-R (KDR/Flk-1), PDGF-R tyrosine kinases	51079	O13
658552	epidermal growth factor receptor kinase	51095	O03
658440	epidermal growth factor receptor tyrosine kinase	51107	M17
658390	EGFR kinase	51117	M13
658401	epidermal growth factor receptor kinase autophosphorylation	51133	M15
124012	Akt1,Akt 2, Akt3	120450	A09
121790	PDGFR	477648	A05

124011	Akt	477649	A07
124018	Akt1, Akt2, Akt3	477650	A11
124020	Akt	477651	A13
189404	Aurora Kinase Inhibitor II	477652	A17
189405	Aurora Kinase Inhibitor III	477653	A22, O19
197221	Bcr-abl	477654	A19
203290	PKC	477655	A21
203297	PKC	477656	C03
203696	EGFR	477657	C05
234505	EGFR	477658	C09
260961	DNA-PK	477659	C11
260962	DNA-PK	477660	C13
266788	Diacylglycerol Kinase Inhibitor II	477661	C17
260964	DNA-PK	477662	O17
317200	B-PDGFR	477663	C19
324673	EGFR	477664	C21
324674	EGFR	477665	E03
324840	EGFR, ErbB-2, ErbB-4	477666	E05
343020	Flt-3	477667	E07
343021	Flt-3 Inhibitor II	477668	E09
343022	Flt-3 Inhibitor III	477669	E21
344036	CFMS	477670	E11
365250	PKC	477671	E13
365251	PKCα, PKCβ	477672	E15
371806	class III receptor tyrosine kinase	477673	E17
375670	P60v-src	477674	E19
407248	IGF-1R Inhibitor II	477675	G03
407601	Interleukin-1 Receptor	477676	G05
420099	JAK1	477677	G07
420104	JAK-3	477678	G09
420126	JAK-3	477679	G13
440203	specific phosphatidylinositol 3-kinase inhibitor	477680	G19

448101	Met Kinase	477681	G21
513035	EGFR	477682	I03
513040	EGFR tyrosine kinase	477683	I05
521231	PDGFR	477684	I07
521232	α-PDGFR	477685	I09
521233	PDGFR Tyrosine Kinase Inhibitor IV	477686	I11
521234	PDGFR Tyrosine Kinase Inhibitor V	477687	I13
521275	PDK1/Akt/Flt Dual Pathway Inhibitor	477688	A15
527450	PKR	477689	I15
527455	PKR	477690	I17
528100	DNA-PK, PI3-K, mTOR	477691	C15
528106	PI 3-Kg	477692	I19
528108	PI 3-Kg	477693	I21
529574	EGFR kinase	477694	K03
529581	T339G	477695	K05
539648	Staurosporine	477696	K17
539652	EGFR	477697	K07
539654	PKCβII, βI	477698	K09
553210	p70 S6 kinase	477699	K11
555553	ROCK	477700	K13
555554	ROCK II	477701	K15
567805	Src	477702	K19
569397	PKA	477703	O16, O21
572660	PDGFRB	477704	K21
574711	Syk	477705	M03
574712	Syk	477706	M05
574713	Syk Inhibitor III	477707	M07
616451	TGF-β receptor I kinase	477708	M09
616453	TGF-β RI Kinase Inhibitor III	477709	M11
658550	PDGFR kinase	477710	M19
658551	PDGFR kinase	477711	M21
676480	(RTK) inhibitor	477712	O05

676481	KDR, Flt-1, c-Kit	477713	O07
676482	VEGFR Tyrosine Kinase Inhibitor III	477714	O09
676485	VEGF-R2	477715	O11
676489	VEGFR-2 KDR/Flk-1	477716	O15
121767	IGF-1	477717	A03
688000	p160ROCK	477718	G04
118501	ATM, ATR Kinase Inhibitor	477719	A08
126870	Cdk1/cyclin B, Gsk-3β	477720	A10
126871	Cdk1/cyclin B, Gsk-3β	477721	A12
128125	Cdk1/cyclin B, Cdk2/cyclinA, Cdk2/cyclin E, Cdk5/p25	477722	A14
128135	Cdkl1, Cdkl5, GSK-3	477723	A16
164640	Cdk1/cyclin B	477724	A18
171260	AMPK	477725	A20
189406	Aurora Kinase, Cdk Inhibitor	477726	B04
196870	TNF-α-inducible phosphorylation of IκBα	477727	C08
203600	CDK	477728	C10
217695	Cdk1	477729	C12
217714	Cdk1/cyclin B, Cdk2/cyclin A	477730	C16
217720	Cdk1/cyclin B, Cdk5/p25	477731	C18
218696	CK1, CK1d	477732	C20
218710	Casein Kinase II Inhibitor III	477733	C22
219476	CDK4/D1	477734	E04
219477	CDK4/D1	477735	E06
219478	Cdk4 Inhibitor III	477736	E08
219491	Cdk, Crk Inhibitor	477737	E20
220486	Chk2	477738	E12
234503	Cdc28p	477739	E14
238803	Cdk2/A, Cdk2/E	477740	E16
238804	Cdk1/cyclin B	477741	E18
328007	ERK Inhibitor II	477742	G06
328008	ERK Inhibitor II	477743	G08
341251	CDK4/D1	477744	G10

361540	Gsk-3β	477745	G12
361541	Gsk-3β	477746	G14
361549	Gsk-3β	477747	G16
361550	GSK-3α/β	477748	G18
361551	GSK-3α/β	477749	G20
361553	Gsk-3β	477750	G22
361554	Gsk-3β	477751	M16
361555	GSK-3	477752	I06
371957	Chk1	477753	I08
371970	PKA	477754	O06
401481	IKK-2	477755	I12
402081	Src	477756	I14
420123	JNK 1, JNK 2, JNK 3	477757	I18
420129	hJNK 1, hJNK 2, hJNK 3	477758	I20
420135	JNK	477759	K06
420136	JNK	477760	I22
420298	CaM kinase II	477761	K08
422706	cam kinase 2	477762	A04
422708	cam kinase 2	477763	K12
444937	MEK	477764	K14
444938	MEK1	477765	K16
444939	MEK1, MEK2	477766	K18
454861	MNK1	477767	K20
475863	MK2a	477768	K04
481406	NF-κB transcriptional activation	477769	K22
506121	p38 MAP kinase	477770	M04
506126	p38 MAP kinase	477771	M06
513030	p38 MAP kinase	477772	M10
540500	Cdc2/cyclin B	477773	M14
559387	p38β	477774	M20
559389	P38MAPK	477775	O04
559396	P38MAPK	477776	M12
559402	Chk1	477777	O08

565625	p38MAPK	477778	O10
567305	(LPS)-stimulated IL-1, TNF-α production	477779	O12
567731	GST-hsk	477780	O14
570250	CaM-KKα	477781	O18
572650	Cdk2/A	477782	O20
616373	Tpl2 Kinase	477783	O22
118500	ATM Kinase	477784	A06
217707	Cdc7, Cdk9	598016	B21
116890	Adenosine Kinase	619344	B01
124029	Akt Inhibitor	619345	B03
181305	CDK4/D1	619346	B05
191500	GSK-3β	619347	B07
203294	PKC	619348	B09
203303	PKC	619349	B11
203881	CDK/CK1	619350	B13
203882	CDK/CK1	619351	B15
208922	CaMKII	619352	B17
217699	Cdk1	619353	B19
218697	CK2	619354	D01
218699	CK2	619355	D03
218713	CK2	619356	D05
219445	CDK2	619357	D07
219448	CDK2/cyclin E, Cdk5	619358	D09
219457	CDK1, CDK2	619359	D11
220485	Chk2	619360	D13
234501	DNA-PK	619361	D15
238806	Cdk2/E, Cdk9/T1	619362	D17
238811	Cdk9/T1	619363	D19
238900	PKA	619364	D21
324515	eEF-2 Kinase	619365	F01
361556	GSK-3	619366	F03
365252	PKC	619367	F05
371964	PKA	619368	F09

401482	IKK-2	619369	F11
401483	IKK-2	619370	F13
401486	IKK	619371	F15
401487	IKK-2	619372	F17
401488	IKK-3	619373	F19
401489	IKK	619374	F21
401490	IKK-2	619375	H01
402086	Gsk-3β	619376	H03
406170	IP 3-K	619377	H05
407900	ERK2	619378	H07
420320	PKA	619379	H09
422709	cam kinase 2	619380	H11
440206	p110	619381	H13
444965	MEK1/2	619382	H15
475864	MK-2	619383	H17
480065	necroptosis	619384	H21
495621	Cdk1/B	619385	J03
506106	p21-Activated Kinase	619386	J05
506153	p38 MAP Kinase	619387	J07
506157	p38 MAP Kinase	619388	J09
506158	p38 MAP Kinase	619389	J11
506163	p38 MAP Kinase	619390	J13
524611	PIKfyve	619391	J15
526520	PIM1	619392	J17
526522	PIM1 Kinase	619393	J19
526523	PIM1, PIM2 Kinase	619394	J21
526524	PIM1, PIM2 Kinase	619395	L03
528111	PI 3-Kα	619396	L05
528112	PI 3-Kγ, CKII	619397	L07
528113	PI 3-Kβ	619398	L09
528116	PI 3-Kα	619399	L13
528282	plk1	619400	L15
528283	plk1	619401	L17
539644	UCN-01	619402	L19

551590	PIM1	619403	L21
553509	Ras/Rac Transformation	619404	N03
554717	Reversine	619405	N05
555550	ROCK	619406	N07
555551	ROCK	619407	N09
555555	ROCKII	619408	N11
557362	p34cdk1/cyclin B	619409	N15
557520	PKC	619410	N17
559285	RSK	619411	N19
559399	P38K	619412	N21
559404	MAPKp38α & β	619413	P03
565715	Plk1	619414	P05
569615	c-kit	619415	P07
570100	Ste11 MAPKKK	619416	P09
616404	Tpl2 Kinase	619417	P11
655203	eEF2-K	619418	P13
681500	Cdk2	619419	P15
681637	Wee1, Chk1	619420	P17

References

Affolter, M., Bellusci, S., Itoh, N., Shilo, B., Thiery, J.P., and Werb, Z. (2003). Tube or not tube: remodeling epithelial tissues by branching morphogenesis. Dev Cell *4*, 11-18.

Akhurst, R.J. (2004). TGF beta signaling in health and disease. Nat Genet *36*, 790-792.

Allen-Gipson, D.S., Spurzem, K., Kolm, N., Spurzem, J.R., and Wyatt, T.A. (2007). Adenosine promotion of cellular migration in bronchial epithelial cells is mediated by the activation of cyclic adenosine monophosphate-dependent protein kinase A. J Investig Med *55*, 378-385.

Andrew, D.J., and Ewald, A.J. (2010). Morphogenesis of epithelial tubes: Insights into tube formation, elongation, and elaboration. Dev Biol *341*, 34-55.

Asherson, R.A., and Cervera, R. (1992). The antiphospholipid syndrome: a syndrome in evolution. Ann Rheum Dis *51*, 147-150.

Belibi, F.A., Reif, G., Wallace, D.P., Yamaguchi, T., Olsen, L., Li, H., Helmkamp, G.M., Jr., and Grantham, J.J. (2004). Cyclic AMP promotes growth and secretion in human polycystic kidney epithelial cells. Kidney international *66*, 964-973.

Blomqvist, S.R., Vidarsson, H., Fitzgerald, S., Johansson, B.R., Ollerstam, A., Brown, R., Persson, A.E., Bergstrom, G.G., and Enerback, S. (2004). Distal renal tubular acidosis in mice that lack the forkhead transcription factor Foxi1. J Clin Invest *113*, 1560-1570.

Bouchard, M. (2004). Transcriptional control of kidney development. Differentiation *72*, 295-306.

Bouchard, M., Souabni, A., Mandler, M., Neubuser, A., and Busslinger, M. (2002). Nephric lineage specification by Pax2 and Pax8. Genes Dev *16*, 2958-2970.

Boyle, S., and de Caestecker, M. (2006). Role of transcriptional networks in coordinating early events during kidney development. Am J Physiol Renal Physiol *291*, F1-8.

Briassouli, P., Chan, F., Savage, K., Reis-Filho, J.S., and Linardopoulos, S. (2007). Aurora-A regulation of nuclear factor-kappaB signaling by phosphorylation of IkappaBalpha. Cancer Res *67*, 1689-1695.

Bridgewater, D., Cox, B., Cain, J., Lau, A., Athaide, V., Gill, P.S., Kuure, S., Sainio, K., and Rosenblum, N.D. (2008). Canonical WNT/beta-catenin signaling is required for ureteric branching. Dev Biol *317*, 83-94.

Bush, K.T., Sakurai, H., Steer, D.L., Leonard, M.O., Sampogna, R.V., Meyer, T.N., Schwesinger, C., Qiao, J., and Nigam, S.K. (2004). TGF-beta superfamily members modulate growth, branching, shaping, and patterning of the ureteric bud. Dev Biol *266*, 285-298.
Butcher, E.C. (2005). Can cell systems biology rescue drug discovery? Nat Rev Drug Discov *4*, 461-467

Carmeliet, P., and Tessier-Lavigne, M. (2005). Common mechanisms of nerve and blood vessel wiring. Nature *436*, 193-200.

Cebrian, C., Borodo, K., Charles, N., and Herzlinger, D.A. (2004). Morphometric index of the developing murine kidney. Dev Dyn *231*, 601-608.

Challa, V.R., Thore, C.R., Moody, D.M., Anstrom, J.A., and Brown, W.R. (2004). Increase of white matter string vessels in Alzheimer's disease. J Alzheimers Dis *6*, 379-383; discussion 443-379.

Chen, Y., Wang, Y., Yu, H., Wang, F., and Xu, W. (2005). The cross talk between protein kinase A- and RhoA-mediated signaling in cancer cells. Exp Biol Med (Maywood) *230*, 731-741.

Cheng, H.T., Kim, M., Valerius, M.T., Surendran, K., Schuster-Gossler, K., Gossler, A., McMahon, A.P., and Kopan, R. (2007). Notch2, but not Notch1, is required for proximal fate acquisition in the mammalian nephron. Development *134*, 801-811.

Chi, X., Michos, O., Shakya, R., Riccio, P., Enomoto, H., Licht, J.D., Asai, N., Takahashi, M., Ohgami, N., Kato, M., *et al.* (2009). Ret-dependent cell rearrangements in the Wolffian duct epithelium initiate ureteric bud morphogenesis. Dev Cell *17*, 199-209.

Chihara, T., and Hayashi, S. (2000). Control of tracheal tubulogenesis by Wingless signaling. Development *127*, 4433-4442.

Choi, Y., Tee, J.B., Gallegos, T.F., Shah, M.M., Oishi, H., Sakurai, H., Kitamura, S., Wu, W., Bush, K.T., and Nigam, S.K. (2009). Neuropeptide Y functions as a facilitator of GDNF-induced budding of the Wolffian duct. Development *136*, 4213-4224.

Ciardiello, F., and Tortora, G. (1998). Interactions between the epidermal growth factor receptor and type I protein kinase A: biological significance and therapeutic implications. Clin Cancer Res *4*, 821-828.

Costantini, F. (2010). GDNF/Ret signaling and renal branching morphogenesis: From mesenchymal signals to epithelial cell behaviors. Organogenesis *6*, 252-262.

Costantini, F., and Kopan, R. (2010). Patterning a complex organ: branching morphogenesis and nephron segmentation in kidney development. Dev Cell *18*, 698-712.

Costantini, F., and Shakya, R. (2006). GDNF/Ret signaling and the development of the kidney. Bioessays *28*, 117-127.

Cuadrado, A., and Nebreda, A.R. (2010). Mechanisms and functions of p38 MAPK signalling. Biochem J *429*, 403-417.

Daniel, P.B., Walker, W.H., and Habener, J.F. (1998). Cyclic AMP signaling and gene regulation. Annu Rev Nutr *18*, 353-383.

Dankers, P.Y., Boomker, J.M., Meijer, E.W., Popa, E.R., and van Luyn, M.J. (2011). From kidney development to drug delivery and tissue engineering strategies in renal regenerative medicine. J Control Release.

Delghandi, M.P., Johannessen, M., and Moens, U. (2005). The cAMP signalling pathway activates CREB through PKA, p38 and MSK1 in NIH 3T3 cells. Cell Signal *17*, 1343-1351.

Derynck, R., Akhurst, R.J., and Balmain, A. (2001). TGF-beta signaling in tumor suppression and cancer progression. Nat Genet *29*, 117-129.

Ekins, S., Nikolsky, Y., Bugrim, A., Kirillov, E., and Nikolskaya, T. (2007). Pathway mapping tools for analysis of high content data. Methods Mol Biol *356*, 319-350.

Esteban, V., Lorenzo, O., Ruperez, M., Suzuki, Y., Mezzano, S., Blanco, J., Kretzler, M., Sugaya, T., Egido, J., and Ruiz-Ortega, M. (2004). Angiotensin II, via AT1 and AT2 receptors and NF-kappaB pathway, regulates the inflammatory response in unilateral ureteral obstruction. J Am Soc Nephrol *15*, 1514-1529.

Falk, M., Salmivirta, K., Durbeej, M., Larsson, E., Ekblom, M., Vestweber, D., and Ekblom, P. (1996). Integrin alpha 6B beta 1 is involved in kidney tubulogenesis in vitro. J Cell Sci *109 (Pt 12)*, 2801-2810.

Ganter, B., and Giroux, C.N. (2008). Emerging applications of network and pathway analysis in drug discovery and development. Curr Opin Drug Discov Devel *11*, 86-94.

Georgas, K., Rumballe, B., Valerius, M.T., Chiu, H.S., Thiagarajan, R.D., Lesieur, E., Aronow, B.J., Brunskill, E.W., Combes, A.N., Tang, D., *et al.* (2009). Analysis of early nephron patterning reveals a role for distal RV proliferation in fusion to the ureteric tip via a cap mesenchyme-derived connecting segment. Dev Biol *332*, 273-286.

Giacomotto, J., and Segalat, L. (2010). High-throughput screening and small animal models, where are we? Br J Pharmacol *160*, 204-216.

Giannouli, C.C., and Kletsas, D. (2006). TGF-beta regulates differentially the proliferation of fetal and adult human skin fibroblasts via the activation of PKA and the autocrine action of FGF-2. Cell Signal *18*, 1417-1429.

Goebeler, M., Gillitzer, R., Kilian, K., Utzel, K., Brocker, E.B., Rapp, U.R., and Ludwig, S. (2001). Multiple signaling pathways regulate NF-kappaB-dependent transcription of the monocyte chemoattractant protein-1 gene in primary endothelial cells. Blood *97*, 46-55.

Goldstein, D.M., Gray, N.S., and Zarrinkar, P.P. (2008). High-throughput kinase profiling as a platform for drug discovery. Nat Rev Drug Discov *7*, 391-397.

Gong, K.Q., Yallowitz, A.R., Sun, H., Dressler, G.R., and Wellik, D.M. (2007). A Hox-Eya-Pax complex regulates early kidney developmental gene expression. Mol Cell Biol *27*, 7661-7668.

Grote, D., Souabni, A., Busslinger, M., and Bouchard, M. (2006). Pax 2/8-regulated Gata 3 expression is necessary for morphogenesis and guidance of the nephric duct in the developing kidney. Development *133*, 53-61.

Gupta, I.R., Piscione, T.D., Grisaru, S., Phan, T., Macias-Silva, M., Zhou, X., Whiteside, C., Wrana, J.L., and Rosenblum, N.D. (1999). Protein kinase A is a negative regulator of renal branching morphogenesis and modulates inhibitory and stimulatory bone morphogenetic proteins. J Biol Chem *274*, 26305-26314.

Hiraoka, M., Tsukahara, H., Ohshima, Y., Kasuga, K., Ishihara, Y., and Mayumi, M. (2002). Renal aplasia is the predominant cause of congenital solitary kidneys. Kidney international *61*, 1840-1844.

Horowitz, A., and Simons, M. (2008). Branching morphogenesis. Circ Res *103*, 784-795.

Howe, D.G., Wiley, J.C., and McKnight, G.S. (2002). Molecular and behavioral effects of a null mutation in all PKA C beta isoforms. Mol Cell Neurosci *20*, 515-524.

Hoy, W.E., Bertram, J.F., Denton, R.D., Zimanyi, M., Samuel, T., and Hughson, M.D. (2008). Nephron number, glomerular volume, renal disease and hypertension. Curr Opin Nephrol Hypertens *17*, 258-265.

Ichikawa, I., Kuwayama, F., Pope, J.C.t., Stephens, F.D., and Miyazaki, Y. (2002). Paradigm shift from classic anatomic theories to contemporary cell biological views of CAKUT. Kidney international *61*, 889-898.

Iida, N., Namikawa, K., Kiyama, H., Ueno, H., Nakamura, S., and Hattori, S. (2001). Requirement of Ras for the activation of mitogen-activated protein kinase by calcium influx, cAMP, and neurotrophin in hippocampal neurons. J Neurosci *21*, 6459-6466.

Jin, H., Garmy-Susini, B., Avraamides, C.J., Stoletov, K., Klemke, R.L., and Varner, J.A. (2010). A PKA-Csk-pp60Src signaling pathway regulates the switch between endothelial cell invasion and cell-cell adhesion during vascular sprouting. Blood *116*, 5773-5783.

Kainkaryam, R.M., and Woolf, P.J. (2009). Pooling in high-throughput drug screening. Curr Opin Drug Discov Devel *12*, 339-350.

Kerman, B.E., Cheshire, A.M., and Andrew, D.J. (2006). From fate to function: the Drosophila trachea and salivary gland as models for tubulogenesis. Differentiation *74*, 326-348.

Keshet, Y., and Seger, R. (2010). The MAP kinase signaling cascades: a system of hundreds of components regulates a diverse array of physiological functions. Methods Mol Biol *661*, 3-38.

Kim, S.S., Park, H.J., Han, J., Choi, C.Y., and Kim, B.S. (2003). Renal tissue reconstitution by the implantation of renal segments on biodegradable polymer scaffolds. Biotechnol Lett *25*, 1505-1508.

Kirschner, L.S., Yin, Z., Jones, G.N., and Mahoney, E. (2009). Mouse models of altered protein kinase A signaling. Endocr Relat Cancer *16*, 773-793.

Kispert, A., Vainio, S., Shen, L., Rowitch, D.H., and McMahon, A.P. (1996). Proteoglycans are required for maintenance of Wnt-11 expression in the ureter tips. Development *122*, 3627-3637.

Kobayashi, A., Kwan, K.M., Carroll, T.J., McMahon, A.P., Mendelsohn, C.L., and Behringer, R.R. (2005). Distinct and sequential tissue-specific activities of the LIM-class homeobox gene Lim1 for tubular morphogenesis during kidney development. Development *132*, 2809-2823.

Kohyama, T., Ertl, R.F., Valenti, V., Spurzem, J., Kawamoto, M., Nakamura, Y., Veys, T., Allegra, L., Romberger, D., and Rennard, S.I. (2001). Prostaglandin E(2) inhibits fibroblast chemotaxis. Am J Physiol Lung Cell Mol Physiol *281*, L1257-1263.

Kohyama, T., Yamauchi, Y., Takizawa, H., Itakura, S., Kamitani, S., Desaki, M., Kawasaki, S., and Nagase, T. (2009). Procaterol inhibits lung fibroblast migration. Inflammation *32*, 387-392.

Kwoh, C.K., and Ng, P.Y. (2007). Network analysis approach for biology. Cell Mol Life Sci *64*, 1739-1751.

Landro, J.A., Taylor, I.C., Stirtan, W.G., Osterman, D.G., Kristie, J., Hunnicutt, E.J., Rae, P.M., and Sweetnam, P.M. (2000). HTS in the new millennium: the role of pharmacology and flexibility. J Pharmacol Toxicol Methods *44*, 273-289.

Lee, D.C., Chan, K.W., and Chan, S.Y. (1998). Expression of transforming growth factor alpha and epidermal growth factor receptor in adult polycystic kidney disease. J Urol *159*, 291-296.

Lelongt, B., Trugnan, G., Murphy, G., and Ronco, P.M. (1997). Matrix metalloproteinases MMP2 and MMP9 are produced in early stages of kidney morphogenesis but only MMP9 is required for renal organogenesis in vitro. J Cell Biol *136*, 1363-1373.

Levinson, R.S., Batourina, E., Choi, C., Vorontchikhina, M., Kitajewski, J., and Mendelsohn, C.L. (2005). Foxd1-dependent signals control cellularity in the renal capsule, a structure required for normal renal development. Development *132*, 529-539.

Lieschke, G.J., and Currie, P.D. (2007). Animal models of human disease: zebrafish swim into view. Nat Rev Genet *8*, 353-367.

Lindquist, J.M., Fredriksson, J.M., Rehnmark, S., Cannon, B., and Nedergaard, J. (2000). Beta 3- and alpha1-adrenergic Erk1/2 activation is Src- but not Gi-mediated in Brown adipocytes. J Biol Chem *275*, 22670-22677.

Little, M., Georgas, K., Pennisi, D., and Wilkinson, L. (2010). Kidney development: two tales of tubulogenesis. Curr Top Dev Biol *90*, 193-229.

Loverre, A., Ditonno, P., Crovace, A., Gesualdo, L., Ranieri, E., Pontrelli, P., Stallone, G., Infante, B., Schena, A., Di Paolo, S., *et al.* (2004). Ischemia-reperfusion induces glomerular and tubular activation of proinflammatory and antiapoptotic pathways: differential modulation by rapamycin. J Am Soc Nephrol *15*, 2675-2686.

Lu, B.C., Cebrian, C., Chi, X., Kuure, S., Kuo, R., Bates, C.M., Arber, S., Hassell, J., MacNeil, L., Hoshi, M., *et al.* (2009). Etv4 and Etv5 are required downstream of GDNF and Ret for kidney branching morphogenesis. Nat Genet *41*, 1295-1302.

Lu, P., and Werb, Z. (2008). Patterning mechanisms of branched organs. Science *322*, 1506-1509.

Maeshima, A., Sakurai, H., Choi, Y., Kitamura, S., Vaughn, D.A., Tee, J.B., and Nigam, S.K. (2007). Glial cell-derived neurotrophic factor independent ureteric bud outgrowth from the Wolffian duct. J Am Soc Nephrol *18*, 3147-3155.

Maeshima, A., Vaughn, D.A., Choi, Y., and Nigam, S.K. (2006). Activin A is an endogenous inhibitor of ureteric bud outgrowth from the Wolffian duct. Dev Biol *295*, 473-485.

Magenheimer, B.S., St John, P.L., Isom, K.S., Abrahamson, D.R., De Lisle, R.C., Wallace, D.P., Maser, R.L., Grantham, J.J., and Calvet, J.P. (2006). Early embryonic renal tubules of wild-type and polycystic kidney disease kidneys respond to cAMP stimulation with cystic fibrosis transmembrane conductance regulator/Na(+),K(+),2Cl(-) Co-transporter-dependent cystic dilation. J Am Soc Nephrol *17*, 3424-3437.

McAlees, J.W., and Sanders, V.M. (2009). Hematopoietic protein tyrosine phosphatase mediates beta2-adrenergic receptor-induced regulation of p38 mitogen-activated protein kinase in B lymphocytes. Mol Cell Biol *29*, 675-686.

Mendelsohn, C. (2009). Using mouse models to understand normal and abnormal urogenital tract development. Organogenesis *5*, 306-314.

Mezzano, S., Droguett, A., Burgos, M.E., Ardiles, L.G., Flores, C.A., Aros, C.A., Caorsi, I., Vio, C.P., Ruiz-Ortega, M., and Egido, J. (2003). Renin-angiotensin system activation and interstitial inflammation in human diabetic nephropathy. Kidney Int Suppl, S64-70.

Mezzano, S.A., Barria, M., Droguett, M.A., Burgos, M.E., Ardiles, L.G., Flores, C., and Egido, J. (2001). Tubular NF-kappaB and AP-1 activation in human proteinuric renal disease. Kidney international *60*, 1366-1377.

Michael, L., and Davies, J.A. (2004). Pattern and regulation of cell proliferation during murine ureteric bud development. J Anat *204*, 241-255.

Michos, O. (2009). Kidney development: from ureteric bud formation to branching morphogenesis. Curr Opin Genet Dev *19*, 484-490.

Michos, O., Goncalves, A., Lopez-Rios, J., Tiecke, E., Naillat, F., Beier, K., Galli, A., Vainio, S., and Zeller, R. (2007). Reduction of BMP4 activity by gremlin 1 enables ureteric bud

outgrowth and GDNF/WNT11 feedback signalling during kidney branching morphogenesis. Development *134*, 2397-2405.

Moore, M.W., Klein, R.D., Farinas, I., Sauer, H., Armanini, M., Phillips, H., Reichardt, L.F., Ryan, A.M., Carver-Moore, K., and Rosenthal, A. (1996). Renal and neuronal abnormalities in mice lacking GDNF. Nature *382*, 76-79.

Morizane, R., Monkawa, T., and Itoh, H. (2009). Differentiation of murine embryonic stem and induced pluripotent stem cells to renal lineage in vitro. Biochem Biophys Res Commun *390*, 1334-1339.

Mugford, J.W., Sipila, P., McMahon, J.A., and McMahon, A.P. (2008). Osr1 expression demarcates a multi-potent population of intermediate mesoderm that undergoes progressive restriction to an Osr1-dependent nephron progenitor compartment within the mammalian kidney. Dev Biol *324*, 88-98.

Mugford, J.W., Yu, J., Kobayashi, A., and McMahon, A.P. (2009). High-resolution gene expression analysis of the developing mouse kidney defines novel cellular compartments within the nephron progenitor population. Dev Biol *333*, 312-323.

Muller, U., Wang, D., Denda, S., Meneses, J.J., Pedersen, R.A., and Reichardt, L.F. (1997). Integrin alpha8beta1 is critically important for epithelial-mesenchymal interactions during kidney morphogenesis. Cell *88*, 603-613.

Nadella, K.S., Jones, G.N., Trimboli, A., Stratakis, C.A., Leone, G., and Kirschner, L.S. (2008). Targeted deletion of Prkar1a reveals a role for protein kinase A in mesenchymal-to-epithelial transition. Cancer Res *68*, 2671-2677.

Nakai, S., Sugitani, Y., Sato, H., Ito, S., Miura, Y., Ogawa, M., Nishi, M., Jishage, K., Minowa, O., and Noda, T. (2003). Crucial roles of Brn1 in distal tubule formation and function in mouse kidney. Development *130*, 4751-4759.

Nakayama, K.H., Batchelder, C.A., Lee, C.I., and Tarantal, A.F. (2010). Decellularized rhesus monkey kidney as a three-dimensional scaffold for renal tissue engineering. Tissue Eng Part A *16*, 2207-2216.

Nigam, S.K., and Shah, M.M. (2009). How does the ureteric bud branch? J Am Soc Nephrol *20*, 1465-1469.

Nika, K., Hyunh, H., Williams, S., Paul, S., Bottini, N., Tasken, K., Lombroso, P.J., and Mustelin, T. (2004). Haematopoietic protein tyrosine phosphatase (HePTP) phosphorylation by cAMP-dependent protein kinase in T-cells: dynamics and subcellular location. Biochem J *378*, 335-342.

Ota, K., Stetler-Stevenson, W.G., Yang, Q., Kumar, A., Wada, J., Kashihara, N., Wallner, E.I., and Kanwar, Y.S. (1998). Cloning of murine membrane-type-1-matrix metalloproteinase (MT-1-MMP) and its metanephric developmental regulation with respect to MMP-2 and its inhibitor. Kidney international *54*, 131-142.

Paulucci-Holthauzen, A.A., Vergara, L.A., Bellot, L.J., Canton, D., Scott, J.D., and O'Connor, K.L. (2009). Spatial distribution of protein kinase A activity during cell migration is mediated by A-kinase anchoring protein AKAP Lbc. J Biol Chem *284*, 5956-5967.

Perin, L., Da Sacco, S., and De Filippo, R.E. (2010). Regenerative medicine of the kidney. Adv Drug Deliv Rev.

Pichel, J.G., Shen, L., Sheng, H.Z., Granholm, A.C., Drago, J., Grinberg, A., Lee, E.J., Huang, S.P., Saarma, M., Hoffer, B.J., *et al.* (1996). Defects in enteric innervation and kidney development in mice lacking GDNF. Nature *382*, 73-76.

Pierrat, B., Correia, J.S., Mary, J.L., Tomas-Zuber, M., and Lesslauer, W. (1998). RSK-B, a novel ribosomal S6 kinase family member, is a CREB kinase under dominant control of p38alpha mitogen-activated protein kinase (p38alphaMAPK). J Biol Chem *273*, 29661-29671.

Pinedo, H.M., and Duursma, S.A. (1975). Renovascular hypertension in renal tuberculosis. Neth J Med *18*, 184-190.

Pohl, M., Bhatnagar, V., Mendoza, S.A., and Nigam, S.K. (2002). Toward an etiological classification of developmental disorders of the kidney and upper urinary tract. Kidney international *61*, 10-19.

Pohl, M., Sakurai, H., Bush, K.T., and Nigam, S.K. (2000a). Matrix metalloproteinases and their inhibitors regulate in vitro ureteric bud branching morphogenesis. Am J Physiol Renal Physiol *279*, F891-900.

Pohl, M., Stuart, R.O., Sakurai, H., and Nigam, S.K. (2000b). Branching morphogenesis during kidney development. Annu Rev Physiol *62*, 595-620.

Poladia, D.P., Kish, K., Kutay, B., Bauer, J., Baum, M., and Bates, C.M. (2006). Link between reduced nephron number and hypertension: studies in a mutant mouse model. Pediatr Res *59*, 489-493.

Pollack, A.L., Runyan, R.B., and Mostov, K.E. (1998). Morphogenetic mechanisms of epithelial tubulogenesis: MDCK cell polarity is transiently rearranged without loss of cell-cell contact during scatter factor/hepatocyte growth factor-induced tubulogenesis. Dev Biol *204*, 64-79.

Qi, Y.X., Jiang, J., Jiang, X.H., Wang, X.D., Ji, S.Y., Han, Y., Long, D.K., Shen, B.R., Yan, Z.Q., Chien, S., *et al.* (2011). PDGF-BB and TGF-{beta}1 on cross-talk between endothelial and smooth muscle cells in vascular remodeling induced by low shear stress. Proc Natl Acad Sci U S A *108*, 1908-1913.

Qiao, J., Sakurai, H., and Nigam, S.K. (1999). Branching morphogenesis independent of mesenchymal-epithelial contact in the developing kidney. Proc Natl Acad Sci U S A *96*, 7330-7335.

Romio, L., Wright, V., Price, K., Winyard, P.J., Donnai, D., Porteous, M.E., Franco, B., Giorgio, G., Malcolm, S., Woolf, A.S., *et al.* (2003). OFD1, the gene mutated in oral-facial-

digital syndrome type 1, is expressed in the metanephros and in human embryonic renal mesenchymal cells. J Am Soc Nephrol *14*, 680-689.

Rosines, E., Johkura, K., Zhang, X., Schmidt, H.J., Decambre, M., Bush, K.T., and Nigam, S.K. (2010). Constructing kidney-like tissues from cells based on programs for organ development: toward a method of in vitro tissue engineering of the kidney. Tissue Eng Part A *16*, 2441-2455.

Sakai, D., Suzuki, T., Osumi, N., and Wakamatsu, Y. (2006). Cooperative action of Sox9, Snail2 and PKA signaling in early neural crest development. Development *133*, 1323-1333.

Sakurai, H., Barros, E.J., Tsukamoto, T., Barasch, J., and Nigam, S.K. (1997a). An in vitro tubulogenesis system using cell lines derived from the embryonic kidney shows dependence on multiple soluble growth factors. Proc Natl Acad Sci U S A *94*, 6279-6284.

Sakurai, H., Bush, K.T., and Nigam, S.K. (2005). Heregulin induces glial cell line-derived neurotrophic growth factor-independent, non-branching growth and differentiation of ureteric bud epithelia. J Biol Chem *280*, 42181-42187.

Sakurai, H., and Nigam, S.K. (1997). Transforming growth factor-beta selectively inhibits branching morphogenesis but not tubulogenesis. Am J Physiol *272*, F139-146.

Sakurai, H., Tsukamoto, T., Kjelsberg, C.A., Cantley, L.G., and Nigam, S.K. (1997b). EGF receptor ligands are a large fraction of in vitro branching morphogens secreted by embryonic kidney. Am J Physiol *273*, F463-472.

Sampogna, R.V., and Nigam, S.K. (2004). Implications of gene networks for understanding resilience and vulnerability in the kidney branching program. Physiology (Bethesda) *19*, 339-347.

Santos, O.F., Moura, L.A., Rosen, E.M., and Nigam, S.K. (1993). Modulation of HGF-induced tubulogenesis and branching by multiple phosphorylation mechanisms. Dev Biol *159*, 535-548.

Sanz, A.B., Justo, P., Sanchez-Nino, M.D., Blanco-Colio, L.M., Winkles, J.A., Kreztler, M., Jakubowski, A., Blanco, J., Egido, J., Ruiz-Ortega, M., *et al.* (2008). The cytokine TWEAK modulates renal tubulointerstitial inflammation. J Am Soc Nephrol *19*, 695-703.

Sanz, A.B., Sanchez-Nino, M.D., Ramos, A.M., Moreno, J.A., Santamaria, B., Ruiz-Ortega, M., Egido, J., and Ortiz, A. (2010). NF-kappaB in renal inflammation. J Am Soc Nephrol *21*, 1254-1262.

Schmid, H., Boucherot, A., Yasuda, Y., Henger, A., Brunner, B., Eichinger, F., Nitsche, A., Kiss, E., Bleich, M., Grone, H.J., *et al.* (2006). Modular activation of nuclear factor-kappaB transcriptional programs in human diabetic nephropathy. Diabetes *55*, 2993-3003.

Schmidt-Ott, K.M., Yang, J., Chen, X., Wang, H., Paragas, N., Mori, K., Li, J.Y., Lu, B., Costantini, F., Schiffer, M., *et al.* (2005). Novel regulators of kidney development from the tips of the ureteric bud. J Am Soc Nephrol *16*, 1993-2002.

Segalat, L. (2006). Drug discovery: here comes the worm. ACS Chem Biol *1*, 277-278.
Shah, M.M., Sampogna, R.V., Sakurai, H., Bush, K.T., and Nigam, S.K. (2004). Branching morphogenesis and kidney disease. Development *131*, 1449-1462.

Shakya, R., Jho, E.H., Kotka, P., Wu, Z., Kholodilov, N., Burke, R., D'Agati, V., and Costantini, F. (2005). The role of GDNF in patterning the excretory system. Dev Biol *283*, 70-84.

Simian, M., Hirai, Y., Navre, M., Werb, Z., Lochter, A., and Bissell, M.J. (2001). The interplay of matrix metalloproteinases, morphogens and growth factors is necessary for branching of mammary epithelial cells. Development *128*, 3117-3131.

Singaravelu, K., and Padanilam, B.J. (2009). In vitro differentiation of MSC into cells with a renal tubular epithelial-like phenotype. Ren Fail *31*, 492-502.

Skalhegg, B.S., Huang, Y., Su, T., Idzerda, R.L., McKnight, G.S., and Burton, K.A. (2002). Mutation of the Calpha subunit of PKA leads to growth retardation and sperm dysfunction. Mol Endocrinol *16*, 630-639.

Snowden, M., and Green, D.V. (2008). The impact of diversity-based, high-throughput screening on drug discovery: "chance favours the prepared mind". Curr Opin Drug Discov Devel *11*, 553-558.

Soh, U.J., Dores, M.R., Chen, B., and Trejo, J. (2010). Signal transduction by protease-activated receptors. Br J Pharmacol *160*, 191-203.

Spagnoli, F.M., Cicchini, C., Tripodi, M., and Weiss, M.C. (2000). Inhibition of MMH (Met murine hepatocyte) cell differentiation by TGF(beta) is abrogated by pre-treatment with the heritable differentiation effector FGF1. J Cell Sci *113 (Pt 20)*, 3639-3647.

Spurzem, J.R., Gupta, J., Veys, T., Kneifl, K.R., Rennard, S.I., and Wyatt, T.A. (2002). Activation of protein kinase A accelerates bovine bronchial epithelial cell migration. Am J Physiol Lung Cell Mol Physiol *282*, L1108-1116.

Stork, P.J., and Schmitt, J.M. (2002). Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation. Trends Cell Biol *12*, 258-266.

Sun, C., Chan, F., Briassouli, P., and Linardopoulos, S. (2007). Aurora kinase inhibition downregulates NF-kappaB and sensitises tumour cells to chemotherapeutic agents. Biochem Biophys Res Commun *352*, 220-225.

Sutherland, D., Samakovlis, C., and Krasnow, M.A. (1996). branchless encodes a Drosophila FGF homolog that controls tracheal cell migration and the pattern of branching. Cell *87*, 1091-1101.

Takahashi, T., Sun, W.H., Duan, Z., and Shen, B. (2000). Preparation of benzocycloheptene derivatives from zirconacyclopentadienes. Org Lett *2*, 1197-1199.

Takayama, H., LaRochelle, W.J., Sabnis, S.G., Otsuka, T., and Merlino, G. (1997). Renal tubular hyperplasia, polycystic disease, and glomerulosclerosis in transgenic mice overexpressing hepatocyte growth factor/scatter factor. Lab Invest *77*, 131-138.

Tamura, D.Y., Moore, E.E., Johnson, J.L., Zallen, G., Aiboshi, J., and Silliman, C.C. (1998). p38 mitogen-activated protein kinase inhibition attenuates intercellular adhesion molecule-1 up-regulation on human pulmonary microvascular endothelial cells. Surgery *124*, 403-407; discussion 408.

Tang, M.J., Cai, Y., Tsai, S.J., Wang, Y.K., and Dressler, G.R. (2002). Ureteric bud outgrowth in response to RET activation is mediated by phosphatidylinositol 3-kinase. Dev Biol *243*, 128-136.

Tasken, K., and Aandahl, E.M. (2004). Localized effects of cAMP mediated by distinct routes of protein kinase A. Physiol Rev *84*, 137-167.

Tee, J.B., Choi, Y., Shah, M.M., Dnyanmote, A., Sweeney, D.E., Gallegos, T.F., Johkura, K., Ito, C., Bush, K.T., and Nigam, S.K. (2010). Protein kinase A regulates GDNF/RET-dependent but not GDNF/Ret-independent ureteric bud outgrowth from the Wolffian duct. Dev Biol *347*, 337-347.

Torres, M., Gomez-Pardo, E., Dressler, G.R., and Gruss, P. (1995). Pax-2 controls multiple steps of urogenital development. Development *121*, 4057-4065.

Valdes, F., Alvarez, A.M., Locascio, A., Vega, S., Herrera, B., Fernandez, M., Benito, M., Nieto, M.A., and Fabregat, I. (2002). The epithelial mesenchymal transition confers resistance to the apoptotic effects of transforming growth factor Beta in fetal rat hepatocytes. Mol Cancer Res *1*, 68-78.

Wang, L., Zhu, Y., and Sharma, K. (1998). Transforming growth factor-beta1 stimulates protein kinase A in mesangial cells. J Biol Chem *273*, 8522-8527.

Wang, Y., Xiang, G.S., Kourouma, F., and Umar, S. (2006). Citrobacter rodentium-induced NF-kappaB activation in hyperproliferating colonic epithelia: role of p65 (Ser536) phosphorylation. Br J Pharmacol *148*, 814-824.

Wyatt, T.A., Sisson, J.H., Forget, M.A., Bennett, R.G., Hamel, F.G., and Spurzem, J.R. (2002). Relaxin stimulates bronchial epithelial cell PKA activation, migration, and ciliary beating. Exp Biol Med (Maywood) *227*, 1047-1053.

Yang, Y., Pan, X., Lei, W., Wang, J., Shi, J., Li, F., and Song, J. (2006). Regulation of transforming growth factor-beta 1-induced apoptosis and epithelial-to-mesenchymal transition by protein kinase A and signal transducers and activators of transcription 3. Cancer Res *66*, 8617-8624.

Zent, R., Bush, K.T., Pohl, M.L., Quaranta, V., Koshikawa, N., Wang, Z., Kreidberg, J.A., Sakurai, H., Stuart, R.O., and Nigam, S.K. (2001). Involvement of laminin binding integrins and laminin-5 in branching morphogenesis of the ureteric bud during kidney development. Dev Biol *238*, 289-302.

Zhang, A., Wang, M.H., Dong, Z., and Yang, T. (2006). Prostaglandin E2 is a potent inhibitor of epithelial-to-mesenchymal transition: interaction with hepatocyte growth factor. Am J Physiol Renal Physiol *291*, F1323-1331.

Zhang, L., Duan, C.J., Binkley, C., Li, G., Uhler, M.D., Logsdon, C.D., and Simeone, D.M. (2004). A transforming growth factor beta-induced Smad3/Smad4 complex directly activates protein kinase A. Mol Cell Biol *24*, 2169-2180.

Zhang, X., Mernaugh, G., Yang, D.H., Gewin, L., Srichai, M.B., Harris, R.C., Iturregui, J.M., Nelson, R.D., Kohan, D.E., Abrahamson, D., *et al.* (2009). beta1 integrin is necessary for ureteric bud branching morphogenesis and maintenance of collecting duct structural integrity. Development *136*, 3357-3366.

Zheng, L., Sinniah, R., and Hsu, S.I. (2006). Renal cell apoptosis and proliferation may be linked to nuclear factor-kappaB activation and expression of inducible nitric oxide synthase in patients with lupus nephritis. Hum Pathol *37*, 637-647.

Zhong, H., Voll, R.E., and Ghosh, S. (1998). Phosphorylation of NF-kappa B p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300. Mol Cell *1*, 661-671.