Pathways that Regulate Renal Development, Fibrosis, and Metabolic Disease in Mouse Models

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Personal Statement
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Declaration

I, Abdulsalam Soofi, declare that the work presented in this thesis submitted to the University of Warwick in support of my application for the Doctor of Philosophy. This document has been prepared and edited by myself and has not been previously submitted for application to obtain any degree.

The document presents a comprehensive list of sources of background, published articles and biography that shall constitute original contribution to knowledge in the field. The projects, experimental design, experimental executions, data collections, and data analysis included in the published work were carried out by the author as the first author and signed by the corresponding author Dr. Dressler.

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This work was performed under the supervision of Professor G. R. Dressler his Lab, at the Department of Pathology, Medical School, University of Michigan. This work was supported by NIH grants DK054740 and DK062914 to Dr. Dressler.

Abdul Soofi developed the concepts and experimental approaches, performed the experiments and data analysis, prepared the Figures, and participated in the drafting and editing of the manuscript.

Inna Levitan preformed the whole mount for in situ hybridization shown in Figure 3.

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- Signed Professor G. R. Dressler .................. Date 10-2-17

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Abdul Soofı preformed the conception and design of the project, preformed the experiments, collection and analysis of data, prepared the Figures and participated in the drafting and editing of the manuscript.

Katherine I Wolf assisted with experiments and data analysis,
Egon Ranghini and Mohammad Amin assisted with data analysis.
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Abdul Soffi designed and coordinated the study and performed the experiments, collected and analyzed the data, prepared the Figures and participated in the drafting and editing of the manuscript.

Katherine Wolf assisted with experiments and data analyzes for Figs. 1, 3 and 5;
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ABSTRACT

The kidney is an essential organ that maintains homeostasis, maintains water and mineral balance, and removes metabolic waste products from the body. In mammals, the kidney derives from the intermediate mesoderm (IM) and develops through a multistep process where undifferentiated mesenchyme is converted into a highly complex organ. Several transcriptional regulators, including the Pax2 gene, have been identified in the specification and maintenance of this multistep process. The Pax2 gene marks the IM shortly after gastrulation, when the mesoderm becomes compartmentalized into paraxial, intermediate, and lateral plate. Pax2 expression in the IM distinguishes all of the cells fated to become epithelia in the urogenital tract and is necessary to establish and maintain this phenotype. Pax2 null mutants do develop a nephric duct (Brophy et al., 2001; Soofi et al., 2012), but the duct is completely absent in a Pax2/8 double mutant, suggesting that these Pax genes function redundantly in this early IM domain; however, in Pax2 homozygous mutant mice, the metanephric mesenchyme neither responds to inductive signals nor does the mutant mesenchyme aggregate into early renal vesicles resulting in a lack of kidneys, ureters, and genital tract. We describe two new alleles of Pax2 created by inserting the Enhanced Green Fluorescent Protein coding region into the 5' untranslated leader sequence. One allele is a hypomorph that generates less protein and exhibits structural defects in kidneys and ureters upon homozygosity. A second allele is a true null that can be used to image Pax2 expressing cells in a mutant background. Organ culture and embryo analyses point to a loss of epithelial cell polarity and increased mobility in cells that have deleted Pax2 function. These experiments provide new insight into the role of Pax2 protein levels in determining correct renal architecture and cell fate.

The prevalence of chronic kidney disease (CKD) worldwide is reflected by the increasing number of people with end stage renal disease (ESRD) requiring some form of renal replacement therapy. The overall incidence of ESRD is increasing at an alarming rate and is correlated with the rise of diabetes, obesity, and hypertension. Yet, effective therapies for chronic fibrosis in the kidney and other tissues are still awaited. Among the most extensively studied signaling pathways in renal fibrotic disease are those of the TGFβ superfamily (TGFβ and BMPs). Given the critical roles for TGFβ and BMP
proteins in enhancing or suppressing renal interstitial fibrosis, respectively, the results of this thesis will show how the expression of this secreted protein KCP could diminished renal fibrosis in mouse models of chronic and acute kidney disease.

In vivo, KCP-KO mice are viable and fertile but are more sensitive to tubular injury and exhibit significant pathology after recovery. Also, deletion of KCP sensitized mice to developing obesity and associated complications such as liver steatosis and glucose intolerance. In contrast, transgenic mice that expressed KCP in the kidney, liver, and brown adipose tissues were resistant to developing high fat diet induced obesity and had significantly reduced white adipose tissue. This data demonstrates that modulation of the TGFβ signaling with secreted inhibitors or enhancers can alter the profile of adipose tissue, which reduces obesity and impaired the progression of metabolic disease.

The Metabolic Syndrome is reaching epidemic proportions in the developed world, primarily due to the increased availability of high caloric foods and the decrease in daily physical activity. Energy balance is critical for maintaining normal body weight and homeostasis. When caloric intake chronically exceeds energy expenditure, white adipose tissue stores excess energy in the form of triglycerides, leading to obesity and related complications such as type-2 diabetes, a condition also referred to as metabolic syndrome which is a condition of chronic sub-clinical inflammation.

In mice, the TGFβ superfamily has been implicated not only in the development and differentiation of white and brown adipose tissues, but also in the induction of the pro-inflammatory state that accompanies (Tseng et al., 2008). The work outlined in this thesis suggests that altering the TGFβ superfamily signaling pathway by a secreted protein (KCP) can attenuate renal fibrosis and the negative effects of obesity-associated metabolic syndrome. Providing a conceptual basis for the use of small molecule analogues of KCP to attenuate profibrotic pathways that depend on continued TGFβ signaling and/or counteraction by BMPs may potentially provide a novel approach to translating the protective role of specific BMPs (e.g. BMP-7) into clinical benefit.
Chapter I. General Background

Ia. Pax2 Roles and regulation in Kidney development, disease, and Repair/Regeneration

I.a.1- Mammalian Pax Gene Family

The PAX family is classified into four groups according to their structural similarity, sequence homology, the presence or absence of an octapeptide motif and also according to its homeodomain or partial homeodomain (Dahl et al., 1997) (Table 1). Pax proteins are characterized by the presence of a 128 amino acid sequence in their structure, which constitutes a DNA-binding domain, the paired domain (PD) (Chi and Epstein, 2002). Each Pax protein has a c-terminal region, rich in serine and threonine, that is responsible for transcriptional activation of target genes (Chi and Epstein, 2002; Ward et al., 1994).

All of the Pax genes are expressed in developing structures and control the early specification of specific cell types or the compartmentalization of the embryo into specific regions. These proteins can modulate the expression of diverse genes in a complex pattern, as it is mediated not only by the binding of PD to DNA, but also through interactions with other DNA-binding domains (Dahl et al., 1997). The nine Pax genes, (Pax-1 to Pax-9), described in humans and mice are associated with organogenesis and maintenance of the pluripotency state of stem cell populations during development (Chi and Epstein, 2002).
I.a.2- Pax2 Role in Kidney Development

In mouse embryos, Pax2 is expressed around the 9-somite stage in the nephric duct primordium (Bouchard et al., 2000; Bouchard et al., 2002; Torres et al., 1995). Pax2 mutant embryos initially form a nephric duct, which degenerates by apoptosis during the elongation process, and fail to form normal mesonephric tubules (Dressler et al., 1990). As a result, Pax2-deficient embryos completely lack metanephric kidneys (Bouchard et al., 2002; Torres et al., 1995). Surprisingly, Pax8 null embryos show normal nephric duct and kidney development but die postnatally due to developmental defects in the thyroid gland (Mansouri et al., 1998). However, in the context of Pax2 gene deficiency, Pax8 inactivation exacerbates urogenital defects such that the pro/mesonephros is completely absent and the prospective renal tissue undergoes massive apoptosis (Bouchard et al., 2002). This finding demonstrates the functional redundancy between Pax2 and Pax8 as pro/mesonephros development is initiated normally with either Pax2 or Pax8 present (Bouchard et al., 2002). The fact that only Pax2 is required for later renal development...
may reflect higher expression levels in the nephric duct epithelium. Alternatively, both proteins may have acquired distinct features rendering Pax2 better suited to sustain the renal transcriptional program beyond the pro/mesonephros stage. In this system, Pax2 could also synergize with Lhx1, but since its onset of expression occurs after pronephric induction, it likely plays a later role in pronephric development (Buisson et al., 2015; Carroll and Vize, 1999). Given the crucial role of Pax2 in renal fate specification and morphogenesis, it becomes essential to understand the mechanisms by which they are activated in the intermediate mesoderm. The induction of the prospective kidney field in the intermediate mesoderm is set by secretory morphogens along the mediolateral axis, while regulatory molecules expressed along the rostro-caudal axis seem to define a domain of renal competence. On the mediolateral axis, the intermediate mesoderm is surrounded by the paraxial mesoderm (prospective somitic field), the surface ectoderm and the lateral plate mesoderm. Evidence so far suggests that Bmp4 from the ectoderm activates itself in the lateral plate mesoderm, which in turn is necessary for Pax2 expression in the intermediate mesoderm (James and Schultheiss, 2003, 2005; Obara-Ishihara et al., 1999). In species such as frog and zebrafish, the intermediate mesoderm marker Osr1 has also been identified as a competence factor and regulator of Pax2 expression (Tena et al., 2007). However, Osr1 mutant mice do form a pro/mesonephros, indicating that, by itself, this transcription factor is not a critical regulator of lineage induction (Mugford et al., 2008b). Instead, mouse Osr1 plays an important, but later, role in mesonephric tubules and metanephric kidney induction (Mugford et al., 2008b). Recent studies have explored the gene regulatory networks downstream of Pax genes in the renal system. In the nephric duct, Pax2/8 were found to regulate the transcription factor genes Gata-3 and Lhx1, which together with Pax proteins, turn on the down-stream transcriptional program necessary for renal morphogenesis (Boualia et al., 2013). Gata-3 acts as a driver of nephric duct guidance and morphogenesis in the mouse embryo (Grote et al., 2008), while Lhx1 plays an important role in nephric duct elongation and survival (Kobayashi et al., 2005; Potter et al., 2007; Shawlot and Behringer, 1995; Tsang et al., 2000). Similarly, Pax2/8 co-operate with Hnf1 in the nephric duct and the ureteric bud epithelium. Gene inactivation studies have shown that Hnf1 is an important regulator of nephric duct differentiation and ureteric bud branching (Lokmane et al., 2010). Together,
these findings underline the complexity of the Pax2 regulatory network in the ductal epithelium and further suggest that some regulatory interactions are maintained but are utilized differently in different systems.

The metanephros is the site of adult kidney development in the vertebrate embryo and responds to the concerted action of several genetic regulators. Metanephric development in mice begins at E10.5 by induction of the nephric duct to form the ureteric bud, which invades the metanephric mesenchyme and initiates branching morphogenesis. Ureteric bud formation is initiated by the action of the mesenchymal signal Gdnf that binds the co-receptor complex Ret/GFR 1 expressed in the nephric duct epithelium (Chi et al., 2009). This crucial interaction induces cell shape changes and proliferation that leads to the invasion of the metanephric mesenchyme by the ureteric bud (Chi et al., 2009; Dressler, 2009). Accordingly, inactivation of Gdnf, Ret or Gfr1 prevents ureteric bud formation, leading to renal agenesis (Skinner et al., 2008). Pax genes act on this system at several distinct levels during kidney development. In the nephric duct, Ret is a direct regulatory target of Gata3 (Boualia et al., 2013; Grote et al., 2008; Marcotte et al., 2014). The Pax2/8-Gata3 cascade is therefore necessary to establish the responsiveness of the nephric duct to kidney induction. Among the transcriptional regulators are Osr1, Pax2, Eya1, Hox11 and Six1/2 (Brophy et al., 2001; Wellik et al., 2002; Xu et al., 1999; Xu et al., 2003). Inactivation of each of these genes leads to a down regulation or loss of Gdnf expression in the metanephric mesenchyme which prevents normal kidney development.

I.a.3- Pax2 Role in Kidney Regeneration and Repair

The cellular hallmark of kidney repair is a rapid proliferative response ultimately leading to the restoration of nephron structure and function. The level of Pax gene expression must be finely tuned in renal cells to ensure proper tissue homeostasis. In the mouse urogenital system, Pax2 expression persists in the nephrogenic zone until around ten days after birth, but is normally switched-off as the renal epithelium differentiates (Dressler and Douglass, 1992). Re-activation of Pax2 expression in mature renal
epithelial cells is seen during kidney repair and is also associated with a number of diseases including cancer and polycystic kidney disease (PKD) (Dressler, 2011; Dressler and Douglass, 1992; Esquela and Lee, 2003; Imgrund et al., 1999; Lindoso et al., 2009; Winyard et al., 1996). On the other hand, loss of Pax2 is closely associated with congenital abnormalities of the kidneys and urogenital tract (Hwang et al., 2014). Dysplastic kidneys are a common cause of chronic kidney failure in young children and results from perturbed epithelial-mesenchymal interactions (Yang et al., 2000). In this study, they found that components of the TGFβ1 axis were expressed in these malformations: TGFβ1, mRNA, and protein were up-regulated in dysplastic epithelia and surrounding mesenchymal cells, whereas TGFβ receptors I and II were expressed in aberrant epithelia. They further generated a dysplastic kidney epithelial-like cell line that expressed cytokeratin, and ZO1. They also found that exogenous TGFβ1 inhibited proliferation and decreased expression of Pax2 and BCL2, molecules characterizing dysplastic tubules in vivo. Yang Su P et al., study provided preliminary data to support the hypothesis that TGFβ1 is implicated in the pathogenesis of human renal dysplasia by regulating Pax2 expression. Despite Pax2 transient expression during embryogenesis, its deregulation is associated with several anomalies in mice and humans. Failure in Pax2 expression leads to anephric kidneys, while its continued expression results in kidney malformations (Dressler et al., 1993; Winyard et al., 1996). Therefore, the correct up- and downregulation of this gene are extremely important. It is difficult to list all the Pax2-regulated genes, as the Pax2 binding domain seems to be present in many different genes, but some of them are not related to modulation by Pax2. To date, Pax2-regulated genes have been reported that play a role in kidney development (Brophy et al., 2003; Dehbi et al., 1996; Grote et al., 2006; Sariola and Saarma, 2003; Self et al., 2006; Stark et al., 1994; Stuart et al., 1995; Zhang et al., 2007). However, there is very little known about specific Pax2-regulated genes during kidney repair. The supposition that Pax2 might play a crucial role in renal regeneration was reinforced after Imgrund and coworkers demonstrated that the Pax2 gene was re-expressed in proximal tubule cells after injury (Imgrund et al., 1999). In healthy adult kidneys, Pax2 is detectable only in cells of the collecting ducts (Torban et al., 2000) and the medulla. Later, Maeshima and coworkers using an ischaemia/reperfusion animal model confirmed that Pax2 is
singly re-expressed among other transcription factors, such as Pax8, WT1, Wnt4 and BF-2, which are also present during development (Maeshima et al., 2002a). These studies emphasize that the presence of Pax2 may potentially influence renal regeneration, conducting key events as it does during development, but that actions of Pax2 in renal recovery are still not fully understood. Thus, Pax2 expression would drive tubular kidney cells to proliferate. In addition, the expression of this gene has also been demonstrated to prevent apoptosis. Torban and coworkers (Torban et al., 2000) used different strategies, in vivo and in vitro, to confirm that the primary function of Pax2 is preventing apoptosis, but demonstrated that Pax2 does not lead to proliferation. Most of the literature is in agreement with the view that Pax2 protects cells from apoptosis; however, further studies are necessary to better clarify other features of Pax2 actions in cell biology. There are very few in vivo approaches to directly associate Pax2 with renal recovery after injury, especially showing the participation of Pax2 in key processes related to tissue repair in vivo. It can be considered a growing field of interest as judged by the increasing number of studies showing that different factors known to influence renal tissue regeneration are now being related to Pax-2 gene expression (Maeshima et al., 2002a; Zhang et al., 2004).

I.a.4- Pax2 & TGFβ Superfamily Interaction in Kidney Development

The many processes involved in kidney development are tightly regulated by complex molecular regulatory networks. Members of the TGFβ superfamily of signaling molecules have been shown to play important roles both in vitro (Bush et al., 2004; Plisov et al., 2001; Sims-Lucas et al., 2008) and in vivo to regulate key aspects of kidney development (Dudley et al., 1995; Esquela and Lee, 2003; Michos et al., 2007; Oxburgh et al., 2004; Sakurai and Nigam, 1997). In mice at embryonic day E11.5, transcripts for the three TGFβ prologues (TGFβ 1, 2, 3) the type I and II TGFβ receptors as well as transcripts for many other members of the TGFβ superfamily are present in the mouse kidney (Oxburgh et al., 2004). Recent evidence suggests that the efficacy of TGFβ
prologues in tissue development depends on the presence or absence of specific TGFβ superfamily co-receptors such as the type III TGFβ receptor (TGFBR3), commonly referred to as betaglycan, an accessory receptor (Stenvers et al., 2003). The betaglycan heterozygous kidneys exhibited accelerated ureteric branching with a transient decrease in BMP4 expression at E11.5 and a subsequent cascade of changes in the gene regulatory network that governs metanephric development, including significant increases in Pax2, Eya1, Gdnf, Ret, Wnt4, and WT1 expression. In contrast, betaglycan null kidneys exhibited renal hypoplasia (Walker et al., 2011).

Lindoso R S et al., 2009, reported that activin A and TGFβ1 promote downregulation of Pax2 expression inhibiting cellular proliferation (Lindoso et al., 2009). In kidneys, Activins are expressed during development and re-expressed after injury periods (Maeshima et al., 2001; Tuuri et al., 1994). These proteins act as autocrine factors and play different roles in the kidney, such as activation of renal interstitial fibroblasts (Yamashita et al., 2004).

The putative mechanism of action of activin A is regulation of the expression of transcription factors like Pax2 (Nakamura et al., 1990). Data presented by Maeshima and coworkers showed that Pax2-positive cells present specific activin A receptors (ActR-II) and that administration of activin A leads to a reduction in the number of cells therefore BrdU/Pax2 double positive in vivo (Maeshima et al., 2002a). Activin A leads to reduction of Pax2 expression in the kidney culture system during embryonic development as well as in tubular cell lineages (Maeshima et al., 2002a; Maeshima et al., 2002b; Maeshima et al., 2006). The inhibition of activin A, either by follistatin or by superexpression of a mutant truncated receptor, leads to increases in Pax2 expression and cell growth promotion (Maeshima et al., 2002a). Another member of the TGFβ superfamily, TGFβ1 has been related to the regulation of Pax2. This growth factor is related to important biological processes such as apoptosis, cell growth, tissue regeneration and development (Grande, 1997). Two decades ago, Liu and coworkers demonstrated that TGFβ1 promotes a negative regulation in the expression of the Pax2 protein (Liu et al., 1997). However, in contrast to activin A, TGFβ1 downregulates Pax2 gene expression through a posttranscriptional process (Liu et al., 1997). This mechanism,
known for modulating important growth regulatory gene products, affects the stability of Pax2 mRNA and consequently promotes a reduction of the Pax2 protein in the cell.

I.b- Kidney Biology and function

The kidneys are the central organs of homeostasis in our body. Filtering removes metabolic waste products, and kidney action adjusts water, salt, and pH to maintain the homeostatic balance of tissue fluids (McMahon, 2016). After gastrulation in mammals, the kidney develops from the intermediate mesoderm as a continuum along the anteroposterior axis in a distinct temporal sequence (Figure1) (Dressler, 2006, 2009). Anterior kidney structures include the pro- and mesonephros, whose complexity, size, and duration vary greatly among vertebrate species. In the mouse, the pronephros is barely detectable, whereas mesonephric tubules are well developed with a proximal glomerulus and convoluted tubules that empty into the nephric duct (Dressler, 2009). Specification of the intermediate mesoderm and the epithelial derivatives that will make the mammalian kidney depend on the concerted action of many transcription factors and signaling proteins. Among the earliest genes expressed in the nephric duct and surrounding mesenchyme is Pax2, the function of which is essential for making and maintaining the epithelium (Dressler, 2011; Soofi et al., 2012). The renal collecting system arises from the ureteric bud, a derivative of the intermediate-mesoderm derived nephric duct that responds to inductive signals from adjacent tissues via a process termed ureteric induction. The ureteric bud subsequently undergoes a series of iterative branching and remodeling events in a process called renal branching morphogenesis. The human kidney is composed of an arborized network of collecting ducts, calyces, and urinary pelvis that facilitate urine excretion and regulate urine composition. The renal collecting system is formed in utero, completed by the 34th week of gestation in humans, and dictates final nephron completion (Blake and Rosenblum, 2014).
The kidneys consist of two essential parts: an outer part “cortex” and an inner part “medulla” (Figure 2). Each adult kidney contains about one million nephrons and each nephron contains a glomerulus surrounded by a thin-walled, bowl-shaped structure named Bowman capsule. The nephron also contains a small tube that drains filtrate from the space in the Bowman capsule and a collecting duct that drains urine from the tubule and regulates urine concentration (Kim et al., 2007). Each tubule has three interconnected parts: the proximal convoluted tubule, the loop of Henle, and the distal convoluted tubule. The distal tubule connects to the collecting duct, a continuous highly arborized epithelial network with a quite distinct origin from the contiguous renal tubule. The collecting duct epithelium displays a distinct cortical-medullary axis of branching, and cellular organization. The medullary collecting ducts are highly water permeable in order to facilitate water retention which is critical for sodium retention (Al-Awqati and Gao, 2011; Pearce et al., 2015).

Figure 1. The intermediate mesoderm: its origin and derivatives. A) Cross section embryo at E8.5. B) The Wolffian duct at E9.0. C) Mesonephric tubules at E10. D) Outgrowth of the ureteric bud (UB). E) The UB has bifurcated and induced mesenchyme surrounds the tips. G) Live image of E115.5 eGFP kidney of WT embryos. Images A to E are from Dressler, 2009 and G done by the author.
The primary function of the kidneys is to maintain the proper balance of water and minerals in the body. Mineral balance is maintained by tightly controlled ion fluxes that are external (intestine and kidney) and internal (between bone and other organs), and are regulated and coordinated by many endocrine signals among these organs (Kuro and Moe, 2016). An additional function is filtration and excretion of waste products from the processing of food, drugs, and harmful substances. Blood is filtered through small pores in the glomerulus, leaving behind blood cells and large molecules, such as proteins. The
glomerular basement membrane acts as a filtration barrier, reducing entry of larger molecular weight serum solutes into the nephron (> 15 kDa) such as serum albumin (Miner, 2011; Suh and Miner, 2013). In healthy adults, about 180 liters of fluid is filtered into the kidney tubules each day. Nearly all of this fluid, (and the electrolytes contained in it), is reabsorbed by the kidney. The prevalence of chronic kidney disease substantially increases with increasing metabolic syndrome risk factors (Chen et al., 2004). There are a number of pathologic links between metabolic syndrome and chronic kidney disease (Abrass, 2004). Contemporary research highlights the relationship between hyperinsulinemia and modifications within the kidney, including glomerular hypertrophy, mesangial matrix proliferation, and glomerulosclerosis. These changes are thought to be secondary to glomerular hyperfiltration as well as inflammatory mediators from increased adiposity. Additionally, obesity-related kidney damage has been posited to be due to a series of alterations like hyperlipidemia, increased oxidative stress, increased salt intake, and activation of the sympathetic nervous system (Palatini, 2012). Also hyperglycemia, hypertension (Eckel et al., 2005; Wong et al., 2016) and protein damage due to glycation may contribute to kidney damage (Faria and Persaud, 2017).

**I.c- Chronic Kidney Disease and Fibrosis**

Chronic kidney diseases (CKD) can be due to structural or functional abnormalities typically characterized by active inflammation and renal fibrosis. While the primary pathology leading to most forms of CKD differs significantly, all forms of progressive renal diseases, including glomerulonephritis, chronic interstitial nephritis, and diabetic nephropathy, exhibit interstitial fibrosis (Eddy, 1996; Fogo, 2000). Despite the strong correlation between tubulointerstitial fibrosis and the loss of renal function, the molecular mechanisms underlying fibrosis have remained elusive. However, evidence pointing to the TGFβ superfamily of proteins as primary regulators of fibrosis is accumulating. Indeed, TGFβ1 is generally regarded as the key mediator in the development of renal fibrosis (Flanders, 2004). Transgenic mice overexpressing TGFβ develop interstitial fibrosis, as do mice treated with recombinant TGFβ (Kopp et al.,
Furthermore, inhibition of TGFβ by neutralizing antibodies can improve injury in various models of kidney disease (Ziyadeh et al., 2000). In the normal kidney, the expression of TGFβ is weak; however, many disease states, including diabetes mellitus, increase TGFβ activity (Yamamoto et al., 1996). TGFβ induces resident fibroblasts to produce extracellular matrix components, such as type IV collagen and fibronectin, leading to the formation of tubulointerstitial fibrosis (Marti et al., 1994; Martin et al., 1998). BMPs may play an important role in kidney development and kidney regeneration (Cirio et al., 2014; Tsujimura et al., 2016). Animal studies have shown that systemic administration of BMP7 can reverse damage induced kidney fibrosis (AKI), improve cartilage damage, and inhibit the formation of bone metastases resulting from prostate or breast cancer, and increase energy expenditure by inducing the formation of brown adipocyte tissue. BMP7 thus seems a very promising new therapeutic agent in the treatment of a variety of disease states, including obesity and obesity-related disorders such as type 2 diabetes mellitus and cardiovascular disease. An understanding of the complexities of the interplay between the TGFβ1 signaling pathway and the development of CVD, CKD, and obesity with insulin resistance are important (Figure 3).

**Figure 3:** TGFβ1 is a common target molecule and interactive regulator of pathological conditions. Manipulation of the TGFβ1 signaling pathway may be a useful approach for amelioration of mortality and morbidity in individuals with cardiovascular risk factors, Image done by the author.
I.d- Obesity; Origin and Characterization of adipose tissue

In humans, two types of adipose tissue can be distinguished both histologically and functionally: white adipose tissue (WAT) and brown adipose tissue (BAT). Whereas WAT is the main tissue for storage of triglycerides in the form of fat, BAT has evolved to generate heat through uncoupled mitochondrial fatty acid oxidation (Cannon and Nedergaard, 2004). Much progress has been made toward understanding the developmental origins of brown and white adipocytes, although all aspects have not been resolved. Lineage-tracing studies of adipose tissue and muscle are both considered to be of mesodermal origin (Gesta et al., 2007). Adipocytes develop from mesenchymal stem/progenitor cells which derive from embryonic stem cells. When triggered by appropriate developmental cues, these cells become committed to adipocyte lineages, i.e. the preadipocytes (Figure 4). More recently, Seale et al., used a myogenic marker, myf5, to perform cell fate mapping in the mouse and found that both skeletal muscle and interscapular brown fat, but not white fat, arise from progenitors expressing myf5 (Seale et al., 2008). In addition to these discrete interscapular brown fat cells, uncoupling protein1 (UCP-1-positive) brown adipocytes are also found systemically distributed in the body, especially within white fat depots (Cousin et al., 1992) and between muscle bundles (Almind et al., 2007). Interestingly, these “systemic” brown adipocytes, such as those present in white fat and muscle, are not derived from myf5-expressing precursors (Seale et al., 2008), suggesting different developmental origins for these different pools of brown fat (beige adipose cells). We are still early in the process of understanding the similarities and differences between brown and beige adipose cells, and we do not yet have a clear picture of their relative importance in energy homeostasis.

The understanding of adipose tissue biology has progressed rapidly recently. The development of successful adipose-tissue-based therapeutic strategies to treat metabolic syndrome is reliant on a good understanding of basic adipose-tissue biology. The recent
confirmation that adult humans have brown adipose tissue (BAT) has transformed our understanding of how adipose tissue regulates metabolism and energy balance once again.

(Cypess et al., 2009; van Marken Lichtenbelt et al., 2009; Virtanen et al., 2009).

The relatively new finding that some adult humans have substantial amounts of heat-dissipating brown adipose tissue has raised the prospect that in humans it may be an important contributor to energy balance and a possible therapeutic target for the treatment of metabolic disease. The primary function of BAT is to maintain core body temperature in response to cold stress by generating heat, a process known as non-shivering thermogenesis (Cannon and Nedergaard, 2004). Brown adipocytes are distinct from white adipocytes in that their abundant mitochondria express uncoupling protein 1 (UCP1), which uncouples substrate oxidation from ATP production so that heat is produced (Cannon and Nedergaard, 2004). Consequently, activated BAT has a large

Figure 4. Representation of the origins of white, beige, and brown adipocyte tissue. KCP possible effects on the adipocyte biology. KCP over expression may increase the number of beige cell within the white fat, and KCP deletion has the contrary effect. A modify image from the different shades of fat review (Peirce et al., 2014).
capacity for glucose and lipid uptake per gram of tissue, and may contribute towards the regulation of glycaemia and lipidaemia in mouse models of diabetes and dyslipidaemia (Arbeeny et al., 1995; Bartelt et al., 2011). In line with its remarkable capacity for substrate oxidation, BAT is activated in rodents in response to excess nutrient consumption, such as eating a high-fat diet, a process known as diet-induced thermogenesis (Rothwell and Stock, 1983).

Obesity is a considerable public health problem that affects a sizeable part of the world population across all age and racial/ethnic groups. Obesity is a worldwide epidemic that predisposes individuals to cardiometabolic complications, such as type 2 diabetes mellitus (T2DM) and nonalcoholic fatty liver disease (NAFLD). The obesity spreading patterns around the world are remarkably predictable, low and middle-income countries are presently going through the same rapid transition from normal weight to overweight to obesity as parts of Europe and the United States already have done. According to the Center for Disease Control (CDC), more than 30% of adults are obese in United States (Ogden et al., 2013). The obesity epidemic is multifactorial, but can be mostly attributed to increased consumption of high calorie foods, decreased physical activity, and an acceptance by individuals that being overweight or obese is simply normal. In obesity, adipocytes undergo hypertrophy, which leads to an imbalanced secretion of adipokines. Adipose tissue secretes polypeptides hormones/factors like Leptin, adiponectin and resistin called “adipokines”. Collectively, adipose tissue-secreted factors are involved in energy homeostasis and regulation of glucose and lipid metabolism, immunity, and neuroendocrine systems (Ahima and Lazar, 2008, 2013). Intriguingly, other studies in humans show a very strong and consistent association between resistin and inflammation and/or inflammatory diseases (Senolt et al., 2007). Several developmental regulators hold crucial roles in adipocyte differentiation. Therefore, improved knowledge on the mechanisms underlying the formation of adipose tissue and its role in energy homeostasis is needed for preventing the growing prevalence of obesity and the inappropriate accumulation of ectopic (non-adipose) lipid.

This thesis focuses on the role of transforming growth factor-beta (TGFβ) superfamily members in adipogenesis. TGFβ changes the adipocyte profile from anti-to pro-inflammatory; invading macrophages switch to a pro-inflammatory phenotype
Identification of the pathogenic molecular mechanisms involved, and effective therapeutic approaches are required.

**I.e- Obesity and liver**

The liver is a key metabolic organ which regulates a variety of processes vital for maintaining metabolic homeostasis. These processes include control of glucose production, lipid metabolism, and dysregulation of which are symptomatic of the metabolic syndrome. The liver is a multicellular organ that relies on two highly conserved mechanisms: the ability to store energy to prevent starvation and the ability to fight infection. White Adipose tissue has the potential to store large amounts of triglycerides whereas the liver stores a limited amount of glycogen for use during starvation or to combat stressful situations. During the course of obesity, the adipose tissue’s ability to store excess energy is compromised, leading to ectopic lipid accumulation in non-adipose tissues such as muscle and liver (van Herpen and Schrauwen-Hinderling, 2008). The response of the liver to damage and inflammation is a complex process involving parenchymal and non-parenchymal cells as well as monocyte-derived hepatic macrophages (Gressner and Bachem, 1995; Morinaga et al., 2015). The failure to regulate this inflammation during the progression of obesity causes pathological chronic hepatic inflammation characterized by the advance of fatty liver to steatohepatitis, fibrosis, cirrhosis, and eventually liver failure (Buzzetti et al., 2016; Robinson et al., 2016).

In addition, both adipose tissue and liver are populated with innate and adaptive immune cells. The transforming growth factor beta (TGFβ) family signaling pathways play essential roles in the regulation of different cellular processes including proliferation, differentiation, migration or cell deaths, which are essential for the homeostasis of tissues and organs. Because of the diverse and pleiotropic TGFβ functions, deregulation of its pathways contributes to human disease. In the case of the liver, TGFβ signaling participates in all stages of disease progression, from initial liver injury through inflammation and fibrosis, to cirrhosis and cancer.
I.f- The transforming growth factor β (TGFβ) signaling pathway

I.f.1- TGFβ Signal transduction

Since the purification of its first ligand, TGFβ1, from human platelets in 1983 (Assoian et al., 1983), a considerable body of research has focused on this superfamily and more than 30 ligands have been discovered in humans (Feng and Derynck, 2005; Massague, 2008). According to their sequence similarity and biological effects, the TGFβ superfamily can be divided into two distinct groups, the TGFβ/activin/nodal subfamily and bone morphogenetic proteins (BMPs)/anti-muellerian hormone (AMH)/growth and differentiation factors (GDFs) subfamily. The TGFβ signaling regulates a diverse set of cell processes. For example, TGFβs cause cell cycle arrest in epithelial and hematopoietic cells and control mesenchymal cell proliferation and differentiation, while BMPs are important for the differentiation of osteoblasts and the survival of renal mesenchymal cells (Massague, 1998; Patel and Dressler, 2005; Reddi, 1998). In fact, TGFβ superfamily plays a key role throughout the whole development process and is involved in the formation of nearly all organs.

Although there are a number of ligands and several receptors, the general signaling transduction for TGFβ superfamily is relatively simple as illustrated in (Figure 5). In mammals, the binding of TGFβ ligand to its receptor, TGFβ receptor type II, leads to the recruitment and phosphorylation of TGFβ receptor type I (TGFβRI) (Derynck and Zhang, 2003). The activated TGFβRI is a serine/threonine kinase that transduces the signal through phosphorylating receptor-activated Smad proteins (R-Smads), which are the main mediators for TGFβ signaling. Commonly, for TGFβs, the R-Smads are Smad2 and 3, while for BMPs, they are Smad1, 5, and 8. The phosphorylated R-Smads usually form a heteromeric complex with a common partner, Smad4 (Co-Smads), and translocate into the nucleus. Normally, the Smad complex requires other transcriptional factors to activate or repress target gene expression (Itoh et al., 2000; Labbe et al., 2000; Sano et al.,
Besides R-Smads and Co-Smads, TGFβ signaling can induce the expression of a third group of Smad proteins, Smad 6 and 7 (Inhibitory Smads, I-Smads), which inhibits TGFβ signaling through competitive receptor binding and blocking the interaction between R-Smads and Co-Smads (Hayashi et al., 1997; Imamura et al., 1997). The TGFβ superfamily is widely involved in embryogenesis and subsequent organogenesis, as it interacts with other signaling pathways, such as Wnt and Notch signaling. Since TGFβ superfamily plays critical roles in a variety of biological processes, it is highly regulated at different levels, from ligand releasing to mediator activation, and finally to transcriptional complex formation and target gene expression. In the following section, the mechanism through which TGFβ signaling is regulated and functions synergistically with other signaling pathways in a defined biological context is discussed.
Figure 5. Diagram representing the functional and structural features of the TGFβ Superfamily Signaling include Ligands-Receptors- Smad pathway. TGFβ1 activation occurs with the release from latent TGFβ binding protein (LTBP) complex by proteases. TGFβ1 signaling is initiated upon binding of active TGFβ1 with TGFβ receptor type II (TβRII) and forming the TβRI-TβRII heteromeric complex, leading to phosphorylation of Smad2/3, oligomerization with Smad4, and subsequent nuclear translocation to regulate the transcription of ECM genes. The monoubiquitination turnover of Smad4 mediated by Ectodermin and FAM (Dupont et al., 2009; Massague, 2008, 2012; Soofi et al., 2013) diagram done by the author.
**I.f.2- TGFβ Prologues (TGFβ1, 2, 3) in the Kidneys**

Studies of human kidney specimens have confirmed that three major prologues TGFβ1, TGFβ2, and TGFβ3 are expressed in the kidney (Ito et al., 2010). While functional redundancy between the TGFβ prologues has been long recognized, there is a growing body of evidence for the existence of nonredundant functions in inflammation and organ development (Ren et al., 2009). TGFβ1 is the predominant and best-characterized member, while TGFβ2 and TGFβ3 are less well known. In the normal adult kidney, glomerular expression of TGFβ2 and TGFβ3 is seen mainly in podocytes, whereas TGFβ1 is primarily detected in the tubules but not in the glomeruli (Ito et al., 2010). Interestingly, glomerular expression of TGFβ1, generally with TGFβ2 and TGFβ3, was detected in podocytes in kidney biopsy specimens from patients with proliferative glomerulonephritis and in mesangial cells in diabetic nephropathy and IgA nephropathy (Ito et al., 2010). Moreover, increased expression of TGFβ1 was associated with development of severe glomerulonephritis and glomerulosclerosis (Ito et al., 2010).

Biological actions of TGFβ prologues are mediated by ligand binding to its receptors for the initiation of signaling. Both TGFβ1 and TGFβ3 bind directly with TβRII, whereas TGFβ2 requires the presence of a type III TGFβ receptor (TβRIII) for ligand binding to TβRII (Yu et al., 2003). Given the differences in the expression patterns and the mechanism of ligand binding, together with apparent non-overlapping phenotypes of the three TGFβ proteins knockout mice, it is not unreasonable that some cellular responses may differ among the TGFβ prologues. All three TGFβ prologues have been shown, in vitro, to induce ECM protein production in various renal cells, including glomerular mesangial cells, renal fibroblasts, and renal tubular epithelial cells (Wang et al., 2011; Yu et al., 2003). While most studies have demonstrated similar profibrotic effects of the TGFβ prologues, a number of studies have suggested that TGFβ2 and TGFβ3 can exert antifibrotic effects (Prelog et al., 2005; Ren et al., 2009; Yu et al., 2003). Moreover, TGFβ2 stimulated the expression of ECM proteins and induced EMT in tubular epithelial cells, whereas neutralizing antibody to TGFβ2 or repression of TGFβ2 expression inhibited renal fibrogenesis (Wang et al., 2011). Further investigations are warranted to clarify the seemingly opposite findings regarding the antifibrotic roles of
TGFβ2 and TGFβ3, which carry important implications for therapeutic targeting strategy. One of the major tasks ahead will be to further delineate the roles and specificity of the TGFβ prologues to concrete targets in normal physiology and to aberrant targets in the altered conditions of disease states.

**I.f.3- Regulation of Receptor Activation**

Despite the diversity of the ligands for the TGFβ superfamily, they all share similar sequence and structure features (Feng and Derynck, 2005). As for TGFβ paralogues (TGFβ1, 2, 3), its mature form is cleaved from homodimeric proproteins (pro-TGFβ) and remain associated with its N-terminal peptides, called the latency-associated proteins (LAP), to form the latent TGFβ complex. A family of large secretory glycoproteins known as latent-TGFβ-binding protein (LTBPs) covalently bind to LAP via disulfide linkages to form the TGFβ large latent complex. LTBPs are not required for maintenance of TGFβ latency but may instead facilitate the secretion and storage of the TGFβ–LAP complex, which may be covalently anchored to the extracellular matrix (ECM) from where it can be released in a regulated manner (Figure 5) (Annes et al., 2003; Hyytiainen et al., 2004; Massague, 2012). Whether the ligands from other TGFβ subfamily undergo the same secreting process is not clear.

Based on their structural and functional properties, the TGFβ receptor family is catalogued into two groups: type I receptors and type II receptors. There are seven type I and five type II receptors dedicated to TGFβ signaling in humans (Manning et al., 2002). Both types of the receptors are serine/threonine kinases, sharing a similar structure as an N-terminal extra-cellular ligand binding domain, a transmembrane region, and a C-terminal serine/threonine kinase domain (Shi and Massague, 2003). Compared to the type II receptor, the type I receptors have an extra domain between the transmembrane region, and the kinase domain, termed GS domain (sequence as SGSGSG), which can be phosphorylated by type II receptors and is critical for signaling activation (Souchelnytskyi et al., 1996; Wrana et al., 1994). As for the interaction between the ligands and receptors, there are two distinct modes represented separately by
TGFβ/Activin subfamily and BMP subfamily. TGFβ and Activin showed a high affinity for type II receptors and the type I receptors were recruited only after the ligand-type II receptor complex was formed (Massague, 1998). In contrast, from the analysis of binding affinity, BMPs interacted with the type I receptors first, then the type II receptors (Liu et al., 1995). No matter of this sequential issue, the activation of type I receptors and its interaction with Smad proteins required the phosphorylation of its GS domain by type II receptors (Feng and Derynck, 2005; Massague, 1998; Shi and Massague, 2003).

The regulation of TGFβ receptor activation comprises two aspects: (1) controlling the access of TGFβ ligands to their receptors; (2) controlling the activation of type I receptors. Two classes of molecules with opposing function regulate the access of TGFβ ligands to their receptors. One class consists of a variety of soluble proteins that sequester TGFβ ligands and prevent their binding to the receptors. A separate class consists of membrane-anchored proteins, including betaglycan and endoglin, which may function as accessory receptors to enhance TGFβ signaling (Massague and Chen, 2000; Shi and Massague, 2003).

<table>
<thead>
<tr>
<th>Ligands</th>
<th>Antagonists</th>
<th>Enhancers</th>
<th>Receptor type I</th>
<th>Receptor type II</th>
<th>Coreceptor</th>
<th>Smads</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGFβ1,2,3,5,6</td>
<td>KCP, Follistatin</td>
<td>CTGF</td>
<td>TβR1, ALK4, ALK6, ALK4, 7, ALK4, 7</td>
<td>TβR2, ActβR-II A, B, ActβR-II A, B, ActβR-II A, B, ActβR-II A, B, ActβR-II A, B</td>
<td>Betaglycan, Cripto</td>
<td>Smad 2,3 for all</td>
</tr>
<tr>
<td>ACTIVIN A, B, C</td>
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<td>MYOSTATIN</td>
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<tr>
<td>NODAL GDF 5,6,7</td>
<td>Inhibin Lefty 1,2</td>
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<tr>
<td>BMP 2,4,5,6,7</td>
<td>Generally Noggin/CTGF, Chordin/Sog, Cer1, Corberus, Chordin/Dmp/Germalin, PRDC, DAN, Dancite, CeCan1</td>
<td>KCP</td>
<td>BMPR-1A - B, BMPR-1A - ALK2, BMPR-1A - ALK2</td>
<td>BMPR-1A - ALK2, BMPR-1A - ALK2, BMPR-1A - ALK2, BMPR-1A - ALK2, BMPR-1A - ALK2, BMPR-1A - ALK2, BMPR-1A - ALK2, BMPR-1A - ALK2, BMPR-1A - ALK2</td>
<td>RIMG-a,c,d, Endoglin</td>
<td>Smad 1, 2, 3, 4, 5, 6 for all</td>
</tr>
<tr>
<td>BMP 9, 10</td>
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<td>GDF 5,6,7</td>
<td>AMH/Mis</td>
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Table 2. Representation of ligands, antagonists, receptors, coreceptors and smads proteins relationships to the TGFβ and BMP branches of the TGFβ superfamily signaling pathway (Lin et al., 2005; Massague, 2008; Soofi et al., 2013).

Although the length and structure vary considerably among BMP antagonists, such as Noggin, Chordin/Sog, and DAN family, they all share a common cysteine-rich
region. For example, Noggin contains a carboxy-terminal cysteine-rich (CR) domain, while Chordin contains four cysteine-rich repeats (Massague and Chen, 2000). The CR domain confers the antagonists to form a homodimer to match the structure of BMP ligand homodimers. The crystal structure of the Noggin-BMP7 complex directly showed that Noggin inhibited BMP7 by blocking the surfaces that were required to interact with the type I and type II BMP receptors (Groppe et al., 2002). Those antagonists are expressed during embryogenesis and are critical for the dorsal-ventral patterning and left-right asymmetry. Interestingly, although most of the BMP antagonist shared the CR domain, not all proteins containing CR domain counteract BMP. In this thesis it is shown that instead of blocking BMP signaling, the CR domain protein KCP (Kielin/chordin-like protein) enhanced BMP-receptor interactions and counteract the TGFβ interactions (figure 6) (Lin et al., 2005).

![Diagram](image.png)

**Figure 6. The secreted protein KCP enhances BMP and suppresses TGFβ.**

KCP Interacts with TGFβ and BMPs ligands in a paracrine manner. KCP can increase the binding of BMP to its receptor and inhibits TGFβ binding to its receptor. Results in the increases of P-Smad1 the BMP effectors and decreases of P-Smad2/3 the TGFβ effectors, Image done by the author.
I.g.- Kielin/chordin-like protein (KCP) Characterization

I.g.1. KCP Functions and Attributions

The original description of KCP was made in a series of publications from the Dressler laboratory (Lin et al. 2005; Lin et al. 2006). The newly-described gene, KCP, encodes a protein with homology to the extracellular regulators of the TGFβ superfamily of secreted signaling peptides. KCP is a large secreted protein with 18 repeated cysteine-rich domains. KCP is expressed in the developing kidney at both early and late stages, and its expression is correlated with the formation of early epithelial structures within the intermediate mesoderm, and to the formation of the proximal tubules in the more developed metanephric kidney. In the mammalian kidney, BMP7 plays an essential role in development and disease. BMP7-null mice show arrested renal development at around E14.5, resulting in severe renal hypoplasia (Dudley et al., 1995; Luo et al., 1995). BMP7 is also an anti-fibrotic agent that can reduce interstitial fibrosis, a common pathology in a broad spectrum of chronic renal diseases. Administration of recombinant BMP7 has shown remarkable efficacy in the reduction of glomerular and interstitial fibrosis in mouse models of chronic renal disease (Zeisberg et al., 2003a; Zeisberg et al., 2003b).

BMPs bind to specific type I and type II transmembrane receptors that contain cytoplasmic Ser/Thr kinase domains (Shi and Massague, 2003; Zwijsen et al., 2003). The activated receptor complex then phosphorylates the intracellular Smad proteins, which translocate to the nucleus and activate ligand responsive genes (Nishimura et al., 2003). The regulation of BMP signaling by sequestering ligand availability is a fundamental morphogenetic mechanism during development that establishes the dorsal–ventral pattern in both invertebrates and vertebrates (Capdevila and Belmonte, 1999; Christian, 2000). Numerous proteins such as Noggin, Chordin, Short gastrulation (Sog), Twisted Gastrulation (Tsg), and their related factors Caronte, Cerberus, and Gremlin bind BMP family ligands and prevent their contact with receptors (Garcia Abreu et al., 2002; Shi and Massague, 2003). Chordin and Sog are secreted proteins with repeated cysteine-
rich domains that bind BMPs to inhibit signaling (Larrain et al., 2000).

Unlike previously described CR domain proteins, KCP is a potent paracrine enhancer of BMP signaling. KCP increases the affinity of ligand to receptor and/or enhances the stability of the ligand-receptor complex. Given the role of BMP7 in renal disease, we analyzed the phenotypes of KCP KO mice and the KCP transgenic mice in two independent models of renal injury. Both strains of KCP mice were used in the Diet Induced Obesity (DIO) study. The data point to an important role for KCP to enhance BMP signaling, attenuate the initiation, and progression of fibrotic disease after renal injury. KCP also protects mice from the DIO, fatty liver, and metabolic syndrome. These conditions will be discussed in more detail in Chapter II (Soofi et al., 2016; Soofi et al., 2013).

I.g.2- Original Description of KCP

During the course of conducting a yeast two-hybrid screen (Lin et al., 2005; Lin et al., 2006) with an embryonic kidney cDNA library, several hundred partial cDNA clones were sequenced after primary selection. From the embryonic kidney library, a partial cDNA was identified containing a novel protein coding sequences with multiple domains homologous to Xenopus chordin, Drosophila crossveinless 2 (Cv2), and Drosophila Short gastrulation (Sog). Although this cDNA proved negative for specific protein-protein interactions upon secondary selection, the novelty of the coding region and its potential impact on kidney development prompted the need for further investigation of this gene. A mouse embryonic kidney cDNA library was screened by hybridization and overlapping clones was identified. Upon completion of the cDNA sequence, a coding region was found to be similar to Xenopus Keielin protein (Matsui et al., 2000). Thus, the gene was named KCP for Keilin/chordin-like protein. The KCP protein consists of 1254 amino acids. KCP protein (GenBank Accession AY884211) reveals a signal peptide, 18 cysteine-rich Chordin repeats (CR), and a carboxyl-terminal Von Willebrand Factor Type D domain (Figure 7A).
The KCP tissue-specific expression patterns were confirmed and expanded upon using whole mount of in situ hybridization using embryos. In mouse embryos at E9.5, the limb bud mesenchyme was positive for KCP RNA (Figure 7B). Expression in the kidney region could be detected as early as E9 in the intermediate mesoderm (Figure 7B, arrow). By E10, the mesonephric tubules and nephric ducts were clearly positive for KCP mRNA (Figure 7C and D, arrow). At later stages, high levels of KCP mRNA localized to the developing tubules (Figure 7G, arrows and H).

Figure 7. The Structure and Expression pattern of the endogenous KCP protein. A) KCP protein structure representation. B) Whole mount in situ hybridization of E9 embryo showing staining in limb bud mesenchyme (arrow) and in the nephric duct (arrowhead). C, D) E10 whole mount embryo indicating kcp mRNA expression in the mesonephric tubules (arrow). E) A section taken through an E9 embryo showing kcp mRNA in the forelimb bud mesenchyme (arrowheads) and in the intermediate mesoderm (arrow). F) E10 whole-mount embryo section indicating Kcp mRNA expression in the mesonephric tubules (arrow). G) A bisected E16 kidney with kcp expression in the presumptive proximal tubules (arrows). H) A bisected E16 kidney stained with control sense strand probe. Image A done by the author & B Modify image from Lin et al., 20015.
I.g.3- Generation of KCP KO mice

Generation of The KCP-KO mice was done by homologues recombination in the mouse germline by replacing exons 2-21 of KCP with lacZ and a Geneticin cassette (Figure 8a). By inserting the lacZ gene in frame, its expression was expected to reflect the endogenous pattern of KCP in the embryo. Thus, lacZ was expected to serve as a useful marker to detect KCP expression cells (Lin J., 2005). The targeting vector deletes amino acids 67-774, which includes most of the CR domains. Also, as expected the LacZ/neo cassette inhibits the expression of coding sequences downstream of 774 aa.

Germline chimera’s mice were obtained from the injected ES cells clones. The chimeras were then backcrossed with C57Bl/6J to generate the offspring containing the KCP heterozygote mice. Further homozygous KCP–KO mice were generated by crossing KCP heterozygous mice and genotyped by Southern blotting followed by PCR. KCP-KO mice were viable and fertile without obvious abnormalities.

![Figure 8. Generation KCP-KO](image)

Figure 8. Generation KCP-KO (a) Schematic diagram of the Kcp targeting vector that was designed to delete exons 2–21, spanning amino acids 67–774 of the coding region. The lacZ gene was inserted in frame after amino acid 67 in exon 2 (Lin et al., 2005).

Unlike Chordin, KCP enhances BMP mediated signaling in a paracrine manner by interacting with the type I receptor to facilitate the binding of BMP7 to BMP receptor 1A (Lin et al., 2005). In contrast, mice homozygous for a mutant KCP allele showed no gross developmental abnormalities but exhibited enhanced susceptibility to developing renal interstitial fibrosis in two different animal models, a process known to be regulated by both BMPs and TGFβ (Lin et al., 2005).
The TGFβ pathway can directly transduce extracellular cues from the cell-surface transmembrane receptors to the nucleus through intracellular mediators, known as Smads. The Smad family is well conserved (Feng et al., 1998; Moustakas and Heldin, 2009; Patterson and Padgett, 2000). In most vertebrates, there are eight Smads, compared to six in the Caenorhabditis genus and four in Drosophila species (Huminiecki et al., 2009). Smads proteins can be divided into three functional groups: (1) Receptor-regulated Smads (R-Smads, 1/2/3/5/8); (2) Common Smad (Co-Smad, 4); (3) Inhibitory Smad (I-Smad, 6/7) illustrated in figure 5, page 22.

This thesis will also discuss how TGFβ1 may have a pivotal role in the pathogenesis of obesity and progressive kidney diseases that are characterized by fibrosis. TGFβ1 signal transduction is mainly through the Smads protein system, and it is well known that Smad2/3 play important roles in regulating target genes transcription involved in progressed CKD and extracellular matrix (ECM) metabolism. The blockade of Smad3 attenuates development of TGFβ1-driven renal fibrosis. This was examined in vivo in a transgenic model of TGFβ1-induced chronic kidney disease with or without Smad3 expression and in vitro in mesangial cells and glomerular endothelial cells with Smad2/3 inhibitors or Smad3-knockdown (Kellenberger et al., 2013). In addition, Smad3-deficient mice are protected from diet-induced obesity and diabetes. Interestingly, Smad3 deletion results in white adipose tissue acquiring the bioenergetic and gene expression profile of brown adipocytes (‘beiging’; (Yadav et al., 2011). Together, this demonstrates that TGFβ signaling regulates glucose tolerance and energy homeostasis, and suggests that modulation of TGFβ activity by modifying the expression of Smad proteins might be an effective treatment strategy for obesity, diabetes, livers disease, and especially chronic kidney diseases.
CHAPTER III

III. a. Thesis Conclusions, Reflections, and Future Directions

III. a. 1- Thesis Conclusions

In this thesis, evidence has been presented through the published articles, showing that kidney development in mammals is the final product of three successive embryonic steps that are characterized by the transformation of intermediate mesoderm cells. The development of the first kidney, the transient pronephros, is initiated by signals from the somite and surface ectoderm that induce cells in the intermediate mesoderm to undergo the transition to epithelial cells forming the nephric duct (Mari and Winyard, 2015; Mauch et al., 2000; Obara-Ishihara et al., 1999). The caudal migration of the nephric duct subsequently induces the adjacent nephrogenic mesoderm to aggregate and form the tubules of the mesonephros, the second embryonic kidney. On further extension, the nephric duct reaches the metanephrogenic mesenchyme at the level of the developing hindlimb, where the ureteric bud evaginates from the nephric duct and invades the surrounding mesenchyme. Both the ureter and mesenchyme subsequently undergo reciprocal inductive interactions to form the nephrons and collecting ducts of the metanephros, the third and adult kidney. In humans, new nephron formation, or nephrogenesis, starts during the 5th week of gestation, the first glomeruli appear at the 9th week, and the last new nephron is formed by the 36th week of gestation. In mice, nephrogenesis starts at embryonic day 10.5, with the first glomeruli appearing at embryonic day 14 and the last new nephron approximately appearing 1 week to 10 days after birth (Mari and Winyard, 2015; Saxen and Sariola, 1987; Soofi et al., 2012).

In other parts in this thesis we described that in mammals, Pax genes control the specification of particular cells and tissues, and have also been linked to human congenital malformations (Chi and Epstein, 2002; de Miranda et al., 2014; Robson et al., 2006). The Pax2 gene is crucial for the development of the kidney and the reproductive tract, both of which are derived from the intermediate mesoderm (Dressler, 2006, 2009). Pax2 is among the earliest markers for the intermediate mesoderm, along with the related
gene Pax8 (Bouchard et al., 2002) and the homeodomain protein Lhx1 (Tsang et al., 2000). Kidney development starts when the ureteric bud (UB) invades the metanephric mesenchyme (MM) and transmits inductive signals, such as Wnt9b (Carroll et al., 2005), to promote condensation of the MM around the UB tips. These UB tip associated Cap mesenchyme cells (CM) continue to express Pax2 and are the stem cells of the nephron that generate all of the epithelial derivatives, including distal, proximal, and glomerular epithelium (Kobayashi et al., 2008; Mugford et al., 2008a). The CM undergoes a mesenchymal-to-epithelial transition to generate all the epithelial cells of the developing nephron. However, the Pax2 expression is down-regulated in the podocyte precursor cells and the mature epithelial cells of the nephron as development comes to an end (Ryan et al., 1995). This Pax2 positive intermediate mesoderm generates the nephric, or Wolffian, duct, an outgrowth of the duct called the UB, and the surrounding MM. Pax2 null mutant do develop a nephric duct, but the duct is completely absent in a Pax2; Pax8 double mutants. Pax2 and Pax8 have redundant function in kidney development. Pax8 mutant embryos develop a normal urogenital system, but mice die shortly after birth due to defect in the thyroid gland development. In contrast, a Pax2 mutant results in complete renal agenesis because the nephric duct is abnormal and the metanephric mesenchyme cannot respond to inductive signals.

To assess the fate of Pax2 positive cells during embryonic development, we inserted the enhanced green fluorescent protein (EGFP) coding region into the 5' UTR of the mouse Pax2 gene by homologous recombination. Two different alleles were created, one that carries a PGK-neo cassette and another that has PGK-neo deleted. Surprisingly, the presence of PGK-neo results in a hypomorphic allele that is homozygous viable, whereas the deletion of PGK-neo generates a null allele. We utilized both alleles to study Pax2 expression in normal and mutant embryos and to examine the phenotypes of embryos and adults with reduced Pax2 protein levels in the hypomorphs. The results indicate a critical role for Pax2 in maintaining the epithelial integrity of the nephric duct. Furthermore, reduced levels of Pax2 protein generate a spectrum of structural defects including multiple ureters, cystic kidneys, and fewer nephrons. Both of these novel Pax2 alleles are useful for cell imaging, while the new hypomorphic allele is also a good mouse model that mimics multiple aspects of congenital abnormalities of the kidneys and
urogenital track (CAKUT). Among the causes of CAKUT are heterozygous mutations in the Pax2 gene, which lead to Papillorenal Syndrome, and result in hypoplastic kidneys, vesicoureteral reflux, progressive renal failure, and optic nerve coloboma. Losing of Pax2 function results in complete renal and reproductive tract agenesis in mice (Soofi et al., 2012; Torres et al., 1995). In the absence of Pax2, the IM cells assume a pattern of gene expression more consistent with paraxial mesoderm and its derivatives (Ranghini and Dressler, 2015). Despite its central role in kidney development and renal disease, the biochemistry of Pax2 and its effects on gene regulation are not well characterized in a developing tissue. Few target genes have been identified, including many known kidney developmental regulators, such as Gdnf, c-Ret, Six2, Sal1, and Lhx1, but also affected are genes and proteins associated with glycosylation, cell membranes, cell-cell signaling, and cell adhesion (Ranghini and Dressler, 2015). Furthermore, ectopic or deregulated expression of Pax2 is also seen in Wilms' tumor (Dressler and Douglass, 1992), renal cell carcinoma (Gnarra and Dressler, 1995), and polycystic kidney disease (Ostrom et al., 2000), where it is thought to promote proliferation and/or survival. The reactivation of Pax2 expression is also observed in adult kidneys after acute injury, suggesting a critical role for Pax2 in regenerating the epithelia (Humphreys et al., 2008; Imgrund et al., 1999; Kusaba et al., 2014). We also discussed in Chapter I that Pax2 re-expression is regulated by the TGFβ superfamily singling pathway.

TGFβ and BMP signaling pathways are two main branches of TGFβ superfamily, which are essential for normal development and disease progression. TGFβ signaling is well characterized for its pro-fibrogenic effect in kidney diseases (Liu, 2010). In vitro, TGFβ promoted the transition of epithelial cells to fibroblasts-like cells by downregulating epithelial markers, such as E-cadherin, and activating mesenchymal genes, such as Snail1, Pai1, and Zeb1 (Yang and Liu, 2001). Although the existence of epithelial-mesenchymal transition (EMT) in vivo was challenged in recent years (Kriz et al., 2011), enforced expression of mesenchymal genes, such as Snail1, in epithelial cells induced renal fibrosis in mice (Boutet et al., 2006), suggesting the critical role of the upregulation of mesenchymal genes in epithelial cells in kidney diseases. Recent data have redefined the role of the surviving epithelial cells in fibrosis and attribute myofibroblast expansion to perivascular and interstitial fibroblasts. After damage, the
kidney has the ability to repair itself. With a mild injury, this repair can result in the return to a structural and functional state that is indistinguishable from normal. However, when the repair is more severe or superimposed on baseline kidney abnormalities, the repair process can lead to fibrosis, which can facilitate progression to chronic kidney disease. Acute kidney injury (AKI) can thus result in incomplete repair and persistent tubulointerstitial inflammation, with a proliferation of fibroblasts and excessive deposition of extracellular matrix, a common feature of many different kinds of kidney diseases and a primary determinant of progression to end-stage renal failure (Forbes et al., 2000). Whether AKI is associated with ischemia reperfusion injury, sepsis or toxins, there is a rapid loss of proximal tubular cell cytoskeletal integrity and cell polarity. There is shedding of the proximal tubule brush border, loss of polarity with mislocalization of adhesion molecules, and other membrane proteins such as the Na+K+ATPase and β-integrins (Thadhani et al., 1996; Zuk et al., 1998). Normal cell-cell interactions are disrupted with an injury. When the injury is severe, there is apoptosis and necrosis (Thadhani et al., 1996). Viable and nonviable cells are desquamated leaving regions where the basement membrane remains the only barrier between the filtrate and the peritubular interstitium.

In this thesis, evidence has been presented through the published articles, showing a consistent ability of the secreted kielin/chordin-like (KCP) protein to enhance BMP signaling while suppressing TGFβ signaling. These observations indicate a critical role for KCP in modulating the responses between these anti- and pro-fibrotic roles of these cytokines in the initiation and progression of several diseases including liver disease, renal interstitial fibrosis, and obesity. KCP is a secreted, cysteine-rich (CR) protein, with similarity to mouse Chordin and Xenopus laevis Kielin. KCP is an enhancer of BMP signaling in vertebrates and interacts with BMPs and the BMP type I receptor, to promote receptor-ligand interactions. In contrast to the enhancing effect on BMPs, KCP inhibits both Activin-A and TGFβ mediated signaling through the Smad2/3 pathway. These inhibitory effects of KCP are mediated in a paracrine manner, suggesting that direct binding of KCP to TGFβ or Activin-A can block the interactions with prospective receptors. The ability of KCP to sequester ligands from their receptors and the mechanism of TGFβ inhibition remains to be clearly defined in the future.
Mice homozygous for a KCP null allele are hypersensitive to developing renal interstitial fibrosis, a disease stimulated by TGFβ but inhibited by BMP7. Transgenic mice that express KCP in adult kidneys showed significantly less expression of collagen IV, α-smooth muscle actin, and other markers of disease progression in the unilateral ureteral obstruction model of renal interstitial fibrosis. In an acute tubular necrosis model, mice expressing KCP were more resistant to high doses of folic acid and showed better recovery at lower doses. The data demonstrates that extracellular regulation of the TGFβ/BMP signaling axis by cysteine-rich domain proteins can reduce disease severity in animal models of renal injury (Soofi et al., 2013).

Recently, we examined the effects of KCP loss or gain of function in mice that were maintained on either a regular or a high-fat diet. Loss of KCP sensitized mice to obesity and associated complications such as hepatic steatosis and glucose intolerance. In contrast, transgenic mice that expressed KCP in the kidney, liver, and brown adipose tissues were resistant to developing high-fat diet induced obesity and had significantly reduced white adipose tissue. The data demonstrate that shifting the TGFβ superfamily signaling with a secreted inhibitor or enhancer can alter the profile of adipose tissue to reduce obesity, and can inhibit the initiation, and progression of hepatic steatosis to significantly reduce the effects of high-fat diet induced metabolic disease (Soofi et al., 2016).

The analysis of the mechanisms and the regulation of TGFβ superfamily will enable further insight into TGFβ signaling and may provide new strategies for the treatment of TGFβ associated diseases, such as renal fibrosis, non-alcoholic fatty liver disease, obesity, and metabolic syndrome. Future understanding of the alterations in cell-cell and cell-matrix interactions will prove crucial to deciphering the roles of TGFβ and BMP7 during early and late phases of interstitial fibrosis. An understanding of how the extracellular regulators of latent TGFβ and BMP7 affect the competition between these opposing signals will provide important insights into the progression of chronic disease and perhaps provide new avenues of intervention.
III. b. Thesis Reflection and Future Direction

III.b.1. The ‘how I would do the experiments differently now’ question

All work in this thesis has been peer-reviewed and published in quality Journals. That said, any published work is far from being 100% perfect, and we learn something new on a daily basis. Every day, we do and should become better at what we do. Today I will attempt to discuss some experiments and techniques, and how we may perform them differently. Some of these observations were also made by the examiners, Dr. Paul Winyard and Dr. Peter Hohenstein.

First, we should ensure that all mice and ES cells that are used to inject the targeting vector to generate transgenic or KO mice have the same pure genetic background before carrying out any experiments. To guarantee this, we could make it a general requirement for all future experiments. Furthermore, it is important to note that concepts that may seem logical to us may require a thorough explanation to readers in order to make sense, even if the Journal Peer Reviewers pass it. Indeed, to describe in more details each technique and methods.

Figure 1. Section B, we decided not to show a blot of a negative egfp/egfp (Pax2 mutant), even though there is no Pax2 expression. The internal control (Tubulin) could indicate that they are in the blot.

Figure 3. We should have specified if we were comparing littermates to each other or gene expression independent of the phenotype. It may have been better to compare the different gene expression in the similar phenotype of the Pax2 Eneo/Eneo E11-11,5 embryonic kidneys.

Figure 6. Sections E-F, if we were going to talk about cell planar polarity we should have used better polarity markers.


Figure 2. Section A, to confirm that indeed Myc-KCP is localized to the ER compartment in the epithelial cells; we should have co-stained cells with ER markers and markers of other cell compartments as control. For example, we could use Golgi or mitochondrial markers as the negative compartments for the Myc-KCP expression.

Figure 6. Section D, we should have blotted for the total protein Smad3 and Smad1 to normalized the p-Proteins and determine the ratio of the relative unit for each protein. Furthermore, we could also measure Creatinine and the blood urea nitrogen (BUN) as a secondary indication of kidney damage.


Even though the 5008 Purina lab chow used in the animal facilities were the same for all animal groups, we and others should not utilize it as control diet to the high-fat diet (HFD). The ND 5008 Purina lab chow has many different basic nutrients in comparison to the HFD D12451 from Research Diet Inc. Practice must change in future experiments.

Even though intensive work was done in this project addressing the effect of normal, gain and loss of KCP in white and brown adipocyte tissue, very little was done about other fat depots, such as the subcutaneous fat tissue (Soofi et al., 2017). Experiments will be seriously considered for future projects in this field, yet it is difficult to cover all in one study.

III. b. 2. Further experiments based on this thesis

The Pax2 project provides new insight into the role of Pax2 in determining the correct renal architecture and cell fate. These new Pax2 alleles are valuable genetic reagents for further research on urogenital development, disease and future use of Pax2 as a therapeutic target. Both of these novel Pax2 alleles are useful for cell imaging, whereas the new Pax2 hypomorphic allele is also a good mouse model that mimics multiple aspects of CAKUT. Our lab has recently (Ranghini and Dressler, 2015) used the Pax2-egfp knock-in allele of Pax2. They identified and sorted cells of the intermediate mesodermal lineage, and compared gene expression patterns in egfp positive cells that were heterozygous (Pax2-egfp/+) or homozygous null for Pax2 (Pax2-egfp/egfp). Thus, we identified critical regulators of intermediate mesoderm and kidney development whose expression depended on Pax2 function. In cell culture models, Pax2 is thought to recruit epigenetic modifying complex to imprint activating histone methylation marks through interactions with the adaptor protein PTIP, a project we are following up with in vivo studies. In Pax2 mutants, a set of genes was also identified whose expression was up-regulated in egfp positive cells and whose expression was consistent with a cell fate transformation to paraxial mesoderm and its derivatives. These data provide evidence that Pax2 specifies the intermediate mesoderm and renal epithelial cells through epigenetic mechanisms and in part by repressing paraxial mesodermal fate. We are
interested in continuing these studies using the combination of the Pax2-egfp alleles and the new made Pax2 conditoinal KO mice in kidney development as well as in adult kidney regeneration after injury. Another recent publication from our lab by Gerimely E etal., 2017 used the Pax2-egfp mice as an important model to report that Pax proteins are re-expressed or ectopically expressed in cancer and other diseases of abnormal proliferation, making them attractive targets (Grimley et al., 2017). This new data confirms that small molecules targeting the DNA binding paired domain can be identified and may be good lead compounds for developing tissue and cell-type specific anticancer therapies. We will be focusing on future experiments in pursuing the role of Pax2 in Kidney development, kidney disease, and regeneration, as well as the role Pax2 plays in other organs like the female and male reproductive system.

Given the critical roles for TGFβ and BMP proteins in enhancing or suppressing renal interstitial fibrosis, respectively, and the recently established link of the TGFβ/Smads axis to NAFLD, fibrosis, and HFD-induced obesity, our works confirm that regulating TGFβ signaling through the extracellular proteins that inhibit or enhance receptor/ligand interactions is a viable strategy to attenuate both hepatic steatosis and its long-term effects, such as liver fibrosis and other metabolic disorders. These secreted regulatory proteins may prove to be valuable tools to study liver metabolism, pathology and obesity-related complications in animal models. Acute kidney injury (AKI) and chronic kidney disease (CKD) are among the most common, costly and deadly disease in the USA, and we are committed to keep working on new projects using the knowledge and models developed in this work, using mice that have different gene mutations or small molecules that mimic the effects of the KCP protein to attenuate both AKI and CKD. This work helps people get further insight into the TGFβ signaling pathway and may provide new clues for the medical treatment of TGFβ associated diseases, such as renal fibrosis, NAFLD, metabolic syndrome, and cancer.
List of References

Including text of the thesis and all publications


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Candidate list of Publications

- **Soofi A** et al. The Kielin/Chordin-like Protein KCP can Attenuate High Fat Diet Induced Obesity and Metabolic Syndrome in Mice. Manuscript Under review JCB 2017.


- Moeller MJ, Soofi A, Braun GS, Li X, Watzl C, Kriz W, Holzman LB. Protocadherin FAT1 binds Ena/VASP proteins and is necessary for actin dynamics and cell polarization. EMBO J. 2004 Sep 2


Personal Statement

The prevalence of Chronic Kidney Disease (CKD) and obesity-related conditions such as heart disease, stroke, type 2 diabetes and certain types of cancer, are some of the leading causes of preventable death. In the U.S. alone, CKD prevalence was recently estimated at 336 per million a year and the rate of obesity reached 34.9%. The estimated annual medical cost of obesity in the U.S. was $147 billion in 2008. Renal fibrosis is the major determinant in progression of acute and chronic kidney diseases. The transforming growth factor-β (TGFβ) superfamily has been shown to be an important mediator of progressive fibrosis and to influence the differentiation of preadipocytes to mature adipocytes. Thus, an understanding of interactions between chronic kidney disease and metabolic syndrome is an important issue and attention should be given to TGFβ superfamily as crucial factors for regulation and modulation of those pathological conditions.

This thesis proposal will focus on demonstrating that modification of the TGFβ superfamily signaling pathway with a secreted inhibitor or enhancer can alter the metabolic profile of adipose tissue to reduce obesity and inhibit the initiation and progression of hepatic steatosis. This significantly reduces the effects of metabolic diseases induced by a high fat diet and attenuate kidney disease in animal models. This work will provide a new insight on how a secreted proteins or derivatives could attenuate profibrotic pathways, and may provide a novel approach to translating the protective role into clinical benefit.

Pursuing my PhD degree at Warwick University will enable me to fulfill my personal achievements and career objectives and establish the ideal foundation for my future roles as a researcher. I am committed to improving a deeper public consciousness of crucial health problems like obesity and cancer. Having completed my PhD degree, I would be able to continue working for patient benefit, through my research. Throughout my research career I have pursued the goal of improving the lives of others. During my undergraduate and master programs (1987-1994) I studied biomarkers of alcoholism. As a result of that tremendous personal effort, biomarkers of alcoholism were used for the first time in the diagnosis and treatment of alcoholism in several heath institutions in Cuba thereby improving the health and quality of life of patients and their families. In Yemen, the drug called Khat and the misuse of pesticides represent another kind of epidemic causing health, social and economic problems and eventually leading to destruction of families. When I returned to Yemen from Cuba, in June 1995, I spent one year working diligently on a project to create the Toxicology Center which would serve as a basis to establishing forensic medicine in the country. In addition, I participated in educational programs and, through the use of local and national media, persuaded the public to understand the critical nature of these problems.
At the end of 1996, I moved to the U.S. and I decided to stay there because of the many opportunities for advancing my career. However, in the beginning it wasn’t very easy for many reasons, including getting accustomed to the language, but I dedicated much effort to becoming familiar quickly. Coming from a family with minimal formal education, I had to migrate, first within Yemen, and later to Cuba where I obtained my basic science training. In 1996, I moved to Detroit, Michigan (U.S.) where I noticed that many children of illiterate immigrant parents struggled with their school homework, so I volunteered to participate in after school educational programs to help them and to provide them with a safe environment.

At the beginning of 1997, I started my scientific research work in the U.S. at Wayne State University in aging and DNA repair. Then in 2000, I moved to the University of Michigan where I was hired to run the renal core in the Nephrology Division. We generated several cell and mouse models to study glomerular structure and glomerular diseases. At the end of 2009, I joined Dr. Dressler’s laboratory in the Department of Pathology where, among other responsibilities, I have had the opportunity to have my own projects. Since then, I have been working on interrelated projects and publications of my latest works which will be used to obtain my PhD.

Obtaining my PhD degree represents the culmination of my studies and carries immense personal importance. Above all, I hope to inspire my coworkers, friends and families, but most importantly my 17 years old daughter and 15 years old autistic son. Professionally, it will open new doors to new opportunities and give me the authority to independently pursue my scientific research. This is critical for me to be able to collaborate with scientists in countries facing similar health challenges to tackle these serious illnesses.

*Abdulsalam Ahmed Muthana Soofi*