Abstract

The crosstalk of Hgf and canonical Wnt signaling in kidney repair

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While Wnt and Hgf signaling pathways are known to regulate epithelial cell responses during kidney injury and repair, whether they exhibit functional cross-talk is not well defined. Canonical Wnt signaling is initiated by the phosphorylation of the Lrp5/6 co-receptors and culminates in stabilization of β-catenin, its translocation to the nucleus and activation of gene transcription. In the current study we demonstrate that Hgf stimulates the Met-dependent and Wnt-independent phosphorylation of Lrp5/6 at 3 separate activation motifs in murine renal epithelial cells. This Hgf-induced phosphorylation of Lrp5/6 was restricted to subconfluent, de-differentiated mouse proximal tubular (MPT) cells and down-regulated as the cells become more confluent. We then pursued the mechanism of Hgf-induced Lrp5/6 phosphorylation. This phosphorylation was found to be Gsk3-dependent and Hgf treatment stimulated the selective association of 'active' (tyrosine phosphorylated) Gsk3 with Lrp5/6. In contrast, Akt-phosphorylated 'inactive' Gsk3 is excluded from this association. Subsequently, we conducted studies to determine the significance of our findings. After knocking down Lrp5 and 6 in MPT cells, we found that Hgf mediated Lrp5/6 phosphorylation led to β-catenin stabilization, its nuclear accumulation and increased expression of the Wnt target gene c-myc. We also find that Hgf protects against epithelial cell apoptosis in an Lrp5/6 dependent fashion and increased Survivin gene expression. *In vivo*, the increase in Lrp5/6 phosphorylation and β-catenin stabilization on the first day after renal ischemic injury was significantly reduced in mice lacking Met receptor in the renal proximal tubule. Our results thus identify Hgf as an important transactivator of canonical Wnt signaling that is mediated by Met-stimulated, Gsk3-dependent Lrp5/6 phosphorylation.

The crosstalk of Hgf and canonical Wnt signaling in kidney repair

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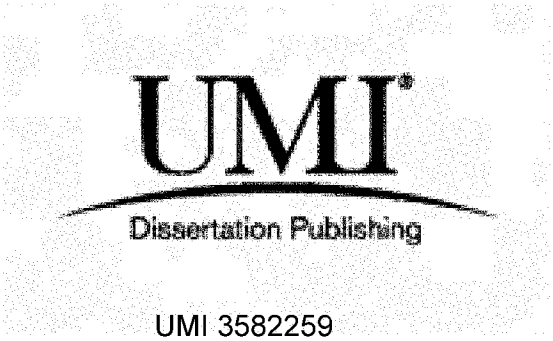

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Chapter 1. Background and Introduction

Introduction:

Renal tubular epithelial cells (TECs) are highly susceptible to injury during episodes of ischemia, and yet must serve as the progenitor cells for tubule repair[1, 2]. This repair process involves survival, dedifferentiation, migration, and proliferation of sub-lethally injured TECs, followed by redifferentiation to a fully functional phenotype. The repair results in the recovery of renal function that is typically seen after acute kidney injury (AKI) in clinical settings. Two signaling cascades that are activated following AKI and are known to regulate epithelial cell differentiation and survival are the hepatocyte growth factor (Hgf)/Mesenchymal-epithelial transition factor (Met) pathway and the integration/wingless (Wnt)/β-catenin (canonical) pathway[3-7].

Overview of Hgf/Met signaling:

Hepatocyte growth factor (Hgf) was originally discovered as a potent mitogenic protein ('hepatotropin') for rat hepatocytes in 1984[8]. Subsequently human Hgf was cloned and its primary structure clarified as a hetero dimer of 697-692 amino acids composed of an alpha and beta chain[9, 10] similar to plasminogen. In 1991, the previously unidentified molecules 'scatter factor'[11], 'tumor cytotoxic factor'[12], '3-D epithelial morphogen'[13] were all identified as being Hgf. Met, a product of the c-met proto-oncogene was identified as the receptor for Hgf at the same time. [14]

Hgf is primarily expressed in mesenchymal tissues and acts in a paracrine manner on its receptor Met that is mostly expressed in epithelia[15]. Hence, Hgf/Met signaling is a great example of epithelial-mesenchymal interactions that play crucial roles in organ development and in the repair process after injury. Besides its role as a mitogen, mitogen and 3-D morphogen[16], Hgf has

also been established as a potent anti-apoptotic[17], anti-fibrotic[18] and anti-inflammatory[19] factor. The dual-chain pre-pro form of Hgf has 728 amino acids which is cleaved to the single-chain biologically active Hgf by proteases including Hgf activator (HGF-A), matriptase, and hepsin[9]. HGF-A and its membrane-bound inhibitor HGF-A inhibitor-1 (HAI-1), are not only involved in the regulation of Hgf activation but their balance play a key role in macrophage activation and wound healing.[20]

Met is a single-pass heterodimer receptor composed of two intracellular (juxtamembrane and tyrosine kinase) domains, a transmembrane domain and three extracellular (Sema, PSI and IPT) domains[21]. When Hgf binds to Met, its kinase activity is switched on by receptor dimerization and *trans*-phosphorylation of Tyr1234 and Tyr1235 in the catalytic region. This is followed by phosphorylation of C-terminal tyrosine residues, Tyr1349 and Tyr1356, in the multifunctional docking site of the Met receptor leading to recruitment of multiple signaling molecules like PI3K (phosphatidylinositol 3-kinase), Grb2 (growth-factor-receptor-bound protein 2), Gab1 (Grb2)-associated binder 1), PLCg (phospholipase Cg), and Shp2 (SH2-domain-containing protein tyrosine phosphatase 2) that mediate downstream signaling[22]. While the a-chain of Hgf binds Met with high affinity, the low-affinity b-chain binding is required for Met activation and biologic responses[23]. Although Hgf is its only known activating ligand, Met can also be activated by semaphorins (when oligomerized with plexins) in an Hgf-independent manner[24].

The signal transduction pathways activated downstream of Met include the phosphoinositide 3-kinase–Akt (PI3K–Akt) pathways, the mitogen-activated protein kinase (MAPK) cascades [extracellular signal-regulated kinase (Erk) 1 and 2, Jun amino-terminal kinases (JNKs) and p38], signal transducer and activator of transcription proteins (STATs), and the nuclear factor-κb (NF-κb) pathways[21]. These pathways are responsible for transmitting signals from the plasma membrane to the nucleus to modulate gene transcription. While the proximal signaling of the Met receptor is unique, these downstream pathways are shared among different tyrosine kinase receptors, for example the epidermal growth factor receptor (EGFR).

In the MAPK cascade, Met activation of Ras through the Grb2–SOS complex or through tyrosine phosphatase SHP2 leads to Mek1/2 and subsequently Erk1/2 activation. The active Erks translocate to the nucleus where they activate several transcription factors including those that are involved in cell cycle. Unlike Erk, the activation of JNK and p38 MAPKs is mediated by upstream stimulation of Rac, a small GTPase that is switched on by a Ras–PI3K-mediated pathway. JNKs and p38s control many cellular processes including cell proliferation, differentiation and apoptosis[21]. Met can directly activate PI3K or indirectly through Ras leading to the formation of phosphatidylinositol-3,4,5-triphosphate, which tethers the Ser/Thr kinase Akt, to the plasma membrane. Once it is recruited to the membrane, Akt is activated via PDK1 (3-phosphoinositide dependent protein kinase-1) mediated phosphorylation. Akt mediates anti-apoptotic signaling via inactivation of pro-apoptotic molecules like Bad and p53. Akt also mediates Gsk3 inhibition and mammalian target of rapamycin (mTOR) activation[21]. Met activation leads to tyrosine phosphorylation of STAT3, which causes STAT3 to dissociate from the receptor, homodimerize and translocate to the nucleus, where it functions as a transcriptional regulator of several genes implicated in cell proliferation and differentiation[21]. Met activation is also known to stabilize NF-κb which translocates to the nucleus to stimulate the transcription of various genes, including mitogenic and anti-apoptotic genes[25].

The signaling pathways downstream of Met are regulated by protein–protein interactions and cellular compartmentalization. Other processes that regulate Met include extracellular shedding, intracellular cleavage and ubiquitin-mediated degradation[21]. After ligand binding, Met undergoes rapid internalization by clathrin-mediated endocytosis leading to Erk activation and focal adhesion complexes to mediate cell migration[26]. Subsequently Met traffics along the microtubule network in a protein kinase Cε (PKCε) dependent fashion to be delivered juxtanuclear where is causes STAT activation and nuclear translocation[27].

One of the key elements in Hgf/Met signaling is the short duration of the signaling response. While the rapid sequestration of the activated Met from the plasma membrane is required

for signaling (as mentioned above), it also ultimately leads to its endosomal sorting for lysosomal degradation; hence preventing sustained activation and modulating the physiologic responses generated[28]. The ubiquitination, endocytosis, and degradation of Met is mediated by Cbl, an E3 ubiquitin ligase[29]. Protein kinase-C can regulate Hgf-induced Met activation through phosphorylation of Ser985 in the juxtamembrane domain of Met[30]. Met is also regulated by several protein-tyrosine phosphatases which dephosphorylate the tyrosine residues to inhibit Met signaling[31]. Decorin, an extracellular proteoglycan, can bind directly to Met with high affinity acting as an antagonistic ligand[32].

Overview of Wnt signaling:

The Wnt signaling network is highly complex with numerous regulatory steps and levels of crosstalk with other signaling pathways. In 1982, Roel Nusse and Harold Varmus identified a new mouse proto-oncogene that was named int-1 (integration 1)[33]. In 1987, Nusse's group subsequently reported that the already known and characterized Drosophila segment polarity gene Wingless (Wg) was identical to this mouse mammary oncogene int-1[34]. Eventually the int/Wingless family was renamed the Wnt family and int-1 became Wnt1[35]

Wnts are evolutionarily conserved secreted Cys-rich glycoproteins proteins that require lipid modification (palmitoylation) of cysteine residues to allow the protein to bind its receptor due to the covalent attachment of fatty acids. They also undergo glycosylation, which attaches a carbohydrate in order to insure proper secretion[36]. In humans there are 19 genes that encode Wnts. Wnt more commonly associated with β-catenin-dependent signaling are Wnt1, Wnt3a and Wnt8 while Wnt5a and Wnt11 are known to be predominantly involved in β-catenin-independent signaling[37].

Numerous Wnt receptors and co-receptors have been identified[37]. These include low-density lipoprotein receptor-related protein 5/6 (Lrp5/6)[38-40], Frizzled (Fzd) [41], protein Tyr kinase 7 (PTK7)[42], receptor Tyr kinase-like orphan receptor (ROR)[43], muscle skeletal receptor Tyr kinase (MUSK)[44], proteoglycans[45] and receptor Tyr kinases (RYKs)[46]. While Fzd is the common receptor for most Wnt signaling pathways, the use of either Lrp5/6 or ROR determines whether canonical or non-canonical Wnt signaling is initiated[47]. Besides the Wnt proteins, other extracellular ligands of these receptors include agonists like Norrin[48] and R-spondin[49]; and anatagonists like including Dickopf-related protein 1 (Dkk1)[50], secreted Frizzled-related protein (SFRP)[51], Cerberus[52], Sclerostin [53] and WNT inhibitory factor (WIF)[54].

Wnt signaling has been traditionally categorized into the canonical Wnt/ β-catenin pathway and the non-canonical β-catenin-independent pathways. In the canonical Wnt pathway, Wnt activation of the receptors Fzd and Lrp5/6 leads accumulation of β-catenin in the cytoplasm and its eventual translocation into the nucleus to act as a coactivator of transcription factors that belong to the TCF (T cell factor)/ LEF (lymphoid enhancer-binding factor) family. This stabilization of β-catenin involves inhibition of a destruction complex, consisting of Axin, adenomatosis polyposis coli (APC), protein phosphatase 2A (PP2A), glycogen synthase kinase 3 (Gsk3) and casein kinase 1α (Ck1α) proteins, which is normally responsible for ubiquitination and proteasome-mediated destruction of β-catenin in the unstimulated cells[37]. This Wnt-mediated inhibition of β-catenin destruction involves inhibition of the activities of Axin and Gsk3, a process that involves their removal from the protein complex, translocation to the plasma membrane and interaction with Lrp5/6 proteins. Besides Wnt, other proteins like biglycan and complement C1q can also activate canonical Wnt signaling[55]. The canonical Wnt pathway is involved in many physiologic processes including cell differentiation, proliferation and survival. The regulation of canonical Wnt signaling will be discussed in the next chapter.

The best-characterized non-canonical Wnt pathway is the planar cell polarity (PCP) pathway[56]. This does not involve Lrp5/6 but instead uses ROR and PTK7 as co-receptors with Fzd.

It is activated by binding of Wnt to Fzd which leads to Dishevelled (Dsh) mediated activation of the small G-protein Rho and subsequently Rho-associated kinase (ROCK) which are major regulators of the cytoskeleton. This also leads to Rac1-mediated JNK activation which is responsible for actin polymerization. PCP and canonical Wnt signaling are known to antagonize each other. Wnt5a, which is a known activator of PCP signaling, competes for and inhibits binding of Wnt3a to Frizzled 2, thereby suppressing the β-catenin-dependent pathway[57]. PCP pathway is responsible for cytoskeletal signaling and cell polarity.

The noncanonical Wnt/calcium pathway is also independent of Lrp5/6, Gsk3 and β-catenin and plays a role in regulating calcium release from the endoplasmic reticulum (ER) to control intracellular calcium levels. As with PCP pathways, this also involves Wnt-Fzd binding. However in this case, besides Dsh there is also activation of G-protein which then leads to the activation of either phospholipase C (PLC) or cGMP-specific phosphodiesterase (PDE). PLC activation results in PIP2 cleavage into diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP3) and calcium release from the ER and activation of calmodulin-dependent kinase II (CAMKII), calcineurin and protein kinase C (PKC), which activate the transcriptional regulator nuclear factor associated with T cells (NFAT). On the other hand PDE activation inhibits calcium release from the ER. This Wnt/ calcium pathway is involved in cancer, inflammation and neurodegeneration[58].

Cross-talk of Wnt, Hgf/Met and other signaling pathways:

Recent studies have put into question this distinction of Wnt signaling into canonical and non-canonical due to increasing evidence of the overlap of these pathways and an integrated rather than linear approach to Wnt signaling has been proposed[59, 60]. Moreover Wnt signaling has been known to crosstalk with Hgf/Met and other signaling pathways in the regulation of downstream responses.

The crosstalk of Met and Wnt signaling has been observed in various cellular and physiologic contexts. Like canonical Wnts, Hgf can induce Gsk3 inhibition and nuclear translocation of β-catenin with activation of TCF/LEF-mediated gene transcription[61]. Loss of HAI-1 resulted in activation of not only Hgf but also β-catenin expression[62]. Several studies provide insight into the possible mechanism and physiologic consequences of Hgf-induced β-catenin activation. In cancer cells, Hgf was shown to increase tyrosine phosphorylation and nuclear transfer of β-catenin in a Wnt-independent fashion[63]. Zeng et al showed that the tyrosine residues 654 and 670 in β-catenin are crucial in regulation of β-catenin interactions with Met and its cellular localization[64]. Hgf was found to induce β-catenin phosphorylation at Y142 and a nuclear localization[65]. The activation of β-catenin by Hgf has been shown to be important for cell scattering [66] as well as Hgf induced hepatomegaly and hepatocyte proliferation[67].

Beside Hgf-mediated β-catenin stabilization, there are other levels of the Met and Wnt crosstalk. The Wnt signaling protein, APC was found to function downstream of Hgf and PI3K and to be involved in the regulation of cell morphology and migration[68]. Met is also known to be a one of the Wnt target genes[69]. Lrp5 inhibition was found to decrease both Met expression and Hgf-induced cell motility[70]. Hgf induced expression of Lef1 via PI3K/Akt and NF-κB signaling was shown to promote tumor invasion[71].

The Hgf/ Met and canonical Wnt signaling are known to cross-talk at the level of Gsk3 which they both inhibit, albeit in different ways. While Hgf-stimulated Met activation is known to result in Akt mediated inhibitory phosphorylation of Gsk3β at serine 9 [72], the mechanism by which Gsk3 is inhibited as a result of Wnt signaling is still under debate. The conventional hypothesis suggests that Gsk3 is directly inhibited by binding to the C-terminus of Lrp5/6 [73] while a more recent study suggests that Gsk3 is sequestered into multi-vesicular bodies and hence separated from its cytosolic substrates, and prevented from phosphorylating (and degrading) β-catenin [74] . This subject is discussed in more detail in Chapter 4.

Wnt signaling is also known to crosstalk with other signaling pathways in which Gsk3 is the focal point of cross communication. Besides β-catenin stabilization, Wnt-mediated Gsk3 inhibition can also lead to activation of the m-TOR pathway by preventing the Gsk3-mediated phosphorylation of TSC2[75]. Since the Gsk3-mediated phosphorylation of TSC2 is primed by the cellular energy sensor AMPK, hence Gsk3 is a focal point of the interaction of the Wnt, m-TOR and AMPK pathways. Similarly, Wnt was shown to decrease Gsk3-meiated SMAD1 phosphorylation, in which a priming phosphorylation was provided by growth factor-dependent MAPK phosphorylation[76]. Hence Gsk3 also plays a central role in the cross-talk of the Wnt, BMP and MAPK pathways. Wnt signaling can also crosstalk with other pathways in ways that are not Gsk3-dependent. Wnt was reported to regulate morphogenesis through CK1-mediated control of Rap1 signaling a β-catenin-independent fashion[77].

Similarly Hgf/ Met signaling is known to communicate with other pathways. Hgf was found to activate the Notch signaling pathway to reduce myocardial injury by reducing cardiomyocyte apoptosis[78]. Met was reported to crosstalk with Axl to promote neuronal cell migration and survival[79]. MUC1 was found to inhibit Hgf-induced c-Met activation and cell motility and invasion in cancer cells[80]. Leucine-rich repeat kinase-2 was found to increase Met activation independent of Hgf and promote tumor cell survival and proliferation[81]. NF-kappaB was observed to mediate the crosstalk between the SDF-1alpha/CXCR4 and Hgf/Met signaling in glioma cell migration[82]. Finally, the crosstalk between Hgf and Tgf-β pathways has been noted in several studies[83].

The physiologic and clinical significance of the crosstalk of Met and Wnt signaling pathways has been noted in many studies. In a model of myocardial injury and repair, over-expression of multiple growth resulted in activation of multiple downstream signaling molecules including Wnt, Akt and MAPK leading to enhanced repair and functional recovery[84]. Met hyperactivation was shown to increase Wnt7a and b levels and Pax2 expression in a polycystic

including Gsk3, juxtaposed with sites phosphorylated by Casein Kinase-1 (CK1). Together these sites are called PPSPXS motifs and they are highly conserved among species and between the Lrp5 and 6 proteins[89]. Upstream of these motifs is an S/T cluster which is also phosphorylated by CK1.

Through very elegant real-time imaging the Niehr group showed that Wnt binding to the Fzd-Lrp6 heterodimer leads to the formation of 'signalosomes', multi-protein complexes containing phosphorylated Lrp6, Fzd, Dvl, Gsk3, and Axin[90]. It is proposed that this 'initiation' phase[91] is followed by an 'amplification' phase where Dvl polymers promote further Lrp6 aggregation[92] creating a high density of phosphorylation sites and hence increasing the efficiency of phosphorylation of Lrp6 by Gsk3 [89]. This signalosome formation eventually leads to inhibition of Gsk3-dependent β-catenin phosphorylation.

Besides their involvement in canonical Wnt signaling, Lrp5 and 6 are also known to play a role in other non-Wnt related pathways. These include PKA-dependent Lrp6 phosphorylation [93]and Lrp6-dependent cAMP production after activation of G protein-coupled receptors (GPCRs)[94] in response to PTH, and Lrp5/6 ECD-mediated inhibition of non-canonical Wnt signaling[95]. Recently receptor tyrosine kinases like FGFR2, FGFR3, EGFR and TRKA kinases were shown to induce phosphorylation of Lrp6 through activation of mitogen-activated protein kinases (MAPKs) leading to augmentation of Wnt activation[96]. As mentioned below, connective tissue growth factor (CTGF) was also shown to mediate phosphorylation of Lrp5/6.

Due to their diverse physiologic roles, Lrp5 and 6 proteins are implicated in various human diseases. Lrp5 mutations are known to result in osteoporosis-pseudoglioma syndrome with defective bone mass and eye development[97] and with hepatic cystogenesis[98], while Lrp6 mutations are associated with Alzheimer's disease[99], and with early coronary artery disease and metabolic syndrome[100]. Lrp5/6 are also involved in aortic calcifications[101] and in osteoarthritis[102]. In the kidney Lrp6 has been implicated in the pathogenesis of diabetic nephropathy mediated by CTGF[103]. Ren et al recently described a central role of Lrp6 in kidney fibrosis. Besides downstream signaling

through β-catenin, Lrp6 was found to crosstalk with multiple other signaling pathways including PDGF-, TGF-β-, and CTGF-activated MAPK and JNK signaling cascades[104]. Germline mutations of Lrp5 result in mammary tumorgenesis[105] and in impaired cholesterol and glucose metabolism[106], while global knockout of Lrp6 leads to neonatal lethality with defects major defects in the cardiovascular and nervous system similar to many Wnt mutant phenotypes.[107] The various kinases, including Gsk3, that are involved in Lrp5/6 phosphorylation will be discussed in Chapter 4.

Based on the data that other receptor tyrosine kinases can mediate Lrp5/6 phosphorylation and based on our previous data that Hgf can promote downstream Wnt signaling through the PI3K/Akt pathway[72], we sought to ask whether Hgf can also promote proximal Wnt signaling through Lrp5/6 phosphorylation.

Hypothesis and specific aims:

Hgf stimulation can lead to Lrp5/6 phosphorylation.

1. To find the time course, stoichiometry and sites of Hgf-dependent Lrp5/6 phosphorylation.
2. To find whether Hgf-dependent Lrp5/6 phosphorylation is dependent on Wnt or Met.

Materials and Methods:

Cell culture and Reagents

Mouse inner medullary collecting duct-3 (mIMCD-3)[108] and mouse proximal tubule (MPT)[109] epithelial cells were maintained using standard cell culture techniques in Dulbecco's modified Eagle's medium (DMEM)-F12 medium containing 10% fetal bovine serum (FBS). Antibodies to β-actin, Met and phospho-Met (Tyr1234) were obtained from Santa Cruz Biotechnology. Antibodies to Lrp6, Lrp5 and phospho-Lrp6 Ser1490 were obtained from Cell

Signaling. Antibodies to phospho-Lrp6 Thr1572 and Ser1607 were obtained from Millipore. Recombinant mouse Wnt3a and Dickkopf related protein 1 (Dkk-1) were purchased from R&D Systems. Recombinant human HGF was purchased from Sigma Aldrich. Met kinase inhibitor-II were purchased from Calbiochem. Met inhibitor PHA-665752 was purchased from Selleck Chemicals. For western blotting secondary antibodies were purchased from Invitrogen. EDTA-free Protease and Phosphatase Inhibitor cocktail was obtained from Thermo Scientific.

Western analysis

Cells were serum starved for 24 h, followed by Hgf (40 ng/ml) or Wnt3a (50ng/ml) stimulation for the indicated time. Cells were lysed in RIPA buffer (50mM Tris-Hcl, 150 mM NaCl, 1.0% deoxycholic acid, 0.1 % SDS, 1.0% TritonX-100 and 2mM EDTA), insoluble material removed by centrifugation, and the supernatant protein content determined using the Bradford assay. The proteins were separated using 7.5% - 20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE] (Bio-Rad), electrophoretically transferred to Immobilon-P membranes (Millipore), immunoblotted with the appropriate antibody overnight, and visualized by enhanced chemiluminescence (Amersham Biosciences, Inc.) or West Femto Maximum Sensitivity Substrate (Thermo Scientific). Quantification of ECL signals was performed using NIH Image J software.

Reverse Transcriptase -Polymerase Chain Reaction

MPT cells were lysed and total RNA isolated using the RNeasy kit (Qiagen Valencia, CA, USA). One microgram of RNA was reverse transcribed using random hexamer primers (SuperScript II, Invitrogen) and PCR was performed using REDTaq DNA Polymerase (Sigma) followed by visualization of amplified DNA through agarose gel electrophoresis.

Statistical methods

The data between two groups was compared using the student's two-tailed *t* test. Significance was determined at $P < 0.05$ and the error bars represent standard deviations.

Results:

The epithelial cells of the proximal tubule are the prime site of injury during ischemia reperfusion injury (IRI)[110]. Since our ultimate objective was to study the cross-talk of Hgf and Wnt signaling after AKI, for our cell-based experiments, we chose an immortalized cell line derived from the proximal tubule of the murine kidney. These mouse proximal tubule (MPT) cells were originally isolated at Boston University,[109] however they have been extensively used in our lab for cell-based experiments[111].

Treatment of non-confluent, serum-starved MPT cells with Hgf (40ng/ml) for 10 minutes lead to activation of the Met receptor and a two-fold increase in phosphorylation of Lrp6 at S1490, with no change in the total amount of Lrp5 and 6 (Fig1A, quantification in Fig 1B). The phosphorylation peaked within 5-10 minutes of stimulation and declined over the next 60 minutes (Fig 1C). Similarly, Met activation resulted in phosphorylation of Lrp6 at T1572 and S1607 (Fig 1D, quantified in E) at this early time point. Of note, PCR revealed that MPT cells express the mRNA for both Lrp5 and Lrp6 (Fig1F), and the commercial antibodies used do not distinguish between the phosphorylated forms of the two proteins. Individual knockdown of Lrp6 or Lrp5 suggests that the homologous phosphorylation motifs on the Lrp5 protein (S1502, T1577 and S1608) are also activated by Hgf (Chapter 5). Hence we presume that Hgf results in phosphorylation of the PPS/TP motifs on both Lrp5 and 6 proteins simultaneously (subsequently referred to as Lrp5/6 phosphorylation).

As expected, treatment of MPT cells with Wnt3a (50ng/ml for 10 minutes) induced phosphorylation of these same motifs on Lrp6 (Figure 1G) and the amount of phosphorylation was

slightly higher (around 3 fold) with Wnt. Wnt3a is the prototype canonical Wnt ligand used to induce Lrp5/6 phosphorylation and has been used extensively in many studies[47].

Our previous *in vivo* data has shown that renal collecting duct cells also express high levels of the Met receptor[112]. Similar to MPT cells, Hgf stimulation of collecting duct mIMCD-3[108] cells induced Lrp5/6 phosphorylation within 10 minutes (Fig 1H). This demonstrates that Hgf-dependent Lrp5/6 phosphorylation was not a cell-line specific phenomenon.

The rapid increase in Lrp5/6 phosphorylation following Hgf stimulation suggested that Wnt expression/secretion may not be required for this pathway of Lrp5/6 activation. To address this, serum starved MPT cells were incubated with the well-known extracellular Wnt-antagonist Dickkopf-1 (Dkk-1, 0.1 μg/ml) followed by stimulation with Hgf or Wnt3a. As previously mentioned, Dkk-1 is a secreted glycoprotein that binds to the ECD of Lrp6 and prevents binding of Wnt and hence the phosphorylation of Lrp5/6[50] and downstream canonical Wnt signaling[113]. Treatment with exogenous Wnt3a resulted in the expected increase in Lrp5/6 phosphorylation, with Dkk-1 pretreatment inhibiting this effect by approximately 70% (Fig 1I, quantified in J). In contrast, Hgf-stimulated Lrp5/6 phosphorylation was not inhibited by Dkk-1 (Figure 1I, quantified in J). Of note, incubation of control cells with Dkk-1 resulted in a modest but significant decrease in basal Lrp5/6 phosphorylation.

To determine if Hgf-stimulated Lrp5/6 phosphorylation required activation of the Met receptor, MPT cells were preincubated with the specific Met kinase inhibitor PHA-665752 (0.2 μM) followed by stimulation with Hgf. PHA-665752 is a highly specific, small molecule, ATP-competitive, active-site inhibitor of the catalytic activity of c-Met kinase[114] and has been shown to prevent Hgf-dependent cell migration and proliferation[115]. Met inhibition reduced Hgf-stimulated Lrp6 phosphorylation at S1490, T1572 and S1607 by greater than 90% (Figure 1K-N). These findings were reproduced with a separate Met inhibitor, Met inhibitor-II (0.5 μM, data not shown)

and demonstrate that Hgf stimulation of Lrp5/6 is Wnt-independent and requires activation of the Met receptor.

Discussion:

Recent studies have shown that growth factors can induce Lrp5/6 phosphorylation and modulate canonical Wnt signaling. Fgf and Egf has been shown to mediate MAPK-dependent phosphorylation of the Wnt co-receptor Lrp5/6 [116] and contribute to downstream signaling in both a β-catenin dependent[96, 116] and independent[117] fashion. CTGF has been shown to lead to phosphorylation of Lrp5/6 and eventually β-catenin mediated gene transcription[103]. We report for the first time that Hgf can mediate phosphorylation of the Wnt co-receptor Lrp 5/6 in renal epithelial cells. This phosphorylation happens rapidly, is short-lived, independent of Wnt and downstream of Met receptor phosphorylation. The reproduction of these findings in another cell line (m-IMCD3) suggests that this was not a cell line restricted phenomenon.

As mentioned above, Met activation by Hgf typically induces a short-lived, acute downstream signaling response due to rapid endocytosis and degradation of the Met receptor[28]. Our finding that Hgf-mediated Lrp5/6 phosphorylation lasts for only 1-2 hours is quite different from Wnt- mediated Lrp5/6 phosphorylation which usually lasts for 24 hours. This phenomenon might be due to the difference in half-life of Hgf versus Wnt3a or due to the difference in the rate of turnover and degradation of the phosphorylated Met or Lrp5/6. In any case, this does suggest Hgf and Wnt are likely to initiate potentially different physiologic response in response to Lrp5/6 activation.

The Lrp5/6 PPS/TP sites are not only phosphorylated by Wnt stimulation but also have constitutive phosphorylation[118] mediated by various kinases[89]. It has been shown that these motifs act synergistically to initiate downstream Wnt signaling and that phosphorylation of one site alone is not enough.[118, 119] Wnt stimulation has been shown to cause activation of multiple motifs

simultaneously.[118, 120, 121]. Since the commercial antibodies are available for only three of the five phosphorylation sites, we not able to confirm phosphorylation at the two remaining motifs; T1530 and S1590. However, the observation that Met activation leads to phosphorylation of at least 3 of the 5 known Lrp5/6 activation sites (including the most proximal-S1490 and most distal-S1607) suggests that this phosphorylation is likely to be physiologically relevant.

Our findings reveal a proximal mode of cross-talk between the Hgf and Wnt signaling pathways in addition to the previously known distal interaction through Met/PI3K/Akt/Gsk3. We further pursued the mechanism and significance of this phosphorylation.

Chapter 3. Hgf-dependent Lrp5/6 phosphorylation is regulated by cell confluency

Introduction:

AKI is characterized by tubular epithelial cell (TEC) death, sub-lethal injury to other TECs, loss of the TEC-TEC and TEC-ECM (extracellular matrix) contacts leading to cell detachment, cast formation and tubular obstruction; all of which contributes to reduced glomerular filtration rate (GFR) and an aggressive immune response in the kidney.[122]

The renal TEC, which is a site of initial injury during AKI, also plays the key role in the subsequent repair process[123]. Sub-lethally injured TECs undergo a process of de-differentiation (from a highly differentiated basal state) with the expression of mesenchymal markers. This change in the phenotype of these cells plays an important role in the repair process after ischemic kidney injury.[124] TEC dedifferentiation enables them to adopt characteristics that are essential for the initial part of the repair process including the ability to proliferate, flatten, elongate and migrate[124]. This leads to cellular restoration which is followed by a process of re-differentiation of these cells to revive their functional capacity and thereby complete the repair process. In this later phase of renal repair, the cells stop their migration and proliferation, reestablish the cell-cell and cell-ECM interactions and apico-basal polarity.[2] Persistence of the de-differentiated phenotype of the TECs and failure to re-establish normal TEC-TEC and TEC-ECM contacts is believed to lead to fibrosis/scarring and development of chronic kidney disease (CKD).[125]

Overview of cell-cell and cell-matrix junctions and cell confluency:

In a healthy adult kidney, the highly differentiated TECs are tightly packed as a result of the cell-cell junctions (CCJs) at their lateral membranes including the tight junctions (TJ) that are towards the apical end of the cell and control paracellular transport and the adherens junctions (AJ) that are localized basally from the TJs and are primarily responsible for cells adherence to each other. In AJ, β-catenin binds to E-cadherin via its central Armadillo domain and to α-catenin via its amino-terminal domain.[126] β-catenin phosphorylation at tyrosine 142 and tyrosine 654 decreases its binding with cadherins at the AJ.[127, 128] β-catenin that is not bound to cadherins, evades the destruction complex and enters the nucleus of the cell to mediate de-differentiation signaling through the LEF/TCF transcription factors.

In an in vitro setting using cell culture systems, the effects of CCJs on differentiation of epithelial cells can investigated by varying cell confluency (density) in the plating dish. Among the key players in this process are factors like Hgf and Wnt.[2] We have previously shown that cells maintained at high confluence selectively down regulate signaling events such as Rac activation and β-catenin-dependent transcription that would otherwise promote cell dedifferentiation and migration[2]. Using mIMCD-3 cells we showed that increasing cell confluency resulted in a greater tendency of cells to organize into epithelial tubes in a 3D Matrix and a significant decrease in migratory responsiveness to Hgf in a cell monolayer.[72] Analysis of downstream signaling revealed that PI3K-dependent activation of Akt and Rac were selectively diminished in confluent cells. In nonconfluent cells treated with Hgf, the high level of Akt activation resulted in inhibitory phosphorylation of Gsk3β and increased β-catenin nuclear signaling. In contrast, confluent cells, in which Hgf-stimulated Akt activation was diminished, displayed less inhibitory phosphorylation of Gsk3β and less nuclear signaling by β-catenin.

Other studies also provide links between Wnt signaling and cell confluency. The AJ protein N-cadherin was found to be associated with the Wnt receptor complex of Lrp5 and Axin.[129] Stewart et al showed that the level of Cadherin 6 is increased with confluency in MDCK cells and was regulated by β-catenin.[130] Maher et al. showed a cadherin-dependent mechanism of regulation of β-catenin stability through Axin, APC and Gsk3[131]. They also showed that cell-cell adhesion increases the turnover of β-catenin though promotion on N-terminal phosphorylation by cadherins.

In MDCK cells at high confluency, the TJ protein Zona Occludens-1 (ZO-1) accumulation at the TJ suppresses cell proliferation by cytoplasmic sequestration of the transcription factor ZONAB and reduced nuclear accumulation of cell division kinase-4.[132] Hence this provides a key mechanism of inhibition of cell proliferation at high confluency known as 'contact inhibition'. At low confluency ZONAB is in the nucleus and transcriptionally active.[132] Lima et al showed that ZO-1 senses epithelial cell density and is involved in the switch between proliferation and differentiation of renal proximal epithelial tubular cells (OK Cells).[133] They showed in this effect in the small islands of sparsely plated OK cells. Interestingly, cells in the center of the islands with cell-cell contact had poor expression of ZONAB and high expression of the apical differentiation markers Megalin/Cubilin. On the other hand, cells on the periphery of the islands, which lacked external tight junctions, strongly expressed nuclear ZONAB and the proliferation marker PCNA (proliferating cell nuclear antigen). These cells failed to differentiate. This study emphasizes the importance of the method of plating epithelial cells for cell culture experiments. Cells plated in small islands can have different signaling (and hence physiologic) responses compared to cells plated uniformly throughout the culture dish.

Besides its communication with CCJs as described previously, Hgf is also known to modulate lamellipodia protrusion, cell spreading and migration, and branching morphogenesis in cultured epithelial cells, events that require regulation of cell-matrix interactions. Using subconfluent mIMCD-3 cells, we studied the effect of Hgf on focal adhesion complexes that are

required for its migratory and tubulogenic responses.[134] The TEC-ECM interactions are also critical in the survival of epithelial cells and absence of these signals triggers cell death through a process called anoikis.[135]

The data above highlights the importance of cell-cell junctions and cell confluency in not only the in epithelial cell signaling and physiology but also in the repair process after AKI. During our cell culture experiments in the study of Hgf-dependent Lrp5/6 phosphorylation, we noted that the degree of Lrp5/6 phosphorylation in both stimulated and unstimulated cells was variable depending on the plating density of the cells in the culture. Due to this observation plus the data that Hgf/Wnt signaling can significantly regulate cell-cell and cell-matrix interactions, we conducted experiments to test the effect of cell confluency on Hgf-dependent Lrp5/6 phosphorylation.

Hypothesis and specific aims:

Hgf-dependent Lrp5/6 phosphorylation is modulated by cell confluency.

1. To find out how Hgf mediates Lrp5/6 phosphorylation in MPT cells plated at standardized confluencies (ranging from very low to maximal)

Materials and Methods:

Cell culture and Reagents

MPT cells were maintained using standard cell culture techniques in DMEM-F12 medium containing 10% FBS. Antibodies to β-actin, Met, phospho-Met (Tyr1234) and Gsk3-β were obtained from Santa Cruz Biotechnology. Antibodies to Lrp6, Lrp5, phospho-Lrp6 Ser1490, total

Erk1/2 and phospho-Erk1/2 (Thr202/Y204) antibodies were obtained from Cell Signaling. Antibodies to phospho-Lrp6 Thr1572 and Ser1607 were obtained from Millipore, while anti-E-cadherin antibodies were from BD Biosciences. Recombinant human HGF was purchased from Sigma Aldrich. For western blotting experiments secondary antibodies were purchased from Invitrogen. EDTA-free Protease and Phosphatase Inhibitor cocktail was obtained from Thermo Scientific.

Cell density

Cells were counted using a hemocytometer. Low, medium and high confluency cells were plated at $2.1x10^3/cm^2$, $4.2x10^4/cm^2$ and $4.2x10^5$ cells/cm^2, respectively. Caution was taken to ensure that all cells are plated uniformly throughout the cell culture dish rather than accumulation of islands of cells. Experiments were performed 24 hours after plating (representative cell morphology as shown in Fig 2A).

Western analysis

Cells were serum starved for 24 h, followed by Hgf (40 ng/ml) stimulation for the indicated time. Cells were lysed in RIPA buffer, insoluble material removed by centrifugation, and the supernatant protein content determined using the Bradford assay. The proteins were separated using 7.5% - 20% SDS-PAGE gels (Bio-Rad), electrophoretically transferred to Immobilon-P membranes (Millipore), immunoblotted with the appropriate antibody overnight, and visualized by enhanced chemiluminescence (Amersham Biosciences, Inc.) or West Femto Maximum Sensitivity Substrate (Thermo Scientific). Quantification of ECL signals was performed using NIH Image J software.

Statistical methods

The data between two groups was compared using the student's two-tailed *t* test. Significance was determined at $P < 0.05$ and the error bars represent standard deviations.

Results:

To determine the impact of confluency on Hgf-stimulated phosphorylation of Lrp5/6, MPT cells were plated at 'low', 'medium' and 'high' confluency (representative images in Fig 2A). This density of cells was chosen based on our previous data[72, 111] and on our observation of Lrp5/6 phosphorylation changes depending on cell morphology during our initial experiments.

At 'low' confluency the cells express low levels of E-cadherin and Lrp6, but respond in a robust fashion to Hgf with Met activation, Erk activation and Lrp6 phosphorylation at S1490 (Fig 2B, quantified in C). At 'medium' confluency, the amount of both total Lrp5/6 and phosphorylated Lrp5/6 increased in unstimulated and Hgf-stimulated cells. However, there was only a minor, non-significant increase in the ratio of phosphorylated Lrp5/6 to total Lrp5/6 following Hgf stimulation. At 'high' confluency Hgf-stimulated Met activation and signaling were completely suppressed, with no change in Lrp5/6 phosphorylation with Hgf stimulation, however the basal Lrp5/6 phosphorylation persisted (Fig 2B).

Hgf-mediated phosphorylation of Lrp6 at T1572 and S1607 demonstrate a similar confluency-dependent pattern (Fig 2D).

Discussion:

Our results show that Hgf maximally phosphorylates the Lrp5/6 coreceptors when MPT cells are plated at a very subconfluent level (Fig 2A). These findings, coupled with our previous demonstration that Hgf-mediated inhibition of cytosolic Gsk3 occurs only in subconfluent cells [72], suggest that Hgf/Met signaling specifically transactivates canonical Wnt signaling in flattened,

spindle shaped, de-differentiated epithelial cells, reminiscent of sub-lethally injured cells after AKI. This pathway is apparently suppressed as cells become more differentiated and confluent, resembling the healthy, uninjured epithelial cells of the renal tubule. It is interesting to speculate why.

As mentioned above, as cells become more confluent ('low to 'medium'), the cell-cell junctions are increased and cell-ECM junctions are reduced. The stabilized (non-degraded) β-catenin is diverted more toward AJ rather than the nucleus. β-catenin-mediated gene transcription and consequently canonical Wnt signaling is reduced which would have otherwise retained the cells in a de-differentiated state. Hence the ability of Hgf to phosphorylate Lrp5/6 also decreases to allow reduction in canonical Wnt signaling and increase in the differentiation status of the cells. Our subsequent results (Fig 4) show that Hgf-dependent Lrp5/6 phosphorylation was indeed responsible for β-catenin stabilization and nuclear localization.

As the cells reach maximal confluency ('high'), the Hgf/Met signaling is completely blocked. While this has other downstream effects (e.g. blockage of Erk phosphorylation), it also blocks the ability of Hgf to phosphorylate Lrp5/6 which is dependent on Met phosphorylation (Fig 1). At this time it's not clear how Hgf's ability to phosphorylate Met stops when cell are highly differentiated and cuboidal with maximal cell-cell and least cell-ECM contacts. However it does make sense. For cells to get fully differentiated, downstream Met signaling (responsible for de-differentiation, migration and proliferation) has to stop. It is possible that at high confluency, there are conformational changes in or mislocalization of the Met and Lrp5/6 receptors leading to changes in Hgf-dependent phosphorylation, however, this is not experimentally proven in our study.

The other interesting finding is that basal Lrp5/6 phosphorylation also increased as the cell confluency increased. Our earlier (Fig 1) and subsequently (Fig 3) findings, suggest that there is a potential activator of Wnt signaling in the serum-free conditioned media of MPT cells, which might

be secreted by these cells. This is because both Dkk-1 (Fig 1) and Gsk3 (Fig 3) inhibition led to a significant reduction in basal Lrp5/6 phosphorylation. It is possible that MPT cells express a Wnt ligand as they become more confluent that can induce an autocrine/paracrine increase in Lrp5/6 phosphorylation. Wnt signaling is also known to be increased during cell cycle activation[136]. Even though we used serum-free conditions, it is possible that as cells go from 'low' to 'medium' confluency, they acquire greater proliferative potential due to autocrine secreted factors.

Since the phenomenon of Hgf-dependent Lrp5/6 phosphorylation was maximal at the 'low' confluency, we restricted all subsequent cell-based experiments to this condition.

Chapter 4: Hgf mediates Lrp5/6 phosphorylation through Gsk3

Introduction:

Lrp5/6 phosphorylation can be mediated by multiple kinases depending on the activating stimulus and cellular context.[89] Zeng et al identified Gsk3 and CK1 as dual kinases that mediate Wnt-dependent Lrp5/6 phosphorylation[121]. CK1 is known to phosphorylate the S/T cluster N-terminal to the first PPS/TP motif at S1490 on Lrp6 and also sites that are 3 or 6 amino acids C-terminal to each PPS/TP motif that is the site of Gsk3 phosphorylation. In addition to Gsk3 and CK1, G protein-coupled receptor kinases (Grk5/6) have also been shown to phosphorylate PPS/TPxS motifs in a Wnt-dependent fashion.[137] On the other hand, Protein kinase A (PKA) in response to parathyroid hormone (PTH) and PFTAIRE protein kinase1 (Pftk1) during cell mitosis can phosphorylate this motifs in a Wnt-independent fashion.[136, 138] Recently, mitogen activated protein kinases (MAPKs) have also been shown to phosphorylate Lrp6 and promote canonical Wnt signaling.[96, 116] Since Gsk3 is known to mediate Wnt-dependent Lrp5/6 phosphorylation[121], and Hgf can regulate canonical Wnt signaling through Gsk3,[72] and also since Gsk3 plays a key role in AKI and repair[139], we chose to study the whether Gsk3 was also the kinase that mediated Hgf-dependent Lrp5/6 phosphorylation.

Overview of Gsk3 signaling:

Gsk3 was first identified in 1980 as a protein kinase that inactivates glycogen synthase[140], however it was later found to play major roles in multiple signaling pathways and be involved in the regulation of key physiologic process including cell proliferation, differentiation and apoptosis

in response to stimulation by growth factors[141]. Due to this central physiologic role, Gsk3 is involved in the pathogenesis of many diseases including bipolar disorder, Alzheimer's disease, diabetes mellitus, disorders of bone mass, vascular calcification, cardiac hypertrophy, obesity, cancer and kidney diseases like acute kidney injury, chronic kidney disease and nephrogenic diabetes insipidus[141]. Hence there is a strong pharmaceutical interest in the development of inhibitors and activators of Gsk3 for therapeutic purposes.

Gsk3 is highly conserved serine/threonine kinase and has two isoforms Gsk-3α (51 kDa) and Gsk-3β (47 kDa) that have almost 98% homology in the catalytic domain, but significant differences in their C- and N-termini.[142] While homozygous knockout of Gsk3β in mice results in an embryonic-lethal phenotype [143], mice with homozygous knockout of Gsk3α[144] are viable with a much milder phenotype. These date suggest major functional differences in the two isoforms. however, they are known to be functioning redundant in many signaling pathways including canonical Wnt signaling[145]. Besides the two major isoforms, there is an alternatively spliced GSK-3b variant (encoding GSK-3β2) which is known to play a role in neurons[146].

Gsk3 is unique in being a constitutively activate kinase that is mostly regulated through inhibition. Its signaling specificity is regulated through phosphorylation, substrate priming, proteolytic cleavage, association with multi-protein complexes, and through intracellular compartmentalization.[146, 147] Gsk3 substrates are known to be phosphorylated by a priming kinase at 4 amino acids C-terminal to the site phosphorylated by Gsk3 (consensus recognition sequence proposed as S/T-X-X-X-pS/pT). For example CK2 is the priming kinase in the Gsk3 mediated phosphorylation of glycogen synthase. Through crystallographic studies it was shown that this priming phosphorylation is directly coupled to catalytic activation[148]. However this mechanism of priming is not known to be involved with some Gsk3 substrates like Lrp5/6 which don't contain this consensus motif in their phosphorylation sites.

Gsk3 is regulated by both serine/threonine and tyrosine phosphorylation. The sites S21 on Gsk3α and S9 on Gsk3β are inhibitory and known to be phosphorylated by various kinases including Akt, PKA, PKC, ILK, S6K and RSK2[146]. T43 and S389/390 can be phosphorylated by Erk[149] and p38MAPK[150] respectively, both of which also result in inhibition. The sites Y279 on Gsk3α and Y216 on Gsk3β are activating and known to be phosphorylated by kinases like Fyn, Pyk2, Mek1 and Zak1. Cole et al showed that tyrosine phosphorylation might also be an autophosphorylation event[151]. Through crystallographic studies it was proposed that tyrosine phosphorylation of Gsk3 resulted in stabilization of the activation loop of the kinase allowing substrate accessibility[152] while the serine phosphorylated N-terminus is proposed to become a pseudo-substrate, which competes with the priming phosphate for substrate binding, leading to GSK3 inactivation[153]. It was also shown that N-terminal cleavage of Gsk3 (with removal of the inhibitory serine residues) by calpain resulted in Gsk3 activation.[154]

In 1995, Cross et al showed that Insulin activates PI3K leading to Akt phosphorylation and activation which then leads to inhibition of Gsk3 through the N-terminal serine phosphorylation[155]. This pathway of Gsk3 inhibition was subsequently shown to employed by other growth factors like Hgf.[72] Since Gsk3 inhibition through Insulin[156] and Akt overexpression[157] doesn't lead to significant β-catenin stabilization, it is thought that canonical Wnt mediated Gsk3 inhibition occurs via different (non-Akt dependent) mechanisms[158]. Wu and Pan proposed that at least 3 pools of Gsk3 might be present in cells that result in specific context and stimulus dependent signaling[142]. The Akt-associated Gsk3 is involved in growth factor but not in Wnt signaling. Wnt signaling involves the Axin-associated Gsk3 and a third pool that is neither associated with Axin nor with Akt.

In canonical Wnt signaling, Gsk3 is known to play a unique dual role. He et al first identified the role of Gsk3 in canonical Wnt signaling by their observation that a dominant negative form in Xenopus laevis embryos resulted in a dorsal ventral axis duplication phenotype[159]; a process

that was subsequently known to be mediated through the Gsk3 substrate β-catenin[160]. As mentioned above, there is a pool of β-catenin that is associated with cadherins at cell junctions and a cytosolic "free" pool of β-catenin. Gsk3 appears to be responsible for controlling the amount of this cytosolic β-catenin, which is the chief mediator of the downstream effects of canonical Wnts. Gsk3 is part of cytosolic destruction complex that is responsible for ubiquitin–proteasome-mediated degradation of β-catenin in the absence of Wnt through an inhibitory phosphorylation of β-catenin that is primed by CK1α. Wnt stimulation leads to Gsk3 inhibition and hence failure of degradation of β-catenin that translocates to the nucleus to activate gene transcription. This is effect of Wnt is recapitulated by pharmacological inhibition of Gsk3[161]. Besides β-catenin, many other proteins (normally inhibited by Gsk3) are stabilized as a result of inhibition of the destruction complex in response to Wnt[162].

Besides the role in β-catenin stabilization, the He lab also identified for the first time that Gsk3 can phosphorylate the PPP(S/T)P phosphorylation motifs on Lrp5/6 following Wnt stimulation, revealing a role of Gsk3 in the initiation of canonical Wnt signaling[121]. It was subsequently found that Gsk3 (in association with Axin) can translocate to the plasma membrane on Wnt stimulation, a process mediated by Fzd and Dvl[91]. This leads to the formation of signalosomes which are known to be responsible for the initiation of proximal Wnt signaling[90]. Whether the phosphorylation by Gsk3 of the multiple PPS/TP sites on Lrp5/6 is processive (single binding) or distributive (requires multiple binding events) is not clear. However the distance between these sites on Lrp5/6 and the end-product inhibition of Gsk3 by Lrp5/6 phosphorylation (discussed below) makes distributive phosphorylation more likely[89].

How this initiation of proximal Wnt signaling eventually leads to Gsk3 inhibition remain an enigma. The most dominant hypothesis is that the direct interaction of phosphorylated[163] or un-phosphorylated[164] Lrp6 with Gsk3 leads to its inhibition and stabilization of β-catenin. Besides this direct inhibition model, other mechanisms proposed for Wnt-mediated Gsk3 inhibition include

Axin degradation (leading to Gsk3 release from the signalosomes) and activation of phosphatases[142]. More recently, Taelman et al proposed an alternative mechanism of Gsk3 inhibition in response to Wnt where Gsk3 is shielded from inactivating b-catenin by being 'trapped' in multi-vesicles formed at the plasma membrane[74]. As previously mentioned, Wnt-mediated Gsk3 inhibition not only leads to β-catenin stabilization, but it also leads to the activation of the m-TOR pathway[75] and inhibition of BMP signaling[76].

Hypothesis and specific aims:

Gsk3 mediates Hgf-dependent Lrp5/6 phosphorylation.

1. To find out whether Gsk3 is the kinase that mediates Hgf-dependent Lrp5/6 phosphorylation
2. To define the mechanism by which Hgf regulates Gsk3.

Materials and Methods:

Cell culture and Reagents

MPT cells were maintained using standard cell culture techniques in DMEM-F12 medium containing 10% FBS. Antibodies to β-actin, Lrp6, Met, and Gsk3-beta were obtained from Santa Cruz Biotechnology. Antibodies to Lrp6, Lrp5, phospho-Lrp6 Ser1490, phospho-Gsk3-beta Ser9, Na/K ATPase and antibodies were obtained from Cell Signaling. Antibodies to phospho-Lrp6 Thr1572 and Ser1607 were obtained from Millipore, while anti-phospho Gsk3-betaY216 antibodies were from BD Biosciences. Recombinant human HGF and lithium chloride were purchased from Sigma Aldrich. The Gsk-3 inhibitor BIO IX and Met kinase inhibitor-II were purchased from Calbiochem. Gsk3 inhibitor CHIR-99021 were purchased from Selleck Chemicals.

For western blotting after immunoprecipitation experiments, light-chain specific anti-mouse and anti-rabbit IgG secondary antibodies were obtained from Jackson Immunoresearch. For all other experiments secondary antibodies were purchased from Invitrogen. EDTA-free Protease and Phosphatase Inhibitor cocktail was obtained from Thermo Scientific.

Immunoprecipitation and Western analysis

Cells were serum starved for 24 h, followed by Hgf (40 ng/ml) stimulation for the indicated time. Cells were lysed in RIPA buffer, insoluble material removed by centrifugation, and the supernatant protein content determined using the Bradford assay. For co-immunoprecipitation experiments, cell were lysed in NP-40 buffer (50 mM Tris-Hcl, 150 mM NaCl, 5mM EDTA and 1% NP40) containing protease and phosphatase inhibitors, pre-cleared with protein G-PLUS or protein A agarose beads (1:1 slurry in PBS; Santa Cruz) for 45 minutes. 0.5-1.0 mg of cell lysate was immunoprecipitated with the appropriate antibody overnight, washed x3 in ice-cold NP-40 buffer. The precipitated proteins were separated using 7.5% - 10% SDS-PAGE, electrophoretically transferred to Immobilon-P membranes (Millipore), immunoblotted with the appropriate antibody overnight, and visualized by enhanced chemiluminescence (Amersham Biosciences, Inc.) or West Femto Maximum Sensitivity Substrate (Thermo Scientific). Quantification of ECL signals was performed using NIH Image J software.

Membrane and Cytosol fractionation.

Cells were lysed in 4ml of solubilization buffer (1 mM sodium fluoride, 1 mM sodium vanadate, 30mM Trizma base, 20mM MES, 100mM NaCl), and then frozen at -80^{0}C for 1 hour, homogenized, and insoluble material removed by low-speed centrifugation. The resultant supernatant was subjected to ultracentrifugation at 100,000xg for 1 hour. The supernatant yielded the cytosolic fraction and the pellet was resuspended in RIPA buffer and yielded the membrane

fraction. Protein content was determined using the Bradford assay. Purity of the membrane preparations was determined by immunoblotting for E-cadherin.

Statistical methods

The data between two groups was compared using the student's two-tailed *t* test. Significance was determined at $P < 0.05$ and the error bars represent standard deviations.

Results:

PTH has been shown to mediate PKA-dependent Lrp5/6 phosphorylation by forming a ternary complex with its receptor PTH1R and Lrp6, eventually leading to β-catenin stabilization and to promote osteoblast differentiation[138]. To find out whether Hgf also mediates Lrp5/6 phosphorylation through a similar mechanism, and based on our previous finding that Met inhibition prevents this phosphorylation, we immunoprecipitated Lrp6 protein and looked for its association with the Hgf receptor Met. However, we did not detect co-immunoprecipitation of Met with Lrp5/6.

Based on the reasons mentioned in the introduction section above, we then looked at Gsk3 as the kinase mediating Hgf-dependent Lrp5/6 phosphorylation. We first showed that Gsk3 was indeed present at the cell membrane by doing a cell fractionation assay separating cytosol and membrane fractions (Fig 3A). MPT cells were then stimulated with Hgf ± pre-incubation with the highly specific and well established Gsk3 inhibitor 6-Bromoindirubin-30-oxime (BioIX). BioIX is an Indirubin belonging to the chemical family of bis-indoles and is an ATP-competitive inhibitor of Gsk3 that targets the ATP-binding pocket of the kinase[165, 166]. Hgf-stimulated Lrp6 phosphorylation at S1490 was inhibited by BioIX (Fig 3B, quantified in C) in a dose dependent manner (Fig 3D). BioIX also reduced the basal phosphorylation of Lrp5/6 by approximately 25% (Figure 3A, quantified in B). This is similar to the effect seen with Dkk-1 (Fig 1J), and consistent

with the possibility that MPT cells express a Wnt ligand that weakly activates Gsk3-dependent Lrp5/6 phosphorylation at baseline as discussed in Chapter 3. Taking into account the inhibition of this basal phosphorylation, there was an almost 80% decrease in the Hgf-stimulated phosphorylation of Lrp5/6 following BioIX treatment (10μM). Gsk3 inhibition also reduced Hgf-dependent Lrp6 phosphorylation at T1572 and S1607 to a comparable degree (data not shown).

To confirm that Hgf-mediated phosphorylation of Lrp5/6 was dependent on Gsk3, two other well-established Gsk3 inhibitors, CHIR-99021 and Lithium Chloride (LiCl), were tested and also found to also inhibit Hgf-stimulated phosphorylation of S1490 in a dose dependent fashion (Fig 3E). CHIR99021 is a highly selective, ATP-competitive GSK-3 inhibitor belonging to the aminopyrimidine class of chemical inhibitors. It was shown to activate glycogen synthase in vitro and lower blood glucose levels in murine diabetic models[167]. Lithium is unique in the family of Gsk3 inhibitors. It works by a non-ATP dependent mechanism (magnesium antagonism)[161], has no activity against cell cycle proteins like the CDK1–cyclin B complex and inhibits Gsk3 directly (as above) and also indirectly through serine phosphorylation[168]. Lithium has been used clinically for decades as a highly effective treatment for bipolar disorder. However it also plays a role in improvement of bone mass [169] and in the reduction of AKI[139], new roles that are the focus of early clinical trials. Gsk3 inhibition plays the central role in mediating all these physiologic effects.

We then went to investigate how Hgf can lead to Gsk-dependent Lrp5/6 phosphorylation. Examination of the phosphorylation state of Gsk3 in whole cell lysates confirmed that inhibitory phosphorylation of Gsk3β at serine 9 was significantly increased by Hgf whereas activating phosphorylation of Gsk3β at Y216 (and Gsk3α at Y279) was unchanged (Fig 3F). Hence, Gsk3 phosphorylation (at least at the whole cell level) was unlikely to be a mechanism of how Hgf 'activates' Gsk3 to mediate Lrp5/6 phosphorylation.

In light of our findings and the previous studies demonstrating separate membrane and cytosolic pools of Gsk3[91 121], Lrp5/6 was immunoprecipitated from MPT cell lysates to assess for

any Gsk3 association and the activation state of the associated Gsk3. These experiments revealed basal association of Lrp5/6 and Gsk3, which likely explained the basal Gsk3-dependent Lrp5/6 phosphorylation in our cell line. However, there was a significant increase in this association following stimulation with Hgf (Fig 3G, quantified in H). Surprisingly, despite the increase in serine 9 phosphorylation of Gsk3 detected in Hgf-stimulated whole cell lysates, there was no detectable serine 9 phosphorylated Gsk3 found in Hgf-stimulated Lrp5/6 immunoprecipitates. In contrast, Y216 phosphorylated Gsk3β and Y279 phosphorylated Gsk3α were readily detected in Lrp5/6 immunoprecipitates, and increased in a similar fashion to total Gsk3 following Hgf treatment (Fig 3G, quantified in H). These findings demonstrate that Hgf selectively recruits the active form of Gsk3 to Lrp5/6 while suppressing the recruitment of the inhibited serine phosphorylated form.

Discussion:

Since the Hgf-dependent Lrp5/6 phosphorylation was rapid, did not involve direct association of Met and Lrp5/6, and since Gsk3 is known to mediate crosstalk with Hgf and β-catenin signaling though PI3K/Akt[72], we investigated the possibility that Gsk3 was the also kinase responsible for Met activated Lrp5/6 phosphorylation.

Similar to previous studies[91] we first confirmed that Gsk3 was present at the membrane in our cells which is also the location of Lrp5/6 co-receptors (Fig 3 A). Then using 3 separate kinase inhibitors, we show that Gsk3 is highly likely to be the kinase responsible for the majority of Hgf-stimulated Lrp5/6 phosphorylation (Fig 3 D-E). , The three inhibitors we chose are well-established, highly specific and work through different mechanisms. Also, using phospho-specific antibodies we find that Hgf-mediated Lrp5/6 stimulation at three of the five known Gsk3 phosphorylation sites (S1490, T1752 and S1607) is significantly reduced by Gsk3 inhibition. Hence we believe that Gsk3 is the predominant (if not the only) kinase used by Hgf to mediate

Lrp5/6 phosphorylation. However, genetic models of Gsk3 inhibition (kinase dead mutants) or activation (S9/21 mutants), would be required to definitively confirm this observation.

As mentioned above, while canonical Wnts, such as Wnt3a, appear to regulate Gsk3 activity by either sequestration into multivesicles or direct inhibition by phosphorylated Lrp6, tyrosine kinase activating growth factors such as Hgf are known to regulate Gsk3 activity via inhibitory serine phosphorylation. Phosphorylation of Gsk3 is not believed to be important for Wnt-dependent Gsk3 regulation. Our finding that Hgf leads to an increase in the inhibitory (serine) while having no effect on the activating (tyrosine) phosphorylation of Gsk3 in whole cell lysates (Fig 3 F), suggests that change in phosphorylation is unlikely to be the method via which Hgf can 'stimulate' Gsk3 to phosphorylate Lrp5/6. Hence we investigated the alternative mechanism of Gsk3 regulation; namely association with other proteins and cellular localization. We find that Gsk3 increases its association with Lrp5/6 in response to Hgf stimulation (Fig 3 G-H), suggesting that activated Met can either recruit cytosolic Gsk3 to the membrane or selectively target membrane-associated Gsk3 to Lrp5/6. This finding is similar to that reported with canonical Wnts[91], and suggests the interesting possibility that Hgf not only targets the Akt-associated Gsk3 pool but also the non-Akt associated pool of Gsk3 involved in Wnt signaling.

This theory is further strengthened by our finding that that stimulation of MPT cells with Hgf results in the selective exclusion of the S9 phosphorylated pool of Gsk3β from association with Lrp5/6, even though there is a marked increase in S9 phosphorylated Gsk3β at the whole cell level. Instead, the pool of Gsk3 that is recruited to Lrp5/6 following Hgf stimulation includes Gsk3β that is phosphorylated at Y216 (and Gsk3α phosphorylated at Y279) (Fig 3 G-H). As mentioned above, these residues are on the activating T-loop and their phosphorylation has been shown to result in a conformational change in the enzyme that facilitates substrate binding to the catalytic site and thus increases kinase activity [146]. Our data suggest that Hgf treatment does not directly stimulate the phosphorylation at these sites at the whole cell level, but instead mediates the recruitment of this active form of Gsk3 to Lrp5/6. As mentioned above, the inhibition of the kinase activity of Gsk3

through serine 21/9 phosphorylation is usually on substrates like glycogen synthase that contain the Gsk3 consensus motif S/T-X-X-X-pS/pT that requires a priming phosphorylation by another kinase[152]. Since this motif is absent from the Lrp5/6 phosphorylation sites, the Gsk3 associated with Lrp5/6 is unlikely to be regulated by serine phosphorylation. On the other hand, the Gsk3 consensus motif and priming phosphorylation probably doesn't play a role in tyrosine phosphorylated Gsk3 activity which we found to be associated with Lrp5/6.

How Met activation leads to the recruitment of active Gsk3 to Lrp5/6 while excluding serine phosphorylated Gsk3β remains unclear. One possibility is that Hgf-mediated Lrp5/6 phosphorylation results in a conformational change in the Gsk3-binding site of Lrp5/6 that doesn't allow serine phosphorylated Gsk3 from binding. However, this has not yet been tested.

Our findings do establish Gsk3 as the predominant kinase involved in Hgf-mediated Lrp5/6 phosphorylation likely through association of its active form with Lrp5/6. This pool of Gsk3 is separate from the Akt-phosphorylated Gsk3 that is inhibited by Hgf stimulation. Since both these pathways of Gsk3 regulation could eventually lead to β-catenin stabilization, we proceeded with our subsequent experiments to study the role of Hgf-mediated Lrp5/6 phosphorylation in β-catenin stabilization and signaling.

Chapter 5. Hgf mediated β-catenin stabilization and anti-apoptotic activity is dependent on Lrp5/6 phosphorylation

Introduction:

Since β-catenin is the master regulator of downstream canonical Wnt signaling and since epithelial cell apoptosis is regulated by both Hgf and Wnt signaling and is a critical physiologic event in AKI, we sought to study the role of Hgf mediated Lrp5/6 phosphorylation in the regulation of β-catenin and apoptosis.

Overview of β-catenin signaling:

β-catenin is not only a major component of AJs and responsible for cell adhesion but also the prime mediator of canonical Wnt signaling in the nucleus[170]. Besides Wnt, β-catenin is also regulated by ligands such as TGF-β[171] and Hgf[172] hence, it is a focal point of crosstalk of Wnt and growth factor signaling similar to Gsk3. It is known to be implicated in various renal physiologic process like nephrogenesis[173]; and disease processes like diabetic nephropathy[174], polycystic kidney disease[175, 176], chronic allograft nephropathy[177], obstructive uropathy[178] and AKI/repair[5, 6, 179].

Since β-catenin plays such a central role in the maintenance of cell physiology and homeostasis, the regulation of its activity is tightly controlled to allow specificity depending on the stimulus and context. Similar to Gsk3, one of the prime methods of β-catenin regulation is through association with other proteins e.g. with E-cadherin in the AJ, with APC in the cytoplasmic

destruction complex and with Tcf/Lef in the nucleus. Crystallography analysis reveals that many of the β-catenin binding partners share a common groove in the central binding region, hence they cannot bind β-catenin simultaneously, thereby creating mutual exclusivity[170].

The other prime method of β-catenin regulation is through phosphorylation. Phosphorylation at T142 and T654 decreases its affinity to E-Cadherin[180], while the phosphorylation at S675 keeps the helix structure open.[181] This promotes release of β-catenin from AJ and its translocation to the nucleus and binding to transcriptional co-activators like CBP (CREB binding protein). On the other hand its phosphorylation at S33/37 and T41 by Gsk3 leads to ubiquitin-mediated degradation of β-catenin[182, 183].

Wnt independent mechanisms of β-catenin stabilization have also been reported. Thorton et al reported an alternate pathway of Gsk3 inactivation through p38-MAPK.[150] Platelet-derived growth factor (PDGF) treatment was shown to lead to c-Abl mediated phosphorylation of p68 which displaced Axin from the β-catenin destruction complex and promoted nuclear translocation of β-catenin in a Wnt-independent pathway[184].

The mechanism of the nuclear transport of stabilized β-catenin is not clear, but the players implicated include nuclear pore complex components such as Nup358[185], Forkhead-box transcription factor FoxM1[186] and Rac1/Jnk2.[187] In the nucleus, β-catenin mediates gene transcription. Since it doesn't have a DNA-binding domain, it uses DNA binding partners like Tcf/Lef to guide it to the promoters of its target genes.[188] β-catenin promotes transcription of many genes in an upstream signal, context and tissue dependent manner. Many of the Wnt target genes are also activated by growth factors.[189]

Deactivation of the β-catenin /Tcf mediated gene transcription is important for regulation of the response. This can be achieved through transcriptional repressors like Reptin[190], SMRT (Silencing Mediator for Retinoid and Thyroid hormone receptor) and NCoR (Nuclear receptor Co-Repressor)[191] or via nuclear export mediated by APC and Axin.[192] Besides Tcf/Lef, β-catenin

mediates gene transcription through other DNA binding transcription factors including the androgen receptor[193] and Hypoxia Induced Factor 1a (HIF1a)[194].

Overview of apoptosis and the role of Hgf and canonical Wnt signaling:

The two major forms of cell death in AKI are necrosis and apoptosis, with the former postulated to be a result of severe insult to the renal tubule. Apoptosis or 'programmed cell death' on the other hand is postulated to be a consequence of less severe insult in the setting of a lack of renal growth factors, and loss of cell-matrix or cell-cell contacts[195]. In *in vivo* models of AKI, it is well-established that the apoptosis that occurs immediately after injury (first 24 hours) is detrimental. Prevention of apoptosis has been shown to significantly reduce injury is murine AKI models[196, 197] and reduction in epithelial cell apoptosis is deemed to be a major mechanism of the beneficial effects of growth factors in experimental models of AKI[198].

Both canonical Wnt and Hgf/Met signaling have been shown to have paradoxical effects on apoptosis depending on the cell type and physiologic context. Hgf was shown to protect renal epithelial cells from apoptosis induced by disruption of cell-cell contacts[199]. Subsequently, Hgf is known to reduce apoptosis in other cell types[200-202] induced by various stimuli. Hgf has been proposed to modulate apoptosis via two major mechanisms[17]. One is the acute induction of the PI3K/Akt pathway leading to the phosphorylation and inactivation of the pro-apoptotic protein Bad. The other, more delayed, pathway is increase in the level of the anti-apoptotic protein Bcl-xl. Other mechanisms proposed are activation of the pro-apoptotic protein BAG-1[203] and potentially Akt-mediated inactivation of the anti-apoptotic like caspase 9 and Forkhead family of transcription factors[204]. Migration of cells requires detachment from cell-cell and cell-ECM junctions which can induce anoikis, a form of apoptotic cell death[21] as previously mentioned. The significance of prevention of apoptosis by Hgf/Met signaling during organ repair is not only in reducing the loss

of injured cells but also to enable cells to migrate, which like cell survival, is essential to repair and regeneration.

Despite acting primarily as a survival factor, Hgf is also known as a 'tumor cytotoxic factor' for its ability to elicit apoptotic responses in certain cancer cells[205]. This paradoxical response to Hgf was also seen in other cell types including n myofibroblasts[206]. These apoptotic responses are believed to be mediated through PKC, JNK and the p38 MAPK pathways[21]. Coltella et al showed that Hgf can sensitize cancer to the pro-apoptotic effects of chemotherapeutic drugs through the p38 MAPK pathway[207]. How Hgf/Met signaling can switch between these opposing roles in survival depending on cellular and physiologic contexts is not clear, however, a potential mechanism has been proposed. The extracellular domain of activated Met interacts with the death receptor FASL preventing its aggregation and limiting apoptosis via the extrinsic pathway. However excessive Hgf can release FASL leading to caspase activation and cleavage of Met. One of the resulting intracellular fragments can trigger cell death[21].

Wnt/β-catenin signaling has also been shown to both reverse[208] and promote[209] apoptosis in various cell types. The mechanisms of anti-apoptotic β–catenin signaling include expression of the anti-apoptotic genes Bcl-2, c-Jun, c-Myc and Survivin [210] and/or inhibition of pro-apoptotic molecules such as Bax [6]. While the role of β-catenin in cell survival in normal adult kidneys, where it is expressed at a low level[178], is not clear, it is upregulated during renal disease including AKI. By generating a renal tubule-selective β-catenin knockout mouse model using the Ksp Cre, Zhou et al showed that β-catenin plays a key role in renal protection during AKI induced by ischemia or folic acid[5]. They found that β-catenin mediates this via reduction in apoptosis and promotion of cell survival through the regulation of pro-apoptotic factors p53 and Bax, and anti-apoptotic factors like Akt and Survivin. While Akt phosphorylation is known to inactivate Gsk3 and stabilize β-catenin in some settings[72], this study shows reciprocal relationship where β-catenin ablation leads to reduction in Akt phosphorylation. Since the β-catenin- knockout mice do not have any

phonotypical defects at baseline, this study also highlights the possible redundancy of β-catenin at cell junction maintenance in the healthy kidney. Wnt was also shown to suppress apoptosis in a non-β–catenin dependent manner[211].

Price et al showed that ATP depletion of renal epithelial cells resulted in loss of β-catenin form the AJ and its translocation into the nucleus[212]. Wang et al showed that β-catenin regulates apoptosis during epithelial cell injury through Akt and Bax.[6] Using constitutively active and dominant negative constructs they showed the β-catenin regulated Bax oligomerization and mitochondrial translocation. They also found that Akt mediated inactivation of Bax. Unlike the study by Zhou et al,[5] Wang et al showed that β-catenin not only regulated Akt phosphorylation but also Akt transcription and protein levels. Bax, a major mitochondrial mediator cell death,[213] has also been known to be regulated by Gsk3[214]. Besides, Bax-mediated apoptosis, Akt and Gsk3 are also known to regulate apoptosis through other pathways including NF-kB, caspases, forkhead transcription, mammalian target of rapamycin (mTOR), and other BH3 proteins, including Bad[3, 215] and Bid[216]. In our recent study we have shown that Akt, downstream of Met signaling, plays a key role in promoting survival in AKI[3]. Similar to Hgf, Wnt signaling also has pro-apoptotic roles especially in cancer cells. In a recent study, Bao et al showed that Lithium, an activator of canonical Wnt signaling, can also promote apoptosis and enhance the chemotherapeutic effect of Cisplatin[217].

We set out a series of experiments to determine whether Hgf-dependent Lrp5/6 phosphorylation alters downstream canonical Wnt/β-catenin signaling including β-catenin stabilization and its transcription of early genes. As mentioned above, renal epithelial cell apoptosis is a considered to be a major pathogenic mechanism in the progression of AKI an reduction in GFR.[218], and Hgf and canonical Wnt signaling are likely to be key players in this process. Therefore, we also conducted experiments to find out whether Hgf-dependent Lrp5/6 phosphorylation has any relevance on the survival of MPT cells.

Hypothesis and specific aims:

Hgf-dependent Lrp5/6 phosphorylation is involved in β-catenin stabilization and renal epithelial cell apoptosis.

1. To knockdown Lrp5 and 6 proteins in MPT cells.
2. To determine if Hgf-dependent Lrp5/6 phosphorylation is affected in the knockdown cells.
3. To determine if β-catenin stability and nuclear transfer is affected in the knockdown cells.
4. To study the effect of Lrp5/6 knock-down on apoptosis/survival.

Materials and Methods:

Cell culture and Reagents

MPT cells were maintained using standard cell culture techniques in DMEM-F12 medium containing 10% FBS. Antibodies to β-actin, Met, phospho-Met (Tyr1234) and Lamin A/C were obtained from Santa Cruz Biotechnology. Antibodies to Lrp6, Lrp5, phospho-Lrp6 Ser1490, HSP-90 antibodies were obtained from Cell Signaling. Antibodies to phospho-Lrp6 Thr1572 and Ser1607 were obtained from Millipore. Recombinant mouse Wnt3a was purchased from R&D Systems. Recombinant human HGF was purchased from Sigma Aldrich. For western blotting experiments secondary antibodies were purchased from Invitrogen. EDTA-free Protease and Phosphatase Inhibitor cocktail was obtained from Thermo Scientific.

Western analysis

Cells were serum starved for 24 h, followed by Hgf (40 ng/ml) or Wnt3a (50ng/ml) stimulation for the indicated time. Cells were lysed in RIPA buffer insoluble material removed by centrifugation, and the supernatant protein content determined using the Bradford assay. The

proteins were separated using 7.5% - 10% SDS-PAGE, electrophoretically transferred to Immobilon-P membranes (Millipore), immunoblotted with the appropriate antibody overnight, and visualized by enhanced chemiluminescence (Amersham Biosciences, Inc.) or West Femto Maximum Sensitivity Substrate (Thermo Scientific). Quantification of ECL signals was performed using NIH Image J software.

Cell Surface Biotinylation

The manufacturer protocol (Pierce Cell Surface Protein Isolation Kit, Thermo Scientific) was followed. Briefly, cells were washed and Sulfo-NHS-SS-Biotin in ice-cold PBS was added to each dish which was then placed on a rocking platform for 30 minutes at 4°C. This was followed by quenching of the reaction, washing and cell lysis by sonication in ice cold lysis buffer with protease and phosphatase inhibitors. After centrifugation, 20% of the supernatant was saved as the whole lysate (WCL) fraction, while the remainder was incubated with NeutrAvidin Agarose beads for 60 minutes at room temperature with mixing using a rotator. This was followed by washing and protein elution. This yielded the plasma membrane (biotinylated) fraction. The two protein fractions were analyzed by Western Blot.

Nuclear and Cytoplasmic protein extraction

Cells were trypsinized, washed with ice-cold PBS, centrifuged and the manufacturer protocol (NE-PER Nuclear and Cytoplasmic Extraction Reagents, Thermo Scientific) was followed. Fresh protease and phosphatase inhibitors were added to the manufacturer's reagents. The cytoplasmic and nuclear extracts were analyzed by Western blot.

Apoptosis assay

To induce apoptosis, hydrogen peroxide (H2O2) at 250 mM concentration [208] in serum-free media was added to the cells after 90 minutes of stimulation with either vehicle or Hgf. Cells

were cultured for 2 hours followed by Terminal deoxynucleotidyl transferase-mediated dUTP nickend labeling (TUNEL) staining based on the manufacturer's protocol (Roche). Briefly the cells were washed, fixed in 4% paraformaldehyde and stained. TUNEL-positive cells were imaged using a Nikon microscopy system and counted in eight randomly selected fields per well at 40X. Results are expressed as the percentage of total (DAPI-positive) cells counted that were apoptotic (TUNEL-positive) cells. An average of the 10 fields per condition in 3 experiments was used for quantification.

Stable Lrp5 and 6 knockdown in MPT cells

Lentivirus containing shRNA against the Lrp5 and 6 proteins and control (scrambled) shRNA were obtained from Santa Cruz Biotech. MPT cells were plated on 48 well dishes one day before infection. Polybrene (Santa Cruz Biotech) at 5ug/ml and lentivirus particles were added into FBS/ antibiotic-free DMEM and left for overnight culture. A concentration of 100 infectious units (IFU) of virus per cell was used. On day two following transduction, the medium was replaced with DMEM supplemented with FBS and 10ug/ml of puromycin (Santa Cruz Biotech). Untransduced dead cells were removed on day three and live cells were further expanded and maintained to generate stable cell lines.

Quantitative PCR

RNA was isolated using the RNeasy Mini Kit per the manufacturer's protocol. One microgram of RNA was reverse transcribed into cDNA using random hexamer primers (SuperScript II, Invitrogen, Carlsbad, CA, USA) and gene expression analysis was determined by qPCR using CFX96 Real-Time System (Bio-Rad). Primers used for PCR were chosen for efficiency of 90-100%. Normalization was to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression from the same PCR reaction (dCt).

The primers used were:

1. Bcl-2:
    a. TGAGTACCTGAACCGGCATCT (forward)
    b. GCATCCCAGCCTCCGTTAT (reverse);
2. Survivin:
    a. ATCGCCACCTTCAAGAACTG (forward)
    b. GGCCAAATCAGGCTCGTTCT (reverse)
3. Cyclin D1:
    a. TGGAGCCCCTGAAGAAGAG (forward)
    b. AAGTGCGTTGTGCGGTAGC (reverse)
4. c-Myc,
    a. CTGTTTGAAGGCTGGATTT (forward)
    b. TCGAGGTCATAGTTCCTGTT (reverse)
5. GAPDH:
    a. GACGGCCGCATCTTCTTGT (forward)
    b. CACACCGACCTTCACCATTTT (reverse)

Statistical methods

The data between two groups was compared using the student's two-tailed *t* test. Significance was determined at $P < 0.05$ and the error bars represent standard deviations.

Results:

We first checked whether Hgf-stimulated Lrp5/6 phosphorylation is taking place at the plasma membrane-bound Lrp5/6 where it functions as a proximal activator Wnt signaling. Using

cell surface protein biotinylation, we found that there was indeed a significant increase in plasma membrane pool of Lrp6 phosphorylated at S1490 in response to Hgf; while there was no change in the total Lrp6 protein levels at the membrane (Fig 4A).

To determine the significance of Hgf-stimulated Lrp5/6 phosphorylation in MPT cells, stable cell lines were created with simultaneous knockdown of both Lrp5 and Lrp6 proteins. MPT cells were infected with lentivirus containing either shRNA against Lrp5 and 6 or the scrambled (scr) control. Stable cell mixtures expressing the appropriate shRNA were selected on puromycin. Knockdown (80%) of Lrp6 protein individually did not result in any change in the level of Lrp5 protein or in the degree of Hgf-stimulated Lrp5/6 phosphorylation (data not shown). A similar data was obtained when Lrp5 protein was individually knocked down. This suggested that either one co-receptor increases the stochiometry of its phosphorylation when the other is reduced and/or that the phospho-specific antibodies being used do not differentiate between the homologous motif on either Lrp5 or 6 proteins. Hence we decided to knockdown both Lrp5 and 6 proteins simultaneously. This observation also confirmed that Hgf was indeed leading to phosphorylation of both Lrp5 and 6 proteins.

Initially we were able to achieve a 60% knockdown of Lrp5 and 65% knockdown of Lrp6 compared to control (Fig 4B, quantified in C), correlating with a 60-65% reduction in Hgf-stimulated Lrp5/6 phosphorylation at the Gsk3 phosphorylation sites (Fig 4D, quantified in E). Both Lrp5 and 6 shRNA had the puromycin-based selection. Therefore, while we were able to estimate an overall reduction in the two proteins as mentioned above, we were not able to determine to what extent were the Lrp5 and Lrp6 proteins knocked down in each individual cell. One cell could potentially have a higher knockdown of one protein and thereby function differently. To resolve this issue and to achieve an even higher level of knockdown, we transfected MPT cells as before and after puromycin-based selection of viable cells we plated single cells in a 96 well plate to achieve individual clones. Of the seven clones recovered, clone 5 and clone 6 had the highest

degree of knockdown of both Lrp5 and 6 (85%) and also the highest reduction (80%) in Hgf-stimulated Lrp5/6 phosphorylation (Fig 4F).

After achievement of an adequate knockdown we proceeded to study the effects on downstream signaling. Hgf treatment of control MPT cells, revealed an increase in the 'activate', non-Gsk3 phosphorylated form of β-catenin (ABC) in both whole cell lysates (Fig 4G) and nuclear fractions (Fig 4I) at 90 minutes after Hgf stimulation. In Lrp5/6 knock-down cells, the stabilization of β-catenin was significantly reduced in both the whole cell lysates (Fig 4G, quantified in H) and isolated nuclear fractions (Fig 4I). In the experiments using Clone 5 and 6, a similar reduction in β-catenin phosphorylation and nuclear translocation was seen (data not shown). To test whether the nuclear translocation of β-catenin as a result of Hgf stimulated Lrp5/6 phosphorylation was involved in the transcription of early genes we tested two of the well-known target genes of canonical Wnt signaling: c-Myc and Cyclin D1. While we found no difference in the transcription of Cyclin D1 (data not shown), we found a significant (1.9 fold) increase in the transcription of c-Myc after Hgf treatment of control MPT cells. This increase was significantly reduced (by 80%) in the Lrp5/6 knock-down cells (Fig 4J).

To test the importance of Lrp5/6 phosphorylation in mediating the Hgf-dependent anti-apoptotic activity, MPT cells were treated with Hgf or vehicle for 90 minutes followed induction of apoptosis with hydrogen peroxide (H_2O_2) based on previously published protocols[208]. Treatment with Hgf reduced the H_2O_2-induced apoptosis by 40% in control cells, but failed to prevent apoptosis in the Lrp5/6 knock-down cells (Fig 4K). To test the potential mechanism of the anti-apoptotic effect of Hgf-stimulated Lrp5/6 phosphorylation, we studied the RNA expression of two other well-known target genes of canonical Wnt signaling involved in anti-apoptotic signaling: Bcl-2 and Survivin. While we found no difference in the mRNA levels of Bcl-2 (data not shown), we found a significant (2.2 fold) increase in the mRNA expression of Survivin after Hgf treatment of

control MPT cells. This increase was significantly reduced (90%) in the Lrp5/6 knock-down cells (Fig 4L).

Discussion:

A recent study showed that Erk-dependent Lpr6 phosphorylation can occur during the Golgi processing of Lrp6 protein in the cytosol, however, the significance of this phosphorylation is not clear[96]. We show through biotinylation experiments that Hgf-dependent Lrp5/6 phosphorylation occurs at the plasma membrane which is the site where Lrp5/6 mediates canonical Wnt signaling. To study the significance of this pathway for downstream signaling, Lrp5 and 6 proteins were knocked down in stable pooled cells. This led to a significant reduction in Hgf-dependent Lrp5/6 phosphorylation and suppressed the Hgf-stimulated increase in the active, non-phosphorylated form of β-catenin at whole cell level and the nucleus and reduced transcription of c-myc.

c-Myc, one of the key Wnt target genes[219], is a transcription factor that activates expression of many genes and also has a direct role in the control of DNA replication[220]. c-Myc is known to be activated by various mitogenic signals such as Wnt, Shh and EGF leading to various biologic effects. These include cell proliferation, cell growth, apoptosis, differentiation, and stem cell self-renewal. Its role in canonical Wnt signaling was first discovered by He et al where they report c-Myc as being a target gene of β-catenin and this was mediated through Tcf-4 binding sites in the c-Myc promoter[221]. C-Myc is known to be an immediate early gene that is also regulated by MAPK/Erk signaling[222].

The results above show that Hgf-dependent Lrp5/6 phosphorylation leads to inhibition of the destruction complex of β-catenin (likely via downstream inhibition of Gsk3, similar to Wnt), allowing β-catenin to be translocated to the nucleus to mediate gene transcription. These results

also support our conclusion that Hgf regulates the pool of Gsk3 involved in mediating both Lrp5/6 and β-catenin phosphorylation.

The functional importance of this signaling pathway for Hgf-mediated cell survival was demonstrated by our finding that the anti-apoptotic activity of Hgf was significantly reduced in Lrp5/6 knockdown cells. Besides our observation of the anti-apoptotic role of Hgf-dependent Lrp5/6 phosphorylation using TUNEL staining, we also found that the mRNA expression of Wnt target gene Survivin was also significantly mediated by this phosphorylation. Survivin, is an intracellular protein that has been implicated in regulation of apoptosis through caspase-dependent and –independent mechanisms[223]. Survivin is known to be highly expressed in renal epithelial cells[224] and was shown to be protective in nephrotoxic murine AKI by suppression of p53 mediated apoptosis[225]. Chen et al showed that renal Survivin expression was increased after ischemic kidney injury and was important is renal repair in a STAT3/Notch2 dependent mechanism[226]. Hgf has been shown to increase gene expression of Survivin[227]. Zhang et al discovered that Survivin was a target gene of canonical Wnt signaling by showing that APC down-regulates Survivin expression via APC/β-catenin/TCF-4 signaling[210].

Our work thus far, highlights the mechanism and significance of Hgf-dependent Lrp5/6 phosphorylation in MPT cells. We subsequently went on the study the role of this phosphorylation in a murine model of ischemic kidney injury.

Chapter 6. Lrp5/6 phosphorylation early after ischemic kidney injury is Met dependent

Introduction:

AKI is very common diagnosis in hospitalized patients, is increasing in incidence and is associated with a high mortality rate in the range of 50-60%.[228] The survivors have poor long term outcomes.[229] AKI is now well recognized as a major risk factor for the development of CKD and the severity of AKI is a predictor for this progression.[230] In most cases AKI is followed by a repair phase involving reconstitution of the injured nephron leading to recovery of renal function. Clinical variables including baseline renal function, severity of AKI, older age, severity of illness and volume overload and biomarkers such as Interleukin-18 (IL-18), Chitinase-3-like protein 1 (YKL-40), Alpha-1-microglobulin (α1M), Insulin-like growth factor-binding protein 7 (IGFBP-7) and Hgf can help predict recovery from AKI [231]. Both Hgf/Met and canonical signaling pathways are known to play key roles in this repair and recovery process after AKI.

The role of canonical Wnt signaling in AKI and other kidney diseases:

Wnt signaling has been implicated as a key player in renal organogenesis[173]. Wnt9b and Wnt 4 play a key role in the mesenchymal to epithelial transition [173] while Wnt 7b is involved in branching morphogenesis[232] in the developing nephron. Besides its role in kidney development, Wnt signaling is reactivated in the adult kidney tissues during various disease states such as diabetic nephropathy, fibrosis, cystic kidney diseases and AKI[179].

Canonical Wnt signaling is known to be activated during acute injury/repair in various organs including the skin and heart[233]. Its beneficial role in the repair process after AKI has been recently brought into light. Using the $Axin2^{lacZ}$ reporter mouse that highlights canonical Wnt signaling, Lin et al found Wnt pathway to be constitutively active in the renal papilla, area of low oxygen tension and high cell turnover even at a basal state[234]. Following ischemic kidney, Wnt signaling was also activated in the cortex and medulla. In mice with heterozygous knockout of Lrp5 and 6 proteins, the injury was more severe (day 2) and the repair process was impaired (day 7)[234]. Other groups have also shown the role of Wnt signaling in AKI. Terada et al showed that Wnt4 expression was highly upregulated within the first day after AKI followed by increase in Cyclin D1 and cell proliferation[235]. As previously mentioned, conditional ablation of the downstream regulator of canonical Wnt signaling, β-catenin, resulted in grater injury after AKI[5]. Lancaster et al showed that the loss of the Jouberin (Jbn) protein in mouse leads defective Wnt/β-catenin signaling and repair after AKI[236]. Similar to the study by Lin et al[234], they found that this defective repair process leads to a cystic phenotype.

Canonical Wnt signaling is known to have anti-apoptotic and proliferative effects on renal epithelial cells that might be responsible for its reparative role after AKI. Another possible mechanism is through macrophage activation which seems to play a dual role in AKI. We have previously shown that while the pro-inflammatory type of macrophage is involved in the enhancing the injury response, the pro-reparative type play an important role in modulating the repair and regeneration[237]. Persistence of the pro-inflammatory type of macrophage, is involved in defective repair and development of CKD[238]. One of the mechanisms through which the macrophages play a pro-reparative after AKI is through the stimulation of Wnt signaling. Through genetic deletion, Lin et al showed that macrophage Wnt 7b was a key player is epithelial regeneration[234].

Besides this potentially beneficial role in repair after AKI, canonical Wnt signaling has been shown to play a detrimental role in many other kidney diseases. While acute activation of Wnt

signaling has a pro-reparative role, its chronic activation is known to be associated with renal fibrosis. Tubulointerstitial fibrosis and glomerular sclerosis is the key feature of all forms of CKD and is the key determinant of kidney function and the progression towards end stage renal disease (ESRD). Inhibition of canonical Wnt signaling by using Dickkopf-1 has also been shown to reduce renal fibrosis in a UUO model.[178] Another group had similar findings using another canonical Wnt inhibitor sFRP4[239]. Both groups found an increased expression of β-catenin in the tubules and the interstitial fibrous area. Duffield's group recently described Lrp6 to be a major regulator of myofibroblast activation in a Wnt/β-catenin dependent and independent (TGFβ-, CTGF- and PDGF-mediated) fashion[104]. Besides the tubulointerstitium, canonical Wnt signaling also seems to mediate podocyte injury. In an interesting study, He et al showed that the clinically used vitamin D analog paricalcitol could ameliorate podocyte injury, proteinuria, and renal fibrosis in a murine model of adriamycin nephropathy[240].

Wnt signaling has been implicated in numerous studies in the pathogenesis of polycystic kidney disease (PKD) which is most common genetic cause of chronic kidney disease[241]. However, the exact role it plays in either cyst formation or disease progression remains a mystery. Defects in the PCP, one of the non-canonical Wnt signaling pathways, which leads to disoriented cell division, has been implicated as a major player in cyst development and PKD[242]. Another study however put this into question[243]. The role of canonical Wnt signaling is also controversial with studies both in support of and against its role in PKD[244]. Mice containing constitutively active mutations of β-catenin[245] or deletion of the *Apc* gene[246] exhibit a cystic phenotype. Kim et al showed that Pkd2 null renal epithelial cells displayed enhanced canonical Wnt signaling through the up-regulation of β-catenin, axin 2 and c-myc[247], However there are other studies that have refuted the involvement of canonical Wnt signaling in the pathogenesis of PKD. Using the TCF/β-catenin–lacZ (BAT-gal) reporter mice, Miller et al showed that mice with Pkd1 and Pkd2 mutations were found to have no

activation of canonical Wnt signaling in the cyst lining cells[175]. The study by Lancaster et al showed reduced (rather than increased) endogenous Wnt activity in cystic kidneys[236].

Promotion of canonical Wnt signaling has been utilized in development of new therapeutic agents. Dkk1 inhibition was shown to be a therapeutic means to stimulate new bone formation in multiple myeloma models[248] and one inhibitor BHQ880 is currently in use in phase 1 clinical trials in multiple myeloma patients (NCT01302886). Since Wnt/b-catenin signaling is involved in both repair after AKI and in the development of renal fibrosis (reminiscent of maladaptive repair), its exact role in renal repair has to be further defined before agents targeting this pathway can be employed for clinical trials in AKI. This pathway is also known to be involved in many cancers which also remains a major concern in therapeutics.

The role of Hgf/Met signaling in AKI and other kidney diseases:

Although it was originally discovered in the liver, Hgf and its receptor Met are highly expressed in the kidney[249]. While Hgf expression is mostly restricted to mesenchyme derived cells like mesangial cells, endothelial cells, interstitial fibroblasts, and macrophages[249], Met is more widely expressed including epithelial cells of the proximal and distal renal tubule[250]. This pattern of expression suggest that Hgf/Met signaling is important in renal physiology, however its role in the normal adult kidney is not defined. Mice with whole body knockout of Hgf or Met die in utero due to severe placental and liver defects[251, 252] as previously mentioned. Mice with conditional knockout of the Met receptor in the proximal tubule (using the GGT-Cre) and distal tubule (using the Hox-B7-Cre) generated in our lab show no developmental defects however both show impaired repair process after injury[3, 253].

A great amount of evidence supports the protective role of Hgf in kidney, both in early cytoprotection and subsequently in effective repair and prevention of fibrosis[254]. Hgf is rapidly

induced following various forms of kidney injury including ischemic and nephrotoxic [15, 255]. Rabkin et al showed that Met was significantly increased in ischemically injured kidneys 12 hours after injury and rose to a maximum after the first day with high levels (above control) persisting up to one week[256]. Moreover the highest Met expression was seen in the nephron regions of the hypoxic outer medulla (the prime site of ischemic kidney injury). A unique aspect of Hgf is that it is not only stimulated locally, but also induced in distant organs like liver and lung after AKI, presumably allowing it to be rapidly mobilized to the injured kidney in an endocrine fashion[15]. The specificity of action of Hgf is likely determined by the expression patterns of Met that are exclusive in the injured kidney[15], and through cleavage of pro-Hgf into the biologically active, double chain form of Hgf which may also be specifically activated in injured kidney[257].

There are various postulated mechanisms that can explain the beneficial effects of Hgf in AKI. The most critical mechanism is the anti-apoptotic effect of Hgf on renal epithelial cells[17, 258] that was also seen *in vivo* after kidney injury[3, 4], and supported by at least three lines of evidence. The early expression of Hgf and Met coincides with the high degree of apoptosis seen early after kidney injury[256]. Secondly, as mentioned above, the lack of Met increases this early apoptosis and degree of injury[3, 4]. Thirdly, Hgf delivery before kidney injury reduces apoptosis and the injury extent[259]. Therefore, it is highly likely that Hgf is a significant component of the anti-apoptotic forces that get activated early after AKI.

We have previously shown that Hgf promotes adhesion of ATP-depleted renal tubular epithelial cells to the ECM[260]. This could be a potential mechanism how Hgf prevents cell detachment during ischemic kidney injury. Another key player in the early protection offered by Hgf after AKI is reduced inflammation. How Hgf reduces inflammation is not clear but potential mechanisms include NFkappaB inhibition through Gsk3[19] and by down-regulating macrophage adhesion to the inflamed endothelium mediated by E-selectin[261]. The Hgf-dependent tubule formation in 3-D cultures is dependent on the Erk, STAT3 and NF-κb pathways[262] in the early

stages and MMPs in the late stages[263]. However it is not currently known whether tubulogenesis takes place in vivo during repair after kidney injury. Lastly, increase in epithelial cell proliferation during the repair phase is another mechanism through which Hgf promotes renal recovery after AKI[3, 264].

Besides its protective role in AKI, Hgf is postulated to be an anti-fibrotic agent that has a potential role in the prevention of CKD. This is in contrast to the role of canonical Wnt signaling in fibrosis described above. Hgf was shown to prevent epithelial to mesenchymal transition (EMT) by preserving E-cadherin in a unilateral ureteral obstruction (UUO) model of renal fibrosis, thereby blocking sustained de-differentiation[18]. Other mechanisms implicated in the anti-fibrotic role of Hgf include countering the actions of Tgf-β and PDGF, matrix degradation and reduction in inflammation[265].

Despite these beneficial effects, sustained activation of Hgf might have a detrimental role in the kidney. Overexpression of Hgf was shown to tubular hyperplasia, glomerulosclerosis, and polycystic kidney disease[266]. In a recent study, failure of Met degradation was shown to lead to sustained mTOR activation and cystic kidney disease[267]. In ICU patients with AKI requiring dialysis, lower levels of Hgf at Day 7 and 14 were associated with higher chances of being free from dialysis at day 60.[268]

The use of Hgf as a therapeutic agent in AKI has to be balanced against these findings along with the well-known role of Met as an oncogene. It is possible that while Hgf, (like Wnt) might be beneficial early in repair phase after AKI, its sustained presence is detrimental. This concern along the obstacles in the optimum method of delivery of Hgf due to its short half-life have slowed its development a therapeutic agent in clinical trials. Hgf has been safely used in early clinical trials as an adjunct therapy to coronary artery disease[269], in diabetic neuropathy[270], limb ischemia[271] and in fulminant hepatitis[272]. There is an ongoing phase 1 trial looking at the use of BB3 (a small molecule mimetic of Hgf) in delayed graft function (NCT01561599) which is usually

as a result of AKI immediately after kidney transplantation. It will be interesting to look at the findings of this trial which can hopefully pave way for more clinical studies of Hgf in other forms of AKI.

Our findings above show that Hgf-mediated Lrp5/6 phosphorylation occurs exclusively in de-differentiated renal epithelial cells which are similar to the surviving cells in the areas of tubular injury in AKI (Fig 2) and that this phosphorylation mediates the apoptotic effects of Hgf (Fig 4). Gsk3, a focal point of the crosstalk of the canonical Wnt and Hgf/Met signaling pathways, is known to a play a major role in the pathogenesis of injury in ischemic[139], septic[273] and nephrotoxic[217] models of AKI. Based on these data, and the evidence that both Hgf and Wnt have a protective role early after AKI, we studied the crosstalk of Hgf/Met and canonical Wnt signaling early after ischemic kidney injury using the γGT-Cre;$Met^{fl/fl}$ and γGT-Cre;$Met^{+/+}$ mice.

Hypothesis and specific aims:

Hgf mediates Lrp5/6 phosphorylation *in vivo* early after kidney injury.

1. To find out whether Lrp5/6 phosphorylation changes early after ischemic kidney injury and whether this is Met dependent.
2. To find out whether β-catenin stabilization early after ischemic kidney injury is Met dependent.

Materials and Methods:

Reagents:

Antibodies to β-actin was obtained from Santa Cruz Biotechnology. Antibodies to Lrp6, Lrp5 and phospho-Lrp6 Ser1490 were obtained from Cell Signaling. Antibodies to phospho-Lrp6

Thr1572 and Ser1607 were obtained from Millipore. For western blotting experiments secondary antibodies were purchased from Invitrogen. EDTA-free Protease and Phosphatase Inhibitor cocktail was obtained from Thermo Scientific.

Western analysis

Kidneys were harvested at 6 and 24 hours after IRI surgery and immediately flash frozen in liquid nitrogen. Tissue was homogenized in RIPA buffer containing protease and phosphatase inhibitors using a Dounce homogenizer while being kept on ice (4°C) throughout. The proteins were separated using 7.5% - 10% SDS-PAGE, electrophoretically transferred to Immobilon-P membranes (Millipore), immunoblotted with the appropriate antibody overnight, and visualized by enhanced chemiluminescence (Amersham Biosciences, Inc.) or West Femto Maximum Sensitivity Substrate (Thermo Scientific). Quantification of ECL signals was performed using NIH Image J software.

Generation of conditional Met knock-out mice and I/R surgery

Met$^{fl/fl}$ mice [253, 274] were mated to *γGT*-Cre mice to induce Met knock-out in the renal proximal tubule [3]. Eight- to ten-week old *γGT-Cre;Met*$^{fl/fl}$ mice or *γGT-Cre;Met*$^{+/+}$ (WT) controls were subjected to 25 minutes of warm left kidney ischemia with contralateral nephrectomy, followed by reperfusion as previously described[3]. Sham animals underwent midline ventral incision without clamping. The mice were sacrificed after 6 hours or 1 day for kidney harvest. All mouse experiments were conducted under a protocol approved by the Yale IACUC.

Statistical methods

The data between two groups was compared using the student's two-tailed *t* test. Significance was determined at $P < 0.05$ and the error bars represent standard deviations.

Results:

It has been previously shown that Lrp6 phosphorylation and subsequent canonical Wnt signaling are activated following AKI [234], so this model was used to assess the relevance of Hgf-stimulated transactivation of the canonical Wnt pathway *in vivo*. Consistent with the published data, phosphorylation of Lrp5/6 was found to be increased in kidney lysates from wild type mice on the first day after renal ischemia-reperfusion (I/R) injury (Fig 5A).

To test the importance of the Hgf/Met signaling pathway in this process, we utilized *GGT-Cre;Met$^{fl/fl}$* mice in which the Met receptor has been conditionally knocked out in the renal proximal tubule [3]. Western analysis of kidney lysates from *GGT-Cre;Met$^{fl/fl}$* mice revealed a significant reduction in I/R-induced Lrp5/6 phosphorylation at both S1490 and T1572 at 1 day after I/R injury as compared to wild-type mice (Fig 5B-D).

Consistent with the *in vitro* findings, this decrease in Met-dependent Lrp5/6 phosphorylation in I/R injured *GGT-Cre;Met$^{fl/fl}$* mice correlated with a decrease in active, non-phosphorylated β-catenin (ABC, Fig 5 E, quantified in 5F) compared to the control (wild type) mice.

Discussion:

The importance of Wnt signaling in tissue regeneration and repair after injury[233] is now well established. Wnt signaling is known to promote recruitment of stem/progenitor cells to the injury site[275]. The activation of canonical Wnt signaling appears to be one of the initial molecular responses to injury[276]. Even in mammals, which have a limited capacity to regenerate, Wnt signaling is involved in wound healing[277]. Besides AKI[234], Wnt signaling is also initiated in other forms of tissue injury where they promote the repair process. These include bone fracture[278], exposure to ionizing radiation[279], inhalation injury to the lung[280], skin wounding and myocardial infarction[233]. An interesting question is how does Wnt signaling get initiated in response to tissue

injury? Mazumdar et al provide a potential explanation to this question. They show that undifferentiated cells respond to low oxygen tension by up-regulating the expression of hypoxia-inducible factor (HIF)-1α which in turn modulates canonical Wnt signaling by enhancing β-catenin activation[281]. Besides this downstream activation, the study by Lin et al show the activation at the level of Lrp5/6 receptor[234].

Renal ischemic injury leads to rapid cell loss and dedifferentiation of the surviving cells. The finding that Hgf-dependent Lrp5/6 phosphorylation is maximal at low cell confluency is consistent with our previous data [72], and argues that this pathway is specifically activated in dedifferentiated cells. Therefore, the *in vivo* relevance of this pathway was assessed using I/R injury.

We recently used the GGT-Cre to create a mice line with conditional knockout of the Met receptor in the proximal tubule of the kidney (γGT-Cre;$Met^{fl/fl}$)[3]. The kidneys of these mice had no developmental defects with normal renal function at baseline. However after ischemia reperfusion injury (IRI), these mice had a significantly greater injury response (greater apoptosis, tubular injury score and levels of serum creatinine) compared to the wild type γGT-Cre;$Met^{+/+}$ kidneys. These findings correlated with a significant reduction in the activation of anti-apoptotic proteins like Akt and p70-S6 kinase and reduced inhibition of pro-apoptotic proteins like Bad[3]. In addition there was reduced proliferation and delayed repair in the γGT-Cre;$Met^{fl/fl}$ mice. Another recent study by Liu's group reproduced our findings. They also generated a knockout mouse with conditional deletion of the Met receptor in the renal tubules using the Ksp-Cre[4]. These mice also had normal kidneys at baseline, however after nephrotoxic (Cisplatin) and ischemic (IRI) AKI, the Ksp-met-/- mice had greater initial injury and apoptosis. This was accompanied by reduced activation of Akt and increased expression of pro-apoptotic proteins like Bax and Fas ligand. There was also an increased inflammatory response noted. Ectopic overexpression of Hgf had a protective effect in the kidneys of the wild type but not Ksp-met-/- mice[4]. These data highlighted the importance of Hgf/Met signaling in the anti-apoptotic/cytoprotective machinery that gets activated early after AKI.

We now show that Lrp5/6 phosphorylation and β-catenin stabilization are detectable in the first day after ischemic kidney injury and are reduced in the *GGT-Cre Met$^{fl/fl}$* mouse. Hence, Lrp5/6 phosphorylation and β-catenin stabilization early after ischemic kidney injury is in part dependent on Met receptor activation in the proximal tubule. These data suggest that the early anti-apoptotic effects of Hgf that are critical for tubular cell survival and ultimate tubule repair may be dependent on coordinated signals derived from both Lrp5/6 and Akt activation. Of note, Wnt-stimulated activation of Lrp5/6 phosphorylation has been shown to occur 2-7 days after injury, and is also required for normal kidney repair[234]. The current findings suggest that Hgf signaling transactivates this pathway prior to Wnt, thus potentially initiating the expression of downstream cell survival signals and contribute to the repair process.

Chapter 7. Conclusions and Future Directions

The normal renal epithelial cell physiology and its alterations in response to injury involve a crosstalk between different signaling pathways. Tyrosine kinases are known to activate canonical Wnt signaling in response to growth factor stimulation. Hgf/Met and canonical Wnt signaling are both up-regulated after AKI and is actively involved in the repair process through similar physiologic processes including survival and proliferation of the epithelial cells. Hence we hypothesized that Hgf could activate canonical Wnt signaling in cultured renal tubular epithelial cells and in the kidney after AKI.

Our data shows that Hgf stimulates Lrp5/6 phosphorylation at at least 3 activation sites in a Wnt-independent and Met-dependent manner. This phosphorylation is mediated by Gsk3 utilizing a previously unidentified mechanism in which 'active' Gsk3 is selectively recruited to Lrp5/6 and the inhibited Gsk3 (serine phosphorylated) form is excluded from this association. This suggests that Hgf regulates at least two separate pools of Gsk3, one that is inhibited by Akt through serine phosphorylation and the other that is associated with Lrp5/6 and mediates its phosphorylation. Hgf-mediated Lrp5/6 phosphorylation leads to β-catenin stabilization and nuclear accumulation, and it is possible that both these Gsk3 pools are involved in this process.

The significance of Hgf stimulated phosphorylation of Lrp5/6 is highlighted by the following observations. We find that Hgf stimulated phosphorylation of Lrp5/6 is restricted to highly sub-confluent, dedifferentiated cells that are phenotypically similar to epithelial cells that are involved in the repair process after AKI. Secondly, Hgf-mediated anti-apoptotic activity is dependent on Lrp5/6 phosphorylation and correlates with increased Survivin expression. Thirdly, consistent with these cell-based findings, *in vivo* studies show that there is a Met-dependent increase in Lrp5/6 phosphorylation and β-catenin stabilization early after renal ischemia/reperfusion (I/R) injury in mice.

Hence overall, our results highlight a novel mechanism of Hgf-dependent transactivation of canonical Wnt signaling that identifies a Wnt-independent pathway for rapid, early *in vitro* and *in vivo* Lrp5/6 activation. Combined with our previous studies [3, 72], this work suggests a dual mechanism through which Hgf/Met signaling cross-talks with canonical Wnt signaling; upstream activation of Lrp5/6 phosphorylation by Gsk3 and downstream inhibition of Gsk3 by Akt, both potentially contributing to β-catenin activation and regulation of apoptosis of renal epithelial cells (Fig 6). Parathyroid hormone has been shown to also stimulate canonical Wnt signaling via two separate mechanisms, similarly involving proximal phosphorylation of Lrp5/6 [138] and distal inhibition of Gsk3[93].

There are several questions that emerge from our data that should be the focus of future studies. Why is Hgf-mediated Lrp5/6 phosphorylation related to cell confluency and differentiation status of MPT cells? We find that the basal phosphorylation of Lrp5/6 (in the absence of Hgf or Wnt) is inhibited by Dkk-1 and is Gsk3 dependent. What regulates this basal phosphorylation of Lrp5/6 and why is this increased with a rise in cell confluency? Does this basal phosphorylation also regulate β-catenin stabilization? Does this β-catenin go to the nucleus to mediate gene transcription or to the AJ to strengthen cell-cell contacts?

Another interesting question is how does Hgf regulate different Gsk3 pools in the cell? How does Hgf increase the association of 'active' Gsk3 with Lrp5/6 while excluding the 'inhibited' (serine phosphorylated) Gsk3 from this association? Is the regulation of different Gsk3 pools by Hgf dependent on the physiologic context or do they work in synergy? Are they both involved in β-catenin regulation? One potential way to answer this question, as previously mentioned, would be to mutate the inhibitory and stimulatory phosphorylation sites of Gsk3 and then look at downstream signaling in response to Hgf.

Another area that merits further investigation is the role of Hgf-mediated Lrp5/6 phosphorylation in reducing apoptosis. Is the β-catenin-mediated Survivin gene expression

involved in this or is it regulated by β-catenin mediated regulation of cytosolic Bax? Does Hgf mediate anti-apoptotic signals through PI3K/Akt/Bad pathways separately from those mediated by Lrp5/6 phosphorylation or are they inter-dependent?

Lastly our *in vivo* data also raises interesting questions that can be further investigated. Is the Lrp5/6 phosphorylation and β-catenin stabilization after AKI regulated differently by Hgf and Wnt? It is possible that while Hgf-mediated Lrp5/6 phosphorylation primarily regulates the initial anti-apoptotic signaling along with Akt, while Wnt-mediated Lrp5/6 phosphorylation primarily regulates the subsequent proliferative responses. How and when is Hgf-mediated Lrp5/6 phosphorylation down-regulated after AKI?

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Figure Legends:

Figure 1. Hgf induces Wnt independent phosphorylation of Lrp5/6:

(A) MPT cells were stimulated ±Hgf for 10 minutes and the lysates immunoblotted using a phosphospecific Lrp6 antibody against S1490 in Lrp6 (and potentially S1502 in Lrp5).

(B) Quantification of Hgf-stimulated pLrp5/6 in MPT cells normalized to total Lrp6 (n=4).

(C) Time course of Hgf-stimulated MPT cells.

(D) Lysates from Hgf-stimulated MPT cells were immunoblotted using phosphospecific Lrp6 antibodies that recognize T1572 and S1607 in Lrp6 (and potentially T1577 and S1608 in Lrp5).

(E) Quantification of pLrp5/6 as in C, D normalized to total Lrp6 (n=3).

(F) PCR analysis of MPT cells reveals mRNA expression for both Lrp5 and Lrp6 genes.

(G) Wnt3a stimulation of MPT cells for 10 minutes with immunoblots using pLrp6 antibodies to the three phosphorylation motifs (S1490, T1572 and S1607).

(H) mIMCD-3 cells stimulated ±Hgf for 10 minutes followed by immunoblotting for pLrp6 S1490.

(I) MPT cells were pre-incubated with Dkk1 or vehicle, followed by stimulation with vehicle, Wnt3a or Hgf for 10 minutes followed by immunoblotting for pLrp6 S1490 and total Lrp6.

(J) Densitometric quantification of 3 separate experiments in (I) using pLrp6 S1490 normalized to total Lrp6. (**$p<0.01$ for control + Dkk1 vs. control; ***$p<0.001$ for Hgf vs. control and Wnt3a vs. control; $^{\$}$p=NS for Hgf + Dkk1 vs. Hgf; $^{\#}p<0.01$ for Wnt + Dkk1 vs. Wnt alone).

(K) MPT cells were pre-incubated with the Met inhibitor PHA-665752 or vehicle, followed by stimulation ±Hgf for 10 minutes followed by immunoblotting for pLrp6 S1490, total Lrp6, pMet, total Met and -actin.

(L) Densitometric quantification of 4 separate experiments as in (K) with pLrp6 S1490 normalized to total Lrp6 (**p<0.01 for Hgf vs. control and #p<0.01 for Hgf + PHA-665752 vs. Hgf alone). (M, N) Immunoblots of pLrp6 T1572 and pLrp6 S1607 from cells treated as in (K).

(**=p<0.01 vs. control, ***=p<0.001 vs. control).

Figure 2. Hgf stimulated phosphorylation of Lrp5/6 is dependent on cell confluency:

(A) 40X bright field microscopy images of morphology of MPT cells plated at 'low' ($2.1x10^3$ cells/cm^2), 'medium' ($4.2x10^4$/cm^2), and 'high' ($4.2x10^5$/cm^2) confluency.

(B) Immunoblot images of MPT cells plated at the indicated confluencies ±Hgf stimulation for 10 minutes showing relatives changes in the levels of total Lrp6, pLrp6 S1490, total Met, pMet, total Erk1/2, pErk1/2, E-cadherin and β-actin.

(C) Quantification of the relative phosphorylation of pLrp6 S1490 as in (B) normalized to total Lrp6 (n=3. **=p<0.01 vs. control at 'low' confluency, NS= non-significant vs. control at 'medium' confluency).

(D) MPT cells treated as in (B) were immunoblotted for pLrp6 T1572 and S1607.

Figure 3. Hgf stimulated phosphorylation of Lrp5/6 is dependent on Gsk3:

(A) Gsk-3b is present in both the cytosol and membrane fractions of MPT cells. E-cadherin was used as a membrane marker.

(B) MPT cells were pre-incubated with the Gsk3 inhibitor BioIX (10μM) or vehicle, followed by treatment ± Hgf for 10 minutes and immunoblotting for pLrp6 S1490 and total Lrp6.

(C) Densitometric quantification of pLrp6 S1490 as in (A) using total Lrp6 for normalization (n= 4; *p<0.05 BioIX vs. control; **p<0.01 Hgf vs. control; #p<0.001 Hgf +BioIX vs. Hgf alone).

(D) Dose response of MPT cell pLrp6 S1490 phosphorylation by Hgf at the indicated doses of Bio IX (in μM).

(E) MPT cells were pre-incubated with 3 different Gsk3 inhibitors: BIOIX at 10uM, Lithium chloride (LiCl) at 10mM and 100mM and CHIR-99021 (CHIR) at 20M and 80uM or vehicle, followed by treatment ± Hgf for 10 minutes and immunoblotting for pLrp6 S1490 and total Lrp6.

(F) MPT cells were stimulated ±Hgf for 10 minutes, followed by immunoblotting of whole cell lysates for the inhibitory pS9 site on Gsk3β and activating pY279/216 sites on Gsk3α and β, respectively.

(G) MPT cells were stimulated ±Hgf for 10 minutes, the lysates immunoprecipitated with anti-Lrp6 or control IgG and then immunoblotted for total Lrp6, total Gsk3, pGsk3α/β Y279/216 and pGsk3β S9. Immunoblots from whole cell lysates (WCL) prior to IP are shown on right.

(H) Densitometric quantification of the amount of total Gsk3 and pGsk3α/β T279/216 that coimmunoprecipitated with Lrp5/6 normalized to the amount of immunoprecipitated Lrp6. (total Gsk3: n=3,*p< 0.05 Hgf vs. control; pGsk3α/β Y279/216: n=5, ** p< 0.01 Hgf vs. control).

Figure 4: Hgf mediated b-catenin stabilization and anti-apoptotic activity is dependent on Lrp5/6 phosphorylation:

(A) MPT cells were stimulated ±Hgf for 10 minutes followed by biotinylation. WCL and lysates from biotinylated fractions were immunoblotted for pLrp6 S1490 and total Lrp6. β-actin was used a negative while Na/K ATPase was used as positive marker for the biotinylated fraction.

(B)Lysates of MPT cells expressing shRNA against both Lrp5 and 6 proteins (5/6 kd) or a scrambled shRNA control (scr) were immunoblotted for Lrp5, Lrp6 and β–actin.

(C) Densitometric quantification as in (B) using β-actin for normalization (n= 4; ***$p<0.001$ Lrp5/6 kd vs. scr for both Lrp5 and 6).

(D) 5/6 kd or scr cells were stimulated ±Hgf for 10 minutes and lysates immunoblotted for pLrp6 S1490, T1572, S1607 and β-actin.

(E) Densitometric quantification of pLrp6 S1490 from 4 separate experiments normalized to β-actin (***$p<0.001$ for Hgf vs. control in scr cells; # $p<0.01$ for Hgf in 5/6kd cells vs. Hgf in scr cells).

(F) 5/6 kd (Clone 5 and 6) or scr cells (clone mix) were stimulated ±Hgf for 10 minutes and lysates immunoblotted for pLrp6 S1490, pMet and β-actin. Densitometric quantification of pLrp6 S1490 from 3 separate experiments normalized to β-actin. (**$p<0.001$ for Hgf vs. control in scr cells; # $p<0.001$ for Hgf in 5/6kd cells vs. Hgf in scr cells).

(G) Lrp5/6 kd or scr cells were stimulated ±Hgf for 90 minutes and the lysates immunoblotted for active β-catenin (ABC) and β-actin.

(H) Densitometric quantification of 4 separate experiments as in (F) using ABC normalized to β-actin (***$p<0.001$ for Hgf vs. control in scr cells; # $p<0.01$ for Hgf in 5/6kd cells vs. Hgf in scr cells).

(I) Lrp5/6 kd or scr cells were stimulated ±Hgf for 90 minutes followed by isolation of nuclear fractions and immunoblotting for ABC, HSP-90 (to detect cytosolic contamination) and Lamin A/C (to detect nuclear fractions).

(J) Q-PCR quantification of c-myc normalized to GAPDH from 3 separate experiments as in (F) with y-axis showing the fold change. (*p<0.05 for Hgf vs. control in scr cells; # p<0.05 for Hgf in 5/6kd cells vs. Hgf in scr cells).

(K) Lrp5/6 kd or scr cells were stimulated ±Hgf for 90 minutes followed by culture in 250mM H202 for 2 hours. Control cells were cultured in the same media without H202, The cells were fixed and processed for TUNEL staining. The quantification represents 5 experiments. (**p<0.01 for Hgf+H2O2 in scr control vs. H2O2 alone; *p<0.05 for Hgf+H2O2 in scr vs. Hgf+H2O2 in 5/6 kd).

(L) Q-PCR quantification of Survivin normalized to GAPDH from 3 separate experiments as in (K) with y-axis showing the fold change. (*p<0.05 for Hgf vs. control in scr cells; # p<0.01 for Hgf in 5/6kd cells vs. Hgf in scr cells).

Figure 5. Lrp5/6 phosphorylation and b-catenin stabilization immediately after ischemic kidney injury are Met dependent:

(A) Immunoblot of kidney lysates from the wild type (WT) mouse kidney before (0), 6 hrs after (0.25) and one day after I/R injury showing relative amounts of pLrp6 S1490 and total Lrp6.

(B) Representative immunoblot from kidney lysates from 2 *GGT-Cre;Met$^{fl/fl}$* mice and 2 WT mice one day after I/R injury showing relative amounts of pLrp6 S1490 and total Lrp6.

(C) Densitometric quantification of pLrp6 S1490 one day after I/R as in (B) normalized to total Lrp6. n=5 mice in each group, *p<0.05 *GGT-Cre;Met$^{fl/fl}$* vs. WT.

(D) Kidney lysates from *GGT-Cre;Metfl/fl* and WT mice 6 hrs and one day after I/R injury immunoblotted for pLrp6 T1572.

(E) Representative immunoblot from kidney lysates from *GGT-Cre;Met$^{fl/fl}$* mice and WT mice one day after I/R injury showing relative amounts of active β-catenin (ABC) and β-actin.

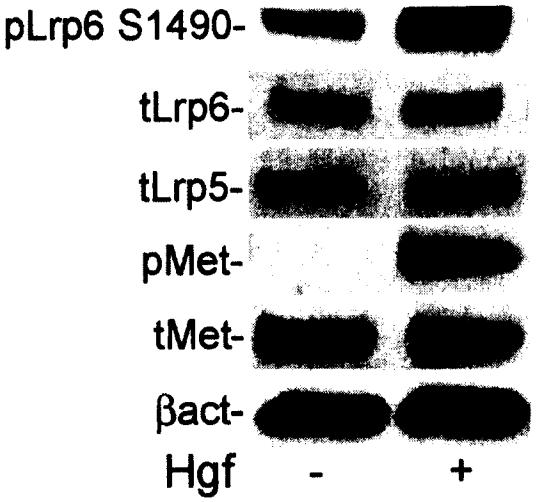
(F) Densitometric quantification of ABC one day after I/R as in (E) normalized to β-actin. n=5 mice in each group, *$p<0.05$ *GGT-Cre;Met$^{fl/fl}$* vs. WT.

Figure 6. A schematic diagram illustrating the potential mechanisms of the cross-talk between Hgf/Met and canonical Wnt signaling pathways

Figures:

Figure 1

Figure 1A



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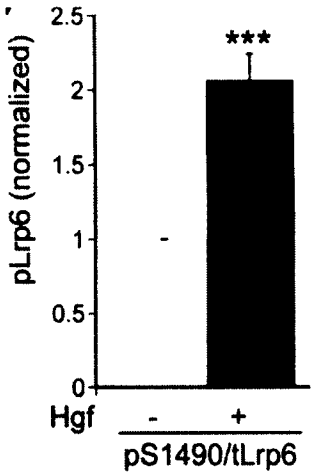
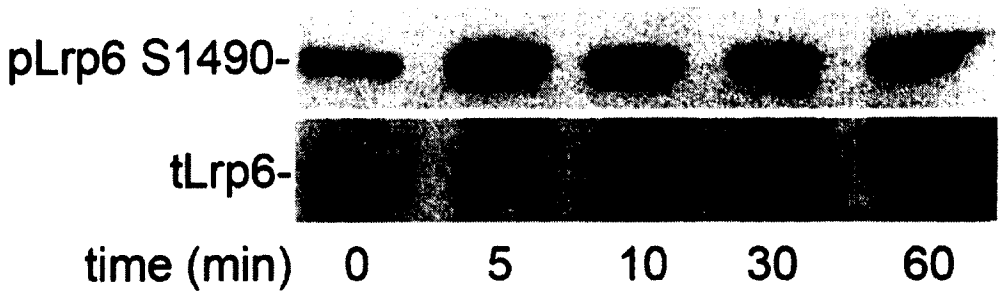


Figure 1C



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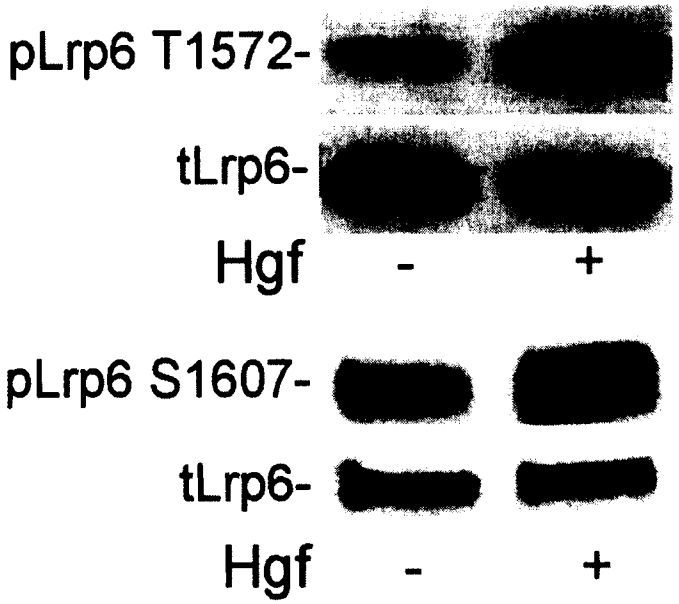

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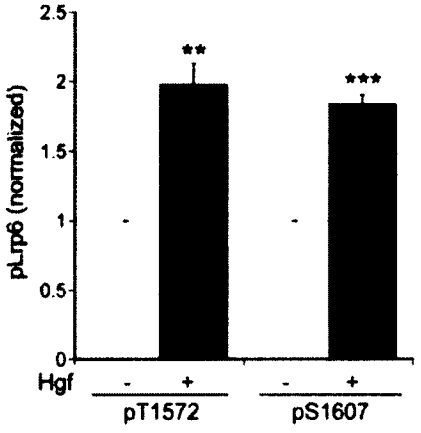

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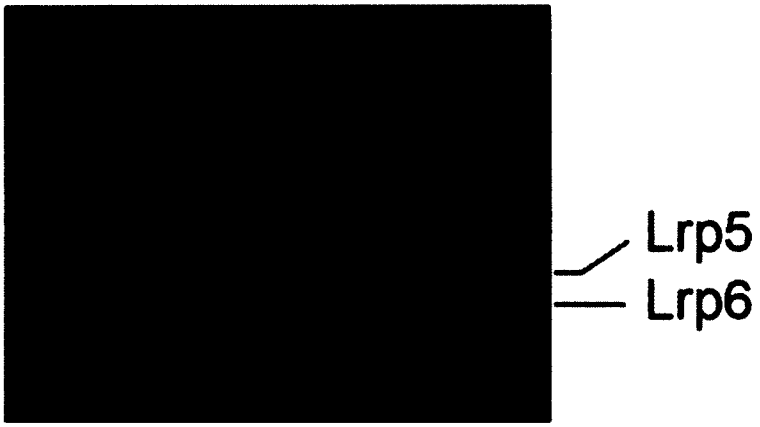

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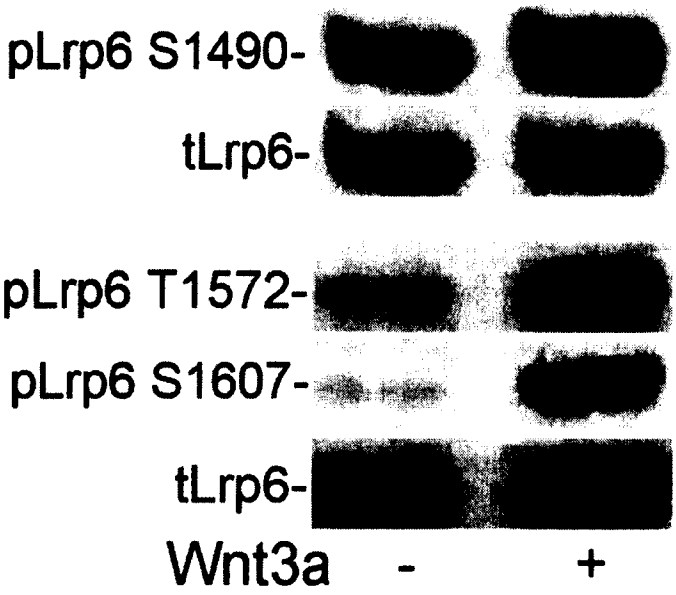

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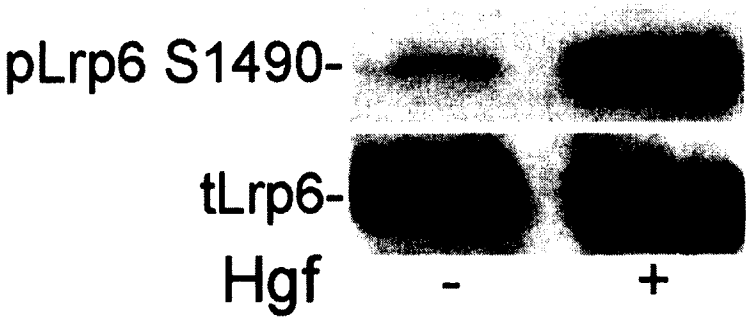

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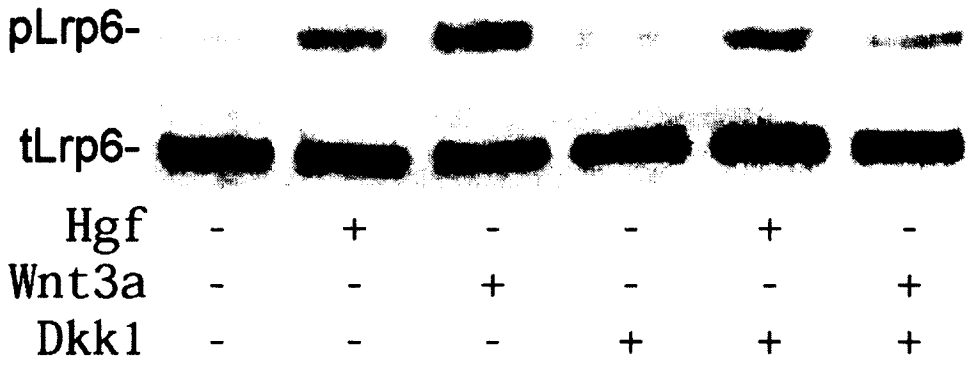

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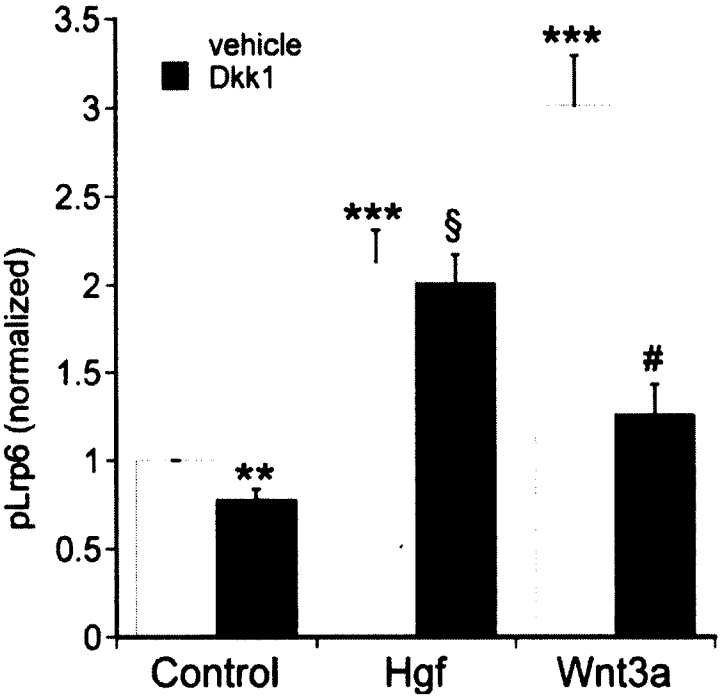

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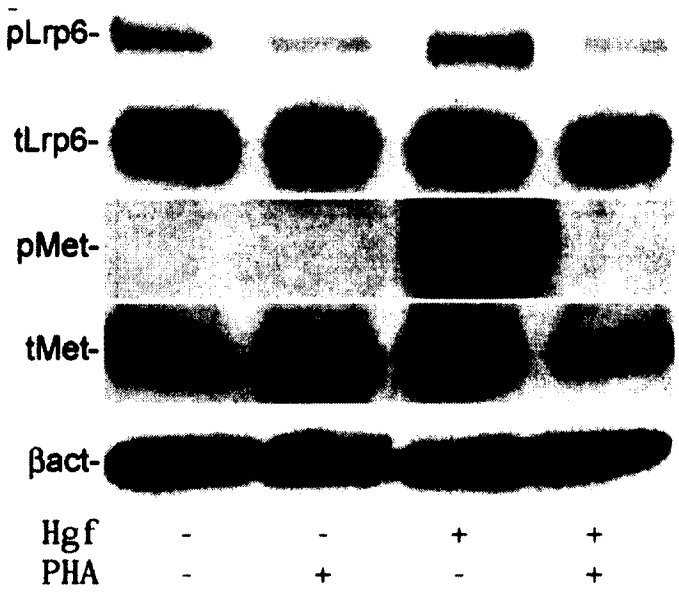

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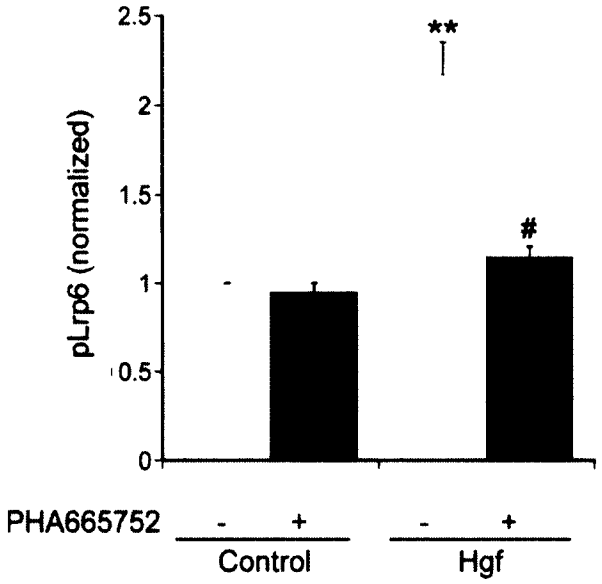
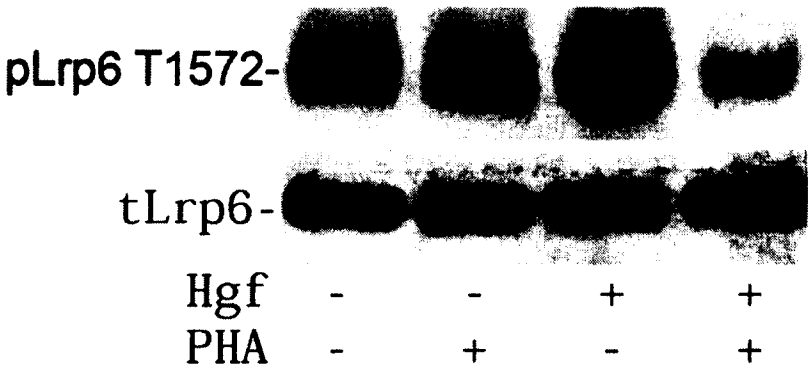


Figure 1M



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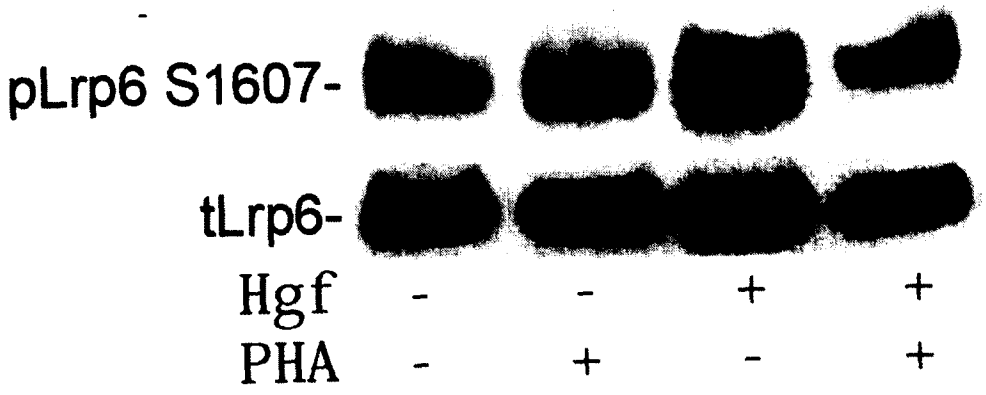

Figure 2

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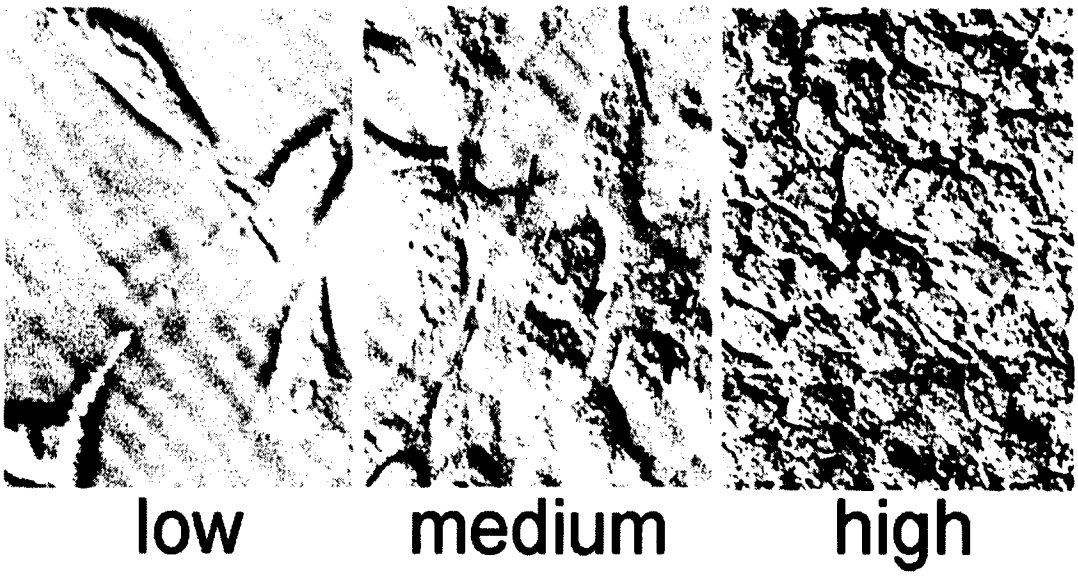

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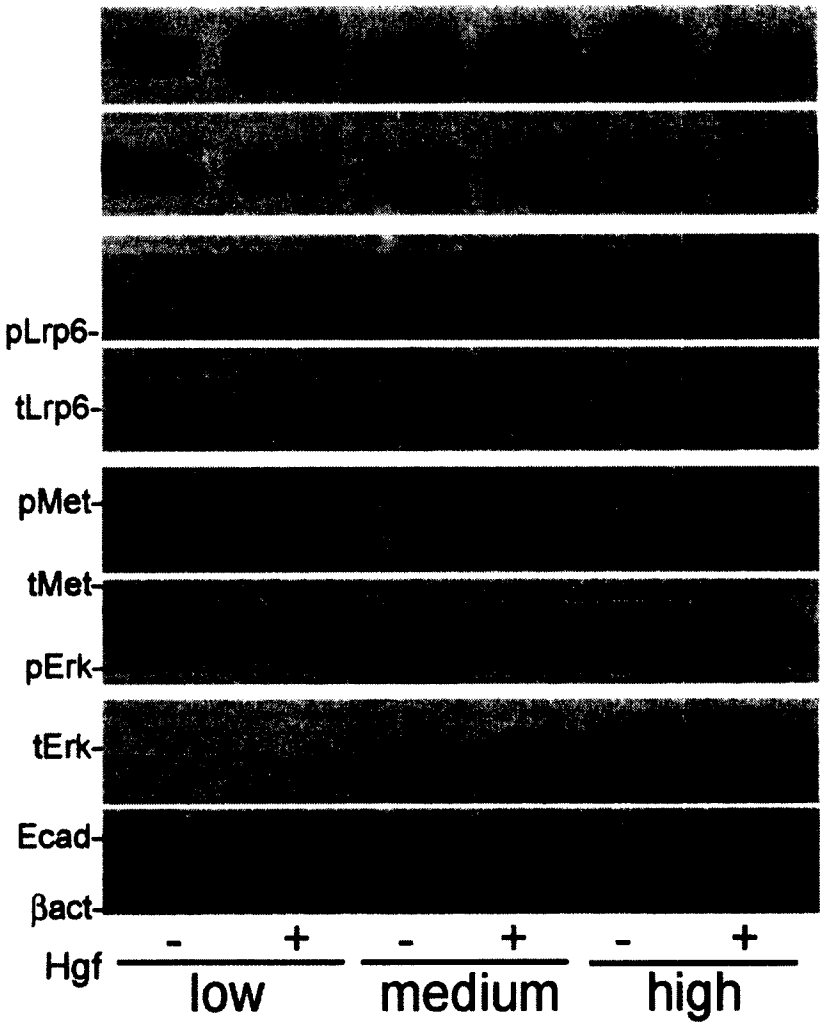
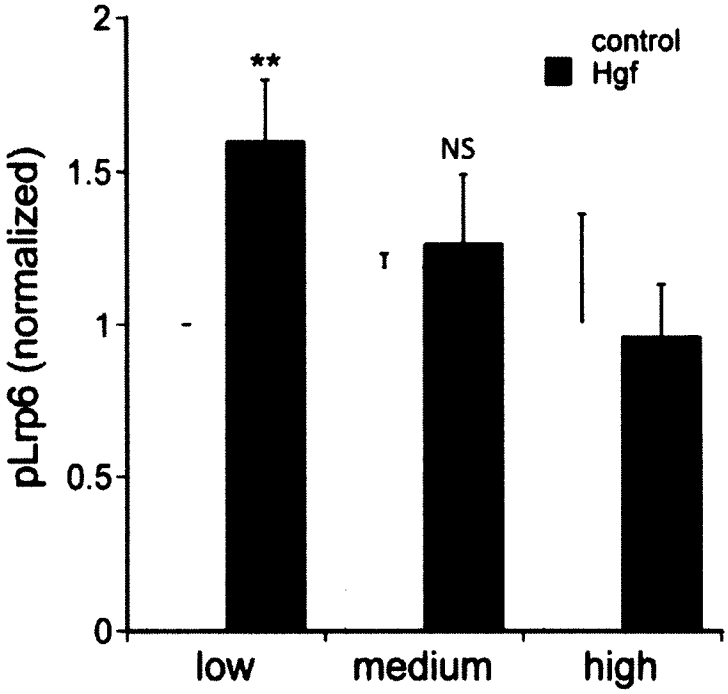

pLrp6-
tLrp6-
pMet-
tMet-
pErk-
tErk-
Ecad-
βact-
Hgf
-
+
-
+
-
+
low
medium
high

Figure 2C



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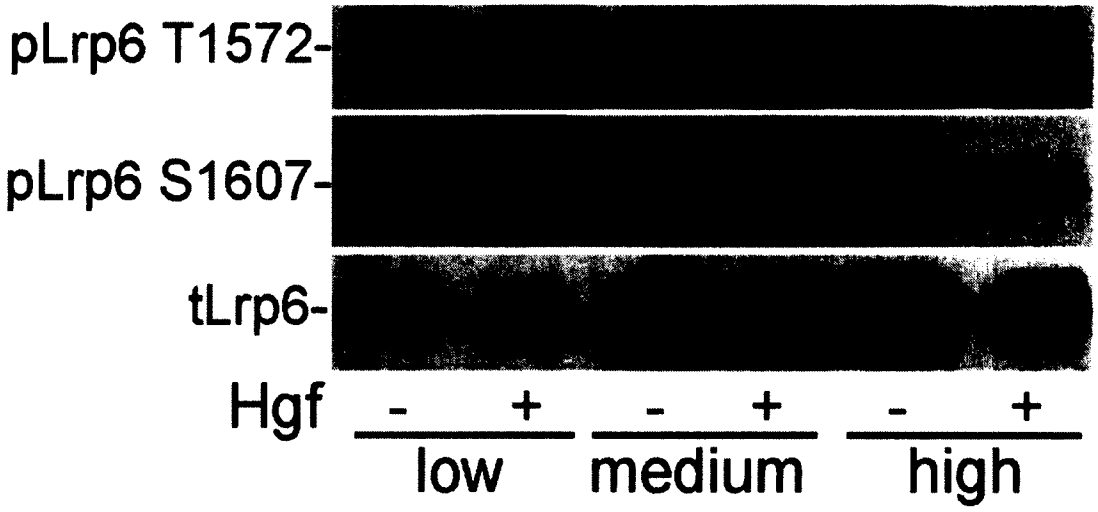
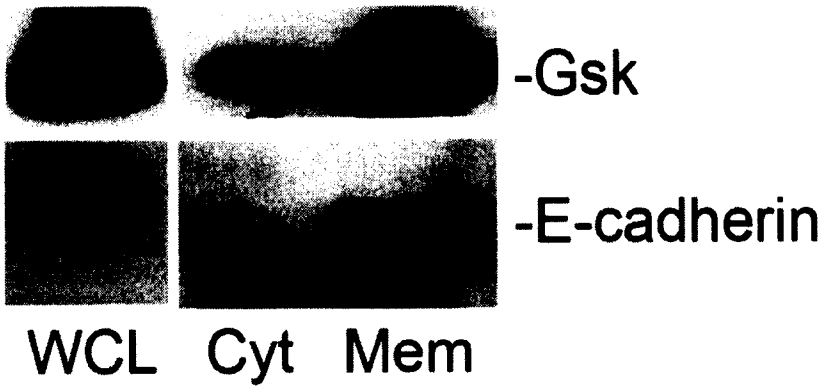


Figure 3

Figure 3A



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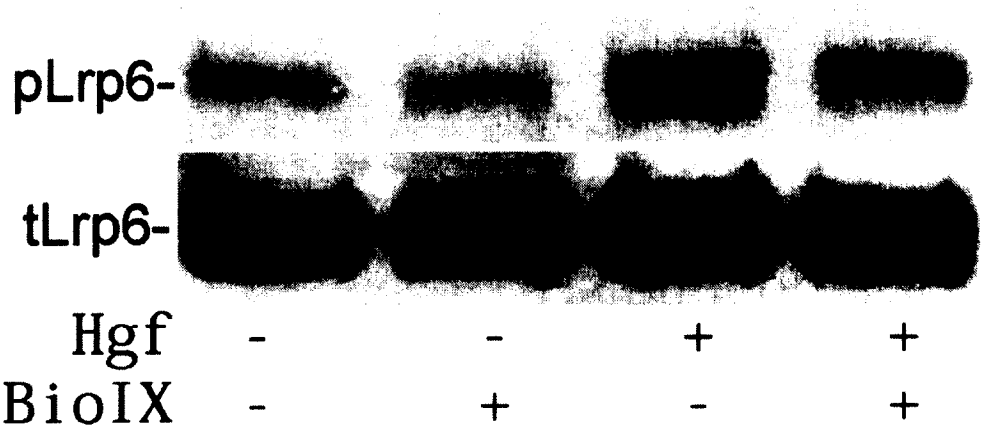

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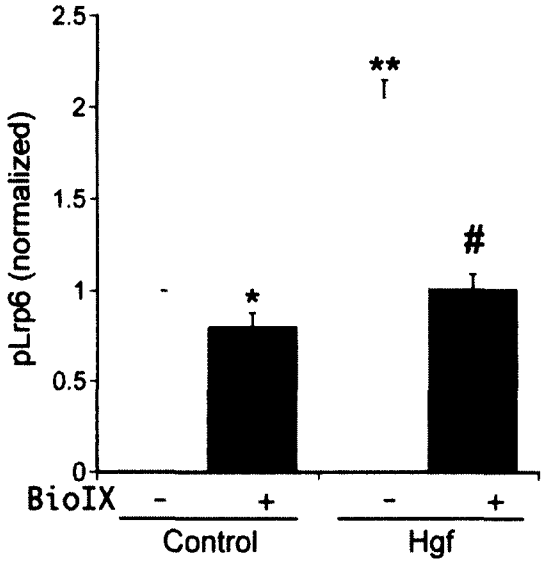

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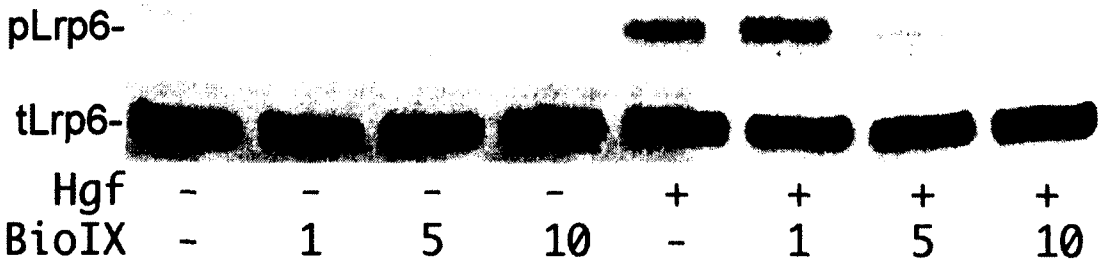

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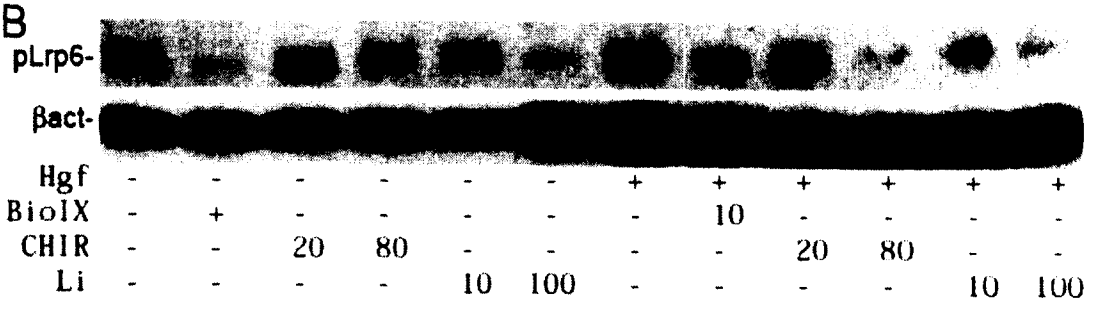

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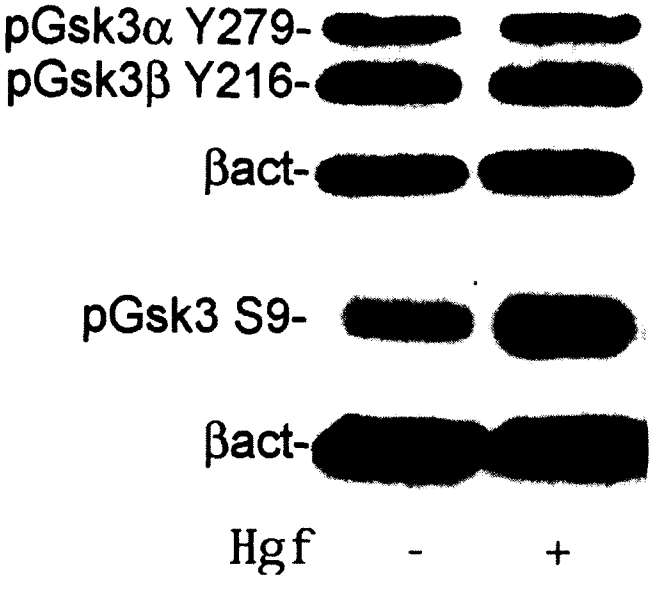

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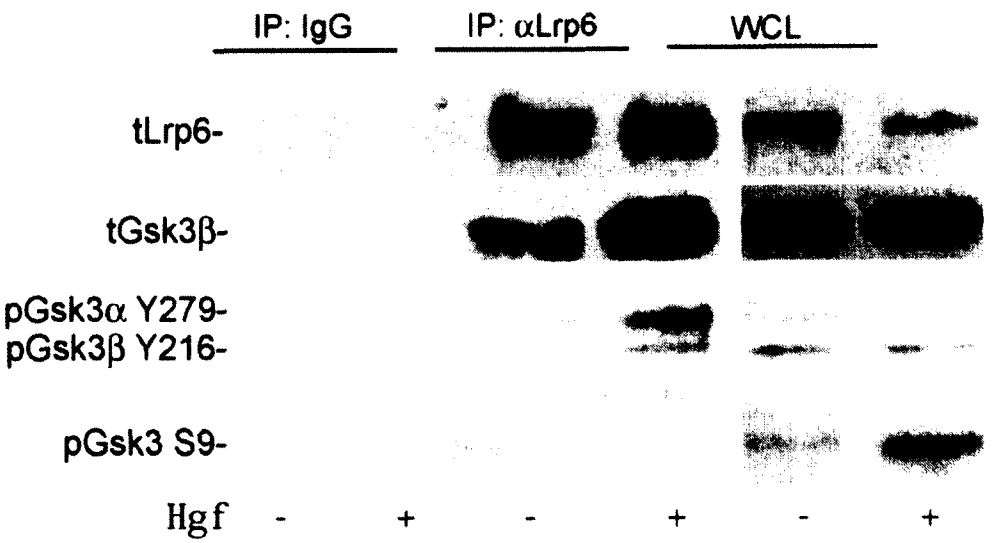

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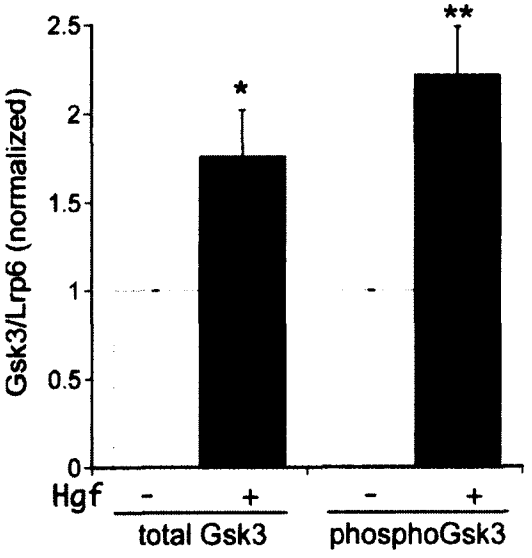
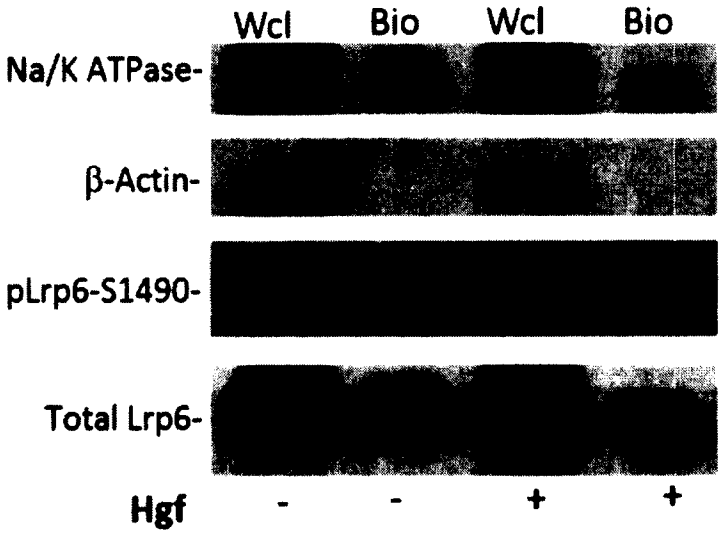


Figure 4

Figure 4A



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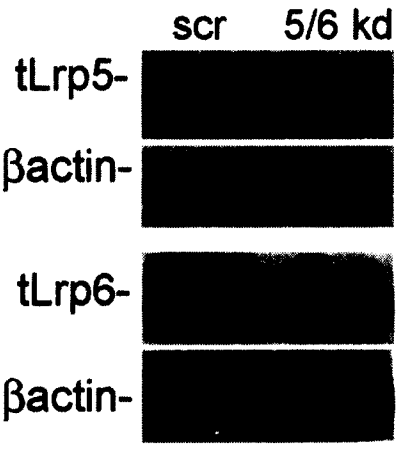

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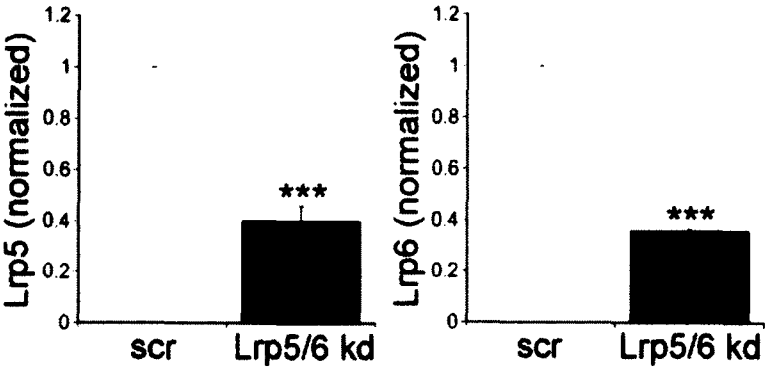

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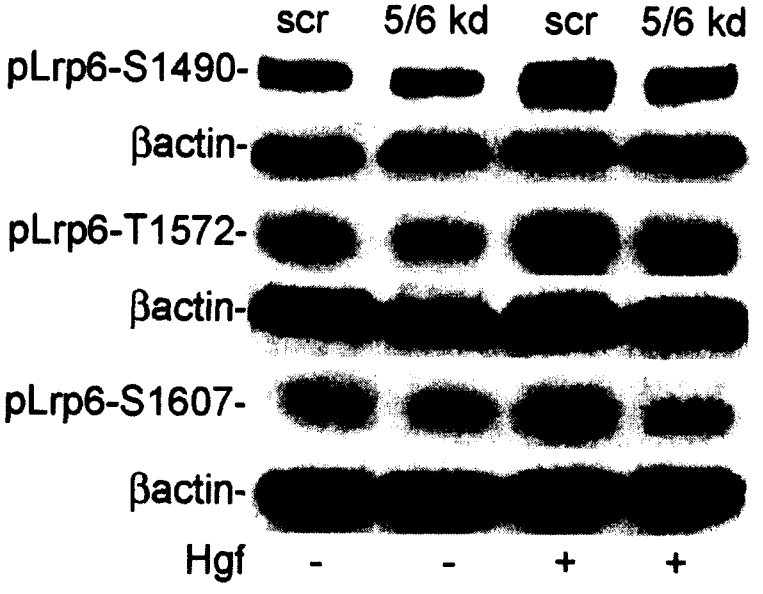

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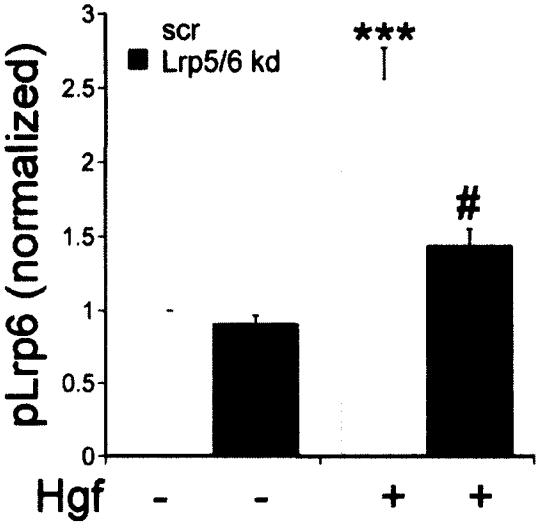

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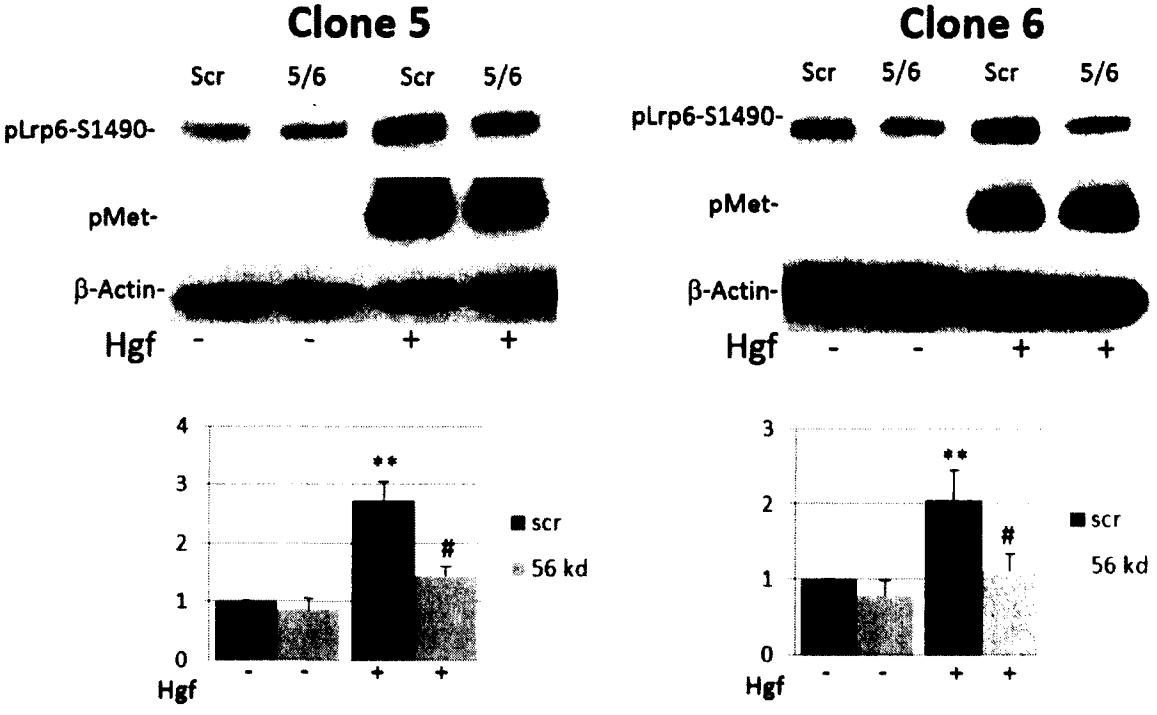

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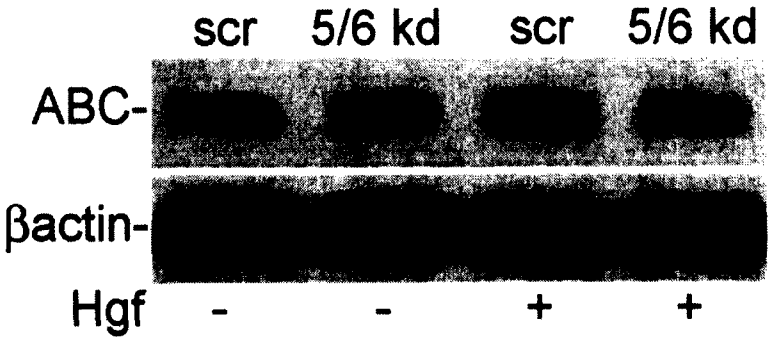

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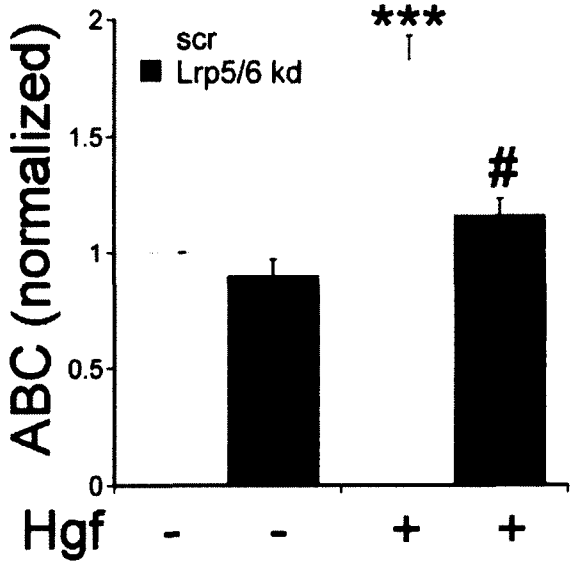

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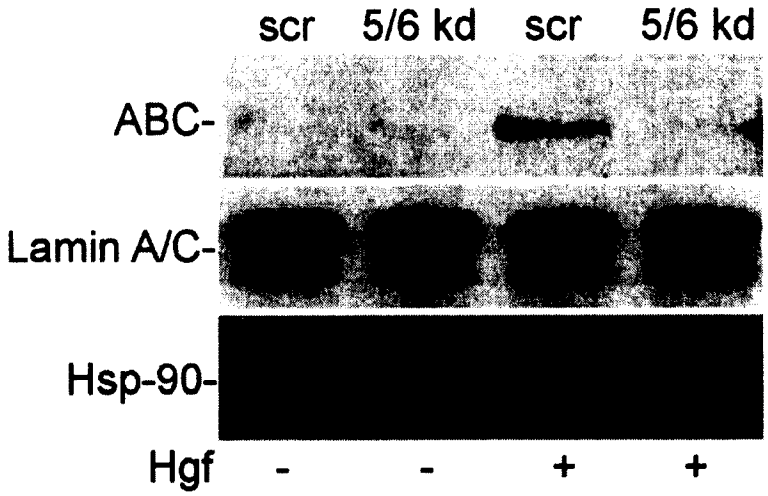

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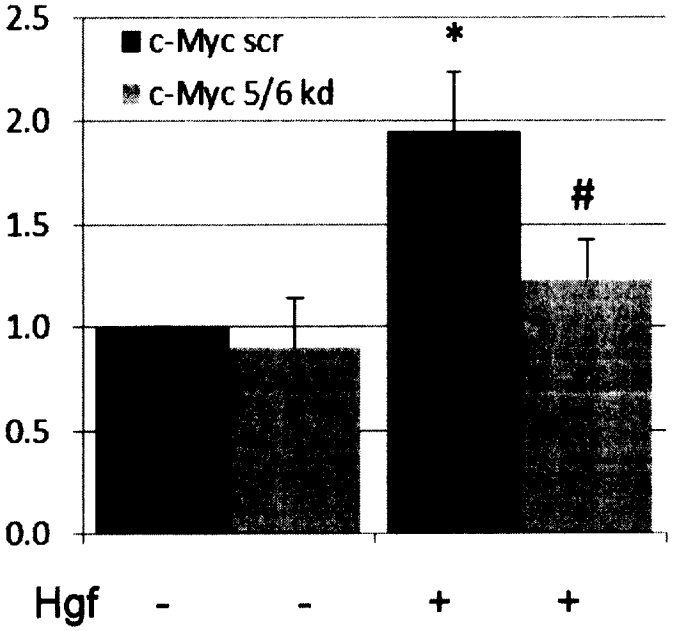

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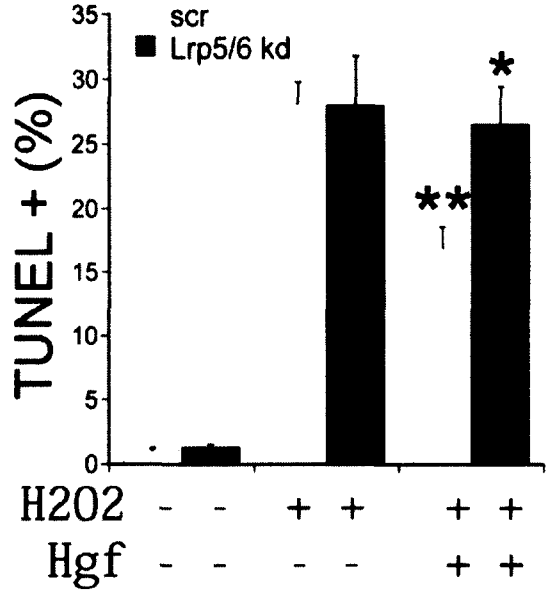

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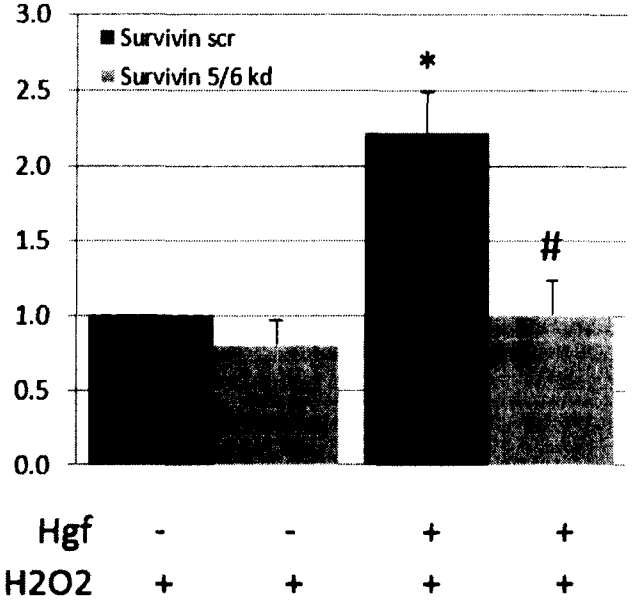
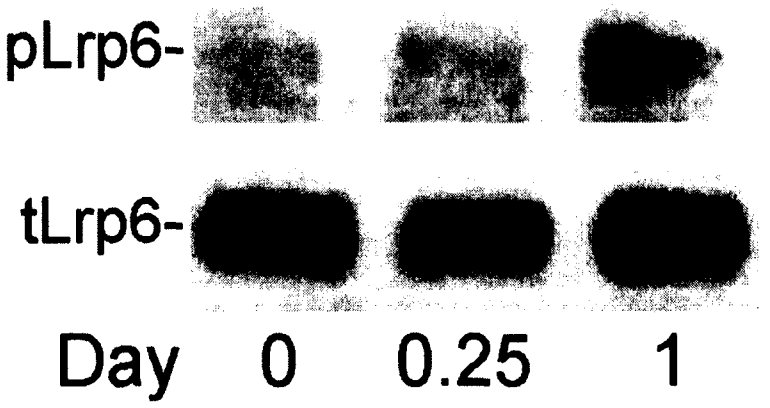


Figure 5

Figure 5A



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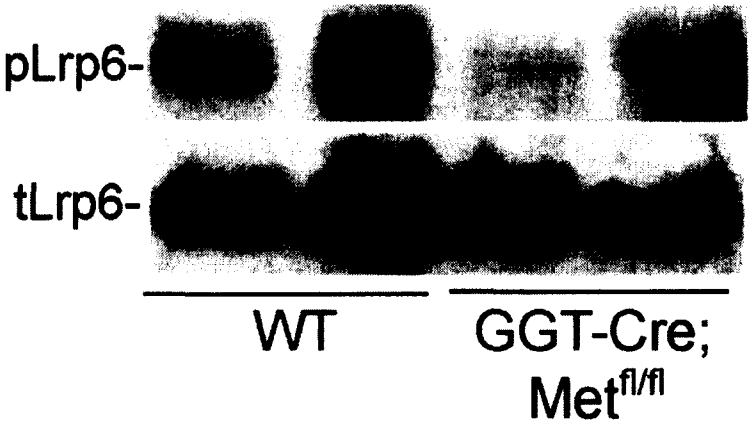

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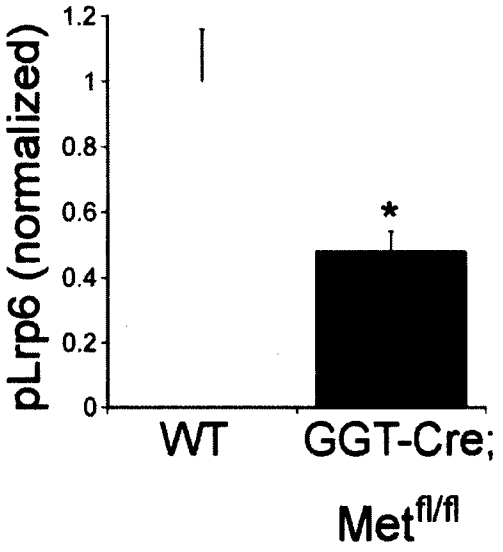

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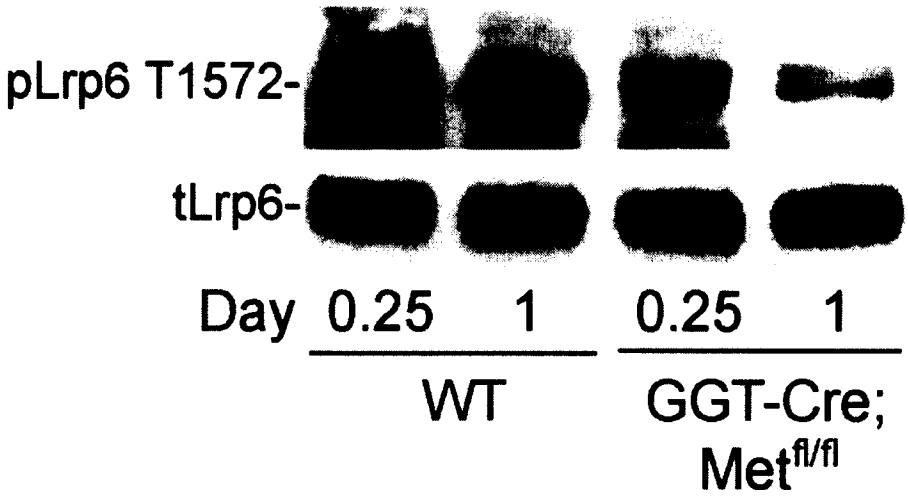
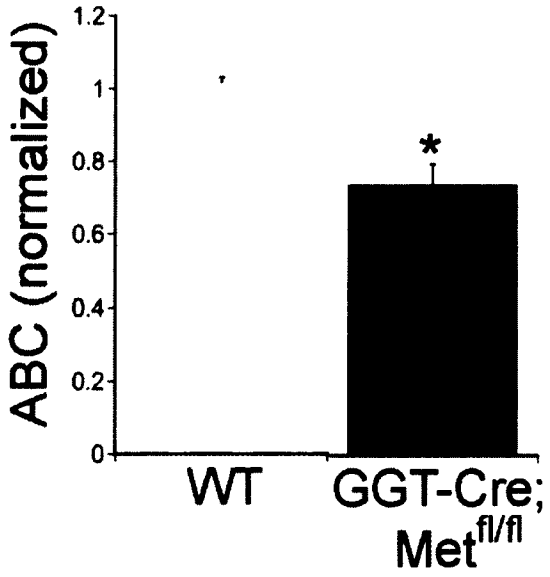


Figure 5E

Figure 5F



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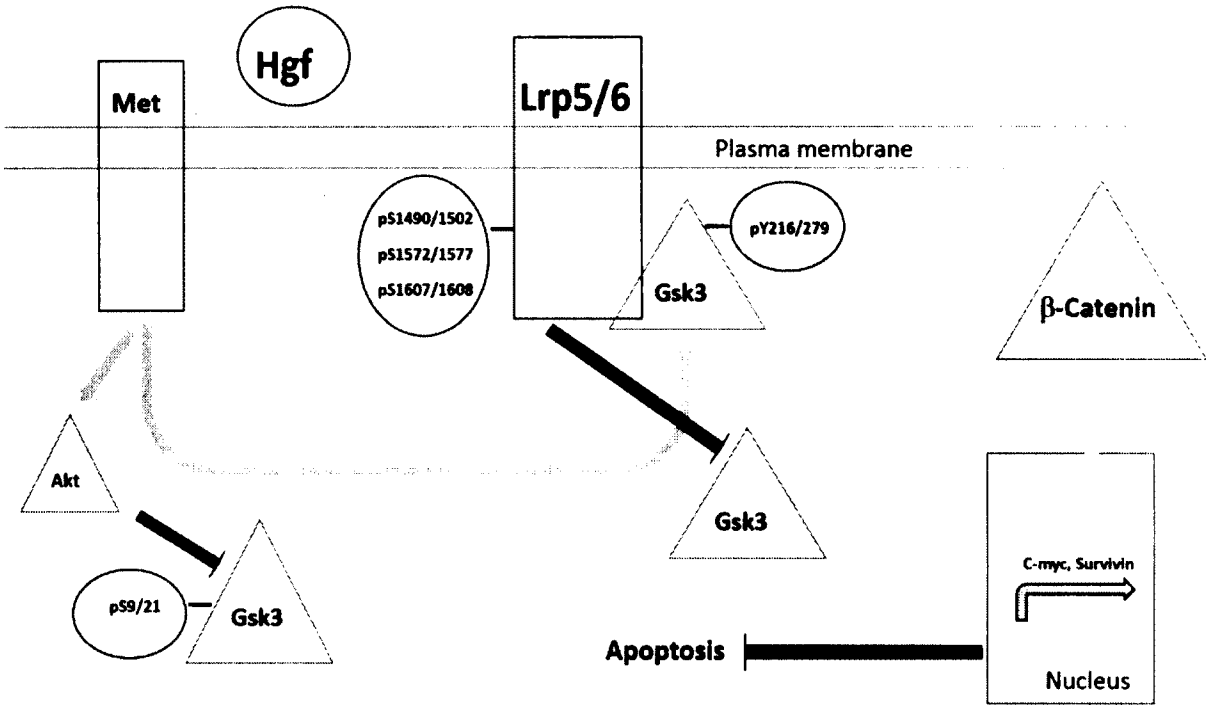
Hgf
Met
Lrp5/6
Plasma membrane
pS1490/1502
pS1572/1577
pS1607/1608
pY216/279
Gsk3
β-Catenin
Akt
Gsk3
pS9/21
Gsk3
C-myc, Survivin
Apoptosis
Nucleus

kidney disease model[85]. In a recent study, β-catenin was found to be the central point of the crosstalk of the EGFR/ERK, PI3K/AKT and canonical Wnt signaling pathways in modulating epithelial tissue regeneration[86].

This cross communication between Wnt and Hgf signaling highlights the complexity of signal transduction in mammalian physiology. Signaling occurs not only in a linear fashion from membrane receptor down to the nuclear transcription factor but also in a 'horizontal' fashion forming a network with other pathways where each protein can be regulated by multiple different proteins leading to diverse downstream responses depending on the cellular and physiologic context. Unravelling these complexities in signal transduction will lead to a greater understanding of normal physiology and its alteration during disease states like kidney injury.