Deleting the TGF-beta Receptor in Proximal Tubules Impairs HGF Signaling

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Transforming growth factor-β (TGF-β) and hepatocyte growth factor (HGF) play key roles regulating the response to renal injury but are thought to mediate divergent effects on cell behavior. However, how TGF-β signaling alters the response to HGF in epithelia, the key site of HGF signaling in the injured kidney, is not well studied. We showed that deleting the TGF-β type II receptor in conditionally immortalized proximal tubule cells unexpectedly impaired HGF-dependent signaling. This reduced signaling was due to decreased transcription of c-Met, the HGF receptor, and both the TGF-β-dependent c-Met transcription and increased response to HGF in proximal tubule cells were mediated by the Notch pathway. The interactions of TGF-β, HGF, and Notch pathways had biologically significant effects in branching morphogenesis, cell morphology, migration, and proliferation. In conclusion, epithelial TGF-β signaling promotes HGF signaling in a Notch-dependent pathway. These findings suggest that TGF-β modulates proximal tubule responses not only by direct effects, but also by affecting other growth factor signaling pathways.

Key words: TβRII, growth factors, proximal tubule, Notch signaling
Introduction

Growth factors are critical modulators of the kidney’s response to all forms of injury. After renal injury, growth factors are upregulated and coordinate cellular events such as de-differentiation, migration, and proliferation that lead to repair. In the injured kidney, growth factors affect the responses of the injured epithelium, surrounding fibroblasts, endothelium, and infiltrating inflammatory cells, and the balance of these growth factors impacts how the kidney recovers. Two growth factors that play key roles in the response to injury are hepatocyte growth factor (HGF) and transforming growth factor-β (TGF-β).

Both TGF-β and HGF signaling are increased in the damaged epithelium(15, 21). All three TGF-β ligands (-β1, -β2, -β3) bind to the ubiquitously expressed TGF-β type II receptor (TβRII) which heterodimerizes with the type I receptor and transduces intracellular signaling through canonical Smads as well as non-canonical pathways such as MAPK and GTPases. HGF exerts its effects through the tyrosine kinase receptor c-Met which becomes autophosphorylated upon ligand binding and then activates intracellular signaling mediators such as ERK and PI 3-kinase(30). The diverse downstream signaling pathways of both growth factors lead to multiple cellular responses such as cell growth and differentiation. TGF-β and HGF affect some of these cellular events in a similar manner while other responses are divergent. Both growth factors promote epithelial de-differentiation, spreading, and migration after injury: HGF has pro-proliferative and anti-apoptotic effects on epithelium(41), while TGF-β is cytostatic and increases susceptibility to apoptosis(10). Consistent with this, HGF plays a predominantly protective role in renal injury(11, 16, 21, 25), whereas excess TGF-β adversely impacts renal injury(17, 18, 23). Many studies show that TGF-β and HGF negatively regulate each other’s production during kidney injury(8, 24). While several investigations have focused on how TGF-
β signaling affects HGF production, few have examined how TGF-β alters HGF signaling in renal epithelia, the main target of HGF in the injured kidney and a critical compartment for renal regeneration(41).

The Notch signaling pathway, like TGF-β and HGF, is integral to epithelial cell differentiation and is upregulated in renal injury. In canonical mammalian Notch signaling, the Delta-like and Jagged ligands bind to one of four Notch receptors(13). After ligand binding, γ-secretase cleaves the Notch intracellular domain which translocates into the nucleus and activates target genes(13). TGF-β stimulates Notch activity in renal tubules which is important in TGF-β-mediated cell de-differentiation(22, 37). Although there is little information on how Notch impacts HGF signaling in the kidney, crosstalk between these two signaling pathways alters the response to cardiac injury as well as tumor development(1, 12, 38). TGF-β, Notch, and HGF pathways are upregulated in injured proximal tubules, but how these signaling pathways interact to alter epithelial responses is not clear.

Our group previously showed that blocking TGF-β signaling in proximal tubular epithelium protected against mercuric chloride-induced acute kidney injury in mice(10). Given the antagonistic relationship between TGF-β and HGF described above, we hypothesized that inhibiting TGF-β may be protective, in part, by augmenting HGF signaling. Unexpectedly, we found that TGF-β signaling in proximal tubules augmented responsiveness to HGF through transcriptional upregulation of c-Met. This TGF-β-mediated increase in HGF signaling was dependent upon Notch signaling. These findings elucidate novel interactions among TGF-β, Notch, and HGF signaling pathways and suggest that blocking TGF-β signaling also impairs epithelial responses to HGF.

Methods
**Cell culture:** Proximal tubule (PT) cells were generated from the Immortomouse crossed with the Tgfr2^{fl/fl} mice as described previously(10). PT cells were grown at 33°C in DMEM/F12 supplemented with 2.5% fetal bovine serum, hydrocortisone, insulin/transferrin/selenium, triiodothyronine, and penicillin/streptomycin (complete PT media) with interferon-γ. Prior to experiments, PT cells were moved to 37°C and interferon-γ removed to induce differentiation. Cortical fibroblasts (CF) were isolated from renal cortices of Tgfr2^{fl/fl} mice, immortalized by transfection with psv40 as previously described(7) and grown in DMEM/F-12 supplemented with 10% FBS and penicillin/streptomycin. TβRII deletion in PT and CF was achieved by adeno-Cre treatment in vitro as previously verified with immunoblots showing absence of both TβRII and response to TGF-β1(10, 27).

**HGF Stimulation:** Cells were serum starved overnight prior to treatment with HGF (Millipore, 40ng/mL). Unless otherwise noted, cells were subconfluent (<60%) at the time of HGF treatment which lasted 20 minutes for all experiments. For phosphatase inhibition, PT cells were treated with 2 mM sodium orthovanadate (Na₃VO₄) 30 minutes prior to and during HGF stimulation. For the low calcium assay, cells were serum starved in DMEM containing either normal calcium (1mM CaCl₂) or low calcium (5μM CaCl₂) 12 hours prior to HGF treatment. To block the TGF-beta type I receptor, PT cells were plated with either 10μM of a specific ALK5 inhibitor (SB431542, Tocris Biosciences) or equal volume of DMSO for 5 days prior to HGF stimulation. To inhibit Notch cleavage, cells were incubated with 10 μM γ-secretase inhibitor (Selleckchem) or equal volume of DMSO for 2 days prior to HGF stimulation.

**Real time PCR Analysis:** Total RNA was extracted from PT cells according to the Qiagen RNeasy Kit's instructions, reverse transcription performed using the iScript cDNA Synthesis Kit (Biorad). Quantitative PCR was performed with 100ng cDNA, 400nM primers, and SYBER
green supermix (Biorad) using the CFX96 Thermocycler (Biorad). Relative mRNA expressions were determined by $\Delta\Delta^{CT}$ equation and, after validation with a panel of housekeeping genes, GAPDH was used as a reference gene. Primers are as follows: GAPDH: 5’AGGTCAAGTTGAACGGATTTG (forward) and 5’TGTAGACCCTGTAGGTAGTGTTCA (reverse), HGF: 5’CACCACTTGAGGTATTGTGC (forward) and GGGACATCAGTCTCTATTACAG (reverse), c-Met: 5’CCCTGAGAAAACACTTTCC (forward) and 5’TGTAGAAGGGAGATGAGA (reverse), Sp1: 5’TGGCAAGACAGTGAAGGAAG (forward) and 5’AGTGTGCTCCGAGATGTGAG (reverse), Sp3: 5’TTGGTCCTCTGTACAGTTAGG (forward) and 5’GGAAGGACATAACTCAGCCCACG (reverse), Hes-1: 5’CCGCTACACCAACAGAT (forward) and 5’CACATGGAGTCCGAGTAC (reverse), Hey-1: 5’GCAGATGAATGTGGATTACC (forward) and 5’CCAAAACCTCGTAGTATCCCAT (reverse), Jagged1: 5’GGATTCGACTTTCAGACT (forward) and 5’TATTGCAGCAAAGCGCAT (reverse).

**Immunoblots:** Cells were lysed in ice-cold RIPA buffer plus protease and phosphatase inhibitors (Sigma cocktail), homogenized by shearing through a syringe, clarified by centrifugation and quantified using BCA protein Assay (Thermo Scientific). For tissue lysates, the $\gamma$T-Cre;Tgfb1r2flox/flox mice, in which recombination was previously confirmed, were injured with a single injection of HgCl2 (30μmol/kg in saline) and cortical lysates generated as discussed previously (10). For both cell and tissue lysates, proteins were separated by SDS-PAGE gel, transferred onto polyvinylidene difluoride or nitrocellulose membranes, blocked in 5% milk or bovine serum albumin (BSA) and incubated with the following primary antibodies: c-Met, pc-Met, p-ERK, p-Akt, total Akt, cNotch1 (Cell Signaling), and total ERK (Santa Cruz), and E-cadherin (BD Biosciences). GAPDH, FAK (Santa Cruz), and $\alpha$-Tubulin (Cell Signaling) were validated as loading controls (data not shown), chosen based upon the size of the target protein,
and used to control for protein loading. Bands on autoradiography film were quantified using Java-based image processing software (Image J).

3D Culture Assay: PT cells (20,000) were plated in gels as described previously(2, 3) containing collagen I and Matrigel and, once gels solidified, 100 microliters of complete PT media (see above) +/- HGF was added. After 5 days, gels were washed, fixed with 4% paraformaldehyde, and either stained with rhodamine phalloidin (after permeabilization with 0.025% saponin, quenching with 75mM NH₄Cl and 20mM glycine in PBS with CaCl₂ and MgCl₂) for confocal imaging or were photographed with an inverted scope and camera, and 10 random tubules imaged per sample with branches measured by Image J.

Cell migration assay: PT cells (20,000) in serum-free media were plated on transwells (8μM) precoated with Matrigel and incubated for 6 hours. Cells on top of the membrane (i.e. did not migrate) were removed with a cotton swab, and the bottom was fixed in 4% PAFA for 45 minutes. The membrane was stained with 2% crystal violet overnight and pictures were taken at 200x with a Nikon Eclipse TE300 inverted microscope (10 randomly chosen fields per sample), and the number of migrated cells was counted and quantified in a blinded fashion. HGF-treated samples were pretreated for 24hr with 40ng/mL which was continued during migration. Cells treated with γ-secretase inhibitor (10uM) were pretreated for 3 days (controls received equivalent volumes of DMSO).

Cell morphology: PT cells were plated on Matrigel (BD Biosciences)-coated chamberwell slides in serum free media +/- HGF (40ng/mL) for 24 hours and then stained with rhodamine phalloidin. For γ-secretase studies, PT cells were incubated with the inhibitor or equal amounts of DMSO for 2 days before plating on chamberwell slides and stimulation with HGF as described above. Pictures were taken using a fluorescent microscope (OLYMPUS BX51).
MTS cell proliferation assay: PT cells were plated in 12 wells plates, serum starved overnight, then treated with HGF for 24 hours. To ensure equal number of cells, cell numbers were quantified at the time of HGF stimulation using the CellTiter 96 Aqueous One Solution (Promega) and again after 24 hours +/- HGF.

Isolation of membrane proteins: Subconfluent, serum starved (overnight) PT cells were put on ice, washed with PBS (pH 8.0) plus CaCl₂ and MgCl₂ (PBS-CM) and incubated with 1 mM of EZ-Link Sulfo-NSS-SS-Biotin (Thermo Scientific) in DMEM/F12 supplemented with protease and phosphatase inhibitors (Sigma) for 1 hour at 4°C. After washing PT cells, un-bound biotin was quenched by incubating with 0.1% BSA in PBS-CM at 4°C, washed in PBS-CM, lysed in basic lysis buffer (20Mm Tris-HCl pH 8, 150Mm NaCl, 5Mm EDTA, 1% Triton X-100 and proteases and phosphatases inhibitors), scraped and centrifuged 15 min at 13,000 rpm at 4°C. 50-60 µg protein/sample was incubated 16 hours with streptavidin-agarose beads (Thermo Scientific) at 4°C, washed, centrifuged, and the pellet was saved.

Isolation of cytosolic and nuclear proteins: Cytosolic and nuclear fractions were isolated from subconfluent, serum-starved PT cells using a protocol previously published(33).

Statistics: The student’s t-test with unequal variance was used to compare two sets of data with p<0.05 considered statistically significant. Each experiment was repeated three times, and data are shown as means ± standard error.

Results

Blocking TGF-β signaling in PT cells impairs the response to HGF

We used proximal tubule (PT) cells, the target of acute kidney injury, to determine how TGF-β signaling affects epithelial responsiveness to HGF. PT cells, with and without TβRII(10), were exposed to HGF for different time periods. TβRII⁻/⁻ PT cells had reduced activation (i.e.
phosphorylation) of the HGF receptor c-Met, compared to TβRIIflox/flox PT cells (Figure 1A, 1B). TβRII−/− PT cells also had reduced total expression of the c-Met receptor (Figure 1C) which was not significantly different from the difference in c-Met activation (Figure 1B). Although TGF-β signaling has been shown to alter HGF expression in fibroblasts(5), there was no significant difference in HGF transcript levels between TβRIIflox/flox and TβRII−/− PT cells (Figure 1D), and minimal HGF protein expression was detected in PT cells’ conditioned media as measured by ELISA (data not shown). Consistent with decreased c-Met phosphorylation, TβRII−/− PT cells had impaired activation of downstream signaling proteins Akt and ERK in response to HGF (Figure 1E-H). We stimulated renal cortical fibroblasts +/- TβRII(27) with HGF and observed that TβRII−/− fibroblasts had augmented c-Met activation (Figure 1I, 1J), suggesting that TGF-β signaling alters the response to HGF in a cell-specific manner.

**TβRII−/− PT cells have reduced c-Met membrane expression and transcript levels**

We then examined whether these TβRII-dependent changes in c-Met expression and phosphorylation were present in proximal tubules in vivo. Mice lacking TβRII in proximal tubules (γGT-Cre;Tgfbr2fl/fl)(10) had significantly reduced expression and phosphorylation of c-Met compared to floxed controls after mercuric chloride-induced acute kidney injury (Figure 2A-C), an injury model previously shown to increase HGF transcript levels and activity(14). As TβRII-dependent changes in c-Met expression and activation were present in vivo and in vitro, we further investigated how TβRII alters c-Met expression in vitro. Compared to TβRIIflox/flox PT cells, TβRII−/− PT cells had reduced c-Met expression and membrane localization (Figure 2D-F). c-Met expression in TβRII−/− PT cells was decreased to a similar extent in whole cell lysates (47%±6 (SE)) compared to membrane preparations (45%±6) (Figure 2E, 2F), suggesting that
decreased c-Met expression in TβRII−/− PT cells was not due to increased endocytosis of the receptor. TβRII−/− PT cells had decreased c-Met transcript levels as measured by qPCR (Figure 2G), indicating that the reduced c-Met protein expression is due to transcriptional changes.

To determine whether TβRII−/− PT cells’ decreased c-Met expression and activation is due to impaired TGF-β signaling, we used a well-characterized inhibitor of ALK5, the TGF-β type I receptor. ALK5 inhibition significantly reduced HGF activation of c-Met in both cell populations (Figure 2H, 2I), but only reduced c-Met transcript levels in TβRIIflox/flox PT cells (Figure 2J). These results imply that TβRII−/− PT cells’ reduced c-Met expression is due to impaired signaling through the TGF-β receptors.

We also investigated whether there were other mechanisms, apart from altering c-Met transcription, that reduced HGF signaling in TβRII−/− PT cells. We investigated the role of E-cadherin since this adherens junction protein can inhibit tyrosine kinase growth factor signaling(29) and is suppressed by TGF-β. Consistent with this, TβRII−/− PT cells had augmented E-cadherin expression and membrane localization (Figure 3A-C). Furthermore, the difference in HGF signaling was present only in subconfluent conditions during which time E-cadherin is suppressed (Figure 3D, 3E). We disrupted E-cadherin using low calcium (5μM) media and confirmed that this augmented c-Met activation in confluent TβRIIflox/flox PT cells (Figure 3F, 3G). However, low calcium media did not alter differences in HGF signaling between TβRII−/− and TβRIIflox/flox PT cells in subconfluent conditions (Figure 3H, 3I). Thus, augmented E-cadherin expression does not play a role in TβRII−/− PT cells’ reduced HGF signaling.

We also investigated whether altered phosphatase activity might account for differences in HGF signaling as TGF-β alters other tyrosine kinase growth factor signaling pathways
through phosphatases(35). As expected, pre-treatment of PT cells with sodium orthovanadate, a tyrosine phosphatase inhibitor, augmented c-Met phosphorylation in response to HGF in TβRII−/− and TβRIIflox/flox PT cells (Figure 3J, 3K). However, TβRII−/− PT cells’ decreased responsiveness to HGF persisted even with the phosphatase inhibitor (Figure 3J, 3K). Taken together, our data show that inhibiting TGF-β signaling either genetically or pharmacologically reduces HGF signaling through transcriptional changes in c-Met and not by altering E-cadherin expression or phosphatase activity.

Notch signaling mediates TβRII−/− PT cells’ reduced HGF signaling

Our data indicated that TβRII−/− PT cells’ decreased c-Met expression was transcriptionally mediated, so we investigated putative transcription factors that link TGF-β signaling and c-Met expression. The transcription factors Sp1 and Sp3 have been shown to regulate c-Met expression(39), but we found no significant differences in their transcript levels between TβRIIflox/flox and TβRII−/− PT cells by qPCR (Figure 4A, 4B). We then investigated whether the Notch signaling pathway may be involved since this pathway is a downstream target of TGF-β signaling(22) and interacts with the HGF/c-Met pathway in other organs(1, 12). TGF-β has been shown to induce expression of the ligand Jagged1(22, 28), and, consistent with this, TβRII−/− PT cells had suppressed Jagged1 mRNA expression (Figure 4C). Notch transcriptional targets, Hes1 and Hey1, were also diminished in PT cells lacking TβRII (Figure 4D, 4E). Furthermore, cleaved Notch1, an indicator of Notch signaling, was significantly decreased in nuclear isolates of TβRII−/− PT cells (Figure 4F, 4G). Thus, our data indicate that Notch signaling is reduced in TβRII−/− compared with TβRIIflox/flox PT cells.
To determine whether the suppressed Notch signaling in TβRII−/− PT cells accounts for its reduced responsiveness to HGF, we inhibited Notch activity using a γ-secretase inhibitor. As expected, the γ-secretase inhibitor blocked Notch1 cleavage and Hes1 transcription but also reduced c-Met protein expression and transcription in TβRIIflox/flox PT cells (Figure 5A-E). Furthermore, addition of the γ-secretase inhibitor reduced the HGF-induced c-Met activation to levels similar to those of TβRII−/− PT cells (Figure 5F, 5G), implying that the difference in c-Met activation between TβRIIflox/flox and TβRII−/− PT cells is Notch-dependent.

**TβRII−/− PT cells have reduced biological responses to HGF**

We defined the biological significance of reduced HGF signaling in TβRII−/− PT cells by investigating responses to cellular events known to be modulated by HGF signaling. Initially, we explored how TGF-β and HGF signaling interact to regulate branching morphogenesis. TβRII−/− PT cells grown in 3D gels (collagen I/Matrigel) had significantly more branching compared with TβRIIflox/flox PT cells (Figure 6A-C). However, HGF stimulation significantly increased branching in TβRIIflox/flox PT cells (5.8 branches per tubule increased to 10.3) contrasted with a minimal change in TβRII−/− PT cells (10.1 branches to 11.8) (Figure 6A-C). HGF is known to induce morphologic changes whereby the cell de-differentiates and has a more fibroblast-like shape(19, 32). We observed that TβRII−/− PT cells spread less at baseline compared to TβRIIflox/flox PT cells (Figure 6D). After HGF treatment, TβRIIflox/flox PT cells had a more fibroblast-like appearance, consistent with HGF-induced de-differentiation, whereas there was little change in the TβRII−/− PT cells (Figure 6D). We next defined how HGF altered epithelial migration of PT cells with and without TβRII on Matrigel-coated transwells (Figure 6E, 6F). HGF treatment dramatically increased TβRIIflox/flox PT cell migration, but this response was not
present in HGF-treated TβRII−/− PT cells (Figure 6E, 6F). Similarly, HGF promoted more
proliferation, and there was a greater increase in the number of TβRII<sup>flox/flox</sup> PT cells after HGF
treatment (>60%), compared to a 20% increase in HGF-treated TβRII−/− PT cells (Figure 6G).
Thus, consistent with the decreased signaling in response to HGF, TβRII−/− PT cells had reduced
functional responses as well.

γ Secretase Inhibitor Abrogated HGF Signaling in TβRII<sup>flox/flox</sup> PT Cells

We then investigated whether inhibiting Notch signaling using the γ secretase inhibitor could
block the biological responses to HGF in TβRII<sup>flox/flox</sup> PT cells. The HGF-induced increase in
cell de-differentiation and lamellopodia formation in TβRII<sup>flox/flox</sup> PT cells was reduced by
treatment with the γ secretase inhibitor whereas there was little change in the actin cytoskeleton
of TβRII−/− PT cells (Figure 7A). Similarly, pre-treatment with a γ secretase inhibitor dramatically
reduced TβRII<sup>flox/flox</sup> but not TβRII−/− PT cell migration (Figure 7B, 7C). These data suggest that
TβRII−/− PT cells’ reduced responsiveness to HGF is mediated through suppressed Notch
signaling.

Discussion

TGF-β directly alters many epithelial responses to injury but may also modulate cellular events
through affecting other key growth factor pathways. We showed that deleting TβRII in proximal
tubules significantly suppressed HGF signaling and biological responses due to transcriptional
reduction of the c-Met receptor. Our data suggest that Notch signaling plays an important role
regulating the TGF-β-dependent increase in HGF signaling. Thus, our data indicate that the
interaction between TGF-β and downstream Notch signaling pathways in proximal tubule cells is
necessary for a full biological response to HGF.
Our finding that TGF-β signaling promotes epithelial HGF signaling was somewhat surprising as others have shown that these growth factors mediate divergent responses to renal injury(20, 24). These differences may be explained by our data showing that the TGF-β-dependent changes in HGF signaling were cell-type specific. TGF-β signaling inhibited HGF signaling in cortical fibroblasts which is consistent with other reports showing that blocking TβRII in mesenchymal cells augments HGF signaling(5). However, our data that TGF-β does not alter HGF production in epithelial cells contrasts with TGF-β’s suppressive effect on HGF synthesis in fibroblasts reported by others(26), further showing the cell-specific interactions of TGF-β and HGF signaling. Although mesenchymal cells are important sources of HGF, injured renal epithelia are considered the most important sites of HGF signaling(41). Perhaps our finding that epithelial TGF-β promotes HGF-dependent signaling is not too surprising given that both HGF and TGF-β promote cell de-differentiation, spreading, and migration. Thus, it is possible that these two growth factors cooperatively interact to regulate cellular responses critical to the injured epithelia.

Deleting TβRII in proximal tubules reduced c-Met expression both in cells in vitro and in mercuric chloride-injured kidneys. It is unclear if the reduced c-Met expression was due to abrogated TGF-β signaling or because they sustained less injury as we previously reported(10). Since epithelial injury upregulates c-Met, reduced injury could also cause reduced c-Met expression; however, our in vitro data suggest that impaired TGF-β signaling contributes to decreased c-Met expression. Our finding that TGF-β augmented HGF signaling through a transcriptional increase in the c-Met receptor is consistent with a study that showed exogenous TGF-β upregulates c-Met transcription in human PT cells(40). However, this earlier study showed that the transcription factor Sp1 was critical to c-Met transcription induced by exogenous
TGF-β1(40), while we showed no difference in levels of Sp1 expression between TβRIIfl/fl and TβRII−/− PT cells. Differences in the cells used (conditionally immortalized murine PT cells versus immortalized human PT cells) may account for discrepant mechanisms, but the relationship between renal epithelial TGF-β and HGF appears conserved between species.

To confirm that the reduced HGF signaling in PT cells lacking TβRII was due to suppressed TGF-β signaling, we pharmacologically inhibited the type I receptor using the well-established ALK5 inhibitor. This inhibitor reduced both responsiveness to HGF and c-Met expression in the TβRIIfl/fl PT cells, but also reduced responsiveness to HGF in TβRII−/− PT cells without significantly changing c-Met expression. TGF-β-independent ligands (e.g. activins) may activate ALK5 in a TβRII-independent manner. Our data suggest that these TGF-β ligand-independent signaling pathways also alter HGF signaling but not through c-Met transcription.

Our studies also illustrate how deleting TβRII from proximal tubules fundamentally changes the cells: reduced cell spreading, altered actin cytoskeleton, and augmented E-cadherin expression. Some of these changes are consistent with our previous work showing that deleting TβRII in collecting duct epithelia altered stress fiber formation and augmented Rho GTPase activity(9). Though many studies have focused on how excessive TGF-β signaling dramatically alters epithelial cytoskeleton as part of an epithelial to mesenchymal transition (EMT), our results suggest that even basal TGF-β signaling is integral to epithelial cell structure and organization.

Our data convincingly show that TGF-β signaling in PT cells increases Notch activity which is consistent with previous reports showing that exogenous TGF-β1 augments epithelial Notch signaling (22, 34, 36, 37). The mechanism whereby TGF-β signaling upregulates Notch
activity was beyond the scope of this paper, but our results showing that TβRII−/− PT cells have decreased Jagged1 transcription are consistent with other studies indicating that TGF-β increases Notch signaling through transcriptional upregulation of Jagged1(22, 28, 37). Others have shown that Smad3 is critical to TGF-β-induced Jagged1/Notch signaling in renal epithelia(37). Taken together with earlier studies showing that TGF-β induced c-Met expression through Smads(40), it is possible that Smad-dependent induction of Jagged1 augments Notch activity, which is key to TGF-β-dependent c-Met expression and augmented HGF signaling (Figure 8). While little is known about Notch interactions with c-Met in the kidney, there is evidence of crosstalk between c-Met and Notch signaling pathways in cancer biology(38). However, there are conflicting results regarding how Notch affects c-Met transcription as suppression of Notch1 reduced c-Met expression in one study but constitutively active Notch repressed c-Met transcription in another (1, 31). To block the effects of Notch, we used a γ secretase inhibitor which prevented Notch cleavage in our studies and has been widely used by others to inhibit Notch signaling(4, 6, 34). However, γ secretase inhibitors are not specific for Notch signaling, and it is possible that another γ secretase target mediates TGF-β-dependent c-Met transcription.

The interactions between TGF-β and HGF in our studies were shown to be biologically relevant as TβRII−/− PT cells had impaired HGF-mediated cellular responses. Furthermore, we showed that TGF-β enhances the response to HGF through Notch signaling as both HGF-dependent morphologic changes and migration in TβRIIfl/fl PT cells were blocked with the γ secretase inhibitor. The signaling interactions among TGF-β, HGF, and Notch pathways in epithelial cells have not been well studied previously, but each of these pathways has been shown to be upregulated in tubular injury and to promote epithelial de-differentiation. In the
injured renal epithelium, coordinated signaling among growth factor pathways that control
differentiation may be important to allow regeneration after renal injury.

In conclusion, this study demonstrates biologically significant interactions between TGF-
β and HGF signaling pathways in proximal tubule epithelial cells. In addition, we show that
Notch signaling plays an important role regulating these interactions. These findings suggest that
intact epithelial TGF-β signaling is necessary for a full biological response to HGF.
Furthermore, TGF-β signaling alters epithelial cell behavior not only by direct effects, but also
by changing how these cells respond to other growth factors.

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**Figure Legends**

**Figure 1.** TβRII−/− PT cells have impaired HGF signaling. TβRII^{flox/flox} and TβRII^{−/−} PT cells were treated with 40ng/ml HGF for various time points, and c-Met expression and phosphorylation were measured with immunoblots (A) and quantified using α-tubulin as loading control (B, C). qPCR analysis of HGF transcription by TβRII^{flox/flox} and TβRII^{−/−} PT cells normalized to GAPDH (D). Immunoblots and quantification of Akt (E, F) and ERK (G, H) phosphorylation were performed on TβRII^{flox/flox} and TβRII^{−/−} PT cells stimulated by HGF (40ng/mL) for 20 minutes. Lysates of TβRII^{flox/flox} and TβRII^{−/−} cortical fibroblasts treated with 40ng/ml HGF for various time points were immunoblotted for c-Met phosphorylation (I) which was quantified using α-tubulin as a control (J). Quantifications are expressed as means of three separate experiments ± standard error. (n=3) * p<0.05; **p<0.01; ***p<0.0001.

**Figure 2.** Proximal tubules lacking TβRII in vitro have reduced c-Met membrane localization and transcript levels. Cortical tissue lysates of γGT-Cre;Tgfbr2^{fl/fl} (conditional deletion of TβRII in proximal tubule) and Tgfbr2^{fl/fl} (floxed controls) mice after mercuric chloride-induced acute
kidney injury were immunoblotted (A) for c-Met expression and phosphorylation with α-tubulin as a loading control, and protein expression was quantified (B, C). Lysates of whole cell and membrane preparations (see Methods) were immunoblotted for c-Met expression with α-tubulin used to show the purity of membrane preparations and β1 integrin used as a loading control for membrane expression (D). Quantification of c-Met expression in whole cell lysates (E) and membrane preparations (F) is shown with TβRII^flox/flox PT cells’ expression in each experiment (n=3) adjusted to 1. qPCR analysis on TβRII^flox/flox and TβRII^-/- PT cells shows c-Met mRNA normalized to GAPDH expression (n=3) (G). PT cells pre-treated for 4 days with an ALK5 inhibitor (SB431542) or vehicle control (DMSO) were either stimulated with HGF and immunoblotted for c-Met phosphorylation (H) with expression quantified using α-tubulin as loading control, n=3 (I) or RNA isolated, converted to cDNA and c-Met transcription quantified with qPCR (J). Results are expressed as means ± standard error, * p<0.05.

**Figure 3.** E-cadherin expression and phosphatase activity do not account for reduced HGF responsiveness in TβRII^-/- PT cells. Total cell lysates and membrane preparations of TβRII^flox/flox and TβRII^-/- PT cells were immunoblotted for E-cadherin expression with α-tubulin, and integrin β1 as loading controls (A), and E-cadherin expression in whole cell (B) and membrane preparations (C) was quantified. TβRII^flox/flox and TβRII^-/- PT cells were stimulated with HGF in confluent (>90%) or subconfluent (<60%) conditions, and c-Met phosphorylation was measured by immunoblots (D) and the differences in confluent versus subconfluent conditions were quantified (E). Confluent TβRII^flox/flox PT cells were incubated in low (5uM) or normal (1μM) calcium-containing media (see Methods), stimulated with HGF, and immunoblotted for c-Met phosphorylation expression (F) and quantified using densitometry (G). Subconfluent TβRII^flox/flox
and TβRII−/− PT cells incubated in low or normal calcium-containing media were treated with HGF and immunoblotted for c-Met phosphorylation (H) and expression was quantified (I). PT cells were pre-treated with a tyrosine phosphatase inhibitor (Na₃VO₄) 20 minutes prior to HGF stimulation, and c-Met phosphorylation was measured by immunoblot (J) and quantified with FAK as a loading control (K). Results are expressed as means ± standard error. (n=3) * p<0.05, and white lines indicate where lanes within the same blot have been moved.

**Figure 4.** Notch signaling is impaired in TβRII−/− PT cells. qPCR was used to quantify transcription of Sp1, Sp3, Jagged1, Hes-1, and Hey1 (A-E). TβRIIflox/flox and TβRII−/− PT cells were fractionated into cytosolic and nuclear components (see Methods), and cleaved Notch1 (cNotch1) was measured with GAPDH and PARP1 as loading controls for cytosolic and nuclear compartments, respectively (F). Nuclear expression of cleaved Notch was quantified, n=3, and compared between TβRIIflox/flox and TβRII−/− PT cells (G).

**Figure 5.** Blocking Notch with γ secretase reduces c-Met activation in TβRIIflox/flox PT cells. PT cells +/- γ secretase inhibitor (γSI) immunoblotted for cleaved Notch1 (cNotch1) and c-Met expression with α-tubulin as a loading control (A). The effects of γSI on c-Met protein expression (B) and Notch1 cleavage (C) were quantified. qPCR quantified Hes-1 and c-Met transcripts in PT cells pre-treated with either γSI or vehicle control (DMSO), n=3 (D,E). PT cells pre-treated with either γSI or DMSO were then stimulated with HGF and immunoblotted for c-Met phosphorylation (F) and quantified using α-tubulin as a loading control, n=3 (G). All experiments were repeated 3 times, and results are expressed as means ± standard error. * p<0.05.
Figure 6. TβRII\(^{-/-}\) PT cells have diminished biological responses to HGF. TβRII\(^{\text{flox/flox}}\) and TβRII\(^{-/-}\) PT cells were grown in 3D gels containing Matrigel and collagen IV for branching morphogenesis studies (see Methods). Representative confocal images of PT cells +/- HGF (40 ng/ml) are shown (A) and the number of branches (B) and tubule length (μm):branch number (C) quantified using Image J on 10 tubules per experiment (n=3). HGF-induced effects on cell morphology were assessed by rhodamine phalloidin staining of TβRII\(^{\text{flox/flox}}\) and TβRII\(^{-/-}\) PT cells plated on Matrigel-coated chamber slides +/- HGF, 400x, scale bar = 50 μM (D). Migration was assessed by plating TβRII\(^{\text{flox/flox}}\) and TβRII\(^{-/-}\) PT cells on Matrigel-coated transwells +/- 40 ng/ml HGF and stained with crystal violet with a representative picture at 200x (E) and scale bars of 50 μM. The number of migrated cells was counted in a blinded manner using 10 pictures at 200x per sample, n=3, with means ± standard error (F). HGF-induced increase in proliferation was measured by the MTS assay (see Methods) and reported as the % increase of HGF-treated cells compared to untreated cells after 24 hrs (G). For statistical significance * = p<0.05, ** = p<0.01, and *** = p<0.001.

Figure 7. γ-secretase inhibitor reduces TβRII\(^{\text{flox/flox}}\) but not TβRII\(^{-/-}\) PT cells’ response to HGF. Representative images of PT cells, pre-treated with γSI or DMSO and all stimulated with HGF (see Methods), plated on Matrigel-coated chamber slides and stained with rhodamine phalloidin were taken at 400x with scale bar in white = 50 μM for all panels (A). Migration on Matrigel-coated transwells was repeated with all cells stimulated by HGF plus either γSI or DMSO. Representative images taken at 200x are shown with black scale bars = 50 μM (B) and migrated cells were counted in a blinded manner using 10 fields per sample, n=3 (C). Results are expressed as means ± standard error. * p<0.05.
Figure 8. A schematic of how TGF-β signaling increases epithelial responsiveness to HGF through the Notch signaling pathway.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 7
Figure 8