Age dependent shifts in renal response to injury relate to altered BMP6/CTGF expression and signaling

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Abstract

Age is associated with an increased prevalence of chronic kidney disease (CKD), which, through progressive tissue damage and fibrosis, ultimately leads to loss of kidney function. Although much effort is put into studying CKD development experimentally, age has rarely been taken into account. Therefore, we investigated the effect of age on the development of renal tissue damage and fibrosis in a mouse model of obstructive nephropathy (i.e. unilateral ureter obstruction; UUO). We observed that after 14 days, obstructed kidneys of old mice had more tubulointerstitial atrophic damage but less fibrosis than those of young mice. This was associated with reduced connective tissue growth factor (CTGF), and higher BMP6 expression and pSMAD1/5/8 signaling, while TGF-β expression and transcriptional activity were no different in obstructed kidneys of old and young mice. In vitro, CTGF bound to and inhibited BMP6 activity.

In summary, our data suggest that in obstructive nephropathy atrophy increases and fibrosis decreases with age, and that this relates to increased BMP signaling, most likely due to higher BMP6 and lower CTGF expression.
Abbreviations:

BMP (6/7) - Bone Morphogenetic Protein (family member 6 or 7)
CTGF - Connective Tissue Growth Factor
TGFβ - Transforming Growth Factor β
UUO - Unilateral Ureter Obstruction
CLK - Contralateral Kidney
OBK - Obstructed Kidney
CKD - Chronic Kidney Disease
KW - Kidney Weight
BW - Body Weight
Chronic kidney disease (CKD) primarily affects the aging population (11, 37, 42). Decline of kidney function with age might result from multifactorial changes in kidney physiology primarily due to “senescence” itself, and a more adverse response to injury (1, 5, 22, 43, 45, 47). As such, it has been proposed that the aging kidney increasingly accumulates extracellular matrix, leading to glomerulosclerosis and interstitial fibrosis (1, 25) and ultimately to loss of renal mass and reduced glomerular and tubular function (17, 51). Parameters such as diabetes, vitamin D deficiency, alterations in Renin Angiotensin and Aldosteron (RAAS) signaling, oxidative stress by reactive oxygen species (ROS) production and advanced glycation end (AGE) products are thought to underlie these morphological and functional changes of the kidney parenchyma (8).

Furthermore, the age associated gradual decline in Klotho, a transmembrane FGF23 co-receptor expressed in the convoluted distal tubule under physiological condition renders kidneys more susceptible to injury, dysregulation of mineral homeostasis and fibrosis (28).

In young mice, kidney response to injury is profoundly influenced by the Transforming Growth Factor β-superfamily (TGF β), including TGFβ1, Bone Morphogenetic Protein 7 (BMP7), and BMP6 (14, 35). Canonical signaling of the TGF β superfamily is accomplished via Activin like kinase (ALK) activation and subsequent downstream SMAD phosphorylation. Activation of ALK4, 5 or 7 by TGFβ leads to fibrosis associated SMAD2/3 phosphorylation. ALK1-3 or 6 activation by BMP’s leads to renoprotection associated SMAD1/5/8 phosphorylation (10). Under specific conditions, TGFβ has been shown to activate ALK1 (36). Upon injury, TGFβ levels rise and BMP levels drop rapidly (34). The shift in these factors is regarded as major early event in ensuing tissue damage with subsequent fibrosis following injury.
Connective Tissue Growth Factor is another immediate early factor shown to greatly influence the response to kidney injury. CTGF is a matricellular protein involved in various fibrosis associated phenomena such as extracellular matrix production, proliferation and myofibroblast differentiation (19). Although no exclusive CTGF receptor has been identified, CTGF has been known to interact with TGFβ and BMP7 thereby modulating signaling in favor of pro-fibrotic TGFβ whilst inhibiting BMP7 signaling (2, 35). As such, TGFβ, BMP6 and 7, and CTGF are regarded as major factors influencing the renal response to injury. The age associated production of reaction oxygen species (ROS) is related to increased levels of pro-fibrotic growth factors (41), but little is known about the impact of aging on the regulation of these factors in the kidney. It has been reported that pro-fibrotic TGFβ signaling generally increases with age, which might at least in part be due to the gradual decline of Klotho (15).

The effect of ageing on renal CTGF expression is largely unknown, but CTGF/CCN2 was found to be reduced in aged skin in association with loss of collagen (39). Interestingly, conditional overexpression of CTGF prevented age-related degenerative changes in epiphyseal cartilage of rats (27). In contrast, ageing is associated with increased cardiac CTGF expression in mice (40, 48), suggesting that age related differential expression of CTGF is context dependent.

Reports on age-associated changes in expression and signaling of the (anti-fibrotic) BMPs are scarce, but it has been noted that BMP7 expression declines in aging cartilage (3), while increased expression of BMP6 was found in Alzheimer brains (12), and early aging in Klotho-deficient mice was associated with increased BMP-signaling and vascular calcification (23). To the best of our knowledge, there are no previous data on age-related changes in renal BMP expression and signaling. Based on our find in the
current study, we hypothesize that old and young kidneys respond differently to injury in association with differential TGFβ/BMP/CTGF signaling.

Unilateral Ureter Obstruction (UUO) is a commonly used model of renal injury characterized by inflammation, extensive morphological damage and fibrosis (7, 29). Upon obstruction, the quick rise in TGFβ levels and subsequent phosphorylation of SMAD2/3 and PAI1 upregulation are regarded as key events ultimately leading to fibrosis (26, 44). We studied morphological damage and fibrosis following 14 days of UUO and observed a shift from a largely fibrotic phenotype in young, to a more atrophic phenotype of tubulointerstitial damage in old kidneys, although BMP7 and TGFβ were not different. This phenotypic shift might derive from a synergistic effect of the observed decrease of CTGF and increase of BMP6, even more so since we noted in vitro that CTGF directly binds to BMP6 and inhibits its signaling activity. Together these findings provide further understanding of the age associated response to injury, and might help to identify better diagnostic methods and therapeutic interventions in the aging population.
**Materials and Methods**

*Animals*

Two groups of C57Bl6/J mice (16 week old (n=6; Young) and 50 week old (n=6; Old)) were housed under standard conditions and subjected to UUO. Under general isoflurane anesthesia the left ureter was ligated with silk suture through the left flank, after which the wound was closed and stitched. One young and one old mouse deteriorated in condition rapidly after surgery and both were sacrificed within a week. These mice were excluded from further analysis. After 13 days the remaining mice were housed in metabolic cages for 16 hours for urine collection. At day 14 they were killed and organs and plasma were collected for analysis. Animal experiments were carried out with approval of the Experimental Animal Ethics Committee of the University of Utrecht conform Dutch law.

*Immunohistochemistry*

Fresh kidney tissue was fixed in buffered 4% paraformaldehyde solution and embedded in paraffin. 3µm sections were cut, embedded on object slides and incubated in a stove at 60°C for 16 hours. Sections were deparaffinised and rehydrated in xylene, 100% & 70% ethanol respectively, after which the sections were rinsed in de-mineralized water. Periodic acid Schiff and Masson-trichrome staining was performed using standard procedures. For quantification of morphological damage, 10 arbitrary cortical fields per kidney were scored in PAS-stained sections with regards to atrophy and dilatation (0=<1%, 1=1-25%, 2=25-50%, 3=50-75%, 4=75-100%). For immunohistochemistry, antigen retrieval consisted of 20 minute boiling in either citrate buffer (pH=6), EDTA buffer (pH=9) or 10 minute pepsin digestion.
depending upon primary antibody. Slides were incubated with the following antibodies:

- αSMA (EDTA, 1:200, Abcam, Cambridge, UK), CTGF (Citrate, 1:200, Santa-Cruz Biotechnology, Santa Cruz, CA), pSMAD2/3 (Pepsin, 1:400, Santa-Cruz Biotech.), or
- pSMAD1/5/8 (Citrate, 1:50, Cell signaling tech., Danvers, MA). To determine positive area percentages of Masson-trichrome and CTGF stained slides, 10 random fields per section were chosen and photographed. Using Photoshop (version 12.0) positive staining areas were selected and quantitated using ImageJ software (NIH, Baltimore, MD).


**Hydroxyproline:** Paraffin sections were analyzed for hydroxyproline levels as a measurement of collagen content using HPLC (18); proline ratio was taken as a measure of relative abundance of collagen. **Plasma urea:** Plasma urea levels were measured by colorimetric assay using standard procedures (DiaSys, Holzheim, Germany). **Urinary protein:** Urinary protein was measured by BCA assay (BioRad, Hercules, CA). **Senescence Associated-β-Galactosidase:** SA-β-gal activity was detected as described (13).

**Western blot**

Snap frozen renal cortex was homogenized and lysed using NP-40 lysis buffer containing Na-Orthovanadate, Na-Fluoride and complete protease inhibitor cocktail. Lysates were spun down and pellets were discarded. Total protein concentration in the supernatant was measured using BCA (Pierce Thermo, Rockford, IL). 20ug of protein was boiled with Laemli/DTT and run for 90 minutes on 10% SDS-PAGE gels (BioRad). Gels were subsequently blotted for 90 minutes on PVDF membrane using a wet blotting transfer system (BioRad). For p-Smad1/5/8 analysis membranes were incubated with primary
antibody (pSMAD1/5/8, 1:2000, CST; SMAD1/5/8, 1:1000, Santa-Cruz) in TBS-Tween containing 3% BSA overnight. After thorough rinsing membranes were incubated with secondary HRP conjugated antibody and imaged using chemiluminescence substrate (GE healthcare lifescience, Buckinghamshire, UK).

**RT-qPCR**

RNA was isolated from both tissue homogenate and cell cultures using TRIzol (Life technologies, Carlsbad, CA). RNA was reversely transcribed to cDNA using standard procedures. Expression of target genes was determined using commercially available pre-designed TaqMan probes (Bmp6, Mm00432095_m1; Bmp7, Mm00432102_m1; Col1α2, Mm00483888_m1; Ctgf, Mm00515790_g1; Hsp47, Mm00438058_g1; Klotho, Mm00502002_m1; Pai1, Mm00435860_m1; Tgfβ1, Mm01178820_m1; Id1: Mm00775963_g1; Tbp: Mm01277042_m1; Thermo Fisher, Waltham, MA). Samples were run on a Lightcycler 480 (Roche, Basel, Switzerland) and relative expression was determined by the ΔΔCT method. Application of GeNorm identified TATA box binding protein (tbp) as the most stable reference gene (out of gapdh, yhwaz, actb and tbp).

**Solid-phase BMP6/CTGF binding assay**

Microtiter plates were coated with fixed concentration of 200 ng/ml full length rhCTGF (BioVendor, Modrice, Czech republic) at 4°C overnight. Plates were rinsed and blocked with 1% BSA for 2h. After rinsing, a range of 0-1000 ng/ml rhBMP6 (R&D Systems) was added. Bound BMP6 was detected by using a BMP6 antibody (Santa Cruz).

**Cell culture**
HK-2 cells were maintained in DMEM (Gibco/Thermo, Waltham, MA) with 10% FCS, penicillin and streptomycin in humidified air with 5% CO$_2$ at 37 °C. HK-2 cells were plated at a density of $1 \times 10^5$ cells in 6-well plates. Cells were serum starved for 24 hours and subsequently incubated with serum-free medium alone, 50 ng/ml rhBMP-6 (R&D Systems, Minneapolis, MN) with or without 400 ng/ml rhCTGF. Cells were harvested after 1 h for Western blot analysis and after 2 h for quantitative PCR.

Statistics

Data was analyzed using GraphPad Prism version 6.02 (Graphpad software inc., La Jolla, CA). All data was statistically tested with Student-T test for two groups, or two-way ANOVA followed by post-hoc Tukey correction for multiple testing when more groups were compared, unless stated otherwise. p< 0.05 was considered statistically significant. Error bars represent SEM.
RESULTS

General characteristics

To investigate potential age related differential responses to injury, we performed UUO in both groups for 14 days. Post UUO, kidney weight loss, diuresis, plasma urea and proteinuria were similar at both ages (data not shown). However, old mice lost more body weight compared to young mice (Figure 1A). Old contralateral kidneys (50 weeks) showed sporadic senescence associated-β-Galactosidase activity whereas this was not detected in any of sections from kidneys of young mice (16 weeks) (data not shown). Glomerulosclerosis, a phenomenon associated with ageing, was not seen in old CLKs (Figure 1B).

Ureteral ligation induces a more severe morphological phenotype in aged kidneys

To assess the extent of injury, dilatation and atrophy (two hallmarks of UUO induced renal damage) were scored. Morphological interstitial damage after obstruction was more severe in old Obstructed Kidneys (OBK), as exemplified by higher kidney tubular morphological damage scores for dilatation and atrophy in old OBKs compared to young OBKs (Figure 1B and 1C).

Development of fibrosis is reduced in aged kidneys

Since fibrosis is a second major phenomenon occurring during UUO mediated renal injury, we studied the level of ECM deposition and associated myofibroblast accumulation in obstructed kidney of both age groups. Quantification of Masson Trichrome (MTC) staining, a staining for fibrillary collagen, showed a reduced area positivity (%) in old OBKs compared to young OBKs (Figure 2A). An increase in MTC positive surface area in young OBKs was observed, whereas no significant increase was
seen in old OBKs compared to CLKs (Figure 2B). Furthermore, myofibroblast numbers as assessed by α Smooth Muscle Actin (αSMA) were also lower in old OBKs compared to young OBKs (Figure 2A lower panels B&C). No difference was observed in CLKs (data not shown). Old OBKs show a reduced Col1α2 up regulation compared to young OBKs (Figure 2D). Correspondingly, a significant increase in the message for collagen chaperone Hsp47 was seen in young kidneys upon obstruction whereas this was not observed in old obstructed kidneys (Figure 2E). Hydroxyproline levels were higher in old CLKs compared to young CLKs, but were similar in OBKs of both age groups (Figure 2F). Fold increase (OBK/CLK) of hydroxyproline was reduced in old compared to young kidneys (Figure 2 F; right panel). Taken together this suggests a decreased de novo synthesis of collagen in old compared to young kidneys upon ureteral obstruction.

Age is associated with an altered pro-fibrotic/regenerative balance

To gain insight in potential underlying differences in pro-fibrotic signaling resulting in the altered phenotype, we studied several important known mediators of ageing or fibrosis. For assessment of pro-regenerative gene expression, we investigated Klotho, Bmp6 and Bmp7 mRNA expression levels (Figure 3 A-C). The renoprotective factor Klotho is strongly associated with aging, and interacts with the TGFβ pathway during fibrogenesis in the kidney (4, 31, 50). Despite the 38 week age difference, Klotho expression was not differentially regulated in unobstructed CLKs of 50 week and 12 week old mice (Figure 3A). Also, Klotho was similarly down regulated in OBKs of both age groups, and no significant change in Klotho expression was observed between old and young OBKs (Figure 3A). In young mice, obstructed kidneys had significantly reduced gene expression levels of Bmp6 and Bmp7 (Figure 3B&C). In old and young
OBKs, Bmp7 expression was similarly suppressed (Figure 3B). However, unlike young OBKs, old OBKs showed no reduction of Bmp6 expression (Figure 3C).

The increase of TGFβ1 as a key pro-fibrotic regulator, and PAI-1 as important downstream mediator of TGFβ1 in kidney fibrosis (24), were not different in old and young OBKs (P=0.96, P=0.36 respectively; Figure 3D&E). To further evaluate downstream signaling of TGFβ1 we performed pSMAD2/3 IHC on kidney cortex. However, the number of cortical cells showing TGFβ associated nuclear SMAD2/3 phosphorylation was not significantly different between old and young OBKs (P=0.95; Data not shown). CTGF greatly influences the fibrotic/regenerative balance by positively modulating TGFβ and negatively modulating BMP signaling, and is commonly regarded as pro-fibrotic (2, 35). In CLKs, Ctgf mRNA expression levels were identical in old and young mice (Figure 3F). In OBKs mean Ctgf gene expression tended to be higher in young OBK as compared to old OBK, and compared to young CLK (P=0.07); the mean increase vs. age-matched CLK was 7 fold in young OBKs, compared to 3.5 fold in old OBKs (Figure 3F). This increase was significant in young OBKs but not in old OBKs (p=0.013 vs 0.54 respectively). Furthermore, CTGF IHC showed a decrease in CTGF positive area both in CLK and OBK old kidneys compared to CLK and OBK young kidneys respectively (Figure 3G&H: P<0.05), while the increase in OBK compared to CLK was significant only in young mice. Signal loss occurred mainly in cortical tubules. Bmp6/Ctgf ratio’s in individual OBKs was significantly higher in old than in young OBKs (12.1 SEM 2.6 vs 4.1 SEM 0.4; p<0.05).

**Canonical BMP signaling is better preserved in cortical tubular epithelium of old mice**

Given the increased BMP6/CTGF ratio, we next studied whether this resulted in an increase in canonical BMP signaling. In old OBKs, pSMAD1/5/8 signal was better...
preserved compared to young OBKs (Figure 4A & 4B). Immunostaining for pSMAD1/5/8 shows that glomerular and interstitial pSMAD1/5/8 is similar in young and old OBKs (Figure 4C & 4D). However, preservation of pSMAD1/5/8 signal occurred in the tubuli.

CTGF binds BMP6 and inhibits canonical downstream signaling

CTGF inhibits canonical BMP7 signaling in proximal tubular (HK-2) cells via direct interaction (35). Whether CTGF holds similar potential with regards to BMP6 is unknown. By solid phase assay we observed concentration dependent binding of rhBMP6 in rhCTGF coated microtiter plates (Figure 5A). Stimulating HK-2 cells with rhBMP6 increased downstream transcriptional Id1 expression significantly (p<0.05) (Figure 5B). When co-stimulating HK-2 cells with rhBMP6 and rhCTGF however, this increase was significantly less profound (Figure 5B). Consistently, Western blot analysis showed increased SMAD1/5/8 phosphorylation upon rhBMP6 stimulation, which was less profound when rhBMP6 was pre-incubated with rhCTGF prior to stimulation (Figure 5C). Thus, in vivo the increased BMP6 expression and associated SMAD1/5/8 phosphorylation might be further complemented by reduced physical inhibition by CTGF.
Discussion

In this study, we revealed an age related differential response to persistent renal injury. We show that age-associated changes in the fibrotic response to kidney injury, occur already prior to the appearance of typical senescence markers like SA-β-Gal activity, Klotho loss, and spontaneous glomerulosclerosis. In particular, increased morphological damage and a reduced fibrotic response were observed in 1-year-old mice without alteration of canonical TGFβ transcriptional activity. Instead, a decrease of CTGF and increase of BMP6 expression was found, associated with increased downstream pSMAD1/5/8 activity in cortical tubules, which might at least in part explain the observed reduced fibrogenesis to kidney injury in aging.

In human diagnostics and experimental animal research interstitial fibrosis and tubular atrophy (IFTA) is often assessed together and thought to "go hand in hand" (21). We show that following injury in the aged kidney, the proportion of fibrosis and atrophy can be shifted in favour of atrophy suggesting ramifications for clinical assessment of IFTA in the ageing kidney. Previously, it has been shown that renal damage in response to Ischemia Reperfusion Injury (IRI), in terms of GFR loss, morphological injury, and fibrosis, is increased in ageing (9, 46). Inverse correlations have been reported previously (38). Age-related acceleration of progressive kidney senescence and CKD development, after the initial acute injury has subsided, is becoming a widely recognized phenomenon, and considered to be due to decrease of reparative capacity (20). It remains to be established whether, in addition to decreased regenerative capacity, also the observed “weaker” but possibly more persistent fibrotic renal response to injury might be involved in more progressive loss of function upon transient injury in old kidneys.
The kidney is a major contributor to Klotho production and Klotho loss is strongly associated with ageing and the renal response to damage (6, 16, 30, 31, 33). Klotho is an important inhibitor of TGFβ, one of the most important mediators of fibrotic renal response to damage and ageing (34, 43). However, we found that at the age of 50 weeks Klotho and Tgfβ expression were not yet affected by ageing but diminished and increased, respectively, to a similar extent in young obstructed kidneys. Thus the observed differential renal damage response occurred prior to the well-established changes of baseline Klotho and TGFβ expression at a more advanced age.

Interestingly, expression of the well-established anti-fibrotic and pro-regenerative BMP7-gene was also not different in old as compared to young OBKs, but the older OBKs had an increased BMP6/CTGF ratio, resulting from retained BMP6 expression and suppressed CTGF expression. The finding that preserved Bmp6 expression in old OBK was associated with less fibrosis is congruent with our previous observation that loss of BMP6, together with the ensuing overexpression of CTGF, aggravated renal fibrosis and myofibroblast (αSMA) accumulation (14). This study by Dendooven et al. also showed that the level of tubular dilatation was unaltered suggesting BMP6 to be unrelated to dilatation. We propose that direct BMP6-effects might mainly attenuate fibrosis, while the other morphological differences observed might be secondary to this.

In our experiment, the increased BMP6 expression might at least partially account for the 50% reduction of CTGF in old OBK. Figure 5 shows that CTGF directly interacts with BMP6 and as such inhibits canonical signaling. Since old OBKs show less CTGF but more BMP6 expression, this increased BMP6/CTGF ratio might underlie the found pSMAD158 increase in cortical tubules, especially since we have an indication that the inhibitory effects of CTGF on pSMAD158 might be due to direct physical interaction
In the cortex, mainly distal tubules and collecting ducts display canonical BMP signaling, and it has been noted previously that upon UUO, signaling decreases (32). The phenomenon of epithelial to mesenchymal transition (EMT) is a large contributor to the development of renal fibrosis (49). Possibly the increase in tubular pSMAD1/5/8 seen in figure 4 reflects reduced EMT rate underlying the reduction in fibrosis.

Previously, we reported that a 50% reduction of CTGF as such is not sufficient to hamper the phenotype observed in 14 day UUO and other severe models of CKD (18). In conjunction with the present observations this might suggest that, at least in the UUO model, BMP6/CTGF balance is more important for fibrosis control than the absolute CTGF level.

One might speculate that, in conjunction with less pronounced fibrosis, increase of morphological damage in terms of atrophy and dilatation in old OBKs, might result from less fibrogenic growth factor activity, with the resulting reduction of matrix deposition hampering generation of sufficient structural support to withstand increased pressure developing upon obstruction. As previously mentioned, there are many factors playing a role in the process of renal aging. However, the production of pro-fibrotic cytokines is a common end point (e.g. TGFβ). Since there is no differential regulation of TGFβ or downstream PAI1 expression, the observed effects might have different drivers than the usual suspects of aging.

In conclusion, our studies have revealed an age-dependent shift in renal response to injury, developing a more atrophic and less fibrotic phenotype. This change is associated with altered BMP6/CTGF balance and already occurs before mice have lived through half of their life span and before the appearance of classical signs of senescence namely spontaneous loss of Klotho, increase in TGFβ expression and SA-β-Gal. Figure 6
depicts a proposed mechanism distilled from the observations presented in this manuscript. While most experimental studies addressing CKD have been performed in young rodents, these might not appropriately reflect renal response to injury in ageing patients. This should be taken into account when interpreting existing and designing future studies addressing CKD progression in the ageing population.
Disclosures

RG has performed contract research for, and received research support, from FibroGen Inc.; a company involved in development of anti-CTGF therapy. RG has been employed by FibroGen Inc. from August 2008 till August 2009.
References


**Titles and legends to figures**

**Figure 1:** *Unilateral Ureter Obstruction (UUO) causes more morphological damage in old kidneys.*

- **A.** Total bodyweight (BW) prior to UUO (Start) and after sacrifice (End);
- **B.** Representative micrographs of PAS stained slides of contralateral kidney (CLK) and obstructed kidney (OBK) in both age groups (200x);
- **C.** Quantification of microscopically observed atrophy and dilatation observed; Error bars represent SEM; **p<0.01, ***p<0.005. Statistics used in A: two-way ANOVA with Sidak correction for multiple comparison, C&E: non-paired two-way ANOVA with Tukey correction.

**Figure 2:** *Old kidneys show less ECM deposition upon UUO.*

- **A.** Representative micrographs of Masson-Trichrome (MTC) and αSMA stained kidneys of both age groups (200x);
- **B.** Morphometric quantification of fibrosis as seen with MTC staining;
- **C.** Morphometric quantification of myofibroblasts as seen with αSMA staining;
- **D.** *Col1a2* expression levels in CLK and OBK;
- **E.** *Hsp47* expression levels in CLK and in OBKs;
- **F.** Hydroxyproline/proline ratio; Error bars represent SEM; * p<0.05, ***p<0.005. Statistics used in B-D: non-paired two-way ANOVA with Tukey correction.

**Figure 3:** *CTGF/BMP6 ratio is altered prior to differential Klotho or TGFβ regulation in old obstructed kidneys.*

- **A.** *Klotho* expression levels in CLK and OBK of both age groups;
- **B.** *TGFβ* expression levels in CLK and OBK kidneys;
- **C.** *Pai-1* expression levels in CLK and OBK kidneys;
- **D.** *Bmp7* expression levels in CLK and OBK;
- **E.** *Bmp6* expression levels in CLK and OBK;
- **F.** *Ctgf* expression levels in CLK and OBK;
- **G.** Representative micrographs of CTGF stained slides of CLK and OBK (200x);
- **H.** Morphometric quantification of CTGF positive staining area. Arrowheads indicate positive tubular staining; Error bars represent SEM; * p<0.05, **p<0.01, ***p<0.005. Statistics used A-F & H: non-paired two-way ANOVA with Tukey correction.

**Figure 4:** *Tubular pSMAD1/5/8 signaling is preserved in old OBKs*

- **A.** Western blot of OBK cortical lysates showing pSMAD1/5/8 and SMAD1/5/8 levels band intensity;
- **B.** pSMAD1/5/8 band intensity quantification corrected for total SMAD1/5/8 levels;
- **C.** Representative micrographs of pSMAD1/5/8 OBKs (200x magnified). Arrowheads indicate pSMAD1/5/8 positive tubular
cells. D. Quantification of the number of cortical pSMAD158 positive nuclei in glomerular, tubular and interstitial cells; Error bars represent SEM; * p<0.05, **p<0.01, ***p<0.005. Statistics used A&C: non-paired two-way ANOVA with Tukey correction, E: Student-T test.

Figure 5: CTGF binds BMP6 and inhibits canonical BMP signaling

A. Absorbance levels of CTGF/BMP6 solid phase assay B. Western blot of HK-2 cell lysate 1 hour after stimulation with rhBMP6 and/or rhCTGF. Upper panel pSMAD1/5/8, Lower panel SMAD1/5/8. C. Id1 expression levels of HK-2 cells stimulated with rhBMP6 and/or rhCTGF; Error bars represent SEM; * p<0.05, **p<0.01, ***p<0.005. Statistics: non-paired two-way ANOVA with Tukey correction.

Figure 6: Proposed model for age associated change in the response to renal injury

In the aged kidneys there is an increased expression of BMP6 that in itself is capable of reducing CTGF expression. Both the increase of BMP6 and the reduction of CTGF lead to increased levels of pSMAD1/5/8 (partially due to loss of physical binding to and inhibition of BMP6 by CTGF). The increase in pSMAD1/5/8 leads to reduced fibrosis, possibly via inhibition of EMT.
Figure 5

A. CTGF solid phase assay

B. Id1

C. Western blot analysis

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