Dact1, a Wnt-Pathway Inhibitor, Mediates Human Mesangial Cell TGF- β 1-Induced Apoptosis

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Chronic kidney disease (CKD) is a worldwide public health problem that affects millions of men and women of all ages and racial groups. Loss of mesangial cells (MC) represents an early common feature in the pathogenesis of CKD. Transforming growth factor- β 1 (TGF- β 1) is a key inducer of kidney damage and triggers several pathological changes in renal cells, notably MC apoptosis. However, the mechanism of MC apoptosis induced by TGF- β 1 remains elusive. Here, we demonstrate for the first time a novel regulatory pathway in which the disheveled-binding antagonist of β -catenin 1 (Dact1) gene is upregulated by TGF- β 1, inducing MC apoptosis. We also show that the inhibitory effect of Dact1 and TGF- β 1 on the transcriptional activation of the pro-survival Wnt pathway is the mechanism of death induction. In addition, Dact1 mRNA/protein levels are increased in kidney remnants from 5/6 nephrectomized rats and strongly correlate with TGF- β 1 expression. Together, our results point to Dact1 as a novel element controlling MC survival that is causally related to CKD progression.

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In the past few years, the incidence and prevalence of chronic kidney disease (CKD) have reached alarming levels worldwide (Coresh et al., 2003; Lameire et al., 2005). Regardless of the initial cause, CKD results in progressive loss of renal function due the ensuing fibrotic process and later, end-stage renal disease (ESRD) (Khwaja et al., 2007). Increasing rates of morbidity and mortality have been associated to CKD and ESRD (Levey et al., 2007). There is an urgent need for the development of specific tools that prevent renal disease progression.

In human and animal models of CKD, the glomerulus is the primary target in most cases (Kriz and LeHir, 2005). Mesangial cells (MCs) are responsible for proper glomerular architecture and play a critical role in the glomerular filtration rate (Abboud, 2012). MCs actively contribute to CKD pathogenesis as well (Marshall and Shankland, 2006; Lee and Song, 2009). Metabolic insults, such as hypertension and hyperglycemia, stimulate MCs to overproduce extracellular matrix (ECM) with consequent aberrant deposition, the main feature of the glomerular sclerotic process (Lee and Song, 2009). In a specific diabetic nephropathy murine model (BTBR ob/ob), it was recently demonstrated that MC loss is the earliest detectable histological feature of disease progression (Hudkins et al., 2010). In fact, MC apoptosis has been associated with CKD progression in different scenarios (Marshall and Shankland, 2006; Fenton, 2015). However, the effect of MC apoptosis selective inhibition on the development of renal injury has not been tested to date.

A variety of elements that interact to promote kidney fibrosis have already been identified. Among these, Transforming Growth Factor-Beta 1 (TGF- β 1) has been shown to be particularly important (Ziyadeh, 2004; Chuang et al., 2012; Meng et al., 2015). The increased expression of TGF- β 1 has been documented in human as well as various experimental models of CKD (Border and Noble, 1994; Loeffler and Wolf, 2013). It has been shown that TGF- β 1 signaling promotes ECM overproduction and deposition, while inhibiting its degradation (Loeffler and Wolf, 2013). Additionally, a growing body of

evidence indicates that TGF- β 1 triggers a number of other renal cell phenotypic changes, in particular MC apoptosis (Kume et al., 2007; Sato et al., 2011; Loeffler and Wolf, 2013). However, the mechanisms underlying TGF- β 1's effects are not fully understood.

Several studies have demonstrated that TGF- β 1 effective blockade prevents and/or reduces glomerular and tubular damage in various models of CKD, pointing to TGF- β 1 as a promising therapeutic target (Border and Noble, 1998; Huang et al., 2006; Santibanez et al., 2011; Ling et al., 2004). However, due to the important physiologic roles of that cytokine in different tissues, blocking TGF- β 1 systemically does not seem to be a reasonable alternative for kidney disease control. The identification of TGF- β 1 signaling mediators specifically associated with renal cell phenotypic changes can produce more attractive therapeutic targets.

Wnt signaling is extremely important for proper embryo formation (Cheyette et al., 2002; Kispert et al., 1998) and for adult tissue regeneration after injury (Terada, 2003). At least three branches of Wnt signaling were identified in mammals: the canonical Wnt/ β -catenin, the noncanonical planar cell polarity (PCP), and Ca²⁺-dependent pathways (Kikuchi et al., 2007).

Canonical Wnt/ β -catenin signaling is initiated by the binding of Wnt ligands to the Frizzled (FZ) transmembrane receptor

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and LRP5/6 co-receptor, leading to the recruitment and activation of Disheveled (Dvl) (Kikuchi et al., 2007). Dvl causes the disruption of the GSK-3 β -Axin- β -catenin complex by inhibiting GSK-3 β , resulting in increased β -catenin stability and nuclear translocation (Kikuchi et al., 2007). In the nucleus, β -catenin interacts with the transcription factors TCF/LEF and regulates the expression of several target genes.

The cross-talk between the Wnt/ β -catenin and TGF- β pathways has been studied in the fibrogenesis of different organs (Cisternas and Vio, 2014; Guo et al., 2012). In an experimental model of obstructive nephropathy, inhibition of β -catenin gene transcription induction improved kidney lesions and abrogated TGF- β 1-induced profibrotic gene overexpression (Hao et al., 2011). Inappropriate activation of β -catenin has been implicated in the pathogenesis of CKD in other contexts as well (He et al., 2009; von Toerne et al., 2009; Dai et al., 2009; Liu, 2011). On the other hand, there is substantial evidence that this same pathway is related to MC survival control. By forcing agonist expression, Wnt activation prevents MC apoptosis in a high glucose environment. MCs growing under the same conditions showed Wnt4 and Wnt5a down-regulation and impaired β -catenin nuclear translocation (Lin et al., 2006). Accordingly, simvastatin (Lin et al., 2008) and spironolactone (Zhu et al., 2013) drugs, commonly used in clinical practice, also had positive effects on MC survival by activating the Wnt/ β -catenin pathway. Thus, it seems that well-controlled β -catenin signaling is essential for renal cell homeostasis.

The Dact1 gene is a Wnt signaling modulator from the Dapper/Frigo family. Three members of the Dapper family have been described in mammals: Dact1, Dact2, and Dact3 (Meng et al., 2008; Suriben et al., 2009; Wen et al., 2010; Xue et al., 2013). Dact1, in particular inhibits the Wnt pathway by promoting Dvl lysosomal degradation (Zhang et al., 2006; Chen et al., 2011) and is also able to negatively regulate Wnt/ β -catenin signaling activity in the nucleus by promoting the association of the transcription factor LEF1 to the histone deacetylase I (HDAC1), keeping LEF1 in a repressive state (Gao et al., 2008).

Dact1 is essential during embryogenesis (Suriben et al., 2009; Wen et al., 2010). In adult age, Dact1 acts as a tumor suppressor in a variety of malignancies (Yin et al., 2013; Wang et al., 2012). To date, there has been no evidence of the role of Dact1 in renal diseases.

In previous experiments using microarray technology (not shown), we identified Dact1 as an upregulated gene in response to TGF- β 1 in immortalized human mesangial cells (ihMC). In the present study, we investigated the role of Dact1 in TGF- β 1-induced ihMC apoptosis. We demonstrate that Dact1-forced overexpression enhances apoptosis of ihMCs and its knockdown significantly inhibits TGF- β 1-induced ihMC apoptosis. Furthermore, Dact1 expression is increased in kidney remnants from 5/6 nephrectomized rats and its expression strongly correlates with TGF- β 1 levels, indicating that our *in vitro* findings have potential relevance in CKD progression.

Experimental Procedures

Cell culture and treatment

Immortalized human mesangial cells (ihMC), kindly provided by Dr. Bernhard Banas (Ludwig-Maximilians University, Munich, Germany), and were maintained in DMEM low glucose supplemented with 10% fetal bovine serum. ihMCs were serum-starved for 24 h, then treated with TGF- β 1 (2 ng/ml), LiCl (10 mM), Angiotensin II (Ang II— 10^{-6} M), PDGF (10 ng/ml) or bFGF (10 ng/ml) for periods of time indicated in the figure legends. When applicable, the pSMAD3 inhibitor SIS3 (Sigma-Aldrich, St. Louis, MO) was pre-incubated for 30 min, followed by the addition of TGF- β 1 (2 ng/ml).

Dact1 overexpression

Adenoviral vectors were built using the ViralPower™ Adenoviral Expression System kit (Invitrogen, Carlsbad, CA) and the pCMV-Myc-Dact1 vector, kindly provided by Dr. Ye-Guang Chen (Department of Biological Sciences and Biotechnology, Tsinghua University, China). ihMCs were infected at a multiplicity of infection of 60 (MOI 60). After 24 h, the culture medium was changed. Analyses were performed 48 or 72 h after infection and the LacZ vector was used as negative control.

Dact1 gene silencing

Specific interference RNA sequences (sense: 5'-CAAGCAGCCUCGCAAGAA-3' and antisense: 5'-UUCUUGCGAGGCUGGCUUG-3' [Sigma-Aldrich, SASI_Hs02_00313430]) and the negative control (Mission Universal Negative Control siRNA #1, Sigma-Aldrich) were obtained commercially. ihMCs were transfected using the N-TER Nanoparticle siRNA Transfection System kit (Sigma-Aldrich). After 24 h, the culture medium was changed. Analyses were performed 48 h after transfection.

Quantitative RT-PCR

Gene expression was quantified using Rotor-Gene™ 6000 (Qiagen, Düsseldorf, Germany) and the Quantitect SYBR Green I kit (Qiagen), according to the manufacturer's recommendations. Specific primer pairs were designed using the PrimerSelect DNASTAR software. Target gene expression was normalized by GAPDH mRNA levels.

Luciferase assays

Luciferase assays were performed as previously described elsewhere (Sato et al., 2011). Briefly, ihMCs were transfected by the non-liposomal lipid-based technique (Attractene, Qiagen) with various plasmids, as indicated in the figure legends. At 48 h post-transfection, cells were harvested and luciferase activity was measured in cell lysates through a non-homogeneous assay using Glo-Lysis Buffer and the Bright-Glo Luciferase Assay System (Promega, Fitchburg, WI). The transcriptional activity of the Canonical Wnt pathway was measured with the reporter vector M50 8x super TOPFlash (Addgene, Cambridge, MA). pDsRed-Mito (Clontech Lab, Palo Alto, CA) was cotransfected as a fluorescent internal control. The FARGyte Plate Reader (Amersham/GE, Fairfield, CT) was used for fluorescence (544-nm excitation and 595-nm emission wavelengths) and luminescence quantification.

Chromatin morphology analysis

In order to quantify the number of apoptotic ihMCs, chromatin morphology was analyzed using the nuclear stain Hoechst 33342 (0.5 mg/ml; Molecular Probes, Eugene, OR). Adherent and floating cells were collected, fixed in 4% paraformaldehyde and analyzed under UV fluorescence microscopy ($\times 400$). Two hundred and fifty nuclei were analyzed per sample.

Caspase-3 activity assay

Adherent and floating ihMCs were collected and lysed using RIPA Buffer (Sigma-Aldrich). Protein concentration was quantified with the BCA Protein Assay kit (BioAgency, Sao Paulo, Brazil) and an equivalent to 30 μ g of protein was subjected to analysis. Caspase-3 activity was accessed through the Fluorometric Caspase-3 Assay kit (Sigma-Aldrich). Fluorescence was measured with an excitation wavelength of 360 nm and an emission wavelength of 460 nm.

Experimental model of 5/6 nephrectomy (5/6 nx)

This study was previously approved by the Ethics Committee on Animal Experimentation of the Hospital Israelita Albert Einstein (permit number: 1315/11). After general anesthesia (Ketamine 100 mg/Kg and Xylazine 10 mg/Kg, intraperitoneally), male Wistar rats underwent a midline abdominal incision and had the left kidney exteriorized and the renal artery exposed. The lower and upper branches of the renal artery were ligated and the kidney was repositioned in the abdominal cavity. The right kidney was removed and then the skin was sutured. Animals from the sham group had their abdominal cavities opened and kidneys manipulated without compromising blood flow. Four weeks after surgery, kidney remnants (from the 5/6 group) or whole organs (sham group) were removed for analysis. Part of the kidney samples was fixed in 10% formaldehyde, followed by paraffin embedding for histologic and immunohistochemical studies. The remaining kidney tissue was frozen in liquid nitrogen and stored at -80°C for RNA and protein extraction.

Histology and immunohistochemical staining

Immunohistochemistry assays were performed as previously described (Simoes Sato et al., 2014). Briefly, paraffin-embedded rat kidney sections (5 mm thickness) were incubated with rabbit polyclonal anti-Dact1 (ab51260; Abcam, Cambridge, MA) and mouse monoclonal anti-TGF- β 1 (ab64715; Abcam). Detection was performed with DAB chromogen. Sections were counterstained with hematoxylin (Dako, Carpinteria, CA) and mounted onto glass slides. Control sections were treated with rabbit or mouse isotype control antibodies (Santa Cruz Biotechnology, Dallas, TX) to determine the specificity of the staining. ECM deposition was evaluated by Masson trichrome staining.

Statistical analysis

The results were presented as means $\pm$ SE. Comparisons between two groups were performed with Student's *t*-tests. For multiple comparisons, we used ANOVA followed by the Tukey test. At least three different samples were analyzed in each experimental group. $P < 0.05$ was considered significant.

Results

Dact1 is upregulated by TGF- β 1, but does not alter matrix gene expression

TGF- β 1 induced a concentration-dependent upregulation of Dact1 (Fig. 1A). Of note, a very low concentration (0.2 ng/ml) could produce significant increments in Dact1 mRNA levels. In addition, Dact1 mRNA levels were rapidly increased following the administration of TGF- β 1, with significant changes after 1 h of treatment. Peak upregulation was seen at 6 h (Fig. 1B). We also evaluated whether Dact1 expression could be regulated by other growth factors known to be relevant to mesangial cell biology. However, bFGF, PDGF, and ANGII failed to promote any significant change in Dact1 mRNA levels (Fig. 1C). It is known that the majority of TGF- β 1 actions are mediated by SMAD pathway activation. Accordingly, pSMAD3 blockade abrogated TGF- β 1's ability to induce Dact1 expression (Fig. 1D). In order to elucidate the functional role of Dact1 in ihMCs, gain- and loss-of-function experiments were performed (online suppl. fig. S1). It is well-established that TGF- β 1 promotes the progressive accumulation of ECM in CKD, but Dact1-forced overexpression did not change the mRNA levels of fibronectin and collagen IV, nor of the protease inhibitor Plasminogen Activator Inhibitor-1 (PAI-1), which is responsible for

preventing ECM degradation (Fig. 1E). Similar findings for ECM gene regulation were obtained following Dact1 gene silencing, except for a significant elevation in PAI-1 mRNA levels (Fig. 1F–H).

Dact1 mediates TGF- β 1-induced apoptosis through inhibition of the pro-survival Wnt pathway activation

We found that Dact1 overexpression induced a significant increase in apoptotic ihMC number, even in the presence of fetal bovine serum (FBS; Fig. 2A and online suppl. fig. S2). These data were confirmed by caspase-3 activity assays (Fig. 2B), indicating that Dact1 is an effective pro-apoptotic agent. On the other hand, Dact1 mRNA knockdown using siRNA showed no significant reduction in basal rates of ihMC apoptosis (Fig. 2C and D). Under the same experimental conditions, however, Dact1 gene silencing markedly inhibited TGF- β 1-induced ihMC death (Fig. 2C and D). It is noteworthy that even partial reductions of Dact1 expression levels ($\sim 50\%$) were sufficient to significantly promote ihMC survival.

Lithium chloride (LiCl), a GSK-3 β inhibitor, promotes β -catenin stabilization and consequent Wnt signaling activation (Abu-Baker et al., 2013). In agreement with previous studies (Lin et al., 2006), we observed that LiCl inhibits serum-withdrawal-induced ihMC apoptosis and prevents TGF- β 1-induced ihMC apoptosis (Fig. 2E and F). In a separate series of gene reporter experiments, Dact1 prevented Wnt signaling transcriptional activation by LiCl (Fig. 2G) and similar effects were also observed after TGF- β 1 treatment (Fig. 2H). In addition, TGF- β 1 also prevented Wnt signaling transcriptional activation induced by the constitutively active β -catenin vector pVL- β -catenin-deltaN90 (Addgene) (Fig. 2I). Dact1 gene silence not only restored the Wnt signaling basal activity inhibited by TGF- β 1, but it also induced its transcriptional activation (Fig. 2J), indicating that Wnt signaling inhibition is the mechanism for TGF- β 1-induced, Dact1-mediated ihMC apoptosis.

Dact1 expression levels are increased in 5/6 nephrectomy model

Next, we evaluated Dact1 expression in vivo in a chronic nephropathy model. In comparison to normal kidneys, remnant kidneys from 5/6 nephrectomized animals presented, as expected, augmented glomerular areas and matrix deposition, as well as decreased glomerular cell counts (Fig. 3A, upper part). Immunohistochemistry assays revealed increased Dact1 protein levels in the glomerulus and in particular, in tubular regions in the 5/6 Nx group (Fig. 3A, middle part). As expected, TGF- β 1 protein expression was markedly elevated in both compartments in the same group (Fig. 3A, lower part). qPCR analysis disclosed a significant upregulation of both Dact1 (Fig. 3B) and TGF- β 1 (Fig. 3C) mRNA levels in samples from kidney remnants. Moreover, the amount of Dact1 mRNA strongly correlated with TGF- β 1 expression levels (Fig. 3D). These data suggest that our in vitro results may also have implications for CKD progression.

Discussion

Renal cell apoptosis has been associated to the pathogenesis of many nephropathies (Goilav, 2011; Fenton, 2015). Renal function improvement was observed after apoptosis inhibition in different models (Yang et al., 2003; Docherty, 2005; Hruby et al., 2008).

It is known that MCs are the primary target in clinical conditions such as IgA nephropathy, lupus nephritis, and diabetic nephropathy (Marshall and Shankland, 2006; Abboud, 2012). In fact, it has been shown that MC loss is the earliest

Fig. 1. Dact1 is upregulated by TGF- β 1, but does not alter matrix gene expression: Gene expression analysis. ihMC were serum-starved for 24 h and treated with TGF- β 1 for 24 h (A), with TGF- β 1 (2 ng/ml) for the indicated time (B), bFGF (10 ng/ml), PDGF (10 ng/ml), ANGII (10^{-6} M) and TGF- β 1 (2 ng/ml) for 6 h (C), and specific Smad3 phosphorylation inhibitor SIS3 (10 μ M) pre-incubated for 30 min, followed by the addition of TGF- β 1 (2 ng/ml) for 6 h (D), ihMCs were infected with pAd-Dact1 or pAd-LacZ (control) in MOI 60 (E), or transfected with siDact1 or siControl (45 nm) and treated with TGF- β 1 (2 ng/ml) or vehicle (F–H). Data were normalized by GAPDH expression levels and expressed as fold-change relative to respective controls. N = 3. *** P < 0.001 and * P < 0.05 vs. control. 4496

histological feature detectable in DN progression. Mesangial matrix accumulation is preceded and accompanied by some degree of mesangiolysis in this model (Hudkins et al., 2010).

Here, we demonstrate for the first time the role of Dact1 in MC apoptosis (Fig. 4). Dact1 overexpression in vitro induced ihMC death even in a prosurvival environment (Fig. 2A and B). In addition, Dact1 knockdown was able to partially inhibit TGF- β 1-induced ihMC apoptosis (Fig. 2C and D). As mentioned, only partial Dact1 gene silencing was achieved and, perhaps, a more robust phenotype would be observed with more effective Dact1 knockdown. On the other hand, it is known that TGF- β 1 can induce mesangial apoptosis by other mechanisms (Negulescu et al., 2002; Kume et al., 2007). Thus, we did not expect to abolish TGF- β 1-induced apoptosis by suppressing only one downstream element. Therefore, Dact1 knockdown-triggered apoptosis inhibition is a relevant finding.

The dual functions of TGF- β 1 in the pathogenesis of CKD have been discussed in the literature. TGF- β 1 has been highlighted as a key inducer of several pathological changes in renal cells, notably mesangial cell apoptosis (Kume et al., 2007; Sato et al., 2011; Loeffler and Wolf, 2013). Conversely, there is evidence that TGF- β 1 induces autophagy and

protects mesangial cells from undergoing apoptosis during serum deprivation (Ding et al., 2010). However, in this study, a significant reduction in the number of viable cells is observed in the group serum starved and treated with TGF- β 1 compared to the group maintained in the presence of 15%, which suggests that even with the protective effect of TGF- β 1, the cells die. Besides autophagy has been considered as a cytoprotective mechanism, it has also been considered an alternative cell-death pathway. Autophagy and apoptosis may be triggered by common upstream signals, and sometimes this results in combined autophagy and apoptosis (Maiuri et al., 2007).

Both TGF- β 1 and Dact1 inhibited transcriptional activation of the Wnt/ β -catenin pathway (Fig. 2G–I). Dact1 gene silencing not only restored the Wnt basal activity previously inhibited by TGF- β 1, but also had additional effects on its transcriptional activation (Fig. 2J). Given that the Wnt pathway was associated with apoptosis blockade, we conclude that this is the mechanism for TGF- β 1-triggered apoptosis induction in ihMCs. Our findings point to Dact1 as a downstream molecule in TGF- β 1 signaling and may represent a link between the canonical pathways of TGF- β 1 and Wnt in controlling cell survival.

Fig. 2. Dact1 mediates TGF- β 1-induced apoptosis, through inhibition of the pro-survival Wnt pathway activation: ihMCs were infected with pAd-Dact1 or pAd-LacZ, in the presence or absence of serum for 24 h. Chromatin morphology analysis, N = 6 (A), Caspase3 activity assay. Data are expressed as means $\pm$ SEM of arbitrary fluorescence units (AFU), N = 4 (B), ihMCs were transfected with siDact1 or siControl (45 nm) followed by TGF- β 1 (2 ng/ml) treatment or vehicle. Chromatin morphology analysis was carried out in the presence (C), or absence of 10% FBS, N = 4 (D), ihMCs were treated with LiCl (10 mM) for 24 h and serum starved or not by the same period of time (E), or treated with LiCl (10 mM) plus TGF- β 1 (2 ng/ml) for 24 h and serum starved by the same period of time (F), data were expressed as percentage of apoptotic cells (A, C, D–F) * P < 0.05 vs. respective control. ihMCs were transfected with Wnt reporter plasmid (TopFlash M50) and infected with pAd-Dact1 or treated with TGF- β 1 (2 ng/ml) and LiCl (10 mM) for 24 h (G and H). ihMCs were cotransfected with TopFlash M50 and pVL- β -catenin-deltaN90 vectors and treated with TGF- β 1 (2 ng/ml) for 24 h (I). ihMCs were transfected with siDact1 or siControl after 24 h transfected with TopFlash M50 and then treated with TGF- β 1 (2 ng/ml) for 24 h (J). After 48 h of transfection, cells were processed for gene reporter luciferase assay (G–J). pDS-RedMito plasmid was co-transfected as an internal control for transfection efficiency. Luciferase activity is expressed as relative arbitrary units of luminescence, normalized by fluorescence. N = 4. *** P < 0.001 and * P < 0.05.

Apoptosis is a remarkably orchestrated process. The resulting apoptotic fragments enveloped by cell membranes are quickly removed by macrophages or neighboring cells, preventing an immune response (Elmore, 2007). Scavenging after cell death hampers the in vivo evaluation of apoptosis rates. Although we were not able to detect apoptotic cells in samples from our 5/6 nephrectomy model, it was clear that operated animals presented glomerular cell paucity compared with sham-operated animals (Fig. 3A, upper part).

Smad3 is a key mediator of the TGF- β 1 deleterious effects. It has been shown that Smad3 inhibition attenuates renal fibrosis, inflammation and apoptosis in an obstructive nephropathy model (Inazaki et al., 2004). Here, we demonstrated that Dact1 regulation by TGF- β 1 is mediated by Smad3 phosphorylation (Fig. 1D). At the same time, no effect on ECM components was observed after manipulation of Dact1 expression. This is an interesting observation, since TGF- β 1-induced matrix accumulation is usually mediated by the same pathway (Poncelet and Schnaper, 2001; Schnaper et al., 2003). It is possible that

Smad3 phosphorylation leads to the activation of distinct sets of mediators responsible for controlling cell survival and ECM synthesis and degradation. Additional studies outside the scope of this manuscript are in progress to evaluate this issue.

Distinct publications have demonstrated the interaction between TGF- β 1 and the Wnt pathway in the fibrogenesis of various organs (Akhmetshina et al., 2012; Zhang et al., 2012; Zhou et al., 2012). In fact, the Wnt pathway has been described as a mediator of the fibrogenic effects of TGF- β 1, through physical interaction with Smads (Tian et al., 2012). Furthermore, Wnt inhibition was effective in preventing and reverting renal fibrosis in different models (Dai et al., 2009; He et al., 2011). A recent study demonstrated that loss of Dact3, also a Wnt signaling inhibitor, enhanced ECM overproduction in a mouse model of CKD. This aggravated fibrotic phenotype was accompanied by accumulation of Wnt pathway members and Wnt-responsive fibrogenic gene overexpression (Meng et al., 2008), reinforcing the idea that a well-controlled regulation of the Wnt pathway is essential to renal ECM

Fig. 3. Dact1 expression levels are increased in 5/6 nephrectomy model: Five-sixths nephrectomy stimulates ECM deposition (in blue), glomerular enlargement and cell paucity. Masson trichrome stain (A, upper part). rDact1 (A, middle part) and rTGF- β 1 (A, lower part) protein expression were analyzed by immunohistochemistry. Positive cells were stained in brown. Nuclei were counterstained with hematoxylin. qPCR from sham and Nx 5/6 kidney lysate. rDact1 mRNA levels (B), rTGF- β 1 mRNA levels (C), Scatter plot of rTGF- β 1 vs. Dact1 expression levels (D). Data were normalized by GAPDH expression levels and expressed as fold-change relative to control group. N = 9 (sham group) and N = 16 (Nx 5/6 group). ** $P < 0.001$ and * $P < 0.05$.

homeostasis. Moreover, there is evidence that Wnt inhibition promotes ECM synthesis in MCs subjected to high glucose concentrations (Lin et al., 2010). Those studies did not test whether the observed effects were related to cell death. This is a matter that deserves further investigation. In this sense, the manipulation of Dact1 expression in vivo could represent a useful strategy to explore Wnt pathway effects on chronic renal disease in more detail. Dact1 loss-and-gain of function would potentially affect cell survival, but not the expression of ECM components. Specific tools would have to be designed to address this question. Dact1 is essential during embryogenesis (Wen et al., 2010) and plays important roles in different organs. In the central nervous system, Dact1 promotes autophagy and its ablation results in motor coordination defects as well as the accumulation of p62 and ubiquitinated proteins (Ma et al., 2014). In the heart, Dact1 is essential for cardiomyocyte hypertrophy induction, an adaptive response to pathological stress (Hagenmueller et al., 2014). It also acts as a tumor suppressor in different malignancy types (Wang et al., 2012; Yin et al., 2013). Thus,

selective, conditional, and cell type specific models of in vivo Dact1 loss-and-gain of function should be sought.

To conclude, the identification of TGF- β 1 signaling mediators associated with phenotypic changes of renal cells appears to be essential for the development of specific and selective therapies that are to date unavailable. The continued analysis of the interrelationships between the TGF- β 1 signaling and the Wnt pathway is promising. This is the first description in the literature of a novel regulatory pathway in which Dact1 is overregulated by TGF- β 1, inducing apoptosis in MCs (Fig. 4). It may be assumed that this effect is causally related to the progression of glomerular damage. Dact1 and associated proteins may represent markers and/or therapeutic targets in CKD. More complex complementary in vivo studies will be necessary to define the full implications of the findings here described.

Disclosure

No conflicts of interest, financial or otherwise, are declared by the authors.

Fig. 4. General view of the data presented. Representative scheme of the novel regulatory pathway involved in the control of ihMC survival.

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