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Review

Involvement of small Ras GTPases and their effectors in chronic renal disease

C. Martínez-Salgado[a,b], A. B. Rodríguez-Peña[c] and J. M. López-Novoa[d,b,*]

[a] Unidad de Investigación, Hospital Universitario de Salamanca, Salamanca (Spain)
[b] Red Cooperativa de Investigación Renal del Instituto Carlos III (RedinRen), Salamanca (Spain)
[c] Centro de Investigación del Cáncer CSIC-USAL, Campus Miguel de Unamuno, Salamanca (Spain)
[d] Instituto "Reina Sofía" de Investigación Nefrológica, Departamento de Fisiología y Farmacología, Universidad de Salamanca, Avda. Campo Charro s/n, 37007 Salamanca (Spain), Fax: +34 923 294669, e-mail: jmlnovoa@usal.es

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Abstract. The mechanisms involved in the development of renal fibrosis are poorly understood. Small Ras GTPases control cell proliferation, differentiation, cellular growth and apoptosis, with cell-specific expression in the kidney. Cytokines, high glucose medium or advanced glycation end-products activate Ras in different renal cells. Increased Ras activation has been found in experimental tubulointerstitial fibrosis. Transforming growth factor-β1 (TGF-β1) and Ras signalling pathways are close related: TGF-β1 overcomes Ras mitogenic effects, and Ras counteracts TGF-β signalling. However, Ras activation is also an intracellular signal transduction point for several molecules (*e.g.* TGF-β1) involved in kidney damage. Ras isoforms play different roles in regulating extracellular matrix synthesis in fibroblasts and mesangial cells. These data give evidence for a role for Ras in renal fibrosis, but no reviews are available on the role of p21 Ras in this process. Thus, our goal is to review the role of Ras activation and signalling in renal fibrosis.

Keywords. Mesangial cells, Ras, renal fibroblasts, renal fibrosis, transforming growth factor-β1, tubular epithelial cells.

Introduction

Hypertension, diabetes and several renal diseases frequently lead to end-stage renal disease [1], which is characterized by cell proliferation and progressive deposition of extracellular matrix (ECM) in glomeruli (glomerulosclerosis) and/or interstitial space (tubulointerstitial fibrosis), resulting in progressive decline of renal function [2]. The intracellular signalling pathways involved in fibrogenesis remain relatively unknown. The relevance of transforming growth factor-β1 (TGF-β1) in the origin and maintenance of glomerulosclerosis and tubulointerstitial fibrosis has been shown in the last years [3–5]. These effects of TGF-β1 are a consequence of its effects on cell proliferation [6, 7] and renal ECM synthesis and degradation [3, 8, 9]. There is a close relationship between TGF-β and Ras signalling pathways: TGF-β1 overcomes Ras mitogenic effects [10–12], and Ras can counteract TGF-β signalling, altering the expression of TGF-β1 type II receptor [13]. On the other

* Corresponding author.

hand, renal Ras activation serves, at least in part, as an intracellular signal transduction pathway for molecules involved in obstructed kidney damage, such as TGF-β1 [14].

Ras proteins are small GTPases with two conformational states, a guanosine diphosphate (GDP)-bound inactive state and a guanosine triphosphate (GTP)-bound active state [15]. Ras proteins function as intracellular switches in signal transduction cascades that control a wide number of biological actions including proliferation and apoptosis in physiological conditions [16]. Cell proliferation and apoptosis also play relevant roles in the genesis and progression of renal fibrosis. The roles of members of the Ras superfamily such as Rho in renal fibrosis have been recently reviewed [17–19]. However, no reviews are available on the role of p21 Ras in chronic renal injury. Thus, the purpose of this manuscript is to review the most recent data on the role of Ras activation in fibrotic nephropathies. This has direct clinical implications, as in the last years an impressive arsenal of drugs devoted to interference with Ras pathways and its effectors has been developed for use in the treatment of cancer. It is possible that at least some of these drugs can also be used to block renal fibrosis and renal failure progression.

Ras proteins

The classical Ras proteins (p21 Ras: H-, K- and N-Ras), together with M-Ras, R-Ras (R-Ras, TC21, R-Ras 23), Rap (Rap 1A, Rap 1B, Rap 2A, Rap 2B) and Ral (Ral A, Ral B), are the prototype members of the Ras subfamily that is included in the Ras superfamily of small monomeric GTP-binding (G) proteins; this superfamily also includes the Rho, Ran, Rab, Rac, Rheb, Arf and Kir/ReM/Ras subfamilies [15]. p21 Ras proteins include three closely related members with a molecular mass of ~21 kDa: H-Ras (or Ha-Ras), K-Ras (or Ki-Ras) and N-Ras. H- and N-Ras result from the transcription of *Hras-1* and *Nras* genes, respectively; K-Ras occurs in two alternatively spliced forms, Ki(A)-Ras (or K-Ras4A) and Ki(B)-Ras (or K-Ras4B), deriving from *Kras-2* gene expression [15]. Due to their ability to modulate transcription, Ras proteins control cell growth and proliferation as well as other aspects of cell biology including senescence/cell cycle arrest, differentiation and survival.

Mechanisms of Ras activation. Many different extracellular signals induce Ras activation through binding to several transmembrane receptors, including protein tyrosine kinase receptors (PTKRs), G protein-coupled receptors (GPCRs) and integrins [15, 16]. PTKRs autophosphorylate tyrosine residues in their cytoplasmic domains, leading in many cases to tyrosine phosphorylation of other proteins and resulting in activation of Ras. GPCRs signal to membrane-localized heterotrimeric G proteins, which transform from the GDP (inactive) state to the biologically active GTP state following GDP/GTP exchange. Proline-rich tyrosine kinase 2 (PYK2), also termed calcium-dependent tyrosine kinase, is a non-receptor tyrosine kinase whose activation is Ca^{2+}- and/or PKC-dependent and is also induced by several GPCR agonists and cytokines; upon stimulation, PYK2 signals to mitogen-activated protein kinase (MAPK) family members such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) or p38 MAPK, depending on a given cell type [21]. GPCRs also induce transactivation of PTKRs, and PYK2 is a candidate to link GPCRs with PTKRs; activated PYK2 regulates ERK-dependent cascades through Ras activation [21–23].

As shown in Fig. 1, Ras proteins need post-translational modifications for their localization at the inner surface of the plasma membrane and their biological activity [24]. A lipid modification indispensable for functional anchorage of Ras proteins into the plasma membrane is the addition of a C-terminal prenyl group, either farnesyl or geranylgeranyl. The enzyme 3-hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) reductase, the rate-limiting step of cholesterol biosynthesis, is involved in the synthesis of prenyl groups. H-, N- and K-Ras4A undergo palmitoylation and traffic to the plasma membrane *via* the Golgi complex, but K-Ras4B is routed directly from the endoplasmic reticulum.

In its inactive state, Ras is bound to GDP; exchange of GDP for GTP causes a conformational change turning the molecular switch to the "on" position. Guanine nucleotide exchange factors (GEFs) enhance the extremely slow spontaneous GDP/GTP exchange reaction and promote the formation of the active Ras-GTP state. Intrinsic GTPase activity, which returns Ras to the GDP state, terminates Ras-GTPase signalling. The slow spontaneous hydrolysis rate of Ras-GTP is dramatically enhanced by interaction with GTPase-activating proteins (GAPs) (Fig. 2).

The embryonic lethality observed in the *K-ras*- [25, 26] but not *H-ras*-, *N-ras*- or double *H-ras*/*N-ras*-knockout (KO) mice [27] shows the distinct roles for each Ras protein. There is additional evidence for unique roles of H-, K-, and N-Ras: many tumours are associated with mutations in one Ras family member [28], and the levels of Ras mRNA in mice appear to be regulated both temporally and spatially, with certain tissues preferentially expressing one or more

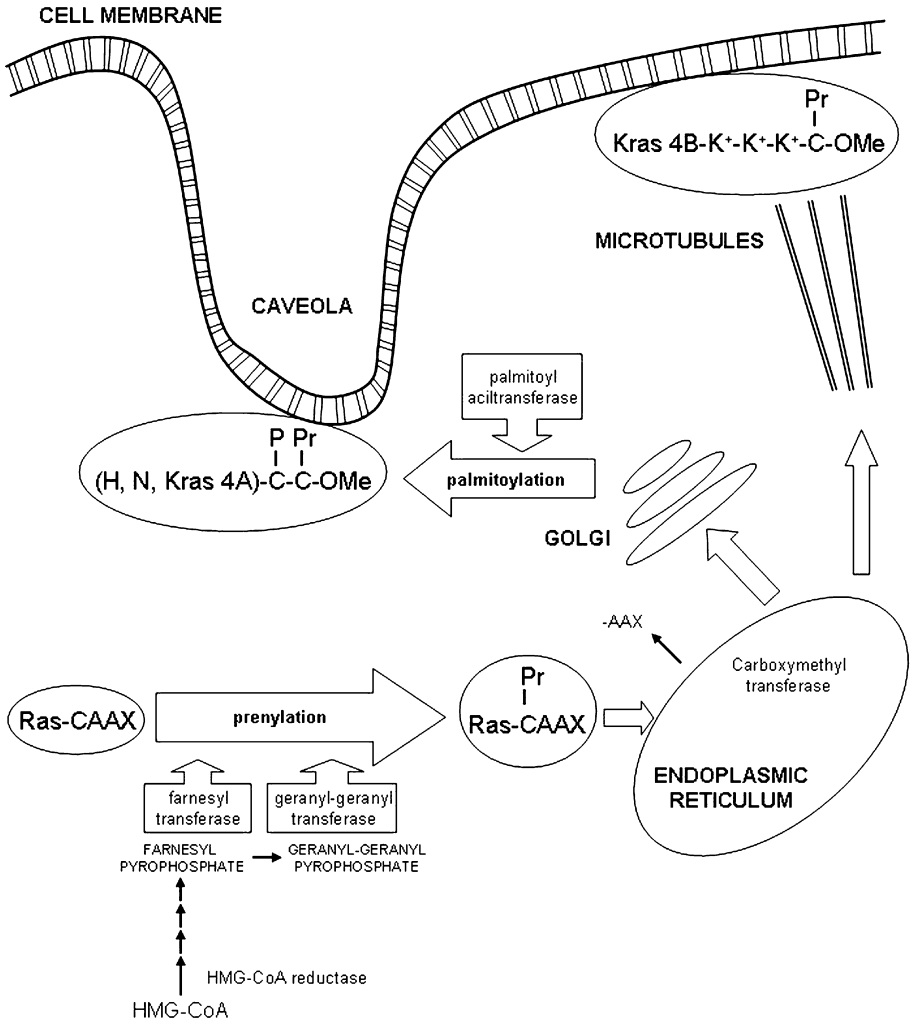

Figure 1. Ras post-translational modifications and trafficking to the plasma membrane. Farnesyl or geranylgeranyl transferases catalyze the addition of the C-terminal prenyl group; the rate-limiting step of the synthesis of prenyl groups is the enzyme HMG-CoA reductase. Enzymes associated with the endoplasmic reticulum catalyze the proteolytic removal of the AAX residues and carboxyl methylation of the now terminal prenylated cysteine residue. H-Ras, N-Ras and K-Ras4A undergo palmitoylation of cysteine residues, which completes their binding to the plasma membrane *via* the Golgi complex; palmitate modification might target Ras proteins to cholesterol-rich plasma membrane pits (caveola, lipid rafts). The polybasic domain (lysine-rich sequence) of K-Ras4B provides a second signal to complete its binding to the plasma membrane by an undefined pathway (from [16]) (A, aliphatic amino acid; C: cysteine; HMG-CoA, 3-hydroxy-3-methylglutaryl Coenzyme A; K, lysine; P, palmitoyl group; Pr, prenyl group; X, serine or methionine).

members of the family [29]. Few studies in the scientific literature show inhibition of Ras isoforms by interference RNA due to the partial inhibition observed of the targeted Ras isoforms and to the undesired unspecific inhibition of other isoforms. Antisense oligonucleotides against Ras isoforms seem to be more effective and specific in reducing the levels of their target mRNA and could be used for therapeutic intervention; oligonucleotides can be targeted to inhibit Ras isoforms in different cell types within the kidney, thereby increasing the specificity of the system and reducing unwanted side effects [30, 31].

Ras effectors. In their activated form, Ras proteins stimulate a wide group of downstream signalling pathways with a multitude of effector proteins. The protein kinase Raf [32], the lipid kinase phosphatidylinositol 3-kinase (PI3K) [33] and the GEF for the Ral subfamily Ral-GDS [34] are the most well known Ras effectors. Phosphoinositide-specific phospholipase Cε has also been identified as a novel class of Ras effector [35] (Fig. 2).

Raf/ERK signalling pathway. Active Ras (Ras-GTP) binds cytoplasmic members of the Raf family of serine/threonine kinases and translocates to the plasma membrane to become activated [36]. Specific Raf phosphorylation by cAMP-dependent protein kinase (PKA) [37, 38] and probably by Akt [39] counterbalances the activating phosphorylations by reducing Raf activity and/or modifying the ability of Raf to interact with Ras-GTP. Activated Raf phosphorylates the intermediate MAPK kinases MEK1 (MKK1) and MEK2 (MKK2), which in turn phosphorylate ERK1 (p44-MAPK or MAPK1) and ERK 2 (p42-MAPK or MAPK2), respectively. ERK phosphorylation promotes its homodimerization and translocation to the nucleus, stimulating the activity of different proteins by direct phosphorylation [40, 41]. It is unclear whether ERK1 and ERK 2 have different biological roles. ERK1/2 have several sub-

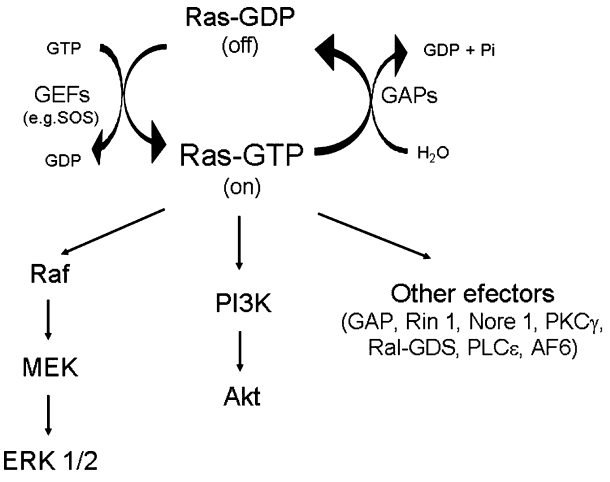

Figure 2. Ras activation and its effectors (ERK, extracellular signal regulated kinase; GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; GDP, guanosine diphosphate; GTP, guanosine triphosphate; MEK, mitogen-activated protein kinase kinase; PI3K, phosphatidylinositol 3-kinase; PKCγ, protein kinase C gamma; PLCε, phospholipase C epsilon; Ral-GDS, GEF for the Ral subfamily; SOS, Son of Sevenless).

strates including protein kinases and transcription factors such as Elk-1, which is involved in regulation of c-fos expression [42]. Other substrates include Ets 1, Sap1a, m-Myc, signal transducers and activators of transcription proteins (STAT), adapter proteins (Sos), enzymes (p90Rsk S6 kinase, phospholipase A_2), and cell surface and nuclear receptors such as epidermal growth factor (EGF) and estrogen receptors.

Elucidation of physiological consequences of ERK signalling has been facilitated by the availability of pharmacological inhibitors of ERK action, PD-98059 and U-0126. Both inhibitors appear to indirectly block ERK signalling through inhibition of its immediate upstream activators, MEK1 and MEK2. However, the specificity of these inhibitors is not absolute; PD-98059 is also a potent inhibitor of cyclooxygenase-1 and -2 [43]. A recently described highly MEK1-specific derivative of PD-98059, PD-184352, has been shown to be functional *in vivo*, effectively suppressing tumour growth [44].

PI3K-Akt signalling pathway. Other well-known effectors of Ras are the PI3K family of cytoplasmic lipid kinases. PI3K is recruited to the plasma membrane upon receptor stimulation, where it phosphorylates the D3 position of localized membrane phosphoinositides, generating phosphatidylinositol (3,4)-bisphosphate [PtdIns(3,4)P2] and phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P3] (Fig. 3). These inositol lipids act as second messengers by binding to and activating different target proteins involved in the regulation of cell growth, proliferation, survival, differentiation and cytoskeletal changes [33]. PI3K has to be localized in the membrane to allow its lipid kinase domain of the catalytic subunit to interact with Ras [33]. The localization of PI3K in the membrane allows its binding with either Ras-GTP (formed in a PTKR- or GPCR-dependent manner) or its membrane substrates, the phosphoinositides. Activated Ras interacts with the p110 catalytic subunit of PI3K to stimulate its lipid kinase activity. The second messengers PtdIns(3,4)P2 and PtdIns(3,4,5)P3, released by PI3K kinase activity, stimulate the phosphoinositide-dependent kinases PDK-1 and PDK-2, which then activate the serine-threonine kinase Akt (or protein kinase B, PKB) (Fig. 3). The Akt pathway controls different cellular processes such as promotion of cell survival and protein synthesis [16, 45, 46].

Once activated, Akt phosphorylates numerous targets in the cytosol, where it interacts with chaperone proteins including heat shock protein 90 (Hsp90), APPL and others [47]. Activated Akt is also transported to the nucleus where it phosphorylates target proteins [48].

Much research has been performed looking for inhibitors of the PI3K/Akt pathway [49]. Class I PI3K inhibitors such as wortmannin and LY294002 compete at the ATP-binding site of the lipid kinase catalytic domain of all PI3Ks. However, these inhibitors have not been developed for clinical use because of the broad specificity of kinase inhibition, poor pharmacokinetics and relatively weak inhibition [50]. Moreover, the recent finding that LY294002 binds and inhibits the activity of Pim-1 suggests that LY294002 can no longer be considered a selective tool to study PI3K-dependent biology. IC87114 is a p110δ-specific PI3K inhibitor that has been shown to prevent anaphylaxis in a mouse model [51]. Development of a specific inhibitor of Akt has proven difficult because of toxicity. More recently, API-2, also known as triciribine, was shown to be a non-isoform-specific Akt inhibitor at much lower doses than previously tested [52]. Another Akt inhibitor that has now entered phase II trials is the orally bioavailable alkylphospholipid perifosine, which interferes with proper Akt membrane localization, leading to Akt dephosphorylation [53].

Ras expression in the kidney

Almost 20 years ago, Barbacid [20] described the expression of Ras proteins in normal tissue. In human kidney, Ras was detected in glomerular cells, proximal convoluted tubule (PCT) cells, distal convoluted tubule (DCT) cells, cortical collecting tubule (CCT) cells, medullar collecting duct (MCD) cells, interstitial

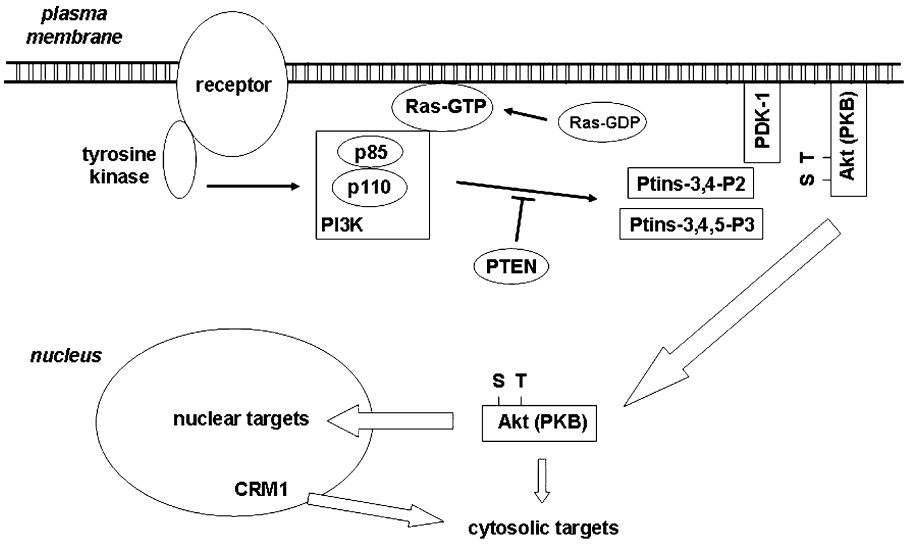

Figure 3. Akt activation by phosphatidylinositol 3-kinase (PI3K) and transport to the nucleus. PI3K is a heterodimer of regulatory (p85) and catalytic (p110) subunits and is recruited to the plasma membrane after receptor stimulation, where it generates phosphatidylinositol (3,4)-bisphosphate and phosphatidylinositol (3,4,5)-trisphosphate; these lipids stimulate phosphoinositide-dependent kinases, which then activate the serine-threonine kinase Akt (or protein kinase B). PTEN, with phosphatase activity, reduces subsequent Akt activation. Activated Akt is transported to the nucleus where it phosphorylates target proteins and is also exported from the nucleus *via* interactions with CRM1 at a nuclear export sequence localized in its kinase domain (CRM1, nuclear export receptor; PDK-1, phosphoinositide-dependent kinase-1; PKB, protein kinase B, Akt; PTEN, phosphatase and tensin homolog deleted on chromosome ten; Ptins-3,4-P2, phosphatidylinositol (3,4)-bisphosphate; Ptins-3,4,5-P3, phosphatidylinositol (3,4,5)-trisphosphate; S, T, serine-threonine kinase).

cells and subcapsular fibroblasts (SCF) [54]. These authors also showed cell-specific expression of the three Ras isoforms. Mesangial cells stained positively for H-Ras and K-Ras but not for N-Ras, although we have observed the presence of N-Ras in cultured human mesangial cells by both Western blot and PCR, which show a higher sensitivity to detect these isoforms than the immunohistochemistry analysis (Martinez-Salgado et al., unpublished observations). Ras expression was not detected in podocytes. PCT cells express all Ras isoforms, with predominant expression of K-Ras. Predominant expression of N-Ras and slight expression of K-Ras and H-Ras was shown in DCT cells. N-Ras was not expressed in the SCF cytoplasm, and it was also not detected in interstitial cells. In the renal medulla, MCD cells showed predominant expression of K-Ras (in the cell plasma membrane), with slight expression of the other isoforms. CCT cells showed slight expression of the three isoforms. The study of Kocher et al. [54] also showed a characteristic brush border distribution for K-Ras but not for H- or N-Ras. PCT cells showed a marked and distinctive brush border staining for K-Ras when compared with DCT cells. Similarly, marked plasma membrane staining for K-Ras was found in MCD cells, with no staining observed for the other isoforms.

Our group also performed immunohistochemical studies to detect the expression of Ras isoforms in murine renal tissue [55]. In contrast with the results reported by Kocher et al. in human renal tissue [54], all three Ras isoforms were found to be absent in tubular epithelial cells from kidneys of normal mice along all the segments of the nephron (Fig. 4). H-Ras and N-Ras but not K-Ras expression was detected in glomerular endothelial cells. Cortical interstitial cells stained for K-Ras and N-Ras but not for H-Ras, with the same pattern of expression found in medullary interstitial cells. H-Ras, N-Ras and K-Ras were detected in medullary endothelial cells. The differences between our results and those of Kocher et al. [54] may be explained by the use of renal tissues from different species, the efficiency of the Ras isoform antibodies or differences in tissue processing. The expression patterns of Ras isoforms in human and murine renal kidney are summarized in Table 1.

Role of Ras in renal cell proliferation and extracellular matrix synthesis *in vitro*

ECM synthesis and proliferation of interstitial fibroblasts, glomerular mesangial cells and tubular epithelial cells play essential roles in the development of renal fibrosis [56–58].

Fibroblasts. Therapy of fibroproliferative diseases has largely been relegated to blocking inflammation. However, despite the excellent anti-inflammatory

Table 1. Expression patterns of Ras isoforms in human and murine renal kidney.

	Human kidney			Murine kidney		
	H-Ras	K-Ras	N-Ras	H-Ras	K-Ras	N-Ras
glomerular endothelial cells	+	+	+	+	–	+
mesangial cells	+	+	+	?	?	?
podocytes	–	–	–	?	?	?
PCT cells	+	+	+	–	–	–
DCT cells	+	+	+	–	–	–
CCT cells	+	+	+	–	–	–
MCD cells	+	+	+	?	?	?
medullary endothelial cells	?	?	?	+	+	+
medullary interstitial cells	?	?	?	–	+	+
cortical interstitial cells	?	?	?	–	+	+
SCF	+	+	–	?	?	?

CCT, cortical collecting tubule; DCT, distal convoluted tubule; MCD, medullar collecting duct; PCT, proximal convoluted tubule; SCF, subcapsular fibroblasts; +, expression; –, absent; ?, not described in studies reported to date.

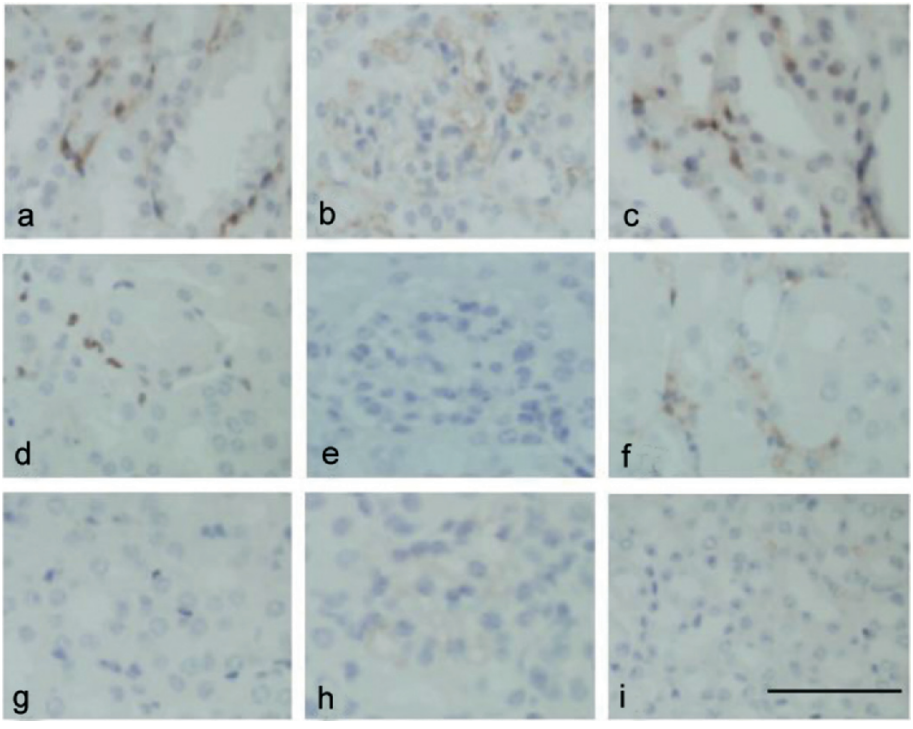

Figure 4. Immunohistochemistry of Ras isoforms in kidneys from normal C57BL/6J mice. (*a–c*) Representative images of N-Ras immunostaining in cortical tubular areas, glomeruli and medullary areas, respectively. (*d–f*) Representative K-Ras immunostaining in cortical tubular areas, glomeruli and medullary areas, respectively. (*g–i*) H-Ras immunochemistry in cortical tubular areas, glomeruli and medullary areas, respectively (black bar: 200 microns; reproduced from [55] with permission from SIN ITALIA).

efficacy of the agents used, clinical benefit is limited, suggesting that therapy specifically targeting fibroblast activation is essential. Fibroblast activation leads to a myofibroblast phenotype, cell proliferation and an increase in ECM deposition; thus, renal fibroblast proliferation is involved in the pathophysiology of interstitial fibrosis. Different growth factors (*e.g.* EGF), cytokines and integrins involved in proliferation also activate intracellular signalling pathways that converge on Ras proteins [59]. EGF receptor is present on fibroblasts [60], and Buday and Downward [61] showed that EGF treatment induces rapid activation of Ras proteins in rat fibroblasts. In a primate renal fibroblast cell line, K-Ras was the predominantly expressed isoform and was involved in EGF- and serum-stimulated proliferation [62]. These authors also detected mRNA for H-Ras, K-Ras4B and N-Ras but not for K-Ras4A and showed that K-Ras was the predominantly expressed isoform ($>95\%$ of total Ras protein), with N- and H-Ras detected in small amounts [31]; they also showed different roles for K- and H-Ras in the control of proliferation.

TGF-β1 is a pro-fibrotic cytokine that is capable of inducing expression of ECM proteins as collagen, fibronectin, tenascin and laminin [63–65] as well as inhibiting production of ECM proteases and inducing inhibitors of ECM proteases [66]. It was recently shown that Ras and TGF-β signalling mediate antagonistic effects on ECM gene expression in fibroblasts. Using gene expression profiling of 3T3 fibroblasts transformed by Ras and Raf, Wisdom et al. [67] identified the down-regulation of ECM gene expression as a common target of oncogenic signalling through the Ras/Raf/ERK pathway; the list of repressed genes after Ras-fibroblast transformation includes a number of genes that encode ECM components, including lysil oxidase, OSF-2, fibronectin and several collagen isoforms, showing an important role for reduced ECM synthesis in generating the transformed state and suggesting that there is substantial overlap between the repression program elicited by Ras and Raf and the panel of transcripts induced by TGF-β. In particular, TGF-β was able to reverse many of the changes in ECM gene expression that are characteristic of Ras transformation. Moreover, among the genes repressed by Ras transformation, 21% were induced by TGF-β treatment, again showing that the antagonistic regulation of gene expression by TGF-β treatment and Ras transformation is substantially higher than the level expected just by chance. These authors also suggested that Ras oncogenic transformation and TGF-β counterregulate ECM gene expression by controlling trans-acting factors different from Smads or AP-1. The observation that TGF-β can reverse many of the transcriptional changes and phenotypic traits that are elicited by Ras oncogenic transformation is broadly consistent with genetic evidence that many components of the TGF-β signalling pathway are tumour suppressors.

Recent data from our laboratory show that H- and N-*ras* KO fibroblasts (H-$ras^{-/-}$/N-$ras^{-/-}$) have a higher ECM accumulation and a higher Akt activation than heterozygote fibroblasts (H-$ras^{+/-}$/N-$ras^{+/-}$) used as controls [68]. Akt activation may be related to the increases in ECM synthesis, because PI3K inhibition decreased fibronectin and collagen type 1 expression [67]. PI3K/Akt activation is involved in increases in TGF-β-induced collagen mRNA synthesis in the lung [69] and in PDGF-induced collagen synthesis in cultured fibroblasts [70].

The MEK/ERK pathway can also play a role in TGF-β-mediated ECM synthesis. This pathway can have different effects depending on the cell type and conditions [71–75]. It has been described that ERK1/2 inhibits collagen type I synthesis in human skin fibroblasts after mitogenic stimulations [76], and it was recently shown that a lack of H- and N-Ras isoforms induces a reduction in ERK activation together with the observed increases in fibronectin and collagen expression [68]. Stratton et al. [77] showed that activation of the Ras/MEK/ERK pathway is required for the induction of connective tissue growth factor (CTGF) by TGF-β2 and that the *in vitro* and *in vivo* antifibrotic effects of prostacyclin derivatives were due to inhibition of the Ras/MEK/ERK pathway, suggesting that specific inhibition of this pathway in fibroblasts might prevent fibrosis while leaving other physiological effects of TGF-β unaffected.

Several authors have demonstrated that TGF-β stimulates proliferation of fibroblasts [78–80] and mesangial cells [7]. We have observed that cell proliferation and proliferating cell nuclear antigen (PCNA) expression in response to TGF-β1 are diminished in H-$ras^{-/-}$/N-$ras^{-/-}$ fibroblasts. This reduction may be in part related to the lower ERK phosphorylation observed in H-$ras^{-/-}$/N-$ras^{-/-}$ fibroblasts compared with control cells [79]. MEK1/2–ERK1/2 pathway activation is capable of promoting different – and sometimes conflicting – cellular processes [81]. It has been described that elevated ERK activation can induce cell cycle stop together with senescence, apoptosis and differentiation processes, whereas a slow but sustained level of activation is a common characteristic of proliferative cells in many systems [82–85]. Moreover, it was recently demonstrated in fibroblasts that insulin growth factor-1-induced proliferation is regulated, at least in part, by the MEK-ERK pathway [86]. ERK activation has also been linked to increases in cultured renal cell proliferation [87]. The proliferative effect of TGF-β1 was not observed when H-$ras^{-/-}$/N-$ras^{-/-}$ fibroblasts were treated with U0126, an inhibitor of ERK1/2 phosphorylation. These data confirm that ERK1/2 pathway activation is involved in the regulation of TGF-β-induced proliferation, because when its activation is reduced in H-$ras^{-/-}$/N-$ras^{-/-}$ fibroblasts, proliferation is also reduced with respect to controls. Moreover, these data suggest that H- and N-Ras isoforms are involved, at least in part, in ERK activation and stimulation of proliferation induced by TGF-β1, because in the absence of these isoforms, there is a clear reduction in TGF-β1-induced proliferation and ERK1/2 phosphorylation [68].

Mesangial cells. Accumulation of ECM proteins in glomerular basement membrane and glomerular spaces is the common end-stage in glomerulosclerosis induced by hypertension, diabetes or intrinsic primary diseases. Renal mesangial cells are largely responsible of this deposition of glomerular matrix, and their proliferation rate is known to be increased under

pathological conditions. The loss of renal filtration function is a result of mesangial cell hypertrophy and glomerular fibrosis [88]. TGF-β also plays a major role in the hypertrophic and fibrogenic response of mesangial cells in glomerular diseases and in diabetic nephropathy [8]. High glucose, protein glycation products, angiotensin II, endothelin-1 and platelet-derived growth factor (PDGF) amplify the synthesis of TGF-β and/or the expression of its receptors in the diabetic state [88]; moreover, the effect induced by TGF-β1 in mesangial cells is due, at least in part, to EGF receptor transactivation [89]. Different agonists activate Ras in cultured mesangial cells, including lysophosphatidylcholine [90], endothelin-1 [91, 92] and PDGF [93]. Exposure of mesangial cells to high glucose levels increases mesangial cell proliferation and membrane-associated Ras and Rho GTPase protein expression [94]. H-Ras and K-Ras appear to be required for cell cycle progression and proliferation in human mesangial cells [95]. Co-treatment of mesangial cells with simvastatin, an HMG-CoA reductase inhibitor that prevents prenyl group synthesis, reversed high glucose-induced Ras and Rho membrane translocation and inhibited high glucose-induced mesangial cell proliferation. Khwaja et al. [96] showed that the Ras inhibitor S-trans, trans-farnesylthiosalicylic acid (FTS) inhibits human mesangial cell proliferation, and this effect appeared to be mediated by inhibitory effects on Ras-dependent signalling that occur as a result of the dislodgment of Ras from its membrane-anchorage sites by FTS. Advanced glycation end-products (AGEs) are derived from a non-enzymatic reaction between reducing sugars and proteins or lipids; elevated concentrations have been found in inflammatory diseases and in diabetes mellitus. Elevated formation of AGEs participates in diabetes-related complications such as peripheral vascular disease [97]. Administration of AGEs to cultured glomerular endothelial cells leads to increased autocrine production and release of TGF-β that may stimulate matrix protein production in surrounding mesangial cells [98], and Ras modulation of superoxides mediates high glucose- and AGE-induced TGF-β1 and fibronectin expression in mesangial cells [99]. Diabetic mice overexpressing AGE receptors develop a fast renal disease that is reversed by AGE inhibitors [100]. In cultured mesangial cells, AGEs stimulate ERK phosphorylation mediated by activated K-Ras, but not H-Ras, in a PI3K-dependent manner [101]; PI3K participates in most Ras signalling pathways [102], and MAPK and PI3K effectors appear to function largely in parallel to execute cellular signalling programs.

It has been described that ERK1/2 inhibits collagen type I synthesis in human mesangial cells after mitogenic stimulation [76]. However, other authors have described that an ERK inhibitor or dominant-negative ERK1 block TGF-β-induced collagen synthesis in human mesangial cells, indicating that this pathway is involved in TGF-β-mediated collagen type I transcription [74]. Moreover, *in vitro* studies with renal cells have linked ERK activation to excessive fibronectin synthesis [87], proliferation and trans-differentiation [103]. In rat mesangial cells, ERK pathway activation is associated with fibronectin accumulation [104], and Ras induction of superoxide was previously described to activate ERK-dependent fibronectin expression in rat mesangial cells [99]. A down-regulatory role of ERK1/2 in the modulation of MMP-2 activity was recently described: blockade of ERK phosphorylation promotes pro-MMP2 and membrane type 1-MMP activation and reduces the production of tissue inhibitor of metalloproteinase-2 [105]. CTGF plays a fundamental role in the development of tissue fibrosis by stimulating matrix deposition and mediating many of the pro-fibrotic effects of TGF-β1. Chen et al. [106] have shown that the induction of CTGF by TGF-β in kidney mesangial cells also requires Ras/MEK/ERK, suggesting that Ras/MEK/ERK may be generally involved in CTGF gene regulation.

Abnormalities in lipid and lipoprotein metabolism are commonly observed in patients with chronic renal disease. Specifically, hyperlipidemia and the glomerular deposition of atherogenic lipoproteins (*e.g.* low density lipoprotein/LDL and its oxidized variants) are implicated in key pathobiological processes involved in the development of glomerular disease, including stimulation of monocyte infiltration into the mesangial space, mesangial cell hypercellularity and mesangial ECM deposition [107]. Generally, the mitogenic intracellular signalling pathways are regulated by the activation of a series of transmembrane and cytoplasmic protein tyrosine kinases that converge into the activation of Ras and downstream MAPK. Studies from Kamanna [108] in glomerular mesangial cells showed that the accumulation of LDL and, more potently, its oxidized forms within the glomerulus through the activation of membrane receptor tyrosine kinases (*e.g.* EGF receptor) activates Ras and the MAPK signalling cascade, leading to DNA synthesis and subsequent mesangial cell proliferation and matrix deposition, thus providing further evidence for the role of Ras activation in chronic glomerular disease.

Tubular epithelial cells. As discussed previously, CTGF plays a relevant role in the development of tissue fibrosis due to its stimulation of matrix deposition and mediation of many pro-fibrotic effects

induced by TGF-β. Induction of CTGF by TGF-β in renal proximal tubule epithelial cells (PTECs) is likely to play an important role in the development of tubulointerstitial fibrosis [109]: TGF-β1 induced CTGF promoter activity, mRNA and protein in human PTECs, and TGF-β1-dependent CTGF promoter activity was reduced by inhibiting Ras and MEK activation. MEK inhibition also resulted in inhibition of the TGF-β1-induced secreted CTGF protein. These data show that TGF-β1-induced expression of CTGF in human PTECs is dependent on Ras/MEK/ERK [109] and suggest that inhibition of TGF-β-induced CTGF by targeting Ras/MEK/ERK signalling pathways could be of therapeutic value in renal fibrosis.

ERK1 and ERK2, the MKKKs, Raf-1, the MKKs/MEKs and the ERK effector p90 S6 kinase (Rsk) are expressed in all nephron segments [110]. Repeated tubular ischemia plays a major role in chronic renal fibrosis [111]. Renal ischemia *in vivo* induces ERK activation [112, 113], and this activation has been implicated as a modulator of ischemia/reperfusion injury in the kidney [114, 115]. The PI3K/Akt pathway has also been reported to be activated by ischemia/reperfusion in various organs, including the kidney [114, 116–118], and after mechanical injury in renal proximal tubular cells [119]. Kwon et al. [120] observed that both ERK and Akt are activated by hypoxia/reoxygenation (H/R) in renal epithelial cells; their results suggest that H/R induces activation of MEK/ERK and PI3K/Akt survival signalling pathways through the reactive oxygen species-dependent EGFR/Ras/Raf cascade. These signals were associated with proliferation of epithelial cells during the early time of reperfusion after hypoxia. Arany et al. [121] showed recently that mouse proximal tubular epithelial cell survival depends on sustained activation of the Ras effector ERK, and lack of this activation was associated with death during oxidative stress after H/R. Thus, it can be deduced that activation of these kinases may be involved in the repair process during ischemia/reperfusion and may also play a role in control of cell proliferation/apoptosis during the progression of chronic renal damage.

Epithelial-mesenchymal transition (EMT) of tubular epithelial cells is an important mechanism involved in tubulointerstitial fibrosis [122]. EMT is characterized by loss of epithelial cell characteristics and gain of ECM-producing myofibroblast characteristics. EMT is an orchestrated, highly regulated process involving four key steps: (1) loss of epithelial cell adhesion, (2) *de novo* α-smooth muscle actin (α-SMA) expression and actin reorganization, (3) disruption of tubular basement membrane and (4) enhanced cell migration and invasion into the interstitium [123]. Tubular EMT can be induced by TGF-β1 [124], advanced-glycation end-products [125] and angiotensin II [126], all agonists that activate ERK1/2, as previously described. However, TGF-β1 is probably the key inducer of EMT, because TGF-β1 signalling is sufficient to induce EMT in cultured epithelial cells [124]. The intracellular signalling responsible for TGF-β-induced EMT includes ERK1/2 activation [127, 128], p38 MAPK [129] and JNK [130]. ERK activation seems to be a necessary step in the induction of EMT [131, 132].

Ras activation in *in vivo* models of experimental fibrosis

Mesangial cells and renal fibroblasts express activated Ras induced by proliferative signals such as EGF and AGEs, suggesting a role for Ras as a mediator of renal fibrosis. Angiotensin II [133], TGF-β1 [14], PDGF [134], EGF [135], endothelin-1 [136] or thrombin [137] can activate Ras and are upregulated in human renal fibrosis as well as in several models of experimental renal fibrosis, where they play a major role in the genesis of renal damage [138–143]. Thus, renal Ras activation serves, at least in part, as an intracellular signal transduction point for the effects of molecules involved in obstructed kidney damage.

Tubulointerstitial fibrosis. Studies from our laboratory have shown a marked increase in renal Ras activation in a model of tubulointerstitial fibrosis induced by 3 days of unilateral ureteral obstruction (UUO) in C57BL/6J mice [55]. Fibronectin immunostaining confirmed an increased fibrotic interstitium in obstructed kidneys (ligated, L) when compared with non-obstructed (non-ligated, NL) kidneys. N-Ras and K-Ras staining were detected in NL kidneys, but their expression was dramatically increased in UUO. In L kidneys, H-Ras staining intensity was notably lower than in NL kidneys [55]. Ras activation was accompanied by increases in phosphorylated forms of the Ras effectors ERK1/2 and Akt [144]. Renal ERK activation has also been reported after UUO in rats [145], and it has been related to tubular proliferation in the obstructed kidney [146]. Treatment with the MEK inhibitor U0126 induced a 50% decrease in the number of proliferating tubular cells in L kidneys when compared with control mice (Rodriguez-Peña and Lopez-Novoa, unpublished results). These data suggest a role for the renal ERK1/2 signalling pathway in early obstruction-induced tubular cell proliferation. ERK activation has been related to proliferative responses

in rats with experimental glomerulonephritis [147, 148].

Ureteral ligation induces a marked up-regulation of both activation and expression of Akt (Rodriguez-Peña and Lopez-Novoa, unpublished data). Tubular apoptosis constitutes a typical feature of renal damage in obstructed kidney [149], and pAkt localization may be associated with PI3K/Akt-mediated survival mechanisms [150]. Further support for this hypothesis is given by the fact that dilated tubules of L kidneys presented reduced activity of Akt and increased staining for activated caspase-3, indicative of apoptosis. Moreover, *in vivo* inhibition of PI3K-Akt resulted in increased obstruction-induced damage of altered tubules (Rodriguez-Peña and Lopez-Novoa, unpublished data), and Akt up-regulation prevented apoptosis in proximal tubular cells [151]. Taking all these data together, it could be suggested that Akt activation plays an important role in protecting against obstructive injury by contributing to survival signalling in renal tubules of obstructed kidney. Figure 5 summarizes the involvement of Ras activation in experimental fibrosis.

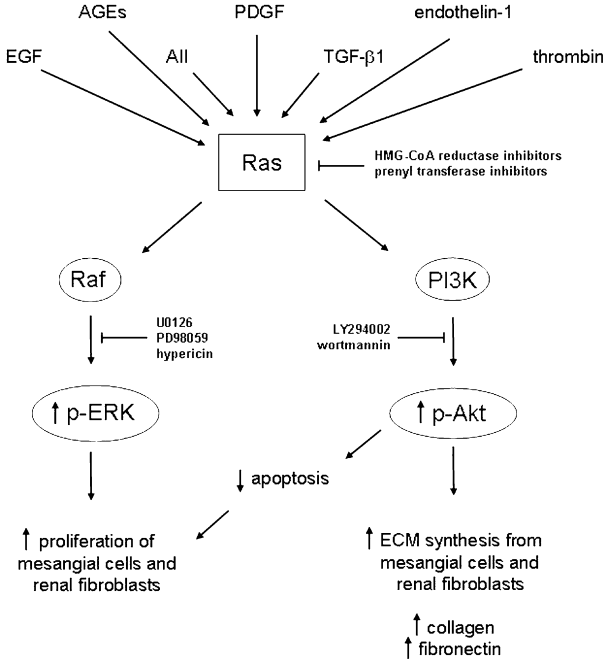

Figure 5. Involvement of Ras activation in experimental fibrosis (AGEs, advanced glycation end-products; AII, angiotensin II; ECM, extracellular matrix; EGF, epithelial growth factor; PDGF, platelet-derived growth factor; p-Akt, phosphorylated form of Akt; p-ERK, phosphorylated form of ERK; TGF-β1, transforming growth factor-β1).

Glomerular damage. In an experimental model of rat anti-Thy1 nephritis, Clarke et al. [30] have shown that Ras inhibition with FTS reduces glomerular cellular proliferation. Moreover, inhibition of ERK resulted in amelioration of the renal damage in a rat model of experimental mesangioproliferative glomerulonephritis by suppressing the proliferation of mesangial cells [152]. In addition, ERK inhibition slowed the progression of renal disease in DBA/2-pcy/pcy (pcy) mice, a murine model of polycystic kidney disease [153].

Akt phosphorylation has also been described in a rat model of anti-Thy1 nephritis [154] as well as in diabetic rats [155, 156]. Akt activation is observed in remnant kidneys in rats after 5/6th nephrectomy, a model of glomerulosclerosis [157]. In this model, the protective effect of an erythropoietin analogue on renal structure and function is mediated by activation of the PI3K/Akt pathway in endothelial cells [158].

Tubular damage. Administration of the MEK inhibitor U0126 is able to partially protect the kidney against tubular damage induced by cisplatin [159]. In addition, increased ERK1/2 activation has been observed in the kidneys of cadmium-intoxicated rats, probably as a consequence of Ras activation [160].

Phosphorylation of Akt was increased after ischemia/reperfusion in the mouse kidney and reduced by wortmannin administration. Renal cell proliferation, which increased after ischemia/reperfusion injury in mouse, was inhibited by wortmannin, suggesting that activation of the PI3K/Akt signalling pathway maintains cell viability after ischemia, thus playing an important role in the regulation of renal repair after ischemia/reperfusion injury [161]. Treatment with erythropoietin partially prevents renal damage by preventing epithelial cell apoptosis. The antiapoptotic effects of erythropoietin were dependent on JAK2 signalling and the phosphorylation of Akt by PI3K [162].

Conclusion and perspectives

Activation of Ras and its effectors ERK and/or Akt may mediate certain pathological effects of the molecules involved in renal fibrogenesis. Pharmacological inhibitors of the Ras signalling pathway or its effector cascades, which have been extensively studied as potential drugs in cancer therapy, may represent a new therapeutic approach to prevent or even revert the progression of fibrosis and the consequent end-stage renal failure (Fig. 5). Ras prenylation can be inhibited by prenyl transferase inhibitors, which were designed to interfere with the signal transduction of cancer cells containing Ras gene mutations [163]. Farnesyl transferase inhibitors were designed to prevent farnesylation of Ras and other intracellular proteins, which has a protective effect on malignant

cell proliferation and survival [163]. Prenylation blockade seems to be an important mechanism by which statins (hydroxyl-3-methylglutaryl coenzyme A reductase inhibitors) exert antiproliferative and anti-inflammatory effects [164]. Administration of statins in experimental disease reduces glomerular injury and atherosclerosis [165, 166], showing the relevance of prenylation in the development of progressive renal injury. Prenylation inhibitors show potential for use in the treatment of renal disease, as described previously by several authors [167, 168]. Preliminary data from our laboratory show that both statins and farnesyl transferase inhibitors reduce interstitial fibrosis in an experimental model of tubulointerstitial renal disease (Rodriguez-Peña and Lopez-Novoa, unpublished data).

Another functional Ras antagonist, FTS, impairs Ras-membrane interactions, dislodges the protein from its anchorage domains and facilitates its degradation, thus reducing cellular Ras content and Ras activation [169, 170]. FTS has been shown to decrease inflammation and fibrosis in experimentally induced liver cirrhosis in rats [171]. As described above, the antiproliferative effect of FTS in mesangial cells *in vitro* [96] and the beneficial effects of FTS in an experimental model of Thy-1 nephritis [30] suggest that it may be a valuable therapeutic in mesangioproliferative renal disease and in proliferative nephritis. As previously reported, many chemicals have been developed to block the MEK/ERK and PI3K/Akt pathways. Sorafenib (previously known as BAY 43-9006), a multikinase inhibitor designed to target the Raf/MEK/ERK pathway, has been approved for use in the treatment of renal cell carcinoma [172]. Although the effects of many inhibitors have been well tested in cellular models of renal disease or even in animal models of the disease, their application to humans is still in the first phases of development; much basic and clinic research is necessary before these drugs can be used for effective therapy of chronic renal disease. The sustained activation of ERK and Akt may be involved in the repair processes during ischemia/reperfusion or during renal damage progression; for these reasons, inhibition of Ras and its signalling pathways may be therapeutic, but in some pathophysiological circumstances it may also hinder the repair processes. Furthermore, supporting data from large clinical trials on the use of these drugs for treating chronic renal disease are required before they can be used in the clinical setting.

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