

Angiotensin II and EGF receptor cross-talk in chronic kidney diseases: a new therapeutic approach

Alexandre Lautrette¹, Shunqiang Li², Rohia Alili¹, Susan W Sunnarborg², Martine Burtin¹, David C Lee², Gérard Friedlander¹ & Fabiola Terzi¹

Mechanisms of progression of chronic renal diseases, a major healthcare burden, are poorly understood. Angiotensin II (AngII), the major renin-angiotensin system effector, is known to be involved in renal deterioration, but the molecular pathways are still unknown. Here, we show that mice overexpressing a dominant negative isoform of epidermal growth factor receptor (EGFR) were protected from renal lesions during chronic AngII infusion. Transforming growth factor- α (TGF- α) and its sheddase, TACE (also known as ADAM17), were induced by AngII treatment, TACE was redistributed to apical membranes and EGFR was phosphorylated. AngII-induced lesions were substantially reduced in mice lacking TGF- α or in mice given a specific TACE inhibitor. Pharmacologic inhibition of AngII prevented TGF- α and TACE accumulation as well as renal lesions after nephron reduction. These findings indicate a crucial role for AngII-dependent EGFR transactivation in renal deterioration and identify in TACE inhibitors a new therapeutic strategy for preventing progression of chronic renal diseases.

Regardless of etiology, most human kidney diseases are characterized by an initial injury, followed by progression of renal lesions to complete parenchymal destruction and end-stage renal failure¹. Clinical and experimental studies have shown that angiotensin II (AngII), the major renin-angiotensin system effector, has an important role in the biological process leading to renal deterioration. Indeed, pharmacological inhibition of the renin-angiotensin system attenuates development of renal lesions in several experimental models of renal injury² and retards progressive loss of renal function in individuals with chronic kidney disease (CKD)³. Conversely, individuals with genetic variants associated with higher renin-angiotensin system activity are at increased risk for progression of chronic renal failure⁴. It has been suggested that AngII causes renal injury through renal hemodynamic effects and stimulation of kidney growth and matrix deposition⁵, but the molecular pathways underlying these phenomena remain largely unidentified.

AngII acts on at least two structurally and pharmacologically distinct G-protein-coupled receptors (GPCRs), AT₁ and AT₂ (ref. 6). Renal cells predominantly express AT₁ receptors, which mediate the majority of known AngII actions⁷. AT₁ receptors activate Gq-phospholipase C to generate inositol triphosphate and diacylglycerol, thereby increasing intracellular calcium and stimulating protein kinase C⁸. Additionally, activation of AT₁ receptors promotes tyrosine phosphorylation and stimulates mitogen-activated protein kinases and proliferation^{9,10}. How AT₁ receptors, which lack intrinsic tyrosine kinase activity, induce these events is unclear, but recent evidence suggests that 'transactivation' of the epidermal growth factor receptor (EGFR) is involved¹¹, and may require several intermediary signaling molecules including

Ca²⁺, protein kinase C and cytosolic tyrosine kinases⁹. Recently, it has been shown that metalloprotease-dependent release of EGFR ligands from cells is also involved in GPCR-induced EGFR transactivation¹². Whether and by which molecular mechanisms AngII transactivates EGFR in renal cells during kidney diseases is unknown.

EGFR binds members of a family of growth factors, comprised of EGF, transforming growth factor- α (TGF- α), heparin-binding EGF-like growth factor, amphiregulin, epiregulin, betacellulin and epigen¹³. All family members are synthesized as membrane-anchored precursors that can be processed by specific metalloproteases to release soluble bioactive factors from the cell surface¹³. In the kidney, both EGFR and its ligands are abundantly expressed along the nephron, suggesting a paracrine-autocrine system^{14,15}. Addition of EGFR ligands to tubular cells promotes several biological responses *in vitro*, including cell proliferation¹⁶, mesenchymal-epithelial transdifferentiation¹⁷ and collagen production¹⁸. *In vivo*, activation of EGFR is thought to be involved in the evolution of renal diseases¹⁹. Overexpression of the EGFR-related c-erb-B2 receptor induces tubular hyperplasia and the development of renal cysts in transgenic mice²⁰. Conversely, expression of a dominant negative isoform of EGFR in proximal tubules inhibits tubular cell proliferation and interstitial collagen accumulation, leading to reduced renal lesions after nephron reduction²¹. Other genetic and pharmacological approaches have confirmed the role of EGFR in tubular cyst formation and cell proliferation in polycystic kidney diseases^{22,23}, and in renal fibrosis in an experimental model of hypertension²⁴. Collectively, these results suggest that EGFR could be a major determinant in the development of renal lesions, possibly through enhanced cell proliferation and matrix deposition.

¹INSERM U426, Hôpital Necker Enfants Malades, IFR 94, Université Paris 5, 149 Rue de Sèvres, 75015 Paris, France. ²Department of Biochemistry & Biophysics, 405 Mary Ellen Jones Building UNC School of Medicine, Chapel Hill, North Carolina, 27599-7260, USA. Correspondence should be addressed to F.T. (terzi@necker.fr).

Published online 24 July 2005; doi:10.1038/nm1275

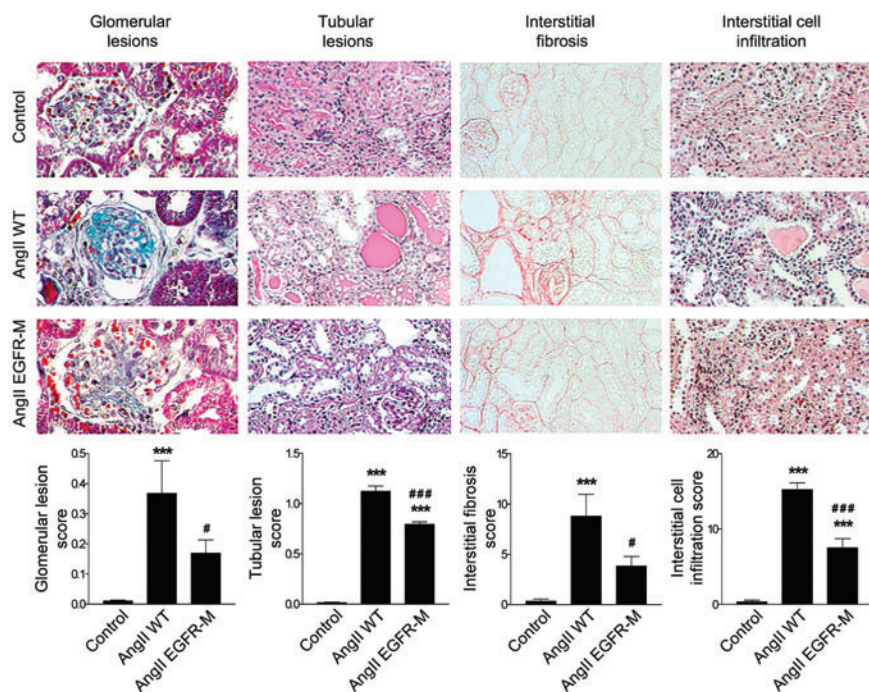


Figure 1 Overexpression of a dominant negative isoform of EGFR prevents lesion development in AngII-treated mice. Morphology and lesion scores of kidneys from control (upper panels), AngII-treated wild-type (middle panels) and AngII-treated transgenic EGFR-M (lower panels) mice (original magnification, $\times 200$). Because no differences were detected between wild-type and transgenic control mice, only one group is shown. Data are means \pm s.e.m.; $n = 5-6$ for each experimental group. ANOVA, followed by Tukey-Kramer test. AngII versus control: *** $P < 0.001$; EGFR-M versus wild-type mice: # $P < 0.05$, ### $P < 0.001$.

We hypothesized that EGFR transactivation is crucial to the lesion-promoting effects of AngII in the kidney. Using transgenic mice and pharmacological inhibitors, we show that inhibition of the EGFR pathway prevents development of AngII-induced renal lesions. Furthermore, we provide evidence that TACE (tumor necrosis factor- α converting enzyme, also known as ADAM17) and TGF- α are crucial intermediates between AngII signal and EGFR transactivation during kidney diseases, and we identify a new therapeutic target.

RESULTS

EGFR is required for AngII-induced renal lesions

To analyze the role of EGFR activation in angiotensin-induced renal lesions, we infused AngII into wild-type or transgenic EGFR-M mice, which selectively express a dominant negative isoform of EGFR in proximal tubular cells.

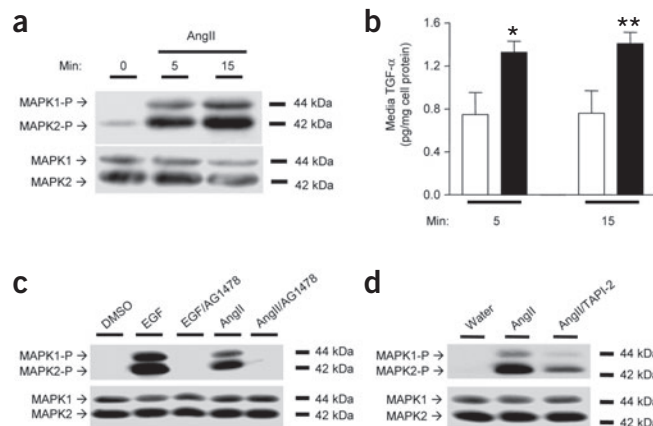
In wild-type littermates, AngII infusion over 2 months produced severe renal lesions, mainly comprising glomerulosclerosis, tubular atrophy and/or dilation with little microcyst formation, mild interstitial fibrosis and multifocal mononuclear cell infiltration (Fig. 1). In contrast, the frequency and severity of renal lesions were substantially reduced in AngII-infused EGFR-M mice (Fig. 1). Development of renal lesions resulted in severe proteinuria in wild-type mice (15.8 ± 9.2 versus 3.2 ± 0.7 mg/d in AngII-treated and control mice, respectively). Proteinuria was notably decreased in EGFR-M mice (8.4 ± 2.0 mg/d).

Figure 2 AngII stimulates TGF- α shedding and MAP kinase phosphorylation in GN5 cells. (a) MAPK1 and MAPK2 activation in AngII-treated cells. (b) Soluble TGF- α in conditioned media from cells treated with (black bars) or without (white bars) AngII. (c) MAP kinase activation in EGF- and AngII-treated cells after preincubation with or without the specific EGFR inhibitor AG1478. (d) MAP kinase activation in AngII-treated cells after preincubation with or without the specific TACE inhibitor TAPI-2. Data are means \pm s.e.m.; $n = 4$ experiments. Mann-Whitney test: AngII versus no treatment: * $P < 0.05$; ** $P < 0.01$.

rather than hypertension accounts for the development of renal lesions during AngII pathway activation.

AngII induces TACE-mediated shedding of TGF- α

We next investigated the mechanisms by which AngII transactivates EGFR during kidney disease. Previous *in vitro* data suggested that GPCR transactivation of EGFR is mediated by ADAM protease-dependent shedding of EGF family ligands^{12,25}. Our recent finding that TGF- α might be involved in renal deterioration²⁶ prompted us to evaluate whether AngII treatment rapidly induces shedding of TGF- α . We addressed this issue *in vitro* using GN5 epithelial-cell cultures, which show rapid activation of MAPK in response to AngII treatment (Fig. 2a). Notably, levels of TGF- α in the media were increased approximately twofold, at 5 and 15 min after addition of AngII (Fig. 2b). Furthermore, treatment of GN5 cells with either the EGFR antagonist AG1478 or a specific inhibitor of the TGF- α sheddase TACE²⁷, TAPI-2, prevented AngII-induced phosphorylation of MAPK1 and MAPK2 (Fig. 2c,d).



TGF- α is essential for AngII-induced renal damage

We then assessed the contribution of AngII-induced TGF- α shedding to lesion development *in vivo*. Immunohistochemical analysis showed TGF- α staining only in a few cortical distal tubules and the ascending limb of loops of Henle in control mice (Fig. 3a). But 2 months after AngII infusion, TGF- α staining increased markedly in kidneys from treated mice (Fig. 3a). Western blot analysis detected a specific, immunoreactive band of 37 kDa in kidneys from AngII-infused mice (Fig. 3b), but not in control mice. Real-time RT-PCR showed similar levels of *Tgfa* mRNA (which encodes TGF- α) in kidneys of vehicle- and AngII-treated mice (Fig. 3c). In contrast, there was a decrease, though statistically not significant, of EGF staining after AngII infusion in cortical distal tubules (data not shown), indicating that AngII selectively affects expression of EGFR ligands.

We then infused AngII into *Tgfa*^{-/-} mice²⁸ and showed that the development of renal lesions was substantially reduced in mice lacking TGF- α as compared to wild-type *Tgfa*^{+/+} littermates (Fig. 3d). Similarly, proteinuria was markedly decreased in AngII-treated *Tgfa*^{-/-} mice to levels indistinguishable from those of control mice receiving vehicle alone (2.3 \pm 0.4 versus 4.8 \pm 1.1 mg/d in AngII- and vehicle-treated *Tgfa*^{-/-} mice, respectively). In contrast, absence of TGF- α did not prevent the increase of blood pressure in mutant mice (185 \pm 3 mmHg after 2 months of AngII infusion). Collectively, these data suggest that TGF- α is a key mediator of AngII-induced renal damage.

TACE is induced and redistributed in AngII-damaged kidneys

We next examined the ability of AngII to stimulate TACE production in mice. Western blot analysis detected a specific anti-TACE reactive band of approximately 115 kDa comigrating with TACE transfected into TACE-deficient cells (Fig. 4a). TACE protein levels rose substantially in kidneys of mice receiving AngII compared with vehicle-treated controls (Fig. 4a). A similar increase in TACE protein levels was observed in AngII-treated EGFR-M and *Tgfa*^{-/-} mice (Fig. 4a). *Adam17* mRNA (which encodes TACE) levels, determined by real-time RT-PCR, were similar in vehicle- and AngII-treated mice (Fig. 4b), suggesting that TACE is induced through a post-transcriptional mechanism.

In mammalian cells, the immature form of TACE is predominantly intracellular,

whereas the mature (and active) form is detectable both within the cell and on the cell surface²⁹. Immunofluorescence analysis showed weak perinuclear staining in only a few cortical distal tubules of control animals. In contrast, TACE immunostaining was markedly enhanced in kidneys of AngII-treated mice (Fig. 4c). Additionally, the pattern of staining was altered so that TACE was detected in the cytoplasm and on apical membranes (Fig. 4d). TACE was not detected in glomeruli. To further characterize TACE distribution, we performed colocalization experiments using Tamm-Horsfall, a well-known glycoprotein expressed in the ascending limb of loops of Henle and distal tubules³⁰, the nephron segments that synthesize TGF- α ¹⁴. TACE was mainly expressed in Tamm-Horsfall-positive tubules (Fig. 4c).

Inhibition of TACE blunts AngII-induced renal lesions

Our results suggested that TACE could be a potential target for the treatment of CKD. WTACE2 is a new metalloprotease inhibitor specific for TACE³¹. Daily administration of WTACE2 substantially reduced the development of renal lesions. The majority of glomeruli and tubules

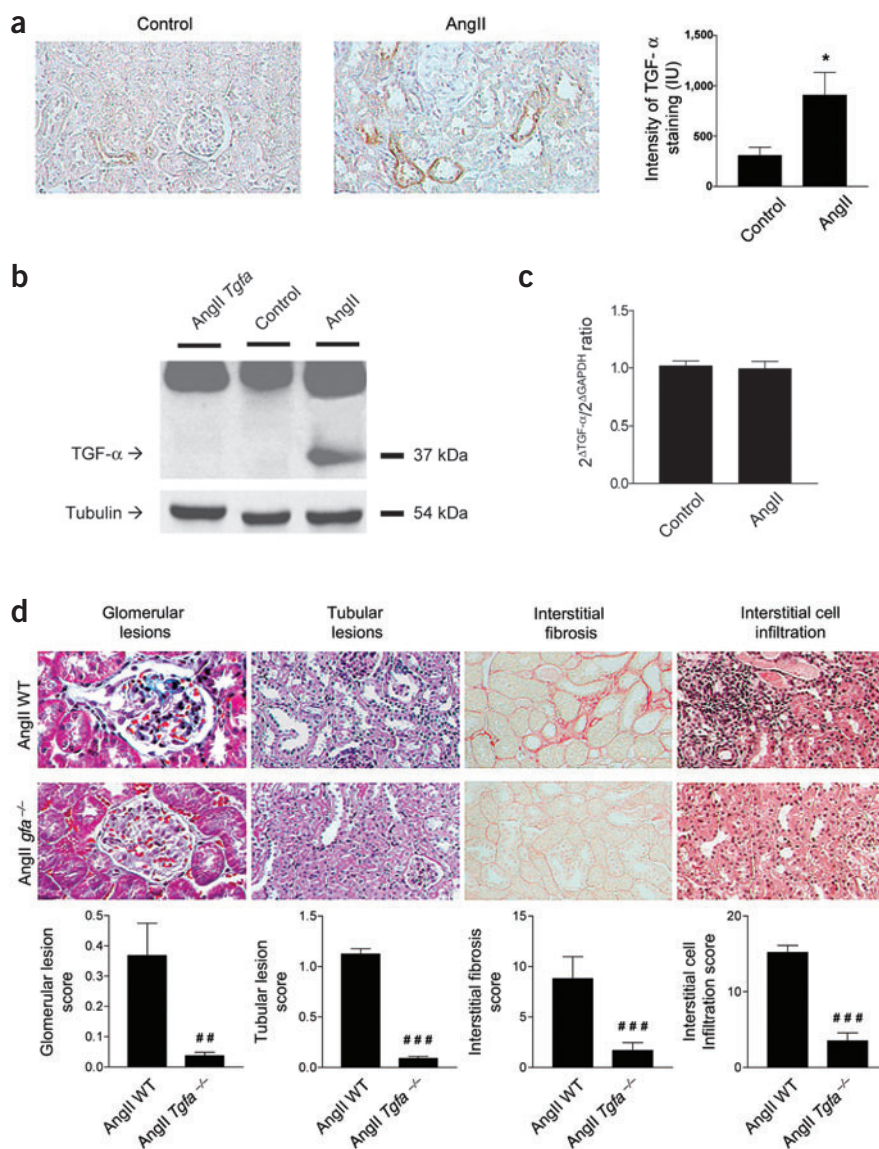


Figure 3 TGF- α is increased and essential for AngII-induced renal damage. (a–c) TGF- α protein and *Tgfa* mRNA expression in control and AngII-treated (AngII) mice, evaluated by (a) immunohistochemistry (original magnification, $\times 200$), (b) western blot and (c) real-time RT-PCR. (d) Morphology and lesion scores of kidneys from AngII-treated wild-type (AngII WT, upper panels) and AngII-treated *Tgfa*^{-/-} mice (AngII *Tgfa*^{-/-}, lower panels). Data are means \pm s.e.m.; $n = 5$ –6 for each experimental group. mRNA was assayed in triplicate for each point. Mann-Whitney test: AngII versus control: * $P < 0.05$; *Tgfa*^{-/-} versus wild-type mice: ## $P < 0.01$; ### $P < 0.001$.

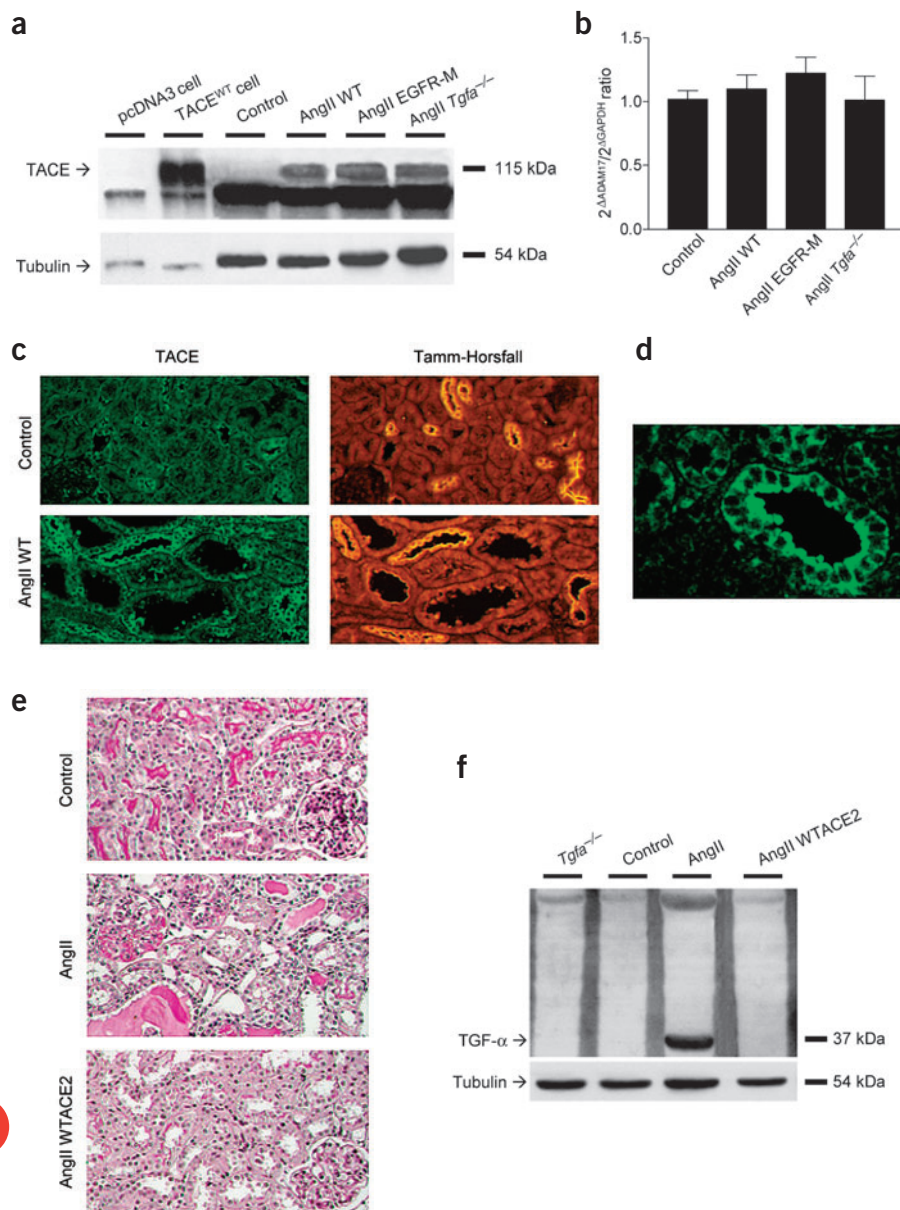


Figure 4 TACE activation is crucial for AngII-induced TGF- α overexpression in lesion development. **(a,b)** TACE protein and *Adam17* mRNA expression in control, AngII-treated wild-type (AngII WT), transgenic EGFR-M (AngII EGFR-M) and *Tgfa*^{-/-} (AngII *Tgfa*^{-/-}) mice, evaluated by western blot and real-time RT-PCR. **(c)** Colocalization experiments of TACE (left) and Tamm-Horsfall (right) in serial sections of kidneys from control (upper panels) and AngII-treated (AngII WT, lower panels) mice (original magnification, $\times 200$). **(d)** Apical localization of TACE by confocal microscopy. **(e)** Morphology and **(f)** TGF- α western blot analysis of kidneys from control and AngII-treated mice receiving either the vehicle or WTACE2 (original magnification, $\times 200$). Data are means \pm s.e.m.; $n = 5-6$ for each experimental group.

AngII inhibition prevents EGFR transactivation after nephrectomy

Finally, we investigated whether TACE-TGF- α -EGFR transactivation mediates the deleterious effect of AngII after nephron reduction, a pathological condition characterized by renin-angiotensin system activation⁵ and thought to be involved in the evolution of most CKD³². Hence, we performed 75% reduction of renal mass in FVB/N mice and treated mice either with vehicle or the AngII inhibitor losartan. Two months after nephron reduction, FVB/N mice developed severe renal lesions, whereas daily administration of losartan markedly protected remnant kidneys from the deterioration process (Fig. 6a). Quantification showed that losartan-treated mice had considerably fewer glomerular, tubular and interstitial lesions as compared with vehicle-treated counterparts (data not shown). Moreover, inhibition of AT₁ receptor prevented the development of proteinuria and hypertension in subtotal nephrectomized mice. Daily protein excretion and mean arterial blood pressure were 3.9 ± 0.9 , 23.6 ± 6.1 and 3.6 ± 0.4 mg/d ($P < 0.01$) and 127 ± 5 , 162 ± 6 and 118 ± 5 mmHg ($P <$

were normal, and very few areas of interstitial fibrosis and mononuclear cell infiltration were observed in WTACE2-treated animals after a 2-month AngII infusion (Fig. 4e). These observations were confirmed by scoring of glomerular, tubular and interstitial lesions (data not shown). WTACE2 also abolished the increase in TGF- α protein in AngII-treated mice (Fig. 4f), but did not prevent hypertension (178 ± 9 mmHg after 2 months of treatment).

AngII induces EGFR phosphorylation in damaged kidneys

We then evaluated the phosphorylation of the receptor *in vivo* and showed that in wild-type mice, AngII infusion markedly stimulated EGFR phosphorylation without affecting EGFR protein levels (Fig. 5). As expected²¹, the overexpression of the dominant negative EGFR completely prevented EGFR activation in response to AngII in transgenic EGFR-M mice. Notably, AngII-induced EGFR phosphorylation was abolished in *Tgfa*^{-/-} mice as well as in wild-type WTACE2-treated mice (Fig. 5).

0.001) in control, nephrectomized vehicle-treated and nephrectomized losartan-treated mice, respectively.

Immunohistological and western blot analyses showed that both TGF- α and TACE protein levels increased substantially 2 months after nephron reduction in vehicle-treated but not losartan-treated mice (Fig. 6b-e). Notably, increased TACE expression in damaged remnant kidneys was also accompanied by redistribution of the metalloprotease to apical membranes (Fig. 6e). But administration of losartan prevented the protein apical redistribution (Fig. 6d). In contrast, EGF levels were unaffected by either nephron reduction or losartan administration (data not shown).

DISCUSSION

The mechanisms by which AngII, the most important mediator of CKD, induces kidney destruction are largely unknown. By applying genetic and pharmacological approaches to experimental models of renal injury, we have established a pivotal role for EGFR

transactivation in AngII-induced renal lesions. Furthermore, we have defined an important pathophysiologic mechanism by which AngII causes induction and redistribution of the metalloprotease TACE (also known as ADAM17) to the apical membranes of distal renal tubules, consistent with a role in cleaving surface-localized TGF- α precursor. Inhibition of this pathway through overexpression of a dominant negative isoform of EGFR, inactivation of the *Tgfa* gene or use of a specific metalloprotease inhibitor, WTACE2, prevented lesion development in AngII-treated mice. Conversely, inhibition of AngII receptors by losartan abrogated apical localization of TACE and overexpression of TGF- α and prevented development of lesions in mice after 75% nephron reduction. Collectively, these results establish the importance of the TACE-TGF- α -EGFR pathway in AngII-induced renal damage, and they suggest that TACE targeting to apical membranes is an early step in the development of lesions.

Cross-talk between GPCRs, such as AngII receptors, and the EGFR signaling pathway is widely established in a variety of *in vitro* cell-culture systems³³. But the role of this phenomenon in the complex pathogenesis of diseases has not been adequately investigated. Here, we show that EGFR acts as a central integrator of the AngII signaling pathway, stimulating the development of renal lesions. We previously showed that inhibiting EGFR prevented the development of renal lesions after nephron reduction²¹. In parallel, other studies have shown a similarly beneficial effect of AngII inhibitors^{2,3}. Our observations that chronic AngII infusion activated the EGFR pathway (although conversely, AngII

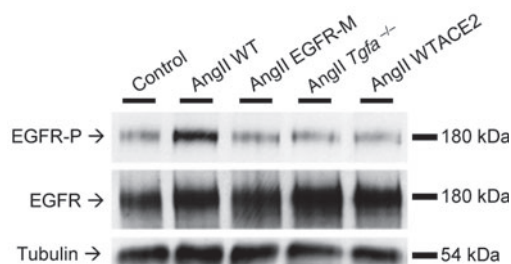


Figure 5 AngII-induced EGFR phosphorylation is abolished by EGFR, TGF- α or TACE inhibition in mice. Western blot analysis of EGFR phosphorylation (upper panel), EGFR expression (middle panel) and tubulin (lower panel) in control, AngII-treated wild-type (AngII WT), transgenic EGFR-M (AngII EGFR-M) and *Tgfa*^{-/-} (AngII *Tgfa*^{-/-}) and WTACE2-treated (AngII WTACE2) mice. Blots are representative samples of four animals for each experimental group.

inhibitors prevented EGFR transactivation after nephron reduction) establish a clear mechanistic relationship between these two molecular pathways, expanding the understanding of pathogenesis of CKD. Different levels of EGF pathway activation could account for the heterogeneity among the mouse models used. In particular, the complete absence of the ligand in the *Tgfa*^{-/-} mice might explain the greatest protection observed in this strain. It is noteworthy that proteinuria, a key mediator of renal deterioration³⁴, almost undetectable in *Tgfa* mutant mice, was present to a small extent in EGFR-M mice, suggesting that increased urinary protein excretion accounted for greater tubular damage in these animals.

The mechanisms by which GPCRs transactivate EGFR are not well understood. Recent studies have suggested the crucial role of metalloproteases in the enzymatic conversion of EGF family precursors to mature, soluble ligands that activate EGFR through autocrine or paracrine mechanisms^{12,25,35}. Our study provides the first *in vivo* evidence for a similar mechanism in the pathogenesis of CKD and, in particular, identifies TACE and TGF- α as crucial transactivation components required for kidney destruction after AngII infusion

inhibitors prevented EGFR transactivation after nephron reduction) establish a clear mechanistic relationship between these two molecular pathways, expanding the understanding of pathogenesis of CKD. Different levels of EGF pathway activation could account for the heterogeneity among the mouse models used. In particular, the complete absence of the ligand in the *Tgfa*^{-/-} mice might explain the greatest protection observed in this strain. It is noteworthy that proteinuria, a key mediator of renal deterioration³⁴, almost undetectable in *Tgfa* mutant mice, was present to a small extent in EGFR-M mice, suggesting that increased urinary protein excretion accounted for greater tubular damage in these animals.

The mechanisms by which GPCRs transactivate EGFR are not well understood. Recent studies have suggested the crucial role of metalloproteases in the enzymatic conversion of EGF family precursors to mature, soluble ligands that activate EGFR through autocrine or paracrine mechanisms^{12,25,35}. Our study provides the first *in vivo* evidence for a similar mechanism in the pathogenesis of CKD and, in particular, identifies TACE and TGF- α as crucial transactivation components required for kidney destruction after AngII infusion

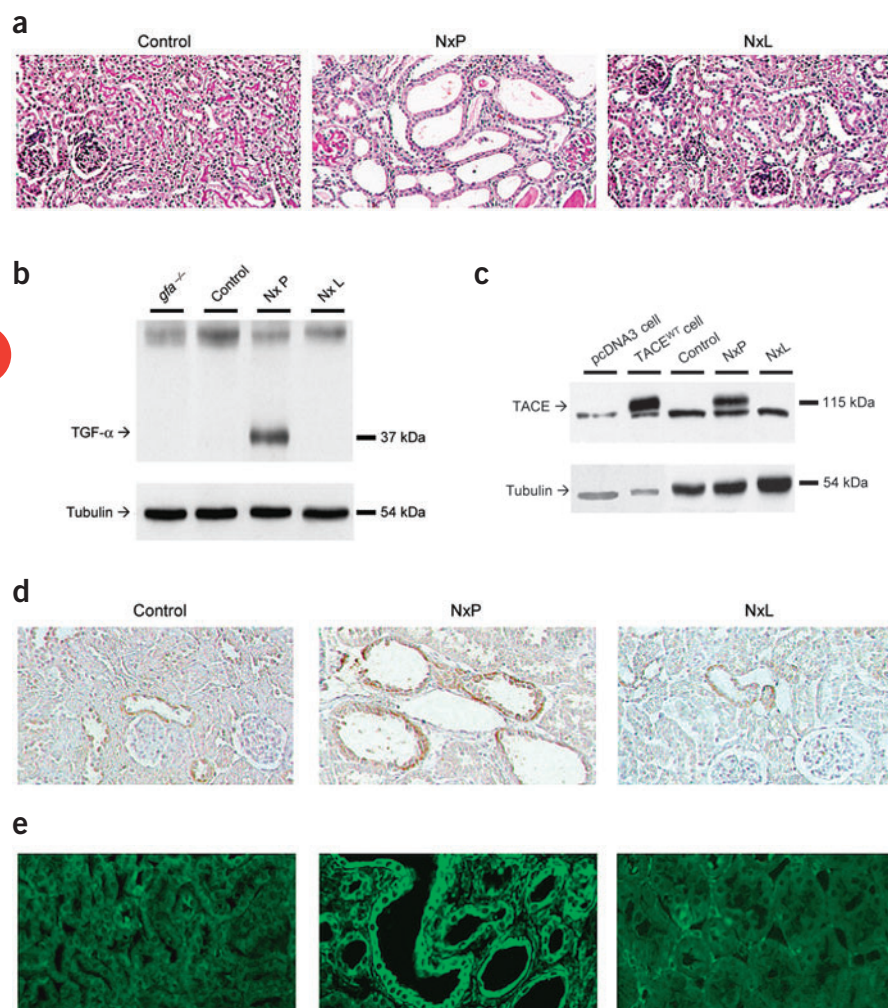


Figure 6 Inhibition of AngII signaling pathway attenuates development of lesions and abrogates overexpression of TGF- α and TACE after nephron reduction. **(a)** Morphology of kidneys from control (left panels), vehicle-treated nephrectomized (NxP, middle panels) and losartan-treated nephrectomized (NxL, right panels) mice (original magnification, $\times 200$). **(b,c)** Immunoblot analysis and **(d,e)** immunohistochemistry (original magnification, $\times 200$) of TGF- α (**b,d**) and TACE (**c,e**) expression in control, vehicle-treated nephrectomized (NxP) and losartan-treated nephrectomized (NxL) mice. Blots and pictures are representative samples of six mice for each group. Because no differences were detected between vehicle- and losartan-treated control mice, only one group is shown.

or 75% nephron reduction. Although technical limitations prevent us from confirming AngII-enhanced shedding of TGF- α *in vivo*, our *in vitro* results establish that AngII stimulates shedding of TGF- α in a well-studied model³⁶. The 37 kDa TGF- α protein detected in kidney from AngII-treated mice probably corresponds to a high molecular weight form of secreted TGF- α , as we detected it in urine of both AngII-treated and nephrectomized mice (data not shown), and large (18–68 kDa), bioactive forms of TGF- α species have been observed in conditioned media and urine^{37,38}. The previous observation that heparin-binding EGF-like growth factor shedding by ADAM12 is required for AngII-induced cardiac hypertrophy³⁹ suggests that different molecular pathways may be triggered by AngII to transactivate EGFR during pathological conditions, possibly in a cell type-specific manner^{35,40}. Conceivably, context-specific differences in cell compartmentalization of ADAMs and various EGF family precursors could account for these distinctions. Notably, TACE and TGF- α are both expressed in ascending limbs of loops of Henle and distal tubules during lesion development, consistent with the roles identified by our findings. The autocrine-paracrine link between distal tubular cells, where TGF- α is synthesized and probably secreted into the lumen, and proximal tubular cells, the major target of this growth factor, remains to be elucidated.

TACE belongs to the ADAM family of integral membrane proteins synthesized in the rough endoplasmic reticulum and mature in the late Golgi compartment⁴¹. TACE protein resides in a perinuclear region but appears on the cell surface in a catalytically active form upon processing²⁹. Our observations provide the first evidence of a role for GPCRs in regulating TACE trafficking from the perinuclear compartment to apical membranes, where it can presumably act on TGF- α precursor. It is noteworthy that the cell-surface pool of the enzyme is long-lived compared to the cytoplasmic pool⁴², which could account for the post-transcriptional control of TACE in our experimental conditions.

TACE cleavage mediates the shedding of several EGFR ligands, including TGF- α , heparin-binding EGF-like growth factor and amphiregulin^{27,43}. But our finding that AngII does not induce renal lesions in *Tgfa*^{-/-} mice suggests a distinct and essential role for this particular EGFR ligand, which is supported by additional evidence. First, inhibition of AngII receptors prevented both TGF- α overexpression and renal lesions after nephron reduction. Second, we previously showed that development of renal lesions in *Jund1* knockout mice was associated with TGF- α upregulation and that the inhibition of EGFR blocked this renal phenotype²⁶. Third, transgenic mice overexpressing TGF- α develop severe renal lesions⁴⁴, whereas transgenic mice broadly expressing EGF do not⁴⁵. On the other hand, EGF, the principal EGFR ligand expressed in adult kidney¹⁴, was unchanged by either AngII infusion or nephron reduction.

It has been suggested that AngII may cause renal injury by inducing systemic or glomerular hypertension and by stimulating renal overgrowth⁵, but the relative contribution of each has been difficult to discern. Our finding that inhibition of the EGFR pathway prevents the formation of renal lesions without ameliorating systemic hypertension in AngII-treated mice suggests renal hypertrophy as the dominant pathogenic mechanism. Similarly, KB-R7785, an ADAM12 inhibitor, prevented cardiac hypertrophy in AngII-infused mice without affecting hypertension³⁹. It is noteworthy that EGFR acts as a potent mitogen in renal cells¹⁶ and that EGF-induced proliferation in proximal tubular cells is potentiated by AngII¹⁶. In cardiomyocytes, as well as in ACHN (a human kidney adenocarcinoma cell line), the mitogenic effect of AngII is mediated through EGFR transactivation^{35,39}. It remains to be established whether the same phenomenon mediates the growth-promoting effects of AngII in kidney diseases.

Several studies have shown that pharmacological inhibition of AngII pathway delays the progressive loss of renal function in individuals with

CKD⁴⁶. But the risk of developing potentially life-threatening complications, most often related to the hemodynamic effects of these drugs, precludes their use in some individuals⁴⁷. Furthermore, the long-term renoprotective effect of AngII inhibition shows a marked heterogeneity between individuals⁴⁸. Our study shows that pharmacological inhibition of TACE by WTACE2 is beneficial in preserving kidneys from chronic injury. Others have previously reported that inhibition of TACE attenuates the extent of renal cyst formation in *bpk* mice, a model of polycystic kidney disease³¹. Further studies are required to investigate whether the concerted actions of TACE inhibitors and AngII blockers will show an additive effect on renal preservation.

In conclusion, our study highlights the importance of EGFR transactivation during progressive kidney diseases. This is the first demonstration that TACE-mediated shedding of TGF- α is instrumental in AngII-induced renal lesions. Moreover, we have identified a new therapeutic strategy to treat the growing number of individuals with chronic renal failure.

METHODS

Mice. For these studies, we used control FVB/N mice (Iffa Credo), transgenic EGFR-M mice expressing a dominant negative isoform of EGFR under the control of kidney-specific type 1 γ -glutamyl transpeptidase promoter²¹ and *Tgfa*^{-/-} mice²⁸. EGFR-M and *Tgfa*^{-/-} mice were on pure FVB/N and mixed 129/Sv \times C57Bl6 genetic backgrounds, respectively. We performed all experiments on 9-week-old female mice. Animal procedures were conducted in accordance with French government policies (Services Vétérinaires de la Santé et de la Production Animale, Ministère de l'Agriculture).

Angiotensin II-induced nephropathy. We subjected FVB/N and transgenic mice to unilateral nephrectomy and treated them with either AngII (1.4 mg/kg/d; Sigma-Aldrich) or vehicle (0.9% NaCl) through subcutaneous osmotic minipumps (2004, Alzet) for 2 months. Transgenic studies used 12 EGFR-M or *Tgfa*^{-/-} mice and 12 wild-type littermates originating from the same litters; for each group, 6 mice were treated with AngII and 6 with the vehicle (0.9% NaCl, control). For TACE/TGF- α studies, 12 FVB/N mice were infused either with AngII ($n = 6$) or the vehicle ($n = 6$). For WTACE2 studies, we studied four groups of six FVB/N mice receiving: (i) AngII + metalloprotease inhibitor WTACE2 (100 mg/kg/d by daily intraperitoneal injection; a gift from Wyeth-Ayerst Research); (ii) AngII + vehicle of WTACE2 (0.9% NaCl, 0.5% methylcellulose, 2% Tween); (iii) vehicle of AngII (0.9% NaCl) + WTACE2; and vehicle of AngII + vehicle of WTACE2. For EGFR phosphorylation study, we used four mice of each experimental group. Mice were killed after 2 months of infusion. One week before killing, we collected urine samples and recorded blood pressure in five awake mice of each experimental group as described²⁶. For EGFR phosphorylation study, mice were killed at day 7 (for details, see **Supplementary Methods** online).

Subtotal nephrectomy. We subjected FVB/N mice to subtotal nephrectomy or sham operation as previously described⁴⁹, then treated them with either AngII type 1 receptor antagonist losartan (30 mg/kg/d in drinking water; MSD Laboratory) or vehicle (water). We studied six sham-operated mice and nine nephrectomized mice for each experimental group. Mice were killed 2 months after surgery. We collected blood pressure and urine samples 1 week before killing.

Renal function. We measured proteinuria using an Olympus multiparametric autoanalyzer (Instrumentation Laboratory).

Renal morphology. We fixed kidneys in 4% paraformaldehyde, embedded them in paraffin, and stained sections of 4 μ m with periodic acid-Schiff, Masson trichrome, hematoxylin and eosin or picosirius red. We quantified the degree of renal lesions using a Nikon digital camera Dx/m/1200 and Lucia software (Laboratory Imaging Ltd.) as reported²⁶.

Immunohistochemical and immunofluorescence analysis. TGF- α and EGF immunostaining were performed as described²⁶. For TACE and Tamm-Horsfall staining, we incubated serial sections of 4 μ m with a rabbit antibody to TACE (1:100; AbCys Biology) or a sheep antibody to Tamm-Horsfall (1:50; Valbiotech),

followed by a goat IgG FITC-conjugated antibody to rabbit and a donkey IgG TRITC-conjugated antibody to sheep (1:200; Molecular Probes), respectively.

Western blot analysis. We performed immunoblotting as described previously²⁶ using a goat antibody to TGF- α (1:100; Santa Cruz Biotechnology Inc.), a rabbit antibody to TACE (1:1000; AbCys Biology), a rabbit antibody to phosphorylated EGFR (1:500; Santa Cruz Biotechnology Inc.) or a rabbit antibody to EGFR (1:200; Santa Cruz Biotechnology Inc.) followed by donkey horseradish peroxidase-linked secondary antibody (Amersham Pharmacia Biotech) diluted 1:2,000 or 1:5,000 for TGF- α and phosphorylated EGFR or TACE and EGFR, respectively. We used a mouse monoclonal antibody to α -tubulin (Sigma-Aldrich) as a control. We used protein extracts from kidneys of *Tgfa*^{-/-} mice²⁸ and from TACE (Δ Zn/ Δ Zn) cells transfected with either *Adam17* cDNA (TACE^{WT}) or the empty vector (pcDNA3)²⁷ to confirm antibody specificity.

Real-time RT-PCR. We isolated total RNAs from kidneys using the Rneasy Protect Midi Kit (Qiagen). We carried out quantitative RT-PCR on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Applied) using SYBR green. We used the following primers (MWG Biotechnology): *Tgfa* primers: 5'-GCCAGATTCCCACTCAG-3' and 5'-CACGGCACCCTCACAGTG-3'; *Adam17* primers: 5'-CACTTTGGTGCTTCGTCCT-3' and 5'-CGTAGTCTGAGAGCAAAGAATCAAGC-3'; *Gapdh* primers: 5'-TGCACCACCACTGCTTAG-3' and 5'-GGATGCAGGGATGATGTTTC-3'. GAPDH was used as the normalization control.

In vitro experiments. The GN5 clone of transformed rat liver epithelial cells³⁶ was serum-starved for 24 h in Richter improved minimal essential medium with 0.1% FBS and 0.1 mM insulin. We treated cells with AngII (2 μ M) for 5 or 15 min. We then collected media, concentrated it using Sep-Pak C18 cartridges (Waters Corp) and analyzed it for TGF- α levels using a specific radioimmunoassay²⁸. MAPK1 and MAPK2 phosphorylation were assayed after pretreatment with or without the EGFR inhibitor AG1478 (10 μ M in DMSO, final concentration of DMSO 0.3%; Sigma) or the TACE inhibitor TAPI-2 (100 μ M in water; EMD Biosciences).

Data analysis and statistics. Data were expressed as means \pm s.e.m. Differences between the experimental groups were evaluated using ANOVA, followed when significant ($P < 0.05$) by the Tukey-Kramer test, or when only two groups were compared, the Mann-Whitney test.

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGMENTS

We are grateful to S. Le Corre, M. Muffat-Joly and Y. Xiong. We thank W. Russell and M. Stevenson for TGF- α RIA measurements and E. Esquivel, M. Pontoglio and D. Laouari for critical advice. We are grateful to Wyeth-Ayerst Research Laboratories and MSD Laboratories for WTACE2 and losartan, respectively. This work was supported by INSERM, Université René Descartes, Laboratoires de Recherches Physiologiques and Centre de Recherche Industrielle et Technique and by US National Institutes of Health/National Cancer Institute grants CA43793 and CA85410 (to D.C.L.).

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 14 December 2004; accepted 23 June 2005

Published online at <http://www.nature.com/naturemedicine/>

- Hostetter, T.H. Progression of renal disease and renal hypertrophy. *Annu. Rev. Physiol.* **57**, 263–278 (1995).
- Lafayette, R.A., Mayer, G., Park, S.K. & Meyer, T.W. Angiotensin II receptor blockade limits glomerular injury in rats with reduced renal mass. *J. Clin. Invest.* **90**, 766–771 (1992).
- Lewis, E.J. *et al.* Renoprotective effect of the angiotensin-receptor antagonist irbesartan in patients with nephropathy due to type 2 diabetes. *N. Engl. J. Med.* **345**, 851–860 (2001).
- Pei, Y., Scholey, J., Thai, K., Suzuki, M. & Catran, D. Association of angiotensinogen gene T235 variant with progression of immunoglobulin A nephropathy in Caucasian patients. *J. Clin. Invest.* **100**, 814–820 (1997).
- Brewster, U.C. & Perazella, M.A. The renin-angiotensin-aldosterone system and the kidney: effects on kidney disease. *Am. J. Med.* **116**, 263–272 (2004).
- Ardailou, R. Angiotensin II receptors. *J. Am. Soc. Nephrol.* **10** Suppl. 11, S30–S39 (1999).

- Siragy, H.M. AT1 and AT2 receptor in the kidney: role in health and disease. *Semin. Nephrol.* **24**, 93–100 (2004).
- de Gasparo, M., Catt, K.J., Inagami, T., Wright, J.W. & Unger, T. International union of pharmacology. XXIII. The angiotensin II receptors. *Pharmacol. Rev.* **52**, 415–472 (2000).
- Eguchi, S. & Inagami, T. Signal transduction of angiotensin II type 1 receptor through receptor tyrosine kinase. *Regul. Pept.* **91**, 13–20 (2000).
- Bokemeyer, D., Schmitz, U. & Kramer, H.J. Angiotensin II-induced growth of vascular smooth muscle cells requires an Src-dependent activation of the epidermal growth factor receptor. *Kidney Int.* **58**, 549–558 (2000).
- Eguchi, S. *et al.* Calcium-dependent epidermal growth factor receptor transactivation mediates the angiotensin II-induced mitogen-activated protein kinase activation in vascular smooth muscle cells. *J. Biol. Chem.* **273**, 8890–8896 (1998).
- Prenzel, N. *et al.* EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature* **402**, 884–888 (1999).
- Harris, R.C., Chung, E. & Coffey, R.J. EGF receptor ligands. *Exp. Cell Res.* **284**, 2–13 (2003).
- Nouwen, E.J. & De Broe, M.E. EGF and TGF- α in the human kidney: identification of octal cells in the collecting duct. *Kidney Int.* **45**, 1510–1521 (1994).
- Breyer, M.D., Redha, R. & Breyer, J.A. Segmental distribution of epidermal growth factor binding sites in rabbit nephron. *Am. J. Physiol.* **259**, F553–F558 (1990).
- Norman, J. *et al.* EGF-induced mitogenesis in proximal tubular cells: potentiation by angiotensin II. *Am. J. Physiol.* **253**, F299–F309 (1987).
- Okada, H., Danoff, T.M., Kalluri, R. & Neilson, E.G. Early role of Fsp1 in epithelial-mesenchymal transformation. *Am. J. Physiol.* **273**, F563–F574 (1997).
- Creely, J.J., DiMari, S.J., Howe, A.M., Hyde, C.P. & Haralson, M.A. Effects of epidermal growth factor on collagen synthesis by an epithelioid cell line derived from normal rat kidney. *Am. J. Pathol.* **136**, 1247–1257 (1990).
- Terzi, F., Burtin, M. & Friedlander, G. Early molecular mechanisms in the progression of renal failure: role of growth factors and protooncogenes. *Kidney Int. Suppl.* **65**, S68–S73 (1998).
- Stocklin, E., Botteri, F. & Groner, B. An activated allele of the c-erbB-2 oncogene impairs kidney and lung function and causes early death of transgenic mice. *J. Cell Biol.* **122**, 199–208 (1993).
- Terzi, F. *et al.* Targeted expression of a dominant-negative EGF-R in the kidney reduces tubulo-interstitial lesions after renal injury. *J. Clin. Invest.* **106**, 225–234 (2000).
- Richards, W.G. *et al.* Epidermal growth factor receptor activity mediates renal cyst formation in polycystic kidney disease. *J. Clin. Invest.* **101**, 935–939 (1998).
- Sweeney, W.E., Chen, Y., Nakanishi, K., Frost, P. & Avner, E.D. Treatment of polycystic kidney disease with a novel tyrosine kinase inhibitor. *Kidney Int.* **57**, 33–40 (2000).
- Francois, H. *et al.* Prevention of renal vascular and glomerular fibrosis by epidermal growth factor receptor inhibition. *FASEB J.* **18**, 926–928 (2004).
- Schafer, B., Gschwind, A. & Ullrich, A. Multiple G-protein-coupled receptor signals converge on the epidermal growth factor receptor to promote migration and invasion. *Oncogene* **23**, 991–999 (2004).
- Pillebout, E. *et al.* JunD protects against chronic kidney disease by regulating paracrine mitogens. *J. Clin. Invest.* **112**, 843–852 (2003).
- Sunnarborg, S.W. *et al.* Tumor necrosis factor- α converting enzyme (TACE) regulates epidermal growth factor receptor ligand availability. *J. Biol. Chem.* **277**, 12838–12845 (2002).
- Luetkeke, N.C. *et al.* TGF α deficiency results in hair follicle and eye abnormalities in targeted and waved-1 mice. *Cell* **73**, 263–278 (1993).
- Schlondorff, J., Becherer, J.D. & Blobel, C.P. Intracellular maturation and localization of the tumour necrosis factor α convertase (TACE). *Biochem. J.* **347**, 131–138 (2000).
- Ivanyi, B. & Olsen, T.S. Immunohistochemical identification of tubular segments in percutaneous renal biopsies. *Histochemistry* **95**, 351–356 (1991).
- Dell, K.M. *et al.* A novel inhibitor of tumor necrosis factor- α converting enzyme ameliorates polycystic kidney disease. *Kidney Int.* **60**, 1240–1248 (2001).
- Anderson, S. Mechanisms of injury in progressive renal disease. *Exp. Nephrol.* **4** Suppl. 1, 34–40 (1996).
- Gschwind, A., Zwick, E., Prenzel, N., Leserer, M. & Ullrich, A. Cell communication networks: epidermal growth factor receptor transactivation as the paradigm for inter-receptor signal transmission. *Oncogene* **20**, 1594–1600 (2001).
- Zoja, C., Benigni, A. & Remuzzi, G. Cellular responses to protein overload: key event in renal disease progression. *Curr. Opin. Nephrol. Hypertens.* **13**, 31–37 (2004).
- Schafer, B., Marg, B., Gschwind, A. & Ullrich, A. Distinct ADAM metalloproteinases regulate G protein-coupled receptor-induced cell proliferation and survival. *J. Biol. Chem.* **279**, 47929–47938 (2004).
- Earp, H.S. *et al.* Angiotensin II activates at least two tyrosine kinases in rat liver epithelial cells. Separation of the major calcium-regulated tyrosine kinase from p125FAK. *J. Biol. Chem.* **270**, 28440–28447 (1995).
- Luetkeke, N.C. *et al.* Characterization of high molecular weight transforming growth factor α produced by rat hepatocellular carcinoma cells. *Biochemistry* **27**, 6487–6494 (1988).
- Aladib, W., Yoshida, H. & Sato, M. High molecular weight type- α transforming growth factor in the urine of patients with surgical bone wound involved in mandibular osteotomy. *Bone Miner.* **9**, 59–70 (1990).
- Asakura, M. *et al.* Cardiac hypertrophy is inhibited by antagonism of ADAM12 processing of HB-EGF: metalloproteinase inhibitors as a new therapy. *Nat. Med.* **8**, 35–40 (2002).
- Gschwind, A., Hart, S., Fischer, O.M. & Ullrich, A. TACE cleavage of proamphiregulin regulates GPCR-induced proliferation and motility of cancer cells. *EMBO J.* **22**, 2411–2421 (2003).

ARTICLES

41. Black, R.A. Tumor necrosis factor-alpha converting enzyme. *Int. J. Biochem. Cell Biol.* **34**, 1–5 (2002).
42. Doedens, J.R. & Black, R.A. Stimulation-induced down-regulation of tumor necrosis factor-alpha converting enzyme. *J. Biol. Chem.* **275**, 14598–14607 (2000).
43. Sahin, U. *et al.* Distinct roles for ADAM10 and ADAM17 in ectodomain shedding of six EGFR ligands. *J. Cell Biol.* **164**, 769–779 (2004).
44. Lowden, D.A. *et al.* Renal cysts in transgenic mice expressing transforming growth factor-alpha. *J. Lab. Clin. Med.* **124**, 386–394 (1994).
45. Wong, R.W. *et al.* Overexpression of epidermal growth factor induced hypospermatogenesis in transgenic mice. *J. Biol. Chem.* **275**, 18297–18301 (2000).
46. Mackenzie, H.S., Ziai, F., Omer, S.A., Nadim, M.K. & Taal, M.W. Angiotensin receptor blockers in chronic renal disease: the promise of a bright clinical future. *J. Am. Soc. Nephrol.* **10** Suppl. 12, S283–S286 (1999).
47. Navis, G., Faber, H.J., de Zeeuw, D. & de Jong, P.E. ACE inhibitors and the kidney. A risk-benefit assessment. *Drug Saf.* **15**, 200–211 (1996).
48. Laverman, G.D., de Zeeuw, D. & Navis, G. Between-patient differences in the renal response to renin-angiotensin system intervention: clue to optimising renoprotective therapy? *J. Renin Angiotensin Aldosterone Syst.* **3**, 205–213 (2002).
49. Terzi, F. *et al.* Reduction of renal mass is lethal in mice lacking vimentin. Role of endothelin-nitric oxide imbalance. *J. Clin. Invest.* **100**, 1520–1528 (1997).