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Snail activation disrupts tissue homeostasis and induces fibrosis in the adult kidney

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During embryonic development, the kidney epithelium originates from cells that undergo a mesenchymal to epithelial transition (MET). The reverse process, epithelium to mesenchyme transition (EMT), has been implicated in epithelial tumor progression and in the fibrosis that leads to end-stage kidney failure. Snail transcription factors induce both natural and pathological EMT, but their implication in renal development and disease is still unclear. We show that *Snail* genes are downregulated during the MET that occurs during renal development and that this is correlated with Cadherin-16 expression. Snail suppresses Cadherin-16 via the direct repression of the kidney differentiation factor HNF-1β, a novel route by which Snail disrupts epithelial homeostasis. Indeed, Snail activation is sufficient to induce EMT and kidney fibrosis in adult transgenic mice. Significantly, *Snail* is also activated in patients with renal fibrosis. Thus, *Snail* expression is suppressed during renal development and it must remain silent in the mature kidney where its aberrant activation leads to fibrosis.

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Introduction

During embryonic development, epithelial and mesenchymal phenotypic transitions are necessary for the correct formation of different tissues. However, these processes are not only involved in development but they are also thought to be implicated in pathological conditions such as cancer and fibrosis (Nieto, 2002; Kalluri and Neilson, 2003; Liu, 2004; Thiery and Sleeman, 2006). One striking example is in kidney development and disease. During development, mesenchymal cells are initially formed by epithelium to mesenchyme transition (EMT) and subsequently, some of these cells undergo mesenchymal to epithelial transition (MET) to form the epithelia of the pronephros, mesonephros and metanephros (Dressler, 2002). In the adult, the reverse process (EMT) is implicated in the progress of carcinomas and in organ fibrosis. Fibrosis is regarded as the main event leading to end-stage kidney failure in most progressive renal diseases (Liu, 2004). Thus, while kidney formation involves reciprocal transformations, suppression of this plasticity in the adult is critical to maintain normal tissue architecture and homeostasis.

The *Snail* gene family of transcription factors is best known for its ability to trigger EMT, converting epithelial cells into mesenchymal cells with migratory properties. *Snail* genes influence both tissue formation during embryonic development and the acquisition of invasive properties in epithelial tumors (Barrallo-Gimeno and Nieto, 2005). Of the three vertebrate Snail family members, *Snail* and *Slug* (recently been renamed as *Snail1* and *Snail2*, respectively; Barrallo-Gimeno and Nieto, 2005) are functionally equivalent (del Barrio and Nieto, 2002; Bolós *et al*, 2003).

Prompted by our observation that Snail1 is a strong suppressor of kidney-specific cadherin (Cadherin-16) expression in cell culture, we have studied the role of *Snail* genes in the kidney. We found that *Snail* genes are repressed during kidney differentiation and identified that they regulate Cadherin-16 expression by repressing HNF-1β. To determine to what extent plasticity may be retained in the adult, we developed a system allowing us to reactivate Snail expression. We show that Snail activation induces renal fibrosis in transgenic mice and report the presence of pathological *Snail* expression in human fibrotic kidneys. Hence, the activation of Snail in the adult has profound effects on epithelial homeostasis in the kidney, which can be considered as a process of reverse embryogenesis likely to be pivotal in the development of renal fibrosis.

Results

To gain insight into the mechanisms used by Snail to repress the epithelial phenotype, we established an *in vitro* model of Snail1-induced EMT in the mouse mammary gland-derived epithelial cell line NMuMG. These cells typically display a cobblestone-like phenotype in culture (Figure 1A), yet they acquire a mesenchymal phenotype when stably transfected with Snail1 (Figure 1B). This morphological change is accompanied by the loss of E-Cadherin and a reorganization of the actin cytoskeleton (Supplementary Figure 1). Likewise, Snail1 transfectants become motile and acquire invasive properties (Supplementary Figure 1). Indeed, the transcription of many epithelial genes was strongly repressed in Snail1 transfectants, including that of *E-Cadherin* (Figure 1C) and other known Snail1 targets such as mucin-1, claudins and occludins (data not shown and Barrallo-Gimeno and Nieto, 2005; Huber *et al*, 2005). Thus, this cell-culture system provides a potent tool to investigate EMT driven by Snail.

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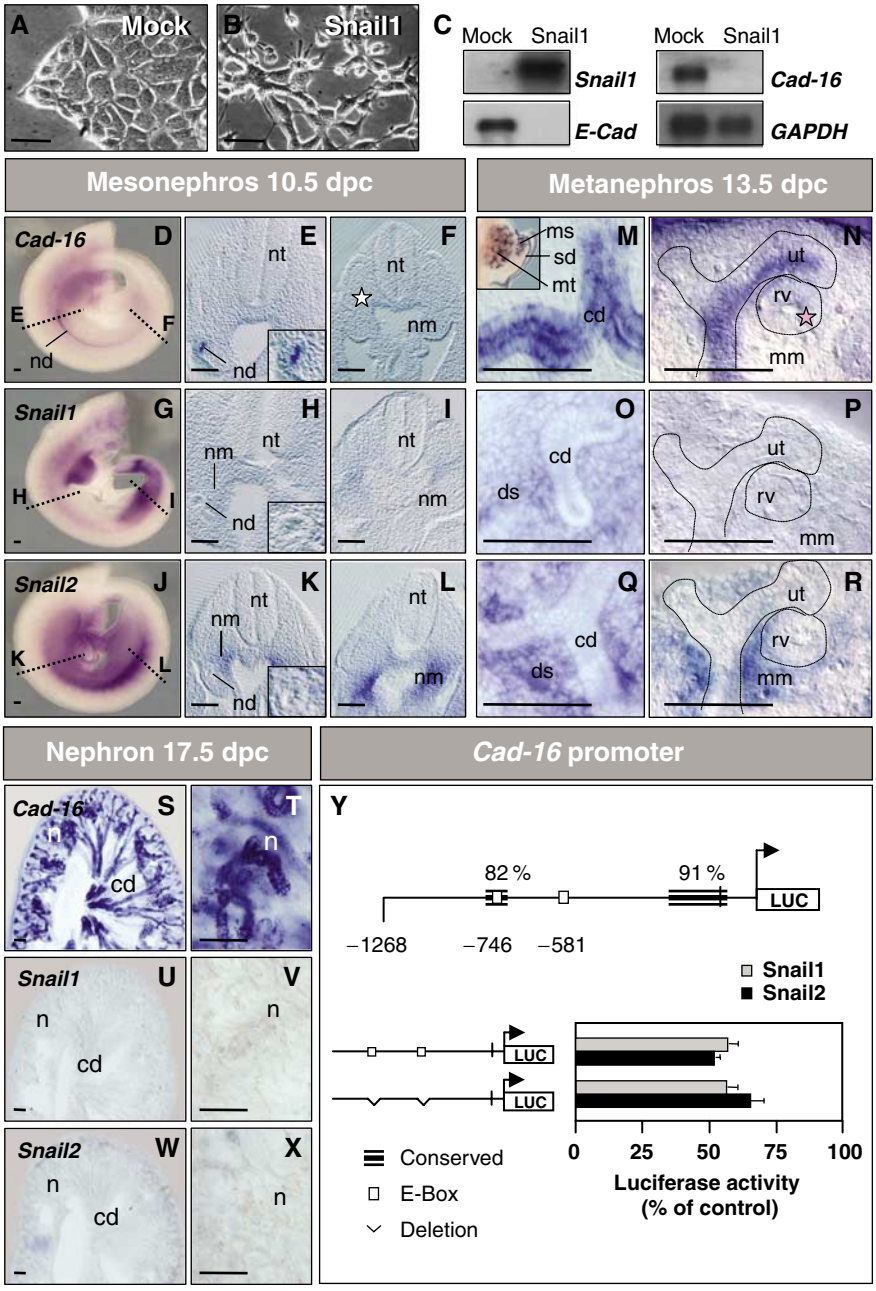

Figure 1 Snail1 represses the kidney epithelial *Cadherin-16* both in cell culture and in the embryo. (**A**, **B**) Phase-contrast images and (**C**) *Snail1*, *E-Cadherin* and *Cadherin-16* expression in stable mock- and *Snail1*-transfected cells. Snail1 expression represses *E-cadherin* and *Cadherin-16* transcription. *GAPDH* levels are shown as a control. (**D**–**X**) ISH for C*adherin-16*, *Snail1* and *Snail2* in whole-mount mouse embryos and transverse sections taken at the mid (E, H, K) and posterior (F, I, L) trunk levels. *Cadherin-16* is expressed in the newly formed nephric duct epithelium (nd, E) that no longer expresses *Snail* genes (H, K insets). *Snail* transcripts are observed in the undifferentiated anterior (H, K) and posterior (I, L) nephrogenic mesenchyme (nm). Dissected urogenital system (see M, inset) or gelatin sections (M–R) hybridized with *Cadherin-16* and *Snail* probes. *Cadherin-16* is expressed in the collecting duct epithelia (M) and their ureteric tips (ut, N) of the developing metanephros but it is absent from the newly forming renal vesicle (pink star, rv; N). Expression is also detected in the sexual ducts and in the tubules of the transient mesonephros (sd and ms; M, inset). *Snail1* and *Snail2* expression is restricted to the metanephric mesenchyme and to the deep stroma (mm and ds, O–R). Nephrons (n) appear after the complete epithelialization of the metanephric mesenchyme. The nephron epithelia and the collecting ducts (cd) strongly express *Cadherin-16* (S, T), whereas *Snail1* and *Snail2* expression disappears when the mesenchyme transforms into epithelia (U–X). nt, neural tube. Scale bar, 100 μm. (**Y**) Snail proteins repress the *Cadherin-16* promoter. Schematic representation of the mouse *Cadherin-16* promoter showing the regions of high similarity between mouse and human, and the location of the consensus Snail-binding sequences (white boxes). Luciferase reporter constructs carrying the wild-type mouse *Cadherin-16* promoter (−1268) or deletions in the two E-boxes were assayed in the NMuMG cells together with either the mouse Snail1 or Snail2 expression vectors or an empty vector as a control (pcDNA3). Luciferase activity was measured 24 h after transfection and the activity is expressed relative to that of the wild-type construct. The results are the mean values ± S.E. of duplicates from four independent experiments. Deletions of the Snail-binding sites do not relieve the repression of the *Cadherin-16* promoter activity.

Mock-transfected NMuMG cells were found to contain a high level of *Cadherin-16* transcripts, and its expression was completely downregulated in stable Snail1 transfectants (Figure 1C). Cadherin-16 is a kidney-specific cadherin expressed in renal tubular epithelial cells and collecting ducts of the differentiating and mature kidney (Thomson

et al, 1995). Hence, *Cadherin-16* expression was unexpected in a mammary gland-derived cell line and indeed, it was not expressed in the mouse mammary gland or in human mammary gland-derived cell lines (Supplementary Figure 2), and we concluded that Cadherin-16 expression was merely a peculiarity of NMuMG cells. Given the importance of MET and EMT in renal development, this result prompted us to determine whether Snail genes regulate Cadherin-16 expression in the kidney.

Inverse correlation between Cadherin-16 and Snail gene expression during kidney development

To determine whether Snail1 could repress Cadherin-16, we first examined the expression of each gene at different stages during murine kidney development. As Snail2 also represses *E-Cadherin* and it is considered functionally equivalent to Snail1 (del Barrio and Nieto, 2002; Bolós *et al*, 2003), we also analyzed its expression in the kidney. *Cadherin-16* expression commences during the differentiation of the developing kidney, concomitant with the MET that gives rise to the different renal epithelial cell populations (Wertz and Herrmann, 1999). The first renal epithelium to form is the nephric duct, derived from the intermediate mesoderm in the caudal part of the trunk region. Although the early mesoderm expresses *Snail1* in mouse embryos, the expression of the *Snail1* and *2* genes becomes more complex as the mesoderm segregates into distinct populations. As such, when *Cadherin-16* is not yet expressed in the embryo (not shown), *Snail2* expression is upregulated in the intermediate and lateral mesoderms (Sefton *et al*, 1998). Once the nephric duct became epithelialized, *Snail* genes expression was downregulated and *Cadherin-16* transcripts could be detected (Figure 1E, H and K). The mesenchyme adjacent to the nephric duct (nm, nephrogenic mesenchyme) expresses both *Snail* genes (Figure 1H and K) and later epithelializes to form the transient mesonephros. The resulting mesonephric tubules express high levels of *Cadherin-16* (Figure 1M, inset).

In the posterior embryo, *Snail* transcripts are expressed in the nephrogenic mesenchyme where the metanephros will form (Figure 1I and L). Strong *Cadherin-16* expression is observed in the collecting ducts of the metanephros that arise from the branching of the nephric duct, whereas *Snail* genes expression is maintained in the deep stroma (Figure 1M, O and Q). The ureteric tips of the collecting ducts also express *Cadherin-16* unlike the condensing mesenchyme that gives rise to the renal vesicle and is still devoid of transcripts (Figure 1N). In the metanephric mesenchyme that surrounds the renal vesicle and that gives rise to the nephrons, *Snail2* and weaker *Snail1* expression was detected (Figure 1P and R). Again, the epithelialization of this tissue was correlated with *Snail* downregulation (Figure 1M–R).

At 17.5 days post-coitum (dpc), the nephron displayed robust *Cadherin-16* expression (Figure 1S and T), having downregulated *Snail* genes by this stage (Figure 1U–X). This situation was maintained throughout adulthood (Supplementary Figure 3). Thus, the expression of the *Snail* and *Cadherin-16* genes is complementary during kidney ontogenesis and tissues that express *Cadherin-16* are derived from *Snail1*- and *Snail2*-expressing mesenchyme. These results are compatible with *Snail* genes acting as repressors of *Cadherin-16 in vivo*.

Direct repression of HNF-1β transcription by Snail genes prevents Cadherin-16 expression

Given the precedent of *E-Cadherin* (Batlle *et al*, 2000; Cano *et al*, 2000), we examined whether Snail proteins could directly repress *Cadherin-16* transcription. Two consensus E-boxes for Snail binding were identified at −581 and −746 bp in the mouse *Cadherin-16* promoter (Figure 1Y). A construct containing these two boxes within sequences able to recapitulate the physiological expression of *Cadherin-16* in the developing kidney (Whyte *et al*, 1999; Shao *et al*, 2002) was cotransfected with either *Snail1* or *Snail2* constructs. Promoter activity decreased to 61 and 56%, respectively, indicating that Snail proteins do indeed repress *Cadherin-16* transcription. However, the modification or deletion of the individual E-boxes (Supplementary Figure 4) or simultaneous deletion of both boxes did not impair this repression (Figure 1Y), indicating that Snail1 and 2 do not directly downregulate *Cadherin-16* expression.

As Snail proteins act as transcriptional repressors (Nieto, 2002), we speculated that they might downregulate an activator of *Cadherin-16*. The HNF-1β transcription factor promotes the epithelial phenotype in the kidney by strongly activating *Cadherin-16* expression (Bai *et al*, 2002). Thus, we compared *HNF-1*β expression with that of *Cadherin-16* during kidney development. *HNF-1*β was expressed in the differentiated epithelia of the anterior nephric duct (Figure 2B), where *Snail* genes had been downregulated and *Cadherin-16* transcripts were found at 10.5 dpc (Figure 1E, H and K, insets). At this stage, *HNF-1*β transcripts were also detected in the differentiating posterior nephric duct (dnd, white star; Figure 2C), where *Cadherin-16* was not yet expressed (white star; Figure 1F). In the cortex of the metanephros at 13.5 dpc, *HNF-1*β expression was observed in the ureteric tips of the collecting ducts and in the forming renal vesicles that were still devoid of *Cadherin-16* transcripts (Figures 1N and 2D and G). However, *Cadherin-16* was expressed strongly in the previously differentiated collecting ducts (Figure 2E), while the tips of the collecting ducts and the renal vesicles are surrounded by *Snail2*-expressing cells yet to condense (Figure 2F). Accordingly, *HNF-1*β transcripts were detected in the areas of epithelializing mesenchyme (Figure 2D and G), some of which do not yet express *Cadherin-16* (compare Figures 1F with 2C, white stars, and Figures 1N with 2G, pink stars). The differentiated nephrons that give rise to functional epithelial structures of the definitive kidney express both *HNF-1*β (Figure 2H and *Cadherin-16* (Figure 1S and T). Thus, the expression of HNF-1β is compatible with it acting as an activator of *Cadherin-16*.

We detected *HNF-1*β transcripts in NMuMG cells, explaining the high levels of *Cadherin-16* expression, but both genes were completely downregulated in stable Snail1 transfectants (not shown and Figure 1C). To determine if activating Snail was sufficient to repress the expression of these genes, we used a chimeric construct in which Snail1 could be activated by tamoxifen (Snail1-ER; see Materials and methods). Snail activation in stable transfectants triggered EMT within 24 h (Figure 3A) analyzed by the loss of E-cadherin, reorganization of the actin cytoskeleton and the acquisition of motile and invasive properties (not shown, but see Supplementary Figure 1). These phenotypic changes were concomitant with the downregulation of *HNF-1*β and *Cadherin-16* (Figure 3C). Interestingly, within 6 h of Snail1 activation *HNF-1*β levels fell

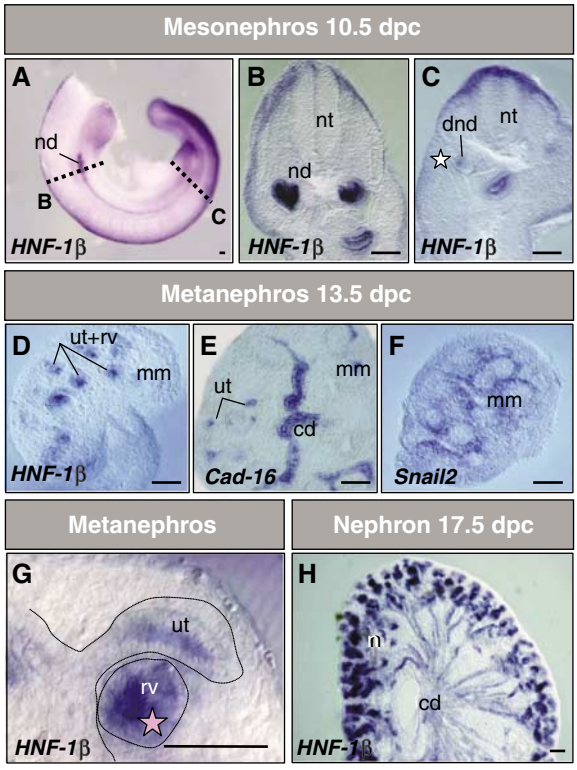

Figure 2 *HNF-1*β expression precedes that of *Cadherin-16* in the developing kidney epithelia. (**A–H**) ISH of embryos or dissected kidneys. *HNF-1*β expression was observed in the epithelia at all stages of kidney development. At 10.5 dpc, *HNF-1*β transcripts are seen in the nephric duct (nd, B) and in the condensing mesenchyme of the differentiating posterior nephric duct (dnd, C), which is still devoid of *Cadherin-16* transcripts (white star in Figure 1F). At 13.5 dpc, in addition to the collecting duct (cd), the ureteric tips of the ducts (ut) and the renal vesicle (rv) contain *HNF-1*β transcripts (D, G), some of which remain negative for *Cadherin-16* (E and pink star in Figure 1N). *Snail2* is expressed in the metanephric mesenchyme (mm) but not in the areas expressing *HNF-1*β. (H) At 17.5 dpc, the epithelia of the nephrons (n) and the collecting ducts express high levels of *HNF-1*β and both *Snail* genes have been downregulated (Figure 1U–X). nt, neural tube; scale bars, 100 μm.

by 20%, whereas *Cadherin-16* transcripts remained unaffected (not shown), indicative of the sequential downregulation of *HNF-1*β and *Cadherin-16* and suggesting that Snail1 inhibits Cadherin-16 expression by repressing *HNF-1*β. Indeed, the two consensus sequences for Snail binding in the *HNF-1*β promoter indicate that Snail proteins could directly repress *HNF-1*β transcription. The most proximal of these was conserved and lies in a highly conserved region in mouse, rat and human (Figure 3D). Accordingly, both Snail proteins repressed *HNF-1*β promoter activity when assayed using a reporter construct that included both these boxes (Figure 3D). Moreover, while deletion of the distal nonconserved E-box did not affect repression, deleting the conserved box abrogated inhibition (Figure 3D).

Chromatin immunoprecipitation (ChiP) further demonstrated that Snail1 binds to the *HNF-1*β promoter (Figure 3E), repressing its transcriptional activity and surely accounting for the subsequent downregulation of *Cadherin-16* expression. This interaction is specific for the fragment containing the conserved E-box, as *HNF-1*β promoter sequences were not recovered when a nonconserved promoter region was used. Indeed, Snail1 bound in a similar manner to the *HNF-1*β promoter sequences as to *E-cadherin* promoter sequences (Figure 3E). As NMuMG cells are derived from the mammary gland, we examined whether this repression also occurred in a kidney cell line. Indeed, *HNF-1*β and *Cadherin-16* transcription was completely downregulated by Snail1 in MDCK cells (Supplementary Figure 5).

Snail activation induces renal fibrosis in transgenic mice

As *Snail* genes repress *HNF-1*β expression in culture and consequently the epithelial phenotype, we assessed whether they behave similarly *in vivo*. More importantly, we assessed whether they could induce full EMT in the kidney and thus, the loss of the epithelial homeostasis. A transgenic mouse expressing the same tamoxifen-inducible Snail1-ER construct was generated identifying a line that specifically expressed significant levels of transgenic protein in the kidney (Supplementary Figure 6). This inducible system is appropriate to analyze transcription factor activity since despite its constitutive expression, the exogenous protein is only active after nuclear translocation upon tamoxifen administration (Figure 4A–H). In these animals, nuclear exogenous Snail1 protein was observed after two weeks of subcutaneal tamoxifen administration (Figure 4B, D, F and H). Both *HNF-1*β and *Cadherin-16* were downregulated in the cortex and the medulla of kidneys from tamoxifen-treated mice (Figure 4I–P and U). Interestingly, we also observed the induction of *Snail2* expression (Figure 4Q–T and U), the family member whose expression is prominent during kidney development. These data are in agreement with our previous finding that Snail1 induces *Snail2* transcription during neural crest development in *Xenopus* embryos (Aybar *et al*, 2003).

The downregulation of *Cadherin-16 in vivo* can be explained by Snail1 repressing *HNF-1*β transcription and the loss of both markers should be associated with the loss of epithelial characteristics. Indeed, upon Snail activation, collecting duct cells in the medulla seemed to acquire a fibroblast-like morphology (Figure 4F). The phenotype of the transgenic kidneys with active Snail1 was further analyzed and the collecting ducts in the medulla were clearly disorganized (Figure 5B). This disorganization correlated with the morphological changes characteristic of EMT (Figure 5B inset) and the repression of epithelial markers such as *HNF-1*β, *Cadherin-16* (Figure 4) and also *E-Cadherin* (Figure 5C and D). In addition, cells expressed mesenchymal markers such as the intermediate filament vimentin (Figure 5E and F) and smooth muscle actin (SMA, Figure 5G and H). These histological changes are reminiscent of those observed after experimentally induced or pathological fibrosis (Li *et al*, 2003; Sato *et al*, 2003; Zeisberg *et al*, 2003; McMorrow *et al*, 2005; Slattery *et al*, 2005). The deposition of Collagen I is a hallmark of renal fibrosis (Alexakis *et al*, 2005) and focal deposition was observed after the activation of *Collagen I* transcription by Snail1 (Figure 5I–L). Epithelial disorganization was also observed in the cortex, where dilated tubules and disrupted basement membranes were readily apparent as well as Collagen I deposition (Figure 5M–T). The mice died after about 2 weeks of treatment, presumably of renal failure given the distorted morphology of the kidney and the high levels of urea in their serum (not shown).

As the postnatal kidney may be considered immature or prone to revert to the embryonic phenotype, it was important to check whether Snail1 activation alone could induce

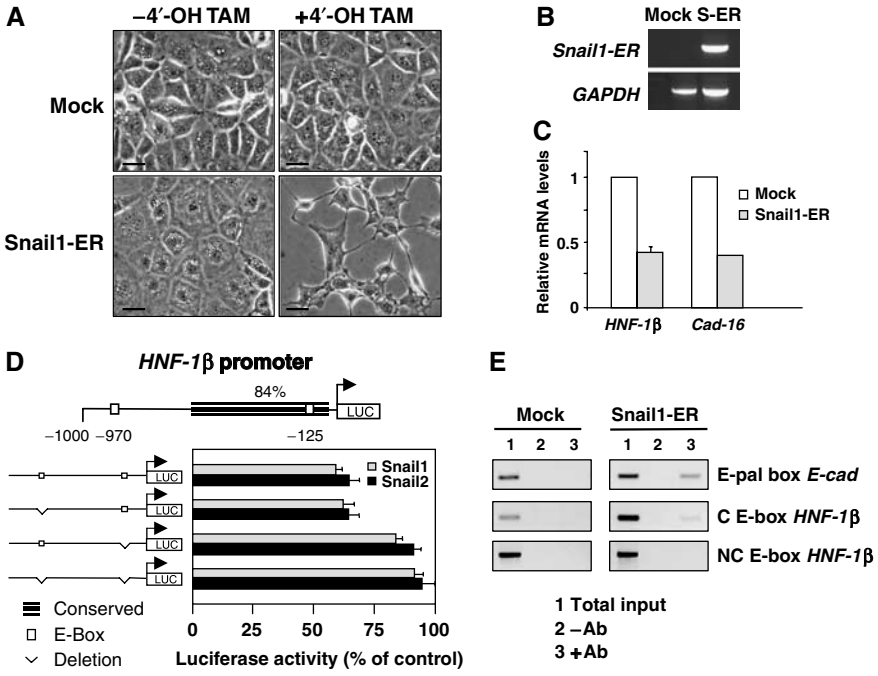

Figure 3 *Snail* genes directly repress *HNF-1*β transcription, which in turn impairs *Cadherin-16* expression. (**A**) NMuMG cells stably transfected with an inducible Snail1 construct (Snail1-ER) or with the empty vector (Mock) were analyzed 24 h after induction. Note the phenotypic change of the Snail1-transfected cells upon 4′-OH-tamoxifen (4′-OH-TAM) administration. Scale bar, 25 μm. (**B**) Transgene expression visualized by RT–PCR in Mock and Snail1-ER transfectants (S-ER). (**C**) Quantitative RT–PCR for *Cadherin-16* and *HNF-1*β 24 h after 4′-OH-tamoxifen administration. (**D**) Diagram of the 1 kb region upstream of the translational initiation site in the mouse *HNF-1*β gene showing regions highly conserved between mouse and human. Of the two consensus E-boxes for Snail binding, only one lies within the conserved region. Snail1 and Snail2 repressed the activity of the wild-type *HNF-1*β promoter (measured as described for the *Cadherin-16* promoter), but they did not affect the promoter constructs in which the conserved E-box was deleted. (**E**) ChiP analyses show that Snail1 binds directly to the *HNF-1*β promoter. ChIP assays were carried out with anti-ER antibodies on Mock- and Snail1-ER cells 24 h after induction. As a positive control, the interaction of Snail with the E-pal element of the *E-cadher*in promoter is shown. Snail does not bind to the nonconserved (NC) E-box in the *HNF-1*β promoter. Amplifications of the indicated promoter regions in the input (1), nonimmunoprecipitated (2) and immunoprecipitated (3) fractions are shown. The data presented are representative of three independent experiments.

fibrosis in the adult kidney. When tamoxifen was administered for 2 months commencing 2 months after birth (Figure 6), the collecting ducts and renal tubules became disorganized and dilated (Figure 6A–D), and Collagen I deposits were formed (Figure 6E–H). A feature frequently associated with renal fibrosis is the appearance of cysts, a phenomenon that was incipient in the postnatal transgenic kidneys following Snail activation. As the young mice died very early, we could not follow the progress of this putative polycystic kidney disease. Nevertheless, we observed the appearance of scattered cysts in the adult kidneys when Snail1 was activated over several weeks (Figure 6D).

The loss of epithelial features perfectly correlated with Snail activation since the transgenic protein was expressed in the renal tubules and the collecting ducts, but it was absent from the glomeruli and the interstitial cells (Figure 6O). Accordingly, Snail1 activation did not induce morphological changes or *Snail2* expression in the glomeruli of postnatal animals (yellow diamond in Figure 4D, G and T) and adult mice (yellow arrow in Figure 6O). In the medulla of adult mice, the transgenic protein displayed a mosaic distribution. A tubular structure was maintained in ducts that did not express Snail1 or when the protein was not efficiently translocated to the nucleus upon tamoxifen administration (Figure 6N, yellow diamond and triangle, respectively). However, when Snail1 reached the nucleus, the tubules were disorganized and the epithelial structure was lost in both the medulla and the cortex (Figure 6N and P, yellow stars). The mosaic expression observed in the adult tubules explains why epithelial structures are less disorganized than in the young animals. These results provide an internal control of the activity of the Snail1 protein and highlight its ability to repress the epithelial phenotype in a cell autonomous manner. Indeed, the activation of Snail1 is sufficient to induce the features of renal fibrosis in both young and adult animals.

Snail genes are pathologically active during human renal fibrosis

We next analyzed normal and fibrotic human kidney tissue obtained from patients subjected to nephrectomy (see Materials and methods). Features of renal fibrosis were observed in the tissue from patients affected by renal failure, including dilation and tubule disorganization in the medulla and cortex (Figure 7B and H). Strong vimentin expression (Figure 7D and J) was accompanied by deposition of Collagen I in the sclerotic areas of the tubule and massive interstitial fibrosis in both the cortex and the medulla (Figure 7F and L). While no *Snail1* or *Snail2* expression was observed in the normal human tissue (Figure 7M; $n=4$, see also Supplementary Figure 3), high levels of *Snail1* were detected in patients with renal fibrosis (Figure 7M). As in the experimental mouse model, *Snail2* was also expressed in the fibrotic tissue. Interestingly, the presence of fibrotic and

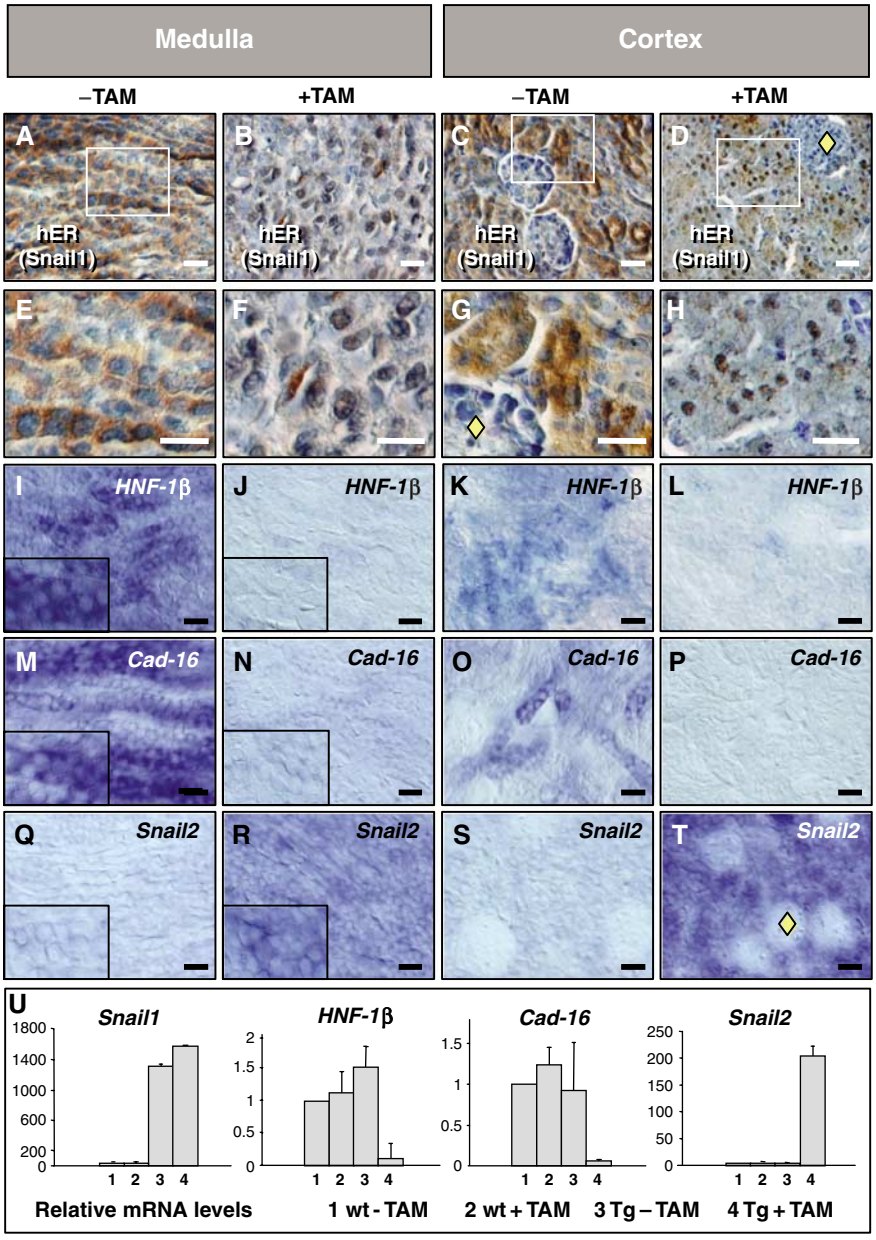

Figure 4 Snail expression induces the loss of the epithelial character in postnatal transgenic kidneys. (**A**–**H**) Exogenous Snail1 protein was translocated to the nucleus upon tamoxifen (TAM) administration, as seen with an anti-hER antibody in both the medulla and the cortex. Note the absence of the transgenic protein in the glomeruli (yellow diamonds). (I–P) *HNF-1*β (**I**–**L**) and *Cadherin-16* expression (**M**–**P**) in sections from 2-week-old transgenic kidneys show the loss of the two epithelial and differentiation markers. (**Q**–**T**) Snail1 activation also induces *Snail2* expression but not in the glomeruli (yellow diamond in T). (**U**) Quantitative RT–PCR analysis of *Snail1, HNF-1*β, *Cadherin-16* and *Snail2* expression in wild-type and transgenic kidneys in the absence or presence of TAM. Transcription is normalized to *GAPDH* mRNA expression and the error bars represent the standard error of the mean.

nonfibrotic tissue in the same kidney showed that Snail activation was only associated with fibrosis (Figure 7M). Thus, human fibrosis is accompanied by the aberrant activation of *Snail* expression.

Discussion

The data presented here implies that Snail not only represses the prototypical epithelial cadherin, E-Cadherin, but also the kidney-specific *Cadherin-16*. This repression is indirect, via the well-characterized regulator of renal differentiation, *HNF-1*β, in contrast with Snail's direct transcriptional suppression of *E-Cadherin* through promoter binding. This defines a new pathway contributing to Snail's activity as a general epithelial repressor and in the disruption of tissue homeostasis, leading to the appearance of pathological features common to end-stage renal failure.

Genetic observations in mice have shown that HNF-1β is important for normal kidney function. Polycystic renal disease is seen in transgenic mice expressing either a mutant HNF-1β under the control of the *Cadherin-16* promoter (Hiesberger *et al*, 2004) or carrying a kidney-specific deletion (Gresh *et al*, 2004). HNF-1β inhibits the autosomal-recessive polycystic kidney disease gene, *Pkdh1* (Hiesberger *et al*,

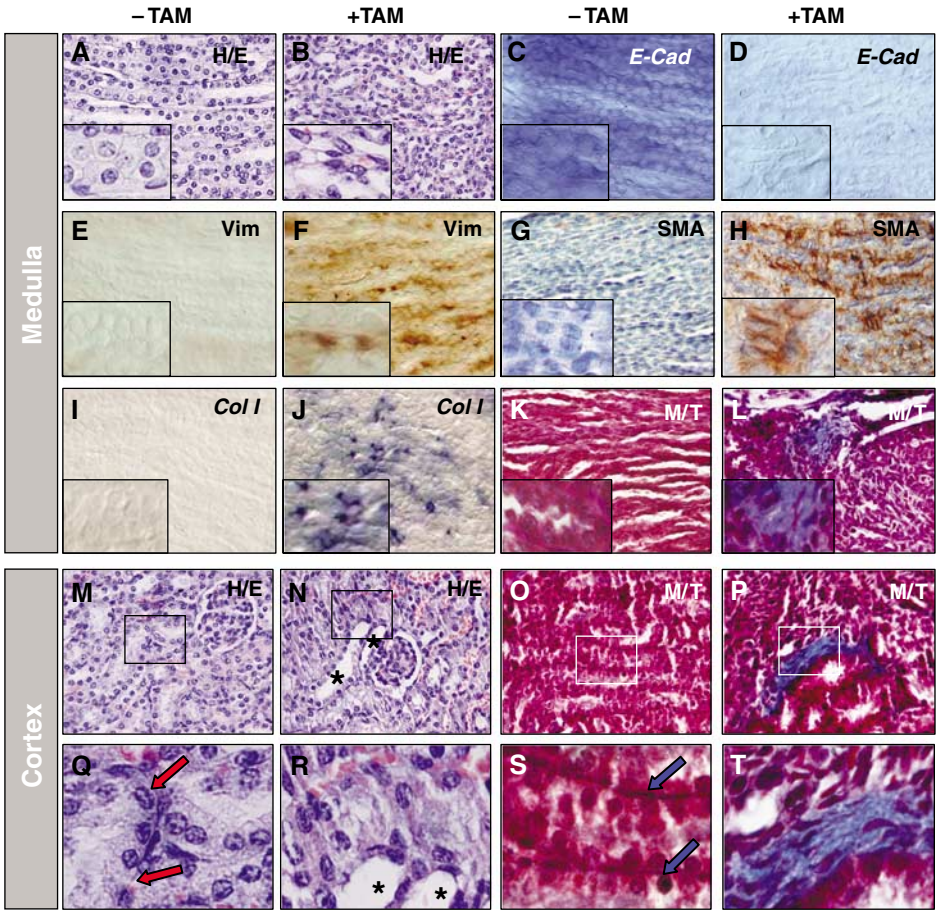

Figure 5 Snail expression induces EMT and features of fibrosis in postnatal transgenic mice. (**A**, **B**) Hematoxylin/eosin staining of sections from the kidneys shown in Figure 4. Note the depolarized and fibroblast morphology of the collecting duct cells in the medulla region of animals treated with tamoxifen (+TAM). (**C**, **D**) *E-Cadherin* expression is completely downregulated in the kidneys of transgenic mice upon Snail1 activation, confirming the loss of epithelial character. (E–H) Snail 1 activation also induces the expression of vimentin (**E, F**) and smooth muscle actin (SMA, **G, H**), both indicating the appearance of mesenchymal characteristics. (I–L) *Collagen I* transcripts (**I, J**) and Collagen fibrotic deposits (**K, L**) detected by Masson–Trichrome staining in the transgenic kidneys upon Snail1 activation. (M–T) Similar signs of epithelial disruption and fibrosis are also observed in the cortex of the transgenic kidneys from tamoxifen-treated mice. Hematoxylin/eosin staining (**M, N**) and collagen deposits (**O, P**). (**Q–T**) High-power images to better assess the dilation of renal tubules (asterisks) and the disruption of basement membranes upon Snail1 activation. Basement membranes that can be clearly observed in the untreated transgenic kidneys (arrows).

2004) and other genes in which mutations are associated with cystic diseases (Gresh *et al*, 2004). Thus, *Snail* genes can now be added to this transcriptional network, as upstream effectors and the first known repressors of HNF-1β. Interestingly, mutations in HNF-1β cause abnormalities in human renal development (Edghill *et al*, 2006), including cysts, familial hypoplastic glomerulocystic disease, malformations and atypical familial hyperuricemic nephropathy. Our finding that *Snail* genes repress the expression of HNF-1β suggests that genetic or epigenetic alterations influencing Snail expression or activity can cause renal developmental abnormalities independently of HNF-1β mutations. Interestingly, our adult mice in which Snail gene expression was reactivated showed a milder cystic phenotype than that described when *HNF-1*β function is lost in mouse models (Gresh *et al*, 2004; Hiesberger *et al*, 2004). This is very likely due to the fact that in those models the inactivation of *HNF-1*β occurs at an early developmental stage, as the transgene is under the control of the *Cadherin-16* promoter. However, in our model, inactivation was induced in the mature kidney that had developed normally but nevertheless, we show that Snail1 activation in the adult kidney was sufficient to drive epithelial to mesenchymal transition and to favor the deposit of Collagen. Thus, Snail is a good candidate to coordinate the fibrosis and cyst formation that occurs in human renal disease.

Kidney fibrosis and scarring is thought to be a key process linking progressive loss of renal function with a wide range of primary diseases such as glomerulonephritis (including IgA nephropathy), diabetes, toxic injury, congenital abnormalities, urinary tract obstruction and chronic rejection of transplanted kidneys (Kalluri and Neilson, 2003; Liu, 2004; Zeisberg and Kalluri, 2004; Vongwiwatana *et al*, 2005). Although renal fibrosis was believed to be associated with the activation of interstitial fibroblasts, myofibroblasts also appear to originate from renal tubular epithelial cells that undergo an EMT (Strutz *et al*, 1995; Iwano *et al*, 2002). There is evidence that tubular EMT occurs in the kidneys of patients with renal fibrosis (Jinde *et al*, 2001; Rastaldi *et al*, 2002) and in animal models where as much as 36% of the fibroblasts involved seem to be derived from local tubular epithelial cells (Kalluri and Neilson, 2003).

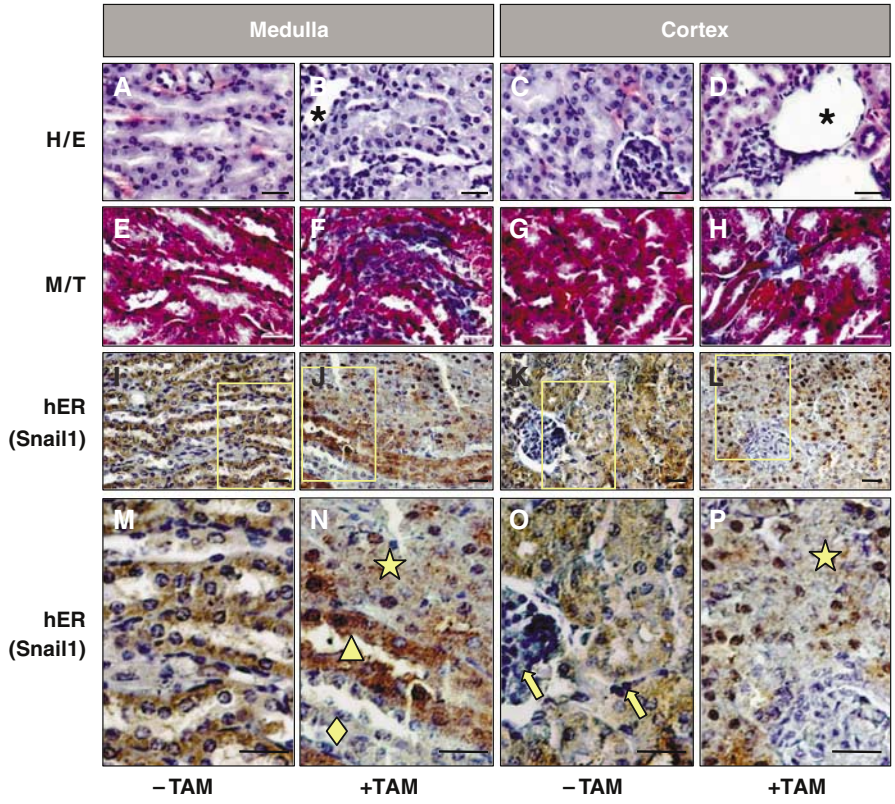

Figure 6 Snail activation is sufficient to induce renal fibrosis in adult transgenic mice. (**A–D**) Hematoxylin/eosin staining of sections from 4-month-old transgenic mice kidneys. Tamoxifen treatment in mice (+TAM) was initiated 2 months after birth. Note the defective overall morphology including the presence of dilated tubules. (**E–H**) Fibrotic deposits can be observed by Masson–Trichrome staining in tamoxifen-treated mice. Fibrosis is also manifested by the presence of cysts (D). (**I–P**) Detection of the transgenic Snail1 protein with an anti-hER antibody (brown) in paraffin sections counterstained with hematoxylin (blue). Note the absence of transgenic protein in the glomeruli and in the interstitial cells (yellow arrows in O) and in some ducts in the medulla (yellow diamond in N). The yellow triangle in (N) indicates ducts in which the Snail1 protein has not been efficiently translocated to the nucleus (note the blue nuclei and brown cytoplasms). The ducts and tubules with Snail1 nuclear expression lost the epithelial character and present a completely disorganized structure (yellow star in N and P). Scale bars, 25 μm.

We show that Snail1 activation in the kidney promotes EMT of both tubular and collecting duct cells inducing fibrosis. Although studies *in vitro* suggest that medulla cells are resistant to EMT (Liu, 2004), it does not strike us as strange to find both the cortex and the medulla affected by Snail *in vivo*. Both epithelial cells from the renal collecting duct and the tubular epithelium are derived from Snail-expressing embryonic mesenchyme (the intermediate mesoderm and the metanephric mesenchyme, respectively). Hence, pathological activation of Snail in the adult could be regarded as a return to the embryonic phenotype, evidence of the reversibility of these processes and the restoration of a plastic state. Importantly, this also highlights the relevance and consequences of the reactivation of Snail in the adult, capable of inducing EMT and fibrosis in a cell-autonomous manner.

Although *Snail* genes play important roles during embryonic development, it seems critical that they are maintained silent in the adult. Epigenetic mechanisms are probably fundamental in this silencing, and are presumably reversed when Snail is activated in renal fibrosis and cancer. Indeed, we have found aberrant Snail expression in the fibrotic areas of human kidneys and higher levels of Snail expression due to promoter demethylation have been correlated with the increase in the invasive properties of carcinoma derived cell lines (Fraga *et al*, 2004). This supports the idea that the reversal of silencing or transcriptional regulation rather than irreversible genetic loss contributes to tumor progression (Cheng *et al*, 2001) and perhaps, to organ fibrosis. Interestingly, changes in expression related to epigenetic mechanisms have been associated with aging (Burzynski, 2005) and the aged kidney is much more susceptible to fibrosis (Melk, 2005).

It seems likely that the pathological activation of Snail may contribute to other fibrotic diseases. Thus, it would be interesting to analyze Snail's involvement in lung and cavernous fibrosis (Chilosi *et al*, 2003; Ryu *et al*, 2005) and even in the EMT of epithelial cells, pericytes and other progenitors from the circulatory pool during systemic sclerosis and related rheumatic diseases (Postlethwaite *et al*, 2004). In relation to this, Snail is upregulated during the EMT undergone by mesothelial cells in patients treated with peritoneal dialysis (Yañez-Mo *et al*, 2003), renal cells following unilateral ureteral obstruction in mice (Sato *et al*, 2003) and hepatocytes (Valdés *et al*, 2002). Interestingly, these pathological processes can be induced by TGF-β, a potent Snail activator (Barrallo-Gimeno and Nieto, 2005). As abnormal high levels of TGF-β have been associated with renal fibrosis (Liu, 2006) and activation of Snail is sufficient to trigger fibrosis in the adult, we propose that Snail may be the key transducer of TGF-β signalling in these pathologies. Importantly, the antagonism of TGF-β by BMP7 seems to be

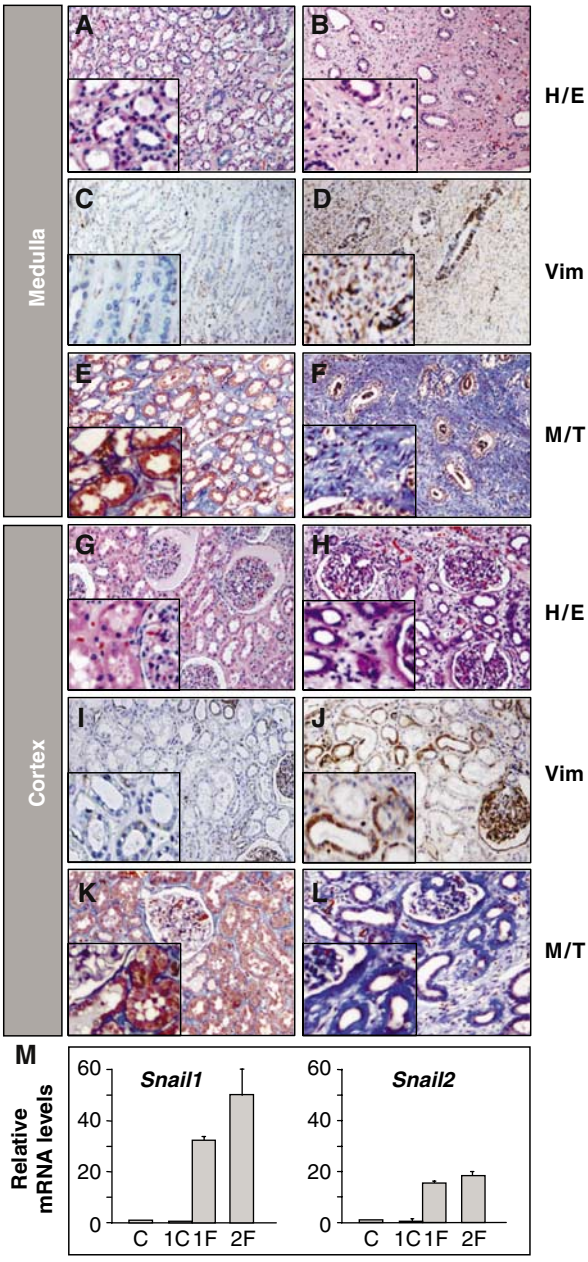

Figure 7 Fibrotic human kidneys show strong *Snail* expression. Sections from normal human kidney tissue (**A**, **C**, **E**, **G**, **I**, **K**) and from a patient subjected to nephrectomy due to urinary obstruction and kidney failure (**B**, **D**, **F**, **H**, **J**, **L**) showing hematoxylin/eosin staining (H/E), vimentin expression (Vim) and fibrotic deposits in blue following Masson–Trichome staining (M/T). (**M**) Quantitative RT–PCR analysis of *Snail1* and *Snail2* expression in normal human kidney tissue (C, $n=4$), nonfibrotic (1C) and fibrotic tissue from patient 1 (1F) and patient 2 (2F). Transcript levels are normalized to GAPDH mRNA expression and the error bars represent the standard error of the mean.

a promising strategy to treat renal fibrosis as BMP7 can reverse TGF-β-induced renal fibrosis in mice (Zeisberg *et al*, 2003; Zeisberg and Kalluri, 2004), and mice mutant for the BMP signaling activator kielin are more susceptible to the disease (Lin *et al*, 2005). Signalling molecules such as TGF-β and BMPs trigger complex transduction cascades. Thus, if Snail transduces the pathological TGF-β signal and its sole activation induces all the features of renal fibrosis, inhibiting Snail activity could provide a more specific way to prevent or reverse the disease. In summary, we show that the reactivation of otherwise silent developmental pathways leads to loss of adult tissue homeostasis, and may make a major contribution to the progression of pathological conditions, in particular those associated with aging and degenerative processes.

Materials and methods

Plasmids and antibodies

Expression plasmids. pcDNA3-*Snail1* and pcDNA3-*Snail2* contain the mouse *Snail1* and *Snail2* cDNAs, respectively. pcDNA3-Snail1-ER (CMV promoter) contains the Snail1-coding sequence fused to a mutated version of the ligand-binding domain of the human estrogen receptor that recognizes the synthetic ligand 4′-OH-Tamoxifen. The fragment corresponding to the binding site was obtained from pCre-ER^{T2} kindly provided by P Chambon (Feil *et al*, 1996). This construct was used to generate the stable Snail1-inducible NMuMG transfectants and for the generation of Snail1-inducible transgenic mice (see below). *Reporter plasmids*. The mouse *Cadherin-16* promoter reporter construct pKsp(1268F)-*Luc* was a kind gift from Dr Peter Igarashi (U Texas Southwestern Medical Center, Dallas, TX). The mouse *HNF-1*β promoter sequence containing 1072 bp upstream of the ATG was amplified by PCR from NMuMG cells DNA using the primers: 5′-ggtaccATCTACACATTCAC TACTAGA-3′ and 5′-acgcgtTTTCCAAGGACGGAAAAAGAA-3′ (GenBank™ sequence X55842 plus *Kpn*I and *Mlu*I restriction sites, respectively). The purified PCR product was subcloned into the pGL3-basic vector (Promega). The Quickchange Site Directed Mutagenesis kit (Stratagene) was used to mutate the E-boxes in the mouse *Cadherin-16* promoter. The core 5′-CA(G/C)(G/C)TG-3′ sequence was mutated to 5′-AA(G/C)(G/C)TA-3′. The sequences of the oligonucleotides used to delete the E-boxes on the *Cadherin-16* and *HNF-1*β promoters are available upon request.

Antibodies. Anti-E-cadherin (1:200, Takara), anti-vimentin (1:200, Dako), anti-smooth muscle actin (1:200, Dako). The Snail1-ER fusion protein was detected by immunoblotting or immunohistochemistry using an anti-human estrogen receptor antibody (1:100, Santa Cruz). F-actin was detected using FITC-phalloidin (1:10, Sigma).

Cell culture and generation of stable Snail1- and Snail1-ER-transfected cells

For transfection studies, the NMuMG and MDCK lines were plated in six-well plates (5×10^4 cells per well) in a 1:1 mixture of Ham's F12 Medium and DMEM supplemented with 100 IU/ml penicillin, 0.1 mg/ml streptomycin, 2 mM glutamine, 2.5 μg/ml amphotericin B, 10% fetal bovine serum and 10 μg/ml insulin. DNA constructs for constitutive or inducible Snail1 expression or control vectors (500 ng) were added to the cells 24 h after seeding in the presence of LIPOFECTAMIN (Roche) and the transfected cells were selected for neomycin resistance 24 h later using G-418 (Calbiochem, 400 μg/ml). Two independent *Snail1*- and *Snail1-ER* transfectant clones were isolated in each case.

Migration and invasion assays

Cells were seeded in six-well culture dishes at a density of 3×10^5 cells/well. A wound was made in the center of the confluent culture 24 h later, and after washing and adding fresh medium, phase-contrast pictures were taken of the wounded area at different intervals. Invasion assays on collagen type IV gels were carried out using the two-compartment Boyden chambers (Cano *et al*, 2000). Having carefully removed the cells from the upper surface after 8 h, the fixed cells on the lower surface of the filters were stained with DAPI.

Analysis of gene transcripts

Poly(A)$^+$ mRNA was isolated using the Microfast Track kit (Invitrogen). For Northern blots, 1.5 μg aliquots of Poly(A)$^+$ oligo(dT)-cellulose-purified mRNA were transferred to nylon membranes and hybridized with [α-^{32}P]dCTP-labelled probes (*redi*prime II, Amersham Biosciences) for mouse *Snail1*, *E-Cadherin*, *Cadherin-16* and *GAPDH*. RT–PCR for *Snail1* and

GAPDH was carried out as described (Cano *et al*, 2000). Quantitative RT–PCR was carried using an ABI PRISM® 7000 sequence detection system and TaqMan® probes. The primers and probes were obtained from Applied Biosystems Assays-on-Demand as follows: Mm-99999915-g1 (GAPDH), Mm-00441533-g1 (Snail1), Mm 00441531-m1 (Snail2), Mm-00483196-m1 (Cadherin-16), Mm-00447452-m1 (HNF-1β), Hs 99999905-m1 (GAPDH), Hs 00193591-m1 (SNAI1), Hs 00161904-m1 (SNAI2), Hs 00187880-m1 (CADH16) and Hs 00172123-m1 (HNF1 β). RNA expression was calculated using the comparative C_t method normalized to GAPDH. Data are expressed relative to a calibrator (mock cells or wild-type mice) using the $2^{-(\Delta\Delta Ct)} \pm$SD formula. RNA was extracted and cDNA synthesized from cultured Snail1-ER and mock-transfected cells at different times after 4′-OH-Tamoxifen administration and from the kidneys of wild-type and transgenic mice after different periods of tamoxifen or vehicle administration.

In situ hybridization

Mouse embryos were obtained from Balb-C mice and their ages established in dpc, the day on which the vaginal plug was detected being designated 0.5 dpc. The urogenital systems or isolated kidneys were removed at stages 13.5 and 17.5 dpc, respectively, fixed in 4% paraformaldehyde and either processed directly for *in situ* hybridization (ISH), or gelatin embedded to obtain 50 μm vibratome sections. ISH was performed as described previously (Cano *et al*, 2000), using mouse *Snail1*, *Snail2* and *E-Cadherin* probes (Sefton *et al*, 1998; Cano *et al*, 2000). The mouse *Cadherin-16* and *HNF-1*β probes were obtained by RT–PCR from NMuMG cells cDNA using the primers (sense/antisense): *Cadherin-16*, 5′-ACTGTAGAGATCTCCATAGTA-3′/5′-CTCCACTAGTATAGTCACTGT-3′; *HNF-1*β, 5′GACACTCCTCCCATCCTCAA-3′/5′-TGTAGCGCACTCCT GACATC-3′. The human *Cadherin-16* and *Snail2* probes were obtained by RT–PCR from human kidney and lung cDNA obtained from BD Biosciences. Amplification was performed with the primers (sense/antisense): human *CDH16*, 5′-GCAGAGCTGTCT GTGGAAGTT-3′/5′-CTCCAGGTGATAGTGCACATC-3′; human *SNAI2*, 5′-CTGTAGGGACCGCCGTGT-3′/5′-GCTTCGGAGTGAAGAAATGC-3′. Human *Snail1*, *Snail2* and *E-Cadherin* were analyzed in renal biopsies as described (Blanco *et al*, 2002). After hybridization, whole embryos or free-floating slices were processed as described (Cano *et al*, 2000).

Promoter analyses

Cadherin-16 and *HNF-1*β promoter activities were measured by cotransfecting NMuMG cells with 50 ng of pcDNA3-*Snail1*, pcDNA3-*mSnail2* or empty pcDNA3 vector and with 300 ng of pKsp(1268F)-*Luc* or 400 ng of pmHNF-1β-*Luc*. A *Renilla reniformis* luciferase plasmid (phRL-CMV-*Luc* from Promega) was also cotransfected as a control of efficiency. At 24 h after transfection, firefly luciferase (Luc) and *renilla* luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Luc activity was normalized to *renilla* luciferase activity. In all experiments, the total amount of DNA transfected was standardized by adding empty vector. The results are presented as the percentage of luciferase activity relative to controls (luciferase values in cells cotransfected with empty vector).

ChIP assays

Cells were crosslinked with formaldehyde before DNA sonication. The chromatin was immunoprecipitated with the anti-human ER antibody (see above) and sheared to an average length of 0.5–1 kb. A 252-bp fragment of the mouse *HNF-1*β promoter containing the conserved E-box was amplified with the primers (sense/antisense) 5′-ATCTTGCCGAAAGCTGAGCCC-3′/5′-TTTCCAAGGACGGAAAAA GAAG-3′ and a more distal 500-bp fragment of the promoter carrying the nonconserved E-box with 5′-CACATTCACTACTAG AAAGGA-3′/5′-CTCCAAATCTTCTCTACCGAT-3 to be used as a negative control. As a positive control, a 191-bp fragment from the *E-cadherin* promoter carrying the Snail1-binding E-pal element (Cano *et al*, 2000) was amplified with the primers: (sense/antisense) 5′-CATGCCACCAACTACAGACAG-3′/5′-AGTTCTTGGGA ACTCAGTAGT-3′.

Transgenic mice

A tamoxifen-inducible Snail1 transgenic mouse was generated according to standard procedures (Hogan *et al*, 1994) with the pcDNA3-Snail1-ER (see above) and a line was selected that expressed significant levels of the transgenic protein in the kidney. The Snail1-ER protein is constitutively expressed, but it is only functional when translocated into the nucleus upon tamoxifen administration. The subcellular localization of the protein and the level of expression were assessed by immunohistochemistry using a human estrogen-receptor antibody also used to assay the amount of Snail1-ER protein in the tissues obtained from 2-month-old transgenic mice in Western blots. A solution of Tamoxifen 30 mg/ml was sonicated and 30 or 200 μg of tamoxifen/gram body weight was injected subcutaneously into neonatal or intraperitoneally into 2-month-old animals every 3 days over different periods. The animals were killed and their kidneys processed for mRNA extraction, ISH or immunohistochemistry, or paraffin sections were stained with hematoxylin–eosin or Masson–Trichrome.

Human tissue samples

Normal kidney tissue was obtained from the unaffected areas of tumor nephrectomies (four cases) and fibrotic tissue from patients affected by kidney failure caused by urinary obstruction. Specimens approximately 1 cm^3 were fixed in 10% formaline or snap frozen in liquid nitrogen-cooled isopentane. The Research Ethics Committees of Sant Joan d' Alacant, Guy's and St Thomas's Hospitals approved the protocols to analyze the tissues, which were processed for real-time RT–PCR, ISH, immunohistochemistry and histology as described above.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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