High doses of TGF-β potently suppress type I collagen via the transcription factor *CUX1*

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ABSTRACT **Transforming growth factor-β (TGF-β) is an inducer of type I collagen, and uncontrolled collagen production leads to tissue scarring and organ failure. Here we hypothesize that uncovering a molecular mechanism that enables us to switch off type I collagen may prove beneficial in treating fibrosis. For the first time, to our knowledge, we provide evidence that *CUX1* acts as a negative regulator of TGF-β and potent inhibitor of type I collagen transcription. We show that *CUX1*, a CCAAT displacement protein, is associated with reduced expression of type I collagen both in vivo and in vitro. We show that enhancing the expression of *CUX1* results in effective suppression of type I collagen. We demonstrate that the mechanism by which *CUX1* suppresses type I collagen is through interfering with gene transcription. In addition, using an in vivo murine model of aristolochic acid (AA)-induced interstitial fibrosis and human AA nephropathy, we observe that *CUX1* expression was significantly reduced in fibrotic tissue when compared to control samples. Moreover, silencing of *CUX1* in fibroblasts from kidneys of patients with renal fibrosis resulted in increased type I collagen expression. Furthermore, the abnormal CUX1 expression was restored by addition of TGF-β via the p38 mitogen-activated protein kinase pathway. Collectively, our study demonstrates that modifications of *CUX1* expression lead to aberrant expression of type I collagen, which may provide a molecular basis for fibrogenesis.**

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INTRODUCTION

Fibrosis can affect most major organs of the body and is characterized by extensive tissue remodeling, end-stage organ failure, and lethality (Trojanowska *et al.*, 1998; Bartram and Speer, 2004). The primary cause of fibrosis is not yet fully understood but is likely to involve cell, organ, and environment-specific components (Wynn, 2007). Excessive type I collagen deposition is a common biological finding that leads to progressive scarring (Shi-Wen *et al.*, 1997; Wynn, 2008). TGF-β is a key player in fibrogenesis (Verrecchia and Mauviel, 2007) via promoting activation and proliferation of resident fibroblasts (Postlethwaite *et al.*, 2004), which leads to excessive synthesis of type I collagen (Cutroneo, 2003). TGF-β regulates *COL1A2* expression at the level of transcription via several mechanisms that involve both TGF-β canonical and noncanonical signaling (Inagaki *et al.*, 1994; Ponticos *et al.*, 2009).

Work from several laboratories, including our own, has studied the mechanisms of type I collagen regulation at the level of transcription. The transcription of *COL1A2* is tightly regulated by combinatorial interactions of specialized proteins known as transcription factors. The proximal promoter of *COL1A2* is under the control of a canonical CCAAT motif that is located at −80 base pairs relative to the transcriptional start site (TSS) and is recognized by a protein called CCAAT binding factor (CBF/nuclear factor [NF]-Y) (Maity *et al.*, 1988). Additionally, Stimulatory protein 1 and 3 (Sp1, 3) recognize the TCC-rich box that is located at −125 base pairs upstream of the TSS (Ihn *et al.*, 2001). Moreover, three GCC-rich sequences in the −300 base pair region are associated with binding of Sp1 (Inagaki *et al.*, 1994). Studies using point mutations of the CCAAT motif of

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Abbreviations used: AA, aristolochic acid; BSA, bovine serum albumin; C/EBP, CCAAT-enhancer binding protein; CBF, CCAAT binding factor; CMV, cytomegalovirus; dpi, days postinjection; ECM, extracellular matrix; EMSA, electrophoretic mobility shift assay; EV, empty vector; JNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein; NF, nuclear factor; PBS, phosphate-buffered saline; qPCR, quantitative PCR; Sp, Stimulatory protein; TGF-β, transforming growth factor-β; TSS, transcriptional start site.

COL1A2 revealed that single nucleotide base changes in the genetic code within the –80 base pair region of the COL1A2 promoter led to defective transcription of type I collagen gene in transgenic animals (Tanaka *et al.*, 2004). In addition to positive regulation of the gene, portions of the *COL1A2* promoter that have been shown to be involved in negative regulation of the *COL1A2* gene include: a methylation-responsive CpG site located at +7 base pairs, which is recognized by Regulatory Factor X proteins (Xu *et al.*, 2003), and a TCC-rich box located at –160 base pairs, which has been associated with Fli1 protein binding (Ihn and Trojanowska, 1997; Czuwara-Ladykowska *et al.*, 2001).

CCAAT displacement protein, also known as Cut-like, CUTL1, and *CUX1*, is a member of the family of homeobox transcription factors involved in the regulation of cellular growth and differentiation (Jack *et al.*, 1991; Liu *et al.*, 1991; Nepveu, 2001). It is an evolutionarily conserved protein and contains four DNA binding domains. Its structure and function have been well documented (Harada *et al.*, 1995). *CUX1* has been shown to bind promoters of target genes and act as a transcriptional modifier. The majority of published studies describe *CUX1* as a transcriptional repressor (Sansregret and Nepveu, 2008). *CUX1* has been reported to carry a CCAAT displacement activity that enables it to compete for binding with CBF in relevant promoter/enhancers of genes. The CCAAT-displacement activity of *CUX1* has been documented in the human thymidine kinase (Kim *et al.*, 1997) and sperm H2B gene transcription (Barberis *et al.*, 1987). The role of *CUX1* in regulating type I collagen transcription via displacing CBF from critical regions of the promoter remains unexplored. In this study we show that *CUX1* acts as a repressor of type I collagen in response to high doses of transforming growth factor-β (TGF-β). We suggest that *CUX1* mediates suppression by binding to the proximal promoter and directly down-regulating *COL1A2* transcription. We provide evidence that these effects are through displacement of CBF from the promoter of collagen. The originality in our work is that TGF-β, which is a cytokine commonly associated with the production of profibrotic genes, at high doses suppresses type I collagen via the induction of the transcription factor *CUX1*. This finding suggests the existence of a TGF-β negative feedback loop that can be paralleled to the induction of the inhibitory Smad7. Overall, our data suggest that *CUX1*, or domains of this protein, may be potential targets for limiting fibrogenesis.

RESULTS

CUX1 is a novel negative regulator of type I collagen

Using liposome-mediated transfection assays, we overexpressed both the full-length (p200) and the short isoform (p75) of *CUX1* in three fibroblastic cell lines that include kidney, lung, and skin. We chose to overexpress p200 and p75 based on the well-documented experimental findings that different isoforms of *CUX1* exhibit different transcriptional and physical binding properties. The p200 isoform of *CUX1* binds DNA rapidly but transiently and is regarded as a repressor, whereas the p75 *CUX1* isoform exhibits slow yet prolonged DNA binding kinetics and has been shown to act as both an activator and repressor of transcription (see Figure 1A for a diagrammatic representation of the structure of *CUX1* isoforms). Transient transfection of p200 and p75 expression vectors led to significant overexpression of *CUX1* mRNA levels as measured by quantitative PCR (qPCR) (Figure 1B) and increased protein levels as measured by Western blotting (Figure 1C). Enhanced *CUX1* expression was associated with a potent reduction of type I collagen mRNA when compared to an empty vector (EV) or baseline measurement from nontransfected cells (Figure 1B, $p = 0.001$). In addition to causing a decrease of *COL1A2* mRNA, overexpression of CUX1 also led to potent inhibition of type I collagen production, thus confirming the qPCR results (Figure 1C). To validate our results, we also examined the effects that *CUX1* overexpression has on human lung– and skin–derived fibroblasts. Expression of both *CUX1* isoforms significantly inhibited collagen production in lung (Figure 1D) and skin fibroblasts (Figure 1E).

CUX1 suppresses type I collagen transcriptionally

Type I collagen is mainly regulated at the level of transcription. We have previously identified and characterized the human untranslated *cis*-acting regulatory sequences that correspond to the *COL1A2* gene promoter/enhancer. Here we performed bioinformatics analyses of the *COL1A2* promoter/enhancer that led to the identification of two putative sites for CUX1 (Figure 2A). We therefore hypothesized that *CUX1* suppresses type I collagen by inhibiting normal transcription of the gene. To test this hypothesis, we generated three stable transfectant lines. The lines were selected on the basis of the amount of *CUX1* expression. Thus we generated a *CUX1* low-expressing line-α, an intermediate line-β, and a high-expressing transfectant line-γ. We validated *CUX1* expression levels in the transfectant lines by Western blotting for *CUX1* and lamin A/C (loading control) as well as type I collagen protein (Figure 2B). The increasing concentrations of nuclear *CUX1* correlated with a dose-dependent reduction of type I collagen. These results were quantified using densitometry (Figure 2B). We then used the transfectant lines to measure promoter activity of *COL1A2* by using the *COL1A2* reporter gene constructs. We found that, in all three transfectant lines, *CUX1* suppressed *COL1A2* promoter activity in a dose-dependent manner (Figure 2C). A DNA vector of the *COL1A2* promoter with a designed point mutation specifically at the CBF/NF-Y –80 base pair site (Figure 2A) revealed that the overexpression of *CUX1* failed to further suppress collagen expression in the absence of an intact CBF site (Figure 2C, bottom panel). We then went on to test the interaction of *CUX1* with the *COL1A2* promoter by using electrophoretic mobility shift assays (EMSAs). We generated two probes (probes 1 and 2), which can be seen in Figure 2A. *CUX1* was found associated with the promoter when using probe 1 (see arrows pointing at "shifts"; Figure 2D). This interaction was specific for *CUX1* because competition for binding with excess concentration of an unlabeled *CUX1* consensus oligonucleotides removed the binding (Figure 2D, left, lane 4, removal of "shift"), whereas competition with CBF, CCAAT-enhancer binding protein (C/EBP), SP1, and NF-κB consensus oligonucleotides did not affect the binding (lanes 5–8). *CUX1* was also found to bind the *COL1A2* promoter when using probe 2 (Figure 2E). We and others have shown that CBF binds the *COL1A2* promoter at position –80 base pairs, which corresponds to probe 2 shown in Figure 2A. The binding of CBF to probe 2 can be seen in Figure 2E (arrowhead). Here we report for the first time to our knowledge that, in addition to CBF, *CUX1* (when overexpressed) also binds to the *COL1A2* promoter at position –80 base pairs (asterisk, Figure 2E). This binding is specific because it is partially removed by competition with unlabeled *CUX1* consensus oligonucleotides (lane 3) and partially by CBF oligonucleotides (lane 4); however, the "shifts" remain unaffected with SP1 and C/EBP competition (lanes 5 and 6, respectively). These data indicate that *CUX1* acts as a type I collagen suppressor via a physical interaction with the *COL1A2* proximal promoter, which leads to partial competition for binding occupancy with CBF (probe 2).

TGF-β induction of *CUX1* suppresses type I collagen

CUX1 has been shown to be a TGF-β–responsive gene in NIH3T3 mouse embryonic fibroblasts (Michl *et al.*, 2005). We therefore

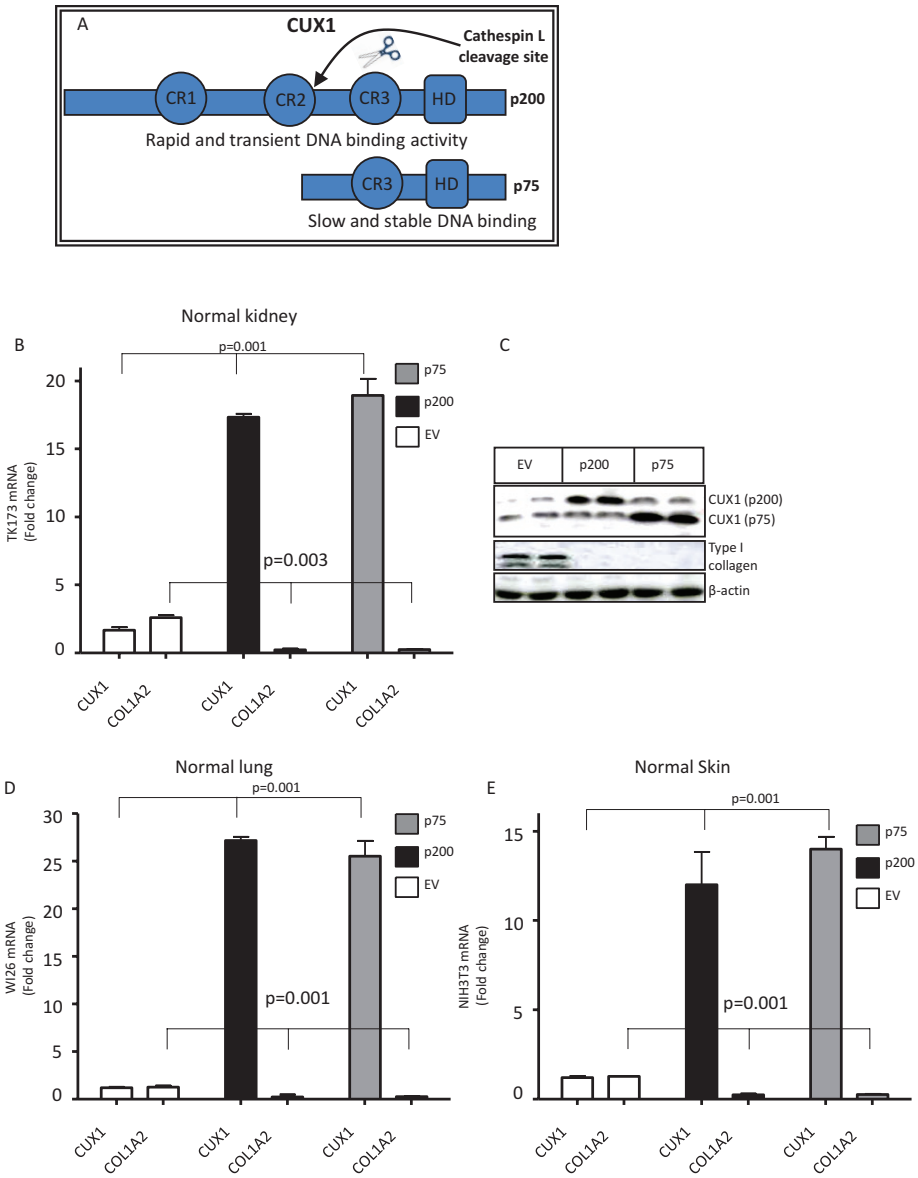

FIGURE 1: *CUX1* suppresses type I collagen in collagen-producing cells. Diagrammatic representation of DNA constructs used for enhancing *CUX1* expression in vitro is shown in (A); p200 and p75 are isoforms of *CUX1*. qPCR analysis was carried out to measure *CUX1* and *COL1A2* mRNA levels in normal kidney fibroblasts (TK173). TK173 were transfected with a p200 or p75 expression vectors or an EV. The results are expressed as fold change increase when compared to nontransfected cells, which serve as baseline (B). By using Western blotting techniques, we measured the protein level of *CUX1* (both isoforms), type I collagen, and β-actin (loading control) (C). To validate our results, *CUX1* and *COL1A2* mRNA expression was also measured in normal lung (D) and normal skin fibroblasts (E).

studied the ability of TGF-β to induce *CUX1* in human renal cells. To test this, we stimulated human renal fibroblasts with varying concentrations of TGF-β (1–10 ng/ml) and measured *CUX1* and type I collagen mRNA production by qPCR (Figure 3A) and protein by Western blotting (Figure 3B). We found that 10 ng/ml of TGF-β caused a significant increase of *CUX1* mRNA and protein, which correlated with no increase of the collagen gene. We then studied the expression level of collagen and found that TGF-β increased collagen expression in a dose-dependent manner, as expected. TGF-β at high doses (10 ng/ml), however, failed to up-regulate collagen production as measured by mRNA and protein (Figure 3, A and B). Cells were then stimulated with a low and a high TGF-β concentration (2 and 10 ng/ml, respectively). These two concentrations were chosen because the low dose of TGF-β showed a potent induction of collagen but had no significant effect on *CUX1*. TGF-β at the high concentration had the reverse effect (increased *CUX1* but no effect on collagen). Using these two concentrations of TGF-β, we additionally overexpressed *CUX1* by using a mammalian transfection system, and found that overexpression of *CUX1* could reverse the low-dose TGF-β effects (2 ng/ml) but had no further silencing effects when high doses of TGF-β were used (Figure 2C). We then performed immunofluorescence staining to detect *CUX1* expression and localization. We found that cells stimulated with TGF-β at 5 ng/ml exhibited an increased expression of *CUX1* when compared with control cells. Additionally, CUX1 at this concentration of TGF-β exhibited a predominantly cytoplasmic localization, whereas in cells stimulated with high TGF-β concentration (10 ng/ml), *CUX1* was predominantly nuclear, suggesting that CUX1 was present in an active form. The nuclear localization was measured and quantified (Figure 2D). To validate the role of *CUX1* in mediating silencing of *COL1A2*, we have used two distinct siRNA oligonucleotides to silence *CUX1*. These siRNA were transfected, and mRNA levels for *CUX1* were measured in cells stimulated with TGF-β (Figure 3E). Silencing of *CUX1* was validated using Western blotting. We found that stimulation of cells with TGF-β at high doses could now activate collagen production in the absence of *CUX1*. We blotted for *CUX1*, type I collagen, and β-actin (loading control) (Figure 3F). These results suggest that high TGF-β concentrations fail to induce collagen because CUX1 is induced, thus providing a "protective" role for CUX1 in fibrotic conditions.

Smad3 to p38 mechanistic switch controls *CUX1* induction in low and high TGF-β doses

We observed that TGF-β induced *CUX1* expression in a dose-dependent manner, hence we set out to investigate the mechanism by which low, intermediate, and high doses (i.e., 2, 5, and 10 ng/ml) determine *CUX1* induction. We stimulated endogenous CUX1 expression by incubating cells with 2 or 10 ng/ml of TGF-β followed by DNA/protein binding assays (EMSAs). The EMSA showed that, while there was no detectable binding of CUX1 to the relevant site of the COL1A2 promoter at low doses of TGF-β, there was binding at the high concentrations (Figure 4A). This binding was specific as it was selectively competed out with CUX1 oligonucleotides but remained unaffected by competition with nonspecific oligonucleotides (Figure 4A). We then went on to study Smad signaling as it is downstream of TGF-β. Smad3 is a main member of the canonical TGF-β signaling cascade; we therefore studied its

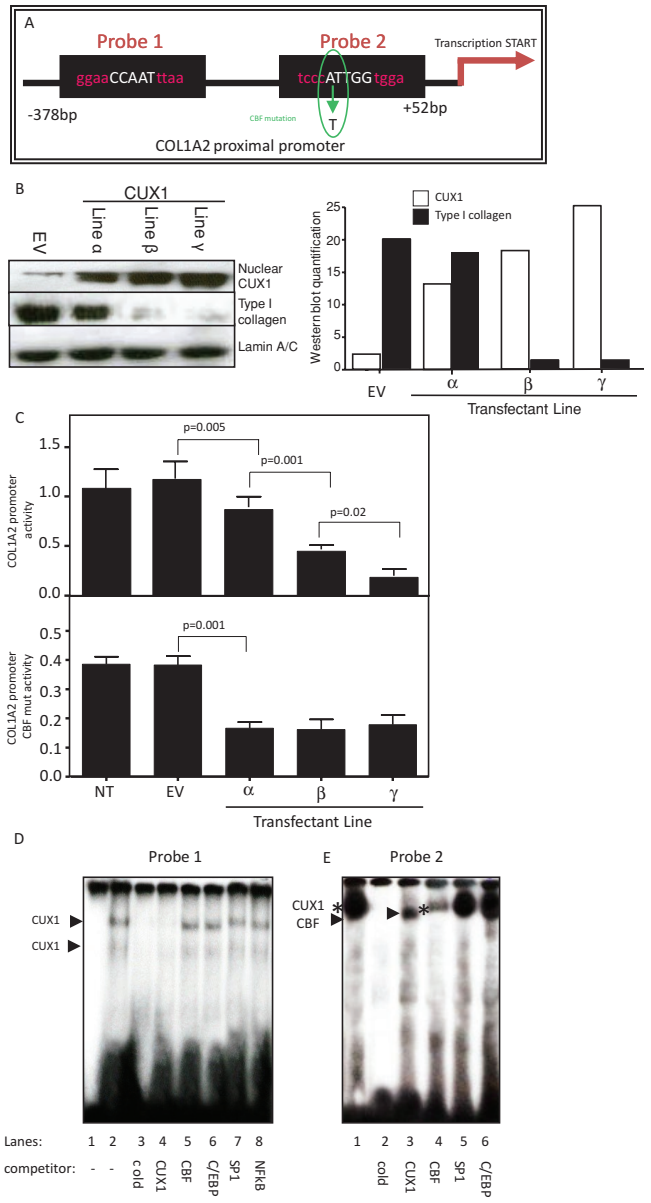

FIGURE 2: *CUX1* binds to and regulates *COL1A2* at the level of transcription. Two putative consensus sites for *CUX1* binding were identified in the *COL1A2* promoter using web-based bioinformatics analysis (Genomatix, Ann Arbor, MI). The location and sequence information of DNA probes used for EMSA is shown in (A) (probes 1 and 2). The CBF point mutation is highlighted in green (A). Three stable transfectant lines, α, β, and γ, were generated overexpressing *CUX1* at different concentrations. Nuclear *CUX1* and lamin A/C (loading control) were measured to confirm the overexpression of *CUX1* (B). Type I collagen, from cell supernatant, was also screened (middle panel, B). Densitometry was carried out to quantify the Western blot findings (B). The activity of the *COL1A2* promoter was measured in the transfectant *CUX1* lines α, β, and γ and in cells transfected with an EV using a specially designed DNA construct that carries the untranslated regulatory sequences of the *COL1A2* gene linked to *LacZ* to allow measurement of promoter activity. *COL1A2* promoter activity was also measured in conditions where the promoter of *COL1A2* was genetically modified by introducing a point mutation at –80 base pairs (see A for sequence information of mutation), termed CBF mutant construct (C). Two double-stranded DNA probes, shown in (A), were used in EMSA experiments. *CUX1* was found bound to the promoter of *COL1A2* when using probe 1 (arrows) in TK173 cells overexpressing *CUX1*. *CUX1* binding was removed with specific *CUX1* oligonucleotide competition (lane 4) but was unaffected with control competition with CBF, C/EBP, SP1, and NF-κB (lanes 5–8) (D). *CUX1*, when overexpressed, was also found bound to an alternative site of the *COL1A2* promoter, using probe 2. This binding was partially competed with *CUX1* competition (lane 3, arrowhead) and partially with CBF (lane 4, asterisk). Control SP1 and C/EBP competition had no effect (lanes 5 and 6) (E).

expression and localization in response to TGF-β stimulation. We found that, in nonstimulated cells, phosphorylated Smad3 was not present or was below detection level; however, 5 ng/ml of TGF-β caused a significant up-regulation of phospho-Smad3 that localized primarily in the nucleus of the TK173 cells. With high doses of TGF-β we observed that CUX1 expression was increased but it retained a predominantly cytoplasmic localization (Figure 4B, quantification right-hand-side panel). We then tested Smad3 localization and induction in TK188 cells, lung cells, and skin cells, and the same phenomenon was observed in all cell types tested (unpublished data). We went on to study the relative contribution of Smad2 and Smad3 (both members of the canonical signaling cascade) by silencing their expression using specific siRNA oligonucleotides. Knockdown efficiency was measured by qPCR, and only experiments with more than 70% knockdown were presented. siRNA against Smad2 and 3 successfully blocked expression of Smads and reversed the TGF-β effects seen at an intermediate dose (5 ng/ml) (Figure 4C). Smad2 or 3 knockdown did not affect, however, *CUX1* production at the high TGF-β concentration (Figure 4C). This finding led us to study the relative role of mitogen-activated protein (MAP) kinase in the induction of *CUX1* in response to intermediate and high concentrations of TGF-β stimulation. Using pharmacological inhibitors to block either p38 or c-Jun N-terminal kinase (JNK), we found that, although JNK did not contribute to this pathway significantly, when p38 was blocked the high-dose TGF-β effect on CUX1 production was abolished (Figure 4D).

CUX1 expression is reduced in chronic renal fibrosis

We generated a model of aristolochic acid (AA)-induced interstitial kidney fibrosis (Sato *et al.*, 2004). Under normal conditions, *CUX1* is highly expressed in kidneys (Debelle *et al.*, 2002). In this fibrotic model, we observed that at 28 and 56 d after induction of fibrosis there was a significant increase in extracellular matrix (ECM) deposition as measured by picrosirius red stain followed by histological scoring (Figure 5A). We examined the expression of *CUX1* in this model of AA-induced progressive fibrosis and found that the mRNA level of *CUX1* expression decreased significantly at days 28 and 56 of disease (Figure 5B). We then validated the mRNA results by measuring protein levels and found that, similarly to RNA, protein levels of *CUX1* reduced in expression, and by day 56 *CUX1* protein was undetectable by Western blotting (advanced fibrosis stage, Figure 5C). This decrease of *CUX1* was concomitant with an increase in type I collagen, which reached a maximum at day 56 (Figure 5C). To investigate whether the effects that we observed in the mouse when we induced fibrosis were consistent with the situation in humans, we examined *CUX1* expression in human fibroblasts derived from kidney with established fibrosis. We found that *CUX1* mRNA is expressed at low levels, and we could enhance its expression by using transfection with the p200 and p75 vectors (Figure 5D). We then assessed the enhanced expression of *CUX1* by Western blotting and also found that overexpression of *CUX1* in fibrotic human cells resulted in a reduction of type I collagen (Figure 5E), indicating that *CUX1* could be used as a therapeutic target. To

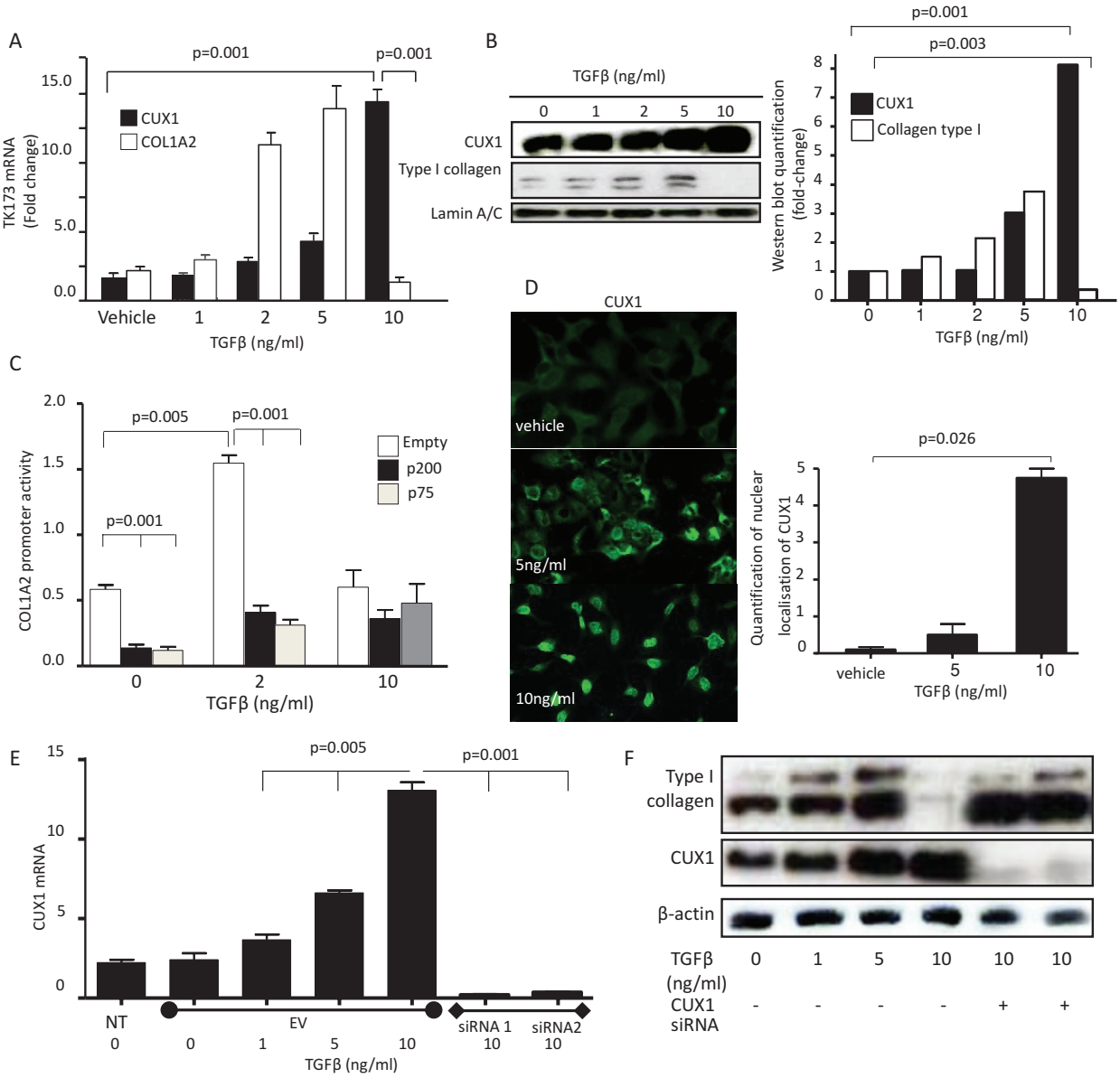

FIGURE 3: TGF-β suppresses type I collagen via induction of *CUX1* at high doses. We measured the mRNA expression of *CUX1* and *COL1A2* by qPCR in normal collagen-producing fibroblastic cells (TK173) stimulated with increasing concentrations of TGF-β (1, 2, 5, and 10 ng/ml). *CUX1* mRNA increased in response to TGF-β in a dose-dependent manner, reaching maximum stimulation at 10 ng/ml of TGFβ (A). *COL1A2* mRNA increased in response to TGF-β (up to 5 ng/ml), but addition of TGF-β at high doses (10 ng/ml) had no stimulatory effects (A). These results were validated by measuring protein expression of *CUX1* and collagen using Western blotting and by densitometry to quantify the findings (B). *COL1A2* promoter activity was then measured in cells overexpressing either the p200 or the p75 isoforms of *CUX1* and compared with EV-transfected kidney cells. TGF-β increased promoter activity of *COL1A2* in EV cells at 2 ng/ml but consistently had no effect at 10 ng/ml. Overexpression of the *CUX1* isoforms resulted in abolishing the TGF-β effects in promoting *COL1A2* promoter activation (C). Using immunofluorescence, analysis of *CUX1* intensity and localization was investigated in response to stimulation with 2 or 10 ng/ml of TGF-β and compared with vehicle-treated cells (left panel, D). Quantification of nuclear localization of *CUX1* and pSMAD3 in response to TGF-β treatment was quantified (right panel, D). *CUX1* was knocked down using two specific siRNA oligonucleotides, and efficiency of siRNA silencing was tested by qPCR in TK173 cells stimulated with TGF-β (E). Protein levels of type I collagen, *CUX1*, and β-actin were studied in TGF-β–stimulated cells with and without specific siRNA (F).

determine whether CUX1 could occupy the endogenous COL1A2 *cis*-acting element in vivo we studied the binding of CUX1 in mouse kidney whole lysates from 0, 28, and 56 d postinjection (dpi) of AA toxin. Here we provide evidence that even though at basal conditions CUX1 occupies the COL1A2 promoter as disease develops there is less binding (28 dpi), and when disease is established (i.e., 56 dpi) there is no binding of CUX1 that can be detected with EMSA (Figure 5F).

DISCUSSION

Collectively in this study we provide in vitro and in vivo data that show that *CUX1* is a potent repressor of type I collagen and that it

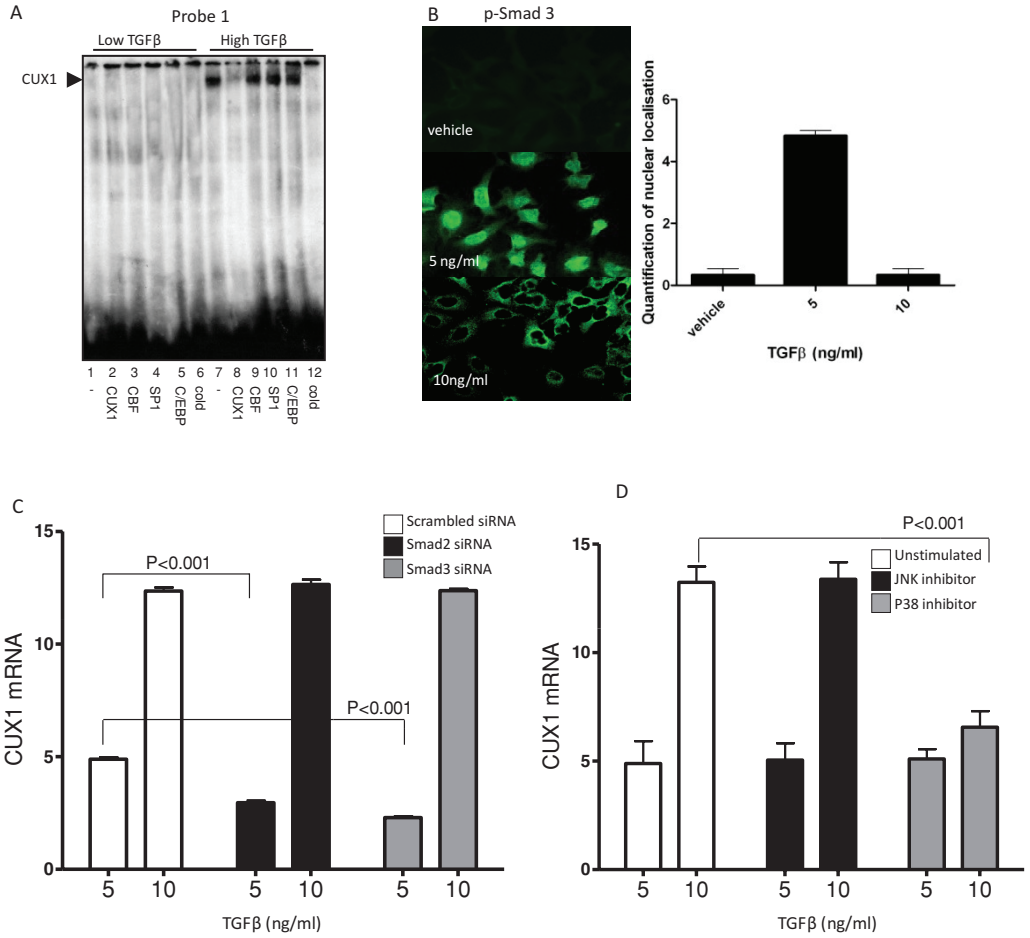

FIGURE 4: TGF-β drives a mechanistic switch from Smad3- to p38-dependent activation of *CUX1*. The ability of TGF-β to increase binding of *CUX1* to the COL1A2 promoter was investigated. TK173 stimulated with low (2 ng/ml) and high (10 ng/ml) TGF-β concentration were subjected to EMSA. *CUX1* was found bound to the COL1A2 promoter only at the high TGF-β concentrations (A). The localization and intensity of the phosphorylated form of Smad3 was studied in TK173 cells in response to TGF-β stimulation. Low TGF-β (2 ng/ml) caused an induction of phospho-Smad3 that was primarily nuclear; however, high TGF-β (10 ng/ml) failed to trigger nuclear localization of phopsho-Smad3 (B). Then Smad 2 or 3 was selectively knocked down using specific siRNA oligonucleotides, and the effects that the knockdown had on *CUX1* induction in response to TGF-β were studied. Smad2 and 3 knockdown significantly reduced TGF-β–induced *CUX1* expression at the lower range (5 ng/ml) but had no effect at the higher concentrations of TGF-β (C). We then investigated the relative contribution of MAP kinases using pharmacological inhibition of JNK and p38. JNK and p38 inhibitors had no effect at all. P38 reduced TGF-β-induced *CUX1* expression selectively at the high concentrations of TGF-β (10 ng/ml) but had no effect at the low concentrations (D).

exerts its effects by transcriptionally switching off the collagen gene. *CUX1* expression is reduced during fibrosis, which may be a cause for a shift in the balance toward increased collagen production and fibrosis. Because *CUX1* is a negative regulator of type I collagen, it could be speculated that its role is to maintain the equilibrium of collagen expression; when *CUX1* is significantly reduced, fibrosis is aggravated as a consequence. Moreover, our results suggest that restoration of *CUX1* in human fibrotic fibroblasts may have beneficial effects due to the reduction of the expression of type I collagen.

One of the novel findings presented here is the observation that the homeobox transcription factor *CUX1* is a potent regulator of type I collagen transcription. We present data to show that *CUX1* is acting as a transcriptional repressor by competing with CBF/NF-Y for binding on the *COL1A2* promoter. Using human fibroblastic cell lines, we show that enhancing the expression of *CUX1* resulted in a significant decrease of type I collagen protein level and *COL1A2* promoter activity concomitant with endogenous mRNA down-regulation of the gene. This inhibition was effective in comparable levels using either the p200 full-length protein or the p75, which is a shorter isoform. *CUX1* has been reported to transcriptionally suppress several other genes (Skalnik *et al.*, 1991; Higgy *et al.*, 1997; van Gurp *et al.*, 1999; Pacheco-Sanchez *et al.*, 2007). In these studies, investigators report that the shorter isoform (i.e., p75) is a more potent inhibitor of transcription. We did not find an enhanced suppression of type I collagen with the shorter isoform. We postulate that in our system *CUX1* is overexpressed over a short period of time (transient transfections of *CUX1*) and therefore even rapid binding of the p200 to the promoter of *COL1A2* was able to facilitate silencing of gene expression.

A central finding in this study is that TGF-β promoted not only a potent activation of *CUX1* mRNA and protein at high doses but also a movement of the molecule to the nucleus, where it then becomes bioavailable to exert its inhibitory effects. Moreover, we provide, for the first time, evidence for a cross-talk between TGF-β–induced *CUX1* and suppression of type I collagen synthesis. We report that

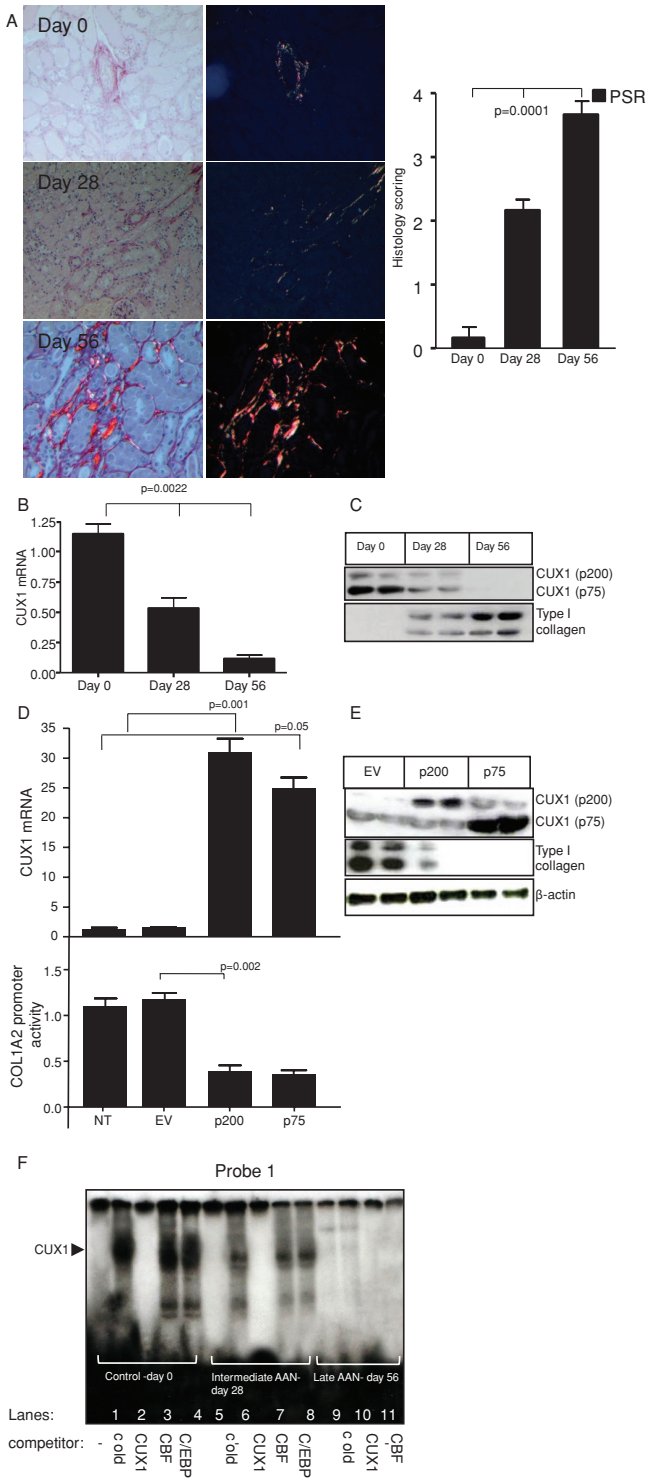

FIGURE 5: *CUX1* is reduced in progressive interstitial fibrosis in vivo. AA was injected intraperitoneally into male C57BL6 mice to induce kidney fibrosis, and animals were killed at 0, 28, and 56 dpi. Their kidneys were collected, and histological sections were stained with picrosirius red to detect fibrillar collagen deposition (A). *CUX1* mRNA was measured in whole kidney lysates from the AA model (B). Protein levels of *CUX1* and type I collagen were also studied in the model (C). *CUX1* was then overexpressed in fibroblasts derived from fibrotic regions of human kidney (TK188), where *CUX1* expression is low. *CUX1* mRNA and *COL1A2* promoter activity were measured (D). *CUX1*, *COL1A2*, and β-actin protein expression was then investigated in fibrotic fibroblasts (E). The occupancy of the CUX1 site was studied in whole kidney lysates from control mice or 28 and 56 dpi. Using EMSA, we showed that CUX1 binds to the COL1A2 promoter in control animals on day 0, and this binding is diminished by 28 dpi and by day 56 of established fibrosis (F).

TGF-β, at high doses, can suppress type I collagen production via the induction of a negative regulator of collagen transcription (i.e., *CUX1*). With the use of RNAi experiments we efficiently blocked *CUX1* and therefore validated its role as a transcriptional repressor of the collagen gene. In normal collagen-producing cells we found to our surprise that TGF-β could activate collagen synthesis at high doses only in the absence of *CUX1*. This key evidence highlights the importance of *CUX1* in regulating a balance between TGF-β–promoted collagen transcription and *CUX1*-mediated silencing. Moreover when we performed further experiments we showed that Smad2 and Smad3 are important in inducing *CUX1* at low doses of TGF-β, whereas it is through p38 MAP kinase that TGF-β exerts its effects at high concentrations. Using a TGF-β–driven model of AA-progressive fibrosis (Li *et al.*, 2009), we found that type I collagen levels increased, whereas *CUX1* levels decreased during disease progression. TGF-β has been characterized as a cytokine that plays a vital role in driving fibrosis via promoting induction of matrix proteins, including type I collagen (Holmes *et al.*, 2001). The TGF-β effects are normally accompanied by a decrease of modifying enzymes such as metalloproteinases and an increase in their inhibitors to further perpetuate the fibrotic pathophysiology in tissues (Bou-Gharios *et al.*, 1994). These changes shift the balance toward overproduction of ECM proteins and scarring. Even though TGF-β is considered as a pleiotropic cytokine with multiple functions that are tissue and context dependent, this is the first report to our knowledge that provides a molecular explanation of TGF-β–directed suppression of collagen.

Indeed the DNA/protein binding assays showed that *CUX1* also binds at the CBF/NF-Y site only when it is overexpressed and that when overexpressed *CUX1* competes with CBF/NF-Y for the binding occupancy. Displacing CBF/NF-Y is expected to result in reduction of *COL1A2* transcriptional activity. CBF/NF-Y is an activator of type I collagen gene expression, and a single point mutation introduced at the promoter of collagen at the site of CBF interaction has been shown to result in the inability of CBF/NF-Y to associate with the promoter followed by a significant fourfold decrease in promoter activity both in vitro (Hasegawa, 1996) and in vivo (Tanaka *et al.*, 2004). These previous studies, together with our report, highlight the importance of CBF/NF-Y in promoting type I collagen activation. Therefore the concept of identifying a factor able to compete with CBF/NF-Y for binding occupancy that results in down-regulation of collagen can be conceived as a beneficial strategy against fibrosis. We have also demonstrated that CUX1 is not able to bind to the COL1A2 promoter and suppress its expression during established fibrosis probably because the levels of CUX1 are suppressed in fibrosis and therefore there is no availability of CUX1 to occupy the COL1A2 promoter. We also reported that in the absence of CUX1 the relevant site on the COL1A2 promoter remains unoccupied as we do not detect binding by another transcription factor at this critical site. The lack of binding of an alternative transcription factor to the relevant CUX1 *cis*-acting element of the COL1A2 promoter could be explained by the high sequence specificity that certain transcription factors exhibit. It is therefore not surprising that, in the absence of CUX1, there is not another transcription factor that replaces it.

CUX1 binds to and suppresses collagen; type I collagen transcription, however, cannot be permanently switched off because it is required during normal development and is necessary in tissue remodeling. Thus having a mechanism to switch off type I collagen

when desired is important to either treat or prevent scar formation. *CUX1* has been shown to act as gene repressor in other gene settings in vivo, including for the sperm histone gene promoter, H2B (Barberis *et al.*, 1987). The human γ-globin gene (Superti-Furga *et al.*, 1989) and factor inhibiting hypoxia (FIH)-1 (Li *et al.*, 2007). We have not investigated, in this study, whether the binding of *CUX1* at the –200 base pair box influences the Smad binding activation of *COL1A2*. It has previously been illustrated that overexpression of *CUX1* under the control of the cytomegalovirus (CMV) promoter leads to a spontaneous expansion in renal mesangial cells that is followed by increased type IV collagen deposition that eventually leads to glomerulosclerosis in old animals (Brantley *et al.*, 2003). Limited interstitial fibrosis was reported. The mechanism by which *CUX1* led to enhanced type IV collagen production was not fully explored, and this transgenic approach elicited ectopic expression in a broad range of cell types and also caused cell proliferation. It is therefore likely that the increased cell number was one of the causes of increased type IV collagen production. Our finding that *CUX1* is highly expressed in a normal human kidney and this expression diminishes in AAN fibrosis argues that dysregulation of *CUX1* may be a key factor leading to renal abnormalities, and thus targeting the regulation of *CUX1* is likely to be a new approach to combat kidney fibrosis.

In conclusion, we report that *CUX1* is activated at high doses by TGF-β and exerts its protective effects by acting as a negative regulator of type I collagen transcription. Using both in vitro and in vivo systems, we have delineated the molecular mechanisms by which *CUX1* facilitates type I collagen suppression. It will be of interest to test whether CUX1 is a negative regulator of type I collagen in other murine and human models of fibrosis, such as those in the lung and skin.

MATERIALS AND METHODS

Mice and AA nephropathy

C57BL6 wild-type mice were obtained from Charles River Laboratories International (Wilmington, MA). AA-induced nephropathy was established by intraperitoneal injection of the animals with 5 mg/kg AA once a day for five consecutive days (Sigma, St. Louis, MO). Mice that received injections with either AA or citrate buffer were killed at 0, 28, and 56 dpi.

Cell culture

All cell lines used were maintained in DMEM supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. The cells were cultured at 37°C in a humidified 5% CO_2 atmosphere and were subcultured as needed.

Plasmid/siRNA transfection and reporter assays

Cells were seeded in six-well plates before transfection and 24 h later were transfected using FuGENE 6 (Roche, Basel, Switzerland), according to the manufacturer's instructions; this method was used to transfect *CUX1* isoforms and/or *COL1A2* promoter reporter constructs. A cotransfection with a CMV-driven luciferase construct (CMV/Luc) was performed to measure and normalize transfection efficiency. β-galactosidase and luciferase were measured using the Dual-Light Tropix kit (Applied Biosystems, Warrington, UK) and quantified using a Tropix TR717 microplate luminometer. siRNA transfections were performed with Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. siRNA, HPLC purity, was purchased from Ambion (Foster City, CA) with sequences for Smad2 (5′ ACUCCUGAUUAG 3′) and Smad3 (5′ GCAGAACAGGUAGUAUUA 3′). Stable lines α, β, and γ were generated by transfecting cells with *CUX1* vector and propagating single colonies after selection with G418 antibiotic for 4–6 wk.

Western blot and antibodies

Western blot was performed as previously described using the Mini Protean system from Bio-Rad (Hercules, CA). Primary antibodies against total Smad, phosho-Smad, and β-actin were purchased from Cell Signaling (Danvers, MA). Antibodies against *CUX1* were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and antibody against *CUX1* used for immunohistochemistry from obtained from Abgent (San Diego, CA).

Immunofluorescence/limmunohistochemistry

Cells grown on poly-L-lysine–treated coverslips were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), permeabilized with 0.2% Triton X-100 in PBS, blocked with 4% bovine serum albumin (BSA), and subjected to primary and fluorescently labeled secondary antibodies in 4% BSA in PBS. Coverslips were mounted on DAPI containing solution and analyzed using a deconvolution microscope. Immunohistochemistry was performed according to Wester *et al.* (2000). All further image processing (level adjustments, brightness, contrast settings, and overlaying) was performed using Adobe Photoshop 7.0.

Semiquantitative PCR and EMSA

For semiquantitative PCR, RNA was extracted using Trizol according to the manufacturer's instructions (Invitrogen). Real-time PCR was performed using SYBR Green according to the manufacturer's instructions (Abgene). EMSA was performed as described by Hellman and Fried (2007). Probes used for EMSA were as follows:

-200 base pairs 5′ AGCCCTCCCATTGGTGGAGG 3′
-80 base pairs 5′ TCCACCAATGGGAGGGCT 3′

Primers used for PCR were as follows:

CUX1 primer pair 1: 5′ CCT GCA GAG TGA GCT GGA C 3′ and 5′ GCT TGC TGA AGG AGG AGA AG 3′; CUX1 primer pair 2: 5′ GAG AGC ATG GAG AGG GAC TG 3′ and 5′ TTC TCG TGG AAC TTG TGC AG 3′; COL1A2 primer pair: 5′ AGA GGA CCA CGT GGA GAA AG 3′ and 5′ GGC CTG TGG GAC CAT CTT 3′.

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