Type IV Collagen Is Transcriptionally Regulated by Smad1 under Advanced Glycation End Product (AGE) Stimulation*

Received for publication, September 22, 2003, and in revised form, January 15, 2004
Published, JBC Papers in Press, January 19, 2004, DOI 10.1074/jbc.M310427200

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Prolonged exposure to hyperglycemia is now recognized as the most significant causal factor of diabetic complications. Excessive advanced glycation end products (AGEs) as a result of hyperglycemia in tissues or in the circulation may critically affect the progression of diabetic nephropathy. In diabetic nephropathy, glomerulosclerosis is a typical pathologic feature characterized by the increase of the extracellular matrix (ECM). We have reported previously that a1 type IV collagen (Col4) is one of the major components of ECM, which is up-regulated by AGEs, and that the overexpression of Col4 is transcriptionally regulated by an unknown transcription factor binding to the promoter. Here we identified this protein as Smad1 by yeast one-hybrid screening. Using chromatin immunoprecipitation and reporter assay, we observed that Smad1 directly regulated transcription for Col4 through the binding of Smad1 to the promoter of Col4. Smad1 was significantly induced along with Col4 in AGE-treated mesangial cells. Moreover, suppression of Smad1 by antisense morpholino resulted in a decrease of AGE-induced Col4 overproduction. To elucidate the interaction between transforming growth factor-β and Smad1, we investigated whether activin receptor-like kinase1 (ALK1) was involved in this regulation. AGE stimulation significantly increased the expression of the ALK1 mRNA in mesangial cells. We also demonstrated that Smad1 and ALK1 were highly expressed in human diabetic nephropathy. These results suggest that the modulation of Smad1 expression is responsible for the initiation and progression of diabetic nephropathy and that blocking Smad1 signaling may be beneficial in preventing diabetic nephropathy and other various diabetic complications.

Diabetic nephropathy is the leading cause of end-stage renal disease and a major contributing cause of morbidity and mortality in patients with diabetes throughout the world. There is accumulating evidence that AGEs have a pathogenic role in the development of diabetic glomerulosclerosis. Excessive AGEs produced as the result of hyperglycemia are known to stimulate the production of the ECM and inhibits its degradation. AGEs have a wide range of chemical, cellular, and tissue effects through changes in charge, solubility, and conformation that characterize molecular senescence. AGEs induce a variety of cellular events in vascular cells and other cells, possibly through several functional AGE receptors, thereby modulating the disease processes (1–5). AGEs have recently been accepted as having an important role, not only in diabetic complications but also in aging and old age-related diseases, including atherosclerosis (6, 7). Moreover, a truncated, soluble form of the receptor for AGEs was reported to suppress the development of accelerated diabetic atherosclerosis (8). Exposure of cultured mesangial cells to AGEs results in a receptor-mediated up-regulation of mRNA and protein secretion of Col4 (5, 9). However, there is little information regarding the mechanisms that underlie this regulation.

It was recently shown that TGF-β plays an important role in the AGE response of the glomeruli (10). Transgenic mice over-expressing TGF-β develop severe glomerulosclerosis (11). Thus, TGF-β is a central mediator of the sclerosing process in diabetic nephropathy. Members of the TGF-β superfamily bind to two different types of serine/threonine kinase receptors, termed type I and type II receptors (12). Type II receptors activate type I receptors, and signals are mediated through the type I receptors. Ligand-specific Smads are direct substrates of type I receptor kinases. Thus, the specificity of the signals is determined by type I receptors. ALK1 is one of the type I receptor members for TGF-β family proteins and has been linked to the inherited multisystemic vascular disorder, hereditary hemorrhagic telangiectasia 2 (HHT2). Although ALK1 has been known as an orphan receptor, recent reports show that ALK1 transduces TGF-β signals via Smad1 (13, 14).

Morphologically, the development of diabetic nephropathy is characterized by progressive thickening of the glomerular basement membrane (GBM) and, by expansion, of the mesangial ECM. During the process of glomerular injuries, mesangial cells can overproduce Col4 and secrete type I collagen (Col1) and osteopontin (OPN), which are not normally present in the mesangial matrix (15). In particular, Col4 is a major component of the thickened GBM and expanded ECM, but no molecules that directly regulate the Col4 expression have been found. The 130-bp bidirectional promoter of Col4 contains a large stem-loop structure (CIV), which has been shown to interact with several DNA-binding proteins (16). Using a gel mobility shift assay, we reported previously that an unknown protein binding to the CIV site directly regulates Col4 expression only when exposed to AGEs (9).
that binds to the CIV site in the promoter region of the mouse Col4 gene, we constructed a cDNA library from mouse mesangial cells treated with AGEs. In this study, we used a yeast one-hybrid system to isolate a clone that encodes a specific transcription factor from the library, and we identified the clone as the cDNA that encodes Smad1.

EXPERIMENTAL PROCEDURES

Cell Culture—A glomerular mesangial cell line was established from glomeruli isolated from normal, 4-week-old mice (C57BL/6JxSJLJ) and was identified according to the method described previously (17). The mesangial cells were maintained in B medium (a 3:1 mixture of minimal essential medium/F12 modified with trace elements) supplemented with 1 mM glutamine, penicillin at 100 units/ml, streptomycin at 100 mg/ml, and 20% fetal calf serum. The cultured cells fulfilled the criteria generally accepted for glomerular mesangial cells previously (17). AGE or bovine serum albumin (BSA) exposure was carried out as described previously (9).

Preparation of AGEs—AGE-BSA was prepared by incubating BSA in phosphate-buffered saline (10 mm, pH 7.4) with 50 mm glucose 6-phosphate for 8 weeks at 37 °C as described previously (5). Unmodified BSA was incubated under the same conditions without glucose 6-phosphate as control. Protein concentrations were measured by the Bradford method. All AGE-protein-specific fluorescence intensities were measured at a protein concentration of 1 mg/ml. AGE-BSA and control BSA were incubated with the appropriate preimmune serum for 20 min at 4 °C overnight. PCR was performed with primers to amplify the region containing the CIV-1 motif. The 5′-primer was 5′-GGAGGTCCCTCCTGAGGAGGCGCCGGCCG-3′; CIV-1) from the mouse type IV collagen gene was ligated into the yeast integration and reporter vector pHISi or pLacZi to generate pHISi-CIV-1 or pLacZi-CIV-1, respectively (15). Each pHISi-CIV-1 and pLacZi-CIV-1 reporter construct was linearized and integrated into the genome of competent yeast YM4271, sequenced, and contained 61.3 and 8.31 units of AGE per milligram of protein, respectively.

cDNA Library Construction and Yeast One-hybrid Screening—We prepared cDNA from mouse mesangial cells exposed to AGEs and inserted it into the pGAD10 vector. Yeast one-hybrid screening was carried out according to the MATCHMAKER one-hybrid protocol (Clontech). Briefly, tandem repeats of the 26-bp sequence (5′-TTTCCCTTCTGGAGGAGGCCGGCCGG-3′; CIV-1) from the mouse type IV collagen gene were ligated into the yeast integration and reporter vector pHISi or pLacZi to generate pHISi-CIV-1 or pLacZi-CIV-1, respectively (15). Each pHISi-CIV-1 and pLacZi-CIV-1 reporter construct was linearized and integrated into the genome of competent yeast YM4271, sequenced, and contained 61.3 and 8.31 units of AGE per milligram of protein, respectively.

RESULTS

Smad1 Regulates Type IV Collagen under AGE Stimulation

Chromatin Immunoprecipitation Assay—Chromatin immunoprecipitation assays were performed essentially as described previously by Luo et al. (16). We used anti-Smad1 antibody, anti-Smad4 antibody (Santa Cruz Biotechnology), or normal control IgG at 4 °C overnight. PCR was performed with primers to amplify the region containing the CIV-1 motif. The 5′-primer was 5′-GGAGGTCCCTCCTGAGGAGGCGCCGGCCG-3′; CIV-1) from the mouse type IV collagen gene was ligated into the yeast integration and reporter vector pHISi or pLacZi to generate pHISi-CIV-1 or pLacZi-CIV-1, respectively (15). Each pHISi-CIV-1 and pLacZi-CIV-1 reporter construct was linearized and integrated into the genome of competent yeast YM4271, sequenced, and contained 61.3 and 8.31 units of AGE per milligram of protein, respectively. The resulting yeast cells with the integrated pHISi-CIV-1 and pLacZi-CIV-1 were used for one-hybrid screening with the AGE-stimulated mouse mesangial cell library. Positive colonies were selected on pLacZi-CIV-1 were used for one-hybrid screening with the AGE-stimulated yeast cells with the integrated pHISi-CIV-1 and pLacZi-CIV-1 reporter constructs. Each pHISi-CIV-1 and pLacZi-CIV-1 reporter construct was linearized and integrated into the genome of competent yeast YM4271, sequenced, and contained 61.3 and 8.31 units of AGE per milligram of protein, respectively.

Reporter Assay—1.3 × 106 COS7 cells in 10% fetal bovine serum/Dulbecco’s modified Eagle’s medium were seeded into 6-well plates. Eight hours later, the cells were transfected with 750 ng of CIV-1-LacZ reporter construct along with either 750 ng of vector encoding wild type Smad1 or the mock vector and 75 ng of CMV-LUC (Firefly luciferase under the control of cytomegalovirus promoter) as an internal control. Transfection was performed with FuGENE6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer’s instructions. Forty-eight hours later, the cells were harvested in reporter lysis buffer, and β-galactosidase and luciferase activities were then measured using the Luminescent β-galactosidase reporter system (BD Biosciences) and the luciferase reporter assay system from Promega. β-galactosidase results were normalized for luciferase activity.

RNAse Protection Assay—Total RNA was isolated from mesangial cells using the TRIsol reagent (Invitrogen), and an RNase protection assay was performed as described previously (20). Briefly, the RNA probes were prepared by linearizing the pPuF vector fragment of Col4 from p1294, the ApaI fragment of Col1 from pGM101, and the EcoRI fragment of mouse ribonuclease inhibitor and τ-phosphatase dehydrogenase (GAPDH) from pMGAP1. In addition, mouse riboprobe for Smad1 (5′-GCAAGGTTCAGAGCATGCCAGG-3′ and 5′-CCCATGTCATGAAAGCCG-3′) were amplified by reverse transcription PCR. The PCR fragments were sequenced to confirm that they were the respective cDNAs and then were cloned into a pGEM-T plasmid. After digesting the plasmid with SalI, an antisense riboprobe was synthesized in vitro using T7 RNA polymerase. The RNA probes and the test RNA were hybridized overnight at 45 °C. RNAse A (40 μg/ml) and RNAse T1 (2 μg/ml) were added to each tube, and the tubes were incubated for 1 h at 30 °C. The RNase resistant fragments were analyzed by 5% polyacrylamide/8 M urea gel electrophoresis and autoradiography. The protected bands for each RNA probe had the same size as the coding sequence for the specific mRNA, thus providing evidence for their specificity, and were evaluated by densitometric analysis.

Immunostaining of Cultured Cells—Cultured cells were fixed in 4% paraformaldehyde. The antibodies used were anti-Smad1 antibody, 1:100 (Santa Cruz Biotechnology), and anti-pSmad1, 1:100 (Calbiochem). An appropriate fluorescein isothiocyanate-conjugated secondary antibody was used for visualization, and imaging was done using a confocal laser microscope and a fluorescence microscope (Olympus). Smad1 Morpholino—A sense/antisense Oligonucleotide—The antisense oligonucleotide used was a 25-nucleotide morpholinoo oligo (Genetools LLC) with the base composition 5′-CAACCGTGTCCACATCCATGCCCGT-3′. A standard morpholinoo oligo with the base composition 5′-CAGCTGTCACATCCAAGCGC-3′ (points of mismatch are shown by small letters) was used as a control. Microinjection of in vitro transcribed RNA was performed as described previously (21).

Histology—Histopathological studies were performed on human tissues. This study was in accordance with the Declaration of Helsinki, and we obtained approval from the institutional review board. All patients gave their informed written consent. Diabetic kidney specimens (n = 5) were obtained from renal biopsies. Control human tissue specimens (n = 5) were obtained from normal renal cortex harvested from kidney donors removed for renal malignancy. Tissues for analysis were fixed in the paraformaldehyde. Cryopreserved kidney tissues were cut in 5-μm-thick sections and fixed in acetone for 5 min. Endogenous peroxidase activity was quenched by a 20-min incubation in the dark with 1% H2O2 in methanol. To eliminate nonspecific staining, sections were incubated with the appropriate preimmune serum for 20 min at room temperature, followed by incubation with primary antibodies against Smad1 (Santa Cruz Biotechnology) and anti-ALK1 (R&D Systems) antibodies.

Results

Smad1 Is Identified as a Binding Protein to Col4—To identify the protein binds to the CIV site in the promoter region of the mouse Col4 gene, we constructed a cDNA library from mouse mesangial cells treated with AGEs. We then used a yeast one-hybrid system to isolate a clone that encodes a specific transcription factor from the library. We identified this clone as the cDNA that encodes Smad1. Smad1 is well known for transducing the bone morphogenetic protein (BMP) signal (22) and is essentially important in the development of kidney (23). However, the expression of Smad1 is not detected in glomeruli in adult mouse (24).

To confirm the binding of Smad1 to the Col4 promoter in vivo, we performed a chromatin immunoprecipitation assay. Precipitated DNA was purified, and the promoter of the Col4 gene was detected by PCR. The anti-Smad1 antibody precipitated chromatin containing the CIV-1 site from cells stimulated with AGEs (Fig. 1). In contrast, considerably less binding was observed in BSA-exposed cells. We also detected Smad1 on the CIV-1 site (Fig. 1). These observations suggest that Smad1 and Smad4 can target the CIV motif in mesangial cells, especially when exposed to AGEs.

Smad1 Transcriptionally Regulates Col4 Expression—Next, we examined the transcriptional activity of the Col4 gene by a reporter assay. We constructed a vector by fusing the CIV-1 promoter in front of the LacZ reporter and then cotransfected

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with a wild-type Smad1 vector in COS7 cells. First, we confirmed the expression of Smad1 by Western blot analysis (Fig. 2a). Phosphorylated Smad1 (pSmad1) was detected in cell lysates that had been tranfected with wild-type Smad1 vector. Cotransfection of the wild-type Smad1 resulted in an 18-fold increase in a relative β-galactosidase activity of Col4 compared with that of the vector alone (Fig. 2b, mock). The CIV-1 promoter has a GC-rich sequence in its 3’ end, which has been identified as a binding site for Smad1 (25). We then constructed two mutant reporter plasmids, the deletion mutant of GC rich in CIV-1 (5’-TTCTCCCTGAGGAGC-3’; Mut1) and the trinucleotide substitution mutant of GC rich motif in CIV-1 (5’-TTCTCCCTTGGAGGACCTG-3’; Mut2) (points of mutation are shown by small, underlined letters). The promoter activities of Mut1 and Mut2 were reduced to 4.9- and 4.3-fold increase, respectively (data not shown). β-galactosidase activity was normalized to luciferase activity and standardized as fold changes relative to cells cotransfected with the mock vector. In contrast, mock had no effect on the β-galactosidase activity in cotransfected cells. These results suggest that Smad1 is significantly involved in the induction of Col4 gene transcription.

**Activation and Translocation of Smad1 under AGE Exposure**—To determine whether Smad1 is transcriptionally up-regulated by AGEs, we examined the expression of Smad1 in mesangial cells with or without AGEs stimulation. The levels of Smad1 mRNA were proportionally increased in a time-dependent manner (Fig. 3a). Similarly, the levels of Col4 mRNA increased in parallel with the up-regulation of Smad1 transcripts. After BSA treatment, however, no change in the mRNA expression of Smad1 or Col4 was detected. Smad1 is well known to be phosphorylated and translocated into the nucleus, where it participates in the transcriptional regulation of target genes (22, 26). Therefore, we next examined the issue of whether the phosphorylation and translocation of Smad1 is affected by AGE treatment in mesangial cells (Fig. 3b). Consistent with the RNase protection assay, Smad1 and pSmad1 were distributed throughout mesangial cells with a preferential cytoplasmic localization after a 72-h-incubation in the presence of AGEs. Furthermore, the nuclear accumulation of Smad1 and pSmad1 in response to AGEs was observed in the cells 120 h after AGE stimulation, whereas BSA treatment led to little expression of Smad1 and pSmad1. The cells were counterstained with DAPI, and the nuclei were identified (data not shown). Similarly, both the Smad1 and pSmad1 proteins were detected in extracts from AGE-treated, but not BSA-treated, cells (Fig. 3c). These findings indicate that the regulation of Col4 is correlated with the expression of Smad1 under AGE exposure.

**The Blocking of Smad1 Attenuates ECM Protein Overproduction**—To examine the importance of the Smad1 signaling pathway for the AGE-induced overexpression of Col4, we selectively inhibited this pathway by the antisense (AS) gene. The AGE-mediated induction of Smad1 was completely abolished in the presence of AS, but not in the presence of control oligos (4-mismatch) (Fig. 4, a and b). The overexpression of Col4 was strongly attenuated, consistent with the inhibition of Smad1. Similarly, both Col1 and OPN mRNA levels were significantly

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**Fig. 1. Binding of Smad1 to Col4 promoter.** Chromatin immunoprecipitation was carried out with mesangial cells under treatment with AGEs (A) or BSA (B) using the indicated antibodies. PCR was performed using primers for the CIV-1 motif. After sonication, DNA from 10% of each sample was saved as input fraction (Input). One of three independent experiments is shown.

**Fig. 2. Effects of Smad1 on Col4 transcriptional activity.** a, cells were cotransfected with CIV-1-lacZ reporter plasmid and with either the vector encoding wild-type Smad1 (Smad1-Wt) or the vector alone (Mock), along with CMV-LUC as an internal control. Whole cell lysates were analyzed by Western blot with the anti-Smad1 and anti-pSmad1 antibody. One of three independent experiments is shown. b, after 48 h, cells were lysed, and β-galactosidase (β-gal) and luciferase (Luc) activities were measured. Values are the averages of triplicate determinations ± S.D.

**Fig. 3. Exposure to AGEs causes dynamic changes in Smad1 expression.** a, RNase protection assay analysis of Smad1 and Col4 mRNA expression in total RNA lysates from mesangial cells treated with AGEs or BSA for the indicated time periods. Chronic stimulation of AGEs promotes Smad1 continuous expression, paralleled with expression of Col4. One of three independent experiments is shown. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. b, immunofluorescence of mesangial cells after 72 or 120 h of treatment with AGEs or BSA. Data from one of three representative experiments is shown. c, Smad1 and pSmad1 were monitored by Western blot in response to a 72-h treatment with AGEs or BSA. One of three independent experiments is shown.
Smad1 Regulates Type IV Collagen under AGE Stimulation

Changes in GBM structure occur very earlier in diabetic nephropathy, even before microalbuminuria is apparent. Although Col4 is the principal component of the GBM, the cellular and molecular mechanisms involved in the up-regulation of Col4 in diabetic conditions are, as yet, poorly understood. We have reported previously that an unknown protein binds to the Col4 promoter under AGE exposure (9). Here, we identified the protein as Smad1 using a yeast one-hybrid system. It is generally acknowledged that Smad1 transduces BMP signals, inducing formation of bone and cartilage (22). Moreover, signaling by Smad1 is modulated by various other proteins such as signal transducers and activators of transcription 3 (STAT3) (27) and Smurf1 (28), allowing the TGF-β superfamily ligands to elicit diverse effects on target cells. Recently, mesangial cells have been shown to produce TGF-β when exposed to AGES (29). We observed that chronic exposure of AGES, inducing the sustained increase in Smad1 gene activation and expression, leads to Col4 overproduction, suggesting that Smad1 is a critical modulator in diabetic conditions.

Targeted gene disruption of the Smad1 gene in mice results in embryonic lethality, suggesting that Smad1 plays critical roles in early embryogenesis (30). However, because of the early embryonic lethality, not much is known about the role of Smad1 in vivo, particularly in the adult. A recent study has shown that Smad1 is absent in renal glomeruli in normal adult mouse (24). We show for the first time that AGES induce the expression of Smad1 in adult mouse glomeruli. Therefore, Smad1 may be the earliest indicator of renal dysfunction.

Development of diabetic kidney disease in diabetic patients is a huge clinical problem associated with increased morbidity and mortality. It is also clear that the current therapy, optimal glycemic control, can slow (1, 2) but not prevent the development or progression of diabetic nephropathy in most patients. Previous studies have shown that TGF-β is a key mediator of ECM accumulation in experimental and human kidney disease, leading to progressive glomerular scarring and renal failure (10, 11). Therapeutic approaches to down-regulate TGF-β signaling under diabetic conditions provide one strategy for inhibiting the progression of diabetic nephropathy. For example, the use of the endogenous proteoglycan decorin (natural inhibitor of TGF-β) (31) and the use of a neutralizing TGF-β antibody (32) have been shown to prevent the development of diabetic glomerulosclerosis. However, prolonged inhibition of TGF-β may lead to unwanted adverse effects, because TGF-β has anti-proliferative effect in some cancers and, in one report, Smad3-deficient animals found metastatic colon tumors (33). Therefore, inhibitors for specific responses of TGF-β will lead to
a novel therapeutic approach. We have demonstrated here that the morphologic antisense oligo specific for Smad1 strongly attenuated the overproduction of Col4 induced by AGEs. Similarly, Col1 and OPN mRNA expressions were partially inhibited. It is reported that Smad1 dissociates the repressor Hoxc-8 from the OPN promoter, thereby inducing OPN transcription (34). Thus, Smad1 may be a novel therapeutic target in diabetic complications and be useful in combination with the current therapy.

TGF-β evokes its biological effects by signaling through two different types of serine/threonine kinase receptors. Type II receptor activates type I receptors, which transduce various signals via the Smads (22, 14). Therefore, we investigated the expression of ALK1 in mouse mesangial cells and human kidney tissues. We have also shown that ALK1 and Smad1 are expressed in renal glomeruli, corresponding to the progression of diabetic conditions. These results lead not only to a better understanding of the mechanisms responsible for the initiation and progression of diabetic conditions but also to the development of novel therapeutic strategies for the treatment of diabetic complications in various organs by suppressing the pathologically activated production of collagen. Both Smad1 and ALK1 are nearly absent in normal mesangial cells. In this study, we first demonstrated that ALK1 as well as Smad1 participate in the development of diabetic change in kidney, suggesting that ALK1/Smad1 acts upstream of the excessive production of Col4.

AGEs are known to induce a variety of cellular events in vascular cells and other cells, possibly through the functional several AGEs receptors, thereby modulating the disease processes. AGEs have been recently accepted as having an important role, not only in diabetic complications but also in aging and old age-related diseases, including atherosclerosis (6, 7). Col4 is also a major component of the vascular basement membrane that lies beneath the endothelium, surrounds medial smooth muscle cells, and undergoes significant nonenzymatic glycosylation (glycation). Glycation leads ultimately to increased cross-linking of collagen, resulting in increased arterial stiffness (35). We report here that AGE-induced Col4 overproduction is mediated by Smad1 signaling. Recent reports show that Smad1 is expressed in endothelial cells of some blood vessels and is at the site of vasculogenesis in the developing yolk sac during blood island formation (36). Furthermore, ALK1 is highly expressed in vascular endothelial cells (22, 37) and may be essential for vascular maturation and stabilization (38, 39). Inactivating mutations of ALK1 result in human hereditary hemorrhagic telangiectasia 2, also known as Osler-Rendu-Weber syndrome (40). In addition, recent evidence indicates that Smad1 transcriptionally regulates the osteopontin gene (33), which is a key factor of the progression of renal injuries and atherosclerosis. Accordingly, we speculate that the ALK1/Smad1 signaling may mediate the development of atherosclerosis, both in diabetic patients and in the aged, by inducing an overproduction of ECM. Because diabetic renal disease in the human is a process that occurs slowly over many years, it is likely that a very detailed evaluation of this phenomenon will be required to determine the interaction of Smad1 and ALK1 in this condition. Further work is in progress to clarify the role of ALK1/Smad1 in diabetic kidney using animal models.

Acknowledgments—We thank Dr. K. Miyazono (The University of Tokyo, Japan) for providing a plasmid encoding Smad1 and Dr. Y. Takishita (Tokushima Prefectural Central Hospital, Japan) for his assistance with histological analysis. We also thank the members of our laboratory for discussions.

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Smad1 Regulates Type IV Collagen under AGE Stimulation
Type IV Collagen Is Transcriptionally Regulated by Smad1 under Advanced Glycation End Product (AGE) Stimulation

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J. Biol. Chem. 2004, 279:14201-14206.
doi: 10.1074/jbc.M310427200 originally published online January 19, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M310427200

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