Modifications of Chromatin Dynamics Control Smad2 Pathway Activation in Aneurysmal Smooth Muscle Cells

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Rationale: The activation of the Smad2 signaling pathway is thought to play an important role in human aneurysmal diseases as described by an important body of research. We previously showed that constitutive Smad2 activation is associated with Smad2 mRNA overexpression in aneurysmal vascular smooth muscle cells (VSMCs), which is dependent on epigenetic regulation of the SMAD2 promoter involving histone modifications. However, the underlying molecular mechanisms controlling Smad2 overexpression are currently unknown.

Objective: The aim of the present study is to understand the mechanisms regulating the constitutive Smad2 overexpression in VSMCs by identification of the histone-modifying enzymes, transcription factors, and cofactors responsible for Smad2 promoter activation in aneurysmal disease.

Methods and Results: This study was performed on medial tissue extracts and primary cultures of VSMCs of human thoracic aneurysms (n=17) and normal thoracic aortas (n=10). Here, we demonstrate that the activation of SMAD2 promoter is driven by the recruitment of a multipartner complex, including the transcription factor p53 and histone acetyltransferases. Remarkably, the transcriptional regulatory network of the SMAD2 promoter is dramatically altered in human aneurysmal VSMCs in vitro and in situ with a switch from Myc-dependent repression of SMAD2 in normal vessel to a p53-dependent activation of SMAD2 in aneurysms. Furthermore, histone acetyltransferases p300 and P300/CBP-associated protein play a major role in SMAD2 promoter activation by acting on histone acetylation, p53 recruitment, and acetylation.

Conclusions: These results provide evidence for a major role of p53 and the complex composed of p300 and p300/CBP-associated protein in Smad2 activation in human aneurysmal VSMCs. (Circ Res. 2013;113:881-890.)

Key Words: epigenetics ■ epigenomics ■ marfan syndrome ■ smooth muscle cells ■ tumor suppressor protein p53

The transforming growth factor (TGF)-β signaling pathway is a ubiquitous system involved in the regulation of many aspects of cell homeostasis, including survival, differentiation, matrix production, antiprotease secretion, inflammation reduction, and the healing process.1 The TGF-β signaling pathway is divided into 3 subcellular areas: (1) extracellular TGF-β that binds to latent TGF-β-binding protein-1 and adhesive matrix proteins, such as fibronectin and fibrillin. Its bioavailability, including storage, secretion, and activation, depends on extracellular matrix integrity and the activation of proteases; (2) signal transduction at the plasma membrane via the TGF-β receptors 1/2 and their respective phosphorylation and oligomerization; and (3) signaling from cytoplasm to nucleus, mainly dependent on Smad phosphorylation and nuclear translocation.4 The complexity of the TGF-β/Smad pathway is important, considering that noncanonical and alternative pathways have been described.1,5 Importantly, development of aneurysms of the ascending aorta in human patients has been linked to TGF-β pathway dysregulation, mutations in genes coding for key actors of the TGF pathway, including FBN1 (fibrillin-1), TGFBR1/R2 (TGF-β receptor type 1 and type 2),5,7 SMAD3,8 and TGFB2 (TGF-β type 2),5,10 as well as tissue fibrinolytic system activation11,12 and constitutive Smad2 overexpression and activation.13

The role of TGF-β signaling in aneurysms is a source of controversy, arising from the comparison between observational data in human tissues and experimental results obtained in mouse models.14,15 It has been widely assumed that the increase in TGF-β release from the degraded extracellular matrix and consecutive TGF-β/Smad2 pathway activation were the main causes of aortic dilation. Nevertheless, the homogeneity of activation of Smad2 signaling in patients bearing loss-of-function mutations of the TGF-β receptors (TGFBR1 and TGFBR2) or SMAD3 has suggested implication of alternative signaling pathways or compensatory mechanisms. Furthermore, in a previous study, we observed a discordance between the spatially
restricted retention of TGF-β and the homogeneous widespread nuclear distribution of phosphorylated Smad2 in vascular smooth muscle cells (VSMCs) of human aneurysmal tissues.\textsuperscript{19} We established that the increase in Smad2 phosphorylation was constitutive and independent of TGFβ1 in human aneurysmal VSMCs and was associated with the overexpression of Smad2 at the mRNA and protein levels. Importantly, we observed that Smad2 overexpression and activation were restricted to VSMCs in the aneurysmal tissue. Finally, Smad2 overexpression was linked to modifications in the histone code on the SMAD2 promoter in nuclei of aortic VSMCs.\textsuperscript{11} However, molecular mechanisms controlling SMAD2 promoter activation were not well characterized in this previous study. The purpose of the present study is to identify the molecular mechanisms controlling SMAD2 promoter activation in human aneurysms.

Methods

An expanded Materials and Methods section is available in Online Data Supplement.

Patients and Aortic Specimens

The clinical research protocol was approved by the local ethical committee (CPP 05 04 32, Ambroise Pare, Boulogne, France, April 2005; updated in March 2008) and was in accordance with the principle of the Declaration of Helsinki.\textsuperscript{20} All patients provided informed consent. Aneurysmal ascending aortic specimens were collected during aortic surgery (Hôpital Bichat). Forty-six aneurysmal specimens were divided into 3 groups according to their clinical features and genetic background: Marfan (Marfan syndrome; n=5; mean age, 39±15 years), associated with bicuspid aortic valve (n=3; mean age, 39±15 years) and degenerative ascending aortic aneurysm (n=6; mean age, 68±20 years). The clinical data associated with this series of patients have been reported.\textsuperscript{19} All specimens were from aneurysms of >5 cm in diameter. Normal ascending aortas were obtained from organ transplant donors (n=10) with the authorization of the French Biomedicine Agency (PFS09-007) and in accordance with the Declaration of Helsinki.\textsuperscript{20} Aneurysmal tissues were sampled in the outer curvature, the most dilated part of the ascending aorta, and in Valsalva for Marfan patients. Aortic tissue preparation consisted of an immediate dissection of the aortic wall, with the separation of the medial and adventitial layers at the junction of the external elastic lamina and the adventitia. This procedure was followed by either freezing or enzymatic digestion to obtain VSMC cultures. The immediate separation of the medial and adventitial layers avoids contamination of the medial VSMC cultures with adventitial fibroblasts.

Primary Cultures and In Vitro Stimulation

Medial samples were incubated in collagenase 0.1% and elastase 0.1% solution (3 hours, 37°C) to obtain VSMCs to be cultured.\textsuperscript{7} All cells were routinely cultured in smooth muscle cell medium (Promocell) containing 5% fetal calf serum, gentamicin sulfate (50 mg/mL), amphotericin B (50 µg/mL), insulin (5 mg/mL), and growth factors (human epidermal and fibroblast growth factors, 0.5 and 2 µg/mL, respectively). Passages 3 to 6 were used for experiments. All stimulations were performed after 24 hours of incubation in serum-free smooth muscle cell medium. VSMCs were incubated with garcinol (p300/300/CBP-associated protein [PCAF] inhibitor) 10 µmol/L (Sigma Aldrich) or cyclopentylidene-[4-(4-chlorophenyl) thiazol-2-yl)hydrazone (CPTH2) (KAT2A [K (lysine) acetyltransferase 2A] [GCN5]) 10 µmol/L (Sigma Aldrich) for 5 hours.

Aortic Tissue Extraction

Extractions (protein, mRNA, and chromatin) were performed directly from frozen aortic media stored at −80°C. The aortic samples were first cryogenically pulverized in liquid nitrogen using a freezer mill (model 6750 SPEX SamplePrep). Approximately 100 to 300 mg of crushed aortic powder was subsequently used.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) assay was performed with aneurysmal (Marfan: n=3; degenerative: n=3; bicuspid aortic valve: n=3) and control (n=5) frozen aortic media. Tissue homogenates were cross-linked with 1% formaldehyde for 15 minutes at room temperature. Glycine was added to obtain a final concentration of 125 mmol/L for 10 minutes at room temperature. Chromatin shearing was performed using a standardized sonication protocol (Bioruptor, Diagenode). The quality of chromatin shearing was assessed by agarose gel migration, and chromatin fragment length between 200 and 800 base pairs was obtained. Immunoprecipitations (IP) were performed with Smad2, p53, myc, PCAF, GCN5, transformation/transcription domain–associated protein (TRRAP), H3K9/14ac, H3 antibodies, and mouse or rabbit control IgG antibodies (2 µg/IP) and using Magna CHiP G or Magna CHiP A kits (Millipore). After IP, DNA was purified. DNA from nonimmunoprecipitated sheared chromatin (INPUT) was also purified. Polymerase chain reaction was then performed on immuno-precipitated (IP) and nonimmunoprecipitated (Input) samples using specific primers (Online Table I). All ChIP data are representative of 3 independent experiments, with quantification for IP and INPUT performed in duplicate by real-time polymerase chain reaction. Results are expressed as the ratio of IP/INPUT for each primer set.

Small Interfering RNA Knockdown

Human VSMCs were transfected with 0.5 µmol/L Accell pool small interfering RNAs (siRNAs; Dharmacon) in Accell delivery media (B-005000). Accell siRNAs were as follows: Accell pool Non-Targeting siRNA (D-001910-10), Accell Red Cyclophilin B siRNA as positive control (D-001975-01-20), Accell pool siRNA PCAF (E-005055-00-0020), and Accell pool siRNA EP300 (E-003486-00-0020). After 72 hours of transfection, proteins and RNAs were extracted as previously described.

Statistical Analysis

Values are expressed as means±SEM. The significance of differences between groups was tested using the Mann–Whitney nonparametric test. A value of P≤0.05 was considered significant.

Results

SMAD2 Activation Is Dependent on p53 Binding to the Proximal Promoter in Aneurysmal VSMCs

The overexpression of Smad2 and its subsequent activation are remarkable features associated with human thoracic aneurysms of various causes.\textsuperscript{13} The increased expression of Smad2 was associated with modifications of the histone code within its promoter. Using in silico analysis of the Smad2 promoter, p53 and Myc consensus binding sites seemed to be of relevant interest. The involvement of p53 in SMAD2 activation was investigated, considering its potential effect on aneurysmal cell survival and apoptosis and the accumulation of p53 in the zones of cystic medial degeneration.\textsuperscript{20,21} Two p53 consensus DNA-binding sites were identified in the SMAD2 promoter: one upstream to the transcription start site (−300 bp) and one

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
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<tr>
<td>HAT</td>
<td>histone acetyl transferase</td>
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<tr>
<td>PCAF</td>
<td>P300/CBP-associated protein</td>
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<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
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<td>TAA</td>
<td>thoracic aortic aneurysm</td>
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<td>TGF-β1</td>
<td>transforming growth factor-β1</td>
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<tr>
<td>TRRAP</td>
<td>transformation/transcription domain–associated protein</td>
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<td>VSMC</td>
<td>vascular smooth muscle cell</td>
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localized in the coding sequence (+750 bp; Online Table II). An increase in p53 binding on the SMAD2 promoter was observed in aneurysmal medial chromatins extracted compared with control (P<0.01; Figure 1A). Similar binding of p53 was found in aneurysmal VSMC cultures, suggesting a persistent recruitment of p53 in vitro after several passages. Of major interest, a decrease in Myc recruitment on the SMAD2 promoter was observed in aneurysmal tissues and VSMCs (Figure 1A). The increase in p53 recruitment in thoracic aortic aneurysm (TAA) VSMCs was specifically detected in the proximal SMAD2 promoter containing the p53-binding site, whereas no binding was found in the intronic region of SMAD2 (Online Figure IA and IB). With the same expression of p53 in control and TAA VSMCs, its recruitment on the SMAD2 promoter was associated with its translacation and accumulation in aneurysmal VSMC nuclei (Figure 1B and 1C). Remarkably, no differences in p53 and Myc at the mRNA and protein levels were observed in control and TAA medial extracts, suggesting that a switch in recruitment takes place on the SMAD2 promoter (Figure 1C). To determine how p53 or Myc binding modulates the SMAD2 promoter activity, we used a series of promoter-reporter plasmids containing various lengths of the SMAD2 promoter (SMAD2-Luc) or site-directed mutations of p53- and Myc-binding sites (Figure 1D and Online Figure II). Rat VSMCs were transfected with the different SMAD2-Luc, and promoter activity was analyzed by luciferase assay. Deletion and mutation of the Myc-binding site induced a significant increase in SMAD2 promoter activity. In contrast, deletion and mutation of the p53-binding site were associated with decreased SMAD2 promoter activity (Figure 1D). These results provide compelling evidence that Myc and p53 play a major role in SMAD2 promoter regulation by controlling its repression and activation, respectively. Furthermore, these results demonstrated that a switch in recruitment occurs between Myc and p53 on the SMAD2 promoter, leading to a change from repression to activation in aneurysmal VSMCs.

Histone H3 Hyperacetylation and Activation of H3 Acetyltransferases p300/PCAF and GCN5 in Aneurysmal VSMCs

Involvement of epigenetic mechanisms, including modifications of the histones on the SMAD2 promoter, has been

Figure 1. Switch between Myc and p53 enrichment on the SMAD2 promoter. A, Chromatin immunoprecipitation (ChIP) assay assessing p53 and Myc enrichment on the SMAD2 promoter in medial extracts or cultured vascular smooth muscle cells (VSMCs) from thoracic aortic aneurysm (TAA) and control aortas. Left, An increase in p53 enrichment on the SMAD2 promoter is observed in aneurysmal tissue compared with control (TAA: 13.77±4.4 vs control: 4.45±3.52; *P<0.01). A significant decrease in Myc recruitment on the SMAD2 promoter is also detected in the aneurysmal media compared with control (P<0.01). Right, Representative images of p53 or Myc enrichment on the SMAD2 promoter after ChIP assays. B, No significant modification of p53 and Myc mRNA (top) or protein (bottom) levels is observed in VSMCs. C, Immunostaining of p53 in aneurysmal and control VSMCs reveals a constitutive nuclear translocation of p53 in TAA VSMCs. Scale bar, 50 µm. D, SMAD2 promoter-reporter luciferase assay. Constructs of mutant SMAD2 promoters for Myc- or p53-binding sites (BS) were transfected in rat VSMCs. Mutation of Myc BS induces a significant increase in the Smad2 promoter activity (*P<0.01). In contrast, mutation of the p53 BS reduces activation of the SMAD2 promoter (*P<0.01). Results are expressed as the ratio of mutant/wild-type (WT) SMAD2-luciferase reporter vector. NS indicates nonsignificant.
observed in human thoracic aneurysms. Here, we investigated the changes in histone H3 hyperacetylation on the Smad2 promoter observed in aneurysms compared with control (P<0.001; Figure 2A and 2B). The increase in the acetylation of H3 is observed in TAA compared with control at positions −600 bp (aneurysms: 21.7±5 vs control: 10.1±6; P<0.001) and −400 bp (TAA: 36.3±5 vs control 13.6±1; P<0.001). The representative gel picture of the samples (top bands) and input (bottom band) is shown below the graph (bottom). Pictures of immunoprecipitation (IP) and Input samples are from the same gel. B, Mapping of H3K9/14ac enrichment along the SMAD2 promoter. All results are expressed as the ratio of immunoprecipitated sample (IP)/nonimmunoprecipitated sample (Input). C and D, Quantification of nonmodified H3 by ChIP at different positions on the SMAD2 promoter in control and TAA aortic tissues. C, A decrease in H3 density is observed by qPCR in TAA compared with control at positions −200 bp (TAA: 13.1±7.4 vs control: 40±10.3; P<0.001) and +100 bp (TAA: 6.7±1.5 vs control: 17.7±5.1; P<0.01). The representative gel picture of the samples (top bands) and input (bottom band) is shown below the graph (IP and INPUT are from the same gel). D, Mapping of nonmodified H3 enrichment along the SMAD2 promoter. Results are expressed in percentage of IP/INPUT.

Figure 2. Chromatin remodeling on the SMAD2 promoter. A and B, Quantification of H3K9/14ac by chromatin immunoprecipitation (ChIP) assay on the SMAD2 promoter in control and thoracic aortic aneurysm (TAA) aortic tissues. A, Assessment of H3K9/14ac enrichment at 4 different locations of the SMAD2 promoter by quantitative polymerase chain reaction (qPCR; graph, top). An increase in the acetylation of H3 is observed in TAA compared with control at positions −600 bp (aneurysms: 21.7±5 vs control: 10.1±6; P<0.001) and −400 bp (TAA: 36.3±5 vs control 13.6±1; P<0.001). The representative gel picture of the samples (top bands) and input (bottom band) is shown below the graph (bottom). Pictures of immunoprecipitation (IP) and Input samples are from the same gel. B, Mapping of H3K9/14ac enrichment along the SMAD2 promoter. All results are expressed as the ratio of immunoprecipitated sample (IP)/nonimmunoprecipitated sample (Input). C and D, Quantification of nonmodified H3 by ChIP at different positions on the SMAD2 promoter in control and TAA aortic tissues. C, A decrease in H3 density is observed by qPCR in TAA compared with control at positions −200 bp (TAA: 13.1±7.4 vs control: 40±10.3; P<0.001) and +100 bp (TAA: 6.7±1.5 vs control: 17.7±5.1; P<0.01). The representative gel picture of the samples (top bands) and input (bottom band) is shown below the graph (IP and INPUT are from the same gel). D, Mapping of nonmodified H3 enrichment along the SMAD2 promoter. Results are expressed in percentage of IP/INPUT.

The acetylation of lysines K9 and K14 of histone H3 was remarkably increased in aneurysmal VSMC nuclei compared with control (P<0.001; Figure 3A). This observation was confirmed by immunoblotting showing a global increase in lysine acetylation and H3K9/14 acetylation in TAA medial extracts compared with healthy aortas (Figure 3B). These results suggest an increase in the activity of histone acetyl transferases (HATs), including the HAT responsible for H3K9/14 acetylation. In accordance with this hypothesis, an increase in H3 HAT activity was detectable in aneurysmal medial extracts compared with control (Figure 3C; P<0.01). In contrast, no difference in H4 HAT or histone deacetylase activity was observed (Figure 3C; Online Figure III). In aneurysmal VSMC cultures, a significant increase in H3 HAT activity was also observed compared with control VSMCs (P<0.001; Figure 3D). VSMCs were then treated with 2 H3 HAT inhibitors, garcinol and CPTH2, respectively, responsible for the inhibition of p300/PCAF and GCN5. Importantly, p300/PCAF and GCN5 are the major enzymes responsible for the acetylation of histone 3 tail. The global level of H3 HAT activity was strongly repressed by garcinol treatment in aneurysmal VSMCs (garcinol: 0.02±0.014 versus nonstimulated: 0.115±0.04; P<0.05; Figure 3D). In contrast, a limited effect of CPTH2 was observed. These data provide convincing evidence of an increase in H3 HAT activity and the involvement of p300/PCAF in SMAD2 overexpression in TAA tissue extracts and VSMCs in culture.

The p300/PCAF Complex Plays a Crucial Role in SMAD2 Promoter Activation

Co-operative interactions between p53 and transcriptional complexes, including enzymatic subunits such as p300/PCAF and GCN5, have been previously described. These
complexes are also composed of nonenzymatic cofactors, which act as nuclear scaffold proteins by their ability to bind transcription factors and histone-modifying enzymes. Considering the proteins involved in our study, the recruitment of the cofactor TRRAP was suspected.\textsuperscript{25,26} By ChIP, we assessed the enrichment of PCAF, GCN5, and TRRAP on the SMAD2 proximal promoter, in the vicinity of the p53-binding site (Figure 4A). An important increase in PCAF and TRRAP enrichment was observed in aneurysmal medial extracts compared with control. In contrast, no difference in GCN5 recruitment on the SMAD2 promoter was observed in aneurysmal and control tissues (Figure 4A). It is of major interest that the recruitment process of HATs and cofactors is totally independent of variations in their expression at the protein and mRNA levels (Figure 4B, Online Figure IV). Furthermore, we observed a modification in the intracellular localization of PCAF in aneurysmal VSMCs compared with control. Indeed, an accumulation of PCAF within the nucleus was observed in TAA VSMCs, whereas the subunit p300 was largely localized within the nuclei of both control and aneurysmal VSMCs.

To evaluate the involvement of p300/PCAF and GCN5 in Smad2 expression, we quantified Smad2 mRNA levels after treatment of control and aneurysmal VSMCs with garcinol or CPTH2. In the absence of inhibitors, we observed an overexpression of total Smad2 mRNA in aneurysmal VSMCs (aneurysms: 0.1±0.013 versus control: 0.038±0.004; \#P<0.001; Figure 5A). Treatment with H3 HAT inhibitors affected the expression of Smad2 in aneurysmal VSMCs but not in control cells. Indeed, both garcinol (*P<0.01) and CPTH2 (*P<0.05) induced a decrease in the expression of Smad2 mRNA. Interestingly, we previously reported that Smad2 isoforms (Online Figure VA)\textsuperscript{27} were differentially expressed in thoracic aneurysms, with a specific increase in the Smad2 1a mRNA.\textsuperscript{13} In accordance with these observations, we show here that the inhibition of H3 HAT by garcinol or CPTH2 induced a specific decrease in Smad2 1a expression in aneurysmal VSMCs (Online Figure VB), but the Smad2 1b variant was not modified (Online Figure VC).

These results indicate that H3 HATS control the Smad2 expression observed specifically in aneurysmal media and cultured VSMCs. Furthermore, nuclear translocation of p300/PCAF and the increased enrichment on the SMAD2 promoter suggest that p300/PCAF could be a major actor in SMAD2 regulation. To determine rigorously the role of both PCAF and p300 in SMAD2 promoter activation, we treated human TAA VSMCs with siRNA targeting PCAF or p300, respectively. It is well recognized that primary cultures of

Figure 3. Histone and nonhistone protein hyperacetylation in thoracic aortic aneurysm (TAA) vascular smooth muscle cells (VSMCs). A, Immunofluorescent staining of H3K9/14ac reveals hyperacetylation in TAA VSMC nuclei. Staining of acetylated lysine residues in aneurysmal and control VSMC cultures shows that acetylated lysine is exclusively present in nuclei (4',6-diamidino-2-phenylindole). A basal increase in lysine acetylation is observed in aneurysmal VSMCs compared with control. Scale bar, 50 μm. B, Immunoblotting of acetylated lysine and H3K4ac in media tissue extracts. A global hyperacetylation of lysine is observed in TAA tissue extracts compared with control. The same results were observed for H3 acetylation that is slightly increased in TAA. H3 was used as a protein loading control. C, Histone acetyltransferases (HAT) activity in TAA and control aortic tissues. A specific H3 HAT activity increase is detected in TAA (results expressed as optical density (OD)/μg of proteins; TAA: 0.0085±0.0006 vs control: 0.0045±0.0004; P<0.001). D, H3 HAT activity in TAA and control VSMCs treated with garcinol or CPTH2. A basal increase in H3 HAT activity was observed in TAA VSMCs (TAA: 0.116±0.042 vs control: 0.066±0.001; *P<0.05). Treatment with garcinol induces an important decrease in H3 HAT activity (nontreated: 0.116±0.042 vs garcinol: 0.02±0.01; #P<0.005). A more limited decrease in H3 HAT activity is observed with CPTH2 treatment.
human VSMCs are not easily transfectable. We first validated our modified siRNA transfection procedure (Online Figure VI) that presented a high efficiency. Then, we assessed the expression of PCAF or p300 after transfection with corresponding siRNA or control siRNA (Figure 5B). A significant decrease in p300 or PCAF was induced by specific siRNA transfection in TAA VSMCs, whereas control siRNA did not modify p300 or PCAF expression. A decrease in Smad2 mRNA level was observed in TAA VSMCs after treatment with siRNA targeting p300 or PCAF, providing evidence that these HATs are responsible for Smad2 overexpression in TAA-VSMCs (Figure 5C). Furthermore, treatment of aneurysmal VSMCs with garcinol or CPTH2 clearly decreased H3K9/14ac enrichment of the SMAD2 promoter, indicating that PCAF/p300 and GCN5 are indeed responsible for H3K9/14ac enrichment of the SMAD2 promoter (Figure 5D).

In addition to their role in histone acetylation, HATs can also acetylate transcription factors and induce their activation. Consequently, our next step was to investigate the role of PCAF and GCN5 on p53 binding and acetylation. First, p53 binding on the SMAD2 promoter was assessed in control and aneurysmal VSMCs after treatment with garcinol and CPTH2 (Figure 6A). Treatment with garcinol drastically decreased the binding of p53 on the SMAD2 promoter in TAA VSMCs. Several residues of p53 can undergo acetylation. An estimation of the global acetylation of p53 was performed by IP with a p53 antibody and immunoblotting with acetyl lysine antibody. An increase in p53 acetylation was observed in aneurysmal medial extracts compared with control extracts (*P<0.01; Figure 6B), as well as in nontreated aneurysmal VSMCs compared with control (*P<0.01; Figure 6C). Treatment with garcinol revealed that p53 acetylation was caused by p300/PCAF in aneurysmal VSMCs (nontreated: 1.6±0.2 versus garcinol: 0.55±0.07; #P<0.01). Finally, we tested the effect of PCAF and p300 knockdown on the p53 acetylation level in control and TAA VSMCs by transfecting PCAF, p300, or control siRNA (Figure 5D). It is of major interest that a significant decrease in p53 acetylation was found after treatment with p300-targeting siRNA, demonstrating that p300 plays a crucial role in p53 acetylation.

Together, these results identified the mechanisms of activation of the SMAD2 promoter in aneurysmal VSMCs consisting of the recruitment of a transcriptional complex, including p53, PCAF, GCN5, and TRRAP (Figure 7). The p300/PCAF complex plays a crucial role in the recruitment and activation of p53 by its ability to acetylate histone H3 and p53.

**Discussion**

This study identifies for the first time the molecular mechanisms controlling the constitutive overexpression of Smad2 in human VSMCs and the chromatin remodeling associated with aneurysmal disease. This process includes a switch between Myc and p53 recruitment that profoundly modulates the SMAD2 promoter activity. The SMAD2 promoter activation is strongly dependent on the presence of histone acetyltransferases p300/PCAF, which play a crucial role in histone and p53 acetylation and p53 enrichment on the Smad2 promoter. This activation of the SMAD2 promoter observed in aneurysmal tissue extracts is constitutively maintained once aortic VSMCs are isolated and cultured, independently of environmental cues.

Histone acetylation has been largely associated with transcription activation, causing an increase in promoter accessibility and the recruitment of nuclear factors. In this study, the increase in histone acetylation is correlated with global hyperacetylation of the lysine residues of histones and nonhistone proteins. Studies have already suggested that general nuclear hyperacetylation could influence cell proliferation and differentiation and could be implicated in chronic human diseases, such as cancer and atherothrombosis. Here, we show that histone acetylation and, in particular, the acetylation of H3K9/14 are global processes probably affecting a set of genes (including SMAD2) in the aneurysmal VSMCs.

The process described in our present study presents several levels of specificity. First, overexpression of Smad2 is specific of aneurysmal VSMCs and is not detected in aneurysmal adventitial fibroblasts. Second, Smad2 overexpression only concerns the Smad2 1a transcriptional variant. In parallel, the multiplicity of partners and mechanisms...
involved underlines the complexity of the transcriptional activation of the SMAD2 promoter. Our data also suggest that recruitment and formation of a multipartner complex are essential in such an epigenetic regulatory process. This complex associates a catalytic subunit (HAT, eg, p300/PCAF), the transcriptional machinery, and a cofactor (eg, TRRAP; Figure 7). TRRAP, which is devoid of enzymatic properties, seems to act as a nuclear scaffold protein and is responsible for a concerted and context-dependent recruitment of HATs and the coordination of distinct chromatin-based processes. Importantly, the activation and recruitment of these partners (eg, p53, p300/PCAF, and TRRAP) occur in aneurysmal VSMCs without any modification of their expression, but our results suggest that the enrichment of the Smad2 promoter is associated with nuclear translocation of some of these partners.

In vascular diseases, compelling evidence has been provided for the involvement of p300 in the acetylation of several lysine residues on the H3 and H4 tails. Importantly, a recent study demonstrated that the regulation of TGF-β1–mediated transcription is dependent on p300/CBP, p300 is also responsible for the acetylation of the Smad2 protein and increases its transcriptional activation function.

It is widely known that p300/CBP and PCAF form a complex regulating gene activity. In contrast, less attention has been paid to the potential cooperation of p300/PCAF and GCN5, which acetylate specifically H3K9/14. Even if ChIP analysis showed that both p300/PCAF and GCN5 are recruited to the SMAD2 promoter, our data suggest a predominant role of the p300/PCAF complex in the activation of SMAD2 in aneurysmal VSMCs. Indeed, p300/PCAF recruitment on the SMAD2 promoter controls p53 binding and acetylation of H3 tails and p53. Thus, we can speculate that p300/PCAF and GCN5 have distinct functions. Indeed, GCN5, basally present on the SMAD2 promoter, could be responsible for the basal acetylation of the SMAD2 promoter under physiological conditions. However, during aneurysmal disease progression, a VSMC-specific recruitment of p300/PCAF on the SMAD2 promoter occurs and induces severe hyperacetylation of H3K9/14, as well as p53 recruitment. Histone acetylation per se could have multiple potential actions by direct modification of the chromatin conformation or by recruitment of nuclear factors. Indeed, acetylated lysine can be recognized by proteins with a bromodomain, including PCAF.

A major feature described in our studies is the switch from Myc to p53 binding on the SMAD2 promoter of aneurysmal

Figure 5. p300 and P300/CBP-associated protein (PCAF) histone acetyltransferases are directly involved in Smad2 overexpression and induce histone hyperacetylation of the SMAD2 promoter in thoracic aortic aneurysm (TAA) vascular smooth muscle cells (VSMCs). A, In cultured VSMCs, garcinol and CPTH2 treatments induce a decrease in Smad2 expression in aneurysmal VSMCs (Smad2–relative mRNA level: nontreated: 1±0.013, garcinol: 0.4±0.003; P<0.01, CPTH2: 0.68±0.002, #P<0.01: TAA vs control VSMC; *P<0.01, *P<0.05: garcinol or CPTH2 vs nontreated; †P<0.05 garcinol vs CPTH2). B, Specific knockdown of PCAF or p300 was performed by transfection of specific small interfering RNA (siRNA) in human TAA or control VSMCs. siRNAs targeting PCAF or p300 induce a specific and significant decrease in PCAF and p300, respectively, whereas no effect is observed with control siRNA (*P<0.05). C, siRNA-induced knockdown of PCAF or p300 decreases expression of Smad2, providing direct evidence of their participation in Smad2 overexpression in TAA (P<0.05). D, Chromatin immunoprecipitation of H3K9/14ac in cultured VSMCs treated with garcinol and CPTH2. H3 acetylation level was estimated specifically on the SMAD2 promoter. A basal increase in H3 acetylation is observed in aneurysmal VSMCs, and treatment with garcinol or CPTH2 decreases H3 acetylation level on the Smad2 promoter. Inhibitors have no effect on H3 acetylation in control VSMCs.
VSMCs. The association of ChIP and promoter-reporter luciferase assays provides compelling evidence that Myc is an important regulatory factor limiting Smad2 expression in normal VSMCs. Myc is a transcription factor that can both activate and repress genes depending on its interaction with cofactors.41 In aneurysmal VSMCs, the considerable increase in p53 expression and SMAD2 promoter activation is because of abolishment of the repressive effect of Myc and the recruitment of p53. The tumor suppressor gene p53 is a transcription factor with pleiotropic effects influencing cell survival, proliferation, and differentiation.42 Recent studies have highlighted the involvement of p53 in regulating vascular VSMCs in cardiovascular diseases such as atherosclerosis.43 Nevertheless, controversial results relating to the exact impact of p53 activation on VSMCs and their resistance to apoptosis have been published, thus suggesting context-dependent effects of p53.21,44 Our study demonstrates the implication and the major role of p53 in aneurysmal VSMCs, but the balance between beneficial and deleterious effects of p53 activation and the subsequent expression of Smad2 remain unknown.

Aneurysmal diseases are defined by aortic dilation associated with thinning of the aortic medial layer. In application of the Laplace law (stress=pressure×radius/thickness), the stress/strain relationship to which VSMCs are submitted is enhanced in the aneurysmal media in relation to dilation without compensatory hypertrophy. There is recent evidence that chromatin remodeling can be influenced by strain in adherent cells.45,46 These studies showed that mechanotransduction, via the structural continuum of the cell and biochemical signaling to the nucleus, can control gene expression.45 More precisely, the authors described how increased mechanical strain causes a decrease in histone deacetylase activity.46 This hypothesis merits further exploration in aneurysmal VSMCs.

The importance of epigenetic reprogramming and how chromatin modulation regulates the VSMC expression repertoire might provide new insights into the onset and progression of aneurysms and other chronic vascular diseases.47,48
None.

References
28. Barlev NA, Liu L, Chehab NJ, Mansfield K, Harris KG, Halazonetis TD, Berger SL. Acetylation of p53 activates transcription through...
44. Mercer J, Mahmoudi M, Bennett M. DNA damage, p53, apoptosis and vascular disease. Mutat Res. 2007;621:75–86.

Novelty and Significance

What Is Known?

• Human aneurysms of the ascending aorta, whatever their cause are characterized by progressive dilation of the aorta associated with overexpression and increased nuclear translocation of Smad2 in vascular smooth muscle cells of the media.
• This constitutive overexpression of Smad2 has been reported to be due to epigenetic modifications involving histone acetylation at the level of the Smad2 promoter.

What New Information Does This Article Contribute?

• The induced change in Smad2 promoter accessibility causes a switch in binding of transcription factors from Myc, a repressor to p53, an activator.
• This switch is under the control of histone acetyltransferase complex formation, with p300 and p300/CBP-associated protein, on the promoter of Smad2.

During the formation of aortic aneurysms, blood-borne proteases degrade the extracellular matrix, leading to aortic dilation and rupture. In response to such dilation, smooth muscle cells of the media change their phenotype to limit proteolytic injury. This phenotypic switch involves the independency of Smad2 expression, activation, and consequences with regard to extracellular transforming growth factor-β. This autonomy is controlled by an epigenetic phenomenon of histone acetylation changing the accessibility of the Smad2 promoter to transcription factors. In the present study, we examined the molecular mechanisms of this constitutive Smad2 overexpression and nuclear translocation. We found that histone acetyltransferases, p300 and P300/CBP-associated protein, are involved in altered transcription factor binding to the Smad promoter, resulting in a switch from Myc-dependent repression to p53 activation. These results demonstrate that aneurysmal dilation of the human aorta is associated with changes in chromatin dynamics within smooth muscle cells of the proteolysed media, independently of transforming growth factor-β. Epigenetic activation of the Smad2-dependent pathway controls different patterns of genes, including genes encoding for fibrillar extracellular matrix and antiproteases, partly protecting the aortic wall by slowing down the proteolytic damage.
Modifications of Chromatin Dynamics Control Smad2 Pathway Activation in Aneurysmal Smooth Muscle Cells

Delphine Gomez, Ketty Kessler, Jean-Baptiste Michel and Roger Vranckx

_Circ Res._ 2013;113:881-890; originally published online July 3, 2013; doi: 10.1161/CIRCRESAHA.113.301989

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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Supplemental Material

Detailed Methods

**Immunofluorescent staining**

Cells were fixed in 3.7% paraformaldehyde (20 min at room temperature), then incubated with methanol (10 minutes, -20°C). Cells were blocked with 1% BSA for 1 hour and incubated with H3K9/14ac, HP-1 (5µg/mL, Diagenode), PCAF (10µg/mL, Santa Cruz), p300 (2 µg/mL, Santa Cruz) and p53 (10µg/mL, Millipore) antibodies for 1h at room temperature. Secondary antibodies coupled with Alexa-555 and Alexa-488 were used.

**Reverse Transcription - PCR**

Total RNA was extracted from frozen media samples or cultured VSMCs using the EZNA kit (Omega Biotek), according to the manufacturer’s directions. Reverse Transcription was performed using kits from Invitrogen. Real-time PCR was performed in the LightCycler system with SYBR Green detection (Roche Applied Science) using specific primers ([Online Table I](#)).

**Immunoblotting and immunoprecipitation**

Protein extracts from medial tissues were used after homogenization in lysis buffer [50 mM Tris (pH 8), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 5 mM EDTA] and containing protease inhibitors (Sigma Aldrich). Protein extracts (20µg) were separated in reducing conditions by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The membrane was blocked with 5% milk or BSA (Bovine Serum Albumin) powder in TBS-T [Tris-buffered saline (pH 7.4)-0.1% Tween 20] and was then incubated overnight (4°C) with primary antibodies against: acetylated lysine (Cell Signaling), PCAF (Cell Signaling), TRRAP (Cell Signaling), GCN5 (0.25 µg/mL; Millipore) or p53 (1µg/mL; Santa Cruz). The membrane was then incubated with peroxidase-conjugated anti-mouse or anti-rabbit IgG (Jackson Laboratories) for 1 h. The signal was detected using a chemiluminescence kit (ECL + kit; Amersham). For immunoprecipitation, samples (300µg) were incubated with beads Bio-Adembeads Protein G (Ademtech) and anti-p53 antibody at 4µg/mL at 4°C overnight. Immunoblotting was then performed following the same protocol.

**HAT and HDAC activity assay**

Assay of HAT and HDAC activity was performed according to the manufacturer’s instructions (Millipore), using aneurysmal and control medial extracts or aneurysmal and control cell extracts (100µg).

**Luciferase assay**

$SMAD_2$ promoter-luciferase constructs ($SMAD_2$-Luc) consisting of1500, 420, 338 or 131 bp of the proximal $SMAD_2$ promoter were generated using basic restriction enzyme cloning methods. 1500 bp $SMAD_2$-Luc with site-directed mutations of p53 and Myc binding sites were generated by directed mutagenesis according to the manufacturer’s instructions (Stratagene). Mutations were confirmed by sequencing. Briefly, $SMAD_2$-Luc (p1500, p420, p338 and p131) were amplified by PCR with specific primers ([Online Table I](#)), using Taq High Fidelity polymerase (Invitrogen). Fragments were digested by restriction enzymes $EcoRI$ and $HindIII$ and inserted into the pMetLuc2-Reporter vector (Clontech). The reporter plasmid encodes for the secreted Luciferase protein. $SMAD_2$-Luc constructs were transfected in rat VSMCs growing in DMEM medium containing 10% Fetal Bovine...
Serum. Briefly, cells having reached 70% confluency were used. Transfections were performed with the liposomic reagent Fugene (Roche) at 6µl/ml and with 1µg/ml of the SMAD2-Luc plasmid. Promoter-reporter plasmids were pre-incubated with the transfecting reagent (30 minutes at room temperature). Complexes were added to cells drop-wise. The cell medium was sampled 24h after transfection and luciferase activity was measured (in relative luciferase units).

**SMAD2 promoter in silico analysis**

An analysis of the SMAD2 promoter was performed (from -1500 to 1000 of the human SMAD2 promoter). The biocomputing analysis of the transcription factor binding sites was performed using Genomatix Software. Sequence, position and score corresponding to putative p53 and Myc binding sites were listed in Online Figure I. The presence and location of Transcription Start Sites (TSS) CAAT box were also investigated.

Reference List


Online Figure I. Mapping of p53 and Myc binding on the SMAD2 promoter

A. Schematic representation of the SMAD2 proximal promoter and localization of p53 and myc consensus binding sites. Several sites have been found in the vicinity of the Transcription Start Sites (arrows).

B. ChIP analysis of p53 and Myc enrichment along the SMAD2 promoter (-800 and +1000bp). A significant increase in p53 binding is observed in aneurysmal VSMCs, around -300bp corresponding with the location of the putative p53 binding site. In this region, a decrease in Myc binding has been found. In contrast, no binding of p53 is quantified in both control and TAA around +800 corresponding with a putative p53 binding site.
Online Figure II. p53 and myc binding sites modulate the SMAD2 promoter activity
Schematic representation of the SMAD2 promoter-reporter constructs used for luciferase assay. Deletion or site-directed mutation constructs of the SMAD2 promoter have been generated. A significant increase in the SMAD2 promoter activity is quantified when the Myc binding site is deleted or mutated (* p<0.01). Opposed resulted are observed with deletion or mutation of the p53 binding site with significant decrease in SMAD2 promoter activity (# p<0.01).
Online Figure III. HDAC global activity in aneurysmal and control aortic media. HDAC activity assay shows no difference in aneurysm compared with control (results expressed as OD/µg of proteins).
Online Figure IV. mRNA expression of GCN5, PCAF and TRRAP in aortic VSMCs. No difference in GCN5, PCAF and TRRAP expression is observed in control and aneurysmal VSMCs. Results are normalized to GAPDH mRNA level.
Online Figure V. Smad2 alternative transcript expression.

A. Schematic representation of SMAD2 promoter. Two TSSs were identified on the Smad2 promoter by Takenoshita et al. Alternative TSSs lead to the expression of two main Smad2 mRNA variants: Smad2 1a and Smad2 1b. The two Transcription Start Sites (TSS) are represented upstream to the 1a and 1b exons, respectively. Alternative promoter and splicing lead to the expression of two main mRNA variants: 1a and 1b. 

B & C. Quantification of the Smad2 1a (B) and Smad2 1b (C) mRNA by qPCR after treatment with Garcinol or CPTH2. As previously described, a basal increase in the Smad2 1a mRNA is observed. The decreased expression of Smad2 induced by Garcinol and CPTH2 concerns only the transcriptional variant 1a but not the variant 1b (p<0.01). The effects of HAT inhibitors are specific of aneurysmal VSMCs and are not observed in control VSMCs.
Online Figure VI. Validation of SiRNA transfection in primary culture of human VSMCs.

A. The efficiency of the transfection is evaluated by a red tagged Accell Red Cyclophilin B siRNA (PPIB). The siRNA transfected in the hVSMC is red.

B & C. The PPIB siRNA transfected decreases significantly its mRNA and protein expression in the hVSMC (p<0.005). The control siRNA does not affect the mRNA and protein expression.
**Online Table I.** Primer list and sequences used to qPCR, ChIP and **SMAD2** promoter-reporter constructions.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMAD2 1a variant</td>
<td>forward 5'-ACACCTTATACCTGGGATCCCTACC-3'</td>
</tr>
<tr>
<td></td>
<td>reverse 5'-GGACGGGCGCTGAGAGAGAAGA-3'</td>
</tr>
<tr>
<td>SMAD2 1b variant</td>
<td>forward 5'-GAGTGTCTTCAATCCGCTTCCCA-3'</td>
</tr>
<tr>
<td></td>
<td>reverse 5'-GCAGCGGCACTGAGACAGC-3'</td>
</tr>
<tr>
<td>SMAD2 promoter</td>
<td>forward 5'-GCCTAATCGTCGCAACAGTGGGAG-3'</td>
</tr>
<tr>
<td></td>
<td>reverse 5'-CGGGTAGGGAAGTGCCAGGTA-3'</td>
</tr>
<tr>
<td></td>
<td>forward 5'-ACACCTTATACCTGGGACCCCTACC-3'</td>
</tr>
<tr>
<td></td>
<td>reverse 5'-GGACGGGCGCTGAGAGAAGAA-3'</td>
</tr>
<tr>
<td></td>
<td>forward 5'-GGAGGACCCCTTTTCTTCTCAG-3'</td>
</tr>
<tr>
<td></td>
<td>reverse 5'-CCGCGCATTAAGACGATTTCC-3'</td>
</tr>
<tr>
<td></td>
<td>forward 5'-GGAGATGGAAGACGAGGGAG-3'</td>
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<td></td>
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<td></td>
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<tr>
<td></td>
<td>reverse 5'-GGAGGACCCCTTTTCTTCTCAGGAG-3'</td>
</tr>
<tr>
<td></td>
<td>forward 5'-GCCGCGCCACTAGTGACAGC-3'</td>
</tr>
<tr>
<td></td>
<td>reverse 5'-CCGCGCATTAAGACGATTTCC-3'</td>
</tr>
</tbody>
</table>
**Online Table II.** The p53 and myc consensus binding sites on the *SMAD2* promoter with sequence position and strand.

<table>
<thead>
<tr>
<th>Transcription Factor</th>
<th>Putative binding sites (Sequence)</th>
<th>position (base pairs)</th>
<th>strand</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53</td>
<td>tgaggt CCTGtaagggcct gcgc</td>
<td>-318 -295</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>cctg CAAG gtggcg ccaggt cccc</td>
<td>758 782</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>tgag caCGTG tcc</td>
<td>-362 -349</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>ccgc ccGC GCgc</td>
<td>137 150</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>aagccg CGGCGg</td>
<td>134 153</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>gtgc ctCGG Gccc</td>
<td>162 176</td>
<td>+</td>
</tr>
</tbody>
</table>

- **TSS 1a**: 1
- **TSS 1b**: 600
- **CAAT box**: -105