Tet3-Mediated Hydroxymethylation of Epigenetically Silenced Genes Contributes to Bone Morphogenic Protein 7-Induced Reversal of Kidney Fibrosis

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ABSTRACT

Methylation of CpG island promoters is an epigenetic event that can effectively silence transcription over multiple cell generations. Hypermethylation of the Rasal1 promoter contributes to activation of fibroblasts and progression of kidney fibrosis. Here, we explored whether such causative hypermethylation could be reversed through endogenous mechanisms and whether such reversal of hypermethylation is a constituent of the antifibrotic activity of bone morphogenic protein 7 (BMP7). We show that successful inhibition of experimental kidney fibrosis through administration of BMP7 associates with normalization of Rasal1 promoter hypermethylation. Furthermore, this reversal of pathologic hypermethylation was achieved specifically through Tet3-mediated hydroxymethylation. Collectively, our findings reveal a new mechanism that may be exploited to facilitate therapeutic DNA demethylation to reverse kidney fibrosis.


In recent years, epigenetics have emerged as determinants of fibrosis in the kidney (and other tissues as well).1–5 Furthermore, epigenetics have been implied to contribute to the individual susceptibilities of CKD patients to develop fibrosis.1–3 Among the known epigenetic mechanisms, methylation of CpG island promoters (referred to as DNA methylation) is the most potent to silence transcription of affected genes.6 Because transcriptional silencing of affected genes has been shown to causally contribute to fibroblast activation and fibrogenesis, inhibition or reversal of such aberrant methylation is considered beneficial for the kidney.5 Although in recent years, evidence has emerged that DNA methylation is less stable than previously thought, because the methylome is widely erased during zygote formation, little is yet known about the dynamics of DNA methylation in adult somatic cells.7 Here, we aimed to investigate if the adult kidney possesses endogenous mechanisms to normalize aberrant DNA methylation and explore the possibility that such endogenous mechanisms could be therapeutically used to protect the kidney.

We first aimed to establish a common methylation mark, which would allow us to monitor methylation and possible demethylation across various mouse models of kidney fibrosis. Based on our previous studies, in which we had identified RASAL1 (which encodes for rasGAP-activating–like protein 1, a suppressor of Ras-GTP function) in a genome-wide methylation screen to be selectively hypermethylated in fibrotic human renal fibroblasts, as well as experimental kidney fibrosis of folic acid–induced nephropathy and nephrotoxic serum nephritis,3 we now expanded our analysis to mouse models of unilateral ureteral obstruction (UUO), CD1 mice that developed diabetic nephropathy through administration of streptozotocin (DN), COL4A3-deficient Alport mice, and 5/6 nephrectomy.8–10 In each of these analyzed models, experimental kidney fibrosis was associated with
Figure 1. Ameliorated experimental renal fibrosis upon BMP7 treatment is associated with reversal of aberrant Rasal1 promoter methylation. (A) Histology of UUO-challenged kidneys. The panels display representative photomicrographs of Masson’s trichrome-stained (MTS) kidney sections from control and UUO kidneys treated with either vehicle or BMP7. Original magnification, ×10; scale bar, 200 μm. (B) Rasal1 methylation in UUO kidneys. We performed methylated DNA immunoprecipitation (MeDIP) to assess the effect of antifibrotic BMP7 treatment on Rasal1 methylation in mouse kidneys that were challenged with UUO. In this assay, fragmented total kidney DNA (input DNA) was exposed to antibodies that specifically capture 5mC DNA. The captured DNA is eluted and analyzed by primers specific
Rasal1 hypermethylation and decreased Rasal1 expression, providing additional evidence for a role of Rasal1 hypermethylation in experimental renal fibrosis, irrespective of the underlying disease model (Figure 1, A–F, Supplemental Figure 1). To further corroborate our findings, we analyzed Rasal1 methylation and mRNA expression levels in kidney biopsies and corresponding primary fibroblast cultures from a small cohort of patients (Supplemental Table 1). In whole-kidney biopsies and corresponding fibroblasts, severe fibrosis was associated with Rasal1 hypermethylation (Supplemental Figure 2, A and C) and transcriptional silencing of Rasal1 (Supplemental Figure 2, B and D). In this regard, transcriptome analysis data on larger cohorts available through the Nephromine database (www.nephromine.org) reveal that CKD caused by minimal change nephropathy and hypertensive nephrosclerosis correlated with decreased Rasal1 expression.11–13 In summary, our results suggest that Rasal1 is hypermethylated in kidney fibrosis, irrespective of the underlying cause, and based on our data, testing the use of Rasal1 methylation as a biomarker of CKD in larger cohorts of patients may deserve consideration.

To gain insights into possible reversal of aberrant hypermethylation, we next analyzed Rasal1 methylation in mice with experimental CKD, which had been successfully treated with antifibrotic bone morphogenetic protein 7 (BMP7).9 We decided to focus on BMP7-treated mice, because it had been previously established to be antifibrotic in all the models of kidney fibrosis studied above,8,14,15 it acts as an antagonist of the profibrotic TGF-β1 (a known inducer of aberrant Rasal1 methylation),16 and it has been shown to normalize the profibrotic phenotype of activated renal fibroblasts (in which Rasal1 is hypermethylated).17 Reduced renal fibrosis on BMP7 treatment in mice challenged with UUO and DN correlated with normalization of Rasal1 promoter methylation and expression levels (Figure 1, A–F, Supplemental Figure 1, B and C).

We next aimed to gain insights into the possible mechanisms that underlie normalization of Rasal1 promoter methylation on BMP7 treatment. Because the covalently linked methyl group cannot be simply removed from methylated cytosine bases (5mC), this process requires removal of 5mC and subsequent replacement with unmethylated naked cytosine bases.18 Under physiologic conditions, such demethylation occurs during formation of zygotides through an oxidative process, which involves the addition of an OH group to the methyl group of 5mC; the product is called hydroxymethylcytosine.19,20 Such hydroxymethylation of methylated CpG island promoters increases transcriptional activity of affected genes and also serves as a starting point for subsequent base excision repair and incorporation of unmethylated cytosine.21,22 To gain insights into the existence of possible endogenous demethylation...
Figure 2. BMP7 normalizes TGF-β1–induced methylation of Rasal1 through hydroxymethylation in primary renal fibroblasts. (A) Rasal1 methylation (5mC). Rasal1 methylation in primary mouse kidney fibroblasts in response to TGF-β1 and/or BMP7 for 1, 2, 5, and 10 days was analyzed by MeDIP. After 2 days of TGF-β1 stimulation, Rasal1 methylation was detected. After removal of TGF-β1 (after 5 days when Rasal1 was robustly methylated), Rasal1 remained hypermethylated and transcriptionally silenced (assessed at day 10 of tissue culture).
mechanisms in the adult kidney, we next analyzed Rasal1 hydroxymethylation in mice that had been challenged with experimental renal fibrosis and treated with antifibrotic BMP7 (normalized Rasal1 methylation) through hydroxyl-methylated DNA immunoprecipitation, enabling us to discriminate between methylated and hydroxymethylated DNA (which is not possible through BGS). We observed de novo Rasal1 hydroxymethylation in kidneys of BMP7-treated mice, which correlated with the observed decreased Rasal1 methylation (Figure 1, G and H).

Because physiologic hydroxymethylation in zygotes is mediated by the 10–11 translocation enzymes (Tet1, Tet2, and Tet3),19,20 we next hypothesized that enzymes of the Tet family are similarly involved in BMP7-induced erasure of aberrant Rasal1 methylation. Experimental renal fibrosis in mice challenged with UUO, DN, nephrotoxic serum–nephritis, COL4A3 knockout, and 5/6 nephrectomy was associated with decreased expression of Tet3, but not Tet1 and Tet2 (Figure 1, I–K, Supplemental Figure 3, A–C). These results are in line with existing data on Nephromine, which reveal decreased Tet3 expression in cohorts of DN, hypertensive nephrosclerosis, IgA nephropathy, and lupus nephritis patients (www.nephromine.org).12,13,23,24 Effective BMP7 treatment of UUO and DN mice (decreased fibrosis) correlated with normalization of Tet3 expression levels (Figure 1K). In summary, fibrosis was associated with Rasal1 promoter hypermethylation, suppressed Rasal1 expression, and suppressed Tet3 expression (expression of Tet1 and Tet2 were not substantially altered), whereas amelioration of fibrosis was associated with normalization of Rasal1 promoter methylation and Rasal1 expression, de novo hydroxymethylation of Rasal1 promoter, and increased Tet3 expression (Tet1 and Tet2 expression levels were not increased).

CONCISE METHODS

To further explore the link between antifibrotic BMP7 treatment, Rasal1 methylation, hydroxymethylation, Rasal1 expression levels, and Tet proteins, we next used an inducible cell culture system in which sustained exposure to TGF-β1 induces Rasal1 hypermethylation, transcriptional repression, and fibroblast proliferation (Supplemental Figure 3, D–F).3 In this cell culture system, exposure to TGF-β1 caused Rasal1 hypermethylation (Figure 2A) and suppressed Rasal1 mRNA expression (Figure 2B). It correlated with increased proliferative fibroblast activity (Figure 2C), induction of α-smooth muscle actin, and collagen 1A1 mRNA expression levels (Supplemental Figure 3, G and H). After removal of TGF-β1 (after 5 days when Rasal1 was robustly methylated), Rasal1 remained hypermethylated and transcriptionally silenced (assessed at day 10 of tissue culture, when cells had been exposed to 5 days of TGF-β1 and an additional 5 days of growth factor-free media) (Figure 2, A and B). When TGF-β1 incubation was followed by exposure to BMP7 for 5 days, Rasal1 was demethylated, correlating with rescue of Rasal1 mRNA expression, normalization of fibroblast proliferation, and mRNA expression levels of α-smooth muscle actin and collagen 1A1 (Figure 2, A–C, Supplemental Figure 3, G and H).

Loss of Rasal1 methylation on BMP7 exposure correlated with de novo Rasal1 hydroxymethylation (Figure 2D), suggesting that BMP7 facilitates normalization of aberrant Rasal1 methylation through an active, hydroxymethylation-dependent mechanism.

Based on our observations in kidneys of BMP7-treated mice, we next explored regulation of Tet proteins in cultured fibroblasts in response to TGF-β1 and BMP7. Although exposure to TGF-β1 or BMP7 did not affect mRNA expression levels of Tet1 and Tet2 (Figure 2, E and F), suppressed Tet3 mRNA expression and protein levels were observed after treatment with TGF-β1, whereas BMP7 restored Tet3 expression levels (Figure 2, G–I). Although knockdown of Tet1 and Tet2 when cells had been exposed to 5 days of TGF-β1 and 5 additional days of growth factor-free media). When TGF-β1 incubation was followed by exposure to BMP7 for 5 days, Rasal1 was no longer found to be methylated. (B) Rasal1 expression in response to TGF-β1 and BMP7. Primary mouse kidney fibroblasts were subjected to TGF-β1 for 1, 2, 5, and 10 days. Exposure to TGF-β1 caused rapid suppression of Rasal1 mRNA within 1 day without Rasal1 methylation. Prolonged exposure to TGF-β1 (starting after 2 days) causes Rasal1 methylation, and Rasal1 mRNA expression levels remain suppressed, even when the TGF-β1 was removed after 5 days. Exposure to BMP7 normalized Rasal1 mRNA expression levels. Experiments were done in triplicate, and data are presented as means. ***P<0.001. P values were calculated respective to 5 days TGF-β1+5 days untreated. (C) Proliferation of primary mouse kidney fibroblasts. Primary mouse fibroblasts were seeded into six-well plates at a density of 60,000 cells per well, trypsinized, and counted after 1, 2, 5, and 10 days. Compared with fibroblasts cultured in growth factor-free media, TGF-β1 increased fibroblast proliferation, even when the TGF-β1 was replaced with growth factor-free media after 5 days. Treatment with BMP7 decreased TGF-β1–induced fibroblast proliferation. Experiments were replicated four times, and data are presented as means. **P<0.01. P values were calculated respective to 5 days TGF-β1+5 days untreated. (D) Rasal1 hydroxymethylation (5hmC) in response to TGF-β1 and BMP7. The upper panel shows a virtual gel image of Rasal1 PCR products of immunoprecipitated 5hmC DNA, and the lower panel shows PCR products of input DNA as controls for equal loading in hydroxy-MeDIP. BMP7 induced Rasal1 hydroxymethylation. (E–G) Tet mRNA expression on treatment with TGF-β1 and BMP7. Tet expression levels were analyzed by quantitative RT-PCR. Whereas Tet1 and Tet2 were not significantly regulated on exposure to TGF-β1 and/or BMP7, treatment with TGF-β1 suppressed Tet3, whereas BMP7 normalized mRNA expression levels. Experiments were done in triplicate, and data are presented as means±SDs. *P<0.05; ***P<0.001. (H) Tet3 protein levels on TGF-β1 and/or BMP7 treatment. On exposure to TGF-β1 and/or BMP7 for 2 days, Tet3 analyzed by Western blot was suppressed after treatment with TGF-β1, whereas BMP7 restored altered protein levels. (I) Quantification of Tet3 protein levels on treatment with TGF-β1 and/or BMP7. Tet3 band density was quantified relative to α-tubulin as control for equal loading. Measurements were done in triplicate, and data are presented as means±SD. ***P<0.001; ****P<0.0001. n.s., not significant.
Figure 3. BMP7 normalizes TGF-β1–induced methylation of Rasal1 through Tet3-dependent hydroxymethylation. Fibroblasts were maintained in serum-free control media (Rasal1 unmethylated) or media supplemented with TGF-β1 (Rasal1 hypermethylated) for 5 or 10 days. For analysis of possible Rasal1 demethylation, TGF-β1–containing media were removed after 5 days and replaced with either serum-free control media or media containing BMP7 for additional 5 days. For analysis of possible involvement of Tet proteins in Rasal1
demethylation and Rasal1 hydroxymethylation, cells were transfected with small interfering RNA (siRNA) oligonucleotides specifically targeting Tet1, Tet2, or Tet3 or scrambled control nucleotides. (A) Impact of Tet knockdown on Rasal1 methylation (5mC). The upper panel shows virtual gel images of Rasal1 PCR products of immunoprecipitated 5mC DNA, and the lower panel shows PCR products of input DNA as controls for equal loading. Panels display representative analysis of cells that had been transfected with scrambled siRNA or siRNAs targeting Tet1, Tet2, or Tet3. Tet3 knockdown but not Tet1 or Tet2 knockdown prevents Rasal1 demethylation in response to BMP7. (B) Impact of Tet3 knockdown on Rasal1 hydroxymethylation (5hmC). The upper panel shows a virtual gel image of Rasal1 PCR products of immunoprecipitated 5hmC DNA, and the lower panel shows PCR products of input DNA as controls for equal loading in hydroxy-MeDIP. BMP7-induced hydroxymethylation is only observed when Tet3 is present. (C) Impact of Tet3 knockdown on Rasal1 mRNA expression. Rasal1 mRNA expression levels were analyzed by quantitative RT-PCR. Bar graphs summarize relative Rasal1 mRNA expression in control cells (transfected with scrambled siRNA) on Tet1 and Tet2 knockdown as well as Tet3 knockdown. Rasal1 mRNA expression was only restored by BMP7 when Tet3 was present. Experiments were done in triplicate, and data are presented as means. **P<0.01. P values were calculated respective to 5 days TGF-β1+5 days untreated. (D) Impact of Tet3 knockdown on fibroblast proliferation. The bar graphs summarize relative cell counts after 5 and 10 days. Cells were transfected with either scrambled or siRNA-specific for Tet1, Tet2, or Tet3. BMP7 induced fibroblast proliferation when Tet3 was knocked down. Experiments were replicated four times, and data are presented as means. *P<0.05; **P<0.01; ***P<0.001. P values were calculated respective to 5 days TGF-β1+5 days untreated.

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DISCLOSURES
None.

REFERENCES


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