MeCP2 Controls an Epigenetic Pathway That Promotes Myofibroblast Transdifferentiation and Fibrosis

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BACKGROUND & AIMS: Myofibroblast transdifferentiation generates hepatic myofibroblasts, which promote liver fibrogenesis. The peroxisome proliferator-activated receptor γ (PPARγ) is a negative regulator of this process. We investigated epigenetic regulation of PPARγ and myofibroblast transdifferentiation. **METHODS:** Chromatin immunoprecipitation (ChIP) assays assessed the binding of methyl-CpG binding protein 2 (MeCP2) to PPARγ and chromatin modifications that silence this gene. MeCP2$^{-/y}$ mice and an inhibitor (DZNep) of the epigenetic regulatory protein EZH2 were used in the carbon tetrachloride model of liver fibrosis. Liver tissues from mice were assessed by histologic analysis; markers of fibrosis were measured by quantitative polymerase chain reaction (qPCR). Reverse transcription PCR detected changes in expression of the microRNA miR132 and its target, elongated transcripts of MeCP2. Myofibroblasts were transfected with miR132; PPARγ and MeCP2 expressions were analyzed by qPCR or immunoblotting. **RESULTS:** Myofibroblast transdifferentiation of hepatic stellate cells is controlled by a combination of MeCP2, EZH2, and miR132 in a relay pathway. The pathway is activated by down-regulation of miR132, releasing the translational block on MeCP2. MeCP2 is recruited to the 5′ end of PPARγ, where it promotes methylation by H3K9 and recruits the transcription repressor HP1α. MeCP2 also stimulates expression of EZH2 and methylation of H3K27 to form a repressive chromatin structure in the 3′ exons of PPARγ. Genetic and pharmacologic disruptions of MeCP2 or EZH2 reduced the fibrogenic characteristics of myofibroblasts and attenuated fibrogenesis. **CONCLUSIONS: Liver fibrosis is regulated by an epigenetic relay pathway that includes MeCP2, EZH2, and miR132. Reagents that interfere with this pathway might be developed to reduce fibrogenesis in chronic liver disease.**

Myofibroblasts are sufficient and essential for wound repair; they appear in injured tissues at an early stage and stimulate both wound contraction and formation of a temporary scar (granulation tissue) that protects against infection and further damage.[1] Persistence and proliferation of myofibroblasts in chronic injury is associated with progressive deposition of collagen-rich extracellular matrix leading to tissue fibrosis. This fundamental role in tissue homeostasis necessitates an improved understanding of myofibroblast generation, function, and fate. Myofibroblasts are absent in normal tissue but appear on injury as a consequence of stimulation/transdifferentiation of resident fibroblasts, epithelia, and pericytes.[2] Despite the different cellular origins, myofibroblast transdifferentiation generates a similar phenotype, including de novo expression of smooth muscle α-actin and secretion of the profibrogenic growth factor, transforming growth factor β1 (TGFβ1). In addition, the cell will secrete copious amounts of types I and III collagen and the collagenase inhibitor, tissue inhibitor of metalloproteinase-1 (TIMP-1), which in combination promotes the net deposition of cross-linking fibril-forming collagen.[3] Myofibroblast transdifferentiation is therefore a highly conserved physiologic process that must be tightly regulated to ensure limited and controlled scar formation.

Myofibroblast transdifferentiation is underpinned by changes in expression of hundreds of different genes that combine to generate the myofibroblast epigenome. Although numerous candidate transcription factors have been identified, the early regulatory events that trigger and orchestrate reprogramming of the myofibroblast epigenome are poorly understood. We recently described a role for DNA (CpG) methylation as a blueprint for the global alterations in the epigenome that drive transdifferentiation.[4] Methylated CpGs act as a signal for transcrip-

Abbreviations used in this paper: BDL, bile duct ligation; CCl_4, carbon tetrachloride; ChIP, chromatin immunoprecipitation; CREB, adenosine 3′,5′-cyclic monophosphate response element-binding protein; HSC, hepatic stellate cell; MeCP2, methyl-CpG binding protein 2; PPARγ, peroxisome proliferator-activated receptor-γ; PRC2, Polycomb repressor complex 2; qHSC, quiescent hepatic stellate cell; qPCR, quantitative polymerase chain reaction; RT, reverse transcriptase; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; siRNA, small interfering RNA; TGFβ1, transforming growth factor β1; TIMP-1, tissue inhibitor of metalloproteinase-1; UTR, untranslated region; Wt, wild-type.

0016-5085/10/$36.00
doi:10.1053/j.gastro.2009.10.002

tional repression and silencing. Transcriptional silencing of the peroxisome proliferator-activated receptor-gamma (PPARγ) gene is required for conversion of hepatic stellate cells (HSCs) into myofibroblasts.[5,6] PPARγ expression is associated with the adipogenic features of quiescent HSCs (qHSCs) and must be silenced for the cell to adopt its myofibroblastic characteristics. Forced overexpression of PPARγ in hepatic myofibroblasts results in reversion of transdifferentiation, with down-regulation of type I collagen, loss of proliferation, and reacquisition of their adipogenic characteristics. Similar data are reported for lung fibroblasts in which expression of a constitutive active PPARγ protein inhibits the ability of TGFβ1 to induce myofibroblastic characteristics, including expression of collagen I.[7] TGFβ1 stimulation of myofibroblastic conversion of ocular fibroblasts is also prevented by adenoviral-mediated overexpression of PPARγ.[8] PPARγ agonists can also partially reverse epithelial to mesenchymal transition in anaplastic thyroid cancer cells.[9] Taken together, these studies suggest a pivotal regulatory role for PPARγ in transdifferentiation for cells of distinct origins and phenotypes. As such, we reasoned that investigation into how transcriptional silencing of PPARγ is regulated in HSCs would lead to the discovery of novel and critical epigenetic regulators of fibrogenesis.

Gene silencing can be achieved by ≥3 epigenetic mechanisms: (1) DNA methylation and the activities of the methyl-CpG binding proteins; (2) remodelling of the histone code by enzymes that either add or remove acetyl and methyl groups to the lysine and arginine tails of the core histones; and (3) the actions of microRNAs.[10] Here, we describe how all 3 mechanisms combine to generate a multicomponent epigenetic relay pathway that regulates repression of PPARγ transcription in the hepatic myofibroblast. Furthermore, by disrupting the activities of 2 key components, the methyl-CpG binding protein 2 (MeCP2) and the histone methyltransferase EZH2,[11,12] we provide in vitro and in vivo evidence that the epigenetic relay has a wider function in controlling the myofibroblast phenotype and liver fibrosis.

Materials and Methods

Animals

Mecp2$^{-/y}$ mice were obtained from The Jackson Laboratory (Bar Harbor, ME) (strain B6.129P$_2$(C)-Mecp2$^{tm1.1Bird}$/J) and are a cross of a constitutive CMVCre strain and a strain carrying an Mecp2 gene containing loxP sites around exons 3 and 4. The crossed line (pure C57BL/6) lack expression of Mecp2 mRNA and protein; they appear normal at birth but develop mobility problems and have an expected lifespan of 50–60 days. Authors hold licences for work relating to all experiments carried out in animals issued/approved by local ethical committee and UK Home Office.

Chronic CCl_4 Liver Injury Model

Fibrogenesis was induced by 3 weeks of carbon tetrachloride (CCl_4) treatment of 6-week-old *Mecp2*$^{-/y}$ or age-matched wild-type (Wt) littermates. Mice were injected intraperitoneally twice weekly with CCl_4/olive oil in a 1:1 (vol/vol) ratio at 1 μL/g body weight. Twenty-four hours after the final CCl_4 administration, animals were humanely killed, and liver samples were prepared.

Acute CCL_4 Liver Injury

3 Deazaneplanocin A was administered intraperitoneally to 2 groups of 5 C57Bl6 mice (at 15 mg/m^2) 2 hours before a single dose of CCl_4 (prepared as in "Chronic CCl_4 Liver Injury Model"). Mice were killed 24 hours after injury, blood samples were taken for assessment of liver enzyme concentrations, and tissues were harvested for histologic and biochemical analyses.

Cell Isolation

Rat and mouse HSCs (from C57Bl6 Wt or *Mecp2*$^{-/y}$ livers) were isolated as previously described.[13] The protocol for isolation of activated HSCs from rat livers injured by bile duct ligation (BDL) for 10 days or acute or repeated injury with CCl_4 for 3 weeks is provided in the supplemental material.

Immunohistochemistry

Mouse liver tissue was fixed in 10% formalin in phosphate-buffered saline, and liver sections were stained with Sirius Red as previously described.[14]

siRNA Transfection

Cells were transfected with 2 μg small interfering RNA (siRNA) targeting EZH2, MeCP2, or negative control with the use of a square wave electroporator and allowed to grow for 48 hours before preparation of RNA and/or whole-cell extracts.

Micro RNA Detection, Amplification, and Transfection

Micro RNAs were isolated from cells and reverse transcribed; rat miR132 was detected with the use of the miScript primer assay. To assess the effect of miR132 presence in myofibroblasts, 2 μg miR132 mimic was transfected into myofibroblasts (as outlined for siRNA).

SDS-PAGE and Immunoblotting

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were done as previously described.[14] Antibody recognizing MeCP2 (Abcam, Cambridge, MA) was used at 1 μg/mL; EZH2 (Active Motif Inc, Carlsbad, CA) at 1/500 dilution; and β-actin (Sigma, St Louis, MO) at 1/1000 dilution.

Quantitative Reverse Transcriptase–Polymerase Chain Reaction

Total RNA was obtained as in Oakley et al[14]; cDNA was generated by using a random hexamer primer