Increased Reelin Promoter Methylation Is Associated With Granule Cell Dispersion in Human Temporal Lobe Epilepsy

Katja Kobow, MSc, Ina Jeske, BSc, Michelle Hildebrandt, MD, Jan Hauke, MSc, Eric Hahnen, PhD, Rolf Buslei, MD, Michael Buchfelder, MD, Daniel Weigel, MD, Hermann Stefan, MD, Burkhard Kasper, MD, Elisabeth Pauli, PhD, and Ingmar Blümcke, MD

Abstract
Mesial temporal sclerosis (MTS) is the most common lesion in chronic, intractable temporal lobe epilepsies (TLE) and characterized by segmental neuronal cell loss in major hippocampal segments. Another histopathological hallmark includes granule cell dispersion (GCD), an architectural disturbance of the dentate gyrus encountered in approximately 50% of patients with mesial temporal sclerosis. Reelin, which plays a key role during hippocampal development and maintenance of laminar organization, is synthesized and released by Cajal-Retzius cells of the dentate molecular layer, and previous studies have shown that Reelin transcript levels are downregulated in human temporal lobe epilepsies specimens. To investigate whether epigenetic silencing by Reelin promoter methylation may be an underlying pathogenetic mechanism of GCD, DNA was harvested from 3 microdissected hippocampal subregions (i.e. molecular and granule cell layers of the dentate gyrus and presubiculum) from 8 MTS specimens with GCD, 5 TLE samples without GCD, and 3 autopsy controls. Promoter methylation was analyzed after bisulfitet treatment, cloning, and direct sequencing; immunohistochemistry was performed to identify Cajal-Retzius cells. Reelin promoter methylation was found to be greater in TLE specimens than in controls; promoter methylation correlated with GCD among TLE specimens (p < 0.0002). No other clinical or histopathological parameter (i.e. sex, age, seizure duration, medication or extent, of MTS) correlated with promoter methylation. These data support a compromised Reelin-signaling pathway and identify promoter methylation as an epigenetic mechanism in the pathogenesis of TLE.

Key Words: Epigenetic, Hippocampus, Mesial temporal sclerosis, neuropathology, Reelin, seizures.

INTRODUCTION
Epigenetic mechanisms are self-perpetuating, posttranslational modifications of nuclear proteins (acetylation, methylation, phosphorylation, etc.) and DNA (methylation) that can produce lasting alterations in chromatin structure and gene expression patterns. They are increasingly recognized as fundamental regulatory processes in central nervous system development, synaptic plasticity, and memory (1). Whereas covalent modifications of histone proteins occur rapidly and are highly diverse in their effects on gene expression, DNA methylation is long-lasting, heritable, and associated with stringent gene silencing and stabilization of the genome (2). DNA methylation is established by several DNA methyltransferases (DNMTs). Epigenetic programming occurs in distinct developmental stages, cell types, and genomic regions (4). The precise mechanisms linking DNA methylation with histone tail modifications remain to be elucidated, but DNMTs are components of protein complexes including methyl CpG binding proteins, histone methyltransferases, and histone deacetylases (HDACs) (3).

In brain development, fate determination of newly generated neurons is tightly regulated by epigenetic mechanisms; they also play roles in the induction of synaptic plasticity, memory formation, and cognition (1, 5). Moreover, alterations in DNA methylation patterns have been increasingly associated with neurological disorders including schizophrenia (6), Alzheimer disease (7), and autism (8). In rodent models of chronic seizures, the function of repressor element-1 transcription factor/neuron restrictive silencer factor (REST/NRSF), a major transcriptional repressor in neuronal gene regulation, has been associated with neuronal hyperactivity (9). Increased expression of REST/NRSF is observed in rats after status epilepticus, followed by a decrease in mRNA levels of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptor subunit 2 GLUR2 gene (10). Treatment with HDAC inhibitors reversed deacetylation of (GluR2) associated histones, thereby blunting seizure-induced downregulation of GluR2. Further evidence that neuronal activity in epilepsies induces epigenetic changes and has significant impact on gene expression patterns is indicated in studies in which kainic acid-induced status epilepticus and electroconvulsive seizures increased expression of cAMP responsive element binding protein (CREB) binding protein (CBP and p300). The associated histone acetyltransferase
activity is mainly responsible for histone H4 hyperacetylation at gene promoter regions in rat hippocampus. Specific inhibition of the acetyltransferase activity of CREB binding protein suppressed the induction of histone modification and reduced the severity of epilepsy (11, 12).

Medically refractory epilepsies such as temporal lobe epilepsies (TLE) frequently respond to dietary manipulations such as the ketogenic diet. The ketogenic diet is a high-fat, high-protein, low-carbohydrate diet that may act through glycolytic inhibition. Because the full anticonvulsant effect of the ketogenic diet requires up to weeks to develop, it has been suggested that altered gene expression may be involved in the beneficial dietary effects (13). Small metabolic intermediates are thought to act as regulators of transcriptional corepressors and coactivators including REST/NRSF, thereby linking energy availability to chromatin structure and transcriptional output (14). Nevertheless, it has not yet been shown in vivo whether DNA methylation is directly targeted by chronic recurrent seizures in epilepsy patients.

Reelin is an extraacellular matrix protein targeting the major effector pathway for cortical lamination in the hippocampus; deficient Reelin signaling has been associated with architectural abnormalities in the hippocampus in TLE patients (15–17). By binding to membrane Apolipoprotein E receptor 2 (ApoER2)- and very low density lipoprotein receptor (VLDLR) receptors, Reelin initiates tyrosine phosphorylation of the intracytoplasmic docking protein Dab1 by src-family kinases (18). Dab1 can also be phosphorylated by Ser/Thr kinase CDK5, which is independent of Reelin (19). Tyrosine-phosphorylated Dab1 has numerous binding partners downstream the Reelin signaling cascade that modulate either microtubule stability or dynamics (20), as well as cell adhesion by integrins (18), N-methyl-D-aspartate (NMDA) receptor-mediated Ca++ influx (21) and CREB-initiated transcription of genes that are important for synaptic plasticity, neurite outgrowth, and dendritic spine development (22). Biochemical and functional studies using either organotypic slice cultures or knockout mice corroborate the relevance of Reelin signaling for development and plasticity in the adult brain. Reelin controls the proper positioning of migrating hippocampal neurons particularly by acting on the radial glial scaffold (16). Stripe choice assays support the notion that radial glial fibrillary acidic protein (GFAP) positive glial cells than neurons or microglia move to and accumulate on a Reelin-enriched matrix. Furthermore, Reelin promotes branching and collateral extension of GFAP-positive glial processes (23, 24). Although no influence of Reelin on the radial orientation of the glial scaffold was apparent, cocultures of Reelin-deficient (reeler) and wild-type hippocampal slices demonstrated a directed growth of radial glial fibers toward the source of Reelin (i.e. the dentate gyrus [DG]), suggesting that Reelin is required in a normotopic position to exert its function. Finally, the formation of a densely packed granule cell layer (GCL) and subsequent rescue of laminar specificity of commissural fibers were seen in the reeler slice after proper time- and region-specific exposition/rescue with Reelin (16). Here, we studied epigenetic modifications in patients with chronic TLE and controls to elucidate the contribution of deficient Reelin signaling in the pathogenesis of human TLE.

MATERIALS AND METHODS

Patients

Resection samples for this study were obtained from 13 adult patients (ages [mean ± SEM], 41.33 ± 1.57 years) with chronic intractable TLE who underwent surgical treatment after extensive presurgical monitoring. Presurgical evaluation included interictal and ictal video-electroencephalogram monitoring, using 32 to 64 electroencephalogram channels, neuropsychological examination, magnetic resonance imaging (1.5-T Sonata Siemens), visual field testing, and psychiatric evaluation. Functional magnetic resonance imaging and magnetic source imaging, positron emission tomography, or single photon emission computed tomography was applied in selected cases. Details of the Erlangen presurgical monitoring protocol have been published elsewhere (25).

Informed and written consent was obtained from all patients. All studies were conducted in accordance with the Declaration of Helsinki and were approved by the local medical ethics committee. Patients with TLE underwent resection of the hippocampus as part of the surgical procedure. Selective amygdalo-hippocampectomy, resection of anterior-medial structures, or en bloc “standard resections” were carried out depending on the characterization of the seizure focus and its relation to functionally eloquent areas, memory capacity of the affected hippocampus, or reserve capacity of the contralateral hippocampus.

Surgical specimens underwent systematic evaluation by 2 experienced neuropathologists (I.B. and M.H.) to determine the presence of mesial temporal sclerosis (MTS) and granule cell dispersion (GCD) of the DG. Mesial temporal sclerosis was classified according to recently published data by Blümcke et al (26). Eight patients displayed MTS either of type 1a (classic hippocampal sclerosis, n = 3) or 1b (severe type, n = 5). Granule cell dispersion was histopathologically defined following the International League Against Epilepsy commission report (27), taking into account more than 10 cell layers in vertical direction, measured in regions without curvatures of the DG to avoid artifacts. Additional patterns included enlarged granule cell bodies, ectopic granule cells, or granule cell clusters and a bilaminated DG; these features can be focal. Both observers agreed on the presence of GCD in the MTS cases. Five TLE patients (later referred to as “no GCD”) showed no MTS and associated GCD or other lesions (e.g. tumor and cortical malformation; Table 1). At the time of surgery, all epilepsy patients received antiepileptic medication and all had previous periods of Valproate (VPA) treatment.

Three age-matched autopsy samples served as controls (ages [mean ± SEM], 45.67 ± 3.53 years). Criteria for including these brain specimens as control tissue were the patient ages, a postmortem range less than 48 hours, no history of neurological deficits, and no evidence of autolysis upon histopathological examination.

Tissue Preparation, Neuronal Cell Counts, and Measurement of GCD

Tissue preparation, including hematoxylin and eosin staining and neuronal cell counts for MTS classification using NeuN immunohistochemistry were performed as previously
TABLE 1. Clinical Data

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Note: Patient identification number (\#); sex (m, male; f, female); ages at time of surgery, onset of temporal lobe epilepsy, duration of disease, and age at initial precipitating insult (age IPI) are given in years; precipitating events (if known) are listed: FS, febrile seizure; GCD, granule cell dispersion (++ severe dispersion with bilaminar granule cell layer; + dispersion with more than 10 granule cell layers); lesions of temporal lobe epilepsies biopsy patients in addition to mesial temporal sclerosis in biopsy patients and major causes of death in autopsy controls are indicated. L, left; R, right.

Microdissection

After neurosurgical resection of the human hippocampus, a 15-mm-thin coronal slice was taken from the mid hippocampal body, snap-frozen into liquid nitrogen, and stored at −80°C until further use. For microdissection, 15 sections (each 20 μm thick) were cut with a cryomicrotome (Microm HM 560) at −20°C. The MCL and GCL of the DG as well as presubiculum adjacent to CA1 were microdissected at 10-fold enlargement under a stereomicroscope (Olympus SZX9) to obtain separate DNA samples from distinct anatomical regions from each patient (Figs. 1A–C). Dentate gyrus MCL was chosen as a target area harboring Reelin synthesizing/releasing C-R cells. The presubiculum was chosen as an “internal control” because it was always present in the same surgical specimen and displayed no neuronal cell loss in MTS. All microdissected areas were documented by hematoxylin and eosin staining after formalin fixation (Figs. 1A–C).

DNA Isolation and Methylation-Specific Sequencing

Microdissected cells from DG MCL, GCL, and presubiculum were collected in buffer ATL (QIAamp DNA Micro Kit; Qiagen, Hilden, Germany) and digested overnight with proteinase K at 56°C. Further purification of genomic DNA was performed the next day according to the manufacturer’s protocol. Bisulfite modification was carried out using a total of up to 2 μg DNA according to the manufacturer’s protocol using the EpiTec Bisulfite Kit (Qiagen). Modified DNA was used
for subsequent nested polymerase chain reaction (PCR) amplification. The primers were used as published previously (30). Platinum Taq High Fidelity (2.5 U) and PCR-Enhancer System (both from Invitrogen, Carlsbad, CA) were used at appropriate cycling conditions. Polymerase chain reaction products from each sample set were used for TA cloning according to manufacturer’s instructions using the TOPO TA Cloning Kit with One Shot TOP10 Chemically Competent Escherichia coli (Invitrogen). At least 8 clones with proven insertion of the PCR fragment (white colonies confirmed by PCR analysis) were sequenced for each sample using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). The BioEdit v7.0.5 software was used for sequence analysis, DNA editing, and alignments for each clone. The Genomatix MatInspector software was used to identify putative transcription factor binding sites (31).

**Statistical Analysis**

The statistical evaluation was performed using the Statistical Package for the Social Sciences (SPSS version 14). Cell counts and methylation specific sequencing data were analyzed performing a 2-tailed t-test. Statistical correlations were calculated using the χ² test and univariate analysis (analysis of variance). P values less than 0.05 were considered as significant.

**RESULTS**

**Increased Reelin Promoter Methylation in TLE**

The Reelin promoter region contains a large CpG island covering more than 2000 bp and is, therefore, likely to be modified by DNMTs under physiological and pathological conditions. Here, we investigated the methylation status of the human Reelin promoter in 13 surgical specimens obtained from TLE patients and 3 age-matched autopsy control patients with no neurological history. Harvested DNA from microdissected tissue was used for amplification and sequencing of the human Reelin promoter including 5’UTR (positions −525 to +127) after bisulfite treatment. Position +1 was designated as the transcriptional start site. All microdissected areas were identified by hematoxylin and eosin staining after formalin fixation (Figs. 1A–C). Alignments and positional nucleotide numerical analysis from 8 subclones of each sample were used to generate methylation maps of the Reelin promoter (Fig. 2A). Because the patient groups differed in sample size (Table 1), the sum of methylated cytosines was normalized against the number of all sequences included in each cohort alignment (Fig. 2B). Comparison of general methylation patterns between resection and autopsy specimens revealed significant differences with higher overall methylation levels in the group of TLE patients (p = 0.0249; Fig. 2B). Table 2 summarizes all positions, in which ≥50% of clones per subject contained methylated cytosines.

**Correlation Analysis Between Promoter Methylation and Clinical Histories in TLE Patients**

We then assessed possible correlations of clinical parameters, including age, year of onset, duration of epilepsy, and seizure frequency (complex partial seizures) per month and approximate seizure number experienced in lifetime (complex partial seizures/life), with the methylation patterns...
but identified no correlations (data not shown). In addition, there were no significant differences between male and female patients in the methylation profiles along the Reelin promoter (data not shown). Interestingly, however, region-specific analysis for the dentate molecular and GCL or presubiculum obtained from all specimens revealed different methylation patterns for each patient and for each anatomically distinct area (Table 2).

Reelin Promoter Methylation and GCD
Comparison of methylation patterns between MTS patients with and without GCD revealed a remarkable difference. Mesial temporal sclerosis patients with GCD showed significantly higher methylation levels than the no GCD patients over the entire promoter region (p = 0.0002; Fig. 3B). The numbers of identified hotspots per subject in each group were significantly greater in the GCD cohort.
(mean_{GCD} \pm SEM: 9.875 \pm 1.246, n = 8; mean_{no \ GCD} \pm SEM: 3.400 \pm 1.568, n = 5; p = 0.008). Intriguingly, all patients displayed individual methylation patterns along the promoter, whereas distinct anatomical regions of the same subject showed broad differences in hotspot allocation (Table 2).

**Immunohistochemical Analysis**

Cajal-Retzius cells were specifically labeled by immunohistochemistry either for calretinin/p73 (28) or Reelin (29); they had bipolar or fusiform shapes and horizontally oriented processes in the outer dentate molecular layer (Figs. 4A, B). After semiquantitative measurements in 3 adjacent visual fields at 100× magnification, representing 0.75 mm², mean number of labeled cells/mm² \pm SEM of all subjects per group were calculated for GCD (CR/p73-ir [mean \pm SEM], 1.83 \pm 1.999; Reelin-ir [mean \pm SEM], 11.30 \pm 0.468) and no GCD specimens (CR/p73-ir [mean \pm SEM], 12.61 \pm 1.336; Reelin-ir [mean \pm SEM], 9.91 \pm 1.997). Using both detection protocols, no significant difference was apparent between our patient cohorts (p = 0.748 and p > 0.05, respectively; Fig. 5). In

**FIGURE 3.** Methylation levels of human Reelin promoter in temporal lobe epilepsy specimens. (A) Increased methylation levels were evident at nearly all sites of the Reelin promoter region in patients with mesial temporal sclerosis (MTS) and granule cell dispersion (GCD; orange) compared with “no GCD” specimens (green). TSS, transcription start site. (B) MTS patients with GCD had significantly higher methylation levels over the entire promoter region than “no GCD” patients (p = 0.0002).

**FIGURE 4.** Immunoreactivity of Cajal-Retzius cells in the outer molecular layer of the dentate gyrus by confocal and conventional imaging. (A, C, D) Confocal imaging. Cajal-Retzius cells of the dentate gyrus molecular layer (MCL) are immunoreactive for Calretinin (CR-ir; green) and p73 (red). Panels C and D are single-channel images obtained from panel A. (B, E) Immunoperoxidase staining. Arrows in panel B indicate Reelin-immunoreactive cells within the outer molecular layer. High-power magnification of a Reelin-positive Cajal-Retzius cell in a temporal lobe epilepsy specimen. Scale bar = (A) 50 \mu m, applies also to B. Scale bar = (E) 20 \mu m, applies also to C and D.
Granule cell dispersion is a major histopathological hallmark of human MTS (32) and is recognized in approximately 50% of surgical specimens (33). Whereas previous studies demonstrate deficient Reelin expression to associate with GCD in the human and rat DG (15, 16), the population of C-R cells is well preserved (34) or even increased (35). Our present data suggest that increased promoter methylation of the Reelin gene may contribute to lower Reelin expression levels in MTS specimens with GCD (15, 16) and, therefore, that epigenetic regulation is likely to play a role in this chronic seizure disorder.

Control of Hippocampal Architecture by Reelin-Secreting C-R Cells

Reelin-synthesizing and secreting C-R cells are the first neurons that enter the cortical plate; they play a major role in cortical layer formation (16, 36). Cajal-Retzius cells reside in the marginal zone in the hippocampus, the prospective molecular layer of the DG, and seem to be specifically regulated during ontogenesis. In the prenatal human hippocampus, the numbers of C-R cells increase until the 36th week of gestation and decline rapidly after birth (35). In rats, it has been estimated that once neuronal migration has been completed, more than 95% of C-R cells die after the first postnatal week in rats (36, 37). Low resting potential and postnatal upregulation of NMDA receptors are likely mechanisms by which C-R cells decline during cortical maturation and the onset of functional network activity (38). Here, we confirmed our previous report of a persisting C-R cell population within the molecular layer of hippocampal specimens obtained from TLE patients (35). By contrast, Haas et al (15) describe an inverse correlation between the degree of GCD and Reelin mRNA expression in epilepsy patients with hippocampal sclerosis. Although compact layer organization could be associated with abundant Reelin mRNA expression, TLE patients with pronounced GCD showed significantly reduced Reelin mRNA levels (15). Reelin is known to be a key molecule during hippocampal development and maintenance of laminar organization in the hippocampal “gatekeeper” region, that is, the DG (16).

Epigenetic Regulation of Reelin Expression in CNS Disorders

Reelin-mutant mice display severe layering alterations in most brain regions including the hippocampus and tend to develop epileptic seizures (39). Autosomal recessive lissencephaly, a severe neuronal migration disorder of the hippocampus and neocortex, is linked to Reelin mutations that inhibit proper function and secretion of the Reelin protein (40). Mutational analysis of the Reelin gene in epilepsy patients with hippocampal sclerosis, however, has not established that a genetic pathogenesis is involved (Blütmcke, Hildebrandt, Kobow, Jeske, Buslei, unpublished data 2006). A familiar predisposition for epileptic seizures in TLE, which would further indicate a genetic background independent of Reelin, was neither evident in the present nor similar TLE patient cohorts. In conclusion, therefore, negative transcriptional regulation of Reelin expression through repressive transcription factor binding and/or epigenetic mechanisms inducing changes in chromatin structure are likely to play a role in epilepsy-associated architectural abnormalities of the hippocampus.

Studies on human Reelin regulation mechanisms have also been performed in the context of schizophrenia in which Reelin function is associated with plasticity of dendritic spines and synapses in the cortex (6). Indeed, schizophrenic patients reveal significantly reduced Reelin mRNA levels and hypermethylation of the Reelin promoter. Our data extend these findings with respect to chronic focal epilepsies. Methylation hotspots could be identified both upstream and downstream of the transcriptional start site (Table 2), which may be crucial for silencing the promoter. Our data are, therefore, compatible with an epigenetic regulation mechanism of Reelin expression. Identified hotspots for methylated cytosines correspond to putative transcription factor binding sites (Fig. 6), including CREB, cell cycle dependent element (CDE), GC box factor Sp1 (Sp1), paired box 6 (Pax6), or nuclear respiratory factor 1 (NRF1) and partially overlap with previously identified hotspots in schizophrenic patients (6). The impact of epigenetic alterations in the initiation and progression of neurological disorders among others has challenged novel therapeutic targets and resulted in the development of pharmacological inhibitors of DNMTs and HDACs. By inducing DNA demethylation and histone acetylation, these compounds can reverse pathological epigenetic silencing, resulting in reactivation of genes and restoring of crucial cellular pathways (41). Valproate, one of the most commonly used anticonvulsants, achieved Food and Drug Administration approval in 1987, but its influence on epigenetic processes was only discovered in 2001 (42). Interestingly, VPA has been shown to act as an HDAC inhibitor and inducer of active

FIGURE 5. Semiquantitative analysis of Reelin-immunoreactive Cajal-Retzius cells. There was no significant difference in numbers of Reelin-positive cells per mm² in granule cell dispersion (GCD) and “no GCD” specimens.

addition, there was no correlation between the thickness of the GCL (extent of GCD) and respective C-R cell numbers (data not shown).

DISCUSSION

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DNA demethylation (43). In addition, Valproate decreases Reelin promoter methylation in vitro (44). In the present series, all patients had received long-term anticonvulsant medication including VPA, which may have affected the results obtained but does not sufficiently explain the correlations of the pathology of MTS and associated GCD. In addition, achievable VPA serum levels may not be sufficient to exert the entire spectrum of antiepileptic propensity (45).

Epigenetic Regulation of Gene Expression in Focal Epilepsies?

Clonal analysis revealed increased promoter methylation in chronic TLE patients compared with autopsy controls. The pathogenetic mechanism of this intriguing finding remains to be clarified. We hypothesize that there is an epilepsy-associated susceptibility for promoter methylation in a specific cohort of MTS patients that affects the Reelin gene. Within the cohort of TLE patients, there was a striking association between Reelin promoter methylation and the presence of GCD. No other clinical or histopathological parameter (e.g. sex, age [onset vs operation], antiepileptic medication, seizure frequencies, or MTS classification) reached this level of significance. Intriguingly, our data showed increased Reelin promoter methylation in the 3 anatomical subregions (i.e. MCL, GCL, and presubiculum) of TLE patients with GCD. Each of these subregions is composed of different glial and neuronal/interneuronal cell elements. The presubiculum, however, did not show MTS-associated cell loss and/or gliosis and was considered, therefore, as “internal” control. GABAergic interneurons residing in the dentate molecular layer may provide mossy fiber-evoked feedback inhibition to granule cell dendrites. Interestingly, these neurons are known to be readily killed by seizures with a corresponding reduction of feedback inhibition to granule cells (46). That makes them unlikely to have influenced the methylation patterns observed in MCL. On the other hand, the influence of DNA obtained from glial cells should be carefully taken into consideration. This cell population is likely to represent the largest cell group in the molecular layer and may also be found interspersed with granular neurons from dispersed granular layer as well as in the presubiculum. Only the population of C-R cells is known to functionally release Reelin, however. This suggests that decreased Reelin mRNA levels in GCD (as convincingly shown by Haas et al [15]) most likely result from promoter methylation in this particular cohort of patients.

The intriguing question remains as to whether Reelin promoter methylation increases with hippocampal seizure activity in TLE patients. In our present series, the duration of seizures tended to be associated with GCD (p = 0.053). The second (“no GCD”) cohort, however, also experienced a long history of epilepsy without Reelin downregulation and architectural abnormalities. Recent global and loci-specific analysis of methylation patterns in different human tissues excluded an association between age and methylation levels (47). This pattern would be rather compatible with an early manifestation of promoter methylation. It is tempting to speculate, therefore, that initial precipitating injuries, which are frequently associated with MTS, represent a trigger for this epigenetic regulatory machinery. Further studies, including in experimental animals, will be required to further clarify this issue.

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