Promoter Methylation Analysis Reveals That KCNA5 Ion Channel Silencing Supports Ewing Sarcoma Cell Proliferation

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Abstract

Polycomb proteins are essential regulators of gene expression in stem cells and development. They function to reversibly repress gene transcription via posttranslational modification of histones and chromatin compaction. In many human cancers, genes that are repressed by polycomb in stem cells are subject to more stable silencing via DNA methylation of promoter CpG islands. Ewing sarcoma is an aggressive bone and soft-tissue tumor that is characterized by overexpression of polycomb proteins. This study investigates the DNA methylation status of polycomb target gene promoters in Ewing sarcoma tumors and cell lines and observes that the promoters of differentiation genes are frequent targets of CpG-island DNA methylation. In addition, the promoters of ion channel genes are highly differentially methylated in Ewing sarcoma compared with nonmalignant adult tissues. Ion channels regulate a variety of biologic processes, including proliferation, and dysfunction of these channels contributes to tumor pathogenesis. In particular, reduced expression of the voltage-gated Kv1.5 channel has been implicated in tumor progression. These data show that DNA methylation of the KCNA5 promoter contributes to stable epigenetic silencing of the Kv1.5 channel. This epigenetic repression is reversed by exposure to the DNA methylation inhibitor decitabine, which inhibits Ewing sarcoma cell proliferation through mechanisms that include restoration of the Kv1.5 channel function.

Implications: This study demonstrates that promoters of ion channels are aberrantly methylated in Ewing sarcoma and that epigenetic silencing of KCNA5 contributes to tumor cell proliferation, thus providing further evidence of the importance of ion channel dysregulation to tumorigenesis. Mol Cancer Res; 14(1); 1–9. © 2015 AACR.

Introduction

A fundamental trait of cancer cells is their ability to sustain proliferation (1). Cancer cells accomplish this by hijacking physiologic pathways and silencing or mutating tumor-suppressor genes that control cell-cycle progression (1). Moreover, the proliferative phenotype of cancer cells is known to contribute to tumor relapse (2–4), a major impediment to cancer cures. Therefore, continued elucidation of the mechanisms that promote cancer cell proliferation is key for developing novel anticancer therapies.

Disruptions to gene expression in cancer can be achieved by both genetic and epigenetic mechanisms that lead to either aberrant induction or repression of target transcripts (reviewed in refs. 5, 6). Although genetic loss of function is usually achieved by gene deletion or inactivating mutations, epigenetic loss of function is most often associated with aberrant repression of gene transcription mediated by posttranslational histone modifications and DNA methylation. In normal stem cells, polycomb group proteins reversibly repress target genes and, in so doing, prevent differentiation, maintain stemness, and control development until appropriate developmental cues are received (7, 8). Importantly, the promoters of these stem cell targets of polycomb complexes are frequently targeted for DNA hypermethylation in cancer (9–11). In this way, more stable, and potentially irreversible, gene silencing is effected, and cancer cells become locked in a stem cell–like state.

Ewing sarcoma is an aggressive bone and soft-tissue tumor that presents most often in adolescents and young adults. It is a tumor of presumed stem cell origin and is characterized by an undifferentiated cellular phenotype and the presence of a tumor-initiating oncogenic fusion gene, most commonly EWS–FLI1 (12). Ewing sarcoma is genetically quiet, with a low rate of genetic mutation (13–15), and tumor pathogenesis is largely mediated by EWS–FLI1 and its impact on the epigenome (16, 17). Overexpression of polycomb proteins, in particular EZH2 and BMI-1, is evident in
nearly all Ewing sarcoma tumor cells, and both proteins contribute to the tumor phenotype (18–21). In addition, altered DNA methylation of cancer-associated gene promoters has been described, and preclinical studies demonstrate that exposure of Ewing sarcoma cells to hypomethylating agents inhibits tumor growth (22, 23).

In the current study, we used a custom-designed DNA methylation array to investigate whether the promoters of polycomb target genes are targeted for abnormal DNA methylation in Ewing sarcoma. Our results reveal that, like other cancer, CpG islands in the promoters of differentiation-associated genes are targets of DNA methylation. In addition, they uncover the unanticipated finding that promoters of ion channel genes are frequent targets of aberrant DNA methylation. In particular, these studies demonstrate that DNA hypermethylation contributes to epigenetic repression of the \( \text{KCNA5} \) locus and that the resulting suppression of the \( \text{Kv1.5} \) ion channel supports cancer cell proliferation.

### Materials and Methods

**Cell lines and tumor tissues**

Ewing sarcoma cell lines were provided by Dr. Timothy Triche Children’s Hospital Los Angeles (CHLA, Los Angeles, CA) and the Children’s Oncology Group cell bank (www.cogcell.org). Human vascular endothelial cells (HuVEC) were obtained from Lonza (191027). MRC-5 cells were obtained from the ATCC (CCL-171). Human bone marrow–derived mesenchymal stem cell (MSC) lines and human embryonic stem-cell (hESC)-derived neural crest stem cells (NCSC) were obtained as previously described (24). Identities were confirmed by short tandem repeat profiling. All cell lines were cultured in standard cell culture media, supplemented with 10% FBS (Atlas Biologicals, Inc.; F-500-A) at 37°C in 5% CO\(_2\). Ewing sarcoma tumor specimens were acquired from the Vanderbilt University pathology archives. Approval from the Vanderbilt University Institutional Review Board was granted prior to tissue acquisition.

**Proliferation analysis**

Cells were plated at a density of 200,000 cells per 6-well plate and left for 24 hours prior to a 72-hour drug treatment where the cells were treated every 24 hours. Brightfield images of the cells were captured on the Olympus CKX41 microscope on the 10× objective by the Lumenera Infinity 3-1 1.4 Megapixel camera. Proliferation was determined by trypsin-blue exclusion and EdU incorporation. 5-ethynyl-2’-deoxyuridine (EdU) was added to fresh medium of the cells after the 72-hour incubation for 2 hours before harvest at a concentration of 10 \( \mu \text{mol/L} \). EdU incorporation was determined with the Click-IT Plus EdU Alexa Fluor 488 flow cytometry assay kit (Life Technologies; C10632) following the manufacturer’s instructions, performed on the Accuri C6 flow cytometer and analyzed using FlowJo V10.

**Pharmacologic studies**

Diphenyl phosphate oxide-1 (DPO-1; 310 nmol/L; Tocris Bioscience; 2533) was prepared in ethanol, 4’Aminopyridine (4’AP; 50 \( \mu \text{mol/L} \); Sigma-Aldrich; 275875) was prepared in an aqueous solution, and 5-aza-2’-deoxycytidine (5’AZA-CdR; decitabine; 100 nmol/L; Sigma-Aldrich; A3656) was diluted in dimethyl sulfoxide (Dish; D128-500). Cells were pretreated at these concentrations for 72 or 96 hours.

**Quantitative real-time PCR**

Total RNA extraction was performed using the RNeasy Plus Mini Kit (Qiagen; 74136) or Quick-RNA Miniprep (Zymo; R1055), and cDNA was generated using iScript (Bio-Rad; BIO1708891). qRT-PCR was performed using validated SYBR primers (\( \text{KCNA5}, \text{GAPDH}, \text{HPRT}, \text{IDT} \)). Analysis was performed in triplicate using the Lightcycler 480 System (Roche Applied Science). Data were analyzed by normalizing average \( C_\text{T} \) values of the gene of interest (\( \text{KCNA5} \)) to the geometric mean of reference genes (\( \text{HPRT} \) and \( \text{GAPDH} \)) within each sample using the \( \Delta \Delta C_\text{T} \) method. Primers for \( \text{KCNA5} \) were as follows: Forward: 5’-CTG AGG CCA AGA GC-3’; Reverse: 5’-TCC CAT TCC CTA CTC CAC TG-3’.

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism 6. \( P \) values of less than 0.05 across at least 3 independent experiments were considered significant.

**DNA methylation**

DNA methylation was interrogated on a previously custom-designed Illumina Golden-Cone bead array. This array consists of 1,536 probes designed to detect DNA methylation at CpG islands within the promoters of >700 genes that are established targets of polycomb regulation in human embryonic stem cells (8). Two independent batches of samples were analyzed using this array, the first included 4 Ewing sarcoma tumors and 5 Ewing sarcoma cell lines and the second comprised 52 samples from 11 different nontransformed adult tissues that were obtained at rapid autopsy (25). Analysis of DNA methylation (as determined by beta values) was restricted to 1,297 probes that were not targeted to promoters of X- or Y-linked genes and were successfully and reproducibly detected in over 75% of the samples. Probes that contained repetitive sequence of more than 10 base pairs or that contained SNPs within CpG islands were also excluded, resulting in evaluable data for 1,204 loci. Differential methylation was defined as a median difference in beta value of at least 0.2 and a Bonferroni multiple test-corrected \( P \) value of less than 0.05 between Ewing sarcoma and nonmalignant adult tissues. Gene ontology was determined using DAVID and enriched categories determined relative to all genes, as well as relative to the 1,204 evaluable loci (26).

**MethyLight studies**

MethyLight analyses were carried out as previously described (27). Briefly, genomic DNA was isolated using the Genomic DNA Clean & Concentrator Kit (Zymo Research; D4011). Sodium bisulfite conversion of genomic DNA was performed using the EZ DNA Methylation Kit (Zymo Research; D5002). After sodium bisulfite conversion, the genomic DNA was amplified using MethyLight, a fluorescence-based real-time quantitative PCR, as previously described (27, 28), with the use of the EpiTect MethyLight PCR Kit (Qiagen; 59496) and EpiTect PCR control DNA set (Qiagen; 59695). The assay for \( \text{KCNA5} \) consisted of Forward primer: 5’-ATCGTAATCGGTTAGTTTCGACG-3’; Reverse primer: 5’-ATCGTAATCGGTTAGTTTCGACG-3’; Probe: 6FAM-CTGAAACCCCGCTCACAGCC-BAQ (IDT; Applied Biosystems; and Biosearch Technologies). Control probes and conditions have been published previously (27). Analysis was performed in triplicate using the Lightcycler 480 System (Roche Applied Science), and the average percentage of methylated
Results

DNA methylation of polycomb target gene promoters in Ewing sarcoma differs from normal adult tissues

Ewing sarcoma is characterized by an undifferentiated histology, and transcriptional profiling and tumor biology studies suggest that this primitive state is a consequence of both cellular origin and a block in cellular differentiation (18, 20, 21, 24). DNA hypermethylation at the promoters of developmentally critical polycomb target genes has been observed in many adult tumors and is associated with transcriptional silencing of genes that direct cell differentiation (refs. 10, 11 and reviewed in ref. 6). Given the high level of polycomb protein expression that is evident in Ewing sarcoma, and the critical roles of these proteins in tumor pathogenesis, we hypothesized that abnormal DNA methylation of polycomb target genes might be a feature of Ewing sarcoma.

To begin to address this, we investigated the methylation status of gene promoters using a custom-designed Illumina GoldenGate bead array (see Materials and Methods). Five Ewing sarcoma cell lines and four primary tumors were analyzed, and their DNA methylation data were compared with a panel of 52 normal adult tissues. As shown, despite their diverse origins, promoter DNA methylation was remarkably similar in all adult tissues, but was distinct from Ewing sarcoma (Fig. 1A). Notably, Ewing sarcoma tumors and cell lines clustered together and showed a similar overall pattern of DNA methylation, demonstrating that cell culture was not the primary driver of the abnormal DNA methylation profiles. Comparison of Ewing sarcoma with normal tissues identified 547 probes with differences in median beta value of ≥0.2, and 498 of these probes reached statistical significance, as described in Materials and Methods (Supplementary Table S1). A total of 265 loci showed increased methylation in Ewing sarcoma, and 222 loci had lower levels of methylation than in adult tissues. As expected, an increase in DNA methylation was significantly more likely at CpG islands than nonislands. Specifically, 378 differentially methylated probes were located within CpG islands, and nearly two thirds of them (N = 243, 64%) showed increased methylation in Ewing sarcoma. Conversely, 120 differentially methylated probes were not located within annotated CpG islands, and the vast majority (N = 98, 82%) showed reduced levels of methylation in Ewing sarcoma (Fig. 1B).

Promoters of ion channel encoding genes are differentially methylated in Ewing sarcoma

In order to better characterize the biology of the genes with differentially methylated promoters, we performed gene ontology analyses to identify enriched biologic processes and molecular functions. To begin, we assessed enrichment of the gene ontology categories relative to the whole genome. As expected, given that the array was developed to directly interrogate polycomb targets, genes with differentially methylated promoters were highly enriched for embryonic transcription factors involved in morphogenesis, differentiation, and development (Supplementary Table S2). In particular, numerous HOX genes were differentially methylated in Ewing sarcoma (Supplementary Table S1). This is consistent with our recent studies, which showed that expression of HOX genes is widely abnormal in Ewing sarcoma compared with adult tissues (29).

In addition to the expected enrichment of developmental transcription factors, this analysis also identified an unanticipated enrichment of genes with molecular functions related to potassium ion binding, transport, and channel activity (Supplementary Table S2B). To determine if enrichment of these functional categories was merely a reflection of the array design rather than a true enrichment, gene ontology analysis was repeated and enrichment determined relative to the set of 1,204 evaluable probes rather than the whole genome. This analysis confirmed that biologic and molecular processes involved in ion homeostasis were the most significantly enriched categories among differentially methylated genes (Fig. 1C and D). In particular, genes involved in calcium homeostasis were most prominently associated with increased promoter methylation (Fig. 1C), whereas potassium-associated genes were overrepresented among loci with reduced levels of DNA methylation (Fig. 1D). Thus, in addition to the expected pattern of altered DNA methylation at developmental transcription factors, this analysis also identified ion regulatory genes as prime targets of differential methylation in Ewing sarcoma.

The promoter of the voltage-gated potassium ion channel gene KCNA5 is DNA methylated in cancer

The flux of potassium, sodium, chloride, and calcium ions across the cytoplasmic membrane is regulated by a complex array of voltage-gated channels, and control of intracellular ion levels by these channels is essential for cell proliferation and survival. In particular, voltage-gated ion channels play critical roles in the maintenance of cellular homeostasis (30, 31), and specific deregulation of potassium ion channels has recently been broadly implicated in cancer pathogenesis (32). Twenty-two potassium ion channel genes have been reported to be differentially expressed in a wide variety of human cancers, and 20 of these were found to be overexpressed in the context of malignant disease (reviewed in ref. 32). In contrast, expression of KCNQ1 and of Kv1.5 has been reported to be inversely correlated with clinical aggression in gastrointestinal tumors (33) and in lymphoma and gliomas (34–36), respectively. Thus, deregulated expression of potassium ion channels is common in cancer, but the mechanisms underlying their deregulation remain largely ill-defined. Significantly, we recently reported that expression of the KCNA5 gene, which encodes Kv1.5, is reduced in Ewing sarcoma and that the locus is reversibly epigenetically repressed by the polycomb proteins BMI-1 and EZH2 (37). Therefore, we hypothesized that more permanent silencing of the KCNA5 locus by DNA methylation may be an additional mechanism of more stable channel suppression in Ewing sarcoma. To address this, we first identified potassium ion channel genes among the list of 498 differentially methylated loci in Ewing sarcoma. Twenty potassium ion channel gene promoters were included in the list, and fourteen showed a relative reduction in DNA methylation in tumors compared with nonmalignant adult tissues. Conversely, six showed an increase in DNA methylation (Fig. 2A). Among relatively hypomethylated loci, increased expression of channels encoded by KCNA1 (Kv1.1), KCN4 (Kv3.4), and KCNK2 (K2P2.1) has been reported in cancer (32). Whether a relative reduction in DNA methylation at these gene promoters contributes to channel overexpression remains to be elucidated. With respect to the six loci with increased methylation in Ewing sarcoma, only downregulation of KCNA5 (Kv1.5) has been implicated in cancer pathogenesis (34–37), thus leading us to continue to focus our studies on this channel. Significantly,
Promoter methylation was detected in both Ewing sarcoma tumors and cell lines, confirming that, in at least some Ewing sarcoma cells, polycomb-dependent repression of the locus is complemented by CpG-island DNA methylation (Fig. 2B). To validate the data from the smaller array-based study, we analyzed the KCNA5 locus in a larger and completely independent set of primary tumors and cells using MethyLight analysis. A methylation-specific probe was designed that allowed quantification of DNA methylation at the KCNA5 locus, at a site that was several hundred bases downstream of the GoldenGate array probe site, but still within the promoter-associated CpG island (Fig. 2C). This analysis confirmed that the KCNA5 locus is methylated in Ewing sarcoma cell lines and tumors compared with both nonmalignant fibroblasts and endothelial cells as well as bone marrow–derived MSC, a putative cell of Ewing sarcoma origin (Fig. 2D). In contrast, the KCNA5 locus was highly methylated in NCSCs, another putative cell of tumor origin (Fig. 2D). Thus, these data suggest that differences in DNA methylation exist between stem cells of different developmental origins. It is noteworthy that, compared with the GoldenGate array probe, the MethyLight probe detected more abundant DNA methylation in the cell lines than in the primary...
tumors. This suggests that broader methylation of locus might be an adaptive response of cells to prolonged in vitro culture, or that Ewing sarcoma cells with more extensive DNA methylation of the KCNA5 gene are more amenable to generating a cell line (Fig. 2D). However, given that MSCs, fibroblasts, and endothelial cell lines showed no evidence of significant DNA methylation, it is clear that cell culture alone cannot explain increased methylation of the KCNA5 locus in Ewing sarcoma cells. In support of this, data from the MethHC database (38) shows significant tumor-specific KCNA5 promoter DNA methylation in all of the 18 different tumor types analyzed (Supplementary Fig. S1). Thus, DNA methylation at the KCNA5 promoter is a common attribute of multiple tumor types in vivo, thereby providing a potential mechanism to explain prior observations that Kv1.5 is downregulated in cancer.

Decitabine induces loss of DNA methylation at the KCNA5 locus and an increase in gene expression

Having established that KCNA5 is methylated in Ewing sarcoma cells, we next sought to determine if exposing cells to the hypomethylating agent, decitabine, would reverse DNA methylation and lead to increased gene expression. At high doses, decitabine is cytotoxic, but at low doses, it prevents DNA methylation without inducing cell death, and the efficacy of decitabine as an epigenetic therapy is best achieved with subcytotoxic doses, which in vitro usually range from 20 to 300 nmol/L (39). Exposure of Ewing sarcoma cells to 100 nmol/L decitabine did not induce cell death and led to reduced methylation of KCNA5 in all three Ewing sarcoma cell lines (Fig. 3A). Consistent with DNA methylation being a mechanism of gene repression, we also observed
that expression of the KCNA5 transcript increased concomitantly with loss of DNA methylation (Fig. 3C). However, the correlation between loss of methylation and derepression of gene expression was not linear over time, indicating that other mechanisms of gene regulation also contribute to modulation of KCNA5 expression in these cells.

**Kv1.5 channel function inhibits Ewing sarcoma cell proliferation**

We next evaluated whether DNA methylation of KCNA5 affects the proliferative phenotype of Ewing sarcoma cells. Exposure of Ewing sarcoma cells to low-dose decitabine for 72 hours had a profound impact on cell expansion (Fig. 4A). Trypan-blue exclusion displaying either total live cell count (Fig. 4B) or total dead cell count (Fig. 4C) and EdU incorporation (Fig. 4D) were performed to determine whether this effect was due to an increase in cell death or a decrease in proliferation. As shown, no significant loss of viability was observed (Fig. 4C). In contrast, total live cell count was significantly reduced (Fig. 4B), and incorporation of EdU was reduced (loss of 2nd peak in histogram; Fig. 4D), revealing that decitabine inhibits cell-cycle progression and proliferation of Ewing sarcoma cells. To determine if reduced proliferation in decitabine-treated cells was a result of loss of KCNA5 repression, we used the pharmacologic compounds 4′AP and DPO-1 (Fig. 5A). These two compounds are highly specific inhibitors of the Kv1.5 channel when used at pharmacologically validated doses (40–43). Notably, they function to block the Kv1.5 channel function by inhibiting the flux of potassium ions across the channel pore and altering transmembrane current, without altering the levels of channel expression. Significantly, blocking the Kv1.5 channel with either agent partially restored the proliferative phenotype of decitabine-treated Ewing sarcoma cells (Fig. 5B and C). Together, these data reveal that epigenetic silencing of the Kv1.5 channel, by DNA methylation of KCNA5, contributes to Ewing sarcoma cell proliferation and that this effect is mediated, at least in part, by the function of Kv1.5 as a negative regulator of cell-cycle progression (Fig. 6).

**Discussion**

Cancer cells hijack and dysregulate epigenetic mechanisms to dynamically alter gene expression and drive tumor pathogenesis. In the current studies, we assessed the DNA methylation profile of polycomb target gene promoters in the context of Ewing sarcoma. From these studies, we discovered that, like other human cancers, CpG islands in the promoters of polycomb target genes are selectively methylated in Ewing sarcoma. However, we also discovered the unanticipated finding that genes involved in calcium and potassium homeostasis are also differentially methylated relative to nonmalignant adult tissues. More specifically, our studies identified potassium ion channels as frequent targets of aberrant DNA methylation in Ewing sarcoma and revealed that the promoter of KCNA5 is relatively hypermethylated in tumor cells. In addition, interrogation of public databases showed that the KCNA5 locus is reproducibly hypermethylated, relative to adjacent normal tissues, in a wide variety of human cancers (38). We recently showed that polycomb-dependent repression of KCNA5 contributes to Ewing sarcoma cell survival under conditions of hypoxia (37). Thus, the current study validates our prior observation that the Kv1.5 channel suppression contributes to the
cancer phenotype and provides new evidence that cancer cell proliferation in ambient conditions is promoted, in part, by stable DNA methylation–dependent repression of the KCNA5 locus.

Potassium channels are transmembrane proteins that control cellular ion concentrations and homeostasis. Given that cell proliferation and survival are intimately linked to intracellular potassium ion levels (44–46), dysregulation of potassium channels can affect the cancer phenotype. Consistent with this, aberrant expression of ion channels is prevalent in cancer (47, 48). Potassium-conducting channels also regulate the biophysical properties of a cell, controlling intracellular potassium concentrations and maintaining resting membrane potential (31). Thus, hijacking of potassium ion channel function by cancer cells has the potential to affect cell proliferation, cell survival, migration, and differentiation (32). Interestingly, however, although most potassium channels have been found to be overexpressed in cancer, Kv1.5 is one of only two potassium channels that is downregulated (32).

Knowledge regarding the physiologic functions of Kv1.5 in normal cell biology provides insights into why this channel in particular would be selectively silenced in cancer. In myoblasts, high Kv1.5 expression leads to accumulation of cyclin-dependent kinase inhibitors and impairs cell-cycle progression at the G1 to S transition (49), suggesting the channel is a cell-cycle checkpoint regulator and could function as a tumor-suppressor gene. Our studies begin to illuminate the potential importance of this function in the context of Ewing sarcoma. Specifically, we have found that DNA methylation and repression of KCNA5 contribute to cell-cycle progression and that reversion of promoter methylation and KCNA5 de-repression is associated with growth inhibition and cell-cycle arrest. Moreover, specific pharmacologic inhibition of the Kv1.5 channel function partially restores proliferation in Ewing sarcoma cells that have been exposed to decitabine. Thus, these studies together provide evidence that reactivation of silenced Kv1.5 channels can inhibit cancer proliferation, likely by permitting efflux of K+ ions and inhibiting G1 to S transition. Alternatively, emerging data suggest that there are...
other functions for potassium channels that do not depend on their roles as ion conductors (32, 50). The known effect of both 4'AP and DPO-1 is to block K⁺ efflux and alter transmembrane current. Whether noncanonical roles exist for Kv1.5 outside of regulating K⁺ influx and membrane depolarization exist and whether they contribute to its function as a tumor suppressor remain to be elucidated.

The contribution of ion channel deregulation to tumor pathogenesis remains a relatively unexplored area of investigation in cancer biology. We have now shown that the Kv1.5 channel is reproducibly targeted for epigenetic repression, by both polycomb proteins (37) and by DNA methylation (this study), and that repression contributes to cancer cell survival and proliferation. These findings provide compelling evidence to support the designation of KCNA5 as a tumor-suppressor gene in human cancers, and suggest that further studies into its mechanism of action as a cell-cycle regulator will reveal how loss of the Kv1.5 function supports the proliferative cancer phenotype.

Disclosure of Potential Conflicts of Interest

D.J. Weisenberger is consultant at Zymo Research Corporation, has ownership interest (including patents) in USC patent royalty payment; and is a consultant/advisory board member for Zymo Research Corporation. P.W. Laird has received honoraria from the Speakers Bureau of Merck SD. No potential conflicts of interest were disclosed by the other authors.

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