Targeted methylation of two tumor suppressor genes is sufficient to transform mesenchymal stem cells into cancer stem/initiating cells

I-Wen Teng1,*, Pei-Chi Hou1,*, Kuan-Der Lee2, Pei-Yi Chu3, Kun-Tu Yeh3, Victor X. Jin4, Min-Jen Tseng1, Shaw-Jenq Tsai5, Yu-Sun Chang6, Chi-Sheng Wu6, H. Sunny Sun7, Kuen-daw Tsai1,8, Long-Bin Jeng8, Kenneth P. Nephew9, Tim H.-M. Huang10, Shu-Huei Hsiao1,+ and Yu-Wei Leu1,+

1Human Epigenomics Center, Department of Life Science, Institute of Molecular Biology and Institute of Biomedical Science, National Chung Cheng University, Chia-Yi, 621, Taiwan;
2Chang Gung Memorial Hospital, Chia-Yi, 613, Taiwan;
3Department of Pathology, Changhua Christian Hospital, Changhua, 500, Taiwan;
4Department of Biomedical Informatics, The Ohio State University, Columbus, OH 43210, USA;
5Department of Physiology, College of Medicine, National Cheng Kung University, Tainan, 701, Taiwan;
6Basic Medical Sciences, Chang Gung University, Tao-Yuan, 333, Taiwan;
7Institute of Molecular Medicine, College of Medicine, National Cheng Kung University,
Tainan, 701, Taiwan;

8Department of Internal Medicine, China Medical University Beigang Hospital, Yunlin, 651, Taiwan.

9Medical Sciences and Department of Cellular and Integrative Physiology, Indiana University Simon Cancer Center, School of Medicine, Bloomington, IN 47405, USA.

10Division of Human Cancer Genetics, Department of Molecular Virology, Immunology, and Medical Genetics, and the Comprehensive Cancer Center, The Ohio State University, Columbus, OH 43210, USA

Running Title: Methylated HIC1 and RassF1A transformed MSC.

Keywords: DNA methylation, MSC, and Tumorigenesis.

*Note: I-W. Teng and P.-C. Hou contributed equally to this work.

†To whom correspondence should be addressed: E-mail: bioywl@ccu.edu.tw (Y.W.L) or bioshh@ccu.edu.tw (S.H.H.).
Abstract

Although DNA hypermethylation within promoter CpG islands is highly correlated with tumorigenesis, it has not been established whether DNA hypermethylation within a specific tumor suppressor gene (TSG) is sufficient to fully transform a somatic stem cell. In this study, we addressed this question using a novel targeted DNA methylation technique to methylate the promoters of $HIC1$ and $RassF1A$, two well-established TSGs, along with a two-component reporter system to visualize successful targeting of human bone marrow-derived mesenchymal stem cells (MSC) as a model cell system. MSCs harboring targeted promoter methylations of $HIC1/RassF1A$ displayed several features of cancer initiating/cancer stem cells, including loss of anchorage dependence, increased colony formation capability, drug resistance and pluripotency. Notably, inoculation of immunodeficient mice with low numbers of targeted MSC resulted in tumor formation, and subsequent serial xenotransplantation and immunohistochemistry confirmed the presence of stem cell markers and MSC lineage in tumor xenografts. Consistent with the expected mechanism of TSG hypermethylation, treatment of the targeted MSC with a DNA methyltransferase inhibitor reversed their tumorigenic phenotype. To our knowledge, this is the first direct demonstration that aberrant TSG hypermethylation is sufficient to transform a somatic stem cell into a fully malignant cell with cancer initiating/cancer stem properties.
Introduction

DNA methylation, a tightly regulated process during normal development, frequently becomes dysregulated during disease development, including cancer (1-3). Although methylation-induced tumorigenesis has yet to be recapitulated experimentally, during somatic cell proliferation, environmental and extracellular signals can initiate changes in DNA methylation that contribute to clonal selection, altered cellular behavior, and ultimately tumorigenesis (4-6). Hypomethylation and/or hypermethylation of specific loci, including tumor suppressor loci, were strongly associated with transformation and carcinogenesis (7, 8), and genetic knockout of the DNA methyltransferases (DNMTs), resulted in global hypomethylation and tumorigenesis (9, 10). Although a causative role for altered methylation at specific loci, particularly as an initiating neoplastic event, remains poorly understood, dormant stem cells, either pre-existing in tissues or arising from somatic cells, may play a role in cancer origin and prognosis (4). Furthermore, because DNA hypermethylation of TSG has been documented in many cancers, and its spreading correlates with cancer progression (4), we hypothesized that abnormal DNA hypermethylation can disrupt somatic stem cell proliferation and differentiation, resulting in the development of neoplasia.

To directly examine the effect of aberrant DNA methylation on cellular physiology, we established a Targeted DNA Methylation method called “TDM” (11, 12), in which transfection
established an *in vitro* methylated DNA complementary to the target gene promoter region initiated recruitment of DNMT to the endogenous target loci. Consequently, DNMT-mediated methylation spreads within the promoter region of the target loci, ultimately silencing the target gene after cellular passages. In order to monitor progression of TDM and cellular transformation, we also developed a two-component system (11, 12). The first component consisted of regulation of tetracycline repressor (*Tet*) expression by the cloned target promoter sequence. A CMV promoter driving expression of a reporter (*enhanced green fluorescence protein or EGFP*) comprised the second component. A *Tet* repressor binding site, *Tet operator*, placed between the CMV and the EGFP, regulated EGFP expression. In the absence of DNA methylation, *Tet* expression was observed, and the expression of EGFP was silenced. Induction of DNA methylation silenced the Tet repressor and activated EGFP expression, allowing us to observe progression of DNA methylation in a living cell. Furthermore, if target loci DNA methylation was sufficient to induce cellular transformation, an EGFP-expressing cell behaved like a tumor (11, 12). It was of interest to use this system to initiate TDM in a somatic stem cell and monitor the subsequent effects on cellular transformation. Although spontaneous transformation of human MSC *in vitro* was recently described (13, 14), genetic disruptions of the p53 pathway, but not retinoblastoma (Rb), was sufficient to transform a fat-derived MSC (15), supporting the possibility that sarcoma could
be initiated from MSC.

In the current study (work flow is illustrated in Supplementary Figure S1A), we aimed to test the hypothesis that targeted DNA methylation is sufficient for cellular transformation, thus the promoter regions of \textit{HIC1} (hypermethylated in cancer 1) and \textit{RassF1A} (ras associated family protein isoforms 1A), two TSGs reported to be frequently silenced by DNA methylation in cancer (16-18), were cloned, methylated \textit{in vitro}, and then transfected into the MSCs, individually or concurrently. \textit{HIC1} and \textit{RassF1A} are involved in highly diverse, interacting cellular networks (16, 19, 20), and their loss of function could result in the recently described phenomenon of oncogenic addiction through p53 pathway (5). We thus hypothesized that hypermethylation of \textit{HIC1} and \textit{RassF1A} would not only to directly suppress their tumor suppressor function, but also disrupt multiple cellular networks, resulting in tumorigenesis and cancer progression.

\textbf{Materials and Methods}

\textbf{MSC isolation and characterization.} Human MSC isolation and culture were performed as described by K.D. Lee \textit{et al}. (21). MSC expansion medium, passages and culture condition were as described by Hsiao \textit{et al}. (11).

\textbf{In vitro DNA methylation.} Four micrograms of PCR-amplified \textit{HIC1} and \textit{RassF1A}
promoters were incubated with 20 Units of CpG methyltransferase (New England BioLabs) at 37°C for 4 hrs in the presence of 160 μM S-adenosylmethionine to induce methylation.

**Validation of in vitro DNA methylation.** Methylated DNA showing resistance to methylation-sensitive restriction enzymes (BstUI) indicated completed conversion (Supplementary Figure S2).

**Cy5 labelling of the HIC1 promoter fragment.** HIC1 DNA was labelled with LabelIT tracker Reagents (Mirus) according to the manufacture’s instruction.

**Transfection.** The methylated PCR products (0.4 μg) were denatured at 95°C and then transfected into 5×10⁵ cells using DMRIE-C (Invitrogen), according to the manufacturer’s instructions. Unmethylated PCR products were transfected as control. Cells were transfected three times at day 1, 3, and 5.

**Semi-quantitative real-time methylation specific PCR (qMSP).** The qMSP experiment was conducted and products were quantified according to the protocol described in Yan et al. (22). Briefly, bisulfite converted genomic DNAs (0.5 μg) were subject to real-time PCR with methylation specific primers (Supplementary Table 2). The qMSP reactions were performed using the SYBR Green I PCR Kit (Toyobo) in an iQ5 Real-Time PCR instrument (Bio-Rad). Melting analysis was performed followed by all of the PCR reactions to ensure a specific amplicon was generated. Col2A1 was used for standard curve construction and as loading
control. Serial dilution of Col2A1 amplified bisulfite-converted DNA was used to generate standard curve. Methylation percentage was calculated as: [Means of target gene]/[Means of Col2A1]; fold change was calculated as: [TDM methylation percentage]/[Mock methylation percentage]. Endogenous and exogenous HIC1 promoters were discerned by the reverse primers indicated in Supplementary Figures S1B and S3A.

**Differential Methylation Hybridization, DMH.** All procedures for the DMH microarray were performed as described in Leu et al. (18) using a human CpG island microarray (Agilent). Briefly, me_H&R-treated and control MSC genomic DNAs were digested into small fragments and then ligated with designed adaptors. Methylation-sensitive restriction enzymes (BstUI and HpaII) were used to discriminate the methylated and the unmethylated DNA fragments. Differences in methylation status were then amplified by PCR using the adaptor as primer. The amplicons from control and me_H&R-transfected cells were labeled with Cy3 and Cy5, respectively, then co-hybridized onto the CpG microarray. After measuring the Cy3 and Cy5 intensity, the M value \[M = \log_2(Cy5 \text{ intensity}/Cy3 \text{ intensity})\] was used to indicate the difference in DNA methylation between two sources, and the L value \[L = 0.5x\log_2(Cy5xCy3)\] was used to indicate the intensity of individual loci. Both values were adjusted and normalized by LOWESS. A cut-off of 4 based on the M value was used to identify the target loci.
Immunostaining. Cells were fixed in 2% formaldehyde/PBS, then permeabilized with 0.5% NP40/PBS. After blocked with horse serum/PBS (1:100), the slides were incubated with primary antibody in 3% BSA/PBS followed by three times of PBS washes. The cells were incubated with secondary antibodies conjugated with Fluorescein or Texas Red (Vector Lab) in 3% BSA/PBS. After several PBS washes, the slides were mounted in mounting medium with 4’,6-diamidino-2-phenylindole (DAPI, Vector Lab). The primary antibodies used were: anti-HIC1 (Millipore), anti-RassF1A (Bioscience), anti-CD133 (Abcam), anti-Oct4 (Cell Signaling), and anti-Neuronal nuclei (NeuN, Chemicon).

5-aza-dc-2’-deoxycytidine (5-aza-dc) treatment. Control and me_H&R transfected MSCs were treated with either 20 μM (Figure 1) or 5 μM (Supplementary Figure S6D) of 5-aza-dc or an equal final volume of DMSO for 5 consecutive days.

Cloning of the human HIC1 and RassF1A promoters. Primer sequences for human HIC1 and RassF1A promoters are listed in Supplementary Table 1. Genomic DNA purified from human MSCs served as a template for PCR. Purified PCR products were ligated into the pyT&A cloning vector (Yeastern Biotech) according to the manufacturer’s protocol. Inserts were confirmed by restriction digests and sequencing. Cloning and TDM for the Salvador-Warts-Hippo (SWH) signaling pathway components were performed using the same protocols and the primers were listed in Supplementary Table 1.
Semi-quantitative RT-PCR, qRT-PCR. Total RNA isolation, first-strand cDNA synthesis, and detection of the transcripts were carried out as described (18). Briefly, total RNA (2 μg) was reverse transcribed using the SuperScript III reverse transcriptase (Invitrogen). The qRT-PCR was then performed by SYBR Green I PCR Kit (Toyobo) in an iQ5 Real-Time PCR instrument (Bio-Rad). A serial dilution of GADPH amplified cDNA was used as control to generate standard curve and GAPDH from each samples was used as loading control. The primers are listed in Supplementary Table 2.

Cell survival assay. In 96-well plates, 20 μl of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (Sigma-Aldrich, 5 mg/ml) was added to each well containing different number of cells and incubated at 37°C for 5 hrs. The reaction was terminated by adding 200 μl of DMSO, and absorbance was measured at 595 nm.

Soft agar assay. Cells were plated at a density of 5×10⁴/well in soft agar. After 2 weeks of culture, cells were stained with 0.01% crystal violet, and the number of spheres (more than 50 cells) from each dish was counted.

Trans-well study. Cells were plated at a density of 5×10⁴/well into the hanging cell culture insert (Millipore), and the redistribution of the cells on the other side of the insert was observed, stained and quantified.

In vivo tumorigenesis and serial transplantation of xenografts. Six-week-old nude mice
(Narl:ICR-Foxn1nu) were inoculated subcutaneously with $1 \times 10^4$ me_H&R-transfected or control MSCs. Growth of tumours was monitored until they reached 0.8 cm in diameter. Then, tumors were surgically removed and sub-cultured in MSC medium on low attachment plates until spheres were observed again. The same number of subcultured cells were inoculated into a new nude mouse, and the entire procedure (n=9) was repeated four more times (n=36 in total).

**Immunohistochemistry.** Tumor masses surgically removed from nude mice inoculated with me_H&R-transfected MSCs were paraffin-embedded and sectioned at 4 μm or embedded in O.C.T. and sectioned on a cryostat (Leica) at 12 μm. Sections were stained with the indicated antibodies, and detection was performed with Vectastain (Vector Lab) for the paraffin sections and Fluorescein or Texas red-conjugated anti-mouse or rabbit IgG (Vector Lab) for the cryo-sections, followed by DAPI staining. Sections were also stained with Hematoxylin and Eosin (H&E staining, Vector Lab) for pathological exams.

**Lineage-specific induction of MSCs.** Transfected MSCs (mock or me_H&R) were plated onto 6-well plates at $5 \times 10^4$ cells/well. After attachment, the medium was replaced with neuronal pre-induction medium (DMEM with 20% FBS, 10 ng/ml bFGF, and 1 mM β−mercaptoethanol) for 24 hrs, followed by neuronal induction medium (DMEM with 100 μM BHA, 10 μM forskolin, 2% DMSO, 25 mM KCl, 2 mM valproic acid, 1× B27 supplement, 10
ng/ml bFGF, 10 ng/ml PDGF). Morphological changes and NeuN expression were used to validate neuronal induction. Osteocyte induction medium consisted of DMEM, 10% FBS, 10 mg/ml penicillin/streptomycin, 100 nM dexamethasone, 10 mM β-glycerophosphate, and 50 μM L-ascorbic acid-2-phosphate. Cells were treated with the osteocyte induction medium for 10 days and then subject to alkaline phosphatase (Sigma-Aldrich) staining.

**Construction of two-component reporter system.** The construction of \( HIC1 \) two-component reporter system is described and illustrated in Supplementary Figure S4. Both constructs were sequence validated and used to transfect the MSCs and transfected cells were selected with hygromycin and G418 resistance. PCR were performed to validate the integrations of both constructs (\( HIC1-TR \) and \( EG1 \), Supplementary Figure S4B). MSC clones carrying both reporter constructs were treated with doxycycline (Dox) (Supplementary Figure S4B). Dox-induced EGFP expression indicates the reporter system is functional.

**Human subjects.** Isolation and characterization of human MSCs were conducted under IRB regulations of the Chang Gung Memorial Hospital, Chia-Yi, Taiwan.

**Animals.** The use of mice followed the regulations and protocols reviewed and approved by the Institutional Animal Care and Use Committee at the National Chung Cheng University.
Results

Targeted HIC1 and RassF1A methylation transforms MSCs. In vitro methylated (validation of in vitro methylation is shown in Supplementary Figure S2) or unmethylated (control) HIC1 and RassF1A promoter DNA fragments were transfected into human bone marrow-derived MSCs mixed population (MSC_MP) alone or in combination. Transfection of methylated HIC1 (me_HIC1) or methylated RassF1A (me_RassF1A) increased endogenous HIC1 or RassF1A promoter methylation, as detected by qMSP (Figure 1A, upper panels. MSP amplification was confirmed by sequencing in Supplementary Figure S3A & S3B) and the expression of endogenous HIC1 or RassF1A decreased accordingly, as detected by immunostaining (Figure 1A, lower panels). TDM was confirmed by bisulfite sequencing (Supplementary Figure S3C and S3D).

Loss of contact inhibition was used as a screening criterion for MSC transformation (Figure 1B, left panels. Transfection and selection of transformed MSCs are illustrated in Supplementary Figure S6A). Co-transfection of methylated HIC1 and RassF1A (me_H&R) caused formation of anchorage-independent aggregates, similar to the transformed phenotype in attached cultures, while the controls and cells transfected with either me_HIC1 or me_RassF1A alone remained contact-inhibited (Figure 1B, left center two). When cultured in low attachment dishes, both methylated- and mock-transfected MSCs formed spherical
aggregates (Figure 1B, right panels), suggesting that these me_H&R transfected MSCs retained their self-renewal property.

To confirm that loss of anchorage dependence was due to DNA hypermethylation, cells were treated with the DNMT inhibitor 5-aza-dc. 5-aza-dc treatment decreased the hypermethylation level of endogenous HIC1 and RassF1A in the me_H&R-transfected MSCs (Figure 1C and Supplementary Figure S6D) and abrogated the formation of me_H&R-induced aggregates (Figure 1D right and Supplementary Figure S6D upper). These data indicate that DNA hypermethylation of both HIC1 and RassF1A potentially transformed somatic MSCs and rendered them anchorage-independent. Moreover, the requirement for methylation of both HIC1 and RassF1A for MSC transformation supports the notion that cancer initiation and development is a multistep process.

Validation of TDM by two-component reporter system.  In order to confirm that the TDM caused silencing at the transcriptional level and induced aggregates within the targeted cells, we used a two-component reporter system (11, 12) to mark and track methylation-mediated silencing of the HIC1 promoter in live cells (single colonies, SC). In this system, the HIC1 promoter regulates expression of the EGFP reporter construct (Figure 2A flow diagram on left; reporter system construction is illustrated in Supplementary Figure S4). Methylation of the
HIC1 promoter alone did not result in phenotypic changes but caused HIC1 promoter silencing, as indicated by increased EGFP expression (Figure 2A, right center). However, concomitant methylation of RassF1A and HIC1 led to cell aggregates containing EGFP-expressing (i.e., HIC1-silenced) cells (Figure 2A, right and Supplementary Figure S6B & S6C).

To track the distribution of transfected DNAs, me_HIC1 construct was labeled with Cy5 prior to transfection. Cy5 signals localized mainly in the nuclear region (Figure 2B and Supplementary Figure S5), indicating that the me_HIC1 entered the MSC nuclei. EGFP was detected in these Cy5-containing/transfected MSC (Figure 2B, columns 3, 7, 8). When me_HIC1 was used to transflect the cells, qMSP analysis further demonstrated increased methylation of exogenous HIC1 promoter (HIC1-TR) (Figure 2C, left), and EGFP expression increased accordingly (Figure 2C, right). The me_RassF1A, RassF1A or control (unmethylated) HIC1 DNAs failed to induce the EGFP expression, further indicating that we successfully and specifically methylated the HIC1 promoter. Targeted HIC1 methylation had no effect on the methylation state of the endogenous RassF1A promoter and vise versa (Figure 2D), consistent with our previous report that the locus-specific TDM (11, 12). Combined transfection of me_HIC1 and me_RassF1A resulted in methylation of both endogenous loci, as determined by qMSP (Figure 2D, left panels), as well as concomitant
silencing of both genes (qRT-PCR, Figure 2D right panels). Data from the two-component reporter system further confirmed that our targeting method caused gene silencing at the transcriptional level and that aberrant DNA methylation of both HIC1 and RassF1A might transform normal somatic stem cells. Furthermore, our data indicated that methylation of HIC1 initiated the accumulation of epigenetic changes in MSCs and increased the potential for transformation. In normal cells, the transformation process can be prohibited by gatekeepers or roadblocks, such as RassF1A; thus, gatekeeper silencing can allow for excess cell proliferation and/or genome corruption, increasing the probability of neoplastic transformation.

**Transfection of me_H&R induces genome-wide DNA methylation changes in MSC.** To further investigate changes in DNA methylation following me_H&R targeting, genome-wide methylation profiling of me_H&R-transfected and control MSCs was carried out using DMH microarrays (23). As shown in the methylation heatmap, transfection of me_H&R induced extensive disruption in the MSC epigenome (Figure 3A, left). Individual loci (Figure 3A, right) displaying hypermethylation (SOX7, ADAM9, and GATA6) or hypomethylation (CXXC1, OSBP, and TBX2) were validated by qMSP (Figure 3B). Based on the wide range of cellular functions associated with HIC1 and RassF1A (16, 19, 24), it was not surprising to observe
global changes in the MSC methylome. Furthermore, as both HIC1 and RassF1A were reported to function as TSGs that act via p53, we conducted unsupervised ontological analysis of the array target loci and found that p53 and its associated signaling components were significantly altered by me_H&R transfection (Figure 3C and Supplementary Figure S7, target loci with the p53 binding domain are listed in Supplementary Table 3). Our finding that MSC transformation is strongly associated with altered p53 function is consistent with a previous report using genetic approaches (15).

**Characteristics of me_H&R-transformed MSCs.** To determine whether the me_H&R-transformed MSCs retained stemness, me_H&R transfected MSCs were labeled with antibodies against stem cell surface markers CD133 and Oct4 and induced to differentiate. Expression levels of CD133 and Oct4 were unaffected by me_H&R transfection (Figure 3D, upper left). Multipotency was assayed by neuronal induction and osteogenic differentiation. MSCs transfected with me_H&R were able to differentiate into neuron-like cells (Figure 3D, right, indicated by NeuN staining) and osteoblasts (Fig. 3D, left bottom, alkaline phosphatase-positive). These data indicate that although concurrent methylation of HIC1 and RassF1A tumor suppressor genes was sufficient to transform MSCs, as indicated by altered anchorage dependence and methylation patterns, the transformed
MSCs retained somatic stem cell characteristics.

The proliferation and invasion capability of transformed MSCs were evaluated by using colony formation and trans-well studies. Colony formation was substantially increased (~8-fold, n=6) in me_H&R-transfected cells vs. controls (Figure 4A and Supplementary Figures S8A and S9A), and this was inhibited by 5-aza-dc treatment (Figure 4A and Supplementary Figure S8A). In trans-well studies, MSCs transfected with me_H&R exhibited greater migratory capability (Figure 4B and Supplementary Figures S8B and S9B). Taken together, these results indicated that MSCs with concurrent methylation of HIC1 and RassF1A acquired a cancer phenotype.

Acquired drug resistance is a hallmark of malignancy and a characteristic of a cancer stem cell (CSC) phenotype. Cisplatin treatment induced cell death in control MSCs in a dose-dependent manner, but the drug was much less effective in me_H&R-transfected MSCs, even at a very high dose (100 μM; Figure 4C), further indicating that concurrent HIC1 and RassF1A methylation transformed normal somatic stem cells into CSC-like.

To examine the tumorigenic capacity of the me_H&R-transfected MSCs in vivo, immunodeficient nude mice were inoculated with me_H&R-treated MSC. As shown in Figure 4D, 100% of these mice developed tumors (n=9), while the mice inoculated with control MSCs remained tumor-free. Immunohistochemistry revealed the presence of
CD133+ stem cells in the tumors (Figure 4D, lower left). Furthermore, the tumors were soft tissue sarcomas-like (Figures 4D, right and 5A and 5B), consistent with this type of malignancy in mice with heterozygous disruption of HIC1 (17).

Expression of stem cell markers in me_H&R MSC-derived tumors. We examined xenografts from serial transplantations for expression of known stem cell markers by immunohistochemistry. As shown in Figure 5A, expression of CD44, CD133, and Oct4 was observed, substantiating the CSC-like phenotype. Vimentin expression was also detected, confirming the mesenchymal origin of the xenografts. Clonal expression of a panel of epithelial markers, including NSE, S-100, cytokeratin, desmin, and LCA (Figure 5B), further demonstrated the ability of CSC to differentiate into heterogeneous tumors. In addition, expression of both stem cell (CD133) and mesenchymal (vimentin) markers in sparsely scattered cells of me_H&R tumors demonstrated that the xenografts were derived from inoculated me_H&R MSCs (Figure 5C). Although we observed slight enrichment of CD133+/vimentin+ cells in serial transplantation experiments (Supplementary Figure S10A), the overall percentage remained low, in agreement with previous observations (25) that CSC continue to form a small proportion of the overall tumor even after in vitro enrichment and xenograft transplantation.
Transformation specificity induced by concurrent methylated of HIC1 and RassF1A. To establish that MSC transformation was due to concomitant methylation of HIC1 and RassF1A and not accumulation of targeted genes, we simultaneously methylated nine genes in the Salvador-Warts-Hippo (SWH) signaling pathway by TDM in MSCs (20) (Figure 6A). Abnormal SWH signaling pathway has been associated with tumorigenesis in both mammalian cells and Drosophila (26, 27), and RassF1A is a SWH pathway component controlling cellular proliferation, survival, anti-apoptosis, organ size and cell contact inhibition (20, 28). Because loss-of-function of these nine genes (Figure 6A) has been reported in various cancers (20), we hypothesized that methylation of these SWH signaling pathway genes (i.e., “me_SWH”) could cause MSC transformation. me_SWH-treated MSC displayed loss of contact inhibition (Figure 6B), increased colony formation in soft agar assays (Supplementary Figure S9A), and were invasive in trans-well assays (Supplementary Figure S9B). Furthermore, me_SWH-treated MSC retained stemness markers, could be induced to differentiate (Figure 6C). However, these cells were not tumorigenic in nude mice assay (Figure 6D). Taken together, these results support a unique role for the combination of methylated HIC1 and RassF1A in MSC transformation. Furthermore, increasing the number of methylated loci within the SWH pathway did not result in transformation of MSC, even though RassF1A was
included in the me_SWH loci.

Collectively, our data demonstrated that concurrent methylation of HIC1 and RassF1A was sufficient to transform MSCs. The observation that pluripotency was maintained suggests that the cells had acquired a CSC phenotype. Increased proliferation is often accompanied with increased genetic and/or epigenetic mutations (29), which further enhance transformation and clonal selection during mesenchymal-to-epithelial transition (EMT) (30-32). In more permissive environments, including nude mice, subpopulations of inoculated me_H&R produced NSE- and LCA-positive epithelial cells (Figure 5C) or other cell types (Supplementary Figure S10B). The acquired differentiation capacity, and perhaps later migratory capability, may allow these CSC-like cells to either remain in their original location or migrate to form secondary tumors (Figure 5D, bottom scheme). Bioinformatic analysis further revealed the presence of p53 binding elements within the targets identified by DMH (Supplementary Table 3), suggesting a central role for p53 in MSC oncogenic transformation. The tumor suppressor activities of both HIC1 and RassF1A are due, in part, to p53 activation, in agreement with previous genetic findings (17). Concordant silencing of HIC1 and RassF1A by DNA methylation may impair p53-mediated apoptosis and contribute to the tumorigenic ability of MSCs.
Discussion

Here, we elucidated the role of DNA methylation in the transformation of somatic stem cells into CSC-like cells. Bone marrow-derived MSCs are known to play important roles in cancer progression and metastasis (33-35). By providing a microenvironment that enhances primary tumor growth, invasiveness, and metastasis, MSCs have the capacity to mobilize to other organs, providing a niche suitable for the disseminated cancer cells to metastasize to distant tissues (33). Our data demonstrate that in addition to these two supporting roles, MSCs may play a previously unidentified role in tumorigenesis, as abnormal DNA hypermethylation of HIC1 and RassF1A, two tumor suppressor genes involved in functionally diverse, interacting networks, transformed MSCs from normal somatic stem cells to cancer-like stem cells.

Concordant methylation of HIC1 and RassF1A has been identified in advanced ovarian cancer (36), and HIC1 shows increased concurrent hypermethylation with other genes in advanced myelodysplasia syndrome (37), suggesting that disturbance of HIC1-associated networks may be essential for tumor initiation. Unlike RassF1A, which can be inactivated by either genetic or epigenetic mechanisms, repression of HIC1 is mainly caused by DNA methylation (16). Thus, DNA hypermethylation of HIC1 could predispose cells to cancer development (Figure 2A). A second epigenetic hit, such as RassF1A methylation, may then
permit more efficient cancer development. However, it is also possible that hypermethylation of RassF1A results in further epigenomic disturbances, rendering a cell more susceptible to cancer-causing insult(s). Our data further demonstrate that DNA methylation is a cause rather than a consequence of malignancy, and p53 may be at the center of this oncogenic transformation. p53-dependent apoptosis plays an integral part in tumor growth, progression, and drug resistance development (38), and the tumor suppressor ability of both HIC1 and RassF1A is due to p53 activation. Thus, concordant silencing of HIC1 and RassF1A by DNA methylation may synergistically disrupt the p53-mediated apoptotic pathway and contribute to the observed tumorigenic ability of MSCs.

The origin of cancer stem cells or cancer initiating cells has been widely debated. These cells may arise either from de-regulated somatic stem cells or from de-differentiated mature cells (4, 7, 8, 39-42). It has been reported that several hypermethylated genes, including HIC1 and RassF1A, in adult cancer are unmethylated in embryonic stem cells and only partially methylated in embryonic carcinomas (43). In this study, we demonstrate that forced epigenetic silencing of HIC1 and RassF1A is sufficient to confer normal somatic stem cells with malignant properties, including loss of contact inhibition, increased colony formation, migration capability, drug resistance, and tumor formation in inoculated mice. Moreover, the cells retained sensitivity to neuron- and osteocyte-induction and displayed both
lineage-specific markers and stem cell markers in xenografts. Thus, we reason that methylation of both \textit{HIC1} and \textit{RassF1A} triggers the transformation of normal somatic stem cells to CSC-like cells. As proposed in Figure 5D, this transition may promote additional transforming events and further cellular selection. We further propose that under the influence of different environmental niches, these transformed stem cells could give rise to tissue-specific cancers.

References


Acknowledgments

Yu-Wei Leu (NRPGM, NSC-98-3112-B-194-001 and NSC-97-2320-B-194-003-MY3), Shu-Huei Hsiao (NSC-96-2320-B-194-004), and Shaw-Jenq Tsai and Yu-Wei Leu (NSC-97-2627-B-006-003) are supported by the National Science Council, Taiwan. Kenneth P. Nephew and Tim H.-M. Huang are supported in part by NIH grants CA085289 and CA113001.

Figure legends

Figure 1. Concurrent HIC1 and RassF1A methylation in transformed MSCs. (A) HIC1 and RassF1A TDM. Denatured methylated or unmethylated (mock control) HIC1 and/or RassF1A promoter DNAs were transfected individually or together into human MSCs. The promoter methylation of endogenous HIC1 (upper left) and endogenous RassF1A (upper right) were detected by qMSP and protein expression was detected by immunostaining (lower panels).
(B) Transformation of MSCs by concurrent HIC1 and RassF1A methylation. MSCs transfected with unmethylated DNA only (control), me_HIC1, me_RassF1A, or me_H&R were cultured in attachment (left) or low attachment dishes (right). Spheroid formation was observed for all four treatments on low attachment dishes; however, only the me_H&R MSCs showed loss of contact inhibition. (C) 5-aza-dc treatment reverses me_H&R-induced endogenous HIC1 and RassF1A hypermethylation as measured by qMSP. (D) 5-aza-dc treatment represses me_H&R-induced MSC aggregates. Loss of DNA methylation after 5-aza-dc treatment was correlated with reversion of the loss of contact inhibition phenotype in me_H&R treated MSCs (right bottom).

**Figure 2.** Visualization of targeted DNA methylation and MSC transformation. (A) Schematic diagram of the reporter system (left; construction and validation of the obtained clones in Supplementary Figure S4). Targeted HIC1 methylation was visualized by EGFP fluorescence (right center). Including RassF1A TDM led to EGFP-expressing cell aggregation (right). (B) Tracking TDM. Unmethylated (control) and/or methylated HIC1 and RassF1A DNA fragments were transfected into the MSCs harboring both constructs shown in (A). me_HIC1 was labeled with Cy5 in order to track the distribution of transfected DNA in the MSCs, after induction of EGFP expression. Only the Cy5-containing cells expressed EGFP,
confirming the specificity of the targeted methylation. (C) Detecting the methylation state of
the exogenous HIC1 promoter (HIC1-TR) and the expression of EGFP. qMSP was performed
to quantify exogenous HIC1 promoter region methylation using HIC1-specific and
vector-specific primers (left). Increased HIC1-TR methylation was observed only in the
me_HIC1-targeted MSCs. EGFP expression increased accordingly, as detected by qRT-PCR
(right). (D) Validation of HIC1 and/or RassF1A TDM. MSCs were treated with methylated
HIC1 and/or RassF1A as (C). qMSP was used to detect methylation changes at endogenous
HIC1 or RassF1A promoter (left panels). qRT-PCR was used to determine changes in
expression of endogenous HIC1 or RassF1A (right panels).

Figure 3. Methylation changes and stemness of me_H&R-transfected MSCs. (A) Left:
Altered methylation levels of the MSC methylome depicted by the heatmap of the DMH data.
Mock-transfected cells were labeled with Cy3, me_H&R-transfected cells were labeled with
Cy5. Red and green lines correspond to hyper- and hypo-methylation, respectively. Right:
arrows highlight hypermethylated genes in selected array blocks. (B) Validation of altered
methylation by qMSP. (C) Visualization of affected loci by unsupervised pathway finding
(PathVisio) (44). (D) Stemness of me_H&R-transfected MSCs. Immunostaining for Oct4
and CD133 stem cell markers (top left). Phase-contrast and immunostaining of MSCs
during neuronal induction (right) and osteocyte induction (lower left). pre-NIM, neuronal pre-induction medium; NIM, neuronal induction medium. The neuronal differentiation was indicated by NeuN immunostaining. AP: alkaline phosphatase.

Figure 4. *In vitro and in vivo* tumorigenesis of *me_H&R*-transfected MSCs. (A) In soft agar assays, *me_H&R*-transfected MSCs formed a greater number of colonies vs. controls (5th and 7th columns), and colony formation was inhibited by 5-aza-dc treatment (6th column). (B) Quantification of migrated cells. *Me_H&R*-transfected MSCs migrated to the bottom of the trans-well inserts (control cells did not) and this migration was blocked by 5-aza-dc treatment. (C) Increased drug resistance in *me_H&R*-transfected MSCs. Cisplatin-induced cell death was measured by MTT assay. High dose cisplatin treatment (100 μM) caused massive cell death in control MSCs but not in *me_H&R*-transfected MSCs (histogram). Representative images of cisplatin-induced cell death (right panel). (D) Tumor development in mice inoculated with *me_H&R*-transfected MSCs (n=9) (left top). H&E staining (right panels) and immunohistochemistry of CD133 expression (bottom left) were performed on xenograft tumors.

Figure 5. Immunohistochemistry of xenografts from *me_H&R*-transfected MSCs. (A)
Stem cell marker expression in xenografts. Representative images of cells positive for CD44, CD133, or Oct4 from the serial sections of xenograft tumors. (B) Representative images from xenografts (n=36) containing heterogeneous cell types of either epithelial or mesenchymal lineage (vimentin). (C) Expression of lineage-specific markers (left: NSE, right: LCA) was observed in many CD133-positive cells, or lineage-specific marker expressing cells surrounded CD133-positive cells. (D) Upper: images of co-expression of CD133 and vimentin in a subpopulation of control MSCs (top), me_H&R-transfected MSCs (middle), and subcultures derived from me_H&R-transfected MSC xenografts (bottom). Lower: Simplified model for mechanisms by which normal somatic stem cells (primary) can gradually become tissue-specific tumor cells, via transformation and clonal selection occurring at niches in primary or secondary sites. Colored circles represent different lineages. EMT: epithelial-to-mesenchymal transition; MET: mesenchymal-to-epithelial transition.

**Figure 6.** TDM of SWH signaling pathway is not sufficient for full MSC transformation. *In vitro* methylation of the main components within SWH pathway (*me_SWH*) was performed and transfected cells were characterized. (A) Methylation of the nine SWH pathway components was detected by qMSP. In the physical maps, short, filled boxes indicate the target sites in each promoter and the arrow heads indicate the primer sites used to detect the
TDM on the left. (B) Images: methylation of the SWH pathway caused the MSC to lose contact inhibition (top right). Treatment with 5-aza-dc reversed the aggregate phenotype caused by TDM (bottom right). Histograms: me_SWH-treated MSCs exhibited higher growth rate in soft agar assay vs. mock-treated cells. (C) Me_SWH-treated MSCs retained stemness properties. Me_SWH-treated MSCs expressed stem cell markers CD133 and Oct4 (top left) and neuronal (right panels) and osteogenic (bottom left) lineages could be induced. (D) No tumor formation was observed after subcutaneous (s.c.) injection of me_SWH treated MSCs in nude mice (center; n=8); in contrast, s.c. implanted me_H&R-treated MSCs formed tumors (right).
Figure 2
Figure 3
Figure 6
Targeted methylation of two tumor suppressor genes is sufficient to transform mesenchymal stem cells into cancer stem/initiating cells

I-Wen Teng, Pei-Chi Hou, Kuan-Der Lee, et al.

Cancer Res  Published OnlineFirst April 25, 2011.