Histone Methylase MLL1 plays critical roles in tumor growth and angiogenesis and its knockdown suppresses tumor growth in vivo

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| Gene               | Forward primer (5’→3’)                      | Reverse primer (5’→3’)                      |
|--------------------|---------------------------------------------|---------------------------------------------|
| MLL1 (ORF)         | GAGGACCCCGGATTAAACAT                       | GGAGCAAGAGGTTTCAGCATC                      |
| MLL2 (ORF)         | AGGAGCTGCAAGAAGAGCAG                       | CAGCCAAACTGGAGAACAGAG                      |
| Cyclin A (ORF)     | AAGAAGCAGCCAGCATACCGGAA                   | AGCTGCAAGTTTCCCCCTCAGAACA                 |
| Cyclin A (Promoter)| TCCACACTCAGTTTCTGAG                        | AGGTGACTGCAACAGCCTGCTTCCTCCCTCCGCT       |
| Cyclin B (ORF)     | TTAGATATTCCCTGCCCGCCAGCC                 | TTTCCGTGCCATTTCCTAGTTTCCTGCCCTGG          |
| Cyclin B (Promoter)| ACAATTGGCCTTGAGAACTGACAGA                 | GGTATGAGAAGGCTGTCGAGCTGTGTCGATGAG         |
| Cyclin D (ORF)     | ATGAGATTGAGTTGAGAACTGACAGA                | GGTATGAGAAGGCTGTCGAGCTGTGTCGATGAG         |
| Cyclin E (ORF)     | TTTCAGGGTATCACCTGAGTTGCAGGAC             | ACAACATGACTTTCTATCTGGTCCCTG              |
| P15 (ORF)          | ATGGCGGAGAGAACAAG                         | CCCAGCTACCTGGAGATCG                       |
| p18 (ORF)          | ACTTGGAGAAGACTGAGACTCGAGAGA               | GTCGCTCTCTATTCCTGAGCATT                   |
| p27 (ORF)          | ACTTGGAGAAGACTGAGACTCGAGAGA               | GTCGCTCTCTATTCCTGAGCATT                   |
| p57 (ORF)          | ATCCACCAGATGAGAGCTCTCTCT                  | CCTGGCTGGAAGAGCTGATCC                     |
| p57 (Promoter)     | GACCAGGAGGTCTGACTGACTGAGA                 | GGTATGAGAAGGCTGTCGAGCTGTGTCGATGAG         |
| CD31 (ORF)         | GAGTACTTGTGAGACTGAGA                       | TCGGCTTTCTCTTCCTCGAGT                   |
| CD31 (Promoter)    | GCCATTTTGAGGAAATGCA                       | TTTTTCCAGTCTCTTCCCTTC                    |
| HIF1α (ORF)        | CCACCATATGACCTGCTGCTGCTG                  | TATCCAGGCTCGTGCAGT                        |
| HIF1α (Promoter)   | GACAAGCAGCGTCCCGAGAGAGC                   | GAAAGGCAAGCGTCCCGAGAG                      |
| VEGF (ORF)         | AAGGAGAAGGAGGAGAACTGA                      | ATCTGATGAGGATTGATGAG                     |
| VEGF (Promoter)    | TGCCAGGCTCTCTCGGG                          | TCTGGCTGCTCATCACCCATCC                    |
| α-Actin (ORF)      | AGAGCTAGAGGCTGCTGCTGC                     | GATCTTGCGCTCAGGAG                        |
| MLL1-A1            | CTCTTTCCTTGAGCCTCCTCCGGCT                 |                                      |
| MLL1-A2            | ATGCAGCTTGCTCTGCCCTGCCA                   |                                      |
| MLL1-A3            | TGCCAGGCTGCTCTCCCTCAC                     |                                      |
| MLL1-A4            | GCCTGTACCTGCTCTCCTCCTCTC                |                                      |
| MLL1-A5            | TTGCGGTCTCCACCACACTCTCTC                 |                                      |

Antisense oligonucleotides specific to MLL1 (all linkages are phosphorothioate linkages)
Figure S1. Sequence information and level of knockdown efficiency of MLL1 antisenses. (a) The nucleotide sequences and positions (corresponding to different regions of MLL1 mRNA) of the four different MLL1 specific phosphorothioate antisense oligonucleotides (MLL1-A1 to MLL1-A5). (b, c, d, e and f) Knockdown of MLL1 in HeLa cells. HeLa cells were transfected with varying concentrations of MLL1-A1, MLL1-A2, MLL1-A3, MLL1-A4 and MLL1-A5 antisenses, respectively, for 48 h. RNA was extracted and subjected to RT-PCR analysis using primer specific to MLL1 and MLL2 (antisense specificity control) and rRNA is shown as loading control.)
Figure S2. Knockdown of MLL1 by using MLL1-antisense in different cell lines. Human cervical cancer (HeLa), lung cancer (H358), colon cancer (SW-480), breast cancer (MCF7) choriocarcinoma placenta (JAR), normal breast epithelial (MCF10) cell and normal colon (CCD-18Co) cell, were grown up to 60% confluency, and then transfected with MLL1-antisense for 48 h using ifect (MolculA) transfection reagent. Control cells were treated with equivalent amount of a scramble antisense. The level of MLL1 expression was analyzed by RT-PCR using primer specific to MLL1. Expression of MLL2 was done to confirm specificity of MLL1 antisense towards MLL1 gene. β-actin was used as loading control.
Figure S3. Knockdown of MLL1 induced apoptosis in cervical cancer cell (HeLa). Cells are grown up to 60% confluency and then transfected with MLL1-antisense for 48 h. Cells were then either fixed in 4% formaldehyde for immuno-fluorescence studies or harvested for caspase analyzing by using cell lysates. (A) Immuno-staining of cytochrome-c. MLL1-antisense treated and control cells were immuno-stained with cytochrome-c antibody and detected by using FITC-labeled secondary antibody (top panel). Corresponding DAPI stained nucleus is shown in bottom panel. (B) Depletion of MLL1 induced activation of caspase-3/7. HeLa cells were treated with MLL1-antisense (or scramble antisense) for 48 h, cell lysates were analyzed for caspase activity using Caspase-3/7 Assay Kit (AnaSpec Inc). This experiment was done twice with five replicates each time (n = 10). Bars indicated standard error.
Figure S4. Effect of MLL1 antisense treatment on HeLa (cervical cancer), MCF10 (normal breast epithelial) and CCD-18Co (normal colon) cells. Cells are grown up to 60% confluency and then transfected with MLL1-antisense (MLL1-A3) for 48 h and then subjected to TUNEL and caspase assay. (a-c) TUNEL assay. MLL1-antisense treated and control cells were fixed in 70% EtOH and subjected to terminal nicked end-labeling using fluorescent dUTP. In parallel cells were also stained with DAPI (nuclear staining, blue fluorescence) and propidium iodide (PI that stains nucleus of dead cells, red color). dUTP stained green speckles represent apoptotic cells with fragmented nuclei. (d) Cell lysates were subjected to caspase activity using Caspase-3/7 Assay Kit (AnaSpec Inc). This experiment was done twice with five replicates each time (n = 10). Bars indicated standard error.
Figure S5. Effect of MLL1-A5 antisense treatment on HeLa cells. Cells are grown up to 60 % confluency and then transfected with MLL1-A5 antisense for 48 h. (a) The RNA was isolated and analyzed by RT-PCR analysis with primers specific to MLL1 and MLL2. rRNAs were used as control. The real-time PCR quantification of the genes is shown in the right panel. (b) Cell viability: MLL1-A5 antisense treated and control cells were subjected to MTT assay. The relative (%) cell viability (MLL1-A5 antisense vs scramble) was plotted for different cell lines. Bars indicated standard errors. (c) Microscopic analysis of MLL1-A5 antisense treated and control cells. (d) TUNEL assay. MLL1-antisense treated and control cells were subjected to terminal nicked end-labeling using fluorescent dUTP, DAPI and PI staining and analyzed under a fluorescence microscope. (e) Cell lysates were subjected to caspase activity using Caspase-3/7 Assay Kit (AnaSpec Inc). This experiment was done twice with five replicates each time (n = 10). Bars indicated standard error.
Figure S6. Roles MLL1 in vasculogenesis: Para-formaldehyde perfused tumor xenograft tissue were sectioned and subjected to co-immuno-chemical staining with CD31 and MLL1 antibodies, followed by staining with FITC and rhodamine conjugated secondary antibodies. Nuclear counter staining was done with DAPI and then visualized under fluorescence microscope. Representative images of the xenografted tumor tissue showing the blood vessels sections are shown.
Supplementary Materials and Methods

Cell culture and transfection with antisense

Human cervical cancer (HeLa), human bronchialveolar carcinoma (H358), choriocarcinoma placenta (JAR), colorectal adenocarcinoma (SW-480), nonmalignant colon fibroblast (CCD-18Co), human adenocarcinoma mammary (MCF7), and nonmalignant mammary gland fibrocystic cell (MCF10) were obtained from American type cell culture collection (ATCC). All other cell lines (except H358) were grown and maintained in DMEM media, supplemented with 10% FBS, 2 mM l-glutamine, and penicillin/streptomycin (100 unit and 0.1 mg/mL, respectively) in presence of 5% CO₂ at 37 °C. The H358 cells were grown and maintained in RPMI-1640 with all supplements as added to DMEM1-3.

For antisense-mediated knockdown experiments, cells were grown up to 60% confluency and transfected with varying amounts (0.6−1.8 μg/mL) of different MLL1 antisense oligonucleotides (custom synthesized from IDT-DNA) in FBS-free media using infect transfection reagent (Molecular) and following manufacturer’s instruction. In brief, antisense and infect transfection reagents were mixed in 300 μL of DMEM without any supplements and applied to cells (60 mm plate) in the presence of 1.7 mL of supplement-free medium. The cells were incubated for overnight followed by addition of 2 mL media containing all supplements and 20% charcoal stripped FBS. Cells were then incubated for an additional 48 h. Antisense-treated cells were harvested for RNA/protein extraction or fixed in 4% formaldehyde for ChIP assay.

Reverse transcription-PCR (RT-PCR) and western blot analysis

Antisense-treated and control cells were harvested by centrifugation at 500 g, resuspended in diethyl pyrocarbonate-treated buffer A (20 mm Tris/HCl, pH 7.9, 1.5 mm MgCl₂, 10 mm KCl, 0.5 mm dithiothreitol and 0.2 mm phenylmethanesulfonyl fluoride), incubated on ice for 10 min and then centrifuged at 3500 g for 5 min. The supernatant containing the cytoplasmic extracts was subjected to phenol–chloroform extraction followed by LiCl precipitation of cytoplasmic mRNA by incubating overnight at −80 °C. The mRNA was washed with diethyl pyrocarbonate treated 70% EtOH, air dried and resuspended in diethyl pyrocarbonate-treated water.

The reverse transcription reactions were performed with 1 μg of total RNA in a 25 μL reaction cocktail containing 2.4 μM of oligo dT (Promega), 100 units of MMLV reverse transcriptase, 1× first strand buffer (Promega), 100 μM each of dATP, dGTP, dCTP, and dTTP (Invitrogen), 1 mM dithiothreitol (DTT), and 20 units of RNaseOut (Invitrogen). The cDNA was diluted to 100 μL final volume. For semiquantitative PCR the cDNA was PCR amplified by using Taq DNA Polymerase (Genscript) and primers as described in Table S1.

For real-time PCR analysis, the cDNA was amplified using SsoFast EvaGreen supermix (Bio-Rad) using CFX96 real-time PCR detection system. The real-time PCR results were analyzed using the CFX Manager software. The experiments were repeated at least twice with three replicates each time. The normality of the data was analyzed by using t-test and analyses of the variants (ANOVA) were performed at 5% level of significance.
Nuclear proteins extracts were prepared from the nuclear pellets, as described previously\(^2\). For Western blot analysis equivalent amount of proteins were analyzed in SDS/PAGE and subjected to western blot analysis with specific antibodies against MLL1 and MLL2, (Abgent) and β-actin (Sigma).

**Analyzing cell viability, morphology and cytotoxicity**

The cytotoxicity of MLL1 antisenses were determined by using MTT assay as described by us previously (1-3). In brief, approximately 10,000 cells (in 150 μL DMEM) were seeded into each well of a 96 well microtiter plate and incubated for 24 h. Cells were treated with antisense (MLL1 or scramble)-transfection reagent cocktail (final antisense concentration was 1 μg/mL) and incubated for 48 h. Then 20 μL MTT (stock 5 mg/mL in PBS) was added into each wells and incubated for 2 h under normal growth condition to allow the viable cell to convert MTT to formazan. Then the media was discarded, formazan crystals were dissolved by adding 100 μL DMSO and incubating 2 h with continuous shaking. The absorbance of the lysates was directly measured at 560 nm using a micro plate reader (Fluostar-omega, BMG Labtech). The percent viable cells (calculated based on absorbance of the control untreated sample) were plotted. The each experiment was repeated at least twice (with five replicates each time).

For analyzing effect of MLL1-antisense on cell viability and cell morphology, cells were grown in 60 mm culture plates and transfected with MLL1-antisense (or scramble) for 48 hrs, stained with trypan blue (10 min) and visualized under differential interference contrast (DIC) setting of a microscope (Nikon Eclipse TE2000-U, Japan).

**Flow-cytometry analysis.**

HeLa cells were grown to 60% confluence and transfected with MLL1 and scramble antisense oligonucleotides separately using Maxfect transfection (MoleculA) reagents, and incubated for 48 h. Control and transfected cells were harvested, fixed in 70% ethanol for 2 h, washed twice with 1× NaCl/Pi and stained with propidium iodide (final concentration, 0.5 μg·mL\(^{-1}\)). The cells were analyzed by flow cytometry, using a Fusing Beckman Coulter (Fullerton, CA, USA) Cytomics FC500 Flow Cytometry Analyzer.

**TUNEL assay**

The TUNEL (terminal dUTP nicked end labeling) assay was performed using ApoAlert DNA Fragmentation Assay Kit (Clontech) as described previously (2). In brief, cells were transfected with MLL1 antisense for 48 h, washed with PBS, fixed in 1 % formaldehyde (4 °C for 25 min), permeabilized in 0.2 % Triton X-100 (15 min on ice), equilibrated in equilibration buffer. Terminal deoxynucleotidyl (dUTP) end labeling reaction and termination were done as instructed by the manufacturer. Cells were then incubated with 10 μL of DAPI (to stain the nucleus, 5 mg/mL) and propidium iodide (PI that stains dead cells, 0.5 mg/mL) for five minutes, mounted on microscope slide and visualized under fluorescence microscope.

**Analysis of Caspase-3/7 activity**

For caspase-3/7 activation assay HeLa cells were transfected with MLL1 antisense for 48 h, lysed and centrifuged (2500 g for 10 min at 4 °C). The supernatant was diluted (to 1 μg
μL−1 of protein). Caspase-3/7 activity was assessed by using a SensoLyte TM Homogenous AMC Caspase-3/7 Assay Kit (AnaSpec Inc.). Briefly, 150 μL extract was mixed with 50 μL of assay buffer containing caspase-3/7 substrate and incubated for 15 min at 37 °C and then fluorescence intensity (350EX/440EM) was measured at 10 min intervals (up to 2 h). The concentration of activated caspase-3/7 was calculated by using a calibration curve and finally expressed relative to untreated control cells.

**Chromatin immuno-precipitation (ChIP) assay**

Cells were transfected with MLL1 and scramble antisense for 48 h. Cells were fixed with 1 % formaldehyde for at least 15 min, washed thrice in cold PBS, resuspended in 1ml of lysis buffer (1 % SDS, 10 mM EDTA, 50 mM Tris/HCl, pH 8, 1X protease inhibitors and 0.2 mM PMSF). Cells were then sonicated until chromatin was sheared to an average DNA fragment length of 0.2 - 0.5 kb. The sonicated chromatin was pre-cleaned, quantified and subjected to immuno-precipitation (as described previously2, 3 using different antibodies as needed. The immuno-precipitated chromatin were sequentially washed, eluted and subjected to de-crosslinking by incubating at 65 °C for 4 hr followed by proteinase K digestion. The chromatin was subjected to pheno:chloroform extraction followed by precipitation over night. The immuno-precipitated DNA fragments were subjected to PCR analysis with primer specific to promoter sequence of different gene as needed.

**Immuno-staining with anti-cytochrome-c**

For the immuno-fluorescent staining HeLa cells transfected with MLL1 and scramble antisenses for 48 h and subjected to immuno-staining with anti-cytochrome-c antibody and DAPI and visualized under fluorescence microscope as described by us previously (1, 4). In brief, cells were fixed for 10 min with 4% formaldehyde, washed twice with PBS, and permeabilized for 10 min using 0.2 % Triton X-100 and blocked with 10 % goat serum for 1 h prior to incubation with anti-cytochrome-c antibody for additional 1 h. Cells were then washed thrice with 0.02 % Tween-20 and then stained with FITC conjugated secondary antibody for 30 min, washed thrice with cold PBS. Nuclear counterstaining was performed with DAPI. Immuno-stained cells were mounted and observed under a fluorescence microscope (Nikon Eclipse TE2000-U, Japan).

**Animal toxicity and cervical cancer xenografts**

All the animal experiments and xenograft experiments were carried out using the IACUC approved protocol (PI: Subhrangsu Mandal Protocol no: A07.007) using similar protocols as described previously4, 5. Every care and precautions were taken to minimize the pain/stress on the animal. Animals were hosted in the institutional (UTA) animal care facility under the supervision of trained personal. Prior getting into the xenograft experiments, the animal toxicity of each of the antisense oligonucleotides were examined using nude mouse. Six weeks old Athymic nude (nu/nu) mice (obtained from Harlan, Indianapolis, IN) were used for these experiments. For the toxicity analysis, we injected intraperitoneally three different doses (100, 300 and 500 μg/ 20 gm body weight) of MLL1 and scramble antisense into six week old nude mouse (3 replicates each) and then monitored the health of each mouse on a daily basis for a month. We monitored the body weight every 4 days intervals after treatment with antisense.
For the xenograft experiments, $2 \times 10^6$ human cervical cancer cells (HeLa cell in $100 \mu l$ of PBS) were injected subcutaneously (near the right back limb). Animals were examined daily for signs of tumor growth and behavior. Once the tumor size reached ~32 mm$^2$ (2 to 3 weeks after injection of cells) we administered MLL1-antisense intraperitoneally (PBS solution, twice in a week, 300 µg/20 gm body weight, in three parallel replicates, continued for a month). Experiments were repeated at least two times. Control mice were injected with equal volume of the diluents (PBS) or scramble antisense alone. Tumor sizes were measured every two days intervals and bi-dimensional measurements were carried out using calipers and cross-sectional area (tumor size) were plotted. The normal growth habit of the animals were observed and body weight of the animals were recorded over the experimental period.

**RNA and protein analysis in tumor tissue**

For RNA and protein analysis, tumors were directly excised from euthanized mice, flash frozen in liquid nitrogen, homogenized and subjected to RNA extraction (ZyGEM kit) and protein extraction that were then subjected to qPCR and western blotting as described above.

**Immuno-histological analysis of tumor xenograft (Fluorescent staining)**

Immuno-histochemical staining of the xenografted tumor sections were done as described by us previously. In brief, sections were blocked by incubating in presence of goat serum, incubated (2 h) with the respective primary antibodies (CD31, MLL1, HIFα), washed and incubated with fluorescein isothiocyanate (FITC) or rhodamine (Jackson Immuno Research Laboratories, West Grove, PA, USA) conjugated secondary antibodies. Nuclear counterstaining was performed with DAPI. Immuno-stained cells were mounted and observed under a fluorescence microscope (Nikon Eclipse TE2000-U; Nikon, Melville, NY, USA).

**Immunohistochemistry analysis (DAB staining)**

The cryoprotected xenografted tumor sections were incubated in blocking buffer (containing donkey serum) and then with primary antibodies specific to MLL1 and CD31, HIFα. Sections were then incubated with biotinylated donkey secondary antibody flowed by and avidin–biotin complexes, followed by peroxidase labeling using a DAB substrate kit (Vector Laboratories). Sections were examined under a differential interference contrast (DIC) setting of a microscope (Nikon Eclipse TE2000-U, Japan).

**Chromatin immuno-precipitation analysis of tumor xenograft**

For chromatin immuno-precipitation and immuno-hostochemical staining mice were sequentially perfused with cold 1× PBS, followed by 4% paraformaldehyde in 1× PBS at a pH of 7.4, and the xenografted tumors were removed, cryoprotected in PBS containing 30% sucrose and sectioned. ChIP assay of the tumor sections were done using antibodies against MLL1, RNAPII (Abcam), H3K4-tri-methyl (Upstate) and β-actin as described above.
Supplementary references

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