CHAPTER III

MATERIALS AND METHODS

3.1 Cell lines and media

HPV-positive cervical cancer cell lines, HeLa and SiHa will be maintained in Eagle's modified essential medium (Hyclone, England) and CaSki will be maintained in RPMI-1640 medium (Hyclone, England) containing 10% fetal bovine serum (v/v) (Hyclone, England), 100 U/ml penicillin (General Drugs House Co., Ltd., Thailand), 0.4 mg/ml streptomycin (M & H Manufacturing Co., Ltd., Thailand), 1% sodium pyruvate (Hyclone, England) and 1% HEPES (Hyclone, England) at 37°C and incubated in humidified 5% CO_2 incubator (Thermo Electron Corporation, USA). Confluent cell monolayers will be maintained and used for experiments by treating with 0.25% (w/v) Trypsin - 0.53 mM EDTA (Hyclone, England).

3.2 Cell culture and treatment

3.2.1 Cell culture

HPV-positive cervical cancer cell line was detached from a tissue culture treated flask by treating with 0.25% (w/v) Trypsin - 0.53 mM EDTA (Hyclone, England). Complete media was added and cells were resuspended by gently pipetting. Single cell suspension was centrifuged at 1000 rpm for 5 min (Profuge, USA). After centrifugation, complete media was discarded and MEM or RPMI-1640 complete media was added. Cell viability was assessed by trypan blue dye (Hyclone, England) exclusion method using a haemocytometer. Cell was diluted to appropriate cell concentration and plated in tissue culture plates for further experiments.

3.2.2 Cell preservation for storage

Freezing media were prepared by adding 10% DMSO (v/v) (Sigma Aldrich, USA) to MEM or RPMI-1640 complete media. After collecting cells by centrifugation, cells were resuspended in 1 ml cold freezing media and stored in cryogenic vial (Corning Incorporation, USA). The frozen cells were immediately stored in -80°C refrigerator overnight and moved for long term storage in Liquid Nitrogen Tank 34 HC Taylor Wharton Cryogenic (Harsco Corporation, USA) the next day.

3.2.3 Thawing cell for use

Frozen cells in cryogenic vials in liquid nitrogen were thawed in 37°C water bath (Memmert, Germany). Cell suspensions were added to 9 ml serum-free media and centrifuged at 1,000 rpm for 5 min. Freezing media in serum-free media was removed and MEM or RPMI-1640 complete media was added. Cells in complete media were plated in a tissue culture treated flask for experiments.

3.3 Plasmids and transfection

3.3.1 Plasmid preparation

Plasmids were transformed into competent *E. coli* DH5α by heat-shock technique. Briefly, fifty µl of competent from -80°C was thawed on ice. One µl of plasmid was added to competent cells and the plasmid-competent cell mixture was incubated on ice for 30 min. This mix was heated shock at 42°C for 90 sec and immediately placed on ice for 2 min. After the heat shock process, cells were transferred to 1 ml of LB broth (Appendix) and incubated at 37°C for 1 hr by shaking at 200 rpm. After that, fifty to one hundred µl of LB broth containing the cells was plated on LB agar (Appendix) plates containing 50 µg/ml of ampicillin. The plate was incubated at 37°C for 16-24 hr and the colony was picked for plasmid preparation.

3.3.2 Plasmid isolation

A few colonies were picked up and cultured for 16-18 hr at 37°C in 2 ml LB broth containing 50 μg/ml of ampicillin. One ml of the culture was sterilely moved to 1.5 ml microcentrifuge tube, and cells were pelleted by centrifugation at 13,000 rpm for 1 min at 4°C. The supernatant was discarded and the plasmid was extracted using QIAprep Spin Miniprep kit or QIAGEN plasmid Midi kit (Qiagen, Germany) according to the manufacturer's instructions. The obtained plasmid was eluted in 50 μl or 100 μl sterile HPLC water and stored at -20°C until use.

3.3.3 Plasmid quantitation

Plasmid stored at -20°C was diluted in sterile deionized water at 50 fold dilution. The absorbance was measured at wavelength 260 and 280 nm. One OD_{260} corresponds to a concentration of 50 μg/ml plasmid DNA. Therefore, the concentration of plasmid was calculated by using the following equation.

$$\text{Plasmid } (\mu g/ml) = OD_{260} \times 50 \times \text{dilution factor } (\mu g/ml)$$

The purity of plasmid was evaluated from a ratio of OD_{260}/ OD_{280}. The ratio of appropriately purified plasmid was 1.8-2.0.

3.3.4 Bacterial glycerol stock

Bacterial culture was added 0.5 ml of sterile 60% glycerol (total concentration of glycerol is 20% v/v) and vortex the culture to ensure that the glycerol is evenly dispersed. The culture was transferred to a labeled storage tube equipped with a screw cap. The tube was kept at –70°C for long term storage.

To recover the bacteria, the frozen surface of the culture was scraped with a sterile inoculating loop, and then immediately streaked onto the surface of an LB agar plate containing the appropriate antibiotic. The frozen culture was returned to storage at –70°C and incubated the plate overnight at 37°C.

3.3.5 Transient transfection using FuGene® HD transfection reagent

HPV-positive cervical cancer cell lines (5×10^4 cell/ml) in 0.5 ml culture media was plated overnight in 24-well tissue culture treated plates. Preparing the FuGene® HD transfection reagent (Roche, USA) was conducted according to the manufacturer's instructions. Optimal FuGene® HD transfection reagent and plasmid ratio was chosen at 6:2. Briefly, FuGene® HD transfection reagent was brought to room temperature prior to use for 15 min. Volume of serum free media Opti-MEM (Invitrogen, England) and plasmid were calculated to dilute the plasmids and they were prepared in a 1.5 ml microcentrifuge tube. To form the transfection complex, FuGene® HD transfection reagent was directly added to the diluted plasmids without being contacted with the wall of the plastic tubes and the mixtures were gently vortexed for 1-2 sec. They were incubated at room temperature for 15 min and the complex mixture was added dropwise to cells. Finally, the plate was swirled and incubated for 12-48 hr. After this incubation, cells were used for further experiments.

3.4 Retroviral vector construction and transduction

3.4.1 Preparing HPV-positive cervical cancer cell lines and 293T cells

HPV-positive cervical cancer cell lines (1×10^5 cell/ml) in 2 ml culture media were plated overnight in 12-well tissue culture treated plates and 293T cells (1×10^6 cell/ml) in 4 ml culture media were plated in 60 mm tissue culture treated plate a night before transfection (at least 12 hr).

3.4.2 Preparing retroviral supernatant

The retroviral vector and packaging construct pCL-Ampho were cotransfected into 293T cells using FuGene® HD transfection reagent (Roche, USA), according to the manufacturer's instructions. The retroviral vector constructs are MSCV-IRES-GFP (Addgene, USA) and MSCV-Mam (12-74)-EGFP (a kind gift from Professor Warren Pear, University of Pennsylvania, USA). Twelve µl of FuGene® HD transfection reagent was diluted in 90 µl of

serum free media Opti-MEM (Invitrogen, England) in 1.5 ml microcentrifuge tube. They were resuspended well and incubated at room temperature for 5 min. For preparation of diluted DNA mixture, 2 μg of retroviral vector and packaging construct pCL-Ampho were added in 90 μl of serum free media Opti-MEM into another 1.5 ml microcentrifuge tube. Diluted DNA mixture was added to diluted FuGene® HD transfection reagent. They were resuspended well and incubated at room temperature for 15 min, to let DNA:FuGene form complex. After incubation, 293T cells media was removed and washed once with cold PBS. Two ml of fresh complete media was added in 60 mm tissue culture treated plate and incubated. Eight hundred μl of complete media was added into DNA:FuGene complex and resuspended well. The complex mixture was added dropwise to 293T cells. Finally, the plate was rocked gently and incubated in incubator.

The next day, GFP^+ cells were observed under Fluorescent microscopes (Olympus DP71, USA). Forty-eight hours post transfection, viral supernatant was harvested and fresh complete media was added in 293T cells. Viral supernatant was centrifuged at 1,200 rpm for 10 min and filtered through a 0.45 μm acrodisc syringe filter (Pall, USA).

3.4.3 Retroviral infection

Six μl of FuGene® HD transfection reagent was added to retroviral supernatant and mixed well. Forty-eight hours post transfection, HPV-positive cervical cancer cell lines media were removed and the retroviral supernatant mixture was added dropwise to cells. The plate was rocked gently and centrifuged at 2,200 rpm for 1 hr at 30°C using Benchtop Centrifuge Model Universal 320 (Hettich, Germany). After incubation for 1 hr, complete media was added into 2 ml in each wells and plate was incubated for 2 days. This procedure was repeated the following day using 72 hr post transfection viral supernatant. Forty-eight hours post infection, GFP^+ cells were observed under Fluorescent microscopes.

3.5 RNA extraction

HPV-positive cervical cancer cell lines treated with GSI or transduced DN-MAML1 (1×10^5 cell/ml in 1 ml culture) was plated in 12-well tissue culture plate overnight. The culture supernatant was removed and one ml of TriZol reagent (Invitrogen, England) was added directly to cells. The mixture was incubated for 5 min at room temperature. TriZol reagents containing RNA were transferred to 1.5 ml microcentrifuge tubes and 0.2 ml of chloroform (Lab-Scan, Ireland) was added. All tubes were vigorously mixed by hands for 15 sec and incubated at room temperature for 3 min. The samples were centrifuged using Refrigerated Centrifuge Model 1920 (Kubota, Japan) at 12,000xg for 15 min at 4°C. Only colorless aqueous phase was carefully transferred to fresh tubes. RNA was precipitated by gently mixing with 0.5 ml of Isopropanol (Merck, Germany). The samples were incubated at room temperature for 10 min and centrifuged at 12,000xg for 10 min at 4°C. The RNA pellets on the bottom side of each tube were visible at this stage. The supernatants were rinsed and the RNA pellets were washed once with 1 ml of ice cold 75% ethanol in 0.01% DEPC water (Appendix). The samples were mixed by vortex mixer model G560E (Scientific Industries, USA) and centrifuged at 7,500xg for 5 min at 4°C. RNA pellets were dried for 5-10 min, dissolved in 20 µl of 0.01% DEPC water, and incubated for 10 min at 55°C. RNA samples were kept at –70°C until use for further experiments.

3.5.1 Quantitation of RNA using spectrophotometer

RNA was diluted to 50 to 100-fold dilution in 0.01% DEPC-treated water. The diluted RNA was subjected to absorbance measurement at 260 and 280 nm in the spectrophotometer. An OD_{260} corresponds to a concentration of 40 µg/ml single stranded RNA. Therefore, the concentration of RNA was calculated in µg/ml by using the following equation.

$$\text{RNA } (\mu g/ml) = OD_{260} \times 40 \times \text{dilution factor}$$

The purity of RNA was evaluated from a ratio of OD_{260}/ OD_{280}. The ratio of appropriately purified RNA was in the range of 1.8-2.0.

3.5.2 Quantitation of RNA using Quanti iT Assays (Invitrogen, England)

Measuring amount of RNA using Quanti iT were performed according to the manufacturer's instruction. Briefly, Quanti-iT reagent and Quanti-iT buffer (Invitrogen, England) were calculated and prepared to be Quanti-iT working solution. Ten µl of RNA standards composing of 0 ng/µl of RNA and 10 ng/µl of RNA were mixed with 190 µl of working solution. RNA samples was diluted to 10-fold dilution in Hypure® water PCR grade (Hyclone, England) and 2 µl of diluted RNA was mixed with 198 µl of working dilution. Calibrations of RNA standard were performed by Quanti-iT and concentrations of RNA samples were measured. The concentrations of RNA were calculated in µg/ml by using the following equation.

RNA (µg/ml) = measured concentration × dilution factor

3.6 cDNA synthesis by reverse transcriptase

Obtained RNA 1 µg was used for converting to cDNA. Total RNA was mixed with 0.2 µg of random hexamer (Qiagen, Germany), and the volume was adjusted to 12.5 µl by 0.01% DEPC treated water. The RNA mixture was heated at 65°C for 5 min and placed on ice for 5 min. Then, 1×Reverse transcriptase buffer (Fermentus, Canada), 1 mM dNTP mix (Fermentus, Canada) and 20 U of RNase Inhibitor (Fermentus, Canada) were added in the mixture and followed by incubation at room temperature for 5 min. Reverse transcriptase (Fermentus, Canada) was added to final amount of 200 U per reaction, and the reaction was carried out in Bioer Life Express (Bioer technology, China) at 25°C for 10 min, 42°C for 60 min, 70°C for 10 min and 25°C for infinity. The cDNA was stored at -20°C until use.

3.7 Polymerase chain reaction (PCR)

The components of PCR reactions were as follows: 1xTag buffer (Fermentus, Canada), 0.64 mM dNTP mix (Fermentus, Canada), 2mM $MgCl_2$ (Fermentus, Canada),0.2 μM forward and reverse primers, 25 U of Tag polymerase and Hypure® water PCR grade adjusted to volume 20-22.5 μl per reaction. Obtained cDNAs were used 2.5-5 μl as templates to amplify, human *Notch1*, *Notch2*, *Notch3*, *Notch4, Hes1*, *MAML1*, *TP53*, HPV16 *E6*, HPV16 *E7*, HPV18 *E6* and HPV18 *E7*. *β-actin* was used as loading control. RT-PCR without reverse transcriptase was used as negative control. The forward and reverse primers used for PCR amplification are as follows: human *Notch1* (5'-CAGCCTGCACAACC AGACAGA-3' and 5'-TGAGTTGATGAGGTCCTCCAG-3'); *Notch2* (5'-TGAGTAGGCTCCATCCA GTC-3' and 5'-TGGTGTCAGGTAGGGATGCT-3'); *Notch3* (5'-TCTTGCTGCTGGT CATTCTC -3' and 5'-TGCCTCATCCTCTTCAGTTG-3'); *Notch4* (5'-CACTGAGCCA AGGCATAGAC-3' and 5'-ATCTCCACCTCACACCACT G -3'); *Hes1* (5'-ACGA CACCGGATAAACCAAA-3' and 5'-CGGAGGTGCTTCACTGTCAT-3'); *MAML1* (5'-CAGCATCAGTTGCTTTTGGA-3' and 5'-CTGCTCTG AGGCATGTTTTG-3'); *TP53* (5'-TCCACTACAACTACATGTGTAAC-3' and 5'-GTGAAATATTCTCCATCCAGTG-3'); HPV16 *E6* (5'-TCAAAAGCCACTGTGTCCTGA-3' and 5'-CGTGTTCTTGATGATC TGCAA-3'); HPV16 *E7* (5'-ATGACAGCTCAGAGGAGGAG-3' and 5'-TCCTAGT GTGCCCATTAACAG-3'); HPV18 *E6* (5'-TGGCGCGCTTTGAGGA-3' and 5'-TGT TCAGTTCCGTGCACAGATC-3'); HPV18 *E7* (5'-TAATCATCAACATTTACCAGCC CG-3' and 5'-CGTCTGCTGAGCTTTCTACTACTA-3'); and *β-actin* (5'-ACCAACTGG GACGACATGGAGAA-3' and 5'-GTGGTGGTGAAGCTGTAGCC-3'). The PCR reactions were carried out using Bioer Life Express® by condition as follows; 94°C 5 min, 94°C for 1 min, 54°C (human HPV18 *E6*) or 55°C (human *Notch1,-2,Hes1*, HPV16 *E7*, HPV18 *E7*,

β-actin) or 56°C (human *Notch3*, *-4*, *TP53*) or 57°C (human HPV16 *E6*) or 59°C (human *MAML1*) for 1 min, 72°C for 1 min and 72°C for 10 min. PCR were amplified for 30 cycles.

The PCR products were analyzed on 2% agarose gel by Mini Gel Electrophoresis Unit for DNA, RNA and Proteins Mupid-2 Advance (Cosmo Bio, Japan). The amplified DNA bands were detected after staining with ethidium bromide using Gel Documentation and Quantity one 4.4.1 (BioRad, USA).

3.8 Semi-Quantitative RT-PCR (qPCR)

Total RNA was extracted from cells treated with GSI or transduced DN-MAML1 as indicated using TriZol reagent (Invitrogen). The qPCR amplification was performed with 1x Maxima™ SYBR Green/ROX qPCR Master Mix, with 0.3 μM forward and reverse primer, RNase Free water and 2 μl obtained cDNAs according to the manufacturer's protocol (Fermentus, Canada). The forward and reverse primers used for qPCR amplification are as follows: human *Notch1* (5'-CAGCCTGCACAACCAGACAGA-3' and 5'-TGAGTTGATG AGGTCCTCCAG-3'); *Hes1* (5'-ACGACACCGGATAAACCAAA-3' and 5'-CGGAGGT GCTTCACTGTCAT-3'); *MAML1* (5'-CAGCATCAGTTGCTTTTGGA-3' and 5'-CTGCTC TGAGGCATGTTTTG-3'); *TP53* (5'-TCCACTACAACTACATGTGTAAC-3' and 5'-GTG A AATATTCTCCATCCAGTG-3'); HPV16 *E6* (5'-TCAAAAGCCACTGTG TCCTGA-3' and 5'-CGTGTTCTTGATGATCTGCAA-3'); HPV16 *E7* (5'-ATGACAGCTCAGAGGAG GAG-3' and 5'-TCCTAGTGTGCCCATTAACAG-3'); HPV18 *E6* (5'-TGGCGCGCTTTG AGGA-3' and 5'-TGTTCAGTTCCGTGCACAGATC-3'); HPV18 *E7* (5'-TAATCATCAAC ATTTACCAGCCCG-3' and 5'-CGTCTGCTGAGCTTTCTACTACTA-3'); and *β-actin* (5'-ACCAACTGGGACGACATGGAGAA-3' and 5'-GTGGTGGT GAAGCTGTAGCC-3'). *β-actin* was used as a reference gene. Reaction without cDNA was used as negative control. The qPCR was carried out using MJ Mini personal Thermal cycler (Biorad, USA) by

condition as follows 95°C for 10 min, 95°C for 15 sec, 54°C (human HPV18 *E6*) or 55°C (human *Notch1,Hes1*, HPV16 *E7*, HPV18 *E7*, *β-actin*) or 56°C (human *TP53*) or 57°C (human HPV16 *E6*) or 59°C (human *MAML1*) for 30 sec and 72°C for 30 sec, follows by repeating for 40 cycles. The relative expression levels were calculated and analyzed by $2^{-\Delta\Delta CP}$.

$$\Delta CP_x = CP_x - CP_{\beta\text{-actin average}} \quad (x = \text{sample } 1, 2, 3, \ldots..)$$

$$\Delta\Delta CP = \Delta CP_x - \Delta CP_{\text{control average}}$$

$$\text{Relative expression level} = 2^{-\Delta\Delta CP}$$

The PCR products were randomly analyzed on 2% agarose gel by electrophoresis and visualized after staining with ethidium bromide using the Gel Documentation System.

3.9 Western blot

3.9.1 Protein extraction

Cell lysates from cell lines treated with GSI or transduced DN-MAML1 were prepared as described previously (Suwanjunee *et al.*, 2008). Briefly, cells were washed once each in PBS and added an appropriate volume of RIPA buffer (Appendix) and protease inhibitor. Cells were incubated on ice or in a refrigerator (2–8 °C) for 5 min. Cells were rapidly scraped the plate with a cell scraper to removed and lysed residual cells. The cell lysate were transferred to a tube on ice and clarified the lysate by centrifugation at 8,000 x *g* for 10 min at 4 °C to pellet the cell debris. Carefully transfer the supernatant containing the soluble protein to a tube on ice and stored at –70 °C for future analysis.

3.9.2 Protein assay

Protein concentrations were measured using BCA (bicinchoninic acid) ™ protein assay (PIERCE, USA), according to manufacturer's instruction. The working reagent composed of reagent A and reagent B mixed at ratio 50: 1 was prepared and BSA (1 mg/ml) was used as protein standard. BSA was diluted in sterile deionized water at 0, 31.25, 62.5,

125, 250, 500 and 1,000 µg/ml in 96-well microtiter plate (Corning Incorporation, USA). The samples were diluted at 1:10 (1 µl of sample in 9 µl of sterile deionized water) and 200 µl of working reagents was added to each well. The plates were incubated for 30 min at 37°C. After incubation, absorbance at 540 nm (A_{540} nm) was measured using microplate reader Elx 800 (Bio-Tek instrument, Canada). Fifteen micrograms of proteins were equally applied for separation by SDS-PAGE in all experiments.

3.9.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide gels were prepared following the formulas shown in Appendix. Fifteen micrograms of proteins sample and 2×Laemmli buffer (Appendix) were mixed in an equal volume in 1.5 ml microcentrifuge tube. The samples were heated at 100°C for 5 min on Thermomixer Compact (Eppendorf, Germany). Heated samples and prestained molecular weight markers (Fermentas, Canada) were loaded to the gels. The samples were separated at 100 volt for at least 120 min in Western blot running buffer (Appendix) using Protein III system (BioRad, USA).

3.9.4 Protein transfer

After separation process, the stacking gel was removed and size of the separating gel was measured. The gels were equilibrated in transfer buffer (Appendix) for 5 min. Six pieces of Whatman filter paper and a PVDF membrane (GE Healthcare, USA) were prepared. PVDF membranes were next soaked in absolute methanol (Merck, Germany) and rinsed with deionized water twice and immersed in transfer buffer. Gels, PVDF membrane and filter papers were placed in a semi-dry transfer Trans-Blot® SD (BioRad, USA) apparatus and air bubbles were eliminated by gently rolling a glass test tube on the top of stacks of filter paper. The semi-dry transfer was carried out under the following condition; current at 80 or 130 mA for 90 min for one gel or two gels, respectively.

3.9.5 Antibody probing

The PVDF membranes after protein transfer were blocked in blocking solution (Appendix) twice for 5 min each on Labnet Rocker 25 (Labnet International Inc, USA). After blocking, the PVDF membranes were probed with 1:2,000 dilution of rabbit anti Notch1 (Santa Cruz Biotechnology, USA), 1:1,000 dilution of rabbit anti cleaved Notch1 (Cell Signaling Technology, USA) and 1:10,000 dilution of mouse anti β-actin (Chemicon International, USA) which are primary antibodies at 4°C refrigerator overnight. The probed membranes were further incubated on a rocker for 1 hr at room temperature. The primary antibody solution was discarded, and the membrane was washed with PBST (Appendix) for 5 min 2 times and 15 min 2 times. After washing, PBST was discarded, and 1:4,000 dilution of donkey anti rabbit and 1:5,000 dilution of sheep anti mouse which are secondary antibody against mouse or rabbit immunoglobulins conjugated with horseradish peroxidase (HRP) (Amersham Biosciences, England) were added. The PVDF membranes were incubated for 1 hr with rocking before washing with PBST as follow described above.

3.9.6 Signal detection by chemiluminescence and autoradiography

The substrates were prepared using the formula shown in Appendix. Briefly, solution A was mixed with solution B. PBST was removed from the container carrying the PVDF membranes. The mixture of solution A and B was poured directly on the membranes and incubated for 1 min. The membranes were wrapped in the plastic wrap and placed in Hypercassette (Amersham Biosciences, England) to expose to High Performance Chemilumunescence Film: Amersham Hyperfilm™ ECL (Amersham Biosciences, England) in the dark. Exposure time for Notch1 or cleaved Notch1 and β-actin (as loading control) was 5 min and 10 sec, respectively. Exposed film was developed for 5 sec in X-ray film developer, washed with tap water, fixed for 3 min in the fixer and finally washed with tap water.

3.10 MTT assay

Cells (1×10^5 cell/ml in 100 µl culture) were treated with GSI or transduced DN-MAML1 and control vehicle for the time indicated in 96-well tissue culture plate. At the end of treatment, 10 µl of MTT (5 mg/ml) (USB, USA) (Appendix) in PBS was added and incubated in 37°C and 5% CO_2 incubator for 4 hr. To dissolve the formazan crystal generated by the reduction reaction, 100 µl of isopropanol (0.04 N HCl) (Appendix) were added to each well and mixed by pipetting. Absorbance was measured using a microtiter plate reader at 540 nm (A_{540} nm). Percentage of viable cells was calculated using the following formula:

$$\% \text{ viability} = \frac{(\text{OD test average} - \text{OD blank average}) \times 100}{\text{OD control cell average} - \text{OD blank average}}$$

3.11 Cell cycle analysis

Cells were treated with GSI or transduced DN-MAML1 and control vehicle for the time indicated in 12-well tissue culture treated plate were prepared in a 1.5 ml microcentrifuge tube. Briefly, cells were fixed in 200 µl of 70% cold ethanol at 4°C for 4 hr. After incubation, 500 µl of PBS was added into cells and centrifuged at 10,000 rpm for 5 min at 4°C and PBS was removed. One hundred µl of PBS was added and cells were treated with 1 µl of RNaseA (10 mg/ml) at 37°C for 30 min. After washing, cells were stained with 50 µl of propidium iodide (1 mg/ml) (Sigma Aldrich, USA) (Appendix) at 37°C for 30 min in the dark and subjected to FACS analysis (BD Biosciences, USA). Acquired data was analyzed by Summit 5.0 software program.

3.12 Detection of GFP$^+$ cells upon retroviral transduction

Cells were transduced DN-MAML1 and control vehicle for the time indicated in 12-well tissue culture treated plate were prepared in a 1.5 ml microcentrifuge tube. After removing completed media, cell was treated with 0.25% (w/v) Trypsin - 0.53 mM EDTA (Hyclone, England) and centrifuged at 10,000 rpm for 5 min at 4°C. After washing with PBS, cells were fixed in 500 µl of 4% Paraformaldehyde (Sigma Aldrich, USA) (Appendix) and subjected to FACS analysis (BD Biosciences, USA).

3.13 Clonogenic proliferation assay

Cell lines were treated with GSI or transduced DN-MAML1 and control vehicle. Briefly, cells were washed once each in PBS, trypsinized, and plated at cell densities of 300 or 400 cells/well (three wells each) in 12-well plates. Cells were then allowed to form colonies by incubation in drug-free medium for 10 days. The resulting colonies were stained with 2% crystal violet in methanol (Appendix) and incubated at room temperature for 10 min. After washing with PBS, wells consisting of more than 50 colonies per well were counted.

3.14 Statistical analysis

Mean ± SD of independent experiments will be analysed. All data will be analysed using an independent *t*-test of SPSS software. A P value of < 0.05 will be considered statistically significance.