CHAPTER IV

RESULTS

4.1 Expression of *Notch receptors*, *MAML1*, *Hes1*, Notch1 and cleaved Notch1 in HPV-positive cervical cancer cell lines

To examine mRNA expression of *Notch receptors*, *MAML1* and *Hes1* in HPV-positive cervical cancer cell lines, RT-PCR was performed using specific primers for *Notch1-4*, *MAML1* and *Hes1* genes. In all cell lines tested, *hNotch1*, *hNotch2*, *hMAML1* and *hHes1* were clearly detectable, whereas *hNotch4* was undetectable in all samples. Expression of *hNotch3* was found in CaSki and SiHa, but not in HeLa. All cervical cancer cell lines expressed at least one *Notch receptor*, *MAML1* and *Hes1*, suggesting that Notch signaling is active in these cervical cancer cell lines (Figure 4.1).

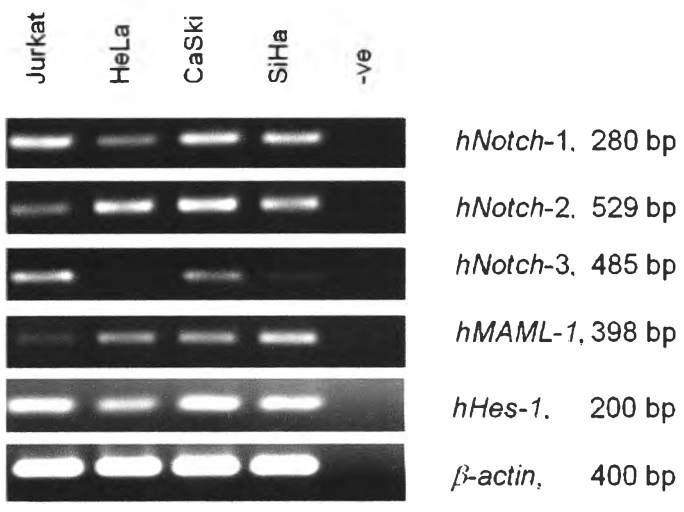


Figure 4.1 Expressions of *hNotch receptors*, *hMAML1* and *hHes1* in HPV-positive cervical cancer cell lines, HeLa, CaSki and SiHa were analyzed by RT-PCR. *β-actin* was used as loading control. RNA from human T cell leukemia Jurkat was used as a positive control.

To determine whether HPV-positive cervical cancer cell lines express proteins of Notch1 and active form of Notch1 (cleaved Notch1), Western blots using antibodies directed against the intracellular domain of Notch1 and cleaved form of Notch1 (Val1744) were performed. In all cells tested in this study, Notch1 was clearly detected, but at different levels, whereas only CaSki expressed detectable cleaved Notch1 (Figure 4.2A and B).

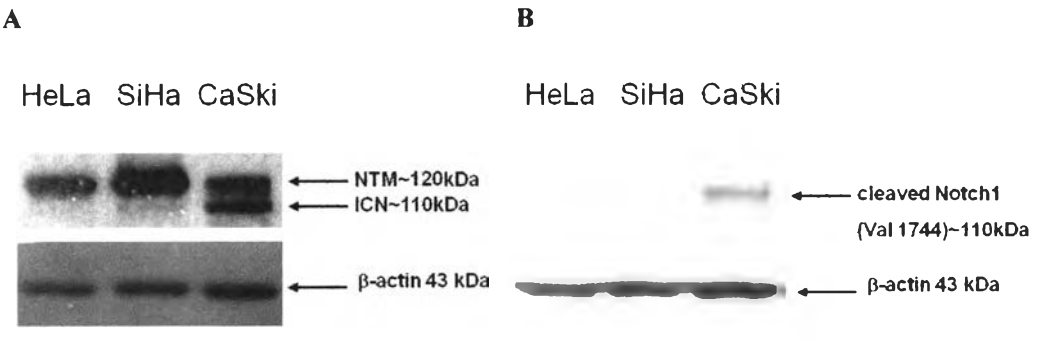


Figure 4.2 Expressions of Notch1 and cleaved Notch1 in HeLa, SiHa and CaSki. All cervical cancer cell lines were analyzed for Notch1 and cleaved Notch1 expression by Western blot. Antibodies specific for intracellular Notch1 domain (A) and cleaved Notch1 (Val1744) (B) were used. β-actin was used as loading control. ICN, intracellular Notch; NTM, intracellular Notch with transmembrane domain.

4.2 Effects of DAPT treatment on Notch1 and cleaved Notch1 expression and viability of HPV-positive cervical cancer cell lines

To examine the effects of suppression of Notch signaling on these cell lines, the effect of DAPT treatment on Notch1 and cleaved Notch1 expression was first investigated by Western blot. The inhibition of Notch signaling by DAPT treatment in CaSki resulted in higher level of Notch1 (Figure 4.3A). This result may be due to the accumulation of Notch1 protein on the cell surface as it is not processed by γ-secretase when treated with DAPT, and

is consistent with previous studies (Suwanjunee *et al.*, 2008). On the other hand, this phenomenon was not observed in other two cell lines. When cleaved Notch1 was examined, DAPT treatment completely abrogated the presence of this form of Notch1 in CaSki, suggesting that DAPT treatment is effective in inhibiting γ-secretase activity and processing of Notch1 (Figure. 4.3B).

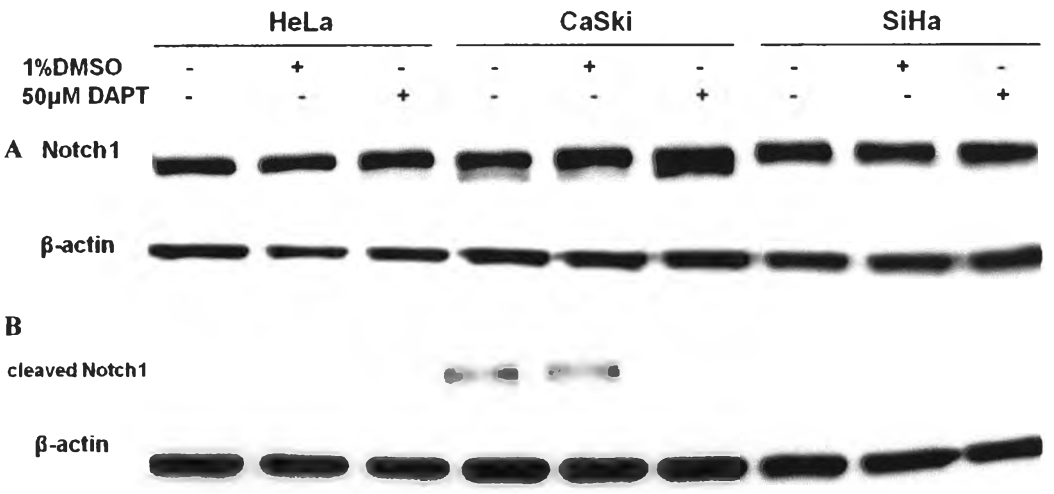
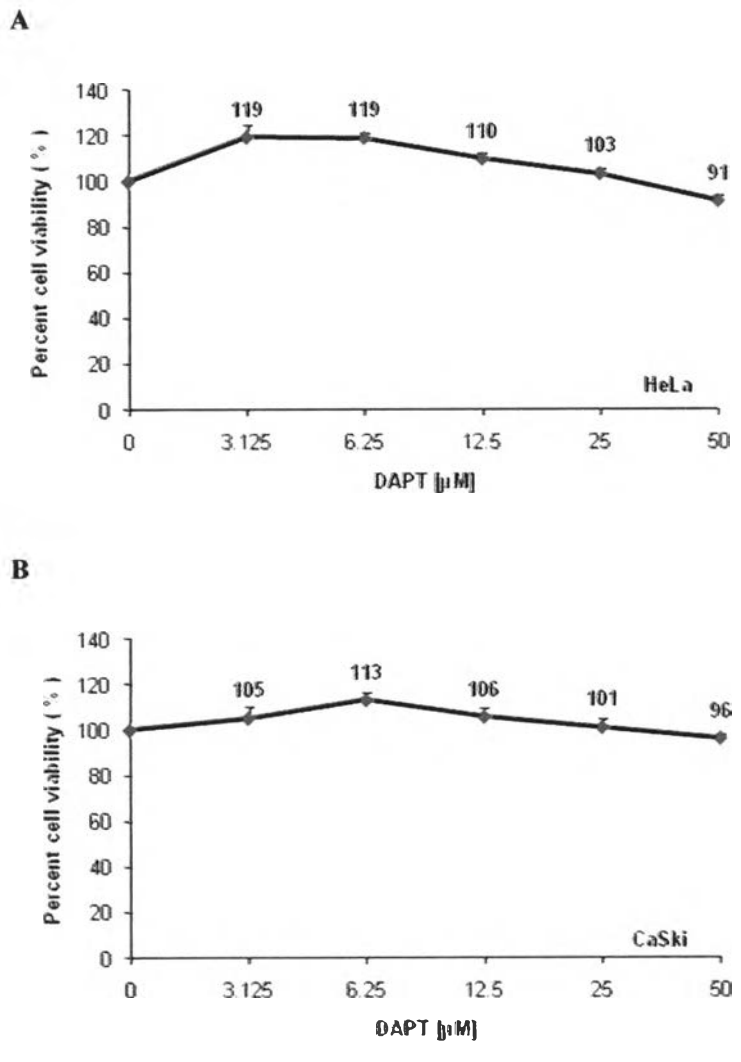


Figure 4.3 Expressions of Notch1 and cleaved Notch1 in HeLa, CaSki and SiHa treated with DAPT, DMSO or left untreated for 4 days. Expression of Notch1 (A) and cleaved Notch1 (B) were analyzed by Western blot. Antibodies specific for intracellular Notch1 domain and cleaved Notch1 (Val1744) were used. β-actin was used as loading control.

Next, we treated these cell lines with DAPT at various concentrations for 4 days and measured cell viability by MTT assay. As shown in Figure 4.4A-C, DAPT treatment did not affect cell survival in any cell line tested. Therefore, our data confirmed that Notch1 is commonly expressed in cervical cancer cells, but cell survival was not compromised by DAPT treatment. It is possible that Notch signaling alone is not directly involved in

regulating cell viability in these cell lines. Treatment with 50 μM of DAPT was chosen for further experiments.



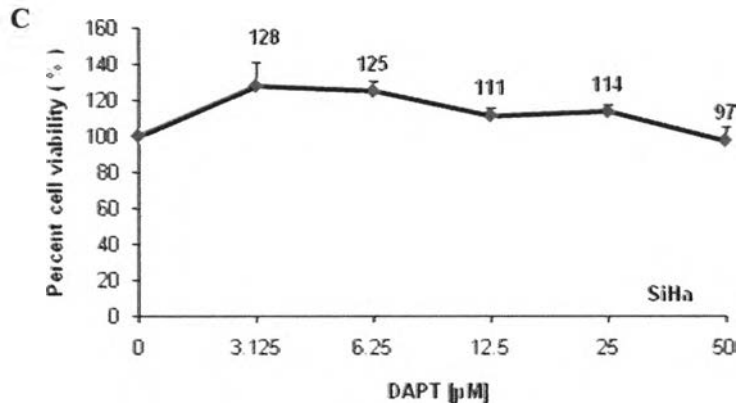


Figure 4.4 The effect of DAPT treatment on viability of HPV-positive cervical cancer cell lines. HeLa (A), CaSki (B) and SiHa (C) were treated with various concentrations of DAPT, as indicated, for 4 days. Cell viability was assayed by MTT method. The results represent two independent experiments in triplicate and are shown in triplicate±SD.

4.3 Effects of DAPT treatment on expression of *Notch1*, *Hes1*, *MAML1*, *E6*, *E7* and *TP53*

As DAPT treatment did not affect cell survival, we observed cellular morphology of cells under inverted microscope (Figure 4.5). Upon DAPT treatment, CaSki showed vacuolated features in cytoplasm (Figure 4.5F) but other two cell lines did not show any changes. To examine the effect of DAPT treatment on gene expression in these cell lines, quantitative real-time PCR analysis was performed. There was no difference in expression level of *Hes1*, *Notch1*, *MAML1*, *E6*, *E7* and *TP53* in HeLa between treated and untreated cells (Figure 4.6A). In CaSki, however, DAPT treatment led to significant decrease in *Notch1* expression, whereas expression of *Hes1*, *MAML1* and *TP53* were increased. Since *Hes1* is one of the target genes of Notch signaling pathway, failure to reduce *Hes1* expression by DAPT treatment in CaSki implies that other Notch receptors or other undefined Notch unrelated pathways may control *Hes1* expression in this cell line (Figure 4.6B). In DAPT-

treated SiHa, expression of *MAML1* was significantly decreased (Figure 4.6C). Because there was no change in gene expressions in HeLa, further analysis of HeLa was not carried out. In addition, since the types of HPV in CaSki and SiHa were reported to be of the same type, they were used for further investigation in this study.

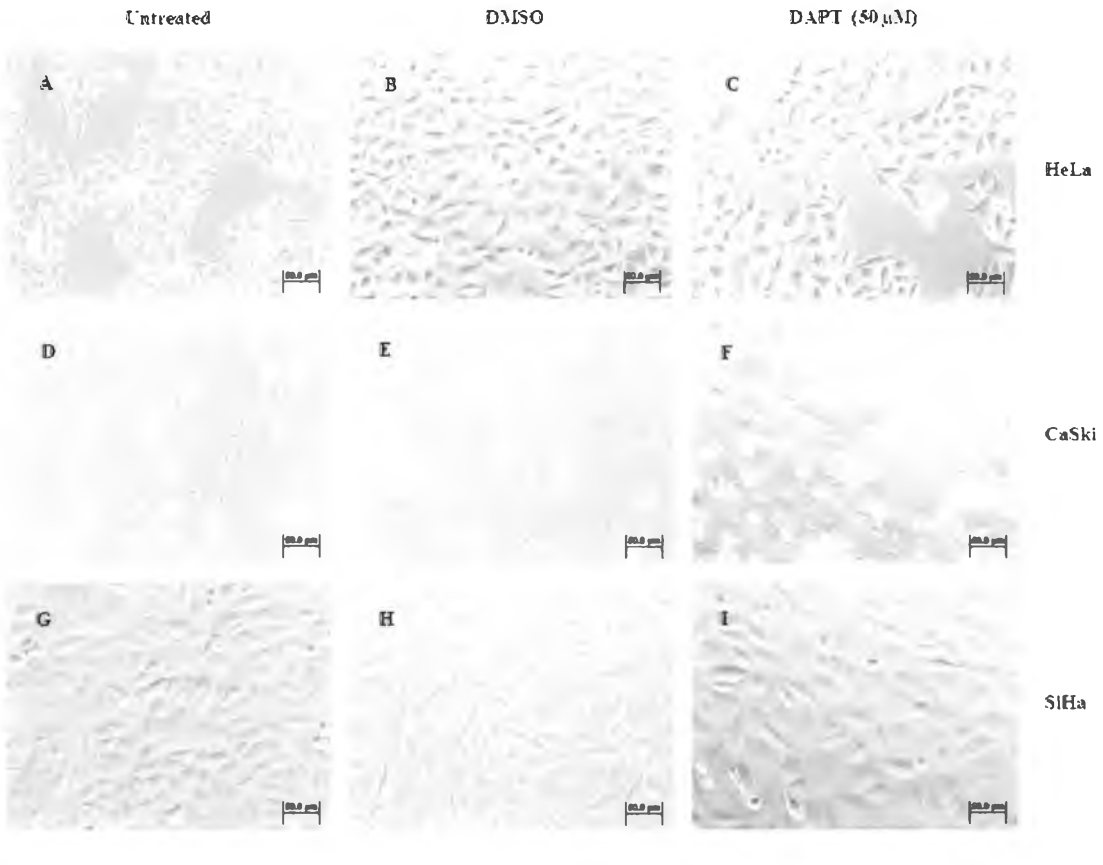
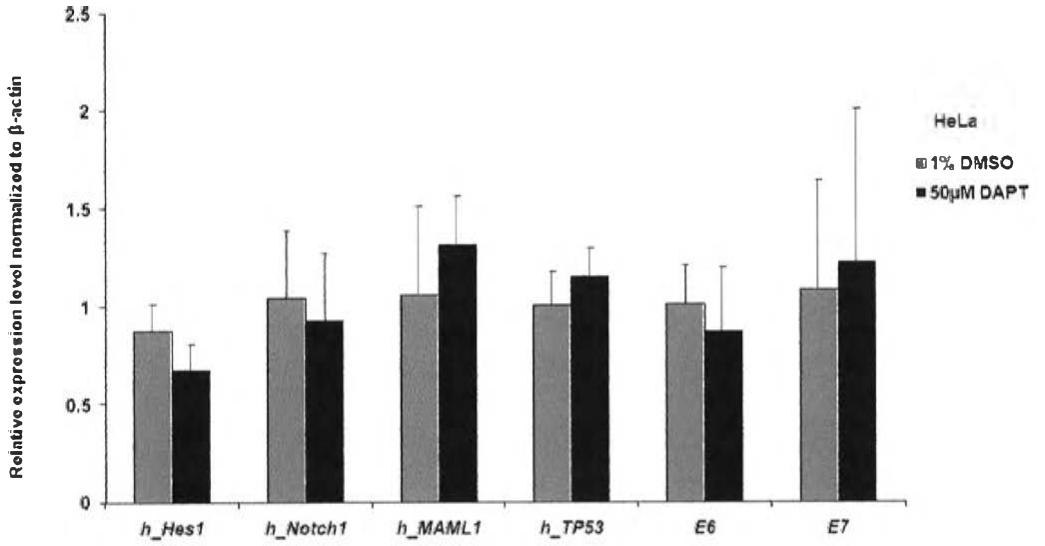
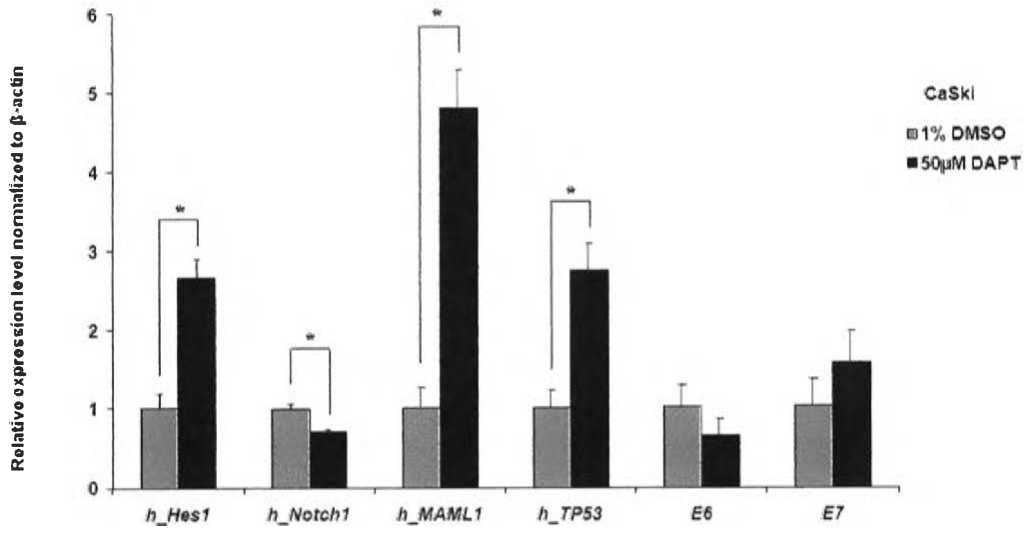


Figure 4.5 Cellular morphology of HPV-positive cervical cancer cell lines treated with DAPT, DMSO or left untreated for 4 days. A, D, and G showed untreated HeLa, untreated CaSki and untreated SiHa, respectively. Panels B, E and H showed HeLa, CaSki and SiHa upon DMSO treatment. Panels C, F and I showed HeLa, CaSki and SiHa upon DAPT treatment.

A


Relative expression level normalized to β-actin
2.5
2
1.5
1
0.5
0
h_Hes1
h_Notch1
h_MAML1
h_TP53
E6
E7
HeLa
1% DMSO
50μM DAPT

B


Relative expression level normalized to β-actin
6
5
4
3
2
1
0
*
*
*
*
h_Hes1
h_Notch1
h_MAML1
h_TP53
E6
E7
CaSki
1% DMSO
50μM DAPT

C

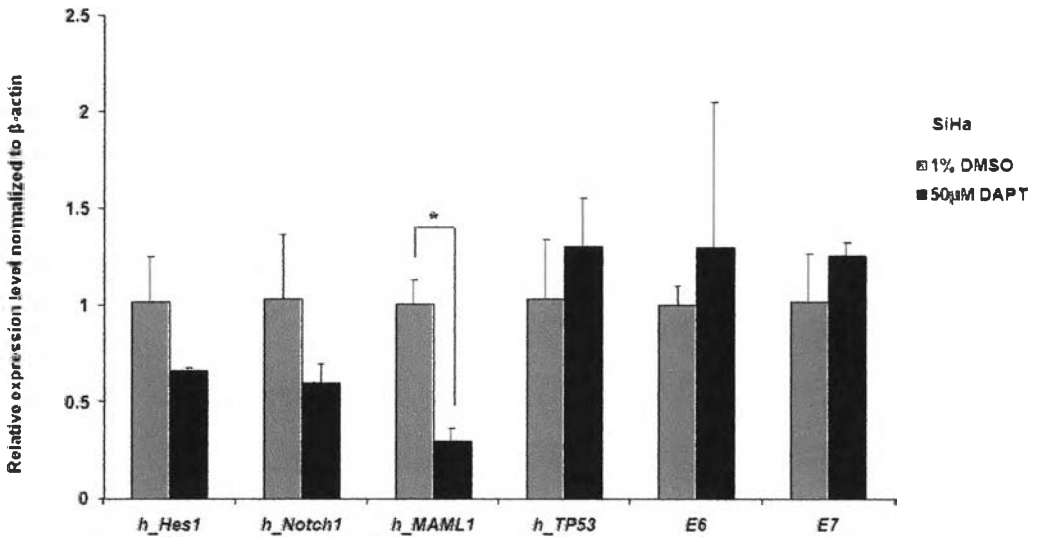


Figure 4.6 Expressions of *hHes1*, *hNotch1*, *hMAML1*, *hTP53*, HPV type 18 *E6* and *E7*, and HPV type 16 *E6* and *E7* upon treatment with DAPT. HeLa (A), CaSki (B) and SiHa (C) cells were treated with DAPT and DMSO for 4 days. mRNA expression was examined by Real-time PCR analysis. The results represent two independent experiments in triplicate and are shown in triplicate ± SD. *β-actin* was used as loading control. * $p < 0.05$ indicates statistical significance.

4.4 Effects of DAPT treatment on cell cycle and cell proliferation

To determine the effects of DAPT treatment on the cell cycle progression, cell cycle analysis was performed. DAPT treatment in CaSki and SiHa for 4 days did not affect distribution of cells in each phase of cell cycle, compared to the vehicle control treatment (Figure 4.7). Cells in G2/M phase was slightly decreased upon DAPT treatment but did not reach statistical significance. An additional investigation on the role of Notch signaling in promoting proliferation of HPV-positive cervical cancer cells were examined by clonogenic assay. When DAPT treatment was carried out in CaSki, the number of colonies significantly

increased to 120% as compared to DMSO treatment, whereas no difference was seen in SiHa (Figure 4.8C).

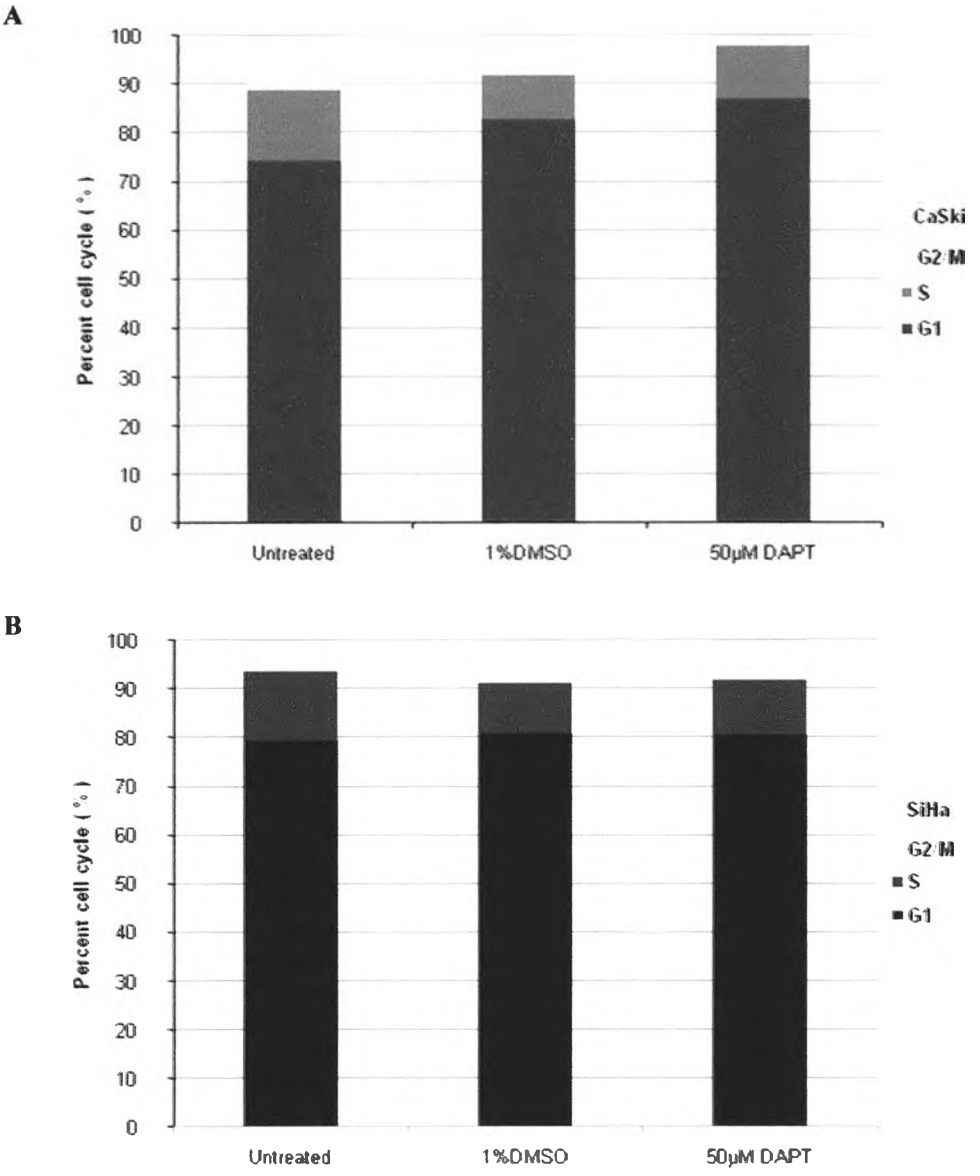
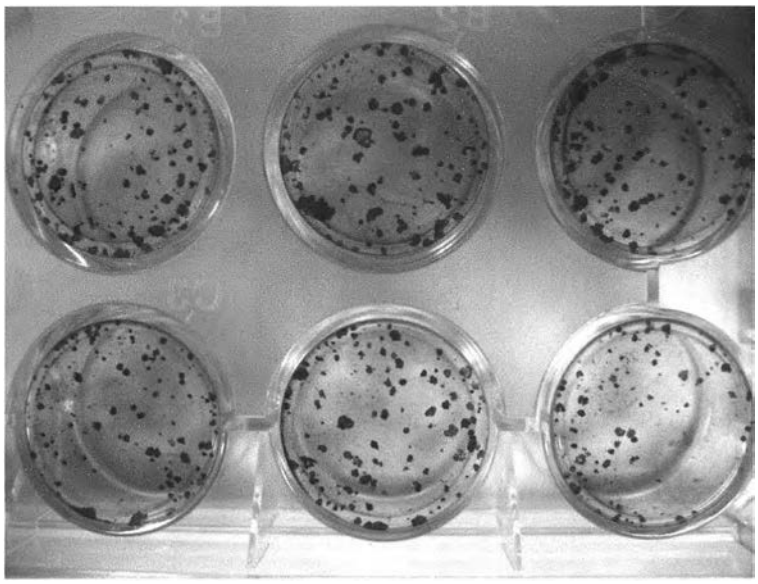


Figure 4.7 Cell cycle analysis in DAPT-treated CaSki and SiHa. CaSki (A) and SiHa (B) cells were treated with DAPT (50 μM), DMSO, or left untreated for 4 days. Cell cycle analysis was performed as described in materials and methods using FACS. Data was analyzed by Summit 5.0 software program. The results represent two independent experiments.

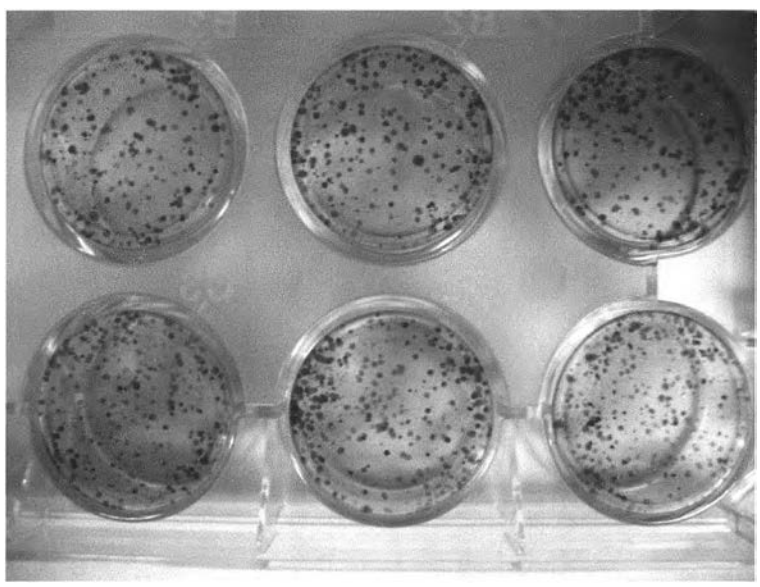
A **CaSki**



DMSO

DAPT

B **SiHa**



DMSO

DAPT

C

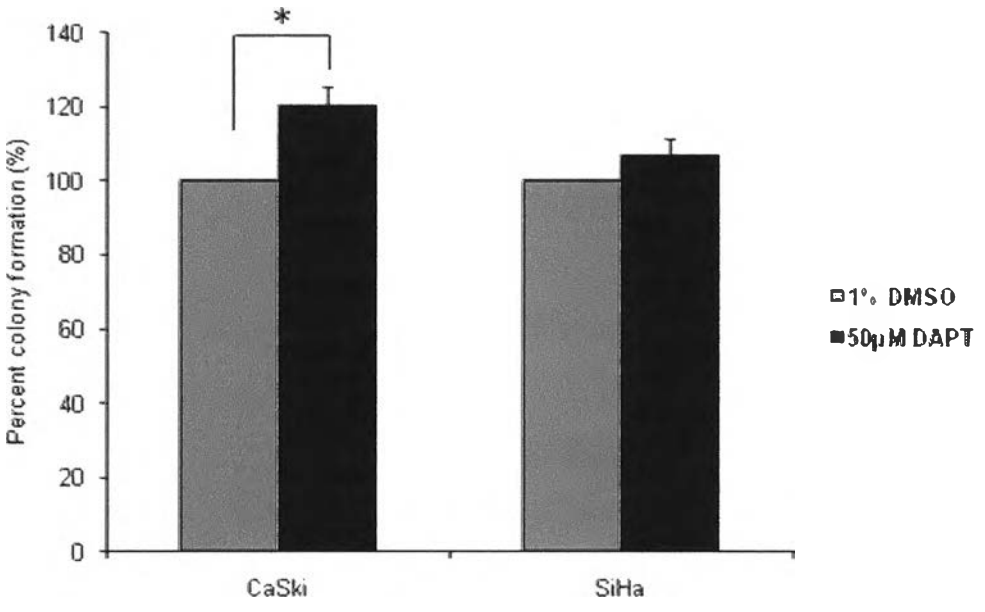


Figure 4.8 Clonogenic assay of HPV-positive cervical cancer cells in DAPT-treated. CaSki (A) and SiHa (B) cells were treated with DMSO (top panel) and DAPT (bottom panel) for 4 days. The proliferation was examined as described in materials and methods by clonogenic assay (C). The results represent two independent experiments in triplicate and are shown in triplicate ± SD. * $p < 0.05$ indicates statistical significance.

Taken together, inhibition of Notch signaling by DAPT treatment resulted in accumulation of Notch1, abrogation of cleaved Notch1 and growth promotion in CaSki. These studies provided evidences that Notch signaling is constitutively active in this cell line but by itself may not sufficient in regulating cell survival in CaSki. In addition, Notch signaling may partially repress cell proliferation.

4.5 Retroviral transduction of CaSki with DN-MAML1 or control plasmid vector

MAMLs are co-activators playing an essential role in regulating Notch-mediated transcriptions by functioning as scaffold protein for transcriptional activation complex. DN-MAML1, a truncated form of MAML1, harboring an N-terminal ICN/CSL interaction domain but lacking a C-terminal TAD, was used as a pan-Notch inhibitor at the stage of transcriptional activation of target genes without affecting γ-secretease activity. CaSki was retrovirally transduced with retroviral vector harboring DN-MAML1 (MSCV-Mam (12-74)-EGFP) or control vector (MSCV-IRSE-GFP) to inhibit Notch signaling pathway. Because these plasmids contained GFP as a marker for transduction, transduction efficiency was monitored by GFP expression. The percentage of GFP^+ in transduced-CaSki was quantitated by FACS. As shown in Figure 4.9, more than 95% of GFP^+ cells were found in cells transduced with either plasmid (96%, 98% respectively). Therefore, these cells were used for further experiments without sorting.

4.6 Effects of DN-MAML1 on expression of *Notch1*, *Hes-1*, *MAML1*, *E6*, *E7* and *TP53* in CaSki

To determine the effects of DN-MAML1 expression on CaSki, mRNA expressions of Notch1 and Notch target gene were examined by quantitative real-time PCR after 48 hr of viral transduction. Expression of DN-MAML1 resulted in significantly decreased *Hes1* and *Notch1* expressions, as compared to control MSCV-IRES-GFP (Figure 4.10). As expected, inhibition of Notch signaling by DN-MAML1 led to decrease in *Hes1* expression, suggesting that Notch signaling was effectively suppressed by overexpression of truncated MAML1.

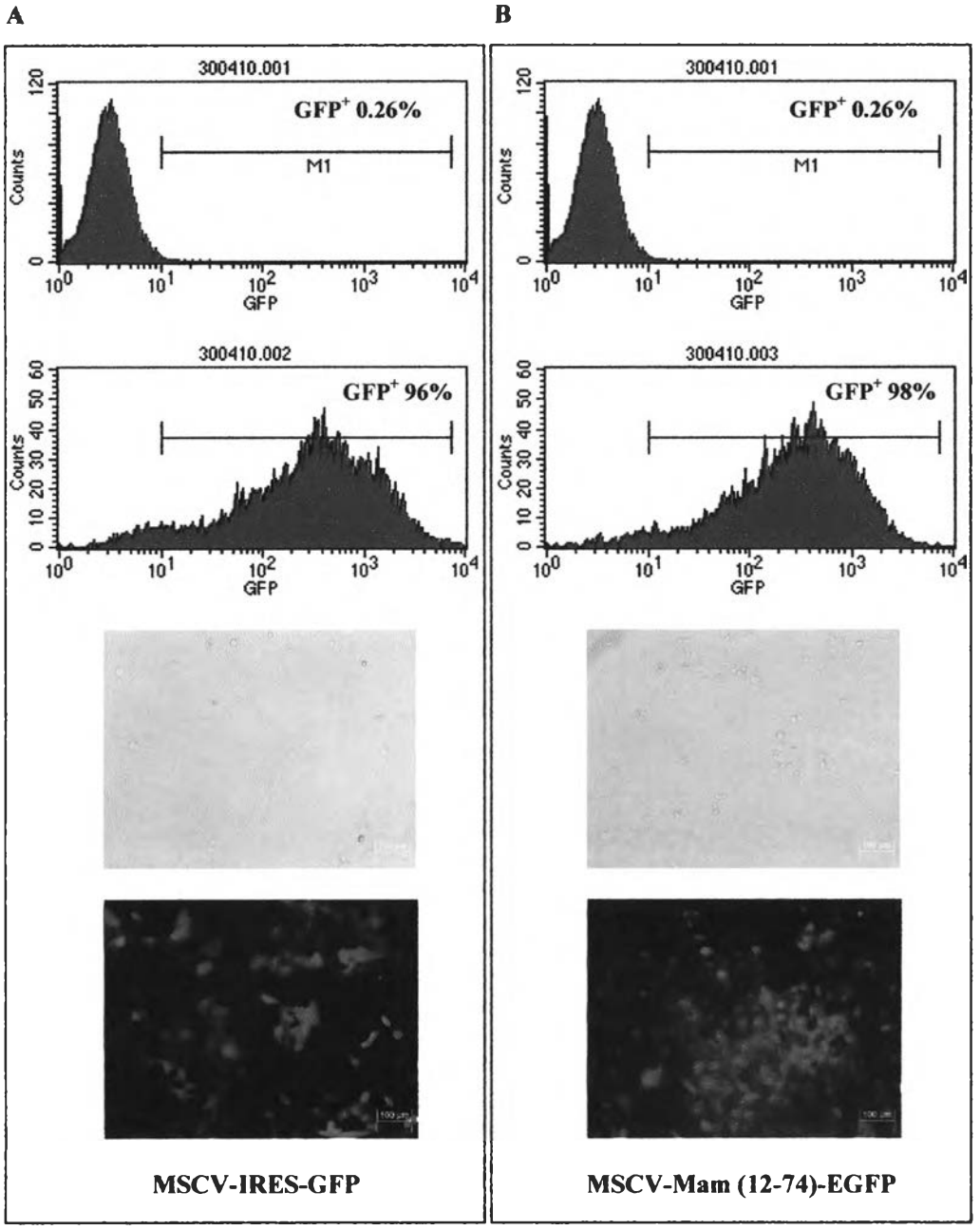


Figure 4.9 Expression of GFP or EGFP in CaSki transduced with MSCV-Mam (12-74)-EGFP (DN-MAML1) or control plasmid vector (MSCV-IRES-GFP). CaSki was transduced with control MSCV-IRES-GFP (A) or MSCV-Mam (12-74)-EGFP (B) and GFP^+ cells were analyzed by FACS on day 2 post transduction. The results represent two independent experiments.

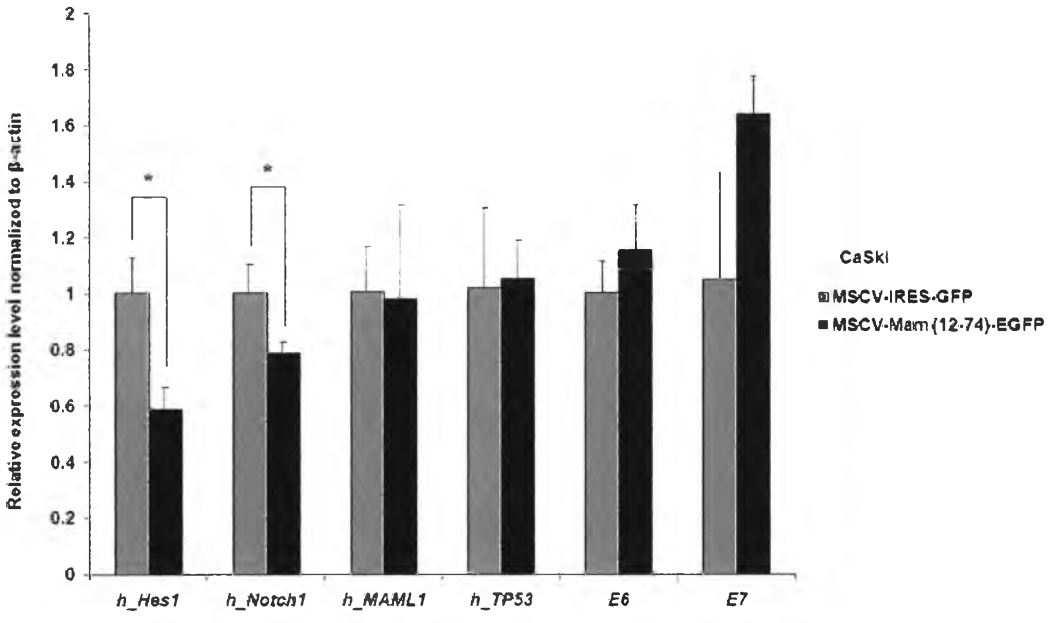


Figure 4.10 Expressions of *hHes1*, *hNotch1*, *hMAML1*, *hTP53*, HPV type 16 *E6* and HPV type 16 *E7* in CaSki transduced with control MSCV-IRES-GFP or MSCV-Mam (12-74)-EGFP. mRNA expression in these cells were analyzed on day 2 post transduction by real-time PCR analysis. The results represent two independent experiments in triplicate and are shown in triplicate ± SD. *β-actin* was used as loading control. * $p < 0.05$ indicates statistical significance.

4.7 Effects of DN-MAML1 expression on cell viability in CaSki

The effects of DN-MAML1 expression on viability of CaSki by MTT assay was performed at day 3 after transduction. Inhibition of Notch signaling by DN-MAML1 led to statistic increase in the percentage of viable cells in CaSki at 114% (Figure 4.11). Therefore, suppression of Notch signaling by DN-MAML1 resulted in more viable cells in CaSki.

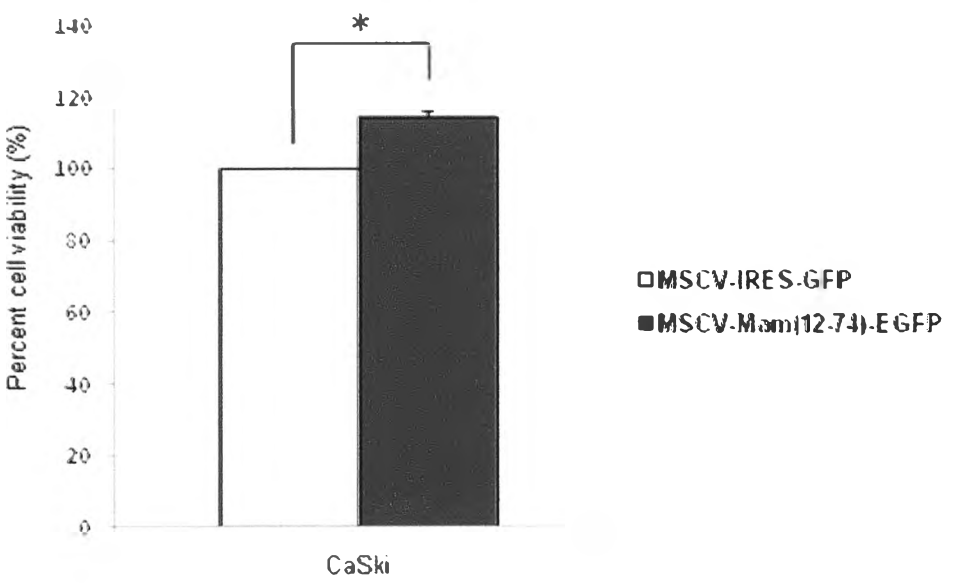


Figure 4.11 Effect of DN-MAML1 on viability of CaSki. CaSki was transduced with control MSCV-IRES-GFP or MSCV-Mam (12-74)-EGFP. Cell viability was assayed on day 3 post transduction by MTT assay as described previously. The results represent two independent experiments in triplicate and are shown in triplicate±SD. * $p < 0.05$ indicates statistical significance.

4.8 Effects of DN-MAML1 expression on cell cycle and cell proliferation of CaSki

To investigate the effects of DN-MAML1 expression on cell cycle progression, cell cycle analysis on transduced-CaSki was carried out. As shown in Figure 4.12, there was no difference in distribution of cells in each cell cycle phase at day 2 after transduction between control vector and MSCV-Mam (12-74)-EGFP. These results are consistent with phenotypes obtained in DAPT treatment in the previous experiment.

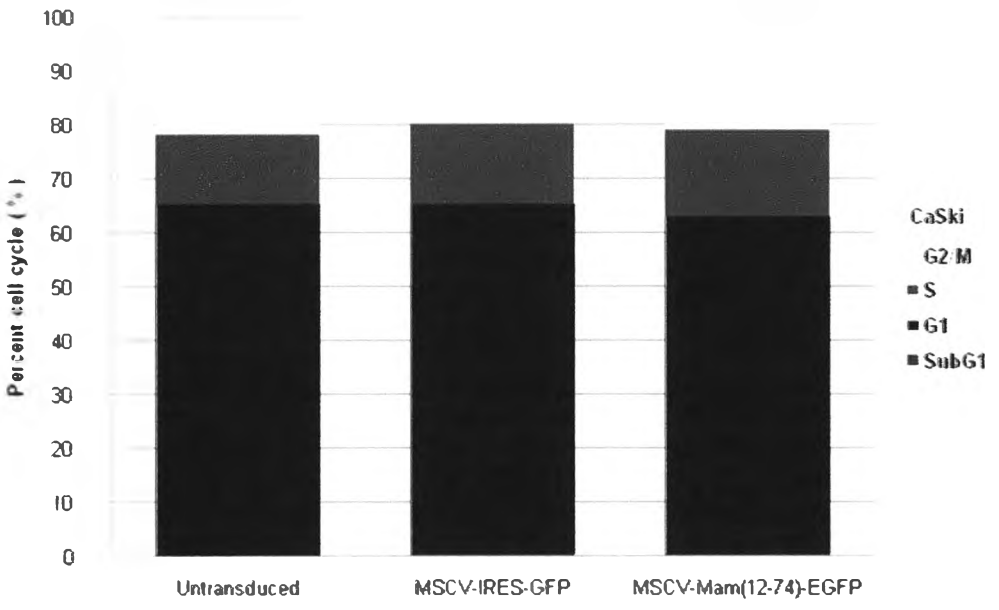
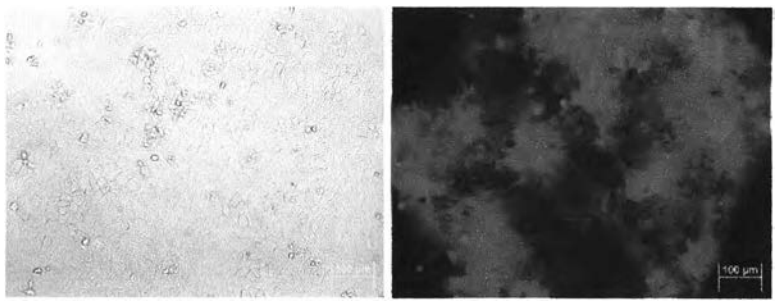


Figure 4.12 Effect of DN-MAML1 on cell cycle in CaSki. CaSki was transduced with control MSCV-IRES-GFP or MSCV-Mam (12-74)-EGFP. Transduced cells were subjected to cell cycle analysis on day 2 post transduction as described in materials and methods using FACS. Acquired data was analyzed by Summit 5.0 software program. The results represent two independent experiments.

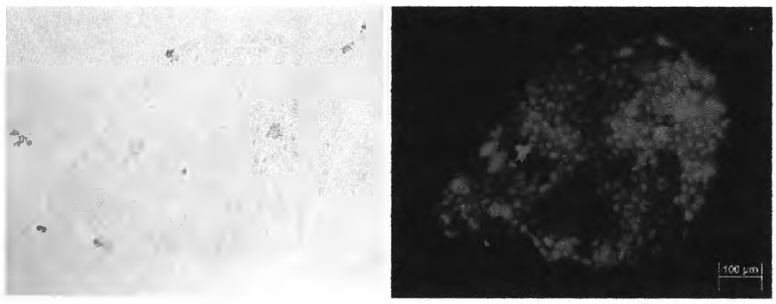
To determine the effects of DN-MAML1 expression on cell proliferation, clonogenic assay was carried out. The inhibition of Notch signaling by DN-MAML1 led to promotion of proliferation in CaSki, as numbers of colonies were significant increased to 148% (Figure 4.13D). This result is consistent with DAPT treatment in previous experiment. Interestingly, transduced MSCV-Mam (12-74)-EGFP-CaSki cells formed smaller colonies, compared to MSCV-IRES-GFP control cells (Figure 4.13A-C). These findings suggested that Notch signaling plays a negative regulatory role in controlling cell proliferation in CaSki.

A



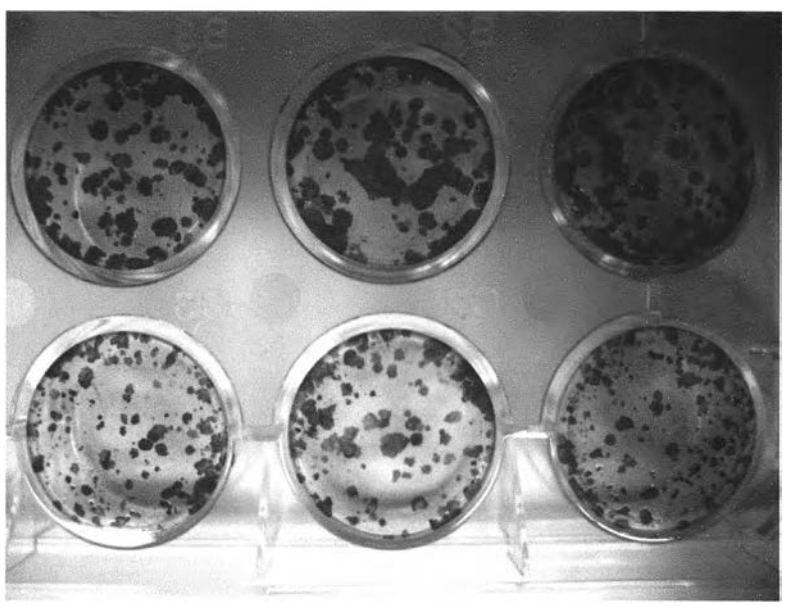
MSCV-IRES-GFP

B



MSCV-Mam (12-74)-GFP

C



MSCV-IRES-GFP

MSCV-Mam (12-74)-GFP

D

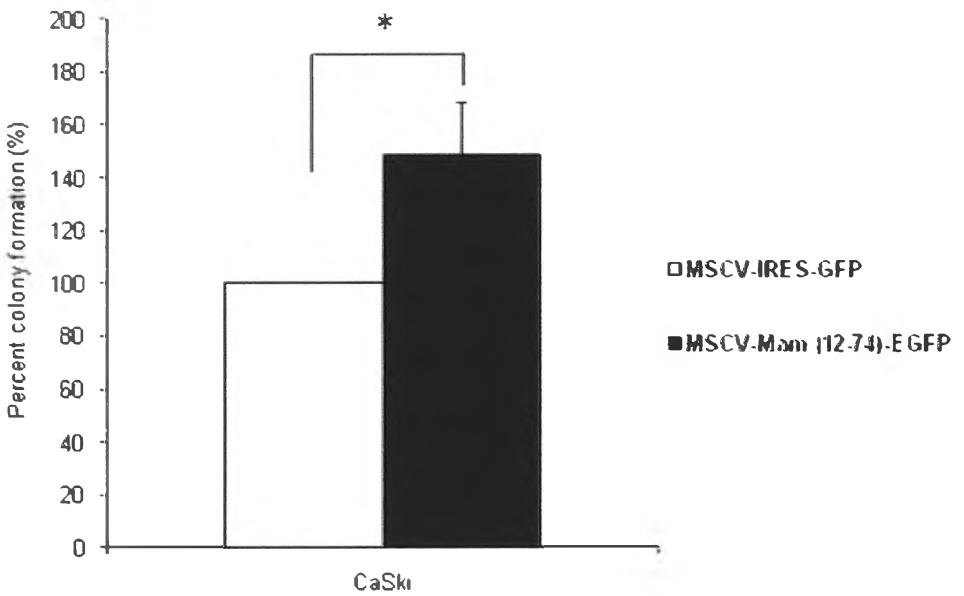


Figure 4.13 Clonogenic assay of CaSki transduced with DN-MAML1 vector or control vector. CaSki was transduced with control MSCV-IRES-GFP (A) or MSCV-Mam (12-74)-EGFP (B) and observation of GFP^+ cells were performed on day 12 post transduction by fluorescent microscope. The proliferation was examined by clonogenic assay as described in materials and methods (C) and the obtained data was analyzed and shown in D. The results represent two independent experiments in triplicate and are shown in triplicate±SD. * $p < 0.05$ indicates statistical significance.