CHAPTER V

DISCUSSION

Aberrant Notch signaling is linked to multiple developmental disorders and cancers. Although the links between Notch signaling and HPV-positive cervical cancer are well established, the exact roles of Notch signaling remain controversial. In 1995, overexpression of Notch1 was detected in invasive cervical adenocarcinomas (Zagouras *et al.*, 1995). Deregulated Notch1 activation caused cell death in CaSki, suggesting that Notch1 is necessary for maintenance of the transformed phenotype in HPV-positive cervical cancer cells (Weijzen *et al.*, 2002). Two key viral oncogenes, E6 and E7, are persistently expressed in HPV-associated cervical cancer. Chakrabarti *et al.* found that E6 amino acid 83 variants enhance MAPK signaling through a Ras-dependent manner. These variants inhibit oncogenic Ras-mediated transformation that are potentially permissive for cervical tumor progression (Chakrabarti *et al.*, 2004). In addition, Veeraraghavalu *et al.* compared the expression patterns and the role of two Notch ligands, Jagged1 and Delta1 in the progression of human cervical cancer and relationship with E6/E7. They found that Jagged1 is preferentially upregulated in human cervical tumors, and this upregulation sustains transformation by HPV 16 E6-E7 oncogenes and confers resistance to anoikis (Veeraraghavalu *et al.*, 2005). In contrast, Talora *et al.* showed that high levels of Notch1 exerted specific protective effects against HPV-induced transformation through suppression of *E6/E7* expression, suggesting that overexpression of Notch1 may play an important role in late stages of HPV-induced carcinogenesis (Talora *et al.*, 2002). Subsequently, they showed that activating Notch signaling resulted in cell cycle arrest in cervical cancer cell lines (Talora *et al.*, 2005). Consistent with this finding, activated Notch1 resulted in growth inhibition of HeLa by inducing G2-M cell cycle arrest and apoptosis and the growth inhibition was correlated with inhibition of nuclear factor kappa B (NF-κB) p50 activation (Yao *et al.*, 2007). Furthermore,

Song *et al.* found that Notch1 regulated NF-κB by associating with the IKK signalosome through IKKα in CaSki (Song *et al.*, 2008). Taken together, both gain of function and loss of function of Notch1 have been reported in cervical cancer and cell lines. It remained unclear, however, whether the role of Notch signaling in HPV cervical cancer is oncogenic or tumor suppressive. The purpose of this research is to study the effects of suppressing of Notch signaling in HPV-positive cervical cancer cell lines, using GSI and DN-MAML1 approaches, and investigate these effects on phenotypes of cancer cell lines. While GSI treatment interferes with cleavage of Notch receptors by γ-secretase, overexpression of DN-MAML1 interferes with transcription of Notch target genes without any effect on Notch receptor processing.

We have studied three HPV-positive cervical cancer cell lines representing three different cervical lesions and containing either HPV16 or HPV18. We found that they all expressed *Notch1*, *MAML1* and *Hes1*, suggesting that Notch signaling is active. Our data show that Notch1 protein is present in all HPV-positive cervical cancer cell lines, in agreement with Lathion *et al.* (Lathion *et al.*, 2003) and Song *et al.* (Song *et al.*, 2008). While only CaSki expressed cleaved Notch1, in agreement with Veeraraghavalu *et al.* (Veeraraghavalu *et al.*, 2004), the other two cell lines did not. The status of other Notch rececptors are currently unknown in these cell lines, since there is no antibody specific for each active form available. In the cases of HeLa and SiHa, cleaved Notch1 were not detected. It is possible that these two cell lines might express cleaved Notch1 at a low level.

Previously, inhibition of Notch signaling by DAPT in cancer cell lines have been reported. Some studies observed the effect of DAPT treatment on cancer cell lines at varying periods of incubation. Suwanjunee *et al.* showed that DAPT treatment for 4 days decreased *Hes1* expression, cell proliferation and increased total Notch1 expression in human T cell leukemia cell line , Jurkat, and human hepatocellular carcinoma cell line, HepG2 (Suwanjunee *et al.*, 2008). As reported previously, Ramdass *et al.* showed that the NF-κB activity, pAKT and pIKK levels were decreased by DAPT treatment while they significantly

increased with overexpression of activated Notch1 (AcN1) in CaSki. They suggested that Notch signaling is associated with NF-κB activity in CaSki (Ramdass *et al.*, 2007). Consistently, treatment of CaSki with cbz-Leu-Leu-Nle-CHO, another γ-secretase inhibitor, suppressed NF-κB activity in a dose-dependent manner (Song *et al.*, 2008). Even though the inhibition of Notch signaling is reported in cervical carcinoma as well as other carcinomas, a detailed analysis of gene or protein expression, cell viability, cell cycle and cell proliferation has not been reported in HPV-positive cervical cancer cell lines.

Using DAPT treatment to suppress Notch signaling, we found increased total Notch1 level in CaSki upon DAPT treatment for the duration of 4 days. This result is likely due to the accumulation of Notch1 protein on the cell surface as it is not processed by γ-secretase. When cleaved Notch1 was examined, DAPT treatment completely abrogated the presence of this form of Notch1 in CaSki, suggesting that DAPT is effective in inhibiting the activity of γ-secretase.

Yu *et al.* demonstrated that RNA interference–mediated silencing of Notch1 or PS-1 blocked the Notch signaling and inhibited the growth of tumor cells both *in vitro* (HeLa) and *in vivo* (Yu *et al.*, 2007a). In contrast, DAPT treatment did not affect cell growth nor cell cycle progression in any cell line tested in this study. This may be because of difference in treatment and duration of treatment. Unexpectedly, our data show that the inhibition Notch signaling by DAPT treatment led to subtle but significant promotion of CaSki proliferation, consistent with the data reported by Talora *et al.* (Talora *et al.*, 2005). They showed that inhibition of Notch signaling resulted in promotes cell proliferation in HeLa and SiHa.

Interestingly, when CaSki was treated with DAPT, while mRNA of *Notch1* decreased, increased *Hes1* mRNA was detected. This result suggested that other Notch receptors may contribute to *Hes1* expression or *Hes1* expression is controlled by other unknown signaling pathway in a Notch independent manner. Consistently, Curry *et al.* showed elevated levels of Hes1 while expression of Notch receptors were decreased in confluent endothelial cell treated with γ-secretase inhibitor (Z-Leu-Leu-Nle-CHO) (Curry *et*

al., 2006). Since a number of other transmembrane proteins, such as ErbB4 (Ni *et al.*, 2001, Lee *et al.*, 2002), E-cadherin (Marambaud *et al.*, 2002) and CD44 (Lammich *et al.*, 2002), are also γ-secretase substrates, DAPT treatment may inhibit other substrates besides Notch receptor. In CaSki, when Notch signaling was decreased upon DAPT treatment, mRNA expressions of *MAML1* and *TP53* were increased. This may be due to the upregulation of TP53 by MAML1 (Zhao *et al.*, 2007) and/or due to the mechanisms described by Nair *et al.*. They found that activated Notch1 in HaCaT, an HPV negative human keratinocyte cell line, inhibited p53-induced apoptosis through a PI3K-PKB/Akt-dependent pathway (Nair *et al.*, 2003b).

In a second set of experiments, dominant-negative peptides of MAML1 were used to study the effects of suppressing Notch signaling in CaSki. DN-MAML1 is a pan-Notch receptor that inhibits Notch transcriptional activation without any effect on the step of processing of Notch receptor. In this study, DN-MAML1 expression led to decrease *Notch1* and *Hes1* mRNA expressions, in contrast to DAPT treatment. These results confirm that Hes1 transcription is controlled by Notch signaling and DN-MAML1 expression was sufficient in inhibiting *Hes1* expression. In addition, it also suggested that in DAPT treated CaSki, other Notch receptors may bypass the requirement of γ-secretase processing and constitutively active. This may be due to the mutation in *Notch receptor* gene.

Furthermore, inhibition of Notch signaling by DN-MAML1 expression caused more cell survival and promoted cell proliferation in CaSki, consistent with the results of DAPT treatment without affecting cell cycle. These findings showed that Notch signaling controlled growth and survival of CaSki. This result is in contrast with Weng *et al.* that reported in T-ALL cell lines (Weng *et al.*, 2003). It is possible that differences in tumor cell origins and cellular context which Notch signaling operated may result in the opposite outcomes.

An unexpected finding in the *in vitro* proliferation assay is the striking difference in the colony size between cells transduced with control MSCV-IRES-GFP and DN-MAML1 in clonogenic assay. CaSki which were transduced with DN-MAML1 formed smaller colonies,

whereas those transduced with MSCV-IRES-GFP resulted in bigger colonies in a clonogenic assay (Figure 4.13), consistence with Srivastava *et al.*. They studied Rhoc that is an effector of Notch1 that regulates invasion, metastasis, epithelial-to-mesenchymal transition (EMT) and angiogenesis in various carcinomas (Sequeira *et al.*, 2008, Wang *et al.*, 2008, Boone *et al.*, 2009) and found the effect of dominant negative RhoC caused significantly smaller tumors in CaSki. In addition, they found that inhibition of Notch and PI3K with pharmacological agents led to reduce RhoC activity, in which Notch1 has been shown to regulate RhoC activity in CaSki (Srivastava *et al.*, 2010). Our data and those of Srivastava *et al.* suggest that Notch signaling may regulate features of tumor progression in CaSki, but we can not rule out the role of other signaling pathways interacting with Notch signaling in CaSki.

In this study, we undertook two different approaches in suppressing Notch signaling and obtained partially, but not completely, overlapping results. This discrepancy may suggest the possibility that targeting Notch signaling at the γ-secretase level and the transcriptional level may yield different outcomes. More importantly, this also implies that there may have a transcriptional activity independent role of Notch receptor. When Notch receptors are allowed to be cleaved but unable to form a functional transcriptional complex with MAML/CSL, it may interact with other signaling cascade such as NF-κB and cause unexpected phenoptypes.