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Communication

Epigenetic Silencing of TNFSF7 (CD70) by DNA Methylation during Progression to Breast Cancer

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To escape the immune system, tumor cells may remove surface molecules such as the major histocompatibility complex (MHC) and co-stimulatory molecules, which are essential for recognition by lymphocytes. Down-regulation of the co-stimulatory molecules CD70 (TNFSF7) and CD80 may contribute to tumor cell survival; however, the mechanism of down-regulation of the *TNFSF7* gene during tumorigenesis is poorly understood. Here we present evidence indicating that *TNFSF7* gene expression is epigenetically down-regulated via DNA hypermethylation within its promoter region during progression in breast cancer cells in the isogenic MCF10 model. Bisulfite sequencing revealed that the CpG pairs at the proximal region of the *TNFSF7* promoter are heavily methylated during progression of breast cancer cells but that methylation of the more distal sequences was not changed considerably. Thus, this epigenetic silencing of the *TNFSF7* gene via hypermethylation of its proximal region may allow the benign and invasive MCF10 variants to escape immune surveillance.

INTRODUCTION

The escape of tumor cells from the immune system is crucial for the survival and progression of these cells. The escape mechanisms used by tumor cells include loss of tumor antigen expression, down-regulation of surface major histocompatibility complex (MHC) molecules and/or co-stimulatory molecules, and induction of lymphocyte apoptosis (Borst et al., 2005; Cormary et al., 2004; Douin-Echinard et al., 2000; 2003; Pardoll, 1998). Recent studies demonstrated that overexpression of the co-stimulatory molecules CD70 (TNFSF7) and CD80 by tumor cells may induce an anti-tumor response by T lymphocytes (Cormary et al., 2004; Douin-Echinard et al., 2000; 2003). Thus, tumor cells down-regulate these co-stimulatory molecules to escape immune response. Indeed, epigenetic inactivation of tumor suppressor genes frequently occurs during tumor progression. In particular, DNA hypermethylation in the promoter regions of tumor suppressors may contribute to the development of cancer (Baylin and Jones, 2007; Robertson, 2005). More specifically, epigenetic down-regulation of the aquaporin-5 (*AQP5*), *BubR1*, regenerating islet-derived 3 alpha (*REG3A)*, and *RUNX3* genes via DNA methylation was observed in certain cancer cell types (Choi et al., 2007; Motegi et al., 2005; Park et al., 2005; 2007). In addition, methylation of CpG islands in the 7-dehydrocholestrol reductase (*Dhcr7*) promoter is related to its suppression of transcription (Kim et al*.,* 2005), and heat stress can induce aberrant DNA methylation of imprinted genes in mouse (Zhu et al., 2008). The mechanism of down-regulation of co-stimulatory molecules such as CD70 and CD80, however, is largely unknown.

The MCF10 cell lines may provide an opportunity to study breast cancer initiation, development, and progression systemically (Hurst et al., 2009; Marella et al., 2009; Rhee et al., 2008; Worsham et al., 2006). The isogenic MCF10 breast cancer model, which includes normal, benign, non-invasive, and invasive carcinomas, was originally developed from a mortal cell line (MCF-10M) (Santner et al., 2001). Previously, we reported that at least five genes, including *TNFSF7* and the S100 family genes, were epigenetically down-regulated during progression to breast cancer in this model (Rhee et al., 2008).

Here, we report that epigenetic down-regulation of the *TNFSF7* gene occurs via DNA hypermethylation of the promoter region in a series of isogenic human breast cancer cell lines. We determined the DNA methylation pattern of CpG pairs in the promoter regions of the *TNFSF7* gene in the isogenic MCF10 model. These data suggest that the proximal region of the *TNFSF7* gene promoter may contribute to the epigenetic silencing of this gene during progression to breast cancer. Thus, these findings suggest that epigenetic silencing of *TNFSF7* may allow the benign and invasive MCF10 variants to escape immune surveillance.

MATERIALS AND METHODS

Cell culture and cell lines

A series of MCF10 cell lines including MCF-10A (non-tumorigenic, non-metastatic), MCF-10AT1 (benign proliferation stage), MCF-10CA1a cl1 (an invasive carcinoma stage), and MCF-10CA1d cl1 (an invasive carcinoma stage) were obtained from The Barbara Ann Karmanos Cancer Institute (USA). MCF-10DCIS.com (the carcinoma *in situ* stage) cell line was purchased from Asterand, Inc. (USA). Cells were cultured in Dul-

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becco's modified Eagle's medium-F12 medium (Cambrex, USA) at 37°C in a 5% CO_2 humidified incubator. The DMEM/F12 medium was supplemented with 100 ng/ml cholera toxin (BIMOL, g-117), 20 ng/ml epidermal growth factor(Sigma, USA; E-4127), 10 μg/ml insulin (Sigma, I-1882), 0.5 μg/ml hydrocortisone (Sigma, H-0888), 0.028 M sodium bicarbonate, and 5% horse serum.

5′-Aza-2′-deoxycytidine (5′-Aza-dC) treatment

For extraction of total RNA from drug-treated cells, the cell lines were incubated with 1 μM DNA methyltransferase inhibitor 5′-aza-2′-deoxycytidine (5′-aza-dC; Sigma) for 24 h. Then, the medium was changed, and cells were harvested after 4 days (Rhee et al., 2008).

RNA isolation and semi-quantitative RT-PCR analysis

Total cellular RNA was extracted using the RNeasy Mini Kit (Qiagen, USA) according to the manufacturer's instructions. RNA samples were treated with DNase I (Invitrogen, USA). Then, 5 μg total RNA was converted to cDNA via priming with oligo dT primers using the Superscript III first-strand synthesis system (Invitrogen) according to the manufacturer's protocol. The following primers were used: TNFSF7 forward, 5′-AATCACACAGGACCTCAGCAG-3′; TNFSF7 reverse, 5′-CACTGCACTCCAAAGAAGGTC-3′; GAPDH forward, 5′-TGATGACATCAAGAAGGTGGTGAAG-3′; GAPDH reverse, 5′-TCCTTGGAGGCCATGTGGGCCAT-3′. The expected sizes of amplified products and number of amplification cycles for RT-PCR are described in the previous report (Rhee et al., 2008).

Bisulfite sequencing

Genomic DNA was isolated using the LaboPass™ Genomic DNA Extraction Kit (COSMO Genetech, Korea) and the cytosine bases of genomic DNA (500 ng) were converted to uracil according to the protocol described in the EZ DNA Methylation Kit™ (Zymo Research Corporation, USA). To determine the location of the CpG islands in the promoter region of *TNFSF7*, we used a program available online, Methyl Primer Express® Software v1.0. The *TNFSN7* promoter regions containing CpG islands were amplified from the bisulfite-modified DNA using two sets of primers: for region 1 CpGs, forward, 5′-GGTTAAGGCGGG-TAGATTA-3′; reverse, 5′-TTTTTTTTTTTTTTAAAACAAAATC-TC-3′; and for region 2 CpGs, forward, 5′-GGGTGGATTATTTAAGGTTAGG-3′; reverse, 5′-ATACCCCTCTCCTACATTTTTTTA-3′. PCR amplification was performed with Hot Star Taq DNA polymerase (Qiagen, USA). The cycling conditions were as follows: initial activation of Hot Star Taq DNA polymerase at 95°C for 15 min, 35 cycles of 94°C for 30 s, 60 °C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR products were cloned into pUCm-T using the TA cloning system (RBC, Banqiao). After transformation of *Escherichia coli*, seven subclones were selected and sequenced using M13F primers.

RESULTS

Epigenetic silencing of the *TNFSF7* gene is reversed by treatment with 5′-aza-dC

In our previous report, we demonstrated that two members of the TNF gene family, *TNFSF7* and *TNFSF10*, were down-regulated during progression of breast cancer in the MCF10 cell lines. The down-regulation of *TNFSF7* and *TNFSF10* recovered following treatment with silencing inhibitors, such as the DNA methyltransferase inhibitor 5′-aza-2′-deoxycytidine (5′-aza-dC), suggesting a potential role of epigenetic silencing in regulation of expression of these genes (Rhee et al., 2008; Xu et al., 2007). In this study, we performed semi-quantitative RT-PCR to confirm the effects of treatment with 5′-aza-dC on the transcript levels of *TNFSF7* in transformed and tumor cell lines as well as the normal MCF-10A cells. Following treatment, the transformed and tumor cell lines exhibited a reversal of down-regulation at the gene level as determined by RT-PCR (Fig. 1). Our results are consistent with the previous report, suggesting that epigenetic silencing of the cytokine *TNFSF7* via DNA methylation may be involved in the transformation and progression of breast cancer cells (Fig. 1).

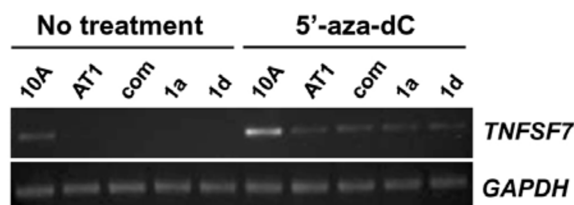

Fig. 1. *TNFSF7* mRNA expression is down-regulated during progression to breast cancer in MCF10 cell lines, and the expression pattern was reversed by treatment with a DNA methyltransferase inhibitor. The transcriptional expression of the *TNFSF7* gene was examined following treatment with the epigenetic silencing inhibitor 5′-aza-dC via semi-quantitative RT-PCR. The following cell lines were used: 10A, MCF-10A (untransformed) as control; AT1, MCF-10AT1 (benign proliferation); com, MCF-10DCIS.com (carcinoma *in situ*); 1a, MCF-10CA1acl1 (invasive); and 1d, MCF-10CA1d cl1 (invasive carcinoma). GAPDH was utilized as an internal control for the PCR.

Down-regulation of the *TNFSF7* gene is correlated with hypermethylation of CpG pairs near its promoter region

Next, we determined whether the DNA methylation inhibitor affected the methylation pattern of CpG islands within the *TNFSF7* promoter. Based on the sequences of the *TNFSF7* promoter and 5′-flanking region previously reported (Lu et al., 2005), DNA methylation patterns were analyzed for the 33 CpGs within the two upstream regions shown in Figs. 2A and 2B. To compare the methylation patterns among the genetically related MCF10 cell lines including normal (MCF-10A), benign proliferation (MCF-10AT1), ductal carcinoma *in situ* (MCF-10DCIS.com), and invasive carcinoma (MCF-10CA1a cl1 and MCF-10CA1d cl1) breast epithelial cells, PCR products were subcloned, and each of seven individual subclones was sequenced. Relatively little change was observed in DNA methylation status of the 12 CpG pairs in the promoter region between -993 and -754; however, the 7th CpG in the benign and cancer cell lines was moderately increased compared to the normal cell line (Fig. 2C).

In contrast, the 11 CpG pairs in the region between -588 and -323 were hypermethylated with about 1.6-fold increase during progression to breast cancer in the isogenic MCF10 model. In particular, the 4th and 10th CpG of benign and invasive MCF10 variants were heavily methylated compared with the normal MCF-10A cells (Fig. 2D). Thus, the bisulfite sequencing analysis suggests that methylation at the 4th and/or 10th CpG between -588 and -323 plays a major role in the epigenetic silencing of *TNFSF7* gene expression. Collectively, these studies suggest that the methylation of the CpG pairs between -588 and -323 may suppress promoter function of *TNFSF7* to a greater degree than the methylation of the more distal sequences between -993 and -754.

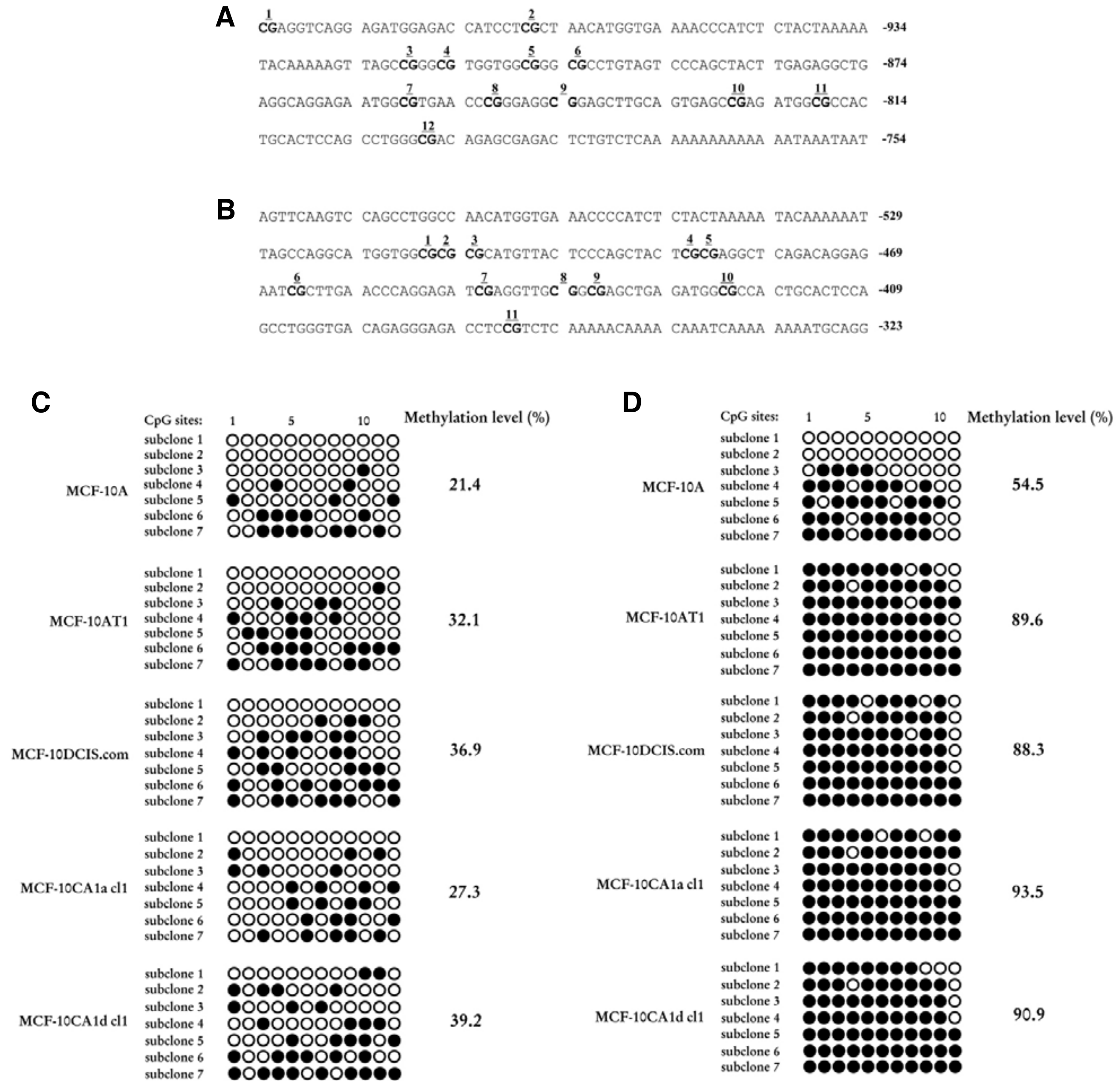

Fig. 2. Methylation patterns of CpG islands in the *TNFSF7* promoter revealed by bisulfite sequencing. (A) The CpG islands of upstream sequences of the *TNFSF7* promoter were slightly modified and adopted from Lu et al. (2005). This sequence spans 240-bp between positions -993~-754 relative to the transcription start site, including 12 CpGs (the distal region). (B) This sequence spans 240-bp between positions -588~-323 relative to the transcription start site, including 11 CpGs (the proximal region). (C, D) Genomic DNA from a series of MCF10 cell lines was treated with sodium bisulfite, and then the *TNFSF7* promoter was amplified by PCR. The PCR products were cloned into pUCm-T vectors with the TA cloning system. Seven subclones, each from five different MCF10 variants, were randomly selected, and the sequences of the purified plasmid DNAs were determined. (C) indicates the bisulfite sequencing data for the distal region (-993~-754), and (D) indicates the bisulfite sequencing data for the proximal region (-588--323). ○, unmethylated cytosines; ●, methylated cytosines.

DISCUSSION

The epigenetic regulation of *TNFSF7* (CD70) gene expression during progression to breast cancer is poorly understood. *TNFSF7* is normally expressed on normal breast epithelial cells (MCF-10A), but its expression is significantly down-regulated in the isogenic tumor cell lines (Rhee et al., 2008; Fig. 1). A correlation between the *TNFSF7* expression patterns and the DNA methylation pattern of CpG pairs nearby its promoter region is unknown. In the present report, we determined DNA methylation patterns of CpG pairs within the upstream sequences of the *TNFSF7* promoter using bisulfite sequencing. Our data indicated that the CpG pairs at the proximal region (-588~-323) are heavily methylated during progression to breast cancer while the methylation of the more distal sequences (-993~-754) remained relatively unchanged during this progression. Thus, these data suggest that the methylation of the CpG pairs in the *TNFSF7* gene promoter between -588 and -323 play an important role in epige-

netic silencing of *TNFSF7* gene expression during progression to breast cancer in the isogenic MCF10 model.

Tumors escape attack by the immune system via multiple contrasting mechanisms: (1) Removal of surface molecules essential for recognition by lymphocytes, and (2) induction of lymphocyte apoptosis. The first mechanism includes removal of co-stimulatory molecules, loss of tumor antigen expression, and decreased expression of surface MHC molecules (Douin-Echinard et al., 2000; Pardoll, 1998). On the contrary, tumors may utilize much more aggressive mechanism to escape the immune response through the induction of lymphocyte apoptosis. One interesting example occurs in renal cell carcinoma (RCC) cells. Overexpression of *CD70* (*TNFSF7*) in RCC promotes lymphocyte apoptosis via interaction with its receptor CD27 and with the intracellular receptor-binding protein SIVA, implying a positive role of CD70 in progression of RCC (Diegmann et al., 2006). Several studies, however, demonstrated that the expression of co-stimulatory molecules such as CD70 (TNFSF7) or CD80 by tumor cells induces an anti-tumor response based mainly on T lymphocytes (Cormary et al., 2004; Douin-Echinard et al., 2000; 2003). Yet another report also demonstrated that overexpression of histone deacetylase 5 (HDAC5) inhibited tumor cell growth and induced apoptosis through activation of the tumor necrosis factor death receptor pathway that includes TNFR1, TNFSF7, caspase-8, and DAPK1 (Huang et al., 2002). Thus, down-regulation of the *TNFSF7* gene may be required for escape from spontaneous apoptosis of tumor cells. In addition, *TNFSF7* gene expression was significantly down-regulated in an anti-cancer drug doxorubicin-resistant acute myelocytic leukemia cells (Song et al., 2006). Consistent with these data, our presented data showed that *TNFSF7* gene expression is epigenetically down-regulated via the hypermethylation of CpG islands in its promoter region during breast cancer progression. Therefore, accumulating evidence supports the idea that tumor cells may escape immune response via the down-regulation of *TNFSF7*. Together, our data suggest a negative role for CD70 in progression of MCF10 breast cancer cells in stark opposition to the demonstrated positive role for CD70 in progression of RCC.

Interestingly, our bisulfite sequencing data indicate that the CpG pairs in the *TNFSF7* promoter region were heavily methylated in the breast tumor cells compared to the related normal cell line. The hypermethylated region of *TNFSF7* identified in the breast cancer progression corresponds to that of *TNFSF7* in $CD4^+$ T cells (Lu et al., 2005). Usually *TNFSF7* is down-regulated in normal $CD4^+$ T cells but overexpressed in Lupus T cells and T cells treated with Lupus-inducing drugs such as 5′-aza-dC, procainamide (DNA methyltransferase inhibitor), and hydralazine (a blocker for the ERK signaling pathway) (Lu et al., 2005). Since reduction of *TNFSF7* gene expression was reversed by treatment of 5′-aza-dC (Fig. 1), the anti-tumor effect of 5′-aza-dC may occur through reactivation of co-stimulatory molecules such as CD70 (TNFSF7).

In conclusion, we demonstrate that the down-regulation of the *TNFSF7* gene during progression to breast cancer in the MCF10 cell lines occurs via DNA hypermethylation of CpG pairs in its promoter region. More specifically, our data indicate that DNA methylation of the CpG pairs at the proximal region of the upstream sequences of the *TNFSF7* promoter contributes to the epigenetic silencing of *TNFSF7* gene expression. Thus, this epigenetic down-regulation of *TNFSF7* gene may allow the benign and invasive MCF10 variants to escape the immune response.

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REFERENCES

Baylin, S.B., and Jones, P.A. (2007). Epigenetic determinants of cancer. In Epigenetics, C.D. Allis, T. Jenuwein, and D. Reinberg, eds. (New York, USA: Cold Spring Harbor, Cold Spring Harbor Laboratory Press), pp. 457-476.

Borst, J., Hendriks, J., and Xiao, Y. (2005). CD27 and CD70 in T cell and B cell activation. Curr. Opin. Immunol. *17*, 275-281.

Choi, B., Suh, Y., Kim, W.H., Christa, L., Park, J., and Bae, C.D. (2007). Down-regulation of regenerating islet-derived 3 alpha (REG3A) in primary human gastric adenocarcinomas. Exp. Mol. Med. *39*, 796-804.

Cormary, C., Gonzalez, R., Faye, J.C., Favre, G., and Tilin-Mariame, A.F. (2004). Induction of T-cell antitumor immunity and protection against tumor growth by secretion of soluble human CD70 molecules. Cancer Gene Ther. *11*, 497-507.

Diegmann, J., Junker, K., Loncarevic, I.F., Michel, S., Schimmel, B., and Eggeling, Fv. (2006). Immune escape for renal cell carcinoma: CD70 mediates apoptosis in mymphocytes. Neoplasia *8*, 933-938.

Douin-Echinard, V., Bornes, S., Rochaix, P., Tilkin, A.F., Peron, J.M., Bonnet, J., Favre, G., and Coudere, B. (2000). The expression of CD70 and CD80 by gene-modified tumor cells induces an antitumor response depending on the MHC status. Cancer Gene Ther. *7*, 1543-1556.

Douin-Echinard, V., Peron, J.M., Lauwers-Cances, V., Favre, G., and Couderc, B. (2003). Involvement of CD70 and CD80 intracytoplasmic domains in the co-stimulatory signal required to provide an antitumor immune response. Int. Immunol. *15*, 359-372.

Huang, Y., Tan, M., Gosink, M., Wang, K.K.W., and Sun, Y. (2002). Histone deacetylase 5 is not a p53 target gene, but its overexpression inhibits tumor cell growth and induces apoptosis. Cancer Res. *62*, 2913-2922.

Hurst, D.R., Xie, Y., Edmonds, M.D., and Welch, D.R. (2009). Multiple forms of BRMS1 are differentially expressed in the MCF10 isogenic breast cancer progression model. Clin. Exp. Metastasis *26,* 89-96.

Kim, J.H., Hwang, E.H., Park, H.J., Paik, Y.K., and Shim, Y.H. (2005). Methylation of CpG islands in the rat 7-dehydrocholesterol reductase promoter suppresses transcriptional activation. Mol. Cells *19*, 279-282.

Lu, Q., Wu, A., and Richardson, B.C. (2005). Demethylation of the same promoter sequence increases CD70 expression in Lupus T cells and T cells treated with Lupus-inducing drugs. J. Immunol. *174*, 6212-6219.

Marella, N.V., Malyavantham, K.S., Wang, J., Matsui, S., Liang, P., and Berezney, R. (2009). Cytogenetic and cDNA microarray expression analysis of MCF10 human breast cancer progresssion cell lines. Cancer Res. *69*, 5946-5953.

Motegi, K., Azuma, M., Tamatani, T., Ashida, Y., and Sato, M. (2005). Expression of aquaporin-5 in and fluid secretion from immortalized human salivary gland ductal cells by treatment with 5-aza-2′-deoxycytidine: a possibility for improvement of xerostomia in patients with Sjogren's syndrome. Lab. Invest. *85*, 342-353.

Pardoll, D.M. (1998). Cancer vaccines. Nat. Med. *4*, 525-531.

Park, W.S., Cho, Y.G., Kim, C.J., Song, J.H., Lee, Y.S., Kim, S.Y., Nam, S.W., Lee, S.H., Yoo, N.J., and Lee, J.Y. (2005). Hypermethylation of the RUNX3 gene in hepatocellular carcinoma. Exp. Mol. Med. *37*, 276-281.

Park, H.Y., Jeon, Y.K., Shin, H.J., Kim, I.J., Kang, H.C., Jeong, S.J., Chung, D.H., and Lee, C.W. (2007). Differential promoter methylation may be a key molecular mechanism in regulating BubR1 expression in cancer cells. Exp. Mol. Med. *39*, 195-204.

Rhee, D.K., Park, S.H., and Jang, Y.K. (2008). Molecular signatures associated with transformation and progression to breast cancer in the isogenic MCF10 model. Genomics *92*, 419-428.

Robertson, K.D. (2005). DNA methylation and human disease. Nat. Rev. Genet. *6*, 597-610.

Santner, S.J., Dawson, P.J., Tait, L., Soule, H.D., Eliason, J., Mohamed, A.N., Wolman, S.R., Heppner, G.H., and Miller, F.R.

(2001). Malignant MCF10CA1 cell lines derived from premalignant human breast epithelial MCF10AT cells. Breast Cancer Res. Treat. *65*, 101-110.

Song, J.H., Choi, C.H., Yeom, H.J., Hwang, S.Y., and Kim, T.S. (2006). Monitoring the gene expression profiles of doxorubicin-resistant acute myelocytic leukemia cells by DNA microarray analysis. Life Sci. *79*, 193-202.

Worsham, M.J., Pals, G., Schouten, J.P., Miller, F.R., Tiwari, N., van Spaendonk, R., and Wolman, S.R. (2006). High-resolution mapping of molecular events associated with immortalization, transforma-tion, and progression to breast cancer in the MCF10 model. Breast Cancer Res. Treat. *96*, 177-186.

Xu, J., Zhou, J.Y., Tainsky, M.A., and Wu, G.S. (2007). Evidence that tumor necrosis factor-related apoptosis-inducing ligand induction by 5-Aza-2'-deoxycytidine sensitizes human breast cancer cells to adriamycin. Cancer Res. *67*, 1203-1211.

Zhu, J.Q., Liu, J.H., Liang, X.W., Xu, B.Z., Hou, Y., Zhao, X.X., and Sun, Q.Y. (2008). Heat stress causes aberrant DNA methylation of H19 and Igf-2r on mouse balstocysts. Mol. Cells *25*, 211-215.