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SMRT 及 HDAC3 在癌症進展過程扮演互斥而多樣性的角色 (第一階段研究)

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計畫主持人：蔡坤志
共同主持人：吳志雄
計畫參與人員：　曲有爲

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中文摘要

外遺傳 (Epigenetic) 變化，包括 DNA 甲基化 (Methylation)、組織蛋白 (Histone) 修飾以及染色質 (Chromatin) 重組等，在惡性腫瘤的發生以及演化上扮演樞紐的地位。我們最近發現一個重要的外遺傳調控蛋白，SMRT (Silencing mediator for retinoic acid and thyroid hormone receptor)，是人類乳癌多重抗藥性表現型的關鍵致因。運用基因表現剖析 (Gene expression profiling)，我們進一步發現 SMRT 另外並調節許多與癌細胞侵犯 (Invasion) 及轉移 (Metastasis) 過程扮演重要地位的基因之表現，意味著 SMRT 有著先前未被發現的功能, 此一功能是與其在細胞抗藥性的地位有所區別的。在本研究中我們發現 SMRT 促進腫瘤乳腺上皮細胞的生長同時卻抑制其侵犯，這些作用是肇因於 SMRT 抑制與乳癌轉移有關的因子包括 fibronectin-1，thrombospondin-1 及 matrix metalloproteinase-1 同時卻促進與細胞黏合 (Adhesion) 有關的 desmosomal cadherins 的表現。與這些基因表現一致的發現是，SMRT 的過度表達 (Overexpression) 可使得腫瘤乳腺上皮細胞在一類似體內的微環境中進行部份的組織結構分化 (Structural differentiation)。我們更進一步發現 SMRT 抑制這些與細胞侵犯有關的基因之表現是仰賴著與其結合的去乙醯基酶 Histone deacetylase 3 (HDAC3) 的活性。釐清 SMRT 在惡性腫瘤致病機轉中的多樣性角色將有助於我們對外遺傳控制腫瘤惡性進展 (Malignant progression) 的認識同時並有助於針對此一外遺傳機轉的治療方式的設計。

中文關鍵詞

外基因變化，細胞核輔抑制因子，乳癌，侵犯，黏著，腫瘤進展

英文摘要

Epigenetic changes, including DNA methylation, histone modifications and chromatin remodeling, play a pivotal role in the initiation as well as evolution of malignant tumors. We previously showed that an important epigenetic regulator, SMRT (Silencing mediator for retinoic acid and thyroid hormone receptors), mediates multidrug resistance and contributes to poor therapeutic outcome of human breast cancers. We now demonstrate a previously unidentified function of SMRT in tumor progression, which is distinct from its role in cell death resistance. We could show that SMRT enhances the proliferation of neoplastic mammary epithelial cells (MECs) and inhibits their invasion *in vitro*. We verified the findings from gene expression profiling in which SMRT represses the expression of several genes implicated in breast cancer invasion and metastasis, including fibronectin 1 (*FN1*), thrombospondin-1 (*THBS1*) and matrix metalloproteinase-1 (*MMP1*), while upregulates the expression of desmosomal cadherins that mediate cell-cell adhesions. Consistent with these gene expression changes, SMRT overexpression forces partial structural differentiation and phenotypic reversion of neoplastic MECs in an in vivo-like tissue organization model. We further demonstrated that SMRT transcriptionally represses pro-invasive gene expression by modulating the activity of histone deacetylase 3 (HDAC3). The finding that N-CoR2 modulates tumor cell growth, survival, treatment resistance and invasion implies that the N-CoR2-dependent epigenetic pathway is a pleiotropic regulator of cancer progression.

英文關鍵詞

Epigenetic changes, nuclear corepressor, breast cancer, invasion, adhesion, tumor progression

報告內容

前言

It is increasingly recognized that epigenetic changes, including DNA methylation, histone modifications and chromatin remodeling, play a pivotal role in the initiation as well as evolution of malignant tumors. Histone modifications elicited by the dynamic actions of histone acetyltransferases (HATs) and histone deacetylases (HDACs) represent one of the major ways whereby chromatin is remodeled and gene transcription is regulated. We have recently shown that an important epigenetic regulator, SMRT (Silencing mediator for retinoic acid and thyroid hormone receptor), is critically involved in the acquisition of multi-drug resistance phenotype of human malignant tumors, such as breast cancers, which is functionally dependent on the nuclear deacetylase activity of its binding partner HDAC3. Using gene expression profiling, we further demonstrated that SMRT also regulates the expressions of many genes involved in the invasion and metastasis of malignant cells, suggesting a previously unidentified function of SMRT which is distinct from its role in cell death resistance. The findings that SMRT mediates death resistance while suppresses invasion of malignant cells raised an interesting possibility that SMRT may have "antagonistic pleiotropic effects" on the progression of cancers.

研究目的

To determine whether SMRT-dependent epigenetic pathway modulates the invasive and metastatic capacity of cancer cells, we will adopt *in vitro* as well as *in vivo* approaches. We will verify the candidate genes that have been shown by gene expression profiling to be regulated by SMRT, which play important roles in tumor progression (*Aim 1*). We will simultaneously adopt gain-of-function (*i.e.*, overexpression of SMRT and/or HDAC3) and loss-of-function (*i.e.*, knockdown of SMRT and/or HDAC3) approaches to address the causal relationship between the SMRT/HDAC3-mediated transcription-regulatory activities and cell invasive potential *in vitro* (*Aim 2*). Finally, to gain clinical relevance of these findings, we will conduct animal studies and tissue array experiments to examine the effect of SMRT/HDAC3 on the metastatic potential of malignant tumors *in vivo* (*Aim 3*).

文獻探討

Cell development/differentiation and stem cell fate are governed by the hierarchical order of gene activation and repression controlled at the level of chromatin structures by epigenetic mechanisms. Disruptions in the epigenetic regulation of chromatin structures and gene expressions would therefore lead to dysregulated cell growth, dedifferentiation and cancer. Consistent with this view, there are now circumstantial evidences supporting the epigenetic progenitor model in favor of the classical clonal genetic model of cancer (1). Epigenetic alterations, such as global DNA hypomethylation and chromatin hyperacetylation, are found at very early stages of tumorigenesis. On the other hand, hypermethylation and chromatin hypoacetylation on selective promoters are common strategies which tumor used to silence selective tumor-suppressor genes, such as retinoblastoma 1 (*RB1*), p16 (*CDKN2A*), von Hippel-Lindau tumor suppressor (*VHL*) and MutL protein homologue 1 (*MLH1*). Histone hypoacetylation can be caused by inactivation of histone acetylase (HAT) activity due to gene mutations, inhibitory action of viral oncoproteins and chromosomal translocations. For instance, mutations in CBP and P300 are associated with cancer predisposition (2, 3). Fusion proteins involving MLL (mixed-lineage leukemia) or MORF (monocytic-leukemia-zinc-finger-protein related factor) and p300 or CBP have been associated with acute myelogenous leukemia (AML) (4, 5). On the other hand, histone hypoacetylation and tumorigenesis can be also caused by altered histone deacetylase (HDAC) activities. For instance, chromosomal translocation events in acute promyelocytic leukemia (APL) produce fusion proteins

that contain retinoid acid receptor (RAR)α and PML (promyelocytic leukemia protein), and RARα and PLZF (promyelocytic zinc finger), which recruit HDACs with high affinity and result in constitutive repression of RAR-targeted genes (6). Moreover, the fusions proteins AML1-ETO and TEL-AML1 expressed in AML and acute lymphoblastic leukemia recruit HDACs and repress the AML1 transcriptional factor (7). Inappropriate transcriptional repression mediated by HDACs may also operate in the tumorigenesis of solid tumors, although the precise mechanisms remain incompletely understood.

Epigenetic changes not only play important roles in tumor initiation but may also contribute to malignant progression. Phenotypic plasticity mediated by epigenetic mechanisms has now been recognized as an important source of cancer-cell heterogeneity that drives phenotypic evolution of tumors. For example, DNA hypomethylation can drive genomic instability as a result of decondensation of centromeric heterochromatin and the formation of new centromeres (8). A reduction in heterochromatin-associated protein 1 (*HP1HSα*), a nonhistone chromosomal protein that mediates transcriptional repression, is directly associated with breast tumor cell invasion and metastasis (9). Recently, the polycomb group protein *EZH2*, a histone methyltransferase that causes gene silencing, is found to overexpress in metastatic prostate cancer or invasive breast cancer and promote the proliferation and invasion of tumor cells through its interaction with HDAC2 (10, 11). EZH2 was also found to be an independent predictor of prostate and breast cancer recurrence and death. Moreover, it was reported that the gene expression pathway associated with Bmi-1, a component of the chromatin remodeling complex PRC1 (polycomb repressive complex 1) which mediates ubiquitination of histone H2A, strongly predicts recurrence, metastasis and death in various types of human cancers (12). If epigenetic plasticity is a common strategy used by tumor cells to evolve into more advanced malignant states, it's likely that more epigenetic regulators will be identified as contributors to tumor progression.

The silencing mediator for retinoic acid and thyroid hormone receptors (SMRT) or nuclear corepressor 2 (*NCOR2*) and its paralog N-CoR (*NCOR1*) are epigenetic regulators that mediate transcriptional repression by recruiting and activating various histone deacetylases (HDACs) (13). SMRT and N-CoR originally were identified as transcriptional corepressors of unliganded nuclear receptors, such as reteinoic acid and thyroid hormone receptors (14). It was increasingly recognized latter that SMRT and N-CoR also mediate repression of a wide array of non-receptor transcriptional factors, including the myogenic specific bHLH protein MyoD (15), B-Myb (16), the Pbx family of homeobox genes (17), the signal transducers and activators of transcription-5 (STAT5) (18), the oncoproteins PLZF-RAR (19) and LAZ3/BCL6 (20), serum response factor (SRF), activating protein-1 (AP-1), and nuclear factor-κB (NFκB) (21). Biochemical purification of the N-CoR2/N-CoR complexes demonstrated that both N-CoR2 and N-CoR exist in large protein complexes comprising GPS2 (G-protein pathway suppressor 2), which mediates inhibition of the JNK pathway (22), TBL-1 (transducin β-like protein 1) and TBL-R1, which serve as E3 ligases that recruit the ubiquitin conjugating/19S proteosome complex thereby degrades the SMRT/N-CoR complex (23, 24), and HDAC3, which exhibits histone deacetylase activities. Interestingly, the purified SMRT-HDAC3 complex possesses deacetylase activity, whereas HDAC3 alone does not function as a HDAC, suggested that SMRT or N-CoR not only serves as the adaptor but also the activator of the HDAC3 enzymatic activity (25).

Aside form its nuclear receptor corepressor functions, N-CoR has been found to play important roles in differentiation (15) and stem cell maintainence (26). Recently, SMRT was also found to be involved in forebrain development and in maintenance of the neural stem cell state in mice (27). However, most of the studies on SMRT have focused at its protein biochemistry and its role in hormone receptor signaling and much less was known about its biological functions and its role in tumorigenesis. Using three-dimensional (3D) tissue reorganization models in the presence of extracellular matrices (ECM), we have recently identified a novel role of SMRT in the regulation of cell sensitivities to exogenous death stimuli (manuscript in preparation). Compared to cells cultures

as monolayers, N-CoR2 is upregulated in mammary acini formed in response to reconstituted basement membrane (rBM) at both the mRNA and protein levels. In contrast, N-CoR expression was not context-dependent. Using both loss-of-function and gain-of-function approaches, we confirmed that SMRT was necessary and sufficient for the architecture-dependent death resistance in non-neoplastic and neoplastic epithelial cells. SMRT mediates death resistance by concordantly modulating the expressions of multiple pro-apoptotic and anti-apoptotic mediators. Importantly, we found that both SMRT and HDAC3 expressions were correlated with probabilities of disease relapse and survival of breast cancer patients. These results demonstrated a novel role of SMRT/HDAC3 in therapeutic response and clinical prognosis of breast cancers.

研究方法

1. **Establish N-CoR2-overexpressing breast cancer cell lines**:
   The retroviral construct inducibly expressing HA- and EGFP-epitope tagged N-CoR2 was prepared by subcloning murine *NCOR2* cDNA (e isoform, NCBI RefSeq #NM_011424) into pBluescriptII KS+ (Stratagene) and then recloned into pLZRS-MFG-*tet*-EGFP to generate the final expression construct pLZRS-MFG-*tet*-HA-EGFP-NCOR2. HMT3522 T4-2 or MDA-MB-231 breast carcinoma cells were spin infected with retrovirus carrying N-CoR2 constructs, followed by infection with a high titer MFG virus expressing the tetracycline-controlled transcriptional transactivator. The transduced cells were sorted for EGFP positive cells and the sorted cell were expanded in the presence of tetracycline until 2-3 days before the experiments.
2. **Transcript expression analysis of SMRT-regulated genes:** Total RNA will be extracted from SMRT-overexpressing or SMRT-knockdown cancer cells and then converted to cDNA by reverse transcription. Quantitative analysis of cDNA in respective cell lines will be performed using quantitative real-time PCR (RT-PCR). Oligonucleotide primers will be designed using the Primer Bank database (http://pga.mgh.harvard.edu/primerbank/index.html) or similar online tools. On the other hand, we will also analyze protein expression levels of candidate SMRT-regulated genes by standard Western blot assay using commercial antibodies against respective proteins.
3. ***In vitro* cell invasion assay**: We will investigate the invasive capacity of SMRT-overexpressing T4-2 and MDA-MB-231 cells using the modified Boyden chamber apparatus with Matrigel filters. Briefly, polycarbonate filters, 0.4 μm pore size (6-well Transwell inserts, Corning) are coated with 50 μg/ml of growth factor-reduced Matrigel matrix (Becton Dickinson Labware) and dried under a hood. Cells ($1\text{-}2 \times 10^5$) are seeded in the insert and allowed to settle onto the Matrigel-coated membrane. The lower compartments of the Transwells are filled with 2 ml of fibroblast-conditioned medium and the coated inserts are mounted in the chamber. After an incubation period of 6 hours at 37°C, the cells on the upper surface of the filter are removed with a cotton swab. The filters are then fixed with 3% glutaraldehyde solution and stained with 0.5% crystal violet solution. Invasive cells adhering to the under-surface of the insert are counted using a phase contrast microscope (400×). The data will be expressed as the summation of the number of invasive cancer cells in 5 representative fields.
4. **Three-dimensional (3D) organotypic culture assay**: HMT-3522-T4-2 cells that overexpress SMRT or control vectors were propagated as monolayers on plastics or within 3D rBM (Matrigel, BD Biosciences) in chemically defined medium consisting of DMEM:F12 medium (Invitrogen GIBCO), containing 250 ng/ml insulin (Boehringer Mannheim), 10 μg/ml transferrin (Sigma, St. Louis, MO), 2.6 ng/ml sodium selenite (Collaborative Research), 10^{-10} M estradiol (Sigma), 1.4×10^{-6} M hydrocortisone (Collaborative Research), and 5 μg/ml prolactin (Sigma). The three-dimensional (3D) cultures were grown in 8-well chamber slides (Nalgene Nunc) for 10 days before image or biochemical analysis. MDA-MB-231 cells were

grown as monolayers in DMEM supplemented with 10% fetal bovine serum and antibiotics.

結果與討論

1. Verification of SMRT-regulated genes that are implicated in tumor invasion:

Using gene expressing profiling, we have previously identified a list of 304 genes (represented by 350 Affymetrix probe sets) whose expressions were significantly altered by SMRT overexpression in the neoplastic breast epithelial cells HMT3542 T4-2 (data not shown). Functional annotations of these genes further identified a list of genes involved in extracellular matrix assembly and remodeling (*e.g., FN1*, *SDC2*, *TIMP3*, *MMP1*, *COL4A1*, *THBS1*, *TAGLN*, *TNC*, *COL6A2*, *ITGA6*, *ITGB4*) and cytoskeleton and cell-cell adhesion (*e.g., TPX2*, *SPOCK1*, *ARHGAP1*, *PAK1*, *WASL*, *DSC2*, *DSG3*). Specifically, SMRT significantly represses the transcriptions of several genes that are associated with invasion and progression of cancers, including fibronectin 1 (*FN1*), matrix metalloproteinase 1 (*MMP1*), thrombospondin-1 (*THBS1*), tenascin-C (TNC) and α6 and β4 integrins (*ITGA6*, *ITGB4*). In contrary to the downregulation of genes associated with invasion, two major components of the intercellular desmosome junctions, desmocollin 2 (*DSC2*) and desmoglein 3 (*DSG3*), were significantly upregulated in response to SMRT overexpression, which may strengthen cell-cell adhesions and relate to the formation of compact cellular "islands" as shown in monolayer cultures.

To verify the findings from gene expression profiling experiments, we employed real-time PCR to examine the differential expressions of the selected SMRT-regulated genes which have been implicated in breast cancer invasion and progression (Fig. 1). Consistently, we found that the expressions of fibronectin 1, thrombospondin-1 and MMP1 were markedly downregulated upon overexpression of SMRT in neoplastic mammary epithelial cells. The expressions of components of type IV collagen were also repressed by SMRT expression. On the contrary, the two desmosomal cadherins DSG3 and DSC2 were upregulated in SMRT-overexpressing cells. It should be noted that these SMRT-mediated changes in gene expressions were probably not cell type- or malignant state-specific, as similar changes were seen in the noninvasive T4-2 cells and the highly invasive MDA-MB-231 cells.

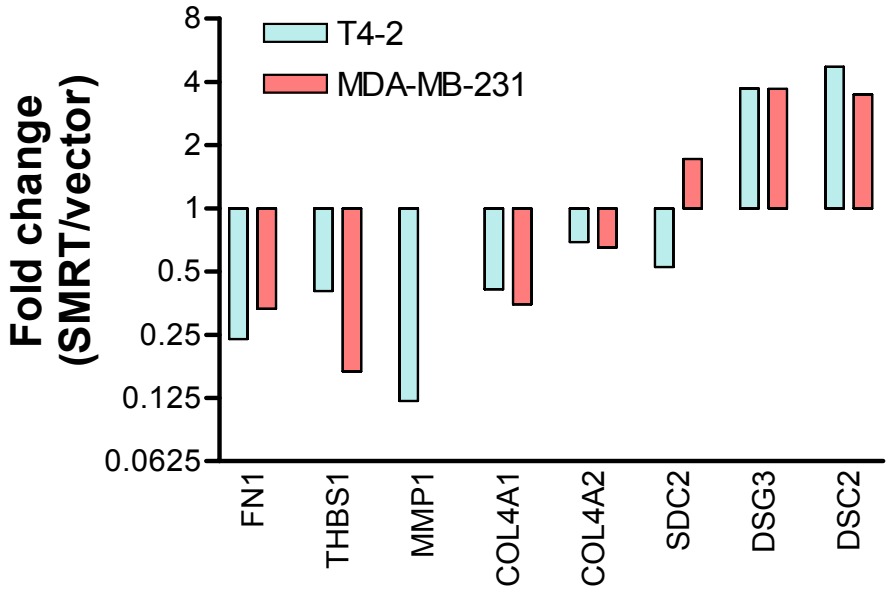

Figure 1 Verification of SMRT-mediated changes in gene expressions. Fold changes in the mRNA expression levels of selected SMRT-regulated genes comparing between SMRT-overexpressing T4-2 cells or MDA-MB-231 cells and the vector control cells as quantified

by RT-PCR.

We further verified the changes in the protein expression level of fibronectin, the gene whose expression was most pronouncedly repressed upon SMRT overexpression, using immunoblotting and immunohistochemistry. Consistent with its changes in the mRNA level, the protein abundance of fibronectin was markedly lower in the SMRT-overexpressing T4-2 cells as compared with the vector control cells (Fig. 2A). When grown as multicellular spheroids in three-dimensional reconstituted basement membrane (rBM), T4-2 cells expressing the control vector deposited a thick layer of fibronectin surrounding the tumor spheroid, while only a faint and thin layer of fibronectin was seen around the tumor spheroid formed by the SMRT-overexpressing cells (Fig. 2B).

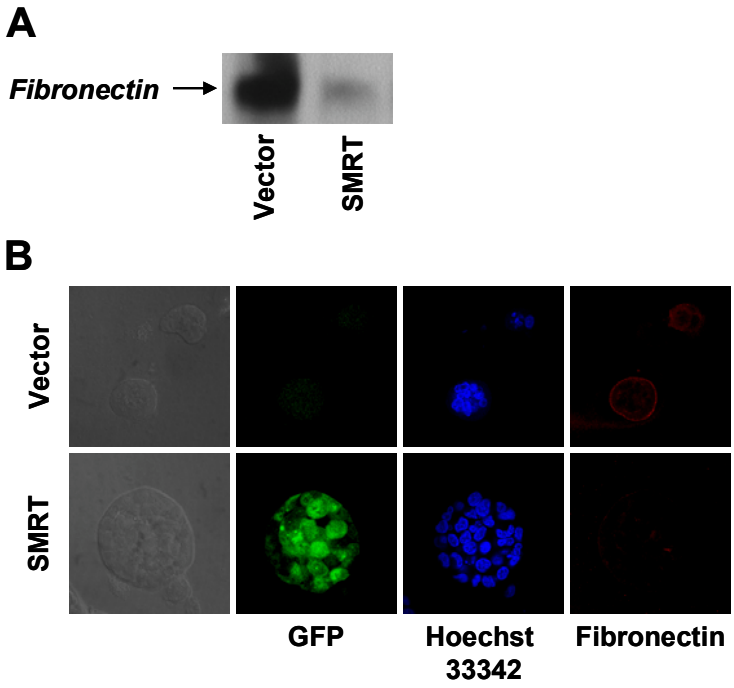

Figure 2 SMRT downregulates the protein expression of fibronectin in neoplastic MECs. A. Protein abundances of fibronectin, as analyzed by immunoblotting, in T4-2 cells expressing SMRT or an empty vector. B. Phase-contract micrographs (left panels) and confocal fluoresence microscopy (middle and right panels) of T4-2 cells expressing SMRT or an empty vector grown as multicellular structures in 3D rBM. The structures were immunostained with anti-fibronectin and nuclei were counterstainied with Hoechst 33342. Note the GFP-tagged SMRT protein was mainly nucleus-localized.

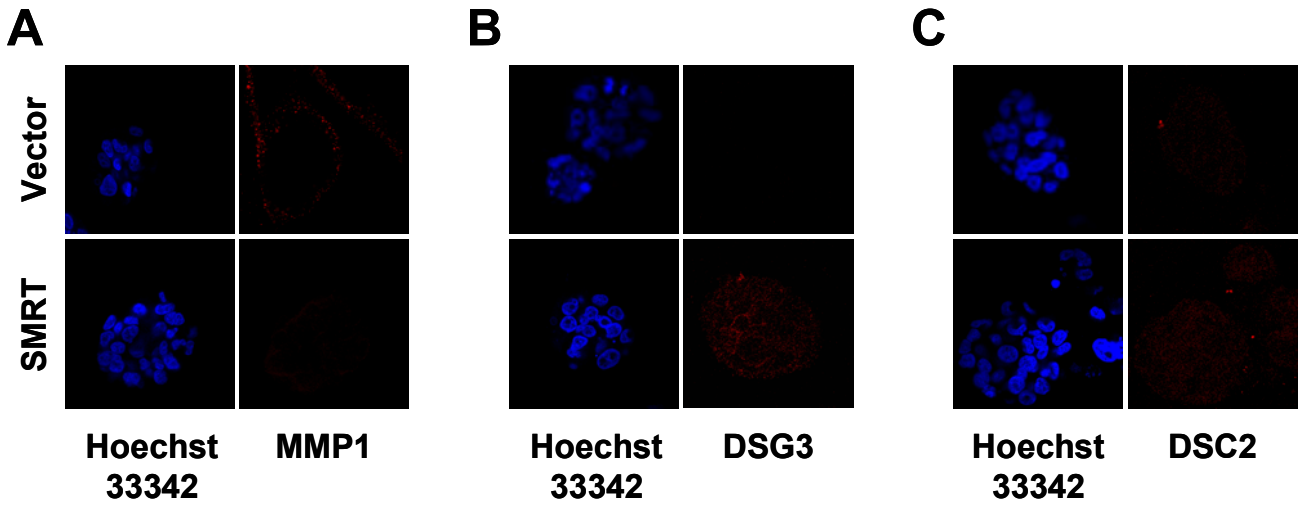

Figure 3 SMRT downregulates the expression of MMP1 while upregulates the expression of desmosomal cadherins. SMRT-overexpressing T4-2 cells and the vector control cells were grown in 3D rBM for 6 days and the expressions of desmocollin 2 (Left) or desmoglein 3 (Right) were examined using immunohistochemistry.

2. Effect of SMRT on the *in vitro* invasive potentials of neoplastic MECs:

As described earlier, SMRT suppressed the expression of genes important for cell invasion while up-regulated cell desmosome junction components, we speculate that tumor cells that retain the expression of SMRT, compared with those losing it expression, may have impaired ability of remodeling their surrounding ECM and tissue invasion. To test this possibility, we investigated the invasive capacity of SMRT-overexpressing cancer cells using the modified Boyden chamber apparatus with Matrigel filters. This *in vitro* assay is used because it mimics the three-step process of invasion: adhesion, proteolytic dissolution of the ECM and migration. In addition, it has been demonstrated that invasiveness in this assay correlates well with the metastatic potential of a given tumor cell line (28). Briefly, polycarbonate filters, 0.4 μm pore size (6-well Transwell inserts, Corning) are coated with 50 μg/ml of growth factor-reduced Matrigel matrix (Becton Dickinson Labware) and dried under a hood. Cells ($1\text{-}2 \times 10^5$) are seeded in the insert and allowed to settle onto the Matrigel-coated membrane. The lower compartments of the Transwells are filled with 2 ml of fibroblast-conditioned medium and the coated inserts are mounted in the chamber. After an incubation period of 6 hours at 37°C, the cells on the upper surface of the filter are removed with a cotton swab. The filters are then fixed with 3% glutaraldehyde solution and stained with 0.5% crystal violet solution. Invasive cells adhering to the under-surface of the insert are counted using a phase contrast microscope (400×). The data will be expressed as the summation of the number of invasive cancer cells in 5 representative fields.

Consistent with our hypothesis, neoplastic MECs, including T4-2 cells and the highly invasive MDA-MB-231 cells were rendered less invasive through rBM when SMRT was overepressed (Fig. 4). In contrast, verexpression of a mutant SMRT (K449A) that failed to activate the nuclear deacetylase activity of its associating HDAC3 protein restored the invasive capacity of T4-2 cells to an extent slightly higher than the vector control cells. These results reinforced the idea that SMRT suppresses the invasive capacity of neoplastic MECs through activation of HDAC3.

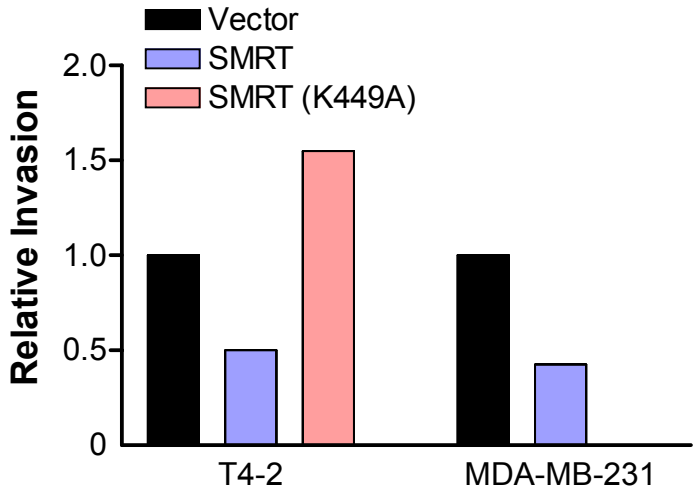

Figure 4 Effect of SMRT on the invasive capacities of neoplastic MECs. T4-2 cells or MDA-MB-231 cells that stably express SMRT or an empty vector were allowed to invade through a thin-layer of rBM using the modified Boyden chamber assay and invaded cells were quantified by counting cell nuclei labeled with DAPI.

3. Effect of SMRT on the tissue organization of neoplastic MECs:

Cancers are disrupted in their tissue organization and cell polarity which are linked to altered cell-cell or cell-matrix adhesions and aberrantly activated signal transduction pathways such as the epidermal growth factor receptor (EGFR) and MAPK signaling cascade (29). Interestingly, recent findings have clearly demonstrated that cancer can re-establish their tissue organization and polarity upon inhibition of EGF or MAPK signaling or β1-integrin (30), suggesting that the malignant phenotypes of cancers can be reverted by certain cell-intrinsic or microenvironmental cues. Consistent with this paradigm, we found that, when cultured in 3D rBM, T4-2 cells formed disorganized multi-cellular structures with nonpolarized expression of the basal surface marker α6-integrin (Fig. 5A). In comparison, T4-2 cells that stably expressed SMRT grew into more organized and mass-like structures with a relatively abundant α6-integrin expression at the basal surface of the cells located at the outer margin. Surprisingly, SMRT overexpression also markedly attenuated the invasive capacity of the highly invasive MDA-MB-231 SMRT cells, which instead formed partially polarized multicellular spheroids (Fig. 5B). Taken together, our results suggest that SMRT may force partial structural differentiation and phenotypic reversion of neoplastic MECs. The finding of the ability of SMRT to drive tissue organization in i*n vivo*-like 3D contexts corroborates the findings obtained from the dual-chamber invasion assay as described in Fig. 4 and further implies its clinical relevance.

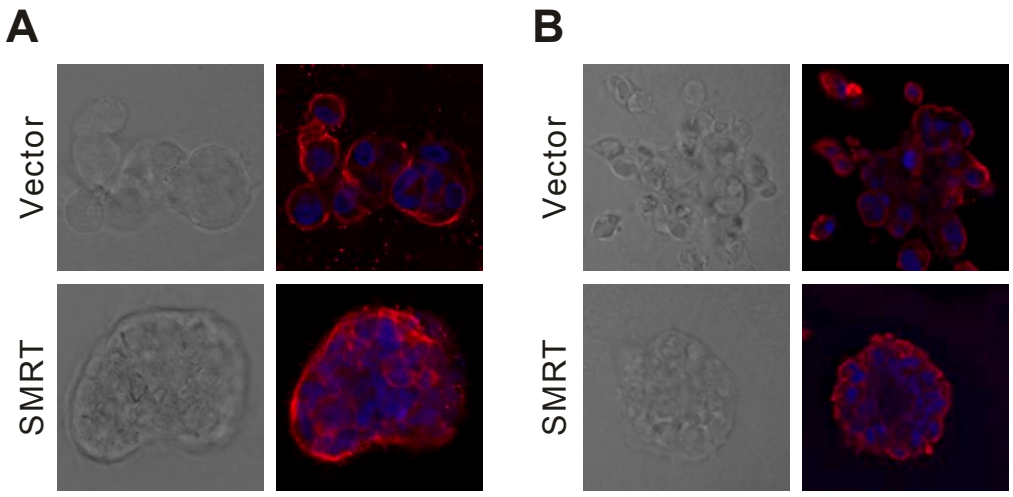

Figure 5 Overexpression of SMRT results in partial reestablishment of tissue organization. Phase-contrast and confocal fluorescence microscopic images of the multicellular structures formed by SMRT-overexpressing T4-2 cells (A) or MDA-MB-231 cells (B) and those expressing an empty vector in 3D rBM. The structures were immunostained with anti-α6-integrin (red) and the nuclei were counterstained with Hoechst 33342 (blue).

4. Effects of SMRT on the proliferative potential of neoplastic MECs:

As SMRT-dependent epigenetic regulations of gene expressions may have diverse effects on breast cancer progression, we further evaluated the effect of SMRT overexpression on the growth of neoplastic MECs. In contrast with its effect of suppressing cell invasion, overexpression of SMRT resulted in a small (~30 %) but reproducible increase in their growth in neoplastic MECs (Fig. 6). This effect was not cell type- or malignant state-dependent as the increased proliferation was observed both in T4-2 cells and the invasive MDA-MB-231 cells. It is conceivable that the growth-promoting effect of N-CoR2 is likely used by initiated cells as a strategy for tumor initiation and progression. Taken together, our results demonstrated previously unidentified functions of SMRT in the malignant progression of breast cancer and reinforced our model that SMRT-dependent epigenetic regulations have pleiotropic influences on different malignant traits of neoplastic MECs (Fig. 7).

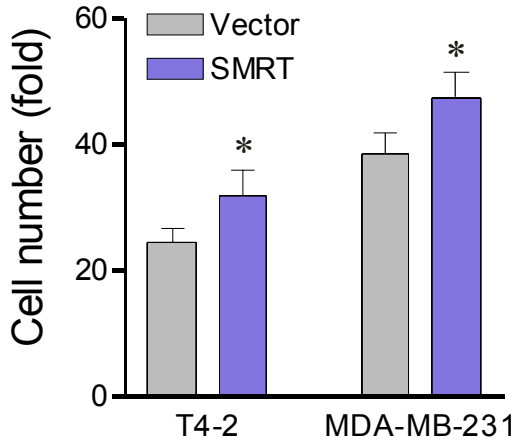

Figure 6 Effect of SMRT on the proliferative capacity of neoplastic MECs. HMT3522 T4-2 cells or MDA-MB-231 cells that stably express SMRT or empty vector were seeded on collagen-coated culture plastics and the fold increase in the cell number were measured at day 7. $^{*}P$

< 0.05, SMRT- versus vector-expressing cells.

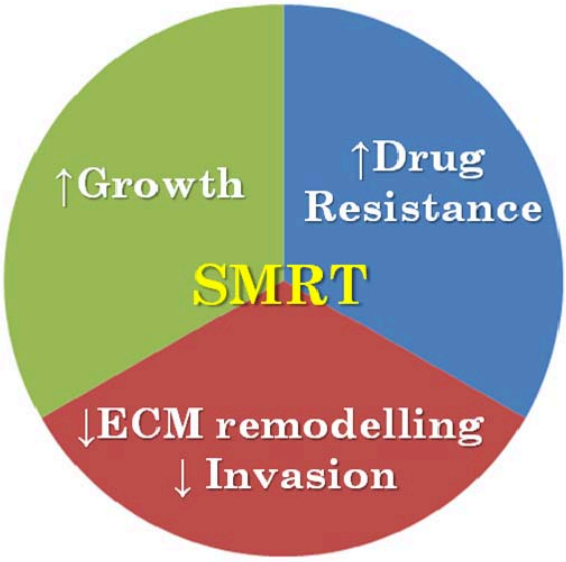

Figure 7　Summary diagram of the proposed model of the pleiotropic control of malignant traits of neoplastic MECs by SMRT. Neoplastic MECs that retain the expression of SMRT display increased proliferative potentials and resistance to therapeutics while have lower capacities of remodeling and invading surrounding ECM.

計畫成果自評

The current project represented the first step of the long-term interest of my lab in the molecular mechanisms and *in vivo* significance of the epigenetic control of tumor progression. Using quantitative real-time PCR assay, we have confirmed that the expressions of several pro-invasive genes, such as *FN1* and *THBS1*, are significantly repressed upon overexpression of SMRT in breast carcinoma cells. On the other hand, desmosomal cadherions are significantly upregulated in response to SMRT overexpression in at least two neoplastic breast epithelial cell lines that represent breast carcinoma cells at different transformation stages. Consistent with these changes in gene expressions, we confirmed that forced expression of SMRT in breast carcinoma cells prominently compromised their abilities to invade through rBM. Moreover, using a novel 3D organotypic culture technique, we demonstrated that overexpression of SMRT partially reverted the invasive and disorganized structures formed by breast carcinoma cells to a more organized multicellular spheroids of considerably larger size. These data collectively serve as the basis of the more in-depth studies of the role of the epigenetic regulator SMRT in breast cancer progression.

In the follow-up experiments, we will provide key molecular effectors that mediate the SMRT-dependent changes in tumor phenotypes. In particular, we will focus on several of the major target genes of SMRT, fibronectin 1 (*FN1*) and thrombospondin 1 (*THBS1*), whose expressions were significantly inhibited upon SMRT overexpression. Notably, both fibronectin-1 and thrombospond-1 have been shown to play an important role in breast cancer invasion. For instance, fibronectin can elicit MMP1-dependent invasion of mammary epithelial cells and breast cancer cells (31). On the other hand, purified THBS1 protein dose-dependently promoted the invasion of breast carcinoma cell lines *in vitro* (32). Moreover, *THBS1* has recently been associated with breast cancer metastasis in a mouse knock-out model (33). In our next step of study, we will use chromatin immunoprecipitation to verify that the promoter of *FN1* and *THBS1* is bound by SMRT, which thereby directly regulates the expression of *THBS1*. To ask whether the decreased expression of

FN1 and/or *THBS1* contributes to the SMRT-mediated anti-invasive effect on breast cancer cells, we plan to downregulate the expression of fibronectin and/or THBS1 in the vector control T4-2 or MDA-MB-231 cells using a retroviral-mediated RNA interference technique. On the other hand, we will stably overexpress THBS1 in SMRT-overexpressing cells. We will employ the same *in vitro* assays (*i.e.,* dual-chamber invasion assay and 3D organotypic culture) to verify the causal relationship between the expression levels of FN1 or THBS1 and the impaired invasive potential of SMRT-overexpressing malignant MECs.

In the next step of our studies, to further explore the *in vivo* significance of the above findings, we will label breast carcinoma cells that express SMRT or empty vector with a retroviral-luciferase reporter and the extent of lung or bone metastasis will be visualized and quantitatively measured using bioluminescence imaging (34). Conceivably, elucidation of the pleiotropic roles of SMRT in breast tumor progression may improve our understandings of the epigenetic control of tumor progression and may facilitate the design of more targeted therapies toward this mechanism. After completing these studies we plan to publish these results in high-profile cancer research journals such as *Cancer Research* or *Oncogene*.

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