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Tumorigenesis and Neoplastic Progression

Epigenetic Modulation of Cancer-Germline Antigen Gene Expression in Tumorigenic Human Mesenchymal Stem Cells

Implications for Cancer Therapy

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Cancer-germline antigens are promising targets for cancer immunotherapy, but whether such therapies will also eliminate the primary tumor stem cell population remains undetermined. We previously showed that long-term cultures of telomerized adult human bone marrow mesenchymal stem cells can spontaneously evolve into tumor-initiating, mesenchymal stem cells (hMSC-TERT20), which have characteristics of clinical sarcoma cells. In this study, we used the hMSC-TERT20 tumor stem cell model to investigate the potential of cancer-germline antigens to serve as tumor stem cell targets. We found that tumorigenic transformation of hMSC-TERT20 cells induced the expression of members of several cancer-germline antigen gene families (ie, GAGE, MAGE-A, and XAGE-1), with promoter hypomethylation and histone acetylation of the corresponding genes. Both *in vitro* cultures and tumor xenografts derived from tumorigenic hMSC-TERT20 single cell subclones exhibited heterogeneous expression of both GAGE and MAGE-A proteins, and similar patterns of expression were observed in clinical sarcomas. Importantly, histone deacetylase and DNA methyltransferase inhibitors were able to induce more ubiquitous expression levels of cancer-germline antigens in hMSC-TERT20 cells, while their expression levels in primary human mesenchymal stem cells remained unaffected. The expression pattern of cancer-germline antigens in tumorigenic mesenchymal stem cells and sarcomas, plus their susceptibility to enhancement by epigenetic modulators, makes them promising targets for immunotherapeutic approaches to cancer treatment. ***(Am J Pathol 2009, 175:314–323; DOI: 10.2353/ajpath.2009.080893)***

A small population of tumor cells with stem cell-like properties may sustain the tumor growth, and effective cancer treatments may depend on the ability to target these cells. Cancer-germline (CG) antigens are suitable targets for immunotherapy of cancer, but whether they are also expressed in tumor stem cell populations remain unresolved.

CG antigens can be found in a large variety of tumor types, but their expression in normal cells is restricted to a number of fetal tissues and immune-privileged sites such as testis and placenta.[1–3] Both natural cellular and humoral immune responses against CG antigens can be observed in cancer patients, indicating that they are immunogenic and appropriate targets for cancer immunotherapy.[4–13] Accordingly, disease regression and improved survival in several cancer types have been achieved following CG antigen immunization.[14,15] Epigenetic regulation is important for expression of CG antigen genes, and drugs that affect epigenetic processes can be used to modulate CG antigen expression in tumor cells.[14,15] The association between expression of CG antigen genes and DNA demethylation is well established.[16–20] However, little is known about the role of histone modifications in regulation of CG antigen genes, although preliminary results have shown that both histone acetylation and methylation may be involved.[21,22] The use of DNA methyltransferase (DNMT) and histone

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deacetylase (HDAC) inhibitors as inducers of CG antigen expression for enhancement of cancer cell immunotargeting is being investigated.[23,24] However, the specific sequences of epigenetic events required for induction of CG antigen gene expression has yet to be determined.

We recently studied telomerase immortalized adult human mesenchymal stem cells (hMSCs) and found that long-term cultures (eg, hMSC-TERT20) showed loss of contact inhibition, anchorage dependence, and tumor formation in mice.[25,26] Despite maintaining a normal karyotype, the tumorigenic hMSC-TERT20 cells exhibited genetic changes often seen in sarcomas, including *INK4a/ARF* gene locus deletion and *DBCCR1* locus hypermethylation. The hMSC-TERT20 tumor stem cell model may support an hMSC origin for Ewing's sarcoma, and offer a model to study the tumorigenic progression of hMSCs.[27] Although the role of hMSCs in development of cancer is controversial, several studies have shown that hMSCs are permissive for transformation.[25,28–31] Furthermore, a small population of self-renewing cancer cells, expressing mesenchymal stem cell surface markers, exist in bone sarcomas.[25]

In this study, we show that CG antigens are heterogeneously expressed by tumorigenic hMSCs and that epigenetic modulation of CG antigen genes can induce a more ubiquitous expression, indicating that CG antigens may be useful targets for elimination of cancer stem cells.

Materials and Methods

Cell Culture

The establishment of primary hMSCs and hMSC-TERT20 cultures from bone marrow aspirates has been described previously.[25,26,32] Non-tumorigenic and tumorigenic cells were derived from population doubling levels (PDL) 102 and 282 of hMSC-TERT20, respectively. While hMSC-TERT20 cells up until PDL123 have maintained a normal phenotype, hMSC-TERT20 cells at PDL 256 show loss of contact inhibition, anchorage dependence, and form tumors when implanted into mice.[25,27] hMSC-TERT20 subclones: BB3, BC8, BC6, BD11, CE8, and DB9 originated from population doubling level 440 hMSC-TERT20. All cell lines and primary hMSCs were grown in Minimal Essential Medium (Invitrogen, Taastrup, Denmark) supplemented with 10% fetal bovine serum (Gibco Invitrogen), L-glutamine and antibiotics in a humidified incubator at 37°C and 5% CO_2. Three-dimensional multicellular cultures (spheroids) were formed by seeding 1 to 2×10^6 cells/well in ultra low adhesion 6-well culture dishes (Corning, Biotech Line A/S, Slangerup, Denmark) and allowing the cells to aggregate for 24 hours. For the CG antigen gene induction experiments, primary hMSCs and hMSC-TERT20 were treated with 1 μmol/L 5-aza-2′-deoxycytidine (5-AZA-CdR) (Sigma-Aldrich, Brondby, Denmark) for 48 hours and/or with 500 nmol/L and Trichostatin (TSA) (Sigma-Aldrich) for 24 hours.

RNA Isolation and RT-PCR

Total RNA was extracted using Trizol reagent (Invitrogen) and reverse transcribed with Superscript III (Invitrogen). Semiquantitative PCR was performed with TEMPase DNA polymerase (Ampliqon, Rodovre, Denmark) for 35 cycles and products were analyzed by 1% agarose gel electrophoresis. Relative quantification using real-time PCR was performed in triplicate using SYBR Green PCR Master mix (Applied Biosystems, Foster City, CA) in accordance with the manufacturer's instructions. The relative expression levels were normalized with endogenous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels. MAGE-A1 and NY-ESO-1 RT-PCR primers were purchased from Superarray, Frederick, MD. Other primers are listed in Table 1.

Bisulfite Sequencing

Bisulfite treatment of genomic DNA was performed using the EZ DNA Methylation kit (Zymo Research, Orange, CA). Promoter regions of interest were PCR amplified from bisulfite-treated DNA (primers are listed in Table 1), TA-cloned into the pCR2.1 vector (Invitrogen), and subjected to DNA sequencing. Each sample was analyzed twice.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) analysis was performed using the EZ-ChIP kit (Millipore, Copenhagen, Denmark) in accordance with the manufacturer's instructions. Briefly, cells were fixed in 1% formaldehyde and lysed in SDS buffer. Genomic DNA was sheared by sonication and subjected to immunoprecipitation using anti-acetyl-H3 (Millipore), or normal rabbit IgG. Crosslinking was reverted and precipitated GAGE DNA was quantified using real-time PCR or semiquantitative PCR with primers recognizing the promoter or intron 1 of the GAGE genes (primers are listed in Table 1). Quantification of GAPDH in input DNA was used for normalization of results. ChIPs were performed twice and in duplicates.

Immunostaining

Mouse tumors and cell cultures for immunostaining were fixed in 4% formaldehyde. Cell cultures were then prepared as cell blocks using Shandon Cytoblock (Thermo Electron Corporation, Pittsburg, PA) or as thrombin clots and embedded in paraffin.

Tumor sections were obtained from specimens collected for diagnostic purposes and deposited in the tissue bank of Odense University Hospital. All experiments were approved by the ethical committee of Funen and Vejle County (VF20050069).

Immunohistochemical and immunocytochemical staining procedures were performed as previously described.[33]

Table 1. Primers

Gene	Assay	Sequence
BORIS	RT-PCR	5′-CAGGCCCTACAAGTGTAACGACTGCAA-3′ 5′-GCATTCGTAAGGCTTCTCACCTGAGTG-3′
GAGE-pan	BS-1	5′-GTGATTGGTAGGATTATGTATTTTGT-3′ 5′-CACCCCCAACCCAAAAC-3′
	BS-2	5′-GTAGGTGTGTAGGTTAGTTATTT-3′ 5′-AAAATAAAACCTTACAATACTTCTCACT-3′
	BS-3	5′-AGGGGAGTATTATTTGAGTTTTAG-3′ 5′-CACCTACCAAATAAATCTCAATAAAAA-3′
	ChIP-pro	5′-AAGGGGCCGGGCAGAAGACG-3′ 5′-TGAGGATGGGCATGGAGGGTTTG-3′
	ChIP-in1	5′-AGGCGGTCAGGGGCTCAGGT-3′ 5′-GGTGCCAGGCCCCTTCTTCA-3′
MAGE-A1	BS	5′-TTGGGGGTAGTTTAGGTTGAGA-3′ 5′-ACTAAAAATCCCTAAAACCCCACT-3′
NY-ESO-1	BS	5′-GGTGAGGGGTTTAAGTTGGTATT-3′ 5′-CACCAAAACTATCAAAACAAAACAA-3′
SSX1-9	RT-PCR	5′-CCTCAGATGACTTTCGGCAGGC-3′ 5′-GCTATGCACCTGATGACGAGGG-3′
XAGE1	RT-PCR	5′-TCCCAGGAGCCCAGTAATGGAGA-3′ 5′-CAGCTTGTCTTCATTTAAACTTGTGGTTGC-3′
HAGE	RT-PCR	5′-CCTTTCAATGTTATCCTGAG-3′ 5′-TATTCTTCAGATTGACGAAG-3′
BAGE	RT-PCR	5′-TGGCTCGTCTCACTCTGG-3′ 5′-CCTCCTATTGCTCCTGTTG-3′
GAPDH	RT-PCR	5′-TGCACCACCAACTGCTTAGC-3′ 5′-GGCATGGACTGTGGTCATGAG-3′

Results

Induced Expression of CG Antigens in Tumorigenic hMSC-TERT20

To evaluate whether the transformation process in long-term cultures of telomerase-immortalized hMSCs (hMSC-TERT20) (Figure 1A)[25,26] was associated with alterations in CG antigen expression, we used semiquantitative RT-PCR to investigate the expression of several members of different CG antigen gene families, including the GAGE family, MAGE-A1, HAGE, BAGE, XAGE-1, NY-ESO-1, the SSX family and BORIS in early-passage, non-tumorigenic hMSC-TERT20 (PDL 102), and late-passage tumorigenic hMSC-TERT20 (PDL 282) (Figure 1B). In primary hMSCs (passage 3) and non-tumorigenic hMSC-TERT20, which were phenotypically similar to primary cells and retained osteoblastic differentiation potential,[25] SSX family members were the only CG antigen genes expressed. In contrast, the tumorigenic hMSC-TERT20 exhibited high expression levels of GAGE family members, MAGE-A1 and XAGE-1, in addition to SSX1–9. Transcripts of HAGE, BAGE, NY-ESO-1, and BORIS were neither detected in primary hMSCs nor in hMSC-TERT20, although these gene products were amplified from a positive control human testis cDNA library. Real-time-based RT-PCR quantification of the expression levels showed that in tumorigenic versus non-tumorigenic hMSC-TERT20 cells GAGE members, MAGE-A1, XAGE-1, and SSX members were up-regulated 123-, 8-, 51- and 9-fold, respectively (Figure 1C). Equivalent expression of the above CG antigen genes was observed in primary hMSCs and non-tumorigenic hMSC-TERT20.

Next, we examined the changes in GAGE and MAGE-A expression at the protein level (Figure 1D). Immunohistochemistry showed that MAGE-A and GAGE proteins were exclusively expressed in tumorigenic hMSC-TERT20, confirming the previous quantitative PCR data. Surprisingly, not all tumorigenic hMSC-TERT20 cells were positive for GAGE and MAGE-A. GAGE family proteins were expressed in more than 95% of tumorigenic hMSC-TERT20 cells, while MAGE-A family proteins were only present in less than 1% of the cells. Such percentages indicate that the heterogeneity did not reflect a cell cycle-specific pattern of expression. No significant differences in staining intensities or patterns were observed between the spheroid and monolayer cultures. Thus, aberrant CG expression was not an artifact restricted to monolayer culture, nor affected by differences in cell shape or cell-to-cell contacts.

Heterogenic Expression of GAGE and MAGE-A Family Proteins in Single Cell Subclones of Tumorigenic hMSC-TERT20

A panel of single-cell clones derived from the late-passage tumorigenic hMSC-TERT20 (PDL 440), which exhibited differences in growth kinetics and tumorigenicity,[26] were also tested for expression of GAGE and MAGE-A family proteins by immunocytochemistry (Figure 2, A and B). A significant difference in the percentage of cells that expressed the two antigen families, as well as in the overall expression levels was observed between the clones. Four clones, BC8, BD6, CE8, and BD11, exhibited strong expression of GAGE in more than 95% of the

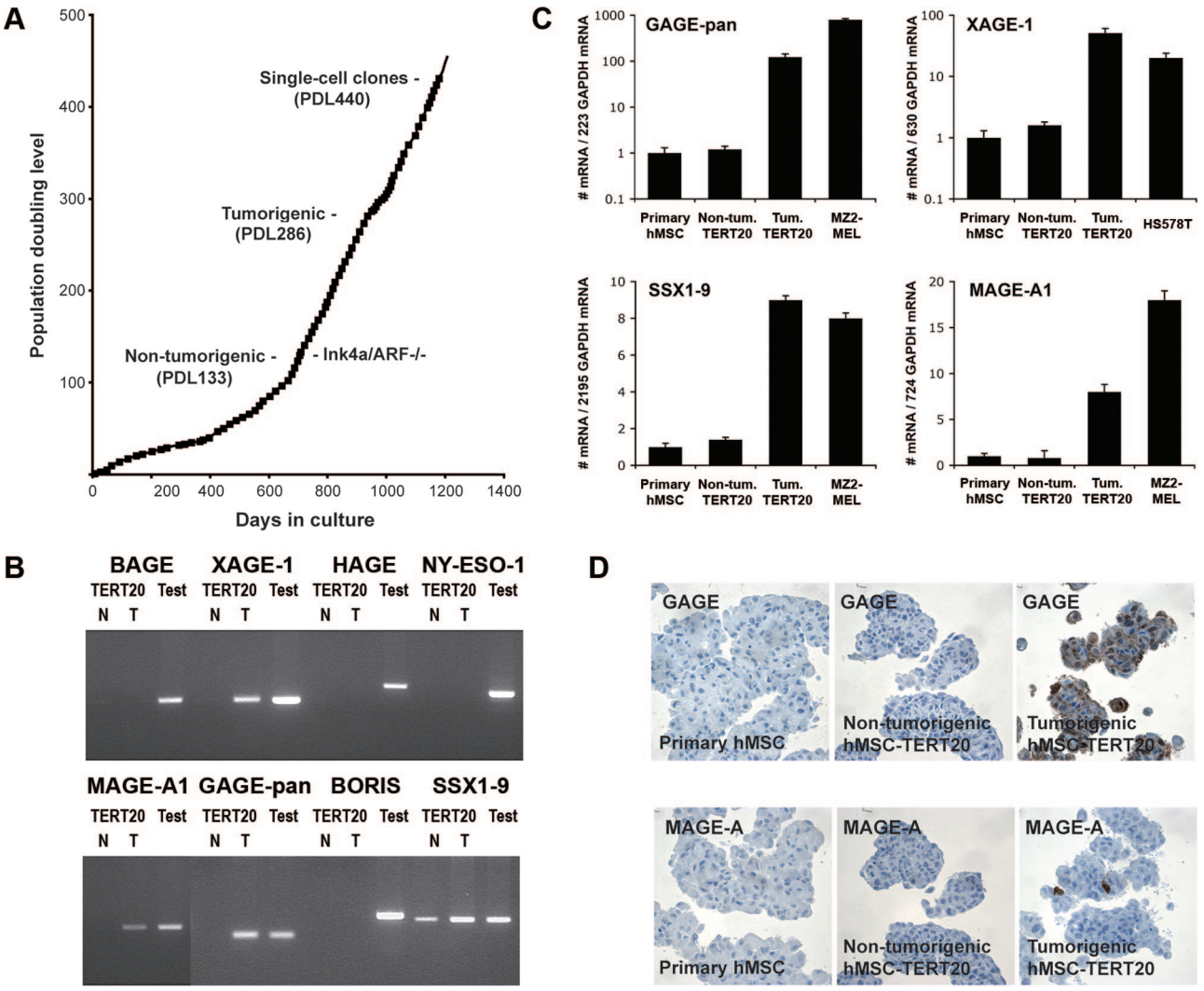

Figure 1. Expression of CG antigens in tumorigenic hMSC-TERT20. **A:** Growth curve of hTERT-transformed human mesenchymal stem cells with time-points indicating specific phenotypes. **B:** RT-PCR analysis of CG antigen gene expression in non-tumorigenic (N) and tumorigenic (T) hMSC-TERT20. Test = testis cDNA library (positive control). **C:** Quantitative RT-PCR analysis of CG antigen gene expression in primary hMSCs and non-tumorigenic and tumorigenic hMSC-TERT20 showing induced expression of CG antigens in tumorigenic hMSC-TERT20. **D:** Immunohistochemical analysis of GAGE and MAGE-A family proteins in primary hMSCs, and non-tumorigenic and tumorigenic hMSC-TERT20 cells (magnification = original ×20).

cells, similar to the parental population. In contrast, the expression in BB3 and DB9 was confined to approximately 1% and 0.1%, respectively. Like tumorigenic hMSC-TERT20, MAGE-A was expressed in less than 1% of the BD6, BD11, CE8, and DB9 cells, yet it was expressed in approximately 5% and 40% of BC8 and BB3 cells, respectively. The differences in the frequencies of GAGE- and MAGE-A-positive cells among the single cell clones demonstrate that GAGE and MAGE-A were not consistently co-expressed. In addition, the levels of GAGE or MAGE-A expression could not be correlated with phenotypic differences between the clones, including anchorage-independent growth, serum dependence, xenograft tumorigenicity or spheroid growth (Table 2).

The GAGE and MAGE-A expression in mouse xenograft tumors derived from the single-cell clones was found to be similar in both intensity and frequency to that in the *ex vivo* spheroids; indicating that the expression was not influenced by the surrounding tissue or other external factors (Figure 2, A and B). Interestingly, in tumors established from clones that expressed GAGE or MAGE-A only in a small percentage of cells, the GAGE- and MAGE-A-positive cells were localized in foci, suggesting that this phenotype was inherited by daughter cells.

Expression of GAGE and MAGE-A Proteins in Sarcomas

Next, we examined the expression of GAGE and MAGE-A CG antigens in a panel of 35 sarcomas, including the major subtypes, such as fibrosarcoma, liposarcoma, leiomyosarcoma, and rhabdomyosarcoma (Figure 3, A–D; Table 3). GAGE family proteins were expressed in 15 of the 35 sarcomas (including specimens of liposarcoma, malignant fibrous histiocytoma, schwannoma, epithelial sarcoma, leiomyosarcoma, synovial sarcoma, an-

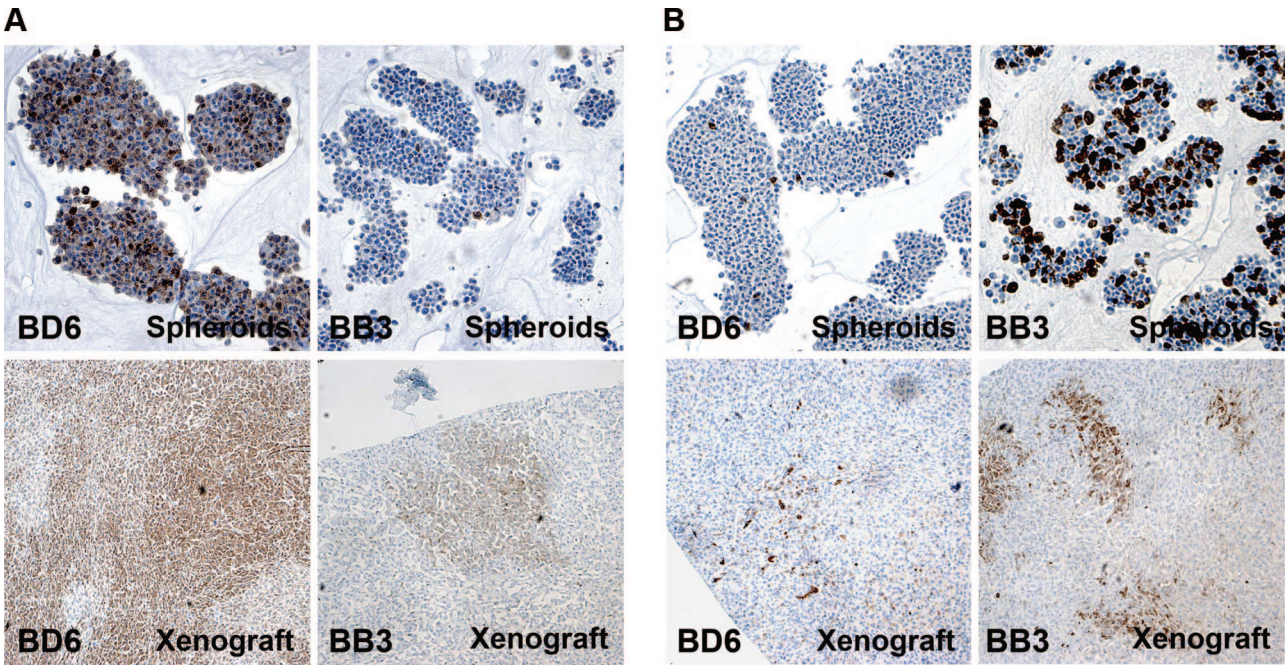

Figure 2. GAGE (**A**) and MAGE-A (**B**) expression in tumorigenic hMSC-TERT20 single-cell clones. GAGE and MAGE-A expression in hMSC-TERT20 subclones BD6 and BB3 grown as spheroid cultures or mouse xenograft tumors (magnification = original ×10 (**top** panels), and ×20 (**bottom** panels)).

giosarcoma, fibrosarcoma) (Figure 3, A–D; Table 3). In 13 of these specimens, the staining was confined to a rare population of cells with a disseminated localization within the tumor, while most cancer cells were stained in the remaining two specimens. GAGE proteins were localized to both the cytoplasm and nucleus of the cancer cells. Ten sarcomas of different subtypes were MAGE-A-positive (including specimens of liposarcoma, malignant fibrous histiocytoma, schwannoma, epithelial sarcoma, leiomyosarcoma, rhabdomyosarcoma, angiosarcoma, fibrosarcoma) (Figure 3, E–H; Table 3), and in 7 of these 10, the staining was also limited to a subset of cancer cells with a diffuse localization similar to that observed for GAGE, while most cancer cells were positive in the remaining three specimens. All MAGE-A-positive specimens exhibited strong staining of the nucleus, and in some specimens, cytoplasmic staining was also observed. The nuclear localization of MAGE-A proteins was consistent with a previous report of MAGE-A2 down-regulation of p53 transactivating function in U2OS osteosarcoma cells through recruitment of HDACs.[34]

Expression of GAGE and MAGE-A was not associated with a specific subtype of sarcoma. Five sarcomas were positive for both GAGE and MAGE-A.

Methylation Status of CG Antigen Promoters

Based on earlier reports showing that CG antigen genes are regulated by promoter methylation, we investigated whether induction of GAGE and MAGE-A1 expression in tumorigenic hMSC-TERT20 was associated with a decrease in methylation of the corresponding gene promoters (Figure 4). Bisulfite sequencing showed that the proximal promoter and intron 1 of the GAGE family genes exhibited decreased methylation in both non-tumorigenic (26% CG methylation) and tumorigenic (28%) hMSC-TERT20, but there was no considerable difference in the level of DNA methylation or in the methylation pattern between non-tumorigenic and tumorigenic cells. This indicates that hypomethylation may be required for induction of the GAGE genes, but it was not sufficient in itself for induction of expression.

Table 2. CG Antigen Expression and Growth Characteristics of hMSC-TERT20 Late-Passage Clones

								GAGE†		MAGE-A†	
Cells	DT (d)*	Saturation density (10^{-4} cells/ml)*	Contact inhibited*	Soft agar colonies (%)*	Monolayer Ki-67 (%)*	Tumors (n/n)*	Viability in 0.1% serum (%)*	Positive cells (%)	Intensity	Positive cells (%)	Intensity
hMSC-TERT20	1.20 ± 0.00	26.3 ± 1.6	No	8.6 ± 2.3	ND	10/10	ND	>95	+++	<1	+++
-BB3	1.06 ± 0.08	23.2 ± 2.0	No	0	66.3 ± 4.6	5/5	39 ± 4.6	~1	++	~40	+++
-BC8	1.32 ± 0.03	21.5 ± 1.8	No	0	71.1 ± 8.5	3/10	16 ± 1.5	>95	+++	~ 5	+++
-BD6	1.21 ± 0.11	36.8 ± 1.8	No	3 ± 0.5	69.6 ± 3.5	5/5	37 ± 6.1	>95	++	<1	++
-BD11	1.25 ± 0.14	21.5 ± 1.9	No	16 ± 2.9	68.4 ± 3.4	5/5	90 ± 6.4	>95	+++	<1	++
-CE8	1.19 ± 0.14	14.7 ± 0.5	No	0	58.1 ± 4.3	5/5	59 ± 4.5	>95	+++	<1	+++
-DB9	1.10 ± 0.08	24.4 ± 2.2	No	12 ± 2.9	76.3 ± 7.1	5/5	91 ± 3.0	<1	++	<1	++

*The phenotype of hMSC-TERT20 and derived clones have been published previously.[26]
†Determined by immunohistochemical staining.

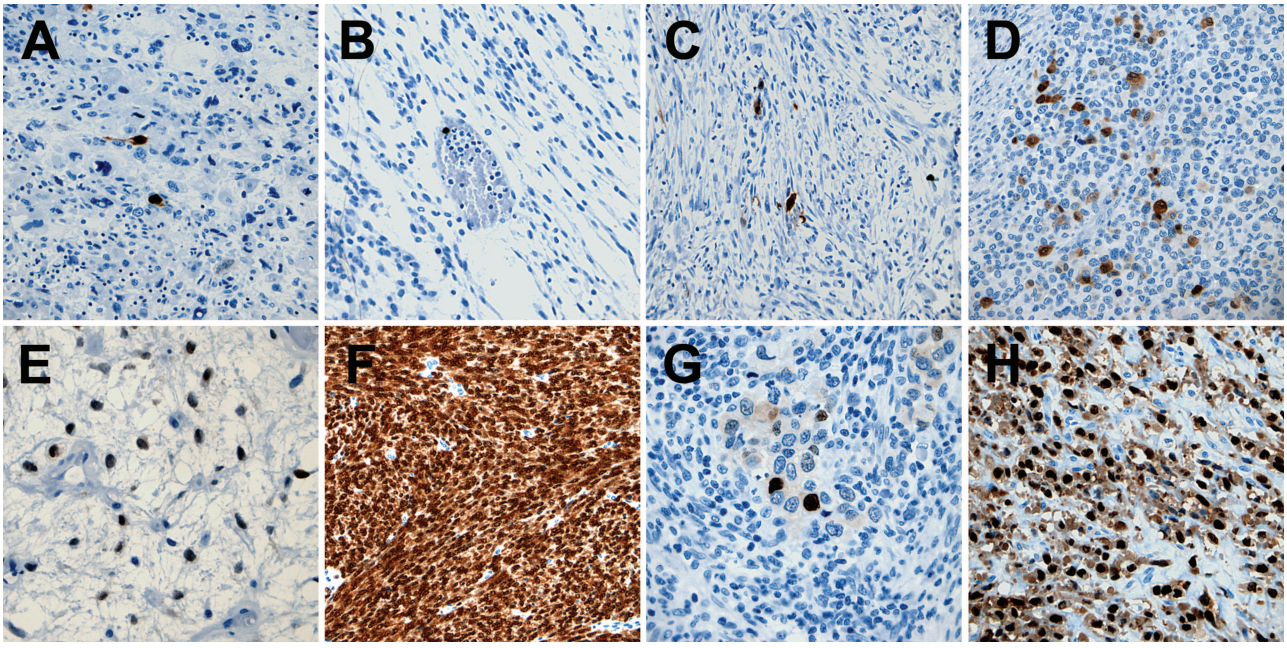

Figure 3. Expression of GAGE and MAGE-A family proteins in sarcoma. GAGE expression in malignant fibrous histiocytoma (**A**), round cell liposarcoma (**B**), leiomyosarcoma (**C**), and epithelial sarcoma (**D**). MAGE-A expression in myeloid liposarcoma (**E**), fibrosarcoma (**F**), epithelial sarcoma (**G**), and leiomyosarcoma (**H**). Magnification = original ×40 (**A**, **D**, **G**), magnification = original ×20 (**B**, **C**, **E**, **F**, **H**).

Similar to the GAGE genes, the DNA methylation of the *MAGE-A1* proximal promoter was significantly reduced in both non-tumorigenic (48%) and tumorigenic hMSC-TERT20 (46%), with no notable difference between the two (Figure 4). In contrast, methylation of the CGs proximal to the transcription initiation site (−63 to +32) was maintained in both. However, these data are not conclusive as *MAGE-A1* was expressed in less than 1% of cells of tumorigenic hMSC-TERT20, and thus all of the *MAGE-A1* alleles analyzed by bisulfite sequencing may be derived from MAGE-A1-negative cells, which may have maintained methylation of these important CGs. Previous reports have demonstrated an association between *MAGE-A1* transcription and demethylation of the CGs spanning the transcription initiation site.[35,36] In the hMSC-TERT20-subclone BB3 the percentage of MAGE-A-positive cells was 40%. Nonetheless, bisulfite sequencing did not show an increase in MAGE-A1 promoter demethylation, as compared with the parental hMSC-TERT20.

In primary hMSCs the level of *GAGE* and *MAGE-A1* DNA methylation was significantly higher than in hMSC-TERT20 (71% and 81%, respectively), but lower than what has been observed in other CG antigen-negative tissues (Figure 4).[16,20]

Bisulfite sequencing of the *CTAG1* (NY-ESO-1) promoter showed that the level of DNA methylation was relatively high in both primary hMSCs (86%) and tumorigenic hMSCs (84%), consistent with the lack of NY-ESO-1 expression in both (Figure 4).

Effect of DNMT and HDAC Inhibitors on CG Antigen Expression in hMSCs

To study the epigenetic mechanisms controlling the expression of CG antigen genes in hMSCs and to examine the potential use of DNMT and HDAC inhibitors to modulate CG antigen gene expression, non-tumorigenic hMSC-TERT20 and primary hMSCs were treated with

Table 3. Immunohistochemical Analysis of GAGE and MAGE-A Expression in Sarcomas

Sarcoma type	GAGE expression	Characteristics of expression	MAGE-A expression	Characteristics of expression
Liposarcoma	3/8	3 disseminated	2/8	2 disseminated
Malignant fibrous histiocytoma	3/4	3 disseminated	1/4	1 disseminated
Rhabdomyosarcoma	0/3	—	1/3	1 disseminated
Schwannoma	1/1	1 extensive	1/1	1 disseminated
Epithelial sarcoma	2/3	2 disseminated	1/3	1 extensive
Leiomyosarcoma	2/3	1 extensive 1 disseminated	2/3	1 extensive 1 disseminated
Synovial sarcoma	1/3	1 disseminated	0/3	—
Angiosarcoma	1/3	1 extensive	1/3	1 disseminated
Fibrosarcoma	2/4	2 disseminated	1/4	1 disseminated

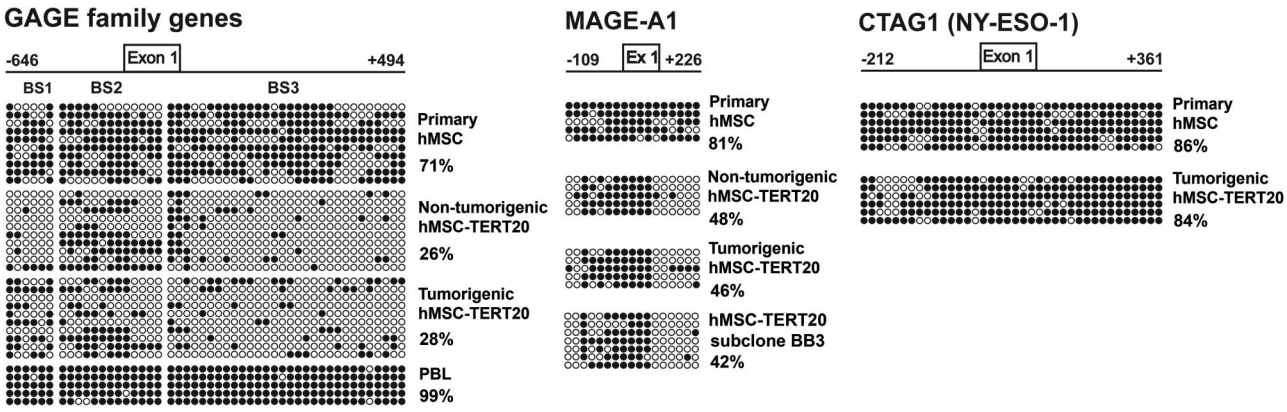

Figure 4. CG methylation of GAGE, MAGE-A1, and NY-ESO-1 promoters in primary hMSCs (passage 3), and non-tumorigenic (PDL 102) and tumorigenic hMSC-TERT20 (PDL 282). CG antigen promoter gene fragments were amplified from sodium bisulfite-modified DNA and subsequently 10 GAGE products, 5 MAGE-A1 products, and 5 NY-ESO-1 products were sequenced for each DNA sample. GAGE promoter methylation was examined in peripheral blood lymphocytes (PBL) as negative control. Black dots = methylated cytosines; white dots = un-methylated cytosines.

5-AZA-CdR and TSA (Figure 5) and expression was evaluated by RT-PCR. Treatment of the former with the HDAC-inhibitor TSA resulted in a 10.5-fold increase in GAGE expression, indicating that histone acetylation may be required for induced expression. In contrast, the DNMT-inhibitor 5-AZA-CdR had little effect on the GAGE expression, consistent with the result of the bisulfite sequencing showing that the GAGE genes was hypomethylated in these cells. The expression of MAGE-A1 was increased 12.6 times by 5-AZA-CdR and was unaffected by TSA. The effect of 5-AZA-CdR on MAGE-A1 expression in non-tumorigenic hMSC-TERT20, despite a significant degree of MAGE-A1 hypomethylation, may be associated with additional demethylation of the CGs proximal to the transcription start site. Similar to MAGE-A1, the NY-ESO-1 gene (CTAG1) was highly induced by 5-AZA-CdR, whereas TSA treatment resulted in only a small increase.

Due to the clinical implication of using 5-AZA-CdR and TSA to enhance the expression of CG antigen genes in cancer cells, we evaluated whether these agents would have similar effects on normal stem cells (Figure 5). Treatment of primary bone marrow-derived hMSC with 5-AZA-CdR and TSA under the same conditions as described above, did not cause significant changes in the expression of GAGE, MAGE-A1, and NY-ESO-1 CG antigen genes. This cancer cell-specific selectivity of 5-AZA-CdR and TSA may be important for their clinical use.

The tumorigenic hMSC-TERT20 subclones BB3 and DB9 contained only subsets of GAGE-positive cells (Figure 6, A and C). To investigate whether a more ubiquitous GAGE expression could be achieved in these cells, they were treated with TSA and assessed by immunocytochemical staining. Interestingly, the TSA treatment resulted in expression of GAGE proteins in all cells of BB3 and DB9 (Figure 6, B and D). In addition, TSA seemed to exhibit enhanced cytotoxicity in BB3 and DB9 cells compared with primary hMSCs and non-tumorigenic hMSC-TERT20 (data not shown).

Induction of GAGE Gene Expression Is Associated with Histone H3 Lysine Acetylation

The results presented above suggested that histone acetylation was involved in regulation of the expression of GAGE genes in hMSC-TERT20. To investigate this association further, we compared the levels of GAGE gene histone acetylation in non-tumorigenic and tumorigenic hMSC-TERT20 using ChIP-PCR, and found that the *GAGE* promoter and intron 1 histone H3 lysine acetylation was increased 2.5 and 3.8 fold in the tumorigenic cells (Figure 6, E and F). This further demonstrated a role for histone acetylation in regulation of GAGE gene expression.

Discussion

CG antigens are promising targets of cancer-specific immunotherapy, and their expression in numerous types of cancer has been extensively examined. However, CG antigens have not been evaluated in the context of tumor stem cells. In this study we investigated whether the molecular changes associated with the spontaneously acquired tumorigenicity of cultivated hMSCs (ie, hMSC-TERT20) included alterations in CG antigen expression. We found that several families of CG antigens (ie, GAGE, MAGE-A1, and XAGE-1) were highly expressed in tumorigenic hMSC-TERT20, but absent from primary hMSCs and non-tumorigenic hMSC-TERT20. In addition, we found that the induction of CG antigen expression occurred at approximately the same population doubling level as the tumorigenic transformation of hMSC-TERT20. Our results indicate that CG antigen expression may be associated with tumorigenic transformation of stem cells and further suggest that CG antigen-based immunotherapy could selectively target a tumor stem cell population, but not the normal stem cells.

CG antigens are often heterogeneously expressed in tumors, including sarcomas, and are frequently found only in a small percentage of cancer cells in a given

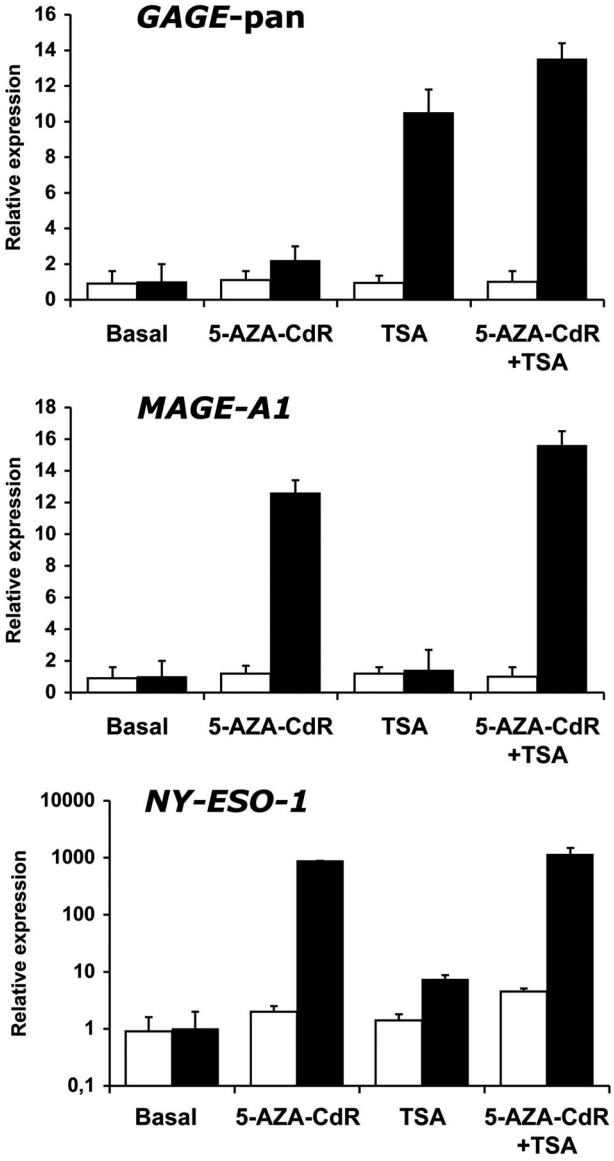

Figure 5. Effect of DNMT-inhibitor 5-AZA-CdR and HDAC-inhibitor TSA on the expression levels of GAGE, MAGE-A1, and CTAG1 (NY-ESO-1) in primary hMSCs (passage 3) (white columns) and non-tumorigenic hMSC-TERT20 (PDL 133) (black columns). Relative expression was evaluated by RT-PCR.

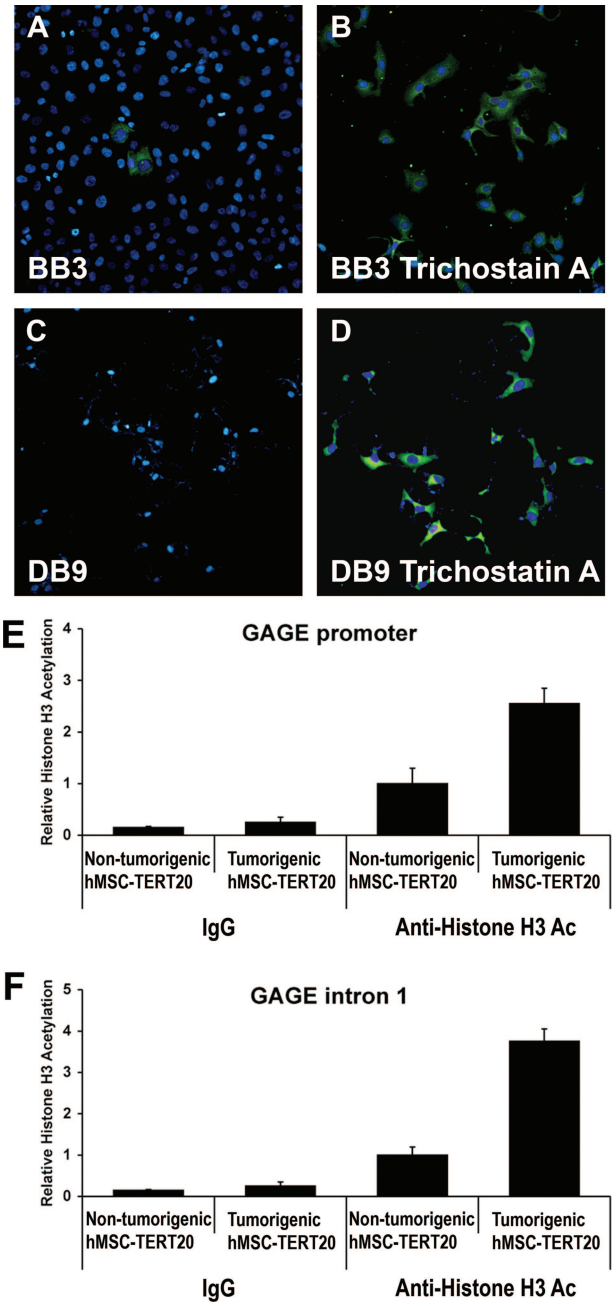

Figure 6. Induction of GAGE gene expression is associated with histone acetylation. HDAC inhibitor TSA induced expression of GAGE (fluorescein isothiocyanate, green) in all cells (>90%) of hMSC-TERT20 subclones BB3 (**A**, **B**) and DB9 (**C**, **D**) (Nuclear stain = 4,6-diamidino-2-phenylindole, blue; magnification = original ×20). ChIP analysis of GAGE gene-associated histone H3 acetylation in non-tumorigenic and tumorigenic hMSC-TERT20 (parental cell line) (**E**, **F**). Chromatin was precipitated with anti-acetylated histone H3 antibody or normal rabbit IgG (negative control) and subjected to quantitative PCR using two sets of primers each recognizing the promoter and intron 1 of the GAGE genes.

tumor. The reason for this heterogeneity is not known. We found that tumorigenic hMSC-TERT20 also exhibited heterogeneity in expression of GAGE and MAGE-A CG antigens. A persistent variation in expression was found among cells of single-cell subclones, indicating that the heterogeneity was unlikely to be due to genetic variation. We found no indication that the subpopulation-specific expression of GAGE and MAGE-A was related to the cell cycle. Xenograft tumors, formed from hMSC-TERT20 clones, also exhibited heterogeneity in GAGE and MAGE-A expression. The positive cells were located in foci, suggesting that the phenotype was passed on to daughter cells. This genotype-independent inherited expression of CG antigens in hMSC-TERT20 subclones could represent a hierarchical cancer stem cell model, in which GAGE- and MAGE-A-expression would be restricted to undifferentiated cancer stem cells or cancer cells undergoing a specific step of the differentiation process. Consistent with this notion the tumorigenic hMSC-TERT20 single-cell subclones exhibited heterogeneous expression of differentiation biomarkers.[27] These data are interesting seen in the context of recent studies, suggesting that both embryonic and adult stem cells are

heterogeneous, with cells moving between two or more metastable states.[37] In addition, these cell states exhibited a biased differentiation potential correlating with expression of specific transcription factors and patterns of epigenetic modification.

Epigenetic factors have been shown to be important for regulation of CG antigen genes, and often promoter CG hypomethylation alone can induce expression of different types of CG antigens. We investigated whether gene hypomethylation was involved in induction of GAGE and MAGE-A1 expression in tumorigenic hMSC-TERT20. Bisulfite sequencing revealed that significant hypomethylation of GAGE and MAGE-A1 promoters was present in both non-tumorigenic and tumorigenic hMSC-TERT20 and thereby preceded induction of GAGE and MAGE-A1 expression. Thus promoter hypomethylation may be required, but was not sufficient, for induction of expression. To further understand the epigenetic regulation of these genes in hMSC-TERT20, we treated non-tumorigenic, CG antigen-negative cells with the DNMT-inhibitor 5-AZA-CdR and the HDAC-inhibitor TSA. This suggested that while DNA methylation was the main mechanism for regulation of the MAGE-A1 gene, histone acetylation was also required for induction of GAGE gene expression. The involvement of histone acetylation in GAGE gene expression was further indicated by ChIP analysis showing that tumorigenic cells exhibited increased lysine acetylation of GAGE gene-associated histones compared with non-tumorigenic cells. The sequence of events leading to activation of GAGE gene transcription in HEK293 cells was recently addressed by D'Alessio et al,[38] who showed that DNA demethylation was preceded by histone acetylation and was also dependent on RNA transcription. Our results demonstrate that another scenario is possible, since in our model DNA demethylation preceded transcription, and histone acetylation seemed to be among the final events that allowed transcription to occur.

Tumor heterogeneity in CG antigen expression may limit its exploitation as therapeutic targets. The heterogeneity in GAGE and MAGE-A expression in tumorigenic hMSC-TER20 suggests that this can also apply to cancer stem cells. However, DNMT and HDAC inhibitors potently induce the expression of CG antigen genes in tumor cells *in vitro* and are promising therapeutic agents for enhancement of CG antigen expression in tumors. As described above, these agents can also modulate the expression of CG antigens in hMSC-TERT20 cells. In addition, we found that the heterogeneous GAGE expression in tumorigenic hMSC-TERT20 single-cell derived subclones (ie, BB3 and DB9) could be changed to a more ubiquitous expression by TSA. Importantly, we also demonstrated that neither 5-AZA-CdR nor TSA could induce expression of CG antigen genes in primary hMSCs. These results suggest that the effect of TSA and 5-AZA-CdR on CG antigen gene expression may be limited to cells with unidentified pre-tumorigenic/tumorigenic alterations, or that cycling cells in general are susceptible to induction of CG antigen expression by these agents. 5-AZA-CdR is a nucleoside analogue of cytosine and a substrate of DNMTs. When incorporated into DNA, it covalently attaches to DNMT and depletes it from the cell.[39] Therefore, 5-AZA-CdR probably affects the gene expression in all proliferating cells. In contrast, modification of histones by HDACs is independent on cell proliferation, although replication-dependent *de novo* modification of histones may also occur.[40] Thus, the restriction of TSA-mediated inhibition of HDACs to pre-tumorigenic/tumorigenic cells remains to be explained.

Our results show that CG antigens are potential targets of tumor stem cells and that epigenetic drugs may induce expression and overcome heterogeneity in expression of CG antigens. However, additional work is required to demonstrate the therapeutic efficacy of such an approach.

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