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Regulation of cancer germline antigen gene expression: implications for cancer immunotherapy

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Abstract

Cancer germline (CG; also known as cancer-testis) antigen genes are normally expressed in germ cells and trophoblast tissues and are aberrantly expressed in a variety of human malignancies. CG antigen genes have high clinical relevance as they encode a class of immunogenic and highly selective tumor antigens. CG antigen-directed immunotherapy is undergoing clinical evaluation for the treatment of a number of solid tumor malignancies and has been demonstrated to be safe, provoke immune responses and be of therapeutic benefit. Achieving an improved understanding of the mechanisms of CG antigen gene regulation will facilitate the continued development of targeted therapeutic approaches against tumors expressing these antigens. Substantial evidence suggests epigenetic mechanisms, particularly DNA methylation, as a primary regulator of CG antigen gene expression in normal and cancer cells as well as in stem cells. The roles of sequence-specific transcription factors and signal transduction pathways in controlling CG antigen gene expression are less clear but are emerging. A combinatorial therapeutic approach involving epigenetic modulatory drugs and CG antigen immunotherapy is suggested based on these data and is being actively pursued. In this article, we review the mechanisms of CG antigen gene regulation and discuss the implications of these mechanisms for the development of cancer immunotherapy approaches targeting CG antigens.

Keywords

cancer germline antigen; cancer testis antigen; cancer vaccine; DNA hypomethylation; DNA methylation; epigenetics; immunotherapy; NY-ESO-1

Over a century ago, John Beard proposed a ‘trophoblastic theory of cancer’ based on the similar biological features of trophoblasts and cancer cells. This theory predicts that cancers may arise from germ cells that fail to complete their embryonic migration to the gonads [1]. Recent evidence supports an association between germ-cell development and cancer, including the observation that a range of human cancers produce human chorionic gonadotropin [1].

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Additional evidence for the trophoblastic theory of cancer is provided by the discovery of protein antigens that are present only in germ cells, trophoblasts and tumors. These antigens were initially described as cancer-testis (CT) antigens based on their expression pattern; however, recent data suggest that these genes may be more accurately considered as cancer-germline (CG) antigens, as they are commonly expressed in the fetal ovary [1–3]. The discovery of CG antigens supports the theory that one cause of tumorigenesis is the abnormal activation of a gametogenic program in somatic cells.

Cancer germline antigen genes have been classified as genes expressed in tumors as well as in testicular and ovarian germ cells and/or the placenta, with expression in no more than two nongermline normal tissues [1]. In the cases where nongermline normal tissue expression is detected, it is a fraction of the level detected in the testis. The general characteristics of CG antigen genes include: existence in multigene families that are frequently localized on the X chromosome (CG-X antigens), immunogenicity in cancer patients, heterogeneous expression in cancer tissues, *in vitro* activation in cancer cell lines by epigenetic modulatory drugs and increased expression during tumor progression [4]. CG-X antigens comprise approximately 10% of the genes on the X chromosome, are grouped into multigene families and are frequently expressed in premeiotic spermatocytes [5,6]. Non-X CG antigens are located on autosomal chromosomes, do not appear to exist as multigene families and are often expressed during meiosis [6]. Unlike CG-X antigens, non-X CG genes show less restriction of expression to the testis, and their immunogenicity has not yet been clearly validated [5,7]. CG antigen gene classification also includes subgroups: testis restricted, testis/brain restricted and testis selective [5]. CG antigen genes are referred to by their original given names as well as their assigned CT number identifier [6,8]. Over 130 CG antigen genes have now been described; these belong to 83 gene families that separate into 12 distinct groups based on sequence homology [5,7].

Melanoma antigen-1 (MAGE-A1), the first CG antigen identified, was discovered by Boon and colleagues from a human melanoma patient using autologous typing [9]. Using this strategy, the gene encoding the tumor antigen MZ2-E was cloned and termed *MAGE-1* (later re-named *MAGE-A1*). Additional studies showed that *MAGE-A1* is expressed in the testis but not normal somatic tissues, and its expression is not limited to melanoma; it is expressed in other cancer types, including breast cancer [4]. Using T-cell epitope cloning, MAGE-A2, MAGE-A3, BAGE and GAGE antigens were also discovered as antigens recognized by cytotoxic T lymphocytes (CTL) in melanoma patients [4]. Later genetic studies revealed 12 closely related genes, comprising the *MAGE-A* family, located at Xq28 [1]. A second cluster of *MAGE* genes, the *MAGE-B* family at Xp21.3, was discovered in an investigation of the gene responsible for the sex-reversal phenotype [1]. A third cluster of *MAGE* genes is the *MAGE-C* family at Xq26–27 [1]. Expression of *MAGE-A*, *-B* and *-C* genes is restricted to germ cells and cancer, while other clusters of *MAGE* genes (*MAGE-D* to *MAGE-L*) are also expressed in normal somatic tissues [1,10].

The difficulty of the T-cell epitope cloning approach led to the development and use of serological analysis of recombinant tumor cDNA expression libraries with autologous serum (SEREX) to identify novel tumor antigens [4]. This method allows for the isolation of tumor antigens that provoke a high-titer IgG response in human hosts. A number of novel CG antigens, including NY-ESO-1, have been discovered using SEREX [4]. Additional CG antigen genes have been discovered using mRNA expression profiling, by searching for genes expressed in the testis tissues and cancer but not normal somatic tissues [5]. Technologies used in this approach include representational difference analysis, differential display and microarray analysis [4]. Although transcript profiling alone cannot establish tumor-antigen-coding genes that are immunogenic in cancer patients, genes that show cancer- and germline-specific expression are generally referred to as CG antigen genes [7].

Some cancers frequently express CG antigen genes while others only rarely express them. For example, melanoma, lung and bladder cancers frequently express CG antigen genes, while colon, renal and leukemia/lymphoma rarely express these genes [1,4,5]. Another common observation is that tumors that express CG antigen genes tend to express multiple CG antigen genes simultaneously, suggesting a common regulatory mechanism [1,11,12]. Finally, CG antigens often show heterogeneous expression within individual tumors, as determined by immunohistochemistry (IHC). This has significant consequences for the therapeutic targeting of these antigens using immunotherapy [1,13–15].

Epigenetic regulation of CG antigen genes

The coordinate expression of CG antigen genes suggests a common molecular mechanism of gene activation. A large body of work suggests that epigenetic regulation is a key mechanism in the transcriptional regulation of CG antigen genes [16]. Epigenetic regulation refers to heritable gene expression states that are not directly controlled by DNA base sequence; this control mechanism prominently includes cytosine DNA methylation (hereafter referred to as DNA methylation) and covalent histone modifications [17].

DNA methylation

DNA hypomethylation (both global and promoter specific) is associated with CG antigen gene expression in normal somatic tissues and cancer [18,19]. Importantly, global DNA hypomethylation occurs during gametogenesis, the normal setting for CG antigen gene expression [1]. Global DNA hypomethylation, which classically affects repetitive DNA sequences, occurs in a variety of human cancers and also leads to hypomethylation at the normally methylated CpG-rich promoters of CG antigen genes [14,18,20]. The first evidence for the activation of CG antigen genes by DNA hypomethylation was provided by Weber *et al.*, who demonstrated that 5-aza-2'-deoxycytidine (decitabine), one of the DNA methyltransferase inhibitors (DNMTi), induced *MAGE-A1* gene expression in cultured melanoma cells [21]. In addition to decitabine, other DNMTi, including zebularine, have been shown to activate CG antigen gene expression [16]. Key mechanistic evidence for an important role for DNA methylation in CG antigen gene suppression is provided by studies demonstrating that hypomethylated CG antigen promoter transgenes show high activity in cells in which the corresponding endogenous CG antigen gene and promoter is repressed and hypermethylated [22]. Thus, DNA hypermethylation is sufficient to suppress CG antigen gene promoter activity in the presence of endogenous factors that would otherwise lead to gene activation.

Most CG antigen gene promoters contain either CpG islands [23] or CpG-rich regions that are regulated by DNA methylation. A high density of CpG sites at CG antigen promoter regions may indicate the relative importance of DNA methylation in the regulation of these genes, while other regulatory factors may be more important at CpG-poor promoters. DNA methylation exerts a repressive effect on transcription by preventing the binding of transcription factors and by recruiting methyl-CpG-binding domain proteins (MBDs), which attract repressor complexes that modify chromatin structure by deacetylating or methylating specific residues on histones [24]. MBD1, a member of the MBD protein family, appears to play an important role in the repression of *MAGE-A* genes [25].

DNA methylation patterns are acquired during development by a process that involves a phase of extensive demethylation in the early embryo prior to blastulation, followed by a stage of *de novo* methylation at the time of embryo implantation [26]. Methylation patterns are inherited through cell generations by a maintenance process that copies pre-existing methylation patterns onto the nascent DNA strand. Three enzymatically active DNA methyltransferases are present in mammals: DNMT1, DNMT3a and DNMT3b. DNMT1 can recognize and methylate both unmethylated and hemimethylated CpG duplexes, but is thought to be primarily responsible

for the maintenance of DNA methylation [27]. By contrast, DNMT3a and DNMT3b preferentially recognize and methylate unmethylated CpG substrates and are responsible for the *de novo* methylation during early embryonic development [27]. Importantly, both classes of DNMTs have been demonstrated to be involved in CG antigen gene regulation [28]. Knockout of both *DNMT1* and *DNMT3b*, but neither gene alone, is associated with CG antigen gene activation and promoter hypomethylation in the colon cancer cell line HCT116 [28]. Loss of *DNMT1* and *DNMT3b* in HCT116 similarly induces both X-linked and autosomal CG genes [28,29]. By contrast, in another colon cancer cell line (RKO), loss of *DNMT1* alone is sufficient to induce CG antigen gene expression [28]. Finally, downregulation of DNMT1, but not DNMT3a or DNMT3b, results in the activation of a methylated *MAGE-A1* transgene in melanoma cells [30]. Taken together, the data suggest that DNMT1 is the key enzyme involved in CG antigen gene methylation, but that the DNMT3 enzymes play a role in a cell context-dependent manner.

Unlike the majority of CG antigen genes, whose protein products have an unclear function in cancer, *MAGE-A11* appears to directly contribute to the development of androgen independent prostate tumor growth by stimulating the activity of the androgen receptor [31]. Like other *MAGE-A* genes, *MAGE-A11* expression is regulated by DNA methylation status. In castration-recurrent prostate tumors, *MAGE-A11* is upregulated, correlating with hypomethylation of discrete CpG sites adjacent to the transcriptional start site of the gene [31]. By contrast, the methylation status of other regions of the *MAGE-A11* promoter CpG island does not correlate with gene expression [31]. Furthermore, treatment of prostate cells with decitabine causes upregulation of *MAGE-A11* expression [31]. It remains to be determined whether *MAGE-A11* upregulation in prostate cancer is a specifically selected event or whether it correlates with a global epigenetic defect impacting other CG antigen genes. Regardless, DNA hypomethylation-mediated activation of *MAGE-A11* provides an illustration of how epigenetic activation of CG antigen genes directly contributes to oncogenesis.

Histone modifications

Histone modifications are also involved in epigenetic regulation of CG antigen gene expression [16,28,29,32,33]. Histones are basic proteins that are assembled with DNA into nucleosomes, the basic unit of chromatin. N-terminal histone protein tails protrude from the nucleosome, are post-translationally modified and interact with other proteins [34]. A plethora of histone modifying enzymes, which post-translationally modify specific amino acid residues of the histone tails, have been described [35].

Histone deacetylases (HDACs) alter chromatin structure by promoting the interaction between positively charged histone tail lysines and the negatively charged DNA phosphodiester backbone. This has the overall effect of compacting chromatin structure and preventing the accessibility of DNA to transcription factors and RNA polymerase. Inhibition of HDACs by specific inhibitors (HDACi) in cells reverses this process and consequently leads to upregulation of many genes, including CG antigens [16,36]. It has been observed that combined treatment with DNMTi and HDACi can synergistically activate CG antigen gene expression [16]. Most studies using HDACi have observed that these drugs are able to augment the activation of CG antigen genes by DNMTi, but as single agents, they do not cause significant induction of CG antigen genes [16,37,38].

Histone methylation also regulates gene expression. Unlike histone acetylation, histone methylation can correlate with either gene activity or gene repression, depending on the lysine residue that is methylated. For example, while methylation of K9 or K27 of histone H3 (H3K9 or H3K27) are repressive marks, methylation of H3K4 is an active mark [39]. Direct evidence of a role for histone methylation in CG antigen gene regulation was revealed by the discovery that mouse embryonic stem (ES) cells sustaining knockout of the H3K9 methyltransferase

enzymes G9a or GLP show induced expression of *MAGE-A* genes [40,41]. These findings prompted an investigation of the role of G9a/GLP enzymes in CG antigen gene regulation in human cancer [33]. Genetic knockdown of *G9a* and/or *GLP* in human colon cancer cells reduces H3K9 methylation both globally and at CG antigen gene promoters but does not induce CG antigen gene expression [33]. Treatment of tumor cells with the G9a inhibitor BIX-01294 does not induce CG gene expression either [33]. However, G9a inhibition synergizes with decitabine to induce CG gene expression in cancer cells, suggesting that histone methylation plays an important accessory role to DNA methylation by repressing CG antigen gene expression [33]. Importantly, *G9a*- or *GLP*-null mouse ES cells are DNA hypomethylated, including at *MAGE-A* genes, implicating G9a in DNA methylation control in ES cells [33, 42,43]. By contrast, no effect of *G9a/GLP* knockdown on DNA methylation was observed in human cancer cells [33]. Taken together, these observations again support DNA methylation as a critical epigenetic regulator of CG antigen gene expression.

Enhancer of zeste homolog (EZH)2 is a histone methyltransferase and polycomb group protein that is linked to transcriptional repression via the propagation of the H3K27 trimethylation histone mark. In a recent study, EZH2 was implicated in the repression of *GAGE*-type CG antigen genes in breast cancer cell lines [44]. Combination pharmacological treatment with 3-deazaneplanocin A (DZNep), a potent inhibitor of EZH2, along with DNMTi and HDACi, led to the robust induction of *GAGE* genes [44]. Interestingly, these effects showed specificity to tumor cells relative to normal breast cells [44]. Notably, one breast cancer cell line was identified that showed low levels of DNA methylation along with low *GAGE* gene expression and EZH2-mediated H3K27 trimethylation was shown to influence repression in this cell type [44]. These data reveal that, in some cases, histone modifications can silence CG antigen expression in the absence of DNA hypermethylation.

CG antigen gene expression heterogeneity in cancer

One defining characteristic of CG antigen gene expression in human cancer is that the expression is heterogeneous [1]. This heterogeneity pertains to comparisons between different tumor types, different tumors of the same type (intertumor heterogeneity) and different cells or regions of the same tumor (intratumor heterogeneity). While intertumor heterogeneity has been linked to promoter DNA methylation status in a number of studies, until recently, the mechanisms regulating intratumor heterogeneity were unclear. An early study using clonal cell lines established from a human melanoma indicated that *MAGE-A3* expression differences correlated with promoter DNA methylation status *in vitro* [45]. More recently, microdissection of individual ovarian tumors based on NY-ESO-1 IHC staining status was performed, followed by promoter DNA methylation analysis [14]. Importantly, this study revealed a correlation between intratumor NY-ESO-1 expression heterogeneity and promoter DNA methylation [14]. This heterogeneity also extended to global DNA methylation status, as determined by total 5-methyl-2'-deoxycytidine measurement or methylation of the LINE-1 repetitive element [14]. These data provide definitive *in vivo* evidence that intratumor CG antigen expression heterogeneity is associated with promoter and global genomic DNA methylation status.

CG antigen gene expression in normal & cancer stem cells

The nature of CG antigen gene expression and epigenetic regulation in stem cells has been the subject of recent work. Unlike human cancer cell lines, in cultured murine ES cells, methylated *MAGE-A1* promoter transgenes undergo spontaneous hypomethylation and gene activation [30,46]. Consistent with a role for specific transcriptional activators in this process, mutation of the ETS transcription factor binding sites in the transgene leads to reduced DNA hypomethylation [46]. These observations suggest that transcriptional activation of hypomethylated CG antigen gene promoters protects against *de novo* DNA methylation [30]. The characteristic property of murine ES cells to promote DNA hypomethylation is evidenced

by the observation that *in vitro* differentiation of these cells into embryoid bodies promotes re-methylation of a *MAGE-A1* transgene [46]. While promoter transgenes show activity in murine ES cells, it is important to note that endogenous CG antigen genes are often inactive and hypermethylated in murine ES cells [33,40,41]. However, murine ES cells appear to show a high degree of epigenetic plasticity that can lead to CG antigen gene expression and promoter hypomethylation under experimental conditions that alter the epigenetic machinery [33,42, 43].

It has been hypothesized that the expression of CG antigen genes is a hallmark of stem cells and is linked to normal stem cell biology [47]. This idea is based on observations that undifferentiated human mesenchymal stem cells express several CG antigen genes [48]. This stem cell model proposes that CG antigen gene expression in tumors is not due to a specific gene activation event driven by oncogenesis, but rather is caused by the expansion of constitutively expressing cancer stem cells [47,48]. However, recently published studies have argued against this model [15,49]. Quantitative measurement of mRNA expression of CG antigen genes in a variety of human ES and mesenchymal stem cell lines reveal little-to-no CG antigen gene expression, coinciding with hypermethylation of these genes [49]. In another study, both primary and nontumorigenic mesenchymal stem cells showed little-to-no CG antigen gene expression [15]. However, immortalization of mesenchymal stem cells with telomerase sometimes led to tumorigenicity, coinciding with activation of CG antigen gene expression [15]. Both *in vitro* cultures and tumor xenografts of tumorigenic mesenchymal cell clones show heterogeneous expression of CG antigen genes by IHC, similar to human tumors [15]. CG antigen gene expression in tumorigenic mesenchymal stem cells is also induced by treatment with DNMTi and HDACi. Most interestingly, both tumorigenic and nontumorigenic immortalized mesenchymal stem cells display hypomethylated CG antigen gene promoters, despite the fact that only tumorigenic mesenchymal stem cells express these antigens [15]. This finding argues that specific oncogenic events are required for the activation of CG antigen gene promoters, even when these genes are hypomethylated. These data are consistent with earlier findings demonstrating that hypomethylation of CG antigen genes is insufficient for CG antigen gene expression in normal cell types, including human mammary epithelial cells and melanocytes [22].

Observations on the nature of CG antigen gene regulation in melanoma stem cells provide somewhat contrasting data to that previously described. Sigalotti *et al.* reported that cultured nonadherent melanoma cells, which are enriched for cells with stem-like properties, express CG antigen genes [50]. For *MAGE-A3*, expression in melanoma stem cells coincided with promoter DNA hypomethylation, suggesting that epigenetic regulation may also be an important mechanism of CG antigen gene regulation in cancer stem cells [50]. Geyde *et al.* investigated CG gene expression in putative melanoma stem cells isolated on the basis of CD133⁺ expression and made a number of interesting observations [51]. CG antigens were expressed at significantly higher levels in CD133⁺ cells compared with CD133⁻ cells, in a variety of melanoma cell lines. Furthermore, chemotherapy treatment *in vitro* selected for both CD133⁺ cells and enrichment of CG antigen gene expression. Finally, CD133⁺ cells were efficiently recognized by CTLs directed against NY-ESO-1 but not by CTLs targeting melanoma differentiation antigens [51]. These data suggest that CG-antigen-targeted therapies may be particularly useful in melanoma as compared with immunotherapy directed against differentiation antigens, owing to targeting of the putative cancer stem cell population. Outside of melanoma, enhanced expression of CG antigen genes in the cancer stem cell compartment was recently reported in glioma [52]. Putative glioma stem cells were isolated on the basis of tumor sphere formation and, in these cell populations, expression of CG antigen genes was frequently observed [52]. In addition, CG gene expression in these cells correlated with histone hypoacetylation and DNA hypomethylation, reinforcing the notion that epigenetic control processes are critical for the regulation of CG antigen genes in cancer stem cells [52].

Additional mechanisms of CG antigen gene regulation

While epigenetic mechanisms clearly play an important role in regulating CG antigen gene expression, there is mounting evidence for additional nonepigenetic mechanisms of CG antigen gene regulation. Although far less is known regarding these mechanisms, they appear to involve sequence-specific transcription factors as well as signal transduction pathways, including activated tyrosine kinases.

ETS factors

The ETS transcription factor sites have been shown to play a key role in regulating the expression of *MAGE-A1* as well as other *MAGE-A* genes. Two inverted ETS motifs near the transcriptional start site of *MAGE-A1* were shown to drive approximately 90% of the activity of the unmethylated promoter [53,54]. Luciferase assays demonstrated that *MAGE-A1* promoter transgenes are highly active in cells in which the endogenous *MAGE-A1* promoter is methylated and inactive, and that this activity is largely dependent on ETS sequences [53, 54]. The ETS consensus sequences in *MAGE-A* promoters contain a centrally located CpG, and methylation of this site results in a protrusion into the major groove of the DNA duplex, which inhibits ETS protein binding [22,55]. Electrophoretic mobility shift assays detected complexes that bind to the *MAGE-A1* ETS sites, although the identity of the protein(s) bound is unknown; the ETS protein family contains many members that could potentially bind to these sites [22,53,54]. Now that specific antibodies to most ETS family members are available, systematic electrophoretic mobility shift assay supershift analyses could be used to identify the specific ETS proteins that bind to *MAGE-A1* and other CG antigen gene promoters. When the CpG sites within the ETS sequences are methylated, nuclear proteins fail to bind *in vitro* [22,53,54]. Notably, the relative levels of *MAGE-A1*-promoter-binding factors is distinct between tumor and normal epithelial cells, providing a potential explanation for why DNMTi preferentially activate CG antigen genes in tumor cells [22]. Although the ETS factor(s) that drive *MAGE-A* expression *in vivo* remain unclear, it was recently shown that overexpression of ETS1 activates the expression of *MAGE-A* class genes in a mechanism dependent both on DNA hypomethylation and lack of MBD1 binding [25]. Overall, the data demonstrate that the epigenetic state of CG antigen gene promoters interplays with sequence-specific transcriptional activators to regulate CG antigen gene expression.

BORIS protein

The BORIS protein is a recently described paralog of the imprinting regulator and chromatin insulator protein CTCF [56,57]. Recent studies have implicated BORIS as playing a key role in promoting the expression of CG antigen genes in cancer [56,58,59]. Specifically, BORIS was observed to be recruited to the promoters of CG antigen genes, including *MAGE-A1* and *NY-ESO-1*, during activation induced by treatment with DNMTi; BORIS recruitment coincided with loss of CTCF binding to these regions [58,59]. BORIS overexpression was additionally shown to activate various CG antigen genes in normal human dermal fibroblasts [59]. CTCF/BORIS binding sites were noted to be present in a number of *MAGE-A* genes that showed co-expression in head-and-neck cancer [11]. In addition, BORIS overexpression in TERT-immortalized oral keratinocytes led to *MAGE-A* gene induction and DNA hypomethylation [11].

A potential mechanism to explain the activating role of BORIS at CG antigen gene promoters is that it binds the SET1A H3K4 methyltransferase, which propagates histone modifications associated with transcriptional activity [60]. Importantly, BORIS itself is an autosomal CG antigen that is activated by DNA hypomethylation in cancer [29,61]. A model has been suggested wherein the activation of BORIS leads to the coordinate induction of other CG antigen genes in cancer [11]. It is possible that other CG genes, such as *BRDT* and *SPO11*, that

regulate epigenetic states, act in concert with BORIS to amplify genome-wide epigenetic defects in cancer [62]. While these are attractive models supported by a number of observations, conflicting data exist. For example, De Smet and colleagues have shown that BORIS expression in human melanoma is not associated with *MAGE-A1* activation [63]. Moreover, in glioma stem cells, frequent expression of numerous CG antigen genes was observed, despite the absence of BORIS expression [52]. In addition, our group has observed that BORIS overexpression does not activate CG antigen gene expression or alter DNA methylation in ovarian cancer cells [Woloszynska-Read A *et al.*, Submitted]. It remains plausible that co-expression of BORIS with other epigenetically acting CG antigens may be required to fully induce CG antigen genes in specific cell or tissue contexts [62]. To summarize, BORIS is an intriguing potential global regulator of CG antigen gene expression in human cancer, but further studies will be necessary to clarify this role.

Tyrosine kinases

The KIT protein is a receptor tyrosine kinase (TK) with oncogenic activity that is expressed and/or mutated in a variety of malignancies [64]. Two recent studies identified a possible link between KIT and CG antigen gene expression [65,66]. Yang *et al.* found that treatment of mast cell lines with imatinib, a tyrosine kinase inhibitor that affects KIT as well as other TK targets, results in *MAGE-A*, -B and -C gene and protein downregulation and possibly also alters DNA methylation levels [66]. Another study found that ovarian germ cell tumors show frequent *KIT* mutations, as well as heterogeneous expression of CG antigen genes, including *MAGE-A4* [65]. These data provide evidence for a link between an oncogenic TK and CG antigen gene expression in cancer.

In addition to KIT, two recent papers have reported that FGF receptor (FGFR)2 has profound effects on the expression of *MAGE-A3* in thyroid and pituitary cancers [67,68]. FGFR2 is a member of the FGF family of high-affinity receptor TKs. FGFR2 activation was shown to downregulate *MAGE-A3* expression in association with the remodeling of histone H3 marks at the *MAGE-A3* promoter region [67]. Thus, the FGFR2 TK appears to have an opposing effect on CG antigen gene expression as compared with the KIT TK discussed earlier. It was not determined whether the FGFR2-induced histone changes correlated with DNA hypermethylation at the *MAGE-A3* promoter. Consistent with a negative regulatory role for FGFR2 in *MAGE-A3* expression, in thyroid carcinomas, it was noted that the *FGFR2* and *MAGE-A3* gene promoters show reciprocal DNA methylation patterns [67]. In pituitary tumors, it was additionally noted that tumors from females displayed increased *MAGE-A3* expression relative to males [68]. This prompted an investigation of the effects of estrogen (estradiol) and the estrogen receptor (ER) on *MAGE-A3* expression in mouse pituitary tumor cells. Robust activation of *MAGE-A3* was observed upon estradiol treatment and ER activation [68]. Whether this activation was through direct or indirect effects of ER on the *MAGE-A3* promoter was not determined. Overall, these studies reveal a potentially critical regulatory circuit governing *MAGE-A3* expression in cancer and suggest an important function for *MAGE-A3* in tumorigenesis. In agreement with this view, prior data suggest that MAGE proteins, including *MAGE-A3*, can repress p53 activity, and other data suggest that *MAGE-A* genes can promote cancer cell growth [11,68–70].

cAMP, Sp1 & p53

Other factors that have been reported to regulate CG antigen gene expression include cAMP, Sp1 and p53 [31,71,72]. cAMP upregulation in the cycling endometrium was shown to parallel increased *MAGE-A11* expression, suggesting that it may contribute to *MAGE-A11* expression [73]. Later studies revealed that cAMP treatment of prostate cells increases *MAGE-A11* expression, which consequently activates the androgen receptor, as *MAGE-A11* is an androgen receptor co-activator [31]. cAMP-mediated activation of *MAGE-A11* is dependent on the DNA

methylation status of the promoter, as a hypermethylated promoter blocked the activation [31].

Sp1 was shown to contribute to the activation of the CG antigen gene *NY-ESO-1* in lung cancer cells [71]. Decitabine treatment causes nuclear translocation of Sp1 and subsequent binding of both Sp1 and BORIS to the *NY-ESO-1* promoter [71]. Mutation of Sp1 sequences diminishes *NY-ESO-1* promoter activity, validating the functional role of Sp1 motifs in *NY-ESO-1* expression [71]. The fact that many CG antigen genes contain GC-rich promoter sequences suggests that Sp1 could play a role in the regulation of other members of this gene family.

p53 was reported to play a negative regulatory role in the promoter activity of the autosomal CG antigen gene *BORIS* [72]. An inverse relationship between wild-type p53 and *BORIS* expression was noted in a panel of 31 cancer cell lines [72]. This led to a functional examination of the role of p53 in *BORIS* regulation, which revealed that overexpression of wild-type, but not mutant, p53 strongly repressed *BORIS* expression [72]. Along with prior work demonstrating that CG antigens inhibit p53 function [69,70], these data suggest the existence of an autoregulatory loop involving p53 and CG antigens.

A model for CG antigen gene expression in human cancer

Based on the information described earlier, we present a model to account for the CG antigen gene-expression patterns observed in human cancer (Figure 1). In most normal somatic cells (diagramed in blue), CG antigen genes are not expressed, and this correlates with promoter and global DNA hypermethylation, the latter of which is primarily a read-out of the methylation status of repetitive DNA sequences in the genome. Following oncogenic transformation, two opposing forces may impact CG antigen gene-expression status:

- Activating signals for CG antigen gene expression include the expression of specific proteins, such as ETS transcription factors, KIT and BORIS. In addition, CG antigen expression is often associated with the global DNA hypomethylation phenotype sometimes observed in human cancer;
- Repressive signals for CG antigen gene expression include the expression of FGFR2 and p53.

In addition, immunological suppression via CG antigen-directed CTLs may limit the expansion of CG-antigen-positive cells. These opposing forces result in an equilibrium state in which heterogeneous intratumor expression of CG antigen genes is frequently observed. CG-antigen-expressing tumor cells (diagramed in pink in Figure 1) are distinguished by promoters that are DNA hypomethylated, show increased levels of active histone marks (e.g., H3K9 acetylation and H3K4 methylation) and are bound by sequence-specific DNA-binding transcription factors, including Sp1 and ETS. In at least some tumors, CG-antigen-expressing cells also appear to express markers associated with cancer stem cells (e.g., CD133). Conversely, CG-antigen-negative tumor cells (diagramed in purple in Figure 1) have DNA hypermethylated promoters associated with increased levels of repressive histone marks (e.g., H3K9 methylation and H3K27 methylation) and MBD protein occupancy.

CG antigen immunotherapy & epigenetic modulatory drugs CG antigen vaccine therapy

The development of strategies for actively stimulating immunological rejection of tumors, previously an elusive goal, has been accelerated by recent improved understanding of the molecular basis of immune recognition and immune regulation of cancer cells. As a consequence, several human tumor antigens have been defined, of which CG antigens are among the most immunogenic. Many CG antigens elicit spontaneous humoral and cellular

immune responses in cancer patients [1]. An ideal cancer antigen would be expressed specifically in tumor cells, would be critical for the survival of the cancer cell and would trigger a vigorous response by the human immune system. CG antigens are ideal targets for vaccine therapy because of their limited expression in normal somatic tissue and widespread expression in human malignancies. In addition, certain CG antigen genes have emerging roles in oncogenesis, as discussed earlier. MAGE-A1, MAGE-A3 and NY-ESO-1 are currently under investigation as vaccine targets; the vaccines are formulated to stimulate T-cell responses against tumors. In clinical trials, MAGE-A1, MAGE-A3 and NY-ESO-1 vaccines have been demonstrated to induce both humoral and cell-mediated immune responses. In addition, some patients have shown clinical benefit, with regards to disease stabilization and prolonged time to recurrence [1,74,75].

The CG antigen NY-ESO-1, initially defined by SEREX in esophageal cancer, is expressed at a variable frequency in many tumors, including 40% of epithelial ovarian cancers [13,76]. NY-ESO-1 elicits both humoral and cellular immune responses in a high proportion of patients with NY-ESO-1-expressing tumors [75,77,78]. Several NY-ESO-1 HLA class I- and II-restricted epitopes (recognized by CD8⁺ cytotoxic and CD4⁺ helper T cells, respectively) have been characterized, including those recognized in conjunction with HLA-A2 [79], A24 [80], B35 [81], DR4 [82–84], DP4 [85] and other epitopes still being described. Based on these characteristics, the NY-ESO-1 antigen has recently been selected as a prototypic human cancer antigen for the development of antigen-specific human cancer vaccines by the National Cancer Institute [86].

Multiple strategies are currently under investigation in clinical trials to determine optimal immunogenic vaccines of CG antigens, including the use of peptides, proteins, DNA, RNA and viral and bacterial vectors. The goal of the optimal vaccine is to induce consistent effector and memory CD8⁺ and CD4⁺ T-cell responses with high affinity for processed CG antigens and the ability to specifically recognize tumors. While it is clear that antitumor immune cells can be generated against tumors, the rate of regression of growing tumors has been infrequent. Explanations for the disappointing clinical results of immunotherapy trials include: tumor antigen loss [87], intratumor heterogeneity of CG antigen expression, upregulation of inhibitory molecules, such as CTLA-4, on tumor-infiltrating T cells [88], tumor overproduction of indoleamine 2,3-dioxygenase, which arrests T-cell proliferation [89], increased tumor infiltration by regulatory CD4⁺CD25⁺ T cells [90,91] and induction of certain types of natural killer T cells that inhibit tumor immune destruction [92]. To enhance the antitumor potential of immunotherapy, it will be essential to target immune evasion mechanisms and to test these approaches in the clinic.

Epigenetic modulatory drugs & CG antigen gene expression

The discovery of the critical role of epigenetic change in tumorigenesis has led to great interest in utilizing epigenetic modulatory drugs (predominantly DNMTi and HDACi) in cancer therapy [24,93]. While most efforts have focused on tumor suppressor gene re-activation to alter the oncogenic phenotype, an alternative and potentially equally important mechanism and use for epigenetic drugs is the activation of CG antigen gene expression to promote anticancer immunity [16,24,94]. Pre-clinical studies suggest that decitabine-mediated activation of CG antigen gene expression and CTL recognition show specificity to tumor cells over normal epithelial cells [22,38]. The effects of decitabine on CG antigen and HLA expression are prolonged and relatively stable, suggesting that the epigenetic changes provoked by these agents are heritable [95]. Recent clinical observations also support this approach. Myelodysplastic syndrome and acute myeloid leukemia patients treated with decitabine show *de novo* expression of *MAGE-A1*, *SSX* and *NY-ESO-1* [96]. In addition, approximately a third of lung, esophageal and mesothelioma cancer patients treated with decitabine show

upregulation of *NY-ESO-1* and *MAGE-A3* expression [97]. Notably, NY-ESO-1 antibody responses post-treatment also occur in a patient subset [97]. These data suggest that one potential mechanism of action of decitabine and other epigenetic therapies is the activation of anticancer immunity as a consequence of CG antigen induction.

Combinatorial use of CG antigen vaccines & epigenetic modulatory drugs

Patient selection for existing CG antigen vaccine approaches is based on the expression of CG antigens in tumor biopsies (using reverse transcription PCR or IHC analyses), and patients are enrolled even if expression is heterogeneous or focal [74]. A drawback of the selection criteria is that CG-negative neoplastic cells exist within most or all tumors, suggesting that they will be unresponsive to CG-antigen-specific immune recognition. As previously mentioned, recent studies have demonstrated that intratumor CG antigen expression heterogeneity is associated with DNA methylation status, and that treatment of cell lines with DNMTi augments CG antigen expression [14,45]. DNMTi treatment also causes upregulation of HLA class I molecules in various cell types [16,22]. HLA class I molecules upregulated by DNMTi are functional and facilitate tumor cell lysis by CTLs [98]. By causing simultaneous activation of CG antigens and HLA molecules, the immunogenicity of tumor cells may be greatly amplified by DNMTi treatment. Importantly, epigenetic modulators can enhance CTL-directed CG-antigen-restricted killing of tumor cells, providing validation that CG antigen gene activation by treatment with epigenetic modulators leads to functional antigen presentation and immune cell recognition [16,38,99]. Taken together, the data suggest that DNMTi, alone or in combination with other epigenetic modulatory drugs, could be used to augment CG antigen expression in patients undergoing CG antigen vaccine therapy. One potential drawback of this approach is that induction of CG antigens, some of which play roles in oncogenesis, could provide cancer cells with an increased malignant phenotype. For this reason, it will be important to carefully monitor patients receiving epigenetic modulators, either alone or in combination with CG-antigen-directed vaccines.

Implementation of a combinatorial therapeutic approach using decitabine and immunotherapy for the treatment of bulky solid tumors is likely to involve concurrent use of cytotoxic chemotherapy. The major limitation for combining cytotoxic chemotherapy and immunotherapy is that cytotoxic drugs are generally regarded as immunosuppressive because of toxicity to the dividing immune cells in the bone marrow and peripheral lymphoid tissues [100]. Although some chemotherapy regimens clearly cause lymphopenia severe enough to predispose patients to opportunistic infections, most cytotoxic therapies cause mild or no lymphopenia. Indeed, the rebound thymic response that occurs following chemotherapy may compensate for any lymphopenia occurring during chemotherapy [101]. In addition, some agents may promote a desirable cytokine profile in the tumor microenvironment or systemic circulation of the host. Recent evidence suggests that chemotherapy might:

- Increase the range of antigens delivered for presentation;
- Increase the dose of antigens delivered for presentation;
- Enhance the antitumor T-cell response in the draining lymph node through crosspriming;
- Affect T-cell traffic;
- Affect target cell destruction;
- Affect the generation of immunological memory [100].

Taken together, the available information indicates that both conventional treatment and immunotherapeutic strategies might be combined to control tumor growth, while allowing vaccine-induced immune responses to develop and eliminate residual disease.

In an ongoing clinical trial for recurrent ovarian cancer at Roswell Park Cancer Institute (NY, USA), we are testing a multimodal therapeutic strategy that includes:

- An immunogenic valid ovarian tumor antigen vaccine to enhance presentation of a CG antigen (NY-ESO-1) to the immune system;
- Granulocyte macrophage colony-stimulating factor to enhance the efficacy of antigen-presenting cells;
- Decitabine to enhance tumor antigen expression and effector differentiation of T cells.

This regimen is used in conjunction with standard chemotherapy using liposomal doxorubicin (Doxil®, Centocor Ortho Biotech, Inc., PA, USA). The goals of the multimodal strategy consisting of NY-ESO-1 vaccine therapy in combination with decitabine are to:

- Enhance antigenic dose to antigen-presenting cells and promote clonal expansion of antigen specific CD8⁺ T cells;
- Promote effector differentiation of vaccine-induced NY-ESO-1-specific CD8⁺ T cells;
- Minimize immune escape via tumor antigen loss. An additional advantage of this approach could be decitabine-mediated sensitization of ovarian cancer to chemotherapy, a concept that has been pursued previously [102].

One consideration in this regimen is drug sequencing. Our preclinical studies indicate that decitabine must be administered prior to Doxil in order to achieve promoter hypomethylation and *NY-ESO-1* gene activation, because initial treatment with Doxil causes cell cycle arrest, thus preventing decitabine incorporation into DNA. Based on these data, our clinical regimen involves decitabine treatment at day 1, Doxil at day 8 and vaccination with an emulsion of NY-ESO-1 protein, granulocyte macrophage colony-stimulating factor and adjuvant at day 15. This regimen is repeated monthly. If the study demonstrates strong immune responses, it will provide compelling motivation to begin further studies in patients with primary ovarian cancer disease, in order to determine whether immune responses can prolong disease-free and overall survival.

Adoptive transfer therapy & epigenetic modulatory drugs

Adoptive transfer of T cells (ACT) represents another promising immunotherapy strategy that may be enhanced by epigenetic modulatory agents. ACT involves the transfer of *ex vivo* expanded tumor-infiltrating effector cells as a means of augmenting the antitumor immune response and has been employed in the context of human melanoma [103]. In contrast to tumor vaccination strategies, ACT can allow for far greater control over the magnitude and avidity of the targeted response by appropriate manipulation and selection *in vitro* of the T cells used for therapy [104,105]. Tumor-reactive effector cells of a desired specificity and phenotype can be identified *in vitro*, selected based on expression of a high-affinity receptor and specific surface markers, and expanded to very high numbers. The required numbers of high-affinity effectors can be routinely achieved, producing frequencies of antigen-specific T-cell clones in the peripheral blood that can be several-fold higher than that possible by current immunization regimens alone. A recently developed variation of ACT is the use of patient peripheral blood lymphocytes genetically engineered to express tumor-reactive T-cell receptors (TCRs) via viral transduction [106]. This strategy was used to introduce MART-targeted TCR-expressing lymphocytes into patients with metastatic melanoma and led to dramatic tumor regression in a few patients [106]. More recently, this strategy was used to target NY-ESO-1 with an improved vector design to improve TCR avidity and tumor reactivity [37]. Notably, decitabine treatment, alone or in combination with the HDACi depsipeptide, enhances the reactivity of NY-ESO-1-targeted TCR-engineered lymphocytes to a variety of tumor cell lines [37]. Thus,

pre- or concurrent treatment of patients with epigenetic modulatory drugs may enhance the efficacy of ACT involving CG-antigen-specific TCR-transduced peripheral blood lymphocytes [37].

A synopsis of epigenetic therapy-mediated augmentation of CG antigen-directed immunotherapy

Figure 2 summarizes how epigenetic therapy may augment CG-antigen-targeted immunotherapy. DNMTi, including decitabine, are the most potent and efficacious inducers of CG antigen gene expression. Other epigenetic modulatory drugs, including HDACi, histone methyltransferase inhibitors and EZH2 inhibitors, can be used in combination with DNMTi to promote greater levels of activation of these genes in tumor cells. Since histone methyltransferase and EZH2 inhibitors are currently in the early stages of development, HDACi will have more utility in this regard for the near future. The effects of epigenetic drug treatment include CG antigen gene activation, as well as the activation of HLA class I and other immune costimulatory molecules, which further enhances immune presentation of CG antigens. Epigenetic modulators of CG antigen gene expression show specificity towards tumor cells. This specificity may be accounted for by the activation of sequence-specific transcription factors preferentially in tumor cells that act on hypomethylated CG antigen gene promoters and also by the increased proliferation rate of tumor cells relative to normal cells. However, epigenetic modulatory drugs may activate CG antigen gene expression in some normal cell types. Epigenetic drugs can be used to augment immunotherapy utilizing CG-antigen-directed vaccines or ACT therapy targeting CG antigens. Enhanced antitumor immune responses will include both humoral and cell-mediated pathways.

Conclusion

Cancer germline antigen genes are a large family of genes whose expression is mainly limited to germ cells and cancer. Based on their expression pattern, immunogenicity and emerging roles in oncogenesis, they have emerged as important cancer immunotherapy and therapeutic targets. These genes are regulated by epigenetic mechanisms and, in particular, DNA methylation is closely linked to gene-expression status. Furthermore, CG antigen genes are often co-expressed in human cancer. CG antigen gene expression commonly shows both intertumor and intratumor heterogeneity, which is linked to DNA methylation status. CG antigen genes also appear to be expressed and epigenetically regulated in cancer stem cells. Recent data indicate that specific signal transduction pathways and transcription factors also regulate CG antigen genes, in concert with or in addition to epigenetic mechanisms. Epigenetic modulatory drugs, including DNMTi and HDACi, robustly activate CG antigen gene expression *in vitro* and *in vivo*. In fact, one potential mechanism of action of epigenetic drugs in human cancer patients is the induction of anticancer immunity as a by-product of CG antigen gene induction. Vaccines targeting distinct CG antigens have been developed, and these approaches are safe and promote immune and clinical responses. Combined use of epigenetic modulatory drugs and CG antigen vaccines or ACT therapy may lead to increased immune and clinical responses in cancer patients, and this approach is being pursued.

Future perspective

The next few years will shed light on the role of specific epigenetic marks and regulators in controlling CG antigen gene expression. The recent discovery and characterization of a variety of novel histone-modifying enzymes, as well as proteins that reverse these modifications, will drive investigations into the role of these epigenetic networks in CG antigen gene regulation. Outside of epigenetics, studies will begin to elucidate the role of specific signal transduction pathways in the regulation of CG antigen gene expression in human cancer. Probably most notable will be the investigation of direct links between classical oncogenic pathways (e.g.,

those involving TKs) and CG antigen gene expression. It is also likely that the next few years will bring additional testing of the role of specific CG antigen gene products in tumorigenesis. As one example, the functional contribution of MAGE-A11 to castration-recurrent prostate cancer should become clearer.

Clinically, it is likely that CG antigen-directed vaccine(s) will be approved for the treatment of human cancer within 5–10 years. Additional epigenetic modulatory drugs will also be approved during this timeframe. Detailed molecular pharmacological studies will focus on determining the extent to which CG antigen gene activation contributes to the therapeutic activity of epigenetic modulatory drugs. Finally, clinical approval of CG antigen immunotherapy and epigenetic modulatory drugs will lead to investigations of combinatorial regimens involving these approaches.

Executive summary

Epigenetic regulation of cancer germline antigen genes

- Global genomic and promoter-specific DNA hypomethylation is associated with cancer germline (CG) antigen gene expression
- Specific histone modifications are associated with CG antigen gene expression
- DNA methylation status is linked to CG antigen expression heterogeneity in cancer.
- CG antigen genes can be expressed in normal and cancer stem cells.

Additional mechanisms of CG antigen gene regulation

- The ETS transcription factors are sequence-specific methylation-dependent regulators of CG antigen gene expression
- BORIS is a CG antigen that plays a key role in the activation of other CG genes in a cell context-dependent manner.
- Receptor tyrosine kinases KIT and FGF receptor 2 regulate *MAGE-A* gene expression.
- cAMP, Sp1 and p53 regulate CG antigen gene expression.

CG antigen immunotherapy & epigenetic modulatory drugs

- CG antigens are ideal targets for cancer immunotherapy, and several CG-antigen-directed vaccines are in clinical trials
- DNA methyltransferase and histone deacetylase inhibitors induce CG antigen gene expression *in vitro* and *in vivo*, and promote immune recognition of tumor cells expressing CG antigens.
- Combination therapies using CG antigen vaccines or adoptive transfer of T cell therapy in conjunction with epigenetic modulatory drugs are in clinical development.

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Figure 1. Cancer germline antigen gene regulation in human cancer

CG: Cancer germline; FGFR: FGF receptor; MBD: Methyl-CpG-binding domain proteins; me: Methylation.

Figure 2. Epigenetic therapy augmentation of cancer germline antigen immunotherapy

CG: Cancer germline; DNMTi: DNA methyltransferase inhibitors; DZnep: 3-deazaneplanocin A; EZH2i: Enhancer of zeste homolog 2 inhibitors; HDACi: Histone deacetylase inhibitors; HMTi: Histone methyltransferase inhibitors.